Kinetic Properties of Three Isoforms of Trypsin Isolated from the Pyloric Caeca of Chum Salmon (*Oncorhynchus keta*)

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Three isoforms of anionic chum salmon trypsin (ST-1, ST-2, and ST-3) were purified from the pyloric caeca of chum salmon (*Oncorhynchus keta*). The molecular weights of the three isoforms were about 24 kDa as determined by SDS-PAGE. The isoelectric points of ST-1, ST-2, and ST-3 were 5.8, 5.4, and 5.6, respectively. The apparent K_m values of two isoforms (ST-1 and ST-2) for BAPA (benzoyl-L-arginine-*p*-nitroanilide) hydrolysis at 5, 15, 25 and 35 °C were slightly higher than that of the main isoform ST-3, depending on temperature. The turnover numbers, k_{cat}, of ST-1 and ST-2 were about twice as high as that of ST-3. Consequently, the catalytic efficiencies (k_{cat}/K_m) of ST-1 and ST-2 were more efficient than ST-3. There were marked differences in both apparent K_m and k_{cat} values of three anionic chum salmon trypsins as compared to bovine cationic trypsin. K_m values of all chum salmon trypsins were approximately 10 times lower than those of bovine trypsin, depending on the temperature. The k_{cat} values of all chum salmon trypsins were about 2- to 5-fold higher than those of bovine trypsin; therefore, the catalytic efficiencies (k_{cat}/K_m) of chum salmon trypsin were 20- to 40-fold more efficient than those of bovine trypsin. On the other hand, k_{cat}/K_m values of ST-1 for TAME (tosyl-L-arginine methyl ester) hydrolysis were lower than those of bovine trypsin, whereas k_{cat}/K_m values of ST-2 and ST-3 were comparable to those of bovine trypsin, depending on the temperature.

Key words trypsin; isoform; chum salmon; *Oncorhynchus keta*; characterization; catalytic efficiency

Trypsin, an important enzymes belonging to the large and diverse family of serine proteases, has been found in numerous higher mammals, fish, plants, insects and bacteria. Several studies on the isolation and characterization of trypsins from cold-adapted fishes, such as Atlantic salmon (*Salmo salar*),[1,2] chum salmon (*Oncorhynchus keta*),[3—5] Atlantic cod (*Gadus morhua*),[6] Greenland cod (*Gadus ogac*),[7,8] Antarctic fish (*Paraotohhthenia magellanica*),[9] Japanese anchovy (*Engraulis japonicus*),[10] and anchovy (*Engraulis encrasicholus*)[11] have been reported. These fish trypsins resemble mammalian trypsins in many respects, but are also different in some respects. For example, most of the fish trypsins studied are more unstable than those from mammals at acidic pH and high temperatures.[2,4,6,9,11] Moreover, trypsins from cold-adapted fish have higher catalytic efficiencies (measured by the k_{cat}/K_m ratio) than those from bovine and porcine.[2,5—7,9—11] Furthermore multiple isoforms of trypsin have been found in many fish species,[2—6,10,11] while one cationic and one anionic form of trypsin have been found in humans,[12] bovines,[13] porcine,[14] and dogs.[15]

Trypsin from chum salmon (*Oncorhynchus keta*) was first reported by Uchida *et al.*[3,4] Chum salmon trypsin is composed of seven anionic trypsins and one cationic trypsin, and they were separated by ion-exchange resin. Their functional properties were similar to those of bovine cationic trypsin; however, the detailed catalytic properties have not been reported. Thus, in our previous study, we isolated an anionic trypsin from chum salmon and measured the catalytic properties toward some substrates. The catalytic efficiency (k_{cat}/K_m) of an anionic chum salmon trypsin for benzoyl-L-arginine-*p*-nitroanilide (BAPA) hydrolysis is 22-fold higher than that of bovine trypsin at 5 °C.[5] To improve our understanding of the higher level of catalytic efficiency at a lower temperature of enzymes from cold-adapted fish compared to mammalian enzymes, we also examined the X-ray crystallographic structure of an anionic chum salmon trypsin.[16] The result suggested that a higher level of catalytic efficiency of anionic chum salmon trypsin might be achieved from the lower electrostatic potential of the S1-binding pocket.

Studies on related enzymes from organisms adapted to living in a wide range of environments have revealed general patterns for how variations in sequence and structure allow enzymes to perform similar catalytic functions under different environmental conditions. These general patterns suggest mechanisms for how specific changes in structure may result in changes in enzymatic functions. Our studies are directed at the synthesis of non-covalent inhibitors of trypsin or trypsin-like enzymes (*e.g.* thrombin, kallikrein and urokinase) that may be used as scaffolds for the development of more specific drugs[17,18]; therefore we are interested in the catalytic properties and three-dimensional structures of other isoforms of chum salmon trypsin. These data may be useful for the design of more potent inhibitors of trypsin or trypsin-like enzymes.

In this study, we examined the isolation of isoforms of trypsin from pyloric caeca of chum salmon, and three isoforms of anionic chum salmon trypsin were obtained. Their catalytic properties were determined as the binding affinity (K_m) and turnover number (k_{cat}) of enzymes toward BAPA and TAME substrates. The present report describes the purification and characterization of two newly isolated isoforms from chum salmon, and compares the physical properties and kinetic properties with main isoform, which was reported previously,[5] and with cationic bovine trypsin, respectively.

MATERIALS AND METHODS

Materials Bovine trypsin (EC 3.4.21.4) was purchased from Worthington Biochemical Co. Benzoyl-L-arginine-*p*-nitroanilide (BAPA) and tosyl-L-arginine methyl ester (TAME) were purchased from the Peptide Institute Inc. 4-Nitrophenyl-4′-guanidinobenzoate hydrochloride (NPGB) was

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purchased from MERCK. DEAE-Sepharose, Benzamidine-Sepharose 6B, and Sephadex G-75 were purchased from GE Healthcare Bio-Sciences Corp.

Isolation and Purification of Chum Salmon Trypsin Anionic salmon trypsin was isolated from pyloric caeca of chum salmon by ammonium sulfate fractionation followed by acetone precipitation, affinity chromatography on Benzamidine-Sepharose 6B, gel filtration on Sephadex G-75, and ion-exchange chromatography on DEAE-Sepharose according to our reported procedure.[5] Stepwise purification of the enzyme was performed from the pyloric caeca of chum salmon as a measure of BAPA amidase activity. The overall purification of anionic chum salmon trypsin was 20-fold and recovery was approximately 16%. The purified anionic trypsin was further separated into at least six peaks by DEAE anion exchange chromatography, as shown in Fig. 1. Elution was carried out with 10 mM Tris–HCl buffer having a linear gradient of 0—0.1 mol/l NaCl. Enzymes from peaks 1—3 were hardly observed in trypsin activity. Three isoforms of anionic trypsins (ST-1, ST-2, and ST-3) were obtained from peaks 4, 5 and 6, respectively. All purification steps were carried out at 5 °C, and purified anionic chum salmon trypsins were stored at −30 °C.

Enzyme Assays Protein concentrations were determined using a DC protein assay kit (BIO-RAD) with bovine γ-globulin as the standard protein.[19] Trypsin activity was determined by observing amidase activity using BAPA as a substrate by following the increase of absorbance at 410 nm for 10 min at 25 °C. One enzyme unit was defined as the amount of enzyme hydrolyzing 1 mmol of BAPA per min under the conditions described above.

Active Site Titration The concentration of active sites of various trypsins was determined with NPGB according to the method of Chase and Shaw.[20]

Electrophoresis SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli.[21] Protein samples (10 μg) were denatured by heating (100 °C) for 3 min in 1 w/v% SDS, 10 w/v% sucrose and 2 v/v% 2-mercaptoethanol in 50 mM Tris–HCl buffer (pH 8) and then applied to 20 w/v% polyacrylamide gel. The molecular weight standards used were carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa), cytochrome C (12.5 kDa) and bovine lung trypsin inhibitor (6.5 kDa).

Isoelectric points of the isolated trypsins were determined by analytical electrofocusing in thin-layer polyacrylamide flat gel (LKB Ampholine PAGE plate) containing ampholine (pH range 4—6). As a reference, an isoelectric focusing calibration kit containing 11 standard proteins was used. The protein bands were stained with Coomassie brilliant blue R-250.

Kinetic Measurements Amidase activity was determined by using BAPA as a substrate according to the method of Erlanger *et al.*[22] Assay buffer consisted of 50 mM Tris–HCl (pH 8.1) and 10 mM $CaCl_2$. The amount of *p*-nitroaniline liberated from BAPA was determined by the increase in absorbance at 410 nm. Esterase activity was determined by using TAME as a substrate according to the method of Hummel,[23] measuring the change in absorbance at 247 nm. K_m was determined at five substrate concentrations in the range of 0.02—0.1 mmol/l for BAPA and 0.05—0.25 mmol/l for TAME at each temperature. Kinetic constants were obtained by least-squares fitting of initial velocity data to Lineweaver–Burk transformation of the Michaelis–Menten equation.

RESULTS

Purification and Physical Characteristics of Three Isoforms of Anionic Chum Salmon Trypsin The overall purification of ST-1, ST-2, and ST-3 was 28-fold, 27-fold, and 24-fold, and recovery was 2.1%, 1.9%, and 6.3%, respectively, as shown in Table 1.

The molecular weights of ST-1, ST-2 and ST-3 were estimated from SDS-PAGE to be about 24 kDa, similar to those reported for other fish trypsins.[1—10] Isoelectric points (pI) were estimated from analytical isoelectric focusing in a pH gradient from pH 4—6, as shown in Fig. 2. The isoelectric points of the isoforms of chum salmon trypsin were 5.8 for ST-1, 5.4 for ST-2 and 5.6 for ST-3. These pI values were not in accord with the values of anionic chum salmon trypsins

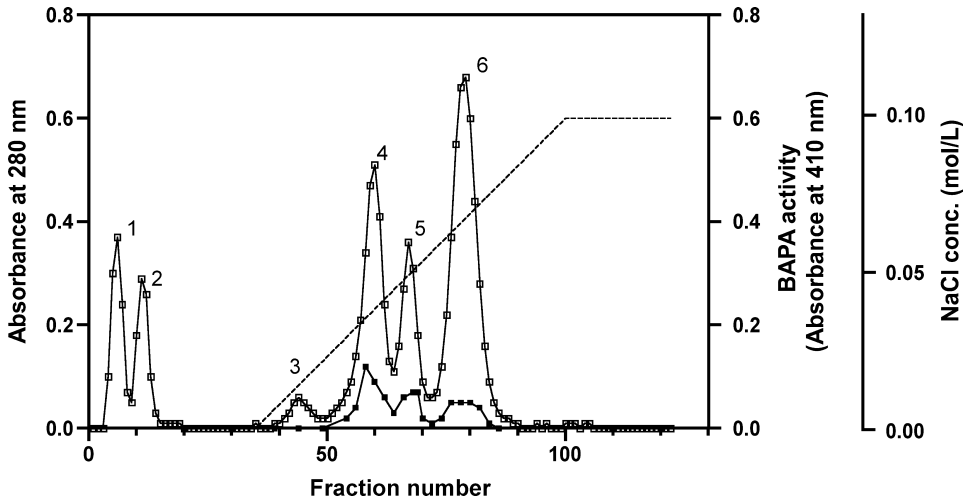

Fig. 1. DEAE-Sepharose Chromatography of the Chum Salmon Trypsin from the Pyloric Caeca

The elution was carried out with 10 mmol/l Tris–HCl buffer (pH 7.0 containing 5 mmol/l $CaCl_2$) having a linear gradient of 0—0.1 mol/l NaCl. –□–: absorbance at 280 nm; –■–: BAPA activity.

Table 1. Purification of Three Isoforms of Anionic Chum Salmon Trypsin[a)]

Fraction	Total protein (mg)	Total activity (unit)[b)]	Specific activity (unit/mg)	Recovery (%)	Purification (-fold)
Clarified extract	3083	708	0.23	100	1.0
Benzamidine-Sepharose	24	112	4.66	15.8	20.3
DEAE-Sepharose					
ST-1	2.3	14.8	6.43	2.1	28.0
ST-2	2.2	13.7	6.23	1.9	27.1
ST-3	8.0	44.5	5.56	6.3	24.2

a) Activities are calculated based on the hydrolysis of BAPA at 25 °C. *b*) One unit of amidase was defined as the amount of enzyme that hydrolysis 1 μmol/l of BAPA per min.

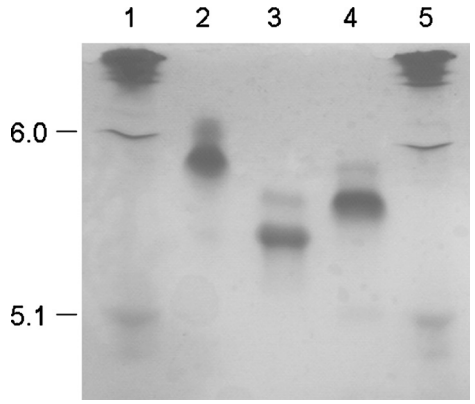

Fig. 2. Isoelectric Focusing over pH 4—6 of Isoforms of Anionic Chum Salmon Trypsin

Lanes 1 and 5: standard pI marker; lane 2: ST-1; lane 3: ST-2; lane 4: ST-3.

(pI=4.1—4.3) reported by Uchida *et al.*,[3)] Atlantic salmon trypsins (pI=4.5—4.7),[2)] and anchovy trypsins (pI=4.6—4.9).[11)]

Kinetic Properties The kinetic parameters of BAPA and TAME hydrolysis by three isoforms of anionic chum salmon trypsins and cationic bovine trypsin were compared over a temperature range from 5 °C to 35 °C. As shown in Table 2, the apparent K_m values of two isoforms (ST-1 and ST-2) for BAPA hydrolysis were slightly higher than that of main isoform (ST-3), depending on the temperature. Turnover numbers (k_{cat}) of ST-1 and ST-2 were about twice as high as those of ST-3, depending on the temperature. Consequently, the catalytic efficiencies (k_{cat}/K_m) of ST-1 and ST-2 were more efficient than ST-3, depending on the temperature.

There were marked differences in both apparent K_m and k_{cat} values of three chum salmon trypsins (ST-1, ST-2, and ST-3) as compared to bovine trypsin when the amidase activity was considered. The K_m values of chum salmon trypsins for BAPA hydrolysis were approximately 10 times lower than bovine trypsin, depending on the temperature. The k_{cat} of chum salmon trypsins was about 2- to 6-fold higher than that of bovine trypsin, depending on the temperature. The difference in catalytic efficiency (k_{cat}/K_m) was therefore 20- to 40-fold higher for salmon trypsin over bovine trypsin. Moreover, the k_{cat}/K_m values of three isoforms of chum salmon trypsin at 35 °C did not increase more than those at 25 °C, whereas the k_{cat}/K_m values of bovine trypsin increased with a rise of temperature. Furthermore, the difference in k_{cat}/K_m values between anionic chum salmon trypsins and bovine trypsin was larger at 5 °C than at 35 °C, *i.e.*, the k_{cat}/K_m of ST-1 was about 28-fold higher than that of bovine trypsin at 5 °C, whereas 20-fold higher than that of bovine trypsin at 35 °C.

Table 2. Kinetic Properties of Chum Salmon Trypsins for Bz-L-Arg-*p*NA (BAPA) and Tos-L-Arg-OMe (TAME) Compared to Bovine Trypsin at Various Temperatures

Substrate	Trypsin source	Temp. (°C)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
BAPA	Chum salmon				
	ST-1	5	0.02	1.06	53.0
		15	0.03	2.74	91.3
		25	0.03	4.06	135
		35	0.07	7.70	110
	ST-2	5	0.02	0.83	41.5
		15	0.03	2.72	90.7
		25	0.04	5.54	139
		35	0.05	6.74	135
	ST-3	5	0.02	0.64	32.0
		15	0.02	1.27	63.5
		25	0.02	2.00	100
		35	0.04	3.86	96.5
	Bovine	5	0.20	0.30	1.50
		15	0.27	0.76	2.81
		25	0.26	0.94	3.62
		35	0.44	2.43	5.52
TAME	Chum salmon				
	ST-1	5	0.02	41.3	2070
		15	0.03	64.5	2150
		25	0.04	94.3	2360
		35	0.05	94.3	1890
	ST-2	5	0.02	61.7	3090
		15	0.02	105	5250
		25	0.02	234	11700
		35	0.03	200	6670
	ST-3	5	0.02	68.4	3420
		15	0.03	154	5130
		25	0.02	159	7950
		35	0.04	189	4730
	Bovine	5	0.01	46.2	4620
		15	0.01	69.2	6920
		25	0.01	109	10900
		35	0.02	139	6950

The catalytic efficiency of chum salmon trypsins for TAME hydrolysis was also compared with bovine trypsin between 5—35 °C (Table 2). The K_m values of ST-1, ST-2 and ST-3 for TAME hydrolysis were compared with those of ST-1, ST-2 and ST-3 for BAPA hydrolysis. The k_{cat} values of ST-1, ST-2 and ST-3 for TAME hydrolysis indicated that these isoforms degraded ester bonds at a faster rate than amide bonds; however, the k_{cat} values of ST-1 were apparently lower

than those of ST-2 and ST-3. The catalytic efficiency (k_{cat}/K_m) of ST-1 therefore appeared to be inferior to those of ST-2 and ST-3.

Although the K_m values of bovine trypsin for TAME hydrolysis were approximately 2 times lower than ST-2 and ST-3, the k_{cat} values of bovine trypsin were slightly lower than those of ST-2 and ST-3, depending on the temperature. Thus, differences in the esterase activity of chum salmon and bovine trypsins were not as obvious as for the amide substrate.

DISCUSSION

The molecular weights of three isoforms of chum salmon trypsin (ST-1, ST-2, and ST-3) were estimated to be about 24 kDa by SDS/PAEG. This is within the range of molecular weitht, similar to those reported for other fish trypsins,[1—10] and to that of bovine trypsin in this study. The pI values of isoforms were 5.8 for ST-1, 5.4 for ST-2 and 5.6 for ST-3 at neutral pH. This result suggested that the three isoforms are all anionic forms, however, the amounts of basic and acidic amino acid residues are different. To our knowledge cationic trypsins have not been isolated from fish species, except for from Atlantic salmon[2] and chum salmon.[3] Cationic trypsins are the most common isoform in mammalian species (*e.g.* pI=10.1 for bovine trypsin; 10.8 for porcine trypsin).[24]

The equation for an enzyme-catalyzed hydrolysis involving an acyl-enzyme intermediate is shown in Eq. 1. This formulation includes ES, the enzyme-substrate complex; ES′, the acyl-enzyme; P_1 and P_2, the alcohol and acid portions

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} \begin{matrix} ES' \\ + \\ P_1 \end{matrix} \xrightarrow{k_3} E + P_2 \qquad (1)$$

of the substrate, respectively.[25] Literature data indicate that while K_m of specific amide substrates is a true binding constant, K_m of specific ester substrates is a combination of an equilibrium constant and rate constants. Furthermore, k_{cat} of specific amide substrates is controlled by the acylation step, k_2, while k_{cat} of specific ester substrates is controlled by the deacylation step, k_3.[26] The measured K_m and k_{cat} values of chum salmon trypsins and bovine trypsin for BAPA substrate should therefore approximate the true binding affinities (K_s) and acylation rates (k_2), respectively. The catalytic efficiencies (k_{cat}/K_m) of all anionic chum salmon trypsins (ST-1, ST-2 and ST-3) were 20—40 times higher than that of bovine trypsin. These differences were caused by a dramatic decrease in K_m values for anionic chum salmon trypsins. Thus, the increased catalytic efficiency observed for anionic chum salmon trypsins compared to bovine trypsin is due to a higher binding affinity of the BAPA substrate. This behavior was observed between bovine trypsin and other fish trypsins.[1,2,6,9,11] On the other hand, chum salmon trypsins have a higher efficiency with ester substrate (TAME) than with amide substrate (BAPA). This difference was caused by an increase in k_{cat} values for TAME. The esterase activity of chum salmon trypsin and bovine trypsin were not as obvious as for the amide substrate.

The differences in catalytic efficiency (k_{cat}/K_m) between anionic chum salmon trypsin and cationic bovine trypsin are larger at 5 °C than at 35 °C. This difference may be due to inactivation of the anionic chum salmon trypsin at higher temperatures. The k_{cat}/K_m values of three isoforms of chum salmon trypsin at 35 °C for BAPA hydrolysis did not increase more than those at 25 °C, but the k_{cat}/K_m values of bovine trypsin increased with a rise of temperature. Therefore, it seems that at 25 °C fish trypsins are better suited than bovine trypsin for their catalytic function.

The three isoforms of anionic chum salmon trypsin showed slightly different behavior for catalytic efficiency toward BAPA and TAME substrates. Unfortunately we cannot explain this observation without further experiment, such as X-ray crystallographic analyses of three isoforms.

Several studies by Smalås and co-workers suggest that the increased substrate affinity (lower K_m) of trypsins from cold-adapted species may be achieved by the lower electrostatic potential of the S1-binding pocket.[27] The lower electrostatic potential in the substrate-binding cleft of anionic salmon trypsin may help to orient the positively charged P1 arginine side-chain of a substrate and to guide the substrate into the binding cleft. In our previous paper,[16] we described that bovine trypsin has two positively charged residues (Lys222 and Lys224) in the S1-binding pocket, but anionic chum salmon trypsin has one negatively charged residue (Glu221B). In this study, we obtained the three isoforms of anionic chum salmon trypsin. From the data of isoelectric points, these isoforms have different overall net charge and/or the number of charged amino acid residues. In order to understand how small differences in sequence and structure may be responsible for significant changes in enzymatic activity, we will elucidate the amino acid sequence and crystal structure of all isoforms. Comparative studies of the amino acid sequence and structure of trypsins from different origins are necessary for understanding the relationship between the isoelectric point of the protein and tryptic catalytic function.

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