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Fast Isoelectric Focusing and Antipeptide Antibodies for Detecting Bovine Casein in Adulterated Water Buffalo Milk and Derived Mozzarella Cheese

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Plasmin hydrolysis of water buffalo casein (CN) can liberate a peptide comigrating with bovine γ_2-CN. Occurrence of this peptide may lead to false-positive detection of cow's milk for a genuine water buffalo cheese when it is analyzed by applying a fast version of the European official method for detecting bovine casein in water buffalo cheese. After isoelectric focusing of CN plasminolysates, performed according to the official method, immunoblot analysis with antipeptide antibodies was assayed to distinguish between γ_2-CN and the interfering bovine γ_2-CN-like peptide. Small, synthetic peptides containing partial sequences of bovine γ_2-CN were used as immunogens for antipeptide antibodies raised in rabbits. The antibody preparation directed toward the synthetic peptide containing the first five amino acid residues of γ_2-CN cross-reacted with native and in vitro generated γ_2-CN from bovine and water buffalo CN, but it did not recognize the bovine γ_2-CN-like band in the electrophoretic profile of pure water buffalo CN.

KEYWORDS: Cheese adulteration; water buffalo mozzarella cheese; γ_2-casein; antipeptide antibodies; PAGIF; immunoblotting

INTRODUCTION

Analytical methods used by the forensic disciplines require intrinsically powerful capacity, knowing that the accuracy of the analytical response will primarily be used to determine the judge's verdict. A number of methods have been proposed for the identification of the animal species products present in milk and dairy products. Many of these methods are based on the analysis of either casein or whey protein components and include electrophoretic, chromatographic, and immunological techniques (*1*−*3*). European communities have established the official analysis method for detecting bovine casein in water buffalo cheese (*4*). This method is based on the different isoelectric points of bovine and water buffalo γ_2- and γ_3-casein, two of the pH 4.6 insoluble peptides generated from plasmin hydrolysis of β-CN, corresponding to β-CN fragments 106−209 and 108−209, respectively (*5*). Accordingly, the method mainly consists of polyacrylamide gel isoelectric focusing analysis of the CN plasminolysate, Coomassie Brilliant Blue G-250 staining, and identification of the bovine CN marker. Bovine and water buffalo γ_2- and γ_3-CN split into two unique subgroups that migrate distinctly from one another on the basis of their different p*I* values. Using two milk reference samples, that is, water buffalo milk containing 0 and 1% of cow's milk, a cheese sample would be adulterated if the bovine γ_2-CN and γ_3-CN amount is greater than or equal to 1%. A routine, fast, and low-cost procedure for the detection of bovine milk in ovine, caprine, and water buffalo milk or cheese is currently applied in some of the world's laboratories using precast or homemade IEF mini-gels on a PhastSystem apparatus (GE Healthcare, Uppsala, Sweden). The time from loading the gel to detection is <1 h, and the resolution is similar to that of larger conventional gels of the European communities' method (*6*). The current fast analytical method claims to be sensitive to an amount of bovine milk as low as 0.5% (v/v). Unfortunately, some genuine water buffalo mozzarella cheese samples have been determined to be adulterated due to apparent bovine casein exceeding 1%, which is assumed as the maximum intrinsic error tolerated by the current analytical procedure. In these cases, just one peptide having a p*I* similar to that of bovine γ_2-CN was responsible for the false-positive response. Water buffalo CN plasminolysate contained this peptide at a similar, nearly reproducible, concentration that persisted up to an enzyme to CN ratio range of 1:500. In addition, occurrence of the bovine γ_2-CN-like peptide in plasmin hydrolysates of pure water buffalo CN was irrespective of the CN genetic trim of individual water buffalo milk samples. In contrast, immunological analysis techniques are more specific, more sensitive, easier to handle and better capable of distinguishing milks from different animal species (*7*−*9*). Historically, immunoblotting has made it easier to identify membrane-immobilized proteins with polyclonal or monoclonal antibodies. In the past decade, this technique has been widely used as a rapid and straightforward method to identify casein variants (*10*). The success of developing an immunochemical method is largely

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determined by the binding specificity of the antibody preparation used as the primary reagent. The crucial step in setting the antibody reactivity spectrum is the choice of an appropriate immunogen. Immunization with synthetic peptides containing exact amino acid sequences present in stretches within a specific protein allows for the specificity of the antibody response to the selected regions of the protein (*11*). Therefore, we raised protein-reactive antipeptide antibodies using synthetic peptides as immunogen. Single casein families are recognized by specific antipeptide antibodies raised against short synthetic peptides (approximately 15 amino acids in length) corresponding to exact sequence stretches within the protein regions that are likely to be accessible to solvent, mainly the amino (N)-terminal or carboxy (C)-terminal sequences (*12*−*15*). Antibovine β-CN(1−28) has served as a primary antibody for monitoring β-CN degradation during hard cheese ripening (*16*, *17*).

Immunochemical techniques used to identify the species source of the milk used as an ingredient requires species-specific reagents. As a rule, polyclonal antibodies developed against each of the bovine caseins are also able to recognize the respective counterpart in milk from a different species. Alternatively, valuable advances in the differentiation between related species milk by immunochemical means have been achieved using antipeptide antibody technology. An antibody preparation using a bovine β-CN peptide (corresponding to amino acids 176−185) selectively distinguishes between bovine and water buffalo γ_2- and γ_3-CN from each of the two ovine and caprine counterparts that lack the Pro^{179}−Tyr^{180} peptide (*18*). Unfortunately, an antibody preparation is not available for differentiating water buffalo and bovine cheese CN presently. The antipeptide antibodies directed against bovine κ-CN(155−164) (*19*) are able to detect selectively bovine κ-CN in mixtures of bovine and water buffalo casein, but this antibody preparation is ineffective for cheese analysis due to the fact that the targeted casein-glycopeptide separates out in the whey after milk clotting.

The present study establishes an immunochemical method for quality control of water buffalo milk and derived mozzarella cheese. Antipeptide antibodies used as primary reagents in this study were specially raised against three sequence stretches of bovine γ_2-CN, including peptides in the C-terminus, in the N-terminus, and one within the middle region of bovine γ_2-CN. Antibody preparations were successfully assayed for their ability to recognize bovine versus water buffalo γ_2-CN bands and evaluate milk adulteration.

MATERIALS AND METHODS

Synthetic Peptides and Production of the Antisera. Peptides were chemically synthesized and HPLC purified by Primm (Milano, Italy). The peptides corresponded to the following amino acid sequences of β-CN: 195−209, 176−185, and 106−110. The identity of the peptides was controlled by MALDI-TOF. A cysteine residue was added to each of the peptides for conjugation with ovalbumin according to the coupling procedure described by Mattson et al. (*20*). The cysteine was added to the C-terminus of the peptides corresponding to amino acids 195−209 and 176−185 of β-CN. The cysteine was added to the N-terminus of the peptide containing amino acids 106−110 of β-CN. The ovalbumin conjugates were used to immunize two rabbits. Finally, the antisera were filtered on a 0.45 μm membrane (Millipore, Bedford, MA), split into aliquots of 1 mL, and stored at −20 °C.

Milk and Cheese Samples. Water buffalo and bovine milk samples were from local herds. Standard mixtures of water buffalo milk containing 25, 12.5, 6, 3, 1.5, and 0.75% (w/w) of bovine milk were prepared at laboratory scale. Samples of mozzarella cheese from purely water buffalo milk, bovine milk, or mixtures containing 10, 5, 2, 1, 0.5, of 0.25% (v/v) bovine milk were obtained from a cheese plant from separate vats. Fresh samples were divided into different lots and stored at −20 °C until analysis. Reference casein samples were obtained by isoelectric precipitation of skimmed milk by a 10 min centrifugation at 4500 rpm according to the procedure described by Aschaffenburg and Drewry (*21*). The protein concentration in the samples was preliminarily determined by the biuret method according to Bradford (*22*) using a protein assay dye reagent (Bio-Rad, Hercules, CA).

Plasmin Hydrolysis. A volume of 1 mL of milk was added to an equal volume of 0.2 M NH_4HCO_3, pH 8.0, vortexed for a few seconds, and allowed to stand for 5 min. A 500 μL aliquot of milk in bicarbonate solution was transferred to a plastic culture tube (15 mm × 10 mm), added to 10 μL of a 5 U/mL plasmin suspension (Boehringer, Mannheim, Germany), and incubated in a 37 °C water bath for 1 h. To recover the proteins, an equal volume of 24% (w/v) trichloroacetic acid was added to the sample solutions. The mixed solutions were left standing at room temperature for 10 min, followed by centrifugation at 4500*g* for 10 min at room temperature to pellet the proteins. The precipitate was then solubilized in 250 μL of 9 M urea. For mozzarella cheese, a 200 mg sample was suspended in 1.5 mL of 0.2 M NH_4HCO_3, pH 8.0, and vortexed until the casein was solubilized (about 3 min). Then, a 500 μL aliquot of the cheese solution was treated similarly to the liquid milk and analyzed according to the same procedure.

Electrophoresis and Immunoblotting. Isoelectric focusing was performed on small, ultrathin-layer polyacrylamide gels (thickness, 0.25 mm). A pH gradient in the range 3.5−10 was obtained by mixing Pharmalyte (GE Healthcare, Uppsala, Sweden) 3.0−10 and 5.0−8.0 in a 1:1 (v/v) ratio. Protein separation was carried out on the PhastSystem apparatus (Pharmacia, Uppsala, Sweden) according to the procedure described by Moio et al. (*23*). After gel electrophoresis, proteins were transferred to a nitrocellulose membrane by capillary diffusion. The procedure was similar to that described by Chianese et al. (*9*); however, the blocking solution was replaced with PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136 mM NaCl, 2.7 mM KCl, pH 7.4) containing 10% heat-inactivated horse serum (v/v). Immunostaining was carried out using the antipeptide antisera as the primary reagent and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG polyclonal antibodies as the secondary reagent (Bio-Rad). For CN detection, the nitrocellulose paper was placed in 10 mM Tris-HCl, pH 7.5, containing 0.5 mg/mL of 3,3-diaminobenzidine (Fluka, Buchs, Switzerland) and 0.06% (w/v) $NiCl_2$ hexahydrate (Fluka). To start the reaction between HRP-labeled goat anti-rabbit IgG antibodies and 3,3-diaminobenzidine, 20 μL of 30% (v/v) hydrogen peroxide was added. Incubation of the nitrocellulose membrane was performed at room temperature with agitation and examined periodically until the protein bands became suitably dark (1−5 min). The reaction was stopped by placing the nitrocellulose membrane in distilled water.

RESULTS AND DISCUSSION

Performance of the Antipeptide Antibodies in Immunoblotting Analysis of Milk Mixtures. Preliminarily, plasmin hydrolysates of pure water buffalo casein and cheese were analyzed to select water buffalo samples including a band with a p*I* similar to that of bovine γ_2-CN. These samples were used as the water buffalo milk and cheese reference samples throughout the present study. Plasmin hydrolysates of bovine and water buffalo samples and mixtures thereof containing 0.75−25% (w/w) of bovine milk were separated by polyacrylamide gel isoelectric focusing (PAGIF), and the protein profiles were stained by Coomassie Brilliant Blue G-250. A gel image is shown in **Figure 1A**, where both the bovine and the water buffalo γ_2- and γ_3-CN bands are clearly identified. Occurrence of a band with a p*I* similar to that of bovine γ_2-CN in pure water buffalo casein plasminolysate (lane 8) falsely accounted for an adulteration exceeding 1% bovine casein according to the densitometric evaluation. The antibody raised against the synthetic peptide corresponding to amino acids 195−209 of β-CN was able to detect a number of bands among those stained by Coomassie Brilliant Blue G-250. However, this antibody barely recognizes the presence of the bovine γ_2-CN-like band (**Figure 1B**). A similar false-positive response was obtained using the antibody raised against the peptide corresponding to amino

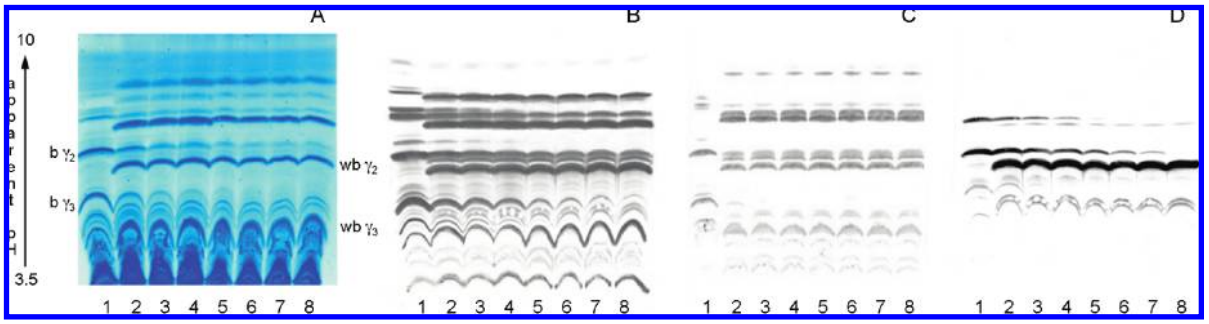

Figure 1. Fast isoelectric focusing and immunoblotting of plasmin hydrolysates from water buffalo milk containing 25% (lane 2), 12.5% (lane 3), 6% (lane 4), 3% (lane 5), 1.5% (lane 6), or 0.75% (lane 7) bovine milk (w/w): (**A**) Coomassie Brilliant Blue G-250 stained profiles of the plasmin hydrolysates; (**B**) immunostaining of the profiles from panel **A** using a 1:1000 (v/v) dilution of anti-β-CN(195−209) antiserum as a primary reagent; (**C**) immunostaining of the profiles from panel **A** using a 1:1000 (v/v) dilution of anti-β-CN(176−185) antiserum as a primary reagent; (**D**) immunostaining of the profiles from panel **A** using a 1:1000 (v/v) dilution of anti-β-CN(106−110) antiserum as a primary reagent. A 1:2000 (v/v) dilution of horseradish peroxidase-labeled goat anti-rabbit IgG antibodies was used as a secondary reagent. Bovine γ_2-CN and γ_3-CN bands are indicated as b γ_2 and b γ_3 and water buffalo γ_2-CN and γ_3-CN bands as wb γ_2 and wb γ_3, respectively. Plasmin hydrolysates of whole casein from bovine milk (lane 1) and water buffalo milk (lane 8) were included as reference samples.

acids 176−185 of bovine β-CN (**Figure 1C**). According to these results, the water buffalo peptide migrating like bovine γ_2-CN was one of the pH 4.6 insoluble peptides generated by β-CN plasminolysis. Similarly to both bovine and water buffalo γ_2-CN, its C-terminal region, including at least the last 30 amino acid residues, is sufficiently exposed at the protein surface to be accessible to antibodies. On the contrary, the antibody targeting amino acids 106−110 of β-CN was able to recognize both bovine and water buffalo γ_2-CN, but did not cross-react with the bovine γ_2-CN-like peptide (**Figure 1D**). This result may suggest that the bovine γ_2-CN-like peptide, even if it originates from water buffalo β-CN, retains the same β-CN amino acid sequence starting from an amino acid residue after 110. Alternatively, the bovine γ_2-CN-like peptide may contain the antibody-targeted sequence, but conformational constraints acting on the protein structure may prevent the antibody from accessing the specified region. Consistent with this last hypothesis, the antibodies also failed to recognize both native bovine and water buffalo β-CN by ELISA (data not shown), likely due to the fact that the 106−110 sequence was not accessible to the antibodies in the parent β-CN. Further studies are ongoing to identify the bovine γ_2-CN-like peptide that hinders the correct labeling of genuine water buffalo milk and cheese according to the PhastSystem procedure (*23*). Thus, the anti-β-CN(106−110) antibody preparation was effective in overcoming interferences due to the presence of the bovine γ_2-CN-like peptide. The same antibody preparation was also able to detect a few other plasmin-mediated fragments of β-CN, but it did not recognize the γ_3-CN peptide. This suggests that the two hydrophilic N-terminal amino acids, His[106] and Lys[107], play a crucial role in antibody recognition.

Immunoblotting Analysis of Water Buffalo Mozzarella Cheese Using Anti-β-CN(106−110). Experimental water buffalo mozzarella cheese samples adulterated by spiking water buffalo milk with bovine milk were analyzed by immunoblotting with the antibody preparation directed against amino acids 106−110 of β-CN (**Figure 2**). No interfering bands migrating similar to bovine γ_2-CN occurred in the electrophoretic profile (lane 8, **Figure 2B**), even if the bovine γ_2-CN-like peptide was included in the reference water buffalo mozzarella cheese, as shown by Coomassie Brilliant Blue G-250 staining (lane 8, **Figure 2A**). A gradual positive response for true bovine γ_2-CN was recorded for 0.25−10% adulteration (lanes from 7 to 2, **Figure 2B**). The positive response strictly corresponded to the actual amount of bovine milk present within the water buffalo mozzarella cheese.

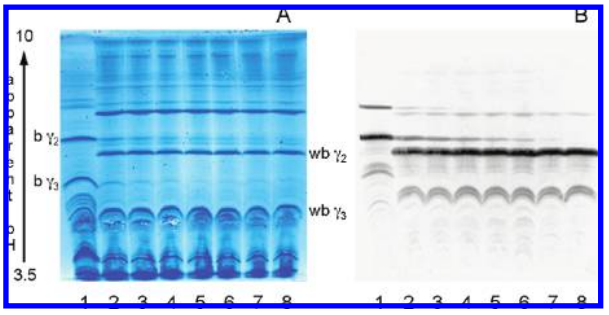

Figure 2. Fast isoelectric focusing and immunoblotting of plasmin hydrolysates of water buffalo cheese containing either 10% (lane 2), 5% (lane 3), 2% (lane 4), 1% (lane 5), 0.5% (lane 6), or 0.25% (lane 7) bovine milk (v/v): (**A**) Coomassie Brilliant Blue G-250 stained profiles of plasmin hydrolysate; (**B**) immunostaining of the profiles shown in panel **A** using a 1:1000 (v/v) dilution of the anti-β-CN(106−110) antiserum as a primary reagent. A 1:2000 (v/v) dilution of horseradish peroxidase-labeled goat anti-rabbit IgG antibodies was used as a secondary reagent. Bovine γ_2-CN and γ_3-CN bands are indicated as b γ_2 and b γ_3 and water buffalo γ_2-CN and γ_3-CN bands as wb γ_2 and wb γ_3, respectively. Plasmin hydrolysates of whole casein from bovine milk (lane 1) and pure water buffalo cheese (lane 8) were included as reference samples.

The presence of bovine milk at an amount as low as 0.25% (v/v) was detected in the water buffalo mozzarella cheese samples (lane 7). Thus, the additional advantage of this analytical method is the improved sensitivity to bovine γ_2-CN. Of note, the observed limit of detection is actually 4-fold lower, at least, due to overloading of the migration gel with protein and prolonged time of color development with the benzidine reagent. In addition to lower detection ability, the number of antigenic bands recognized by the anti-β-CN(106−110) antibody is limited. Therefore, when adulteration, as evaluated according to the current modified European official method (*4*), determines the presence 1% or more of bovine milk, additional confirmation by immunoblotting analysis should be carried out. Accordingly, the present procedure currently used in our laboratory to certify the authenticity of Protected Designation of Origin (PDO) water buffalo *Mozzarella di Bufala Campana* has eliminated any doubt about false-positive results.

In this work it was confirmed once more that targeted peptide-specific antibodies can be especially valuable analytical tools in solving problems relevant to the dairy field. To restrict

immunological responses toward bovine γ_2-CN, three synthetic peptides mimicking three distinct regions of γ_2-CN were evaluated each as immunogen to obtain site-directed immunoreagents. All of these antipeptide antibodies were able to cross-react with water buffalo and bovine γ_2-CN, according to the 99% sequence homology between γ_2-CN from these two animal species. One of these antibody preparations, specifically the anti-β-CN-(106−110), was so selective toward γ_2-CN that it did not recognize the majority of peptides resulting from water buffalo β-casein plasminolysis. At present, immunoblotting procedures based on the use of this antipeptide antibody should be used as the "gold standard" method for detecting bovine CN in water buffalo milk and cheese. Operationally, we suggest that the analytical method used to determine adulteration that is outlined in the current official method of analysis (*4*) be included as a second assurance of water buffalo milk purity and cheese genuineness.

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