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Purification and Characterization of Cold Adapted Trypsins from Antarctic krill (*Euphausia superba*)

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Abstract Three trypsins (TRY-ES) were purified from Antarctic krill (*Euphausia superba*) by ammonium sulfate precipitation, ion-exchange and gel-filtration chromatography, with relative molecular mass of 28.7, 28.8 and 29.2 kDa respectively. The TRY-ES was inhibited by specific trypsin inhibitors (benzamidine, STI, CHOM and TLCK), with optimum temperature at 40 (Trypsin I), 45 (Trypsin II) and 40 °C (Trypsin III) repetitively. The TRY-ES was stabled between 5 and 40 °C, which was consistent with the red shift in fluorescence intensity peak at 40 °C (Trypsin I) and 45 °C (Trypsin II and Trypsin III) and blue shift at 40 °C (Trypsin II and Trypsin III). The K_{cat}/K_m values of the TRY-ES was 14.28, 9.46 and 5.93 $mM^{-1}s^{-1}$ respectively, 1.1–10.2 folds higher than trypsins from other crustacean and mammal, which was supported by the differences in thermodynamics parameters, the free energy, enthalpy, and entropy of benzamidine and the TRY-ES system.

Keywords *Euphausia superba* · Trypsin · Cold adaption · Stability · Catalytic efficiency

Introduction

Antarctic krill (*Euphausia superba*), a kind of psychrophile, is the dominant euphausiid species in the Antarctic and sub-Antarctic sea area. Being wealthy in biomass, Antarctic krill has a wide use in food and feed industries (Nicol and Endo 1997). There are diverse cold-adapted serine-type proteinases and carboxypeptidases in the body of Antarctic krill (*Euphausia superba*), with higher catalytic efficiency than that of most commercialized enzymes (Anheller et al. 1989; Mekkes et al. 1998; Sjödahl et al. 2002). Proteinases from *E. superba* have been tested for ulceration debridement with promising results (Anheller et al. 1989), wound healing (Mekkes et al. 1998) and low value marine protein degrading (Suzuki and Shibata 1990).

Trypsin (EC 3.4.21.4), one of the major proteinases in animal digestive system, acts as a key mediator not only during the food assimilation and zymogen activation of animals, but also during exuviation and immunity improving of insects and crustuceans. The enzyme activity of trypsins from Antarctic krill (*Euphausia superba*) (TRY-ES), is forty percent of the entire proteinase (Sjödahl et al. 2002).

It has been confirmed that the catalytic efficiency of enzymes from cold-adapted or psychrophilic microorganism is higher at lower temperature, with the lower optimum temperature values, lower activating energy and higher flexibility (Marx et al. 2007).

In this paper, trypsins (TRY-ES) were purified from *E. superba* and characterized. The effect of temperature and benzamidine on fluorescence spectra of TRY-ES was measured. The aim of this study was to investigate whether the TRY-ES has distinct properties that could lead to new theoretical explanation for crustacean enzymatic biochemistry and commercial production of enzyme preparation.

Materials and Methods

Materials

Samples of Antarctic krill (*Euphausia superba*) were obtained from South Orkney Islands (60°30′ S, 44°25′W) and stored at −80 °C.

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Purification of Trypsins

Cephalothoraxes of the krill were homogenized in chilled Milli-Q water (1: 2, w/v) for 5 min. The mixture was centrifuged (15000 g, 20 min, 4 °C) twice and the supernatant was collected as the crude extract (CE). Solid ammonium sulfate was then added to the crude extract, and the fraction precipitating between 20 % and 70 % saturation was collected. The sediment was dissolved in chilled Milli-Q water and desalted using Amicon Ultra-4 (Merck Millipore, Germany). The insoluble material was removed after centrifugation (15000 g, 20 min, 4 °C) and then the supernatant was pooled and applied to a DEAE Sepharose Fast Flow column (1.6 × 20 cm) (GE Healthcare, USA). Three active fractions were pooled and applied to a Sephacryl S100 HR column (1.6 × 70 cm) (GE Healthcare, USA) respectively. Active fractions were pooled, concentrated and desalted. The purity and the relative molecular mass of the purified enzymes were determined by SDS-PAGE. The protein bands were stained with Coomassie Blue G-250 (Candiano et al. 2004).

Protein Determination

The total soluble protein was determined by the Bradford method using bovine serum albumin as standard.

Enzyme Assays

The activity of trypsins was evaluated using *N*-α-benzoyl-L-arginine-*p*-nitroanilide (BA*p*NA) (Sigma-Aldrich, USA) as substrate, with phosphate buffers (0.05 mol L^{-1}, pH 8.0) (Erlanger et al. 1961). One BApNA Unit (U) of activity was defined as the amount of trypsin that release 1 μmol *p*-nitroanalide (ε = 8800 mol L^{-1} cm^{-1}) per min at 35 °C, pH 8.0.

Enzymatic Characteristics

The effect of metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+} and Cu^{2+}) and inhibitors, such as phenyl methyl sulfonyl fluoride (PMSF), benzamidine, soybean trypsin inhibitor (STI), chicken egg ovomucoid (CEOM), N-α-tosyl-L-lysine chloromethyl ketone (TLCK) and N-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (Sigma-Aldrich, USA) on the activity of the TRY-ES and bovine trypsin from pancreas of *Bos Taurus* (Sigma-Aldrich, USA) were performed. The temperature optimum (5–65 °C) and stability (5–55 °C), pH optimum (2.0–11.0) and pH stability (2.0–11.0) of the TRY-ES and bovine trypsin were performed.

Enzymatic Kinetics

Michaelis–Menten kinetics was evaluated with 0.05 mol L^{-1} phosphate buffer (pH 8.0) at 35 °C, using six concentrations of BA*p*NA (0-0.3 mmol L^{-1}, dissolved in DMSO). Kinetic constants (K_m) were obtained by least squares fitting of initial velocity data to the Lineweaver–Burk transformation of the Michaelis–Menten equation. The turnover constants (K_{cat}) and catalytic efficiency (K_{cat}/K_m) were calculated.

Effect of Temperature on Fluorescence Spectra of Trypsins

The fluorescence intensities of the TRY-ES (1.0×10^{-5} mol L^{-1}) within phosphate buffer (0.05 mol L^{-1}, pH 8.0) at six temperatures (5, 15, 25, 35, 40 and 45 °C) were measured with a RF-5301PC fluorescence spectrophotometer (Shimadzu, Japan), which was equipped with 1.0 cm quartz cells and a thermostat bath. The excitation wave length (λ_{ex}) was set at 278 and 295 nm and the emission wavelength (λ_{em}) was scanned from 300 to 400 nm. Both the widths of the excitation slit and the emission slit were set to 5.0 nm. Phosphate buffer (0.05 mol L^{-1}, pH 8.0) was used as blanks subtracted to correct background of fluorescence.

Effect of Benzamidine on Fluorescence Spectra of Trypsins

Benzamidine (6.0×10^{-4} mol L^{-1}) were mixed with the TRY-ES (final concentration, 1.0×10^{-5} mol L^{-1}) and phosphate buffer (0.05 mol L^{-1}, pH 8.0) to final concentration of 0, 0.4, 0.8, 1.2, 1.6, 2.0 and 2.4×10^{-4} mol L^{-1}. The fluorescence intensities of mixture solutions at three temperatures (278 K, 288 K and 298 K) were measured with a RF-5301PC fluorescence spectrophotometer (Shimadzu, Japan), which was equipped with 1.0 cm quartz cells and a thermostat bath. The excitation wave length (λ_{ex}) was set at 278 and the emission wavelength (λ_{em}) was scanned from 290 to 450 nm. Both the widths of the excitation slit and the emission slit were set to 5.0 nm with scanning speed at 500 nm min^{-1}. Appropriate blanks corresponding to the buffer were subtracted to correct background of fluorescence.

Data Analysis

All assays were carried out in quadruplicate, and variance analysis (one way ANOVA) and F test ($P < 0.05$) for enzymatic characteristics was performed by SPSS 13.

Results and Discussion

Purification of Trypsins

Three trypsins (Trypsin I, Trypsin II and Trypsin III) were purified from *E. superba*. Isotrypsins have been found from

Table 1 Purification steps of *Euphausia superba* trypsins

Purification step	Total activity (U)	Yield (%)	Protein (mg)	Recovery (%)	Specific activity (U mg^{-1})	Purification (-fold)
CE	3000	100.0	41.3	100.0	72.7	1.0
FP	2762	92.1	28.3	68.7	97.4	1.3
DEAE I	372.7	12.4	1.14	2.8	325.6	4.5
DEAE II	603.4	20.1	0.964	2.3	625.9	8.6
DEAE III	320.5	10.7	0.486	1.2	658.8	9.1
Trypsin I	268.2	8.9	0.659	1.6	406.8	5.6
Trypsin II	443.2	14.8	0.356	0.9	1243.5	17.1
Trypsin III	318.2	10.6	0.317	0.8	1004.3	13.8

Solid ammonium sulfate was added to the crude extract (CE). The dissolved, desalted and centrifuged fraction precipitating (FP) was then applied to a DEAE Sepharose FF column (1.6 × 20 cm). Three active fractions (DEAE I, DEAE II, DEAE III) were pooled and applied to a Sephacryl S100 HR column (1.6 × 70 cm) respectively. Active fractions (Trypsin I, Trypsin II and Trypsin III) were pooled, concentrated and desalted

several other crustaceans including North Pacific krill (*Euphausia pacifica*) (2) (Wu et al. 2008), Red swamp crawfish (*Procambarus clarkii*) (4) (Jeong et al. 2000), Whiteleg shrimp (*Litopenaeus vannamei*) (3) (Sainz et al. 2004), Caribbean spiny lobster (*Panulirus argus*) (5) (Perera et al. 2012), and fish including Atlantic cod (*Gadus morhua*) (3) (Asgeirsson et al. 1989), Atlantic salmon (*Salmo salar*) (4) (Outzen et al. 1996), Japanese anchovy (*Engraulis japonica*) (2) (Ahsan and Watabe 2001), Yellowfin tuna (*Thunnus albacores*) (2) (Klomklao et al. 2006), Skipjack tuna (*Katsuwonus pelamis*) (3) (Klomklao et al. 2007), Chum salmon (*Oncorhynchus keta*) (3) (Toyota et al. 2007) and Round sardinella (*Sardinella aurita*) (3) (Ben Khaled et al. 2011).

The specific activity and the purification fold of Trypsin II and Trypsin III was 3.1 and 2.5 times higher than those of Trypsin I, the total activity and activity recovery of Trypsin II and Trypsin III was 1.7 and 1.2 times higher than those of Trypsin I, while the protein yield of Trypsin I was about 1.8 and 2 times higher than that of Trypsin II and Trypsin III (Table 1).

The relative molecular mass of the TRY-ES was approximately 28.7 kDa, 28.8 kDa and 29.2 kDa (Fig. 1), which were similar to trypsins from other crustaceans, including *L. vannamei* (30.2-32.9 kDa) (Sainz et al. 2004) and *E. pacifica* (32.3-33 kDa) (Wu et al. 2008). The relative molecular mass of crustacean trypsins were higher than those of vertebrate trypsins (24.0 kDa) (Asgeirsson et al. 1989; Ahsan and Watabe 2001; Klomklao et al. 2006, 2007; Toyota et al. 2007; Ben Khaled et al. 2011). Variation of relative molecular mass among isotrypsins from the same species is related to glycosylation of trypsins (Sainz et al. 2004).

Enzymatic Characteristics

The effects of metal ions and inhibitors on the activity of trypsins were determined and the results were showed in

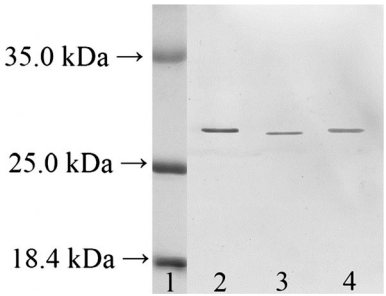

Fig. 1 SDS-PAGE of *Euphausia superba* Trypsins. SDS-PAGE was performed in a 15 % (w/v) gel stained with Coomassie Blue G-250. *Lane 1* molecular mass standard; *Lane 2* Trypsin I; *Lane 3* Trypsin II; *Lane 4* Trypsin III

Table 2. With 0.5 mM of Ca^{2+}, the promotion rate of the bovine trypsin activity (141.7 %) was greater than that of Trypsin I (127.4 %) and Trypsin II (133.2 %) activity, and the promotion rate of Trypsin III activity (113.7 %) was the least ($P < 0.01$). While with 0.5 mM of Mg^{2+}, the promotion rate of the TRY-ES activity (143.3, 129.7 and 119.7 % respectively) was higher than that of the bovine trypsin activity (117.4 %) ($P < 0.05$). With 1 mM (85.6, 88.9 and 91.3 % respectively) and 5 mM (91.7, 83.1 and 98.5 % respectively) of Ca^{2+}, the TRY-ES activity was inhibited, while that of the bovine trypsin was promoted (151.7 and 132.6 % respectively) ($P < 0.05$). With 1 mM (81.1, 84.0 and 87.1 % respectively) and 5 mM (85.9, 89.2 and 88.5 % respectively) of Mg^{2+}, the TRY-ES activity was also inhibited, while that of the bovine trypsin was promoted (115.9 and 112.4 % respectively) ($P < 0.05$).

There is a binding site with higher affinity for calcium iron in bovine trypsin. In the presence of Ca^{2+}, calcium ions bind to the aspartic acid and glutamic acid residues at the specific trypsin sites, and trypsins undergo a conformational change giving stability and activation to the

Table 2 Influence of metal ions and inhibitors on the activity of *Euphausia superba* trypsins and bovine trypsin (Mean ± SD, $n = 4$)

Inhibitor & activator	Incubating Concentration	Trypsin I Activity (%)	Trypsin II Activity (%)	Trypsin III Activity (%)	Bovine Activity (%)
Ca^{2+}	0.5 mM	127.4 ± 1.4	133.2 ± 5.5	113.7 ± 2.6	141.7 ± 6.0
	1.0 mM	85.6 ± 0.3	88.9 ± 1.7	91.3 ± 0.9	151.7 ± 7.6
	5.0 mM	91.7 ± 0.7	83.1 ± 2.7	98.5 ± 0.3	132.6 ± 3.5
Mg^{2+}	0.5 mM	143.3 ± 1.4	129.7 ± 4.9	119.7 ± 3.9	117.4 ± 3.4
	1.0 mM	81.1 ± 2.6	84.0 ± 1.6	87.1 ± 2.2	115.9 ± 8.5
	5.0 mM	85.9 ± 1.8	89.2 ± 2.7	88.5 ± 0.9	112.4 ± 4.2
Zn^{2+}	0.5 mM	77.2 ± 2.6	75.2 ± 1.9	62.4 ± 2.5	76.5 ± 2.4
	1.0 mM	76.8 ± 5.1	77.1 ± 3.3	50.1 ± 1.8	69.3 ± 2.3
	5.0 mM	8.3 ± 2.9	11.5 ± 2.2	15.0 ± 0.4	25.6 ± 1.4
Cu^{2+}	0.5 mM	85.1 ± 4.2	82.1 ± 4.4	80.1 ± 4.4	72.2 ± 3.9
	1.0 mM	58.3 ± 3.8	75.2 ± 0.4	82.9 ± 4.2	67.2 ± 3.4
	5.0 mM	22.3 ± 1.6	29.7 ± 1.9	16.0 ± 3.8	12.9 ± 2.7
PMSF	1 mM	76.4 ± 2.7	64.3 ± 3.8	88.2 ± 3.2	20.0 ± 0.0
STI	10 μg ml^{-1}	10.2 ± 1.6	7.9 ± 2.1	11.8 ± 2.9	57.9 ± 8.4
CEOM	10 μg ml^{-1}	20.0 ± 2.8	2.6 ± 0.9	13.2 ± 3.4	17.8 ± 1.9
Benzamidine	1 mM	12.1 ± 2.1	10.2 ± 1.8	11.3 ± 1.8	1.8 ± 0.3
TLCK	1 mM	9.5 ± 1.2	7.0 ± 1.7	11.0 ± 0.9	12.9 ± 1.3
TPCK	1 mM	98.7 ± 1.7	98.2 ± 0.3	99.3 ± 0.7	96.8 ± 3.4

Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+} were selected for the metal ion assays. The general serine protease inhibitor, phenyl methyl sulfonyl fluoride (PMSF), the specific trypsin inhibitors, benzamidine, chicken egg ovomucoid (CEOM), soybean trypsin inhibitor (STI) and N^{α}-tosyl-L-lysine chloromethyl ketone (TLCK) and the specific chymotrypsin inhibitor, N^{α}-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were selected for the inhibitor assays. Prior to assays, 25 μl of trypsin sample was incubated with 25 μl of metal ions or inhibitors in 800 μl of the buffer solutions for 30 min at 35 °C, pH 8.0. Relative activity is expressed as a percentage of the absolute activity of the control

enzymes (Rypniewski et al. 1994). With 0.5 mM of Ca^{2+}, the promotion rate of the TRY-ES activity was less than that of the bovine trypsin, while with 0.5 mM of Mg^{2+}, the promotion rate of the TRY-ES activity was great than that of the bovine trypsin activity. Further research is needed to illustrate following issues, such as whether there is calcium iron binding sites in invertebrate trypsins, for example the TRY-ES, whether there is magnesium iron binding sites in trypsins, whether the binding sites for calcium were the same for the magnesium and whether the difference in promoting or inhibition rate was due to the difference in binding site affinity for calcium iron or magnesium iron.

With 5.0 mM of Zn^{2+}, the TRY-ES (8.3, 11.5 and 15.0 %) and bovine trypsin (25.6 %) activity was less than those with 1 mM of Zn^{2+} (76.8, 77.1 and 50.1 %, and 69.3 %) ($P < 0.01$). With 5.0 mM of Cu^{2+}, the TRY-ES (22.3, 29.7 and 16.0 %) and bovine trypsin (12.9 %) activity was less than those with 1 mM of Cu^{2+} (58.3, 75.2 and 82.9 %, and 67.2 %) ($P < 0.01$). The inhibition rate of the TRY-ES and bovine trypsin activity was increased with the concentration of Zn^{2+} or Cu^{2+}. Zn^{2+} and Cu^{2+} had been shown to inhibit the activity of *E. pacifica* (Wu et al. 2008) and fish trypsins (Ben Khaled et al. 2011). The inhibition rate of Trypsin I (91.7 %), Trypsin II (88.5 %), Trypsin III (85.0 %) activity with 5 mM of Zn^{2+} was higher than that of Trypsin I (77.7 %), Trypsin II (70.3 %), Trypsin III (84.0 %) activity with 5 mM of Cu^{2+} respectively. While the inhibition rate of the bovine trypsin activity (74.4 %) with 5 mM of Zn^{2+} was less than that with 5 mM of Cu^{2+} (87.1 %) ($P < 0.01$).

The TRY-ES activity was slightly inhibitedby the general serine protease inhibitor, PMSF (23.6, 35.7 and 11.8 % respectively), but with which, 80.0 % of the bovine trypsin activity was inhibited. The TRY-ES activity was strongly inhibited by the specific trypsin inhibitors, such as benzamidine (87.9–89.8 %), CEOM (80.0–97.4 %), SBTI (88.2–92.1 %) and TLCK (89.0–93.0 %). The TRY-ES and bovine trypsin activity were not obviously inhibited by the specific chymotrypsin inhibitors, TPCK (Table 2). PMSF had shown inhibition to trypsins and other serine proteinases (Asgeirsson et al. 1989; Outzen et al. 1996; Jeong et al. 2000; Ahsan and Watabe 2001; Sainz et al. 2004; Wu et al. 2008; Perera et al. 2012). SBTI (Asgeirsson et al. 1989; Jeong et al. 2000; Ahsan and Watabe 2001; Sainz et al. 2004; Wu et al. 2008; Perera et al. 2012), CEOM (Asgeirsson et al. 1989; Wu et al. 2008), Benzamidine (Asgeirsson et al. 1989; Outzen et al. 1996; Ahsan and Watabe 2001; Wu et al. 2008; Perera et al. 2012) and TLCK (Asgeirsson et al. 1989; Jeong et al. 2000; Ahsan and Watabe 2001; Sainz et al. 2004; Wu et al. 2008;

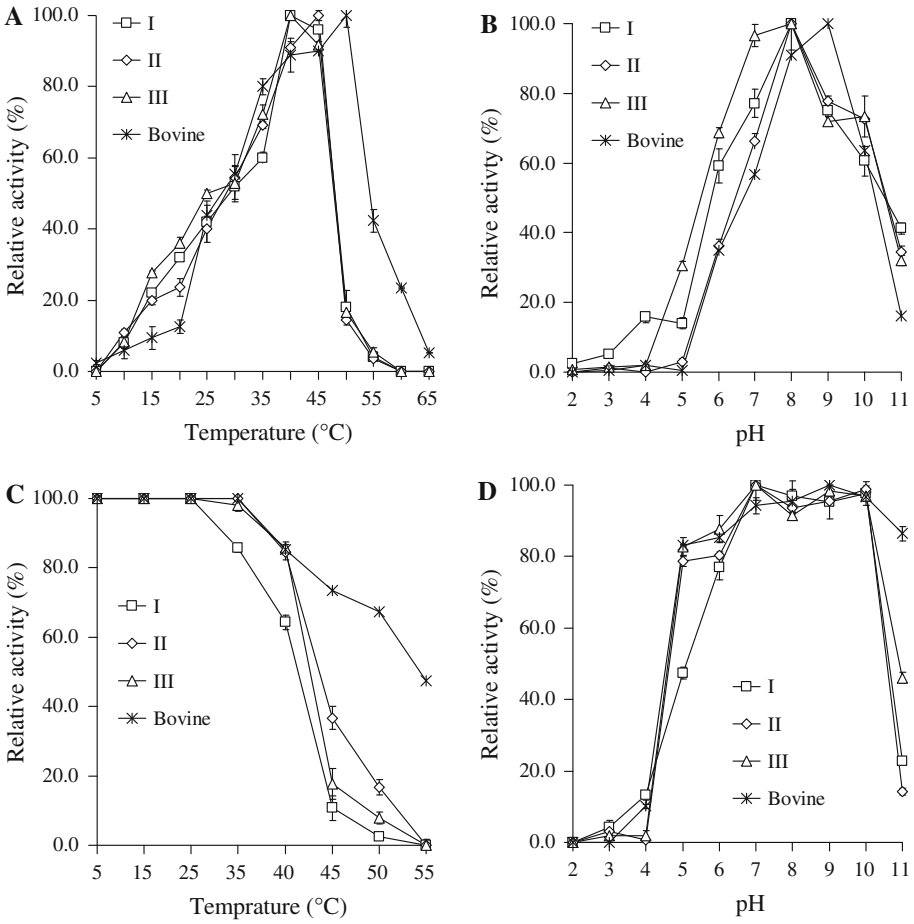

Fig. 2 Effect of temperature and pH on the activity (**A**, **B**) and the stability (**C**, **D**) of the TRY-ES and bovine trypsin (Mean ± SD, $n = 4$). **A** Effect of temperature on the activities were determined at pH 8.0 and at various temperatures (5–65 °C). **B** Effect of pH on the activities were determined at 35 °C and at various pH (2.0–11.0). **C** Effect of temperature on the stability, the TRY-ES and bovine trypsin were stored at pH 8.0 and various temperatures (5–55 °C) for 2 h, the activities were determined at 25 °C and pH 8.0. **D** Effect of pH on the stability, the TRY-ES and bovine trypsin were stored at 25 °C and various pH (2.0–11.0) for 2 h, the activities were determined at 25 °C and pH 8.0. Relative activity is expressed as a percentage of the initial absolute activity. Significant differences ($P < 0.05$) were observed between Trypsin I, Trypsin II, Trypsin III and bovine trypsin between 35 and 55 °C

Perera et al. 2012) had been shown to inhibit crustacean, fish and higher vertebrate trypsins. According to proteinase inhibitors assays, the TRY-ES showed properties of serine proteinases and trypsins.

The effects of various temperatures and pH on the activity and stability of the TRY-ES and bovine trypsin were shown in Fig. 2. The TRY-ES was active from 10 to 50 °C, while bovine trypsin was active from 10 to 65 °C (Fig. 2A). The optimum temperature of the TRY-ES was at 40 °C (Trypsin I), 45 °C (Trypsin II), 40 °C (Trypsin III) respectively, which was lower than that of the bovine trypsin (55 °C) (Fig. 2A). The optimum temperature of the TRY-ES was lower than those of trypsins from Frigid Zone crustacean, Giant Antarctic slater (*Glyptonotus antarticus*) (49 °C) (Dittrich 1992), Frigid-Temperate Zone crustaceans, such as Common hermit crab (*Pagurus bernhardus*) (48 °C) (Dittrich 1992) and *E. pacifica* (50 °C) (Wu et al. 2008), and Frigid Zone fish, such as *G. morhua* (50 °C) (Asgeirsson et al. 1989), *S. Salar* (50 °C) (Outzen et al. 1996), Arabesque greenling (*Pleuroprammus azonus*) (50 °C) (Kishimura et al. 2006a), Brown hakeling (*Physiculus japonicus*) (50 °C) (Kishimura et al. 2006b), Walleye pollock (*Theragra chalcogramma*) (50 °C) (Kishimura et al. 2008) and Pacific cod (*Gadus macrocephalus*) (50 °C) (Fuchise et al. 2009). The optimum temperature of trypsins from the Frigid Zone organisms were lower than those of trypsins from the Temperate Zone organisms, such as *P. clarkii* (60 °C) (Jeong et al. 2000), *L. vannamei* (60 °C) (Sainz et al. 2004), *P. argus* (60 °C) (Perera et al. 2012), *E. japonicus* (60 °C) (Ahsan and Watabe 2001), *S. aurita* (55 °C) (Ben Khaled et al. 2011) and Jacopever (*Sebastes schlegelii*) (60 °C) (Kishimura et al. 2007), and the Tropical Zone organisms, such as *T. albacores* (60 °C) (Klomklao et al. 2006), *K. pelamis* (60 °C) (Klomklao et al. 2007) and Pacific sierra (*Scomberomorus Sierra*) (60 °C) (Valdez-Melchor et al. 2012).

The TRY-ES was active over pH 4.0–11.0 (Trypsin I) or 5.0–11.0 (Trypsin II and Trypsin III), while the bovine trypsin was active over 6.0–11.0 (Fig. 2B). The optimum pH of the TRY-ES was at pH 8.0 (Fig. 2B), which was similar to those of crustacean trypsins (Jeong et al. 2000; Sainz et al. 2004; Wu et al. 2008; Perera et al. 2012), fish trypsins (Asgeirsson et al. 1989; Outzen et al. 1996; Ahsan and Watabe 2001; Kishimura et al. 2006a, 2006b, 2007, 2008; Klomklao et al. 2006, 2007; Fuchise et al. 2009) and porcine pancreatic trypsins (Kishimura et al. 2007). The

optimum pH of the bovine trypsin was at pH 9.0, which was similar to those of *S. aurita* trypsins (Ben Khaled et al. 2011) (Fig. 2B).

The TRY-ES and bovine trypsin remained relative stable after 2 h incubation at 5, 15, 25 and 35 °C, with 85.7 % (Trypsin I), 100.0 % (Trypsin II), 98.2 % (Trypsin III) and 100.0 % (Bovine trypsin) activity at 35 °C respectively (Fig. 2C). The TRY-ES activity was lost rapidly over 40 °C, with only 10.7 % (Trypsin I), 36.7 % (Trypsin II) and 17.7 % (Trypsin III) activity at 45 °C, while that of the bovine trypsin was 73.5 % at 45 °C. The TRY-ES activity was totally lost at 55 °C, but that of bovine trypsin remains 47.5 % at 55 °C (Fig. 2C). The thermal stability of Trypsin II was relatively higher than that of Trypsin III and Trypsin I, which was similar to those of trypsins from Frigid Zone organisms (Wu et al. 2008; Asgeirsson et al. 1989; Outzen et al. 1996; Kishimura et al. 2006a, 2006b, 2008; Fuchise et al. 2009). The stability of trypsins from the Frigid Zone organisms were relatively lower than those from the Temperate Zone organisms (Jeong et al. 2000; Sainz et al. 2004; Ahsan and Watabe 2001; Kishimura et al. 2007; Ben Khaled et al. 2011) and the Tropical Zone organisms (Perera et al. 2012; Klomklao et al. 2006, 2007; Valdez-Melchor et al. 2012).

The TRY-ES in the crude extract can be recovered from crude extracts stored at 37 °C for up to 20 days (Wu et al. 2008). The inactivation of the pure enzymes is probable as a result of the absence of other stabilizing proteins and peptides naturally presented in the crude extract.

For pH stability, the TRY-ES was stable between pH 5.0 and 10.0, which was unstable at below pH 5.0 and above pH 10.0 (Fig. 2D). The bovine trypsin was relatively stable between pH 5.0 and 11.0. The pH stability property of the TRY-ES was similar to those of crustacean trypsins (Jeong et al. 2000; Sainz et al. 2004; Wu et al. 2008; Perera et al. 2012) and fish trypsins (Asgeirsson et al. 1989; Outzen et al. 1996; Ahsan and Watabe 2001; Kishimura et al. 2006a, 2006b, 2007, 2008; Klomklao et al. 2006, 2007; Fuchise et al. 2009; Ben Khaled et al. 2011; Valdez-Melchor et al. 2012).

Enzymatic kinetics

With the use of BApNA as substrate at pH 8.0 and 35 °C, the kinetic parameters of the TRY-ES were determined (Table 3). The K_m value of the TRY-ES was 0.244 (Trypsin I), 0.995 (Trypsin II) and 0.306 mM (Trypsin III) respectively, the K_{cat} value of the TRY-ES was 3.48 (Trypsin I), 9.41 (Trypsin II) and 1.82 s^{-1} (Trypsin III) respectively, and the K_{cat}/K_m value of the TRY-ES is 14.28 (Trypsin I), 9.46 (Trypsin II) and 5.93 $mM^{-1}s^{-1}$ (Trypsin III) respectively.

Compared with other crustacean trypsins at 35 °C and pH8.0, the K_m values of the TRY-ES were higher than those of *E. pacifica* (0.088 and 0.064 mM), *T. orientalis* (0.093 mM), *P. argus* (0.08–0.19 mM), but the K_{cat} values were higher and the K_{cat}/K_m values were 1.2 to 7.1 times higher than those of crustacean trypsins from *E. pacifica* (9.54 $mM^{-1}s^{-1}$), *T. orientalis* (9.78 $mM^{-1}s^{-1}$), *P. argus* (2.02–6.04 $mM^{-1}s^{-1}$).

The K_m values of Trypsin I (0.24 mM) and Trypsin III (0.31 mM) were less than that of bovine (0.44 mM) and porcine (2.07 mM) trypsin. The K_{cat}/K_m values of the TRY-ES were 2.6, 1.7 and 1.1 times higher than that of bovine trypsin respectively, and 10.2, 6.76 and 4.24 times higher than that of porcine trypsin respectively (Ahsan and Watabe 2001; Dallas et al. 2002; Toyota et al. 2007; Wu et al. 2008; Perera et al. 2012).

The K_{cat}/K_m values of trypsins from Fridge Zone fish, such as *G. morhua* Enzyme I (51.9 $mM^{-1}s^{-1}$) and *O. keta* ST-1 (135 $mM^{-1}s^{-1}$), ST-2 (139 $mM^{-1}s^{-1}$) and ST-3 (110 $mM^{-1}s^{-1}$), were higher than those of Temperate Zone fish trypsins, such as *O. mykiss* trypsin (42.6 $mM^{-1}s^{-1}$), *E. Japonicus* aT-I (52.4 $mM^{-1}s^{-1}$) and aT-II (112.1 $mM^{-1}s^{-1}$), and *S. aurita* trypsin A (17.92 $mM^{-1}s^{-1}$), trypsin B (14.61 $mM^{-1}s^{-1}$), trypsin C (28.8 $mM^{-1}s^{-1}$), and were distinctly higher than those of crustacean trypsins (2.03–15.3 $mM^{-1}s^{-1}$) and mammal trypsins (1.40–5.52 $mM^{-1}s^{-1}$).

Effect of Temperature on Fluorescence Spectra of Trypsins

Within protein, the ratio of fluorescence emission intensity by phenylalanine, tyrosine and tryptophan is 0.9:9:100, with intensity peak at about 282 nm, 303 nm and 348 nm respectively. With excitation at 278 nm, both tyrosine and tryptophan excite fluorescence, but only tryptophan excites fluorescence when excited at 295 nm (Lakowicz 2006).

According the NCBI Bank, most trypsins have at least four conserved tryptophans (Trp_{34}, Trp_{121}, Trp_{193} and Trp_{215}, bovine trypsin as example) and ten tyrosine residues (Tyr_5, Tyr_{14}, Tyr_{22}, Tyr_{42}, Tyr_{76}, Tyr_{131}, Tyr_{152}, Tyr_{195}, Tyr_{206}, Tyr_{212}, bovine trypsin as example) that can be used as intrinsic fluorophores.

The result of effect of temperature on the TRY-ES fluorescence spectra were shown in Fig. 3. With excitation both at 278 nm and 295 nm, the fluorescence intensity peaks of the TRY-ES were all at 340–357 nm, indicating that the fluorescence chromophores were tryptophans. But the fluorescence intensity of the TRY-ES with excitation at 278 nm (Fig. 3A, C, E) was all higher than that at 295 nm (Fig. 3B, D, F), meaning that there were two kinds of fluorescence chromophores, tryptophan and tyrosine. Hence, there is energy transfer from tyrosine to tryptophan

Table 3 Comparison of kinetic parameter composition of trypsins from different animals

Habitat	Organism	Trypsin	pH	T (°C)	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($mM^{-1}s^{-1}$)	Reference
Frigid Zone	*E. superba*	Trypsin I	8.0	35	0.24	3.48	14.28	This paper
		Trypsin II	8.0	35	0.99	9.41	9.46	
		Trypsin III	8.0	35	0.31	1.82	5.93	
Frigid-Temperate	*E. pacifica*	TRY I	8.0	35	0.088	0.84	9.54	Wu et al. (2008)
		TRY II	8.0	35	0.064	0.98	15.3	
Tropical Zone	*P. argus*	PaT1	8.0	37	0.12	0.57	4.82	Perera et al. (2012)
		PaT2	8.0	37	0.12	0.62	5.02	
		PaT3	8.0	37	0.11	0.57	4.98	
		PaT4	8.0	37	0.08	0.16	2.02	
		PaT5	8.0	37	0.19	1.15	6.04	
Frigid Zone	*G. morhua*	Enzyme I	8.1	25	0.077	4.0	51.9	Asgeirsson et al. (1989)
		Enzyme II	8.1	25	0.094	1.9	20.2	
		Enzyme III	8.1	25	0.102	0.7	6.8	
	O. keta	ST-1	8.0	25	0.030	4.06	135	Toyota et al. (2007)
		ST-2	8.0	25	0.040	5.54	139	
		ST-3	8.0	25	0.020	2.00	100	
	O. keta	ST-1	8.0	35	0.070	7.70	110	
		ST-2	8.0	35	0.050	6.74	135	
		ST-3	8.0	35	0.040	3.86	96.5	
Temperate Zone	*E. japonicus*	aT-I	8.0	25	0.037	1.94	52.4	Ahsan and Watabe (2001)
		aT-II	8.0	25	0.033	3.70	112.1	
	S. aurita	Trypsin A	9.0	25	0.125	2.24	17.92	Ben Khaled et al. (2011)
		Trypsin B	9.0	25	0.083	1.21	14.61	
		Trypsin C	9.0	25	0.10	5.76	28.8	
	Bos taurus	Bovine trypsin	8.0	25	0.26	0.94	3.62	Toyota et al. (2007)
		Bovine trypsin	8.0	35	0.44	2.43	5.52	
	Sus scrofa	Porcine trypsin	8.0	25	1.72	1.78	1.05	Dallas Johnson et al. (2002)
		Porcine trypsin	8.0	35	2.07	2.89	1.40	

with excitation at 278 nm (Borkman and Phillips 1985; Lakowicz 2006).

With excitation both at 278 nm and 295 nm, the fluorescence intensity of the TRY-ES was declined with the increasing temperature, indicating the quenching function of temperature on TRY-ES fluorescence intensity. Except that the fluorescence intensity of Trypsin I (Fig. 3A) and Trypsin III (Fig. 3E) at 15 °C was a little higher than that at 5 °C, with no significant difference.

The fluorescence peak of Trypsin I was slightly red shifted from 346 nm (5, 15, 25 and 35 °C) to 348 nm (40 and 45 °C) with excitation at 278 nm (Fig. 3A), which was red shifted from 348 nm (5, 15, 25 and 35 °C) to 357 nm (40 and 45 °C) with excitation at 295 nm (Fig. 3B), indicating the increase of microenvironment polarity of tryptophan, increase of Trypsin I flexibility and decrease in Trypsin I thermal stability at 40 and 45 °C. The optimum temperature of Trypsin I was at 40 °C and the thermal stability of Trypsin I lost rapidly over 40 °C, which might be illustrated with the red shift of the fluorescence peak of Trypsin I at 295 nm.

The fluorescence peak of Trypsin II was blue shifted from 345 nm (5 °C) to 340 nm (40 °C) gradually with excitation at 278 nm and from 347 nm (5, 15, 25 and 35 °C) to 344 nm (40 °C) with excitation at 295 nm respectively, indicating the decrease in microenvironment polarity of tryptophans and Trypsin II flexibility, yet increase of Trypsin II stability. Then the fluorescence peak of Trypsin II was red shift to 345 nm (45 °C) (λ_{ex} = 278 nm) (Fig. 3C) and 348 nm (45 °C) (λ_{ex} = 295 nm) (Fig. 3D), indicating the increase of microenvironment polarity of tryptophans and Trypsin II flexibility, yet decrease in Trypsin II stability. The optimum temperature of Trypsin II was at 45 °C and the thermal stability of Trypsin II was higher than that of Trypsin I at 40 °C and lost rapidly over 45 °C, which might be illustrated with the

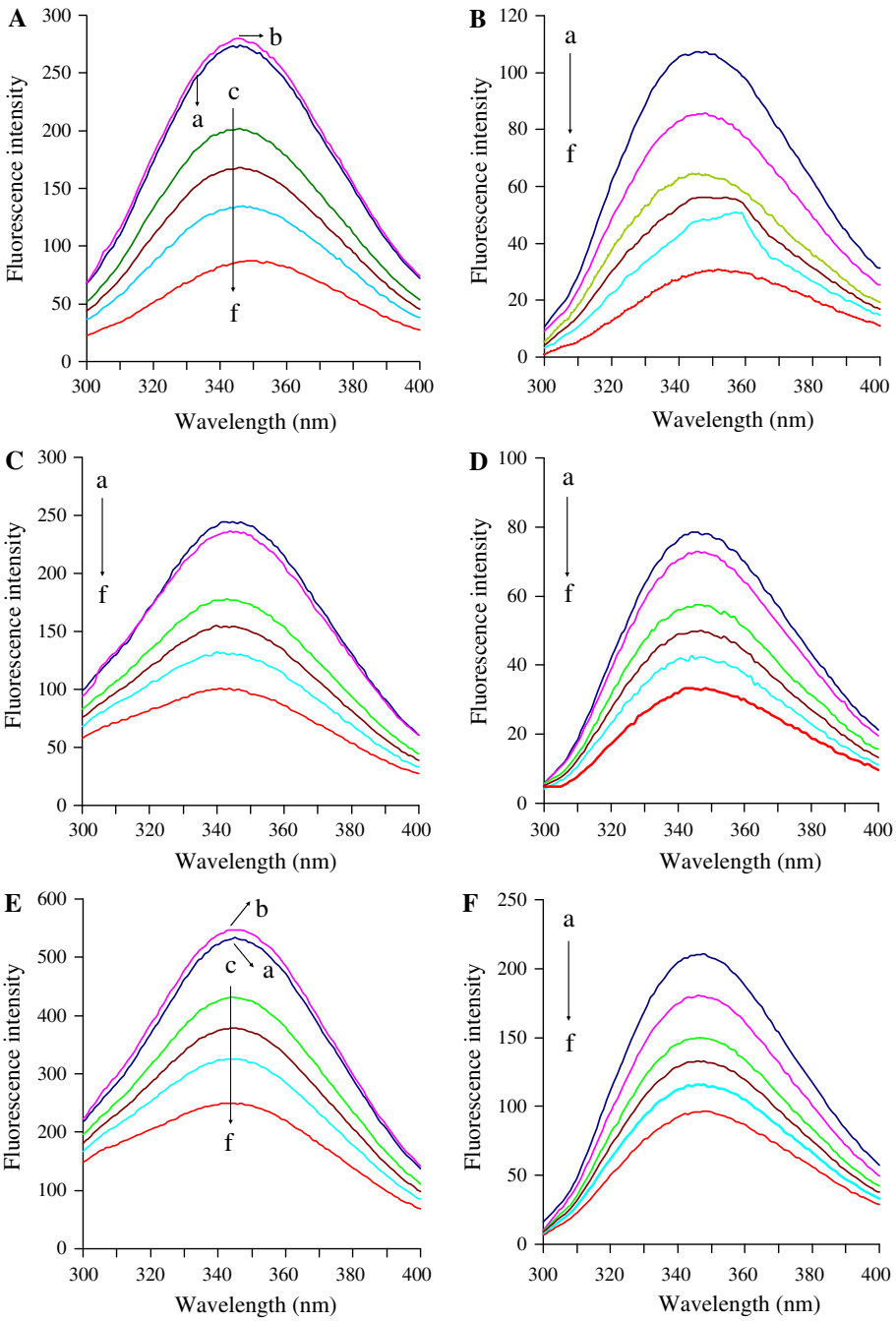

Fig. 3 Effect of temperature on fluorescence spectra of Trypsin I (**A, B**), Trypsin II (**C, D**) and Trypsin III (**E, F**). **A**, **C**, **E** $\lambda_{ex} = 278$ nm; **B**, **D**, **F** $\lambda_{ex} = 295$ nm; a–f: 5, 15, 25, 35, 40 and 45 °C

blue shift of the fluorescence peak of Trypsin II at 40 °C, and then red shift at 45 °C.

The fluorescence peak of Trypsin III was slightly blue shifted from 345 nm (5, 15, 25 and 35 °C) to 343 nm (40 °C) ($\lambda_{ex} = 278$ nm) and from 348 nm (5, 15, 25 and 35 °C) to 346 nm (40 °C) ($\lambda_{ex} = 295$ nm) respectively, which was then red shifted to 345 nm (45 °C) ($\lambda_{ex} = 278$ nm) and 349 nm (45 °C) ($\lambda_{ex} = 295$ nm) (Figs. 3E, 4F). The optimum temperature of Trypsin III was at 40 °C and the thermal stability of Trypsin III was higher than that of Trypsin I at 40 °C and lost rapidly over 45 °C, which might be illustrated with the blue shift of the fluorescence peak of Trypsin III at 40 °C, and then red shift at 45 °C.

It is reported that, with λ_{ex} at 295 nm, the change in emission wavelength (λ_{em}) at 330–332 indicated that trytophan resides are located in the nonpolar region and buried in a hydrophobic cavity in proteins; λ_{em} at 350–353 nm shows that tryptophan residues are exposed to water, on the surface of proteins; λ_{em} at 340–342 nm shows that tryptophan residues is in limited contact with water which is probably immobilized

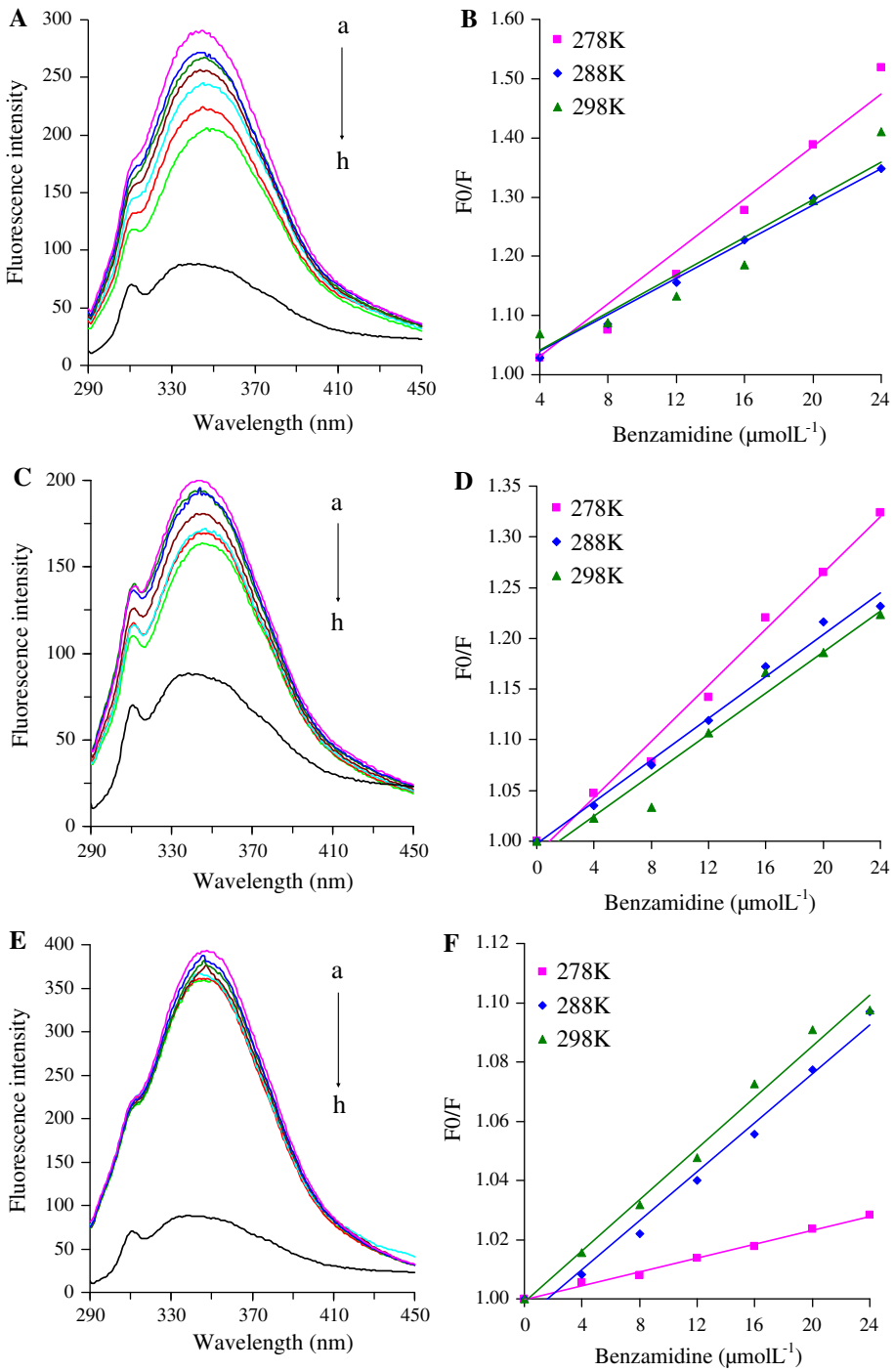

Fig. 4 Effect of benzamidine on fluorescence spectra of Trypsin I (**A, B**), Trypsin II (**C, D**) and Trypsin III (**E, F**). **A**, **C**, **E** fluorescence intensity, $\lambda_{ex} = 278$ nm; a–g: $C_{trypsin} = 1.0 \times 10^{-5}$mol L^{-1}, $C_{benzamidine} = 0.0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4 \times 10^{-5}$mol L^{-1}, h: Benzamidine only, $C_{benzamidine} = 1.2 \times 10^{-5}$mol L^{-1}, the fluorescence intensity of a–g was modified with h; **B**, **D**, **F** Stern–Volmer plots, $\lambda_{ex} = 278$ nm, $C_{trypsin} = 1.0 \times 10^{-5}$mol L^{-1}

by bonding at the macromolecular surface (Burstein et al. 1973). The fluorescence peak of Trypsin I (348 nm), Trypsin II (347, 344 and 348 nm) and Trypsin III (348, 346 and 349 nm) at different temperatures indicated that the tryptophan residues in TRY-ES were in limited contact with water, except that at 40 and 45 °C, the tryptophan residues in Trypsin I ($\lambda_{em} = 357$ nm) was exposed to water totally.

Effect of Benzamidine on Fluorescence Spectra of Trypsins

The effect of benzamidine on fluorescence spectra of the TRY-ES were shown in Fig. 4. The fluorescence intensity of the TRY-ES was quenched by benzamidine and decreased gradually with increasing benzamidine content,

Table 4 Stern-Volmer quenching constants, binding parameters and thermodynamics parameters for the Benzamidine-trypsin system

	T (K)	K_{sv} (L mol^{-1})	K_q (L mol^{-1} S^{-1})	R^2_{sv}	K_a (L mol^{-1})	n	R^2_a	$\triangle Go$ (kJ mol^{-1})	$\triangle Ho$ (kJ mol^{-1})	ΔS^o (JK^{-1}mol^{-1})
Trypsin I	278	2.25×10^4	2.55×10^{12}	0.9916	2.27×10^7	1.65	0.9992	−39.153	−194.698	−559.515
	288	1.54×10^4	1.54×10^{12}	0.9910	1.22×10^6	1.40	0.9975	−33.558	−35.819	−7.853
	298	1.51×10^4	1.51×10^{12}	0.9960	7.38×10^5	1.36	0.9901	−33.479		
Trypsin II	278	1.38×10^4	1.38×10^{12}	0.9903	4.99×10^4	1.12	0.9968	−25.005	−37.922	−46.463
	288	1.03×10^4	1.03×10^{12}	0.9915	2.82×10^4	1.09	0.9957	−24.540	141.667	577.109
	298	1.00×10^4	1.00×10^{12}	0.9928	2.06×10^5	1.28	0.9908	−30.311		
Trypsin III	278	0.117×10^4	0.117×10^{12}	0.9956	3.83×10^2	0.89	0.9979	−13.754	421.523	1565.748
	288	0.446×10^4	0.446×10^{12}	0.9979	2.16×10^5	1.37	0.9992	−29.410	−230.459	−698.008
	298	0.437×10^4	0.437×10^{12}	0.9903	8.54×10^3	1.06	0.9941	−22.431		

Stern–Volmer equation: $F_0/F = 1 + K_q\tau_0$ [Q] $= 1 + K_{sv}$. F_0 and F: the fluorescence intensity of the TRY-ES solution in the presence and absence of quencher; K_{sv}: the Stern–Volmer quenching constant of the biological macromolecule; K_q: the quenching rate constant of the biological macromolecule; [Q]: the concentration of quencher; τ_0: average lifetime of the biological macromolecules without quencher, usually 10^{-8} s. Double logarithmic regression curve: $\log [(F_0\text{-}F)/F] = \log K_a + n \log$ [Q]; F_0 and F: the fluorescence intensity of TRY-ES solution in the presence and absence of quencher; [Q]: the concentration of quencher. van't Hoff equation: $\ln(K_{a2}/K_{a1}) = \Delta H^o (1/T_1\text{-}1/T_2)/R$; thermodynamic equation: $\Delta G^o = \Delta H^o - T\Delta S^o = -RT\ln K_a$; K_{a1} and K_{a2}: the binding constants at T_1 and T_2 respectively; R: the universal gas constant, 8.3144621 JK^{-1}mol^{-1}

indicating a binding reaction between benzamidine and the TRY-ES (Fig. 4A, C, E).

The fluorescence quenching classifies into static quenching and dynamic quenching. For the static quenching, the quencher and fluorophore form a complex, while the dynamic quenching means the quencher collides with the fluorophore in excited state (Lakowicz 2006). According the Stern–Volmer plots of the TRY-ES at three different temperatures (278 K, 288 K and 298 K), there was a linear relationship between the quenching function of benzamidine and the concentration (Fig. 4B, D, F).

Stern–Volmer Quenching Constants for the Benzamidine-Trypsin System

To distinguish the fluorescence quenching type of benzamidine to the TRY-ES, the Stern–Volmer quenching constant (K_{sv}) and quenching rate constant (K_q) were calculated by Stern–Volmer equation (Mátyus et al. 2006) (Table **4**).

The influence of temperature on static and dynamic quenching is different. In static quenching, a higher temperature leads to the instability of the formed complex and the decrease in the K_{sv} value, while in dynamic quenching, a higher temperature results in larger diffusion coefficients as well as the quenching constant K_{sv} (Khan et al. 2008). As shown in Table 4, the K_{sv} value of Trypsin I and Trypsin II decreased with increasing temperatures, which is in consistent with the characteristic of static quenching. The *K*q value of the TRY-ES at 278 K, 288 K and 298 K were much larger than the maximum scatter collision quenching constant (2.0×10^{10} L mol^{-1} s^{-1}) of various quenchers with the biological macromolecules, indicating the static quenching type of benzamidine to the TRY-ES (Khan et al. 2008). These results suggested that the quenching was static rather than dynamic type and benzamidine and the TRY-ES formed a complex during the binding interaction, which was consistent with the quenching type of bovine trypsin with norfloxacin (Lu et al. 2010), clenbuterol (Chai et al. 2013), benzidine (Zhang and Wang 2010) and sodium benzoate (Mu et al. 2011).

Binding Parameters for the Benzamidine-Trypsin System

To determine the binding parameters when benzamidine, a small molecule and noncompetitive inhibitor to trypsin, bind independently to a set of equivalent sites on the TRY-ES, the binding constant (K_a) and the binding sites number (n) is calculated on the double logarithmic regression curve (Khan et al. 2008), and the results were shown in Table 4.

The K_a values of Trypsin I at 278 K, 288 K and 298 K were 4.81×10^7, 5.49×10^5 and 7.38×10^5 L mol^{-1} respectively, indicating that there was an intensive binding

between benzamidine and trypsin and the binding force was weakened at 288 K and 298 K. The K_a values of Trypsin I were higher than that of bovine trypsin with norfloxacin (Lu et al. 2010), clenbuterol (Chai et al. 2013), benzidine (Zhang and Wang 2010) and sodium benzoate (Mu et al. 2011).

The K_a values of Trypsin II at 278 K, 288 K and 298 K were 4.99×10^4, 2.82×10^4 and 2.06×10^5 L mol^{-1} respectively, indicating that there was an intensive binding between benzamidine and trypsin and the binding force was weakened at 288 K and then enhanced at 298 K. The K_a values of Trypsin II were lower than that of bovine trypsin with norfloxacin (Lu et al. 2010), similar to bovine trypsin with clenbuterol (Chai et al. 2013) and benzidine (Zhang and Wang 2010) and higher than that of bovine trypsin with sodium benzoate (Mu et al. 2011).

The K_a values of Trypsin III at 278 K, 288 K and 298 K were 3.83×10^2, 2.16×10^5 and 8.54×10^3 L mol^{-1} respectively, indicating that there was a relative slight binding between benzamidine and trypsin at 278 K, the binding force significantly enhanced at 288 K, then rapidly weakened at 298 K. The K_a value of Trypsin III at 288 K was higher than that of bovine trypsin with norfloxacin (Lu et al. 2010), clenbuterol (Chai et al. 2013), benzidine (Zhang and Wang 2010) and sodium benzoate (Mu et al. 2011).While the K_a value of Trypsin III at 298 K was only higher than that of bovine trypsin with sodium benzoate (Mu et al. 2011), which was the smallest at 278 K.

According the n values of Trypsin I, Trypsin II and Trypsin III, there was one binding site between benzamidine and the TRY-ES, which is similar to that of bovine trypsin with norfloxacin (Lu et al. 2010), clenbuterol (Chai et al. 2013), benzidine (Zhang and Wang 2010) and sodium benzoate (Mu et al. 2011). The K_a value of Trypsin I at 298 K (7.38×10^5 L mol^{-1}) was higher than that of Trypsin II (2.06×10^5 L mol^{-1}) and Trypsin III (2.16×10^5 L mol^{-1}) at 288 K, suggesting the higher affinity of Trypsin I to benzamidine than that of Trypsin II and Trypsin III.

Thermodynamics Parameters for the Benzamidine-Trypsin System

Interaction forces between small organic molecules and biological macromolecules are usually divided into the following four kinds: hydrogen bonds, van der Waals forces, electrostatic forces and hydrophobic interactions. They can be determined according to van't Hoff equation and thermodynamic equation. If $\Delta H^o < 0$, $\Delta S^o < 0$, hydrogen bonding or van der Waals force plays a major role in the reaction; if $\Delta H^o < 0$, $\Delta S^o > 0$, electrostatic force is the dominant force; if $\Delta H^o > 0$, $\Delta S^o > 0$, hydrophobic interaction is the main binding force (Ross and Subramania 1981). To determine the binding forces of the benzamidine and TRY-ES system, the thermodynamic parameters were calculated and shown in Table 4.

The negative value of ΔG^o indicated that the interaction between benzamidine and the TRY-ES was spontaneous, which was similar to bovine trypsin with norfloxacin (Lu et al. 2010), clenbuterol (Chai et al. 2013), benzidine (Zhang and Wang 2010) and sodium benzoate (Mu et al. 2011).

The negative enthalpy change (ΔH^o) and entropy change (ΔS^o) confirmed that hydrogen bonding or van der Waals force was the dominant binding force between benzamidine and Trypsin I. The ΔH^o and ΔS^o increased from −194.698 and −559.515 (between 278 K and 288 K) to −35.819 and −7.853 (between 288 K and 298 K) respectively, which was consistent with that the binding forces, bingding constants and quenching constants were decreased with the rising temperature.

The negative enthalpy change (ΔH^o, −37.922) and entropy change (ΔS^o, −46.463) confirmed that hydrogen bonding or van der Waals forces was the dominant binding force between benzamidine and Trypsin II between 278 and 288 K. While the positive enthalpy change (ΔH^o, 141.667) and entropy change (ΔS^o, 577.109) confirmed that hydrophobic interaction was the dominant binding force between 288 K and 298 K. The ΔH^o and ΔS^o between 288 K and 298 K were increased compared to which between 278 K and 288 K respectively, which was consistent with that the binding forces and quenching constants were decreased, yet the binding constants were increased with the rising temperature.

The positive enthalpy change (ΔH^o, 421.523) and entropy change (ΔS^o, 1565.748) confirmed that hydrophobic interaction was the dominant binding force between benzamidine and Trypsin III between 278 K and 288 K. While the negative enthalpy change (ΔH^o, –230.459) and entropy change (ΔS^o, –698.008) confirmed that hydrogen bonding or van der Waals forces was the dominant binding force between 288 K and 289 K. The ΔH^o and ΔS^o between 288 K and 298 K were decreased compared to which between 278 K and 288 K respectively, which was consistent with that the binding forces, bingding constants and quenching constants were increased with the rising temperature.

The negative enthalpy change (ΔH^o) and entropy change (ΔS^o) of clenbuterol (between 289 K and 310 K, Chai et al. 2013), sodium benzoate (between 277 K and 296 K, Mu et al. 2011) with bovine trypsin was similar to benzamidine to Trypsin I (between 278 K and 298 K), Trypsin II (between 278 K and 288 K) and Trypsin III (between 288 K and 298 K), while that of norfloxacin (Lu et al. 2010), benzidine (Zhang and Wang 2010) was negative enthalpy change (ΔH^o) and positive entropy change (ΔS^o) between 288 K and 298 K. With addition of clenbuterol

and sodium benzoate, the portion of β-sheet was decreased and the portion of α-helix and β-turn was increased, indicating the decrease in flexibility (Chai et al. 2013; Hu et al. 2013).The differences in the free energy, enthalpy, and entropy of activation with BA*p*NA between *G. morhua* trypsins and bovine trypsin, and that of unfolding with guanidine hydrochloride between *S. salar* trypsins and bovine trypsin might explain the large differences in K_m and K_{cat}/K_m values between trypsins from Fridge zone organisms and mammals (Asgeirsson et al. 1989; Outzen et al. 1991).

Conclusions

The purified TRY-ES from *E. superba* had activity with the lower optimum and stalbe temperature, but higher flexibility and higher catalyst efficiency, which had a high thermal stability in crude extracts. The distinct properties of the TRY-ES might be explained by the effect of temperature and benzamidine on fluorescence spectra. Having a catch of 2.15×10^5 t as feed stuff each year (FAO 2012), *E. superba* appears to be a potential large scale resource for the TRY-ES. Furthermore, site-directed mutagenesis and heterogeneous expression provides another promising approach to commercialize *E. superba* TRY-ES with distinct stability and physiological efficiency.

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Conflict of interest Zhiqiang Wu, Junren Wang, Xianming Shang, Zhaoqing Yang, Guoliang Jiang declare that they have no conflict of interest.

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