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Valorisation of low-valued ray-finned fish (*Labeobarbus nedgia*) by enzymatic hydrolysis to obtain fish-discarded protein hydrolysates as functional foods

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ABSTRACT

This study investigated the valorisation of low-valued ray-finned fish *(Labeobarbus nedgia)* (LB) muscle into valuable protein hydrolysates using three commercial enzymes (Alcalase®, Novozym®, and Protease®). After testing different enzyme concentrations (0–3 %, v/w) and temperatures (50–70 °C), the best results, including the highest degree of hydrolysis (DH) and antioxidant activity, were achieved when using Alcalase® at 3 % (v/w) and a temperature of 60 °C. However, Novozym® was found to be more resistant to high temperatures than Alcalase®. Functional properties of freeze-dried protein hydrolysates prepared using Alcalase® and Novozym®, at an enzyme concentration of 3 % (v/w) and a temperature of 60 °C, were determined. Both hydrolysates exhibited similar solubility, water and oil holding capacity, and foaming capacity and stability. This study demonstrated that the low-valued LB muscle can be used to produce a valuable protein hydrolysate, which could be used as a functional ingredient in the food industry.

1. Introduction

Recently, the emphasis on foods has shifted from providing the essential nutrients for sustaining life and growth to preventing or indeed curing various forms of diseases. Moreover, the recent population lifestyle changes, technological advancement, and socio-economic trends throughout the world indicate the need for foods with increased health benefits (Tadesse and Emire, 2020). These are the key determining and driving forces for the growth of the current development and production of functional foods in the global market.

The health benefit of functional foods is derived from the bioactive compounds, such as phytochemicals, vitamins, and peptides, found naturally in them, formed during processing, or extracted from other sources and added to them (Butnariu and Sarac, 2019). Among these bioactive compounds, antioxidant

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peptides have received considerable attention in recent times. Bioactive peptides are specific protein fragments possessing various biological functions, such as antioxidant activity, thus, can be utilized to retain human health by maintaining food safety and quality through the mitigation of oxidative stress conditions (Bashir et al., 2017; Hema et al., 2017).

Various food-derived protein sources have been used to produce bioactive peptides (Tadesse and Emire, 2020). In the search for bioactive peptides, however, protein amount and quality, and the cost of the raw materials have important roles. Therefore, those cheap materials containing high amounts and quality of protein can feasibly be used to produce bioactive peptides. As regards, marine resources, such as low-valued fish and fish byproducts, have been reported as good and inexpensive sources of bioactive peptides. Among these, catfish (Seniman et al., 2014), chub marckerel (Bashir et al., 2017), *Acetes indicus* (Dhanabalan et al., 2017), and *Epinephelus malabaricus* skin (Hema et al., 2017) have been recently reported as sources of bioactive peptides. 38.5 million tons of different fish species are discarded globally as by-catch fish due to their low economic value (Wisuthiphaet et al., 2016), despite the fact that these by-catch fish are considered valuable sources of essential protein. *Labeobarbus spp*. are endemic fish to Africa and constitute a family of about 80 large cyprinid fish species, which are widely distributed in the large rivers in Africa such as Nile, Niger, Congo and Zambezi and in the Great Rift and other Lakes of East Africa, south to KwaZulu-Natal in the east and the Orange and Clan William Olifants Rivers in the west (Skelton and Bills, 2000). However, these fish species are low-valued because of the presence of small bones inside the meat that make them difficult for consumption, thus, unacceptable to consumers in the market (Zebib et al., 2020), which consider these species by-catch fish. Moreover, there is no study conducted so far on the possibility of using these fish species for the production of value-added products. Therefore, taking these considerations into account, *Labeobarbus* fish species could be utilized for the production of bioactive antioxidant peptides helping to create new value chains from by-catch fish species contributing to fish waste valorization.

Proteins from different sources contain amino acid sequences that can give rise to bioactive peptides after hydrolysis. In this way, bioactive peptides can be obtained from proteins using either chemical or biochemical methods (Ozyurt et al., 2018). Although chemical hydrolysis methods are simple and the processes are quick, it is still challenging to get the required properties of bioactive peptides as these methods lack specificity and sensitivity, as well as they leave toxic residues in the products (Daud et al., 2015). Moreover, these methods are susceptible to amino acid damage since the raw material is treated in extreme pH values at high temperature and, in some cases, at high pressure over a given time (Ishak and Sarbon, 2018).

In the food and pharmaceutical industries, however, the enzyme hydrolysis method is preferred over the chemical hydrolysis one due to its GRAS nature (He et al., 2019). Industrial food-grade proteinases derived from microorganisms such as Alcalase® and Protamex® (Bashir et al., 2017), plant sources such as papain (Hema et al., 2017), and animal sources such as pepsin, chymotrypsin, and trypsin (Nakajima et al., 2009) have been widely used for the production of bioactive peptides. Nevertheless, scarce information is available on the performance of enzymatic activity of Novozym® and the corresponding antioxidant activities of protein hydrolysates. Besides the type of enzyme, the required properties of protein hydrolysates are affected by several factors, such as the enzyme to substrate ratio, temperature, pH, and hydrolysis time (Borrajo et al., 2020; Noman et al., 2018). Therefore, this study aimed to evaluate the effect of enzyme type and hydrolysis conditions on the antioxidant and some physicochemical and functional properties of protein hydrolysates from *Labeobarbus nedgia* muscle.

2. Materials and methods

2.1. Materials

Labeobarbus nedgia (LB) was captured in Lake Tana, Ethiopia, and used as a source of protein. The Lake Tana Basin is in the northwestern highlands of Ethiopia. It lies between 10°58′–12°47′ N and 36°45′–38°14′ E, at an altitude of 1800 m above sea level. The fish were collected in the morning time and immediately taken to Bahir Dar University, Food and Chemical Engineering laboratory using ice boxes. The fish, then, were immediately filleted, and the muscle was washed twice with freshwater, freeze-dried, grounded, and packed with polyethylene bags and stored at –20 °C for use.

2.2. Proximate composition analysis

Standard Methods from AOAC International (AOAC, 2016) were used for the determination of moisture content (AOAC 950.46), crude fat (AOAC 976.21), and ash (AOAC 920.153) content of LB muscle. The total nitrogen content of the experimental material determined by elemental analysis was used for protein determination.

2.3. Elemental and inorganic composition

The elemental composition (C, H, N, S) of the LB muscle was determined by Flash 2000 elemental microanalyzer (Thermo Scientific, USA). Oxygen content was determined by mass balance. The inorganic element composition of the LB muscle was determined by inductively coupled plasma mass spectrometry (ICP-MS-Agilent 7500cx, USA). Samples were digested with a HNO_3 solution (2 %) by using a microwave system. The digested samples were cooled and diluted with deionized water. Concentrations of inorganic elements were measured using standard solutions prepared in the same acid matrix.

2.4. Amino acid profile analysis

The amino acid profile of the LB muscle was analyzed by gas chromatography (Hewlett-Packard, 6890 series) with an EZ:faast AAA LC integrated column and FID detector (Trigueros et al., 2021). 1 g of LB muscle was hydrolyzed by mixing with 1 mL of 6 M HCl and incubated for 24 h at 110 °C. Afterward, 1 mL of a 1 M HCl and ethanol solution (1:1 v/v) was added and then filtered with a 0.2 µm cellulose acetate syringe filter. Asparagine and glutamine are converted quantitatively to aspartic and glutamic acid, respectively, during acid hydrolysis. Moreover, tryptophan, cystine, and cysteine could be destroyed by acid hydrolysis, and methionine could be destroyed partially by acid hydrolysis. Therefore, basic hydrolysis was employed to analyze these amino acids by mixing 0.1 g of LB muscle with 7 mL of 4.2 M NaOH. The mixture was incubated for 24 h at 110 °C, cooled, and

neutralized with 6 M HCl to get a pH between 1.5 and 5.5. After hydrolysis, the amino acid profile was analyzed according to the EZ:faast Phenomenex procedure (Trigueros et al., 2021), consisting of a solid phase extraction followed by derivatization and a final liquid/liquid extraction.

2.5. Enzymatic hydrolysis

Three different commercial proteases were used in this work. Protease® from *Bacillus sp*. was purchased from Sigma Aldrich. On the other hand, Novozym® 11028 and Alcalase® 2.4 L from *Bacillus licheniformis* were kindly donated by Novo Industry. Enzymatic hydrolysis was performed using a 500 mL jacketed reactor according to the procedure described by Trigueros et al. (2021). 200 mL of phosphate buffer (pH 8) were added into the reactor equipped with a magnetic stirrer. The hydrolysis was performed at different temperatures (50–70 °C) and enzyme to LB ratios (from 2 % to 3 % v/w). It must be highlighted that in this work, comparison of proteases activity was carried out on equal mass to substrate ratio instead of on equal hydrolytic activity. According to the literature Alcalase® and Novozym® enzymatic activity was determined by the casein method resulting 1157 ± 74 and 826 ± 33 µmol of tyrosine/mL for Alcalase® and Novozym®, respectively (Barea et al., 2023).

When the temperature was reached, 20 g of the LB muscle were added and waited until the mixture was homogenized. At this point, 2 mL of sample were taken and immediately the enzyme was added to the mixture, and hydrolysis was carried out for 4 h. Samples were withdrawn at regular time intervals to follow the kinetics and heated for 10 min at 100 °C to deactivate the enzyme activity and placed immediately in the ice. Samples were centrifuged (Thermo Scientific Sorvall ST16, USA) and filtered through a 0.45 µm cellulose acetate syringe filter, and kept in the refrigerator for further analysis. A control sample was also carried out at the same hydrolysis conditions but with no enzyme addition to the medium.

2.6. Characterization of the liquid enzymatic hydrolysates

2.6.1. Degree of hydrolysis, protein yield, and total hydrolysis yield

The degree of hydrolysis (DH) was estimated by the ninhydrin reaction method according to the Sigma Aldrich protocol. 1 mL of ninhydrin reagent solution was gently mixed with 2 mL of sample and heated for 10 min at 100 °C using a boiling water bath. Afterward, the samples were cooled and 5 mL of 95 % ethanol were added. The absorbance was measured at 570 nm. A calibration curve was constructed using a leucine solution daily prepared (Friedman, 2004). The DH was evaluated according to the equation by Adler-Nissen et al. (1983):

$$\mathrm{DH}(\%) = \frac{h}{h_{tot}} \times 100 \quad (1)$$

Where h is the number of equivalent peptide bonds hydrolyzed, expressed as meq/g protein and h_{tot} is the total amount of millimoles of individual amino acids per gram in the unhydrolyzed protein that can be evaluated from the amino acid profile.

Total protein content in the hydrolysates was determined by Lowry's assay (Lowry et al., 1951). A calibration curve was prepared using bovine serum albumin as standard and absorbance of samples and standards was measured at 750 nm using a Jasco spectrophotometer. The specific protein yield was calculated as:

$$\text{Specific protein yield}(\%) = \frac{\text{Protein in the hydrolysate}(g)}{\text{Protein in LB}(g)} \times 100 \quad (2)$$

The total hydrolysis yield was determined according to the method by Dhanabalan et al. (2017), and calculated using the following equation:

$$\text{Hydrolysis yield}(\%) = \frac{\text{Weight of freeze dried hydrolysate}(g)}{\text{Weight of raw material}(g)} \times 100 \quad (3)$$

2.6.2. Antioxidant capacity

2.6.2.1. Determination of DPPH radical-scavenging capacity. DPPH radical-scavenging capacity was determined according to the method used by Centenaro et al. (2011) with a slight modification. 20 µL of the liquid hydrolysate were added to a 980 µL of DPPH. In a single experiment, the sample was replaced with 20 µL of methanol to perform the control. The mixture was shaken vigorously and allowed to stand for 60 min, and the absorbance of the sample and control solutions were measured at 517 nm with a Jasco spectrophotometer. Trolox was used to prepare the calibration curve. The antioxidant capacity of the protein hydrolysates was expressed in micromoles of Trolox equivalents/g of the LB muscle (µmol TE/g LB).

2.6.2.2. Determination of ABTS radical-scavenging capacity. The ABTS radical cation ($ABTS^{\bullet+}$) decolorization assay method was performed according to Re et al. (1999). $ABTS^{\bullet+}$ was produced by reacting an equal part of ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 16 h before use. The $ABTS^{\bullet+}$ solution was diluted with ultrapure water to get an absorbance of about 0.70 at 734 nm. 20 µL of diluted sample solution were added to 980 µL of the $ABTS^{\bullet+}$ reagent and the absorbance was measured by spectrophotometry after 20 min incubation in the dark. For the blank solution, the sample was replaced with 20 µL of ultrapure water. The difference between the sample and blank absorbance was taken to the calibration curve, which was made by Trolox as standard, to calculate the antioxidant capacity of the protein hydrolysate expressed as micromoles of Trolox equivalents /g of LB (µmol TE/g LB).

2.6.2.3. Determination of ferric-reducing antioxidant power (FRAP). The reducing capacity of protein hydrolysate was determined by the ferric reducing antioxidant power (FRAP) method described by Benzie and Strain (1996). The working reagent was freshly prepared by mixing 25 mL sodium acetate buffer (pH 3.6), 2.5 mL of 20 mM $FeCl_3$, 2.5 mL TPTZ (2,4,6-tris.2-pyridyl-s-triazine), and 3 mL of ultrapure water. 30 µL of sample were added to 970 µL of FRAP reagent and kept for 30 min at 37 °C in a water bath. The absorbance was measured at 593 nm. For the control solution, the sample was replaced with 30 µL ultrapure water. The calibration curve was made with iron (II) sulfate as standard under the same conditions as the samples and results were expressed as µmol of $FeSO_4$ per g of LB, µmol Fe^{2+}/g LB).

2.7. Freeze-drying process

Freeze-dried protein hydrolysates (FDH) were obtained from the liquid hydrolysates obtained with Alcalase® and Novozym® at 60 °C for 4 h using enzyme dose of 3 % (v/w). First, liquid hydrolysates were equilibrated at –80 °C for 2 h and then submitted to freeze-drying in a Labconco FreezeDry System (Labconco Corporation, U.S.A.) at $1.5{\cdot}10^{-4}$ mbar.

2.7.1. Characterization of the freeze-dried protein hydrolysates

2.7.1.1. *Color profile determination*. Color profiles of freeze-dried protein hydrolysates were evaluated by CM-2600d colorimeter. The L*, a* and b* values represent brightness, red to green, and yellow to blue color, respectively, based on the method described by Alahmad et al. (2022). Other conditions were illuminant D65 (daylight source) and a 10° standard observer (perception of a human observer) following the CIE recommendations.

2.7.1.2. *β-carotene bleaching inhibition*. The capacity of freeze-dried hydrolysates to inhibit bleaching of β-carotene was determined as described by Koleva et al. (2002). A 1 mL aliquot of β-carotene solution (5 mg of β-carotene/10 mL of chloroform) was mixed with 25 µL of linoleic acid and 200 µL of Tween 40, using a 50 mL conical flask. The chloroform was evaporated by introducing nitrogen through the flask and 100 mL of distilled water was added. Then, the emulsion was vigorously stirred with magnetic stirrer for about 30 min 2.5 mL of the β-carotene-linoleic acid emulsion were transferred to test tubes containing 0.5 mL different concentration (from 1 to 5 mg/mL) of protein hydrolysates. The emulsion system was immediately placed in water bath and incubated for 2 h at 50 °C and the absorbance of each sample was measured at 470 nm. Butylhydroxyanisole (BHA) was used as positive standard. In the case of the control, 0.5 mL of distilled water instead of the sample solution was added to the β-carotene–linoleic acid emulsion. The antioxidant activity of the hydrolysates was evaluated in terms of bleaching of β-carotene using the following formula:

$$\textit{Inhibition of } \beta-\textit{Carotene bleaching } (\%) = 1 - \frac{(A_o - A_t)}{(A_o' - A_t')} \times 100 \quad (4)$$

where: A_o and A_o' are absorbances of the sample and the control at zero time, respectively, and A_t and A_t' are absorbances of the sample and the control at 2 h of incubation, respectively.

2.7.2. Determination of functional properties of freeze-dried protein hydrolysates

2.7.2.1. *Solubility*. The solubility of the FDH was determined according to the method reported by Yathisha et al. (2022). 200 mg of FDH were dissolved in 20 mL of distilled water, and the pH was adjusted from 2 to 12 using 0.1 M HCl or 0.1 M NaOH. The mixture was stirred continuously at 37 °C for 30 min and then centrifuged at 8000 rpm for 10 min. The protein content of the supernatant and sample was determined following the Kjeldahl method. Finally, the solubility of the samples was calculated using the following equation:

$$\text{Solubility}(\%) = \frac{\text{Protein content in supernatant}}{\text{Total protein content in the sample}} \times 100 \quad (5)$$

2.7.2.2. *Water-holding capacity*. The water-holding capacity (WHC) of FDH was determined according to the method used by Noman et al. (2018) with some modifications. 10 mL of distilled were added to 0.5 g of FDH in a 50 mL of falcon tube and vortexed for 60 s. The dispersion was centrifuged at 5000 g for 30 min after 6 h of standing time at room temperature. The supernatant was then decanted and the residue was weighed. The WHC was calculated using the following equation:

$$\text{WHC}(\text{g water/g of the sample}) = \frac{W_2 - W_1}{W_o} \quad (6)$$

where w_0 is the weight of the sample (g), w_1 is the weight of the sample with falcon tube (g), and w_2 is the weight of sample with falcon tube after decantation.

2.7.2.3. *Oil-holding capacity*. The oil-holding capacity (OHC) of FDH was evaluated according to the method used by Noman et al. (2018) with slight modification. 10 g of corn oil were added to 0.5 g of hydrolysate in a 50 mL of falcon tube and vortexed for 60 s. After 30 min, the mixture was centrifuged at 2800 g for 30 min at 20 °C. The oil was then decanted and the residue was weighed. The OHC was calculated using the following equation:

$$\text{OHC}(\text{g Oil/g of the sample}) = \frac{W_2 - W_1}{W_o} \quad (7)$$

where w_0 is the weight of the sample (g), w_1 is the weight of the sample with falcon tube (g), and w_2 is the weight of sample with falcon tube after decantation.

2.7.2.4. *Foaming capacity and foam stability*. Foaming capacity (FC) and foam stability (FS) of FDH were measured according to the methods of Noman et al. (2018) with some modifications. 1 g of the protein hydrolysate was dissolved in 50 mL of distilled water at room temperature in a volumetric cylinder of 500 mL and the foam was prepared using a homogenizer at 20,000 rpm for 2 min. The foam volume was recorded immediately. FS was determined by measuring the fall in the volume of the foam after 5 min. FC and FS were calculated using the following equations:

$$\text{FC}(\%) = \frac{V_2 - V_1}{V_1} \quad (8)$$

$$\text{Fs}(\%) = \frac{V_3 - V_1}{V_1} \quad (9)$$

where V_1 is the volume before whipping, V_2 is the total volume after whipping, and V_3 is the total volume after standing time of 5 min

2.8. Modeling of the hydrolysis curves

Modeling the hydrolysis curves is a useful tool to optimize the extraction process reducing the cost of the process. The use of empirical models helps to simplify the study of complex systems such as the extraction of bio-compounds from raw materials (Alonso-Riaño et al., 2020). In this work, the Weibull model, which has been previously proposed to model the extraction and recovery of compounds from different types of solid matrix, was used to fit the experimental data obtained from the extraction and try to elucidate the extraction mechanism. Weibull's model can be expressed as:

$$DH = D(1 - \exp(-bt^n)) \quad (10)$$

where DH is the hydrolysis degree, t is the extraction time (min) and D, b and n are the kinetic parameters.

A deep analysis of the kinetic parameters for the models would help to determine the dependence on operating conditions leading to a better design, simulation, optimization, and control of further industrial processes. To estimate the kinetic parameters, non-linear regression was performed by using the SigmaPlot software version 14.5. Experimental results were then compared with those of the model prediction through the values of the Root Mean Square Deviation (RMSD) between experimental and calculated extraction yields:

$$RMSD = \sqrt{\frac{\sum_{i=1}^{n} (DH_{exp} - DH_{calc})^2}{n}} \quad (11)$$

where n is the number of experimental data points in each kinetic curve.

2.9. Statistical analysis

The results were analyzed by ANOVA (one-way) and Fisher's Least Significant Difference (LSD) at a p-value < 0.05 using the R statistical package (version 19.0). The results are expressed as the mean ± standard deviation of at least in duplicates.

3. Results and discussion

3.1. Chemical composition of the raw material

The moisture content of the freeze-dried LB muscle was 3.00 ± 0.01 % (w/w). Table 1 presents the proximate compositions of the freeze-dried LB muscle in a dry basis. The protein, ash, and crude fat content of the LB muscle were 71.9 ± 0.7, 9.5 ± 0.2 and 14.7 ± 0.2 %, respectively (total identified compounds 96 ± 1 % in a dry basis). Carbohydrate analysis were not carried out in this work, since generally fish has a low content of carbohydrates (Ahmed et al., 2022). In general, it has been assessed that carbohydrates are usually neglected during analysis of fish. In a recent review, Ahmed et al. (2022) reported that the main components of fish were 66 %–81 % water, 16 %–21 % protein, 1.2 %–1.5 % mineral, 0.2 %–25 % fat and 0 %–0.5 % carbohydrate (less than 2.8 % in a dry basis). Based on this literature data, the difference up to 100 % of composition might be come from carbohydrate content, but also due to the accuracy and precision of the methods used to determine the moisture, fat, and ash contents. The mean value of the protein content was slightly lower than the content (77.8 % w/w, dry basis) of *Labeobarbus intermedius* reported by Geremew et al. (2020). However, the crude fat and ash contents were higher than the values reported by the same authors, where the fat and ash content were 12 % and 5.1 % (w/w) in a dry basis, respectively.

Table 1 – Proximate and elemental composition in a weight percentage dry basis of the freeze-dried LB muscle in dry basis.

Compound	Composition, %	Element	Composition, %
Proteins	71.9 ± 0.7	C	50.8 ± 0.6
Lipids	14.7 ± 0.2	H	8.1 ± 0.1
Ashes	9.5 ± 0.2	N	13.1 ± 0.1
		O	18 ± 1
		S	0.6 ± 0.1

Crude protein was obtained from the nitrogen content and the corresponding conversion factor calculated from the amino acid profile of the LB muscle (see Table 2) according to the NREL standard protocols. A N-factor of 5.5 was determined for LB muscle. This value agrees with the value recently reported by Barea et al. (2023) for fish meal with a N-factor of 5.0, what indicates the presence of other non-amino acids N-compounds in fish products. The LB muscle contained all amino acids with a total amino acid (TAA) content of 862 ± 20 mg/g_{prot} and a total essential amino acid (TEAA) content of 346 ± 11 mg/g_{prot} (see Table 2). The TAA content obtained in the LB muscle was in line with the value of the Chinese sturgeon muscle, 89 g/100 g_{prot} (Noman et al., 2018). However, the TEAA content in the LB muscle was lower than the value in the Chinese sturgeon muscle, 49 g/100 g_{prot}. Glutamic acid was found as a major amino acid in the LB muscle with a content of 141.3 ± 0.1 mg/g_{prot}. The LB muscle was also rich in aspartic acid (119 ± 4 mg/g_{prot}), alanine (75.6 ± 0.7 mg/g_{prot}), leucine (74.0 ± 0.2 mg/g_{prot}), lysine (70.9 ± 2.6 mg/g_{prot}) and glycine (63.6 ± 0.4 mg/g_{prot}). However, it contained low levels of cysteine (1.8 ± 0.2 mg/g_{prot}) and tryptophan (5.5 ± 0.4 mg/g_{prot}). Shahidi et al. (1995) also reported similar results for Capelin (*Mallotus villosus*), which contained a high amount of glutamic acid, aspartic acid, alanine, and leucine, but low levels of cysteine and tryptophan.

The elemental composition (CHNSO) and inorganic elements of freeze-dried LB muscle are also presented in Tables 1 and 3, respectively. Among the essential elements, K was the most abundant (18 ± 2 g/kg). Traces of some toxic elements such as Hg, Pb, and Cr were found at permissible levels, whereas others such as Cd were not detected.

3.2. Degree of hydrolysis (DH)

The DH is defined as the amount of the total number of peptide bonds that are cleaved during hydrolysis. The degree to which a protein source has been hydrolyzed is a reflection of the number of peptide bonds broken and, therefore, the average size of the peptides present. Thus, the extent of peptide bonds broken down by proteolytic enzymes is significantly related to protein recovery yield, biological activities, and functional properties of the resulting protein hydrolysates (Yarnpakdee et al., 2012).

3.2.1. Effect of type of enzyme

Three different enzymes namely Alcalase®, Novozym®, and Protease® were used to hydrolyze the LB protein. The hydrolysis was performed at similar hydrolysis conditions, namely enzyme concentration on equal mass to substrate ratio of 2 % (v/w), substrate concentration of 10 % (w/v), hydrolysis temperature of 60 °C, and pH 8. DH was evaluated according to Eq. (1), with a h_{tot} value of 7 according to the muscle amino acid profile. As presented in Fig. 1A, the rate of hydrolysis increased rapidly in the first 60 min due to the hydrolysis of a large number of peptide bonds (Yarnpakdee et al., 2012). Thereafter, the hydrolysis rate was decreased gradually and finally reached a plateau. The enzyme added at the start of the process split a bunch of peptide bonds, and the hydrolysis of the most compacted core proteins took place afterwards (Dhanabalan et al., 2017). Moreover, the rate of hydrolysis decreased as the reaction time increased due to

Table 2 – Amino acid profile of freeze-dried LB muscle and free amino acid (FAA) profile of protein hydrolysates obtained using different enzymes at different concentrations and hydrolysis temperatures.

Amino Acid (mg/g_{prot})	LB muscle	Control	Protease®	Alcalase®						Novozym®					
		0 %	2 %	2 %			3 %			2 %			3 %		
		60 °C	60 °C	50 °C	60 °C	70 °C	50 °C	60 °C	70 °C	50 °C	60 °C	70 °C	50 °C	60 °C	70 °C
ALA	75.6 ± 0.7	1.60 ± 0.01 ^{h}	1.53 ± 0.01 ^{h}	2.76 ± 0.00^{b}	2.63 ± 0.05^{c}	2.19 ± 0.08^{e}	2.71 ± 0.07^{bc}	3.1 ± 0.06^{a}	2.08 ± 0.00 ^{f}	2.17 ± 0.04^{ef}	2.37 ± 0.04^{d}	2.36 ± 0.03^{d}	2.21 ± 0.01^{e}	1.94 ± 0.04 ^{g}	2.25 ± 0.08^{e}
GLY	63.6 ± 0.4	5.31 ± 0.00^{i}	5.48 ± 0.00 ^{h}	6.27 ± 0.00 ^{cd}	4.81 ± 0.07^{j}	6.37 ± 0.09^{bc}	6.19 ± 0.18^{de}	5.84 ± 0.06 ^{f}	6.29 ± 0.00^{bcd}	5.89 ± 0.01 ^{f}	6.1 ± 0.03^{e}	6.64 ± 0.06^{a}	6.41 ± 0.03^{b}	5.69 ± 0.01 ^{g}	6.41 ± 0.03^{b}
VAL	37.1 ± 0.2	0.43 ± 0.01^{i}	0.54 ± 0.02^{fg}	0.58 ± 0.00^{de}	0.62 ± 0.02^{bc}	0.51 ± 0.01^{gh}	0.57 ± 0.02^{ef}	0.64 ± 0.02^{b}	0.49 ± 0.00 ^{h}	0.57 ± 0.01^{ef}	0.72 ± 0.04^{a}	0.60 ± 0.00 ^{cd}	0.63 ± 0.01^{bc}	0.60 ± 0.00^{cde}	0.64 ± 0.00^{bc}
LEU	74.0 ± 0.2	0.59 ± 0.01 ^{g}	1.04 ± 0.03 ^{f}	1.54 ± 0.09^{e}	1.74 ± 0.04^{bc}	1.06 ± 0.08 ^{f}	1.53 ± 0.06^{e}	1.8 ± 0.02^{ab}	0.95 ± 0.00 ^{f}	1.52 ± 0.02^{e}	1.88 ± 0.01^{a}	1.62 ± 0.01^{de}	1.66 ± 0.06 ^{cd}	1.74 ± 0.07^{bc}	1.68 ± 0.04 ^{cd}
ILE	30.8 ± 0.2	0.26 ± 0.01 ^{h}	0.33 ± 0.01^{efg}	0.37 ± 0.00^{cdef}	0.4 ± 0.01 ^{cd}	0.31 ± 0.01^{fgh}	0.35 ± 0.04^{defg}	0.47 ± 0.02^{ab}	0.3 ± 0.00 ^{gh}	0.34 ± 0.00^{defg}	0.38 ± 0.03^{cde}	0.38 ± 0.00^{cdef}	0.42 ± 0.06^{bc}	0.32 ± 0.08^{efg}	0.51 ± 0.03^{a}
THR	43.8 ± 2.2	0.63 ± 0.00^{g}	0.55 ± 0.00 ^{h}	1.04 ± 0.04^{bc}	1.04 ± 0.02^{bc}	0.9 ± 0.03^{e}	1.00 ± 0.01 ^{cd}	1.28 ± 0.00^{a}	0.82 ± 0.00 ^{f}	0.96 ± 0.02^{de}	1.07 ± 0.05^{b}	1.09 ± 0.00^{b}	0.99 ± 0.06 ^{cd}	0.97 ± 0.03^{d}	0.97 ± 0.00^{d}
SER	36.4 ± 1.8	0.45 ± 0.01 ^{h}	0.38 ± 0.00 ^{h}	1.07 ± 0.00^{c}	1.23 ± 0.02^{b}	0.73 ± 0.01^{e}	1.07 ± 0.03^{c}	1.63 ± 0.01^{a}	0.56 ± 0.00 ^{g}	0.75 ± 0.00^{e}	0.82 ± 0.05^{d}	0.84 ± 0.00^{d}	0.71 ± 0.05^{e}	0.63 ± 0.05 ^{f}	0.72 ± 0.02^{e}
PRO	35.4 ± 0.9	0.34 ± 0.00 ^{g}	0.27 ± 0.01 ^{h}	0.41 ± 0.00 ^{cd}	0.40 ± 0.00 ^{cd}	0.41 ± 0.00^{bc}	0.42 ± 0.02^{bc}	0.51 ± 0.02^{a}	0.4 ± 0.00^{cde}	0.38 ± 0.00^{def}	0.38 ± 0.03^{ef}	0.44 ± 0.00^{b}	0.41 ± 0.01 ^{cd}	0.36 ± 0.02 ^{f}	0.41 ± 0.01^{bc}
ASP	119 ± 4	0.45 ± 0.01^{hi}	0.31 ± 0.01^{j}	0.53 ± 0.00^{def}	0.53 ± 0.03^{cde}	0.56 ± 0.00^{bcd}	0.50 ± 0.02^{efg}	0.61 ± 0.02^{a}	0.58 ± 0.00^{ab}	0.47 ± 0.01^{gh}	0.48 ± 0.04^{fgh}	0.58 ± 0.01^{abc}	0.49 ± 0.01^{fg}	0.42 ± 0.01^{i}	0.52 ± 0.04^{defg}
MET	32.8 ± 0.5	0.24 ± 0.00 ^{h}	0.36 ± 0.01 ^{g}	0.92 ± 0.00^{e}	1.20 ± 0.14 ^{cd}	0.49 ± 0.00 ^{f}	0.88 ± 0.00^{e}	1.29 ± 0.02^{abc}	0.38 ± 0.00 ^{g}	1.26 ± 0.03^{bcd}	1.37 ± 0.01^{a}	1.33 ± 0.02^{ab}	1.25 ± 0.08^{bcd}	1.19 ± 0.00^{d}	1.18 ± 0.01^{d}
HYP	4.8 ± 0.8	0.15 ± 0.00 ^{g}	0.33 ± 0.02^{c}	0.42 ± 0.01^{b}	0.22 ± 0.01 ^{f}	0.31 ± 0.00 ^{cd}	0.41 ± 0.03^{b}	0.22 ± 0.00 ^{f}	0.26 ± 0.00^{e}	0.44 ± 0.00^{b}	0.28 ± 0.00^{de}	0.61 ± 0.01^{a}	0.59 ± 0.02^{a}	0.22 ± 0.00 ^{f}	0.44 ± 0.04^{b}
GLU	141.3 ± 0.1	0.95 ± 0.00 ^{g}	0.61 ± 0.01^{i}	1.42 ± 0.00^{c}	1.67 ± 0.1^{b}	1.14 ± 0.01^{e}	1.26 ± 0.01^{d}	1.62 ± 0.06 ^{h}	1.07 ± 0.00 ^{f}	1.39 ± 0.02^{c}	1.75 ± 0.02^{a}	1.40 ± 0.01^{c}	0.93 ± 0.01 ^{g}	0.83 ± 0.00 ^{h}	1.16 ± 0.01^{e}
PHE	42.1 ± 1.7	0.30 ± 0.00 ^{h}	0.41 ± 0.02 ^{g}	0.90 ± 0.01^{bc}	0.92 ± 0.05^{b}	0.55 ± 0.01 ^{f}	0.78 ± 0.08^{e}	1.08 ± 0.01^{a}	0.41 ± 0.00 ^{g}	0.80 ± 0.01^{de}	1.07 ± 0.00^{a}	0.86 ± 0.01 ^{cd}	0.92 ± 0.01^{bc}	1.06 ± 0.01^{a}	0.83 ± 0.03^{de}
LYS	70.9 ± 2.6	1.86 ± 0.04^{i}	1.32 ± 0.03^{j}	2.55 ± 0.01 ^{f}	2.58 ± 0.21 ^{f}	2.17 ± 0.03 ^{h}	2.89 ± 0.04^{d}	2.33 ± 0.05 ^{f}	3.15 ± 0.01^{c}	2.81 ± 0.00^{de}	4.45 ± 0.03^{b}	2.78 ± 0.02^{de}	2.73 ± 0.03^{e}	6.71 ± 0.02^{a}	2.78 ± 0.1^{de}
HIS	9.1 ± 2.5	5.61 ± 0.02^{j}	5.13 ± 0.03^{k}	7.52 ± 0.04^{d}	7.15 ± 0.04 ^{f}	6.82 ± 0.01 ^{g}	6.84 ± 0.01 ^{g}	7.98 ± 0.05^{b}	5.15 ± 0.01^{k}	7.25 ± 0.01^{e}	6.62 ± 0.03 ^{h}	8.21 ± 0.04^{a}	7.80 ± 0.00^{c}	5.77 ± 0.02^{i}	8.06 ± 0.1^{b}
HLY	5.3 ± 0.4	0.13 ± 0.00^{k}	0.40 ± 0.02 ^{g}	0.59 ± 0.03^{d}	0.87 ± 0.01^{c}	0.39 ± 0.00 ^{g}	0.58 ± 0.02^{d}	0.54 ± 0.00^{e}	1.00 ± 0.00^{b}	0.29 ± 0.01^{i}	0.35 ± 0.02 ^{h}	0.25 ± 0.00^{j}	0.27 ± 0.00^{ij}	1.79 ± 0.03^{a}	0.51 ± 0.01 ^{f}
TYR	33.5 ± 0.2	0.24 ± 0.01 ^{h}	0.24 ± 0.00 ^{h}	0.68 ± 0.00^{e}	1.04 ± 0.03^{bc}	0.47 ± 0.00 ^{g}	1.22 ± 0.09^{a}	0.99 ± 0.01 ^{cd}	0.60 ± 0.03 ^{f}	0.71 ± 0.02^{e}	1.04 ± 0.00^{bc}	0.74 ± 0.00^{e}	0.98 ± 0.06 ^{cd}	0.94 ± 0.06^{d}	1.08 ± 0.03^{b}
TRP	5.5 ± 0.4	0.00 ± 0.00^{i}	0.26 ± 0.01^{c}	0.12 ± 0.00^{ef}	0.74 ± 0.00^{a}	0.09 ± 0.01^{gh}	0.11 ± 0.01^{fg}	0.14 ± 0.00^{a}	0.21 ± 0.00^{d}	0.09 ± 0.00 ^{h}	0.10 ± 0.00^{fgh}	0.10 ± 0.00^{gh}	0.11 ± 0.02^{fg}	0.20 ± 0.00^{d}	0.31 ± 0.03^{b}
CYS	1.8 ± 0.2	0.00 ± 0.00 ^{i}	0.09 ± 0.01^{k}	0.20 ± 0.00^{j}	0.56 ± 0.01^{c}	0.26 ± 0.00 ^{h}	0.28 ± 0.00 ^{g}	0.37 ± 0.00^{e}	0.23 ± 0.01^{i}	0.57 ± 0.01^{c}	0.65 ± 0.01^{b}	0.41 ± 0.00^{d}	0.64 ± 0.01^{b}	0.4 ± 0.0 ^{f}	0.7 ± 0.0^{a}
AA, FAA	862 ± 20	19.5 ± 0.3^{i}	19.6 ± 0.3^{i}	29.9 ± 0.3^{de}	30.3 ± 0.9^{d}	25.7 ± 0.4 ^{g}	29.6 ± 0.8^{e}	32.4 ± 0.4^{a}	24.9 ± 0.1 ^{h}	28.6 ± 0.2 ^{f}	31.9 ± 0.5^{ab}	31.2 ± 0.2^{bc}	30.1 ± 0.5^{de}	31.0 ± 1.4^{bc}	31.2 ± 0.6^{c}
Yield (%)		2.3 ± 0.1	2.3 ± 0.1	3.5 ± 0.1	3.5 ± 0.1	3.0 ± 0.1	3.4 ± 0.1	3.8 ± 0.1	2.89 ± 0.03	3.3 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	3.5 ± 0.1	3.6 ± 0.2	3.6 ± 0.1
EAA	346 ± 11	9.9 ± 0.2^{i}	9.9 ± 0.2^{i}	15.6 ± 0.2^{e}	16.4 ± 0.5^{d}	12.9 ± 0.2 ^{g}	15.0 ± 0.3 ^{f}	17.0 ± 0.2^{c}	11.86 ± 0.02 ^{h}	15.6 ± 0.1^{e}	17.7 ± 0.2^{b}	17.0 ± 0.1^{c}	16.5 ± 0.3^{d}	17.8 ± 1.2^{a}	17.0 ± 0.4^{c}
EAA/AA (%)	40.1 ± 0.1	50.7 ± 0.2 ^{f}	50.74 ± 0.01 ^{f}	52.0 ± 0.2^{e}	54.0 ± 0.2^{d}	50.1 ± 0.1 ^{g}	50.5 ± 0.5^{fg}	52.4 ± 0.1^{e}	47.6 ± 0.0 ^{h}	54.4 ± 0.1 ^{cd}	55.4 ± 0.1^{b}	54.3 ± 0.2 ^{cd}	54.7 ± 0.3^{c}	58.5 ± 0.2^{a}	54.4 ± 0.3 ^{cd}
HPAA/AA (%)	54.6 ± 0.4	60.3 ± 0.3	65.2 ± 0.1	59.6 ± 0.1	57.83 ± 0.01	60.8 ± 0.5	60.6 ± 0.9	59.0 ± 0.1	61.01 ± 0.01	59.8 ± 0.2	59.6 ± 0.2	59.6 ± 0.2	61.5 ± 0.1	56.1 ± 1.5	60.9 ± 0.5

Values with different letters in each row are significantly different when applying the Fisher's least significant differences (LSD) method at p-value < 0.05. AA amino acids, FAA free amino acids, EAA essential amino acids, HPAA hydrophobic amino acid.

Table 3 – Inorganic composition of the freeze-dried LB muscle.

Element	ppm	Element	ppm	Element	ppm	Element	ppm	Element	ppm	Element	ppm
Li	0.4 ± 0.6	P	5354 ± 925	V	0.08 ± 0.02	Cu	1.0 ± 0.2	Sr	6.3 ± 1.7	La	0.02 ± 0.01
B	1.2 ± 1.6	S	5647 ± 1089	Cr	0.4 ± 0.1	Zn	18.8 ± 4.1	Zr	0.11 ± 0.03	Ce	0.05 ± 0.01
Na	1994 ± 392	Cl	1780 ± 440	Mn	1.7 ± 0.4	Ga	0.3 ± 0.1	Nb	0.02 ± 0.01	Pr	0.01 ± 0.00
Mg	1149 ± 326	K	17588 ± 2213	Fe	50.3 ± 5.8	Se	0.8 ± 0.1	I	0.18 ± 0.05	Nd	0.02 ± 0.00
Al	31.2 ± 9.1	Ca	218 ± 52	Co	0.05 ± 0.01	Br	35.5 ± 0.3	Cs	0.01 ± 0.00	Hg	0.3 ± 0.1
Si	166 ± 44	Ti	0.8 ± 0.2	Ni	0.09 ± 0.04	Rb	8.4 ± 1.2	Ba	1.1 ± 0.3	Pb	0.02 ± 0.01
									Total	34055 ± 5507	

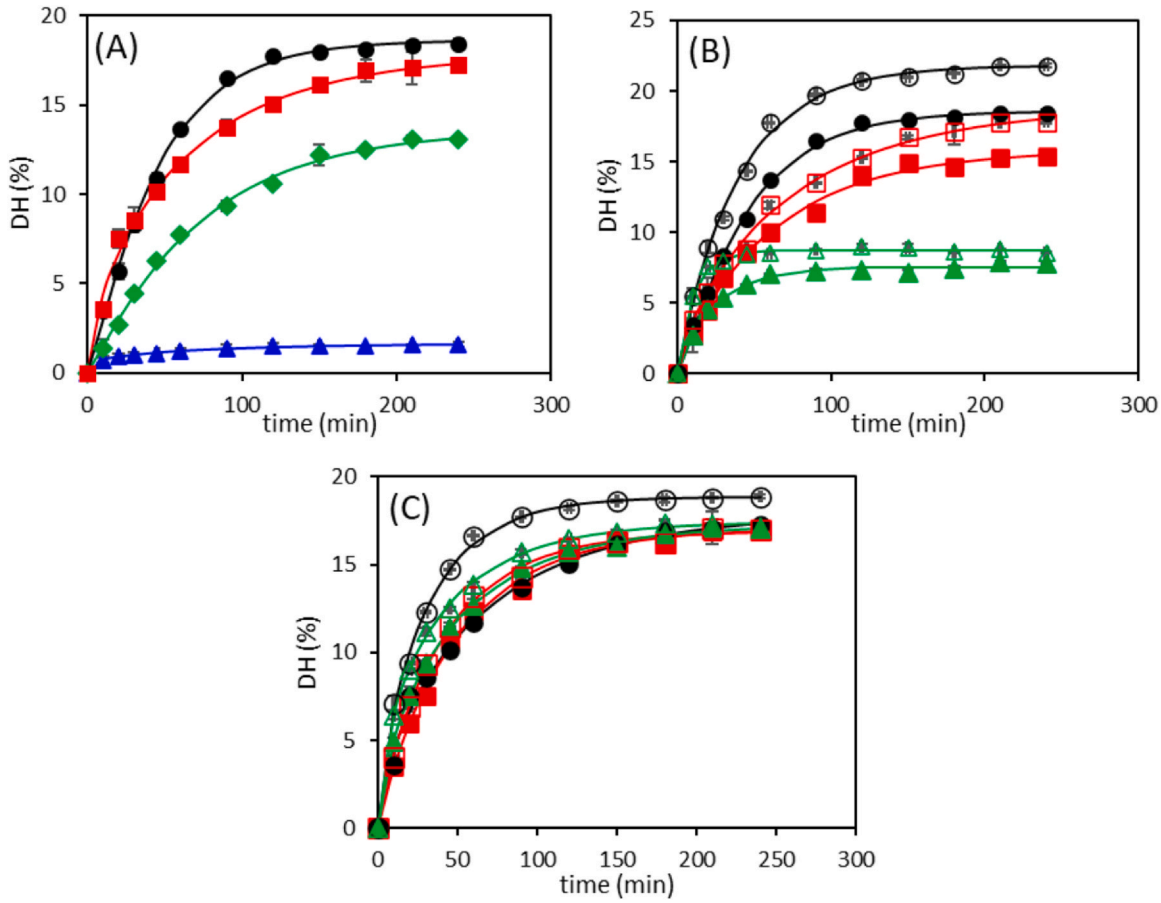

Fig. 1 – Kinetics of LB muscle hydrolysis by using different enzymes at different temperatures and enzyme concentrations. (A) Effect of enzyme type on DH performed at enzyme concentration of 2 % (v/w) and temperature of 60 °C (● Alcalase®, ■ Novozym®, ◆ Protease®, ▲ Control), (B) Effect of Alcalase® concentration and temperature on DH, (C) Effect of Novozym® concentration and temperature on DH. Symbols for (B) and (C): ■ 2 % (v/w) and 50 °C, ● 2 % (v/w) and 60 °C, ▲ 2 % (v/w) and 70 °C, □ 3 % (v/w) and 50 °C, ○ 3 % (v/w) and 60 °C, △ 3 % (v/w) and 70 °C. The continuous lines represent the Weibull model.

a decrease in the number of hydrolysis sites in the substrate, enzyme autodigestion, and/or product inhibition. The shape of the hydrolysis curves obtained in this study is similar to those previously reported for Chinese sturgeon protein hydrolysates (Noman et al., 2018).

At the final hydrolysis time (240 min), a significantly ($p < 0.05$) higher DH value was recorded by Alcalase® (18.4 ± 0.3 %) followed by Novozym® (17 ± 2 %) and Protease® (15.3 ± 0.1 %). This might be due to the enzymatic activity of Alcalase® being more efficient than Novozym® and Protease® for preparing the LB protein hydrolysates, but also to the higher hydrolytic activity of Alcalase® per mL of enzyme commercial solution, since comparison was done based on equal mass to substrate ratio and not on equal hydrolytic activity. This result showed that the susceptibility of the peptide bonds of the LB protein to hydrolysis reaction depends on the type of enzymes. Alcalase® has been testified as the most efficient enzyme to hydrolyze protein from a wide range of different sources such as ribbon fish (Yathisha et al., 2022) and catfish (Seniman et al., 2014). Therefore, based on the lower DH for Protease® compared to Alcalase® and Novozym®, only these two commercial enzymes were used further to investigate the effect of enzyme concentration and incubation temperature on the degree of hydrolysis. Fig. 1 A also shows the control kinetic where no enzyme was added to the medium. DH reached in the control assay was less than 1.6 %, which possibly comes from the action of endogenous enzymes and free amino acid content in the sample. Due to the low DH reached when no external enzymes were added to the reaction medium, control assay was only carried out at 60 °C, focusing the next section on the different behaviour of Alcalase® and Novozym® with temperature and enzyme loading.

3.2.2. *Effect of temperature and enzyme loading*

Fig. 1B shows the degree of hydrolysis for Alcalase® hydrolysates prepared at different enzyme concentrations and hydrolysis temperatures. Both, enzyme concentration and temperature significantly affected the DH. Increasing the enzyme concentration from 2 % to 3 % increased the overall hydrolysis rate and final DH. On the other hand, the DH increased when the hydrolysis temperature increased from 50 to 60 °C. However, further increasing the incubation temperature to 70 °C, the DH values significantly decreased. Obtaining the lowest DH at the highest hydrolysis temperature might be due to the thermal denaturation of the enzyme, which reduced the capacity of Alcalase® to cleave the peptide bonds (Noman et al., 2018). Wisuthiphaet et al. (2016) reported a similar result on the production of protein hydrolysates from low-valued fish by which the DH decreased when the temperature increased from 60 to 70 °C.

Unlike Alcalase® treatment, there were no remarkable differences in DH values between Novozym® hydrolysates when the hydrolysis was carried out at different temperatures (50–70 °C) using an enzyme concentration of 2 % (Fig. 1C). However, a significantly higher DH value was achieved when hydrolyzing the LB muscle at a temperature of 60 °C and a Novozym® to LB ratio of 3 %. In any case, the decrease in the DH from 60 to 70 °C was about 10 %, which was much less than the decrease in DH by using Alcalase®, 60 %.

Generally, at all possible combinations of the hydrolysis conditions, the highest DH value of 21.8 ± 0.1 % was achieved by Alcalase® treatment at an enzyme concentration of 3 % and hydrolysis temperature of 60 °C. On the contrary, the lowest DH value of 7.79 ± 0.01 % was found when hydrolyzing the LB using Alcalase® at a concentration of 2 % and 70 °C.

3.2.3. *Kinetic model*

The Weibull model was applied to fit the hydrolysis curves and estimate its parameters. This model has successfully been applied to different extraction and hydrolysis curves (Alonso-Riaño et al., 2020). The Weibull model fitted the data fairly well, with RMSD values ranging from 0.85 % to 6.68 % (R^2 > 0.993; P < 0.001), and the estimated values of the parameters were generated and presented in Table 4.

The 'D' constant of Weibull model showed a similar trend for Alcalase® and Novozym®, and resulted in very close values to the experimental DH values at the final hydrolysis time since this parameter is considered as the maximum DH at infinity time (see Eq. 10). The D constant and the DH, at the final hydrolysis time, generally increased as the ratio of enzymes to LB increased; although this increase was more remarkable for Alcalase® than for Novozym® probably due to the higher hydrolytic activity of Alcalase® for mL of enzymatic commercial solution. On the other hand, this parameter increased as the hydrolysis temperature increased from 50 to 60 °C. Further increasing the temperature to 70 °C, the D parameter decreased strongly for Alcalase® being this decrease less significant for Novozym® indicating that Novozym® was less sensitive to temperature than Alcalase®, as previously explained.

In general, the 'b' constant, the hydrolysis rate, increased as the concentration of Alcalase® and hydrolysis temperature increased. According to the value of this parameter, the highest value of the b parameter was found at a temperature of 70 °C. This might be due to a higher activity of Alcalase® at the early hydrolysis time and a reduction of the activity of the enzyme as the hydrolysis time further increased resulting in a lower DH value. The parameter 'c' indicates the shape of the hydrolysis curve. Most of the values of 'c' were less than 1, except those treated with 2 % Protease® and Alcalase® at 60 °C with values slightly higher than 1, indicating that the shape is parabolic with a high initial slope followed by an exponential shape.

3.3. *Protein yield and total hydrolysis yield*

Table 5 displays the recovered protein content, specific protein yield, and total hydrolysis yield of proteases hydrolysates prepared using Alcalase® and Novozym® at different concentrations and hydrolysis temperatures after 240 min hydrolysis, as well as the final values of DH.

The specific protein yield is defined as the ratio of protein content recovered during hydrolysis to the total protein content in the source (Eq. 2). As a general trend, the specific protein yield increased as the concentration of enzymes increased; although similar to DH this increase was more remarkable for Alcalase® due to higher hydrolytic activity per mL of sample. This result is aligned with Ramakrishnan et al. (2013) who reported that increasing enzyme concentration from 0.5 % to 2.0 % increased the protein yield because more enzyme molecules are associated with the substrate and cleaved large number of peptide bonds, thus, releasing more soluble protein into the system.

Regarding the effect of temperature on specific protein yield, an optimum working temperature of 60 °C was observed for

Table 4 – Kinetic parameters of the Weibull models for DH.

Enzyme	Enzyme concentration (v/w %)	Hydrolysis Temperature (°C)	Weibull parameters				
			D	b	c	R^2	RMSD
Control	0	60	1.673	0.009	0.336	0.993	0.0291
Protease®	2	60	13.658	0.014	1.054	0.998	0.0438
Alcalase®	2	50	15.821	0.015	0.902	0.996	0.0342
		60	18.607	0.024	1.009	0.998	0.0571
		70	7.557	0.038	0.857	0.994	0.0278
	3	50	18.858	0.012	0.753	0.997	0.0358
		60	21.775	0.025	0.956	0.997	0.0335
		70	8.738	0.078	0.734	0.996	0.0179
Novozym®	2	50	17.045	0.019	0.906	0.997	0.0274
		60	17.899	0.013	0.664	0.995	0.0668
		70	17.298	0.017	0.662	0.999	0.0085
	3	50	16.874	0.022	0.851	0.998	0.0228
		60	18.847	0.028	0.739	0.998	0.0298
		70	17.418	0.019	0.574	0.999	0.0132

Table 5 – Protein content, specific protein yield, hydrolysis yield, and DH of protein hydrolysates prepared using Alcalase® and Novozym® at different concentrations and hydrolysis temperatures, and control obtained no enzyme addition.

Enzyme	Enzyme concentration (v/w, %)	Hydrolysis Temperature (°C)	mg protein/g	Protein yield, %	Hydrolysis yield (%)	DH (%)
No enzyme (Control)	0	60	98 ± 1.6^{h}	13.7 ± 0.2^{h}	12.0	1.6 ± 0.1^{f}
Alcalase®	2	50	525 ± 1^{e}	73.0 ± 0.2^{e}	33.7	15.3 ± 0.1^{d}
		60	611 ± 3^{c}	85.0 ± 0.5^{c}	40.2	18.4 ± 0.3^{b}
		70	382 ± 1^{g}	53.2 ± 0.2^{g}	20.7	7.79 ± 0.03^{e}
	3	50	530 ± 9^{e}	73.7 ± 1.2^{e}	37.1	17.81 ± 0.01^{bc}
		60	659 ± 5^{a}	91.6 ± 0.7^{a}	46.6	21.8 ± 0.1^{a}
		70	459 ± 1^{f}	63.9 ± 0.1^{f}	21.5	8.58 ± 0.01^{e}
Novozym®	2	50	548 ± 5^{d}	76.2 ± 0.7^{d}	34.0	17 ± 0.1^{c}
		60	625 ± 3^{b}	87.0 ± 0.4^{b}	38.0	17.2 ± 1.9^{c}
		70	633 ± 7^{b}	88.0 ± 1.0^{b}	35.0	17.13 ± 0.02^{c}
	3	50	615 ± 1^{c}	85.6 ± 0.1^{c}	36.3	17.0 ± 0.2^{c}
		60	650 ± 1^{a}	90.3 ± 0.1^{a}	39.5	18.9 ± 0.1^{b}
		70	627 ± 6^{b}	87.1 ± 0.8^{b}	36.8	17.04 ± 0.02^{c}

Values with different letters in each column are significantly different when applying the Fisher's least significant differences (LSD) method at p-value < 0.05.

Alcalase® with lower values at 50 °C, but even lower at 70 °C due to enzyme inactivation. These results agree with the values of DH previously discussed. On the other hand, for Novozym® the lowest protein yields were obtained at the lowest temperature essayed in this work, 50 °C, but similar results were obtained at 60 and 70 °C proving that Novozym® was less affected by the high working temperature. Based on the values of the specific protein yield, it can be concluded that Alcalase® is more sensitive to temperature than Novozym®, since also lower protein yields were obtained at 50 °C compared to Novozym®.

Table 5 also lists the total hydrolysis yield evaluated according to Eq. 3. The total hydrolysate yields increased as the concentration of enzymes increased observing maximum hydrolysis yields when operating at 60 °C for both enzymes, with higher sensitivity towards temperature for Alcalase®, but similar to DH and the protein yield the increased was more evident for Alcalase®. The highest total hydrolysis yield was determined for Alcalase® at 60 °C and 3 % of enzyme dose, achieving a value of 46.6 %, much higher than the value reported for Novozym® under similar working conditions, 39.5 %. The highest response for Alcalase® at 60 °C and 3 % v/w of enzyme concentration was also observed in the protein content (659 ± 5 mg/g).

From these results, it is clear that the total hydrolysis and protein yield are correlated with the DH. In general, the highest hydrolysis yield (46.6 %) and protein yield (91.6 %) were recorded at the highest DH (21.8 %) by Alcalase® treatment at a concentration of 3 % (v/w) and 60 °C. On the other hand, the lowest hydrolysis yield (20.7 %) and protein yield (53.2 %) were recorded at the lowest DH (7.79 %) by Alcalase® treatment at 2 % (v/w) enzyme concentration and 70 °C.

Fig. 2 shows the relationship between protein and total hydrolysis yield as a function of the DH. A similar trend was observed for both enzymes, although it must be highlighted the narrower DH range for Novozym® as described in previous sections. A linear relationship was observed for both, protein and total hydrolysis yield, as a function of DH showing that an increase in enzyme activity led to an increase in DH accompanied by a further increase in the protein recovery in the hydrolysate and in the total hydrolysis yield at the experimental conditions essayed in this work. In the progress of the protein hydrolysis, the added enzyme is involved in the breakdown and solubilization of the insoluble

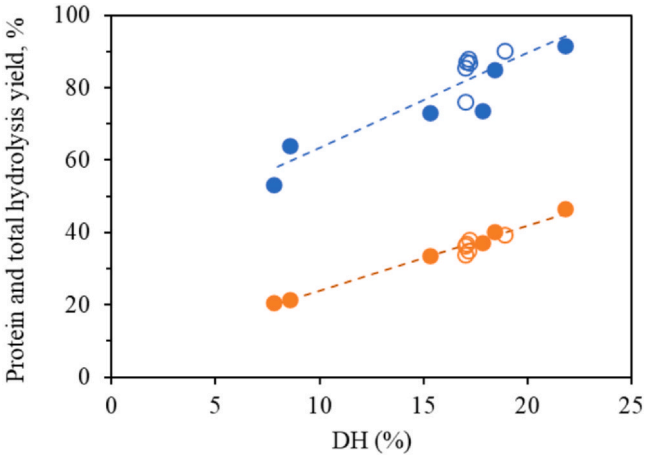

Fig. 2 – Protein and total hydrolysis yield (%) as a function of hydrolysis degree (%): (●, ○) Protein yield for Alcalase® and Novozym®, respectively (protein yield = 2.6161·DH +37.26, r^2 = 0.7985), (●, ○) total hydrolysis yield for Alcalase® and Novozym®, respectively (total hydrolysis yield = 1.7879·DH +6.052, r^2 = 0.9733).

high molecular mass proteins in the substrate. When the dose of an enzyme increases at the optimum hydrolysis temperature, it binds with the peptide bonds in the core protein and severs many peptide bonds, leading to the release of different sizes of soluble proteins. This results in increased protein recovery and hydrolysis yield.

The highest hydrolysis yield obtained in this study is higher than the yield obtained from shortfin scad hydrolysate (14.52 %) prepared using Alcalase® at a concentration of 3 % and temperature of 55 °C (Kang et al., 2018).

3.4. *Free amino acid profile*

Free amino acid (FAA) profiles in the protein hydrolysates after 4 h enzyme hydrolysis using Alcalase®, Novozym®, and Protease® at different hydrolysis conditions are presented in Table 2. The total free amino acid content for Alcalase® ranged from 24.9 ± 0.1–32.4 ± 0.4 mg aa/g_{prot} with the lowest value obtained at 70 °C and enzyme concentration of 3 % and the highest value obtained at 60 °C and enzyme concentration of

3 % (v/w). The FAA content range determined for Novozym® was from 28.6 ± 0.2–31.9 ± 0.5 mg aa/g_{prot} with the lowest value observed at a temperature of 50 °C and enzyme concentration of 3 % (v/w). Although there were statistically significant differences between the free amino acid content, in any of the enzymatic treatments, the values were not very high indicating the hydrolysis by the enzymatic treatment could yield mainly small peptides rather than free amino acids. This can be clearly observed in the values of the free amino acid yield reported for all the treatments carried out in this work ranging from 2.3 % to 3.8 %. FAA values in this work were much lower than the values, 75 and 71 mg/g, of water-soluble protein hydrolysates obtained from tuna fish meal using Alcalase and Novozym, respectively (Barea et al., 2023). This difference might be because of the variation in the substrate used and the structure of the LB proteins.

The essential amino acids in various sources of proteins play significant roles in numerous biological and physiological activities and in maintaining the health of human beings (Ryu et al., 2021). It was observed that the essential free amino acids content of LB muscle was increased after enzymatic hydrolysis. More than 50 % of the FAA content in protein hydrolysates were essential amino acids, which were higher compared to the percent in the LB (40.1 ± 0.1 %). Moreover, enzymatic hydrolysis of LB muscle protein resulted in the production of higher hydrophobic amino acids. The ratio of hydrophobic amino acids on the molar base in hydrolysates was higher (56.1 ± 1.5–65.2 ± 0.1 %) than in the LB (54.6 ± 0.4 %). Hydrophobic amino acids have been reported to play significant roles in improving the antioxidant properties of protein hydrolysates (Aderinola et al., 2018). The hydrophobicity of peptides in protein hydrolysates helps to improve their solubility in a lipid medium, which facilitates the entrance of active peptides into the target organ and provides potent antioxidant capacities (Ryu et al., 2021). Histidine was also found as a major free amino acid in protein hydrolysates with yields ranging from 56 % to 90 % (Barea et al., 2023). Histidine residues are credited with a strong radical scavenging activity in oxidative reactions, especially for enzyme-catalyzed reactions, due to the presence of an imidazole ring as an important proton donor (Zou et al., 2016). Protein hydrolysates also contained relatively higher amounts of glycine, lysine, alanine, and leucine.

3.5. *DPPH radical scavenging activity*

All hydrolysates derived from enzymatic hydrolysis of LB muscle using Alcalase®, Novozym®, and Protease® at different concentrations and hydrolysis temperature and time showed higher scavenging activity than the non-hydrolyzed sample (Figs. 3A,3B and 3C). The scavenging activity of the hydrolysates increased rapidly in the first 60 min and remained constant until 4 h. Wu et al. (2003) reported that the

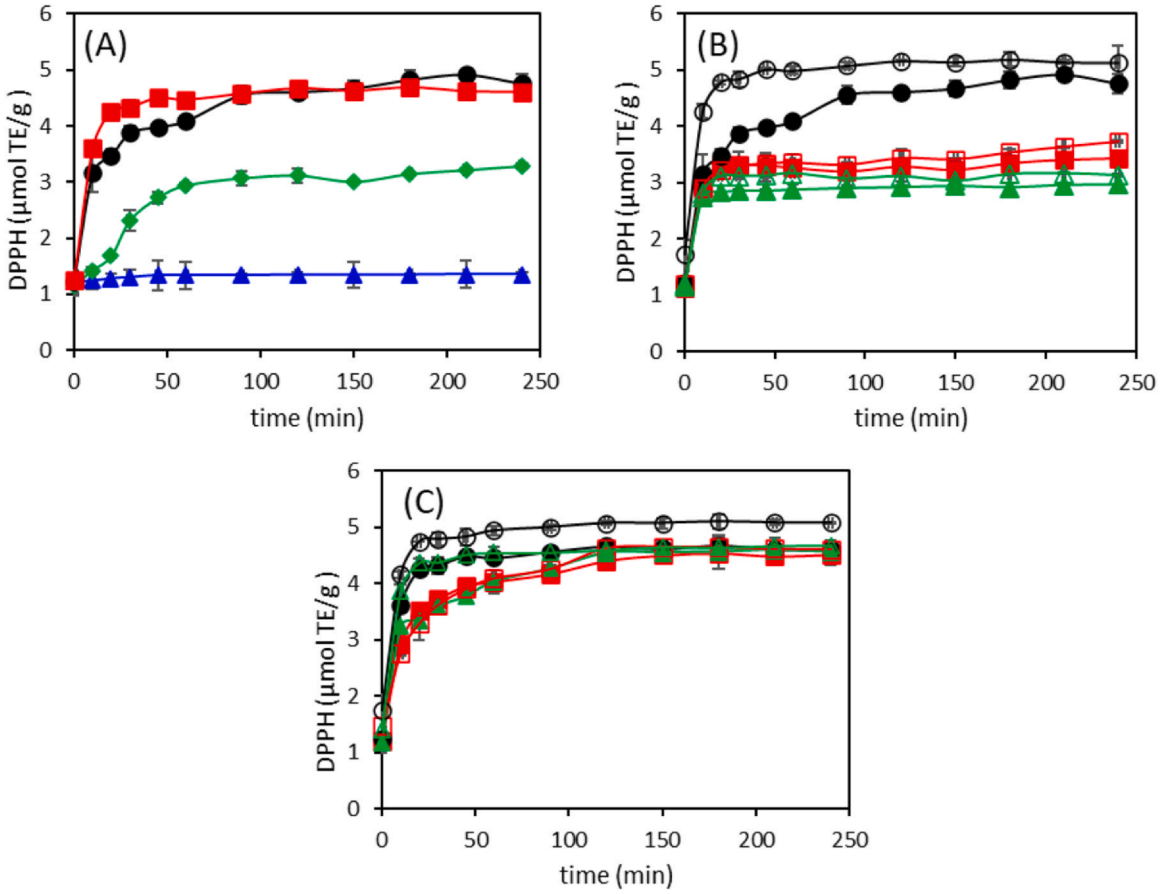

Fig. 3 – DPPH radical scavenging activity of LB muscle protein hydrolysates prepared using different enzymes at different temperatures and enzyme concentration. (A) Effect of enzyme type on DPPH radical scavenging activity of protein hydrolysates prepared at enzyme concentration of 2 % (v/w) and temperature of 60°C (● Alcalase®, ■ Novozym®, ◆ Protease®, ▲ Control). (B) Effect of Alcalase® concentration and temperature, (C) Effect of Novozym® concentration and temperature. Symbols for (B) and (C): ■ 2 % (v/w) and 50 °C, ● 2 % (v/w) and 60 °C, ▲ 2 % (v/w) and 70 °C, □ 3 % (v/w) and 50 °C, ○ 3 % (v/w) and 60 °C, △ 3 % (v/w) and 70 °C.

DPPH scavenging activity of mackerel protein hydrolysate was improved gradually with increasing hydrolysis time. The present result revealed that the LB hydrolysates possibly comprised substances that were electron donors and could react with free radicals, convert them to more stable products, and terminate the radical chain reaction. Comparing the three enzymes, Alcalase® and Novozym® hydrolysates possessed higher scavenging capacity than Protease® hydrolysate (Fig. 3A). Lower antioxidant activity in Protease® hydrolysate might be attributed to the specific enzyme cleavage which could not produce peptides with the ability to scavenge the DPPH radicals. Islam et al. (2021) reported that amino acids such as threonine, isoleucine, and valine strongly contribute to the positive effects on DPPH scavenging activities. In this study, Protease® hydrolysate contained lower level of these amino acids (see Table 2).

Fig. 3B illustrates the DPPH scavenging activity of protein hydrolysates derived from LB muscle treated by Alcalase® for 4 h at different enzyme doses and hydrolysis temperatures. Values of DPPH radical scavenging activity of Alcalase® protein hydrolysates followed similar trends as DH values showed, which indicates that antioxidant activity is correlated with DH. Dhanabalan et al. (2017) also reported a similar result where the DPPH radical scavenging activity of Alcalase® treated protein hydrolysate from *Acetes indicus* increased as the DH increased. In the present study, the highest value of DPPH radical scavenging activity (5.1 ± 0.3 µmol TE/g) was attained from Alcalase® protein hydrolysate at 60°C, with the maximum obtained for an enzyme concentration of 3 % (v/w) at which the maximum DH was observed. In contrast, the lowest value of DPPH radical scavenging activity (2.96 ± 0.02 µmol TE/g) was obtained from protein hydrolysate after 4 h of hydrolysis at an enzyme concentration of 2 % and hydrolysis temperature of 70 °C at which the minimum DH was observed. Similarly, the maximum value (5.08 ± 0.01 µmol TE/g) of DPPH radical scavenging activity of protein hydrolysate treated with Novozym® was observed at similar hydrolysis conditions where the maximum DH was observed (Novozym® concentration of 3 %, 60 °C and 4 h) (Fig. 3C). However, there were no remarkable differences between values of DPPH radical scavenging activity of protein hydrolysates obtained from LB muscle treated with Novozym® at the remaining combination of hydrolysis conditions as was observed in DH values of similar hydrolysates.

A Pearson correlation between DPPH antioxidant capacity of the hydrolysates with the DH (Centurion Statgraphics software) showed a statistically significant non-zero correlations at the 95.0 % confidence level and positive correlation coefficients between DPPH antioxidant capacity and the DH with values of the correlation coefficients of 0.7092 and 0.7437 for Alcalase® and Novozym®, respectively (n = 66 n°. of pairs of data values used to compute each coefficient for each enzyme).

Centenaro et al. (2011) reported that fish and chicken bone hydrolysates with the highest DH values showed higher antioxidant activities, possibly due to factors such as the size and composition of peptides, which play an important role in the ability to delay or inhibit oxidation. Numerous research works reported that hydrolysates with higher content of low molecular weight peptides and free amino acids generally possessed higher DPPH radical-scavenging activity (Centenaro et al., 2011). In the current study, protein hydrolysates with the highest DH had higher proportion of low molecular weight peptides and free amino acids.

3.6. *ABTS radical scavenging activity*

The ABTS radical scavenging activity of hydrolysates prepared by Alcalase®, Novozym®, and Protease® for 4 h at a concentration of 2 %, hydrolysis temperature of 60 °C, is depicted in Fig. 4A. All hydrolysates showed much higher ABTS radical scavenging activity than the control. The ABTS radical scavenging activity of the hydrolysates increased sharply in the first 10 min and slightly up to 60 min of hydrolysis. However, there were no significant differences when the hydrolysis time further increased to 4 h. Protein hydrolysate prepared using Protease® exhibited lower ABTS radical scavenging activity than protein hydrolysates prepared using Alcalase® and Novozym®, which had comparable activity. The differences in antioxidant activity of Protease-treated protein hydrolysates from the Alcalase® and Novozym®-treated hydrolysates might be due to the differences in enzyme specificity and susceptibility of the LB muscle proteins to enzymes, which resulted in the release of different molecular weight peptides, and level and compositions of free amino acids (Klompong et al., 2008). These results agreed with those reported by Islam et al. (2021) who found that some amino acids such as tyrosine, cystine, tryptophan, and especially histidine, exhibit strong radical scavenging activity, which were higher in the Alcalase® and Novozym® hydrolysates.

Fig. 4B & 4C illustrate the ABTS radical scavenging activity of protein hydrolysates derived from LB muscle treated by Alcalase® and Novozym®, respectively, for 4 h at different concentrations of enzymes and hydrolysis temperatures. Even though there were no significant differences between protein hydrolysates prepared using Novozym® at all possible combinations of hydrolysis conditions (Fig. 3C), enzyme concentration, and hydrolysis temperature had a significant effect on the ABTS radical scavenging activity of Alcalase®-treated protein hydrolysates. As shown in Fig. 4B, the ABTS radical scavenging activity of Alcalase® treated protein hydrolysates increased with increasing concentration of enzyme from 2 % to 3 % and increasing hydrolysis temperature from 50 to 60 °C. However, further increasing the hydrolysis temperature to 70 °C resulted in the reduction of the ABTS radical scavenging activity. In general, protein hydrolysate prepared using Alcalase® at 3 % concentration and 60 °C and all Novozym®-treated protein hydrolysates showed the highest mean values of ABTS scavenging activity (66.1–69.5 µmol TE/g) since there were no significant differences observed between them. Enzyme type, degree of hydrolysis, solubility of hydrolysates, type and content of peptides, and free amino acid content, all these parameters influence the ability of the hydrolysates to scavenge ABTS radicals (Borrajo et al., 2020). This study revealed that ABTS scavenging activity is not dependent only on the DH of protein hydrolysates, as these hydrolysates had significantly different DH values. Daud et al. (2015) also reported that different molecular weight fractions of protein hydrolysate could contribute differently for the ABTS scavenging activity.

Similar to DPPH analysis, a Pearson correlation between ABTS antioxidant capacity of the hydrolysates with the DH (Centurion Statgraphics software) showed a statistically significant non-zero correlations at the 95.0 % confidence level and positive correlation coefficients between ABTS

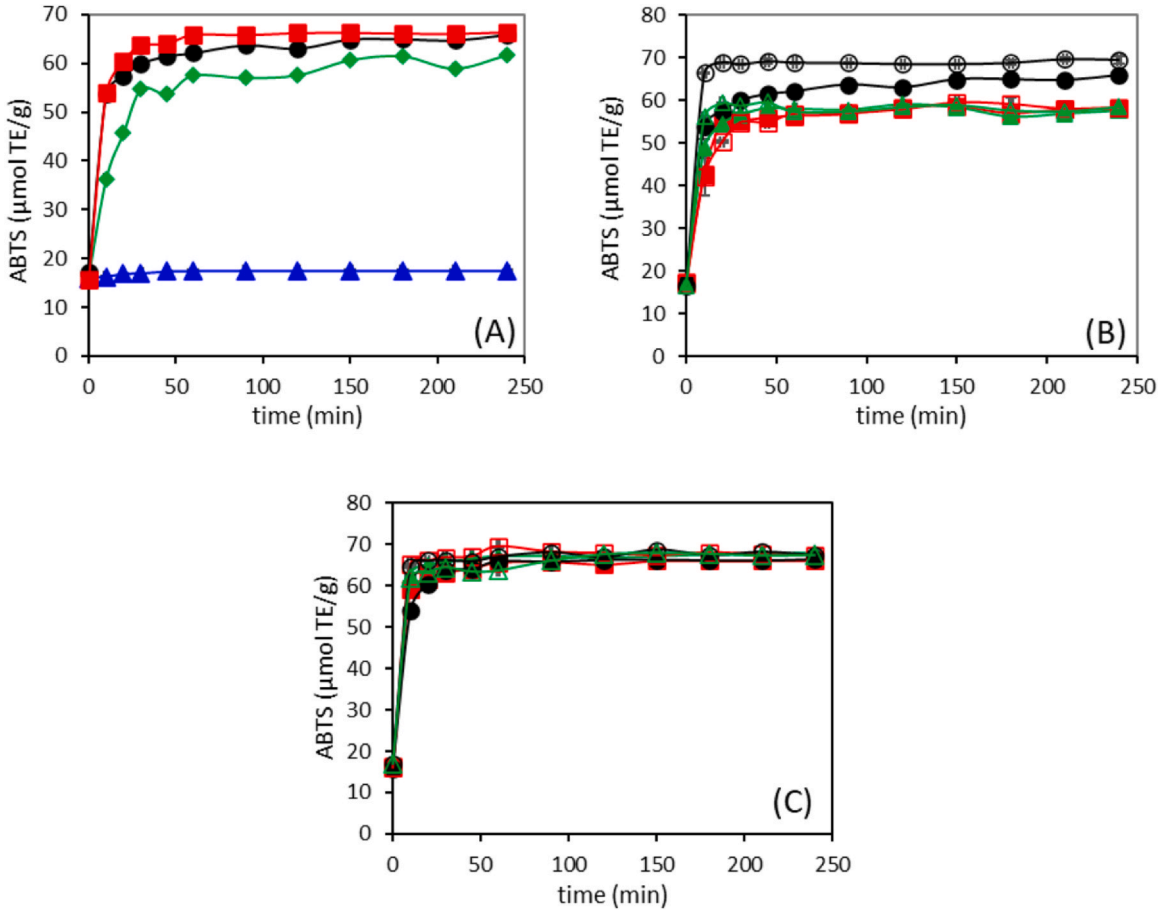

Fig. 4 – ABTS radical scavenging activity of LB muscle protein hydrolysates prepared using different enzymes at different temperatures and enzyme concentration. (A) Effect of enzyme type on ABTS radical scavenging activity of protein hydrolysates prepared at enzyme concentration of 2 % (v/w) and temperature of 60°C (● Alcalase®, ■ Novozym®, ◆ Protease®, ▲ Control). (B) Effect of Alcalase® concentration and temperature, (C) Effect of Novozym® concentration and temperature: Symbols for (B) and (C): ■ 2 % (v/w) and 50 °C, ● 2 % (v/w) and 60 °C, ▲ 2 % (v/w) and 70 °C, □ 3 % (v/w) and 50 °C, ○ 3 % (v/w) and 60 °C, △ 3 % (v/w) and 70 °C.

antioxidant capacity and the DH with values of the correlation coefficients of 0.7708 and 0.8497 for Alcalase® and Novozym®, respectively (n = 66 n°. of pairs of data values used to compute each coefficient for each enzyme).

3.7. *Ferric-reducing antioxidant power (FRAP)*

The capacity of the LB protein hydrolysates to act as a reducing component was assessed by the FRAP assay. Ferric reducing antioxidant power (FRAP) measures the ability of antioxidants to reduce ferric ion (Fe^{3+}) into ferrous through an electron transfer (Sbroggio et al., 2016). The reducing power of protein hydrolysates prepared using the three enzymes increased markedly during the first minutes of treatment (Fig. 5A). All three enzymes provided protein hydrolysates with much higher antioxidant capacity than the control. However, protein hydrolysate obtained using Protease® had a significantly lower reducing power than Alcalase® and Novozyme®, which had comparable reducing power. The lower values of FRAP obtained in Protease® hydrolysate could be due to the lower peptide content in the hydrolysate that lower the ability to reduce ferric ions to their ferrous form, as it was observed in the DPPH and ABTS assays. The structure and size of the peptides of the resulting hydrolysates could also be responsible for the variation in antioxidant activities among hydrolysates (Borrajo et al., 2020). Enzymes that act as an endopeptidase mainly produce small and medium-sized oligopeptides or polypeptides that have a strong reducing power since they cleave peptide bonds at the interior of the polypeptide chain (Liu et al., 2010).

Fig. 5B and 5C shows the results of FRAP assay for the protein hydrolysates obtained using Alcalase® and Novozym®, respectively, at different combinations of enzyme concentration and hydrolysis temperature. Alcalase®-treated protein hydrolysates showed significantly different ferric-reducing antioxidant power with the highest values recorded at the hydrolysis temperature of 60 °C and the lowest values of FRAP observed at 70 °C (Fig. 5B). However, protein hydrolysates obtained using Novozym® at different concentrations and hydrolysis temperatures had no significant differences (Fig. 5C). This clearly shows that Alcalase® is more sensitive to temperature than Novozym®.

A Pearson correlation between reducing capacity of the hydrolysates with the DH (Centurion Statgraphics software) showed a statistically significant non-zero correlations at the 95.0 % confidence level and positive correlation coefficients between FRAP and the DH with values of the correlation coefficients of 0.9039 and 0.9463 for Alcalase® and Novozym®, respectively (n = 66 n°. of pairs of data values used to compute each coefficient for each enzyme). These coefficients are higher than the ones reported for DPPH and ABTS assays.

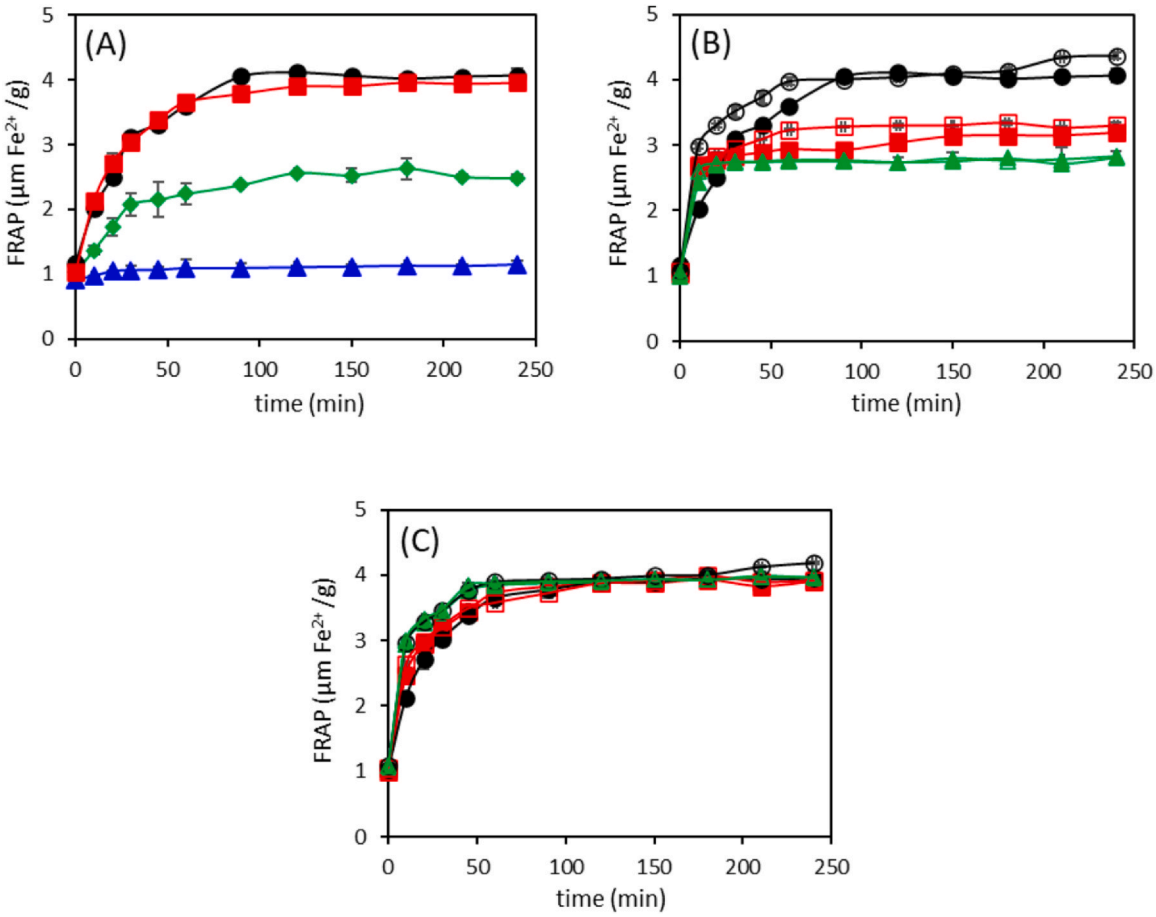

Fig. 5 – Ferric-reducing antioxidant power (FRAP) of LB muscle protein hydrolysates prepared using different enzymes at different temperatures and enzyme concentrations. (A) Effect of enzyme type on FRAP of protein hydrolysates prepared at enzyme concentration of 2 % (v/w) and temperature of 60°C (● Alcalase®, ■ Novozym®, ◆ Protease®, ▲ Control). (B) Effect of Alcalase® concentration and temperature, (C) Effect of Novozym® concentration and temperature. Symbols for (B) and (C): ■ 2 % (v/w) and 50 °C, ● 2 % (v/w) and 60 °C, ▲ 2 % (v/w) and 70 °C, □ 3 % (v/w) and 50 °C, ○ 3 % (v/w) and 60 °C, △ 3 % (v/w) and 70 °C.

The strongest ferric-reducing antioxidant power capacity (4.37 ± 0.04 µmol Fe^{2+}/g) was observed from protein hydrolysate prepared using Alcalase® at 3 % concentration and hydrolysis temperature of 60 °C. The strong reducing power of this protein hydrolysate could be a result of the increase in the availability of hydrogen ions (protons and electrons) due to the cleavage of the peptide bonds (Sbroggio et al., 2016). Moreover, a protein hydrolysate having a higher proportion of < 5000 Da molecular weight, with a higher level of acidic amino acids, such as aspartic acid and glutamic acid, has been reported to possess a strong reducing power (Islam et al., 2021).

3.8. *Freeze dried hydrolysate properties*

3.8.1. *Color profile of freeze-dried hydrolysates*

Color parameters of freeze-dried hydrolysates (FDHs) obtained from LB muscle treated with Alcalase® and Novozym® at different concentrations and hydrolysis temperatures after 4 h of hydrolysis are presented in Table 6. Even though there were no significant differences between the lightness values of FDHs obtained by both enzymes at different concentrations and hydrolysis temperatures, the lightness values (range from 81 ± 3–88.8 ± 0.7) of all FDHs were significantly lower than the control (94 ± 2.0). On the other hand, values of redness ("a" ranged from –0.3 ± 0.1–1.5 ± 0.2) and yellowness ("b" ranged from 9.7 ± 0.3–14.3 ± 0.2) of hydrolysates were significantly higher than the control values (a^* = –0.63 ± 0.04 and b^* = 8.0 ± 0.9). It was observed that significantly higher values of redness and yellowness were obtained at the hydrolysis temperature of 60 °C.

Lightness values in this study are much higher than the values (22.0 – 36.2) of loach protein hydrolysates (You et al., 2009). However, values of lightness, redness, and yellowness are generally in line with the values of lightness (86.6), redness (0.3), and yellowness (12.7) of bighead carp protein hydrolysates (Alahmad et al., 2022). Enzymatic browning reactions are supposed to contribute to the reduction of the luminosity, giving a darker appearance at high DH (Wasswa et al., 2007).

The differences in peptide size and amino acid sequences of fish protein hydrolysate are also responsible for the color differences (You et al., 2009). Variations in color parameters might also be attributed due to differences in fish species. Myoglobin and hemoglobin have been known to be responsible for the color characteristics of fish flesh or their product (Yarnpakdee et al., 2012). Dark-fleshed fish, such as sardine, mackerel, and round scad, contained a high amount of myoglobin (Dong et al., 2008). Furthermore, enzyme types, enzyme to substrate ratio, and differences in color measuring instruments could influence the color of obtained FPH powder.

Table 6 – Color profile of freeze-dried LB muscle (control) and FDH prepared using Alcalase® and Novozym® at different concentrations and hydrolysis temperatures.

Enzyme	Enzyme concentration (%)	Hydrolysis Temperature (°C)	L*	a*	b*
Control			94 ± 2^{a}	-0.63 ± 0.04^{h}	8.0 ± 0.9^{g}
Alcalase®	2	50	86 ± 2^{ab}	0.9 ± 0.1^{d}	10.9 ± 0.9^{cdef}
		60	84 ± 3^{bcd}	1.27 ± 0.03^{abc}	13.5 ± 0.5^{ab}
		70	88.8 ± 0.7^{ab}	0.10 ± 0.05^{f}	10.1 ± 0.5^{ef}
	3	50	85 ± 4^{bc}	1.1 ± 0.1^{cd}	11.5 ± 0.2^{cdef}
		60	81 ± 3^{c}	1.5 ± 0.1^{ab}	14.3 ± 0.2^{a}
		70	86 ± 4^{b}	-0.3 ± 0.1^{g}	12.1 ± 0.2^{bcd}
Novozym®	2	50	85.2 ± 0.1^{bc}	0.9 ± 0.5^{d}	9.7 ± 0.3^{fg}
		60	85.9 ± 0.8^{bc}	1.3 ± 0.1^{abc}	10.5 ± 0.8^{ef}
		70	87 ± 2^{b}	1.5 ± 0.1^{a}	11.8 ± 0.3^{bcde}
	3	50	86 ± 2^{bc}	0.6 ± 0.1^{e}	10.0 ± 0.9^{f}
		60	87 ± 4^{b}	1.5 ± 0.2^{ab}	12.6 ± 0.8^{abc}
		70	86 ± 3^{bc}	1.2 ± 0.2^{bc}	10.8 ± 0.7^{cdef}

Values with different letters in each column are significantly different when applying the Fisher's least significant differences (LSD) method at p-value < 0.05.

3.8.2. *Antioxidant activity measured by the β-carotene bleaching method*

β-carotene linoleate bleaching assay is widely employed to measure the antioxidant activity of bioactive compounds since β-carotene is extremely susceptible to free radical-mediated oxidation of linoleic acid (Khaled et al., 2014). β-carotene undertakes a rapid discoloration in the absence of antioxidants, which results in a reduction of the absorbance of the sample solution (Koleva et al., 2002). Thus, the addition of antioxidants delays the degree of β-carotene bleaching by neutralizing the linoleic hydroperoxyl radicals formed.

Protein hydrolysates, prepared by hydrolyzing the LB muscle for 4 h using Alcalase® and Novozym® at an enzyme to substrate ratio of 3 % and hydrolysis temperature of 60 °C, were tested for their potential to suppress the discoloration of β-carotene and results are presented in Fig. 6. Both hydrolysates prevented the discoloration of β-carotene to different degrees. Alcalase®-treated hydrolysates showed a higher capacity to prevent the bleaching of β-carotene than Novozym® ($p < 0.05$). Furthermore, the antioxidant activity of both hydrolysates increased with increasing concentration of hydrolysates. However, the activity of both protein hydrolysates to prevent the β-carotene bleaching was lower than for butylated hydroxyanisole (BHA) (91.8 ± 0.3 %).

The antioxidant activity of hydrolysates depends on DH, amino acids composition and length, and molecular weight (Centenaro et al., 2011). Hydrolysates rich in hydrophobic amino acids enhance antioxidant activity by increasing the solubility of peptides in the lipid phase (Ryu et al., 2021). In addition, histidine can make reactive oxygen species (ROS) stable through electron/proton transfer (Wiriyaphan et al., 2012). In this study, Alcalase® treated hydrolysate had a higher DH (Table 5) and was richer in histidine and hydrophobic amino acids than Novozym® hydrolysate (Table 2). This could contribute to a higher degree of the inhibition of β-carotene bleaching of Alcalase® hydrolysate.

3.8.3. *Functional properties of FDHs*

3.8.3.1. Solubility of protein hydrolysate. Protein solubility is an important functional and physicochemical property since it

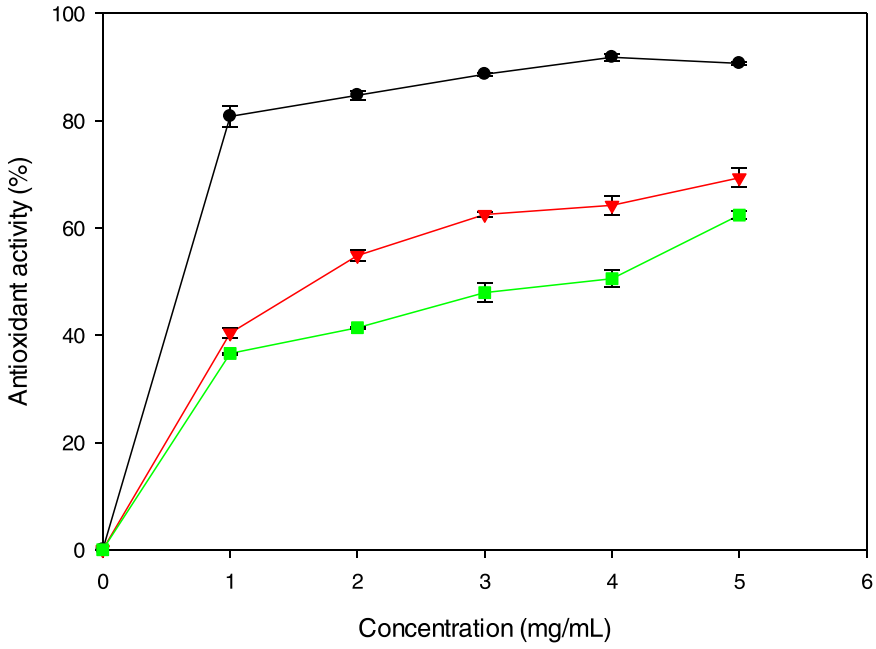

Fig. 6 – β-carotene bleaching inhibition capacity of FDH prepared using Alcalase® and Novozym® at enzyme concentration of 3 %(v/w) and 60 °C (● BHA, ▼ Alcalase®, and ■ Novozym®).

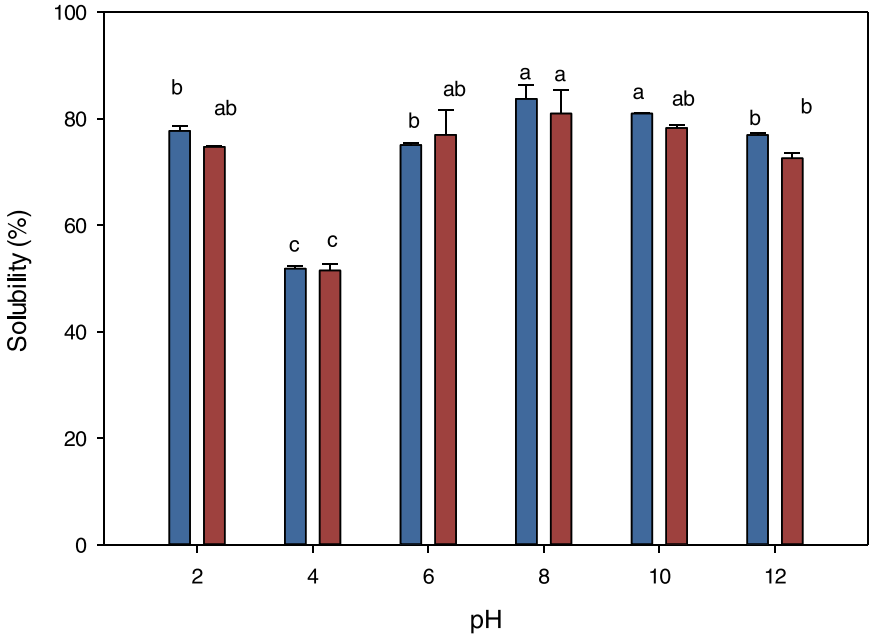

Fig. 7 – Solubility of FDH prepared using Alcalase® and Novozym® at enzyme concentration of 3 % (v/w) and hydrolysis temperature of 60 °C. (■ Alcalase® and ■ Novozym®).

influences the quality and utilization of the final product in many applications (Noman et al., 2018). The solubility of LB protein hydrolysates obtained by Alcalase® and Novozym® at 60 °C and enzyme dose of 3 % was determined at different pH values, ranging from 2 to 12 (Fig. 7). Both hydrolysates showed comparable solubility with a similar trend over the tested pH range. pH showed a significant effect on the solubility of both hydrolysates. Alcalase® and Novozym® hydrolysates exhibited a higher solubility at pH 8 (84 ± 3 and 81 ± 5 %) and pH 10 (81.0 ± 0.1 and 78.2 ± 0.6 %), and a significantly lower solubility at pH 4 (51.8 ± 0.5 and 52 ± 1 %), respectively. These results are supported by Liu et al. (2015) who reported that the Skipjack tuna protein hydrolysate showed a higher solubility at pH 8 and 10, and a lower solubility at pH 4.

Enzymatic hydrolysis reduces higher molecular-weight polypeptides into smaller molecular-weight peptides and forms more hydrophilic groups, thus increasing protein solubility (Alahmad et al., 2022). The lower solubility of protein hydrolysate at pH 4 could be due to the isoelectric point of protein (Yathisha et al., 2022). Solubility of protein decreases at the isoelectric points of proteins, which are between pH 4.5–5.5, due to the high interaction between proteins rather than the interaction between protein and water (Noman et al., 2019).

3.8.3.2. Water- and oil-holding capacity (WHC and OHC). The water- and oil-holding capacity of FDH prepared using Alcalase® and Novozym® under optimal conditions (3 % enzyme concentration and 60 °C) are presented in Table 7. The results revealed no significant differences between Alcalase® and Novozym® hydrolysates in WHC, 2.1 ± 0.2 and 2.3 ± 0.4 g water/g FDH, respectively. The values of WHC in this study were higher than the value (1.58 g/g) of Chinese sturgeon protein hydrolysate (Noman et al., 2019) but lower than values (2.87–4.38 g/g) of Grass Turtle protein hydrolysates (Islam et al., 2021). Enzymatic hydrolysis improves the WHC of the resulting protein hydrolysates by breaking down peptide bonds and, as a consequence, increases the concentration of polar groups, such as -COOH and -NH_2 (Alahmad et al., 2022). However, extensive hydrolysis of proteins decreases the WHC due to the production of extra-small peptides (Alavi et al., 2019).

Table 7 – Functional properties of FDH prepared using Alcalase® and Novozym® at enzyme concentration of 3 % and hydrolysis temperature of 60 °C.

Functional properties	Alcalase® hydrolysate	Novozym® hydrolysate
WHC (g water/g FDH)	2.1 ± 0.2[a]	2.3 ± 0.4[a]
OHC (g oil/g of FDH)	3.7 ± 0.2[a]	4.1 ± 0.3[a]
FC (%)	78 ± 3[a]	75 ± 2[a]
FS (%)	27 ± 1[a]	25 ± 2[a]

Values of OHC for Alcalase® and Novozym® hydrolysates were 3.7 ± 0.2 and 4.1 ± 0.3 g oil/g, respectively, with no significant differences. These values are higher than Chinese sturgeon protein hydrolysate (2.56–3.33 g/g) prepared by Alcalase® (Noman et al., 2019) but lower than the value (5.58 g/g) of ribbon fish protein hydrolysates (Yathisha et al., 2022). Enzymatic hydrolysis alters the structure of proteins and promotes the physical entrapment of oil (Leni et al., 2020). The hydrophobicity of proteins influences the capacity of the resulting hydrolysates to absorb oil (Wasswa et al., 2007). The reason that the values of OHC were higher than the WHC of the hydrolysates could be attributed to a higher hydrophobicity of the peptides after treatment.

3.8.3.3. Foaming capacity and foaming stability. The foaming capacity (FC) and foaming stability (FS) of hydrolysates prepared using Alcalase® and Novozym® under optimal conditions (3 % enzyme concentration and 60 °C) are presented in Table 7. The result revealed no significant differences between Alcalase® and Novozym® hydrolysates in FC (78 ± 3 and 75 ± 2 %, respectively) and FS (27 ± 1 and 25 ± 2 %, respectively). Foaming properties are governed by the transportation, penetration, and rearrangement of molecules at the air-water interface (Elavarasan et al., 2014). The surface hydrophobicity of proteins determines the adsorption at the air–water interface. Thus, good foaming properties depend on the ability of

hydrolysates to rapidly absorb into the air-water interface and rearrange their structures, as well as having several molecular characteristics such as good surface balance, charge distribution, and molecular hydrophobicity (Halim and Sarbon, 2020).

4. Conclusions

Based on the obtained results, the type of enzyme, enzyme to substrate ratio, hydrolysis temperature, and time influenced the degree of hydrolysis, antioxidant activities, and other related physicochemical properties of protein hydrolysates. The highest degree of hydrolysis, antioxidant activities, hydrolysate yield, and protein recovery yield were observed from the protein hydrolysate prepared using Alcalase® at an enzyme/substrate ratio of 3 % and hydrolysis temperature of 60 °C. Even though Alcalase® was found to be the most efficient enzyme, among the three enzymes tested in this work, to hydrolyze *Labeobarbus nedgia* muscle, Novozym® could also be used to produce antioxidant peptides since this enzyme was found to be less sensitive to high temperatures. Therefore, the present study revealed the possibilities of utilizing low-valued *Labeobarbus nedgia* as a resource for the production of valuable protein hydrolysates. Further investigations could be carried out on the purification and separation of the particular antioxidant peptides from the protein hydrolysate.

Author contribution

Solomon Abebaw Tadesse wrote the main manuscript text and carried out the experimental part. Shimelis Admassu Emire reviewed the manuscript and planned the experimental work. Pedro Barea contributed to the experimental part and solved technical problems. Alba Ester Illera revised the data and the manuscript text. Rodrigo Melgosa revised the data and the manuscript text. Sagrario Beltrán contributed with the writing process and the supervision of the work, funding acquisition. María Teresa Sanz contributed with the writing process of the manuscript text and figures, supervised the work and funding acquisition.

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Data availability

All data generated or analysed during this study are included in this published article.

Declaration of Competing Interest

The authors declare no competing interests.

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