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The effect of different frequencies of ultrasound on the activity of horseradish peroxidase

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ABSTRACT

Ultrasound technology has been studied by food researchers as an alternative method for thermal processing. The use of ultrasound as a way to inactivate and/or activate enzymes has been widely studied at low frequencies (20–40 kHz), however, little research on the effect of high frequencies has been reported. Thus, the effect of high and low frequency ultrasound on commercial horseradish peroxidase with a concentration of 0.005 mg mL^{-1} is described. Experiments were performed for 60 min using 20, 378, 583, 862, 995, 1144 and 1175 kHz ultrasound at power levels (acoustic energy) between 2.1 and 64 W. Residual activity was monitored using a spectrophotometric method and data analysis was performed using ANOVA. A significant enhancement of enzyme inactivation ($p < 0.05$) was observed at each frequency with an increase of sonication time and power. Inactivation of peroxidase by ultrasound followed first order kinetics and an increase of the rate constant with the power applied was observed for all the frequencies studied. Overall, low frequency (20 kHz) and low power are not effective on the enzyme inactivation and the level of residual activity remained high. The use of 378 and 583 kHz (48 W) is particularly effective for complete enzyme inactivation.

1. Introduction

Peroxidase (POD), a member of a large group of enzymes called oxidoreductases, is commonly found in raw fruit and vegetables (Burnette, 2006). It is a haem-containing enzyme, which can catalyse a large number of reactions in which peroxide is reduced while an electron donor is oxidized. The presence of this enzyme has been associated with food quality degradation leading, for instance, to the appearance of off-flavours and off-colours in raw and unblanched frozen vegetables (Lopez et al., 1994). Therefore, the inactivation of this enzyme increases the shelf life of vegetables during frozen storage and is often used to evaluate the efficiency of vegetable blanching (Barrett & Theerakulkait, 1995; Williams, Lim, Chen, Pangborn, & Whitaker, 1986).

Thermal processes are often used for enzyme inactivation and the kinetics of such processes under heat treatment have been extensively studied (Adams, 1991; Ling, Tang, Kong, Mitcham, & Wang, 2015). However, heat can also cause undesirable changes in the organoleptic characteristics of food, such as loss of colour, flavour and texture as well as in its nutritional value (Cheng, Zhang, & Adhikari, 2013; O'Donnell, Tiwari, Bourke, & Cullen, 2010). For this reason, the food industry is continually searching for alternative methods of food processing with less negative effects. Consequently, several non-thermal technologies have been investigated, including high hydrostatic pressure (HPP), pulsed electric fields (PEF) and ultrasound (US) which aim at extending shelf-life of food products while maintaining their quality, safety and nutritional value. One such alternative method is ultrasound, i.e. sonic waves above 18 kHz (Chandrapala, Oliver, Kentish, & Ashokkumar, 2012; Islam, Zhang, & Adhikari, 2014; Kwiatkowska, Bennett, Akuna, Walker, & Bremner, 2011; Soria & Villamiel, 2010).

There has been considerable interest in the application of ultrasound in food technology as it can be used as a processing, preservation and extraction technique. Chemat, E-Hama, and Muhammed (2011) has reviewed, highlighted and explained the different applications of ultrasound in food processing and the effect of low frequency ultrasound on enzyme activity, including peroxidase, polyphenol oxidase and pectin esterase, has been investigated (Baslar & Ertugay, 2013; Cruz, Vieira, & Silva, 2006; De Gennaro, Cavella, Romano, & Masi, 1999; Huang, Cheng, Hu, & Pan, 2015; Jang & Moon, 2011; Koshani, Ziaee, Niakousari, & Golmakani, 2015; Lopez & Burgos, 1995; Terefe et al., 2009) although the mechanisms that lead to enzyme inactivation have not yet been clarified. Nevertheless, the effect of ultrasound on enzymes seems to be associated with mechanical and chemical processes that occur as a consequence of cavitation (Ercan & Soyal, 2011). This phenomenon refers to the formation, growth and implosion of bubbles causing shock waves, which generate extreme temperatures and

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pressures inside the collapsing bubbles with the concomitant generation of hydroxyl radicals (Xu et al., 2015).

The application of ultrasound in order to either inactivate or enhance enzyme activity has been widely studied but most of the work has been performed at low frequency (20–40 kHz). The effect of high frequency on the inactivation of enzymes, to our knowledge, has been only reported by Grintsevich and Metelitza (2002) and Rachinskaya, Karasyova, and Metelitza (2004).

In fact, there is very limited information about the effect of higher frequency ultrasound on food enzymes and consequently the aim of this study is to investigate the effect of various ultrasonic frequencies (20, 378, 583, 862, 998, 1144 and 1174 kHz) at different acoustic powers on the activity of the commercial enzyme peroxidase. Specifically, the effectiveness of higher frequencies of ultrasound on the residual activity of commercial peroxidase was compared to results obtained with 20 kHz ultrasound and also from purely thermal treatment. Furthermore, the use of similar power levels at different frequencies were investigated in order to determine the optimum energy input required to decrease the residual activity of the enzyme at each frequency studied.

2. Materials and methods

2.1. Enzyme solution and assay

Peroxidase from horseradish (EC 1.11.1.7, RZ ≥ 1.0) was purchased from Sigma-Aldrich, Gillingham, UK and an aqueous solution of 0.005 mg mL^{-1} of horseradish peroxidase was prepared using deionized water. Peroxidase (POD) activity (ΔA_{470} min^{-1} g^{-1} FW) was monitored as an increase in optical density due to the oxidation of guaiacol to tetraguaiacol. The complete reaction mixture contained potassium phosphate buffer (100 mM; 1.0 mL; pH 6.1), guaiacol (96 mM; 0.5 mL), hydrogen peroxide (12 mM; 0.5 mL), enzyme solution (0.005 mg mL^{-1}; 0.1 mL), and deionized water (0.4 mL) (Castillo, Penel, & Greppin, 1984). The enzyme activity was measured at 470 nm in glass cuvettes over a period of 1 min on a UV-Vis spectrophotometer (UV-1650 PC, Shimadzu UK Ltd). The percentage of residual activity (RA) of peroxidase was calculated using RA = (A/A_0) *100, where A and A_0 are, respectively, POD activity after and before the treatment. All the samples were re-analysed after being kept at 4 °C for 24 h in order to investigate if any re-activation of the enzyme occurred after treatment and/or during storage.

2.2. Thermal inactivation (control)

Considering the increase of temperature during sonication (maximum temperature reached was 40 ± 3 °C) the effect of heating at 40 ± 3 °C was studied as a control. Glass test tubes containing the enzyme solution (2 mL) were placed in a thermostatic bath previously equilibrated at the specified inactivation temperature. At predetermined time intervals three test tubes were taken out of the bath and then immersed in an ice bath. After cooling, aliquots (0.1 mL) were pipetted into glass cuvettes containing the substrate solution in order to measure the enzyme activity.

2.3. Ultrasonic treatment

The ultrasound equipment used in these experiments was either a Misonix Ultrasonic Liquid Processor operating at 20 kHz or a Meinhardt Ultraschlltechnik high frequency sonicator with a Meinhardt Power Amplifier. The high frequency sonicator has two transducers: a F701 transducer operating at 378, 995 or 1175 kHz and a F712 transducer operating at 583, 862 or 1144 kHz. Moreover, different amplitudes corresponding to different acoustic powers were selected (see Table 1).

The low frequency experiments were performed using a Misonix Ultrasonic Liquid Processor fitted with a 1.3 cm Titanium probe operating at 20 kHz in the continuous mode at different amplitudes, which correspond to 11, 16 and 35 Watts. The probe was immersed in the peroxidase solution (0.005 mg mL^{-1}; 200 mL) in a 400 mL beaker (the same beaker was used for all the experiments) and the probe was positioned 20 mm from the bottom of the beaker. The starting temperature for all the ultrasonic experiments was 20 ± 2 °C but in order to control the increase of the temperature during sonication the beaker was placed in a 2 L bath filled with ice and water and the temperature profile was recorded.

Table 1
Range of frequencies and power used in the ultrasonication treatments.

Frequency (kHz)	Power Level (W)					
20	n/a	11	16	35	n/a	n/a
378	3.9	10	17	32	48	n/a
583	2.1	8.9	17	34	48	n/a
862	4.3	9.6	n/a	20	n/a	64
995	4.9	9.4	17	24	n/a	n/a
1144	3.4	8.8	17	n/a	49	n/a
1175	3.4	n/a	15	39	n/a	n/a

For the high frequencies experiments, the enzyme solutions at the same concentration as the low frequency experiments (0.005 mg mL^{-1}; 200 mL) were introduced into a glass reaction vessel (62.5 mm internal diameter). The starting temperature for all the ultrasonic experiments was 20 ± 2 °C and cooling was applied through the jacketed reactor (wall thickness 5 mm) by use of water pumped through a cryostatic bath (Fisher Scientific, ISOTEMP Thermostatic). All the ultrasound treatments were performed in triplicate for 60 min and samples were withdraw at 2, 4, 6, 8, 10, 15, 30, 45, and 60 min for analysis. Table 1 shows all the ranges and frequencies investigated in this work.

2.4. Data analysis

A one-factor-at-a-time experimental design was used to evaluate the effect of the individual sonication parameters on the residual activity of peroxidase. The calculation of RA and the plots of RA versus time were performed using Excel®. The inactivation rate constants were calculated by linear regression of the natural logarithm of RA versus time. Further data analysis was performed using two-way ANOVA (Analysis of variance) by IBM SPSS Statistics 22 to examine if there was an interaction between power and time at each frequency studied. Values presented are the mean of experiments done in triplicate and replicated 3 times (n = 9). The values were considered significantly different when $p < 0.05$.

3. Results and discussion

A wide range of frequencies (20, 378, 583, 862, 995, 1114 and 1175 kHz) and different acoustic powers were used in order to investigate their effect on peroxidase activity. In fact, the figures and tables presented on this section aim to show the effect of different powers at the same frequency and similar acoustic powers obtained at different frequencies on the enzyme activity. The overall results are presented in Table 2, however some of this data is also used in the figures to highlight the main findings of this work. It should be pointed out that enzyme reactivation did not occur after the ultrasonication treatments.

The effect of ultrasound on enzyme activity is different according to the acoustic power and frequency used and this is shown in Fig. 1, where the effect of these two variables on the RA after 60 min of ultrasonication is presented.

Generally, reduced acoustic powers (< 16 W) at low (20 kHz) and high frequencies, lead to a significant decrease in enzyme activity ($p < 0.05$) after 60 min, nevertheless it did not totally inactivate POD.