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Characterization of recombinant human serum albumin using matrix-assisted laser desorption ionization time-of-flight mass spectrometry

John Flensburg*, Makonnen Belew
Amersham Biosciences AB, Björkgatan 30, SE-751 84 Uppsala, Sweden

Abstract

Chromatographically purified recombinant human serum albumin (rHSA), produced in genetically transformed yeast cells, was characterized using matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) MS techniques. The molecular mass of the intact protein was determined to be 66 671, in good agreement with that of purified HSA which was used as a standard. The identity of rHSA to its natural counterpart was established with high precision using peptide mass fingerprinting of tryptic peptides. Partial amino acid sequence data for rHSA were obtained using Ettan™CAF™ MALDI Sequencing Kit and post-source decay on the tryptic peptides. The results achieved provide strong evidence that MALDI–TOF-MS is an important analytical technique for characterising gene products and for establishing the identity and bio-compatibility of recombinant proteins relative to their natural counterparts.

Keywords: Post-source decay; Peptide mass fingerprinting; Albumin; Proteins

1. Introduction

Human serum albumin (HSA) is by far the most abundant protein in the circulatory system of human blood accounting for at least 50% of the proteins in human blood plasma. In a healthy individual, this corresponds to a concentration of ~35–40 g HSA/l of blood. It is produced in the liver and has a variety of physiological functions [1]. It is a single chain protein (585 amino acid residues) containing a total of 17 disulphide bridges and a free Cys-34 residue [2]. It is also rich in histidine residues (~18) and contains 1 mol of tryptophan per mol HSA.

Traditionally, HSA has been prepared by the well-known cold ethanol fractionation method of Cohn et al. [3] and column chromatography [4] from plasma obtained from healthy donors and is used for treating a variety of clinical conditions such as burns and maintenance of homeostasis. It is also widely used as an excipient in pharmaceutical preparations to stabilize drugs during processing and/or storage.

Due to the high cost of production and the risk of viral infection, recombinant DNA technology is increasingly used to produce rHSA in bacteria, yeast, etc.

In an effort to demonstrate the bio-equivalence of rHSA produced by transformed yeast cells grown in

*Corresponding author. Fax: +46-18-612-1869.
E-mail address: john.flensburg@amersham.com (J. Flensburg).

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culture, matrix-assisted laser desorption ionization (MALDI) time-of flight (TOF) MS techniques were applied for characterizing a highly purified preparation of rHSA.

2. Experimental

2.1. Materials

Ettan MALDI-TOF Pro, Ettan™CAF™ MALDI Sequencing Kit and NAP-5 columns were obtained from Amersham Biosciences (Uppsala, Sweden). Recombinant HSA (rHSA) was purified to homogeneity from the cell culture supernatant of fermented yeast cells at the Institute for Surface Biotechnology, the Biomedical Center, University of Uppsala, Sweden. Highly purified HSA was kindly provided by I. Andersson at Amersham Biosciences, and was used as a reference in this investigation.

Ile^7-angiotensin III (AngIII), human adrenocorticotropic hormone fragment 18–39 (hACTH 18–39), bovine albumin, α-cyano-4-hydroxycinnamic acid and sinapinnic acid were obtained from Amersham Biosciences. Sequencing-grade trypsin was purchased from Promega (Madison, WI, USA). Acetonitrile (HPLC grade), and trifluoroacetic acid (TFA) were from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris), urea and iodoacetamide were obtained from Merck (Darmstadt, Germany). ZipTip C_{18} was purchased from Millipore (Bedford, MA, USA).

2.2. Methods

2.2.1. MALDI–TOF-MS

All MS analyses were performed using an Ettan MALDI-TOF Pro system equipped with a quadratic field reflectron and a timed ion gate.

Analysis of intact proteins was performed in linear mode with positive ionization at an acceleration voltage of 20 kV. Prior to MS analysis, the samples of purified rHSA and HSA were desalted against ultrapure water using a NAP-5 column. The samples for MS analysis were prepared by the ‘‘dried-droplet’’ method. A 1-μl aliquot of the analyte was mixed with an equal volume of 50% acetonitrile, 0.9% TFA saturated with sinapinnic acid. A 0.3-μl aliquot of the mixture (containing 5 pmol of protein) was loaded onto the stainless steel MALDI slide for analysis.

Protein identification (using peptide mass fingerprinting of trypsin digested rHSA) was conducted in reflectron mode with positive ionization at 20 kV. The sample was in this case mixed with an equal volume of 50% acetonitrile, 0.5% TFA saturated with α-cyano-4-hydroxycinnamic acid and 0.3 μl was dispensed on the slide. AngIII and hACTH 18–39 were used for internal calibration.

Post-source decay (PSD) was performed by first acquiring a reflectron mode spectrum which was internally calibrated. Based on this analysis, peaks were chosen for timed ion gating and the instrument was switched to PSD mode at an acceleration voltage of 20 kV. Due to the quadratic field reflectron of the instrument used, PSD spectra were obtained over the entire m/z range without the need for data stitching.

2.2.2. CAF MALDI sequencing

Derivatization of tryptic peptides of rHSA was performed on ZipTip C_{18} according to the instructions included in the Ettan CAF MALDI sequencing kit. The kit is based on chemistry developed by Keough et al. [5]. The tryptic peptides of rHSA were first guanidinated resulting in the conversion of lysines to homoarginines [6] and an increased peptide mass of 42 u. This was followed by labelling with the CAF reagent which adds a sulphonic acid group to the N-terminal of the peptides, thereby increasing their molecular mass by 136 u. After ionization by MALDI, the sulphonated N-terminus is negatively charged through deprotonation which is balanced by the positive charge on the basic C-terminus (Arg). One additional proton is formed which can move randomly along the backbone of the CAF derivatized peptide resulting in an enhanced fragmentation towards b- and y-fragments during PSD [5]. Due to the negative charge at the N-terminus, the b-fragments become neutral, resulting in a PSD spectrum consisting exclusively of y-ions thus simplifying the interpretation of acquired spectra. The amino acid sequence of a CAF derivatized tryptic peptide can thus be calculated manually from the mass differences between adjacent y-fragments.

The derivatization step in the Ettan CAF MALDI sequencing kit is better than the original chemistry

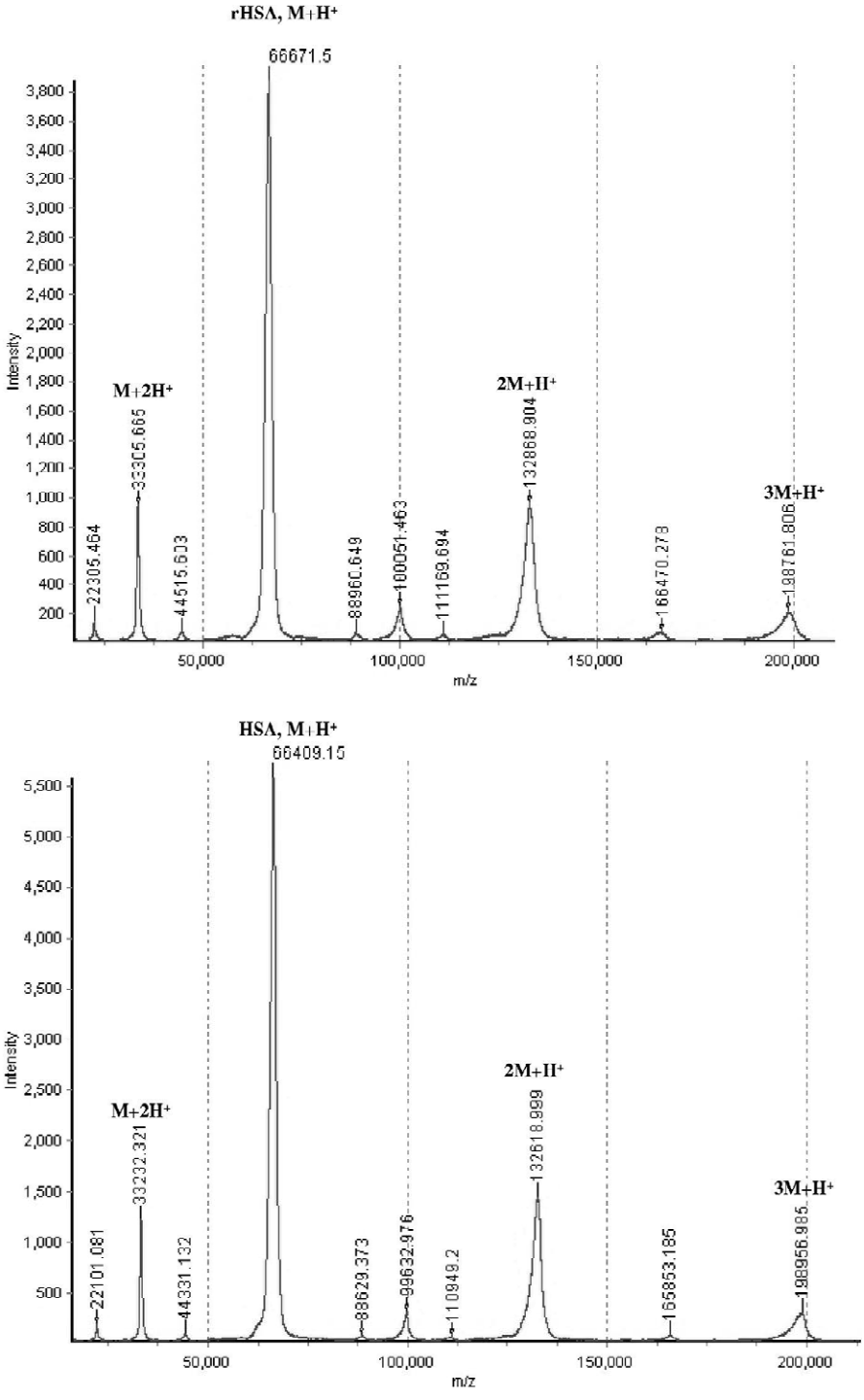
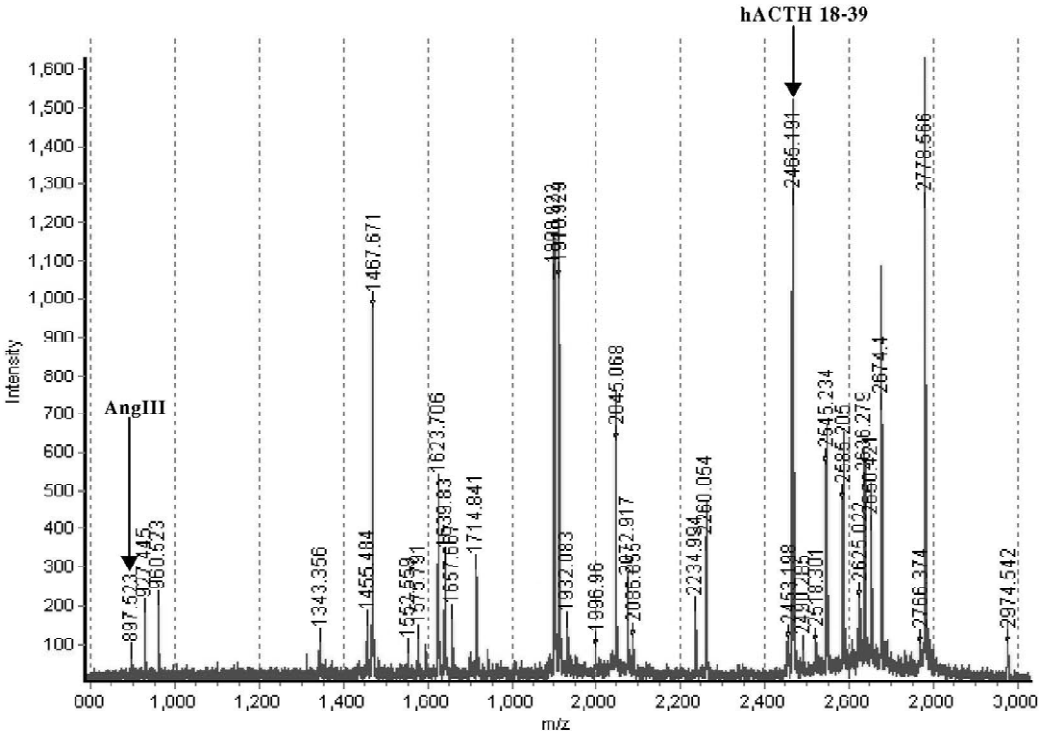


Fig. 1. MALDI–TOF-MS analyses in linear mode of full length rHSA (upper part of figure) and plasma-derived HSA (lower part of figure). Approximately 5 pmol of material was loaded in each instance onto the stainless steel MALDI sample slide. The instrument was externally calibrated with bovine serum albumin and 256 laser shots were accumulated per spectrum.



Identification result

Result name: 010924, Position 15, rHSA Fr 24, jodo,tryp

Run list name: 010924

Rank	Confidence	Protein information	Coverage	pI	kDa
1	0,99	gi|4389275|pdb|1BKE - Human Serum Albumin In A Complex With Myristic Acid And Tri-Iodobenzoic Acid	43,0	5,7	68,01
1 *	-	gi|11513969|pdb|1E7E|A - Chain A, Human Serum Albumin Complexed With Decanoic Acid (Capric Acid)	42,7	5,7	69,45
1 *	-	gi|4502027|ref|NP_000468.1 - albumin precursor; PRO0883 protein [Homo sapiens]	41,1	5,9	71,35
1 *	-	gi|178345|gb|AAA98798.1 - (M12523) alloalbumin Venezia [Homo sapiens]	41,4	6,0	71,21
1 *	-	gi|28592|emb|CAA23754.1 - (V00495) serum albumin [Homo sapiens]	38,9	6,1	71,34
1 *	-	gi|28590|emb|CAA23753.1 - (V00494) reading frame HSA [Homo sapiens]	40,2	5,9	71,27
1 *	-	gi|11493459|gb|AAG35503.1|AF130117_36 - (AF130077) PRO2619 [Homo sapiens]	42,5	6,0	58,53
1 *	-	gi|6013427|gb|AAF01333.1|AF190168_1 - (AF190168) serum albumin precursor [Homo sapiens]	37,6	5,9	71,20
1 *	-	gi|7441762|pir||G01747 - albumin homolog - human (fragment)	45,5	5,7	53,43
1 *	-	gi|6650826|gb|AAF22034.1|AF118094_29 - (AF118090) PRO2044 [Homo sapiens]	49,4	7,0	30,09
1 *	-	gi|7770217|gb|AAF69644.1|AF119917_52 - (AF119890) PRO2675 [Homo sapiens]	38,5	6,1	33,47
1 *	-	gi|7959791|gb|AAF71067.1|AF116721_45 - (AF116645) PRO1708 [Homo sapiens]	33,2	6,6	33,12
1 *	-	gi|2492797|sp|Q28522|ALBU_MACMU - SERUM ALBUMIN PRECURSOR	18,3	5,8	69,86
2	0,00	gi|1168538|sp|P22767|ASSY_ECOLI - ARGININOSUCCINATE SYNTHASE (CITRULLINE--ASPARTATE LIGASE)	23,0	5,2	50,05

Fig. 2. MALDI–TOF-MS analysis in reflectron mode of a trypsin digest of rHSA. The tabulated results at the bottom of the figure show the protein identification result using Ettan MALDI–TOF Pro software. The mono-isotopic m/z values are shown at the top of each peak. AngIII and hACTH 18–39 were used for internal calibration.

reported by Keough et al. since the reactions can be performed in an aqueous environment [7,8].

3. Results and discussion

3.1. Comparison of rHSA with HSA

The molecular mass of purified HSA and rHSA was determined by MALDI–TOF-MS operating in linear mode with positive ionization after external calibration with purified bovine serum albumin (BSA). The intensities and m/z values (mass vs. charge) of both proteins are very similar suggesting that the masses of HSA and rHSA are, for all practical purposes, identical. The molecular mass of the main component (the singly charged monomer of rHSA at an m/z value of 66 671) showed a deviation of 0.4% (262 u) relative to HSA. This variation in molecular mass is very small and might be due to the statistical error that is inherent in the method employed or due to other factors such as polymorphism of the gene coding for HSA and posttranslational modifications as pointed out by Girard et al. [9]. As can be seen from the spectra in Fig. 1, the single charged monomeric form of the proteins as well as double and triple charged monomeric forms are visible. This applies also to the single, double and triple charged dimeric and trimeric forms of these two proteins.

3.2. Analysis of trypsin digested rHSA and HSA

The purified rHSA was also subjected to digestion with trypsin and the peptides so generated were analysed by MALDI–TOF-MS in reflectron mode. The acquired m/z values were transferred to the internal search engine, Profound, which resulted in more than 40% coverage of the protein and assignment of HSA as the highest ranked candidate (Fig. 2). Similar results were obtained for HSA when it was subjected to the same kind of analysis (data not

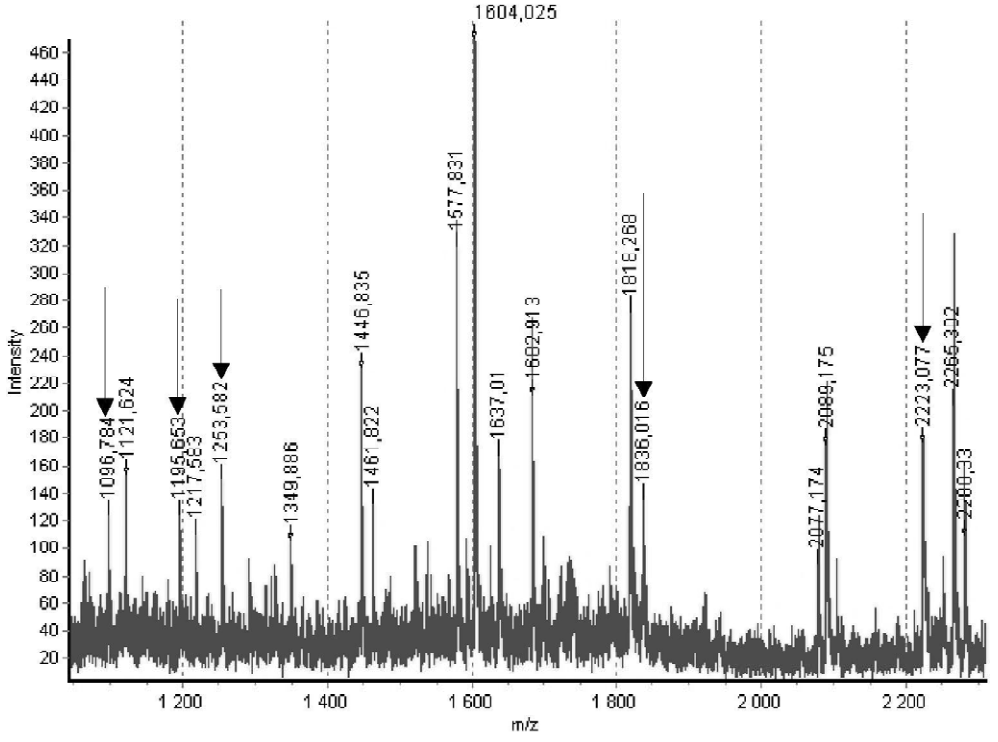


Fig. 3. Spectrum of trypsin digested and CAF derivatized rHSA in the reflectron mode. Peaks denoted with arrows were chosen for sequencing by PSD.

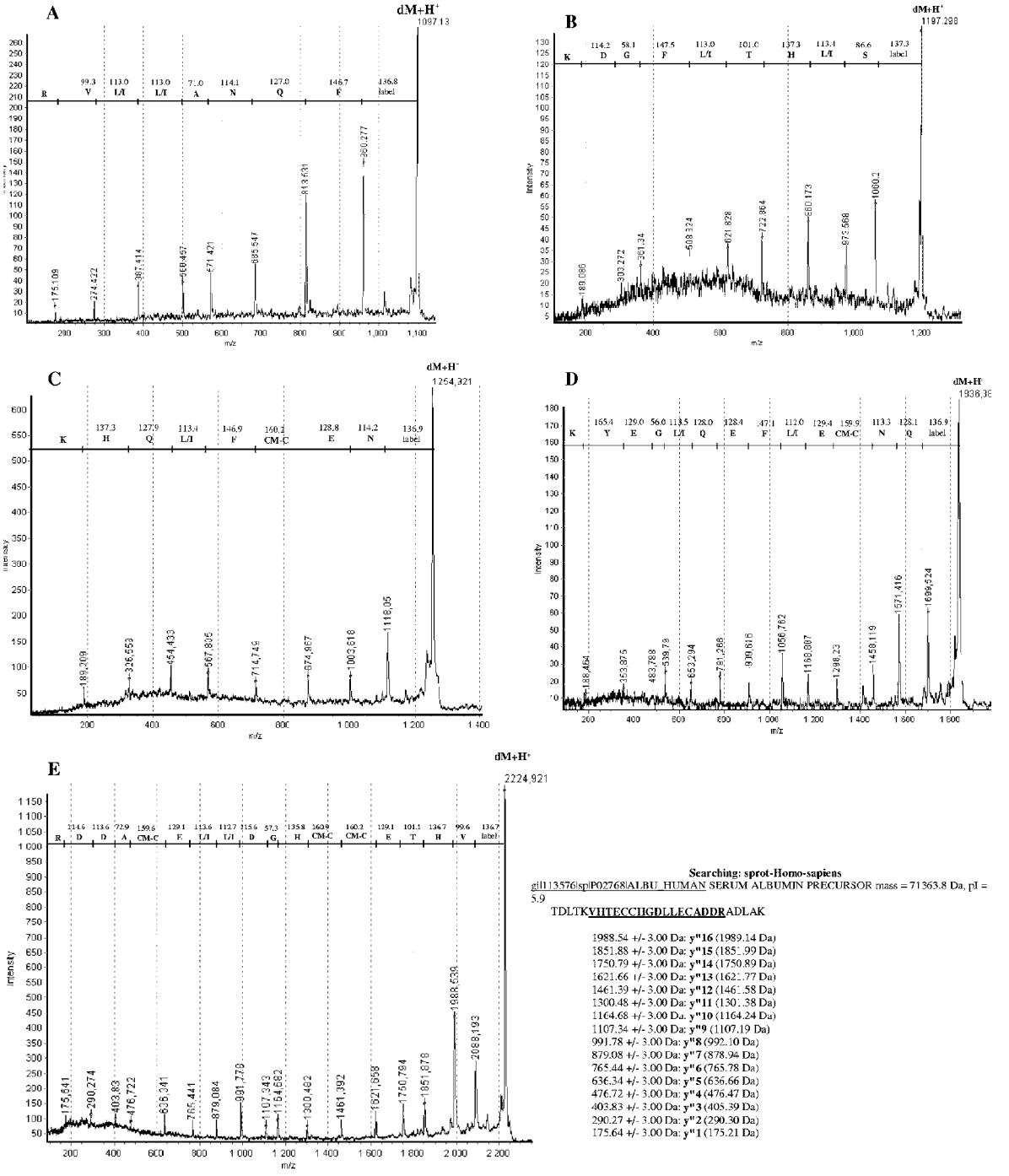


Fig. 4. CAF enhanced MALDI-PSD analyses of the five tryptic peptides from rHSA. The resulting amino acid sequence is inserted on top of each PSD spectrum. (A) PSD spectrum of the CAF labelled 1097 peptide. (B) PSD spectrum of the CAF labelled 1196 peptide. (C) PSD spectrum of the CAF labelled 1254 peptide. (D) PSD spectrum of the CAF labelled 1836 peptide. (E) PSD spectrum of the CAF labelled 2224 peptide and the identification result from PepFrag of the peptide fragments that were analysed.

shown). Taken together, the results from determinations of full length proteins and peptide mass finger printing by MALDI–TOF-MS clearly demonstrate that the natural and recombinant forms of HSA have identical amino acid compositions (a total of 585 residues for each protein).

3.3. Determination of the sequences of five tryptic peptides of rHSA by CAF mediated MALDI–MS

The amino acid sequences of five peptides generated from rHSA after trypsin digestion were determined by MALDI–TOF-MS in PSD mode after chemical derivatization according to the Ettan CAF MALDI sequencing kit. As described in the Methods section, the tryptic digest of rHSA was derivatized by guanidination which was followed by a sulphonation reaction. The resulting reflectron mode spectrum is shown in Fig. 3. Some of the peptides differing in molecular mass with 42 + 136 u (K-terminated peptides) or 136 u (R-terminated peptides) compared with the underivatized peptide masses were chosen for PSD analysis. Due to the quadratic field reflectron of the Ettan MALDI–TOF Pro instrument, all fragments, independent of size, could be focused in a single run. In contrast to a MALDI–TOF-MS instrument utilizing a linear reflectron, no time-consuming stitching procedure of several spectra covering different m/z ranges was necessary in order to acquire a complete PSD spectrum. In Fig. 4A–E, the resulting PSD spectra of the CAF derivatized peptides are shown. The complete sequences of these five peptides were achieved in less than 5 min and from the same sample spot that contained less than 1 pmol of material. The accuracy of the masses for most amino acid residues were within 1 u. CAF and MALDI-PSD could thus be convenient alternatives to other techniques for de novo and confirmative peptide sequencing. Moreover, in vitro induced modifications were easily detected as seen from those peptides containing half-cystine residues. The mass of carboxymethylated cys (CM-C), which was produced as a result of treatment with iodoacetamide prior to digestion with trypsin, was 161 (an increase in mass of 58 relative to half-cystine). The sequences and positions deduced from CAF derivatized peptides using the search engine PepFrag [10] or the internal Ettan MALDI–TOF Pro software were identical to their natural counterparts thus offering further evidence that the natural and recombinant forms of HSA are identical proteins.

4. Conclusion

The results achieved were obtained in less than 1 week. Using traditional methods for obtaining the same information would have taken considerably more time. This in itself is one of the best examples of recent technological advances in the fields of physics and chemistry. The fast generation of data will have wide implications for the approval of new recombinant proteins and peptides intended for clinical use as well as in comparative evolution studies.

References

[1] L.O. Andersson, R. Lundén, in: B. Blombäck (Ed.), Plasma Proteins, KABI, AWE/GEBERS Bohuslänningen, Uddevalla, 1976, p. 31.
[2] D.C. Carter, X. He, S.H. Munson, P.D. Twigg, K.L. Gernert, M.B. Broom, T.Y. Miller, Science 244 (1989) 1195.
[3] E.J. Cohn, L.E. Strong, W.L. Hughes, D.J. Mulford, J.N. Ashworth, M. Melin, H.L. Taylor, J. Am. Chem. Soc. 68 (1946) 459.
[4] J.M. Curling, J.H. Berglöf, L.O. Lindquist, S. Eriksson, I. Andersson, Vox Sang. 33 (1977) 97.
[5] T. Keough, M.P. Lacey, R.S. Youngquist, Proc. Natl. Acad. Sci. USA 96 (1999) 7131.
[6] T. Keough, M.P. Lacey, R.S. Youngquist, Rapid Commun. Mass Spectrom. 14 (2000) 2348.
[7] U. Hellman, R. Bhikhabhai, Rapid Commun. Mass Spectrom. 16 (2002) 1851.
[8] M. Liminga, U. Carlsson, C. Larsson, J.-L. Maloisel, R. Palmgren, T. Keough, R.S. Youngquist, in: Proceedings of the 49th ASMS Conference on Mass Spectrometry and Allied Topics, Chicago, IL, 2001, American Society for Mass Spectrometry, Santa Fe, New Mexico, USA.
[9] M. Girard, H.P. Bietlot, T.D. Cyr, J. Chromatogr. A 772 (1997) 235.
[10] D. Fenyö, J. Qin, B.T. Chait, Electrophoresis 19 (1998) 998.