Journal of Chromatography, 207 (1981) 55–62
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,439

SEPARATION OF α- AND β-TRYPSIN BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY

PETR ŠTROP*
Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Fleming. n. 2, 166 10 Prague 6 (Czechoslovakia)
and
DANA ČECHOVÁ
Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Fleming. n. 2, 166 10 Prague 6 (Czechoslovakia)
(Received October 17th, 1980)

SUMMARY

The separation of α- and β-trypsin by means of hydrophobic chromatography on Spheron P 300 was investigated with respect to the separation conditions, *i.e.*, salt concentration, pH, temperature, sample loading, flow-rate and support particle size. The optimal conditions have been selected at low pH (3.0) where the autodigestion of trypsin is suppressed. This method based on different exposures of hydrophobic amino acid residues of α- and β-trypsin is rapid, simple and effective for both analytical- and preparative-scale separations.

INTRODUCTION

The structure of the trypsin molecule and the mechanism of its action have been studied in considerable detail. At present trypsin is one of the best known proteins. Therefore it provides an important and widely used model system for kinetic as well as physical studies. Such studies require a well defined form of trypsin, preferably the uncleaved, single chain β-trypsin which retains full enzymatic activity. Studies in which trypsin is used for protein fragmentation require enzyme of the same quality[1]. Most commercial trypsin preparation contain some inactive material and ψ-trypsin of low activity[2], in addition to both the active forms, β-trypsin and the double chain α-trypsin. The content of the individual constituents is often unknown, however.

A number of methods for the isolation of α- and β-trypsin has been developed based on ion-exchange[2] and affinity[3–5] chromatography. The method of Schroeder and Shaw[2] using chromatography on SE-Sephadex at neutral pH with *p*-aminobenzamidine in the elution buffer has proved to be the most successful and is frequently employed.

In this paper we introduce a procedure for effective separation of α- and β-trypsin based on differences in the accessibility of the hydrophobic amino acid residues in

0021-9673/81/0000–0000/$02.50 © 1981 Elsevier Scientific Publishing Company

the neighbourhood of the active site[6]. The fractionation of both active forms of trypsin was studied with respect to the type of salt, salt concentration, pH, temperature, sample loading and flow-rate. Optimal conditions for preparative as well as analytical scale separations have been determined.

MATERIALS AND METHODS

Trypsin from bovine pancreas (lyophilized, TRL L AA, 207 U/mg) was obtained from Worthington (Freehold, NJ, U.S.A.), trypsinogen from bovine pancreas (3000 NF E/mg) from Fluka (Buchs, Switzerland) and *p*-aminobenzamidine from Koch-Light (Colnbrook, Great Britain). Spheron P 300 (20–40 μm) was supplied by Lachema (Brno, Czechoslovakia), Spheron P 300 (14.7 $\pm$ 1.6 μm) from Spolek (Ústí n. Labem, Czechoslovakia). Other chemicals (analytical grade) were from Lachema. The sorbent extraction and other characteristics have been reported previously[7].

Equipment

The chromatographic apparatus consisted of a Type 68005 minipump connected to a 2-ml pre-column, a 75-μl sample loop, an injection septum head, a precision bore glass column (8.00 mm I.D.) with adjustable ends and a thermostated water jacket. The absorbance was recorded at 254 or 280 nm on a Type UVM-4 spectromonitor. All these instruments were made by the Instrument Development Workshops of the Czechoslovak Academy of Sciences. Polyacrylamide gel electrophoresis of reduced protein samples in sodium dodecyl sulphate were described previously[6]. The pH value were determined on a digital MV 87 pH meter (Prätcitronic, G.D.R.), using a GK 2301 C electrode (Radiometer, Copenhagen, Denmark). They were not corrected for high salt concentrations. Determination of tryptic activity was done in the usual manner[6].

RESULTS AND DISCUSSION

In previous papers[7,8] Spheron was shown to provide a rigid and highly cross-linked glycolmethacrylate matrix and non-polar binding sites suitable for hydrophobic interaction chromatography of large peptide fragments and particularly of proteins. In contrast to the frequently used Sepharose derivatives which interact also with the hydrophobic surface amino acid residues of proteins in crevices ("pockets"), Spheron displays different mechanical as well as binding properties. It was found to interact entirely with the surface exposed groups of proteins.

Salt concentration dependence of α- and β-trypsin separation

Trypsin is one of the proteins with both types of non-polar surface side chains mentioned above. However, the separation of α- and β-trypsin can be accomplished more easily on Spheron. β-Trypsin appears to exhibit a larger exposed non-polar surface area than α-trypsin, localized in the proximity of the active site[6]. Consequently, β-trypsin was found to be more retained on the Spheron column than α-trypsin under all the conditions examined. Fig. 1 illustrates the elution pattern of a commercial trypsin sample obtained with a Spheron P 300 column in the course of isocratic elution with various concentrations of $(NH_4)_2SO_4$ in the elution buffer. The elution volumes of both active forms at 20°C and 30°C are indicated. Pseudotrypsin (or

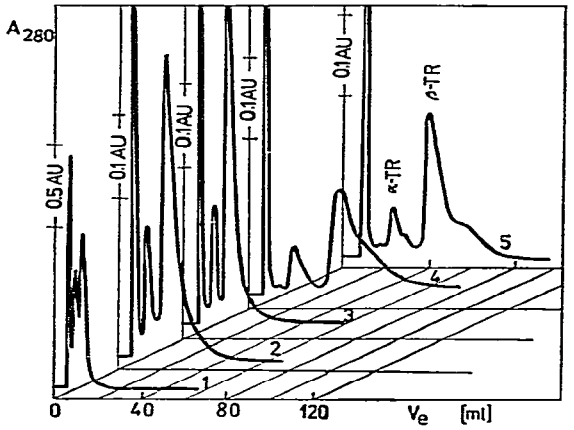

Fig. 1. Elution profiles of a commercial trypsin sample on HIC at pH 3.0 using various concentrations of ammonium sulphate. 1, 0.3 *M* $(NH_4)_2SO_4$, 20°C, 6 mg of trypsin; 2, 0.5 *M* $(NH_4)_2SO_4$, 20°C, 5 mg of trypsin; 3, 0.5 *M* $(NH_4)_2SO_4$, 30°C, 4 mg of trypsin; 4, 0.7 *M* $(NH_4)_2SO_4$, 20°C, 4 mg of trypsin; 5, 0.7 *M* $(NH_4)_2SO_4$, 30°C, 4 mg of trypsin. Trypsin was dissolved in 100 µl of the elution buffer [0.05 *M* formic acid with a given concentration of $(NH_4)_2SO_4$, pH adjusted to 3.0 with 2 *M* NaOH] and applied to a Spheron P 300 (14.7 ± 1.6 µm) column (250 × 8.0 mm I.D.), flow-rate 30 ml/h. A_{280} = Absorbance at 280 nm; V_e = elution volume in ml; α-TR = α-trypsin; β-TR = β-trypsin; AU = absorbance unit.

ψ-trypsin) and products of trypsin degradation as well as some impurities are present in the first peak, α-trypsin is in the second peak and β-trypsin is found in the third one. ψ-Trypsin as well as the majority of impurities present in the sample displays only very low retention even at high ammonium sulphate concentrations. On the other hand, both α- and β-trypsin are markedly retained if the salt concentration is increased; this effect is typical of hydrophobic interaction chromatography (HIC). If the concentration of $(NH_4)_2SO_4$ is raised from 0.3 *M* to 0.7 *M* at pH 3.0 the elution volume of β-trypsin is increased three-fold. An increase in the salt concentration followed by higher retention gives rise to an improvement in the peak resolution. Accordingly, the peak width is reduced at higher temperatures (30°C) because of the higher diffusion velocities. This brings about a remarkable restriction of tailing (Fig. 1). Likewise, a better separation of the small peak following the major peak of β-trypsin seems to contribute to the reduced tailing.

A significant decrease in the retention of β-trypsin can be accomplished by the addition of a low-molecular-weight trypsin inhibitor, *p*-aminobenzamidine to the elution buffer at a concentration of *ca.* $1 \cdot 10^{-3}$ *M*. The HIC pattern of natural trypsin inhibitors and their complexes with trypsin, as well as the above phenomenon, suggest that the hydrophobic amino acids adjacent to the trypsin active site are implicated in the trypsin retention on Spheron[6].

pH dependence

Fig. 2 illustrates the elution pattern of a commercial trypsin sample in 0.5 *M* $(NH_4)_2SO_4$ at various pH of the elution buffer. A pH decrease from 8.4 to 5.5 results in a decrease in the retention of both α- and β-trypsin. In contrast, a further decrease in pH to 4.0 results in a small increase in the retention, whereas a decrease in the

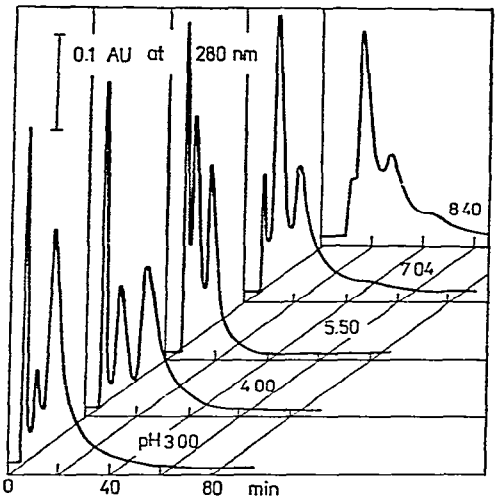

Fig. 2. Elution profiles of a commercial trypsin sample on HIC in 0.3 *M* $(NH_4)_2SO_4$ at different pH values. The elution buffer at pH 3.0 and pH 4.0 was the same as in Fig. 1.1; for pH 5.5, 7.04 and 8.4, 0.03 *M* phosphate buffer containing 0.3 *M* $(NH_4)_2SO_4$ was used. The elution profiles at pH 4.0 and 5.5 were obtained with 0.05 *M* acetate buffer as well. Flow-rate: 60 ml/h at 20°C. Sample: 0.77 mg of trypsin in 70 μl of the elution buffer. For other conditions and symbols see Fig. 1.

retention was again observed when the elution buffer was adjusted to pH 3.0 (Fig. 2). A different pattern of pH-dependence was found when a higher concentration of NaCl in the elution buffers was used to enhance the interaction between trypsin and the support. The relatively flat dependence of the retention upon pH was found in the range pH 8.0–5.5 in 0.5 *M*, 1 *M* and 1.5 *M* NaCl solutions. When the pH was less than 5.5 the retention increased sharply with increasing acidity[6].

The nature of the hydrophobic interaction between a protein and a chromatographic support appears to be complex. Both the salts examined, NaCl and $(NH_4)_2SO_4$, exhibit "salting out" characteristics and are known to enhance hydrophobic interactions[9–11]. The pH dependence, however, is essentially different with the two salts as a result of the character of the anion. The HIC data available for a variety of proteins in buffers containing either $(NH_4)_2SO_4$ or Na_2SO_4 and KCl or NaCl are in agreement with the dominant rôle of the anion.

An apparent increase in the content of α-trypsin as compared to β-trypsin at pH 4.0 is illustrated in Fig. 2. The α-trypsin peak is four times larger than that at pH 3.0, and small changes during sample preparation and chromatography are observed even at pH values as low as 3.5 (not shown in Fig. 2).

For pH $\geqslant$ 5.5, the first peak containing ψ-trypsin and the other inactive impurities is reduced. This is explained by the fact that material responsible for this peak is cleaved by active trypsin into small peptides. Moreover the peptides of higher absorbance at 280 nm, containing aromatic amino acids, show a higher retention as compared to the unaffected material and to the peptides of lower hydrophobicity.

Considering the autolysis of trypsin, the separation procedure at pH 3.0 appears to be optimal for isolation of β-trypsin as well as for a rapid analysis of trypsin samples.

Temperature dependence

The temperature dependence of the trypsin retention in 0.3 *M* $(NH_4)_2SO_4$ at pH 3.0 was investigated in the range 15–50°C. The increase in temperature does not influence the peak size of the main components, *i.e.*, of α- and β-trypsin, but the first peak of inactive constituents is reduced. This peak is influenced even by temperatures as low as 25°C. When the temperature is raised to 35°C, a decrease in the second peak of α-trypsin occurs (Fig. 3). Further increase in temperature brings about a decrease in total recovery and at 50°C only a negligible portion of the material applied was recovered. Within the temperature range 20–45°C the retention of α- and β-trypsin also decreases with increasing temperature. In contrast, the trypsinogen retention in the same elution system [0.3 *M* or 0.5 *M* $(NH_4)_2SO_4$, pH 3.0] is increased as the temperature is raised. Trypsinogen is eluted between the α- and β-trypsin peaks at 20°C (Fig. 4), whereas at the same salt concentration at a higher temperature (30°C) the elution volume of trypsinogen equals that of β-trypsin.

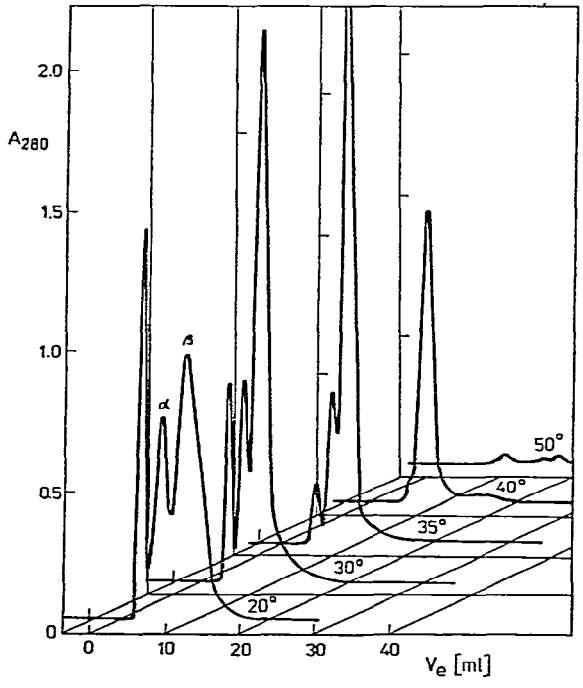

Fig. 3. Elution profiles of a commercial trypsin sample on HIC at different temperatures. The conditions are as in Fig. 1.1, except for temperature.

For the analysis of the activation products of trypsinogen the optimal temperature is 20°C, in 0.5 *M* $(NH_4)_2SO_4$, pH 3.0. Better resolution can be achieved at higher temperatures but is obviously limited by protein stability. A temperature of 30°C was found to be optimal for the rapid separation of α- and β-trypsin and for the analysis of their content in commercial samples.

Flow-rate and particle size dependence

The diffusion constants of proteins are about one order of magnitude lower

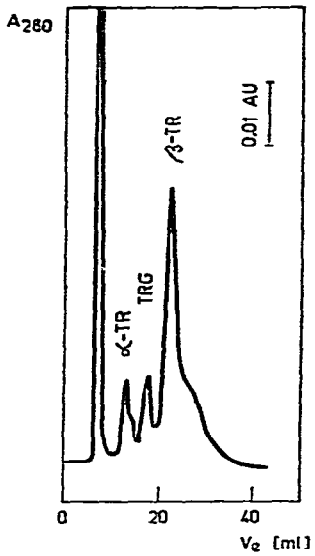

Fig. 4. Separation of components of crude trypsin and trypsinogen. Sample: 1.2 mg of commercial trypsin and 0.15 mg of trypsinogen in 70 μl of the elution buffer. Flow-rate: 7 ml/h at 22°C. Other conditions as in Fig. 1.2. TRG = Trypsinogen.

than those of low-molecular-weight solutes. Accordingly, with respect to the particle size, much higher flow-rates than that at the minimum plate height are often used[12]. The elution profiles shown in Figs. 1–4 were obtained on a column packed with

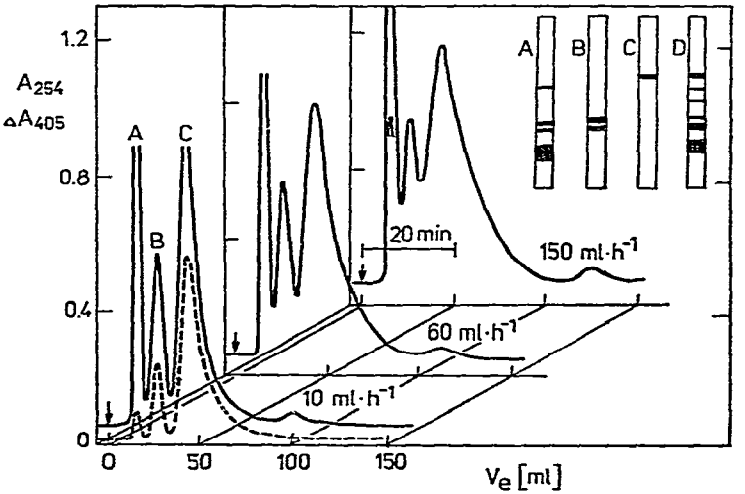

Fig. 5. Elution profiles of α- and β-trypsin at different flow-rates. A 70-mg sample of crude commercial trypsin in 300 μl of the elution buffer was applied to a Spheron P 300 (20–40 μm) column (505 × 8.0 mm I.D.) and eluted with 0.05 *M* formate buffer containing 0.5 *M* $(NH_4)_2SO_4$, pH 3.0, 22°C, at the flow-rates indicated. A_{254} = Absorbance at 254 nm (———); A_{405} = absorbance at 405 nm (– – – – –) indicating the trypsin activity. The peak fractions A, B and C were used for sodium dodecyl sulphate electrophoresis in polyacrylamide gel, as shown in the insert. A = ψ-Trypsin and inactive impurities; B = α-trypsin; C = β-trypsin; D = commercial trypsin sample.

particles of mean size 14.7 μm, whereas Figs. 5 and 6 illustrate the effect for the particles of size 29 μm. Fig. 5 shows the elution profile and resolution corresponding to a 15-fold increase in flow-rate (from 10 ml h^{-1} to 150 ml h^{-1}, *i.e.*, a linear flow-rate of 2.8 mm sec^{-1}).

Influence of sample loading

The influence of the sample loading upon separation of α- and β-trypsin is illustrated in Fig. 6. A low sample loading (*ca.* 0.9 mg/ml) results in a high retention of the individual trypsin forms. With higher loadings, the resolution is poorer but the separation of β-trypsin remains satisfactory, even when the sample loading is as high as 9 mg/ml. Therefore the small column used in these experiments (27 ml) can be used quite effectively for the preparation of β-trypsin.

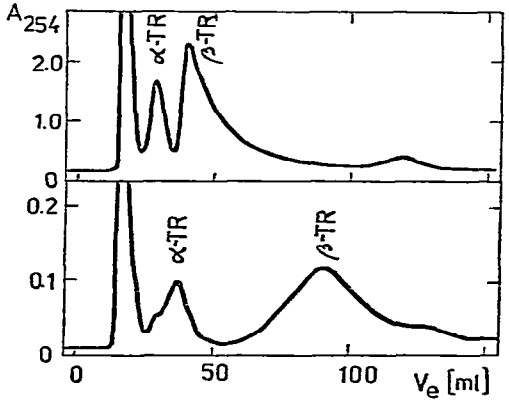

Fig. 6. Dependence of α- and β-trypsin separation on sample loading. Upper curve: 240 mg of crude trypsin in 600 μl of the elution buffer. Lower curve: 24 mg of trypsin in 200 μl of the elution buffer. Flow-rate: 10 ml/h. For other conditions see Fig. 5.

CONCLUSIONS

Separation of α- and β-trypsin by HIC offers many advantages: the simple isocratic elution system prevents autodigestion of trypsin, no inhibitor (*e.g.*, *p*-aminobenzamidine) is needed and the separation can be performed at room temperature. The rigid chromatographic support permits the use of particles of small diameter at high pressures and flow-rates.

REFERENCES

1 V. Keil-Dlouhá, N. Zylbert, N.-T. Tong and B. Keil, *FEBS Lett.*, 16 (1971) 287.
2 D. D. Schroeder and E. Shaw, *J. Biol. Chem.*, 243 (1968) 2943.
3 D. V. Roberts and D. T. Elmore, *Biochem. J.*, 141 (1974) 545.
4 G. W. Jameson and D. T. Elmore, *Biochem. J.*, 141 (1974) 555.
5 K. Kasai and S. Ishii, *J. Biochem. (Tokyo)*, 71 (1972) 363.
6 P. Štrop, D. Čechová and V. Tomášek, to be published.

7 P. Štrop, F. Mikeš and Z. Chytilová, *J. Chromatogr.*, 156 (1978) 239.
8 E. Brynda, P. Štrop, F. Mikeš and J. Kálal, *J. Chromatogr.*, 196 (1980) 39.
9 F. Franks, *CRC Crit. Rev. Biochem.*, 3 (1975) 165.
10 S. Lewin, *Displacement of Water and its Control of Biochemical Reactions*, Academic Press, London, New York, 1974.
11 F. Franks, *Water, a Comprehensive Treatise*, Vol. 4, Plenum, New York, 1975.
12 J. C. Giddings, *Dynamics of Chromatography*, Marcel Dekker, New York, 1965.