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Purification of heparin cofactor II from human plasma

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ABSTRACT

Heparin cofactor II (HCII) is an inhibitor of thrombin in human plasma whose activity is enhanced by heparin and dermatan sulphate. HCII was purified to homogeneity from normal human plasma with an overall yield of 7.5%. After treatment with barium chloride, precipitation with 50% saturated ammonium sulphate and dialysis of the resuspended precipitate against 0.02 *M* Tris–HCl (pH 7.4), the sample was chromatographed on a heparin–Sepharose CL 6B affinity column, DEAE-Sepharose CL 6B ion-exchange gel and an AcA 34 gel permeation column. For the final steps, a high-performance liquid chromatographic system was used which included ion-exchange chromatography on a Mono-Q column and gel permeation using a Superose column. The purified protein was homogeneous by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The specific activity of purified HCII was 12.2 U/mg. The HCII activity was evaluated as antithrombin dermatan sulphate cofactor activity. A specific antiserum against HCII was raised in the rabbit.

INTRODUCTION

Heparin cofactor II (HCII) is a recently described plasma inhibitor of thrombin [1,2], the activity of which is potentiated by heparin and more specifically by dermatan sulphate, another glycosaminoglycan [3]. HCII displays structural and biological similarities with antithrombin III (ATIII) [4,5], the main plasma cofactor of heparin [6]. Nevertheless, whereas ATIII is active towards most of the activated clotting factors [6], HCII is a specific inhibitor of thrombin [1,2,7].

We report here the purification to homogeneity of HCII from human plasma and the production of a specific antiserum against the inhibitor raised in the rabbit.

EXPERIMENTAL

Materials

All reagents were of analytical-reagent grade and were obtained from Merck (Darmstadt, Germany) unless stated otherwise. Heparin–Sepharose CL6B and DEAE-Sepharose CL6B were purchased from Pharmacia (Uppsala, Sweden). The

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anion-exchange chromatographic Mono-Q column and the gel permeation Superose column were obtained prepacked from Pharmacia. The fast protein liquid chromatography (FPLC) system (Pharmacia) was used. The AcA-34 gel permeation column was obtained from IBF (Villeneuve la Garenne, France).

Collection of plasma

Venous blood was collected from at least 30 healthy individuals in evacuated tubes containing 0.129 *M* trisodium citrate (1:9) (Vacutainer 606608; Beckton Dickinson, Orangeburg, NJ, U.S.A.). Blood samples were immediately centrifuged at 2300 *g* and 3°C for 15 min. Plasma samples were pooled and stored frozen at −70°C until used.

Purification of HCII

The thawed plasma (485 ml) was treated with 1 *M* barium chloride solution (1:10, v/v) with constant magnetic stirring for 15 min at room temperature. After a 10-min centrifugation at 5000 *g*, the precipitate was discarded. All subsequent steps were carried out at 4°C, except the two final steps performed using the FPLC system. The supernatant was treated with 50% saturated ammonium sulphate (final concentration) for 20 min with constant magnetic stirring and centrifuged at 10 000 *g* for 20 min. The precipitate was dissolved in 65 ml of 0.02 *M* Tris–HCl buffer (pH 7.4), dialysed overnight against the same buffer, and the solution was centrifuged at 10 000 *g* for 20 min.

The supernatant was applied to a column (20 × 2.5 cm I.D.) of heparin–Sepharose CL6B equilibrated in 0.02 *M* Tris–HCl (pH 7.4). The column was extensively washed with this buffer until the absorbance of the effluent was zero. The column was eluted with 350 ml of a linear gradient from 0.0 to 0.4 *M* NaCl in 0.02 *M* Tris–HCl (pH 7.4). Fractions of 4.2 ml were collected at a flow-rate of 13 ml/h. Fractions containing more than 1.5 U/ml of dermatan sulphate (DS) cofactor activity were pooled (volume $v = 29.4$ ml) and diluted 1:3 in 0.02 *M* Tris–HCl (pH 7.4) to reduce the ionic strength.

This protein solution was applied to a column (30 × 1.5 cm I.D.) of DEAE-Sepharose equilibrated in 0.02 *M* Tris–HCl (pH 7.4). The column was extensively washed until the absorbance of the effluent was zero and eluted with 150 ml of a linear gradient from 0 to 0.4 *M* NaCl in 0.02 *M* Tris–HCl (pH 7.4). Fractions of 1.5 ml were collected at a flow-rate of 9 ml/h. Fractions containing more than 1.5 U/ml of DS cofactor activity were pooled ($v = 10.5$ ml) and concentrated against polyethylene glycol (PEG) to a final volume of 3 ml.

The solution was applied to a column (80 × 1.5 cm I.D.) of AcA-34 equilibrated with 0.15 *M* NaCl buffered with 0.02 *M* Tris–HCl (pH 7.4). Gel permeation chromatography was carried out in this buffer at a flow-rate of 6 ml/h. Fractions of 1.2 ml were collected. Fractions containing more than 2.0 U/ml DS cofactor activity were pooled ($v = 8.4$ ml).

The final steps were performed using the FPLC system at room temperature. The protein solution was applied to a Mono-Q ion-exchange column equilibrated with 0.02 *M* Tris–HCl (pH 7.4). The elution was carried out using a linear gradient from 0.0 to 0.5 *M* NaCl in 0.02 *M* Tris–HCl (pH 7.4). Fractions of 1.0 ml were collected at a flow-rate of 1.0 ml/min. Fractions containing the peak of DS cofactor activity were

pooled (v = 3 ml), concentrated against PEG and applied to a gel permeation (Superose) column equilibrated in 0.02 *M* Tris–HCl (pH 7.4) containing 0.3 *M* NaCl. The chromatography was performed using this buffer and 1.0-ml fractions were collected at a flow-rate of 0.10 ml/min. Fractions containing the peak of DS cofactor activity were pooled (v = 4 ml). This preparation was stored frozen at −70°C in aliquots.

Electrophoretic techniques

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed, according to Laemmli [8], in 7.5% polyacrylamide slab gels containing 0.1% (v/v) SDS. Apparent relative molecular masses (M_r) were obtained by comparison with a mixture of reduced reference proteins (Bio-Rad Labs., Richmond, CA, U.S.A.): β-galactosidase (M_r 116 000 dalton), phosphorylase B (M_r 92 000 dalton), bovine serum albumin (M_r 66 000 dalton), ovalbumin (M_r 45 000 dalton), carbonic anhydrase (M_r 31 000 dalton) and soybean trypsin inhibitor (M_r 22 000 dalton). All gels were stained with Coomassie Brillant Blue R.

Crossed immunoelectrophoresis was performed according to Ganrot [9] in 1% agarose gel containing 1% antisera against HCII.

Determination of proteins

The protein concentrations in the chromatographic fractions were determined by measuring the absorbance at 280 nm [10] in a cell of 1-cm light path. The extinction coefficients at 280 nm ($A_{280}^{1\%}$) used were 10 for a mixture of proteins and 11.7 for purified HCII [2].

HCII activity assay

HCII activity was assayed as antithrombin dermatan sulphate cofactor activity [11].

Production of specific antiserum against HCII

A 50-μg sample of the purified protein mixed with 1 ml of complete Freund's adjuvant was injected subcutaneously into rabbits. The animals received three subsequent injections of 50 μg of HCII mixed with 1 ml of Freund's incomplete adjuvant at 1-week intervals. The rabbits were bled 1 week after the last injection. The blood was allowed to clot at 37°C for 3 h and the serum, obtained by centrifugation at 2300 *g* for 15 min, was stored frozen at −30°C in aliquots.

RESULTS

Purification of HCII

As shown in Fig. 1, the peak of DS cofactor activity was eluted from the heparin–Sepharose column with a mean concentration of 0.18 *M* NaCl in 0.02 *M* Tris–HCl (pH 7.4). The fractions containing more than 1.5 U/ml were pooled, diluted in buffer to reduce the ionic strength and applied to a DEAE-Sepharose column and eluted with a linear gradient from 0 to 0.4 *M* NaCl in 0.02 *M* Tris–HCl (pH 7.4). Fractions containing a DS activity of more than 1.5 U/ml were eluted with a mean sodium concentration of 0.18 *M*, as shown in Fig. 2. These fractions were then pooled,

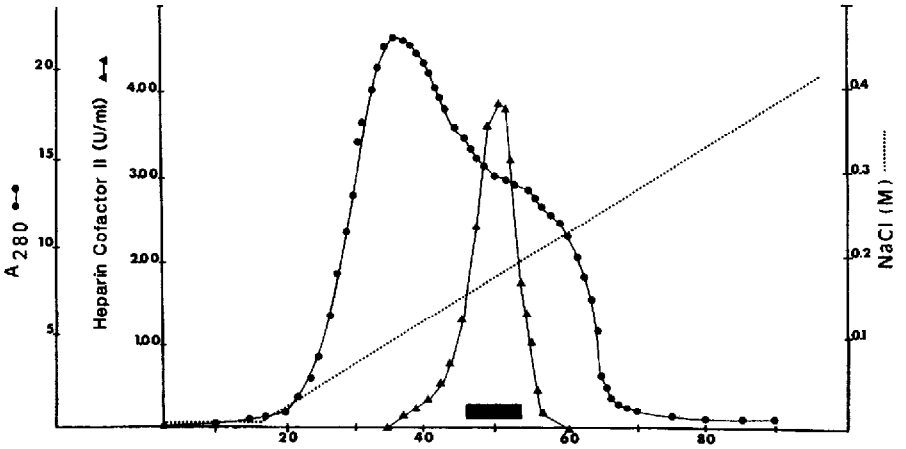

Fig. 1. Elution of HCII activity from heparin–Sepharose. The column (20 × 2.5 cm I.D.) was eluted with a 350-ml linear gradient from 0.0 to 0.4 *M* NaCl in 0.02 *M* Tris–HCl (pH 7.4). Fractions of 4.2 ml were collected at a flow-rate of 13 ml/h. ● = Absorbance at 280 nm; ▲ = HCII cofactor activity evaluated as antithrombin dermatan sulphate cofactor activity. Pooled fractions are indicated by a solid bar.

concentrated against PEG and gel chromatographed on an AcA-34 column. The elution profile is shown in Fig. 3. The DS cofactor activity was eluted with an elution volume of 54.0 ml. This protein solution was then applied to a Mono-Q ion-exchange column connected to the FPLC system. The column was eluted with a linear gradient from 0.0 to 0.5 *M* NaCl in 0.02 *M* Tris–HCl (pH 7.4). As shown in Fig. 4, the peak of DS cofactor activity was eluted with a mean concentration of 0.28 *M* NaCl. The final step consisted of gel permeation using a Superose column. The elution profile is shown in Fig. 5.

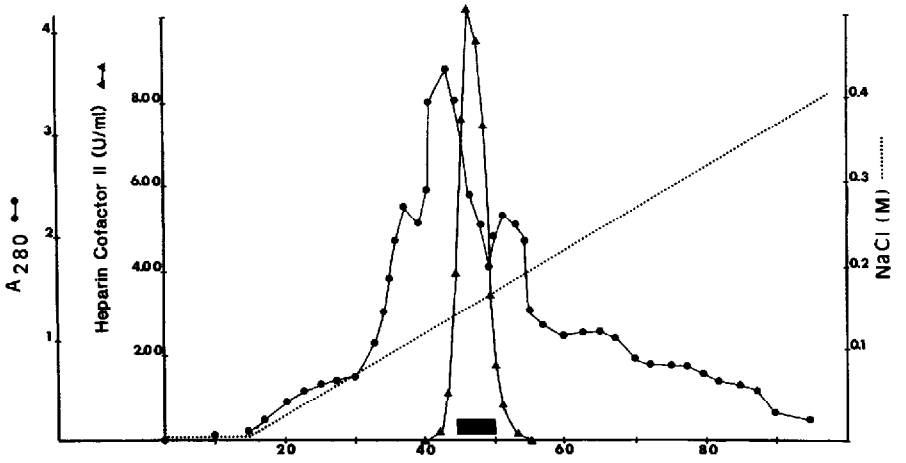

Fig. 2. Elution of HCII activity from DEAE-Sepharose. The column (30 × 1.5 cm I.D.) was eluted with a 150-ml linear gradient from 0.0 to 0.4 *M* NaCl in 0.02 *M* Tris–HCl (pH 7.4). Fractions of 1.5 ml were collected at a flow-rate of 9 ml/h. Symbols as in Fig. 1.

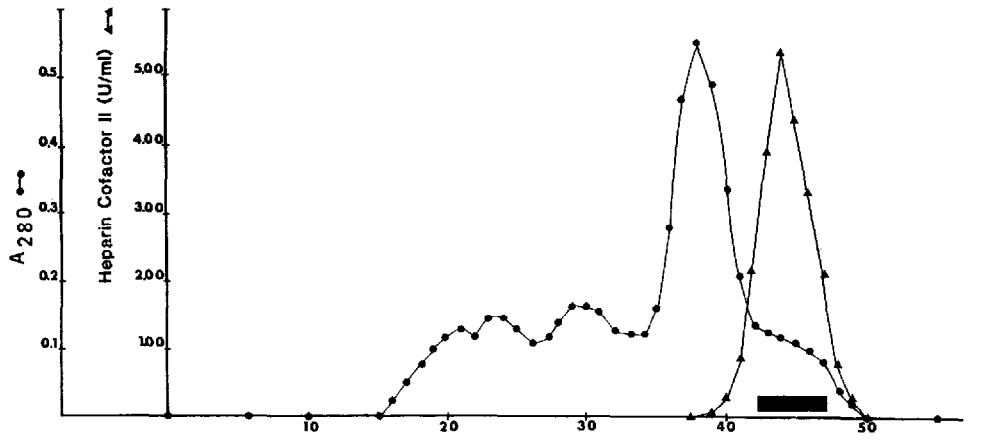

Fig. 3. Gel permeation of HCII activity on AcA 34. The proteins were chromatographed on a column (80 × 1.5 cm I.D.) equilibrated with 0.15 *M* NaCl in 0.02 *M* Tris–HCl (pH 7.4). Fractions of 1.2 ml were collected at a flow-rate of 6 ml/h. Symbols as in Fig. 1.

HCII was purified 760-fold from human plasma with an overall yield of 7.5% (Table I). The final product was homogeneous in SDS-PAGE (Fig. 6). No anti-Xa activity or immunoreactive ATIII could be detected in the protein preparation. Thus the purified HCII preparation was devoid of ATIII. HCII was eluted from the column of heparin–Sepharose (Fig. 1) with a much lower ionic strength (0.18 *M*) than ATIII, which appeared in the effluent at higher saline concentrations (0.80 *M*) (not shown).

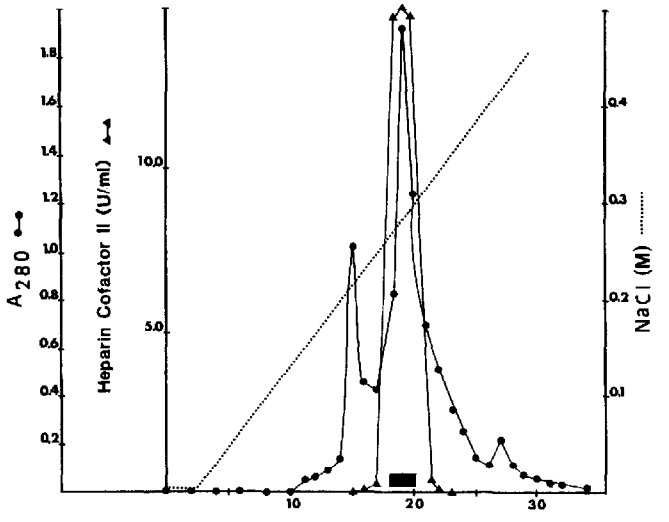

Fig. 4. Elution of HCII activity from a Mono-Q anion-exchange column. The column was eluted with a 35-ml linear gradient from 0.0 to 0.5 *M* NaCl in 0.02 *M* Tris–HCl (pH 7.4). Fractions of 1.0 ml were collected at a flow-rate of 1.0 ml/min. Symbols as in Fig. 1.

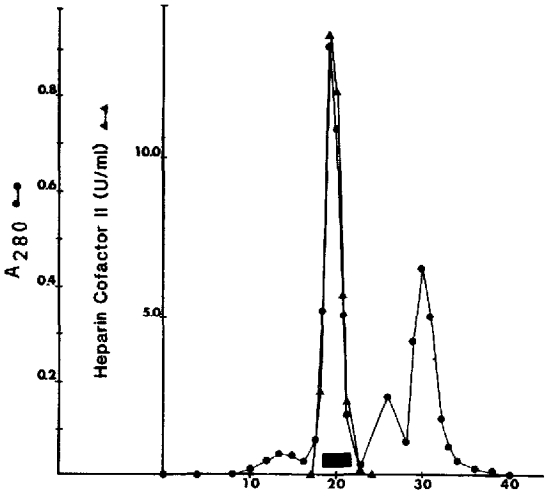

Fig. 5. Gel permeation of heparin cofactor II on a Superose column. The column was eluted with 0.3 *M* NaCl in 0.02 *M* Tris–HCl (pH 7.4). Fractions of 1.0 ml were collected at a flow-rate of 0.10 ml/min. Symbols as in Fig. 1.

Specificity of the antiserum against HCII

The antiserum was found to be monospecific, as evidenced by the single peak pattern of normal human plasma in crossed immunoelectrophoresis (CIE) as shown in Fig. 7. When purified ATIII was submitted to CIE, no immunoreactive protein could be found (not shown).

DISCUSSION

Functionally active heparin cofactor II was purified to homogeneity from human plasma with a recovery of 7.5%. The specific activity of the purified protein

TABLE I

PURIFICATION OF HEPARIN COFACTOR II FROM HUMAN PLASMA

Step	Volume (ml)	Total protein (mg)	Total HCII (U/ml)	Specific activity (U/mg)	Yield (%)	Purification factor
Starting plasma	485	26 389	418.5	0.016	100	1
Barium chloride-treated plasma	495	25 533	413.2	0.016	98.7	1
Ammonium sulphate precipitate	110	5809	176.0	0.030	42.1	1.88
Heparin–Sepharose	29.4	71.3	85.8	1.201	20.5	75.1
DEAE-Sepharose	10.5	12.8	61.5	4.805	14.6	300
AcA-34	8.4	6.8	46.0	6.765	11.0	422
Mono-Q	3	4.1	42.7	10.41	10.2	650
Superose	4	2.57	31.4	12.20	7.5	763

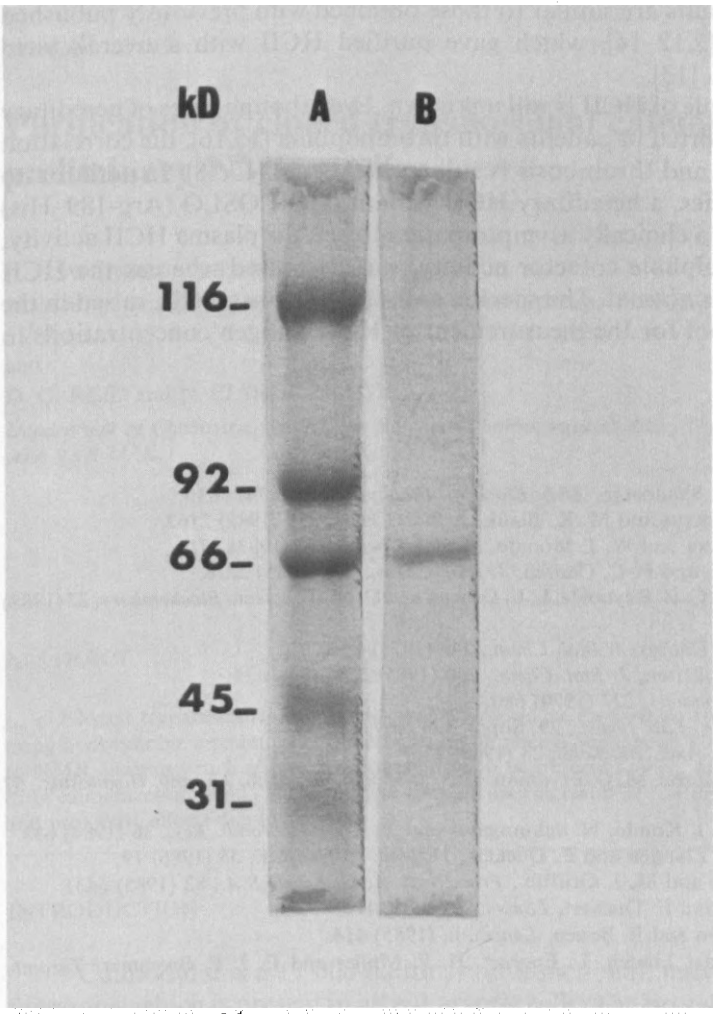

Fig. 6. SDS-PAGE of the final preparation of HCII. The final product was homogeneous and migrated with M_r 66 500 dalton (lane B). The molecular mass standards (M_r in kilodaltons) are in lane A.

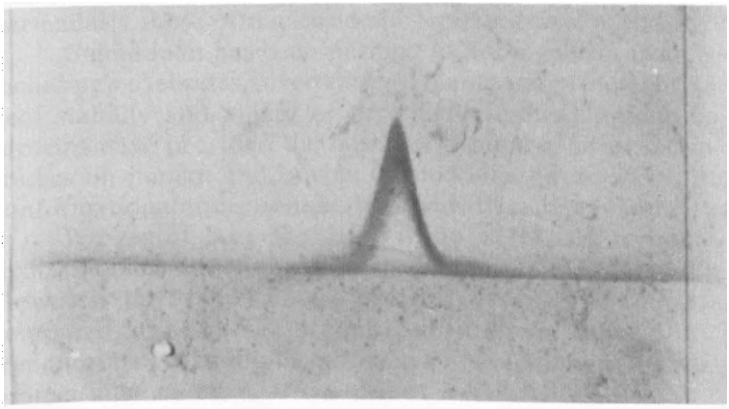

Fig. 7. Crossed immunoelectrophoresis of a plasma sample. The antiserum was used at a concentration of 1% (v/v) in the second dimension. A single peak pattern was found, suggesting the monospecificity of the antiserum.

was 12.2 U/mg. These results are similar to those obtained with previously published purification procedures [2,12–14], which gave purified HCII with a overall yield between 3% [2] and 30% [13].

The physiological role of HCII is still unknown. Even though cases of hereditary HCII deficiency were reported in patients with thrombophilia [15,16], the correlation between HCII deficiency and thrombosis is still controversial [17,18]. In addition to the quantitative deficiencies, a hereditary HCII variant, HCII OSLO (Arg-189–His) was recently found [19] in a clinically asymptomatic subject; the plasma HCII activity, measured as dermatan sulphate cofactor activity, was decreased whereas the HCII antigen concentration was normal. The specific antiserum against HCII, raised in the rabbit, can be a useful tool for the measurement of HCII antigen concentrations in clinical studies.

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