PURIFICATION OF BOVINE PROTHROMBIN BY AFFINITY CHROMATOGRAPHY

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1. Introduction

Bovine prothrombin is a glycoprotein [1–3] of mol. wt about 70 000 [4–7]. Its conversion to thrombin is an event of primary importance in blood coagulation. Several procedures for the isolation of this protein have been described [6,8–12]. They all include time-consuming chromatographic steps with a final yield between 15–50%.

Affinity chromatography is a simple and rapid purification procedure. Furthermore, the molecules of interest are usually obtained in high yield with their biological activity well retained. We report here the isolation of bovine prothrombin by affinity chromatography. This method makes it possible to prepare large quantities of pure prothrombin in a short time.

2. Materials and methods

Bovine blood was collected in plastic containers and immediately mixed with 0.12 vol 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 as antigen for antibody production in a goat. Two injections each of 4 mg prothrombin mixed with Freund's adjuvant (Difco, Detroit, Mich., USA) 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.

2.1. *Purification of anti-prothrombin immunoglobulin fraction*

The goat antiserum was brought to 50% saturation with respect to ammonium sulphate and the precipitate obtained after 30 min at 4°C was dissolved in 0.05 M Tris–HCl buffer pH 7.4. This solution was dialysed against the same buffer until free from sulphate ions. Sepharose 4B (Pharmacia, Uppsala, Sweden) was activated as described by Porath et al. [13], the interstitial water removed by filtration and 2 g of activated gel immediately mixed with 9 mg prothrombin in 10 ml 0.25 M $NaHCO_3$ pH 9.0 and left at room temperature for 24 hr with gentle shaking. The gel was allowed to react with 10 ml ethanolamine in 0.25 M $NaHCO_3$, pH 9.0, washed with the same buffer, then with distilled water and finally with 0.05 M Tris–HCl pH 7.4 containing 5 mM $CaCl_2$ before packing into a column (0.5 × 4 cm). The column was equilibrated at 4°C with the last buffer. The washing fluids contained no material absorbing at 280 nm.

The immunoglobulin fraction (ammonium sulphate precipitate) in 0.05 M Tris–HCl, pH 7.4, was brought to 5 mM in $CaCl_2$ and applied to the column. The column was washed extensively with 0.05 M Tris–HCl pH 7.4 and 0.5 M $NaHCO_3$ before the immunoglobulins were eluted in 2 M NaBr containing 5 mM EDTA.

2.2. *Purification of prothrombin*

Bovine blood was collected in plastic vessels containing potassium oxalate to a final concentration of 0.01 M. The vitamin K-dependent factors were initially adsorbed to barium sulphate and eluted in 0.15 M trisodium citrate as described by Hjort [14]. The eluate was dialysed against 0.05 M Tris–HCl pH 7.4 containing 5 mM EDTA.

The fraction of anti-prothrombin immunoglobulins purified by affinity chromatography was coupled to Sepharose 4B as described above. For coupling, activated Sepharose (80 g) was mixed with 71 ml of protein solution (2.5 mg/ml). The gel was poured into

a plastic column and equilibrated at 4°C with 0.05 M Tris–HCl pH 7.4 containing 5 mM EDTA. Dialysed eluate from barium sulphate was applied to the column followed by washing with the equilibration buffer (step I) and elution with 0.5 M $NaHCO_3$ containing 5 mM EDTA (step II) and 2 M NaBr containing 5 mM EDTA (step III). Aliquots were withdrawn from the effluent fractions and tested for factor II, VII and X activity as described [15,16]. The activity of 1 ml citrated bovine plasma was chosen as one unit. The columns were reused after washing with 2 M NaBr and 0.05 M Tris–HCl pH, 7.4. Protein was measured by the method of Lowry et al. [17] with bovine immunoglobulin G (Sigma, St. Louis, Mo., USA) as standard. Analytical polyacrylamide disc gel electrophoresis was carried out according to Davis [18]. Bovine thrombin (Topostasin) was obtained from Hoffman-La Roche, Basle, Switzerland.

3. Results and discussion

3.1. *Purification of the immunoglobulins*

The antiserum was found to be monospecific to bovine prothrombin which was neutralised and precipitated. The antiserum also neutralized bovine thrombin very effectively, one ml of antiserum neutralized 5–600 NIH units of thrombin. Analytical disc gel electrophoresis of the fractions obtained from the prothrombin-Sepharose immunoadsorbent column showed that a fraction of highly purified immunoglobulins was obtained in the 2 M NaBr eluate (fig.1). With 9 mg of prothrombin coupled to the Sepharose, the immunoglobulin fraction (ammonium sulphate precipitate) was applied to the column in portions of 17–18 ml, each equivalent to about 15 ml of serum. No significant loss in adsorbent capacity was noted in more than 10 runs with the same bed material. The final yield of purified immunoglobulins was 178 mg from 164 ml antiserum.

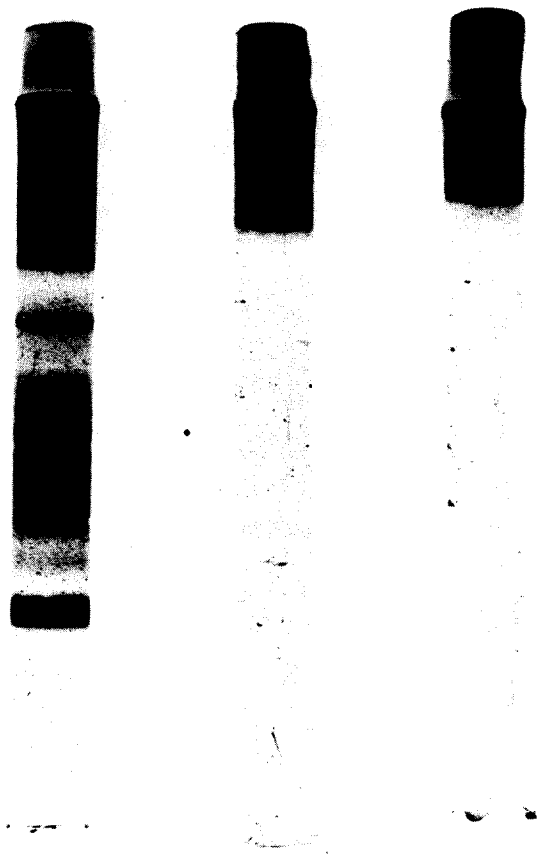

Fig.1. Analytical disc gel electrophoresis of three fractions eluted from the prothrombin-Sepharose column used for the purification of antiprothrombin immunoglobulin. Left: fraction eluted in 0.05 M Tris–HCl pH 7.4. Centre: fraction eluted in 0.5 M $NaHCO_3$. Right: fraction eluted in 2 M NaBr containing 5 mM EDTA. Protein corresponding to 0.03 A_{280} units was applied to each gel.

3.2. *Purification of prothrombin*

The specific immunoglobulins (178 mg) were coupled to 80 g of activated Sepharose. In order to avoid unspecific interaction with the immunoglobulins in the column material it is important [19] to remove the immunoglobulin fraction from plasma before submitting the plasma to immunoadsorbent chromatography. When the vitamin K-dependent factors were adsorbed to and eluted from barium sulphate, most of the immunoglobulins were removed. The eluate from barium sulphate was submitted to the immunoadsorbent column and eluted in three steps (fig.2). Factor VII and factor X were eluted in step I (the non-adsorbed fraction). Some factor II was eluted in step I and step II (0.5 M $NaHCO_3$ containing 5 mM EDTA), but the bulk of the factor II activity came in step III (2 M NaBr containing 5 mM EDTA). (Usually less factor II was found in step I and II than indicated in fig.2). Analytical disc gel electrophoresis showed a

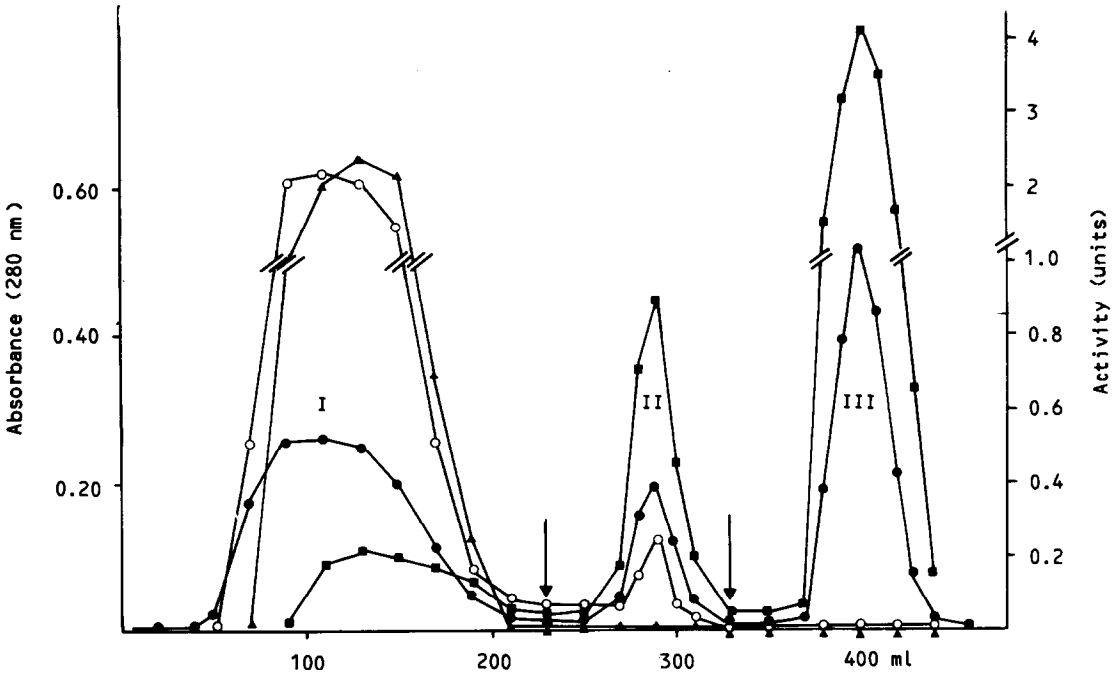

Fig.2. Affinity chromatography of barium sulphate eluate on anti-prothrombin immunoglobulin-Sepharose column. The column was eluted with 0.05 M Tris–HCl, pH 7.4, containing 5 mM EDTA (step I), 0.5 M $NaHCO_3$ (step II) and 2 M NaBr containing 5 mM EDTA (step III). Changes in elution fluid are indicated by arrows. (o——o——o) Factor X (units/ml); (▲——▲——▲) Factor VII (units/ml); (■——■——■) Factor II (units/ml); (●——●——●) Optical density 280 nm.

single band in peak III coinciding with the factor II activity (fig.3). No activity of factor VII or X was eluted in step III.

The results of a representative purification are outlined in table 1. The degree of purification is similar to that obtained by conventional methods and the recovery is better. The affinity column has been reused more than 10 times without any loss of activity.

Table 1
Purification of bovine prothrombin

Stage	Volume (ml)	Protein (mg)	Total activity (units*)	Specific activity (units*/mg)	Yield (%)
Plasma	175	13 000	175	0.0135	100
Eluate from $BaSO_4$ (after dialysis)	50	300	155	0.52	88
Eluate from affinity column	73	16	129	8.1	74

* The activity in one ml of citrated bovine plasma was taken as one unit.

Fig.3. Analytical disc gel electrophoresis of purified prothrombin (30 μg).

The present method has the additional advantages of great rapidity and simplicity and may be adapted very easily to large scale purification of prothrombin.

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References

[1] Miller, K. D. and Seegers, W. H. (1950) Arch. Biochem. Biophys. 60, 398–401.
[2] Magnusson, S. (1965) Arch. Kemi 23, 285–298.
[3] Nelsestuen, G. L. and Suttie, J. W. (1972) J. Biol. Chem. 247, 6090–6102.
[4] Harmison, C. R., Landaburu, R. H. and Seegers, W. H. (1961) J. Biol. Chem. 236, 1693–1696.
[5] Tishkoff, G. H., Williams, L. C. and Brown, D. M. (1968) J. Biol. Chem. 243, 4151–4167.
[6] Ingwall, J. S. and Scheraga, H. A. (1969) Biochemistry 8, 1860–1868.
[7] Cox, A. C. and Hanahan, D. J. (1970) Biochim. Biophys. Acta 207, 49–64.
[8] Moore, H. C., Lux, S. B., Malhotra, O. P., Bakerman, S. and Carter, J. R. (1965) Biochim. Biophys. Acta 111, 174–180.
[9] Malhotra, O. P. and Carter, I. R. (1968) Thromb. Diathes. Haemorrh. Suppl. 19, 178.
[10] Mann, K. G., Heldebrandt, C. M. and Ross, D. N. (1971) J. Biol. Chem. 246, 6106–6114.
[11] Stenn, K. S. and Blout, E. R. (1972) Biochemistry 11, 4502–4515.
[12] Bajaj, S. P. and Mann, K. G. (1973) J. Biol. Chem. 248, 7729–7741.
[13] Porath, J., Aspberg, K., Drevin, H. and Axén, R. (1973) J. Chromat. 86, 53–56.
[14] Hjort, P. F. (1957) Scand. J. Clin. Lab. Invest. Suppl. 27, pp.183.
[15] Gladhaug, A. and Prydz, H. (1970) Biochim. Biophys. Acta 215, 105–111.
[16] Prydz, H. and Gladhaug, Å. (1970) Thromb. Diathes. Haemorrh. 24, 601–602.
[17] Lowry, H. C., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[18] Davis, B. S. (1964) Ann. N. Y. Acad. Sci. 121, 404–427.
[19] Palmer, W. C. (1972) Biochim. Biophys. Acta 278, 299–304.