Title	Cold-adapted structural properties of trypsins from walleye pollock (Theragra chalcogramma) and Arctic cod (Boreogadus saida)
Author(s)	Kanno, Gaku; Kishimura, Hideki; Yamamoto, Jun; Ando, Seiichi; Shimizu, Takeshi; Benjakul, Soottawat; Klomklao, Sappasith; Nalinanon, Sitthipong; Chun, Byung-Soo; Saeki, Hiroki
Citation	European Food Research and Technology, 233(6), 963-972 https://doi.org/10.1007/s00217-011-1592-8
Issue Date	2011-12
Doc URL	http://hdl.handle.net/2115/50775
Rights	The original publication is available at www.springerlink.com
Type	article (author version)
File Information	EFRT233-6_963-972.pdf

Cold-adapted structural properties of trypsins from walleye pollock (*Theragra chalcogramma*) and Arctic cod (*Boreogadus saida*)

Gaku Kanno · Hideki Kishimura · Jun Yamamoto · Seiichi Ando · Soottawat Benjakul · Sappasith Klomklao · Sitthipong Nalinanon · Byung-Soo Chun · Hiroki Saeki

G. Kanno · H. Kishimura (*) · H. Saeki
Laboratory of Marine Products and Food Science, Research Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan
e-mail: kishi@fish.hokudai.ac.jp

J. Yamamoto
Field Science Center for Northern Biosphere, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

S. Ando
Department of Natural Sciences, Faculty of Health and Welfare Science, Nayoro City University, Nayoro, Hokkaido 096-8641, Japan

S. Benjakul
Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University,
Hat Yai, Songkhla 90112, Thailand

S. Klomklao
Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phattalung Campus, Phattalung 93110, Thailand

S. Nalinanon

Faculty of Agro-Industry, King Mongkut's Institute of Technology Ladkrabang, Choakhunthaharn Building, Choakhunthaharn Rd., Ladkrabang, Bangkok 10520, Thailand

B.-S. Chun

Faculty of Food Science and Biotechnology, Pukyong National University, Busan 608-737, Republic of Korea

Abstract Complementary DNA clones encoding trypsins were isolated from pyloric ceca of cold-adapted fish, walleye pollock (*Theragra chalcogramma*) (WP-T) and Arctic cod (*Boreogadus saida*) (AC-T). The isolated full-length cDNA clones of WP-T and AC-T were 852 bp and 860 bp, respectively, and both cDNAs were contained an open reading frame of 726 bp. WP-T and AC-T seemed to be synthesized as preproenzyme that contains a signal peptide, an activation peptide and a mature trypsin. Although the amino acid sequence identities of WP-T and AC-T to that of bovine trypsin were 64% and 63%, respectively, they completely conserved the structural features for catalytic function of trypsin. On the other hand, WP-T and AC-T possessed the four Met residues (Met135, Met145, Met175 and Met242) in their molecules and the deletion of Tyr151 and substitution of Pro152 for Gly in their autolysis loops when aligned with the sequences of tropical-zone fish and bovine trypsins. In addition, the contents of charged amino acid residues at the *N*-terminal regions (positions 20-50) of WP-T and AC-T were extremely higher than those of other fish and bovine trypsins. Moreover, one amino acid (Asn72) and two amino acids (Asn72 and Val75) coordinating with Ca^{2+} in bovine trypsin were exchanged for another amino acids in WP-T (His) and AC-T (His and Glu), respectively, and the contents of negative charged amino acids at their Ca^{2+}-binding regions were lower than those of tropical-zone fish and bovine trypsins. Therefore, it was considered that these structural characteristics of WP-T and AC-T are closely related to their lower thermo stability.

Keywords Trypsin, Walleye pollock, *Theragra chalcogramma,* Arctic cod, *Boreogadus saida*, cDNA cloning, Primary structure, Thermo stability, Cold-adaptation

Introduction

Trypsin (EC 3.4.21.4) is a member of serine protease and specifically cleaves peptide bond on the carboxyl side of Lys and Arg residues. Since bovine trypsin was isolated and analyzed as the first proteolytic enzyme, it has been studied in a broad range of species from bacteria to human [1]. So, trypsin is an excellent model to study structure-function relationships and considerable researches have been done to elucidate the structural properties of mammalian pancreatic trypsin [2-7].

On the other hand, at 1980s, it was found that fish trypsin has unique properties, which are substantially higher catalytic efficiency (*kcat*/*Km*) at low temperatures and lower thermo stability than its mammalian counterparts. In 1982, Hjelmeland and Raa obtained the results that two trypsin-like enzymes from Arctic fish capelin (*Mallotus villosus*) have lower temperature optimums for hydrolysis of Bz-Arg-NH-Np and lower thermo stability than bovine trypsin [8]. Simpson and Haard also showed that the optimum temperature of Greenland cod (*Gadus ogac*) trypsin is lower than that of bovine trypsin [9]. Then, Asgeirsson et al. found that the *kcat*/*Km* value of trypsin from Atlantic cod (*Gadus morhua*) for hydrolysis of Bz-Arg-NH-Np at 25 ^{o}C is about 17 times higher than that of bovine trypsin [10]. Later on, Kristjansson [11] and Outzen et al. [12] also reported the same results using trypsins from rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*), respectively. In addition, fish trypsin is more sensitive to inactivation by heat, low pH and autolysis than that of mesophilic analogues [8-13]. These enzymatic properties of fish trypsin are interesting for several industrial applications, such as in certain food processing operations that require low processing temperatures. Indeed, Atlantic cod trypsin has already been used in industrial applications [14, 15]. But, the extension of its application, it is significant to know more detail information for the structure-function relationship of fish trypsin.

Recently, we measured thermo stability of trypsins from various species of marine fish [16-27] and found an interest phenomenon of the strong positive correlations between habitat temperature of marine fish and thermo stability of these fish trypsins [26]. We also found the percentage of hydrophobic amino acid in the *N*-terminal 20 amino acids sequences of cold-zone fish trypsins is lower (28%) than those of temperate-zone fish trypsins (34%), tropical-zone fish trypsins (37%) and mammalian trypsins (34%) [27]. So, in the previous study, we investigated a primary structure of trypsin from cold-adapted fish, arabesque greenling (*Pleurogrammus azonus*) [28]. As a result, trypsin of arabesque greenling possessed the deletion of Tyr151 and substitution of Pro152 for Gly in the autolysis loop. In addition, the ratio between positive and negative charged amino acid residues at the calcium-binding region and the ratio between charged and hydrophobic amino acid residues at the *N*-terminal region of the trypsin were higher than those of temperate-zone fish trypsins, tropical-zone fish trypsins and bovine trypsin.

Walleye pollock (*Theragra chalcogramma*) and Arctic cod (*Boreogadus saida*) belong to cod species and are the representatives of cold-adapted fish. In this study, we inspected primary structures of trypsins from walleye pollock (WP-T) and Arctic cod (AC-T) to confirm the relationships between structural characteristics of fish trypsin and its thermal stability.

Materials and methods

Materials

Walleye pollock (*Theragra chalcogramma*) were collected by rod angling in the mouth of Funka Bay, southwest of Hokkaido, Japan, on January 2009. Arctic cod (*Boreogadus saida*) were collected from the bottom trawl surveys which were conducted in the south of St. Lawrence Island, northern Bering Sea, on July 2009, by T/S Oshoro-Maru of Hokkaido University. Pyloric ceca dissected from live specimens steeped into RNAlater solution (Applied Biosystems, CA, USA) and stored at -80 ^{o}C.

cDNA cloning by reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE)

The cDNA cloning for WP-T and AC-T was carried out as previously described except for some primers (Table 1) [28]. In brief, total RNA was extracted from pyloric ceca with TRIzol reagent (Invitrogen, CA, USA), and mRNA was isolated using Oligotex^{TM}-dt30 <Super> mRNA Purification Kit (From Total RNA) (TaKaRa, Kyoto, Japan). The mRNA was reverse-transcribed by using a RT-RACE primer and a SuperScript II^{TM} (Invitrogen, CA, USA). PCR was carried out using the first strand cDNA, Amplitaq Gold (Applied Biosystems, CA, USA), and a set of primer (forward primer: RT-RACE F1, reverse primer: RT-RACE R1) under the following conditions: 1 cycle of 95 ^{o}C for 9 min, 45 cycles of 94 ^{o}C for 15 s, 54 ^{o}C for 30 s and 72 ^{o}C for 60 s, followed by 1 cycle of 72 ^{o}C for 7 min. The PCR products were subcloned in a pDrive Cloning Vector (QUIAGEN, Duesseldorf, Germany) and transformed into JM109 Competent Cells (Promega, WI, USA). Plasmid DNA was purified from the positive clone using a Wizard SV Gel and PCR Clean-Up System (Promega, WI, USA).

To recover the full-length cDNA sequence, 3'- and 5'-RACE were performed. The 3'-terminal cDNA fragments were amplified using the above first strand cDNA, an

Amplitaq Gold (Applied Biosystems, CA, USA), and a set of primer (forward primer: 3’-RACE F1, reverse primer: 3’-RACE R1) under the conditions: 1 cycle of 95 ^{o}C for 9 min, 45 cycles of 94 ^{o}C for 15 s, 54 ^{o}C for 30 s, 72 ^{o}C for 60 s, and 1 cycle of 72 ^{o}C for 7 min. 5’-RACE was performed using 5’ Full RACE Core Set (TaKaRa, Kyoto, Japan). The mRNA was reverse-transcribed by using a RT primer and AMV Reverse Transcriptase XL, and then the first strand cDNA was ringed with T4 RNA Ligase. The first PCR was carried out using the ringed cDNA, Premix Taq*TM*, and a set of primer (forward primer: 5’-RACE F1, reverse primer: 5’-RACE R1) under the conditions: 1 cycle of 94 ^{o}C for 3 min, 25 cycles of 94 ^{o}C for 30 s, 65 ^{o}C for 30 s, 68 ^{o}C for 30 s. The second PCR was carried out using the first PCR products, Premix Taq, and a set of primer (forward primer: 5’-RACE F2, reverse primer: 5’-RACE R2) under the conditions: 27 cycles of 94 ^{o}C for 30 s, 65 ^{o}C for 30 s, 68 ^{o}C for 30 s.

Nucleotide sequencing

Nucleotide sequence was determined using ABI PRISM 310 Genetic Analyzer (Applied Biosystems, CA, USA) after labeling the DNA with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

Results and discussion

cDNA clones of WP-T and AC-T

The isolated cDNA clones encoding WP-T (Accession No.: AB506710 in DDBJ) and AC-T (Accession No.: AB530319 in DDBJ) were composed of 852 bp (Fig. 1) and 860 bp (Fig. 2), respectively, with an open reading frame of 726 bp from the ATG start codon through to the TAA stop codon. The 5’-noncoding region of WP-T was 23 bp long and that of AC-T was 22 bp long. These lengths are almost the same as those of arabesque greenling (24 bp) [28], anchovy (23 bp) [29], but different from Atlantic cod (9 bp) [30], Antarctic fish (11 bp) [31], topmouth culter (25 bp) [32] and grass carp (28 bp) [32]. The polyadenylation signals (AATAAA) of both cDNAs occurred at 22 bp upstream from the first adenine of poly (A) track. The 3’-noncoding region of WP-T was 103 bp long and that of AC-T was 112 bp nucleotides. The open reading frames of WP-T and AC-T encoded 241 amino acids starting from the first Met, and both trypsins seemed to be synthesized as preproenzyme that contains hydrophobic signal peptide of 15 amino acids, acidic activation peptide of 4 amino acids and mature trypsin of 222 amino acids.

Signal and activation peptides of WP-T and AC-T

As shown in Fig. 3, the signal peptides of WP-T and AC-T were composed by fifteen amino acids and their sequences were identical with each other as well as that of Atlantic cod trypsin I [30]. These signal peptides had a hydrophobic core containing seven contiguous hydrophobic residues, and the result indicates that the cleavage site between signal peptide and activation peptide of these trypsins is at the *C*-terminal of Ala residue (amino acid position 15). The amino acid sequences of WP-T and AC-T signal peptides were compared to those of frigid-zone fish (Atlantic cod, Arabesque greenling, Antarctic fish and Atlantic salmon), temperate-zone fish (anchovy and flounder), tropical-zone fish (zebrafish and tilapia) and bovine trypsins. The signal peptide of bovine trypsin contains a hydrophobic core terminated by a helix-breaking Gly residue, and the hydrophobic core falls into two clusters interrupted by a less hydrophobic Ala residue at position 8 [33]. The signal peptides of zebrafish and tilapia (tropical-zone fish) showed the same structural composition as that of bovine trypsin. However, the signal peptides of frigid-zone and temperate-zone fish trypsins had seven contiguous hydrophobic residues.

The activation peptides of WP-T and AC-T were tetrapeptide composed by a poly-anionic cluster of two Glu and one Asp residues (positions 6-8) and a Lys residue (position 9) located at the *C*-terminal end (Fig. 4). Generally, the activation peptide of mammalian trypsin is consisted by octapeptide containing a hydrophobic cluster of three amino acid residues followed by a poly-anionic cluster of four contiguous Asp residues. Similarly to WP-T and AC-T, the sequences of activation peptides of frigid-zone and temperate-zone fish trypsins required the deletion of two amino acid residues (positions 1 and 2) in the hydrophobic cluster when aligned with those of tropical-zone fish and bovine trypsins. The number of amino acid in the poly-anionic cluster of fish trypsins except for zebrafish was one residue shorter than that of bovine. Louvard & Puigserver [34] revealed that enterokinase or trypsin itself of mammals recognize the poly-anionic cluster to cleave the activation peptide from the proenzyme. So, the cleavage sites between activation peptides and mature enzymes of WP-T and AC-T are predicted at the *C*-terminal of Lys residue.

General structures of WP-T and AC-T

Predicted amino acid sequences of mature WP-T and AC-T are shown in Fig. 5. WP-T and AC-T were both composed of 222 amino acid residues, and their molecular weights were calculated at 23,885 and 24,011, respectively. Gudmundsdottir et al. [30] also clarified that mature trypsins (trypsin I and trypsin X) from Atlantic cod are consisted of 222 amino acids and the calculated molecular sizes of the isozymes are 23,459 and 23,819, respectively. Theoretical isoelectric point (pI) of WP-T and AC-T were estimated to 5.6 and 5.5, respectively, in analogy with three anionic trypsin isozymes (trypsins I, II and III) from Atlantic cod with pI of 6.6, 6.2 and 5.5, respectively [10]. Amino acid sequence identities of WP-T and AC-T show in Table 2. Cod trypsins, WP-T, AC-T and Atlantic cod trypsin, showed high sequence identities (91-95%) one another, and the identities of WP-T and AC-T to frigid-zone fish and temperate-zone fish trypsins (79-85%) were higher than those to tropical-zone fish trypsins and bovine trypsin (63-70%). WP-T and AC-T could have six disulfide-bridges (Cys15-Cys145, Cys33-Cys49, Cys117-Cys218, Cys124-Cys191, Cys156-Cys170 and Cys181-Cys205), because they possessed twelve Cys residues at the appropriate positions in bovine trypsin [35].

WP-T and AC-T completely conserved the catalytic triad (His57, Asp102 and Ser195), the consensus sequence (GDSGG) around the Ser195, S1 substrate-binding pocket (positions 189-195, 214-220 and 225-228), loop 1 (positions 184-188), loop 2 (positions 221-225) and Tyr172 residue. The catalytic activity of trypsin is due to the ability of His57 to transfer a proton from Asp102 to Ser195 [35], and the consensus sequence is common to a serine protease [36]. On the other hand, the steric and electrostatic characteristics of the S1 substrate-binding pocket are need for substrate specificity [3]. The specificity of trypsin for Lys and Arg residues results from the presence of Asp189 residue at the bottom of S1 pocket, and the Tyr172 residue also interacts synergistically with the residues in the S1 pocket and two surface loops (loop 1 and loop 2) to determine substrate specificity [4].

Cold-adapted structures of WP-T and AC-T

WP-T and AC-T had six and seven Met in their molecules, respectively (Fig. 5). Although, the structural role of Met residue is generally poorly understood, Gudmundsdottir et al. [30] found that Atlantic cod trypsins, as well as the other fish trypsins, have a higher Met content than mammalian enzymes. Later, Leiros et al. [37] described that four Met residues, Met135, Met145, Met175 and Met242, are observed in the psychrophilic fish trypsins, and the conservation of the four Mets seems to introduce flexibility in the cold-adapted trypsins. In this study, these four Met residues also existed in WP-T and AC-T.

It was already investigated by Gable and Kasche [38] that the cleavage of single-chain bovine trypsin (β-trypsin) at Lys145 residue in the autolysis loop (positions 143-153) results α-trypsin which shows less thermal stability, and it is thought that the structure of autolysis loop is strongly related to thermo stability of trypsin. However, fish trypsin including WP-T and AC-T had not the Lys145 residue (Fig. 5). On the other hand, WP-T and AC-T had the deletion of Tyr151 residue and substitution of Gly for Pro152 residue in the autolysis loop when aligned with the sequences of bovine trypsin. These structural differences were also found in frigid-zone and temperate-zone fish trypsins, but not in tropical-zone fish and bovine trypsins. Thus, the deletion of Tyr151 residue and substitution of Gly for Pro152 could lead to a flexible structure of the autolysis loop in cold-adapted trypsins. Relating to the Tyr151 and Pro152 residues, Ahsan et al. [29] considered that Gln192 residue located at the entrance of the S1 substrate-binding pocket in anchovy trypsin is much more freedom with lack of the bulky ring of Tyr151 residue, and substitution of Gly for Pro152 residue in anchovy trypsin could result in a completely different orientation of the autolysis loop between anchovy trypsin and bovine trypsin. In addition, WP-T and AC-T also had Asp150 and Lys154 residues in their autolysis loops. Leiros et al. [37] demonstrated that the ion-pair of Asp150-Lys154 is in the crystal structure of Atlantic salmon trypsin and the Lys154 is closely associated with the ion-pair of Glu21-His71, and they proposed the ion-pair network is a possible cold-adaptation determinant for trypsin.

Genicot et al. [31] proposed that the thermal stability and flexibility of fish trypsin are affected by its overall decrease in hydrophobicity and an increase in surface hydrophilicity as compared to mammalian counterparts. As shown in Table 3, WP-T and AC-T also showed the higher contents of charged amino acid residues (Lys, Arg, Aps, Glu and His) compared with those of tropical-zone fish trypsins and bovine trypsin, and more remarkable was the extremely high contents of charged amino acids at the *N*-terminal region (positions 20-50) of the cod trypsins (WP-T, AC-T and Atlantic cod trypsin). In contrast, at the *N*-terminal region, the ratios between the number of hydrophobic amino acid residues (Trp, Phe, Leu, Ile, Val, Tyr and Pro) to the number of charged amino acid residues of the cod trypsins (1.00-1.33) were lowest followed by other frigid-zone fish and temperate-zone fish trypsins (1.60-1.80), tropical-zone fish trypsins (3.67) and bovine trypsin (10.00) (Table 3). In the previous study, we aligned the *N*-terminal 20 amino acid sequences of various fish and mammalian trypsins and verified that the charged amino acid contents of frigid-zone fish trypsins (mean: 19.2%) were higher than those of temperate-zone fish trypsins (mean: 11.0%), tropical-zone fish trypsins (mean: 12.1%) and mammalian trypsins (mean: 5.0%) [39]. Therefore, it was confirmed that the structure of *N*-terminal region is closely related to thermo stability of trypsin.

It is well known that bovine trypsin requires Ca^{2+} to thermally stabilize and to resist to self-degradation, and the stabilizing effect is accompanied by a conformational change in the trypsin molecule resulting in a more compact structure [40, 41]. The calcium-binding site of bovine trypsin is located at the external loop of molecule, and five amino acid residues (Glu70, Asn72, Val75, Glu77 and Glu80) coordinate with calcium ion [41]. These residues were completely conserved in tropical-zone fish trypsins (Fig. 5). However, the Asn72s in WP-T and AC-T was exchanged for His, and the Val75 in AC-T was exchanged for Glu, as well as other frigid-zone and temperate-zone fish trypsins (Atlantic cod, Asn72-His; arabesque greenling, Val75-Ala; Antarctic fish, Asn72-His; anchovy, Asn72-His and Val75-Gln; flounder, Asn72-Lys). In addition, there was an interesting correlation between the thermo stability of fish trypsins and the negative charge at their calcium-binding regions. That is, at the calcium-binding region (positions 68-84), the proportions of the negative charged amino acids to the charged amino acids of WP-T and AC-T including frigid-zone fish and temperate-zone fish trypsins (43-67%) were lower than those of tropical-zone fish trypsins (75-80%) and bovine trypsin (100%). These results show that the combination of Ca^{2+} with the cold-adapted trypsins could be weaker than those of tropical-zone fish and bovine trypsins.

4. Conclusions

To confirm the relationships between structural characteristics of fish trypsin and its thermal stability, we inspected primary structures of trypsins from walleye pollock (WP-T) and Arctic cod (AC-T), which are the representatives of cold-adapted fish, and compared the structures with those of other fish and mammalian trypsins. Consequently, it was confirmed that the Met residues at positions 135, 145, 175 and 242 and the structures of autolysis loop, *N*-terminal region and calcium-binding region are closely related to thermo stability of trypsin. It is considered that these structural characteristics of fish trypsin together with the enzymatic ones are the important information for its industrial application, such as in certain food processing operations that require low processing temperatures.

Acknowledgement

We would like to thank crew and officers of the T/S *Oshoro Maru*, Hokkaido University, for in collecting Arctic cod. This research was partly supported by the grant from HOKUSUI Association.

References

1. Rypniewski, W.R., Perrakis, A., Vorgias, C.E., & Wilson, K.S. (1994). Evolutionary divergence and conservation of trypsin. *Protein Engineering*, 7, 57-64.
2. 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.
3. Hedstrom, L., Szilagyi, L., & Rutter, W.J. (1992). Converting trypsin to chymotrypsin: the role of surface roops. *Science*, 255, 1249-1253.
4. Hedstrom, L., Perona, J., & Rutter, W.J. (1994). Converting trypsin to chymotrypsin: residue 172 is a substrate specificity determinant. *Biochemistry*, 33, 8757-8763.
5. Hedstrom, L., Lin, T., & Fast, W. (1996). Hydrophobic interactions control zymogen activation in the trypsin family of serine proteases. *Biochemistry*, 35, 4515-4523.
6. Pasternak, A., Liu, X., Lin, T., & Hedstrom, L. (1998). Activating a zymogen with out proteolytic processing: mutation of Lys15 and Asn194 activates trypsinogen. *Biochemistry*, 37, 16201-16210.
7. Szabo, E., Bocskei, Z., Naray-Szabo, G., & Graf, L. (1999). The three-dimensional structure of Asp189Ser trypsin provides evidence for an inherent structural plasticity of the proteases. *European Journal of Biochemistry*, 263, 20-26.
8. 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.
9. Simpson, B.K., & Haard, N.F. (1984). Trypsin from Greenland cod, *Gadus ogac*. Isolation and comparative properties. *Comparative Biochemistry and Physiology*, 79B, 613-622.

10. 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.
11. 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.
12. Outzen, H., Berglund, G.I., Smalas A.O., & Willassen N.P. (1996). Temperature and pH sensitivity of trypsins from Atlantic salmon (*Salmo salar*) in comparison with bovine and porcine trypsin. *Comparative Biochemistry and Physiology*, 115B, 33-45.
13. 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.
14. Bjarnason, J.B. (2000). Fish serine proteases and their pharmaceutical and cosmetic use. Patent PCT, WO 00/78332 A2, December 28, 2000.
15. Bjarnason, J.B., & Benediktsson, B. (2001). Protein hydrolysates produced with the use of marine proteases. Patent PCT, WO 01/28353 A2, April 26, 2001.
16. 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.
17. Kishimura, H., Hayashi, K., Miyashita, Y., & Nonami, Y. (2006a). Characteristics of trypsins from the viscera of true sardine (*Sardinops melanostictus*) and the pyloric ceca of arabesque greenling (*Pleuroprammus azonus*). *Food Chemistry*, 97, 65-70.
18. Kishimura, H., Tokuda, Y., Klomklao, S., Benjakul, S., & Ando, S. (2006b). Enzymatic characteristics of trypsin from the pyloric ceca of spotted mackerel (*Scomber australasicus*). *Journal of Food Biochemistry*, 30, 466-477.
19. Kishimura, H., Tokuda, Y., Klomklao, S., Benjakul, S., & Ando, S. (2006c). Comparative

study on 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.

20. Kishimura, H., Tokuda, Y., Yabe, M., Klomklao, S., Benjakul, S., & Ando, S. (2007). Trypsins from the pyloric ceca of jacopever (*Sebastes schlegeli*) and elkhorn sculpin (*Alcichthys alcicornis*) : Isolation and characterization. *Food Chemistry*, 100, 1490-1495.
21. 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.
22. Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., & Simpson, B.K. (2006b). Purification and characterization of trypsin from spleen of tongol tuna (*Thunnus tonggol*). *Journal of Agricultural Food and Chemistry*, 54, 5617-5622.
23. Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., & Simpson, B.K. (2007a). Purification and characterization of trypsins from skipjack tuna (*Katsuwonus pelamis*) spleen. *Food Chemistry*, 100, 1580-1589.
24. Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., & Simpson, B.K. (2007b). Trypsin from the pyloric ceca of bluefish (*Pomatomus saltatrix*). *Comparative Biochemistry and Physiology*, 148B, 382-389.
25. Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., & Simpson, B.K. (2007c). A 29 kDa protease from the digestive glands of Atlantic bonito (*Sarda sarda*): recovery and characterization. *Journal of Agricultural and Food Chemistry*, 55, 4548-4553.
26. Kishimura, H., Klomklao, S., Benjakul, S., & Chun, B.-S. (2008). Characteristics of trypsin from the pyloric ceca of walleye pollock (*Theragra chalcogramma*). *Food*

Chemistry, 106, 194-199.

27. Fuchise, T., Kishimura, H., Sekizaki, H., Nonami, Y., Kanno, G., Klomklao, S., Benjakul, S., & Chun, B.-S. (2009). Purification and characteristics of cold-zone fish trypsin, Pacific cod (*Gadus macrocephalus*) and saffron cod (*Eleginus gracilis*). *Food Chemistry*, 116, 611-616.
28. Kanno, G., Kishimura, H., Ando, S., Nalinanon, S., Klomklao, S., Benjakul, S., Chun, B.-S., & Saeki, H. (2011). Structural properties of trypsin from cold-adapted fish, arabesque greenling (*Pleurogrammus azonus*). *European Food Recearch and Technology*, 232, 381-388.
29. Ahsan, M.N., Funabara, D., & Watabe, S. (2001). Molecular cloning and characterization of two isoforms of trypsinogen from anchovy pyloric ceca. *Marine Biotechnology*, 3, 80-90.
30. 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.
31. 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.
32. Ruan, G.-L., Li, Y., Gao, Z.-X., Wang, H.-L., & Wang W.-M. (2010). Molecular characterization of trypsinogens and development of trypsinogen gene expression and tryptic activities in grass carp (*Ctenopharyngodon idellus*) and topmouth culter (*Culter alburnus*). *Comparative Biochemistry and Physiology*, Part B, 155, 77-85.
33. Watson, M.E.E. (1984). Compilation of published signal sequences. *Nucleic acids research*, 12, 5145-5164.

34. Louvard, M.N., & Puigserver, A. (1974). On bovine and porcine anionic trypsinogens. *Biochimica et Biophysica Acta*, 371, 177-185.

35. Huerou, I.L., Wicker, C., Guilloteau, P., Toullec, R. & Puigserver, A. (1990). Isolation and nucleotide sequence of cDNA clone for bovine pancreatic anionic trypsinogen: structural identity within the trypsin family. *European Journal of Biochemistry*, 193, 767-773.

36. Krem, M.M., Rose, T., & Cera, E.D. (1999). The C-terminal sequence encodes function in serine proteases. *Journal of Biological Chemistry,* 274, 28063-28066.

37. Leiros, H.-K.S., Willassen, N.P., & Smalas, A.O. (2000). Structural comparison of psychrophilic and mesophilic trypsins: elucidating the molecular basis of cold-adaptation. *European Journal of Biochemistry*, 267, 1039-1049.

38. Gable, D., & Kasche, V. (1973). Autolysis of β-trypsin: influences of calcium ions and heat. *Acta Chemistry of Scandinavia*, 27, 1971-1981.

49. Kanno, G., Yamaguchi, T., Kishimura, H., Yamaha, E., & Saeki, H. (2010). Purification and characteristics of trypsin from masu salmon (*Oncorhynchus masou*) cultured in fresh-water. *Fish Physiology and Biochemistry*, 36, 637-645.

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

41. Bode, W., & Schwager, P. (1975). The single calcium-binding site of crystalline β-trypsin. *FEBS LETTERS*, 56, 139-143.

42. 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.

43. Hartley, B.S., & Kauffman, D.L. (1966). Corrections to the amino acid sequence of bovine chymotrypsinogen A. *Biochemical Journal*, 101, 229-231.

(Captions to figures)

Fig. 1. Nucleotide and deduced amino acid sequences of WP-T. The deduced amino acid sequence and the residue numbers are shown above the codons. The ATG initiation codon and the TAA termination codon are asterisked. Putative polyadenylation signal is written in bold-italic.

Fig. 2. Nucleotide and deduced amino acid sequences of AC-T. The deduced amino acid sequence and the residue numbers are shown above the codons. The ATG initiation codon and the TAA termination codon are asterisked. Putative polyadenylation signal is written in bold-italic.

Fig. 3. Comparison of the signal peptides of WP-T and AC-T with those of other fish and bovine trypsins. Walleye pollock, walleye pollock (*Theragra chalcogramma*) trypsin; Arctic cod, Arctic cod (*Boreogadus saida*) trypsin; Atlantic cod I, Atlantic cod (*Gadus morhua*) trypsin I [30]; Arabesque greenling, arabesque greenling (*Pleurogrammus azonus*) trypsin [28]; Antarctic fish, Antarctic fish (*Paranotothenia magellanica*) trypsin [31]; Atlantic salmon I, Atlantic salmon (*Salmo salar*) trypsin I [42]; Anchovy I, anchovy (*Engraulis japonicus*) trypsin I [29]; Flounder I, flounder (*Paralichthys plivaceus*) trypsin I (Accession No.: AB029750 in GenBank); Zebrafish, zebrafish (*Danio rerio*) trypsin (Accession No.: AF541952 in DDBJ); Tilapia, tilapia (*Oreochromis niloticus*) trypsin (Accession No.: AY510093 in DDBJ); Bovine cat, bovine cationic trypsin (Accession No.: BC134797 in DDBJ).

Fig. 4. Comparison of the activation peptides of WP-T and AC-T with those of other fish and bovine trypsins. The names of trypsins are the same in Fig. 3. Dashes indicate deletions introduced for maximizing the sequence similarity.

Fig. 5. Alignment of the deduced amino acid sequences of WP-T and AC-T with the sequences of other fish and bovine trypsins. The amino acids are numbered by the standard chymotrypsin numbering system [43]. The names of trypsins are the same in Fig. 3. Dashes indicate deletions introduced for maximizing the sequence similarity. The residues of catalytic triad (His57, Asp102 and Ser195) and obligatory Asp189 are marked with asterisks.

Fig. 1

```
5’-  ATGAGCCTGATCACAAAGCAACC                                                    23
1                                   10                                      20
*** Lys Ser Leu Ile Phe Val Leu Leu Leu Gly Ala Val Phe Ala Glu Glu Asp Lys Ile
ATG AAG TCT CTT ATC TTC GTT CTG CTC CTC GGA GCT GTC TTC GCT GAG GAG GAC AAG ATC  83

                                    30                                      40
Val Gly Gly Tyr Glu Cys Thr Arg His Ser Gln Ala His Gln Val Ser Leu Asn Ser Gly
GTC GGA GGG TAT GAG TGT ACG AGG CAC TCC CAG GCC CAC CAG GTG TCT CTG AAC TCT GGA 143

                                    50                                      60
Tyr His Phe Cys Gly Gly Ser Leu Val Ser Lys Asp Trp Val Val Ser Ala Ala His Cys
TAC CAC TTC TGT GGA GGC TCC CTG GTC AGC AAG GAC TGG GTG GTG TCT GCT GCT CAC TGC 203

                                    70                                      80
Tyr Lys Ser Arg Ile Glu Val Arg Leu Gly Glu His His Ile Arg Val Asn Glu Gly Thr
TAC AAG TCC CGT ATT GAG GTG CGT CTG GGC GAG CAC CAC ATC AGG GTC AAC GAG GGA ACC 263

                                    90                                      100
Glu Gln Phe Ile Ser Ser Ser Ser Val Ile Arg His Pro Ser Tyr Ser Ser Tyr Asn Ile
GAG CAG TTC ATC TCC TCC TCC AGC GTC ATC CGT CAC CCC AGC TAC AGC TCC TAC AAC ATC 323

                                    110                                     120
Asn Asn Asp Ile Met Leu Ile Lys Leu Ser Lys Pro Ala Thr Leu Asn Gln Tyr Val Gln
AAC AAC GAC ATC ATG CTG ATC AAG CTG AGC AAG CCC GCC ACC CTG AAC CAG TAT GTG CAG 383

                                    130                                     140
Pro Val Ala Leu Pro Thr Glu Cys Ala Ala Asp Gly Thr Met Cys Thr Val Ser Gly Trp
CCT GTG GCC CTT CCC ACC GAA TGT GCT GCT GAT GGC ACC ATG TGC ACA GTG TCT GGC TGG 443

                                    150                                     160
Gly Asn Thr Met Ser Ser Val Asp Asp Gly Asp Lys Leu Gln Cys Leu Asn Leu Pro Ile
GGA AAC ACC ATG AGC TCC GTT GAT GAC GGG GAC AAG CTT CAG TGC CTG AAC CTG CCC ATC 503

                                    170                                     180
Leu Ser His Ala Asp Cys Asp Asn Ser Tyr Pro Gly Met Ile Thr Gln Ser Met Phe Cys
CTC TCC CAC GCC GAC TGT GAC AAC TCC TAC CCT GGC ATG ATC ACC CAG TCC ATG TTC TGC 563

                                    190                                     200
Ala Gly Tyr Leu Glu Gly Gly Lys Asp Ser Cys Gln Gly Asp Ser Gly Gly Pro Val Val
GCT GGC TAC CTG GAG GGA GGC AAG GAC TCT TGC CAG GGT GAC TCC GGT GGT CCC GTG GTG 623

                                    210                                     220
Cys Asn Gly Val Leu Gln Gly Val Val Ser Trp Gly Tyr Gly Cys Ala Glu Arg Asp His
TGC AAC GGT GTG CTG CAG GGT GTT GTG TCC TGG GGA TAC GGA TGT GCC GAG AGG GAC CAC 683

                                    230                                     240
Pro Gly Val Tyr Ala Lys Val Cys Val Leu Ser Gly Trp Val Leu Asp Thr Met Ala Ser
CCC GGT GTC TAC GCC AAG GTC TGC GTT CTC TCG GGC TGG GTT CTC GAT ACC ATG GCA AGT 743

    242
Tyr ***
TAT TAA                                                                         749

ATGATCCTCTTCAGATTCCTGTAGCAGCTTCACATCAGGCTGTTAATGCAGAAAATGAATATGATCAATAAAGTTTAAA 828
AGAGAAATCAAAAAAAAAAAAAAA-3’                                                     852
```

Fig. 2

```
5'-  TGAACCTGATCACAAAGCAACC                                                     22
1                                  10                                       20
*** Lys Ser Leu Ile Phe Val Leu Leu Leu Gly Ala Val Phe Ala Glu Glu Asp Lys Ile
ATG AAG TCT CTT ATC TTC GTT CTG CTC CTC GGA GCT GTC TTC GCT GAG GAG GAC AAG ATC  82

                                   30                                       40
Val Gly Gly Tyr Glu Cys Thr Lys His Ser Gln Ser Tyr Gln Val Ser Leu Asn Ala Gly
GTC GGA GGG TAT GAG TGT ACA AAG CAC TCC CAG TCC TAC CAG GTG TCT CTG AAC GCT GGA 142

                                   50                                       60
Tyr His Phe Cys Gly Gly Ser Leu Val Ser Lys Asp Trp Val Val Ser Ala Ala His Cys
TAC CAC TTC TGT GGA GGC TCC CTG GTC AGC AAG GAC TGG GTG GTG TCT GCT GCT CAC TGC 202

                                   70                                       80
Tyr Lys Ser Arg Ile Glu Val Arg Leu Gly Glu His His Ile Arg Glu Asn Glu Gly Thr
TAC AAG TCC CGT ATT GAG GTG CGT CTG GGC GAG CAC CAC ATC AGG GAG AAC GAG GGA ACC 262

                                   90                                      100
Glu Gln Phe Ile Ser Ser Ser Met Val Ile Arg His Pro Thr Tyr Ser Ser Tyr Asn Ile
GAG CAG TTC ATC TCC TCC TCC ATG GTC ATC CGT CAC CCC ACC TAC AGC TCC TAC AAC ATC 322

                                  110                                      120
Asn Asn Asp Ile Met Leu Ile Lys Leu Ser Lys Pro Ala Thr Leu Asn Gln Tyr Val Gln
AAC AAC GAC ATC ATG CTG ATC AAG CTG AGC AAG CCC GCC ACC CTG AAC CAG TAT GTG CAG 382

                                  130                                      140
Pro Val Ala Leu Pro Thr Glu Cys Ala Ala Asp Gly Thr Met Cys Thr Val Ser Gly Trp
CCT GTG GCC CTT CCC ACC GAA TGT GCT GCT GAT GGC ACC ATG TGC ACC GTG TCT GGC TGG 442

                                  150                                      160
Gly Asn Thr Met Ser Ser Val Asp Asp Gly Asp Lys Leu Gln Cys Leu Asn Leu Pro Ile
GGA AAC ACC ATG AGC TCC GTT GAT GAC GGG GAC AAG CTT CAG TGC CTG AAC CTG CCA ATC 502

                                  170                                      180
Leu Ser His Ala Asp Cys Glu Asn Ser Tyr Pro Gly Met Ile Thr Pro Ser Met Phe Cys
CTC TCC CAC GCC GAC TGT GAG AAC TCC TAC CCT GGC ATG ATC ACC CCG TCC ATG TTC TGC 562

                                  190                                      200
Ala Gly Tyr Leu Glu Gly Gly Lys Asp Ser Cys Gln Gly Asp Ser Gly Gly Pro Val Val
GCT GGC TAC CTG GAG GGA GGC AAG GAC TCC TGC CAG GGA GAC TCC GGT GGC CCC GTG GTG 622

                                  210                                      220
Cys Asn Gly Val Leu Gln Gly Val Val Ser Trp Gly Tyr Gly Cys Ala Glu Arg Asp His
TGC AAC GGT GTG CTG CAG GGT GTT GTG TCC TGG GGA TAC GGA TGT GCC GAG AGG GAC CAC 682

                                  230                                      240
Pro Gly Val Tyr Ala Lys Val Cys Val Leu Ser Gly Trp Val Arg Asp Thr Met Ala Thr
CCC GGT GTC TAC GCC AAG GTC TGC GTT CTC TCG GGC TGG GTT CGC GAT ACC ATG GCA ACT 742

    242
Tyr ***
TAT TAA                                                                       748

ATGATCCTCTTCAGATTCCTGTAGCAGCTTCACATCAGGCTGTTAATGCAGAAAATGAATATGATCAATAAAGTTTCAA 827
AGAGAAATCAAAAAAAAAAAAAAAAAAAAAAAA -3'                                          860
```

Fig. 3

	1				5					10					15
Walleye Pollock	Met	Lys	Ser	Leu	Ile	Phe	Val	Leu	Leu	Leu	Gly	Ala	Val	Phe	Ala
Arctic cod	Met	Lys	Ser	Leu	Ile	Phe	Val	Leu	Leu	Leu	Gly	Ala	Val	Phe	Ala
Atlantic cod I	Met	Lys	Ser	Leu	Ile	Phe	Val	Leu	Leu	Leu	Gly	Ala	Val	Phe	Ala
Arabesque greenling	Met	Met	Ser	Leu	Val	Phe	Val	Leu	Leu	Ile	Gly	Ala	Ala	Phe	Ala
Antarctic fish	Met	Arg	Ser	Leu	Val	Phe	Val	Leu	Leu	Ile	Gly	Ala	Ala	Phe	Ala
Atlantic salmon I	Met	Ile	Ser	Leu	Val	Phe	Val	Leu	Leu	Ile	Gly	Ala	Ala	Phe	Ala
Anchovy I	Met	Arg	Pro	Leu	Val	Phe	Leu	Val	Leu	Leu	Gly	Ala	Ala	Phe	Ala
Flounder II	Met	Arg	Ser	Leu	Val	Phe	Val	Leu	Leu	Ile	Gly	Ala	Ala	Phe	Ala
Zebrafish	Met	Lys	Ala	Phe	Ile	Leu	Leu	Ala	Leu	Phe	Ala	Val	Ala	Tyr	Ala
Tilapia	Met	Lys	Tyr	Phe	Ile	Leu	Leu	Ala	Leu	Phe	Ala	Ala	Ala	Tyr	Ala
Bovine cationic	Met	Lys	Thr	Phe	Ilu	Phe	Leu	Ala	Leu	Leu	Gly	Ala	Ala	Val	Ala

Fig. 4

	1				5				9
Walleye pollock	---	---	---	---	---	Glu	Glu	Asp	Lys
Arctic cod	---	---	---	---	---	Glu	Glu	Asp	Lys
Atlantic cod I	---	---	---	---	---	Glu	Glu	Asp	Lys
Arabesque greenling	---	---	Leu	---	---	Glu	Glu	Asp	Lys
Antarctic fish	---	---	Thr	---	---	Glu	Glu	Asp	Lys
Atlantic salmon I	---	---	Thr	---	---	Glu	Asp	Asp	Lys
Anchovy I	---	---	---	---	---	Glu	Asp	Asp	Lys
Flounder II	---	---	Leu	---	---	Glu	Asp	Asp	Lys
Zebrafish	Ala	Pro	Leu	Gly	Asp	Asp	Asp	Asp	Lys
Tilapia	Ala	Pro	Ile	---	---	Glu	Asp	Asp	Lys
Bovine cat	Phe	Pro	Val	---	Asp	Asp	Asp	Asp	Lys

Fig. 5

```
                                    16            30   37          50                69
Walleye Pollock                     IVGGYECTRHSQAHQVSLNSGYHFCGGSLVSKDWVVSAAHCYKSRIEVRLGEHHIR
Arctic cod                          IVGGYECTKHSQSYQVSLNAGYHFCGGSLVSKDWVVSAAHCYKSRIEVRLGEHHIR
Atlantic cod I                      IVGGYECTKHSQAHQVSLNSGYHFCGGSLVSKDWVVSAAHCYKSVLRVRLGEHHIR
Arabesque greenling                 IVGGYECTPHTQAHQVSLNSGYHFCGGSLVSADWVVSAAHCYKSRVEVRLGEHNIR
Antarctic fish                      IVGGKECSPYSQPHQVSLNSGYHFCGGSLVNENWVVSAAHCYKSRVEVRMGEHHIR
Atlantic salmon I                   IVGGYECKAYSQTHQVSLNSGYHFCGGSLVNENWVVSAAHCYKSRVEVRLGEHNIK
Anchovy I                           IVGGYECQAHSQPHTVSLNSGYHFCGGSLVNENWVVSAAHCYKSRVEVRLGEHHIG
Flounder II                         IVGGYECTPHSQAHQVSLNSGYHFCGGSLVNENWVVSAAHCYKSRVEVRMGEHKIR
Zebrafish                           IVGGYECTKNGVPYQVSLNSGYHFCGGSLISNLWVVSAAHCYKSRVQVRLGEHNID
Tilapia                             IIGGYECAKNSVPYMVSLNIGYHFCGGSLISSTWAVSAAHCYQSSIQLRLGEHNIA
Bovine cationic                     IVGGYTCGANTVPYQVSLNSGYHFCGGSLINSQWVVSAAHCYKSGIQVRLGEDNIN
                                                                                   *

                                                   90                  110             127
Walleye pollock                     VNEGTEQFISSSSVIRHPSYSSYNINNDIMLIKLSKPATLNQYVQPVALPTECAAD
Arctic cod                          ENEGTEQFISSSMVIRHPTYSSYNINNDIMLIKLSKPATLNQYVQPVALPTECAAD
Atlantic cod I                      VNEGTEQYISSSSVIRHPNYSSYNINNDIMLIKLTKPATLNQYVHAVALPTECAAD
Arabesque greenling                 ATEGNEQFIRSSRVIRHPEYSSYNINNDIMLIKLSKPATLNRYVQTVALPTSCAPA
Antarctic fish                      VTEGKEQFISSSRVIRHPNYSSYNIDNDIMLIKLSKPATLNQYVQAVALPSSCAPA
Atlantic salmon I                   VTEGSEQFISSSRVIRHPNYSSYNIDNDIMLIKLSKPATLNTYVQPVALPTSCAPA
Anchovy I                           QNENTEQFIDSSRVIRHPQYSSYNIDNDVMLIKLSTPATLNQYVQPVALPSRCASA
Flounder II                         VNEGTEQFVSSSRVIRHPNYDSWNIDNDIMLIKLSKPATLNQYVKTVALPSSCAPA
Zebrafish                           VTEGTEQFINSEKVIRHPSYNSNTLDNDVMLIKLSSSAQINSYVKTVSLPSSCASS
Tilapia                             VNEGTEQFISSSRVIRHQSYNSYTLDNDIMLIKLSQPATLNSYVKTVSLPSGCAGA
Bovine cationic                     VVEGNEQFISASKSIVHPSYNSNTLNNDIMLIKLKSAASLNSRVASISLPTSCASA
                                                                           *

                                    133              150                 170            184+
Walleye pollock                     GTMCTVSGWGNTMSSVDD-GDKLQCLNLPILSHADCDNSYPGMITQSMFCAGYLEG
Arctic cod                          GTMCTVSGWGNTMSSVDD-GDKLQCLNLPILSHADCENSYPGMITPSMFCAGYLEG
Atlantic cod I                      ATMCTVSGWGNTMSSVAD-GDKLQCLSLPILSHADCANSYPGMITQSMFCAGYLEG
Arabesque greenling                 GTMCKVTGWGNTMSSTAD-GDKLQCLNIPILSEADCENSYPGMITKAMFCAGYLEG
Antarctic fish                      GTMCTVSGWGSTQSSSAD-GNKLQCLNIPILSDRDCDNSYPGMITDAMFCAGYLQG
Atlantic salmon I                   GTMCTVSGWGNTMSSTAD-SNKLQCLNIPILSYSDCNNSYPGMITNAMFCAGYLEG
Anchovy I                           GTMCLVAGWGNTMSNVS--GDKLQCLQIPILSDRDCDNSYPGMITDAMFCAGYLEG
Flounder II                         GTMCKVSGWGNTMSSADN-GDLLQCLDIPILSFSDCNNAYPGMITDSMFCAGYLEG
Zebrafish                           GTSCLISGWGNMSAGSSNYPSRLMCLNAPILSDSTCRNAYPGQISSNMFCAGFMEG
Tilapia                             GTSCLISGWGNTSTSGSNYPDRLMCLNAPILSDTDCRNSYPGEITNNMFCAGFLEG
Bovine cationic                     GTQCLISGWGNTKSSGTSYPDVLKCLKAPILSDSSCKSAYPGQITSNMFCAGYLEG

                                     188+        200  209      219221+     230       240
Walleye pollock                     GKDSCQGDSGGPVVCNGVLQGVVSWGYGCAERDHPGVYAKVCVLSGWVLDTMASY
Arctic cod                          GKDSCQGDSGGPVVCNGVLQGVVSWGYGCAERDHPGVYAKVCVLSGWVRDTMATY
Atlantic cod I                      GKDSCQGDSGGPVVCNGVLQGVVSWGYGCAERDHPGVYAKVCVLSGWVRDTMANY
Arabesque greenling                 GKDSCQGDSGGPVVCNGELQGVVSWGYGCAQRDNPGVYAKVCLFNEWLETTMASY
Antarctic fish                      GKDSCQGDSGGPVVCNGELQGVVSWGYGCAERDHPGVYAKVCLFNDWLETSMANY
Atlantic salmon I                   GKDSCQGDSGGPVVCNGELQGVVSWGYGCAEPGNPGVYAKVCIFNDWLTSTMASY
Anchovy I                           GKDSCQGDSGGPVVCNGELQGVVSWGYGCAERDHPGVYAKVCIFTDWLQSTMASN
Flounder II                         EKDSCQGDSGGPVICNGELQGVVSWGYGCAERGNPGVYAKVCLFNDWLESTMASY
Zebrafish                           GKDSCQGDSGGPVVCNNQLQGIVSWGYGCAQRNKPGVYAKVCTSPPGSETP
Tilapia                             GKDSCQGDSGGPVVCNGQLQGIVSWGYGCAQRDRPGVYTKVCNYNSWISNTMANN
Bovine cationic                     GKDSCQGDSGGPVVCSGKLQGIVSWGSGCAQKNKPGVYTKVCNYVSWIKQTIASN
                                                  *     *
```

Table 1

Table 1 Primers for cDNA cloning of WP-T and AC-T

RT-RACE	5’-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3’
RT-RACE F1	5’-ATCGTCGGAGGGTATGAGTG-3’
RT-RACE R1	5’-AGTCACCCTGGCAAGAGTCC-3’
3’-RACE F1	5’-TCTGCGCTGGATACCTGGAG-3’
3’-RACE R1	5’-GGCCACGCGTCGACTAGTAC-3’
RT	5’-(p)CACACCGTTGCA-3’
5’-RACE F1	5’-ATGTTCTGCGCTGGCTACCT-3’
5’-RACE R1	5’-AGACGCACCTCAATACGGGA-3’
5’-RACE F2	5’-AAGGACTCTTGCCAGGGTGA-3’
5’-RACE R2	5’-TGTAGCAGTGAGCAGCAGAC-3’

Table 2

Table 2 Amino acid sequence identities of WP–T and AC–T to other trypsins

	Walleye pollock	Arctic cod
Walleye pollock	–	95
Arctic cod	95	–
Atlantic cod I	93	91
Arabesque greenling	85	83
Antarctic fish	82	81
Atlantic salmon I	82	80
Anchovy	82	80
Flounder II	81	79
Zebrafish	65	63
Tilapia	69	70
Bovine cat	64	63

The names of trypsins are the same in Fig. 3.

Table 3

Table 3 Contents of charged amino acids in WP–T and AC–T

	Whole (%)[*1]	*N*–terminal region (%)[*2]	Hyd/Cha[*3]
Walleye pollock	19	24	1.00
Arctic cod	19	21	1.33
Atlantic cod I	19	24	1.00
Arabesque greenling	19	17	1.60
Antarctic fish	19	17	1.80
Atlantic salmon I	15	17	1.60
Anchovy	19	17	1.60
Flounder II	19	17	1.60
Zebrafish	14	10	3.67
Tilapia	14	10	3.67
Bovine cat	13	3	10.00

[*1] The contents of charged amino acid residues in the whole trypsin molecule.

[*2] The contents of charged amino acid residues at the *N*-terminal region (positions 20-50) of trypsin.

[*3] The ratio between the number of hydrophobic amino acids to the number of charged amino acids at the *N*-terminal region.

The names of trypsins are the same in Fig. 3.

Table 4

Table 4 Contents of negative charged amino acids at the Ca^{2+}–binding regions of trypsins

	Nega/(Nega+Posi) (%)*
Walleye pollock	50
Arctic cod	57
Atlantic cod I	50
Arabesque greenling	50
Antarctic fish	43
Atlantic salmon I	60
Anchovy	67
Flounder II	50
Zebrafish	80
Tilapia	75
Bovine cat	100

* The proportion of negative charged amino acids to charged amino acids at the Ca^{2+}–binding region of trypsin.

The names of trypsins are the same in Fig. 3.