IDENTIFICATION AND PURIFICATION OF AN ASPARTIC PROTEINASE FROM HUMAN SEMEN

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ABSTRACT

To purify and evaluate the molecular changes associated with an aspartic protease (Cathepsin D) in human semen from infertile subjects. Cathepsin D was purified from normo-, oligo- and azoospermic semen, by a procedure involving detergent solubilisation, affinity chromatography and gel filtration chromatography. The enzyme from normo-, oligo- and azoospermic samples was purified 86, 60 and 44 fold respectively. The purified enzyme appeared as a single band on SDS as well as on native PAGE irrespective of the pathological conditions. The molecular weight of Cathepsin D from oligospermic and normospermic samples was 40 kDa while that of azoospermic sample was found to be 43 kDa. The enzyme was inhibited by pepstatin while other proteinase inhibitors and metal ions did not have any effect. Purified Cathepsin D from azoospermic sample differs from normospermia and oligospermia.

KEY WORDS

Purification, Cathepsin D, aspartic protease, semen.

KEY MESSAGES

1. Semen is a mixture of complex functional proteases, which can mediate protein degradation.
2. The purified aspartic protease showed a bimodal pH optima at 1.6 and 2.5.
3. The semen aspartic protease was identified as cathepsin D.
4. The enzyme from normal and pathological semen differs in specific activity and molecular properties.

INTRODUCTION

The presence of proteinases and peptidases in human seminal plasma is known since long (1). One of the suggested roles of these enzymes is their fibrinolytic activity responsible for the liquification of ejaculated semen (2). These enzymes may also be important in sperm transport (3). Proteolytic enzymes have been found to dissolve

the zona pellucida of the mammalian egg and high semen viscosity is associated with low proteinase activity.

Although acrosin is regarded as the key zona lysin, it has been proposed that other enzymes might act singly or in concert with acrosin. Studies carried out on acrosome reveal that delayed penetration of zona pellucida by the sperm results from the altered rate of protein dispersal from the acrosome (4). Acrosomal enzymes such as glycohydrolases and proteases are reported to be important factors involved in regulating the penetration of zona pellucida (5).

Low pH is an important requirement for dissolving the zona pellucida (6). Acrosome being a modified lysosome, its hydrolytic enzymes are similar to those associated with lysosomes, possessing optimum activity at acidic pH. Aspartic proteases are a major group of lysosomal enzymes postulated to play an important role in protein degradation under various physiopathological conditions (7). Since semen is a mixture of complex functional proteins, aspartic proteases which can mediate protein degradation could play a vital role in determining the semen quality (8).

Aspartic proteases, especially Cathepsin D from human semen have not been adequately studied. Hence, in the present communication, an attempt has been made to purify the enzyme to homogeneity by affinity chromatography and to

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characterize it from the semen of normal and pathological subjects.

MATERIAL AND METHODS

Pepstatin - A Agarose, hemoglobin, casein, bovine serum albumin (BSA), pepstatin A, TPCK (L-1-Tofylamido-2-phenyl-ethyl chloro methyl ketone), TLCK (N- p- tofyl- α - lysine chloromehtyl ketone), leupeptin, EDTA (Ethylene diamine tetra acetate), PMSF (Phenyl methyl sulphonyl fluoride), Tris (Tris (hydroxy methyl)- amino methane, 2-mercaptoethanol, Brij 35, SDS, ammonium persulphate were procured from Sigma Chemicals Co., St Louis, USA. Sephadex-G-100 was procured from Pharmacia fine Chemicals, Uppasala, Sweden. Acrylamide and N,N-methylene bisacrylamide were purchased from Merck, Germany. SDS - PAGE (Sodium Dodecyl Sulphate - Polyacrylamide gel electrophoresis), low molecular weight markers were purchased from BIORAD laboratories, Richmond, USA. All the other chemicals were of Analar Grade.

Semen Samples

Semen samples were collected in a sterile container from patients (20 - 40 years of age), consulting at the infertility clinic. The patients and normal subjects were asked to abstain from sexual activity for at least 72 hours to enhance optimal quality and quantity of semen. Samples were then obtained by masturbation. The freshly ejaculated samples were allowed to liquefy for 30 minutes at room temperature before evaluation for sperm characteristics according to guidelines laid down by W. H. O. (1992) (9). The specimens of each category (normal and pathological) were pooled immediately after characterization in a properly labeled sterile container and stored at - 40°C.

For studying the properties of the aspartic protease (cathepsin D) samples from 12 to 15 subjects of each group (normo-, oligo- and azoospermic) were pooled separately. The pooled volume of each sample was 35 to 40 ml. The pooled specimens were homogenized in presence of Triton X - 100 in a final concentration of 0.1% using a polytron homogenizer at a setting of 5 for 2 minutes and subjected to seven freeze - thaw cycles using dry ice and acetone mixture. The homogenized specimens were diluted 1: 2 times with distilled water and centrifuged at 15,000 rpm for 15 minutes at 4°C. The supernatant was adjusted to pH 3.8 with 5M HCl and the resultant precipitate was removed by centrifugation at 12,000 rpm for 10 minutes at 4°C.

Affinity Chromatography

The supernatant was chromatographed on a

pepstatin agarose gel column pre - equilibrated with 50 mM acetate buffer pH 3.8, containing 0.02% Brij 35 and 5% glycerol. The enzyme was eluted with 50 mM Tris / HCl buffer pH 7.0 containing 0.02% Brij 35 and 5% glycerol. Active fractions of aspartic proteinase were pooled and concentrated. The same procedure was repeated for separation of enzymes from oligo- and azoospermic samples.

Gel Filtration Chromatography

Fractions obtained after affinity chromatography were pooled, concentrated by ultrafiltration and applied to Sephadex-G-100, in 20 mM acetate buffer pH 3.8 containing 0.02% Brij 35 and 5% glycerol. The active fractions were collected and concentrated. Homogeneity of the enzyme was determined by polyacrylamide disc gel electrophoresis. Unstained gels were sliced into 12 - 15 segments and the enzyme activity was assayed. Molecular weight was determined by SDS - PAGE.

Protein Estimation

The proteolytic end products and protein content were determined by the method of Miller (10) DL-tyrosine and crystalline Bovine serum albumin were used as standards. The unit of enzyme activity was expressed as nmoles of tyrosine liberated / minute.

Enzyme Assay

Haemoglobin splitting activity of aspartic proteinase was determined by the method of Barrett and Heath (11).

RESULTS

Purification and partial characterization of aspartic protease

Data presented in Table 1 reveals that aspartic protease was purified 86, 60 and 44 fold with 0.6%, 1.1% and 1.2% recovery from normo-, oligo- and azoospermic semen respectively.

The diluted acid extract was charged on a pepstatin agarose gel column. Pepstatin, a powerful inhibitor of carboxyl proteinase, retards acid proteinase covalently on an affinity column. The bound enzyme was eluted as a sharp peak by changing the pH from 3.8 to 7.0. The elution patterns of the enzyme obtained from normospermic, oligospermic and azoospermic semen samples, after affinity chromatography are depicted in Fig. 1a, 1b, 1c respectively.

Gel filtration brought about a high degree of purification Fig. 2a, b, c. The enzyme eluted from the gel permeation column as a single peak, which

Table 1. Purification of an acid proteinase from human semen

Step	Specimen	Specific Activity (nmole/mg/protein/min)	RSA*	Yield# (%)
Homogenized with Triton X - 100	Normospermic	81.0	1.0	100.0
	Oligospermic	73.0	1.0	100.0
	Azoospermic	62.0	1.0	100.0
Solubilized & centrifuged supernatant	Normospermic	78.0	0.96	97.0
	Oligospermic	72.0	0.90	97.0
	Azoospermic	61.0	0.98	98.0
Pepstatin agarose chromatography	Normospermic	2016.0	25.0	1.87
	Oligospermic	1821.0	25.0	3.2
	Azoospermic	1491.0	24.0	3.7
Sephadex G-100	Normospermic	6940.0	86.0	0.57
	Oligospermic	4363.0	60.0	1.06
	Azoospermic	2739.0	44.0	1.18

* Relative Specific Activity is calculated as the ratio of specific activity in the fraction to that in the homogenate.

Yield % is computed on the basis of the activity in homogenate which is considered as 100%.

Fig. 1a. Affinity chromatography :
Purification of acid proteinase from
normospermic human semen sample

Fig. 1b. Affinity chromatography :
Purification of acid proteinase from
normospermic human semen sample

Table 2. Influence of inhibitors on an acid protease purified from Normospermic (N), Oligospermic (O) and Azoospermic (A) human semen

Compounds	Concentration	Activity retained (%)		
		N	O	A
No addition	-	100	100	100
TPCK	1 mM	82	76	85
ZnSO ₄	1 mM	100	100	100
Pepstatin	10 µM	47	63	72
PMSM	5 mM	100	100	100
Iodoacetamide	10 mM	89	100	100
Cobalt Chloride	1 mM	92	97	99
Mercuric chloride	1 mM	86	80	80
Urea	1 mM	100	100	100
EDTA	10 mM	82	80	80
Zinc acetate	1 mM	88	100	100
Leupeptin	100 nM	90	80	85

The enzyme was preincubated with the inhibitors at room temperature in 0.1 M sodium acetate buffer pH 3.8 for 60 minutes and then the remaining activity as assayed using haemoglobin.

Fig. 3. Native PAGE pattern of Cathepsin D from semen samples

1 = Normospermic 2 = Oligospermic
3 = Azoospermic

Fig. 4. SDS PAGE pattern of Cathepsin D from semen samples

N = Normospermic O = Oligospermic
A = Azoospermic
M = Standard Molecular Weight Markers

on Native PAGE migrated as a single band (Fig. 3), indicating that the preparation was homogenous. When the segments of the unstained gel were examined for aspartic proteinase activity, the region corresponding to the protein band matched with the zone of highest enzyme activity. The apparent molecular weight of aspartic proteinase from normospermic and oligospermic categories was found to be 40 kDa whereas azoospermic samples exhibited a molecular weight of 43 kDa, when compared with standard molecular markers by SDS-PAGE (Fig. 4).

The enzyme from all the three sources showed a temperature optimum at 50°C. The enzymes exhibited bimodal pH optima at 1.6 and 2.5 with haemoglobin as substrate. The enzymes from all the three sources were stable upto 45°C for 2 hours, but were completely inactivated at 65°C within 2 hours.

The enzymes shows preferential activity towards haemoglobin followed by casein and BSA. The enzymes from normo-, oligo- and azoospermic samples were inhibited 53%, 37% and 28% respectively by pepstatin. Other protease inhibitors were without effect (Table 2).

DISCUSSION

We have been successful in isolating cathepsin D to homogeneity from normo, oligo and azoospermic human semen samples by pepstatin agarose affinity chromatography. Although the yield during this step is reduced drastically from 97- 98% to 3-4%, the affinity step is of importance for getting the enzymes in a highly pure form. The primary aim in the present investigation was the qualitative assessment of the influence of reproductive defects on the molecular properties of cathepsin D. Hence the pepstatin agarose step was used despite the fall in yield. The aspartic protease purified from human semen of normospermic, oligospermic and azoospermic samples showed temperature optimum at 50°C and a bimodal optima at 1.6 and 2.5. A molecular weight of 40 kDa was deduced for the enzyme in normospermic and oligospermic samples, while that from azoospermic samples exhibited a molecular weight of 43 kDa. The enzymes degraded haemoglobin and were inhibited by pepstatin. Based on these characteristics, the enzyme can be identified as Cathepsin D. Cathepsin D is a major lysosomal proteolytic enzyme and has been purified from various animal tissues. Although Cathepsin D has been routinely assayed at 37°C, a higher temperature optimum and stability has been reported for a purified preparation from fish skeletal muscle (11). The purified enzyme when stored at -40°C was found to be stable for three months.

Optimal activities at two different pH values 1.6 and 2.5 has been observed for the Cathepsin D in human semen. Bimodal pH dependencies at pH 3.4 and 3.8 have been also reported by Cunningham and Tang for Cathepsin D from porcine spleen. This property could be an intrinsic property of haemoglobin - Cathepsin D system presumably determined by the source of enzyme and the substrate.

The specific activities of Cathepsin D in the oligospermic and azoospermic groups were significantly lesser when compared with normospermia (Table 1); 37% less in the case of oligospermia and 60% less in the case of azoospermia. This decrease in specific activity could not be ascribed to the presence of contaminating non-enzymatic proteins because the enzyme preparations have been found to be homogenous, showing a single band seen in native as well as SDS PAGE. It is probable that the relative catalytic efficacy of the enzymes from the pathological group could be significantly retarded. This contention is supported by the observation that the yield of the enzymes is considerably more in the pathological groups in spite of the low specific activity. The observed difference in the molecular weight of the enzyme from azoospermics could be due to structural changes in the post-translational processing of the enzyme. It is well known that Cathepsin D could undergo post-translational modifications (12). Such changes could indeed lead to apparent differences in molecular weight.

The purified protease under study, preferentially cleaved protein substrates in the order haemoglobin > casein > bovine serum albumin. It is reported that at acidic pH values, the conformation of haemoglobin favours the exposure of several hydrophobic areas, which are preferentially cleaved by Cathepsin D (13). This renders the protein sensitive to cleavage. Inhibitors such as TLCK, TPCK, leupeptin, EDTA, urea, iodoacetamide, cobalt chloride, mercuric chloride and zinc acetate were found to be ineffective.

Studies using pepstatin, a specific inhibitor of aspartyl proteases indicates that the proteolysis of the seminal proteins at acidic pH is carried out by these proteases (14). Not all men have enough concentration of aspartic proteases in their seminal plasma for proteolysis. Since, Cathepsin D exerts its effects only under acidic conditions, women with an abnormally high vaginal pH may be more likely to produce antibodies towards seminal protein having even low levels of Cathepsin D. Earlier findings had shown that Cathepsin D, along with other lysosomal marker enzymes, exhibit marked variation in their levels in normospermic and

azoospermic men (15). It may therefore, be concluded that the function of the human seminal Cathepsin D is to degrade vaginally introduced seminal proteins which may in turn prevent the development of immuno-infertility in women and the degraded low molecular weight proteins may be useful in oocyte and sperm fusion by zone pellucida permeation (16, 17).

Although the enzymes from normal and oligospermic pathological semen samples exhibit identical properties, the purified enzymes, could differ in their biological functions. Further studies using purified enzymes from normal and pathological semen samples will be needed to precisely assess their role in sperm migration through cervical mucus.

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