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Trypsins from the pyloric ceca of jacopever (*Sebastes schlegelii*) and elkhorn sculpin (*Alcichthys alcicornis*): Isolation and characterization

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Abstract

Trypsins from the pyloric ceca of jacopever (*Sebastes schlegelii*), TR-J, and elkhorn sculpin (*Alcichthys alcicornis*), TR-E, were purified by gel filtration on Sephacryl S-200 and Sephadex G-50. The molecular weights of TR-J and TR-E were estimated to be 24,000 Da by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. TR-J and TR-E revealed optimum temperatures of 60 and 50 °C, respectively, and showed the same optimum pH (pH 8.0) for hydrolysis of N^{α}-*p*-tosyl-L-arginine methyl ester. TR-J and TR-E were unstable at above 50 and 40 °C, respectively, and were more stable at alkaline pH than at acidic pH. Thermal stabilities of TR-J and TR-E were highly calcium dependent. These purified trypsin enzymes were inhibited by serine protease inhibitors, such as TLCK and soybean trypsin inhibitor. The N-terminal amino acid sequences of TR-J and TR-E were also investigated. The N-terminal amino acid sequences of TR-J, IVGGYECKPYSQPHQVSLNS, and TR-E, IVGGYECTPHSQAHQVSLNS, were found, and these sequences showed highly homology to other fish trypsins.
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Keywords: Jacopever; *Sebastes schlegelii*; Elkhorn sculpin; *Alcichthys alcicornis*; Pyloric ceca; Trypsin; Characteristics

1. Introduction

Fish viscera, accounting for 5% of total mass, have wide biotechnological potential as a source of digestive enzymes, especially digestive proteases, that may have some unique properties of interest for both basic research and industrial applications (Simpson & Haard, 1999). Fishes are poikilothermic, so their survival in cold waters required adaptation of their enzyme activities to the low temperatures of their habitats. Enzymes from cold-adapted fish species thus often have higher enzymatic activities at low temperatures than their counterparts from warm-blooded animals (Asgeirsson, Fox, & Bjarnason, 1989; Kristjansson, 1991). High activity of fish enzymes at low temperatures may be interesting for several industrial applications of enzymes, such as in certain food processing operations that require low processing temperatures. Furthermore, relatively lower thermal stability, often observed with fish enzymes, may also be beneficial in such applications' as the enzymes can be inactivated more readily, with less heat treatment, when desired in a given process (Simpson & Haard, 1987).

One of the main digestive proteases, which is detected in the pyloric ceca and intestine of fish, is trypsin (EC 3.4.21.4). Trypsin is a member of a large family of serine proteases and cleaves the peptide bond on the carboxyl side of arginine and lysine. So far, trypsins have been isolated from some fish and characterized. These fish include the following: capelin (Hjelmeland & Raa, 1982), catfish (Yoshinaka, Suzuki, Sato, & Ikeda, 1983), Greenland cod

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(Simpson & Haard, 1984), anchovy (Martinez, Olsen, & Serra, 1988), Atlantic cod (Asgeirsson et al., 1989), rainbow trout (Kristjansson, 1991), Monterey sardine (Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, & Toro, 2005), and Japanese anchovy (Kishimura, Hayashi, Miyashita, & Nonami, 2005). Recently, we isolated trypsins from the viscera of true sardine (*Sardinops melanostictus*) and from the pyloric ceca of arabesque greenling (*Pleurogrammus azonus*) (Kishimura, Hayashi, Miyashita, & Nonami, 2006). 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. Jacopever (*Sebastes schlegelii*) and elkhorn sculpin (*Alcichthys alcicornis*) are important fish in Japan and are used for food production. No information regarding the characteristics of trypsins from the pyloric ceca of either species has been reported. Therefore, this study aimed to purify trypsins from the pyloric ceca of jacopever (*S. schlegelii*) and elkhorn sculpin (*A. alcicornis*) and compare their characteristics to those of porcine pancreatic trypsin.

2. Materials and methods

2.1. Materials

The jacopever (*S. schlegelii*) and elkhorn sculpin (*A. alcicornis*) were caught off Hakodate, Hokkaido Prefecture, Japan. Sephacryl S-200 and Sephadex G-50 were purchased from Pharmacia Biotech (Uppsala, Sweden). N^{α}-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) was obtained from Wako Pure Chemicals (Osaka, Japan). Ethylenediaminetetraacetic acid (EDTA), 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-phenylalanine chloromethyl ketone (TPCK), and pepstatin A were purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

2.2. Preparation of crude enzyme

Defatted powders of the pyloric ceca of jacopever and elkhorn sculpin were prepared by the method of Kishimura and Hayashi (2002). Trypsin was extracted by stirring the defatted powder in 50 volumes of 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ at 5 °C for 3 h. The extract was centrifuged (H-200, Kokusan, Tokyo, Japan) at 10,000*g* for 10 min, and then the supernatant was lyophilized and used as crude trypsin.

2.3. Purification of trypsin

The crude trypsin 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 $CaCl_2$ and the proteins were eluted with the same buffer. The main trypsin fraction was concentrated by lyophilization. Then the concentrated fraction was 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 $CaCl_2$ and the proteins were eluted with the same buffer. Trypsin was eluted as single peak on the gel filtrations. Trypsin fractions were pooled and used for further studies.

2.4. Assay for trypsin activity

Trypsin activity was measured by the method of Hummel (1959) using TAME as a substrate. One unit of enzyme activity was defined as the amount of the enzyme hydrolyzing 1 mM of TAME in a minute. The effect of inhibitors on trypsin was determined according to the method of Klomklao, Benjakul, and Visessanguan (2004) by incubating trypsin with an equal volume of proteinase inhibitor solution, to obtain the final designated concentration (0.1 mM E-64, 1 mM *N*-ethylmaleimide, 1 mM iodoacetic acid, 1 mg/ml soybean trypsin inhibitor, 5 mM TLCK, 5 mM TPCK, 1 mM pepstatin A and 2 mM EDTA). After incubation of the mixture at 25 °C for 15 min, the remaining activity was measured and percent inhibition was then calculated. 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 or 10 mM $CaCl_2$.

2.5. Polyacrylamide gel electrophoresis

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

2.6. Analysis of amino acid sequence

To analyze the N-terminal sequence of 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).

2.7. Protein determination

The protein concentration was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard.

3. Results and discussion

In this study, two trypsins (TR-J and TR-E) were purified from the pyloric ceca of jacopever and elkhorn sculpin, respectively, by gel filtration on Sephacryl S-200 and Sephadex G-50. The final preparations from jacopever and elkhorn sculpin were purified 62-fold and 31-fold, respectively, from the crude trypsin (Table 1). TR-J and TR-E were nearly homogeneous in SDS–PAGE (Fig. 1).

The molecular weights of both TR-J and TR-E were estimated as approximately 24,000 Da using SDS–PAGE (Fig. 1). Similar results can also be found in trypsins or trypsin-like serine proteinases from mammalian pancreas (24,000 Da), Japanese anchovy (24,000 Da) (Kishimura et al., 2005), true sardine (24,000 Da) (Kishimura et al., 2006), arabesque greenling (24,000 Da) (Kishimura et al., 2006), Greenland cod (23,500 Da) (Simpson & Haard, 1984), and Atlantic cod (24,200 Da) (Asgeirsson et al., 1989).

Table 1
Purification of trypsins from jacopever (*S. schlegelii*) and elkhorn sculpin (*A. alcicomis*)

Purification stages	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purity (fold)	Yield (%)
Jacopever					
Crude enzyme	4392	3514	0.8	1	100
Sephacryl S-200	1138	2504	2.2	3	71
Sephadex G-50	34	1615	47.5	59	46
Elkhorn sculpin					
Crude enzyme	6594	7253	1.1	1	100
Sephacryl S-200	2002	4404	2.2	2	61
Sephadex G-50	67	2345	35.0	32	32

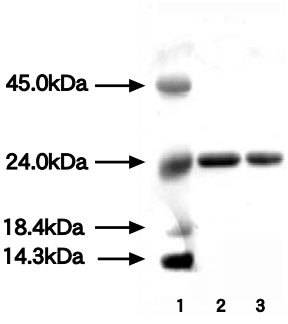

Fig. 1. Electrophoresis of purified trypsins from jacopever (*S. schlegelii*) and elkhorn sculpin (*A. alcicornis*). Electrophoresis was performed using a 0.1% SDS–12.5% polyacrylamide slab-gel. Lane 1 contains protein standards; egg albumin (molecular weight, 45,000 Da), bovine milk β-lactoglobulin (18,400 Da), and egg-white lysozyme (14,300 Da). Lane 2 contains trypsin from jacopever (TR-J). Lane 3 contains trypsin from elkhorn sculpin (TR-E).

The effects of various proteinase inhibitors on the activity of TR-J and TR-E were determined (Table 2). Both trypsins were effectively inhibited by serine proteinase inhibitor, such as soybean trypsin inhibitor and TLCK (77–97%), while specific inhibitors of cysteine proteinase (E-64, *N*-ethylmaleimide, iodoacetic acid), chymotrypsin (TPCK), aspartic proteinase (pepstatin A), and metalloproteinase (EDTA) had no inhibitory effect on the activities of TR-J and TR-E. These results indicated that the two enzymes are trypsin.

The effect of pH on the trypsin activity of TR-J and TR-E are depicted in Fig. 2a. Both enzymes hydrolyzed TAME effectively at alkaline pH with an optimum activity at about pH 8.0, similar to those of porcine pancreatic trypsin (Fig. 2b) and other fish trypsins (Asgeirsson et al., 1989;

Table 2
Effects of various inhibitors on the activity of trypsins from jacopever (*S. schlegelii*) and elkhorn sculpin (*A. alcicomis*)

Inhibitors	Concentration	% Inhibition	
		Jacopever	Elkhorn sculpin
Control		0	0
E-64	0.1 mM	0	0
N-Ethylmaleimide	1 mM	0	0
Iodoacetic acid	1 mM	1	0
Soybean trypsin inhibitor	1 mg/ml	95	97
TLCK	5 mM	77	95
TPCK	5 mM	5	3
Pepstatin A	0.01 mM	0	0
EDTA	2 mM	1	4

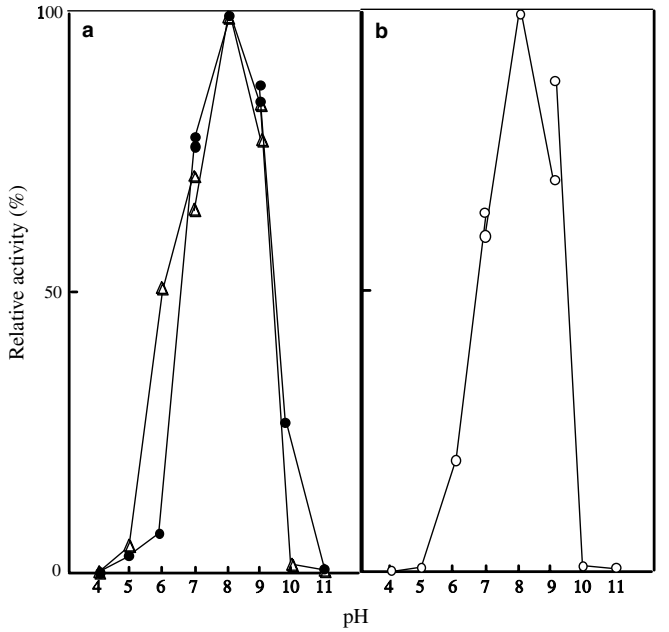

Fig. 2. Effects of pH on the activity of trypsins from jacopever (*S. schlegelii*) and elkhorn sculpin (*A. alcicornis*). The activities were determined in 50 mM buffer solutions [acetic acid–sodium acetate (pH 4.0–7.0), Tris–HCl (pH 7.0–9.0), and glycine-NaOH (pH 9.0–11.0)] at 37 °C. (a): trypsin from jacopever (TR-J) (closed circle); trypsin from elkhorn sculpin (TR-E) (open triangle), (b): porcine pancreatic trypsin.

Castillo-Yanez et al., 2005; Hjelmeland & Raa, 1982; Kishimura et al., 2005, 2006; Martinez et al., 1988; Simpson & Haard, 1984; Yoshinaka, Sato, Suzuki, & Ikeda, 1984). Fig. 3a shows the temperature dependencies of TR-J and TR-E. TR-J and TR-E revealed optimum temperatures of 60 and 50 °C, respectively, which were lower than that of porcine pancreatic trypsin (60–70 °C, Fig. 3b). TR-J examined in this study had an optimum temperature of 60 °C similar to those of rainbow trout trypsin (Kristjansson, 1991), Japanese anchovy trypsin (Kishimura et al., 2005), true sardine trypsin (Kishimura et al., 2006). On the other hand, the optimum temperature of TR-E (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., 2006). The pH stability of TR-J and TR-E are shown in Fig. 4a. TR-J was stable at 30 °C for 30 min in the pH range from pH 5.0 to 11.0, whereas TR-E was stable between pH 6.0 and 8.0. Diminished stability at acidic pH has been observed for trypsin from several fish species (Asgeirsson et al., 1989; Kishimura et al., 2005, 2006; Kristjansson, 1991; Martinez et al., 1988), but is in marked contrast to the pH stability of porcine pancreatic trypsin which is most stable at pH 4 (Fig. 4b). Fig. 5a shows the temperature stabilities of TR-J and TR-E. TR-J was stable below 40 °C, but its activity quickly fell above 50 °C. The temperature stability of TR-J was similar to that of Monterey sardine trypsin (Castillo-Yanez et al., 2005) and Japanese sardine trypsin (Kishimura et al., 2006). Whereas TR-E was stable when heated up to 30 °C, and its activity quickly fell above 40 °C. TR-E was more unstable than TR-J, other fish trypsins (Kristjansson, 1991; Martinez et al., 1988), and porcine pancreatic trypsin (Fig. 5b). Genicot, Rentier-Delrue, Edwards, Vanbeeumen,

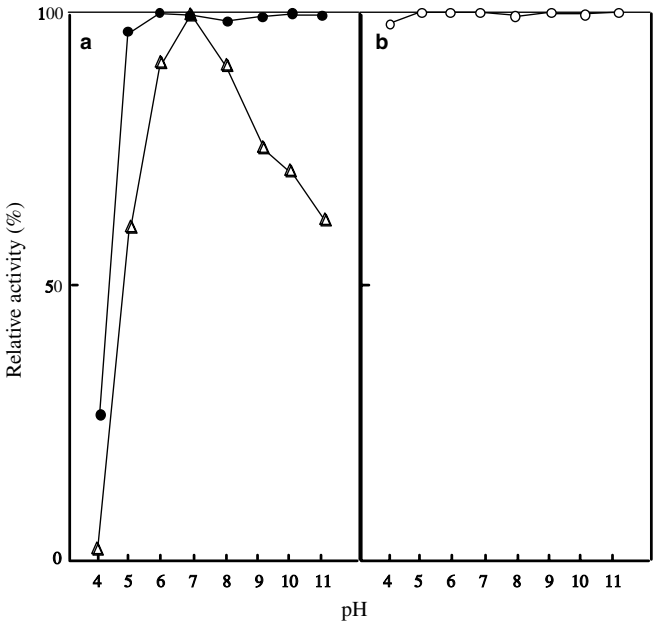

Fig. 4. pH stability of trypsins from jacopever (*S. schlegelii*) and elkhorn sculpin (*A. alcicornis*). The enzymes were kept at 30 °C for 30 min and pH 4.0–11.0, and then the remaining activities at 30 °C and pH 8.0 were determined. (a): trypsin from jacopever (TR-J) (closed circle); trypsin from elkhorn sculpin (TR-E) (open triangle), (b): porcine pancreatic trypsin.

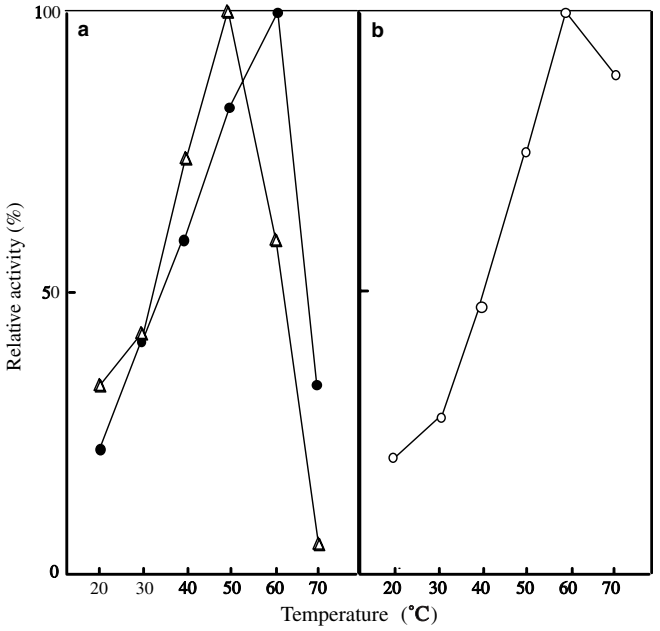

Fig. 3. Effects of temperature on the activity of trypsins from jacopever (*S. schlegelii*) and elkhorn sculpin (*A. alcicornis*). The activities were determined at pH 8.0 and at various temperatures. (a): trypsin from jacopever (TR-J) (closed circle); trypsin from elkhorn sculpin (TR-E) (open triangle), (b): porcine pancreatic trypsin.

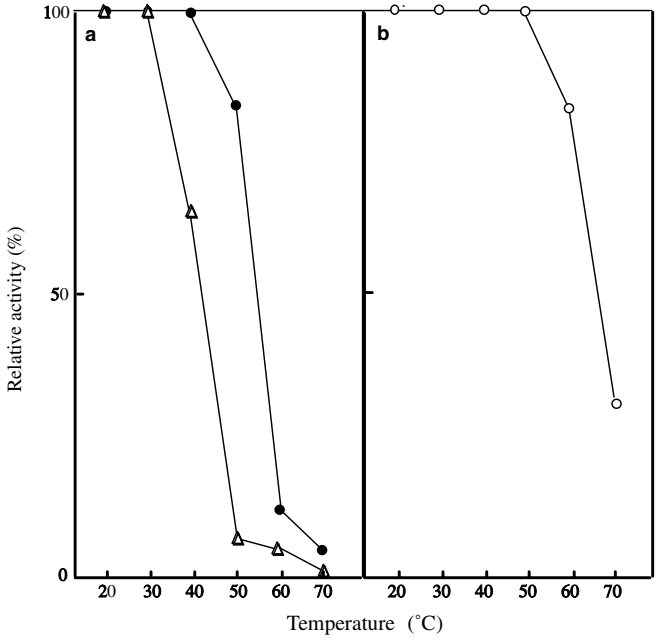

Fig. 5. Thermostability of trypsins from jacopever (*S. schlegelii*) and elkhorn sculpin (*A. alcicornis*). The enzymes were kept at 20–70 °C for 15 min and pH 8.0, and then the remaining activities at 30 °C and pH 8.0 were determined. (a): trypsin from jacopever (TR-J) (closed circle); trypsin from elkhorn sculpin (TR-E) (open triangle), (b): porcine pancreatic trypsin.

and Gerday (1996) considered that the overall decrease of hydrophobicity and increase in surface hydrophilicity of fish trypsin, as compared to its bovine counterparts, have effects on thermostability and flexibility. Therefore, such structural characteristics of TR-E may contribute to its lower temperature stability.

The effects of $CaCl_2$ on TR-J and TR-E were found in the presence of 10 mM EDTA or 10 mM $CaCl_2$. Thermal stabilities of both enzymes were highly dependent on the presence of calcium ion (Fig. 6a), similar to porcine pancreatic trypsin (Fig. 6b). Stabilization against thermal inactivation by calcium has been reported for the trypsins from catfish (Yoshinaka et al., 1984), eel (Yoshinaka, Sato, Suzuki, & Ikeda, 1985), and rainbow trout (Kristjansson, 1991). There are two calcium-binding sites in bovine trypsinogen (Kossiakoff, Chambers, Kay, & Stroud, 1977). The primary site, with a higher affinity for calcium ions, is common in trypsinogen and trypsin, and the secondary site is only in the zymogen. Occupancy of the primary calcium-binding site stabilizes the protein toward thermal denaturation or autolysis. TR-J and TR-E were stabilized by calcium ion from thermal denaturation. These findings suggest that TR-J and TR-E possess the primary calcium-binding site, like mammalian pancreatic trypsin and other fish trypsins (Male, Lorens, Smalas, & Torrissen, 1995; Genicot et al., 1996).

The N-terminal amino acid sequences of TR-J and TR-E were analyzed and twenty amino acids, IVGGYECKPYSQPHQVSLNS (TR-J) and IVGGYECTPHSQAHQVSLNS (TR-E), were found. It was indicated that the N-termini of TR-J and TR-E were unblocked. The N-terminal amino acid sequences of TR-J and TR-E were aligned with the sequences of other animal trypsins (Fig. 7). N-terminal 4 amino acid sequences of TR-J and TR-E (IVGG) were identical with those of other animal trypsins in this study (Fig. 7). Being similar to other fish trypsins, TR-J and TR-E had a charged Glu residue at position 6, where Thr is most common in mammalian pancreatic trypsins (Fig. 7). On the other hand, bovine pancreatic trypsin has a disulfide bond between Cys-7 and Cys-142 (Stroud, Kay, & Dickerson, 1974), and other vertebrate trypsins also possess the Cys-7 (Emi et al., 1986, 1993, 1996; Hermodson, Ericsson, Neurath, & Walsh, 1973 Kishimura et al., 2005, 2006; Male et al., 1995; Walsch, 1970). In this study, the Cys residue was characteristically conserved in TR-J and TR-E (Fig. 7). These results indicate that TR-J and TR-E may also have a disulfide bond between the corresponding residues (between Cys-7 and Cys-142) of bovine pancreatic trypsin.

In conclusion, TR-J showed almost the same characteristics as of porcine pancreatic, trypsin except for being unstable below pH 5.0. These results suggest that the viscera of jacopever (*S. schlegelii*) would be a potential source of trypsin for food processing operations. On the other hand, TR-E showed a lower optimum temperature than that of porcine pancreatic trypsin and was more unstable than porcine pancreatic trypsin below pH 5.0 and above 40 °C. These results suggest that the pyloric ceca of elkhorn sculpin (*A. alcicornis*) can be used as a novel source of trypsin for certain food processing operations that require low processing temperatures, and the relatively low thermal stability of TR-E may also be beneficial in such applications where the enzymes need to be readily inactivated.

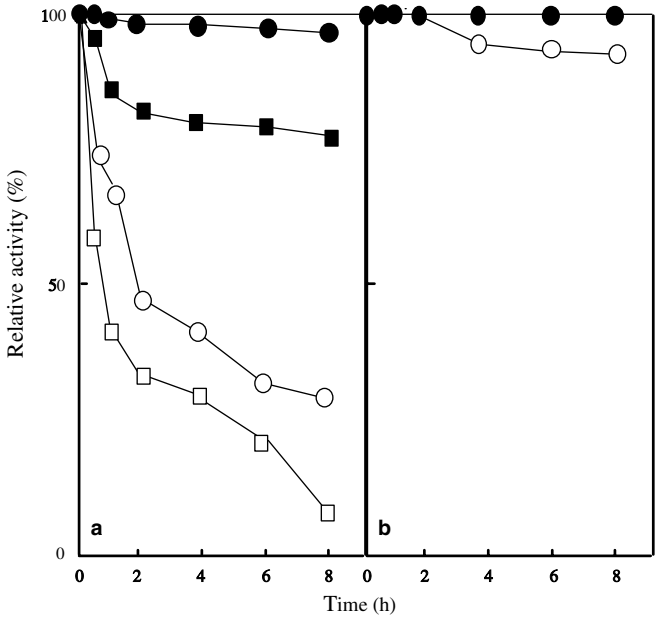

Fig. 6. Effect of calcium ion on the stability of trypsins from jacopever (*S. schlegelii*) and elkhorn sculpin (*A. alcicornis*). The enzymes were kept at 30 °C and pH 8.0 for 0–8 h in the presence of 10 mM $CaCl_2$ (closed symbol) or 10 mM EDTA (open symbol), and then the remaining activities at 30 °C and pH 8.0 were determined. (a): trypsin from jacopever (TR-J) (circle); trypsin from elkhorn sculpin (TR-E) (square), (b): porcine pancreatic trypsin.

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                          1        10        20
Jacopever                 IVGGYECKPYSQPHQVSLNS
Elkhorn sculpin           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
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Fig. 7. Comparison of the N-terminal amino acid sequences of trypsins from jacopever (*S. schlegelii*) and elkhorn sculpin (*A. alcicornis*) with those of other vertebrates. True sardine (Kishimura et al., 2006); Arabesque greenling (Kishimura et al., 2006); Japanese anchovy (Kishimura et al., 2005); 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).

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