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Influence of the extent of enzymatic hydrolysis on the functional properties of protein hydrolysate from grass carp (*Ctenopharyngodon idella*) skin

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Abstract

Protein hydrolysates from grass carp skin were obtained by enzymatic hydrolysis using Alcalase®. Hydrolysis was performed using the pH-stat method. The hydrolysis reaction was terminated by heating the mixture to 95 °C for 15 min. At 5.02%, 10.4%, and 14.9% degree of hydrolysis (DH), the hydrolysates were analyzed for functional properties. The protein hydrolysates had desirable essential amino acid profiles. Results demonstrated that the hydrolysates had better oil holding and emulsifying capacity at low DH. The water holding capacity increased with increased levels of hydrolysis. Enzymatic modification was responsible for the changes in protein functionality. These results suggest that grass carp fish skin hydrolysates could find potential use as functional food ingredients as emulsifiers and binder agents.

Keywords: Grass carp; Enzymatic hydrolysis; Protein hydrolysates; Functional properties

1. Introduction

Vast amounts of protein rich byproduct materials from the seafood industry are discarded without any attempts of recovery. At the same time processors can no longer discard offal directly into waters, resulting in high disposal costs (Kristinsson & Rasco, 2000a). Processing discards from fisheries account for as much as 70–85% of the total weight of catch and 30% of the waste is in the form of bones and skins (Shahidi, 1994).

The use of fish waste has been of increasing interest in the past years as the biomass of marine origin is considered a safe material and provides proteins with high nutritional properties and a good pattern of essential amino acids (Guerard, Dufossé, Broise, & Binet, 2001). Traditionally, fish wastes have been used as fish meal for animal feeding. Another way of upgrading for fish proteins has been the production of fish protein hydrolysates (FPH) in controlled conditions. Many of these protein-rich seafood byproducts have a range of dynamic properties and can potentially be used in foods as binders, emulsifiers and gelling agents.

Many studies have demonstrated that selective enzymatic hydrolysis of shark protein (Diniz & Martin, 1997), salmon protein (Gbogouri, Linder, Fanni, & Parmentier, 2004; Kristinsson & Rasco, 2000a; Sathivel, Smiley, Prinyawiwatkul, & Bechtel, 2005), herring (Liceaga-Gesualdo & Li-Chan, 1999; Sathivel et al., 2003), capelin (Shahidi, Han, & Synowiecki, 1995), sardine (Quaglia & Orban, 1987, 1990), soy protein (Don, Pilosof, & Bartholomai, 1991; Jung, Murphy, & Johnson, 2005; Tsumura et al., 2005) and whey protein (Kuehler & Stine, 1974; Sinha, Radha, Prakash, & Kaul, 2007) improved their functional properties, including solubility, water holding, oil holding, emulsifying and foaming characteristics. Proteins extracted

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from fish processing byproducts can be modified to improve the quality and functional characteristics (solubility, fat absorption, water holding capacity, foaming properties, emulsifying properties and sensory properties) by enzymatic hydrolysis (Kristinsson & Rasco, 2000b; Shahidi, 1994).

Not much work has been done recently on fish protein hydrolysates (FPH), but some research has been directed towards the potential of using powdered hydrolysates in food formulations. By careful control of the hydrolysis process at well defined stages, it is possible to produce hydrolysates with different degrees of hydrolysis and different functional properties (Quaglia & Orban, 1987). The choice of substrate, the protease enzyme employed and the degree of hydrolysis (DH) can greatly affect the physico-chemical properties of the resultant hydrolysate.

Grass carp (*Ctenopharyngodon idella*) is herbivorous freshwater fish that originated in the rivers draining East Asia and is cultured in many parts of the world. Grass carp byproducts, including viscera, heads, cut-offs, bone and skin, like many other fish processing byproducts, have been used for production of fish meal. Grass carp skin is a good source of high quality proteins that can be used as protein ingredients for food. In a previous study, optimization of the production of hydrolysates from grass carp skin using Alcalase was done. The freeze dried hydrolysate had high protein content (90.8%). The hydrolysate produced was highly soluble with good water holding, oil binding and emulsifying properties (unpublished results). However, there have been no reported attempts to study the effect of enzymatic hydrolysis on the functional properties fish skin protein. The objective of the present study was to investigate the influence of the degree of enzymatic hydrolysis on the functional properties of protein hydrolysate from grass carp skin.

2. Materials and methods

2.1. Raw materials

Fresh grass carp (*Ctenopharyngodon idella*) skin was obtained from the local seafood market of Wuxi (Jiangsu, China). It was finely ground, homogenized, vacuum packed and kept frozen at −20 °C until needed for the experiment. Prior to the hydrolysis process, a portion of the mince was thawed overnight in a refrigerator at 4 ± 1 °C.

2.2. Enzymes and other chemicals

Alcalase® (a declared activity of 2.4 AU/kg and a density of 1.18 g/ml) is a bacterial endoproteinase from a strain of *Bacillus licheniformis* and it was provided by Novo Nordisk (Denmark) and stored at 5 °C until it was used for the hydrolysis experiments.

Other chemicals and reagents were obtained from local manufacturers and made available at the university chemical store. All chemicals and/or reagents used in this work were food grade or reagent grade.

Table 1
Characteristics used in the preparation of samples used in the evaluation

Sample code	Sample characteristics				
	T	pH	*t*	E/S	DH
DH:5.02	59	8	75	0.12	5.02
DH:10.4	58	8	110	0.57	10.4
DH:14.9	60	9	120	1.08	14.9

T, temperature (°C); *t*, time (minutes); E/S, enzyme/substrate ratio (%); DH, degree of hydrolysis (%).

2.3. Preparation of protein hydrolysates

Three hydrolysed samples with varying degree of hydrolysis (DH) were produced using the characteristics described in Table 1. All reactions were done in a 1 L polyethylene-jacketed glass vessel in a thermostatically controlled water bath with constant agitation (200 rpm). The vessel was covered with a close fitting lid which had openings for an automatic temperature compensator (ATC) probe, a pH electrode, a mixer shaft and for the addition of alkali. During the reactions, the pH was maintained at the desired value by addition of 0.2 N NaOH. The reaction vessel with 100 g of thawed grass carp skin mince and a measured volume of deionised water was placed in a heated water bath. A 5 min homogenisation took place to allow the adjustment of pH (through addition of 0.2 N NaOH) and temperature to the desired value. After equilibrium was reached, the enzyme (Alcalase®) was added and the reaction was allowed to proceed. The amount of alkali added to keep the pH constant during the hydrolysis was recorded and used to calculate the degree of hydrolysis (DH).

The reactions were terminated by immersing the reaction vessel into water at 95 °C for 15 min with stirring to ensure the inactivation of the enzyme and the separation of oil from the substrate. The resultant slurry was directly cooled on ice and then centrifuged at 2800*g* for 20 min at 2 °C. The supernatant was collected, concentrated and freeze dried. The freeze dried hydrolysates were stored in a desiccator until required for functional property analyses. Fig. 1 outlines the process used in the preparation of protein hydrolysate from grass carp skin.

2.4. Estimation of degree of hydrolysis

The hydrolysis was carried out using the pH-stat method (Adler-Nissen, 1977). Degree of hydrolysis (DH) is defined as the percentage ratio between the number of peptide bonds cleaved (h) and the total number of peptide bonds in the substrate studied (h_{tot}). The degree of hydrolysis was determined based on the consumption of base necessary for controlling system pH during the batch assay as depicted in Eq. (1) (Adler-Nissen, 1986).

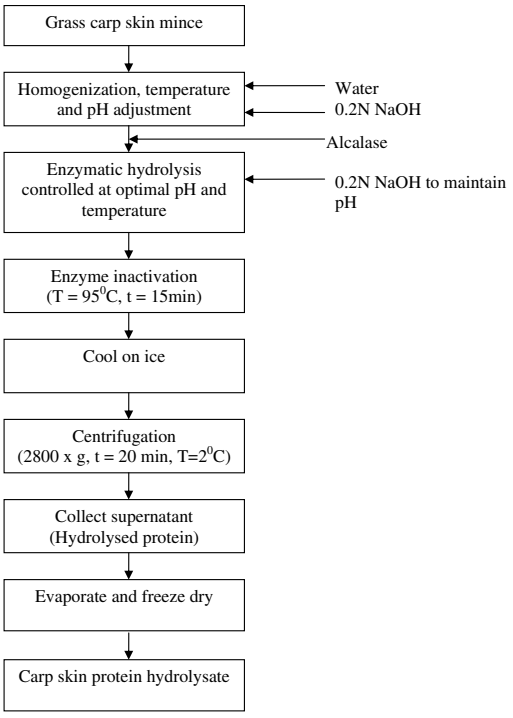

Fig. 1. Flow sheet for Alcalase-assisted preparation of grass carp skin protein hydrolysate.

$$\mathrm{DH} = \frac{h}{h_{\mathrm{tot}}} \times 100\% = B \times N_{\mathrm{b}} \times \frac{1}{\alpha} \times \frac{1}{\mathrm{MP}} \times \frac{1}{h_{\mathrm{tot}}} \times 100\% \quad (1)$$

h_{tot} is the total number of peptide bonds in the protein substrate, in mmol/g_{protein}; h is the number of hydrolyzed peptide bonds; B is the base consumption in ml; N_{b} is base normality; α is the average degree of dissociation of the α-NH groups and MP is the mass of protein in g.

The functional properties were studied, especially as a function of percent DH at 5.02, 10.4 and 14.9.

2.5. Proximate composition

Moisture content was determined by placing approximately 2 g of sample into a pre-weighed aluminum dish. The samples were then dried in a forced-air convection oven at 105 °C until a constant weight was reached (AOAC, 1995). Ash content was estimated by charring in a crucible at 600 °C until the ash had a white appearance (AOAC, 1995). The total crude protein ($N \times 6.25$) content of the samples was determined using the Kjeldahl method (AOAC, 1995). The extraction and determination of total lipids from the samples was performed (Bligh & Dyer, 1959).

2.6. Water holding capacity

Water-holding capacity (WHC) was determined using the centrifugation method (Diniz & Martin, 1997). Duplicate samples (0.5 g) of hydrolysate were dissolved with 20 ml of water in centrifuge tubes and dispersed with a vortex mixer for 30 s. The dispersion was allowed to stand at room temperature for 6 h, and it was then centrifuged at 2800*g* for 30 min. The supernatant was filtered with Whatman No. 1 filter paper and the volume recovered was accurately measured. The difference between the initial volume of distilled water added to the protein sample and the volume of the supernatant was determined, and the results were reported as ml of water absorbed per gram of protein sample.

2.7. Oil-holding capacity

Oil-holding capacity (OHC) was measured as the volume of edible oil held by 0.5 g of material (Haque & Mozaffar, 1992). The sample 0.5 g was added to 10 ml of soybean oil in a centrifuge tube, and mixed for 30 s in a vortex mixer in duplicate. The oil dispersion was centrifuged at 2800*g* for 30 min. The volume of oil separated from the hydrolysate was measured and OHC was calculated as the ml of oil absorbed per gram of protein sample.

2.8. Emulsification capacity

Emulsification capacity (EC) was measured by an oil titration method (Diniz & Martin, 1997) with slight modification. The freeze-dried sample and 30 ml of soybean oil were added to 60 ml of NaCl solution (30 g/l) and mixed using Fluko FA25 homogenizer (Shanghai, China) at 9500 rpm for 10 min. After this period, another 30 ml of soybean oil were added over 1.5 min and then mixed for a further 30 s. The mixture was transferred to centrifuge tubes, held in a water bath at 85 °C for 15 min, and then centrifuged at 2800*g* for 30 min. Emulsification capacity was calculated by the,

$$\mathrm{EC} = \frac{O_{\mathrm{A}} - O_{\mathrm{R}}}{W_{\mathrm{S}}} \quad (2)$$

O_{A} is the volume of oil added to form an emulsion, O_{R} is the volume of oil released after centrifugation and W_{S} is the weight of the sample.

2.9. Colour measurements

The color of the hydrolysate powders were evaluated using the Hunter Lab colorimeter (WSC-S Colour Difference Meter) and reported as L^*, a^* and b^* values, in which L^* is a measure of lightness, a^* represents the chromatic scale from green to red and b^* represents the chromatic scale from blue to yellow. The instrument was standardized to measure the colour difference with an L^* value of 91.32, an a^* value of 0.03 and a b^* value of 0.01.

2.10. Molecular weight distribution of hydrolysates

The molecular weight distribution of hydrolysate was determined using a Waters™ 600E Advanced Protein Purification System (Waters Corporation, Milford, MA, USA).

A TSK gel, 20005 μ × L, (6.5 × 300 mm) column was used with 10% acetonitrile + 0.1% TFA in HPLC grade water as the mobile phase. The calibration curve was obtained by running bovine carbonic anhydrase (29,000 Da), horse heart cytochrome C (12,400 Da), bovine insulin (5800 kDa), bacitracin (1450 Da), gly–gly–tyr–arg (451 kDa) and gly-gly-gly (189 Da). The results were obtained and processed with the aid of Millennium32 Version 3.05 software (Waters Corporation, Milford, MA 01757, USA).

2.11. Amino acid analysis

The hydrolysates were dissolved in 3.5% 5-sulfosalicylic acid (SSA). After filtration and centrifugation, the supernatants were submitted for online derivatization by *o*-phthaldialdehyde (OPA) and reverse phase high performance liquid chromatography (RP-HPLC) analysis in an Agilent 1100 (Agilent Technologies, Palo Alto, CA 94306, USA) assembly system using a Zorbax 80A C_{18} column (4.6 i.d. × 180 mm) using the conditions set by the equipment manufacturer.

2.12. Statistical analysis

Experiments on the functional properties were done in duplicate. The data obtained was subjected to a one-way analysis of variance (ANOVA). Duncan's new multiple range test (DNMRT) was performed to determine the significant difference between samples at the 5% probability level (SAS, 2002).

3. Results and discussion

The composition of fresh grass carp skin and its hydrolysate is given in Table 2.

Several studies have shown that fish protein hydrolysates have excellent water holding capacity (WHC) and can increase the cooking yield when added to minced meat (Kristinsson & Rasco, 2000b; Shahidi et al., 1995). WHC increased with increased hydrolysis and the values were statistically different at 5% level for the samples DH:5.02, DH:10.4 and DH:14.9 as shown in Table 3. A similar trend in water absorption was observed for whey protein hydrolysate with increased degree of hydrolysis from 16 to 34 ml per 100 g of sample (Sinha et al., 2007). The presence of polar groups such as COOH and NH_2 that increased during enzymatic hydrolysis had a substantial effect on the amount of adsorbed water (Kristinsson & Rasco, 2000b).

Grass carp skin hydrolysates at different degree of hydrolysis (DH) exhibited excellent oil holding capacity (OHC) as reported in Table 3. OHC expresses the quantity of oil directly bound by the protein and is of great interest as it is an important functional characteristic, especially expected by the meat and confectionary industry (Gbogouri et al., 2004). The hydrolysates at 5.02% DH had significantly higher OHC (3.6 ml oil/g hydrolysate) followed by 10.4% DH (3.2 ml oil/g hydrolysate) and then 14.9% DH (2.4 ml oil/g hydrolysate) due to the larger particle sizes. A decrease in OHC with DH increase has been reported for shark muscle protein hydrolysis (6.8–4.8 ml/g of sample) (Diniz & Martin, 1997) and red salmon head (5–3.9 ml/g of sample) (Sathivel et al., 2005). The hydrolysates can potentially be used as functional ingredients for the meat and confectionary industry. The decrease in the OHC as DH increased might have been due to the hydrolytic degradation of the protein structure. Protein forms a network which is degraded by hydrolysis with consequent diminishing of the sample's oil absorption capacity (Don et al., 1991). Other studies indicated that hydrophobic interactions are primarily responsible for this (Haque & Mozaffar, 1992; Liceaga-Gesualdo & Li-Chan, 1999).

The emulsifying capacities of the hydrolysates decreased with increasing protein hydrolysis. An inverse relationship of DH to emulsifying capacity has been reported (Quaglia & Orban, 1990). This may be a result of the presence of smaller peptides, which are less effective in stabilizing emulsions. At 14.9% DH, the emulsion value (20.8 ml/0.5 g) was relatively comparable to that of shark muscle hydrolysate (23.09 ml/g) obtained at 18.8% DH (Diniz & Martin, 1997). Similar results on emulsion capacity of whey protein hydrolysate showed a decreasing trend with increasing degree of hydrolysis (45 ml of oil/g of control to 35 and

Table 2
Proximate composition (%) of the fresh grass carp skin and its hydrolysate

Sample	Moisture	Crude protein	Fat	Ash
Fresh skin	64.1 ± 0.10	22.6 ± 0.60	1.10 ± 0.30	0.27 ± 0.02
Hydrolysate	2.87 ± 0.05	92.0 ± 1.75	0.2 ± 0.10	5.86 ± 0.60

Table 3
Functional properties of hydrolysed grass carp fish skin

Sample	WHC	OHC	EC
DH:5.02	2.0 ± 0.3[a]	3.6 ± 0.2[a]	38.0 ± 3.0[a]
DH:10.4	3.8 ± 0.2[b]	3.2 ± 0.1[b]	27.4 ± 2.0[ab]
DH:14.9	4.9 ± 0.2[c]	2.4 ± 0.2[c]	20.8 ± 2.5[b]

WHC = Water-holding capacity (ml of water absorbed/g of sample), OHC = Oil-holding capacity (ml of oil absorbed/g of sample), EC = Emulsification capacity (ml of oil/0.5 g of sample).
[a–c] Values columns with different superscripts are significantly different at $\alpha = 0.05$. Values are means of duplicate determinations.

Table 4
Hunter colour parameter values of hydrolysed grass carp fish skin

Sample	Hunter colour parameters		
	L^*	a^*	b^*
DH:5.02	68.9 ± 0.9[a]	−3.73 ± 1.2[a]	18.4 ± 0.7[a]
DH:10.4	59.6 ± 0.2[b]	−2.46 ± 0.3[ab]	22.0 ± 0.5[b]
DH:14.9	59.3 ± 0.7[b]	−1.38 ± 0.8[b]	26.6 ± 0.9[c]

L^*: measure of lightness, a^*: chronic scale from green ($-a$) to red ($+a$), b^*: chronic scale from blue ($-b$) to yellow ($+b$).
[a–c] Values columns with different superscripts are significantly different $\alpha = 0.05$. Values are means of triplicate determinations.

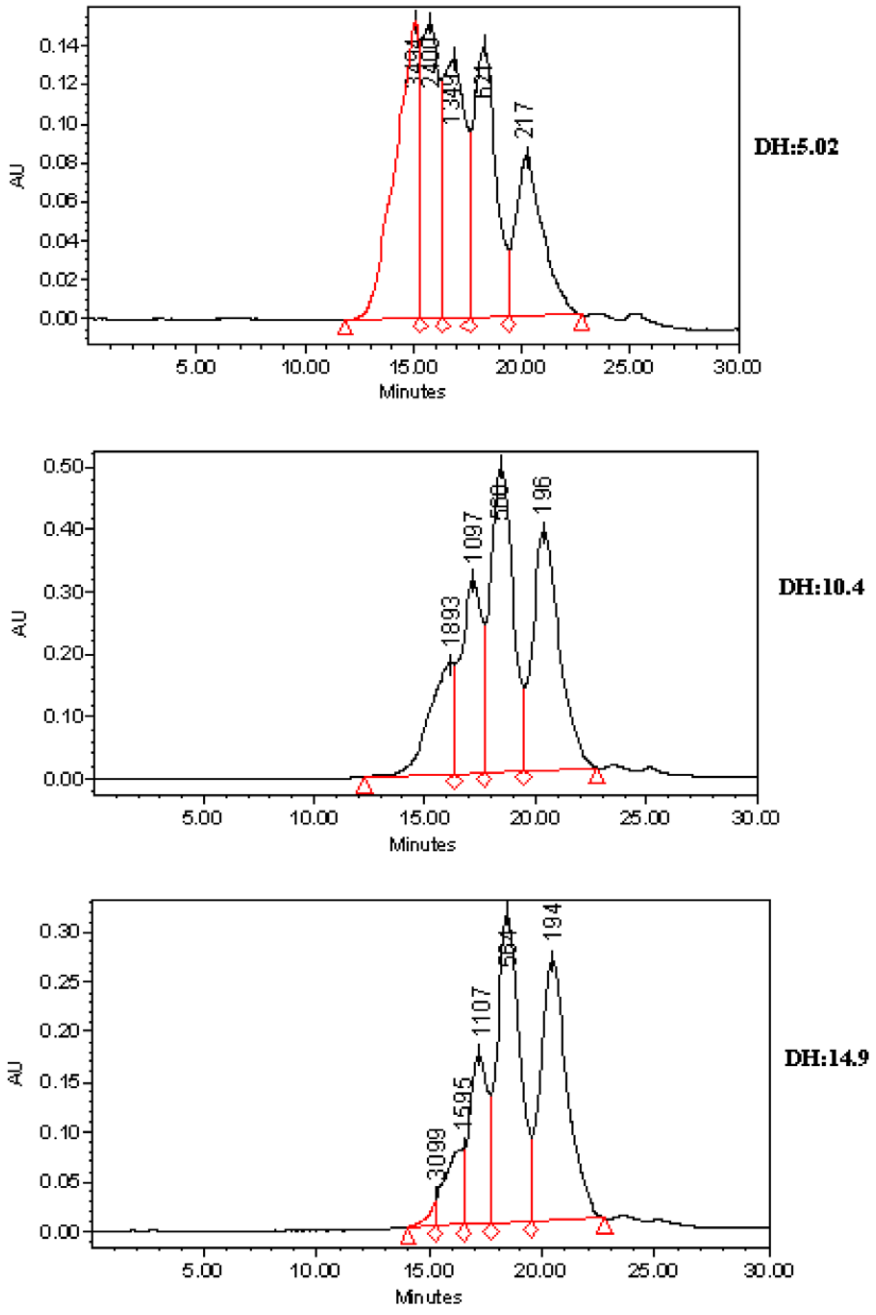

Fig. 2. Size exclusion chromatography profiles grass carp fish skin hydrolysates.

30 ml of oil/g using fungal protease and papain respectively) (Sinha et al., 2007). A decrease in emulsifying capacity of casein with increased DH, in the range of 25–65%, using porcine pancreatin has been reported (Mahmoud, Malone, & Cordle, 1992). However, they did not study the effect at DH between 0% and 25%. In this study, at 5.02% hydrolysis, the emulsification value (38 ml/0.5 g hydrolysate) was not significantly different from 10.4% hydrolysis (27.4 ml/0.5 g hydrolysate) but was significantly different from 14.9% hydrolysis (20.8 ml/0.5 g hydrolysate). The higher EC value obtained at 5.02% hydrolysis can be attributed to the low level of degradation of the protein molecules by Alcalase®, which brought about the increase in the availability of large peptide units at the oil-water interface hence providing a larger surface area (Diniz & Martin, 1997). The significant decrease in EC at 14.9% hydrolysis was presumably due to the effect of pH which was increased from 8 to 9 during the hydrolysis, reduced peptide chain length and increased hydrophobicity (Sathivel et al., 2003). Generally, the pH of protein solutions during emulsification affects their emulsifying properties via charge effects (Nielsen, 1997). Addition of salts improves the emulsion properties of peptide fractions (Turgeon, Gauthier, & Paquin, 1992). Several factors such as blend-

ing speed, protein source, temperature, pH, type of oil added and water content influence emulsion capacity (Linder, Fanni, & Parmentier, 1996). The mechanism of the emulsification process of hydrolysates is attributed to adsorption, to the surface, of freshly formed oil droplets during homogenization and this forms a protective membrane that prevents droplets from coalescening. Hydrolysates are surface active materials and promote oil-in-water emulsions because of their hydrophilic and hydrophobic groups (Gbogouri et al., 2004).

Colour influences the overall acceptability of food products. Hydrolysis of grass carp skin produced protein powders that were light yellow in colour (Table 4). DH:14.9 sample was the darkest ($L^* = 59.3$) and most yellowish ($b^* = 26.56$) whereas DH:5.02 sample was the lightest ($L^* = 68.85$) and least yellowish ($b^* = 18.38$). Increased time of hydrolysis resulted in increased enzymatic browning reactions. Enzymatic browning reactions are assumed to have contributed to reduction in the luminosity, giving a darker appearance at high DH. The b^* value was significantly different for all the samples at 5% level. The changes appear to indicate that the colour of grass carp skin protein hydrolysate powder is positively influenced by the enzymatic treatment with Alcalase®.

Chain length of peptides, which are dependant on DH, is of special interest because properties such as emulsion capacity and bitterness depend at least in part on molecular size (Mohr, 1980). Fig. 2 shows the size exclusion chromatography profiles for grass carp fish skin hydrolysates. The major components in the hydrolysates had a molecular weight of 150–2500 Da. The molecular weight distribution of the 3 hydrolysates was similar although the relative proportions of the peaks varied accordingly with DH. From chromatographic data (Table 5), it can be seen that the average molecular weight of the peptides is below 3500 Da and that the peptide range repartition depends on DH. Hydrolysates with lower DH (5.02%) were characterized by a higher percentage of high molecular weight peptides ranging from 2500 to 3500 Da compared with hydrolysates with higher DH (14.9%), that were characterized by low molecular weight peptides ranging from 150 to 500 Da. The proportion of the high molecular weight peptides decreased as the DH increased as evidenced with salmon byproducts (Gbogouri et al., 2004; Sathivel et al., 2005).

Table 5
Molecular weight distribution (percent of total area) of grass carp fish skin hydrolysate

Molecular weight (Da)	Area		
	DH:5.02	DH:10.4	DH:14.9
>2500	25.4	–	1.00
1500–2500	17.7	13.4	7.36
1000–1500	19.4	19.7	16.2
500–1000	21.8	34.7	37.7
<500	15.8	32.3	37.7

Table 6
Total amino acid composition of native grass carp skin and its hydrolysate (g 100 g^{-1} Protein)

Amino acid	Native skin protein	Skin hydrolysate DH 14.9%	EAA[a]
Glycine	12.8	17.1	
Alanine	5.64	15.6	
Valine	1.65	2.26	1.3
Isoleucine	1.19	1.60	1.3
Leucine	2.01	2.86	1.9
Proline	5.49	8.34	
Cysteine	1.05	1.86	
Phenylalanine	1.53	2.18	
Tyrosine	5.58	3.78	
Serine	2.17	3.14	
Threonine	1.41	2.03	0.9
Methionine	1.18	1.54	1.7[b]
Lysine	2.58	3.44	1.6
Histidine	4.42	5.95	1.6
Arginine	4.85	7.22	
Aspartic acid	3.66	5.54	
Glutamic acid	6.43	9.44	

[a] Suggested profile of essential amino acid requirements for adults (FAO/WHO, 1990).
[b] Methionine + cysteine.

The amino acid profiles of grass carp native skin protein and its hydrolysate are given in Table 6. The amino acid composition did differ significantly between the native skin protein, sampled before hydrolysis, and the resultant hydrolysate. The results showed that the amino acid profiles of the skin hydrolysates were generally higher in essential amino acid profiles compared with the suggested pattern of requirement by FAO/WHO for adult humans (FAO/WHO, 1990).

4. Conclusions

Protein hydrolysates have been obtained from grass carp skin using a commercial enzyme (Alcalase®) preparation. It is noticeable that functional properties of fish protein hydrolysates could be modified according to the DH value. Hydrolysis with proteolytic enzymes provides the possibility of controlling the degree of cleavage of the protein in the substrate. The results suggested that the extent of the enzymatic modification should be determined by the intended application of the new food ingredient, since enzyme treatment can cause substantial changes in protein functionality. Hydrolysates from grass carp skin protein may potentially serve as a good source of desirable peptides and amino acids. The functional properties (fat absorption, water absorption and emulsification stability) of the grass carp skin hydrolysates are consistent with potential applications as emulsifiers and binder agents. The grass carp skin based hydrolysate powders can be commercially incorporated into foods for human consumption. This makes them potential competitors with dairy based and plant based protein hydrolysates currently being used.

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