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Desalting Fish Skin Protein Hydrolysates Using Macroporous Adsorption Resin

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Abstract: Macroporous Adsorption Resin (MAR) DA 201-C was used to desalt different Fish Skin Protein Hydrolysates (FSPHs). The FSPHs were obtained by hydrolysis of fish skin using Alcalase in a batch reactor a 60°C and pH 8.25. The ash was removed by adsorbing FSPHs onto MAR. Desorption was achieved by washing with alcohol at different concentrations. Ash content of the FSPHs was reduced from 4.69-5.57 to 1.07-2.48% range. The protein content was enriched from 89.07-90.82 to 94.89-96.38% range. MAR has good hydrolysate recoveries. The use of MAR showed promising results in decolourization and fishy flavour reduction. Nile tilapia and Nile perch skin protein hydrolysates were moderately bitter compared to Grass carp skin protein hydrolysates. The bitter taste in FSPHs was reduced to slightly detectable levels by our sensor panel. The hydrolysates had relatively low molecular weight. The process of applying MAR to desalt and debitter FSPHs is feasible.

Key words: Enzymatic hydrolysis, fish skin protein hydrolysate, desalting, macroporous adsorption resin, bitterness

INTRODUCTION

Many studies have demonstrated that enzymatic hydrolysis of fish and fish by-products including, shark protein (Diniz and Martin, 1997), salmon protein (Gbogouri *et al*., 2004; Kristinsson and Rasco, 2000; Sathivel *et al*., 2005), herring (Liceaga-Gesualdo and Li-Chan, 1999; Sathivel *et al*., 2003), capelin (Shahidi *et al*., 1995) and sardine (Quaglia and Orban, 1990, 1987) improved their functional properties, including solubility, water holding, oil holding, emulsifying and foaming characteristics. Protein hydrolysis produces peptides with functional, bioactive and sensory properties that are better than those of the native proteins from which they are obtained (Cheison and Wang, 2003).

Fish by-products, including viscera, heads, cut-offs, bone and skin, like many other fish processing by-products, have been used for production of fish meal. Fish skin is a good source of high quality proteins that can be used as protein ingredients for food. In a previous study, Fish Skin Protein Hydrolysates (FSPHs) were produced from Grass carp (*Ctenopharyngodon idella*), Nile perch (*Lates niloticus*) and Nile tilapia (*Oreochromis niloticus*). The freeze dried hydrolysates had a high protein content (above 90%). The hydrolysates produced were highly soluble with good water holding, oil binding, foaming and emulsifying properties (unpublished results).

Substrate susceptibility to enzyme hydrolysis is influenced by the pH. There is, therefore, need for the pH to be regulated, hence the pH-stat method is used to achieve this (Adler-Nissen, 1986). To achieve higher DH, or if hydrolysis is done at higher pH, more alkali is required to neutralise

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the pH-depressing hydrolysis products. This leads to undesirably higher ash build-up in the hydrolysates, which lowers product quality.

In protein purification, it is common to reach a desired purity acceptable for product consumption. Various techniques have been used. Traditionally, desalting of large biomolecules is performed using dialysis, which is slow besides requiring large buffer volumes. Additionally, material loses have been reported as a result of the protein adsorption to the dialysis membranes. Proteins have been desalted using either nanofiltration membranes (Cuartas *et al*., 2004) or gel permeation chromatography using the desalting Sephadex™ gels which are expensive. Desalting and debittering of fish skin protein hydrolysates enhances their value-added qualities as well as processing safety into the product because of consumer sensitivity and attitude to chemicals in food formulations. Cheaper desalting options are therefore invited to lower the production costs while giving higher hydrolysate recoveries.

Macroporous Adsorption Resins (MARs) have been used for desalting biological samples, casein non-phosphorylated peptides (Zhao *et al*., 2002) and taurine from industrial manufacture (Tang *et al*., 2001) with good hydrolysate recoveries. MAR is a non-polar adsorbent resin used mainly for adsorption of organic substances and decolourisation.

It is important to select a cheaper desalting process which is simple and easy to operate. While peptide bitterness is of both academic and technological interest, no reports exist on desalting of FSPHs on MAR, nor are there any reports of debittering with the same. This study was intended to investigate the use of MAR in simultaneous desalting and debittering different freshwater FSPHs.

MATERIALS AND METHODS

Materials

Fresh skin of Grass carp (*Ctenopharyngodon idella*) were obtained from local seafood market of Wuxi, China in September 2006. Skins of Nile perch (*Lates niloticus*) and Nile tilapia (*Oreochromis niloticus*) were obtained from Fishways (U) Limited, Uganda in September 2006. The skin samples were transported to the School of Food Science laboratory of Southern Yangtze University, Wuxi, China. Each of the samples was scrapped off any meaty and fatty tissue, chopped into pieces and dried at temperatures not exceeding 40°C. The dried samples were finely ground, vacuum packed and kept frozen at -20°C till when needed for the experiments. Alcalase® (declared activity of 2.4 AU kg^{-1} and a density of 1.18 g mL^{-1}) a bacterial endoproteinase from a strain of *Bacillus licheniformis* was provided by Novo Nordisk (Denmark) and stored at 5°C till it was used for hydrolysis experiments. A non-polar styrene-based Macroporous Adsorption Resin (MAR), branded DA201-C was sourced from Jiangsu Suqing Water Treatment Engineering Group (Jiangying, Jiangsu, China). All other chemicals and reagents were of analytical grade commercially available locally.

Protein Hydrolysis

Hydrolysis experiments were carried out in a 1-l thermostatically stirred-batch reactor vessel using the pH-stat method. 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. The reaction vessel with 200 g of each of ground skin sample (Nile perch, Grass carp and/or Nile tilapia) was independently mixed with 200 mL of distilled water placed in a previously heated water bath at 60°C. All reactions were performed at pH 8.25 and 60°C following the instructions of maximum activity and stability of the enzyme Alcalase provided by provided by Novo Nordisk (Denmark). The enzyme/protein substrate ratio used was 1.7% (v/w protein). The enzyme was added and the reaction allowed to proceed for 85 min under set conditions of pH and temperature with constant agitation at 500 rpm. The volume of 0.2 N NaOH solution needed to keep the pH constant

during hydrolysis was recorded to allow calculation of degree of hydrolysis (%DH). Reactions were terminated by heating the solution to 95°C for 20 min, ensuring for inactivation of the enzyme. The resulting slurry was centrifuged at 22,000 x g for 15 min at 2°C. The supernatant was collected. The hydrolysates were adsorbed directly following hydrolysis.

Batch Desalting in a Beaker

Due of the length of time taken to desalt and the desalting efficiency, adsorption of the peptides in a beaker was adopted. In this procedure, about 500 mL MAR was stirred with about 1 L fish skin protein hydrolysates (supernatant) for 24 h. At the end of adsorption, the contents were allowed to settle, the top layer was skimmed off and the resins were washed with five-bed volumes of deionised water. The washing was continued with stirring over a magnetic stirrer. At the end of washing, the resins were washed with alcohol to desorb the peptides.

Desorption with Alcohol

Step-wise desorption was used by initially washing with 25% alcohol. The alcohol concentration was varied from 25 to 50% and finally, was washed with 95% alcohol, followed by deionised water. The collected fractions were concentrated under vacuum and freeze-dried. The resin was regenerated by washing with 1 N NaOH to wash off any remaining peptides followed by 1 N HCl with thorough deionised water rinsing in between, until the pH returned to neutral. The cycle could be repeated as desired.

Proximate Composition

Moisture and ash content of freeze dried samples were analysed in triplicates using AOAC standard methods 930.15 and 942.05, respectively (AOAC, 1995). The total crude protein (N×6.25) content of the samples was determined using the Kjeldahl method (AOAC, 1995).

Molecular Weight Distribution of Hydrolysates

The molecular weight distribution of hydrolysates was determined using a Waters™ 600 E 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 Millennium 32 Version 3.05 software (Waters Corporation, Milford, MA 01757, USA).

Amino Acid Analysis

The hydrolysates were dissolved in 3.5% 5-sulfosalicylic acid (SSA). After filtration and centrifugation, the supernatants were submitted to online derivatization by o-phthaldialdehyde (OPA) and reversed 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 id×180 mm) as by the conditions set by the equipment manufacturer.

Colour Measurements

The colour 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.

Sensory Evaluation

Sensory evaluation for the fishy flavour, salt taste and bitter taste in FSPHs powders was conducted by 9 panelists between the ages of 23 and 31. The powdered FSPH samples were served at ambient temperature coded with three digit random numbers. A descriptive test conventional profiling with two replicates was carried out. A profile consisting of 6 attributes, that is, total intensity of smell, stock fish smell, salt taste, bitter taste, stock fish taste and after taste were judged according to a linear scale of intensity increasing from 1 to 9 for none to extremely strong. The assessments were performed in a room specially designed for sensory analysis.

Statistical Analysis

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).

RESULTS AND DISCUSSION

The MAR properties are summarized in Table 1. Desorption of FSPHs from MAR by alcohol shows that the interaction involved between the resin and the hydrolysates is indeed hydrophobic in nature. This is due of the presence of both the hydrophobic and a hydrophilic zone in alcohol. The non-polar amino acid residues will not contact with the water while the polar side chains point out towards water (Tanford, 1962). In this way, the hydrolysates interacted with the resins hydrophobically to achieve a favourable configuration during desalting and rinsing. The ability of alcohol recovery by a process such as low pressure evaporation is of advantage as it may lower the cost of the process in the long term.

The ash and protein content in the desalted fractions as they were enriched in the range 1.07-2.48 and 94.89-96.38%, respectively (Table 2). In a previous study, the ash and protein contents of the samples not desalted were in the range of 4.69-5.57 and 89.07-90.82%, respectively (unpublished results). Similar observations were reported when MAR was used for simultaneous desalting and debittering of Whey Protein Hydrolysates (WPH) (Cheison *et al*., 2006). The protein contents where not significantly different from each other in the desalted samples. Total protein recovery in the three FSPHs varied from about 44 to about 70%. Grass carp skin had the least protein recovery of 43.73% while Nile tilapia had the highest protein recovery of 69.96%. This is rather low, but not unexpected considering that not all the peptides will be adsorbed into the MAR. Low protein recoveries have been reported during desalting and debittering of WPH with MAR using alcohol concentrations of 20, 40 and 75% (v v^{-1}) for desorption (Cheison *et al*., 2006). The protein recovery could be improved by another adsorption process on the same hydrolysate but this may be time consuming.

Table 1: DA201-C macroporous adsorption resin properties[a]

Polarity	None
Pearl size	0.4-1.25 (mm)
Surface area	1000-1300 ($m^2 g^{-1}$)
Average pore diameter	30-40 (nm)
Pore volume	1.0-1.1 ($cm^3 g^{-1}$)

[a]Data supplied with product in producer's manual manufactured from styrene based material

Table 2: Proximate composition (%) of Nile perch, Grass carp and Nile tilapia skin hydrolysate

Sample	Protein	Moisture	Ash	Protein recovery
Nile perch	95.74±1.26[a]	2.84±0.03[a]	2.48±0.04[a]	57.43
Grass carp	94.89±0.92[a]	3.83±0.14[a]	1.07±0.08[b]	43.73
Nile tilapia	96.38±1.15[a]	2.87±0.04[a]	1.83±0.05[b]	69.96

Same letter superscripts in a column denote no significant difference ($\alpha = 0.05$)

From the chromatographic data, (Fig. 1) it was observed that all hydrolysates were composed of low molecular weight peptides. Size exclusion chromatogram of the hydrolysate powders prepared showed a much higher of peptides with a molecular weight below 3500 Da. This molecular weight size corresponds well with the size of peptides extracted from cod bone using different bacterial enzyme preparations (Gildberg *et al*., 2002).

There were no remarkable differences in the content of amino acids for the three FSPHs (Table 3). The results showed that the amino acid profile of the skin hydrolysates were generally higher in essential amino acid profile compared with the suggested pattern of requirement by FAO/WHO for adult humans (FAO/WHO, 1990).

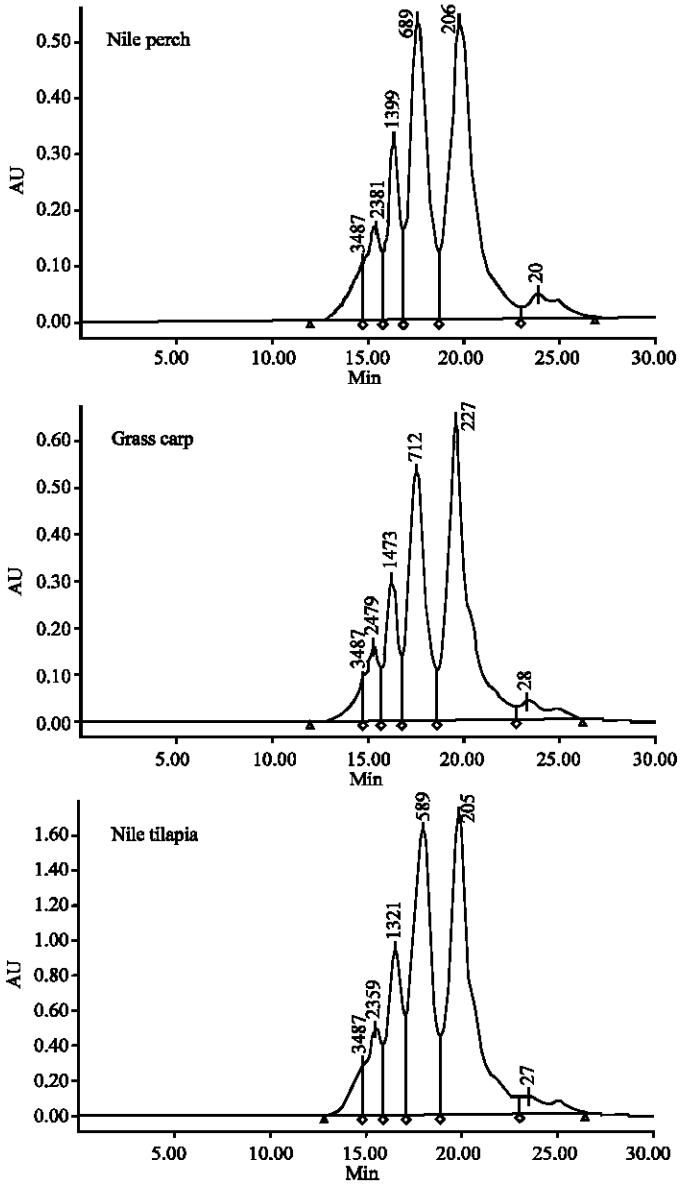

Fig. 1: Size exclusion chromatography profiles of Nile perch, Grass carp and Nile tilapia skin hydrolysate

Colour influences the overall acceptability of food products. Hydrolysis of FSPHs produced protein powders that were light yellow in colour (Table 4). The L* and b* values were significantly different for all samples at 5% probability level. In measurement of colour of prepared FSPHs, samples from Nile perch were darkest with corresponding high scores of redness and yellowness, while Nile tilapia hydrolysates scored highest for lightness. In a previous study, the hunter colour parameters observed for the FSPHs which had not undergone the desalting procedure were darker than the present study (unpublished results). In contrary, no adverse effects in colour we observed in desalted milk hydrolysates (Ma *et al*., 1983). This study, therefore, proved the MAR was able to eliminate some colour from the FSPHs.

Sensory evaluation of the FSPHs (Table 5) showed that they were bitter. Nile tilapia and Nile perch fractions had moderate to moderately strong bitterness while the Grass carp fraction had slight bitterness and was significantly different at 5% probability level. The bitterness in FSPHs can be

Table 3: Total amino acid composition of Nile perch, Grass carp and Nile tilapia skin hydrolysate

	Composition (g/100 gprotein)			
Amino acid	Nile perch	Grass carp	Nile tilapia	EAA[a]
Glycine	17.86	17.11	18.40	
Alanine	17.42	15.59	17.03	
Valine	2.52	2.26	2.33	1.3
Isoleucine	1.52	1.60	1.55	1.3
Leucine	2.93	2.86	3.24	1.9
Proline	8.58	8.34	10.56	
Cysteine	1.74	1.86	1.97	
Phenylalanine	2.22	2.18	2.25	
Tyrosine	4.32	3.78	4.30	
Serine	2.86	3.14	3.26	
Threonine	2.20	2.03	2.23	0.9
Methionine	1.71	1.54	1.46	1.7[b]
Lysine	3.64	3.44	3.42	1.6
Histidine	8.93	5.95	8.96	1.6
Arginine	7.58	7.22	7.82	
Aspartic acid	5.69	5.54	5.73	
Glutamic acid	9.89	9.44	10.00	

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

Table 4: Hunter colour parameter values of Nile perch, Grass carp and Nile tilapia skin hydrolysate

	Hunter colour parameters		
Samples	L*	a*	b*
Nile perch	92.32±0.13[a]	-0.89±0.52[a]	20.96±0.12[a]
Grass carp	96.64±0.90[b]	-1.31±0.93[a]	18.76±0.20[b]
Nile tilapia	100.03±0.32[c]	-3.81±0.02[b]	17.15±0.16[c]

[a-c]Values in a column with different superscripts are significantly different $\alpha = 0.05$. Values are means of triplicate determinations. L* Measure of lightness, a* Chronic scale from green (-a) to red (+a), b* Chronic scale from blue (-b) to yellow (+b)

Table 5: Sensory attributes of the three FSPHs analysed by ANOVA

	Skin protein hydrolysates		
Organoleptic parameters	Nile perch	Grass carp	Nile tilapia
Intensity of smell	3.43[a]	5.21[a]	3.50[a]
Stock fish smell	3.29[ab]	4.64[a]	2.86[b]
Salt taste	2.07[a]	3.43[b]	1.50[a]
Bitter taste	5.57[a]	3.64[b]	7.00[a]
Stock fish taste	3.86[a]	4.21[a]	2.87[a]
After taste	4.93[a]	4.36[a]	4.36[a]

[a-b]Value means in a row with different superscripts are significantly different $\alpha = 0.05$

attributed to highly hydrophobic, short peptides composed largely of a good supply of essential amino acids (Kanekanian *et al*., 2000). High bitterness has been reported in enzymatically hydrolysed cod by-products (Daukšas *et al*., 2003). The acceptance of fish protein hydrolysates in food has been precluded because of the problems with fishy off-flavours (Adler-Nissen, 1986). In the present study, the fishy flavour was almost undetectable showing that MAR was capable of reducing the fishy flavour in the three fractions. The use of MAR in this study hence provided an upgradeable process that can be utilized in debittering and fishy flavour removal from FSPHs. Related results have been reported when MAR was used in debittering of WPH (Cheison *et al*., 2006).

CONCLUSIONS

The present results showed that the hydrolysate salt was significantly removed with adsorption of FSPHs on MAR followed by rinsing with deionised water to wash out the salt during which instance the peptides remained adsorbed onto the MAR resins. It also provides an exciting technological manipulation to reduce the fishy flavour and debitter FSPHs. MAR, therefore, presents technological importance to remove salt in protein hydrolysates. Although Grass carp skin hydrolysate powder gave the least bitter hydrolysates, low protein recovery was observed. The best protein recovery was observed with Nile tilapia skin hydrolysate powders. The alcohol used in desorption could be recovered and reused cutting down the process costs. The FSPHs obtained after the desalting process generally had a low molecular weight and could be incorporated into foods for human consumption making them potential competitors with dairy based and plant based protein hydrolysates currently being used.

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