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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 minutes using 20, 378, 583, 862, 995, 1144 and 1175 kHz ultrasound at power levels (acoustic energy) between 2.1 to 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.

Keywords – Peroxidase; High-frequency ultrasound; Enzyme inactivation; Food enzymes.

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 et al., 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 et al., 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 (O'Donnell, Tiwari, Bourke & Cullen, 2010; Cheng, Zhang & Adhikari, 2013). 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 (Soria & Villamiel, 2010; Kwiatkowska et al., 2011; Chandrapala, Oliver, Kentish & Ashokkumar, 2012; Islam, Zhang & Adhikari, 2014).

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 et al. (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 (De Gennaro, Cavella, Romano & Masi, 1999; Terefe et al., 2009; Jang & Moon, 2011; Lopez & Burgos, 1995; Cruz, Vieira & Silva, 2006; Baslar & Ertugay, 2013; Huang, Cheng, Hu & Pan, 2015; Koshani, Ziaee, Niakousari & Golmakani, 2015) 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 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 & Metelitza (2002) and Rachinskaya, Karasyova & 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, 2015). 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

96 are, respectively, POD activity after and before the treatment. All the samples were
97 re-analysed after being kept at 4 °C for 24 h in order to investigate if any re-
98 activation of the enzyme occurred after treatment and/or during storage.

99

100 *2.3 Thermal inactivation (Control)*

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

108

109 *2.4 Ultrasonic treatment*

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

116 The low frequency experiments were performed using a Misonix Ultrasonic Liquid
117 Processor fitted with a 1.3 cm Titanium probe operating at 20 kHz in the continuous
118 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 ^{o}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.

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 ^{o}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.

2.5 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 Figure 1, where the effect of these two variables on the RA after 60 minutes 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 minutes, nevertheless it did not totally inactivate POD. In fact, for all the frequencies applied, when the acoustic power was between 2.4 to 11 W the RA at the end of the treatment was still relatively high, varying from 93.6% (3.4 W; 1175 kHz) to 38.3 % (10 W; 378 kHz). It should be pointed out that when using this low range of acoustic powers 378 kHz seems to be the most effective in order to decrease enzyme activity. Nevertheless, the decrease of the enzyme activity, under these conditions, is not

166 effective enough for preservation purposes. On the other hand, when high
167 frequencies and high acoustic powers are applied the enzyme activity decreased
168 significantly and complete inactivation is achieved when using 378 kHz and 583 kHz
169 (at 48 W) and at 1144 kHz (49 W).

170 The effect of similar acoustic powers (15-17, 32-39 and 48-49 W) obtained at low (20
171 kHz) and high frequency (378, 583, 995, 1114 and 1175 kHz) was also investigated.
172 Frequencies of 1175 kHz (15 W) and 20 kHz (16 W) lead to similar residual enzyme
173 activity (59 and 62% respectively) at the end of the 60 minutes, while the application
174 of 378, 583, 995 and 1144 kHz, with an acoustic power of 17 W, lead to RA of 15.4,
175 34.2, 30.3 and 41.7 %, respectively.

176 The effect of high / low frequency at acoustic powers of 32, 34, 35 and 39 W on POD
177 activity is presented in Figure 2 (this figure also shows, as an example, the linearity
178 of the decrease of RA in function of the sonication time). The use of 378 and 583
179 kHz (32 and 34 W, respectively) seems to be most effective on the decrease of
180 enzyme activity leading to an RA <11% after 45 minutes of sonication (at 60 min no
181 RA was observed). On the other hand, the RA of POD at the end of sonication (60
182 minutes) was still above 25% when 20 kHz (35 W) and 1175 kHz (39 W) were
183 applied.

184 Considering the frequencies and wattages studied, the most effective conditions for
185 enzyme inactivation are the use of either 378 or 583 kHz at 48 W. As a control
186 experiment, and taking into account the maximum increase of temperature of the
187 enzyme solution during sonication at high wattages being approximately 40 °C (± 3
188 °C), a thermal treatment at this temperature was performed to rule out the possible
189 effect of the temperature on the enzyme activity. Under thermal conditions the

190 enzyme showed a typical biphasic behaviour which has been found in previous work,
191 but after 60 minutes the residual activity was still approximately 79%.

192 Residual activity was plotted against time for each of the frequencies and wattages
193 and rate constants were calculated from the slopes of the curves. Horseradish
194 peroxidase inactivation by ultrasound followed first order kinetics and the overall
195 results (rate constant and residual activity) are presented in Table 2. Analysis of
196 data using ANOVA (analysis of variance) was performed to investigate differences
197 and interactions between time and wattage, for each frequency studied. In all
198 experiments, a significant ($p<0.05$) decrease in the enzyme activity and interaction
199 between wattage and time was observed.

200 The kinetics of peroxidase inactivation against different parameters (e.g.
201 temperature, high pressure, ultrasonication) have previously been reported using
202 first, order, biphasic, fractional conversion and Weibull models. For instance,
203 biphasic behaviour of peroxidase treated thermally has been observed and this was
204 associated and explained by the existence of 2 different fractions of the enzyme – a
205 stable and a labile form (Lemos, Oliveira & Saraiva, 2000; Cruz, Vieira & Silva,
206 2006). Tomato peroxidase has been investigated under ultrasonication conditions
207 (23 kHz) and its inactivation follows first order kinetics (Ercan & Soyal, 2011).
208 Similar enzyme behaviour was found after thermosonication of water cress, but in
209 this case when the enzyme was submitted to heat treatment a biphasic behaviour
210 was observed (Cruz, Vieira & Silva, 2006). A first order behaviour of peroxidase
211 under thermosonication has also been observed but, no increase of the rate constant
212 at higher power levels was seen (De Gennaro, Cavella, Romano & Masi, 1999).
213 Conversely, others observed that the rate constant was elevated with an increase of
214 ultrasonication power at 23 kHz (Ercan & Soyal, 2011) and this is in accordance with

the findings on the present study within the range of all frequencies investigated. In fact, it was observed that higher wattage at each frequency studied lead to an increase of the rate constant.

The current study aimed at looking at the effect of frequency, acoustic power and duration of the sonication on the activity of a commercial peroxidase. As it can be seen from the results presented above all these variables affect enzyme activity and consequently, it is possible to state that ultrasound induces changes in the enzyme structure which lead to its partial or total inactivation. In fact, increasing power at the same frequency leads to a decrease of the enzyme activity, but the extension of inactivation is very much dependent upon the frequency used. It is not possible to conclude, even using the same power that an increase of frequency can, proportionally, lead to a decrease of the enzyme activity. However, at each frequency, the increase of power induces a higher level of enzyme inactivation (lower residual activity). Delgado-Povedano and Castro (2015) stated in their review article that a longer sonication time can be responsible for an increase of bubble formation and at higher powers there is a larger number of bubbles formed which will collapse and will create an adverse environment for the enzyme structure (high temperature and pressure). The use of ultrasound has been reported to inactivate enzymes and different mechanisms have been proposed, including cavitation and the formation of free radicals due to sonolysis of water (Kadkhodaee & Povey, 2008) which results in the denaturation of proteins and enzyme inactivation (O'Donnel, Tiwari, Bourke & Cullen, 2010). The inactivation mechanism induced by ultrasonication is dependent on the enzyme amino acid composition and the resulting conformational structure Ozbek & Ulgen, 2000). Moreover, the formation of free radicals caused by cavitation can alter the enzyme structure, because of the

reactions between hydroxyl radicals and amino acids present in the enzyme (Terefe et al., 2009; Terefe, Buckow & Versteeg, 2015; Raviyan, Zhang & Feng, 2005).

Overall, there are three main processes which have been considered to be involved in the inactivation of peroxidase: dissociation of the haem group from the holoenzyme (active enzyme system), conformational changes in the apo-enzyme (the protein part of the enzyme) and/or modification or degradation of the prosthetic group (Lemos, Oliveira & Saraiva, 2000). The inactivation of peroxidase by manothermosonication has been attributed to the detachment of the haem group (Lopez & Burgos, 1995). An insight into a potential mechanism for the inactivation of POD has been proposed in our previous work (Tsikrika et al., 2017). After treatment of a dilute solution of POD with 378 kHz or 583 kHz ultrasound (the same conditions used in the present study), the samples were examined to determine any conformational or chemical changes using fluorescence-emission from the Tyr and Trp amino acid residues present in the enzyme. The effect of the ultrasound was to produce new fluorescent species, which can possibly be attributed to the formation of di-tyrosine within the enzyme. It is postulated that monitoring the formation of this fluorescence can be indicative of changes in enzymatic structure and hence the enzymatic activity. A possible explanation for the origin of this fluorescence is the reaction of neighbouring Tyr residues in the presence of the haem and hydrogen peroxide, produced by sonication of the water. UV-visible spectroscopic measurements also showed the removal of the haem moiety which also leads to enzymatic inactivation. Further work on the precise mechanism and possible different effects of higher and lower frequencies of ultrasound is continuing of POD and other enzymes.

Little work has been performed on the effect of power and ultrasonic frequency on enzymes although many reports have concentrated on dosimeters and chemical pollutants. Erzsébet-Szabó and Csiszar (2013) studied the effect of amplitude of 40 kHz ultrasound on commercial cellulase and found increasing amplitude (power) resulted in higher levels of enzyme inactivation. Higher ultrasonic power increased the amount of degradation of methylene blue due to greater cavitation and hence enhanced generation of hydroxyl radicals (Kobayashi et al., 2012) and these effects were also observed in the current work. It has been shown that higher frequencies favour the production of hydroxyl radicals over the usual 20 kHz (Petrier, Jeunet, Luche & Reverdy, 1992) and the formation of hydrogen peroxide and the decolourisation of dyes was found to be most effective using 850 kHz ultrasound (Comeskey, Larparadsudthi, Mason & Paniwnyk, 2012). Higher frequencies increase the number of cavitation bubbles but on collapse these smaller bubbles release less energy than those formed at lower frequencies but it should be noted the polarity of a particular substrate has a major effect on whether the species are able to enter into the cavitating bubble or reside on the outside surface (Petrier & Francony, 1997). Overall it can be concluded that there is a complex interplay of factors which determine the optimum frequency for the degradation of peroxidase and include: size and lifetime of the cavitating bubbles, geometry of the ultrasonic reactor, frequency, power, efficiency of hydroxyl radical production; cavitation threshold; nature of the substrate, size and polarity of the enzyme. Indeed, it was recently suggested that, “there may be a unique frequency of maximum efficiency or reaction rate for every set of conditions and geometries” (Balachandran et al., 2016).

Conclusion

Ultrasonic treatment at different frequencies and amplitude levels caused a decrease in POD residual activity, while complete deactivation of the enzyme was most efficiently achieved with the application of 378 kHz and 583 kHz at a power level of around 48 W for 60 min. In addition, the experimental findings suggest that there exists a frequency/amplitude combination to which corresponds to the maximum efficiency of the treatment. Inactivation of the enzyme follows first order kinetics and probably involves dissociation of the haem group due to loss of iron facilitated by hydroxyl radicals as a result of cavitation. Further research is needed in order to determine the precise nature of the mechanism of peroxidase inactivation and this is currently being studied.

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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

Table 2 – Rate constants (min^{-1}) and % of POD residual activity (±STDEV) after 60 minutes of ultrasonication (unless stated differently) at various frequencies and power.

Frequency (KHz)	Power (W)	Rate Constant ($x10^{-3}$ min^{-1})	Residual activity (%) ±STDEV (R^2)
20	11	6.4	68 ±2 (R^2= 0.994)
	16	7.8	62±2 (R^2= 0.997)
	35	20.9	28±2 (R^2= 0.989)
378	3.9	2.5	86±1 (R^2= 0.855)
	10	15.5	38±3 (R^2= 0.995)
	17	30.7	15±3 (R^2= 0.988)
	32	50.9	10±1 (R^2= 0.987)*
	48	96.1	6±1 (R^2= 0.974)**
583	2.1	4.6	79 ±11 (R^2= 0.917)
	8.9	10.1	61±6 (R^2= 0.975)
	17	18.0	34±6 (R^2= 0.999)
	34	49.2	10±1 (R^2= 0.994)*
	48	89.4	7±2 (R^2= 0.967)**
862	4.3	2.2	90±8 (R^2= 0.951)
	9.6	5.5	72±3 (R^2= 0.979)
	20	14.2	42±6 (R^2= 0.997)
	64	46.2	12±6 (R^2= 0.995)*
995	4.9	4.4	76±2 (R^2= 0.990)
	9.4	6.9	65±3 (R^2= 0.988)
	17	19.0	30±2 (R^2= 0.996)
	24	31.5	13±2 (R^2= 0.995)
1114	3.4	3.4	81±6 (R^2=0.977)
	8.8	7.4	64±3 (R^2=0.993)
	17	14.2	42±6 (R^2=0.998)
	49	44.9	6±1 (R^2=0.983)
1175	3.4	1.1	93.58±0.93 (R^2=0.964)
	15	7.6	59±4 (R^2=0.959)
	39	20.7	27±4 (R^2=0.998)

* Residual activity after 45 minutes ** Residual activity after 30 minutes

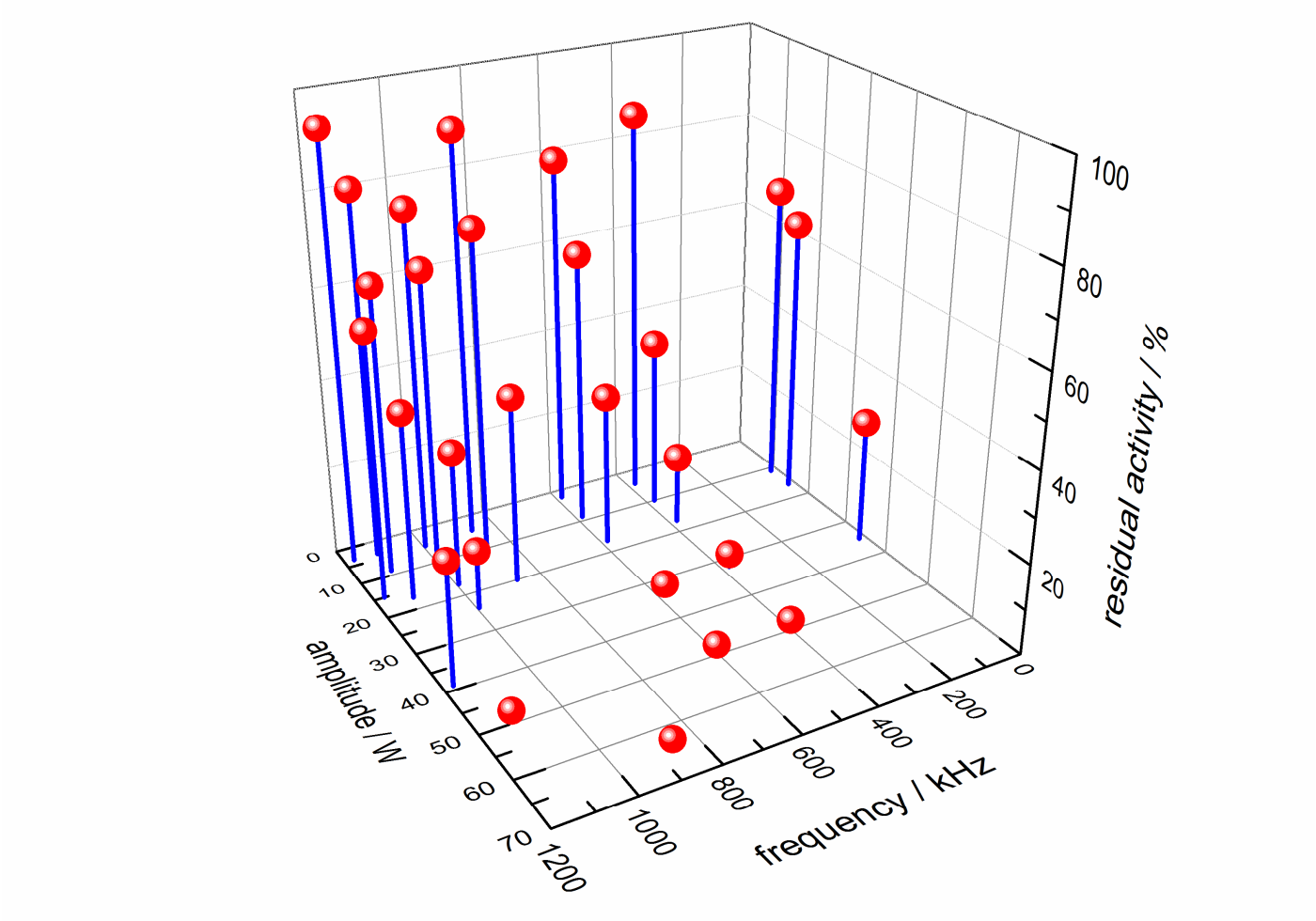

Figure 1 – Residual activity (%) after 60 minutes of ultrasonication at different power levels (amplitude, W) and frequency (kHz).

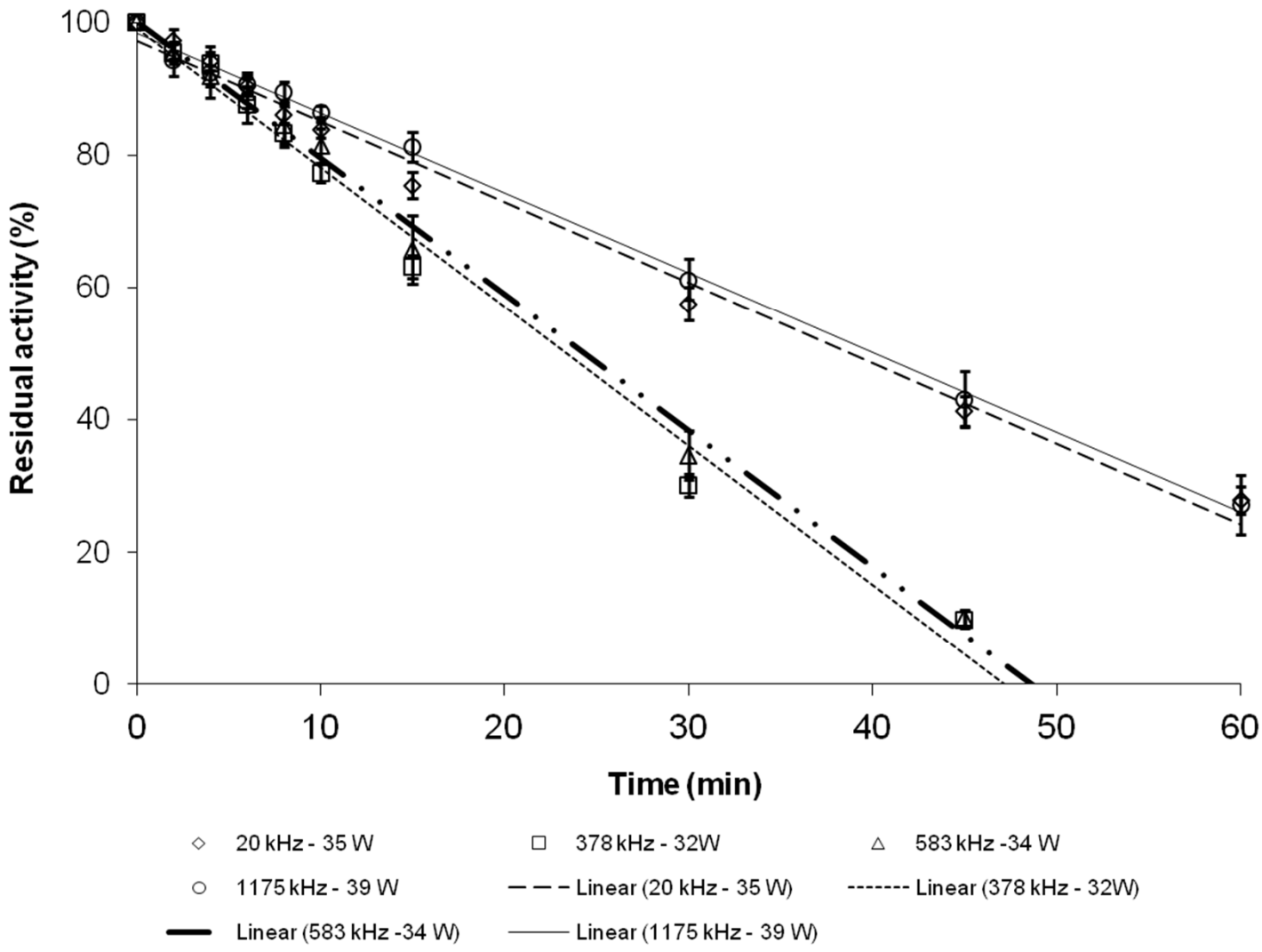

Figure 2 – Residual activity of POD treated at high (378, 583 and 1175 kHz) and low frequency (20 kHz) at 32-39 W. Values presented are the average (n=9)±STDEV.

Highlights

- Inactivation of peroxidase by ultrasound follows a first order kinetic;
- The rate constant increases with an increase of the power applied;
- High frequency ultrasound is more effective in the inactivation of peroxidase;
- 378 and 583 kHz (48 W) are particularly effective on the enzyme inactivation;