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JMS Letters

Dear Sir,

Characterization of Cyanogen Bromide Fragments of Reduced Human Serum Albumin by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

Human serum albumin (HSA) is the most abundant protein in the circulatory system. HSA consists of a single, non-glycosylated, polypeptide chain of 585 amino acid residues, and has many physiological functions. However, the 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]

Methods for the structural characterization of HSA appear of interest in view of possible applications, including the structural characterization of genetic variants, the characterization of chemical modifications following exposure to xenobiotics, the determination of the nature and topology of binding sites for ligands and the standardization of analytical procedures for the validation of HSA preparations obtained from genetic engineering techniques. For these reasons, different approaches for HSA mapping have been reported, based upon combination of chemical and enzymatic methods of protein degradation with chromatographic separation procedures and identification of the fragments.[2–6]

In recent years, the development of 'soft' desorption/ionization methods of mass spectrometry, such as fast atom bombardment (FAB) and more recently electrospray (ES) and matrix-assisted laser desorption/ionization (MALDI), have provided powerful tools for protein characterization.[7–11] Previous work in our laboratory has been concerned with the structural characterization of HSA by integration of chemical and enzymatic degradation methods with chromatographic separation and FAB/MS analysis.[12,13] MALDI/MS appeared of interest for the structural characterization of HSA because of its high sensitivity and the possibility of analysing high molecular mass fragments. The 'micro-heterogeneity' of HSA,[1] however, precludes a direct analysis by MALDI/MS of the intact protein. The structural characterization of HSA by combination of CNBr cleavage of the unreduced protein, chromatographic separation of the fragments and MALDI/MS analysis has been reported previously.[14] In the present study we have characterized HSA by a combination of CNBr cleavage of the reduced and carboxymethylated protein, high-performance liquid chromatographic (HPLC) separation of the fragments produced and MALDI/MS analysis.

HSA (Cohn Fraction V) was purchased from Sigma (St Louis, MO, USA). The procedure employed for obtaining the HSA fragments subjected to MALDI/MS analysis is summarized in Scheme 1. The protein (2 mg) was first reduced with dithiothreitol and the resulting thiol groups were blocked with iodoacetic acid. Reduced and carboxymethylated HSA was dissolved in 70% (v/v) formic acid at a concentration of 10 mg ml^{-1}. CNBr (100-fold molar excess over protein concentration) was added and the mixture allowed to react for 24 h at 4 °C in the dark under a nitrogen atmosphere. The solution was then diluted 20-fold with water and lyophilized. The latter procedure was repeated twice.

Because of the presence of six methionine residues in the sequence, the cleavage with CNBr produces seven fragments. The partial sequences and calculated average molecular masses for the homoserine and homoserine lactone forms of proteolytic fragments I–VII are summarized in Table 1. Direct MALDI analysis of the mixture arising from the CNBr cleavage resulted in a spectrum which showed only the signals for the fragments II and IV, and not for the other fragments. The seven fragments produced were then fractionated by HPLC using an RP-Vydac C_{18} column (25 × 1.0 cm i.d.) equilibrated with 80% of solvent A (0.05% trifluoroacetic acid (TFA)) and 20% of solvent B (acetonitrile–propan-2-ol (2:1) containing 0.08% TFA). The column was eluted at room temperature with a linear gradient of solvent B in A from 20% to 60% in 35 min; the flow-rate was 3 ml min^{-1}. Peaks were detected by measuring their UV absorption at 224 nm. The resulting chromatogram is shown in Fig. 1. Complete isolation of five fragments (II, III, IV, VI and VII) was obtained, whereas fragments I and V co-eluted in a single peak. Since the subsequent MALDI/MS analysis did not require the complete separation of the fragments, no further optimization of the chromatographic separation was necessary.

MALDI mass spectra were obtained with a Bruker Biflex time-of-flight spectrometer (Bruker-Franzen, Bremen, Germany) equipped with a UV nitrogen laser (337 nm), a dual-channel plate detector and x-mass data system for spectral acquisition and instrument control. The instrument was operated in the linear mode. Sample preparation was carried out using the thin-layer method.[15] Spectra were obtained at an acceleration voltage of 20 kV using the minimum laser power necessary to obtain the molecular ion signal ($\sim 5 \times 10^6$ W cm^{-2}) and a 10 Hz pulse frequency (3 ns). Spectra were

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Table 1. Partial sequences, calculated average M_r and M_r determined by MALDI of the CNBr fragments of reduced and carboxymethylated HSA

Fragment	Partial sequence	Calculated average M_r (Da)		M_r determined by MALDI (Da)
		Homoserine	Homoserine lactone	
I	1–87	10041.2	10023.1	10022
II	88–123	4354.8	4336.8	4334
III	124–298	20699.3	20681.3	20704
IV	299–329	3372.8	3354.7	3357
V	330–446	13905.9	13887.9	13902
VI	447–548	11959.6	11941.6	11941
VII	549–585	4097.6		4097

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HSA

↓ Reduction with 1,4 dithiothreitol in 6M guanidine hydrochloride, 1M tris-HCl pH 8.5

↓ Blocking by iodoacetic acid

↓ Dialysis and lyophilisation

↓ CNBr cleavage in 70% formic acid

Fractions	I	II	III	IV	V	VI	VII
Partial sequences	(1-87)	(88-123)	(124-298)	(299-329)	(330-446)	(447-548)	(549-585)

↓ Lyophilisation

↓ HPLC separation

MALDI-MS

Scheme 1

calibrated internally or externally using insulin, trypsinogen, lysozyme and cytochrome *c* as standards.

All fragments with their M_r and partial sequences identified are summarized in Table 1. The MALDI mass spectrum of the HPLC fraction I–V is reported in Fig. 2. A sharp molecular ion signal corresponding to the calculated M_r was obtained for the *C*-terminal fragment VII, which does not contain a homoserine residue. The molecular ion signals for all other fractions were broad and composed by overlapping of the molecular ion signals of the homoserine and homoserine

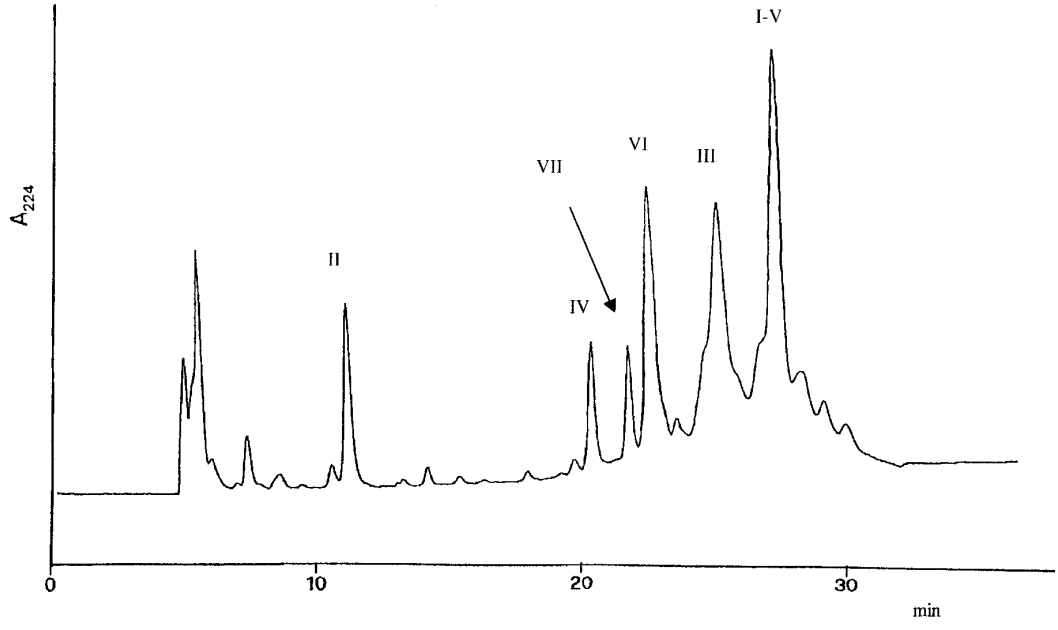

Figure 1. HPLC separation of CNBr fragments of reduced and carboxymethylated HSA.

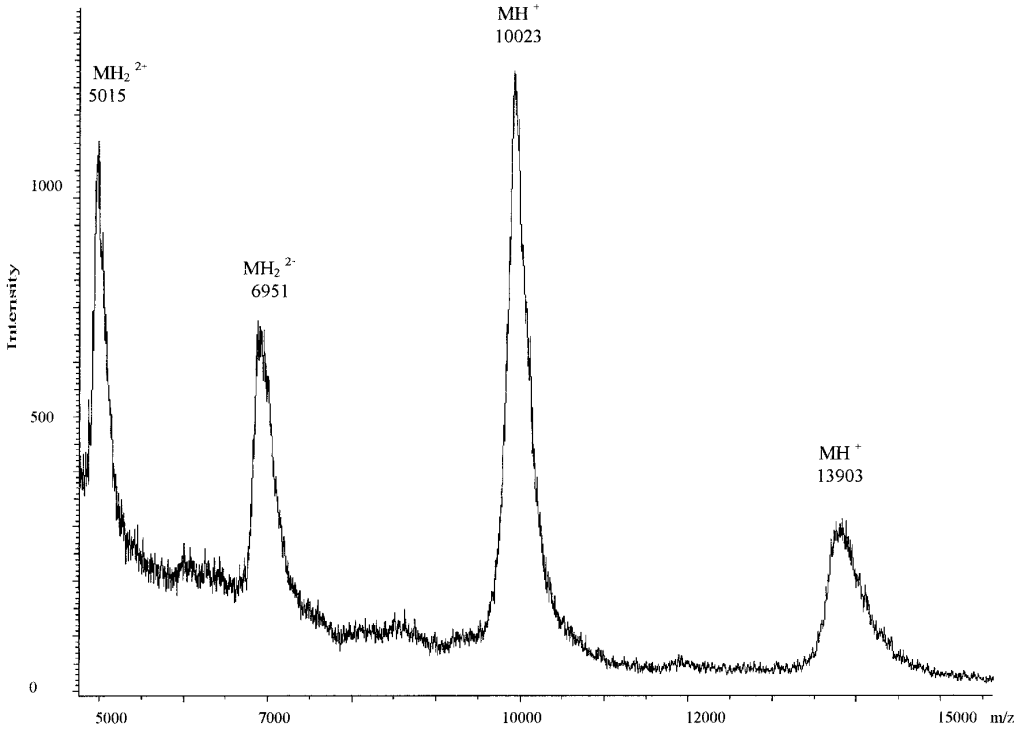

Figure 2. MALDI mass spectrum of fragments I–V.

lactone forms of each fragment, which co-elute in a single HPLC peak. The observed masses of the signals corresponded closely to the homoserine lactone forms of the fragments, probably because this is the most abundant species in the mixtures. The determined M_r are in all cases almost coincident with the calculated values (Table 1), thus allowing an exact characterization of the entire HSA sequence.

Moreover, these results show that the MALDI/MS determination of the M_r of the seven CNBr fragments was not affected by the intrinsic heterogeneity of the HSA molecule. In fact, two major sources of the HSA heterogeneity are (i) uptake of ligands (such as fatty acids and bilirubin) with the formation of non-covalent complexes and (ii) covalent modification of the HSA structure by formation of mixed disulphide bonds between the free thiol group of cysteine-34 and other thiol-containing molecules (free cysteine, glutathione).[1] In the procedure described here, reduction and carboxymethylation of the protein eliminate heterogeneity due to the formation of mixed disulphides and the formation of non-covalent complexes, because the complex-forming capability of HSA is almost completely lost upon destruction of the tertiary structure. Therefore, the present procedure appears suitable for the fast detection of covalent modifications of HSA originated by site-specific reactions or by exposure to xenobiotics, and also for detecting genetic variants, provided that the mass shift introduced by the structural abnormality is greater than the accuracy of mass determination by MALDI/MS.[10,11]

The procedure described here presents several advantages in comparison with the MALDI/MS analysis of the CNBr fragments of unreduced HSA reported previously.[14] In fact, the HPLC isolation of the CNBr fragments is much faster, produces purer samples and requires a much smaller amount of protein with respect to the combination of gel permeation and ion-exchange chromatography employed for the isolation of the CNBr fragments of unreduced HSA. In addition, using the procedure described here, fragments are produced without the occurrence of side reactions, such as that observed between the homoserine lactone end-groups and Tris in the CNBr cleavage of unreduced HSA.

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References

1. T. Peters, Jr, *All About Albumin: Biochemistry, Genetics and Medical Applications*. Academic Press, San Diego, CA (1996).
2. L. Minchiotti, M. Galliano, M. C. Zapponi and R. Tenni, *Eur. J. Biochem.* **214**, 437 (1993).
3. P. Iadarola, L. Minchiotti and M. Galliano, *FEBS Lett.* **180**, 85 (1985).
4. M. Galliano, L. Minchiotti, P. Iadarola, M. Stoppini, G. Ferri and A. A. Castellani, *FEBS Lett.* **208**, 364 (1986).
5. L. Minchiotti, M. Galliano, P. Iadarola, E. Zepponi and G. Ferri, *Biochim. Biophys. Acta* **1039**, 204 (1990).
6. N. Takahashi, Y. Takahashi, N. Ishioka, B. S. Blumberg and F. W. Putnam, *J. Chromatogr.* **359**, 181 (1986).
7. K. Biemann and S. A. Martin, *Mass Spectrom. Rev.* **6**, 1 (1987).
8. H. R. Morris and F. M. Grier, *Trends Biotechnol.* **6**, 140 (1988).
9. S. A. Carr, M. E. Hemling, M. F. Bean and G. D. Roberts, *Anal. Chem.* **63**, 2802 (1991).
10. U. Bahr, M. Karas and F. Hillenkamp, *Fresenius' J. Anal. Chem.* **348**, 783 (1994).

11. M. Karas, U. Bahr and U. Giessmann, *Mass Spectrom. Rev.* **10**, 335 (1991).
12. A. Compagnini, S. Fisichella, S. Foti, R. Saletti, L. Sardo, *Rapid Commun. Mass Spectrom.* **8**, 459 (1994).
13. S. Fisichella, S. Foti, G. Maccarrone and R. Saletti, *J. Chromatogr.* **693**, 33 (1995).
14. A. Compagnini, S. Fisichella, S. Foti, M. Jetschke, G. Maccarrone, M. Przybylski and R. Saletti, in *Proceedings of the Convegno Nazionale su Orientamenti e Metodologie in Chimica Farmaceutica, Organica e Bioorganica*, Numana, AN, Italy, June 2–6, 1995, P51.
15. M. Kussmann, E. Nordhoff, H. Rahbek-Nielsen, S. Haebel, M. Rossel-Larsen, L. Jakobsen, J. Gobom, E. Mirgorodskaya, A. Kroll-Kristensen, L. Palm and P. Roepstorff, *J. Mass Spectrom.* **32**, 593 (1997).