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PREPARATION, PROPERTIES, AND *IN VITRO* THROMBOGENIC CHARACTERIZATION OF UROKINASE–COLLAGEN MEMBRANE

ISAO KARUBE,[1] SHUICHI SUZUKI,[1] TOMOKO KUSANO,[2] and TOSHIO SATO[2]

[1] *Research Laboratory of Resources Utilization*
Tokyo Institute of Technology
Nagatsuta-cho, Midori-ku, Yokohama
[2] *Faculty of Pharmacology*
Tokushima University of Art and Science
Yamashiro-cho, Tokushima, Japan

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Urokinase (EC 3.4.4.a) was immobilized on collagen membrane. The urokinase–collagen membrane gave a flat pH profile from 7.5 to 9.5. It was more stable against heat than native urokinase. Furthermore, the stability of urokinase in the pH range of 7.0–8.8 was increased with immobilization. The collagen fibril network might stabilize urokinase. The diffusion coefficients of urea, uric acid, and creatinine through the urokinase–collagen membrane were in the range of $2.5–4.5 \times 10^{-7}$ cm^3/sec. The diffusion coefficients decreased to the range of $6.9–8.2 \times 10^{-8}$ cm^2/sec when fibrin clot was formed on the membrane *in vitro*. Immobilized urokinase activates plasmin which lyzes fibrin clot. Therefore, fibrin clot formed on the membrane could be lyzed during prolonged incubation at 37°C and the diffusion coefficients restored to the initial values. The fibrin clot formed *in vivo* may be lyzed with immobilized urokinase.

INTRODUCTION

Recently, advancements have been made in techniques for immobilization of enzymes and bacteria. Many enzymes have been immobilized by various methods and these immobilized enzymes have been applied to industrial production (1) and clinical analysis (2,3). Collagen membranes are to be expected for a dialysis membrane in the artificial kidney (4,5). However, fibrin clot formed on the membrane during hemodialysis prevents the permeation of toxic compounds such as urea, creatinine, and uric acid. Urokinase is known as an activator of plasminogen to plasmin. If the immobilized urokinase reacts with plasminogen in blood, the fibrin clot formed on the membrane is lyzed with plasmin. Therefore, the urokinase–

collagen membrane has a potential application as a dialysis membrane in the artificial kidney.

Urokinase was entrapped in collagen membranes, and enzymatic properties and diffusional properties of the urokinase–collagen membrane were studied.

MATERIALS AND METHODS

Materials

Purified collagen was prepared from Holstein skin as described previously (6). Urokinase (EC 3.4.4.a, 500 IU/mg protein) was obtained from Towa Kako Company. Casein (from milk) was chosen for the substrate of plasmin and purchased from E. Merck Company. Other reagents were commercial analytical reagents or laboratory grade materials. Deionized water was used in all procedures.

Preparation of Urokinase–Collagen Membrane

To 3 ml of urokinase solution (1500 IU), 40 g of 0.8% collagen fibril suspension (pH 4.3) was added. The urokinase–collagen membrane was prepared by casting the suspension on a Teflon plate (7) and drying it at room temperature. The urokinase–collagen membrane was treated with 1% glutaraldehyde solution (in 0.1 M phosphate buffer, pH 7.0) for 1 min at 20°C. The activity of the urokinase–collagen membrane was 0.2 IU/mg membrane.

Enzyme Assay

Unless otherwise noted, standard assays of the native urokinase and the urokinase–collagen membrane were carried out as follows: the reaction mixture was 3 ml of 8% casein solution (in 0.1 M phosphate buffer, pH 7.4) containing 0.2 units of plasminogen and 2 μg of native urokinase or 6–9 mg of the urokinase–collagen membrane (50 μg urokinase). It was incubated for 30 min at 37°C. The activity of urokinase was calculated from the relationship between the proteolytic activity determined by the method of Lowry et al. (8) and the urokinase activity determined by the standard method (9).

Diffusion Coefficient Measurement

The diffusion coefficients of urea, uric acid, and creatinine through the urokinase–collagen membrane and the collagen membrane were determined at 20°C. The apparatus and procedures for measurement of diffusion coefficients were similar to those used previously (10). Urea was determined colorimetrically by the method of Fearon (11) and creatinine by the method of Kostir and Sonka (12). Uric acid was determined enzymatically (13).

RESULTS AND DISCUSSION

pH–Activity Profiles

Urokinase was immobilized on the collagen membrane. The activity yield of the urokinase–collagen membrane (4%) was lower than that of other enzyme–collagen membranes reported previously (14). Since the molecular weight of the native urokinase was low (31,500), the enzyme leaked from the collagen membrane during incubation. Therefore, the urokinase–collagen membrane was tanned with 1% glutaraldehyde solution for 1 min. Since urokinase is a labile enzyme, glutaraldehyde treatment of the membrane might cause the inactivation of urokinase.

Figure 1 shows the pH versus activity profiles of the urokinase–collagen membrane and native urokinase in the neutral pH range (pH 7–9). The activity of the native urokinase and the urokinase–collagen membrane was expressed as relative to that determined at pH 9.0. The highest activity of the native urokinase in the pH range just described was observed at pH 9.0. The activity of the native urokinase decreased with lowering pH. On the other hand, the urokinase–collagen membrane gave a flat pH profile from 7.5 to 9.0.

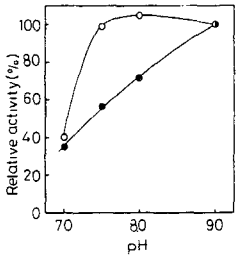

FIG. 1. pH versus activity profiles of the urokinase–collagen membrane and native urokinase. The enzyme assay was carried out under standard conditions except for pH of buffer (0.1 M phosphate buffer) employed. ●, Native urokinase; ○, the urokinase–collagen membrane.

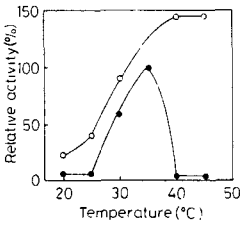

FIG. 2. Temperature versus activity profiles. The enzyme assay was carried out under standard conditions except for temperature employed. ●, Native urokinase; ○, the urokinase–collagen membrane.

Activity–Temperature Profiles

Temperature versus activity profiles are shown in Fig. 2. The activity of the native urokinase and the urokinase–collagen membrane was expressed as relative to that determined at 37°C. The optimum temperature of native urokinase was 37°C and no activity was observed at 45°C. On the other hand, the optimum temperature of the urokinase–collagen membrane was from 40 to 45°C. Since heat caused the urokinase–collagen membrane to shrink, the exact activity of the immobilized urokinase could not be determined above 45°C. The immobilized urokinase was more stable against heat than native urokinase.

pH Stability of Urokinase–Collagen Membrane

Figure 3 shows the pH stability of the urokinase–collagen membrane and the native urokinase. The native urokinase and the urokinase–collagen membrane were incubated in various pH solutions for 1 h. The activity was expressed on the basis of the activity before incubation as being the relative activity. The activity of the native urokinase decreased to about 30% of the original activity during incubation. On the other hand, the activity of the urokinase–collagen membrane was 80% of the original activity at pH 7.5.

The urokinase–collagen membrane has a potential application as a dialysis membrane in the artificial kidney. Therefore, the stability of the

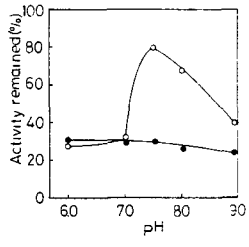

FIG. 3. pH stability of the urokinase–collagen membrane and native urokinase. To 3 ml of various pH of 0.1 M phosphate buffer, the urokinase–collagen membrane (6–9 mg) or native urokinase (6 μg) was added. The reaction mixtures were incubated for 1 h at 37°C. Then the enzyme assay was carried out under standard conditions. ●, Native urokinase; ○, the urokinase–collagen membrane.

urokinase is required for practical use. As described earlier, the urokinase–collagen membrane showed good stabilities under various conditions. The collagen fibril network might stabilize urokinase in the membrane.

Diffusional Properties of Urokinase–Collagen Membrane

As described earlier, fibrin clot formed on the membrane during blood dialysis prevents the permeation of toxic compounds. The apparent diffusion coefficients for urea, uric acid, and creatinine through the urokinase–collagen membrane and the collagen membrane are shown in Table 1. The diffusion coefficients were determined by the method described previously (10). Experiments were performed as follows: The urokinase–collagen membrane or the collagen membrane (1 cm^2) was fixed to the center of the apparatus as described previously (10). The diffusion coefficients of urea, uric acid, and creatinine through the urokinase–collagen membrane and the collagen membrane were about $3–4 \times 10^{-7}$ cm^2/sec. There is no statistically significant difference between the D values for the cellulose membranes. Fibrin clot was formed on the membrane at 37°C by casting 0.1 ml of a solution containing 0.5 unit of fibrinogen and 0.4 mg of thrombin *in vitro*. Then the diffusion coefficients were determined at 20°C. The diffusion coefficients decreased to $7–8 \times 10^{-8}$ cm^2/sec when fibrin clot was formed on the urokinase-collagen membrane. The fibrin clot–membrane was incubated for 19 h at 37°C. The diffusion coefficients of these compounds restored to the original values. Therefore, fibrin clot formed on the membrane was lyzed during incubation. This result suggests that fibrin clot formed during blood dialysis can be lyzed with urokinase immobilized on the collagen membrane.

TABLE 1. Diffusion Coefficients of Membranes

	Diffusion coefficient (cm^2/sec)[a]			
Compound	C	UK–C	F+UK–C[b]	F+UK–C[c]
Urea	3.7×10^{-7}	3.0×10^{-7}	6.9×10^{-8}	3.1×10^{-7}
Uric acid	2.6×10^{-7}	3.2×10^{-7}	7.1×10^{-8}	3.6×10^{-7}
Creatinine	4.3×10^{-7}	2.8×10^{-7}	8.2×10^{-8}	2.9×10^{-7}

[a] C, Collagen membrane (thickness 50 μm, wet); UK–C, urokinase–collagen membrane (thickness 60 μm, wet); F, fibrin clot. Relative standard deviation 8%.
[b] After 1 h incubation at 37°C.
[c] After 19 h incubation at 37°C.

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REFERENCES

1. WEETALL, H. H., and SUZUKI, S. (eds.) (1975) *In:* Immobilized Enzyme Technology, Plenum Press, New York.
2. SATOH, I., KARUBE, I., and SUZUKI, S. (1977) Biotechnol. Bioeng. 19:1095.
3. SATOH, I., KARUBE, I., and SUZUKI, S. (1977) J. Solid-Phase Biochem. 2:1.
4. RUBIN, A. L., RIGGIO, R. R., NACHMAN, R. L., SCHWARTZ, G. H., MIYATA, T., and STENZEL, K. H. (1968) Am. Soc. Artif. Int. Organs 14:169.
5. STENZEL, K. H., SULLIVAN, J. F., MIYATA, T., and RUBIN, A. L. (1969) Am. Soc. Artif. Int. Organs 15:114.
6. KARUBE, I., SUZUKI, S., KINOSHITA, S., and MIZUGUCHI, J. (1971) Ind. Eng. Chem. Prod. Res. Develop. 10:160.
7. KARUBE, I., NAKAMOTO, Y., and SUZUKI, S. (1976) Biochim. Biophys. Acta 445:774.
8. LOWRY, O. H., RESEROUGH, N. J., FARR, A. U., and RANDALL, R. J. (1951) J. Biol. Chem. 193:265.
9. JOHNSON, A. J. (1969) Thromb. Diath. Haemor. 21:259.
10. NAKAMOTO, Y., KARUBE, I., and SUZUKI, S. (1975) J. Ferment. Technol. 53:595.
11. FEARON, W. R. (1939) Biochem. J. 33:902.
12. KOSTIR, J. V., and SONKA, J. (1952) Biochim. Biophys. Acta 8:86.
13. FEICHTMEIR, T. V., and WERENN, W. T. (1955) Am. J. Clin. Pathol. 25:833.
14. SUZUKI, S., KARUBE, I., and SATOH, I. (1977) *In:* Biomedical Applications of Immobilized Enzymes and Proteins, CHANG, T. M. S. (ed.), Plenum Press, New York, pp. 177–189.