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Animal species milk identification by comparison of two-dimensional gel map profile and mass spectrometry approach

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ABSTRACT

The aim of the current study was to present the primary protein profile of cow, goat, camel, yak and buffalo milk, along with binary mixtures of these milks through two-dimensional gel electrophoresis coupled with mass spectrometry for detection of specific milks in mixtures. Distributions of α-lactalbumin and/or β-lactoglobulin spots on gel maps were used to detect goat, camel, yak and buffalo milk adulterated with cow milk. Appearance of α-lactalbumin and β-lactoglobulin protein spots were helpful for detection of camel, yak and buffalo milk adulteration with goat milk. α_{S1}-Casein from cow and goat milk was also used to determine camel milk adulteration. In particular, β-lactoglobulin from cow, goat, yak and buffalo milk, and α-lactalbumin from camel milk were useful to detect adulteration of specific milk mixtures at levels as low as 0.5%. These results highlight applicability of this method for characterisation of milk proteome and detection of specific milk in mixtures.

1. Introduction

Variation in environmental conditions and the natural selection process have promoted diversification of animal species and breeds, which has resulted in differing milk composition and production (Murgiano, D'Alessandro, Zolla, Valentini, & Pariset, 2012). Milk from minor dairy species, such as goat, buffalo, yak and camel, has high nutritional and economical value (Medhammar et al., 2012). For financial profit, higher priced milk (such as yak and camel milk) substituted by lower priced milk (such as cow milk) has been found in dairy products. To protect consumer benefits and product authenticity, it has been necessary to develop analytical procedures able to detect fraudulent substitution.

The development of analytical techniques, such as capillary electrophoresis (Herrero-Martínez, Simó-Alfonso, Ramis-Ramos, Gelfi, & Righetti, 2000), polymerase chain reaction (Bai et al., 2009; Guerreiro, Fernandes, & Bardsley, 2012), chromatography (Bramanti, Sortino, Onor, Beni, & Raspi, 2003; Cordeiro, Bordin, Rodriguez, & Hart, 2001), enzyme-linked immunosorbent assay (Hurley, Coleman, Ireland, & Williams, 2004), infrared spectroscopy (Kasemsumran, Thanapase, & Kiatsoonthon, 2007; Nicolaou, Xu, & Goodacre, 2010), as well as nuclear magnetic resonance (Lamanna, Braca, Di Paolo, & Imparato, 2011), has enabled their use to evaluate content of mixtures of milk of different species. Recently, mass spectrometry methods have been developed for investigating milk adulteration. In particular, liquid chromatography–tandem mass spectrometry (Chen, Chang, Chung, Lee, & Ling, 2004; MacMahon, Begley, Diachenko, & Stromgren, 2012) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI–TOF MS) (Calvano, De Ceglie, Monopoli, & Zambonin, 2012; Cozzolino et al., 2001; Nicolaou, Xu, & Goodacre, 2011) have been utilised to improve milk product analysis for quantification of specific proteins.

Two-dimensional gel electrophoresis (2-DE) is a powerful technique used to acquire protein profiles and allow protein spots to be visualised on gels. This method resolves proteins into spots, and the map of protein spots can be considered as the "protein profile" of a specific sample (Yarmush & Jayaraman, 2002). Thus, 2-DE maps of milk were constructed and used to characterise the protein profile of different mammalian milk (D'Auria et al., 2005; Hinz, O'Connor, Huppertz, Ross, & Kelly, 2012). Roncada et al. (2012) reviewed the most recent progress in characterisation of the milk proteome that allows the milk proteome of farm animal species to be easily distinguished. However, literature data have hardly been concerned with changes of protein profile on gel maps

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from different animal species. We hypothesised that milk mixtures from different animal species would result in alteration of distribution of protein spots on the 2-DE gels. Thus, 2-DE coupled with MALDI–TOF MS was used to investigate detection markers and distinguish milk authentication including goat, yak, buffalo and camel milk adulteration with cow milk, as well as yak, buffalo and camel milk adulteration with goat milk.

2. Materials and methods

2.1. Sample preparation

Thirty cow milk samples were collected from a dairy farm in Beijing city; 27 goat milk samples collected from a farm in Shanxi province; 21 camel milk samples collected from a farm in Urumchi city in Xinjiang; 30 yak milk samples collected from a farm in Qinghai province; and 21 buffalo milk samples collected from a farm in Yunnan province. These raw milk samples were collected from animals free of disease, as based on veterinarian records.

Milk samples from each farm were pooled for three fractions. Binary mixtures were prepared as in Table 1. Different milk types were pooled according to volume ratios and mixed by vortexing for 5 min. Then 2 mL milk samples were centrifuged at 3000 × *g* for 10 min at 4 °C to separate the skimmed milk and fat layer. Skimmed milk, as milk protein, was recovered from each sample and centrifuged again at 100,000 × *g* for 1 h at 4 °C to recover the supernatant. Concentration of protein was determined by the modified Bradford method with bovine serum albumin as a standard (Bio-Rad, Hercules, CA, USA).

2.2. Two-dimensional gel electrophoresis separation

Two hundred and 50 μg of protein of skimmed milks or ultracentrifugal supernatants were mixed in 350 μL immobilised pH gradient (IPG) rehydration buffer comprising 8 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 0.5% (v/v) pH 4–7 IPG buffer and trace bromophenol blue. First dimensional isoelectric focussing was carried out using 17 cm pH 4–7 IPG strips (Bio-Rad) at 20 °C. The IPG strips were rehydrated for 12 h and then taken through a series of focussing. They were focused at 0–500 V for 1 h, 500–1000 V for 1 h, 1000–9000 V for 5 h, and then 9000 V for 80,000 Vh.

After being focused, IPG strips were equilibrated with 2% (w/v) dithiothreitol, 50 mM Tris–HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, and 2% (w/v) sodium dodecyl sulphate at room temperature for 12 min followed by incubation with 2.5% (w/v) iodoacetamide, 50 mM Tris–HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, and 2% (w/v) sodium dodecyl sulphate at room temperature for another 12 min. Subsequently, strips were transferred onto gels and sealed with 0.5% (w/v) low melting point agarose and separated in a second dimension with 12% polyacrylamide gels.

Table 1
List of milk binary mixtures of cow, goat, camel, yak and buffalo milk.

Milk binary mixtures	Volume ratio of specific milk mixtures			
Cow milk adulterated with goat milk	95:5	98:2		
Cow milk adulterated with camel milk	95:5	98:2		
Cow milk adulterated with yak milk	95:5	98:2		
Cow milk adulterated with buffalo milk	95:5	98:2		
Goat milk adulterated with cow milk	95:5	98:2	99:1	99.5:0.5
Camel milk adulterated with cow milk	95:5	98:2	99:1	99.5:0.5
Yak milk adulterated with cow milk	95:5	98:2	99:1	99.5:0.5
Buffalo milk adulterated with cow milk	95:5	98:2	99:1	99.5:0.5
Goat milk adulterated with camel milk	95:5	98:2	99:1	99.5:0.5
Goat milk adulterated with yak milk	95:5	98:2	99:1	99.5:0.5
Goat milk adulterated with buffalo milk	95:5	98:2	99:1	99.5:0.5
Camel milk adulterated with goat milk	95:5	98:2	99:1	99.5:0.5
Yak milk adulterated with goat milk	95:5	98:2	99:1	99.5:0.5
Buffalo milk adulterated with goat milk	95:5	98:2	99:1	99.5:0.5

After electrophoresis, gels were stained with Coomassie Brilliant Blue G-250 solution, as described by Candiano et al. (2004). Gel images were analysed using ImageMaster 5.0 software (GE Healthcare, Piscataway, NJ, USA). Each pooled sample was repeated 3 times. For comparison of relative abundance of proteins among gels, protein spots were automatically detected and manually confirmed with "all or none" as the determining criterion that protein spot was only detected in milk binary mixtures compared with control milk.

2.3. In-gel digestion

Selected protein spots were excised manually from the gels and washed twice with MilliQ water for 15 min. Then, gel piece was washed with 50% (v/v) acetonitrile and destained with 50% (v/v) acetonitrile in 0.1 M ammonium bicarbonate for 15 min and then dried to completeness in pure acetonitrile. Subsequently, dried gel pieces were rehydrated with a small volume of digestion buffer (50 mM ammonium bicarbonate containing 0.01% (w/v) sequence-grade trypsin) and then 20 μL 10 ng μL^{-1} digestion buffer was added to cover the gel piece. Finally, the gel piece was incubated at 37 °C overnight and then digestion was terminated by addition of 2 μL of 5% (v/v) trifluoroacetic acid.

2.4. Protein identification and database search

Extracted peptide mixtures were loaded onto one spot of a MALDI target plate. Mass spectra and tandem mass spectra (MS/MS) were obtained by using 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA) with 355 nm Nd:YAG (Neodymium-doped Yttrium Aluminium Garnet) laser, and at an acceleration voltage of 20 kV. Positively charged ions were obtained in the reflector mode by delayed extraction. Peptide mass fingerprinting was gained in the mass range of 800–4000 Da. Eight highest precursor ions with a signal-to-noise ratio of at least 50 from each sample were analysed using MS/MS mode with 2500 laser shots, and at collision energy of 20 keV.

Protein identification was performed with MASCOT (Matrix Science) to search the NCBI nonredundant database (http://www.ncbi.nlm.nih.gov). All peaks with a signal-to-noise ratio of at least 15 were included in the search. Peptide mass fingerprinting search parameters were set as follows: monoisotopic mass accuracy < 100 ppm, allowance for one missed cleavage, carbamidomethylation of cysteine as a fixed modification, and methionine oxidation as a variable modification. Fragment mass tolerance and peptide mass tolerance were set to a ±0.4 Da and ±100 ppm, respectively.

3. Results

In the present study, 2-DE gels were repeated 3 times for each sample to detect reproducibility. Image analysis showed that these 2-DE maps were reproducible. To construct a 2-DE map, it was important to have a representative sample. Thus, master gel maps were constructed by comparing the 2-DE maps from the same samples with ImageMaster 2-DE analysis software, and then master gel maps were used to perform differential expression analysis. Protein profiles on the gel maps of cow, yak and buffalo milk were similar; however, protein profiles of goat and camel milk presented obvious differences.