Purification of human seminal acrosin inhibitor and its kinetics

ADITI CHATTERJEE, SAGARIKA KANJILAL* and ASOK K BHATTACHARYYA**

Reproductive Biology Laboratory, Department of Biochemistry, Calcutta University College of Science, 35, Ballygunge Circular Road, Calcutta 700 019, India

**Department of Veterinary Pathobiology, University of Minnesota, Twin Cities, MN 55108, USA*

***Corresponding author (Fax, 91-33-4764419 ; Email, ashokbha@cal2.vsnl.net.in).*

A low molecular mass, naturally occurring acrosin inhibitor has been identified and purified (490·7-fold) from human semen, and kinetic studies have been performed on the association characteristics as well as for the determination of affinity constants (K_i values). The results show that K_i value ($3{\cdot}34 \times 10^{-2}$) of the inhibitor towards human acrosin is almost three times lower than that of pancreatic trypsin, indicating a much higher specificity and inhibitory property for acrosin. The purified human seminal acrosin inhibitor has a molecular mass of 5·5 kDa and shows a single band using 10–20% gradient SDS PAGE. The work is of great significance for the development of more specific, nontoxic and irreversible inhibitors for human acrosin.

1. Introduction

Spermatozoa possess a multitude of enzyme systems, and one of them is acrosin which is present in acrosome and plays a major role in fertilization (Bartoov *et al* 1994). The acrosome is an exocytotic vesicle of Golgi origin that lies over the anterior portion of the sperm head and contains several hydrolytic enzymes (Bhattacharyya and Kanjilal 1991; Bhattacharyya and Zaneveld 1982) apart from proacrosin-acrosin system. Indirect immunoperoxidase staining has revealed that of them at least proacrosin, the zymogen of the trypsin-like enzyme, is distributed in the acrosome in granules rather than in the homogenous form (Phi-Van *et al* 1983). These enzymes are released during fertilization in a membrane fusion event called the acrosome reaction (Liw and Baker 1990), presumably immediately after contact with the egg investments.

Both *in vivo* (Zaneveld *et al* 1970) and *in vitro* (vander Ven *et al* 1985; DeJonge *et al* 1993) studies in the presence of acrosin inhibitors indicate that acrosin plays an essential role in the sperm-egg interaction system as well as in the penetration of zona – the most species specific component of egg. Acrosin has been shown to be inhibited by various proteinase inhibitors, namely soyabean trypsin inhibitors, and aprotinin (Zaneveld *et al* 1972; Kobayashi *et al* 1991). Particularly effective are the microbial inhibitors leupeptin and antipain (Fritz *et al* 1973). Apart from these, inhibitors like acrosome reaction inhibiting glycoprotein (ARIG) a 74 kDa protein (Drisdel *et al* 1995) and a protein C inhibitor of acrosin (Zheng *et al* 1994) with a molecular mass of 57 kDa have been isolated from seminal plasma. The active acrosin quickly associates with the naturally occurring inhibitors present in acrosomal extracts and masks much of the enzyme activity (Bhattacharyya and Zaneveld 1982).

Though the occurrence of highly active low molecular mass natural inhibitors has also been documented, no significant work has been done in regard to the purification of this low molecular mass human seminal inhibitor (HSI). The observation is that low molecular mass inhibitors can also prevent fertilization (Zaneveld *et al* 1973), encouraged us for further study on the proteinase inhibitors, particularly on its purification and kinetics of association with the active form of the enzyme.

2. Materials and methods

2.1 *Materials*

Fresh semen samples were obtained from selected healthy doners possessing normal sperm count and motility with an abstinence of 3–5 days from SSKM Hospital, Calcutta

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and Repose Nursing Home and Fertility Clinic, Calcutta. Pancreatic Trypsin, α-N-benzoyl-L-arginine ethyl ester (BAEE), acrylamide, bis-acrylamide, TEMED, Coomassie brilliant blue R-250 and centricon 10 and 30 were obtained from Sigma Chemical Company, USA. The spectrpor membrane tubings were obtained from Spectrum Medical Industries Inc., Los Angeles, USA. Bromophenol blue was the product of BDH chemicals, England. Sephadex G-50 was purchased from Pharmacia Biotech, Sweden. The standard markers used in SDS PAGE were from Bio-Rad, USA. Bicinchoninic acid was obtained from Pierce Chemical Company, USA. Other chemicals (Tris, conc. HCl, $CaCl_2$, SDS, glycerol etc.) were of analytical or guaranteed reagents grade and were purchased from E Merck, Germany or from BDH, England or from Spectrochem, India.

2.2 *Collection and separation of seminal plasma and spermatozoa*

Ejaculates, obtained from healthy doners, were centrifuged at 6,000 *g* to separate the spermatozoa from seminal plasma. The seminal plasma was processed for isolation of the inhibitor and the sperm pellet was used for extraction of acrosin. The human seminal plasma (HSP) thus obtained was centrifuged at 12,000 *g* in CR2B2 Himac centrifuge for 1 h. The pellet was discarded and the supernatant was centrifuged at 80,000 *g* in Hitachi SCP70H centrifuge for 20 h using RP65T rotor. The supernatant thus obtained was then dialysed overnight (cut off size 1200–1500 daltons) against deionized water at 0–4°C with three changes. The dialysed material was then lyophilized in Lyolab BII model lyophilizer. The lyophilized powder was stored at –70°C or used immediately for further purification. The lyophilized powder was dissolved in 50 mM Tris-HCl buffer, pH 7·4 (dialysed soluble supernatant-DSS). This was processed for 10 kDa cut with centricon 10. The fraction obtained below 10 kDa was retained and was named human seminal inhibitor (HSI) and the one above 10 kDa was discarded.

The HSI was divided into two parts: (i) The first part was precipitated with 10% TCA (final concentration) and centrifuged at 10,000 *g* to obtain the required protein. The precipitate was washed repeatedly with chilled acetone to remove any adhering TCA. The precipitate thus obtained was air dried and redissolved in Laemine sample buffer pH 6·8 and run in a 10–20% gradient SDS PAGE to determine the molecular mass of the purified protein. Along with the purified HSI, crude seminal plasma (CSP); dialysed soluble supernatant (DSS) as well as standard markers were run in both SDS and native PAGE. The concentration of the protein in each lane was 30 μg and the electrophoresis was run for two hours at a constant voltage of 70 volts at 15°C. The protein content was estimated using bicinchoninic acid (Smith *et al* 1985). After destaining, the gels (lanes 3 and 4) were scanned using an LKB Bromma 2202 Ultrascan Laser densitometer.
(ii) The second part of HSI containing 0·91 mg protein per ml was serially diluted with Tris-HCl buffer, pH 7·4 to various concentrations (from 9 : 1 to 1 : 1– inhibitor : buffer) which were used to determine the K_i values of the purified inhibitor with pancreatic trypsin (1 mg/ml; activity-14,000 units/mg) and purified human acrosin (1420 mIU/150 × 10^6 cells/ml) as well as the K_m and V_{max} for both the enzymes.

2.3 *Isolation of acrosin*

The human sperm pellet containing 150 × 10^6 spermatozoa, were washed twice with 10 mM phosphate buffer, pH 7·4 and were separated at 6,000 *g* for 10 min at 0–4°C. The washed spermatozoa were then used for extraction of acrosin with 1 ml of HCl at pH 3 at 4°C. Throughout the process inert plastic tubes were used as acrosin sticks to glass unless siliconized. Extraction was carried out for 1 h at 4°C with continuous stirring using magnetic stirrer. The acid extracted spermatozoal suspension was then centrifuged at 27,000 *g* in Hitachi SCP70H model centrifuge for 45 min at 4°C. The supernatant thus obtained contains acrosin at pH 3 at which the enzyme is relatively stable. This was concentrated by ultrafiltration through centricon 30 and the fraction above 30 kDa was passed through Sephadex G-50 for further purification which was equilibrated at pH 3. The Sephadex G-50 column had a bed volume of 9·5 ml and fractions were collected at an interval of 10 min with a flow rate of 9·4 ml/h with 1 mM HCl. Active fractions appeared between tube numbers 3 and 5. This resulted in a 50-fold purified acrosin which was used for studying the kinetics of the human seminal inhibitor.

As stated earlier the isolated inhibitor was serially diluted for its kinetic studies. The buffered substrate used was BAEE at variable concentrations in the presence of 50 mM $CaCl_2$ and 50 mM Tris-HCl at pH 8. For acrosin assay the concentration of BAEE was varied from 0·5 mM to 0·188 mM and for trypsin the concentration was varied from 0·5 mM to 0·125 mM. Below these concentrations complete inhibition of the enzyme occurred due to very low substrate concentrations and above 0·5 mM no significant change in inhibition pattern was observed. The residual activity i.e., the inhibited activity of both the enzymes were measured by observing the change in absorbance for 5 min at 253 nm at 25°C in a Hitachi U-3210 spectrophotometer after allowing maximum enzyme-inhibitor complex formation by incubation at 25°C for 10 min at pH 8 in Tris-HCl buffer. A molar absorbance difference of 1150 M^{-1} Cm^{-1} was used to convert the change in optical density to micromoles of BAEE hydrolysed. One mIU of activity was defined as the amount of enzyme which causes the hydrolysis of 1 nmole

Table 1. Purification steps of human seminal inhibitor.

Steps	Fractions	Residual activity[a] of acrosin in mIU/7·5M[b]/min	Activity of fractions in mIU/ml/min	Protein content in mg/ml	Specific activity	Fold purification	Yield in (%)
I	HSP	69·5	30	35·00	0·86	—	100·0
II	DSS	60·2	216	5·90	36·61	42·6	16·9
III	HIS	51·8	384	0·91	421·98	490·7	2·6

[a]The enzyme was incubated with different fractions containing natural inhibitors for 10 min at 25°C and the residual activity was assayed as described in § 2.
[b]M indicates million (cells).

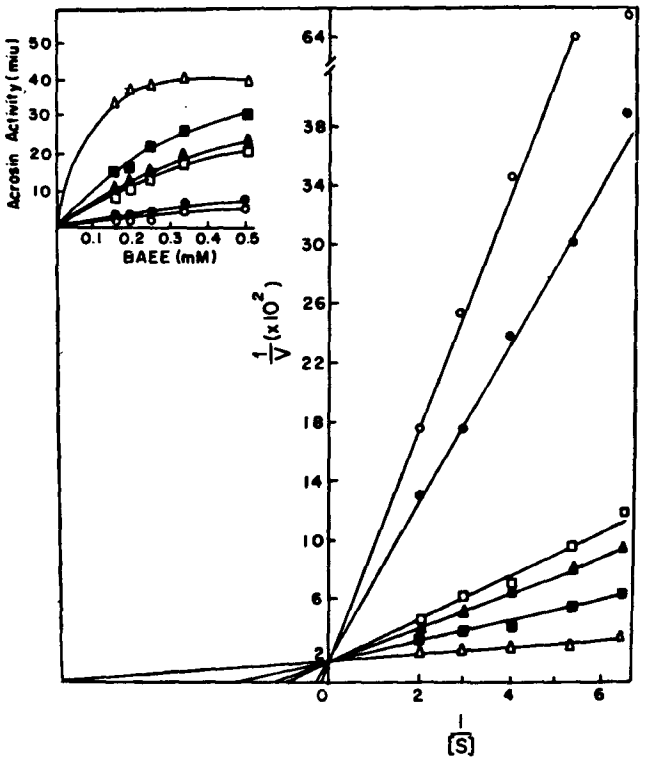

Figure 1. Double reciprocal (Lineweaver–Burk) plot for the inhibition of human acrosin by HSI using BAEE concentrations from 0·188 mM–0·5 mM. (Δ), No inhibitor; (■), 1 : 1 HSI; (▲), 3 : 2 HSI; (□), 4 : 1 HSI; (●), 9 : 1 HSI; (O), undiluted HSI. *Inset*: Michaelis Menten plot of acrosin against varying concentrations of substrate.

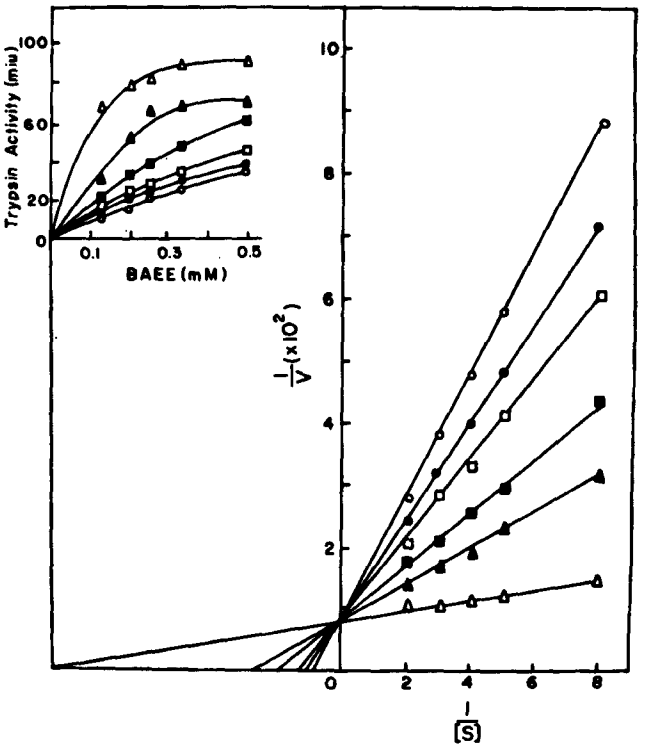

Figure 2. Double reciprocal (Lineweaver–Burk) plot for the inhibition of pancreatic trypsin by HSI using BAEE concentrations from 0·125 mM–0·5 mM. (Δ), No inhibitor; (▲), 1 : 1 HSI; (■), 7 : 3 HSI; (□), 4 : 1 HSI; (●), 9 : 1 HSI; (O), undiluted HSI. *Inset*: Michaelis Menten plot of pancreatic trypsin.

of BAEE in 1 min at 25°C (Goodpasture *et al* 1980). For each substrate concentration a blank was run prior to experiments. From the results thus obtained the K_m and V_{max} values of the enzyme as well as the affinity constant (K_i) of the inhibitor were calculated.

3. Results

The inhibitor from human seminal fluid has been purified using two step methodology and a 490·7-fold purification has been achieved (table 1). The 50-fold purified human acrosin with an activity of 71 mIU/7·5 × 10^6 cells/min was used for determining the kinetic properties of the natural inhibitor. The crude acid extract of human sperm acrosome had an acrosin activity of 212·2 mIU/150 × 10^6 cells/min with a specific activity of 620·4. An activity of 918·3 mIU/150 × 10^6 cells/min with specific activity of 3,748·0 (6·0-fold purified) was obtained after centricon 30 cut, and employing Sephadex G-50 exclusion chromatography a single peak with 1,420 mIU/150 × 10^6 cells/min activity and a specific activity of 31,119·9 could be obtained. Over-all purification was 50·2-fold and the enzyme was stored at pH 3 for all kinetic studies.

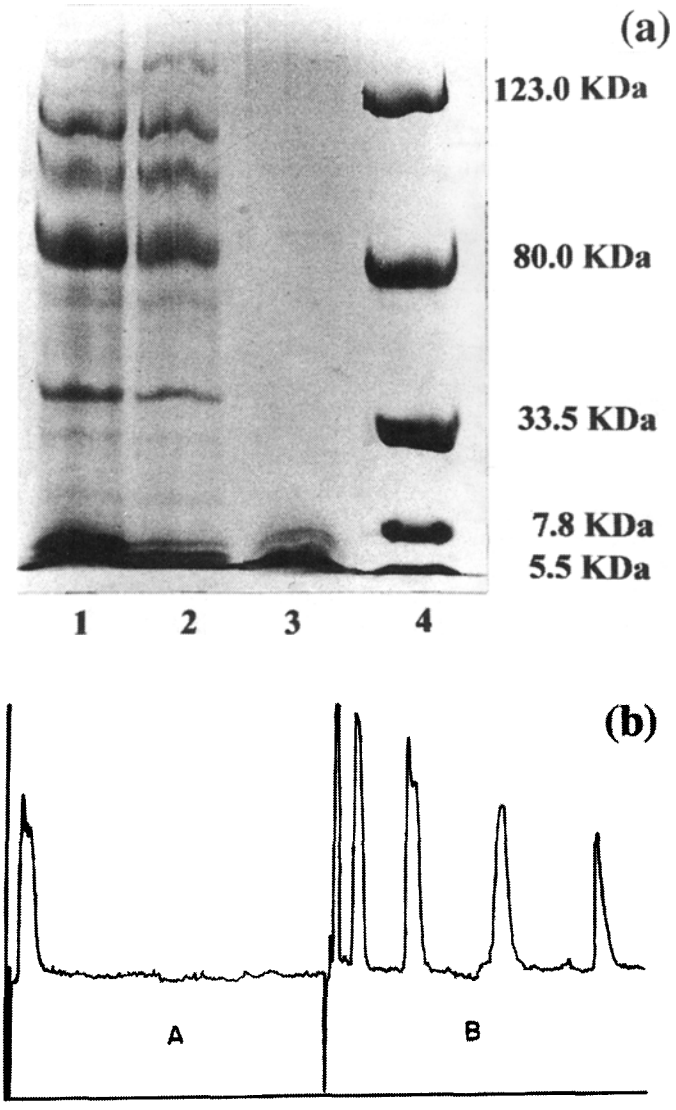

Figure 3. (a) SDS-PAGE pattern (10%–20%) of the purified HST. Lane 1, human seminal plasma (HSP, crude); lane 2, dialyzed soluble supernatant (DSS); lane 3, purified human seminal inhibitor (HSI, 5·5 kDa); lane 4, standard protein markers (123·0 kDa: β-galactosidase; 80·0 kDa: bovine serum albumin; 33·5 kDa: carbonic anhydrase; 7·8 kDa: aprotinin). (b) Laser densitometry scan. (A), Lane 3 of SDS PAGE showing a single peak; (B), lane 4 of SDS PAGE identifying the protein markers.

A 42·6-fold purification of inhibitor was achieved by prolonged ultra centrifugation at 80,000 *g* with an yield of 16·9% from the crude seminal fluid. Subsequently, centricon 10 cut yielded 11·5-fold purification from step II with an overall purification of 490·7-folds. The inhibition kinetics study of HSI showed that it is competitive in nature. This has been concluded from the

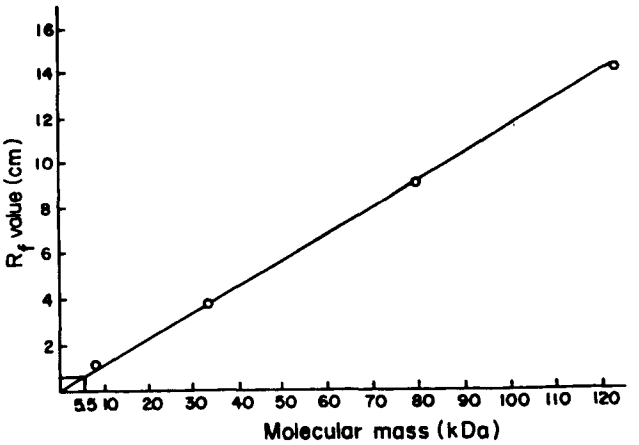

Figure 4. Plot of R_f value against molecular mass (kDa) of standard protein markers and purified HSI.

Lineweever-Burk (double reciprocal) plots (figures 1, 2), where the control and the experimental curves intersected at the ordinate. The kinetic characteristics of the inhibitor obtained with acrosin and trypsin revealed that the inhibitor has a higher affinity for acrosin than towards trypsin, and its K_i value (affinity constant) is almost three times lower than that of trypsin. K_i value for trypsin is $9{\cdot}84 \times 10^{-2}$ where as that for acrosin it is $3{\cdot}43 \times 10^{-2}$. The V_{max} values of trypsin and acrosin were 125 mIU and 55·5 mIU respectively. Following the same order, the K_m of the two enzymes are 0·112 mM and 0·166 mM. These reveal that the complex formation between the acrosin and the inhibitor takes place at a faster rate than pancreatic trypsin.

The molecular mass of HSI as determined from the R_f value of the 10–20% SDS as well as native PAGE was 5·5 kDa.(figures 3a, 4) and the inhibitor is peptide in nature. Barring the gel pictures, the laser densitometry scan (figure 3b) of the gel (lanes 3 and 4) clearly showed a single peak for the purified inhibitor.

4. Discussion

This work represents the first report on the purification of a stable, low molecular mass acrosin inhibitor of human semen. The brief two-step procedure yields 490·7-fold purified protein with relatively high affinity towards human spermatozoal acrosin in comparison to pancreatic trypsin.The presence of this kind of inhibitor in human ejaculate/spermatozoa (Zaneveld *et al* 1973; Bhattacharyya and Zaneveld 1978) is of physiological significance for the spontaneous inhibition of excess enzyme which may be released in the female upper genital tract particularly during acrosome reaction and zona reaction.

Soyabean trypsin inhibitor (SBTI), a natural inhibitor, also inhibits human acrosin activity (Liw and Baker 1993). But in the *in vitro* fertilization experiment in mouse, these inhibitors were almost ineffective (Bhattacharyya *et al* 1979). However, the present purified human seminal inhibitor, apart from being of low molecular mass and non-toxic due to its presence in the physiological system, has very high affinity towards human acrosin. Though earlier reports have been documented on the characteristics of similar low molecular mass natural inhibitors (Zaneveld *et al* 1973), as well as a large component inhibiting acrosin (Zheng *et al* 1994) with a molecular mass of 57 kDa which has been purified to homogeneity (Mandal and Bhattacharyya 1994) and are presumably cleaved to low molecular mass protein after completion of spontaneous liquefaction (Lilja and Laurrel 1984), the present investigation could establish the homogeneity of an active low molecular mass protein. This is apparent from the appearance of a single 5·5 kDa protein band on SDS PAGE and the component may easily be the most active breakdown product of the high molecular mass components that exist before liquefaction (Mandal and Bhattacharyya 1994). The molecular mass of the present inhibitor is in good agreement with the earlier findings which indicate an approximation of molecular mass between 5·4–6·2 kDa (Zaneveld *et al* 1973; Schumacher *et al* 1974).

The natural inhibitor being competitive and reversible in nature gets detached from the sperm cells in the secretions of the female genital tract (Zaneveld *et al* 1973). As such, the naturally occurring inhibitor isolated in its present physiological form needs a modification before it is considered as potential contraceptive. For this the inhibitor is to be converted into an uncompetitive or a non-competitive agent. This can be achieved by changing their characteristics through the attachment of the protein with some ligands. The fact that potential contraceptives should be irreversible in nature, had diverted the attention of eminent scientists on synthetic inhibitors. One such well documented inhibitor is p-nitro phenyl p′ guadinobenzoate (NPGB) which is uncompetitive in nature and inhibits at a very low concentration (Bhattacharyya *et al* 1976) as well as has higher specificity towards acrosin in comparison to goat acrosin and bovine pancreatic trypsin (Bhattacharyya *et al* 1986). However the compound being toxic could not be used as a contraceptive agent (Zaneveld *et al* 1979). The present study on the purification of human seminal inhibitor is of great significance for the possibility of developing these small molecular mass peptides into irreversible inhibitors for acrosin.

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