A simple chromatographic procedure for enhancing the purity of commercial preparations of crystalline trypsin

It has been reported[1-5] that commercial preparations of crystalline trypsin may be frequently contaminated with appreciable amounts (25 % to 50 %) of inactive protein which is presumably produced during the activation of trypsinogen[2] or as a consequence of autolysis[6]. The purity of such preparations may be increased by chromatography on carboxymethyl-cellulose[3,4,7], but, because of the pK ($\sim$4.5) of this cellulose derivative[8], maximum resolution is achieved only at pH's which are not optimal for the stability of trypsin[9]. Trypsin has also been purified by chromatography on Amberlite IRC-50, but in this case 8 M urea must be used to stabilize the enzyme[3]. In this note we wish to report a simple chromatographic procedure for enhancing the purity of trypsin which involves the use of sulfoethyl (SE)-Sephadex. Because of its low pK[8] ($\sim$ 0.5), SE-Sephadex is fully dissociated and hence can display maximum exchange capacity in a pH range (pH 2 to 3) where the autolysis of trypsin is negligible[9].

A column (2.6 × 50 cm) of SE-Sephadex G-25 (Pharmacia, Piscataway, N.J.) was equilibrated with 2.5 × 10^{-3} M HCl–0.05 M $CaCl_2$, pH 2.6. One hundred mg of crystalline bovine trypsin (2 × crystallized, Worthington, lot TRL 6256, or Novo Industri A/S, lot 41019) were dissolved in 5 ml of the same solution and applied to the column. A gradient involving an increase in salt concentration at a constant pH was produced by introducing the same starting solution containing 0.5 M NaCl (the pH being readjusted to 2.6 if necessary) into a closed mixing chamber containing 250 ml of the starting solution. Ten ml fractions were collected at a flow rate of 35 ml/h at 4°. The effluent was monitored for protein by measuring the absorbance at 280 mμ. Aliquots of each tube were assayed for tryptic activity using the substrate benzoyl-L-arginine ethyl ester (BAEE)[10], and activity was expressed in terms of BAEE units where 1 BAEE unit equals an absorbance change of 0.001/min at 253 mμ at 25°.

Fig. 1 (A and B) shows the chromatographic patterns obtained with the Worthington and Novo preparations of crystalline trypsin. In both cases an inactive peak was well separated from an active peak, although the relative proportions of these two peaks were quite different. With the Worthington enzyme the inactive peak comprised about one-third of the total protein, whereas only 10 % of the protein of the Novo preparation was inactive. Tubes corresponding to the active peaks were pooled, dialyzed against 10^{-3} M HCl, and lyophilized. A portion (20 mg) of this preparation was rechromatographed on SE-Sephadex in the same manner as before (Fig. 1, C and D). In addition to measuring trypsin activity on BAEE[10], the percent purity of this preparation was calculated on the basis of the specific titration of the active site of trypsin using p-nitrophenyl N^2-benzyloxycarbonyl-L-lysinate[11].

From the data of Table I it is evident that as a consequence of the removal of inert protein, the specific activity of Worthington trypsin increased from 13 000 to 23 000 BAEE units/mg. This activity was only slightly higher than that of the original unchromatographed sample of Novo trypsin which had an activity of 22 000 BAEE units/mg. Chromatographic removal of the 10 % inert component of Novo trypsin resulted in only a slight increase in activity. It should be noted that rechromatography of the active peaks of both trypsins resulted in the further separation of

inert protein, 6% and 13% for Worthington and Novo enzymes respectively. This inactive material may represent inert protein still remaining in the active fraction and/or that which has been engendered by subsequent manipulations of the active fraction (dialysis, lyophilization, and chromatography).

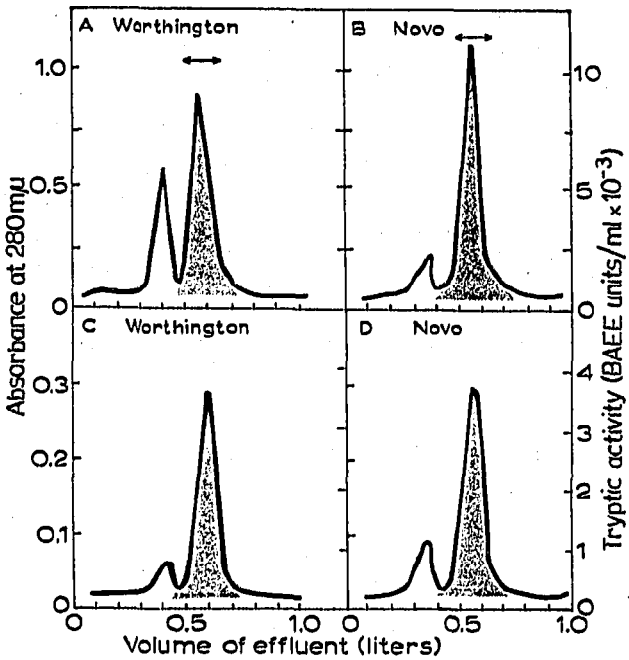

Fig. 1. Chromatography of crystalline trypsins on SE-Sephadex. A and B, original preparation of Worthington and Novo enzymes respectively; C and D, rechromatography of active peaks (pooled tubes denoted by double-headed arrow) from Worthington and Novo enzymes respectively. Solid curve denoted protein measured by absorbance at 280 mμ. Trypsin activity is shown by shaded portion of curves. See text for other details.

As shown in Table I the purity of Worthington trypsin, as determined by titration of its active site, was 56%, a value which is in very close agreement with the value of 57% reported by BENDER *et al.*[11] for another lot of Worthington trypsin. Following chromatographic purification, the titratable purity of this enzyme was increased to 82%. The distribution of proteins observed in the chromatographic

TABLE I

COMPARISON OF TRYPSIN PREPARATIONS BEFORE AND AFTER CHROMATOGRAPHY

Preparation	*Specific activity (BAEE units/mg)*	*% Purity*	
		Chromatography[a]	*Titration of active sites*[b]
Worthington, original	13,000	67	56
Worthington, chromatographed	23,000	94	82
Novo, original	22,000	90	82
Novo, chromatographed	22,500	87	82

[a] Per cent of total protein which is active based on the areas of each peak in the chromatographic patterns shown in Fig. 1.

[b] Based on titration with *p*-nitrophenyl N-benzyloxycarbonyl-lysinate[11].

patterns of Worthington trypsin before and after chromatography indicated a purity of 67% and 94% respectively. Chromatography of the Novo trypsin did not significantly enhance its state of purity which was essentially the same as the chromatographically purified sample of Worthington trypsin.

A lack of correspondence will be noted between the purity revealed by chromatography and that determined by actual titration of the active sites. Part of this discrepancy could perhaps be ascribed to the presence of a residual amount of inactive protein which is not separated from the active enzyme during chromatography. Disc gel electrophoresis[12], however, of the chromatographically purified trypsins disclosed the presence of only one component. Another possibility is that the chromatographically purified fraction is actually a mixture of closely related, enzymatically active components[5,13] which, because of differences in molecular weight and specific activity[5], might have different titration values. Therefore, although the procedure described here may serve the practical purpose of enhancing the purity of certain commercial preparations of crystalline trypsin, it may not be capable of resolving various molecular species of the active enzyme.

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Department of Biochemistry,
University of Minnesota,
St. Paul, Minn. 55101 (U.S.A.)

STAMATIOS PAPAIOANNOU
IRVIN E. LIENER

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