BIOCHEMISTRY

Rivanol, Resin and the Isolation of Thrombins

Human, cow, and horse thrombins were quickly fractionated from citrate- or bio-activated prothrombin preparations by precipitation of residual prothrombin and inert protein(s) with the cationic dye 6,9-diamino-2-ethoxyacridine lactate (often referred to as rivanol, available under the trade name 'Ethodin' from the Winthrop Laboratories, New York, New York). This is a general procedure previously applied in purifying γ-globulin[1], β1-metal-combining globulin[2], and cæruloplasmin[3]. A more efficient yet rapid fractionation, however, was achieved by the adsorption of preparations containing thrombin on filter cakes or short columns of '*IRC*-50' ('*XE*-64-Rivanol'), this resin form being prepared by stirring '*XE*-64-Na^+' with an excess of rivanol. After the impurities, including other rivanol-soluble proteins, were washed off, the thrombins were eluted with 0·15 *M* calcium chloride. The thrombins were recovered from all eluates by acetone precipitation.

As thrombin sources, prothrombin preparations from the plasmas of various species[4] were activated both autocatalytically[5] and with bioactivators prepared by special treatment of acetone powders of brain tissue from the species to be studied. Five hundred mgm. of the brain powders[6] were first incubated with 10·0 ml. of serum from the same species for 30 min. at 25° C. They were then washed twice with 0·15 *M* magnesium chloride and finally suspended in 10·0 ml. of 0·15 *M* calcium chloride. Within 30 min. one volume of the suspensions completely activated 5 vol. of prothrombin solutions containing about 4,000–5,000 units/ml. After activation the thromboplastin was removed by high-speed centrifugation and the proteins were precipitated with cold acetone.

The batch purification of these thrombin preparations by precipitation of impurities with rivanol was studied in various solutions. Maximum purification was obtained over a broad range of conditions. Rivanol concentrations of 0·1–1·0 per cent, ionic strengths of 0·1 to 0·5, and *p*H's between 7·0 and 9·5 did not affect the degree of purification. Anion type did not influence the fractionation. The only important variable was the protein concentration. Quantitative recoveries of thrombin were obtained when the protein concentrations were held in the range of 1–5 mgm./ml., whereas some losses occurred with more dilute or concentrated solutions. The results in Table 1 demonstrate changes in the specific activities of some citrate- and bio-activated horse, cow, and human prothrombins brought about by the batch precipitation of impurities with rivanol. These fractionations were carried out at 0° C. in 0·05 *M* potassium citrate, *p*H 8·5, which was 0·3 per cent with respect to rivanol. After centrifugation to remove the insoluble impurities, the thrombins were precipitated by adding two volumes of cold (−10° C.) acetone and the precipitates were dissolved in dilute magnesium chloride solutions. The inert proteins were recovered from the dye-protein precipitate by dissolving the material in 0·5 *M* trisodium citrate and then adsorbing the rivanol on '*XE*-64-Na^+'.

Table 1. Purification of Thrombins with Rivanol and '*XE*-64-Rivanol'

Method	Species	Type activation	Specific Activity*	
			Before purification	After purification
Batch Rivanol	Horse	Autocat-	12,900	28,500
	Horse	Bio-	10,700	19,400
	Cow	Autocat-	17,700	28,000
	Human	Bio-	6,900	12,100
XE-64-Rivanol	Horse	Bio-	8,200	30,800
	Cow	Bio-	5,100	61,100
	Human	Bio-	6,900	42,000

* 'Iowa' units/mgm. tyrosine (Folin-Ciocalteau).

Subsequent to these preliminary batch experiments, a highly efficient and rapid fractionation technique was devised by superimposing the rivanol precipitation phenomenon on the very effective ion exchange chromatography of thrombin first accomplished by Rasmussen[7]. Up to 12-fold purifications were achieved. '*XE*-64-Rivanol' was washed repeatedly with 0·1 *M* sodium acetate. Filter cakes or columns of the resin 5–7 cm. high were prepared and the thrombin preparations were applied and washed with 0·1 *M* sodium acetate until the effluents were protein-free. The thrombins were then eluted with 0·15 *M* calcium chloride, and the effluent fractions were collected with an automatic device. Only 2–3 hr. were required for the entire procedure, including activation of the prothrombin, separation of the thrombin on '*XE*-64-Rivanol', and, finally, precipitation of the thrombin fraction with acetone. Data indicating the degree of purification of several species of thrombin by this method appear in Table 1. Samples of the horse thrombin containing 8,200 units/mgm. tyrosine, which in quantitative yield was purified to 30,800 units/mgm. tyrosine on the '*XE*-64-Rivanol' column, were also purified independently by the batch rivanol treatment and by ion exchange chromatography[7]. The batch technique gave an increase in specific activity to only 16,800 units/mgm. tyrosine, while chromatography produced a product with only 22,800 units/mgm. tyrosine. To emphasize the great efficiency of the purification method, the activated prothrombin preparations selected for this study were purposely of a very low activity compared to their theoretical maxima[4]. However, except for its rapidity, the author doubts that the '*XE*-64-Rivanol' technique is superior to ion exchange chromatography in the isolation of thrombin prepared from completely activated, homogeneous prothrombin preparations[8]. Preliminary studies indicate that the horse and cow thrombins isolated using short '*XE*-64-Rivanol' columns approach homogeneity. If this should be true, it is noteworthy that a twofold difference in specific activities exists between the isolated thrombins of the two species just as between the respective prothrombins[4].

The '*XE*-64-Rivanol' fractionation method is being applied to other protein mixtures, and the several factors governing the separation process are under study.

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