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Characteristics of threadfin bream *(Nemipterus japonicas)* hydrolysate produced using bilimbi (*Averrhoa bilimbi* L.) protease and alcalase

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Abstract

Threadfin bream *(Nemipterus japonicas)* muscle was hydrolysed using protease extracted from bilimbi (*Averrhoa bilimbi* L.) fruit. This study was performed in order to compare the efficiency of bilimbi protease in producing threadfin bream protein hydrolysate with the commercial protease; alcalase 2.4 L. Initially, protease was extracted and then purified using 40% ammonium sulfate precipitation method. The proteolytic activity of the crude extract and purified protease was determined. Precipitation using 40% ammonium sulfate resulted in bilimbi protease specific activity of 2.36 U/mg and 23.13% recovery. Threadfin bream hydrolysate was prepared based on the pH-stat method by hydrolysis for 2 hrs. Hydrolysis using bilimbi protease produced 34.76% degree of hydrolysis (DH) and 3.75% yield while hydrolysis using alcalase resulted in 86.6% DH with 22.78% yield. Alcalase hydrolysate showed higher solubility than bilimbi protease hydrolysate at pH 7 with 70.87 and 32.16% solubility, respectively. Results also showed that protein content of threadfin bream hydrolysate produced using alcalase was higher (86.86%) than those produced using bilimbi protease (22.12%). However, both hydrolysates showed low moisture content between 3.93 to 7.00%. The molecular weight distribution analysis using SDS–PAGE indicated the distribution of smaller peptides especially in alcalase hydrolysate. Overall, the results showed that alcalase is more efficient enzyme choice than bilimbi protease for preparing threadfin bream hydrolysates. However, both hydrolysates could play an important role thus contribute to the food industry.

Introduction

Alcalase is not only a common enzyme but ought to be the best enzyme used to hydrolyse protein for producing fish protein hydrolysate. This can be seen through numerous studies indicating alcalase as a better protease for most protein sources, for example, hydrolysates from fish by-product prepared using alcalase has been shown to have greater amount of protein (82.66%) than those prepared using flavourzyme (73.51%) (Muzaifa *et al*., 2012). In terms of solubility, emulsifying and foaming properties, hydrolysate prepared using alcalase was also better than those prepared using flavourzyme (Sathivel *et al.*, 2005). However, it is also suggested that at the same DH, protein hydrolysate functionalities depend on the type of enzyme used (Klompong *et al*., 2007). Besides, alcalase shows its effectiveness in the production of hydrolysate with appropriate amino acids composition and protein efficiency ratio (PER) values (Ovissipour *et al*., 2010). Research has also been done on the optimization of fish protein hydrolysate from catla viscera, pacific whiting solid waste, threadfin bream and grass carp skin by using alcalase (Nilsang *et al.*, 2005; Normah *et al.*, 2005a; Bhaskar and Mahendrakar, 2008; Wasswa *et al.*, 2008).

Threadfin bream has been labelled as 'trash' fish (Matsushita and Ali, 1997). The fish are often caught by accident in the shrimp trawler and due to its small size and sorting difficulty, the fish is normally discarded at sea (Alverson *et al*., 1994). However, nowadays threadfin bream is among one of the valued fish used in surimi production (Benjakul *et al*., 2003). The flesh contains approximately 19% protein (Karthikeyan *et al*., 2006).

Protein hydrolysate has been studied for the purpose of improving food functionalities and technological properties, nutritional and medical purposes and also for improvement of food taste (Ovesen, 1991; Haslaniza *et al.*, 2010). Hydrolysate from threadfin bream has been studied in terms of optimization for its production and physico-chemical properties, functional properties and sensory characteristics (Normah *et al*., 2005a; Normah *et al*., 2005b; Nalinanon *et al*., 2011).

Bilimbi (*Averrhoa bilimbi* L.) is a local fruit which is easily obtain, very cheap and affordable. It has both the food value and medicinal uses. Bilimbi is a potential fruit to be used widely in food industry.

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However, there is not much research been done to develop and promote this fruit. Bilimbi protease extraction and purification has been reported, however, the detail study on the protease is still limited (Normah and Nur' Ain, 2013). Proteases extracted from natural sources are very scarce including papain, chymopapain and peptidase A from the latex of young papaya fruit, ficin from fig as well as bromelain from the fruit and stem of pineapple (Mazumdar & Majumder, 2003; Whitehurst & van Oort, 2010). Furthermore, studies on the production of hydrolysate using bilimbi protease have never been reported before. Therefore, in this study proteolytic activity of bilimbi protease was determined and its efficiency in hydrolysing threadfin bream protein was compared with alcalase.

Materials and Methods

Materials

Bilimbi was obtained from a local farmer in Shah Alam, Selangor. Only fruit with uniformity in size and color (dark-green, maturity stage 1) as well as free from defects was selected. Threadfin bream *(Nemipterus japonicus)* was purchased from the nearest wet market in Selangor, Malaysia. Alcalase 2.4 L, a food-grade enzyme with a declared activity of 2.4 AUg^{-1} and a density of 1.18 g ml^{-1} was obtained from Novo Nordisk Industries (AS, Bagsvaerd, Denmark).

Extraction and purification of bilimbi protease

All the extraction and purification steps were performed at low temperature (4°C) according to Normah and Nur' Ain (2013). Initially, the fruit was washed thoroughly and cut into dice. The juice was then extracted in a juice extractor and filtered through four layers of muslin cloth. To the collected juice, ammonium sulfate at 40% (w/v) was added until precipitate formed. The mixture was then centrifuged at 5000 rpm (Heraeus, Biofu ge pico, Belgium) for about 20 mins at 4°C in order to separate the precipitate from the supernatant which was then dialyzed against Tris-HCL buffer (0.02 M, pH 7.5) overnight.

Determination of proteolytic activity

Proteolytic activity was measured using casein as a substrate according to Ahmad *et al*., (2012) with slight modification. About 9 ml casein solution (1% w/v in 0.2 M sodium phosphate buffer, pH 7) and 1 ml sample were added into a vial. The mixture was incubated at 37°C for 30 mins. Then, 3 ml trichloroacetic acid (5% w/v) was added and subsequently filtered through 0.45 µm membrane filter to remove any insoluble particles. About 2 ml of the filtrate was mixed with 1 ml of 2 N Folin-Ciocalteu reagent and 5 ml of 500 mM sodium carbonate solution and incubated for 30 min at 37°C. The absorbance was measured at 660 nm. One unit of protease activity (U) was defined as the amount of protease that can hydrolyse casein into 1 µg of tyrosine in 1 min at 37°C.

Total activity of bilimbi protease

The total activity was determined according to Ahmad *et al.*, (2012) with slight modification using the following formula:

$$\text{Total activity (Unit/ml)} = IU \times \frac{100}{V_t} \times \frac{1}{0.1} \times DF$$

where:

IU = µmole tyrosine/min
DF = dilution factor
V_t = volume of sample
0.1 = volume of enzyme used

Protein content

Protein content was determined using Lowry method with slight modification (Lowry *et al.*, 1951). Bovine serum albumin (BSA) was used as a standard at a concentration ranged from 0-100 mg/L. The absorbance was measured at 750 nm.

Preparation of threadfin bream hydrolysate using alcalase and bilimbi protease

The fish was degutted, washed thoroughly under running tap water and deboned. About 30 g fish flesh was mixed with 120 mL distilled water. The mixture was hydrolyzed with alcalase according to Normah *et al.*, (2005a). The hydrolysis conditions were set at pH 8.5, 60°C, enzyme/substrate ratio of 2% and duration of 120 min. Sodium hydroxide (2 M) was added to maintain a constant pH. At the end of the hydrolysis, the mixture was heated in a water bath for 20 min at 90°C and then cooled at room temperature. This was followed by centrifugation at 5000 rpm, temperature of 4°C for 40 min using a refrigerated centrifuge (Heraeus, Biofu ge pico, Belgium). The supernatant was adjusted to pH 6 with 2 M HCl and dried in a freeze drier (SANYO- Biomedical freeze drier). As for the preparation of threadfin bream hydrolysate using bilimbi protease, similar method was applied except that different pH and temperature were used which are pH 4 and 40°C, respectively.

Degree of hydrolysis

Degree of hydrolysis (DH) was calculated using

a pH-stat method based on the amount of NaOH used in hydrolysis for maintaining the constant pH. The DH was computed according to Adler-Nissen (1986) as follows:

$$DH\ (\%) = \frac{\beta \times N_{\beta}}{\alpha \times M_p \times h_{tot}} \times 100$$

where :

β = volume of NaOH
N_{β} = molarity of NaOH
α = average degree of dissociation of NH_2
M_p = mass of protein
h_{tot} = total number of peptide bonds in the protein substrate

Chemical analysis of the hydrolysate

Moisture and protein content were determined according to the AOAC (2005). The protein content was determined using the Kjeldahl method. For moisture content determination, approximately 2 g sample was placed into a pre-weighed aluminium dish. The sample was dried in a forced-air convection oven at 105°C until a constant weight was achieved.

Yield

The yield (%) was determined by the ratio of the mass of hydrolysate obtained and the total weight of threadfin bream muscle used as follows:

$$Yield\ (\%) = \frac{\text{Weight of powdered hydrolysate}}{\text{Wet weight of threadfin bream muscle}} \times 100$$

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weight distribution was determined according to Normah and Nur' Ain (2013). About 0.05g/ml threadfin bream hydrolysate was mixed with NuPage sample buffer and NuPage reducing agent and then heated at 70°C for 10 mins. About 10 µl of the mixture was then loaded into the gel. Benchmark™ protein ladder ranging from 3 to 188 kDa was used as a marker. Electrophoresis was run for 50 mins at 100-125 mA/gel using the XCell Surelock electrophoresis cell (Consort, Model EV231, Germany). The gel was then stained in Coomassie Brilliant Blue solution and destained using ultrapure water.

Solubility

Solubility was determined according to Normah and Nurfazlika Nashrah (2013) with slight modification. Threadfin bream protein hydrolysate (0.5 g) was dispersed in 50 ml of 0.1 M NaCl and the pH was adjusted to pH 7. The mixture was stirred for 1 hr at room temperature and then centrifuged (Heraeus, Biofu ge pico, Belgium) at 10,000 rpm for 30 min. The supernatant was filtered through Whatman filter paper No.1 and nitrogen content was analyzed by Lowry method (Lowry *et al*., 1951). Solubility of the hydrolysate was calculated as follows:

$$Solubility\ (\%) = \frac{\text{Protein content in the supernatant}}{\text{Protein content in the mixture}} \times 100$$

Statistical analysis

Data was analyzed using the Analysis of Variance (ANOVA) to determine significance at 5% level. Duncan Multiple Range Test (DMRT) was used to identify differences between means. The statistical program used was Statistical Analysis System (SAS Institute Inc., 2004).

Results and Discussion

Proteolytic activity of bilimbi protease

Bilimbi protease was extracted from unripe bilimbi fruit (*Averrhoa bilimbi* L.) at maturity stage 1 (dark green) and purified using 40% ammonium sulfate precipitation method. The maturity stages of bilimbi were as follows; Stage 1 : a completely dark green skin (about >90%), firm and immature; Stage 2 : light green (70%), mature, semi unripe, with slightly yellow (30%) on one side; Stage 3 : mature, ripe, harvest stage, fully yellow skin (about >90%); and Stage 4 : mature, fully-ripe, harvest stage, soft and deep-yellow skin. Bilimbi protease purified by 40% ammonium sulfate precipitation showed approximately three times proteolytic activity (2.36 U/mg) and purification fold (3.06) than its crude extract (0.77 U/mg). In previous studies, purification using 20 to 40% ammonium sulfate resulted in highest total activity which was more than 44% compared to purification using ethanol and poly (ethylene glycol) (Soares *et al*., 2011). Table 1 showed that purification of bilimbi protease with 40% ammonium sulfate precipitation method resulted in 23.13% recovery while recovery of proteases from kachri fruit (*Cucumis trigonus Roxburghi)* and *Artocarpus integer* leaf were 35 and 15%, respectively (Asif-Ullah *et al.*, 2006; Siti Balqis and Rosma 2011). Both studies used 60% ammonium sulfate precipitation. Therefore, the percent recovery of bilimbi protease was within the reported range. The extent of protein precipitation is a function of ammonium sulfate concentration and the degree of purification decreased with increased in the concentration of ammonium sulfate (Roy and Gupta, 2000; Narayan *et al*., 2008).

Table 1. Proteolytic activity of bilimbi (*Averrhoa bilimbi* L.) crude extract and bilimbi protease purified using 40% ammonium sulfate precipitation (40% asp)

Sample	Total protein (mg)	Total Activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	1905.5	1470	0.77	1.00	100
40% asp	144.07	340	2.36	3.06	23.13

Table 2. Yield, degree of hydrolysis (DH), solubility, protein and moisture content of threadfin bream hydrolysate produced using alcalase and bilimbi protease

Protease	Yield (%)	DH (%)	Solubility (%)	Protein (%)	Moisture (%)
Alcalase	22.78±0.65^{a}	86.60±2.95^{a}	70.87±0.04^{a}	86.86±0.07^{a}	3.93±0.06^{a}
Bilimbi	3.75±1.05^{b}	34.76±2.25^{b}	32.16±1.26^{b}	22.12±0.48^{b}	7.00±1.73^{b}

Values expressed on samples basis are average of triplicate analysis ±std dev. Different superscripts within the same column indicate significant difference at $p < 0.05$

Analysis of threadfin bream hydrolysate

Threadfin bream hydrolysate was produced by hydrolysing threadfin bream flesh using alcalase and bilimbi protease resulting in yield of 22.78% and 3.75%, respectively (Table 2). Yield can be improved by properly monitoring the hydrolysis conditions (Normah and Nurfazlika Nashrah, 2013). It also depends on the type of enzyme used as showed in the previous studies where yield of meriga *(Cirrhinus mrigala)* egg hydrolysate was higher (41.2%) when alcalase was used instead of only 9.7% using papain (Chalamaiah *et al.*, 2010). Higher yield was due to higher degree of solubilisation. The reported yield of fish protein hydrolysate falls between 10 to 15% (Quaglia and Orban, 1990).

Threadfin bream hydrolysate produced using alcalase had higher DH (86.60%) compared to using bilimbi protease (34.76%) (Table 2). As DH increases, the pH of the hydrolysis process increased and subsequently the volume of NaOH used will also increase (Severin and Xia, 2005). In this study, the pH used to produce threadfin bream hydrolysate using alcalase was greater which is 8.5 compared to only 4 when bilimbi protease was applied. Thus, hydrolysis using alcalase to produce threadfin bream hydrolysate consumed more NaOH compared to bilimbi protease. It has been suggested that the differences in DH are essentially due to the difference in the specificity of enzyme used during hydrolysis (Ktari *et al*., 2012). A higher DH exhibited by alcalase compared to flavourzyme during the hydrolysis of silver carp protein was suggested to be due to higher affinity of alcalase although similar amount of enzyme was used (Dong *et al*., 2008). Alcalase also caused random cleavage during the hydrolysis as compared to enzyme such as papain which is more selective and as a result alcalase produced higher DH (62%) than papain (17.1%) (Chalamaiah *et al*., 2010). Alkaline proteases such as alcalase have been reported to exhibit higher activities than neutral or acid proteases, such as papain, pepsin and flavourzyme (Rebeca *et al*., 1991; Klompong *et al*., 2007).

Solubility

Solubility is one of the most important physico-chemical and functional properties of protein hydrolysates (Kristinsson and Rasco, 2000). One of the most important characteristics of protein in many functional food applications is good solubility mainly for emulsion, foam and gel purposes because soluble protein provide a homogeneous dispersibility of the molecules in colloidal systems and enhance the interfacial properties (Zayas, 1997). The solubility of the threadfin bream hydrolysate produced using alcalase was significantly higher ($p<0.05$) than those using bilimbi protease (Table 2). Alkaline proteases had greater capability to dissolve protein compared to neutral and acidic proteases (Zhao *et al.*, 2012). Furthermore, at higher DH, solubility increases where the protein fraction was characterised by lower molecular mass (Dong *et al*., 2008). This was in agreement with molecular weight distribution

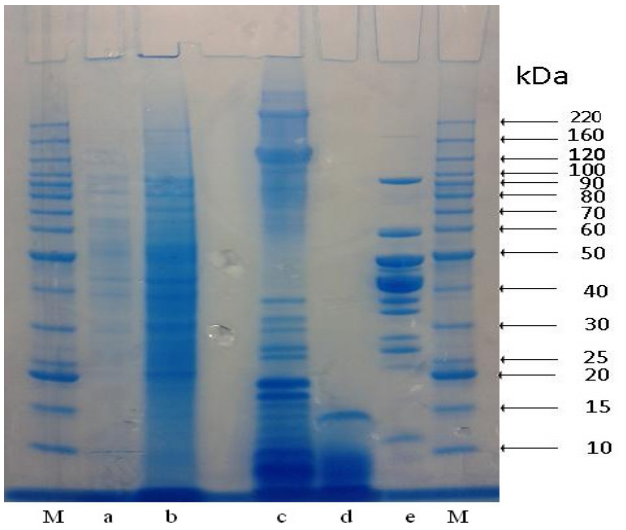

Figure 1. SDS-PAGE of a) bilimbi crude extract, b) bilimbi protease purified at 40% ammonium sulfate precipitation, c) threadfin bream hydrolysate produced using bilimbi protease, d) threadfin bream hydrolysate produced using alcalase, e) threadfin bream flesh and M) protein marker

results of alcalase hydrolysate that shows the existence of mainly low molecular weight peptides (Figure 1). Higher DH produced lower molecular weight polypeptides, thus, increasing their solubility. Smaller peptides are expected to have proportionally more polar residues with the ability to form hydrogen bonds with water and increase solubility (Gbogouri *et al*., 2004). It has also been shown that in the hydrolysis of pacific whiting *(Merluccius productus)* muscle as DH increased so did the soluble protein (Pacheco-Aguilar *et al*., 2008). High solubility of protein hydrolysates was due to the size reduction, formation of a smaller and more hydrophilic and more solvated polypeptide units (Chalamaiah *et al*., 2010).

Protein content

High protein content of fish protein hydrolysates demonstrates its potential use as protein supplements for human nutrition (Chalamaiah *et al*., 2012). Threadfin bream hydrolysate produced using alcalase had significantly higher ($p<0.05$) protein content compared to bilimbi protease hydrolysate (Table 2). Similar findings were also observed in meriga *(Cirrhinus mrigala)* egg hydrolysate production where the protein content of hydrolysate produced by alcalase was 85% compared to only 70% with papain (Chalamaiah *et al.*, 2010). High protein content was due to the solubilisation of protein during hydrolysis and the removal of insoluble undigested non-protein substances (Benjakul and Morrissey, 1997).

Moisture

Most studies demonstrated that protein hydrolysates from various fish proteins contain less than 10% moisture (Chalamaiah *et al.*, 2012). The low moisture content of protein hydrolysates was related to the type of sample and higher temperatures employed during the process of evaporation and spray drying where during these processes, the sample lost most of its moisture (Bueno-Solano *et al.*, 2009). Moreover, hydrolysate with low moisture content improved its stability during storage (Normah and Nurfazlika Nashrah, 2013). Based on this study, the moisture content for hydrolysate produced using alcalase do not show remarkable variation with bilimbi protease hydrolysate (Table 2). Previous studies also showed that fish egg protein hydrolysates produced by alcalase had lower moisture content (5.2%) than papain (8.2%) (Chalamaiah *et al.*, 2010). The hydrolysate prepared from the frame meat of striped catfish *(Pangasianodon hypophthalmus)* produced from bromelain and papain showed low moisture content of 5.97 and 4.81%, respectively (Tanuja, 2012). They stated that moisture content of <6% enhance hydrolysate stability.

Molecular weight distribution

Molecular weight distribution is one of the most important properties in producing protein hydrolysate to be used as functional materials which have a direct impact on their functional properties (Zheng *et al*., 2006). The electrophoretic pattern of bilimbi crude extract, bilimbi protease, threadfin bream hydrolysate and flesh is shown in Figure 1. The molecular weight distribution and average peptide size is affected by enzyme specificity (Kristinsson and Rasco, 2000). Molecular weight distribution of bilimbi crude extract was almost similar with bilimbi protease purified with 40% ammonium sulfate which range from 10 to 100 kDa. However, the bands in the purified bilimbi protease are more intense. More intense bands exhibited by bilimbi protease were due to the purification process using 40% ammonium sulfate leaving behind the protein component that has been purified. Threadfin bream flesh had bands with molecular weight range from 10 to 90 kDa. As for the threadfin bream hydrolysate produced using bilimbi protease, the band can be visualized between <10 kDa to 220 kDa whereas threadfin bream hydrolysate produced using alcalase were visible at the lower range which was <15 kDa. It can be seen that high molecular weight bands (>15 kDa) seems to be disappeared. Nevertheless, very intense bands appeared at <15 kDa. This shows that alcalase has the ability to produce low molecular weight peptides

by means of a high DH. In the previous studies, characterization of the molecular weight of tilapia by-product hydrolysate powder by SDS-PAGE showed the presence of strong bands ranging between 3.5 to 26.6 kDa which indicated that alcalase was able to produce small-sized peptides (Roslan *et al*., 2014). Fish protein hydrolysate with high nutritional values should be rich in low molecular weight peptides (Bhaskar and Mahendrakar, 2008). Hydrolysate containing small size peptides has potential application in functional food products (Roslan *et al*., 2014). Unlike the band showed by the threadfin bream hydrolysate produced using alcalase, there were various bands visible from the highest until the lowest molecular weight for the threadfin bream hydrolysate produced using bilimbi protease. The high molecular weight bands especially above 90 kDa in threadfin bream hydrolysate produced using bilimbi protease basically derived from the high molecular weight peptides presence in the bilimbi protease (Figure 1). It can be seen that these high molecular weight bands were very much alike for both bilimbi protease hydrolysate and bilimbi protease. Generally, each protease selectively hydrolyses a specific peptide bond within protein substrates (Nakamura, 2006).

Conclusion

Bilimbi protease purified by 40% ammonium sulfate precipitation produced approximately three times proteolytic activity (2.36 U/mg) than its crude extract (0.77 U/mg). This shows that the purification using 40% ammonium sulfate precipitation method was able to produce high specific activity for bilimbi protease compared to crude extract. The physico-chemical properties for both hydrolysates would contribute important role in food industry in terms of solubility. The low moisture content suggested the storage stability of the hydrolysate. Threadfin bream hydrolysate produced using alcalase had higher yield and DH compared to threadfin bream hydrolysate produced using bilimbi protease. Thus, alcalase is more efficient enzyme choice than bilimbi protease for preparing threadfin bream hydrolysate.

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