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Detection and Elimination of Serum Protein Contaminants during the Purification of Human Urinary Kallikrein[1)]

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Antiserum against purified human urinary kallikrein (HUK) found to be a homogeneous preparation by disc electrophoresis was produced in rabbits. However, this antiserum was found to react slightly with some serum protein components, such as albumin or albumin-like protein, as well as HUK. It is conceivable that some small amount of serum protein detectable only by the immunochemical method was contained in the electrophoretically homogeneous HUK. Although a trace amount of serum protein contained in the purified HUK could not be completely removed by physicochemical procedures, highly purified homogeneous HUK was successfully obtained by the application of affinity absorption treatment using anti-human whole serum. Both in immunodiffusion and in immunoelectrophoresis, antiserum against this preparation gave only one line of precipitin with the crude or the finally purified HUK and no line with human whole serum. Our antiserum was found to be immunochemically monospecific.

Keywords——purification of kallikrein; antiserum of kallikrein; human urine; serum proteins; antigenicity of albumin; affinity absorption

Introduction

In order to study the physiological and pathological roles of kallikrein, it seems to be important to carry out an immunochemical investigation or to establish a sensitive assay method for kallikrein in the body, for instance, radioimmunoassay of HUK. The purification of HUK has already been studied and physicochemically pure kallikrein has been prepared.[2)] Accordingly, antiserum against this preparation was produced. However, in the radioimmunoassay of HUK, anti-HUK serum was found to react slightly with the serum protein components, particularly serum albumin. In the present paper, the cause of the reaction between anti-HUK serum and serum protein components was clarified and a method capable of providing immunochemically pure HUK was developed.

Experimental

Materials——The crude HUK powder[3)] (3.09 KU/A_{280}, 0.12 EU/A_{280}) and human serum albumin (250 mg/ml) were supplied by the Green Cross Co., Osaka, Japan. DEAE-cellulose (0.92 meq/g) and Column Lite (30—100 mesh) were purchased from Brown Co., Berlin, U.S.A., and Fuji Chem. Co., Toyama, Japan, respectively. Sephadex G-25, G-75 (40—120 μ), QAE-Sephadex A-50 (3.0±0.4 meq/g, 40—120 μ) and Sepharose 4B were products of Pharmacia Fine Chemicals, Uppsala, Sweden. Freund's complete adjuvant from Iatron Lab., Tokyo, Japan, and Agar Noble from Difco Lab., Detroit, U.S.A. were also used. Anti-human whole serum (1 mg antibody/ml) and anti-human serum albumin (4.3 mg antibody/ml) were obtained from Miles Chem. Co., Elkhart, U.S.A. Anti-α_1-antitrypsin (0.55 mg antibody/ml), anti-Ba-α_2-glycoprotein (0.35 mg antibody/ml), anti-haptoglobin (1.5 mg antibody/ml), anti-α_1-acid glycoprotein (0.9 mg antibody/ml), anti-α_2-macroglobulin (3.5 mg antibody/ml), anti-β-lipoprotein (2.0 units/ml), anti-transferrin (1.1 mg antibody/ml), and anti-fibrinogen (2.5 mg antibody/ml) were obtained from Boehringer Mannheim GmbH, Mannheim, West Germany. N^{α}-Toluenesulfonyl-L-arginine methyl ester (Tos–Arg–OMe) and N^{α}-benzoyl-L-arginine ethyl ester (Bz–Arg–OEt) were supplied by the Protein Research Foundation, Osaka, Japan. Other chemicals used were commercial guaranteed-grade reagents.

Assay of Kallikrein Activities——The vasodilator activity of kallikrein was determined by measuring the increase of blood flow in the dog femoral artery according to the method of Moriya *et al.*[4] The activity was expressed in kallikrein units (KU).

Esterolytic activity toward Tos–Arg–OMe was measured at 30°, pH 8.0, by the colorimetric method with chromotropic acid.[5] The activity was expressed in esterase units (EU) which were identical in terms of μmoles of substrate hydrolyzed per min. Esterolytic activity on immunodiffusion plates was detected by immersing the plates in a solution of Bz–Arg–OEt–formazan staining system for 15 min at 37° as reported previously.[6]

Double Immunodiffusion——The method of Ouchterlony was employed.[7] Agarose solution (1.5% (w/v) in 0.05 M phosphate buffer, pH 7.0) was poured onto a glass plate (2.6×7.5 or 7.5×11 cm) to provide a 1 mm thick gel layer. Antigens and antibodies (5 μl) were separately added to the wells (diameter; 2 mm) and kept for 5 hr at 4° in a humid chamber. Thereafter the precipitin lines formed were observed directly. If necessary, the plates were treated with 0.1% (w/v) tannic acid after being washed in distilled water for a week in order to eliminate soluble proteins.

Immunoelectrophoresis——This was carried out on 1.5% (w/v) agarose gel in 0.05 M veronal buffer, pH 8.6, for 2 hr at 20° at a constant current of 3 mA/cm as described by Scheidegger.[8] The development with antibody was carried out for 10 hr at 4°.

Counterimmunoelectrophoresis——This electrophoresis was performed according to the method of Gocke *et al.*[9] in 1.0% (w/v) agarose gel (8.5×12 cm) for 50 min at 4—5 V/cm (constant) at room temperature, using veronal acetate buffer, pH 8.2. Antigens and antibodies (5 or 10 μl) were separately applied to wells of 2 or 3 mm in diameter.

Immunoabsorption——Various doses of human whole serum (0—11 A_{280}/ml) were added to an equal volume of anti-HUK serum (0.2 ml) and kept for 12 hr at 4°. The immunocomplex formed was eliminated by centrifugation (3000 rpm, for 20 min, at 4°). The supernatant was used in double immunodiffusion.

Single Radial Immunodiffusion[10]——Anti-human serum albumin was diluted 100 times with 1.5% (w/v) agarose solution in 0.05 M phosphate buffer, pH 7.0, and 1 mm thick agarose diffusion plates were prepared. The sample solution or various concentrations of human serum albumin (5 μl) were separately added to wells of 2 mm diameter and immunoreaction was carried out for 3 days at 4°. The concentration of albumin-like protein in a sample was estimated by measuring the diameter of rings formed.

Preparation of HUK——Two different lots of purified HUK, named 501-M and 705-M were obtained according to the method of Matsuda *et al.*[2] The esterolytic activities of 501-M and 705-M were 8.7 and 8.6 EU/A_{280}, respectively. The crude HUK powder was also purified by chromatography on Column Lite in addition to the previous method and two different preparations thus obtained, 707-M and 711-M, showed esterolytic activities of 13.6 and 14.5 EU/A_{280}, respectively.

Preparation of Anti-HUK Serum——HUK (501-M, 7.5 A_{280}/ml, 8.7 EU/A_{280}) solution in saline was emulsified with an equal volume of Freund's complete adjuvant. One ml of this emulsion was injected intracutaneously into all foot pads and the backs of rabbits weighing about 2.5 kg. After 4 weeks, 1.5 ml of the same emulsion was injected subcutaneously into the backs of the rabbits. Further, 2.0 ml of it was given to the rabbits 3 weeks after the second injection. The whole blood was collected 5 days after the last injection. The serum was obtained by centrifugation (3500 rpm, for 20 min, at 4°) and was heated for 30 min at 56°. Insoluble materials formed were removed by centrifugation (8000 rpm, for 30 min, at 4°) and the antiserum obtained from 3 rabbits was stocked at 4° after filtration through a Millipore filter for sterilization. The titers were 6 (48 ml) and 3 μg/ml (36 and 52 ml) of antigen solution (501-M) as determined by the ring test.

Preparation of Sepharose Covalently Linked with Anti-Human Whole Serum——The procedures of Cuatrecasas were used.[11] Two ml of anti-human whole serum was added to 20 g of activated Sepharose 4B in 20 ml of 0.2 M sodium bicarbonate buffer, pH 8.9 and the mixture was stirred gently for 6 hr at 25°. Anti-human whole serum conjugated Sepharose 4B was washed with 50 ml of 0.5 M Tris-HCl buffer, pH 8.0. Such a preparation was capable of binding 5 μg of human whole serum protein per g of Sepharose 4B.

Results

Double Immunodiffusion

The immunodiffusion of the various preparations of HUK was performed by using the antiserum against electrophoretically homogeneous HUK (501-M) (Fig. 1). The samples, 501-M, 711-M, 707-M, and 705-M, formed 4, 3, 2, and 3 lines of precipitin with anti-HUK serum, respectively. Two preparations, 501-M and 711-M, gave a precipitin line which fused with that of human serum albumin. Other preparations (707-M and 705-M) showed no apparent line corresponding to serum albumin (Fig. 1), since the concentration of these preparations (0.06 A_{280}/ml) was about a quarter of that of 501-M or 711-M (0.25 A_{280}/ml).

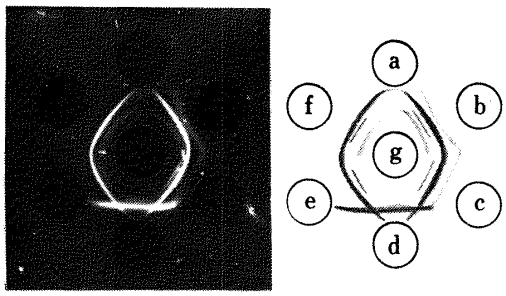

Fig. 1. Double Immunodiffusion Pattern of Various Purified HUK Preparations

a, 0.05 M phosphate buffer, pH 7.0 (5 μl); b, 501-M (0.25 A_{280}/ml, 5 μl); c, 711-M (0.25 A_{280}/ml, 5 μl); d, human serum albumin (0.1 mg/ml, 5 μl); e, 707-M (0.06 A_{280}/ml, 5 μl); f, 705-M (0.06 A_{280}/ml, 5 μl); g, antiserum against 501-M (5 μl).

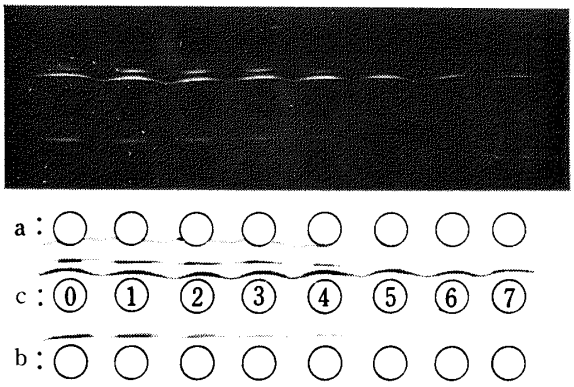

Fig. 2. Immunoabsorption Pattern of HUK and Anti-HUK Serum Treated with Human Whole Serum

Five μl of antigen or antibody was poured into each well. a: 711-M (0.25 A_{280}/ml). b: human whole serum (2 A_{280}/ml). c: 0, anti-HUK serum only (not treated); 1, 2, 3, 4, 5, 6, and 7, anti-HUK serum treated with 11, 5.5, 2.75, 1.38, 0.69, 0.34, and 0.17 A_{280}/ml solutions of human whole serum, respectively. For details, see "Experimental."

Immunoabsorption

Against anti-HUK serum treated with low doses of human whole serum (0—0.69 A_{280}/ml), 711-M gave 3 lines of precipitin. On the other hand, two of these disappeared upon treatment with high doses of serum (1.38—11 A_{280}/ml) (Fig. 2). After activity staining of that plate with Bz-Arg-OEt-formazan system,[6] only the precipitin line unabsorbed with human serum was stained. Thus, the remaining one line of precipitin possessed esterolytic activity toward Bz-Arg-OEt (one of the kallikrein activities). Consequently, this finding indicated that 501-M (used as the antigen in the preparation of antiserum) and 711-M still contained small amounts of serum protein components.

Counterimmunoelectrophoresis

The presence of protein components derived from human blood was examined in the crude

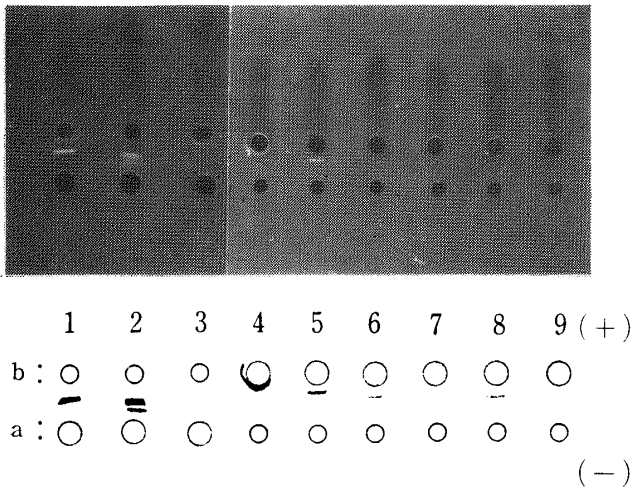

Fig. 3. Counterimmunoelectrophoresis of Crude HUK Powder

a: crude HUK powder (30 mg/ml, 5 or 10 μl). b: 1, anti-α_1-acid glycoprotein (5 μl); 2, anti-Ba-α_2-glycoprotein (5 μl); 3, anti-haptoglobin (5 μl); 4, anti-human serum albumin (10 μl); 5, anti-α_1-antitrypsin (10 μl); 6, anti-α_2-macroglobulin (10 μl); 7, anti-β-lipoprotein (10 μl); 8, anti-transferrin (10 μl); 9, anti-fibrinogen (10 μl). For details, see "Experimental."

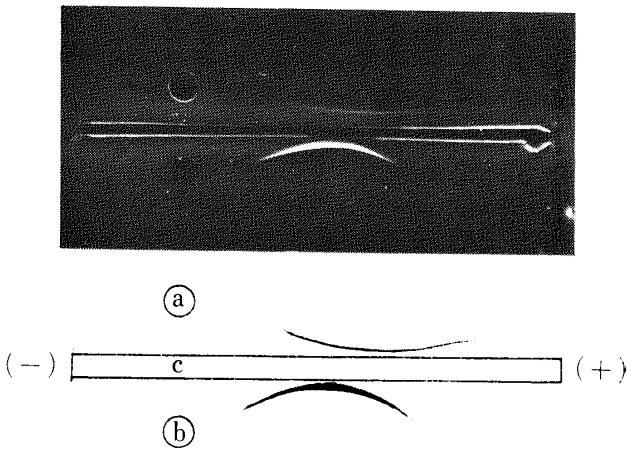

Fig. 4. Immunoelectrophoretic Pattern of Crude HUK Powder with Anti-Human Serum Albumin

a, crude HUK powder (5 mg/ml, 5 μl); b, human serum albumin (1 mg/ml, 5 μl); c, anti-human serum albumin (100 μl).

HUK powder, the starting material, by the counterimmunoelectrophoresis technique (Fig. 3).[9] Numerous serum protein components, α_1-acid glycoprotein, Ba-α_2-glycoprotein, human serum albumin, α_1-antitrypsin, α_2-macroglobulin, and transferrin were detected in it. In particular, the precipitin line formed between the crude HUK and anti-human serum albumin was observed near the well of the antibody (Fig. 3). Thus, it appeared that the content of albumin-like protein in the crude HUK powder was high as compared with those of the other components.

Immunoelectrophoresis

Anti-human serum albumin was used in order to compare the albumin-like protein in the crude HUK powder with albumin in human plasma. The immunoelectrophoretic pattern of the crude HUK powder is depicted in Fig. 4. The crude HUK gave a line of precipitin with slightly higher mobility than that of human serum albumin. This result suggested that the albumin-like protein in the crude HUK powder was not identical with albumin itself in human plasma.

Elimination of Serum Protein Components in the Crude HUK Powder

As the crude HUK powder contained a large amount of albumin-like protein and anti-HUK serum reacted with human serum albumin, an improved purification was developed by the use of the following procedures in which albumin was checked as an index of the elimination of the contaminating serum protein components.

The crude HUK powder (1 g) was dissolved in 0.05 M phosphate buffer, pH 7.0, at 2 mg/ml concentration and applied to a column of DEAE-cellulose (1.7×85 cm) equilibrated with the same buffer. The column was eluted with an exponential gradient system of 0.05 M phosphate buffer, pH 7.0, containing 0 to 0.5 M NaCl, and the active fractions were pooled and concentrated.

The concentrated sample was filtered through a Sephadex G-75 column (2.5×100 cm) equilibrated with 0.05 M phosphate buffer, pH 7.0.

Active fractions were applied to a QAE–Sephadex A-50 column (2.5×80 cm) equilibrated with 0.05 M phosphate buffer, pH 7.0. The elution was carried out with the same exponential gradient procedure used in DEAE-cellulose chromatography.

The sample was adsorbed to a column (2.4×45 cm) of Column Lite after being dialyzed against distilled water. The column was eluted with a linear gradient elution system from 0 to 0.25 M phosphate buffer, pH 7.0.

Albumin content was found to decrease gradually during the course of purification. However, Sephadex G-75 gel filtration was not effective for the elimination of albumin or albumin-like protein.

For further purification after Column Lite chromatography, affinity absorption treatment with anti-human whole serum was performed as follows.

Sepharose 4B linked with anti-human whole serum (20 g) and the HUK preparation (3.9 A_{280} protein) were mixed and stirred mildly for 3 hr at 25°. The unabsorbed fraction was concentrated. In the final preparation, albumin or albumin-like protein could not be detected immunochemically. Its activities were 707 KU/A_{280} and 16.2 EU/A_{280} and the recovery of activity toward Tos–Arg–OMe was 59%. The results of the above procedures are summarized in Table I. When the same affinity absorption treatment was carried out against the purified HUK (711-M, albumin content: 4%) obtained by the previous method,[2] the resulting preparation was found to be also immunochemically homogeneous.

Antiserum against this preparation was prepared as described in "Experimental." As shown in Fig. 5 (A) and (B), the anti-HUK serum gave only a single precipitin line against the crude HUK or the final preparation on both immunoelectrophoresis and immunodiffusion, and formed no precipitin line with human whole serum. Thus, our highly purified HUK was immunochemically homogeneous and its antiserum was monospecific.

Table I. Elimination of Serum-Like Protein Components from Crude HUK Powder

Procedure	Total A_{280}	Total Tos-Arg-OMe	Tos-Arg-OMe EU/A_{280}	Albumin content (%)
1. Crude HUK powder	1010	322	0.121	28
2. DEAE-cellulose	344	134	0.39	15
3. Sephadex G-75	160	88	0.55	16
4. QAE-Sephadex A-50	56.6	47	0.85	7.6
5. Column Lite	3.9	34	9.75	2.9
6. Affinity absorption	1.23	20	16.2	Not detected

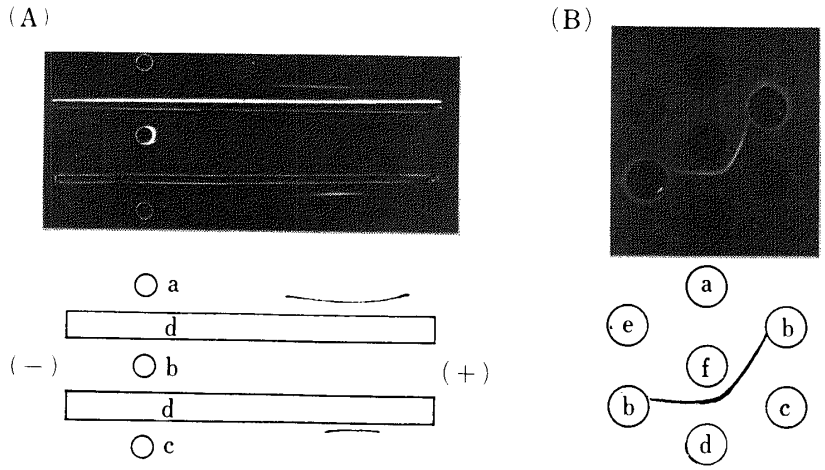

Fig. 5. Immunochemical Properties of the finally Purified HUK and Its Antiserum

Immunoelectrophoresis and double immunodiffusion were carried out as described in "Experimental." Immunoelectrophoresis (A): a, the finally purified HUK (1.5 A_{280}/ml, 5 μl); b, human whole serum (68 A_{280}/ml, 5 μl); c, crude HUK powder (50 mg/ml, 5 μl); d, antiserum against the finally purified HUK (100 μl). Double immunodiffusion (B): a, 0.05 M phosphate buffer, pH 7.0 (5 μl); b, human whole serum (2 A_{280}/ml, 5 μl); c, the finally purified HUK (0.75 A_{280}/ml, 5 μl); d, crude HUK powder (10 mg/ml, 5 μl); e, human serum albumin (0.2 mg/ml, 5 μl); f, antiserum against the finally purified HUK (100 μl).

Discussion

HUK preparations (4.6—9.9 EU/A_{280}) that were homogeneous on disc electrophoresis have already been prepared by Matsuda *et al.*[2] Thus, purification from crude HUK powder was tried according to our previous method, and purified HUKs (501-M: 8.7 EU/A_{280}, 705-M: 8.6 EU/A_{280}, 707-M: 13.6 EU/A_{280}, 711-M: 14.5 EU/A_{280}) with nearly the same purity as that previously obtained were prepared. Nevertheless, antiserum against the purified HUK (501-M) was observed to give more than one precipitin line with the above HUK preparations. After treatment of the anti-HUK serum with human whole serum, only one precipitin line which possessed Bz-Arg-OEt-hydrolyzing activity was formed between 711-M and anti-HUK serum. Judging from these results, we consider that HUK that was found to be physicochemically homogeneous may still have contained some serum protein components, and that antibodies against these components may have been produced. A similar phenomenon was reported in the case of ceruloplasmin.[12]

Rigas and Heller have reported that most protein excreted into the urine of normal persons originated from the blood, and that albumin or albumin-like protein accounted for a large part.[13] Our present results obtained by counterimmunoelectrophoresis are in good agreement with their observations. According to the studies of Peterson, the molecular weight of albumin-like protein found in urine was equal to that of serum albumin (molecular weight: 6.9×10^4).[14] However, if the molecular weight of albumin-like protein in the urine is nearly equal

to that of serum albumin, HUK (molecular weight: 4.05×10^4)[15] and albumin-like protein in the crude HUK powder could be separable to some extent by Sephadex gel filtration or other methods. In our present experiments, however, the content of albumin-like protein in HUK was never decreased after Sephadex G-75 gel filtration (Table I). On the other hand, Friberg[16] and Mori[17] discovered low molecular weight albumin-like proteins with molecular weights of $(2—3) \times 10^4$ and about 1×10^4 in urine. These albumin-like proteins had the same antigenicity as native serum albumin and moved with slightly higher mobility than serum albumin in immunoelectrophoresis. In these respects (antigenicity and mobility), their observations are in good agreement with our results (Figs. 1 and 4). However, regarding the molecular weight, it appears that the molecular weight of albumin-like protein in the crude HUK powder is slightly larger than those of the albumin-like proteins reported by them; namely, near the molecular weight of HUK. Moi Yoi *et al.* reported that a HUK preparation obtained after isoelectric focusing contained albumin-like protein as the main contaminant.[18] We also observed that HUK-I (isoelectric point: 3.9), having a different isoelectric point from albumin (isoelectric point: 4.7), reacted with anti-human serum albumin. Our present results suggest that the molecular weight and the isoelectric point of HUK are very similar to those of albumin-like protein present in our crude HUK powder, so that it is very difficult to separate HUK and albumin-like protein by conventional physicochemical procedures. Affinity absorption treatment was applied to the purification of HUK by using the reaction between anti-human whole serum and serum protein components in the crude HUK powder. In this way, protein contaminants in the purified HUK preparations (501-M, 705-M, 707-M, and 711-M) were completely eliminated.

By using this homogeneous HUK as an antigen, extremely high quality antiserum against HUK was successfully obtained. Thus, highly purified homogeneous HUK is now available for biochemical studies related to human kallikrein.

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