STUDIES ON ACROSIN. I. PURIFICATION AND CHARACTERIZATION OF BOAR ACROSIN

SATORU KANEKO AND CHIAKI MORIWAKI

Laboratory of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo, Ichigaya Funagawaracho, Shinjuku-ku, Tokyo, 162, Japan

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Acrosin was extracted from boar sperm and purified by Sephadex gel filtration and affinity chromatography on Phe-Phe-Arg Sepharose 4B in acidic condition. Its enzymic properties were characterized in comparison with trypsin.

The oligopeptides with Arg at the carboxy-termini were used as the ligands for affinity chromatography. Phe-Phe-Arg adsorbed acrosin at pH 5 and released it at pH 3. To adsorb acrosin, it was found that the ligand should be longer than tripeptide with Arg in the carboxy-termini. Disc gel electrophoretogram of purified boar acrosin gave a broad band consisted from three fractions which hydrolysed N- α -benzoyl-arginine ethylester (BAEE). The pH optimum and inhibition spectra were similar to those of trypsin, however, the influence of urea on them were very different among each other. Calcium ion decreased K_m for BAEE, and increased K_i of aprotinin. The kinetic analysis of acrosin for its substrate and products resulted that K_m for BAEE was minimal at around pH 8 and maximal at pH 5, on the contrary, K_i of the product was low at pH 5, but progressively increased along the elevation of pH. The same tendency was observed for trypsin. From the attitudes on the affinity chromatographies and the pH profiles of kinetic parameters, it was concluded that the active sites of acrosin and trypsin were similar to each other.

Keywords—acrosin; trypsin; product analogue; active site; sperm

INTRODUCTION

The acrosome, an organelle located at the head of mammalian sperm, plays an important role on sperm penetration into the ovum in the fertilization. There are two acrosome enzymes which participate in the sperm penetration. One is hyaluronidase¹⁾ which is located in acrosome vesicle and facilitates sperm penetration through the cumulus oophorus. The other is acrosin which is thought to be bound to the inner acrosomal membrane or equatorial segment and dissolves the zona pellucida.^{2,3)}

This paper describes the purification of acrosin from the boar sperm by employing affinity chromatography, and characterization of its enzymic properties in comparison with bovine trypsin as a related enzyme.

MATERIAL AND METHODS

Boar Semen — Freshly ejaculated boar semen was obtained from Kanagawa Life Stock Improvement Association (Kanagawa, Japan).

Chemicals — N- α -benzoyl-arginine ethylester (BAEE) was obtained from the Peptide Research Co. (Osaka, Japan). Sephadex G-150 and Sepharose 4B were from Pharmacia Fine Chem. (Uppsala, Sweden). Soybean trypsin inhibitor (SBTI), limabean trypsin inhibitor (LBTI), benzoyl-arginine (Bz-Arg), benzoyl-glycyl-arginine (Bz-Gly-Arg) and bovine trypsin (3 x crystalized) were from Sigma Chemical Co. (St. Louis, M.O., U.S.A.). Aprotinin was obtained from Bayer A.G. (Germany). *Trans*-aminomethyl cyclohexylic acid (*t*-AMCHA) was provided by Eisai Co. (Tokyo, Japan). Potato kallikrein inhibitor (PKI) was purified by the method of Hojima *et al.*^{4,5)} Leucyl-arginine (Leu-Arg) was a gift of Prof. N. Yanaihara (Shizuoka

College of Pharmacy). Phenylalanyl-phenylalanyl-arginine (Phe-Phe-Arg) was obtained from Nittobo Co. (Tokyo, Japan). The other chemicals used were guaranteed reagents.

Siliconized glass or plastic wares were used throughout the experiments.

Determination of Protein Concentration — Protein concentration was estimated spectrophotometrically from the absorbance at 280 nm (Hitachi Spectrophotometer Type 181) taking $E_{280}^{1\%} = 11.1$.

Preparation of Affinity Adsorbents — Arg, Leu-Arg and Phe-Phe-Arg were coupled with BrCN activated Sepharose 4B according to the method of Cuatrecasas.⁶⁾ Arg (60 mg) was dissolved in a small amount of 0.2 M NaHCO_3 , pH 8.1 and coupled to 30 ml of BrCN activated Sepharose 4B. Leu-Arg (100 mg) and Phe-Phe-Arg (500 mg) were coupled in the same condition to 30 ml and 100 ml of activated Sepharose 4B, respectively. The coupling reaction was carried out for 2 hr at 25°. The gel was treated with 0.1 M glycine in 0.3 M NaHCO_3 , pH 8.1 for several hr at 25°.

Disc Gel Electrophoresis — Disc gel electrophoresis was performed with 7.5% (w/v) polyacrylamide gel at pH 4.3. The purified acrosin was run in duplicates with 2 mA/tube for 3 hr. One gel was stained with Coomassie Brilliant Blue and the electrophoretogram was analysed with a Shimazu Dual Densitometer CS 901 and the other gel was sliced into 2 mm width by gel slicer (Hotta Rika, Tokyo) to determine enzymic activity. Each sliced gel piece was suspended in 0.3 ml of 1 mM HCl overnight at 4°, acrosin activity in the extract was determined against BAEE.

Measurement of Acrosin Activity — Acrosin activity was determined by the measurement of the esterolytic activity against BAEE according to the method of Schwert and Takenaka.⁷⁾ The sample (0.2 ml) was mixed with 2.8 ml of 1 mM BAEE in 0.3 M Tris-HCl, containing 0.05 M CaCl_2 , pH 8.3 at 25°, and the increase of absorbance at 253 nm was measured by Hitachi Spectrophotometer Type 124. The activity was expressed in terms of BAEE unit (BAEE U): 1 unit of activity hydrolysed 1 μmol of BAEE in 1 min.

RESULTS

Purification of Acrosin

All purification procedure was carried out at 4° unless otherwise mentioned. Freshly ejaculated boar semen (140 ml) was centrifuged ($11000 \times g$, 20 min at 4°), and the sperm pellet was stored at -20° . The frozen sperm pellet was thawed and extracted with 30 ml of 2% (v/v) acetic acid containing 10% (v/v) glycerin and the supernatant was separated by centrifugation. Further extraction from the pellet was repeated twice in the same manner. The combined extracts was centrifuged ($105000 \times g$, 1 hr at 4°) and the supernatant was concentrated to 20 ml using polyethyleneglycol 20000. Acrosin activity was not detected in this step. This was due to the contamination of considerable amount of seminal plasma acrosin inhibitor in the extract. The concentrated extract was applied to Sephadex G-150 column (3.0×90 cm) equilibrated with 2% (v/v) acetic acid (pH 2.6). Acrosin was separated from the inhibitor and was eluted in the fraction of 37–54 (total volume 174 ml) on the Sephadex G-150 column (Fig. 1). The total BAEE U in the combined active fractions was 1190.

Preliminary Experiments for Affinity Chromatography

Further purification of acrosin was carried out

FIG. 1. Sephadex G-150 Gel Filtration of Acid Extract of Boar Sperm

Elimination of acrosin inhibitor from the active fractions (No. 37–54) was confirmed by the measurement of the activity after neutralization. : This mark indicates the elution of the inhibitor.

by employing affinity of the enzyme to oligopeptides with Arg at the carboxy-termini which have analogous amino acid sequence to the products produced by acrosin. As preliminary experiment for acrosin purification, three types of ligands, *i.e.*, Arg, Leu-Arg and Phe-Phe-Arg were tested. Sepharose 4B coupled with each ligand was packed in a small column and equilibrated with 0.05 M acetate buffer containing 0.5 M NaCl, pH 5.3. An aliquot (20 BAEE U) of pooled fraction of acrosin from Sephadex G-150 gel filtration adjusted to pH 5.3 by 1 N NaOH was applied to the affinity column, and washed with the same buffer until the absorbance at 280 nm became negligible, then the buffer was changed to 5 mM HCl, pH 2.6. Acrosin was efficiently adsorbed by Phe-Phe-Arg Sepharose 4B and eluted out with 5 mM HCl, but it failed to be adsorbed by Arg and Leu-Arg Sepharose 4B. According to the result of preliminary experiment, Phe-Phe-Arg Sepharose 4B was used for the larger scale purification of acrosin.

Purification of Acrosin with Phe-Phe-Arg Sepharose 4B

The pooled active fraction from the gel filtration column was concentrated 2-fold with polyethyleneglycol 20000 and adjusted the pH 5.3, and then applied to the affinity column (2.0 × 15 cm) equilibrated with 0.1 M acetate buffer, pH 5.3. The column was first washed with 0.01 M ammonium formate, 0.3 M NaCl, pH 5.3, then with the same solution containing 10% (v/v) dioxane until absorbance at 280 nm of effluent became negligible, and finally with 5 mM HCl. The acrosin activity was eluted by the final solu-

FIG. 2. *Affinity Chromatography of Acrosin on Phe-Phe-Arg Sepharose 4B following Sephadex G-150 Gel Filtration*

Arrows indicate the replacement of elution solvents.

A: 0.01 M HCOONH_4 , 0.3 M NaCl, pH 5.3.

B: 0.01 M HCOONH_4 , 0.3 M NaCl, 10% (v/v) dioxane, pH 5.3.

C: 5 mM HCl.

tion with a recovery of 96.6% (Fig. 2).

The result of purification was given in Table I. Assuming that all the activity was recovered in the acid extract, sperm contained in 1 ml of semen had 8.5 BAEE U of acrosin activity.

Disc gel electrophoretogram of purified acrosin at pH 4.3 gave a broad protein staining band with relatively slow mobility, but the densitometry analysis of gel indicated the broad band consisted of three peaks of protein staining (Fig. 3). The profile of BAEE activity extracted from the gel slices coincided with the density of protein staining as shown in Fig. 3.

Effects of pH, Metal Ions, Urea and Inhibitors on the Esterolytic Activity of Acrosin

The optimum pH of acrosin for BAEE

TABLE I. *Purification of Boar Acrosin*

	Total A_{280}	Total BAEE U	S.A. ^{a)}	Yield %
Acid extract	435	—	—	—
Sephadex G-150	210	1190	5.67	100
Phe-Phe-Arg-Sepharose 4B	39.8 (35.9 mg)	1149	28.9 (32.1) ^{b)}	96.6

a): Specific activity, U/A_{280} .

b): U/mg .

esterolytic activity was 9.0 (Fig. 4). This was similar to that of trypsin.⁸⁾

The effects of various metal ions on the esterolytic activity of acrosin were tested (Table II). Relatively high concentrations of Co^{2+} (10^{-3} M) and Hg^{2+} (10^{-2} M) inactivated the enzyme. Potentiation of the activity was observed by the addition of Ca^{2+} and was found to be concentration dependent. Similar results were reported for trypsin.⁸⁾

Therefore, the influence of Ca^{2+} on the inhibitory effects of urea on acrosin was examined. This study was done in the presence of

0.05 M CaCl_2 . The activity of acrosin decreased linearly along with the increased concentrations of urea and inactivated completely with 8 M urea, whereas trypsin was not at all affected by 6 M urea and 70% remained active even with 8 M urea (Fig. 5). Thus, the effect of urea on these enzymes was different, and Ca^{2+} did not alter the response of them for urea (Fig. 5).

The inhibitor spectra of acrosin and trypsin were very similar. Inhibition of acrosin was observed with SBTI, LBTI, PKI and aprotinin, which were known as trypsin or kallikrein inhibitors, but not with *t*-AMCHA, a plasmin inhibitor

FIG. 3. Disc Gel Electrophoresis of Purified Acrosin
Gel was stained with Coomassie Brilliant Blue and densitometric tracing was carried out with dual wave length scanner at 750 nm (sample) and 550 nm (reference).

FIG. 4. Effect of pH on the Esterolytic Activity of Acrosin

Substrate solution was 1.0 mM BAEE in 3.0 ml of 0.1 M Britton-Robinson's wide range buffer (pH 3.5–11.0). Purified acrosin (0.5 U, determined at pH 8.3) was used and activity measurement was carried out as mentioned in Method. Maximum activity was expressed as 100%.

TABLE II. Effects of Metal Ions on the Esterolytic Activity of Acrosin

Metal	Relative activity					(M)
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	
HgCl_2	100	113	72	0	—	
NiSO_4	98	98	104	109	—	
CoCl_2	95	81	0	0	—	
CaCl_2	102	106	112	146	150	
ZnCl_2	97	101	105	102	—	

The substrate solution contained 1.0 mM BAEE in 0.25 M Tris-HCl, pH 8.3, containing 10^{-5} – 10^{-1} M metal ions.

Activity of purified acrosin (0.5 U, determined in metal free substrate) was assayed without preincubation.

(Table III). Then, the influence of Ca^{2+} on the inhibitory effect of aprotinin against acrosin was examined. As shown in Table III, the inhibitory activity of aprotinin was decreased in the presence of 0.05 M CaCl_2 .

In order to investigate this finding in more

FIG. 5. Effect of Urea on the Esterolytic Activity of Acrosin

The substrate solution contained 1.0 mM BAEE, 0–8 M urea and 0 or 0.05 M CaCl_2 in 0.3 M Tris-HCl, pH 8.3. Purified acrosin and trypsin (1.0 U, determined in 0.3 M Tris-HCl, pH 8.3) were assayed without preincubation.

○ : Acrosin, ● : Trypsin, Dotted and solid lines indicate the results with and without 0.05 CaCl_2 , respectively.

detail the kinetic parameters of acrosin and aprotinin were measured with and without Ca^{2+} . Calcium ion altered the K_m value of acrosin against BAEE from 0.33 to 0.88 mM and the K_i value of aprotinin against acrosin from 5.8×10^{-4} to 2.0×10^{-2} mM. These results were in good agreement with the results shown in Table II and III.

Kinetic Analysis of Acrosin Activity

The properties of acrosin and trypsin were compared by measuring the kinetic parameters for substrate and the inhibitory effect of enzyme products at pH 5.3–8.3. Bz-Arg and Bz-Gly-Arg, the esterolytic product of BAEE and its related compound, were the model compounds for affinity ligands. Their K_i values against acrosin at various pH were determined and changes of the K_i were compared with those of the K_m values of acrosin against BAEE. As shown in Fig. 6, the K_m of acrosin against BAEE was minimal at around pH 8, indicating the affinity of the enzyme and substrate reached maximal at neutral pH. On the contrary, the K_i values of both Bz-Arg and Bz-Gly-Arg were low at acidic pH (around pH 5) but progressively increased at neutral pH. The enzyme had higher affinity with those compounds at acidic pH and lesser affinity

TABLE III. Effects of the Inhibitors on the Esterolytic Activity of Acrosin

(a)	Inhibition (%)				μg inhibitor
	1	10	10^2	10^3	
LBTI	70.6	87.3	86.3	86.9	
SBTI		1.9	82.9	87.3	
PKI		1.8	15.2	87.2	
<i>t</i> -AMCHA		0	0	0	
(b)	Inhibition (%)				KIU
	CaCl_2 (M)	10	10^2	10^3	
Aprotinin	0	50.2	76.8	85.9	
	0.05	0	13.7	64.4	

KIU: Kallikrein inhibitor unit.

(a) Equal volume of purified acrosin (1.0 U/ml, 0.3 M Tris-HCl, pH 8.3) and the inhibitors 20–2000 $\mu\text{g}/\text{ml}$ were mixed and incubated for 10 min at 25°. The remaining activity was measured and the results were expressed by % inhibition.

(b) Acrosin and aprotinin (20–2000 KIU) were treated as above with or without Ca^{2+} .

FIG. 6. Kinetic Parameters of Acrosin for Substrate and Products at Various pH's

One unit of acrosin (with 1.0 mM BAEE in 0.2 M Tris-HCl, pH 8.3, 25°) was used for kinetic studies. Tris-HCl buffer (0.2 M) was used for pH's 8.3 and 7.3, and acetate buffer (0.2 M) was for pH's 6.3 and 5.3.

(a) The K_m values of acrosin at various pH's were obtained by the Lineweaver-Burk plots using 0.083–0.25 mM BAEE.

(b) The K_i values of Bz-Arg (○) and Bz-Gly-Arg (●) were obtained by using the concentration of 0.2 mM and 0.1 mM of the respective compounds. The range of BAEE concentration was the same as (a) and K_i values were calculated by the Lineweaver-Burk plots.

at neutral environments. This result supports the fact that acrosin was adsorbed by Phe-Phe-Arg Sepharose 4B which had a product analogue type ligand at relatively low pH. The K_i values of Bz-Gly-Arg at various pH were always lower than those of Bz-Arg, this result indicates that insertion of Gly into the Bz-Arg bond induces an increase of affinity. The same tendency was also observed for trypsin.⁹⁾

DISCUSSION

Proteolytic activity in acrosome was first described by Stambaugh and Buckley (1968) in rabbit sperm,²⁾ and this enzyme was named acrosin by Zaneveld *et al.* (1972).³⁾ Schleuning *et al.* (1973) purified boar acrosin by affinity chromatography on *p*-aminobenzamidine (an inhibitor of acrosin)cellulose.¹⁰⁾ Acrosin was adsorbed on this ligand at pH 7–8 and eluted at pH 3. We

employed product analogue ligand, Phe-Phe-Arg coupled to Sepharose 4B, for acrosin purification; this ligand adsorbed acrosin at pH 5 and released it at pH 3.

Kasai *et al.* have described affinity chromatography of trypsin on the product analogue ligands which were oligopeptides with Arg at carboxy-termini.¹¹⁾ Their experiment was useful to discuss about the active site of trypsin. They found that the pH dependency of the affinity for its substrate and products were reversed.¹²⁾ Trypsin bound to the product analogue ligands at pH 5 and dissociated at pH 3.¹¹⁾ The ligands required were no shorter than tripeptides to absorb trypsin.¹¹⁾ Ligand elongation increased the affinity of trypsin due to the lesser steric hindrance by Sepharose beads.^{6,13)} This relates to the depth of substrate binding pocket of trypsin molecule; they suggested that the depth of binding pocket of trypsin seemed to correspond roughly with the length of tripeptides.⁹⁾ Acrosin hydrolysed BAEE and this activity was inhibited by SBTI, hence acrosin is recognized as one of trypsin-like enzyme. In this investigation, the product analogue ligands of different length were employed for study of the nature of its active site. Both acrosin and trypsin were adsorbed by tripeptides-Sepharose (Phe-Phe-Arg Sepharose 4B), which indicates that the depth of binding pockets of acrosin and trypsin are similar to each other. Thus, we could demonstrate the similarity of the active sites of acrosin and trypsin by use of the product analogue ligand in affinity chromatography.

The disc gel electrophoretogram of purified acrosin showed a broad band consisted of three protein peaks, all of which showed acrosin activity. Schleuning *et al.* also found similar observation in their acrosin preparation, and they suggested that these multiple forms were produced by autolysis corresponding to α , β and γ forms of trypsin.¹⁴⁾ They incubated α and β forms of acrosin preparation at pH 8.0 and observed their conversion to the γ form. This conversion was prevented by addition of acrosin inhibitor, such as *p*-nitrophenyl-guanidinobenzoate.¹⁵⁾ Schleuning *et al.* performed affinity chromatography at pH

8,¹⁰) so it might have been possible to have generated multiple forms by autolysis during purification. However, as we carried out all the purification procedures below pH 5.3 at 4°, it is unlikely that the formation of multiple forms occurred in the purification procedures. Those multiple forms might have been produced in the activation process of proacrosin, although the mechanism still remains to be answered.

Stambaugh and Smith reported that the amino acid composition of rabbit acrosin was similar to that of bovine and human pancreatic trypsin, but acrosin lacked half cystine.¹⁶⁾ The different effects of urea on acrosin and trypsin (Fig. 5) suggests that the denaturation may be related to the rigidity of the enzyme molecule by disulfide bonds.

The results obtained in this investigation supported that the enzymic properties of acrosin and trypsin were very similar. The fact that alteration of K_m and K_i values of acrosin according to the change of pH (Fig. 6) is similar to those of trypsin suggests that these enzymes have a very similar type of active site. Such pH dependencies of kinetic parameters are characteristic for trypsin and its related serine enzymes.

In the fertilization processes, capacitation and acrosome reaction take place and Ca^{2+} have been proven to be essential for the latter. Yanagimachi *et al.*^{17,18)} reported that guinea pig sperm could be capacitated in Ca^{2+} free medium, whereas acrosome reaction failed to occur in this medium. The addition of Ca^{2+} to the medium, however, induced the reaction. For the sperm penetration through the ovum membranes, two enzymes, hyaluronidase and acrosin, are known to be required. Hyaluronidase, however, is insensitive to Ca^{2+} .¹⁾ In this report we have investigated the possible function of Ca^{2+} to acrosin activity with the relationship to its inhibitor. Calcium ion decreased K_m value of acrosin and increased K_i value of aprotinin against acrosin. Our results agreed the finding of Brown *et al.*¹⁹⁾ that Ca^{2+} suppressed the inhibitory activity of Kunitz pancreatic trypsin inhibitor on acrosin but not on trypsin. Acrosin inhibitor is located on the surface

of the ejaculated sperm and the release of the inhibitor from the sperm might be a part of capacitation process.^{20,21)} The fact that Ca^{2+} increased the K_i value of aprotinin against acrosin suggests that the role of Ca^{2+} in the fertilization processes may be not only to initiate the acrosome reaction but also to regulate the acrosin activity along with the endogeneous acrosin inhibitor.

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