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Identification of a new genetic variant of bovine β-casein using reversed-phase high-performance liquid chromatography and mass spectrometric analysis

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Abstract

Various components of the β-casein fraction from bovine milk were separated by preparative reversed-phase high-performance liquid chromatography (RP-HPLC). They included the genetic variants βA^1, βA^2, βA^3, and an unknown component previously denoted βX [S. Visser et al., J. Chromatogr. 548 (1991) 361–370]. Tryptic digests of these components were compared by RP-HPLC and most peaks were analysed by mass spectrometry (MS). The tryptic map of βX was closest to that of βA^1, but with a few mutually different peak components. Electrospray ionisation MS revealed that in the βX map these components had relative molecular masses of 16 higher than the corresponding ones in the βA^1 map. The main differential peaks represented the 114–169 fragments of βA^1 and βX, respectively, which were both purified and then cleaved with cyanogen bromide. In the resulting mixtures, each of which contained three fragments, the corresponding peptides representing the 145–156 sequence showed the 16 relative molecular mass difference. In βX this sequence contained a Leu residue at position 152 instead of the Pro-152 in βA^1, as established by fast-atom bombardment MS–MS. The Leu could be discriminated from an Ile residue by the presence of a side-chain-specific, D-type fragment ion in the MS–MS spectrum of the βX CNBr peptide. The sequence of the two homologous 145–156 fragments was confirmed by regular amino acid sequence analysis. In accordance with internationally accepted guidelines for the nomenclature of milk proteins, the new genetic variant has been named β-casein F-5P.

1. Introduction

The protein fraction of milk consists of caseins and whey proteins, which differ in their solubility behaviour at pH 4.6. At this pH the caseins can be selectively isolated by isoelectric precipitation. The casein fraction can be subdivided into α_{s1}-, α_{s2}-, β- and κ-caseins, representing proteins with different structures and physicochemical properties. Further, all caseins and also the major whey proteins, β-lactoglobulin and α-lactalbumin, demonstrate genetic polymorphism, which can influence the composition of milk and/or some milk-processing parameters (for recent reviews see Refs. [1–4]). Milk protein genetic variants are therefore thought to be a potential selection criterion in dairy cattle breeding [3].

Common techniques for investigating genetic

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polymorphism of milk proteins are classical gel electrophoresis [5–7], isoelectric focusing [8,9] and high-performance liquid chromatography (HPLC) [10–12]. Recently, capillary electrophoresis was also used for this purpose [13].

In a previous report on the separation of genetic variants of milk proteins by reversed-phase (RP) HPLC we encountered an unknown β-casein type, which was provisionally named βX [10]. It was found to occur in varying amounts in whole caseins from bovine milks.

In the present paper we report the isolation of this component by preparative RP-HPLC and its identification by tryptic mapping followed by mass spectrometric (MS) analysis of peptide fragments.

2. Experimental

Whole caseins were isolated from milk of two or more cows by isoelectric precipitation at pH 4.6 with dilute hydrochloric acid at room temperature. The cows belonged to the MRY breed, a red-and-white population accounting for roughly one fifth of the Dutch milk production.

TPCK-treated trypsin and cyanogen bromide were purchased from Sigma (Product Nos. T-8642 and C-6388, respectively).

RP-HPLC was carried out with the equipment described previously [10]. Depending on the separation problem and the amount of material available, one of three differently sized HiPore RP-318 columns (Bio-Rad Labs.) was used with a C_{18} cartridge (Bio-Rad) as guard column. For each kind of separation the elution conditions were adapted accordingly using linear gradients of several steps, as described below. The solvent system was acetonitrile–water–trifluoroacetic acid in the volume ratios 100:900:1 (solvent A) and 900:100:0.7 (solvent B). Column temperature was 30°C throughout. Peak detection was at 220 nm and occasionally also at 280 nm (serial coupling of detectors).

Analytical separation of caseins was performed on a 250 mm × 4.6 mm I.D. column as described elsewhere [10].

For the isolation of β-caseins a 250 mm × 21.5 mm I.D. column was used, which was loaded with 400-μl portions of whole casein (10 mg/ml of solvent A). Starting from 38% solvent B in solvent A, a gradient was generated immediately after sample injection, going to 40% B over 25 min, eluting isocratically for 5 min, continuing to 70% B over 3 min, eluting isocratically for 5 min and returning to the starting conditions over 5 min. The flow-rate was 11 ml min^{-1}.

Tryptic hydrolysis was carried out at 15°C for 24 h in 20 m*M* ammonium hydrogencarbonate buffer, pH 7.5, at a 1:50 (m/m) enzyme/substrate ratio. After hydrolysis the samples were freeze-dried.

Tryptic digests of the various β-casein types were compared and fractionated by RP-HPLC on a 250 mm × 10 mm I.D. column, which was loaded with 300-μl portions containing 5 mg of digest per ml of solvent A. Immediately after sample injection, a gradient was started from 0% to 55% B over 50 min, and then to 70% B over 5 min, followed by isocratic elution for 5 min before returning to starting conditions over 3 min. The flow-rate was 3 ml min^{-1}.

For the isolation of differential peak fractions from the relevant regions of the elution patterns, the 250 mm × 4.6 mm I.D. column was used with a loading of 150 μl containing 2 mg digest per ml of solvent A. A gradient was started 3 min after sample injection, going from 23 to 34% B over 27 min, eluting isocratically for 5 min, continuing to 38% B over 16 min, then to 80% B over 3 min, eluting isocratically for 5 min and returning to the starting conditions over 3 min. The flow-rate was 0.8 ml min^{-1}.

Isolated components were analysed by electrospray ionisation (ESI) or fast atom bombardment (FAB) MS. In FAB-MS–MS, precursor ions selected in MS-1 were further fragmented in a collision cell located in the third field-free region of the tandem mass spectrometer, a Jeol JMS-SX102/102A four-sector instrument of a B_1E_1–B_2E_2 geometry. In that way the amino acid sequences of peptides of molecular masses up to ca. 2400 can be established. ESI-MS was performed on a VG Platform quadrupole mass spectrometer (VG Biotech, Cheshire, UK). Further experimental conditions for both MS techniques have been reported elsewhere [14].

Cyanogen bromide cleavage was performed by

incubating peptide material in the dark for 20 h at room temperature in 70 vol.-% formic acid (nitrogen-saturated) with a molar ratio of CNBr to Met of ca. 100 [15]. The digest was diluted ten-fold with distilled water and then freeze-dried. CNBr cleavage products were purified by RP-HPLC on the 250 mm × 4.6 mm I.D. column generating a multi-step gradient from 0 to 70% B in A over 55 min at a flow-rate of 0.8 ml min^{-1}.

Amino acid analysis was performed on a Type 4151 Alpha Plus amino acid analyser (Pharmacia–LKB) after hydrolysis of the sample with 6 *M* HCl in vacuo for 24 h at 110°C.

Amino acid sequence analysis was carried out using a gas-phase sequencer, Type 476A, with an on-line PTH-amino acid analyser (Applied Biosystems).

Table 1
Molecular mass of isolated β-casein A variants and βX as determined by ESI-MS

	Molecular mass[a]		n[b]
	Calculated[c]	Observed[d]	
βX	-[e]	24041 ± 2	2
$βA^1$	24023	24031 ± 5	4
$βA^2$	23983	23988 ± 2	2
$βA^3$	23974	23979 ± 3	3

[a] Values (in relative molecular mass units) rounded off to nearest integers.
[b] Experimental data averaged from n determinations.
[c] Derived from the amino acid sequence [17,18].
[d] Averages of unresolved mass clusters.
[e] Identification described in this paper.

3. Results

In Fig. 1 the analytical RP-HPLC patterns of three whole caseins are shown. From earlier work [10,16] the elution position of the different genetic variants was known. In alkaline gel electrophoresis without the use of sodium dodecylsulfate, the unknown βX component was separated from βB, but co-migrated with the βA components; in capillary electrophoresis under the conditions used in Ref. [13] βX co-migrated

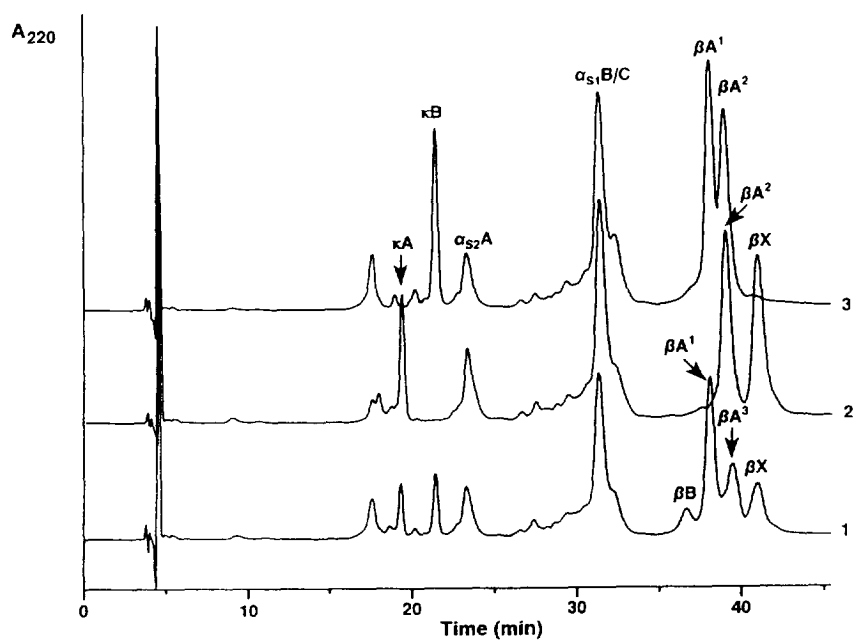

Fig. 1. RP-HPLC patterns of three whole caseins containing different amounts of the βX component identified in this study. (1) Casein from bulk milk collected at a local farm; (2, 3) caseins from milks of two cows representing high and low (or negligible) amounts of βX, respectively. βA components and βX were isolated from 1 and 2. For details of the separation, see Experimental.

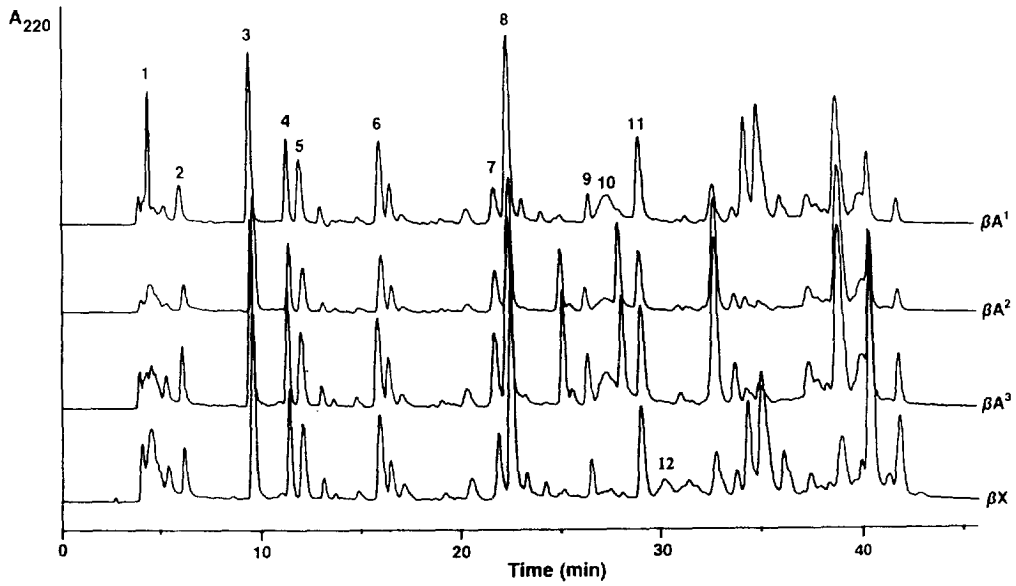

Fig. 2. RP-HPLC patterns showing tryptic maps of βX and the various βA variants. Peak components numbered 1–12 were isolated and then identified by mass spectrometric analysis (Table 2). For separation conditions, see Experimental.

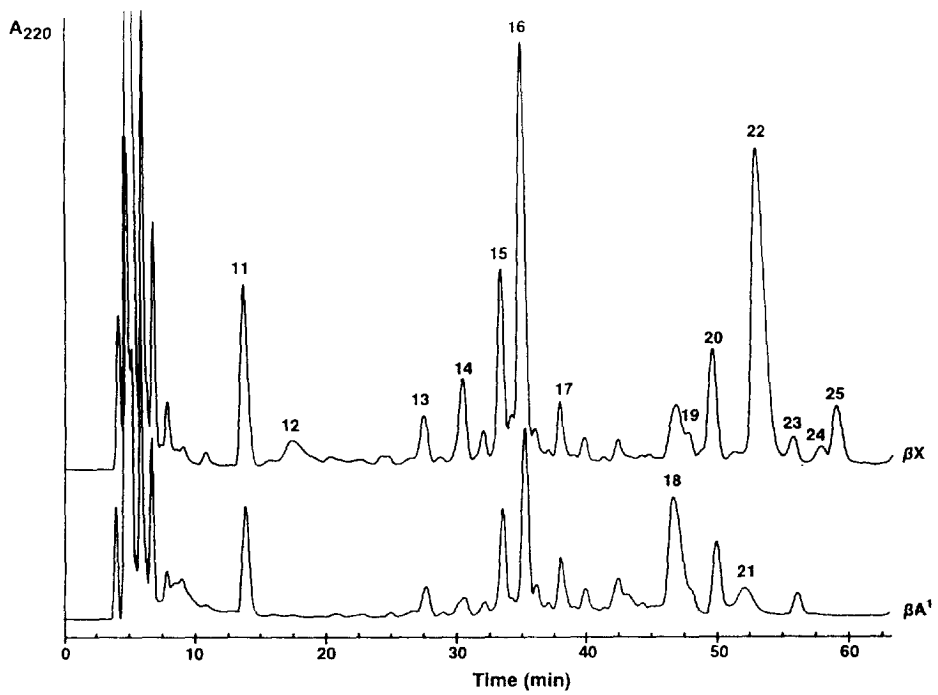

Fig. 3. Further RP-HPLC separation of the last parts of the βA^1 and βX tryptic maps of Fig. 2 with an adapted solvent gradient; see Experimental. Isolated peak components numbered 11–25 were identified by mass spectrometric analysis (Table 2).

with βA^1, but was separated from βB, βA^2 and βA^3 (results not shown). Therefore, in the present study we further compared βX with the βA variants, focusing more particularly on βA^1.

The various β-caseins were isolated by preparative RP-HPLC, taking whole caseins (Fig. 1, samples 1 and 2) as starting materials. Table 1 gives the molecular masses of the isolated components, as established by ESI-MS.

Tryptic maps of the β-caseins are shown in Fig. 2. The tryptic map of βX most closely resembles that of βA^1, especially as far as the

Table 2
Results of FAB-MS and ESI-MS analyses of isolated peak components 1–25 from the tryptic maps of βA^1 and βX (see Figs. 2 and 3)

Peak No.[a]	FAB/ESI-MS	Relative molecular mass[b]		β-Casein fragment
		Observed	Calculated	
1	FAB-MS–MS	245	245.2	98–99
		283	283.2	106–107
		373	373.2	26–28
		388	388.2	30–32
2	FAB-MS	645	645.3	100–105
3	FAB-MS–MS	829	829.5	177–183
4	FAB-MS	2062	2062.0	33–48
	ESI-MS	2063	2062.0	
5	FAB-MS–MS	779	779.5	170–176
6	FAB-MS–MS	747	747.4	108–113
7	ESI-MS	3123	3123.0	1–25
8	FAB-MS–MS	1382	1382.8	191–202
9	ESI-MS	2263	2263.6	49–68
10* (βA^1)	ESI-MS	2912	2912.4	144–169
		2781	2781.2	145–169
11	FAB-MS–MS	742	741.5	203–209
	ESI-MS	742	741.9	
12* (βX)	ESI-MS	2928	2928.5	144–169[c]
		2798	2797.3	145–169[c]
13	ESI-MS	3114	3113.7	69–97
14	ESI-MS	2186	2186.6	184–202
15	ESI-MS	4864	4864.7	53–97
16	ESI-MS	5359	5359.3	49–97
17	ESI-MS	4988	4987.9	49–93
18* (βA^1)	ESI-MS	6363	6362.4	114–169
19	ESI-MS	3736	3736.3	114–145
20	ESI-MS	3468	3467.9	114–143
21* (βA^1)	ESI-MS	5731	5731.7	114–163
22* (βX)	ESI-MS	6378	6378.5	114–169[c]
23	ESI-MS	3599	3599.1	114–144
24* (βX)	ESI-MS	6164	6163.1	114–167[c,d]
25* (βX)	ESI-MS	5748	5747.8	114–163[c]

[a] Peak components were identified in both tryptic maps, except those indicated by an asterisk, which occurred in only one of the two maps, as indicated.

[b] ESI-MS and FAB-MS results have been converted to uncharged masses (M). For FAB measurements average mass values (M) of unresolved isotopic ion clusters are given for $M > 1500$ (mono-isotopic masses for $M < 1500$). In the case of ESI-MS the isotopic clusters were no longer resolved at $M > 500$.

[c] With relative molecular mass 16 units higher than that of the corresponding βA^1 fragment ($Pro_{152} \rightarrow Leu_{152}$).

[d] No corresponding fragment observed in the βA^1 map.

lack or presence of certain peaks in the 25–28 min and 33–36 min regions is concerned. Since βA^1 differs from βA^2 only at position 67 of the sequence (His instead of Pro), the resemblance of the βX tryptic map to that of βA^1 already suggested a His-67 in βX as well. As seen in Table 1, the total mass of βX differed from that calculated for βA^1 by only 18 ± 2 relative molecular mass units. The tryptic maps of βX and βA^1 were identical as far as the first part of the RP-HPLC pattern (Fig. 2, peaks 1–9) was concerned. In the βX map some extra peaks occurred in the region following peak 11. Since ESI-MS performed on isolated components from that region showed that several peaks contained more than one component, the tryptic digests of βX and βA^1 were rechromatographed, focusing on a satisfactory separation in the last part of the pattern (Fig. 3, peaks 11–25). Peak components 1–25 of Figs. 2 and 3 were isolated and identified by their molecular mass (ESI-MS and/or FAB-MS) and by their amino acid sequence, determined either directly by FAB-MS–MS or by deduction, via their mass, from the primary structure of β-casein (Table 2). A number of atypical cleavage positions were observed, probably due to some chymotryptic activity present in the trypsin preparation used. In those cases, for fragment identification based solely on mass values the presence of a regular tryptic cleavage site at either end of the sequence was considered a precondition. In Fig. 4 the primary structure of β-casein [17,18] is depicted with the positions of enzymic cleavage indicated. The fragments identified (Table 2) together accounted for the complete β-casein sequence. Monophosphorylation of fragment 33–48 could be easily determined from its positive ESI-MS and FAB-MS spectra. Similarly, the presence of four phosphate groups in fragment 1–25 was confirmed on the basis of positive and negative ESI-MS spectra.

Three peaks in the tryptic map of βA^1 had a corresponding (differential) component with 16 relative molecular mass units difference in the βX tryptic map. It concerned the following pairs of peaks in Figs. 2 and 3: 10 versus 12, 18 versus 22 and 21 versus 25, representing the sequences 144/145–169, 114–169 and 114–163, respectively. The spectral data reflect the mass increment of 16 relative molecular mass units between the corresponding fragments of βA^1 and βX (Table 2). Of one minor peak component (No. 24) in the βX map no corresponding fragment was found in the βA^1 map. Its molecular mass was compatible with fragment 114–167 having a mutation of 16 relative molecular mass units difference. Peak 18 in the βX map could be neglected as it represented the tryptic fragment 114–169 from βA^2; the latter was, by analytical RP-HPLC and capillary electrophoresis [13], found to be a contaminant in the original βX fraction. Since the 114–145 (peak 19) and the 114–144 (peak 23) fragments appeared to be

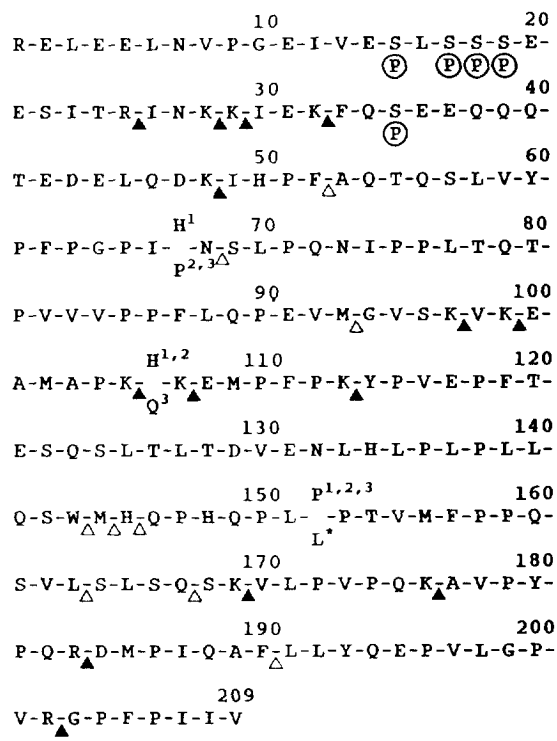

Fig. 4. Amino acid sequence of bovine β-casein [17,18] with the various substitutions by which the genetic variants described in this paper are differentiated. Positions of enzymic cleavage found in this study (see fragments in Table 2) are indicated (▲ for regular tryptic and △ for atypical cleavage sites). 1, 2 and 3 represent residues present in the βA^1, βA^2 and βA^3 variants, respectively; the asterisk represents the residue found in the βX variant; the encircled P represents phosphate.

identical for βA^1 and βX and the mass difference was found in the 114–163 fragment (peaks 21 and 25, see Fig. 3 and Table 2), the location of the mutation could be further narrowed to the 146–163 sequence.

The main differential peaks, i.e. 18 and 22, both representing fragment 114–169, were isolated by RP-HPLC and subjected to CNBr digestion. The RP-HPLC patterns of the resulting digests (not shown) contained three peptide fragments each. The retention time differed between the two digests for only one of these.

The relevant parts of the FAB-MS spectra representing the whole CNBr digests of components 18 and 22 are given in Fig. 5. In the digest of component 18 an MH^+ ion at m/z 1334 is observed, representing the 145–156 sequence of βA^1. The digest of component 22 shows the corresponding 145–156 fragment of βX at m/z 1350, indicating that the mutation is situated in this fragment. The digested components 18 and 22 both gave an MH^+ ion at m/z 1418, which corresponds to the 157–169 fragment of β-casein; this could be confirmed by FAB-MS–MS.

In the FAB-MS spectra of CNBr peptides the MH^+ ions are followed by one or more ions with mass increments of 28 (Fig. 5). The latter could be ascribed to different levels of formylation of

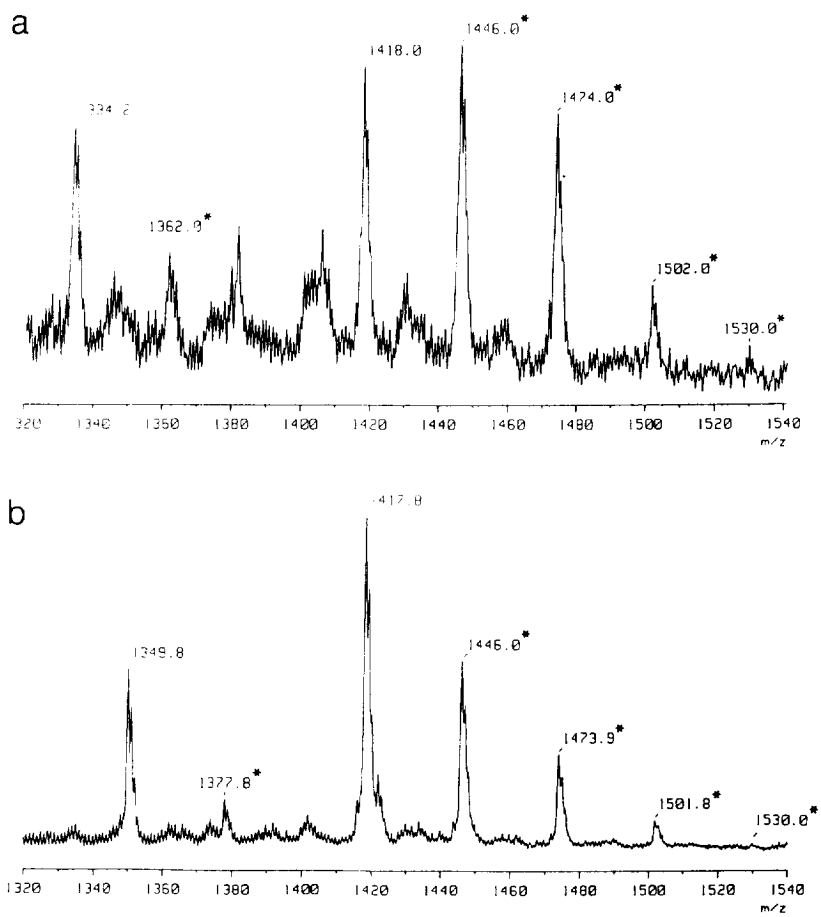

Fig. 5. Part of FAB-MS spectra representing the whole CNBr digests of 114–169 fragments from βA^1 (a) and βX (b). The peaks at m/z 1418 represent β-casein fragment 157–169, identical for both digests. Those at m/z 1334 and 1350 are the 145–156 fragments of βA^1 and βX, respectively. Peaks indicated by asterisks originate from the same peptides but have formylated Ser or Thr residues (see the text).

the reaction products by the 70% formic acid used as solvent during CNBr digestion. Such a chemical modification has been reported earlier [19] for reactions in aqueous formic acid over long periods of time. In our case it is clear from Fig. 5 that the number of formylated components correlates with the number of Ser and Thr residues present in the cleavage product concerned (i.e. one in fragment 145–156 and four in fragment 157–169).

The FAB-MS–MS spectra (Fig. 6) of the protonated peptides at m/z 1334 and 1350 show unequivocally the mutation Pro(βA^1)→Leu(βX) at position 152 of the β-casein sequence. The spectra are in both cases dominated by ions resulting from fragmentations of the peptide bond between a Pro and its preceding amino acid residue in the sequence (Pro–Pro bonds being relatively resistant), leaving the charge at the N-terminal (B-type) or C-terminal (Y″-type) ion [20]. This preferential fragmentation of a peptide bond between Pro and its preceding amino acid is known from the literature [21]. At position 152 of the βX sequence

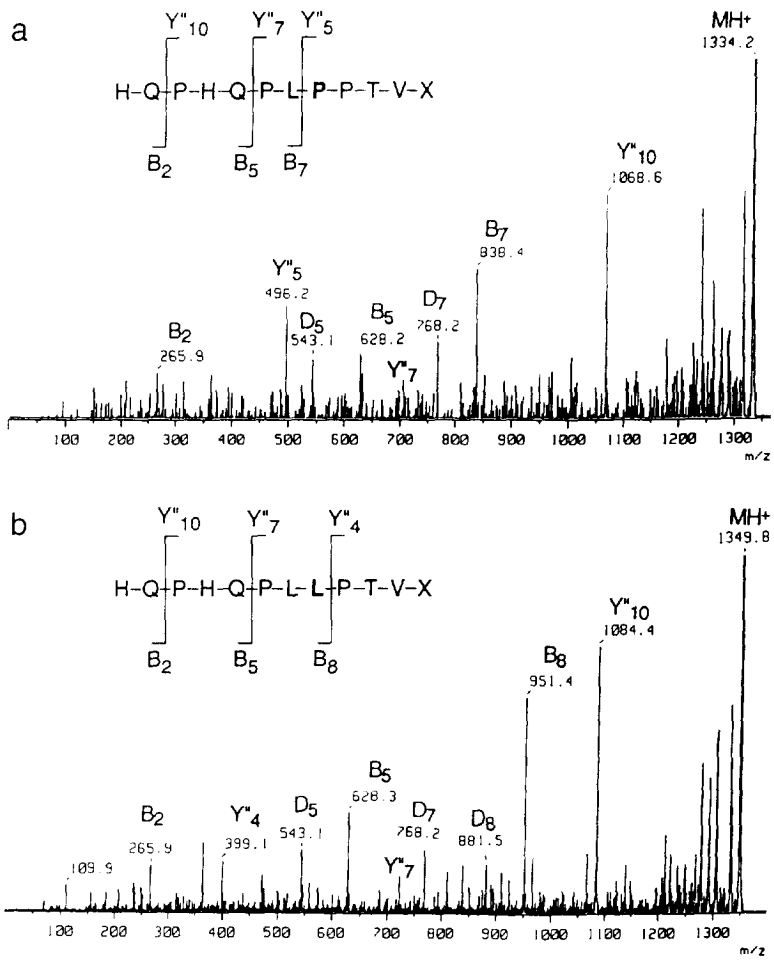

Fig. 6. FAB-MS–MS spectra of the components with m/z 1334 (a) and 1350 (b) from Fig. 5. Sequence ions are indicated using the notation of Roepstorff and Fohlman [20]. In the proposed amino acid sequences (see insets) X stands for homoserine lactone. The side-chain-specific D_δ ion at m/z 881 discriminates Leu from the isomeric Ile at position 152 of the βX sequence (Fig. 4).

Table 3
Amino acid composition (mol/mol) and sequence of fragments 145–156 isolated from the βA^1 and βX variants[a]

Amino acid	βA^1(145–156)		βX(145–156)
Thr	1.0	(1)	0.9
Glx	2.1	(2)	2.0
Pro	**3.9**	(4)	**2.7**
Val	1.2	(1)	1.1
Met[b]	1.0	(1)	1.0
Leu	**0.9**	(1)	**1.9**
His	1.8	(2)	1.9
Sequence[c]	HQPHQPLPPTVX		HQPHQPLLPTVX

Values in parentheses are according to the sequence reported for β-casein [17].
[a] For isolation procedure, see text.
[b] Determined as homoserine plus homoserine lactone.
[c] Determined by regular amino acid sequence analysis.

Leu could be differentiated from its isomer Ile by the side-chain-specific D_δ ion at m/z 881 in the spectrum of Fig. 6b [22].

The identity of the two homologous 145–156 fragments was further confirmed by amino acid analysis and regular amino acid sequence determination. The results of these analyses are given in Table 3.

4. Discussion

Until recently, genetic variants of bovine β-caseins had been primarily differentiated by electrophoretic techniques [23], owing to charge differences between the variants at alkaline or acidic pH. The development of RP-HPLC for the phenotyping of milk proteins [10,16] led us to the variant which was found in the present study to have an electro-neutral Pro→Leu substitution at position 152 of the amino acid sequence. Probably this variant was observed earlier by Carles [24], who isolated an atypical β-casein by RP-HPLC of a whole casein at neutral pH. On the basis of amino acid analysis of tryptic fragments this author concluded that a Pro→Leu substitution was present, but the exact position was not identified.

RP-HPLC followed by ESI-MS and FAB-MS appeared to be a successful approach to the analysis of intact β-casein variants and of the peptides isolated from the tryptic and CNBr digests. In the latter some artifactual products owing to formylation of Thr and Ser residues by solvent molecules were demonstrated by FAB-MS. By using other methods of analysis this side-reaction would probably have remained undetected. In one of the CNBr peptides (i.e. sequence fragment 145–156) the Pro→Leu mutation could be located by FAB-MS–MS and further confirmed by regular amino acid sequence analysis. The substitution found only requires a point mutation (CCT→C*T*T) in the known DNA sequence of the β-casein gene [25–27].

The 1–25 and 33–48 fragments from the tryptic map of βX were found to be identical to the corresponding (phosphate-containing) fragments from βA^1 on the basis of retention time in RP-HPLC and molecular mass determined by either of the two MS techniques used. From this it can be inferred that βX contains the same five serine-bound phosphate groups as present in most β-casein variants (Fig. 4). Apart from the three A variants mentioned in this paper, four other β-casein types have been identified, known as B, C, D and E variants. Therefore, in accordance with the internationally recognized guidelines of the American Dairy Science Association Committee on the Nomenclature and Meth-

odology of Milk Proteins [28] we propose to name the present βX variant β-casein F-5P.

It would be interesting to study the frequency with which this new variant occurs in milk of cows of various breeds and the possible consequences for milk processing and properties of the resulting products [1,2].

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