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Isolation and characteristics of trypsin from
pyloric ceca of the starfish *Asterina pectinifera*

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Abstract

Trypsin was purified from pyloric ceca of the starfish *Asterina*

pectinifera by ammonium sulfate precipitation, gel filtration, and cation-exchange chromatography. Final enzyme preparation was nearly homogeneous in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and its molecular weight was estimated as approximately 28,000. Optimum pH and temperature of *A. pectinifera* trypsin for hydrolysis of N^α-p-Tosyl-L-arginine methyl ester hydrochloride were about pH 8.0 and 55 °C, respectively. *A. pectinifera* trypsin was unstable at above 50 °C and below pH 5.0 and was not activated by adding Ca²⁺. The N-terminal amino acid sequence of *A. pectinifera* trypsin, IVGGHEF, was found.

Keywords: *Asterina pectinifera*, Calcium ion, Isolation, Marine invertebrate, pH stability, Pyloric cecum, Starfish, Trypsin.

1. Introduction

Trypsin (EC 3.4.21.4) is an important pancreatic serine protease synthesized as a proenzyme by pancreatic acinar cells and is secreted into the

intestine of mammals. Mammalian pancreatic trypsin and its proenzyme have been extensively characterized (Walsh, 1970; Kossiakoff, 1977). Fish trypsin is similar to mammalian pancreatic trypsin in its molecular weight, amino acid composition, Ca^{2+} requirement, and reaction with substrates and inhibitors. cDNAs encoding trypsin from Atlantic cod and salmon have been isolated (Gundmundsdottir et al., 1993; Male et al., 1995). Fish trypsin has distribution patterns of charged and hydrophobic amino acid residues similar to mammalian trypsin, indicating similar three-dimensional structures. Trypsins from some marine invertebrates have been purified and characterized (Gates and Travis, 1969; Winter and Neurath, 1970; Kishimura and Hayashi, 1989). The characteristics of enzymes from marine invertebrates resemble those of mammalian pancreatic trypsin in molecular weight, cleavage specificities, and reaction with inhibitors. From a structural study on active site of trypsin of the starfish *Dermasterias imbricata*, the amino acid composition of the peptide from the active site of the trypsin is similar to the amino acid composition of mammalian pancreatic trypsin (Camacho et al., 1976) . However, marine invertebrate trypsins were unstable at acidic pH and were not activated or stabilized by adding calcium ions, unlike mammalian pancreatic trypsin. These findings suggest that notable structural differences exist between mammalian pancreatic and starfish trypsins.

In this study, we purified a trypsin from the pyloric ceca of the starfish *Asterina pectinifera* and we examined the characteristics and N-terminal amino acid sequence of trypsin.

2. Materials and methods

2.1. Materials

The starfish *A. pectinifera* was caught off Hakodate, Hokkaido Prefecture, Japan. Sephacryl S-200 was purchased from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was purchased from Whatman (Maidston, England). Sephadex G-50, carboxymethyl (CM)-cellulose and *N*^α-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Preparation of crude trypsin

Pyloric ceca of *A. pectinifera* were homogenized in three volumes of acetone at -20 °C for 30 min, and the homogenate was filtrated *in vacuo* on ADVANTEC No. 2 filter paper. Similarly, the residue was homogenized in two volumes of acetone at -20 °C for 30 min, and then the residue was air-dried at room temperature. Trypsin was extracted by stirring from the delipidated powder in 50 volumes of 50 mM Tris-HCl buffer (pH 8.0) at 5 °C for 3 h. The extract was centrifuged at 10,000 × g for 10 min, and then the supernatant was fractionated between 40% and 75 % saturation with ammonium sulfate solution. The trypsin fraction was dissolved in the minimum amount of 50 mM Tris-HCl buffer (pH 8.0) and was dialyzed against the same buffer into crude trypsin.

2.3. Purification of A. pectinifera trypsin

The crude trypsin was applied to a column of Sephacryl S-200 (3.9×44 cm) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0) and the proteins were eluted with the same buffer. The main trypsin fraction was concentrated by lyophilization and was dialyzed against 10 mM Tris-HCl buffer (pH 8.0). The dialyzate was applied to a DEAE-cellulose column (2.2×18 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) and the proteins were eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. Trypsin activity was detected mainly in the non-adsorbed fraction. This fraction was concentrated and was applied to a Sephadex G-50 column (3.9×64 cm) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The proteins were eluted with the same buffer. A single trypsin fraction was obtained and the fraction was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) and was applied to a CM-cellulose column (1.1×18 cm) pre-equilibrated with the same buffer. The proteins were eluted with a linear gradient of 0–0.5 M NaCl. Trypsin was eluted mainly with 0.15–0.25 M NaCl and the protein in this fraction showed a nearly single band on SDS-PAGE (Fig. 1). The final enzyme preparation was purified 36-fold from the crude trypsin (Table 1).

2.4. Enzyme assay

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 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 30–70 °C and by incubating the enzyme at 30 °C for 30 min at a range of pH 4.0–9.0, respectively.

The effect of CaCl₂ on the activity of the enzyme was found by incubating the enzyme at 30 °C and at pH 8.0 in the presence of 2 mM ethylenediaminetetraacetic acid (EDTA) or 2 mM CaCl₂.

2.5. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using a 0.1 % SDS–10 % polyacrylamide slab gel by the method of Porzio and Pearson (1977). 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 purified *A. pectinifera* trypsin, the enzyme was electroblotted to polyvinylidenedifluoride membrane after SDS–PAGE. The amino acid sequence of the enzyme was found by using a protein sequencer, Procise 492 (Perkin Elmer, Foster City, CA, USA).

2.7. Protein concentration

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

3. Results and discussion

3.1. Properties of *A. pectinifera* trypsin

Trypsin was purified from pyloric ceca of the starfish *Asterina pectinifera* by ammonium sulfate precipitation, gel filtration, and cation-exchange chromatography. The *A. pectinifera* trypsin was adsorbed CM-cellulose column and was not adsorbed DEAE-cellulose column, so the enzyme was presumed cationic trypsin. Two forms of trypsin, anionic and cationic, are in vertebrate pancreas (Walsh and Neurath, 1964; MacDonald et al., 1982; Yoshinaka et al., 1983; Pinsky et al., 1985; Fletcher et al., 1987; LeHuerou et al., 1990; Gudmundsdottir et al., 1993). Recent molecular work has revealed that trypsins are encoded by multi-gene families in vertebrate pancreas (MacDonald et al., 1982; Emi et al., 1986; Gudmundsdottir et al., 1993; Male et al., 1995). It is possible that starfish, like vertebrate, may have both anionic and cationic trypsins. However, up to the present, cationic trypsin has not been found in the starfish tissues. Therefore, this is the first report of starfish cationic trypsin.

The molecular weight of *A. pectinifera* trypsin was estimated as approximately 28,000 using SDS-PAGE (Fig. 1) similar to that of *A. amurensis* trypsin (28,000) (Kishimura and Hayashi, 1989), which was larger than trypsin

molecular weights of the starfishes *D. imbricata* (25,000–26,000) (Camacho et al., 1970), *L. anthosticta* (25,000) (Kozlovskaya and Elyakova, 1974), *P. giganteus* (26,300) (Bundy and Gustafson, 1973), *P. brevispinus* (23,000–24,000) (Gilliam and Kitto, 1976).

Figs. 2a and b show the pH and temperature dependencies of *A. pectinifera* trypsin, respectively. The enzyme hydrolyzed the TAME substrate effectively at alkaline pH with an optimum activity at about pH 8.0 (Fig. 2a). The optimum pH of *A. pectinifera* trypsin was similar to those of the starfishes *E. trochелиi* (pH 8.1) (Winter and Neurath, 1970), *D. imbricata* (pH 8.0–8.5) (Camacho et al., 1970), *L. anthosticta* (about pH 8.5) (Kozlovskaya and Elyakova, 1974), and *A. amurensis* (about pH 8.0) (Kishimura and Hayashi, 1989). The trypsin of *A. pectinifera* examined in this study had an optimum temperature of about 55 °C (Fig. 2b) similar to that of *A. amurensis* trypsin (about 55 °C) (Kishimura and Hayashi, 1989), which was higher than that of *L. anthosticta* (about 45 °C) (Kozlovskaya and Elyakova, 1974). The optimal temperature of these enzymes far exceeds the physiological temperature of the organisms. Although this is a very interesting phenomenon, we do not know the reason. Figs. 2c and d show the temperature and pH stabilities of *A. pectinifera* trypsin, respectively. The enzyme was stable below 40 °C, but its activity quickly fell above 50 °C (Fig. 2c). *A. pectinifera* trypsin was stable between pH 6.0 and 9.0 (Fig. 2d). However, it was unstable below pH 5.0, and the remaining activity at pH 4.0 was about 60 %. These properties of the pH stability of *A. pectinifera* trypsin were similar to those of other starfish trypsins (Camacho et al., 1970; Winter and Neurath, 1970; Bundy and Gustafson, 1973; Kozlovskaya and Elyakova, 1974; Gilliam and Kitto, 1976;

Kishimura and Hayashi, 1989), but were unlike mammalian pancreatic trypsin (Walsh, 1970).

The effect of CaCl_2 on *A. pectinifera* trypsin was found at pH 8.0 and 30 °C in the presence of 2 mM EDTA or 2 mM CaCl_2 . The enzyme was not activated by adding Ca^{2+} (Fig. 2e). Similarly, trypsins of other starfish species were neither activated nor stabilized by calcium ions (Camacho et al., 1970; Winter and Neurath, 1970; Bundy and Gustafson, 1973; Kozlovskaya and Elyakova, 1974; Gilliam and Kitto, 1976), but unlike mammalian pancreatic trypsin (Walsh, 1970). Two calcium binding sites are in bovine trypsinogen (Kossiakoff, 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. However, starfish trypsins were unstable below pH 5.0 and were not activated by Ca^{2+} . These findings suggest that a notable difference in the structure of the primary calcium binding site may exist between mammalian pancreatic trypsin and starfish trypsin.

3.2 N-terminal amino acid sequence of *A. pectinifera* trypsin

The N-terminal amino acid sequence of *A. pectinifera* trypsin was analyzed. We sequenced seven amino acids, IVGGHEF, indicating that the N-terminus of trypsin was unblocked. The N-terminal amino acid sequence of *A. pectinifera* trypsin was aligned with the sequences of other animal trypsins (Fig. 3). The N-terminal amino acids of trypsins from the starfish *L.*

anthosticta are Glx or Asx (Kozlovskaya and Elyakova, 1974). However, the N-terminal 4 amino acid sequence of *A. pectinifera* trypsin (IVGG) was identical with those of other animal trypsins in this study (Fig. 3). Being similar to fish trypsins, *A. pectinifera* trypsin had a charged Glu residue at position 6, where Thr is most common in mammalian pancreatic trypsins (Fig. 3). The starfish trypsin resembles mammalian pancreatic trypsin in cleavage and inhibitor specificities (Winter and Neurath, 1970; Bundy and Gustafson, 1973; Kozlovskaya and Elyakova, 1974; Gilliam and Kitto, 1976). Camacho et al. (1975) studied the structure of the active site of the starfish *D. imbricata* trypsin. They reported that the amino acid composition of the peptide from the active site of the trypsin is found to be similar to the amino acid composition of mammalian pancreatic trypsin. On the other hand, bovine pancreatic trypsin has a disulfide bond between Cys-7 and Cys-142 (Stroud et al., 1974), and other vertebrate trypsins has conserved the Cys-7. However, in this study, the Cys residue was characteristically replaced by Phe in *A. pectinifera* trypsin (Fig. 3). Recently, we extracted total RNA from the pyloric ceca of the starfish *A. pectinifera* and obtained first strand cDNA library. cDNA cloning and sequencing of the *A. pectinifera* trypsin are now under way.

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(captions to figures)

Fig. 1. Electrophoresis of purified the starfish *A. pectinifera* trypsin.

Electrophoresis was performed using a 0.1 % SDS-10 % polyacrylamide slab-gel. Lane 1 is *A. pectinifera* trypsin. Lane 2 is protein standards: cytochrome C dimer (24,800), cytochrome C trimer (37,200), cytochrome C

tetramer (49,600), and cytochrome C hexamer (74,400).

Fig. 2. Effects of pH, temperature, and CaCl_2 on activity or stability of the starfish *A. pectinifera* trypsin.

a: Effect of pH on the activity of *A. pectinifera* trypsin. An assay was performed by incubating mixtures of The enzyme and 50 mM of a buffer solution (acetic acid–sodium acetate (pH 4.0–5.0), sodium phosphate monobasic–dibasic (pH 6.0–7.0), and Tris–HCl (pH 8.0–9.0)), and then the activity at 30 °C was determined.

b: Effect of temperature on the activity of *A. pectinifera* trypsin. An assay was performed by incubating mixtures of the enzyme and 50 mM Tris–HCl (pH 8.0) at 20–60 °C.

c: Effect of temperature on the stability of *A. pectinifera* trypsin. The enzyme was kept at 30–70 °C for 15 min and at pH 8.0, and then the remaining activity at 30 °C and at pH 8.0 was determined.

d: Effect of pH on the stability of *A. pectinifera* trypsin. The enzyme was kept at 30 °C for 30 min and at pH 4.0–9.0, and then the remaining activity at 30 °C and at pH 8.0 was determined.

e: Effect of CaCl_2 on activity of *A. pectinifera* trypsin. The enzyme was kept at 30 °C and at pH 8.0 for 0–60 min in the presence of 2 mM CaCl_2 (●) and 2 mM EDTA (○), and then the remaining activity at 30 °C and at pH 8.0 was determined.

Fig. 3. Alignment of the N-terminal amino acid sequence of the starfish *A. pectinifera* trypsin with the sequences of trypsins from other animals.

Residues identical with *A. pectinifera* trypsin are shaded. Starfish, *A. pectinifera* trypsin (present paper); Dogfish; spiny Pacific dogfish *Squalus acanthias* pancreatic trypsin (Titani et al., 1975); Antarctic fish, antarctic fish *Paranotothenia magellanica* trypsin (Genicot et al., 1996); Cod anionic I, Atlantic cod anionic trypsin I (Gudmundsdottir et al., 1993); Cod anionic X, Atlantic cod anionic trypsin X (Gudmundsdottir et al., 1993); Salmon, Atlantic salmon *Salmo salar* trypsin I A (Male et al., 1995); Lungfish, African lungfish *Protopterus aethiopicus* pancreatic trypsin (Hermodson et al., 1971); Rat anionic, rat pancreatic anionic trypsin I (MacDonald et al., 1982); Rat cationic, rat pancreatic cationic trypsin (Fletcher et al., 1987); Dog anionic, dog pancreatic anionic trypsin (Pinsky et al., 1985); Dog cationic, dog pancreatic cationic trypsin (Pinsky et al., 1985); Porcine, porcine pancreatic trypsin (Hermodson et al., 1973); Bovine anionic, bovine pancreatic anionic trypsin (LeHuerou et al., 1990); Bovine cationic, bovine pancreatic cationic trypsin (Walsh and Neurath, 1964); Human, human pancreatic trypsin (Emi et al., 1986).

Fig. 1. Chromatography for purification of the trypsin-like enzyme from the pyloric ceca of the starfish *A.pectinifera*.

a: Gel filtration on Sephacryl S-200 (first) of the crude enzyme. The crude enzyme was applied on a column (2.7×45 cm) preequilibrated with 50 mM Tris-HCl buffer (pH 8.0), and protein was eluted with the same buffer at a flow rate of 40 ml/h. Each 3.5 ml fraction was collected.

b: DEAE-cellulose chromatography of the active fraction (A2) obtained from gel filtration in Fig. 1a. The active fraction was applied on a column (2.2×18 cm) preequilibrated with 10 mM Tris-HCl buffer (pH 8.0), and protein was eluted with a linear gradient of NaCl from 0 to 0.8 M of the total volume

of 500 ml at a flow rate of 60 ml/h. Each 3.5 ml fraction was collected.

c: Gel filtration on Sephacryl S-200 (second) of the active fraction (B1) obtained from DEAE-cellulose chromatography in Fig. 1b. The active fraction was applied to a column (2.7×45 cm) preequilibrated with 50 mM Tris-HCl buffer (pH 8.0), and protein was eluted with the same buffer at a flow rate of 40 ml/h. Each 2.0 ml fraction was collected.

d: Gel filtration on Sephadex G-50 of the active fraction (C1) obtained from gel filtration Fig. 1c. The active fraction was applied on a column (2.7×45 cm) preequilibrated with 50 mM Tris-HCl buffer (pH 8.0), and protein was eluted with the same buffer at a flow rate of 40 ml/h. Each 2.0 ml fraction was collected.

Curves are designated as follows: absorbance at 280 nm, — ; tryptic activity, —●—; NaCl concentration, ∙∙∙.

Fig. 2. CM-Cellulose chromatography for purification of the trypsin-like enzyme from the pyloric ceca of the starfish *A. pectinifera*.

CM-Cellulose chromatography of the active fraction (D1) obtained from gel filtration in Fig. 1d. The active fraction was applied on a column (1.1×18 cm) preequilibrated with 10 mM Tris-HCl buffer (pH 8.0), and protein was eluted with a linear gradient of NaCl from 0 to 0.5 M of the total volume of 400 ml at a flow rate of 20 ml/h. Each 3.3 ml fraction was collected. Curves are designated as follows: absorbance at 280 nm, — ; tryptic activity, —●—; NaCl concentration, ∙∙∙.

Table 1
Purification of the starfish *A. pectinifera* trypsin

Purification step	Protein (mg)	Total activity (U)*	Specific activity (U/mg)	Purity (fold)	Yield (%)
Crude enzyme	4,120	2,833	0.7	1	100
Sephacryl S-200	421	1,236	2.9	4	44
DEAE-Cellulose	27	412	15.3	22	15
Sephadex G-50	4.0	94	23.5	34	3
CM-Cellulose	1.0	25	25.0	36	1

* One unit (U) of activity was defined as the amount of the enzyme hydrolyzing one micromole of TAME in a minute.

DEAE: diethylaminoethyl; CM: carboxymethyl.

Fig. 1

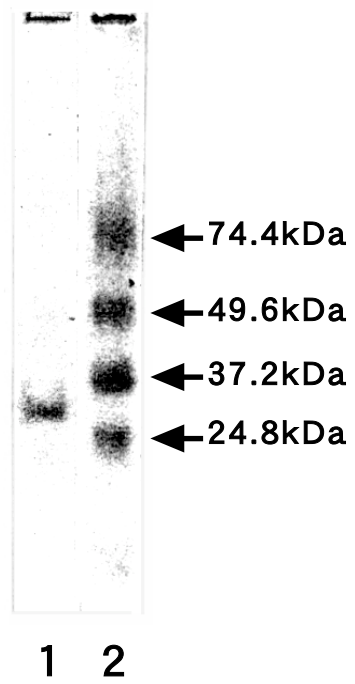
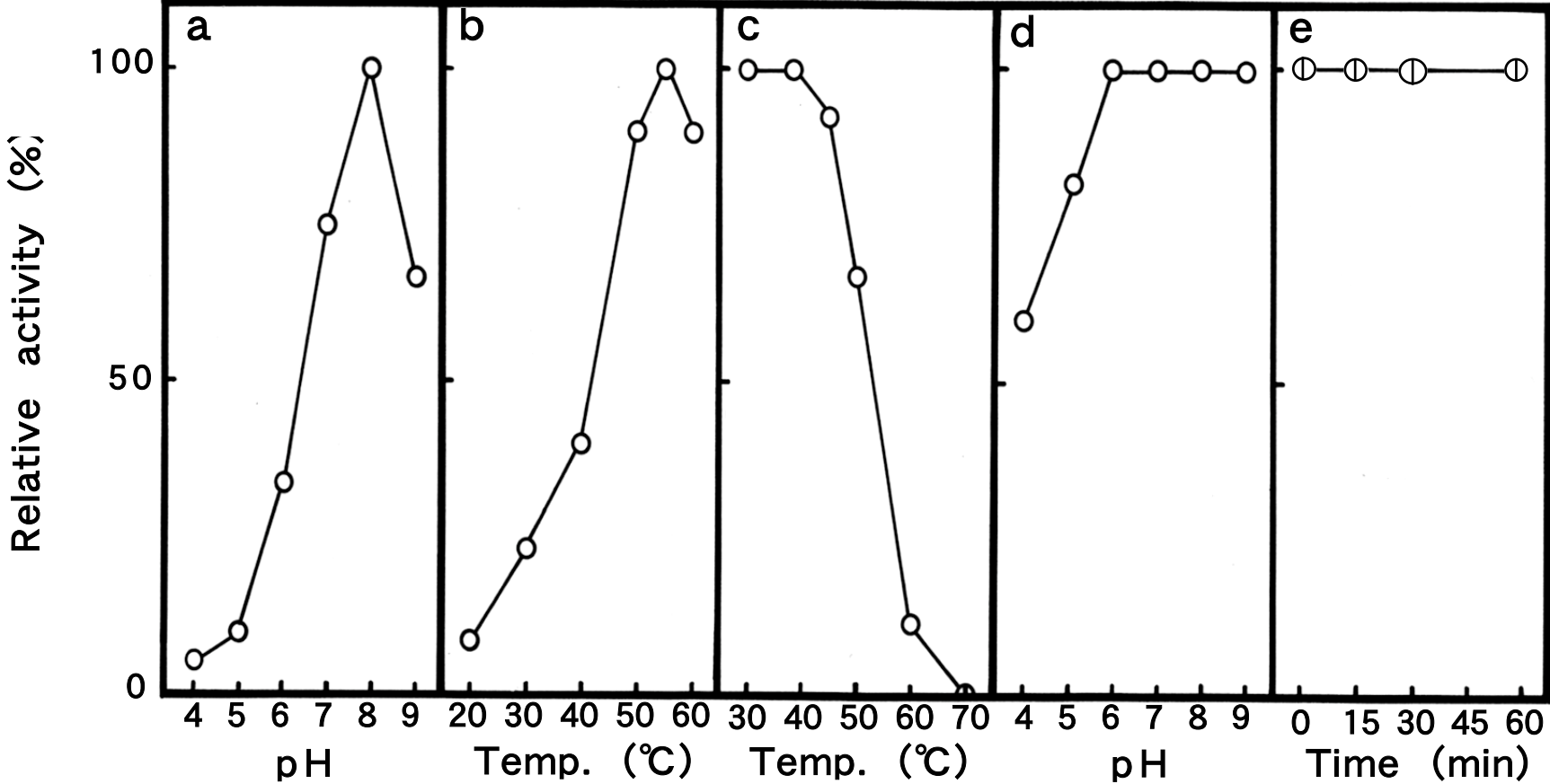


Fig. 2



	1
Starfish	IVGGHEF
Dogfish	IVGGYEC
Antarctic fish	IVGGKEC
Cod anionic I	IVGGYEC
Cod anionic X	IVGGYEC
Salmon	IVGGYEC
Lungfish	IVGGYEC
Rat anionic	IVGGYTC
Rat cationic	IVGGYTC
Dog anionic	IVGGYTC
Dog cationic	IVGGYTC
Porcine	IVGGYTC
Bovine anionic	IVGGYTC
Bovine cationic	IVGGYTC
Human	IVGGYNC