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Characteristics of carboxypeptidase B from pyloric ceca of the starfish
Asterina pectinifera

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Abstract

Carboxypeptidase B was purified from the pyloric ceca of the starfish *Asterina pectinifera*. The final enzyme preparation was nearly homogeneous in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and its molecular weight was estimated as approximately 34,000. The value of the specificity constant (kcat/Km) for hydrolysis of benzoyl-glycyl-L-arginine by the purified enzyme was $1.72 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$. The optimal pH and the optimal temperature of the enzyme were pH 7.5 and 55 °C, respectively. The enzyme was unstable above 50 °C and below pH 5.0. The enzyme was activated by Co^{2+} , and inhibited by EDTA. The N-terminal amino acid sequence of the enzyme was determined as ATFDYNKYHSYQEIMDWVTN.

Keywords: *Asterina pectinifera*; Carboxypeptidase B; 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 and/or peptides. CPBs from porcine and bovine pancreases have been isolated and well characterized (Folk et al., 1960; Folk et al., 1962; Wolff et al., 1962; Cox et al., 1962; Wintersberger et al. 1962; Schmid & Herriott, 1976; Clauser et al., 1988; Edge et al., 1998; Ventura et al., 1999). The CPBs from mammalian pancreas have been used as diagnostic biochemical reagents. Markvicheva et al. (2000) applied immobilized trypsin and CPB to prepare human insulin from recombinant proinsulin. Also, Winter et al. (2002) reported the production of human proinsulin in *Escherichia coli* and the efficient conversion of the renatured proinsulin to mature insulin by treatment with trypsin and CPB. Betancourt et al. (2001) developed a method for the easy isolation and direct sequencing of N-terminally blocked peptide in proteins refractory to N-terminal sequencing. It is based essentially on tandem enzymatic treatments of the protein with trypsin and CPB. Recently, neurochemical studies suggested that human brain CPB may play a significant role in the elimination of the intracellular accumulation and toxicity of amyloid-beta in the human brain and thereby protect the neurons from degeneration (Papp et al., 2003).

On the other hand, utilizations of mammalian pancreatic CPBs for nutrition research and food processing were attempted. Nihei et al. (2001) examined whether or not elimination of L-arginine from the standard 20% casein diet would improve proteinuria and other symptoms in glomerulonephritic rats. The arginine-reduced casein prepared by enzymatically digesting with trypsin and CPB. The arginine-reduced feed ameliorated proteinuria and hyperlipidemia without restriction of protein intake. Yusep et al. (2002) reported the influence of CPB from porcine pancreas on the free amino acid, peptide and methylpyrazine contents of under-fermented cocoa beans. Their findings indicate that porcine CPB was more prominent in the formation of

cocoa-specific aroma.

In contrast, few studies exist on the enzymology and application of protease from marine invertebrates. In a previous study, we isolated trypsin from the pyloric ceca of starfish (*Asterina pectinifera*), and compared its enzymatic properties with those of mammalian pancreatic trypsin (Kishimura & Hayashi 2002). In this study, we have isolated CPB from the pyloric ceca of the starfish (*A. pectinifera*) and have examined the characteristics of the enzyme.

2. Materials and methods

2.1. Materials

The starfish (*A. pectinifera*) was caught off Hakodate, Hokkaido Prefecture, Japan. These specimens were stored at -20 °C until used. Benzoyl-glycyl-L-arginine (Bz-Gly-L-Arg) was purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of crude enzyme solution

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

2.3. Purification of CPB

The crude enzyme solution prepared was applied to a Sephacryl S-200 column (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 major CPB fraction was concentrated by lyophilization and dialyzed against 10 mM Tris-HCl buffer (pH 8.0). The dialyzates were 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. CPB activity was found mainly in the non-adsorbed fraction. This CPB fraction was concentrated and 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, and the CPB fraction obtained.

2.4. Assay for CPB activity

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

The effects of temperature and pH on stability of the enzyme were found by incubating at pH 8.0 for 15 min over the temperature range 30-70 °C and by incubating at 30 °C for 30 min over the range of pH, 4.0-11.0, respectively.

The effects of EDTA and/or Co^{2+} on the activity of the enzyme were found by incubating at 25 °C and at pH 7.5 in the presence or absence of 1 mM EDTA or 1 mM $CoCl_2$.

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 *A. pectinifera* 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

The purification procedure of the *A. pectinifera* CPB is summarized in Table 1. The final enzyme preparation was purified 418-fold from the crude enzyme solution in a yield of 23 %. The enzyme was found to be nearly homogeneous using SDS-PAGE (Fig. 1). The molecular weight of the *A. pectinifera* CPB was estimated as approximately 34,000 using SDS-PAGE (Fig. 1). The value of the specificity constant (kcat/Km) for hydrolysis of Bz-Gly-L-Arg by the *A. pectinifera* CPB was $1.72 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ (Table 2).

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

The effect of temperature on stability of the *A. pectinifera* CPB was examined between 30 and 70 °C by incubating the enzyme for 15 min at each temperature. The results are shown in Fig. 2c. The enzyme was stable between 30 and 45 °C, whereas about 70 % of the enzymic activity was lost after 15 min of incubation at 60 °C. The effect of pH on the stability of the *A. pectinifera* CPB was examined between pH 4 and 11 by incubating the enzyme for 30 min at each pH (Fig. 2d). The enzyme was stable between pH 6 and 10, whereas about 60 % of the enzymic activity was lost after 30 min of incubation at pH 4.

The effect of EDTA on the activity of the *A. pectinifera* CPB 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 activity (Fig. 3). About 90 % of the enzymic activity was lost after 6 h of incubation in 1 mM EDTA. The effect of Co²⁺ on the activity of the *A. pectinifera* CPB was examined. The enzyme was pre-incubated with 1 mM CoCl₂ in 25 mM Tris-HCl buffer (pH 7.5) at 25 °C for various times. The activity was measured at 25 °C and pH 7.5 with Bz-Gly-L-Arg, and was expressed as percent of the initial activity. The enzymic activity was enhanced 1.7-fold by Co²⁺ (Fig. 3).

The N-terminal amino acid sequence of the *A. pectinifera* CPB was determined. A sequence of 20 amino acid residues, ATFDYNKYHSYQEIMDWVTN, was found.

4. Discussion

The starfish CPB was purified from the crude enzyme solution prepared from the pyloric ceca of *A. pectinifera*. 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 substrate, respectively, no activities of these enzymes were found (results not shown). The value of the specificity constant (kcat/Km) for hydrolysis of Bz-Gly-L-Arg by the *A. pectinifera* CPB ($1.72 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$) was similar to the corresponding values of CPB from pig ($5.0 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$) (Wolff et al., 1962), cow CPB ($3.1 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$) (McKay et al., 1979), camel CPB ($5.21 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$) (Al-Ajlan & Bailey, 1999), African lungfish ($2.69 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$) (Reeck & Neurath, 1972), and dogfish ($2.59 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$) (Hajjou et al., 1995).

The *A. pectinifera* CPB isolated in this study showed a molecular weight of about 34,000 on SDS-PAGE, being similar to those in other animal species (33,000-35,000) (Folk et al., 1960; Wintersberger et al., 1962; Prahl & Neurath, 1966; Reeck & Neurath, 1972; Marinkovic et al., 1977; Cohen et al., 1981; Yoshinaka et al., 1984a; Bradley et al., 1996). However, lower molecular weights of CPB were reported for cod (26,000) (Overnell, 1973). The optimum pH of the *A. pectinifera* CPB (pH 7.5) was similar to those of other animal species (pH7-8.2) (Marinkovic et al., 1977; Ajlan & Bailey, 1999; Bradley et al., 1996; Hajjou et al., 1995; Yoshinaka et al., 1984b). The optimal temperature of these CPB enzymes far exceeds the physiological temperature of the organisms (Sagara & Ino, 1954). Although this is a very interesting phenomenon, we do not know the reason. The pH stability of the *A. pectinifera* CPB was similar to those of the carp (Cohen et al., 1981) and catfish (Yoshinaka et al., 1984b). On the other hand, the *A. pectinifera* CPB was inhibited by the metal-chelating agent (EDTA) similar to those of the mammalian species. About

90 % of the enzymic activity was lost after 6 h of incubation with 1 mM EDTA. With the catfish and dogfish CPBs, on the other hand partial inhibition of the enzymic activity was obtained with 1 mM EDTA, and total inhibition occurred at higher metal chelator concentration or in the presence of 1 mM *o*-phenanthroline (Hajjou et al., 1995; Yoshinaka et al., 1984b). Hence, the metal ion required for the enzymic activity seems to be tightly bound to the CPB.

In this study, the N-terminal amino acid sequence of the *A. pectinifera* CPB was determined. A sequence of 20 amino acid residues was readily determined, indicating that N-terminus of the *A. pectinifera* CPB was unblocked. The N-terminal amino acid sequence of the *A. pectinifera* CPB was aligned with the sequence of other animal CPBs (Fig. 4). The sequence homology in the N-terminal region of the *A. pectinifera* CPB to other animal CPBs was relatively low (30-35 %). As shown in Fig. 4, one amino acid deletion was required for the *A. pectinifera* CPB to align the N-terminal region of the mammalian pancreatic CPB, whereas the lungfish CPBs showed an amino terminal deletion of three residues. On the other hand, all the mammalian CPBs had same residues in positions 5 (tyrosine), 8 (tyrosine), 14 (isoleucine) and 17 (tryptophan). Further comparison of the homologies has to await additional sequence data of the starfish CPB.

In conclusion, it may be pointed out that the *A. pectinifera* CPB is closely related to the mammalian pancreatic CPB, and these results suggest that the pyloric ceca of the starfish (*A. pectinifera*) would be a potential source of CPB.

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(captions to figures)

Fig. 1. Electrophoresis of the purified carboxypeptidase B of the starfish *A. amurensis*. 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).

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 the *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 the *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 the *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 the *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 of 1 mM EDTA (▲) or 1 mM CoCl₂ (●) and in the absence of 1 mM EDTA and 1 mM CoCl₂ (○), 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 the carboxypeptidase B of the starfish *A. pectinifera* with the sequences of carboxypeptidase Bs from other animals. Residues identical with the *A. pectinifera* carboxypeptidase B are shaded. *A. pectinifera*, *A. pectinifera* carboxypeptidase B (present paper); Lungfish, African lungfish *Protopterus aethiopicus* carboxypeptidase B (Reeck & Neurath, 1972); Ostrich, ostrich *Struthio camelus* carboxypeptidase B (Bradley et al., 1996); Cow, bovine pancreatic carboxypeptidase B (Titani et al., 1975); Pig, porcine pancreatic carboxypeptidase B (Ventura et al., 1999); Rat, rat pancreatic carboxypeptidase B (Clauser et al., 1988); Man, human pancreatic carboxypeptidase B (Marinkovic et al., 1977).

Fig. 1

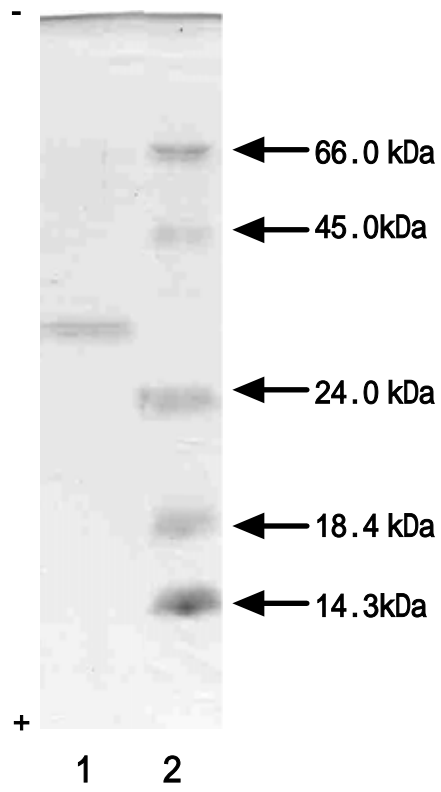


Fig. 2

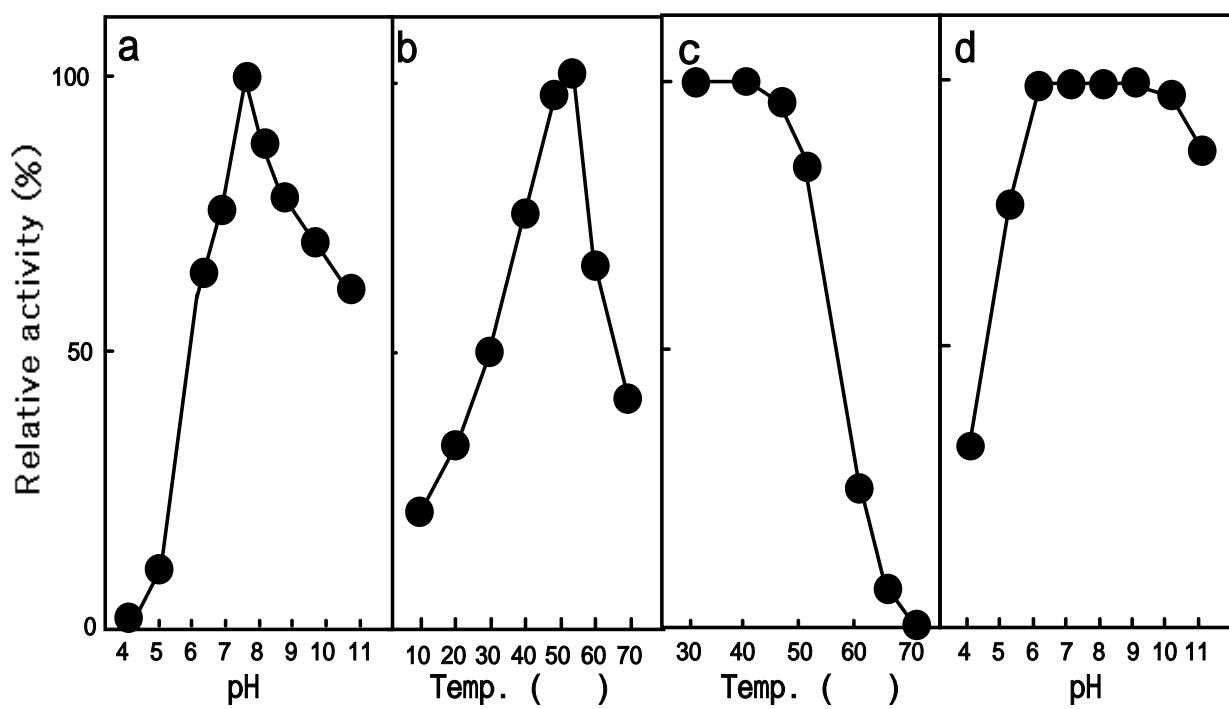


Fig. 3

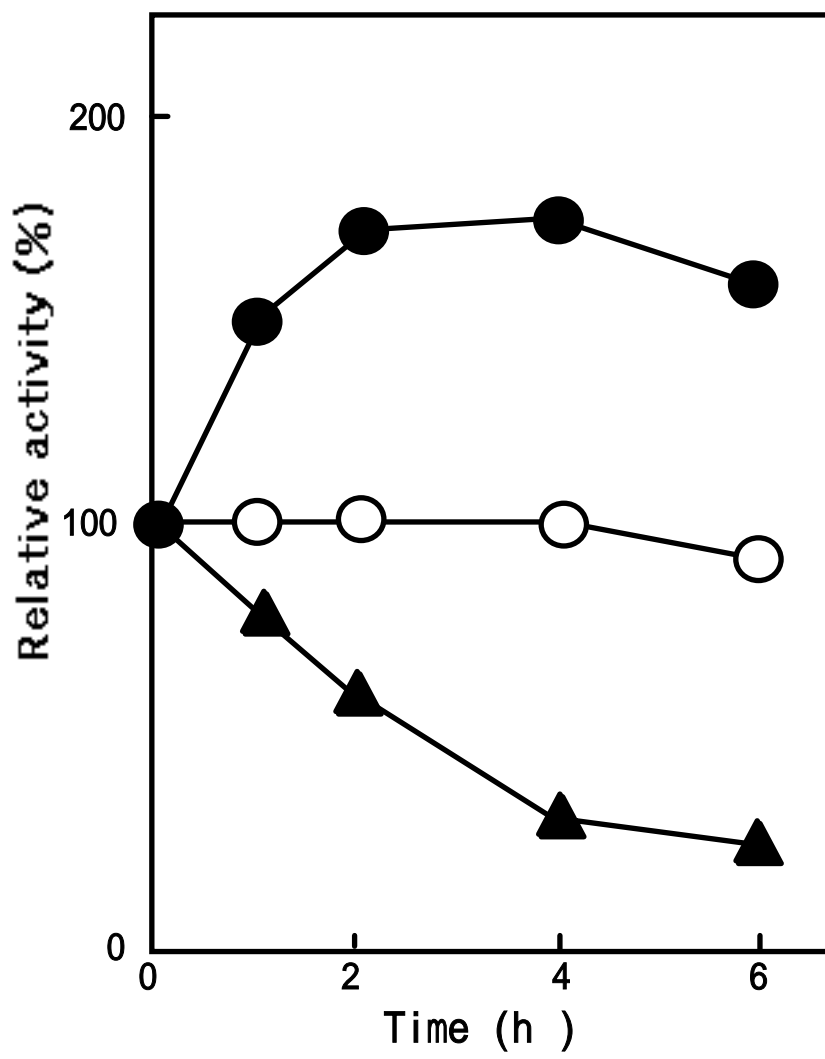


Fig. 4

	1	10	20
<i>A. pectinifera</i>	AT-FDYNKYHSYQEIMDWVTN		
Lungfish		YSYEKYNTWEI	AAWTXE
Ostrich		NAGHYEKYNKWEA	
Rat	ASGHYTKYNKWEI		EAWIQQ
Pig	TTGHSYEKYNNWEI		EAWTKQ
Cow	TTGHSYEKYNNWEI		EAWTEQ
Man	ATGHSYEKYNNWEI		EAWTQ

Table 1
Purification of carboxypeptidase B of the starfish *Asterina pectinifera*

Purification step	Protein (mg)	Total activity (U)*	Specific activity (U/mg)	Purity (fold)	Yield (%)
Crude enzyme solution	2,384	880	0.4	1	100
Sephacryl S-200	138	690	5	13	78
DEAE-Cellulose	10	370	37	93	42
Sephadex G-50	1.2	200	167	418	23

*One unit (U) of activity was defined as the amount of enzyme hydrolysing one micromol of Bz-Gly- L-Arg in a minute.

DEAE: diethylaminoethyl.

Table 2

Comparison of kinetic parameters of carboxypeptidase B of the starfish *Asterina pectinifera* with those from other animals

	Km (mM)	kcat (s ⁻¹)	kcat/Km (M ⁻¹ s ⁻¹)	References
Starfish <i>A. pectinifera</i>	0.58	100	1.72×10^5	Kishimura & Hayashi (present study)
Catfish I	0.76	-	-	Yoshinaka et al. (1984b)
Catfish II	0.66	-	-	Yoshinaka et al. (1984b)
Lungfish	0.32	86	2.69×10^5	Reek & Neurath (1972)
Dogfish	0.34	88	2.59×10^5	Hajjou et al. (1995)
Cow	0.23	60	2.61×10^5	Alter et al. (1977)
Pig	0.21	105	5.00×10^5	Wolff et al. (1962)
Camel	0.19	99	5.21×10^5	Al-Ajlan & Bailey (1999)

Table 3
 Effect of metal ions on the activity of carboxypeptidase B of the starfish *Platichthys stellatus*

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.