Plasminogen Activator from Porcine Heart Tissue [*], [**]
Isolation and Partial Characterization

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Summary

By ion exchange chromatography, precipitation with $(NH_4)_2SO_4$ and gel filtration highly enriched tissue activator of plasminogen is prepared from pig heart tissue. Further purification can be achieved by isoelectric focusing. With the same technique five different forms of the activator were observed with isoelectric points between *p*H 7.2 and 8.9. The existence of multiple forms was also confirmed by disc polyacrylamide gel electrophoresis at *p*H 3.8. From gel filtration parameters a molecular weight of 58000 was obtained for the tissue activator.

Introduction

Tissue activator undoubtedly plays an important role in the physiological activation of plasminogen. Despite its significance tissue activator has received only moderate interest and the molecular parameters are only poorly characterized. This is, at least to a certain extent, due to the difficulties which are encountered during the isolation and purification of this substance. It is associated with certain structural elements of the cell [1], thus requiring special conditions for its solubilization. Moreover, since it is a potent activating substance, even minute quantities of it in the tissues are sufficient to guarantee an adequate activating potential in vivo.

Tissue activator is present in most organs (except liver) [2,3]. The richest sources are highly vascularized organs, such as uterus, prostate and heart tissue. The first step in an isolation procedure of tissue activator is its solubilization. For this purpose several methods have been proposed over the past years, such as extraction with 2M potassium isothiocyanate [4], extraction under nearly physiological conditions [5] or at an elevated temperature (for the isolation of activator from the vascular bed) [6] and solubilization in the presence of urea [7].

In previous work [8] some of these methods were investigated and compared with each other. We came to the conclusion that—at least for our purpose—solublization with urea was most suitable, since it gave a reasonable yield of activator activity and the amount of spontaneous protease activity concomitantly extracted was lower than in the other methods.

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Material and Methods

Tissue homogenization

The ground tissue was suspended in ice-cold 5M urea solution [7] (2 liters per kg tissue) and the mixture was first treated in an ordinary household mixer. The *p*H of the resulting suspension was brought to 5 and 0.02 M acetate buffer, containing 0.05M NaCl, *p*H 5.0 was added (to a total volume of 8.8 liters). Homogenization was then carried out in a Manton-Gaulin press (manufactured by Manton-Gaulin, Hilversum, Holland). In this apparatus the pre-mixed tissue suspension is forced through an adjustable nozzle at a working pressure between 600 and 650 atmospheres. Microscopic examination revealed that the treatment with the mixer alone caused the rupture of only less than half of the cells, whereas after two or three consecutive treatments with the Manton-Gaulin press virtually no intact cells were detectable. With this combined homogenization technique the yield of solubilized activator was increased by a factor of about 3 as compared to homogenization in the ordinary mixer.

Activity measurements

Activator activity was measured quantitatively using human fibrinogen which had been labeled with dansylchloride (5-dimethylamino-1-naphthalene sulfonyl chloride) by the method of Rinderknecht [10]. The assay mixture contained 0.5 ml 0.5% fibrinogen solution, 0.3 ml activator solution, 0.1 ml 0.06% plasminogen solution (plasminogen was isolated by affinity chromatography [11]) and 0.1 ml 0.1% thrombin solution (Thrombin «Roche», 60 to 70 NIH U/mg).

After incubation for 6 min at 37°C the samples were put into an ice-bath and 5 ml of cold saline were added. The residual clot was broken up and then removed by centrifugation. The fluorescence in the supernatant was measured in an Eppendorf photometer with an attachment for fluorescence measurements, using a 313 to 366 nm primary filter, a 500 to 3000 nm secondary filter and calibrated with fluorescence standard Nr. 6637. Activator activity was expressed in CTA units using a calibration curve obtained with a standard urokinase preparation[1] in the range of 5 to 40 CTA units.

For serial measurements the fibrin plate method [12, 13] was applied using both unheated and heated fibrin plates to test for unspecific protease activity. This was necessary only in the first two steps of the isolation procedure.

Protein determination

Protein was usually determined by the method of Warburg and Christian [14]. Occasionally the Lowry-method [15] was also applied.

Gel electrophoresis

Disc polyacrylamide gel electrophoresis was performed with 7.5% polyacrylamide gels at *p*H 3.8 according to Maurer [16].

Chemicals

Chemicals and reagents were of the «pro analysi» grade, obtained from Merck, Darmstadt.

Results

Isolation procedure

1. Crude extract

Fresh pig hearts, cleaned by removing fat and larger blood vessels, were first put through a meat grinder and the meat was frozen at −60°C. Homogenization was carried out as described under «Materials and Methods». Care was taken to provide adequate cooling during the treatment in the Manton-Gaulin press. The homogenate was centrifuged at 5000 × g for 1 hr at 4°C. The sediment was discarded.

2. Adsorption on CM-Sephadex C-50

Adsorption on CM-Sephadex C-50 was chosen to recover the tissue activator from the large volume of homogenate. This was done in the batch-procedure by adding 3 g of equilibrated CM-Sephadex per liter of crude extract. After 2 to 3 hrs the ion exchanger was separated on a Buchner funnel and washed with 0.02M acetate buffer, containing 0.26M NaCl, *p*H 5.0. Activator activity was eluted by increasing the NaCl concentration in the buffer to 0.6M.

3. Precipitation with $(NH_4)_2SO_4$

The slightly reddish eluate was subjected to a precipitation with $(NH_4)_2SO_4$ at 30% saturation. The sediment of precipitated activator was then extracted with 0.02M acetate buffer, *p*H 5.0, containing 0.18M $(NH_4)_2SO_4$. Two or three such extractions yielded «concentrate I».

4. Gel filtration on Sephadex G-200

Gel filtration was carried out on Sephadex G-200 in 0.02M acetate buffer, *p*H 5.0, containing 0.33M $(NH_4)_2SO_4$ in a column 7 × 90 cm. Activator activity was eluted in a small peak after the appearance of a large peak containing essentially inert protein (see Fig. 1).

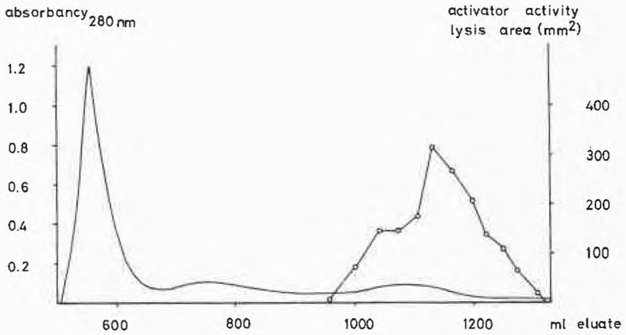

Fig. 1: Gel filtration of concentrate I on Sephadex G-200 ——— absorbancy at 280 nm, —O—O— activator activity

[1] We are indebted to Drs. *R. Strässle* and *R. Studer* of F. Hoffmann-La Roche, Basel, for the generous gift of urokinase.

Table 1: Yields and purities of tissue activator observed at the individual steps of the purification. Starting material was 1 kg of porcine heart tissue

	Protein content, grams	Specific activity CTA units/mg	Total activity	Purification factor
Crude extract	ca. 19	7	135000	
CM-Sephadex eluate	1.63	76	124000	11
Concentrate I	0.52	164	85000	23
Gel filtrate	0.02	2800	56000	400
Electrofocusing (peak fractions)	0.001	15000	15000	2150

In Table 1 yields and purities after each step of the procedure are summarized. The data were obtained from a typical experiment using 1 kg of heart tissue as the starting material.

One of the most important steps in the fractionation method is the adsorption of the tissue activator on CM-Sephadex. This allows an efficient recovery of the desired substance from the homogenate and enables the removal of a significant amount of contaminating protein by washing the adsorbent at a moderately increased ionic strength. At the same time this washing procedure ensures a practically quantitative elimination of unspecific protease activity. Tissue activator preparations which were carried through the CM-Sephadex adsorption step are free of any measurable protease activity, which means that the activator activity can be determined with unheated fibrin substrate. The most efficient purification is achieved in the gel filtration step. The separation is greatly affected by the ionic strenght of the medium. At low ionic strength we observed a considerable overlapping between the two peaks. The separation was improved by an increase in ionic strength which apparently counteracts associative tendencies between tissue activator and other components of the mixture. Satisfactory separation was obtained at an ionic strength of 1. High ionic strength of the medium had a similar influence on the nucleic acid content of the preparation. According to the 260/280 nm absorbance ratio nucleic acid material is, in variable amounts, a companion of the tissue activator. By working in the presence of relatively high salt concentrations the content of nucleic acid material in the active fractions at this stage was lowered to less than 1%.

Estimation of the molecular weight

The molecular weight of the tissue activator was determined from gel filtration parameters obtained with a column (5 × 80 cm) of Sephadex G-200 in 0.02 M acetate buffer, *p*H 5.0, containing 0.33 M $(NH_4)_2SO_4$. As reference proteins human transferrin, bovine serum albumin, porcine pepsin and bovine β-lactoglobulin (proteins obtained from Sigma Chemicals Co., Saint Louis, USA) were used, and from their elution parameters a calibration curve was obtained in the form of a straight line. With the aid of this curve a molecular weight of 58000 was determined for the tissue activator.

Isoelectric focusing

For the estimation of the isoelectric point highly purified tissue activator was subjected to isoelectric focusing in a LKB-8101 column. The *p*H and density gradients were formed according to the instructions of the manual supplied by LKB, using *p*H 3 to 10 Ampholine solution and saccharose. In order to prevent the protein from precipitating in the sharpening zones urea (recrystallized in the cold) had to be incorporated in the medium at a concentration of 3,5 M. Isoelectric focusing was carried out at 2 °C for about 20 hrs with an initial voltage of 500 V. The column content was then collected in 2 ml fractions, the *p*H in the fractions was measured and the protein was finally dialyzed against 0.05 M phosphate buffer, *p*H 7.4, containing 0.15 M NaCl. The determination of activator activity gave at least five maxima within the *p*H range between 7.2 and 8.9 with individual isoelectric points at *p*H 7.2, 7.65, 8.0, 8.4 and 8.9. On re-electrofocusing of the individual peak fractions under identical conditions the activity maxima again appeared in their corresponding isoelectric points. These individual forms did not differ markedly in their specific acticities which, on the average, reached a level of 15000 CTA units/mg protein.

Isoelectric focusing can be considered as an additional step of the purification procedure, as indicated in Table 1.

The existence of individual activator forms was confirmed by polyacrylamide gel electrophoresis at *p*H 3.8. By this technique the pooled peak fractions which were dialyzed and concentrated by ultrafiltration showed 5 to 6 bands closely following each other.

Discussion

The described procedure allows the preparation of highly enriched tissue activator. The modified homogenization technique has the advantage of solubilizing about three times as much activator activity from a given amount of tissue as compared to the previously used method [9]. The yield of activator after the gel filtration step is 40 to 45% which is an agreement with the original method. After gel filtration, however, the specific activity is only 50 to 60% of the value reported earlier [9]. It is possible that the more efficient homogenization produces a different spectrum of contaminating protein with componentes which are only incompletely removed by the applied purification steps. The molecular weight value of 58000 agrees well with reports from other laboratories. Bachmann [17], and also Kok and Astrup [18] obtained from gel filtration parameters values between 56000 and 60000. Isoelectric focusing indicated the presence of tissue activator forms which differ in their isoelectric points. This may indicate the occurence of isoforms. At present, however, we do not have sufficient information to decide whether tissue activator really exists in multiple active forms in the cells. It is also feasible that the observed multiplicity may represent an artifact introduced, for instance, by a partial proteolytic degradation under the action of cell proteases in the first stage of the purification procedure.

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