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ACID PROTEASE AND ITS PROENZYME FROM HUMAN SEMINAL PLASMA

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An acid protease with an optimum pH of 2.5 was first described in human seminal plasma as pepsin and pepsinogen (1), but had not been purified or characterized. Recently, we have purified the acid protease and its proenzyme from human seminal plasma (2,3). In many respects, the properties of seminal plasma acid protease are similar to those of gastric pepsin. Since the proenzyme is more stable than the active enzyme in alkaline solution and can be converted into its active form in acidic solution, the acid protease is likely to exist in seminal plasma, at the physiological pH around 7.5 (4), in proenzyme form.

It seems improbable for the proenzyme to be activated in the slightly alkaline pH of seminal plasma. Under physiological conditions, however, when the semen is freshly deposited in the vagina and mixed with the vaginal discharge, a slightly acidic environment is created (5). The pH of vaginal fluid is changed from 4.3 to 7.2, and from 3.5 to 5.5, in normal and low fertility respectively, after mixing with semen. Therefore, it is of particular interest to investigate the acid dependency of activation of the proenzyme.

It has been suggested that the proteolytic activity of seminal plasma is an essential prerequisite to sperm penetration through the cervical mucus (6); thus, the possible physiological function of the acid protease is also discussed in this report.

EXPERIMENTAL PROCEDURE

Purification of Acid Protease

The proenzyme and active form of acid protease were purified as described previously (3).

Assays for Acid Protease Activity

The activity of both proenzyme and active acid protease were assayed with acid-denatured hemoglobin substrate by the method of Kassell and Meitner (7); the proenzyme is converted to the active form in the acid conditions of the assay. The 2 ml assay mixture contained 0.1 M citrate-phosphate buffer, pH 2.5, 25 mg hemoglobin and appropriately diluted enzyme concentration. The assays were performed for 30 min at 37° and terminated by addition of 2 ml of 5% trichloroacetic acid; the absorbance at 280 nm of soluble fraction was then measured. To assay proenzyme, the pH of the incubation was raised to 8 with 1 M Tris to destroy the active form. This solution was then acidified to activate and assay for the proenzyme.

Activation of Proenzyme of Acid Protease

The proenzyme solution was incubated at 37° in 0.1 M citrate-phosphate buffer at the desired acid pH. After an appropriate time interval, aliquots were transferred into 1 M Tris, pH 8.0 and further incubated for 30 min, stopping activation as well as destroying the active enzyme. The remaining proenzyme was then assayed at pH 2.5 as described.

Hydrolysis of Cervical Mucus Protein by Acid Protease

The cervical mucus protein, containing 1-2 mg protein, was incubated with 5 mg of acid protease in 0.1 M citrate-phosphate buffer, pH 2.5 at 37° for 1 hr. After stopping the reaction by raising the pH of the incubating mixture to 7 with NaOH, it was then lyophilized. Digestion of the protein was detected by using SDS-gel electrophoresis (8).

Protein Determination

Protein concentration was determined by the method of Lowry *et al.* (9) using bovine serum albumin as standard.

RESULTS

Acid protease activity in seminal plasma at pH 3.0 and 7.5. Previous results (3) have shown that the acid protease, pH optimum of

2.5, is unstable at pH 7.5, the physiological pH of human seminal plasma. Thus, an experiment on pH stability of an acid protease in human seminal plasma was performed. As shown in Table I, the proteolytic activity found before and after adjusting seminal plasma to pH 3 appeared to be the same; but when the latter was readjusted to its original pH of 7.5, its activity was lost. Since the proenzyme of the acid protease was stable at pH 7.5, this finding clearly indicated that the acid protease occurred in seminal plasma as a proenzyme which could be converted into its active form when the pH of seminal plasma was lowered to 3. In addition, the active enzyme was destroyed when pH of seminal plasma was raised to 7.5. This was supported by result shown in Figure 1; the protease activity was completely destroyed within 90 sec after exposure to pH 8.

Conversion of the proenzyme into its active form. We concluded that only the proenzyme form of acid protease can exist in human seminal plasma at the physiological pH of around 7.5; therefore, we investigated activation of proenzyme in acid medium. The purified proenzyme was incubated in 1 mM HCl, pH 3, for 1 hr, and then chromatographed on Sephadex G-50 column. The proenzyme was converted into an active form and some peptide of small molecular weight was released (Fig.2). As shown in Table II, when the amino acid analyses of the proenzyme, the active form, and activation peptide were carried out, the number of each amino acid residue of the proenzyme agreed well with the additive value between the number of that amino acid in the active form and activation peptide. This supported the conversion of the proenzyme to an active form. The amino acid composition of the active protease and of the proenzyme were comparable to those of bovine pepsin (10) and pepsinogen (11). However, definite differences are present. About forty residues which carried most of the basic amino acids, were released from pepsin, while sixty-nine residues, which carried about 30% of basic amino acid of the precursor were liberated from the proenzyme of seminal plasma acid protease.

pH-dependency of the proenzyme activation. As shown in Figure 3, both the rate and extent of the proenzyme activation were dependent on pH. The activation was essentially completed after 10 min at pH 2 and 3; but more of the proenzyme remained at 30 min when the activation was performed at higher pH value, from 4 to 5. At these high pH values, the percentage of proenzyme remaining did not change when the activation continued for more than 30 min. At the end of 30 min, if the pH of the activation was brought down to 2, the activation would immediately accelerate to a completion.

Between pH 5 and 2, as the pH was lowered, the rate of activation increased. For activation at pH 2, 3 and 4, if the remaining proenzyme were plotted semilogarithmically against incubation time, a linear line would be observed, suggesting a first-order reaction.

Table I

Acid protease activity in human seminal plasma

The activity of acid protease in seminal plasma was determined at 37° for 30 min using hemoglobin as substrate. 2 ml of reaction mixture contains 15 mg Hb, 20 λ seminal plasma (corresponding to 0.6 - 0.7 enzyme unit) and 0.1 M citric acid-phosphate buffer, pH 2.5.

Treatment of Seminal Plasma Prior to Assay	Acid Protease Activity (ΔA_{280}/30 min)
None	0.65
None, 37°/2 hr	0.66
Adjusted to pH 3, 37°/1 hr*	0.66
Readjusted to pH 7.5, 37°/1 hr**	0.05

*Seminal plasma, pH 7.5, was adjusted to pH 3 with 4 N HCl and incubated at 37° for 1 hr before assaying.

**The pH 3 treated-seminal plasma was readjusted to pH 7.5 with 4 N NaOH and incubated at 37° for 1 hr before assaying.

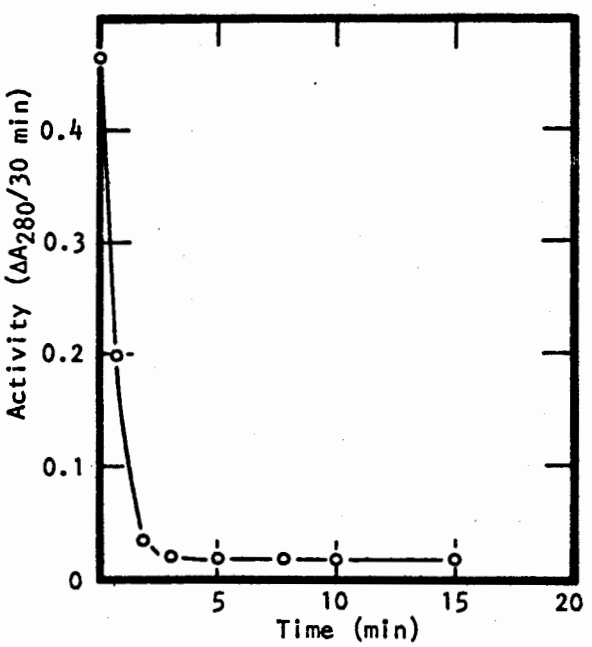

Figure 1. Stability of the active acid protease at pH 8. The enzyme was incubated in 0.1 M sodium phosphate buffer, pH 8 at 37°. Aliquots were removed at different time intervals and assayed for proteolytic activity at pH 2.5, using hemoglobin as substrate.

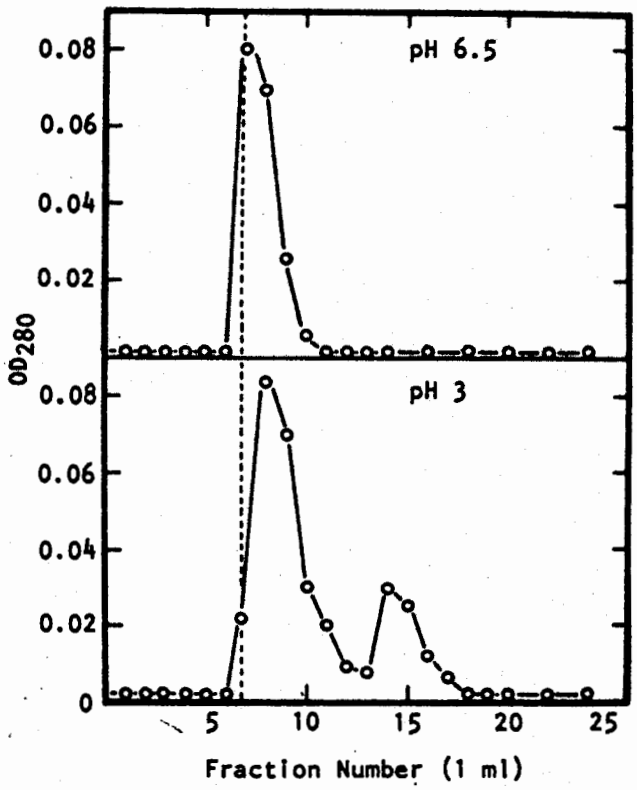

Figure 2. Gel filtrations on Sephadex G-50 (0.9 x 50 cm): (Top) proenzyme in 0.02 M sodium phosphate buffer, pH 6.5. (Bottom) proenzyme was incubated in 1 mM HCl at 37° for 1 hr and then chromatographed in 1 mM HCl, pH 3.

Table II

Amino acid composition of the proenzyme, the acid protease, and the activated peptide of human seminal plasma.

Each sample containing 5 nmoles of protein was hydrolyzed in a sealed and evacuated tube at 110° for 22 hr with 6 N HCl. The analysis was performed with a Beckman automatic amino acid analyzer.

Amino acid	Proenzyme[a]	Activation peptide	Acid protease	Human pepsin (15)
		residues/mole		
Aspartic acid	38 (36)	6	30	40
Glutamic acid	55 (53)	10	43	31
Threonine	29 (30)	4	26	27
Serine	35 (37)	6	31	43
Proline	23 (23)	4	19	19
Glycine	41 (43)	6	37	35
Alanine	26 (26)	5	21	18
Valine	27 (25)	3	24	27
Half cystine	8 (6)	1	5	6
Methionine	4 (4)	0	4	5
Isoleucine	15 (15)	3	12	25
Leucine	34 (35)	6	29	22
Tyrosine	20 (20)	3	17	15
Phenylalanine	19 (20)	3	17	15
Tryptophan	N.D.[b]	N.D.[b]	N.D.[b]	5
Lysine	15 (14)	4	10	0
Histidine	4 (4)	1	3	1
Arginine	11 (10)	4	6	3
Total	406 (404)	69	335	337

[a]Number in parenthesis is the sum of the values found in the acid protease and in the activation peptide.

[b]N.D. = not determined

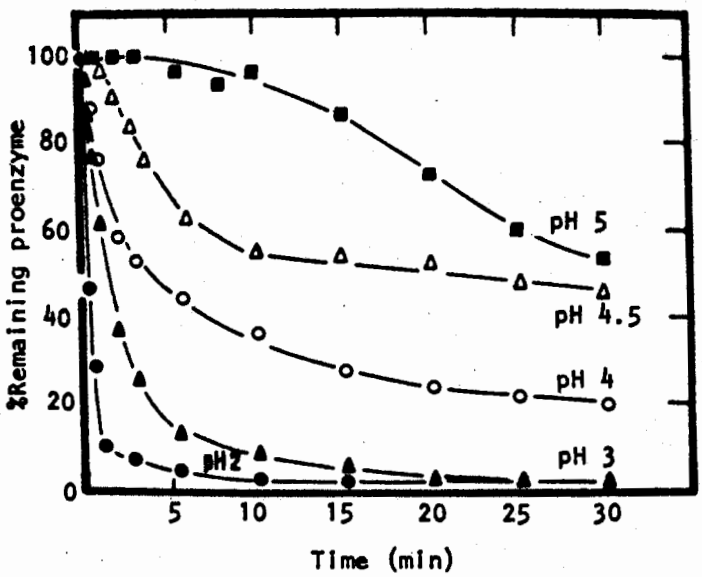

Figure 3. The time course of activation of the proenzyme at various pH values. The proenzyme (8μg/ml) was incubated in 0.1 M citrate-phosphate buffer of the desired pH. The proenzyme remaining at various time intervals was assayed as described in "Experimental Procedure".

At pH 4.5 and 5, there was an initial lag period followed by a more rapid decline in enzyme concentration, suggesting a deviation from a first order to a more complex reaction. A detailed discussion of this activation mechanism appears in a separate paper (12) and in chapter 6 of this book.

Hydrolysis of protein substrate by acid protease at various pH. Protein substrates other than hemoglobin were digested at pH values higher than its optimum pH. The results obtained in Table III were in agreement with those observed in pH profile (2); i.e. that none of the proteins could be hydrolyzed at pH above 4.5.

Hydrolysis of cervical mucus protein by the acid protease. The pattern of cervical mucus protein before and after hydrolyzing by acid protease were shown in Figure 4. The major protein band was lost in cervical mucus treated with acid protease. The appearance of new bands of smaller molecular weight indicated that hydrolysis had occurred. However, this protein band was also lost in cervical mucus hydrolyzed with chymotrypsin at pH 7.8, suggesting a rather nonspecific hydrolysis. The cervical mucus protein seemed to be hydrolyzed to a greater extent by the seminal plasma acid protease than by chymotrypsin.

DISCUSSION

Clearly, the proenzyme is the only form of acid protease that exists in human seminal plasma at its physiological pH of 7.2-7.8. If, on the other hand, the proenzyme form could be converted into its active form in seminal plasma, the latter would have then been destroyed at the slightly alkaline pH of seminal plasma. Thus, the protease activity would not have remained constant, but would have decreased as the time of incubation increased. Since this was not observed, the activation must not be taking place in the seminal plasma.

Upon activation in acid, the proenzyme converts into the active form probably by a limited proteolysis, which results in the release of a peptide, containing sixty-nine amino acid residues (Table II). Our other results (12) suggested that it seems to involve a single cleavage of a specific peptide bond in the proenzyme molecule, releasing only one activation peptide. In contrast, activation of bovine gastric zymogen, pepsinogen (13) and prochymosin (14) produce a multiplicity of peptides by the cleavage of 7-8 bonds. However, cleavage of only one of these bonds seems to be required to release active enzyme, and the other 6-7 bonds may be susceptible linkage sensitive to proteolysis in general. It should be noted that the amino acid composition of acid protease from human seminal plasma (335 residues) compares favorably with that of human pepsin (337 residues) (15).

Table III
Digestion of bovine serum albumin, ovalbumin and dimethylcasein at various pH by acid protease from seminal plasma

The reaction mixtures contained 2μg enzyme, 15 mg protein substrate and 0.1 M citric acid-sodium phosphate buffer in a total volume of 2 ml. The assays were performed at 37° for 30 min.

pH	Protein Substrates		
	Bovine Serum Albumin	Ovalbumin	Dimethylcasein
		ΔA280/30 min	
2.5	0.06	0.04	0.35
3.5	0.01	0.01	0.10
4.5	0	0	0.05
5.5	0	0	0
6.5	0	0	0

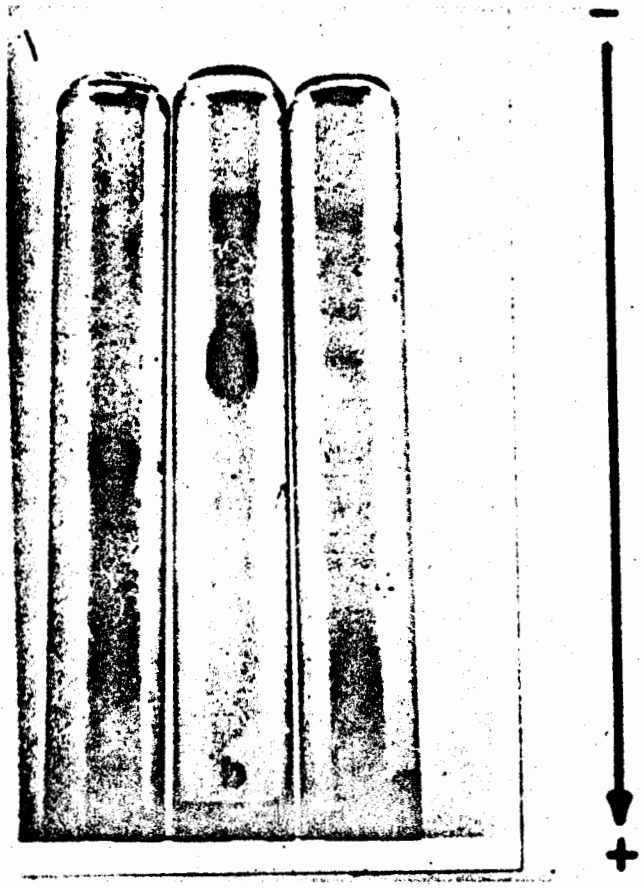

Figure 4. Polyacrylamide gel electrophoresis in 0.1% SDS of; a) acid protease-hydrolyzed cervical mucus in citric-phosphate buffer, pH 2.5; b) non-hydrolyzed cervical mucus; and c) chymotrypsin-hydrolyzed cervical mucus in phosphate buffer, pH 7.8. The electrophoresis was performed, using 7% gel, at the current of 8 ma per tube. Proteins were stained with coomassie brilliant blue and destained in acetic:methanol:H_2O (75:50:875).

The activation process of the proenzyme is a complicated reaction which is influenced by the pH of the medium. The kinetics of activation shift from the first order between pH 2 and 4 to more complexity with a lag period between pH 4.5 and 5 (Fig.3). Nevertheless, the mechanism of activation involving at least two steps has been proposed (12) and represented as $P \rightleftharpoons P' \rightleftharpoons A + t$. The first step is a pH-dependent conformational change of the native proenzyme (P) into a form capable of self-activation (P'). The second step is a unimolecular hydrolytic release of an activation peptide (t) from P'; the remainder of P' becomes the active acid protease (A). This proposed mechanism can explain the incompleteness of activation at high pH (Fig.3) in that the equilibrium of the second step may favor P' at high pH value and shift to A at low pH.

Since the proenzyme is progressively converted into its active form at pH below 6 and the pH of vaginal fluid is fairly acidic to bring about the activation, it is conceivable that the proenzyme can be activated when it comes into contact with the acidic vaginal fluid during ejaculation. The physiological role of seminal plasma acid protease is difficult to elucidate, although it can hydrolyze the proteins of cervical mucus (Fig.4). The hydrolysis seems to be nonspecific, since the proteins can also be hydrolyzed by seminal plasma neutral protease, chymotrypsin, and trypsin (6). Moreover, when the pH profile of enzyme activity, stability, activation as well as the pH in the female reproductive tract are considered together, it appears that the pH in the female reproductive tract is low enough to activate the proenzyme and to maintain the enzyme activity, but not low enough to allow proteolytic activity. On the other hand, the enzyme might function at physiological pH higher than its *in vitro* pH optimum, but the result in Table III seems not to support this possibility. Extensive evidence is available, however, that the acid protease is not the only enzyme involved in reproduction that requires acid pH for its action (16,17). Thus, the possibility might exist that the acid environment might somehow reside in the female reproductive tract so that the acid protease could exert its activity towards certain protein substrates.

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SUMMARY

An acid protease, with an optimum pH of 2.5, exists in seminal plasma in a proenzyme form. In acidic pH, the proenzyme is converted into the active form, resulting in the release of small molecular

weight peptide. The extent and rate of proenzyme-active enzyme conversion is absolutely dependent on pH. Between pH 5 and 2, as the pH is lowered, the extent of conversion increases and reaches a maximum between pH 3 and 2. The kinetics of activation shift from first-order between pH 2 and 4 to more complexity with a lag period between pH 4.5 and 5. Under physiological conditions, the proenzyme might be activated by coming into contact with acidic vaginal fluid during ejaculation. The acid protease can hydrolyze the cervical mucus protein.

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