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Running title: TRYPSIN ISOZYMES OF ANCHOVY

**CHARACTERISTICS OF TWO TRYPSIN ISOZYMES FROM THE
VISCERA OF JAPANESE ANCHOVY (*ENGRAULIS JAPONICA*)**

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ABSTRACT

Two isozymes of trypsin (TR-I and TR-II) were purified from the viscera of Japanese anchovy (Engraulis japonica) by gel filtration and anion-exchange chromatography. Final enzyme preparations were nearly homogeneous in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the molecular weights of both enzymes were estimated to be 24,000 Da by SDS-PAGE. The N-terminal amino acid sequences of the TR-I, IVGGYECQAHSQPHTVSLNS, and TR-II, IVGGYECQPYSQPHQVSLDS, were found. Both TR-I and TR-II had maximal activities at around pH 8.0 and 60 C for hydrolysis of N^α-p-tosyl-L-arginine methyl ester. The TR-I and TR-II were unstable at above 50 C and below pH 5.0, and were stabilized by calcium ion.

key wards: Japanese anchovy, *Engraulis japonica*, Purification, Trypsin, Isozyme, Thermal stability

INTRODUCTION

The development of the fisheries industry will depend on effective utilization of the available raw materials. Fish viscera are non-edible parts produced in large quantities by the fish processing industry (about 450,000 t of the catch of Japanese anchovy). These materials, however, are rich sources of various enzymes 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 requires adaptation of their enzyme activities to low habitat temperatures. Enzymes from cold adapted fish species thus often have higher enzymatic activities at low temperatures than their counterparts from warm-blooded animals (Asgeirsson et al. 1989; Kristjansson 1991). High activities 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).

E. japonica is one of the important fish harvested from cold and temperate water in Japan and is used mostly for food. Postmortem anchovy sometimes autolyzes very quickly due to enzymes leaking from the digestive organs. Martinez and Serra (1989) reported the distribution of protease activities in the viscera of *E. encrasicolus*. Serine proteases, mainly trypsin and chymotrypsin, seemed to play a decisive role in protein digestion. In this study, we purified two isozymes of trypsin from the viscera of *E. japonica* and compared the characteristics to those of porcine pancreatic trypsin.

MATERIALS AND METHODS

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

Defatted powder of viscera of *E. japonica* was 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 CaCl₂ 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.

The crude trypsin was applied to a column of Sephacryl S-200 (3.9 × 64 cm) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl₂ and the proteins were eluted with the same buffer. The main trypsin fraction was concentrated by lyophilization and was dialyzed against 500 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl₂ at 5 C. The dialyzate was applied to a DEAE-cellulose column (2.2 × 18 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl₂ and the proteins were eluted with a linear gradient of 0-0.5 M NaCl in the same buffer. Two types of trypsin were eluted mainly with 0.3-0.35 M NaCl (TR-I) and 0.4-0.45 M NaCl (TR-II). TR-I and TR-II fractions were dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl₂ and these dialysates were concentrated by lyophilization. Then TR-I and TR-II fractions were applied to a Sephadex G-50 column (3.9 × 64 cm) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl₂, respectively, and the proteins were eluted with the same buffer.

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 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 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 10 mM ethylenediaminetetraacetic acid (EDTA) or 10 mM CaCl₂.

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. To analyze the N-terminal sequence of the purified enzyme, protein band 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). The protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

In this study, two anionic isozymes of trypsin (TR-I and TR-II) were purified from the viscera of *E. japonica* by gel filtration and anion-exchange chromatography. TR-I and TR-II were

eluted as single peak on gel filtrations (G-50), respectively, and showed a nearly single band on SDS-PAGE (Fig. 1). The final TR-I and TR-II preparations were purified 38-fold and 73-fold, respectively, from the crude trypsin (Table 1). Simpson and Haard (1985) isolated trypsin and trypsin-like enzyme from the pancreas of the stomachless cunner. Hjelmeland and Raa (1982) and Martinez et al. (1988) purified two anionic trypsins from capelin and anchovy (*E. encrasicolus*), respectively, by DEAE-chromatography.

The molecular weights of both TR-I and TR-II were estimated as approximately 24,000 Da using SDS-PAGE (Fig. 1) similar to those of mammalian pancreatic trypsin (24,000 Da), Greenland cod trypsin (23,500 Da) (Simpson and Haard 1984) and Atlantic cod trypsin (24,200 Da) (Asgeirsson et al. 1989), which were smaller than trypsins of capelin (28,000 Da) (Hjelmeland and Raa 1982), catfish (26,000 Da) (Yoshinaka et al. 1983), anchovy (*E. encrasicolus*) (27,000-28,000 Da) (Martinez et al. 1988), and rainbow trout (25,700 Da) (Kristjansson 1991). The N-terminal amino acid sequences of the TR-I and TR-II were analyzed and twenty amino acids, IVGGYECQAHSQPHTVSLNS (TR-I) and IVGGYECQPYSQPHQVSLDS (TR-II), were found. These results indicated that the TR-I and TR-II were single proteins. It was indicated that the N-termini of the TR-I and TR-II were unblocked. Four positions (position 9, 10, 15, and 19) of amino acids differed between the TR-I and the TR-II. The N-terminal amino acid sequences of the TR-I and TR-II were aligned with the sequences of other animal trypsins (Fig. 2). Being similar to other fish trypsins, the TR-I and TR-II had a charged Glu residue at position 6, whereas Thr is most common in mammalian pancreatic trypsins (Fig. 2).

Fig. 3a shows the pH dependencies of TR-I and TR-II. 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. 3b) and other fish trypsins (Hjelmeland and Raa 1982; Simpson and Haard 1984; Yoshinaka et al 1984; Martinez et al. 1988; Asgeirsson et al. 1989). Fig. 4a shows the temperature dependencies of the TR-I and TR-II. Both enzymes examined in this study had an

optimum temperature of about 55-60 C similar to those of other fish trypsins (Asgeirsson et al. 1989; Kristjansson 1991), which were lower than that of porcine pancreatic trypsin (60-70 C; Fig. 4b). Figs. 5a shows the pH stabilities of the TR-I and TR-II. Both enzymes were stable between pH 6.0 and 11.0, but there were unstable below pH 5.0. These properties of the pH stability of the TR-I and TR-II were similar to those of other fish trypsins (Martinez et al. 1988; Asgeirsson et al. 1989; Kristjansson 1991), but not to porcine pancreatic trypsin (Fig. 5b). Although this is a very interesting phenomenon, we do not know the reason. Possibly the structure of fish trypsin may differ from that of mammalian pancreatic trypsin. Figs. 6a shows the temperature stabilities of the TR-I and TR-II. Both enzymes were stable below 50 C, but their activity quickly fell above 60 C. The properties of the thermostabilities of TR-I and TR-II were similar to those of other fish trypsins (Martinez et al. 1988; Kristjansson 1991), which were less stable than porcine pancreatic trypsin (Fig. 6b).

The effects of CaCl₂ on the TR-I and TR-II were found in the presence of 10 mM EDTA or 10 mM CaCl₂. Both enzymes were stabilized by calcium ion and were unstabilized by EDTA (Fig. 7a) similar to porcine pancreatic trypsin (Fig. 7b). Maybe the calcium ions of these trypsins were chelated by EDTA. 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. The TR-I and TR-II were stabilized by calcium ion from thermal denaturation. These findings suggest that the TR-I and TR-II possess the primary calcium binding site like mammalian pancreatic trypsin and other fish trypsins (Male et al. 1995; Genicot et al. 1996).

In conclusion, the TR-I and TR-II showed almost same characteristics with that of porcine pancreatic trypsin except for unstable below pH 5.0. These results suggest that the viscera of *E. japonica* would be a potential source of trypsin for food processing operations.

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(captions to figures)

FIG. 1. ELECTROPHORESIS OF PURIFIED TRYPSIN ISOZYMES OF *E. JAPONICA*.

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 TR-I. Lane 3 contains TR-II.

FIG. 2. COMPARISON OF THE DEDUCED AMINO ACID SEQUENCES OF *E. JAPONICA* TRYPSIN ISOZYMES WITH THOSE OF OTHER VERTEBRATES.

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. 3. EFFECTS OF PH ON THE ACTIVITY OF TRYPSIN ISOZYMES OF *E. JAPONICA*.

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 30 C. a: TR-I (closed circle); TR-II (open triangle), b: Porcine pancreatic trypsin.

FIG. 4. EFFECTS OF TEMPERATURE ON THE ACTIVITY OF TRYPSIN ISOZYMES OF *E. JAPONICA*.

The activities were determined at pH 8.0 and at various temperatures. a: TR-I (closed circle); TR-II (open triangle), b: Porcine pancreatic trypsin.

FIG. 5. PH STABILITY OF TRYPSIN ISOZYMES OF *E. JAPONICA*.

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: TR-I (closed circle); TR-II (open triangle), b: Porcine pancreatic trypsin.

FIG. 6. THERMOSTABILITY OF TRYPSIN ISOZYMES OF *E. JAPONICA*.

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: TR-I (closed circle); TR-II (open triangle), b: Porcine pancreatic trypsin.

FIG. 7. EFFECT OF CALCIUM ION ON THE STABILITY OF TRYPSIN ISOZYMES OF *E. JAPONICA*.

The enzymes were kept at 30 C and pH 8.0 for 0-8 h in the presence of 10 mM CaCl₂ (closed symbol) or 10 mM EDTA (open symbol), and then the remaining activities at 30 C and pH 8.0 were determined. a: TR-I (circle); TR-II (triangle), b: Porcine pancreatic trypsin.

TABLE 1.
PURIFICATION OF TRYPSIN ISOZYMES OF *E. JAPONICA*

| Purification stages | Fraction | Protein (mg) | Total activity (U) | Specific activity (U/mg) | Purity (fold) | Yield (%) |
|---------------------|----------|--------------|--------------------|--------------------------|---------------|-----------|
| Crude enzyme | | 7,438 | 2,529 | 0.34 | 1 | 100 |
| Sephacryl S-200 | | 398 | 1,672 | 4.2 | 12 | 66 |
| DEAE-cellulose | TR-I | 57 | 612 | 10.8 | 32 | 24 |
| | TR-II | 21 | 357 | 17.0 | 50 | 14 |
| Sephadex G-50 | TR-I | 45 | 576 | 12.8 | 38 | 23 |
| | TR-II | 11 | 273 | 24.8 | 73 | 11 |

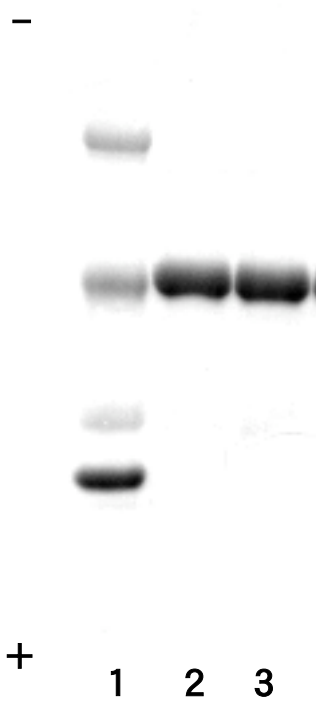


Fig.2

| | 1 | 10 | 20 |
|--------------------------|---|---------------------|----|
| Japanese anchovy (TR-I) | I | VGGYECQAHSQPHTVSLNS | |
| Japanese anchovy (TR-II) | I | VGGYECQPYSQPHQVSLDS | |
| Antarctic Fish | I | VGGKECSPYSQPHQVSLNS | |
| Cod | I | VGGYECTKHSQAHQVSLNS | |
| Salmon | I | VGGYECKAYSQTHQVSLNS | |
| Flounder Fish | I | VGGYECTPYSQPHQVSLNS | |
| Porcine | I | VGGYTCAANSVPYQVSLNS | |
| Bovine | I | VGGYTCGANTVPYQVSLNS | |
| Human | I | VGGYNCEENSVPYQVSLNS | |

Fig.3

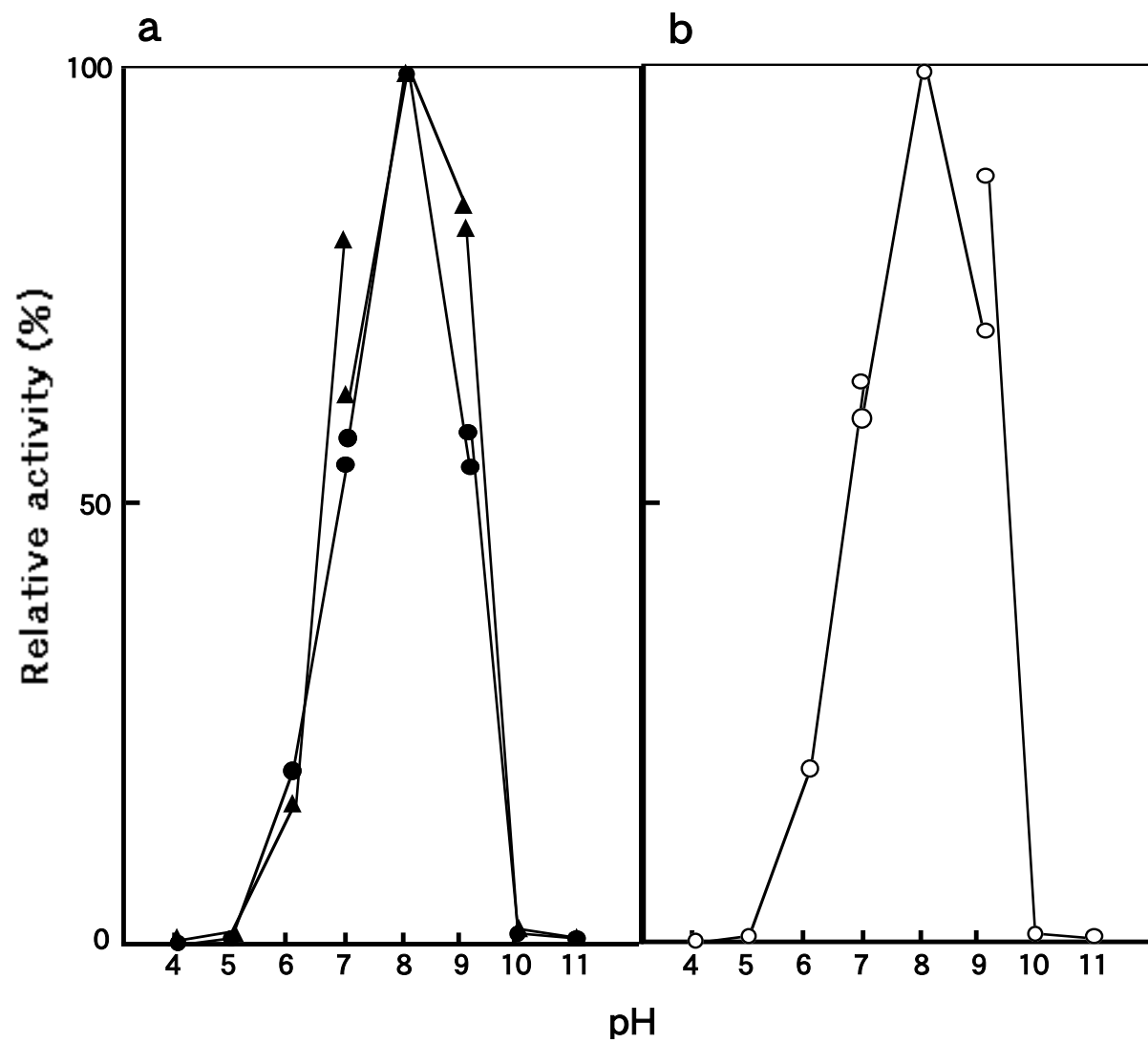


Fig.4

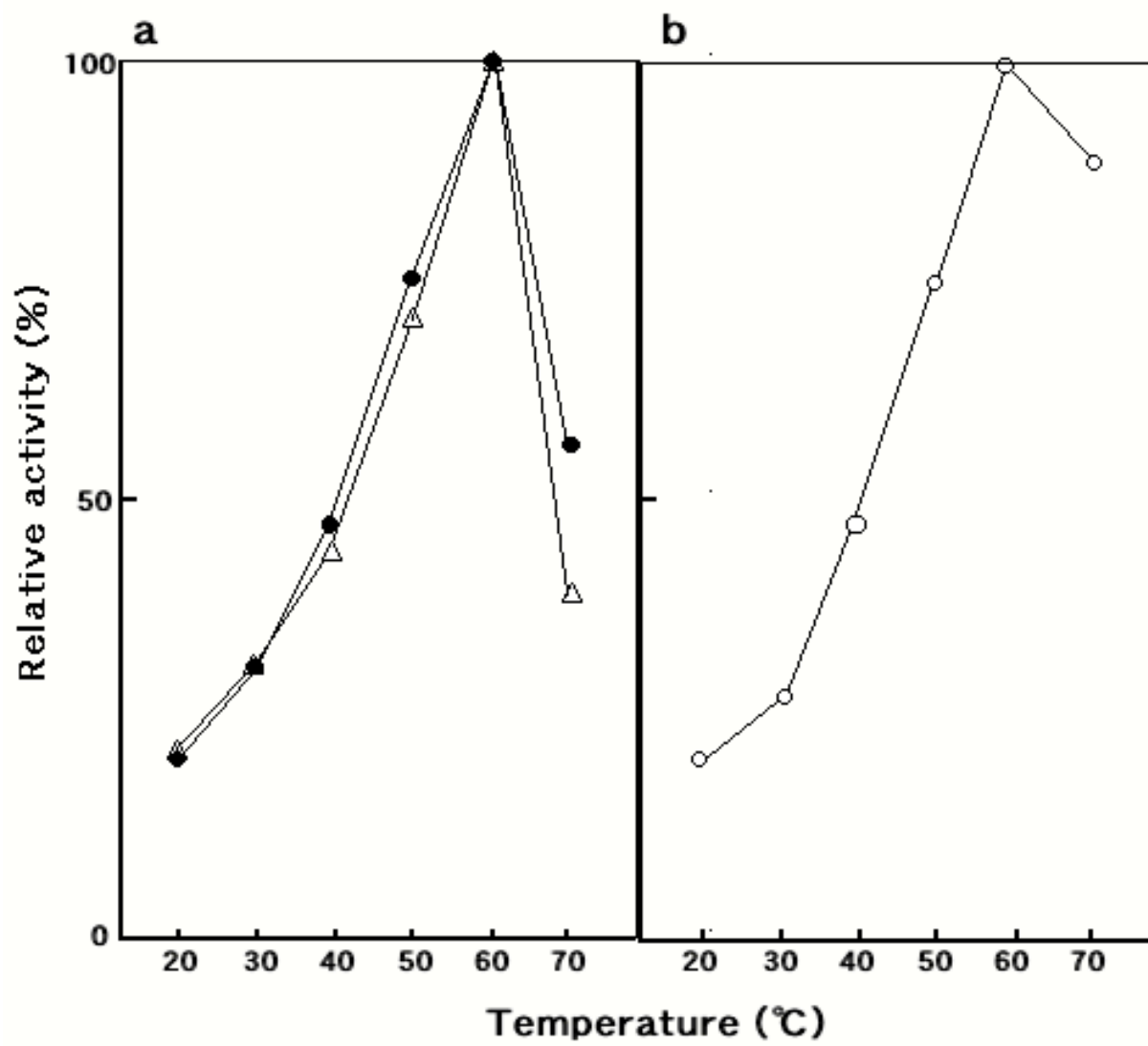


Fig.5

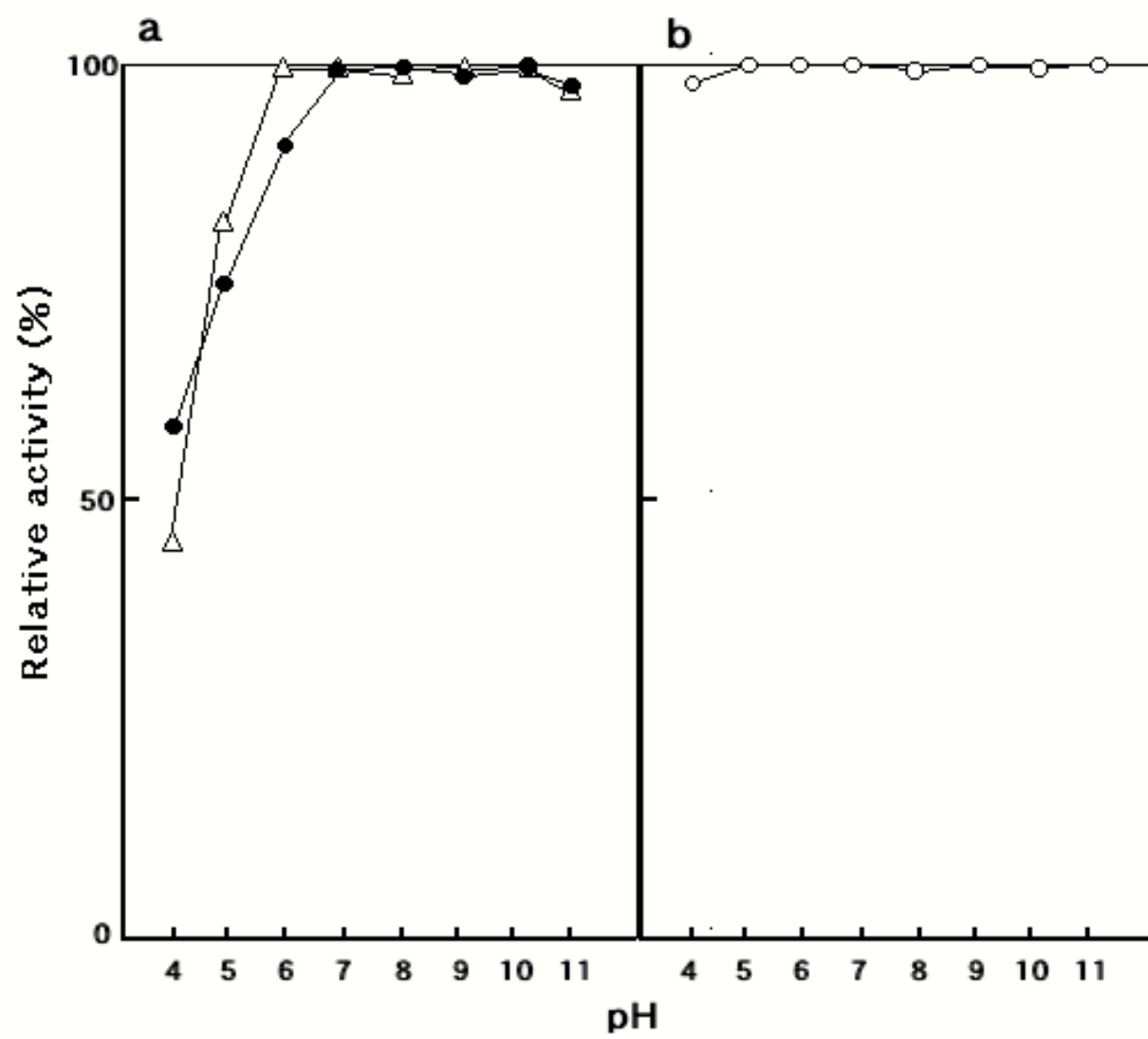


Fig.6

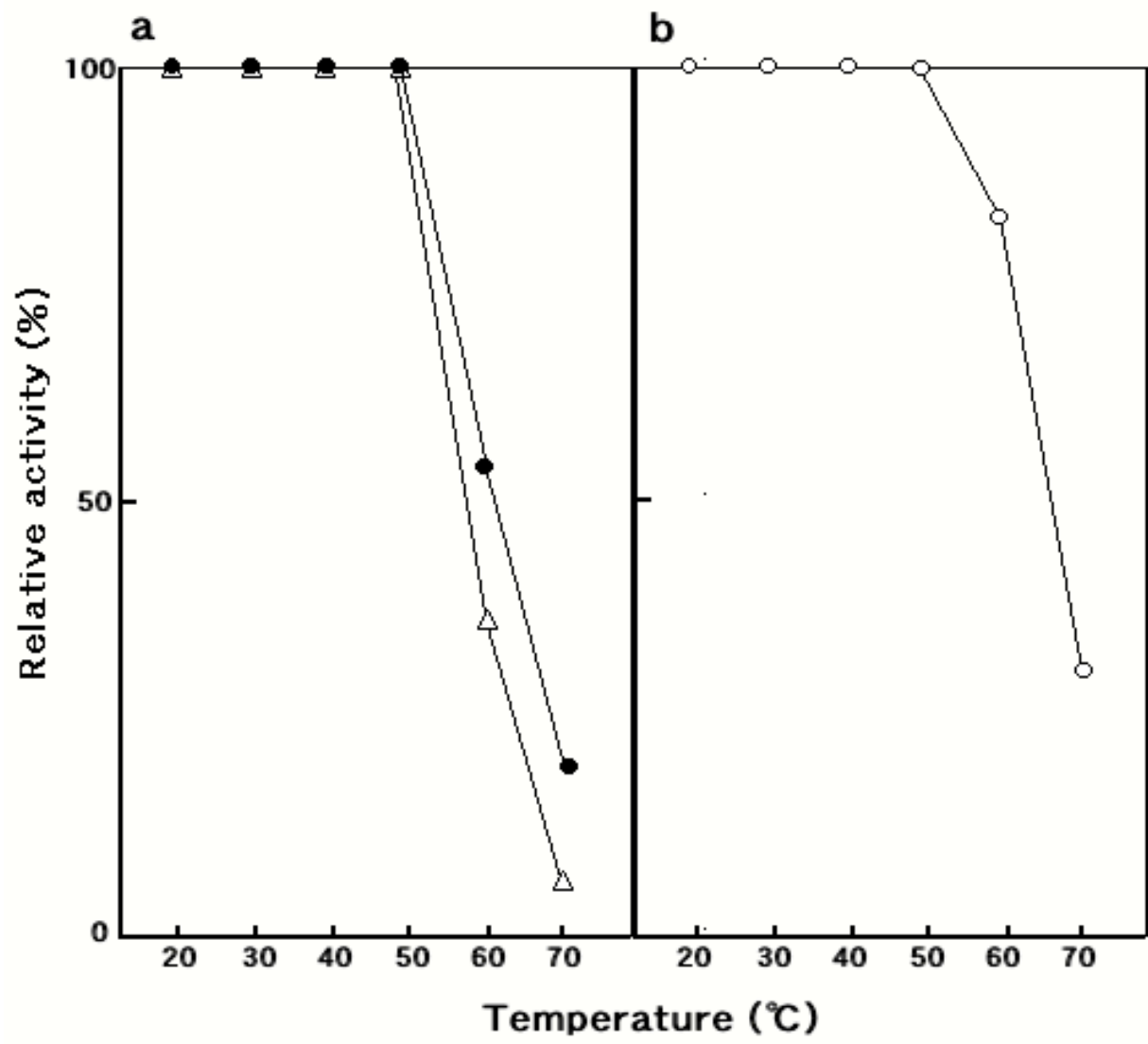


Fig.7

