Potential of bovine kappa - casein as biomarker for detection of adulteration of goat's milk with cow's milk

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Abstract

Using two-dimensional (2-D) electrophoresis it was shown that bovine kappa-casein could be an appropriate biomarker of adulteration of goat's milk with cow's milk not only in raw milk, but also for milk thermally processed by pasteurization or treated with ultra-high-temperature. The presence of cow's milk in goat's milk was detected at level of 2 %. Furthermore, position of bovine kappa-casein spots on 2-D gels remained unchanged even with samples from two different geographical origins, Belgium and Slovenia. These results show that neither thermal processing nor different geographical area seem to affect the position of bovine kappa-casein spots on 2-D gels.

Key words: **adulteration, goat's milk, kappa-casein, geographical origin, thermal processing**

Introduction

Adulteration of milk is commonly recognized as dilution with water and it can be detected by determining the freezing point. Another kind of possible fraud is the adulteration of more expensive milk and milk products (e.g. goat's) with milk from other dairy animals (e.g. cow's) (Mininni et al., 2009; Dąbrowska et al., 2010; Rodrigues et al., 2012; Jürg et al., 2013). When the addition of cow's milk is not declared in milk or dairy products made from goat's milk such products are not suitable for people with allergic reactions to cow's milk (Spink and Moyer, 2011). The presence of cow's milk was found in 30 % of ewe and goat cheeses in markets of Croatia (Špoljarić et al., 2013) and in 29 % of ewe and goat cheeses in markets of Slovenia (Klančnik et al., 2016)

During the last decade, proteomics technologies have been applied to different research areas within food technology including detection of food adulteration (Ortea et al., 2016). To detect adulteration of goat's milk with cow's milk the reference method is used, which is based on the differentiation of isoelectric points of cow and goat γ_2- and γ_3- caseins originating from the hydrolysis of the belonging β-caseins (Commision Regulation 2008; EC 273/08). But there are many studies, where mass spectrometry (MS) (Chen et al., 2004; Calvano et al., 2012; Girolamo et al., 2014) or gel

based approaches (Hinz et al., 2012; Špoljarić et al., 2013; Yang et al., 2014) have been used to detect adulteration of goat's milk with cow's milk or just for comparing protein profiles of different animal species milks. Although the general tendency nowadays is to move to gel-free workflows, protein electrophoresis has been used extensively (Ortea et al., 2012) and continues to be used (mainly 2-DE) in food authentication studies (Ortea et al., 2016). However, data provided by protein electrophoresis are often used as complementary to data obtained from MS-based approaches (Ortea et al., 2016). In our previous study (Jeršek et al., 2014) in order to detect the addition of raw cow's milk to raw goat's milk a 2-DE was used. It was found that bovine kappa-casein was an appropriate biomarker of adulteration. In the present study the potential to use a bovine kappa-casein as a biomarker for detecting the adulteration of goat's milk that has been thermally processed by pasteurization and ultra-high-temperature (UHT) treatment was examined. Additionally, samples of different geographical origins (Slovenia, Belgium) were compared to test if the results were geographically dependent.

Materials and methods

Milk samples

In this study raw, pasteurized, UHT cow and goat milk as well as their mixtures (2 % cow milk, 98 % goat milk) were used. Samples of raw goat milk and cow milk were obtained from Inex nv., Bavegem, Belgium. Half of the milk was pasteurized at the temperature of 62 °C for 30 min and cooled down on ice. UHT goat's milk and UHT cow's milk from the same milk batches was prepared at Inec nv. Furthermore, additional samples of goat's milk and cow's milk were obtained from two different farms in Slovenia. Aliquots of 40 mL of each milk sample were stored at -20 °C prior to use.

Chemicals and reagents

Immobilized pH gradient (IPG) buffer and 3-[(3-cholamidoprpyl)dimethylammonio]-1-propanesulfonate (CHAPS) and a 2-D clean up kit were from GE Healthcare (Little Chalfont, UK). Sodium dodecyl sulphate (SDS), glycerol, thiourea, urea, 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), dithiothreitol (DTT), iodoacetamide (IAA), bovine serum albumin and bromophenol blue were from Sigma (St. Louis, MO, USA), from(GE Healthcare). SYPRO Ruby was from Invitrogen (Carlsbad, CA, USA).

Two-dimensional electrophoresis

Concentration of proteins in the milk was determined by the method of Bradford (Bradford, 1976). Proteins were precipitated using a 2-D Clean up kit according to the manufacturer instructions.

Two-dimensional electrophoresis (2-DE) was performed according to Görg (1991), with minor modifications. Samples (100 μg of precipitated proteins) were solubilised in rehydration solution (7 M urea, 2 M thiourea, 2 % [w/v] CHAPS, 2 % [v/v] immobilised pH gradient [IPG] buffer [pH 3-10], 18 mM DTT and a trace of bromophenol blue) and applied to 13-cm NL pH 3 to 10 IPG strips. After rehydration, the first dimension (isoelectric focusing) was carried out on a Multiphore II system (GE Healthcare, Little Chalfont, UK). Before the second dimension, the IPG strips were equilibrated in equilibration buffer (75 mM Tris HCl, pH 8.8, 6 M urea, 30 % [v/v] glycerol, 2 % [w/v] SDS and a trace of bromophenol blue), containing 1 % DTT for 15 min, and for an additional 15 min in equilibration buffer containing 4.8 % IAA. SDS polyacrylamide gel electrophoresis was carried out using 12 % running gels on a vertical SE 600 discontinuous electrophoretic system (Hoefer Scientific Instruments, Holliston, MA, USA) at a constant 20 mA/gel for 15 min and then at a constant 40 mA/gel until the bromophenol blue reached the bottom of the gel. The 2-D gels were stained with SYPRO Ruby. For each sample, two 2-D gels were run under the same conditions. The 2-D gels were documented using a CAM-GX-CHEMI HR system (Syngene, Cambridge, UK). The gel images were aligned using the 2-D Dymension software, version 2.02 (Syngene Cambridge, UK).

Protein identification and amino acid sequences aligning

Spots of interest were excised from the gel and identified using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF-TOF) mass spectrometry at the University of York. The resulting data were searched against the IPI_Bovine database (34274 sequences). The following search parameters were applied: *Bos taurus* as a species, tryptic digest with maximum one missed cleavage site. The peptide mass tolerance was set to ±100 ppm, the fragment mass tolerance was set to ± 0.5 Da. Carbamidomethylation and oxidation of methionine were considered as possible modifications.

Aligning of amino acid sequences of kappa-casein from different species (cow, goat) were done using Clustal Omega program (Sievers et al., 2011).

Results and discussion

In our previous research (Jeršek et al., 2014) it was shown that the addition of cow's milk to goat's milk can be detected by a 2-DE coupled by MS, where cow and goat kappa-caseins can be differentiated depending on their post-translational modifications. Raw milk samples of the Slovenian origin were used and a mixture of 10 % of cow's milk and 90 % of goat's milk was tested for the detection of adulteration. In the present study the analysis of thermally processed milk samples from other geographical origin (Belgium) and with lower quantity of cow's milk (2 %) in the adulterated mixtures were included. In order to detect adulteration, 2-D gels were compared to find protein/s that is/ are present only in cow's milk and not in goat's milk.
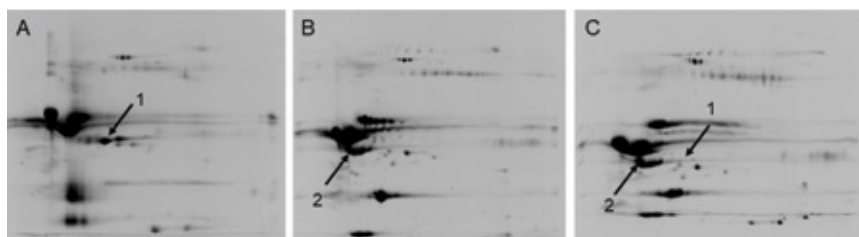

FIGURE 1. Protein profiles of raw cow's milk (A), goat's milk (B) and their mixture (cow, 2 % + goat, 98 %; cow - C). Proteins corresponding to cow's (1) and goat's (2) kappa-casein are marked with arrow

FIGURE 2. Protein profiles of pasteurized cow's milk (A), goat's milk (B) and their mixture (cow, 2 % + goat, 98 %; cow - C). Proteins corresponding to cow's (1) and goat's (2) kappa-casein are marked with arrow
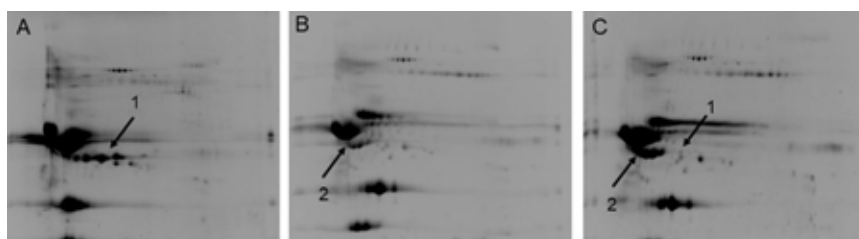
FIGURE 3. Protein profiles of UHT treated cow's milk (A), goat's milk (B) and their mixture (cow, 2 % + goat, 98 %; cow - C). Proteins corresponding to cow's (1) and goat's (2) kappa-casein are marked with arrow
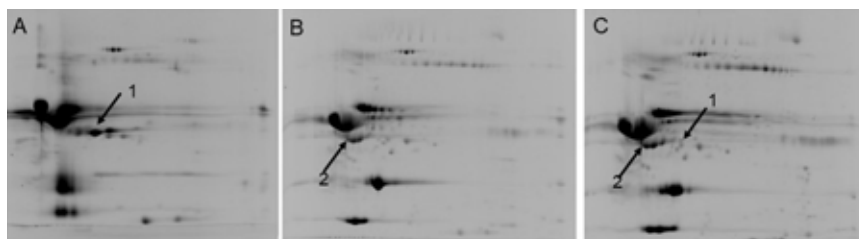
By comparing protein profiles of pure cow's milk to the pure goat's milk samples, in all samples regardless of the applied thermal processing, two proteins specific for cow's milk that were not detected on the same position on a 2-D gel of goat's milk were observed (Fig. 1, 2, 3). The same two spots with the same location on 2-D gels were observed in our previous study (Jeršek et al., 2014), where raw milk samples of Slovenian origin were analyzed (Fig. 4). Both two spots were identified as

kappa-casein from *Bos taurus*. Although raw, pasteurized and UHT milk have the same composition, protein modification can occur due to the high temperatures applied durng heating. During thermal processing free amino groups of proteins can be glycated by lactose through the Maillard reaction. The early stable products resulting from the rearrangement of the primary adducts are named the *Amadori* compounds (Finot et al., 1981). The extent of the reaction depends on the intensity of the heat treatment applied to milk. By aligning 2-D gels

and comparing the position of two spots related to the bovine kappa-casein, no changes concerning the isoelectric point or molecular weight were observed (Fig. 1, 2, 3). Although, the presence of intact kappa-casein does not exclude that it can be partially lactosylated during thermal processing (Milkovska-Stamenova and Hoffmann, 2016). These results indicate that the intact bovine kappa-casein remained in measurable quantities at temperatures of pasteurization as well as at conditions of UHT treatment.

FIGURE 4. Protein profiles of raw cow's milk and goat's milk from different geographical origin (A - Belgian cow's milk, B - Belgian goat's milk, C - Slovenian cow's milk, D - Slovenian goat's milk). Proteins corresponding to cow's (1) and goat's (2) kappa-casein are marked with arrow

FIGURE 5. Alignment diagrams of kappa casein from *Bos Taurus* (P02668) and *Capra hircus* (P02670) was obtained using Clustal Omega (Sievers et al., 2011). Amino acids that are 100 % conserved (identical and similar) are shown with (*) and (:), 80 % conserved amino acids are shown with (.)

Kappa-casein is a common protein in the mammalian milk that determines the size and specific function of milk micelles (Gutierrez-Adan et al., 1996). Aligning of amino acid sequences of kappa-casein from different species showed differenc-

es (The UniProt Consortium, 2017). Thus, bovine and goat kappa-casein have 84.375 % identity and 6.25 % similarity (Fig. 5). Additionally, they differ in post-translational modifications (PTM), specifically in degree of phosphorylation and glycosylation. Bo-

vine has eight and four positions where O-linked glycosylation and phosphorylation occur, respectively. In the case of goat kappa-casein there are seven positions with O-linked glycosylation and three positions which are phosporylated (The UniProt Consortium, 2017). Thus differences in the PTM degree result in different positions of bovine and goat kappa- casein on 2-D gels (Fig, 1-4). Hinz et al. (2012) compared protein profiles of milk from different animal species and highlighted significant interspecies differences. Concerning kappa-casein it exhibits different spots positions on 2-D gel in the case of goat's as well as camel's milk compared to cow's milk, but they were located on the same positions in case of buffalo's and cow's milk. For the latter two samples also general protein profile was similar. In the study of Yang et al. (2014) distributions of α-lactalbumin and/or β-lactoglobulin spots on 2-D gel were used to detect the adulteration of goat's, camel's, yak's and buffalo's milk with cow milk. On the other side, the reference method for determining the presence of cow's milk in goat's milk is based on the differentiation of isoelectric points of cow and goat γ_2- and γ_3- caseins originated from the hydrolysis of β-casein using isoelectric focusing (Commision Regulation 2008; EC 273/08).

The position of both bovine kappa-casein spots of on 2-D gel remained unchanged, when milk samples from other geographical region were analyzed. Here milk samples were from Belgium compared to previous study, where samples were from Slovenia (Jeršek et al., 2014). This indicates that the presence and location of both bovine kappa casein spots on 2-D gel is not geographically dependent (Fig. 4).

Complementary to our previous study (Jeršek et al., 2014) where a mixture (10 % cow's milk + 90 % goat's milk) was investigated, here a less adulterated mixture (2 % cow's milk + 98 % goat's milk) was analyzed and two proteins that belong to bovine kappa-casein were still detected in all cases (Fig. 1, 2, 3). It has to be highlighted that Sypro ruby was used to stain the gels, which is more sensitive compared to Coomassie brilliant blue, as most common stain used (Berggren et al., 2000).

Conclusion

None of proteomic gel based studies/methods published so far focused on the location in 2-D gel of kappa-casein of different species (cow, goat) as a possible biomarker for the detection of cow milk as adulterant of goat milk. In the present study it was shown that neither thermal processing nor different geographical origin had an effect on the position of both bovine kappa casein protein spots on 2-D gel. Using the described approach, the presence of cow's milk in goat's milk can be detected at level of 2 %.

Acknowledgement

Potencijal kravljeg kapa-kazeina kao biomarkera za utvrđivanje patvorenja kozjega mlijeka kravljim

Sažetak

Korištenjem dvodimenzionalne elektroforeze dokazano je da kravlji kapa-kazein može biti odgovarajući biomarker za utvrđivanje patvorenja kozjeg mlijeka kravljim mlijekom, ne samo u sirovom mlijeku, već i za mlijeko koje se termički obrađuje pasterizacijom ili ultra-visokom temperaturom. Prisutnost kravljeg mlijeka u kozjem mlijeku moguće je detektirati na razini od 2 %. Nadalje, položaj proteinskih mrlja kapa-kazeina na 2-D gelovima ostao je nepromijenjen uspoređujući uzorke mlijeka različitog geografskog podrijetla, Belgije i Slovenije. Ovi rezultati pokazuju da ni toplinska obrada niti različito geografsko podrijetlo ne utječu na položaj kravljih proteinskih mrlja kapa-kazeina na 2-D gelovima.

Ključne riječi: patvorenje, kozje mlijeko, kapa-kazein, zemljopisno podrijetlo, toplinska obrada

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