Food Chemistry 141 (2013) 597–603

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

A signature protein-based method to distinguish Mediterranean water buffalo and foreign breed milk

Simonetta Caira [a,*], Gabriella Pinto [a], Valentin A. Balteanu [b], Lina Chianese [a], Francesco Addeo [a]

[a] Università degli Studi di Napoli "Federico II", Facoltà di Agraria Via Università, 100 80055 Portici, Napoli, Italy
[b] Department of Biotechnology, Faculty of Animal Husbandry and Biotechnology, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Manastur Road, 400372, Cluj-Napoca, Romania

ARTICLE INFO

Article history:
Received 19 April 2012
Received in revised form 12 September 2012
Accepted 10 February 2013
Available online 15 March 2013

Keywords:
Signature protein
Water buffalo alpha-s1-casein
Internally deleted variant
Primary structure
HPLC-ESI-MS method

ABSTRACT

A novel genetic variant at the α_{s1}-casein *locus* of water buffalo (WB), 8-residue shorter than its wild-type has been found and sequenced. The internal deletion of the peptide $E^{35}KVNELsT^{42}$ was confirmed by the isolation of the junction peptide. The 8-residue deletion mutant has a molecular weight that is 919 Da less than that of the wild-type. The novel isoform with a unique f35-42 deletion could be the result of the skipping of exon 6, generating an exon 6-deleted variant of α_{s1}-casein. The wild-type and its shortened α_{s1}-casein forms were found to co-exist in many individual milk samples. In contrast, the 8-residue, internally deleted α_{s1}-casein variant did not occur in water buffaloes of the Mediterranean breed reared in Italy. Wild-type α_{s1}-casein has 6 to 8 phosphate groups (P) while the internally deleted form 6 and 7P per molecule.

1. Introduction

Water buffalo (WB) milk is made into cheese, yogurt and lactic-acid-fermented beverages. In India, 28% of the total milk is transformed into ghee and approximately 20% into curd, milk powder and a variety of milk sweets (Ganguly, Bandopadhyay, & Kumar, 1999). There are 170 million WB in the world today: 97% in Asia, 2% in Africa (mainly Egypt), and 0.2% in Europe (mainly Italy) (FAO, 2004). The indigenous Mediterranean WB breed to which the Italian WB belongs produces milk richer in total solids, fat and protein than cow's milk (Borghese & Moioli, 2003). WB casein (CN) has been recognized *via* species-specific peptides even in ternary/quaternary milk mixtures using MALDI-TOF for analysis of tryptic peptides (Cuollo et al., 2010). Quantification of adulterated milk has been carried out using synthetic peptide analogs as internal standards.

Although WB is a potential germplasm reservoir useful for crosses (i.e. the example of 18 Indian water buffalo breeds), the data available on the occurrence of milk protein variants are limited. A few data refer to Italian WB samples collected during either the morning or evening milking. (Chianese et al., 2008; Ferranti et al., 1998). To extend the data in the literature, we investigated casein polymorphism in Romanian, Canadian and Venezuelan breeds. The most complete study using a mass spectrometry-based proteomic approach combines the separation of WB milk proteins by 2DE and individual protein identification by MS (Scaloni et al., 2008) . However, the analysis of a single milk sample did not allow the detection of any additional information on CN polymorphism beyond the scope of the study (Scaloni et al., 2008). WB α_{s1}-CN is one of the major CN components and represents approximately 37% of the total CN. Although the polymorphism of α_{s1}-CN was investigated in four WB breeds, only the amino acid sequence was determined for the Romanian WB. The molecular markers are useful tools for an assisted selection as well as for preservation of the genetic diversity of the local breeds/populations. Genetic variation of milk proteins depends on the adaptation of WB to the unfavorable living conditions of the Mediterranean basin where they have undergone natural selection.

The marshy habitat would have significantly improved WB survival and reproduction. We present a few examples of α_{s1}-CN polymorphisms found via a proteomics-based method that uses LC–ESI–MS and q TOF ESI–MS. This study represents a robust tool for linking phenotypes with α_{s1}-CN variants according to the country of origin.

2. Materials and methods

2.1. Milk samples and casein preparation

The data were obtained from milk collected from 139 individuals belonging to 3 Romanian buffalo populations from

* Corresponding author. Address: Food Science Institute, National Research Council, via Roma, 52-83100 Avellino (AV), Italy. Tel.: +39 081 2539346; fax: +39 081 7762580.
E-mail addresses: caira@unina.it, scaira@isa.cnr.it (S. Caira).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.02.033

Transylvania, belonging to the National Genetic Patrimony. Samples of Mediterranean CN were taken as controls. Two bulked WB milks were sampled: one from twenty herds of the Mediterranean breed located in the Salerno district (Italy), which is representative of approximately 6000 animals, and the other from the Caserta district (Italy) from a tank containing approximately 60,000 liters.

Skimmed isoelectric casein was prepared according to two procedures. In the first procedure, milk reconstituted from powder (1 mL), 100% ice-cold acetone (8 mL) and 100% trichloroacetic acid (1 mL) were mixed together. After incubation at room temperature for 1 h, the precipitate was collected by centrifugation at 3000×*g* for 10 min at 4 °C. The supernatant was discarded, and the protein was recovered after washing with 1 mL ice-cold acetone. This treatment was repeated twice, and the resulting protein pellet was washed three times with diethyl ether and dried under a ventilated hood. In the second procedure applied to fresh raw milk, casein was prepared from skimmed milk and treated according to the procedure of Aschaffenburg and Drewry using acetic acid and sodium acetate buffer as the precipitating agent (Aschaffenburg & Drewry, 1959).

2.2. Chemical and enzymatic hydrolyses

Modified trypsin, sequencing grade, was purchased by Promega (Madison, WI, USA), alkaline phosphatase (ALP) by Roche (Mannheim, Germany). Trypsin digestion and ALP hydrolysis were carried out in 0.4% ammonium bicarbonate, pH 8.0, at 37 °C for 4 and 16 h, using a ~50:1 substrate-to-enzyme ratio (w/w). Cyanogen bromide (CNBr) (Sigma–Aldrich, Oakville, ON, Canada) hydrolysis was carried out in 70% trifluoroacetic acid at room temperature for 18 h using a ~100-fold molar reagent excess.

2.3. HPLC separation of α_{s1}-casein

The α_{s1}-CN samples were isolated by Reversed-Phase-High Performance Liquid Chromatography (RP-HPLC) performed using an HP 1100 modular system (Agilent Technology, Palo Alto, CA, USA) equipped with a Vydac (Hesperia, CA, USA) C4 column (214TP54, 5 μm, 250 × 4.6 mm) by applying a linear gradient from 30% to 50% of 0.1% trifluoroacetic acid (TFA) in acetonitrile, (v/v), (solvent B), over 60 min at a flow rate of 1 mL/min; solvent A was 0.1% TFA in water (v/v). Dephosphorylated caseins were dissolved in 1 mL of 10 mM dithiothreitol (DTT) and incubated for 1 h at 37 °C; for each injection, 100 μL of the resulting solution was used. Column effluents were monitored by UV detection at 220 and 280 nm. Protein fractions were manually collected and either used for mass spectrometry analysis by flow injection (FIA) method, directly or after concentration under vacuum, or they were lyophilized before enzymatic digestion.

2.4. Liquid chromatography electrospray ionization mass spectrometry (LC–ESI–MS)

For LC–ESI–MS analysis, tryptic digests of caseins were fractionated using the same instrument as that used for RP-HPLC separation of intact proteins. In the case of peptides, a Vydac C18 218TP52 column (5 μm, 250 × 2.1 mm) was utilized at a constant flow rate of 0.2 mL/min. Elution was carried out with a linear gradient from 5% to 60% of solvent B applied over 60 min (solvent A and B as above) after 5 min of isocratic elution at 5% B. Approximately 100 μg of the peptide mixture, dissolved in 0.1% TFA, was injected for each analysis. The liquid effluent from the column was directly injected into the source of an Agilent 1100LC/MSD single quadrupole instrument via a 0.25 mm PEEK tube connection.

The ESI mass spectra scans were acquired from *m*/*z* 1600–400 at a scan cycle of 1 s/scan and 0.1 s inter-scan delay. The source temperature was 350 °C. Spectra were acquired in positive ion mode, with a capillary voltage of 3.5 kV. N2 was used as both the drying and nebulizing gas. The LC/MS pattern was elaborated using the LC/MSD ChemStation Data Analysis Software A.08.03, (Agilent Technology, Palo Alto, CA, USA) which was supplied with the instrument.

2.5. Electrospray-quadrupole, time-of-flight mass spectrometry (ESI-QTOF–MS)

Native and dephosphorylated whole CN as well as derived tryptic digests, dissolved at a concentration of 1 μg μL^{-1} in acetonitrile–water (v/v) containing 0.1% TFA, were analyzed by MS. Analysis was carried out using ESI Q-TOF™ hybrid quadrupole/time-of-flight mass spectrometer (Micromass Ltd., Manchester, UK) and a Z-spray ion source in the positive ion mode. The nano flow was accomplished with a syringe pump at 0.5 μL min^{-1}, and the TOF mass analyzer was used to acquire data in both the MS and MS/MS modes. The source temperature was 100 °C, and the desolvation temperature was 200 °C. The TOF operated at an acceleration voltage of 9.1 kV, a cone voltage of 100 V, a cone gas (N_2) of 13 L h^{-1} and a collision energy in MS mode of 10 eV. Collision-induced dissociation (CID) spectra were acquired in a data-dependent method on the most abundant ions having mass to charge ratios (*m*/*z*) ranging from 600 to 1800. The collision energy was dependent on the *m*/*z* ratio and the charge state of the parent ion and was generally between 25 and 40 V. The collision cell was pressurized with 10.34 Pa ultra-pure Ar (99.999%). The raw MS/MS data were combined and processed using the MaxEnt 3 algorithm prior to de novo sequence analysis using the Mass Seq software (Waters, Manchester, UK).

3. Results and discussion

3.1. Proteomics of α_{s1}-CN

Rapid detection of putative casein variants and post-translational modifications of caseins is ordinarily performed using proteomics-based methods with first-dimension HPLC separation and second-dimension MS. Therefore, due to their similar elution times, the α_{s1}- and β-CN variants are often confused with each other. An alternative means is to submit CN to dephosphorylation with ALP and then to treat the protein sample according to the procedure described in the Methods section prior to LC–MS. In this manner, the CN samples lost the original heterogeneity of native CN, enabling acquisition of the molecular mass value for the dephosphorylated samples. This technique has previously allowed researchers to demonstrate the presence of a deleted protein variant in ovine CN (Ferranti et al., 2001).With HPLC–ESI–MS, CN was fractionated into four major peaks, and each correlated with the presence of a different CN fraction (Fig. 1).

Changes occurred in the amino acid sequence of the α_{s1}-CN mutant involving the electrophoretically silent amino acid substitution of Ser for Leu. Using a gradient of aqueous acetonitrile and acetonitrile with the addition of a low concentration of an ion-pairing reagent, such as trifluoroacetic acid, silent protein variants were eluted from the C4 column in order of increasing hydrophobicity. Therefore, α_{s1}-CN Leu^{178} was retarded on the C4 column with respect to the Ser^{178} mutant. ESI–MS analysis and data processing generated a normalized molecular mass for dephosphorylated α_{s1}-CN (Supplementary Fig. S1, panel a). Therefore, we could assign unknown peaks to various α_{s1}-CN species, but primary sequence validation will ultimately be needed. In particular, it was