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FIBRINOLYSIS BY UROKINASE ENDOWED WITH MAGNETIC PROPERTY

Y. Inada, K. Ohwada, T. Yoshimoto, S. Kojima, K. Takahashi, Y. Kodera, A. Matsushima and Y. Saito

Laboratory of Biological Chemistry, Tokyo Institute of Technology, Ookayama, Meguroku, Tokyo 152, Japan

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SUMMARY The activated magnetic modifier was synthesized from magnetite, α, ω-dicarboxymethylpoly(oxyethylene) and N-hydroxysuccinimide (Biochem. Biophys. Res. Commun., 145, 908-914, 1987). Urokinase was directly coupled with the activated magnetic modifier to obtain magnetic urokinase. The magnetic urokinase dispersed in saline and exerted high fibrinolytic activity (13.8×10^4 IU/mg protein), and was readily recovered from saline by magnetic force of 250 Oe. By applying magnetic force, the urokinase was attracted at our will and local fibrinolysis was achieved on fibrin gel in a petri dish.

Enzymes can be modified by chemically binding an amphipathic and non-immunogenic macromolecule, polyethylene glycol, to the surface. This can counter some of drawbacks of native molecules and improve their properties; reduction of immunoreactivity, prolongation of clearance time and solubilization in organic solvents (1, 2). It became possible recently to further endow a modified enzyme with magnetic property (3). Yoshimoto et al., however, devised a method to prepare magentic enzymes in one step without much loss of activity using an activated magnetic modifier (4). The modifier was synthesized by activating free carboxyl groups of α, ω-dicarboxymethylpoly(oxyethylene) bound with magnetite using N-hydroxysuccinimide and dicyclohexylcarbodiimide. It was directly coupled with amino groups on the surface of enzyme molecules without any cross-linking among enzyme

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molecules. The magnetic lipase dispersed both in aqueous solution and in organic solvents, and was readily recovered from reaction mixture by magnetic force without loss of enzymic activity (3-5). This kind of modified enzymes should be useful not only for industrial but also clinical applications.

Patients with thrombosis or embolism are treated with urokinase, a plasminogen activator (6). However, because of its very short half-life and susceptibility toward its inhibitor, usually a huge dosage of urokinase has to be administered. This therapy, therefore, is accompanied with a risk of unnecessary bleeding and other side effects.

The present communication deals with a basic study to achieve effective localization of urokinase to an affected part by providing the enzyme with magnetic property.

MATERIALS AND METHODS

A purified urokinase (EC 3. 4. 21. 31) with molecular weight of 55,000 obtained from human urine was kindly provided from Green Cross Co. (Osaka, Japan). Its specific activity was 15.8×10^4 IU/mg protein. The molar extinction coefficient was 7.48×10^5 $M^{-1}cm^{-1}$ at 280 nm (6). Fibrinogen, plasminogen and thrombin obtained from humans were also given from Green Cross Co. (Osaka, Japan). α,ω-Dicarboxymethylpoly(oxyethylene) with average molecular weight of 3000 was kindly supplied by Nippon Oil & Fat Co. (Tokyo, Japan). Other chemicals used were of analytical grade.

The activated magnetic modifier was prepared by the method described previously (3). By coupling of the activated magnetic modifier to amino groups of urokinase, magnetic urokinase was prepared. To 1 ml of urokinase (3.0 mg/ml) dissolved in phosphate buffered saline (pH 7.0) were added 6 mg of the activated magnetic modifier to form acid-amide linkage. The mixture was sonicated and gently shaken for 30 min at room temperature. Then the magnetic urokinase was recovered by magnetic force (250 Oe) and washed extensively with saline.

Fibrinolytic activity was determined using fibrin plate containing plasminogen by the method of Ploug and Kjeldgaard (7). Fibrinolysis with magnetic urokinase was performed as follows. A fibrin plate was prepared by adding 70 µl of thrombin (10 IU/ml) to 4 ml of fibrinogen (10 mg/ml) solution containing 5 mM calcium ions in a petri dish (diameter: 5.5 cm). To the plate, 2.5 ml of plasminogen (10 IU/ml) were poured and mixed, and then 100 µl of magnetic urokinase (1670 IU/ml) dispersed in saline were added. It was gently swung in the first 3 min and was kept standing for 1 hr at room temperature. Fibrinolysis on the petri dish was observed with or without a round Sm-Co permanent magnet (diameter: 1 cm) placed at the center of the reverse side of the dish. Protein concentration was determined by the method of Lowry et al. (8) after removing magnetite from magnetic enzyme with concentrated hydrochloric acid. Contents of magnetite were estimated by measuring amount of iron with atomic absorption analysis using a Varian AA-875 atomic absorption spectrophotometer.

RESULTS AND DISCUSSION

The activated magnetic modifier, which can render magnetic property to proteins, was coupled with amino groups in urokinase molecule to form acid-amide linkage. The magnetic urokinase prepared by mixing with the weight ratio (2 : 1) of activated magnetic modifier to urokinase has following properties. The weight percentages of magnetite (Fe_3O_4), protein and polyethylene glycol in the magnetic urokinase were 37.7%, 0.54% and 61.8%, respectively. The value of 61.8% was estimated by subtracting the sum of values of magnetite and protein from 100%. The magnetic enzyme dispersed in saline and exerted high fibrinolytic activity of 13.8×10^4 IU/mg protein (750 IU/mg magnetic urokinase). These results were summarized in Table 1.

The next test was conducted to see whether magnetic urokinase particles were magnetically recovered from an aqueous solution. The result was shown in Table 2. Magnetic urokinase particles dispersed in saline and exhibited the fibrinolytic activity of 1870 IU/ml. They were completely recovered by magnetic force of 250 Oe in 20 min. No enzymic activity was detected in residual saline after removing the magnetic enzyme from the saline. The recovered magnetic enzyme dispersed again in saline with almost no loss of enzymic activity (1670 IU/ml). These results indicate that urokinase molecules are tightly bound with magnetite through α,ω-dicarboxymethylpoly(oxyethylene), and all of magnetic urokinase particles are easily recovered by magnetic force.

Using the magnetic urokinase, next experiments in vitro were performed as a basic study for the local dissolution of thrombus. The magnetic

Table 1. Characteristics of Magnetic Urokinase

Composition (Weight %)[a]	
Magnetite (Fe_3O_4)	: 37.7 %
Protein	: 0.54%
Polyethylene Glycol	: 61.8 %
Fibrinolytic Activity	
13.8×10^4 IU/mg protein	
750 IU/mg magnetic urokinase	

a) The value (%) of polyethylene glycol was estimated by subtracting the sum of values of magnetite and protein from 100%.

Table 2. Magnetic Separation of Magnetic Urokinase

Solution	Fibrinolysis Activity (IU/ml)
Colloidal Solution of Magnetic Urokinase	1870
Residual Solution after Magnetic Separation	0
Magnetic Urokinase Recovered by Magnetic Separation	1670

The magnetic separation was carried out in a magnetic field of 250 Oe for 20 min in saline containing magnetic urokinase (12.1 μg/ml). The fibrinolysis activity was determined using fibrin plate containing plasminogen.

urokinase dispersed in saline containing plasminogen was poured into the petri dish in which thin fibrin gel was uniformly sheeted. Then, a small and round magnet was placed at the center of the reverse side of the dish. In the first 3 min, the dish was given a gentle swing and then kept standing at room temperature. Almost all of magnetic enzyme particles were attracted at the center of the dish by magnetic force in 3 min. And the fibrinolytic activity was extensively exhibited at the center. Fig. 1 A represents the photograph of fibrinolysis obtained in the presence of the magnet 60 min after adding the magnetic enzyme. A round hole was observed at the center of the petri dish. This is due to the local fibrinolysis caused by the activation of plasminogen with magnetic urokinase. Fig. 1 B represents the photograph obtained without the magnet under the same conditions as above. As urokinase was distributed uniformly throughout the gel in low concentration, no obvious fibrinolysis took place under the experi-

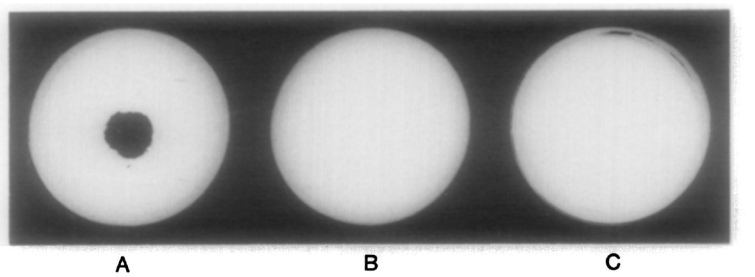

Fig. 1. Local and effective fibrinolysis with magnetic urokinase on fibrin plate in a petri dish. A, Magnetic urokinase with a magnet; B, Magnetic urokinase without a magnet; C, Without magnetic urokinase and a magnet.

mental condition, and fibrinolysis was observed uniformly on fibrin plate at later times. Without magnetic urokinase and magnet, no fibrinolysis was observed at all on fibrin plate (Fig. 1 C). These results indicate that local and effective fibrinolysis on thrombus would be possible by using a combination of magnetic urokinase and a magnet.

This study may give a clue to the treatment of thrombus in vivo and also open up a novel avenue to the development of biomedical field.

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