Biochimica et Biophysica Acta, 658 (1981) 17—26

BBA 69214

STUDIES ON PROTEINASES FROM THE DIGESTIVE ORGANS OF SARDINE

I. PURIFICATION AND CHARACTERIZATION OF THREE ALKALINE PROTEINASES FROM THE PYLORIC CAECA

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(Received May 19th, 1980)
(Revised manuscript received October 7th, 1980)

Key words: Proteinase; Alkaline proteinase; (Sardine)

Summary

Three alkaline proteinases designated I, II and III were found in the pyloric caeca of sardine and isolated by $(NH_4)_2SO_4$ fractionation, DEAE-cellulose chromatography and gel filtration on Sephadex G-100. The final preparations were judged homogeneous by multiple criteria. The molecular weights of the enzymes I, II and III were determined by the sedimentation equilibrium method to be 22 900, 28 700 and 27 000, respectively. The isoelectric points were 5.45, 5.30 and 4.85, and the sedimentation coefficients ($s^0_{20,w}$) were 2.91, 3.06 and 2.94, respectively. Enzymes II and III had similar amino acid compositions which were different from that of enzyme I, especially in the content of lysine, valine and tyrosine. All the enzymes belonged to a group of serine proteases. Enzymes II and III were found to be an anionic α-chymotrypsin-like enzyme and an anionic trypsin-like enzyme, respectively. Although these fish enzymes had properties in common with those of bovine pancreatic cationic trypsin and chymotrypsin, they are distinctly different in their optimum pH, pH stability, net charge and immunological properties.

Introduction

Sea-foods are a major source of dietary protein in many countries. However, the rapid deterioration of fish, even under refrigeration, hampers its use for

Abbreviations: TPCK, *N*-*p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone; TLCK, *N*-*p*-toluenesulfonyl-L-lysine chloromethyl ketone.

human nutrition. Since ancient times fermentation has been used to preserve sea-foods, especially in the orient [1,2]. Fish sauce is prepared by proteolysis of fish. NaCl is added to check bacterial spoilage and proteolytic enzymes are provided by halophilic bacteria or by the fish [1]. Fish proteinases have not been extensively studied although trypsin-like proteinases have been detected in a few species of fish and shrimps [3—9]. In the present paper we have described the isolation and some of the properties of three alkaline proteinases that are present in the pyloric caeca of the sardine (*Sardinops melanosticta*).

Materials and Methods

Materials. Trypsin (bovine pancreas, twice crystallized) and α-chymotrypsin (bovine pancreas, three times crystallized) were obtained from Sigma Chemical Co. Diisopropylfluorophosphate was obtained from Wako Pure Chemical Industries, Ltd. *N-p*-Toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK) and *N-p*-toluenesulfonyl-L-lysine chloromethyl ketone (TLCK) were obtained from Nakarai Chemicals, Ltd. Soybean trypsin inhibitor (five times crystallized) was obtained from General Biochemicals Inc.

Sardine. Sardines were caught near Choshi on the Pacific coast of Japan in March, 1977. In all, about 2 h elapsed from the time the fish were caught until they were completely frozen at —20°C. They were held in frozen storage for 3—4 months until their use in our experiments.

Proteinase activity. Proteinase activity of the enzymes was determined according to the modification of Anson's method [10] as follows: 0.6% milk casein (Merck Co.) was dissolved in 0.1 M bicarbonate buffer, pH 10.0 and used as a substrate. 1 ml enzyme solution was added to 5 ml substrate and incubated at 37°C for 10 min. After incubation, the reaction was terminated by adding 5 ml of a reagent containing 0.11 M trichloroacetic acid/0.22 M sodium acetate/ 0.33 M acetic acid. The resulting precipitate was filtered by Toyo No. 5C filter. To 2 ml filtrate were added 5 ml 0.55 M sodium carbonate solution and 1 ml of a 5-fold diluted solution of phenol reagent (Wako Pure Chemical Ind. Japan). The mixture was incubated at 30°C for 20 min and proteinase activity was determined by absorbance at 660 nm of the incubation mixture. 1 unit of proteinase activity was defined as the absorbance equivalent of 1 μg tyrosine produced per min under the above experimental condition. Our experiment indicated the following equation: a unit of proteinase activity = absorbance at 660 nm × 124.2 × dilution factor of enzyme. 10—15 μg purified enzyme were used for the determination of optimal pH and temperature or of thermal and pH stability.

Protein concentration. Protein concentration was determined spectrophotometrically according to the method of Lowry et al. [11] using egg albumin as a standard.

Amino acid analysis. The pure enzyme preparations were subjected to amino acid analysis in a Hitachi KLA-5 type analyzer after hydrolysis in 6 N HCl at 110°C for 24, 48 and 72 h. Amino acid values of serine, threonine and tyrosine were corrected by extrapolating to zero time of hydrolysis. The values of valine and isoleucine represented an average of 48- and 72-h hydrolysates. The values of other amino acids represented the average of three samples. Tryptophan was

determined by the method of Goodwin and Morton [12]. Cysteine was determined as cysteic acid after the HCl hydrolysis in 0.2 M dimethylsulfoxide according to the method of Spencer and Wald [13].

Molecular weight. Sedimentation equilibrium studies were performed with a Hitachi model UCA-1 analytical ultracentrifuge according to the procedure of Yphantis [14]. About a 1% solution of samples (alkaline proteinases I, II and III) dialyzed against 0.01 M phosphate buffer, pH 6—7/0.1 M NaCl, was used. Equilibrium scans were made at 5° C at a rotor velocity of 12 290 rev./min (Hitachi rotor No. RA-72T). In calculating the average molecular weight, the partial specific volume of protein was estimated to be 0.71—0.72 using the amino acid composition. The molecular weight was also estimated by electrophoresis in a 10% polyacrylamide gel (7 cm long) with 0.1% sodium dodecyl sulfate (SDS) and 2% mercaptoethanol.

Sedimentation coefficient. The sedimentation coefficient was determined by the method of Schachman [15] at 5° C with a Hitachi UCA-I type ultracentrifuge and was corrected at 20° C in water.

Immunization. Pure alkaline proteinase III, emulsified with an equal volume of Freund's complete adjuvant, was injected intradermally at multiple sites on the back of rabbits. An initial injection of 1 mg enzyme was followed 2 weeks later by a 500 μg booster injection. Once an antibody response was elicited, another 500 μg proteinase III were injected intravenously.

Fish protein substrates. Three fish proteins i.e., sarcoplasmic, myofibril and stroma proteins were fractionated from sardine muscle according to the method of Connell, and used as fish protein substrate. Sarcoplasmic [16,17] and myofibril proteins [18] were soluble in the 0.02 M phosphate buffer, pH 7.5 and in the same buffer containing 0.45 M KCl, respectively. Stroma protein which was a residue after the extraction of the above two proteins was used in a suspension for a substrate. All the muscle proteins were pretreated at 100° C, for 5 min, before use.

Extraction of alkaline proteinases. All steps of extraction and purification were carried out at 4° C, unless otherwise stated. Approx. 500 ml distilled water were added to 100 g of digestive organs including mainly pyloric caeca, obtained from sardine body and homogenized in a Waring Blender for 3 min. The homogenate was centrifuged at 9800 × *g* for 10 min, the supernatant fluid was filtered through celite. Proteinase activity in the clear filtrate was collected by precipitation with 30—70% saturation of $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, dissolved in a minimum amount of distilled water and dialyzed overnight against distilled water. The dialyzed solution was centrifuged and the precipitate discarded. The supernatant fluid was used as crude enzyme solution.

Results

Purification. Alkaline proteinases were extracted and concentrated and the concentrated solution (crude enzyme solution) was applied to the first DEAE-cellulose column (Fig. 1). The three alkaline proteinases were eluted from this column and designated alkaline proteinase I, II and III. Each of alkaline proteinases was further purified by the ion-exchange chromatography on the

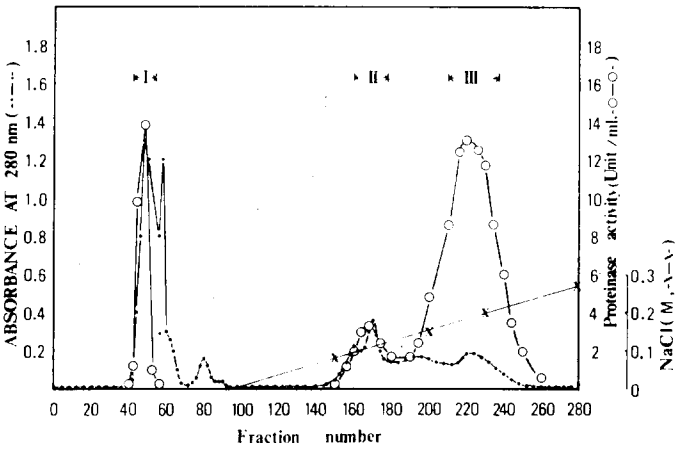

Fig. 1. Chromatography of sardine alkaline proteinases on the first DEAE-cellulose column. 25 ml of the crude enzyme solution (967 mg protein) were prepared as described in Materials and Methods, equilibrated with 0.01 M phosphate buffer (pH 7.0) and applied to a DEAE-cellulose column (3 X 100 cm, Brown Co.) previously equilibrated with the same buffer. This column was washed with the same buffer and further eluted using a 750 ml linear gradient of 0—0.6 M NaCl in the same buffer. Each fraction of alkaline proteinases II and III, shown in parentheses, was concentrated and purified on the second DEAE-cellulose column under the same condition as described above.

second DEAE-cellulose and/or the gel filtration on Sephadex G-100 (Fig. 2). The outline and the results of the purification of the alkaline proteinase are summarized in Table I. Three alkaline proteinases (I, II and III) were isolated in reasonable quantities by the present purification procedures starting with 100 g of digestive organ: 45.2 mg alkaline proteinase I, 14.2 mg proteinase II and 47.2 mg proteinase III were isolated, at a combined yield of 17%. Enzyme I and III were major alkaline proteinases. A 50—120-fold purification was achieved. The final products after the first or the second Sephadex G-100 were stored at −15°C and used for subsequent characterization studies as reported in this paper.

TABLE I

PURIFICATION OF SARDINE ALKALINE PROTEINASES I, II AND III

Procedure	I		II		III	
	Spec. act. (unit/mg protein)	Yield (%)	Spec. act. (unit/mg protein)	Yield (%)	Spec. act. (unit/mg protein)	Yield (%)
Homogenate	0.77	100	0.77	100	0.77	100
Crude extract	2.4	69.5	2.4	69.5	2.4	69.5
$(NH_4)_2SO_4$ fractionation	11.5	29.9	11.5	29.9	11.5	29.9
First DEAE-cellulose	6.3	5.48	6.1	1.6	44.3	16.3
Second DEAE-cellulose	—	—	11.9	1.58	45.2	12.8
First Sephadex G-100	20	5.29	40.1	1.53	88.5	10.3
Second Sephadex G-100	42	5.1	—	—	—	—

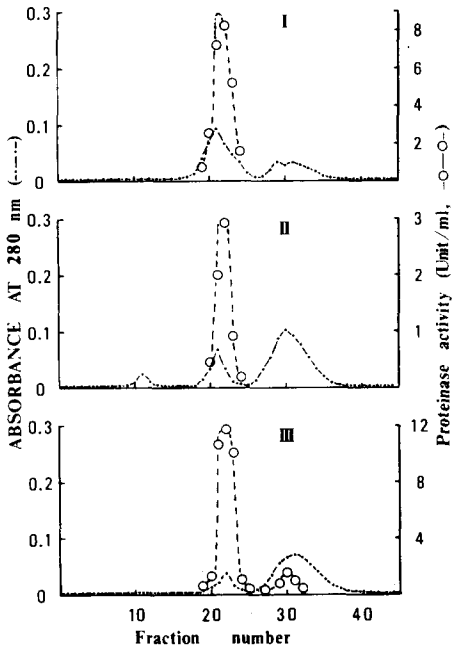

Fig. 2. Gel filtration of alkaline proteinases I, II and III on Sephadex G-100. Alkaline proteinase, I, II and III fractions from the first or the second DEAE-cellulose column were concentrated to 5 ml by ultrafiltration and further purified by gel filtration on a Sephadex G-100 column (2 X 100 cm) using 0.01 M phosphate buffer (pH 7.0). Sample for I: alkaline proteinase I from the first filtration. Samples for II and III: alkaline proteinases II and III from the second DEAE-cellulose.

Evidence for purity of alkaline proteinases I, II, and III. When the purified proteinases were examined by polyacrylamide disc-gel electrophoresis, at pH 9.5, each gave rise to one discrete band after the staining for protein (Fig. 3). Likewise, electrophoresis of each preparation on SDS-polyacrylamide gel electrophoresis yielded a single protein band (see below). Isoelectric focussing also yielded a single protein band from each proteinase and this band coincided with proteinase activity. Isoelectric points of proteinases I, II and III are 5.45, 5.30 and 4.85, respectively. Each proteinase gave rise to a single symmetrical sedimentation peak (Fig. 4) from which sedimentation coefficients ($s^0_{20,w}$) were found to be 2.91, 3.06 and 2.94 for enzymes I, II and III, respectively.

Molecular weight. The molecular weights determined by the sedimentation equilibrium method were calculated to be 22 900, 28 700 and 27 000 for alkaline proteinases I, II and III, respectively. Electrophoresis in SDS-polyacrylamide gel columns in the presence of mercaptoethanol produced a single band at a position corresponding to the molecular weights of 23 300, 26 000 and 27 600 for proteinases I, II and III, respectively. In the estimation of the molecular weights good agreement was obtained between the sedimentation equilibrium and SDS-polyacrylamide gel electrophoresis methods.

Amino acid composition. The amino acid compositions of proteinases I, II and III (Table II) show similarities, but they differ mainly in terms of lysine, aspartic acid, cysteine, valine and tyrosine. The relative proportions of acidic,

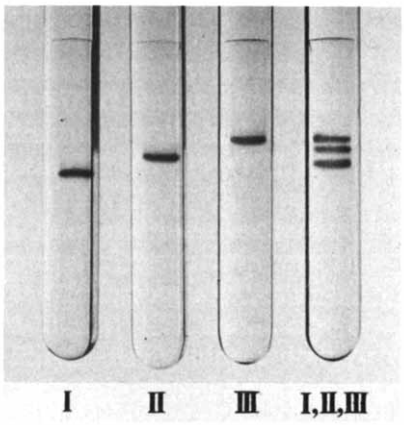

Fig. 3. Polyacrylamide gel electrophoresis of alkaline proteinases I, II and III (top figure). The electrophoresis was performed at pH 9.5, 4 mA per tube, 5°C for 30 min in 0.5 × 7 cm columns of 7.5% polyacrylamide gel according to Davis [19]. I, II and III represent purified alkaline proteinases I, II and III, respectively. Protein bands were stained by Coomassie brilliant blue R-250 solution.

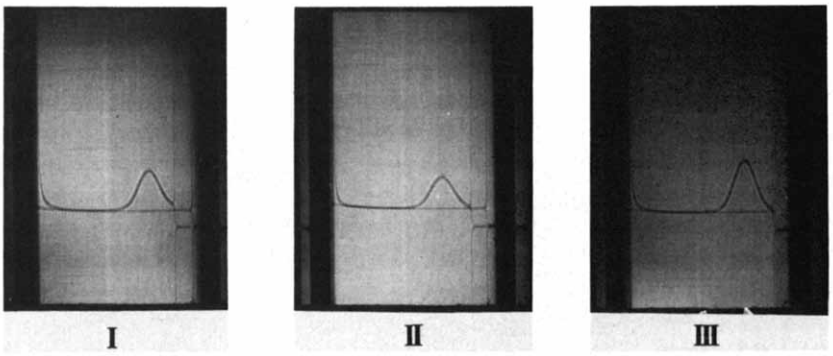

Fig. 4. Sedimentation patterns of alkaline proteinases I, II and III (bottom figure). The purified enzymes were dissolved in 0.01 M phosphate buffer (pH 6—7)/0.1 M NaCl to give the concentration of 1.35% for I, 0.88% for II and 1% for III. The photographs were taken 100 min after attainment of full speed (55 430 rev./min, Hitachi rotor No. RA-72T). Temperature: 5°C, bar angle: 70°.

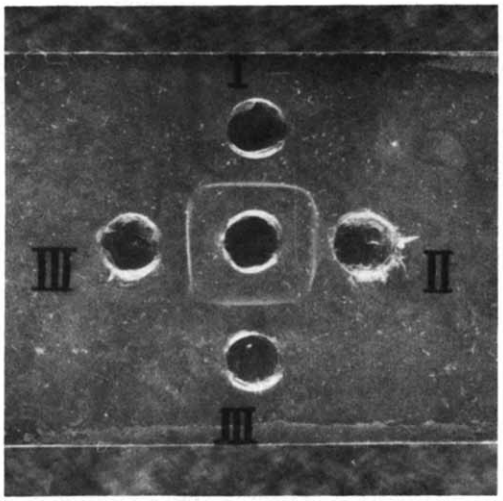

Fig. 5. Double diffusion precipitin reaction of alkaline proteinases I, II and III against antisera of alkaline proteinase III (top right figure). Anti-alkaline proteinase III plasma was placed in the central well. Pure alkaline proteinases I, II and III (μg quantities) were placed in wells I, II and III, respectively. The diffusion was allowed to take place for 24 h at 30°C.

TABLE II

AMINO ACID COMPOSITION OF SARDINE ALKALINE PROTEINASES I, II AND III

Amino acid composition of alkaline proteinases and various other proteinases. The compositions were expressed as amino acid residues/mol, based on a molecular weight of alkaline proteinase I (22 900), II (28 700) and III (27 000). The values in parentheses are the nearest integer.

Amino acid	Sardine Alkaline Proteinase			Trypsin	α-Chymotrypsin
	I	II	III		
Lysine	10.2(10)	18.6(19)	17.4(17)	14	14
Histidine	3.2(3)	2.8(3)	3.2(3)	3	2
Arginine	5.6(6)	6 (6)	6 (6)	2	3
Aspartic acid	19.1(19)	23 (23)	30.6(31)	22	20
Threonine	17.5(18)	17.1(17)	18.6(19)	10	22
Serine	12.9(13)	16.2(16)	16.9(17)	34	27
Glutamic acid	20.4(20)	18.6(19)	16.4(16)	14	15
Proline	9.9(10)	13 (13)	9.5(10)	8	8
Glycine	21.1(21)	29.7(30)	27.4(27)	25	23
Alanine	14 (14)	15 (15)	14.8(15)	14	22
Half cystine	6.9(7)	12.6(13)	7.8(8)	12	10
Methionine	0.7(1)	0.9(1)	0.8(1)	2	2
Valine	12.2(12)	21.1(21)	17.9(18)	15	22
Leucine	12.8(13)	10.1(10)	9.5(10)	13	18
Isoleucine	9.7(10)	7.7(8)	10.7(11)	13	10
Tyrosine	6.9(7)	14 (14)	13 (13)	10	4
Phenylalanine	4.1(4)	3.5(4)	4.3(4)	3	6
Tryptophan	4.3(4)	4.9(5)	4.4(4)	3	6

basic and neutral residues in the three proteinases are similar to those in bovine trypsin and α-chymotrypsin.

Immunological properties. Double diffusion of rabbit anti-alkaline proteinase III and each of alkaline proteinases I, II and III on an Ouchterlony plate produced a single precipitin band (Fig. 5), whereas the antibodies did not cross-react with bovine pancreatic trypsin, α-chymotrypsin and antarctic krill trypsin-like enzyme (Murakami, K. and Kimoto, K., unpublished data).

Proteinase activity. The pH optimum for alkaline proteinases I, II and III is 10—11 for proteolysis of casein and hemoglobin, but around 9.5 for proteolysis of sarcoplasmic protein of sardine muscle.

The optimal temperature of three alkaline proteinases for the maximum rate of hydrolysis toward milk casein was 45° C.

Although each alkaline proteinase could hydrolyze protamine, fibrinogen, fibrin and collagen at different rates, milk casein and hemoglobin were the best substrates. Sardine muscle proteins i.e., sarcoplasmic, myofibril and stroma proteins were also good substrates for sardine enzymes, especially for enzyme III.

Synthetic substrates. The results of hydrolysis of synthetic substrates by the three proteinases were shown in Table III. Enzyme III, like trypsin, could hydrolyze benzoyl-arginine ethyl ester, tosyl-arginine methyl ester and benzoyl-arginine amide but could not hydrolyze *N*-acetyl-tyrosine ethyl ester. On the other hand, enzyme II, like α-chymotrypsin, could hydrolyze *N*-acetyl-tyrosine ethyl ester but could not hydrolyze any other synthetic substrates. Enzyme I could scarcely hydrolyze any synthetic substrates except that it hydrolyzed benzoyl-arginine amide slowly.

TABLE III

HYDROLYSIS OF SYNTHETIC SUBSTRATES BY SARDINE ALKALINE PROTEINASES I, II AND III

Esterolytic activities were determined spectrophotometrically according to Hummel's modification [20] of the method of Schwert and Takenaka [21] as follows. 35 μg of each of the purified enzymes were incubated with 1 ml 0.1 M borate buffer (pH 8.0)/0.94 mM substrate for several min at 20°C. Esterolytic activities were expressed as absorbance/min per mg enzyme. Amidase activity was determined as follows. 80 μg of each enzyme were incubated with 1 ml 0.1 M phosphate buffer (pH 7.0)/1 mM benzoyl-arginine amide for 30 min, at 30°C. After incubation, the reaction was stopped by addition of ninhydrin reagents [22] and absorbances at 570 nm was measured. Amidase activity was expressed as absorbance at 570 nm/min per mg enzyme.

Synthetic substrates	Alkaline proteinase		
	I	II	III
N-Acetyl-L-tyrosine ethyl ester	0	0.32	0
Tosyl-L-arginine methyl ester	0	0	2.1
N-Benzoyl-L-arginine ethyl ester	0	0	0.5
Benzoyl-arginine amide	0.04	0	0.12

TABLE IV

PROPERTIES OF SARDINE ALKALINE PROTEINASES I, II AND III

1: Molecular weight by sedimentation equilibrium method. 2: Molecular weight by SDS-polyacrylamide gel electrophoresis.

	I	II	III
Specific activity for casein (unit/mg protein)	42	40.1	88.5
Optimum pH	10	10	10
Optimum temperature	45°C	45°C	45°C
pH stability	6~9	6~9	7~9
Thermal stability	65%	42%	32%
Sedimentation coefficient ($s^{\circ}_{20\,w}$)	2.91	3.06	2.94
Isoelectric point	5.45	5.30	4.85
Molecular weight (1)	22 900	28 700	27 000
Molecular weight (2)	23 300	26 000	27 600

TABLE V

EFFECT OF VARIOUS REAGENTS ON SARDINE ALKALINE PROTEINASES I, II AND III

Each of the purified enzymes was preincubated with each reagent in 0.01 M phosphate buffer, pH 7.0, at 30°C for 20 min and assayed. Enzyme activities are expressed as percentages of the activity in the absence of reagents.

Reagents	Concn. (M)	Activity (%)		
		I	II	III
None		100	100	100
p-Chloromercuribenzoate	10^{-4}	100	108	100
o-Phenanthroline	10^{-3}	98	107	100
Ethylendiaminetetraacetate	10^{-4}	85	100	101
Mercaptoethanol	10^{-3}	100	100	98
L-Cysteine-hydrochloride	10^{-3}	100	100	100
Diisopropylfluorophosphate	10^{-3}	35	0	0
Trypsin inhibitor	0.25mg/ml	100	35	0

TABLE VI

EFFECT OF TLCK AND TPCK ON THE PROTEOLYTIC ACTIVITY OF SARDINE ALKALINE PROTEINASES I, II AND III

$1.0 \cdot 10^{-6}$ M of each of the purified enzyme and the 150 molar excess of each inhibitor in 0.1 M phosphate buffer, pH 7.0 (with 10^{-3} M $CaCl_2$ in the case of trypsin and α-chymotrypsin), were mixed and incubated for 60 min at 30° C. Then, activities were assayed.

Enzyme	Relative activity (%)		
	None	TLCK	TPCK
Trypsin	100	8	102
α-Chymotrypsin	100	102	34
I	100	96	68
II	100	100	45
III	100	12	98

Stability. No loss of proteinase activity of the three enzymes was noted when stored at −15° C for 1 month after freeze-drying of the purified enzyme solution at pH 7.0. Almost no loss of activity of the three enzymes was observed at pH 6—9, when they were incubated at 37° C for 2 h. Thermal stability was tested after the three enzymes were preincubated for 5 min in 0.5 ml 0.1 M phosphate buffer (pH 7.0) at 20—55° C. The remaining activities after the preincubation at 55° C were 65%, 42% and 32% for enzymes I, II and III, respectively.

Molecular and enzymatic properties of the three enzymes are summarized in Table IV.

Inhibitors. Alkaline proteinases I, II and III were neither inhibited nor activated by metal chelating agents and sulfhydryl reagents. They were inhibited strongly by diisopropylfluorophosphate. Soybean trypsin inhibitor did not inhibit enzyme I but did inhibit enzymes II and III. These results (Table V) indicate that enzymes I, II and III belong to a group of serine proteinases although they have different molecular properties. Active site-oriented inhibitors for trypsin or α-chymotrypsin were used to elucidate the difference among these enzymes more clearly (Table VI). Enzyme III, like mammalian trypsin, was inhibited strongly by TLCK but not by TPCK. In contrast, enzymes I and II, like α-chymotrypsin, were inhibited by TPCK but not by TLCK.

Discussion

The three alkaline proteinases resemble each other in such properties as pH stability, molecular weight and sedimentation constants, optimum pH and temperature and immunological reactions. These enzymes can be classified as serine proteinases with essential histidine residues. Enzyme III, the major alkaline proteinase of the pyloric caeca, is a trypsin-like proteinase since it is inhibited by soybean trypsin inhibitor and by TLCK. In addition, it hydrolyzes the synthetic trypsin substrates. Enzymes I and II are sensitive to TPCK but only the latter catalyses the hydrolysis of the chymotrypsin substrate, acetyl-tyrosine ethyl ester. Thus, while enzyme II can be considered as a chymotrypsin-

like enzyme, enzyme I seems to be of the unique type which can hydrolyze trypsin substrate, benzoyl-arginine amide, but is not inactivated by a trypsin-specific inhibitor, TLCK. However, these three enzymes seem to have a common precursor judging from their immunological similarity.

The three alkaline proteinases show an optimum pH of about 10 which is well above the optima for mammalian trypsin [3] and for trypsin-like proteinases from some lower organisms, including some fish [8,23]. Other properties of these enzymes from sardine are acidic isoelectric points and instability at low pH values. In these respects they resemble trypsin-like enzymes from lower organisms (e.g., starfish [24], crayfish [25] and shrimps [26]) but are distinct from mammalian pancreatic trypsin and α-chymotrypsin [3] which have basic isoelectric points and are stable at pH 3.

Acknowledgements

The authors would like to acknowledge the continuing guidance and encouragement of Professor Y. Ogasawara, University of Tokyo Domestic Science and Dr. N. Iguchi, Central Research Laboratories of Kikkoman Shoyu Co., Ltd.

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