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5 **Characteristics of trypsin from the pyloric ceca of**
6 **walleye pollock (*Theragra chalcogramma*)**
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Abstract

Trypsin was purified from the pyloric ceca of walleye pollock (*Theragra chalcogramma*) by gel filtration on Sephacryl S-200 and Sephadex G-50. The final enzyme preparation was nearly homogeneous in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the molecular mass of the enzyme was estimated to be 24 kDa by SDS-PAGE. Trypsin activity was effectively inhibited by serine protease inhibitors, such as soybean trypsin inhibitor and TLCK. Trypsin had maximal activities at around pH 8.0 and 50 °C for the hydrolysis of *N*^α-*p*-tosyl-L-arginine methyl ester hydrochloride. Trypsin was unstable above 30 °C and below pH 5.0, and was stabilized by calcium ions. Walleye pollock trypsin was more thermo unstable than trypsin from the Temperate Zone fish and Tropical Zone fish. The *N*-terminal amino acid sequence of the trypsin, IVGGYECKHSQAHQVSLNS, was found, and the sequential identity between the walleye pollock trypsin and Frigid Zone fish trypsin was higher (85-100 %) than with Temperate Zone fish trypsin (75-90 %), Tropical Zone fish trypsin (75-85 %), or mammalian trypsin (60-65 %).

Keywords: Walleye pollock; *Theragra chalcogramma*; Pyloric caecum; Trypsin; *N*-terminal amino acid sequence; Thermo stability; Frigid Zone fish

1. Introduction

The development of the fisheries industry will depend on the effective utilization of the available raw materials. Fish viscera are non-edible parts produced in large quantities by the fisheries industry and represent as waste or pollution. These materials, however, are rich potential sources of various enzymes that may have some unique properties of interest for both basic research and industrial applications (Simpson & Haard, 1999).

Fishes are poikilothermic, so their survival in cold waters required adaptation of their enzyme activities to the low temperatures of their habitats. Enzymes from cold adapted fish species thus often have higher enzymatic activities at low temperatures than their counterparts from warm-blooded animals (Asgeirsson, Fox, & 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 & Haard, 1987).

Walleye pollock (*Theragra chalcogramma*) is one of the important fish-catches of Japan and its muscle is used almost entirely for “surimi” production. However, postmortem walleye pollock autolyzes very quickly due to enzymes leaking from the digestive organs and its viscera are largely underutilized and are discarded as waste. It is reported that Monterey sardine (*Sardinops sagax caerulea*) (Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, & Toro, 2005), true sardine (*Sardinops melanostictus*) (Kishimura, Hayashi, Miyashita, & Nonami, 2006a), and spotted mackerel (*Scomber australasicus*) (Kishimura, Tokuda, Klomklao, Benjakul, & Ando, 2006b) are also susceptible to abdominal autolytic degradation after death, and they isolated and characterized trypsin in its viscera. Trypsin (EC 3.4.21.4), which is detected in the pyloric ceca and intestine of

fish, is one of the main digestive proteases. It is a member of a large family of serine proteases and cleaves the peptide bond on the carboxyl side of arginine and lysine (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007). In this study, we purified trypsin from the pyloric ceca of walleye pollock and compared its characteristics with those of other fish and porcine pancreatic trypsins.

2. Materials and methods

2.1. Materials

The walleye pollock (*T. chalcogramma*) was 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) and porcine pancreatic trypsin were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Preparation of crude enzyme

Defatted powder of the pyloric ceca of walleye pollock was prepared by the same method of Kishimura & 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 °C for 3 h. The extract was centrifuged (H-2000B, Kokusan, Tokyo, Japan) at $10,000 \times g$ for 10 min, and then the supernatant was lyophilized and used as crude trypsin.

2.3. Trypsin Purification

1 The crude trypsin of walleye pollock was applied to a column of Sephacryl S-200 (3.9×64
2 cm) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl_2 and the proteins
3 were eluted with the same buffer at a flow rate of 0.5 ml/min at 4 °C. The main trypsin fractions
4 were concentrated by lyophilization and then dissolved in distilled water prior to size exclusion
5 chromatography. The sample was chromatographed on a Sephadex G-50 column (3.9×64 cm)
6 previously equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM CaCl_2 and the
7 proteins were eluted with the same buffer at a flow rate of 0.5 ml/min. The fractions with TAME
8 activity were pooled and used for further studies.

9 10 *2.4. Assay for trypsin activity*

11
12 Trypsin activity was measured by the method of Hummel (1959) using TAME as a
13 substrate. One unit of enzyme activity was defined as the amount of the enzyme hydrolyzing one
14 micromole of TAME in a minute. The pH dependencies of the enzyme were determined in 50 mM
15 buffer solutions [acetic acid-sodium acetate (pH 4.0-7.0), Tris-HCl (pH 7.0-9.0), and glycine-NaOH
16 (pH 9.0-11.0)] at 30 °C. The temperature dependencies of the enzyme were determined in 50 mM
17 Tris-HCl buffer pH 8.0 and at various temperatures. The effects of temperature and pH on the
18 stability of the enzyme were found by incubating the enzyme in 50 mM Tris-HCl buffer pH 8.0 for
19 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
20 4.0-11.0 (50 mM buffer solutions), respectively. The effect of CaCl_2 on the activity of the enzyme
21 was found by incubating the enzyme at 30 °C and in 50 mM Tris-HCl buffer pH 8.0 with the
22 presence of 10 mM ethylenediaminetetraacetic acid (EDTA) or 10 mM CaCl_2 .

23 24 *2.5. Polyacrylamide gel electrophoresis*

1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out
2 using a 0.1 % SDS-12.5 % polyacrylamide slab-gel by the method of Laemmli (1970). The gel
3 was stained with 0.1 % Coomassie Brilliant Blue R-250 in 50 % methanol-7 % acetic acid and the
4 background of the gel was destained with 7 % acetic acid.

5 6 *2.6. Analysis of amino acid sequence*

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8 To analyze the *N*-terminal sequence, the purified enzyme was electroblotted on to
9 polyvinylidenedifluoride (PVDF) membrane (Mini ProBlott Membranes, Applied Biosystems, CA,
10 USA) after SDS-PAGE. The amino acid sequence of the enzyme was analyzed by using a protein
11 sequencer, Procise 492 (Perkin Elmer, Foster City, CA, USA).

12 13 *2.7. Protein determination*

14
15 The protein concentration was determined by the method of Lowry, Rosebrough, Farr, &
16 Randall (1951) using bovine serum albumin as a standard.

17 18 19 **3. Results and discussion**

20
21 In this study, trypsin was purified from the pyloric ceca of walleye pollock by gel filtration
22 on Sephacryl S-200 and Sephadex G-50, and the purity was increased to 83 fold with approximately
23 28% yield (Table 1). The molecular weight of the trypsin was estimated as approximately 24 kDa
24 using SDS-PAGE (Fig. 1) similar to those of mammalian pancreatic, Atlantic cod (Gudmundsdottir,
25 Gudmundsdottir, Oskarsson, Bjarnason, Eakin, & Craik, 1993), true sardine (Kishimura et al.,

1 2006a), arabesque greenling (Kishimura et al., 2006a), jacobever (Kishimura, Tokuda, Yabe,
2 Klomklao, Benjakul, & Ando, 2007), elkhorn sculpin (Kishimura et al., 2007) trypsins, which were
3 smaller than trypsins of capelin (28 kDa) (Hjelmeland & Raa, 1982), catfish (26 kDa) (Yoshinaka,
4 Suzuki, Sato, & Ikeda, 1983), anchovy (27-28 kDa) (Martinez, Olsen, & Serra, 1988), rainbow trout
5 (25.7 kDa) (Kristjansson, 1991), and Monterey sardine (25 kDa) (Castillo-Yanez et al., 2005). The
6 effects of various proteinase inhibitors on the activity of the walleye pollock trypsin were
7 determined (Table 2). The trypsin was strongly inhibited by serine proteinase inhibitor, such as
8 soybean trypsin inhibitor and TLCK (82-91 %). While, specific inhibitors of cysteine proteinase
9 (E-64, *N*-ethylmaleimide, iodoacetic acid), chymotrypsin (TPCK), aspartic proteinase (pepstatin A),
10 and metallo proteinase (EDTA) had almost no inhibitory effect on the activities of walleye pollock
11 trypsin. These results for molecular weight and effects of inhibitors confirmed that the purified
12 enzyme was serine proteinase, most likely trypsin.

13 The effect of CaCl_2 on the stability of the walleye pollock trypsin was determined in the
14 presence of 10 mM EDTA or 10 mM CaCl_2 . The trypsin was stabilized by calcium ion (Fig. 2a)
15 similar to porcine pancreatic trypsin (Fig. 2b). Two calcium binding sites are in bovine
16 trypsinogen (Kossiakoff, 1977). The primary site, with a higher affinity for calcium ions, is
17 common in trypsinogen and trypsin, and the secondary site is only in the zymogen. Occupancy of
18 the primary calcium binding site stabilizes the protein toward thermal denaturation or autolysis.
19 The walleye pollock trypsin was stabilized by calcium ion from thermal denaturation. This
20 finding suggests that the walleye pollock trypsin possesses the primary calcium binding site like
21 mammalian pancreatic trypsin and other fish trypsins (Male, Lorens, Smalas, & Torrissen, 1995;
22 Genicot, Rentier-Delrue, Edwards, Vanbeeumen, & Gerday, 1996; Kishimura et al., 2006a, 2006b;
23 Kishimura, Tokuda, Klomklao, Benjakul, & Ando, 2006c; Kishimura et al., 2007).

24 Fig. 3a shows the pH dependence of the walleye pollock trypsin. The enzyme hydrolyzed
25 the TAME effectively at alkaline pH with an optimum activity at pH 8.0 similar to those of porcine

1 pancreatic trypsin (Fig. 3b) and other fish trypsins (Hjelmeland & Raa, 1982; Simpson & Haard,
2 1984; Yoshinaka, Sato, Suzuki, and Ikeda, 1984; Martinez et al., 1988; Asgeirsson et al., 1989;
3 Castillo-Yanez et al., 2005; Kishimura, Hayashi, Miyashita, & Nonami, 2005; Kishimura et al.,
4 2006a, 2006b, 2006c, 2007). For pH stability, the enzyme was stable between pH 6.0 and 11.0,
5 but it was unstable below pH 6.0 (Fig. 4a). The property of the pH stability of the walleye pollock
6 trypsin was similar to those of other fish trypsins (Martinez et al., 1988; Asgeirsson et al., 1989;
7 Kristjansson, 1991; Kishimura et al., 2005, 2006a, 2006b, 2006c, 2007), but not to porcine
8 pancreatic trypsin (Fig. 4b).

9 The effects of various temperatures on the activity of trypsin from walleye pollock are
10 shown in Fig. 5a. Optimum temperature of the trypsin was 50 °C, which was lower than that of
11 porcine pancreatic trypsin (60-70 °C; Fig. 5b). The walleye pollock is a Frigid Zone fish and its
12 trypsin had an optimum temperature of 50 °C (Fig. 5a) similar to those of other trypsins from Frigid
13 Zone fish, such as arabesque greenling, brown hakeling, and elkhorn sculpin (Kishimura et al.,
14 2006a, 2006b, 2007). Optimum temperature of the walleye pollock trypsin was lower than those
15 of porcine pancreatic trypsin (60-70 °C; Fig. 5b), Temperate Zone fish trypsin (60 °C) (Kishimura et
16 al., 2005, 2006a, 2006b, 2006c, 2007), and Tropical Zone fish trypsin (55-65 °C) (Klomklao,
17 Benjakul, Visessanguan, Kishimura, Simpson, & Saeki, 2006a; Klomklao, Benjakul, Visessanguan,
18 Kishimura, & Simpson, 2006b; Klomklao et al., 2007). Fig. 6a shows the temperature stability of
19 the walleye pollock trypsin. The trypsin was stable below 30 °C, but its activity quickly fell above
20 40 °C. Similar to those of other trypsins from Frigid Zone fish (Kishimura et al., 2006a, 2006b,
21 2007), the temperature stability of the walleye pollock trypsin was unstable than trypsins from the
22 Temperate Zone fish (Kishimura et al., 2005, 2006a, 2006b, 2006c, 2007), Tropical Zone fish
23 (Klomklao et al., 2006a, 2006b, 2007), and porcine pancreas (Fig. 6b). Then, it was investigated
24 that the relationship between habitat temperature of fish and thermo stability of the fish trypsin.
25 As shown in Fig. 7, the relationship between habitat temperature of fish and thermo stability of the

1 fish trypsin indicated strong positive correlation. This is the first result on the relationship
2 between habitat temperature and thermo stability for the trypsin. Furthermore, the *N*-terminal
3 amino acid sequence of the walleye pollock trypsin was also analyzed. The *N*-terminal amino acid
4 sequence of the trypsin was determined to be IVGGYECTKHSQAHQVSLNS (Fig. 8). It was
5 indicated that the *N*-termini of the trypsin was unblocked. The *N*-terminal amino acid sequence of
6 the walleye pollock trypsin was aligned with the sequences of other animal trypsins. Being
7 similar to other fish trypsins, the walleye pollock trypsin had a charged Glu residue at position 6,
8 where Thr is most common in mammalian pancreatic trypsins (Fig. 8). Bovine pancreatic trypsin
9 has a disulfide bond between Cys-7 and Cys-142 (Stroud, Kay, & Dickerson, 1974), and other
10 vertebrate trypsins also possess the Cys-7. As shown in Fig. 8, the Cys residue at position 7 was
11 characteristically conserved in the walleye pollock trypsin. The *N*-terminal amino acid sequence
12 of the walleye pollock trypsin was completely identical with that of cod trypsin, and the identity
13 between the walleye pollock trypsin to Firigid Zone fish trypsin was relatively higher (85-100 %)
14 than Temperate Zone fish trypsin (75-90 %), Tropical Zone fish trypsin (75-85 %), and mammalian
15 trypsin (60-65 %) (Fig. 8).

16 In conclusion, the walleye pollock trypsin showed a lower optimum temperature than that of
17 porcine pancreatic trypsin and was more unstable than porcine pancreatic trypsin below pH 6.0 and
18 above 40 °C. These results suggest that the pyloric ceca of walleye pollock could be a potential
19 source of trypsin for certain food processing operations that require low processing temperatures,
20 and the relatively low thermal stability of the walleye pollock trypsin may also be beneficial in such
21 applications as the enzymes can be inactivated more readily.

22 23 **Acknowledgment**

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References

- Asgeirsson, B., Fox, J.W., & Bjarnason, J.B. (1989). Purification and characterization of trypsin from the poikilotherm *Gadus morhua*. *European Journal of Biochemistry*, 180, 85-94.
- Castillo-Yanez, F.J., Pacheco-Aguilar, R., Garcia-Carreno, F.L., & Toro, M.A.N. (2005). Isolation and characterization of trypsin from pyloric caeca of Monterey sardine *Sardinops sagax caerulea*. *Comparative Biochemistry and Physiology*, 140B, 91-98.
- Emi, M., Nakamura, Y., Ogawa, M., Yamamoto, T., Nishide, T., Mori, T., & Matsubara, K. (1986). Cloning, characterization and nucleotide sequences of two cDNAs encoding human pancreatic trypsinogens. *Gene*, 41, 305-310.
- Genicot, S., Rentier-Delrue, F., Edwards, D., Vanbeeumen, J., & Gerday, C. (1996). Trypsin and trypsinogen from Antarctic fish: molecular basis of cold adaptation. *Biochimica et Biophysica Acta*, 1298, 45-57.
- Gudmundsdottir, A., Gudmundsdottir, E., Oskarsson, S., Bjarnason, J.B., Eakin, A.K., & Craik, C.S. (1993). Isolation and characterization of cDNAs from Atlantic cod encoding two different forms of trypsinogen. *European Journal of Biochemistry*, 217, 1091-1097.
- Hermodson, M.A., Ericsson, L.H., Neurath, H., & Walsh, K.A. (1973). Determination of the amino acid sequence of porcine trypsin by sequenator analysis. *Biochemistry*, 12, 3146-3153.
- Hjelmeland, K., & Raa, J. (1982). Characteristics of two trypsin type isozymes isolated from the Arctic fish capelin (*Mallotus villosus*). *Comparative Biochemistry and Physiology*, 71B, 557-562.
- Hummel, B.C.W. (1959). A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. *Canadian Journal of Biochemistry and Physiology*, 37, 1393-1399.
- Kishimura, H., & Hayashi, K. (2002). Isolation and characteristics of trypsin from pyloric ceca of the starfish *Asterina pectinifera*. *Comparative Biochemistry and Physiology*, 132B, 485-

- Kishimura, H., Hayashi, K., Miyashita, Y., & Nonami, Y. (2005). Characteristics of two trypsin isozymes from the viscera of Japanese anchovy (*Engraulis japonica*). *Journal of Food Biochemistry*, 29, 459-469.
- Kishimura, H., Hayashi, K., Miyashita, Y., & Nonami, Y. (2006a). Characteristics of trypsin from the viscera of true sardine (*Sardinops melanostictus*) and the pyloric ceca of arabesque greenling (*Pleuroprammus azonus*). *Food Chemistry*, 97, 65-70.
- Kishimura, H., Tokuda, Y., Klomklao, S., Benjakul, S., & Ando, S. (2006b). Enzymatic characteristics of trypsin from pyloric ceca of spotted mackerel (*Scomber australasicus*). *Journal of Food Biochemistry*, 30, 466-477.
- Kishimura, H., Tokuda, Y., Klomklao, S., Benjakul, S., & Ando, S. (2006c). Comparative study of enzymatic characteristics of trypsins from the pyloric ceca of yellow tail (*Seriola quinqueradiata*) and brown hakeling (*Physiculus japonicus*). *Journal of Food Biochemistry*, 30, 521-534.
- Kishimura, H., Tokuda, Y., Yabe, M., Klomklao, S., Benjakul, S., & Ando, S. (2007). Trypsins from the pyloric ceca of jacobever (*Sebastes schlegelii*) and elkhorn sculpin (*Alcichthys alcicornis*): isolation and characterization. *Food Chemistry*, 100, 1490-1495.
- Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., Simpson, B. K., & Saeki, H. (2006a). Trypsins from yellowfin tuna (*Thunnus albacores*) spleen: purification and characterization. *Comparative Biochemistry and Physiology*, 144B, 47-56.
- Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., & Simpson, B. K. (2006b). Purification and characterization of trypsin from the spleen of tongol tuna (*Thunnus tonggol*). *Journal of Agricultural and Food Chemistry*, 54, 5617-5622.
- Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., & Simpson, B. K. (2007). Purification and characterization of trypsins from the spleen of skipjack tuna (*Katsuwonus*

1 *pelamis*). *Food Chemistry*, 100, 1580-1589.

2 Kossiakoff, A.A., Chambers, J.L., Kay, L.M., & Stroud, R.M. (1977). Structure of bovine
3 trypsinogen at 1.9Å resolution. *Biochemistry*, 16, 654-664.

4 Kristjansson, M.M. (1991). Purification and characterization of trypsin from the pyloric caeca of
5 rainbow trout (*Oncorhynchus mykiss*). *Journal of Agricultural and Food Chemistry*, 39,
6 1738-1742.

7 Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of
8 bacteriophage T4. *Nature*, 227, 680-685.

9 Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951). Protein measurement with the
10 Folin phenol reagent. *Journal of Biological Chemistry*, 193, 265-273.

11 Martinez, A., Olsen, R.L., & Serra, J.L. (1988). Purification and characterization of two trypsin-like
12 enzymes from the digestive tract of anchovy *Engraulis encrasicolus*. *Comparative*
13 *Biochemistry and Physiology*, 91B, 677-684.

14 Male, R., Lorens, L.B., Smalas, A.O., & Torrissen, K.R. (1995). Molecular cloning and
15 characterization of anionic and cationic variants of trypsin from Atlantic salmon. *European*
16 *Journal of Biochemistry*, 232, 677-685.

17 Simpson, B.K., & Haard N.F. (1984). Trypsin from Greenland cod, *Gadus ogac*. Isolation and
18 comparative properties. *Comparative Biochemistry and Physiology*, 79B, 613-622.

19 Simpson, B.K., & Haard N.F. (1987). Cold-adapted enzymes from fish. In D. Knorr (Ed.), *Food*
20 *Biotechnology*, (pp. 495-528). New York: Marcel Dekker.

21 Simpson, B.K., & Haard N.F. (1999). Marine enzymes. In F.J. Francis (Ed.), *Encyclopedia of Food*
22 *Science and Technology*, Second edition, Vol. 3. (pp. 1525-1534). New York: John Wiley
23 and Sons Inc.

24 Stroud, R.M., Kay, L.M., & Dickerson, R.E. (1974). The structure of bovine trypsin: electron
25 density maps of the inhibited enzyme at 5 Å and 2.7 Å resolution. *Journal of*

1 *Molecular Biology*, 83, 185-208.

2 Walsch, K.A. (1970). Trypsinogens and trypsins of various species. *Methods in Enzymology*, 19, 41-
3 63.

4 Yoshinaka, R., Suzuki, T., Sato, M., & Ikeda, S. (1983). Purification and some properties of anionic
5 trypsin from the catfish pancreas. *Bulletin of Japanese Society of Scientific Fisheries*, 49,
6 207-212.

7 Yoshinaka, R., Sato, M., Suzuki, T., & Ikeda, S. (1984). Enzymatic characterization of anionic
8 trypsin of the catfish (*Parasilurus asotus*). *Comparative Biochemistry and Physiology*,
9 80B, 475-480.

(captions to figures)

Fig. 1. Electrophoresis of purified trypsin from walleye pollock. Electrophoresis was performed using a 0.1 % SDS-12.5 % polyacrylamide slab-gel. Lane 1 contains protein standards; bovine pancreatic trypsinogen (molecular weight, 24 kDa), bovine milk β -lactoglobulin (18.4 kDa), and egg-white lysozyme (14.3 kDa). Lane 2 contains walleye pollock trypsin.

Fig. 2. Effect of Calcium ion on the stability of trypsin from walleye pollock. The enzyme was kept at 30 °C 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 °C and pH 8.0 were determined. a: walleye pollock trypsin, b: porcine pancreatic trypsin.

Fig. 3. Effect of pH on the activity of trypsin from walleye pollock. The activity was 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 °C. a: walleye pollock trypsin, b: porcine pancreatic trypsin.

Fig. 4. pH stability of trypsin from walleye pollock. The enzyme was kept at 30 °C for 30 min and pH 4.0-11.0, and then the remaining activity at 30 °C and pH 8.0 was determined. a: walleye pollock trypsin, b: porcine pancreatic trypsin.

1 Fig. 5. Effect of temperature on the activity of trypsin from walleye pollock. The activity was
2 determined at pH 8.0 and at various temperatures. a: walleye pollock trypsin, b: porcine
3 pancreatic trypsin.

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6 Fig. 6. Thermostability of trypsin from walleye pollock. The enzyme was kept at 20-70 °C for
7 15 min and pH 8.0, and then the remaining activity at 30 °C and pH 8.0 was determined. a:
8 walleye pollock trypsin, b: porcine pancreatic trypsin.

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11 Fig. 7. Relationship between habitat temperature of fish and thermo stability of the fish trypsin.
12 The 50 % denaturing temperature shows the temperature that the enzyme was denaturated 50 %
13 by incubation at pH 8.0 for 15 min at a range of 20-70 °C. Open triangle, walleye Pollock trypsin;
14 Closed triangle, brown hakeling trypsin (Kishimura et al., 2006c); Open square, spotted mackerel
15 trypsin (Kishimura et al., 2006b); Closed square, Japanese anchovy trypsin (Kishimura et al., 2005);
16 Open circle, skipjack tuna trypsin (Klomklao et al., 2007); Closed circle, yellowfin tuna trypsin
17 (Klomklao et al., 2006a); Open diamond, porcine pancreatic trypsin.

1 Fig. 8. Comparison of the *N*-terminal amino acid sequences of trypsins from walleye pollock (*T.*
2 *chalcogramma*) with those of other vertebrates. Cod (Gudmundsdottir et al., 1993); Elkhorn
3 sculpin (Kishimura et al., 2007); Brown hakeling (Kishimura et al., 2006c); Arabesque greenling
4 (Kishimura et al., 2006a); Spotted mackerel (Kishimura et al., 2006b); Yellow tail (Kishimura et
5 al., 2006c); Jacopever (Kishimura et al., 2007); True sardine (Kishimura et al., 2006a); Japanese
6 anchovy (Kishimura et al., 2005); Skipjack tuna (Klomklao et al., 2007); Tongol tuna (Klomklao et
7 al., 2006b); Yellowfin tuna (Klomklao et al., 2006a); Porcine (Hermodson et al. 1973); Bovine
8 (Walsch, 1970); Human (Emi et al., 1986). Amino acid residues different from the walleye
9 pollock trypsin are shaded.

Table 1
Purification of trypsin from walleye pollock

Purification stages	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purity (fold)	Yield (%)
Crude enzyme	8,220	4,932	0.6	1	100
Sephacryl S-200	219	2,321	10.6	18	47
Sephadex G-50	28	1,400	50.0	83	28

Table 2
Effects of various inhibitors on the activity of trypsin from walleye pollock ^a

Inhibitors	Concentration	% Inhibition
Control		0
E-64	0.1 mM	0
<i>N</i> -ethylmaleimide	1 mM	0
Iodoacetic acid	1 mM	0
Soybean trypsin inhibitor	1 mg/ml	91
TLCK	5 mM	82
TPCK	5 mM	11
Pepstatin A	0.01 mM	3
EDTA	2 mM	9

^a The enzyme solution was incubated with the same volume of inhibitor at 25 °C for 15 min and residual activity was analysed using TAME as a substrate for 20 min at pH 8.0 and 30 °C.

Fig.1

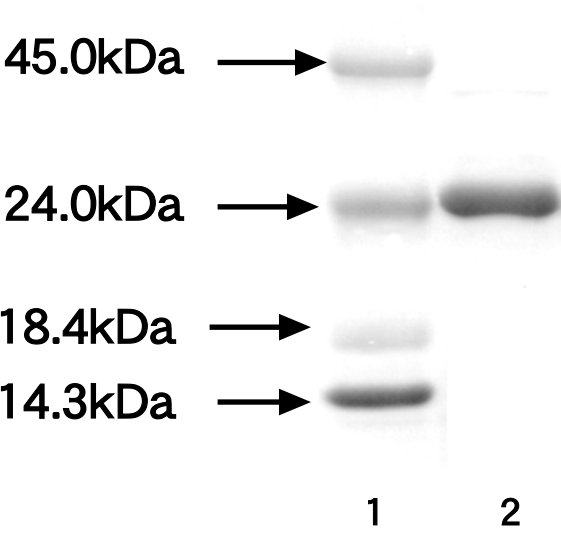


Fig.2

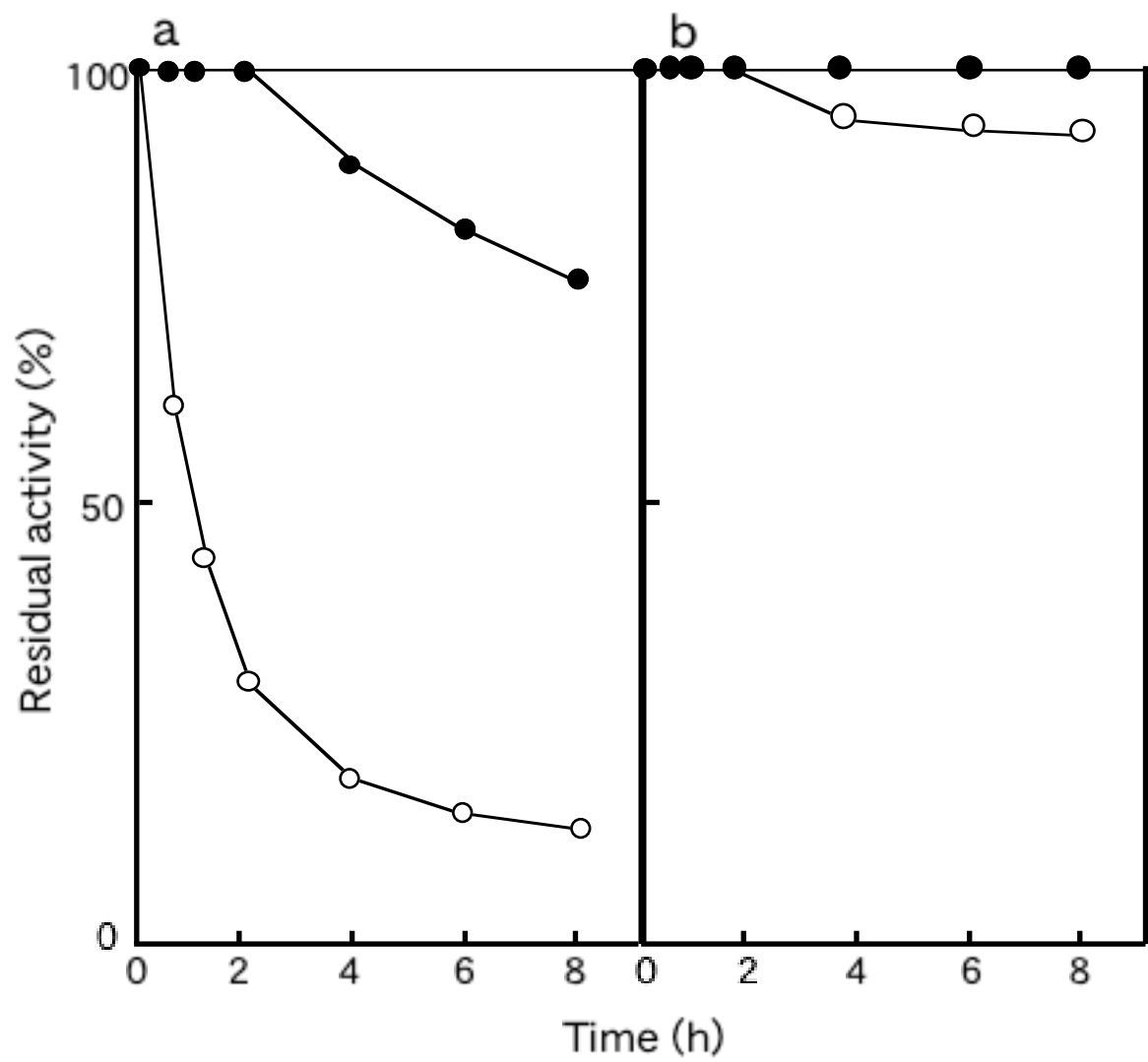


Fig.3

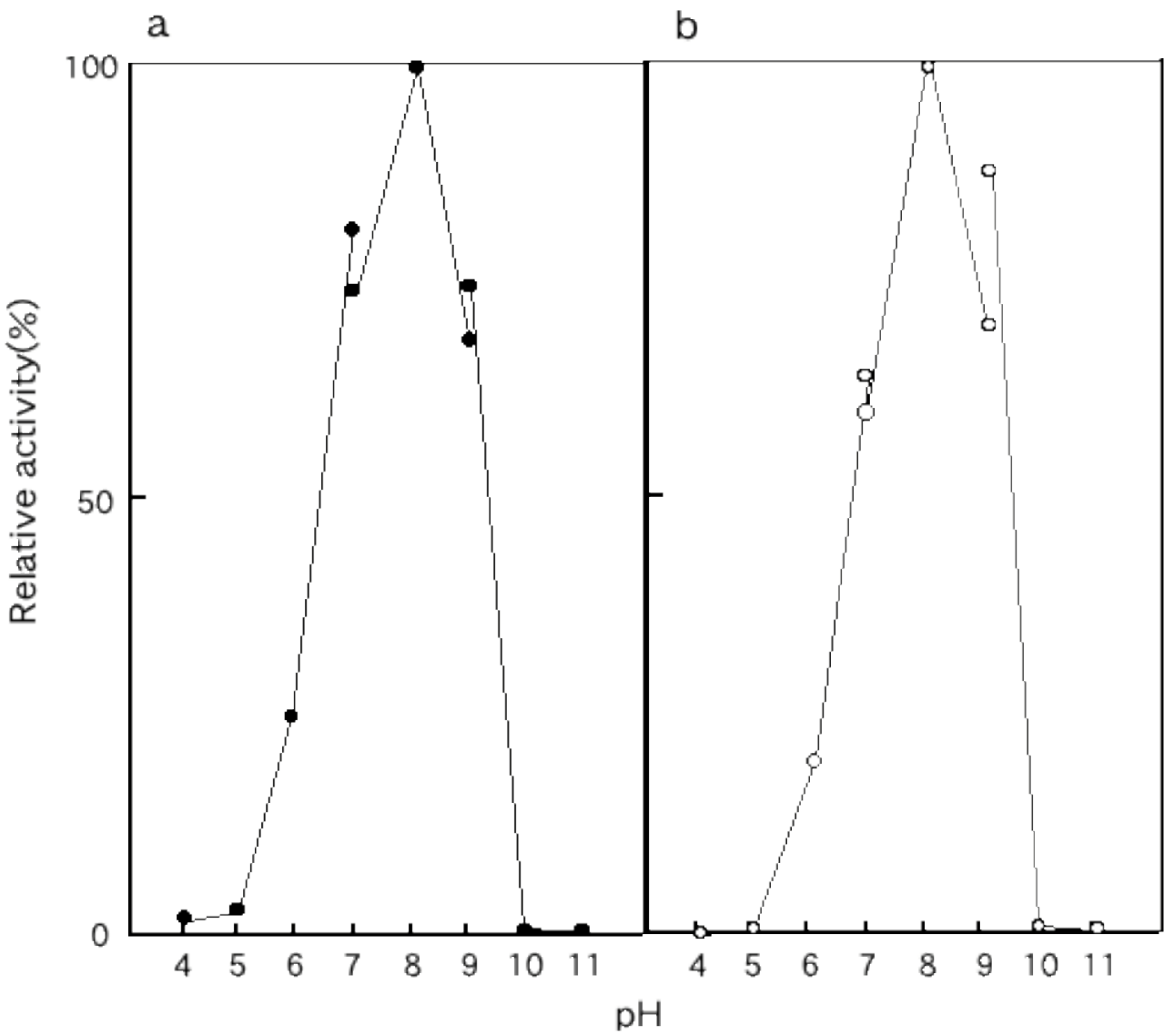


Fig.4

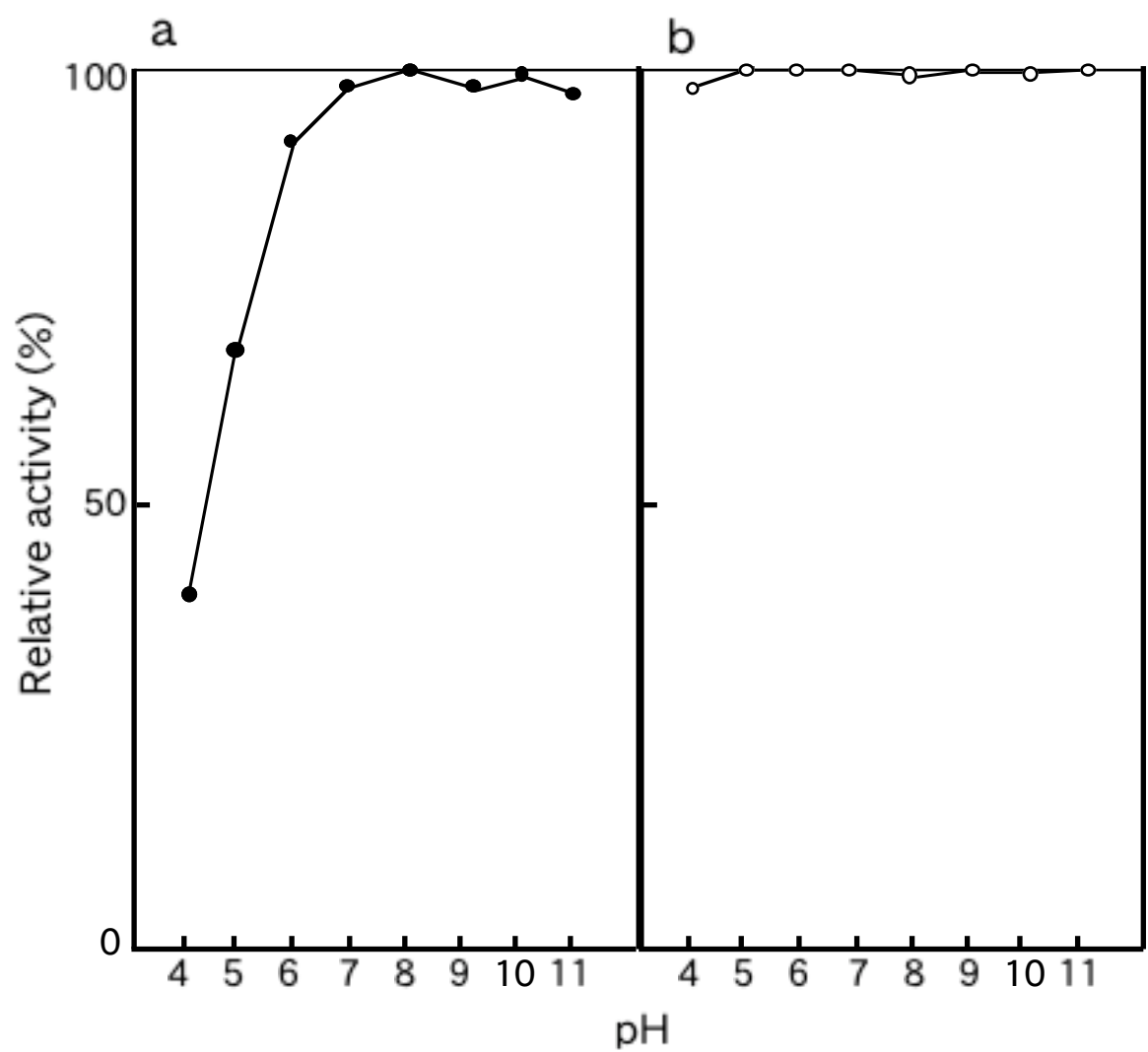


Fig.5

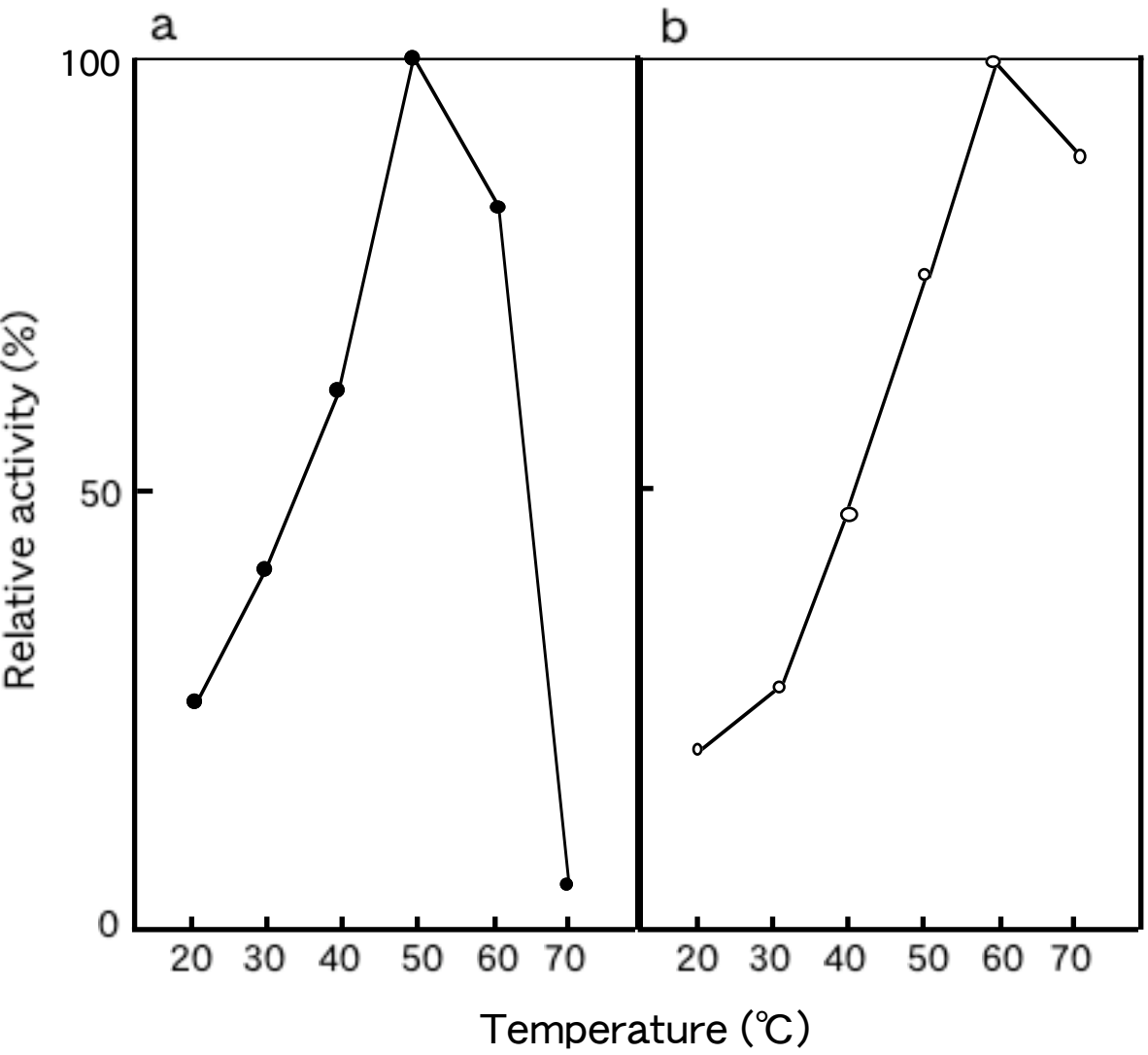


Fig.6

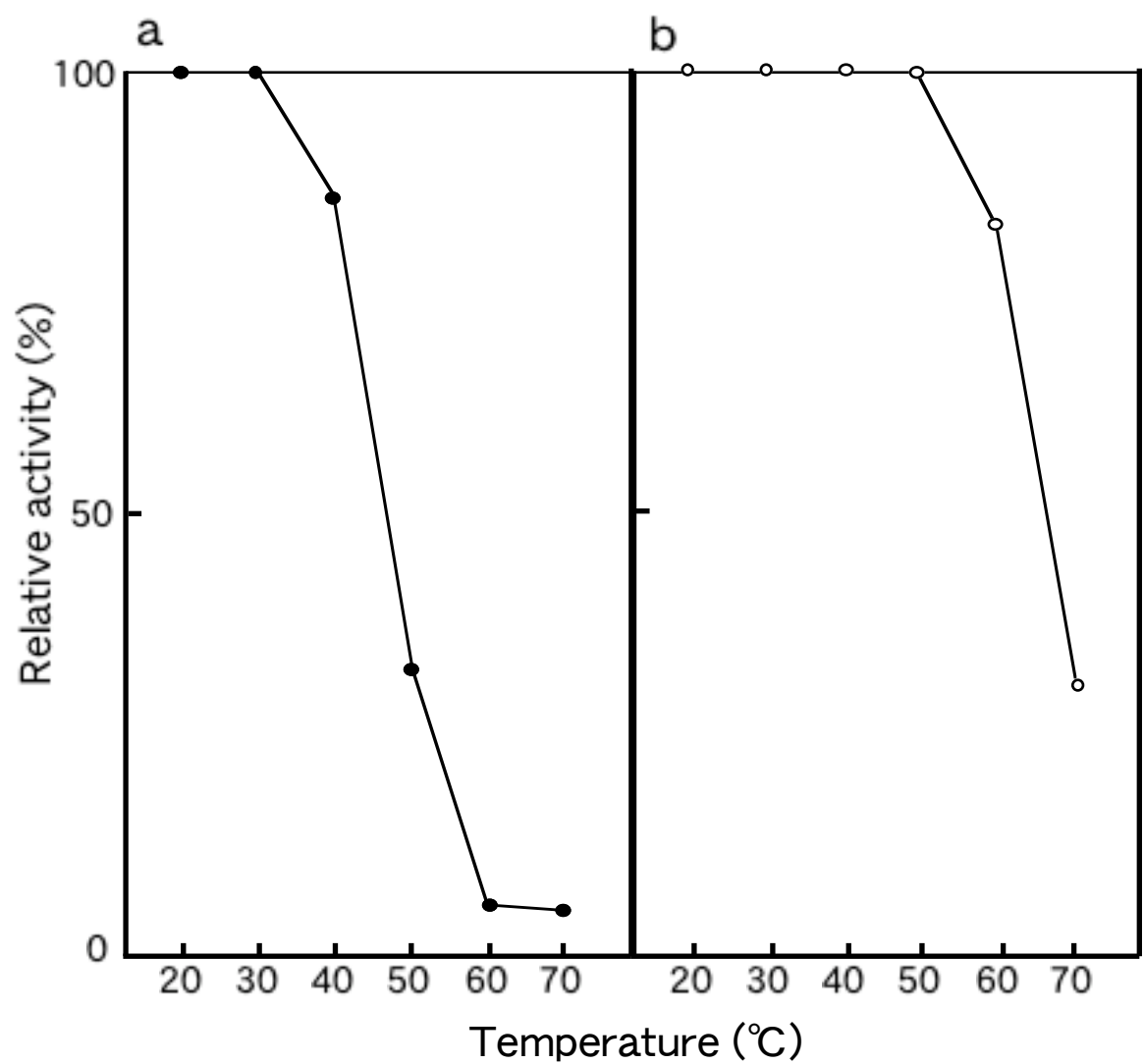
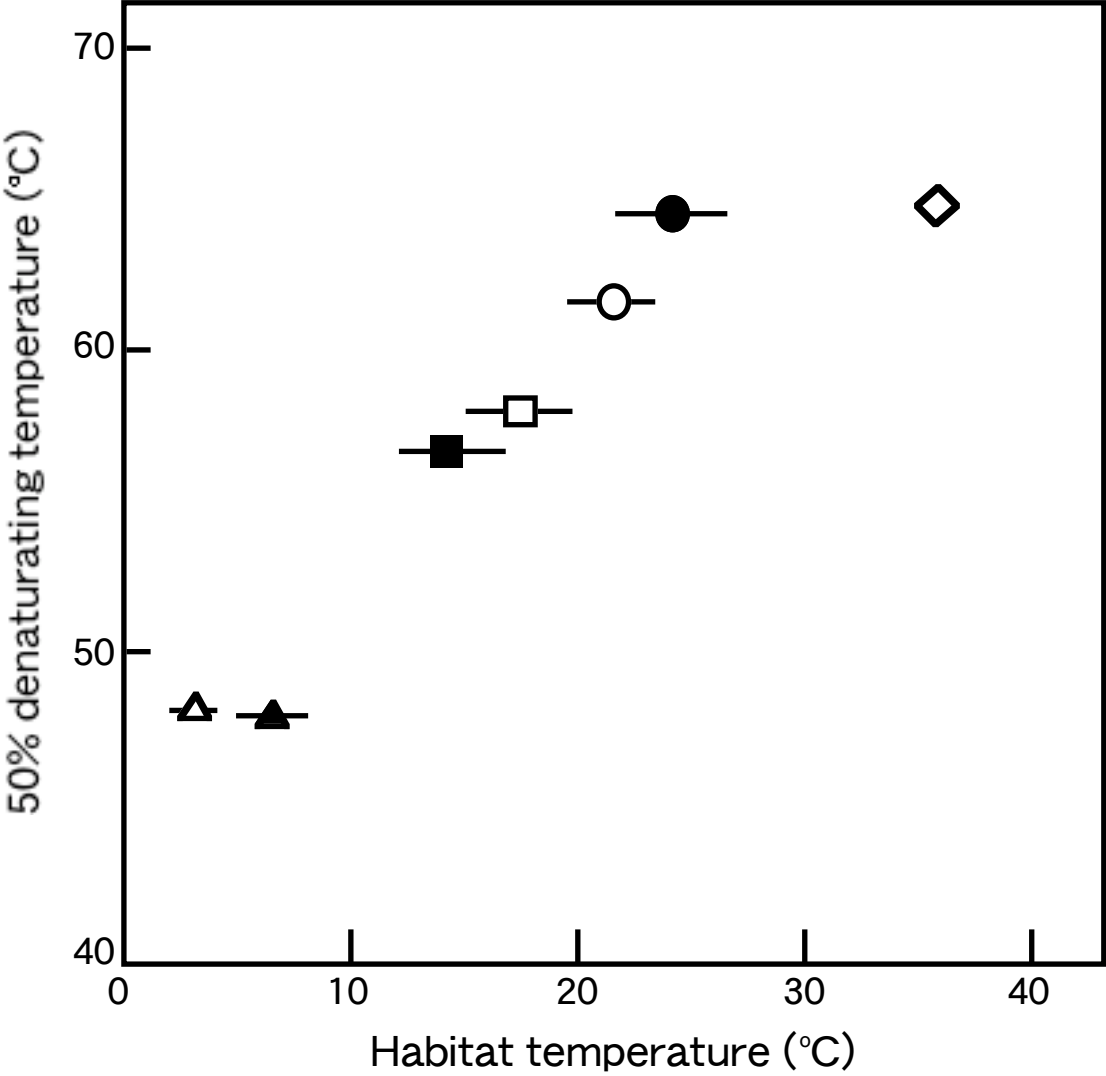


Fig.7



1 Fig.8

2

3

4

5

6

Frigid Zone Fish

7

Walleye pollok

8

Cod

9

Elkhorn sculpin

10

Brown hakeling

Arabesunque greenling

11

Temperate Zone Fish

12

Spotted mackerel

13

Yellow tail

14

Jacopever

15

True sardine

Japanese anchovy

Tropical Zone Fish

Skipjack tuna

Tongol tuna

Yellowfin tuna

Mammal

Porcine

Bovine

Human

1

10

20

IVGGYECTKHSQAHQVSLNS

IVGGYECTKHSQAHQVSLNS

IVGGYECTPHSQAHQVSLNS

IVGGYECTPKHSQPHQVSLNS

IVGGYECTPHTQAHQVSLDS

IVGGYECTAHSQPHQVSLNS

IVGGYECTPYSQPHQVSLNS

IVGGYECKPYSQPHQVSLNS

IVGGYECKAYSQPWQVSLNS

IVGGYECQPYSQPHQVSLDS

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IVGGYECQAHSQPHQVSLNA

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