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Extraction, characterization and activity of digestive enzyme from Nile tilapia *(Oreochromis niloticus)* viscera waste

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Abstract

Nile tilapia viscera extracts were prepared using water, NaCl solution and acetone homogenization. The saline solution (5% w/v) yielded the extract with the highest protease and amylase specific activities of 1,300 and 1,800 unit/mg protein, respectively. These two enzymes were active at the identical optimum pH (8.0) and temperature (60°C). At this condition, protease activity was gradually lost and remained at 50% of its initial value after 30 min. In contrast, amylase activity had risen 5 times before declining to about 3 times of its initial activity after 90 min. Precipitation of other protein by using 30 or 35% ammonium sulphate effectively improved specific activity of both enzymes of the extract. This partial purified fraction and the crude exhibited three protein bands by using SDS-PAGE assigned as 56.22, 31.33 and 26.71 kDa. Thus, the results revealed that active digestive enzymes could be prepared from Nile tilapia viscera.

Introduction

Nile tilapia *(Oreochromis niloticus)* is one of the most important fish species in Thailand's aquaculture (Nurit, 2012). The mass industrial processing of Nile tilapia, frozen fillets as a priority, generates large amount of waste especially viscera (Nurit, 2012; Freitas-Júnior *et al*., 2012). Fish viscera have been reported to be a good source of digestive enzymes (Simpson *et al.*, 1991; Bezerra *et al*., 2005; Klomklao *et al*., 2009; Khantaphant and Benjakul, 2010) and their properties are highly valued in a wide range of industrial applications and processes since some proteases are stable and active under harsh conditions (high temperature and pH) and in the presence of oxidizing agents or surfactants (Klomklao *et al.,* 2005). Proteases are primary enzymes which have been isolated and characterized from various parts of Nile tilapia digestive tract (Tengjaroenkul *et al*., 2000, Hinsui *et al.*, 2006). Enzyme from Nile tilapia viscera is however likely to contain more than one type of enzyme. Because of Nile tilapia is a herbivorous fish and its digestive system also displays greater activity of amylase than protease and a lesser lipase activity (Tengjaroenkul *et al*., 2000).

Digestive proteases from fishes are hydrolytic in their action and catalyze the cleavage of peptide bonds with the participation of water molecule as reactants (Klomklao, 2008). Proteases found in the viscera of fish include trypsin, chymotrypsin, collagenase, elastase, carboxypeptidase and carboxyl esterase (Haard, 1994). Amylases from the intestinal cavity of tilapia species, Sarotherodon melanotheron, are active similar to those of human and porcine pancreatic α-amylase (Al Kazaz *et al.*, 1996). Only three tissues (liver, mesenteric tissue and intestine) were found to contain any significant amount of amylase. No activity was recovered from the stomach (Moreau *et al*., 2001).

Extraction of enzymes from whole digestive tract is more practicable for this soft and perishable by-product. Optimization of extraction and separation in order to obtain active those enzymes are thus concerned. Until now, no information has been reported on preparation of crude visceral extract containing protease and amylase especially from tilapia viscera. Hence, it was the primary objective of this study to optimize an extraction of crude enzyme from Nile tilapia viscera and to evaluate properties of this mixed enzyme.

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Materials and Methods

Sample preparation

The tilapia viscera were collected from a local market and a frozen tilapia manufacturer in Songkhla province, Thailand. They were transported to the laboratory at Prince of Songkla University on ice within 2 hrs. The visible fat and gallbladder was removed manually before being washed with water and stored at -20ºC until used for enzyme extraction.

Crude tilapia enzyme preparation

The minced tilapia viscera were subjected to homogenization with different media; distilled water, acetone, and 50 mM NaCl solution at the ratio of 1:3 (w/v). The mixtures were centrifuged at 10,000 × g for 15 min at 4ºC. The supernatants were then collected and used as "crude enzyme extract" for further studies.

Effect of NaCl on enzyme extraction and activity was determined by homogenized the minced tilapia viscera with different concentration of NaCl (0.1%, 0.5%, 1%, 5%, 10%, 15%, 20%w/v) at the ratio of 1:3. The suspended particle was removed by centrifugation at 10,000 g for 15 min. The supernatant (crude extract) was then collected and determined enzyme activity at 37ºC pH 7.0

Protein content

The protein content of the crude enzyme extract was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Protease activity

The protease activity of the crude extract was determined as described by Banik and Prakash (2006) with some modifications and using casein as a substrate. To initiate the reaction, 1,000 µl of 100-fold diluted viscera crude extract with Tris-HCl buffer pH 8.0 was added into 1,000 µl of 1% casein and incubated at 37ºC for 15 min. The enzymatic reaction was terminated by adding 2,000 µl of stop buffer (0.1M TCA: 0.22 M sodium acetate: 0.33 M acetic acid, 1:1:1). The mixture was allowed to stand for 30 min and then centrifuged at 8,000 x g for 10 min at 4ºC. The supernatant was measured using spectrophotometer at 275 nm. One unit of protease activity against casein was defined as the amount of enzyme catalyzed an increase of one unit per minute in the absorbance at 275 nm under the assay conditions. Specific activity was expressed as the amount of unit activity per mg of protein.

Amylase activity

Amylase activity of the crude extract was determined by the starch hydrolysis method, described by Pongsawadi and Yagisawa (1988) with some modifications. To initiate the reaction, 1,000 µl of 100-fold diluted viscera crude extract with Tris-HCl buffer pH 8.0 was added into 3,000 µl of 1% starch solution and incubated at 60ºC 30 min. Then 200 µl of the mixed solution was transferred into 5,000 µl of iodine solution. The absorbance of the solution at 540 nm was measured using spectrophotometer. One unit of amylase activity was defined as the amount of enzyme catalyzed an increase of one unit per minute in the absorbance at 540 nm under the assay conditions. Specific activity was expressed as the amount of unit activity per mg of protein.

Effect of pH and temperature on crude enzyme extract

The effect of pH on enzyme activity was determined at 37ºC, over the pH range of 2.0-11.0 (0.1 M sodium citrate buffer for pH 2.0-5.0, 10 mM Tris-HCl buffer for pH 6.0-9.0, 0.05 M carbonate-bicarbonate buffer for pH 10.0-11.0). And the effect of temperature on enzyme activity was determined at the optimum pH over the thermal range of 30-80ºC.

Stability of crude enzyme extract

The stability of crude enzyme was examined by pre-incubation the crude enzyme at optimum pH and temperature for 6 hrs. Samples were removed at intervals of 30 min and residual activities of protease and amylase were examined.

Partial removal of non-enzyme protein

The crude enzyme extract was first submitted to ammonium sulphate precipitation at 10%, 20%, 30%, 40%, 50% or 60% w/v. The supernatant obtained after centrifugation at 10,000 x g and 4ºC for 15 min was analyzed for protein content and enzyme activities. The supernatant from the optimum precipitation was later dialyzed by using a hollow-fiber membrane (molecular weight cut off 30 kDa) at ambient temperature (28-30ºC), pressure of 1.3 bar and flow rate of 17 ml/sec. The operational modes included a pre-diafiltration and post-concentration processes. Protein profile of desalted extract was investigated by using SDS-PAGE (Laemmli, 1970).

Discussion

Effects of extract media on crude Nile tilapia viscera extract

Effects of different extract media on protein content and specific activity of protease and amylase

Table 1. Effect of extractants on protein content and specific activity of protease and amylase enzyme of crude extracts of Nile tilapia viscera

extractant	protein content (mg/ml)*	specific activity of protease (unit/mg protein)*	specific activity of amylase (unit/mg protein)*
acetone	4.82 ± 0.03	471.88 ± 2.70	1,360.40 ± 4.27
water	4.88 ± 0.06	410.04 ± 2.98	1,414.69 ± 3.78
NaCl (50mM)	5.47 ± 0.06	551.55 ± 3.78	1,453.82 ± 2.68

* The values are expressed as mean ± standard deviation from triplicate determinations. There are significant differences among means within the same column at $p \leq 0.05$.

of tilapia viscera extract are shown in Table 1. Crude extract with the highest protein contents and specific activity of both enzymes was derived by using saline solution as an extract medium. This was an unexpected result based on the fact that enzyme is known as a water soluble protein. Decreasing on ionic strength of endogenous solution of the visceral system after its homogenization with water is however expected.

This may however consequently lead to precipitation of salt soluble protein as supported by a low protein content of the aqueous extract. This is possibly the result of co-precipitation of water soluble protein including enzyme accounted by the lowest specific activity of protease. By using salt solution, on the other hand, alteration of ionic strength of the sample may limit and provide a condition, not only reduce the co-precipitation, but also enhance some extraction efficiency. This proposition is, at least, supported by the observation that both total protein and specific enzyme activity were increased by the saline extraction. However, it is important to note that beneficial effects of the salt solution was prevalence on protease extraction and relative to that of the amylase.

Pre-extraction of viscera with organic solvent aimed for fat and lipid removal is used as a material pretreatment by some research groups (Kishimura and Hayashi, 2002; El-Beltagy *et al.*, 2004; Khaled *et al*., 2011). Thus, acetone was used to investigate how this fat removal medium affects enzyme extraction. The result revealed that it caused significant loss on both protein content and specific enzyme activity. Existence of colloidal particles facilitated by visceral proteins may account for the loss.

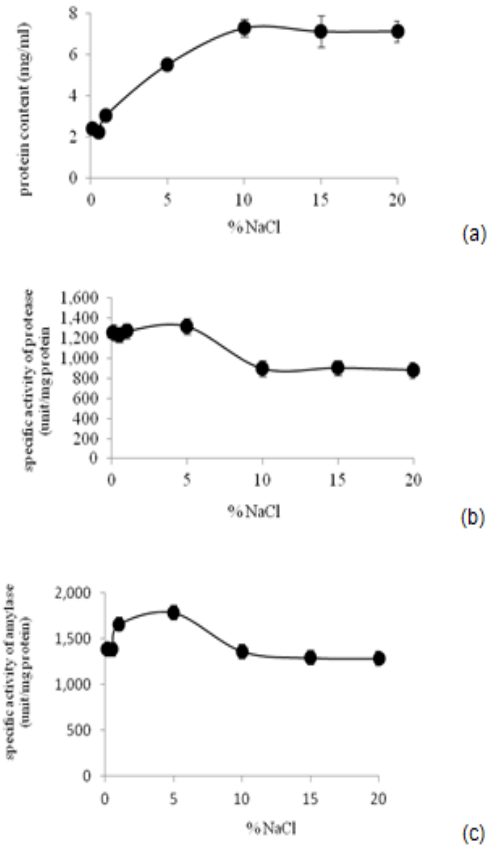

Figure1. Effect of NaCl concentration on protein content (a) and specific activities of protease (b) and amylase (c)

Effects of concentration of NaCl solution on Nile tilapia viscera extract

Effects of NaCl concentration on protein content and protease and amylase activities of the tilapia extracts are shown in Figure 1. Protein content of the extract was increased gradually with an increase of salt concentration reaching the highest value of 7.31 mg/ml at 10% NaCl. Thereafter a plateau of protein content was found with a further increase of salt concentration. Effect of salt concentration on specific activity of both enzymes however differed from its effect on protein content. The enzyme activity compared to that of the water extract was increased only by using 5% NaCl. The difference between these two responds (protein content and specific enzyme activity) against salt concentration suggested at least that extraction of other proteins is enhanced when salt concentration is higher than 5%. As well as, amylase activity of redfish *(Sebastes mentella)* was enhanced by NaCl concentration at certain range up to 50 mM (Munilla-Moran and Saborido-Rey, 1996). Moreover, a continuous decrease in aspartic protease activity with increasing NaCl concentration was reported (Khaled *et al*., 2011).

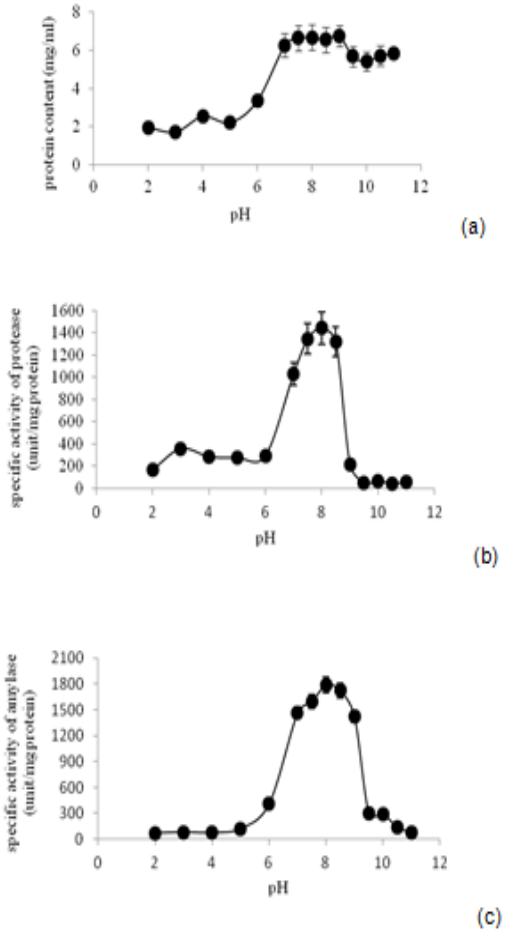

Figure 2. pH profiles of protein content (a) and specific activities of protease (b) and amylase (c)

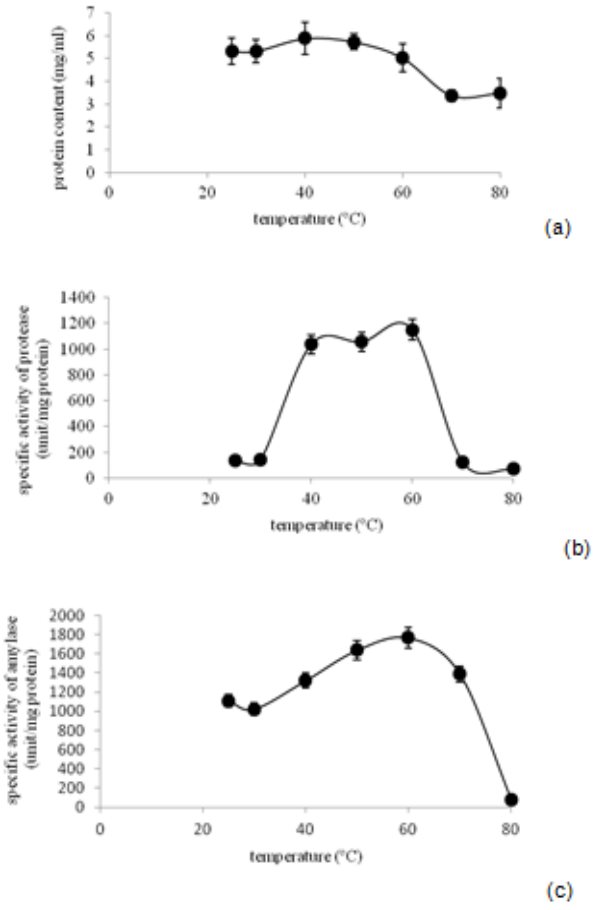

Figure 3. Temperature profiles of protein content (a) and specific activities of proteas (b) and amylase (c)

pH and temperature profiles of tilapia viscera extract

Effects of pH on protein content and specific activity of crude extract derived from the tilapia viscera were determined over the pH range of 2.0-11.0 as shown in Figure 2. An increase in protein content was observed with increasing pH from 2.0-7.0 and later stable at pH 7.0-9.0. However, its protein content was slightly decreased if pH was higher than 9.0. This profile may be relevant to the net charge of the protein at various pH values (Klomklao *et al*., 2009).

The protease and amylase of the extract were highly active within pH ranges of 7.5-8.5 and 7.0-9.0, respectively, with the maximum activity at identical pH 8.0 (Figure 2b and Figure 2c). The optimum pH value of protease was in accordance with the value reported by Bezerra *et al*., (2006). As well as, Klahan *et al.*, (2009) reported that the optimal pH of amylase from liver, stomach and intestine of tilapia were 7.0, 8.0 and 7.0, respectively.

Effects of temperature on protein content and specific activity of crude enzyme extract at pH 8.0 was determined at temperature range of 30-80ºC as shown in Figure 3. Decrease of protein content was noted after temperature rose above 40ºC. Thermal precipitation is generally accepted as a primary cause (Klomklao, 2008). In contrast, the protease and amylase of the tilapia crude extract were highly active at temperature ranges of 40-60ºC and 50-65ºC, respectively. Their optimal temperature was identical at 60ºC. The result thus suggested that both enzymes is thermally stimulated. As well as that, thermally labile and non-enzyme protein was coagulated within these temperature ranges. The optimum temperature of protease is slightly higher than the values of the intestine extract of Nile tilapia (50ºC) and hybrid Tilapia nilotica/aurea (40ºC) (Bezerra *et al.*, 2005). The amylase optimal temperature was higher than the value (40ºC) reported by Tsao *et al.*, (2004). In addition, optimal temperature ranges of amylase activities of the stomach and intestine extracts of the Mekong Giant Catfish were 25-50ºC and 25-30ºC, successively (Tongsiri *et al*., 2010).

The appreciable decrease in specific activity of both enzymes was observed at temperatures above 60ºC which is presumably a result of partial unfolding of the enzyme molecule due to thermal inactivation (Klomklao *et al.*, 2006 and Villalba *et al*., 2011).

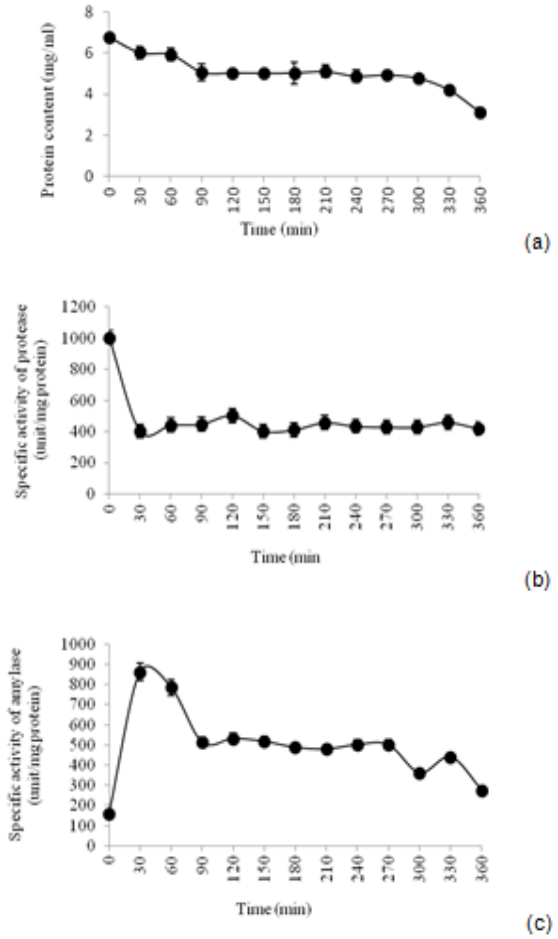

Figure 4. Stability of protein content (a) and specific activities of protease (b) and amylase (c)

Stability of protease and amylase enzyme

The stability of tilapia viscera enzymes at 60ºC and pH 8.0 is shown in Figure 4. Protease activity of the crude extract decreased significantly after 30 min incubation thereafter exhibited a plateau phase at about 50% of its initial activity with extension of incubation time up to 360 min. In contrast, amylase activity of the extract rose 5 times after incubation for 30 min. It later declined gradually reaching a stationery phase of about 3 times its initial activity after 90 min incubation. The results thus suggest a difference between these two enzymes. Protease activity obviously has low heat tolerance while amylase activity is not only more heat tolerance but also well heat stimulate. Since only about 15 and 30% drop of protein content of the extract was noted due to the 30 and 90 min incubation, respectively, thus thermal coagulation of the enzymes could not primarily account for those changes of the enzyme specific activity. On other hand conformation changes of the enzymes due to thermal activation is likely responsible for the observation made.

Effect of ammonium sulphate precipitation

Removal of soluble protein of the crude extract occurred progressively with increasing of ammonium sulphate concentration from 10% to 60%. According to specific enzyme activity the result suggested that the appropriated ammonium sulphate concentration ranges for partial recovery protease and amylase are 10-35% and 30-40%, respectively. Thus, the extract with improved specific activity of both enzymes could be obtained by precipitation of other protein by using a very narrow range (30-35%) of ammonium sulphate concentration. At ammonium sulphate concentration higher than 35% a decreased on specific activity of the protease in the residue extract was noted which reflected a better precipitation of the enzyme with relative to other non-enzyme proteins. Rawdkuen *et al*. (2012) found that the sediment with the highest proteases activity of farmed giant catfish *(Pangasianodon gigas)* could be obtained by partitioning with 50% ammonium sulphate. In contrast, Temiz *et al.* (2013) reported that 20 to 40% ammonium sulphate precipitation of anchovy digestive tract extract yielded the pellet richen of alkaline protease. The result also revealed that partial separation of amylase from protease and other proteins could be possible by using 40% w/v ammonium sulphate whereas partial separation of protease from amylase and other proteins could be performed at ammonium sulphate concentration lower than 30% w/v.

Protein patterns of the crude extract and the partial purified extracts obtained by using 35% ammonium sulphate under SDS-PAGE. The major protein bands with the molecular weight of 56.22, 31.33 and 26.71 kDa were found in both extracts. Molecular weight of protease derived from several fish visceral fall within a range of 23- 28 kDa (Freitas-Júnior *et al.*, 2012). And, Moreau *et al.* (2001) reported that the molecular weight of α-amylases derived from the intestinal cavity of two tilapia species, *Oreochromis niloticus* and *Sarotherodon melanotheron,* were 56.6 and 55.5 kDa, respectively.

Conclusions

The tilapia crude enzyme extract showed high protease and amylase activities in the 5% NaCl solution and exhibited a maximal activity at 60ºC and pH 8.0. The protease activity of the tilapia crude enzyme extract decreased significantly after 30 min incubation while amylase activity increased rapidly by about 5 times within 30 min incubation. The optimum concentration of ammonium sulphate precipitation for both protease and amylase recovery was 30 to 35%. The major protein bands with the

molecular weight of 56.22, 31.33 and 26.71 kDa were found in both crude enzyme extract and the partial purified extract.

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