Purification from bovine blood of the warfarin-induced precursor of prothrombin

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SUMMARY

A simple and rapid method is described for the purification of the warfarin-induced precursor of prothrombin from bovine blood. The immunoglobulin fraction from a monospecific antiserum against prothrombin was coupled to Sepharose 4 B and used as an immunoadsorbent. This step is a convenient substitute for the adsorption to insoluble barium salts used in the purification of prothrombin.

INTRODUCTION

PIVKA-II is the inactive precursor of factor II which is produced by the liver in vitamin K absence or after administration of vitamin K antagonists (1). Much interest has been focused on this protein recently to reveal its structural differences from prothrombin as a clue to the mode of action of vitamin K (2 - 10). Unlike prothrombin, PIVKA-II does not adsorb onto insoluble barium salts. Hence, such adsorptions cannot be used for the purification of PIVKA-II and as a result, more time-consuming procedures with low overall yields have been described (5, 8). We report here the isolation of homogeneous bovine PIVKA-II in good yield by a quick and easy purification procedure involving immunoadsorption.

MATERIALS AND METHODS

Bovine blood was collected in plastic containers and immediately mixed with 0.12 volume of 2.85 % sodium citrate. Plasma was prepared within 2 hr and prothrombin was purified by the method of Stenn and Blout (11). The final product was electrophoretically homogeneous and used to raise an antiserum in a goat. Two injections, each of 4 mg prothrombin mixed with Freund's adjuvant (Difco, Detroit, Mich., U.S.A.), were given at multiple subcutaneous sites with a one month interval. One week after the last injection 300 ml blood was drawn from the goat.

Preparation of γ-G-immunoglobulin from antiserum

100 ml antiserum was brought to 50 % saturation with respect to ammonium sulphate and the precipitate obtained after 30 min at 4 °C was dissolved in 0.01 M sodium phosphate buffer pH 8.0. This solution was dialysed against the same buffer until free from sulphate ions. The immunoglobulin fraction was further purified by chromatography on a column of DEAE-cellulose (1.9 x 10 cm) as described by Fahey (12).

Preparation of immunoadsorbent

The immunoglobulin fraction (about 1.5 g) was dialysed against 0.2 M $NaHCO_3$ pH 9.5. Sepharose 4 B (Pharmacia, Uppsala, Sweden) (80 ml packed volume) was activated with CNBr (13), transferred to a plastic vessel containing the immunoglobulin fraction and allowed to react at 4 °C for 20 hr with gentle stirring. The gel was then

reacted with 1 M glycine for 4 hr at room temperature. Before use the gel was washed with the solutions recommended by March et al. (13).

Preparation of warfarin-plasma

Warfarin (Marevan, Nyegaard & Co., Oslo, Norway) (0.9 - 1 g) was injected into the jugular vein of a cow (450 kg). Decay in prothrombin activity was followed each day by the assay described earlier (14). After 4 days the activity had reached 50 % of normal and blood (4500 ml) was drawn from the cow into plastic containers and immediately mixed with 0.12 volume of 2.85 % sodium citrate. Normal prothrombin was removed by absorbing the plasma once with barium citrate (11) and once with barium sulphate (15). This final product is here referred to as warfarin-plasma.

Purification of PIVKA-II

Affinity chromatography. The immunoadsorbent was poured into a plastic column to form a gel bed of 1.6 x 7 cm and equilibrated with 0.05 M Tris-HCl pH 7.4 at room temperature. Warfarin-plasma was applied and the column subsequently washed with the equilibration buffer and then with 0.5 M $NaHCO_3$ before the antigen was eluted in 0.1 M sodium acetate-buffer pH 4.0 containing 4 M urea and 0.5 M NaCl. This eluate was immediately dialyzed against 0.1 M sodium phosphate-buffer pH 6.0.

DEAE-Sephadex chromatography. The dialyzed antigen-containing fraction from the affinity column was applied to a column of DEAE-Sephadex A-50 (1.6 x 11 cm)

equilibrated with 0.1 M sodium phosphate-buffer pH 6.0 and eluted with a 500 ml linear gradient of NaCl (0 to 1 M) in the same buffer.

Gel filtration. The relevant fractions from the DEAE-Sephadex column were pooled and concentrated by ammonium sulphate precipitation at 66 % saturation and dissolving the precipitate in 3 ml of distilled water. This solution was subjected to gel filtration on a column of Bio-gel P-100 (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) (1.6 x 35 cm) in 0.05 M Tris-HCl pH 7.4.

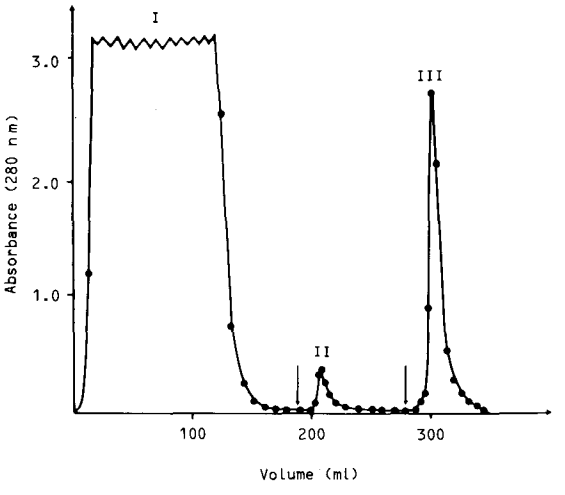

Figure 1

Chromatography of warfarin-plasma (120 ml) on an immunoadsorbent column (1.6 x 7 cm).

Stepwise elution by

I 0.05 M Tris-HCl pH 7.4

II 0.5 M $NaHCO_3$

III 0.1 M sodium acetate pH 4.0 containing 4 M urea and 0.5 M NaCl

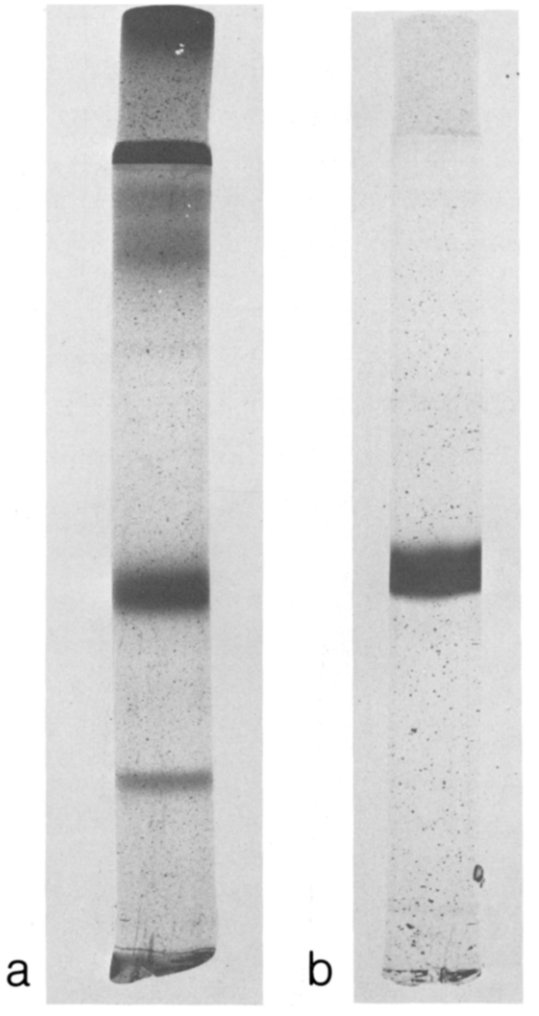

Figure 2

Polyacrylamide disc gel electrophoresis of protein from:

a) fraction III (figure 1) 30 µg

b) fraction I (figure 3) 30 µg

Immunodiffusion and immunoelectrophoresis were carried out as described by Ouchterlony (16) and Scheidegger (17) respectively. Protein was measured by the method of Lowry et al. (18). Analytical polyacrylamide

disc gel electrophoresis was carried out according to Davis (19). The activation of PIVKA-II by venom from Echis carinatus (kindly given by F. Kornalik, Institute of Patophysiology, Prague, Czeckoslovakia) was carried out as described by Nelsestuen and Suttie (8).

RESULTS AND DISCUSSION

Preparation of immunoadsorbent. The goat antiserum was monospecific in immunodiffusion and immunoelectrophoresis against prothrombin which was neutralized and precipitated. About 95 % of the purified immunoglobulin fraction obtained from this antiserum was coupled to Sepharose.

Purification of PIVKA-II

The adsorption with barium citrate and barium sulphate

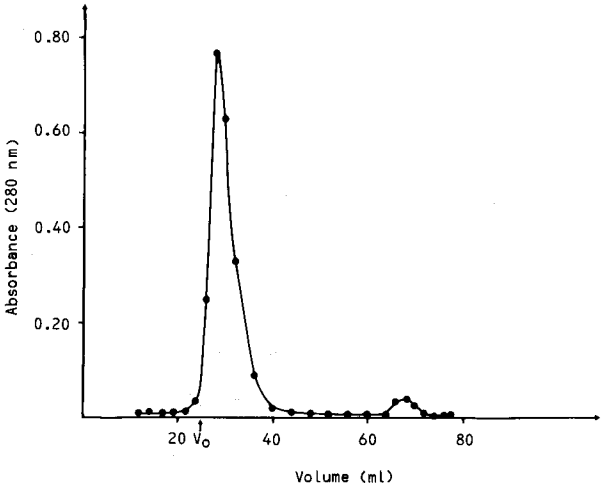

Figure 3

Chromatography of the concentrated PIVKA-II fraction from DEAE-Sephadex on Bio-gel P-100 (1.6 x 35 cm) in 0.05 M Tris-HCl pH 7.4.

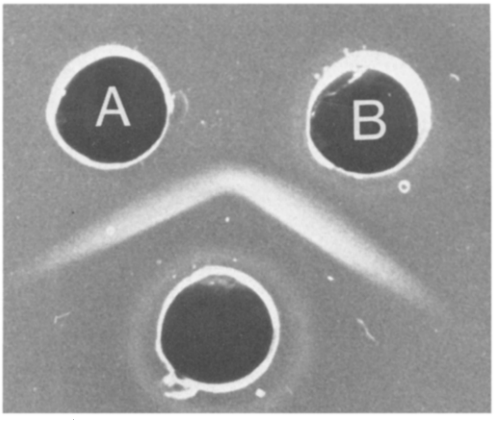

Figure 4

Immunodiffusion of purified PIVKA-II (A) and normal plasma (B) against anti-bovine prothrombin serum.

removed prothrombin completely from plasma. No factor II activity was present after this treatment. In agreement with results of others (5, 8), immunodiffusion gave a single line thus suggesting a complete immunological identity between the antigen in warfarin-plasma and that in normal plasma.

The capacity of the immunoadsorbent was such that 1 ml of coupled Sepharose removed all antigenic material from 15 ml of warfarin-plasma. The antigenic material could then be eluted with 0.1 M sodium acetate-buffer pH 4.0 containing 4 M urea and 0.5 M NaCl (Fig. 1). However, 2 M NaBr, 2 M urea containing 0.5 M NaCl or 0.1 M Tris-glycine pH 2.8 containing 1 M NaCl did not release the antigen. Since prothrombin is released from a similar column by elution with 2 M NaBr, PIVKA-II binds more tightly to the column.

Polyacrylamide disc gel electrophoresis of the protein preparation at this step showed the presence of 3 - 4 bands in addition to the band corresponding to PIVKA-II (Fig. 2 a). One of the contaminants, albumin, was removed by DEAE-Sephadex chromatography. Ammonium sulphate precipitation and gel filtration on Bio-gel P-100 (Fig. 3) resulted in an electrophoretically homogeneous protein (Fig. 2 b) which was without procoagulant activity in the ordinary factor II-test system but which gave full thrombin activity when activated by Echis carinatus venom. One precipitation line was observed in immunodiffusion against the anti-prothrombin serum. A reaction of identity was seen between this line and that given by factor II in plasma (Fig. 4). From 100 ml bovine plasma about 3 mg of PIVKA-II was obtained. Considering that the prothrombin activity of this plasma as drawn from the cow was 50 %, the yield is high compared with that obtained by other methods (5, 8).

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