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Original Article

Extraction, Purification, and Characterization of Trypsin Obtained from the Digestive System of Yellowfin Seabream (*Acanthopagrus latus)*

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ABSTRACT

The development of the marine aquaculture industry has led to the generation of significant amounts of fish wastes. Marine farm wastes exert adverse effects on the surrounding area of the cages. On the other hand, wastes of fish and other aquatic animals are regarded as major sources of valuable natural bioactive compounds, including enzymes, proteins, bioactive peptides, oil, amino acids, collagen, gelatin, calcium, biopolymers, and water-soluble minerals. To investigate the potential of marine fish waste, the whole digestive system of yellowfin seabream (*Acanthopagrus latus*) was extracted for extraction and identification of trypsin enzyme. Fish (179.93±93.67 g; 184±28.17 cm) were caught from the Persian Gulf and stored at -20 °C. Yellowfin seabream were dissected and their whole digestive systems were removed. Samples were thoroughly washed with distilled water and purified through defatting using acetone and ammonium sulfate precipitation. The following issues were assessed: the total and specific activity of trypsin, protein determination, molecular weight, enzyme activity and stability in different pH values and temperatures. The obtained results indicated that specific activity and protein content of trypsin enzyme were 4.4 U and 3.4 mg/ml, respectively. The molecular weight of 23 kDa was reported for trypsin using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method. Maximum activity and stability of trypsin were observed at 60°C and 45°C, respectively. Trypsin demonstrated maximum activity and stability at a pH value of 8.0. In general, the results of the current study suggested that trypsin extracted from the digestive system of yellowfin seabream has considerable potential for industrial applications, such as the food industry, owing to its characteristics and stability under alkaline conditions.

Keywords: Enzyme purification, Yellowfin seabream (*Acanthopagrus latus*), Trypsin, Fish waste

Extraction, Purification et Caractérisation de la Trypsine Obtenue à Partir du Système Digestif du Pagre à Nageoires Jaunes (*Acanthopagrus latus*)

Résumé: Le développement de l'industrie de l'aquaculture marine a conduit à la génération de quantités importantes de déchets de poisson. Les déchets de la ferme marine ont des effets néfastes sur les abords des cages. De plus, les déchets de poisson et d'autres animaux aquatiques sont considérés comme des sources majeures de précieux composés bioactifs naturels, notamment des enzymes, des protéines, des peptides bioactifs, des huiles, des acides aminés, du collagène, de la gélatine, du calcium, des biopolymères et des minéraux hydrosolubles. Pour étudier le potentiel des déchets marins des poissons, l'ensemble du système digestif du pagre à nageoires jaunes (*Acanthopagrus latus*) a été extrait dans le but d'extraire et d'identifier l'enzyme trypsine. Des poissons (179,93 ± 93,67 g; 184 ± 28,17 cm) ont été capturés dans le golfe persique et conservés à

-20 °C. Le pagre à nageoires jaunes a été disséqué et son système digestif entier a été prélevé. Les échantillons ont été soigneusement lavés à l'eau distillée et purifiés après dégraissage à l'aide de précipitations à l'acétone et au sulfate d'ammonium. Les paramètres suivants ont été évalués: l'activité totale et spécifique de la trypsine, l'identification des protéines, le poids moléculaire, l'activité enzymatique et la stabilité à différentes valeurs de pH et de températures. Les résultats obtenus indiquent que l'activité spécifique et la teneur en protéines de l'enzyme trypsine sont de 4,4 U et de 3,4 mg/ml, respectivement. Un poids moléculaire de 23 kDa a été observé pour la trypsine par la méthode d'électrophorèse sur gel de polyacrylamide en présence de dodécylsulfate de sodium (SDS-PAGE). Les activités et stabilités maximales de la trypsine ont été observées à 60 °C et 45 °C, respectivement. A pH 8,0, la trypsine montrait une activité et une stabilité maximales. En somme, les résultats de cette étude suggèrent que la trypsine extraite du système digestif du pagre à nageoires jaunes a un potentiel considérable pour les applications industrielles, telles que l'industrie alimentaire, en raison de ses caractéristiques et de sa stabilité dans des conditions alcalines.

Mots-clés: Purification d'enzymes, Pagre à nageoires jaunes (*Acanthopagrus latus*), Trypsine, Déchets de poisson

INTRODUCTION

Based on the publication of Food and Agriculture Organization FAO (2016) on the state of world fisheries and aquaculture, aquaculture production was more than 73.8 million tons and capture fishery production was more than 93.4 million tons in 2014. About 78% of total aquaculture and captured fish around the world (146.3 million tons in 2014) are used for human consumption. Developments in aquaculture industry have led to the production of high amounts of wastes from fish which pollute water sources. Nitrates and phosphates are the main organic wastes that reduce oxygen levels and destruct aquatic life due to algal bloom stimulation (Canada, 2009). Waste produced during fish processing can be solid or liquid. Liquid wastes include wastewater and solid fish wastes consist of gut, head, skin, tails, frames, and fins. Fish wastes are the significant sources of valuable products, namely enzymes, proteins, bioactive peptides, amino acids, oil, collagen, gelatin, calcium, biopolymers, and water-soluble minerals (Lordan et al., 2011). Protease enzymes constitute one of the most important groups of industrial enzymes being extensively used these days. It is estimated that approximately 60% of the total enzyme market is shared by proteases nowadays (Sawant and Nagendran, 2014). Proteases are extracted from different sources, such as animal, plant, and microbial sources (Rani et al., 2012). The most common proteases of animal origin include pancreatic trypsin, chymotrypsin, pepsin, and rennin (Sawant and Nagendran, 2014). Furthermore, digestive enzymes could be extracted from fish viscera which are a rich source of these enzymes (Ketnawa et al., 2013). In general, the extraction of enzymes from fish has more advantages over the extraction from chemicals or other methods. Moreover, since they are obtained from animal tissues, it is not necessary to perform such tests as raw materials toxicological. In addition, catalytic activities are higher in an alkaline pH and could be different from mammalian enzymes which have more stability under acidic pH (Shahidi and Janak Kamil, 2001). Trypsin plays a significant role in the hydrolysis of peptide bonds in the carboxyl-terminal ends of lysine and arginine residues (Yazawa and Numata, 2014). Special characteristics, such as the acceptable activity and stability of trypsin under difficult conditions, have remarkably increased the use of fish trypsin (temperatures of 38-70 °C, pH value of 8-11) (Khangembam and Chakrabarti, 2015). The present investigation was the first study which aimed to investigate the extraction, purification, and characterization of trypsin from the visceral waste of a carnivorous species, yellowfin seabream (*Acanthopagrus latus*). This species belongs to

the Sparidae family and lives in the Persian Gulf. In 2013, about 5,410 tons of Sparidae were caught in the coastal areas of the Persian Gulf (Vahabnezhad et al., 2017). In addition, the production of this species has increased along with the development of cage culture in Iran.

MATERIAL AND METHODS

Sample preparation and enzyme extraction. Yellowfin seabream (*Acanthopagrus latus*) were caught from the Persian Gulf (179.93±93.67 g, 184±28.17 cm) and stored at -20 °C. Fish were dissected and their digestive systems were then removed (3.9±1% of total weight) to be later used for enzyme extraction. Fish digestive systems (88 g) were thoroughly washed with distilled water, defatted by acetone (300 cc), and homogenized with 0.02 M Tris-HCl (a ratio of 1 to 50; pH=9.0). Thereafter, the homogenate was centrifuged at 8000 rpm for 45 min.

Purification of trypsin. The crude trypsin was precipitated from the supernatant by the addition of ammonium sulfate (36.1 ammonium sulfate per 100 ml of crude enzyme), followed by centrifugation at 8,000 rpm for 45 min. Subsequently, the precipitate was dissolved in 38 cc of Tris-Nacl buffer (pH=8.0) and dialyzed with dialysis tubing (typical molecular weight cut-off =10000 KD) at 4 °C for 24 h.

Trypsin activity. Amidase activity of trypsin was spectrophotometrically determined using BAPNA (Na-benzoyl-DL-argenine-p-nitroanilide-HCL) as the substrate. The enzyme crude extract (25µl) was added to a tube containing 1250 µl of substrate-buffer (43.5 mg BAPNA dissolved in 1ml of dimethyl sulfoxide (DMSO) to reach a volume of 100 ml by the addition of 50 mM Tris-HCl containing 10 mM CaCl2, pH=8.0 as the buffer) and incubation at 60 °C for 20 min, followed by reading at 410 nm. The enzyme unit activity (U) is defined as 1µmol p-nitroaniline released from BAPNA /ml/min.

$$\text{Trypsin activity (U/ml)} = \frac{A1 - A0 \times 1000 \times Mix\ V\ (ml)}{8800 \times reaction\ time\ (min) \times V\ sample\ (ml)}$$

$$\text{Trypsin activity (U/mg protein)} = \frac{A_{min} \times 1000 \times Mix\ V\ (ml)}{8800 \times Protein\ mg\ assay}$$

where 8800 (cm-1 M-1) is the extinction coefficient of P-nitroaniline, and A1 and A0 are A410 of the sample and blank, respectively (Khantaphant and Benjakul, 2010). Specific enzyme activity is defined as units per milligram of protein (Candiotto et al., 2018).

Protein determination. Protein concentration was determined using Lowry's method (Lowry et al., 1951).

Determination of molecular weight. The molecular weight of trypsin enzyme was estimated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) (12.5% acrylamide electrophoresis gel). Moreover, it was determined based on comparing its Rf with protein standards. Relative mobility is defined as:

$$Rf = \frac{Distance\ migrated\ by\ protein}{Distance\ migrated\ by\ marker}$$

Temperature profile. The effect of temperature on trypsin activity was examined using substrate buffer (1mM BAPNA in 50mM Tris–HCl buffer containing 10 mM $CaCl_2$) at 25, 35, 45, 50, 60, and 70 °C in pH=8 for 20 min and measured at 410 nm (Khantaphant and Benjakul, 2010).

Temperature stability. Purified trypsin was incubated at 4, 25, 35, 45, 50, 60, and 70 °C for 30 min. Samples were mixed with substrate buffer (1mM BAPNA in 50mM Tris-HCl buffer containing 10 mM CaCl2), incubated at 60 °C for 30 min, and measured at 410 nm (Khantaphant and Benjakul, 2010).

pH profile. Using 1mM BAPNA as the substrate, the activity of trypsin was measured at different pH values ranging from 4-11. The reaction mixture pH was altered using different buffers: 50 mM sodium acetate (pH 4-6), 50 mM Tris–HCl buffer (pH 7-9), and 50 mM glycine buffer (pH 10-11), all of which contained 10 mM of $CaCl_2$. The samples were incubated at 60 °C for 30 min and trypsin activity was measured at 410 nm (Khantaphant and Benjakul, 2010).

pH stability. Purified trypsin was mixed with an equal volume of different buffers containing 10 mM of $CaCl_2$ with different pH levels. Prior to assay, the mixture was placed at room temperature (25 °C) for 30 min with an optimal pH level using BAPNA as a

substrate. Samples were incubated at 60 °C for 30 min and trypsin activity was measured at 410 nm (Khantaphant and Benjakul, 2010).

RESULTS

Table 1 depicts the characteristics of purified trypsin from the digestive system of yellowfin seabream. Trypsin was purified from the digestive system of yellowfin seabream. In the first steps, the specific and total activities were measured at 0.96 U and 20 U, respectively. In the next steps (i.e. ammonium sulfate fractionation), the purity of trypsin increased with a higher specific activity (4.4 U), moreover, protein content increased from 0.95 to 3.4 mg/ml. The purified trypsin migrated in the SDS-PAGE with the molecular mass of 23 kDa (Figure 1).

Table 1. Characteristics of purified trypsin from the digestive system of yellowfin seabream

Fish	Sample	Total volume (ml)	Protein content (mg/ml)	Trypsin activity (U)	Specific activity (U)
Yellowfin seabream	Crude	670	0.95	20	0.96
	Ammonium sulfate precipitation	32	3.4	19.2	4.4

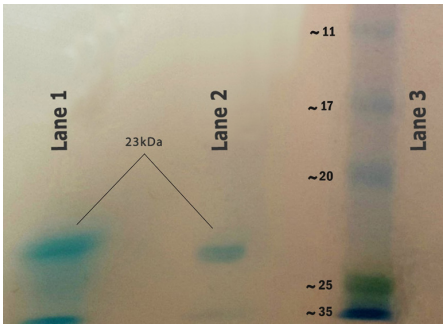

Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified trypsin from the digestive system of Yellowfin seabream (12.5% acrylamide gel).
Lane1: Commercial Trypsin, Lane 2: Dried Trypsin, Lane 3: Protein Ladder

The effect of different temperatures on purified trypsin activity was assessed using BAPNA as a substrate (figures 2 and 3). Temperature for the maximum activity of trypsin was within 50-60 °C. Thermal stability within 45-50 °C is more than 90% of its original activity; however, temperatures less than 50 °C led to a reduction in the stability.

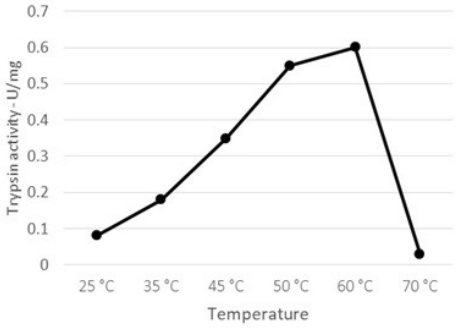

Figure 2. Purified trypsin from digestive system of yellowfin seabream, optimum temperature.

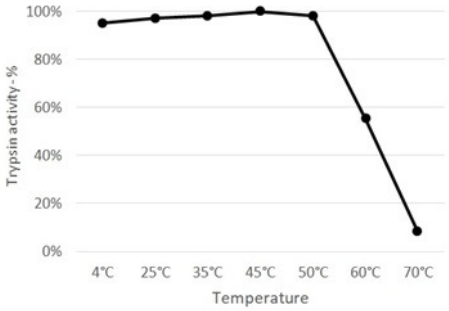

Figure 3. Purified trypsin from the digestive system of Yellowfin seabream, thermal stability.

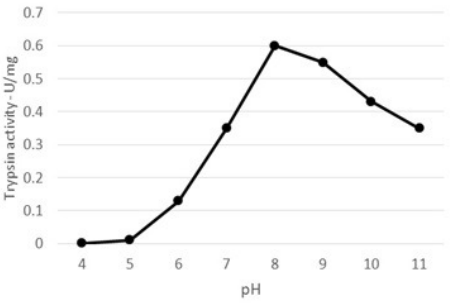

Figure 4. Purified trypsin from digestive system of Yellowfin seabream, optimum pH.

Activity and stability of purified trypsin in different pH were determined using BAPNA as a substrate at a pH range of 4.0-11.0 (figures 4 and 5). It was observed that pH levels of 4.0 and 5.0 indicated no significant

activity. The optimal pH for trypsin activity was found to be 8.0. Nonetheless, pH levels of 8.0 and 9.0 were optimal for trypsin stability with an activity of more than 90%.

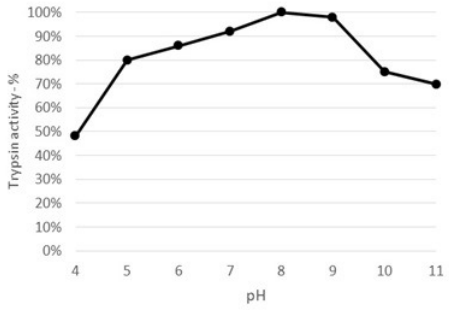

Figure 5. Purified trypsin from the digestive system of yellowfin seabream, stability at pH.

DISCUSSION

The digestive system of yellowfin seabream (*Acanthopagrus latus*) was used as a source of trypsin to assay some important characteristics of the extracted trypsin. As evidenced by the results of the current study, trypsin activity is acceptable and fractionation by ammonium sulfate precipitation increased trypsin specific activity and protein content. Ammonium sulfate precipitation is a method commonly used to eliminate other proteins from the crude purified matter (Khantaphant and Benjakul, 2010). Trypsin is considered a one-polypeptide enzyme since only a single band is indicated on the SDS-PAGE with a molecular mass of 23 kDa. In general terms, the molecular weight of trypsin from fish species was within the range of 23-28 kDa (Silva et al., 2011). However, it was measured at 23 kDa in golden grey mullet (*Liza aurata*) (Bkhairia et al., 2016). Moreover, the molecular weight of trypsin was obtained at 27 kDa in some other studies performed on catfish (*Luphiosilurus alexandri*) (Dos Santos et al., 2016) and zebra blenny (*Salaria basilisca*) (Ktari et al., 2012). In addition, the same results were reported for Oil Sardine (*Sardinella longiceps*) (Khandagale et al., 2017) and red snapper (*Lutjanus vitta*) (Khantaphant and Benjakul, 2010). Furthermore, the studies carried out on silver mojarra (*Diapterus rhombeus*) the molecular mass was measured at 26.5 kDa (Silva et al., 2011). Thermal activity and stability were similar to those reported in other studies. Optimal temperature of purified trypsin from silver mojarra (*Diapterus rhombeus*) was reported to fall within 50-55°C with a peak at 60°C (Silva et al., 2011). The trypsin highest activity in Brazilian flounder (*Paralichthys orbignyanus*) was observed at 50°C. However, the stability of the enzyme was defined within 40-55°C with 80% of its original activity (Candiotto et al., 2018). The high optimal temperature may be attributed to living in warm water. This optimal temperature was detected in zebra blenny (*Salaria basilisca*) (Ktari et al., 2012) and some other warm-water fish, such as Indian mackerel (*Rastralliger kanagurta*) (Khandagale et al., 2015) and red snapper (Lutjanus vitta) (Khantaphant and Benjakul, 2010). The differences in the thermal stability of trypsin could be ascribed to environmental and genetic features (Silva et al., 2011). Trypsin belongs to the alkaline proteinase group and is not able to properly bind to the substrate under acidic conditions (Dos Santos et al., 2016). The results of this study are comparable to other studies. Trypsin activity in silver mojarra (*D. rhombeus*) peaked at a pH of 8.5 (Silva et al., 2011) with enzyme stability in pH values within the range of 8.5-11. Trypsin extracted from Chinook salmon (*O. tshawytscha*) had the highest activity in ranges of 8.5-10.5; however, it was inactive at pH $\leq$ 4.0. Enzyme stability in pH values within the range of 7.0-9.0 was more than 70%. Generally, trypsin stability is higher in pH values ranging from 7.5-10.5 (Khantaphant and Benjakul, 2010). Same results were reported in catfish (*Luphiosilurus alexandri*) (Dos Santos et al., 2016), Flatfish (*Paralichthys olivaceus*) (Kim and Jeong, 2013), Brazilian flounder (*Paralichthys orbignyanus*) (Candiotto et al., 2018), Oil Sardine (*Sardinella longiceps*), (Khandagale et al., 2017), and red snapper (*L. vitta*) (Khantaphant and Benjakul, 2010).

As a conclusion, trypsin is one of the most valuable proteases as a bioactive compound. It was purified and characterized by the digestive system of yellowfin seabream in the present research. The purified enzyme demonstrated distinct optimum levels of alkaline pH, temperature, and molecular weight. As evidenced by the obtained results, low temperature and acidic pH can inactivate trypsin. Therefore, trypsin has considerable potential for industrial applications, such as the food industry, owing to its characteristics and stability under alkaline conditions. Furthermore, using fish waste as a source of trypsin can reduce environmental pollution regarding the disposal of such wastes

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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