A new bovine β-casein genetic variant characterized by a Met_93 → Leu_93 substitution in the sequence A^2

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Lait 82 (2002) 171–180

DOI: 10.1051/lait:2002002

Original article

A new bovine β-casein genetic variant characterized by a $Met_{93} \rightarrow Leu_{93}$ substitution in the sequence A^2

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(Received 13 February 2001; accepted 16 May 2001)

Abstract – A new variant of bovine β-casein was isolated from individual milk and characterized. It is proposed for nomenclature as β-CN H. The variant H differed from the variant A^2 by the substitution of the residue methionine at position 93 by a leucine residue, by the substitution of the residue glutamine at position 72 by a glutamic acid residue and by another equivalent (Gln→Glu) substitution within the sequence 114–169. The leucine residue at position 93 was identified in the plasmin-induced peptide (f49–99) by electrospray ionization tandem mass spectrometry (ESI-MS^2), and confirmed by the amino acid composition of this peptide C-terminus. This mutation would correspond to the substitution of the codon ATG by CTG, originally reported in cDNA by Jimenez-Flores et al. [11]. The molecular mass of β-casein H was measured as 23 969.1 ± 2.4 $g \cdot mol^{-1}$.

β-casein / genetic variant / on-line HPLC/ESI-MS / peptide mapping

Résumé – **Un nouveau variant génétique de la caséine β bovine caractérisé par une substitution $Met_{93} \rightarrow Leu_{93}$ dans la séquence A^2**. Un nouveau variant de la caséine bovine β a été isolé d'un lait individuel, et caractérisé. Il est proposé de le nommer β-CN H. Le variant H diffère du variant A^2 par la substitution du résidu méthionine en position 93 par un résidu leucine, par la substitution du résidu glutamine en position 72 par un résidu d'acide glutamique, et par une seconde substitution équivalente (Gln→Glu) dans la séquence 114–169. Le résidu leucine en position 93 a été identifié dans un fragment d'hydrolyse par la plasmine, le peptide (f49–99), par spectrométrie de masse tandem (MS^2) à source d'ionisation electrospray, et confirmé par la composition de la séquence C terminale de ce

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peptide. Cette mutation correspond à la substitution du codon ATG par CTG, décrite dans l'ADNc par Jimenez-Flores et al. [11]. La masse moléculaire de la caséine β H a été déterminée à 23 969,1 ± 2,4 g·mol^{-1}.

caséine β / variant génétique / couplage HPLC/ESI-MS / cartographie peptidique

1. INTRODUCTION

Since the pioneer work of Ashaffenburg and Drewry [3] on the polymorphism of bovine milk proteins, all six main proteins from cow's milk, i.e. α-La, β-Lg, and the four caseins α_{S1}, α_{S2}, κ and β have been found polymorphic. β-casein (β-CN) has this unique characteristic that all known variants arise from genetic polymorphism, and not from post-transcriptional modifications. Ng-Kwai-Hang and Grosclaude [16] reviewed the genetic variants of β-CN resulting from substitutions of charged residues and which could therefore be separated by various electrophoretic techniques. Five electromorphs, labeled A^1, A^2, A^3, B and C, compose the major part of the genetic pool of European cattle. Biochemical characterizations were carried out on these variants, allowing the location of their mutated residues. Sequences for variants A^2 and A^1 were essentially confirmed by sequencing of the coding genes [4, 20]. The variant A^2 is regarded as the original β-CN type [10]. Its consensual sequence was reported by Swaisgood [21]. In addition, rare variants D, E, A', A^4, $A^3_{MONGOLIE}$ and B^2 were isolated from the milk of rare European or exotic cattle, but only the sequences of the variants D and E have been reported so far [16]. Genetic mutation causes defect of phosphorylation in variants C and D [16]. A study of the gene sequence [11], later corrected [21], and a second one restricted to a part of the sequence [15] suggested additional variability. Both works agreed with a single substitution of the codon ATG, coding for a methionine residue at position 93, by the codon CTG, coding for a leucine residue. The corresponding mutated protein has not yet been characterized, nor fully recognized as a variant.

Using reverse phase – high performance liquid chromatography (RP-HPLC), Carles [6] resolved the β-CN fraction of a supposed homozygous β-CN A^1 cow into a variant A^1 and a second variant with similar electrophoretic mobility that was therefore termed "silent". These variants differed by the mutation of a neutral residue (proline) by another one (leucine). One fourth of random single-point mutations in exons would not be expressed in the protein, one fourth would lead to substitutions of charged residues, and one half to the "silent" exchange of neutral residues [10]. There is therefore potentially twice as many silent variants of β-CN as electrophoretic variants already known. However, only the silent variants F [23] and G [8] have been reported so far, and the latter variant has been recently questioned [22].

We report an additional silent variant mutated with respect to β-CN A^2 by a leucine residue at position 93. Methods used in this study essentially corresponded to the RP-HPLC peptide-mapping described by Carles [6]. However, the systematic use of mass-spectrometry for detection contributed to significantly simplifying this method. Whereas the elution times of peptides from unknown and reference variants separated in HPLC had to be compared [6], in the present work the relative molecular masses (M_r) of the variant peptides could be determined with sufficient accuracy to refer only to theoretical standards. According to the current nomenclature [9], the new variant described thereafter should be referred to as β-CN H.

2. MATERIALS AND METHODS

2.1. Reagents

Except trifluoro acetic acid (TFA), amino acids (Sigma, Sigma-Aldrich, St Quentin-Fallavier, France) and 9-fluorenylmethyl chloroformate (FMOC) (Fluka, Sigma-Aldrich), all other chemicals of analytical grade were purchased from Prolabo (Prolabo, Merck Eurolab, Darmstadt, Germany). Acetonitrile (Merck Eurolab, Darmstadt, Germany) and tetrahydrofurane were of chromatographic grade. HPLC buffers were made of ultra-pure water with a resistivity of 18 MΩ (Elga, ODIL, Talant, France), filtered through 0.45 μm and degassed.

Inactive plasminogen was isolated from fresh bovine blood [7], and kept frozen at – 20 °C. Immediately before use, aliquots were treated with urokinase (EC 3.4.21.73) (Choay, Sanofi Winthrop, Gentilly, France) at an enzyme/substrate ratio of 1/100 IU·mg^{-1} for 10 min at 37 °C, and thus activated to plasmin (EC 3.4.21.7).

Chymotrypsin (EC 3.4.21.1) and carboxypeptidase A (EC 3.4.17.1, type II-phenylmethylsulfonyl fluoride-treated) were purchased from Sigma.

2.2. Preparation of β-casein

Individual milk was obtained from the phenotyped Normande herd of the Coopérative Graindorge S.A. (Livarot, France). A milk found homozygous for β-CN by isoelectric focusing was selected. The milk top fat layer was removed after centrifugation (1800 *g*, 4 °C, 15 min). Whole casein was obtained from the skim milk by isoelectric precipitation at pH 4.6 [24].

Crude β-CN was obtained in gram amounts by batch-wise ion exchange [12], dialyzed against water, and lyophilized. The purity determined by urea-polyacrylamide gel electrophoresis (urea-PAGE) [1] was ~ 90%.

Homogeneous β-CN was obtained from crude β-CN by fast protein liquid chromatography ion exchange [2], scaled down to fit a HR16/10 column (Pharmacia Biotech. AB, Uppsala, Sweden). Briefly, 600 mg of crude β-CN were loaded onto 20 mL of Q Sepharose FF (Pharmacia Biotech. AB), and eluted at 1 mL·min^{-1} with a linear gradient of NaCl over 0.09–0.32 mol·L^{-1} in buffer 25 mmol·L^{-1} Tris-HCl, 4.5 mol·L^{-1} urea, 0.08 mmol·L^{-1} DTT, pH 7. Fractions collected over 5 min were checked by urea-PAGE [1] for homogeneity, pooled, dialyzed against water, and lyophilized.

2.3. Enzymatic hydrolysis

2.3.1. Digestion with plasmin

Plasmin was used to perform limited cleavage of β-CN, specific for the bonds Lys-X and Arg-X. Activated plasmin was added at 1/100 (w/w) to pure β-CN at 10 g·L^{-1} in 75 mmol·L^{-1} ammonium acetate, pH 6.5, and the mixture incubated at 37 °C for 8 h. The reaction was stopped by boiling for 10 min at 100 °C. The hydrolysate was diluted in an equal volume of 8 mol·L^{-1} urea HPLC buffer A, and filtered through 0.45 μm.

2.3.2. Digestion with chymotrypsin

The peptide β-CN (f49–99) (~ 5 nmol) was dissolved in 200 μL of 20 mmol·L^{-1} ammonium acetate, pH 8.0, with added chymotrypsin in a molar ratio 1/100, and incubated for 1 h at 37 °C. The reaction was stopped by adding formic acid at a final concentration 5/1000 (v/v).

2.3.3. Digestion with carboxypeptidase

About 5 nmol of β-CN (f49–99) were dissolved in 200 μL of 0.2 mol·L^{-1} ammonium bicarbonate, pH 8.0, with added carboxypeptidase A in a molar ratio of

1/20, and incubated at 37 °C for 18 h. The reaction was stopped by decreasing the pH to below 1.8 with TFA. The amino acids solution was centrifuged (20 000 *g*, 5 min) and vacuum dried at room temperature (~ 25 °C).

2.4. Reverse phase – high performance liquid chromatography

2.4.1. Separation of peptides

Peptides and proteins were analyzed on a C18 Zorbax column (4.6 × 150 mm, 300 Å, 3.5 μm, Agilent Technologies, Newport, USA). Analysis was carried out at 37 °C using Waters equipment (St Quentin-en-Yvelines, France) composed of a 625 LC pump and a photodiode array detector 991. About 5 nmol of intact or digested β-CN were manually loaded onto the previously equilibrated column. The solvent system was composed of water/acetonitrile/TFA in the volume ratio 1000/0/1.05 (solvent A) and 400/600/1 (solvent B). A 5 min isocratic elution at 5% B and 0.8 mL·min^{-1} enabled the elution of salts, while the MS detector was by-passed, and was followed by a linear gradient going from 5% to 60% (v/v) B over 55 min for peptide elution. The eluent was simultaneously monitored by uv absorbance (214 nm) and on-line MS analysis.

All peaks in β-CN digest with plasmin were manually collected, and dried at 30 °C in a vacuum concentrator.

2.4.2. Separation of amino acid derivatives

Dried amino acids released from peptide β-CN (f49–99) by C-terminal limited digestion were dissolved in 100 μL of buffer A [5]. To 10-μL samples, 50 μL of 0.4 mol·L^{-1} sodium borate, pH 10.4 and then 10 μL FMOC at 2.5 g·L^{-1} in acetonitrile were successively added, and the mixture incubated for 5 min. The derivatives were immediately analyzed by RP-HPLC on a Hypersyl ODS column (4.6 × 250 mm, 120 Å, 5 μm, Supelco, Sigma-Aldrich) at 40 °C on a LC5000 Varian equipment (St Quentin-en-Yvelines, France). Ten microliters of the derivative mixture were automatically loaded onto the equilibrated column. The elution was immediately carried out at a flow rate of 0.8 mL·min^{-1} using a binary buffer system [5] and the following gradient (expressed as % of B): 0 min = 33, 1 min = 35, 2 min = 39, 3 min = 42, 30 to 35 min = 100. Fluorimetric detection on Gynkotek RF 2 000 (Dionex, Voisins-le-Bretonneux, France) was at 266 nm (excitation) and 345 nm (emission). The peaks were identified and quantified, in duplicate, by reference to the mean (n = 3) retention time and peak height of 21 amino acids (Sigma) each injected at 5 × 10^{-10} mol·L^{-1} after a treatment similar to that of the initial peptide.

2.5. Electrospray ionization – mass spectrometry (ESI-MS)

Electrospray ionization (ESI) uses a pneumatically assisted source to produce intact multicharged gas phase ions directly from high molecular mass analytes in solution. Peptides were delivered to the source either as HPLC eluent (on-line LC-MS), or as mixtures, in infusion mode. Positive ionization was used. Mass spectrometry was carried out on a Sciex API III (Perkin-Elmer, Toronto, ON, Canada) composed of three quadrupoles, Q1, Q2 and Q3. Q3 was used for detection by scanning the mass-to-charge ratio (m/z) with a dwell-time of 1 ms and a step-size of 0.33 throughout both LC-MS and tandem MS analysis.

2.5.1. On-line LC-MS

On-line MS was performed using a single quadrupole configuration (Q3). During peak elution, multiple scans were acquired over the m/z range 600–2 200, and averaged. Collected data were processed

by MasSpec 3.3 and BioMultiview 1.2 softwares. The deconvoluted mass spectra of multicharge ions allowed us to get the M_r of the polypeptide. Monoisotopic M_r were used below 1 500. The accuracy achieved in M_r determination under these conditions was typically 0.01% [13]. Observed M_r were matched with theoretical M_r of tryptic/ chymotryptic peptides from β-CN A^2 [21] by the MacPro Mass program. The peptides were thus identified according to two criteria: the agreement of their termini with the applied cleavage specificity, and the agreement of their M_r with calculated values.

2.5.2. Tandem MS in infusion mode

In tandem MS, the parent ion produced by ESI is further fragmented, either once (MS^2) or twice (MS^3), to measure the M_r of the constitutive amino acid residues. Chymotryptic digest of ~ 5 nmol of the peptide β-CN (f49–99) in 200 µL water containing 0.5% (v/v) formic acid was delivered to the ionization source at 5 µL·min^{-1} by a syringe pump (Harvard Apparatus, South Natick, USA).

For MS^2, a charged peptide was selected through Q1 and underwent collision-induced dissociation (CID) in Q2 by colliding with Argon molecules. Fragment ions were analyzed through Q3 by scanning m/z over the range from 60 to the M_r of the parent ion + 50. CID occurs especially at peptidic bonds, and thus produces charged fragment peptides. Ions corresponding to respectively C-terminal and complementary N-terminal fragment peptides are labeled y_n and b_n. The M_r increments between two successive y_n or b_n ions indicate the M_r of the fragments of the missing amino acid residues. Observed y_n and b_n ions in the CID spectrum were matched with theoretical M_r for a putative amino acid sequence. Pseudo MS^3 was carried out by subjecting to MS^2 the charged fragments of a peptide spontaneously fragmented during ESI.

3. RESULTS

3.1. Determination of β-casein M_r

Figure 1 displays LC-MS analysis of the purified β-CN. The reconstructed M_r spectrum displayed one major peak and two

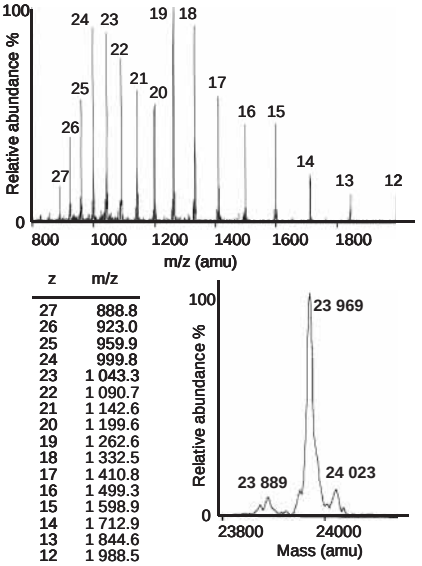

Figure 1. Spectrum (top), m/z values (bottom left), and deconvoluted spectrum (bottom right) from LC/ESI-MS analysis of bovine β-CN. Above main peaks are reported the net charges (z) of the ions corresponding to multiproto-nated forms (top) or the average M_r of the main species (bottom).

minor ones. The main component was found to have a M_r = 23 969.1 ± 2.4, which differed from that of all previously reported variants of β-CN. It was in particular lower by 16 than that of the A^2 variant [13]. This component was labeled β-CN H. One minor component displayed a M_r lower by 80 than that of β-CN H, and thus corresponded unequivocally to a mono-dephosphorylated form of β-CN H. The second minor component displayed a M_r = 24 023, consistent with the reported M_r for the variant A^1 [13].

3.2. Peptide mapping

Substitution(s) potentially accounting for the observed molecular mass discrepancy were investigated in the fragments of β-CN H. The M_r of nine peptides obtained by limited cleavage of β-CN H with plasmin were measured in LC-MS, and compared to the theoretical M_r of β-CN A^2 fragments (Tab. I). Eight peptides accounting for the segments 1–48 and 100–209 were thus identified. The peptide β-CN H (f114–169) displayed a ΔM_r (observed M_r – theoretical M_r for A^2 variant) = + 1. This discrepancy was consistently observed for three additional longer peptides that encompassed this segment (not shown): (f106–169), (f106–176) and (f108–176). One peptide displayed a ΔM_r = – 17.4 when compared to its probable match, β-CN A^2 (f49–99). We hypothesized that a mutation within the stretch 49–99 of β-CN H was responsible for this discrepancy, and tentatively labeled this unknown peptide as β-CN H (f49–99).

The peptide β-CN H (f49–99) was extensively digested with chymotrypsin. Chymotrypsin cleaved the peptidic bonds 52–53, 56–57, 58–59, 72–73, and 93–94, in agreement with the reported specificity [18], and in addition the bond 68–69 which resulted in the release of nine peptides. Peptides β-CN H (f49–52), β-CN H (f53–58), β-CN H (f57–68), β-CN H (f59–68) and β-CN H (f94–99) were readily identified from their M_r. Peptides β-CN H (f57–72) and β-CN H (f59–72) displayed ΔM_r = + 1. The two remaining peptides displayed ΔM_r = – 18 and – 17 respectively. These peptides were tentatively identified as, respectively, the mutated peptides β-CN H (f73–93) with M_r = 2 298.3 ± 0.1 and β-CN H (f69–93) with M_r = 2 723.9 ± 0.1.

Table I. Identification of plasmin-induced β-CN H peptides by on-line LC/ESI-MS.

Peptides	Observed M_r	Theoretical M_r [21]	Assigned sequence
1	3 478.3 ±0.7	3 478.6	1–28
2	2 560.1 ±0.4	2 560.6	29–48
3	5 529.2 ±0.1	5 546.6	49–99 [1]
4	645.5[2]	645.8	100–105
5	1 012.9 ±0.1	1 012.5	106–113
6	6 363.7 ±0.3	6 362.3	114–169
7	779.7 ±0.2	779.5	170–176
8	829.6 [2]	829.5	177–183
9	2 910.1 ±0.2	2 910.5	184–209

[1] With ΔM_r = – 17.
[2] Measured for a single charge number (z = 1).

3.3 Tandem MS analysis of mutated peptides

The mutated peptide β-CN H (f69–93) was analyzed by MS^2 in infusion mode, by selecting the corresponding double charged ion with m/z 1363. The M_r for the fragment residues 69 to 93 (results not shown) matched the theoretical M_r for the residues of the A^2 variant, except at position 72 and 93. The M_r of the fragment residue 93 was determined as 113.5, which corresponds to either Leu or Ile residue, and the M_r of the fragment residue 72 as 129.3, which corresponds to Glu residue. The fragmentation spectrum of this peptide, i.e. 25 amino acid residues, is too complex to be adequately assigned. Hence a shorter mutated peptide, producing a simpler spectrum, was generated and analyzed by pseudo-MS^3.

Proline-containing polypeptides have been reported to undergo specific fractionation of Pro-X bonds at low energy [14]. The potential voltage conditions in the field free skimmer-orifice region preceding the first quadrupole (Q1) elicit low-energy fragmentation, that typically increases with increasing orifice potential voltage [19]. The increase of the orifice potential from 60 to 100 V resulted in the fractionation of the peptide β-CN H (f69–93), with as a main product the peptide β-CN (f85–93) with M_r = 1039. Pseudo MS^3 enabled the determination of the M_r of the fragments of the residues 85 to 93 (Fig. 2). The M_r of the fragment residues 85 to 93 were consistent with that measured in MS^2.

3.4 Amino acid composition of the C-terminal sequence of the mutated peptide β-CN H (f49–99)

Leucine residue was released by digestion of the peptide β-CN H (f49–99) C-terminus, whereas isoleucine and methionine were not. Nine amino acids released from β-CN H (f49–99) by carboxypeptidase A were identified with reference to standards, whereas two peaks could not be identified. The identified residues were found from their relative proportions to originate from two distinct sequences (Tab. II). A minor set matched the sequence Phe_{52}-Ala-Gln-Thr_{55}, and highlighted probable endopeptidase activity in the commercial enzyme. The major set corresponded to the peptide's C-terminal sequence with a leucine residue

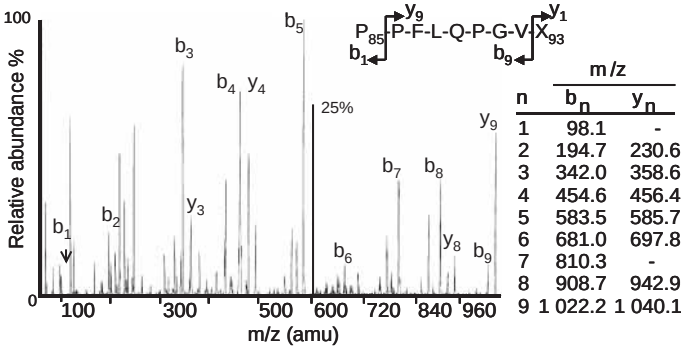

Figure 2. Collision induced dissociation spectrum (left) and m/z (right) of the peptide fragment ions y_n and b_n (see top) observed for the fragment ion (m/z 1 040.1) of the peptide (f69–93) by tandem mass spectrometry (pseudo-MS^3). Predominant y_n and b_n ions are labeled above peaks. X stands for either Leu or Ile residues in the reconstructed sequence (top) for the analyzed fragment (f85–93).

Table II. Amino acid residues released from peptide β-CN H (f49–99) treated with carboxypeptidase A for 18 h expressed as a molar ratio to serine residue.

Amino acid residues	Theoretical [21]		Observed (mean n = 2)
	β-CN A^2	mutated Leu_{93}	
Sequence X_{93}-Gly-Val-Ser-Lys-Val-Lys_{99}			
lysine	2	2	1.6
valine	2	2	1.7
serine	1	1	1.0
glycine	1	1	1.1
leucine	0	1	0.8
methionine	1	0	0.0
threonine	0	0	0.4
glutamine	0	0	0.3
alanine	0	0	0.2
phenylalanine	0	0	0.2

at position 93, i.e. Leu_{93}-Gly-Val-Ser-Lys-Val-Lys_{99}.

4. DISCUSSION

In addition to the $Met_{93} \rightarrow$ Leu substitution in the sequence of β-CN H characterized by MS^2 and pseudo-MS^3, two Gln→ Glu substitutions were detected. The substitution $Gln_{72} \rightarrow$ Glu was assessed by MS^2. The substitution of another Gln residue at one of the positions 123, 141, 146, 149, 160 or 167 is suggested by a consistent $\Delta M_r = +1$ for all the peptides encompassing the segment (f114–169). The Gln→ Glu substitutions had not been found previously in association with the main $Met_{93} \rightarrow$ Leu genetic mutation [11, 15]. The combination of two or three of these mutations accounted for the observed variations of the ΔM_r (– 16, – 17 or – 18) of mutated β-CN H peptides with various termini. The addition of all three substitutions made up for the $\Delta M_r = -16$ for the intact casein.

The M_r of the studied peptides indicated that, except for the identified mutated residues, all residues were apparently consistent with the A^2 sequence. In particular, the M_r of the peptides β-CN H (f1–28) and β-CN H (f29–48) matched that of the peptides β-CN A^2-4P (f1–28) and β-CN A^2-1P (f29–48), which assessed the conservation of the phosphoserine residues. However, single-point mutations that would conserve mass (Ile↔Leu, and Gln↔Lys), or conservative multiple substitutions might have been undetected. The probability of detecting a possible multiple substitution conserving the total M_r of the casein was maximized by measuring the M_r of overlapping fragments (not shown). The identity of 43 residues was directly assessed: residues 56 to 93 were checked by MS^2 of peptides β-CN (f56–68) and β-CN (f69–93) (not shown), and residues 93 to 99 by direct sequencing.

The present work provides experimental support to the cDNA mutation observed originally by Jimenez-Flores et al. [11].

The occurrence of the variant H is not known, since electrophoretic routine techniques for genetic phenotyping of cattle do not allow the resolution of variants A^2 and H. Prior to this study, the variant H had probably been studied twice by independent groups [11, 15]. This suggests that a significant proportion of the so-called variant A^2 might actually be found identical to the variant H by applying adequate techniques.

Besides its molecular mass, the main substitution in variant H should result in an altered cyanogen bromide cleavage pattern. Indeed, variant A^2 was shown to release seven peptides accounting for the six methionine residues [17], whereas variant H contains only five methionine residues. This discrepancy may be used for the rapid screening of supposed A^2 β-casein, provided that a separative method were optimized.

ACKNOWLEDGEMENT

The authors are grateful to D. Le Bars (INRA, Jouy-en-Josas) for helpful discussion. They wish to thank H. Lamprell for linguistic revision, and Y. Noel for commenting the manuscript (INRA, Poligny).

This work was supported by grant nº 99 Y0098-13, and D. Senocq was a recipient of Ph.D. grant nº 555, from the French Ministry for Education, Research and Technology.

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