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Physicochemical properties of powdered protein hydrolysate from Yellowstripe scad *(Selaroides leptolepis)* fish

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Abstract

Yellowstripe scad fish (YSF) or *Selaroides leptolepis* belongs to the small pelagic group that is abundantly found in South China Sea and is categorised as low value fishes. This study is designed to explore the physicochemical properties of YSF protein hydrolysate extracted using sodium phosphate buffer followed by 0.5%-2.0% of Alcalase at a series of hydrolysis time (1 hr and 2 hr). The properties of freeze and spray dried protein hydrolysate were evaluated for yield, degree of hydrolysis, protein content, microstructure and water holding capacity. Results showed that prolonged hydrolysis time exhibited increasing yield (0.6%-1.6% for spray drying and 12-16% for freeze drying) and high degree of hydrolysis (80-95%). Protein content recovered from hydrolysis process is within 20-29%. Microstructure of freeze dried YSF protein hydrolysate had 'collapsed-building' structure (irregular shapes with edges) while spray dried had small and spherical structure. Freeze dried protein hydrolysates were significantly ($p<0.05$) higher than spray dried hydrolysates in water holding capacity.

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Introduction

Yellowstripe scad fish or *Selaroides leptolepis* belongs to the small pelagic group which is categorised as low value fishes, is one of the plentiful marine source in South China Sea (Vietnam sea area) (Bui and Toshiaki, 2014). This species is distinguished by its prominent lateral yellow band and smaller eye, differing from scads of Selar. In order to increase the value and utilization of low value proteinacious fish, processes such as protein hydrolysis via enzymatic hydrolysis is used to produce a more marketable and functional protein hydrolysate (Aspmo *et al.*, 2005).

Unutilized fish, under-utilized fish or fish waste can be used to produce fish protein concentrate or hydrolysate since they contain so much amino acids and functional protein (Ramakrishnan *et al.*, 2013). Fish protein hydrolysate produced by controlled enzymatic hydrolysis, is considered to be the best fish protein hydrolysate due to its nutritional properties of well-balanced amino acids composition and these hydrolysate is highly digestible by consumers (Kristinsson and Rasco, 2000). Protein hydrolysate with different degree of hydrolysis and different functional properties could be produced by proper control during hydrolysis process. Physicochemical properties of protein hydrolysate are greatly affected by the degree of hydrolysis, type of substrate and protease enzyme used (Amiza *et al.*, 2013).

There are many different types of proteolytic enzymes that can be used to produce protein hydrolysate (Liceaga -Gesualdo and Li-Chan, 1999). The most common source of proteolytic enzymes is found to be either plant or microorganisms, which are suitable for the production of fish protein hydrolysate (Bhaskar *et al.*, 2008). Alcalase is a commercially obtainable enzyme which is widely used in protein hydrolysis because of its thermostability (50°C) and high optimal pH (pH 8.5) where it can minimise the growth of microorganisms along hydrolysis process (Salwanee, 2013). Alcalase is originated from a strain of *Bacillus licheniformis*, subtilisin A (Subtilisin carlsberg) which act as the main enzyme component. This enzyme is an endopeptidase, also available in food grade form that complies with FAO/WHO (Novo Nordisk, 1995).

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Yellowstripe scad contains high amount of protein (19.98%) (Nurnadia *et al*., 2011), which is prone to degradation, oxidation and other undesirable processes, however limited studies regarding the proper handling techniques and parameters were reported. Hence, there should be a handling technique and parameters reported in order to obtain protein from this fish. This study could widen the usage of Alcalase enzyme to produce fish protein hydrolysate rather than only domestically used or processed into feeds and serve as a reference for further study. This study is aimed to determine the physicochemical properties of Yellowstripe scad fish or Selaroides leptolepis powdered fish protein hydrolysate.

Materials and Method

The raw material used in this study was fresh Yellowstripe scad fish obtained from fish market in Pulau Kambing, Kuala Terengganu, Terengganu. The fish had approximately 5-10 cm long and weighed in the range of 35-45 g. They had a variety of maturity since these fishes were caught randomly from South China Sea within May to June. The ground edible portion was used to produce protein hydrolysate.

Protein extraction

Protein was extracted from the edible portion of fish (without the head, viscera, tails and fins). Fifty grams of fish meat was heated in water bath at 90°C for 10 mins to deactivate enzyme originally found in fish meat. The sample was then mixed with 100ml of sodium phosphate buffer at pH 8. Four different concentrations of Alcalase enzymes (Merck, USA) were used, namely 0.5%, 1.0%, 1.5% and 2.0%. The hydrolysis was conducted for 1h and 2 h at 55°C. The resulted hydrolysate was centrifuged at 10000 rpm for 20 mins and filtered. The liquid hydrolysate was subjected to drying (freeze drying or spray drying) prior to further analysis.

Drying process

Freeze drying was conducted using a Labconco Freeze Dryer –Stoppering tray (USA) operated at -54°C while the vacuum was set at 0.250 mbar. Samples were frozen at -80°C prior to the freeze drying procedure. However, spray drying had opposite operational concept. Spray drying was conducted using Ultrasonic Spray Dryer (YKN 01, Kulim Malaysia) at which liquid sample was fed (1L/h) into the atomizer, sprayed into chamber that contained hot air. The temperature of hot air was set at 80°C.

Yield of protein hydrolysate

Yield of powdered protein hydrolysate was obtained by weighing the powder collected after spray drying or freeze drying. The percentage of yield after drying was calculated as shown in Equation 1.

$$\text{Yield of powder hydrolysate} = \frac{\text{powder collected after drying (b)}}{\text{liquid protein hydrolysate (a)}} \times 100\% \quad \text{[Equation 1]}$$

Degree of hydrolysis (DH)

Degree of hydrolysis was calculated according to Hoyle and Merritt (1994). The first sample was added with 10% Trichloroacetic acid (TCA) while the second sample was subjected to Kjeldal method (AOAC, 2000) directly.

The powdered protein hydrolysate was weighed approximately 0.5 g. Ten millilitres of buffer solution and 5 ml of 10% Trichloroacetic acid (TCA) were added to the sample. The solution was held in room temperature for 30 mins. The samples were then centrifuged at 4000 rpm for 15 mins. The supernatant was filtered directly into the digestive tube using a cellulose filter paper (12-15 µm, Filtres Fioroni, Ingré) and the following processing steps were similar to protein determination. The values from the titration are calculated using Equation 2 and Equation 3.

$$\text{Percentage of nitrogen (\%)} = \frac{(T-B)\times N\times 14.007\times 100}{\text{weight of the sample (mg)}} \quad \text{[Equation 2]}$$

$$\text{Percentage of DH (\%)} = \frac{\text{Percentage of nitrogen with TCA}}{\text{Total percentage of nitrogen}} \times 100 \quad \text{[Equation 3]}$$

Protein content protein hydrolysate

Protein content was measured using Kjeldahl method to determine the ammonium compound present in the solution (AOAC, 2000). Briefly, one gram of protein hydrolysate sample was weighed and placed into the digestion tube of the instrument, while powdered protein hydrolysate used was only approximately 0.5 g. Two tablets of Kjeltabs catalyst, Cu 3.5 and 12 ml of the concentrated sulphuric acid was added consecutively. The tubes were then connected to the digester (2006 Digester, FOSS, Sweden). This process of digestion was continued until green or light blue solution was formed. Then distillation was continued using distillation unit (2100 Kjeltec Distillation Unit, FOSS, Sweden, 2002). The values from the titration was calculated using Equation 4 and Equation 5 given below.

$$\text{Percentage of nitrogen (\%)} = \frac{(T-B)\times N\times 14.007\times 100}{\text{weight of the sample (mg)}} \quad \text{[Equation 4]}$$

$$\text{Percentage of protein (\%)} = \text{percentage of nitrogen} \times F \quad \text{[Equation 5]}$$

Where,
T = Titration volume for the sample (ml)
B = Titration volume for the control (ml)
N = Concentration of hydrochloric acid (HCl)
F = Protein factor (6.25)

Microstructure of protein hydrolysate

Microstructure determination was conducted using Scanning Electron Microscope (Jeol-6360, USA). The powdered sample was applied on the surface of sticker on a specimen holder. Then, the sample was coated with 99% pure gold using JFC 1600 Auto fine coater, before being analysed using SEM. The specimen was viewed using 90 times magnification for freeze dried samples and 250 times magnification for spray dried samples (modified method of Moreira *et al*., 1997).

Water holding capacity

Water holding capacity analysis was conducted according to a modified method of Medcalf and Gilles (1965). A suspension of 5 g protein hydrolysate (dry weight) was added to 75 ml of distilled water. The mixture was agitated at 25°C for 1hr, and centrifuged at 3000 rpm for 10 mins. Free water was removed and drained for 10 mins. The pallet left in the centrifuge tube was weighed. The value of water holding capacity was calculated using Equation 6.

$$\text{WHC of FPH (\%)} = \frac{\text{Weight pallet}}{\text{Weight powder}} \text{ X } 100 \quad \text{[Equation 6]}$$

Statistical analysis

The statistical analysis for protein hydrolysate analysis was completed using SPSS software at the confidence level at $p \leq 0.05$. The samples had three different parameters, namely time of hydrolysis, enzyme concentrations and drying methods, thus the data obtained were analyzed using two-way ANOVA. Comparisons of means were carried out using Tukey HSD.

Results and Discussion

Yield of protein hydrolysate

The efficiency of drying on protein hydrolysate was determined using yield of powdered protein hydrolysate. Table 1 depicts that freeze drying gave higher yield of powdered protein hydrolysate than that of ultrasonic spray drying. The yield was between 0.6% to 1.6% for ultrasonic spray drying and 12% to 16% for freeze drying. The highest yield was achieved by freeze drying protein hydrolysate with hydrolysis condition of 2 h and 2% of enzyme with 16.2%±0.001.

Table 1. Percentage of yield of powdered protein hydrolysate

Sample	Yield (%)
S1A	1.63±0.02
S1B	0.66±0.02
S1C	0.70±0.05
S1D	1.19±0.12
S2A	0.97±0.13
S2B	0.78±0.07
S2C	0.91±0.01
S2D	1.08±0.01
F1A	12.93±0.65
F1B	14.01±0.48
F1C	14.50±1.11
F1D	14.76±1.24
F2A	14.82±0.07
F2B	15.32±0.45
F2C	15.81±0.08
F2D	16.17±0.14

Note: S= ultrasonic spray dried, F= freeze dried [Drying method] 1= 1 hr, 2=2 hr [Hydrolysis time] A= 0.5%, B=1.0%, C=1.5%, D=2.0% [Enzyme concentration] All values given are means of triplicate results. Standard deviation (mean ± SD) is included for each average.

There was statistically significant three way interaction ($p<0.05$) between the drying methods, hydrolysis time and concentration of enzymes used, $F(3,32) = 4.99$, $p = 0.006$. Generally, the data showed that the yield of freeze dried hydrolysate in every hour increased as the concentration of enzyme used increased, but ultrasonic spray dried hydrolysate were not consistent. Table 1 also shows that the yield increased as enzyme concentration and hydrolysis time increased. This result is aligned with Ramakrishnan *et al.* (2013) who reported that increasing enzyme concentration from 0.5% to 2.0% increased protein yield for all fish parts because more enzyme molecule associate with fish, releasing more protein molecules into system (Kristinsson and Rasco, 2000). Kristinsson and Rasco (2000) reported that Alcalase enzyme was selected in hydrolysis because it had relatively high degree of hydrolysis in relative short time. Freeze dried samples higher yield probably due to the theory of freeze drying at which the samples were dried using the sublimation of frozen water into vapour, without losing any other components. However, in ultrasonic spray drying, when sample was sprayed into hot chamber, only big compounds that have mass such as protein was dried into powder and collected in the collection beaker (unpublished data from manufacturer). Liquid containing other particle was collected in waste container of the ultrasonic spray dryer.

Degree of hydrolysis

Degree of hydrolysis (DH) plays a vital role in determining important properties of a protein hydrolysate. Table 2 shows that DH was in the range of 39%-48%. A significant difference was observed in 1.5% and 2.0% of enzyme used (Figure 1). On the other hand, Norma *et al.* (2005) and Guerard *et*

Table 2. Percentage of degree of hydrolysis (%DH) of powdered protein hydrolysate

Sample	DH (%)
S1A	44.16±1.98
S1B	43.98±5.05
S1C	42.38±3.44
S1D	42.60±0.73
S2A	41.27±1.46
S2B	43.12±3.28
S2C	39.58±2.54
S2D	46.94±4.86
F1A	47.93±3.59
F1B	42.98±4.21
F1C	45.11±3.34
F1D	48.71±2.50
F2A	43.94±2.43
F2B	43.14±2.96
F2C	40.00±4.43
F2D	47.72±4.80

Note: S= ultrasonic spray dried, F= freeze dried [Drying method] 1= 1 hr, 2=2 hr [Hydrolysis time] A= 0.5%, B=1.0%, C=1.5%, D=2.0% [Enzyme concentration] All values given are means of triplicate results. Standard deviation (mean ± SD) is included for each average.

Table 3. Protein content of powdered protein hydrolysate

Sample	Protein (%)
S1A	40.77±0.69
S1B	40.06±1.14
S1C	51.76±1.65
S1D	50.15±0.69
S2A	41.35±2.90
S2B	39.94±3.34
S2C	47.13±1.86
S2D	42.46±0.68
F1A	52.86±1.37
F1B	60.23±5.39
F1C	55.07±1.73
F1D	50.90±1.41
F2A	55.20±3.51
F2B	55.30±4.52
F2C	55.43±3.24
F2D	57.59±2.08

Note: S= ultrasonic spray dried, F= freeze dried [Drying method] 1= 1 hr, 2=2 hr [Hydrolysis time] A= 0.5%, B=1.0%, C=1.5%, D=2.0% [Enzyme concentration] All values given are means of triplicate results. Standard deviation (mean ± SD) is included for each average

al. (2002) reported that DH increased as incubation time and enzyme-substrate ratio increased on threadfin bream and yellowfin tuna, respectively. The inconsistent degree of hydrolysis along the hydrolysis might be due to reduction of enzyme activities due to exhaustion the enzyme as substrate as time prolonged. Besides, prolonged hydrolysis time could denature protein molecules. Claver and Huiming (2005) also reported that the decrease in DH could be due to denaturation of protein molecules, subsequently reduces its biological activities. Degree of hydrolysis is also dependent on the availability of susceptible peptide bonds on which primary attack is based and the physical structure of the protein molecule (Kanu *et al.*, 2009).

Protein content

Protein content obtained was mostly referred to the nitrogen compound found in the sample (Sheriff *et al.*, 2013). The nitrogen content reflects the yield of protein that can be recovered from the hydrolysis process (Sheriff *et al.*, 2013). Freeze dried protein hydrolysate showed higher percentage of protein as compared to the result shown by ultrasonic spray dried hydrolysate (Table 3). Table 3 also revealed that there statistically significant three way interaction ($p<0.05$) between the drying methods, hydrolysis time and concentration of enzymes used, $F(3,32) = 6.616$, $p = 0.001$. Freeze drying had higher protein content probably due to low temperature drying which reduced protein denaturation (Ratti, 2008). Drying of protein using high temperature induces few stresses that can denature protein by modifying protein structures (Joshi *et al.,* 2011), which resulted in low yield.

Freeze dried protein hydrolysate showed higher percentage of protein compared to the result shown by ultrasonic spray dried hydrolysate (Table 3). Table 3 also portrays that the increase in enzyme concentration from 0.5 to 2.0% had resulted in the increment in protein content. Similar study was reported by Ramakrishnan *et al*. (2013), disclosing that more enzymes molecules were associated with fish particles, releasing more protein molecules during hydrolysis (Shahidi *et al*., 1995; Kristinsson and Rasco, 2000). However, the results were inconsistent, whereby in ultrasonic spray dried sample from 2 hr hydrolysis and freeze dried sample from 1hr hydrolysis. Gildberg (1992) reported that an increase in enzyme concentration increased the rate of reaction but fish tissue is very complex substrates that contains large amount of proteinase inhibitors which make it difficult to explain protein hydrolysis. Kristinsson and Rasco (2000) also reported that the presence of various types of peptide bonds present and their specificity for the attack of enzymes make hydrolysis process complicated.

Microstructure

Structure of dried protein hydrolysate plays a vital role in determining functional properties of protein hydrloysate. Scanning electron microscope (SEM) was used to show microstructure of dried samples, treated with different conditions. Generally, freeze dried protein hydrolysate had "collapsed-building" shape whereas, ultrasonic spray dried protein hydrolysate appeared to be more rounded and symmetrical in shape. Similar results were reported by Qiang *et al*. (2013).

Figure 2 illustrates the images of freeze dried samples with 90X magnification while ultrasonic spray dried samples with 250X magnification. Ultrasonic spray dried samples had smaller size

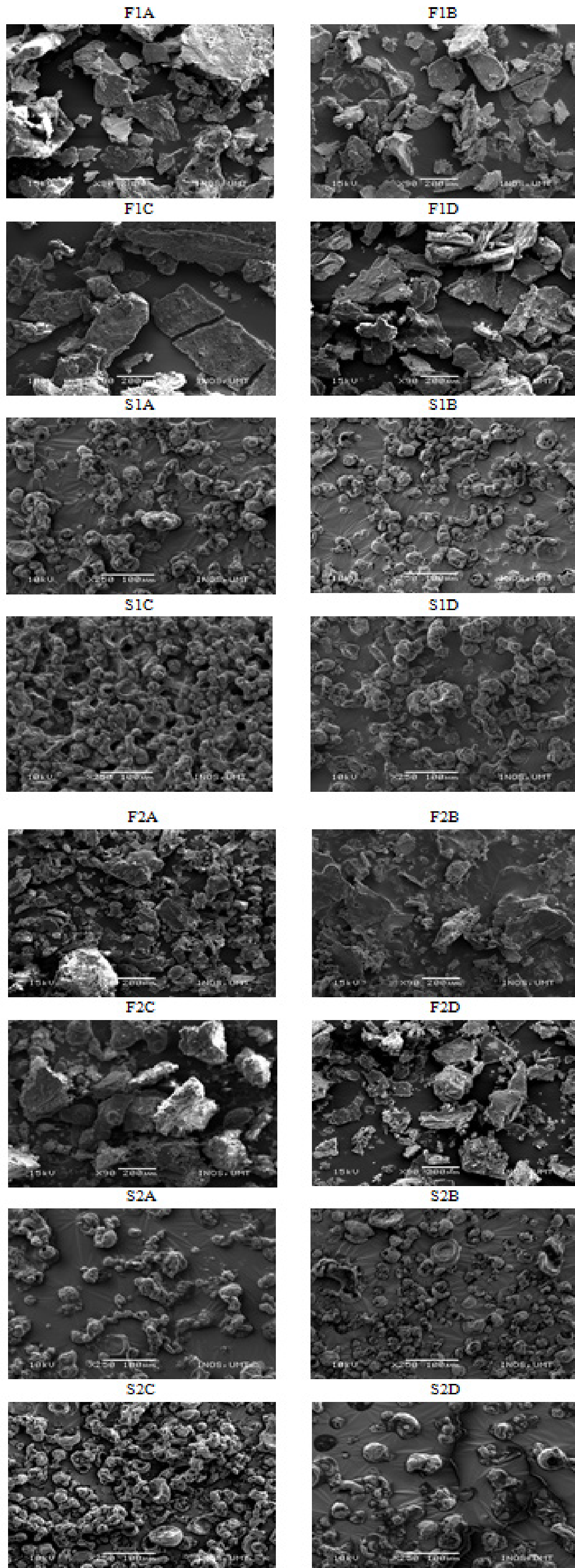

Figure 2. SEM images of powdered protein hydrolysate
Note: S= ultrasonic spray dried, F= freeze dried [Drying method]1= 1hr, 2=2hr [Hydrolysis time] A= 0.5%, B=1.0%, C=1.5%, D=2.0% [Enzyme concentration]

ranged between 30-50 µm while freeze dried samples had bigger size ranged between 180-250µm. Paraman *et al.* (2008) reported that differences in functional properties of protein hydrolysate might be attributed by the diversity of extraction and drying methods as protein concentrate recovered by ultra-filtration and ultrasonic spray drying showed better functional properties such as higher solubility and emulsifying properties as compared to freeze-dried samples. However, drying of protein induce few stresses that can denature protein by modifying protein structures (Joshi *et al*. 2011). Lower reading in protein content and yield, as discussed above, could be caused by exposing protein hydrolysate to high temperature in ultrasonic spray drying.

Table 4. Water holding capacity of powdered protein hydrolysate

Sample	WHC (%)
S1A	12.13±0.26
S1B	10.73±0.50
S1C	13.12±0.38
S1D	10.45±0.27
S2A	17.89±0.74
S2B	13.31±0.67
S2C	10.13±0.29
S2D	11.47±0.49
F1A	32.43±0.74
F1B	37.34±1.20
F1C	24.65±1.21
F1D	31.63±0.33
F2A	28.56±0.72
F2B	34.32±0.50
F2C	32.27±0.68
F2D	37.70±0.95

Note: S= ultrasonic spraydried, F= freeze dried [Drying method] 1= 1hr, 2=2hr [Hydrolysis time] A= 0.5%, B=1.0%, C=1.5%, D=2.0% [Enzyme concentration] All values given are means of triplicate results. Standard deviation (mean ± SD) is included for each average.

Water holding capacity

Water holding capacity is the absorption capacity possessed in protein compounds (Taheri *et al*., 2012). A significant three way interaction ($p<0.05$) between the drying methods, hydrolysis time and concentration of enzymes used, $F(3,32) = 138.97$, $p = 0.00$ (Table 4) was observed. Protein hydrolysate in freeze dried sample was found to have the highest water holding capacity compared to that of ultrasonic spray dried hydrolysate. This might be due to higher concentration of polar groups such as NH_2 and COOH which can absorb higher amount of water (Kristinsson and Rasco, 2000).

Freeze dried protein hydrolysate also indicated the presence of more hydrophilic polar side chain which can hold more water than ultrasonic spray dried protein hydrolysate (Taheri *et al*., 2012). Kristinsson and Rasco (2000) reported that fish protein hydrolysate had excellent water holding capacity, thus they can increase cooking yield, while Chiang *et al.* (1999) suggested that fish protein hydrolysate could be used as an additive to bind water and improve texture in intermediate-moisture food.

Conclusion

The best technique to produced Yellowstripe scad's powdered protein hydrolysate was using 2.0% of Alcalase enzyme at 2 hr of hydrolysis time and subjected to freeze drying. The highest yield (16%) and degree of hydrolysis (95%) was successfully obtained by this condition. High degree of hydrolysis gives better functional properties of protein hydrolysate. The optimum extraction condition also produces the best protein recovery of the extracted Yellowstripe scad's protein hydrolysate. Furthermore, freeze dried samples also portray significantly higher water holding capacity than spray dried protein hydrolysate. These freeze dried protein hydrolysate with better functional properties could be used as a part of ingredients in batter for fried food as the oil reducer agent.

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