Journal of Dairy Research (2004) **71** 14–19. © *Proprietors of Journal of Dairy Research 2004*
DOI: 10.1017/S0022029903006551 *Printed in the United Kingdom*

Primary structure of water buffalo α-lactalbumin variants A and B

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Received 25 October 2002 and accepted for publication 20 January 2003

A novel electrophoretic α-lactalbumin (α-la) variant was detected in the Italian water buffalo breed. The isoelectric point of the variant, labelled A, was lower than the most frequent variant B. It presented an allelic frequency of 0·5% compared with the 97·1% of the BB allele. From Liquid Chromatography-Electrospray Ionization/Mass spectrometry, the molecular mass of the two α-la A and B variants were measured as 14235·1 ± 0·8 and 14236·1 ± 0·9 Da, respectively. The two proteins were sequenced and differentiated from one another by a single amino acid substitution, Asn^{45}(B)→Asp^{45}(A). As this amino acid substitution altered the N-glycosylation sequence consensus Asn45–X–Ser^{46} it may be deduced that the protein glycosylation level of the α-la A would decrease.

Keywords: Galactosyl transferase, bovine, β-lactoglobulin.

It is believed that water buffalo α-lactalbumin (α-la) and β-lactoglobulin (β-lg) are each monomorphic as no polymorphism has yet been reported for these proteins. The bovine α-la counterpart, a 123 amino acid residue protein including four disulphide bridges, has a molecular mass of 14·2 kDa (Gordon, 1971). As part of the enzyme galactosyl transferase, it plays a role in lactose biosynthesis (Brew et al. 1968). Two genetic variants, α-la A and B, differing by a single residue in the 10th position, Gln^{10}(A)→Arg^{10}(B) have been identified in bovine milk. Partially sequenced, water buffalo α-la presented Arg^{10} (Addeo et al. 1976). The newly reported amino acid sequence deduced from DNA analysis (Fan et al. 2000), indicates that water buffalo α-la differs from bovine variant B solely in the amino acid replacement (bovine B) Gly^{17}→(water buffalo) Asp^{17}. Therefore, the compact ellipsoidal structure with a small hydrophobic box (Acharya et al. 1989; Calderone et al. 1996) could also be maintained in water buffalo α-la since one bound Ca^{2+} ion and four disulphide bridges are conserved. Acidification to pH<4 causes the release of Ca^{2+}, thus rendering apo-α-la more flexible and more prone to hydrolysis than native protein. Human apo-α-la with bound oleic acid induces apoptosis in both immature and tumour cells, whilst healthy cells resist such an event (Svensson et al. 2000). This work implies that the capacity for apoptosis might very well be inherent to α-la. The scarce availability of data on milk protein polymorphism makes the search interesting as a focus of study as it concerns the Italian water buffalo, a breed probably introduced into Italy several centuries ago and protected since from any continental cross-breeding. Bearing in mind this lack of information, we submitted a number of individual samples of water buffalo whey proteins to further analysis by means of gel isoelectric focusing and polyclonal antibodies raised against single whey protein components for detection of protein polymorphism. The present work reports the occurrence of water buffalo α-la variants A and B and their determined amino acid sequence.

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Materials and Methods

Whey protein preparation

A total of 207 individual milk samples were taken from water buffalo reared in Campania, a region in the south of Italy. Each sample was skimmed by centrifugation, 10 min at 4500 ***g***, and treated according to the procedure described by Aschaffenburg & Drewry (1959) to separate casein and acid whey. Acid whey was dialysed against distilled water and freeze-dried.

Gel electrophoresis

Isoelectric focusing on ultra thin-layer polyacrylamide gel (0·25 mm) (UTLIEF) and immunoblotting with anti-α-la polyclonal antibodies sera were carried out according to the procedure of Chianese et al. (1992).

Densitometric analysis

The Coomassie-stained gel was submitted to densitometric scanning on an Ultroscan XL (Pharmacia-LKB, Uppsala, Sweden) equipped with a GelScan 2.0 integration program managed using an IBM PC PS/2. Integration of the peaks was performed using the parameter SIGNAL.

Reversed-phase HPLC of whey proteins

Whey proteins were fractionated by reversed-phase HPLC on Kontron equipment (Kontron Instruments, Milan, Italy) consisting of two model 420 pumps, a rheodyne sample injector (a 250-µl loop) and a model 491 solvent programmer. A reversed-phase Vydac C4 column (214 TP, 1 × 25 cm) was used for protein separation. Whey proteins were dissolved in water (0·1%) and filtered through a 0·2 µ filter (Millipore Corp., Bedford, MA01730, USA) and injected into the loop. Solvents A and B were trifluoracetic acid (1 ml/l) in water and in acetonitrile, respectively. A linear gradient from 0 to 36% B was applied at a constant flow rate of 0·5 ml/min for 5 min, followed by a linear gradient from 36 to 46% for 25 min. Proteins were detected at 220 nm using a Kontron variable wavelength detector (Model 430).

Electrospray mass spectrometry

Electrospray (ESI) Mass Spectrometry analysis of α-la was performed by means of a Platform apparatus (Micromass, UK), a single quadrupole mass spectrometer following the procedure of Ferranti et al. (1998). Mass spectra were obtained in an interval ranging from 600 to 1800 m/z with a 5 s/scan frequency by a computer system using a software program provided by MassLinks (Micromass). The source temperature was 85 °C and the orifice voltage was 40 V. Mass values are reported as average masses.

Reduction and carboxymethylation procedure

Purified α-la was dissolved in 300 ml of 0·3 M-Tris–HCl, pH 8·0, containing 6 M-guanidine–HCl, 1 mM-EDTA, and treated with dithiothreitol (10 : 1 molar excess with respect to cysteinyl residues) at 37 °C for 2 h. Carboxymethylation was carried out with a five-fold molar excess of iodoacetic acid with respect to dithiothreitol, at pH 8·0, at room temperature for 30 min in the dark. The sample was desalted by gel filtration through a PD-10 G-25 column (Bio-Rad) in 50 mM-ammonium bicarbonate, pH 8·5, and freeze-dried.

Enzymic hydrolysis

Hydrolysis by trypsin was carried out in 0·4% (w/w) ammonium bicarbonate, pH 8·5, at 37 °C for 6 h at a substrate to enzyme ratio of 50 : 1 (w/w). Hydrolysis with endoproteinase Asp-N was carried out at an enzyme to substrate ratio of 1 : 100 (w/w) in 50 mM-ammonium bicarbonate, pH 8·5, at 37 °C overnight. Reaction was stopped by rapid freezing and freeze-drying.

Reversed-phase HPLC of whey protein hydrolysate

Trypsin hydrolysate was fractionated by HPLC on a 218TP52, 5-µm reversed-phase Vydac C18, 250 × 2·1 mm column (Vydac, Hesperia, CA, USA). Solvent A was 0·3 ml of trifluoroacetic acid (TFA)/l water. Solvent B was 0·2 ml TFA/l acetonitrile. Samples (100 µg) were dissolved in 200 µl of water and injected onto the HPLC column equilibrated in solvent A. A linear gradient from 0% to 70% solvent B was applied at a flow rate of 0·5 ml/min for 90 min. HPLC fractions, manually collected after measurement of absorbance at 220 nm, were analysed by ESI/MS using a Platform (Micromass, Manchester, UK) single quadruple mass spectrometer. ESI mass spectra were scanned from 1800 to 400 at a scan cycle of 5 sec/scan. Source temperature was 85 °C and orifice voltage was 40 V. Mass values are reported as average masses.

Electrospray–time-of-flight tandem mass spectrometry (ESI-TOFMS/MS)

Electrospray MS/MS analysis was performed using a Q-star Pulsar quadrupole time-of-flight (TOF) hybrid (Sciex, Ontario, Canada) mass spectrometer equipped with an Ionspray source operating in positive ion mode. The sample was dissolved in 1 : 1 (v/v) 5% acetic acid in water/acetonitrile and introduced into the source using a syringe pump integrated into the spectrometer, at a flow rate of 4 µl/min. Ionization conditions were ESI electrode voltage 5500 V, air used as nebulizer gas and curtain gas at indicated pressure of 30 and 20 psi, respectively, and orifice ring voltage was 80 V. The voltage for first quadrupole (QO) was 60 V and for quadrupole 2 (RO2) was 9·9 V. The resulting product-ion spectrum was acquired by TOF analyser.

Results and Discussion

Polyacrylamide gel isoelectric focusing and immunoblotting of whey proteins

To compare the mobility of the different α-la occurring in individual samples of bovine and water buffalo whey proteins, an Ultra Thin Layer Iso-Electric Focusing (UTLIEF) plate was developed with polyclonal antibodies produced against α-la (Fig. 1a). Bovine α-la B exhibited a pI lower than that of the most common variant water buffalo α-la B (lane 5). The novel water buffalo α-la A variant with a higher pI was detected along profile 6. Each water buffalo α-la variant B (lane 5) and A (lane 6) presented a heterogeneity, which possibly depended on the presence of glycated forms of α-la.

Water buffalo and bovine β-lg bands were also identified along the profiles shown in Fig. 1b by using polyclonal antibodies against this protein (results not shown).

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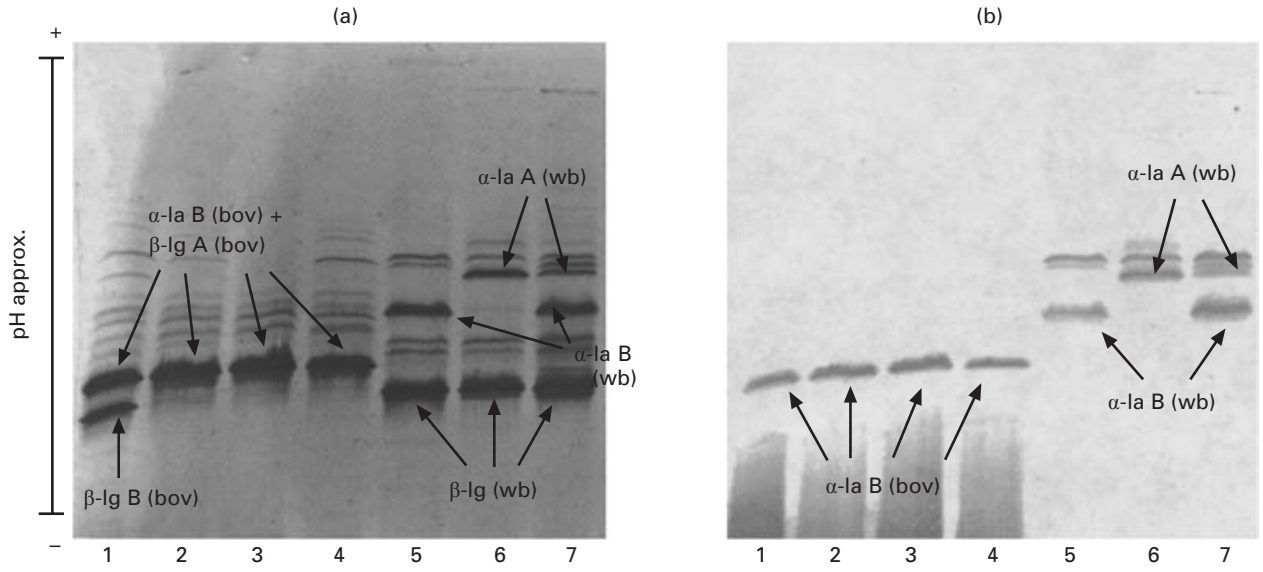

Fig. 1. UTLIEF profiles of bovine (bov) (lanes 1–4) and water buffalo (wb) (lanes 5–7) whey: (a) Coomassie-stained and (b) anti α-la polyclonal antibodies stained.

Table 1. Relative percentage of α-la† and β-lg in individual water buffalo samples shown in Fig. 1‡

Protein	Lane 5	Lane 6	Lane 7
α-la A		26	8
α-la B	30		23
β-lg	50	48	49

† Only the α-la main band is reported
‡ The level of other whey proteins is not reported

Thus, once the UTLIEF plate with Coomassie Blue G stain was developed, we were able to label single bands (Fig. 1b) and to evaluate the relative proportions of water buffalo α-la and β-lg in the samples by means of densitometric analysis (Table 1).

α-La accounted for about 30% whey proteins irrespective of the variant. However, in the heterozygous form (lane 7) α-la A was expressed at a lower level, about 8% of that expected, whereas the B variant was at a higher level, i.e., 23% (lane 7). The allelic frequency of water buffalo α-la was: BB = 97·1%; AA = 0·5%; and AB = 2·4%, hence explaining why this rare variant had not been detected previously within the Italian water buffalo breed.

Structural study on water buffalo α-la

HPLC fractionation of the whey proteins from two individual samples allowed us to isolate α-la A and B. Each main peak corresponding to a glycosylated α-la form was manually collected and submitted to ESI/MS analysis (results not shown). The molecular mass of α-la A and α-la B was respectively, 14236·1 ± 0·9 and 14235·1 ± 0·8 Da, implying that a single amino acid residue substitution might have occurred, i.e., Asn or Gln replaced by Asp or Glu, each accounting for a difference of 1 Da. Such a substitution could also justify the pI difference between the two α-la variants. As the molecular mass difference might also depend on multiple amino acid substitutions, it was necessary to determine the primary structure of both water buffalo α-la variants.

Determination of primary structure of water buffalo α-la A and B

Present knowledge of the primary structure of the most frequent α-la form of water buffalo does not extend beyond the definition of 36 N-terminal and two C-terminal residues (Addeo et al. 1976). Each water buffalo α-la was submitted to carboxymethylation and then digested by AspN enzyme specifically breaking the X-Asp bond. Protein digests were fractionated by HPLC (Fig. 2) and the C18 column eluate on line was analysed by ESI/MS. Each variant gave rise to 16 peaks whose molecular mass and primary structure are reported in Table 2. Peptide sequence was determined by ESI-TOFMS/MS analysis of each collected HPLC peak. By this procedure, the complete primary structure of both α-la variants was achieved, as reported in Fig. 3. Mass spectrometric sequencing showed that each A and B variant contained an Asp^{17} residue, agreeing with the nucleic acid sequence reported by Fan et el. (2000), which alone justifies the lower pI of water buffalo α-la with respect to Gly^{17} residue of bovine counterparts. A further amino acid substitution, Asn^{45}(B)→Asp^{45}(A), differentiated the two water buffalo α-la, which justifies the lower pI of A variant relative to that of B. The presence of Asp^{45} generated, in the endoproteinase AspN digest of the A variant, the peptides 37–44 and 45–62, which were a unique peptide 37–62 in the digest from the B variant. The formation of these two peptides itself represents the identification of the substitution Asn^{45}→Asp^{45}. Direct evidence of this amino

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Table 2. Identification by ESI/MS of the HPLC-purified peptides from reduced and carboxymethylated water buffalo α-la variant B and A hydrolysed with Endoproteinase Asp-N

RT†, min Variant B	RT, min Variant A	Molecular mass, Da Measured	Molecular mass, Da Expected	Peptide	Amino acid sequence
27·9	25·0	463·1 ± 0·0	462·2	D1(83–86)	DDLT
30·0	27·3	578·0 ± 0·0	577·2	D2(82–86)	DDDLT
32·8		1002·2 ± 0·7	1002·5	D3(37–45)	DTQAIVQNN
	30·6	889·8 ± 0·0	887·4	D3(37–44)	DTQAIVQN
35·3	34·6	1483·8 ± 0·4	1481·6	D4(17–29)	DYGGVSLPEWVC‡T
38·2		1530·2 ± 0·7	1527·7	D5(46–58)	DSTEYGLFQINNK
	36·6		1643·7	D5(45–58)	DDSTEYGLFQINNK
40·2	38·1	636·4 ± 0·0	636·3	D6(78–82)	DKFLD
41·6	39·5	1762·1 ± 0·5	1759·7	$D7_1$(63–77)	DDQNPHSSNICNISC
		1647·3 ± 0·5	1644·7	$D7_2$(64–77)	DQNPHSSNICNISC
44·6	42·9	1309·9 ± 0·1	1308·7	D8(1–10)	EQLTKCEVFR
48·4	47·0	1680·4 ± 0·6	1678·9	D9(1–13)	EQLTKCEVFRELK
51·4		931·0 ± 0·8	930·4	D10(46–53)	DSTEYGLF
	49·7	1046·0 ± 0·5	1045·4	D10(45–53)	DDSTEYGLF
52·2	50·8	1092·1 ± 0·4	1090·5	D11(116–123)	DQWLCEKL
53·2	52·3	1234·7 ± 0·4	1233·7	D12(87–96)	DDIMCVKKIL
54·2	53·5	2246·1 ± 0·7	2445·2	D13(97–115)	DKVGINYWLAHKALCSEKL
57·1		2117·8 ± 0·8	2116·0	D14(46–62)	DSTEYGLFQINNKIWCK
	56·7	2231·5 ± 0·2	2231·0	D14(45–62)	DDSTEYGLFQINNKIWCK
61·1	61·0	3206·3 ± 0·9	3204·6	D15(97–122)	DKVGINYWLAHKALCSEKLDQWLCEK
62·8	62·6	2276·4 ± 0·3	2276·0	D16(17–36)	DYGGVSLPEWVCTTFHTSGY
67·9	68·4	2631·7 ± 0·6	2633·9	D17(14–36)	DLKDYGGVSLPEWVCTTFHTSGY

† RT, Retention time
‡ C, carboxymethylated cysteine

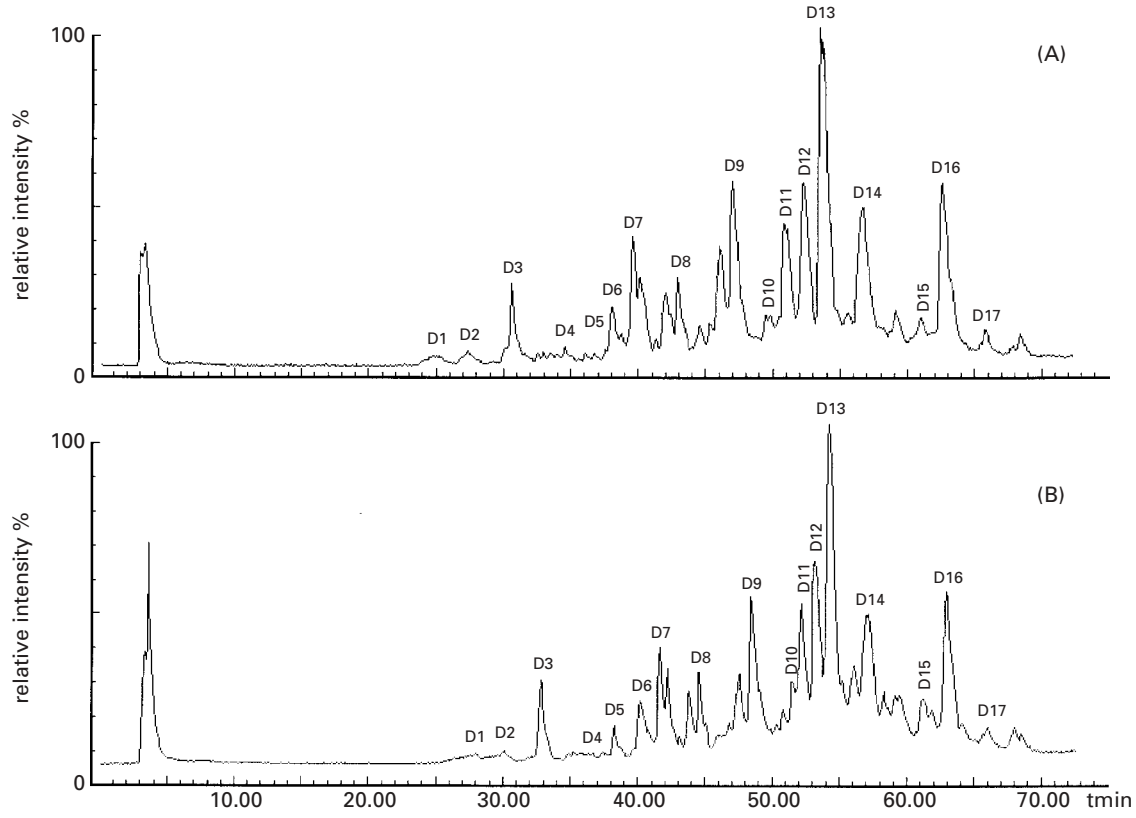

Fig. 2. Reversed-phase HPLC of AspN digest water buffalo α-la (a) A and (b) B. Experimental details are given in the text. D, peptides derived from protein hydrolysis by AspN action; for identification see Table 2.

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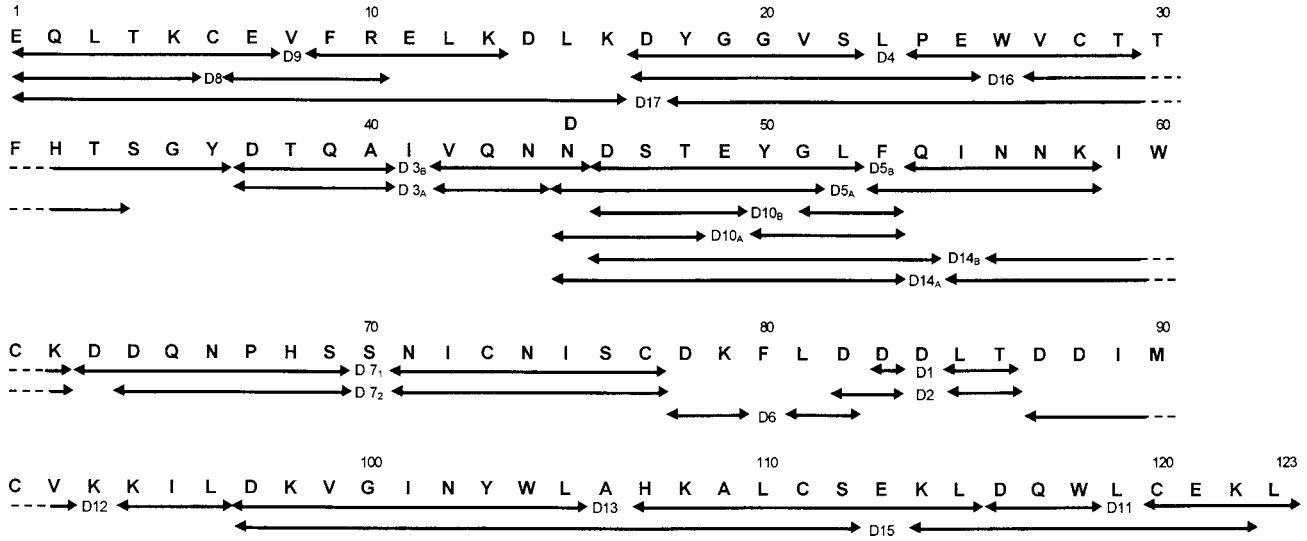

Fig. 3. Amino acid sequence of α-la A and B. D, as in Table 2.

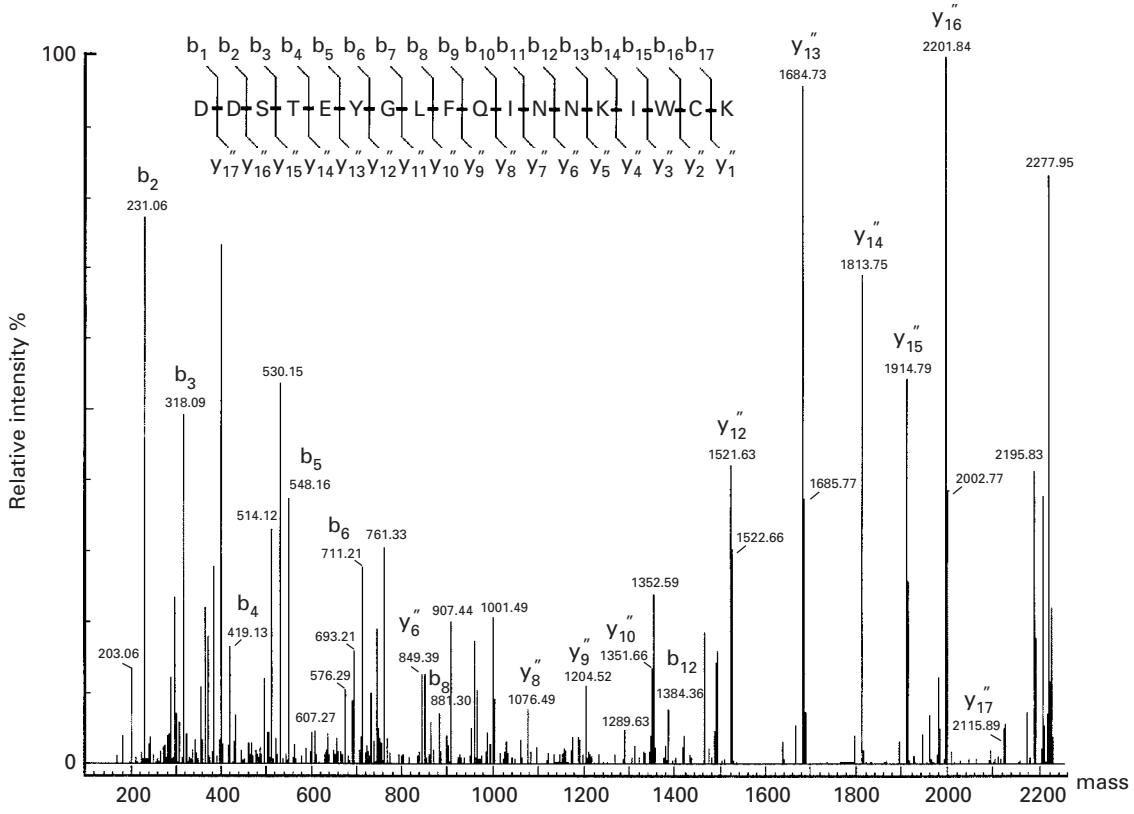

Fig. 4. ESI-TOFMS/MS spectrum of the endoproteinase Asp-N peptide D15 in Fig. 2, identified as the fragment 45–62 of water buffalo α-la variant A containing N-terminal Asp.

acid substitution, however, was obtained by ESI-TOFMS/MS analysis of peptide 45–62 (Fig. 4). Here, signals are indicative of a sequence DDSTGYGLFQINNKIWCK with N-terminus Asp[45].

It is worth noting that this amino acid substitution affects the consensus for N-linked carbohydrate chains sequence (Asn–X–Ser/Thr), which changed in the A variant into a non-consensus sequence Asp45–Asp–Ser[47]. An additional putative consensus sequence for N-linked carbohydrate chains, Asn[74]–Ile–Ser[76], instead, was not affected by amino acid substitution in the water buffalo, bovine, sheep or goat α-la, each containing about 10% glycosylated α-la. Rabbit and rat α-la are completely glycosylated at site 45 with a Asn–Ser–Gly sequence at this site, being more

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effective as a substrate for glycosylation system than the corresponding sequence, Asn–Asp–Ser (Prasad et al. 1982). It is reported that the carbohydrate moiety does not seem to be involved in the function of α-la in lactose biosynthesis, since the glycosylated and nonglycosylated forms are equally active as lactose synthase specifier proteins (Barman, 1970). Asn^{45} is reported to be located in solvent-exposed loop of the α-la molecule (Pike et al. 1996). Slangen & Visser (1999) confirmed, through mass spectrometric studies, that some glycan residues are located at Asn^{45} and no apparent N-linked carbohydrate chains at Asn^{74}. Water buffalo α-la A containing the altered consensus sequence Asp^{45}–Asp–Ser^{47} for N-linked carbohydrates might present a different glycosylation pattern from the water buffalo B variant and bovine counterparts. This was not apparent, either from the electrophoretic patterns of whey proteins or from ESI spectra of the two water buffalo α-la. An approach similar to that used by Slangen & Visser (1999) implying concentration of glycosylated α-la forms could clarify this aspect. Specific studies are in progress in our laboratory to characterize the glycosylated forms and to localize the carbohydrate moiety on the protein chain.

In any case, considering that the novel variant A of α-la is expressed at a reduced rate in the few milk samples analysed to date, it is also important to continue investigations on other milk samples representing Italian herds so as to find possible relationships between casein and α-la variant A content.

This research was supported by Operative Project ''Research, Technological Developments and Higher Education'' of the National Research Council (CNR) – Italy (1994–1999) to L.C.

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