

Comparison between Thermal Hydrolysis and Enzymatic Proteolysis Processes for the Preparation of Tilapia Skin Collagen Hydrolysates

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Abstract

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The tilapia (*Oreochromis niloticus*) skin hydrolysate was produced by thermal or enzymatic hydrolysis processes. Several product characteristics were studied such as the average molecular weight, 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity, yield, and protein content, in order to compare thermal hydrolysis and enzymatic proteolysis processes for the hydrolysed tilapia skin collagen production. The effects of the following hydrolysis parameters (retorting time and pH, protease combination, and proteolysis time) were studied. Compared with the thermal hydrolysis process, the enzymatic proteolysis process needed less time and milder conditions, under which hydrolysates could be obtained as low molecular weight antioxidant peptides.

Keywords: fish skin gelatin; hydrolysate; antioxidant activity; autoclaving; proteases

Global production of tilapia is dominated by China. According to China Fishery Yearbook 2010, by 2009 the annual China production of tilapia had risen to nearly 1.25 mill. t and the volume of tilapia processing was 0.59 mill. ton.

Large quantities of fish waste including skins and bones from tilapia fillet processing account for about one half of the unprocessed fish weight. If these collagen-containing wastes are dumped, they may cause pollution and the protein resources are squandered.

Collagen from fish skins can meet religious needs (Jewish or Muslim) and avoid bovine spongiform encephalopathy (BSE) or the foot-and-mouth disease (FMD) in the collagen of land animal origins.

GROSSMAN and BERGMAN (1992) explored the process for the production of gelatin from tilapia skin. JAMILAH and HARVINDER (2002) and ZENG *et al.* (2009) studied the characteristics of gelatin obtained from the skin of tilapia, which manifested that the gelatin from tilapia skins has broad pros-

pects for application. In recent years, numerous studies appeared on hydrolysed fish skin collagen (YANG *et al.* 2008; JIA *et al.* 2010; YIN *et al.* 2010). Hydrolysed collagen of fish origin is currently used in various fields including functional food, beverages, and dietary supplements. Chances are that hydrolysed collagen coming from fish can improve bone metabolism (GUILLERMINET *et al.* 2010) and prevent skin aging (TANAKA *et al.* 2009).

YANG *et al.* (2009) optimised the process for the production of tilapia retorted skin collagen hydrolysates with antioxidant properties. WASSWA *et al.* (2008) prepared protein hydrolysates from the skin of Nile tilapia hydrolysed with protease, which exhibited satisfactory functional properties required in food processes.

The objective of this study was to compare thermal hydrolysis and enzymatic hydrolysis of tilapia skin collagen in order to introduce a better process for hydrolysed tilapia skin collagen production.

MATERIAL AND METHODS

Frozen skin of Nile tilapia (*Oreochromis niloticus*) was obtained from a processing plant located in Maoming, Guangdong, China.

Pretreatment. A previously described pretreatment procedure was applied with some modifications (GROSSMAN & BERGMAN 1992; YANG *et al.* 2009). Frozen tilapia skins were thawed at room temperature. The scales were removed and the skin samples were cut into pieces (about 50 mm × 10 mm) and washed under running tap water. The skin samples were then immersed in 0.25 mol/l NaOH and 1% (v/v) hydrogen peroxide (1:7, w/v) and held at room temperature for 40 minutes. The skin samples were drained through cotton cloth, which was squeezed by hand. The alkali pretreated skin samples were then washed under running tap water for 40 min and drained, and subsequently soaked in 0.2% sulphuric acid (1:7, w/v) for 40 minutes. The liquid was drained and the samples were flushed with tap water. Finally, the skin samples were soaked in 0.3 mol/l acetic acid (1:7, w/v) for 40 min and flushed with tap water to remove the remaining acid. The skin samples were then drained and weighed.

Thermal hydrolysis. A previously described procedure for thermal hydrolysis was applied with some modifications (YANG *et al.* 2009). Distilled water (pH 6.5) or buffer (pH 4 or pH 9) was added (water skin ratio 1:2, w/w) and the skin samples were thermally hydrolysed by retorting in an autoclave at 121°C for 3 hours.

Enzymatic hydrolysis of tilapia skins. Enzymatic hydrolysis of tilapia skins was performed at 50°C using three different proteases: 894 alkaline protease prepared in our laboratory, Alcalase 2.4L FG (Novozyme, Bagsvaerd, Denmark), and papain (Sigma, St. Louis, USA). The optimum hydrolysis conditions used for these enzymes were as follows: 894 (pH 10), Alcalase (pH 8), and papain (pH 6). The skin samples were added to different buffers (10 mmol/l NaCO₃-NaHCO₃ pH 10, 10 mmol/l NaH₂PO₄-Na₂HPO₄ pH 8 or 6). The ratio of the skins to buffers was then adjusted to 2:1 (w/w). The hydrolysis was started with stirring on the addition of enzyme to give the desired enzyme/substrate ratio (300 U protease/1 g thawed tilapia skin). One unit (U) of protease activity was defined as the amount of enzyme required to produce 1 µg of tyrosine from casein per minute under the optimal condition for the respective enzyme. At the end of the hydrolysis, the enzyme was inactivated by heating at 100°C for 10 minutes.

Filtration and lyophilisation. The extract was filtered through a metal sieve (100 mesh) to remove skin residues. Then fine residues were removed by centrifugation at 10 000× *g* for 30 min at 4°C. The resulting solution was adjusted to pH 7 with saturated NaOH or HCl. Finally, the neutralised solution was filtered through Whatman No. 4 filter paper. The filtered solution was then freeze-dried in a lyophiliser. The freeze-dried product was stored at –20°C until use.

All experiments were carried out in triplicate. The yield (%) was calculated as (freeze-dried hydrolysates (g)/wet fish skin (g)) × 100.

Determination of average molecular weight. The average molecular weight was determined by gel permeation chromatography (GPC) using a TSK gel G2000 SW column (7.5 mm × 30 cm; Tosoh, Tokyo, Japan) and Agilent GPC data analysis software (Santa Clara, USA). The mobile phase was water/acetonitrile/trifluoroacetic acid (55:45:0.1, v/v/v). The determination was carried out at room temperature at a flow rate of 0.5 ml/min and with the wavelength of the UV detector set at 220 nm. The calibration curve was obtained using the following standards: glycine (75 Da), Gly-Gly-Gly (189 Da), L-glutathione (308 Da), oxytocin acetate (1007 Da), bacitracin zinc (1486 Da), glucagon (3485 Da), bovine insulin (5733 Da), and cytochrome C (12500 Da).

1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging activity. The scavenging effects of tilapia hydrolysates on the DPPH free radical were measured basically as described by YANG *et al.* (2009). Briefly, a 1.5 ml aliquot of the hydrolysate solution (10 mg/ml) was added to 1.5 ml of 0.1mM DPPH in ethanol. The mixture was vortexed and left for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm (Shimadzu UV2550 Spectrophotometer; Kyoto, Japan). 8 µg/ml ascorbic acid was used as positive control. The scavenging effect was expressed as:

$$\text{DPPH radical scavenging activity (\%)} = (\text{blank absorbance} - \text{sample absorbance}) / \text{blank absorbance} \times 100$$

where: DPPH blank is the value of 1.5 ml of deionised water mixed with 1.5 ml of ethanol containing 0.1mM DPPH. All experiments were carried out in triplicate.

Proximate composition. The moisture of freeze dried samples was determined in triplicates using the moisture loss-on-drying method (2 g sample dried at 105°C for 2 h) (ILELEJI *et al.* 2010). The total crude protein (N × 6.25) content of the samples was determined using the Kjeldahl method (VILASOA-MARTÍNEZ *et al.* 2007).

Table 1. The property of the tilapia skin hydrolysates obtained at various pH in thermal hydrolysis

Thermal hydrolysis	Typical average molecular weight (Da)	Yield (%)	Protein content (%)	DPPH scavenging (%)
pH 4	3000 ± 500	13	94 ± 4	74 ± 6
pH 9	7000 ± 1000	14	89 ± 3	71 ± 3
pH 6.5	6000 ± 500	11	90 ± 5	54 ± 5

RESULTS AND DISCUSSION

Typical average molecular weight is an important property of collagen hydrolysates and, in general, the average molecular weight of the fish hydrolysed collagen is below 5 kDa. DPPH radical proved to be quite useful in the determination of the antioxidant properties of natural compounds. Several specifications were studied such as average molecular weight, DPPH radical scavenging activity, yield, and protein content in order to compare thermal hydrolysis and enzymatic proteolysis processes for the hydrolysed tilapia skin collagen production.

Thermal hydrolysis

As is shown in Table 1, the hydrolysates obtained in the pH 4 condition had a lower average molecular weight and a higher DPPH scavenging effect than those obtained at pH 9 or 6.5, thus the acid process used to prepare fish skin hydrolysates appeared as preferable. YANG *et al.* (2009) prepared hydrolysed collagen by retorting in neutral condition which provided a lower average molecular weight than those obtained under pH 6.5 condition in this study, because we adopted a shorter alkali pretreatment time and sulphuric and acetic acids (not phosphoric acid) in the pretreatment, considering the cost. However, the hydrolysates obtained in the acid condition had properties similar to those in Yang's study. The 3 h thermal hydrolysis time was chosen, for shorter time (2.5 h) led to incompletely hydrolysed products with a high average molecular weight and low DPPH radical scavenging ability (data not shown).

Enzymatic proteolysis

Single protease could not completely hydrolyse tilapia skin collagen in 30 min (data not shown). So a process including two-protease hydrolysis in turn was used to obtain the hydrolysates (LIN & LI 2006; HOU *et al.* 2009). Three kinds of two-protease hydrolysis combination are shown in Table 2. After the first enzymatic hydrolysis, the first protease was inactivated by heat and the second protease was directly applied. When the enzymatic proteolysis time was double 30 min, the average molecular weight of the product ranged from 400 Da to 1000 Da and its scavenging effect on DPPH radical was from 53% to 76%. (Table 2).

As shown in Table 2, when the enzymatic proteolysis time was reduced to double 15 min, the average molecular weights of the products were from 800 Da to 2000 Da, which were higher than those of the products obtained by double 30-min hydrolysis. The yields or protein contents of the hydrolysates produced by different processes, however, did not significantly differ. And the hydrolysates produced by double 15-min hydrolysis had a greater DPPH radical scavenging effect than those produced by double 30-min hydrolysis. Tables 1 and 2 demonstrate that the molecular mass distribution was not correlated with the antioxidant activity of tilapia skin collagen hydrolysate, which is comparable with the results reported by YANG *et al.* (2009)

WASSWA *et al.* (2008) hydrolysed tilapia skin collagen with Alcalase (1.7 g enzyme/100 g ground dried skin) at 60°C for 85 min and obtained hydrolysates, of which 77% of the molecular mass distribution was below 1000 Da. The process in

Table 2. The property of the tilapia skin hydrolysates obtained under various protease hydrolysis conditions

Protease	Hydrolysis time (min)	Typical average molecular weight (Da)	Yield (%)	Protein content (%)	DPPH scavenging (%)
894-papain	30–30	900 ± 100	15	88 ± 4	55 ± 2
Alcalase-papain	30–30	500 ± 100	15	89 ± 5	72 ± 4
894-alcalase	30–30	600 ± 100	15	87 ± 3	62 ± 2
894-papain	15–15	1200 ± 200	14	95 ± 3	70 ± 4
Alcalase-papain	15–15	800 ± 100	13	85 ± 5	71 ± 3
894-alcalase	15–15	1700 ± 300	13	91 ± 2	70 ± 2

this study is more concise and economical (60 mg enzyme/100 g wet skin, 50°C, less than 70 min) with a similar average molecular weight of the product.

It was previously reported that the yield of the tilapia skin collagen was 15% (GROSSMAN & BERGMAN 1992), which was consistent with the yields of the hydrolysates obtained by the thermal hydrolysis and enzymatic proteolysis in this study. The wet tilapia skin in this study contained 65% water and 26% protein, so the yield of the hydrolysates approached 50% on the basis of dry weight, which is in agreement with the previous findings (NAGAI & SUZUKI 2000; ZENG *et al.* 2009).

The thermal hydrolysis process under acid condition could produce the peptides with average molecular weight above 3000 Da, while the processes using two-protease hydrolysis combination could control the molecular mass distribution of the hydrolysates by changing the proteases. Compared with the thermal hydrolysis process, the enzymatic proteolysis process needed a shorter time and milder condition, resulting in hydrolysates with similar specifications such as the yield, protein content, and DPPH radical scavenging ability. This demonstrates that the enzymatic proteolysis is preferable to thermal hydrolysis for the production process of the tilapia skin collagen hydrolysates.

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