Purification of thrombin by chromatography

In a previous paper[1] it was shown that testicular hyaluronidase may be successfully chromatographed on a column of Amberlite IRC-50. Although the isoelectric range of purified bovine thrombin preparations has been found to be about pH 4.1–4.7[2], thrombin is adsorbed on glass[3] as is hyaluronidase[4]. For this reason, chromatography of thrombin on the cation exchanger Amberlite IRC-50 was also attempted.

The thrombin used was a bovine, freeze-dried preparation from Leo Pharmaceutical Products. Thrombin activity was measured at 38° C as follows: To 1.0 ml of prewarmed 0.1 % fibrinogen in 0.1 *M* sodium phosphate buffer at pH 6.8 was added from 0.095 to zero ml of the same phosphate buffer plus from 0.005 to 0.1 ml thrombin solution to give a final volume of 1.1 ml. The tube was shaken and immediately replaced in the water bath; it was then rotated quickly by hand and the time in seconds noted until fibrin thread formation occurred. A standard curve with known amounts of thrombin, 10–150 γ, as abscissa and the corresponding coagulation times in seconds as ordinate was prepared each day determinations took place.

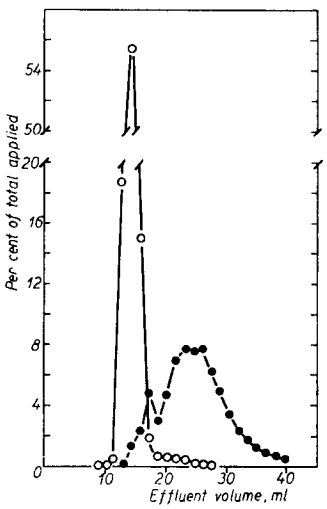

Fig. 1. Chromatography of 30 mg thrombin preparation on a 0.9 × 38 cm column of IRC-50. Eluant: 0.3 *M* sodium phosphate buffer, pH 7.42. Volume of fractions 1.5 ml. ○-protein content of fractions in % of total applied. ●-thrombin content in % of total activity.

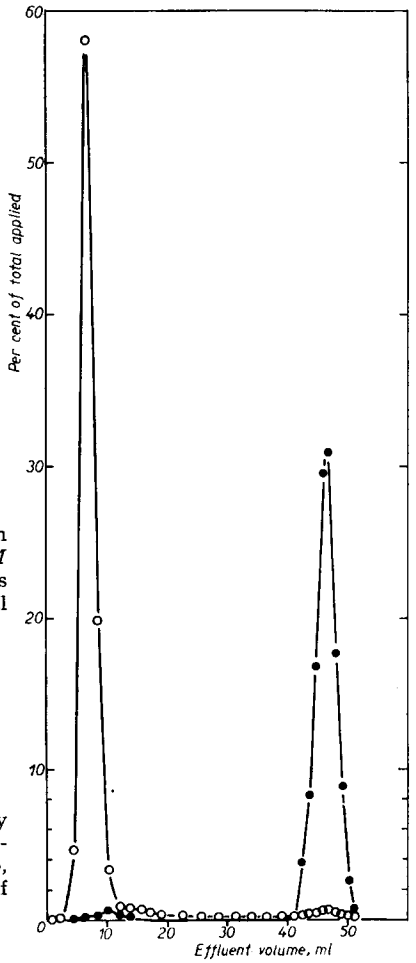

Fig. 2. Purification of thrombin (100 mg applied) by adsorption on a 0.9 × 15 cm column of IRC-50 conditioned with 0.05 *M* sodium phosphate buffer of pH 7.0, and elution with 0.3 *M* sodium phosphate buffer of pH 8. ○-protein; ●-thrombin.

Amberlite IRC-50 of about 250 mesh was treated successively with 2 *N* HCl, water, 2 *N* NaOH, water, and thereafter with primary sodium phosphate of the same molarity as that for the experiment until the pH of the supernatant was at the desired value. The resin was then treated with the appropriate buffer.

Fig. 1 shows an experiment with a 0.9 × 38 cm column at room temperature and under circumstances (0.3 *M* sodium phosphate buffer, pH 7.42) where the adsorption of thrombin was found

reversible. 30 mg of the thrombin preparation was dissolved in *ca.* 0.5 ml of the buffer and applied on the column. Buffer was run through at a flow rate of 3 ml per hour and fractions were collected in 1.5 ml increments. The protein levels were measured by absorption in the ultraviolet at 280 mμ. Both the protein content and the thrombin activity of each fraction in Fig. 1 is given in per cent of total amount applied.

It is seen from Fig. 1 that most of the protein passes directly through the column in a narrow band at about 14 ml of effluent. The thrombin activity eluted more slowly, attaining its peak at about the 25th ml. The activity per fraction in the peak region was 7.8% of the total whereas the protein was only about 0.2% of the total thus giving a 40-fold purification. Although the thrombin peak is not very sharp, most of the eluted activity was found between the 15th and 35th ml of effluent. Only 68% of the activity and 94% of the protein applied could be accounted for in the effluent. It was found, however, that purified thrombin is adsorbed on glass at this buffer concentration and pH. Elution from glass could be effected by 1 *M* secondary potassium phosphate. The yield of only 68% in this case is probably to some extent a result of adsorption on the glass tubes.

Other experiments may be summarized as follows: With sodium phosphate buffers 0.1 *M* pH 6.0, 0.2 *M* pH 6.8, 0.2 *M* pH 7.25 and 0.2 *M* pH 7.5 no thrombin activity escaped from the columns. At 0.25 *M* pH 7.55 and 0.3 *M* pH 7.2 elution of thrombin took place, but so slowly that the activity curve was much more flat than in Fig. 1.

For purification of thrombin on a more preparative scale, adsorption at a low ionic strength and elution at a higher one is useful. Fig. 2 illustrates this possibility. A 0.9 × 15 cm column of IRC-50 conditioned with 0.05 *M* sodium phosphate buffer of pH 7.0 was used. 100 mg of thrombin preparation in *ca.* 2 ml of this buffer was applied. When the solution had run into the column 0.3 *M* sodium phosphate buffer of pH 8.0 was run through at a flow rate of 7.2 ml per hour. Later at 40–50 ml effluent the flow rate had decreased to 4.4 ml per hour. Four fractions were collected per hour. Again, most of the protein passed immediately through the column; a very small amount of thrombin activity was contained in this peak. Between the 41st and 51st ml of effluent the main thrombin activity was eluted; these fractions showed very little protein present. The pH was found here to increase slowly from the initial value of 7. The most active fractions showed a 50-fold purification. Yield with respect to thrombin activity was 120%; 95% of the protein could be accounted for by absorption at 280 mμ. In very similar experiments activity yields of 97 and 115% have been found. A yield of more than 100% activity might be due to removal of an inhibitor, but mixing of samples from the large protein peak with purified thrombin did not inhibit the activity of the latter. It is more likely that a yield of more than 100% is due to experimental error. Only 0.005 ml of the most active fractions were used in the determination and the coagulation time was but 11–12 seconds. A difference of 1 sec in this range corresponds to about 12% difference in activity; duplicate determinations, however, gave identical results. The very small volume was preferred instead of a larger one from a diluted sample, because of the activity loss which occurs on dilution. Dilution with stabilizing agents such as glycerol, carbohydrates and the like have not been tried.

During dialysis and freeze drying of the purified fractions, containing less than 0.5 mg of protein per ml, much of the activity was lost. Most of the loss is probably due to adsorption upon the cellophane membrane and the glass. Some of the lost activity could be eluted from the dialysis membrane by 1 *M* secondary potassium phosphate.

The conditions used in the adsorption–elution experiment of Fig. 2 have up to now given the greatest purification and concentration of the thrombin. When adsorption took place at pH 6, other conditions being the same, more of the inert protein was adsorbed which then eluted with the thrombin activity, yielding only about a 15-fold purification. Adsorption at pH 6.6 resulted in a 30-fold purification.

The finding that thrombin can be chromatographed on Amberlite IRC-50 poses the question whether thrombin is really an "acid" protein. If this is so, then not only some basic and neutral proteins but also certain acid proteins may be chromatographed on IRC-50.

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