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MODEL STUDY OF HYDROPHOBIC INTERACTIONS OF α- AND β-TRYPSIN AND α-CHYMOTRYPSIN

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SUMMARY

The hydrophobic interactions of α- and β-trypsin as a function of ionic strength and pH were studied by hydrophobic chromatography. Evidence was obtained that in spite of the identical specificities and similar activities of α- and β-trypsin, the cleavage of the Lys–Ser bond induces conformational changes in the neighbourhood of the active site. Over a wide range of pH and salt concentration the non-polar residues on the surface of the molecule of β-trypsin are more exposed to an external environment than on the molecule of α-trypsin. In the trypsin(chymotrypsin)–inhibitor complexes the majority of hydrophobic amino acids are buried; other hydrophobic residues localized on the surface contribute only very slightly to the interaction with the chromatographic support.

The retention of trypsin, chymotrypsin and their diisopropylphosphoryl derivatives on a support with flexible hydrophobic ligands bonded to the matrix through a spacer (octyl-Sepharose) was correlated with the retention on a support with hydrophobic binding sites incorporated into the rigid matrix of the resin (Spheron). The native enzymes are always more retained; this indicates that the substitution results in the shielding of the non-polar residues in the neighbourhood of the active site. The differences in the slope of individual proteins, resulting from the correlation of the retention values obtained with both supports at several sodium chloride concentrations are explained by differences in the accessibility of the surface non-polar residues in the individual proteins. In experiments with model peptides the contribution of the individual hydrophobic amino acids to the retention was investigated.

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INTRODUCTION

Proteinases of the serine type (trypsin, chymotrypsin, elastase, etc.) belong to enzyme systems widely studied from the viewpoint of activation mechanism, cleavage, structure and physico-chemical properties. The activation of trypsinogen to active trypsin is a multi-step process. Trypsinogen is first converted into β-trypsin, an active single-chain protein[1]; its primary structure has been determined[2,3]. β-Trypsin is not stable over a wide pH range; α-trypsin is formed by spontaneous autodigestion of the peptide bond between Lys(145)* and Ser(146)* according to Schroeder and Shaw[4]. α-Trypsin consists of two polypeptide chains cross-linked by disulphide bridges[4]. The individual active forms of trypsin (α and β) differ in their properties[4–7]. Earlier modification studies and spectroscopic measurements have shown that certain tyrosine and tryptophan residues are localized on the surface of the trypsin molecule in the neighbourhood of the active site[8,9]; it has been suggested that α- and β-trypsin differ in the accessibility of these non-polar residues to solvents[9].

As the structure of trypsin and chymotrypsin is known and data on their complexes with inhibitors are also available[10–16], these proteins may serve as suitable models. This study was designed to investigate whether hydrophobic chromatography can serve as an appropriate tool for the sensitive detection of both conformational changes around hydrophobic residues and of protein–protein interactions in experiments with proteins studied in less detail than trypsin and chymotrypsin.

EXPERIMENTAL

Proteins

Trypsin (lyophilized TRL L AA, 207 U/mg) and chymotrypsinogen A were obtained from Worthington (NJ, U.S.A.), equine myoglobin and cytochrome *c* from Serva (G.F.R.) and basic pancreatic trypsin inhibitor (Antilysin, 5200 U/mg) from Léčiva (Czechoslovakia). Diisopropylphosphoryltrypsin and diisopropylphosphorylchymotrypsin[17], cow colostrum inhibitor[18], bull seminal proteinase inhibitor[19] and α-chymotrypsin from bovine pancreas (crystallized three times)[20] were prepared as described elsewhere. The samples of α-trypsin and β-trypsin were also isolated on SE-Sephadex by the method of Schroeder and Shaw[4].

Column supports and reagents

Spheron P 300 (20–40 μm) from Lachema (Brno, Czechoslovakia) was purified as described earlier[21]. SE-Sephadex C-50 and Sepharose CL-4B were purchased from Pharmacia (Uppsala, Sweden). Octyl-Sepharose CL-4B of a higher (*ca.* 1.5–2 times) degree of substitution than that of the commercial product was prepared according to an earlier procedure[22]. Acrylamide, sodium dodecyl sulphate, Coomassie Brilliant Blue R 250 and N,N′-methylenebisacrylamide were obtained from Koch-Light (Great Britain); 2-mercaptoethanol was purchased from Fluka (Switzerland). N^{α}-benzoyl-D,L-arginine-*p*-nitroanilide was synthetized according to Tuppy *et al.*[23]. The model peptides were supplied by J. Zbrožek.

* The numbers correspond to chymotrypsin numbering, based on the homology of the two proteins[10–16].

Chromatographic experiments

The hydrophobic interactions of proteins and model peptides were examined by hydrophobic chromatography on a macroreticular resin (Spheron P 300) and on hydrophobized soft agarose gel (octyl-Sepharose CL-4B) under conditions where the solutes are still completely displaced by isocratic elution and where for most experiments the distribution coefficient (K') was less than 10. The hydrophobicity of the proteins was compared in terms of the elution volumes and distribution coefficients K or K':

$$K = (V_e - V_0)(V_t - V_0)^{-1}$$

$$K' = (V_e - V_0)V_t^{-1}$$

where V_e is the elution volume under the conditions given, V_0 corresponds to the elution volume of a protein of identical molecular weight not retarded by hydrophobic interactions and V_t is the total bed volume. K' is used where the retention on octyl-Sepharose is compared as it is difficult, especially with small molecules, to obtain sufficiently precise values of $V_t - V_0$ (V_0 approaches V_t). The experiments were carried out in a jacketed precision glass column (245 × 6.24 mm I.D. for the Spheron P-300 resin and 130 × 8.00 mm I.D. for octyl-Sepharose CL-4B) using a precision piston micropump. The elution was monitored at 220, 254 or 280 nm with a Model UVM4 UV spectrometer; the peptides were also detected with a Knauer differential refractometer. The pH values were measured with a GK 2301 C combined glass electrode (Radiometer, Copenhagen, Denmark), without correction for high salt concentrations. The experimental conditions are given in the legends to the figures.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate was carried out according to Weber *et al.*[24] in 10% acrylamide gel. The proteins were reduced with 1% 2-mercaptoethanol in 0.01 *M* phosphate buffer (pH 7.0) containing 1% of sodium dodecyl sulphate. The mixture was heated for 2 h at 40°C. The concentration of proteins was 0.1 mg per 0.5 ml of solution. A volume of 20–100 μl of the protein solution was used per tube. The gels were stained with Coomassie Blue R 250.

Trypsin activity assay and N-terminal amino acid analysis

Trypsin activity was measured by the standard photometric method at 405 nm with N^{α}-benzoyl-D,L-arginine-*p*-nitroanilide as substrate[25] in 0.1 *M* Tris–HCl buffer (pH 7.8) containing 0.02 *M* calcium chloride. Aliquots of 10 μl of the column fractions were incubated at 25°C for 10 min. The N-terminal amino acids were determined as the 1-dimethylaminonaphthalene-5-sulphonyl derivatives as described elsewhere[26,27].

RESULTS

The differences in the hydrophobic interactions of the individual forms of trypsin become apparent on testing commercial trypsin preparations by hydrophobic chromatography (Fig. 1). The binding affinity of the chromatographic support in-

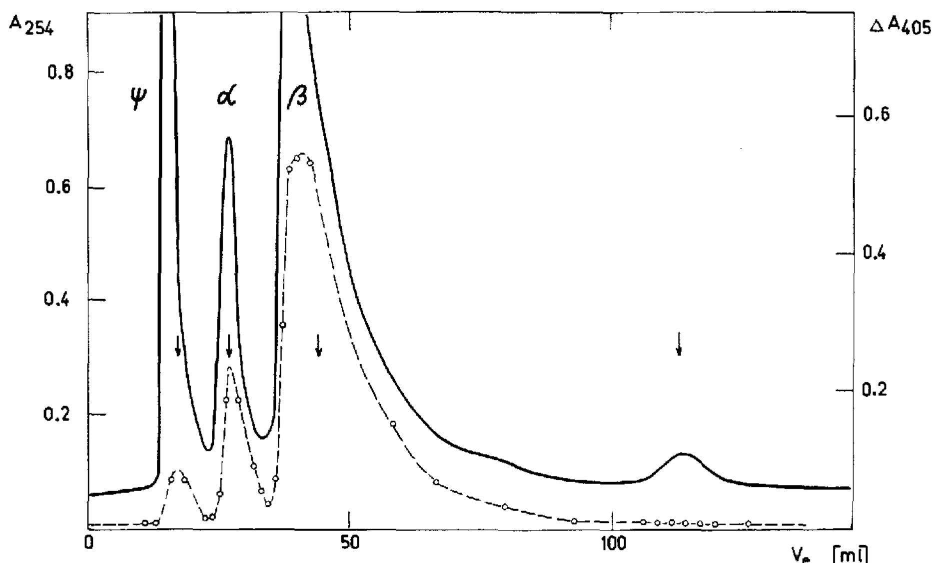

Fig. 1. Fractionation of commercial trypsin by hydrophobic interaction chromatography on Spheron P-300. Full line, absorbance at 254 nm; broken line, trypsin activity; V_e, elution volume (ml). Conditions: trypsin (70 mg) was dissolved in 300 μl of the elution buffer [0.5 *M* $(NH_4)_2SO_4$ in 0.05 *M* formic acid, adjusted to pH 3.0 with NaOH] and applied on to the column of Spheron P-300 (505 × 8 mm I.D.). Fractions of 1.7 ml were collected during 10 min at 22°C. The enzymatic activity was measured as described under Experimental. The arrows indicate fractions used for SDS electrophoresis (see Fig. 2).

creases in the order: ψ- < α- < β-trypsin, as evidenced by the elution pattern. The individual components of the elution profile (Fig. 1) were identified by chromatography of pure α- and β-trypsin prepared by the procedure of Schroeder and Shaw[4] under identical conditions. The homogeneity of α- and β-trypsin was demonstrated by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (Fig. 2). β-Trypsin from the third peak gave a single zone of the same mobility as that of trypsinogen and isoleucine was the only N-terminal residue found. Two zones corresponding in mobility to peptides of molecular weight 11,000–13,000 were observed when reduced α-trypsin was subjected to electrophoresis. Two N-terminal amino acids, isoleucine and serine, were found. This is in agreement with the reported additional cleavage of the Lys(145)–Ser(146) bond in α-trypsin, as reported before[4]. The first peak of the elution profile (Fig. 1) showed a low amidase activity, probably caused by the presence of ψ-trypsin. Isoleucine, serine, aspartic acid, phenylalanine and traces of valine and leucine were detected as N-terminal amino acids. The material from the first peak probably contains a mixture of peptides corresponding to reduced ψ-trypsin and contaminants present in commercial trypsin.

Hydrophobic interactions of α- and β-trypsin

pH and salt concentration dependence. The pH dependence of the hydrophobic interactions of α- and β-trypsin with the non-polar matrix of Spheron was studied over the pH range 3.0–7.7 at various sodium chloride concentrations. At all pH values and sodium chloride concentrations β-trypsin is more retained than α-trypsin (Figs.

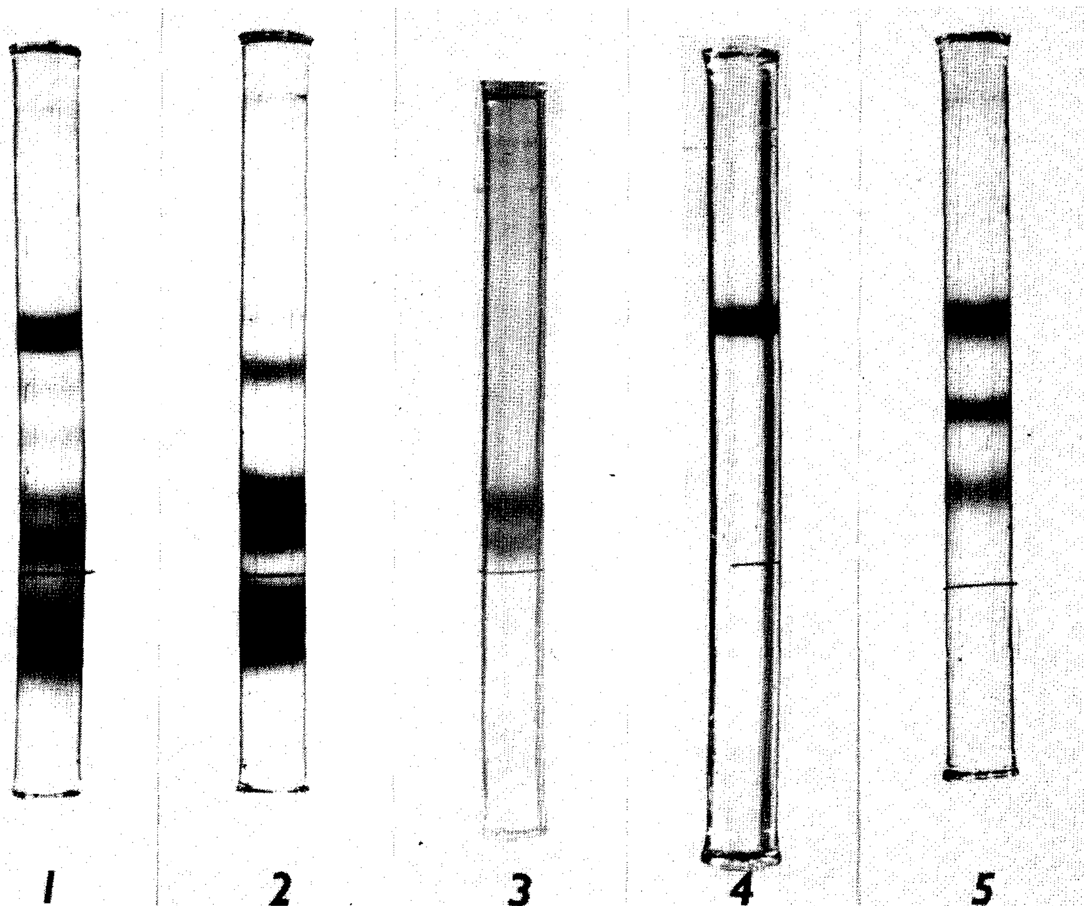

Fig. 2. SDS electrophoresis in polyacrylamide gel. 1, Trypsin (commercial), 0.025 mg; 2, first peak from the separation of trypsin in Fig. 1, 0.05 mg; 3, α-trypsin, 0.005 mg; 4, β-trypsin, 0.01 mg; 5, reference proteins (trypsinogen, 0.003 mg; myoglobin, 0.002 mg; cytochrome *c*, 0.001 mg). Conditions: 3 mA per tube; for other experimental details see Experimental.

3 and 4). The elution volume as a function of sodium chloride concentration at pH 3 is shown in Fig. 3. The interaction of both trypsin forms with the matrix is very weak in buffers lacking the salt and the elution volume is almost identical with the elution volume observed in chromatographic experiments where the gel permeation effect only plays a role and α- and β-trypsin remain unresolved. The retention of both forms, however, increases almost linearly with increasing sodium chloride concentration; the increase is steeper with β-trypsin than with α-trypsin. In acidic media the hydrophobic interaction is relatively strong even at low salt concentrations (Fig. 4); thus, *e.g.*, the elution volume of β- and α-trypsin in 0.25 *M* sodium chloride at pH 3.0 is three and two times higher, respectively, than the elution volume in the absence of salt. When sulphates such as sodium or ammonium sulphate were used the same results were obtained: α-trypsin was always less retained than β-trypsin[28]. A marked pH dependence of the hydrophobic interactions of α- and β-trypsin in

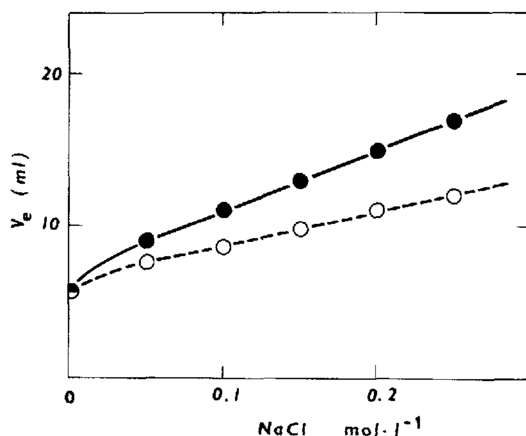

Fig. 3. Dependence of the elution volume of α- and β-trypsin on NaCl concentration in elution buffer at pH 3.0. V_e, elution volume (ml). Full line, β-trypsin; broken line, α-trypsin. Conditions: trypsin (0.02–0.1 mg) was applied on to the Spheron P-300 column in 75 μl of 0.02 M glycine hydrochloric acid buffer (pH 3.0) and eluted with the same buffer a flow-rate of 40 ml h^{-1} at 20°C. The other conditions are given under Experimental.

sodium chloride at pH lower than 5.5 was observed. The retention of both trypsins is reduced at higher pH values (Fig. 4). Thus, *e.g.*, at pH 4.5 and with 1.5 M sodium chloride the retention volume of β-trypsin is twice that at pH 5.50.

Comparison of the retentions of trypsin and α-chymotrypsin on octyl-Sepharose and Spheron. A comparison of the retentions of α- and β-trypsin, diisopropylphosphoryltrypsin, α-chymotrypsin and diisopropylphosphorylchymotrypsin on octyl-Sepharose and Spheron is shown in Table I. The retention of proteins on both

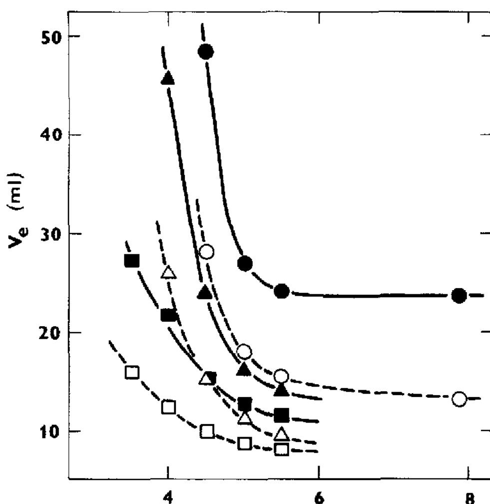

Fig. 4. Dependence of the elution volume of α- and β-trypsin on pH at various NaCl concentrations.■, □, 0.5 M NaCl in elution buffers; ▲, △, 1.0 M NaCl; ●, ○, 1.5 M NaCl. Full line, β-trypsin; broken lines, α-trypsin. Buffers used: 0.02 M glycine–hydrochloric acid (pH 3.5), 0.05 M acetate and 0.05 M phosphate for higher pH values. For other conditions see Fig. 3 and Experimental.

TABLE I

DEPENDENCE OF THE RETENTION OF β-TRYPSIN, DIISOPROPYLPHOSPHORYLTRYPSIN, α-CHYMOTRYPSIN AND DIISOPROPYLPHOSPHORYLCHYMOTRYPSIN DURING HYDROPHOBIC CHROMATOGRAPHY ON OCTYL-SEPHAROSE CL-4B AND SPHERON P-300 AT VARIOUS SODIUM CHLORIDE CONCENTRATIONS OF THE ELUTION BUFFER

The retention of proteins is expressed by the distribution coefficient K'. Conditions: 0.02–0.1 mg of protein was applied on to the column of octyl-Sepharose (flow-rate 15 ml h^{-1}), or the Spheron column at the same flow-rate as described in Fig. 3. The elution buffer was 0.05 M phosphate with the given NaCl concentration, 20°C, pH 5.50.

	K'					
	Molarity of NaCl in octyl-Sepharose			*Molarity of NaCl in Spheron*		
	0.5	*1.0*	*1.5*	*0.5*	*1.0*	*1.5*
Diisopropylphosphoryltrypsin	0.27	0.40	0.62	0.43	0.88	1.63
β-Trypsin	0.29	0.85	1.69	0.28	1.18	2.24
Diisopropylphosphorylchymotrypsin	0.17	0.70	1.49	0.13	0.20	0.44
α-Chymotrypsin	0.81	3.8		0.19	0.36	0.89

chromatographic supports can be ascribed to hydrophobic interactions of non-polar amino acid side-chains of the proteins with the hydrophobic binding sites of the support[28–31]. α-Chymotrypsin is more retained than trypsin on octyl-Sepharose. The retention on Spheron decreases in the order β-trypsin, α-trypsin, diisopropylphosphoryltrypsin, α-chymotrypsin, diisopropylphosphorylchymotrypsin at any concentration of sodium choride or ammonium sulphate in the elution buffer. The retention of the diisopropylphosphoryl derivatives was always lower than that of the unmodified enzymes (Table I). A correlation of the retention parameter K' of β-trypsin, diisopropylphosphoryltrypsin, α-chymotrypsin, diisopropylphosphorylchymotrypsin, chymotrypsinogen and basic pancreatic trypsin inhibitor on octyl-Sepharose and Spheron (Fig. 5) indicates that the retention on both supports is proportional; however, the individual curves differ markedly in slope.

Hydrophobic interaction of the trypsin–inhibitor complex. The formation of complexes of trypsin with its naturally occurring trypsin inhibitors brings about a drastic change in the elution profile during hydrophobic interaction chromatography (Fig. 6). We investigated the retention of six proteinase inhibitors and their complexes with bovine trypsin. If the inhibitor itself is retained only weakly, the complex of the inhibitor with trypsin is also retained only slightly. Table II summarizes the retention data for the inhibitor from cow colostrum, the proteinase inhibitor from bull seminal plasma and also for the basic pancreatic trypsin inhibitor. The complexes of β-trypsin with the colostrum and bull seminal plasma inhibitors are more than ten times less retained than β-trypsin and five times less than α-trypsin. There was no difference in the retention of the complexes of these inhibitors with α- and β-trypsin. The complex trypsin–basic pancreatic trypsin inhibitor is more retained than the complexes of trypsin with the inhibitor from cow colostrum and with the seminal plasma inhibitor, but compared with β-trypsin and especially with the basic pancreatic inhibitor the retention of the complex is greatly reduced (Table II). The same

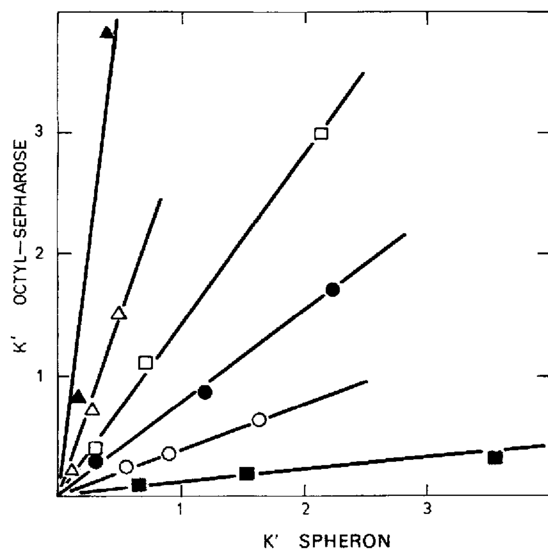

Fig. 5. Correlation of the retentions (expressed by K') of β-trypsin, diisopropylphosphoryltrypsin, α-chymotrypsin and diisopropylphosphorylchymotrypsin, chymotrypsinogen and basic pancreatic trypsin inhibitor on octyl-Sepharose and Spheron P-300 at various NaCl concentrations. ●, β-Trypsin; ○, diisopropylphosphoryltrypsin; ▲, α-chymotrypsin; △, diisopropylphosphorylchymotrypsin; □, chymotrypsinogen; ■, basic pancreatic trypsin inhibitor. For detailed conditions see Table I.

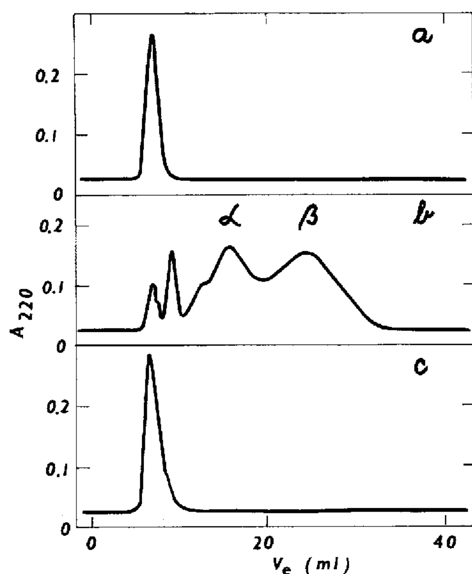

Fig. 6. Elution profile of cow colostrum trypsin inhibitor, trypsin and their complex on hydrophobic chromatography. Full line, absorbance at 220 nm (A_{220}); V_e, elution volume (ml). (a) Trypsin inhibitor from cow colostrum, 0.044 mg; (b) commercial trypsin, 0.21 mg; (c) trypsin–inhibitor complex (1:1), 0.056 mg. Conditions: the protein was applied on to a Spheron P-300 column (248 × 6.24 mm I.D.) in 75 μl of the elution buffer and eluted with 0.05 M phosphate–1.0 M NaCl (pH 5.5). Flow-rate, 40 ml h^{-1}; 20°C. The complex was obtained by mixing equal amounts of equimolar solutions of the inhibitor and commercial trypsin; the sample was applied after standing for 15 min.

TABLE II

RETENTION OF α- AND β-TRYPSIN, α-CHYMOTRYPSIN, COW COLOSTRUM TRYPSIN INHIBITOR, BASIC TRYPSIN INHIBITOR FROM BULL SEMINAL PLASMA AND BASIC PANCREATIC TRYPSIN INHIBITOR AND OF COMPLEXES OF TRYPSIN AND α-CHYMOTRYPSIN WITH THESE INHIBITORS DURING HYDROPHOBIC INTERACTION CHROMATOGRAPHY

The retention is expressed by the distribution coefficient, K. The elution buffer was 0.05 M phosphate containing 1.5 M NaCl, pH 7.85, 20°C. The other conditions are given in the legend to Fig. 6 and under Experimental.

Protein	*K*
α-Trypsin	2.5
β-Trypsin	6.3
α-Chymotrypsin	3.2
Cow colostrum trypsin inhibitor	0.85
Bull seminal plasma trypsin inhibitor	0.43
Basic pancreatic trypsin inhibitor	8.7
α-Trypsin (β-trypsin)–cow colostrum trypsin inhibitor complex	0.49
α-Trypsin (β-trypsin)–bull seminal trypsin inhibitor complex	0.53
α-Trypsin (β-trypsin)–basic pancreatic trypsin inhibitor complex	1.7
α-Chymotrypsin–cow colostrum trypsin inhibitor complex	0.28
α-Chymotrypsin–bull seminal plasma trypsin inhibitor complex	0.60
α-Chymotrypsin–basic pancreatic trypsin inhibitor complex	0.80

TABLE III

DEPENDENCE OF THE DISTRIBUTION COEFFICIENT K ON CONCENTRATION OF Na_2SO_4 AND $(NH_4)_2SO_4$ IN HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF MODEL PEPTIDES

Conditions: 0.05 mg of peptide was applied on to a Spheron P-300 column; the elution buffer was 0.05 M phosphate of the given salt concentration, final pH 5.50, 20°C, flow-rate 40 ml h^{-1}. For other conditions see Experimental.

Peptide	*K*					
	Molarity of Na_2SO_4			*Molarity of* $(NH_4)_2SO_4$		
	0.4	*0.6*	*0.89*	*0.5*	*1.0*	*1.5*
Gly–Gly–Gly			0.05			0.09
Gly–Leu	0.154	0.241	0.30		0.281	0.303
Ala–Tyr	0.427	0.597	0.722	0.436	0.624	0.965
Tyr–Gly	0.543	0.623	0.799	0.531		0.969
Pro–Leu–Gly	0.312	0.521	0.728	0.335	0.686	1.36
Ala–Pro–Leu–Gly	0.298	0.562	0.733			1.68
Gly–Trp	1.49	1.95	2.55	1.37	2.18	3.64

behaviour as β-trypsin and α-trypsin is also shown by α-chymotrypsin and its complexes (Table II).

Hydrophobic interactions of model peptides. The contribution of the individual non-polar amino acid side-chains to the hydrophobic interactions of proteins was studied by hydrophobic interaction chromatography of model oligopeptides on octyl-Sepharose and Spheron. The results in Table III show that the tryptophan-containing peptide is significantly more retained than peptides of other hydrophobic amino acids. As regards the size of the surface of the non-polar side-chain[32], it is largest with the tryptophanyl residue and also the retention is maximal. However, the difference in the size of the tryptophanyl non-polar indole residue is not as large as the difference in the retention of Gly–Trp and of other peptides used.

DISCUSSION

Hydrophobic chromatography of proteins on two supports

Globular proteins in native conformation differ markedly as regards the strength of their interaction with a support containing hydrophobic binding sites, such as Spheron[29], even though the content of hydrophobic amino acids of various proteins differs only slightly on average[33]. Certain proteins, such as α-amylase and serum albumin in the F-conformation (acidic pH), are very strongly retarded and can be eluted only after the polarity of the eluent has been decreased by the addition of a considerable amount of an organic solvent[29]. Other proteins, such as the two proteinase inhibitors from cow colostrum and bull seminal plasma and likewise ribonuclease A and serum albumin in the N-conformation (neutral pH)[29], interact only weakly, even at a relatively high concentration of the salting-out salt (*e.g.* 2–3 *M* sodium chloride). The proteins investigated in this study, *i.e.*, trypsin, α-chymotrypsin and also the basic pancreatic trypsin inhibitor, belong neither to the strongly "hydrophobic" nor "hydrophilic" proteins. Their interaction with both supports is weak in the absence of salt yet it is strongly enhanced by higher concentrations of sodium chloride, sodium sulphate, ammonium sulphate, etc., in the eluent.

It has been suggested that the retention during hydrophobic chromatography can be related to the non-polar contact area[34,35], *i.e.*, to the area where the water molecules are excluded upon binding of the solute.

The retention of the protein (or another related parameter such as the distribution coefficient K or K')* can therefore be utilized for a comparison of the non-polar regions existing on the surface of protein molecules. The chromatographic behaviour of the small peptides (Table III) indicates that the amino acid which contributes most to the retention is tryptophan, whose side-chain possesses the largest contact area of all the hydrophobic amino acids[32]. The most striking effect on the retention of proteins may be ascribed to large hydrophobic domains on the surface created by several non-polar amino acid side-chains.

The interaction of proteins in the native state, possessing a fixed conformation, will also depend on the localization of the surface non-polar residues of the protein, *i.e.*, it will be different with residues in crevices and with those exposed to an external environment.

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* K is related to the true equilibrium binding constant[36]. For the definition see Experimental.

The accessibility of the surface non-polar residues of proteins of similar size can be compared if the retention of the proteins is assayed on two different chromatographic supports under identical conditions. We used octyl-Sepharose CL-4B, in which the flexible non-polar octyl ligand is bonded through a five-atom spacer to the polysaccharide matrix thus made capable of interaction even with the protein residues localized in crevices. The other chromatographic material (Spheron P 300) is a highly cross-linked macroreticular resin in which the hydrophobic binding sites are formed by the aliphatic polymer backbone of very limited mobility and firmly incorporated into the polymer network in the macropore walls[21]. We assume that this material interacts preferentially, because of its structure, with the "exposed" type of non-polar side-chains of proteins.

Differences between α- and β-trypsin in solution

The remarkable decrease in the retention of α-trypsin compared with β-trypsin (Figs. 1, 3 and 4; Table II) observed over a wide range of pH and concentrations of sodium chloride and ammonium sulphate on Spheron[28] indicates that the conformational changes occur in solution after cleavage of the susceptible bond Lys(145)–Ser(146) in α-trypsin. Some of the surface hydrophobic side-chains of α-trypsin formed are much less exposed to the external environment than those of β-trypsin. These results are consistent with earlier spectroscopic observations[37] indicating changes in the tryptophan environment and β-pleated structure content of α- and β-trypsin.

We have observed no difference in the behaviour of α- and β-trypsin on octyl-Sepharose even at high salt concentrations; the hydrophobic groups of α- and β-trypsin are almost equally accessible to flexible ligands attached by a spacer to the Sepharose matrix. Therefore, conformational changes following the conversion of β- to α-trypsin are probably not extensive and bring about just local surface shielding.

The experiments with the complexes of α- and β-trypsin with inhibitors indicate that the non-polar residues of trypsin responsible for these differences are probably localized in its contact region for inhibitors, as there is no difference in the retentions of the complexes of α- and β-trypsin with the same inhibitor.

Comparison of trypsin, chymotrypsin and their diisopropylphosphoryl derivatives with chymotrypsinogen

The retentions of β-trypsin, α-chymotrypsin and their diisopropylphosphoryl derivatives on both supports used are comparable (Fig. 5), even though different types of binding are involved. A reasonable correlation of the distribution coefficients (K') was obtained in 0.5, 1.0 and 1.5 M sodium chloride for octyl-Sepharose and Spheron; for all five proteins as well as for the basic pancreatic trypsin inhibitor straight lines were obtained. The individual lines, however, differ markedly in slope. The steepest slope in Fig. 5, obtained with α-chymotrypsin, can be explained by the "pocket" nature of the interacting residues. Adjacent to the catalytic site of α-chymotrypsin there is a deep hydrophobic pocket; a hydrophobic group occurring in this area can make numerous hydrophobic contacts, especially with Val(213), Trp(215) and Tyr(228)[38,39]. This pocket is absent in chymotrypsinogen[39], which has a much less steep slope (Fig. 5) than α-chymotrypsin. Trypsin is known to differ from chymotrypsin in the binding pocket suitable for the positively charged side-chain of basic amino acids; therefore, its interaction with the hydrophobic octyl ligand of octyl-Sepharose is weaker and the slope of the correlation of K' on both

supports again is less steep than with α-chymotrypsin. Both diisopropylphosphoryl derivatives are not only less retained on both the supports but also show a less steep slope (Fig. 5). Labelling with diisopropylfluorophosphate obviously brings about a shielding of some of the partially buried residues in the pockets. The evidence obtained from chromatographic experiments is consistent with the results obtained in difference spectroscopy studies[9].

Non-polar amino acids on the surface of the molecule of trypsin and basic pancreatic trypsin inhibitor

The hydrophobic residues localized in the contact area of the trypsin–trypsin inhibitor complexes are responsible for most of the hydrophobic contacts with the chromatographic support since the complexes are considerably less retained than the enzymes themselves (Table II; Fig. 6). In bovine trypsin the active site area involves the hydrophobic residues of Trp(141), Tyr(151), Val(154) and Leu(155). The other hydrophobic residues localized on the surface near to the active site are Trp(215), Tyr(228) and Leu(99). There are three other hydrophobic side-chains, of Tyr(39) and Tyr(59) and of Phe(41)[15,16] situated in the contact area (Fig. 7). The contribution of the other non-polar surface residues to the retention of trypsin is almost negligible, probably because these residues are secluded, with the exception of residues located near the C-terminal part of the molecule (Fig. 7). Additional evidence revealed enzymes inhibited by diisopropyl fluorophosphate. Substitution of the serine residue in trypsin or chymotrypsin by diisopropyl fluorophosphate or the presence of *p*-aminobenzamidine in the elution buffer considerably reduced the retention.

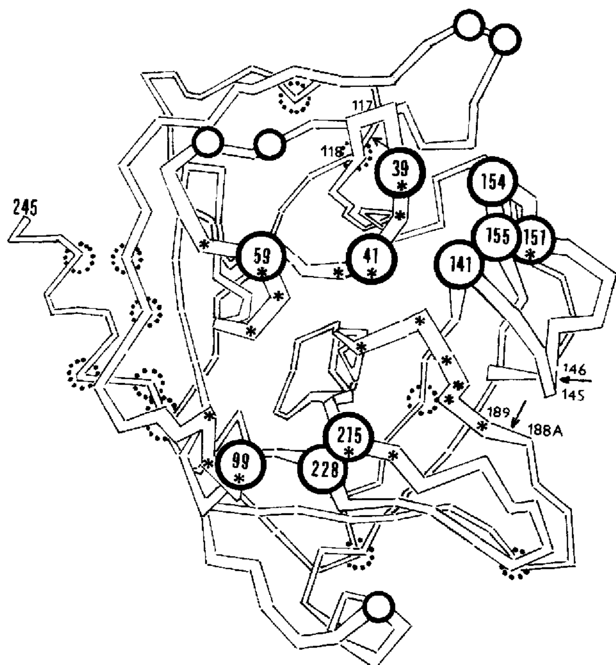

Fig. 7. Schematic drawing of the positions of α-carbons of the polypeptide chain of β-trypsin. The positions of the α-carbons of surface hydrophobic amino acids are indicated by circles; the numbers indicate residues mentioned in the text. The residues interacting with BPTI[15,16] are marked with an asterisk, surface non-polar residues located on the opposite side of the active site are designated by dotted circles; the sites of cleavage in α- and β-trypsin are marked by arrows. The positions of α-carbons are according to ref. 12.

Basic pancreatic trypsin inhibitor and cow colostrum trypsin inhibitor

The basic pancreatic trypsin inhibitor is retained most ($K = 8.7$ in 1.5 *M* sodium chloride) on Spheron of all the proteins tested in this study; the correlation of K' for octyl-Sepharose and Spheron (Fig. 5) is represented by a straight line with the smallest slope. This phenomenon can be accounted for (a) by the compact molecular structure of the inhibitor lacking any deeper hydrophobic pockets or (b) by the smaller dimensions of the inhibitor molecule compared with trypsin or chymotrypsin. The volume accessible to the inhibitor in Spheron or Sepharose columns is about 20–30% larger than that to trypsin or chymotrypsin. We have also compared the distribution coefficients (K) of the basic pancreatic trypsin inhibitor and of other proteins of equal molecular weight. The points characterizing the pancreatic inhibitor lay very near to the abscissa (Fig. 5). Therefore, the explanation of the strong retention of the inhibitor given under (a) seems more plausible.

The colostrum inhibitor is retarded only slightly by both supports even though its primary structure shows a pronounced homology with the structure of the basic pancreatic trypsin inhibitor[40]. It has been postulated that the three-dimensional structure of both proteins is homologous also[41]. The colostrum inhibitor is a glycoprotein and its polypeptide chain is nine amino acid residues longer than that of the pancreatic inhibitor. The polysaccharide moiety, which is more hydrophilic and bulkier than the polypeptide moiety itself, is bound to one asparagine residue [Asn(24)[42,43]]. The hydrophobic contacts of the residues adjacent to this asparagine are therefore shielded. Nuclear magnetic resonance studies have shown that the environment of two aromatic residues at least, namely Tyr(21) and Tyr(23)*, is affected by the polysaccharide moiety[43]. The presence of the hydrophilic residues, which extend the polypeptide chain of the colostrum inhibitor, as well as the replacement of certain non-polar residues lying on the surface of the molecule of the pancreatic inhibitor by polar residues [Tyr(10) by Ala, Pro(9) by Gln, Leu(6) by Glu, Val(34) by Thr] could also contribute to the weaker retention of the colostrum inhibitor and also of its corresponding complexes with α- and β-trypsin.

CONCLUSION

We conclude that the results obtained with the hydrophobic chromatography of α- and β-trypsin and of their complexes with inhibitors can be explained on the basis of a knowledge of the structure of these proteins. Consequently, the method introduced can be employed for the characterization of proteins and for the investigation of their interactions when little is known about their structure and where a special role of hydrophobic interactions can be assumed, as for example with membrane proteinases. Such a system (acrosin-seminal inhibitors) is being studied at present.

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* The residues are numbered according to the basic pancreatic trypsin inhibitor.

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