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

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Abstract

Trypsins from the pyloric ceca of jacopever (*Sebastes schlegeli*), 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 the TR-J and TR-E were estimated to be 24,000 Da by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The TR-J and TR-E revealed optimum temperatures of 60 ^{o}C and 50 ^{o}C, respectively, and showed the same optimum pH (pH 8.0) for hydrolysis of N^{α}-*p*-tosyl-L-arginine methyl ester. The TR-J and TR-E were unstable at above 50 ^{o}C and 40 ^{o}C, respectively, and were more stable at alkaline pH than at acidic pH. Thermal stabilities of the TR-P 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 the TR-J and TR-E were also investigated. The N-terminal amino acid sequences of the TR-J, IVGGYECKPYSQPHQVSLNS and TR-E, IVGGYECTPHSQAHQVSLNS were found, and these sequences showed highly homology to other fish trypsins.

Keywords: Jacopever, *Sebastes schlegeli*, 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 and Haard, 1999). 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 emzymatic activities at low temperatures than their counterparts from warm-blooded animals (Asgeirsson, Fox and 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 and Haard, 1987).

One of the main digestive protease, which is detected in 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 include the following: capelin (Hjelmeland and Raa, 1982), catfish (Yoshinaka, Suzuki, Sato and Ikeda, 1983), Greenland cod (Simpson and Haard, 1984), anchovy (Martinez, Olsen and Serra, 1988), Atlantic cod (Asgeirsson et al., 1989), rainbow trout (Kristjansson, 1991), Monterey sardine (Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno and Toro, 2005), and Japanese anchovy (Kishimura, Hayashi, Miyashita and Nonami, 2005a). Recently, we isolated trypsins from the viscera of true sardine (*Sardinops melanostictus*) and from the pyloric ceca of arabesque

greenling (*Pleuroprammus azonus*). (Kishimura, Hayashi, Miyashita and Nonami, 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. On the other hand, jacopever (*Sebastes schlegeli*) and elkhorn sculpin (*Alcichthys alcicornis*) are one of the important fish-catches of Japan and are used almost for food production. Also, no information regarding the characteristics of trypsins from the pyloric ceca of both species has been reported. Therefore, this study aimed to purified trypsins from the pyloric ceca of jacopever (*S. schlegeli*) and elkhorn sculpin (*A. alcicornis*) and compared the characteristics to those of porcine pancreatic trypsin.

2. Materials and methods

2.1. Materials

The jacopever (*S. schlegeli*) 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-phenyl-alanine 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 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 ^{o}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 one millimole 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 concentration designated (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 the mixture at 25 ^{o}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 ^{o}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 ^{o}C and by incubating the enzyme at 30 ^{o}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 ^{o}C and at pH 8.0 in the presence of 10 mM ethylenediaminetetraacetic acid (EDTA) 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 pancreatic (24,000 Da), Japanese anchovy (24,000 Da) (Kishimura et al., 2005a), true sardine (24,000 Da) (Kishimura et al., 2005b), arabesque greenling (24,000 Da) (Kishimura et al., 2005b), Greenland cod (23,500 Da)

(Simpson and Haard, 1984), and Atlantic cod (24,200 Da) (Asgeirsson et al., 1989).

The effects of various proteinase inhibitors on the activity of the 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 metallo proteinase (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 the TR-J and TR-E are depicted in Fig.2a. Both enzymes hydrolyzed the 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 (Hjelmeland and Raa, 1982; Simpson and Haard, 1984; Yoshinaka, Sato, Suzuki and Ikeda, 1984; Martinez et al., 1988; Asgeirsson et al., 1989; Castillo-Yanez et al., 2005; Kishimura et al., 2005a; Kishimura et al., 2005b). Fig. 3a shows the temperature dependencies of the TR-J and TR-E. The TR-J and TR-E revealed optimum temperatures of 60 ^{o}C and 50 ^{o}C, respectively, which were lower than that of porcine pancreatic trypsin (60-70 ^{o}C; Fig. 3b). The TR-J examined in this study had an optimum temperature of 60 ^{o}C 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, optimum temperature of the TR-E (50 ^{o}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 stability of the TR-J and TR-E are shown in Fig.4a. The TR-J was stable at 30 ^{o}C for 30 min in the pH range from pH 5.0 to 11.0, whereas the 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 (Martinez et al., 1988; Asgeirsson et al., 1989; Kristjansson, 1991; Kishimura et al., 2005a; Kishimura et al., 2005b), but is in marked

contrast to the pH stability of porcine pancreatic trypsin that is most stable at pH 4 (Fig. 4b). Fig. 5a shows the temperature stabilities of the TR-J and TR-E. The TR-J was stable below 40 ^{o}C, but its activity quickly fell above 50 ^{o}C. The temperature stability of the TR-J was similar to that of Monterey sardine trypsin (Castillo-Yanez et al., 2005) and Japanese sardine trypsin (Kishimura et al., 2005b). Whereas the TR-E was stable when heated up to 30 ^{o}C, and its activity quickly fell above 40 ^{o}C. The TR-E was unstable than the TR-J, other fish trypsins (Martinez et al., 1988; Kristjansson, 1991), and porcine pancreatic trypsin (Fig. 5b). Genicot, Rentier-Delrue, Edwards, Vanbeeumen and Gerday (1996) considered that overall decrease of hydrophobicity and increase in surface hydrophilicity of fish trypsin as compared to its bovine counterparts affect to thermostability and flexibility. Therefore, such structural characteristics of TR-E may contribute its lower temperature stability.

The effects of $CaCl_2$ on the 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 and Ikeda, 1985), and rainbow trout (Kristjansson, 1991). Two calcium-binding sites are in bovine trypsinogen (Kossiakoff, Chambers, Kay and 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. The TR-J and TR-E were stabilized by calcium ion from thermal denaturation. These findings suggest that the TR-J and TR-E possess the primary calcium-binding site like mammalian pancreatic trypsin and other fish trypsins (Male, Lorens, Smalas and Torrissen, 1995; Genicot, 1996).

The N-terminal amino acid sequences of the 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 the TR-J and TR-E were unblocked. The N-terminal amino acid sequences of the TR-J and TR-E were aligned with the sequences of other animal trypsins (Fig. 7). N-terminal 4 amino acid sequences of the 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, the 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 and Dickerson, 1974), and other vertebrate trypsins also have possessed the Cys-7 (Walsch, 1970; Hermodson, Ericsson, Neurath and Walsh, 1973; Emi et al., 1986; Gudmundsdottir, Gudmundsdottir, Oskarsson, Bjarnason, Eakin and Craik, 1993; Male et al., 1995; Genicot et al., 1996; Kishimura et al., 2005a; Kishimura et al., 2005b). In this study, the Cys residue was characteristically conserved in the TR-J and TR-E (Fig. 7). The results indicate that the TR-J and TR-E may also have a disulfide bond to the corresponding residues (between Cys-7 and Cys-142) of bovine pancreatic trypsin.

In conclusion, the TR-J showed almost same characteristics with that of porcine pancreatic trypsin except for unstable below pH 5.0. These results suggest that the viscera of jacopever (*S. schlegeli*) would be a potential source of trypsin for food processing operations. On the other hand, the TR-E showed lower optimum temperature than that of porcine pancreatic trypsin and were unstable than porcine pancreatic trypsin below pH 5.0 and above 40 ℃. 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 relatively lower thermal stability of the TR-E may also be beneficial in such applications as the enzymes can be inactivated more readily.

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(captions to figures)

Fig. 1. Electrophoresis of purified trypsins from jacopever (*S. schlegeli*) and elkhorn sculpin (*A. alcicornis*). Electrophoresis was performed using a 0.1 % SDS-12.5 % polyacrylamide slab-gel. Lane 1 contains protein standards; bovine pancreatic trypsinogen （molecular weight, 24,000 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).

Fig. 2. Effects of pH on the activity of trypsins from jacopever (*S. schlegeli*) 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 ℃. a: trypsin from jacopever (TR-J) (closed circle); trypsin from elkhorn sculpin (TR-E) (closed triangle), b: Porcine pancreatic trypsin.

Fig. 3. Effects of temperature on the activity of trypsins from jacopever (*S. schlegeli*) 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) (closed triangle), b: Porcine pancreatic trypsin.

Fig. 4. PH stability of trypsins from jacopever (*S. schlegeli*) and elkhorn sculpin (*A.*

alcicornis). The enzymes were kept at 30 ℃ for 30 min and pH 4.0-11.0, and then the remaining activities at 30 ℃ and pH 8.0 were determined. a: trypsin from jacopever (TR-J) (closed circle); trypsin from elkhorn sculpin (TR-E) (closed triangle), b: Porcine pancreatic trypsin.

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

Fig. 6. Effect of calcium ion on the stability of trypsins from jacopever (*S. schlegeli*) and elkhorn sculpin (*A. alcicornis*). The enzymes were kept at 30 ℃ 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 ℃ and pH 8.0 were determined. a: trypsin from jacopever (TR-J) (circle); trypsin from elkhorn sculpin (TR-E) (square), b: Porcine pancreatic trypsin.

Fig. 7. Comparison of the N-terminal amino acid sequences of trypsins from jacopever (*S. schlegeli*) and elkhorn sculpin (*A. alcicornis*) 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

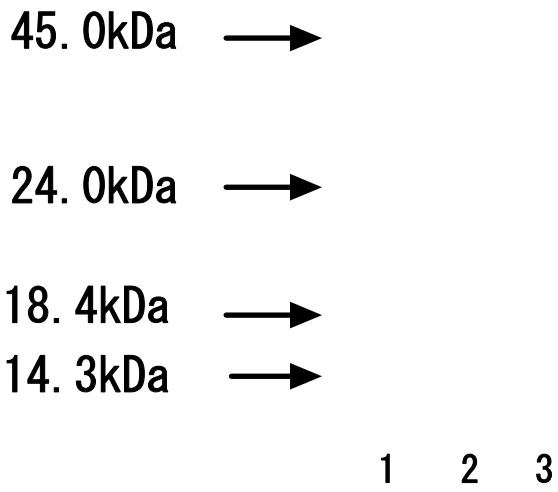
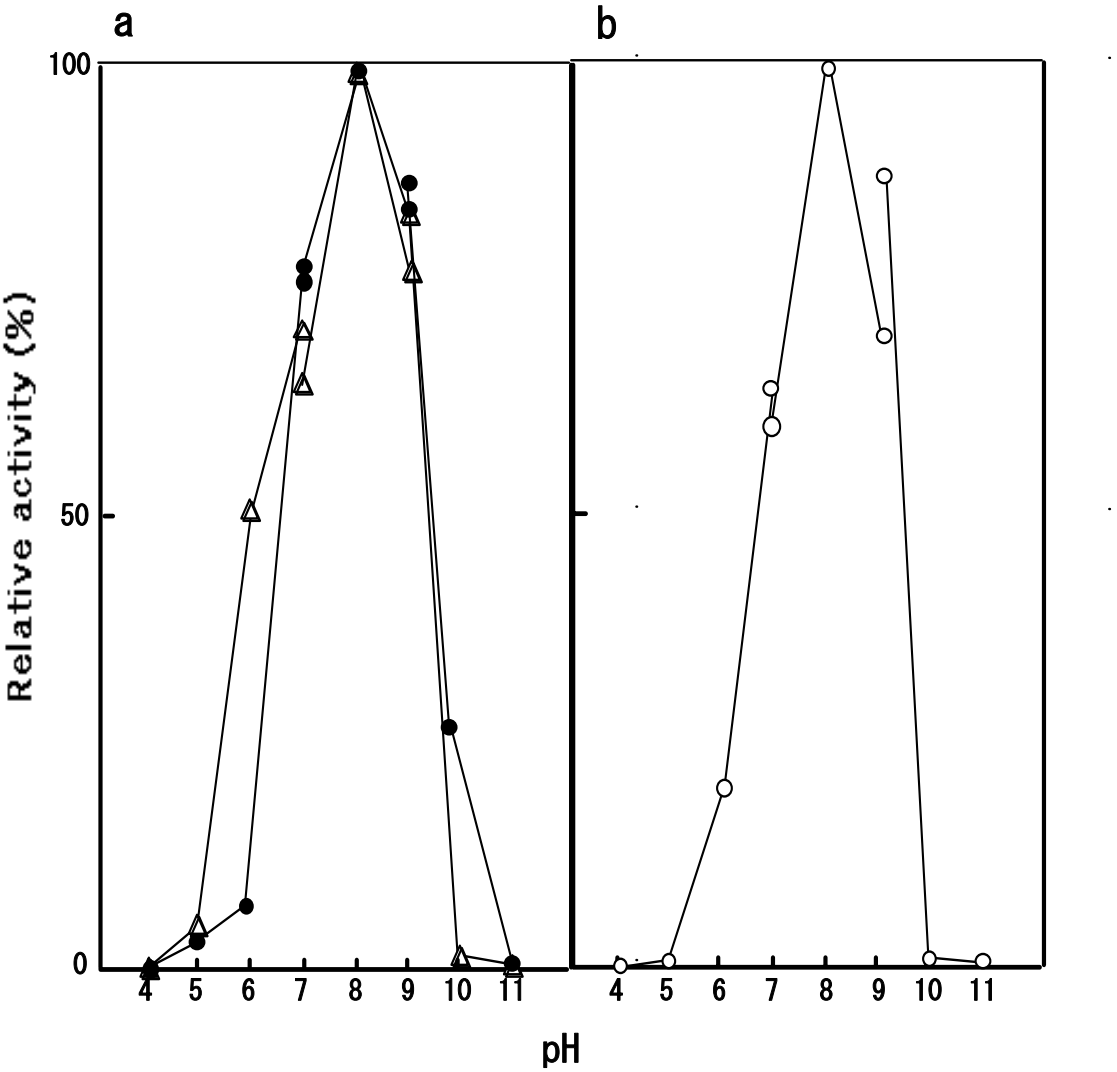


Fig.2



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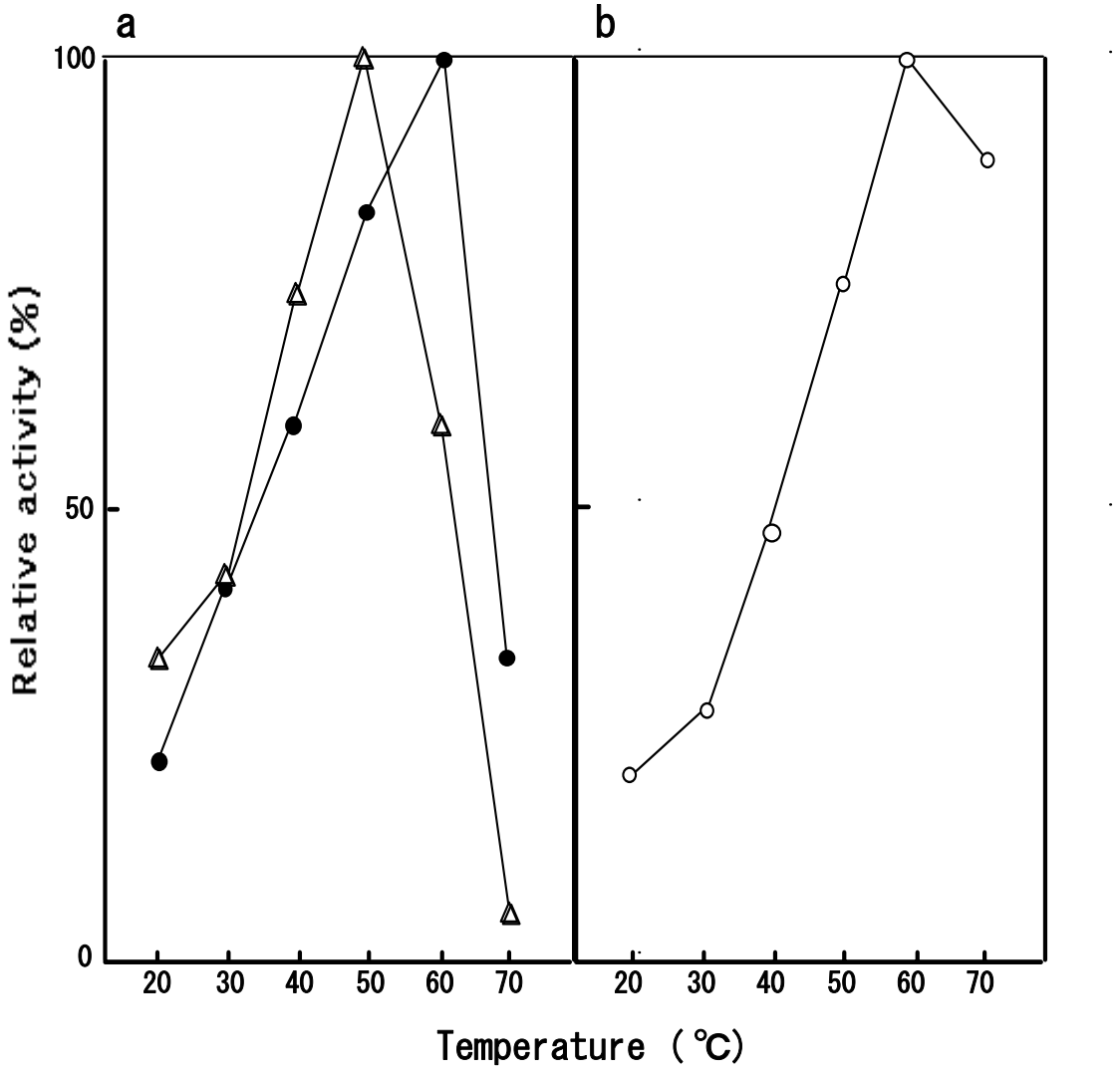

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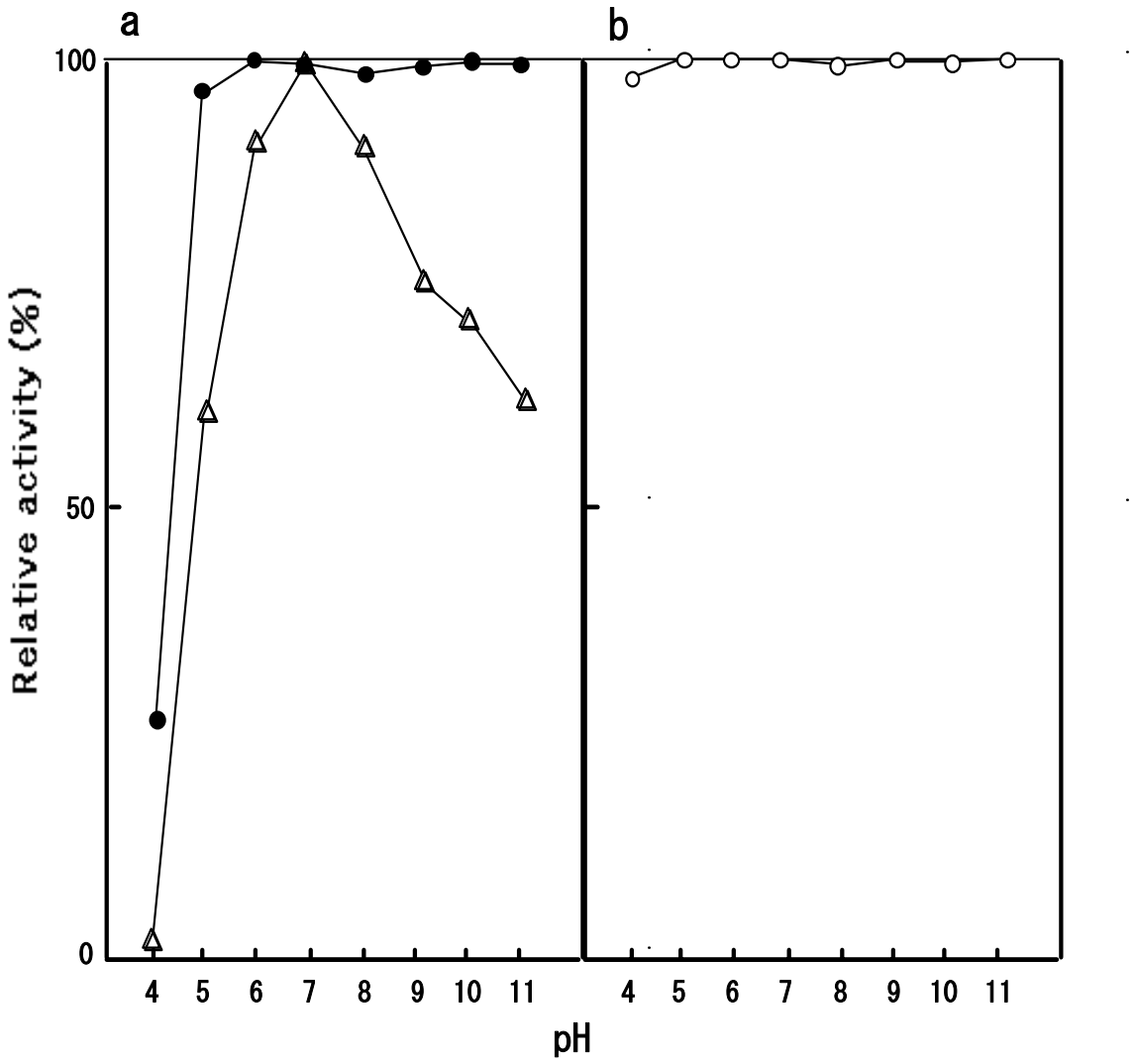

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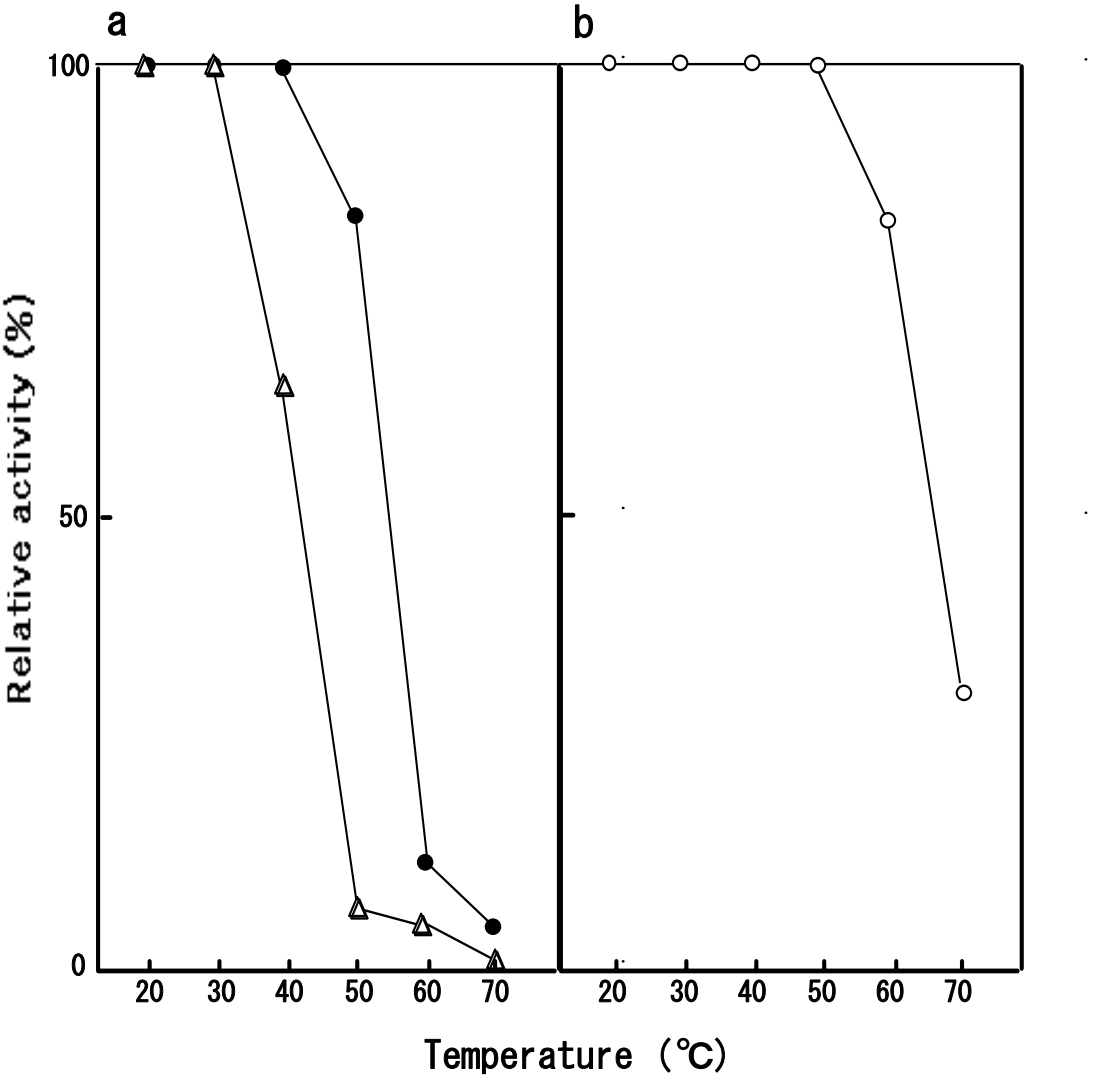

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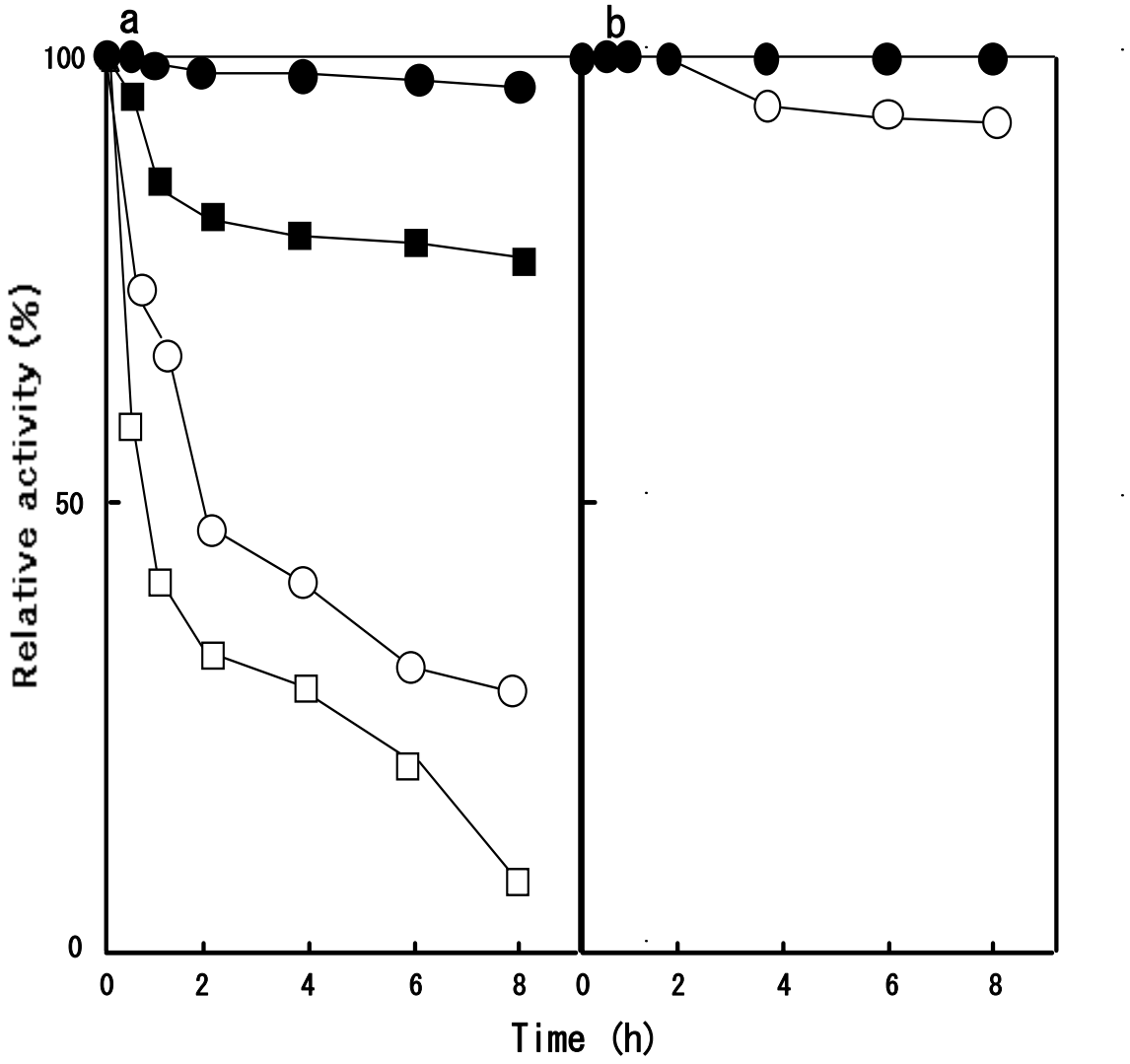

Fig.7

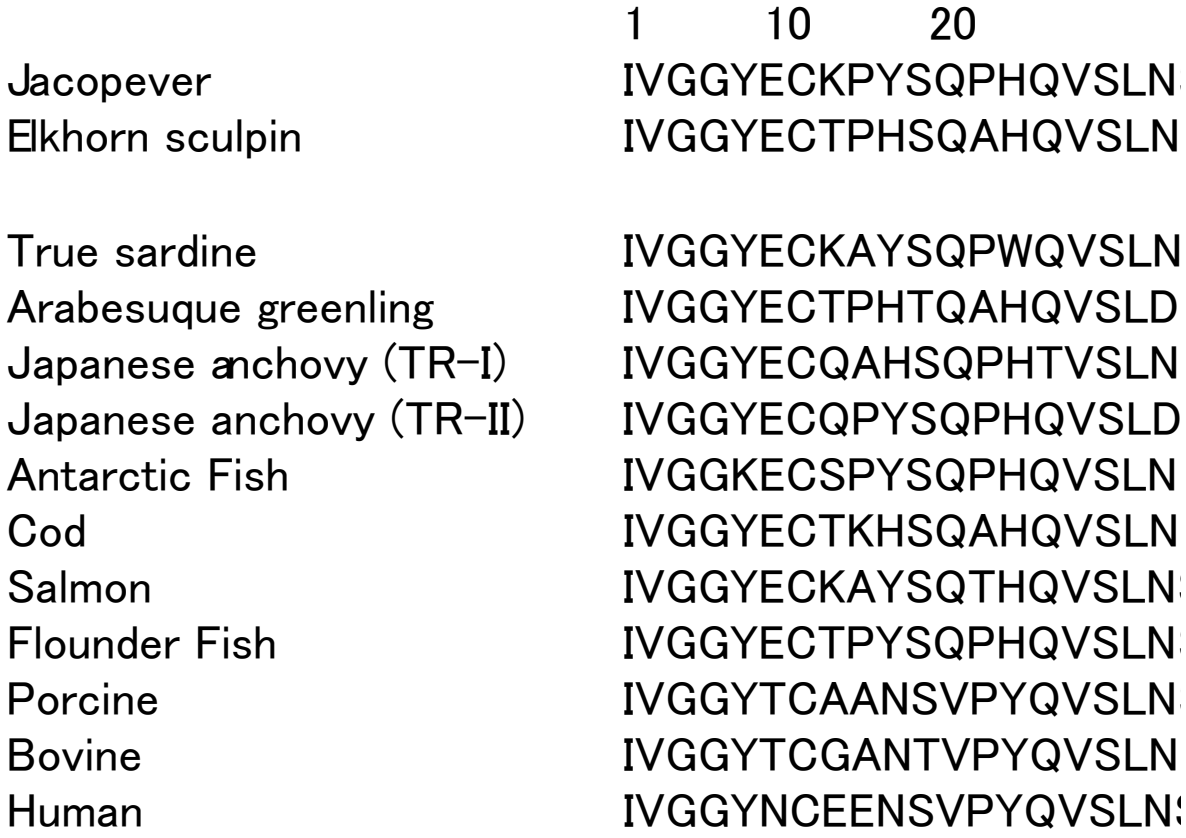
1 10 20
Jacopever IVGGYECKPYSQPHQVSLN
Elkhorn sculpin IVGGYECTPHSQAHQVSLN
True sardine IVGGYECKAYSQPWQVSLN
Arabesuque greenling IVGGYECTPHTQAHQVSLD
Japanese anchovy (TR-I) IVGGYECQAHSQPHTVSLN
Japanese anchovy (TR-II) IVGGYECQPYSQPHQVSLD
Antarctic Fish IVGGKECSPYSQPHQVSLN
Cod IVGGYECTKHSQAHQVSLN
Salmon IVGGYECKAYSQTHQVSLN
Flounder Fish IVGGYECTPYSQPHQVSLN
Porcine IVGGYTCAANSVPYQVSLN
Bovine IVGGYTCGANTVPYQVSLN
Human IVGGYNCEENSVPYQVSLN

Table 1

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

Purification stages	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purity (fold)	Yield (%)
Jacopever					
Crude enzyme	4,392	3,514	0.8	1	100
Sephacryl S-200	1,138	2,504	2.2	3	71
Sephadex G-50	34	1,615	47.5	59	46
Elkhorn sculpin					
Crude enzyme	6,594	7,253	1.1	1	100
Sephacryl S-200	2,002	4,404	2.2	2	61
Sephadex G-50	67	2,345	35.0	32	32

Table 2

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

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