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Purification and characteristics of trypsins from cold-zone fish, Pacific cod (*Gadus macrocephalus*) and saffron cod (*Eleginus gracilis*)

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ABSTRACT

Trypsins from the pyloric ceca of Pacific cod (*Gadus macrocephalus*) (GM-T) and saffron cod (*Eleginus gracilis*) (EG-T) were purified by gel filtration on Sephacryl S-200 and Sephadex G-50. The final enzyme preparations were nearly homogeneous on SDS–PAGE and the molecular weights of both enzymes were estimated to be approximately 24 kDa by SDS–PAGE. The specific trypsin inhibitors, soybean trypsin inhibitor and TLCK, strongly inhibited the activities of GM-T and EG-T. The optimum pH and optimum temperature of both trypsins were around pH 8.0 and 50 °C, respectively, using N^{α}-*p*-tosyl-L-arginine methyl ester as substrate. The GM-T and EG-T were unstable above 30 °C and below pH 5.0, and they were stabilised by calcium ion. The *N*-terminal amino acid sequences of GM-T (IVGGYECTRHSQAHQVSLNS) and EG-T (IVGGYECPRHSQAHQVSLNS) were found. The percentage of hydrophobic amino acid in the *N*-terminal 20 amino acids sequences of these cold-zone fish trypsins was lower (28%) than those of temperate-zone fish trypsins (34%), tropical-zone fish trypsins (37%) and mammalian trypsins (34%). Whereas the content of charged amino acids in the GM-T and EG-T was relatively higher than those of trypsins from temperate-zone fish, tropical-zone fish and mammals. Moreover, the GM-T catalyzed synthesis of N^{α}-(*tert*-butoxycarbonyl)-L-alanyl-L-alanine-*p*-nitroanilide (N^{α}-Boc-L-Ala-L-Ala-*p*NA) has been studied by using N^{α}-(*tert*-butoxycarbonyl)-L-alanine-*p*-guanidinophenyl ester [N^{α}-Boc-L-Ala-O*p*Gu (inverse substrate)] as acyl donor and L-alanine-*p*-nitroanilide (L-Ala-*p*NA) as acyl acceptor, respectively.

1. Introduction

Fish viscera are a source of digestive enzymes that may have some unique properties of interest to both basic research and industrial applications. Especially, proteinases from cold adapted fish often have higher enzymatic activity at low temperatures than those from warm-blooded animals (Asgeirsson, Fox, & Bjarnason, 1989; Kristjansson, 1991). High activity of these fish proteinases at low temperatures may be interesting for several industrial applications, such as in certain food processing operations that require low processing temperatures. Furthermore, proteinases from cold adapted fish are inactivated at relatively low temperatures, which makes such enzymes potentially useful in food applications where ready and rapid denaturation is desirable (Simpson & Haard, 1987).

One of the main digestive proteinases that is present in the pyloric ceca and intestine of fish is trypsin (EC 3.4.21.4). Trypsin is a member of a large family of serine proteinases and acts by cleaving the ester and peptide bonds involving the carboxyl groups of arginine and lysine. Recently, we isolated and characterised fish trypsins from the following species: Japanese anchovy (Kishimura, Hayashi, Miyashita, & Nonami, 2005), true sardine (Kishimura, Hayashi, Miyashita, & Nonami, 2006), arabesque greenling (Kishimura, Hayashi, et al., 2006), yellowfin tuna (Klomklao et al., 2006), spotted mackerel (Kishimura, Tokuda, Klomklao, Benjakul, & Ando, 2006a), yellow tail (Kishimura, Tokuda, Klomklao, Benjakul, & Ando, 2006b), brown hakeling (Kishimura et al., 2006b), tongol tuna (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2006), jacopever (Kishimura et al., 2007), elkhorn sculpin (Kishimura et al., 2007), skipjack tuna (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007a), bluefish (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007b), Atlantic bonito (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007c), and walleye pollock (Kishimura, Klomklao, Benjakul, & Chun, 2008). Consequently, the relationship between habitat temperature of fish and thermostability of fish trypsin indicated strong positive correlation

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(Kishimura et al., 2008). The trypsins from frigid-zone fish showed a lower optimum temperature and lower heat stability than those of temperate-zone fish, tropical-zone fish and mammalian trypsins. The characteristics suggested that the viscera of frigid-zone fish would be a potential source of trypsin for food processing operations.

In this study, we purified trypsins from the pyloric ceca of two species of frigid-zone fish, Pacific cod (*Gadus macrocephalus*) and saffron cod (*Eleginus gracilis*), and investigated their enzymatic characteristics. Especially, Pacific cod is one of the economically important fish species in Hokkaido Prefecture, Japan. Recent harvests of Pacific cod in Hokkaido Prefecture are estimated at about 30,000 metric tons per year. However, its viscera are largely underutilised and are discarded as waste. So, we carried out the utilisation of Pacific cod trypsin as the catalyst of enzymatic peptide synthesis.

2. Materials and methods

2.1. Materials

Pacific cod (*G. macrocephalus*) and saffron cod (*E. gracilis*) were 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) was obtained from Wako Pure Chemicals (Osaka, Japan). Ethylenediamine tetraacetic acid (EDTA), 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), *N*-ethylmaleimide, iodoacetic acid, soybean trypsin inhibitor, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), N^{α}-tosyl-L-phenyl-alanine chloromethyl ketone (TPCK), and pepstatin A were purchased from Sigma Chemical Co. (Mo, USA). L-Ala-*p*NA was purchased from Peptide Institute, Inc. (Osaka, Japan). Boc-L-Ala-O*p*Gu was prepared according to our previous paper (Sekizaki, Itoh, Toyota, & Tanizawa, 1996a).

2.2. Preparation of crude enzyme

Defatted powders of the pyloric ceca of Pacific cod and saffron cod were 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 $CaCl_2$ 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 lyophilised and used as crude trypsin.

2.3. Purification of trypsin

The crude trypsin was applied to a column of Sephacryl S-200 (3.9 × 64 cm) pre-equilibrated with 10 mM tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and the proteins were eluted with the same buffer. The main trypsin fraction was concentrated by lyophilisation. Then the concentrated fraction was applied to a Sephadex G-50 column (3.9 × 64 cm) pre-equilibrated with 10 mM tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and the proteins were eluted with the same buffer. Trypsin was eluted as single peak on the gel filtrations. The trypsin fractions were pooled and used for further studies.

2.4. Assay for trypsin activity

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 hydrolysing one micromole of TAME in a minute. The effect of inhibitors on trypsin was determined according to the method of Klomklao, Benjakul, and Visessanguan (2004) by incubating trypsin with an equal volume of proteinase inhibitor solution to obtain the final concentration designated (0.1 mM E-64, 1 mM *N*-ethylmaleimide, 1 mM iodoacetic acid, 1 mg/ml soybean trypsin inhibitor, 5 mM TLCK, 5 mM TPCK, 1 mM pepstatin A and 2 mM EDTA). After incubation of the mixture at 25 °C for 15 min, the remaining activity was measured and percent inhibition was then calculated. The pH dependence of the enzyme 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 30 °C. The temperature dependence of the enzyme was 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 $CaCl_2$ 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 ethylenediamine tetraacetic acid (EDTA) or 10 mM $CaCl_2$.

2.5. Pacific cod trypsin-catalyzed dipeptide coupling reaction

A solution of 40 μl of 50 mM MOPS buffer (containing 20 mM of $CaCl_2$, pH 8.0), 30 μl of DMSO, 10 μl of acyl acceptor [200 mM L-Ala-*p*NA (**2**) in DMSO] and 10 μl of trypsin solution (2.9 mg/ml solution in MOPS buffer) were mixed. To this mixture, 10 μl of acyl donor [10 mM N^{α}-Boc-L-Ala-O*p*Gu (**1**) in DMSO] was added and incubated at 25 °C. The progress of the coupling reaction was monitored by HPLC under the following conditions: Shim-pack CLC-ODS (M) (column id 4.6 × 250 mm), isocratic elution at 1 ml/min, 50% acetonitrile containing 0.1% trifluoroacetic acid. An aliquot of the reaction mixture was injected and the eluate was monitored at 310 nm (chromophore due to the *p*-nitroanilide moiety). Peak identification was made by correlating the retention time with that of authentic sample which was chemically synthesized (Bieth & Weremath, 1973). Observed peak area was used for the estimation of sample concentration.

2.6. Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate–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.

2.7. Analysis of amino acid sequence

To analyse the *N*-terminal sequence, the purified enzyme was electroblotted to polyvinylidene difluoride (PVDF) membrane (Mini ProBlott Membranes, Applied Biosystems, CA, USA) after SDS–PAGE. The amino acid sequence of the enzyme was analysed by using a protein sequencer, Procise 492 (Perkin Elmer, CA, USA).

2.8. Protein determination

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

3. Results and discussion

The two trypsins (GM-T and EG-T) were purified from the pyloric ceca of Pacific cod and saffron cod, respectively, by gel filtration on Sephacryl S-200 and Sephadex G-50. The final preparations

from Pacific cod and saffron cod were purified 33-fold (with a recovery of 19%) and 28-fold (17%), respectively, from the crude trypsin (Table 1). Purified trypsins, GM-T and EG-T, were nearly homogeneous on SDS–PAGE (Fig. 1). The effects of various proteinase inhibitors on the activities of the GM-T and EG-T were determined (Table 2). The specific trypsin inhibitors, soybean trypsin inhibitor and TLCK strongly inhibited (77–97%) the activities of the GM-T and EG-T. While, specific inhibitors of cysteine proteinase (E-64, *N*-ethylmaleimide, iodoacetic acid), chymotrypsin (TPCK), aspartic proteinase (pepstatin A), and metalloproteinase (EDTA) had no inhibitory effect on the activities of the GM-T and EG-T. According to proteinase inhibitors assays, the purified enzymes were revealed as trypsin. The molecular weights of the GM-T and EG-T were estimated as approximately 24 kDa using SDS–PAGE (Fig. 1). The relative molecular masses of the GM-T and EG-T were similar to those of other fish trypsins (Asgeirsson et al., 1989; Kishimura et al., 2005; Kishimura, Hayashi, et al., 2006; Kishimura et al., 2006a, 2006b, 2007, 2008; Klomklao et al., 2006; Klomklao, Benjakul, et al., 2006; Klomklao et al., 2007a; Simpson & Haard, 1984) except for those of Atlantic bonito (29 kDa) and bluefish (28 kDa) (Klomklao et al., 2007b, 2007c).

The influences of pH on the activity of the GM-T and EG-T are shown in Fig. 2a. Both enzymes hydrolysed the TAME effectively between pH 7.0 and 9.0, with an optimum around pH 8.0. The optimum pH of the GM-T and EG-T was the same as those of other fish trypsins (Asgeirsson et al., 1989; Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, & Toro, 2005; Hjelmeland & Raa, 1982; Kishimura et al., 2005; Kishimura, Hayashi, et al., 2006; Kishimura et al., 2006a, 2006b, 2007, 2008; Klomklao et al., 2006; Klomklao, Benjakul, et al., 2006; Klomklao et al., 2007a; Martinez, Olsen, & Serra, 1988; Simpson & Haard, 1984), but lower than those of Atlantic bonito (pH 9.0) and bluefish (pH 9.5) (Klomklao et al., 2007b, 2007c).

Table 1
Purification of trypsins from Pacific cod and saffron cod.

Purification stages	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purity (fold)	Yield (%)
Pacific cod					
Crude enzyme	3353	2400	0.72	1	100
Sephacryl S-200	509	1416	2.8	4	59
Sephadex G-50	19	456	24.0	33	19
Saffron cod					
Crude enzyme	3948	2541	0.64	1	100
Sephacryl S-200	795	1652	2.1	3	65
Sephadex G-50	24	432	18.0	28	17

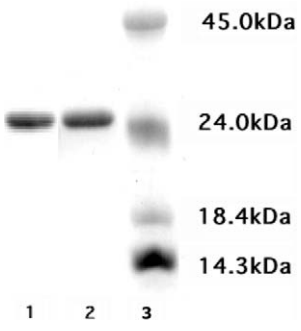


Fig. 1. Electrophoresis of purified trypsins from Pacific cod and saffron cod. Electrophoresis was performed using a 0.1% SDS–12.5% polyacrylamide slab-gel. Lane 1 contains Pacific cod trypsin. Lane 2 contains saffron cod trypsin. Lane 3 contains protein standards; egg albumin (molecular weight, 45.0 kDa), bovine pancreatic trypsinogen (24.0 kDa), bovine milk β-lactoglobulin (18.4 kDa) and egg-white lysozyme (14.3 kDa).

Table 2
Effects of various inhibitors on the activities of trypsins from Pacific cod and saffron cod.[a]

Inhibitors	Concentration	Inhibition (%)
Pacific cod		
Control		0
E-64	0.1 mM	0
N-ethylmaleimide	1 mM	0
Iodoacetic acid	1 mM	6
Soybean trypsin inhibitor	1 mg/ml	80
TLCK	5 mM	89
TPCK	5 mM	8
Pepstatin A	0.01 mM	1
EDTA	2 mM	3
Saffron cod		
Control		0
E-64	0.1 mM	0
N-ethylmaleimide	1 mM	1
Iodoacetic acid	1 mM	3
Soybean trypsin inhibitor	1 mg/ml	84
TLCK	5 mM	79
TPCK	5 mM	2
Pepstatin A	0.01 mM	0
EDTA	2 mM	4

[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. 2b shows the temperature dependencies of the GM-T and EG-T. The GM-T and EG-T were active over a broad temperature range (20–60 °C) with the optimum at about 50 °C. Pacific cod and saffron cod are frigid-zone fish and their trypsins had an optimum temperature as did other trypsins from frigid-zone fish, such as Atlantic cod (Asgeirsson et al., 1989), arabesque greenling (Kishimura, Hayashi, et al., 2006), brown hakeling (Kishimura et al., 2006c), elkhorn sculpin (Kishimura et al., 2007), and walleye pollock (Kishimura et al., 2008). However, the optimum temperature of the frigid-zone fish trypsins was lower than those of temperate-zone fish trypsins (60 °C) (Kishimura et al., 2005; Kishimura, Hayashi, et al., 2006; Kishimura et al., 2006b, 2006c, 2007) and tropi-

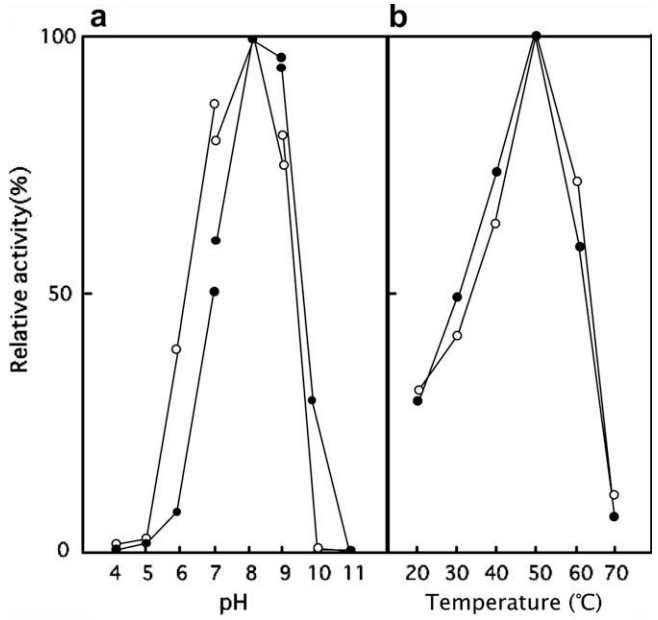


Fig. 2. Effects of pH and temperature on the activity of Pacific cod and saffron cod trypsins. (a) The activities were determined in 50 mM buffer solutions [acetic acid–sodium acetate (pH 4.0–7.0), tris–HCl (pH 7.0–9.0) and glycine–NaOH (pH 9.0–11.0)] at 37 °C. (b) The activities were determined at pH 8.0 and at various temperatures. Closed circle: Pacific cod trypsin; open circle: saffron cod.

cal-zone fish trypsins (55–65 °C) (Klomklao et al., 2006; Klomklao, Benjakul, et al., 2006; Klomklao et al., 2007a, 2007b, 2007c). The pH stabilities of the GM-T and EG-T are shown in Fig. 3a. The GM-T was stable at 30 °C for 30 min in the pH range from 7.0 to 10.0, whereas the EG-T was stable between pH 6.0 and 10.0. Unlike mammalian trypsins, diminished stability of both trypsins was more pronounced after exposure at acidic pH. Instability at acidic pH was also observed for trypsins from other fish species (Asgeirsson et al., 1989; Kishimura et al., 2005; Kishimura, Hayashi, et al.,

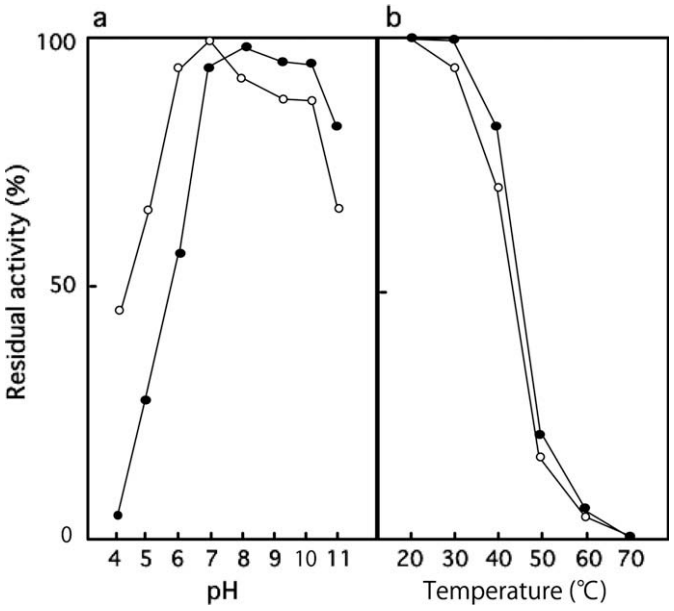


Fig. 3. pH and temperature stabilities of Pacific cod and saffron cod trypsins. (a) The enzymes were kept at 30 °C for 30 min and pH 4.0–11.0, and then the remaining activities at 30 °C and pH 8.0 were determined. (b) The enzymes were kept at 20–70 °C for 15 min and pH 8.0, and then the remaining activities at 30 °C and pH 8.0 were determined. Closed circle: Pacific cod trypsin; open circle: saffron cod.

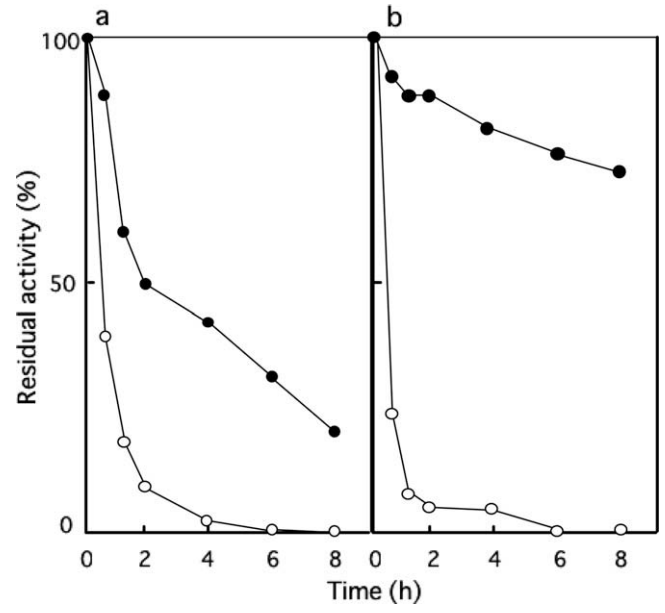
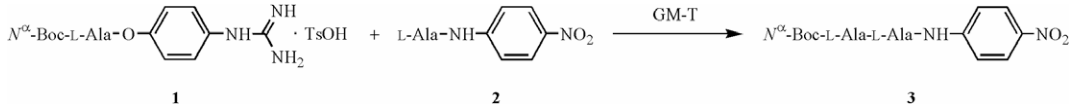


Fig. 4. Effect of calcium ion on the stability of Pacific cod and saffron cod trypsins. The enzymes were kept at 30 °C and pH 8.0 for 0–8 h in the presence of 10 mM $CaCl_2$ (closed symbol) or 10 mM EDTA (open symbol), and then the remaining activities at 30 °C and pH 8.0 were determined. (a) Pacific cod trypsin; (b) saffron cod trypsin.

	1 10 20
Frigid-zone fish	
Pacific cod	IVGGYECTRHSQAHQVSLNS
Saffron cod	IVGGYECPRHSQAHQVSLNS
Atlantic cod	IVGGYECTKHSQAHQVSLNS
Walleye pollok	IVGGYECTKHSQAHQVSLNS
Elkhorn sculpin	IVGGYECTPHSQAHQVSLNS
Arabesuque greenling	IVGGYECTPHTQAHQVSLDS
Brown hakeling	IVGGYECPKHSQPHQVSLNS
Atlantic salmon	IVGGYECKAYSQTHQVSLNS
Antarctic fish	IVGGKECSPYSQPHQVSLNS
Temperate-zone fish	
Spotted mackerel	IVGGYECTAHSQPHQVSLNS
Yellow tail	IVGGYECTPYSQPHQVSLNS
Jacopever	IVGGYECKPYSQPHQVSLNS
Japanese anchovy	IVGGYECQPYSQPHQVSLDS
True sardine	IVGGYECKAYSQPWQVSLNS
Japanese dace	IVGGYECTPYSQPWTVSLNS
Lamprey	IVGGYECAAHSQPWQVSLNI
Tropical-zone fish	
Skipjack tuna	IVGGYECQAHSQPHQVSLNS
Tongol tuna	IVGGYECQAHSQPHQVSLNA
Yellowfin tuna	IVGGYECQAHSQPPQVSLNA
Atlantic bonito	IVGGYECQAHSQPWQPVLNS
Zebrafish	IVGGYECTKNGVPYQVSLNS
Bluefish	IVGGYECKPKSAPVQVSLNL
Tilapia	IIGGYECAKNSVPYMVSLNI
Mammal	
Rat	IVGGYTCQENSVPYQVSLNS
Dog	IVGGYTCEENSVPYQVSLNA
Porcine	IVGGYTCAANSIPYQVSLNS
Bovine	IVGGYTCGANTVPYQVSLNS
Human	IVGGYNCEENSVPYQVSLNS

Fig. 5. Comparison of the *N*-terminal amino acid sequences of Pacific cod and saffron cod trypsins with those of other vertebrates. Atlantic cod (Gudmundsdottir et al., 1993); walleye pollock (Kishimura et al., 2008); elkhorn sculpin (Kishimura et al., 2007); arabesque greenling (Kishimura et al., 2006a); brown hakeling (Kishimura et al., 2006c); Atlantic salmon (Male et al., 1995); Antarctic fish (Genicot et al., 1996); spotted mackerel (Kishimura et al., 2006b); yellow tail (Kishimura et al., 2006c); jacopever (Kishimura et al., 2007); Japanese anchovy (Kishimura et al., 2005); true sardine (Kishimura, Hayashi, et al., 2006); Japanese dace (DDBJ accession number AB445492); lamprey (Roach, Wang, Gan, & Hood, 1997); skipjack tuna (Klomklao et al., 2007); tongol tuna (Klomklao, Benjakul, et al., 2006); yellowfin tuna (Klomklao et al., 2006); Atlantic bonito (Klomklao et al., 2007c); zebrafish (DDBJ accession number AF541952); bluefish (Klomklao et al., 2007b); tilapia (DDBJ accession number AY510093); rat (Roach et al., 1997); dog (Roach et al., 1997); porcine (Hermodson, Ericsson, Neurath, & Walsh, 1973); bovine (Walsh, 1970); and human (Emi et al., 1986). Amino acid residues different from the Pacific cod trypsin are shaded.

2006; Kishimura et al., 2006a, 2006b, 2007, 2008; Klomklao et al., 2006; Klomklao, Benjakul, et al., 2006; Klomklao et al., 2007a, 2007b, 2007c; Kristjansson, 1991; Martinez et al., 1988). For temperature stability, the GM-T and EG-T were stable below 30 °C, but their activities quickly fell above 40 °C (Fig. 3b). The GM-T, EG-T and other frigid-zone fish trypsins were relatively less stable than trypsins from the temperate-zone fish and tropical-zone fish (Kishimura et al., 2008). As shown previously, there is strong relationship between the habitat temperature of fish and the optimum temperature and thermostability of fish trypsin (Kishimura et al., 2008).



Thermal stabilities of the GM-T and EG-T were investigated in the presence of 10 mM EDTA or 10 mM $CaCl_2$. The stabilities of both enzymes were highly dependent on the presence of calcium ion (Fig. 4). Similar results have been reported for the various fish trypsins (Kishimura et al., 2005; Kishimura, Hayashi, et al., 2006; Kishimura et al., 2006a, 2006b, 2007, 2008; Klomklao et al., 2006; Klomklao, Benjakul, et al., 2006; Klomklao et al., 2007a, 2007b, 2007c; Kristjansson, 1991). Two calcium-binding sites are in bovine trypsinogen (Kossiakoff, Chambers, Kay, & Stroud, 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 GM-T and EG-T were stabilised by calcium ion from thermal denaturation. These findings suggest that the GM-T and EG-T possess the primary calcium-binding site like mammalian pancreatic trypsin and other fish trypsins (Genicot, Rentier-Delrue, Edwards, Vanbeeumen, & Gerday, 1996; Male, Lorens, Smalas, & Torrissen, 1995).

Fig. 5 shows the *N*-terminal amino acid sequences of the GM-T and EG-T aligned with those of other animal trypsins. The *N*-terminal amino acid sequences of the GM-T and EG-T were found to be IVGGYECTRHSQAHQVSLNS and IVGGYECPRHSQAHQVSLNS, respectively. The results indicated that the *N*-termini of the GM-T and EG-T were unblocked. The *N*-terminal five amino acid sequences of the GM-T and EG-T (IVGGY) were identical to those of other animal trypsins except for Antarctic fish and tilapia trypsins (Fig. 5). Also, nearly all the trypsins shared the sequence (QVSLN) at position 15-19 (Fig. 5). The GM-T and EG-T had a charged Glu residue at position 6 similar to other fish trypsins, whereas Thr is most common in mammalian pancreatic trypsins (Fig. 5). Furthermore, the GM-T and EG-T characteristically conserved Cys residue at position 7 like the all other trypsins. The result indicates that the GM-T and EG-T may also have a disulphide bond to the corresponding residues (between Cys-7 and Cys-142) of bovine pancreatic trypsin (Stroud, Kay, & Dickerson, 1974). These data lend confidence to the notion that the GM-T and EG-T belong to the trypsin family of enzymes. On the other hand, a percentage of hydrophobic amino acid in frigid-zone fish trypsins (mean: 28%) was relatively lower than those of temperate-zone fish trypsins (34%), tropical-zone fish trypsins (37%) and mammalian trypsins (34%) in Fig. 5. Whereas a percentage of charged amino acid in frigid-zone fish trypsins (mean: 19%) was relatively higher than those of temperate-zone fish trypsins (12%), tropical-zone fish trypsins (13%) and mammalian trypsins (5%). Genicot et al. (1996) considered that overall decrease of hydrophobicity and increase in surface hydrophilicity of fish trypsin as compared to its bovine counterparts affect its thermostability and flexibility. Therefore, such structural characteristics of frigid-zone fish trypsins may contribute their lower thermostability.

The GM-T catalyzed peptide coupling reaction has been studied by using synthetic inverse substrate (**1**) as acyl donor. The coupling reaction was carried out by incubating an acyl donor (**1**) (1 mM) with an acyl acceptor (**2**) (20 mM) and GM-T in a mixture of MOPS buffer (50 mM, pH 8.0, containing 20 mM $CaCl_2$) and DMSO (1:1) at 25 °C.

The progress of the coupling reaction was monitored by HPLC. The elution peak was correlated to that of authentic sample which was chemically synthesized according to the reported procedure (Bieth & Weremath, 1973). The yield of the coupling reaction (60%) was almost same that of bovine trypsin-catalyzed coupling reaction (64%) (Sekizaki, Itoh, Toyota, & Tanizawa, 1996b).

In conclusion, the GM-T and EG-T showed lower optimum temperature and lower thermostability than that of mammalian pancreatic trypsin properties, similar to other frigid-zone fish trypsins. These results suggest that the pyloric ceca of Pacific cod and saffron cod can be used as a novel source of trypsin for certain food processing operations that require low processing temperatures. Furthermore, the catalytic efficiency of GM-T for peptide synthesis using inverse substrate was almost equivalent that of bovine. This result suggested that the GM-T was effective for catalyst of peptide synthesis. More detailed application of the GM-T catalyzed peptide synthesis will be reported elsewhere.

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