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Isolation and characteristics of carboxypeptidase B from
the pyloric ceca of the starfish *Asterias amurensis*

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Abstract

Carboxypeptidase B was purified from the pyloric ceca of the starfish *Asterias amurensis*. The final enzyme preparation was nearly homogeneous in polyacrylamide gel electrophoresis and its molecular weight was estimated as approximately 34,000. The optimum pH and temperature of the enzyme for hydrolysis of benzoyl-glycyl-L-arginine were at around pH 7.5 and 55 °C, respectively. The enzyme was unstable at above 50 °C and at below pH 5.0. The enzyme was activated by Co^{2+} , but was inhibited by EDTA and Hg^{2+} . The N-terminal amino acid sequence of *A. amurensis* carboxypeptidase B, ASFDYNVYHSYQEIMNWITN, was found.

Keywords: *Asterias amurensis*; Carboxypeptidase B; Isolation; Marine invertebrate; Metalloenzyme; N-terminal amino acid sequence; Pyloric cecum; Starfish.

1. Introduction

Carboxypeptidase B (CPB) (EC 3.4.17.2) is a metalloenzyme which

contains one zinc atom per molecule and selectively hydrolyzes arginine and lysine from the carboxyl terminus of proteins or peptides. CPB has been isolated and well characterized from porcine and bovine pancreases (Folk et al., 1960; Folk et al., 1962a, b; Wolff et al., 1962; Cox et al., 1962; Wintersberger et al., 1962; Schmid and Herriott, 1976; Ventura et al., 1999). CPB has been also purified and characterized from human (Marinkovic et al., 1977; Edge et al., 1998), camel (Al-Ajlan and Bailey, 1999), ostrich (Bradley et al., 1996), spiny Pacific dogfish *Squalus acanthias* (Prahl and Neurath, 1966), dogfish *Scyliorhinus canicula* (Hajjou et al., 1995), African lungfish *Protopterus aethiopicus* (Reek and Neurath, 1972), carp *Cyprinus carpio* (Cohen et al., 1981), catfish *Parasilurus asotus* (Yoshinaka et al., 1984a, b), white shrimp *Penaeus setiferus* (Gates and Travis, 1973), and crayfish *Astacus fluviatilis* (Zwilling et al., 1979; Titani et al., 1984).

However, few reports exist on CPB from the digestive gland of marine invertebrates. Ferrell et al. (1974) purified CPB-like enzyme from the pyloric ceca of the starfish *Dermasterias imbricata*. This CPB-like enzyme had a molecular weight of approximately 34,000 and showed high activity toward the substrates, benzoyl-glycyl-L-lysine and benzoyl-glycyl-L-arginine (Bz-Gly-L-Arg). The optimum pH of the CPB-like enzyme was at pH 7–7.5 and seemed to be quite stable, and a little loss of its activity was observed on incubation for 15 min at temperatures up to 50 °C. These properties of the starfish CPB-like enzyme were similar to those of mammalian pancreatic CPB. However, the starfish CPB-like enzyme was found to retain 100 % of its activity when it was incubated with 1 mM ethylenediaminetetraacetic acid (EDTA) for 1 h at 0 °C.

In the previous study, we found high proteolytic activity in the crude enzyme solution extracted from delipidated powder of the pyloric ceca of the starfish *Asterias amurensis* (Kishimura and Hayashi, 1989a). Further, we isolated and characterized a trypsin (Kishimura and Hayashi, 1989b) and a carboxypeptidase A (Kishimura and Hayashi, 1991) from the pyloric ceca of the starfish *A. amurensis*. In this study, as a part of investigation of echinoderm proteases, we isolated CPB from the pyloric ceca of the starfish *A. amurensis* and examined the characteristics and N-terminal amino acid sequence of the CPB.

2. Materials and methods

2.1 Materials

The starfish *A. amurensis* were caught off Hakodate, Hokkaido Prefecture, Japan. The specimens were stored at -20 °C for several months. Bz-Gly-L-Arg was purchased from Sigma (St. Louis, MO, USA).

2.2 Preparation of crude enzyme solution

Crude enzyme solution was prepared by the same method as described by Kishimura and Hayashi (2002).

2.3 Purification of starfish CPB

The crude enzyme solution 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 CPB 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 30 mM NaCl in the same buffer. The main CPB 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 CPB fraction was obtained and the fraction was applied to gel filtration on Sephadex G-50 under the same conditions.

2.4 Assay for CPB activity

CPB activity was measured by the method of Folk and Schirmer (1963) with Bz-Gly-L-Arg as a substrate. One unit of enzyme activity was defined as the amount of the enzyme hydrolyzing one micromole of Bz-Gly-L-Arg in a minute. Values of the kinetic parameters k_{cat} and K_m over the concentration ranges 0.1–1.0 mM for Bz-Gly-L-Arg were calculated from Lineweaver-Burk plot.

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 4.0–11.0, respectively.

The effect of EDTA on the activity of the enzyme was found by incubating the enzyme at 25 °C and at pH 7.5 in the presence or absence of 1 mM EDTA.

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). PAGE was carried out using a 10 % 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.

2.6 Analysis of amino acid sequence

To analyze the N-terminal sequence of purified *A. amurensis* CPB, the enzyme was electroblotted to polyvinylidenedifluoride (PVDF) 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 determination

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

3. Results

Purification procedure of starfish CPB is summarized in Table 1. The final enzyme preparation was purified 920-fold from the crude enzyme solution in a yield of 13 %. The enzyme was found to be nearly homogeneous using SDS-PAGE and PAGE (Fig. 1a, b). The molecular weight of the starfish CPB was estimated as approximately 34,000 using SDS-PAGE (Fig. 1a). The value of the specificity constant (k_{cat}/K_m) for hydrolysis of Bz-Gly-L-Arg by the starfish CPB was $6.01 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$ (Table 2).

The effect of pH on the activity of the starfish CPB was examined at 25 °C with Bz-Gly-L-Arg at several pH values. As shown in Fig. 2a, the maximum activity was found at pH 7.5. Fig. 2b shows the effect of temperature on the activity of the starfish CPB measured at pH 7.5 with Bz-Gly-L-Arg as substrate. The optimum temperature was near 55 °C.

The effect of temperature on the stability of the enzyme was examined at 30–70 °C by incubating the enzyme for 15 min at each temperature. The results are shown in Fig. 2c. The enzyme was stable at 30–45 °C, whereas more than 60 % of the enzyme activity were lost after 15 min of incubation of that at 60 °C. The effect of pH on the enzyme stability was examined at pH 4–11 by incubating the enzyme for 30 min at each pH. The results are shown Fig. 2d. The enzyme was stable at pH 6–10, whereas more than 50 % of the enzyme activity were lost after 30 min of incubation of that at pH 4.

The effect of EDTA on the starfish CPB activity was examined. The enzyme was pre-incubated with or without 1 mM EDTA in 25 mM Tris-HCl

buffer (pH7.5) at 25 °C for various times. The activity was determined with Bz-Gly-L-Arg at pH 7.5 and 25 °C and was expressed in percent of initial CPB activity. The results are shown in Fig. 3. About 90 % of the enzyme activity were lost after 6 h of incubation of that with 1 mM EDTA. The effect of metal ions on the activity of CPB was examined. The enzyme was pre-incubated with 1 mM of each metal ion in 25 mM Tris-HCl buffer (pH 7.5) at 25 °C for 2 h. The activity was measured at 25 °C and pH 7.5 with Bz-Gly-L-Arg and was expressed as percent of CPB activity in the absence of metal ions. The results are shown in Table 3. The enzyme was enhanced by Co^{2+} . On the other hand, the starfish CPB activity was slightly inhibited by Mg^{2+} and more strongly by Hg^{2+} .

The N-terminal amino acid sequence of the starfish CPB was determined after separation by SDS-PAGE and was electroblotted to PVDF membrane. A sequence of 20 amino acid residues, ASFDYNVYHSYQEIMNWITN, was determined.

4. Discussion

CPB was purified from crude enzyme solution prepared from the pyloric ceca of the starfish *A. amurensis*. Although a possible contamination of trypsin, carboxypeptidase A and other proteases was tested with N^{α} -*p*-Tosyl-L-arginine, benzoyl-glycyl-L-phenylalanine and casein as substrates, respectively, no activities of these enzymes were found (results not shown). The value of the specificity constant ($k_{\text{cat}}/K_{\text{m}}$) for hydrolysis of

Bz-Gly-L-Arg by the *A. amurensis* CPB ($6.01 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$) was similar to the corresponding values of $5.0 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$ (Wolff et al., 1962), $3.1 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$ (McKay et al., 1979), and $5.21 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$ (Al-Ajlan and Bailey, 1999) reported for porcine, bovine, and camel CPB, respectively.

A. amurensis CPB showed a molecular weight of about 34,000 on SDS-PAGE being similar to those of other species (33,000–35,000) (Folk et al., 1960; Wintersberger et al., 1962; Prah1 and Neurath, 1966; Reeck and Neurath, 1972; Gates and Travis, 1973; Ferrell et al., 1975; Marinkovic et al., 1977; Cohen et al., 1981; Titani et al., 1984; Yoshinaka et al., 1984a; Bradley et al., 1996). However, lower molecular weights were reported for cod (26,000) (Overnell, 1973), krill (31,000) (Osnes and Mohr, 1986). The optimum pH of *A. amurensis* CPB (pH 7.5) was similar to those of other species (pH 7–8.2) (Ferrell et al., 1975; Marinkovic et al., 1977; Yoshinaka et al., 1984b; Hajjou et al., 1995; Bradley et al., 1996; Al-Ajlan and Bailey, 1999), although the optimum temperature of the *A. amurensis* CPB (about 55 °C) was higher than that of krill (45 °C) (Osnes and Mohr, 1986). The optimal temperature of these enzymes far exceeds the physiological temperature of the organisms (Sagara and Ino, 1954). Although this is a very interesting phenomenon, we do not know the reason. The pH stability of *A. amurensis* CPB was similar to those of carp (Cohen et al., 1981) and catfish (Yoshinaka et al., 1984b) and the temperature stability of the *A. amurensis* CPB was similar to that of the starfish *D. imbricata* (Ferrell et al., 1975). On the other hand, *A. amurensis* CPB was inhibited by metal-chelating agents (EDTA) similar to those of other species. About 90 % of the enzyme activity were lost after 6 h in incubation of that with 1 mM EDTA. On catfish and dogfish CPBs, partial inhibition of the

enzyme activity is obtained with 1 mM EDTA, and total inhibitions occur at higher metal chelator concentration or the presence of 1 mM o-phenanthroline (Yoshinaka et al., 1984b; Hajjou et al., 1995). Hence, the metal ion required for the enzyme activity seems to be tightly bound to the CPB. *A. amurensis* CPB was strongly, but not completely, inhibited by Hg^{2+} like catfish and dogfish CPBs (Yoshinaka et al., 1984b; Hajjou et al., 1995). However, the activity of the catfish and dogfish CPBs was not affected by monoiodoacetate and 4-hydroxymercuribenzoate (Yoshinaka et al., 1984b; Hajjou et al., 1995). These results suggested that SH groups were not directly involved in the enzyme activity.

In this study, the N-terminal amino acid sequence of *A. amurensis* CPB was determined. A sequence of 20 amino acid residues was readily determined, indicating that N-terminus of the *A. amurensis* CPB was unblocked. The N-terminal amino acid sequence of the *A. amurensis* CPB was aligned with the sequence of other animal CPBs (Fig. 4). The sequence homology in N-terminal region of the *A. amurensis* CPB to other animal CPBs was relatively low (20–35 %). As shown in Fig. 4, one amino acid deletion was required for the *A. amurensis* CPB to align the N-terminal region of mammalian pancreatic CPB, whereas crayfish and lungfish CPBs showed an amino terminal deletion of three residues. On the other hand, almost CPBs had identical residues in position 5 (tyrosine), 8 (tyrosine), 14 (isoleucine) and 17 (tryptophan). Further comparison of the homologies has to await additional sequence data of the *A. amurensis* CPB.

Although the isolated starfish *A. amurensis* CPB has some particular biochemical properties, it may be pointed out that the starfish enzyme is

closely related to mammalian pancreatic CPB.

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(captions to figures)

Fig. 1. Electrophoresis of the purified carboxypeptidase B of the starfish *A. amurensis*. (a) Electrophoresis was performed using a 0.1 % SDS–12.5 % polyacrylamide slab-gel. Lane 1 contains starfish carboxypeptidase B. Lane 2 contains protein standards; bovine plasma albumin (molecular weight,

66,000), ovalbumin (45,000), bovine trypsinogen (24,000), bovine milk β -lactoglobulin (18,400), and egg-white lysozyme (14,300). (b) Electrophoresis was performed using a 10 % polyacrylamide slab-gel at pH 8.9. Lane 1 contains starfish carboxypeptidase B.

Fig. 2. Effects of pH and temperature on activity or stability of carboxypeptidase B of the starfish *A. amurensis*. (a) Effect of pH on the activity of *A. amurensis* carboxypeptidase B. An assay was performed by incubating mixtures of the enzyme and 25 mM of a buffer solution (acetic acid-sodium acetate pH 4.0–5.0 (▲), sodium phosphate monobasic-dibasic pH 6.0–7.5 (△), Tris-HCl pH 7.5–9.0 (○), and sodium borate-sodium hydroxide pH 9.0–11.0 (□)), and then the activity at 25 °C was determined. (b) Effect of temperature on the activity of *A. amurensis* carboxypeptidase B. An assay was performed by incubating mixtures of the enzyme and 25 mM Tris-HCl (pH 7.5) at 15–70 °C. (c) Effect of temperature on the stability of *A. amurensis* carboxypeptidase B. The enzyme was kept at 30–70 °C for 15 min and at pH 8.0, and then the remaining activity at 25 °C and at pH 7.5 was determined. (d) Effect of pH on the stability of *A. amurensis* carboxypeptidase B. The enzyme was kept at 30 °C for 30 min and at pH 4.0–11.0, and then the remaining activity at 25 °C and at pH 7.5 was determined.

Fig. 3. Effect of EDTA on activity of carboxypeptidase B of the starfish *A. amurensis*. The enzyme was kept at 25 °C and at pH 7.5 for 0–6 h in the presence (△) or absence (○) of 1 mM EDTA, and then the remaining activity at 25 °C and at pH 7.5 was determined.

Fig. 4. Alignment of the N-terminal amino acid sequence of carboxypeptidase B of the starfish *A. amurensis* with the sequences of carboxypeptidase Bs from other animals. Residues identical with *A. amurensis* carboxypeptidase B are shaded. Starfish, *A. amurensis* carboxypeptidase B (present paper); Crayfish, crayfish *Astacus fluviatilis* carboxypeptidase B (Titani et al., 1984); Lungfish, African lungfish *Protopterus aethiopicus* carboxypeptidase B (Reeck and Neurath, 1972); Ostrich, ostrich *Struthio camelus* carboxypeptidase B (Bradley et al., 1996); Bovine, bovine pancreatic carboxypeptidase B (Titani et al., 1975); Porcine, porcine pancreatic carboxypeptidase B (Ventura et al., 1999); Rat, rat pancreatic carboxypeptidase B (Clauser et al., 1988); Human, human pancreatic carboxypeptidase B (Marinkovic et al., 1977).

Table 1
Purification of carboxypeptidase B of the starfish *A. amurensis*

Purification step	Protein (mg)	Total activity (U)*	Specific activity (U/mg)	Purity (fold)	Yield (%)
Crude enzyme solution	12,400	6,200	0.5	1	100
Sephacryl S-200 47	723	2,900	4	8	
DEAE-Cellulose 29	12	1,800	150	300	
Sephadex G-50	2.7	1,030	380	760	17
Sephadex G-50	1.8	830	460	920	13

*One unit (U) of activity was defined as the amount of the enzyme hydrolyzing one micromols of Bz-Gly-L-Arg in a minute.
DEAE: diethylaminoethyl.

Table 2

Kinetic parameters of carboxypeptidase B of the starfish *A. amurensis*

Substrate	K_m	k_{cat}	k_{cat}/K_m
	(mM)	(sec ⁻¹)	(M ⁻¹ sec ⁻¹)
Bz-Gly-L-Arg	0.71	427	6.01×10^5

Table 3

Effect of metal ions on the activity of carboxypeptidase B of the starfish *A. amurensis*

Compound	Final concentration (mM)	Relative activity (%)*
Non	0	100
CaCl ₂	1	97
MgCl ₂	1	89
CoCl ₂	1	181
HgCl ₂	1	3

*The activities were expressed as percentage of that with the absence of metal ion.

Fig. 1

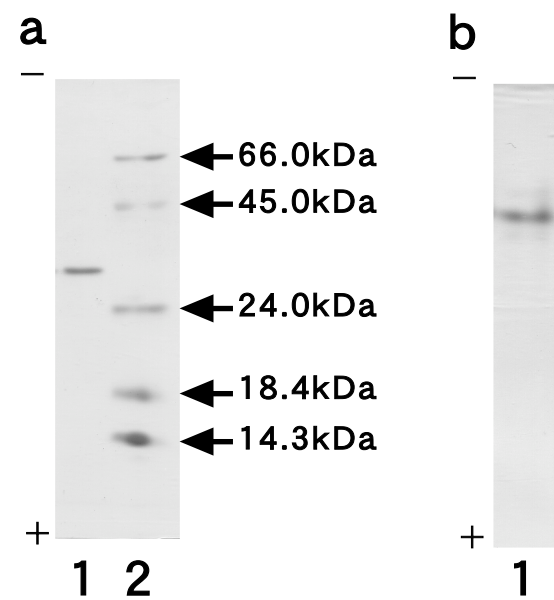


Fig. 2

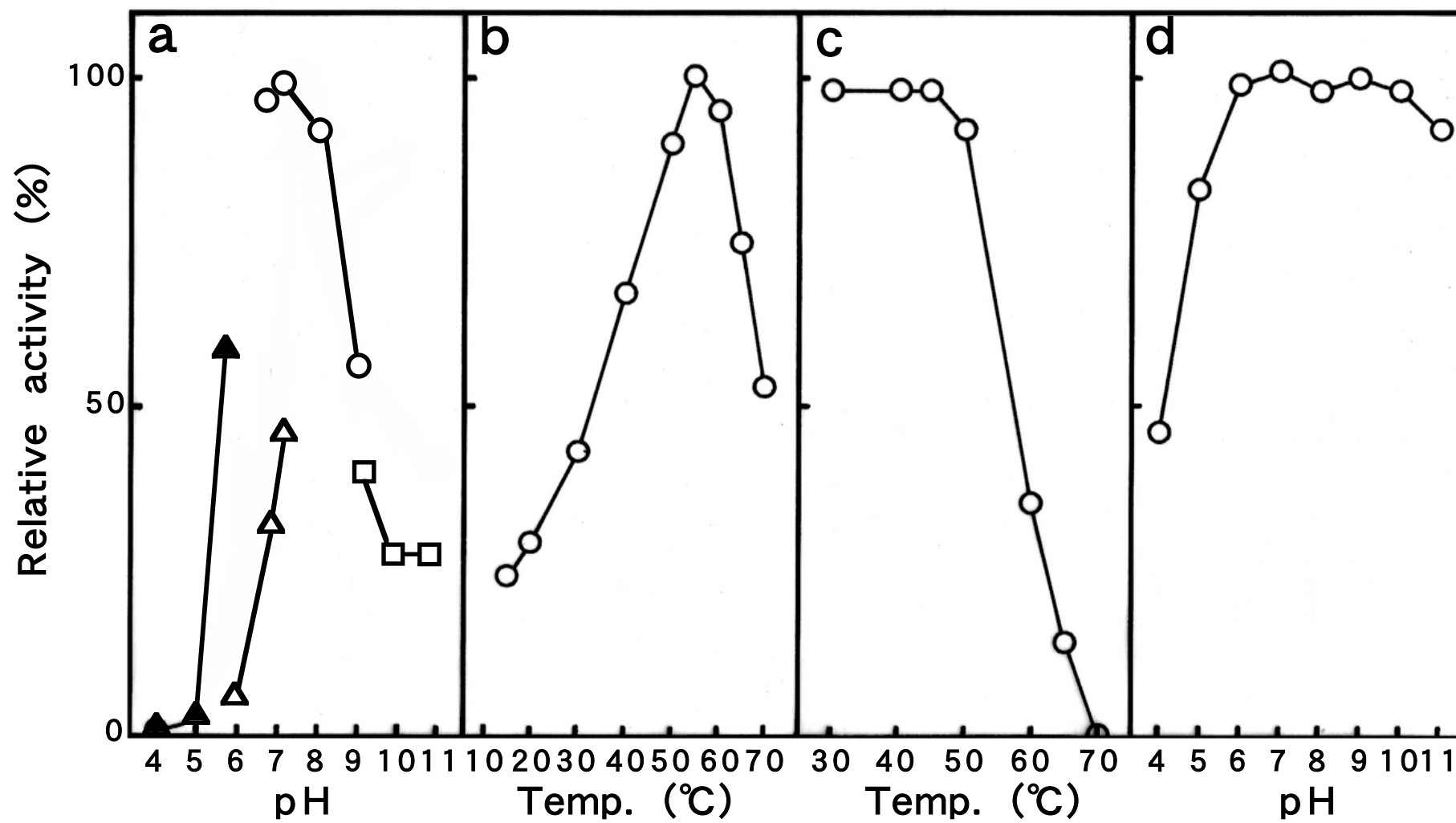
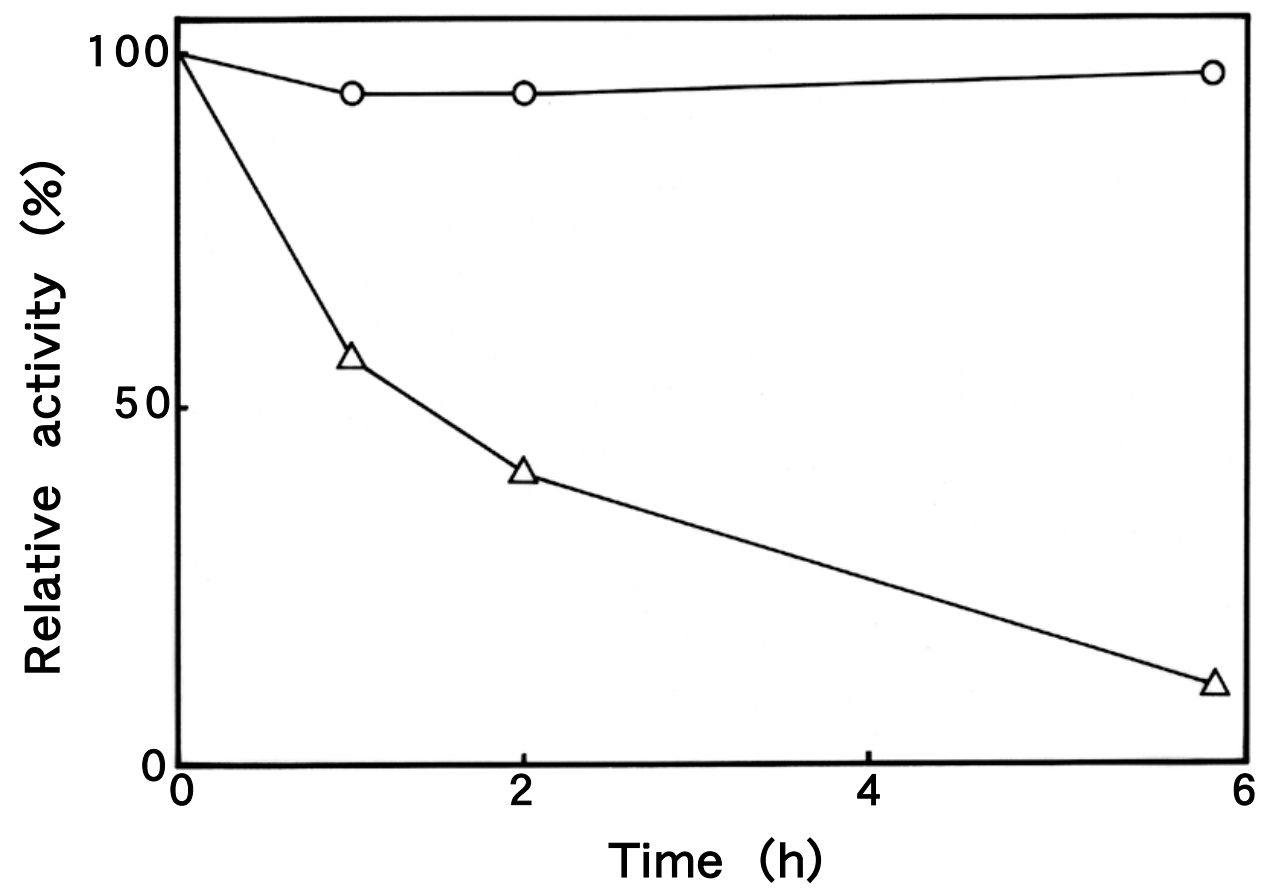


Fig. 3



	1	10	20
Starfish	AS-FDYNVYHSYQEIMNWITN		
Crayfish	MDWTSYHDYDEINAWLDS		
Lungfish	YSYEKYNTWDEIAAWTXE		
Ostrich	NAGHSYKYNKWEA		
Bovine	TTGHSYKYNWETIEAWTEQ		
Porcine	TTGHSYKYNWETIEAWTKQ		
Rat	ASGHSYTKYNKWETIEAWIQQ		
Human	ATGHSYKYNWETIEAWTQ		