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Tryptic peptide mapping of sequence 299–585 of human serum albumin by high-performance liquid chromatography and fast atom bombardment mass spectrometry

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Abstract

The determination of the tryptic peptide mapping of sequence 299–585 (cyanogen bromide fragment A) of human serum albumin (HSA) by chemical and enzymatic cleavages and combined use of HPLC and FAB-MS is described. Reduction and carboxymethylation of A gave four subfragments which were separated by HPLC and digested with trypsin. Tryptic fragments were separated by HPLC and identified by FAB-MS. A total coverage of about 95% of the entire sequence was obtained. Tryptic fragments not identified include mostly single amino acids and very hydrophilic peptides which were absent in the chromatograms. The high reproducibility of the experiments and the satisfactory yield of the tryptic fragments identified demonstrate the great potential of the combined use of HPLC separation and FAB-MS analysis for the structural investigation of HSA.

1. Introduction

Human serum albumin (HSA) is the most abundant protein in the circulatory system. It consists of a single, non-glycosilated, polypeptide chain containing 585 amino acid residues and has many physiological functions. However, the most peculiar property of this protein is its unique ability to bind many endogenous and exogenous ligands, which range from inorganic cationic species to a large variety of organic molecules [1].

Peptide mapping of HSA appears of interest in view of several possible applications, which include the structural characterization of genetic variants, the characterization of chemical modifications, and the determination of the nature and topology of binding sites of HSA for ligands. Different approaches involving various methodologies have been reported [2–6].

Over recent years direct fast atom bombardment mass spectrometry (FAB-MS) of proteolytic mixtures of peptides (FAB mapping) and the combination of FAB-MS with reversed-phase high-performance liquid chromatography (RP-HPLC) have proved to be powerful tools for peptide mapping of proteins [7–12].

A CNBr fragment of bovine serum albumin was partially mapped using these techniques [13], but applications of such procedures to HSA have not yet been reported.

As a part of a research programme concerning the investigation of the interaction of HSA with textile dyes used as ligands for dye–protein

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affinity chromatography [14,15], we have undertaken the determination of tryptic peptide mapping of HSA by combined use of HPLC and FAB-MS and have previously reported the results relative to the sequence 1–298 [16]. We describe here the determination of the tryptic map of the remaining sequence 299–585.

2. Experimental

HSA (Cohn Fraction V), dithiothreitol (DTT), iodoacetic acid and trypsin TPCK treated were purchased from Sigma (Milan, Italy), Spectra Por 6 dialysis membrane was obtained from Roth (Karlsruhe, Germany), trifluoroacetic acid (TFA) was provided by Janssen (Beerse, Belgium), HPLC grade H_2O and CH_3CN were obtained from Carlo Erba (Milan, Italy). All other chemicals were of the highest purity commercially available and were used without further purification. A, B and C cyanogen bromide fragments of unreduced HSA were obtained as described elsewhere [14].

HPLC separations were performed on a Varian 9010 equipped with a Varian 9050 detector and a Varian 4400 integrator. Amino acid analyses were carried out by ion-exchange chromatography with post-column ninhydrin derivatization according to Moore and Stein [17] on a Carlo Erba 3A30 automatic amino acid analyser.

Peptide samples were hydrolysed in 6 *M* hydrochloric acid for 24 h at 105°C in evacuated sealed tubes.

2.1. Reduction and carboxymethylation of fragment A

1 μmol of fragment A was dissolved in 1 *M* Tris–HCl containing 6 *M* guanidine, at a concentration of 10 mg/ml. The pH was adjusted to 8.5 by HCl and a 10-fold molar excess of DTT over the SH concentration, dissolved in 2 ml of the same buffer, was added. The mixture was allowed to react for 3 h at 25°C in the dark under nitrogen atmosphere. Iodoacetic acid (0.95-fold molar excess over total thiols), dissolved in 3 ml of 1 *M* Tris–HCl containing 6 *M* guanidine, was then added and the pH raised to 8.5 by addition of NaOH. The mixture was kept in the dark at room temperature for 1 h maintaining the pH above 8. At the end of the reaction time, excess of iodoacetic acid was eliminated by addition of 10 μl of 2-mercaptoethanol to the reaction mixture. The solution was then dialysed against water and freeze-dried.

2.2. HPLC separation of the subfragments of A

The freeze-dried residue from the reduction and carboxymethylation reaction was dissolved in aqueous 0.05% TFA, filtered on Millex-HV (Millipore, Milan, Italy) and applied onto a RP-Vydac C18 (25 × 1.0 cm) column. About 3 mg of mixture were injected for each run. The column was eluted at room temperature with a linear gradient of solvent B [CH_3CN–isopropanol (2:1) containing 0.08% TFA] in A (H_2O–TFA 0.05%) from 20% to 50% in 35 min. Flow rate was 4 ml min^{-1}. Peaks were detected by their absorption at 224 nm. Four fractions were collected and freeze-dried.

2.3. Tryptic cleavage of reduced and carboxymethylated subfragments of A

Each reduced and carboxymethylated subfragment of A (1 μmol) was dissolved in 20 m*M* ammonium acetate, pH 8.3, containing 1 m*M* calcium chloride, at a concentration of 1 mg/ml. Trypsin was dissolved in the same buffer and added at an enzyme:substrate ratio of 1:50 (mol/mol). The solution was incubated at 37°C for 4 h. The digestion was stopped by cooling in liquid nitrogen and the mixture was immediately freeze-dried.

2.4. HPLC separation of the tryptic peptide mixture of subfragments of A

Lyophilized tryptic peptide mixture of each subfragment of A was dissolved in aqueous 0.1% TFA, filtered on Millex-HV and applied onto a reversed-phase Vydac C18 (1.0 × 25 cm) column. The column was eluted at room temperature with 95% of solvent A (H_2O–TFA 0.1%) and

5% of solvent B (95% CH_3CN–5% H_2O containing 0.08% TFA) for 5 min, and then with a linear gradient of B in A to a final concentration of 47% of B in A in 55 min. Flow rate was 3 ml min^{-1}. Peaks were detected by their absorption at 224 nm. Peaks were collected manually, freeze-dried and subjected to FAB analysis.

2.5. FAB mass spectrometry

FAB mass spectra were recorded on a ZAB-2SE mass spectrometer, equipped with a standard FAB source and a caesium ion gun. The sample was deposited by evaporation from methanol solution onto the probe tip and 2 μl of a 80:20 glycerol–thyoglicerol mixture containing 1% HCl were added. To increase MH^+ signal intensities for peptides containing multiple carboxylic groups, *p*-toluenesulphonic acid was added to the matrix. The sample was bombarded with a beam of 35 KeV caesium ions.

3. Results and discussion

Because HSA consists of a single polypeptide chain of 585 amino acids, its direct digestion with trypsin would give rise to a complex mixture of 79 peptides, which would be difficult to separate by HPLC. In order to obtain a better separation and therefore an extended reproducibility of the tryptic fragments, a combination of chemical and enzymatic cleavages was employed.

Unreduced HSA was initially blocked at the free thiol group of Cys 34 by iodoacetic acid and cleaved by cyanogen bromide. This treatment gives rise to three large fragments which, according to the naming convention of McMenamy et al. [18] are called A, B and C. The mixture of these fragments was separated by gel permeation and ion-exchange chromatography [14].

Tryptic peptide mapping of sequence 1–298 (cyanogen bromide fragments B and C) has been reported previously [16].

Fragment A has a molecular weight of 32 391 and, due to the presence of three methionine residues in its sequence, it is composed of four subfragments held together by disulphide bonds (Table 1). Reduction of the disulphide bridges and carboxymethylation of the resulting free thiol groups produces four independent subfragments.

Table 1
Molecular mass and sequence position of the four subfragments of A

Fragment	Sequence	M_r
A1	299–329	3372
A2	330–446	13905
A3	447–548	11959
A4	549–585	4168

Because of the relatively high molecular weight of fragment A, separation of these component subfragments, prior to tryptic digestion, was carried out. Attempts to separate the four subfragments by ion-exchange chromatography [18] produced unsatisfactory homogeneous fractions. HPLC separation [19,20], instead, resulted in the isolation of pure fractions of the four subfragments, as demonstrated by amino acid analyses and subsequent enzymatic cleavages. HPLC separation of the four subfragments of A is shown in Fig. 1.

The first fraction in the chromatogram (Fig. 1), corresponding to subfragment A1, is formed by two peaks, probably due to the presence of both the free homoserine and homoserine lactone as C-terminal amino acid residue in the peptide. Thus two peptides, slightly differing in retention times, are formed [21]. A2 and A3 may also occur in both forms, but these subfragments appear as single peaks in the chromatogram. It is likely that, because of the higher molecular weight of these peptides, the difference in the retention time of the two forms is smaller so that they are not separated. Peaks not labelled in Fig. 1 are due to partial cleavages at methionine residues and were not further characterized.

The tryptic peptides which should be produced by a completely specific cleavage of the four reduced and carboxymethylated subfragments with trypsin, were predicted using the sequence reported by several authors, obtained from direct sequence analysis [22,23] or from com-

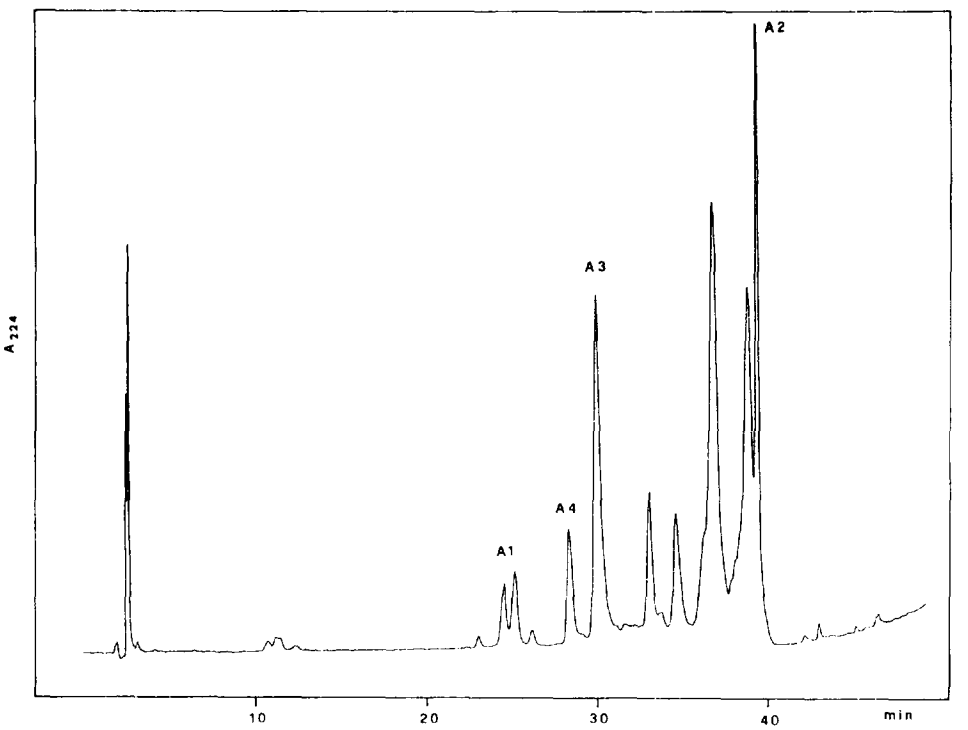

Fig. 1. HPLC separation of the four subfragments of fragment A.

plementary DNA studies [24,25]. These sequences are all coincident within fragment A with the exception of that reported by Lawn et al. [24] in which Glu 396 is substituted by Lys. As will be seen in the following, our results are consistent with the presence of Glu as residue 396. Tryptic fragments derived from A are numbered progressively starting from the N-terminal residue of the whole HSA chain. The tryptic peptides containing methionine result divided in two parts. These are named adding (1) and (2) to the number which identifies the tryptic peptide to indicate the N-terminal and C-terminal moiety, respectively.

Separation of the tryptic peptide fragments was achieved by RP-HPLC. Since FAB-MS identification of the fragments does not require complete separation of the peptides, optimization of the chromatographic conditions was not attempted and the same gradient programme was used in all cases. Retention times of the expected tryptic peptides, as well as retention times of additional peptides identified by FAB-MS in the HPLC of the tryptic mixtures, were calculated according to the equation

$$t_R = a\ln(1 + H) + c$$

where H indicates the hydrophobicity of the peptides, calculated using for the constituent amino acids the weighted retention constants reported by Sasagawa et al. [26,27], and adopting 19.3 and −5.3 for a and c, respectively. The calculated curves are reported in Fig. 2, together with the experimental retention time values. Calculated retention times were in general in good agreement with the experimental values with the notable exception of carboxymethylcysteine containing peptides, for which calculated values were systematically higher than the experimental ones, possibly because the retention constant reported for the carboxymethylcysteine is higher than the real value. Comparison of the experimental retention times of the peptides with the calculated values simplifies and further substantiates the FAB-MS

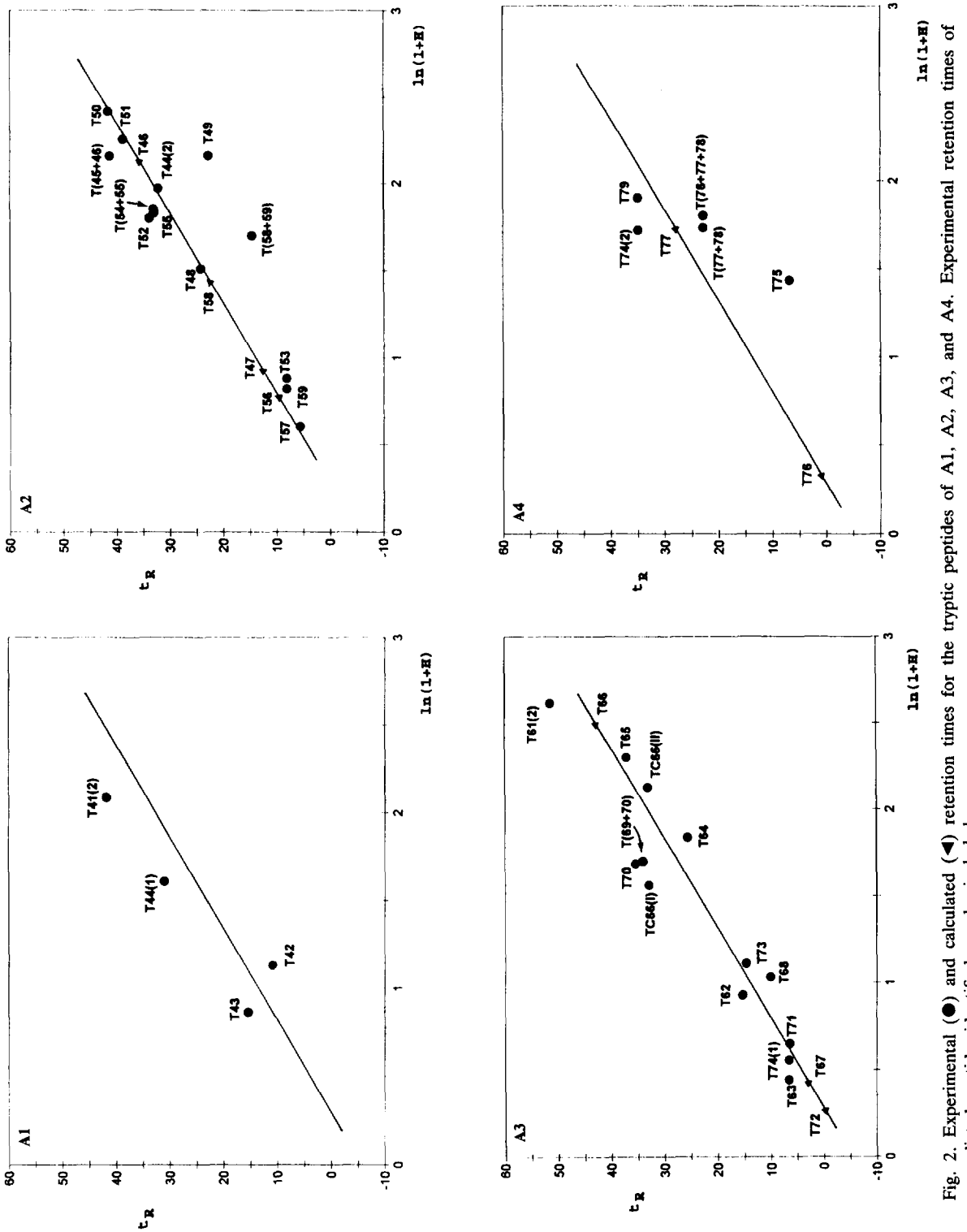

Fig. 2. Experimental (●) and calculated (◀) retention times for the tryptic peptides of A1, A2, A3, and A4. Experimental retention times of unpredicted peptides identified are also included.

Table 2
Sequence, position and identification by HPLC–FAB-MS of the expected tryptic peptides of subfragment A1

Fragment	Position in the sequence	Sequence	M_r	Identification (+) by HPLC–FAB-MS
T41(2)	299–313	PRO–ALA–ASP–LEU–PRO–SER–LEU–ALA–ALA–ASP–PHE–VAL–GLU–SER–LYS	1558.8	+
T42	314–317	ASP–VAL–CMCYS–LYS	521.2	+
T43	318–323	ASN–TYR–ALA–GLU–ALA–LYS	694.3	+
T44(1)	324–329	ASP–VAL–PHE–LEU–GLY–OMO	650.3[a]	+

[a] Free homoserine.

Table 3
Sequence, position and identification by HPLC–FAB-MS of the expected tryptic peptides of subfragment A2

Fragment	Position in the sequence	Sequence	M_r	Identification (+) by HPLC–FAB-MS
T44(2)	330–336	PHE–LEU–TYR–GLU–TYR–ALA–ARG	960.5	+
T45	337	ARG	174.1	as T(45 + 46)
T46	338–348	HIS–PRO–ASP–TYR–SER–VAL–VAL–LEU–LEU–LEU–ARG	1310.7	as T(45 + 46)
T47	349–351	LEU–ALA–LYS	330.2	–
T48	352–359	THR–TYR–GLU–THR–THR–LEU–GLU–LYS	983.5	+
T49	360–372	CMCYS–CMCYS–ALA–ALA–ALA–ASP–PRO–HIS–GLU–CMCYS–TYR–ALA–LYS	1554.5	+
T50	373–389	VAL–PHE–ASP–GLU–PHE–LYS–PRO–LEU–VAL–GLU–GLU–PRO–GLN–ASN–LEU–ILE–LYS	2044.1	+
T51	390–402	GLN–ASN–CMCYS–GLU–LEU–PHE–GLU–GLN–LEU–GLY–GLU–TYR–LYS	1657.7	+
T52	403–410	PHE–GLN–ASN–ALA–LEU–LEU–VAL–ARG	959.5	+
T53	411–413	TYR–THR–LYS	410.2	+
T54	414	LYS	146.1	asT(54 + 55)
T55	415–428	VAL–PRO–GLN–VAL–SER–THR–PRO–THR–LEU–VAL–GLU–VAL–SER–ARG	1510.8	+ also as T(54 + 55)
T56	429–432	ASN–LEU–GLY–LYS	430.2	–
T57	433–436	VAL–GLY–SER–LYS	389.2	+
T58	437–439	CMCYS–CMCYS–LYS	468.1	as T(58 + 59)
T59	440–444	HIS–PRO–GLU–ALA–LYS	580.3	+ also as T(58 + 59)
T60	445	ARG	174.1	–
T61(1)	446	OMO	119.1[a]	–

[a] Free homoserine.

identification of the HPLC peaks in many instances. Moreover, calculated retention times account for the absence of some very hydrophilic peptides in the chromatograms.

HPLC separation of the tryptic peptides of subfragment A1 is illustrated in Fig. 3. Since A1 contains 3 Lys, the formation of 4 tryptic fragments can be anticipated (Table 2). Three chromatographic peaks are identified as T42, T43, and T41(2).

In accordance with previous observation [21], peptide T44(1), which has homoserine as C-terminal amino acid, gives two different HPLC peaks, a faster eluting one, corresponding to the peptide ending in free homoserine, and a second slower eluting peak due to the same peptide with the homoserine in the lactone form. Furthermore the chromatogram (Fig. 4) shows an additional peak at a retention time slightly lower than that corresponding to the free homoserine ending peptide. The FAB spectrum of this peak presents an intense MH^+ signal at m/z 754 which differs by 121 from the MH^+ of the T44(1) peptide containing homoserine in the lactone form. As illustrated previously [16], this signal corresponds to the MH^+ of the product produced by the chemical reaction of tris(hydroxymethyl)aminomethane with the terminal homoserine lactone of the peptide T44(1), in the disulphide reduction step. The fragments identified constitute 100% of the sequence of subfragment A1.

HPLC separation of the tryptic peptides mixture of subfragment A2 is shown in Fig. 4. A2 contains 12 Lys and 6 Arg residues. Because the bond between Lys (378) and Pro (379) is not cleaved by trypsin, 18 tryptic peptides can be predicted. Four fragments [T45, T54, T60, and T61(1)] consist of free amino acids and therefore it is not possible to detect them by HPLC (Table 3).

Ten of the potential tryptic fragments were identified by FAB-MS. Two of them (T53 and T59) are eluted at the same retention time and were collected and identified in one fraction. Peptides T46 and T58 are not found, but two peaks were identified as peptides T(45 + 46) and T(58 + 59). These fragments are produced by incomplete cleavage of Arg (337)–His (338) and Lys (439)–His (440). Incomplete cleavage of the

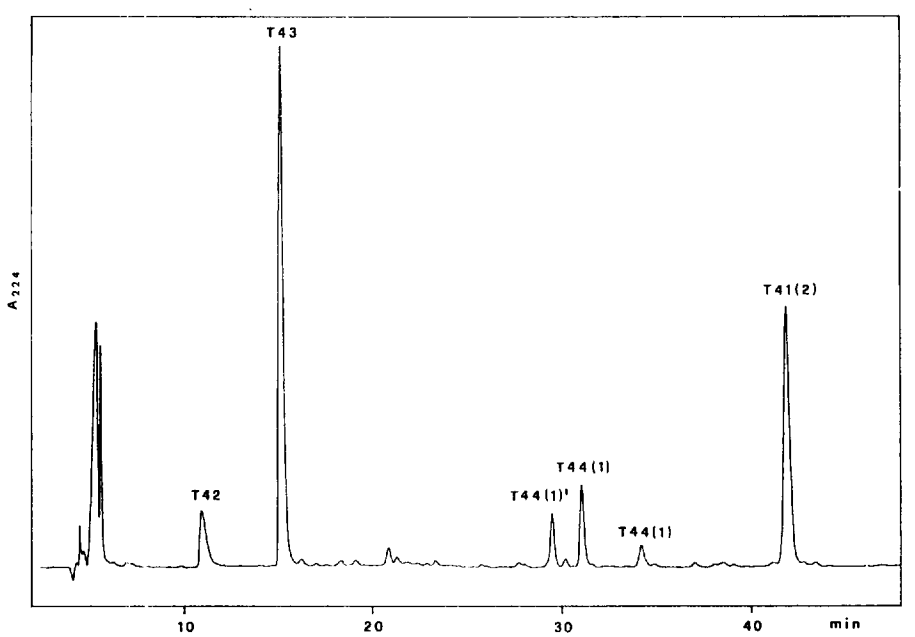

Fig. 3. HPLC separation of the tryptic digest of subfragment A1.

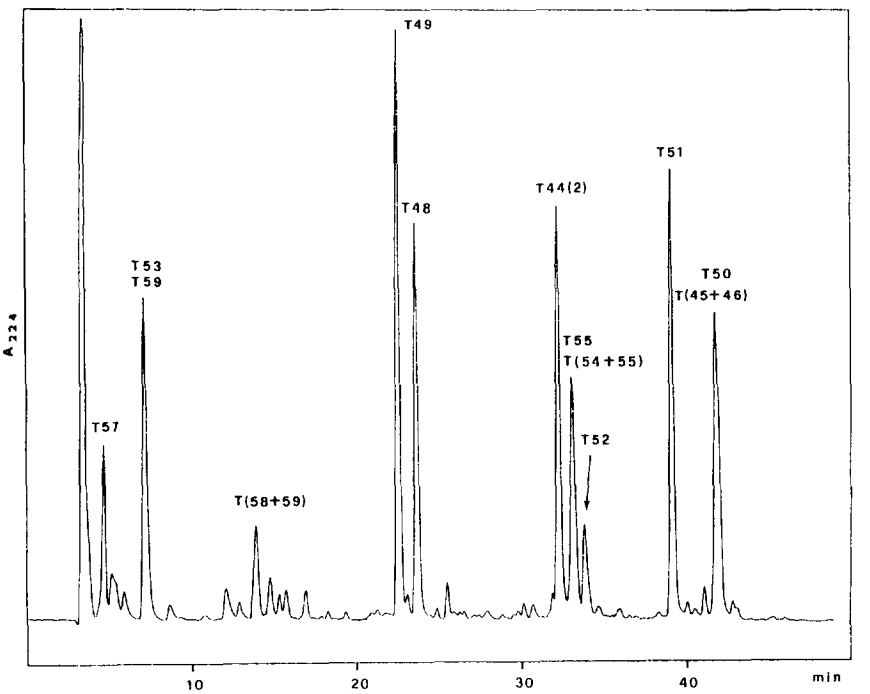

Fig. 4. HPLC separation of the tryptic digest of subfragment A2.

Lys (414)–Val (415) bond accounts for the presence of peptide T(54 + 55), which coelutes with peptide T55. Peptide T51 contains the amino acid in which the sequence reported by Lawn [24] differs from that reported by other authors [22,23,25]. In fact, in the sequence of Lawn the amino acid 396 is a Lys residue, whereas in the others the amino acid residue in this position is Glu. The presence of the peptide T51, found in the present work, confirms the correctness of Glu as residue 396. In all, 92% of the sequence of subfragment A2 was identified.

HPLC separation of the tryptic peptides mixture of subfragment A3 is shown in Fig. 5. This subfragment possesses 10 Lys and 3 Arg, so that a potential yield of 14 tryptic peptides is expected. T69 is a free amino acid and therefore it does not appear in the HPLC chromatogram. Ten of the expected tryptic peptides were identified by FAB-MS (Table 4).

Three of them [T63, T71, and T74(1)], which are very hydrophilic, are eluted at the same retention time. They were collected in one fraction and identified as mixture of peptides. The lactone form of the T74(1) peptide gives a small HPLC peak at 8.20 min. One unexpected peptide was identified as T(69 + 70) originating from incomplete cleavage of Lys (525)–Gln (526) sequence. T70 is also found separately at a slightly higher retention time. The highly hydrophilic peptides T67 and T72 were not found, very likely because of their extremely low retention times (Fig. 2). Peptide T66 does not appear in the chromatogram. However, FAB-MS analysis of a minor peak at 32.95 min indicates that it is composed of two peptides which are produced from peptide T66 by chymotryptic cleavage of Phe (507). The fragments identified constitute about 95% of the sequence of A3.

The chromatographic pattern of the tryptic digest of subfragment A4 is reported in Fig. 6. Subfragment A4 contains 5 Lys giving rise to a potential yield of 6 tryptic fragments, one of which is a single lysine (T78) that is not detected

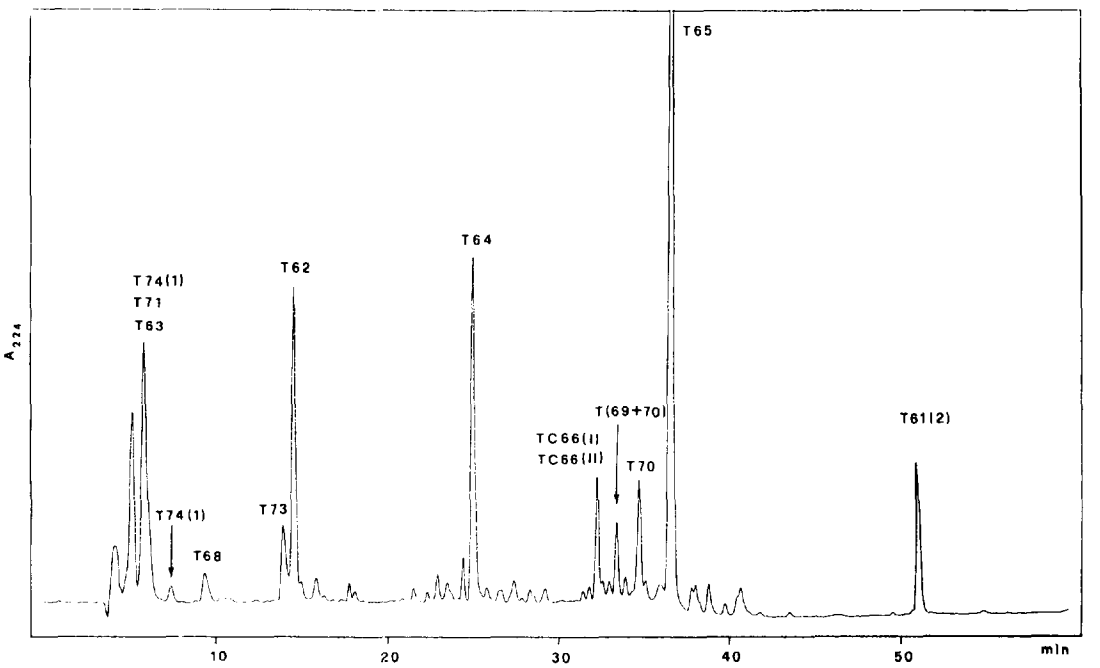

Fig. 5. HPLC separation of the tryptic digest of subfragment A3.

in the HPLC chromatogram. Of the remaining 5 potential peptides, 3 [T74(2), T75, and T79] were identified by FAB-MS (Table 5).

Peptides T74(2) and T79 appear as two almost unresolved peaks at the end of the chromatogram. Peptides T76 and T77 are not detected as such, but the FAB spectrum of an intense HPLC peak eluting at ~23 min shows two intense signals at m/z 1199 and 1628 corresponding to the MH^+ of T(77 + 78) and T(76 + 77 + 78), respectively. These fragments are obviously produced by lack of cleavage of the consecutive Lys

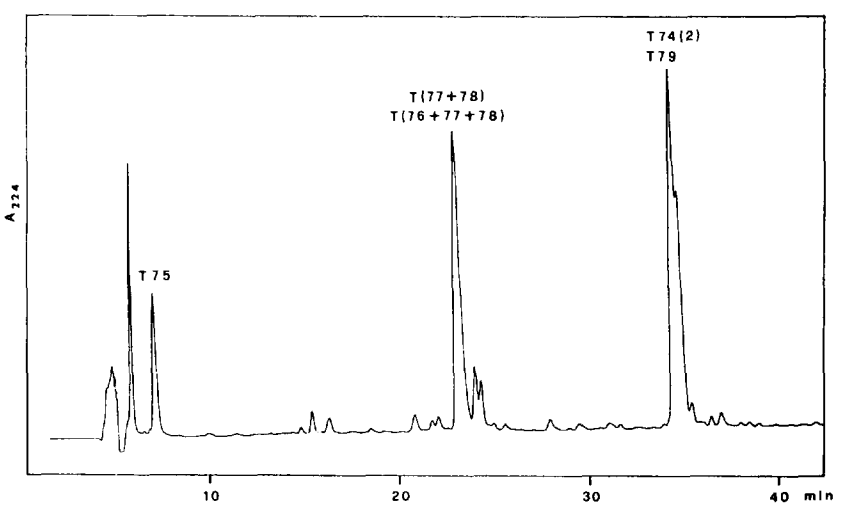

Fig. 6. HPLC separation of the tryptic digest of subfragment A4.

Table 4
Sequence, position and identification by HPLC–FAB-MS of the expected tryptic peptides of subfragment A3

Fragment	Position in the sequence	Sequence	M_r	Identification (+) by HPLC–FAB-MS
T61(2)	447–466	PRO–CMCYS–ALA–GLU–ASP–TYR–LEU–SER–VAL–VAL–LEU–ASN–GLN–LEU–CMCYS–VAL–LEU–HIS–GLU–LYS	2388.1	+
T62	467–472	THR–PRO–VAL–SER–ASP–ARG	673.3	+
T63	473–475	VAL–THR–LYS	346.2	+
T64	476–484	CMCYS–CMCYS–THR–GLU–SER–LEU–VAL–ASN–ARG	1139.5	+
T65	485–500	ARG–PRO–CMCYS–PHE–SER–ALA–LEU–GLU–VAL–ASP–GLU–THR–TYR–VAL–PRO–LYS	1910.9	+
T66	501–519	GLU–PHE–ASN–ALA–GLU–THR–PHE–THR–PHE–HIS–ALA–ASP–ILE–CMCYS–THR–LEU–SER–GLU–LYS	2260	as TC66(I) and TC66(II)
T67	520–521	GLU–ARG	303.1	
T68	522–524	GLN–ILE–LYS	387.2	+
T69	525	LYS	146.1	as T(69 + 70)
T70	526–534	GLN–THR–ALA–LEU–VAL–GLU–LEU–VAL–LYS	999.6	+ also as T(69 + 70)
T71	535–538	HIS–LYS–PRO–LYS	508.3	+
T72	539–541	ALA–THR–LYS	318.2	–
T73	542–545	GLU–GLN–LEU–LYS	516.3	+
T74(1)	546–548	ALA–VAL–OMO	289.2[a]	+

[a] Free homoserine.

Table 5
Sequence, position and identification by HPLC–FAB-MS of the expected tryptic peptides of subfragment A4

Fragment	Position in the sequence	Sequence	M_r	Identification (+) by HPLC–FAB-MS
T74(2)	549–557	ASP–ASP–PHE–ALA–ALA–PHE–VAL–GLU–LYS	1040.5	+
T75	558–560	CMCYS–CMCYS–LYS	468.1	+
T76	561–564	ALA–ASP–ASP–LYS	447.2	as T(76 + 77 + 78)
T77	565–573	GLU–THR–CMCYS–PHE–ALA–GLU–GLU–GLY–LYS	1070.4	as T(76 + 77 + 78) and T(77 + 78)
T78	574	LYS	146.1	as T(76 + 77 + 78) and T(77 + 78)
T79	575–585	LEU–VAL–ALA–ALA–SER–GLN–ALA–ALA–LEU–GLY–LEU	1012.6	+

(573)–Lys (574) bond and of Lys (564) which is surrounded by three acidic amino acid residues. Considering these latter peptides originating from lack of cleavage at lysine residues, the sequence of subfragment A4 results completely covered.

4. Conclusions

Tryptic peptide mapping of sequence 299–585 (cyanogen bromide fragment A) of HSA has been obtained by chemical and enzymatic cleavage and combination of HPLC and FAB-MS. Reduction of A and carboxymethylation of the resulting free thiol groups gave four subfragments which were separated by HPLC and digested with trypsin. Tryptic fragments were separated by HPLC and identified by FAB-MS. A total coverage of about 95% of the entire sequence was achieved. The method allowed easy identification of unexpected peptides originating from chymotryptic or incomplete tryptic cleavage, and also permitted identification of chemically modified peptides produced by reactions with buffer components. Calculated retention times of the peptides simplified and further substantiated the FAB-MS identification of the HPLC peaks in many, but not in all cases. Most importantly, the calculated retention times enabled explanation of the absence of some tryptic fragments in the chromatograms in terms of their extreme hydrophilicity. The reproducibility of the experiments was excellent and the yield of the tryptic fragments identified satisfactory, thus indicating the great potential of the combined use of HPLC and FAB-MS for the structural investigation of HSA.

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References

[1] T. Peters, Jr, in C.B. Anfisen, J.T. Edsall and F.M. Richards (Editors), *Advances in Protein Chemistry*, Vol. 37, Academic Press, London, 1985, p. 161.
[2] L. Minchiotti, M. Galliano, M.C. Zapponi and R. Tenni, *Eur. J. Biochem.*, 214 (1993) 437.
[3] P. Iadarola, L. Minchiotti and M. Galliano, *FEBS Lett.*, 180 (1985) 85.
[4] M. Galliano, L. Minchiotti, P. Iadarola, M. Stoppini, G. Ferri and A.A. Castellani, *FEBS Lett.*, 208 (1986) 364.
[5] L. Minchiotti, M. Galliano, P. Iadarola, E. Zepponi and G. Ferri, *Biochim. Biophys. Acta*, 1039 (1990) 204.
[6] N. Takahashi, Y. Takahashi, N. Ishioka, B.S. Blumberg and F.W. Putnam, *J. Chromatogr.*, 359 (1986) 181.
[7] K. Biemann and S.A. Martin, *Mass Spectrom. Rev.*, 6 (1987) 1.
[8] H.R. Morris and F.M. Grier, *Trends in Biotechnol.*, 6 (1988) 140.
[9] S.A. Carr, M.E. Hemling, M.F. Bean and G.D. Roberts, *Anal. Chem.*, 63 (1991) 2802.
[10] V. Amico, S. Foti, R. Saletti, A. Cambria and G. Petrone, *Biomed. Environ. Mass Spectrom.*, 16 (1988) 431.
[11] M. Svoboda, M. Przybylski, J. Schreurs, A. Miyajima, K. Hogeland and M. Deinzer, *J. Chromatogr.*, 562 (1991) 403.
[12] D. Suckau, M. Mák and M. Przybylski, *Proc. Natl. Acad. Sci. USA*, 89 (1992) 5530.
[13] V. Ling and A.L. Burlingame, in A.L. Burlingame and N. Castagnoli, Jr. (Editors), *Mass Spectrometry in the Health and Life Sciences*, Elsevier, London, 1985, p. 11.
[14] A. Compagnini, M. Fichera, S. Fisichella, S. Foti and R. Saletti, *J. Chromatogr.*, 639 (1993) 341.
[15] S.B. McLoughlin and C.R. Lowe, *Rev. Prog. Coloration*, 18 (1988) 16.
[16] A. Compagnini, S. Fisichella, S. Foti, L. Sardo and R. Saletti, *Rapid Commun. Mass Spectrom.*, 8 (1994) 459.
[17] S. Moore and W. Stein, *J. Biol. Chem.*, 211 (1954) 893.
[18] R.H. McMenamy, H.M. Dintzis and F. Watson, *J. Biol. Chem.*, 246 (1971) 4744.
[19] P. Iadarola, G. Ferri, M. Galliano, L. Minchiotti and M.C. Zapponi, *J. Chromatogr.*, 298 (1984) 336.
[20] P. Iadarola, M.C. Zapponi, M. Stoppini, M.L. Meloni, L. Minchiotti, M. Galliano and G. Ferri, *J. Cromatogr.*, 443 (1988) 317.
[21] P. Pucci, R. Borgia and G. Marino, *J. Chromatogr.*, 331 (1985) 425.
[22] P.Q. Behrens, A.M. Spiekerman and J.R. Brown, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 34 (1975) 591.
[23] B. Meloun, L. Moravek and V. Kostka, *FEBS Lett.*, 58 (1975) 134.

[24] R.M. Lawn, J. Adelman, S.C. Bock, A.E. Franke, C.M. Houck, R.C. Najarian, P.H. Seeburg and K.I. Wion, *Nucleic Acids Res.*, 9 (1981) 6103.
[25] A. Dugaiczyk, S.W. Law and O.E. Dennison, *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 71.
[26] T. Sasagawa, T. Okuyama and D.C. Teller, *J. Chromatogr.*, 240 (1982) 329.
[27] T. Sasagawa and D.C. Teller, in W.S. Hancock, (Editor), *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*, Vol. II, CRC Press, Boca Raton, FL, 1984, p. 53.