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Influence of ultrasonic application on the enzymatic formation of zinc protoporphyrin

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ABSTRACT

Ferrochelatase (FeCH), present in pork liver, catalyses the formation of zinc-protoporphyrin (ZnPP), a stable purple-red pigment found in Parma ham. Kinetics of ZnPP formation is especially slow. Thus, the aim of this study was to improve the ZnPP production using power ultrasound (US) at low (7.05 W/L) and moderate power (36.53 W/L) in homogenised pork liver (HLi) and with added oxyhemoglobin (HLi + OxyHb). ZnPP formation was performed at 37 °C, under anaerobic conditions, for different reaction times (6–48 h). When US was applied at low power, it shortened the time needed to reach maximum ZnPP formation by 50% and increased the yield by 25.77% in the case of HLi and by 4.42% in that of HLi + OxyHb, when compared to the control experiments; however, for the moderate power, ZnPP formation was hindered. Therefore, low power US proved to be an effective method with which to intensify enzymatic ZnPP production.

1. Introduction

The characteristic homogeneous stable redness of raw and dry-cured meat products is a determining factor in their quality, playing a key role in consumer preferences. This stable colour is usually achieved in dry cured meat products by adding nitrates and nitrites as colour enhancers and also as antimicrobial agents. The reaction of nitrite with the myoglobin of the meat muscle gives rise to nitrosylmyoglobin, a characteristic red compound. However, in long aged dry cured Italian hams (Parma ham), the red is formed without adding any nitrifying agent due to an enzymatic reaction by which zinc-protoporphyrin (ZnPP) is produced. Thus, ZnPP is the natural pigment that gives rise to the characteristic redness of Italian Parma ham (Wakamatsu et al., 2004). Furthermore, ZnPP is stable to light, so its addition to dry cured or fermented meat products would enhance the formation of their typical colour, minimising the use of nitrates and nitrites (De Maere et al., 2017).

ZnPP formation consists of two sequentially occurring mechanisms: i) the iron (Fe^{2+}) is released from the heme group of oxymyoglobin to form protoporphyrin IX (PPIX), and ii) the ion zinc (Zn^{2+}) is inserted into the porphyrin ring to form ZnPP (Ishikawa et al., 2007). Wakamatsu et al. (2015) studied pork liver as a substrate for ZnPP formation, obtaining a high concentration of pigment. Pork liver is a coproduct obtained from the meat industry. Therefore, the revaluation of this low-value slaughterhouse coproduct would reduce the cost of disposal and the environmental associated impact (Verma et al., 2022). Furthermore, it should be noted that previous studies examined the ZnPP formation in pork loin by adding an exogenous source of porphyrins from horse myoglobin, resulting in a greater amount of ZnPP (Wakamatsu et al., 2007). The aforementioned mechanisms were also studied *in vitro* in mitochondria, and it was observed that the exogeneous oxymyoglobin porphyrins were a good substrate for the enzyme ferrochelatase (FeCH), a protein associated with the inner mitochondrial membrane that catalyses the ZnPP formation (Ishikawa et al., 2007). Furthermore, Zhai et al. (2022) added commercial porcine hemoglobin (Hb) to Parma ham, which produced a higher formation of ZnPP, this fact indicated that Hb was an interesting substrate to generate ZnPP. In this regard, the addition of an exogenous substrate such as Hb, obtained from pork blood, to the ZnPP formation reactions would be of great interest, since pork blood is a coproduct of the meat industry characterized by its high volume and environmental impact (Alvarez et al., 2012). Parolari et al. (2016) postulated that the ZnPP formation in the Italian Parma ham curing process is catalysed by FeCH. However, there was also evidence that ZnPP formation could take place through other mechanisms, such as non-enzymatic reactions (Becker et al., 2012; Parolari et al., 2016). Although the mechanisms related to the ZnPP

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formation have not been fully elucidated, not only does the presence of endogenous enzymes, such as FeCH (Wakamatsu et al., 2004), play a key-role, but the physicochemical parameters, such as pH and temperature, also seem to be of paramount importance (Benedini et al., 2008; Ishikawa et al., 2007).

Bou et al. (2022) studied the ZnPP formation from FeCH in Serrano ham, observing that the formation began after 12 days of salting, in the so-called resting period, once the temperature was rose from 4 to 16 °C, and was continued during curing (12 months). Therefore, the ZnPP formation catalysed by FeCH could be considered a slow process dependent on both extrinsic (temperature, salt concentration and time) and intrinsic factors of the meat (pH and concentration and availability of enzymes and substrates). Thus, the search for alternatives to accelerate the ZnPP formation process, such as the use of emerging technologies, would be of great interest for subsequent industrial applications.

Power ultrasound (US) has been used to intensify enzymatic reactions (Córdova et al., 2022). It was used to improve the enzymatic hydrolysis of different substrates (Magalhães et al., 2022; Liang et al., 2021), and has also been applied to improve the extraction of enzymes. In this sense, in a previous study, Abril et al. (2021) observed that high power US application improved the FeCH extraction from pork liver compared to mechanical stirring. In these applications, the great energy release caused by the acoustic waves (Khan et al., 2021) helps to improve both hydrolysis and the enzyme extraction from the inner cell (Yao et al., 2020), but the high power may also cause enzyme denaturation. However, another plausible, but scarcely studied (Bansode and Rathod, 2017) strategy by which to improve the FeCH enzymatic reaction, is low or moderate power US application in order to induce a mild cavitation, or only a micro-stirring, and promote the binding of the $ZnSO_4$ and protoporphyrin IX with the FeCH active sites, or even ZnPP diffusion without altering the enzyme structure. Abril et al. (2021) postulated that the rate of ZnPP formation catalysed by the FeCH, using exogenous porphyrins and Zn^{2+}, was controlled by the product diffusion in the steady phase. In this context, this study aims to assess the feasibility of using power ultrasound, applied at moderate and low intensities, to improve the enzymatic reaction of the ZnPP formation catalysed by FeCH using different substrates: homogenised pork liver, and homogenised pork liver with added oxyhemoglobin from pork blood.

2. Materials and methods

2.1. Preparation of homogenised pork liver

Raw pork liver (from Large White x Duroc pigs) were transported at below 4 °C from an industrial slaughterhouse to the laboratory and processed in less than 2 h after slaughter. Afterwards, the pork livers were homogenised for 5 min (Blixer 2, Robot Coupe, Vincennes Cedex, France), immediately vacuum packaged (30 g portions) in plastic film 200 × 300 PA/PE (Sacoliva, Castellar del Vallès, Barcelona) and stored at −20 °C until use.

2.2. Preparation of oxyhemoglobin from hemoglobin standards

The oxyhemoglobin (OxyHb) solution was prepared as described by Bou et al. (2010). Firstly, 0.16 g of porcine hemoglobin (Hb) lyophilised powder (H4131, Sigma Aldrich, Canada) were dissolved in 3 mL of cold phosphate buffer (50 mM, pH 7.3, 4 °C). Hb was reduced to OxyHb by the addition of sodium dithionite crystals (ratio 1:0.8 w/w) and vortexing at 4 °C. In order to remove dithionite, 2.5 mL of the OxyHb solution were passed through a disposable PD-10 desalting column (17-0851-01, GE Healthcare Life Sciences). Then, 4 mL of phosphate buffer (50 mM, pH 7.3, 4 °C) was passed into the stationary phase to elute the OxyHb out of the column. The amount of Hb was calculated by measuring the absorbance at 523 nm (isosbestic point), as described by Snell and Marini (1988), whereas the percentage of OxyHb was calculated as described by Benesch et al. (1973). Only those solutions containing a minimum yield of 90% of OxyHb were used in subsequent experiments.

2.3. Preparation of oxyhemoglobin from pork blood

Blood was removed from pigs sacrificed according to standard procedures. Approximately 100 mL of blood was collected in a bottle containing sodium triphosphate to avoid coagulation. Hb was extracted from red blood cells following the procedure described by Bou et al. (2019). In order to reduce Hb solution to OxyHb, sodium dithionite crystals were added (ratio 1:0.3 w/w). The mixture was vortexed at 4 °C, and it was removed by passing 2.5 mL of OxyHb solution to a disposable PD-10 desalting column. Then, 4 mL of 50 mM Tris buffer (pH 8.5) was passed into the stationary phase to elute the OxyHb out of the column. Finally, the percentage of OxyHb was calculated as described in section 2.2 and only samples with a minimum OxyHb content of 90% were considered.

2.4. Preparation of the different reaction media

In order to investigate the ZnPP formation, 4 types of samples were prepared. Homogenised pork liver (HLi) with two different types of preservatives, antibiotics and organic acids, to study their influence on the ZnPP formation reaction. In addition, and using organic acids as preservatives, homogenised pork liver with added oxyhemoglobin from hemoglobin standards and homogenised pork liver with added oxyhemoglobin extracted from pork blood, were considered.

2.4.1. Homogenised pork liver with added antibiotics

The homogenised pork liver, previously prepared and stored at −20 °C, was kept at 4 °C for 1 h before preparation. From preliminary experiments, the final homogenate contained 20% pork liver; therefore, it was diluted with distilled water and a mixture of three antibiotics: potassium penicillin G (140 μg/mL), streptomycin sulfate (500 μg/mL), and gentamicin sulfate (100 μg/mL), to control microbial growth. The homogenised pork liver was protected from the light and kept on ice. The final solution was homogenised (DI 25 Basic Homogenizer, IKA, Germany) for 1 min at 4 °C and 8000 rpm. Finally, from preliminary experiments, the pH of the 20% liver homogenate was adjusted to 4.8 ± 0.05 with HCl 1 N.

2.4.2. Homogenised pork liver with organic acids

Antibiotics are not allowed in the food industry, and in order to eliminate them from the final homogenate, it was prepared with added organic acids. Therefore, ascorbic and acetic acids, preservatives which are accepted in the food industry as a means of preventing microbial growth, were considered. In order to obtain a final amount of liver in the homogenate of 20%, a solution containing 0.1 g ascorbic acid and 250 μL acetic acid/100 mL distilled water was prepared, and the pH was adjusted to 4.16 with NaOH 1 N. Subsequently, 20 g of liver that had been previously homogenised and kept at 4 °C for 1 h were weighed in a glass beaker and made up to 100 mL of the organic acid solution. The final solution was homogenised (DI 25 Basic Homogenizer, IKA, Germany) for 1 min at 4 °C and 8000 rpm. Finally, the pH of the 20% liver homogenate was adjusted to 4.8 ± 0.05 with HCl 1 N.

2.4.3. Homogenised pork liver with added oxyhemoglobin from hemoglobin standards and from pork blood

To prepare the homogenate of pork liver with added OxyHb, OxyHb obtained from commercial Hb as described in section 2.2 was first used, to better understand the effect of adding this pure and controlled substrate to the ZnPP formation reaction. Subsequently, OxyHb was obtained from pork blood (section 2.3), with the aim of studying the possibility of using this important meat co-product in the ZnPP

formation. 2.5 mL of the OxyHb solution, obtained as described in sections 2.2 and 2.3, respectively, were added to the 100 mL of homogenised pork liver reaction medium with organic acids previously described in section 2.4.2. The final solution was homogenised using a vortex for 45 s and the pH was adjusted to 4.8 ± 0.05 with HCl 1 N.

2.5. Formation of zinc-protoporphyrin: conventional (CV) and ultrasonic-assisted (US)

The kinetics of ZnPP formation were carried out following the experimental methodology previously described by Bou et al. (2022), with minor modifications. ZnPP formation requires an anaerobic medium and an optimal temperature of 37 °C. Thus, the reaction media described in section 2.4 were placed in opaque glass bottles (10 cm height x 2 cm diameter) of 15 mL capacity. Afterwards, the bottles were immersed in water (800 mL) placed inside a 3.5 L Anaerobic Jar (HP0011, OXOID, Argentina) in which anaerobic conditions were forced (Anaerobic System BR 38, Oxoid Ltd., Hampshire, England). The jar was placed (Fig. 1) at 6 cm from the bottom of an ultrasonic bath (15 L ATG15160, ATU, Spain). The samples were incubated at different times (0, 6, 12, 18, 24 and 48 h) in order to study the ZnPP formation kinetics.

The temperature control in conventional (CV) and ultrasonic-assisted (US) experiments was carried out using a similar experimental set-up to that described by Contreras et al. (2018) (Fig. 1), which was based on water recirculation using the upper and lower connections of the ultrasonic bath (5, Fig. 1). The control system acted in two modes: heating and cooling. In the case of ultrasonic (US) assisted ZnPP formation, the heating mode only worked until the temperature of 37 °C was reached in the glass bottle, since after that, the heat provided by US made only the cooling mode necessary. The ZnPP formation kinetics in CV and US modes were performed in triplicate.

As far as the kinetics of US-assisted ZnPP formation are concerned by, two different ultrasonic powers were tested by modulating the wave amplitude in the ultrasonic generator (GAT600W ATU, Spain) (9, Fig. 1). Thus, the actual powers supplied in the reaction media were 7.05 W/L (low) and 36.53 W/L (moderate) and the application was carried out in both cases in pulsed mode (30 min on and 30 min off). The low power US level was selected because it allowed cavitation in the medium (assessed by aluminium foil) with a stable operation and the moderate power US was the one that consumed twice the amperage of the low ultrasonic power density. The actual power supplied in the reaction media was experimentally assessed using the calorimetric method (Cárcel et al., 2007; Ahmad-Qasem et al., 2013). For that purpose, the change in the temperature in the opaque glass bottles was measured by placing a type-K thermocouple in the centre of one bottle filled with 15 mL of water. The thermocouple was connected to a datalogger unit (34970 A, Agilent, U.S.A.), and the data were saved and analysed in a PC (Agilent BenchLink Data Logger 3). Equation (1) was used to determine the ultrasonic power.

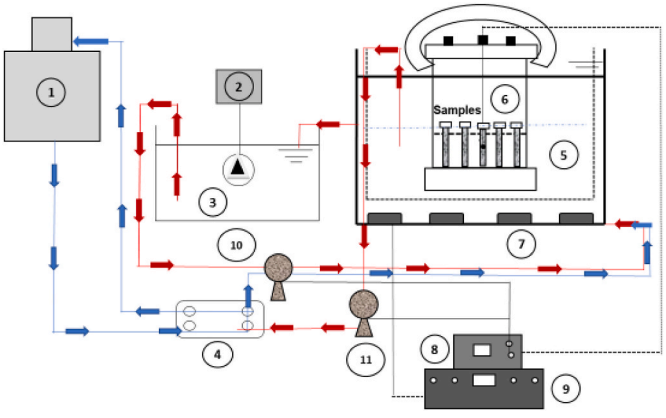

Fig. 1. Scheme of the ultrasonic bath with temperature control. 1, Cooling unit; 2, Circulating thermostat; 3, Water reservoir tank; 4, Plate heat exchanger; 5, Ultrasonic bath; 6, Anaerobic jar; 7, Ultrasonic transducers; 8, Process Controller; 9, Ultrasonic generator and amplifier; 10, Heat pump; 11, Cooling pump.

$$P = (M \times Cp) \times \left(\frac{dT}{dt}\right) \quad (1)$$

where P (W) is the ultrasonic power, M (kg) the mass of the reaction media, Cp (J/kg °C) the heat capacity (water) and dT/dt the rate of temperature change (Equation (1)). The ultrasonic power was measured 5 times for every power tested and expressed as power density (W/L).

2.6. Extraction and quantification of zinc-protoporphyrin

The measurement of ZnPP formation requires its prior separation from the reaction media. For that purpose, 1 g of the reaction media was placed into solvent-resistant 38 mL centrifuge tubes (Nalgene Centrifuge Ware, PPCO), which were kept on ice and protected from the light. In each tube, 10 mL of the extraction solvent (dimethyl sulfoxide (DMSO), ethyl acetate (AcOEt) and glacial acetic acid in a ratio of 1:10:2 v/v/v) were added and the mixture was homogenised for 30 s with a vortex (Velp scientifica, 2 × 3 Advanced Vortex Mixer, Italy) and kept cold at 4 °C and in the dark for 20 min. Then, it was centrifuged for 20 min at 3100 rpm and 4 °C (Medifriger BL-S, SELECTA, Spain) and the supernatant was filtered (Whatman597, GE LIFE SCIENCE, USA). Subsequently, the filtrate was collected in 10 mL amber volumetric flasks. The filtrate was made up to a volume of 10 mL with the extraction solvent. Finally, the ZnPP concentration was measured following the methodology proposed by Wakamatsu et al. (2004). A 96-well plate fluorimeter (Infinite 200 Microplate Reader, TECAN, Switzerland) was used, which was adjusted to excitation and fluorescence emission peaks of 420 nm and 590 nm, respectively.

In order to quantify the ZnPP concentration (mg/L), a calibration curve of ZnPP was obtained. For that purpose, different dilutions of up to 2 mg/L were prepared from concentrated ZnPP (Sigma-Aldrich) using the reaction media as dilution medium. The calibration curve (r^2 = 0.998) is shown in Equation (2).

$$ZnPP = \frac{F - 8724}{22935} \quad (2)$$

where F is the fluorescence (RFU) and ZnPP is the concentration of the product formed (mg/L).

2.7. Statistical analysis

An analysis of variance (ANOVA) was carried out to examine the effect of the reaction medium (with antibiotics and with organic acids) and the reaction time on the ZnPP concentration. In addition, a one-way ANOVA was used to determine whether the effect of the US application had a significant effect on the ZnPP concentration of each homogenate (HLi and HLi + OxyHb). Fisher's least significant difference (LSD) procedure was used to discriminate between means ($p < 0.05$). The analyses were performed using Centurion XVI software (Statpoint Technologies Inc., Warrenton, VA, USA).

3. Results and discussion

3.1. Conventional ZnPP formation from homogenised pork liver

Experiments were carried out on two types of homogenised pork liver: i) with antibiotics and ii) with ascorbic and acetic acids as preservatives. The two reaction media sought to inhibit the microbial growth during the ZnPP formation kinetics. Fig. 2 shows the ZnPP formation kinetics carried out under anaerobic conditions for 48 h. For HLi

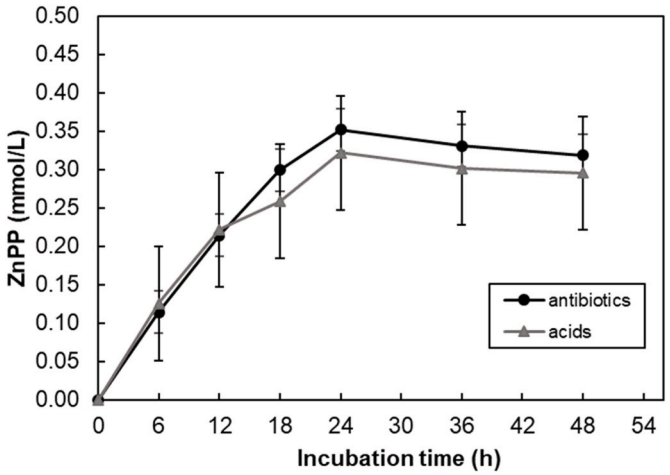

Fig. 2. Conventional (without ultrasound) kinetics of ZnPP formation in homogenised pork liver reaction medium with antibiotics and acids. Average values ± LSD intervals are shown for each experimental incubation time (t).

with antibiotics, the ZnPP concentration increase during incubation followed an almost linear pattern up to 24 h (r = 0.991), when the maximum concentration ($ZnPP_{max}$) was reached (0.352 mmol/L). The average ZnPP formation rate from 0 to 24 h was 0.0151 mmol/L·h. After 24 h, the ZnPP concentration decreased slowly (0.0014 mmol/L·h). Wakamatsu et al. (2015) also studied the ZnPP formation in pork liver at 20% in a reaction medium with antibiotics, at pH 4.5, at 37 °C and during 5 days of anaerobic incubation. They observed that after 24 h of incubation the maximum concentration of ZnPP was reached. When studying homogenised Parma ham *(Biceps femoris)*, Becker et al. (2012) reported that the maximum ZnPP concentration was reached after 48 h. Meanwhile, Wakamatsu et al. (2007) reported that the $ZnPP_{max}$ in homogenised pork loin samples increased rapidly up to 72 h, which is consistent with the time reported for the pork *Longissimus lumborum* (Khozroughi et al., 2017). However, the results obtained regarding the ZnPP formation in the HLi of the present study were obtained in less time (24 h). This could be due to the fact that the amount of substrate (porphyrins and Zn^{2+}) and/or FeCH in pork liver is higher than in pork muscles, such as *Longissimus* and *Biceps femoris* (Wakamatsu et al., 2007; Becker et al., 2012; Khozroughi et al., 2017). Moreover, the higher rate could also be ascribed to the state/structure of the reaction medium, which is solid (intact muscle fibers) in the muscles and liquid (dissolved unstructured tissue) in the liver homogenate. After 24 h, there is a trend towards lower ZnPP, which may be attributed to the instability of the ZnPP, leading to its degradation. On the other hand, Wakamatsu et al. (2019) studied how the pH of the reaction medium affected the formation of ZnPP in twenty types of pork muscles, observing that the optimal value for ZnPP formation was 4.75, a similar value to the pH considered in the present study (the HLi remained at pH 4.8 ± 0.05 throughout the incubation). However, Wakamatsu et al. (2019) postulated that the endogenous myoglobin (source of prophyrins for the ZnPP formation) was degraded during incubation at pH 4.75, limiting the formation of ZnPP.

As observed in Fig. 2, the addition of acetic and ascorbic acids in the reaction medium, as microbial inhibitors, had an almost negligible impact on the ZnPP formation kinetics. Thus, the ZnPP concentration reached in the medium with organic acids was slightly lower (but not significantly, $p > 0.05$) than that reached in the medium with antibiotics after 12 h of incubation, i.e., the ZnPP reached after 24 h was 0.321 mmol/L with acids compared to 0.352 mmol/L with antibiotics. Thereby, it could be postulated that the addition of organic acids to the HLi reaction medium could be very interesting as a substitute for antibiotics for the purposes of microbial growth control. Although it is true that microbial growth quantification has not been carried out in this study, if there has been, it has been minimal since the microorganisms have not interfered with the ZnPP formation. Nonetheless, the microbiological safety of the ZnPP formation process with organic acids has been addressed in previous studies and it was found that the initial total viable counts (Bou et al., 2021) were maintained. Therefore, it would be of great interest for future complementary studies to analyse the microbiota of the homogenate after anaerobic incubation and its likely inactivation using emerging techniques (Mañas and Pagán, 2005).

3.2. *Effect of power ultrasound (US) on the kinetics of ZnPP formation from homogenised pork liver*

The effect of US application on the ZnPP formation kinetics was studied at moderate-36.53 W/L (Fig. 3) and low-7.05 W/L power (Fig. 4) in HLi with organic acids. As Fig. 3 illustrates, when a moderate power (36.53 W/L) was applied, the temperature control system was not able to prevent the temperature increase in the reaction medium related to US application. Thus, the temperature rose above the set-point of 37 °C and fluctuated at around 50 °C in the reaction medium, despite the temperature in the bulk water of the ultrasonic bath being kept at 37 °C. This lack of temperature control was due to the fact that the system controlled the temperature of the external medium (ultrasonic bath water) and the ultrasound energy delivered inside the sample opaque glass bottles was not dissipated fast enough through the glass bottles and anaerobic jar to the heating/cooling medium. The increase in temperature and the ultrasonic application hindered the ZnPP formation, as observed in Fig. 3, and only 0.037 mmol/L was obtained after 24 h of incubation. These results were consistent with those reported by Becker et al. (2012) when analysing homogenised pork meat, who observed that above 60 °C no ZnPP formation was manifested. However, Numata & Wakamatsu (2005) reported that the optimum temperature of ZnPP formation in the liver was 55 °C (maximum ZnPP formation in only 3 h), but the optimum pH for that temperature was found to be 6. Therefore, the results found at moderate power in the present study could be due to the temperature-pH combination, but also to a negative effect of the ultrasonic field, which could alter the enzyme integrity and the enzyme-substrate interaction.

In addition, Dailey et al. (1994) studied the denaturation temperature profile of mammalian FeCH, showing that it denatures from 40 °C upwards. Therefore, the temperature profile (Fig. 3) at moderate US power could have led to a thermal denaturation of FeCH during the process of ZnPP formation. Tian et al. (2004) also observed the same result in the enzymatic reaction of trypsin when they increased the US power (from 20 to 100 W/mL) for short treatment times (from 1 to 20

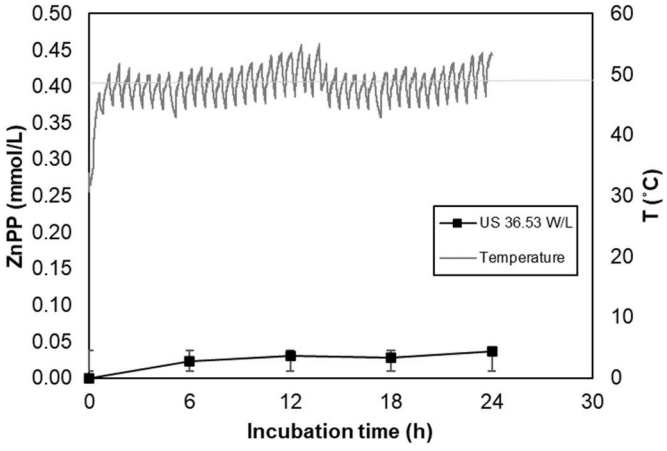

Fig. 3. Kinetics of moderate power (36.53 W/lL) ultrasound (US) assisted ZnPP formation in homogenised pork liver reaction medium (HLi) with organic acids and temperature (T) evolution in the reaction medium. Average values ± standard deviation are shown for each experimental time (t).

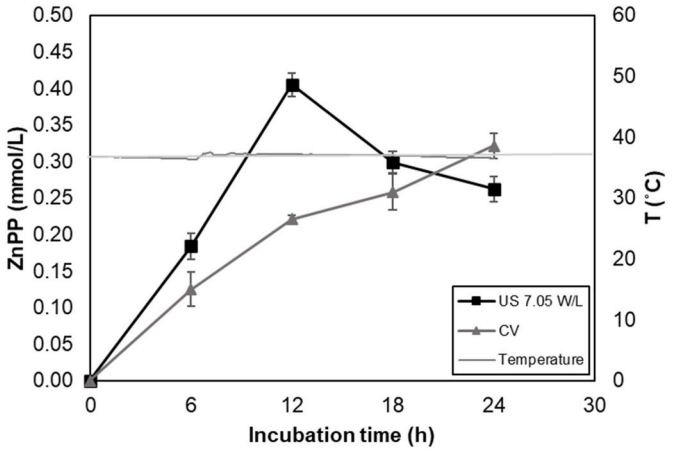

Fig. 4. Kinetics of low power (7.05 W/L) ultrasound (US) assisted and conventional mode (CV), without US, ZnPP formation in homogenised pork liver reaction medium with organic acids (HLi) and temperature (T) evolution in the reaction medium. Average values ± standard deviation are shown for each experimental time (t).

min), due to the rise in the temperature and pressure and to the formation of free radicals induced by the thermal dissociation of water due to US application, which affected the conformation of the enzyme and its stability. Therefore, depending on the US power applied, the structure of the enzymes could be altered, affecting their stability and leading to their denaturation (Nadar and Rathod, 2017).

However, when the applied ultrasonic power was low (7.05 W/L), heat generation was less intense, and the temperature control system was able to keep a constant temperature of 37 °C in the reaction medium, as observed in Fig. 4. Thus, low power (7.05 W/L) US application permitted the acceleration of the ZnPP formation when compared to CV mode. Therefore, the $ZnPP_{max}$ (0.405 mmol/L) was reached after 12 h, which represents 50% less time than when using the CV mode ($ZnPP_{max}$ at 24 h). This could be linked to the fact that US energy improved the FeCH activity and promoted the ZnPP diffusion, obtaining a higher concentration in a shorter time by applying US (Abril et al., 2021).

Thus, low power US application caused an ultrasonic microagitation of the reaction medium that could favour the contact of FeCH with the substrates, as well as the ZnPP diffusion. Conversely, in the case of moderate power (36.53 W/L), the denaturation or modification of the FeCH structure could take place due to the cavitation mechanism or to the temperature increase. The fact that US improves the enzymatic activity by facilitating the enzyme-substrate contact was previously reported by Yu et al. (2013) when studying the tyrosinase enzyme; this was activated with the US treatment, shortening the time of the first phase of the enzymatic reaction, which consists of the binding of the substrates with the enzyme. In addition, as illustrated in Fig. 4, the $ZnPP_{max}$ reached when the low power (0.405 mmol/L) US was applied was significantly higher, 25.77% more than in the case of CV kinetics (0.321 mmol/L). This could be explained by considering the simultaneous processes of formation and degradation of the ZnPP; thus, the concentration measured would be the net concentration, computed from the difference between the accumulated ZnPP formed and degraded at that time. Thereby, the $ZnPP_{max}$ did not account for the total amount formed, but to the net, and its time location marked the moment when the rate of degradation was higher than that of formation. Another hypothesis that could explain this behaviour considers that the degradation occurs to the substrates of the reaction, the porphyrins, which degrade over time (Wakamatsu et al., 2019). This substrate degradation could partly explain why $ZnPP_{max}$ in CV experiments is not reached, as they have slower kinetics than US processing. If Figs. 2 and 4 are compared, it may be elucidated that the net rate of ZnPP degradation was almost one order higher when US was applied; in CV mode, 0.0011 mmol/Lh in Hli was obtained, with respect to low power US application, in which 0.0119 mmol/Lh was found. Therefore, in the same way that US stimulates FeCH activity (Abril et al., 2021), ZnPP degradation processes are also accelerated. So, when the $ZnPP_{max}$ is achieved, the degradation of ZnPP is accelerated more drastically when US is applied. Further studies should address the mechanisms for ZnPP degradation and why US intensifies them. Moreover, the effect of different combinations of temperature/pH and US should be studied.

3.3. *Conventional and ultrasonic-assisted kinetics of ZnPP formation from homogenised pork liver with added oxyhemoglobin*

Once the kinetics of ZnPP formation from homogenised pork liver were addressed, the impact on ZnPP formation of the addition of oxyhemoglobin (OxyHb), as a substrate of protoporphyrin IX (PPIX), to the HLi with organic acid medium, was analysed. Two different external sources of OxyHb were used, one produced from a commercial pure chemical standard (Fig. 5) and one extracted from pork blood (Fig. 6). Thereby, conventional (CV) and low power ultrasonic-assisted (US) ZnPP formation kinetics were tested in the HLi + OxyHb reaction medium.

As for CV ZnPP formation kinetics, HLi + OxyHb was found to behave similarly to HLi, as illustrated in Figs. 5 and 6. Thus, the highest ZnPP concentration was reached after 24 h and no significant differences ($p > 0.05$) were found between the OxyHb from commercial pure chemical standards (0.415 mmol/L) (Fig. 5) and the OxyHb extracted from pork blood (0.430 mmol/L) (Fig. 6). Thereby, the addition of the OxyHb to HLi involved a moderate increase in the quantity of ZnPP formed (0.321 mmol/L for only HLi). Therefore, for any future industrial application, it could be of great interest to use the OxyHb extracted from pork blood, since as happens for liver, it is a co-product of low commercial value in the meat industry (Muflih et al., 2017).

The US assisted ZnPP formation kinetics in HLi + OxyHb followed the same pattern as in HLi. Once again, low power US application (7.05 W/L) shortened the time needed to reach the $ZnPP_{max}$ (12 h) by 50%, compared to CV kinetics (24 h). As in the case of CV kinetics, no significant ($p > 0.05$) differences were found between the $ZnPP_{max}$ obtained from HLi medium with added commercial pure chemical standards and that extracted from pork blood OxyHb (0.439 and 0.449 mmol/L, respectively). In addition, it should be noted that unlike the kinetics obtained from HLi, US application led only to a slight increase (4.42%) in the $ZnPP_{max}$ reached from pork blood OxyHb with respect to $ZnPP_{max}$ obtained from CV mode (0.430 mmol/L in HLi OxyHb). This

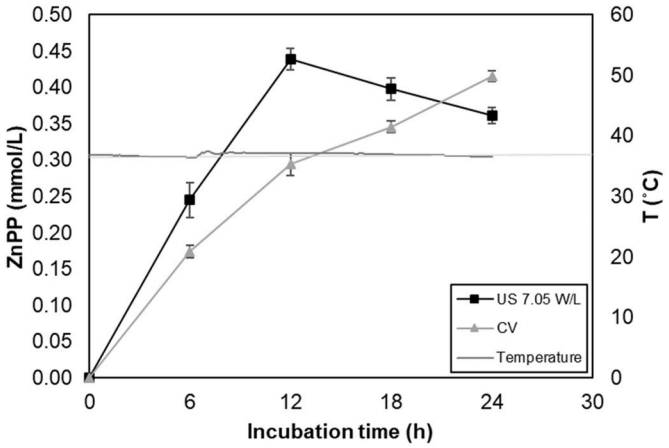

Fig. 5. Kinetics of low power (7.05 W/L) ultrasound (US) assisted and conventional mode (CV), without US, ZnPP formation in homogenised pork liver with added OxyHb from commercial pure chemical standards (HLi + OxyHb) and temperature (T) evolution in the reaction medium. Average values ± standard deviation are shown for each experimental time (t).

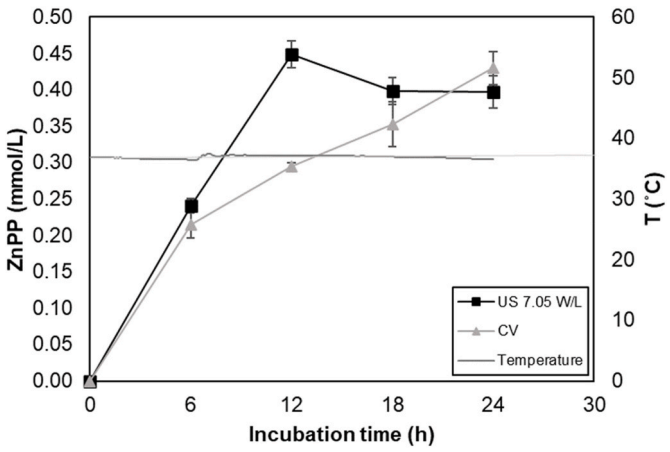

Fig. 6. Kinetics of low power (7.05 W/L) ultrasound (US) assisted and conventional mode (CV), without US, ZnPP formation in homogenised pork liver with added OxyHb extracted from pork blood (HLi + OxyHb) and temperature (T) evolution in the reaction medium. Average values ± standard deviation are shown for each experimental time (t).

could be related to an additional degrading effect of OxyHb (Wakamatsu et al., 2019) associated with US application, since low power US application intensifies the chemical reactions, shortening the process times (Wang et al., 2018). In this regard, the oxidation reaction of OxyHb to MetHb could be limited, since the ZnPP formation took place in anaerobiosis. However, the US application could have promoted the reactions of the OxyHb with the hydrogen (H) present in the reaction medium and, consequently, could have given rise to other degradation products of the heme group (Nagababu et al., 2010).

4. Conclusions

US application could be considered an interesting alternative method to enhance the FeCH enzymatic activity, promoting ZnPP formation, in both pork liver homogenates (HLi and HLi + OxyHb). However, the effect of US mainly depends on the applied ultrasonic power. In this regard, ultrasonic power levels lower than those applied in this work should be studied in order to know if they could improve the effectiveness in the ZnPP enzymatic formation. Thus, moderate power US reduced the ZnPP formation. However, low power US was an effective method for the intensification of the enzymatic reaction, shortening the ZnPP formation time by 50% and obtaining a higher ZnPP.

The present study could contribute to the sustainable production of a new natural ingredient from two co-products of the meat industry (liver and blood) that would offer interesting colouring properties. For the ZnPP production on an industrial scale, the development of continuous reactors assisted by US would be necessary. In this sense, economic factors such as the energy cost of US application and the purification and stabilization of the pigment should be considered. The extracted pigment could be added to meat products such as salami, pâté or fuet, among others. On the other hand, US treatments could also be used on pieces of loin or ham to intensify the formation of ZnPP and reduce the quantity of chemical additives added (nitrates/nitrites) during the manufacturing of meat products.

Author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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