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Purification and characteristics of trypsin from masu salmon (*Oncorhynchus masou*) cultured in fresh-water

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Abstract Trypsin from the pyloric ceca of masu salmon (*Oncorhynchus masou*) cultured in fresh-water was purified by a series of chromatographies including Sephadex S-200, Sephadex G-50 and diethylaminoethyl-cellulose to obtain a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native-PAGE. The molecular mass of the purified trypsin was estimated to be approximately 24,000 Da by SDS-PAGE. The enzyme activity was strongly inhibited by phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, and *N*^α-*p*-tosyl-L-lysine chloromethyl ketone. Masu salmon trypsin was stabilized by calcium ion. The optimum pH of the masu salmon trypsin was around pH 8.5 and the trypsin was unstable below pH 5.0. The optimum temperature of the masu salmon trypsin was around 60 °C and the trypsin was stable below 50 °C, like temperate-zone and tropical-zone fish trypsins. The *N*-terminal 20 amino acids sequence of the masu salmon trypsin was IVGGYECKAYSQPHQVSLNS, and its charged amino acid content was lower than those of trypsins from frigid-zone fish and similar to those of trypsins from temperate-zone and tropical-zone fish. In phylogenetic tree, the masu salmon trypsin was classified into the group of temperate-zone fish trypsin.

Keywords Fresh-water fish • Masu salmon • *Oncorhynchus masou*• Pyloric ceca • Trypsin • Thermal stability • Phylogenetic tree

Introduction

Trypsin (EC 3.4.21.4) is an important pancreatic serine protease synthesized as a proenzyme in the pancreatic acinar cells and secreted into the intestine of mammals. Trypsin acts as a digestive enzyme in the intestine, and it is also responsible for activating all the pancreatic enzymes including itself (Rypniewski et al. 1994). Enzymatic and structural properties of mammalian pancreatic trypsin have been extensively characterized (Walsh 1970; Kossiakoff et al. 1977).

Trypsin has been identified in a wide variety of organisms from prokaryotes to eukaryotes (Rypniewski et al. 1994). Recently, we isolated and characterized fish trypsins from the following marine species: 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), and walleye pollock (Kishimura et al. 2008). Consequently, fish trypsin was similar to mammalian pancreatic trypsin in its molecular weight, Ca^{2+} requirement, and reaction with substrates and inhibitors. However, fish trypsin was unstable and denatured irreversibly at acidic pH, unlike mammalian pancreatic trypsin. We also found that the trypsins from frigid-zone fish showed a lower optimum temperature and lower heat stability than those of temperate-zone fish, tropical-zone fish and mammals. Moreover, the relationship between habitat temperature of marine fish and thermostability of the fish trypsin indicated strong positive correlation (Kishimura et al. 2008).

On the other hand, there are few informations regarding trypsin from fresh-water

fish (Kristjansson 1991; Lu et al. 2008). Masu salmon is one of Salmonidae and inhabits the Asian side of the northern Pacific Ocean (Kato 1991). It usually spends one year in fresh water prior to seaward migration as a smolt. Then, masu salmon stays in the coastal sea for one or two years until beginning a spawning up its parent river. However, some specimens are stays in river during their all life. In this study, we purified a trypsin from the pyloric ceca of masu salmon (*O. masou*) cultured in fresh-water and investigated its enzymatic characteristics.

Materials and methods

Materials

Masu salmon (*Oncorhynchus masou*) was cultured in Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University. Sephadryl S-200 and Sephadex G-50 were purchased from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was purchased from Whatman (Maidston, England). N^{α} -*p*-Tosyl-L-arginine 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-lysine chloromethyl ketone (TLCK), and Pepstatin A were purchased from Sigma Chemical Co. (Mo, USA). All other chemicals were of reagent grade and were used without further purification.

Preparation of crude enzyme

Defatted powder of the pyloric ceca of masu salmon was prepared by the same method of Kishimura and Hayashi (2002). Trypsin was extracted by stirring from the defatted powder (10 g) in 50 volumes of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl₂ at 5 °C for 3 h. The extract was centrifuged (H-2000B, Kokusan, Tokyo, Japan) at 10,000 X g for 10 min, and the supernatant was lyophilized and used as crude trypsin.

Purification of trypsin

The crude trypsin (10 ml) was applied to a column of Sephadryl S-200 (3.9 X 64 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl₂ and the proteins were eluted with the same buffer. The main trypsin fraction was concentrated by lyophilization. The concentrated fraction was then applied to a Sephadex G-50 column (3.9 X 64 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl₂ and the proteins were eluted with the same buffer. 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 CaCl₂. The dialysate was applied to a DEAE-cellulose column (2.2 X 18 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl₂ and the proteins were eluted with a linear gradient of 0-0.5 M NaCl in the same buffer. Trypsin was eluted with 0.25-0.3 M NaCl, and the fraction was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl₂. The dialysate was concentrated by lyophilization and used for further studies as purified trypsin.

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 effect of protease inhibitors on trypsin activity was determined according to the method of Klomklao et al. (2004). Briefly trypsin was incubated with an equal volume of the protease inhibitor solution to obtain the final concentration designated (1 mM PMSF, 1 mg/ml soybean trypsin inhibitor, 5 mM TLCK, 0.01 mM E-64, 0.01 mM Pepstatin A and 2 mM EDTA). The remaining activity was measured after incubation of the mixture at 25 °C for 15 min, and the residual activity (%) was then calculated. The pH dependence of the enzyme 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 30 °C. The temperature dependence of the enzyme was determined at pH 8.0 and at various temperatures. The effects of temperature and pH on the stability of the enzyme were studied by incubating the enzyme at 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, respectively. The effect of CaCl₂ on the trypsin activity was examined by incubating the enzyme at 30 °C and at pH 8.0 in the presence of 10 mM EDTA or 10 mM CaCl₂.

Polyacrylamide gel electrophoresis

SDS-PAGE was carried out using a 0.1 % SDS-13.75 % polyacrylamide slab-gel according to the method described by 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 the background of the gel was destained with 7 % acetic acid.

Analysis of amino acid sequence

To analyze the *N*-terminal sequence, the purified enzyme was electroblotted to polyvinylidene difluoride (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, CA, USA).

Protein determination

The protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Results and discussion

Masu salmon trypsin was purified from the pyloric ceca by a series of chromatographies including Sephadex G-50, Sephadex G-200, and DEAE-cellulose. The purified enzyme from masu salmon was purified 24-fold with a recovery of 24 % from the crude trypsin (Table 1). The purified trypsin appears as a single band on SDS-PAGE and native-PAGE analyses (Fig. 1). The effects of various proteinase inhibitors on the activity of the masu salmon trypsin were determined (Table 2). PMSF, a serine protease inhibitor, and specific trypsin inhibitors, soybean trypsin inhibitor and TLCK, strongly inhibited (100 %) the activity of the Masu salmon trypsin. While, specific inhibitors of cysteine proteinase (E-64), aspartic proteinase (Pepstatin A), and metalloproteinase (EDTA) had no inhibitory effect on the trypsin activity. These protease inhibitory assays indicated that the purified enzyme was

revealed as trypsin. The molecular weight of the masu salmon trypsin was estimated as approximately 24,000 Da using SDS-PAGE (Fig. 1). The relative molecular mass of the masu salmon trypsin was similar to those of other fish trypsins (Simpson and Haard 1984; Asgeirsson et al. 1989; 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; Kishimura et al. 2008) except for those of rainbow trout (25,700 Da) (Kristjansson 1991), mandarin fish (21,000 Da and 21,500 Da) (Lu et al. 2008), Atlantic bonito (29,000 Da) (Klomklao et al. 2007b), and blufish (28,000 Da) (Klomklao et al. 2007c).

Thermal stability of the masu salmon trypsin was investigated in the presence of 10 mM EDTA or 10 mM CaCl₂. The enzyme showed high stability in the presence of CaCl₂, that is one of the typical characteristic of trypsin (Fig. 2). Similar results have been reported for the various kinds of fish trypsins (Kristjansson 1991; 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). Kossiakoff et al. (1977) reported that one calcium-binding site exists in bovine trypsin and the calcium-binding site stabilizes the protein toward thermal denaturation or autolysis. From crystallographic studies of bovine trypsin, Bode and Schwager (1975) have identified the five amino acid residues (Glu70, Asn72, Val75, Glu77, and Glu80) involved in calcium ion binding. Trypsin from Atlantic salmon conserved these amino acid residues (Male et al. 1995). Since the masu salmon trypsin obtained thermal stability by calcium ion, the trypsin seems to possess the primary calcium-binding site.

The influence of pH on the trypsin activity of the masu salmon is shown in Fig. 3a. TAME was hydrolyzed effectively by the enzyme in the pH range from 7.0 to 9.0, with an optimum around pH 8.5. The optimum pH of the masu salmon trypsin was the same as

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), but lower than those of rainbow trout (pH 9-10) (Kristjansson 1991) and bluefish (pH 9.5) (Klomklao et al. 2007c). The pH stability of the masu salmon trypsin is shown in Fig. 3b. The trypsin was stable at 30 °C for 30 min in the pH range from 6.5 to 10.0. Unlike mammalian trypsins, diminished stability of the trypsin was more pronounced after exposure at acidic pH. Instability at acidic pH was also observed for trypsins from other fish species (Martinez et al. 1988; Asgeirsson et al. 1989; Kristjansson 1991; 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; Lu et al. 2008).

Fig. 4a shows the temperature dependence of the masu salmon trypsin activity. The masu salmon trypsin was active over a broad temperature range (20-70 °C) with the optimum at about 60 °C. The optimum temperature of the masu salmon trypsin was similar to those of temperate-zone fish trypsins (60 °C) (Kishimura et al. 2005; Kishimura et al. 2006a; Kishimura et al. 2006b; Kishimura et al. 2006c; Kishimura et al. 2007), tropical-zone fish trypsins (55-65 °C) (Klomklao et al. 2006a; Klomklao et al. 2006b; Klomklao et al. 2007a; Klomklao et al. 2007b; Klomklao et al. 2007c), and rainbow trout trypsin (60 °C) (Kristjansson 1991). For thermal stability, the masu salmon trypsin was stable below 50 °C, but the activity was impaired quickly above 60 °C (Fig. 4b). As shown in Fig. 5, the relationship between habitat temperature of fish and thermal stability of the fish trypsin indicated strong positive correlation, and the thermal stability of the masu salmon trypsin was similar to those of temperate-zone fish and tropical-zone fish trypsins. The masu salmon is

cultured in fresh-water that temperature changes from 1 °C (winter season) to 20 °C (summer season), and the temperature in the summer season is similar to inhabitant temperature of temperate-zone and tropical-zone fish. Therefore, we thought that the masu salmon trypsin must have relatively higher thermal stability.

Fig. 6 shows the *N*-terminal amino acid sequence of the masu salmon trypsin aligned with those of other animal trypsins. The *N*-terminal amino acid sequence of the masu salmon trypsin was found to be IVGGYECKAYSQPHQVSLNS. The result indicates that the *N*-terminal of the masu salmon trypsin is unblocked. The *N*-terminal five amino acid sequences of the masu salmon trypsin (IVGGY) were identical to those of other animal trypsins except for tilapia trypsin (Fig. 6). Also, nearly all the trypsins shared the sequence (QVSLN) at position 15-19 (Fig. 6). The masu salmon trypsin had a charged Glu residue at position 6 similar to other fish trypsins, whereas Thr is most common in mammalian pancreatic trypsins (Fig. 6). Furthermore, the masu salmon trypsin characteristically conserved Cys residue at position 7 like the all other trypsins. The result indicates that the masu salmon trypsin may also have a disulfide bond to the corresponding residues (between Cys-7 and Cys-142) of bovine pancreatic trypsin (Stroud et al. 1974). These data lend confidence to the notion that the masu salmon trypsin belongs to the trypsin family of enzymes. On the other hand, the rate of the charged amino acid in the masu salmon trypsin (15.0 %) was similar to those of temperate-zone fish trypsins (11.0 %) and tropical-zone fish trypsins (12.1 %), whereas it was higher than those of mammalian trypsins (5.0 %) and lower than those of frigid-zone fish trypsins (19.2 %). Genicot et al. (1996) considered that thermal stability and flexibility of fish trypsin is affected by its overall decrease in hydrophobicity and increase in surface hydrophilicity as compared to mammalian counterparts. Therefore, such structural characteristics of the masu salmon trypsin may contribute its higher thermal stability. We also made the phylogenetic tree using *N*-terminal

20 amino acids sequences of the masu salmon trypsin and other vertebrate trypsins. As a result, the masu salmon trypsin should be classified into the group of temperate-zone fish trypsin in phylogenetic tree (Fig. 7). The result also indicates that the *N*-terminal 20 amino acids sequences of fish trypsins strongly relate with their thermal stability.

Conclusion

We purified a trypsin from the pyloric ceca of masu salmon cultured in fresh-water. The optimum temperature and thermal stability of the masu salmon trypsin were similar to those of temperate-zone and tropical-zone fish trypsins. The masu salmon trypsin also resembled in charged amino acid contents with those of trypsins from temperate-zone and tropical-zone fish. The reason why was masu salmon trypsin has high thermal stability is that the temperature of the fresh-water in summer season is similar to inhabitant temperature of temperate-zone and tropical-zone fish.

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Fig. 1. Electrophoresis of purified trypsin from masu salmon. (a) SDS-PAGE: Lane 1 contains protein standards [egg albumin (molecular weight, 45,000), bovine pancreatic trypsinogen (24,000), bovine milk β -lactoglobulin (18,400) and egg-white lysozyme (14,300)]. Lane 2 contains masu salmon trypsin; (b) Native-PAGE: Lane 1 contains masu salmon trypsin.

Fig. 2. Effect of calcium ion on the stability of masu salmon trypsin. The enzyme was kept at 30 °C and pH 8.0 for 0-8 h in the presence of 10 mM CaCl₂ (closed symbol) or 10 mM EDTA (open symbol), and then the remaining activities at 30 °C and pH 8.0 were determined.

Fig. 3. Effect of pH on the activity and stability of masu salmon trypsin. (a) 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 30 °C. (b) 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.

Fig. 4. Effect of temperature on the activity of masu salmon trypsin and its thermal stability. (a) The activity was determined at pH 8.0 and at various temperatures. (b): 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.

Fig. 5. Relationship between habitat temperature of fish and thermal stability of the fish trypsin. The 50 % denaturating temperature shows the temperature that the enzyme was denatured 50 % by incubation at pH 8.0 for 15 min at a range of 20-70 °C. Closed star; masu salmon trypsin (present study); Open triangle, walleye Pollock trypsin; Closed triangle, arabesque greenling trypsin (Kishimura et al., 2006a); Open square, true sardine trypsin (Kishimura et al., 2006a); Closed square, spotted mackerel trypsin (Kishimura et al., 2006b); Open circle, skipjack tuna trypsin (Klomklao et al., 2007); Closed circle, yellowfin tuna trypsin (Klomklao et al., 2006a); Open diamond, porcine pancreatic trypsin.

Fig. 6. Comparison of the *N*-terminal amino acid sequences of masu salmon trypsin with those of other vertebrates. Pacific cod (Fuchise et al., 2009); Saffron cod (Fuchise et al., 2009); Walleye pollock (Kishimura et al., 2008); Elkhorn sculpin (Kishimura et al., 2007); Arabesque greenling (Kishimura et al., 2006a); Brown hakeling (Kishimura et al., 2006c); Yellow tail (Kishimura et al., 2006c); Jacopever (Kishimura et al., 2007); Japanese anchovy (Kishimura et al., 2005); True sardine (Kishimura et al., 2006a); Japnese dace (DDBJ accession number AB445492); Skipjack tuna (Klomklao et al., 2007a); Tongol tuna (Klomklao et al., 2006b); Yellowfin tuna (Klomklao et al., 2006a); Lamprey (Roach et al., 1997); Piranha (DDBJ accession number AB470275); Zebrafish (DDBJ accession number AF541952); Tilapia (DDBJ accession number AY510093); Rat (Roach et al., 1997); Dog (Roach et al., 1997); Porcine (Hermodson et al. 1973); Bovine (Walsch, 1970); Human (Emi et al., 1986). Amino acid residues different from the masu salmon trypsin are shaded.

Fig. 7. Radical rootless phylogenetic tree of trypsins. The phylogenetic tree was made using the *N*-terminal 20 amino acid sequences of trypsins in Fig.6 by the programs of CLUSTAL W (Thompson et al. 1994) and Tree View (Page 1996). The branch length represents the evolutionary distance between the proteins. M-salmon (masu salmon; present study); P-cod (Pacific cod; Fuchise et al., 2009); S-cod (saffron cod; Fuchise et al., 2009); W-pollock (walleye pollok; Kishimura et al., 2008); E-sculpin (Elkhorn sculpin; Kishimura et al., 2007); A-greenling (arabesque greenling; Kishimura et al., 2006a); B-hakeling (Brown hakeling; Kishimura et al., 2006c); Y-tail (yellow tail; Kishimura et al., 2006c); Jacopever (Kishimura et al., 2007); J-anchor (Japanese anchovy; Kishimura et al., 2005); T-sardine (true sardine; Kishimura et al., 2006a); J-dace (Japanese dace; DDBJ accession number AB445492); S-tuna (skipjack tuna; Klomklao et al., 2007a); T-tuna (tongol tuna; Klomklao et al., 2006b); Y-tuna (yellowfin tuna; Klomklao et al., 2006a); Lamprey (Roach et al., 1997); Piranha (DDBJ accession number AB470275); Zebrafish (DDBJ accession number AF541952); Tilapia (DDBJ accession number AY510093); Rat (Roach et al., 1997); Dog (Roach et al., 1997); Porcine (Hermanson et al. 1973); Bovine (Walsch, 1970); Human (Emi et al., 1986).

Fig.1

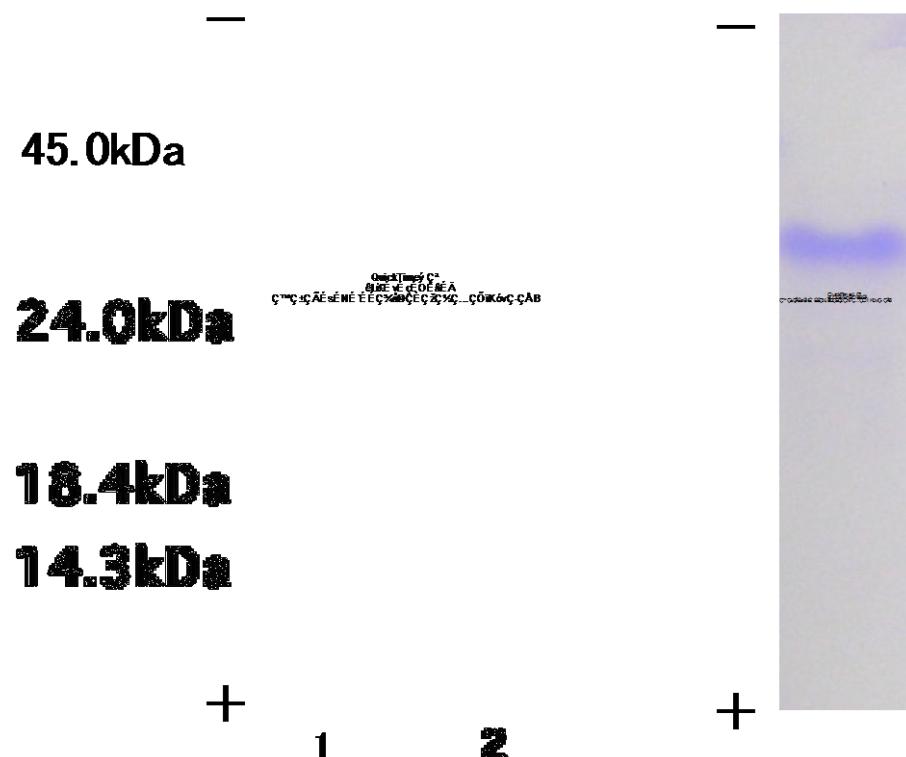


Fig.2

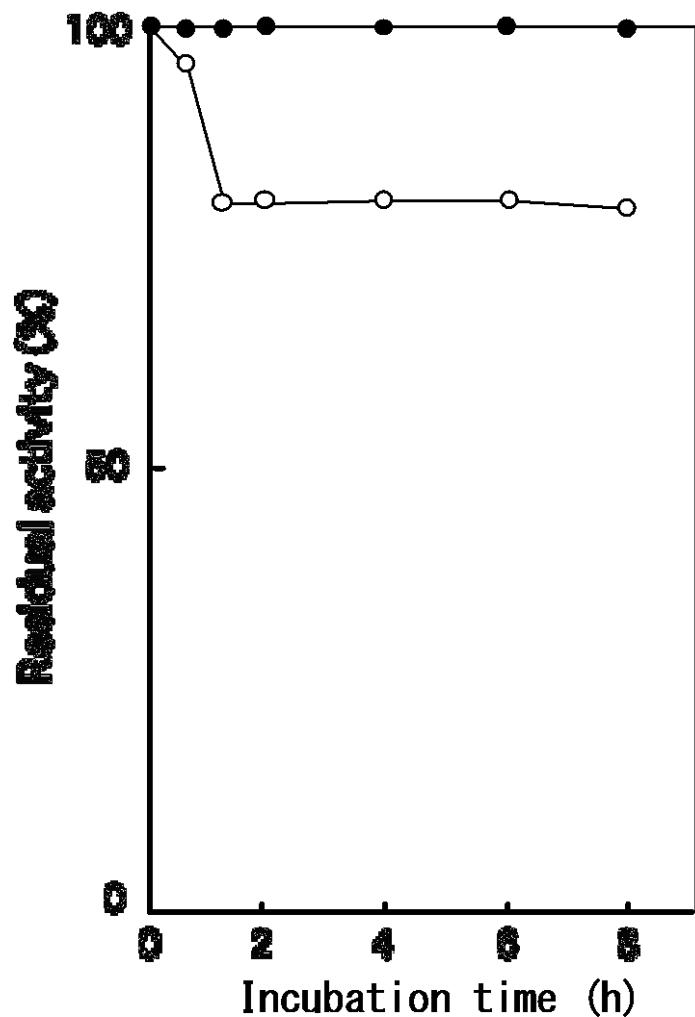


Fig.3

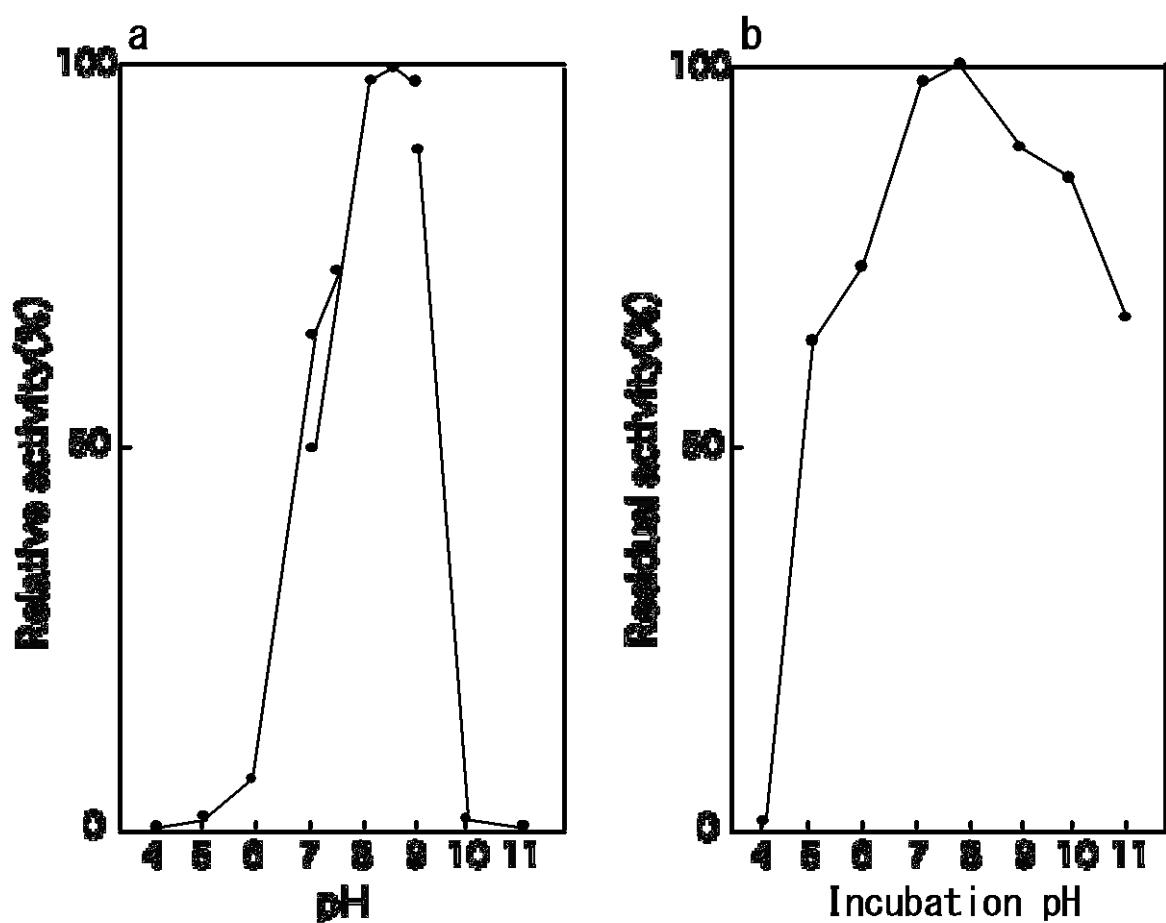


Fig.4

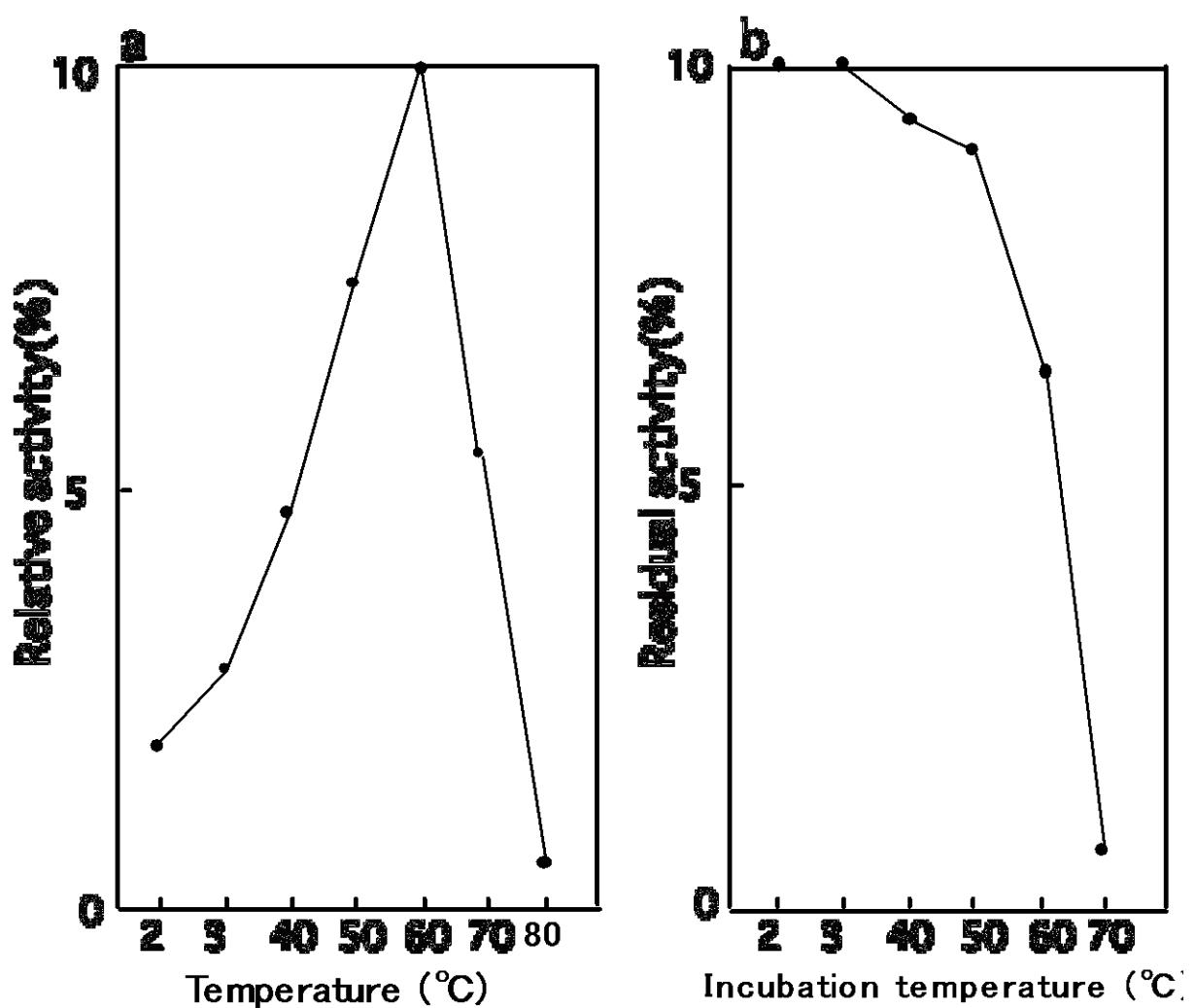


Fig.5

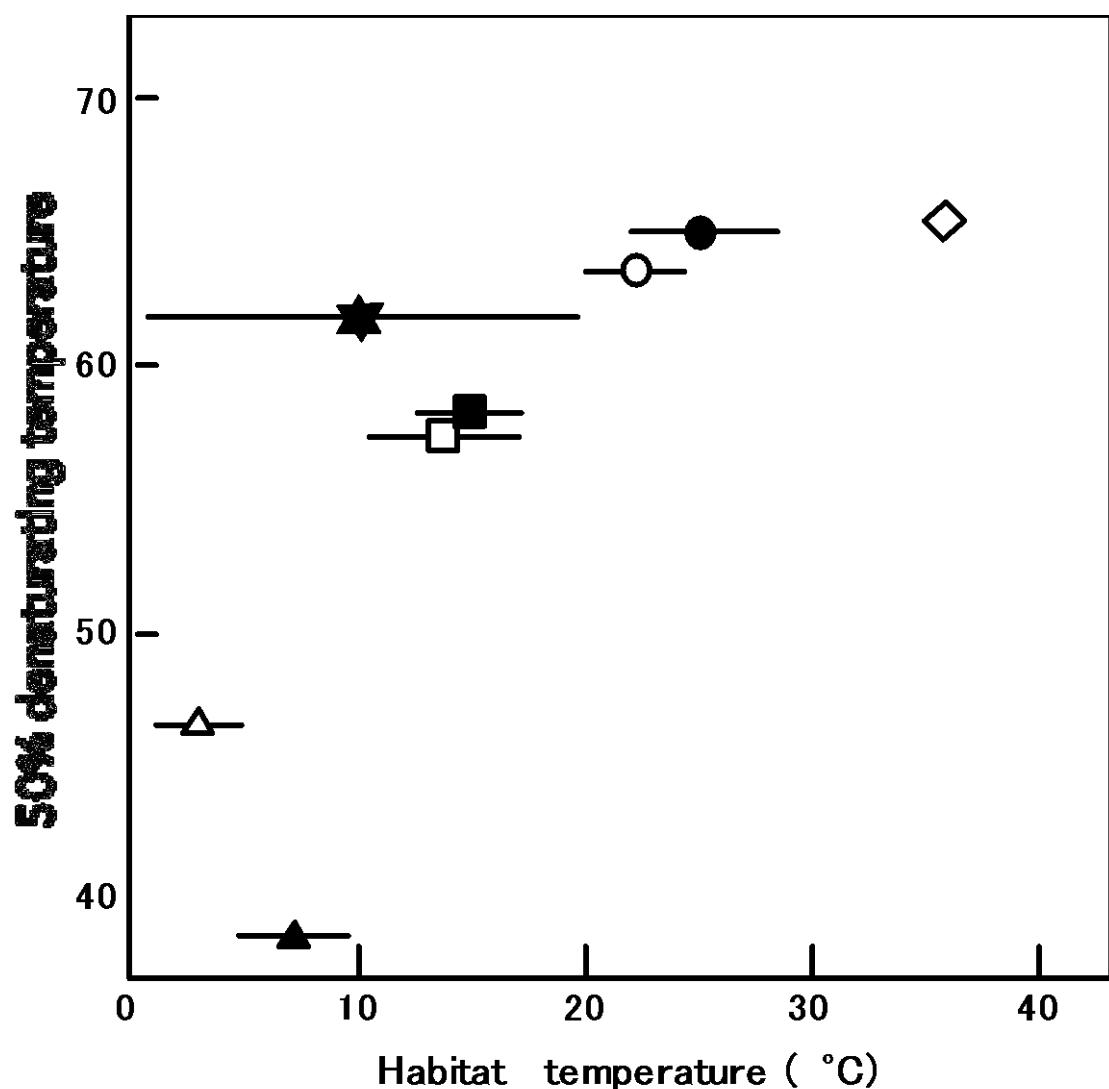


Fig.6

Masu salmon IVGGYECKAYSQPHQVSLNS

Frigid-zone fish

Pacific cod IVGGYECTRHSQAHQVSLNS

Saffron cod IVGGYECPRHSQAHQVSLNS

Walleye pollock IVGGYECTKHSQAHQVSLNS

Elkhorn sculpin IVGGYECTPHSQAHQVSLNS

Arabesque greenling IVGGYECTPHTQAHQVSLDS

Brown hakeling IVGGYECPKHSQPHQVSLNS

Temperate-zone fish

Yellow tail IVGGYECTPYSQPHQVSLNS

Jacopever IVGGYECKPYSQPHQVSLNS

Japanese anchovy IVGGYECQPYSQPHQVSLDS

True sardine IVGGYECKAYSQPWQVSLNS

Japanese dace IVGGYECTPYSQPWTVSLNS

Tropical-zone fish

Skipjack tuna IVGGYECQAHSQPHQVSLNS

Tongol tuna IVGGYECQAHSQPHQVSLNA

Yellowfin tuna IVGGYECQAHSQPPQVSLNA

Lampray IVGGYECAAHSQPWQVSLNI

Piranha IVGGYECKAHSQPWQVSLNV

Zebrafish IVGGYECTKNGVPYQVSLNS

Tilapia IIGGYECAKNNSVPYQMVSNI

Mammal

Rat IVGGYTCQENSVPYQVSLNS

Dog IVGGYTCEENSVPYQVSLNA

Porcine IVGGYTCAANSIPYQVSLNS

Bovine IVGGYTCGANTVPYQVSLNS

Human IVGGYNCEENSVPYQVSLNS

Fig.7

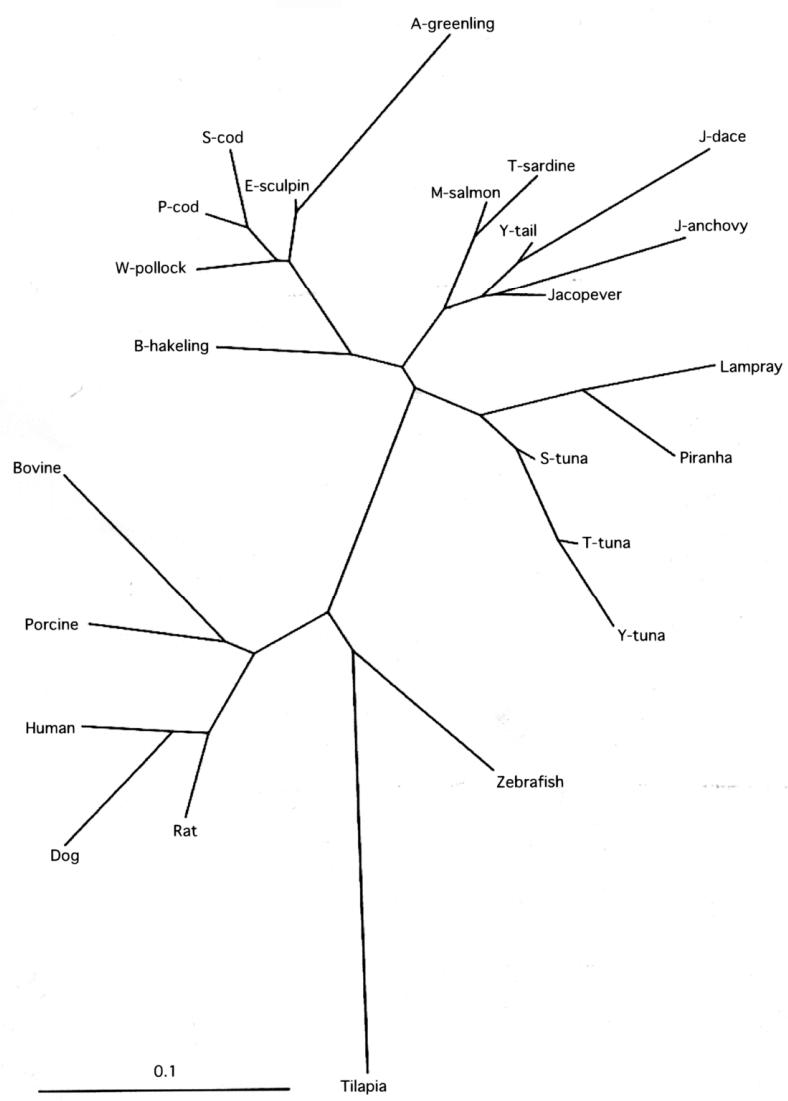


Table 1**Table 1**
Purification of trypsin from masu salmon

Purification stages	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purity (fold)	Yield (%)
Crude enzyme	1,294	8,411	6.5	1	100
Sephadryl S-200	398	6,066	15	2	70
Sephadex G-50	75	5,554	74	11	66
DE-52	13	2,035	156	24	24

Table 2**Table 2**
Effects of various inhibitors on the activity of trypsin from masu salmon^a

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

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