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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF COMPLEX MIXTURES OF CYANOGEN BROMIDE-PRODUCED PEPTIDES FROM DIFFERENT PROTEINS

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SUMMARY

A variety of approaches have been required in order to achieve the resolution of large fragments from cyanogen bromide (CNBr) digests of Inc k chain (an immunoglobulin light chain), human serum albumin (HSA) and four of its mutants. Reversed-phase high-performance liquid chromatography (RP-HPLC) under different conditions failed to resolve the Inc k chain digest; the three CNBr fragments (3.1, 6.7 and 13.7 kDa) were separated in a homogeneous form by gel HPLC. Five of the seven CNBr fragments (ranging from 3.4 to 20.0 kDa) obtained from CNBr cleavage of HSA can be resolved by a single reversed-phase HPLC step; separation of the other two requires modification of the eluent composition. Some structural features of the peptides seem to influence their chromatographic behaviour; by examining the elution patterns from albumin mutants, the sequence-related contribution of single amino acid residues is apparent.

INTRODUCTION

Considerable progress has been achieved in recent years in the resolution of small peptides by reversed-phase high-performance liquid chromatography (RP-HPLC). Standard procedures are now available, and the role of a variety of mobile-phase parameters (such as the pH, buffer ion composition and concentration of organic solvent modifier) on peptide resolution is well documented[1–6].

RP-HPLC has also been extended to the purification of large denatured peptides; although the successful use of large pore diameters and short-chain bonded phases[7–9] and also of ternary solvent systems[4,9,10] in resolving some mixtures has been reported, the technique still requires optimization on a case-by-case basis. Much more than small peptides, the larger peptides may have a hydrophobic nature and similar physico-chemical properties which make them interact with each other rather than establishing selective interactions with the stationary and/or mobile phases; in addition, a single fragment, undergoing partial refolding, may generate multiple peaks. High-performance size-exclusion chromatography (HPSEC) offers a potential advantage in resolving components that have the same hydrophobic character and

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differ in size only. However, its resolving power may be limited, either by the similarity in size of the cleavage products or by their interaction with the matrix, which causes a non-ideal size-exclusion behaviour[11–15]. Hence, most of the reported applications of HPSEC to the separation of chemical and enzymatic digests of proteins have yielded only partial resolution of the mixtures[14,16].

The failure to resolve mixtures through a single HPLC approach and to predict the chromatographic behaviour of large fragments on the basis of general rules appears to be largely dependent on their structural features, which in turn reflect the molecular properties of the protein from which they originated. In order to evaluate the general usefulness and reliability of RP-HPLC and HPSEC for the resolution of large fragment mixtures, and possibly to obtain some insight into the structure–retention relationship, we compare in this paper the chromatographic behaviour of CNBr digests from an immunoglobulin light chain (Inc k chain, 23.5 kDa), human serum albumin (HSA, 66.5 kDa) and four of its genetic mutants. Using different approaches, all of the fragments have been resolved.

The results reported here show that some structural features of the fragments may be related to their retention behaviour; in particular, positional effects of specific amino acids residues were demonstrated by examining the elution patterns of the fragments from albumin mutants.

EXPERIMENTAL

High-performance liquid chromatography system

A Waters Assoc. (Milford, MA, U.S.A.) high-performance liquid chromatograph was used, which included two M 6000 solvent delivery units, an M 680 automated solvent programmer and a U6K sample injector, coupled to a Jasco (Japan Spectroscopic, Tokyo, Japan) variable-wavelength detector. Absorbance was recorded with an Omni Scribe chart recorder (Houston Instruments, Austin, TX, U.S.A.). The two TSK G 2000-SW columns (each 30 cm × 7.5 mm I.D.), connected in series, used for HPSEC were obtained from Altex (Berkeley, CA, U.S.A.). For RP-HPLC, the following columns were used: a Vydac C_{18} column (25 cm × 4.6 mm I.D., 10 μm, 300 Å pore size) from Separation Group (Hesperia, CA, U.S.A.), an Aquapore Butyl column (22 cm × 4.6 mm I.D., 10 μm, 300 Å pore size) from Pierce (Rockford, IL, U.S.A.), a μBondapack C_{18} column (30 cm × 3.9 mm I.D., 10 μm, 100 Å pore size) from Waters Assoc. and an Ultrasphere ODS column (25 cm × 4.5 mm I.D., 5 μm) from Beckman (Berkeley, CA, U.S.A.).

Reagents

All reagents were of analytical-reagent grade. Acetonitrile, supplied by Merck (Darmstadt, F.R.G.), was filtered through a 0.45-μm Millipore filter (Milford, MA, U.S.A.) and degassed under vacuum prior to use. Water was glass-distilled and purified by passing it through an UHQ Elgastat purification system (Elga, High Wycombe, U.K.). Sequenal-grade trifluoroacetic acid (TFA) was obtained from LKB (Bromma, Sweden) and iodoacetic acid from Eastman Kodak (Rochester, NY, U.S.A.); cyanogen bromide (CNBr) and dithiothreithol were purchased from Sigma (St. Louis, MO, U.S.A.). The reagents used for automated sequential analyses were supplied by Beckman (Palo Alto, CA, U.S.A.). All other chemicals were purchased from Carlo Erba (Milan, Italy).

Sample preparation

Normal human serum albumin and its variants (Mi/Fg, Tagliacozzo, Vr/Vr, and Vanves) were purified from serum of donors according to Winter *et al.*[17]. Inc k chain was purified from the urine of a patient by standard procedures[18]. All proteins were completely carboxymethylated with iodoacetic acid following the method of Swenson *et al.*[19]. The extent of S-carboxymethylation of each protein was checked by standard amino acid analysis. CNBr cleavage was carried out on 0.25 μmol of carboxymethylated protein at a concentration of 10 mg/ml in 70% formic acid, as described by Gross[20].

Amino acid and sequence analyses

Protein and peptide samples were hydrolysed in 6 *M* hydrochloric acid for 24 h at 105°C in evacuated sealed tubes. Amino acid analyses were carried out by ion-exchange chromatography with post-column ninhydrin derivatization according to Moore[21] on a Cromakon 500 automatic analyser (Kontron, Zurich, Switzerland). The tryptophan content was determined according to Liu and Chang[22]. The amino acid sequences were determined by liquid-phase sequence degradation in a Beckman System 890 M instrument, using the fast-protein Quadrol program with precycled Polybrene. Amino acid derivatives were identified by RP-HPLC on a Beckman Ultrasphere ODS column, as described by Pucci *et al.*[23]. Hydrophilicity profiles were calculated according to Hopp and Woods[24]. CNBr fragments are designated by Roman numerals, starting from the N-terminal end of the molecule.

RESULTS AND DISCUSSION

CNBr digests were obtained from fully reduced carboxymethylated Inc protein, HSA and its genetic mutants Mi/Fg, Vr/Vr, Tagliacozzo and Vanves.

Inc protein is an immunoglobulin k light chain of 23.5 kDa, which consists of 219 amino acids. From its known primary structure[18], three fragments of 3.1, 6.7 and 13.7 kDa (starting from the N-terminal end of the molecule) are expected after subjecting the protein to CNBr treatment. Most aqueous solvents at different pH values [0.1% TFA–0.01 *M* ammonium acetate (pH 6.8)] failed to dissolve the components from freeze-dried CNBr digests completely and equally. The addition of 5 *M* urea did not improve their solubility. As the fragments were soluble in formic acid, the digestion mixture was diluted 10-fold (final formic acid concentration 7%), and samples were loaded directly on to the column. Resolution of this mixture was difficult, despite the limited number and the large difference in size of its components. The digest was poorly resolved by RP-HPLC under different conditions. Fig. 1A shows the resolution obtained using a C_4 column.

Amino acid and partial sequence analyses of the collected material showed that all the major peaks eluted between 22 and 25 min contained homogeneous CNBr I (3.1 kDa). The distribution of a fragment in several peaks may be due to modification of some amino acyl residues after CNBr treatment of the protein, or to its partial refolding. CNBr II (6.7 kDa) and CNBr III (13.7 kDa) are unresolved under the broader peak which appeared at 38–42 min. Fractions of this peak were separately collected and analysed; the ascending portion of the peak was shown to contain mainly CNBr II and the descending part mainly CNBr III. The C_{18} column was

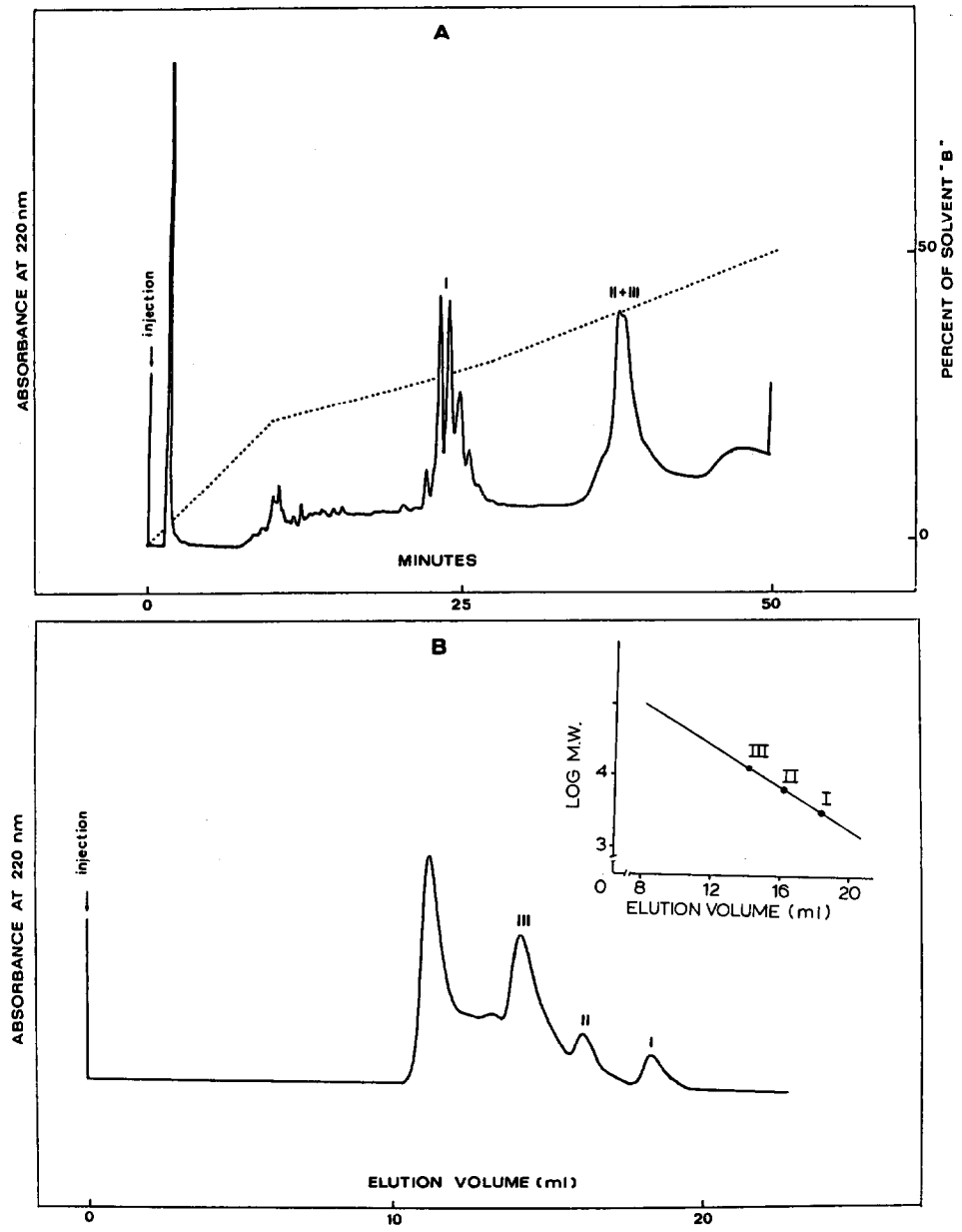

Fig. 1. Elution pattern of CNBr fragments of the whole reduced and carboxymethylated Inc k chain. The digest was dissolved at a concentration of 4.0 mg/ml in 7% formic acid solution. (A) Samples of 80 μl (corresponding to *ca.* 14 nmol of protein) were injected into an Aquapore Butyl column (22 cm × 4.6 mm I.D., 10 μm, 300 Å pore size) equilibrated with 0.05% aqueous TFA (solvent A). Acetonitrile–2-propanol (2:1, v/v) containing 0.05% TFA was solvent B. The elution was performed at room temperature and at a flow-rate of 2 ml/min, using the gradient indicated by the dashed line; 0.64 a.u.f.s. (B) Samples of 250 μl were injected into two TSK G 2000-SW columns (each 30 cm × 7.5 mm I.D.), connected in series. The eluent system used was 35% acetonitrile in 1% aqueous TFA; flow-rate, 0.4 ml/min; 0.64 a.u.f.s. Inset: Relationship between elution volume and logarithm of the MW of the CNBr fragments.

ineffective in improving separation of the mixture; the elution pattern obtained with this column was similar to that in Fig. 1A, the unique difference being the elution of CNBr I in a single, broader peak (data not shown). The separation was not substantially affected by changing the initial composition and/or the slope, or the relative proportion of acetonitrile and 2-propanol in the elution gradient.

The chromatographic behaviour of Inc k chain CNBr digest in HPSEC was then examined. Taking into account the advantages of adding volatile organic solvents to the eluent[15,25,26], the separation was carried out in acetonitrile–1% aqueous TFA (35:65); the elution pattern obtained is shown in Fig. 1B.

The determination of the material eluted under each peak, by amino acid and partial sequence analyses, showed that the fragments were eluted in order of decreasing molecular weight; a linear relationship was obtained by plotting log MW *versus* retention time (inset in Fig. 1B). As judged by N-terminal analysis, the homogeneity of the collected material was unequal; CNBr I was recovered in pure form, while CNBr II and III were slightly contaminated. However, as shown by the data reported in Table I, homogeneous fragments are eluted in the central part of the corresponding

TABLE I

AMINO ACID COMPOSITION OF THE INTACT AMYLOID Inc k CHAIN AND OF ITS CNBr PEPTIDES (RESIDUES PER MOLECULE)

CNBr fragments were obtained as described in Fig. 1B.

Amino acid	*Inc k chain*		*CNBr I*		*CNBr II*		*CNBr III*	
	*A**	*B**	*A*	*B*	*A*	*B*	*A*	*B*
Cys**	4.8	5	0.7	1	0.9	1	2.7	3
Asp	18.3	18	1.3	1	6.7	7	9.6	10
Thr***	15.6	16	1.9	2	2.8	3	10.6	11
Ser***	32.4	33	6.5	7	9.4	10	15.7	16
Glu	23.1	23	2.8	3	4.1	4	15.8	16
Pro	13.2	13	4.0	4	2.6	3	5.7	6
Gly	17.5	17	1.2	1	9.1	9	7.1	7
Ala	11.2	11	0.9	1	2.0	2	8.0	8
Val§	17.6	18	1.6	2	4.7	5	10.6	11
Met	2.0	2	–	1	–	1	–	–
Ile§	6.6	7	1.7	2	2.7	3	2.0	2
Leu§	17.8	18	3.8	4	4.7	5	9.0	9
Tyr	8.6	9	–	–	4.8	5	4.0	4
Phe	6.6	7	–	–	1.9	2	4.6	5
Lys	11.7	12	1.0	1	1.1	1	9.7	10
His	2.9	3	–	–	1.0	1	1.9	2
Arg	4.1	4	–	–	1.0	1	2.8	3
Trp§§	2.8	3	–	–	1.0	1	2.0	2
Hse	–	–	0.8	–	0.9	–	–	–
NH_2 terminus		Asp		Asp		His		Gln

* A, found values; B, values expected from the sequence[18].

** Determined as carboxymethylcysteine and cysteic acid.

*** Corrected for destruction during acid hydrolysis.

§ Corrected for slow release during acid hydrolysis.

§§ Determined after hydrolysis with 3 *N p*-toluenesulphonic acid[22].

peaks. The amino acid content of the isolated fragments accounts for the amino acid composition of the whole protein.

The recovery of injected material was satisfactory (> 75%), but the recovery of the homogeneous fragments was low. As shown in Fig. 1B, about 40% of the injected material is found in a peak eluted at 10.8 ml with an apparent MW of about 42 kDa; both N-terminal analysis and reinjection of the material in the same chromatographic system proved it to consist mainly of aggregate fragments. A small amount of undigested protein (apparent MW 23 kDa, a single N-terminal; Asp) was also recovered under the shoulder which was eluted between 12 and 13 ml. Difficulties encountered in solubilizing fragments limited the use of other elution systems. Attempts to achieve resolution with 0.01 *M* ammonium acetate (pH 6.8) resulted in a minor resolution of the solubilized components.

The structural contribution to chromatographic behaviour has been elucidated in several studies by using synthetic peptides of known amino acid sequence[6,27–29] and three-dimensional structure[25,30]. Such parameters may be tentatively evaluated, when dealing with natural large-fragment mixtures, by examining some structural features of the protein; as a useful approach, the evaluation of the hydrophilicity pattern has been suggested recently[29].

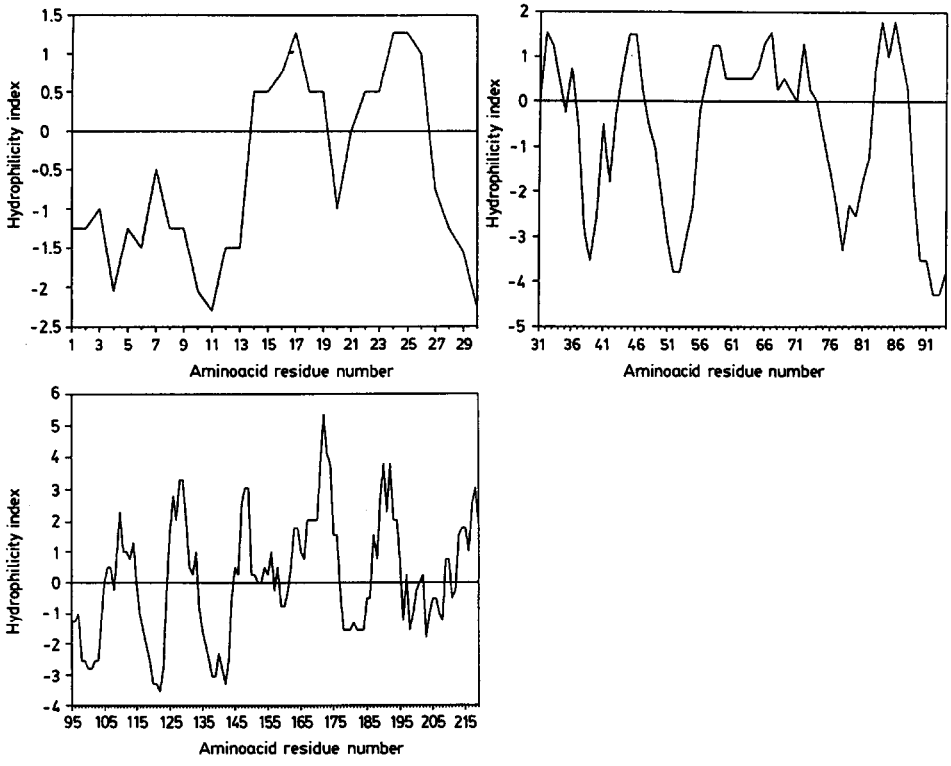

Fig. 2. Hydrophilicity profiles of Inc k chain CNBr peptides. CNBr I (top, left), II (top, right) and III (bottom) profiles are reported. Hydrophilicity and hydrophobicity are indicated by the positive and negative values, respectively.

A particular hydrophobic character was brought to light by analysing the amino acid sequence of Inc k chain CNBr peptides; as shown in Fig. 2, all peptides possess hydrophobic regions. However, the points of highest average hydrophobicity are mainly localized in CNBr II and III. The highly hydrophobic nature of these peptides, particularly of II and III, may explain our failure to obtain a good resolution in both chromatographic systems. These properties, which are known to play

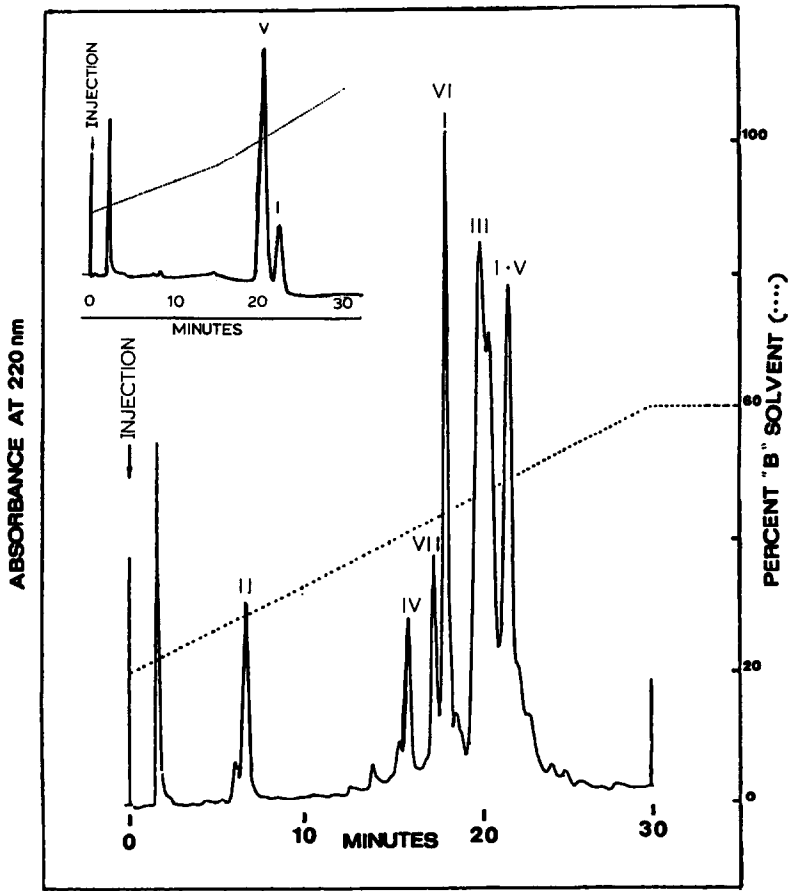

Fig. 3. Elution pattern of CNBr fragments of the whole reduced and carboxymethylated normal human serum albumin (HSA). The digest was dissolved in 0.05% aqueous TFA (pH 2.3) (solvent A). Samples of 150 μl (corresponding to *ca.* 10 nmol of protein) were injected into a Vydac C_{18} column (25 cm × 4.6 mm I.D., 10 μm, 300 Å pore size), equilibrated with 80% solvent A and 20% acetonitrile–2-propanol (2:1, v/v) containing 0.05% TFA (solvent B). Elution was performed at room temperature, using a 30-min linear gradient from 20 to 60% of solvent B; flow-rate, 2 ml/min; 1.28 a.u.f.s. Inset: Elution pattern of CNBr V and CNBr I fragments. The mixture of peptides was dissolved in 0.05% aqueous TFA; 100 μl (corresponding to *ca.* 5 nmol of peptides) were injected into a μBondapak C_{18} column (30 cm × 3.9 mm I.D., 10 μm, 100 Å pore size), equilibrated with 80% of solvent A and 20% of acetonitrile–0.05% TFA (solvent B). Elution was performed at room temperature, using a linear gradient from 20 to 35% of solvent B over a period of 15 min; the concentration of solvent B was then linearly increased to 60% in an additional period of 15 min; flow-rate, 2 ml/min; 0.64 a.u.f.s.

an essential role in RP-HPLC, make II and III behave identically under the experimental conditions used. Also, the finding of high-MW aggregates in HPSEC may be attributable to their tendency to be tightly associated through hydrophobic interactions. The higher number and larger size of the components did not prevent the complete resolution of HSA CNBr digest.

HSA (66.5 kDa) consists of a single polypeptide chain of 585 residues[31,32], from which seven large fragments (ranging between 31 and 175 residues) are obtained following CNBr cleavage. The freeze-dried digest of the protein is completely soluble in 0.05% aqueous TFA and five of the mixture components were isolated in homogeneous form and in good yield (>70%) after a single pass through the Vydac C_{18} column. Using the conditions described in Fig. 3, the elution order of most fragments appears to be correlated with the summed relative hydrophobic contributions of the component amino acids. In Table II the elution order, as calculated on the basis of the constants suggested by Guo *et al.*[28], is reported. Similar data are obtained by using other proposed constants[27,33]. These data suggest that, even with very large peptides, the retention properties appear to be properly correlated with amino acid composition, the sequence-induced contribution being negligible. In fact, the fragments, with the exception of I and V, elute in the expected order. CNBr I (85 residues) and CNBr V (117 residues), which are expected to be eluted before CNBr VI and III, respectively, were more strongly retained by the stationary phase and were eluted together in the last peak of the chromatogram. Although their resolution can be easily achieved by changing the gradient slope and the polarity of the solvent (inset in Fig. 3), their elution order is still different from the expected, CNBr I being eluted later than CNBr V.

This behaviour could be accounted for by a different distribution of polar/apolar residues through the primary structure of the two peptides. In fact, examination of the profiles in Fig. 4 shows, on the whole, a rather hydrophilic profile of CNBr I (A), the presence of a large hydrophobic region corresponding to a cluster of 15 apolar or slightly polar residues (from Phe 19 to Gln 33 of the HSA sequence). In

TABLE II

CHARACTERISTICS OF THE CNBr FRAGMENTS FROM NORMAL HUMAN SERUM ALBUMIN

Elution order		*Number of residues***	*Molecular weight*** (kDa)*
Found	*Calculated**		
II	II	36	4.1
IV	IV	31	3.4
VII	VII	37	4.0
VI	I	87	9.4
III	VI	102	11.6
V + I	V	117	13.5
	III	175	20.0

* The constants reported by Guo *et al.*[28] were used to calculate the summed hydrophobicities of the component amino acids.

** Values refer to the fragment numbers given in column 2.

TABLE III

AMINO ACID SEQUENCE OF CNBr FRAGMENTS FROM NORMAL AND VARIANT HUMAN SERUM ALBUMINS

	300 *310* *320*
CNBr IV	P ADLPSLAADF VES KDVCKNYAEADVFLGM
Tagliacozzo	N
	550 *560* *570* *580*
CNBr VII	DDFAAFVEKCCKADDKETCFA EEGKKLVAASQAALGL
Mi/Fg	E
Vr/Vr	K
Vanves	N

contrast, CNBr V has similar properties, but a more symmetrical distribution of the hydrophobic amino acids through its sequence (B). Sugita *et al.*[16] used HPSEC to separate HSA CNBr digest. Although an ideal size-exclusion behaviour was obtained, the similarity in size of the cleavage products prevented their resolution, and only peptide families were recovered.

The use of HPSEC, often required as a preliminary step in the resolution of complex mixtures, seems to be unnecessary in this instance, because of the excellent resolution that can be achieved by RP-HPLC. Comparison of the chromatographic behaviour of CNBr fragments from normal albumin and its genetic mutants allowed us to evaluate the positional contribution of specific amino acid residues. All four albumin variants that were examined arise from single-residue replacement occurring in different positions of the CNBr VII and CNBr IV sequence[34]. The primary structures of normal and abnormal fragments are indicated in Table III.

As shown by the elution profiles in Fig. 5, Mi/Fg CNBr VII, which contains the Lys 573 → Glu replacement, is eluted 2 min later than normal CNBr VII (Fig. 5A and B); on the other hand, the Glu 570 → Lys substitution, as observed in Vr/Vr CNBr VII, results in the elution 2 min earlier than the normal fragment (Fig. 5A and

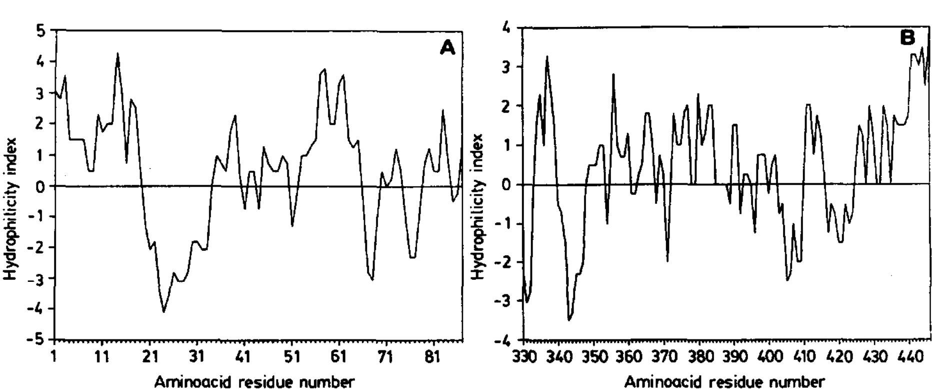

Fig. 4. Hydrophilicity profiles of normal HSA fragments CNBr I (A) and CNBr V (B).

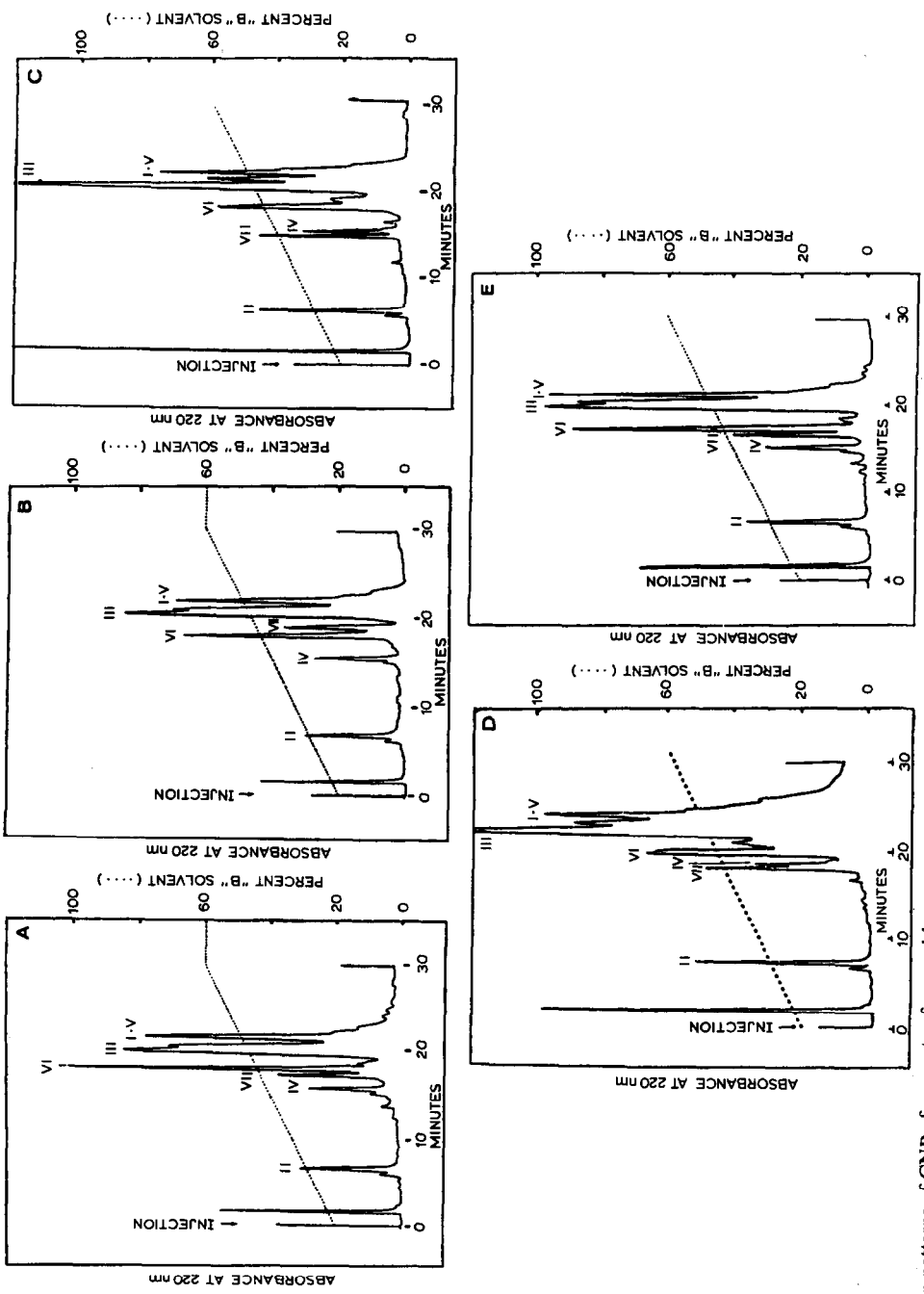

Fig. 5. Elution patterns of CNBr fragments of normal human serum albumin and its genetic variants. (A) Normal serum albumin; (B) Mi/Fg variant; (C) Vr/Vr variant; (D) Tagliacozzo variant; (E) Vanves variant. Experimental conditions as in Fig. 3.

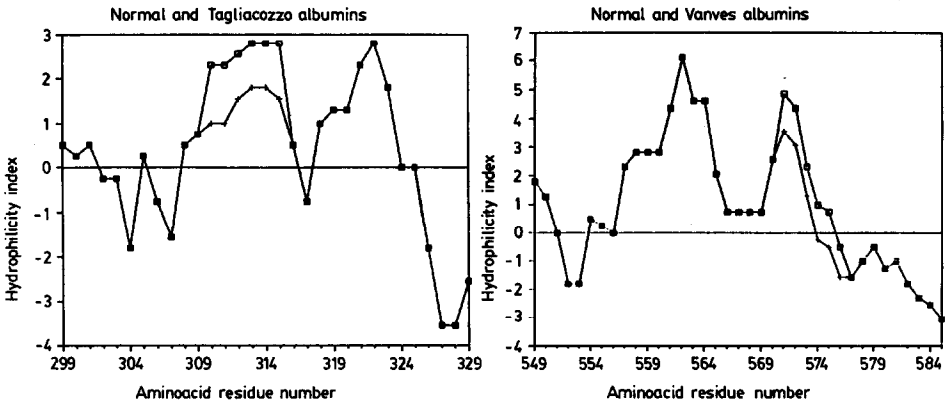

Fig. 6. Hydrophilicity profiles of fragment CNBr IV from variant Tagliacozzo and of fragment CNBr VII from variant Vanves, compared with normal human serum albumin. (□) Normal; (+) Tagliacozzo and Vanves.

C). Both the direction and the extent of the shift are in accordance with the observed substitution, which involves the loss (Mi/Fg) or the acquisition (Vr/Vr) of the same hydrophylic Lys residue, respectively.

On comparing the elution behaviour of Vanves CNBr VII and Tagliacozzo CNBr IV, it appears that the same amino acid replacement, when occurring in a different region of the sequence, may produce different effects. Both albumins originated from a Lys → Asn substitution, which takes place at position 574 in Vanves and at position 313 in Tagliacozzo. Vanves and normal CNBr VII behave identically in RP-HPLC (see Fig. 5A and E), while Tagliacozzo CNBr IV is eluted 1.7 min later than the normal one (Fig. 5D). Accordingly, the hydrophilicity profile of Tagliacozzo CNBr IV appears more extensively modified than that of Vanves CNBr VII (Fig. 6). The Lys → Asn replacement, which makes the first fragment less hydrophilic, does not appreciably affect the polarity of the second.

From the results reported here, it appears that structural features of the sample components may affect the resolving power of a chromatographic system, making it extremely efficient, as in the HSA digest, or ineffective, as in the Inc k chain digest. Apart from the size and summed hydrophobicity contributions of the component amino acids, sequence-related hydrophobic parameters seem to influence the retention properties of large fragments. In fact, the extent of a specific amino acid contribution appears to be determined not only by its peculiar properties, but also by the microenvironment in which it is situated; the Lys → Asn substitution, while having no effect on the Vanves CNBr VII elution behaviour, makes it different in the Tagliacozzo CNBr IV fragment. As a practical consequence, the chromatographic behaviour may in some instances be inadequate for recognizing abnormal fragments in a mutated protein digest.

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