

Ultrasound assisted enzymatic protein hydrolysis by defatted *Protaetia brevitarsis* larvae powder

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Introduction

Edible insects are gaining popularity as future food items because of low production costs and high nutrition. Furthermore, they are a good source of protein from a nutritional standpoint and a good source of lipids for biodiesel production (Motoki et al., 2020). In comparison to livestock (e.g., cattle, swine, and poultry), which are the most consumed sources of protein today, edible insects have an advantage in that they can be mass-produced on a small patch of land and produce low levels of methane gas (Grau et al., 2017). Beetles, bees, ants, grasshoppers, and crickets are the most consumed edible insects. These insects can be used in each development stage, such as egg, larvae, pupae, and imagoes (Van der Spiegel et al., 2013). Nonetheless, edible insects have an awkward appearance for consumers to accept, and it is a relatively foreign concept for most people to consider as a food item. Therefore, for insects to be more readily used as food, necessary elements must be extracted, pulverized, and hydrolyzed to hide their appearance (Mintah et al., 2019).

Currently, ten insect species are officially recognized as edible food ingredients in Korea, including *Protaetia brevitarsis* larvae, *Tenebrio molitor*, and *Gryllus bimaculatus* (Kim et al., 2025). Among them, *Protaetia brevitarsis* (PB) larvae contain approximately 58% protein, 18% fat, and 17% carbohydrates. It has been reported that they possess various beneficial properties, including high-quality protein, antioxidant activity, antithrombotic efficacy, neuroinflammation-inhibitory effect, and liver function improving effect (Choi et al., 2019; Lee et al., 2017).

Proteins function as a nutrient and gain other biologically active functions by breaking down into small amino acid units, which break up the peptide bonds during the digestive process. In the process of the protein breaking down, free amino acids, oligopeptides, and low molecular protein are the most representative materials that have biologically active

functions (Hwang et al., 2001). Further, some proteins and peptides have been reported to increase antioxidant activity within the body and have anti-inflammatory and anti-bacterial properties (Cicero et al., 2017; Li-Chan, 2015). Additionally, protein hydrolysates within food products may increase their shelf life (Di Bernardini et al., 2011). Some protein hydrolysates consist of different mixtures of various amino acids and increase the umami and saltiness. Thus, they could be used as sodium substitutes (Zhou et al., 2021).

The production of peptides with biologically active functions is usually created through enzymes' hydrolysis. Enzyme selection can be chosen based on the purpose, and a positive quality of the manufactured products is that they do not have by-products except for amino acids. Therefore, a positive quality is that they do not create poisonous compounds such as furfural, phenolic, and weak acids (Sasmal and Mohanty, 2018). Another positive quality of enzyme hydrolysis is that it has different reaction conditions from acid or alkaline hydrolysis (Szentner et al., 2019).

However, the negative aspect of hydrolysis using enzymes is that peptide production may be limited based on protein structure (Kim and Imm, 2018). To solve this, recently, there have been technological attempts to create changes in protein before hydrolysis by using external heat, physical force, pH, and ions. In particular, ultrasound technology has been widely utilized as a non-thermal processing method, effectively preserving the original quality and nutritional components of food products (Ma et al., 2016). Furthermore, it creates cavitation, which may incite the protein's physical and chemical changes (Alizadeh and Aliakbarlu, 2020). Moreover, the mechanical shear generated by acoustic cavitation provides key operational advantages by facilitating enzymatic access to solid surfaces, enhancing extraction yield, and improving the aqueous solubility of target compounds (Mintah et al., 2019).

Therefore, this study applied ultrasound-assisted enzymatic hydrolysis to enhance the potential of edible insects as a sustainable protein resource. Furthermore, the physicochemical properties of the insect proteins were evaluated as a function of ultrasound treatment and enzymatic hydrolysis duration.

Materials and Methods

Materials

Enzymes were purchased from DAEJONGZYMES Co., Ltd. (Seoul, Korea). 2,2-Diphenyl-1-picrylhydrazyl was obtained from Alfa Aesar (Massachusetts, USA). Hydrogen peroxide solution, trichloroacetic acid, potassium persulfate, and iron(III) chloride, 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, ($\pm$)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, and 2,4,6-Tris (2-pyridyl)-s-triazine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid and iron(II) sulfate heptahydrate were purchased from Daejung chemicals & metals Co., Ltd (Siheung, Korea) and Wako Pure Chemical Industries, Ltd (Osaka, Japan), respectively.

Sample preparation

The third instar larval stage of PB larvae were obtained from Jeju insect farming association corporation. PB larvae were sacrificed by blanching (95 °C, 1 min), vacuum-packaged, and stored in a freezer at -20 °C until use. Then, PB larvae were hot-air dried at 60 °C (PS-100C, Shiniltech, Gimhae, Korea) to a moisture content of $15 \pm 5\%$ or lower and ground to a fine powder using a grinder (SHMF-3500G, Hanil, Seoul, Korea). The

resulting powder was then passed through a 40-mesh sieve. The insect sample was defatted using *n*-hexane. The powder was mixed with *n*-hexane at a ratio of 1:5 (w/v) and stirred for 18 h. Every 6 h, the mixture was centrifuged at $3,134 \times g$ for 10 min (Labo-Gene 1248R, GYROZEN Co., Ltd., Daejeon, Korea), and the supernatant was replaced with fresh *n*-hexane. The defatted samples were then dried under a fume hood to evaporate the residual *n*-hexane.

Ultrasonic treatment

The defatted insect powder was suspended in 10 mM phosphate buffer (pH 7.4) and hydrated during stirring for 10 min at room temperature. The suspension was divided into two groups: an untreated control and an ultrasonicated treatment. Sonication was performed at 750 W and $20 \text{ kHz} \pm 50 \text{ Hz}$ using a pulse cycle of 15 s on and 10 s off for a total duration of 15 min (VCX750, Sonic & Materials, Inc., Newtown, CT, USA).

Preparation of protein hydrolysates

Prior to enzymatic hydrolysis, both control and ultrasound-treated sample were heated in a water bath at 90°C for 20 min to inactivate endogenous enzymes. Alcalase or Neutrase was then added at an enzyme-to-substrate ratio of 1% (w/w). Hydrolysis was carried out at 55°C and 150 rpm for 0–24 h. After hydrolysis, heat treatment was performed at 90°C for 20 min for complete enzyme inactivation. The hydrolysate was cooled to room temperature and centrifuged at $3,134 \times g$ for 20 min. To fractionate low-molecular-weight peptides, the supernatant was passed through Amicon® Ultra centrifugal filter units with a 3 kDa molecular weight cut-off (MWCO) (Merck Millipore,

Billerica, MA, USA). The resulting hydrolysates were lyophilized and stored at -80°C until analysis. The hydrolysis yield was calculated as follows:

$$\text{Yield (\%)} = \frac{\text{Sample after processing weight (g)}}{\text{Sample before processing weight (g)}} \times 100$$

pH

The pH of the hydrolysates was determined digital pH-meter (S470 SevenExcellenceTM, Mettler Toledo, Zurich, Switzerland). All measurements were obtained in triplicate.

Degree of hydrolysis

The degree of hydrolysis (DH) was calculated as described by Hoyle and Merritt (1994), with some modification. The hydrolyzed suspension was centrifuged at $3,134 \times g$ for 10 min. Subsequently, 1 mL of supernatant was mixed with 1 mL of 20% trichloroacetic acid (TCA) and centrifuged at $3,134 \times g$ for 10 min. The BCA assay was used to determine the protein content in the TCA-soluble supernatant. The degree of hydrolysis was estimated as follows:

$$\text{DH(\%)} = \frac{\text{20\% TCA soluble protein content in the sample}}{\text{Total protein content in the sample}} \times 100$$

Protein solubility

Protein solubility of the hydrolysates was measured as described by Maryia et al. (2019). Briefly, sample (0.2 g) was dispersed in distilled water (10 mL), and the pH adjusted within the range of 3.0 to 9.0 using 1 M NaOH or 1 M HCl. The dispersion was stirred on a shaker for 1 h at 40°C . The mixture was then centrifuged at $3,134 \times g$ for 20

min, and the supernatant was collected to determine the protein concentration using the BCA assay. Protein solubility was calculated as follows:

$$\text{Protein solubility (\%)} = \frac{\text{Protein content in supernatant}}{\text{Protein content in hydrolysates}} \times 100$$

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

For the measurement of *in vitro* antioxidant activities, the protein hydrolysates were diluted in distilled water to a concentration of 100 mg/mL. The DPPH radical scavenging activity of the hydrolysates was measured as described by Mensor et al. (2001) with minor modifications. Briefly, 2 mL of sample was mixed with 800 µL of 0.3 mM DPPH in 95% ethanol. The mixture was incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using a microplate reader (EpochTM, BioTek Instruments, Inc., Winooski, VT, USA). Ascorbic acid was used as a positive control. The DPPH radical scavenging activity (%) was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = \left[1 - \frac{A_1}{A_0} \right] \times 100$$

where A_1 is the absorbance of the sample mixture, and A_0 is the absorbance of the blank control (i.e., DPPH solution mixed with distilled water).

ABTS radical scavenging activity

The ABTS radical scavenging activity of the hydrolysates was measured as described by Pellegrini et al. (1999), with some modifications. A 7 mM ABTS solution was mixed with 140 mM potassium persulfate and incubated in the dark for 24 h. Prior to use, the absorbance of the solution at 734 nm was adjusted to 0.70 ± 0.02 with absolute ethanol. Then, 50 µL of the sample and 1 mL of the diluted ABTS solution were mixed and kept

in the dark for 2.5 min. The absorbance of all samples was measured. The activity was expressed as mg Trolox equivalents/mL based on calibration curve prepared with Trolox.

Ferric ion reducing antioxidant power (FRAP assay)

The ferric reducing antioxidant power of the hydrolysates was measured as described by Benzie and Strain (1996) with minor modifications. The FRAP reagent was prepared by mixing 300 mM sodium acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, and 20 mM iron (III) chloride hexahydrate in a 10:1:1 (v/v/v) ratio. A 3 mL aliquot of FRAP reagent, 300 μ L of distilled water, and 100 μ L of the sample were mixed and warmed at 37 °C in a water bath for 4 min. The absorbance was measured at 593 nm. The ferric reducing power was expressed as mg iron (II) sulfate heptahydrate equivalents/mL using a calibration curve established with iron (II) sulfate heptahydrate.

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of the hydrolysates was determined as described by Yu et al. (2017) with minor modifications. Briefly, 20 μ L of the sample, 100 μ L of sodium phosphate buffer (pH 7.4), and 20 μ L of 1 mM H₂O₂ were mixed and incubated at 37 °C in a water bath for 4 min. Subsequently, 30 μ L of 1.25 mM ABTS and 30 μ L of 1 U/mL peroxidase were mixed and incubated at 37 °C for 10 min. The absorbance at 405 nm. The activity was expressed as mg Trolox equivalents/mL using a Trolox standard calibration curve.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using polyacrylamide gels at concentrations of 12.5% and 16.5%. The hydrolysate samples were centrifuged at $3,134 \times g$ for 10 min. The supernatant was mixed with 2% SDS and 6× sample loading dye, followed by heating at 95 °C for 5 min. Two protein molecular weight markers were used as standards: a 5–245 kDa marker (EZ-Perfect Marker Plus, DOGEN, Seoul, Korea) and 1–29 kDa low molecular weight marker (EzStandard LMW, ATTO, Tokyo, Japan). Electrophoretic separation was performed using a PageRun Ace system (WSE-1150, ATTO Co., Tokyo, Japan) at a constant current of 21 mA for 120 min. Running buffer consisted of 100 mM Tris, 0.1% SDS, and 100 mM Tricine. After electrophoresis, the gels were stained using Coomassie Brilliant Blue R-250 and destained with distilled water.

Contact angle

The contact angles of the hydrolysate samples were determined using a contact angle meter (Phoenix-150, SEO Co., Suwon, Korea) and Surfaceware software. For the measurements, glass cover slides were immersed in the hydrolysate solution (2% w/v) for 1 h and subsequently air-drying at 40 °C for 24 h. A drop of deionized water (approximately 6 µL) was gently deposited onto the surface of the hydrolysate-coated glass cover slide using a syringe. Each sample was measured at three different locations and the contact angle of each droplet was recorded every second for 15 s. The average values were reported.

Amino acid composition

Samples pretreated with sonication were collected at specified time intervals during enzymatic hydrolysis, centrifuged ($3,134 \times g$, 10 min), and the supernatant was used for

amino acid analysis. The amino acid composition was determined using an amino acid analyzer (L-8900, HITACHI, Tokyo, Japan) according to the official method of the Korea Food and Drug Administration (Ministry of Food and Drug Safety, 2020).

Statistical analysis

All experiments were carried out in triplicate, and the data are expressed as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using Minitab 18 (Minitab Inc., State College, PA, USA). Significant differences between groups were determined by one-way ANOVA with Tukey's multiple comparison test ($p < 0.05$)

Results and Discussion

Visual appearance and yield

The visual appearance of PB powder solutions varied depending on whether ultrasound was applied prior to enzymatic hydrolysis (Fig. 1). Before ultrasound treatment, the agitated mixture exhibited a darker color owing to the dissolved powder, with a distinct separation layer between the solvent and insoluble solid residues. In contrast, following ultrasound application, the solution became turbid (murky) and the boundary of insoluble components became less distinct, indicating an enhanced degree of dispersion. Ultrasound technology increases the solubility of insoluble substances that have solidified, precipitates the chemical reaction, and promotes dispersion, surface activity, and reactivity. These visual changes are attributed to increased solubility induced by physical forces—such as acoustic cavitation—acting on the insoluble particles (Stefanović et al., 2017). Fig. 2 shows the visual appearance of the hydrolysates after enzymatic hydrolysis and subsequent ultrafiltration using a 3 kDa molecular weight cut-off (MWCO)

membrane filter. Overall, the hydrolysates exhibited a dark coloration, likely influenced by the intrinsic pigments of the insect powder. Following 3kDa membrane filtration, the permeate showed increased clarity (translucency) and a noticeably lighter color. Variations in color were observed between Alcalase and Neutrase treatments, suggesting that the intrinsic color of each enzyme formulation also affected the hydrolysates (Jeon et al., 2016).

Table 1 shows the overall yields at different processing stages. The primary hydrolysis yield ranged from 89.19% to 91.25% after removing unreacted insoluble residues. Following 3 kDa fractioning, the yield decreased to 66.35–77.68%, as higher molecular weight peptides were retained by the membrane filter. The final yield after freeze-drying was 2.38–2.84%. The research verified that the yield without moisture through freeze-drying. There was no significant tendency observed in relation to the enzyme type or ultrasound treatment. Although ultrasound enhances extractability by disrupting microscopic matrix structures and enzyme efficiency varies by type, the extended reaction time (24 h) likely allowed hydrolysis to reach near-complete equilibrium, masking noticeable differences between treatments.

pH

The pH values of the PB larvae protein hydrolysates are shown in Table 1. A significant decrease in pH was observed following enzymatic hydrolysis. Prior to hydrolysis, the initial pH was 7.30 ± 0.07 , which subsequently decreased to 4.18–4.76 after hydrolysis. This reduction is consistent with previous reports indicating that proteolysis cleaves proteins into peptide units, exposing acidic carboxyl groups ($-\text{COOH}$) as well as releasing protons, thereby lowering the solution pH (Franco and Filho, 2011; Jang et al., 2008).

Additionally, the acidification can be attributed to the accumulation of lactic acid,

which is produced via the breakdown of glycogen—the primary carbohydrate storage molecule in insects (Miyazaki and Park, 2020). Among the treatments, hydrolysates generated with Alcalase exhibited a lower pH compared to those produced with Neutrase. This difference is likely due to the higher activity of Alcalase 2.4L (2.4 AU/g) relative to Neutrase 0.8L (0.8 AU/g), resulting in a higher degree of cleavage and exposure of acidic groups. A similar trend was reported by Chung et al. (2006).

Degree of hydrolysis

The degree of hydrolysis (DH) is defined as the percentage of cleaved peptide bonds relative to total number of peptide bonds in a protein substrate (Navarrete del Toro and García-Carreño., 2002). Based on previous studies demonstrating high hydrolytic efficiency and antioxidant activity in protein hydrolysates (Lee et al., 2017), Alcalase and Neutrase were selected to evaluate the hydrolysis characteristics of PB larvae hydrolysates under different pretreatment conditions.

The progression of DH in PB larvae hydrolysates during enzymatic hydrolysis is presented in Fig. 3. For all treatments, the DH increased rapidly during the initial 0.5 h and continued to rise steadily up to 8 h, after which no significant changes were observed until the termination of the reaction. This tendency is consistent with previous research (Cho et al., 2019; Jang et al., 2019; Lee et al., 2017). This plateauing effect is primarily attributed to the gradual reduce in pH during hydrolysis, which alters the active site conformation and activity of the enzymes (Komoda and Matsunaga, 2015). Furthermore, highly reactive peptide bonds are rapidly cleaved during the initial phase, whereas less accessible or lower-affinity bonds are hydrolyzed at a much slower rate (Kim, 2010).

Specifically, Alcalase hydrolysates reached a DH plateau as early as 0.5 h, whereas Neutrase hydrolysates showed no statistically significant differences () after 8 h. This

indicates that Alcalase achieved optimal hydrolysis substantially faster than Neutrase, suggesting that the extended reaction time required for Neutrase may limit its efficiency for industrial-scale processing (Suh et al., 2017). These results are largely driven by the higher specific activity of Alcalase (2.4 AU/g) compared to Neutrase (0.8 AU/g), as well as their distinct catalytic mechanisms and substrate specificities (Kim, 2010). Alcalase, a broad-specificity serine endopeptidase, preferentially cleaves peptide bonds adjacent to hydrophobic and aromatic amino acid residues, whereas Neutrase is a zinc-dependent metalloendopeptidase with a narrower substrate specificity. These mechanistic differences likely account for the faster hydrolysis rate and higher DH observed with Alcalase. In the ultrasound treatment group, the initial DH increment during 0–0.5 h was noticeably higher than that in non-sonicated control groups. Ultrasonic irradiation induces acoustic cavitation—the rapid formation, growth, and violent collapse of microbubbles within the liquid medium. The resulting localized shear forces and physical impacts disrupt non-covalent interactions, such as hydrogen bonding and electrostatic attractions, facilitating the partial unfolding of protein structures (Marciniak et al., 2018; Pino et al., 2020). Moreover, compact native structures (such as globular and α -helix) undergo conformational transitions into more open coiled or β -sheet-like structures, making the substrates more accessible to the enzymes (Alizadeh and Aliakbarlu, 2020; Garcia LA et al., 2016; Ochoa-Rivas et al., 2016).

Thus, ultrasound-induced cavitation disrupts the dense secondary and tertiary structures of proteins and exposes internal peptide bonds that are inaccessible to enzymes, thereby promoting enzymatic attack rather than acting through a direct extraction mechanism (Umego et al., 2021). This structural unfolding effect is considered the primary factor contributing to the accelerated DH observed under ultrasound-assisted

conditions, supporting the practical value of ultrasound as a pretreatment for enzymatic hydrolysis.

Protein solubility

The solubility of protein is an important category that intersects with functional properties such as emulsification, foaming, and gelling. It is determined by the interactions between protein-protein, protein-water, protein-ion, and ion-water (Trevino et al., 2008).

The pH-dependent solubility profiles of the PB larvae hydrolysates are shown in Table 2. Although insect proteins are generally reported to exhibit minimum solubility near their isoelectric points (pI, pH 4.0–6.4) (Bußler S et al., 2016; Foo et al., 2006), the generated hydrolysates maintained remarkably high solubility (84.36–91.60%) across all evaluated pH ranges, including the pI range. After the hydrolysis, protein is broken down to low molecular peptides, which is align well with the DH trends (Fig. 3) and molecular weight distributions (Fig. 8). In addition, subsequent ultrafiltration using a 3 kDa membrane filter effectively enriched low-molecular-weight peptides, thereby further enhancing overall solubility. These findings according to the results of Zhang and Romero (2020), who observed a direct positive correlation between the degree of hydrolysis and protein solubility in soy protein hydrolysates.

Comparing the enzymes used, Alcalase-treated hydrolysates exhibited higher solubility overall than those treated with Neutrase. This observation is consistent with previous findings indicating that Alcalase produces hydrolysates with superior solubility and cleavage efficiency compared to other proteases (Ahmadifard et al., 2016; Lee et al., 2017). This difference is mainly driven by the higher catalytic activity and broad substrate specificity of Alcalase, a potent endopeptidase that extensively cleaves peptide bonds to

generate a high proportion of low-molecular-weight peptides and free amino groups, ultimately contributing to enhanced solubility.

DPPH radical scavenging activity

The DPPH radical scavenging activities of the PB larvae hydrolysates fractionated below 3 kDa are presented in Fig. 4. Ascorbic acid (0.01 mg/mL) was employed as a positive control for comparison. The non-hydrolyzed protein ($42.19 \pm 2.12\%$) exhibited lower scavenging activity, whereas enzymatic hydrolysis significantly enhanced the antioxidant capacity, showing higher activities for Alcalase ($50.47 \pm 0.52\%$) and Neutrase ($47.97 \pm 1.01\%$) hydrolysates than for the ascorbic acid ($46.15 \pm 2.08\%$). This enhancement highlights the efficacy of enzymatic cleavage in releasing bioactive peptides from PB larvae proteins, consistent with observations in *Gryllodes sigillatus* hydrolysates where enzymatic hydrolysis markedly elevated DPPH radical scavenging potential.

Compared to the control group, ultrasound pretreatment significantly increased ($p < 0.05$) the DPPH radical scavenging activity from 42.19% to 46.15%. When ultrasound was combined with enzymatic hydrolysis, slight increases in DPPH radical scavenging activity were observed—from 50.47% to 50.73% for Alcalase and from 47.97% to 48.40% for Neutrase—although these changes were not statistically significant ($p > 0.05$). Ultrasound-induced cavitation promotes partial structural unfolding of proteins, exposing hydrophobic amino acid residues to the molecular surface (Pino et al., 2020). This is because ultrasound-induced structural unfolding exposes hydrophobic residues from the protein interior to the surface, thereby increasing their accessibility to free radicals and enhancing antioxidant activity (Chalamaiah et al., 2012). Furthermore, extensive enzymatic cleavage breaks peptide bonds to generate low-molecular-weight peptides, which are known to play a key role in radical scavenging.

The relationship between molecular weight and DPPH radical scavenging activity observed in this study aligns with previous literature on animal-derived protein hydrolysates. Kim et al. (2013b) reported that sub-3 kDa peptide fractions from Jeju horse leg bone hydrolysate (20 mg/mL) exhibited superior DPPH radical scavenging activity (0.14 mM Trolox equivalent) compared to higher molecular weight fractions. Similarly, low-molecular-weight pork skin extracts (50 mg/mL) demonstrated 91.97% radical scavenging activity, equivalent to 1 mg/mL of vitamin C, outperforming fractions above 3 kDa (Kim et al., 2013a). These findings reinforce that ultrafiltration-derived low-molecular-weight peptides from animal and insect proteins possess superior DPPH radical scavenging activity.

Among the enzymes evaluated, Alcalase hydrolysates consistently showed higher scavenging activity than Neutrase hydrolysates. This result is consistent with the findings of Yang et al. (2013) on silkworm protein hydrolysates, where Alcalase exhibited the highest DPPH radical scavenging activity among various proteases. Although both DPPH and ABTS assays evaluate radical scavenging activity via electron transfer or hydrogen donation, DPPH measures lipophilic free radical scavenging, whereas ABTS evaluates hydrophilic/lipophilic cationic radical neutralization (Meir et al., 1995). Variations in outcomes between these methods are primarily attributed to differing degrees of interaction between the substrate and the reactive species (Lee et al., 2012).

ABTS radical scavenging activity

The ABTS radical scavenging assay measures antioxidant capacity through the reduction of blue-green ABTS radical cations generated via potassium persulfate oxidation (Lee et al., 2017). Although the required radical generation step necessitates

prior preparation, the assay offers rapid spectrophotometric measurement and excellent stability across a broad pH range (Yoo et al., 2007).

The ABTS radical scavenging activities of PB larvae hydrolysates are presented in Fig. 5, with Trolox (0.02 mg/mL) used as a positive control (74.82%). All enzymatic hydrolysates exhibited a significant increase in radical scavenging capacity—more than 4-fold higher than that of the control ($22.53 \pm 0.79\%$) and ultrasound-pretreated control ($25.64 \pm 1.87\%$) groups—while also significantly outperforming the positive control ($p < 0.05$). Similarly, ultrasound pretreatment prior to hydrolysis showed no significant enhancement ($p > 0.05$) in ABTS radical scavenging activity compared to hydrolysis alone. This aligns with findings by Mintah et al. (2019) on *Hermetia illucens* larvae, where ultrasound pretreatment alone showed minimal impact on ABTS radical scavenging activity. Ultrasonic cavitation facilitates protein structural expansion and exposes hydrophobic amino acid residues, thereby modifying substrate accessibility for enzymatic cleavage (Pino et al., 2020). However, the lack of a distinct sonication effect in the final hydrolysates in this study is likely; attributable to the extended hydrolysis duration, which allowed enzymatic cleavage to reach a maximum plateau regardless of pretreatment.

Overall, the dramatic elevation in ABTS radical scavenging activity following enzymatic cleavage strongly correlates with the DH trends (Fig. 3), as previously reported by Kumar et al. (2016). Enzymatic cleavage converts intact insect proteins into short-chain peptides and free amino acids, increasing the exposure of electron- and proton-donating functional groups (Jang et al., 2019). Differences in antioxidant capacity among individual hydrolysates are primarily governed by the specific sequence, composition, and structural positioning of hydrophobic and aromatic amino acid residues generated during proteolysis.

411

412 **Ferric Ion Reducing Antioxidant Power (FRAP assay)**

413 Unlike the DPPH and ABTS assays, which primarily rely on radical scavenging via
414 hydrogen or electron transfer, the FRAP assay evaluates antioxidant activity by
415 measuring the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) through a single
416 electron transfer mechanism (Johnson et al., 2007). This colorimetric method measures
417 the formation of an intense blue Fe^{2+} -TPTZ complex at 593 nm, offering high
418 repeatability and analytical simplicity (Benzie and Strain, 1996).

419 The FRAP values of the PB larvae hydrolysates are shown in Fig. 6. The control and
420 ultrasound treatment groups exhibited low ferric reducing capabilities of 10.6 μM
421 FeSO_4/mg and 14.4 μM FeSO_4/mg , respectively, with no statistically significant
422 difference ($p > 0.05$). Furthermore, ultrasound pretreatment prior to enzymatic hydrolysis
423 did not induce a significant enhancement in reducing power ($p > 0.05$). Although Mintah
424 et al. (2019) observed an increase in FRAP values following sonication, the lack of a
425 prominent ultrasound effect in this study suggests that sonication parameters (e.g., power
426 intensity and duration) and the extent of subsequent enzymatic hydrolysis strongly
427 modulate the overall exposure of electron-donating residues.

428 In contrast to the DPPH and ABTS assay results, Neutrase hydrolysates displayed
429 significantly higher reducing power (46.4 μM FeSO_4/mg) than Alcalase hydrolysates
430 (35.9 μM FeSO_4/mg) ($p < 0.05$). This can differ based on the antioxidant activity
431 measuring methods, and the peculiarities of the substrate of enzymes created differences
432 in amino acid arrangements or composition (Pihlanto, 2006). Jang et al. (2019) previously
433 evaluated the antioxidant activities of sub-3 kDa peptide fractions from various edible
434 insects, reporting a FRAP value of 84 μM FeSO_4/mg for PB larvae Alcalase hydrolysates
435 —higher than the 35.9 μM FeSO_4/mg observed in this study. Furthermore, sub-3 kDa

fractions from *Allomyrina dichotoma* (rhinoceros beetle) larvae and *Tenebrio molitor* (mealworm) larvae exhibited FRAP values of 30.17 $\mu\text{M FeSO}_4/\text{mg}$ and 15.95 $\mu\text{M FeSO}_4/\text{mg}$, respectively. Despite using identical ultrafiltration cut-offs ($< 3 \text{ kDa}$), variations in insect species, rearing or processing conditions, and pretreatment methods contribute to differences in peptide profiles and resultant ferric reducing capabilities.

Hydrogen peroxide scavenging activity

Hydrogen peroxide (H_2O_2), a major non-radical reactive oxygen species (ROS), induces oxidative damage to biomolecules such as proteins and lipids, leading to cellular dysfunction and accelerated tissue aging (Martindale and Holbrook, 2002). The H_2O_2 scavenging capacities of PB larvae hydrolysates are shown in Fig. 7. Compared to the control group (2.08 mg TE/g), both ultrasound treatment alone (2.56 mg TE/g) and ultrasound-assisted enzymatic hydrolysis (2.87–2.99 mg TE/g) resulted in a significant increase ($p < 0.05$) in H_2O_2 scavenging activity.

Similarly, Zhang et al. (2021) also demonstrated that ultrasonic pretreatment significantly improves the radical scavenging activity of protein hydrolysates. One reason may be attributed that acoustic cavitation disrupts the compact tertiary structure of native proteins, increasing the exposure of hydrophobic amino acid residues that contribute to ROS neutralization (Ajibloa et al., 2013; Zhang et al., 2021). In addition, sonication promotes the exposure of reactive sulfhydryl ($-\text{SH}$) groups. Chandrapala et al. (2012) reported an increase in free sulfhydryl content in milk serum proteins following ultrasound treatment, attributing this change to physical shear forces that expose thiol groups. Generally, thiol-containing amino acid residues (e.g., cysteine) play a critical role in scavenging H_2O_2 and maintaining intracellular redox homeostasis (Je et al., 2009; Kinnula et al., 2004). Furthermore, the sulfhydryl group is a coenzyme of amino acids

with hydrophobic qualities and has high reactivity for free radicals, which has good scavenging capabilities in oxidative environments (Gracia et al., 2016).

Enzymatic hydrolysates treated with ultrasound (both Alcalase and Neutrase) exhibited significantly higher ($p < 0.05$) H₂O₂ scavenging activity than the group treated with ultrasound alone. However, Alcalase hydrolysates showed no significant difference ($p > 0.05$) in H₂O₂ scavenging activity, regardless of ultrasound pretreatment, suggesting that extensive enzymatic cleavage by Alcalase alone generates sufficient small peptides and exposed active residues to reach a scavenging efficiency plateau.

Electrophoresis patterns (SDS-PAGE)

SDS-PAGE was conducted to evaluate the molecular weight (MW) distribution and hydrolysis kinetics of PB larvae proteins treated with Alcalase following ultrasound pretreatment, which were selected based on previous results (Fig. 8).

PB larvae protein (unhydrolyzed control) exhibited distinct bands spanning a broad MW range from >245 kDa down to 5 kDa. Upon Alcalase hydrolysis, these high-MW bands rapidly disappeared, giving rise to low-MW peptide fragments below 14.3 kDa. Both the electrophoresis profiles and the DH trends (Fig. 3) confirm effective proteolysis, which proceeded rapidly as early as 0.5 h after enzyme addition.

In the native substrate profile prior to ultrasound treatment, prominent protein bands were detected at 100–140 kDa, 35–60 kDa, 20 kDa, and 10 kDa. Bands in the 14–35 kDa range are likely attributable to insect cuticle protein (Anderson et al., 1995). Protein bands observed above 35 kDa may correspond to functional insect proteins, including melanin-inhibiting protein (43 kDa), β -glucosidase (59 kDa), trypsin-like protein (59 kDa), and melanin-engaging protein (85 kDa) (Cho et al., 1999; Ferreira et al., 2001; Prabhakar et al., 2007; Zhao et al., 2005). Furthermore, high MW bands in the 100–140 kDa range

likely represent vitellogenin-like lipoglycoproteins (160 kDa) synthesized in larval fat bodies (Lee et al., 2000).

To better resolve low-MW peptides appearing in the 5–10 kDa range during enzymatic cleavage, higher-concentration acrylamide gels were utilized. With extending hydrolysis duration, the staining intensity of peptide bands in the 3.5–14.3 kDa region progressively decreased, indicating further breakdown into sub-3 kDa fragments or free amino acids.

In the ultrasound control group, overall band intensity increased across all MW regions, accompanied by high-MW bands (>245 kDa) and dense staining within the loading wells. This was similar to previous findings where physical pretreatments (e.g., ultrasound, thermal processing, and microwave exposure) enhanced protein solubility and structural dispersion, yielding darker electrophoretic bands (Ahmadifard et al., 2016; Hall and Liceaga, 2020; Zielinska et al., 2017). Compared to non-sonicated samples, ultrasound pretreatment improved the solubility of high-MW proteins, facilitating subsequent enzymatic action. Immediately upon Alcalase addition, rapid cleavage shifted the entire protein population toward lower MW ranges, similar to previous studies (Jang et al., 2019; Jin et al., 2020; He et al., 2021). Based on the research conducted by Yi et al. (2013), regarding water-soluble protein extracts that conducted SDS-PAGE, only proteins with < 95 kDa appeared. Therefore, it is estimated that enzyme hydrolysis helps in the creation of water-soluble proteins.

Contact angle

The contact angle (θ) was evaluated to determine the surface wettability and amphiphilic characteristics of the protein samples. A contact angle of $\theta < 90^\circ$ indicates high surface wettability and hydrophilicity, making the particles suitable for stabilizing oil-in-water (O/W) emulsions. Conversely, $\theta > 90^\circ$ indicates a hydrophobic surface

favorability for water-in-oil (W/O) emulsions (Sharkawy et al., 2021). The dynamic contact angles of the non-hydrolyzed and 0.5 h Alcalase hydrolysate are shown in Fig. 9. At 1 s post-deposition, the contact angles of the non-hydrolyzed control and 0.5 h hydrolysate were 52.95° and 34.57°, respectively. Over time, both values progressively declined, reaching 25.05° and 12.09° at 15 s due to droplet spreading and surface adsorption. Although both samples exhibited hydrophilic properties ($\theta < 90^\circ$), the Alcalase hydrolysate demonstrated a distinctly lower contact angle, indicating enhanced surface wettability. These values suggest that the Alcalase enzymatic hydrolysis increased the hydrophilicity of the hydrolysates derived from PB larvae protein. The enhanced affinity for water is primarily attributed to the progressive exposure of hydrophilic amino acid residues and the generation of low-molecular-weight (M_w) bioactive peptides during proteolysis (Table 3). Consequently, these results demonstrate that PB larvae protein hydrolysates possess favorable surface properties for application as hydrophilic emulsifiers in food emulsion systems.

Amino acid composition

The functional and nutritional properties of protein hydrolysates are largely determined by their primary structure and amino acid profiles. The amino acid composition of PB larvae protein hydrolysates, prepared via Alcalase treatment following ultrasonic pretreatment across different hydrolysis time points, is shown in Table 3.

The PB larvae protein contained all essential amino acids (EAAS) except for tryptophan. In the 24 h hydrolysate, the six most abundant amino acids were Glu (216.44 mg/100 g), Asp (142.27 mg/100 g), Pro (141.13 mg/100 g), Tyr (119.25 mg/100 g), Gly (97.35 mg/100 g), and Lys (95.94 mg/100 g). Total hydrophobic amino acids (HAAs) and branched-chain amino acids (BCAAs) accounted for 633.81 mg/100 g and 233.02 mg/100

g, respectively. The concentrations of all individual amino acids significantly increased with extending hydrolysis duration ($p < 0.05$).

Since EAA content is a fundamental indicator of nutritional quality, the significant elevation of EAAs (including lysine, leucine, valine, isoleucine, threonine, phenylalanine, and histidine) demonstrates that ultrasonic-assisted enzymatic hydrolysis markedly enhances the nutritional value of PB larvae proteins (Kim and Ahn, 2014). In particular, BCAAs (leucine, isoleucine, and valine) serve as critical substrates for skeletal muscle protein synthesis and energy production via mitochondrial oxidation during physiological stress or metabolic exertion (Churchward-Venne et al., 2013; Wagenmakers et al., 1990). Although total BCAA levels increased significantly compared to the non-hydrolyzed control, no significant difference ($p > 0.05$) was observed between the early (0.5–1 h) and final (24 h) stages of hydrolysis, suggesting rapid proteolysis kinetics.

Furthermore, specific amino acid residues, including methionine, tyrosine, histidine, and lysine, strongly contribute to antioxidant activity (Samaranayaka and Li-Chan, 2011). The observed rise in radical scavenging capacity following sonication and hydrolysis is largely governed by the liberated free amino acids and exposed active terminal groups (Mintah et al., 2019). However, antioxidant capacity is governed not only by amino acid composition but also by peptide chain length and spatial conformation. Concurrently, the progressive accumulation of polar and charged residues (e.g., serine, glutamic acid, aspartic acid, and threonine) enhances water-binding capacity, thereby promoting protein solubilization (Trevino et al., 2008).

Conclusion

In conclusion, ultrasound pretreatment prior to enzymatic hydrolysis significantly

enhanced the antioxidant properties, degree of hydrolysis, and protein solubility of *Protaetia brevitarsis* (PB) larvae. The results indicated that sonicated enzymatic hydrolysates had a significant impact on the antioxidant properties, degree of hydrolysis and solubility. In addition, each of the Alcalase and Neutrase enzymes had various impacts on the research indicators. The products resulted from the enzymatic hydrolysis with Alcalase higher DPPH and ABTS radical scavenging activity than the Neutrase hydrolysis. Therefore, considering the physicochemical aspect, it was determined that hydrolysis using Alcalase after ultrasonic treatment is a suitable method. Based on the above results, according to the electrophoretic analysis was confirmed that low molecular weight peptides were produced over time when hydrolyzed with Alcalase. Moreover, content of essential amino acids, branched chain amino acids, and hydrophilic amino acids were increased through hydrolysis. Consequently, bioactive peptides obtained through sonicated enzymatic hydrolysis of PB larvae have potential to have an antioxidant property, along with the ability to produce food products to enhance the general health. In addition, their improved surface wettability suggests that PB larvae protein hydrolysates can serve as effective hydrophilic emulsifiers in food emulsion systems.

Conflicts of Interest

The authors declare no potential conflicts of interest.

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Table 1. Effect of ultrasound treatment on yield (%) and pH of different enzymatic hydrolysis of *Protaetia brevitarsis* larvae powder at each process step

	Yield (%)			pH
	Hydrolysis	3 kDa membrane filter	Freeze drying	
Alcalase	91.25±0.24 ^{a2)}	75.90±0.20 ^a	2.47±0.01 ^b	4.76±0.25
Neutrase	90.64±0.06 ^a	68.39±3.42 ^b	2.38±0.12 ^b	4.40±0.43
US ¹⁾ +Alcalase	91.12±0.57 ^a	66.35±0.42 ^b	2.50±0.02 ^b	4.70±0.16
US+Neutrase	89.19±0.14 ^b	77.68±0.12 ^a	2.84±0.00 ^a	4.18±0.21

All values are mean±SD.

¹⁾ US: Ultrasound treatment

²⁾ Different superscripts within the column represent significantly different at $p < 0.05$ by Tukey's multiple range test.

810 **Table 2. The solubility of *Protaetia brevitarsis* larvae powder hydrolysates produced with different enzymatic hydrolysis and ultrasound treatment**
811 **at various pH levels**

Samples	pH						
	3	4	5	6	7	8	9
Control	56.73±2.18 ^{C3)ab4)}	67.28±1.64 ^{Bab}	96.32±2.01 ^{ANS2)}	94.96±0.62 ^{Ans}	96.74±0.40 ^{Ans}	98.83±4.27 ^{Ans}	96.44±0.72 ^{Ans}
US ¹⁾	54.13±3.01 ^{Bb}	58.02±6.07 ^{Bb}	74.65±4.29 ^{AB}	85.00±6.78 ^A	89.67±6.78 ^A	85.54±7.80 ^A	83.91±7.70 ^A
Alcalase	88.07±7.19 ^{NSa}	87.64±6.84 ^a	89.56±7.04	88.80±7.02	89.09±7.14	88.24±9.55	88.54±6.89
Neutrase	86.63±1.94 ^{ABa}	88.28±1.17 ^{ABa}	88.04±0.87 ^{AB}	88.38±0.72 ^A	87.62±0.06 ^{AB}	85.73±0.44 ^{AB}	84.36±0.49 ^B
US+Alcalase	90.80±7.27 ^{NSa}	90.61±8.08 ^a	90.90±8.46	91.60±8.77	91.22±6.96	90.20±7.54	90.97±6.63
US+Neutrase	88.86±7.96 ^{NSa}	89.63±8.53 ^a	89.19±8.30	89.28±8.75	88.41±8.08	87.12±7.50	85.42±7.31

812 All values are mean±SD.

813 ¹⁾ US: Ultrasound treatment

814 ¹⁾ NS: Not statistically significant

815 ³⁾ Different superscripts within rows represent significantly different at $p < 0.05$ by Tukey's multiple range test.

816 ⁴⁾ Different superscripts within the column represent significantly different at $p < 0.05$ by Tukey's multiple range test.

Table 3. Amino acid composition of *Protaetia brevitarsis* larvae powder hydrolysates during enzymatic hydrolysis by alcalase

Amino acid (mg/100g)	Time (h)				
	0	0.5	1	8	24
Tyrosine	25.07±0.62 ^d	109.55±3.35 ^c	121.19±1.24 ^{bc}	111.06±0.17 ^{ab}	119.25±3.56 ^a
Glycine	36.71±0.25 ^c	94.21±0.78 ^b	95.71±0.02 ^{ab}	96.47±0.40 ^{ab}	97.35±1.20 ^a
Serin	32.74±0.30 ^d	103.48±0.30 ^c	107.33±0.25 ^b	107.34±0.90 ^a	88.22±0.93 ^a
Alanine	40.02±0.14 ^b	93.19±0.48 ^a	92.49±0.31 ^a	94.18±0.48 ^a	94.87±1.48 ^a
Glutamic acid	87.16±1.50 ^b	212.73±0.50 ^a	217.32±0.42 ^a	216.25±0.58 ^a	216.44±3.30 ^a
Lysine	29.92±1.48 ^b	90.93±0.14 ^a	93.42±0.13 ^a	95.56±0.89 ^a	95.94±2.28 ^a
Leucine	26.86±2.34 ^b	91.94±2.05 ^a	95.29±0.54 ^a	94.24±0.47 ^a	94.96±0.85 ^a
Methionine	5.78±0.14 ^b	21.90±0.76 ^a	23.75±1.80 ^a	21.44±1.38 ^a	23.07±1.64 ^a
Valine	22.68±0.05 ^b	73.94±1.96 ^a	76.64±0.26 ^a	75.91±2.38 ^a	79.14±0.52 ^a
Arginine	30.36±1.18 ^b	73.20±0.43 ^a	76.73±0.76 ^a	75.17±1.17 ^a	71.79±2.04 ^a
Asparagine	46.39±2.18 ^b	138.72±0.17 ^a	141.50±0.21 ^a	142.19±0.45 ^a	142.27±2.43 ^a
Isoleucine	18.36±1.51 ^b	53.41±2.41 ^a	56.25±1.09 ^a	55.88±2.60 ^a	58.92±0.07 ^a
Threonine	22.97±0.93 ^b	70.46±0.44 ^a	71.90±0.18 ^a	72.78±0.79 ^a	72.07±1.05 ^a
Phenylalanine	21.66±1.76 ^c	63.21±0.82 ^b	65.72±0.28 ^{ab}	67.24±0.59 ^{ab}	67.45±0.99 ^a
Proline	79.11±0.86 ^b	135.82±5.52 ^a	133.03±0.77 ^a	138.32±1.15 ^a	141.13±3.30 ^a
Histidine	22.92±1.56 ^b	45.93±0.96 ^a	48.37±0.45 ^a	47.20±0.06 ^a	46.79±0.26 ^a
EAA ¹⁾	165.34±9.52 ^b	489.81±8.50 ^a	507.57±2.38 ^a	508.80±7.66 ^a	515.27±6.02 ^a
BCAA ²⁾	67.89±3.80 ^b	219.28±6.42 ^a	228.17±1.90 ^a	226.02±5.44 ^a	233.02±1.44 ^a
HAA ³⁾	245.38±6.02 ^c	605.70±2.02 ^b	615.11±1.18 ^{ab}	622.23±8.06 ^{ab}	633.81±8.41 ^a

¹⁾EAA(Essential amino acid) - Lysine, Leucine, Valine, Isoleucine, Threonine, Phenylalanine, Histidine

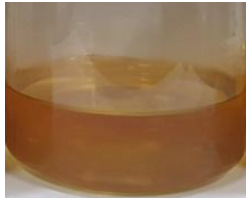
²⁾BCAA(Branched-chain amino acid) - Leucine, Valine, Isoleucine,

³⁾HAA(Hydrophobic acid) - Glycine, Alanine, Leucine, Valine, Isoleucine, Phenylalanine, Proline

^{a-b}The mean in each row followed by the letter are not significantly by Tukey's multiple range test at $p < 0.05$



Fig. 1. Production of protein hydrolysates from *Protetia brevitarsis* larvae powder by enzymatic hydrolysis.

Non-ultrasound treatment			Ultrasound treatment		
Non-filtration	3 kDa membrane Filtration		Non-filtration	3 kDa membrane Filtration	
	Liquid form	Freeze-dried form		Liquid form	Freeze-dried form
Alcalase					
Neutrase					

828 Fig. 2. The appearance of hydrolysate after Alcalase and Neutrase enzymatic hydrolysis depending on ultrasound treatment and filtration.

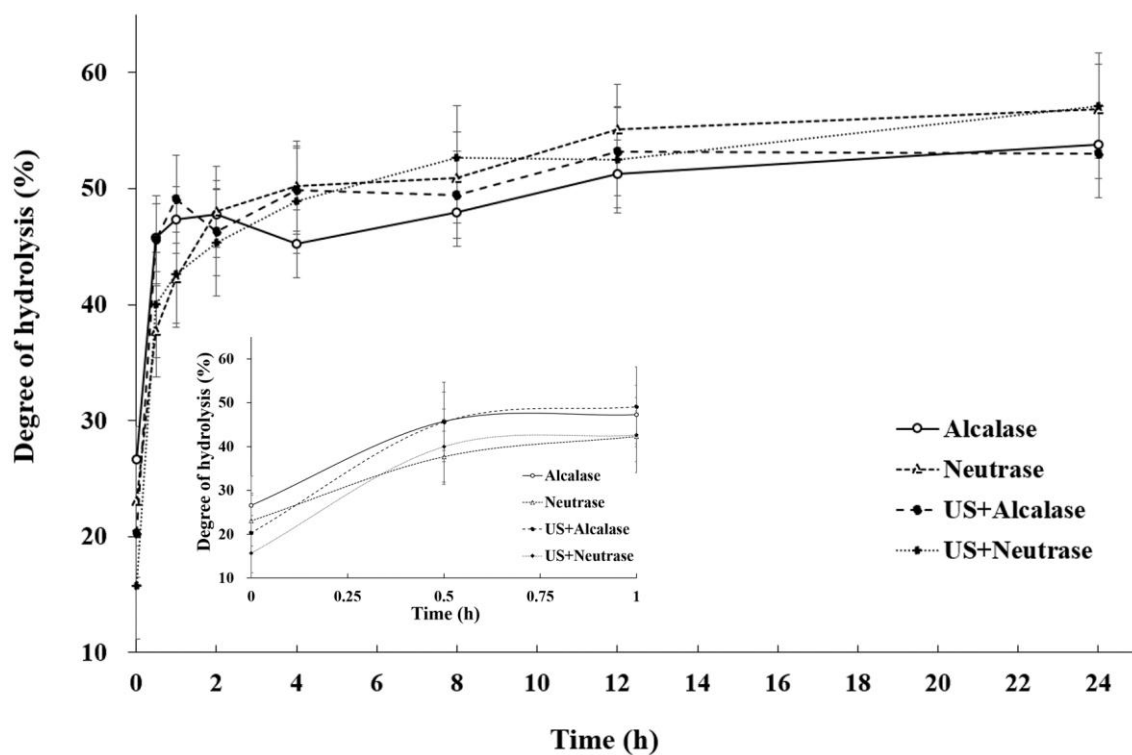


Fig. 3. Degree of hydrolysis of *Protactia brevitaris* larvae powder hydrolysates produced with different enzymatic hydrolysis and ultrasound treatment.

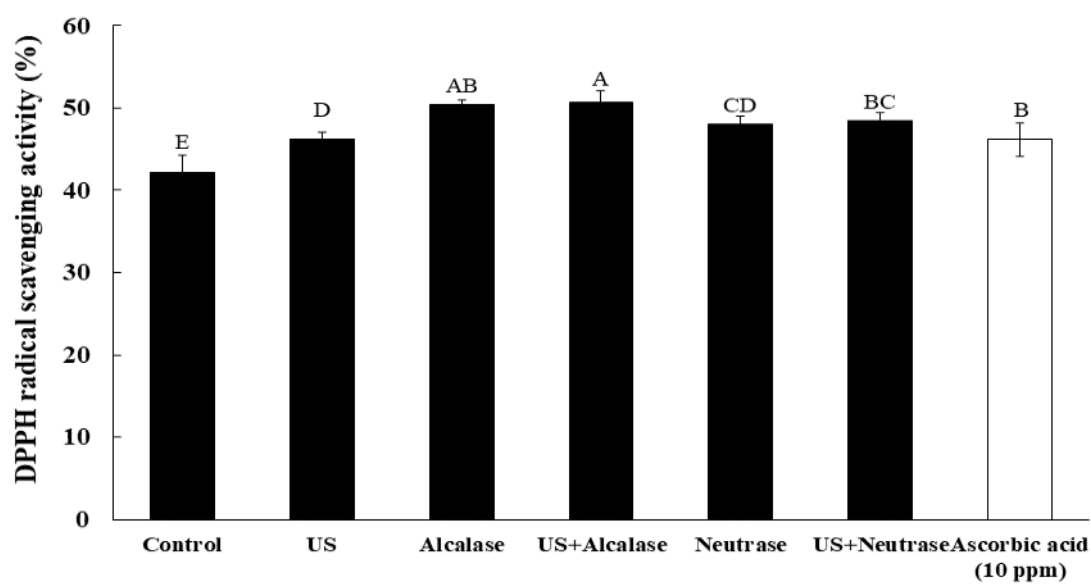


Fig. 4. DPPH radical scavenging (%) of *Protactia brevitaris* larvae powder hydrolysates using different enzymatic hydrolysis and ultrasound treatment.

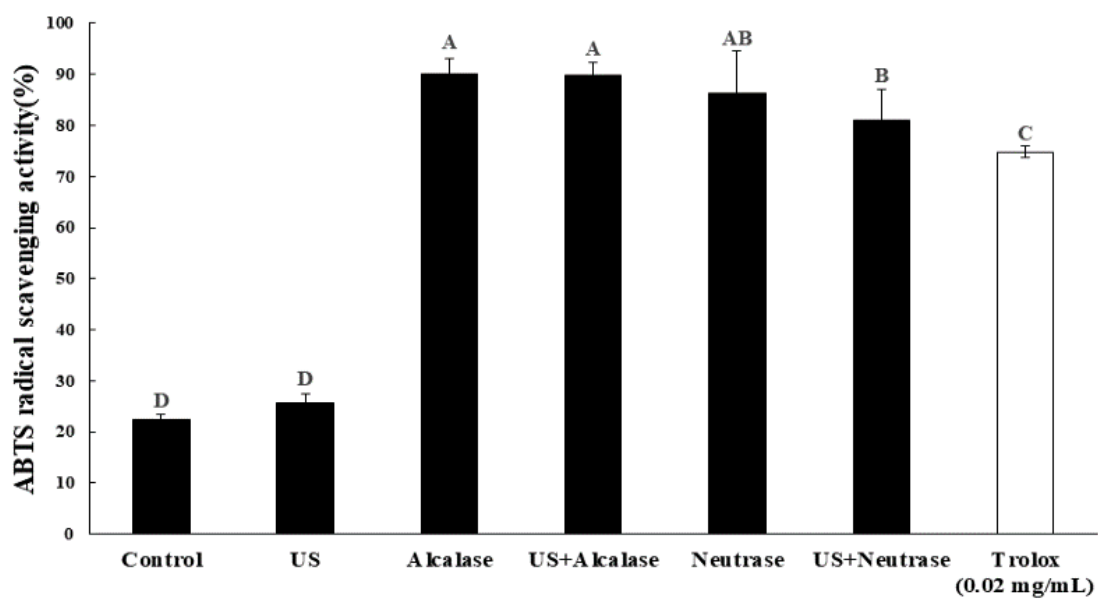


Fig. 5. ABTS radical scavenging activity of *Protaetia brevitarsis* larvae powder hydrolysates by using different enzymatic hydrolysis and ultrasound treatment.

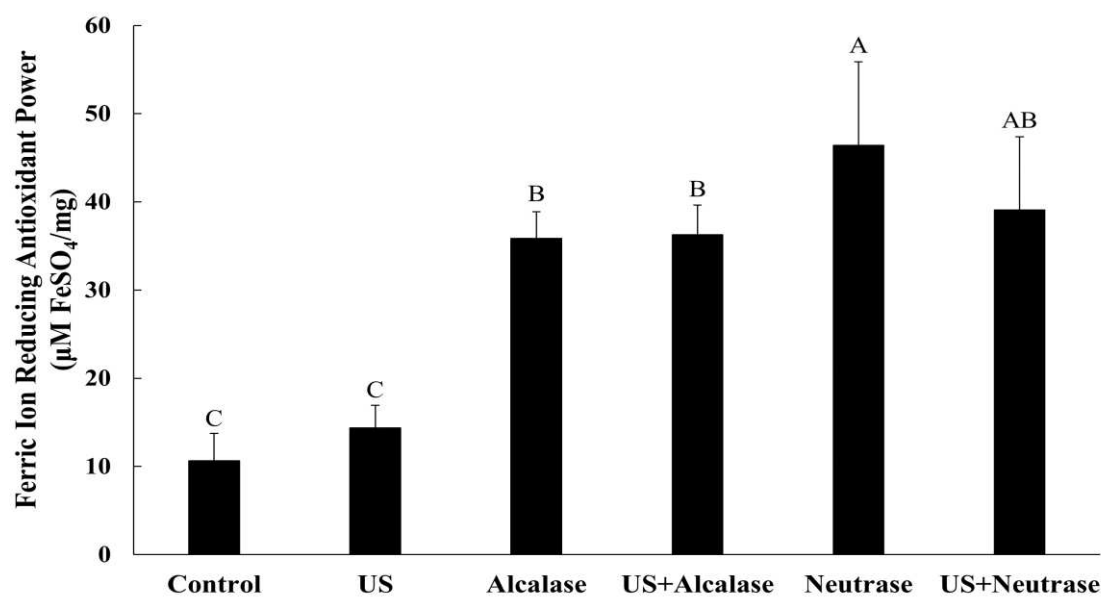


Fig. 6. FRAP of *Protactia brevitorsis* larvae powder hydrolysate by further enzymatic hydrolysis and ultrasound treatment.

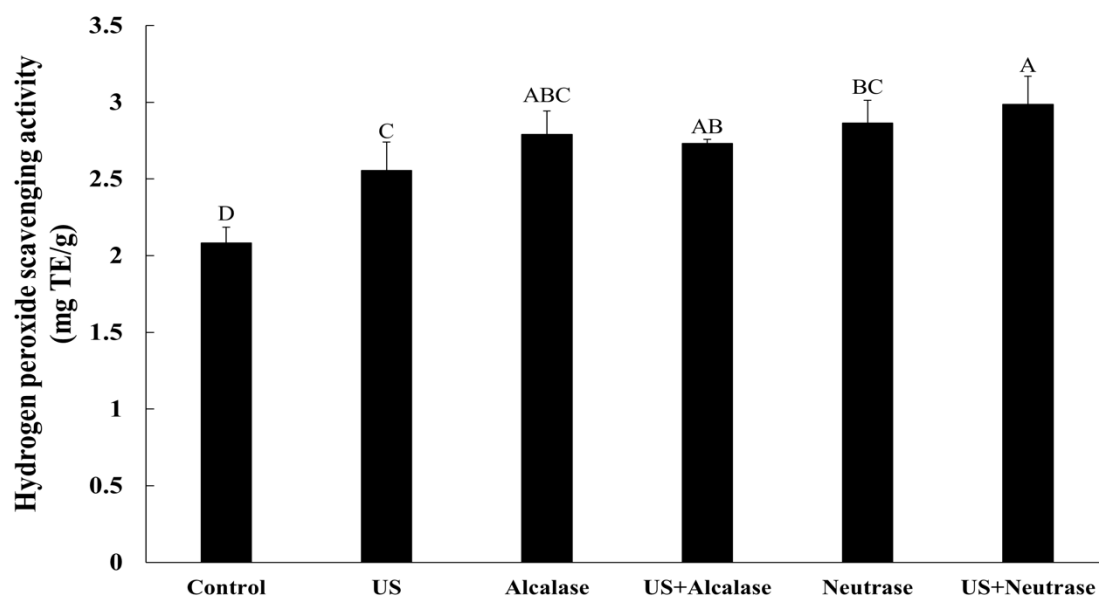


Fig. 7. Hydrogen peroxide radical scavenging activity of *Protactia brevitaris* larvae powder hydrolysate by further enzymatic hydrolysis and ultrasound treatment.

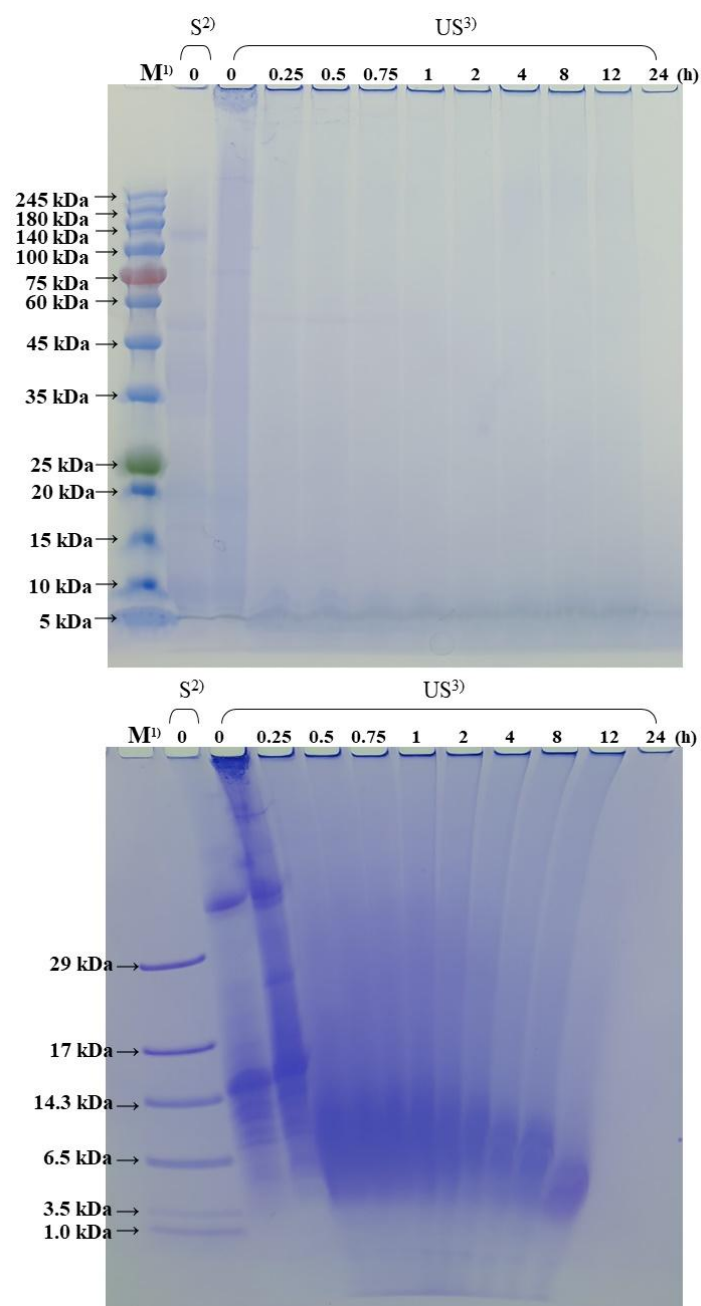


Fig. 8. SDS-PAGE electrophoretic profiles at (A) broad molecular weight range (5-245 kDa) or (B) low molecular range (1-29 kDa) of *Protactia brevitarsis* larvae powder hydrolysates during enzymatic hydrolysis by Alcalase.

¹⁾ M-standard marker

²⁾ S-stirring

³⁾ US-ultrasound treatment

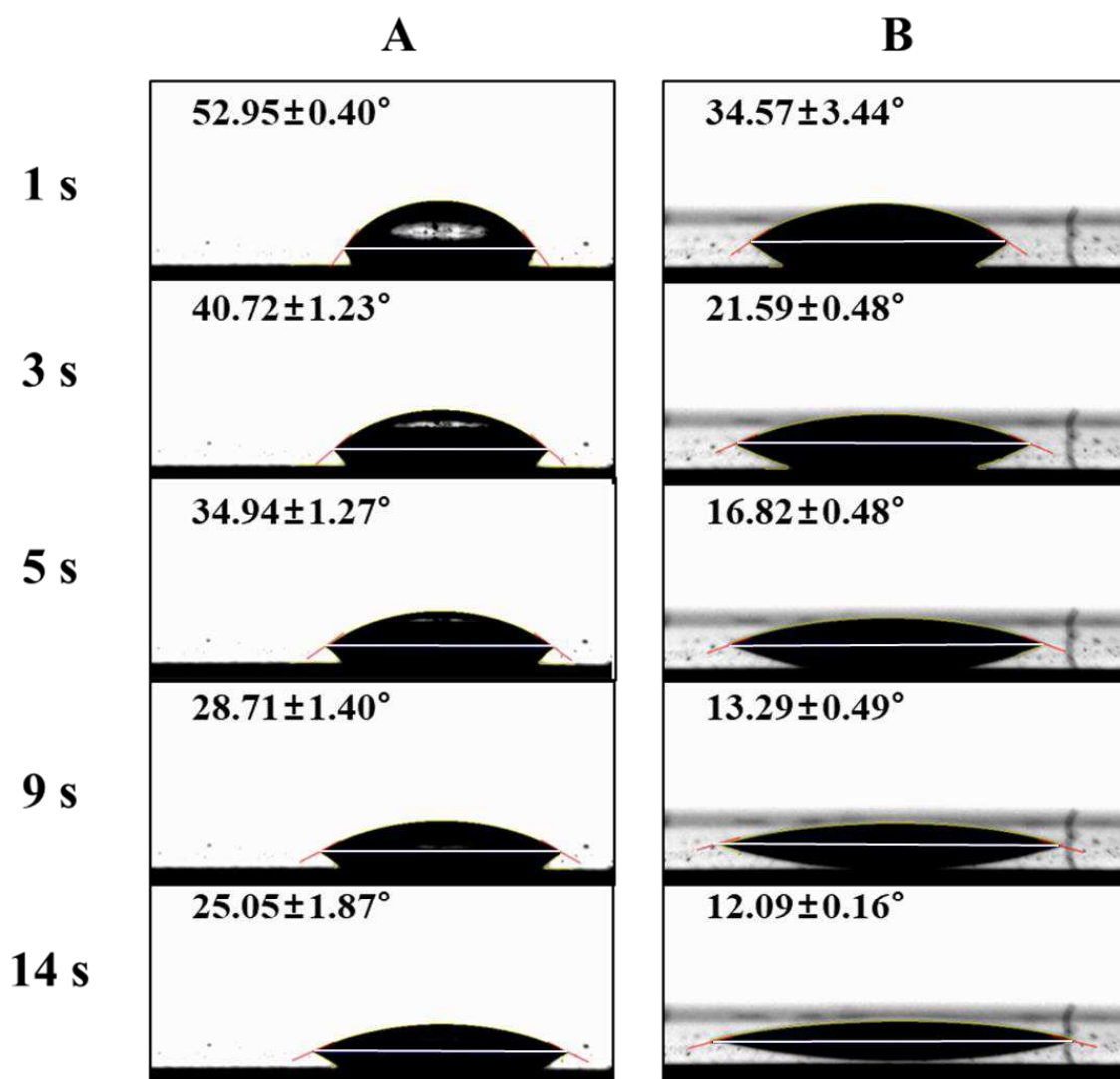


Fig. 9. Contact angle in the air environment of *Protætia brevitarsis* larvae hydrolysates; (A) non-hydrolyzed or (B) 0.5 h hydrolyzed.