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Research Article

Proteolytic action in *Valamugil seheli* and *Ilisha melastoma* for fish sauce production

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Abstract

Protease action on fish protein hydrolysis during the production of Malaysian fish sauce, *Budu*, was studied using *Valamugil seheli* and *Ilisha melastoma* as the fermentation substrate. Results indicated that protease activity and degree of hydrolysis of *Ilisha melastoma* were significantly higher ($p< 0.05$) than in *Valamugil seheli*. The liquid percentages (yield) of fish sauce produced by *Valamugil seheli* were significantly higher ($p<0.05$) than that by *Ilisha melastoma* during the two months fermentation. The pH values of the liquid from *Ilisha melastoma* was 5.83 and decreased with time while the pH values of liquid from *Valamugil seheli* was initially 5.68, but increased with time.

Keywords: Fish sauce; fermentation; protease; Malaysia; *Budu*

Introduction

Fermented fish products have been consumed since ancient times. Fish sauce is the proteineous product obtained through natural hydrolysis by endogenous enzymes and microorganism [1]. Fish sauce constitutes an important part of the diet of more than 250 million people in Southeast Asia [2]. It is consumed by all socioeconomic groups, but is more important to those in the lower income group, providing a substantial part of the protein requirements of these people [3, 4]. During fermentation, proteolysis of fish proteins results in an increase of soluble protein [3].

"Budu", a dark brown-to-black condiment, is a fish sauce produced mainly on the east coast of West Malaysia, namely the states of Kelantan and Terengganu [5]. Fish sauce is made by mixing

fish of the *Stolephorus* species or anchovy, with salt and is left to ferment for 6-12 months [4]. The resultant liquid is then added with tamarind, palm sugar, monosodium glutamate and flavouring compounds prior to bottling [5].

The factors that greatly influence the compositional and nutritional quality of fish sauce are the ratio of salt to fish, fermentation temperature, fish species and minor ingredients [1]. One of these factors, the species of fish used in the manufacturing of fish sauce, varies from country to country. *Nampla* (Thai fish sauce), which is the most dominant fish sauce in the world market, is mainly produced from anchovies (*Stolephorus spp*.), mackerel (*Ristrelliger spp*.) and herring (*Clupea spp*.) [6].

Unfortunately, there have been fluctuations in the abundance of anchovies and sardines, associated with large-scale changes in ocean temperatures [7]. Fishing and fish abundance are also affected by monsoons, synonymous with heavy seas and high winds, leading to a decline in catch rate [8]. Lopetcharat *et al* [1] stated that although anchovies and sardines are most frequently used for fish sauce production, other raw materials can also be used for production of good quality fish sauce. Therefore, the objective of this work was to investigate the feasibility of producing fish sauce from an alternative raw material. In our study, *Ilisha melastoma* (anchovy) and an underutilized fish, *Valamugil seheli* (commonly known as blue spot mullet) were used as the fish source.

Materials and Methods

Sample preparation

Fresh anchovies (*Ilisha melastoma*) and blue spot mullet *(Valamugil seheli*) were purchased from a local market in Penang, Malaysia and transported back to the laboratory in iceboxes. Both fish were separately washed, drained and then 240 g fish was mixed with 160 g salt in 500 ml ceramic containers. Twelve containers each were prepared for both types of fish. Plastic mosquito netting was cut to the size and shape of the container opening and placed at the top, weighed down by clean pebbles, to ensure that the fish were submerged in liquid at all times. The containers were then placed in a 40°C incubator and triplicate containers were withdrawn at the interval sampling times of 0 day, 14 days, 28 days and 60 days. Samples were analysed for liquid yield, pH, degree of hydrolysis, protease activity and soluble protein concentration. All analyses were carried out in duplicates.

Liquid yield

Samples were centrifuged at 710g for 5 minutes (centrifuge Kubota 5100; Tokyo) to obtain the liquid portion. The volume of liquid obtained was measured and the liquid was used for further analysis.

pH

The pH of the liquid portion was determined using a pH Meter (Delta 320, Mettler Toledo; Switzerland).

Protease activity

Protease activity of the fish sauce was determined according to the method of Wang, *et al* [9]. Sample was diluted by adding 9 ml distilled water to 1 ml of sample. Then, 0.1 ml diluted samples were transferred to a test tube, added with 1 ml 1% bovine serum albumin in Tris buffer (pH 7.0) and then incubated at 38°C for 20 minutes. Subsequently, 3 ml Trichloroacetic acid solution, 5%

(w/v) was added to stop the reaction. The mixture was centrifuged at 7000 rpm for 10 minutes and the supernatant was used to determine L-tyrosine by spectrophotometric method at 280 nm. Standard curve of absorbance values versus L-tyrosine concentration (0-180 µg/ml) was plotted. One unit of enzyme activity represents the amount of enzyme required to liberate one µmole of L-tyrosine per min under standard assay conditions.

Degree of hydrolysis

Degree of hydrolysis was calculated from the ratio of α-amino nitrogen and total nitrogen. The amino content was determined by a modified formol titration method [10]. Sample (10 ml) was mixed with 10 ml distilled water and titrated to pH 7.0 with 0.1 M NaOH. Then 10 ml of formaldehyde solution (38%) was added. The titration was continued to pH 9.5 with 0.2 M NaOH. Total nitrogen for fish was determined by using Kjeldahl method [11].

Soluble protein

Soluble protein content was measured using Folin Ciocalteau/Lowry method [12]. An amount of 1 ml of sample was taken and added with 5 ml of reagent C (50 ml 2% Na_2CO_3 in 0.1M NaOH and 1 ml 0.5% $CuSO_4.5H_2O$ in 1% sodium or potassium tartrate). Folin reagent was added and the solution was left at room temperature for 30 minutes. Soluble protein concentration was determined from a standard curve of bovine serum albumin (Sigma; Steinheim Germany) with range from 0-1500 µg/ml. Absorbance at 750 nm versus concentration of protein (mg/ml) was plotted.

Statistical analysis

Data of analysis was carried out with SPSS Inc. Software (version 15.0) (SPSS Inc., Chicago, U.S.A). One way analysis of variance was used to determine the significant difference between means (n=3), with a significance level of $\alpha=0.05$. Tukey' test was used to perform multiple comparisons between means. All data presented are mean values of triplicates, obtained from 3 batches of samples, unless stated otherwise.

Results and Discussion

Liquid yield of fish sauce

The liquid percentages (w/w) produced during the 8 week fermentation of the *V. seheli* and *I. melastoma* are shown in Table 1. The initial (0 day) liquid percentages from *I. melastoma* fermentation were significantly ($p<0.05$) higher than that of *V. seheli*. This was probably due to the difference in fish size; *I. melastoma* is smaller in size, thus having bigger surface area to volume ratio, leading to an increased rate of osmosis of fluid from the fish. Studies have shown that expanding the surface area by grinding through mechanical disruption of cells has resulted in a higher liquid yield in less time [13].

However, as the fermentation progressed, more liquid extract ($p<0.05$) was obtained from the fermented *V. seheli* than from *I. melastoma,* possibly because *V. seheli* is much larger than *I. melastoma*. The maximum size (length) of *V. seheli* was about 60 cm whilst that for *I. melastoma* was only 20 cm [14], therefore more liquid can be collected from *V. seheli* fish. The rate of salt penetration into fish tissue is known to depend on various factors including species of the fish, skin of fish, temperature, muscle structure, fat content and others [15].

Table 1. The liquid percentages, pH, protease activity and degree of hydrolysis data of fermented *Ilisha melastoma* and *Valamugil seheli*.

Ferm. period (Day)	*Ilisha melastoma*					*Valamugil seheli*				
	Liquid (%, w/w)*	pH *	Protease Activity* (IU/ml)	Degree of hydrolysis* (%)	Soluble Protein* (mg)	Liquid (%, w/w)*	pH *	Protease Activity* (IU/ml)	Degree of hydrolysis* (%)	Soluble Protein* (mg)
0	10.30 ± 0.46^{a}	5.83 ± 0.01^{c}	0.38 ± 0.04^{a}	12.80 ± 0.65^{a}	31.71 ± 0.30^{a}	3.94 ± 0.55^{A}	5.68 ± 0.01^{A}	0.015 ± 0.00^{A}	10.77 ± 0.87^{A}	1.59 ± 0.03^{A}
14	14.05 ± 0.60^{b}	5.82 ± 0.01^{c}	1.59 ± 0.10^{b}	53.03 ± 0.65^{b}	83.84 ± 1.02^{b}	22.92 ± 2.28^{C}	5.49 ± 0.01^{A}	0.11 ± 0.00^{B}	11.77 ± 0.38^{A}	2.70 ± 0.00^{B}
28	13.88 ± 0.34^{b}	5.67 ± 0.01^{b}	1.65 ± 0.35^{b}	71.31 ± 0.32^{c}	122.65 ± 0.85^{c}	18.65 ± 3.22^{BC}	5.89 ± 0.04^{AB}	0.13 ± 0.00^{B}	16.91 ± 3.57^{AB}	3.67 ± 0.32^{C}
60	9.76 ± 1.04^{a}	5.49 ± 0.01^{a}	1.61 ± 0.19^{b}	73.83 ± 0.97^{c}	1.49.93 ± 6.48^{d}	11.86 ± 0.94^{AB}	6.12 ± 0.12^{B}	0.15 ± 0.01^{C}	20.92 ± 0.92^{B}	1.71 ± 0.07^{A}

* Results are expressed as means ± S.D; values are means of duplicate from three independent samples.
Means in the same column followed by different lowercase and uppercase letters are significantly different ($p<0.05$).

Apart from the fluid drawn out of the fish by osmosis, the liquid yield from fish fermentation can also be due to the enzymatic breakdown of fish by proteolysis [10, 16]. During fermentation, the fish tissue is disintegrated gradually and the final product is a liquid [4]. High concentrations of salt and the subsequent onset of fish hydrolysis during the first stages of fermentation result in the formation of liquid [4]. The yield of liquid is about 75 ml/100 g of fish [3].

pH

Statistical analysis indicated that there was significant difference between pH of the fish sauce produced from the fish, *I. melastoma* and *V. seheli* (Table 1). There was an increase in pH value for fish sauce from *V. seheli* (pH 5.68 to 6.12) during fermentation, whereas the pH values for that of *I. melastoma* decreased (pH 5.83 to 5.48) during the two months fermentation period. The decrease in pH values could be due to the release of free amino acids from proteins and large polypeptides [1]. Leroi and Joffraud [17] in their study also explained that pH decrease in flesh of fish by addition of salt is due to the increase of ionic strength of the solution inside the cells.

In contrast, the pH of fish sauce from *V. seheli* increased during the experiment. This may be due to the presence of some volatile nitrogen compounds like ammonia and trimethylamine (TMA) during fermentation [10]. TMA and ammonia contain basic properties and will react with acidic compounds to form more stable compounds.

Mclver [3] stated that the pH of *Budu* is approximately 5.6. Saisithi, *et al* [18] in an earlier study on fish sauce also revealed that the pH never exceeded 6.8. However, different species of fish affects the biological properties differently and therefore produces different kinds of peptides and amino acids [1]. Thus, the pH profile of the two species of fish during fermentation varies depending on the biological properties of the fish.

Protease activity

During fermentation of fish sauce, fish tissue is degraded by endogenous and exogenous protease enzymes and these enzymes hydrolyze protein into smaller peptide units or free amino acids [19, 20]. The most important enzymes in fish sauce fermentation are proteases, catalyzing amino acid degradation. Results indicated that the protease activity of *I. melastoma* was significantly ($p<0.05$) higher than that of *V. seheli* (Table 1). Poralla [10] stated that during fermentation, the proteins that

have been well autolysed were released and the cellular tissues were broken down, the protease activity is said to be affected by the amount of protein released. Ordinarily, the presence of proteolytic enzymes will cause texture softening and lower the value of fresh whole fish [13].

The change of protease activity was related mainly to the salt concentration and other factors during fermentation [21]. The activation of protease in fish are highly dependant on the salt concentration, as high salt concentration retards enzymatic activity, increases the osmotic pressure thus causing the fish tissue to harden, and inhibits attack by proteolytic enzymes [21].

Degree of hydrolysis

Fish sauce is the result of complete hydrolysis of muscle protein of fish in a saturated salt concentration [1]. During fermentation, proteins are hydrolyzed, mainly as a result of autolytic action by the digestive proteases in fish [22, 23]. Thus, generally, the degree of hydrolysis reflected the protease activity in the samples [24]. The conventional method to produce fish sauce normally takes 9 to 12 months in order to complete hydrolysis [1].

Degree of hydrolysis of *I. melastoma* was significantly higher ($p<0.05$) than *V. seheli* throughout the eight week fermentation time (Table 1). This is in tandem with higher protease activity shown by *I. melastoma*. As fermentation progressed, protein hydrolysis is gradually increased by the action of proteinases [16].

Soluble protein

Brining caused solubilisation of some of the muscle proteins, which were released into the brine [25, 26]. For *budu*, the fish sauce from Malaysia, approximately 56% of the total fish protein is converted to soluble protein [10]. Insoluble protein in fish is digested by protease enzyme to form soluble nitrogen compounds, therefore proteolysis of fish proteins results in increasing soluble proteins. During fermentation, the solid material is progressively digested, the protein being solubilised gradually by enzymatic action, leading to the increase in peptides and amino acids in the liquid component [27]. Soluble protein present in the fish sauce is the product of hydrolysis of fish tissue by protease enzymes. The soluble protein of *I. melastoma* was significantly higher ($p<0.05$) than that of *V. seheli* (Table 1). This is in tandem with the higher protease activity produced in the *I. melastoma* fish during fermentation, thus producing more soluble protein in fish sauce samples.

Conclusion

Different types of fish influences the physiochemical properties of fish sauce. Based on the biochemical properties, fish sauce made from *I. melastoma* had a higher soluble protein content and therefore would be the preferable fish for production of fish sauce. On the other hand, *V. seheli* can serve as alternative raw material for the production of fish sauce as it produced a higher liquid yield.

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