Improved Procedure for the Isolation of Human Plasminogen*

Daniel L. Kline and Jacob B. Fishman

From the Department of Physiology, Yale University School of Medicine, New Haven, Connecticut

(Received for publication, June 30, 1961)

Although plasminogen purification methods based on cellulose column chromatography have been described (1-4), the procedure based on acid extraction of Cohn's plasma Fraction III (5) has continued to be widely used because of its simplicity, reproducibility and the absence of a limitation on the amount of Fraction III which can be processed. With the Fraction III available in 1953, plasminogen with a specific activity of 45 to 60 Remmert and Cohen (7) C.U./per mg of N could be obtained by the acid method, but more recently, it has not been possible to obtain purities exceeding 40 C.U. per mg of N.

In this paper we described a modification of the original method and the addition of two steps which permit the isolation of plasminogen with a specific activity of 110 to 173 C.U. per mg of N. The over-all procedure can be applied to large quantities of material, requires no special apparatus or techniques, is reasonably reproducible, and yields a product which is homogeneous in the ultracentrifuge and in electrophoretic analyses.

EXPERIMENTAL PROCEDURE

Fraction III was obtained from E. R. Squibb and Sons through the courtesy of Dr. James H. Port of the American National Red Cross. Caseinolytic activity was measured by a modification (8) of the method of Remmert and Cohen (7). One unit represents the liberation of 450 μg of trichloroacetic acid-soluble tyrosine equivalents per hour. The unit of activity obtained with the modified procedure agrees within experimental error with that obtained with the original Remmert and Cohen method.2 Protein concentration was measured by the biuret reaction from a curve which had been established by micro-Kjeldahl analysis of purified plasminogen.

Streptokinase (Varidase, Lederle Laboratories Division) was kindly supplied by Dr. E. C. De Renzo and Dr. B. L. Hutchings.

RESULTS

The published acid extraction method (Method 1) involved an initial extraction of Fraction III with 0.05 N H₂SO₄ (1 g/20 ml) for 10 minutes at room temperature, followed by centrifugation at 2500 r.p.m. for 10 minutes. The supernatant solution was decanted through glass wool and adjusted to pH 11 with N NaOH. All acid and alkali additions were run in by pipette while the solution was stirred by hand. The pH was then immediately brought to 5.3 with N HCl and the preparation placed in the refrigerator for a minimum of 3 hours (usually overnight). The pH of the suspension was then adjusted to 2 with N HCl, and the material was centrifuged for 1 hour at 2700 r.p.m. The clear supernatant solution containing plasminogen was carefully decanted from the gelatinous residue. At this point, the new procedure (Method 2) deviated from the original method. The acid solution of plasminogen was adjusted to pH 9 with N NaOH and the volume was measured. One milliliter of 0.02 M Na₂HPO₄ solution, pH 8.5, was added per 100 ml of plasminogen solution, and the enzyme was permitted to precipitate overnight at 4°C. The plasminogen was collected by centrifugation at 2500 r.p.m. for 10 minutes and was dispersed in 0.5 ml of distilled water per g of Fraction III used, which gave a protein concentration of about 4 to 6 mg per ml at this point. Complete solution was obtained by the addition of a drop or two of N HCl after dispersal of the precipitate. The yield and specific activity varied with the batch of Fraction III used. As an average, a yield of 36.5% of the starting activity was observed with an activity of 48.5 to 74 C.U. per mg of N (Table I).

Hagan (8) has described the solubilizing effect of lysine on plasminogen at pH 5.5. We have used this observation in the next step of the purification. To the acidified plasminogen solution obtained as described above, L- or D-lysine·HCl (Mann Research Laboratories, Inc.) is added in solid form to make a solution which is 0.1 M with respect to lysine. The pH of the solution is then adjusted to 9.5 and immediately to 5.5. After 10 minutes at room temperature with occasional stirring, the insoluble material is removed by centrifugation and the solution containing plasminogen is dialyzed against 0.001 M HCl overnight at 4°C to remove the lysine. The loss of activity in this step was 10%, and the specific activity of the product varied from 75 to 112 casein units per mg of N (Table I). The dialysate is then adjusted to pH 2 with N HCl and solid NaCl is added to produce a concentration of 1.0 M. During precipitation, the mixture is placed at 4°C for 15 minutes or longer and is then centrifuged at 2700 r.p.m. for 15 minutes. The purified plasminogen is then dissolved in distilled water to any desired concentration and dialyzed against 0.001 M HCl to remove the salt. The pH of the final solution is about 3. The product contained 29% of the activity originally extractable from the Fraction III, and the specific activity varied from 110 to 173 C.U. per mg of N (Table I).

The purified plasminogen obtained by Method 2 was relatively stable in the frozen state at pH 3 or as a lyophilized powder. During a period of 2 months, 25% of the activity disappeared slowly. The lyophilized powders had been kept under room conditions.

Plasminogen which had deteriorated was reprocessed by treatment with lysine and NaCl as described above, and the original
TABLE I

Purification of plasminogen*

<table>
<thead>
<tr>
<th>Start</th>
<th>End</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity C.U./mg. N</td>
<td>Casatin units</td>
<td>Specific activity C.U./mg. N</td>
</tr>
<tr>
<td>Treatment with lysine—Step 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>1925</td>
<td>91</td>
</tr>
<tr>
<td>56</td>
<td>4510</td>
<td>75</td>
</tr>
<tr>
<td>60</td>
<td>1235</td>
<td>112</td>
</tr>
<tr>
<td>60</td>
<td>258</td>
<td>96</td>
</tr>
<tr>
<td>49</td>
<td>485</td>
<td>70</td>
</tr>
<tr>
<td>64†</td>
<td>105†</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>60.4</td>
<td>92</td>
</tr>
<tr>
<td>NaCl precipitation after lysine—Step 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>91</td>
<td>254</td>
<td>130</td>
</tr>
<tr>
<td>89</td>
<td>249</td>
<td>117</td>
</tr>
<tr>
<td>73</td>
<td>370</td>
<td>117</td>
</tr>
<tr>
<td>105†</td>
<td></td>
<td>120†</td>
</tr>
<tr>
<td>Average</td>
<td>90</td>
<td>123</td>
</tr>
<tr>
<td>Lysine and NaCl—Steps 1 and 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>97</td>
<td>1998</td>
<td>151</td>
</tr>
<tr>
<td>64</td>
<td>1950</td>
<td>123</td>
</tr>
<tr>
<td>84</td>
<td>2775</td>
<td>155</td>
</tr>
<tr>
<td>77</td>
<td>2795</td>
<td>153</td>
</tr>
<tr>
<td>80</td>
<td>4350</td>
<td>150</td>
</tr>
<tr>
<td>79</td>
<td>4520</td>
<td>173</td>
</tr>
<tr>
<td>63</td>
<td>4500</td>
<td>140</td>
</tr>
<tr>
<td>50</td>
<td>5200</td>
<td>115</td>
</tr>
<tr>
<td>53</td>
<td>2350</td>
<td>110</td>
</tr>
<tr>
<td>Average</td>
<td>73</td>
<td>137</td>
</tr>
</tbody>
</table>

* Starting material was obtained from Fraction III by two acid extractions and precipitation by addition of phosphate buffer (see text).
† Fraction III prepared by Cutter Laboratories.

specific activity was regained. In fact, less pure materials, after a second processing, equalled those obtained in the most successful experiments.

Spontaneous plasmin activity amounted to 2.1% of the total obtainable after activation with optimal concentrations of streptokinase. This quantity of presumably nonplasminogen contamination was not detected in Tiselius electrophoretic or in ultracentrifuge studies. All preparations of purified plasminogen showed a single peak in electrophoretic studies at pH 2.0 to 4.0 at enzyme concentrations from 2.0 to 10 mg per ml despite variations in specific activity from 100 to 175 C.U. per mg of N. Evidently, such analyses are not decisive as a criterion of plasminogen purity.

Studies in the ultracentrifuge likewise indicated a single protein type to be present (Fig. 1). The sedimentation constant determined at 22.7°C in 0.1 M glycine buffer, pH 2.9, was 3.38 S. Corrected to water at 20°C, the value is 3.2.

FIG. 1. Sedimentation pattern of human plasminogen. Concentration of protein 1%, buffer 0.1 M glycine, pH 2.9. Photographs were taken 32 and 96 minutes after rotor reached speed of 59,780 r.p.m. in a Spinco model E ultracentrifuge. Direction of sedimentation is to the left.

The purified material contained no organic ash, 14.9% nitrogen, and 0.91% reducible carbohydrate. During the conversion of plasminogen to plasmin with streptokinase, none of the carbohydrate could be detected in the protein-free supernatant solution in agreement with the finding of Shulman, Alkjaersig, and Sherry (9). We did not confirm a conflicting report of the release of carbohydrate during the activation of plasminogen (10). The purified plasminogen exhibited the same solubility characteristics as less purified material. It was easily soluble only below pH 4.0 or above 8.6.

Upon the addition of 10,000 streptokinase units per C.U., the purified plasminogen was completely converted to plasminogen activator as measured in lysine methyl ester and bovine clot assays, supporting our finding with less pure plasminogen that human plasminogen or plasmin have proactivator potential (6).

Purification of plasminogen beyond the specific activities obtained with the original acid method has been described. Norman (3) used diethylaminoethyl cellulose, eluting with aminoethanol. Using Method 2 described in this paper, he has obtained material which was slightly more active than that isolated from his cellulose columns.3 Hagan, Ablondi, and De Renzo (4) have reported purification after separation on a carbosymethyl cellulose column and elution at acid pH. It is not pos-

3 P. S. Norman, personal communication.
Purification of Plasminogen

Vol. 236, No. 12

possible to compare directly the activities obtained by them with those obtained with Method 2 since different assays were used. Calculations, however, indicate that the products possessed a similar specific activity if the best of each are considered. The carboxymethyl cellulose columns, however, in addition to imposing severe limitations on the quantity of enzyme which can be handled, are also laborious to use and have proved to be uncertain because of variations between batches of either cellulose or Fraction III.

The nitrogen value obtained, 14.9%, is lower than that reported for less pure plasminogen (9); carbohydrate, 0.94%, was the same. The sedimentation constant, 3.2 S, agrees with the previously reported figure (4, 9) after correction for the concentration of protein used.

Since streptokinase can react with plasmin to form a plasminogen activator (6), it is noteworthy that spontaneous plasmin activity was found in these highly purified preparations. The possibility that streptokinase can also react with plasminogen to form an activator is not excluded by these results. The most highly purified preparations are converted to plasmin by streptokinase or urokinase, and “autocatalytic” conversion occurs in 50% glycerol. Plasmin has been obtained by the use of small amounts of streptokinase (50 units per C.U.) with a specific activity equal to that of the plasminogen used. The plasmin formed, however, is unstable in the frozen or lyophilized state. Activity is maintained in 50% glycerol solution.

SUMMARY

A modification of the acid extraction method of plasminogen purification is described and two additional steps have been added. The modification consists in a precipitation by the addition of phosphate buffer, pH 8.5 to the plasminogen solution which has been adjusted to pH 9.0 instead of precipitation at pH 6.0. The two additional steps involve (a) extraction of plasminogen with 0.1 M lysine at pH 5.5 followed by dialysis against 0.001 M HCl to remove the lysine, and (b) precipitation of plasminogen at pH 2.0 by the addition of solid NaCl to a final concentration of 1.0 M. With this new procedure, Method 2, the increase in specific activity as compared to serum is about 400 times. The simplicity of the original method has been retained and reproducibility is satisfactory.

REFERENCES

Improved Procedure for the Isolation of Human Plasminogen
Daniel L. Kline and Jacob B. Fishman