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Functional and antioxidative properties of fish protein hydrolysate (FPH) produced from the frame meat of striped catfish *Pangasianodon hypophthalmus* (Sauvage, 1878) using alkaline protease alcalase

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ABSTRACT

The striped catfish *Pangasianodon hypophthalmus* has gained importance as a potential candidate for aquaculture in India. It is an abundant and underutilised resource that can be used as a unique protein source to make fish protein hydrolysates (FPH). The objective of the present study was to prepare FPH from the frame meat of *P. hypophthalmus* using the alkaline protease alcalase at different enzyme-substrate concentrations of 0.5%, 1.5% and 2.5% (v/w) of the protein content of the substrate and to compare the yield, functional and antioxidative properties. Among the functional properties, foaming capacity, emulsification capacity, oil binding capacity and peptide solubility were studied. 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, as well as reducing property assay and metal chelating ability study were done to assess the antioxidative property. The increase in concentration of alcalase enzyme was found to have a significant effect ($p<0.05$) on increasing the degree of hydrolysis. The hydrolysate showed better functional properties at low degree of hydrolysis. Antioxidative property also decreased significantly ($p<0.05$) with increase in degree of hydrolysis. The study proved that the protein hydrolysate produced from *P. hypophthalmus* is having excellent functional and antioxidative properties especially at lower enzyme concentration of 0.5% (v/w) and has potential use as functional food ingredient.

Keywords: Antioxidative property, DPPH, Fish protein hydrolysate, Functional property, *Pangasianodon hypophthalmus*

Introduction

Fish processing wastes comprising head, viscera, skin, bones and frame meat constitute around 30-40% of the original material. These protein rich byproducts are discarded without any attempt of recovery or being converted into low market value products like fish meal or fertilizer. The global weighted discard rate is 8% of the total annual fish landings (Kelleher, 2005). Recovering the fish muscle proteins from processing waste and converting it into high end products such as protein hydrolysates is the need of the hour. This provides an opportunity for increasing revenue in addition to reducing environmental pollution.

Enzymatic modification of proteins using selected proteolytic enzyme preparations to cleave specific peptide bonds is more frequently used in the food industry to produce hydrolysates with excellent functional properties (Mullally *et al*., 1994). Protein hydrolysates are produced for a wide variety of uses in the food industry, including milk replacers, animal food, media for growing microorganisms, protein supplements, stabilisers in beverages and flavour enhancers in confectionery products (Clemente, 2000) Fish protein hydrolysates also reported to have antioxidant properties (Sathivel *et al.,* 2003). A number of scientific journals have cited the antiproliferative activity of fish protein hydrolysate, which makes it eligible for listing as a nutraceutical too.

Presently catfish farming is gaining importance among Indian farmers as an alternative to carps in different parts of the country; accordingly its contribution to overall fish production is on the rise. *P. hypophthalmus*, popularly referred to as pangasius or striped catfish is

a prime species farmed in Vietnam and other South-east Asian countries. It was first introduced to India in West Bengal in 1995. In 2000, the species was introduced into Andhra Pradesh. Because of its remarkable growth rate (almost one kg in 90 days), there has been much enthusiasm among fish breeders and farmers particularly in West Bengal and Andhra Pradesh for its culture and propagation. But recently the farmers have suffered a setback due to the low market price realised for the fish. The low price is attributed mainly to the yellowing of fish flesh. Hence conversion of the meat into high end products like protein hydrolysates can be an option. Moreover, the processing wastes like viscera, skin, scales, bones and frame meat can be converted into useful byproducts. Of these frame meat can be used as an excellent rawmaterial for hydrolysate production.

There is a little information regarding protein hydrolysates from the meat of striped catfish (*P. hypophthalmus*) and their antioxidative activity and functional properties as affected by enzymes used. Hence the aim of this study was to produce protein hydrolysate from the frame meat of *P. hypophthalmus* using different concentrations of the alkaline protease alcalase and to study the difference in functional properties and antioxidative activity.

Materials and methods

Frame meat from *P. hypophthalmus* procured from a farm near Kodungallur, Kerala was collected after gutting and filleting. The meat was packed in polythene bags, blast frozen and kept at -20 ^{0}C until used in the hydrolysis experiments. The proteases employed in the study *viz*., Alcalase 2.4L (Sigma, Alkaline protease). Standard L-Leucine, 2,4,6-trinitrobenzene sulfonic acid (TNBS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Laboratories. 3-(2-pyridyl)-5-6-diphenyl-1,2,4-triazine- 4'4'-disulphoinc acid sodium salt (ferrozene) was obtained from Merck.

Production of protein hydrolysate from P. hypophthalmus frame meat

The frame meat from *P. hypophthalmus* was minced and 50 g each was suspended in distilled water in the ratio 1:1. The mixture was adjusted to the optimum pH, *i.e*, 8.5 for alcalase activity. The mixtures were preincubated at the defined temperatures of 55 ^{0}C for 20 min prior to enzymatic hydrolysis. The protein hydrolysis was initiated by addition of the alcalase at the level of 0.5% v/w, 1.5%v/w and 2.5% v/w of the protein content in the mince. The enzymatic hydrolysis proceeded for 90 min at 55 ^{0}C with continuous stirring. After 90 min, the enzyme activity was terminated by keeping the mixture in a water bath at 90 ^{0}C for 10 min. When the mixture was cooled down, it was filtered and centrifuged (Hermle Z36HK) at 6500 *g* at 4 ^{0}C for 15 min. The supernatant was collected and vacuum dried (Heraeus vacutherm, Germany). The hydrolysates were sealed in polythene bags and kept at 4 ^{0}C for further experiments.

Yield and degree of hydrolysis (DH)

Yield was calculated as a percentage of the weight of the substrate. Degree of hydrolysis was determined by the TNBS method (Benjakul and Morrissey, 1997). All samples and standard solutions were prepared in 1% (w/v) SDS. Five hundred milligram of the sample was dissolved in 1% SDS and heated at 50 ^{0}C for 15 min. Duplicate aliquots of 250 µl samples (5mg ml^{-1}) were added to test tubes containing 2 ml sodium phosphate buffer (0.2 M, pH 8.2), 2 ml 0.1% (v/v) TNBS reagent was then added to each tube followed by mixing and incubation at 50 ^{0}C for 60 min in a covered water bath. The reaction was stopped by addition of 0.1 N HCl (4 ml) to each tube. Samples were then allowed to cool to room temperature and the absorbance was measured at 420 nm using a Spectronic® Genesys™ 5 spectrophotometer. L-leucine (0-2.0 mM) was used to generate a standard curve and DH was calculated as follows:

$$DH = [(L_t - L_0)/(L_{max} - L_0)] x100$$

where L_t is the amount of α amino acid released at time t. L_o is the amount of α amino acid in the original *P. hypophthalmus* meat before enzyme hydrolysis L_{max} is the total α-amino acid in the original *P. hypophthalmus* meat after acid hydrolysus (6N HCl at 110 ^{0}C for 24 h).

Determination of functional properties

Peptide solubility

Nitrogen solubility Index (NSI) was used to determine the solubility of protein hydrolysate (Morr, 1985). Two hundred milligram of protein hydrolysate was dispersed in 20 ml distilled water. The pH was adjusted to 3, 7 and 10 using 2M HCl and 2N NaOH as required. The mixture was stirred at room temperature for 30 min and later centrifuged at 4500 *g* for 30 min at 4 ^{0}C. The supernatant was filtered through Whatman no.1 filter paper and the nitrogen content in the total fraction and in the soluble fraction was analysed by AOAC method (2000). The nitrogen solubility index (NSI) was calculated as:

$$\% \text{ NSI} = \frac{\text{Protein content in the supernatant}}{\text{Total protein content in the sample}} \times 100$$

Emulsifying properties

The emulsifying capacity of the protein hydrolysate was determined following the method of Swift and Sulzbacher (1963) with some modifications. Two hundred milligram of hydrolysate sample was dissolved in 0.1 M NaCl. The mixture was homogenised for 20 seconds and weighed. Oil was added to the suspension at a flow rate of 0.5 ml sec^{-1}. A multimeter was used to measure the maximum resistance point achieved. The difference in weight was measured and emulsifying capacity was expressed as ml of oil g^{-1} sample by correcting for oil density (0.9112g l^{-1})

Foaming properties

Five hundred milligram of the fish protein hydrolysate was dispersed in 100 ml distilled water and stirred for 10 min using a homogeniser. The contents along with foam were poured immediately into a 250 ml measuring cylinder and foam volume was measured after 1 min. Foam volume was recorded after 15, 30 and 60 min. Foaming ability was expressed as foam expansion at 1 min, while foam stability was expressed as foam expansion during 60 min. Foam expansion was calculated according to Lawhon *et al.* (1972) using the following equation:

Foam expansion % = [(A-B)/B] x 100

where A = volume after whipping (ml) and B = initial volume.

Oil binding capacity

Five hundred milligram of hydrolysate was taken in a centrifuge tube and 10 ml sunflower oil was added to it. It was thoroughly vortexed for 1 min and centrifuged at 4500 *g* for 30 min at 4 ^{0}C. The unbound oil was decanted off. Oil absorption capacity was determined from the difference in weight of the hydrolysate samples and expressed as gram of oil bound per gram sample (Shahidi *et al.,* 1995).

Determination of antioxidative activities

DPPH radical scavenging capacity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH radical scavenging activity was determined by the method described by Shimada *et al.* (1992) with some modifications. Two millilitres of the sample solution (10 mg ml^{-1}) was mixed with 2 ml of 100 µM freshly prepared DPPH (1,1-diphenyl-2-picrylhydrazyl) methanolic solution and vortexed for about 10s. The resulting solution was then left to stand for 30 min, prior to being spectrophotometrically measured at 517 nm. A low absorbance at 517 nm indicates a high scavenging activity. DPPH without sample was used as blank. The DPPH scavenging activity was calculated according to the following equation:

$$\text{DPPH Scavenging capacity (\%)} = \frac{\text{Absorbance (control) - Absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

Reducing power

Reducing power was determined according to the method of Yildrim *et al.* (2001), with modifications. Sample solution (0.5 ml, 40 mg protein ml^{-1}) was mixed with 2.5 ml of 0.2 m phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. Mixture was incubated at 50 ^{0}C for 20 min. An aliquot of 2.5 ml was mixed with 2.5 ml distilled water and 2.5 ml of 0.1% $FeCl_3$ and the absorbance was read at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Chelating activity on Fe^{2+}

Metal chelating activity was determined according to the method of Decker and Welch (1990). Distilled water (3.7 ml) was added to the sample solution (5mg ml^{-1} which was then made to react with a solution containing 0.1 ml 2 mm $FeCl_2$ and 0.2 ml of 5 mm Ferrozene. The mixture was vortexed and the absorbance was measured at 562 nm after 10 min. In the control, distilled water was used instead of sample and in the blank distilled water was used instead of Ferrozene. The chelating activity was calculated as follows:

$$\text{Chelating capacity (\%)} = \frac{\text{Absorbance (control) - Absorbance) (sample)}}{\text{Absorbance (control)}} \times 100$$

Statistical analysis

Mean and standard deviation of each treatment with respect to the parameters were calculated. The data were analysed by analysis of variance (ANOVA) to study the effect of treatments on the functional and antioxidant properties using SAS 9.2.

Results and discussion

Yield and DH

The increase in concentration of alcalase enzyme was found to have a significant effect ($p<0.05$) on increasing the degree of hydrolysis (Fig. 1). The yield of FPH increased from 7.03 $\pm$ 0.83% to 9.85 $\pm$ 0.25% with the increase in

concentration of enzyme. The result indicates that peptide bonds are more extensively cleaved in the presence of a higher amount of enzyme. The peptides released are hydrolysed further into amino acids and smaller peptides by the enzymes. Beak and Cadwallader (1995) also shown that a linear relationship exists between the concentration of enzyme used and the degree of hydrolysis.

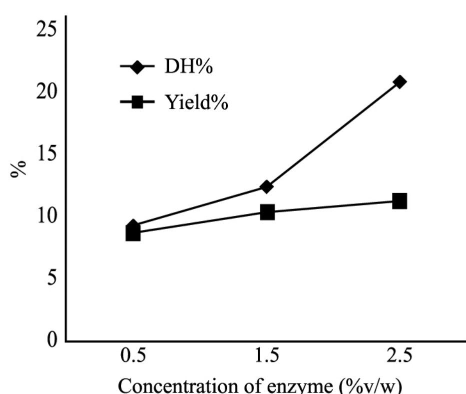

Fig. 1. Degree of hydrolysis (%) and yield of fish protein hydrolysate prepared with enzyme concentrations (1) 0.5%v/w (2) 1.5%v/w and (3) 2.5% v/w of protein concentration in substrate. Values represent mean ± SD of 3 replications.

Antioxidant activity

Because of the development of undesirable off flavours and potentially toxic reaction products proven to be carcinogenic, lipid peroxidation is of concern to the food industry. The synthetic antioxidants used in food industry are under strict control because of the potential health hazards posed by them. Therefore, the search for natural antioxidants as alternatives to synthetic ones is of great interest among researchers.

DPPH radical scavenging activity

DPPH, a stable free radical has an absorbance maximum at 517 nm. When DPPH encounters a proton-donating substance, such as an antioxidant, the radical is scavenged and the absorbance is reduced (Shimada *et al.,* 1992). Antioxidative activity of protein hydrolysates depends on the proteases (Jun *et al.,* 2004) and hydrolysis conditions employed (Jao and Ko, 2002; Jun *et al.,* 2004). Changes in size, level and composition of free amino acids and small peptides also affect the antioxidative activity (Wu *et al.,* 2003). The DPPH radical scavenging activity of the hydrolysates decreased significantly ($p<0.05$) when the enzyme concentration was increased from 1.5 to 2.5% (Fig. 2). This may be due to breakdown of antioxidative peptide sequences formed during early stages of the hydrolysis process. Similar results were reported by Klampong *et al.,* (2007) in the antioxidative properties of protein hydrolysates produced from yellow stripe travelly using alcalase enzyme where the DPPH radical scavenging capacity decreased with increase in DH. The lowest DPPH radical scavenging capacity of 61.15% was higher than that obtained in sardine hydrolysates prepared with alcalase enzyme (Ali *et al.,* 2010) The result reveals that the striped catfish hydrolysates potentially contained substances which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

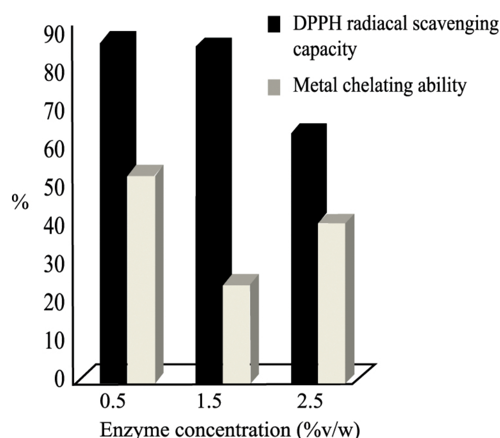

Fig. 2. DPPH radical scavenging capacity (%) and metal chelating ability (%) of striped catfish proetein hydrolysates prepared with enzyme concentrations, (1) 0.5%v/w (2) 1.5%v/w and (3) 2.5% v/w of protein concentration in substrate *i.e.,* fish mince. Values represent mean ± SD of 3 replications.

Metal chelating activity

Initially the metal chelating activity decreased significantly with increase in enzyme concentration ($p<0.05$) and DH (Fig. 2) but when the enzyme concentration increased from 1.5-2.5% it increased significantly. Klompong *et al.* (2007) and Naliananon *et al.* (2011) reported that the metal chelating activity increases with increase in DH. None the less, the presence of specific peptide sequences also determine the rate of metal chelation. This may explain the decrease in metal chelation with increase in enzyme concentration to 1.5%. Carboxyl and amino groups in the side chains of acidic and basic amino acids are thought to play an important role in chelating metal ions (Saiga *et al.,* 2003). Metal ions like Fe, Cu and Co in foods react very quickly with peroxides by acting as one-electron donors to form alkoxyl radical (Gordon, 2001). Peptides in hydrolysates could chelate these prooxidants, leading to decreased lipid oxidation. The FPH produced with the lowest enzyme concentration of 0.5% (v/w) had a metal chelating ability

of 51.02% ± -0.24. Thus the results indicate that striped catfish protein hydrolysates have a pronounced capacity of iron binding.

Reducing property

With increase in enzyme concentration, the reducing property decreased significantly ($p<0.05$) (Table 1). This is in correlation with the decrease in DPPH radical scavenging capacity with increase in DH. Klompong *et al*. (2007) also reported a similar result with the yellow stripe travelly protein hydrolysate prepared with alcalase enzyme. The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron or hydrogen (Yildirim *et al.,* 2000). Many reports have revealed that there is a direct correlation between antioxidant activities and reducing power of bioactive peptides. Ali *et al.* (2010) reported that sardine hydrolysates produced with alcalase enzyme had a potent reducing power of 2.2 ± 0.48, whereas the highest reducing power of striped cat fish hydrolysate was only 0.472 ± 0.007. Yellow stripe travelly protein hydrolysates had a reducing capacity of 0.55 nm (DH 5%) which is comparable to that obtained for the striped catfish protein hydrolysates (DH 7.64 ± 0.39) (Klompong *et al.,* 2007).

Table 1. Reducing property of striped catfish proetein hydrolysates prepared with varying enzyme concentrations

Sample	Absorbance (nm)
FPH1	0.472 ± 0.007A
FPH2	0.289 ± 0.002B
FPH3	0.259 ± 0.001C

FPH1- FPH prepared with 0.5% v/w concentration of alcalase
FPH2 - FPH prepared with 1.5% v/w concentration of alcalase
FPH3 - FPH prepared with 2.5% v/w concentration of alcalase
Values represent Mean ± SD of 3 replications
Different letters within the same parameter indicate significant difference

Functional properties.

Peptide solubility

Solubility is one of the most important functional properties of FPH. The solubility of peptides increased significantly ($p<0.05$) (Table 2). with increase in enzyme concentration and the DH. All hydrolysates were soluble over a wide pH range with more than 98% solubility and the solubility increased with increase in degree of hydrolysis. This may be due to the degradation of proteins to smaller peptides which leads to more soluble products (Gbogouri *et al.,* 2004; Linder *et al*., 1996). The smaller peptides from myofibrillar proteins are expected to have proportionally more polar residues, with the ability to form hydrogen bonds with water and augment solubility (Gbogouri *et al.,* 2004). The findings of Shahidi *et al*., (1995) and Quaglia and Orban (1987) also support the observation. Solubility of hydrolysed protein in a broad pH range is one of the most desirable physicochemical and functional properties and it also determines the other functionalities like emulsifying and foaming in a food system. Hydrolysates were found to be more soluble at neutral and alkaline pH than acididc pH. This is because the hydrolysates generally show low solubility at their isoelectric points (Chobert *et al.,* 1988; Linder *et al*., 1996). There was no marked increase in solubility when the enzyme concentration increased from 1.5 to 2.5% at the 3 different pH (Fig. 3). This may be because almost all the (99.5%) peptides became soluble at the enzyme concentration of 1.5%.

Emulsification capacity

An emulsion is formed when the peptides in the hydrolysate get adsorbed to the surface of freshly formed

Table 2. Functional properties of striped catfish protein hydrolysate prepared with varying enzyme concentrations

Samples	Emulsifying capacity (ml oil 200 mg $^{-1}$ hydrolysate)	Peptide solubility (%) at pH 7	Foaming capacity (%)	Oil binding capacity (ml oil g) hydrolysate $^{-1}$)
FPH1	55.19 ± 2.15A	98.72 ± 0.07A	69.72 ± 0.65A	1.16 ± 0.15A
FPH2	17.39 ± 2.67B	99.43 ± 0.12B	11.38 ± 0.51B	0.88±0.03B
FPH3	15.59 ± 1.03B	99.57 ± 0.12B	9.96 ± 0.46B	0.82 ± 0.03B

FPH1- FPH prepared with 0.5% v/w concentration of alcalase
FPH2 - FPH prepared with 1.5% v/w concentration of alcalase
FPH3 - FPH prepared with 2.5% v/w concentration of alcalase
Values represent the mean ± SD of 3 replications
Diferent alphabets within the same parameter indicate significant difference

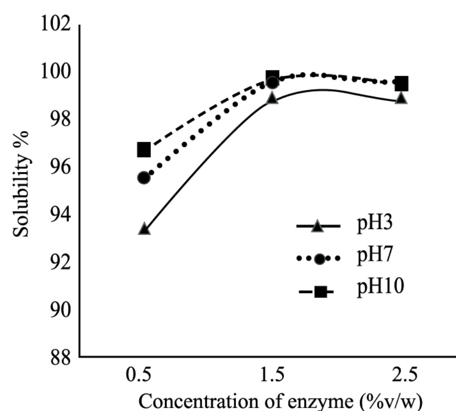

Fig. 3. Solubility of striped cat fish protein hydrolysate prepared with enzyme concentrations (1) 0.5%v/w (2) 1.5%v/w and (3) 2.5% v/w of protein concentration in the substrate *i. e*., fish mince at pH 3, 7 and 10. Values represent mean ± SD of 3 replications

oil droplets during homogenisation and by the formation of a protective membrane that inhibits coalescence of the oil droplet. Hydrolysates are surface-active materials and promote oil-in-water emulsion because of their charged hydrophilic and hydrophobic groups (Rahali *et al.,* 2000; Gbogouri *et al.,* 2004). Emulsification capacity of striped catfish protein hydrolysate decreased significantly ($p<0.05$) (Table 2) with increase in enzyme concentration. At low DH (7.64 ± 0.39) attained with an enzyme concentration of 0.5%, the hydrolysate exhibited strong emulsifying properties. This is because higher contents of larger molecular weight peptides or more hydrophobic peptides contribute to the stability of the emulsion. Small peptides produced by excessive hydrolysis migrate rapidly and adsorb at the interface, but they are less efficient in decreasing the interface tension since they cannot unfold and reorient at the interface like large peptides to stabilise emulsions (Rahali *et al.,* 2000; Gbogouri *et al.,* 2004). The difference in emulsifying activity was less significant when the enzyme concentration increased from 1.5 to 2.5%. This may be because the chain length of peptides might have been reduced to less than 20 with an enzyme concentration of 1.5% to function as good emulsifiers (Lee *et al.,* 1978). At a DH of $7.64\% \pm 0.39$, the striped catfish protein hydrolysate had an emulsifying capacity of 55.19 ± 2.15 ml oil 0.2 g^{-1} which indicates its potential for use in food industry.

Foaming capacity

Proteins in dispersion cause lowering of the surface tension at the water–air interface, thus creating foam (Surowka and Fik, 1992). The foaming capacity was higher (69.72 ± 0.65) at a lower DH (DH $7.64\% \pm 0.39$) and it decreased significantly ($p<0.05$) with increase in DH (Table 2). This is because, the larger the molecular size of the protein better is the foaming capacity. Similar results have been reported by Klompong *et al.* (2007) and Nalinanon *et al.* (2011). Poor foaming properties at high DH is due to the small size of peptides, which would hinder the formation of a stable film around the gas bubbles. This is in line with previous findings that larger the molecular size of the protein better is the foaming capacity.

Oil binding capacity

Oil binding capacity is an important functional characteristic of ingredients used in the meat and confectionery industries. It is an important attribute that will influence the taste of a product. The mechanism of fat absorption is attributed mostly to physical entrapment of the oil, and thus the higher the bulk density of the protein the more will be the fat absorption. Fat-binding capacity of proteins correlates with surface hydrophobicity (Kristinsson and Rasco, 2000). The striped cat fish protein hydrolysate showed good oil binding capacities at all the 3 enzyme concentrations. The capacity decreased significantly with increase in DH ($p<0.05$) (Table 2). This may be due to the proteolytic degradation of protein structure (Diniz and Martin, 1997). There was no significant change in the fat binding capacity when the enzyme concentration increased from 1.5 to 2.5%. Similar results have been reported by Wasswa *et al.* (2007) with grass carp skin protein hydrolysates and Diniz and Martin (1997) with shark protein hydrolysates where the oil binding capacities decreased with the increase in DH.

The results of the present study indicate that the protein hydrolysates prepared by enzyme alcalase exhibited good antioxidant activity and functional property at low enzyme concentrations. The optimisation of enzymatic production, the effects of hydrolysates on food taste and quality as well as identification of the specific peptides in striped catfish protein hydrolysate responsible for its overall antioxidative capability need to be further studied.

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