Studies on Elastase and Elastin

II. PARTIAL PURIFICATION AND PROPERTIES OF ACTIVATION OF PROELASTASE*

Francois Lamy and Stuart Tauber

From the Department of Anatomy, University of Pittsburgh School of Medicine, Pittsburgh 13, Pennsylvania

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Elastase is a proteolytic enzyme that hydrolyzes the peptide bonds of elastin (1, 2). It was puzzling to early investigators that elastase activity could be demonstrated only with difficulty, if at all, in fresh extracts of pancreas or pancreatic juice (3, 4), although it was readily extracted from dried, defatted, powdered pancreas. In 1955 Grant and Robbins (5, 6) clarified this phenomenon by demonstrating that freshly extracted pancreas contained an inactive proenzyme capable of elastolytic activity when activated by trypsin. We shall describe in this communication the partial purification of proelastase and some features of its activation.

EXPERIMENTAL PROCEDURE

In addition to the analytical reagents necessary for the preparation of buffers, the following chemicals were used. Tris(hydroxymethyl)aminomethane (primary standard) was purchased from the Sigma Chemical Company. Twice crystallized trypsin, salt-free and containing 50% magnesium sulfate, purified soybean trypsin inhibitor, and the synthetic substrates benzoyl-L-arginine methyl ester and acetyl-L-tyrosine ethyl ester were purchased from Mann Research Laboratories.

Crystalline trypsinogen and DFP-trypsin were kindly provided by Dr. C. Martin, Department of Biochemistry, University of Pittsburgh School of Medicine, and by Dr. Sam Seifter, Department of Biochemistry, Albert Einstein School of Medicine, respectively.

The elastin used as a substrate for elastase was the defatted, alkali-treated product described previously (2).

Protein Determination—The protein content of various fractions obtained during purification and the amount of degraded elastin in solution after elastase action were determined by the phenol-biuret reagent of Lowry et al. (9). The color developed was related to the amount of elastin solubilized by the relation, optical density = 0.0245 x (milligrams of elastin solubilized) (2). The concentration of trypsin was obtained spectrophotometrically at 280 μm by using a value of ε1% = 14.3 for the specific extinction coefficient (10).

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1 The abbreviations used are: DFP-trypsin, trypsin treated with the inhibitor, diisopropyl fluorophosphat; PEI.U., proelastolytic unit; (T.U.), trypsin unit according to Kunitz (7); (U.T.) and (U.C.), trypsin and chymotrypsin units defined as change of absorbancy per minute X 10⁸ (at 253 μm) when the enzymes hydrolyze benzoyl-L-arginine methyl ester and acetyl-L-tyrosine ethyl ester (8).

Protoelastase Activity—Trypsin activity was measured both by the spectrophotometric technique of Schwert and Takenaka (8) and by the casein digestion method of Kunitz (7). Chymotrypsin was determined spectrophotometrically (8).

RESULTS

Protoelastase Activity in Pancreas—The existence of proelastase can be demonstrated easily in hog pancreas. The glands collected at the slaughterhouse are immediately refrigerated, transported to the laboratory, and frozen at -20°. Ten grams of frozen pancreas are trimmed of fat and homogenized in a cold Waring Blendor with 100 ml of 0.06 M Tris buffer at pH 8.6. The insoluble material is removed by centrifugation, and the supernatant fluid is assayed for elastase activity in both the absence and the presence of 0.5 mg of added trypsin. Table I shows the composition of the reaction mixtures and the results of such an experiment. Comparison of Flasks 3, 4, and 5 demonstrates that in the absence of trypsin no hydrolysis of elastin takes place. Trypsin itself (Flask 6) has no activity toward elastin. When the extract and trypsin are mixed, as in Flasks 1 and 2, however, 10.5 mg of the original 50 mg of elastin become soluble.

Extracts of fresh pancreas of various animals were tested for their proelastolytic activity according to the same method. Expressed in milligrams of elastin dissolved in 20 minutes per 100 mg of dry tissue, the results are as follows: rat, 20 mg; hog, 89 mg; goosefish, 200 mg (11); rabbit, 0; man, 0. Human and rabbit plasma were also assayed and showed no activity.

Assay for Proelastase and Definition of Proelastolytic Unit—One unit of proelastase is defined as the amount of the proenzyme that, when completely activated by trypsin, will hydrolyze 1 mg of elastin in 20 minutes at 37° in 0.06 M Tris buffer at pH 8.6.

The activation by trypsin is extremely rapid, as will be shown later, so that the assay for proelastase is essentially the same as the 20-minute assay for elastase (2), which is performed under conditions in which the hydrolysis of elastin by elastase follows zero order kinetics. Table I summarizes the conditions under which the assays are performed, and Fig. 1 shows the linear relationship that exists between the concentration of proenzyme and the amount of elastin solubilized after activation by trypsin.

Fractionation Procedure—The starting material was freshly frozen hog pancreas. In the development of the fractionation procedure, only 10 g of the gland were processed at a time, but the same method can be applied to as much as 1 kg of starting material. All procedures were carried out in the cold. Table II summarizes the method. The specific activity of the product
obtained at the intermediate steps is somewhat variable, but that of the final product is quite constant, ranging between 13 and 16 PELU per mg of protein.

Ten grams of frozen pancreas were trimmed of fat and ground in 100 ml of 0.05 M Tris buffer at pH 8.6, containing 10 mg of soybean trypsin inhibitor. The inhibitor is necessary to prevent the spontaneous activation of the proenzyme by trypsin. The classic extraction of the pancreas by 0.25 M sulfuric acid (7) is not feasible because proelastase is completely inactivated at low pH.

The extract was centrifuged at a low speed to remove insoluble material, and the supernatant solution was defatted with butanol. To 100 ml of extract, 8 ml of cold butanol were added drop by drop with stirring. The solution was centrifuged at 2500 x g for 20 minutes. A heavy fatty layer covered the active solution, which was recovered with a syringe. The same treatment with butanol was repeated until a layer of pure butanol appeared after centrifugation. At this stage, 50 ml of the extract remained, possessing approximately the same specific activity as the original extract.

The activity in the defatted extract was precipitated by dialysis against an 0.01 M acetate buffer at pH 5.6. At this molarity a precipitate can be obtained between pH 4.5 and pH 8.3. However, the maximal yield of activity occurs at pH 5.6. The yield decreases sharply below pH 5.0 and above pH 7.0.

The precipitate was collected by centrifugation and washed twice with distilled water. It was dissolved in 50 ml of 0.06 M Tris buffer at pH 8.6. The resulting solution was cloudy and could be clarified by high speed centrifugation.

An ammonium sulfate precipitation was then carried out. Preliminary experimentation showed that 60% of the activity could be recovered between 25 and 40% saturation, whereas only 40% of the protein precipitated. The precipitate was washed with saturated ammonium sulfate and redissolved in 25 ml of 0.08 M Tris-maleic acid buffer at pH 6.5. The solution was again dialyzed against 0.01 M acetate buffer at pH 5.0, and the final precipitate was redissolved in Tris-maleic acid buffer at pH 6.5.

The proenzyme was kept frozen in this buffer, because it loses its activity completely upon lyophilization and progressively becomes inactive when stored frozen at a slightly alkaline pH.
tyrosine ethyl ester by trypsin itself. A sample of crystalline chymotrypsin showed an activity of 11.2 (U.C.) per μg of protein when tested under the same conditions. It is possible, therefore, that in a typical proelastase preparation chymotrypsinogen represents approximately 30% by weight of the protein present.

By contrast, no trypsinogen activity was detected, even after a long period of activation (Table III). Trypsin alone progressively loses its activity over a period of 3 days. With a mixture of proelastase and trypsin, no increase in tryptic activity is noticed, as would be expected if trypsinogen were present. The activity also decreases with time but is always lower than that observed for trypsin alone. On the average 5 (U.T.) per 0.2 ml of the reaction mixture, or 48 (U.T.) per PEl.U., seem to disappear.

**Activation of Proelastase by Trypsin**—Trypsin, at the concentration used in the 20-minute assay, is adequate as an activator since a linear relationship exists between the amount of elastin hydrolyzed and the concentration of proelastase. Some of the features of this activation were studied further in order to determine whether the 20-minute assay is carried out under optimal conditions, as well as to try to understand the mechanism by which proelastase is transformed into active elastase by trypsin.

![Fig. 2. Elastase formed from 20 PEl.U., by different amounts of trypsin, as a function of time. The figures appearing on each of the four curves refer to the concentration of trypsin used in 3 ml of reaction mixture.](http://www.jbc.org/)

**Fig. 2.** Elastase formed from 20 PEl.U., by different amounts of trypsin, as a function of time. The figures appearing on each of the four curves refer to the concentration of trypsin used in 3 ml of reaction mixture.

**TABLE III**

**Stability of trypsin in presence of proelastase**

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Temperature</th>
<th>Activity in reaction mixture</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 min</td>
<td>25°</td>
<td>46</td>
<td>9.2</td>
</tr>
<tr>
<td>10 min</td>
<td>24</td>
<td>44</td>
<td>8.8</td>
</tr>
<tr>
<td>60 min</td>
<td>24</td>
<td>33</td>
<td>6.6</td>
</tr>
<tr>
<td>3 days</td>
<td>4</td>
<td>22</td>
<td>4.4</td>
</tr>
<tr>
<td>Trypsin + proelastase</td>
<td>24</td>
<td>41</td>
<td>8.2</td>
</tr>
<tr>
<td>10 min</td>
<td>25</td>
<td>44</td>
<td>8.8</td>
</tr>
<tr>
<td>60 min</td>
<td>25</td>
<td>50</td>
<td>3.5</td>
</tr>
<tr>
<td>3 days</td>
<td>18</td>
<td>3.6</td>
<td></td>
</tr>
</tbody>
</table>

Since there seems to be a relationship between the amount of trypsin and the amount of elastase produced, we tried to determine the minimal amount of trypsin necessary to activate 1.0 PEL.U. The experimental data are shown in Fig. 3 (curve with open circles). To a series of aliquots containing 7.5 PEL.U. were added various amounts of trypsin, and the elastase produced was estimated by the 20-minute assay. The curve obtained is linear at the origin and ends up parallel to the abscissa. If these two lines are extended, they meet at the point at which 7.5 PEL.U. are activated by 8.5 μg of trypsin. We conclude that it takes roughly 1.13 μg of trypsin to activate fully 1.0 PEL.U.
In the standard proelastase assay, trypsin, the proenzyme, and elastin are added together, and the mixture is placed immediately at 37°. Two reactions occur. First, proelastase is activated to form elastase, after which the enzyme hydrolyzes the substrate. If the assay is to measure the amount of the proenzyme, then the rate of activation must be much faster than the rate of hydrolysis of the substrate by the active enzyme.

We checked this point in the experiment described in Table IV. The experimental plan was to incubate trypsin and proelastase, to stop the activation reaction with soybean trypsin inhibitor after various times of contact, and finally to carry out a 20-minute assay to measure the amount of elastase produced during the first reaction. The data in Table IV demonstrate that the activation is very rapid. At 37°, it is essentially complete after 1 minute of contact with trypsin. At 1°, it seems to be as rapid, but the accuracy of the assay is not adequate to detect a slight difference in the rate of activation.

When an excess of trypsin is used to insure that all of the proelastase is activated, the amount of elastin hydrolyzed is proportional to the amount of proelastase in the sample up to a concentration of 20 PELU. with purified proelastase and 10 PELU with a pancreatic extract. The smaller capacity in the assay of pancreatic extracts is a result of the presence in the substances used in place of trypsin or in addition to it.

Specificity of Trypsin as Activator of Proelastase—The experiments described so far show that trypsin is a very efficient activator of proelastase, but do not demonstrate that the activation process involves a trypsin-catalyzed hydrolysis of a peptide bond (or bonds) in proelastase. In order to explore this question, the experiments described in Lines 1 to 6 of Table V were performed. In general, it can be said that when the proteolytic activity of trypsin is inhibited, the ability of the enzyme to

### Table IV

**Rate of production of elastase from trypsin-activated proelastase at 1° and 37°**

A series of flasks containing 9 PELU. per 3 ml of 0.06 M Tris buffer at pH 8.6 were set up at the two temperatures with 250 µg of trypsin. The reactions were stopped by addition of 250 µg of soybean trypsin inhibitor. This amount of inhibitor was found sufficient to prevent 250 µg of trypsin from hydrolyzing benzoyl-L-arginine methyl ester. Elastin, 50 mg, was then added, and the 20-minute assay for elastase was performed. The control consisted of a standard 20-minute assay performed on the same proelastase. The amounts of elastin hydrolyzed in the reaction mixtures are given as percentages of that hydrolyzed in the control.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>Activity at 1°</th>
<th>Activity at 37°</th>
</tr>
</thead>
<tbody>
<tr>
<td>sec</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>10</td>
<td>59</td>
<td>56</td>
</tr>
<tr>
<td>30</td>
<td>79</td>
<td>85</td>
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<tr>
<td>60</td>
<td>77</td>
<td>92</td>
</tr>
<tr>
<td>120</td>
<td>97</td>
<td>95</td>
</tr>
<tr>
<td>300</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>600</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>300°</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**In this flask, soybean trypsin inhibitor and proelastase were added before trypsin and incubated for 300 seconds. The soybean inhibitor-trypsin complex is not an activator of proelastase, since no elastin is hydrolyzed.**

### Table V

**Ability of various compounds to activate proelastase**

A standard 20-minute proelastase assay was performed with each of the given activators. The amount of elastin hydrolyzed is expressed as the percentage of that obtained by using trypsin alone with the same proenzyme preparation.

<table>
<thead>
<tr>
<th>Activator</th>
<th>Concentration of activator in reaction mixture*</th>
<th>Trypsin activity</th>
<th>Elastase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Trypsin</td>
<td>250 µg/3 ml</td>
<td>0.98</td>
<td>100</td>
</tr>
<tr>
<td>2. DFP-trypsin</td>
<td>250 µg/3 ml</td>
<td>0.12</td>
<td>75</td>
</tr>
<tr>
<td>3. Trypsin, heated at 56°</td>
<td>950 µg/3 ml</td>
<td>0.004</td>
<td>10</td>
</tr>
<tr>
<td>4. Trypsinogen</td>
<td>500 µg/3 ml</td>
<td>0.002</td>
<td>8</td>
</tr>
<tr>
<td>5. Trypsin + soybean</td>
<td>250 µg/3 ml</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6. Chymotrypsin</td>
<td>500 µg/3 ml</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7. Protamine</td>
<td>500 µg/3 ml</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>8. Trypsin + cysteine</td>
<td>10⁻² M</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>9. Trypsin + Ca²⁺</td>
<td>3 × 10⁻³ M</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>10. Trypsin + EDTA</td>
<td>3 × 10⁻³ M</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>11. Trypsin + EDTA + Ca²⁺</td>
<td>3 × 10⁻³ M each</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

* In this column, after the first line, it is to be understood that trypsin, when used, was always at a concentration of 250 µg per 3 ml of reaction mixture. The concentrations listed are those of the substances used in place of trypsin or in addition to it.
activate proelastase is also impaired. For example, the soybean inhibitor-trypsin complex is completely inactive. Similarly, DFP-trypsin, heat-denatured trypsin, and trypsinogen have a reduced ability to activate proelastase. Although heat-denatured trypsin and trypsinogen have a negligible proteolytic activity, they still have a slight capacity to activate proelastase. Similarly, DFP-trypsin, which exhibited only 13% of the specific proteolytic activity of crystalline trypsin, was 75% active toward proelastase.

The properties of DFP-trypsin and of heat-denatured trypsin were further examined in the following experiments. Fig. 3 (curve with solid circles) shows the amount of elastase produced when DFP-trypsin (0.12 T.U., per mg) acts on a solution of proelastase (8.0 PEL.U. per 3 ml of reaction mixture). The curve has the same initial slope as that obtained with trypsin and, if it is extrapolated in the same fashion, indicates that 60 µg of DFP-trypsin are needed to activate 6.0 PEL.U., whereas it took 8.5 µg of trypsin to produce 7.5 PEL.U. In other words, 1.13 µg of crystalline trypsin and 10 µg of DFP-trypsin (containing 1.30 µg of active enzyme) are needed for each PEL.U.

The activation of proelastase with DFP-trypsin can, therefore, be accounted for by the active enzyme in the preparation. Even with a large excess of DFP-trypsin, however, the full potential activity of the proelastase was not realized. Since only 6 elastase units were obtained instead of the 8 produced by activation with trypsin, it is possible that DFP-trypsin acts as an inhibitor of elastase.

In Fig. 4 is shown the rate of heat denaturation of trypsin as measured by its remaining proteolytic and proelastase-activating properties. A solution of trypsin (350 µg per ml) was heated at 56°. At different times, aliquots were assayed for their proteolytic activity against casein, and, in a 20-minute assay with proelastase and elastin, their ability to activate the proenzyme was also measured. Trypsin loses its proteolytic activity much more rapidly than it does its ability to activate proelastase. After 60 minutes at 56°, practically no proteolytic activity is detectable, whereas 10% of the proelastase can be transformed into active enzyme by the heated product. This does not imply that the denatured enzyme can activate proelastase. In these experiments a large excess of trypsin was used over the amount just sufficient to activate proelastase, but when the trypsin was assayed against casein it was diluted sufficiently to fall in the range of concentration adequate for the casein assay. In the heated preparation enough residual native enzyme would be present to activate a portion of the proelastase, whereas after dilution, this trypsin would not be detected by the casein assay.

Lines 7 to 11 of Table V show that two other basic proteins, chymotrypsinogen and protamine, cannot activate proelastase and that the addition of various compounds to trypsin does not enhance or inhibit the activating ability of the enzyme.

**DISCUSSION**

The experiments described in this work confirm the findings of Grant and Robbins (3, 6) that an inactive form of elastase exists in the pancreas of several animal species. A 40-fold concentration of this substance was achieved. Its properties clearly are those of a protein, and it can be purified by methods that are effective for proteins in general. The product is contaminated by chymotrypsinogen, as were partially purified preparations of elastase by chymotrypsin (2). Apparently the two zymogens and the two enzymes have very similar physical properties. In this connection it is worth mentioning that Naughton and Sanger (12) have obtained an elastase preparation very low in chymotrypsin activity by chromatography on a carboxymethyl cellulose column at pH 4.5. Preliminary experiments with proelastase under the same conditions have led to an almost total loss of proelastolytic activity.

Although proelastase was demonstrated in the pancreas of the hog, rat and goosefish, it could not be found in the pancreas of rabbit and man. Bala and Banga (13) and Lewis, Williams, and Brink (3) reported the presence of elastase in human pancreas, but Hall, Reel, and Turnbridge (14) could not find it. Since the authors were assaying for elastase and not for proelastase, any activity detected would be expected to be very small. Indeed, from the data of Lewis, Williams, and Brink it can be calculated (cf. (2)) that the human pancreas tested contained between 0.00015 and 0.0031 elastase unit per mg of protein, as compared to 60 to 70 units for crystalline elastase. Even after activation with trypsin, we were unable to detect any activity in human pancreas. This does not necessarily mean that the proenzyme is absent in the gland. The lack of activity could be due to a potent elastase inhibitor or to the fact that human trypsin has a specific ability to activate human proelastase, which is lacking in the bovine trypsin used in this work.

The activation of proelastase by trypsin presents interesting characteristics, which can be accounted for by the following hypothesis. Trypsin breaks some specific and particularly exposed bond in the proelastase molecule with the formation of two fragments. One is the active elastase; the other is an inhibitor of trypsin that inactivates it very rapidly.

The bond(s) broken in proelastase must be peptide bond(s) involving the carboxyl groups of lysine or arginine residues, since only native trypsin will activate the proenzyme. Chymotrypsin and the soybean inhibitor-trypsin complex are not activators of proelastase. Trypsinogen exhibits only a very weak activity. The activity of DFP-trypsin and of heat-denatured trypsin can be accounted for by the residual, native trypsin in the preparations. The bond(s) attacked must be particularly exposed, since the reaction proceeds so rapidly (Table IV). Similarly susceptible bonds are known to exist in myosin, which is also attacked very rapidly by trypsin (fast reaction of Mihalyi and Harrington (15)).

One of the fragments cleaved from the proelastase must be able to associate very tightly with trypsin and to inhibit it completely. This is why we found that on the average 48 (U.T.) are inactive for each PEL.U. produced, that a minimal amount of trypsin does not produce an increasing amount of elastase with time (Fig. 2), and that the production of elastase is proportional to the concentration of trypsin (up to 75% of complete activation, Fig. 3).

The experimental data presented in this paper are in accord with this hypothesis. A more rigorous test of its validity must await the preparation of pure proelastase.

**SUMMARY**

A 40-fold purification of proelastase of hog pancreas has been achieved. The product is contaminated with chymotrypsinogen, but not with trypsinogen.

The activation of proelastase to elastase requires active trypsin. This reaction is rapid and is proportional to proelastase concentration (with an excess of trypsin) and to trypsin (up to 75% of complete activation). When a minimal amount of trypsin
is used, the elastase formed does not increase with time. Approximately 1.1 \mu g of trypsin are needed for each proelastolytic unit activated.

Based upon these properties an assay for proelastase has been developed. Trypsin in large excess, proelastase, and elastin are mixed, and the amount of soluble polypeptides liberated by the action of the newly formed elastase on elastin is measured.

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