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Purification and characterization of trypsin from Luphiosilurus alexandri pyloric cecum

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ABSTRACT

Trypsin from *L. alexandri* was purified using only two purification processes: ammonium sulfate precipitation and anion exchange liquid chromatography in DEAE-Sepharose. Trypsin mass was estimated as 24 kDa through SDS-PAGE, which showed only one band in silver staining. The purified enzyme showed an optimum temperature and pH of 50 °C and 9.0, respectively. Stability was well maintained, with high levels of activity at a pH of up to 11.0, including high stability at a temperature of up to 50 °C after 60 min of incubation. The inhibition test demonstrated strong inhibition by PMSF, a serine protease inhibitor, and Kinetic constants km and kcat for BAPNA were 0.517 mM and 5.0 S^{-1}, respectively. The purified enzyme was also as active as casein, as analyzed by zymography. Therefore, we consider trypsin a promising enzyme for industrial processes, owing to its stability in a wide range of pH and temperature and activity even under immobilization.

1. Introduction

Studies by the Food and Agriculture Organization of the United Nations (FAO) indicate that by 2030, the world population will increase from 7 billion to 8.3 billion [28], with a greater population density in Asian, African, and South American countries. Based on these estimates, it is clear that large-scale adaptations are necessary, as the supply to such a large population will represent an enormous challenge to each country. Facing this reality, commercialization of fish is an activity that has been increasing at a faster rate in the food production chain, particularly those derived from aquaculture and represented by species grown in captivity. For example, Brazil is a strong candidate to become the major producer and exporter in this category because of its wide diversity and proper geographic and climate conditions (12% of the fresh water available on the planet) [28]. In addition to the economic benefits assured to aquaculture producers, it is necessary to emphasize the possibility of disseminating native species with real extinction risks [26]. One of the major obstacles faced by breeders is related to production cost, particularly food costs that, depending on the species, can reach 70% of total expenses. To counterbalance the excessive costs of production, breeders should dispose all of the animal mass potential and not just the commercialization of muscle fiber, because approximately 30% of the assessed weight corresponds to innards, primarily categorized as subproducts [10]. These residues have significant biotechnological potential by providing, for example, a source of enzymes that can be applied in several industries.

In the past few years, proteases from different sources have been increasingly analyzed and used, with approximately 50% of total industrial sales of enzymes consisting of proteases. In order to offer a greater variety of proteases, particularly those with unique properties, new sources of proteolytic enzymes have been explored, including proteases derived from fish, particularly from fish innards [30]. Fish proteases have shown higher catalytic activities over a wide range of pH and temperature conditions [27]. Proteases have been applied in industries for several uses such as detergents, food, pharmaceutical products, leather, and silk industry [19]. Trypsin is a serine protease responsible for the hydrolysis of peptide bonds in the carboxyl terminal ends of lysine (K) and arginine (R) residues. Normally, trypsin isolated from either fresh or salt water fish shows satisfactory levels of reactivity in extreme conditions, such as high alkalinity and temperature as well as in the presence of surfactant agents. Consequently, studies have been conducted on the purification and characterization of these molecules from biological sources in order to identify low-cost methodologies with high level of recuperation: spotted goatfish (*Pseudupeneus maculatus*) [19], brown stripe red snapper (*Lutjanus vitta*) [13], hybrid catfish (*Clarias microcephalus* and

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Clarias gariepinus) [16], sardines (*Sardinella aurita*) [10], silver mojarra (*Diapterus rhombeus*) [29], and zebra blenny (*Salaria basilisca*) [20].

The present investigation is a pioneering study of an isolated enzyme from *Lophiosilurus alexandri*, a native fish from São Francisco River (Brazil). *L. alexandri* is a carnivorous catfish belonging to the *Pseudopimelodidae* family of the order Siluriformes, which includes fish such as Surubim (*Pseudoplatystoma fasciatum*) and Pintado (*P. corruscans*) [26]. It has a sedentary behavior, with preference for lentic environments and sandy or rocky bottoms. It is highly valued for its organoleptic properties, such as tasty meat and absence of spines in the inside of muscles, due to which the fish has a high aggregate value in the international cuisine [25]. Considering this scenario, the objective of this investigation was to purify and characterize trypsin from the pyloric cecum of *L. alexandri*.

2. Material and methods

2.1. Obtaining pyloric cecum

The fish were obtained from the Company for Development of São Francisco and Parnaíba Valleys (CODEVASF), which is specialized in species nutrition and reproduction. All the experiments received previous approval of the Ethics Committee on Animal Experimentation of the Federal University of Alagoas, number 23/2013. After slaughtering the fish, the innards were removed using forceps, scissors, and scalpels, and the pyloric ceca were isolated at 0 °C.

2.2. Crude extract preparation

Five pyloric ceca were collected and transferred to a beaker containing 8.0 mL of extraction buffer (50 mM Tris-HCl, pH 8.0). Using scissors, the tissues were grinded in small portions to facilitate maceration through a tissue homogenizer (HOMOMIX, Ser. No.: 175315) in high rotation at 0 °C cooling. Subsequently, the homogenate was transferred to eppendorf tubes and centrifuged at 14,000 rpm for 40 s at 4 °C (Centrifuge HT, model MCD-2000). The resulting supernatant was defined as crude extract.

2.3. Trypsin purification

The crude extract was subjected to saline precipitation (0–20, 20–40, 40–60, 60–80, and 80–100% of ammonium sulfate), and all the obtained fractions were centrifuged (HERMLE- Z236K) at 15,000*g* for 10 min at 4 °C. The precipitates were solubilized and stored using the same extraction buffer at 4 °C. The fraction with the greatest activity was subjected to dialysis process with the extraction buffer for 24 h at 4 °C (Dialysis bags, Sigma D9777-100FT, 12.4 kDa, St. Louis, MO, USA).

After the dialysis process, the sample was subjected to liquid chromatography using DEAE-Sepharose column (30 mL) attached to FPLC Akta M1 previously equilibrated with 50 mM Tris-HCl buffer pH 8.0. After the washing process, the adsorbed proteins were eluted by applying a saline gradient with 50 mM Tris-HCl buffer pH 8.0 + 0.5 M NaCl at a flow rate of 0.5 mL/min.

2.4. Enzyme assay

All the assays used 500 μM of BAPNA (Nα-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride) substrate concentration and reaction was stopped by adding 500 μL of 20% (v/v) citric acid. An absorbance at 410 nm was used to measure the activity of enzyme. BAPNA hydrolysis units (U) were calculated with the following equation: $U = (A410/min) \times 1000 \times (1/8800 \times mg\ enzyme)$, where 8800 $M^{-1}\ cm^{-1}$ is the p-nitroanilide molar extinction coefficient at 410 nm.

2.5. Electrophoresis (SDS-PAGE), native electrophoresis, and zymogram

The approximate mass of the enzyme was estimated using PAGE in the presence of sodium dodecyl sulfate (SDS-PAGE) on a 12% (w/v) gel using the Mini-Protean 3 Electrophoresis System (Bio-Rad), with a molecular weight marker [Amersham™ Full-Range Rainbow™ (12,000–225,000 Da)]. The gels were stained with silver nitrate. The same protocol was followed for native electrophoresis, except that the sample was not heated and SDS and reducing agent were left out. The zymogram had the gel polymerized with the addition of 0.2% casein, which was washed with 2.5% Triton X-100 for 30 min under stirring. Subsequently, 100 mL of extraction buffer was added and kept for 48 h at 25 °C and stained by Coomassie Brilliant Blue.

2.6. Protein quantification

Protein concentration was assessed according to Bradford method [4] using bovine serum albumin (BSA) as standard (5, 10, 20, 40, and 80 μg BSA/mL).

2.7. Effect of pH on activity and stability

For assessment of the optimum pH, we performed the above mentioned enzyme assay but with different buffers (pH 4.0–13.0) at a concentration of 100 mM for 30 min at 50 °C. For the stability test, the enzyme was incubated for 60 min at 50 °C at different pH values. Finally, the reaction was stopped as described in the enzyme assay method. The highest detected activity was attributed as 100% activity and used to determine the relative activity at different pH.

2.8. Effect of temperature on activity and stability

The above mentioned enzyme assay was performed at different temperatures (20–70 °C) for assessment of the optimum temperature. For the thermal stability test, the enzyme was pre-incubated for 60 min at different temperatures (20–70 °C). Subsequently, the reaction was continued as described for the enzyme assay. The highest detected activity was attributed as 100% activity and used to determine the relative activity at different temperature.

2.9. Inhibition test

The purified enzyme was preincubated with inhibitors (PMSF, EDTA and 2-mercaptoethanol) and for 10 min at 25 °C, and then the remaining enzyme activity was estimated using BAPNA (37 °C for 7 h) as a substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as 100%.

2.10. Kinetic studies

The activity of the purified enzyme was assessed for 90 min at optimum temperature and pH at different concentrations of BAPNA (0–6.0 mM). The determinations were repeated twice and the respective kinetic parameters, including Km and Vmax, were calculated from Lineweaver–Burk plots. The value of the turnover number (kcat) was calculated from the following equation: $kcat = Vmax/[E]$, where [E] is the active enzyme concentration and Vmax is the maximal velocity.