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Properties and Structures of Commercial Polygalacturonase with Ultrasound Treatment: Role of Ultrasound in Enzyme Activation

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Abstract

Polygalacturonase (PG) is one of the most commonly used enzymes during fruit and vegetable processing in the food industry. Ultrasound has the potential to enhance enzyme activity, modify the PG enzyme and enlarge its application range. This study investigated the enzymatic properties of commercial PG under ultrasound treatment, including enzyme activity, kinetic and thermodynamic properties and temperature stability. These properties were investigated with the aid of a chemical reaction kinetics model, Michaelis–Menten equation, Arrhenius equation, Eyring transition state theory and biphasic inactivation kinetics model. PG structures were also studied using fluorescence spectroscopy and circular dichroism (CD) spectroscopy. The maximum activity of PG was observed at 4.5 W/ml intensity and ultrasound duration of 15 min, under which the enzyme activity increased by 20.98% over the control. Results of degradation kinetics and thermodynamics of hydrolysis reactions catalysed by PG certified that ultrasound treatment could make PG exhibit higher reaction ability, which was evidenced from the increased rate constants and reduced thermodynamic parameters. Meanwhile, after ultrasound treatment, the value of V_{max} in the enzymatic reaction increased, whereas K_m decreased as compared with the control. These results demonstrated that the substrate was converted into the product at a higher rate and efficiency, and the enzyme displayed better affinity to the substrate. Ultrasound improved the temperature stability of PG and prolonged its lifetime without affecting its optimum temperature. Fluorescence spectra and far-UV CD spectra revealed that ultrasound treatment irreversibly decreased the amount of

tryptophan on the PG surface but increased the β-sheet in PG secondary conformation, possibly by the exposure of more active sites.

1. Introduction

Ultrasound, characterized by its high efficiency and eco-friendly properties, has been frequently applied in food industries, including homogenization, viscosity alteration, extraction, drying, crystallization, defoaming and degradation [1–3]. Currently, applications of ultrasound to modify enzyme properties are attracting considerable attention [4]. Ultrasound has been used as a method for enzyme inactivation for several years, but studies devoted to its activation effect on the enzyme have recently emerged [5]. The effects of ultrasound on biological systems are mainly due to the cavitation phenomenon [2]. During ultrasound, the distance between contiguous molecules can surpass the critical molecular distance of the liquid, creating microbubbles or cavities. These microbubbles grow during the compression cycles and then collapse violently in extremely small time intervals [6]. Cavitation results in the release of large amounts of energy and the formation of intense shear forces and highly reactive free radicals in the liquid system [7].

Mild ultrasound conditions do not only improve enzyme activity but also prolong its lifetime. Wang et al. [8] evaluated the effect of ultrasound on alliinase activity and observed that ultrasound at an intensity of 0.5 W/cm^2 can increase alliinase activity by 47.1% and improve its thermostability in the temperature range of 20 °C–60 °C. However, the effect of ultrasound on enzyme activity is strongly dependent on its intensity and duration. Several reports [9–13] showed that the activity of free enzymes

increases under mild ultrasound treatment but decreases under intense conditions. Dextranase enzyme activity under ultrasound treatment was investigated by Bashari et al. [9]. The maximum dextranase activity was achieved with ultrasound treatment at 40 W for 15 min and increased by 13.4% over the control. On the contrary, dextranase was inactivated by ultrasound treatment when the ultrasound power exceeded 60 W or the treatment time was over 25 min.

Low-intensity pulsed ultrasound helps disintegrate the bulky enzyme molecular aggregates into smaller fragments, exposing more active sites and directly increasing the activity of enzymes [14]. The notable effect of acoustic streaming on the improvement of heat and mass transfer in heterogeneous systems also promotes the accessibility of enzymes [15, 16]. Nevertheless, under extreme ultrasound conditions (excessively high intensity or prolonged time), a large increase in free radicals and the strong shear force will destroy the enzyme structure and lead to inactivation [14].

Polygalacturonase (PG; EC 3.2.1.15) is a member of the pectinase family that can randomly hydrolyse the α-(1-4) glycosidic bonds of de-esterified pectate into small segments [17]. It is one of the most commonly used enzymes during fruit and vegetable processing in the food industry, because of its high enzyme activity and optimum operating conditions at a low pH range [18]. However, applications of PG under high temperatures are often restricted by its poor heat tolerance. Most PG enzymes are irreversibly thermally deactivated at approximately 60 °C [19]. The ability of ultrasound to enhance the activity and thermostability of enzymes makes it a potential option for the modification of PG under mild conditions, which can enlarge

the application range of PG enzymes. The current work aimed to evaluate the effect of ultrasound treatment on the activity, kinetic and thermodynamic properties, thermal behaviours and structures of the commercial PG.

2. Materials and Methods

2.1. Chemicals

The enzyme preparation from *Aspergillus niger* (EC Number 3.2.1.15, PG) and the substrate pectin from citrus peel (galacturonic acid, 74.0%) were purchased from Sigma–Aldrich (Shanghai, China) and used without further purification. All other chemicals were of analytical grade.

2.2. Sample treatments

Both the enzyme and substrate samples were prepared in 1 mol/l citric acid-phosphate buffer at pH 4.0. The final concentrations of PG and pectin were 1 and 5 mg/ml, respectively.

2.3. Assay of PG activity

PG activity was determined through the 3,5-dinitrosalicylic acid (DNS) method as described by Miller [20] with slight modification. The prepared PG (50 μl) and pectin (950 μl) were mixed and reacted at 30 °C for 5 min in a 10 ml colorimetric tube. Subsequently, 2 ml of DNS reagent was immediately added to terminate the incubation, and the mixture was then boiled for 5 min. After cooling, 7 ml of water was added, and the absorbance of the yellow-brown mixture was measured at 540 nm. One unit of PG activity (U) was the amount of enzyme that degrades pectin to produce reducing sugar equivalent to 1 μmol galacturonic acid per minute under the

105 assay conditions.

106 **2.4. Ultrasound treatment**

107 The device used for ultrasound treatment of enzyme was a probe sonicator
108 (JY92-IIDN, Ningbo Scientz Biotechnology Co., Ningbo, China). The ultrasonic
109 processor had a maximum power of 900 W and it was operated at a frequency of
110 22 kHz. The horn microtip had a diameter of 10 mm. The enzyme solution (20 mL)
111 was placed in a cylindrical glass reactor with an inner diameter of approximately
112 2.77 cm. The ultrasound generator probe was embedded about 1 cm from the top of
113 the mixed liquor to introduce ultrasonic field. The solution was then processed with
114 the ultrasound at different amplitudes (2%–20%) for 5–40 min. During sonication, the
115 solution was maintained at 30 ℃ with a low-temperature thermostatic water bath
116 (DC-1006, Safe Corporation, Ningbo, China).

117 The ultrasound intensity emitted from the probe tip into the solution was
118 calculated according to Eq. (1):

119 $$I = P/V \quad (1)$$

120 where I is the ultrasound intensity (W/ml), P is the input power (W) and V is the
121 volume of the solution (ml). Amplitudes were adjusted to 2%, 4%, 6%, 8%, 10%,
122 12%, 14%, 16%, 18% and 20% of the total power (900 W) to obtain the
123 corresponding ultrasound intensities of 0.9, 1.8, 2.7, 3.6, 4.5, 5.4, 6.3, 7.2, 8.1 and
124 9 W/ml.

125 **2.5. Hydrolysis reaction**

126 The prepared PG sample (50 μl) with or without ultrasound treatment and the

pectin solution (950 μl) were mixed in 10 ml colorimetric tubes, which were placed in a shaking water bath at different temperatures (20 °C–70 °C) for different incubation times (5–60 min). After hydrolysis, the colorimetric tubes were immediately placed in a boiling water bath for 3 min to denature the enzyme and then cooled on ice.

2.6. Degradation kinetics of pectin

Given the difficulty in measuring the decrement of pectin, the degradation kinetics of pectin can be demonstrated by the increased amount of galacturonic acid released by pectin as follows:

$$\ln(V_\infty - V_t) = -kt + \ln V_\infty \quad (2)$$

where V_t is the concentration of galacturonic acid in the reactant at time t (μM), and V_∞ is the ultimate concentration of galacturonic acid after thorough degradation of pectin (μM). Concentrated H_2SO_4 (6 ml) was added to the substrate (950 μl), and the mixture was boiled for 10 min to completely hydrolyse pectin and obtain the ultimate concentration of galacturonic acid.

2.7. Thermodynamic parameters of pectin degradation

The activation energy (E_a) can be calculated from the Arrhenius equation as follows [10, 12]:

$$k = Ae^{-\frac{E_a}{RT}} \quad (3)$$

where A is the pre-exponential factor, E_a is the activation energy (J/mol) and R is the universal gas constant (8.314 J/mol K).

Eyring transition state theory was used to understand the effect of temperature on PG activity and obtain the thermodynamic parameters [10, 12]:

$$k = \frac{k_B T}{h} \exp\left(-\frac{\Delta G}{RT}\right) = \frac{k_B T}{h} \exp\left(-\frac{\Delta H}{RT} + \frac{\Delta S}{R}\right) \quad (4)$$

where T is the absolute temperature (K), k_B is Boltzman constant (1.38×10^{-23} J/K) and h is Planck constant (6.6256×10^{-34} J/s). ΔG, ΔH and ΔS are the changes in free energy, enthalpy and entropy of the reaction, respectively.

2.8. Enzymatic kinetics of PG

Pectin with different initial concentrations (7.60–75.96 μM) was incubated with the untreated and ultrasound-treated PG at 30 °C for 10 min. The reaction rates for the two enzymes at different substrate concentrations were measured. Values of the Michaelis–Menten constant (K_m) and maximum rate of reaction (V_{max}) were attained from Lineweaver–Burk plots.

2.9. Optimum temperature and temperature stability of PG

After treatment under optimum ultrasound conditions, PG was applied to the hydrolysis reactions conducted at different temperatures ranging from 20 °C to 70 °C (at 10 °C intervals). Enzyme activity was measured to determine the optimum temperature. The temperature stability of PG was investigated by incubating the reactants at different temperatures (20 °C–70 °C) for different times (10–60 min), with their residual enzyme activities measured. The initial enzyme activities were measured from the hydrolysis experiment conducted within 1 min at different temperatures and were all designated a relative activity of 100%.

2.10. Inactivation kinetics of PG

In the current study, the first-order kinetics model and the biphasic model were used to depict the inactivation process for PG. The first-order kinetics model was

described as follows:

$$A = A_0 e^{-k_d t} \quad (5)$$

where A is the residual activity at time t (U), A_0 is the initial activity (U) and k_d is the inactivation rate constant (min^{-1}) at the temperature studied.

In the biphasic model, the thermal inactivation process can be reflected as bifurcated curves: heat-labile fraction and heat-stable fraction [14]. Deactivation of both fractions abides by the first-order kinetics model:

$$A = A_s e^{-k_s t} + A_L e^{-k_L t} \quad (6)$$

where A_s and A_L are activity fractions of the stable and labile fractions of PG (%), respectively; k_s and k_L are the corresponding inactivation rate constants (min^{-1}).

The half-life ($t_{1/2}$) and D value of inactivation are mathematically expressed as Eqs. (7) and (8), respectively:

$$t_{1/2} = \ln 2 / k_d \quad (7)$$

$$D = \ln 10 / k_d \quad (8)$$

2.11. Intrinsic fluorescence analysis

Intrinsic fluorescence spectra of enzyme samples with different treatments were recorded at room temperature (20 °C ± 1 °C) with a fluorescence spectrophotometer (Varian Inc., Palo Alto, USA; Model Cary Eclipse) at 280 nm (excitation wavelength, slit = 5 nm), 300–500 nm (emission wavelength, slit = 5 nm) and scanning speed of 1200 nm/s. Buffer used to dissolve PG was applied as blank solution for the sample.

2.12. Circular dichroism (CD)

The CD spectra of the samples were measured with a spectropolarimeter (French

193 Biological Company, Noble, France; Model MOS-450), using a quartz cuvette of
194 1 mm optical path length at room temperature (20 °C ± 1 °C). Scanning was
195 conducted in the far-UV range of 190–250 nm at 30 nm/min with 0.1 nm as
196 bandwidth. The CD data were expressed in the form of mean residue ellipticity $[\theta]$
197 (deg cm^2 $dmol^{-1}$). The secondary structures of PG with or without ultrasound
198 treatment were analysed using DICHROWEB.

199 **2.13. Statistical analysis**

200 All experiments described above were conducted in triplicate, and the mean
201 ± standard deviation was used in the analysis. The experimental data were analysed
202 using ANOVA ($p < 0.05$) and Duncan's multiple range tests by SPSS 17.0 (SPSS Inc.,
203 Chicago, IL, USA). The figures were plotted using Origin Software Version 9
204 (OriginLab Corp., MA, USA).

205 ## 3. Results and discussions

206 **3.1. Effect of ultrasound factors on PG activity**

207 Fig. 1 shows the changes in PG activity after treatment under different
208 ultrasound conditions. The enzyme activity of PG solution sonicated for 15 min at
209 ultrasound intensities of 0–9 W/ml is illustrated in Fig. 1(a). The enzyme activity
210 increased from 0.98 U to 1.19 U, and it was positively correlated with the ultrasound
211 intensity before achieving the maximum value at 4.5 W/ml. The initial enhancement
212 of PG activity might be attributed to the mechanical effects of ultrasound. Shear
213 forces generated from the cavitation bubbles could disperse enzyme aggregates [10] and
214 directly alter the enzyme configuration [21], leading to the exposure of more active sites.

Additionally, a more homogeneous reaction mixture obtained with ultrasound was also conducive to the enhancement of enzyme activity [22].

However, when the ultrasound intensity exceeded 4.5 W/ml, PG activity began to decrease (Fig. 1(a)). Furthermore, when the intensity was over 8.1 W/ml, PG activity was lower than that of the control, demonstrating that enzyme deactivation occurred. The accelerated production of free hydroxyl and hydrogen radicals during high-intensity sonication might be the most important explanation for this phenomenon [14, 23]. These free radicals can react with amino acid residues of the enzymes, which are responsible for enzyme structural stability, substrate binding affinity or catalytic activity, adversely leading to enzyme aggregation [24, 25], reduction in disulphide bonds [26] and destruction of enzyme conformations. Meanwhile, intense shear forces generated from extreme ultrasound conditions can also destroy polypeptide chains, inhibiting the catalytic functions of the enzyme [27]. The effect of ultrasound duration on PG activity is shown in Fig. 1(b). Similar to Fig. 1(a), PG activity firstly increased and then decreased with the prolonged ultrasound duration. PG activity was significantly increased by 20.98% compared with the control at 15 min. However, PG was inactivated when the treatment time exceeded 35 min. Mechanisms for this entire process were also ascribed to the chemical and mechanical effects of ultrasound as mentioned above. Results demonstrated that low-intensity pulsed ultrasound had a positive effect on PG activation, whereas high-intensity, prolonged ultrasound could induce the inactivation process.

As observed from previous studies [9, 10, 12, 13, 28], the intensification of enzyme

activity for different enzymes under their optimum conditions ranges from 5.8% to 200%. In the current study, the obtained optimum ultrasound conditions (output power: 90 W; duration: 15 min) were more intense when compared with those applied to dextranase (40 W, 15 min) [9], alcalase (80 W, 4 min) [10], cellulase (15 W, 10 min) [28] and lipase (60 W, 9 min) [13]. These findings might be attributed to the discrepancies in the enzyme structures. Compared with the β-sheet conformation, the α-helix conformation seemed to be more susceptible under an ultrasonic field. For example, the number of α-helix in dextranase increased by 15.74% after ultrasound treatment, whereas the number of β-sheet only changed by 2.49% [9]. In cellulase [12], alterations in the number of α-helix and β-sheet after ultrasound treatment were 12.38% and 6.58%, respectively. Therefore, the relatively intense ultrasound conditions in the present study were supposed to be ascribed to the high β-sheet contents in the PG structures.

3.2. Effect of ultrasound on the degradation kinetics

In the enzymatic reactions, rate constant is closely related to the catalytic ability of enzymes. The kinetic curves for PGs (untreated and with ultrasound treatment at 4.5 W/ml intensity for 15 min) are presented in Fig. 2. The rate constants at a certain temperature are summarised in Table 1. Within the tested temperature range, the degradation kinetics of different PGs all fitted first-order kinetics well ($R^2 > 0.96$). Obviously, the rate constant k increased as the temperature rose from 20 °C to 50 °C, which was ascribed to the promotion of collision frequency between the pectin molecule and PG enzyme at higher temperature [10]. Kinetic constants for both treated and untreated enzymes peaked at 50 °C, signifying that 50 °C was the optimum

temperature of PG. The sharp decline in the degradation rate at 60 °C implied a serious inactivation process. Furthermore, Table 1 shows that the rate constants of ultrasound-treated PG were always higher than that of the control at each tested temperature, proving that ultrasound increased the catalytic ability of PG.

3.3. Effect of ultrasound on the thermodynamic parameters

Activation energy (E_a) is the threshold energy barrier between the transition state and the starting reagents, and it determines the sensitivity of the reaction rate to temperature [29]. Chemical reaction rate is closely related to E_a, and a lower value of E_a generally indicates a faster reaction procedure. For enzymatic reactions, E_a is influenced by the enzyme species [30], substrate species, reaction temperatures, etc. [31]. Ultrasound treatment could change the PG activity so as to affect the E_a of the hydrolysis process. Arrhenius plots of $\ln k$ against $1/T$ (K^{-1}) for untreated and ultrasound-treated PGs are depicted in Fig. 3 (a); the correlation coefficients of the untreated and ultrasound-treated PGs are 0.9738 and 0.9873, respectively. E_a values were estimated from the slope of the curves and listed in Table 2. The E_a for the ultrasound-treated PG decreased by 40.94% compared with that of the untreated enzyme, revealing an increased rate in molecular collision of reactants and lower necessary potential barrier for the reaction under ultrasound treatment. This phenomenon contributed to the easier occurrence of the enzymatic reactions.

Eyring plots of $\ln(k/T)$ versus $1/T$ (K^{-1}) (Fig. 3(b)) represented good linear relations with correlation coefficients of 0.9637 and 0.9748 for untreated and treated PG, respectively. ΔH values were inferred from slopes of the Eyring plots and ΔS

values were determined by the intercepts. Thermodynamic parameters including ΔH, ΔS and ΔG were calculated and listed in Table 2. Enthalpy strongly depends on enzyme structures, such as the formation and disruption of hydrogen bond and hydrophobic cores [32]. According to previous reports [17, 33], the stable structure of PG is generally held by hydrogen and disulfide bonds. Therefore, the 48.65% decrease in ΔH can be attributed to the ultrasound-induced structural extension of PG, probably including the decomposition of hydrogen bonds and the internal hydrophobic cores, which resulted in the collapse of the ground-state conformations of the protein [10]. Entropy is commonly understood as a measure of disorder in a thermodynamic system. After the ultrasound process, ΔS was reduced by 7.50%, which was possibly due to the original interaction between free radicals and the amino acid residues, and the consequent process of enzyme agglomeration [10]. However, compared to the thermodynamic studies on the ultrasound-treated Alcalase [10] and cellulase [12] (in which the ΔS decreased by 34.01% and 30.49%, respectively), reduction in ΔS of the ultrasound-treated PG was significantly lower, which implied a more slight oxidative denaturation process. This was beneficial for the ultrasonic activation of PG enzyme. Finally, ΔG for ultrasound-treated PG decreased compared with that of the control, suggesting that the enzyme exhibited increased activity and became more available for the pectin hydrolysis process after ultrasound treatment. Changes in thermodynamic parameters ascertained the increase in activity and accessibility of PG under ultrasound treatment, with major favourable contributions of exothermic enthalpy, thereby increasing the enzyme–substrate bond.

3.4. Effect of ultrasound on the enzymatic parameters

The effect of varying substrate concentrations on pectin enzymatic hydrolysis displayed Michaelis–Menten properties with correlation coefficients of 0.9929 and 0.9953 for the untreated and ultrasound-treated PGs, respectively (Fig. 4). Four kinetic parameters, including the maximum rate of reaction (V_{max}), Michaelis constant (K_m), catalytic constant (K_{cat}), and specificity constant (K_{cat}/K_m), were obtained and summarized in Table 3. V_{max} represents the limiting reaction rate achieved at saturating substrate concentration, and K_m demonstrates the enzyme's affinity to the substrate. Linear regression analysis of the double reciprocal plot showed that the V_{max} of the enzymatic reaction had increased, whereas the K_m for the PG decreased after ultrasound treatment. The increase of V_{max} manifested an increased binding efficiency of pectin–PG complexes and an accelerated release of product to the medium, which was probably caused by the expedited mass transfer in a more homogenous system under an ultrasonic field [21]. Meanwhile, the decrease in K_m suggested that the pectin bound to the ultrasound-treated PG with a higher affinity. Ultrasound cavitation effects could propel the exposure of enzyme substrate binding site and catalytic sites, making PG more accessible to the substrate [34]. On the other hand, K_{cat}/K_m values were calculated to estimate the catalytic efficiency of enzymes. A significant increase of 27.10% in K_{cat}/K_m was observed for ultrasound-treated PG compared with that of the control, which indicated that the product formation was processed at an increased rate and higher efficiency under ultrasound treatment.

3.5. Effect of ultrasound on the optimum temperature and temperature stability of PG

3.5.1. Effect of ultrasound on the optimum temperature of PG

Enzymatic reaction rate is related with operating temperatures. In general, higher temperature can increase the enzymatic reaction rate because of the enhanced intermolecular collision frequency; however, excessive heat leads to thermal inactivation of enzymes and slumped reaction rate [35]. As shown in Fig. 5, the optimal temperatures of the PGs with and without ultrasound treatment remain unchanged at 50 °C. This result was in accordance with the reported optimum temperature of some other commercial PGs [19]. Within the experimental temperature range, the PG activity under sonication was higher than that of the control at each temperature. Results indicated that ultrasound treatment could increase the activity of PG within the examined temperature range without affecting the enzyme's optimum temperature; this result is in line with previous reports [8, 9, 11].

3.5.2. Effect of ultrasound on the temperature stability of PG

In industrial applications, enzymatic reactions are often processed at elevated temperatures for higher productivity and lower adverse effects of microbiological contamination [19]. Free radicals and shear forces generated from ultrasound cavitation can seriously affect the enzyme stability [36], which can be applied as a strategy to enhance the temperature stability of PG. As shown in Fig. 6, the increase of PG stability was more remarkable within 30 min than longer period. At 30 °C, for example, the relative activity of ultrasound-treated PG preserved for 30 min increased

by 26.61% compared with the control, whereas the increment reduced to only 9.92% when the retention time was 60 min. In addition, ultrasound treatment generally played a more significant role in the improvement of PG stability at lower temperatures (20–40 °C) than higher temperatures (50–70 °C). At 20, 30, and 40 °C, the relative activity of untreated PG retained for 30 min was 86.63%, 75.89%, and 43.56%, whereas the enzyme with ultrasound treatment maintained 100%, 96.08%, and 57.83% of the initial activity. However, at 50–60 °C, the disparity of the relative activities between PG with and without ultrasound treatment obviously narrowed down, yet the effects of ultrasound on the PG stability remained positive. In addition, at the optimum temperature of PG, i.e., 50 °C, the PG activity with and without ultrasound treatment for 30 min retained only 33.24% and 30.06% of the initial activity, revealing the poor thermostability of PG at its optimum temperature. At 70 °C, both PGs with and without ultrasound treatment were completely inactivated within 20 min. Thus, we proposed that the enzyme structure bunched up seriously at high temperatures (>50 °C), which eliminated the favourable changes brought about by ultrasound treatment. In conclusion, ultrasound treatment could promote the stability of PG at the tested temperature range of 20–60 °C.

3.6. Effect of ultrasound on the inactivation kinetics of PG

The kinetics of thermal inactivation process of the PGs with and without ultrasound treatment at 40–60 °C for 30 min were investigated, and the semi-logarithm graphs of residual PG activity versus heating time are shown in Fig. 7. Single first-order reaction equation is known to be competent in interpreting the

369 inactivation kinetics of most enzymes [14, 37–39]. However, in the present study, it failed
370 to fit the inactivation kinetics of PGs with or without ultrasound treatment considering
371 the low coefficients (Table A.1). Nevertheless, a two-fraction property for the curves
372 was obvious with high coefficients of determination (>0.99).

373 The biphasic (two-fraction) behaviour of PG inactivation process has been
374 widely reported [19, 40, 41]. The non-linear inactivation nature is generally attributed to
375 the existence of different constituents in the enzyme system with diverse unwinding
376 rates during thermal deactivation, resulting in the coexistence of two thermal
377 behaviours. These two components are often interpreted as the heat-stable and
378 heat-labile fractions, respectively [19]. The activity of the heat-stable fraction tends to be
379 higher at lower temperatures, whereas the activity of the heat-labile fraction often
380 increases with the increase in temperature. Ortega et al. [19] applied the biphasic model
381 to the inactivation kinetics of Pectinase CCM (a commercial PG) and found that,
382 when the operating temperature is increased from 40 °C to 55 °C, the activity of the
383 heat-labile fraction increased from 18.7% to 39.3%, whereas that of the heat-stable
384 fraction decreased from 82.7% to 62.7%. This report is similar to the results in the
385 present study. At 40 and 60 °C, an evident increase in activity of the heat-stable
386 fraction can be observed for the PG under ultrasound treatment; at 50 °C, activity of
387 the two fractions were almost the same for the untreated and ultrasound-treated PGs.
388 On the other hand, the inactivation rate constants of both fractions (k_S and k_L) for
389 PG with ultrasound treatment were all lower than those for untreated enzymes at the
390 temperature range tested, demonstrating that the ultrasound-treated PG has stronger

thermostability. Thus, we proposed that both the increase in activity of the heat-stable fraction and the decrease in inactivation rate constants of the two fractions might have a combined contribution to the slower inactivation process of PG under ultrasound treatment.

Half life ($t_{1/2}$) and decimal reduction time (D-value) are frequently-used indexes in the identification of enzyme thermostability [42]. The values of these parameters for each fraction of both untreated and ultrasound-treated PGs are listed in Table 5. The $t_{1/2}$ value and D-value of PG with ultrasound treatment for both fractions were higher than that of the untreated enzyme at 40 and 50 °C. With the increase in temperature from 40 °C to 50 °C, the increments of D-value for the heat-labile fraction were 28.78% and 54.21%, respectively, and corresponding increments for the heat-stable fraction were 24.82% and 23.40%. At 60 °C, however, the values for heat-labile fractions of both enzymes were identical, while only a 9.33% increment was observed for the heat-stable fraction under ultrasound treatment. Ortega et al. [19] hypothesized that $t_{1/2}$ values of two phases can simply be added to obtain the total $t_{1/2}$ value for the enzyme. In this case, ultrasound-treated PG was observed to have higher thermostability with $t_{1/2}$ increasing by 25.04%, 25.19% and 8.73% compared with that of the untreated enzyme at 40, 50 and 60 °C, respectively. Results demonstrated that ultrasound treatment improved the thermostability of PG and prolonged its lifetime.

3.7. Effect of ultrasound on the structures of PG

Shear forces, free radicals and heat generated from an ultrasonic field could act

413 on the amino acid residues of PG and in turn influence its catalytic activity; the
414 change can be reversible or irreversible. In the present study, the intrinsic fluorescence
415 and CD spectra of PG were obtained to investigate the effect of ultrasound treatment
416 on the tertiary and secondary conformations of PG. Meanwhile, the structures of PG
417 with or without ultrasound treatment preserved at 4 °C for 24 h were also elucidated
418 to determine whether the denaturation was reversible or not.

419 The intrinsic fluorescence of protein is mainly contributed by Trp (tryptophan),
420 Tyr (tyrosine) and Phe (phenylalanine) residues, especially the Trp residue [10]. As can
421 shown in Fig. 8(a), PG with ultrasound treatment showed lower fluorescence intensity
422 (the maximum fluorescence emission wavelength was 336 nm) than the untreated PG,
423 which indicated a decreased amount of Trp on the PG surface under ultrasound
424 treatment [10]. In addition, a redshift or blueshift was not detected. This phenomenon
425 was ascribed to the structural unfolding of PG under an ultrasonic field. An increased
426 number of PG internal areas exposed outside buried the previous Trp on the surface,
427 and thus led to the reduction in the fluorescence intensity. Comparison of the PG
428 measured immediately to that preserved for 24 h showed little variation in
429 fluorescence spectra. Thus, renaturation process did not happen within 24 h, which
430 implied that the ultrasound-induced changes in PG tertiary structure were probably
431 irreversible.

432 Fig. 8(b) shows the CD spectra of PG with different treatments, and Table 6
433 summarizes the contents of α-helix, β-sheet, turn and random coil in PG secondary
434 structure. Notably, β-sheet was predominant in PG secondary structures with a

pronounced peak at 210 nm. As shown in Table 6, ultrasound treatment increased the amount of the α-helix and β-sheet conformation, whereas the treatment decreased turn and random coil in PG secondary structures. This configuration transformation was iunder an ultrasonic field [43]. According to a previous study, the substrate binding site of endo-PG on the exterior of the β-helix consists of β-sheet conformations [33]. Therefore, the slight increase in β-sheet possibly implied the formation of more active sites and could be a feasible explanation for the improvement in the PG enzymatic properties. However, changes in PG secondary structures were smaller when compared with that of ultrasound-treated Alcalase [10], dextranase [9] and cellulase [12, 28], possibly due to the high β-sheet contents of the PG structure as mentioned before. Moreover, little differences in CD spectra and enzyme activity were found between PG measured immediately after ultrasound treatment and that preserved for 24 h, suggesting the irreversible denaturation in PG secondary structures under ultrasound treatment.

4. Conclusions

In the present work, low-intensity and short-duration pulsed ultrasonic field was proved to increase the activity of PG. The increased reaction rate constants and the reduced thermodynamic parameters for the ultrasound-treated PG indicated that ultrasound treatment increased the reaction ability of PG. Meanwhile, the increased V_{max} and the reduced K_m values ascertained that PG with ultrasound treatment displayed better catalytic activity and higher affinity to the substrate. In addition, thermal behaviours of both treated and untreated PGs were studied. Under the optimal

conditions, ultrasound could improve the temperature stability of PG without affecting the optimum temperature. A biphasic first order model was chosen to fit the inactivation process, and parameters proved that ultrasound could prolong PG lifetime. Finally, the fluorescence and far-UV CD spectra revealed that ultrasound treatment favourably exposed more active sites of PG, and the change was irreversible. This research suggested a feasible activation method for PG by applying ultrasound during pretreatment in the enzyme preparation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in Table A. 1.

References

1. L. Zhang, X. Ye, T. Ding, X. Sun, Y. Xu and D. Liu, *ULTRASON SONOCHEM*, 2013, **20**, 222–231.
2. A. C. Soria and M. Villamiel, *TRENDS FOOD SCI TECH*, 2010, **21**, 323–331.
3. V. S. Sutkar and P. R. Gogate, *CHEM ENG J*, 2009, **155**, 26–36.
4. B. Xie, L. Wang and H. Liu, *ULTRASON SONOCHEM*, 2008, **15**, 775–781.
5. P. R. Gogate and A. M. Kabadi, *BIOCHEM ENG J*, 2009, **44**, 60–72.
6. P. R. Gogate and A. B. Pandit, *J CHEM TECHNOL BIOT*, 2008, **83**, 1083–1093.
7. F. Chemat, Zill–e–Huma and M. K. Khan, *ULTRASON SONOCHEM*, 2011, **18**, 813–835.
8. J. Wang, Y. Cao, B. Sun, C. Wang and Y. Mo, *ULTRASON SONOCHEM*, 2011, **18**, 534–540.
9. M. Bashari, A. Eibaid, J. Wang, Y. Tian, X. Xu and Z. Jin, *ULTRASON SONOCHEM*, 2013,

20, 155–161.
10. H. Ma, L. Huang, J. Jia, R. He, L. Luo and W. Zhu, *ULTRASON SONOCHEM*, 2011, **18**, 419–424.
11. M. Sakakibara, D. Wang, R. Takahashi, K. Takahashi and S. Mori, *ENZYME MICROB TECH*, 1996, **18**, 444–448.
12. P. B. Subhedar and P. R. Gogate, *J MOL CATAL B–ENZYM*, 2014, **101**, 108–114.
13. S. H. Jadhav and P. R. Gogate, *IND ENG CHEM RES*, 2014, **53**, 1377–1385.
14. M. N. Islam, M. Zhang and B. Adhikari, *FOOD REV INT*, 2014, **30**, 1–21.
15. E. V. Rokhina, P. Lens and J. Virkutyte, *TRENDS BIOTECHNOL*, 2009, **27**, 298–306.
16. I. Goncalves, C. Silva and A. Cavaco–Paulo, *GREEN CHEM*, 2015, **17**, 1362–1374.
17. S. W. Cho, S. Lee and W. Shin, *J MOL BIOL*, 2001, **311**, 863–878.
18. J. D. DZIEZAK, *FOOD TECHNOL–CHICAGO*, 1991, **45**, 78.
19. N. Ortega, S. de Diego, M. Perez–Mateos and M. D. Busto, *FOOD CHEM*, 2004, **88**, 209–217.
20. G. L. MILLER, *ANAL CHEM*, 1959, **31**, 426–428.
21. J. Wang, Q. Huang, F. Huang, J. Wang and Q. Huang, *Sheng wu gong cheng xue bao = Chinese journal of biotechnology*, 2007, **23**, 1121–1128.
22. P. B. Subhedar and P. R. Gogate, *IND ENG CHEM RES*, 2013, **52**, 11816–11828.
23. M. V. Potapovich, A. N. Eremin and D. I. Metelitza, *APPL BIOCHEM MICRO+*, 2003, **39**, 140–146.
24. M. T. Zhong, X. W. Ming, P. W. Su and Q. K. Ju, *ULTRASON SONOCHEM*, 2004, **11**, 399–404.
25. M. Barteri, M. Diociaiuti, A. Pala and S. Rotella, *BIOPHYS CHEM*, 2004, **111**, 35–42.
26. H. Huang, K. Kwok and H. Liang, *ULTRASON SONOCHEM*, 2008, **15**, 724–730.
27. B. Ozbek and K. O. Ulgen, *PROCESS BIOCHEM*, 2000, **35**, 1037–1043.
28. Z. Wang, X. Lin, P. Li, J. Zhang, S. Wang and H. Ma, *BIORESOURCE TECHNOL*, 2012, **117**, 222–227.
29. J. M. Steinweg, S. Jagadamma, J. Frerichs and M. A. Mayes, *PLOS ONE*, 2013, **8**.
30. C. Trasar–Cepeda, F. Gil–Sotres and M. C. Leiros, *SOIL BIOL BIOCHEM*, 2008, **40**, 269.
31. M. Wallenstein, S. D. Allison, J. Ernakovich, J. M. Steinweg and R. Sinsabaugh, in *Soil Biology*, eds. G. Shukla and A. Varma, Editon edn., 2011, vol. 22, pp. 245–258.
32. E. Kazlauskas, V. Petrikaite, V. Michailoviene, J. Revuckiene, J. Matuliene, L. Grinius and D. Matulis, *PLOS ONE*, 2012, **7**.
33. G. van Pouderoyen, H. J. Snijder, J. Benen and B. W. Dijkstra, *FEBS LETT*, 2003, **554**, 462–466.
34. D. Knorr, M. Zenker, V. Heinz and D. U. Lee, *TRENDS FOOD SCI TECH*, 2004, **15**, 261–266.
35. P. Resa, L. Elvira, C. Sierra and F. Montero De Espinosa, *ANAL BIOCHEM*, 2009, **384**, 68–73.
36. C. Basto, C. J. Silva, G. Guebitz and A. Cavaco–Paulo, *ULTRASON SONOCHEM*, 2007, **14**, 355–362.
37. P. Raviyan, Z. Zhang and H. Feng, *J FOOD ENG*, 2005, **70**, 189–196.
38. L. De Gennaro, S. Cavella, R. Romano and P. Masi, *J FOOD ENG*, 1999, **39**, 401–407.
39. R. Cruz, M. C. Vieira and C. Silva, *J FOOD ENG*, 2006, **72**, 8–15.

40. L. Ceci and J. Lozano, *FOOD CHEM*, 1998, **61**, 237–241.
41. P. LOPEZ, J. L. DELAFUENTE and J. BURGOS, *ANAL BIOCHEM*, 1994, **220**, 346–350.
42. X. F. Cheng, M. Zhang and B. Adhikari, *ULTRASON SONOCHEM*, 2013, **20**, 674–679.
43. I. Gulseren, D. Guezey, B. D. Bruce and J. Weiss, *ULTRASON SONOCHEM*, 2007, **14**, 173–183.

Tables

Table 1

Rate constants of hydrolysis reactions catalysed by PG with or without ultrasound treatment (mean ± SD).

Temperature (°C)	Hydrolysis process	Rate constants $\times 10^{-3}$ (min^{-1})	R^2
20	Control	1.20 ± 0.02	0.9858
	With ultrasound	1.82 ± 0.00	0.9961
30	Control	1.54 ± 0.01	0.9706
	With ultrasound	2.09 ± 0.02	0.9654
40	Control	1.75 ± 0.01	0.9857
	With ultrasound	2.45 ± 0.04	0.9913
50	Control	2.28 ± 0.01	0.9737
	With ultrasound	2.56 ± 0.04	0.9817
60	Control	1.26 ± 0.11	0.9808
	With ultrasound	1.70 ± 0.00	0.9851

Table 2

Thermodynamic parameters of hydrolysis reactions catalysed by PG with or without ultrasound treatment (mean ± SD).

Treatment	E_a (kJ/mol)	ΔH (kJ/mol)	ΔS (J/mol K)	ΔG (kJ/mol)
Control	16.16 ± 0.38	13.60 ± 0.37	-254.27 ± 1.22	90.68 ± 0.01
With ultrasound	9.54 ± 0.49	6.98 ± 0.48	-273.33 ± 1.68	89.84 ± 0.02

Table 3

Enzymatic kinetic parameters of hydrolysis reactions catalysed by PG with or without ultrasound treatment (mean ± SD).

	V_{max} ($\mu M \cdot min^{-1}$)	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m ($\mu M^{-1} \cdot min^{-1}$)
Control	1724.14 ± 5.90	26.05 ± 0.08	1320.72 ± 4.52	50.71 ± 0.01
With ultrasound	1831.50 ± 1.67	21.77 ± 0.07	1402.96 ± 1.28	64.45 ± 0.26

Table 4

Biphasic inactivation kinetic parameters of PG with or without ultrasound treatment at temperature range of 40 °C to 60 °C (mean ± SD).

Temperature (°C)	PG process	Biphasic model kinetic parameters				R^2
		A_L	A_S	k_L	k_S	
40	Control	31.05 ± 3.09	68.79 ± 2.82	0.2661 ± 0.0506	0.0156 ± 0.0017	0.9972
	With ultrasound	16.61 ± 4.86	83.19 ± 4.81	0.2066 ± 0.0967	0.0125 ± 0.0022	0.9937
50	Control	44.34 ± 2.60	55.58 ± 2.02	0.3407 ± 0.0425	0.0210 ± 0.0002	0.9982
	With ultrasound	44.54 ± 1.68	55.46 ± 1.61	0.2210 ± 0.0141	0.0170 ± 0.0011	0.9996
60	Control	75.70 ± 0.75	24.36 ± 0.34	0.3729 ± 0.0061	0.0252 ± 0.0006	0.9999
	With ultrasound	70.94 ± 1.88	28.90 ± 0.97	0.3731 ± 0.0175	0.0231 ± 0.0014	0.9994

Table 5

Effect of ultrasound treatment on the $t_{1/2}$ and D-value of different fractions (mean ± SD).

Temperature (°C)	PG process	$t_{1/2}$ (min)		D (min)	
		Heat-labile	Heat-stable	Heat-labile	Heat-stable
40	Control	2.61 ± 0.51	44.46 ± 4.79	8.65 ± 1.71	147.70 ± 15.91
	With ultrasound	3.35 ± 2.01	55.50 ± 9.94	11.14 ± 6.68	184.35 ± 33.03
50	Control	2.03 ± 0.26	32.94 ± 2.44	6.76 ± 0.86	109.44 ± 8.11
	With ultrasound	3.14 ± 0.20	40.65 ± 2.61	10.42 ± 0.67	135.05 ± 8.67
60	Control	1.86 ± 0.03	27.50 ± 0.66	6.18 ± 0.10	91.37 ± 2.18
	With ultrasound	1.86 ± 0.09	30.07 ± 1.87	6.17 ± 0.29	99.90 ± 6.26

Table 6

Secondary structures of PG with or without ultrasound treatment (measured immediately or after being preserved at 4 °C for 24 h).

	α-Helix (%)	β-Sheet (%)	Turn (%)	Random coil (%)	Pectinase activity (U)
Untreated	2.60	40.94	19.42	37.04	0.98±0.06
Untreated (24 h)	2.60	40.86	19.48	37.06	0.98±0.04
With ultrasound	2.70	41.16	19.28	36.86	1.19±0.05
With ultrasound (24 h)	2.70	41.14	19.22	36.94	1.18±0.02

Figures

Figure 1

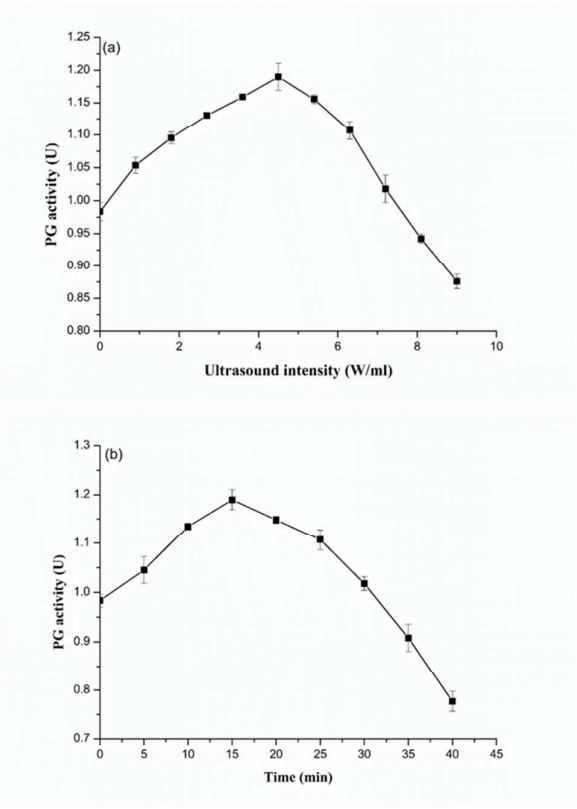
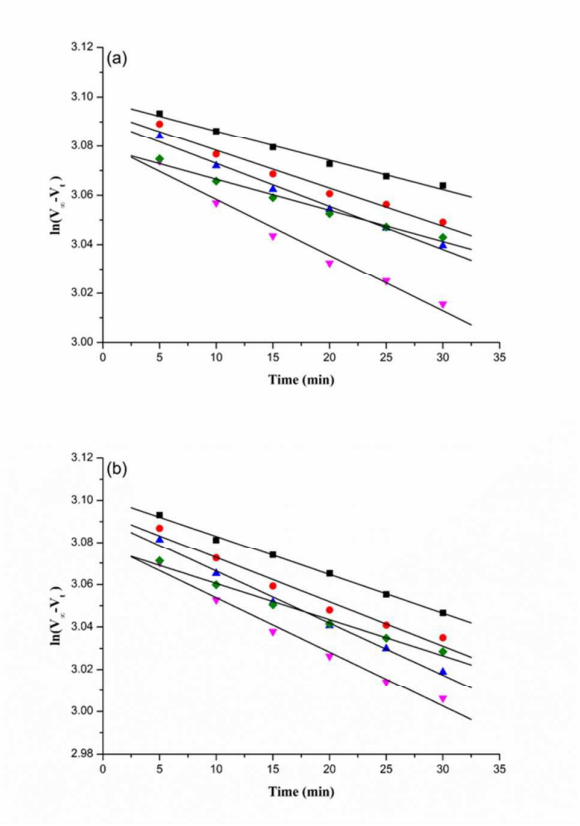


Fig. 1 Effect of (a) ultrasound intensity and (b) ultrasound duration on PG activity

581 **Figure 2**



582

583 **Fig. 2** The degradation kinetics curves of pectin processed with (a) untreated PG and (b)
584 ultrasound-treated PG. ■20 °C ●30 °C ▲40 °C ▼50 °C ♦60 °C

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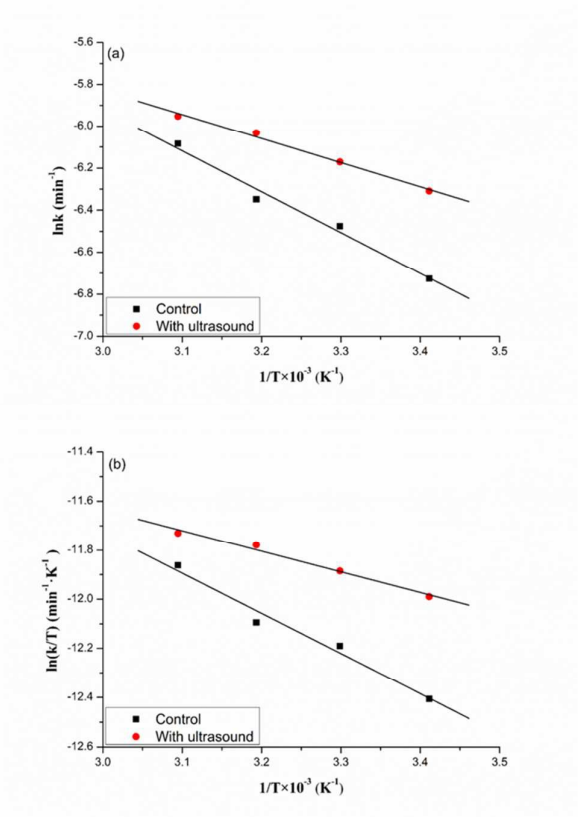
586

587 **Figure 3**



588

589 **Fig. 3** Relationship between (a) $\ln k$ and $1/T$; (b) $\ln(k/T)$ and $1/T$ for hydrolysis
590 reactions catalysed by PG with and without ultrasound treatment.

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Figure 4

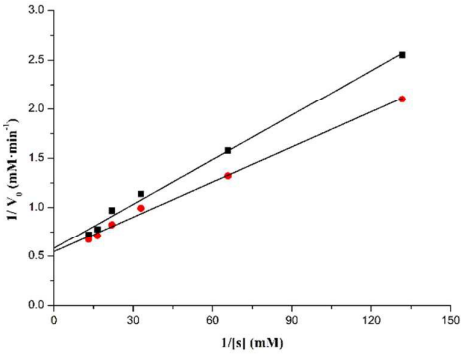
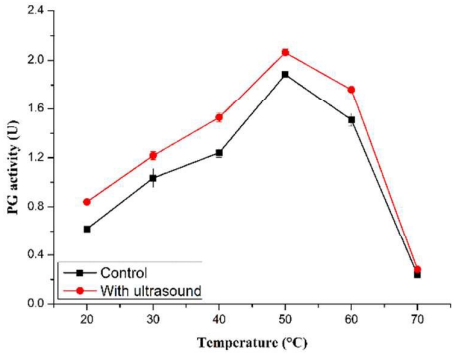


Fig. 4 Plots of the initial velocity values obtained as a function of the correspondent substrate concentration values using Lineweaver–linearization for untreated and ultrasound-treated PG.

600 **Figure 5**



601

602 **Fig. 5** Effect of temperature on the enzyme activity of PG with and without ultrasound treatment.

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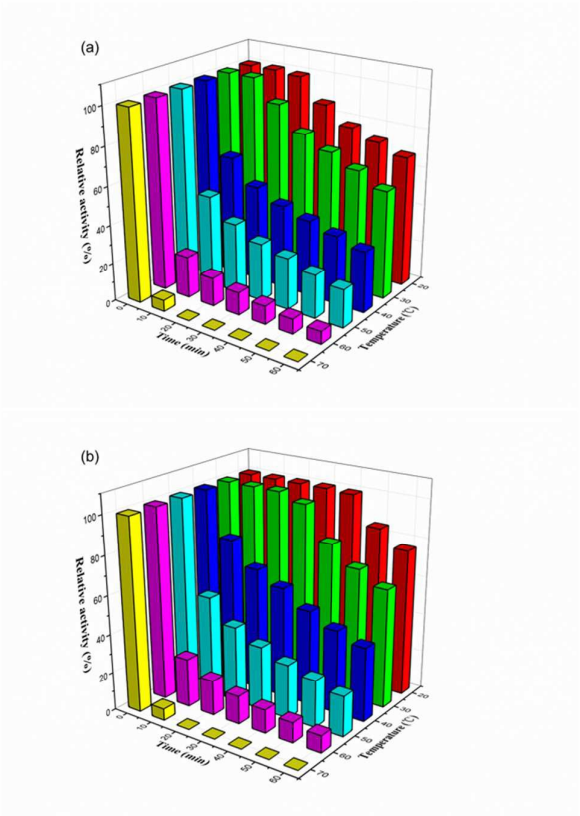
605

606 **Figure 6**



607

608 **Fig. 6** Effect of temperature on the stability of (a) untreated and (b) ultrasound-treated PG.

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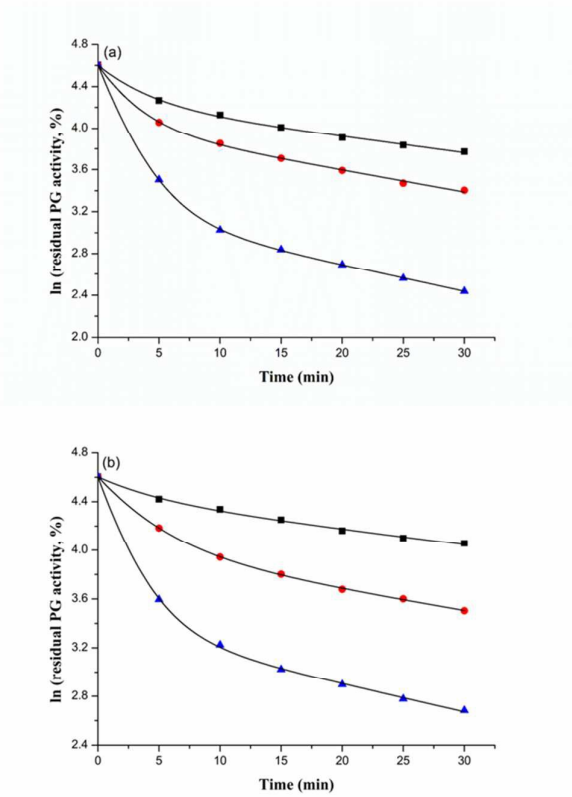
611

612 **Figure 7**



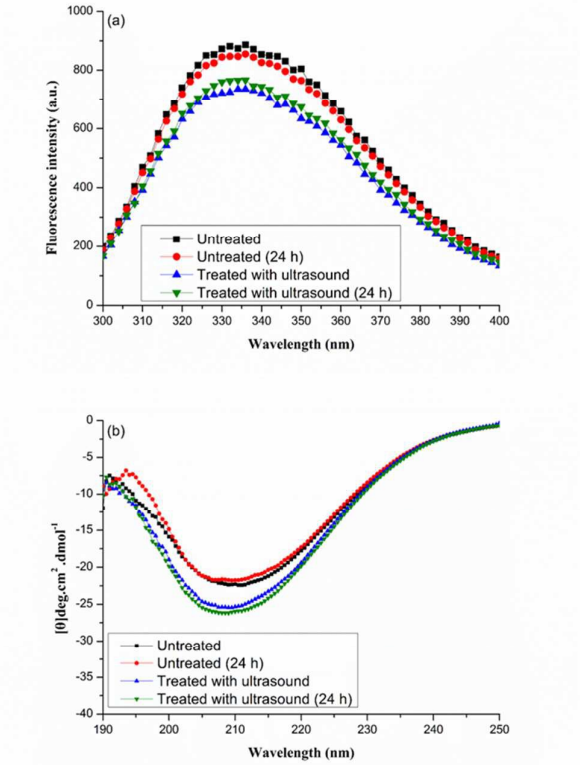
613

614 **Fig. 7** Biphasic inactivation curves of (a) untreated and (b) ultrasound-treated PG. ■40 °C ●50 °C

615 ▲60 °C

616

617 **Figure 8**



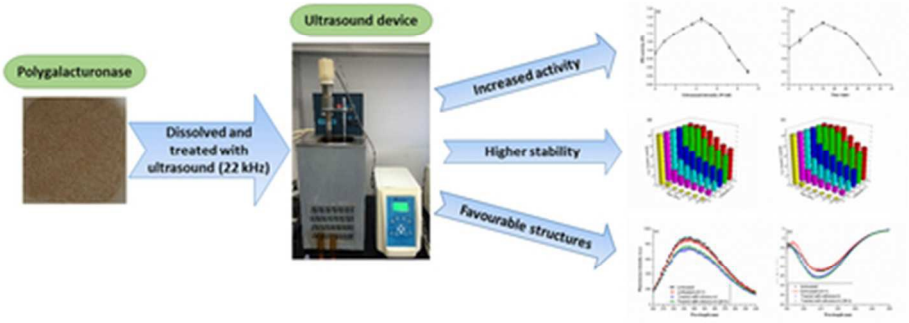
618

619 **Fig. 8** (a) Intrinsic fluorescence spectra and (b) CD spectra of the untreated and ultrasound-treated
620 PG (measured immediately or after being preserved at 4 °C for 24 h).

621

Novelty of the work

The work investigated the effect of ultrasound on the enzymatic properties and structures of polygalacturonase for the first time.



38x19mm (300 x 300 DPI)