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Journal of Chromatography A, 1007 (2003) 47–53
JOURNAL OF CHROMATOGRAPHY A
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Mass mapping analysis as a tool for the identification of genetic variants of bovine β-casein ☆

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Received 7 March 2003; received in revised form 14 May 2003; accepted 22 May 2003

Abstract

Matrix-assisted laser desorption ionisation time-of-flight mass spectrometric analysis of the tryptic digest of β-casein A^2 and β-casein B was performed before and after the separation of the peptides by LC. The overlapping of the chromatograms showed that all peaks were present in both samples, except for one only found in the tryptic digest of the A^2 variant and two in the B variant. Experimental masses could be assigned to those peptides produced by tryptic digest of β-casein variant. This peptide mapping strategy and current methodological improvements represent a promising tool for the identification of milk genetic variants with the difference of an amino acid substitution.

Keywords: Mass mapping; Caseins; Proteins

1. Introduction

Since the discovery of the genetic variants of β-lactoglobulin by Aschaffenburg and Drewry [1] great effort has been made in the identification and separation of different genetic variants of milk proteins, because of the possible link of milk protein polymorphism with production of milk, milk composition and milk quality. Bovine milk protein variants have been identified by electrophoretic, chromatographic and molecular techniques that have been tested and optimised for the identification of variants at the DNA level [2]. An increasingly popular approach to determine the primary structure of proteins, concurrent with the application of large-scale DNA sequencing is the use of mass spectrometry (MS). Current MS technologies are sufficient sensitive to detect and discriminate mutant or variant forms, including post-transcriptional modifications. By digesting the protein with a site-specific protease, the molecular masses of the expected peptides can be predicted and it is straightforward to assign the molecular masses of the MS-measured peptides to those expected. This procedure is called the peptide map fingerprint [3]. The peptide map fingerprint is effective in establishing changes in amino acid sequences resulting from DNA sequencing errors or frame-shift errors [4].

The MS technique providing the highest degree of

☆Presented at the 2nd Meeting of the Spanish Society of Chromatography and Related Techniques, Barcelona, 26–29 November 2002.

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0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00955-5

specificity and sensitivity for proteins is liquid chromatography (LC) combined with electrospray MS–MS. However, initial profiling of the masses of peptides generated by enzymatic hydrolysis is most commonly performed by matrix-assisted laser desorption ionisation (MALDI) time-of-flight (TOF) MS. This technique is advantageous because of the rapid and direct analysis of complex mixtures that can be performed and its tolerance to buffer salts or detergents of the sample [5]. It allows an off-line analysis that provides data from a large number of relevant peptides from each individual target position and avoids the complication of multiply charged ions derived from each constituent as compared with electrospray ionisation (ESI) MS [6]. Its use in combination with LC has proved to be a powerful analytical strategy for many proteomic laboratories as well as a rapid and complementary technique for peptide characterisation by Edman degradation [7,8]. In the field of polymorphism of milk proteins, MALDI-TOF-MS has been applied in combination with other techniques for the identification of short forms of β-lactoglobulin [9], the characterisation of phosphorylation sites in native casein micelles [10] and the identification of peptide fractions of β-casein [11]. Although this mass mapping strategy has been used for the characterisation of a new genetic variant of β-casein differing in one amino acid position from β-casein A^1 [12], no MALDI-TOF mass mapping analysis has been reported to distinguish milk genetic variants with this difference. These variants are often indistinguishable by electrophoresis because the mutation gives rise to no change in the net charge of the protein.

The objective of this paper was to assess the use of MALDI-TOF-MS combined with protein digestion in the analysis of milk genetic variants, specifically the analysis of β-casein A^2 and B of bovine milk, which differ in one amino acid substitution.

2. Experimental

2.1. Samples

Bovine caseins (α_{s1}-casein, β-casein A^2, β-casein B and κ-casein) were purified from whole casein, obtained from defatted milk from individual phenotyped cows at the Hannah Research Institute, by fractionation with cation-exchange chromatography [13].

2.2. Reagents

Bis–Tris propane {1,2-bis[tris(hydroxymethyl)-methylamino]propane}, 2-mercaptoethanol and trifluoroacetic acid (anhydrous) (TFA) were from Sigma (Poole, UK). Urea and acetonitrile were from Fisons (Loughborough, UK). α-Cyano-4-hydroxycinnamic acid (CHCA matrix) was from Aldrich (Gillingham, UK). L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin from bovine pancreas (12 000 BAEE units mg^{-1} of dry mass; 12 200 BAEE units mg^{-1} of protein) was from Sigma.

2.3. Digestion with trypsin

A 1-mg amount of each casein was dissolved in 1 ml of buffer imidazole–NaN_3 (pH 7.0). A 1-mg amount of TPCK-trypsin was dissolved in 1 ml of the same buffer and 4 μl added to each casein solution. After incubation overnight at room temperature the reaction was stopped by freeze–drying. The lyophilised residue was dissolved in water–methanol (50:50).

2.4. Reversed-phase high-performance liquid chromatography (RP-HPLC)

Separation of peptides was performed by RP-HPLC on an APEX WP ODS (C_{18}) column (7 μm, 250×4.6 mm I.D.; Jones Chromatography, Hengoed, UK), using an automated Spectraphysics system. Samples (150 μl) were loaded onto the column and peptides eluted using a linear gradient 0–50% of acetonitrile in 0.1% TFA over 60 min. The flow-rate was 1 ml min^{-1} and UV absorbance was detected at 214 nm [14]. Eighteen fractions were manually collected, freeze–dried and dissolved in water–methanol (50:50).

2.5. Mass spectrometry

MS was performed on a Lasermat 2000 MALDI-TOF mass spectrometer (Finnigan MAT, Paradise,

Hemel Hempstead, UK). Sample desorption was achieved using a 337 nm nitrogen laser, acceleration voltage was 20 kV and peptide spectra were obtained by summing 50 laser pulses at a fixed slide target position.

Freeze–dried samples were dissolved in water–methanol (50:50). Aliquots of 0.5 μl of sample and 0.5 μl of the working matrix solution (10 mg of CHCA matrix ml^{-1} in acetonitrile–water, 70:30, v/v), were mixed on the target slide and allowed to air dry prior to analysis [15]. Casein samples were dissolved in a denaturing buffer (7 *M* urea, 20 m*M* Bis–Tris propane, 0.5%, v/v, mercaptoethanol, pH 7.5) and were mixed with the working matrix solution as described above.

The MS instrument was daily calibrated by using α-lactalbumin and the fragment 1–25 of bovine β-casein isolated at the Hannah Research Institute or a standard solution of peptides of molecular masses of 1046.1, 1060.2, 1182.3, 1619.9 and 1664.9 from Bio-Rad (Hercules, CA, USA). The accuracy of the measurement was always below 0.5%.

Theoretical peptide masses were calculated using the Protein Abacus software program, version 2.0 (Lighthouse data, Finnigan MAT). The primary structures of the caseins and their variants were taken from Swaissgood [16].

3. Results and discussion

3.1. Direct ionisation of caseins

Since desorption/ionisation is dependent on the size and nature of individual proteins, the preparation of different caseins for MALDI mass analysis was studied. 0.2, 1, 4 and 10 mg ml^{-1} solutions of α_{s1}-, β- and κ-casein in water and denaturing buffer were mixed with the matrix solution and submitted to analysis. The most favourable ionisation was achieved with a concentration of 1 mg ml^{-1} of casein in denaturing buffer. Nevertheless, the spectrum of β-casein presented a peak too broad for an accurate mass measurement (results not shown). This width can be due to the possible formation of adducts produced by the capture of small fragments arising from the photochemical decomposition of the matrix. The intensity of these peaks increases with the molecular mass of the analysed protein and can make them indistinguishable [17]. The occurrence of broad peaks in MALDI-TOF-MS is also attributed to a high degree of heterogeneity of proteins such as glycosilation [18,19], although different phosphorylation degrees could be responsible, as it has been observed by electrospray MS in the analysis of ovine caseins [20]. The poor resolving power can also be attributed to metastable ions. Current high-performance TOF mass spectrometers equipped with delayed extraction and a reflectron would enable the ionisation of the intact molecule.

3.2. Digestion of β-casein variants with trypsin and MS analysis without chromatographic fractionation

Variants A^2 and B of β-casein differ in one amino acid at position 122: Ser in β-casein A^2 and Arg in β-casein B. The digestion of β-casein A^2 with trypsin produces, among others, the fragment 114–169. The substitution of Ser by Arg at position 122 gives rise to an additional site of trypsin hydrolysis in β-casein B. Therefore, it is expected that the tryptic digest of this variant contains the peptides 114–122 and 123–169. The tryptic digests of both variants were analysed by MALDI-TOF-MS (Fig. 1). Most of the peaks in the spectrum of the tryptic digest of β-casein A^2 could be assigned to single fragments predicted from the protein sequence and the specificity of trypsin, including the peptide 114–169, with a theoretical M_r of 6361.3 (Fig. 1a, peak 6). However, in the tryptic digest of β-casein B, although several peaks corresponded to known fragments of β-casein, the peptides 114–122 (M_r = 1136.3) and 123–169 (M_r = 5312.2) could not be found (Fig. 1b). Fragments of β-casein identified in the tryptic digests are marked with an asterisk in Table 1. As the analysis of the unfractionated digest provided only partial information, the separation of the peptides by LC was performed.

3.3. Isolated fractions from RPLC

Fig. 2 displays the chromatograms for the β-casein variants A^2 (Fig. 2a) and B (Fig. 2b). Overall, most of the same peaks were detected in each of these chromatograms. However, four unique peaks were

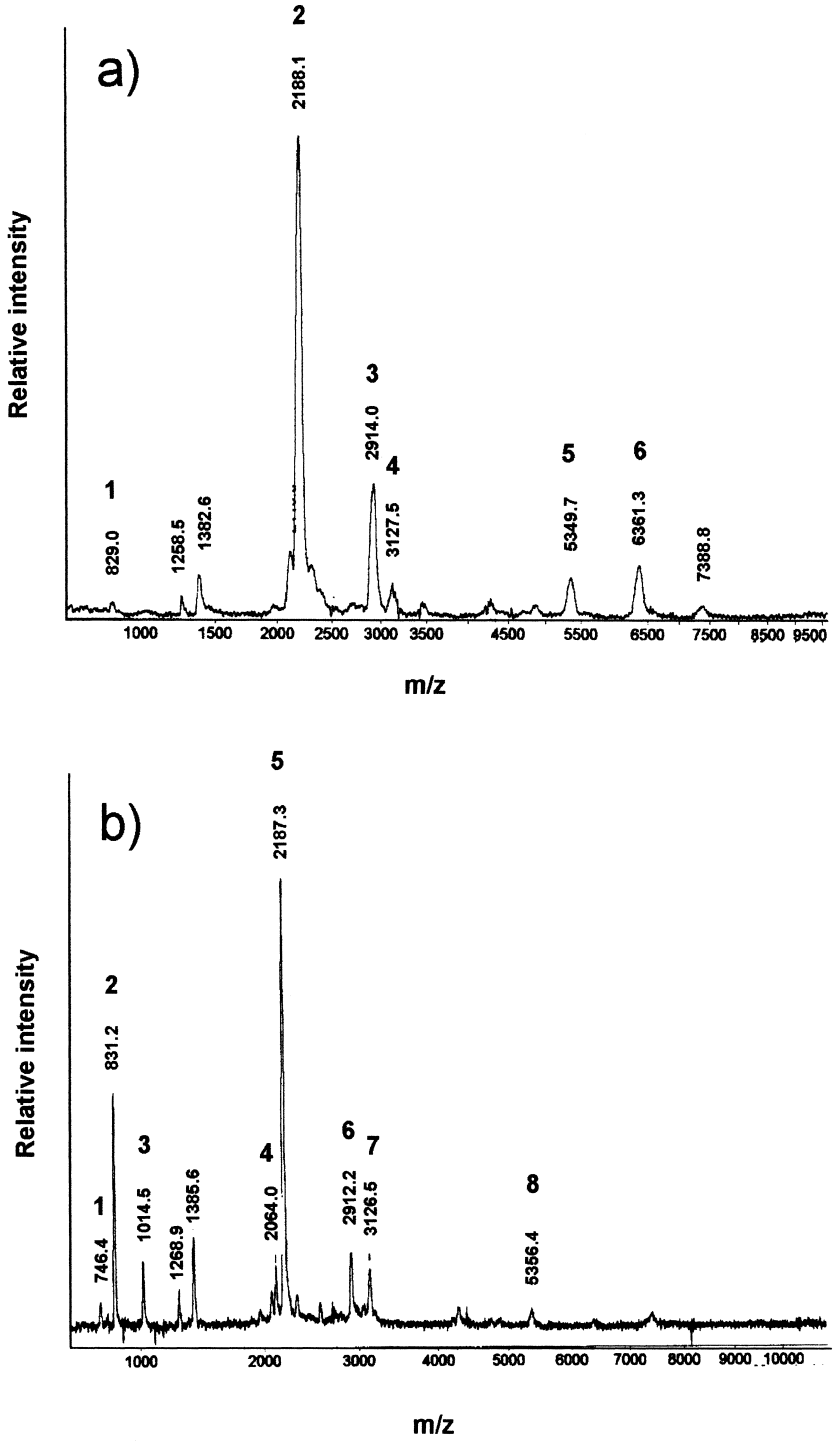

Fig. 1. MALDI-TOF mass spectra of the tryptic digest of (a) β-casein A^2. Peaks: 1=fragment 177–183, 2=fragment 184–202, 3=fragment 184–209, 4=fragment 1–25, 5=fragment 49–97; 6=fragment 114–169; (b) β-casein B; peaks: 1=fragment 203–209, 2=fragment 177–183, 3=fragment 106–113, 4=fragment 33–48, 5=fragment 184–202, 6=fragment 184–209, 7=fragment 1–25, 8=fragment 49–97.

Table 1
MALDI-TOF-MS analysis of the fractionated peptide mixture of tryptic digestion of β-casein A^2 and β-casein B

Fragment of β-casein	Measured (β-casein A^2)	Measured (β-casein B)	Calculated	Amino acid sequence
1–25*	3127.5	3132.4	3123.1	RELEELNVPGEIVESLSSSEESITR
2–25	–	2953.2	2966.9	ELEEL . . . SSSEESITR
2–25 deP	–	2886.1	2886.9	ELEEL . . . SSSEESITR
8–25	2226.2	–	2233.2	VPGEIVESLSSSEESITR
26–28	nd	nd	373.5	INK
26–48	–	2904.5	2916.2	INK . . . TEDELQDK
29–32	nd	nd	516.7	KIEK
33–48*	2064.8	2063.0	2062.0	FQSEEQQQTEDELQDK
33–97	–	7366.4	7402.3	FQSEE . . . PEVMGVSK
33–48 deP*	1979.4	–	1982.0	FQSEEQQQTEDELQDK
49–97*	5356.4	5349.7	5359.3	IHPFA . . . PEVMGVSK
98–99	nd	nd	245.33	VK
100–105	650.8	644.2	645.8	EAMAPK
106–113	746.9	746.8	747.9	HKEMPFPK
108–113*	1014.5	1010.2	1013.2	EMPFPK
114–122	–	1130.4	1136.3	YPVQPFTER
123–169	–	5305.4	5312.2	QSLTLDVENLHLPPLLLSWMHQPHQPLPPTVMFPPQSVLSLSQSK
114–169*	6361.3	–	6361.3	YPVQ . . . VLSLSQSK
170–176	778.2	779.2	781.0	VLPVPEK
177–183*	830.0	830.6	829.9	AVPYPQR
184–202*	2187.3	2188.3	2186.6	DMPIQAFLLYQQPVLGPVR
184–209*	2912.0	2911.8	2910.5	DMPIQ . . . RGPFPIIV
203–209*	743.8	746.4	741.9	GPFPIIV

nd = Not detected.
deP = Dephosphorylated.
* Fragments of β-casein whose m/z have been found in the unfractionated peptide mixture.
Note: S = sites of phosphorylation.

observed in for β-casein variant A^2 and were labelled as peaks 1–4 (Fig. 2a). Two unique peaks (peaks 5 and 6 in Fig. 2b) were observed for β-casein variant B. All fractions were manually collected, lyophilised and submitted to MALDI-TOF-MS analysis in order to check the identity of the separated peptides. The comparison of the experimental masses with the peptide masses of the theoretic digest permitted us to match most peaks with the corresponding fragments in the sequence of both β-casein A^2 and B (Table 1). Differences between experimental and calculated were in the range 0.01 to 0.5%. The m/z of peak 4 corresponded to the fragment 114–169 of β-casein A^2. Peaks 5 and 6 corresponded to fragments 114–122 and 123–169 of β-casein B, respectively. Peaks of β-casein A^2 marked as 1, 2 and 3 corresponded to the peptides 8–25 (m/z: 2226.2), 1–25 (m/z: 3127.5) and an unknown peptide (m/z: 5299.8) probably issued from a missed cleavage. Small peptides were not identified, as peptides with relative molecular masses <600 are not likely to be detected in the MALDI mode.

3.4. Detection of dephosphorylated peptides

β-Casein contains five serine positions that are phosphorylated, four in the 2–25 tryptic peptide and one in the 33–48 peptide. In order to assess the possible detection of these post-translationally modified proteins, those peaks corresponding to the above mentioned peptides whose observed masses were 80 u (or multiples of 80 u) lower than that calculated for a predicted tryptic peptide were tentatively assigned as dephosphorylated forms [18]. The dephosphorylated form of peptide 33–48 (m/z 1979.4) and one dephosphorylated form of peptide 2–25 (m/z 2886.1) were found (Table 1). Therefore, by

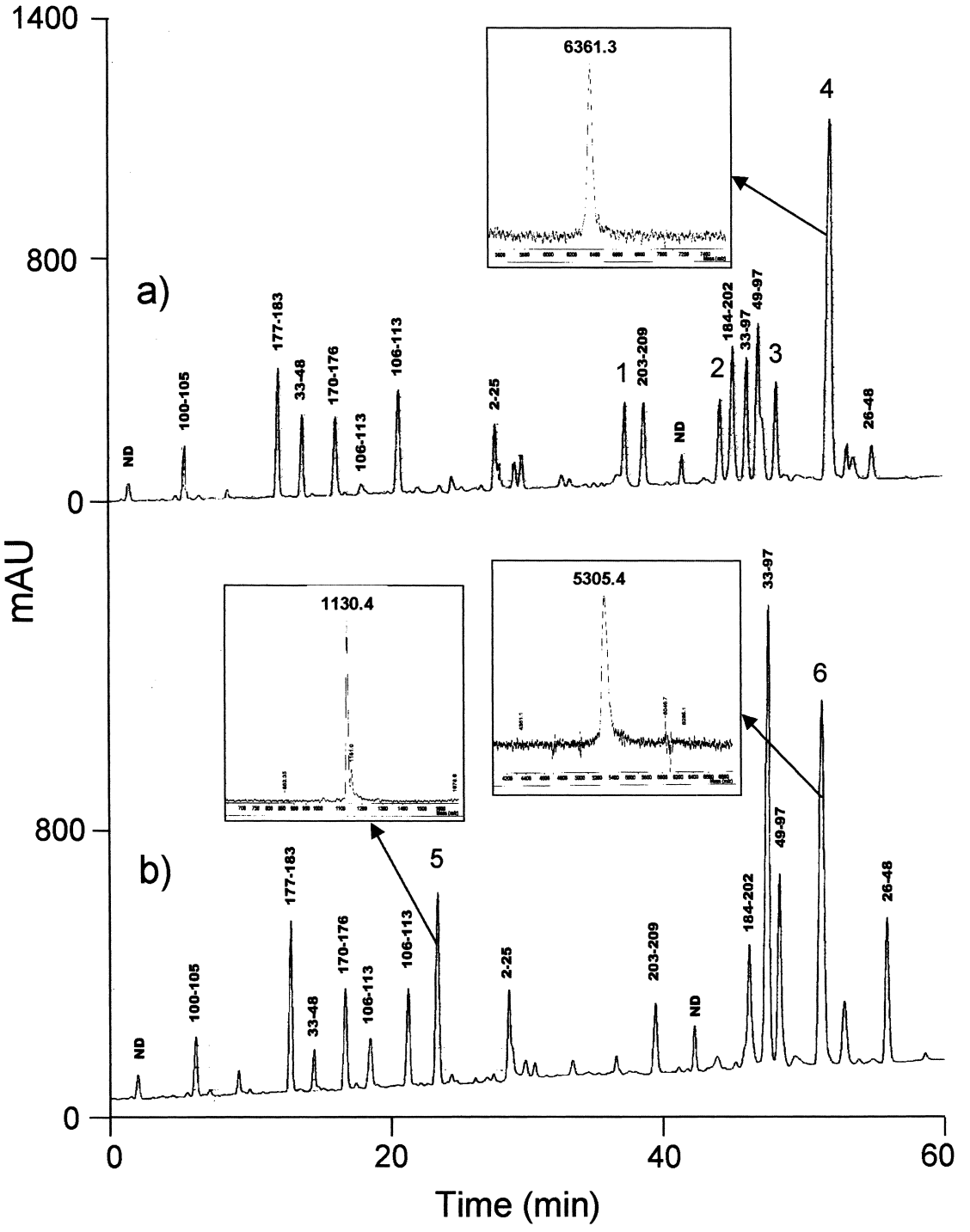

Fig. 2. Chromatograms of the tryptic digest of (a) β-casein A[2]. Peaks: 1 = fragment 8–25, 2 = fragment 1–25; 3 = unknown peptide; 4 = fragment 114–169; (b) β-casein B; peaks: 5 = fragment 114–122, 6 = fragment 184–209, 7 = fragment 123–169. For separation conditions see the Experimental section.

using the tryptic digest of β-casein it was possible to detect two dephosphorylated peptide fragments.

4. Conclusion

The described strategy was proved to be suitable for distinguishing two β-casein variants differing in one amino acid position, as well as being able to detect some dephosphorylated forms. MALDI-TOF-MS following protein digestion and LC represents a valuable tool for performing mass mapping analysis in the case of known variants, although sensitivity and accuracy should be higher for the analysis of unknown proteins. Delayed extraction (time-lag focusing) implemented on the MALDI instrument would yield substantial improvements in mass accuracy.

Acknowledgements

The authors thank the CICYT for the financial support through Research Project AGL-2002-1261. B.M. wishes to express her appreciation to the Spanish Ministry of Education for the grant for the stay at the Hannah Research Institute and the kind welcome at this institution.

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