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Isolation of plasmin-free human plasminogen with N-terminal glutamic acid

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SUMMARY

Lysine-coupled polyacrylamide was synthesized as an adsorbent for the isolation of human plasminogen directly from plasma by affinity chromatography With this adsorbent a practically quantitative removal of plasminogen from plasma was achieved The adsorbed proenzyme was subsequently eluted with phosphate buffered ϵ-aminocaproic acid, in a yield of 10 mg per 100 ml of citrated plasma The specific activity of the plasminogen after activation with streptokinase amounted to 32–42 modified caseinolytic Remmert-Cohen units per mg protein The preparations were free of spontaneous plasmin (EC 3 4 4 14) activity, as determined with the caseinolytic assay. Polyacrylamide gel electrophoresis demonstrated electrophoretic homogeneity of the preparations at acid pH and revealed several electrophoretically different forms at alkaline pH The only N-terminal amino acid detectable in this plasminogen was glutamic acid in an amount close to 1 mole per mole protein

A large number of methods has been developed for the isolation and purification of plasminogen, the precursor of the protease plasmin (EC 3 4 4 14) Some of these methods yield preparations which, based on chemical, physical and immunochemical criteria, are essentially homogeneous However, it is not possible to deduce from this information whether the isolated plasminogen reflects the native state of the molecule as it exists in plasma Most of the known preparations are reported to be contaminated with variable amounts of active plasmin which is capable of activating plasminogen as well as of proteolytically altering the structure of the proenzyme molecule Plasmin activity can develop during the isolation procedure which frequently implies working conditions favorable for the generation of enzymic activity, *i e* exposure to moderately alkaline pH Recent work of Wallén and Wiman[1] has shown that even plasma Fraction III[2] is contaminated with altered plasminogen, most likely due to traces of plasmin For the

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isolation of unaltered plasminogen it is therefore an indispensable requirement to start with fresh human plasma and to avoid the possibility of plasminogen activation by completing the isolation within the shortest possible time, preferably in the presence of an inhibitor The achievement of such requirements calls for a highly specific procedure

Among the known preparative techniques in protein chemistry, affinity chromatography[3-5] appeared to be the most promising method It was already used by Deutsch and Mertz[6] for the isolation of plasminogen By substituting agarose with lysine, they obtained an inhibitor-adsorbent containing the inhibitory functions of ϵ-aminocaproic acid, an inhibitor of the plasminogen activation to plasmin We have tried to reproduce this method but have failed to obtain a satisfactory yield and purity of plasminogen Since polyacrylamide permits a higher degree of substitution, we decided to develop a new adsorbent using this as a carrier We attempted to couple polyacrylamide with lysine in such a way that the substitution reaction primarily involves the α-NH_2 group of lysine, leaving free its α-COOH and ϵ-NH_2 groups as the functional groups of ϵ-aminocaproic acid

Lysine-coupled polyacrylamide (Biogel P-300) was prepared from polyacrylamide over its hydrazide and azide according to the method of Inman and Dintzis[5] Lysine was then coupled to polyacrylamide azide under conditions described by Inman and Dintzis[5] and by Cuatrecasas[4] for the coupling of primary amines

To 100 ml of settled polyacrylamide-lysine gel were added 340 ml of fresh, citrated plasma* which was previously diluted with an equal volume of 0 15 M phosphate buffer, pH 7 4 The mixture was stirred overnight at 2° The gel was washed free of unadsorbed proteins on a Buchner funnel with several portions of 0 3 M phosphate buffer, pH 7 4 The elution of plasminogen from the adsorbent was effected in a chromatographic column with 0 3 M phosphate buffer, containing 0 5 M ϵ-aminocaproic acid, pH 7 4 Washing of the adsorbent and elution of the plasminogen were carried out at room temperature The protein was collected by precipitation with 2 M $(NH_4)_2SO_4$ at pH 5 5 The centrifuged precipitate was dissolved in and dialyzed against either cold 0 1 M NaCl, 0 05 M Tris, 0 02 M lysine buffer, pH 8 3, or 0 05 M phosphate buffer containing 0 1 M NaCl, pH 7 4

This method yielded 10 mg of plasminogen per 100 ml of citrated plasma The supernatant plasma after adsorption was, upon addition of streptokinase, entirely inactive on heated fibrin plates, whereas the original plasma gave clear-cut evidence of lysis We have already applied this method with equal success to the adsorption of plasma batches of 1 5 and 2 5 l In this work we deliberately used an excess of adsorbent to ensure complete adsorption of the plasminogen Preliminary experiments showed that the amount of adsorbent can be further reduced without impairing the yield of proenzyme The adsorbent can be used repeatedly

Plasminogen activity, after activation with streptokinase was determined by a caseinolytic assay according to the method of Remmert and Cohen[7] as modified by Robbins and Summaria[8] Devitaminized, acid-treated[9] Hammarsten casein was used as a substrate Specific plasminogen activity from four different preparations amounted to 32–42 Remmert-Cohen units/mg protein (200–260 units/mg N) The preparations were

*Kindly supplied by the Central Laboratory of the Blood Transfusion Service of the Swiss Red Cross

always entirely free of spontaneous plasmin activity, as determined by the caseinolytic assay The absence of protease activity is apparently responsible for the observed stability of the plasminogen A sample which was stored for 13 days at 2° in 0 1 M NaCl, 0 05 M Tris, 0 02 M lysine buffer, pH 8 3, could be activated to its original potency, and no spontaneous plasmin activity was detectable The protein was readily soluble near neutrality in 0 05 M phosphate-buffered saline in the absence of lysine

In polyacrylamide gel electrophoresis (sample and spacer gels omitted), plasminogen migrated as a single band in the presence of 5 M urea and 37% acetic acid In Tris-glycine buffer[10], pH 8 3, six–seven anodic bands were observed This electrophoretic multiband pattern has been reported by several authors[1,6,11]

N-terminal amino acid analysis was carried out with 2,4-dinitrofluorobenzene as described by Sanger[12] in the presence of 6 M urea The acid hydrolysate of the dinitrophenylated protein was analyzed for N-terminal amino acid derivatives by two-dimensional thin-layer chromatography on silica gel G The solvent systems were 'toluene solvent' (ref 13) in the first dimension and chloroform–benzyl alcohol–acetic acid (70 30 3, by vol)[14] in the second dimension The chromatographed dinitrophenylated amino acids were recovered from the plate, and the concentration was determined from absorbance measurements at 360 nm The only amino acid derivative found was dinitrophenylglutamic acid Dinitrophenyllysine, -valine and -methionine were not detectable In one experiment a hardly visible spot was observed near the theoretical position of dinitrophenylmethionine The scarcity of material, however, did not allow a unequivocal identification After correction for losses during the analytical procedure, determined with a known amount of the same dinitrophenylated amino acid we obtained a yield of N-terminal dinitrophenylglutamic acid of 0 8 mole/mole of protein (mol wt 90 000, ref 1)

In contrast to the agarose derivative, polyacrylamide–lysine showed no unspecific retention of plasma proteins and also gave a much better yield of plasminogen The difference in specificity is probably due to the different nature of the two carriers, since the attached prosthetic groups are the same in both cases Polyacrylamide is well known for its chemical inertness towards most proteins and is therefore often used as a gel medium, for instance in electrophoresis On the other hand, the yield of plasminogen is expected to be a function of the degree of lysine substitution on the carrier Experiments for the determination of this parameter and of the binding capacity for plasminogen are in progress

Most of the properties of the plasminogen obtained by our procedure are very similar to the characteristics determined by Deutsch and Mertz[6] on their preparations The most outstanding feature is in both cases the absence of protease activity and the concomitant stability of the plasminogen The specific activities are not directly comparable due to different assay systems and enzyme units However, the activity of the proenzyme isolated by the above described procedure is about 50% higher than of highly purified plasminogen which we prepared by conventional methods from a residual globulin fraction (Precipitate B)[15] Our findings which clearly indicate glutamic acid as the N-terminal amino acid,of proteolytically unaltered plasminogen are in agreement with the data obtained by Wallén and Wiman[1] These authors isolated plasminogen with a low content of spontaneous plasmin activity (0 1 to 0 3% of the total activity) from plasma

Fraction III by conventional methods Some of this plasminogen had close to 1 mole of N-terminal glutamic acid per mole of protein and as much as 0 2 mole of N-terminal methionine In a preparation with 4% plasmin activity, the dominating N-terminal amino acid was lysine (0 53 mole/mole of protein), followed by decreasing amounts of N-terminal valine, glutamic acid and methionine Our results contradict the report of Robbins *et al* [16] on the N-terminal amino acid composition of human plasminogen These authors purified the proenzyme from Cohn Fraction $III_{2,3}$ The resulting plasminogen was contaminated with 5% plasmin activity and had, besides some valine, N-terminal lysine We obtained essentially the same result with plasmin-contaminated proenzyme from Precipitate B[15] Muramatu *et al* [17] also found N-terminal lysine in human plasminogen

As Wallén and Wiman[1] have already stated, plasmin contaminations of the reported order of magnitude are apparently responsible for structural alterations in the proenzyme molecule One of these changes becomes evident by the disappearance of N-terminal glutamic acid and a concomitant appearance of N-terminal lysine (and valine) The change in N-terminal amino acid composition must be due to the cleavage of a peptide moiety from the N-terminal portion of the polypeptide chain However, the size, nature and function of the cleaved fragment are as yet unknown Also, the molecular structure as well as the activation mechanism of unaltered plasminogen remain to be elucidated

Our procedure is well suited for the isolation of highly purified and proteolytically unaltered plasminogen The isolation is performed under gentle conditions and can be completed within a very short time In small batches we obtained the purified proenzyme within 36 h after blood donation The method allows the isolation of plasminogen from plasma of single blood donors as well as large scale preparative work

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