A thermostable trypsin from freshwater fish Japanese dace (*Tribolodon hakonensis*): a comparison of the primary structures among fish trypsins

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Abstract

Trypsin from Japanese dace (*Tribolodon hakonensis*) (JD-T) living in freshwater (2-18 ºC) was purified. JD-T represented typical fish trypsin characteristics regarding the effects of protease inhibitor, calcium-ion and pH. For the effect of temperature, JD-T quite resembled to the trypsins from tropical-zone marine fish and freshwater fish (the catfish cultured in Thailand), i.e., the optimum temperature was 60 °C, and it was stable below 60 °C at pH 8.0 for 15 min incubation. From the data, it seemed that the trypsin from freshwater fish is thermostable in spite of the fact that their habitat temperatures are low. So, we determined the primary structure of JD-T to discuss its thermostability-structure relationship. JD-T possessed basic structural features of fish trypsin such as the catalytic triad, the Asp189 residue for substrate specificity, twelve Cys residues forming six disulfide-bridges, and the calcium-ion-binding loop. On the other hand, the contents of charged amino acid residues in whole JD-T molecule (16.2 %) and N-terminal region (13.8 %) were similar to those of tropical-zone marine fish and other freshwater fish trypsins. Then, JD-T conserved the five amino acid residues (Glu70, Asn72, Val75, Glu77 and Glu80) coordinate with calcium-ion, and the proportion of negatively charged amino acids to charged amino acids in the calcium-ion-binding region of JD-T (75.0 %) was equivalent to those of tropical-zone marine fish and freshwater fish trypsins. Therefore, it was suggested that the high thermostability of JD-T are stemmed from these structural specificities.

Keywords

Freshwater fish • Japanese dace • Primary structure • Thermostability • *Tribolodon hakonensis* • Trypsin

Introduction

Trypsin (EC 3.4.21.4) is an important serine protease, and it exists in a wide variety of organisms from prokaryotes to eukaryotes (Rypniewski et al. 1994). In mammals, trypsin is synthesized as a precursor in the pancreatic acinar cells and secreted into the intestine. In the intestine, trypsin acts as a digestive enzyme, and it is also responsible for activating all the pancreatic enzymes including itself (Rypniewski et al. 1994). Catalytic

mechanism and structural properties of mammalian pancreatic trypsin have been extensively studied (Walsh 1970; Kossiakoff et al. 1977). Previously, we characterized many kinds of marine fish trypsins from Japanese anchovy (Kishimura et al. 2005), true sardine (Kishimura et al. 2006a), arabesque greenling (Kishimura et al. 2006a), yellowfin tuna (Klomklao et al. 2006a), spotted mackerel (Kishimura et al. 2006b), yellow tail (Kishimura et al. 2006c), brown hakeling (Kishimura et al. 2006c), tongol tuna (Klomklao et al. 2006b), jacopever (Kishimura et al. 2007), elkhorn sculpin (Kishimura et al. 2007), skipjack tuna (Klomklao et al. 2007a), bluefish (Klomklao et al. 2007b), Atlantic bonito (Klomklao et al. 2007c), walleye pollock (Kishimura et al. 2008), Pacific cod (Fuchise et al. 2009), saffron cod (Fuchise et al. 2009), threadfin hakeling (Kishimura et al. 2010), and Pacific saury (Klomklao et al. 2010). Then, it was clarified the strong positive correlation between habitat temperature of marine fish and thermostability of their trypsins (Kishimura et al. 2008). That is, the trypsins from cold-zone marine fish showed a lower optimum temperature and thermostability than those of temperate-zone marine fish, tropical-zone marine fish, and mammals. In addition, we investigated their structural properties to discuss thermostability-structure relationship (Kanno et al. 2011a; Kanno et al. 2011b). As a result, it was suggested that the structural properties of N-terminal region and calcium-ion-binding region of marine fish trypsins are closely related to their thermostability. On the other hand, some researchers reported the studies regarding purification and enzymatic characterization of trypsins from freshwater fish (Cao et al. 2000; Liu et al. 2007; Lu et al. 2008; Zhou et al. 2012; Khangembam and Chakrabarti 2015). However, they had not deeply discussed the structure-function relationship.

In the previous study, we reported that the characteristics of trypsin from the viscera of hybrid catfish cultured in Thailand; depth of the culture pond is about 0.7 m, and the water temperature measuring 20-30 cm in depth is 25-40 ºC on April-May or 23-28 ºC on December (Klomklao et al. 2011). The purified catfish trypsin showed high optimum temperature and thermostability, i.e., it retained approximately 60 % of enzymatic activity after the incubation at 60 °C, pH 8.0 for 15 min like tropical-zone marine fish trypsins. Therefore, in this study, we purified a trypsin (JD-T) from the intestine of Japanese dace (*Tribolodon hakonensis*) captured in freshwater of lower water temperature (2-18 ºC) and examined its enzymatic properties, especially thermostability to

discuss the relationship between habitat temperature of freshwater fish and thermostability of the freshwater trypsin. Then, we determined the primary structure of JD-T to consider thermostability-structure relationship of freshwater fish trypsin.

Materials and methods

Materials

Japanese dace (*T. hakonensis*) was captured at the river of Hakodate, Hokkaido prefecture, Japan, in May. Water temperature of the river is the range from 2 ºC (in winter) to 18 ºC (in summer). The intestine samples for purification were stored at -30 °C and the samples for RNA extraction (about 300 mg) immersed into RNAlater solution (Applied Biosystems; Thermo Fisher Scientific, Inc., CA, USA) were stored at -80 °C. Sephacryl S-200 and Sephadex G-50 were purchased from GE healthcare (Little Chalfont, UK). Diethylaminoethyl (DEAE)-cellulose was purchased from Whatman; GE healthcare (Maidston, UK). N^{α}-*p*-Tosyl-L-Arg methyl ester hydrochloride (TAME) and phenylmethansulfonyl fluoride (PMSF) were obtained from Wako Pure Chemicals (Osaka, Japan). Ethylenediamine tetraacetic acid (EDTA), 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), soybean trypsin inhibitor, N^{α}-*p*-Tosyl-L-lys chloromethyl ketone (TLCK), and Pepstatin A were purchased from Sigma Chemical Co. (Mo, USA).

Purification of trypsin

Defatted powder of the intestine from Japanese dace was prepared by the method of Kishimura and Hayashi (2002). Crude protein 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 solution was centrifuged (H-2000B, Kokusan, Tokyo, Japan) at 10,000 × g for 10 min, and then the supernatant was concentrated by lyophilization into crude enzyme.

The crude enzyme was applied to a Sephacryl S-200 gel filtration (3.9 × 64 cm) column pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and the proteins were eluted with

the same buffer. After measuring the trypsin activity of each fraction, the main trypsin fraction was concentrated by lyophilization. The concentrated fraction was then applied to a Sephadex G-50 gel filtration column (3.9 × 64 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and the proteins were eluted with the same buffer. After measuring the trypsin activity of each fraction, the main trypsin fraction was concentrated by lyophilization. The concentrated fraction was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$. The dialysate 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 0-0.4 M NaCl in the same buffer. The fraction containing the trypsin activity was eluted at around 0.3-0.45 M NaCl, and the fraction was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$. The dialysate was concentrated by lyophilization and used for further studies as purified trypsin.

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. Purity of the enzyme was checked by polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 0.1 % SDS-13.75 % polyacrylamide slab-gel by the method of Laemmli (1970). Native-PAGE was carried out using a 12.5 % polyacrylamide slab-gel with a Tris-HCl buffer at pH 8.9. The gel was stained with 0.1 % Coomassie Brilliant Blue R-250 in 50 % methanol-7 % acetic acid, and background of the gel was destained with 7 % acetic acid. To analyze the *N*-terminal sequence, the purified trypsin was electroblotted to polyvinylidene difluoride (PVDF) membrane (Mini ProBlott Membranes, Applied Biosystems; Thermo Fisher Scientific, Inc., CA, USA) after SDS-PAGE. The amino acid sequence of the trypsin was analyzed by using a protein sequencer, Procise 492 (Perkin Elmer, CA, USA).

Assay for enzyme activity

Trypsin activity was measured by the method of Hummel (1959) using TAME as a substrate, and one unit of the activity was defined as the amount of enzyme hydrolyzing one micromole of TAME in a minute. The effect of protease inhibitors was determined according to the method of Klomklao et al. (2004), i.e. the

trypsin was incubated with an equal volume of the proteinase inhibitor solution (final concentration of 1 mM PMSF, 1 mg/ml soybean trypsin inhibitor, 5 mM TLCK, 0.01 mM E-64, 0.01 mM Pepstatin A, or 2 mM EDTA). After the incubation at 25 °C for 15 min, the remaining activity was measured and the inhibition rate (%) was then calculated.

Characterization of trypsin

The pH dependence of the trypsin was determined in 50 mM buffer solutions (acetic acid-sodium acetate: pH 4.0-7.0; Tris-HCl: pH 7.0-9.0; glycine-NaOH: pH 9.0-11.0) at 30 °C. The temperature dependence of the trypsin was determined at pH 8.0 and at the range of 30-80 °C. The effects of temperature and pH on the stability of the trypsin were determined by incubating the trypsin at pH 8.0 for 15 min at a range of 30-80 °C and by incubating the enzyme at 30 °C for 30 min at a range of pH 4.0-11.0, respectively. The effect of calcium-ion was examined by incubating the trypsin at 30 °C and at pH 8.0 in the presence of 10 mM EDTA or 10 mM $CaCl_2$. The remaining activities were determined at 30 ºC and pH 8.0.

Data collection related to thermostability of fish trypsins

To clarify the relationship between habitat temperature of fish and thermostability of the fish trypsin, the T_{50} values, showing the temperature that 50% enzyme activity after incubation at pH 8.0 for 15 min, were obtained. The trypsin used in this study were as follows: Japanese dace (this study); anchovy trypsin I and anchovy trypsin II (Kishimura et al., 2005); arabesque greenling and Japanese sardine (Kishimura et al., 2006a); Spotted mackerel (Kishimura et al., 2006b); brown hakeling and yellowtail (Kishimura et al., 2006c); Elkhorn sculpin (Kishimura et al., 2007); walleye pollock (Kishimura et al., 2008); Pacific cod (Fuchise et al., 2009); threadfin hakeling (Kishimura et al., 2010); Pacific saury (Klomklao et al., 2010); tongol tuna and yellowfin tuna (Klomklao et al., 2006a); skipjack tuna (Klomklao et al., 2007a) and; hybrid catfish (Klomklao et al., 2011).

Sequence comparison

The amino acid sequences of trypsin were obtained as follows: Grass carp (*Ctenopharyngodon idella*: Accession No. AB698820.1); Topmouth culter (*Culter alburnus*: Ruan et al. 2010); Snakehead (*Channa argus*: Zhou et al. 2012); Zebrafish (*Danio rerio*: Accession No. NP_571783); Tilapia (*Oreochromis niloticus*: Accession No. AY510093); Arabesque greenling (*Pleurogrammus azonus*: Kanno et al. 2011a); Walleye pollock (*Theragra chalcogramma*: Kanno et al. 2011b); Arctic cod (*Boreogadus saida*: Kanno et al. 2011b); Atlantic cod I (*Gadus morhua*: Gudmundsdottir et al. 1993); Anchovy I (*Engraulis japonicus*: Ahsan et al. 2001); Anchovy II (*E. japonicus*: Ahsan et al. 2001); Flounder I (*Paralichthys plivaceus*: Accession No. AB029750); Barramundi (*Lates calcarifer*: XP_018542607); Mefugu (*Takifugu obscurus*: Accession No. GQ227559); Bovine cationic trypsin (Accession No. BC134797); Rat cationic trypsin (Accession No. EDM15437); and Dog cationic trypsin (Accession No. XP_532744). The alignment was prepared using the Molecular Evolutionary Genetics Analysis version X (MEGA X) software (www.megasoftware.net).

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

The cDNA cloning for the trypsin was carried out as described previously (Kanno et al. 2011a; Kanno et al. 2011b). Total RNA was prepared from pyloric ceca with a TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., CA, USA), and mRNA was purified using an Oligotex^{TM}-dt30 <Super> mRNA Purification Kit (TaKaRa, Kyoto, Japan). First strand cDNA was synthesized by reverse transcription using a SuperScript II^{TM} enzyme (Invitrogen, CA, USA) with the mRNA as a template and a primer of RT-RACE (Table 1). Then, PCR was carried out using an Amplitaq Gold enzyme (Applied Biosystems; Thermo Fisher Scientific, Inc., CA, USA) with the first strand cDNA as a template and a set of primer (forward primer: RT-RACE F1, reverse primer: RT-RACE R1: Table 1) 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, and 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 isolated from the positive clone using a Wizard SV

Gel and PCR Clean-Up System (Promega, WI, USA). To recover the full-length of cDNA sequence, 3'- and 5'-RACE were performed. The 3'-terminal cDNA fragments were amplified using an Amplitaq Gold enzyme with the first strand cDNA as template and a set of primer (forward primer: 3'-RACE F1, reverse primer: 3'-RACE R1: Table 1) 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, 72 ^{o}C for 60 s, and 1 cycle of 72 ^{o}C for 7 min. Then, 5'-RACE was performed using 5' Full RACE Core Set (TaKaRa, Kyoto, Japan) according to manufacture protocol using the primers in Table 1.

Nucleotide sequence was determined using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems; Thermo Fisher Scientific, Inc., CA, USA) with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc., CA, USA).

Results and discussion

Purification of Japanese dace trypsin

Japanese dace trypsin (JD-T) was purified 137-fold with a recovery of 34 % from the crude enzyme (Table 2), and it was not found any isozymes during the purification. JD-T showed a single band on SDS-PAGE and native-PAGE analyses, and its molecular weight was estimated to be approximately 24,000 using SDS-PAGE (Fig. 1). The activity of purified JD-T was completely inhibited (100 %) by PMSF, soybean trypsin inhibitor, and TLCK, while specific inhibitors of cysteine proteinase (E-64), aspartic proteinase (Pepstatin A), and metalloproteinase (EDTA) had no inhibitory effect (0 %). Then, the effect of calcium-ion on the thermostability of JD-T was investigated in the presence of 10 mM EDTA or 10 mM $CaCl_2$. The enzyme was fully stabilized at 30 °C and pH 8.0 up to 8 h incubation in the presence of $CaCl_2$, although the activity decreased in approximately 80 % after 8 h incubation in the presence of 10 mM EDTA (Fig. 2a). These data indicated that JD-T is a typical fish trypsin, and similar results have been reported in other fish trypsins (Kishimura et al. 2005; Kishimura et al. 2006a; Kishimura et al. 2006b; Kishimura et al. 2006c; Klomklao et al. 2006a; Klomklao et al. 2006b; Kishimura et al. 2007; Klomklao et al. 2007a; Klomklao et al. 2007b; Klomklao et al. 2007c; Kishimura et al. 2008; Fuchise et al. 2009; Kishimura et al. 2010; Klomklao et al. 2010; Kanno et al. 2010; and Klomklao

and Benjakul 2018).

Effect of pH and temperature on JD-T

JD-T effectively hydrolyzed TAME in the pH range from 7.0 to 9.0 with the optimum at pH 8.5 and was stable at 30 °C for 30 min in the pH range from 6.0 to 11.0 (Fig. 2b). The optimum pH and the instability at acidic pH of JD-T were closed to those of other fish trypsins (Hjelmeland and Raa 1982; Simpson and Haard 1984; Martinez et al. 1988; Asgeirsson et al. 1989; Castillo-Yanez et al. 2005; Kishimura et al. 2005; Kishimura et al. 2006a; Kishimura et al. 2006b; Kishimura et al. 2006c; Klomklao et al. 2006a; Klomklao et al. 2006b; Kishimura et al. 2007; Klomklao et al. 2007a; Klomklao et al. 2007b; Kishimura et al. 2008; Lu et al. 2008; Fuchise et al. 2009; Kishimura et al. 2010; Klomklao et al. 2010; Kanno et al. 2010; Bkhairia et al. 2016; and Klomklao and Benjakul 2018).

The influence of temperature on the activity of JD-T was assessed in the range of 30-80 °C (Fig. 2c). JD-T was active over a broad temperature range (20-80 °C) with the optimum at 60 °C. The optimum temperature of JD-T was similar to that of temperate-zone (60 °C) and tropical-zone marine fish trypsins (55-65 °C) (Kishimura et al. 2005; Kishimura et al. 2006a; Kishimura et al. 2006b; Kishimura et al. 2006c; Klomklao et al. 2006a; Klomklao et al. 2006b; Kishimura et al. 2007; Klomklao et al. 2007a; Klomklao et al. 2007b; Klomklao et al. 2007c; Klomklao et al. 2010; Bkhairia et al. 2016; and Klomklao and Benjakul 2018;). For thermostability, JD-T was stable below 60 °C at pH 8.0 for 15 min, but the activity was impaired quickly above 70 °C (Fig. 2c).

As shown in Fig. 3, the strong positive correlation was found in the habitat temperature of marine fish and the thermostability of the trypsin (T_{50} value in the figure shows the temperature at which enzyme activity is reduced 50% by incubation at pH 8.0 for 15 min). In the previous study, we reported that the trypsin from the viscera of hybrid catfish cultured in Thailand shows high optimum temperature (60 °C) and thermostability (T_{50} value: 62 °C in Fig. 3) (Klomklao et al. 2011). In the present study, JD-T indicated considerably high optimum temperature (60 °C) and thermostability (T_{50} value: 66 °C in Fig. 3), although

Japanese dace lives in the river where has low water temperature (2-18 ºC). From the results, it seemed that the trypsin from freshwater fish is thermostable in spite of their habitat temperature. Then, we investigated the primary structure of JD-T to reveal why JD-T possesses thermostable characteristics.

Structural property of JD-T

The isolated a cDNA clone encoding JD-T (Accession No. AB445492 in DDBJ) was composed of 871 bp, with an open reading frame of 726 bp from the ATG start codon to the TAA stop codon. It was not found any cDNA of isozymes during the cloning steps. The 5'-noncoding region of JD-T was 27 bp long. The polyadenylation signal (AATAAA) of JD-T occurred at 13 bp upstream from the first adenine of poly (A) track. The 3'-noncoding region of JD-T was 115 bp long. The open reading frame of JD-T encoded 242 amino acids starting from the first methionine, and the trypsin seemed to be synthesized as preproenzyme that contains hydrophobic signal peptide of 15 amino acid residues, acidic activation peptides of 5 amino acid residues, and mature trypsin of 222 amino acid residues (Fig. 4).

The signal peptide of JD-T had seven contiguous hydrophobic residues (Fig. 4). The Ala8 was substituted by Val residue in JD-T, which was reported that the hydrophobic core falls into two clusters (Watson 1984). The activation peptide of JD-T possessed a poly-anionic cluster of three Asp residues at positions 6–8 (Fig. 4). Louvard and Puigserver (1974) revealed that mammal's enterokinase or trypsin recognize the poly-anionic cluster to cleave the activation peptide from the proenzyme. Therefore, it was thought that the activation mechanism of JD-T is similar to that of mammalian trypsin. The primary sequence of JD-T showed the typical trypsin features (Fig 4). JD-T possessed twelve Cysteine residues, which formed disulfide-bridges in bovine trypsin (Huerou et al. 1990) (Cys22-Cys157, Cys42-Cys58, Cys128- Cys232, Cys136-Cys201, Cys168-Cys182, and Cys191-Cys220). Then, the catalytic triad (His57, Asp102 and Ser195), the consensus sequence (193-GDSGG-197) around Ser195, and the Asp189 residue for substrate specificity (Huerou et al. 1990; Krem et al. 1990) were conserved in JD-T. The S1 substrate-binding pocket (positions: 189–195, 214–220 and 225–228), loop 1 (positions: 184–188), loop 2 (positions: 221–225), and Tyr172 were also conserved, which

were related to substrate specificity (Hedstrom et al. 1992).

Structural properties related to high-thermostability of JD-T

Genicot et al. (1996) proposed that the thermostability and flexibility of fish trypsin are affected by a decrease in hydrophobicity of the protein and an increase in surface hydrophilicity as compared to mammalian counterparts. As shown in Table 4, JD-T showed the lower content of charged amino acid residues (Lys, Arg, Aps, Glu and His) (16.2 %) in the whole primary sequence compared to that of cold-zone (average: 18.9 %) and temperate-zone marine fish trypsins (average: 18.8 %), and the ratio was identical with that of tropical-zone marine fish trypsins. In the previous study, we clarified that the structure of N-terminal region (positions 20–50) is strongly related to the thermostability of marine fish trypsin (Kanno et al. 2011a; Kanno et al. 2011b). In the present study, the percentage of charged amino acids at the N-terminal region of JD-T (13.8 %) was equivalent to that of tropical-zone marine fish trypsins, which is lower than that of cold- (average: 21.5%) and temperate-zone marine fish trypsins (average: 14.9 %) (Table 4). Additionally, the ratio of hydrophobic amino acid residues (Trp, Phe, Leu, Ile, Val, Tyr and Pro) to charged amino acid residues at the N-terminal region of JD-T (the ratio of Hyd/Cha: 2.8) was nearest to that of tropical-zone marine fish trypsins (average: 2.4), as compared to cold- and temperate-zone marine fish trypsins (average: 1.2 and 2.2, respectively) (Table 4).

It is known that bovine trypsin requires calcium-ion for thermostability and the resistance of self-degradation. The stabilizing effect is accompanied by a conformational change in the trypsin molecule, resulting in a more compact structure (Walsh 1970; Bode et al. 1975). The calcium-ion-binding site of bovine trypsin is located at the external loop of the molecule, and five amino acid residues (Glu70, Asn72, Val75, Glu77 and Glu80) coordinate with calcium-ion (Bode et al. 1975). As shown in Fig 4, the calcium-ion coordination residues were conserved in JD-T and tropical-zone marine fish trypsins, whereas the substitutions of one or two amino acid residues existed in cold- and temperate-zone marine fish trypsins. Previously, we also found an interesting correlation between the thermostability and the proportion of negatively charged amino acids to charged amino acids (Nega/Cha) in the calcium-ion-binding region (Kanno et al. 2011a; Kanno et al. 2011b).

The Nega/Cha value of JD-T (75.0 %) was equal to those of tropical-zone marine fish trypsins, although the cold- and temperate-zone marine fish trypsins showed the lower values (average: 51.8 % and 61.1 %, respectively).

The structural specificities of JD-T were similar to those of other freshwater fish trypsins. Generally, the habitat environments of freshwater fishes are more variable and severe than those of marine fish, e.g., small area, shallow, fast flow, changeable water temperature, etc. Therefore, it was considered that freshwater fishes including Japanese dice might have thermostable trypsin to resist the change of habitat environments.

Conclusion

In this study, we purified a trypsin (JD-T) from Japanese dace living in freshwater (2-18 ºC) and examined its characteristics. JD-T showed general enzymatic properties in fish trypsin such as effect of protease inhibitor, effect of calcium-ion, optimum pH and pH stability, while the optimum temperature and thermostability of JD-T were the same as those of tropical-zone marine fish and freshwater fish (the catfish cultured in Thailand). From the results, it seemed that the trypsin from freshwater fish is thermostable in spite of their habitat temperature. In addition, it was clarified that JD-T possesses similar structural properties to those of tropical-zone marine fish and freshwater fish trypsins, i.e., the contents of charged amino acid residues in whole JD-T molecule and N-terminal region were similar to those of tropical-zone marine fish and other freshwater fish trypsins. Then, JD-T conserved the five amino acid residues coordinate with calcium-ion, and the proportion of negatively charged amino acids to charged amino acids in the calcium-ion-binding region of JD-T was equivalent to those of tropical-zone marine fish and freshwater fish trypsins. Therefore, it was suggested that the high thermostability of JD-T are stemmed from these structural specificities.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure Captions

Fig. 1 Electrophoresis of purified JD-T. (a) SDS-PAGE: Lane 1, protein standards and Lane 2, JD-T. (b) Native-PAGE of JD-T.

Fig. 2 Basic character of JD-T. (a) Effect of calcium-ion on the stability of JD-T. Symbols: the remaining activity in the presence of 10 mM $CaCl_2$ (closed circle) or 10 mM EDTA (closed triangle). (b) Effect of pH on the activity and stability of JD-T. Symbols: the optimum pH (closed circle) and the pH stability (closed triangle). (c) Effect of temperature on the activity and stability of JD-T. Symbols: the optimum temperature (closed circle) and the thermal stability (closed triangle).

Fig. 3 Relationship between habitat temperature of fish and thermostability of the fish trypsin. Trypsin sources: 1, Japanese dace; 2, hybrid catfish; 3, arabesque greenling; 4, walleye pollock; 5, Pacific cod; 6, threadfin hakeling; 7, Elkhorn sculpin; 8, brown hakeling; 9, Japanese sardine; 10, Spotted mackerel; 11, Pacific saury; 12, yellowtail; 13, anchovy trypsin I; 14, anchovy trypsin II; 15, skipjack tuna; 16, tongol tuna; and 17, yellowfin tuna. Symbols: Closed rhomboid (freshwater), Closed triangle (cold-zone marine), Closed square (temperate-zone marine) and Closed circle (tropical-zone marine).

Fig. 4 Comparison of the primary structure of JD-T with those of other fish and mammalian trypsins. The amino acids are numbered by the standard chymotrypsin numbering system (Hartley and Kauffman 1966). Dashes with gray highlight indicate the deletions. The dots show the same residues as JD-T. The black highlight means completely conserved residues within the alignment. The dotted bar indicates N-terminal region (positions 20-50) and one dot chain line indicates calcium-ion-binding region (positions 69-84). The residues of catalytic triad (His57, Asp102 and Ser195) and obligatory Asp189 are marked with asterisks. The trypsins from freshwater, cold-zone, temperate-zone, and tropical-zone were highlighted in green, blue, orange, and red, respectively.

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Table 1 Primers for cDNA cloning of JD-T

Name	Sequence
RT-RACE	5’- GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTT -3’
RT-RACE F	5’- ATCGTCGGAGGGTATGAGTG -3’
RT-RACE R	5’- AGTCACCCTGGCAAGAGTCC -3’
3’-RT-RACE F	5’- TCTGCGCTGGATACCTGGAG -3’
3’-RT-RACE R	5’- GGCCACGCGTCGACTAGTAC -3’
RT	5’- (P)TGGCCATGGTCT -3’
5’-RACE F1	5’- TGTGCTCAGAGAGACAACCC -3’
5’-RACE R1	5’- TGTAGCAGTGAGCAGCAGAC -3’
5’-RACE F2	5’- AAGGACTCTTGCCAGGGTGA -3’
5’-RACE R2	5’- AGAAGTGGTAGCCGGAGTTC -3’

(P) means Phosphorylation.

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Table 2 Purification of JD-T

Purification stages	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purity (fold)	Yield (%)
Crude enzyme	4,340	3,158	0.7	1	100
Sephacryl S-200	1,207	2,865	2	3	90
Sephadex G-50	48	2,245	47	67	71
DE-52	11	1,058	96	137	34

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Table 3 Effects of various inhibitors on the activity of JD-T

Inhibitors	Concentration	Inhibition (%)
Control	-	0
PMSF	1 mM	100
Soybean trypsin inhibitor	1 mg/mL	100
TLCK	5 mL	100
E-64	0.01 mM	0
EDTA	2 mM	0
Pepstatin A	5 mM	0

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

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Table 4 Contents of charged amino acids in fish and mammalian trypsins

Sources of trypsin	Whole (%)[a]	N-terminal region (%)[b]	Hyd/Cha[c]	Nega/Cha (%)[d]
Fresh water fish	Average: 14.6	Average: 12.1	Average: 3.3	Average: 74.5
Japanese dace	16.2	13.8	2.8	75.0
Grass carp	15.3	10.3	4.0	75.0
Topmouth culter	13.4	13.8	3.3	66.7
Snakehead	14.8	13.8	2.5	75.0
Zebrafish	14.2	10.3	3.7	80.0
Tilapia	13.5	10.3	3.7	75.0
Cold-zone marine fish	Average: 18.9	Average: 21.5	Average: 1.2	Average: 51.8
Arabesque greenling	18.9	17.2	1.6	50.0
Walleye pollock	18.9	24.1	1.0	50.0
Arctic cod	19.4	20.7	1.3	57.1
Atlantic cod I	18.5	24.1	1.0	50.0
Temperate-zone marine fish	Average: 18.8	Average: 14.9	Average: 2.2	Average: 61.1
Anchovy I	18.6	17.2	1.6	66.7
Anchovy II	18.9	13.8	2.5	66.7
Flounder	18.9	13.8	2.5	50.0
Tropical-zone marine fish	Average: 16.2	Average: 13.8	Average: 2.4	Average: 75.0
Barramundi	16.6	13.8	2.5	75.0
Mefugu	15.7	13.8	2.3	75.0
Mammal	Average: 13.7	Average: 5.7	Average: 6.7	Average: 94.4
Bovine cat	13.0	3.4	10.0	100.0
Rat cat	14.3	6.9	5.0	83.3
Dog cat	13.9	6.9	5.0	100.0

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

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

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

[d] The proportion of negatively charged amino acids to charged amino acids in the calcium-ion-binding loop.

The names of trypsin sources are the same as Fig. 4.

Fig. 1

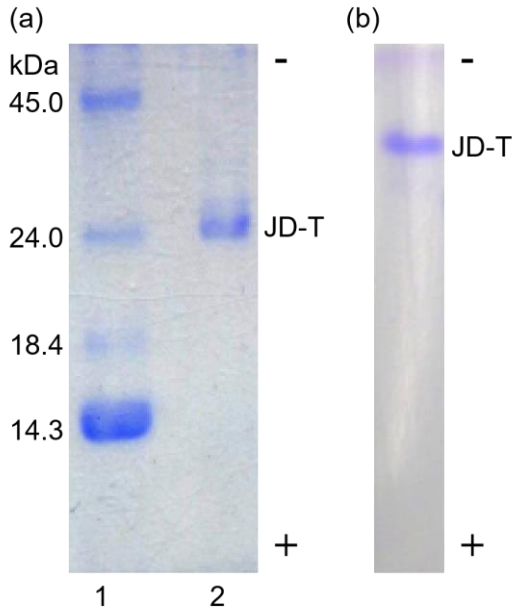


Fig. 2

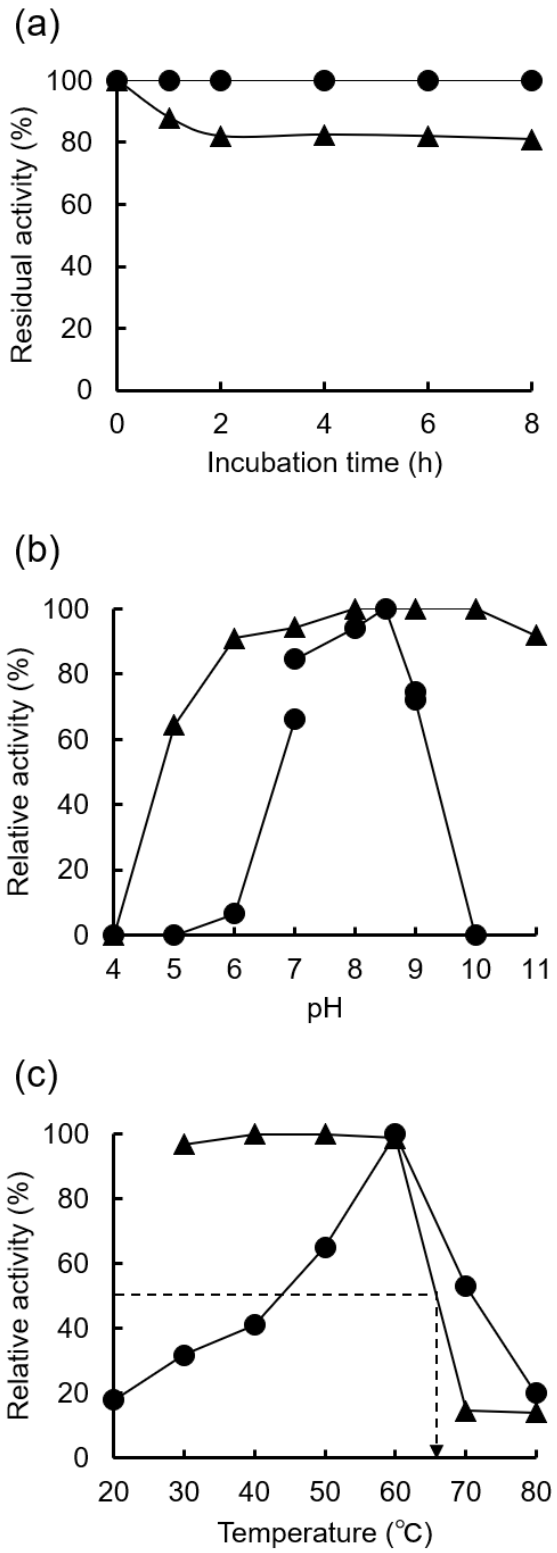

(a)
Residual activity (%)
100
80
60
40
20
0
0
2
4
6
8
Incubation time (h)
(b)
Relative activity (%)
100
80
60
40
20
0
4
5
6
7
8
9
10
11
pH
(c)
Relative activity (%)
100
80
60
40
20
0
20
30
40
50
60
70
80
Temperature (°C)

Fig. 3

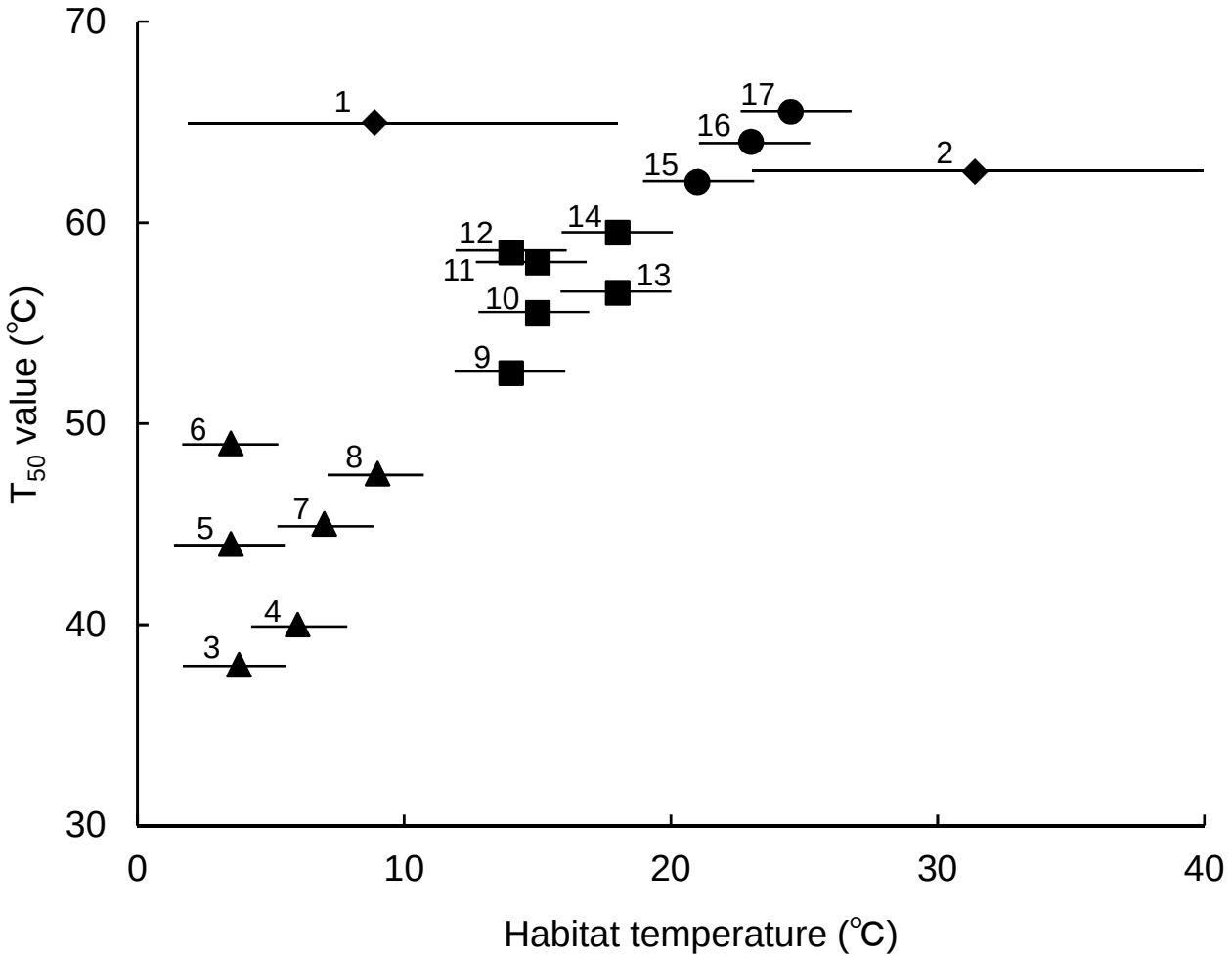
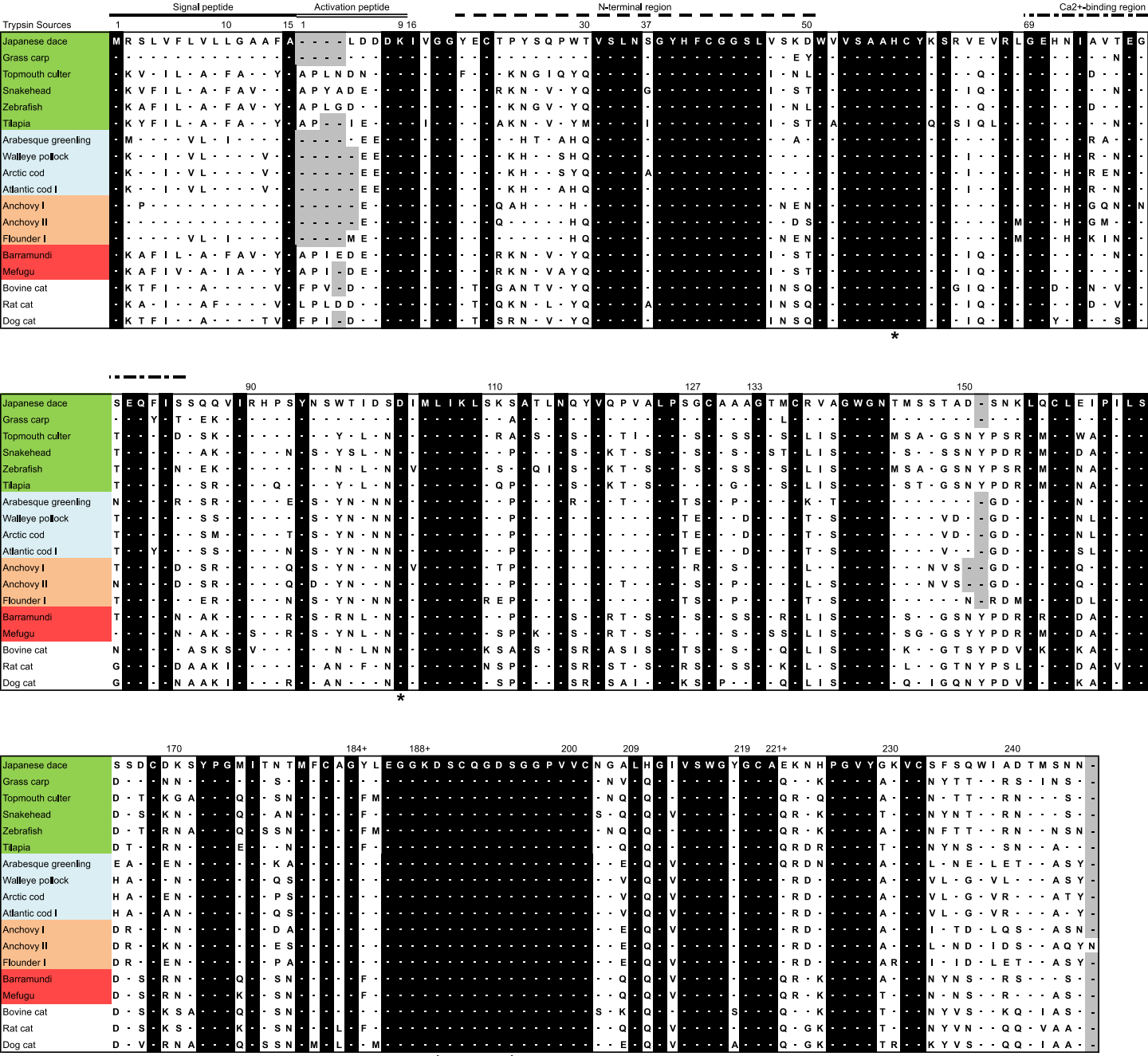

70
60
50
40
30
0
10
20
30
40
T50 value (℃)
Habitat temperature (℃)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

Fig. 4


Signal peptide
Activation peptide
N-terminal region
Ca2+-binding region
Trypsin Sources
Japanese dace
Grass carp
Topmouth culter
Snakehead
Zebrafish
Tilapia
Arabesque greenling
Walleye pollock
Arctic cod
Atlantic cod I
Anchovy I
Anchovy II
Flounder I
Barramundi
Mefugu
Bovine cat
Rat cat
Dog cat