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Studies on Properties of Immobilized Urokinase

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The stability of immobilized human urokinase (UK; EC 3.4.21.31) is an important factor for the clinical application of the enzyme as an antithrombotic material. The stabilities of human UK immobilized on a polymeric support to heat and enzyme inhibitors were compared with those of the original soluble UK.

UK was immobilized on an ethylene vinyl acetate copolymer (EVA) tube which has maleic anhydride as a functional group on its surface. Immobilized UK showed greater stability to thermal degradation than soluble UK. Comparative kinetic studies of these two forms of UK toward enzyme inhibitors revealed that immobilization of the enzyme did not result in any change of Michaelis constants (K_m), though a significant increase of inhibition constants (K_i) was observed.

Furthermore, UK immobilized on the EVA tube showed resistance to thermal denaturation. Therefore, an EVA tube carrying immobilized UK represents a stable and easy-to-handle material for clinical use.

Keywords——immobilized urokinase; heat stability; inhibitor stability; conformational change; serine protease

Introduction

The main feature of an immobilized enzyme is its improved stability, and this is a major reason for utilizing immobilized enzymes industrially or clinically. Melrose[1] selected 50 types of immobilized enzymes, compared the stabilities of the immobilized enzymes with those of the original enzymes, and reported that the stability was increased in 60% of the immobilized enzymes.

Human urokinase (UK) is a serine protease, with a molecular weight of 51 to 55 kilo dalton (kDa). It is produced in the kidneys, and is one of the fibrinolytic enzymes which activate plasminogen in the blood.[2,3]

An attempt to immobilize UK was first reported by Kusserow and Larrow[4] in 1972, and clinical applications of immobilized UK were examined by Ohshiro *et al.*[5] and Sugitachi *et al.*[6,7] The stability and hence the durability of immobilized UK are desirable features for the clinical application of the enzyme as an antithrombotic material.

In this paper, we have examined the change of the stability of UK to heat and enzyme inhibitors associated with immobilization on a polymer support.

Materials and Methods

Materials——Ethylene vinyl acetate copolymer (EVA) was manufactured by Mitsui Polychemical Co., and an EVA tube, 1.7 mm in inside and 2.0 mm in outside diameter, was used as the immobilization support. Human UK (with a specific activity of 127000 IU/mg) was purchased from Daiichi Pure Chemicals Co. Other reagents were obtained as follows: aminoacetal from Aldrich Co., Ltd., U.S.A., maleic anhydride methylvinyl ether copolymer (MAMEC) from GAF Co., Ltd., U.S.A., and glutaryl-Gly-Arg-4-methylcoumarin-7-amide (peptide-MCA) from the Peptide Institute Inc., Japan.

As synthetic enzyme inhibitors, *N*-α-benzoyl-L-argininamide (BAA; Sigma Chemical Co.), *N*-α-tosyl-L-

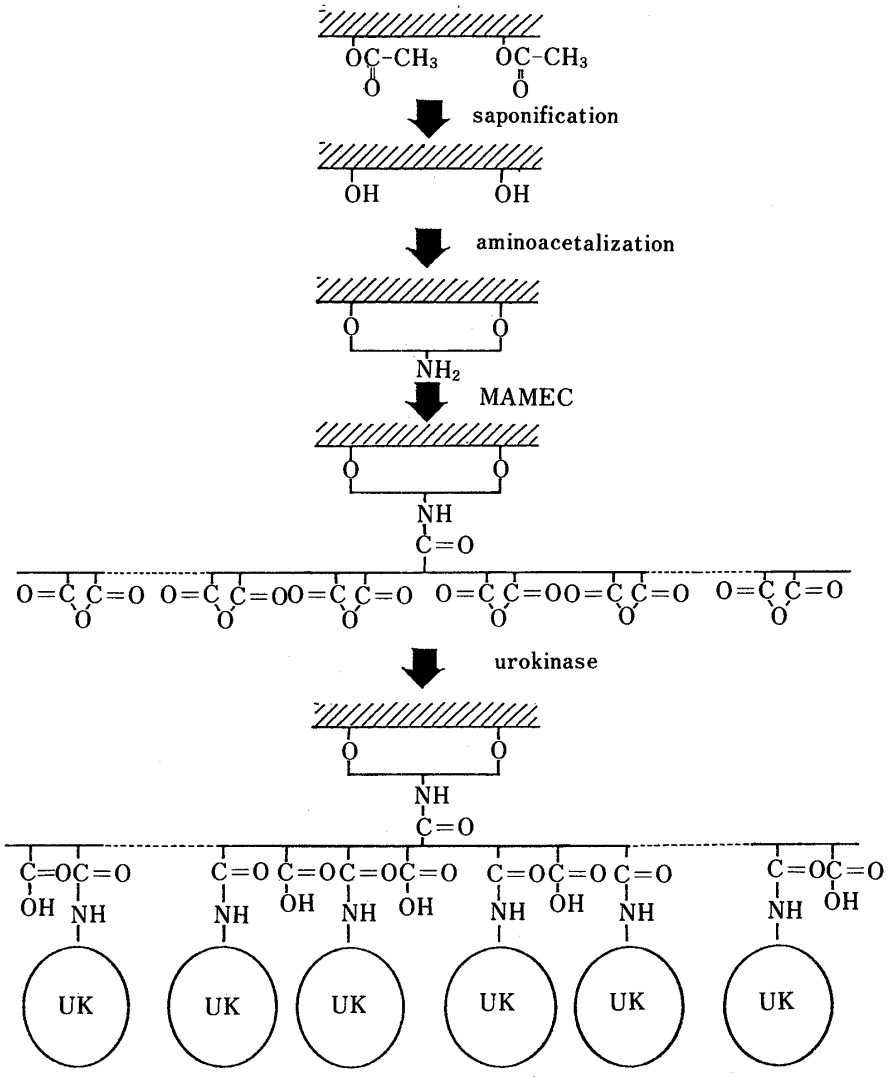

Fig. 1. Immobilization of Urokinase on an EVA Surface

lysylchloromethylketone (TLCK; Sigma Chemical Co.) and *N*-ethylmaleimide (NEM; Sigma Chemical Co.) were used. As a natural inhibitor to UK,[8] human placental urokinase inhibitor (α-globulin, M_r 43000, 500 units/10 mg, Green Cross Corp.) was used.

Methods——Immobilization of UK: UK was immobilized on an EVA tube according to the method described by Ohshiro *et al.*[9] and Sugitachi *et al.*[10] The EVA surface was hydrolyzed with 15% (w/w) NaOH in H_2O–methanol (20—80%) for 2 h at 50 °C, and was further treated in 2% (v/v) aminoacetal in 0.5 N HCl solution for 5 h at 58 °C. Successively, the tube was treated in 4% (w/v) MAMEC in acetone for 2.5 h at ambient temperature, throughly washed with acetone, and treated with a UK (600 unit/ml) solution in 0.01 M acetate buffer (pH 4) for 48 h at 7 °C. The resulting UK immobilized on the tube showed an activity of 10 IU/cm^2 both on the inner and outer wall (Fig. 1).

UK Assay: A synthetic substrate, peptide-MCA, was used as the substrate of UK, and the activity was determined by measuring the fluorescence of 7-amino-4-methylcoumarin (AMC) liberated by the hydrolysis of peptide-MCA.

The method proposed by Morita *et al.*[11] was employed. One milliliter of a substrate solution (50 mM Tris–HCl buffer, pH 8.0, containing 0.1 mM peptide-MCA, 100 mM NaCl and 10 mM $CaCl_2$) was preincubated at 37 °C for 2.5 min. UK solution or immobilized UK was added, and the mixture was allowed to react for 10 min at 37 °C. The reaction was terminated by adding 1.5 ml of 17% acetic acid. The fluorescence of AMC was measured at an excitation wavelength of 380 nm and emission wavelength of 460 nm. The UK activity was expressed in terms of the international unit (IU). The reaction was calibrated by using the UK standard product (1050 IU/vial) of the National Institute of Hygienic Sciences as a control.

Stability to Heat: A physiological scaline solution (1 ml) containing approximately 1000 IU/ml of soluble UK, or slices of EVA tube with approximately 1000 IU of UK was incubated at 45 °C, and after 8 h the remaining UK activity was determined.

Stability to Enzyme Inhibitors: Enzyme inhibitors at the concentrations shown below were added to physiological saline solution (1 ml) containing 1000 IU/ml of soluble UK, or slices of immobilized EVA tube with 1000 IU of UK. The mixtures were incubated at 37 °C for 30 min, and the remaining UK activity was measured. The final concentrations of the inhibitors for the soluble UK were 1.9 mM for BAA, 13.6 mM for TLCK, 34 mM for NEM and 11.6×10^{-4} mM (25 units) for human placental urokinase inhibitor. The final concentrations of the inhibitors for the immobilized UK were 150 mM for BAA, 40 mM for TLCK, 100 mM for NEM and 17.4×10^{-3} mM (375 units) for human placental urokinase inhibitor.

Results

Stability to Heat

The results of a comparison of the stability to heat between immobilized and soluble UK are shown in Fig. 2. Immobilized UK in physiological saline retained more than 50% of its initial activity after 8 h at 45 °C, while soluble UK lost most of its activity in 3 h.

Stability to Enzyme Inhibitors

The inhibitory effects of NEM on immobilized UK and soluble UK are shown as double-reciprocal plots (1/v *vs.* 1/s) in Fig. 3. The Michaelis constant (K_m) and inhibition constant (K_i) were determined from these plots. K_m was 0.16 mM for both immobilized UK and soluble UK. The value of K_i of NEM for soluble UK was 20 mM, while that for immobilized UK was 40 mM, 2 times greater than that of soluble UK. NEM brought about changes in v for both immobilized UK and soluble UK without changes in K_m, demonstrating that the inhibition pattern was noncompetitive.

Figure 4 shows double-reciprocal plots of the inhibitory effects of human placental urokinase inhibitors. The value of K_i of human placental urokinase inhibitor for soluble UK was 5.9×10^{-5} mM (1.3 units), and K_i for immobilized UK was 6.9×10^{-4} mM (14.9 units), 12

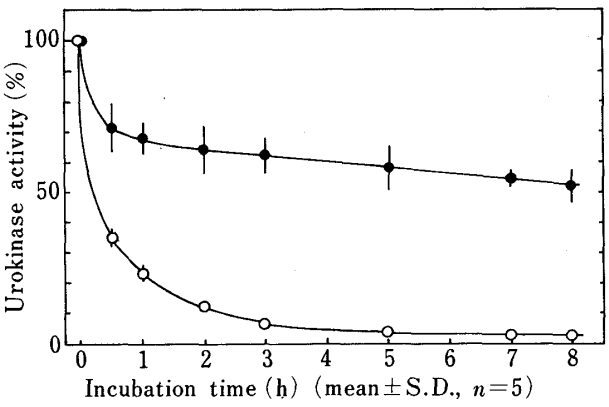

Fig. 2. Heat Stability of Immobilized Urokinase and Soluble Urokinase in Saline at 45 °C

●, immobilized urokinase; ○, soluble urokinase.

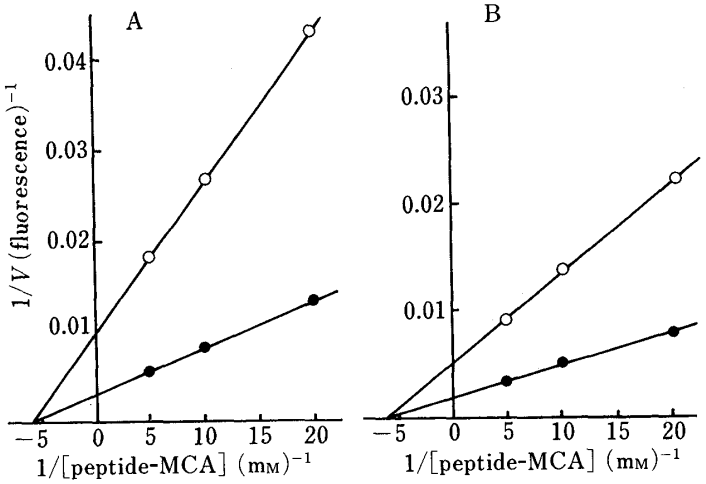

Fig. 3. Inhibition of Immobilized Urokinase (A) and Soluble Urokinase (B) by *N*-Ethylmaleimide

A: —○—, 100 mM; —●—, 0 mM. B: —○—, 34 mM; —●—, 0 mM.

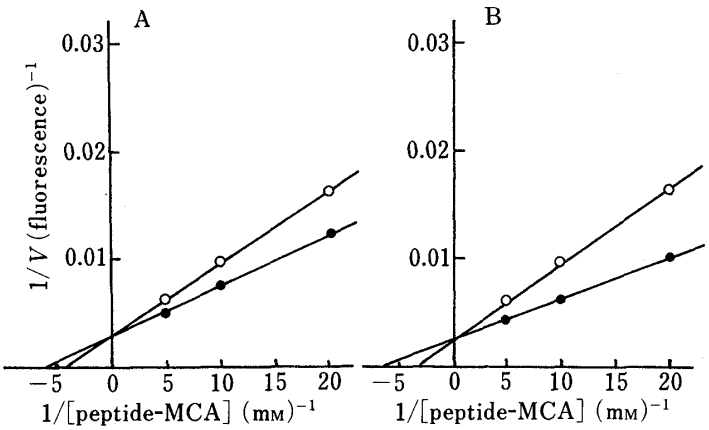

Fig. 4. Inhibition of Immobilized Urokinase (A) and Soluble Urokinase (B) by Human Placental Urokinase Inhibitor

A: —○—, 17.4×10^{-3} mM; —●—, 0 mM. B: —○—, 11.4×10^{-4} mM; —●—, 0 mM.

TABLE I. Inhibition Pattern and Rate Constants for the Inhibition of Urokinase by Various Proteinase Inhibitors

	Substrate	Inhibitor	Inhibition pattern	K_m (mM)	K_i (mM)
Immobilized UK	MCA	NEM	Noncompetitive	0.16	40
	MCA	BAA	Competitive	0.16	8.5
	MCA	TLCK	Competitive	0.16	1.9
	MCA	UK Inhibitor	Competitive	0.16	6.9×10^{-4} (14.9 unit)
Soluble UK	MCA	NEM	Noncompetitive	0.16	20
	MCA	BAA	Competitive	0.16	1.7×10^{-1}
	MCA	TLCK	Competitive	0.16	6.5×10^{-1}
	MCA	UK Inhibitor	Competitive	0.16	5.9×10^{-5} (1.3 unit)

UK: urokinase. MCA: glutaryl-Gly-Arg-4-Methylcoumarin-7-amide. NEM: *N*-ethylmaleimide. BAA: *N*-α-benzoyl-L-argininamide. TLCK: *N*-α-tosyl-L-lysyl chloromethyl ketone. UK inhibitor: α-globulin, human placental, 50 unit/mg.

times greater than that of soluble UK. The human placental urokinase inhibitor brought about changes in apparent K_m in both immobilized UK and soluble UK, and the inhibition pattern was competitive.

The inhibitory effects of other enzyme inhibitors are summarized in Table I.

The K_i value of NEM for immobilized UK was twice that of soluble UK. On the other hand, the difference of K_i value of BAA between the immobilized and soluble UK was as high as 50 fold.

Discussion

We have already reported clinical applications of immobilized UK in intravascular catheters and surgical drains.[6,7] The stability and hence the durability of immobilized UK are essential for the clinical application of the enzyme as an antithrombotic material. In the present study, we have used an EVA tube having maleic anhydride moieties on its surface as an immobilization support and examined the effects of the immobilization on the properties of UK.

The active site of an enzyme contains plural functional groups responsible for the catalytic action in a specific configuration, and the catalytic efficiency of the active site depends on a delicate balance of the rigidity and the elasticity of the enzyme.[12] When an enzyme is immobilized on a carrier, it is generally believed that the conformational rigidity of

the enzyme is increased as a result of multiple covalent bondings between the enzyme and polymeric support.[13]

The thermal denaturation of an enzyme is usually a result of irreversible conformational changes of the enzyme. When incubated in saline at 45 °C for 8 h, the immobilized UK retained an activity many times higher than that of soluble UK. It is reasonable to assume that the enzyme becomes less susceptible to conformational change as a result of immobilization, *i.e.*, an increase of rigidity of the enzyme occurs.

Many UK inhibitors, such as α_2-plasmin inhibitor, α_2-macroglobulin, α_1-antitrypsin and human placental urokinase inhibitor are reported to be present in the blood.[8] Therefore we have examined the effect of immobilization of UK on the kinetic parameters with respect to various enzyme inhibitors.[14-16]

Human placental urokinase inhibitor, a natural inhibitor of UK, and BAA and TLCK, synthetic enzyme inhibitors of serine protease, showed strong inhibitory effects on immobilized UK, while NEM, and SH and NH_2 group inhibitor, showed a weaker effect. As shown in Table I, the inhibition pattern of NEM was noncompetitive, while those of BAA, TLCK and human placental urokinase inhibitor were competitive. NEM showed a relatively small change of K_i upon immobilization of UK. Apparently, NEM does not act directly on the active site of UK (noncompetitive inhibitor).

On the other hand, BAA, TLCK, or human placental urokinase inhibitor showed greater differences of K_i's between soluble and immobilized UK. In all of the experiments in Table I, an increase of K_i's on immobilization and no change of K_m's were observed.

These changes of the kinetic parameters of UK on immobilization will be advantageous for the clinical application of this important enzyme. Immobilization of UK on an EVA tube with maleic anhydride functional groups on its surface improved the resistance of the enzyme to thermal denaturation, and increased K_i for four kinds of inhibitors. Therefore, it is reasonable to assume that a similar effect would be seen with various inhibitors present in the blood. Immobilization of UK is easy and straightforward and yields an antithrombotic material with an activity high enough for practical use. Detailed examination of the properties of the immobilized UK and its application as a biomedical material is in progress.

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