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Purification and Characterization of an Acid Proteinase from Human Uterine Cervix

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Acid and neutral proteinases are distributed in the cervical stroma of the human uterus, and the former activity is about 10 times that of the latter. The acid proteinase was completely separated from the neutral enzyme by Sephadex G-200 gel filtration and the acid enzyme was purified about 630-fold to homogenity by ion-exchange chromatography on DEAE-Sephadex A-50 and rechromatography on Sephadex G-200. The purified acid proteinase showed the optimum pH in the range from 3.4 to 3.6 for acid- and urea-denatured bovine hemoglobins, and was completely inhibited by pepstatin and N-bromosuccinimide. Its molecular weight was calculated to be 4.2×10^4 by gel filtration on Sephadex G-75. From these results, this enzyme could be identified as cathepsin D.

Keywords——acid proteinase; cathepsin D; human uterus; uterine cervix; cervical stroma

Cathepsin D (EC 3.4.4.23) has been known to play an important role in cell physiology, *e.g.*, in the destruction of tissue during inflammation[2] and in the degradation of articular matrix.[3,4] The enzyme has been also shown to be concerned with the loss of collagen in the involuting rat uterus postpartum.[5]

We investigated the mechanism of obvious changes observed in the uterine cervical stroma during dilatation and effacement in pregnancy at term, *e.g.*, a significant decrease in collagen content[6] and an increase in PZ-peptidase activity,[7] respectively. However, little is known about the enzymes concerned with the process in the cervix. During these studies on enzymes in the human uterine cervix, we found a significant amount of acid proteinase in the cervical tissue, and purified this proteinase to homogeneity, characterized it, and compared it with other acid proteinase described so far.

Materials and Methods

Enzyme Source——Gervical tissues from women of child-bearing age were used and obtained from patients scheduled for hysterectomy in fibromyoma not involving the cervix. Cervical epithelium and stroma were completely separated, and the stroma was repeatedly rinsed in a cold saline solution to remove blood and mucus, and the stroma was kept at $-20°$ until use.

Reagents used——The following reagents were commercially obtained: bovine hemoglobin (enzyme substrate powder) from Miles Laboratories, U.S.A., Sephadex G-75 and 200, DEAE-Sephadex A-50 and blue dextran 2000 from Pharmacia, Sweden; bovine serum albumin (5×, crystallized) from Nutritional Biochem., U.S.A.; egg albumin from ICN Pharmaceuticals, U.S.A.; α-chymotrypsinogen A (bovine pancreas, type II) and cytochrome *c* (horse heart, type IV) from Sigma, U.S.A.; casein (Hammarsten grade) from E. Merck,

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2) G. Weissman, R.B. Zurier, and S. Hoffstein, *Am. J. Pathol.*, **68**, 539 (1972).
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Japan and pepstatin from Protein Research Foundation, Japan. Other reagents used were of analytical reagent grade.

Assay for Acid Proteinase——Acid proteinase was analyzed according to the method of Anson[8] with slight modification by using denatured hemoglobin as a substrate. The incubation mixture contained (in final concentration) 40 mM sodium citrate buffer (pH 3.5), 0.8% (w/v) acid denatured hemoglobin and an appropriate amount of the enzyme to a total volume of 2.5 ml. After incubation of the mixture at 37° for 30 min, 2.5 ml of 5% (w/v) trichloroacetic acid was added to terminate the reaction, and the mixture was filtered through Toyo filter paper No. 2. To the digested peptides in the filtrate, was added the Folin-Ciocalteau reagent and the absorbance at 660 nm was measured. Neutral proteinase (pH 7.4) was also analyzed by the same procedure with casein instead of hemoglobin as a substrate. One unit of the enzyme is expressed as an amount which catalyses the liberation of TCA-soluble peptide equivalent to 1 μmol of tyrosine per 30 min under these conditions.

Disc Gel Electrophoresis and Protein Determination——Disc gel electrophoresis was performed by the method of Davis[9] and protein content was also determined by the method of Lowry *et al.*[10] by using crystalline bovine serum albumin as a standard.

Results

Solubility of Acid Proteinase Activity

The solubility of acid proteinase was examined by differential centrifugation. The thawed uterine cervix was mixed with in 0.25 M sucrose buffered with 25 mM Tris-HCl buffer (pH 7.4) and homogenized in a VirTis 45 homogenizer in an ice cold bath 3 times for 30 sec each time. After homogenization three subcellular fractions were obtained by different centrifugation: the nuclear fraction (700×***g*** for 10 min), the granule fraction (20000×***g*** for 20 min at 4°) and the post granule supernate. Over 85% of total acid proteinase was constantly distributed in the post granule supernate, whereas N-acetylglucosaminidase, a novel lysosomal enzyme, and neutral proteinase distributed in the supernate were less than about 60% and 40% of the total activity, respectively. The solubilized acid proteinase activity was about 10-times higher than that of neutral proteinase.

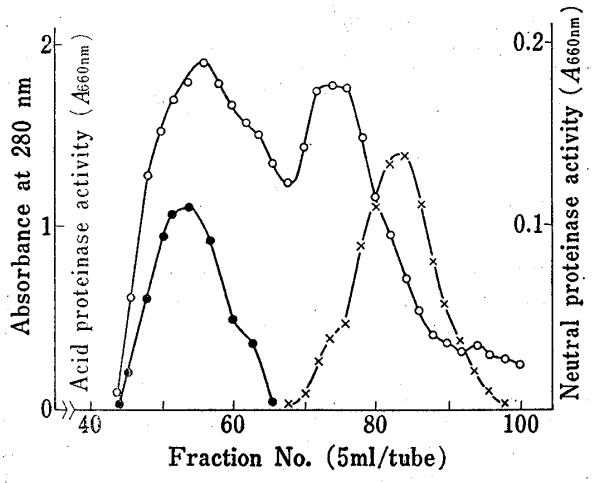

Fig. 1. Gel Filtration of Acid Proteinase Preparation on Sephadex G-200 Column

The enzyme preparation was applied to a column (3.5×80 cm) of Sephadex G-200 equilibrated with 25 mM Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl and eluted with the same buffer at a flow rate of 6 ml/hr. ○—○: absorbance at 280 nm, ●—●: neutral proteinase activity, ×—×: acid proteinase activity.

Purification of Acid Proteinase from Human Uterine Cervix

All purification procedures were carried out at 4°. The thawed uterine cervix (10.8 g) was minced with scissors and homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl as above, and 10% (w/v) homogenate was finally prepared. The homogenate was centrifuged at 20000×***g*** for 20 min and the precipitate was discarded. Solid $(NH_4)_2SO_4$ was added to the supernate to 80% saturation with gentle stirring, and the solution was allowed to stand overnight. The precipitate of this crude enzyme collected by centrifugation was dissolved in about 3 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl and dialyzed against the same buffer (500 ml) for 12 hr. A small precipitate formed was discarded after centrifugation. The acid proteinase fractionated with $(NH_4)_2SO_4$ was ap-

8) M.L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).
9) B.J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).
10) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

plied to a column of Sephadex G-200 equilibrated previously with the above buffer. Figure 1 presents its elution pattern. Acid and neutral proteinases were separated completely by the following procedure. Neutral proteinase activity was eluted at near void volume, but acid enzyme activity appeared late. Fractions 70—80 were collected together, and then desalted and concentrated by Amicon PM-10 membrane. The enzyme was further chromatographed on DEAE-Sephadex A-50 column (2.2×40 cm) equilibrated previously with 25 mM Tris-HCl buffer (pH 7.4). The acid proteinase was eluted stepwise with NaCl, but the pattern of its activity did not coincide with the protein peak. Acid proteinase was thus purified about 60-fold. Disc gel electrophoresis suggested the enzyme preparation was composed of two clear protein bands and a trace of a more rapid moving protein as shown in Fig. 2A. Active fractions were collected together and concentrated as above, and then rechromatographed on a small Sephadex G-200 column (2×78 cm). As shown in Fig. 3, acid proteinase was eluted in a minor symmetrical protein peak and fractions 61—71 were collected together. Analytical disc gel electrophoresis suggested that the preparation was composed of only one

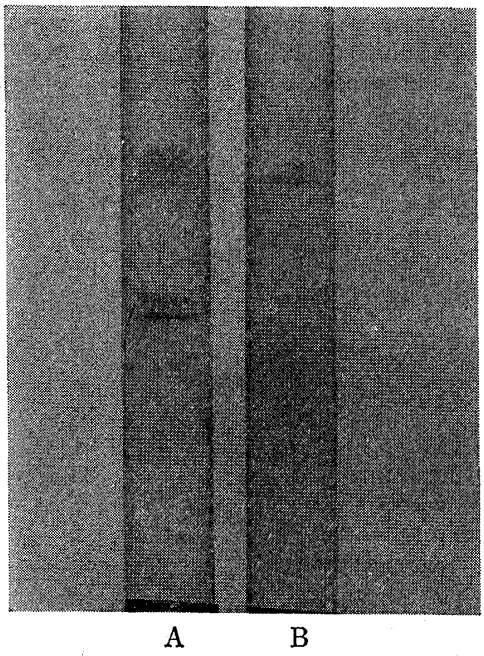

Fig. 2. Polyacrylamide Disc Electrophoresis Patterns of Acid Proteinase from Human Uterine Cervix

About 30 μg of protein was applied to a column of pH 9.4 gel. A constant current of 4 mA/column was applied for 80 min. Staining was done by Amidoschwartz 10B. A; active fraction of DEAE-Sephadex A-50 elute, B; purified acid proteinase.

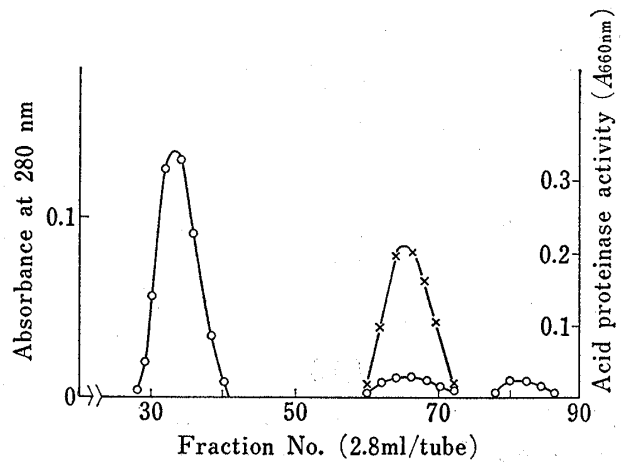

Fig. 3. Rechromatography of Acid Proteinase on Sephadex G-200

The acid proteinase concentrated by Amicon PM-10 was applied to a column (2×78 cm) of Sephadex G-200 equilibrated with 25 mM Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl and eluted with the same buffer at a flow rate of 5 ml/hr.
○—○: absorbance at 280 nm,
×—×: acid proteinase activity.

Table I. Purification Procedure of Acid Proteinase from Human Uterine Cervix

Step	Protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification factor
Homogenate[a)]	831	108.8	0.13	1
$(NH_4)_2SO_4$	636	114.1[b)]	0.18	1.4
Sephadex G-200	62.7	70.9	1.13	8.7
DEAE-Sephadex A-50	2.4	17.8	7.41	57.0
Second Sephadex G-200	0.087	7.1	81.50	626.9

a) Human uterine cervix 10.8 g.
b) By the procedure, over 100% of recovery was usually observed.

protein band (Rf=0.25) and corresponded to the upper band obtained by DEAE-Sephadex A-50 chromatography (Fig. 2B). The specific activity of the purified enzyme was about 630-fold that of the original tissue homogenate and the final recovery of the enzyme was also about 7%. Table I summarizes the purification procedures.

Effect of pH on the Reaction Rate and the Stability of Acid Proteinase

As shown in Fig. 4, purified acid proteinase had its maximal activity in the pH 3.4 to 3.6 region, and below pH 2.0 and above 6.2 the enzyme was virtually inactive on acid-denatured hemoglobin. The pattern of activity was also similar to that observed on urea-denatured hemoglobin, but the hydrolysis rate for acid-denatured bovine serum albumin was only 20% of that for hemoglobin. Figure 4 also shows the stability of this enzyme. When the enzyme was incubated at indicated pH in the absence of the substrate, it was very stable at about pH 7 but lost its activity rapidly in the pH range 3 to 4 for the suitable reaction rate.

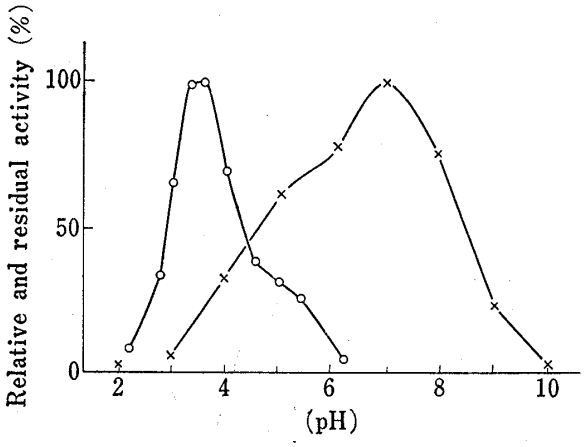

Fig. 4. Effect of pH on the Reaction Rate and the Stability of Purified Acid Proteinase

Buffers used were; pH 2—6: 50 mM citrate-phosphate, pH 7—8: 50 mM Tris-HCl and pH 9—10: 50 mM carbonate-borate. Reaction rate; ○—○: the substrate was acid denatured bovine hemoglobin. Stability; ×—×: the enzyme was incubated with a buffer at 37° for 30 min, and then diluted 10-fold with 50 mM sodium-citrate buffer (pH 3.5) and the residual activity was assayed.

Table II. Effect of Reagents on Purified Acid Proteinase

The enzyme was incubated with a reagent (1 mM) in 25 mM Tris-HCl buffer (pH 7.4) at 37° for 30 min, and the residual activity was determined.

Reagent	Residual activity (%)
Control	100
$CaCl_2$	80
$MnCl_2$	81
$HgCl_2$	38
$CoCl_2$	55
$MgSO_4$	82
$ZnSO_4$	58
$CuSO_4$	78
EDTA 2Na	91
o-Phenanthroline	83
Monoiodoacetic acid	101
N-Bromosuccinimide	2
p-Chloromercuribenzoate	86
Cysteine	113
Dithiothreitol	41
Pepstatin[a]	0
Soybean trypsin inhibitor[b]	105
Human serum[c]	100

a) 0.018 mM, *b*) 1 mg/ml, *c*) 50% (v/v).

Effect of Various Compounds on Acid Proteinase

When the purified acid proteinase was preincubated with each reagent, the enzyme was almost completely inactivated by pepstatin (0.018 mM) and N-bromosuccinimide, and partially inhibited by $HgCl_2$ and dithiothreitol. On the other hand, human serum (50%, v/v), soybean trypsin inhibitor (1 mg/ml), EDTA and *o*-phenanthroline had no effect on it (Table II).

Molecular Weight of Acid Proteinase

The molecular weight of acid proteinase was determined by gel filtration on Sephadex G-75 according to the method of Whitaker.[11] The void volume was estimated with blue dextran 2000 and the molecular weights of bovine serum albumin, egg albumin, α-chymotrypsinogen A and cytochrome *c* were taken as 6.9×10^4, 4.5×10^4, 2.5×10^4 and 1.3×10^4, respectively. The V/V_0 value of them were 1.06, 1.15, 1.35 and 1.56, respectively and gave

11) J.R. Whitaker, *Anal. Chem.*, 35, 1950 (1963).

a linear relation to their log (molecular weights). The value of 1.18 indicated that the molecular weight of the acid proteinase is about 4.2×10^4.

Discussion

Acid proteinase purified from human uterine cervix is similar to cathepsin D reported so far in the optimum pH on acid-denatured hemoglobin and in the pattern of pH stability in the absence of substrate. The molecular weight of about 4.2×10^4 for the cervix acid proteinase is also similar to those of human cethepsin D from the liver, leucocytes and erythrocytes of molecular weight of 4.5×10^4, 4.2×10^4 and 5.0×10^4, respectively.[12–14] The acid proteinase of the human uterine cervix was completely inactivated at a low concentration of pepstatin, whereas it was not inhibited by the human serum and other reagents examined. These properties of acid proteinase suggested that the enzyme might be cathepsin D.

Cathepsin D has been purified from many mammalian tissues. In particular, the enzyme from the human liver,[12] leucocytes,[13] erythrocytes[14] and articular cartilage[15] have been investigated in detail in recent years. In those tissues, cathepsin D was detected as a lysosomal enzyme and distributed in the granular fraction. However, the solubilized amount of cervical cathepsin D in the granular fraction is smaller than that in the post granular fraction. This result suggested that the solubility of cathepsin D in the cervix differs from those of enzymes previously reported and is similar to that of cathepsin D in rat thoratic duct lymphocytes.[16]

Multiple forms of cathepsin D have been reported in many tissues such as the bovine spleen, uterus[5] and human liver.[12] On the other hand, the enzymatic properties of cervical cathepsin D on DEAE-Sephadex A-50, gel filtration and disc gel electrophoresis suggested that any isoenzyme patterns were not confirmed as an enzyme of human leucocytes.[13]

We previously reported on the significant decrease in collagen content in the human uterine cervix in pregnancy at term[6] and also on the increase in the activity of PZ-peptidase which appears to be parallel to that of collagenase.[7] On the other hand, cathepsin D was confirmed to be decreased significantly to about 50% of the control (per wet wt tissue and mg protein, $p<0.001$, unpublished data). These results do not agree with the fact that the rapid breakdown of collagen in the uterus relates to an elevation in the activity of lysosomal enzymes.[5] Further investigations are necessary to discuss the relationship between cathepsin D and such obvious change as a decrease in collagen content observed in the cervix stroma in pregnancy at term.

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16) W.E. Bowers, *J. Exp. Med.*, **136**, 1394 (1972).