Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 106 (2008) 194–199
Food Chemistry
www.elsevier.com/locate/foodchem

Characteristics of trypsin from the pyloric ceca of walleye pollock (*Theragra chalcogramma*)

Hideki Kishimura [a,*], Sappasith Klomklao [b], Soottawat Benjakul [c], Byung-Soo Chun [d]

[a] *Research Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan*
[b] *Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phattalung Campus, Phattalung 93110, Thailand*
[c] *Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*
[d] *Faculty of Food Science and Biotechnology, Pukyong National University, Busan 608-737, South Korea*

Received 19 February 2007; received in revised form 26 March 2007; accepted 28 May 2007

Abstract

Trypsin was purified from the pyloric ceca of walleye pollock (*Theragra chalcogramma*) by gel filtration on Sephacryl S-200 and Sephadex G-50. The final enzyme preparation was nearly homogeneous in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the molecular mass of the enzyme was estimated to be 24 kDa by SDS–PAGE. Trypsin activity was effectively inhibited by serine protease inhibitors, such as soybean trypsin inhibitor and TLCK. Trypsin had maximal activities at around pH 8.0 and 50 °C for the hydrolysis of N^{α}-*p*-tosyl-L-arginine methyl ester hydrochloride. Trypsin was unstable above 30 °C and below pH 5.0, and was stabilized by calcium ions. Walleye pollock trypsin was more thermally unstable than trypsin from the Temperate Zone fish and Tropical Zone fish. The N-terminal amino acid sequence of the trypsin, IVGGYECTKHSQAHQVSLNS, was found, and the sequential identity between the walleye pollock trypsin and Frigid Zone fish trypsin was higher (85–100%) than with Temperate Zone fish trypsin (75–90%), Tropical Zone fish trypsin (75–85%), or mammalian trypsin (60–65%).

Keywords: Walleye pollock; *Theragra chalcogramma*; Pyloric cecum; Trypsin; N-terminal amino acid sequence; Thermostability; Frigid zone fish

1. Introduction

The development of the fisheries industry will depend on the effective utilization of the available raw materials. Fish viscera are non-edible parts produced in large quantities by the fisheries industry and represent a significant source of waste or 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).

Fish 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). 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 processing 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).

Walleye pollock (*Theragra chalcogramma*) is one of the important fish-catches of Japan and its muscle is used almost entirely for "surimi" production. However, post-mortem walleye pollock autolyzes very quickly due to

* Corresponding author. Tel./fax: +81 138 40 5519.
E-mail address: kishi@fish.hokudai.ac.jp (H. Kishimura).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.05.056

enzymes leaking from the digestive organs and its viscera are largely underutilized and are discarded as waste. It has been reported that Monterey sardine (*Sardinops sagax caerulea*) (Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, & Toro, 2005), true sardine (*Sardinops melanostictus*) (Kishimura, Hayashi, Miyashita, & Nonami, 2006), and spotted mackerel (*Scomber australasicus*) (Kishimura, Tokuda, Klomklao, Benjakul, & Ando, 2006a) are also susceptible to abdominal autolytic degradation after death. In addition, trypsin was isolated from its viscera and characterized. Trypsin (EC 3.4.21.4), which is detected in the pyloric ceca and intestine of fish, is one of the main digestive proteases. It is a member of a large family of serine proteases and cleaves the peptide bond on the carboxyl side of arginine and lysine (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007). In this study, we purified trypsin from the pyloric ceca of walleye pollock and compared its characteristics with those of other fish and porcine pancreatic trypsins.

2. Materials and methods

2.1. Materials

The walleye pollock (*T. chalcogramma*) was caught off Hakodate, Hokkaido Prefecture, Japan. Sephacryl S-200 and Sephadex G-50 were purchased from Pharmacia Biotech (Uppsala, Sweden). N^{α}-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) and porcine pancreatic trypsin were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Preparation of the crude enzyme

Defatted powder of the pyloric ceca of walleye pollock was prepared by the same method of Kishimura and Hayashi (2002). Trypsin was extracted by stirring the defatted powder 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-2000B, Kokusan, Tokyo, Japan) at 10,000×*g* for 10 min, and then the supernatant was lyophilized and used as crude trypsin.

2.3. Trypsin purification

The crude trypsin of walleye pollock 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 at a flow rate of 0.5 ml/min at 4 °C. The main trypsin fractions were concentrated by lyophilization and then dissolved in distilled water prior to size exclusion chromatography. The sample was chromatographed on a Sephadex G-50 column (3.9 × 64 cm) previously equilibrated with 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and the proteins were eluted with the same buffer at a flow rate of 0.5 ml/min. The fractions with TAME activity were pooled and used for further studies.

2.4. Assay for trypsin activity

Trypsin activity was measured by the method of Hummel (1959) using TAME as a substrate. One unit of enzyme activity was defined as the amount of the enzyme hydrolyzing one micromole of TAME in a minute. 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 in 50 mM Tris–HCl buffer 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 in 50 mM Tris–HCl buffer pH 8.0 for 15 min at a range of 20–70 °C and by incubating the enzyme at 30 °C for 30 min at a range of pH 4.0–11.0 (50 mM buffer solutions), respectively. The effect of $CaCl_2$ on the activity of the enzyme was found by incubating the enzyme at 30 °C and in 50 mM Tris–HCl buffer pH 8.0 with 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 onto 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, trypsin was purified from the pyloric ceca of walleye pollock by gel filtration on Sephacryl S-200 and Sephadex G-50, and the purity was increased to 83-fold with approximately 28% yield (Table 1). The molecular weight of the trypsin was estimated to be approximately 24 kDa using SDS–PAGE (Fig. 1) similar to those of mammalian pancreatic, Atlantic cod (Gudmundsdottir et al., 1993), true sardine (Kishimura, Hayashi, et al., 2006),

Table 1
Purification of trypsin from walleye pollock

Purification stages	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purity (fold)	Yield (%)
Crude enzyme	8220	4932	0.6	1	100
Sephacryl S-200	219	2321	10.6	18	47
Sephadex G-50	28	1400	50.0	83	28

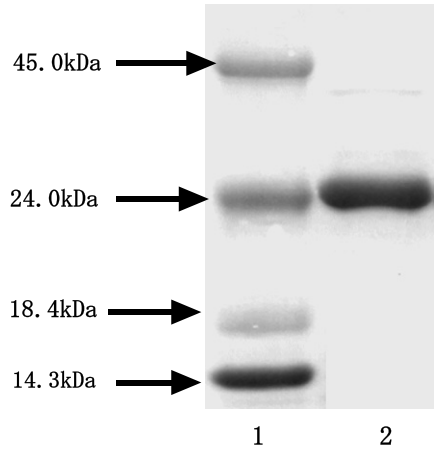

Fig. 1. Electrophoresis of purified trypsin from walleye pollock. Electrophoresis was performed using a 0.1% SDS–12.5% polyacrylamide slab-gel. Lane 1 contains protein standards; bovine pancreatic trypsinogen (molecular weight, 24 kDa), bovine milk β-lactoglobulin (18.4 kDa), and egg-white lysozyme (14.3 kDa). Lane 2 contains walleye pollock trypsin.

arabesque greenling (Kishimura, Hayashi, et al., 2006), jacopever (Kishimura et al., 2007), and elkhorn sculpin (Kishimura et al., 2007) trypsins, which were smaller than trypsins of capelin (28 kDa) (Hjelmeland & Raa, 1982), catfish (26 kDa) (Yoshinaka, Suzuki, Sato, & Ikeda, 1983), anchovy (27–28 kDa) (Martinez, Olsen, & Serra, 1988), rainbow trout (25.7 kDa) (Kristjansson, 1991), and Monterey sardine (25 kDa) (Castillo-Yanez et al., 2005). The effects of various proteinase inhibitors on the activity of the walleye pollock trypsin were determined (Table 2). The trypsin was strongly inhibited by a serine proteinase inhibitor, such as soybean trypsin inhibitor and TLCK (82–91%). While, specific inhibitors of cysteine proteinase (E-64, *N*-ethylmaleimide, iodoacetic acid), chymotrypsin (TPCK), aspartic proteinase (pepstatin A), and metallo proteinase (EDTA) had almost no inhibitory effect on the activities of walleye pollock trypsin. These results for molecular weight and effects of inhibitors, confirmed that the purified enzyme was serine proteinase, most likely trypsin.

Table 2
Effects of various inhibitors on the activity of trypsin from walleye pollock[a]

Inhibitors	Concentration	% Inhibition
Control		0
E-64	0.1 mM	0
N-Ethylmaleimide	1 mM	0
Iodoacetic acid	1 mM	0
Soybean trypsin inhibitor	1 mg/ml	91
TLCK	5 mM	82
TPCK	5 mM	11
Pepstatin A	0.01 mM	3
EDTA	2 mM	9

[a] The enzyme solution was incubated with the same volume of inhibitor at 25 °C for 15 min and residual activity was analyzed using TAME as a substrate for 20 min at pH 8.0 and 30 °C.

The effect of $CaCl_2$ on the stability of the walleye pollock trypsin was determined in the presence of 10 mM EDTA or 10 mM $CaCl_2$. The trypsin was stabilized by calcium ion (Fig. 2a) similar to porcine pancreatic trypsin (Fig. 2b). Two calcium binding sites are 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 occurs only in the zymogen. Occupancy of the primary calcium binding site, stabilizes the protein toward thermal denaturation or autolysis. The walleye pollock trypsin was stabilized by calcium ion from thermal denaturation. This finding suggests that the walleye pollock trypsin, possesses the primary calcium binding site like mammalian pancreatic trypsin and other fish trypsins (Genicot, Rentier-Delrue, Edwards, Vanbeeumen, & Gerday, 1996; Kishimura, Hayashi, et al., 2006; Kishimura et al., 2006a; Kishimura, Tokuda, Klomklao, Benjakul, & Ando, 2006b; Kishimura et al., 2007; Male, Lorens, Smalas, & Torrissen, 1995).

Fig. 3a shows the pH dependence of the walleye pollock trypsin. The enzyme hydrolyzed the TAME effectively at alkaline pH with an optimum activity at pH 8.0 similar

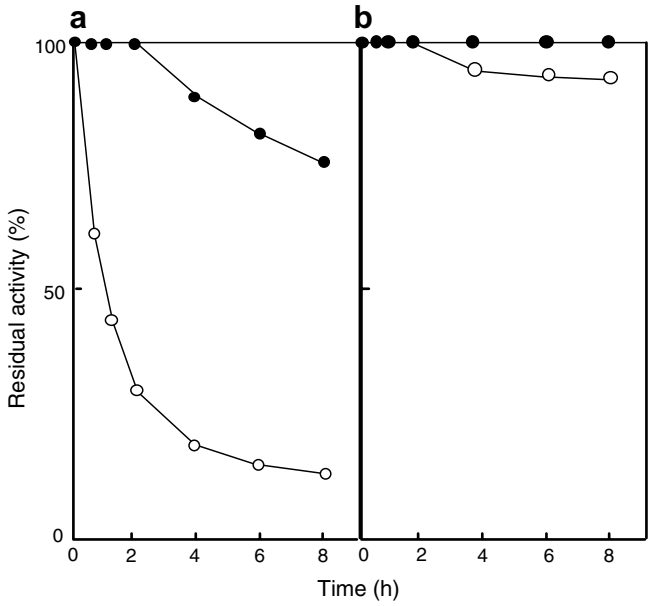

Fig. 2. Effect of calcium ion on the stability of trypsin from walleye pollock. The enzyme was kept at 30 °C and pH 8.0 for 0–8 h in the presence of 10 mM $CaCl_2$ (closed symbol) or 10 mM EDTA (open symbol), and then the remaining activities at 30 °C and pH 8.0 were determined. (a) Walleye pollock trypsin, (b) porcine pancreatic trypsin.

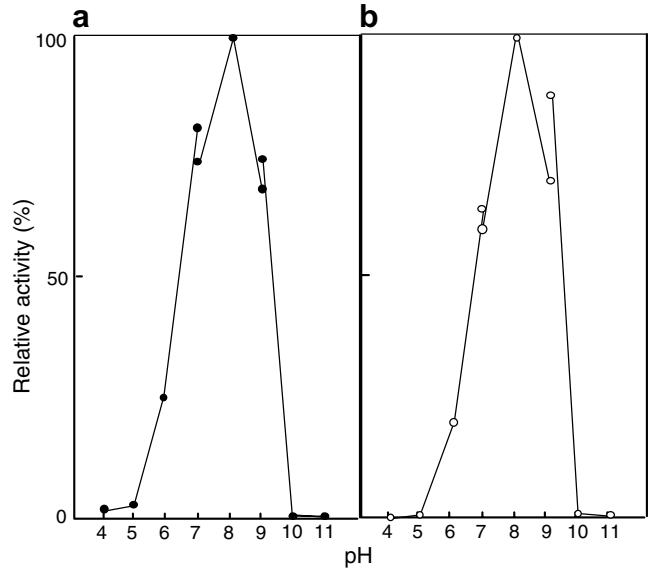

Fig. 3. Effect of pH on the activity of trypsin from walleye pollock. The activity was determined in 50 mM buffer solutions [acetic acid–sodium acetate (pH 4.0–7.0), Tris–HCl (pH 7.0–9.0), and glycine–NaOH (pH 9.0–11.0)] at 37 °C. (a) Walleye pollock trypsin, (b) porcine pancreatic trypsin.

to those of porcine pancreatic trypsin (Fig. 3b) and other fish trypsins (Asgeirsson et al., 1989; Castillo-Yanez et al., 2005; Hjelmeland & Raa, 1982; Kishimura, Hayashi, Miyashita, & Nonami, 2005; Kishimura, Hayashi, et al., 2006, Kishimura et al., 2006a, Kishimura et al., 2006b, Kishimura et al., 2007; Martinez et al., 1988; Simpson & Haard, 1984; Yoshinaka, Sato, Suzuki, & Ikeda, 1984). For pH stability, the enzyme was stable between pH 6.0 and 11.0, but it was unstable below pH 6.0 (Fig. 4a). The property of the pH stability of the walleye pollock trypsin was similar to those of other fish trypsins (Asgeirsson et al., 1989; Kishimura et al., 2005, Kishimura, Hayashi, et al., 2006, Kishimura et al., 2006a, Kishimura et al., 2006b, Kishimura et al., 2007; Kristjansson, 1991; Martinez et al., 1988) but not to porcine pancreatic trypsin (Fig. 4b).

The effects of various temperatures on the activity of trypsin from walleye pollock are shown in Fig. 5a. Optimum temperature of the trypsin was 50 °C, which was lower than that of porcine pancreatic trypsin (60–70 °C; Fig. 5b). The walleye pollock is a Frigid Zone fish and its trypsin had an optimum temperature of 50 °C (Fig. 5a) similar to those of other trypsins from Frigid Zone fish, such as arabesque greenling, brown hakeling, and elkhorn sculpin (Kishimura, Hayashi, et al., 2006, Kishimura et al., 2006a, Kishimura et al., 2007). Optimum temperature of the walleye pollock trypsin was lower than those of porcine pancreatic trypsin (60–70 °C; Fig. 5b), Temperate Zone fish trypsin (60 °C) (Kishimura et al., 2005, Kishimura, Hayashi, et al., 2006, Kishimura et al., 2006a, Kishimura et al., 2006b, Kishimura et al., 2007), and Tropical Zone fish trypsin (55–65 °C) (Klomklao et al., 2006a; Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2006b; Klomklao et al., 2007). Fig. 6a shows the temperature stability of the walleye pollock trypsin. The trypsin was stable below 30 °C, but its activity quickly fell above 40 °C. Similar to those of other trypsins from Frigid Zone fish (Kishimura, Hayashi, et al., 2006, Kishimura et al., 2006a, Kishimura et al., 2007), the temperature stability of the walleye pollock trypsin was more unstable than trypsins from the Temperate Zone fish (Kishimura et al., 2005, Kishimura, Hayashi, et al., 2006, Kishimura et al., 2006a, Kishimura et al., 2006b, Kishimura et al., 2007), Tropical Zone fish (Klomklao et al., 2006a, 2006b, 2007), and

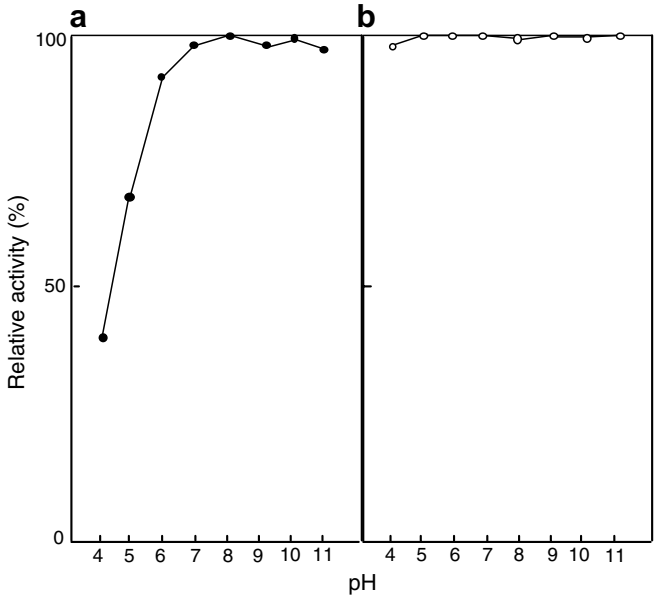

Fig. 4. pH stability of trypsin from walleye pollock. The enzyme was kept at 30 °C for 30 min and pH 4.0–11.0, and then the remaining activity at 30 °C and pH 8.0 was determined. (a) Walleye pollock trypsin, (b) porcine pancreatic trypsin.

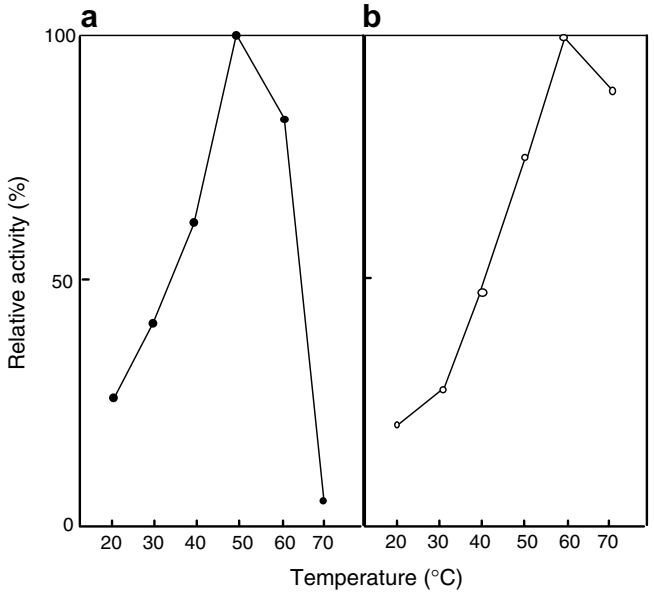

Fig. 5. Effect of temperature on the activity of trypsin from walleye pollock. The activity was determined at pH 8.0 and at various temperatures. (a) Walleye pollock trypsin, (b) porcine pancreatic trypsin.

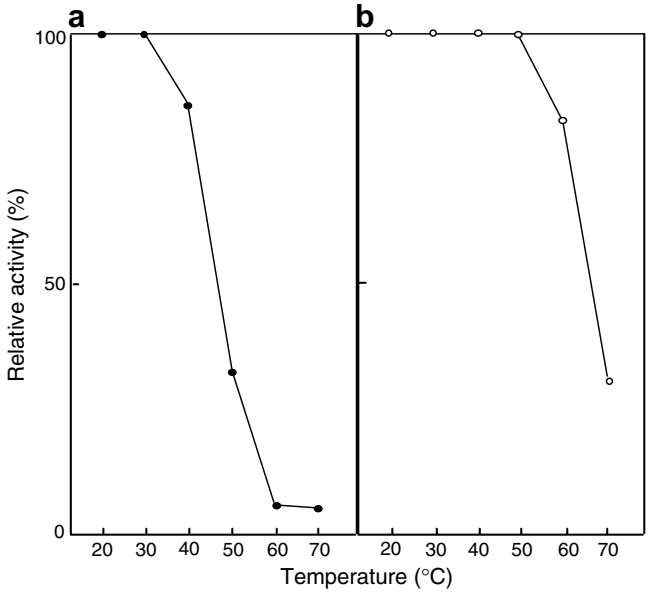

Fig. 6. Thermostability of trypsin from walleye pollock. The enzyme was kept at 20–70 °C for 15 min and pH 8.0, and then the remaining activity at 30 °C and pH 8.0 was determined. (a) Walleye pollock trypsin, (b) porcine pancreatic trypsin.

porcine pancreas (Fig. 6b). The relationship between habitat temperature of fish and thermostability of the fish trypsin was then investigated. As shown in Fig. 7, the

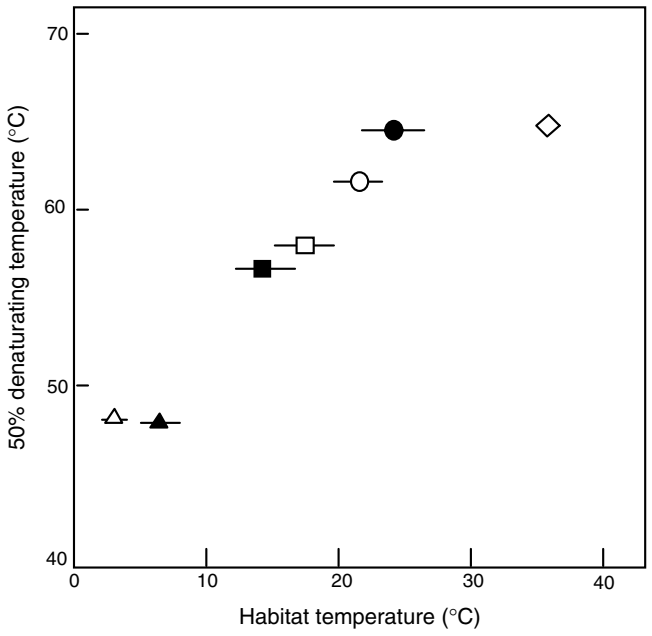

Fig. 7. Relationship between habitat temperature of fish and thermostability of the fish trypsin. The 50% denaturating temperature shows the temperature that the enzyme was denaturated 50% by incubation at pH 8.0 for 15 min at a range of 20–70 °C. open triangle, walleye Pollock trypsin; closed triangle, brown hakeling trypsin (Kishimura et al., 2006b); open square, spotted mackerel trypsin (Kishimura et al., 2006a); closed square, Japanese anchovy trypsin (Kishimura et al., 2005); open circle, skipjack tuna trypsin (Klomklao et al., 2007); closed circle, yellowfin tuna trypsin (Klomklao et al., 2006a); open diamond, porcine pancreatic trypsin.

relationship between habitat temperature of fish and thermostability of the fish trypsin indicated strong positive correlation. This is the first result on the relationship between habitat temperature and thermostability for the trypsin. Furthermore, the N-terminal amino acid sequence of the walleye pollock trypsin was also analyzed. The N-terminal amino acid sequence of the trypsin was determined to be IVGGYECTKHSQAHQVSLNS (Fig. 8). It was indicated that the N-termini of the trypsin was unblocked. The N-terminal amino acid sequence of the walleye pollock trypsin was aligned with the sequences of other animal trypsins. Being similar to other fish trypsins, the walleye pollock trypsin had a charged Glu residue at position 6, where Thr is most common in mammalian pancreatic trypsins (Fig. 8). Bovine pancreatic trypsin has a disulfide bond between Cys-7 and Cys-142 (Stroud, Kay, & Dickerson, 1974), and other vertebrate trypsins also possess the Cys-7. As shown in Fig. 8, the Cys residue at position 7 was characteristically conserved in the walleye pollock trypsin. The N-terminal amino acid sequence of the walleye pollock trypsin was completely identical to that of cod trypsin, and the identity between the walleye pollock trypsin to Fri-

```
                          1         10        20
Frigid Zone Fish
Walleye pollok            IVGGYECTKHSQAHQVSLNS
Cod                       IVGGYECTKHSQAHQVSLNS
Elkhorn sculpin           IVGGYECTPHSQAHQVSLNS
Brown hakeling            IVGGYECPKHSQPHQVSLNS
Arabesuque greenling      IVGGYECTPHTQAHQVSLDS

Temperate Zone Fish
Spotted mackerel          IVGGYECTAHSQPHQVSLNS
Yellow tail               IVGGYECTPYSQPHQVSLNS
Jacopever                 IVGGYECKPYSQPHQVSLNS
True sardine              IVGGYECKAYSQPWQVSLNS
Japanese anchovy          IVGGYECQPYSQPHQVSLDS

Tropical Zone Fish
Skipjack tuna             IVGGYECQAHSQPHQVSLNS
Tongol tuna               IVGGYECQAHSQPHQVSLNA
Yellowfin tuna            IVGGYECQAHSQPPQVSLNA

Mammal
Porcine                   IVGGYTCAANSVPYQVSLNS
Bovine                    IVGGYTCGANTVPYQVSLNS
Human                     IVGGYNCEENSVPYQVSLNS
```

Fig. 8. Comparison of the N-terminal amino acid sequences of trypsins from walleye pollock (*T. chalcogramma*) with those of other vertebrates. Cod (Gudmundsdottir et al., 1993); Elkhorn sculpin (Kishimura et al., 2007); brown hakeling (Kishimura et al., 2006b); Arabesque greenling (Kishimura, Hayashi, et al., 2006); Spotted mackerel (Kishimura et al., 2006a); Yellow tail (Kishimura et al., 2006b); Jacopever (Kishimura et al., 2007); True sardine (Kishimura, Hayashi, et al., 2006); Japanese anchovy (Kishimura et al., 2005); Skipjack tuna (Klomklao et al., 2007); Tongol tuna (Klomklao et al., 2006b); Yellowfin tuna (Klomklao et al., 2006a); Porcine (Hermodson et al., 1973); Bovine (Walsch, 1970); Human (Emi et al., 1986). Amino acid residues different from the walleye pollock trypsin are shaded.

gid Zone fish trypsin was relatively higher (85–100%) than that of Temperate Zone fish trypsin (75–90%), Tropical Zone fish trypsin (75–85%), and mammalian trypsin (60–65%) (Fig. 8).

4. Conclusion

In conclusion, the walleye pollock trypsin showed a lower optimum temperature than that of porcine pancreatic trypsin and was more unstable than porcine pancreatic trypsin below pH 6.0 and above 40 °C. These results suggest that the pyloric ceca of walleye pollock could be a potential source of trypsin for certain food processing operations that require low processing temperatures, and the relatively low thermal stability of the walleye pollock trypsin may also be beneficial in such applications as the enzymes can be inactivated more readily.

Acknowledgement

The authors thank Mr. T. Hirose, the Center for Instrumental Analysis, Hokkaido University, for amino acid sequence analysis.

References

Asgeirsson, B., Fox, J. W., & Bjarnason, J. B. (1989). Purification and characterization of trypsin from the poikilotherm *Gadus morhua*. *European Journal of Biochemistry, 180*, 85–94.

Castillo-Yanez, F. J., Pacheco-Aguilar, R., Garcia-Carreno, F. L., & Toro, M. A. N. (2005). Isolation and characterization of trypsin from pyloric caeca of Monterey sardine *Sardinops sagax caerulea*. *Comparative Biochemistry and Physiology, 140B*, 91–98.

Emi, M., Nakamura, Y., Ogawa, M., Yamamoto, T., Nishide, T., Mori, T., et al. (1986). Cloning, characterization and nucleotide sequences of two cDNAs encoding human pancreatic trypsinogens. *Gene, 41*, 305–310.

Genicot, S., Rentier-Delrue, F., Edwards, D., Vanbeeumen, J., & Gerday, C. (1996). Trypsin and trypsinogen from Antarctic fish: Molecular basis of cold adaptation. *Biochimica et Biophysica Acta, 1298*, 45–57.

Gudmundsdottir, A., Gudmundsdottir, E., Oskarsson, S., Bjarnason, J. B., Eakin, A. K., & Craik, C. S. (1993). Isolation and characterization of cDNAs from Atlantic cod encoding two different forms of trypsinogen. *European Journal of Biochemistry, 217*, 1091–1097.

Hermodson, M. A., Ericsson, L. H., Neurath, H., & Walsh, K. A. (1973). Determination of the amino acid sequence of porcine trypsin by sequenator analysis. *Biochemistry, 12*, 3146–3153.

Hjelmeland, K., & Raa, J. (1982). Characteristics of two trypsin type isozymes isolated from the Arctic fish capelin (*Mallotus villosus*). *Comparative Biochemistry and Physiology, 71B*, 557–562.

Hummel, B. C. W. (1959). A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. *Canadian Journal of Biochemistry and Physiology, 37*, 1393–1399.

Kishimura, H., & Hayashi, K. (2002). Isolation and characteristics of trypsin from pyloric ceca of the starfish *Asterina pectinifera*. *Comparative Biochemistry and Physiology, 132B*, 485–490.

Kishimura, H., Hayashi, K., Miyashita, Y., & Nonami, Y. (2005). Characteristics of two trypsin isozymes from the viscera of Japanese anchovy (*Engraulis japonica*). *Journal of Food Biochemistry, 29*, 459–469.

Kishimura, H., Hayashi, K., Miyashita, Y., & Nonami, Y. (2006). Characteristics of trypsin from the viscera of true sardine (*Sardinops melanostictus*) and the pyloric ceca of arabesque greenling (*Pleuroprammus azonus*). *Food Chemistry, 97*, 65–70.

Kishimura, H., Tokuda, Y., Klomklao, S., Benjakul, S., & Ando, S. (2006a). Enzymatic characteristics of trypsin from pyloric ceca of spotted mackerel (*Scomber australasicus*). *Journal of Food Biochemistry, 30*, 466–477.

Kishimura, H., Tokuda, Y., Klomklao, S., Benjakul, S., & Ando, S. (2006b). Comparative study of enzymatic characteristics of trypsins from the pyloric ceca of yellow tail (*Seriola quinqueradiata*) and brown hakeling (*Physiculus japonicus*). *Journal of Food Biochemistry, 30*, 521–534.

Kishimura, H., Tokuda, Y., Yabe, M., Klomklao, S., Benjakul, S., & Ando, S. (2007). Trypsins from the pyloric ceca of jacopever (*Sebastes schlegelii*) and elkhorn sculpin (*Alcichthys alcicornis*): Isolation and characterization. *Food Chemistry, 100*, 1490–1495.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., Simpson, B. K., & Saeki, H. (2006a). Trypsins from yellowfin tuna (*Thunnus albacores*) spleen: Purification and characterization. *Comparative Biochemistry and Physiology, 144B*, 47–56.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., & Simpson, B. K. (2006b). Purification and characterization of trypsin from the spleen of tongol tuna (*Thunnus tonggol*). *Journal of Agricultural and Food Chemistry, 54*, 5617–5622.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., & Simpson, B. K. (2007). Purification and characterization of trypsins from the spleen of skipjack tuna (*Katsuwonus pelamis*). *Food Chemistry, 100*, 1580–1589.

Kossiakoff, A. A., Chambers, J. L., Kay, L. M., & Stroud, R. M. (1977). Structure of bovine trypsinogen at 1.9 A resolution. *Biochemistry, 16*, 654–664.

Kristjansson, M. M. (1991). Purification and characterization of trypsin from the pyloric caeca of rainbow trout (*Oncorhynchus mykiss*). *Journal of Agricultural and Food Chemistry, 39*, 1738–1742.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–273.

Martinez, A., Olsen, R. L., & Serra, J. L. (1988). Purification and characterization of two trypsin-like enzymes from the digestive tract of anchovy *Engraulis encrasicholus*. *Comparative Biochemistry and Physiology, 91B*, 677–684.

Male, R., Lorens, L. B., Smalas, A. O., & Torrissen, K. R. (1995). Molecular cloning and characterization of anionic and cationic variants of trypsin from Atlantic salmon. *European Journal of Biochemistry, 232*, 677–685.

Simpson, B. K., & Haard, N. F. (1984). Trypsin from Greenland cod, Gadus ogac. Isolation and comparative properties. *Comparative Biochemistry and Physiology, 79B*, 613–622.

Simpson, B. K., & Haard, N. F. (1987). Cold-adapted enzymes from fish. In D. Knorr (Ed.), *Food biotechnology* (pp. 495–528). New York: Marcel Dekker.

Simpson, B. K., & Haard, N. F. (1999). Marine enzymes. In F. J. Francis (Ed.). *Encyclopedia of food science and technology* (Vol. 3, pp. 1525–1534). New York: John Wiley and Sons Inc.

Stroud, R. M., Kay, L. M., & Dickerson, R. E. (1974). The structure of bovine trypsin: Electron density maps of the inhibited enzyme at 5 Å and 2.7 Å resolution. *Journal of Molecular Biology, 83*, 185–208.

Walsch, K. A. (1970). Trypsinogens and trypsins of various species. *Methods in Enzymology, 19*, 41–63.

Yoshinaka, R., Suzuki, T., Sato, M., & Ikeda, S. (1983). Purification and some properties of anionic trypsin from the catfish pancreas. *Bulletin of Japanese Society of Scientific Fisheries, 49*, 207–212.

Yoshinaka, R., Sato, M., Suzuki, T., & Ikeda, S. (1984). Enzymatic characterization of anionic trypsin of the catfish (*Parasilurus asotus*). *Comparative Biochemistry and Physiology, 80B*, 475–480.