Hiroshima Journal of Medical Sciences
Vol. 30, No. 4, December, 1981
HIJM 30-38

IMMUNOCHEMICAL PROPERTIES AND MOLECULAR FORMS OF UROKINASE*)

By

Naotika TOKI[1], Sumiyoshi TAKASUGI[2] and Hiroyuki SUMI[3]

1) *Deparment of Dermatology, Hiroshima Univeisity School of Medicine, Hiroshima 734, Japan*
2) *Department of 2nd Surgery, Hiroshima University School of Medicine, Hiroshima734, Japan*
3) *Biochemistry Section, Michael Reese Research Foundation, Chicago Ill. USA.*

(Received August 28, 1981)

ABSTRACT

1. By the double immunodiffusion technique, highly purified high molecular weight urokinase (HMW-UK; MW 53,000, 124,000 IU/mg protein) and low molecular weight urokinase (LMW-UK; MW 32,000, 230,000 IU/mg protein) revealed the same antigenicity, and their fibrinolytic activities were equally quenched with rabbit antisera against both enzymes.
2. Specific antisera against the functionally-active HMW-UK heavy chain (H-chain; MW 31,000, 201,000 IU/mg protein) and the HMW-UK light chain (L-chain; MW 18,000) were first prepared. By double immunodiffusion anti H-chain serum reacted to all of the active urokinase species (HMW-UK, LMW-UK and H-chain), whereas anti L-chain serum did not react to any of the active enzymes of low molecular form.
3. Anti H-chain serum quenched the fibrinolytic activities of all of the active enzymes in contrast to anti L-chain serum, which had no effect on LMW-UK and H-chain. Anti L-chain serum was found to exert an anti-fibrinolytic effect on HMW-UK.

INTRODUCTION

Urokinase (UK) [EC 3.4.99.26], a serine protease produced in the kidney and found in urine, is a potent activator of plasminogen and has been employed as a thrombolytic agent[1-3]. Forms with molecular weights of about 54,000 (HMW-UK) and 32,000 (LMW-UK), respectively, have been purified[1-5]. HMW-UK has been regarded as the native enzyme, which is a two chain molecule probably connected by one interchain disulfide bond, while LMW-UK is an enzymatically degraded form of HMW-UK composed of a single polypeptide chain[2,6]. Recently, we succeeded in separating the functionally-active HMW-UK heavy chain (H-chain; MW 31,000) and the light chain (L-chain; MW 18,000) after mild reduction and alkylation of HMW-UK[6]. Since Kucinski et al. immunized guinea pigs with partially purified human UK[7], many researchers have prepared the UK antisera[8-11]. Bernik and Kwaan[8] used the UK antiserum to establish the immunochemical identity of UK with that of activators produced in human kidney cultures. It was also found recently that human UK antisera react not only to human UK but also to several malignant tumour plasminogen activators[12]. However, to

*) 土岐尚親，高杉純好，須見洋行：ウロキナーゼ分子異性体の免疫特性

the author's knowledge, no other report on the comparative immunological properties of HMW-UK and LMW-UK has yet been published.

In the present study, we first prepared the rabbit antisera against HMW-UK, LMW-UK and each of the two polypeptide chains of HMW-UK, and then studied the immunological properties of both enzymes.

MATERIALS AND METHODS

Materials:

The following commercial preparations were used: pyro-Glu-Gly-Arg-pNA (S-2444; Kabi Diagnostica), N^{α}-acetylglycyl-L-lysine methylester (AGLMe; Sigma Chemical Co), bovine fibrinogen (Armour Pharmaceutical Co.), bovine thrombin (Mochida Pharmaceutical Co.) Sephadex G-100 (Pharmacia Fine Chemicals), and p-nitrophenyl-p'-guanidinobenzoate (NPGB; U. S. Biochemical Co.).

Preparations of UK and its component polypeptide chains:

Four different preparations were made as follows. HMW-UK and LMW-UK were highly purified from commercial sources (Green Cross Co. and Mochida Pharmaceutical Co.) using affinity chromatography on [N^{α}-ε-aminocaproyl)-DL-homoarginine hexylester]-Sepharose, as described previously[5]. The specific activity of the products was 124,000 IU/mg protein for HMW-UK and 23,000 IU/mg protein for LMW-UK. The H-chain and L-chain of HMW-UK were purified as reported previously[6]. After reduction of 5.2 mg highly purified HMW-UK with 0.01 M 2-mercaptoethanol for 10 hours at 25°C and alkylation with 0.015 M iodoacetate for 15 min at 25°C, the HMW-UK molecule was dissociated into two polypeptide molecules, H-chain and L-chain, still retaining more than 75% of the initial activity. The polypeptide chains were separated using an affinity chromatography column (1.8×5.0 cm), and further purified by Sephadex G-100 gel filtration (1.0×153 cm; 0.1 M phosphate buffer containing 0.2 M NaCl and 0.2 M urea, pH 7.4). The H-chain revealed enzyme activity, with a specific activity of about 201,000 IU/mg protein, whereas the L-chain had no enzyme activity.

All these materials were homogeneous on analytical and sodium dodecylsulfate polyacrylamide gel electrophoresis. They were concentrated by ultrafiltration (PM-10, Amicon Co.), lyophilized and stocked at −70°C untill use.

Amidolysis:

The amidolytic activity of the purified UK was determined on the substrate, pyro-Glu-Gly-Arg-pNA, by the method of Claeson et al.[13] with a UK standard curve. Urokinase Reagent (MW 003) was empolyed as the standard.

Esterolysis:

Hydrolysis of AGLMe was determined by Hestrin's method, as described previously[14].

Fibrinolysis:

The preparation of fibrin plates and assay procedures were as reported by Day et al.[9] For neutralization experiments, equal volumes of several diluted antisera and of UK (200 IU/ml control plasma diluted) were combined, and 50 μl portions of the mixtures were applied to the fibrin plates and the residual activity tested[9].

Protein determination:

Protein concentration was determined by the method of Lowry et al.[15] using bovine serum albumin (Armour) as the standard.

Immunization:

Purified UK preparation (1 mg) was dissolved in 1.0 ml of sodium chloride solution (150 mmol/l), mixed with an equal volume of Freund's complete adjuvant (Difco) and injected into the toe pad of rabbits (2.6-3.1 kg). After 4 weeks the rabbit received, as a booster dose, half the amount of enzyme injected subcutaneously into the back. After 1 week, a blood sample was drawn and incubated at 37°C for 3 hours and at 4°C for 18 hours before serum separation by centrifugation at 24,000×g. Preinjected serum from each rabbit was also collected and used as a control. These materials were heat-inactivated at 56°C for 30 min and stocked at −70°C untill use.

Immunodiffusion:

Double immunodiffusion analysis was carried out by the method of Ouchterlony[16] at room temperature for 24 hours. Agarose gel (Behringwerke) was prepared at a concentration of 1.0% in veronal buffer (I=0.07, pH 8.6) containing 0.05% sodium azide.

RESULTS

Immunodiffusion of anti HMW-UK and anti LMW-UK sera with various UK preparations and human plasma:

The precipitin titration of the rabbit HMW-

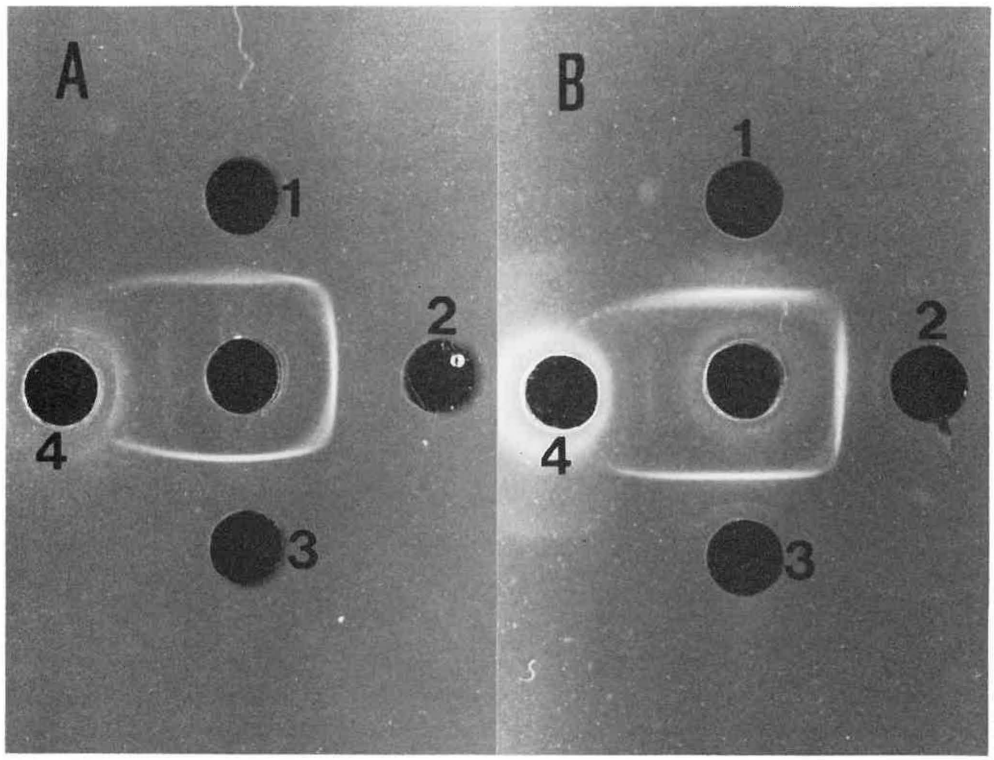

Fig. 1. Immunological analysis of several UK preparations and normal human plasma with anti HMW-UK and anti LMW-UK sera.
A. Center well: 5 μl HMW-UK antiserum (1 : 50 dilution); peripheral wells: 1.5 μl containing 5μg HMW-UK, 2.5 μl containing 4.8 μg LMW-UK, 3.5 μl containing 5 μg H-chain, and 4.5 μl normal human plasma.
B. Center well: 5 μl LMW-UK antiserum (1 : 50 dilution); peripheral wells: 1.5 μl containing 5 μg HMW-UK, 2.5 μl containing 4.8 μg LMW-UK, 3.5 μl containing 5 μg H-chain, and 4.5 μl normal human plasma.

UK and LMW-UK antisera in agarose gel by double immunodiffusion is shown partially in Fig. 1. The anti HMW-UK serum formed a single immunoprecipitin line with the purified enzymes, HMW-UK, LMW-UK and H-chain, but did not react with normal human plasma (Fig. 1A). The results were the same even if higher concentration antisera were used. As shown in Fig. 1B, the results with anti LMW-UK were all identical to those with anti HMW-UK.

In another experiments (not shown), employing the fibrin plate method, we also assessed the quenching effect of the antisera for the fibrinolysis of each enzyme. Both the antisera were found to exert strong inhibitory effects on the active enzymes; they neutralized about 2–5 $\times$ 10^4 IU/ml antiserum, as calculated by 50% inhibition. Such inhibitory effects of the antisera were not observed with AGLMe and pyro-Glu-Gly-Arg-pNA as UK substrates.

Immunodiffusion of anti H-chain and anti L-chain sera with various UK preparations:

To elucidate which polypeptide chain possessed the antigenic site of the UK molecule, we prepared the specific antisera against highly purified H-chain and L-chain, respectively. As illustrated in Fig. 2. A,B, in all cases observed the precipitin lines were a single line. Anti H-chain serum diffused not only to H-chain but also to HMW-UK and LMW-UK. However, it did not react L-chain (Fig. 2A). On the other hand, as shown in Fig. 2B, anti L-chain serum formed a single immunoprecipitin line with L-chain and HMW-UK but did not react with LMW-UK and H-chain. Of course, both

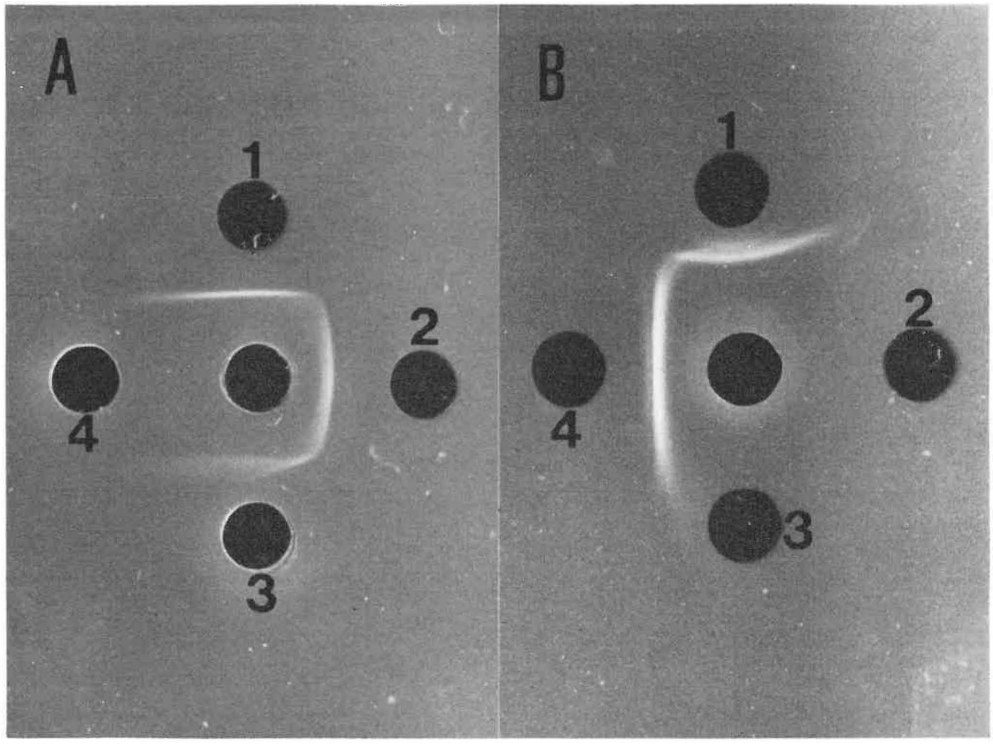

Fig. 2. Immunological analysis of several UK perparations with anti H-chain and L-chain sera.
A. Center well: 5 μl H-chain antiserum (1 : 10 dilution); peripheral wells: 1.5 μl containing 5 μg HMW-UK, 2.5 μl containing 4.8 μg LMW-UK, 3.5 μl containing 5 μg H-chain, and 4.5 μl containing 5 μg L-chain.
B. Center well: 5 μl L-chain antiserum (1 : 10 dilution); peripheral wells: 1.5 μl containing 5 μg HMW-UK, 2.5 μl containing 4.8 μg LMW-UK, 3.5 μl containing 5 μg H-chain, and 4.5 μl containing 5 μg L-chain.

antisera did not react with normal human plasma.

Inhibitory effects of anti H-chain and anti L-chain sera on various UK preparations:

The fibrinolytic activity of the UK molecules was completely quenched by anti H-chain serum (Fig. 3A), as observed by anti HMW-UK and anti LMW-UK sera. On the other hand, anti L-chain serum did not affect the fibrinolytic activities of LMW-UK and H-chain (Fig. 3B). However, anti L-chain serum was found to have an inhibitory effect on HMW-UK, although it was not complete (maximum about 76% inhibition). Inhibitions were also not observed when AGLMe and pyro-Glu-Gly-Arg-pNA were used as UK substrates.

DISCUSSION

Kucinski et al.[7], Day et al.[9], Ogawa et al.[4], Andrassy and Ritz[10], and more recently Shakespeare and Wolf[11] have previously described the antisera against UK molecules, although their immunized UK preparations (except that of Ogawa et al.) were all impure (20,000-45,000 IU/mg protein) or inaccurate. Some materials showed double or triple immunoprecipitin lines on immunodiffusion[9,10]. Ogawa et al. employed a highly purified antigen (224,000 IU/mg protein), although their antiserum was only for the LMW-UK molecule. In the present study, we highly purified four different UK or component polypeptide chain preparations: HMW-UK, LMW-UK, H-chain and L-chain. These were homogeneous on analytical and sodium dodesylsulfate polyacrylamide gel electrophoresis and the specific activities were 124,000 IU/mg for HMW-UK, 230,000 IU/mg for LMW-UK, and 201,000 IU/mg for

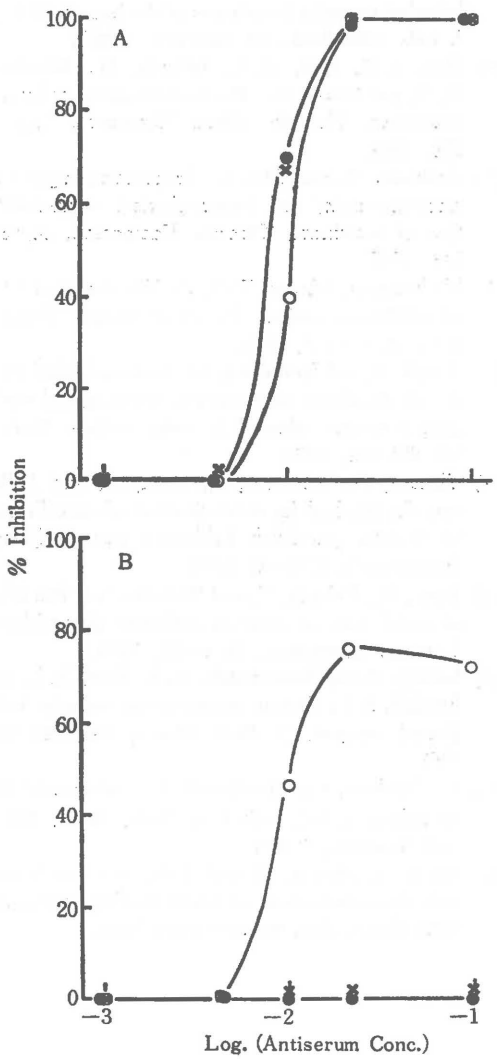

Fig. 3. Inhibitory effects of anti H-chain and L-chain sera on several molecular forms of UK.
A. Effect of anti H-chain serum on the fibrinolysis of HMW-UK (○——○), LMW-UK (●——●), and H-chain (×——×).
B. Effect of anti L-chain serum on the fibrinolysis of HMW-UK (○——○), LMW-UK (●——●), and H-chain (×——×).
Equal amounts of the several diluted antisera and UK solution (200 IU/ml) were mixed, and 50 μl portions of the mixtures were used for the estimation of fibrinolysis (fibrin plate method).

H-chain, which was much higher than the values for the corresponding molecular forms reported previously[7,9,11]. The antisera against these preparations revealed a single precipitin line with purified UK preparations. Furthermore, none of our antisera showed an immunoprecipitin line with human plasma protein, in contrast to the previous results of Shakespeare and Wolf[11]. Using our anti HMW-UK and anti LMW-UK sera, no immunological differences between HMW-UK and LMW-UK were observed (Fig. 1). By double immunodiffusion anti H-chain serum was found to react to all of the active urokinase species, whereas anti L-chain serum did not react to any of the active enzymes of low molecular form. It diffused to both L-chain and HMW-UK. The UK antisera did not show any inhibitory effects on AGLMe and pyro-Glu-Gly-Arg-pNA hydrolysis but had effects on fibrinolysis. We employed the fibrin plate method for the quenching experiments with the antisera against each parent UK and component polypeptide chain. Anti HMW-UK and anti LMW-UK sera, as well as anti H-chain serum, strongly inhibited the fibrinolytic activity of all the active UK molecules, HMW-UK, LMW-UK, and H-chain. On the other hand, anti L-chain serum exerted no effect on LMW-UK and H-chain, although, it partially inhibited the HMW-UK activity (Fig. 3).

On the basis of these results, anti L-chain may represent a useful tool for identifying the molecular forms of UK. HMW-UK has been found to have two polypeptide chains, linked probably by one disulfide bond, in contrast to LMW-UK which comprises a single polypeptide chain[2,6]. Since the L-chain is the inactive polypeptide chain, the inhibitory effect of anti L-chain serum on HMW-UK may be due to some conformational change of the HMW-UK molecule after complexation with the antibody molecule, or the active site (in the H-chain) may thereby be blocked by the antibody molecule. Recently, we found that HMW-UK is more strongly bound to the surface of fibrin than LMW-UK using fibrin monomer-Sepharose[17]. Assuming the fibrin-binding site of the UK molecule to be in the L-chain of HMW-UK, competitive inhibition by anti L-chain may also be present.

REFERENCES

1) White, W. F., Barlow, G. H. and Mozen, M. M.: The isolation and characterization of plasminogen activator (urokinase) from human urine. Biochemistry, 5, 2160-2169, 1966.
2) Soberano, M. E., Ong, E. B., Johnson, A. J., Levy, M. and Schoellmann, G.: Purification and characterization of two forms of urokinase. Biochim. Biophys. Acta, 445, 763-773, 1976.
3) Urokinase Pulmonary Embolism Trial Study Group: Urokinase-streptokinase pulmonary embolism trial. J. Am. Med. Assoc., 229, 1606-1613, 1974.
4) Ogawa, Y., Yamamoto, H., Katamine, T. and Tajima, H.: Purification and some properties of urokinase. Thromb. Diath. Haemorrh., 34, 194-209, 1975.
5) Sumi, H., Sasaki, K. and Muramatu, M.: A simple and rapid purification method of urokinase using [N^{α}- (ε-aminocaproyl) -DL-homoarginine hexylester] -Sepharose column. Acta Haem. Jap., 41, 766-770, 1978.
6) Sumi, H. and Robbins, K. C.: The preparation and characterization of a functionally-active urokinase heavy chain prepared from high molecular weight urokinase. Abstracts of 5th International Conference on Synthetic Fibrinolytic Thrombolytic Agents, Malmö, Sweden, p. 4, 1980.
7) Kucinski, C., Fletcher, A. and Sherry, S.: Effect of urokinase antiserum or plasminogen activators: Demonstration of immunologic dissimilarity between plasma plasminogen activator and urokinase. J. Clin. Invest., 47, 1238-1253, 1968.
8) Bernik, M. B. and Kwaan, H. C.: Origin of fibrinolytic activity in cultures of the human kidney. J. Lab. Clin. Med., 70, 650-661, (1967).
9) Day, E. B., Ball, A. P., Jeffords, D., Woodard, W. T. and Silver, D.: The immunoassay of human urokinase. Thromb. Diath. Haemorrh, 21, 273-286, 1969.
10) Åndrassy, K. and Ritz, E.: Preparation (agar zone electrophoresis) and immunological characterization of urokinase. Thromb. Haemostas., 38, 536-543, 1977.
11) Shakespeare, M. and Wolf, P*: The demonstration of urokinase antigen in whole blood. Thromb. Res., 14, 825-835, 1979.
12) Åstedt, B. and Holmberg, L.: Immunological identity of urokinase and ovarian carcinoma plasminogen activator released in tissue culture. Nature, 261, 595-596, 1976.
13) Claeson, G., Friberger, P., Knös, M. and Eriksson, E.: Method for determination of prekallikrein in plasma, glandular kallikrein and urokinase. Haemostasis, 7, 76-78, 1978.
14) Sumi, H., Takada, Y. and Takada, A.: The effect of metal ions on esterase activities of urokinase. Thromb. Haemostas., 39, 46-52, 1978.
15) Lowry, O. H., Rosebrough, H. J., Farr, A. L. and Randall, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193 265-275, 1951.
16) Ouchterlony, O.: Handbook of Experimental Immunology p. 665, edited by Weir, D. M., Blackwell Scientific, (1967).
17) Sumi, H., Mihara, H. and Toki, N.: Purification and characterization of fibrin-binding urokinase. Acta Haem. Jap., 44, 1044-1049, 1981.