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Research Article

Effects of Enzyme Types and Extraction Conditions on Protein Recovery and Antioxidant Properties of Hydrolysed Proteins Derived from Defatted *Lemna minor*

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Abstract
Lemna minor (*L. minor*), the common duckweed, contains a high protein substance and is considered as a good source of potential bioactive peptides. The objective of this study is to investigate the effect of enzymatic hydrolysis times (60–180 min) and enzyme concentrations (0.5–3.5%v/w) with Alcalase and Flavourzyme on the recovery, hydrolysis degree (DH), and antioxidant properties of peptides derived from defatted *L. minor*. The protein recovery, hydrolysis degree (DH), and antioxidant activities obtained by enzymatic were compared with the alkaline treatment method. The results showed that the protein recovery, DH values, and antioxidant activities were enhanced by increasing the enzyme concentration and hydrolysis time. Specifically, the recovery of protein and DH values reached the highest level after the enzymatic hydrolysis by Flavourzyme or Alcalase at 1.5 v/w enzyme for 120 min. At the same enzymatic hydrolysis condition, the samples hydrolyzed by Flavourzyme had a higher inhibitory effect on the $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ radical scavenging than those hydrolyzed by Alcalase and the alkaline treatment. Further study also showed that the DH values, amino acid contents, and antioxidant activities of the protein extracts were positively correlated. Thus, the extractions with Flavourzyme and Alcalse were a good method to produce a significant amount of amino acids and smaller peptides.

Keywords: *L. minor*, Alcalase, Flavourzyme, Antioxidant properties, Hydrolysis degree

1 Introduction

L. minor grows naturally in the water of warm climates, and is primarily cultivated in ponds and small lakes. It can produce approximately 10 tons of protein per hectare per year [1]. *L. minor* has a high nutritive value because of its high quality protein, fat, carbohydrates and fiber. *L. minor* contains about 23–36% protein, 4–8% fat, and 34–46% carbohydrate [2], [3]. Previous studies have proved that *L. minor* consists of 16–18 amino acids and can be compared with flours from soybean, chickpea, lupine, or pea [3]–[5]. Nowadays, soybeans, chickpeas, lupines, and peas are considered as a cheap sources of protein. However, they contain various anti-nutritional factors (i.e., agglutinins, saponins, cyanogenic glucosides) and related to Genetically modified organism (GMO). Meanwhile, *L. minor* is rich in amino acids, especially a good source

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of lysine (very low in the grains), and can be instead of soybean protein [1]. *L. minor* is mostly utilized as animal feed or discarded. The smallest of duckweeds (*Wolffia arrhzia*) has been used as a nutritious vegetable by Laotians and the people of Northern of Thailand [6]. Thus, the *L. minor* protein is considered as a potential source of low cost vegetable protein for human consumption and less allergenicity [3], [4].

Alkaline protein extraction is a conventional method used to produce protein extracts from *L. minor* [4]. The alkaline protein extraction has some advantages such as low cost and feasibility for scaling up. Unfortunately, it has a low protein recovery and high cost [7]. Chirinos *et al.* reported that the enzyme-assisted protein extraction is an alternative protein extraction method with higher yields than the traditional alkaline method [8]. Accordingly, the protein obtained from the alkaline extraction is not suitable for feed, however it can be used as feedstock for amino acid derived bulk chemical production [9]. Thus, there is a need for extraction methods that allow an increase in the extraction yield and a decrease in hazard content. Nowadays, the enzyme-assisted extraction can be considered as a promising method to extract proteins from microalgae due to its high recovery and specificity, and environmentally friendly method [10]. However, the enzyme-assisted extraction has yet to be tested on duckweed, especially *L. minor*.

Enzyme-assisted extraction conditions and the specificity of the enzyme are key factors for protein extraction. During enzyme assisted extraction, plant cell wall matrix and bound protein are broken-down and released soluble proteins. Furthermore, proteins are cleaved into amino acids and eventually smaller peptides indicating biological activities (i.e., antioxidant, antimicrobial and antihypertensive properties) [11]–[14]. Some previous studies involved the use of commercial enzymes (Alcalase and Flavourzyme) for microalgae biomass [7], [15]. Nowadays, Alcalase and Flavourzyme are promising candidates for industrial hydrolysate production. Alcalase has broad specificity endo-protease activity, low cost, and high tolerance for alkaline pH [16], [17]. Meanwhile, Flavourzyme has been known as a mixture of exopeptidase and endo-protease complex, which can produce a large of number of peptide bonds and hence very high DH and high antioxidant activity. According to Zhang *et al.*, the antioxidant activities of hydrolysates increased with increasing the DH value [17].

Until now, there exists no study on the extraction protein and the bioactivities from *L. minor* using Aalcalase and Flavourzyme. Therefore, the aim of this research is to investigate the effect of Alcalase and Flavourzyme hydrolysis conditions on the protein recovery and antioxidant activities of *L. minor*.

2 Materials and Methods

2.1 *Materials*

Milled *Lemna* meal was collected from Long An province, Vietnam. Two enzyme mixtures: flavourzyme (activity = 500 $U.mL^{-1}$) and Alcalase (activity = 6.22 $U.mL^{-1}$) were obtained from Genencor (Danisco) International Oy, Denmark. All the solvents and reagents were of analytical grade and used without any further process.

2.2 *Methods*

2.2.1 Sample preparation

L. minor powder was defatted by extracting it twice with hexane at a ratio of 1 : 4 (w/v) and was stirred at the room temperature for 3 h [18]. Then, the defatted *L. minor* was air-dried overnight in a fume hood, ground and sieved through a 40 mesh sieve [19].

The phenolic compounds in the defatted *L. minor* were extracted following Do Evangelho *et al.* method with minor modifications [11]. The defatted *L. minor* was shaken with 50% acetone (v/v), for 2 h. After centrifugation at 6000 rpm for 20 min, the solvent was vacuum-evaporated to dryness at 40°C, 500 mmHg. The residue was ground and kept at –20°C in a dark plastic bag until use.

The composition of the defatted *L. minor* was on dry basis: moisture content of 5.10 ± 0.40% and protein content of 36.50 ± 0.95%. The moisture content was analysed using a vacuum oven at 70°C for 72 h [20]. The Kjeldahl method was used for determination of the crude protein content [10]. The lipid content was determined by using a Soxhlet extraction method with hexane for 6 h [21]. All the analyses were carried out in triplicate. The obtained results were expressed in % dry matter.

2.2.2 Extraction procedure

The enzymatic hydrolysis of protein was performed by using individual enzyme preparation (Alcalase and Flavourzyme). For this, 1.6 kg of water was added to the *L. minor* powder (400 g), and the mixtures were then stirred vigorously in a water bath for 30 min. The experiments were carried out at 60°C and pH 9.0 for Alcalase, and 55°C and pH 7.0 for Flavourzyme based on the preliminary experiment. The hydrolysis conditions are described in Table 1. Alkaline treatment was performed in the absence of enzyme at pH 12 (adjusting with 1 mol.L^{-1} NaOH) and 70°C for 16 h [10]. The supernatant containing the proteins and peptides was filtered using Whatman paper to remove small particles. A blank measurement was performed to subtract the added enzyme content to the protein content of *L. minor* for each experiment

Table 1: Experimental conditions of hydrolysis for the different enzyme systems

Hydrolysis Condition			
Hydrolysis Time (min)	60	120	180
Enzyme Concentration (% v/w)	0.5	1.5	2.5

2.2.3 Protein extract recovery

The Lowry method was used to measure protein content in the *L. minor* extract following Barbarino & Lourenço [22]. The Folin–Ciocalteu reagent was purchased from Sigma-Aldrich (St. Louis, MO, USA), and was diluted in two volumes of ultra-pure water (1 : 2) before use. To construct the standard curve for Lowry protein quantification, the standard solution of bovine serum albumin (0 to 1,000 µg/mL) was used. In order to measure protein content, 2 mL of protein extracts were mixed with 1 ml of modified Lowry reagent. The mixtures were then vortexed and incubated for 10 min. After incubation, 100 µL of Folin-Ciocalteu reagent (1 N) were added and vortexed and incubated for 30 min. The mixture was then measured by a UV spectrophotometer/NIR at 750 nm (Shimazu, UV-2600, Japan). The protein content in each sample was calculated as mg/ml BSA equivalents by interpolation in a standard curve. The protein extract recovery was calculated as [Equation (1)]:

% Protein recovery = g protein in the extract×100/g protein proximal composition (1)

2.2.4 Degree of hydrolysis analysis

The degree of hydrolysis analysis of the extracts was determined following Zhang *et al.* with some modifications [23]. 15 µL of the native sample or the protein extract diluted in 42 µL of 1% SDS (sodium deodecysulfate) was added 250 µL 0.21 M sodium phosphate buffer pH 8.2 in a test tube. Then, 45µL of 0.05% TNBS (2,4,6-Trinitrobenzenesulfonic acid) was added and shaken for 1 min. The 500 µL 0.1 M HCl was added to stop reaction. The test tube was covered with aluminium foil and incubated at 50°C for 60 min. After incubation, the solution was measured at the wavelength absorption of 340 nm. Leucine (0.0–1.5 mM) was used to generate a standard curve.

2.2.5 $DPPH^{\bullet+}$ radicals scavenging assay

The DPPH radical scavenging activity was determined by the method of do Evangelho *et al.* with a slight modification [11]. The sample (2.0 mL) was mixed 1 : 1 (v/v) with 0.15 mM DPPH that dissolved in 95% ethanol. The mixture was then shaken vigorously using a vortex mixer and incubated at 30°C for 30 min under light protection. The samples were measured at the wavelength absorption of 516 nm by using a UV spectrophotometer/NIR (Shimadzu, Kyoto, Japan). The antioxidant activity of the samples was expressed based on the following Equation (2).

$$I\ (\%) = [A_{blank} - A_{sample}/A_{blank}] \times 100 \quad (2)$$

where A_{sample} is absorbance of sample; and A_{blank} is absorbance values of the DPPH solution without sample.

2.2.6 Determination of $ABTS^{\bullet+}$ radical scavenging ability

The $ABTS^{\bullet+}$ assay was based on the method of Shahi *et al.*, with some modifications [18]. ABTS+• radical solution was prepared by combining 5 mL of 7 mM ABTS solution and 88 µL of 140 mM potassium persulfate solution. The mixture was placed in a dark place at 10 ± 2°C for 12–18 h before use. Prior testing, the mixture (100 µL) was diluted with ethyl alcohol to achieved an absorbance of 0.7 ± 0.02 at 734 nm. Then, 60 µL was added to 4 mL of diluted $ABTS^{\bullet+}$ solution

and stirred for 30 s. After that, the mixture was kept in a dark place for 10 min and measured the absorbance at 734 nm. The percentage of inhibition of the $ABTS^{\bullet+}$ radical was then calculated according to the following Equation (3).

$$AA\ (\%) = [A_{blank} - A_{sample}/A_{blank}] \times 100 \quad (3)$$

Where, A_{blank} is control absorbance and A_{sample} is absorption of the hydrolyzed sample.

2.2.7 Electrophoresis test

Tris-Tricine-Sodium dodecyl sulfate gel electrophoresis (Tris-Tricine SDS-page) was carried out to characterize the proteins in the *L. minor* extracted with different extraction methods following Garcia-Vaquero *et al.* [24]. Briefly, the samples were diluted with a buffer containing 200 mM Tris-HCl (pH 6.8), 2% SDS, 40% glycerol, and Coomassie blue followed by heating in boiling water for 5 min. Then, polyacrylamide gel was prepared between two glass plates. After that, 10 µL of the supernatant was loaded into a 4–15% gradient gel. The separating gels ran at a constant current of 20 mA for 3 h. The Precision Plus Protein Dual Xtra Standards from Merck (MW: 0.1–26.6 kDa) were used as a molecular mass marker. The gels were washed with three times in ultrapure water for 5 min and then stained with Coomassie blue G-250 for 30 min and detained with the mixture of 10% acetic acid and 5% ethanol. The gels were left overnight and then recorded with an electronic scanner (Umax Power Look 2100, UMAX Technologies, Fremont, CA).

2.2.8 Amino acid analysis

The amino acid profiles of the *L. minor* were determined according to the method of AOAC with a slight modification [20]. The samples (150 mg) were mixed with 8 mL performic acid (hydrogen peroxide: formic acid, 1 : 9) and then placed in a water bath at 80°C for 3 min. Then, sodium metabisulfite (3 g) was added for the performic acid decomposition. The amino acids were liberated from the protein hydrolysis with 50 mL 6 M HCl under reflux for 24 h at 110–120°C. Remove the digestion tubes from heat to room temperature. Add 20 mL internal standard solution 2.5 nM (Sigma-Aldrich, Inc., Milkawkee, USA) containing 23 amino acids to each test solution. The mixtures were evaporated at 60°C under vacuum of 550 mbar. After that, the hydrolysates were diluted with sodium citrate buffer and pH was adjusted to 2.20. The individual amino acid components were separated on amino acid analyzer Biochrom 30+ (Biochrom Co., Cambridge, UK). The mobile phase was ninhydrin (A): sodium citrate buffer (B): 60% : 40% for 10 min and 50% : 50% for 60 min and 40% : 60% for 20 min. The solvent was delivered to the column at a flow rate of 25 mL/h for the total running time of 90 min. Detection was performed with the UV-visible detector set a wavelength at 570 and 440 nm. The concentration of the amino acid in the sample was calculated using the internal standard.

2.3 *Statistical analysis*

The experimental results were expressed as mean ± SD. The statistical analyses were performed using Stagraphic Centrution XV (Statsoft Inc., Umeå, Sweden). The one-way analysis of variance (ANOVA) with a 95% confidence level was used for the determination of differences among the group means.

3 Results and Discussion

3.1 *Enzyme-assisted extraction of proteins from L. minor*

The influence of enzymes in assisting protein extraction is reflected by the percentage of the protein recovery. As shown in Figure 2, without using enzyme the protein recovery was only 15.12%. The result could be explained that alkaline treatment displayed low selectivity releasing multiple components, which resulted in a low protein recovery. Meanwhile, the recovery of protein obtained with the enzymatic treatment was significantly higher than that of the absence of enzymatic hydrolysis. Under Flavourzyme and Alcalase assisted extractions, the protein recovery was 45.12–60.94% and 38.95–53.13%, respectively.

In this work, the increase in enzyme concentration (0.5–1.5%v/w) and extended extraction time (60–120 min) enhanced the protein recovery. After 120 min incubation, the maximum protein recovery for Flavourzyme and Alcalse were 60.94% and 53.13%, respectively at the enzyme concentration of 1.5%v/w. The excessive enzyme concentration and longer

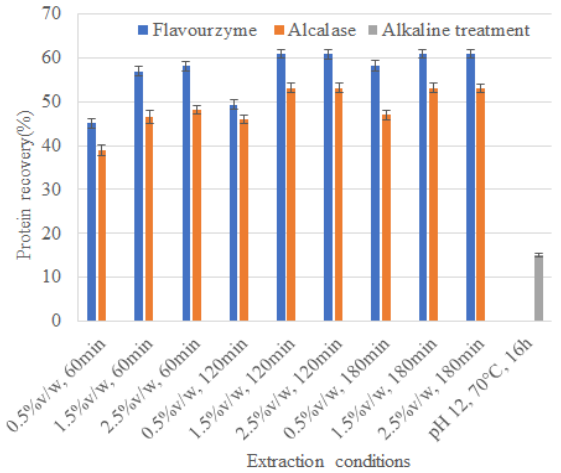

Figure 1: Effect of different enzymatic extraction conditions on the *L. minor*.

treatment time (2.5%v/w; 180 min) did not improve the protein content in the extract (Figure 1). Similar results were also reported for Okara treated with Flavourzyme and Alcalse [14]. In 2019, Verdasco-Martín *et al.* documented that the cell wall matrix and the bound protein were easily broken down by the use of Alcalse [15]. D'Hondt *et al.* reported that Flavourzyme and Alcalse converted proteins of cell membrane into amino acids, resulting in cell wall disruption [7].

In Figure 1, it can be seen that the recovery of *L. minor* protein produced by Flavourzyme was higher than that of Alcalse at the same extraction condition. The result could be explained that Flavourzyme, a mixture of an endo-protease and exopeptidase, cleave of protein molecules into small peptides of various sizes, and thus a higher protein recovery is expected. By contrast, Alcalase is an endopeptidase, which can hydrolyze a protein bond on the inside of protein molecular. Thus, it releases two peptides with a substantial molecular weight.

3.2 *Degree of hydrolysis (DH) and Antioxidant activity*

In the hydrolysis experiments, the DH value is used as an indicator to determine the effectiveness of enzymatic protein hydrolysis [25]. As shown in Table 2, the lowest DH value of the native samples was obtained, which indicated that the native samples did not contain any the smaller peptides. The alkaline hydrolysis was significantly improved the DH value (5.52%). However, this result was still approximately 1 to 3-fold lower than the enzymatic hydrolysis (Flavourzyme and Alcalase). It could be explained that the use of NaOH during the process of protein extraction in which very minor hydrolysis happened and a small amount of amino acids and peptides was released. Similar results were reported by Hou *et al.* [26].

Under Flavourzyme and Alcalse hydrolysis, the DH values increased from 10.56 to 16.34% and 9.45 to 13.56%, respectively in increasing of the hydrolysis time (60 to 120 min) and enzyme concentration (0.5%v/w to 1.5%v/w). The highest DH values were 16.34% and 13.56% for Flavourzyme and Alcalse at 1.5 %v/w of enzyme for 120 min incubation. As shown in Table 2, longer treatment time (120–180 min) and higher enzyme concentration (1.5–2.5%v/w) did not improve the DH value. The different DH values could be attributed to a difference in enzyme specificity. Flavourzyme is an exopeptidase-endo-protease complex, thus can generate higher DH than Alcalase (endopeptidase). Same results were also reported by Bamdad, Wu, and Chen for hydrolysis of barley hordein [27]. These authors suggested that the protein produced by Flavourzyme and Alcalase, can be potentially used as a natural antioxidant ingredient in food and pharmaceutical industries.

In Table 2, the protein hydrolysis with Flavourzyme and Alcalase contributed to enhance the $DPPH^{\bullet+}$ and $ABTS^{\bullet+}$ radical scavenging activities when compared the native samples and non-hydrolyzed samples. For Flavourzyme, when the DH value increased from 10.56 to 16.34%, the ABST and DPPH values increased significantly from 58.60 to 89.01% and 47.64 to 54.15%, respectively. For Alcalase, as the DH value of sample rose from 9.45% to 13.56%, the $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ radical scavenging activities increased and reached maximal values of 70.15% and 50.18%, respectively. This result also verified that the samples extracted with Flavourzyme had a stronger reducing power than Alcalase at the similar extraction condition. The difference could be explained by the specific peptides obtained by different enzymatic hydrolysis. The finding is similar with Karamać *et al.*, who extracted protein from peanut and flaxseed cake proteins using five different enzymes (papain, trypsin, pancreatin, Alcalase and Flavourzyme) [28].

From present study, it can be concluded that the Flavourzyme hydrolysis of the *L. minor* was relatively

Table 2: Effect of different enzymatic extraction conditions and Alkaline hydrolysis on the DH value and $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ free radical scavenging

Enzyme	Extraction Condition	DH (%)	ABTS (%)	DPPH (%)
Flavourzyme	0.5 %v/w, 60 min	$10.56^{e} \pm 0.89$	$58.60^{de} \pm 1.56$	$47.64^{d} \pm 1.56$
	1.5 %v/w, 60 min	13.98 ± 0.75	$71.15^{bc} \pm 2.01$	$49.14^{c} \pm 1.78$
	2.5 %v/w, 60 min	$14.75^{b} \pm 0.56$	$77.98^{b} \pm 2.09$	$50.45^{b} \pm 1.79$
	0.5 %v/w, 120 min	$11.23^{d} \pm 0.67$	$60.21^{d} \pm 1.78$	$48.26^{cd} \pm 1.91$
	1.5 %v/w, 120 min	$16.31^{a} \pm 0.78$	$89.01^{a} \pm 2.13$	$54.15^{a} \pm 1.76$
	2.5 %v/w, 120 min	$16.31^{a} \pm 0.56$	$88.91^{a} \pm 2.01$	$54.14^{a} \pm 1.79$
	0.5 %v/w, 180 min	$14.01^{bc} \pm 0.55$	$77.96^{b} \pm 2.01$	$50.12^{b} \pm 1.91$
	1.5 %v/w, 180 min	$16.01^{a} \pm 0.56$	$88.95^{a} \pm 2.00$	$53.98^{a} \pm 1.67$
	2.5 %v/w, 180 min	$16.34^{a} \pm 0.68$	$88.97^{a} \pm 2.01$	$54.09^{a} \pm 1.58$
Alcalase	0.5 %v/w, 60 min	$9.45^{f} \pm 0.56$	$47.64^{f} \pm 1.68$	$41.21^{e} \pm 1.56$
	1.5 %v/w, 60 min	$11.59^{d} \pm 0.59$	$59.65^{d} \pm 1.67$	$47.96^{d} \pm 1.54$
	2.5 %v/w, 60 min	$12.01^{d} \pm 0.69$	$60.11^{d} \pm 2.00$	$48.97^{cd} \pm 1.68$
	0.5 %v/w, 120 min	11.45 ± 0.71	$58.67^{de} \pm 1.89$	$47.12^{d} \pm 1.56$
	1.5 %v/w, 120 min	$13.51^{c} \pm 0.68$	$69.74^{c} \pm 1.78$	$50.18^{b} \pm 1.75$
	2.5 %v/w, 120 min	$13.35^{c} \pm 0.69$	$70.01^{c} \pm 1.87$	$50.13^{b} \pm 1.54$
	0.5 %v/w, 180 min	$11.98^{d} \pm 0.81$	60.15 ± 1.92	$47.91^{d} \pm 1.55$
	1.5 %v/w, 180 min	$13.56^{c} \pm 0.58$	$70.00^{c} \pm 1.98$	$50.14^{b} \pm 1.67$
	2.5 %v/w, 180 min	$13.51^{c} \pm 0.59$	$70.25^{c} \pm 1.93$	$50.11^{b} \pm 1.54$
Alkaline hydrolysis	-	$2.52g \pm 0.21$	$29.14g \pm 1.06$	$28.91^{f} \pm 1.40$

Different letters in each column denote statistically significant differences between treatments ($p < 0.05$). The values are the mean of three replications SD.

more extensive than the hydrolysis by Alcalase and Alkaline treatment. The enzyme concentration of 1.5%v/w and hydrolysis time of 120 min were selected as the optimal hydrolysis condition for both enzymes.

3.3 *Amino acid and the molecular-weight distribution of protein hydrolysates*

In this study, the protein extract obtained from the native *L. minor*, alkaline treatment, Enzyme-assisted extraction (Flavourzyme and Acalase hydrolysis) at optimal condition (1% enzyme and 120 min) were determined and compared. As shown in Table 3, 17 amino acids were found in the *L. minor* protein extract. Especially, the samples were extracted by Flavourzyme and Acalase at optimal condition contained higher amino acid than the native sample and alkaline hydrolysis. The greatest percentage of essential amino acid (45.34%) was found in the protein isolated from *L. minor* using Flavourzyme, followed Alcalase (45.24%). The lowest percent (43.24%) was found in the extracts obtained from native material. Meanwhile, no significant difference in the total of the non-essential amino acid among these samples were observed. As shown in Table 3, it can be seen that the hydrolyzed samples obtained from Flavourzyme and Acalase were rich in Leu, Asp, and GLu, and poor in Trp and Cys. In addition, Tyr, His, and Lys in the hydrolyzed samples were significantly higher than that those in the native and alkaline hydrolysis. According to Elias *et al.* and You *et al.*, several amino acids such as Tyr, Met, His, Lys, and Trp. were considered to have antioxidant properties [13], [29]. Those authors also concluded that the antioxidant activity of proteins associated with their amino acid composition and their structures. Thus, our results indicated that the hydrolyzed samples with high Tyr, His, and Lys content contributed to the high antioxidant activities.

In order to determine the relationship among DH, the antioxidant activity, and the molecular weight subunits of the *L. minor* protein, SDS-PAGE were used (Figure 2). The first column of the gel (Std) represents the standard molecular weight. The others columns present the molecular weight subunits of the native

L. minor (lane A), non-enzymatic hydrolysis (lane B), and those from the hydrolysates of the *L. minor* by Alcalse (lane C) and Flavourzyme (lane D) at optimal extraction condition (1.5% enzyme and 120 min incubation), respectively. The electrophoretic patterns showed that the native *L. minor* protein and alkaline hydrolysis of the *L. minor* protein were rich in 7.25–30.00 kDa fraction. Meanwhile, the hydrolysates with Flavourzyme and Alcalase at optimal condition were composed of peptides with molecular weights below 0.35–10.04 kDa [Figure 2(D)]. Je *et al.* reported that the peptides with a molecular weight of less than 1.00 kDa showed the stronger in vitro antioxidant activity than those of the 3.00 and 5.00 kDa peptides [30]. From Table 2 and Figure 1, it can be seen that Flavourzyme vs Alcalase can produce low molecular weight peptides via a high degree of hydrolysis, and these peptides significantly influence the antioxidant activities of the hydrolyzed samples.

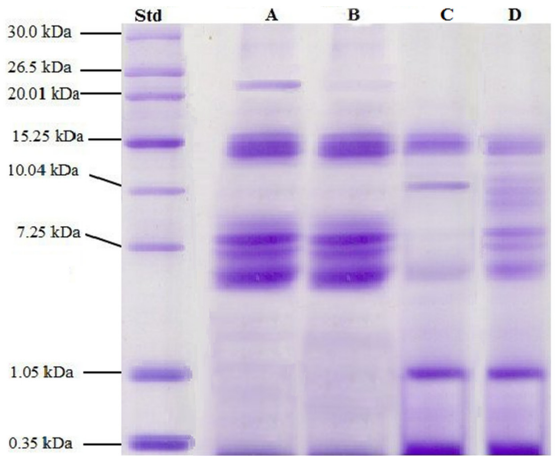

Figure 2: SDS/PAGE of standard molecular weight (Std), native *L. minor* (A), Alkaline hydrolysis (B), hydrolyzed by Alcalase (C), hydrolyzed by Flavourzyme for 120 min and 1.5%v/w (D).

Table 3: Amino acid residue composition of native *L. minor*, Alkaline hydrolysis, and *L. minor* hydrolysates at the optimal condition (g amino acid residues/100 g protein)

Amino Acid		Native *L. minor*	Non-enzymatic Hydrolysis	Alcalase	Flavourzyme
Non-essential Amino Acid	Asp	$3.390^{b} \pm 0.12$	$3.260^{bc} \pm 0.05$	$3.981^{a} \pm 0.06$	$4.012^{a} \pm 0.07$
	Glu	$5.891^{a} \pm 0.11$	$5.119^{a} \pm 0.09$	$5.019^{a} \pm 0.07$	$4.912^{ab} \pm 0.07$
	Ser	$2.348^{b} \pm 0.09$	$2.448^{a} \pm 0.07$	$2.561^{a} \pm 0.04$	$2.574^{a} \pm 0.06$
	Gly	$2.456^{a} \pm 0.08$	$2.856^{a} \pm 0.07$	$2.931^{a} \pm 0.04$	$2.985^{a} \pm 0.09$
	Cys	$0.381^{b} \pm 0.04$	$0.385^{b} \pm 0.06$	$0.410^{a} \pm 0.06$	$0.419^{a} \pm 0.04$
	Arg	$2.918^{a} \pm 0.09$	$2.985^{a} \pm 0.08$	$2.981^{a} \pm 0.03$	$3.012^{a} \pm 0.05$
	Tyr	$2.012^{b} \pm 0.07$	$2.056^{b} \pm 0.06$	$2.883^{ab} \pm 0.07$	$3.010^{a} \pm 0.05$
	Ala	$2.981^{a} \pm 0.08$	$2.987^{a} \pm 0.09$	$2.911^{a} \pm 0.06$	$2.989^{a} \pm 0.09$
	Pro	$1.284^{b} \pm 0.05$	$1.380^{a} \pm 0.05$	$1.451^{a} \pm 0.07$	$1.413^{a} \pm 0.05$
Total of the Non-essential Amino Acid		20.337 (56.76%)	23.130 (56.22%)	24.761 (45.76%)	25.328 (54.66%)
Essential Amino Acid	Thr	$1.956^{ab} \pm 0.04$	$1.887^{ab} \pm 0.05$	$2.019^{a} \pm 0.09$	$2.010^{a} \pm 0.10$
	Val	$2.656^{b} \pm 0.09$	$2.656^{b} \pm 0.07$	$2.915^{a} \pm 0.08$	$2.891^{a} \pm 0.06$
	Met	$0.969^{b} \pm 0.03$	$0.959^{b} \pm 0.06$	$1.051^{ab} \pm 0.08$	$1.085^{a} \pm 0.04$
	His	$1.091^{d} \pm 0.11$	$1.391^{c} \pm 0.06$	$2.021^{b} \pm 0.06$	$2.145^{a} \pm 0.01$
	Ile	$2.049^{b} \pm 0.06$	$2.041^{b} \pm 0.07$	$2.313^{a} \pm 0.07$	$2.451^{a} \pm 0.05$
	Leu	$3.123^{c} \pm 0.06$	$4.120^{b} \pm 0.08$	$4.156^{a} \pm 0.05$	$4.551^{a} \pm 0.07$
	Phe	$2.576^{a} \pm 0.07$	$2.571^{a} \pm 0.07$	$2.591^{a} \pm 0.05$	$2.419^{ab} \pm 0.07$
	Lys	$1.779^{c} \pm 0.08$	$2.773^{b} \pm 0.06$	$2.945^{a} \pm 0.07$	$2.955^{a} \pm 0.08$
	Trp	$0.345^{b} \pm 0.04$	$0.391^{b} \pm 0.05$	$0.4510^{a} \pm 0.04$	$0.413^{a} \pm 0.05$
Total of the Essential Amino Acid (g/100 g)		15.453 (43.24%)	18.789 (43.78%)	20.462 (45.24%)	21.010 (45.34%)
Total Amino Acid (g/100 g)		35.830	42.919	45.232	45.338

Different letters in each row denote statistically significant differences between treatments ($p < 0.05$). The values are the mean of three replications SD

4 Conclusions

This study investigates the effect of enzymatic hydrolysis (Alcalase and Flavourzyme) on a protein extraction from *L. minor* and their antioxidant properties. The results showed that the enzymatic assisted extraction of protein from *L. minor* has a significant impact on the protein recovery, DH value, and antioxidant properties. The enzymatic extraction with Flavourzyme and Alcalase exhibits higher protein recovery, $DPPH^{\bullet+}$ and $ABTS^{\bullet+}$ scavenging activities than the non-enzymatic extraction. Specifically, Flavourzyme showed the highest protein recovery, DH value and antioxidant scavenging at 1.5%v/w enzyme and 120 min for incubation. The antioxidant activities of the *L. minor* protein extract were correlated to the DH value and enzyme used. The protein extract with higher DH values had the higher antioxidant properties. This study also showed that Flavourzyme and Alcalase significantly influenced the molecular weight and amino acid residue composition of the *L. minor* protein. and further influenced the antioxidant activities. Therefore, the Flavourzyme assisted extraction of *L. minor* protein was a potential process for obtaining protein with high antioxidant activity.

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