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# Separation of fragments from human serum albumin and its charged variants by reversed-phase and cation-exchange high-performance liquid chromatography

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ABSTRACT

Reversed-phase high-performance liquid chromatography (RP-HPLC) and ion-exchange chromatography on poly(2-sulphoethylaspartamide)–silica (SCX) were compared as alternative approaches in characterizing charged genetic variants of human serum albumin. The chromatographic behaviour of cyanogen bromide (CNBr), tryptic and V8 protease digests from normal and mutant albumins were examined. The results showed that substituted site-containing CNBr fragments are successfully resolved by RP-HPLC; in most instances SCX and RP-HPLC are equally adequate in identifying the modified tryptic peptides from CNBr fragments; although generally useful, SCX chromatography is specifically needed in all instances where amino acid replacement is occurring in a small hydrophilic tryptic fragment and choosing *Staphylococcus aureus* V8 protease instead of tryptic digestion is advantageous.

## INTRODUCTION

More than 100 genetic variants of human serum albumin (HSA) have been identified by routine clinical electrophoresis or during population surveys and classified as slow or fast with respect to the electrophoretic behaviour of the normal protein[1,2]. The structural characterization of these variants has recently been stimulated by interest in protein genetics and evolution and by the knowledge of the complete protein[3] and genomic[4] sequence of HSA. The availability of high-resolution chromatographic techniques has so far allowed the identification of the molecular defects causing more than twenty different abnormal albumins (alloalbumins)[5–10].

A procedure suggested by Takahashi *et al.*[11] consists in the peptide mapping of the whole HSA tryptic digest through an automated tandem high-performance liquid chromatographic (HPLC) system, which is a combination of anion-exchange (IE) and reversed-phase (RP) column chromatography. Although applied to the structural studies of some variants, this method seems inadequate for identifying the substitution

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site in all mutants, as only 80% of tryptic peptides can be properly characterized[11]. Another method is based on the separation of all the seven fragments obtained after cyanogen bromide (CNBr) digestion of the whole protein, by high-performance size-exclusion chromatography (HP-SEC) combined with peptide mapping of the tryptic digest of the modified fragment by RP-HPLC[7]. The resolving power of SEC seems poor, however, and is probably limited either by the similarity in size of the cleavage products or by their interaction with the matrix.

The strategy we have developed also involves cleavage of the fully alkylated albumins by CNBr, but the isolation of CNBr produced fragments is achieved by RP-HPLC[12]. Despite the large number and size of the components (ranging between 31 and 175 residues), the complete resolution of the CNBr digest is easily accomplished. Moreover, the fragment containing the amino acid substitution may be immediately identified as the retention time of most fragments on the RP support used can be correlated with their amino acid composition[13]. Sequential analysis of the abnormal peptide isolated from the tryptic digest of the modified CNBr fragment allowed us to elucidate the molecular lesion of all the variants studied in our laboratory[5,8,9]. However, the separation by RP-HPLC of tryptic peptides was sometimes difficult, especially when small and extremely hydrophilic peptides are involved. In these cases RP-HPLC is not sufficient to resolve these peptides, so an alternative chromatographic method is needed that provides the required selectivity.

A relatively new material, poly(2-sulphoethylaspartamide)–silica, has been reported to possess good selectivity and efficiency in the purification of peptides as a strong cation-exchange (SCX) packing material[14]. As suggested by Alpert and Andrews[15], retention of peptides on this material is proportional to the number of basic residues; moreover, the selectivity can be affected by addition of various levels of organic solvents to the mobile phases. This material thus displays a selectivity complementary to that of reversed phases and has also been successfully used to resolve peptides containing modified amino acids[14,15].

This paper describes the application of cation-exchange chromatography to the resolution of tryptic and *Staphylococcus aureus* V8 protease digests obtained from CNBr fragments of normal and abnormal albumins. Also, data are presented which show that SCX chromatography in some instances may be a unique means for the unambiguous identification of the molecular defect of HSA charged variants. The results obtained may indicate general utility of the sulphoethylaspartamide sorbent for the routine characterization of proteins containing charged amino acid substituents.

## EXPERIMENTAL

### *Apparatus*

The chromatographic system consisted of two Waters Assoc. (Milford, MA, U.S.A.) Model M 6000 pumps equipped with one M 680 automated gradient controller and a U6K sampling valve with a 2-ml sample loop. A Jasco (Japan Spectroscopic, Tokyo, Japan) Uvidec 100-III variable-wavelength UV detector was used to monitor the column effluent at 220 nm. Chromatograms were recorded with an Omni Scribe chart recorder (Houston Instruments, Austin, TX, U.S.A.).

*Columns*

Peptide mixtures were separated on RP- and SCX-HPLC columns. For RP-HPLC the following columns were used: an Aquapore RP-300 (10 μm) column (25.4 cm × 7 mm I.D.) from Brownlee Labs. (Santa Clara, CA, U.S.A.), a μBondapak $C_{18}$ (10 μm, 100 Å pore size) column (30 cm × 3.9 mm I.D.) from Waters Assoc. and a Vydac (10 μm, 300 Å pore size) column (25 cm × 4.6 mm I.D.) from Separation Group (Hesperia, CA, U.S.A.). For cation-exchange chromatography a poly-(2-sulphoethylaspartamide) column (20 cm × 4.6 mm I.D.) was used, purchased from Nest Group (Southborough, MA, U.S.A.).

*Chemicals*

Acetonitrile of HPLC grade was obtained from Carlo Erba (Milan, Italy) and filtered through a 0.45-μm Millipore filter prior to use. Doubly distilled water was purified by passage through an UHQ Elgastat purification system (Elga, High Wycombe, U.K.). Trifluoroacetic acid (TFA) of sequenal grade was purchased from LKB (Bromma, Sweden) and CNBr 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 reagents were of analytical-reagent grade and were obtained from Carlo Erba. Trypsin [L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK-treated] was supplied by Worthington Biochemicals (Freehold, NY, U.S.A.) and *Staphylococcus aureus* V8 protease by Boehringer (Mannheim, F.R.G.).

*Sample preparation*

CNBr fragments from HSA and its charged variants (Roma, Verona, Castel di Sangro) were obtained as previously described[12]. Digestion of these peptides with TPCK-treated trypsin was performed according to Swenson *et al.*[16]. *Staphylococcus aureus* V8 protease digestion was carried out on CNBr VI from normal and Castel di Sangro albumins for 24 h at room temperature under conditions in which this enzyme is specific for glutamic acid residues (enzyme-to-substrate ratio, 1:20, w/w; 0.05 *M* $NH_4HCO_3$, pH 7.8)[17].

*Identification and nomenclature of peptides*

All peptides were identified by their amino acid composition or amino acid sequence. Amino acid analyses were carried out by ion-exchange chromatography with post-column ninhydrin derivatization according to Moore[18] on a Cromakon 500 automatic analyser (Kontron, Zurich, Switzerland). The amino acid sequences were determined by liquid-phase sequence degradation on 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.*[19].

Tryptic and V8 protease peptides are designated T and S, respectively, and numbered consecutively in their predicted order in the sequence[20].

## RESULTS AND DISCUSSION

In our previous structural studies of HSA mutants, both recognition and preparative isolation of the substituted-site-containing fragments have been successfully

obtained by comparatively mapping on RP-HPLC CNBr digests from S-carboxymethylated normal and variant albumins[12,13]. Unlike all other cases, identification of the molecular defect of a new variant named Castel di Sangro has required a modification of our strategy. Castel di Sangro is a fast-migrating albumin which arises from a point mutation occurring at position 536 (Lys → Glu), in CNBr VI (residues 447–548)[21].

Under the conditions that we proposed previously, amino acid substitution appears to be related to the retention time of the abnormal fragment on RP-HPLC[13]; in accordance with the loss of a hydrophilic Lys residue, fragment CNBr VI from Castel di Sangro albumin is eluted 2 min later than the normal counterpart, partially overlapping CNBr III fragment. However, its resolution was obtained by slightly changing the gradient slope, as shown in Fig. 1. Identification of the recovered fragments was checked by N-terminal and amino acid analyses; the amino acid composition of Castel di Sangro CNBr VI, showing a decrease of one lysine and the presence of an additional glutamic acid residue, confirms this as the variant fragment.

Purified CNBr VI from the normal and Castel di Sangro albumins were then cleaved with trypsin and the digests were mapped by RP-HPLC on an Aquapore RP-300 column. This procedure was ineffective in identifying the variant peptide; as shown in Fig. 2A and B, the elution patterns of the two digests are indistinguishable. Analyses of the recovered material showed that only seven of the fourteen peptides

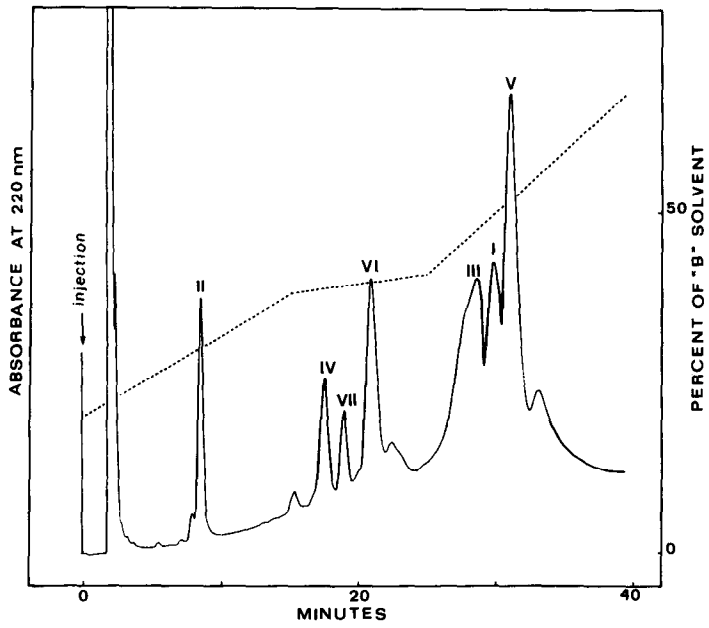


Fig. 1. Elution pattern of CNBr fragments of Castel di Sangro albumin. The digest was dissolved in 0.05% TFA (pH 2.3) (solvent A); samples of 100 μl (corresponding to *ca.* 10 nmol of protein) were injected into a Vydac $C_{18}$ (10 μm, 300 Å pore size) column (25 cm × 4.6 mm I.D.), equilibrated with 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 the gradient indicated by the dashed line. Flow-rate, 2 ml/min; absorbance range, 0.64 full-scale.

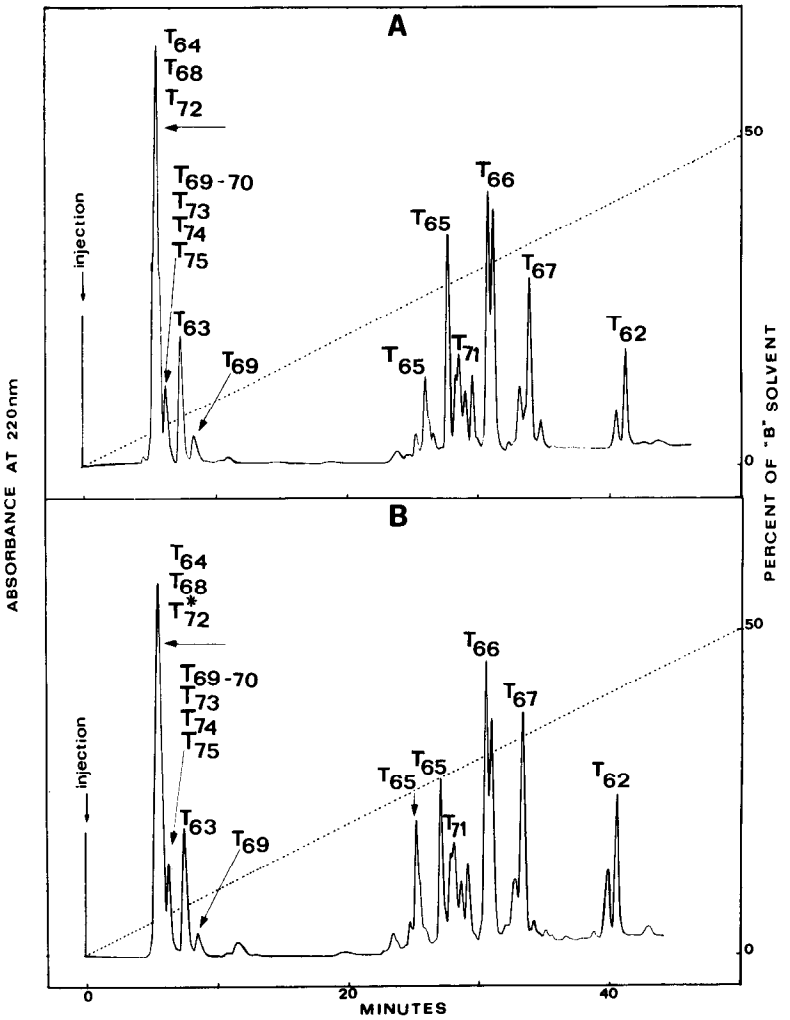


Fig. 2. Comparative tryptic peptide maps of CNBr VI from normal human serum albumin (A) and Castel di Sangro variant (B). 5 nmol of each CNBr digest were chromatographed on an Aquapore RP-300 (10 $\mu$m) column (25 cm × 7.5 mm I.D.) under the following conditions: eluent A, 0.05% aqueous TFA; eluent B, 0.05% TFA in acetonitrile. Elution was performed at room temperature and at a flow-rate of 1.5 ml/min using the gradient indicated by the dashed line. Absorbance range, 0.64 full-scale. T = tryptic peptides. Peptides are designated by arabic numerals following the predicted order in the albumin sequence[20]. Abnormal peptides are marked with asterisks. The presence of two peptides with the same amino acid composition is due to different extents of cysteine alkylation.

expected on the basis of the known sequence of CNBr VI (Table I) are obtained as homogeneous species, all the others, including the modified one, $T^*_{72}$, being eluted unresolved under the first two peaks. The use of a $\mu$Bondapak $C_{18}$ column and of supports with short-chain bonded phases did not substantially improve the resolution of the small fragments.

TABLE I

AMINO ACID SEQUENCE OF TRYPTIC FRAGMENTS FROM CNBr VI OF HSA

$T_{62}$: P–C–A–E–D–Y–L–S–V–V–L–N–Q–L–C–V–L–H–E–K (450, 460)

$T_{63}$: T–P–V–S–D–R (470)

$T_{64}$: V–T–K

$T_{65}$: C–C–T–E–S–L–V–N–R (480)

$T_{66}$: R–P–C–F–S–A–L–E–V–D–E–T–Y–V–P–K (490, 500)

$T_{67}$: E–F–N–A–E–T–F–T–F–H–A–D–I–C–T–L–S–E–K (510)

$T_{68}$: E–R (520)

$T_{69}$: Q–I–K

$T_{70}$: K

$T_{71}$: Q–T–A–L–V–E–L–V–K (530)

$T_{72}$: H–K[a]–P–K

$T_{73}$: A–T–K (540)

$T_{74}$: E–Q–L–K

$T_{75}$: A–V–M

[a] Lys 536 is substituted by Glu in Castel di Sangro variant.

As an alternative method to achieve purification of the peptide mixture, we submitted the two tryptic digests to ion-exchange chromatography. The strong cation exchanger poly(sulphoethylaspartamide) was chosen; in agreement with its excellent selectivity[14,15], this sorbent allowed the identification of the variant peptide. Profiles obtained by SCX chromatography are compared in Fig. 3A and B. Normal and CNBr VI tryptic patterns show the same number of peaks, and all but one eluted with similar retention times. The shift of $T^*_{72}$ relative to $T_{72}$ indicates the former to be the variant peptide. This was confirmed by sequence analysis of the two fragments: $T^*_{72}$ gave the sequence H–E–P–K, instead of H–K–P–K as determined for the normal fragment $T_{72}$. The elution order was as expected on the basis of the nominal net positive charge, $T_{72}$ (+4) being eluted about 8 min later than the modified $T^*_{72}$ (+3).

Owing to their similarity in net positive charge, many of the tryptic peptides from

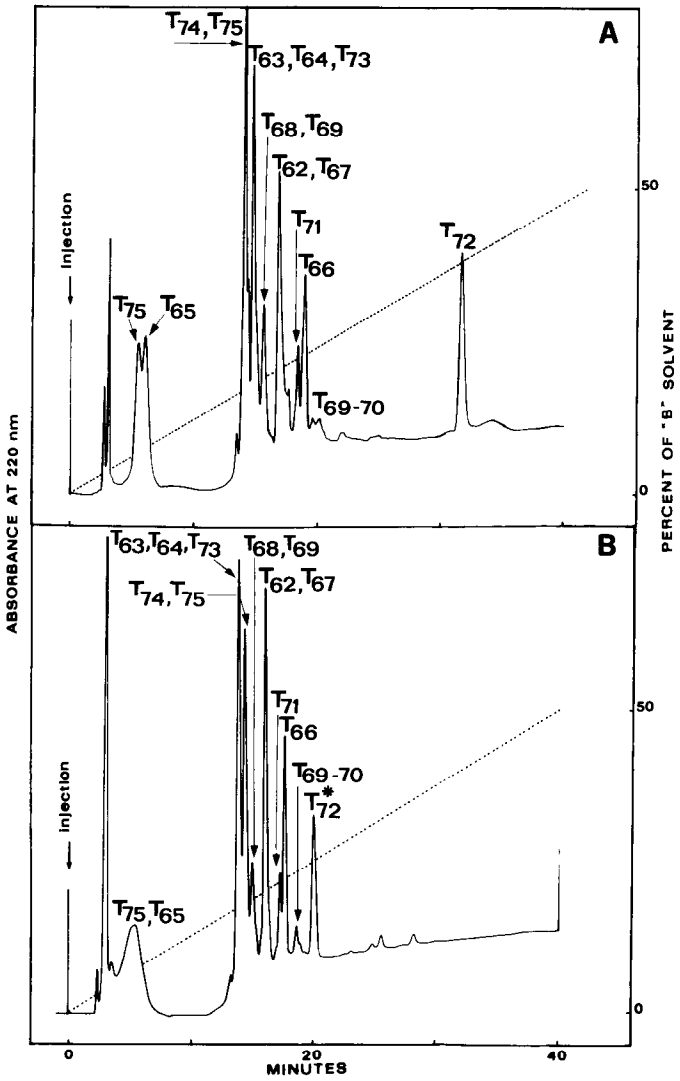


Fig. 3. Comparative tryptic peptide maps of CNBr VI from normal (A) and Castel di Sangro (B) human serum albumins. 5 nmol of each CNBr digest were chromatographed on a poly(sulphoethylaspartamide) SCX column (20 cm × 4.6 mm I.D.) under the following conditions: eluent A, 5 m*M* potassium phosphate (pH 3.0)–acetonitrile (75:25); eluent B, 5 m*M* potassium phosphate +0.5 *M* KCl (pH 3.0)–acetonitrile (75:25). Elution was performed at room temperature and at a flow-rate of 1 ml/min using the gradient indicated by the dashed line. Absorbance range, 0.64 full-scale. The presence of two peptides with the same amino acid composition is due to homoserine–homoserine lactone equilibrium.

CNBr VI are coeluted, but by examining the elution profiles of Fig. 3 it is of interest to stress the different selectivity of the exchanger toward fragments of the same size and net positive charge. Tetrapeptides Q–I–K–K ($T_{69-70}$) and H–E–P–K ($T^*_{72}$) are in fact eluted under different peaks, the first about 1 min earlier than the second, thus suggesting a positional effect of charged residues.

Although reliable identification and excellent resolution of the substituted peptides was obtained, SCX chromatography was inadequate in resolving many of the remaining components of the tryptic mixture. The failure to obtain all tryptic peptides in homogeneous form prompted us to experiment with a different enzymatic cleavage of CNBr VI, in order to characterize the complete primary structure of the variant fragment.

Both recognition of the abnormal fragment and complete resolution of the mixture components were achieved by submitting *S. aureus* V8 protease digests of normal and variant CNBr VI to SCX chromatography. Fig. 4 shows the elution

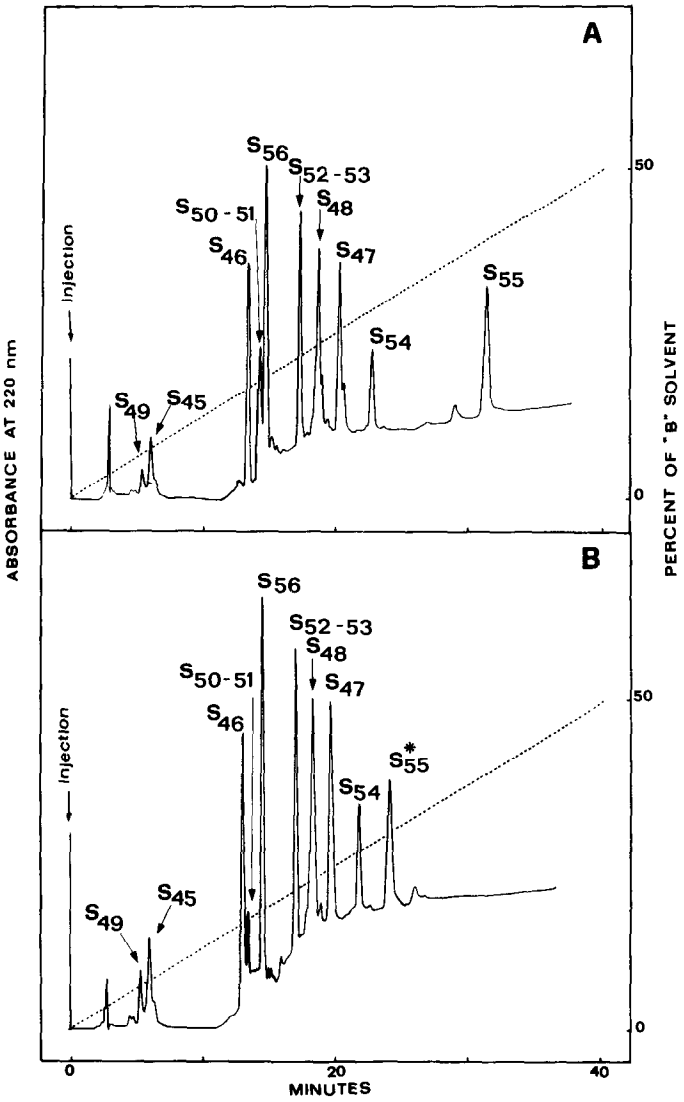


Fig. 4. Comparative peptide maps of *Staphylococcus aureus* V8 protease digests from CNBr VI of normal (A) and Castel di Sangro (B) human serum albumins. HPLC was performed with the stationary and mobile phases described in Fig. 3.

patterns of the *S. aureus* fragments. The total number of *S. aureus* fragments is not modified by Lys 536 → Glu replacement because the Glu 536–Pro 537 bond is not cleaved by *S. aureus* V8 protease. All the fragments expected on the basis of the listed sequence (Table II) are recovered in a homogeneous form. Peptides, indicated as $S_{50-51}$ and $S_{52-53}$ in Fig. 4, result from lack of the cleavage at the bonds Glu 501–Phe 502 and Glu 518–Lys 519, respectively.

TABLE II

AMINO ACID SEQUENCE OF *S. AUREUS* $V_8$ PROTEASE FRAGMENTS FROM CNBr VI OF HSA

$S_{45}$: P–C–A–E (450)

$S_{46}$: D–Y–L–S–V–V–L–N–Q–L–C–V–L–H–E (460)

$S_{47}$: K–T–P–V–S–D–R–V–T–K–C–C–T–E (470)

$S_{48}$: S–L–V–N–R–R–P–C–F–S–A–L–E (480, 490)

$S_{49}$: V–D–E

$S_{50}$: T–Y–V–P–K–E (500)

$S_{51}$: F–N–A–E

$S_{52}$: T–F–T–F–H–A–D–I–C–T–L–S–E (510)

$S_{53}$: K–E (520)

$S_{54}$: R–Q–I–K–K–Q–T–A–L–V–E (530)

$S_{55}$: L–V–K–H–K[a]–P–K–A–T–K–E (540)

$S_{56}$: Q–L–K–A–V–M

[a] Lys 536 is substituted by Glu in Castel di Sangro variant.

Because of the difference in their net positive charge, the substituted fragment $S^*_{55}$ (+5) is eluted about 8 min earlier than the normal $S_{55}$ (+6). The elution order of $S_{46}$ (13 min) and $S_{56}$ (15 min), both possessing the same net positive charge (+2), can be explained by the presence in the former of three additional acidic groups which, as previously suggested[14,15], may affect the chromatographic behaviour. However, the difference in additional acidic residues[15] (four *versus* two) does not prevent fragment $S_{47}$ from being eluted later than $S_{48}$. Again, the positional effect of charged residues seems clear, as the presence of two adjacent arginine residues in $S_{48}$ may cause it to interact loosely with the exchanger.

The greater selectivity of SCX chromatography for *S. aureus* than for tryptic fragments is obviously dependent on a more favourable distribution of positive charge in the former species. In fact, because of the enzyme specificity, most of the fragments originating from tryptic cleavage share the same positive net charge at pH 3, being distinct in two main families, the larger one consisting of the peptides which have the N-terminal and the C-terminal basic residue (Arg or Lys) as positively charged groups, and the second containing those which possess additional positive charges, namely

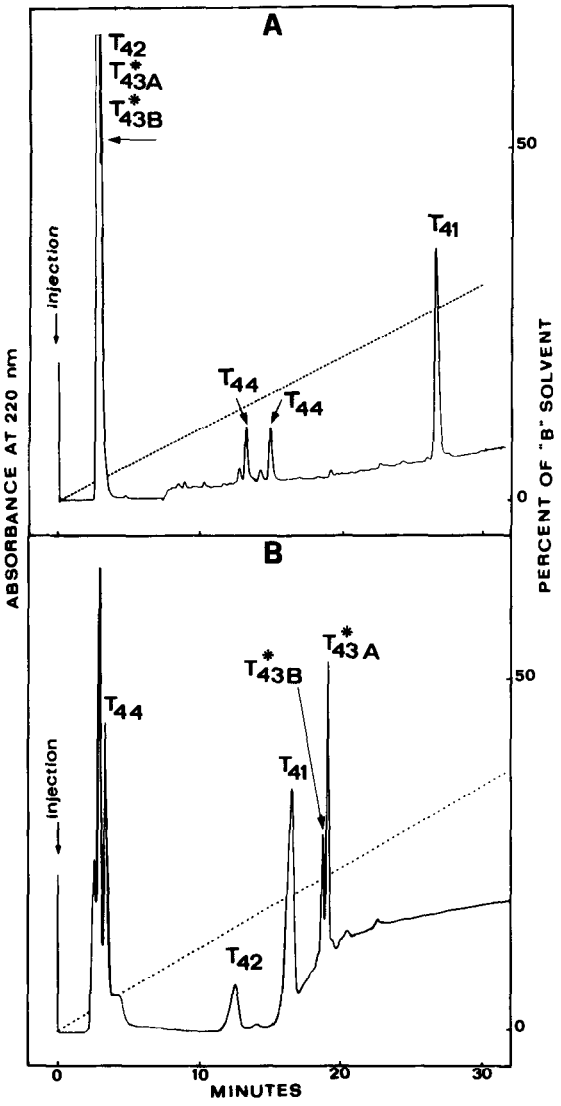


Fig. 5. (A) RP-HPLC profile of CNBr IV tryptic digest from Roma albumin. Column and chromatographic conditions as in Fig. 2. The presence of two peptides with the same amino acid composition is due to homoserine–homoserine lactone equilibrium. (B) SCX-HPLC profile of CNBr IV tryptic digest from Roma albumin. Column and mobile phase as in Fig. 3.

hystidine residues or Lys/Arg–Pro uncleaved bonds. On the other hand, the number and distribution of arginine and lysine residues through the sequence, resulting in a greater charge differentiation of *S. aureus* fragments, make them suitable to interact more selectively with the exchanger.

SCX chromatography has also been used to resolve tryptic digests from CNBr IV and CNBr VII of two slow variants, namely Roma (Glu 321 → Lys)[8] and Verona (Glu 570 → Lys)[5]. Because of the amino acid replacement, the total number of the expected tryptic peptides from the variant fragments is increased in both instances by one unit (five from Roma CNBr IV and seven from Verona CNBr VII). Both RP and SCX chromatography were equally efficient in resolving the tryptic digest of Verona CNBr VII, giving all peptides in a homogeneous form. Three modified fragments were formed because of the known incomplete cleavage at the Lys–Glu bonds; all of these are separately eluted in both RP and SCX chromatography (data not shown).

As a consequence of the Glu 321 → Lys replacement occurring in Roma CNBr IV, two small peptides are formed: the tetrapeptide N–Y–A–K ($T^{*}_{43A}$) and the dipeptide A–K ($T^{*}_{43B}$). Both fragments were resolved by RP-HPLC on a μBondapak $C_{18}$ column, $T^{*}_{43B}$ being eluted as a shoulder of the signal peak[8]. As shown in Fig. 5, the two modified fragments and $T_{42}$, the tetrapeptide D–V–C–K, were coeluted in the void volume when the tryptic digest was passed through an Aquapore RP-300 column (Fig. 5A). As expected, SCX chromatography allowed the separation of these fragments, and also that of all the other components of the tryptic mixture (Fig. 5B).

The results reported here lead to the conclusion that in all instances, characterization of variant tryptic fragments from charged mutants of human serum albumin may be usefully approached through SCX chromatography. In fact, ion-exchange chromatography may offer an additional advantage over the RP mode in identifying substituted-site-containing fragments, as all of these differ in charge from the normal counterpart. In most instances, as in Roma and Verona mutants, RP and SCX chromatography may be equally selective and efficient: however, situations may arise where, as with Castel di Sangro albumin, ion-exchange chromatography should be the method of choice for isolating the modified fragments. The demonstrated utility of poly(sulphoethylaspartamide) in characterizing serum albumin charged variants suggests a more general use of this sorbent in structural studies of abnormal proteins.

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