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FULL ARTICLE

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Sonochemical action and reaction of edible insect protein: Influence on enzymolysis reaction-kinetics, free-Gibbs, structure, and antioxidant capacity

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Abstract

We investigated the impact of sonochemical action and the reaction of *Hermetia illucens* larvae meal protein (HILMP) as regards enzymolysis under varied enzyme concentration and temperature to explain the mechanism and effect of sonication on molecular conformation, limits of kinetics, free-Gibbs energy, and antioxidative capacity. Control treatment was used for comparison. The results showed sonochemical treatment enhanced HILMP-enzymolysis efficiency at various enzyme volume, and temperature. Enzymolysis-kinetics revealed sonochemical treatment increased the rate constant ($p < .05$) by 17.21%, 25.06%, 26.91%, and 41.38% at 323, 313, 303, and 293 K, respectively. On free-Gibbs, sonochemical treatment reduced the reactants-reactivity energy, enthalpy, and entropy by 30.53%, 35.05%, and 10.71%, respectively ($p < .05$). Changes in spectra of UV and fluorescence, and micrographic imaging indicated alterations of HILMP by sonochemical treatment. Antioxidative activity of sonochemically-treated HILMP increased, compared to control. Thus, sonochemical treatment may be beneficial in the production of edible insect proteins with smaller molecular weights for different food and/or pharmaceutical applications.

Practical applications

Sonochemical pretreatment of HILMP positively impacted it enzymolysis rate-reaction, stability of reaction products, structure, and bioactivity. Thus, the technique may be beneficial to industry in the processing/development of new (bioactive/pharmaceutical) products involving enzymolysis of edible insects (e.g., *Hermetia illucens*) protein; particularly at such a time where edible insects are projected to be a source of protein for human nutrition and livestock in the next few years.

KEYWORDS

antioxidant activity, edible insect protein, enzymolysis reactivity energy, molecular conformation, sonochemical treatment

1 | INTRODUCTION

The global demand for protein in human and livestock nutrition is projected to increase in the next two/three decades (Aiking, 2011). Already, existing appraisal point to it that millions of tons of proteins are on demand each year (Jin et al., 2015). At the same time, a vast number of protein resources are underutilized. One such resource is *Hermetia illucens*, an edible insect. The larvae of *H. illucens* is rich in protein—about 38%–60% dry weight (Barragan-Fonseca, Dicke, & van Loon, 2017). This can be extracted for food applications, with the aim of improving the nutritional and techno-functional properties of food products. This technique is deemed very appropriate since it masks the appearance of insects and thus creates an atmosphere for increased consumption across the world (Sosa & Fogliano, 2017). However, just as in the case of proteins from plant sources (Jin et al., 2015) as well as for most insect species (Nongonierma & FitzGerald, 2017), protein extract from the larvae of *H. illucens* has limited utilization in food applications due to its poor solubility in water. There are reports suggesting that the solubility of *H. illucens* larvae meal protein (HILMP) could be augmented by hydrolysis (Hall, Jones, O'Haire, & Liceaga, 2017).

Hydrolysis of proteins by enzymatic means results in the generation of bioactive peptides (Lin, Deng, & Huang, 2014; O'Sullivan, Lafarga, Hayes, & O'Brien, 2017; Sae-Leaw et al., 2017; Saisavoey, Sangtanoo, Reamtong, & Karnchanatat, 2016), and has been judged most adequate compared to alkaline or acid hydrolysis (Clemente, 2001; McCarthy, O'Callaghan, & O'Brien, 2013; Panyam & Kilara, 1996). This is attributed to the fact that the reaction conditions are controllably milder, product quality is high, and the enzymes/proteases are commercially available (Clemente, 2001; McCarthy et al., 2013; Panyam & Kilara, 1996). Nonetheless, conventional enzymolysis has been noted to limit the enzymolysis of proteins owing to the incompatible conformation of protein which makes it hard for the protease to attack the enzyme-driven bonds of a protein (Jian, Wenyi, & Wuyong, 2008; Qu et al., 2013). As a consequence, the duration of enzymolysis is extended and the volume of energy consumed is increased (Qu et al., 2013). It follows that, improving on the conventional enzymolysis approach will benefit industry in many applications such as in the production of functional bioactive products with reduced energy cost. In that regards, ultrasound application may be a preferred option.

The use of ultrasound, a contemporary green-processing technology, has been shown to augment the process of enzymolysis of proteins in preparing bioactive compounds (Jian et al., 2008; Pan, Qu, Ma, Atungulu, & McHugh, 2012; Qu et al., 2013; Qu, Pan, & Ma, 2010; Wang, Sun, Cao, Tian, & Li, 2008). This is linked to the cavitation effect of ultrasound (acoustic in nature) which results in intense shear thrusts, vibration, and shockwaves (Chandrapala, Oliver, Kentish, & Ashokkumar, 2012). Accordingly, the use of ultrasound for/to treat samples prior to enzymolysis has been shown by some researchers to improve the bioactivity of a number of products (Jia et al., 2010; Jian et al., 2008; Jin et al., 2015; Mintah et al., 2019; Zhang et al., 2015), as well as the rate of reaction, and conversion of substrates (Dabbour, He, Mintah, Tang, & Ma, 2018; Jin et al., 2015). This improvement, has been associated with the role of ultrasonication pretreatments in altering the protein structure (Dabbour et al., 2018) and consequently reducing the free-Gibbs ($\triangle G$) and Activation (E_a) energy of reaction (Jin et al., 2015). It is also reported that sonication has significantly positive impact on enzymolysis kinetics as it improves the preliminary rate of reaction through reduction of Michaelis constant (Jin et al., 2015).

Literature shows several studies on the preparation of bioactive hydrolysates from several products/produce (milk, cheese, yoghourt, bean, and rice) using conventional enzymolysis technique (Gobbetti, Stepaniak, Angelis, Corsetti, & Cagno, 2002; Megías et al., 2007). Studies on the techno-functional attributes of insect derived protein-hydrolysates obtained, again, by conventional enzymolysis are also documented (Hall et al., 2017; Purschke, Meinlschmidt, Horn, Rieder, & Jäger, 2018). There are also some studies on edible insect protein hydrolysates obtained through traditional hydrolysis for bioactive functions (Zhang et al., 2016; Zielińska, Baraniak, & Karaś, 2017), but no data has been documented to explain the effect of ultra-sonication (multi frequency control ultrasound [MFCU]) action on the enzymatic hydrolysis of HILMP. Data on proteolysis reaction kinetics is imperative for the hydrolysis of proteins and enhancing the utilization of edible insect protein in food applications thereof. As a consequence, in this study (as the first), the influence of ultrasound (sonochemical) pretreatment (*action*) on the enzymolysis of HILMP (*reaction*) under varied enzyme concentration, and temperature was considered to explain the mechanism and effect of sonication on the enzymolysis efficacy, molecular conformation, and the limits of kinetics and free-Gibbs energy (thermodynamics). The antioxidative capacity of hydrolysates were also, explored. The findings of the present study, therefore, could be beneficial in setting the scientific basis with technological backing for extended research/application in the production of edible insect proteins with smaller molecular weights for different food and/or pharmaceutical applications.

2 | MATERIALS AND METHODS

2.1 | Materials

Defatted *H. illucens* larvae meal (<60 mesh-size, stored at 4°C, protein content by Kjeldahl technique 51.62%) was provided by the RH-research group (Jiangsu University—School of Food and Biological Engineering, China). The larvae were previously obtained from the Difei Bio-Technology Company, Jiangsu (China), and had been freeze-dried prior to milling and defatting. Endopeptidase (Alcalase) with density 1.17 g/ml and activity 150,000 U/ml was acquired from a Biotechnology company in China (Novozymes). Other reagents, including sodium hydroxide (NaOH), used in this work were of analytical rating.

2.2 | MFCU pretreatment of HILMP

MFCU pretreatment of HILMP preceded the enzymolysis reaction. The MFCU equipment (Fanbo Bio-Engineering Co., Ltd., Wuxi, China) consisted of a sonication tank (30.0 × 36.0 × 11.4 cm), two operational frequency modes—sweeping (SFU) and fixed (FFU), and two sonication plates (lower and upper) with acoustic operating power of 600 W. Each frequency mode has five frequency (f) options (22, 28, 33, 40, and 68 kHz). Compared to the FFU, the SFU has unstable frequency work model (i.e., from [$f - x$] to [$f + x$] and vice-versa) around a central frequency with constant linear speed (Ren, Ma, Mao, & Zhou, 2014). Accordingly, the SFU frequency is expressed as $f \pm x$ (where $x = 2$). It follows that the FFU operates under a stable frequency (f). In studying the effect of sonication on HILMP, the SFU with lower sonication plate was used. The sweep-cycle, pulse-on time, pulse-off time, frequency, sonication-time, and temperature were 500 s, 15 s, 5 s, 40 ± 2 kHz, 30 min, and 50°C, respectively; and HILMP suspension (14.0 g/L) was transferred into 0.50 L sample/sealed bags and placed in the ultrasonic tank containing water (5.5 L). The temperature of the water tank is controlled by a digital water heating probe. A control (without sonication treatment) was studied with the aid of an impeller-JJ1 agitator ((J. Xichengxinrui Instrument Co., Jiangsu, China) at 100 rpm.

2.3 | Enzyme hydrolysis of HILMP

The hydrolysis set-up consisted of a digital acidity/basicity meter (10-PB, Sartorius Science instruments, China), a JJ1-impeller agitator (J. Xichengxinrui Inst. Co., China) at a 100 rpm, and a thermostatic bath (6A-HH, Zhongzheng apparatus manufacturing Co., Jintan, China), and centrifuge (16-TGL, HS-Tabletop, Pioway, Nanjing, China). Subsequent to the sonication (SFU) and control pretreatments, the HILMP suspensions were adjusted to pH 9.0 using 1 M NaOH after preconditioning (17 min heating) at specified temperature (20, 30, 40, and 50°C), the respective enzyme volumes (0.56, 0.70, 0.84, and 0.98 ml/L) were added to initiate the hydrolysis reaction. The pH of the respective suspensions were maintained, over an enzymolysis time of 100 min, with the NaOH.

2.4 | Measure of hydrolysis

The extent of hydrolysis, also hydrolysis degree (D_H), of HILMP was determined using the pH-stat technique (Nissen, 1987):

$$D_H(\%) = \frac{B_c N_{sh}}{\alpha M_{hp} h_{tp}} \times 100$$

where, B_c is the amount of alkaline (NaOH) consumed (ml); N_{sh}, alkaline concentration (mol/L); α, mean extent of dissociation of alpha-amino group (α-NH_2) in HILMP, M_{hp}, mass (g) of hydrolyzed protein; and h_{tp}, total content of peptide bonds in the protein. The h_{tp} (7.6) and α (1.0) indicated by Adler-Nissen (1986), and Nielsen, Petersen, and Dambmann (2001) were used.

2.5 | Estimation of hydrolyzed protein concentration (H_{pc})

In order to evaluate the enzymolysis reaction (kinetic) limits of HILMP, the hydrolyzed protein (HILMP) concentration was measured using the method described by Galvão, Pinto, Jesus, Giordano, and Giordano (2009), computed as:

$$H_{pc} = C_s \times D_H \times 0.01$$

where, H_{pc} is the hydrolyzed HILMP concentration (g/L), and C_s the starting HILMP concentration (g/L).

2.6 | Enzymolysis reaction-rate (k)

The first-degree (1°) reaction-rate model was used to measure the rate constant k of HILMP (Kadkhodaee & Povey, 2008). The reaction-rate (kinetic) equation was given as:

$$\frac{dC_t}{dt} = -kC_t$$

Following integration, the kinetic model was expressed as:

$$\ln C_t = -kt + \ln C_s$$

where, k denotes the rate constant (min^{-1}), whereas C_t is the concentration (g/L) of HILMP at a given time t (min).

Since it is difficult to measure the reduction of HILMP, the rate-reaction was estimated from the increase in amount of peptides released in solution.

At a set temperature and pressure, $C_s = V_{max}$ and $C_t = (V_{max} - V_t)$. Hence, the equation $\ln C_t = -kt + \ln C_s$ could be rearranged as:

$$\ln\,(V_{max} - V_t) = -kt + \ln V_{max}$$

where V_{max} is the maximal peptide concentration (μg/ml) obtained under optimal SFU pretreatment conditions and the optimum enzyme conditions (pH 9.0, and 323 K for 5 hr), V_t the peptide concentration at time t. The k value was computed from the gradient of the plot $\ln (V_{max} - V_t)$ against t (i.e., enzymolysis time).

As sonication aided enzymolysis reaction could be influenced by cavitation and thermal effect, likened to control (traditional enzymolysis), the k in the sonication aided enzymolysis was expressed as:

$$k = k_o + k_{se}$$

where k_o and k_{se} denotes the rate-reaction constant attributable to thermal effect in the control-enzymolysis, and sonication-aided enzymolysis, respectively.

2.7 | Thermodynamics of HILMP enzymolysis

The Arrhenius Svante equation was used to describe the association between the rate-reaction constant, k, and hydrolysis temperature, T (Parkin, 2007):

$$k = Ae^{\frac{-E_a}{RT}}$$

where A is the pre exponential factor per minute; T, the absolute (thermodynamic) temperature (K); E_a, the minimal (activation) energy for reaction (J/mol); R, molar gas constant (8.3144 J/K mol).

The Arrhenius Svante equation was transformed to:

$$\ln k = -\frac{E_a}{RT} + \ln A$$

And the plot of ln(k) to 1/T was used to estimate E_a; whereas the Eyring transition state (ETS) theory was used to determine changes in the thermodynamic parameters (ΔG, ΔH, and ΔS) of the hydrolysis process (Parkin, 2007):

$$k = \frac{k_B T}{h} exp\left(-\frac{\Delta G}{RT}\right)$$

where, k_B is the Boltzmann value (1.38×10^{-23} J/K); h, the Planck constant (6.6256×10^{-34} J s); ΔG, Gibbs energy (J/mol).

The free (Gibbs) energy (ΔG) was quantified as:

$$\Delta G = \Delta H - T\Delta S$$

where, ΔH, activation enthalpy (J/mol); and ΔS, the activation entropy (J/mol K).

By combining the ETS and Gibbs equation, a transformation of the ETS equation was given as:

$$\ln \frac{k}{T} = -\frac{\Delta H}{R} \times \frac{1}{T} + \left[\ln \frac{k_B}{h} + \frac{\Delta S}{R}\right]$$

A linear plot of $\ln \frac{k}{T}$ and $\frac{1}{T}$ was used to determine the values of ΔS (from intercept) and ΔH (slope).

2.8 | Hydrolysate peptide concentration

The peptide concentration (μg/ml) of HILMP hydrolysate was determined with method of Ledoux and colleague (Ledoux & Lamy, 1986). HILMP hydrolysate was reacted with 15% trichloroethanoic acid (2:1 volume ratio) and kept at 25°C (30 min); and then centrifuged (4,000 ×g, 15 min).The absorbance was estimated at 680 nm with TU-1810 spectrophotometer (Pui-General Insts. Co. Ltd., Beijing, China). Sample blank was deionized water.

2.9 | Preparation of HILMP isolates

A 500 ml HILMP suspension (14.0 g/L) was pretreated under the set sonication (SFU) conditions (Section 2.2). The pretreated (sonicated) suspension was adjusted to pH 11.5 (achieved with 1 M NaOH), and then stirred continuously (100 rpm) at 50°C (for 60 min), and the slurry was centrifuged at (4,500 ×g, 15 min, 4°C). The resultant (supernatant) was precipitated at isoelectric pH 4.4, allowed to stand (30 min) and again centrifuged (idem). The precipitate (protein isolate) was lyophilized (1.2-ALPHA, Martin Christ Inc., Osterode, Germany) and kept at −20°C prior to analysis. The control isolate was obtained with a JJ1-impeller agitator (100 rpm) instead (i.e., without sonication treatment).

2.10 | Ultraviolet-visible spectra analysis

The prepared HILMP samples were dissolved in 0.01 mol/L (pH 8.0) buffer solution (phosphate—PBS), to 1.5 mg/ml. The ultraviolet-visible spectra (UV-VS) of the resultants were examined in the range 200–400 nm using a Varian-100 Cary UV-Spectrophotometer (Varian Incorporated, Palo-Alto, USA), 10.0 mm quartz cell path-length, at 25°C. The scan-rate and band-width settings were 60 nm/min and 2.0 nm, respectively. The blank spectrum was realized with the PBS solution.

2.11 | Intrinsic fluorescence analysis

Intrinsic fluorescence analysis (IFA) of the prepared HILMP samples (control, and sonication treated) was done at 24 ± 1°C using 0.05 mg/ml in 0.1 mol/L PBS (pH 80). Fluorescence Cary-Eclipse spectrophotometer (Varian Incorporated, Palo-Alto, USA), path cell length 1 cm was used. Sample excitation wavelength, 279 nm; and emission wavelength, 280–450 nm was used; while the slit width and scan speed were 5 nm, and 10 nm/s, respectively. PBS was used as spectrum blank, and 10 scan spectra expressed in mean values were applied.

2.12 | Micrographic imaging analysis (MIA)

The microstructure of the HILMP samples (sonication treated, and control), were analyzed using a digital light-microscope (BX43-Olympus, Tokyo, Japan) installed with a V35-0D camera. The method described by Alenyorege and colleagues (Alenyorege et al., 2018) was used; and micrographs were taken at ambient temperature (24 + 1°C) with 400× magnification.

2.13 | Scavenging activity—hydroxyl radical analyses

The scavenging activity—hydroxyl radical (SAHR) of the HILMP hydrolysates (SFU treated, and control) were estimated at various

enzymolysis time with the protocol outlined by Juan Wang, Wang, Dang, Zheng, and Zhang (2013) with little modifications. Briefly, 1,000 µl of $FeSO_4$ solution (6 mM) was mixed with 1,000 µl of hydrolysates and 1,000 µl of H_2O_2 (6 mM) solution. The resultant mix was vortexed and left for 15 min (25°C). Subsequently, to the mixture, 1,000 µl of a lipophilic monohydroxybenzoic acid (salicylic acid, 6 mM) was added and the absorbance (at wavelength 510 nm) was read after 30 min. The blank preparation was done by substituting the sample with distilled H_2O (water). The SAHR of the hydrolysate was calculated using:

$$\text{SAHR}\ (\%) = \left(1 - \frac{(A_{hs} - A_{ds})}{A_{wb}}\right) \times 100$$

where A_{hs} represent the absorbance of sample, A_{ds} absorbance of sample devoid of salicyclic acid, and A_{wb} the blank.

2.14 | Statistical analysis

All experiments were in triplicate, and the outcomes were presented as average (±) standard deviations. ANOVA (analysis of variance) was done (with Minitab 17.0 Software) to measure the effect of sonication treatment at $p < .05$. One-way ANOVA (using Tukey's pairwise test) was utilized to liken the differences between individual groups. The computations and graphs were done with Microsoft Excel (MSE 2016) and Origin Pro (v. 8.0) Software, respectively.

3 | RESULTS AND DISCUSSION

3.1 | Influence of sonication and control pretreatments on HILMP enzymolysis

The influence of each treatments (sonication and control) on hydrolyzed protein (g/L) at various enzyme concentration, and temperature are displayed in Figures 1 and 2, respectively.

Under varying enzyme concentrations, Figure 1 showed that the hydrolysed HILMP concentration increased correspondingly with increases in enzyme concentration for the duration of enzymolysis. Compared to the control, however, the sonication treated samples showed a relatively sharp increase from 0 to 16 min when the enzyme concentration was 0.84 and/or 0.98 ml/L. This observation, according to Gülseren, Güzey, Bruce, and Weiss (2007), is as a result of the sonication treatment causing the protein molecules to unfold; an outcome which was conducive for improved enzyme action. It also indicate that as more of the enzyme got into the system, collision among the molecules increased, which resulted in commensurate levels of hydrolysed HILMP.

When the intensity of heat in a system is raised, the reacting molecules gain more energy and bounces around. As a consequence, the molecules are most likely to collide and as well combine. The reverse is, however, true when the temperature in a system is decreased.

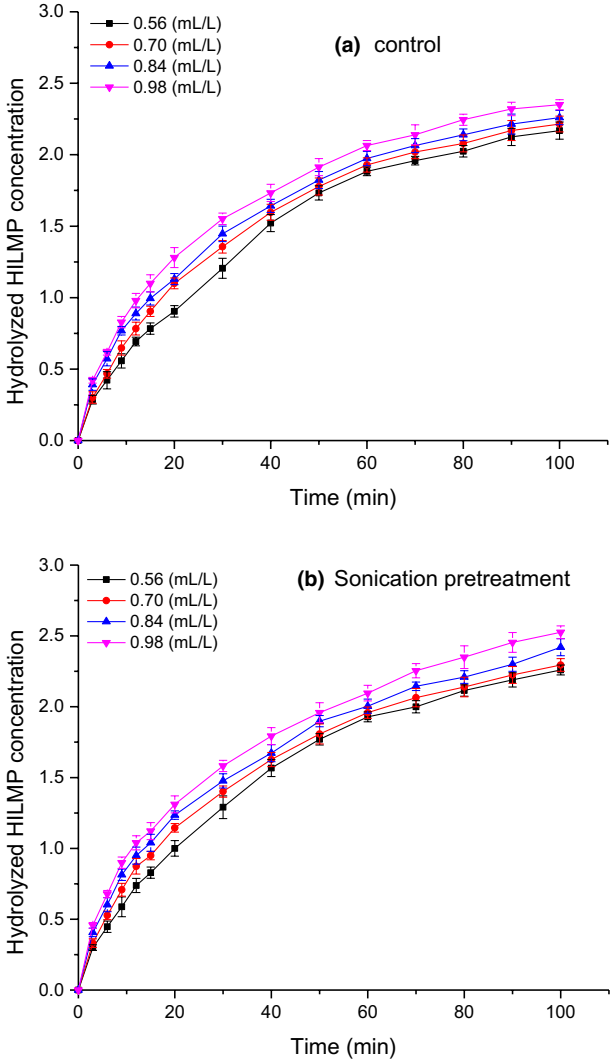

FIGURE 1 Hydrolyzed HILMP concentration in (a) traditional (control) and (b) sonication assisted enzymolysis at various enzyme concentrations (pH = 9.0, C_s = 14 g/L, and T = 323 K)

In the present study, the influence of temperature (293, 303, 313, and 323 K) was shown in Figure 2a,b. From the curves, a direct relation between temperature and concentration of hydrolyzed protein was observed (up to about 80 min). That is, increasing temperature caused an increase in the concentration of hydrolyzed protein for both samples (control and sonication-aided enzymolysis). The sonication pretreatment resulted in noticeable increase in hydrolyzed protein than the control at the set temperatures, particularly at 313 and/or 323 K. At these temperatures, the curves revealed that the hydrolyzed protein concentration obtained through sonication pretreatment increased by 12.49% and 14.01% ($p < .05$) when compared to the control treatment. This observation, for hydrolysis time of 100 min, suggest that the sonication pretreatment resulted in an enhanced enzymolysis than the control, through collision and combination effect of the reacting molecules. Our findings agree with that reported by Jin et al. (2015).

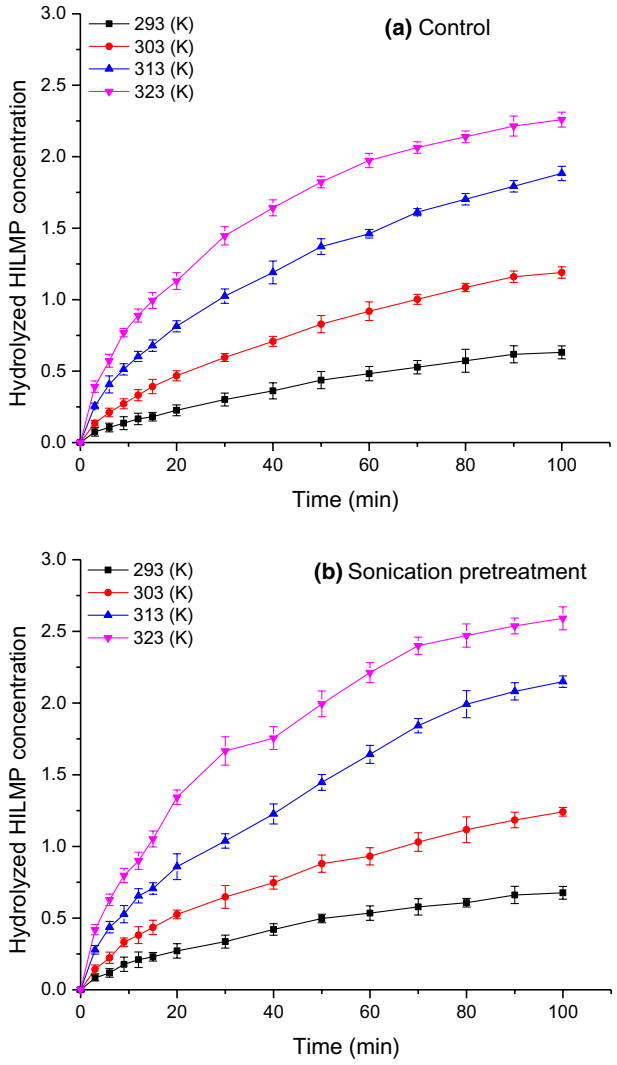

FIGURE 2 Hydrolyzed HILMP concentration in (a) traditional (control) and (b) sonication assisted enzymolysis at different temperature (pH = 9.0, Enzyme = 0.42 ml/L, and C_s = 14 g/L)

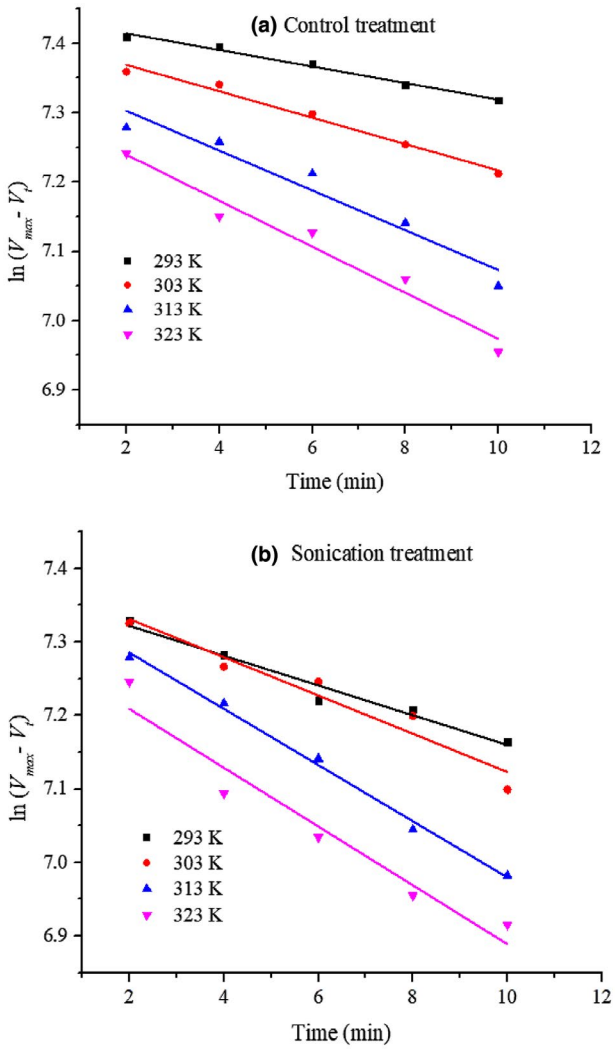

FIGURE 3 Values of ln $(V_{max} - V_t)$ against enzymolysis time (t) for sonication treatment and control (conventional enzymolysis) at various temperatures with substrate and enzyme concentrations 14 g/L and 0.42 ml/L, respectively

3.2 | Effects of sonication pretreatment and control enzymolysis on rate-reaction constant k

As indicated earlier, the ETS and Arrhenius equations were employed in calculating the thermodynamic parameters. The rate-reaction constant is dependent on the enzymolysis temperature, making it an important variable in the said equations. Accordingly, the changes in the rate-reaction (k) values due to the sonication pretreatment and control (conventional) enzymolysis was studied under hydrolysis reaction using: HILMP (14 g/L), enzyme concentration (0.84 ml/L), at temperatures (293–323 K), and pH 9.0; taken into consideration the linear plots between 2 and 10 min. The plots of ln $(V_{max} - V_t)$ against enzymolysis reaction time in control and sonication pretreatment samples at the set temperatures are presented in Figure 3. The curves for both sonication-aided enzymolysis and control (conventional) of the HILMP demonstrated good linear association with reaction time at set temperatures. This relationship is supported by the determination coefficient (R^2) values which were greater than 0.90 for both sonication pretreatment and Control samples. The results support our use of the primary kinetic model in determining the rate-reaction constant fit. Table 1 shows the rate-reaction constant k_{se} (sonication aided enzymolysis) and k_o (control) at varied temperature. The k_{se} and k_o were estimated from the gradient of the linear plots of ln $(V_{max} - V_t)$ against hydrolysis time.

For both control and ultrasonic-treated samples (Table 1), the k values increased (but gradually) with increases in enzymolysis reaction temperature (from 293 to 323 K). Implicit from this is that, the thermal effect on the hydrolysis reaction of HILMP was positive. According to Ma et al. (2011), such increases in k could be due to increased enzyme activity resulting from increased collisions among enzyme and substrate at increased temperature. That is, as more collisions occurred in the enzymolysis system, more combinations of the reacting molecules occurred as they bounced into each other, allowing the molecules to complete the reaction. Compared to the

TABLE 1 Rate-reaction constants and R^2_{Adj} in sonochemical treatment and conventional (control) enzymolysis at varied absolute temperatures

Enzymolysis technique	T (K)	k (min^{-1})	k_o	k_{se}	R^2_{Adj}
Conventional	293	0.0119 ± 0.0007[a]	0.0119 ± 0.0007	0	0.985
	303	0.0190 ± 0.0014[cd]	0.0190 ± 0.0014	0	0.978
	313	0.0287 ± 0.0041[b]	0.0287 ± 0.0041	0	0.924
	323	0.0332 ± 0.0038[b]	0.0332 ± 0.0038	0	0.951
Sonication-aided	293	0.0203 ± 0.0021[bcd]	0.0119 ± 0.0007	0.0084	0.958
	303	0.0260 ± 0.0038[bc]	0.0190 ± 0.0014	0.0070	0.920
	313	0.0383 ± 0.0016[a]	0.0287 ± 0.0041	0.0096	0.993
	323	0.0401 ± 0.0056[a]	0.0332 ± 0.0038	0.0069	0.927

Note: Means ± *SD* (n = 3), average values with different character set within a column implies significant difference at $p < .05$; k_o is the rate-reaction constant in control-enzymolysis, k_{se} is the constant for sonication-aided enzymolysis; k, enzymolysis reaction-rate constant.

traditional (control), the sonication-aided enzymolysis increased by 41.38%, 26.90%, 25.07%, and 17.21% at 293, 303, 313, and 323 K, respectively ($p \leq .05$). Meaning, the reaction was faster with sonication than the conventional (control enzymolysis). Similar outcomes have been reported in literature for Alcalase suspension (Ma et al., 2011) and cellulase (Subhedar & Gogate, 2014). Drawing from our study findings, it could be concluded that the sonication-treated enzymolysis, compared to control, was improved since the k values increased significantly (indicating an enhanced enzyme action).

3.3 | Effects of sonication pretreatment and control enzymolysis on thermodynamic limits

The minimum energy required to start a reaction (E_a) impacts strongly on enzymolysis reaction kinetics. That is, E_a causes stable molecules to react, and consequently impact on reaction rate (Qu et al., 2010). In most cases, E_a ranged from 40 to 400 kJ/mol. At a particular instance where E_a falls below 40 kJ/mol, the reaction goes to completion faster (Qu et al., 2013). The E_a for the sonication treated and control enzymolysis was estimated as slope from Arrhenius curves—ln(k) against 1/T (Figure 4). The values for E_a, however, were shown in Table 2. The E_a for the sonication-aided enzymolysis was 19.14, whereas that for the control was 27.55 kJ/mol. It was obvious that the sonication-aided enzymolysis required less E_a, leading to a rapid reaction among the reactants (HILMP and the enzyme). Accordingly, the sonication-aided enzymolysis caused a reduction in the E_a (30.53%, $p < .05$). It follows that the sonication-aided enzymolysis reaction was faster than the control, inference to the experimental data. In agreement, other scholars also reported decreases in E_a following ultrasonication pretreatment (Ma et al., 2011; Qu et al., 2013). Thus, sonochemical pretreatment may well advance the enzymolysis process of HILMP.

Regarding changes in the entropy (ΔS), free/Gibbs energy (ΔG), and enthalpy (ΔH), the ETS equation was utilized. The line-plot of ln(k/T) against 1/T (Figure 4b) was used to estimate the values of ΔS (i.e., intercept) and ΔH (i.e., slope). Table 2 shows the thermodynamic results. The equation $\Delta G = \Delta H - T\Delta S$ was used in computing

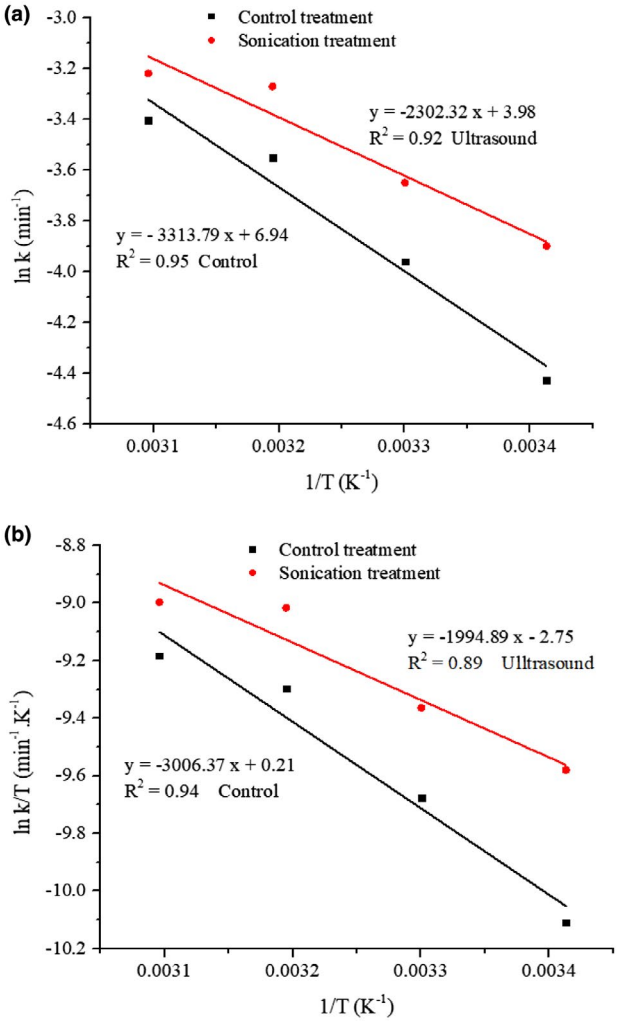

FIGURE 4 Linear-fit curves of (a) ln$k$ against 1/T, and (b) lnk/T against 1/T for sonication treatment and conventional (control) enzymolysis

the ΔG values at varying temperature. From the results, the noticeable decrease in ΔH is indicative that sonication changed the protein structure or precisely, disrupted the hydrophobic interactions,

TABLE 2 Thermodynamic limits for conventional (control) enzymolysis and sonochemical pretreatment

Enzymolysis technique	E_a (kJ/mol)	ΔH (kJ/mol)	ΔS (J/mol K)	ΔG (kJ/mol)			
				293 K	303 K	313 K	323 K
Conventional	27.55 ± 0.41[a]	24.99 ± 0.35[a]	−229.83 ± 1.25[a]	92.33	94.65	96.95178	99.25008
Sonication-aided	19.14 ± 0.23[b]	16.23 ± 0.21[b]	−254.44 ± 1.02[b]	90.78	93.34	95.886	98.43035
Decrease (%)	−30.53	−35.05	−10.71	−1.68	−1.38	−1.10	−0.83

Note: Means ± *SD* (n = 3), average values with different character set within a column implies significant difference at $p < .05$; E_a, activation energy; *H*, enthalpy; *S*, entropy; *G*, Gibbs.

leading to enhanced enzyme action through the conversion of the enzyme-substrate interaction from a lower energy level to an excited one (Cheng et al., 2017). The low value of ΔH (which is endothermic) implied lower energy cost in driving the enzymolysis reaction to completion (products). The ΔS value also decreased (approximately 10.00%, $p < .05$) when compared to the control (conventional enzymolysis), supporting the assertion that sonication-aided enzymolysis enhances enzyme-substrate interaction. Entropy (*S*), typically explains the local disordering of reactants in a reaction vessel. Thus, the reduction in ΔS (shown by negative values) could be as a result of more orderly arrangement of the reactants (HILMP and enzyme) following the sonication pretreatment, which improved the interaction among the reactants. Regarding changes in Gibbs (ΔG), a relational increase with temperature (from 293 to 323 K) was observed (Table 2). Compared to the conventional hydrolysis (control), the sonication-aided values (ΔG) decreased by 1.68%, 1.38%, 1.10%, and 0.83% when the degree of heat increased from 293 to 323 K, respectively. Further, the observed positive ΔG values shows that the enzymolysis reaction was not spontaneous, resulting in formation of stable products, relative to the starting molecules (reactants). From the current findings, it could be inferred that sonication pretreatment enhanced the enzymolysis process of HILMP, when compared to the control (conventional enzymolysis).

3.4 | UV-VS analysis

The UV-VS of the HILMP subjected to sonication and control (without sonication) are displayed in Figure 5a. The typical absorption at 278 nm may be ascribed to the combined effect of tryptophan (~279 nm), tyrosine (~275 nm) and phenylalanine (~257 or 262 nm) (Grimsley & Pace, 2004; Zhou et al., 2013).

From the spectra (Figure 5a), the absorbance intensity of HILMP pretreated by sonication was strengthened, implying that the hydrophobic groups which were characteristically buried (at the interior) got exposed. Thus, the sonication treatment might have brought about the unfolding of the HILMP molecule as a result of breaking the bonds/interactions between them, which eventually exposed more protein to increase the absorption (UV) intensity. This may support why the hydrolyzed protein concentration, rate-reaction/thermodynamic (free energy) property, and antioxidant capacity of the sonication-treated samples improved, compared to the control.

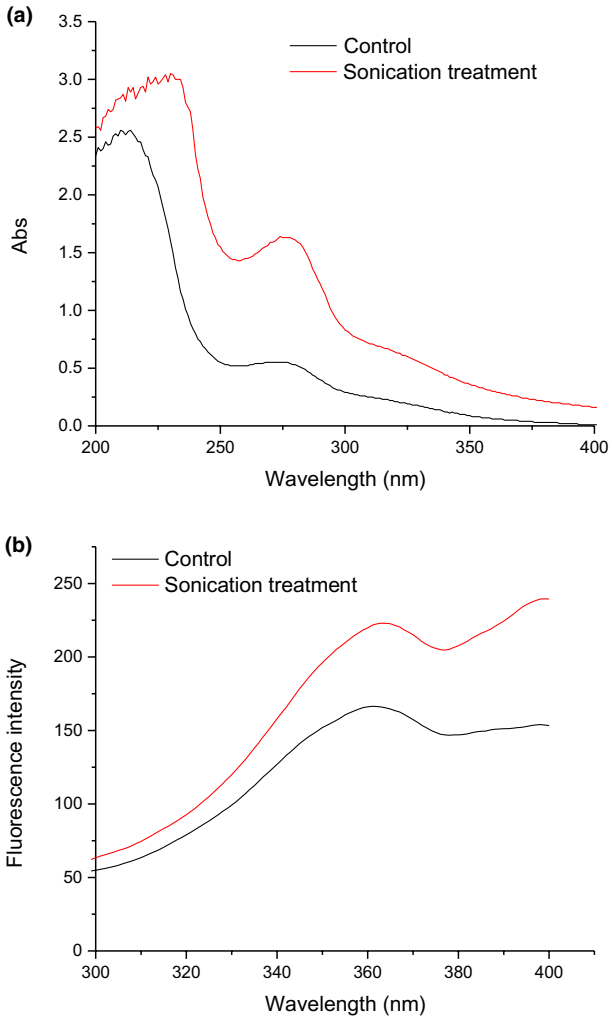

FIGURE 5 (a) UV and (b) fluorescence spectra of conventional (control) and sonication-treated HILMP

Hence, sonication treatment preceding enzymatic hydrolysis may be a better option to conventional techniques.

3.5 | Intrinsic fluorescence analysis

IFA provides spectra that aids the observation of protein attributes and conformational changes during processing and/or storage (Keerati-u-rai, Miriani, Iametti, Bonomi, & Corredig, 2012). Spectra from IFA are attributable to the residues: Tyr, Phe, and Trp (Ma et al., 2011). In most

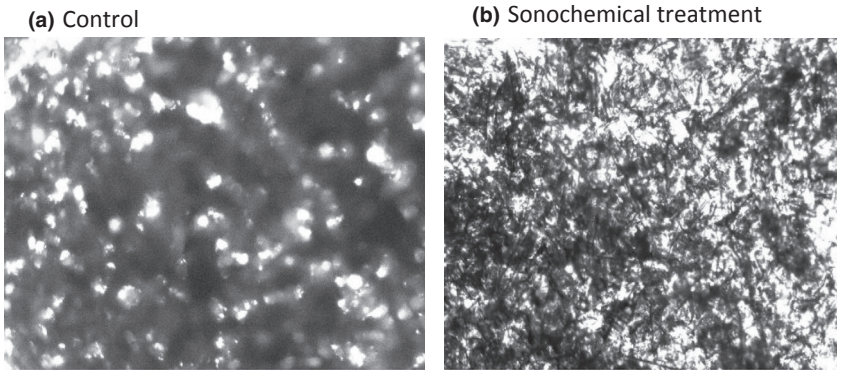

FIGURE 6 Microstructure of (a) conventional (control) and (b) sonochemically treated HILMP

instance, the Trp residues are more sensitive to the polarity of solvent. Thus, the quantum yield of Trp fluorescence could be employed in characterizing the tertiary structural alterations of proteins (Zhao, Dong, Li, Kong, & Liu, 2015). As shown in Figure 5b, the fluorescence spectra of the control HILMP and that subjected to sonication (excited at 279 nm) were indicative at 360 nm. It was observed that fluorescence peak (intensity) of the sonochemically treated HILMP was more intense than the untreated (control). Deducing from this is that, sonication pretreatment altered the protein (HILMP) conformation; and this alteration caused molecules (hydrophobic groups) in the interior to unfold/exposed, thus resulting in the intense fluorescence of HILMP. This could explain why the hydrolysis, kinetics/thermodynamic, and antioxidative attributes of the sonication-treated samples were enhanced than the conventional (control). On another observation (Figure 5b), there seems to have been a second fluorescence peak appearing at 395 nm for the sonication-treated sample. This probable shift of emission fluorescence to extended wavelengths (red-shift), compared to the control, could be indicative of fluorophores being exposed to hydrophilic environment (Keerati-u-rai et al., 2012).

3.6 | Micrographic imaging analysis

Microstructures (photomicrographs) of the HILMP pretreated with sonication, and without sonication (control) are displayed in Figure 6. The micrograph of the control showed distinctive compact or intact morphology/particles (Figure 6a). On thecontrary, spaces between particles of the sonication treated samples appear loose. Inferring from this is that, the cavitation effect produced by the sonochemical pretreatment was effective to create a sponge impact (alternating compression and expansion) resulting in the loose structure of the sonochemically treated samples; and this could further clarify why the sonicated samples demonstrated superior attributes, as regards the study variables, in the current investigation.

3.7 | Scavenging activity (SAHR) of HILMP hydrolysate samples

Hydroxyl radical ($OH^{\bullet}$) is a major oxygen-containing free radical in various disease forms, since it cable of destroying important bio-molecules (e.g., DNA, starches, fats, and proteins) (Young & Woodside, 2001), particularly when it accumulate or it is unchecked over a long period (Aluko, 2012). To control their action in humans (and/or food products), antioxidants from external sources may be applied. In the current study, the SAHR of HILMP hydrolysates (sonochemically treated and control) were thus investigated at various enzymolysis time (Figure 7). The results indicated that the inhibition capacity of the hydrolysates varied with time, and was highest at 80 min with a slight decrease at 100 min for both sonication-aided and control (conventional) enzymolysis. Deducing from this is that, the scavenging activity of HILMP hydrolysates may not have direct relationship with different/increasing enzymolysis time. Our findings shows that HILMP hydrolysate obtained at enzymolysis time 60, 80, and/or 100 min may be beneficial in preventing cell damages due to oxidative stress, particularly the sonochemically treated hydrolysates. Protein hydrolysates, due to their generally low molecular weight peptide content, exhibit potent antioxidative effect (Nwachukwu & Aluko, 2019). Thus, the increase in inhibition activity of the sonication-treated samples over the conventional (control) hydrolysates could be due

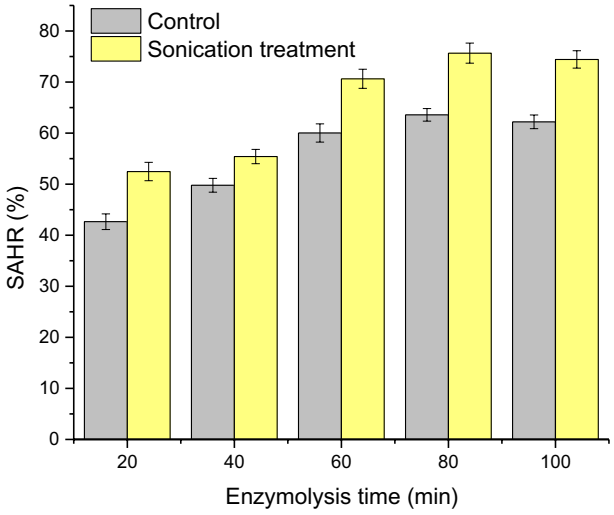

FIGURE 7 Scavenging activity (SAHR) of HILMP hydrolysates pretreated with and without sonication

to the cavitation effect of ultrasound which enhanced the reaction between the reactants (enzyme and HILMP), resulting in the release of more bioactives/proteins with lower molecular weights in solution. This was supported by the already discussed findings on UV and inflorescence spectroscopy, as well as the micrographic imaging in this study.

4 | CONCLUSIONS

Sonochemical (SFU) pretreatment of HILMP enhanced the enzymolysis reaction under different conditions (temperature and enzyme concentration). The efficiency was in line with the reduced E_a, ΔH, ΔS, and ΔG values, which improved the reactivity of the reactants (HILMP and enzyme) and led to desirable outcome. IFA, UV-VS, and MIA buttressed that sonication treatment altered the molecular arrangement/structure, and surface of HILMP for enzyme-substrate interaction; which led to improved enzymolysis reaction as well as antioxidant capacity of the hydrolysates. Sonication pretreatment of HILMP can positively impact enzymolysis rate, stability of reaction products, structure, and bioactivity; and may be beneficial to industry in the development of new products involving enzymolysis of edible insect (*H. illucens*) meal protein.

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CONFLICT OF INTEREST

The authors declare no conflict of interest for this article.

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