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Characteristics of trypsins from the viscera of true sardine (*Sardinops melanostictus*) and the pyloric ceca of arabesque greenling (*Pleuroprammus azonus*)

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Abstract

Trypsins, TR-S and TR-P, from the viscera of true sardine (*Sardinops melanostictus*) and from the pyloric ceca of arabesque greenling (*Pleuroprammus azonus*), respectively, were purified by gel filtration and anion-exchange chromatography. Final enzyme preparations were nearly homogeneous in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the molecular weights of both enzymes were estimated to be 24,000 Da by SDS–PAGE. The N-terminal amino acid sequences of the TR-S, IVGGYECKAYSQPWQVSLNS, and TR-P, IVGGYECTPHTQAHQVSLNS, were found. The TR-S and TR-P had maximal activities at around pH 8.0 for hydrolysis of N^{α}-*p*-tosyl-L-arginine methyl ester. Optimum temperature of the TR-S and TR-P were 60 and 50 °C, respectively. The TR-S and TR-P were unstable at above 50 and 30 °C, respectively, and below pH 5.0. Both TR-S and TR-P were stabilized by calcium ion.

Keywords: Japanese sardine; *Sardinops melanostictus*; Arabesque greenling; *Pleuroprammus azonus*; N-terminal amino acid sequence; Viscera; Pyloric cecum; Trypsin

1. Introduction

The development of the fisheries industry will depend on effective utilization of the available raw materials. Fish viscera are non-edible parts produced in large quantities by the fisheries industry and represent both waste disposal and potential pollution. These materials, however, are rich potential sources of various enzymes that may have some unique properties of interest for both basic research and industrial applications (Simpson & Haard, 1999).

Fishes are poikilothermic, so their survival in cold waters required adaptation of their enzyme activities to the low temperatures of their habitats. Enzymes from cold-adapted fish species thus often have higher enzymatic activities at low temperatures than their counterparts from warm-blooded animals (Asgeirsson, Fox, & Bjarnason, 1989; Kristjansson, 1991). The high activity of fish enzymes at low temperatures may be interesting for several industrial applications of enzymes, such as in certain food processing operations that require low ambient temperatures. Furthermore, relatively lower thermal stability, often observed with fish enzymes, may also be beneficial in such applications, as the enzymes can be inactivated more readily, with less heat treatment, when desired in a given process (Simpson & Haard, 1987).

True sardine (*Sardinops melanostictus*) and arabesque greenling (*Pleuroprammus azonus*) are both important in the fish-catches of Japan and are used almost exclusively for food production. Especially, true sardine autolyzes

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very quickly post-mortem due to enzymes leaking from the digestive organs. Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, and Toro (2005) reported on the isolation and characterization of trypsin from the viscera of Monterey sardine (*Sardinops sagax caerulea*), also susceptible to abdominal autolytic degradation after death. In this study, we purified trypsins from the viscera of true sardine (*S. melanostictus*) and the pyloric ceca of arabesque greenling (*P. azonus*) and compared their characteristics to those of porcine pancreatic trypsin.

2. Materials and methods

2.1. Materials

The true sardine (*S. melanostictus*) and arabesque greenling (*P. azonus*) were caught off Hakodate, Hokkaido Prefecture, Japan. Sephacryl S-200 and Sephadex G-50 were purchased from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was purchased from Whatman (Maidstone, England). N^{α}-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME) was purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Preparation of crude enzyme

Defatted powders of the viscera of true sardine and the pyloric ceca of arabesque greenling were prepared by the same method as that of Kishimura and Hayashi (2002). Trypsin was extracted from the defatted powder by stirring in 50 volumes of 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ at 5 °C for 3 h. The extract was centrifuged (H-200, Kokusan, Tokyo, Japan) at 10,000*g* for 10 min, and then the supernatant was lyophilized and used as crude trypsin.

2.3. Purification of trypsin

The crude trypsin of true sardine was applied to a column of Sephacryl S-200 (3.9 × 64 cm) pre-equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and the proteins were eluted with the same buffer. The main trypsin fraction was concentrated by lyophilization and was dialyzed against 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$. The dialyzate was applied to a DEAE-cellulose column (2.2 × 18 cm) pre-equilibrated with 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and the proteins were eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. Trypsin was eluted mainly with 0.35–0.4 M NaCl. The trypsin fraction was dialyzed against 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and the dialyzate was concentrated by lyophilization. The concentrated fraction was then applied to a Sephadex G-50 column (3.9 × 64 cm) pre-equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and the proteins were eluted with the same buffer. Trypsin was eluted as a single peak on the gel filtrations and showed a nearly single band on SDS–PAGE (Fig. 1). The final preparation (TR-S) was purified 117-fold from the crude trypsin (Table 1).

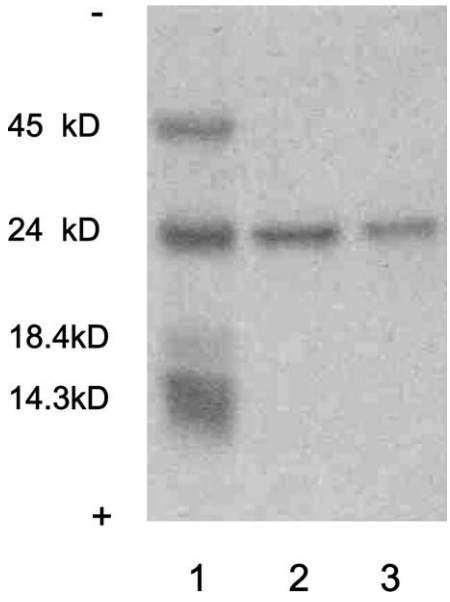

Fig. 1. Electrophoresis of purified trypsins from true sardine (*S. melanostictus*) and arabesque greenling (*P. azonus*). Electrophoresis was performed using a 0.1% SDS–12.5% polyacrylamide slab-gel. Lane 1 contains protein standards: bovine pancreatic trypsinogen (molecular weight, 24,000 Da), bovine milk β-lactoglobulin (18,400 Da), and egg-white lysozyme (14,300 Da). Lane 2 contains trypsin from true sardine (TR-S). Lane 3 contains trypsin from arabesque greenling (TR-P).

The crude trypsin of arabesque greenling was dialyzed against 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$. The dialyzate was applied to a DEAE-cellulose column (2.2 × 18 cm) pre-equilibrated with 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and the proteins were eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. Trypsin was eluted mainly with 0.3–0.4 M NaCl. The trypsin fraction was dialyzed against 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and the dialyzate was concentrated by lyophilization. The concentrated fraction was then applied to a Sephadex G-50 column (3.9 × 64 cm) pre-equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and the proteins were eluted with the same buffer. Trypsin was eluted as single peak on the gel filtrations and showed a nearly single band on SDS–PAGE (Fig. 1). The final preparation (TR-P) was purified 20-fold from the crude trypsin (Table 1).

2.4. Assay for trypsin activity

Trypsin activity was measured by the method of Hummel (1959) using TAME as a substrate. One unit

Table 1
Purification of trypsins from true sardine (S. *melanostictus*) and arabesque greenling (*P. azonus*)

Purification stages	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Relative purity (fold)	Yield (%)
Trude sardine					
Crude enzyme	7240	1014	0.14	1	100
Sephacryl S-200	500	600	1.2	9	59
DEAE-cellulose	47	442	9.4	67	44
Sephadex G-50	18	295	16.4	117	29
Arabesque greenling					
Crude enzyme	6880	2614	0.38	1	100
DEAE-cellulose	382	840	2.2	6	32
Sephadex G-50	22	167	7.6	20	6

of enzyme activity was defined as the amount of the enzyme hydrolyzing 1 mmol of TAME in 1 min. The pH dependencies of the enzyme were determined in 50 mM buffer solutions [acetic acid–sodium acetate (pH 4.0–7.0), Tris–HCl (pH 7.0–9.0), and glycine–NaOH (pH 9.0–11.0)] at 30 °C. The temperature-dependencies of the enzyme were determined at pH 8.0 and at various temperatures. The effects of temperature and pH on the stability of the enzyme were found by incubating the enzyme at pH 8.0 for 15 min over a range of 20–70 °C and by incubating the enzyme at 30 °C for 30 min over a pH range of 4.0–11.0. The effect of $CaCl_2$ on the activity of the enzyme was found by incubating the enzyme at 30 °C and at pH 8.0 in the presence of 10 mM ethylenediaminetetraacetic acid (EDTA) or 10 mM $CaCl_2$.

2.5. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using a 0.1% SDS–12.5% polyacrylamide slab-gel by the method of Laemmli (1970). The gel was stained with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol-7% acetic acid and the background of the gel was destained with 7% acetic acid.

2.6. Analysis of amino acid sequence

To analyze the N-terminal sequence, the purified enzyme was electroblotted to polyvinylidenedifluoride (PVDF) membrane (Mini ProBlott Membranes, Applied Biosystems, CA, USA) after SDS–PAGE. The amino acid sequence of the enzyme was analyzed by using a protein sequencer, Procise 492 (Perkin–Elmer, Foster City, CA, USA).

2.7. Protein determination

The protein concentration was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard.

3. Results and discussion

In this study, anionic trypsins (TR-S and TR-P) were purified from the viscera of true sardine and the pyloric ceca of arabesque greenling, respectively, by gel filtration and anion-exchange chromatography.

The molecular weights of both TR-S and TR-P were estimated as approximately 24,000 Da using SDS–PAGE (Fig. 1) and were similar to those of mammalian pancreatic trypsin (24,000 Da), Greenland cod trypsin (23,500 Da) (Simpson & Haard, 1984) and Atlantic cod trypsin (24,200 Da) (Asgeirsson et al., 1989), which were smaller than trypsins of capelin (28,000 Da) (Hjelmeland & Raa, 1982), catfish (26,000 Da) (Yoshinaka, Suzuki, Sato, & Ikeda, 1983), anchovy (27,000–28,000 Da) (Martinez, Olsen, & Serra, 1988), rainbow trout (25,700 Da) (Kristjansson, 1991), and Monterey sardine (25,000 Da) (Castillo-Yanez et al., 2005). The N-terminal amino acid sequences of the TR-S and TR-P were analyzed and 20 amino acids, IVGGYECKAYSQPWQVSLNS (TR-S) and IVGGYECTPHTQAHQVSLDS (TR-P), were found. Results indicated that the N-termini of the TR-S and TR-P were unblocked. The N-terminal amino acid sequences of the TR-S and TR-P were aligned with the sequences of other animal trypsins (Fig. 2). Being similar to other fish trypsins, the TR-S and TR-P, had a charged Glu residue at position 6, where Thr is most common in mammalian pancreatic trypsins (Fig. 2).

Fig. 3a shows the pH-dependencies of the TR-S and TR-P. Both enzymes hydrolyzed the TAME effectively at alkaline pH with an optimum activity at about pH 8.0, similar to those of porcine pancreatic trypsin (Fig. 3(b)) and other fish trypsins (Asgeirsson et al., 1989; Castillo-Yanez et al., 2005; Hjelmeland & Raa, 1982; Martinez et al., 1988; Simpson & Haard, 1984; Yoshinaka, Sato, Suzuki, & Ikeda, 1984). Fig. 4(a) shows the temperature-dependencies of the TR-S and TR-P. Optimum temperatures of the TR-S and TR-P were 60 and 50 °C, respectively, which were lower than that of porcine pancreatic trypsin (60–70 °C, Fig. 4(b)). The TR-S examined in this study had an optimum temperature of 60 °C similar to those of other fish trypsins

	1 10 20
True sardine	IVGGYECKAYSQPWQVSLNS
Arabesque greenling	IVGGYECTPHTQAHQVSLDS
Antarctic Fish	IVGGKECSPYSQPHQVSLNS
Cod	IVGGYECTKHSQAHQVSLNS
Salmon	IVGGYECKAYSQTHQVSLNS
Flounder Fish	IVGGYECTPYSQPHQVSLNS
Porcine	IVGGYTCAANSVPYQVSLNS
Bovine	IVGGYTCGANTVPYQVSLNS
Human	IVGGYNCEENSVPYQVSLNS

Fig. 2. Comparison of the N-terminal amino acid sequences of trypsins from true sardine (*S. melanostictus*) and arabesque greenling (*P. azonus*) with those of other vertebrates: Antarctic fish (Genicot et al., 1996); Cod (Gudmundsdottir et al., 1993); Salmon (Male et al., 1995); Flounder fish (GenBank accession number AB029750); Porcine (Hermodson et al., 1973); Bovine (Walsch, 1970); Human (Emi et al., 1986).

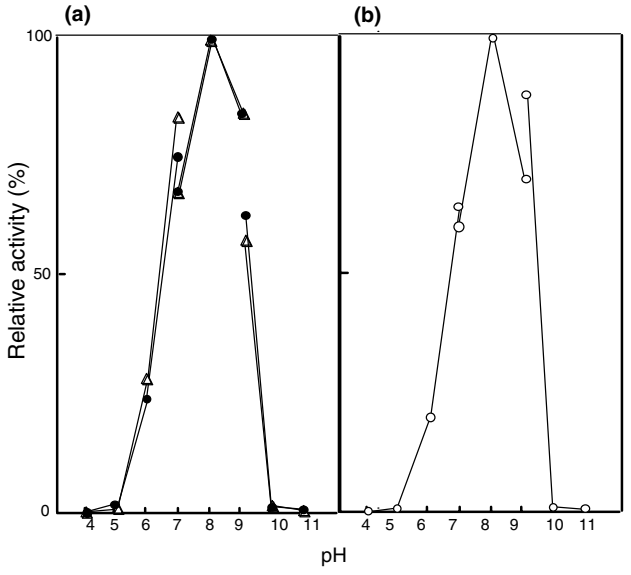

Fig. 3. Effects of pH on the activity of trypsins from true sardine (*S. melanostictus*) and arabesque greenling (*P. azonus*). The activities were determined in 50 mM buffer solutions [acetic acid–sodium acetate (pH 4.0–7.0), Tris–HCl (pH 7.0–9.0), and glycine–NaOH (pH 9.0–11.0)] at 37 °C: (a) trypsin from true sardine (TR-S) (closed circles), trypsin from arabesque greenling (TR-P) (open triangles); (b) porcine pancreatic trypsin.

(Asgeirsson et al., 1989; Kristjansson, 1991). Fig. 5(a) shows the pH stabilities of the TR-S and TR-P. Both enzymes were stable between pH 6.0 and 11.0, but they were unstable below pH 5.0. These properties of the pH stability of the TR-S and TR-P were similar to those of other fish trypsins (Asgeirsson et al., 1989; Kristjansson, 1991; Martinez et al., 1988), but not similar to those of porcine pancreatic trypsin (Fig. 5(b)). Fig. 6(a) shows the temperature stabilities of the TR-S and TR-P. The TR-S was stable below 40 °C, but its activity quickly fell above 50 °C. The temperature stability of the TR-S was

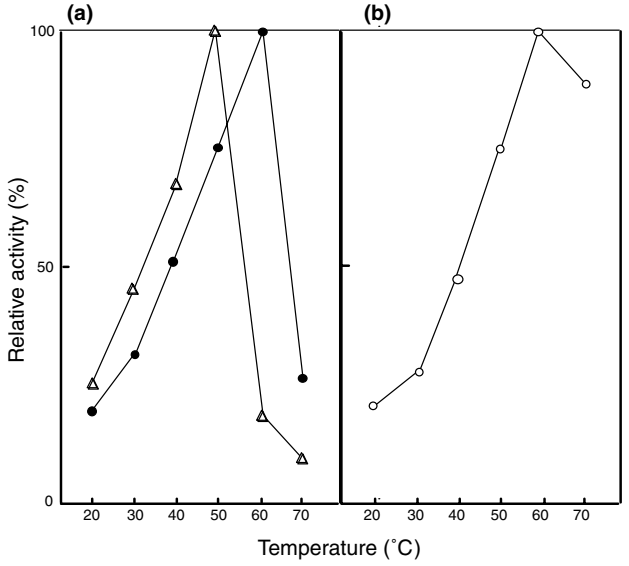

Fig. 4. Effects of temperature on the activities of trypsins from true sardine (*S. melanostictus*) and arabesque greenling (*P. azonus*). The activities were determined at pH 8.0 and at various temperatures: (a) trypsin from true sardine (TR-S) (closed circles), trypsin from arabesque greenling (TR-P) (open triangles); (b) porcine pancreatic trypsin.

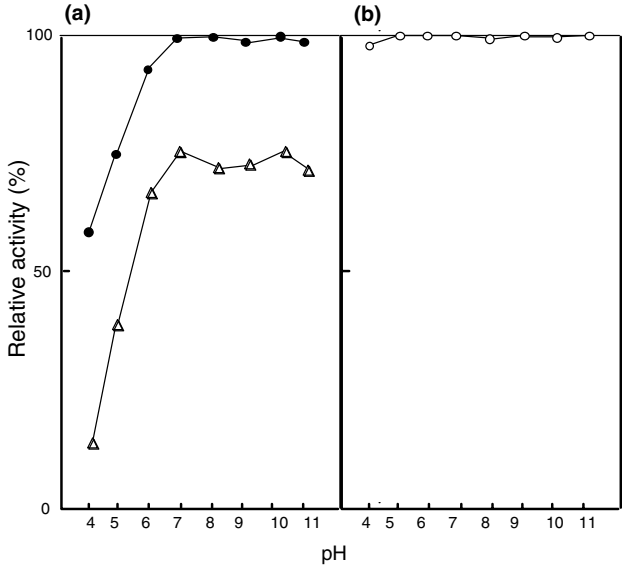

Fig. 5. PH stability of trypsins from true sardine (*S. melanostictus*) and arabesque greenling (*P. azonus*). The enzymes were kept at 30 °C for 30 min and pH 4.0–11.0, and then the remaining activities at 30 °C and pH 8.0 were determined: (a) trypsin from true sardine (TR-S) (closed circles), trypsin from arabesque greenling (TR-P) (open triangles); (b) porcine pancreatic trypsin.

similar to that of Monterey sardine trypsin (Castillo-Yanez et al., 2005). However, the TR-P was stable below 20 °C, but its activity quickly fell above 30 °C. The TR-P was more unstable than the TR-S, other fish trypsins (Kristjansson, 1991; Martinez et al., 1988), and porcine pancreatic trypsin (Fig. 6(b)).

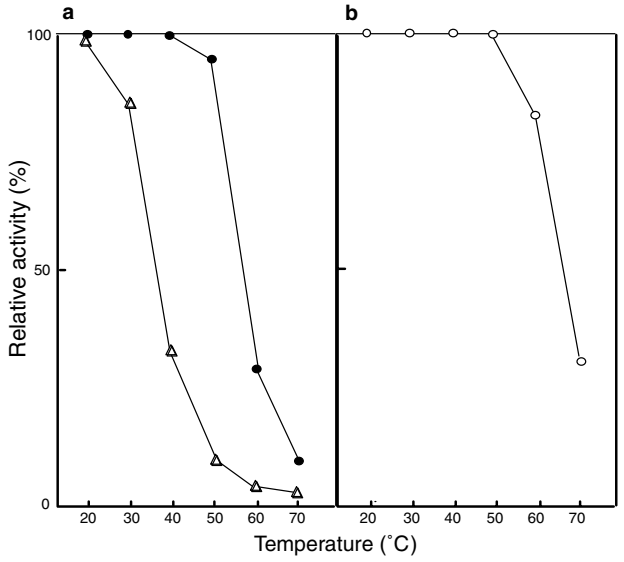

Fig. 6. Thermostability of trypsins from true sardine (*S. melanostictus*) and arabesque greenling (*P. azonus*). The enzymes were kept at 20–70 °C for 15 min and pH 8.0, and then the remaining activities at 30 °C and pH 8.0 were determined: (a) trypsin from true sardine (TR-S) (closed circles), trypsin from arabesque greenling (TR-P) (open triangles); (b) porcine pancreatic trypsin.

The effects of $CaCl_2$ on the TR-S and TR-P were investigated in the presence of 10 mM EDTA or 10 mM $CaCl_2$. Both enzymes were stabilized by calcium ion (Fig. 7(a)) similarly to porcine pancreatic trypsin (Fig. 7(b)). Two calcium binding sites are present in bovine trypsinogen (Kossiakoff, Chambers, Kay, & Stroud, 1977). The primary site, with a higher affinity for calcium ions, is common in trypsinogen and trypsin, and the secondary site is only in the zymogen. Occupancy of the primary calcium-binding site stabilizes the protein toward thermal denaturation or autolysis. The TR-S and TR-P were both stabilized by calcium ion from thermal denaturation. These findings suggest that the TR-S and TR-P possess a primary calcium-binding site like mammalian pancreatic trypsin and other fish trypsins (Genicot, Rentier-Delrue, Edwards, Vanbeeumen, & Gerday, 1996; Male, Lorens, Smalas, & Torrissen, 1995).

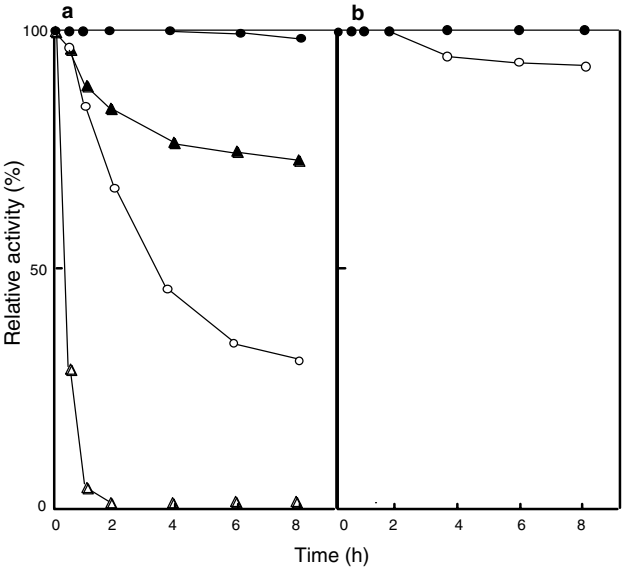

Fig. 7. Effect of calcium ion on the stability of trypsins from true sardine (*S. melanostictus*) and arabesque greenling (*P. azonus*). The enzymes were kept at 30 °C and pH 8.0 for 0–8 h in the presence of 10 mM $CaCl_2$ (closed symbols) or 10 mM EDTA (open symbols), and then the remaining activities at 30 °C and pH 8.0 were determined: (a) trypsin from true sardine (TR-S) (circles), trypsin from arabesque greenling (TR-P) (triangles); (b) porcine pancreatic trypsin.

In conclusion, the TR-S showed almost same characteristics as those of porcine pancreatic trypsin, except for being unstable below pH 5.0. These results suggest that the viscera of true sardine (*S. melanostictus*) would be a potential source of trypsin for food processing operations. On the other hand, the TR-P showed a lower optimum temperature than that of porcine pancreatic trypsin and was more unstable than porcine pancreatic trypsin below pH 5.0 and above 30 °C. These results suggest that the pyloric ceca of arabesque greenling (*P. azonus*) would be a potential source of trypsin for certain food processing operations that require low processing temperatures, and the relatively lower thermal stability of the TR-P may also be beneficial in such applications as the enzymes can ultimately be inactivated more readily.

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