Biochimica et Biophysica Acta, 452 (1976) 525—532

BBA 67970

ACTIVATION OF PROENZYME OF ACIDIC PROTEASE FROM HUMAN SEMINAL PLASMA

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(Received May 5th, 1976)

Summary

The kinetics and the extent of the conversion of the proenzyme into the active acidic protease (EC 3.4.23.—) of human seminal plasma were dependent on acidic pH. Between pH 2 and 4, the initial rate of the activation was first-order with respect to the proenzyme. Between pH 4.5 and 5, the rate deviated from the first-order with an initial lag period which can be abolished by adding an excess amount of the acidic protease or pepsin. The extent of the activation was complete between pH 2 and 3 and became incomplete between pH 4 and 5. Addition of the acidic protease or pepsin did not alter the extent of the activation at the high pH values. According to the chromatographic profile on a Sephadex G-75 column, the activation products (namely active acidic protease and an activation peptide) obtained at pH 3 and those obtained at pH 4.5 were identical. The molecular weight of the activation peptide obtained at pH 3 was 6900; its amino acid composition was analyzed and compared with those of the proenzyme and the acidic protease. Remarkable similarity between the amino acid composition of the acidic protease and that of human pepsin was observed. In the presence of an excess amount of hemoglobin, the conversion of the proenzyme was self-activated and showed an initial lag period. Addition of acidic protease did not change the rate of self activation or the lag period.

Introduction

Our previous reports [1,2] have described the purification and characterization of an acidic protease (EC 3.4.23.—) and its proenzyme in human seminal plasma. Since the proenzyme is stable from pH 6 to 9 while the active acidic

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protease is unstable above pH 6.5, it was concluded that only the proenzyme can exist in human seminal plasma at the physiological pH of 7.0 to 7.5 [3]. For the proenzyme to function, it would have to be activated in an acidic medium [2]. Under physiological conditions, this slightly acidic environment is likely to be found when the semen is freshly deposited in the vagina and mixed with the vaginal discharge [4]. In the case of normal fertility, the vaginal pH changes from 4.3 to 7.2 within 8 s after mixing with semen. In the case of low fertility, the change is smaller from pH 3.5 to 5.5. So it appears that the pH of the vaginal discharge will remain fairly acidic if the volume of the deposited semen is small. Thus, the acid-dependent activation of the proenzyme may play a physiological role in the process of reproduction. So it is of interest to investigate the kinetics of the activation of the proenzyme. Since acid-dependent activation of pepsinogen and prorennin has been well studied (for a recent review, see ref. 5), we shall compare our findings with these studies.

Methods

Purification of Proenzyme of Acidic Protease

The proenzyme of the acidic protease was purified to homogeneity by the procedure described previously [2].

Assay of Acidic Protease and of Proenzyme

The activity of the acidic protease was assayed by the method of Kassell and Meitner [6]. The assay mixture (2 ml) contained 0.1 M citrate/phosphate buffer, pH 2.5, 12.5 mg/ml acid-denatured hemoglobin and an appropriate amount of the enzyme. The proenzyme can be assayed by the same method since it can be totally converted into the active acidic protease under the conditions of the assay. To assay only the proenzyme in a mixture of the proenzyme and the acidic protease, the latter was completely destroyed by incubating in 1 M Tris·HCl buffer, pH 8.0, for 30 min.

Activation of Proenzyme

The activation of the proenzyme was carried out by adjusting the pH of the proenzyme solution with 0.1 M citrate/phosphate buffer to the desired acidic pH. Then, the solution was incubated at 37° C. At appropriate time intervals, aliquots were withdrawn into 1 M Tris, pH 8.0, and further incubated for 30 min. This would stop the activation and completely destroyed the active enzyme. The remaining proenzyme was then assayed at pH 2.5 by the above method.

Electrophoresis

Estimation of the molecular weight of the activation peptide was done by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to the method of Weber and Osborn [7].

Amino Acid Analysis

Amino acid composition was analyzed according to the method of Spackman, Moore and Stein [8] using a Beckman automatic amino acid analyzer.

Determination of Protein

Protein concentration was determined by the method of Lowry et al. [9] using bovine serum albumin as the protein standard. During column chromatography, protein content in each fraction was determined by measuring absorbance at 280 nm.

Results

pH-dependent activation of the proenzyme

When the acid activation of the proenzyme was followed by measuring the amounts of the proenzyme remaining at various time intervals, it was found that both the rate and the extent of the activation increased as the pH decreased (Fig. 1). At pH 2,3 and 4, the semilogarithmic plot of the proenzyme remaining versus time was linear suggesting a first-order reaction. At pH 4.5 and 5, the initial decrease of the proenzyme was slow, followed by a more rapid decline; the activation appeared to have a lag period (Fig. 2) suggesting a deviation from the first-order reaction. The lag period in the activation curve at pH 4.5 can be eliminated upon adding an excess amount of purified acidic protease or pepsin (EC 3.4.23.1) (Fig. 2). However, the addition did not increase the extent of the activation.

Analysis of the Activation Products

Since the kinetics and the extent of the activation at low pH (2 to 3) differed from those at the higher pH (4.5 to 5), it was possible that the products of the activation might also differ in the two pH ranges. After 30 min

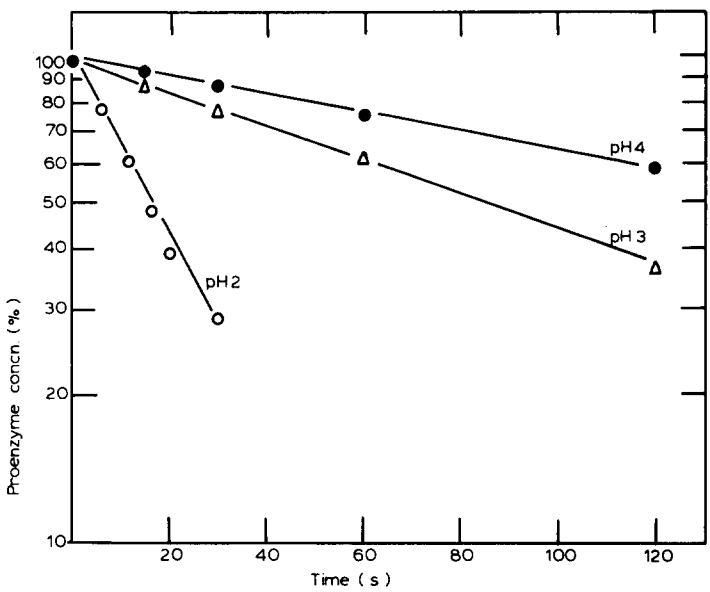

Fig. 1. The time course of activation of the proenzyme at pH 2, 3 and 4. The proenzyme (8 μg/ml) was incubated in 0.1 M citrate/phosphate buffer of the desired pH. The proenzyme remaining at various time intervals was assayed.

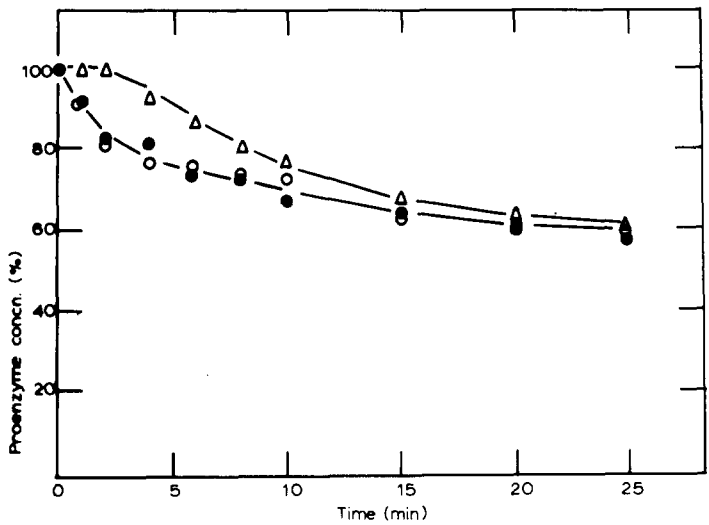

Fig. 2. The effect of addition of acidic protease and of porcine pepsin on the activation of the proenzyme at pH 4.5. The proenzyme (10 μg/ml) was incubated in 0.1 M citrate-phosphate buffer pH 4.5 alone (△), with acidic protease (10 μg/ml) (○), and with porcine pepsin (10 μg/ml) (●). The proenzyme remaining at various time intervals was assayed.

of activation at pH 3 or 4.5, the activation mixture was chromatographed in a Sephadex G-75 column. The protein profile of the pH 3 mixture (Fig. 3A) was essentially identical with that of pH 4.5 mixture (Fig. 3B). Each contained a major peak and a minor peak of smaller molecular weight material. The

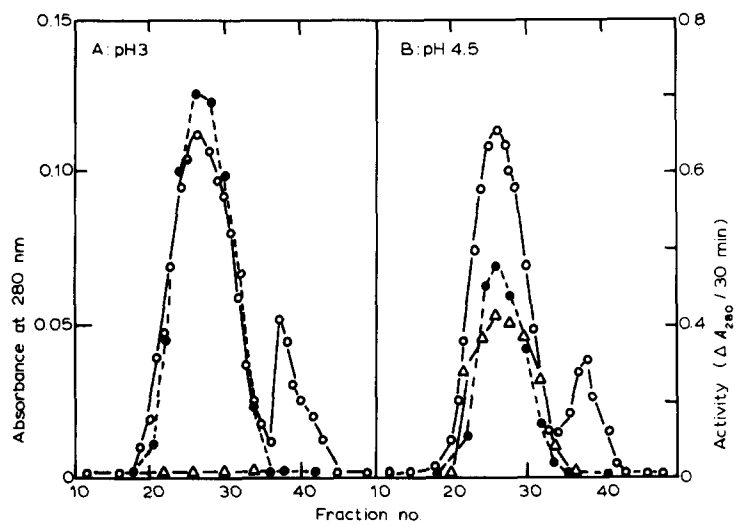

Fig. 3. Gel filtration on Sephadex G-75. The proenzyme activated at pH 3(A) or at pH 4.5 (B) for 30 min. according to the procedure described in Fig. 1 was chromatographed on the column in 0.1 m citrate-phosphate buffer, pH3 (A) or pH 4.5 (B): the protein profile (○), the acidic protease (●) and the proenzyme (△).

position of each peak in both profiles compared quite well. The minor peak showed no proteolytic activity and was, therefore, the activation peptide. The major peak possessed the total proteolytic activity. The major peak obtained at pH 3 contained only the active acidic protease, since the activation at this pH was complete (Fig. 3). In contrast, the major peak obtained at pH 4.5 represented the newly formed acidic protease and the remaining proenzyme (Fig. 3). Thus, the activation products, namely the active acidic protease and the activation peptide obtained at pH 3 and 4.5 did not differ by the chromatographic criteria.

The activation peptide obtained above appeared as a single band in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with an estimated molecular weight of 6900.

Analyses were also carried out on the amino acid compositions of the activation peptide and the newly formed acidic protease obtained at pH 3 (Table I). The activation peptide contained 69 amino acids per molecule while the acidic protease had 335. The sum of each amino acid found in the activation peptide and that in the acidic protease agreed well with the value for the corresponding amino acid found in the proenzyme. This further confirmed that no other product was formed during the activation besides the activation peptide and the acidic protease.

TABLE I

AMINO ACID COMPOSITION OF THE PROENZYME, THE ACIDIC PROTEASE AND THE ACTIVATED PEPTIDE OF HUMAN SEMINAL PLASMA IN COMPARISON WITH HUMAN PEPSIN

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

Amino acid	Residues/mole			
	Proenzyme [a]	Activation peptide	Acidic protease	Human pepsin [19]
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 acidic protease and in the activation peptide.

[b] N.D., not determined.

Activation in the presence of hemoglobin

Pepsinogen was shown to be able to self-activate by a uni-molecular or intramolecular mechanism into active pepsin [10,11]. This was concluded from the observation of pepsin activity when pepsinogen was incubated with a large excess of its substrate, acid-denatured hemoglobin, which would minimize the pepsinogen-pepsinogen (intermolecular) reaction. A similar self-activation process was also observed when the purified proenzyme of the acidic protease was incubated with a large excess of acid-denatured hemoglobin (Fig. 4). Similar to the observation with pepsinogen [10], the self-activation of the proenzyme showed a lag period. In contrast, the same amount of the purified acidic protease incubated with the substrate showed no lag in the proteolytic rate. Moreover, adding the same quantities of the acidic protease and of the proenzyme, without prior mixing of the two components, to the substrate solution resulted in a proteolytic rate which was the sum of the individual rates. Thus, the active acidic protease did not affect the rate of the self-activation of the proenzyme under the condition of excess substrate. It should be noted that the specific activities of the proenzyme and that of the acidic protease were approximately equal while the specific activity of pepsinogen was a quarter that of pepsin [10]. In fact, when the concentration of the substrate was varied from 0.625 to 12.5 mg/ml, the proteolytic rate of the proenzyme was about the same as that of the acidic protease at any of the concentrations of the substrate used. This suggested that the self-activation of the proenzyme gave rise to the newly formed acidic protease which was responsible for the observed proteolytic activity.

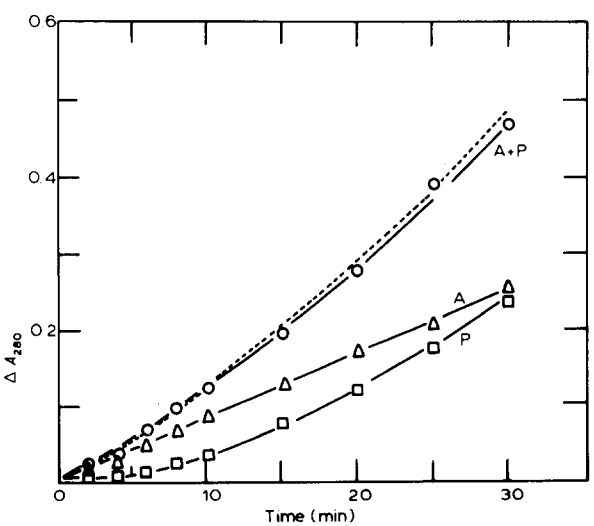

Fig. 4. The time course of digestion of hemoglobin at pH 3 by the acidic protease (curve A), by the proenzyme (curve P) and by the acidic protease and the proenzyme without prior mixing (curve A + P). The dotted line was the numerical sum of curve A and curve P. The concentrations of hemoglobin, the acidic protease and the proenzyme were 2.5 mg, 1.2 μg and 1.2 μg/ml, respectively.

Discussion

The data above clearly show that the activation of the proenzyme is dependent on acidic pH and gives rise to the active acidic protease and an activation peptide. The variations in the order of the activation kinetics suggests a mechanism involving two steps. 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′ and the remainder of the P′ becomes the active acidic protease (A). The second step can also be catalyzed by A if it is present and the pH is favorable for its activity. Thus, the mechanism can be represented as $P \rightleftharpoons P' \rightleftharpoons A + t$. The proposed mechanism fits the data but is not entirely proved. The activation will be of first-order with respect to P when the first step is slower than the second one. This is observed at low pH (Fig. 1) or at high pH in the presence of added pepsin or acidic protease (Fig. 2). Conversely, when the second step is slower than the first, the order will be complex, as seen at high , : (Fig. 2) or at low pH in the presence of a large excess of hemoglobin (Fig. 4). In order to explain the incomplete activation at high pH (Fig. 2), it is necessary to propose that the equilibrium of the second (or hydrolytic) step may favor P′ at high pH values (4 to 5) and shift to right at the low pH conditions (2 to 3).

The proposed mechanism does not require P to possess any proteolytic activity. In fact, the existence of the active pepsinogen during the acid-dependent conversion of pepsinogen into pepsin [5] has been seriously challenged in recent reports [11,12]. The data presented suggest that the activation yields only a single activation peptide. However, this has not yet been confirmed by end-group analysis. In contrast, multiple peptides are released in the activation of porcine [13,14] and bovine [15] pepsinogens and of prorennin [16].

Similar to bovine pepsinogen [17] and pepsin [18], the proenzyme and the acidic protease are rich in acidic amino acids (Table I). However, the amino acid composition of the acidic protease from human seminal plasma (335 residues) compares very favorably with that of human pepsin (337 residues) [19] (Table I). The major differences are as follows. Firstly, the aspartic acid (30 residues) and the glutamic acid (43 residues) contents of the acidic protease are approximately the reverse of the aspartic acid (40 residues) and the glutamic acid (31 residues) contents of human pepsin. Secondly, the smaller isoleucine content in the acidic protease is compensated by the higher content of the closely related amino acid, leucine. Thirdly, the acidic protease has significantly less serine but more lysine and arginine. The remarkable similarity between the amino acid composition of the acidic protease of human seminal and that of human pepsin suggests that there may be some evolutionary relationship between the two enzymes.

Acknowledgement

We wish to thank Dr. Vitoon Osathanondh for the supply of human semen from Ramathibodi Hospital, Dr. Maitri Suthachit for help in the amino acid

analysis. We also acknowledge Drs. Jisnuson Svasti, Bhinyo Panijpan and Yongyuth Yuthavong for valuable criticism of the manuscript.

This study was supported by the Rockefeller Foundation and Funds for Overseas Research Grants and Education, Inc.

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