PURIFICATION AND CRYSTALLIZATION OF HORSE PROTHROMBIN*

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Horse prothrombin is unique for its ease of purification in high yield and for crystallization. Methods include two absorptions on barium citrate, isoelectric precipitation, and chromatography. Crystallization is readily achieved on stirring the lyophilized, salt-free protein with cold chloroform: methanol and from concentrated aqueous solution by conventional technics.

MATERIALS AND METHODS

Purification -- Horse blood (17 parts) is added to 1 part of 0.165 M sodium citrate. Plasma is obtained after centrifugation or, for large volumes, by siphoning after 4-24 hours' settling of the red cells at 3°C.

The citrated plasma is cooled to 2-5°C and cold 1.0 M $BaCl_2$ is added dropwise with stirring to 0.10 M. After 10 minutes' additional stirring, the barium citrate precipitate containing the prothrombin is recovered by centrifugation. The precipitate is gently stirred with cold water (2-5°C) in a Waring Blendor maintained at low speed by a Variac. The suspension is then diluted to one-half the original plasma volume with cold water and stirred, while dry Amberlite IRF-97 (Na^+) (Rohm and Haas, Philadelphia; formerly called XE-64) is added in increments. Usually 3-15 g of resin

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per 100 ml of suspension (amount dependent on the Na^+ equivalent of the resin) are adequate and degradation is complete in 10 minutes. The end point for barium citrate dissolution is clearing of the supernatant on standing 1-2 minutes. The pH of the clear supernatant should be in the range 7.0-9.0. The resin is removed by filtration on fluted Schleicher and Schuell No. 520-B-1/2 papers, or their equivalent.

The filtrate, which contains about 70 per cent of the original prothrombin, is again made 0.1 M with respect to $BaCl_2$ by dropwise addition of a 1.0 M solution. After 10 minutes' stirring at 2-5°C the barium citrate is recovered by centrifugation, blended gently with cold water, and again diluted with water to one-half the original plasma volume. Dissolution of the barium citrate is carried out as described above. Ordinarily half the amount of IRF-97 (Na^+) used to dissolve the first barium citrate absorbent is sufficient to solubilize this second suspension. The resin is again removed by filtration through fluted Schleicher and Schuell No. 520-B-1/2 papers, or their equivalent.

The second barium citrate eluate should contain 60-65 per cent of the original plasma prothrombin. It is adjusted to pH 4.7-4.9 by dropwise addition of 0.5 N HCl with stirring at 3-5°C. After standing 30 minutes at 2-5°C the prothrombin precipitate is recovered by centrifugation at 1200 X g for 20 minutes. The precipitate is suspended in cold water and stirred with either 0.1 N NaOH or dry IRF-97 (Na^+) sufficient to raise the pH to 6.0-6.5. Fifty to sixty per cent of the original horse plasma prothrombin should be recovered in this final product. The zymogen can be crystallized at this point, or chromatographed and then crystallized.

Crystallization -- The prothrombin is dialyzed against at least 8 changes of distilled water, each water volume being at least 150 times that of the prothrombin solution. After lyophilization, the dry protein is suspended in cold (-10°C) chloroform:methanol (2:1). A "sheen" appears in 5-10 minutes of stirring, the suspension composed predominantly of

microscopic needles. An equal volume of cold (-10°C) ethanol is added and the crystals are harvested by centrifugation in glass tubes or on a sintered glass or paper filter. Residual chloroform is removed by 3 washes with cold (-10°C) ethanol and the crystals are dried in vacuo. Cold n-butanol can substitute for the chloroform:methanol.

Crystallization is also accomplished by reducing a 1-per-cent aqueous solution of the prothrombin (at pH 6.0-7.0) to 1/5 to 1/10 its original volume by partial lyophilization. The lyophilization flask and contents are then placed at 2-5°C before the remaining frozen material thaws completely, and seed crystals are added. Further crystallization occurs overnight (Fig. 1).

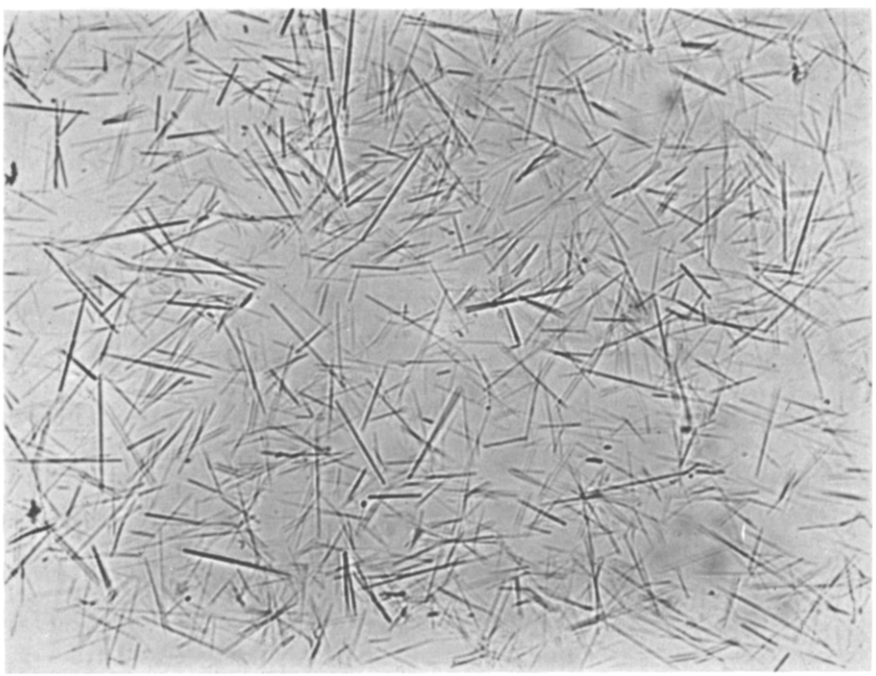

Figure 1.

Chromatography -- Before crystallization, the horse prothrombin can be purified either by chromatography on 100- to 190-cm columns of Sephadex G-200 (63-88 mesh) developed with 0.10 M borate buffer, pH 8.0, at 3°C or by

chromatography on 60-cm columns of Amberlite IR-50 (200-400 mesh) equilibrated and developed with 0.1 M phosphate buffer, pH 5.9-6.0, at 3°C.

Human plasma fractions I, II, III, IV-1, IV-4, and V were prepared by Method 6 of Cohn et al. (1946).

RESULTS AND DISCUSSION

A number of procedures for purifying various species of prothrombin are based on their relatively specific absorption on insoluble barium compounds (Fantl and Nance, 1948; Surgenor et al., 1951; Duckert et al., 1953; Goldstein et al., 1959; Lanchantin et al., 1963). The ingenious method of Lewis and Ware (1953) for absorption of prothrombin on barium citrate in excess barium chloride is fundamental to the procedures described. The initial plasma citrate concentration, about 0.015 M, gives maximum purification without sacrificing yield. The weak carboxylic exchanger, IRF-97, in sodium cycle not only decomposes the barium citrate but also absorbs thrombin-like impurities from the prothrombin and serves as a filter aid. Table I contains yields and specific activities from representative preparations. An occasional preparation will fail for unexplained reasons, usually at the pH 4.7 precipitation step. Also, yields from IRC-50 chromatography are variable and purification is sometimes poor.

Horse prothrombin is remarkably susceptible to purification by these methods; other species require substantial modifications to produce the same high yields. Yields from horse plasma are routinely greater than 50 per cent, while 40-per-cent yields of cow prothrombin are achieved only by ethanol addition at the pH 4.7-4.9 precipitation step. The species differences in solubility characteristics are attributed to marked variations in molecular properties.

Human prothrombin in lyophilized Cohn fraction III was stable to cold chloroform:methanol (2:1) treatment, while impurities and elements of the plasminogen system were denatured (Miller and Copeland, 1965). During similar treatment of purified, lyophilized horse prothrombin, the unusual

Table I

Horse Prothrombin Yields and Activities

Purification Step	No. Preparations	Yield	Specific Activity[a]
1st Ba cit. eluate	10	70-98[b]	210-400
2nd Ba cit. eluate	10	60-85[b]	430-630
After pH 4.7 pptn.	10	50-80[b]	560-900
After IR-50 chromatography	10	45-80[c]	660-1275
After G-200 chromatography	3	90-100[c]	1000-1200
After $CHCl_3$:MeOH crystallization	3	85-99[c]	800-1100
Recrystallization from water	2	57, 85[c]	1100, 1300
Super. after partial solution of crystals	1	39[c]	1300
Residue after partial solution of crystals	1	61[c]	1300

[a] Iowa units per mg. protein.

[b] Percent of starting plasma prothrombin.

[c] Percent of aliquot chromatographed or crystallized.

crystallization phenomenon was first noted. None of the various lyophilized Cohn fractions formed crystals under similar conditions. The prothrombin crystals (Fig. 1) probably form on concentration either during lyophilization or in residual water on stirring with the cold chloroform:methanol. The primary role of concentration is supported by the recrystallization from aqueous solutions above 5 per cent.

Evidence that the crystals are prothrombin is provided by constant specific activity on recrystallization from aqueous medium and on partial and complete solution of the crystals (Table I). The active material from

G-200 or ion-exchange chromatography also produces the same crystal form. Disc electrophoresis of the G-200-chromatographed, crystallized material by the procedure of Hjertén et al. (1965) employing 0.07 M Tris buffer, pH 8.6, demonstrated a sharp major band but with a persistent, slowly migrating contaminant comprising less than 1 per cent of the total protein stained. The nature of this contaminant and its removal are being studied.

A crystalline barium complex of cow prothrombin was recently reported by Tishkoff et al. (1966). The horse zymogen crystals described herein differ in form from those obtained by controlled addition of barium ion.

These horse prothrombin preparations stored at pH 6.0-6.5 at -20°C are stable for at least 18 months, as are lyophilized preparations. No thrombin formed during 10-day storage at 5°C and pH 7.7.

REFERENCES

Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., J. Am. Chem. Soc., 68, 459 (1946).
Duckert, F., Koller, F., and Matter, M., Proc. Soc. Exp. Biol. Med., 82, 259 (1953).
Fantl, P., and Nance, M. H., Med. J. Australia, 1, 128 (1948).
Goldstein, R., Le Bolloc'h, A., Alexander, B., and Zonderman, E., J. Biol. Chem., 234, 2857 (1959).
Hjertén, S., Jerstedt, S., and Tiselius, A., Anal. Biochem., 11, 219 (1965).
Lanchantin, G. F., Friedmann, J. A., DeGroot, J., and Mehl, J. W., J. Biol. Chem., 238, 238 (1963).
Lewis, M. L., and Ware, A. G., Proc. Soc. Exp. Biol. Med., 84, 636 (1953).
Miller, K. D., and Copeland, W. H., Exp. Mol. Pathol., 4, 431 (1965).
Surgenor, D. M., Alexander, B., Goldstein, R., and Schmid, K., J. Phys. Colloid Chem., 55, 94 (1951).
Tishkoff, G. H., Williams, L. M., and Jacobstein, J., Federation Proc. 25, 318 (1966).