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Highly sensitive periodic acid/Schiff detection of bovine milk glycoproteins electrotransferred after nondenaturing electrophoresis, urea electrophoresis, and isoelectric focusing

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Abstract — Due to its lack of sensitivity, periodic acid/Schiff (PAS) staining of gels is not considered to be relevant for the detection of glycoproteins other than mucin-type glycoproteins after nondenaturing or urea polyacrylamide gel electrophoresis (PAGE). A simple and highly sensitive method was developed for the detection of bovine milk glycoproteins separated by nondenaturing PAGE, urea-PAGE, and isoelectric focusing. After electrophoresis, proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane and then glycoproteins were specifically stained by the Schiff's reagent after periodic acid oxidation. This method allowed the detection of the glycosylated forms of bovine α-lactalbumin with sufficient sensitivity after nondenaturing PAGE or isoelectric focusing of whey proteins. Combination of isoelectric focusing and PAS detection on a PVDF membrane provided a resolutive tool in the characterization of the different glycoforms of κ-casein and also of component PP3 of proteose peptones. Distinction between the non-glycosylated and glycoforms of κ-casein separated by urea-PAGE or isoelectric focusing was performed by comparing Coomassie blue staining and PAS staining. Moreover, amino-terminal microsequencing of glycoproteins immobilized on the membrane could be performed after PAS staining.

bovine milk protein / glycoprotein / electrophoresis / isoelectric focusing / electroblotting / Schiff's reagent

Résumé — **Révélation très sensible des glycoprotéines du lait bovin par le réactif de Schiff après électrotransfert des protéines séparées par électrophorèse non dénaturante, électrophorèse en présence d'urée et isoélectrofocalisation.** À cause de sa faible sensibilité, la révélation acide périodique / Schiff (PAS) n'est pas applicable pour la détection des glycoprotéines autres que les

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mucines après électrophorèse en gel de polyacrylamide (PAGE) en conditions non dénaturantes ou en présence d'urée. Une méthode simple et très sensible a été développée pour la détection des glycoprotéines du lait bovin après leur séparation par PAGE non dénaturante, PAGE-urée et focalisation isoélectrique. Après électrophorèse, les protéines sont électrotransférées sur une membrane de polyvinylidène difluoride (PVDF) et les glycoprotéines sont ensuite révélées spécifiquement par le réactif de Schiff après oxydation à l'acide périodique. Ainsi, les formes glycosylées de l'α-lactalbumine ont été détectées avec une bonne sensibilité après PAGE non dénaturante ou focalisation isoélectrique des protéines du lactosérum. L'association de la focalisation isoélectrique et de la révélation PAS sur membrane de PVDF offre une séparation résolutive des glycoformes de la caséine κ ainsi que des glycoformes du composant PP3 des protéoses-peptones. La distinction entre les formes non glycosylées et glycosylées de la caséine κ séparées par PAGE-urée ou focalisation isoélectrique a été réalisée en comparant la révélation PAS à la révélation au bleu de Coomassie. Le microséquençage amino-terminal de glycoprotéines immobilisées sur membrane a pu être réalisé après révélation PAS.

protéine du lait / glycoprotéine / électrophorèse / focalisation isoélectrique / électrotransfert / réactif de Schiff

1. INTRODUCTION

Periodic acid/Schiff (PAS) staining is commonly used for the identification of glycoproteins separated on polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) [13, 27]. The PAS method is specific for glycoproteins since carbohydrates containing *cis*-1,2-diol groups are oxidized by periodic acid and then react with the Schiff's reagent. The PAS method is very sensitive for glycoproteins separated by SDS-PAGE since the carbohydrate moities are easily accessible to the Schiff's reagent after denaturation of the polypeptide chain by SDS [7]. The PAS staining is, however, ineffective in nondenaturing (ND) polyacrylamide gels [7]. The highly sensitive silver staining method developed for glycoproteins [8] can result in staining of non-glycosylated proteins [23]. To visualize glycoproteins, the ND gels must be treated by denaturing agents (i.e., SDS, ethanol, acetic acid, and β-mercaptoethanol) before PAS staining. Direct PAS staining of mucin-type glycoproteins with high carbohydrate content can be performed after ND-PAGE and also after isoelectric focusing (IEF) using the PhastSystem [26]. To our knowledge, a glycoprotein staining method after urea-PAGE separation has not yet been reported.

Several studies show that glycoproteins are easily visualized on a polyvinylidene difluoride (PVDF) membrane after SDS-PAGE and electrotransfer [6, 23]. In this study, the PAS staining of Kapitany and Zebrowski [13] was adapted for the characterization of bovine milk glycoproteins electroblotted onto PVDF membranes after ND-PAGE, urea-PAGE, and IEF separation. This method including electrotransfer and PAS staining was simple and suitable for SDS-PAGE, ND-PAGE, urea-PAGE, and IEF. Highly sensitive and specific detection of N-glycosylated α-lactalbumin, lactophorin (also called component-3 of proteose peptones or PP3), lactoferrin, κ-casein, and glycomacro-peptide (GMP) was obtained.

2. MATERIALS AND METHODS

2.1. Materials

The vertical electrophoresis apparatus model 2001 and the horizontal Multiphor 2117 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The TE-50 Transphor cell was from Hoeffer Scientific Instrument (San Francisco, CA, USA). The Sequi-Blot PVDF membrane was obtained from Bio-Rad Laboratories (Hercules, CA,

USA). The 476A microsequencer was from Applied BioSystems, Foster City, CA, USA).

2.2. Chemicals and proteins

Periodic acid and Schiff's reagent were purchased from Merck (Darmstadt, Germany). Solution containing a mixture of 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide in an acrylamide-to-bisacrylamide ratio of 37.5:1 (Rotiphorese Gel 30) was purchased from Roth (Karlsruhe, Germany). Pharmalytes (pH 3–10, 2.5–5, and 4–6) were from Pharmacia Fine Chemicals. 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) was from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade quality. Bovine κ-casein, bovine GMP, and rennin from calf stomach (EC 3.4.23.4; 51.0 enzyme units·mg^{-1} solid) were obtained from Sigma Chemical Co.

Sodium caseinate was obtained from bovine raw skimmed milk by three steps of isoelectric precipitation at pH 4.6 and dissolution at pH 7.0; the sodium caseinate solution was then dialyzed against ultra-pure water at 4 °C for 72 h (dialysis membrane cut-off 5000 Da) and freeze-dried. After concentration of whey, soluble proteins were directly dialyzed and freeze-dried. Proteose peptones, which are the acid-soluble and heat-stable fraction of milk, were prepared by heating skimmed milk at 95 °C for 30 min followed by cooling at 37 °C and acidification at pH 4.6. The proteose peptones were dialyzed and freeze-dried. Component PP3 was purified by immobilized concanavalin A affinity chromatography of proteose peptones as previously described [10].

2.3. κ-Casein hydrolysis

The commercial κ-casein (10 mg·mL^{-1}) was dissolved in 100 mmol·L^{-1} sodium phosphate buffer (pH 6.5) containing 0.02% (w/v) NaN_3. Rennin (0.23 enzyme units per milligram of κ-casein) was added to the κ-casein solution and the hydrolysis was performed at 37 °C for 6 h. Three volumes of 75 mmol·L^{-1} Tris-HCl, pH 8.9, containing 4 mol·L^{-1} urea, 5% (v/v) β-mercaptoethanol, 50% (v/v) glycerol, and 0.01% (w/v) bromophenol blue were added to one volume of hydrolysate prior to urea-PAGE analysis.

2.4. Electrophoresis methods

ND-PAGE was carried out according to Pâquet et al. [14] with a 9.25% polyacrylamide gel in 0.38 mol·L^{-1} Tris-HCl, pH 8.9. For ND-PAGE, the samples (2 mg·mL^{-1}) were solubilized in 0.38 mol·L^{-1} Tris-HCl, pH 8.9, containing 50% glycerol and 0.01% bromophenol blue. Urea-PAGE was performed according to Sanogo et al. [17] with a 8.2% polyacrylamide gel in 75 mmol·L^{-1} Tris-HCl, pH 8.9, in the presence of 4 mol·L^{-1} urea. For urea-PAGE, the samples (2 mg·mL^{-1}) were incubated at 22 °C for at least 1 h (for a good solubilization) in 75 mmol·L^{-1} Tris-HCl, pH 8.9, containing 4 mol·L^{-1} urea, 5% β-mercaptoethanol, 50% glycerol, and 0.01% bromophenol blue. IEF was carried out according to Sanogo et al. [18] with a horizontal 5% polyacrylamide gel containing 7 mol·L^{-1} urea and 2% (v/v) of each kind of ampholytes (pH 3–10, 2.5–5, and 4–6). For IEF, the samples (2 mg·mL^{-1}) were solubilized in 7 mol·L^{-1} urea containing 5% β-mercaptoethanol. In all cases, aliquots of 25 μL of sample were loaded in each well on the gels for electrophoresis analyses. When gels were not submitted to electrotransfer, proteins were fixed in 12% (w/v) trichloroacetic acid (TCA) for 30 min and stained in 0.1% (w/v) Coomassie blue R250 dissolved in a mixture of 50% (v/v) ethanol and 2% TCA for 60 min. Destaining was performed overnight in a solution of 30% ethanol and 7.5% acetic

acid. In the case of IEF, the ampholytes were eliminated before staining by five successive rinsings of the gel in 12% TCA for 20 h. Staining of proteins was then achieved in 0.25% (w/v) Coomassie blue G250 dissolved in a mixture of 50% ethanol and 12% TCA for 10 min. Destaining was performed for 1 h as described above. All the gels were dried between two cellophane sheets and stored at room temperature.

2.5. Electrotransfer

The polyacrylamide gels were equilibrated for 15 min (5 min in the case of the IEF gel) in 10 mmol·L^{-1} CAPS, pH 11.0, containing 10% methanol before electroblotting proteins onto a PVDF membrane. Before electrotransfer, the PVDF membrane was first prewetted in pure methanol for 2 s, then transferred to water for 2 min, and finally soaked in the CAPS buffer for 10 min. Proteins were electroblotted at 4 °C for 3 h in the case of IEF, and 5 h in the other cases, using a constant voltage of 100 V (the intensity varied in the range 0.5–0.6 A during electrotransfer). The yield of the method depended on the molecular mass and isoelectric point of the proteins electrotransferred. Coomassie blue staining of the polyacrylamide gel did not reveal any protein remaining in the gel after electrotransfer except lactoferrin separated by nondenaturing electrophoresis, for which the yield was estimated close to 50%.

2.6. Glycoprotein characterization

The PAS method of Kapitany and Zebrowski [13] was adapted in order to detect glycoproteins electroblotted onto a PVDF membrane. After electrotransfer, the PVDF membrane was washed in 100 mL of 12% TCA for 5 min. All the further treatments of the membrane were performed in the dark at 4 °C. The membrane was treated by 100 mL of 1% (w/v) periodic acid for 15 min. Three washings with 100 mL of 15% acetic acid were performed for 5 min each. A volume of 50 mL of Schiff's reagent was then added for 30 min and the membrane was washed six times with 100 mL of 7.5% acetic acid for 1 h or more. Finally, the membrane was dried and stored at room temperature.

2.7. Microsequencing

Glycoproteins revealed by the Schiff's reagent were excised from the PVDF membrane and directly submitted to microsequencing. Amino-terminal microsequencing was carried out with an automated protein 476A microsequencer with online identifi cation of the phenylthiohydantoin derivatives.

3. RESULTS AND DISCUSSION

Electrophoretic separation of bovine milk proteins sometimes needs ND-PAGE for whey proteins and urea-PAGE for caseins. While SDS-PAGE and IEF are universal techniques for the separation of all the milk proteins, ND-PAGE is not suitable for casein separation and urea-PAGE is ineffective for whey protein separation. Thus, characterization of whey glycoproteins or of κ-casein needs a PAS protocol relevant for the different kinds of electrophoresis used.

3.1. Nondenaturing electrophoresis

Bovine milk glycoproteins could be directly detected with the PAS staining after separation in SDS-PAGE gels (data not shown). In the present work, however, glycoproteins separated by ND- or urea-PAGE were electrotransferred onto a PVDF membrane before PAS staining in order to be detected, since the PAS protocol was ineffective in the ND- and urea-PAGE gels for the bovine glycoprotein having a low carbohydrate content. The whey proteins

(β-lactoglobulins A and B, α-lactalbumin, serumalbumin, lactoferrin, and γ-immunoglobulins) were identified on the ND gel profile according to Davis [5] and the proteose peptones according to Girardet et al. [9] after Coomassie blue staining. The minor whey glycoproteins (lactoferrin, γ-globulins, and component PP3) were easily visualized on the PVDF membrane after PAS staining (Fig. 1). It was noted that the two glycoforms of α-lactalbumin were detected with sufficient sensitivity on the whey protein profile by the PAS staining. Their identification was performed by amino-terminal micro-sequencing of the five first amino acid residues (Glu-Gln-Leu-Thr-Lys-) after excision of the two corresponding pink bands from the PVDF membrane. The first band with the slowest migration rate corresponds to the N-acetylneuraminic acid-free glycoform, whereas the second band corresponds to the N-acetylneuraminic acid-containing glycoform [20]. Combination of electrotransfer and PAS staining considerably enhanced the sensitivity of the glycoprotein detection since these two glycoforms of α-lactalbumin are not directly detectable in the polyacrylamide gel by PAS staining or Coomassie blue staining of the whey proteins [20].

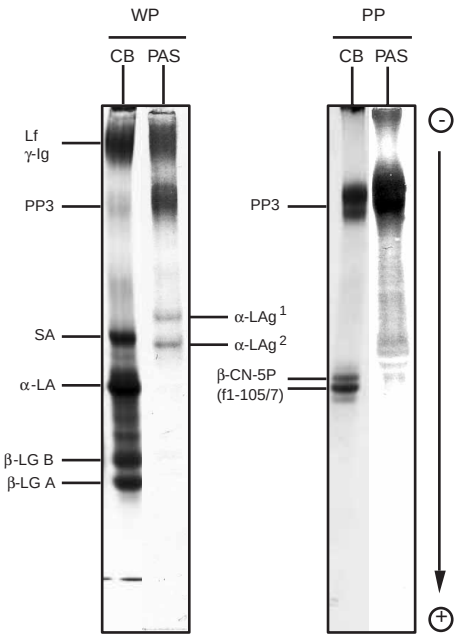

Figure 1. Non-denaturing polyacrylamide gel electrophoresis of whey proteins (WP) and proteose peptones (PP). Protein sample (2 mg·mL^{-1}) aliquots of 25 µL were loaded in each well on the gel. Proteins were directly stained in gel by Coomassie blue (CB) while glycoproteins were specifically revealed by periodic acid/Schiff (PAS) staining after electrotransfer onto a polyvinylidene difluoride membrane. Lf, lactoferrin; γ-Ig, γ-globulins; PP3, component 3 of proteose peptones; SA, serum albumin; α-LA, α-lactalbumin; α-LAg^1, glycosylated α-lactalbumin free of N-acetylneuraminic acid; α-LAg^2, glycosylated α-lactalbumin containing N-acetylneuraminic acid; β-LG A and B, β-lactoglobulin variants A and B; β-CN (f1-105/7), fragments 1-105 and 1-107 of β-casein.

In bovine milk, component PP3 exists as a multimeric aggregate [22]. By hydrolyzing the 53-54 peptide bond of component PP3, plasmin generates peptide PP3 (f54-135) that is N- and O-glycosylated as the whole component PP3 [21]. It was assumed that the multimeric aggregate of component PP3 also included the peptide PP3 (f54-135), since the PAS staining of the ND-PAGE gel revealed a single broad band (Fig. 1). Under denaturing conditions of SDS-PAGE, component PP3 and the peptide PP3 (f54-135) are dissociated from each other and two corresponding bands with equivalent intensities appear after PAS staining [10].

3.2. Urea electrophoresis

κ-Casein represents about 13% of the whole casein [16]. Among the different casein components, only the κ-casein is glycosylated. The proportion of carbohydrates associated with κ-casein was estimated at approximately 3% (w/w) according to the determination of the κ-casein-associated carbohydrate amount by

Guérin et al. [12]. Six putative O-linked glycosylation sites are identified in the 106-169 carboxy-terminal domain corresponding to GMP [15]. The non-glycosylated form of κ-casein is named κ1-casein according to the casein nomenclature. The two A and B genetic variants of κ-casein were identified by anion-exchange fast protein liquid chromatography according to the method of Dalgleish [4]. The B variant-

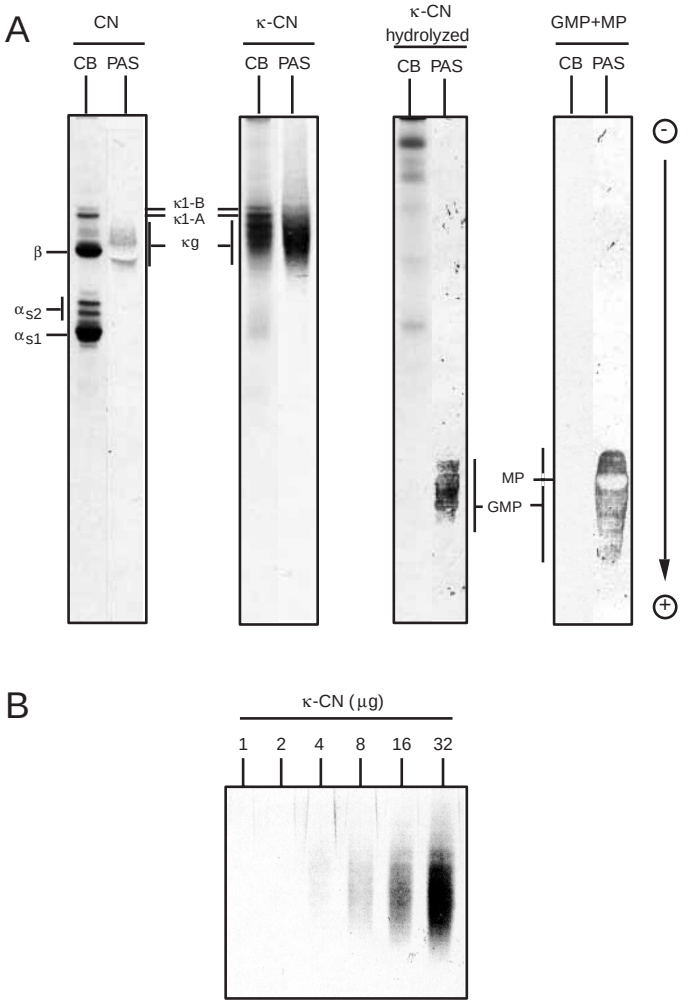

Figure 2. Polyacrylamide gel electrophoresis in the presence of 4.5 mol·L^{-1} urea of sodium caseinate (CN), commercial κ-casein (κ-CN), commercial κ-casein hydrolyzed by rennin (κ-CN hydrolyzed), commercial mixture of glycomacropeptide (GMP) and macropeptide (MP). **A**: Protein sample (2 mg·mL^{-1}) aliquots of 25 μL were loaded in each well on the gel. Proteins were directly stained in gel by Coomassie blue (CB) while glycoproteins were specifically revealed by periodic acid/Schiff (PAS) staining after electrotransfer onto a polivinylidene difluoride membrane. **B**: PAS staining of increasing amounts of commercial κ-casein after electrotransfer. Quantities from 1 to 32 μg protein were loaded in the gel. α_{s1}, α_{s2}, β, κ1-A and -B, α_{s1}-casein, α_{s2}-casein, β-casein, and κ1-casein variants A and B, respectively; κg, glycosylated κ-casein.

to-A variant ratio was higher in the commercial κ-casein (B/A ratio = 0.7) than in the sodium caseinate (B/A ratio = 0.4; chromatographic data not shown). The individual caseins were localized on the urea-PAGE gel profile according to Sanogo et al. [17]. The non-glycosylated κ1-casein A and B were localized according to Trieu-Cuot [24] on the urea-PAGE profile by Coomassie blue staining in gel (Fig. 2A). The κ1-casein variants were well-resolved in two thin bands. The PAS staining could not be directly used with sufficient sensitivity in urea-containing gels (data not shown). Despite the low amount of κ-casein in the whole casein, the glycosylated forms could be detected in the sodium caseinate by PAS staining after electrotransfer without previous purification, whereas the κ1-casein variants were not detected on the PVDF membrane by the Schiff's reagent (Fig. 2A). The sensitivity limit of the PAS staining after urea-PAGE and electrotransfer was found for 4 μg of the commercial κ-casein (Fig. 2B) which was equivalent to approximately 120 ng of carbohydrates. This was close to the sensitivity limit of the detection of ovalbumin electrotransferred after SDS-PAGE (100 ng of ovalbumin-associated carbohydrates; [23]). No detection of the κ-casein could be observed after the PAS staining directly performed in the urea-PAGE gel when 100 μg sodium caseinate were loaded (data not shown).

Action of rennin on the glycosylated κ-casein and on the κ1-casein generates GMP and the non-glycosylated macropeptide (MP), respectively. Their detection in gel by Coomassie blue is very difficult to obtain under the classical staining conditions used. Coolbear et al. [3] use a staining solution containing 5 $g \cdot L^{-1}$ Serva Blue R and a destaining solution only composed of 5% TCA to avoid the disappearance of the dye bound on the GMP or MP molecules. A commercial mixture of GMP and MP (called "glycomacropeptide" by the manufacturer) was separated by urea-PAGE. The classical conditions of Coomassie blue staining were ineffective in detecting the two hydrophilic peptides in gel (Fig. 2A). After electrotransfer and PAS staining, GMP was characterized by a broad pink band and a fast electrophoretic migration rate (Fig. 2A). A negative print of MP was evidenced in the broad pink band since the Schiff's reagent did not stain MP. This method could be a suitable tool for the detection of GMP in order to follow the action of clotting enzymes added to milk (see the urea-PAGE profile of κ-casein hydrolyzed by rennin in Fig. 2A).

3.3. Isoelectric focusing

The IEF gels we used contained 7 $mol \cdot L^{-1}$ urea and should be carefully handled because of their very low thickness (1 mm) and low acrylamide content (5%). While glycoprotein staining was very difficult to realize directly in gel, it was greatly facilitated after electrotransfer onto a PVDF membrane. Caseins were separated by IEF according to their isoelectric point and the different genetic variants were positioned on the IEF profile according to Trieu-Cuot and Gripon [25] in the case of the κ-casein and according to Addeo et al. [1] in the case of the α_{s1}- and β-caseins (lane 1; Fig. 3). In the whole casein sample, the κ-casein glycoforms were slightly revealed by the Schiff's reagent due to the low κ-casein content (about 6 μg). The PAS staining of the commercial κ-casein did not reveal the κ1-casein and was therefore specific of the glycosylated forms (lanes 1–4; Fig. 3). Combination of IEF and PAS staining after electrotransfer could be useful in the characterization of κ-casein of other mammals. Comparison of the IEF profiles of the commercial κ-casein and of the sodium caseinate after PAS staining allowed one to locate the glycoforms of the κ-casein (lanes 2 and 4; Fig. 3). GMP was visualized by PAS staining after electrotransfer as

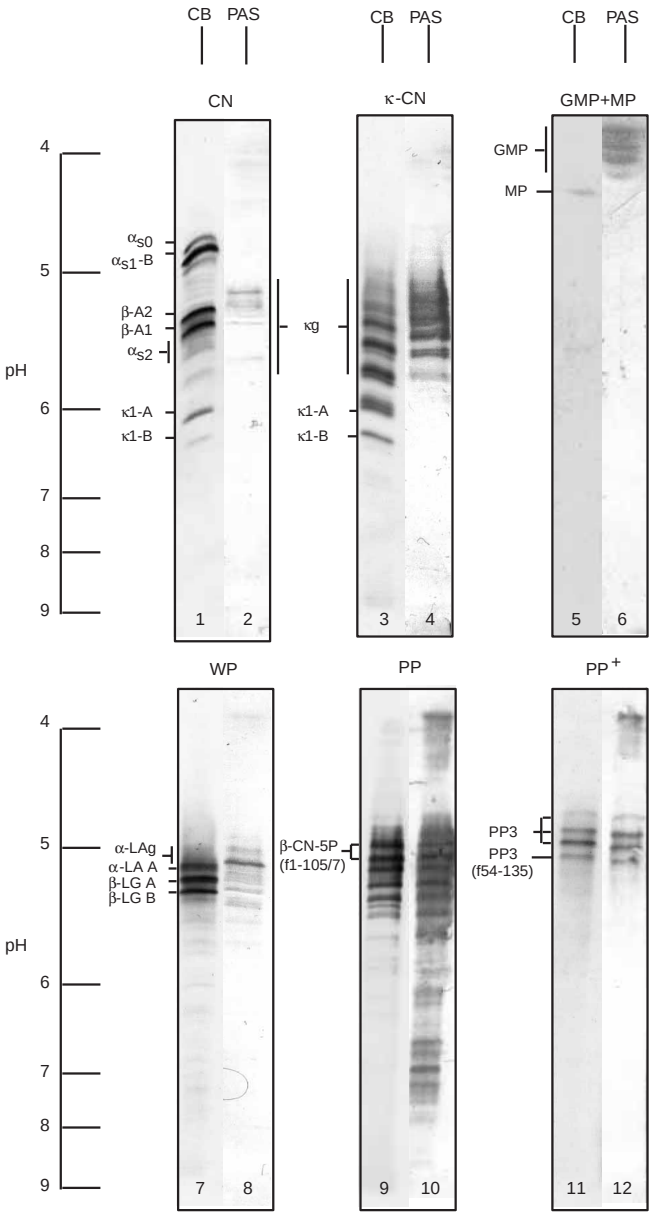

Figure 3. Isoelectric focusing in the presence of 7 mol·L^{-1} urea of sodium caseinate (CN; lanes 1 and 2), commercial κ-casein (κ-CN; lanes 3 and 4), commercial mixture of glycomacropeptide and macropeptide (GMP+MP; lanes 5 and 6), whey proteins (WP; lanes 7 and 8), proteose peptones (PP; lanes 9 and 10), glycoprotein fraction of proteose peptones separated by concanavalin A affinity chromatography (PP^{+}; lanes 11 and 12). Protein sample (2 mg·mL^{-1}) aliquots of 25 µL were loaded in each well on the gel. Proteins were directly stained in gel by Coomassie blue (CB) while glycoproteins were specifically revealed by periodic acid/Schiff (PAS) staining after electrotransfer onto a polivinylidene difluoride membrane. α_{s0}, α_{s1}-B, α_{s2}, β-A1 and -A2, κ1-A and -B, α_{s0}-casein, α_{s1}-casein variant B, α_{s2}-casein, β-casein variants A1 and A2, and κ1-casein variants A and B, respectively; κg, glycosylated forms of κ-casein; α-LA A, α-lactalbumin variant A; α-LAg, glycosylated α-lactalbumin; β-LG A and B, β-lactoglobulin variants A and B, β-CN (f1-105/7), fragments 1-105 and 1-107 of β-casein; PP3, component 3 of proteose peptones.

three or four bands located close to the acidic extremity of the gel, but was not visualized by Coomassie blue, contrary to MP (lanes 5 and 6; Fig. 3).

Whey proteins were separated by IEF (lanes 7 and 8; Fig. 3) and identified according to Zappacosta et al. [28]. The PAS staining revealed several bands among which were the glycosylated forms of α-lactalbumin. However, the distinction of the different glycoforms was not obvious since the glycosylated α-lactalbumin can bear up to 14 different carbohydrate structures [20] leading to a complex separation. The most intense band, whose isoelectric point was slightly lower than that of the non-glycosylated α-lactalbumin, could correspond to the major glycoform identified by Slangen and Visser [20] as a nonsialylated and fucosylated biantennary-type glycan structure having two terminal N-acetylgalactosamine residues. Component PP3 and PP3 (f54-135) were purified from the proteose peptones (PP) by concanavalin A affinity chromatography and were recovered in a fraction called PP^+. The PP and PP^+ fractions were separated by IEF and electrotransferred onto a PVDF membrane prior to PAS staining (lanes 9-12; Fig. 3). Location of the major components in the IEF profile was performed according to previous results [9]. The most abundant components, peptides β-CN-5P (f1-105 and 1-107), were strongly stained by Coomassie blue in gel. These peptides separated by IEF are not revealed by the Schiff's reagent because of their non-glycosylated state [9]. The pink bands located at the same positions as β-CN-5P (f1-105 and 1-107) on the IEF profile corresponded to the isoelectric forms of component PP3. The component PP3 and its carboxy-terminal fragment exhibited three (at least) and one (at least) bands, respectively. Glycosylation, particularly sialylation, and phosphorylation enhance the acidic character of component PP3. Only the N-glycan moiety can be sialylated [11], the O-glycan moiety does not contain any sialic acid residue [2]. Peptide PP3 (f54-135), which does not bear any phosphorylated residue, was less acidic and had a simpler isoelectric profile than component PP3. The numerous bands that were stained by the Schiff's reagent in the PP profile (lane 10; Fig. 3), could not only be explained by the presence of glycosylated component PP3 and PP3 (f54-135). Other bands probably corresponded to proteins lactosylated by milk heating during the proteose peptone preparation. Lactosylated α-lactalbumin and lactosylated β-lactoglobulin have been evidenced in proteose peptones [19]. Bidimensional PAGE combining IEF and SDS-PAGE results in a better separation of β-CN-5P (f1-105/7), component PP3, and its carboxy-terminal fragment, and the glycoproteins are successfully stained by PAS directly in the bidimensional gel [9].

4. CONCLUSION

In conclusion, combination of electrophoretic separation methods with PAS staining after electrotransfer was suitable for the characterization of milk glycoproteins. Due to its remarkable sensitivity, this technique could be applied to glycoproteins having a carbohydrate content lower than mucins and could be extended to other food glycoproteins. Amino-terminal microsequencing of glycoproteins electroblotted could be easily performed after PAS staining and excision of the PVDF fragment containing the glycoprotein of interest. This method presented two other advantages. After electrotransfer and Coomassie blue or PAS staining, the determination of the migration distance of each protein band could be performed with high precision, since the PVDF membrane was not deformed during the successive staining and destaining baths, contrary to the polyacrylamide gel. On the other hand, after PAS staining and glycoprotein location, the PVDF membrane could be stained by Coomassie

blue to reveal all the other proteins. In this manner, only one electrophoretic run and one electrotransfer were needed to discriminate non-glycosylated proteins and glycoproteins.

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