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Running title: Trypsin from yellow tail and brown hakeling

COMPARATIVE STUDY ON ENZYMATIC CHARACTERISTICS OF TRYPSINS FROM THE PYLORIC CECA OF YELLOW TAIL (*SERIOLA QUINQUERADIATA*) AND BROWN HAKELING (*PHYSICULUS JAPONICUS*)

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ABSTRACT

*Trypsins from the pyloric ceca of two fish species, yellow tail (*Seriola quinqueradiata*) and brown hakeling (*Physiculus japonicus*) were purified by a series of chromatographic separations. Purity increased 63- and 106-fold with approximately 55 and 10 % yield for yellow tail trypsin and brown hakeling trypsin, respectively. Final enzyme preparations were homogeneous in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the molecular weights of both enzymes were estimated to be 24 kDa by SDS-PAGE. Yellow tail and brown hakeling trypsins had maximal activity at pH 8.0 for hydrolysis of TAME and was unstable at acidic pH. The optimum temperatures for yellow tail and brown hakeling trypsins were 60 C and 50 C, respectively. Yellow tail trypsin was stable up to 50 C, whereas brown hakeling was stable up to 40 C. Both trypsins were stabilized by calcium ions. The activities of both trypsins were strongly inhibited by soybean trypsin inhibitor and TLCK and partially inhibited by EDTA. The N-terminal amino acid sequences of yellow tail trypsin and brown hakeling trypsin were determined as IVGGYECKPYSQPHQVSLNS and IVGGYECTPHSQAHQVSLNS, respectively.*

INTRODUCTION

Trypsin (EC3.4.21.4) specifically cleaves the peptide bonds on the carboxyl side of arginine and lysine residues and has been identified in a wide variety of organisms (Rypniewski *et al.* 1994). Bovine trypsin was among the first proteolytic enzymes isolated and analyzed. In the pancreas, trypsin not only functions as a digestive enzyme, but also is responsible for activating all the pancreatic enzymes, including itself, by cleaving a short activation peptide from the amino-terminus of inactive zymogens. Mammalian pancreatic trypsin and its proenzyme have been extensively characterized (Walsch 1970; Kossiakoff *et al*. 1977).

On the other hand, the marine environment presents an excellent opportunity for supplying commercial enzymes. Marine animals possess the same functional classes of enzymes as other living organisms, which enable them carry out virtually the same metabolic activities. These enzymes are present in the tissues of animals and may be recovered in active and stable forms for commercial use. In several of the major fish-producing countries, the by-products of seafood harvesting comprise about 50% of the entire harvest. These materials are largely underutilized and discarded as waste. However, this abundant material also includes the enzyme-rich digestive organs, and the enzymes may be recovered and used for a range of commercial applications. Furthermore, fishes are poikilothermic, so their survival in cold waters required adaptation of their enzyme activities to low temperatures of their habitats. Enzymes from cold adapted fish species thus often have higher enzymatic activities at low temperatures than their counterparts from warm-blooded animals (Simpson and Haard 1984; Asgeirsson *et al.* 1989; Kristjansson 1991). High activity of fish enzymes at low temperatures may be interesting for several industrial applications of enzymes, such as in certain food processing operations, i.e. production of cheese, cold fining of beverages and a

digestive aid in fish feed (Haard 1992) that require low processing temperatures. Moreover, relatively lower thermal stability, often observed with fish enzymes, may also be beneficial in such applications as the enzymes can be inactivated more readily, with less heat treatment, when desired in a given process (Simpson and Haard 1987).

Trypsins have been isolated and characterized from several species of fish including capelin (Hjelmeland and Raa 1982), catfish (Yoshinaka *et al.* 1983), Greenland cod (Simpson and Haard 1984), anchovy (Martinez *et al.* 1988), Atlantic cod (Asgeirsson *et al.* 1989), rainbow trout (Kristjansson 1991), Monterey sardine (Castillo-Yanez *et al.* 2005), and Japanese anchovy (Kishimura *et al.* 2005a). In previous studies, we isolated trypsins from the viscera of true sardine (*Sardinops melanostictus*) and from the pyloric ceca of arabesque greenling (*Pleuroprammus azonus*) (Kishimura *et al.* 2005b). The characteristics of these trypsins suggest that the viscera of true sardine and the pyloric ceca of arabesque greenling would be a potential source of trypsin for food processing operations. Such information is not available for trypsin from the pyloric ceca of yellow tail and brown hakeling. In this study, we purified trypsins from the pyloric ceca of yellow tail (*Seriola quinqueradiata*) and brown hakeling (*Physiculus japonicus*) and compared their characteristics to those of porcine pancreatic trypsin.

MATERIALS AND METHODS

The yellow tail (*S. quinqueradiata*) and brown hakeling (*P. japonicus*) were caught off Hakodate, Hokkaido Prefecture, Japan. Sephacryl S-200 and Sephadex G-50 were purchased from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was purchased from Whatman (Maidston, England). N^{α}

-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) and ethylenediaminetetraacetic acid (EDTA) were obtained from Wako Pure Chemicals (Osaka, Japan). 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), N-ethylmaleimide, iodoacetic acid, soybean trypsin inhibitor, *N*-p-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenyl-alanine chloromethyl ketone (TPCK), and pepstatin A were purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

Defatted powders of the pyloric ceca of yellow tail and brown hakeling were prepared by the same method of Kishimura and Hayashi (2002). Trypsin was extracted by stirring from the defatted powder in 50 volumes of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ at 5 C for 3 h. The extract was centrifuged (H-200, Kokusan, Tokyo, Japan) at 10,000×g for 10 min, and then the supernatant was concentrated by lyophilization and used as crude trypsin.

Crude trypsin from yellow tail was applied to a column of Sephacryl S-200 (3.9×64 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ at 5 C and the proteins were eluted with the same buffer at a flow rate of 27 ml/h. Each 4.0 ml fraction was collected. The main trypsin fractions (about 60 ml) were pooled and concentrated by lyophilization to 4.0 ml. The concentrated fraction was then applied to a Sephadex G-50 column (3.9×64 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ at 5 C and the proteins were eluted with the same buffer at a flow rate of 27 ml/h. Each 4.0 ml fraction was collected. Trypsin was eluted as single peak on the gel filtration and showed a single band on SDS-PAGE (Fig. 1).

For brown hakeling trypsin, the crude enzyme was chromatographed on a column of Sephacryl S-200 (3.9×64 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ at 5 C and the proteins were eluted with the same buffer at a flow rate of 27 ml/h. Each 4.0 ml fraction was collected. The main trypsin fractions (about 60 ml)

were pooled and concentrated by lyophilization to 4.0 ml. The concentrated fraction was then dialyzed against 500 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ at 5 C for 6 hours. The dialyzate was applied to a DEAE-cellulose column (2.2×18 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ at 5 C and the proteins were eluted with a linear gradient of 0-0.5 M NaCl in the same buffer at a flow rate of 27 ml/h. Each 4.0 ml fraction was collected. Trypsin was eluted with the non-adsorbed fraction, and was concentrated by lyophilization to 4.0 ml. Then the concentrated fraction was applied to a Sephadex G-50 column (3.9×64 cm) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ at 5 C and the proteins were eluted with the same buffer at a flow rate of 27 ml/h. Each 4.0 ml fraction was collected. Trypsin was eluted as single peak on the gel filtrations and showed a single band on SDS-PAGE (Fig. 1).

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 millimole of TAME in a minute at 30 C and at pH 8.0.

The pH dependencies of the enzyme were determined in 50 mM buffer solutions [acetic acid-sodium acetate (pH 4.0-7.0), Tris-HCl (pH 7.0-9.0), and glycine-NaOH (pH 9.0-11.0)] at 30 C. The temperature dependencies of the enzyme were determined at pH 8.0 and at various temperatures. The effects of temperature and pH on the stability of the enzyme were found by incubating the enzyme at pH 8.0 for 15 min 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 $CaCl_2$ on the activity of the enzyme was found by incubating the enzyme at 30 C and at pH 8.0 in the presence of 10 mM EDTA or 10 mM $CaCl_2$.

The effect of inhibitors on trypsin was determined according to the method of Klomklao *et al*. (2004) by incubating trypsin with an equal volume of the inhibitor solutions, i.e., E-64, N-ethylmaleimide, iodoacetic acid, soybean trypsin inhibitor, TLCK, TPCK,

pepstatin A and EDTA to obtain the final concentrations specified in Table 2. After incubation the mixture at 25 C for 15 min, the remaining activity was measured and expressed as percent inhibition.

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

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

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

RESULTS AND DISCUSSION

In this study, two trypsins were isolated and purified from the pyloric ceca of yellow tail and brown hakeling. The purification steps of both trypsins are summarized in Table 1, and they resulted in 63-fold and 106-fold purification, respectively, for the yellow tail and brown hakeling samples. Sephadex G-50 column chromatography was highly effective in separating trypsin from other proteins. Kishimura *et al*. (2005c) found that the use of Sephacryl S-200 and Sephadex G-50 for purification of trypsin from spotted mackerel (*Scomber australasicus*) led to an increase in trypsin activity by 20-fold. Klomklao *et al*. (2005) also purified the trypsins from skipjack tuna spleen by using Sephacryl S-200 and

Sephadex G-50 an increase in purity by 40.6-fold.

The molecular weights of both yellow tail and brown hakeling trypsins were estimated as approximately 24 kDa using SDS-PAGE (Fig. 1). Trypsin isolated from various fish species have been shown to have molecular weights of 23.5-28 kDa (Hjelmeland and Raa 1982; Yoshinaka *et al*. 1983; Simpson and Haard 1984; Martinez *et al*. 1988; Asgeirsson *et al*. 1989; Kristjansson 1991; Castillo-Yanez *et al*. 2005; Kishimura *et al*. 2005a; Kishimura *et al*. 2005b; Kishimura *et al*. 2005c,). Values obtained are within the accepted range of molecular weights for trypsins from 20 kDa to 28 kDa (Kiel 1971).

Both enzymes hydrolyzed TAME effectively at alkaline pH with an optimum activity at about pH 8.0 (Fig. 2a) similar to those of porcine pancreatic trypsin (Fig. 2b). Similar results have also been reported for trypsin from the hepatopancreas of crawfish which has an optimum pH in the range of 8.0-8.5 (Jeong *et al*. 2000) and for trypsin derived from cunner which has an optimum pH of 8.5 (Simpson and Haard 1985). Figure 3a shows the temperature dependencies of the yellow tail and brown hakeling trypsins. The optimum temperatures of the yellow tail and brown hakeling trypsins for the hydrolysis of TAME were 60 C and 50 C, respectively, which were lower than that of porcine pancreatic trypsin (60-70 C; Fig. 3b). The temperature optimum of yellow tail trypsin observed 60 C, is similar to those of rainbow trout trypsin (Kristjansson 1991), Japanese anchovy trypsin (Kishimura *et al*. 2005a), true sardine trypsin (Kishimura *et al*. 2005b). On the other hand, the optimum temperature of the brown hakeling trypsin for the hydrolysis of TAME (50 C) is similar to those of Atlantic cod trypsin (Asgeirsson *et al*. 1989), Monterey sardine trypsin (Castillo-Yanez *et al*. 2005) and arabesque greenling trypsin (Kishimura *et al*. 2005b).

The effects of pH on the stability of trypsins from the pyloric ceca of yellow tail and brown hakeling are shown in Fig. 4a. Yellow tail trypsin was stable over a broad pH range (pHs 6.0-11.0), whereas brown hakeling trypsin was stable from pH 5.0 to pH 9.0 after 30

min incubation. The trypsin activity of both enzymes was rapidly lost at pH 4.0-5.0. On the other hand, trypsin from porcine pancreas was not inactivated at pH 4.0-5.0(Fig. 4b). The sharp decrease in the fish trypsin activities at low pH may be attributed to the irreversible denaturation of the enzymes (Klomklao *et al*. 2005). A similar inactivation at acidic pH also occurred with trypsins from other fish species (Martinez *et al*. 1988; Asgeirsson *et al*. 1989; Kristjansson 1991; Kishimura *et al*. 2005a, b, c). Figure 5a shows the temperature stabilities of the yellow tail and brown hakeling trypsins. The yellow tail trypsin was stable below 40 C, but its activity quickly fell above 50 C. The temperature stability of the yellow tail trypsin was similar to that of Monterey sardine trypsin (Castillo-Yanez *et al*. 2005) and Japanese sardine trypsin (Kishimura *et al*. 2005b). Whereas the brown hakeling trypsin was stable below 30 C, and its activity quickly fell above 40 C. The results indicated that the brown hakeling trypsin was more heat labile than the yellow tail trypsin, other fish trypsins (Martinez *et al*. 1988; Kristjansson 1991) and porcine pancreatic trypsin (Fig. 5b).

The effects of $CaCl_2$ on the yellow tail and brown hakeling trypsins were investigated in the presence of 10 mM EDTA or 10 mM $CaCl_2$. As shown in Figs. 6a and 6b, both enzymes were stabilized by calcium ion similar to porcine pancreatic trypsin. Two calcium-binding sites are in bovine trypsinogen (Kossiakoff *et al*. 1977). The primary site, with a higher affinity for calcium ions, is common in trypsinogen and trypsin, and the secondary site is only in the zymogen. Occupancy of the primary calcium-binding site stabilizes the protein toward thermal denaturation or autolysis. From the results, the yellow tail and brown hakeling trypsins were stabilized by calcium ion from thermal denaturation. These findings suggest that the yellow tail and brown hakeling trypsins possess the primary calcium binding site like mammalian pancreatic trypsin and other fish trypsins (Male *et al*. 1995; Genicot *et al*. 1996; Kishimura *et al*. 2005a, b).

Protease inhibitors are very important tools to characterize active sites and to

understand enzymatic mechanisms (Klomklao *et al*. 2004). Table 2 presents the effects of eight different inhibitors on the trypsins from the pyloric ceca of yellow tail and brown hakeling. Both enzymes were strongly inhibited by soybean trypsin inhibitor and TLCK, which are widely used to identify trypsins, whereas, TPCK (a synthetic chymotrypsin inhibitor), E-64, N-ethylmaleimide, iodoacetic acid (cysteine protease inhibitors) and pepstatin A (an aspartic protease inhibitor) had no inhibitory effect on both trypsins. However, a metallo protease inhibitor (EDTA) showed partially inhibition. The responses of both enzymes to these eight inhibitors suggest that these enzymes may be classified as trypsins, which possibly require metal ions for activity. Similar results were obtained for other fish trypsins (Hjelmeland and Raa 1982; Simpson and Haard 1984; Kristjansson 1991; Klomklao *et al*. 2004; Kishimura *et al*. 2005c).

The N-terminal amino acid sequences of the purified trypsins from yellow tail and brown hakeling were determined for twenty amino acid residues as, IVGGYECKPYSQPHQVSLNS and IVGGYECPKHSQPHQVSLNS, respectively. It was indicated that the N-termini of both trypsins were unblocked. The N-terminal amino acid sequences of the yellow tail and brown hakeling trypsins were aligned with the sequences of other animal trypsins (Fig. 7). Being similar to other fish trypsins, the yellow tail and brown hakeling trypsins had a charged Glu residue at position 6, whereas Thr is most common in mammalian pancreatic trypsins (Fig. 7). In this study, the Cys residue was conserved in the yellow tail and brown hakeling trypsins (Fig. 7). The results indicate that the yellow tail and brown hakeling trypsins may also have a disulfide bond to the corresponding residues (between Cys-7 and Cys-142) of bovine pancreatic trypsin.

CONCLUSION

Yellow tail trypsin showed similar characteristics as porcine pancreatic trypsin except for instability below pH 5.0. On the other hand, the brown hakeling trypsin showed lower optimum temperature than that of porcine pancreatic trypsin and both was less stable than porcine pancreatic trypsin below pH 5.0 and above 40 C.

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(captions to figures)

FIG. 1. ELECTROPHORESIS OF PURIFIED TRYPSINS FROM YELLOW TAIL (*S. QUINQUERADIATA*) AND BROWN HAKELING (*P. JAPONICUS*).

Electrophoresis was performed using a 0.1 % SDS-12.5 % polyacrylamide slab-gel. Lane 1 contains trypsin from yellow tail (*S. quinqueradiata*). Lane 2 contains trypsin from brown hakeling (*P. japonicus*). Lane 3 contains protein standards; egg albumin (molecular weight, 45,000 Da), bovine pancreatic trypsinogen （24,000 Da）, bovine milk β-lactoglobulin (18,400 Da), and egg-white lysozyme (14,300 Da).

FIG. 2. EFFECTS OF pH ON THE ACTIVITY OF TRYPSINS FROM YELLOW TAIL (*S. QUINQUERADIATA*) AND BROWN HAKELING (*P. JAPONICUS*).

The activities were determined in 50 mM buffer solutions [acetic acid-sodium acetate (pH 4.0-7.0), Tris-HCl (pH 7.0-9.0), and glycine-NaOH (pH 9.0-11.0)] at 30 C. a: trypsin from yellow tail (*S. quinqueradiata*) (closed circle); trypsin from brown hakeling (*P. japonicus*) (closed triangle), b: porcine pancreatic trypsin.

FIG. 3. EFFECTS OF TEMPERATURE ON THE ACTIVITY OF TRYPSINS FROM YELLOW TAIL (*S. QUINQUERADIATA*) AND BROWN HAKELING (*P. JAPONICUS*).

The activities were determined at pH 8.0 and at various temperatures. a: trypsin from yellow tail (*Seriola quinqueradiata*) (closed circle); trypsin from brown hakeling (*P. japonicus*) (closed triangle) (closed triangle), b: porcine pancreatic trypsin.

FIG. 4. pH STABILITY OF TRYPSINS FROM YELLOW TAIL (*S. QUINQUERADIATA*) AND BROWN HAKELING (*P. JAPONICUS*).

The enzymes were kept at 30 C for 30 min and pH 4.0-11.0, and then the remaining activities at 30 C and pH 8.0 were determined. a: trypsin from yellow tail (*S. quinqueradiata*) (closed circle); trypsin from brown hakeling (*P. japonicus*) (closed triangle), b: porcine pancreatic trypsin.

FIG. 5. THERMOSTABILITY OF TRYPSINS FROM YELLOW TAIL (*S. QUINQUERADIATA*) AND BROWN HAKELING (*P. JAPONICUS*).

The enzymes were kept at 20-70 C for 15 min and pH 8.0, and then the remaining activities at 30 C and pH 8.0 were determined. a: trypsin from yellow tail (*S. quinqueradiata*) (closed circle); trypsin from brown hakeling (*P. japonicus*) (closed triangle), b: porcine pancreatic trypsin.

FIG. 6. EFFECT OF CALCIUM ION ON THE STABILITY OF TRYPSINS FROM YELLOW TAIL (*S. QUINQUERADIATA*) AND BROWN HAKELING (*P. JAPONICUS*).

The enzymes were kept at 30 C and pH 8.0 for 0-8 h in the presence of 10 mM $CaCl_2$ (closed symbol) or 10 mM EDTA (open symbol), and then the remaining activities at 30 C and pH 8.0 were determined. a: trypsin from yellow tail (*S. quinqueradiata*) (circle); trypsin from brown hakeling (*P. japonicus*) (square), b: porcine pancreatic trypsin.

FIG. 7. COMPARISON OF THE N-TERMINAL AMINO ACID SEQUENCES OF TRYPSINS FROM YELLOW TAIL (*S. QUINQUERADIATA*) AND BROWN HAKELING (*P. JAPONICUS*) WITH THOSE OF OTHER VERTEBRATES.

True sardine (Kishimura *et al*. 2005b); Arabesque greenling (Kishimura *et al*. 2005b); Japanese anchovy (Kishimura *et al*. 2005a); Antarctic fish (Genicot *et al*. 1996); Cod (Gudmundsdottir *et al*. 1993); Salmon (Male *et al*. 1995); Founder fish (GenBank accession number AB029750); Porcine (Hermodson *et al*. 1973); Bovine (Walsch 1970); Human (Emi *et al*. 1986).

Fig. 1

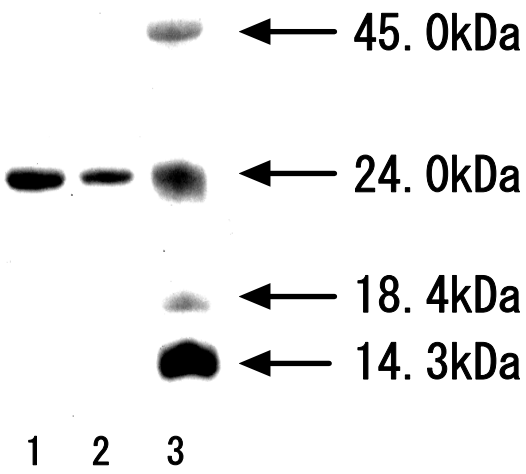

Fig. 2

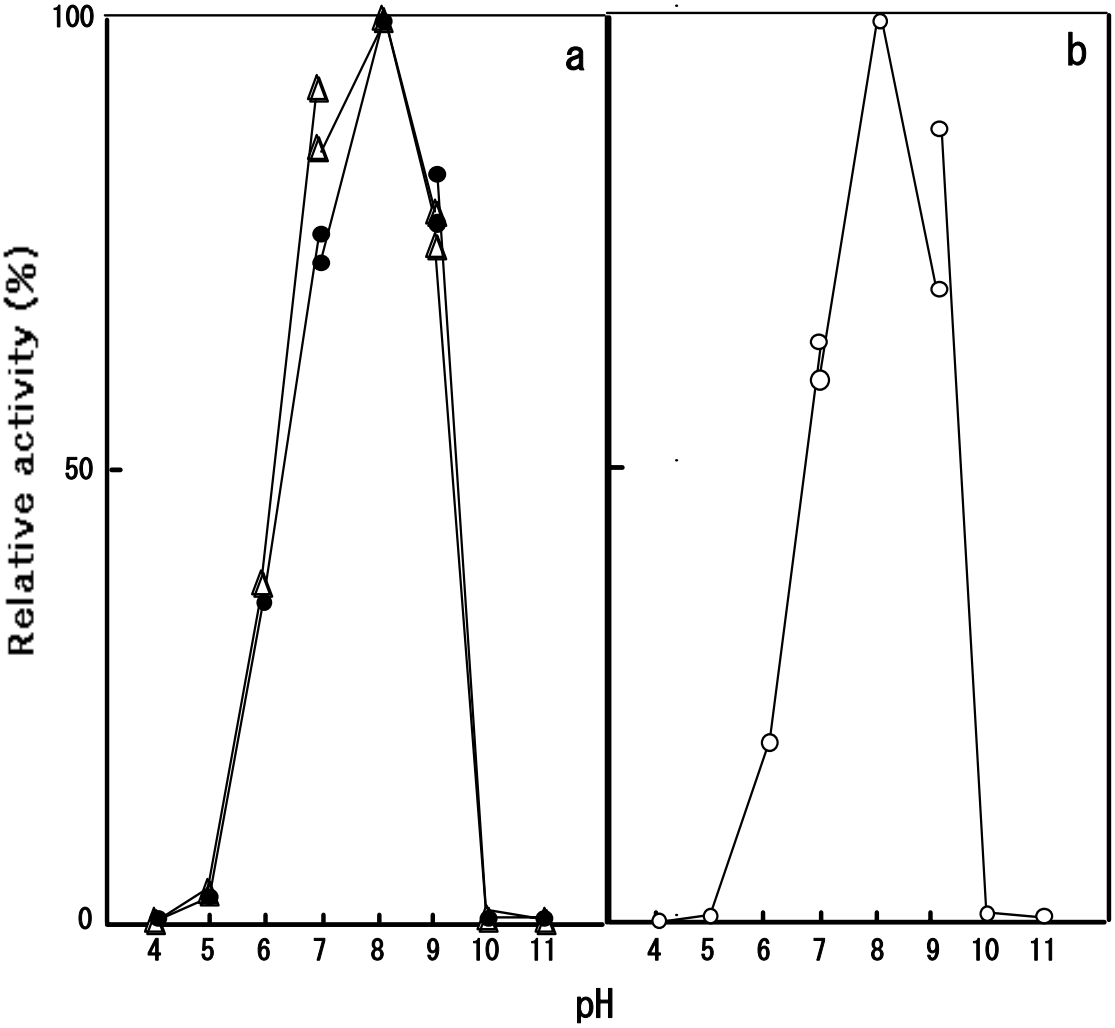

Fig. 3

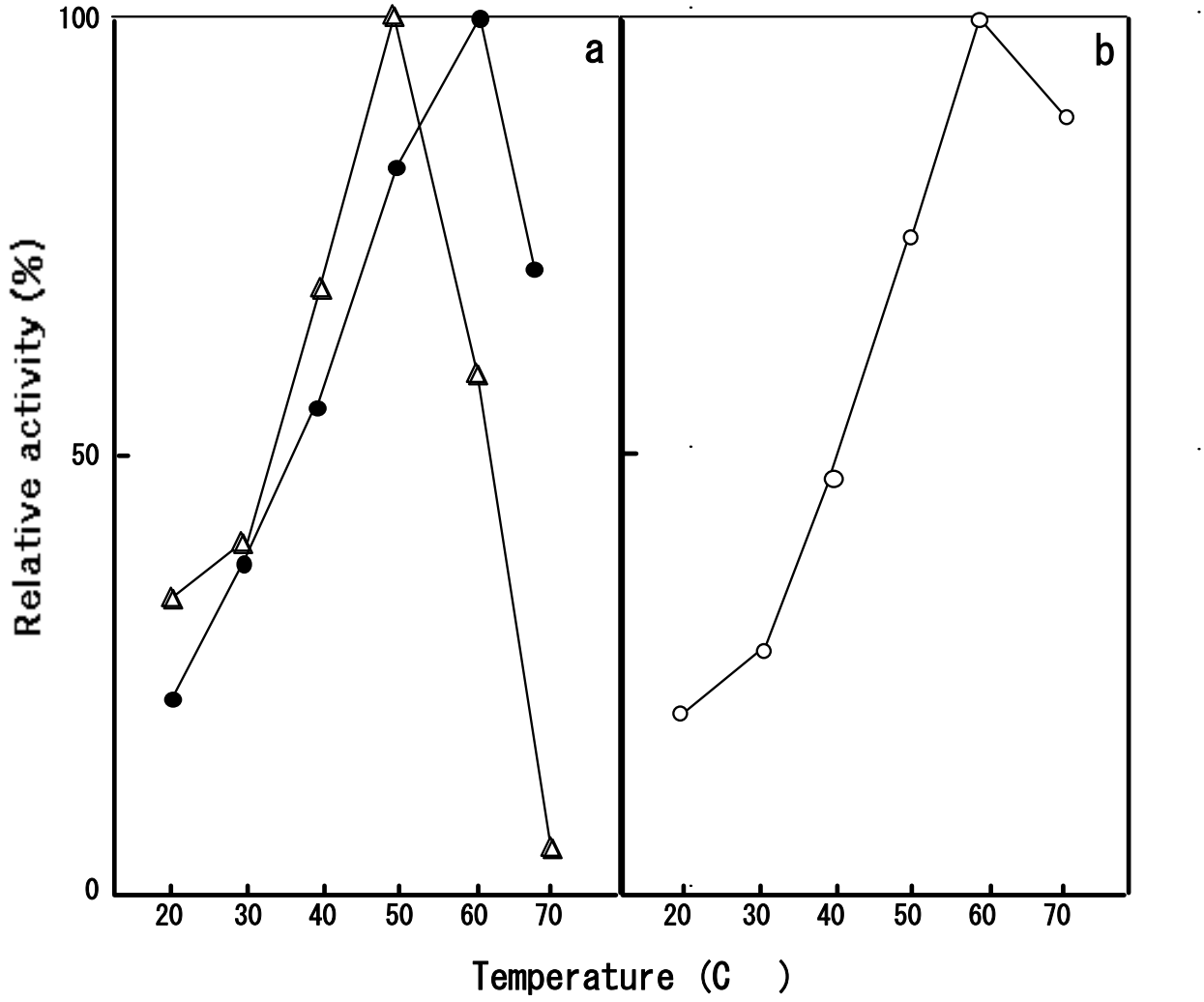

Fig. 4

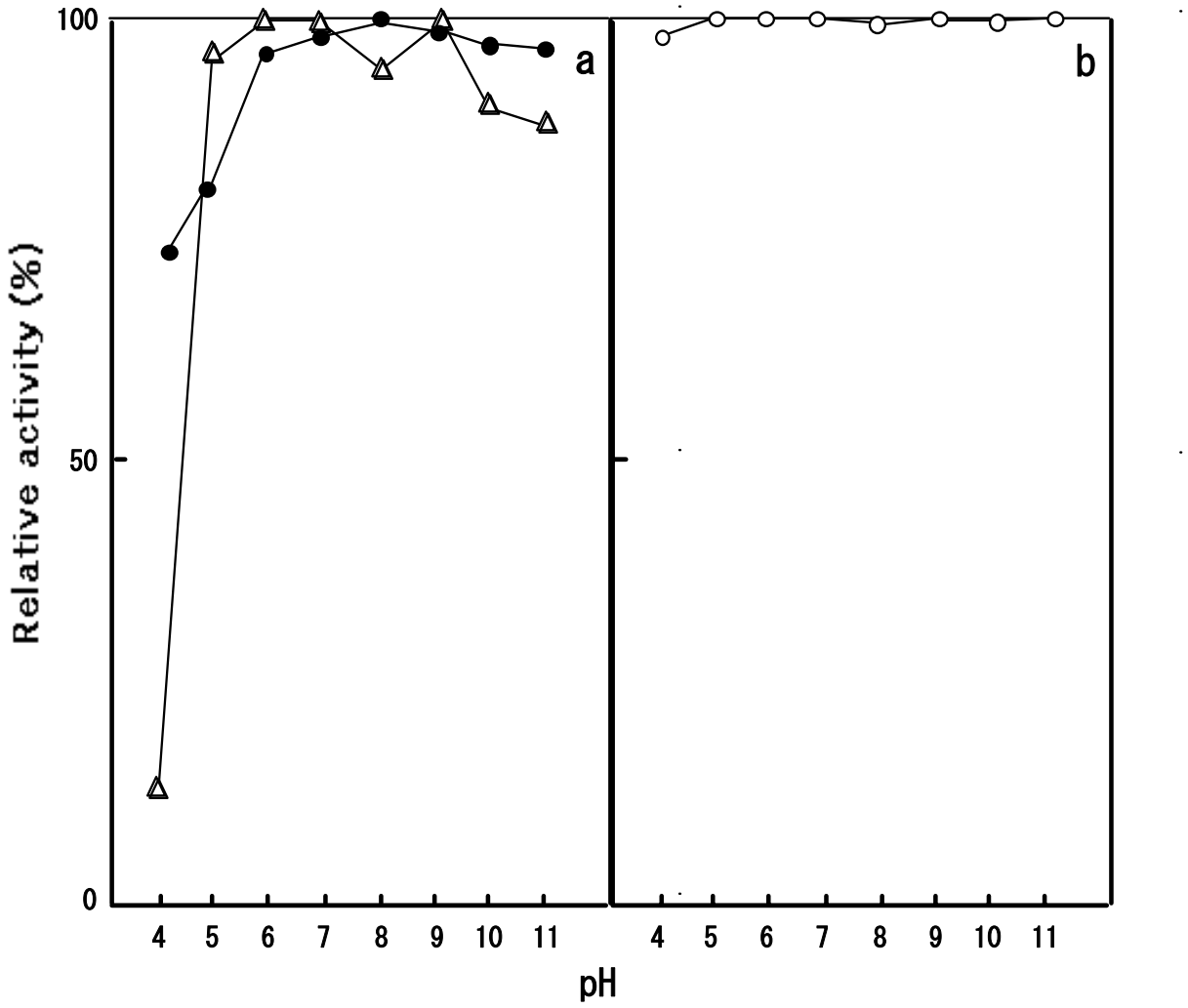

Fig. 5

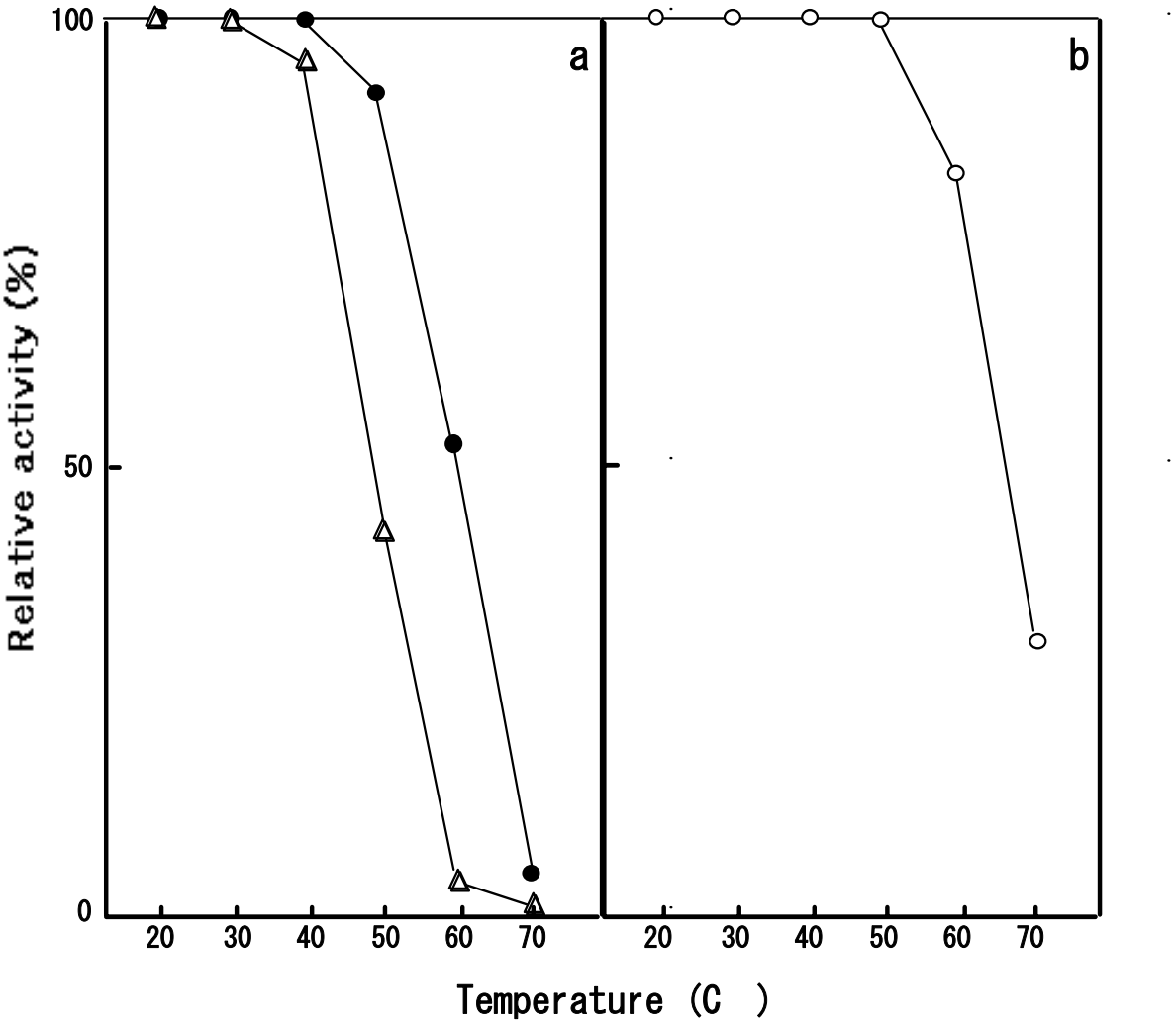

Fig. 6

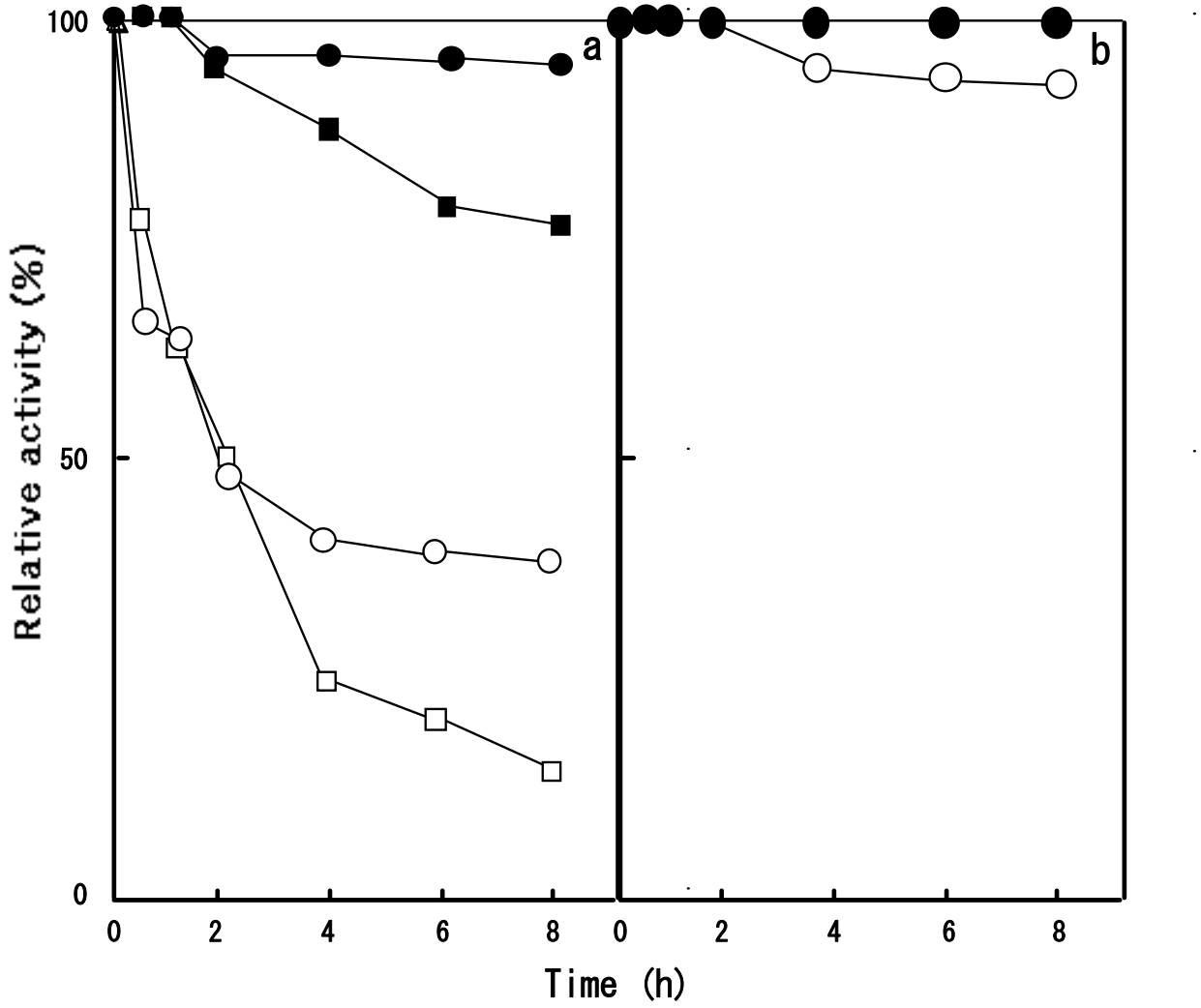

Fig. 7

	1 10 20
Yellow tail	IVGGYECKPYSQPHQVSLNS
Brown hakeling	IVGGYECTPHSQAHQVSLNS
True sardine	IVGGYECKAYSQPWQVSLNS
Arabesuque greenling	IVGGYECTPHTQAHQVSLDS
Japanese anchovy (TR-I)	IVGGYECQAHSQPHTVSLNS
Japanese anchovy (TR-II)	IVGGYECQPYSQPHQVSLDS
Antarctic Fish	IVGGKECSPYSQPHQVSLNS
Cod	IVGGYECTKHSQAHQVSLNS
Salmon	IVGGYECKAYSQTHQVSLNS
Flounder Fish	IVGGYECTPYSQPHQVSLNS
Porcine	IVGGYTCAANSVPYQVSLNS
Bovine	IVGGYTCGANTVPYQVSLNS
Human	IVGGYNCEENSVPYQVSLNS

TABLE 1.
PURIFICATION OF TRYPSINS FROM YELLOW TAIL (*SERIOLA QUINQUERADIATA*) AND BROWN HAKELING (*P. JAPONICUS*)

Purification stages	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purity (fold)	Yield (%)
yellow tail					
Crude enzyme	3,810	346	0.18	1	100
Sephacryl S-200	1,138	283	1.0	6	82
Sephadex G-50	34	190	17.2	96	55
brown hakeling					
Crude enzyme	6,440	773	0.12	1	100
Sephacryl S-200	638	510	0.8	7	66
DEAE-Cellulose	33	310	9.4	78	40
Sephadex G-50	6	76	12.7	106	10

TABLE 2.
EFFECTS OF VARIOUS INHIBITORS ON THE ACTIVITY OF TRYPSINS FROM YELLOW TAIL (*S. QUINQUERADIATA*) AND BROWN HAKELING (*R. JAPONICUS*)

Inhibitors	Concentration	% Inhibition	
		yellow tail	brown hakeling
Control		0	0
E-64	0.1 mM	0	0
N-ethylmaleimide	1 mM	0	0
Iodoacetic acid	1 mM	0	0
Soybean trypsin inhibitor	1 mg/ml	96	93
TLCK	5 mM	80	86
TPCK	5 mM	9	6
Pepstatin A	0.01 mM	0	0
EDTA	2 mM	29	21