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SIĞIR PLAZMASINDAN ELDE EDİLEN PROTROMBİNİN SAFLAŞTIRILMASI VE HPLC İLE İNCELENMESİ

THE PURIFICATION OF PROTHROMBIN FROM BOVINE PLASMA AND ITS EVALUATION WITH HPLC

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SUMMARY

In this study, "prothrombin complex" containing the vitamin K dependent proteins were isolated from bovine plasma by barium citrate adsorption and ammonium sulphate fractionation. Prothrombin was purified from prothrombin complex by DEAE Sephadex A–50 and Heparin Sepharose column chromatography methods. Each purification step was monitored by both SDS–PAGE and isocratic HPLC.

The prothrombin purification procedure was repeated twice. From 11L of a bovine plasma pool, in the first run 880 mg and in the second run 1050 mg prothrombin was obtained. The prothrombin was found to be homogenous by SDS–PAGE, but micro heterogenity was observed by isocratic HPLC. In conslusion, the preparative HPLC could be used as a further purification method after the Heparin Sepharose chromatography step in the purification of protrombin.

ÖZET

Bu çalışmada sığır plazma havuzundan K vitaminine bağımlı plazma proteinleri önce baryum sitrat adsorbsiyonu ve amonyum sülfat fraksiyonlama yöntemi ile "protrombin kompleksi" olarak elde edildi ve bu kompleksden, DEAE–Sephadex A–50 ve Heparin Sepharose kolon kromatografisi yöntemleriyle protrombin saflaştırıldı. Elde edilen ürün SDS–PAGE ve izokratik HPLC ile incelendi.

Bu çalışma iki defa tekrarlandı, 11 L sığır plazmasından 880 ve 1050 mg protrombin elde edildi. Elde edilen protrombinin saflığı SDS–PAGE ve izokratik HPLC ile incelendiğinde, SDS–PAGE'de tek bir band görülürken HPLC'de mikro heterojenite gözlendi. İzokratik HPLC' nin protrombinin daha ileri saflaştırılmasında kullanılabileceği sonucuna varıldı.

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INTRODUCTION

Prothrombin is a glycoprotein found in plasma as the highest concentrated protein of the vitamin K dependent proteins. Its half–life is 72–96 hours in plasma. The other vitamin K dependent proteins involved in coagulation are Factors VII, IX and X. Protein C and protein S are also included in vitamin K dependent proteins but they function as anticoagulants. The other vitamin K dependent proteins which have unknown physiological functions are protein Z and protein M (1)

Vitamin K dependent proteins possess an uncommon amino acid : the gamma carboxyglutamic acid (Gla). Some of the glutamic acid (Glu) residues of newly synthesized proteins are specifically carboxylated to form Gla residues. The carboxylase enzyme responsible for the carboxylation requires vitamin K as a coenzyme. This carboxylation taking place in liver is inhibited by warfarin (2, 3).

Prothrombin was isolated form bovine plasma in 1938 by Seegers (4). Later, different investigators isolated and purified prothrombin from either bovine, human or other sources (5–20). The research topics concentrating on structural and functional relationship of proteins occupy an important place in recent studies. To perform these kinds of studies, highly purified and large quantities, in mg levels of proteins, are required. Therefore in this study isolation and purification of prothrombin was performed by ion exchange chromatography and dye–linked affinity chromatography steps and prothrombin was obtained in mg quantities. The purity of this prothrombin was tested with both isocratic HPLC and SDS–PAGE.

MATERIALS AND METHODS

Chemicals : DEAE Sephadex A–50, Heparin Sepharose CL–6B were obtained from Pharmacia; N, N, N', N' – tetramethylendiamine, mercaptoethanol, bromphenol blue, comassie brillant blue R–250, sodium dodecyl sulphate from Merck; benzamidine HCl, N, N' – methylene – bis – acrylamide from Sigma ; thrombin, Factors II, IX, X and protein C activity determination kits from Stago; fibrinogen from Kızılay Blood Center. The other chemicals were reagent grade provided by Merck.

Instruments : Juan K110 SX centrifuge, Sarsted Centrifuge, Pharmacia Frac 100 fraction collector, PY Unicam UV–VIS–NIR Phillips) spectrophotometer, Millipore Waters HPLC system (model 481 UV dedector, Model U6 K injector, Model 730 data module, Model 510 pump) were used.

Bovine plasma: Bovine blood was obtained from Küçükçekmece slaughterhouse and mixed in 1:10 ratio with 0.11 M sodium citrate having 1 g benzamidine HCl in 1 L. The citrated blood taken from each animal was brought to the laboratory in separeta plastic bottles within 1 hour. They were centrifuged in refrigerated Juan K 110 SX centrifuge at 4°C for 20 min at 3500 rpm. 11 L of platelet poor plasma (PPP) pool was obtained and further purification steps were applied immediately. All of the steps were performed at 4°C.

Methods : A prothrombin complex having all of the vitamin K dependent plasma proteins was obtained from bovine plasma by barium citrate adsorption and ammonium sulphate fractionation procedures. Afterwards, the prothrombin was purified from the prothrombin complex by the two different column chromatography steps.

1 M $BaCl_2$ was added slowly and into 11 L of the plasma at 1:10 ratio with continuous mixing. Stirring was continued for 1 hour. Centrifuged at 3800 rpm for 10 min. Precipitate was washed four times with washing solution containing 0.1 M NaCl, 0.01 M $BaCl_2$, 1 mM benzamidin HCl and 0.02 % g NaN_3 . After washing, the precipitate was suspended in 750 ml of 40 % saturated $(NH_4)_2SO_4$ and stirred gently at 4°C overnight. The precipitate obtained by centrifugation for 1 hour at 3800 rpm was discarded. Saturated $(NH_4)_2SO_4$ was added to the clear supernatant until 67 % saturation was reached. Centrifuged at 3800 rpm for 1 hour. Precipitate was dissolved in 75 ml of 0.05 M sodium phosphate buffer (pH 6.0) containing 0.2 M NaCl and 1 mM benzamidin HCl. This suspension was dialyzed overnight against 15 L of the same buffer and centrifuged at 4000 rpm for 10 min. The clear supernatant obtained was used as the prothrombin complex in further purification steps (16).

The DEAE Sephadex A–50 column chromatography of the prothrombin complex : The DEAE Sephadex A–50 column with a diameter of 3.5 cm and height of 22 cm, was equilibriated with 0.05 M

Na_2HPO_4 / NaH_2PO_4 buffer, pH 6.00, containing 0.2 M NaCl and 1 mM benzamidin HCl. 75 ml of prothrombin complex was applied to the column. The elution rate was adjusted to 1.35 ml/min, and washed with 1500 ml of the equilibriation buffer. Linear salt gradient obtained from 1 L of equilibrium buffer and 1 L of 0.05 M sodium phosphate buffer containing 0.6 M NaCl and 1 mM benzamidin HCl was applied to the column. 16 ml fractions were collected. Fractions were monitored against the equilibrium buffer at 280 nm wave length. According to protein peaks, the fractions were pooled into four groups as A, B., C and D.SDS–PAGE and HPLC analysis were made in the B pool.

The purification of prothrombin from the fraction B pool : The fractions of B peak obtained from the DEAE Sephadex A–50 column chromatography of the prothrombin complex were pooled and concentrated with Minitan S ultrafilitration system using 10,000 NMWL polysulfon membrane. This was dialyzed against 0.05 M imidazole–HCl buffer (pH 6.00) containing 2.5 mM $CaCl_2$, O.25 M NaCl and 1 mM benzamidin HCl, 38 ml of concentrated and the dialyzed pool B was applied to Heparin Sepharose CL–6B column (diameter: 1.6 cm, height : 10 cm) equilibriated with dialysis buffer and washed with 340 ml of the same buffer. The elution rate was 1 ml/min. The linear gradient prepared with 400 ml of equilibrium buffer and 400 ml of 0.05 M imidazole buffer (ph 6.00) containing 2.5 mM $CaCl_2$, 0.7 M NaCl and 1 mM benzamidin HCl, 8.42 ml fractions were collected. The fractions were monitored at 280 nm wave length. Activities were determined in each fraction. SDS–PAGE and HPLC analysis were performed in the pooled protein fractions.

High pressure liquid chromatography (HPLC) : HPLC was performed using Millipore, Waters HPLC system. Samples were dialyzed overnight against 0.05 M sodium phosphate buffer (pH : 7.00), PAK 300 protein column was used. The samples were applied with different protein concentrations and AUF's. Flux rate was 0.6 ml/min. Dialysis buffer was used for elution. The samples were run at room temperature.

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS–PAGE) : SDS–PAGE was performed according to Payne (21) to determine the molecular weights and the purity of the samples.

Protein determination : Purified protein concentrations were determined from the absorbance at 280 nm wave length. $A^{1\%}_{280}$ for prothrombin is 15.5 (16). The concentrations of the proteins in the plasma were measured by the Biuret method and the Lowry method (22) was used to measure the other protein concentrations.

Activity determinations : Two stage prothrombin time was determined according to the Ware and Seegers method (23). The basis of this method depends on conversion of prothrombin completely into thrombin with the help of tissue thromboplastin and Factor V. The thrombin is determined on the basis of fibrinogen determinations in this method.

For the other activities, namely one stage activities, of Factor II, IX, X and protein C, the Stago kits and reactives were used. The normal human plasma pool was used for calibration.

RESULTS

The protrombin complex obtained from 11 L of bovine plasma through barium citrate adsorption, ammonium sulphate fractionation and dialysis was applied to DEAE Sephadex A–50 column chromatography (fig 1). Figure 2 shows SDS–PAGE patterns of each peak of tho chromatogram. Since no commercial antisare were available against bovine prothrombin, immunological methods could not be used for prothrombin characterization. In fraction pool B, prothrombin and to a lesser extent Factor IX activities were detected. The prothrombin containing fraction pool B obtained from DEAE Sephadex A–50 column chromatography was applied to Heparin Sepharose CL–6B column for further purification (Fig 3). Prothrombin did not bind to the column, and eluted in the first peak. This prothrombin was applied to SDS–PAGE and one band was seen with a Mr of 71200 Da (fig 4, band 1). Factor IX activity was found in the 2. peak after the gradient application.

880 mg prothrombin was obtained from 11 L of bovine plasma in the first run and 1050 mg from another 11 L in the second run with a purity of giving single band with SDS–PAGE. Table I shows this purification with specific activities of prothrombin determined by two stage prothrombin.

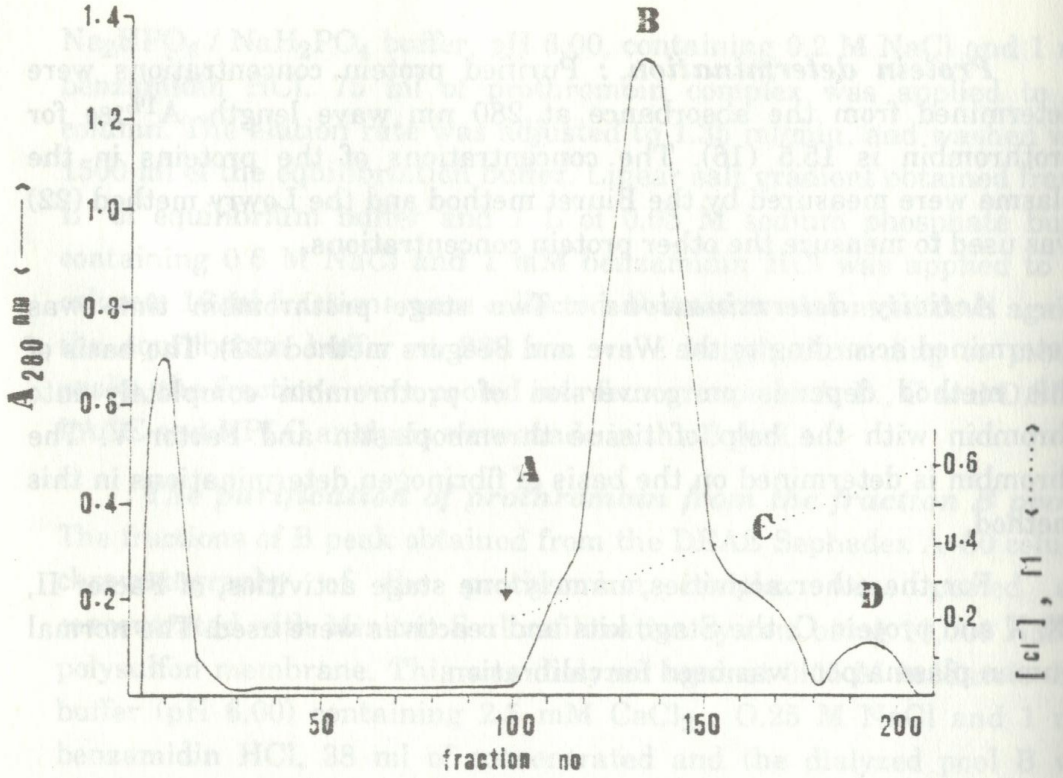

Fig. 1 : DEAE Sephadex A–50 column chromatography of prothrombin complex. Column diameter 3.5 cm; height 22 cm; flow rate: 1.35 ml/min; fraction volume: 16 ml; Equilibrium buffer : 0.2 M NaCl and 1 mM benzamidin HCl containing 0.05 M Na_2HPO_4 / NaH_2PO_4 buffer, pH 6.00; linear gradient : equilibrium buffer and 0.6 M NaCl and 1 mM benzamidin HCL containing 0.05 M Na_2HPO_4 / NaH_2PO_4 buffer, pH 6,00. The arrow shows the start of the gradient; pool A : fractions 102–119; pool B = fractions 120–150; pool C = 151–180; pool D = 181–210).

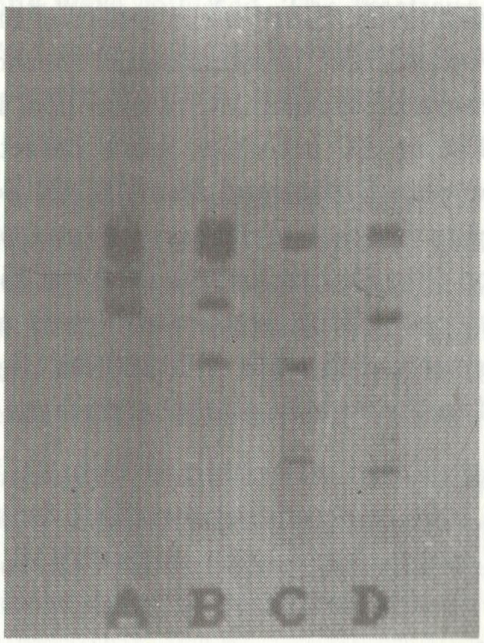

Fig. 2 : The SDS–PAGE of the fractions obtained from DEAE Sephadex A–50 column chromatography of prothrombin complex. (A: fraction pool A; B: fraction pool B ; C: fraction pool C; D: fraction pool D).

TABLE I : Purification of prothrombin. 1 ml of normal bovine plasma prothrombin activity was taken as 1 unit. The activity of prothrombin was measured by the two–stage method. (CC = Column Chromatography)

	Volume ml	Total protein.mg	Total Act. U	Sp. Act. mg	Yield %	Purification (fold)
Initial plasma	11000.0	726000	2618000	4.0	100	1
Prothrombin complex	75.0	1546	1212825	784.0	50	217
DEAE Sephadex A–50 CC	320.0	1030	1133000	1100.0	43	305
Heparin Sepharose CL–6B CC	5.0	880	1754720	1994.0	67	554

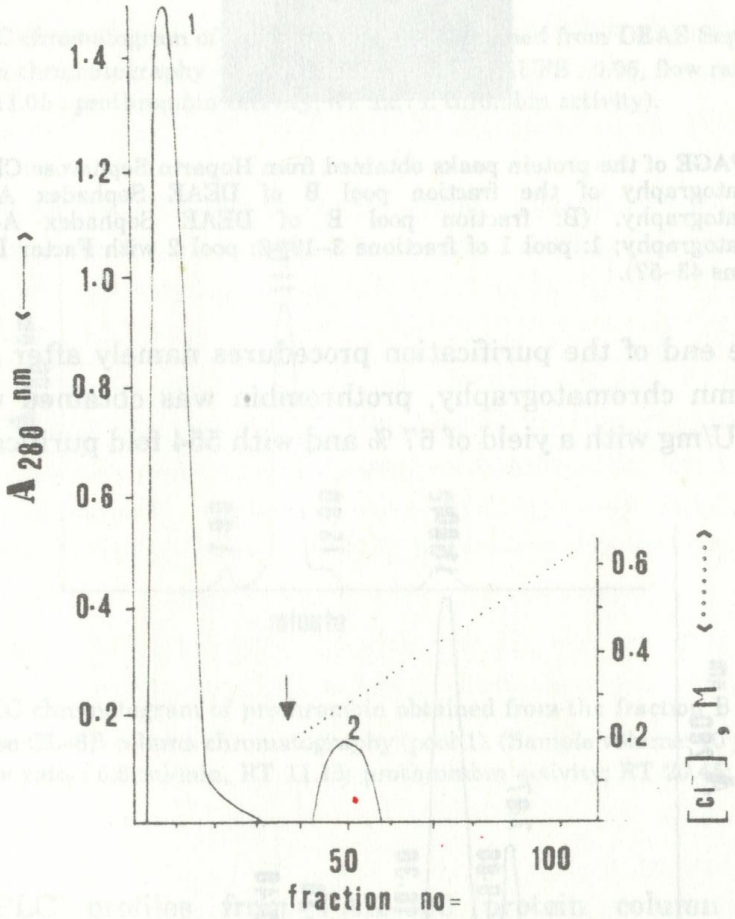

Fig. 3 : The Heparin Sepharose CL–6B column chromatography of the fraction pool B obtained from DEAE Sephadex A–50 column chromatography. (Column diameter: 1.6 cm, height: 10 cm, flow rate : 1 ml/min, fraction volume: 8.42 ml, equilibrium buffer : 2.5 mM $CaCl_2$, 0.25 M NaCl and 1 mM benzamidin HCl containing 0.05 M imidazole HCl, pH 6.00; Linear gradient: equilibrium buffer and 2.5 mM $CaCl_2$, 0.7 mM NaCl and 1 mM benzamidin HCl containing 0.05 M imidazole HCl, pH 6.00. The arrow shows the start of the gradient).

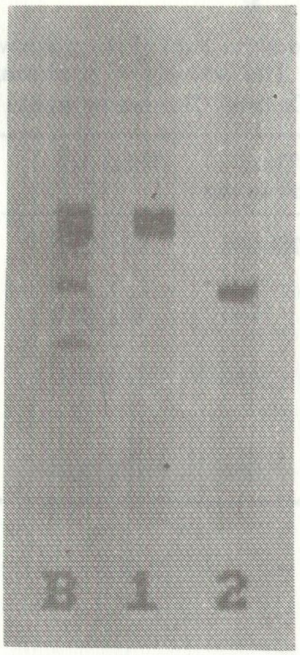

Fig. 4 : SDS–PAGE of the protein peaks obtained from Heparin Sepharose CL–6B column chromatography of the fraction pool B of DEAE Sephadex A–50 column chromatography. (B: fraction pool B of DEAE Sephadex A–50 column chromatography; 1: pool 1 of fractions 3–19; 2: pool 2 with Factor IX activity of fractions 43–57).

At the end of the purification procedures namely after Sepharose CL–6B column chromatography, prothrombin was obtained with a sp. act. of 1994 U/mg with a yield of 67 % and with 554 fold purification.

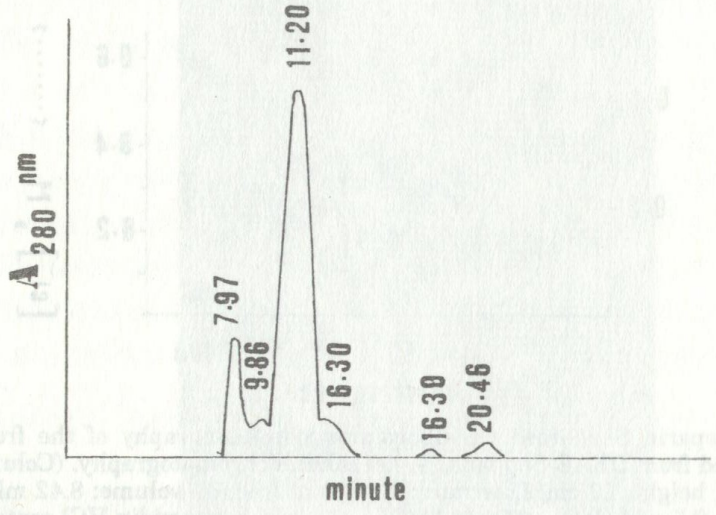

Fig. 5 : The HPLC chromatogram of prothrombin complex. (sample volume 7,5 µl, AUFS: 0.1, flow rate : 0,6 ml/min, RT 11.20: prothrombin activity; RT 12.93 : thrombin activity).

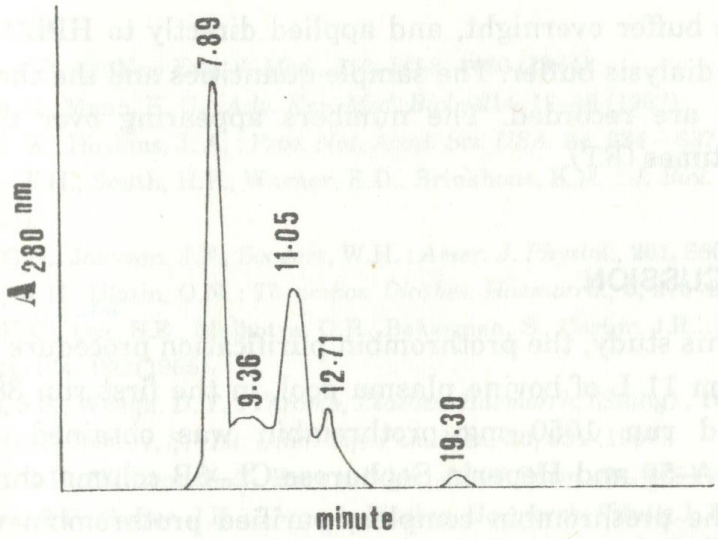

Fig. 6 : The HPLC chromatogram of the B fraction pool obtained from DEAE Sephadex A–50 column chromatography. (Sample volume : 10 µl, AUFS : 0.05, flow rate: 0,6 ml/min; RT 11.05 : prothrombin activity; RT 12.71: thrombin activity).

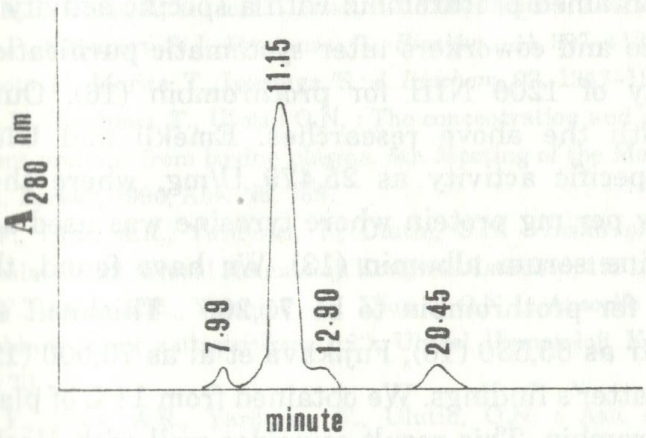

Fig. 7 : The HPLC chromatogram of prothrombin obtained from the fraction B pool after Sepharose CL–6B column chromatography (pool 1). (Sample volume: 10 µl, AUFS : 0.05, flow rate : 0,6 ml/min, RT 11.15: prothrombin activity; RT 20.45: Factor IX activity).

The HPLC profiles from PAK–300 protein column of the prothrombin complex (fig 5), protein fraction pool B of DEAE Sephadex A–50 column chromatography (fig 6), and the purified prothrombin obtained from Heparin Sepharose CL–6B column chromatography (1. protein peak, Fig 7) are shown. In HPLC runs, the samples were not treated with a reducing agent. Samples were dialyzed against 0.05 M

phosphate buffer overnight, and applied directly to HPLC. Elution was done with dialysis buffer. The sample quantities and the chromatography conditions are recorded. The numbers appearing over the peaks are retention times (RT).

DISCUSSION

In this study, the prothrombin purification procedure was repeated twice. From 11 L of bovine plasma pool, in the first run 880 mg and in the second run 1050 mg prothrombin was obtained. After DEAE Sephadex A–50 and Heparin Sepharose CL–6B column chromatography steps of the prothrombin complex, purified prothrombin was obtained with a specific activity of 1994 U/mg. Thishcoff and coworkers, after barium citrate adsorption and after two steps of cristallization, obtained prothrombin with a specific activity of 3000 IOWA U/mg (10), Malhotra and Carter after kaolin–bentonite adsorption and isoelectric precipitation, obtained prothrombin with a specific activity of 3200 U/mg (11), Hashimoto and coworkers after systematic purification obtained a specific activity of 1200 NIH for prothrombin (16). Our results are comparable with the above researches. Emekli and Ulutin reported prothrombin specific activity as 25,479 U/mg, where they calculated specific activity per mg protein where tyrosine was used as a standard instead of bovine serum albumin (13). We have found, through SDS–PAGE the Mr for prothrombin to be 71,200 . Thishkoff et al reported prothrombin Mr as 65,530 (10), Fujikava et al as 70,000 (12). Our result is close to the latter's findings. We obtained from 11 L of plasma, 880 and 1050 mg prothrombin. This result coincides well with Hashimoto et al's results where they reported a yield of 1600 mg prothrombin from 15 L of plasma.

The purified prothrombin was applied to HPLC and the results compared with SDS–PAGE results. Changing either the pH or the ionic strength caused no difference in the HPLC profiles. The electrophoresis results showed single band in SDS–PAGE but in HPLC analysis, micro heterogenity was observed after Heparin Sepharose CL–6B column chromatography. There were three more protein peaks present on the elution profiles. These results made us think that HPLC is a better method in showing the extent of purification. Also with preparative HPLC further purification of prothrombin could be established. HPLC is moreover a more efficient and less time consuming method.

REFERENCES

1. Friedman, P. A. : *New Eng. J. Med.*, **310,** 1458–1460 (1984).
2. Bovill, E. G., Mann, K. G. : *Adv. Exp. Med. Biol.*, **214,** 17–46 (1987).
3. Suttie, J. W., Hoskins, J. A. : *Proc. Nat. Acad. Sci. USA,* **84,** 634 – 637 (1987).
4. Seegers, W.H., Smith, H.P., Warner, E.D., Brinkhous, K.M. : *J. Biol. Chem.*, **123,** 751 – 754 (1938).
5. Ulutin, O.N., Johnson, J.F., Seegers, W.H. : *Amer. J. Physiol.*, **201,** 660–662 (1961).
6. Seegers, W.H., Ulutin, O.N. : *Thrombos. Diathes. Haemorrh.*, **6,** 270–281 (1961).
7. Moore, H.C., Lux, S.E., Malhotra, O.P., Bakerman, S., Carter, J.R. : *Biochim. Biophys, Acta,* **111,** 174–180 (1965).
8. Shapiro, S.S., Waugh, D. F. : *Thromb, Diathes. Haemorrh. (Stuttg)* , **16,** 469–490 (1966).
9. Ulutin, O.N., Balkuv, Ş. : *İst. Üniv. Tıp. Fak. Mec.*, **30**, 651 (1967).
10. Thiskoff, G.H., Williams, L.C., Brown, D.M. : *J.Biol.Chem.*, **24,** 4151–4167 (1968).
11. Malhotra, O.P., Carter, J.R. : *Thromb. Diathes. Haemorrh. (Stuttg.),* **19,** 178–185 (1968).
12. Fujikawa, K., Coan, M.H., Enfield, D.L., Titani, K., Ericsson, L.H., Davie, E. W. : *Proc. Nat. Acad. Sci. USA,* **71,** 427–430 (1974).
13. Emekli, N.B., Ulutin, O.N. : In Recent Progress in Blood Coagulation and Thrombosis Research. (ed. Ulutin, O.N.) *Bibliotheca Haematologica,* **44,** 15–20, S. Karger, Basel (1978).
14. Rabiet, M.J., Elion, J., Labie, D., Josso, F. : *FEBS Letters,* **108,** 287–291 (1979).
15. Bajaj, S.P., Rapaport, S. I., Prodanos, C. : *Biochem.*, **11,** 397–412 (1981).
16. Hashimoto, N., Morita, T., Iwanaga, S.: *J. Biochem,* **97,** 1347–1355 (1985).
17. Uras, A.R., Yardımcı, T., Ulutin O.N. : The concentration and purification of vitamin K dependent proteins from bovine plasma. 5th Meeting of the Mediterranean Blood Club, Antalya, Turkey, 1990, Abs. No. 169.
18. Uras, F., Uras, A.R., Yardımcı, T., Ulutin, O.N. : İnsan plazmasından protrobinin saflaştarılması. 22. Ulusal Hematoloji Kongresi. İstanbul, 1991, Abs. No 66.
19. Uras, F., Aras, A.R., Yardımcı T., Ulutin, O.N. : Absorbe edilmiş asit sıvısından protrombinin kısmi saflaştırılması. 22. Ulusal Hematoloji Kongresi, İstanbul, 1991, Abs. No 70.
20. Uras, F., Uras, A.R., Yardımcı, T., Ulutin, O.N. : Asit sıvısından protrombinin saflaştırılması. 22.Ulusal Hematoloji Kongresi. İstanbul, 1991, Abs. No 71.
21. Payne, J.W. : Electrophoresis of proteins on SDS Gels. In Chromatographic and Electrophoretic Techniques. (ed. Smith, I.) Vol II. Heinemann, London (Fourth edition), 321–332, 1976.
22. Daughhadey, W.H., Lowry, O.H., Rosebrough, N.S., Fields, W.S.: Determination of (protein in cerebrospinal fluid by the Folin–Lowry method. *J.Lab., Clin. Med.,* **39**) 663, 1952, In Clinical Chemistry. Principles and Techniques. (eds. Henry, R.F., Canon, D.C., Winkelman, J.W.) Hagerstown, Maryland, 425–428, 1974.
23. Ware, A.G., Seegers, W.H. : *Amer. J. Clin. Invest.*, **19,** 471–482 (1949).

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