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## Note

# Isolation of human urine urokinase by column chromatography on sawdust and some properties of the enzyme obtained

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The plasminogen activation factor in human urine was discovered by Williams[1] and designated as urokinase (E.C. 3.4.99.26) by Sobel *et al.*[2]. The enzyme has been applied to the therapy of thrombosis, and several papers have been published on its isolation and purification[3–9].

Recently we noticed that urokinase lost its activity in the presence of lignin, but that the addition of sodium chloride to this mixture restored the activity. Sawdust is, in a sense, small pieces of naturally occurring cellulose–lignin complex. The use of sawdust for the isolation and purification of urokinase from urine was studied and was found to be effective.

This paper deals with the conditions for the isolation and purification of urine urokinase using a column of sawdust and some properties of the enzyme obtained.

## MATERIALS AND METHODS

Human urine was freshly collected from about a dozen normal male adults and a small amount of *n*-butanol was added as an antiseptic.

Acetylglycyl-L-lysine methyl ester (AGLMe) as the substrate was purchased from the Foundation for Promotion of Protein Research (Osaka, Japan). Human fibrinogen and thrombin were obtained from Green Cross Corp. (Osaka, Japan). "Uronase", 6000 I.U. (International Units) per vial, as the standard urokinase for plasminogen activation activity was obtained from Mochida Pharmaceutial Co. (Tokyo, Japan). Myoglobin, chymotrypsinogen A, ovalbumin and bovine serum albumin as the standard for the determination of molecular weight by gel chromatography were obtained from Serva (Heidelberg, G.F.R.). The protein standard kit for the determination of molecular weight by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was obtained from Boehringer (Mannheim, G.F.R.).

The sawdust, derived mainly from North American cedar, was sifted to screen pieces of sizes between 32 and 100 mesh, and was suspended in water under reduced pressure to remove air. It was then suspended in about 40 volumes of 2 *N* sodium hydroxide solution with stirring at 40°C until no further brown colour was produced

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(*ca.* 24 h). The sawdust was washed thoroughly with water, then suspended in about 40 volumes of 2 *N* hydrochloric acid with stirring at 40°C for 3–4 h. The acid-treated sawdust was washed thoroughly with water and air dried at 40°C. The washed sawdust was packed in a column after suspension in water. One gram of sawdust on a dry basis gave a packed volume of 6–7 ml.

The ester hydrolytic activity of urokinase was determined by the method of White and Barlow[10], but using AGLMe as the substrate instead of acetyl-L-lysine methyl ester. One unit of esterase activity was defined as the amount of the enzyme that liberated 1 μmole of methanol per minute at 37°C.

Plasminogen activation activity was assayed according to the method of Nishizaki and Kawamura[11] using a fibrin plate, and the activity was expressed in International Units.

Protein concentration was determined spectrophotometrically with a Hitachi Model 124 spectrophotometer assuming that the $A^{1\%}_{280\,nm}$ value of urokinase was 13.6[5]. The specific activity was tentatively expressed by dividing the urokinase activity (I.U./ml) by the protein concentration (mg/ml) of the enzyme solution.

The gel chromatography for the determination of molecular weight was carried out on a Sephadex G-75 column, using 0.02 *M* phosphate buffer (pH 7.4) containing 0.15 *M* sodium chloride and 2 m*M* EDTA as the developing solvent.

SDS (0.1 %) disc electrophoresis in 7.5 % polyacrylamide gel was performed according to the method of Weber and Osborn[12] to check the purity and determine the molecular weight, except that no 2-mercaptoethanol was added as it would have caused complete inactivation of the enzyme. The gel after electrophoresis was stained with 0.1 % Coomassie Brilliant Blue and destained with 7 % acetic acid.

## RESULTS

Human urine, freshly collected and diluted with an equal volume of water, was applied to a sawdust column equilibrated with 0.01 *M* Tris–hydrochloric acid buffer (pH 7.2) containing 0.15 *M* sodium chloride, and the column was washed with the same buffer until no further urine colour was observed in the effluent. The urokinase adsorbed on the column was eluted with 0.02 *M* borate buffer containing 1.0 *M* sodium chloride (pH 10.0). As shown in Fig. 1, most of the activity of urokinase in the fresh urine was adsorbed on the sawdust column, and the activity adsorbed was eluted with a yield of 94 %. A small proportion (15–25 %, depending on the urine employed) of the enzyme activity of urine appeared in the effluent. The specific activity of the eluted enzyme was 5100 I.U./mg protein, an 18,500-fold increase compared with the original urine. The sawdust column used was reactivated by washing successively with water, 0.2 *N* hydrochloric acid, water, 0.2 *N* sodium hydroxide solution and water.

The active fraction obtained by the first chromatography was dialysed against 0.01 *M* phosphate buffer (pH 7.4) containing 0.15 *M* sodium chloride and 2 m*M* EDTA at 4°C, and applied to the sawdust column equilibrated with the same buffer as used for dialysis. After washing the column with the same buffer, the enzyme adsorbed was eluted with 0.02 *M* borate buffer containing 1.0 *M* sodium chloride and 2 m*M* EDTA (pH 10.0) at 4°C. The urokinase recovery was 85 % and the specific activity was 23,000 I.U./mg protein. The urokinase recovered was dialysed and sub-

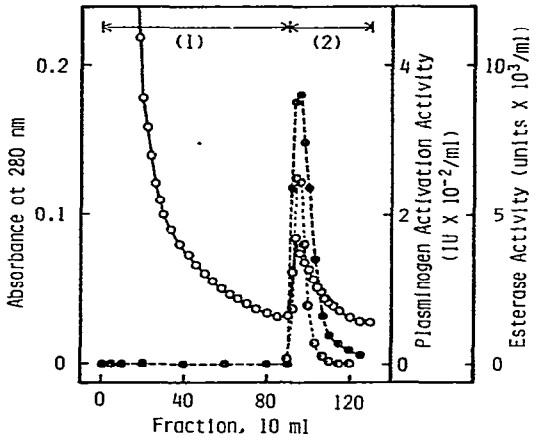


Fig. 1. Elution pattern of urokinase from human urine by chromatography on a sawdust column. Column, 2.0 × 7.5 cm; twice diluted urine, 7900 ml ($A_{280\ nm}$ = 22.1), plasminogen activation activity, 4.5 I.U./ml; flow-rate, 300 ml/h; temperature, 4°C. (1) 0.01 *M* Tris–HCl buffer containing 0.15 *M* NaCl (pH 7.2); (2) 0.02 *M* borate buffer containing 1.0 *M* NaCl (pH 10.0). ○, Absorbance at 280 nm; ●, plasminogen activation activity; ◐, esterase activity.

jected to a third chromatographic step on the same sawdust column as described above. The specific activity increased to 60,000 I.U./mg protein, and the recovery of activity in this step was 85%. The elution patterns of urokinase in this third chromatography are shown in Fig. 2.

As shown in Figs. 1 and 2, the plasminogen activation and esterase activities were eluted in parallel from the sawdust column and their ratio was about 50,000:1 in all of the active fractions.

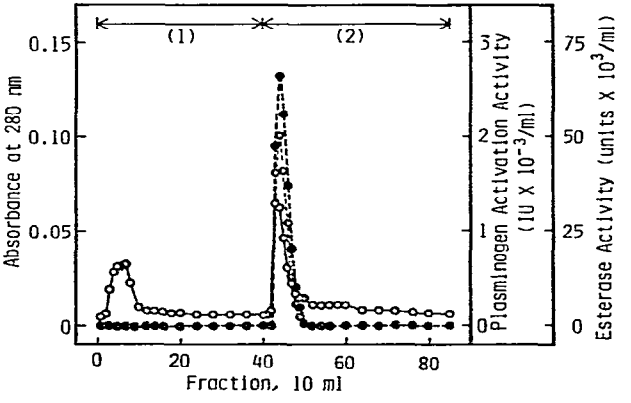


Fig. 2. Elution pattern of urokinase in third chromatographic step on sawdust column. Column as in Fig. 1; enzyme applied, 50 ml ($A_{280\ nm}$ = 0.121), plasminogen activation activity, 2435 I.U./ml, esterase activity, $49.6 \cdot 10^{-3}$ U./ml; flow-rate, 300 ml/h; temperature, 4°C. (1) 0.01 *M* phosphate buffer containing 0.15 *M* NaCl and 2 m*M* EDTA (pH 7.4); (2) 0.02 *M* borate buffer containing 1.0 *M* NaCl and 2 m*M* EDTA (pH 10.0). ○, Absorbance at 280 nm; ●, plasminogen activation activity; ◐, esterase activity.

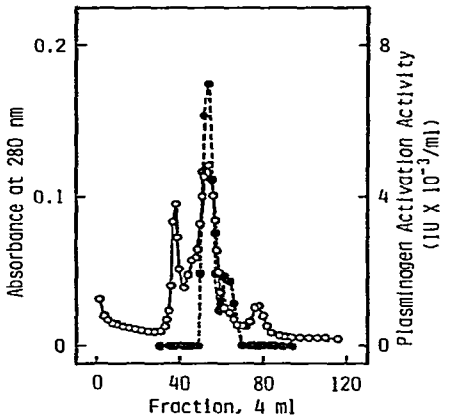


Fig. 3. Gel chromatography of the urokinase on Sephadex G-75. Column, 2.25 × 126 cm; 0.02 *M* phosphate buffer containing 0.15 *M* NaCl and 2 m*M* EDTA, (pH 7.4); enzyme applied, 9.0 ml ($A_{280\,nm}$ = 1.000), plasminogen activation activity, 31,000 I.U./ml; flow-rate, 17 ml/h; temperature, 4°C. ○, Absorbance at 280 nm; ●, plasminogen activation activity.

The protein components of the urokinase preparation obtained by the sawdust column chromatography were examined. The urokinase preparations obtained by performing several series of purifications by triple chromatography were collected and concentrated by ultrafiltration (Model UHP-43, ultrafilter UK-10; Toyo Kagaku Sangyo Co., Osaka, Japan). The specific activity of the enzyme decreased to 70% on ultrafiltration. The concentrate was subjected to gel chromatography using a column of Sephadex G-75. As shown in Fig. 3, protein appeared, separating into peaks for three fractions. The plasminogen activation activity was eluted together with the middle protein peak. However, a small peak of enzyme activity appeared behind the major activity. The molecular weights of the major and minor enzyme active fractions were estimated to be $5.4 \cdot 10^4$ and $3.6 \cdot 10^4$, respectively (Fig. 4).

The concentrated preparation described above was also examined by SDS polyacrylamide gel electrophoresis, as shown in Fig. 5. The protein of the preparation was separated into five components, with molecular weights estimated to be $9.2 \cdot 10^4$

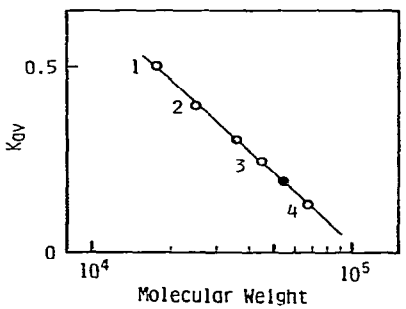


Fig. 4. Determination of molecular weight of urokinase by gel chromatography on Sephadex G-75. Molecular weights of the reference proteins: 1, myoglobin, $1.78 \cdot 10^4$; 2, chymotrypsinogen A, $2.5 \cdot 10^4$; 3, ovalbumin, $4.5 \cdot 10^4$; 4, bovine serum albumin, $6.7 \cdot 10^4$. ●, Urokinase of the major active fraction; ◐, urokinase of the minor fraction. $K_{av}$: partition coefficient.

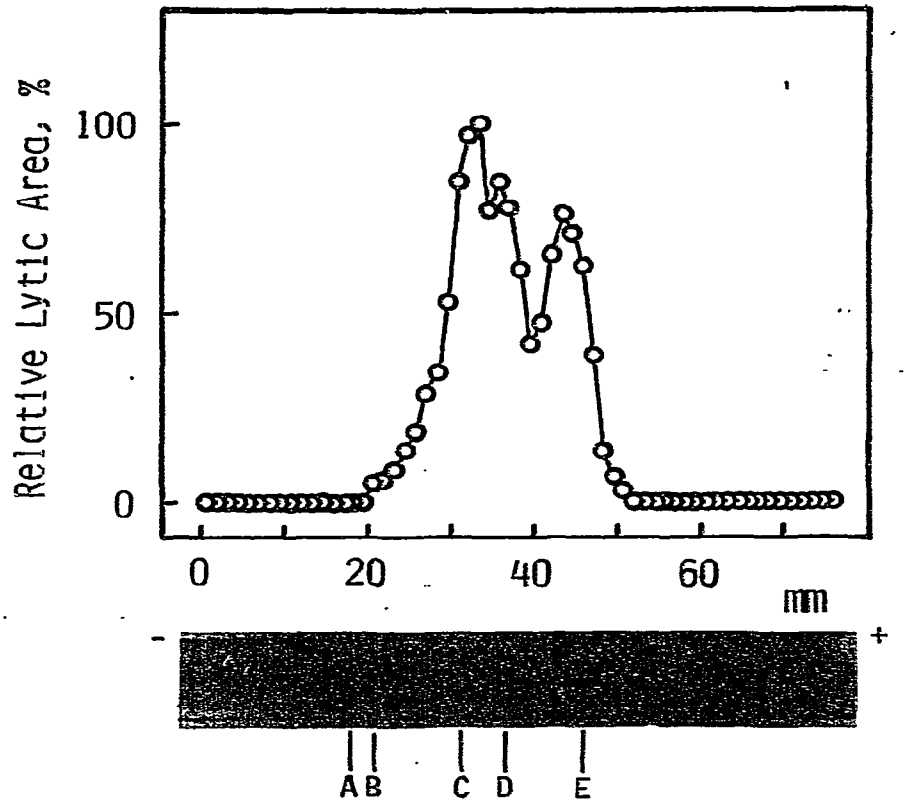


Fig. 5. SDS polyacrylamide gel electrophoresis of urokinase and distribution of urokinase activity on the gel after electrophoresis. The sample was applied after incubation with SDS at 37°C for 24 h but without addition of 2-mercaptoethanol. Molecular weights of the components: A, $9.2 \cdot 10^4$; B, $8.2 \cdot 10^4$; C, $5.4 \cdot 10^4$; D, $4.7 \cdot 10^4$; E, $3.6 \cdot 10^4$. The gels (1.25 mm thick) were placed directly on a fibrin plate and their lytic areas were measured.

(A), $8.2 \cdot 10^4$ (B), $5.4 \cdot 10^4$ (C), $4.7 \cdot 10^4$ (D) and $3.6 \cdot 10^4$ (E). The unstained gel column that had been run at the same time was sectioned into 1.25-mm thick portions, and the sections were placed on a fibrin plate for analysis of the plasminogen activation activity. As shown in Fig. 5, components C, D and E showed plasminogen activation activity, although the method used demonstrated only the presence of enzyme activity and was not effective in establishing the exact relative activities between the components.

## DISCUSSION

The adsorption of urokinase by sawdust seems to be specific, although the adsorption capacity is not as prominent as with various other ion exchangers. The purification of human pancreatic juice elastase II, the isoelectric point of which is 8.8[13], and hen egg white lysozyme has also been performed by chromatography using a sawdust column. It is probable that sawdust adsorbs basic proteins by an ion-exchange reaction. In fact, sawdust activated by washing with dilute hydrochloric

acid and sodium hydroxide solution showed a cation-exchange capacity of about 0.14 mequiv./g. On the one hand, urokinse was found to be markedly inhibited by soluble lignin, which suggested that the enzyme binds to lignin in sawdust.

Two kinds of urokinase are known at present, with molecular weights of about $5.4 \cdot 10^4$ (ref. 5, 9, 14) and $3.2 \cdot 10^4$ (ref. 5, 8, 9, 15, 16). The high-molecular-weight urokinase may change into the low-molecular-weight variety under certain conditions. In an experiment carried out independently, the urokinase of molecular weight $3.2 \cdot 10^4$ was passed through the sawdust column under the conditions described above. The activity ratio of plasminogen activation to esterase of the low-molecular-weight urokinase was half that of the high-molecular-weight urokinase. In this study, the purified enzyme showed a constant activity ratio of esterase and plasminogen activation, indicating that both enzyme activities were attributed to the high-molecular-weight urokinase, although a small amount of the low-molecular-weight enzyme was present. This enzyme may have appeared during the repeated purification by the sawdust adsorption method.

This study also demonstrated that in addition to the above two enzymes, a new urokinase of intermediate molecular weight appeared when examined by SDS polyacrylamide gel electrophoresis. This urokinase may be an artificial product. It is possible that human urokinase exists with several molecular weights with different activities of esterase and plasminogen activation; further study is necessary in order to clarify this aspect.

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