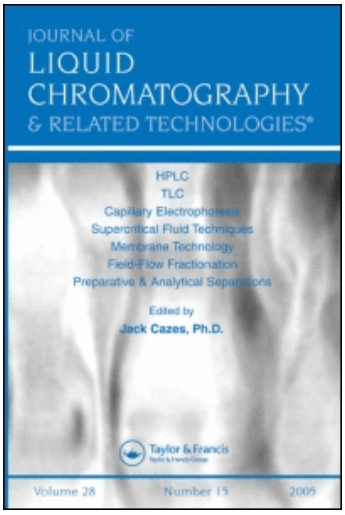

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Purification of Commercially Prepared Bovine Trypsin by Reverse Phase High Performance Liquid Chromatography

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To cite this Article Strickler, M. Patricia , Gemski, M. Judith and Doctor, Bhupendra P.(1981) 'Purification of Commercially Prepared Bovine Trypsin by Reverse Phase High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 4: 10, 1765 — 1775
To link to this Article: DOI: 10.1080/01483918108064845
URL: http://dx.doi.org/10.1080/01483918108064845

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(10), 1765-1775 (1981)

PURIFICATION OF COMMERCIALLY PREPARED BOVINE TRYPSIN BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reverse phase high performance liquid chromatographic method for the purification of milligram amounts of commercially prepared bovine trypsin is described. Increased specific enzymatic activity is observed in the purified material. The advantages of using purified trypsin in protein digestions as well as the potential application of reverse phase high performance liquid chromatography for enzyme characterization are described.

INTRODUCTION

When using reverse phase high performance liquid chromatography (RP-HPLC) as the method for separating peptide fragments, arising from trypsin digestion, impurities in trypsin can interfere with obtaining a true peptide profile. These impurities which are co-eluted with the peptide fragments, may be detected when large amounts of trypsin are required for complete digestion of protein or when the peptide fragment separation is monitored at high levels of sensitivity.

Separation of peptide fragments is commonly used to "fingerprint" or demonstrate the similiarities or differences in the sequences of a series of proteins.(1) The separation process may also be employed to purify the peptide fragments for further characterization.(2) In either case the presence of trypsin impurities co-eluting with the peptide profile could be misleading.

A method is here-by described for the purification of milligram amounts of commercially prepared bovine trypsin.

MATERIAL AND METHODS[1]

Reagents

TCPK-treated trypsin was purchased from Millipore Corp., Bedford, MA, and DPCC treated trypsin from Calbiochem, La Jolla, CA, and Sigma Chemical Co., St. Louis, MO. Acetonitrile and trifluoroacetic acid of HPLC grade were obtained from Fisher Scientific, Silver Spring, MD. The N α- ρ-Tosyl-L- Arginine methyl ester hydrochloride (TAME) was obtained from Aldrich Chemical Co., Milwaukee, WI. All other chemicals were of analytical reagent grade.

Instrumentation

A Waters Associates, (Milford, MA), liquid chromatograph consisting of two M6000A solvent delivery systems, a M660 solvent flow programmer, a U6K universal injector, a M440 absorbance detector set at 280nm, a M450 variable wavelength detector and a M730 data module was utilized. Enzymatic activity was measured using a Beckman Spectrophotometer UV 5230 equipped with an automatic sample changer.

Chromatographic Procedure

A 30 minute linear gradient was run at a flow rate of 2ml/min on a μBondapak C_{18} column (3.9mmx 30cm), Waters Associates. The mobile phase consisted of solution A, 0.1 % aqueous trifluoroacetic acid (TFA) and solution B, acetonitrile-0.1% TFA run from 100% A to 45% B. The column effluent was monitored at 215nm and 280nm. All separations were carried out at ambient temperature.

Enzymatic Activity Procedure

A stock solution of trypsin at a concentration of 5mg/ml was prepared in 0.001N HCl. From this stock 200 μl (0.84mg as calculated by the absorbance at 280nm, E_{280}=14.3) was injected into the liquid chromatograph and eleven fractions were collected as described in the Results and Discussion. After lyophilization the fractions were reconstituted in 1.2ml of 0.001N HCl and the protein concentration of each was calculated as described above. The fractions were then diluted with 0.001N HCl to a concentration range between 10-20 μg/ml. Aliquots (0.3ml) of the substrate TAME, 0.001M in distilled water, and (2.6ml) of 0.046M Tris-HCl buffer, pH = 8.1, mixed with 0.0115M $CaCl_2$ were incubated at 25°C for 3 minutes to establish a baseline at 247nm. At zero time, 0.1ml of the diluted protein fraction was added and reaction rates determined after 3 minutes by the change in absorbance. Activity was calculated in units/mg of protein using the following equation:

$$U/\text{mg protein} = \frac{\Delta A_{247}/\text{min} \times 3 \times 1000}{540 \times \text{mg Trypsin}} \qquad (3)$$

RESULTS AND DISCUSSION

The source of the spurious peaks in the peptide fragment separations was traced to impurities in trypsin by chromatographing solutions of trypsin with concentrations of trypsin equal to that being used in the protein digestions. Three representative commercial solutions of trypsin, at concentrations of 1mg/ml were analyzed by HPLC to detect the presence of impurities. All three samples, exhibited similar impurities as shown in Figures 1, 2 and 3. A

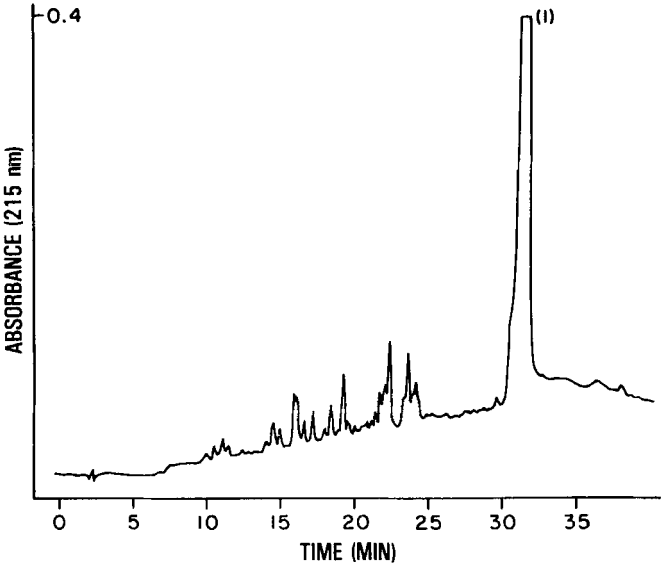

FIGURE 1

Separation of 100 µg of Bovine Trypsin (Millipore), (1) trypsin. Column: µ Bondapak C_{18} (3.9mm x 30cm), Gradient: 30 min., linear. Flow rate: 2.0ml/min. Mobile Phase: 100% aqueous, 0.1% trifluroacetic acid (TFA) to 45% acetonitrile, 0.1% TFA / 55% aqueous 0.1% TFA. Columnm temperature: ambient.

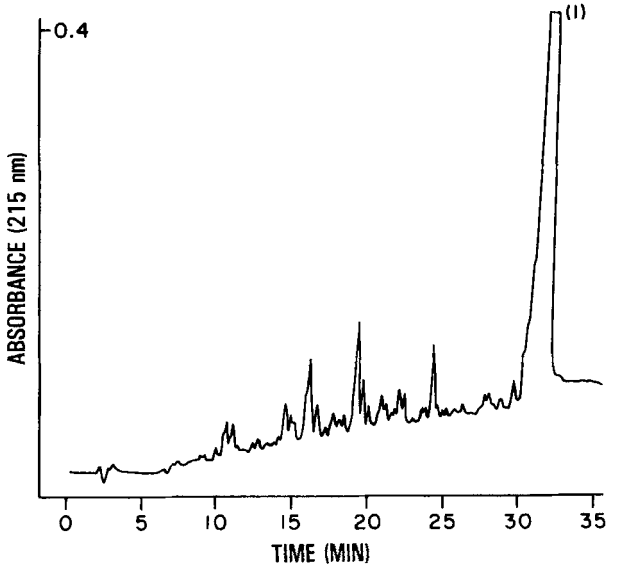

FIGURE 2

Separation of 100 μg of Bovine Trypsin (Sigma), (1) trypsin.

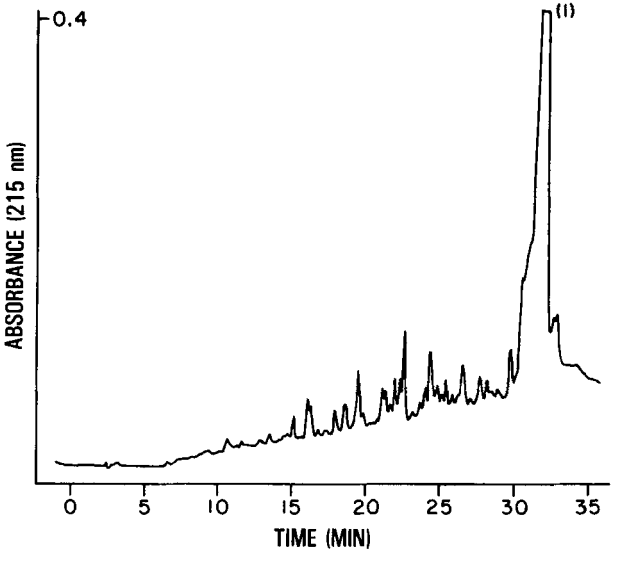

FIGURE 3

Separation of 100 μg of Bovine Trypsin (Calbiochem), (1) trypsin.

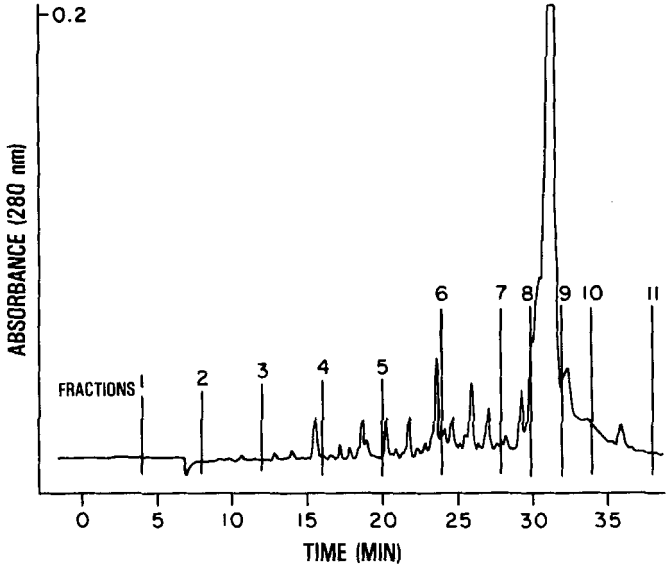

FIGURE 4

Separation of 1mg of Bovine Trypsin (Sigma), collected fractions are indicated by numbers 1-11.

milligram of trypsin (Sigma) was chromatographed (Figure 4) and four milliliter fractions were collected for 28 minutes followed by two milliliter fractions during the next six minutes. After lyophilization and reconstitution the amount of protein in each fraction was calculated using the absorbance at 280nm. The fractions were also assayed for enzymatic activity using the TAME procedure, which is specific for trypsin. The results are summarized in Table 1.

TABLE 1
Protein Concentrations and Enzymatic Activities of the Fractionated Trypsin.

Fraction Number	Time (min)	Concentation (mg/ml calc)	Activity (units/mg protein)
1	4	0.0168	
2	8	0.0133	
3	12	0.0091	
4	16	0.0175	3.7
5	20	0.0231	8.3
6	24	0.0245	
7	28	0.0280	
8	30	0.0637	53.1
9	32	0.2541	151.5
10	34	0.0203	45.1
11	38	0.0147	47.8
Starting sample	———	0.8484	135.8

Fraction 9, which contains the major peak, showed a higher specific activity than the original sample as indicated in Figure 5. A 57% recovery (calculated on the basis of the absorbance at 280nm) was obtained from the chromatographic separation. An aliquot of fraction 9 was rechromatographed to confirm its purification (Figure 6). The purified material used in subsequent digestions gave clean tryptic blank runs.

During the development of the purification method for trypsin several interesting observations were made. A comparison of chromatographic profiles of freshly prepared solutions of trypsin with ones that had been stored showed the major peak, representing trypsin, to change in shape and decrease in area whereas some of the peaks, representing impurities increased in area. Similar

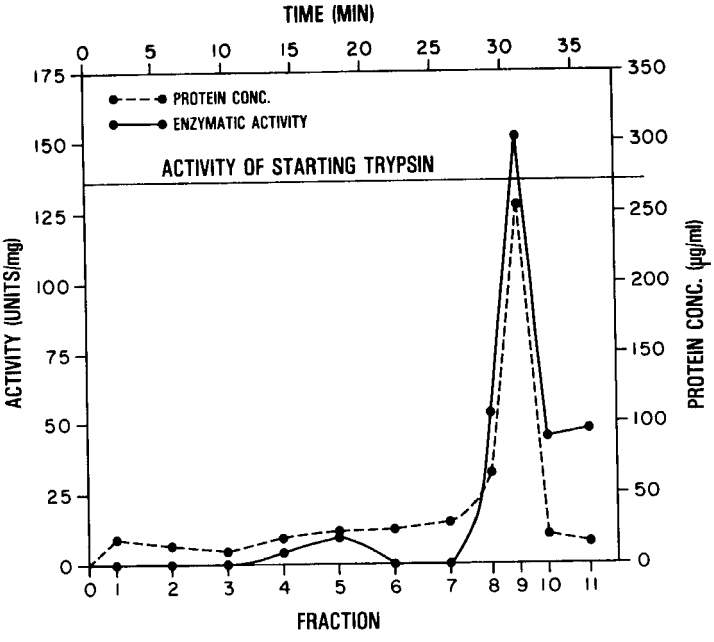

FIGURE 5

Fractionation of Trypsin by HPLC.

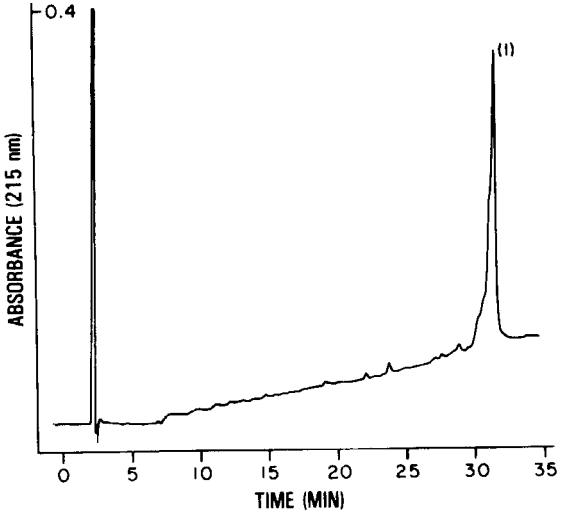

FIGURE 6

Rechromatography of fraction 9, (1) trypsin.

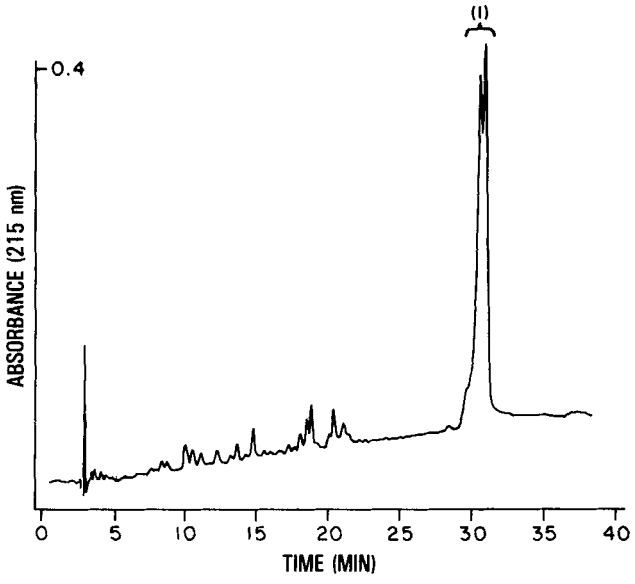

Figure 7

Chromatogram of 50 μg of trypsin (Millipore), (1) possibly the and forms of trypsin. Column: μ Bondapak C_{18} (3.9mm x 30cm), Gradient: 30 min., linear. Flow rate: 1.5ml/min. Mobile Phase: 12% acetonitrile, 0.1% TFA / 88% aqueous, 0.1% TFA to 45% acetonitrile, 0.1% TFA / 55% aqueous, 0.1% TFA. Column temperature: ambient.

chromatographic profiles were also seen in comparing lyophilized samples stored for less than one month to material maintained for four months at 0°C. These observations are consistent with the fact that trypsin not only undergoes autodigestion in solution but in lyophilized samples as well. (4, 5)

The change in shape of the major trypsin peak strongly suggested that this peak was not homogenous. By adjusting the chromatographic conditions, as shown in Figure 7, two distinct peaks were resolved from the single trypsin peak. Multiple forms of trypsin have been separated by others using column chromatography on SE-Sephadex. This technique has demonstrated that the predominant forms, α and β, are always present in commercially available trypsin. (5)

The method described in this paper provides a rapid and simple means of further purifying commercially prepared trypsin. The use of purified trypsin in protein digestions yields a true chromatographic peptide profile. This is especially crucial when large amounts of trypsin are needed for protein digestion or when monitoring at high sensitivities on HPLC. The procedure also demonstrates the potential of RP-HPLC for the final purification of other enzymes as well as the separation of different protein forms and peptide chains.

ACKNOWLEDGEMENTS

We thank Mr. Nesbitt D. Brown and Dr. Richard W. Travis for their technical assistance and Mr. Stewart Galloway for secretarial support.

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1. The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.