A Chromatographic Study of Trypsin

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An analytical procedure for the resolution of trypsin and its derivatives would be useful in searching for active degradation products in trypsin autolysates as well as for other studies of the reactions of trypsin. Analytical procedures are frequently frustrated when applied to this enzyme because of the autolysis which occurs during the analysis. For this reason some previous analyses have been carried out on trypsin which had been stabilized by the conversion of the enzyme to acetyl trypsin (1). For preventing autolysis a more direct method, which would avoid complex side reactions which occur during acetylation (2) and which would allow the measurement of enzymic activity, might be based on the reversible inactivation of trypsin by urea (3). In general any resolving system, for example electrophoresis or chromatography, which maintained the trypsin in concentrated urea could give an analysis uncomplicated by autolysis and, upon dilution or removal of the urea, assays could be made of various fractions. This report describes the development of a technique for the chromatography of trypsin, in which autolysis is prevented by using urea in the eluting buffer.

Experimental Procedure

The trypsin used was twice crystallized and salt-free as obtained from Worthington Biochemical Corporation, Freehold, New Jersey. All lots (TRSF 684-97B, TRSF 685, 815/18) gave similar chromatograms and possessed a specific activity of about 0.23 mmole of TAME hydrolysed per minute per mg of protein under the following conditions.

Enzyme solution (2 to 10 μl) was added to 2.0 ml of 5 mM TAME and the hydrolysis at 25° was followed by maintaining pH 8.0 with 0.1 M NaOH with the use of an autotitrator. The specific activity of these preparations measured by the benzyol arginine ethyl ester method of Schwert and Takenaka (4) at 25° is about 19 as defined by Liener (5). The apparent chymotryptic activity of these preparations was 1.5% as measured by the rate of hydrolysis of ATEE under the conditions of Harris and Hartley (6). In addition to these methods, proteolytic activity was estimated by the hemoglobin assay (7).

The trypsinogen, a once crystallized, 50% MgSO₄ preparation (TG 695A), and the chymotrypsin, a crystalline preparation (CD 5002), were obtained from Worthington Biochemical Corporation.

The operation of the ion exchange columns has already been described (8). Chromatography was carried out on 0.9 × 22-cm columns in 0.13 M phosphate-5 M urea at pH 6.1, and the colorimetric analysis of the effluent fractions was performed according to the procedure of Lowry, Rosebrough, Farr, and Randall (9).

Results

Preliminary experiments (see also (10)) revealed that, even in 8 M urea, autolysis at room temperature was severe enough to inactivate trypsin appreciably in overnight experiments with the result that the chromatograms were very complex. By performing the chromatography at 3 to 5°, however, the trypsin did not autolyse in 8 M urea, and satisfactory chromatograms were obtained. Therefore, the chromatography of trypsin on Amberlite IRC-50 was performed at 3 to 5° with a pH 6.1 buffer of 0.13 M phosphate-8 M urea and gave the chromatogram shown in Fig. 1. That the minor peaks did not arise as chromatographic artifacts was shown by rechromatographing material from the main peak; the results are given in Fig. 2. The recovery of protein (measured colorimetrically) and of activity were tested and found to be quantitative, and so it appears that the trypsin used is about 77% pure. Further support for this estimate of purity is the observation that the specific activity of the main peak measured by either the hemoglobin assay or the TAME assay was 23 + 4% greater than the starting material.

The assay of a chymotryptic activity (measured by the hydrolysis of ATEE) throughout the chromatogram showed a single peak of this activity which exactly coincided with the trypsin activity. That the peak of chymotryptic-like activity did not represent chymotrypsin itself was proved by determining that chymotrypsin was essentially unretained by the column. The rate at which chromatographically pure trypsin hydrolyses ATEE (2.2 μmoles hydrolysed per minute per mg of trypsin) is about 7.5% of the rate at which it hydrolyses TAME (0.28 mmole per minute per mg of trypsin) and about half of the rate at which the starting preparation of trypsin hydrolyses the tyrosine ester.

In order to obtain the results described above it was necessary to dissolve trypsin at 3 to 5°. When the temperature was not carefully controlled during dissolution a much more complex chromatogram was obtained. Contrast the simple chromatogram (Fig. 1) obtained after dissolving trypsin at a carefully controlled temperature of 3 to 5° with the chromatogram presented in Fig. 3 which was obtained by dissolving trypsin in a warm test tube (25°); in both cases the trypsin was dissolved in cold (3 to 5°) buffer, in the cold room, and applied immediately (1 to 2 minutes) to a cold column of resin and eluted at 3 to 5°.

Partially degraded trypsin yielded two new major peaks, both of which were inactive when assayed in the hemoglobin assay and in the TAME assay, whereas the center peak was found to possess a specific enzymic activity equal to the main peak of chromatograms of unmodified trypsin (Fig. 1). The immediate assay of an aliquot of the solution applied to the column compared with the assay of the effluent indicated that no
inactivation occurred while the enzyme was passing through the column. Furthermore, at 3 to 5° in the dilute effluent collected, the trypsin is stable for at least several days. The patterns obtained by rechromatography of material from each of the main inactive peaks indicated that these are stable materials, each appearing as a single peak in its original position on the chromatogram. The conclusion may be drawn from these data that trypsin is severely inactivated as it is dissolving in an inadequately chilled solution of 8 M urea, but that after it is applied to the column further inactivation is negligible. This conclusion was confirmed by measuring the rate of inactivation when trypsin was dissolved under conditions similar to those used in obtaining the chromatogram of Fig. 3 and then chilled as soon as dissolution was complete (1 to 2 minutes). The results of this test showed an initial loss of activity followed by negligible inactivation (at 3 to 5°). In the case of trypsin dissolved in rigorously chilled solution, the rapid inactivation is not observed. (It must be pointed out that the above test was performed on solutions of trypsin which were at least 10 times as concentrated as the effluent fractions from most columns.) These results are in agreement with those of Krudan, Bier, and Nord (10).

The distribution of tryptic activity in a slightly degraded sample of trypsin is given in Fig. 4 and reveals the presence of more than one active peak. That none of these peaks was caused by trypsinogen (rapidly activated during assay) was established by finding that an authentic sample of trypsinogen was hardly retained on this column and coincided with the minor inactive peak shown in Fig. 4 at 12 to 17 ml.

**DISCUSSION**

Two aspects of the homogeneity of trypsin may be considered: first, the fraction of a given preparation which is trypsin and, second, the amount of contaminating activities present in the preparation. The first aspect has already been considered by Perrone, Disitzler, and Domont (11) who found crystalline trypsin to be 80% homogeneous electrophoretically and by Liener (5) whose data (published while the present work was in progress) indicated a purity of 50 to 65%. The present data confirm the estimates of Perrone et al. in showing a main active component of about 77% of the starting material. The discrepancy between these results and Liener's may be caused by the fact that the starting material used by Liener appears to be somewhat less active than the preparations used here.

The second aspect of the purity of trypsin is important for its use in studying protein structure. In releasing peptides from a protein by proteolysis in order to study primary structure, it is critical that the trypsin be pure in order to avoid nonspecific bond cleavage. This is important not only to obtain optimal yields of the peptides but also to assure the validity of the ass-
sumption (e.g. 12) that basic residues in these peptides occur at the carboxyl ends. The method suggested by Harrington, Von Hippel, and Mihalyi (13) for the study of protein secondary structure also depends on the rigorous specificity of the protease. This aspect of the purity of trypsin demands, therefore, that trypsin be free of other proteolytic activities. The present work, however, proved that there was a chymotryptic-like activity which was not only distinct from the usual chymotrypsin, but also was probably an inherent property of the trypsin molecule itself since the ability to hydrolyse ATEE paralleled the trypsin activity. The chymotryptic-like activity which coincided with the trypsin peak accounted for about half of the total chymotryptic-like activity found in the starting material, and so it seems likely that there was also some normal chymotrypsin in these preparations of trypsin and that the chromatographic system accomplished all the purification possible. These findings are similar to those of Liener (5) who has prepared chromatographically pure trypsinogen, and found that on activation it possessed the ability to catalyze the hydrolysis of ATEE. Further support for the notion that this chymotryptic-like activity is not caused by chymotrypsin itself is the finding of McFadden and Laskowski (14) that the specificity of the “chymotryptic-like” activity is not like that of chymotrypsin. Perhaps the strongest proof of the ability of trypsin to catalyze the hydrolysis of ATEE is the work of Inagami and Sturtevant (15). These workers have found that under optimal conditions trypsin hydrolyzes ATEE one-twelfth as fast as does α-chymotrypsin although the contamination of trypsin by α-chymotrypsin was estimated to be less than 1%. The chromatographic system developed here was able to resolve trypsin and some of its derivatives, since trypsinogen and some inactive products were found to have different elution volumes from trypsin itself. The resolving power of this system appears to be greater than that of Liener (5), and, indeed, in the partially inactivated trypsin, active modifications of trypsin were well resolved from the main peak of the enzyme. Although no conclusion can yet be reached on the nature of the inactivation process or the extent of degradation represented by these particular active products, it seems likely that this chromatographic system will prove useful for isolating active fragments of trypsin if they exist, and those peaks showing a higher specific activity than the main peak are being studied now.

Summary

Crystalline trypsin has been submitted to chromatography on Amberlite IRC-50 resin at 3 to 5°, eluting with 0.13 M phosphate-8 M urea buffer of pH 6.1. The trypsin preparations used were about 77% homogeneous, and the main peak possessed a specific activity 23 ± 4% greater than the starting material.

A weak chymotryptic-like activity, the catalysis of the hydrolysis of acetyl tryosine ethyl ester, paralleled the trypsin activity and was shown not to be caused by chymotrypsin itself. Trypsin is easily degraded upon dissolving in slightly warm 8 M urea solution and yields chromatograms which show three or more active peaks.

REFERENCES
