Acidic Protease from Human Seminal Plasma

PURIFICATION AND SOME PROPERTIES OF ACTIVE ENZYME AND OF PROENZYME*

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A procedure to purify to homogeneity the active form as well as the proenzyme form of the acidic protease of human seminal plasma is described. This involved precipitation with ammonium sulfate, chromatography on diethylaminoethylcellulose, Sephadex G-200, and Sephadex G-100. The molecular weights of the active form and of the proenzyme were determined by electrophoresis and gel filtration to be 35,000 and 42,000, respectively. The proenzyme was more stable than the active form in alkaline solution and can be converted into the active enzyme under acidic conditions. The active form of the acidic protease can hydrolyze hemoglobin, N,N'-dimethylcasein, N-acetyl-l-phenylalanyl-l-diiodotyrosine, and N-benzoylcarbonyl-l-glutamyl-l-phenylalanine, but cannot hydrolyze bovine serum albumin, ovalbumin, N-benzoylcarbonyl l-glutamyl l-tyroine. The active form was also inhibited by p-bromophenacyl bromide and 1,2-epoxy-3-(p-nitrophenoxy)propane.

Although several proteases are known to exist in the acrosome of mammalian sperm and in seminal plasma, few have been purified and characterized. A trypsin-like enzyme (acrosin) has been purified from the acrosomes of boar as well as human sperm (1-4). An acidic protease with a pH optimum of 2.8 has been partially purified from the acrosomes of bull and ram sperm (5). A neutral protease with a pH optimum of 8.0 has been purified 25-fold from human seminal plasma (6). An acidic protease in human seminal plasma was originally described as pepsin and its proenzyme as pepsinogen (7). The acidic protease of human seminal plasma is described. This involved precipitation with ammonium sulfate, chromatography on diethylaminoethylcellulose, Sephadex G-200, and Sephadex G-100. The molecular weights of the active form and of the proenzyme were determined by electrophoresis and gel filtration to be 35,000 and 42,000, respectively. The proenzyme was more stable than the active form in alkaline solution and can be converted into the active enzyme under acidic conditions. The active form of the acidic protease can hydrolyze hemoglobin, N,N'-dimethylcasein, N-acetyl-l-phenylalanyl-l-diiodotyrosine, and N-benzoylcarbonyl-l-glutamyl-l-phenylalanine, but cannot hydrolyze bovine serum albumin, ovalbumin, N-benzoylcarbonyl l-glutamyl l-tyroine. The active form was also inhibited by p-bromophenacyl bromide and 1,2-epoxy-3-(p-nitrophenoxy)propane.

EXPERIMENTAL PROCEDURE

Assays for Protease Activity

The activity of the acidic protease was assayed with protein substrates by the method of Kassell and Meitner (11), and with synthetic substrates by the method of Ryles (12).

Determination of Protein

Protein concentration was determined by the method of Lowry et al. (13) using bovine serum albumin as the protein standard. During column chromatography, protein content in each fraction was determined by measuring the absorbance at 290 nm.

Purification of Active Form of Acidic Protease

Pooled fresh human semen was centrifuged at 1,000 x g for 10 min at 4° to remove spermatozoa. The cell-free seminal plasma was kept at -20° until use. When a sufficient amount of the seminal plasma had accumulated, it was thawed and clarified by centrifugation at 20,000 x g for 20 min at 4°. Unless stated otherwise, the following steps were carried out at 4°.

Step 1: Ammonium Sulfate Fractionation—The clear seminal plasma was brought to 40% saturation of ammonium sulfate by the slow addition of the solid salt. After standing for 1 hour, the precipitate was removed by centrifugation at 20,000 x g for 15 min. The protease activity was then precipitated at 70% saturation of ammonium sulfate. The precipitate was collected by centrifugation and redissolved in 0.02 M sodium phosphate buffer, pH 6.5.

Step 2: Column Chromatography on DEAE-cellulose—A concentrated solution of NaCl was added to the protein solution until the final concentration of the salt was 0.2 M. The solution was then applied onto a DEAE-cellulose column (40 x 3.5 cm) which was pre-equilibrated with 0.02 M sodium phosphate buffer, pH 6.5, containing 0.2 M NaCl. A large bulk of protein including a neutral protease was not retained and washed out of the column with the buffer. Then a linear gradient of NaCl was applied to the column. The acidic protease activity was eluted between 0.3 and 0.4 M NaCl. The fractions containing the activity were pooled and concentrated by using Aquacide. A typical chromatographic profile is shown in Fig. 1.

Step 3: Column Chromatography on Sephadex G-200—The concentrated solution was adjusted to pH 3.0 by the addition of 2 M HCl and incubated at 37° for 1 hour. The solution was then applied onto a column of Sephadex G-200 (50 x 2.5 cm) which was pre-equilibrated
with 1 mM HCl containing 0.1 M NaCl. The fractions containing the acidic protease were then pooled and concentrated.

**Step 4: Column Chromatography on Sephadex G-100**—The concentrated solution was further chromatographed on a column of Sephadex G-100 (80 x 1.2 cm) pre-equilibrated with 1 mM HCl containing 0.1 M NaCl. A typical chromatographic profile is shown in Fig. 2. The fractions containing the enzyme activity were pooled, dialyzed against double distilled water, and lyophilized. The protease could then be kept at -20°C for several months without appreciable loss of the enzyme activity.

A summary of a representative purification is shown in Table I.

### Purification of Proenzyme of Acidic Protease

The proenzyme of the acidic protease could be purified by the procedure described above with the following alterations. The protein solution with the activity of the acidic protease from Step 2 was not acidified. The gel filtrations (Steps 3 and 4) were carried out with the columns pre-equilibrated with 0.02 M sodium phosphate buffer, pH 6.5. The fractions containing the proenzyme could be identified by the assay of Kassell and Meitner (11) because the proenzyme is converted into the active form under the acidic conditions of the assay. The chromatographic profiles of the proenzyme were similar to those of the active enzyme. The result of the purification of the proenzyme was similar to that of the active enzyme shown in Table I.

### Table I

| Fraction     | Volume | Protein | Total activity | Specific activity | -Fold Yield |
|--------------|--------|---------|----------------|-------------------|-------------|
| Seminal plasma | 104    | 300     | 8750           | 2.8               | (1) (100)   |
| Ammonium sulfate | 60     | 16.2    | 4250           | 4.3               | 1.5 (49)    |
| DEAE-cellulose | 14     | 1.1     | 1600           | 104               | 137 (18)    |
| Sephadex G-200 | 2.8    | 1.0     | 1100           | 400               | 143 (13)    |
| Sephadex G-100 | 2.0    | 0.47    | 810            | 875               | 310 (9)     |

**Electrophoresis**

Estimation of the molecular weight by polyacrylamide gel electrophoresis was done in the presence of Na dodecyl-\(\text{SO}_4\) according to the method of Weber and Osborn (14).

### RESULTS

**Homogeneity, Molecular Weight, and Stability**—The active form and the proenzyme of the acidic protease of human seminal plasma were purified to homogeneity by the procedures described above. They appeared as single bands in polyacrylamide gel electrophoresis in the presence of Na dodecyl-\(\text{SO}_4\) (Fig. 3). The active form moved slightly faster than the proenzyme. The appearance of the purified proenzyme as a single band in the gel rules out the possibility of any peptide inhibitor noncovalently bound to the enzyme in the preparation. The homogeneity was also confirmed by polyacrylamide gel electrophoresis in 7% gel with Tris-HCl buffer, pH 8.6, performed according to the method of Davis (15).

Mobilities of the active form and of the proenzyme in the polyacrylamide gel electrophoresis in the presence of Na dodecyl-\(\text{SO}_4\) were used to estimate their molecular weights. From the standard curve (Fig. 4), the molecular weights of the

### FRACTION NUMBER

**FIG. 1.** Chromatographic profile of ammonium sulfate fraction on a DEAE-cellulose column (40 x 3.5 cm). The column was equilibrated with 0.02 M sodium phosphate buffer, pH 6.5, containing 0.2 M NaCl. A linear gradient of NaCl from 0.2 to 0.9 M in the same buffer was applied when washing was completed (indicated by the arrow). Protein concentration was determined by absorbance at 280 nm, proteolytic activity by the method of Kassell and Meitner (11) with hemoglobin as substrate, and NaCl concentration by conductivity measurement. Fractions 35 to 70 were diluted 10-fold with buffer prior to the determination of absorbance at 280 nm.

**FIG. 2.** Chromatographic profile of the preparation from Step 3 on Sephadex G-100 (80 x 1.2 cm). The column was pre-equilibrated and eluted with 1 mM HCl, pH 3, containing 0.1 M NaCl. Protein concentrations and proteolytic activity were determined as indicated in Fig. 1.

**FIG. 3.** Polyacrylamide gel electrophoresis in 0.1% Na dodecyl-\(\text{SO}_4\) of (a) proenzyme, (b) active acidic protease, and (c) proenzyme converted into active enzyme. Samples were preincubated in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% Na dodecyl-\(\text{SO}_4\), and 0.1% \(\beta\)-mercaptoethanol at 37°C for 3 hours before electrophoresis which was performed according to the method of Weber and Osborn (14).
active form and of the proenzyme were estimated to be 35,000 and 42,000, respectively. These values were confirmed by gel filtration using Sephadex G-100 chromatography. They were also quite close to those of hog pepsin and of pepsinogen (12).

**pH Stability.** The active form of the acidic protease was quite stable between pH 2 and 6.5. Above pH 6.5 its activity dropped sharply (Fig. 5). The proenzyme was quite stable between pH 6 and 9. Above pH 9, it was quickly denatured. Below pH 6, the proenzyme was converted into the active form of the acidic protease. The data supporting the conversion is given below.

The pH dependence of the activity of the purified acidic protease was similar to that of a partially purified preparation (8) but the pH optimum was slightly shifted from 3.0 to 2.5.

**Conversion of Proenzyme into Active Form.**—When the preparation of the proenzyme was adjusted to pH 3 with 2 M HCl and then incubated at 37° for 1 hour, some small peptides were released. This resulted in an extra peak of a smaller molecular weight material in the elution profile of a Sephadex G-50 column (Fig. 6). Moreover, the acid-treated proenzyme showed a slight reduction in its mobility in the column of Sephadex G-50 (Fig. 6). It also possessed activity and electrophoretic mobility identical with those of the purified active form (Fig. 3). Thus, the proenzyme was converted into its active form at pH 3. Our additional data, not shown here, indicated that acidic conditions (pH 2 to 5) would initiate the conversion.

**Activities of Active Form.**—The purified acidic protease can hydrolyze hemoglobin, N,N'-dimethylcasein, but neither ovalbumin nor bovine serum albumin (Fig. 7). The apparent \( K_m \) values for hemoglobin and N,N'-dimethylcasein were 1.25 and 1.8 mg per ml, respectively. It was noted that a high concentration of N,N'-dimethylcasein was inhibitory to the enzyme. This effect was similar to that observed with trypsin (16). The mechanism of inhibition is unknown. As shown in Table II, the specific activity of the purified acidic protease was quite comparable to that of commercial hog pepsin, when hemoglobin was used as substrate. The sensitivity to inhibition by 1,2-epoxy-3-(p-nitrophenoxy)propane and by \( p \)-bromophenacyl bromide was also similar to that of hog pepsin. However, the acidic protease was 100-fold less active than hog pepsin when \( N \)-acetyl-L-phenylalanyl-L-diodotyrosine was used as substrate. The protease could also hydrolyze \( N \)-benzoyl-L-glutamyl-L-phenylalanine but not \( N \)-benzoyl-L-glutamyl-L-tyrosine.

**DISCUSSION.**

The purification procedure described in this paper differs from that reported earlier (8) mainly in the use of DEAE-cellulose as an ion exchanger instead of CM-cellulose. This change allowed a larger amount of nonenzyme protein to be washed out by the buffer (Fig. 1), resulting in a better purification. In fact, column chromatography on DEAE-cel-
results similar to that of ovalbumin. The concentration of the protease was assayed by the method of Kassell and Meitner (11) with different proteins as substrates. Bovine serum albumin also gave results similar to that of ovalbumin.

**Table II**

Comparison of activities of acidic protease of human seminal plasma and of hog pepsin

| Addition | Acidic protease | Pepsin |
|----------|----------------|--------|
|          | mol/min/mg enzyme |        |
| N-Acetyl-L-phenylalanyl-L-diiodotyrosine (0.35 mM) | 31 | 3520 |
| N-Benzoyl-L-glutamyl-L-phenylalanine (0.35 mM) | 1.3 | 1.9 |
| N-Benzoyl-L-glutamyl-L-tyrosine (0.30 mM) | 0 | 0.9 |
| Hemoglobin (12.5 mg/ml) | ΔA$_{280}$/min/mg enzyme |
|          | 152 | 160 |
| Hemoglobin (12.5 mg/ml) + p-bromophenacyl bromide (0.2 mM) | 20 | 0 |
| Hemoglobin (12.5 mg/ml) + 1,2-epoxy-3-(p-nitrophenoxy)propane (0.2 mM) | 60 | 36 |

Fig. 7. Saturation curves of protein substrates. The active acidic protease was assayed by the method of Kassell and Meitner (11) with different proteins as substrates. Bovine serum albumin also gave results similar to that of ovalbumin.

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