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EFFECT OF ENZYMATIC TREATMENTS ON CARBOHYDRATE MATRICES TOWARDS HEALTHY GLUTEN FREE FOODS APPLICATION

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Hace constar que:

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Dra. Cristina Molina Rosell

No te rindas,
que la vida es eso,
continuar el viaje,
perseguir tus sueños,
destrabar el tiempo,
correr los escombros
y destapar el cielo.

La mariposa recordará por siempre,
Que fue gusano.

Mario Benedetti

A mis padres,
Por enseñarme a no rendirme.
Siempre.

RESUM

El midó és la principal font de reserva d'energia en les plantes, està àmpliament present en diverses aplicacions alimentàries i no alimentàries, i constitueix un dels hidrats de carboni més abundants en la dieta humana. A més de l'ús freqüent i clàssic del midó natiu com a matèria primera en la producció d'aliments, els midons modificats han experimentat gran expansió en el desenvolupament de nombrosos productes a causa del seu caràcter versàtil. Les modificacions enzimàtiques es duen a terme per a realçar la funcionalitat del midó a fi d'esbiaixar les restriccions tecnològiques i millorar la qualitat del producte final. En concret, el midó de dacsa àmpliament produït i consumit, és apropiat com a ingredient principal en la producció d'aliments lliures de gluten. Aquesta Tesi aborda l'estudi de l'efecte individual de tres diferents enzims, α-amylasa fúngica, amiloglucosidasa i ciclodextrina glucosiltransferasa, sobre la temperatura de sub-gelatinización del midó de dacsa. Es van realitzar diferents anàlisi per a ampliar el coneixement dels canvis estructurals i funcionals associats a l'acció de l'enzim. Així mateix, es va seleccionar el midó de dacsa modificat amb ciclodextrina glucosiltransferasa per a investigar la resposta glicémica en ratolins. L'índex glicémic es va relacionar amb l'absència/presencia de productes d'hidròlisis alliberats per l'acció catalítica de l'enzim i les propietats de gelatinización. Els midons de dacsa enzimàticament modificats van presentar alteracions funcionals en els gränuls de midó, la qual cosa els confereix característiques d'interès per a diversos usos alimentaris.

RESUMEN

El almidón es la principal fuente de reserva de energía en las plantas, está ampliamente presente en diversas aplicaciones alimentarias y no alimentarias, y constituye uno de los hidratos de carbono más abundantes en la dieta humana. Además del uso frecuente y clásico del almidón nativo como materia prima en la producción de alimentos, los almidones modificados han experimentado gran expansión en el desarrollo de numerosos productos debido a su carácter versátil. Las modificaciones enzimáticas se llevan a cabo para realzar la funcionalidad del almidón con objeto de soslayar las restricciones tecnológicas y mejorar la calidad del producto final. En concreto, el almidón de maíz ampliamente producido y consumido, es apropiado como ingrediente principal en la producción de alimentos libres de gluten. Esta Tesis aborda el estudio del efecto individual de tres diferentes enzimas, α-amylasa fúngica, amiloglucosidasa y ciclodextrina glucosiltransferasa, sobre la temperatura de sub-gelatinización del almidón de maíz. Se realizaron diferentes análisis para ampliar el conocimiento de los cambios estructurales y funcionales asociados a la acción de la enzima. Así mismo, se seleccionó el almidón de maíz modificado con ciclodextrina glucosiltransferasa para investigar la respuesta glicémica en ratones. El índice glicémico se relacionó con la ausencia/presencia de productos de hidrólisis liberados por la acción catalítica de la enzima y las propiedades de gelatinización. Los almidones de maíz enzimáticamente modificados presentaron alteraciones funcionales en los gránulos de almidón, lo que les confiere características de interés para diversos usos alimentarios.

ABSTRACT

Starch is the major energy reserve in plants, extensively present in many food and non-food applications, and is one of the most abundant carbohydrates in human diet. In addition to starch native form currently used as a raw material for industrial applications, modified starches have become very attractive to develop numerous products that have greatly expanded starch use and utility. Enzymatic modifications are carried out to enhance starch functionality with the aim of overcoming technological constraints and to improve final product quality. Above all starches, corn starch is widely produced and consumed, and used as main ingredient to produce gluten-free products. This Thesis focuses to the study of the individual effect of three different enzymes, fungal α-amylase, amyloglucosidase and cyclodextrin glycosyltransferase, on the above sub-gelatinization temperature of corn starch, in order to modify its properties and extend its applications. Different analyses have been performed to improve the understanding of functional and structural changes promoted by the enzyme action. Moreover, corn starch modified with cyclodextrin glycosyltransferase was selected to investigate the glycemic response in mice. The presence/absence of hydrolysis products released from the enzyme catalytic activity and gelatinization properties were related to glycemic index. Enzymatically modified corn starch resulted in a diversity of functional starch granules that may be used in food applications for many purposes.

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I. Introduction

1. Starch sources and molecular composition of starch granules

Starch is produced by all green plants, occurs in the endosperm in granular form and is the main carbohydrate in the human diet. As the major component in cereals (corn, wheat, rice, barley, sorghum, etc.), tubers (potato, yam, sweet potato), roots vegetables (cassava, taro, etc.) and pulses (pea, lentil, bean, etc.) is contained in many staple foods. Corn, tapioca, potato and wheat starches are the most commonly used starches in the United States (US) and European Union (EU). In selected regions, rice, sorghum, sago and other starches are also used. The intrinsic starch properties (viscosity, stability to processing and distribution and gel strength), which are mainly influenced by the ratio amylose/amylopectin (Srichuwong et al., 2005a,b), determine the application of these starches in foods.

1.1. Polysaccharide components

Starch is a polysaccharide composed of a large number of glucose units joined by glycosidic bonds. It consists of two types of anhydroglucose polymers, amylose and amylopectin. Amylose is a mainly linear and helical polysaccharide made up of 300-3000 glucose units linked via α-(1,4) glycosidic bonds with very few branches involving α-(1,6) glycosidic linkages. Amylopectin is a highly branched polysaccharide consisting of glucose units linearly linked by α-(1,4) glycosidic bonds to form chains of between 6 and >100 glycosyl residues in length, and the branching takes place every 20-30 glucose residues with α-(1,6) glycosidic linkages (Figure 1) (Buléon et al., 1998). The distribution of amylose and amylopectin molecules allows forming the matrix of the starch granule, which consists of alternating, concentric rings of amorphous and semicrystalline domains originated from the hilum toward the periphery. Crystalline layers are mainly amylopectin chains packed into a semi-crystalline structure, whereas the amorphous regions of the growth rings contain mainly amylose. Amylose

molecules are also scattered between the amylopectin in the crystalline layers, disrupting the crystal packing of amylopectin (Vermeylen et al., 2004).

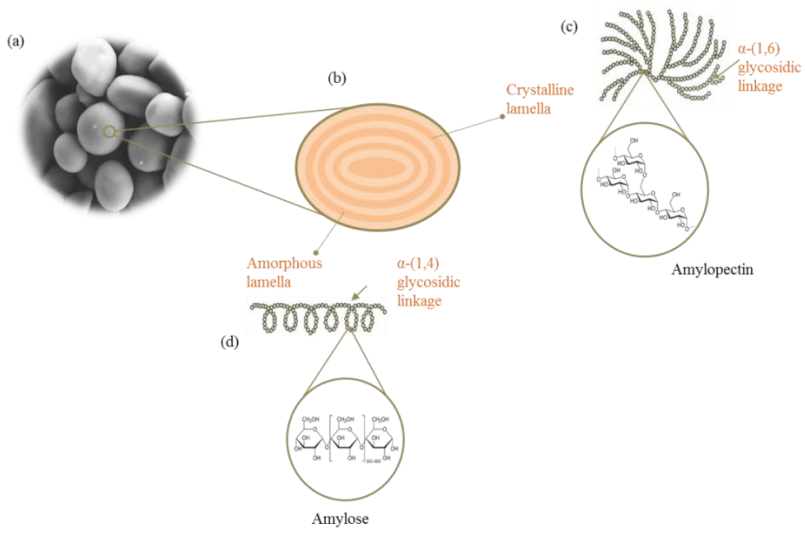

Figure 1. The composition and structure of starch granules: (a) starch native granules from corn, (b) amorphous and semycrystalline growth rings, (c) schematic representation of amylopectin and molecular structure, (d) schematic representation of amylose and molecular structure (Picture composed by A. Dura).

Generalities about structure, granule organization, properties and behavior of starches from different sources are limited. The ratio of amylose to amylopectin varies within the starch source. Most common cereals (corn, wheat, rice, barley, sorghum) range between 20-30% amylose content, while in roots and tubers 19-25% of starch is present as amylose. Higher amylose content, 30-40% is present in pulses. Waxy and high-amylose starches differ in amylose content from normal starches. High amylose starches (corn, barley) have 50-70% of amylose available, whilst waxy varieties (corn, rice, sorghum, barley) have no amylose and contain essentially 100% amylopectin. The amylopectin chain length determines the polymorphism of a starch granule depending on the origin. Starches with A type crystallinity (cereal) have shorter chain length and crystallize with slightly distorted

hexagonal packing with a low water content, while B type starches (tuber and high amylose starches), crystallize in a hexagonal unit cell with a more open structure containing a hydrated helical core. The C-polymorphic structure (pulses and root) is a mixture between A- and B-type patterns. The proportions can vary among pulse and root starches in packing density and structure (Tester et al., 2004).

Starch granules can differ in shape, size and composition, depending on both the botanical source and the environment. Granule size diverges, from rice with only 2-10 μm of diameter, corn with a range of 5-20 μm of diameter, to a 10-35 μm of diameter in different wheat varieties. Starch granule shape, large, spherical, oval, polygonal, round or irregular, also determines the granule size (Lindeboom et al., 2004).

Amylose/amylopectin ratio, granular size and shape are related to starch source and molecular fine structure will influence functionality of starch for their further applications in the food and non-food industries.

1.2. Minor components

Starch granules also contain very small amount of proteins, lipids and phosphorous. The protein content varied in the range 0.1-0.8% (Yusuph et al., 2003). Proteins are either found at the surface of the granules or within the inner parts. The presence of pores or channels as structural features in some starch granules (corn, sorghum and wheat) was first evidenced by Fannon et al. (1992); those extend from the granule surface into the interior parts. Han et al. (2005) showed that the channels were filled with proteins, indicating that proteins were native to the granules and not artifacts of isolation.

Lipids are also found in low amount, especially in cereal starches, located on the surface, mainly triglycerides, followed by free fatty acids, glycolipids and phospholipids. They could be found also inside the granule in the form of

free fatty acids and lysophospholipids (Morrison et al., 1984). Surface and internal lipids are present in free form or bound to starch components, in the form of amylose inclusion complexes or linked via ionic or hydrogen bonding to hydroxyl groups of the starch components (Morrison, 1988). Amylose-lipid complexes range from less than 15% to more than 55% of the amylose fraction in cereal starches, whereas oat starches results especially rich in lipids complexed with amylose (Morrison, 1995).

Starches also contain a small quantity of minerals with low functional significance, with an exception for phosphorous. The phosphorus is found in three major forms: phosphate monoesters, phospholipids and inorganic phosphates. Phosphate monoesters are selectively bound to specific regions within the amylopectin molecule (Blennow et al., 2002) and have diverse effects on starch paste properties from different botanical sources (Jane et al., 1996).

2. Starch production and consumption

The importance of starches lies in their abundant availability, cheapness, renewability, biodegradability and non-toxic nature.

Native starch is mainly isolated from plants. Starch is rarely a uniform product, as it is extracted from different crops offering diverse properties, which starch industry matches to a specific end-use to obtain the desirable product. Starch biosynthesis is subjected to changes with environmental temperature, leading to the formation of different starch structures that can cause different functional properties.

Main sources with high starch content are potatoes, corn, sorghum, wheat, rye, barley, peas, rice, and cassava. Most of the starch produced annually is either directly consumed as food, used as animal feed or in non-food industries as a renewable raw material and bioethanol production. The latest Food and Agriculture Organization (FAO) outlook report for cereal world

production and consumption is displayed in Table 1 (FAO, June 2016). FAO forecasts world cereal production in 2016 at around 2,543 million tons, 0.6 percent higher than in 2015 and only 0.7 percent below the 2014 record high. Compared to 2015, world wheat production is likely to decline, while rice and coarse grains outputs are forecast to increase.

Table 1. World Cereal market

	2014/15	**2015/16** *estimated*	**2016/17** *forecast*
WORLD BALANCE		*million tons*	
Production	**2 561.8**	**2 527.7**	**2 542.9**
Trade[1]	**2 376.1**	**376.2**	**369.1**
Total utilization	**2 501.2**	**2 522.9**	**2 545.7**
Food	1 080.2	1 091.7	1 105.7
Feed	889.8	901.7	914.7
Other uses	531.2	529.5	525.3
Ending stocks	**644.1**	**644.0**	**642.2**
SUPPLY AND DEMAND INDICATORS			
Per caput food consumption:			
World (kg/yr)	148.9	148.8	149.0

Adapted from FAO Food Outlook. Biannual report of global food markets. (FAO, June 2016)

[1] Trade refers to exports based on a July/June marketing season for wheat and coarse grains and on a January/December marketing season for rice.

Worldwide starch production has constantly enlarged due to an increasing demand. Global starch production is led by US with more than a half of starch world production, predominantly corn starch, in conjunction with China, which has increased its corn production over the years. In fact, China, India, Thailand, South Korea and Philippines, are slated to have a growth rate of 9% through 2018 (www.ers.usda.gov). Currently, global corn starch market is the predominant commercial starch, making up about 83% of the world supply. Corn is grown throughout the world, although there are large differences in yields (Figure 2). The ready disposal of corn at relatively low and steady prices, its storability from season to season, its ease of transportation and handling, and its high starch content led naturally to the development of commercial processes for recovery of corn starch. The

expansion of corn starch use for food and non-food applications catalyzed the growth of the global corn starch industry.

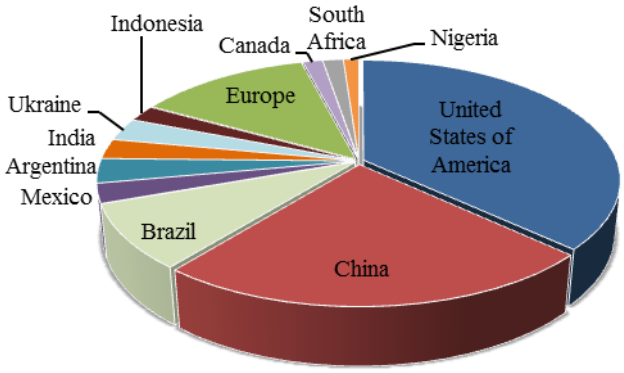

Figure 2. Corn production average 2012-2014 by country. Source: FAO (June 2016).

Nevertheless, the market is moving beyond the traditional sources of starch such as corn, potato and wheat, to consider other economical sources of starch, such as cassava, and sweet potato. There is a significant proportion of starch manufactured from cassava that is recently increasing its production, specifically in developing markets in Asia-Pacific. However, it also exists some starch output derived from rice, barley, oats, sweet potatoes and sago. EU starch production has increased from 8.7 million tons 2004 to 10.7 million tons in 2015, leading corn and wheat starch production, with less than 2 million tons for potato starch (www.starch.eu), whereas North American production of starch is based almost entirely on corn.

Global starch consumption is projected to rise primarily prompted by the growing diversity of starch end-use applications. The largest single outlet for starches has always been for conversion into sweeteners. The global demand for cereal sweeteners is closely related to the development of the soft drink industry, which started several years ago in the US. About 54% goes to sweeteners, 27% goes to fuel alcohol manufacture and 19% is used as starch. The EU consumes 9.3 million tons of starch (excluding starch by-products

totaling around 5 million tons), of which 61% in food, 1% in feed and 38% in non-food applications, primarily paper making and biofuel production (www.starch.eu).

Although new usages of starch have been developed, the food industry has always remained the traditional sustainer for global starch market. Intake of starch is mostly in the form of cereals, particularly breads and breakfast cereals, potatoes and rice. With the increasing demand for low fat and low calorie food, many food companies are replacing fats with gums and carbohydrates, such as starches and hydrocolloids. However, one of the key reasons of the increasing starch demand is the growing consumption of modified starches mainly used in processed and convenience foods.

3. Starch properties

The highly structured nature of the starch granule provides unique properties that depend greatly on the molecular components, distribution and structures that display different physicochemical (gelatinization, retrogradation) and functional (solubility, swelling, water absorption, viscoelastic and rheological behavior of pastes and gels) characteristics.

Isolated starch is typically a dry, soft, white powder. It is insoluble in cold water, alcohol, ether and most organic solvents. Starch, if kept dry, is stable in storage for indefinite periods. However, heating the starch granules in excess of water to progressively higher temperatures enables an irreversible swelling of the granules that contribute to amylopectin-amylose phase separation and crystallinity loss, resulting in amylose leaching and increases the granular size and solubility to lose finally their structural integrity (Singh et al., 2003). The swelling capacity and solubility of starch illustrate the interactions of amylose-amylopectin proportion comprising the amorphous and crystalline domains and vary among starch molecules. These facts, associated with the disruption of the ordered structure, are called gelatinization. Amylose and amylopectin play an important role, where the

functionality of the two main components of starch differs significantly in the gelatinization process. On water dispersion, amylopectin is more stable and produces soft gels and weak films. It is responsible for the swelling power and viscosity development of starch during cooking, whereas amylose, particularly with the presence of lipids, tends to intertwine and restrict the swelling of starch granules. Gelatinization causes large changes in the rheological properties of the system (pasting properties, the viscosity of starch paste and the rheological characteristics of starch gel) and has a major influence on the behavior and functionality of starch-containing systems. Starches that contain high percentage of amylose are less accessible to gelatinize because of the extra energy needed to hydrate and disintegrate the firm interactions. At the opposite, waxy starches gelatinize easily and yield nearly-transparent viscous pastes (Schimer et al., 2015). Upon cooling, starch changes occur during retrogradation, amylose and amylopectin molecules realign (mainly hydrogen bonding) into more ordered structure, which leads to the formation of crystallites that resist enzymatic hydrolysis. Amylose that forms double helical large interactions between glucose units has a high tendency to retrograde, whereas amylopectin occurs by association of the outermost short branches to retrograde slowly. Rate of retrogradation depends on starch varieties. Starches that contain high amylose ≈28%, such as corn starch, retrograde more than starches with lower amylose content ≈20%, such as potato starch (Wang et al., 2015). Starch properties are directly influenced by hydrothermal treatment and processing conditions (pH, rate of heating, amount of agitation and/or time duration), which can affect the final characteristics of the paste or gel formation (viscosity, shear resistance, textures, solubility, thickness, gel stability, cold swelling, hydration properties, retrogradation, etc.)

Granule swelling, gelatinization, pasting and retrogradation are important aspects of starch functionality that vary in degree from one starch source to another depending on the starch granular and molecular composition

(amylose/amylopectin ratio) and may be influenced by the presence of minor components (Ai and Jane, 2015). For instance, normal corn starch, after being cooked, produces an opaque paste which is not sticky and in the end sets to a strong gel. Otherwise, waxy corn and potato starches produce clear but sticky long pastes, and the pastes have fewer tendency to set to gels (Swinkels, 1985). Differences among corn starches in granule swelling, peak temperature, peak viscosity, shear thinning during pasting, and gel firmness during storage, have been mostly attributed to differences in amylopectin structure (Bahnassey and Breene, 1994), whereas differences in setback and final viscosity during pasting have been attributed to amylose structure (Vasanthan and Hoover, 1992b). A wide range of studies have been reported based on the behavioral diversities of starches and other functional characteristics that differ with species and variants (Sasaki et al., 1999; Hoover 2001; Singh et al., 2003; Srichuwong et al., 2005; Sandhu and Singh, 2007; Yoo et al., 2009; Waterschoot et al., 2015).

For many applications, the native granule of starch is not used; instead, the gelatinization process is initiated leading to a destruction of the granule. Improving understanding on how starch structure affects its functionality provides remarkable information to a subsequent end-use of starch and further starch modification that will improve or change its functionality and may be beneficial to an end-product quality. Particularly, corn provides a high-quality starch used widely in the food industry in many applications requiring particular viscosities and textures.

4. Starch modification

Nowadays, industry demand for new technological properties requires the native starch to be physically, chemically or enzymatically modified to extend the range of functionality, and consequently new functional and value added properties (Kaur et al., 2012).

4.1. Physical modifications

Physical modifications of starch combine temperature, moisture, pressure, shear and irradiation and can improve its water solubility and reduce the size of the starch granules. The processing conditions vary considerably between treatments. These treatments can be divided into thermal and non-thermal. Thermal treatments include: pre-gelatinization by spray cooking, drum drying, or solvent-based processing and extrusion. Pre-gelatinization results in a partially gelatinized starch, decreasing hydrogen bonding where the granular integrity of starch is lost; paste viscosity is reduced and gives higher water solubility. An alternative application of pre-gelatinized starch is the preparation of cold water swelling starch produced by a solvent-based method. These starches exhibit smoother texture, greater viscosity and more processing tolerance. They maintain granular integrity but losses its birefringence (Thomas and Atwell, 1999).

High moisture treatment and annealing are common physical modifications regarded as hydrothermal processes that occur below gelatinization temperature. In heat-moisture treatment starches are treated at varying moisture levels (<35%) for a certain period and at a temperature above the glass transition temperature but below the gelatinization temperature. Conversely, the annealing to improve molecular mobility requires an excess of water. The starch integrity is preserved while properties of starches are enhanced to different extends, improving viscosity and stability when subsequently gelatinized and pasted (Zavareze and Dias, 2011).

Non-thermal treatments include: Ultra high-pressure treatment, instantaneous controlled pressure drop, high-pressure homogenizers, dynamic pulsed pressure, pulsed electric field and freezing and thawing. Nowadays, the interest of non-thermal treatment is increasing as alternative processing method to the traditional ones to avoid high temperatures, which can alter organoleptic properties and cause a loss of vitamins and nutrients, and

eliminates pathogenic microorganism and spores. Non-thermal treatments can be used to preserve color, texture, taste, nutrients and other components of food as compared to the traditional thermal processes (Li et al., 2008). Ashogbon et al. (2014) reviewed some of the non-thermal physical treatments, including the effects caused on the starch granules. Non-thermal treatments affected the physicochemical properties of starch in a different way. High hydrostatic pressure permits to achieve starch gelatinization at room temperature. A better preservation of the granular structure was observed after pressure treatment compared to temperature treatment that led to stronger gels (Vallons and Arendt, 2009). Deep freezing and thawing of moistened starch resulted in an increased crystallinity of the granules (Szymonska et al., 2000) while multiple deep freezing and thawing caused an irreversible disruption of the crystalline order (Szymonska et al., 2003). An appropriate selection of the non-thermal treatment is required to obtain the desire effects on the physicochemical properties of starches.

4.2. Chemical modifications

Chemical modification of starch changes the functionality of the starch without affecting the morphology or size distribution of the granules. It is usually performed in a suspension of starch in water, typically 30-45% solids (by weight), then treated with the chemical reagent(s) under proper agitation, temperature and pH. The starch resulted from the complete reaction is brought to the desired pH by a neutralizing agent and then purified by washing with water and recovered as a dry powder. Modified starch presents enhanced molecular stability against mechanical shearing, acidic, and high temperature hydrolysis; obtaining desired viscosity; increasing interaction with ion, electronegative, or electropositive substances; and reducing the retrogradation rate of unmodified starch (Singh et al., 2007).

Modification is generally achieved through derivatization such as etherification, esterification and crosslinking, oxidation, cationization and

grafting of starch. Crosslinking is perhaps the most common type of chemical reaction used, in which starches are produced by introducing a limited number of linkages between the chains of amylose and amylopectin using dysfunctional reagents. By reinforcing the hydrogen bonding, swelling of the starch granule under cooking conditions is restricted and gelatinization is prevented. In order to minimize or prevent retrogradation, substituted starches are produced by etherification or esterification by introducing monofunctional chemical "blocking groups," such as acetyl or hydroxypropyl groups, along the polymer backbone. This reduces the tendency of chains to realign (retrograde) during cooling processing. The most common conversion methods used to reduce molecular weight polymers and to low viscosity in the starch industry include acid hydrolysis, oxidation and pyroconversion. The properties of converted starches can vary widely depending upon the type of base starch used and the conversion process (Ashogbon and Akintayo, 2014).

However, the amount of the starches derived from these techniques allowed in food grade is limited due to consumer´s safety and environment. New trends are brought up to combine chemical modifications with physical methods (microwave, radiation and extrusion) to obtain starches with desirable functional properties (Kaur et al., 2012).

4.3. Enzymatic modifications

Enzymatic modification transforms native starch by modifying the molecular weight to produce end-products with variable structural and functional properties. Enzymes have long been recognized to play a diversified role as a useful tool for improving starch processing behavior and properties in the production of food and several industrial applications. The most common enzymes used to modify starch are the α-amylase family (van der Maarel et al., 2002). The trend from the food industry regarding enzymatic modification has been mostly focus in hydrolyzed starches producing a wide

range of glucose syrups and sweeteners, in the particular case of starch converting enzymes such as amylase (AM) and amyloglucosidase (AMG), and in the production of cyclodextrins by cyclodextrin glycosyltransferase (CGTase). The optimum conditions of hydrolysis depend on the origin of the starch, since they differ in their susceptibilities to be hydrolyzed and on the type of enzyme involved in the modification. Up to now, enzymatically modified starches have been extensively studied from different starch crops to fulfill knowledge. Research was focused at digestibility studies, the effect of the enzyme addition to the final product related to health requirements, degree of crystallinity, hydrolysis products and extent of the reaction (Colonna et al., 1992; Hoover, 2001; Biwer et al., 2002; Gujral and Rosell, 2004; Li et al., 2004; Han et al., 2006; Ao et al., 2007; Zhekova et al., 2009; Gularte and Rosell, 2011; Zhang et al., 2012; van der Maarel and Leemhuis, 2013). However, there is a continuously rising demand for new and improved specific functional traits and it is important to consider the requisites for further investigations to a significant improvement of enzymatically modified starch that could result in important impact in todays advanced technologies and end-use applications. The available information on corn starch granule structure and the individual effect of these enzymes on the starch and improve reaction conditions, still deserves attention.

4.4. Combined treatments of starch

Acquiring of desired functional properties is possible by using various methods of modification, which have specific applications in various industries. Mainly in food applications, starch modification using a combination of chemical, physical and enzymatic methods have grown rapidly to provide an assortment of products with a range of properties. Physical modifications are simple and inexpensive methods, despite energy requirements, which can be produced without chemicals or biological agents. The use of enzymes for modifications presents a wide number of advantages such as higher product quality, lower manufacturing cost, low amount of

enzyme used to achieve a desirable product, reduced energy consumption and environmental pollution. Therefore, they can often replace chemicals, like in the case of syrups replacing sulfuric acid or in baking application as an antistaling agents and dough strengtheners replacing bromate.

Nowadays, many physical and chemical or enzymatic and chemical starch modifications have been combined to improve the functional properties of starch (Jacobs and Delcour, 1998; Sun et al., 2015). Nonetheless, combination of physical and enzymatic modifications result less common to bring about the desire effects for the utilization of starches for the specific application.

5. Industrial applications of starch products

The growing usage of starch as a raw material comes from many reasons: its abundance, it is the cheapest biopolymer, ease for modification and it is very degradable. The variation found in different sources of starch gives great versatility in its uses. Starch derivatives are used in food products as thickeners, gelling agents and encapsulating agents. Besides serving as food, starches are also industrially processed into a series of derivatives by modification treatments that are applied in various industries. Modified starches are also used in papermaking as wet-end additives for dried strength, surface sizes and coating binders, as adhesives (corrugating, bag, bottle labeling, laminating, cigarettes, envelopes, tube-winding and wallpaper pastes), for warp sizing of textiles, for glass fiber sizing, in tableting and cosmetic formulations. Table 2 shows some examples of native and modified starch and its particular application based on the functional characteristics of starch.

Table 2. Applications of native and modified starches in food and non-food industries

Type of application	Application	Reference
Non-food		
Native/Modified starch	-Paper, textile, cardboard and potable alcohol production	Ellis et al., 1998 Sahid et al., 2013 Becerra et al., 2014
Chemical starch modification	-Pharmaceutical industry (excipient)/Drug carrier	Kittipongpatana et al., 2007 Massicotte et al., 2008 Al-karawi et al., 2010
Substituted starches	-Paper: Water treatment (flocculation) -Paper: Oil industry (fluid loss reducer)	Letelier-Gordo et al., 2014 Li Met al., 2010
Acid hydrolysis/ Gelatinized	-Nanoparticles	LeCorre et al., 2010
Combined treatments	-Textile industry -Edible films	Zhang et al., 2014 Jiménez et al., 2012 Dhall RK, 2013
Enzymatically converted starch	-Paper and fermentation industry -Detergents	Van der Maarel and Leemhuis, 2013 Schallmey et al., 2004

Food		
Native starch	-Thickener -Surimi (gel strength) -Encapsulation -Thickener in instantaneous products	Arocas et al., 2009 Hunt et al., 2009 Doade et al., 1990 Wang et al., 2015 Majzoobi et al., 2011
Pre-gelatinized starch	-Cake mixes as softener and retain moisture in the baked product	Takashima, 2005
Extrusion	-Ready-to-eat cereals, snacks, confectionery products, texturized vegetable protein, and macaroni, as well as pet foods	Harper, 1989
Heat-moisture treatment	-Improve baking quality -Freeze-thaw stability and pie filling with good organoleptic properties	Lorenz and Kulp, 1981 Hoover and Manuel, 1996
Chemical starch modifications	-Instant foods, confectionary, coating, texturizers, fat replacer, stabilizers, etc.	Singh et al., 2007
Stabilization	-Frozen foods	Rutenberg and Solarek, 1984
Substituted starches	-Frozen foods	Moore et al., 1984
Cross-linking	-Desserts, bakery products, soups, sauces, baby foods, fruit filling, deep fried foods -Gluten free bread -High amylose starch as a crispiness improver	Poomipuk et al., 2014 Paulus et al., 2005 Sajilata and Singhal, 2004

Etherification (Hydroxypropyl starches)	-Thickeners in fruit pie fillings, puddings, gravies, sauces, and salad dressings	D'Ercole, 1972 Mitan and Jokay, 1969
Acid/Enzyme Hydrolyzed starches	-Food preparation (bulking agent), ice cream industry, beverage, brewing and confectionary industry, canned and frozen fruits or vegetables, baking industry	Homayoumi et al., 2008 Rosicka-Kaczmarek et al., 2013 Daud et al., 2014 Herrero et al., 2014 van der Maarel et al., 2002
	-Maltodextrins (fat replacers and bulking agents, encapsulation of flavor, colorants, in bakery and confectionery, beverages, dairy, desserts, meats and gravies)	Blanchard and Kate, 1995
	-Cyclodextrins (Cholesterol sequestrant, food preservation, encapsulation of flavors, etc.)	Martin del Valle, 2004 Astray et al 2009
	-Production of glucose/fructose syrups and sucrose isomers	Crabb and Shetty, 1999 Buchholz and Seibel, 2008
Enzymatically converted starch	-Fat replacer in low-fat mayonnaise	Mun et al., 2009
	-High amylose starches (process into resistant starch-nutritional benefits)	Vorwerg et al., 2002

Within the properties required for a particular application, availability of the starch and economics play a role in selecting a particular native starch for subsequent modification. Among all kinds of starches, corn starch is one of the most available and accessible starches and an important ingredient in the production of foodstuffs. Corn starch has been widely used as a thickener, stabilizer, colloidal gelling agent, water retention and as an adhesive (Singh et al., 2003). Many different types of hydrolyzate products, such as sweeteners, are produced for a multitude of food products. In addition, ethanol is produced by fermentation of corn starch hydrolyzates and may be used in beverages. Corn starch is also used in non-food industries as paper, adhesive, textile, building materials, packaging, biopolymers, pharmaceutical, biofuel, etc.

It is expected that, the need for starch would continue to increase especially as starch and modify starch continues finding applications among industries.

6. Nutritional implications

Starch plays an essential role in human nutrition as one of the main forms of dietary carbohydrates. The increasing demand from consumers and industry for starch has driven to check the nutrition and health implications regarding the consumption of starch and starch-based foods (Ludwing, 2002). Mann et al. (2007) provided a scientific report from WHO/FAO reviewing health implications of carbohydrates in human nutrition. Starch and starchy food products can be classified according to their digestibility, which is generally characterized by the rate and the duration of the glycemic response. The glycemic response and glycemic index (GI) is a physiological concept used to classify carbohydrate containing foods and refers to the ability of a particular food to elevate postprandial blood glucose concentrations. Diverse scientific studies have established the relationship between starch digestibility and some chronic diseases and some types of cancer (Yokoyama et al., 2002; Jacobsen et al., 2006; Yokoyama and Shao, 2006; Park et al.,

2008; Nazare et al., 2010). Therefore, low glycemic index foods are recommended for modulating the glucose response.

One of the most common methods to classify starches based in their digestibility was suggested by Englyst et al. (1992). Most starches contain a portion that digests rapidly (rapidly digesting starch; RDS), a portion that digests slowly (slowly digesting starch; SDS) and a portion that is resistant to digestion (resistant starch; RS) (Singh et al., 2010). RS has been widely reviewed over the years (Annison and Topping, 1994; Haralamou, 2000; Fuentes-Zaragoza et al., 2010; Perera et al., 2010; Thompson, 2010; Homayouni et al., 2014) and has received considerable attention as a substitute or rather a supplement of dietary fiber and has found a number of applications for improving the functional properties of foods, in particular cakes and other bakery products, where it reduces the tempering step. RS, as a component of dietary fiber, has been has been related to the rate and extent of starch digestion and hence on glucose response. The production of short chain fatty acids (SCFA) in the large intestine (Andoh et al., 2003) may help improve colonic health. Animal studies in pigs and rats have reported that feeding RS increased the caecal and faecal production of total SCFA and the individual concentrations of propionate, butyrate and acetate (Ferguson et al., 2000; Henningsson et al., 2003). The integration of variations in the starch foods, changing the physicochemical and functional properties of starch (altering the amylose/amylopectin ratio, increasing RS content, etc.), could affect and change physiologic responses which could influence satiety and lead to a different glucose/insulin response. These modifications are usually achieved by physical, chemical and enzymatic modifications (Dupuis et al., 2014).

Concerning nutritional implications, nowadays, an extensive world population is avoiding wheat-based products consumption as a result of gluten association with diverse autoimmune diseases. The design of alternative gluten-free baked foods has brought a deep research in the last

decade (Bonet et al., 2007; Marco and Rosell, 2008; Hüttner and Arendt, 2010; Delcour et al., 2012; Gularte et al., 2012). However, nutritional composition and quality regarding gluten-free products is different from its counterparts and is still outlined from traditional wheat-based carbohydrate matrices. These carbohydrate matrices are mainly composed by starch, though less research has been brought concerning the physiological role of the starch in the development of carbohydrate matrix products with quality, shelf life and health benefits. Corn starch, as gluten-free cereal starch and one of the predominant starches produced, becomes suitable to produce foods addressed to celiac patient and attractive for scientific interest. Even more, enzymes have been extensively used as technological aids in cereal-based products (Rosell and Collar, 2008) and in improving gluten-free breads characteristics (Rosell, 2009; Rosell, 2014). Nevertheless, a new approach of the enzymes to change functional and physiological behavior of starches is still under research. Therefore, a better understanding of this modification on corn starch would provide basis for developing new products with enhanced properties.

References

Ai Y., Jane JL. (2015). Gelatinization and rheological properties of starch. Starch/Stärke. 67: 213-224.

Al-Karawi AJM., Al-Daraji AHR. (2010). Preparation and using of acrylamide grafted starch as polymer drug carrier. Carbohydrate Polymers. 79: 769-774.

Andoh A., Tsujikawa T., Fujiyama Y. (2003). Role of dietary fiber and short-chain fatty acids in the colon. Current Pharmaceutical Design. 9: 347-58.

Annison G., Topping DL. (1994). Nutritional role of resistant starch: Chemical structure vs physiological function. Annual Review of Nutrition. 14: 297-320.

Ao Z., Simsek S., Zhang G., Venkatachalam M., Reuhs BL., Hamaker BR. (2007). Starch with a slow digestion property produced by altering its chain length, branch density, and crystalline structure. Journal of Agricultural Food Chemistry. 55: 4540-4547.

Ashogbon AO., Akintayo ET. (2014). Recent trend in the physical and chemical modification of starches from different botanical sources: A review. Starch/Stärke. 66: 41-57.

Astray G., Gonzalez-Barreiro C., Mejuto JC., Simal-Gandara J. (2009). A review on the use of cyclodextrins in foods. Food Hydrocolloids. 23: 1631-1640.

Bahnassey YA., Breene WM. (1994). Rapid viscoanalyzer (RVA) pasting profiles of wheat, corn, waxy corn, tapioca, and amaranth starches (*A. hypochondriacus* and *A. cruentus*) in the presence of konjac flour, gellan, guar, xanthan, and locust bean gums. Starch/Stärke. 48: 134-141.

Becerra V., Odermatt J. (2014). Direct determination of cationic starches in paper samples using analytical pyrolysis. Journal of Analytical and Applied Pyrolysis. 105: 348-354.

Biwer A., Antranikian G., Heinzle E. (2002). Enzymatic production of cyclodextrins. Application of Microbiology Biotechnology. 59: 609-617.

Blanchard PH., Kate FR. (1995). Starch hydrolysates. In: Food polysaccharides and their applications. Stephen AM. (ed). NY: Marcel Dekker, Inc. 99-122.

Blennow A., Engelsen SB., Nielsen TH., Baunsgaard L., Mikkelsen R. (2002). Starch phosphorylation: A new front in starch research. Trends in Plant Science. 7: 445-450.

Bonet A., Rosell CM., Perez-Munuera I., Hernando I. (2007). Rebuilding gluten network of damage wheat by means of glucose oxidase treatment. Journal of the Science of Food and Agriculture. 87: 1301-1307.

Buchholz K., Seibel J. (2008). Industrial carbohydrate biotransformations. Carbohydrate Research. 343: 1966-1979.

Buleon A., Colonna P., Planchot V., Ball S. (1998). Starch granules: Structure and biosynthesis. International Journal of Biological Macromolecules. 23: 85-112.

Colonna P., Lelou V., Buleon A. (1992). Limiting factors of starch hydrolysis. European Journal of Clinical Nutrition. 46: 17-32.

Crabb WD., Shetty JK. (1999). Commodity scale production of sugars from starches. Current Opinion in Microbiology. 2: 252-256.

Daudt RM., Külkamp-Guerreiro IC., Cladera-Olivera F., Thys RCS., Marczak LDF. (2014). Determination of properties of pinhão starch: Analysis of its applicability as pharmaceutical excipient. Industrial Crops and Products. 52: 420-429.

Delcour JA., Joye IJ., Pareyt B., Wilderjans E., Brijs K., Lagrain B. (2012). Wheat gluten functionality as quality determinant in cereal-based food products. Annual Revieiws in Food Science Technology. 3: 469-492.

D'Ercole AD. Process of preparing frozen pudding composition. (1972). U.S. Patent 3, 669,687.

Dhall RK. (2013). Advances in edible coatings for fresh fruits and vegetables: A review. Critical Reviews in Food Science and Nutrition. 53: 435-450.

Doade WM., Maiti S., Wing RE. (1990). Encapsulation by entrapment within matrix of unmodified starch. U.S. Patent, 5670490.

Dupuis JH., Liu Q., Yada RY. (2014). Methodologies for increasing the resistant starch content of food starches. Comprehensive Reviews in Food Science and Food Safety. 3: 1219-1234.

Ellis RP., Cochrane MP., Dale MFB., Duffus CM., Lynn A., Morrison IM., Prentice RDM., Swanston JS., Tiller SA. (1998). Starch production and industrial use. Journal of the Science of Food and Agriculture. 77: 289-311.

Englyst HN., Kingman SM., Cummings JH. (1992). Classification and measurement of nutritionally important starch fractions. European Journal of Clinical Nutrition. 46:33-50.

FAO, June 2016. Food and Agriculture Organization of the United Nations. Food outlook. Biannual report on global food market. ISSN: 0251-1959.

Fannon JE., Hauber RJ., BeMiller JN. (1992). Surface pores of starch granules. Cereal Chemistry. 69: 284-288.

Ferguson LR., Tasman-Jones C., Englyst H., Harris PJ. (2000). Comparative effects of three resistant starch preparations on transit time and short-chain fatty acid production in rats. Nutrition and Cancer. 36: 230-7.

Fuentes-Zaragoza E., Riquelme-Navarrete MJ., Sánchez-Zapata E., Pérez-Álvarez JA. (2010). Resistant starch as functional ingredient: A review. Food Research International. 43: 931-942.

Gularte MA., Rosell CM. (2011). Physicochemical properties and enzymatic hydrolysis of different starches in the presence of hydrocolloids. Carbohydrate Polymers. 85: 237-244.

Gularte MA., de la Hera E., Gomez M., Rosell CM. (2012). Effect of different fibers on batter and gluten-free layer cake properties. LWT-Food Science and Technology. 48: 209-214.

Gujral HS., Rosell CM. (2004). Functionality of rice flour modified with a microbial transglutaminase. Journal of Science Food Agriculture. 84: 1685-1690.

Han XZ., Benmoussa M., Gray JA., BeMiller JN., Hamaker BR. (2005). Detection of proteins in starch granule channels. Cereal Chemistry. 82: 351-355.

Haralampu SG. (2000). Resistant starch-A review of the physical properties and biological impact of RS3. Carbohydrate Polymers. 41. 285-292.

Harper JM. (1989). Food extruders and their applications. In: Extrusion Cooking. Mercier C., and Harper JM. (eds). St. Paul, MN: American Association of Cereal Chemists, Inc. 1-15.

Han XZ., Ao Z., Janaswam S., Jane JL., Chandrasekaran R., Hamaker BR.(2006). Development of a low glycemic corn starch: Preparation and characterization. Biomacromolecules. 7: 1162-1168.

Henningsson AM., Margareta E., Nyman GL., Björck IM. (2003) Influences of dietary adaptation and source of resistant starch in the hindgut of rats. British Journal of Nutrition. 89: 319-28.

Herrero AM., Carmona P., Jiménez-Colmenero F., Ruiz-Capillas C. (2014). Polysaccharide gels as oil bulking agents: Technological and structural properties. Food Hydrocolloids. 36: 374-381.

Homayouni A., Azizi A., Ehsani MR., Yarmand MS., Razavi SH. (2008). Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream. Food Chemistry. 111: 50-55.

Homayouni A., Amimi A., Keshtiban AK., Mortazavian AM., Esazadeh K., Pourmoradian S. (2014). Resistant starch in food industry: A changing outlook for consumer and producer. Starch/Stärke. 66: 102-114.

Hoover R., Manuel H. (1996). The effect of heat-moisture treatment on the structure and physicochemical properties of normal corn, waxy corn, dull waxy corn and amylocorn V starches. Journal of Cereal Science. 23: 153-162.

Hoover R. (2001). Composition, molecular structure, and physicochemical properties of tuber and root starches: A review. Carbohydrate Polymers. 45: 253-567.

Hunt A., Getty KJK., Park JW. (2009). Roles of starch in surimi seafood: A review. Food Reviews International. 25: 299-312.

Hüttner EK., Arendt EK. (2010). Recent advances in gluten-free baking and the current status of oats. Trends in Food Science and Technology. 21: 303-312.

Jacobsen H., Poulsen M., Dragsted LO., Ravn-Haren G., Meyer O., Lindecrona RH. (2006). Carbohydrate digestibility predicts colon carcinogenesis in azoxymethane-treated rats. Nutrition and Cancer-An International Journal. 55: 63-170.

Jane J., Kasemsuwan T., Chen JF., Juliano BO. (1996). Phosphorus in rice and other starches. Cereal Foods World. 41: 827-832.

Jacobs H., Delcour JA. (1998). Hydrothermal modifications of granular starch, with retention of the granular structure: A review. Journal of Agricultural and Food Chemistry. 46: 2895-2905.

Jiménez A., Fabra MJ., Talens P., Chiralt A. (2012). Edible and biodegradable starch films: A review. Food and Bioprocess Technology. 5: 2058-2076.

Kaur B., Ariffin F., Bhat R., Karim AA. (2012). Progress in starch modification in the last decade. Food Hydrocolloids. 26: 398-404.

Kittipongpatana OS., Chaitep W., Kittipongpatana N., Laenger R., Sriroth K. (2007). Physicochemical and pharmaceutical properties of carboxymethyl rice starches modified from native starches with different amylose content. Cereal Chemistry. 84:331-336.

Le Corre D., Bras J., Dufresne A. (2010). Starch nanoparticles: A review. Biomacromolecules. 11: 1139-1153.

Li JH., Vasanthana T., Hoover R., Rossnagel BG. (2004). Starch from hull-less barley: V. In-vitro susceptibility of waxy, normal, and high-amylose starches towards hydrolysis by alpha-amylases and amyloglucosidase. Food Chemistry. 84: 621-632.

Li M., Liu G-L., Chi Z., Chi Z-M. (2010). Single cell oil production from hydrolysate of cassava starch by marine-derived yeast *Rhodotorula mucilaginosa* TJY15a. Biomass and Bioenergy. 34: 101-107.

Li YQ., Chen Q., Liu XH., Chen ZX. (2008). Inactivation of soybean lipoxygenase in soymilk by pulsed electric fields. Food Chemistry. 109: 408-414.

Lorenz K., Kulp K. (1981). Heat-moisture treatment of starches. II. Functional properties and baking potential. Cereal Chemistry. 58: 49-52.

Ludwig DS. (2002). The glycemic index: physiological mechanisms relating to obesity, diabetes, and cardiovascular disease. Journal of the American Medical Association. 287: 2414-2423.

Majzoobi M., Radi M., Farahnaky A., Jamalian J., Tongdang T., Mesbahi G. (2011). Physicochemical properties of pre-gelatinized wheat starch produced by a twin drum drier. Journal of Agricultural Science and Technology. 13: 193-202.

Mann J., Cummings JH., Englyst HN., Key T., Liu S., Riccardi G., Summerbell C., Uauy R., van Dam RM., Venn B., Vorster HH., Wiseman M. (2007). FAO/WHO Scientific Update on carbohydrates in human nutrition: Conclusions. European Journal of Clinical Nutrition. 61: 132-137.

Marco C., Rosell CM. (2008). Breadmaking performance of protein enriched, gluten-free breads. European Food Research and Technology. 227: 1205-1213.

Martin del Valle EM. (2004). Cyclodextrins and their uses: A review. Process Biochemistry. 39: 1033-1046.

Massicotte LP., Baille WE., Mateescu MA. (2008). Carboxylated high amylose starch as pharmaceutical excipients: Structural insights and formulation of pancreatic enzymes. International Journal of Pharmaceutics. 356: 212-223.

Mitan FJ., Jokay L. Process of Coating Food. (1969). U.S. Patent 3, 427,951.

Morrison WR., Milligan TP., Azudin MN. (1984). A relationship between the amylose and lipid contents of starches from diploid cereals. Journal of Cereal Science. 2: 257-271.

Morrison WR. (1988). Lipids in cereal starches: A review. Journal of Cereal Science. 8: 1-15.

Morrison WR. (1995). Starch lipids and how they relate to starch granule structure and functionality. Cereal Foods World. 40: 437-446.

Mun S., Kim YL., Kang CG., Park KH., Shim JY., Kim YR. (2009). Development of reduced-fat mayonnaise using 4-α-GTase-modified rice starch and xanthan gum. Biological Macromolecules. 44: 400-407.

Nazare JA., de Rougemont A., Normand S., Sauvinet V., Sothier M., Vinoy S., Desage M., Laville M. (2010). Effect of postprandial modulation of glucose availability: Short- and long-term analysis. British Journal of Nutrition. 103: 1461-1470.

Letelier-Gordo CO., Holdt SL., De Francisci D., Karakashev DB., Angelidaki I. (2014). Effective harvesting of the microalgae *Chlorella protothecoides* via bioflocculation with cationic starch. Bioresource Technology. 167: 214-218.

Lim S., Seib PA. (1993). Preparation and pasting properties of wheat and corn starch phosphates. Cereal Chemistry. 70: 137-144.

Lindeboom N., Chang PR., Tyler RT. (2004). Analytical, biochemical and physicochemical aspects of starch granule size, with emphasis on small granule starches: A review. Starch/Stärke. 56: 89-99.

Park SJ., Choi MK., Kim JS., Lim HT., Kang MH. (2008). The effect of dietary modified potato starch by chemically denatured treatment and potato starch on the weight loss, lipid metabolism and redox antioxidant system in high fat diet-induced obese rats. Journal of the Korean Society of Food Science and Nutrition. 37: 1251-1257.

Paulus P., Trzasko P., Waring S., Trksak R., Dihel D. (2005). Modified starches for use in gluten-free baked products. U.S. Patent, 20050008761 A1.

Perera A., Meda V., Tyler RT. (2010). Resistant starch: A review of analytical protocols for determining resistant starch and of factors affecting the resistant starch content of foods. Food Research International. 43:1959-1974.

Poomipuk N., Reungsang A., Plangklang P. (2014). Poly-β-hydroxyalkanoates production from cassava starch hydrolysate by *Cupriavidus sp*. KKU38. International Journal of Biological Macromolecules. 65: 51-64.

Rosell CM., Collar C. (2008). Effect of various enzymes on dough rheology and bread quality. In: Recent Research Developments in Food Biotechnology. Enzymes as Additives or Processing Aids. Porta R., P. Di Pierro P., Mariniello L (eds). Research Signpost, Kerala, India. 165-183.

Rosell CM. (2009). Enzymatic Manipulation of Gluten-Free Breads. In: Gluten-Free Food Science and Technology. Gallagher E. (ed). Wiley-Blackwell. 83-98.

Rosell CM. (2014). Enzymes in gluten-free bread. AACC International Annual Meeting 2014. Providence, Rhode Island, US.

Sajilata MG., Singhal RS. (2004). Specialty starches for snack foods. Carbohydrate Polymers. 59: 131-151.

Sandhu KS., Singh N. (2007). Some properties of corn starches II: Physicochemical, gelatinization, retrogradation, pasting and gel textural properties. Food Chemistry. 101: 1499-1507.

Sasaki T., Yasui T., Matsuki J. (1999). Effect of amylose content on gelatinization, retrogradation, and pasting properties of starches from waxy and nonwaxy wheat and their F1 seeds. Cereal Chemistry. 77: 58-63.

Schirmer M., Jekle M., Becker T. (2015). Starch gelatinization and its complexity for analysis. Starch/Stärke. 67: 30-41.

Shahidul I., Shahid M., Mohammad F. (2013). Perspectives for natural product based agents derived from industrial plants in textile applications-A review. Journal of Cleaner Production. 57: 2-18.

Singh N., Singh J., Kaur L., Sodhi NS., Gill BS. (2003). Morphological, thermal and rheological properties of starches from different botanical sources. Food Chemistry. 81: 219-231.

Singh J., Kaur L., McCarthy OJ. (2007). Factors influencing the physico-chemical, morphological, thermal and rheological properties of some chemically modified starches for food applications-A review. Food Hydrocolloids. 21: 1-22.

Singh J., Dartois A., Kaur L. (2010). Starch digestibility in food matrix. Trends in Food Science and Technology. 21: 168-180.

Srichuwong S., Sunarti TC., Mishima T, Isono N., Hisamatsu M. (2005a). Starches from different botanical sources I: Contribution of amylopectin fine structure to thermal properties and enzyme digestibility. Carbohydrate Polymers. 60: 529-538.

Srichuwong S., Sunarti TC., Mishima T, Isono N., Hisamatsu M. (2005b). Starches from different botanical sources II: Contribution of starch structure to swelling and pasting properties. Carbohydrate Polymers. 62: 25-34.

Sun Q., Zhu X., Si F., Xiong L. (2015). Effect of acid hydrolysis combined with heat moisture treatment on structure and physicochemical properties of corn starch. Journal of Food Science Technology. 52: 375-382.

Swinkels JJM. (1985). Sources of starch, its chemistry and physics. In: Starch conversion technology. van Beynum GMA., Roe1s JA (eds). Marcel Dekker Inc., New York. 15-46.

Szymonska J., Krok F., Tomasik P. (2000). Deep-freezing of potato starch. International Journal of Biological Macromolecules. 27: 307-314.

Szymonska J., Krok F., Komorowska-Czepirska E., Rebilas K. (2003). Modification of granular potato starch by multiple deep-freezing and thawing. Carbohydrate Polymers. 52: 1-10.

Tester RF., Karkalas J., Qi X. (2004). Starch-composition, fine structure and architecture. Journal of Cereal Science. 39: 151-165.

Thomas DJ., Atwell WA. (1999). Starch modifications. In: Starch Chemistry and Technology. Thomas DJ., Atwell WA. (eds). St. Paul, MN: American Association of Cereal Chemists. 43-48.

Thompson DB. (2000). Starches for the manufacture of resistant starch. Trends in Food Science and Technologies. 11: 245-253.

Vallons KJR., Arendt EK. (2009). Effects of high pressure and temperature on buckwheat starch characteristics. European Food Research and Technology. 230: 343-351.

van der Maarel M., van der Veen B., Uitdehaag JCM., Leemhuis H., Dijkhuizen L. (2002). Properties and applications of starch-converting enzymes of the α-amylase family. Journal of Biotechnology. 94: 137-155.

van der Maarel M., Leemhuis H. (2013). Starch modification with microbial alpha-glucanotransferase enzymes. Carbohydrate Polymers. 93: 116-121.

Vasanthan T., Hoover R. (1992a). A comparative study of the composition of lipids associated with starch granules from various botanical sources. Food Chemistry. 43: 19-27.

Vasanthan T., Hoover R. (1992b). Effect of defatting on starch, starch structure and physicochemical properties. Food Chemistry. 45: 337-347.

Vermeylen R., Goderis B., Reynaers H., Delcour JA. (2004). Amylopectin molecular structure reflected in macromolecular organization of granular starch. Biomacromolecules. 5: 1775-1786.

Vorwerg W., Radosta S., Leibnitz E. Study of a preparative-scale process for the production of amylose. Carbohydrate Polymers. 47: 181-189.

Wang S., Li C., Copeland L., Niu Q., Wang S. (2015). Starch retrogradation: A comprehensive review. Comprehensive Reviews in Food Science and Food Safety.14: 568-585.

Wang X., Yuan Y., Yue T. (2015). The application of starch-based ingredients in flavor encapsulation. Starch/Stärke. 67: 225-236.

Waterschoot J., Gomand SV., Fierens E., Delcour JA. (2015). Production, structure, physicochemical and functional properties of corn, cassava, wheat, potato and rice starches. Starch/Stärke. 67: 14-29.

Yokoyama WH., Knuckles BE., Wood DF., Inglet GE. (2002). Food processing reduces size of soluble cereal β-Glucan polymers without loss of cholesterol-reducing properties. American Chemical Society Symposium. 8:105-116.

Yokoyama WH., Shao Q. (2006). Soluble fibers prevent insulin resistance in hamsters fed high saturated fat diets. Cereal Foods World. 51:16-18.

Yoo SH., Perera C., Shen J., Ye L., Suh DS., Jane JL. (2009). Molecular structure of selected tuber and root starches and effect of amylopectin structure on their physical properties. Journal of Agriculture and Food Chemistry. 57: 1556-1564.

Yusuph M., Tester RF., Ansell R., Snape CE. (2003). Composition and properties of starches extracted from tubers of different potato varieties grown under the same environmental conditions. Food Chemistry. 82: 283-289.

Zavareze E., Dias A. (2011). Impact of heat-moisture treatment and annealing in starches: A review. Carbohydrate Polymers. 83: 317-328.

Zhang C., Xu D., Zhu Z. (2014). Octenylsuccinylation of cornstarch to improve its sizing properties for polyester/cotton blend spun yarns. Fibers and Polymers. 15: 2319-2328.

Zhekova B., Dobrev G., Stanchev V., Pishtiyski I. (2009). Approaches for yield increase of β-cyclodextrin formed by cyclodextrin glucanotransferase from *Bacillus megaterium*. World Journal of Microbiology and Biotechnology. 25: 1043-1049.

II. Objectives

The main objective of this research was to modulate functional and nutritive value of corn starch through enzymatic strategies for its further effective inclusion in a healthy diet.

In order to achieve this main objective, the following specific goals were proposed to:

1. Provide an updated revision of the current knowledge about cereal constituents involved in bread-making, and classification of the enzymes mostly used in baking, according to the substrate they are acting on, understanding the effect of these enzymes to improve the process.

2. Study the individual effect of fungal α-amylase (AM) or amyloglucosidase (AMG) on corn starch at sub-gelatinization temperature and the subsequent impact of the catalytic activity in starch digestibility, functionality and structural properties.

3. Appraise the resultant corn starch from the enzymatic modification of corn starch granules with cyclodextrin glycosyltransferase (CGTase) under sub-gelatinization conditions, in relation to the biochemical features, thermal and structural changes induced by the enzymatic action.

4. Evaluate possible health benefits of corn starch enzymatically modified by cyclodextrin glycosyltransferase (CGTase) containing hydrolysis products (cyclodextrins), specifically focused on the modulation of the glycemic response. In addition, to investigate the relationship between physical properties of modified corn starch and its *in vivo* glycemic effect in mice.

III. Results and discussion

Chapter 1

Enzymes in bakeries

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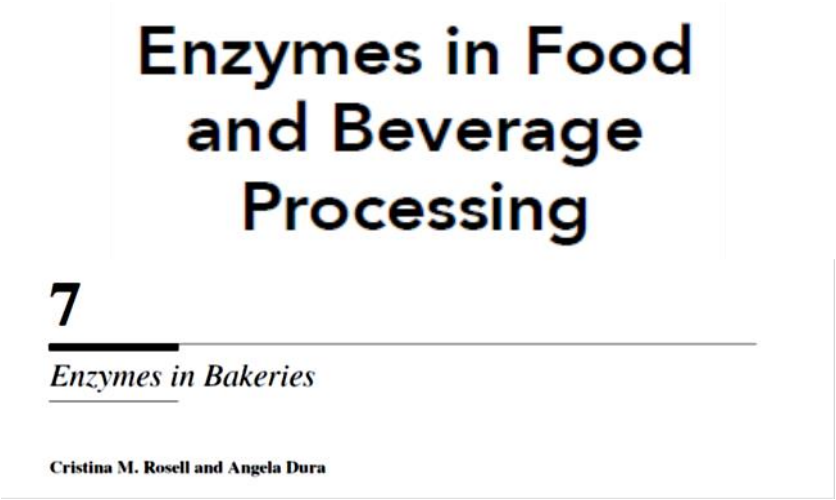
(2015). In: Enzymes in food beverages and processing. Chandrasekaran M. (ed). CRC Press. 171-195.



1. Introduction

Baking industries provide a whole range of bakery products, of which a common characteristic is that they are mainly fermented, cereal-based products subjected to high-temperature processes for increasing their organoleptic properties and shelf life. The main ingredients in baked goods are flours from cereals such as wheat, corn, and sorghum. Other major cereal crops produced include rice and barley, oats, millet, and rye. In general, all flours contain valuable amounts of energy, protein, iron, and vitamins, but the degree of milling will influence the final nutritional content. Cereals still remain important to human nutrition because they lead to nutrient-dense baked goods, which have a worldwide important contribution in the daily intake of macro- and micronutrients. In addition, the consumption of cereal baked foods produces feelings of satiety, and their regular consumption with main meals appears to be a key driver of healthier dietary patterns (Aisbitt et al., 2008).

The almost ubiquitous consumption of baked goods all over the world confers those products a prominent position in international nutrition. Baked products account for a great part of our daily diet through the consumption of bread, breakfast cereals, cookies, snacks, cereal bars, cakes, and so on (Figure 1).

Many different types of bread formulations and many types of bakery goods have been developed so far due to increased awareness on the part of consumers as to the value of eating complex carbohydrates. In addition, bread is handy and very convenient for on-the-go consumers, available in any place and all year around, and affordable, and from a nutritional point of view, it is a source of energy in the form of starch in addition to the supply of dietary fiber and a range of vitamins and minerals. Bread has changed in many ways since the days of our ancestors, going from grainy flat bread to having an

aerated texture. Nevertheless, many types of so-called bread are detected around the world (Rosell and Garzon, 2014).

Figure 1. Different baked products

Sliced pan bread is normally eaten in the United States, but Europe's consumers prefer crispy breads with crusty surface, such as a French baguette. The opposite sensory characteristics can be found in the steamed bread that is consumed in Asian countries. In India, flat bread called chapatti is consumed with the main meals whereas flat bread in Mexico is made of corn, and is named tortilla. In Finland and Germany, a very dark rye bread made of 100% rye flour is the most common bread. In Venezuela, arepas are considered a staple part of the diet, and they are eaten for breakfast, as a snack, or together with a meal. In Brazil, pao de queijo, small, round, cheese-stuffed bread balls, are traditionally served for breakfast. It seems that the term "bread" comprises thousands of different types of breads around the world, and there is a large amount of diversity within each country.

Worldwide bread consumption accounts for one of the largest consumed foodstuff with average consumption ranging from 41 to 303 kg/year per capita, and it is an essential part of the human diet, enjoyed at various times of the day. The most popular kind of bread is white bread made from white wheat flour, which attained great prominence up to the 1960s, but its consumption underwent a steady decrease. On the other hand, a variety of bread products have picked up a market share because they have a superior taste and desirable sensory and nutritional qualities; they include multigrain bread, high fiber bread, cracked wheat bread, sourdough bread, milk bread, composite flour bread, high protein bread, wheat germ bread, and gluten-free bread. Increasing consumer interest in health has had an effect on the types and varieties of breads available, such as whole-grain and multigrain breads. Particularly in Europe, more than 250 types of variety of breads are available, and more than 1000 types of biscuits compete for space on grocer's shelves in Western countries.

In the past, the general market image of baked products and some media negativity impacted the prospects for any growth in the consumption of cereal-based foods related to the prevalence of diets that stress the avoidance of carbohydrate-rich foods. Currently, the global baked goods market has shown rapid recovery following the economic recession, recording strong growth in recent years. Factors fueling market expansion include convenience, affordability, and the health benefits of baked goods products. Interestingly, the rise in cereal-based product consumption that started was also triggered by health concerns. Changing consumer habits have had an important impact on the bread industry. Nowadays, the commercial bakery industry has experienced some recent consolidation and is currently undergoing significant changes in how products are marketed and distributed.

One of the most extensive areas of ongoing research within the industry involves investigating methods of extending shelf life and preserving product freshness.

Bakeries are very active industries in launching innovative foods regarding processing, recipes, shapes, and so on. In this picture, enzymes have played an important role as processing or technological aids. Baking enzymes have become an essential part of the industry throughout the ages. Enzyme supplements have been largely used in baking to make consistently high-quality products by enabling better dough handling, providing antistaling properties, and allowing control over crumb texture and color, taste, moisture, and volume. Lately, a growing interest has been focused on enzymes owing to their generally recognized as safe (GRAS) notation because industries are facing changes due to the greater demand of chemical replacement. Enzymes with some contribution in bakery products can proceed from enzymes naturally present in flour, those associated with the metabolic activity of the dominant microorganism, or those intentionally added during the mixing step as technological or processing aids. The supplementation of flour and dough with enzyme improvers is a usual practice for flour standardization and also as baking aids. Over the years, enzymes gained importance in the bakery industry for their efficiency to modify dough rheology, gas retention, and crumb softness in bread manufacturing; to modify dough rheology in the manufacture of pastry and biscuits; to change product softness in cake making; and to reduce acrylamide formation in bakery products (Rosell and Collar, 2008). The enzymes can be added individually or in complex mixtures, which may act in a synergistic, additive, or antagonistic way in the production of baked goods, and their levels are usually very low. There are many enzymes used to alter major and minor biomolecule structures and to achieve desired functionality (Rosell and Collar, 2008). In baking, the most commonly used enzymes classes are hydrolases, such as amylases, proteases, hemicellulases, and lipases, and oxidoreductases, which comprise, among others, glucose oxidase and lipoxygenase. The enzymes most frequently used in bread making can be classified depending on the substrate on which they are acting. Particularly in bakeries, cereals are the raw commodity, and thus, when deciding on the

inclusion of enzymes as processing aids, it is fundamental to know the cereal constituents that will be present and, consequently, ready to be used as enzyme substrates.

2. Enzymes in baking industries

There are many enzymes used as processing aids to alter major (starch, nonstarch, and proteins) and minor (lipids) cereal structures and to achieve desired functionality (Rosell and Collar, 2008). In baking, the most commonly used enzyme classes fall within the category of oxidoreductases (i.e., glucose oxidase and lipoxygenase) and hydrolases, such as amylases, proteases, hemicellulases, and lipases. Nevertheless, due to the great number of enzymes used in bread making, the next sections will refer to enzymes grouped according to the substrate on which they are acting. Cereal grains contain 66%-76% carbohydrates; thus, this is by far the most abundant group of constituents. The major carbohydrate is starch (55%-70%) followed by minor constituents, such as arabinoxylans (1.5%-8%), β-glucans (0.5%-7%), sugars (~3%), cellulose (~2.5%), and glucofructans (~1%). The second important group of constituents is proteins, which fall within an average range of approximately 8%-11%. With the exception of oats (~7%), cereal lipids belong to the minor constituents (2%-4%) along with minerals (1%-3%) (Rosell, 2012).

Considering the importance of the cereal constituents to understanding the role of enzymes in baking, a description of the chemical structure and main characteristics of carbohydrates, proteins, and lipids is included in the following sections in addition to the description of the enzyme actions (Table 1).

Table 1. Enzymes used in bakery industries (EC numbers and recommended names of baking enzymes according to IUBMB)

EC1	**Oxidoreductases**	(EC 1.1) Acting on the CH-OH group of donors	(EC 1.1.3) With oxygen as acceptor	Glucose oxidase (EC 1.1.3.4)
		(EC 1.10) Acting on diphenols and related substances as donors	(EC 1.10.3) With oxygen as acceptor	Laccase (EC 1.10.3.1)
		(EC 1.13) Acting on single donors with incorporation of molecular oxygen (oxygenases)	Lipoxygenase (EC 1.13.11)	
EC 2	**Transferases**	(EC 2.3) Acyltransferases	(EC 2.3.2) Aminoacyltransferases	Transglutaminase (EC 2.3.2.13)
		(EC 2.4) Glycosyltransferases	(EC 2.4.1) Hexosyltransferases	CGTase (EC 2.4.1.19)
				Amylomaltase (EC 2.4.1.25)
EC 3	**Hydrolases**	(EC 3.1) Acting on ester bonds	(EC 3.1.1) Carboxylic Ester Hydrolases	Lipases (EC 3.1.1.3)
				Phospholipase A (EC 3.1.1.4)
				Hemicellulase EC (3.1.1.73)
			(EC 3.1.3) Phosphoric Monoester Hydrolases	Phytase (EC 3.1.3.26)

	(EC 3.2) Glycosidases	(EC 3.2.1) Enzymes hydrolysing O- and S-glycosyl compounds		α-amylase (EC 3.2.1.1)
				β-amylase (EC 3.2.1.2)
				Glucoamylase (EC 3.2.1.3)
				Xylanase (EC 3.2.1.8)
				Pullulanase (EC 3.2.1.41)
				Isoamylase (EC 3.2.1.68)
	(EC 3.4) Proteases	Acting on peptide bonds: Endopeptidases/Exopeptidases		
	(EC 3.5) Acting on carbon-nitrogen bonds, other than peptide bonds	(EC 3.5.1) In Linear Amides		Asparaginase (EC 3.5.1.1)

Source: http://www.chem.qmul.ac.uk/iubmb/.

2.1. Starch-degrading enzymes

These enzymes are the most extended and commonly used as processing aids in the bakery industry (Table 1). The starch-converting enzymes include α-amylases (EC. 3.2.1.1), β-amylases (EC. 3.2.1.2), and glucoamylases (EC. 3.2.1.3). Some other enzymes involved in starch degrading but not commonly used in bread making are the debranching enzymes isoamylase (EC. 3.2.1.68) and pullanase (EC. 3.2.1.41) and the transferases amylomaltase (EC. 2.4.1.25) and cyclodextrin glycosyltransferase (CGTase) (EC. 2.4.1.19).

2.1.1. Starch

Starch is polymers of glucose linked to one another through the C1 oxygen, known as the glycosidic bond. It is the most important reserve polysaccharide and the most abundant constituent of many plants, including cereals. Two types of glucose polymers are present in starch: amylose and amylopectin. These two glucan polymers are organized into a complex, semicrystalline granular structure with a particle size ranging from 1 to 100 μm in diameter. Amylose and amylopectin have different structures and properties. Amylose is an essentially linear α-1,4-linked molecule containing very few branch points. There are usually between 1500 and 6000 glucose units in a single amylose molecule. Linear glucose chains, much like protein chains, tend to form helices. An important characteristic of amylose is its ability to form helical inclusion complexes with a number of substances-in particular polar lipids-and this can occur in native as well as in gelatinized starch. In contrast, amylopectin is a very large, highly branched polysaccharide of up to 3 million glucose units, consisting of short α-1,4-linked linear chains of 10-60 glucose units and α,1-6-linked side chains with 15-45 glucose units. The average number of branching points in amylopectin is 5%; the ratio of the two polysaccharides varies according to the botanical origin of the starch with typical levels of amylose and amylopectin of 20%-30% and 70%-80%, respectively (van der Maarel et al., 2002). Although an amylopectin molecule

has many nonreducing ends, it has only one reducing end. The "waxy" starches contain less than 15% amylose, "normal" contains 20%-35%, and "high" contains (amylo-) amylose starches greater than approximately 40% (Tester et al., 2004).

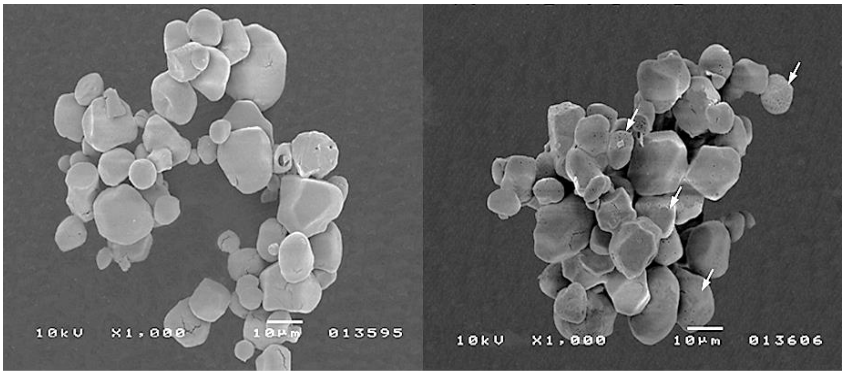
The amylose and amylopectin in starch form a semicrystalline aggregate organized in granules. The size, shape, and morphology of these granules are dependent on the botanical origin. These granules are approximately 30% crystalline. The amylopectin molecules within the granule provide the crystallinity whereas the amylose is present in an amorphous form. The degree of crystallinity ranges from 20% to 40% and is primarily caused by the structural features of amylopectin. It is thought that the macromolecules are oriented perpendicularly to the granule surface (Buleon et al., 1998) with the nonreducing ends of the molecules pointing to the surface. It has long been recognized that the functional properties of starch depend on a number of integrated factors, which include polymer composition, molecular structure, interchain organization, and minor constituents, such as lipids, phosphate ester groups (typical of potato amylopectin), and proteins. Starch is a major component of bread making and plays an important role in the texture and quality of the dough and bread. Heat treatment during the baking process causes structural and often molecular changes in the granular and polymeric structures of starch. Functional properties of starch are directly influenced by hydrothermal treatment or processing conditions. During the gelatinization process, the semicrystalline nature of their structure is reduced or eliminated, and the granules break down; starch granules swell, and the molecular order of the granule is gradually and irreversibly destroyed (and thus the birefringence); a (limited) leaching of the polymer molecules, mainly amylose, and a (partial) granule disruption causes the amylose chains to solubilize, and a starch gel is formed, forming a viscous solution. At this point, starch is easily digestible. Gelatinization of granules in excess water causes large changes in the rheological properties of the system and has a

major influence on the behavior and functionality of starch-containing systems.

2.1.2. Amylases

α- and β-Amylases are naturally present in cereal flours, but although β-amylases are always in a sufficient amount, very often flours require α-amylase supplementation. The α-amylases (α-1,4-glucanohydrolase) are endo-enzymes that randomly catalyze the cleavage of α-1,4-glycosidic bonds in the inner part of amylose or amylopectin chains into a series of branched and linear fragments, called dextrins. Only damage or gelatinized starches are susceptible to enzyme hydrolysis, but after long incubation, even intact starch granules can be attacked, leading to perforated structures (Figure 2). These enzymes can be obtained from cereal, fungal, bacterial, and biotechnologically altered bacterial sources (Rosell et al., 2001). Depending upon their origin, they have significant differences in pH, optimum temperature, thermostability, and other chemical stability. In a comparison study, Rosell et al. (2001) showed that α-amylases from cereals (wheat and malted barley) were less sensitive to the presence of ingredients, additives, and metabolites. Fungal amylase (*Aspergillius oryzae*) is the least temperature stable. Bacterial amylase (*Bacillus subtilis*) has high thermal stability; in consequence, some activity might remain in the bread even after baking. Because of that, an α-amylase of intermediate thermostability was isolated and recommended as an antistaling agent in baked goods. Overall, structural changes induced by α-amylase in dough and bread microstructure are dependent on the α-amylase origin (Blaszczak et al., 2004).

β-Amylases are exo-enzymes that generate maltose by breaking every second α-1,4 linkage from the nonreducing ends of amylose, amylopectin, or dextrins. Therefore, β-amylase needs the previous action of α-amylase that breaks starch into smaller pieces or dextrins for β-amylase efficiency.



Control AM

Figure 2. Scanning electron micrographs of corn starch (control pH 6.0) and after treatment with fungal α-amylase (AM) under the same conditions. Arrows indicated the enzymatic action.

Fungal α-amylases are routinely supplemented in bread making to produce small dextrins for the yeast to use during dough fermentation and the early stage of baking. This results in improved bread volume and crumb texture. In addition, the small oligosaccharides and sugars, such as glucose and maltose, produced by these enzymes enhance the reactions for the browning of the crust and baked flavor. Through starch modification, amylases improve moisture retention, have a crumb-softening effect, and decrease staling. Amylase supplementation can occur at the flour mill or at the bakery in dough and sponges. Addition of amylases mainly aims at optimizing the amylase activity of the flour (i.e., flour standardization) and at retarding bread staling.

2.1.3. Glucoamylase

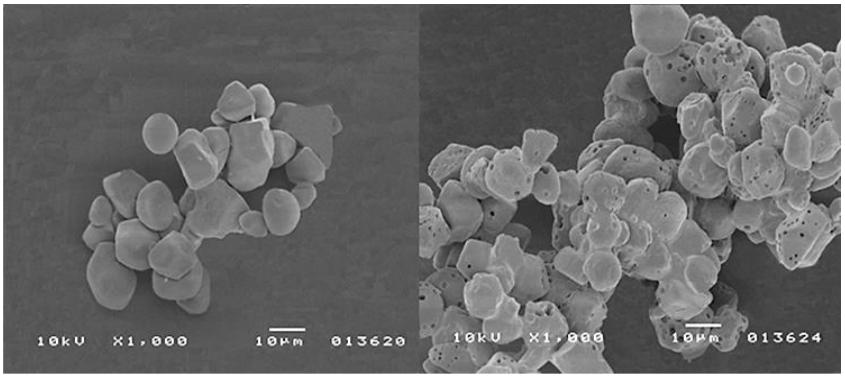
Glucoamylase or amyloglucosidase acts on starch, dextrins, and oligosaccharides by cleaving α-(1,6)-linkages from the nonreducing ends. It is widely used in the manufacture of glucose and for conversion of carbohydrates to fermentable sugars. β-Amylases and glucoamylases have been found in a large variety of microorganisms (Pandey et al., 2000).

Although damaged starch and gelatinized starch are more susceptible to amyloglucosidase attack, Dura et al. (2014) reported the effect of amyloglucosidase on starch at the subgelatinization temperature, revealing that this mechanism might be an alternative to obtaining porous starch. Amyloglucosidase can be more effective than α-amylase for degrading intact or gelatinized starch (Figure 3).

Isoamylase is a debranching amylase that hydrolyzes 1,6-α-D-glycosidic linkages of glycogen, amylopectin, and α- and β-limit dextrins, producing linear malto-oligosaccharides (Rani Ray, 2011). For the same purpose, pullulanases are used to exclusively hydrolyze α,1-6 glycosidic bonds in pullulan (a polysaccharide with a repeating unit of maltotriose), amylopectin, and related oligosaccharides (van der Maarel et al., 2002).

Amylomaltase belongs to the 4-α-glucanotransferase group of the α-amylase family. The enzyme can produce cycloamylose or large-ring cyclodextrin through intramolecular transglycosylation or cyclization reactions of α-1,4-glucan. Amylomaltase and CGTase form a new α,1-4 glycosidic bond while the branching enzyme (EC. 2.4.1.18) forms a new α,1-6 glycosidic bond. CGTase is an enzyme that cleaves α-1,4 glycosidic linkages degrading the starch and produces intramolecular transglycosylation or cyclization reaction. In the process, cyclic and acyclic dextrins are originated, which are oligosaccharides of intermediate size. The cyclic products are cyclodextrins (CDs), namely α-, β-, and γ-CDs consisting of six, seven, and eight glucose monomers in cycles, respectively (Alves-Prado et al., 2008). Cyclodextrin glycosyltransferase catalyzes the conversion of starch and related α,1-4 glucans to cyclodextrins. These molecules have a hydrophilic exterior, which can dissolve in water, and a hydrophobic cavity can form inclusion complexes with a width variety of hydrophobic guest molecules (Astray et al., 2009). In addition to cyclization, the enzyme also catalyzes several intermolecular transglycosylations: coupling (opening of CD rings and transfer of resulting linear malto-oligosaccharides to acceptors),

disproportionation (transfer of linear malto-oligosaccharides to acceptors), and saccharification (hydrolysis of starch). CGTases are found in a wide array of bacteria and archaea living under various environmental conditions, including high temperature (Biwer et al., 2002; Kelly et al., 2009).



Control AMG

Figure 3. Scanning electron micrographs of corn starch (control pH 4.0) and after treatment with amyloglucosidase (AMG).

The effect of CGTase on wheat starch functionality was assessed by Gujral and Rosell, (2004c). When the ability of starch to form a complex with lipids was tested in the presence of CGTase, α-cyclodextrin occurred in the highest concentration followed by β- and γ-cyclodextrins, and the resulting starch exhibited better emulsifying properties owing to the presence of cyclodextrins. In fact, a bakery improver was proposed containing CGTase and, optionally, a starch-debranching enzyme (Rosell and Solis Nadal, 2004).

A bacterial strain designated US132, isolated from Tunisian soil, has shown an ability to produce potent CGTase activity (Jemli et al., 2007) that can be used in bread making because it improves significantly the loaf volume and decreases the firmness of bread during storage.

2.2. Non-starch Polysaccharides Degrading enzymes

2.2.1. Non-starch Polysaccharides (NSP) in cereal flours

Polysaccharides other than starch are primarily constituents of the cell walls and are much more abundant in the outer than in the inner layers of the grains. Therefore, a higher extraction rate is associated with a higher content of NSP. From a nutritional point of view, NSPs are dietary fiber, which has been associated with positive health effects (Goesaert et al., 2008). There are two types of NSP, insoluble and soluble. The insoluble NSP content of most cereals is similar, and the composition of the water-soluble NSP varies. Pentosan refers to a polymer of pentose sugars, comprising xylan, xylobiose, arabinoxylan, and arabinogalactan. The insoluble pentosans are made up of the five-carbon sugars arabinose and xylose. Pentosans present in flour have an important role in bread quality due to their water absorption capability and interaction with gluten, which is vital for the formation of the loaf structure. The arabinoxylans (AX) are the major fraction from nonstarch polysaccharide (85%-90%), which consists of a backbone of β-(1,4)-linked xylose residues, which are substituted with arabinose residues on the C(O)-2 and/or C(O)-3 position (Dornez et al., 2009). They are present in water-extractable (WE-AX) and water-unextractable (WU-AX) forms. The former makes up 25%-30% of total AX in wheat and 15%-25% in rye (Izydorczyk and Biliaderis, 1995). Both WU-AX and WE-AX are polydisperse polysaccharides with one general structure. Endosperm arabinoxylans contain a backbone of 3-1,4-linked o-xylopyranosyl residues, either unsubstituted or substituted at the C(O)-3 and/or the C(O)-2 position with monomeric OL-L arabinofuranose residues. The xylose “backbone” is very insoluble but becomes more soluble as more arabinose is bound. The molecule becomes less soluble as it increases in size (Goesaert et al., 2008). Their structure and aspect result in unique physicochemical properties that strongly determine their functionality in bread making. In particular, WE-AX form highly viscous aqueous solutions because AX are able to absorb 15-20 times more

water than their own weight and, thus, form highly viscous solutions, which may increase the gas-holding capacity of wheat dough via stabilization of the gas bubbles. Numerous studies on the functional role of pentosans in dough development have been performed, studying their effect on bread properties (Ahmad et al., 2014; Butt et al., 2008; Denli and Ercan, 2001; Maeda and Morita, 2003; Oort et al., 1995).

β-Glucans are another type of nonstarchy polysaccharide and are present in a water-extractable and water-unextractable form with one general structure. β-Glucans are composed solely of glucose with β-1,3 and β-1,4 linkages without any branch points. The β-(1,3) linkages interrupt the extended, ribbon-like shape of β-(1,4)-linked glucose molecules, inducing kinks in the chain and making the β-D-glucan chains more flexible, more soluble, and less inert than cellulose (Goesaert et al., 2008). They are also called lichenins and are present particularly in barley (3%-7%) and oats (3.5%-5%). Although present in low concentrations, they can affect the quality of the flour in significant ways because of their ability to bind large amounts of water.

2.2.2. Non-starch hydrolyses

A number of other carbohydrate-degrading enzymes comprise those acting on nonstarch cereal carbohydrates. The use of some enzymes, such as pentosanases (hemicelluloses and xylanases), makes the dough easier to handle, and the resulting bread has a bigger loaf volume and an improved crumb structure (Oort et al., 1995). Pentosanases increase the WE-AX and the ratio of xylose to arabinose due to the enzyme-debranching action on arabinoxylans. The extent of their activity is dependent on the enzyme source; some act during fermentation and others increase their activity during baking (Jimenez and Martinez-Anaya, 2000). For instance, *Bacillus subtilis* endoxylanase has selectivity for WU-AX whereas *Aspergillus aculeatus* endoxylanase has selectivity for WE-AX, and they mainly act during mixing and fermentation, respectively (Courtin et al., 2001). In addition, breads

obtained in the presence of those xylanases confirm that WU-AX is detrimental for bread making whereas WE-AX has a positive impact on bread volume.

Hemicellulases (EC. 3.1.1.73) are a diverse class of enzymes that hydrolyze hemicellulose. Hemicellulose comes in several chemical forms and requires a significantly large number of enzymes and processes to break it down to glucose. In bread making, the hemicellulose reduces the processing time for dough and increases loaf volume without changing the size of the gas cells (Morita et al., 1997). It seems that the presence of hemicellulose slightly increases both gelatinization temperature and enthalpy of starch, but the viscosity coefficient and dough modulus of elasticity decreases.

Xylanase (EC. 3.2.1.8) is a glycosidase that catalyzes the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan and arabinoxylan, yielding arabinoxylo-oligosaccharides. B-D-xylosidases (EC. 3.2.1.37) cleave xylose monomers from the nonreducing end of arabinoxylo-oligosaccharides. The arabinose residues are removed by α-L-arabinofuranosidases (EC. 3.2.1.55), and ferulic acid esterases (EC. 3.1.1.73) cleave ester linkages between arabinose residues and ferulic acid (Collins et al., 2006; Goesaert et al., 2008). Xylanases from families eight and 11 have been found to be effective as baking processing aids (Collins et al., 2006), and within the fungal xylanases, *Aspergillus oryzae* xylanase was the best bread improver and *Trichoderma reesei* xylanase the best antistaling improver (Baskinskiene et al., 2007). In bread making, xylanase increases gluten strength, improves handling and fermentative dough characteristics, and avoids excessive dough hardness and stickiness by interfering with arabinoxylan action on the gluten network (Courtin and Delcour, 2002; Rosell and Collar, 2008), and that effect is even greater in whole wheat bread (Shah et al., 2006). They also play a significant role in increasing the shelf life of bread and reducing bread staling although the extent of this effect depends on their origin (Jiang et al., 2005, 2010). Nevertheless, we must consider the existence of naturally present

endoxylanases inhibitors in flours that are active in the bread making process and can affect the xylanase activity (Trogh et al., 2004).

In rye breads, xylanases make the dough soft and slack and require shorter fermentation times; they induce the fragmentation of cell walls and the release of amylose from the starch granule, and even the release of proteins from aleurone cells is promoted at a high dosage (Autio et al., 1996). These enzymes are also useful for obtaining fiber-enriched breads. Particularly the preliminary treatment of rye bran with hemicelullases or xylanases improves its performance in bread making, reducing the content of total dietary fiber and increasing the amount of soluble pentosans (Laurikainen et al., 1998). Namely, doughs with treated bran are softer and have lower stability, and in general, enzyme mixtures are more effective than individual incorporation of enzymes (Laurikainen et al., 1998).

Endo-glucanases have been also tested in rye breads, resulting in lower proofing times due to their action on the beta-glucans, but they do not influence the content or solubility of the arabinoxylans (Autio et al., 1996).

2.3. Enzymes acting on cereal proteins

The different aspects of gluten functionality can be impacted by different enzymes, such as depolymerizing enzymes (proteases) and enzymes enhancing cross-linking reactions (transglutaminases).

2.3.1. Proteins

The average protein content varies among cereals (8%-15%) although it depends on the genotype (cereal, species, variety), the growing conditions (soil, climate, fertilization), and the time of nitrogen fertilization. A common means of classifying the different proteins of wheat and other grains was devised by Thomas Osborne in the early 1900s. The Osborne classification system (Osborne, 1924) is based on solubility. Albumins, which are water soluble, make up about 15% of the proteins; globulins, which are relatively

minor, make up only about 3% of the total protein and are soluble in salt solutions but insoluble in water; and finally, prolamins and glutenins are soluble in alcohol or acids, respectively. The Osborne fractionation does not provide a clear separation of wheat proteins that differ biochemically or genetically or in functionality during bread making. Nowadays, proteins are preferentially classified from a functional point of view in the nongluten and the gluten proteins (Hui et al., 2006). Gluten proteins (ca. 80%-85% of total wheat protein), the main storage proteins of wheat, are insoluble in water and can be divided into gliadins and glutenins. Gliadin, one of the two major components of the wheat gluten complex, is a prolamin that constitutes about 33% of all the proteins. Those have molecular weights between 30,000 and 80,000 (Veraverbeke and Delcour, 2002) and are single chained and extremely sticky when hydrated. Gliadins are rich in proline and glutamine and have a low level of charged amino acids. Intrachain cystine disulphide bridges are present. The other major component of gluten, glutenins, is insoluble in neutral aqueous solutions, saline solutions, or alcohol. Glutelins make up approximately 55%-70% of the gluten complex and are larger (from 100,000 to several million) than gliadin molecules because of the high number of disulfide bonds connecting the subunits.

Sulfhydryl-disulfide interchanges are the major reactions responsible for the formation of wheat dough. It appears that a specific pattern of interaction between low molecular weight glutenin (<90 KDa) and high molecular weight glutenin (>90 KDa) is important for the development of a viscoelastic gluten (MacRitchie, 1992). The disulfide bonding occurs toward the end of the chains, so in effect, the glutenin molecule is linear. The tertiary structure is thought to be one containing repetitive β-turns, which form a β-spiral structure. This type of structure is stabilized by hydrogen bonding and may explain the elastic nature of glutenin. When stress is applied, this stable conformation is disrupted, but it returns when the stress is absent.

Concerning the amino acid composition of the proteins, glutamine that contains an amide side group constitutes more than 40% of the amino acids comprising these proteins on a molar basis. Another amino acid comprising about 15% of gliadin and 10%-12% of glutenin is proline, which has a cyclic R group structure that puts a bend in a chain of amino acids. Another amino acid of significance is cysteine, which has the ability to form bonds connecting protein chains with their sulfur-containing R group, the so-called disulfide bonds. These three amino acids (glutamine, proline, and cysteine) play a major role in explaining the characteristics of gluten proteins.

2.3.2. Proteases

The reaction catalyzed by proteinases, proteases, or peptidases is not reversible because the peptide bonds and the original rheological condition cannot be restored. Protease from bacteria or fungi can be added to dough to reduce the size of the gluten polymers, making dough easier to mix, but it may compromise the dough's gas-holding ability during fermentation and baking (Goesaert et al., 2005). There are two main types of proteases (EC. 3.4) according to their site of action: exopeptidases and endopeptidases. Exopeptidases cleave the peptide bond proximal to the amino- or carboxy-termini of the substrate whereas endopeptidases cleave peptide bonds distant from the termini of the substrate (Rao et al., 1998). Based on the chemistry of their catalytic mechanism, proteases can be classified as serine, thiol, or cysteine, metallo, and aspartic proteases, which require a hydroxyl group (serine residue), a sulfhydryl group (cysteine residue), a metal ion (e.g., zinc), and a carboxylic function (aspartic acid residue), respectively, at the active site to function properly (Mathewson, 1998). Proteases shorten the flow process time and increase the relaxation rate of the flow processes occurring at long time (Wikstrom and Eliasson, 1998). In bakery products, tyrosinase makes the dough harder and less extendable, which results in breadcrumbs with uneven and large pore size but with a soft texture and increased volume (Selinheimo et al., 2007).

2.3.3. Transglutaminase

Transglutaminase (TGase) is a protein-glutamine γ-glutamyl-transferase (EC. 2.3.2.13), which catalyzes an acyl-transfer reaction between the γ-carboxyamide group of peptide-bound glutamine residues and a variety of primary amines (Motoki and Seguro, 1998). When the ε-amino group of a peptide-bound lysine residue acts as a substrate, the two peptide chains are covalently linked through an ε-(γ-glutamyl)-lysine bond (Folk and Finlayson, 1977). Thus, the enzyme is capable of introducing covalent cross-links between proteins (Nonaka et al., 1989). In the absence of primary amines in the reaction system, water becomes the acyl-acceptor, and the γ-carboxy-amide groups of glutamine residues are deamidated, becoming glutamic acid residues. The enzyme builds up new inter- and intramolecular bonds with the former comprising new covalent nondisulfide cross-links between peptide chains. Transglutaminases for baking applications are usually obtained from microbial origin. Depending on the accessibility of glutamine and lysine residues in the proteins, transglutaminase shows different activity (Gerrard, 2002; Houben et al., 2012).

With regards to cereal proteins, TGase application has shown positive effects on wheat-based baked goods: It reduces the required work input, decreases water absorption of the dough (Gerrard et al., 1998), increases dough stability (Gottmann and Sproessler, 1992), increases volume, improves the structure of breads, and strengthens breadcrumbs (Gerrard et al., 1998) and the baking quality of weak wheat flours (Basman et al., 2002).

TGase action on bread making is readily evident even after 15 min mixing, leading to a decrease in extensibility and an increase in the resistance to stretching due to the stronger protein network (Autio et al., 2005). With TGase supplementation, differences are also observed during proofing when the dough contains more small air bubbles than reference dough, and resulting breads have higher specific volume (Autio et al., 2005).

2.3.4. Asparaginase

Asparaginase (EC. 3.5.1.1) is an enzyme that catalyzes the hydrolysis of the amino acid L-asparagine to L-aspartic acid and ammonia. Free L-asparagine present in food is the main precursor of acrylamide, which is considered to be a probable human carcinogen (World Health Organization, 2006). Acrylamide is formed from L-asparagine and reducing sugars primarily in starchy foods that are baked or fried at temperatures above 120°C. In a fried dough pastry model, Kukurova et al. (2009) confirmed that asparaginase almost completely converted asparagine to aspartic acid, and in consequence, acrylamide formation was reduced up to 90%; this enzyme also converted glutamine in glutamic acid, but neither action affected the browning and Maillard reactions. With that in mind, a enzymatic composition comprising asparaginase and one hydrolyzing enzyme has been reported for decreasing the levels of acrylamide in bakery products such as bread, pastry, cake, pretzels, bagels, Dutch honey cake, cookies, gingerbread, ginger cake, and crispy bread (De Boer and Boer, 2007).

2.4. Lipid enzymes

2.4.1. Lipids

Lipids have great impact on the baking performance despite they are minor constituents of cereals (1%-3% in barley, rice, rye, and wheat; 5%-9% in corn; and 5%-10% in oats on a dry-matter basis). Cereal lipids consist of different chemical structures that vary in their content, depending on the cereal species. Cereal lipids have similar fatty acid compositions in which linoleic acid reaches contents of 39%-69%, and oleic acid and palmitic acid make up 11%-36% and 18%-28%, respectively (Delcour and Hoseney, 2010).

Based on solubility in selective extraction conditions, they are classified as starch lipids and free and bound nonstarch lipids (Hoseney, 1994). Nonstarch

lipids (NSL) comprise about 75% of the total flour lipids and consist predominantly of triglycerides as well as of other nonpolar lipids and digalactosyl diglycerides. The lipids bound to starch (25%) are generally polar. Lysophospholipids, in particular lysophosphatidylcholine (lysolecithin), are the major constituents of the starch lipids. Lipids that are strongly bound in starch granules are essentially unavailable until starch is gelatinized (Van Der Borght et al., 2005). Complexes present in native starch (starch lipids) increase the temperature of gelatinization and, thus, prolong the oven spring. Inclusion complexes between amylose helices and polar lipids are responsible for the antistaling effect. The nonpolar lipids are mainly present in the free NSL fraction, and the glycol and phospholipids are mainly associated with proteins and present in the bound NSL fraction (Hoseney, 1994). In fact, most of the free nonstarch lipids "bind" to gluten during dough mixing (Hoseney, 1994).

Protein-lipid interactions in wheat flour dough also play an important role because both lipids and proteins govern the bread-making quality of flour. Lipids are important components in bread making as they provide a variety of beneficial properties during processing and storage, which reflects their overall diversity. Lipids in bread come from different sources, such as wheat flour lipids, shortenings, and surfactants (Pareyt et al., 2011). Lipids have a positive effect on dough formation and bread volume, namely polar lipids or the free fatty acid component of the nonstarch lipids, whereas nonpolar lipids have been found to have a detrimental effect on bread volume (MacRitchie, 1983).

2.4.2. Lipases

The use of lipases in bread making is quite recent when compared to that of other enzymes. Lipases (EC. 3.1.1.3) possess the unique feature of acting at the interface between an aqueous and a nonaqueous phase, yielding mono- and diglycerides and free fatty acids. In particular, 1,3-specific lipases acting

on 1- and 3-positions improve dough rheological properties and the quality of the baked product when added at 5000 LUS/g, specifically a strengthening effect on the gluten that leads to an increase in dough stability (Jensen and Drube, 1998). Moreover, lipases induce an improvement in breadcrumb structure and freshness retention (Poulson et al., 2006, 2010) in addition to an increase of specific volume when added in combination with vegetable oil (Jensen and Drube, 1998).

Furthermore, a lipase was found to increase expansion of the gluten network, to increase the wall thickness, and to reduce cell density, enhancing volume and crumb structure of high-fiber white bread (Stojceska and Ainsworth, 2008).

Lipase was primarily used to enhance the flavor content of bakery products by liberating short-chain fatty acids through esterification. Along with flavor enhancement, it also prolongs the shelf life of most bakery products. Texture and softness could be improved by lipase as a dough and bread improver, which reduces crumb pore diameter, increases crumb homogeneity, and improves the gluten index in dough (Aravindan et al., 2007; Poulson et al., 2010), and these effects could be enhanced when full-fat soy meal (0.5%) is supplemented (Ertas et al., 2006). More particularly, lipases can be used as a dough strengthener to increase bread oven spring and specific volume (Moayedallaie et al., 2010) or to alter bread crust fracture behavior (Primo-Martin et al., 2008).

Other lipolytic enzymes may also improve bread making, such as phospholipase A (EC. 3.1.1.4), which liberates one fatty acid from phospholipids. This enzyme improves dough-handling properties, suppresses dough stickiness, and increases loaf volume (Inoue and Ota, 1986; Sirbu and Paslaru, 2007).

2.5. Other enzymes that can be involved in bakery performance

2.5.1. Phytase

Phytase (EC. 3.1.3.26) is naturally present in cereal flour, and lately there is a growing interest in its action, owing to its nutritional significance. Much of the phosphate contained in cereals is bound in a molecule called phytate that consists of phosphate groups bound to the six-carbon ring molecule inositol. Inositol is a vitamin, but neither the inositol nor the bound phosphate in phytate is available upon digestion in the human gut. Additionally, phytate chelates (binds) minerals such as calcium and magnesium, rendering them inaccessible to the consumer, and hence it is considered to be an antinutritional compound. Phytase liberates the phosphate and increases the bioavailability of all these nutrients (Afinah et al., 2010). In bread making, the supplementation of phytase results in a shorter fermentation process, an increase of the specific bread volume, and softer crumbs, in addition to the nutritional benefits associated to the reduction of phytate content (Haros et al., 2001a,b).

2.5.2. Oxidases

Different oxidases (lipoxygenase, sulphhydryl oxidase, glucose oxidase, polyphenoloxidase, and peroxidase) have been used for their beneficial effects on dough development and dough quality, mainly ascribed to dough strengthening and stabilization (Oort, 1996), but also as dough-bleaching agents (Gelinas et al., 1998). Glucose oxidase (EC. 1.1.3.4) catalyzes the conversion of β-D-glucose to δ-D- 1,5 gluconolactone, which converts spontaneously into gluconic acid and hydrogen peroxide. The hard oxidizing agent hydrogen peroxide interacts with the very reactive thiol groups of the proteins by forming disulphide bonds and promotes the gelation of water-soluble pentosans, changing the rheological properties of wheat dough (Hoseney and Faubion, 1981; Primo-Martin et al., 2003). Nonetheless, side

activities present in glucose oxidase commercial preparations might have a substantial effect on those reactions (Hanft and Koehler, 2006).

The addition of glucose oxidase produces strengthening of wheat dough and an improvement of fresh bread quality. Studies on gluten proteins at the molecular level by high-performance capillary electrophoresis and at the supramolecular level by cryo-scanning electron microscopy revealed that glucose oxidase modifies gluten proteins (gliadins and glutenins) through the formation of disulfide and nondisulfide cross-links, mainly affecting high molecular weight glutenin subunits (Bonet et al., 2006). Treatment of dough with glucose oxidase increases the gluten macropolymer content due to disulphide and nondisulphide cross-linking (Steffolani et al., 2010) and leads to protein aggregates and broken segments (Indrani et al., 2003). Overdosage of glucose oxidase produces excessive cross-linking in the gluten network with a dramatic effect on the bread-making properties. Glucose oxidase decreases the relaxation time of the flow processes occurring in a short time, whereas it increases the relaxation time of the flow processes occurring over a long time (Wikstrom and Eliasson, 1998).

A similar strengthening effect to the one obtained with glucose oxidase can be reached with hexose oxidase, which causes a dose-responsive reduction of thiol groups (Poulsen and Hostrup, 1998). At the same dosage, hexose oxidase increases dough strength and bread volume more efficiently than glucose oxidase (Gul et al., 2009; Poulsen and Hostrup, 1998).

Lipoxygenase (EC. 1.13.11) is present in high levels in cereal germ and catalyzes the peroxidation of certain polyunsaturated fatty acids containing *cis*, *cis*-1,4-pentadiene systems to produce peroxy-free radicals in the presence of molecular oxygen. Its typical substrate is linoleic acid containing a methylene interrupted, doubly unsaturated carbon chain with double bonds in the *cis*-configuration. Many of the carotenoid pigments of flour are also subject to the action of lipoxygenase. In most flour-based products, the

destruction of these pigments and the resulting bleaching effect are positive. Lipoxygenase from soy flour has been used as a bleaching agent as well as dough conditioner to improve the viscoelastic property of the dough (Junqueira et al., 2007).

Recent research (Zhang et al., 2013) evaluated the effects of a purified recombinant lipoxygenase produced extracellularly in *Bacillus subtilis* on flour, dough, and bread property variations. The textural and structural quality parameters of the enzyme-treated bread showed improved properties, including crumb color, specific volume, resilience, chewiness, and hardness.

Polyphenoloxidases that catalyze the polymerization of the phenolic compounds, such as catechol, pyrogallol, and gallic acid, to quinones by molecular oxygen are naturally present in the outer layers of the grains. When oxygen is present, phenolic compounds polymerize to form very dark pigments that lead to a problem known as "gray dough." Free radicals generated in these reactions are mainly responsible for the protein-protein cross-linking, ferulic acid-mediated, protein-arabinoxylan interactions and diferulated oxidation of arabinoxylans. Based on their substrate specificity, polyphenoloxidases are designated as tyrosinase (EC. 1.14.18.1), catechol oxidase (EC. 1.10. 3.2) and laccase (EC. 1.10.3.1).

Laccase is able to stabilize the dough structure by cross-linking proteins and proteins with arabinoxylans, resulting in a strong arabinoxylan network by oxidative dimerization of feruloyl esters through ferulic acid, and this property is mainly responsible for the improvement of wheat flour dough properties (Houben et al., 2012; Labat et al., 2000). Laccase decreases arabinoxylan extractability due to the crosslinking and increases the oxidation of sulfhydryl groups and the rate of protein depolymerization during mixing (Labat et al., 2000). As a result of laccase action, dough increases in strength and stability as well as reducing stickiness, which

improves machinability and leads to a softer crumb in baked products (Caballero et al., 2007b; Selinheimo et al., 2006).

Laccase catalyzes the oxidation of various aromatic compounds, particularly o-diphenols, producing semiquinones with the concomitant reduction of molecular oxygen to water. The free radical may lead to polymerization of the semiquinones. In wheat flour, laccase catalyzes the polymerization of feruloylated arabinoxylans by dimerization of their ferulic esters (Figueroa-Espinoza and Rouau, 1998). Protein crosslinking may also result from oxidation of sulfhydryl groups, resulting in disulphide bonds (Labat et al., 2000). In wheat flour-based bread-making applications, GO and LAC treatments increase dough strength and stability, increase loaf bread volume, and improve crumb structure and softness (Goesaert et al., 2005; Labat et al., 2000; Si, 1994; Vemulapalli and Hoseney, 1998). Therefore, glucose oxidase and laccase may well promote the formation of a protein and/or nonstarch polysaccharide network in oat batters, resulting in improved bread-making performances.

3. Effect of enzymes in the bread-making process

Bread is considered a staple food worldwide although it must be considered that this term encompasses a wide variety of products that differ in the making process, recipes, shapes, and so on. The common features to all these types of breads are first that they use cereals as a raw commodity and second that mixing, proofing, and baking are the main bread-making stages. In these stages, enzymes play a fundamental role, which might become evident at the dough level and/or in bread quality. However, it must be stressed that in addition to the role of the enzymes intentionally added to dough recipes, enzymes naturally present in the flour (endogenous enzymes) and the yeast enzymes also act on the bread-making process (Rosell and Collar, 2008). Nevertheless, this section is focused on the enzymes used as processing aids in the bakery industry and the effects of those enzymes either in dough or

bread (Table 2). The inclusion of enzymes in bakery processes does not require any changes in the processing line operation. Enzymes are commercialized in powder or liquid forms, they are usually blended with the rest of the ingredients during the mixing stage, and their action occurs during all bread-making stages.

For years, enzymes such as malt and fungal alpha-amylase have been used in bread making. Due to the changes in the baking industry and the awareness of consumers of more natural products, enzymes have gained real importance in bread making.

Bread-making is one of the most common food processing techniques throughout the world. Bread processing can be divided into the following basic operations: mixing of wheat flour and water together with yeast and salt and other specified ingredients in appropriate ratios; fermentation and expansion of the bread dough; and after resting, the dough is divided into loaf-sized pieces, rounded, molded, placed on a baking tray, proofed, and baked. Through the process, a series of physical, chemical (baking time and temperature effect), and biochemical changes (including enzyme-catalyzed reactions) occur to obtain the final baked product. These changes include the development of a gluten network, which traps gas from the yeast fermentation and increases the volume; creation and modification of particular flavor compounds in the dough; evaporation of water; formation of a porous structure; denaturation of protein; gelatinization of starch; crust formation; and browning reactions. In bread making, mixing is one of the key steps that determine the mechanical properties of the dough, which has direct consequences on the quality of the end product. Bread dough is a viscoelastic material that exhibits an intermediate rheological behavior between a viscous liquid and an elastic solid. It is generally known that the formation of gluten, the combination of proteins, which form a large network during dough formation, plays a predominant role in dough development and textural characteristics of the finished bread (Rosell, 2011). The number and type of

sulfuric bonds between gluten proteins have a major effect on the properties of the three-dimensional glutenin network and the dough rheological properties. At optimal mixing, the dough develops because gluten proteins form a continuous network, and within gluten, gliadin and glutenin associate with different free polar lipid types, which contributes to increasing the gas cell stability throughout the bread-making process. During proofing or fermentation, yeast metabolism results in carbon dioxide release and growth of air bubbles previously incorporated during mixing, leading to expansion of the dough during fermentation and baking, and, in consequence, the bread structure and the volume and texture of the baked product. The yeast uses fermentable carbohydrate to produce carbon dioxide and alcohol during alcoholic fermentation, and the enzymes present in yeast and flour also help to speed up this reaction (Rosell, 2011). Baking is the last stage of the bread-making process, in which dough is transformed into an edible final product with excellent organoleptic and nutritive characteristics. The different temperatures reached inside and outside the dough cause the formation of the crust and crumb of bread. The network-like structure of breadcrumb formation is mainly due to starch gelatinization and protein denaturation. The increase in temperature and lower moisture content induce a nonenzymatic browning reaction, which results in the crust formation at the surface of the bread.

Table 2. Brief description of enzymes used in breadmaking and their effects.

Enzymes	Dough	Bread	Staling
α-amylases	Improve fermentation	Improve volume and crumb texture	Anti-staling effect
CGT-ases		Improve volume Decrease firmness	Slower staling kinetics
Pentosanases	Improve handling	Improve volume and crumb texture	
Hemicellulases	Reduce processing time	Increase volume	
Xylanases	Increase gluten strength Improve handling and fermentative characteristics	Increase shelf-life	Reduce bread-staling Increase shelf life
Proteases	Easy mixing May compromise gas-holding ability		
Tyrosinase	Increase hardness Decrease extensibility	Uneven crumbs and large pore size Soft texture and increase volume	
Transglutaminase	Decrease water absorption Increase stability Favors tiny gas cells	Increase volume and improve structure	
Lipases	Improve rheological properties Dough strengthener	Improve crumb structure Flavor enhancement Increase specific volume	Antistaling effect when combined with α-amylase
Phospholipase A	Improve handling and suppress stickiness	Increase volume	
Phytase	Shorten fermentation	Increase specific volume and soften crumbs Improve nutritional values	
Glucose oxidase	Dough strengthener	Improve quality	
Lipoxygenase	Improve visco-elasticity Bleaching agent		
Laccase	Improve stabilization and machinability Dough strengthener	Soften crumb	

Many authors have described the effect of various enzymes and combinations of them that directly or indirectly improve the strength of the gluten network and so improve the quality of the bread (Caballero et al., 2007b; Rosell and Collar, 2008; Selinheimo et al., 2006; Steffolani et al., 2010). Enzymes, such as xylanases, transglutaminases, glucose oxidase, laccase, or a combination of them all, have been used to increase gluten strength, and consequently, to improve dough functionality for bread making. Apart from the application of individual enzymes, such as amylases, proteases, or lipases, the use of enzymes in combination offers a wide range of alternatives to improve bread quality and antistaling or to provide nutritional benefits.

The interactions between enzymes on the various components of bread dough are a very complex subject, and the wrong enzyme combination or even the wrong application rate can result in unfavorable effects on either the dough or the finished baked product (Collar et al., 1998; Rosell et al., 2001c).

3.1. Enzymes added for improving processing

Enzymes have been used extensively for improving dough performance in bread making and bread features. Usually enzymes are blended with the rest of the ingredients during the mixing stage, and their action occurs during all bread-making stages. Additionally, the supplementation of enzymes during grain tempering has been proposed for increasing the milling extraction rate or for extending the period of enzyme action and improving the bread-making wheat flour performance. Rosell et al. (2003) tested the effect of transglutaminase and glucose oxidase supplemented during wheat tempering and investigated the performance of the treated flour. Enzymatically treated flour showed the same behavior as the dough supplemented with those enzymes, confirming the cross-linking effect by capillary electrophoresis and also by empirical rheological analysis. Later on, Yoo et al. (2009) reported the effect of an enzyme cocktail consisting of cellulase, xylanase, and pectinase added at different levels during tempering at the laboratory scale.

Some of those enzymatic treatment combinations affected flour yield from the break rolls more than that from the reduction rolls; nevertheless, results did not allow the confirmation of a significant effect on milling yield. However, the effects observed on the dough and bread characteristics confirmed that enzymes diffuse through the outer layers and remain in the milled flour.

Changes in bread processing have prompted modifications in recipes and processing lines for answering consumer demands. Frozen dough and bake-off technology of frozen dough or frozen partially baked breads have been important changes in bread making, and processing aids have been developed with this change in mind. The dough matrix is subjected to much stress during freezing, frozen storage, and thawing due to ice-crystal formation, and because of this, an extra reinforcement of the gluten matrix is generally required. Glucose oxidase reduces the dough damage caused by frozen storage due to its strengthening action, which counteracts the depolymeration effect of gluten induced by ice-crystal formation and released reducing substances (Steffolani et al., 2012). A frozen dough additive containing protease, glucose oxidase, gluten, and stearoyl lactate has been recommended for producing frozen dough with improved extensibility and workability and also higher dough stability over a long period of time (Anon, 2000).

The combination of oxidases, such as glucose oxidase and peroxidase, has been proposed as an improver to form a stable gluten network structure with resistance to damage of ice crystals in frozen dough (Huang and Jia, 2006). For other enzymes with cross-linking action, the TGase has been combined with a recombinant lipase (*Rhizopus chinensis* lipase) for improving the rheological properties and structure of frozen dough (Li et al., 2011). That enzyme combination increases the water-holding capacity, leading to dough in which starch granules are embedded within the gluten network after 35 days of frozen storage, and after baking, breads have a high specific volume, open network, and uniform crumb structure. Presumably, the new isopeptide

bonds within the proteins catalyzed by TGase give sufficient gluten stability to mitigate the damage caused by dough freezing (Steffolani et al., 2012). For the same application, Hsing et al. (2010) recommended a combination of hemicellulose and/or endoxylanase with ascorbic acid, which delayed the staling and also improved the quality of the freshly baked bread.

3.2. Enzyme combination for improving bread quality

Numerous enzyme combinations have been reported focused on improving bread technological or instrumental quality regarding crumb and crust properties.

For bread made with durum and bread wheat flour, α-amylases, proteases, and a mixture of α-amylaseprotease was suggested by Pena-Valdivia and Salazar-Zazueta, (1997) confirming the dose-dependent effect of the enzymes on the loaf volumes and the best performance of fungal over bacterial α-amylase in durum wheat flour. Those authors recommended a mixture of α-amylase-protease for producing breads from durum wheat flour with more acceptable quality characteristics.

Martinez-Anaya and Jimenez, (1997a) studied nine commercial enzyme preparations and two laboratory designed mixes with amylase and/or pentosanase activity for obtaining bread with improved volume, a soft crumb, and high aroma intensity. The individual or combined addition of commercial amylases, xylanases, lipases, and glucose-oxidases modifies the rheological behavior of dough; in general, enzyme supplementation results in softening and weakening of dough immediately after mixing although it could be counteracted with glucose oxidase (Martinez-Anaya and Jimenez, 1997b). Among the enzymes mentioned previously, pentosan-degrading enzymes cause the main changes, reducing consistency and increasing stickiness (Martinez-Anaya and Jimenez, 1998), and pure lipase preparations are the least significant when compared with unsupplemented dough (Martinez-Anaya and Jimenez, 1997b). Enzymes act quickly and change the textural

properties of dough immediately after mixing and continue during resting (Martinez-Anaya and Jimenez, 1998). Similar effects are observed in sourdough wheat bread making (Martinez-Anaya and Devesa, 2000).

Due to the complexity of finding the right and optimum enzyme combinations, a central composite design was initially proposed by Collar et al. (2000), comprising bacterial α-amylase, intermediate thermostability α-amylase, xylanase, lipase, and glucose-oxidase. Results showed that xylanase resulted in softer, stickier, and less adhesive dough with higher gas retention capability but with weakened gluten, and when combined with intermediate thermostability, amylase decreased fermentation time and increased dough extensibility. According to these authors, the incorporation of xylanase with glucose oxidase is not recommended because glucose oxidase counteracts the softening effect of xylanases (Collar et al., 2000). This antagonistic effect has been elucidated on the basis of the glucose oxidase activity by Primo-Martin et al. (2005), who explained the glucose action through the formation of protein disulfide bonds and cross-links between arabinoxylans; the latter negatively affected the bread quality. The supplementation of xylanases combined with glucose oxidase counteracts that effect, breaking arabinoxylan complexes and releasing small arabinoxylan fragments that interfere with the cross-linking (Primo-Martin et al., 2005). This action could render a redistribution of the water from the nonstarch carbohydrates to gluten, leading to more extensible gluten (Dagdelen and Gocmen, 2007). Conversely, it has been reported that glucose oxidase acts synergistically with phospholipase, ensuring roll shape uniformity and tolerance to process changes in addition to greater bread volume (Novozymes, 2001).

Similar to the effects observed with glucose oxidase, laccase leads to dough hardening, but this effect decreases over the dough resting period due to the laccase-mediated depolymerization of the crosslinked arabinoxylans network, which could be hindered by the addition of xylanase (Selinheimo et al., 2006).

Gluten cross-linking enzymes can actively contribute to confer functional properties to dough. Caballero et al. (2007b) used a number of cross-linking enzymes (TGase, glucose oxidase, and laccase) along with polysaccharide and gluten-degrading enzymes in bread making (α-amylase, xylanase, and protease), systematically analyzing the individual and synergistic effects in bread-making systems. Moreover, they observed a significant synergistic effect within the pairs of glucose oxidase-laccase, glucose oxidase-pentosanase, amylase-laccase, amylase-protease, and pentosanase-protease (Caballero et al., 2007a). Further on, Steffolani et al. (2010) investigated the effect of glucose oxidase, TGase, and pentosanase on wheat protein and bread quality.

Bread-making ingredients greatly affect enzyme activities. For instance, TGase in combination with pectin and diacetyl tartaric acid ester improves water absorption and makes highly cohesive dough and gluten strength in addition to suitable pasting performance during cooking (Collar and Bollain, 2004).

Xylanase in combination with α-amylase and glucose oxidase, along with ascorbic acid, was reported to exhibit an excellent dough-strengthening effect. The effect of this combination of enzymes to weaken wheat flour was evaluated by Shafisoltani et al. (2014). The authors concluded that a combination of optimum levels of the three enzymes resulted in dough with low stickiness and bread with a higher specific volume, higher quality texture, better shape, and higher total score in a sensory evaluation test.

Less attention has been paid to bread crust although lately different enzymes have been tested for extending the crispiness of baked bread. Hamer and Primo-Martin, (2006) proposed the application of an enzyme material with proteolytic activity onto the outside surface of the dough or partially baked bread to retain the crispy perception of the crust for an extended period of time. Lately, amyloglucosidase sprayed onto the partially baked surface has

also been reported for modulating the properties of the bread crust and increasing its crispness (Altamirano-Fortoul et al., 2013). The amyloglucosidase treatment affects the color of the crust and decreases the moisture content and water activity of the crust and the force required for crust rupture. It seems that amyloglucosidase induces the disruption of the crust structure by removing the starchy layer that covers the granules and increasing the number of voids, leading to texture fragility.

3.3. Enzymes acting as antistaling agents

Bread staling is a complex phenomenon that happens during storage, and it is largely caused by water migration and transformations that occur in the starch leading to crumb hardening. Staling implies a relatively short shelf life for fresh bakery products, leading to loss of consumer acceptance. Alterations related to this phenomenon include an increase in moisture in the crust (loss of crispiness), an increase in crystallinity in the starch granule, an increase in crumb firmness, a loss of organoleptic properties in the loaves, and the crumb's loss of water-holding capacity (Gray and BeMiller, 2003; Ribotta and Le Bail, 2007). Specific amylases that produce maltotriose and maltotetraose (Min et al., 1998) or maltopentaose (Hyuck et al., 2005) have been reported as effective antistaling agents for retarding amylopectin retrogradation. It seems that the presence of a low degree of polymerization maltodextrins (DP 3-7) (Rojas et al., 2001) or even with a polymerization degree up to 66 (Defloor and Delcour, 1999) might be responsible for the antistaling effect of α-amylases. However, Hug-Iten et al. (2001) explained the antistaling action of α-amylase is due to its capacity to partially degrade amylopectin, hindering its recrystallization, and to slightly hydrolyze amylose by an endo-mechanism, which induces a part crystallization of amylose that contributes to the kinetic stabilization of the starch network during aging (Hug-Iten et al., 2003). Later on, Palacios et al. (2004) stated that the antistaling action of α-amylase might be a consequence of the decrease in the rate and extent of starch retrogradation that hampers the

formation of double helices, but also the antistaling efficiency of amylases have been related to water immobilization (Goesaert et al., 2009). Additionally, Goesaert et al. (2009) determined the structure of starch in amylase-supplemented breads and correlated these data with crumb firming and amylopectin recrystallization. Results showed that the use of bacterial endo-α-amylase from *B. subtilis* and maltogenic α-amylase from *B. stearothermophilus* reduced crumb firming during storage.

Apart from α-amylase, it has been suggested that CGTase from *B. stearothermophilus* ET1 has potential application in bread making through modulation of the cyclizing and hydrolytic activities (Sung-Ho et al., 2002) because, by protein engineering, it was possible to replace residues Phe191 and Phe255 of CGTase by glycine (Phe191Gly-CGTase) and isoleucine (Phe255Ile-CGTase), respectively, yielding reduced cyclization activity.

α-Amylase antistaling effect can be more effective when combined with lipase, owing to the formation of thermostable amylose-lipid complexes (Leon et al., 2002), which can be enhanced with the addition of distilled monoglyceride, although motivating the formation of amylose-lipid complexes favors the water migration from crumb to crust (Purhagen et al., 2011). In addition, a synergistic effect between intermediate thermostability α-amylase and TGase has been evident in white and whole wheat breads stored up to 20 days (Collar and Bollain, 2005). Supplemented breads with both enzymes had slower staling kinetics, particularly for hardness, cohesiveness, chewiness, resilience, and also sensory deterioration (Collar and Bollain, 2005).

Different combinations of antistaling agents have been suggested for extending the freshness of breads. Armero and Collar, (1996) proposed the combination of emulsifiers, hydrocolloids, and α-amylase for retarding staling in white and whole meal breads, but different synergistic and

antagonistic effects were encountered (DATEM*SSL, α-amylase*SSL, α-amylase*HPMC).

The effect of pentosanase, bacterial α-amylase, and lipase added individually, or in their blends on the shelf life of white pan bread during storage, was reported by Gil et al. (1999), who showed that bacterial α-amylase, specially blended with pentosanase and lipase, increased bread crumb elasticity and reduced firmness during storage up to 72 h. That enzyme mixture has been also effective in high-fiber wheat bread, in which enzymes and sourdough lower the changes in firmness, amylopectin crystallinity, and rigidity of polymers (Katina et al., 2006).

The influence of three different starch-degrading enzymes, a conventional α-amylase, a maltogenic α-amylase, and β-amylase were investigated by Hug-Iten et al. (2003). Results suggested that the enzyme induced fast formation of a starch network contributes to a kinetic texture stabilization, which prevents structure collapse and hinders rearrangements in the starch (amylose) phase, thus contributing to the antifirming effect by preventing further firming over time. Rosell and Collar, (2008) described the effect of different enzymes on bread staling, highlighting the antistaling effect of amylases, the combination of bacterial α-amylase with TGase or xynalases, which led to breads with softer and less chewy fresh crumbs, retarding the starch retrogradation and consequent staling effect on bread samples. Barrett et al. (2005) also confirmed the antistaling effect of two amylases reducing the amount of starch recrystallization, and that effect was even intensified in the presence of 6% glycerol.

Nonstarch carbohydrases (cellulase, xylanase, and glucanase) decrease the starch retrogradation, but xylanases have the greatest antistaling effect (Haros et al., 2002). A kinetic study of the firmness during storage using the Avrami equation showed that carbohydrases reduced the rate of bread firming, but the simultaneous analysis of the hardening and starch retrogradation indicated

that the antistaling effect of xylanase might be due to the retardation in the starch retrogradation although, in the case of cellulase and beta-glucanase, some other mechanism should be also involved (Haros et al., 2002).

Xylanases used at optimum levels play a significant role in increasing the shelf life of bread and reduce read staling (Butt et al., 2008). When xylanase is added in a binary combination with high ester pectin and/or hydroxypropyl methylcellulose, a shortened effect on dough extensional parameters is observed (Rosell and Collar, 2008). Caballero et al. (2007b) studied the changes in dough rheology, quality, and shelf life of breads caused by the use of cross-linking enzymes (TGase, glucose oxidase, laccase) and hydrolytic enzymes (α-amylase, xylanase, protease) and found that bread staling during storage was increased with TGase but was inhibited by amylase, xylanase, and protease.

In a recent study, Oliveira et al. (2014) investigated the influence of the enzyme cocktail with xylanolytic activity from *T. aurantiacus* fungus CBMAI 756 on bread quality and the staling process as well as to identify the specific products released through its activity on wheat flour arabinoxylans. The main product released through enzyme activity after prolonged incubation was xylose, indicating the presence of xylanase; however, a small amount of xylobiose and arabinose also confirmed the presence of xylosidase and α-L-arabinofuranosidase, respectively. The enzyme mixture in vitro mainly attacked water-unextractable arabinoxylan, contributing to a beneficial effect in bread making. The use of an optimal enzyme concentration improved bread quality by increasing specific volume and reducing crumb firmness.

3.4. Enzyme combinations for different bread specialties

Some bread specialties require specific enzyme combinations adapted to the bread-making processes. Wen et al. (2003) reported the effect of α-amylase on steamed bread, showing an improvement in the elongation of the dough

and the elasticity of the bread. They did find a minor effect when flour was supplemented with xylanase. Bacterial maltogenic α-amylase also gave adequate properties to rice-steamed bread, which prevented the staling and maintained the flavor characteristics and the chewy taste (Ahn, 2008). Another improver defined for making this type of bread comprised fungal α-amylase, xylanase, ascorbic acid, monoglycoside, and soybean powder (Jia et al., 2006). Specific thermophilic xylanase (131 U/ml) from *Chaetomium* sp. added in the range of 2.5–5.0 ppm produced up to a 25% increase in the specific volume of steamed bread and almost the same decrease in the firmness (Jiang et al., 2010).

Turkish heart bread has been also supplemented with α-amylases obtaining differences depending on the enzyme source (from cereal and fungal sources) (Dogan, 2003).

Unleavened Indian flat bread, namely South Indian parotta, improves the overall quality score when flour was supplemented with proteinase. It is likely this enzyme breaks large protein fibrils, leading to small ones that improve the continuous gluten formation (Prabhasankar et al., 2004). α-Amylase and xylanase have also been tested as an antistaling agent in other unleavened Indian flat bread made from whole wheat flour, chapatti, and both enzymes inhibited staling to different extents (Shaikh et al., 2008). Moreover, these enzymes can change the nutritional, nutraceutical, and antioxidant properties of chapatti. α-Amylase and xylanase improve the antioxidant properties of this bread, particularly the amylase that increases antioxidant phenolic acids, such as caffeic, gentisic, and syringic acids, in addition to the phenolic acids as well as the soluble dietary fiber (Hemalatha et al., 2012). Nevertheless, at least one starch-degrading enzyme is advisable to be added for retarding flat bread staling and preferably a mixture of an α-amylase and a glucan 1,4-α-glucosidase (Forman and Evanson, 2011).

α-Amylase has been tested as an improver in teftoon, a flat bread prepared from whole wheat flour by hand sheeting and baking (Koocheki et al., 2009). The addition of amylase and emulsifiers decreased the force required to tear the fresh bread, and the best antistaling effect was observed with diacetylated tartaric acid esters of mono- and diglyceride of fatty acids. The optimization of the wheat α-amylase activity determines the softness and staling of the teftoon, and the right α-amylase level can be obtained by germinating wheat flour for a period of 72 h (Moharrami and Shahedi Baghkhandan, 2011).

3.5. Enzyme supplementation for producing nutritional benefits

Other enzymes that can be used in bread making, leading to nutritional improvement are the phytases (Haros et al., 2001a,b). Fungal phytases improve the firmness and chewiness of whole meal wheat bread over a period of 3 days of storage, and the extent of the effect was dose dependent (Zyla et al., 2005). Moreover, the supplementation of phytase induces a decrease of phytate content and an increase in the in vitro digestibility of phosphorus, calcium, carbohydrates, and proteins (Zyla et al., 2005).

The impact of the exogenous fungal phytase on the phytate content of whole meal wheat breads has been reported by comparing different bread-making processes (Rosell et al., 2009). There was significant interaction between the bread-making process and enzyme addition concerning specific volume, crumb lightness, and crumb texture. Freezing and frozen storage of whole meal bread in the presence of fungal phytase decreased significantly the phytate content, independently of the bread-making process that was followed. Furthermore, Penella et al. (2008) studied the effect of a combination of fungal phytase and fungal α-amylase, bran content, and particle size distribution of bran on the technological performance of dough and on the amount of phytic acid remaining in the bread. The authors concluded that the combination of bran with amylolytic and phytate-degrading enzymes could be recommended for overcoming the detrimental

effect of bran on the mineral availability (phytase) and on the performance of the dough (α-amylase). Fungal phytase can improve nutritional and bread-making performance of whole wheat bread; however, not all the phytates are hydrolyzed (Haros et al., 2001a,b).

4. Enzymes in "Gluten-free" products

A variety of cereal foods are produced from wheat flour, such as bread, noodles, bulgur, and couscous, because gluten, which is formed by combining wheat proteins, has unique viscoelastic properties. However, many people suffer from gluten-related disorders, which prompted the development of gluten-free products. One of the main challenging aspects in gluten-free bread making is the production of high-quality bread with good structural properties using gluten-free cereals, such as rice or corn, or some pseudocereals, such as quinoa or amaranth. However, the proteins of those flours are incapable of retaining carbon dioxide during fermentation due to the lack of viscoelastic properties, which forced researchers to find technological alternatives to mimic gluten functionality (Arendt et al., 2008). In this respect, the addition of different groups of enzymes in gluten-free bread production plays an important role in achieving desirable properties to improve the quality of the final product, mainly by cross-linking proteins (Rosell, 2009).

4.1. Transglutaminase

In this respect, transglutaminase has been the enzyme most extensively proposed for creating protein cross-links in rice flour (Gujral and Rosell, 2004a). Nevertheless, the protein network created by the TGase is greatly dependent on the protein origin, its thermal compatibility, and the dosage of the enzyme (Marco and Rosell, 2008c). Gujral and Rosell (2004a) initially proposed the hypothesis that the enzymatic creation of a protein network in gluten-free dough might mimic gluten functionality. With that aim, the authors studied the addition of increasing amounts of TGase (0.5%, 1.0%, or

1.5% w/w) to rice flour obtaining a progressive increase of the viscous and elastic moduli, but a higher bread volume and softer crumb was obtained with 1.0% TGase and further improvement was obtained with the addition of 2% HPMC.

Nonetheless, the flour source has great influence on the extent of activity of TGase as reported by Renzetti et al. (2008) when compared with the effect of that enzyme on six different gluten-free cereals (brown rice, buckwheat, corn, oat, sorghum, and teff). Three-dimensional confocal laser scanning micrographs confirmed the formation of protein complexes by TGase. Batter fundamental rheological analysis and bread quality indicated an improving effect of 10 U TGase on buckwheat and brown rice batters and breads and a detrimental effect on corn batters but with increased specific volume and decreased crumb hardness and chewiness on corn breads. Conversely, transglutaminase was not effective in making breads from oat, sorghum, or teff (Renzetti et al., 2008). Nevertheless, Onyango et al., (2010b) obtained changes in the rheological properties of gluten-free batters of sorghum blended with pregelatinized cassava starch when adding TGase, specifically, the enzyme decreased the resistance to deformation and compliances and augmented zero shear viscosity and elastic recovery.

Surely, the amount and nature of the proteins present in those flours, considering that, for TGase action, lysine and glutamic acid are needed, must explain the differences encountered among flours. In answer to that possible protein deficiency for TGase activity, protein supplementation to gluten-free flours was proposed to increase the amount of substrate for the enzyme (Marco and Rosell, 2008b,c; Marco et al., 2007, 2008).

Marco and Rosell, (2008b) reported the effect of transglutaminase on rice flour functionality when it was blended with protein isolates from different sources (pea, soybean, egg albumen, and whey proteins). The cross-linking action of TGase was confirmed by the decrease in the amount of free amino

acids. Pea, soybean, and whey proteins decreased the final viscosity, thus affecting amylose recrystallization during cooling. Viscoelastic moduli of the dough were significantly modified, but whereas pea and soybean increased this parameter, egg albumen and whey protein dramatically decreased it. Even a combination of soybean and pea protein optimized with an experimental design was recommended for obtaining a better-structured protein network (Marco and Rosell, 2008c). In fact, electrophoretic studies confirmed that TGase action resulted in the formation of isopeptide and disulfide bonds mainly within albumins and globulins and, to a lesser extent, within glutelins, and in consequence, large aggregates between pea and rice proteins were present (Marco et al., 2007). Similarly, soybean proteins were cross-linked with rice proteins through the formation of new intermolecular covalent bonds catalyzed by transglutaminase and the indirect formation of disulfide bonds among proteins, mainly involving β-conglycinin and glycinin of soybean and the glutelins of the rice flour although albumins and globulin also participated (Marco et al., 2008). The combination of gluten-free flours with legume flours has the additional benefit of improving the amino acid balance because the action of TGase does not have any nutritional repercussion.

Concerning the effect of that strategy on bread making, Marco and Rosell, (2008a) proposed the protein enrichment of gluten-free breads with the additional benefit of creating a protein network through the use of transglutaminase. For doing that, those authors optimized the amount of water needed to hydrate the system, the level of protein and enzyme, and also included hydroxypropyl methylcellulose for additional structural strength and a more open aerated structure. Although soybean proteins reduced the specific volume of the bread, scanning electron micrographs confirmed the participation of those proteins in the network created by the TGase.

Houben et al. (2012) proposed the addition of TGase in the presence of skim milk powder and egg protein powder to form protein networks that yielded

stiff crumbs. An experimental design carried out to obtain protein-enriched rice-based gluten-free breads showed that the combination of TGase (1.35 U of enzyme/g of rice flour protein), egg albumen (0.67 g/100 g of flour), and casein (0.67 g/100 g of flour) yielded the highest specific volume of rice-based bread with the lowest crumb hardness (Storck et al., 2013). With the same aim, different extruded gluten-free flours (rice, potato, corn, buckwheat), proteins (egg-white powder, soybean isolate, caseinate), and TGase were included in an experimental design, showing the best quality was in the bread with extruded buckwheat extrudate, egg-white powder, and 10 IU TGase per gram of protein (Smerdel et al., 2012).

Gluten-free jasmine rice bread improved its specific volume with a simultaneous reduction in the crumb hardness when up to 10% rice flour was replaced by pregelatinized tapioca starch and TGase was added up to 1% (Pongjaruvat et al., 2014).

Nevertheless, some concern about the use of microbial TGase arose due to its homology to tissue TGase that is mediated in celiac disease. A study carried out by Cabrera-Chavez et al. (2008) evaluated the reactivity of the IgA of celiac patients to proteins of wheat and gluten-free breads treated with microbial TGase, showing that the reactivity was higher against prolamins of TGase-treated breads due to two individual patients' sera.

4.2. Protease

Even though the creation of a protein network was primarily thought to be the best alternative for improving gluten-free bread quality, proteases also have been proposed as processing aids. Proteases induce the release of low molecular weight proteins from macromolecular protein complexes, which results in lower complex modulus and initial viscosity in addition to a decrease in paste viscosity and breakdown but an increase in the paste stability (Renzetti and Arendt, 2009). Batters show lower resistance to deformation during proofing and in the early stages of baking while

preserving its elasticity. When added to brown rice flour, breads significantly increase the specific volume with a parallel decrease in crumb hardness and chewiness (Renzetti and Arendt, 2009).

Kawamura-Konishi et al. (2013) reported improved crumb appearance, high volume, soft texture, and a low staling rate when a commercial protease from *Bacillus stearothermophilus* (thermoase) was added to rice flour. Presumably, this protease majorly hydrolyzed albumins and globulins, leading to many cellular structures in the breadcrumb.

4.3. Oxidases

Oxidases such as glucose oxidase and laccase could also yield the formation of networks.

Glucose oxidase has been supplemented to rice flour, yielding an increase in specific volume with a simultaneous reduction of the crumb hardness (Gujral and Rosell, 2004b), owing that effect to the protein cross-linking as it reveals the decrease in the amino and thiol groups. In addition, electrophoresis analysis confirmed changes on glutelins (Gujral and Rosell, 2004b).

Renzetti et al. (2010) reported the increased specific volume and softening crumb effect of commercial preparations of laccase (0.01%) and protease (0.001% and 0.01%) containing endo-β-glucanase side activity for making gluten-free oat flour. The authors attributed the improvement due to the increase in batter softness, deformability, and elasticity in part due to the β-glucan depolymerization. In oat based breads, the extensive protein hydrolysis during baking may have improved the functionality of the soluble protein fraction. Flander et al., (2011) also observed an improvement in specific volume of oat bread with the combination of *Trametes hirsute* laccase and xylanase, although crumb softness remained unaltered.

4.4. Other enzymes

A different alternative for improving the quality of gluten-free breads has been the addition of different α-amylases. Gujral et al. (2003a) investigated the use of CGTase as a processing aid in rice-based breads, obtaining a reduction of dough consistency and the elastic modulus and also acted as antistaling additive (Gujral et al., 2003b). At the bread level, using an experimental design, the authors optimized the combination of CGTase, HPMC, and oil amount for producing better specific volume and crumb texture. In addition, CGTase decreased the amylopectin retrogradation during storage (Singh et al., 2003), owing these effects to the hydrolyzing and cyclizing activity of the CGTase. α-Amylase of intermediate stability has been effectively used for antistaling in rice breads but resulted in sticky textures (Singh et al., 2003). Similarly, Onyango et al. (2010a) tested the effect of α-amylase in sorghum-based bread, observing that by increasing enzyme concentration (up to 0.3 U/g) decreased crumb firmness, cohesiveness, springiness, resilience, and chewiness but increased adhesiveness.

Trichoderma reesei tyrosinase has been applied to oat breads, inducing the formation of protein aggregates of high molecular weight, which involved globulins (Flander et al., 2011). That cross-linking resulted in dough hardening and better bread characteristics, and further improvement was obtained when tyrosinase was combined with xylanase.

A complex mixture comprising rice flour, dried albumin, cooking oil, sugar, yeast, emulsifier, salt, and an enzyme improver containing hemicellulase, glucose oxidase, xylanase, and α-amylase has been proposed as a gluten-free bread premix, which requires agitation for degrading the cell wall of rice starch and for improving expansion property (Kim et al., 2009).

5. Use of enzymes in making cakes, cookies, and pastries

Another application of enzymes in baking is in the production of breakfast cereals, biscuits, buns, cakes, and pastries (Table 3). This kind of product, in addition to pasta, forms part of the group of "foods containing fat" and "foods containing sugar," of which the nutritional composition in fat and sugar contents greatly differs from bread and is unique for each elaborated product. Even the process of making these products requires different operations. However, enzymes are also very useful as processing aids in those products although the aim of their application maybe different than the one described for bread making. Mostly, individual enzymes or combinations are added to retard staling and extend freshness perception over time.

Table 3. Enzymes used in sweet baked goods

Enzyme	Cakes	Biscuits or cookies	Pastries and croissants
Endoxynalases	+	-	-
Thermostable 4-alpha-glucanotransferase	+	-	-
α-amylase	+	-	+
β-amylase	+	-	-
Lipase	+	-	-
Xylanase	+	+	+
Proteases	-	+	+
Transglutaminase	-	-	+
Glucose oxidase	-	-	+
Phospholipase	-	-	+
Oxygenases	-	-	+
Oxidases	-	-	+
Peroxidases	-	-	+

5.1. Cakes

In sponge cake systems, protein-foaming properties are fundamental in determining the overall textural quality of the product (Celik et al., 2007). Limited enzymatic hydrolysis of wheat gluten significantly increases its

foaming and emulsifying properties (Drago and González, 2000; Kong et al., 2007), improving cake volume and moistness (Bombara et al., 1997).

Other authors proposed to treat oat or rice bran with endoxylanase (70 and 700 ppm) for improving the properties of the bran when used as an ingredient in cakes (Lebesi and Tzia, 2012). This treatment increased the amount of soluble dietary fiber and reduced the water-holding capacity of bran resulting in a softer cake crumb with high specific volume and better porosity and sensory characteristics. In addition, cakes containing treated bran showed better performance during storage with slower deterioration of the sensory characteristics (Lebesi and Tzia, 2011).

Kim et al. (2012) recommended the use of rice flours previously treated for 48 h with a thermostable 4-α-glucanotransferase from *Thermus aquaticus* to obtain rice cakes with better textural properties and retarded starch retrogradation. The authors explained the extended shelf life due to the action of the enzyme, reducing the chain length of the starch.

The combination of a bacterial α-amylase with gums and pregelatinized starch has been proposed for making devil's fudge cakes (Sozer et al., 2011). By adding these improvers, cakes showed 25% lower toughness and hardness and slower starch retrogradation.

There are some sweet specialties in which enzyme supplementation has been tested leading to improved quality. Specifically, panettone made with the addition of lipase or amylase increases the height, whereas xylanase improves the crumb porosity and dough-handling properties, and the extent of the effect is dose-dependent (Benejam et al., 2009). A steamed, cooked Chinese cake, Mi Gao, stales more slowly when β-amylase, α-amylase, or hydrocolloids were added although considering sensory acceptability the β-amylase was preferred (Ji et al., 2010).

5.2. Biscuits or cookies

Proteases have been very useful for modifying the extensional properties of gluten and improving cookie performance of the wheat flours (Bruno and Oliveira, 1995; Kara et al., 2005; HadiNezhad and Butler, 2009). The action of proteases degrading gliadin and glutenin subunits of flour gluten led to an increase in the spread ratio values of cookies (Kara et al., 2005).

The addition of xylanases is advisable when high fiber content flours are used. In fact, Jia et al. (2011) could make almond cookies adding up to 20%-23% California almond skin flour in the presence of xylanase that acted on the noncarbohydrate polymers without affecting the spread ratio and the breaking force of the cookies.

5.3. Pastries and croissants

Gerrard et al. (2000) first tested the effect of microbial TGase on puff pastries and croissants, observing a similar cross-linking action as mentioned in the case of wheat breads. Particularly, TGase improved the expansion of puff pastry and the volume of yeasted croissants, and this enzyme also conferred stability to the dough during freezing and 90 days of frozen storage. The stabilization effect of the TGase was explained in terms of cross-linking of albumins and globulins, leading to high molecular weight aggregates and also cross-linking of gluten subunits (Gerrard et al., 2001). A similar cross-linking effect on the albumins and globulins was obtained by using glucose oxidase due to disulfide and nondisulfide linkages formation, but this enzyme was less effective in increasing croissant volume due to its minor action on cross-linking glutenins (Rasiah et al., 2005). In the production of pastries, the combination of TGase (1.5 mg/100 g flour) with fermizyme (5 mg/100 g flour) has been recommended for improving sensory quality (Hozova et al., 2003).

Conversely, proteolytic enzyme material has also been advisable for obtaining laminated dough, such as croissants (Hargreaves et al., 2005). The enzyme material is applied on the outside surface of laminated dough or the partially baked laminated dough; in this way, the baked product retains a crispy crust for an extended period after baking and even after reheating.

In bakery products, such as croissants, pastries, pies, and so on, the water in an oil emulsion is fundamental for obtaining laminated dough that is a layered structure. Phospholipase has been recommended for totally or partially replacing emulsifiers such as lecithin, keeping the water-binding capacity of the spread, obtaining a baked product with thin and uniform layers (Nielsen, 2009).

Another improver proposed for increasing the stability during frozen storage of Viennese pastries and brioche dough is comprised of gluten, natural flavoring, bean flours, and a blend of hemicellulose and α-amylase (Muchembled and Julien, 2005). Even more complex enzyme combinations have been reported for pastry production that comprises xylanases, α-amylases, oxygenases, oxidases, peroxidases, phospholipases, and proteases (Anon, 2006). This preparation incorporates xylanases to enhance extensibility and improve hydration; α-amylases to produce fermentable sugars; oxidases, oxygenases, laccases, and peroxidases to improve consistency; glucose oxidases to reinforce dough; phospholipases to increase the volume of the bread and to improve crumb texture; and proteases to improve the effectiveness of kneading.

6. Future trends

Enzymes have been extensively applied in bakery industries, initially for modulating dough performance and improving fresh bread quality, and later on for extending shelf life of baked products. Over the years, fundamental insights about the enzyme activity have been gathered, which help to comprehensively apply individual enzymes or enzymatic combinations for

further applications. Owing to the intrinsic innovation of the bakery industry, future application of enzymes is foresight in different trends. First, is the search for new enzymes or new sources of enzymes with tailored properties for making baked products using mechanized processing and/or different baked specialties; second, a strong tendency is focused on the replacement of chemicals with enzymes moving to green labels in bakery products for answering consumers' demands for more natural products free of chemical additives, and finally, the interest in nutritional aspects is exponentially growing, which is prompting the development of enzymatic applications for improving nutritional and healthy features of baked products.

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References

Afinah S., Yazid AM., Anis SMH., Shuhaimi M. (2010). Phytase: Application in food industry. International Food Research Journal. 17: 13-21.

Ahmad Z., Butt MS., Ahmed A., Riaz M., Sabir SM., Farooq U., Rehman F. (2012). Effect of *Aspergillus Niger* xylanase on dough characteristics and bread quality attributes. Journal of Food Science and Technology. 51: 2445-2453.

Ahn JB. (2008). Method of making rice steamed bread using wheat flour and enzymes to retard shaping and staling together with rice flour. Shany Co Ltd.

Aisbitt B., Caswell H., Lunn J. (2008). Cereals-current and emerging nutritional issues. Nutrition Bulletin 33. 3: 169-185.

Altamirano-Fortoul R., Hernando I., Rosell CM. (2013). Influence of amyloglucosidase in bread crust properties. Food and Bioprocess Technology. 44: 85-94.

Alves-Prado HF., Carneiro AAJ., Pavezzi FC., Gomes E., Boscolo M., Franco CML., da Silva R. (2008). Production of cyclodextrins by CGTase from *Bacillus Clausii* using different starches as substrates. Applied Biochemistry and Biotechnology. 146: 3-13.

Anon. (2000). Frozen dough additive for manufacture of bread, comprises protease enzyme, gluten, stearoyl lactate and glucose oxidase. Showa Sangyo Co.

Anon. (2006). Enzymatic preparation comprises incorporating ascorbic acid, xylanases, alpha-amylases, oxydases, oxygenases, laccases, peroxidases, phospholipases and proteases in the Pastry.

Aravindan R., Anbumathi P., Viruthagiri T. (2007). Lipase applications in food industry. Indian Journal of Biotechnology. 6: 141-158.

Arendt EK., Morrissey A., Moore MM., Dal Bello F. (2008). Gluten-free breads. In: Gluten-Free Cereal Products and Beverages. Arendt EK., Dal Bello F. (eds). San Diego, CA: Academic Press. 289-VII.

Armero E., Collar C. (1996). Antistaling additive effects on fresh wheat bread quality. Food Science and Technology International. 2: 323–333.

Astray G., Gonzalez-Barreiro C., Mejuto JC., Rial-Otero R., Simal-Gandara J. (2009). A review on the use of cyclodextrins in foods. Food Hydrocolloids. 23: 1631-1640.

Autio K., Harkonen H., Parkkonen T., Frigard T., Potanen K., Siika-aho M., Aman P. (1996). Effects of purified endo-beta-xylanase and endo-beta-glucanase on the structural and baking characteristics of rye doughs. Food Science and Technology-Lebensmittel-Wissenschaft and Technologie. 29: 18-27.

Autio K., Kruus K. , Knaapila A., Gerber N., Flander L., Buchert J. (2005). Kinetics of transglutaminase-induced cross-linking of wheat proteins in dough. Journal of Agricultural and Food Chemistry. 53: 1039-1045.

Barrett AH., Marando G., Leung H., Kaletunc G. (2005). Effect of different enzymes on the textural stability of shelf-stable bread. Cereal Chemistry. 82: 152-157.

Baskinskiene L., Garmuviene S., Juodeikiene G., Haltrich D. (2007). Comparison of different fungal xylanases for wheat bread making. Getreidetechnologie. 61: 228-235.

Basman A., Koksel H., Ng PKW. (2002). Effects of increasing levels of transglutaminase on the rheological properties and bread quality characteristics of two wheat flours. European Food Research and Technology. 215: 419-424.

Benejam W., Steffolani ME., Leon AE. (2009). Use of enzyme to improve the technological quality of a panettone like baked product. International Journal of Food Science and Technology. 44: 2431-2437.

Biwer A., Antranikian G., Heinzle E. (2002). Enzymatic production of cyclodextrins. Applied Microbiology and Biotechnology. 59: 609-617.

Blaszczak W., Sadowska J., Rosell CM., Fornal J. (2004). Structural changes in the wheat dough and bread with the addition of alpha-amylases. European Food Research and Technology. 219: 348-354.

Bombara N., Anon MC., Pilosof AMR. (1997). Functional properties of protease modified wheat flours. Food Science and Technology-Lebensmittel-Wissenschaft and Technologie. 30: 441-447.

Bonet A., Rosell CM., Caballero PA., Gomez M., Perez-Munuera I., Lluch MA. (2006). Glucose oxidase effect on dough rheology and bread quality: A study from macroscopic to molecular level. Food Chemistry. 99: 408-415.

Bruno MEC., de Oliveira Camargo CR. (1995). Proteolytic enzymes in the processing of cookies and bread. Boletim da Sociedade Brasileira de Ciencia e Tecnologia de Alimentos. 29: 170-178.

Buleon A., Colonna P., Planchot V., Ball S. (1998). Starch granules: Structure and biosynthesis. International Journal of Biological Macromolecules. 23: 85-112.

Butt MS., Tahir-Nadeem M., Z., Sultan MT. (2008). Xylanases and their applications in baking industry. Food Technology and Biotechnology. 46: 22-31.

Caballero PA., Gomez M., Rosell CM. (2007a). Bread quality and dough rheology of enzyme-supplemented wheat flour. European Food Research and Technology. 224: 525-534.

Caballero PA., Gomez M., Rosell CM. (2007b). Improvement of dough rheology, bread quality and bread shelf-life by enzymes combination. Journal of Food Engineering. 81: 42-53.

Cabrera-Chavez F., Rouzaud-Sandez O., Sotelo-Cruz N., Calderon de la Barca AM. (2008). Transglutaminase treatment of wheat and corn prolamins of bread increases the serum iga reactivity of celiac disease patients. Journal of Agricultural and Food Chemistry. 56: 1387-1391.

Celik I., Yilmaz Y., Isik F., Ustun O. (2007). Effect of soapwort extract on physical and sensory properties of sponge cakes and rheological properties of sponge cake batters. Food Chemistry. 101: 907-911.

Collar C., Bollain C (2004). Impact of microbial transglutaminase on the viscoelastic profile of formulated bread doughs. European Food Research and Technology. 218: 139-146.

Collar C., Bollain C. (2005). Impact of microbial transglutaminase on the staling behavior of enzyme-supplemented pan breads. European Food Research and Technology. 221: 298-304.

Collar C., Andreu P., Martinez-Anaya MA. (1998). Interactive effects of flour, starter and enzyme on bread dough machinability. Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung a-Food Research and Technology. 207: 133-139.

Collar C., Martinez JC., Andreu P., Armero E. (2000). Effects of enzyme associations on bread dough performance. A response surface analysis. Food Science and Technology International. 6: 217-226.

Collins T., Hoyoux A., Dutron A., Georgis J., Genot B., Dauvrin T., Arnaut F., Gerday C., Feller G. (2006). Use of glycoside hydrolase family 8 xylanases in baking. Journal of Cereal Science. 43: 79-84.

Courtin CM., Delcour JA. (2002). Arabinoxylans and endoxylanases in wheat flour bread-making. Journal of Cereal Science. 35: 225-243.

Courtin CM., Gelders GG., Delcour JA. (2001). Use of two endoxylanases with different substrate selectivity for understanding arabinoxylan functionality in wheat flour breadmaking. Cereal Chemistry. 78: 564-571.

Dagdelen AF., Gocmen D. (2007). Effects of glucose oxidase, hemicellulase and ascorbic acid on dough and bread quality. Journal of Food Quality. 30: 1009-1022.

De Boer L., Boer LD. (2007). Enzymatic composition for decreasing acrylamide levels in the food product, e.g., bread and pastry, comprises asparaginase and at least one hydrolyzing enzyme. Dsm Ip Assets Bv, Boer L. D.

Defloor I., Delcour JA. (1999). Impact of maltodextrins and antistaling enzymes on the differential scanning calorimetry staling endotherm of baked bread doughs. Journal of Agricultural and Food Chemistry. 47: 737-741

Delcour JA., Hoseney RC. (2010). Principles of cereal science and technology authors provide insight into the current state of cereal processing. Cereal Foods World. 55: 21-22.

Denli E., Ercan R. (2001). Effect of added pentosans isolated from wheat and rye grain on some properties of bread. European Food Research and Technology. 212: 374-376.

Dogan IS. (2003). Effect of alpha-amylases on dough properties during turkish hearth bread production. International Journal of Food Science and Technology. 38: 209-216.

Dornez E., Gebruers K., Delcour JA., Courtin CM. (2009). Grain-associated xylanases: occurrence, variability, and implications for cereal processing. Trends in Food Science and Technology. 20: 495-510.

Drago SR., González RJ. (2000). Foaming properties of enzymatically hydrolysed wheat gluten. Innovative Food Science and Emerging Technologies. 1: 269-273.

Dura A., Błaszczak W., Rosell CM. (2014). Functionality of porous starch obtained by amylase or amyloglucosidase treatments. Carbohydrate Polymers. 101: 837-845.

Ertas N., Bilgicli N., Turker S. (2006). The effects of soy flour, glucose oxidase and lipase enzymes addition on flour, dough and bread properties. Gida. 31: 143-149.

Figueroa-Espinoza MC., Rouau X. (1998). Oxidative cross-linking of pentosans by a fungal laccase and horseradish peroxidase: Mechanism of linkage between feruloylated arabinoxylans. Cereal Chemistry. 75: 259-265.

Flander L., Holopainen U., Kruus K., Buchert J. (2011). Effects of tyrosinase and laccase on oat proteins and quality parameters of gluten-free oat breads. Journal of Agricultural and Food Chemistry. 59: 8385-8390.

Folk JE., Finlayson JS. (1977). The epsilon-(gamma-glutamyl) lysine crosslink and the catalytic role of transglutaminases. Advances in Protein Chemistry. 31: 1-133.

Forman TM., Evanson DN. (2011). Antistaling process for flat bread. Novozymes North America Inc.

Gelinas P., Poitras E., McKinnon CM., Morin A. (1998). Oxido-reductases and lipases as dough-bleaching agents. Cereal Chemistry. 75: 810-814.

Gerrard JA. (2002). Protein-Protein crosslinking in food: methods, consequences, applications. Trends in Food Science and Technology. 13: 391-399.

Gerrard JA., Fayle SE., Wilson AJ., Newberry MP., Ross M., Kavale S. (1998). Dough properties and crumb strength of white pan bread as affected by microbial transglutaminase. Journal of Food Science. 63: 472-475.

Gerrard JA., Newberry MP., Ross M., Wilson AJ., Fayle SE., Kavale S. (2000). Pastry lift and croissant volume as affected by microbial transglutaminase. Journal of Food Science. 65: 312-314.

Gerrard AJ., Fayle SE., Brown PA., Sutton KH., Simmons L., Rasiah I. (2001). Effects of microbial transglutaminase on the wheat proteins of bread and croissant dough. Journal of Food Science. 66: 782-786.

Gil MJ., Callejo MJ., Rodriguez G., Ruiz MV. (1999). Keeping qualities of white pan bread upon storage: Effect of selected enzymes on bread firmness and elasticity. Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung a-Food Research and Technology. 208: 394-399.

Goesaert H., Brijs K., Veraverbeke WS., Courtin CM., Gebruers K., Delcour JA. (2005). Wheat flour constituents: how they impact bread quality, and how to impact their functionality. Trends in Food Science and Technology. 16: 12-30.

Goesaert H., Courtin CM., Delcour JA. (2008). Use of enzymes in the production of cereal-based functional foods and food ingredients. 237-265. Elsevier Inc.

Goesaert H., Slade L., Levine H., Delcour JA. (2009). Amylases and bread firming-An integrated view. Journal of Cereal Science. 50: 345-352.

Gottmann K., Sproessler B. (1992). Baking agent and baking flour for bread, rolls, etc.-contg. transglutaminase as enzyme with emulsifiers, flour and sugar, 492406-B1: Roehm Gmbh (Rohg).

Gray JA., BeMiller JN. (2003). Bread staling: Molecular basis and control. Comprehensive Reviews in Food Science and Food Safety. 2: 1-21.

Gujral H., Rosell CM. (2004a). Functionality of rice flour modified with a microbial transglutaminase. Journal of Cereal Science. 39: 225-230.

Gujral H., Rosell CM. (2004b). Improvement of the breadmaking quality of rice flour by glucose oxidase. Food Research International. 37: 75-81.

Gujral H., Rosell CM. (2004c). Modification of pasting properties of wheat starch by cyclodextrin glycosyltransferase. Journal of the Science of Food and Agriculture. 84: 1685-1690.

Gujral HS., Guardiola I., Carbonell JV., Rosell CA. (2003a). Effect of cyclodextrinase on dough rheology and bread quality from rice flour. Journal of Agricultural and Food Chemistry. 51: 3814-3818.

Gujral HS., Haros M., Rosell CM. (2003b). Starch hydrolyzing enzymes for retarding the staling of rice bread. Cereal Chemistry. 80: 750-754.

Gul H., Sertac OM., Dizlek H. (2009). Improvement of the wheat and corn bran bread quality by using glucose oxidase and hexose oxidase. Journal of Food Quality. 32: 209-223.

HadiNezhad M., Butler F. (2009). Association of glutenin subunit composition and dough rheological characteristics with cookie baking properties of soft wheat cultivars. Cereal Chemistry. 86: 339-349.

Hamer RJ., Primo-Martin C. (2006). Preparing bread dough/part baked bread for baked bread involves mixing flour, water and bakery ingredients to obtain bread dough, followed by part baking to obtain part baked bread and applying enzyme material. Wageningen Center of Food Science.

Hanft F., Koehler P. (2006). Studies on the effect of glucose oxidase in bread making. Journal of the Science of Food and Agriculture. 86: 1699-1704.

Hargreaves NG., Brinker E., Scharf U., Plijter JJ., Plijter J., van Wilhemus SM., Noort MWJ., Huscroft SC. (2005). Preparation of laminated dough or part-baked laminated dough product for preparing, e.g., croissant, by mixing flour and water, laminating and part-baking resultant dough, and applying enzyme material with proteolytic activity. Csm Nederland Bv, WO2005077194A1.

Haros M., Rosell CM., Benedito C. (2001a). Fungal phytase as a potential breadmaking additive. European Food Research and Technology. 213: 317-322.

Haros M., Rosell CM., Benedito C. (2001b). Use of fungal phytase to improve breadmaking performance of whole wheat bread. Journal of Agricultural and Food Chemistry. 49: 5450-5454.

Haros M., Rosell CM., Benedito C. (2002). Effect of different carbohydrases on fresh bread texture and bread staling. European Food Research and Technology. 215: 425-430.

Hemalatha MS., Bhagwat SG., Salimath PV., Prasada RUJS. (2012). Enhancement of soluble dietary fibre, polyphenols and antioxidant properties of chapatis prepared from whole wheat flour dough treated with amylases and xylanase. Journal of the Science of Food and Agriculture. 92: 764-771.

Hoseney RC. (1994). Principles of cereal science and technology. In: Principles of Cereal Science and Technology, St. Paul, MN: American Association of Cereal Chemists.

Hoseney RC., Faubion JM. (1981). A mechanism for the oxidative gelation of wheat-flour water-soluble pentosans. Cereal Chemistry. 58: 421-424.

Houben A., Hochstotter A., Becker T. (2012). Possibilities to increase the quality in gluten-free bread production: An overview. European Food Research and Technology. 235: 195-208.

Hozova B., Kukurova I., Dodok L. (2003). Application of transglutaminase and fermizyme for sensory quality improvement of pastry. Nahrung-Food. 47: 171-175.

Hsing IL., Faubion JM., Walker CE. (2010). Using enzyme-oxidant combinations to improve frozen-dough bread quality. Getreidetechnologie. 64: 124-134.

Huang W., Jia C. (2006). Anti-freezing fermented dough containing glucose oxidase and peroxidase and its producing method. Univ. Jiangnan.

Hug-Iten S., Escher F., Conde-Petit B. (2001). Structural properties of starch in bread and bread model systems: Influence of an antistaling alpha-amylase. Cereal Chemistry. 78: 421-428.

Hug-Iten S., Escher F., Conde-Petit B. (2003). Staling of bread: Role of amylose and amylopectin and influence of starch-degrading enzymes. Cereal Chemistry. 80: 654-661.

Hui YH., Corke H., de Leyn I., Nip WK., Cross N. (2006). Bakery products. Science and technology. Hui YH., Corke H., de Leyn I., Nip WK., Cross N. (eds). Oxford, UK: Blackwell Publishing.

Indrani D., Prabhasankar P., Rajiv J., Rao GV. (2003). Scanning electron microscopy, rheological characteristics, and bread-baking performance of wheat-flour dough as affected by enzymes. Journal of Food Science. 68: 2804-2809.

Inoue S., Ota S. (1986). Bread or other cereal-based food improver composition involving the addition of phospholipase to the flour. Kyowa Hakko Kogyo Co. Ltd.

Izydorczyk MS., Biliaderis CG. (1995). Cereal arabinoxylans: Advances in structure and physicochemical properties. Carbohydrate Polymers. 28: 33-48.

Jemli S., Ben ME., Ayadi-Zouari D., Naili B., Khemakhem B., Bejar S. (2007). A beta-cyclodextrin glycosyltransferase from a newly isolated *paenibacillus pabulius* 132 strain: purification, properties and potential use in bread-making. Biochemical Engineering Journal. 34: 44-50.

Jensen B., Drube P. (1998). Effect and functionality of lipases in dough and bread. Getreide, Mehl und Brot. 52: 153-157.

Ji Y., Zhu K., Chen ZC., Zhou H., Ma J., Qian H. (2010). Effects of different additives on rice cake texture and cake staling. Journal of Texture Studies. 41: 703-713.

Jia CL., Huang WN., Abdel-Samie MAS., Huang GX., Huang GW. (2011). Dough rheological, mixolab mixing, and nutritional characteristics of almond cookies with and without xylanase. Journal of Food Engineering. 105: 227-232.

Jia Y., Li H., Tian Y. (2006). Enzyme composition as modifier dedicated to flour for steamed bread. Univ. Hebei Agric.

Jiang ZQ., Yang SQ., Tan SS., Li LT., Li XT. (2005). Characterization of a xylanase from the newly isolated thermophilic *thermomyces lanuginosus cau44* and its application in bread making. Letters in Applied Microbiology. 41: 69-76.

Jiang Z., Cong Q., Yan Q., Kumar N., Du X. (2010). Characterisation of a thermostable xylanase from *chaetomium sp* and its application in chinese steamed bread. Food Chemistry. 120: 457-462.

Jimenez T., Martinez-Anaya MA. (2000). Characterization of water soluble pentosans of enzyme supplemented dough and breads. Food Science and Technology International. 6: 197-205.

Hyuck JA., Yong LS., Seok YS., Son HJ., Lee JW., Lee SJ., Kim YB., Park KH. (2005). A novel maltopentaose-Producing amylase as a bread antistaling agent. Food Science and Biotechnology. 14: 681-684.

Junqueira RM., Rocha F., Moreira MA., Castro IA. (2007). Effect of proofing time and wheat flour strength on bleaching, sensory characteristics, and volume of french breads with added soybean lipoxygenase. Cereal Chemistry. 84: 443-449.

Kara M., Sivri D., Koksel H. (2005). Effects of high protease-Activity flours and commercial proteases on cookie quality. Food Research International. 38: 479-486.

Katina K., Salmenkallio-Marttila M., Partanen R., Forssell P., Autio K. (2006). Effects of sourdough and enzymes on staling of high-fibre wheat bread. LWT-Food Science and Technology. 39: 479-491.

Kawamura-Konishi Y., Shoda K., Koga H., Honda Y. (2013). Improvement in gluten-free rice bread quality by protease treatment. Journal of Cereal Science. 58: 45-50.

Kelly RM., Dijkhuizen L., Leemhuis H. (2009). Starch and α-glucan acting enzymes, modulating their properties by directed evolution. Journal of Biotechnology. 140: 184-193.

Kim DC., Kim H., Kim OW., Kim SS., Lee SE., Park JH. (2009). Preparing gluten-free bread premix, involves blending rice flour, dried albumin, cooking oil, sugar, yeast, emulsifier, salt, enzyme conjugate comprising hemicellulase, glucose oxidase, xylanase and amylase, and agitating mixture. Korea Food Res Inst.

Kim Y., Kim YL., Trinh KS., Kim YR., Moon TW. (2012). Texture properties of rice cakes made of rice flours treated with 4-alpha-glucanotransferase and their relationship with structural characteristics. Food Science and Biotechnology. 21: 1707-1714.

Kong XZ., Zhou HM., Qian HF. (2007). Enzymatic preparation and functional properties of wheat gluten hydrolysates. Food Chemistry. 101: 615-620.

Koocheki A., Mortazavi SA., Mahalati MN., Karimi M. (2009). Effect of emulsifiers and fungal alpha-amylase on rheological characteristics of wheat dough and quality of flat bread. Journal of Food Process Engineering. 32: 187-205.

Kukurova K., Morales FJ., Bednarikova A., Ciesarova Z. (2009). Effect of l-asparaginase on acrylamide mitigation in a fried-dough pastry model. Molecular Nutrition and Food Research. 53: 1532-1539.

Labat E., Morel MH., Rouau X. (2000). Effects of laccase and ferulic acid on wheat flour doughs. Cereal Chemistry. 77: 823-828.

Laurikainen T., Harkonen H., Autio K., Poutanen K. (1998). Effects of enzymes in fibre-enriched baking. Journal of the Science of Food and Agriculture. 76: 239-249.

Lebesi DM., Tzia C. (2011). Staling of cereal bran enriched cakes and the effect of an endoxylanase enzyme on the physicochemical and sensorial characteristics. Journal of Food Science. 76: 380-387.

Lebesi DM., Tzia C. (2012). Use of endoxylanase treated cereal brans for development of dietary fiber enriched cakes. Innovative Food Science and Emerging Technologies. 13: 207-214.

Leon AE., Duran E., de Barber CB. (2002). Utilization of enzyme mixtures to retard bread crumb firming. Journal of Agricultural and Food Chemistry. 50: 1416-1419.

Li Z., Tang X., Huang W., Liu JG., Tilley M., Yao Y. (2011). Rheology, microstructure, and baking characteristics of frozen dough containing *rhizopus chinensis* lipase and transglutaminase. Cereal Chemistry. 88: 596-601.

MacRitchie F. (1983). Role of lipids in baking. In: Lipids in Cereal Technology. Barnes PJ (ed). London: Academic Press. 165-188.

MacRitchie F. (1992). Physicochemical properties of wheat proteins in relation to functionality. Advances in Food and Nutrition Research. 36: 1-87.

Maeda T., Morita N. (2003). Flour quality and pentosan prepared by polishing wheat grain on breadmaking. Food Research International. 36: 603-610.

Marco C., Rosell CM. (2008a). Breadmaking performance of protein enriched, gluten-free breads. European Food Research and Technology. 227: 1205-1213.

Marco C., Rosell CM. (2008b). Effect of different protein isolates and transglutaminase on rice flour properties. Journal of Food Engineering. 84: 132-139.

Marco C., Rosell CM. (2008c). Functional and rheological properties of protein enriched gluten free composite flours. Journal of Food Engineering. 88: 94-103.

Marco C., Perez G., Ribotta P., Rosell CM. (2007). Effect of microbial transglutaminase on the protein fractions of rice, pea and their blends. Journal of the Science of Food and Agriculture. 87: 2576-2582.

Marco C., Perez G., Leon AE., Rosell CM. (2008). Effect of transglutaminase on protein electrophoretic pattern of rice, soybean, and rice-soybean blends. Cereal Chemistry. 85: 59-64.

Martinez-Anaya MA., Jimenez T. (1997a). Functionality of enzymes that hydrolyse starch and non-starch polysaccharide in breadmaking. Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung a-Food Research and Technology. 205: 209-214.

Martinez-Anaya MA., Jimenez T. (1997b). Rheological properties of enzyme supplemented doughs. Journal of Texture Studies. 28: 569-583.

Martinez-Anaya MA., Jimenez T. (1997c). Physical properties of enzyme-supplemented doughs and relationship with bread quality parameters. Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung a-Food Research and Technology. 206: 134-142.

Martinez-Anaya MA., Devesa A. (2000). Influence of enzymes in sourdough wheat breadmaking. Changes in pentosans. Food Science and Technology International. 6: 109-116.

Mathewson PR. (1998). Common enzyme reactions. Cereal Foods World. 43: 798-803.

Min BC., Yoon SH., Kim JW., Lee YW., Kim YB., Park KH. (1998). Cloning of novel maltooligosaccharide-Producing amylases as antistaling agents for bread. Journal of Agricultural and Food Chemistry. 46: 779-782.

Moayedallaie S., Mirzaei M., Paterson J. (2010). Bread improvers: comparison of a range of lipases with a traditional emulsifier. Food Chemistry. 122: 495-499.

Moharrami E., Shahedi BM. (2011). Optimization of flour alpha-amylase activity with germinated wheat flour and its effect on staling of taftoon bread. Iranian Journal of Food Science and Technology. 8: 23-33.

Morita N., Arishima Y., Tanaka N., Shiotsubo S. (1997). Utilization of hemicellulase as bread improver in a home baker. Journal of Applied Glycoscience. 44: 143-152.

Motoki M., Seguro K. (1998). Transglutaminase and its use for food processing. Trends in Food Science and Technology. 9: 204-210.

Muchembled JJ., Julien P. (2005). Improver for frozen dough for bread, viennese pastries and brioches comprises gluten, acerola and/or cynorrhodon and/or camu-camu powder and enzymatic preparations providing hemicellulase and alpha-amylase. Lesaffre & Cie Sa.

Nielsen PM. (2009). Producing a water-in-oil emulsion useful for making bakery product, e.g., croissant, danish pastry, pie, pastry involves mixing a composition comprising oil, water, phospholipase, and phosphatide. Hansen as Chr; Novozymes As.

Nonaka M., Tanaka H., Okiyama A., Motoki M., Ando H., Unda K., Matsura A. (1989). Polymerization of several proteins by ca-2+-independent transglutaminase derived from microorganisms. Agricultural and Biological Chemistry. 53: 2619-2623.

Novozymes AS. (2001). Combined use of glucose oxidase and phospholipase for baking. Research Disclosure. 442: 231.

Oliveira DS., Telis-Romero J., Da-Silva R., Franco CML. (2014). Effect of a thermoascus aurantiacus thermostable enzyme cocktail on wheat bread quality. Food Chemistry. 143: 139-146.

Onyango C., Mutungi C., Unbehend G., Lindhauer MG. (2010a). Batter rheology and bread texture of sorghum-Based gluten-free formulations modified with native or pregelatinised cassava starch and alpha-amylase. International Journal of Food Science and Technology. 45: 1228-1235.

Onyango C., Mutungi C., Unbehend G., Lindhauer MG. (2010b). Rheological and baking characteristics of batter and bread prepared from pregelatinised cassava starch and sorghum and modified using microbial transglutaminase. Journal of Food Engineering. 97: 465-470.

Oort M. (1996). Oxidases in baking. A review of the uses of oxidases in bread making. International Food Ingredients. 4: 42-47.

Oort M., van Straaten F., Laane C. (1995). Pentosans and pentosanases in bread making. International Food Ingredients. 2: 23-27.

Osborne TB. (1924). The vegetable proteins, 2nd edition. Longmans Green and Co, London.

Palacios HR., Schwarz PB., D'Appolonia BL. (2004). Effect of alpha-amylases from different sources on the retrogradation and recrystallization of concentrated wheat starch gels: Relationship to bread staling. Journal of Agricultural and Food Chemistry. 52: 5978-5986.

Pandey A., Nigam P., Soccol CR., Soccol VT., Singh D., Mohan R. (2000). Advances in microbial amylases. Biotechnology and Applied Biochemistry. 31: 135-152.

Pareyt B., Finnie SM., Putseys JA., Delcour JA. (2011). Lipids in bread making: Sources, interactions, and impact on bread quality. Journal of Cereal Science. 54: 266-279.

Pena-Valdivia CB., Salazar-Zazueta A. (1997). Effect of amylases and proteases on bread making quality of durum wheat flour. Journal of Food Science and Technology-Mysore. 34: 516-518.

Penella JMS., Collar C., Haros M. (2008). Effect of wheat bran and enzyme addition on dough functional performance and phytic acid levels in bread. Journal of Cereal Science. 48: 715-721.

Pongjaruvat W., Methacanon P., Seetapan N., Fuongfuchat A., Gamonpilas C. (2014). Influence of pregelatinised tapioca starch and transglutaminase on dough rheology and quality of gluten-free jasmine rice breads. Food Hydrocolloids. 36: 143-150.

Poulsen C., Hostrup PB. (1998). Purification and characterization of a hexose oxidase with excellent strengthening effects in bread. Cereal Chemistry. 75: 51-57.

Poulson CH., Soe JB., Rasmussen P., Madrid SM., Zargahi MR. (2006). Lipase and use of same for improving doughs and baked products. Danisco A/S.

Poulson CH., Soe JB., Rasmussen P., Madrid SM., Zargahi MR. (2010). Lipase and use of same for improving doughs and baked products. Danisco A/S.

Prabhasankar P., Indrani D., Jyotsna R., Rao GV. (2004). Influence of enzymes on rheological, microstructure and quality characteristics of parotta-an unleavened indian flat bread. Journal of the Science of Food and Agriculture. 84: 2128-2134.

Primo-Martin C., Valera R., Martinez-Anaya MA. (2003). Effect of pentosanase and oxidases on the characteristics of doughs and the glutenin macropolymer (Gmp). Journal of Agricultural and Food Chemistry. 51: 4673-4679.

Primo-Martin C., Wang MW., Lichtendonk WJ., Plijter JJ., Hamer RJ. (2005). An explanation for the combined effect of xylanase-glucose oxidase in dough systems. Journal of the Science of Food and Agriculture. 85: 1186-1196.

Primo-Martin C., de Beukelaer H., Hamer RJ., Van Vliet T. (2008). Fracture behaviour of bread crust: Effect of ingredient modification. Journal of Cereal Science. 48: 604-612.

Purhagen JK., Sjoo ME., Eliasson AC. (2011). Starch affecting anti-staling agents and their function in freestanding and pan-baked bread. Food Hydrocolloids. 25: 1656-1666.

Rani RR. (2011). Microbial isoamylases: An overview. American Journal of Food Technology. 6: 1-18.

Rao MB., Tanksale AM., Ghatge MS., Deshpande VV. (1998). Molecular and biotechnological aspects of microbial proteases. Microbiology and Molecular Biology Reviews. 62: 597+.

Rasiah IA., Sutton KH., Low FL., Lin HM., Gerrard JA. (2005). Crosslinking of wheat dough proteins by glucose oxidase and the resulting effects on bread and croissants. Food Chemistry. 89: 325-332.

Renzetti S., Arendt EK. (2009). Effect of protease treatment on the baking quality of brown rice bread: From textural and rheological properties to biochemistry and microstructure. Journal of Cereal Science. 50: 22-28.

Renzetti S., Dal BF., Arendt EK. (2008). Microstructure, fundamental rheology and baking characteristics of batters and breads from different gluten-free flours treated with a microbial transglutaminase. Journal of Cereal Science. 48: 33-45.

Renzetti S., Courtin CM., Delcour JA., Arendt EK. (2010). Oxidative and proteolytic enzyme preparations as promising improvers for oat bread formulations: Rheological, biochemical and microstructural background. Food Chemistry. 119: 1465-1473.

Ribotta PD., Le Bail A. (2007). Thermo-physical assessment of bread during staling. LWT-Food Science and Technology. 40: 879-884.

Rojas JA., Rosell CM., Benedito C. (2001). Role of maltodextrins in the staling of starch gels. European Food Research and Technology. 212: 364-368.

Rosell CM. (2009). Enzymatic manipulation of gluten-free breads. In: Gluten-Free Food Science and Technology. Gallagher E. (ed). Wiley-Blackwell. 83-98.

Rosell CM. (2011). The science of doughs and bread quality. In: Flour and Breads and their Fortification in Health and Disease Prevention. Elsevier Inc. 3-14.

Rosell CM. (2012). The nutritional enhancement of wheat flour. In: Breadmaking: Improving Quality, 2nd edition. Cauvain S. (ed), Cambridge, UK: Woodhead Publishing. 687-710.

Rosell CM., Solis Nadal DR. (2004). Composition for improving baking doughs and baked products, comprises a cyclodextrin glucanotransferase and optionally starch debranching enzyme, and particularly has an antistaling effect. Consejo Superior Investigaciones Cientificas. Aplicaciones Enzimaticas Alimentarias.

Rosell CM., Collar C. (2008). Effect of various enzymes on dough rheology and bread quality. In: Recent Research Developments in Food Biotechnology. Enzymes as Additives or Processing Aids. Porta R., P. Di Pierro P., Mariniello L (eds). Research Signpost, Kerala, India. 165-183.

Rosell CM., Garzon R. (2014). Chemical composition of bakery products. In: Handbook of Food Chemistry. Berlin: Springer-Verlag.

Rosell CM., Haros M., Escriva C., Benedito C. (2001). Experimental approach to optimize the use of alpha-amylases in breadmaking. Journal of Agricultural and Food Chemistry. 49: 2973-2977.

Rosell CM., Rojas JA., Benedito C. (2001c). Combined effect of different antistaling agents on the pasting properties of wheat flour. European Food Research and Technology. 212: 473-476.

Rosell CM., Wang J., Aja S., Bean S., Lookhart G. (2003). Wheat flour proteins as affected by transglutaminase and glucose oxidase. Cereal Chemistry. 80: 52-55.

Rosell CM., Santos E., Sanz JM., Haros M. (2009). Wholemeal wheat bread: A comparison of different breadmaking processes and fungal phytase addition. Journal of Cereal Science. 50: 272-277.

Selinheimo E., Kruus K., Buchert J., Hopia A., Autio K. (2006). Effects of laccase, xylanase and their combination on the rheological properties of wheat doughs. Journal of Cereal Science. 43: 152-159.

Selinheimo E., Autio K., Krijus K., Buchert J. (2007). Elucidating the mechanism of laccase and tyrosinase in wheat bread making. Journal of Agricultural and Food Chemistry. 55: 6357-6365.

Shafisoltani M., Salehifar M., Hashemi M. (2014). Effects of enzymatic treatment using response surface methodology on the quality of bread flour. Food Chemistry. 148: 176-183.

Shah AR., Shah RK., Madamwar D. (2006). Improvement of the quality of whole wheat bread by supplementation of xylanase from *aspergillus foetidus*. Bioresource Technology. 97: 2047-2053.

Shaikh IM., Ghodke SK., Ananthanarayan L. (2008). Inhibition of staling in chapati (indian unleavened flat bread). Journal of Food Processing and Preservation. 32: 378-403.

Si JQ. (1994). Use of laccase in baking. Report Novo Nordisk A/S.

Sirbu A., Paslaru V. (2007). Phospholipase using as bread-making improver. Journal of Environmental Protection and Ecology. 8: 1-10.

Smerdel B., Pollak L., Novotni D.,Cukelj N., Benkovic M., Lusic D., Curic D. (2012). Improvement of gluten-free bread quality using transglutaminase, various extruded flours and protein isolates. Journal of Food and Nutrition Research. 51: 242-253.

Sozer N., Bruins R., Dietzel C., Franke W., Kokini JL. (2011). Improvement of shelf life stability of cakes. Journal of Food Quality. 34: 151-162.

Steffolani ME., Ribotta PD., Pérez GT., León AE. (2010). Effect of glucose oxidase, transglutaminase, and pentosanase on wheat proteins: Relationship with dough properties and bread-making quality. Journal of Cereal Science. 51: 366-373.

Steffolani ME., Ribotta PD., Perez GT., Puppo MC., Leon AE. (2012). Use of enzymes to minimize dough freezing damage. Food and Bioprocess Technology. 5: 2242-2255.

Stojceska V., Ainsworth P. (2008). The effect of different enzymes on the quality of high-fibre enriched brewer's spent grain breads. Food Chemistry. 110: 865-872.

Storck CR., Zavareze ED., Gularte MA., Elias MC., Rosell CM., Dias ARG. (2013). Protein enrichment and its effects on gluten-free bread characteristics. LWT-Food Science and Technology. 53: 346-354.

Sung-Ho L., Young-Wan K., Suyong L., Auh JH., Yoo SS., Kim TJ., Kim JW., Kim ST., Rho HJ., Choi JH., Kim YB., Park KH. (2002). Modulation of cyclizing activity and thermostability of cyclodextrin glucanotransferase and its application as an antistaling enzyme. Journal of Agricultural and Food Chemistry. 50: 1411-1415.

Tester RF., Karkalas J., Qi X. (2004). Starch-Composition, fine structure and architecture. Journal of Cereal Science. 39: 151-165.

Trogh I., Sorensen JF., Courtin CM., Delcour JA. (2004). Impact of inhibition sensitivity on endoxylanase functionality in wheat flour breadmaking. Journal of Agricultural and Food Chemistry. 52: 4296-4302.

Van Der Borght A., Goesaert H., Veraverbeke WS., Delcour JA. (2005). Fractionation of wheat and wheat flour into starch and gluten: Overview of the main processes and the factors involved. Journal of Cereal Science. 41: 221-237.

van der Maarel MJEC., van der Veen B., Uitdehaag JCM., Leemhuis H., Dijkhuizen L. (2002). Properties and applications of starch-converting enzymes of the α-amylase family. Journal of Biotechnology. 94: 137-155.

Vemulapalli V., Hoseney RC. (1998). Glucose oxidase effects on gluten and water solubles. Cereal Chemistry. 75: 859-862.

Veraverbeke WS., Delcour JA. (2002). Wheat protein composition and properties of wheat glutenin in relation to breadmaking functionality. Critical Reviews in Food Science and Nutrition. 42: 179-208.

Wen J., Lin J., Wang X., Zhao J. (2003). Effects of enzymes on the rheologic peculiarities and food making properties of flour dough. Food Science and Technology. 4: 44-46.

Wikstrom K., Eliasson AC. (1998). Effects of enzymes and oxidizing agents on shear stress relaxation of wheat flour dough: additions of protease, glucose oxidase, ascorbic acid, and potassium bromate. Cereal Chemistry. 75: 331-337.

World Health Organization, Geneva. (2006). Safety evaluation of certain contaminants in food. Prepared by the Sixty-Fourth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (Jecfa). FAO Food and Nutrition Paper. 82: 1-778.

Yoo J., Lamsal BP., Haque E., Faubion JM. (2009). Effect of enzymatic tempering of wheat kernels on milling and baking performance. Cereal Chemistry. 86: 122-126.

Zhang C., Zhang S., Lu Z., Bie X., Zhao H., Wang X., Lu F. (2013). Effects of recombinant lipoxygenase on wheat flour, dough and bread properties. Food Research International. 54: 26-32.

Zyla K., Mika M., Gambus H., Nowotny A., Szymczyk B. (2005). Fungal phytases in wholemeal breadmaking. I: 3-phytase a improves storage stability and *in vitro* digestibility of nutrients in wheat breads. Electronic Journal of Polish Agricultural Universities 8, no 4.

Chapter 2

Functionality of porous starch obtained by amylase or amyloglucosidase treatments

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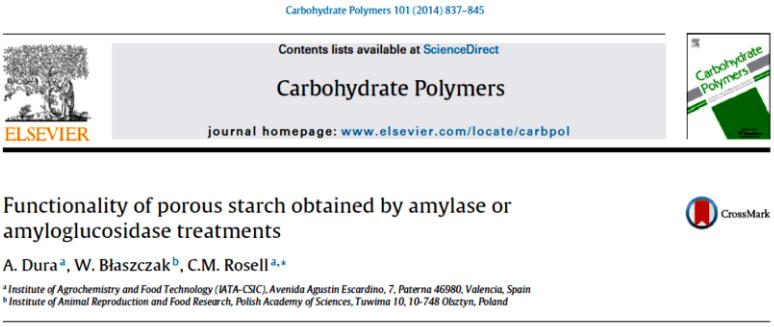
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Functionality of porous starch obtained by amylase or amyloglucosidase treatments

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Abstract

Porous starch is attracting very much attention for its absorption and shielding ability in many food applications. The effect of two different enzymes, fungal α-amylase (AM) or amyloglucosidase (AMG), on corn starch at sub-gelatinization temperature was studied as an alternative to obtain porous starch. Biochemical features, thermal and structural analyses of treated starches were studied. Microscopic analysis of the granules confirmed the enzymatic modification of the starches obtaining porous structures with more agglomerates in the case of AMG treated starches. Several changes in thermal properties and hydrolysis kinetics were observed in enzymatically modified starches. Hydration properties were significantly affected by enzymatic modification being greater influenced by AMG activity, and the opposite trend was observed in the pasting properties. Overall, results showed that enzymatic modification at sub-gelatinization temperatures really offer an attractive alternative for obtaining porous starch granules to be used in a variety of foods applications.

Keywords: corn starch; enzymatic modification; hydration properties; SEM; DSC; hydrolysis kinetics.

1. Introduction

Starch is widely used in food and industrial applications asa thickener, colloidal stabilizer, gelling agent, bulking agent and water retention agent (Singh et al., 2007). In general, native starches produce weak-bodied, cohesive, rubbery pastes when heated and undesirable gels when the pastes are cooled (Abbas et al., 2010). In order to meet its intended function, physical, chemical or enzymatic modification sare applied to achieve the functional properties not found in native starches (Jayakody and Hoover, 2002; Lacerda et al., 2008). The use of enzymatic modification has a number of advantages comprising fewer by-products, more specific hydrolysis products and high yield, besides better control of the process and end products with particular properties. There are many enzymes used to alter starch structure and to achieve desired functionality (Rosell and Collar, 2008). Enzymes hydrolyze (1 → 4) or (1 → 6) linkages between α-D-glucopyranosyl residues. The most common enzymes for starch modification include α-amylase, β-amylase, glucoamylase, pullulanase, and isoamylase. α-Amylase can hydrolyze the (1 → 4)-α-glucosidic bonds of starch in an endo-action. Hydrolysis occurs in a random fashion at any (1 → 4)-linkage within the starch chain to rapidly reduce the molecular size of starch and the viscosity of the starch solution during pasting. Amyloglucosidase is an exo-acting enzyme that catalyzes the hydrolysis of both α-D-(1 → 4) and α-D-(1 → 6)-linkages from the non-reducing ends of the starch chain. Numerous researchers have investigated enzymatic hydrolysis of starches from cereals, roots, tubers, and legumes in terms of enzyme adsorption, action pattern, extent of hydrolysis, degree of crystallinity and hydrolysis products (Colonna et al., 1992; Gallant et al., 1992; Hoover, 2001; Kimura and Robyt, 1996; Li et al., 2004). Taking into particular account that corn starch makes up more than 80% of the world market for starch, many researchers have been focused on the hydrolysis products released from the enzymatic reaction (Huang et al., 2010; Khatoon et al., 2009; Li and Ma, 2011;Miao et al., 2011).

Therefore, kinetics mechanism of the enzymatic reaction and hydrolysis products have been studied by other researchers but the features of the modified starches have been the focus of a few studies. Simultaneous hydrolysis of waxy corn starch with amylase and amyloglucosidase have been reported for modifying the digestion of the starch (Miao et al., 2011), and reducing the digestibility of corn starch by partial α-amylase treatment (Han et al., 2006), or through the action of α-amylase or maltogenic α-amylase with transglucosidase (Ao et al., 2007). In addition, Lacerda et al. (2008) studied the thermal properties of corn starch treated with fungal α-amylase showing higher action on the amorphous region of the granule. Lately, there is an increasing interest for the developing corn porous starch due to it has interesting properties for being used in the areas of food, medicine, chemical industry, cosmetics, agriculture and other fields. In fact, porous starch might be used in foods to ensure a steady release of spices, sweeteners, acid condiments, flavorings, or even to protect from light or oxygen highly oxidized compounds (Zhang et al., 2012). Porous starch is a modified starch that contains micro sized pores on the surface and could be extended to the inner part of the granule. Previously, it has been reported its production by glucoamylase catalysis combined with ultrasonic treatment (Wu et al., 2011) and more recently Zhang et al. (2012) proposed the combination of α-amylase and glucoamylase optimizing the kinetic reaction for increasing the yield. Nevertheless, there is no information about the contribution of each enzyme to the starch changes. Because of modified starch is widely used in food formulations, it is of particular interest to determine biochemical features of starch and how they affect its functional properties. The aim of this research was to determine the independent effect of fungal α-amylase (AM) or amyloglucosidase (AMG) on corn starch at sub-gelatinization temperature, with special emphasis on biochemical features, thermal and structural analyses of treated starches.

2. Materials and methods

2.1. Materials

Corn starch samples were generously supplied by Huici Leidan (Navarra, Spain). The enzymes used were of food grade. Fungal α-amylase (AM) (Fungamyl 2500 SG) and amyloglucosidase (AMG) (Amyloglucosidase 1100L) were provided by Novozymes (Bagsværd, Denmark). Chemical reagents from Sigma-Aldrich (Madrid, Spain) were of analytical grade.

2.2. Methods

2.2.1. Sample preparation

Preliminary assays were carried out for optimizing enzymatic reactions (starch quantity and pH), and pH 4.0 was selected for AMG reaction and pH 6.0 in the case of AM modification. The quantity of enzymes was based on previous experiments, where the amount of enzyme required to hydrolyze 50% of the starch (15%, w/v) at 95 ºC for 10 min was selected (Dura et al., 2013).

Corn starch (5.0 g) was suspended in 25 mL of 20 mM NaH_2PO_4 buffer at pH 6.0 or in sodium acetate buffer at pH 4.0, those starch samples were referred as Control-6 or Control-4, respectively. For obtaining the enzymatic treated starches, enzymes (4 U of AMG /g starch and 5 U of AM /g starch) were added to the starch suspension. Samples were kept in a shaking water bath (50 rpm) at 50 ºC for 24 hours. Then, 50 mL of water were added and suspensions were homogenized with a Polytron Ultraturrax homogenizer IKA-T18 (IKA works, Wilmington, USA) during 1min at speed 3. Samples were centrifuged for 15 min at 7,000×g and 4 ºC. Starches were washed again and centrifuged at the same conditions as before. Supernatants were pooled together and boiled in a water bath for 10 min to inactivate the enzymes before any further analyses (hydration properties and iodine binding values). Sediments containing starch were freeze-dried and kept at -25 ºC for further

thermal, biochemical and microstructural analyses. Four batches were prepared for each treatment.

2.2.2. Scanning electron microscopy (SEM)

The structural properties of the samples were studied using a JSM 5200 scanning electron microscope (JEOL, Tokyo, Japan). The corn starch powders were stick on a specimen holder using cuprum tape, and then coated with gold in a vacuum evaporator (JEE 400, JEOL, Tokyo, Japan). The obtained specimens were examined at an accelerating voltage of 10 kV.

The size of the holes induced by enzymatic action was measured using the image analysis program (Image J, UTHSCSA Image Tool software). Value was the average of 10 independent measurements. Eccentricity (e) was calculated for starch granules according to the equation 1:

$e = [1-(b^2/a^2)] \times 1/2$ Eq. (1)

where a corresponded to the larger diameter (length) and b to the shorter diameter (width) (Rojas et al., 2000). Values of eccentricity approaching 0 indicate very rounded structures, while values approaching 1 describe very elongated structures.

2.2.3. Starch content

Remaining free sugars on the treated starches were determined. Treated starch (0.1 g) placed in 10 mL Pyrex tubes was suspended in 2 mL of ethanol (80%) and incubated at 85 ºC in a shaking water bath (50 rpm) for five min and then centrifuged (1,000×g, 10 min, at room temperature). Supernatant was separated to measured free sugars released. This was performed for two times.

Damage starch was also determined by enzymatic method following the International Association for Cereal Chemistry standard method (ICC, 1996).

The absorbance was measured using an Epoch microplate reader (Biotek Instruments, Winooski, USA) at 510 nm. Starch was calculated as glucose (mg) ×0.9. Replicates (n=4) were carried out for each determination.

2.2.4. Starch hydration properties and iodine binding values

Swelling parameters and water soluble compounds of modified corn starch samples were determined following the method reported by Toyokawa et al. (1989), with slight modification as reported (Rosell et al., 2011). Briefly, the supernatant was decanted into an evaporating dish and the weight of dry solids was recovered by evaporating the supernatant at 70 ºC till constant weight. Four replicates were made for each sample. Residues (Wr) and dried supernatants (Ws) were weighed. Swelling power (SP), solubility index (SI) and swelling capacity (SC) were calculated as follows:

Swelling Capacity (g/g)=Wr/Wi Eq. (2)

Solubility Index (%)=(Ws/Wi) x 100 Eq. (3)

Swelling Power (g/g)=Wr/Wi (100-SI) Eq. (4)

where Wi was the sample weight (g, db).

Iodine binding values are indicative of amylose complex formation. The iodine binding value was determined in the soluble supernatant. The soluble supernatant (40 µL) was mixed with 2 mL of an aqueous solution of 0.2% KI and 0.65% I_2. The absorbance at 690 nm was measured using a spectrophotometer (UV mini-1240, Shimadzu Corporation, Kyoto, Japan). Paste Clarity was directly measured in the supernatant as the absorbance at 650 nm using a spectrophotometer (UV mini-1240, Shimadzu Corporation, Kyoto, Japan). Values were the average from four replicates.

2.2.5. Pasting properties

The pasting properties were determined with rapid visco analyzer (RVA) (Newport Scientific, model 4-SA, Warriewood, Australia) by following the American Association of Cereal Chemists Approved Method (AACC International, 1997), with some minor modifications. Distilled water (25 mL) was added to two grams of corn starches placed into the aluminium RVA canister. RVA settings during assessment were: heating from 50 to 95 ºC in 282 s, holding at 95 ºC for 150 s and then cooling to 50 ºC. Each cycle was initiated by a 10 s, 960 rpm paddle speed for mixing followed by a 160 rpm paddle speed for the rest of the assay. Viscosity was recorded during a heating-cooling cycle using Thermocline software for Windows (Newport Scientific Pty. Limited, Warriewood, Australia).

2.2.6. Thermal Properties

Thermal behavior from starch samples were determined using a differential scanning calorimeter (DSC) from Perkin-Elmer (DSC 7, Perkin-Elmer Instruments, Norwalk, CT), equipped with a thermal analysis data station (Pyris software, Perkin-Elmer Instruments, Norwalk, CT). For the study, corn starch samples were accurately weighed into aluminum DSC pans, and de-ionized water was added by micropipette to achieve a water-sample ratio of 2:1. The sample pans were sealed and equilibrated at room temperature for one hour before analysis. Nitrogen was used to purge analyses cells. Instruments were calibrated with indium, using an empty pan as reference. Thermal analysis consisted on heating from 30 to 120 °C at a rate of 10 °C/min. The onset temperature T_o, peak temperature T_p, and conclusion temperature T_c, were determined from the heating DSC curves. Gelatinization enthalpy (ΔH) was evaluated based on the area of the main endothermic peak, and peak height index (PHI) was calculated as PHI= ΔH/ T_p- T_o. All DSC experiments were replicated two times.

2.2.7. Starch hydrolysis kinetics

Starch hydrolysis was measured following the method described by Gularte and Rosell, (2011) with minor modifications. Briefly, for free sugars removal, corn starch sample (0.1 g) suspended in two milliliters of 80% ethanol was kept in a shaking water bath at 85 ºC for five minutes, and then centrifuged for 10 min at 1000×g. The remaining pellet was incubated with porcine pancreatic α-amylase (6 U/mL) (Type VI-B, ≥ 10 units/mg solid, Sigma Chemical, St. Louis, USA) in 10 mL of 0.1 M sodium maleate buffer (pH 6.9) in a shaking water bath at 37 ºC. Aliquots of 200 µL were withdrawn during the incubation period and mixed with 200 µL of ethanol (96%, w/w) to stop the enzymatic reaction and the sample was centrifuged at 10,000×g for 5 min at 4 ºC. The precipitate was washed twice with 50% ethanol (200 µL) and the supernatants were pooled together and kept at 4 ºC for further glucose enzymatic release.

Supernatant (100 µL) was diluted with 850 µL of 0.1M sodium acetate buffer (pH 4.5) and incubated with 50 µL amyloglucosidase (33 U/mL) at 50 ºC for 30 min in a shaking water bath. After centrifuging at 2000×g for 10 min, supernatant was kept for glucose determination.

The glucose content was measured using a glucose oxidase-peroxidase (GOPOD) kit (Megazyme, Dublin, Ireland). The absorbance was measured using an Epoch microplate reader (Biotek Instruments, Winooski, USA) at 510 nm. Starch was calculated as glucose (mg) ×0.9. Replicates (n=4) were carried out for each determination.

Experimental data were fitted to a first-order equation (Goñi et al., 1997):

$$C_t = C_\infty (1\text{-}e^{-kt}) \quad \text{Eq. 5}$$

Where C_t is the concentration of product at time t, C_∞ is the concentration at the end point, and k is the pseudo-first order rate constant.

2.2.8. Statistical analysis

Experimental data were statistically analyzed for analysis of variance (ANOVA) using Statgraphics Centurion XV software (Bitstream, Cambridge, N). When analysis of variance indicated significant F values, multiple sample comparisons were also performed by Fisher's least significant differences (LSD) test to differentiate means with 95% confidence. The correlation matrix was also performed using Statgraphics Centurion XV software.

3. Results and discussion

Enzymatic modification of the corn starch was carried out independently with α-amylase or amyloglucosidase under optimal pH conditions, 6.0 for α-amylase and 4.0 for amyloglucosidase. The action of each enzyme was compared with their specific control, which was submitted to the same treatment in the absence of enzymes. Results will reflect the effect of the pH and the enzymes on the starch features after being treated at sub-gelatinization temperature (50 ºC). Zhang et al. (2012) found that 50 ºC was the most suitable temperature for obtaining porous starch combining the action of AM and AMG. Also that temperature was useful for keeping the hydrolysis degree under control impeding the collapse and breakage of the granules that is produced during gelatinization.

3.1. Microstructure of the starch

To confirm the enzymatic action on the corn starch granules treated with α-amylase or amyloglucosidase, the microstructure was analyzed using SEM at two different magnifications 2000x (Figure 1) and 3500x (Figure 2) times.

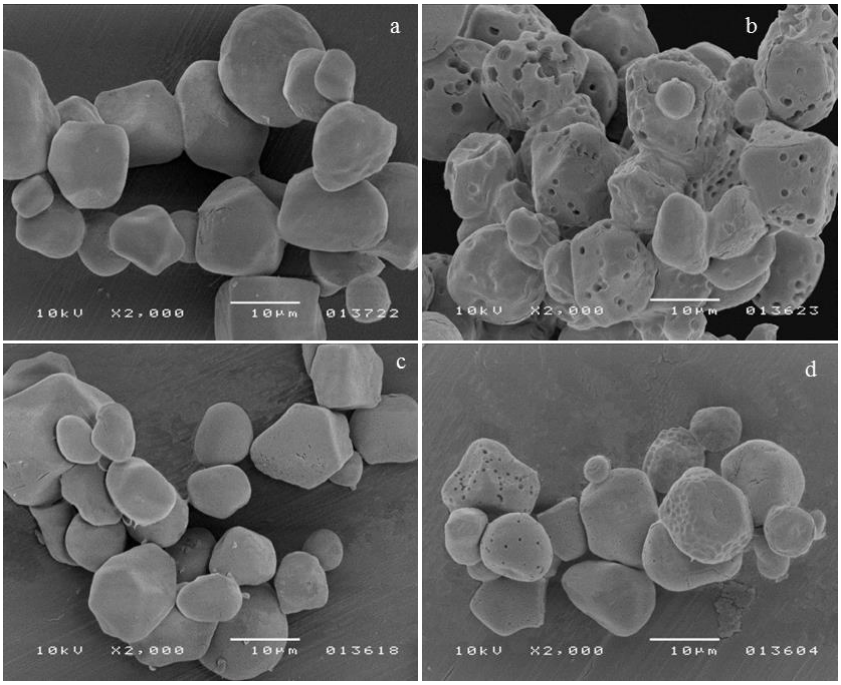

Figure 1. Scanning electron micrograph of corn starch samples treated enzymatically (b, d) and their counterparts controls (a, c). Magnification 2000x. Control pH 4 (a); AMG (b); Control pH 6 (c); AM (d).

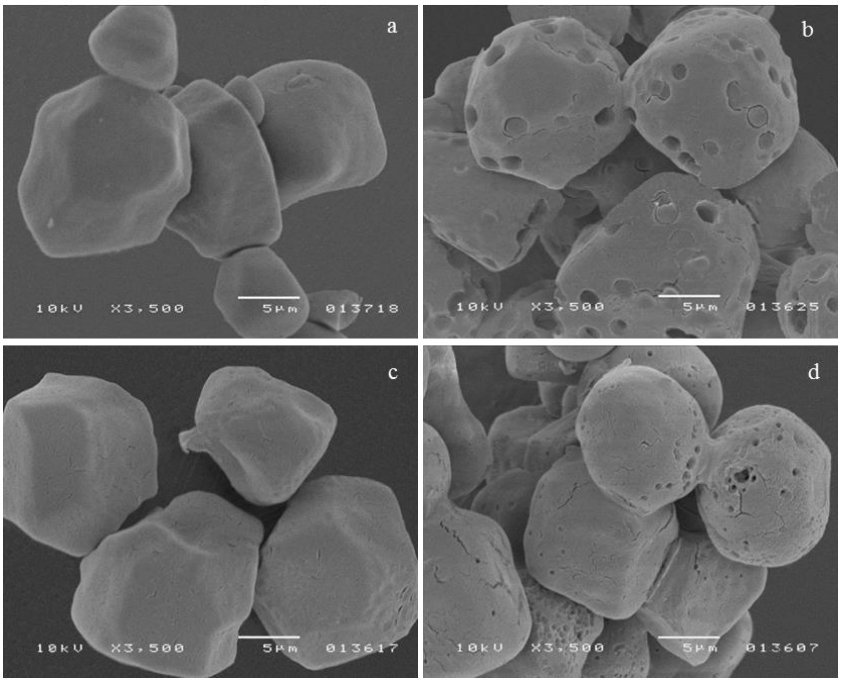

Figure 2. Scanning electron micrograph of corn starch samples treated enzymatically (b, d) and their counterparts controls (a, c). Magnification 3500x. Control pH 4 (a); AMG (b); Control pH 6 (c); AM (d).

Corn starches without enzymatic treatment (Figure 1a,c; Figure 2a,c) showed that pH did not affect the morphology of starch granules. Granules appeared with round and polygonal granular shape, and their surface was relatively smooth with some hollows, attributed to the fingerprints of the native protein bodies. Huber and BeMiller, (2000) explained that the crater-like impression resulted from pressing of protein bodies into the soft endosperm of the growing kernel. At high magnification some tiny cracks were visible. No swelling was observed in the native starch and the starch soaked at 50 ºC for 24 hours (results not shown). At high magnification (Figure 2) it was observed that certain granules appeared pasted to each other, which was explained by the effect of some amylose leached out that acted as gluing material. No holes were visible on the control starches, indicating the absence of endogenous enzymes during the treatment period. Rocha et al. (2012)

found that some holes could appear on the starch surface due to endogenous enzymes action during annealing.

The effect of enzymatic treatment was readily visible in the starches microstructure (Figure 1b,d; Figure 2b,d). Enzymatically treated starches showed changes in the surface but the shape of the granule hardly change. The microstructure of the starch granules was greatly dependent on the enzymatic treatment, but in both cases porous starch granules were obtained. α-Amylase produced some holes on the starch granules and its action was not dependent on the granule size. Amyloglucosidase was greatly active on the starch granules obtaining very perforated granules. At high magnification (Figure 2b) different concentric layers could be distinguished within the holes. Huber et al. (2000) described that in corn and sorghum starch granules, AMG produced openings to channels for providing access to the granule interior, and the surface pores enlarge through channels from hilum region toward the outsides. In the present study, it seems that both enzymes attacked the starch granules on the fingerprint of protein bodies, which seems to be the weaker points more susceptible to enzymatic hydrolysis. In addition, AMG treated starches displayed a more stacked structure, with a kind of gel joining the granules. Likely, the polymers' chains leached out more easily during AMG enzymatic incubation and resulted in more abundant gel in the treated starch granules. Alternatively, it could be that the hydrolyzed amylose chains that leached out to the periphery of the granules were readily able to crystallize after cooling yielding the jelly structure.

The analysis of the size of the holes revealed that α-amylase treatment led to small holes of 0.15 μm ± 0.04 μm, whereas amyloglucosidase action resulted in bigger holes (1.72 μm ± 0.18 μm) and the eccentricity ranged from 0.10 to 0.13, indicating very rounded pores in both cases.

It is generally accepted that granules contain amorphous and crystalline domains arranged in alternating concentric rings that create a semicrystalline

environment within the granule (Ratnayake and Jackson, 2008), which were only evident on the AMG treated starch granules. Considering that the crystalline domains are mainly composed of amylopectin while bulk amorphous domains are made up of amylose traversed by non-crystalline regions of amylopectin, it might be expected that treatment at 50 ºC promotes changes in the amorphous areas of granules (Ratnayake et al., 2008), leading to more structured internal structure.

3.2. Assessing the enzymatic treatment of the corn starch

After the enzymatic treatment, some information about the released compounds was gathered to confirm again that the enzymatic modifications occurred on the starch granules. Because of that, paste clarity, solubility index, swelling power, and apparent amylose content were measured (Table 1). Considering the values obtained for the control at pH 4.0 and 6.0, it was observed that pH did not significantly affect those parameters. Paste clarity, related to the compounds leached out during enzymatic treatment, was only increased with the AMG treatment, which can be explained by the excision activity of this enzyme that hydrolyze α-1,4 and α-1,6 glycosidic linkages from non-reduced extremes releasing free sugars. Therefore, it was expected relatively higher amount of residual free sugars. In fact, the solubility index value was significantly (P<0.05) enhanced by AMG, followed by AM. Solubility index represents the amount of water soluble products obtained from the treated starch. AMG acted breaking the degree of association between intermolecular bonds (Dura et al., 2013), and more soluble compounds were leached. In opposition, it seems that the hydrolysis products released from α-amylase action were less soluble in water. Amylose that leached to the supernatant during incubation and enzymatic treatment was evaluated, but neither the pH nor the enzymatic modification of corn starch did cause changes in the apparent amylose content of the starches.

Table 1. Effect of enzymatic treatment on the hydration properties and chemical composition of the resulting porous starches.

	Control-4	AMG	Control-6	AM
Paste clarity (Abs 650nm)	0.01±0.01a	0.26±0.03a	0.04±0.01a	0.03±0.00a
Solubility index (%)	1.13±0.01a	20.08±2.16c	1.27±0.13a	4.3±0.08b
Swelling power (g/g)	1.96±0.12a	1.93±0.00a	1.82±0.01a	2.68±0.15b
Amylose content (Abs 690nm)	0.03±0.01a	0.01±0.01a	0.04±0.02a	0.02±0.01a
Swelling capacity (g/g)	1.94±0.11b	1.54±0.04a	1.80±0.01b	2.56±0.14c
Free sugars (mg/100mg)	0.10±0.01a	0.63±0.15b	0.11±0.03a	0.18±0.09a
Damaged starch (mg/100mg)	3.16±0.24a	2.49±1.22a	3.40±0.24a	3.03±0.97a

Mean ± standard deviation values followed by different letters within a column denote significantly different levels ($P < 0.05$) (n = 4).

These results indicated that the amount of leached amylose in the supernatant after thermal treatment at 50 ºC was insignificant, and that gelatinization of the starch granules did not occur during the process, which agrees with previous results (Rocha et al., 2012). Taking into account that no differences were observed on the apparent amylose content, thus water soluble compounds but no amylose chains were released during enzymatic treatment.

Swelling power slightly increased in enzymatically modified samples from control samples. Swelling power and solubility can provide evidence in assessing the extent of interaction between starch chains within the amorphous and crystalline domains of the starch granule (Li et al., 2011; Ratnayake et al., 2002).

The enzymatically treated starches were washed to remove the remnant of any hydrolysis product and the swelling capacity of the porous starch granules as well as the amount of damage starch was determined (Table 1). The amount of free sugars was also quantified in the starch granule samples in order to verify the absence of hydrolysis products. The level of free sugars in the samples was very low (<1%). Thus, the washing carried out to remove the released products after hydrolysis was effective, although AMG treated starch had more residual sugars than its respective control. Damage starch content did not differ among samples. Presumably, enzyme treatment modified the structure of corn starch enough to permit the water intake but no the rapid enzyme absorption and penetration into the starch structure, required in the measurement procedure of damage starch. According to Biliaderis, (1991), the crystalline areas of starch maintain the structure of the granule, control its behavior in the presence of water and make it more or less resistant to chemical and enzymatic attack. The amorphous zone of starch granules is the least dense, is more susceptible to enzymatic attacks and absorbs more water at temperatures below the gelatinization temperature (Zavareze and Dias, 2011).

Starch granule swelling is known to begin in the bulk relatively mobile amorphous fraction and, in the more restrained amorphous regions immediately adjacent to the crystalline region (Donovan, 1979). Factors such as temperature, pH and type of enzyme can greatly influence the starch structure. When starch granules are heated in the presence of water, the starch granules absorb water and swell but since the enzymatic reaction occurs at 50ºC, below gelatinization temperature, the water absorption was rather low. Nonetheless, the porous structures obtained by enzymatic treatments showed opposite behavior pertaining swelling capacity, AMG reduced the swelling capacity of the starch granules, in contrast with the increase obtained in the AM treated starches. During annealing, molecules in the amorphous regions of the granule hydrate and increase the mobility of amorphous area, and

cause a limited but reversible swelling of the granule (Perry and Donald, 2000). The annealing results in a more perfectly ordered structure and in an increase in the granule stability by improving the arrangement of double helices, as well as the perfection of starch crystallites (Jayakody and Hoover, 2008). Plausible explanations could be either the enzyme action on the granule surface seems to release the more accessible compounds that bind water molecules or the agglomerates structure reduces the surface area that might be in contact with water molecules.

3.3. Pasting properties

The pasting properties of the porous starches were determined following a heating-cooling cycle (Figure 3). Plots of the pasting portraits showed minor dissimilarities than expected taking into account the great microstructure divergences earlier mentioned. The viscosities of the control starches subjected to different pHs were initially overlapped indicating similar absorption. This agrees with reported results obtained for swelling capacity, but also alike swelling and gelatinization rate (Sandhu and Singh, 2007). The maximum viscosity reached after heating was higher at pH 6.0, and then higher viscosity was observed along the cooling stage. Therefore, pH during incubation affected the annealing process that might take place during incubation at 50 ºC, and lower pH caused larger changes than pH 6.0. Regarding the enzymes action, AMG treated starch showed a delayed pasting formation, likely water absorption and swelling ability of this starch was somewhat hindered after the enzymatic treatment, which was also observed when the hydration ability of the starch was determined. After gelatinization was completed, the AMG treated starch showed higher maximum viscosity, higher cooking stability and viscosity after cooling. Conversely, the AM treated starch showed similar swelling and hydration than its control counterpart, but displayed lower maximum viscosity after cooking and cooling, which has been related to the reduction of the molecular size of starch (Miao et al., 2011).

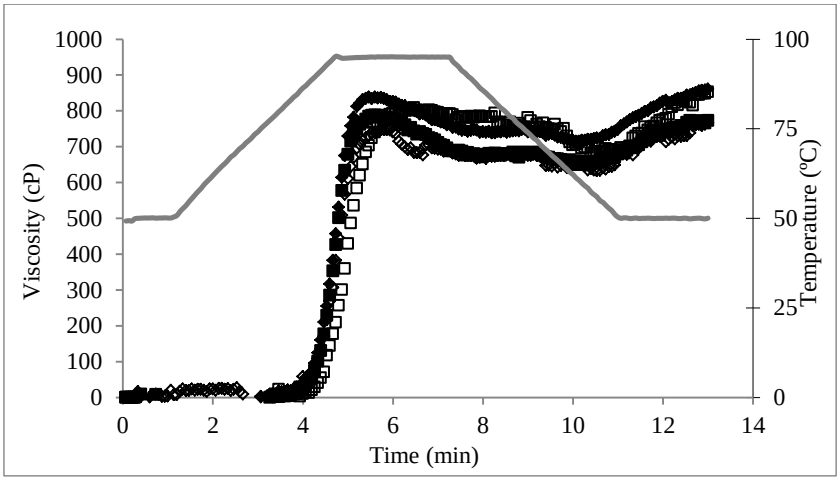

Figure 3. RVA profiles of the corn starches treated with amylase (◊) or amyloglucosidase (□) compared with their respective controls (without enzymatic treatment) in closed symbols (pH 4.0, ■ ; and pH 6.0, ♦).

Parameters recorded from the pasting curves are shown in Table 2. The onset temperature, the temperature where viscosity first increased, was rather similar in the starches kept at different pHs. The trend observed was the same as the one followed by the swelling capacity, which might be expected since the initial stage of the gelatinization is the water absorption and in consequence the starch swelling. Values of the onset temperature for the enzymatically treated starches confirmed that AMG treated starch swelled slowly compared to its counterpart control. Hydrolysis percentage induced by each enzyme was calculated referred to the maximum viscosity given by a known amount of starch obtained after heating and cooling. AM promoted 11% hydrolysis, but AMG did not decrease the viscosity, despite the porous structure of the modified starch. The reduction in the viscosity led by AM was kept beyond heating and cooling. Therefore, although microstructure appearance of AM treated starch seems to be more intact than the one of AMG treated starch, pasting behavior suggested that AM affected the loosely packed internal region of the granules and in lesser extent the densely packed periphery. Miao et al. (2011) studying the digestion of waxy corn starch with a combination of pancreatic α-amylase and amyloglucosidase found that the

end distant from the hilum of native cereal starch was more susceptible to amylolysis, and empty shells were obtained when the hydrolytic reaction progressed in major extent. Nevertheless, although these authors proposed that hydrolytic enzymes act primarily through surface erosion of the starch granules, the results obtained with AM in the present study seem to indicate that AM exerts its major action in the inner core and only small pinholes are necessary for entering.

The opposite trend was observed with AMG that increased the viscosity of starch granules, compared to its control at pH 4.0, and gave lower breakdown related to the cooking stability of the starch granules during heating (Rojas et al., 2001). In addition, this porous starch showed higher setback, related to amylose chains recrystallization, which suggested that higher amount of amylose chains leached out of the treated granule and they were rapidly able to form helical structures responsible of the gel formation.

Table 2. Effect of enzymatic treatment on the pasting parameters of corn starch.

	Control-4	AMG	Control-6	AM
Onset Temp (ºC)	83.2b	86.4c	82.5a	83.2b
Peak Time (min)	5.6a	6.5b	5.5a	5.7a
Peak viscosity (cP)	788a	809b	838c	747a
Trough (cP)	650a	681b	715c	633a
Breakdown (cP)	138b	128a	123a	114a
Final Viscosity (cP)	773a	853b	861b	767a
Setback (cP)	123a	172c	146b	134ab
Hydrolysis 95°C (%)	0	0	0	11
Hydrolysis 50°C (%)	0	0	0	11

Values followed by different letters within a column denote significantly different levels ($P < 0.05$) (n = 4).

3.4. Thermal properties

Thermal properties of the enzymatically modified corn starches were determined by differential scanning calorimetry (DSC) to detect possible changes in physical states of the starch structures (Table 3). The transition temperatures (T_o, T_p and T_c), gelatinization temperature range (T_c–T_o), enthalpies of gelatinization (ΔH) and peak height index (PHI) significantly (P<0.05) differed among samples. Gelatinization temperature (T_p) of the corn starch was 71.4 ºC, which was higher than the 64-69 ºC previously described (Zhang et al., 2012; Rocha et al., 2012). Nevertheless, the increase could be ascribed to the annealing process that took place during incubation at 50ºC that produces an increase in the peak temperature (Rocha et al., 2012). Differences were observed in the thermal parameters derived from the pH effect; gelatinization temperatures were sifted to higher values, with exception of the T_p, when increasing the reaction pH. The effect was even more accentuated when compared the gelatinization temperature range at both pHs; pH 6.0 led to narrow gelatinization range, that has been related to major proportion of crystalline region (Zhang et al., 2012). It is well known that during the endothermic transition, namely gelatinization, primarily water molecules freely diffuse into the amorphous region of starch and secondly they penetrate the crystalline region (Biliaderis, 1991). Since differences were mainly observed on the T_o, it might indicate that amorphous region was more affected at higher pH during incubation.

Enzymatic treatment significantly modified the thermal properties of corn starch, delaying the gelatinization process, which started at higher onset temperature (Table 3). Starch modified with AMG showed the highest value of T_o (67.48 °C) compared to its control (61.43 °C), therefore higher gelatinization temperature is required to initiate gelatinization of the starch, which agrees with previous observations described in the pasting behavior.

Table 3. Thermal properties of enzymatically modified corn starches determined by DSC.

	Control-4	AMG	Control-6	AM
T_o (°C)	61.43a	67.48d	64.71b	66.54c
T_p (ºC)	71.37b	72.18c	71.36b	70.43a
T_c (ºC)	75.00a	77.00c	76.00b	75.00a
T_p-T_o	9.94d	4.70b	6.65c	3.89a
ΔH (J/g)	28.24b	20.40a	28.04b	29.87c
PHI (J/g ºC)	2.84a	4.34b	4.22b	7.68c

T_o = onset temperature, T_p = peak temperature, T_c = conclusion temperature, ΔH = enthalpy change, PHI = Peak High Index.

Values followed by different letters within a column denote significantly different levels ($P < 0.05$) (n = 4).

The overall effect of enzymes on the gelatinization temperatures resulted in significantly lower gelatinization temperature range, mainly in the case of AM. That result was due to a delay in the start of gelatinization more than a shift of the conclusion temperature. α-Amylase is a very common endoenzyme that cannot hydrolyze either the α-1,6 glycosidic bonds that form the branch points in amylopectin, or the α-1,4 glycosidic bonds that are in close proximity to a branch point. Higher transition temperatures in the AMG samples have been explained due to higher degrees of crystallinity, which provide structural stability and make the granules more resistant to gelatinization (Kaur et al., 2009). Despite the different catalytic mechanism of both enzymes, it seems that the modification of starch induced by enzymes mainly affected the swelling behavior, which initially governs the gelatinization.

The higher ΔH of AM enzymatically modified corn starch compared to its respective control suggested that the state of the crystalline and amorphous regions differed from those of the AMG corn starch, in which the gelatinization enthalpy decreased in comparison with the control sample at pH 4. The lower ΔH values indicated that hydrolyzed starch by AMG

required less thermal energy for gelatinization compared to the other samples. Gelatinization involves the uncoiling and melting of the external chains of amylopectin that are packed together as double helices in clusters (Cooke and Gidley, 1992). Crystallinity of starch is caused essentially by amylopectin polymer interactions, with the outer branches of amylopectin molecules interacting to arrange themselves into ‘‘crystallites’’ forming crystalline lamellae within the granule (Tester et al., 2004). Studies carried out on granular starch and model crystallites confirmed that ΔH is mainly due to the disruption of the double helices rather than the long range disruption of crystallinity (Cooke et al., 1992). Therefore, AMG action affected the double helical conformation of the granule, both amorphous and crystalline domains, and likely acting on the amylopectin side chains, due to amylopectin plays a major role in starch granule crystallinity and the presence of amylose lowers the melting point of crystalline regions and the energy for starting gelatinization (Sodhi and Singh, 2003). Conversely, α-amylase acted mainly on the amorphous regions leading to a more crystalline structure that requires higher gelatinization enthalpy.

Values of peak height index (PHI), a measure of uniformity in gelatinization, were clearly influenced by the pH used during the incubation of starch. Again the lower pH (4.0) was more aggressive on the starch granules, giving lower PHI, which suggests narrowed transition range for gelatinization (Kruger et al., 1987). Compared with their respective control starches, enzymatically modified corn samples showed higher PHI values, indicating differences in the granule structure.

Overall, the low transition temperatures of the enzymatically treated starches indicates the reduction in the amorphous domain of the granules, which was accompanied by changes in the crystallite regions following a cooperative process in the case of AMG, but led to more crystalline structures in the AM treated starches.

3.5. Starch hydrolysis kinetics

Even though corn starches are not consumed directly but after being subjected to different processes, the enzymatic in vitro hydrolysis was carried out with the aim to determine the susceptibility of those enzymatically treated starches, with a more porous structure, to the enzymatic digestion. The digestibility curves of the enzymatically treated starches besides their respective controls are displayed in Figure 4. No differences could be envisaged due to the pH difference between the control starches; only at the very end of the digestion reaction was observed that starch kept at pH 4.0 showed higher hydrolysis. In fact, the reaction rate calculated as the slope of the very early hydrolysis stage (0-30 min), showed similar values (0.036 mg $100g^{-1}$ s^{-1}). Again, low pH seems to affect in greater extent the granule structure. It is clearly evident that enzymatically modified corn starches showed greater susceptibility to be digested. Non treated starch granules offer great resistance to enzymatic hydrolysis, thus the possible damage suffered by starch granules due to enzymatically treatment was clearly visible in the digestion. The increase in the enzymatic hydrolysis suggested that the starch granules became more accessible to enzyme hydrolysis. It has been reported that the presence of pores on the surface of annealed granules favored the action of endogenous amylases during annealing, contributing to a greater exposure of the amorphous areas to the exogenous enzymes (Rocha et al., 2012; Zavareze et al., 2011). In the present study, the enzymes used for obtaining the enzymatically treated starches originated porous granules with diverse openings size, which seems to accelerate the enzymatic digestion, and that effect was even greater in the case of AM treated starches. In fact, the reaction rate at early stage was for AMG-starch and AM-starch 0.054 mg 100g-1 s-1 and 0.067 mg $100g^{-1}$ s^{-1}, respectively. The pancreatic α-amylase affinity for digesting starches is dependent on the particle size of starch, due to the enzyme feasibility for binding/absorption and the degree of order of

starch has important influence on the initial rate at which native starch is digested by amylase (Tahir et al., 2010).

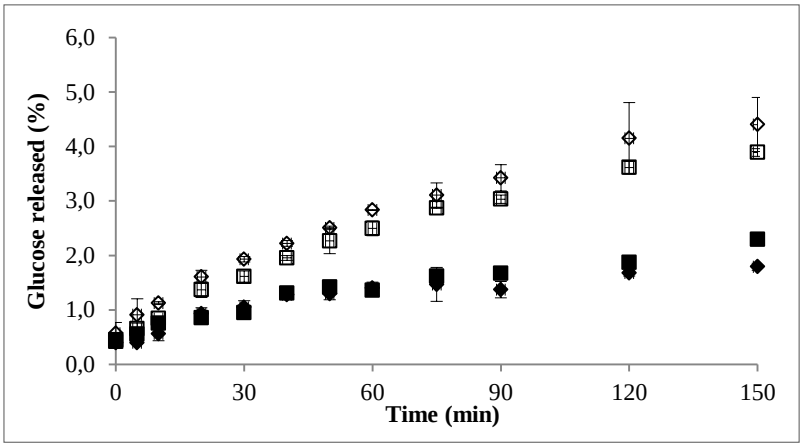

Figure 4. Enzymatic starch hydrolysis of the corn starches treated with amylase (◊) or amyloglucosidase (□) compared with their respective controls (without enzymatic treatment) in closed symbols (pH 4.0, ■; and pH 6.0, ♦).

Starch hydrolysis curves were plotted to obtain the rate constant fitting the values to a first-order equation. Table 4 shows that the pseudo-first order rate constant estimated by the first order model described by Goni et al. (1997). Significant (P<0.05) differences were found on the digestibility constant (k) due to the pH, showing higher rate constant the starch kept at pH 6.0. It has been described that significant differences in k are indicative of structural differences (Butterworth et al., 2012), which agrees with previous observation encountered on pasting and thermal properties. Regarding the enzymatic treatment, porous starches showed lower digestibility constant compared to their respective controls and no differences were observed pertaining the enzyme hydrolytic action. It has been proposed that low k values are related to a slow diffusion of pancreatic amylase into the starch granule as digestion proceeds (Dhital et al., 2010), although considering the microstructure of the porous granules the most plausible explanation to those

values of rate constant seems to be the substrate exhaustion (Butterworth et al., 2011).

Table 4. Kinetic parameters extracted from first-order fitting of the experimental enzymatic hydrolysis of modified corn starches.

	Control-4	AMG	Control-6	AM
k (min^{-1})	0.027±0.009	0.017±0.001	0.039±0.002	0.017±0.003
C_∞ (%)	1.939±0.150	4.114±0.219	1.607±0.065	4.592±0.195

Mean±standard deviation values (n = 4).

The percentage of enzymatic hydrolysis from the samples increased progressively with time during incubation. As described Butterworth et al. (2012), the rate of reaction decreases as the time is extended and plots of the concentration of product formed (or quantity of starch digested) against time are logarithmic. The plots approach an end point where no further reaction is measurable no matter how much longer incubation times are prolonged. This response is predictable based on the assumption that the concentration of available starch substrate decreases with time as starch is converted to products.

The end point values (C_∞) obtained in the hydrolyzed process reflected the concentration at the equilibrium point (Table 4). Results were significantly different, being affected by pH and enzyme treatment. Starch kept at pH 4.0 reached higher C_∞ than that at pH 6.0, indicating again that low pH during annealing affected microstructure of the granule. Enzymatically modified corn starches resulted in higher C_∞ values compared to their respective controls. Higher concentration of final product reflected increased digestibility of starch granules. AM treated starch showed higher C_∞ than AMG treated starch. Therefore, AM action led to more accessible granules that were easily digested.

4. Conclusion

Demands of modified starches are increasing in parallel to the rapid development of food industry. This study showed that enzymatic modification of corn starch by α-amylase or amyloglucosidase at sub-gelatinization temperatures led to porous starch granules that differed in both, the microstructure surface and the internal morphology. Results confirmed that the loss of granular structural order and changes in both amorphous and crystalline domains during sub-gelatinization temperatures can be more influenced by the amyloglucosidase than α-amylase, and changes increased the susceptibility of starches to be digested.

Overall, results showed that enzymatic modification at sub-gelatinization temperatures really offer an attractive alternative for obtaining porous starch granules to be used in a variety of foods applications. The degree of porosity could be controlled by using different enzymes or a combination of enzymes might be considered as well.

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References

Abbas KA., Khalil Sahar K., Meor Hussin AS. (2010). Modified starches and their usages in selected food products: A review study. Journal of Agricultural Science. 2: 2.

AACC International Approved Methods of Analysis, 11th Ed. Method 61-02. Determination of pasting properties of rice with the rapid viscoanalyzer. Approved October 15, 1997. AACC International, St. Paul, MN, U.S.A.

Ao Z., Simsek S., Zhang G., Venkatachalam M., Reuhs BL., Hamaker BR. (2007). Starch with a slow digestion property produced by altering its chain length, branch density, and crystalline structure. Journal of Agricultural and Food Chemistry. 55: 4540-4547.

Biliaderis CG. (1991). The structure and interactions of starch with food constituents. Canadian Journal of Physiology and Pharmacology. 69: 60-78.

Butterworth PJ., Warren FJ., Ellis, PR. (2011). Human α-amylase and starch digestion: An interesting marriage. Starch-Stärke. 63: 395-405.

Butterworth PJ., Warren FJ., Grassby T., Patel H., Ellis PR. (2012). Analysis of starch amylolysis using plots for first-order kinetics. Carbohydrate Polymers. 87: 2189-2197.

Colonna P., Leloup V., Buleon A. (1992). Limiting factors of starch hydrolysis. European Journal of Clinical Nutrition. 46: 17-32.

Cooke D., Gidley MJ. (1992). Loss of crystalline and molecular order during starch gelatinization-Origin of the enthalpic transition. Carbohydrate Research. 227: 103-112.

Dhital S., Shrestha AK., Gidley MJ. (2010). Relationship between granule size and *in vitro* digestibility of corn and potato starches. Carbohydrate Polymers. 82: 480-488.

Donovan JW. (1979). Phase-transitions of starch-water system. Biopolymers. 18: 263-275.

Dura A., Calviño A., Blaszczak W., Rosell CM. (2013). Funcionality of enzymatically treated corn starches above gelatinization temperature. Proceedings of the 7th International Congress "Flour-Bread '13" & 9th Croatian Congress of Cereal Technologists "Brašno-Kruh '13.

Gallant DJ., Bouchet B., Buleon A., Perez S. (1992). Physical characteristics of starch granules and susceptibility to enzymatic degradation. European Journal of Clinical Nutrition. 46: 3-16.

Goni I., Garcia Alonso A., Saura Calixto F. (1997). A starch hydrolysis procedure to estimate glycemic index. Nutrition Research. 17: 427-437.

Gularte MA., Rosell CM. (2011). Physicochemical properties and enzymatic hydrolysis of different starches in the presence of hydrocolloids. Carbohydrate Polymers. 85: 237-244.

Han XZ., Ao Z., Janaswamy S., Jane JL., Chandrasekaran R., Hamaker BR. (2006). Development of a low glycemic corn starch: Preparation and characterization. Biomacromolecules: 7: 1162-1168.

Hoover R. (2001). Composition, molecular structure, and physicochemical properties of tuber and root starches: A review. Carbohydrate Polymers. 45: 253-267.

Huang Q., Xiong F., Xiao-Wei H., Fa-Xing L., Shu-Juan Y., Li, L. (2010). The effect of enzymatic pretreatments on subsequent octenyl succinic anhydride modifications of cornstarch. Food Hydrocolloids. 24: 60-65.

Huber KC., BeMiller JN. (2000). Channels of corn and sorghum starch granules. Carbohydrate Polymers. 41: 269-276.

International Association for Cereal Chemistry. ICC (1996). Official Methods of Analysis, standard method No. 164. Vienna.

Jayakody L., Hoover R. (2002). The effect of lintnerization on cereal starch granules. Food Research International. 35: 665-680.

Jayakody L., Hoover R. (2008). Effect of annealing on the molecular structure and physicochemical properties of starches from different botanical origins-A review. Carbohydrate Polymers. 74: 691-703.

Kaur A., Singh N., Ezekiel R., Sodhi S. (2009). Properties of starches separated from potatoes stored under different conditions. Food Chemistry. 114: 1396-1404.

Kaur B., Ariffin F., Bhat R., Karim AA. (2012). Progress in starch modification in the last decade. Food Hydrocolloids. 26: 398-404.

Khatoon S., Sreerama YN., Raghavendra D., Bhattacharya S., Bhat KK. (2009). Properties of enzyme modified corn, rice and tapioca starches. Food Research International. 42: 1426-1433.

Kimura A., Robyt JF. (1996). Reaction of enzymes with starch granules: Enhanced reaction of glucoamylase with gelatinized starch granules. Carbohydrate Research. 288: 233-240.

Krueger BR., Walker CE., Knutson CA., Inglett GE. (1987). Differential scanning calorimetry of raw and annealed starch isolated from normal and mutant corn genotypes. Cereal Chemistry. 64: 187-190.

Lacerda LG., Menegassi AJA., da Silva MA., Demiate IM., Schnitzler E., Porto VL., Soccol CR. (2008). Thermal characterization of partially hydrolyzed cassava (*Manihot esculenta*) starch granules. Brazilian Archives of Biology and Technology. 51: 1209-1215.

Li DH., Ma Y. (2011). Impact of enzymatic hydrolysis on the crystal structure, and thermal and textural properties of corn starch. African Journal of Biotechnology. 10: 3430-3435.

Li JH., Vasanthana T., Hoover R., Rossnagel BG. (2004). Starch from hull-less barley: V. *In-vitro* susceptibility of waxy, normal, and high-amylose starches towards hydrolysis by alpha-amylases and amyloglucosidase. Food Chemistry. 84: 621-632.

Li W., Shu C., Zhang P., Shen Q. (2011). Properties of starch separated from ten mung bean varieties and seeds processing characteristics. Food and Bioprocess Technology. 4: 814-821.

Miao M., Zhang T., Mu W., Jiang B. (2011). Structural characterizations of waxy corn starch residue following *in vitro* pancreatin and amyloglucosidase synergistic hydrolysis. Food Hydrocolloids. 25: 214-220.

Perry PA., Donald AM. (2000). SANS study of the distribution of water within starch granules. International Journal of Biological Macromolecules. 28: 31-39.

Ratnayake WS., Hoover R., Warkentin T. (2002). Pea starch: Composition, structure and properties-A review. Starch-Starke. 54: 217-234.

Ratnayake WS., Jackson DS. (2008). Advances in Food and Nutrition Research. In: Starch Gelatinization. Elsevier Inc. 221-268.

Rocha TS., Felizardo SG., Jane J., Franco CML. (2012). Effect of annealing on the semicrystalline structure of normal and waxy corn starches. Food Hydrocolloids. 29: 93-99.

Rojas JA., Rosell CM., Benedito C., Pérez-Munuera I., Lluch, MA. (2000). The baking process of wheat rolls followed by cryo scanning electron microscopy. European Food Research and Technology. 212: 57-63.

Rojas JA., Rosell CM., Benedito C. (2001). Role of maltodextrins in the staling of starch gels. European Food Research and Technology. 212: 364-368.

Rosell CM., Collar C. (2008). Effect of various enzymes on dough rheology and bread quality. In: Recent Research Developments in Food Biotechnology. Enzymes as Additives or Processing Aids. Porta R., P. Di Pierro P., Mariniello L (eds). Research Signpost, Kerala, India. 165-183.

Rosell CM., Yokoyama W., Shoemaker C. (2011). Rheology of different hydrocolloids-rice starch blends. Effect of successive heating-cooling cycles. Carbohydrate Polymers. 84: 373-382.

Sandhu KS., Singh N. (2007). Some properties of corn starches II: Physicochemical, gelatinization, retrogradation, pasting and gel textural properties. Food Chemistry. 101: 1499-1507.

Singh J., Kaur L., McCarthy OJ. (2007). Factors influencing the physico-chemical, morphological, thermal and rheological properties of some chemically modified starches for food applications-A review. Food Hydrocolloids. 21: 1-22.

Sodhi NS., Singh N. (2003). Morphological, thermal and rheological properties of starches separated from rice cultivar's grown in India. Food Chemistry. 80: 99-108.

Tahir R., Ellis PR., Butterworth PJ. (2010). The relation of physical properties of native starch granules to the kinetics of amylolysis catalysed by porcine pancreatic α-amylase. Carbohydrate Polymers. 81: 57-62.

Tester RF., Karkalas J., Qi X. (2004). Starch-Composition, fine structure and architecture. Journal of Cereal Science. 39: 151-165.

Toyokawa H., Rubenthaler GL., Powers JR., Schanus EG. (1989). Japanese noodle qualities. 1. Flour components. Cereal Chemistry. 66: 382-386.

Wu Y., Du X.F., Ge H.H., Lv Z. (2011). Preparation of microporous starch by glucoamylase and ultrasound. Starch-Stärke. 63: 217-225.

Zavareze E., Dias A. (2011). Impact of heat-moisture treatment and annealing in starches: A review. Carbohydrate Polymers. 83: 317-328.

Zhang B., Cui D., Liu M., Gong H., Huang Y., Han F. (2012). Corn porous starch: Preparation, characterization and adsorption property. International Journal of Biological Macromolecules. 50: 250-256.

Chapter 3

Physico-chemical properties of corn starch modified with cyclodextrin glycosyltransferase

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Physico-chemical properties of corn starch modified with cyclodextrin glycosyltransferase

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Abstract

Cyclodextrin glycosyltransferase (CGTase) has been used to produce cyclodextrins (CDs) from starches, but their ability to modify starches has been barely explored. The effect of CGTase on corn starch at sub-gelatinization temperature (50 ºC) and at different pH conditions, pH 4.0 and pH 6.0, was evaluated. Biochemical features, thermal and structural analysis, oligosaccharides and CDs content were studied. Microscopic analysis of the granules confirmed the enzymatic modification of the starches obtaining structures with irregular surface and small pinholes. The extent of the starch modification was largely dependent on the pHs, being higher at pH 6.0. This was also confirmed by the low viscosity of the resulting pastes during a heating and cooling cycle. Thermal parameters were not affected due to enzymatic treatment. Modified starches were less susceptible to undergo α-amylase hydrolysis. CDs released were higher for samples treated at pH 4.0. Therefore, CGTase modification of corn starches at sub-gelatinization temperature offers an attractive alternative for obtaining porous starches with different properties depending on the pH conditions.

Keywords: starch, enzymatic modification, pasting properties, cyclodextrin glycosyltransferase.

1. Introduction

Starch is a valuable ingredient being widely used in the food industry owing to its unique thermal, structural and functional properties. However, starch use in the food industry is limited by their weak-bodied, cohesive, poor thermal, shear and acid stability (Abbas et al., 2010). Because of that physical, chemical and enzymatic modifications have been proposed for modulating the functional properties of native starches (Kaur et al., 2012). Beyond all types of modifications, enzymatic modification has a number of advantages comprising replacement of synthetic chemicals, lowering energy consumption levels and fewer by-products. Nowadays, the increasing interest for clean labeled modified starches has prompted the enzymatic modification of starches. Particularly, those catalyzed by amylases and amyloglucosidases (Li and May, 2011; Zhang et al., 2012), looking for understanding starch modification and the released products. Cyclodextrin glycosyltransferase (CGTase) is an endoenzyme, member of the α-amylase family. This enzyme catalyzes four kinds of reactions by cleaving α-1,4 glycosidic bonds present in the inner part of a polysaccharide chain (van der Veen et al., 2000a). CGTase usually has minor hydrolysis activity and mainly catalyzes three transglycosylation reactions: cyclization, coupling and disproportionation. The production of CDs is the specific reaction of CGTase (Leemhuis et al., 2010). The most common CDs are α-, β-, and γ-CD consisting of six, seven, and eight glucose monomers in cycles, respectively. Extensive research has been carried out to optimize catalysis conditions for increasing the CDs yields (Biwer et al., 2002; Szerman et al., 2007; Zhekova et al., 2009). Gujral et al. (2003) proposed CGTase as antistaling agent in gluten free breads owing its action on rice starch and CDs production and their effect on wheat starch pasting behavior and the dynamic rheology was evaluated by Gujral and Rosell (2004). More recently, Han et al. (2014) reviewed the heterologous expression strategies used for enhancing the production of CGTases and discuss the molecular engineering approaches used to improve

the production, secretion, and properties of CGTase. In spite of previous research on the CDs production for many industrial/pharmaceutical applications, there is no information about the contribution of the enzyme to the starch changes. Therefore, the aim of this study was to explore the enzymatic modification of corn starch granules with CGTase under sub-gelatinization conditions, which might open the possibility of obtaining enzymatically modified corn starch with diverse functionality. Special emphasis was placed on understanding the biochemical features, thermal and structural modifications promoted by the enzyme.

2. Materials and methods

2.1. Materials and reagents

Corn starch samples were purchased by Daesang Corporation (Korea). Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) from *Thermoanaerobacter sp* (Toruzyme® 3.0 L) of food grade was provided by Novozymes (Bagsværd, Denmark). Chemical reagents from Sigma-Aldrich (Madrid, Spain) were of analytical grade.

2.2. Methods

2.2.1. Preliminary test to determine the conditions for the enzymatic reaction

Preliminary assays were carried out to determine the impact of pH on the enzymatic reaction of CGTase. 25 mL of 20 mM sodium phosphate buffer (for the range pH 6.0 to pH 8.0) or 20 mM sodium acetate buffer (for the range pH 3.0 to pH 5.0) were added to two grams of corn starch placed into the aluminum canister and then the enzyme (0.32 U of CGTase /g starch) was added. A heating-cooling cycle was applied using the rapid visco analyzer 4500 (RVA) (Perten Instruments SA, Stockholm, Sweden) (RVA), heating from 50 to 95 ºC in 282 s and then cooling to 50 ºC in 282 s. Viscosity was recorded using Thermocline software for Windows provided by Perten

Instruments SA. The level of hydrolysis at 95 ºC and 50 ºC was defined as the %-change in paste viscosity recorded in the RVA at 50 ºC and 95 ºC.

2.2.2. Sample preparation

Corn starch (10.0 g) was suspended in 50 mL of 20 mM sodium phosphate buffer at pH 6.0 or in 20 mM sodium acetate buffer at pH 4.0. Starch samples were referred as C6 or C4, respectively. Enzyme (0.32 U of CGTase /g starch) was added to the starch suspension. Samples were kept in a shaking water bath (50 rpm) at 50 ºC for 24 and 48 hours. Starch suspensions without the addition of enzyme were used as reference. Distilled water (50 mL) was added for washing and suspensions were centrifuged for 15 min at 7,000×g and 4 ºC. Starches were washed twice to remove residual enzyme. No further release of sugars was produced, confirming the complete removal of the enzyme. Supernatants were pooled together and boiled in a water bath for 10 min to inactivate the enzyme before any further analyses. Sediments containing starch were freeze-dried and kept at -25 ºC.

2.2.3. Scanning electron microscopy (SEM)

Corn starch powders were stick on a specimen holder using cuprum tape and then coated with gold in a vacuum evaporator (JEE 400, JEOL, Tokyo, Japan). Structural properties of the samples were assessed at 10 kV accelerating voltage with a SEM (S-4800, Hitachi, Ibaraki, Japan). The microstructure analysis was carried out using image analysis software (Image-Pro Plus 7.0, Media Cybernetics, USA) in the Central Service for Experimental Research of the Universidad de Valencia.

2.2.4. High Performance Anion Exchange Chromatography (HPAEC)

Supernatants containing released hydrolysis compounds were freeze-dried and oligosaccharides and CDs were detected by HPAEC through a CarboPac PA-100 column (250 mm×4 mm), coupled to a pulsed amperometric detector (Dionex). The flow rate was 1.0 mL/min and the volume injection 10 µL.

Using solutions A (water), B (1 mol/L NaOH), C (1 mol/L $C_2H_3NaO_2$), and D (water), the following running profile was applied: time zero, 46.25% A, 5% B, 2.5% C, 46.25% D; 25 min, 42.5% A, 5% B, 10% C, 42.5% D; 1 min, 35% A, 15% B, 15% C, 35% D; 3 min, 33% A, 15% B, 19% C, 33% D; 5 min, 28.5% A, 15% B, 28% C, 28.5% D; 1.5 min, 18.5% A, 15% B, 48% C, 18.5% D. For the identification and quantification of each compound, standards of known concentrations were previously analyzed. Analysis was carried out at least in duplicate.

2.2.5. Starch hydration properties

Swelling parameters and water soluble released compounds of modified corn starch samples were determined following the method reported by Rosell et al. (2011). The iodine binding, indicative of amylose complex formation, was determined in the soluble supernatant. The soluble supernatant (40 µL) was mixed with 2 mL of an aqueous solution of 0.2% KI and 0.65% I_2. The absorbance at 690 nm was measured using a spectrophotometer (UV mini-1240, Shimadzu Corporation, Kyoto, Japan). Paste clarity (PC) was directly measured in the supernatant as the absorbance at 650 nm using a spectrophotometer. Values were the average from four replicates.

2.2.6. Starch hydrolysis kinetics

Starch hydrolysis was measured following the method described by Dura et al. (2014) for gelatinized and non-gelatinized samples. To obtain gelatinized samples previous to starch hydrolysis, corn starch sample (0.1 g) was suspended in 2 mL of 0.1 M sodium maleate buffer (pH 6.9) and incubated 15 min at 100 ºC. Samples were then placed in water bath at 37 ºC. When temperature was reached, porcine pancreatic α-amylase (Type VI-B, ≥10 units/mg solid, Sigma Chemical, St. Louis, USA) 40 CU/g starch and 240 CU/g starch (CU, Ceralpha Units) was added for gelatinized and non-gelatinized samples, respectively.

2.2.7. Pasting properties

The pasting properties were determined with RVA by following the American Association of Cereal Chemists Approved Method (AACC, 1997). Again, the level of hydrolysis at 95 ºC and 50 ºC was defined as the %-change in paste viscosity recorded in the RVA at 50 ºC and 95 ºC.

2.2.8. Thermal Analysis of starch

Thermal behavior of starch samples was determined using a DSC from Perkin-Elmer (DSC 7, Perkin-Elmer Instruments, Norwalk, CT). Corn starch samples were accurately weighed into aluminum DSC pans and de-ionized water was added by micropipette to achieve a water-sample ratio of 3:1. Pans were sealed and equilibrated at room temperature for one hour before analysis. Instrument was calibrated with indium, using an empty pan as reference. Thermal analysis consisted on heating from 30 to 120 °C at a rate of 5 °C/min. The onset temperature T_o, peak temperature T_p, and conclusion temperature T_c, were determined from the heating DSC curves. ΔH was evaluated based on the area of the main endothermic peak, and peak height index (PHI) was calculated as $PHI= \Delta H/ T_p- T_o$. All DSC experiments were replicated three times.

2.2.9. Statistical analysis

Experimental data were statistically analyzed for analysis of variance (ANOVA) using Statgraphics Centurion XV software (Bitstream, Cambridge, N). When analysis of variance indicated significant *F* values, multiple sample comparisons were also performed by Fisher's least significant differences (LSD) test to differentiate means with 95% confidence. The correlation matrix was also performed using Statgraphics Centurion XV software.

3. Results and discussion

Previous analysis were performed to investigate the impact of pH and temperature on the enzymatic reaction and to select suitable reaction conditions for the enzymatic modification of corn starch. The level of hydrolysis obtained at 95 ºC was rather low, as indicated the %-change in paste viscosity recorded in the RVA at 95 ºC (Figure 1). Therefore, despite CGTase belongs to the α-amylase family, it showed rather low hydrolysis activity, which agrees with previous findings (van der Veen et al., 2000b). Enzymatic activity was mainly revealed during the cooling stage of the RVA analysis, resulting in high starch hydrolysis (50 ºC) in the pHs range of 4.0 to 7.0, with two maxima observed at pHs 4.0 and 6.0. Those pHs were selected to perform further enzymatic modification of corn starch.

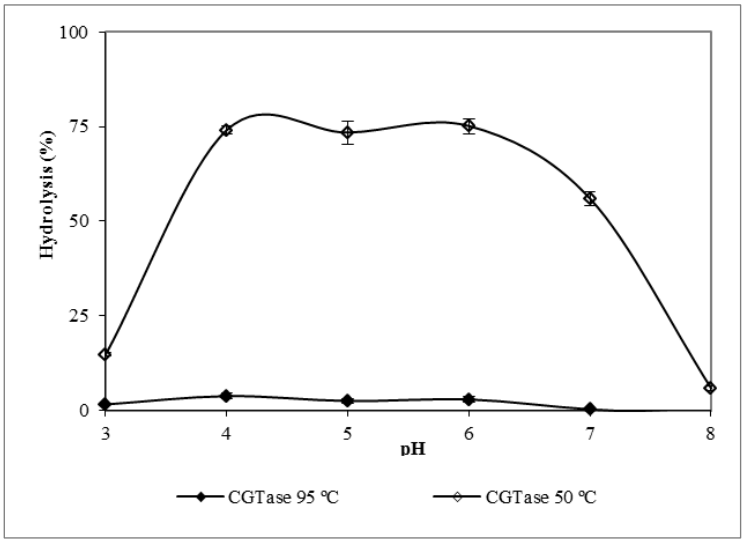


Figure 1. Hydrolysis activity of CGTase at different pHs. The level of hydrolysis at 95 ºC and 50 ºC was defined as the %-change in paste viscosity recorded in the RVA at 50 ºC and 95 ºC.

3.1 Microstructure of the starch

Samples were examined by SEM. No changes in granule size and shape and no holes were visible in C4 and C6 (Figure 2a and b), and surfaces appeared

smooth without any evidence of fissures. When samples were subjected to 50 ºC for 24 and 48 hours (Figure 2c, d, g and h), changes were observed only after 48 hours treatment, starch granules showed shapeless structures, losing its smooth appearance, presumably due to annealing. Jayakody et al. (2008) reviewed the effect of annealing in granules morphology of different tubers and root starches reporting changes on the surface of the granules after treatment. These results were in accordance with Rocha et al. (2012), who found that the structural characteristics of normal and waxy starch granules were affected by annealing.

The effect of enzymatic treatment was readily visible in the starches microstructure as pinholes (Figure 2e, f, i and j). CGTase was greatly active on the starch granules, resulting in uneven superficial porous that augmented as the time of incubation increased. The surface of the granules was extensively eroded with numerous fissures after 48 hours incubation, being less pronounced in samples treated at pH 4.0. It is generally accepted that starch granules have a unique semi-crystalline supramolecular structure with concentric layers of amorphous and crystalline regions radiating from the hilum (Ratnayake and Jackson, 2008). Considering that the crystalline domains are mainly composed of amylopectin while bulk amorphous domains are made up of amylose traversed by non-crystalline regions of amylopectin, it might be expected that treatment with CGTase promotes changes in the amorphous areas of granules, leading to an internally structured morphology. When starch granules are incubated with amylolytic enzymes, the enzymes migrate through the channels (Fannon et al., 1992) and initiate hydrolysis leading to an inside out pattern of digestion (Benmoussa et al., 2006). Surface pores were irregularly distributed, either absent, present in clusters or scattered over the surface (BeMiller, 1997). Micrographs confirmed that CGTase also led to porous corn starch but having smaller and randomly distributed holes.

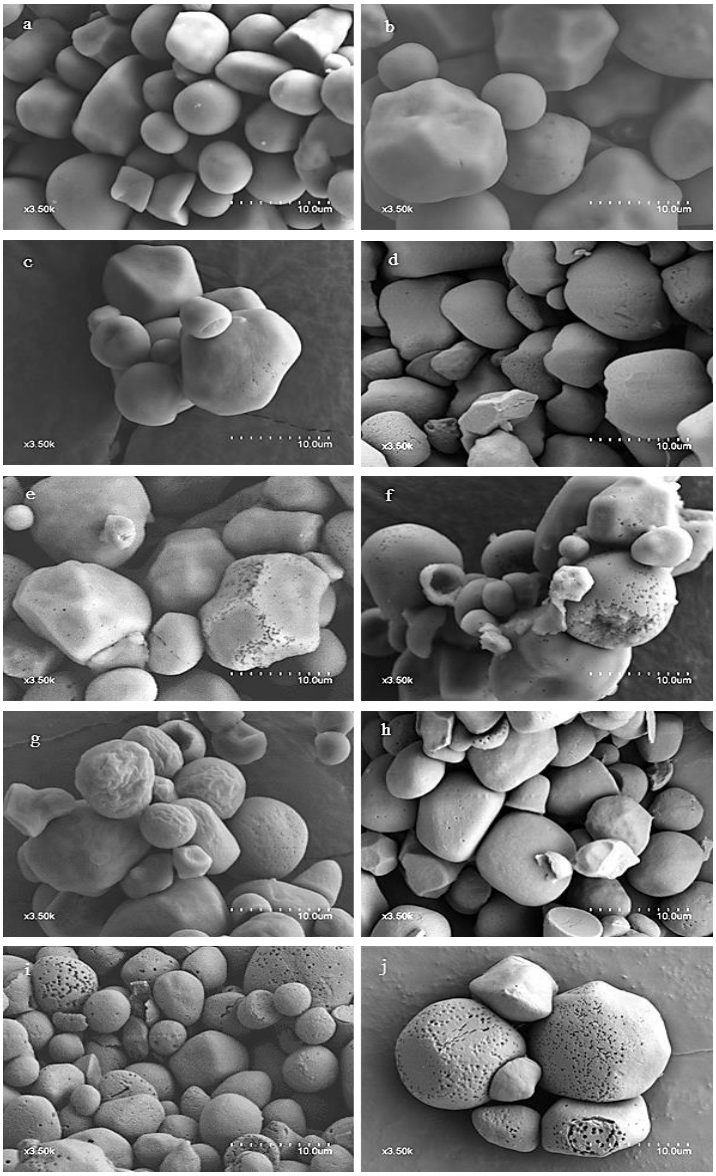


Figure 2. Scanning electron micrograph of corn starch samples (a, b, c, d, g and h) and their counterparts treated enzymatically (e, f, i and j) at pH 4.0, after 24 h (c, e) or 48 h (g, i) and at pH 6.0 after 24 h (d, f) or 48 h (i, j). Magnification 3500×. Control pH 4.0 (a); Control pH 6 (b); Control pH 4.0, 24 h (c); Control pH 6, 24 h (d); CGTase pH 4.0, 24 h (e); CGTase pH 6.0, 24 h (f); Control pH 4.0, 48 h (g); Control pH 6.0, 48 h (h); CGTase pH 4.0, 48 h (i); CGTase pH 6.0, 48 h (j).

3.2. Enzymatic treatment of corn starch

Hydrothermal properties and amylose content were significantly affected (Table 1). The ANOVA indicated a significant effect of the pH on the paste clarity, related to the compounds leached out, which was higher when treated at pH 6.0. The iodine binding value, thus the amount of amylose leached to the supernatant, after thermal treatment at 50 ºC was greater for samples soaked at pH 4.0 for some time. The solubility index (SI) value was significantly ($P < 0.05$) enhanced due to enzymatic treatment and the time of treatment. SI determines the amount of solid compounds leached when breaking intermolecular bonds between amylose and amylopectin. Despite the enzymatic activity at both pH, the amylose released was low, likely due to interaction between amylose-amylose and/or amylose-amylopectin (Waduge wt al., 2006) or reduction in granular swelling (Tester and Debon, 2000). In fact, enzymatically treated corn starches showed significantly lower swelling capacity (SC), thus less amount of water was absorbed by these starches. It seems that either some physical impediment for bounding water molecules or the hydrophobic nature of the internal wall of the pinholes should be responsible of that behavior. Therefore, the enzymatic treatment modified the granular integrity of the starch affecting its swelling capacity.

Overall, CGTase was greatly active breaking the degree of association between molecular bonds for longer time of incubation comparing with the control samples at both pH and more soluble compounds were leached, as was previously reported for amylase and amyloglucosidase (Dura et al., 2013).

Table 1. Effect of enzymatic treatment on the paste clarity, solubility index, amylose content and hydration properties (swelling power and swelling capacity) of the resulting starches.

Sample	Time (h)	Paste clarity (Abs 650nm)	Solubility index (%)	Swelling power (g/g)	Iodine binding (Abs 690 nm)	Swelling capacity (g/g)
C4	0	0.01±0.00	1.04±0.00	0.87±0.00	0.000±0.000	0.86±0.00
C4	24	0.01±0.00	2.80±0.05	0.86±0.01	0.043±0.003	0.84±0.01
C4	48	0.01±0.01	1.58±0.32	0.87±0.00	0.033±0.004	0.85±0.00
CGT4	24	0.00±0.00	3.35±0.07	0.87±0.00	0.000±0.000	0.84±0.00
CGT4	48	0.01±0.00	4.47±0.02	0.86±0.00	0.000±0.024	0.83±0.00
C6	0	0.01±0.00	0.72±0.00	0.86±0.00	0.000±0.009	0.85±0.00
C6	24	0.02±0.01	1.65±0.08	0.86±0.00	0.048±0.003	0.85±0.00
C6	48	0.01±0.00	4.58±0.18	0.90±0.00	0.000±0.005	0.86±0.00
CGT6	24	0.01±0.00	3.87±0.00	0.86±0.00	0.000±0.000	0.83±0.00
CGT6	48	0.02±0.01	6.14±2.00	0.89±0.02	0.006±0.016	0.83±0.00
P value	Time	0.23	0.04	0.05	0.443	0.24
	pH	0.02	0.12	0.09	0.002	0.64
	Enzyme	0.58	0.01	0.79	0.416	0.00

Mean ± standard deviation values (n = 3).

3.3. CDs and oligosaccharides released during enzymatic treatment

Contents of glucose, maltose, maltotriose, maltotetraose, maltopentaose, α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin in mg.100 g^{-1} of starch are presented in Table 2. As it was expected, non-enzymatically treated samples did show neither oligosaccharides nor CDs, with the exception of the sample soaked at pH 4.0 for 48 hours that presented a small amount of glucose. Oligosaccharides and CDs were released from the corn starches when subjected to CGTase hydrolysis at pH 4.0 or 6.0. CGTase is an endo-amylase that cleaves α-1,4-glycosidic bonds present in the inner part of a polysaccharide chain (van der Veen et al., 2000). Results showed that the pattern of released compounds was dependent on the pH. At pH 6.0 the CGTase released mainly oligosaccharides and the production of CDs required longer incubation times (48 hours). Conversely, the treatment carried out at pH 4.0 released major amount of CDs, predominantly ß-cyclodextrin.

At pH 6.0 the primarily cyclodextrin was the α-CD, which agree with Yamamoto et al. (2000) findings, when similar conditions were applied to heat treated potato starch at pH 5.5. Additionally, Kim et al. (1997) reported the production of a small amount of cyclodextrin. It must be stressed that being the CGTase a member of α-amylase family usually reactions are carried out at pH 6.0, but present results showed that specificity was pH-dependent.

Table 2. Oligosaccharides and cyclodextrins released after corn starch hydrolysis by CGTase. Results are expressed in mg.100 g^{-1} of starch.

Sample	Time(h)	Glucose	Maltose	Maltotetriose	Maltotetraose	Maltopentaose	α-CD	β-CD	γ-CD
C4	0	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
C4	24	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
C4	48	0.783	<dl	<dl	<dl	<dl	<dl	<dl	<dl
CGT4	24	3.171	4.204	2.600	1.691	0.461	2.233	3.759	0.300
CGT4	48	5.519	6.967	4.549	4.007	1.234	3.162	4.950	<dl
C6	0	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
C6	24	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
C6	48	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
CGT6	24	5.240	7.075	4.814	0.725	0.041	0.004	<dl	<dl
CGT6	48	5.007	4.933	2.884	0.086	0.383	1.142	1.014	<dl

<dl means under detection limit

3.4. Starch hydrolysis kinetics

Gelatinized samples (Figure 3 C, D) showed faster hydrolysis kinetic than non-gelatinized samples (Figure 3 A, B). Structural changes occurred during gelatinization of starch, losing its original granular structure and crystalline order and leading to more susceptible to enzymatic hydrolysis towards α-amylase (Kimura and Robyt, 1996).

Non-enzymatically treated starches showed higher susceptibility to be hydrolyzed, with the exception of samples soaked for 48 hours. Likely, annealing after 48 hours-soaking induces structural changes that made the granule more resistant to enzymatic attack. Enzymatically treated starches were less susceptible to hydrolysis, exhibiting slower hydrolysis and reaching lower maximum. Despite that the presence of surface porous in the starch granules could suggest an increase in the susceptibility to be hydrolyzed, as occurs with amylase or amyloglucosidase attack (Dura et al., 2014), results indicated the opposite behavior. Enzymatic treatment of the starch by CGTase seems to affect the amorphous zone, releasing sugars from those accessible chains, but leading to a more crystalline structure that was more resistant to the amylase hydrolysis (Kimura and Robyt, 1995). It must be remark at this point that CGTase catalyze mainly transglycosylation reactions, which can lead to starch structures less susceptible to amylase hydrolysis. The annealing after 48 hours led to starches with hydrolysis patterns close to those obtained with enzymatically treated starches.

Faster hydrolysis (0-40 min) was observed in all the gelatinized starches (Figure 3 C, D), rapidly achieving a plateau at higher glucose percentage than the one obtained in non-gelatinized starches. During the gelatinization of starch the molecular order and thus birefringence disappears, the starch granule loses crystallinity, swelling of the granule is followed by leaching of mainly amylose, and when further heated, starch granules are disrupted and partial solubilization is achieved.

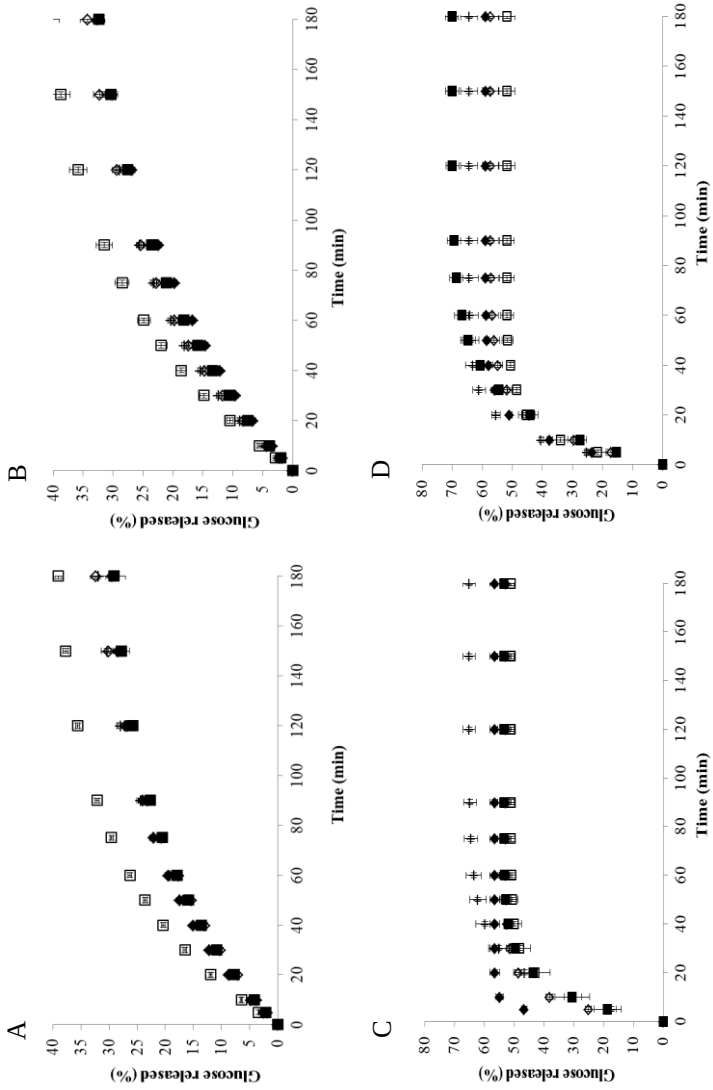


Figure 3. Enzymatic starch hydrolysis profiles of non-gelatinized samples (A, B) and gelatinized corn starches (C, D). Corn starches treated with CGTase at pH 4.0 (A, C) and pH 6.0 (B, D) for 24 hours (■) and 48 hours (♦) were compared with their counterparts without enzymatic treatment after 24 hours (□) or 48 hours (◊). Native starch was included (+).

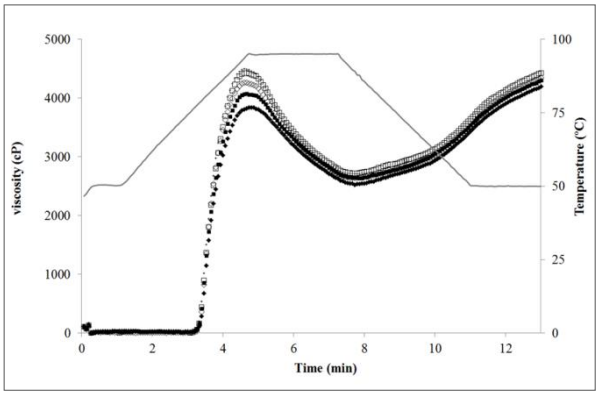
Gelatinization is a process that transforms the native crystalline structure of the starch granules into more amorphous structure, losing their physical integrity, favoring enzymes access to the starch chains. In consequence, enzymatically treated samples after gelatinization showed similar hydrolysis plots to their counterparts without treatment, and that effect was even more pronounced at pH 4.0 (Figure 3 C) than at pH 6.0 (Figure 3 D). Therefore, it seems that when starches loss their crystalline structure, due to gelatinization; structural changes promoted by CGTase were hardly evident.

3.5. Pasting properties

Typical pasting curves were observed when starch suspensions were subjected to a heating and cooling cycle (Figure 4). Enzymatic treatment at different pH promoted differences in the viscosity pattern of the starches especially during cooling, which was lower for the starches treated at pH 6.0. Therefore, pH affected the annealing process that might take place during soaking at 50 ºC. The effect of enzymatic treatment was manifested as a decrease in the maximum peak viscosity. After reaching the maximum viscosity, the swollen starch granules were easily broken and disintegrated by stirring, which was related to the starch stability during heating. Enzymatically treated starches at pH 6.0 showed lower stability than those performed at pH 4.0. PH significantly affected the peak viscosity, breakdown and final viscosity (Table 3). Enzymatic treatment resulted in significant changes on peak viscosity, trough, final viscosity and setback. Enzymatic modification of rice starch with pullulan also affected peak viscosity values, trough and final viscosity (Chen et al., 2015). In addition, enzymatic treatment exerted significant effects on hydrolysis percentage at 95 ºC and 50 ºC. Therefore, the activity of CGTase led to starches with lower maximum peak viscosity and when performed at pH 6.0 also lower viscosity was obtained after cooling. Enzymatically modified samples at pH 6.0 showed lower setback and final viscosity than samples treated at pH 4.0, which suggested that lower amount of amylose chains were able to form helical

structures responsible of the gel formation. Presumably, CGTase at pH 6.0 induced greater hydrolysis of the amylose chains, confirming the different action of this enzyme at both pH, which also agree with micrograph observations that showed erosion and pinholes in the surface of starch granules.

A



B

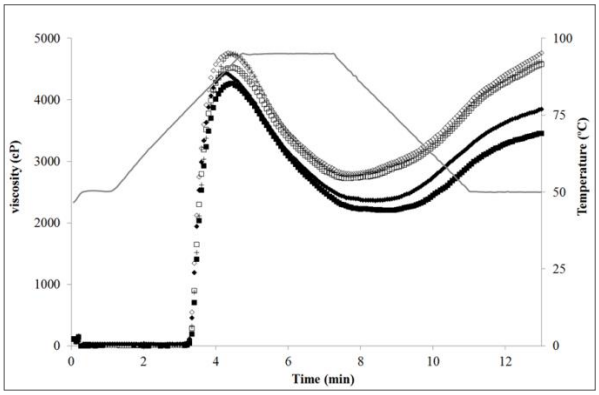


Figure 4. RVA profiles of the corn starches at pH 4.0 (A) and pH 6.0 (B) treated with CGTase at 24 hours, (■) and 48 hours, (♦) compared with their respective controls (without enzymatic treatment) at 24 hours, (□); and 48 hours, (◊). Control sample without any treatment at pH 4.0 and pH 6.0, enclosed symbols, (+).

Table 3. Effect of enzymatic treatment on the pasting parameters of corn starch.

Sample	Time (h)	Onset temp (ºC)	Peak time (min)	Peak viscosity (cP)	Trough (cP)	Breakdown (cP)	Final viscosity (cP)	Setback (cP)	Hydrolysis 95°C (%)	Hydrolysis 50°C (%)
C4	0	76±0	5±0	4499±0	2711±0	1788±0	4320±0	1609±0	0±0	0±0
C4	24	76±0	5±0	4433±192	2719±95	1715±96	4430±122	1712±26	1±4	-3±3
C4	48	72±7	5±0	4263±3	2638±5	1626±2	4346±1	1708±4	5±0	-1±0
CGT4	24	65±16	5±0	4076±66	2639±5	1438±71	4301±44	1663±49	9±1	0±1
CGT4	48	67±14	5±0	3843±57	2527±50	1317±6	4195±44	1669±6	15±1	3±1
C6	0	75±0	5±0	4749±0	2751±0	1998±0	4625±0	1874±0	0±0	0±0
C6	24	77±0	4±0	4530±129	2724±40	1807±89	4576±47	1852±7	5±3	1±1
C6	48	64±17	4±0	4760±107	2791±192	1969±85	4762±187	1971±5	0±2	-3±4
CGT6	24	57±0	4±0	4270±0	2203±0	2067±0	3456±0	1253±0	10±0	25±0
CGT6	48	72±5	4±0	4441±158	2363±133	2078±25	3846±175	1483±42	6±3	17±4
P value	Time	0.90	0.02	0.58	0.95	0.68	0.95	0.69	0.63	0.94
	pH	0.81	0.00	0.00	0.34	0.00	0.72	0.81	0.25	0.02
	Enzyme	0.31	0.93	0.00	0.00	0.39	0.00	0.01	0.00	0.00

Values followed by different letters within a column denote significantly different levels ($P < 0.05$) (n = 3).

3.6. Thermal parameters

Transition temperatures (T_o, T_p and T_c), gelatinization temperature range (T_p-T_o), ΔH and the PHI are presented in Table 4. The pH during treatment significantly affected all thermal parameters. Unexpectedly, enzymatic treatment did not significantly modified thermal behavior of the starches, despite differences observed in the pasting parameters. It seems that changes promoted by CGTase were at the granule surface, whereas pH soaking during prolonged periods led to inner granule changes. Gelatinization temperature (T_p) of the corn starch samples range from 65.01 to 74.11 ºC, being slightly higher at pH 6.0 after the treatment, likely due to the annealing process that took place during soaking at 50 ºC that rose the peak temperature and narrowed the gelatinization temperature range (Dura et al., 2014). However, contradictory results have been reported regarding the gelatinization behavior of starches after annealing. Krueger et al. (1987) observed enhanced enthalpies for wild corn starches and mutant genotypes, while Tester et al. (2000) reported that the enthalpy for corn starch remained unaltered after annealing.

ΔH values were significantly affected by pH and time of the treatment, being lower for samples treated at pH 6.0 for longer period. Considering that the ΔH reflects the disruption of double helices and crystalline order, partial solubilization of amylose and the development of glucan chain-water complexes and chain-chain interactions (Bogracheva et al., 2002), it seems that treatment at pH 6.0 was more aggressive. The decrease observed at pH 6.0 suggests that some of the double helices present in crystalline and in non-crystalline regions of the granule may have disrupted under the conditions treatment, which suggested that annealing was pH dependent. Values of PHI were clearly influenced by the pH used during the incubation of starch. Again the pH 6.0 was more aggressive on the starch granules, giving lower PHI, which suggests narrowed transition range for gelatinization (Krueger et al., 1987)

Table 4. Thermal properties of enzymatically modified corn starches determined by DSC

Sample	Time (h)	T_o (ºC)	T_p (ºC)	T_c (ºC)	T_p-T_o	Area (mJ)	ΔH (J/g)	PHI (J/g ◦C)
C4	0	65.11±0.30	68.54±0.54	73.46±1.39	3.46±0.26	103.19±10.11	11.00±0.79	3.21±0.40
C4	24	65.66±0.27	68.70±0.50	73.63±0.17	3.19±0.28	117.25±8.01	12.19±0.76	3.89±0.29
C4	48	66.28±0.37	69.45±0.33	73.34±0.53	3.17±0.10	113.48±4.85	12.33±0.73	3.86±0.24
CGT4	24	65.01±0.91	68.62±0.79	71.73±1.87	3.73±0.36	108.28±11.75	12.05±1.80	3.20±0.52
CGT4	48	66.34±0.50	69.58±0.57	73.22±1.03	3.27±0.24	120.83±8.74	12.98±1.18	3.87±0.60
C6	0	65.40±0.21	68.75±0.22	74.04±1.23	3.39±0.06	93.21±4.27	10.17±0.27	3.01±0.06
C6	24	65.88±0.91	69.29±1.22	73.00±2.16	3.41±0.32	113.06±7.15	11.75±0.99	3.70±0.51
C6	48	66.39±0.14	69.50±0.27	73.48±0.31	3.05±0.26	107.18±12.95	10.68±0.99	3.49±0.56
CGT6	24	66.14±0.34	69.29±0.28	72.40±0.51	3.19±0.12	115.18±8.26	12.52±0.91	3.92±0.17
CGT6	48	66.78±0.25	69.62±0.28	74.11±0.24	2.8±0.10	97.71±11.17	10.01±0.69	3.53±0.33
P value	Time	0.87	0.44	0.70	0.87	0.70	0.01	0.05
	pH	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Enzyme	0.56	0.61	0.48	0.64	0.41	0.66	0.39

T_o = onset temperature, T_p = peak temperature, T_c = conclusion temperature, ΔH = enthalpy change, PHI = peak height index. Values followed by different letters within a column denote significantly different levels ($P < 0.05$) (n = 4).

4. Conclusions

CGTase offers an alternative for modulating the corn starch properties at sub-gelatinization temperature and it encompasses the applications of this enzyme not only to be used for producing CDs. The extent of starch modification was pH dependent, being more aggressive at pH 6.0, where oligosaccharides were majorly released. Conversely, milder modification could be obtained at pH 4.0, at which greater amount of cyclodextrins were released. CGTase seems to hydrolyze the amorphous part of the starch leading to starches less susceptible to amylase hydrolysis. Porous starches could be obtained with this enzymatic treatment, which keep their thermal characteristics but show different pasting behavior, again dependent on the pHs of the enzymatic treatment.

Acknowledgements

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References

AACC International Approved Methods of Analysis, 11th Ed. Method 61-02. Determination of pasting properties of rice with the rapid viscoanalyzer. Approved October 15, 1997. AACC International, St. Paul, MN, U.S.A.

Abbas KA., Khalil SA. , Hussin ASM. (2010). Modified starches and their usages in selected food products: A review study. Journal of Agricultural Science. 2: 90-100.

BeMiller JN. (1997). Starch modification: Challenges and prospects. Starch-Stärke. 49: 127-131.

Benmoussa M., Suhendra B., Aboubacar A., Hamaker BR. (2006). Distinctive sorghum starch granule morphologies appear to improve raw starch digestibility. Starch-Stärke. 58: 92-99.

Biwer A., Antranikian G., Heinzle E. (2002). Enzymatic production of cyclodextrins. Applied Microbiology and Biotechnology. 59: 609-617.

Bogracheva TY., Wang YL., Wang TL., Hedley CL. (2002). Structural studies of starches with different water contents. Biopolymers. 64: 268-281.

Chen L., Ren F., Yu XP., Zhang ZP., Xu DJ., Tong QY. (2015). Pasting investigation, SEM observation and the possible interaction study on rice starch-Pullulan combination. International Journal of Biology Macromolecules. 73: 45-48.

Dura A., Calviño A., Blaszczak W., Rosell CM. (2013). Funcionality of enzymatically treated corn starches above gelatinization temperature. Proceedings of the 7th International Congress "Flour-Bread '13" & 9th Croatian Congress of Cereal Technologists "Brašno-Kruh '13.

Dura A., Blaszczak W., Rosell CM. (2014). Functionality of porous starch obtained by amylase oramyloglucosidase treatments. Carbohydrate Polymers. 101: 837-845.

Fannon JE., Hauber RJ., Bemiller JN. (1992). Surface pores of starch granules. Cereal Chemistry. 69: 284-288.

Gujral HS., Haros M., Rosell CM. (2003). Effect of cyclodextrinase on dough rheology and bread quality from rice flour. Cereal Chemistry. 80: 750-754.

Gujral HS., Rosell CM. (2004). Modification of pasting properties of wheat starch by cyclodextrin glycosyltransferase. Journal of Science Food Agriculture. 84: 1685-1690.

Han R., Li J., Shin HD., Chen RR., Du G., Liu L., Chen J. (2014). Recent advances in discovery, heterologous expression, and molecular engineering of cyclodextrin glycosyltransferase for versatile applications. Biotechnology Advances. 32: 415-428.

Jayakody L., Hoover R. (2008). Effect of annealing on the molecular structure and physicochemical properties of starches from different botanical origins-A review. Carbohydrate Polymers. 74: 691-703.

Kaur B., Ariffin F., Bhat R., Karim AA. (2012). Progress in starch modification in the last decade. Food Hydrocolloids. 26: 398-404.

Kim TJ., Kim BC., Lee HS. (1997). Production of cyclodextrin using raw corn starch without a pretreatment. Enzyme Microboly and Technology. 20: 506-509.

Kimura A., Robyt JF. (1995). Reaction of enzymes with starch granules: Kinetics and products of the reaction with glucoamylase. Carbohydate Research. 277: 87-107.

Kimura A., Robyt JF. (1996). Reaction of enzymes with starch granules: Enhanced reaction of glucoamylase with gelatinized starch granules. Carbohydate Research. 288: 233-240.

Krueger BR., Walker CE., Knutson CA., Inglett GE. (1987). Differential scanning calorimetry of raw and annealed starch isolated from normal and mutant corn genotypes. Cereal Chemistry. 64: 187-190.

Leemhuis H., Kelly RM., Dijkhuizen L. (2010). Engineering of cyclodextrin glucanotransferases and the impact for biotechnological applications. Applied Microbiology and Biotechnology. 85: 823-835.

Li DH., Ma Y. (2011). Impact of enzymatic hydrolysis on the crystal structure, and thermal and textural properties of corn starch. African Journal of Biotechnology. 10: 3430-3435.

Ratnayake WS., Jackson DS. (2008). Starch gelatinization. In: Advances in Food and Nutrition Research. Elsevier Inc. 221-268.

Rocha TS., Felizardo SG., Jane J., Franco CML. (2012). Effect of annealing on the semicrystalline structure of normal and waxy corn starches. Food Hydrocolloids. 29: 93-99.

Rosell CM., Yokoyama W., Shoemaker C. (2011). Rheology of different hydrocolloids-rice starch blends. Effect of successive heating-cooling cycles Carbohydrate Polymers. 84: 373-382.

Szerman N., Schroh I., Rossi AL., Rosso AM., Krymkiewicz N., Ferrarotti SA. Cyclodextrin production by cyclodextrin glycosyltransferase from *Bacillus circulans* DF 9R (2007). Bioresource of Technology. 8: 2886-2891.

Tester RF., Debon SJJ. (2000). Annealing of starch-A review. International Journal of Biology Macromolecules. 27: 1-12.

van der Veen BA., Uitdehaag JCM., Dijkstra BW., Dijkhuizen L. (2000a). Engineering of cyclodextrin glycosyltransferase reaction and product specicity. Biochimica et Biophysica Acta. 1543: 336-360.

van der Veen BA., van Alebeek G., Uitdehaag JCM., Dijkstra BW., Dijkhuizen L. (2000b). The three transglycosylation reactions catalyzed by cyclodextrin glycosyltransferase from *Bacillus circulans* (strain 251) proceed via different kinetic mechanisms. European Journal of Biochemistry. 267: 658-665.

Waduge RN., Hoover R., Vasanthan T., Gao J., Li J. (2006). Effect of annealing on the structure and physicochemical properties of barley starches of varying amylose content. Food Reserach International. 39: 59-77.

Yamamoto K., Zhang ZZ., Kobayashi S. (2000). Cycloamylose (Cyclodextrin) glucanotransferase degrades intact granules of potato raw starch. Journal of Agriculture Food Chemistry. 48: 962-966.

Zhang B., Cui D., Liu M., Gong H., Huang Y., Han F. (2012). Corn porous starch: Preparation, characterization and adsorption property. International Journal of Biological Macromolecules. 50: 250-256.

Zhekova B., Dobrev G., Stanchev V., Pishtiyski I. (2009). Approaches for yield increase of β-cyclodextrin formed by cyclodextrin glucanotransferase from *Bacillus megaterium*. World Journal of Microbiolgy and Biotechnology. 25: 1043-1049.

Chapter 4

Glycemic response to corn starch modified with cyclodextrin glycosyltranseferase and its relationship to physical properties

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ORIGINAL PAPER

Glycemic Response to Corn Starch Modified with Cyclodextrin Glycosyltransferase and its Relationship to Physical Properties

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Abstract

Corn starch was modified with cyclodextrin glycosyltransferase (CGTase) below the gelatinization temperature. The porous granules with or without CGTase hydrolysis products may be used as an alternative to modified corn starches in foods applications. The amount and type of hydrolysis products were determined, containing mainly β-cyclodextrin (CD), which will influence pasting behavior and glycemic response in mice. Irregular surface and small holes were observed by microscopic analysis and differences in pasting properties were observed in the presence of hydrolysis products. Postprandial blood glucose in mice fed gelatinized enzymatically modified starch peaked earlier than their ungelatinized counterparts. However, in ungelatinized enzymatically modified starches, the presence of β-CD may impede the orientation of amylases slowing hydrolysis, which may help to maintain lower blood glucose levels. Significant correlations were found between glycemic curves and viscosity pattern of starches.

Keywords: corn starch; glucose response; enzymatic modification; CGTase; cyclodextrins.

1. Introduction

Starch is one of the most important glycemic carbohydrates that provides great part of the energy requirement for humans through diet. Starch modification leads to structural changes of the granule varying also properties like pasting, gelling, digestibility and absorption. Enzymatic modification of starch has been proposed as a very attractive way to modulate those properties. Enzymatic modification under gelatinization temperature is less common but it may provide useful properties for some food applications. CGTase is an endoenzyme member of the α-amylase family or glycosyl hydrolase family 13. It catalyzes the cleavage of an interior α-glycosidic bond producing oligosaccharides that subsequently cyclize into cyclodextrins (CDs). The most common CDs are α-, β-, and γ-CD consisting of six, seven, and eight glucose monomers, respectively. CDs have circular conformation in which the glucose units are arranged in a circle with the hydrophilic side facing out, which confers its solubility in water. An internal hydrophobic cavity is formed that can enclose inclusion complexes of hydrophobic compounds (Biwer et al., 2002). Some CDs may sequester cholesterol and reduce its absorption, apart from other applications as food preservatives (Astray et al., 2009).

Moreover, enzymatic modification of starches has been proposed to reduce the glycemic index (GI) and to improve the quality characteristics of the foods. The GI is defined as the area under the curve of the blood glucose concentration in the 2 hour period immediately following consumption of a fixed amount of digestible carbohydrate compared to the *in vivo* digestibility of white bread as a standard (Jenkins et al., 2002). The rate of carbohydrate digestion and absorption affects blood glucose concentration and is of high interest because of its impact on diabetes and other metabolic diseases. Diabetics may benefit from low glycemic index (GI) foods as low GI diets protects against development of type 2 diabetes in general population (Kanellos et al., 2013). For instance the treatment of banana starch with

pullulanase reduced the available starch (Gonzalez-Soto et al., 2004), or α-amylase treatment of corn starch reduced postprandial glycemic response in rats (Han et al., 2006). Moreover, the addition of CDs to soluble starch also contributes to lower the GI, owing the action of γ-CD (Sybuia et al., 2015). Therefore, starch state is crucial to the GI value and the role of some CDs has been reported, but there is no information about the impact of CGTase modified starch and the resulting hydrolysis products on the GI. Further, animal studies offer the possibility to study the GI concept in a near lifelong perspective. Hence, it is on our particular interest to study the effects of enzymatic modification by CGTase on corn starch samples and the possible health benefits of containing CDs and modify the GI in food formulation application. The present study evaluated the effect of enzymatic modification of corn starch by CGTase, under specific sub-gelatinization conditions, focused on the glycemic properties in mice and their relationship to physical properties.

2. Materials and methods

2.1. Materials and reagents

Corn starch samples were generously supplied by Huici Leidan (Navarra, Spain). Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) (Toruzyme® 3.0 L) of food grade was provided by Novozymes (Bagsværd, Denmark). Chemical reagents from Sigma-Aldrich (Madrid, Spain) were of analytical grade.

2.2. Methods

2.2.1. Samples preparation

Corn starch (10.0 g) was suspended in 50 mL of 20 mM sodium phosphate buffer at pH 6.0. Modified starches were prepared by adding CGTase (0.32 U of CGTase/g starch). Samples were kept in a shaking water bath (50 rpm) at 50 ºC for 48 hours. 50 mL of water were added to the suspensions and

homogenized with a Polytron Ultraturrax homogenizer IKA-T18 (IKA works, Wilmington, DE, USA) for 1 min at speed 3. Samples were centrifuged for 15 min at 7,000×g and 4 ºC. The starch pellets were washed with 50 ml of water and centrifuged again with the same conditions. Supernatants were pooled and boiled in a water bath for 10 min to inactivate the enzyme. To assess the role of the water soluble hydrolysis products, two enzymatically treated corn starches were prepared, washed enzymatically treated corn starch (CGT-W) and the enzymatically treated washed corn starch with hydrolyzates added back (CGT-NW). CGT-W sediments were freeze-dried and kept at 4 ºC for further analyses. CGT-NW contained CGT-W and the water soluble compounds from heated and dried washings. The CGT-NW was also freeze-dried and kept at 4 ºC for further analyses. Native corn starch (N) without treatment was used as the control sample.

2.2.2. CDs and oligosaccharides quantification by High Performance Anion Exchange Chromatography.

Supernatants containing released hydrolysis compounds were freeze-dried and oligosaccharides and CDs were detected by HPAEC through a CarboPac PA-100 column (250 mm×4 mm), coupled to a pulsed amperometric detector (Dionex). The flow rate was 1.0 mL/min and the injection volume 10 μL. Using solutions A (water), B (1 mol/L NaOH), C (1 mol/L $C_2H_3NaO_2$), and D (water), the following running profile was applied: time zero, 46.25% A, 5% B, 2.5% C, 46.25% D; 25 min, 42.5% A, 5% B, 10% C, 42.5% D; 1 min, 35% A, 15% B, 15% C, 35% D; 3 min, 33% A, 15% B, 19% C, 33% D; 5 min, 28.5% A, 15% B, 28% C, 28.5% D; 1.5 min, 18.5% A, 15% B, 48% C, 18.5% D. For the identification and quantification of each compound, standards of known concentrations were previously analyzed (Dura and Rosell, 2016). Analysis was carried out at least in duplicate.

2.2.3. Scanning electron microscopy (SEM)

Corn starch powders, held to specimen holders with cuprum tape, were coated with gold in a vacuum evaporator (JEE 400, JEOL, Tokyo, Japan). Samples were scanned at 10 kV accelerating voltage with a SEM (S-4800, Hitachi, Ibaraki, Japan). The microstructure analysis was performed using image analysis software (Image-Pro Plus 7.0, Media Cybernetics, USA) in the Central Service for Experimental Research of the Universidad de Valencia.

2.2.4. Pasting properties

The pasting properties were determined with a rapid visco analyzer (RVA) (model 4500, Perten Instruments, Hägersten, Sweden) using the Approved Methods of the American Association of Cereal Chemists (AACC International, 1997), although 2 g of starch were used. Viscosity was recorded using Thermocline software for Windows (Newport Scientific Pty. Limited). Assays were carried out in triplicate.

2.2.5. Determination of postprandial glucose by glucose tolerance test (GTT)

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) were used for the GTT. The mice were housed individually in an environmentally controlled room (20-22 °C, 60% relative humidity, 12-hours alternating light:dark cycle). Mice were acclimatized and had *ad libitum* access to water and mouse chow diet (LabDiet, PMI International; protein, 239 g/kg; fat, 50 g/kg; non-nitrogenous substances, 487 g/kg; crude fiber, 51 g/kg; ash, 70 g/kg; energy, 17 MJ/kg; and sufficient amounts of minerals and vitamins for healthy maintenance). Samples tested included glucose (G), native corn starch (N), washed corn starch treated with CGTase (CGT-W) and non-washed corn starch treated with CGTase (CGT-NW). The physiological effects of CGTase modified starches on postprandial glucose were assessed. Starch samples were administered by gavage. Starches were suspended in

water, 20% (w/v) for ungelatinized samples and 10% (w/v) for gelatinized samples, due to their high viscosity produced after heating, according to Ayala et al. (2010). Starches were gelatinized by immersion in a boiling water bath for 15 min. Determination of postprandial glucose was performed on 6 mice once a week for 4 weeks. The samples were administered using a 4x4 Latin square design. Mice were fasted for 4 hours prior to starch administration and administered 100 µl/g body weight of a suspension. Blood glucose levels were measured from the tail vein at 0, 15, 30, 60, and 120 min after sample administration using a OneTouch Ultrameter (LifeScan Inc., Milpitas, CA, USA).

Statistical analysis

Experimental data were statistically analyzed for analysis of variance (ANOVA) using Statgraphics Centurion XV software (Bitstream, Cambridge, N). When analysis of variance indicated significant *F* values, multiple sample comparisons were also performed by Fisher's least significant differences (LSD) test to differentiate means with 95% confidence ($P<0.05$). Data was also evaluated using Pearson correlation coefficients to establish relationship among variables.

3. Results and Discussion

3.1. CDs and oligosaccharides in hydrolyzates

Oligosaccharides and CDs content in the starch samples are presented in Table 1. The N and CGT-W did not contain hydrolysis products. The analysis confirms the removal of the water soluble CGTase hydrolysates. The CGT-NW samples contained several oligosaccharides, with a large amount of maltose followed by maltotriose and glucose. CGTase cleaves α-1,4-glycosidic bonds in the inner part of a polysaccharide chain, leading to CDs and oligosaccharides with different degrees of polymerization through disproportionation reactions (van der Veen et al., 2000). α-CD and β-CD

were found in CGT-NW samples, with predominance of β-CD, likely due to the reaction conditions. Blackwood and Bucke (2000) reported that β-CD was the main hydrolysis compound, although the release of α-CD could be favored by adding organic solvents. In the early stage and at low enzyme concentrations CGTase produces predominantly α-CD but with prolonged reaction time or high enzyme dosage, the amount of the other CDs exceeds that of α-CD (Slominska and Sobkowiak, 1997). CDs have been very useful in food formulations to encapsulate flavors, protect against oxidative degradation, and sequestrate cholesterol (Astray et al., 2002).

3.2. Microstructure of starch

Scanning electron micrographs of native and enzymatically modified starches are presented at low magnification (x2000) in Figure 1 and high magnification (x3500) in Figure 2. Changes in microstructures are readily evident. The native starch granules have flat polygonal faces, straight edges and smooth surface with no evidence of cracks or holes in the surface (Figure 1a and 2a). Enzymatically modified starch granules (Figure 1b and c; 2b and c) have a distinctly different surface microstructure. After 48 hours of enzymatic treatment, shapeless structures appear where edges and corners were no longer visible. Native starch granules have a unique semi-crystalline supramolecular structure with concentric layers of amorphous, made up of amylose, and crystalline regions, manly composed of amylopectin, radiating from the hilum. It might be expected that treatment with CGTase at 50 ºC promotes hydrolysis of the amorphous areas of granules, leading to internal fissures whereas the crystalline lamellas are more resistant to hydrolysis. Small and randomly distributed porous starches were obtained when corn starch was treated with CGTase at sub-gelatinization temperature because starch in the interior is more susceptible to enzyme action than the outer part of the granules.

Table 1. Monosaccharides, oligosaccharides and cyclodextrins released by CGTase from corn starch, expressed in mg.100 g^{-1} of starch.

	Glucose	Maltose	Maltotetriose	Maltotetraose	Maltopentaose	α-CD	β-CD	γ-CD
CGT-NW	2.961	4.025	3.097	<dl	0.604	3.884	6.039	<dl
CGT-W	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl

dl means under detection limit. CGT-NW: CGTase non-washed sample; CGT-W: CGTase washed sample.

This mode of degradation differs from those exhibited by the action of other amylase enzymes on corn starch granules that yield bigger and open holes (Dura et al., 2014). This interior hydrolysis confirms the unique CGTase degradation mechanism of hydrolysis activity and transglycosylation reaction. At lower magnification, (Figure 1c), some hydrolysis products can be observed from the action of the enzyme, not present in CGT-W samples (Figure 1b) because those samples were washed. The action of the enzyme and the presence/absence of hydrolysis products are validated by SEM micrographs confirming the existence of hydrolysis products recovered from the supernatant.

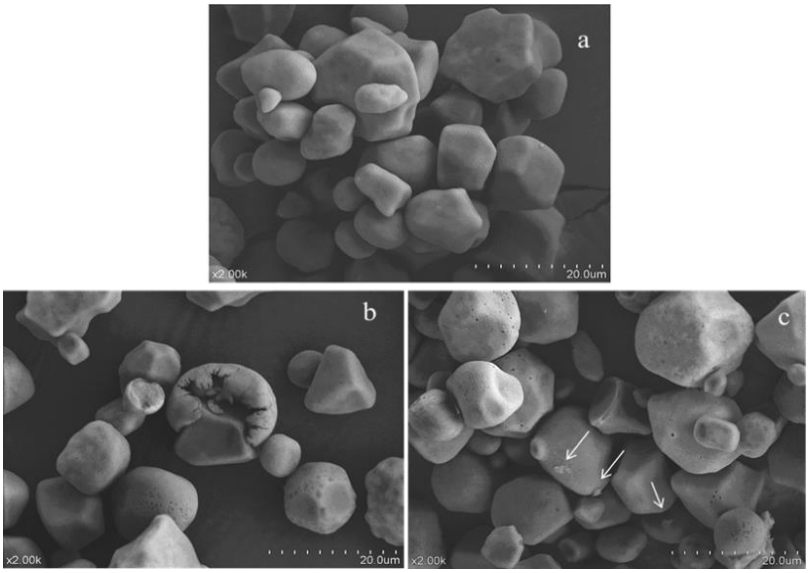


Figure 1. Scanning electron micrograph of corn starch samples treated enzymatically (b and c) and native corn starch sample (a). Magnification 2000×. Native corn starch (a); CGTase washed sample, CGT-W (b); CGTase non-washed sample, CGT-NW (c) (arrows show the hydrolysis products)

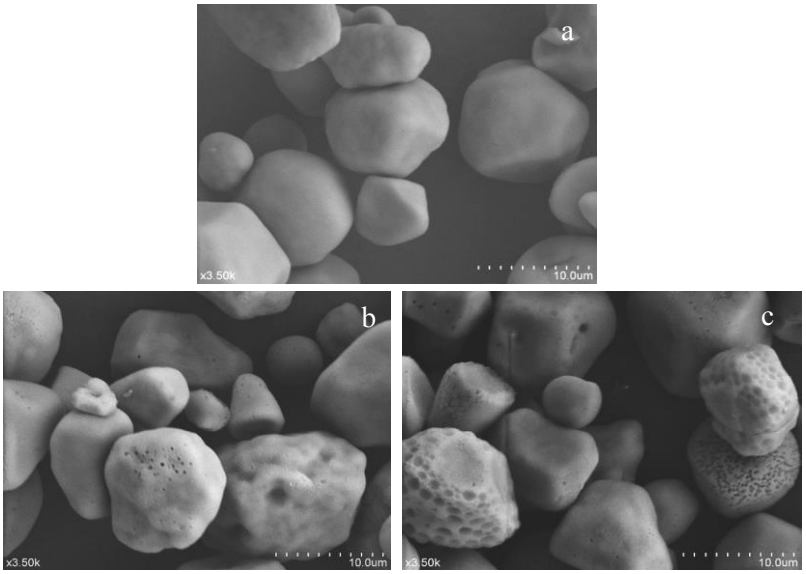


Figure 2. Scanning electron micrograph of corn starch samples treated enzymatically (b and c) and native corn starch sample (a). Magnification 3500×. Native corn starch (a); CGTase washed sample, CGT-W (b); CGTase non-washed sample, CGT-NW (c). Arrows indicate the presence of hydrolysis products

3.3. Pasting properties

RVA curves are plotted in Figure 3. Pasting properties of the enzymatic treatment were observed when compared to native starch (Table 2). Treated starches underwent the action of the enzymatic treatment and also possible rearrangement due to annealing during the preparation process under sub-gelatinization temperature (van der Veen et al., 2000). The CGT-NW treated starch showed significant lowering of the peak time indicating either earlier granule swelling during heating or an effect due to hydrolysis products. The CGT-NW starch also had higher breakdown, indicating lower stability of the samples, and lower viscosity at the end point of the cooling cycle, likely due to steric impediment of the hydrolysis products on the amylose crystallization. The setback viscosity predicts the degree to which the starch polymers are able to form networks resulting in the formation of gels that

have higher viscosity as the starch paste cools. The setback was very low for CGT-NW compared to N and CGT-W samples.

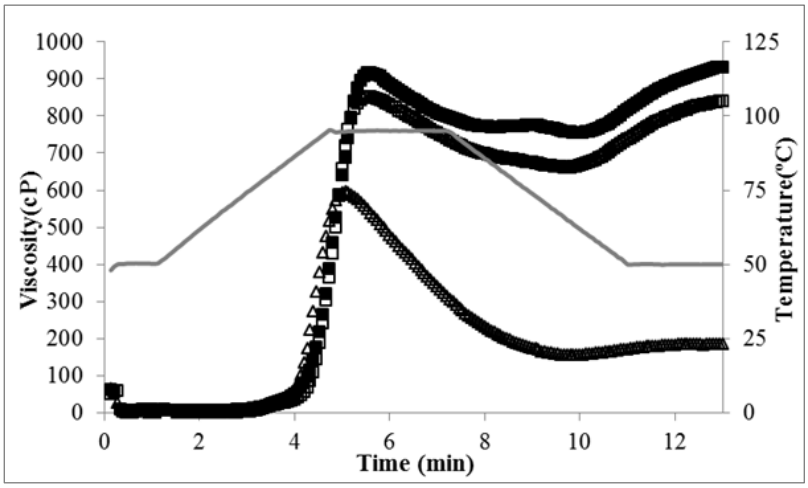


Figure 3. RVA profiles of enzymatically treated corn starches. Legends: Native corn starch, N (■); CGTase washed sample, CGT-W (□); CGTase non-washed sample, CGT-NW (Δ)

The oligosaccharides and CDs from the hydrolysis of amylose might physically interfere with the reorganization (gelling) process (Xiaoming et al., 2002). The final viscosity of the CGT-W sample was lower than the N sample probably because it contained less amylose to form the gel or viscous paste after gelatinization and cooling. Similar results have been described by Gujral and Rosell (2004) when adding CGTase to wheat starch suspensions. They also observed a decrease in peak and final viscosity of the wheat starch suspension. Trough viscosity were higher for N and CGT-W compared to the CGT-NW samples because they were more stable during gelatinization and their peak and final viscosities were higher. Due to CGTase activity hydrolysis products were formed and these products were present in CGT-NW samples resulting in higher hydrolysis percentage at 95 ºC and even higher at 50 ºC when comparing to N or CGT-W samples. The differences in pasting behavior between CGT-W and CGT-NW are attributed to the presence of β-CD and oligosaccharides in the CGT-NW samples. Similar

results were observed by addition of β-CD to cereal starches that caused early granule swelling, decreased onset of pasting temperature and decreased peak viscosity, likely due to its ability to form CD-lipid complex (Astray et al., 2009). β-CD and oligosaccharides may disrupt formation of amylose-lipid complexes and increase the swelling and solubility of starch granules during gelatinization (Gunaratne et al., 2007). Viscosity differences have been linked with the GI of carbohydrates. Guar gum also decreased blood serum glucose in rats during the first month of the experiment (Prias and Sgarbieri, 1998) suggesting that the viscosity of cereal carbohydrates may affect blood glucose and insulin responses.

Table 2. Effect of enzymatic treatment on the pasting parameters of corn starch.

	Native	CGT-W	CGT-NW
Onset temp (ºC)	89±3ab	90±0b	87±0a
Peak time (min)	6±0b	6±0b	5±0a
Peak viscosity (cP)	892±6b	851±33b	584±a
Trough (cP)	749±30c	660±26b	154±11a
Breakdown (cP)	160±3a	191±9a	442±13b
Final viscosity (cP)	883±2b	818±20b	183±13a
Setback (cP)	151±1b	179±14b	29±4a
Hydrolysis 95°C (%)	6±5a	13±3a	39±2b
Hydrolysis 50°C (%)	9±8a	18±4a	82±1b

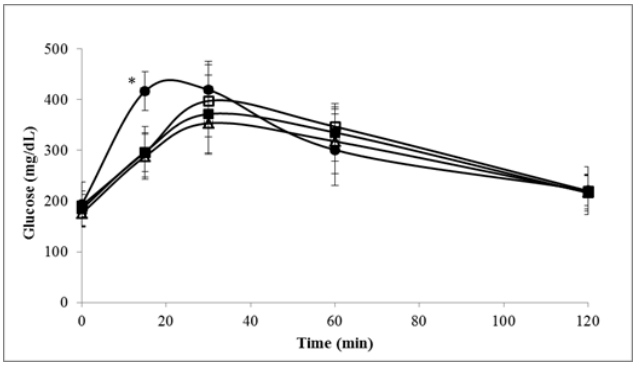
[a, b, c] values followed by different letters within a column denote significantly different levels ($P < 0.05$). Results were presented as mean ± SD (n = 3).

3.4. Glycemic response

Glycemic response is a numerical index that reflects the overall rate of absorption of glucose from the digestive tract into the blood and transfer of glucose from the blood into peripheral tissues. Jenkins et al. (2002) reported that the rate of food digestion was an important determinant of glycemic response. There was a significant difference ($P<0.05$) in blood glucose level

at 15 min between glucose and the nongelatinized N or CGTase modified starches (Figure 4).

A



B

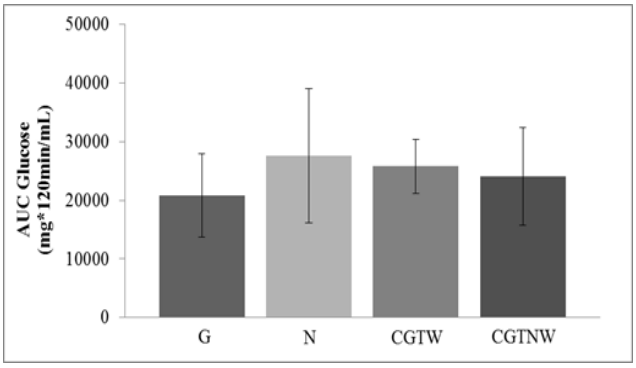


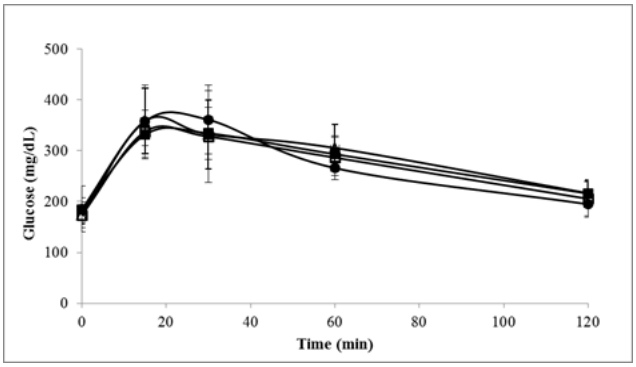
Figure 4. Glucose tolerance in mice gavage with ungelatinized samples. The samples studied were glucose, G (●); native corn starch, N (■); CGTase washed sample, CGT-W (□) and CGTase non-washed sample, CGT-NW (Δ). (A) Glucose tolerance tests (GTT) were performed in the fasting state. (B) Area under the curve (AUC) values. Data are expressed as mean n=6/group

As expected, glucose had the shortest time to peak and the highest peak because it is rapidly absorbed compared to all starches. Glucose also had the

most rapid decrease in blood level suggesting rapid uptake by tissues. All starches had a time to peak of about 30 min, later than glucose, attributed to the slower hydrolysis of amylopectin. The crystalline regions of amylopectin resist the conformational changes necessary for enzymatic hydrolysis. CGT-W had a slightly higher peak viscosity at 30 min compared to the other two starches. SEM had shown that starch granules incubated with amylolytic enzymes at 50 ºC results in changes in the amorphous areas, leading to granules with more ordered internal structure. Hydrolysis of the amorphous regions may lead to more internal surfaces that are accessible to enzyme attack and may be the reason that CGT-W samples seem to be more rapidly digested. CGT-NW had the lowest peak maximum and appeared to be more slowly digested. The presence of CDs may impede the orientation of amylases slowing hydrolysis. Weselake and Hill (1983) showed that β-CD inhibits hydrolysis of starch by α-amylase and intestinal α-glucosidase enzymes resulting in a longer period for absorption. This result suggests that CDs and oligosaccharides decrease the rate of absorption and may help to maintain lower blood glucose levels. Glucose concentration from all samples returned to the base line level 2 hours after the gavage samples. The area under the incremental glucose response curves (AUCs) were calculated from 0 to 2 hours post feeding. No significant differences were found between samples. There is a trend where AUC for the CGT-NW starch appears to be lower than the CGT-W or N starches; this may be due to the rapid hydrolysis and absorption into the blood stream of the hydrolysis products but slower uptake by peripheral tissues due to β-CD inhibitory action. The time to peak of gelatinized starches were shorter and about the same as for glucose (Figure 5). Starch gelatinization resulted in more rapid digestion as shown by the decrease in time to peak from 30 min for ungelatinized to about 17-27 min for gelatinized starches. When starch samples are subjected to extremely boiling temperatures water is absorbed, the granule swells and some amylose is leached, transforming the semicrystalline granules to an amorphous state that increases accessibility to enzymes (Dura and Rosell, 2016). These results

are similar to Collings et al. (1981) findings, who also found that the response of serum glucose to glucose monohydrate and cooked starch were very similar, while that to raw starch was significantly less. No significant differences in AUC were found between starch samples. Correlation between pasting properties and insulinemic responses in mice were analyzed. For ungelatinized samples, there was a negative correlation between setback values and glucose concentration at 2 hours (r=-1.0, P<0.05).

A



B

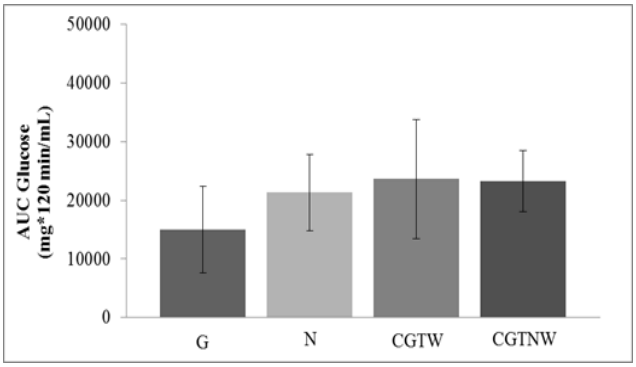


Figure 5. Glucose tolerance in mice gavage with gelatinized samples. The samples studied were glucose, G (●); native corn starch, N (■); CGTase washed sample, CGT-W (□) and CGTase non-washed sample, CGT-NW (Δ). (A) Glucose tolerance tests (GTT) were performed in the fasting state. (B) Area under the curve (AUC) values. Data are expressed as mean n=6/group

Greater values of setback viscosity were related to lower values of glucose at 2 hours. For gelatinized samples there were positive correlations between hydrolysis at 50 ºC and glucose concentration at 60 min (r=0.84, P<0.05) and hydrolysis at 95 ºC and glucose concentration at 60 min (r=0.81, P< 0.05). A negative correlation was also found between peak time and glucose concentration at 60 min (r=-0.85, P<0.05); setback and glucose concentration at 60 min (r=-0.88, P<0.05), and finally trough and glucose concentration at 60 min (r=-0.8218, P<0.05). These correlations support the idea that the different patterns of the glycemic curves are related to the viscosity of the samples.

4. Conclusions

Enzymatic modification at sub-gelatinization temperatures of corn starch by CGTase with or without hydrolysis products offers new possibilities to the modern food industry and the enormous variety of food products looking for healthy properties. Porous starches were obtained by CGTase treatment, which manage to have different pasting behavior and diverse glycemic response owing to the presence of β-CD that inhibits the α-amylase action. Significant correlations were found between glycemic curves and viscosity pattern of starches. Overall, CGTase enzymatically modified corn starch may fill a niche in the diverse uses of starches.

Acknowledgements

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Compliance with Ethical Standards

All authors declare that they have no conflict of interest. All animal procedures were approved by the Animal Care and Use Committee, Western Regional Research Center, USDA, Albany, CA, USA. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

References

AACC International (1997) Approved Methods of Analysis, 11th Ed. Method 61-02. Determination of pasting properties of rice with the rapid viscoanalyzer. Approved October 15, 1997. AACC International, St. Paul, MN, U.S.A.

Astray G., Gonzalez-Barreiro C., Mejuto JC., Rial-Otero R., Gandara JS. (2009). A review on the use of cyclodextrins in foods. Food Hydrocolloid. 23: 1631-1640.

Ayala JE., Samuel VT., Morton GJ.,Obici S., Croniger CM., Shulman GI., Wasserman DH., McGuinness OP. (2010) Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. Disssease Models and Mechanisms. 3: 525-534.

Biwer A., Antranikian G., Heinzle E. (2002). Enzymatic production of cyclodextrins. Application in Microbiology and Biotechnology. 59 :609-617.

Blackwood AD., Bucke C. (2000). Addition of polar organic solvents can improve the product selectivity of cyclodextrin glycosyltransferase-Solvent effects on CGTase. Enzyme Microbiolical Technology. 27: 704-708.

Collings P., Williams C., Macdonald I. (1981). Effects of cooking on serum glucose and insulin responses to starch. British Medical Journal. 282: 1032-1032.

Dura A., Blaszczak W., Rosell CM. (2014). Functionality of porous starch obtained by amylase or amyloglucosidase treatments. Carbohydate Polymers. 101: 837-845.

Dura A., Rosell CM. (2016). Physico-chemical properties of corn starch modified with cyclodextrin glycosyltransferase. International Journal of Biological Macromolecules. 87: 466-472.

Gonzalez-Soto RA., Agama-Acevedo E., Solorza-Feria J., Rendon-Villalobos R., Bello-Perez LA. (2004). Resistant starch made from banana starch by autoclaving and debranching. Starch-Starke. 56: 495-499.

Gujral HS., Rosell CM. (2004). Modification of pasting properties of wheat starch by cyclodextrin glycosyltransferase. Journal of Science Food Agriculture. 84: 1685-1690.

Gunaratne A., Ranaweera S., Corke H. (2007). Thermal, pasting, and gelling properties of wheat and potato starches in the presence of sucrose, glucose, glycerol, and hydroxypropyl β-cyclodextrin. Carbohydate Polymers. 70: 112-122.

Han XZ., Ao ZH., Janaswamy S., Jane JL., Chandrasekaran R., Hamaker BR. (2006). Development of a low glycemic corn starch: Preparation and characterization. Biomacromolecule. 7: 1162-1168.

Jenkins DJA., Kendall CWC., Augustin LSA., Franceschi S., Hamidi M., Marchie A., Jenkins AL., Axelsen M. (2002). Glycemic index: Overview of implications in health and disease. American Journal of Clinical Nutrition. 76: 266-273.

Kanellos PT., Kaliora AC., Liaskos C., Tentolouris K., Perrea D., Karathanos VT. (2013). A study of glycemic response to corinthian raisins in healthy subjects and in type 2 Diabetes Mellitus patients. Plant Food for Human Nutrition. 68: 145-148.

Prias ACD., Sgarbieri VC. (1998). Guar gum effects on food intake, blood serum lipids and glucose levels of Wistar rats. Plant Food for Human Nutrition. 53: 15-28.

Slominska L., Sobkowiak B. (1997). Studies on cyclodextrin synthesis by novel cyclodextrin glucosyl transferase. Starch-Starke 49: 301-305.

Sybuia MF., Guilhermetti M., Mangolim CS., Bazotte RB., Matioli G. (2015). Impact of cyclodextrins on postprandial glycemia: Evaluation in experimental animal model using the real-time continuous glucose monitoring system. Journal of Medicinal Food. 18: 625-630.

van der Veen BA., Uitdehaag JCM., Dijkstra BW., Dijkhuizen L. (2000). Engineering of cyclodextrin glycosyltransferase reaction and product specicity. Biochimica et Biophysica Acta. 1543: 336-360.

Weselake RJ., Hill RD. (1983). Inhibition of alpha-amylase catalyzed starch granule hydrolysis by cycloheptaamylose. Cereal Chemistry. 60: 98-101.

Xiaoming L., King JM., Shih FF. (2002). Pasting property differences of commercial and isolated rice starch with added lipids and beta-cyclodextrin. Cereal Chemistry. 79: 812-818.

IV. General discussion

Bread-making is an extensively investigated process based on many principles originated in ancient times. The process involves mixing flour (mainly wheat), water, salt, and yeast with other ingredients, dough fermentation, kneading, proving and baking, to finally result in a quality end-baked product. Particularly, starch, as the most abundant carbohydrate in cereals, and protein (mainly gluten), which forms a continuous network that gives the structure of the bread dough making it elastic and extensible, play both an important role to achieve the desirable product (Dura and Rosell, 2016). Cereal based products have long been a primary source of carbohydrates in the human diet. However, the consumers' health awareness, besides the demanding of gluten-free products, drive to look for nutritious and added-value starches, since starch is the main player in cereal based foods and also in gluten-free bakery goods. Therefore, the research presented in the previous chapters, responds to the scientific and technological purposes of succeeding in the development and understanding of functional and structural properties of enzymatically modified corn starch. This opens promising ways for the incorporation into cereal-based products of obtained modified starches contributing to healthier and improved gluten-free products.

An updated revision of the current knowledge was performed regarding enzymes involved in the baking process, in order to screen the individual effect of the enzymes according on the substrate they act on. Moreover, the effect from the individual and combined added enzymes as technological aids, on the carbohydrate matrix in the bread-making process was reviewed, along with the enzymes used in gluten-free breads. From that review, the role of carbohydrates, mainly starch, was clearly emphasized as one of the main biopolymers in the bakery matrices, including the enzymes that can act on its structure. Nevertheless, those enzymes have been usually employed as technological or processing aids in bread-making but they have not been

explored as healthy aids (Rosell, 2014), looking for modulating the potential healthy properties of starches.

For further studies, based on preliminary assays, the choice of the three different enzymes, AM, AMG and CGTase, was based on the hydrolyzed substrate, as starch-degrading enzymes, along with corn starch, that was preferred as gluten-free raw material to fulfill the health requirements. Previous analyses were carried out to select the suitable conditions for the enzymatic modification of corn starch (starch quantity, enzyme concentration, pH, time and temperature). Sub-gelatinization temperature (50 ºC) was chosen to modify the corn starch without losing its granular integrity. AM (5 U/g starch) starch modification was accomplished at pH 6.0 while pH 4.0 was selected for AMG (4 U/g starch) starch modification, during 24 hours of incubation. Regarding CGTase (0.32 U/g starch), previous studies suggested to test the catalytic reaction at pHs 4.0 and 6.0, designated for the starch modifications after 24 and 48 hours of incubation. Starch suspensions without the addition of the enzyme at different pHs were used as controls.

To acknowledge that enzymatic reaction occurred on the starch granules and to confirm modification of starch, functional properties, biochemical features, thermal and structural analyses of the treated starches were performed. The starch microstructure analyzed by SEM confirmed the effect of enzymatic treatments in the starch structures leading to different porosity degree depending on the enzyme specificity. AM and CGTase action lead to some uneven pinholes on the granules surface. AMG was readily active on the starch surface resulting in greatly holed granules when compared to the other enzymes. This technique allowed confirming at high magnification that enzyme modification also happened in the internal morphology, related to and inside out digestion pattern (Benmoussa et al., 2006), hydrolyzing the starch granules from the hilum region towards the out-sides. Therefore, less organized amorphous region of the granules could be more accessible to the enzyme attack and subsequent hydrolysis, remaining crystalline domains

resistant to enzymatic action. Nevertheless, slower hydrolysis can take place on the crystalline areas if a longer incubation endures or greater hydrolysis is produced (Wang et al., 1995; Gallant et al., 1997). However, the enzyme action patterns were dependent on the pH and incubation time. Treatment at pH 6.0 for longer period of incubation seemed to be more aggressive disrupting double helices and crystalline order. DSC analysis showed that for AMG-treated starches, gelatinization enthalpy showed lower values denoting that an extensive hydrolysis took place disturbing crystalline areas of the granule (Hoover and Zhou, 2003). Differently, AM-modified starches showed slightly increased gelatinization enthalpy, suggesting that hydrolysis mainly occurred at the amorphous regions while CGTase treatment hardly affected the thermal behavior of the starch, indicating more visible changes at the surface area, as was corroborated by SEM analysis.

Hydrolyzing enzymes have been frequently used for enzymatic modification of different starches as potato, corn, wheat, rice and tapioca (Khatoon et al., 2009; Kaur et al., 2012) to achieve the desired functional properties varying reaction conditions. Simultaneously to those changes in the starch granules, diverse compounds are released from the enzymatic reactions, which have been noticed only when syrup, dextrins and oligosaccharides production is of interest. In the present research, to support the evidence of modifying corn starch with amylolytic enzymes, the released compounds were evaluated. Overall, enzymes were greatly active breaking the degree of association between molecular bonds for longer incubation time, and more soluble compounds were leached out. In the specific reactions catalyzed by CGTase, the quantification of CDs and oligosaccharides released during the enzymatic treatment was performed by HPAEC, confirming a) the action of the enzyme on the starch granules during incubation time, b) the presence of hydrolysis products, and c) the pattern of released compounds was pH-dependent. Oligosaccharide final composition has proven to depend on the concentration at which hydrolysis reaction occurs (Marchal et al., 1999).

Once confirmed the enzymatic modification of the granular starch, its technological and functional properties such as gel formation were evaluated, since they are of key importance to define the industrial uses of starches. Viscosity profiles along a heating-cooling cycle were valuable indicators to characterize different modified starches. A typical viscosity pattern from a native starch, displayed an increase of viscosity up to peak (during heating) followed by a drop of viscosity (during cooling) under agitation and heating. Considering morphological and structural changes promoted by enzymes, pasting profiles were expected to change reducing the peak viscosity. However, changes in the properties were not as prominent as expected. Viscosity values of the enzymatically modified samples changed when comparing to their controls at different pHs. Different trends were observed revealing diverse pasting behavior, related to enzyme action on starch glycosidic linkages and subsequent starch structure modification.

Potential nutritional benefits of the obtained starches were also evaluated. Digestion behavior of the starches is commonly assessed by *in vitro* hydrolysis test using enzymatic analysis. Enzymatic *in vitro* starch hydrolysis was performed to determine the rate and extent of hydrolysis during enzymatic digestion. High susceptibility to be digested was shown by AM- and AMG-treated starches. Enzymes involved in the digestion process had more ready access to hydrolyze enzymatically modified starches and released glucose. Opposite trend was displayed on CGTase-modified samples that resulted less susceptible to be hydrolyzed by digestion enzymes. Transglycosylation reactions, mainly catalyzed by CGTase, probably acted on the exterior surface and led to a more crystalline structure resistant to enzymatic hydrolysis after modification of starch granules (Kimura and Robyt, 1995). Since starch is mostly consumed in gelatinized form, the enzymatic hydrolysis of gelatinized samples was also assessed. It seems that conformation change and disruption of the granule during gelatinization led to structures that were more accessible, enhancing starch susceptibility to

enzymes during digestion (Kimura and Robyt, 1996), and thus leading to faster hydrolysis.

Starch represents one of the most glycemic carbohydrates. Since GI is associated with the development of many diseases (Ludwing, 2002), it is attracting much attention the development of new strategies to produce low GI foods to control and prevent these diseases. Based on previous results, enzymatically modified corn starch samples by CGTase (0.32 U of CGTase/g starch) were selected to study the glycemic response in mice and its relationship to granule physical properties. Reaction conditions were pH 6.0 at longer incubation time (48 hours) and sub-gelatinization temperature (50ºC). Due to the possible role of released compound, two enzymatically treated starches were prepared, washed enzymatically treated corn starch (CGT-W) and thus non-containing hydrolysis products, and enzymatically treated corn starch containing hydrolysis compounds (CGT-NW). HPAEC quantification of oligosaccharides and CDs confirmed the removal of hydrolyzates from CGT-W samples, and the presence of oligosaccharides and predominantly β-CD in CGT-NW samples, resulting from the enzyme action on starch glycosidic bonds and subsequent cyclization (Biwer et al., 2002). SEM-micrographs revealed small and randomly distributed porous on the starch surface and confirmed the presence of hydrolysis products in CGT-NW samples. The presence of oligosaccharides and β-CD modified the pasting behavior. The ability of β-CD to form CD-lipid complexes may disrupt the formation of amylose-lipid complexes and lower viscosity values showing less stable samples during gelatinization (Gunaratne et al., 2007). Physiological effects of CGTase modified starches on postprandial glucose were determined using GTT (Glucose Tolerance Test). Starches were tested as gelatinized (G) and non-gelatinized (NG) to studied the influence of structure changes promoted by gelatinization in glycemic response in mice. Postprandial responses may be changed by altering starch structure in foods using thermal processing (Björck, 1996). Gelatinized samples resulted in

more rapid digestion. Disruption of the starch structure during heating facilitates starch availability for easier water penetration and consequent enzymes action in the digestion of starch in the gastrointestinal tract (Holm et al., 1988). On the contrary, for non-gelatinized samples, the presence of β-CD seemed to inhibit α-amylase starch hydrolysis and intestinal α-glucosidase enzymes resulting in a longer period of absorption for non-gelatinized samples. Significant correlations were found between glycemic curves and viscosity pattern of starches. According to Holm et al. (1988), the degree of gelatinization is positively correlated with *in vivo* plasma glucose and insulin responses. A previous study conducted by Ross et al. (1987) has also related the degree of gelatinization with *in vivo* glycemic response and *in vitro* starch digestibility. Therefore, the different patterns of the glycemic curves were related to the viscosity of the samples. Taking into account the wide range of starchy foods and the presence of residual starch not fully gelatinized during processing, findings result profitable. The consumption of native or minimally processed cereal products could be of particular importance from nutritional science (Kang et al., 2003), and opens the possibility to modulate the gelatinization degree according with low GI.

Increasing demand of modified starches requires developing new strategies to obtain modified starches with enhanced properties. The research conducted provides knowledge about modified functional and structural properties of enzymatically modified corn starch at sub-gelatinization temperature, which offers an alternative for applications in a variety of foods. Possible modulation of the enzymatic modified process permits to obtain desirable modified starch depending on the final application.

References

Biwer A., Antranikian G., Heinzle E. (2002). Enzymatic production of cyclodextrins. Application in Microbiology and Biotechnology. 59 :609-617.

Benmoussa M., Suhendra B., Aboubacar A., Hamaker BR. (2006). Distinctive sorghum starch granule morphologies appear to improve raw starch digestibility. Starch-Stärke. 58: 92-99.

Bjoörck, I. (1996). Starch: nutritional aspects. In: Carbohydrates in Food. Eliasson AC. (ed.). Marcel Dekker, New York. 505-553.

Dura A., Rosell CM. (2016). Enzymes in baking. In: Microbial enzyme technology in food applications. Ray RC & Rosell CM (ed.). CRC Press. Taylor and Francis. 295-314.

Gallant DJ., Bouche B., Baldwin PM. 1997. Microscopy of starch: Evidence of a new level of granule organization. Carbohydrate Polymers. 32: 177-191.

Gunaratne A., Ranaweera S., Corke H. (2007). Thermal, pasting, and gelling properties of wheat and potato starches in the presence of sucrose, glucose, glycerol, and hydroxypropyl β-cyclodextrin. Carbohydrate Polymers. 70: 112-122.

Hwang JK. (2002). Function of uncooked foods. Food Industry and Nutrition. 7: 16-19.

Holm J., Lundquist I., Björck I., Eliasson AC., Asp NG. (1988). Degree of starch gelatinization, digestion rate of starch *in vitro*, and metabolic response in rats. American Journal of Clinical Nutrition. 47: 1010-1016.

Hoover R,. Zhou Y. (2003). *In vitro* and *in vivo* hydrolysis of starches by α-amylase and resistant starch formation in legumes-A review. Carbohydrate Polymers. 54: 401-417.

Kang SM., Shim JY., Hwang SJ., Hong SG., Jang HE., Park MH. (2003). Effect of saengshick supplementation on health improvement in diet-induced hypercholesterolemic rats. Journal of Korean Society Food Science and Nutrition. 32: 906-912.

Khatoon S., Sreerama YN., Raghavendra D., Bhattacharya S., Bhat KK. (2009). Properties of enzyme modified corn, rice and tapioca starches. Food Research International. 42: 1426-1433.

Kaur B., Ariffin F., Bhat R., Karim A. (2012). Progress in starch modification in the last decade. Food Hydrocolloids. 26: 398-404.

Kimura A., Robyt JF. (1995). Reaction of enzymes with starch granules: Kinetics and products of the reaction with glucoamylase. Carbohydrate Research. 277: 87-107.

Kimura A., Robyt JF. (1996). Reaction of enzymes with starch granules: Enhanced reaction of glucoamylase with gelatinized starch granules. Carbohydrate Research. 288: 233-240.

Ludwig DS. (2002). The glycemic index: Physiological mechanisms relating to obesity, diabetes, and cardiovascular disease. Journal of the American Medical Association. 287: 2414-2423.

Marchal LM., Beeftink HH.,Tramper J. (1999). Towards a rational design of commercial maltodextrins. Trends in Food Science and Technology. 10: 345-355.

Rosell CM. Enzymes in gluten free bread. Oral communication in AACCI Conference. 2014. Providence. RI. US.

Ross W., Brand JC., Thorburn AW., Truswell AS. (1987). Glycemic index of processed wheat products. American Journal of Clinical Nutrition. 46: 634-635.

Wang WJ., Powell AD., Oates CG. (1995). Pattern of enzyme hydrolysis in raw sago starch: Effects of processing history. Carbohydrate Polymers. 26: 91-97.

V. Conclusions

1. Enzymes provide a powerful tool for modifying starch granules and thus to change functional properties and nutritional performance, but a careful selection of the enzyme and the conditions for the enzymatic reaction are needed. Modified starch could be matched to a specific end-use to obtain the desirable product.

2. Enzymes are processing aids that act as catalysts for biochemical reactions, providing a wide range of quality improvements in baked goods. Up to now, efforts have been focused on using enzymes as processing aids for improving bakery products, but results show that enzymes' action can be extended supporting the concept of "healthy aids".

3. Enzymatic modification of corn starch by α-amylase or amyloglucosidase at sub-gelatinization temperatures provides starch granules with porous structure that showed specific functional properties. Properties and structure could be modulated by enzymatic conditions (enzyme type, pH, time of reaction). The degree of porosity could be controlled by using different enzymes or a combination of enzymes, which offers a suitable possible application in starch-based food matrices. Amyloglucosidase was more active on starch glycosidic linkages obtaining greater porous starch.

4. Corn starch modified by cyclodextrin glycosyltransferase showed to be pH dependent, being more aggressive at pH 6.0, where oligosaccharides were majorly released. CDs released during enzymatic modification of starch, enhanced the major benefits of the starch granules and thus their further application, taking into account their technological properties. Catalytic transglycosilation reaction of CGTase action on starch granules seemed to act on the amorphous zone, giving porous starches, but leading to a more crystalline structure resulting in more resistant starches to amylase hydrolysis. Nonetheless, after gelatinization, samples showed faster hydrolysis kinetics.

5. The presence of hydrolysis products obtained by enzymatic modification of corn starch with cyclodextrin glycosyltransferase, provoked different pasting behavior. The presence of β-CD in starch containing hydrolysis products hindered the α-amylase action, resulting in longer period required for their absorption affecting the glycemic index obtained after their ingestion. The different absorption patterns of modified starches lead to different glycemic curves, which were related to the viscosity of the samples. Overall, CGTase modified starches could offer a new ingredient to the modern food industry for obtaining food products looking for healthy properties.

Abbreviations

AACC	American Association of Cereal Chemists
AM	α-amylase
AMG	Amylogulosidase
ANOVA	Analysis of variance
AUC	Area under the curve
AX	Arabinoxylans
CD	Cyclodextrin
CGTase	Cyclodextrin glycosyltransferase
CGT-NW	Unwashed enzymatically modified sample with cyclodextrin glycosyltransferase
CGT-W	Washed enzymatically modified sample with cyclodextrin glycosyltransferase
GTT	Glucose tolerance test
C4	Control samples at pH 4.0
C6	Control samples at pH 6.0
DSC	Differential scanning calorimeter
EU	European Union
FAO	Food and Agriculture Organization in the United Nations
G	Glucose
GI	Glycemic index
GO	Glucose oxidase
GOPOD	Glucose oxidase-peroxidase
GRAS	Generally recognized as safe
GTT	Glucose tolerance test
HPAEC	High Performance Anion Exchange Chromatography

HPMC	Hydroxypropylmetil cellulose
ICC	International Association for Cereal Chemistry
LAC	Laccase
LSD	Least significant differences
N	Native corn starch
NSL	Non-starch lipids
NSP	Non-starch polysaccharides
PC	Paste clarity
PHI	Peak height index
RDS	Rapidly digesting starch
RS	Resistant starch
RVA	Rapid visco analyzer
SEM	Scanning electron microscopy
SCFA	Short chain fatty acids
SDS	Slow digesting starch
SEM	Scanning electron microscopy
SC	Swelling capacity
SI	Swelling index
SP	Swelling power
TGase	Transglutaminase
U	Units
US	United States
WE-AX	Water-extractable arabinoxylans
WU-AX	Water-unextractable arabinoxylans
WHO	World Health Organization

List of publications

Dura A., Błaszczak W., Rosell CM. (2014). Functionality of porous starch obtained by amylase or amyloglucosidase treatments. Carbohydrate Polymers. 101: 837-845.

Dura A., Rosell CM. (2016). Physico-chemical properties of corn starch modified with cyclodextrin glycosyltransferase. International Journal of Biological Macromolecules. 87: 466-472.

Dura A., Yokoyama W., Rosell CM. (2016). Glycemic response to corn starch modified with cyclodextrin glycosyltransferase and its relationship to physical properties. Plant Foods Human Nutrition. 71: 252-258.

Rosell CM., Dura A. (2015). Enzymes in bakeries. In: Enzymes in Food and Beverage Processing.Baking Industries. Chandrasekaran M. (ed). CRC Press. Taylor & FrancisGroup. 171-204.

Dura A., Rosell CM. (2016). Enzymes in Baking. In: Microbial Enzyme Technology in Food Applications. Ray R., and Rosell CM. (eds). CRC Press. Taylor & Francis Group. 295-314.

Dura A., Yokoyama W., Rosell CM. Effect of enzymatically modified corn starch by cyclodextrin glycosiltransferase on plasma and liver lipids, energy expenditure and fecal carbohydrates. *In progress.*

Dura A., Rose DJ., Rosell CM. Fecal fermentation of enzymatically modified corn starch. *In progress*.

Ouazib M., Dura A., Zaidi F., Rosell CM. (2016). Effect of partial substitution of wheat flour by processed (germinated, toasted, cooked) chickpea on bread quality. International Journal of Agricultural Science and Technology. 4: 8 - 17.