Lait 80 (2000) 517–526

Note

Plasmin digestion of bovine β-casein dephosphorylated with one protein phosphatase type 2A purified from *Yarrowia lipolytica*

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(Received 26 July 1999; accepted 16 May 2000)

Abstract — Bovine β-casein was dephosphorylated by the catalytic sub-unit of a type 2A protein phosphatase (PP2Ac) purified from the yeast *Yarrowia lipolytica*. Complete dephosphorylation was obtained after 24 to 30 h in Tris buffer (pH 7.5). On the contrary, the activity of PP2Ac was largely inhibited (80%) in the presence of 13 mmol·L^{-1} sodium citrate (pH 6.8). After dephosphorylation of β-casein, plasminolysis was increased and more 3% TCA-soluble peptides were obtained (30% increase). Dephosphorylation led to the disappearance of the f 29-105/107 peptide and to the appearance of a new non-phosphorylated 8 200 g·mol^{-1} peptide, suggested to be f 33-105/107 originated from the middle part of the β-casein sequence and obtained upon plasmin cleavage of the Lys_{32}-Phe_{33} peptide bond.

β-casein / dephosphorylation / protein phosphatase / *Yarrowia lipolytica* / plasmin

Résumé — **Hydrolyse par la plasmine de la caséine β bovine déphosphorylée par une protéine phosphatase de type 2A purifiée chez *Yarrowia lipolytica*.** La caséine β peut être déphosphorylée par la sous-unité catalytique d'une protéine phosphatase de type 2A purifiée chez la levure *Yarrowia lipolytica*. La déphosphorylation complète est obtenue en 24 à 30 h en tampon Tris (pH 7.5). À l'inverse, elle est largement inhibée par le citrate de sodium 13 mmol·L^{-1} à pH 6.8 (80 % d'inhibition). Après déphosphorylation, la plasminolyse de la caséine β est augmentée puisque l'on observe une production supplémentaire de 30 % de peptides solubles dans le TCA à 3 %. La déphosphorylation se traduit par la disparition du peptide f 29-105/107 et l'apparition d'un nouveau peptide non phosphorylé de 8 200 g·mol^{-1}. Il pourrait s'agir du peptide f 33-105/107 provenant de la partie centrale de la séquence protéique de la caséine β et obtenu par attaque de la liaison peptidique Lys_{32}-Phe_{33}.

caséine β / déphosphorylation / protéine phosphatase / *Yarrowia lipolytica* / plasmine

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1. INTRODUCTION

The proteolytic digestion of caseins during cheese ripening is of great importance to the development of the rheological properties of the cheese. The breakdown of β-casein by rennet enzymes is known to affect the yield, texture and flavor of the cheese, and has been studied by HPLC [12, 18] and capillary electrophoresis [19, 20]. Rennet breakdown products of β-casein have been detected in a variety of European cheeses, like Edam, Emmental, Gouda (with no salt added), Pirinees, Tilsit, Beaumont, Munster, Fontina and Taleggio [21]. High water activity and low salt content seem to favour β-casein proteolysis by rennet enzymes. Plasmin, a milk proteinase, also acts on β-casein. The importance of this endogenous enzyme on cheese ripening has been under discussion for a long time. Due to specificity differences and to the particular structure of β-casein, the rennet enzymes, aspartic proteinases (APs), and plasmin (a serine proteinase) act on different regions of the molecule. While plasmin cleaves Lys-X and Arg-X bonds and acts primarily on the N-terminal moiety of β-casein, APs have a marked preference for peptide bonds between bulky, hydrophobic aminoacids and act close to the much more hydrophobic C-terminal. Certain ripening processes previously attributed to rennet enzymes alone may also depend on the plasmin present in milk [24]. However, plasmin activity decreases as temperature and the holding time increase [6]. It has also been observed that plasmin activity could be affected by the presence of proteinase inhibitors in the case of ultrafiltration or addition of whey protein concentrate to increase the yield of cheese [5]. The plasmin system (plasmin and its inactive precursor plasminogen) could also be affected by heat-stable proteases of the psychrotrophic bacteria present in raw milk [10].

In fresh milk, β-casein is converted by plasmin into several large, intermediate degradation products, the γ-caseins, and to the complementary parts called proteose peptones [8, 34]. Casein phosphopeptides containing the phosphoseryl cluster sequence accumulate in cheese because the proteolytic enzymes which are active during ripening seem not to recognize the peptidic bonds involving a phosphoserine residue. These bonds were hydrolyzed when prior dephosphorylation occurred [9]. Phosphatases have then an important role in the release of free amino acids in ripened cheeses like Gruyère, Appenzeller, Emmental, Parmigiano Reggiano and Grana Padano [9]. Pellegrino et al. [25] suggested a reactivation, in the outer part of cheeses, of milk alkaline phosphatase inactivated by pasteurization. On the other hand, Lorient and Linden [17] concluded that milk alkaline phosphatase would possess a very low activity in raw milk due to the presence of endogenous inhibitors (phosphate ions, lactose, β-lactoglobulin). Other protein phosphatases had to be considered. Kyriakidis et al. [15] suggested that a concerted action of proteinase and phosphatase activities expressed in the cell-walls of lactic acid bacteria may be required for the optimum release of peptides from casein. In our laboratory, protein phosphatases from the yeast *Yarrowia lipolytica* have been characterized [13, 14] and used to dephosphorylate food proteins, namely phosvitin and casein [27]. *Y. lipolytica* may occur in cheese technology even if this yeast is rarely desired. In this work, a protein phosphatase type 2A purified from *Y. lipolytica* was used to dephosphorylate bovine β-casein before protein digestion with plasmin. It has been reported [2, 3] that plasmin shows a narrow specificity in its action on β-casein in milk, but the effect of dephosphorylation on the susceptibility to cleavage is unknown.

2. MATERIALS AND METHODS

2.1. Culture and extraction

The yeast *Yarrowia lipolytica* (wild strain W-29 obtained from CLIB*) was grown on

an inorganic phosphate-deficient medium [11]. The cells were sampled during the exponential growth phase (13 h), centrifuged at 4 400 *g* for 15 min and washed with distilled water. Fresh cells were ground in a MSK Braun ball-mill (Braun Sciencetec, France; ball diameter 0.44–0.45 mm) cooled under a stream of CO_2 for 1.5 min in 20 mmol·L^{-1} triethanolamine buffer, pH 7.0, with 50 mmol·L^{-1} NaCl, 1 mmol·L^{-1} ethylene glycol bis(β-aminoethylether) tetraacetic acid, 0.1 mmol·L^{-1} dithiothreitol, 5% (v/v) glycerol, 0.01% (v/v) Brij 35 (hereafter designated as buffer A) and 0.4 mmol·L^{-1} phenylmethylsulfonylfluoride (PMSF). Proportions for grinding were 1/1/3 (w/w/v) cells/balls/buffer A. After centrifugation (48 000 *g* for 45 min at 4 °C), the supernatant was considered as the cell-free extract and contained the soluble endocellular enzymes.

2.2. Purification of protein phosphatase 2A from *Y. lipolytica*

After concentration on a 10 000 g·mol^{-1} Diaflo membrane (Millipore-Amicon Bioseparations, Molsheim, France), the cell-free extract was loaded onto a 5 000–70 000 Ultrogel AcA 54 column (2.6 cm × 91 cm; Sepracor/IBF, France) equilibrated with buffer A. The elution rate was 0.66 mL·min^{-1} and the eluate was collected in 3.3 mL fractions. The first peak of phosphatase activity was eluted in the dead volume and corresponded to the multimeric form of PP2A with a 130 000 g·mol^{-1} apparent molecular mass [13]. The second activity peak was the catalytic sub-unit (37 000 g·mol^{-1}), co-eluted with *Y. lipolytica* proteases which are known to have an apparent molecular mass between 28 000 and 40 000 g·mol^{-1} [1, 23, 30, 35]. Ultrogel fractions corresponding to the first activity peak were pooled and further purified as described previously [13, 27] through ion exchange chromatography (Toyopearl DEAE 650S from TosoHaas, Stuttgart, Germany), treatment with 80% ethanol, in order to unmask the catalytic unit of PP2A [31], and, after concentration, size exclusion chromatography on a Superose 12HR FPLC column (Amersham Pharmacia Biotech Europe GmbH, Saclay, France). After the Superose step, PP2Ac fractions were obtained in buffer A containing 200 mmol·L^{-1} NaCl (purification-fold 120). They were free from contaminating proteases (Fig. 1) and could be used for β-casein dephosphorylation prior to plasmin digestion.

Protein phosphatase activity was assayed through dephosphorylation of [^{32}P]casein

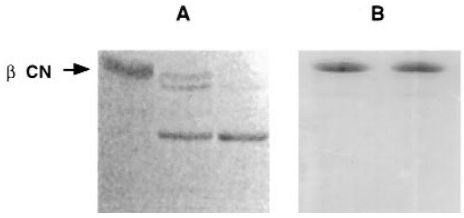

Figure 1. SDS-PAGE of β-casein incubated with PP2Ac for 16 h at 30 °C in the presence of 100 mmol·L^{-1} NaF. (**A**) Superose fractions obtained without the first step of size exclusion chromatography on Ultrogel AcA 54 to remove contaminant proteases. (**B**) Superose fractions obtained after the complete purification protocol. The casein/PP2Ac ratio was 20 (w/w). The sample protein concentration was 0.3 mg·mL^{-1}.

Figure 1. Electrophorèse sur gel de polyacrylamide en milieu dénaturant de la caséine β incubée à 30 °C pendant 16 h en présence de PP2Ac et de NaF 100 mmol·L^{-1}. Les fractions Superose sont obtenues sans la première étape de chromatographie d'exclusion stérique sur Ultrogel AcA 54 destinée à éliminer les protéases contaminantes (**A**) ou après le protocole complet de purification (**B**). Le rapport caséine/enzyme est de 20 (p/p). La concentration en protéine des échantillons est de 0,3 mg·mL^{-1}.

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as described earlier [13, 14, 27]. Labeled casein was obtained through the reaction of chemically dephosphorylated casein (Sigma-Aldrich, Saint-Quentin Fallavier, France) with [γ-^{32}P]ATP (54 kBq·nmol^{-1}; Amersham Pharmacia Biotech, France) in the presence of rabbit cAMP-dependent protein kinase [22]. Labeled casein was freed from [γ-^{32}P]ATP by size exclusion chromatography. Casein dephosphorylation was carried out by incubating aliquots of enzyme fractions at 30 °C with 11 μmol·L^{-1} [^{32}P]casein containing 0.08 μmol·L^{-1} ^{32}P. Proteins were precipitated with cold 20% trichloroacetic acid (TCA). After centrifugation (6 min at 12 000 *g*), radioactivity was counted in the supernatant through liquid scintillation (Tricarb 1500, Packard Instrument S.A., Rungis, France).

2.3. β-casein dephosphorylation by PP2Ac

Casein dephosphorylation was carried out at 30 °C by incubating PP2Ac with solutions of commercial bovine β-casein (Sigma-Aldrich) prepared in 50 mmol·L^{-1} Tris HCl (pH 7.5) or 50 mmol·L^{-1} citrate buffer (pH 6.8). The casein/enzyme ratio (w/w) was 20, the final β-casein concentration was 1 mg·mL^{-1}.

Free phosphate released from β-casein during the reaction was measured by the method of Baykov et al. [4] adapted by Queiroz-Claret and Meunier [26]. Reaction aliquots were directly mixed with color reagent (malachite green), and after 15 min incubation absorbance was measured at 630 nm in a double-beam spectrophotometer (Uvikon 941, Bio-Tek Kontron, Saint-Quentin-en-Yvelines, France). The amount of released phosphate was determined from a calibration curve, using KH_2PO_4 as standard. The dephosphorylation yield was calculated taking into account that β-casein has a molecular mass of 24 000 g·mol^{-1} [28].

Reaction aliquots were also subjected to urea-polyacrylamide gel electrophoresis according to the method described by van Hekken et al. [33] for PhastSystem (Amersham Pharmacia Biotech) using 20% polyacrylamide homogeneous gels. Samples were prepared in denaturing buffer (6.6 mol·L^{-1} urea, 112 mmol·L^{-1} Tris, 112 mmol·L^{-1} sodium acetate, pH 6.4) containing 5% (v/v) 2-mercaptoethanol. Four microliters of sample (0.5 g·L^{-1} protein concentration) were loaded on each lane. Gels were stained with Coomassie Blue R.

2.4. Hydrolysis of β-casein by plasmin and analysis of peptides

β-casein dephosphorylated for 16 h by PP2Ac and β-casein maintained at 30 °C for 16 h without PP2Ac were hydrolyzed with plasmin (Sigma-Aldrich, 5 units·mg^{-1} protein) in 50 mmol·L^{-1} Tris (pH 8.0) for 3 h at 37 °C in the presence of 100 mmol·L^{-1} NaF to stop dephosphorylation. The casein/plasmin ratio was 400 (w/w).

For analysis of peptides by SDS-PAGE, 15 μL aliquots were mixed with Tris buffer (pH 8.0) containing 0.5 mmol·L^{-1} EDTA, 5% (w/v) SDS and 2% (v/v) 2-mercaptoethanol and boiled for 5 min. Gel electrophoresis was performed under denaturing conditions with PhastGel high density gels (Amersham Pharmacia Biotech) designed for the separation of peptides and low molecular weight proteins. For molecular weight determination, the Pharmacia peptide marker kit composed of horse myoglobin peptides (M*r* range 2 512–16 949) was used. Gels were stained with Coomassie Blue R. In order to prevent peptides from being washed out of the gel during staining, a fixation step with 2.5% (v/v) glutaraldehyde in water was added, as preconized by Pharmacia.

At selected times of casein digestion, TCA was added to 50 μL aliquots to a final concentration of 3% (w/v). After 30 min at room temperature, the precipitate was removed by centrifugation at 12 000 *g* for 10 min. The supernatant was filtered through

a 0.22 μm pore size filter and the filtered samples were subjected to reverse phase HPLC.

Analytical rp-HPLC was performed on a Beckman equipment consisting of a System Gold programmable solvent module 126, a Rheodyne sample injector (100 μL loop) and a 166 UV-VIS detector. Peptide separation was achieved on a Ultrasphere ODS 5 μm C_{18} analytical column (4.6 mm × 25 cm, Beckman Coulter, Roissy, France), and the amide bond was detected at 214 nm. The elution was carried out with linear gradients of acetonitrile in 0.1% trifluoroacetic acid (TFA) in water. After a 2 min isocratic run, a gradient from 0 to 30% acetonitrile was developed in 8 min followed by a 30 to 77% acetonitrile gradient over 25 min [18]. The elution rate was 1.0 $mL \cdot min^{-1}$ and the column temperature was 21 °C.

2.5. Other procedures

Protein was measured by the Bradford method [7] using bovine serum albumin as standard.

Contaminating protease activity in PP2A Superose fractions was detected by SDS-PAGE of β-casein incubated with PP2A at 30 °C, in the presence of 100 $mmol \cdot L^{-1}$ NaF in order to avoid concomitant dephosphorylation.

The peptides isolated by HPLC were characterized using matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF HP G2025). Samples were mixed (1/1, v/v) with α-cyano-4-hydroxycinnamic acid on the target and dried in a HP G2024 sample drying chamber. The energy of the nitrogen laser (337 nm) was between 1.7 to 5.5 μJ. Ions were submitted to a 28 kV accelerating voltage towards the detector (–4.75 kV). Analysis was carried out in positive polarity, without a filter, after a calibration with standard peptides in the molecular mass range 1 008–6 968.7 (oxytocin, vasopressin, angiotensin I, somatostatin, insulin, hirudin).

3. RESULTS AND DISCUSSION

3.1. β-casein dephosphorylation by PP2Ac from *Y. lipolytica*

The time-course dephosphorylation of β-casein by PP2Ac purified from *Y. lipolytica* was studied through the determination of released free phosphate. In Tris buffer (pH 7.5), complete dephosphorylation was obtained after 24 to 30 h, without phosphate dialysis. Complete dephosphorylation of isolated β-casein was also obtained with alkaline phosphatase from calf intestine and with acid phosphatase from potato [16], which shows that all the phosphate groups are accessible. However, Lorient and Linden [17] found that the dephosphorylation of whole casein with milk alkaline phosphatase was limited to 20% and that dialysis was required to avoid inhibition by phosphate and let dephosphorylation proceed. It seems that all phosphate groups were cleaved by PP2Ac in our experiments.

In the presence of 13 $mmol \cdot L^{-1}$ sodium citrate (pH 6.8), PP2Ac activity was largely inhibited and a 19% dephosphorylation yield was obtained after 24 h. This low yield cannot be explained by a pH effect alone, since the optimum pH for PP2Ac activity with casein as substrate is 7.2 [13]. The inhibitory effect of citrate on PP2Ac activity from *Y. lipolytica* has not been previously described. The complexation of divalent cations by citrate could account for this effect, but only to a minor extent, as PP2A is known to be only slightly dependent on divalent cations.

Electrophoretic patterns of native and dephosphorylated β-casein were compared using urea-PAGE which is known to separate proteins according to their net charge to mass ratio (Fig. 2). Under these conditions, bands with lower mobility than that of native β-casein appeared upon dephosphorylation due to the removal of negatively charged phosphate groups. Casein bands with a varying number of phosphate groups were visible as reported elsewhere [32, 37].

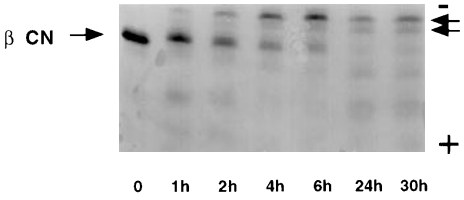

Figure 2. Urea-polyacrylamide gel electrophoresis of β-casein (45 μmol·L^{-1}) and β-casein dephosphorylated with PP2Ac (1.6 μmol·L^{-1}) from *Yarrowia lipolytica* for 1 to 30 h. Samples denaturated in Tris/acetate buffer containing urea (3.3 mol·L^{-1}) and 2-mercaptoethanol (5%, v/v) were deposited on 20% polyacrylamide homogeneous gels (see Materials and Methods).

Figure 2. Electrophorèse sur gel de polyacrylamide en milieu urée de caséine β (45 μmol·L^{-1}) et de caséine β déphosphorylée par la PP2Ac (1.6 μmol·L^{-1}) de *Yarrowia lipolytica* pendant 1 à 30 h. Les échantillons sont dénaturés dans du tampon Tris/acétate contenant de l'urée 3,3 mol·L^{-1} et 5 % (v/v) de 2-mercaptoéthanol et déposés sur des gels homogènes à 20 % de polyacrylamide (voir Matériel et Méthodes).

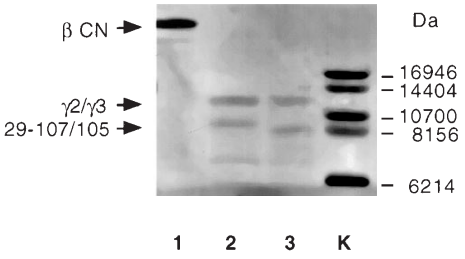

Figure 3. SDS-PAGE of peptides obtained after 3 h plasmin digestion of β-casein (lane 2) and of β-casein dephosphorylated for 16 h with *Y. lipolytica* PP2Ac (lane 3). The casein/PP2Ac ratio was 20 (w/w), the casein/plasmin ratio was 400 (w/w). Lane 1: β-casein. K: peptide marker kit.

Figure 3. Electrophorèse sur gel de polyacrylamide en milieu dénaturant des peptides obtenus après 3 h de plasminolyse de caséine β (puits 2) et de caséine β déphosphorylée pendant 16 h par la PP2Ac de *Y. lipolytica* (puits 3). Le rapport caséine/PP2Ac est de 20 (p/p), celui de caséine/plasmine de 400 (p/p). Puits 1 : caséine β. K : marqueurs de peptides.

3.2. Plasmin hydrolysis of β-casein

3.2.1. SDS-PAGE of peptides obtained by plasmin hydrolysis

Plasminolysis was carried out on native β-casein and on β-casein incubated for 16 h with PP2Ac and, hence, highly dephosphorylated (≃ 90%). SDS-PAGE of peptides was carried out on high density gels, designed for the separation of small proteins, particularly in the M*r* range 1 000–20 000.

The susceptibility of β-casein to plasmin has been established by Andrews [2, 3], Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108} being the main cleavage sites. In the case of native β-casein (results not shown), we identified the major peptides as γ_1-casein (f 29-209, 21 000 g·mol^{-1}), γ_2- and γ_3-casein (f 106-209 and f 108-209, 12 000 g·mol^{-1}) and their complementary fragments (f 1-105/107, 15 000 g·mol^{-1}). Subsequent digestion of γ_1-casein led to the disappearance of γ_1-casein and to the appearance of f 29-105/107 around 8 700 g·mol^{-1}. Only the proteose-peptone component 8F (f 1-28) [3] was not identified, possibly because, owing to its small size, it is more difficult to fix and is easily washed out of the gel during staining. In the same way, the smaller peptide of the Pharmacia kit (2 512 g·mol^{-1}) is often not visible. After 3 h digestion, only γ_2/γ_3-casein and f 29-105/107 were recognized, together with an unidentified 6 900 g·mol^{-1} band (Fig. 3, lane 2). Three hours plasmin digestion of dephosphorylated β-casein led to a slight modification in the electrophoretic pattern. Instead of the 8 700 g·mol^{-1} band (lane 2), a 8 200 g·mol^{-1} band appeared (lane 3). The 500 g·mol^{-1} difference cannot be ascribed to the loss of one phosphoryl group in f 29-105/107 alone and suggests the cleavage of either five aminoacids or four aminoacids plus one phosphoryl group.

3.2.2. rp-HPLC of the 3% TCA-soluble peptides

The chromatogram of the 3% TCA-soluble peptides obtained upon plasmin digestion of β-casein is presented in Figure 4B. β-casein is the most hydrophobic casein due to the C-terminal third of the molecule (residues 136–209) and to a highly hydrophobic β-sheet spiral structure of residues 189 to 207. On the chromatogram, hydrophilic peptides appeared before peak b, eluted at 15 min, which was present in all reference chromatograms. They come from the N-terminal region of β-casein that is hydrophilic owing to its high content of phosphoseryl and other acidic residues: f 1-28, f 1-105/107 and, in a minor degree, f 29-105/107 (peaks 1, 2 and 3). γ_1-casein and hydrophobic peptides coming from the C-terminal moiety of the molecule (γ_2- and γ_3-caseins) eluted between 16 and 20 min (peaks 4, 5 and 6). The kinetics of appearance of the major peptides was followed for 3 h of β-casein plasminolysis. Peptide formation increases with time but the rate of production slows after 1 h. Figure 4C shows the chromatogram of the 3% TCA-soluble peptides obtained after 3 h plasmin digestion of β-casein dephosphorylated by *Y. lipolytica* PP2Ac. The determination of peak areas indicated that more 3% TCA-soluble peptides were obtained from dephosphorylated β-casein (30% increase). Plasminolysis accelerates with dephosphorylation as reported for chymosin and pepsin digestion [29, 36]. The removal of phosphate groups from a peptide should increase its hydrophobicity and hence its retention time in reverse phase chromatography. The disappearance of the important peak observed at 14.34 min in the plasmin digest of native β-casein (peak 3, Fig. 4B), and the appearance of a new more hydrophobic peak at 18.14 min in the digest of dephosphorylated β-casein (peak 7, Fig. 4C) were observed. The elution of hydrophobic γ-caseins was not affected by dephosphorylation (peaks 4, 5, 6).

The molecular masses of peaks 3 and 7 differ by 580 $g \cdot mol^{-1}$, as indicated by MALDI-TOF mass spectrometry analysis. This difference could be explained by the loss of one phosphoryl group and the cleavage of the tetra-peptide Lys-Ile-Glu-Lys.

4. CONCLUSION

In the whole, our results show that a higher amount of 3% TCA-soluble peptides are formed upon plasminolysis of β-casein dephosphorylated by *Y. lipolytica* PP2Ac (30% increase). The dephosphorylation leads to the disappearance of the f 29-105/107 peptide and to the appearance of a non-phosphorylated peptide, probably f 33-105/107, obtained by cleavage of the Lys_{32}-Phe_{33} bond. It has been reported [34] that plasmin specificity could be broader. The dephosphorylation of $PSer_{35}$ could increase the susceptibility of the Lys_{32}-Phe_{33} bond to cleavage. As β-casein used in this work was 90% dephosphorylated, other phosphopeptides must be dephosphorylated as well: f 1-105/107 and f 1-29. No behaviour modification was visible, because, on the one hand f 1-105/107 rapidly disappeared with time for the benefit of f 29-105/107, and on the other hand f 1-29 was not detected on SDS-PAGE and rp-HPLC in our experimental conditions.

β-casein is largely dephosphorylated by PP2Ac purified from *Yarrowia lipolytica*. After this dephosphorylation, plasmin digestion is accelerated and induces the attack of a new peptide bond, Lys_{32}-Phe_{33}. It is suggested that phosphatases from microorganisms could be involved in cheese ripening and could modify digestion processes. As phosphatase activity could be inhibited by milk endogenous components (phosphate, citrate), this enzymatic activity could take place after curd formation and syneresis and then throughout the ripening process.

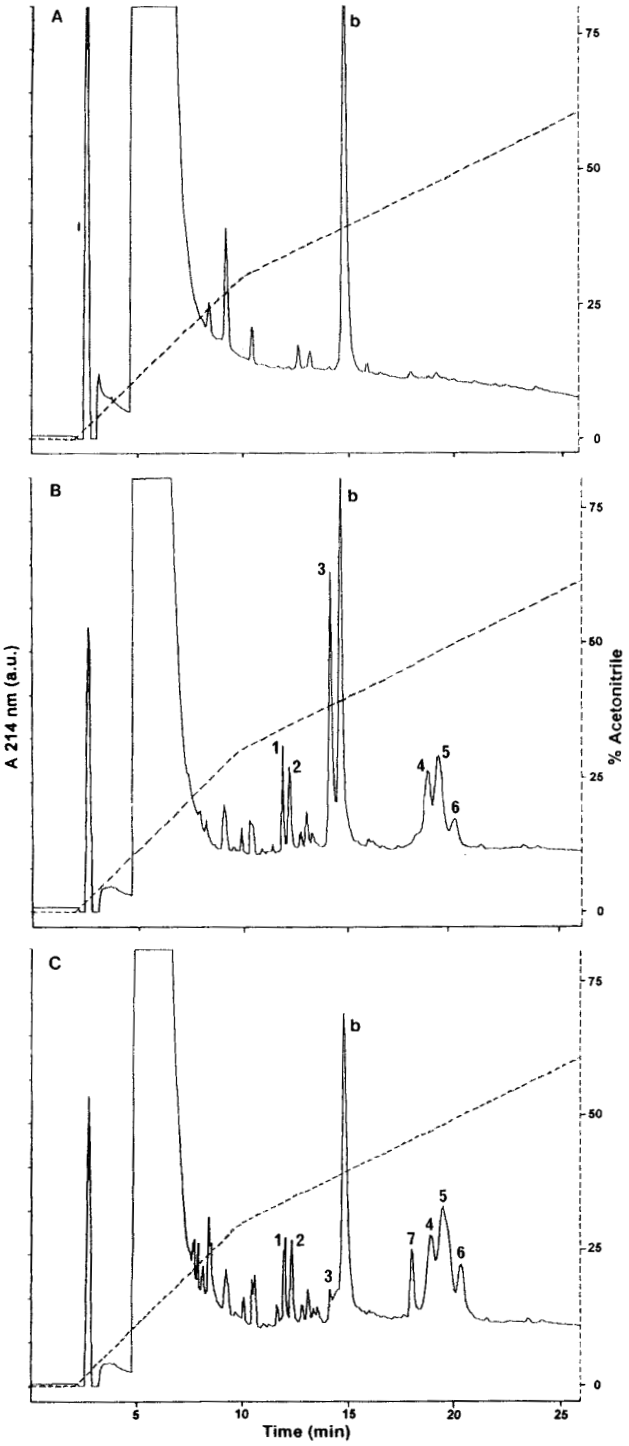

Figure 4. Reverse phase HPLC of the 3% TCA-soluble peptides obtained from β-casein prior to plasmin addition (**A**), from 3 h plasmin digestion of β-casein (**B**), from 3 h plasmin digestion of β-casein dephosphorylated for 16 h with PP2Ac (**C**). The casein/PP2Ac ratio was 20 (w/w). The casein/plasmin ratio was 400 (w/w). rp-HPLC conditions were as described in Materials and Methods.

Figure 4. Analyse chromatographique en CLHP en phase inverse des peptides solubles à 3 % de TCA obtenus à partir de β-caséine sans addition de plasmine (**A**), après 3 h de plasminolyse de caséine β (**B**), après 3 h de plasminolyse de caséine β déphosphorylée pendant 16 h par la PP2Ac (**C**). Le rapport caséine/PP2Ac est de 20 (p/p). Le rapport caséine/plasmine est de 400 (p/p). Les conditions chromatographiques sont décrites dans Matériel et Méthodes.

ACKNOWLEDGEMENTS

The authors wish to thank C. Beauvallet and J.-C. Huet (Unité de biochimie et de structure des protéines, INRA, Jouy-en-Josas, France) for MALDI-TOF analysis.

REFERENCES

[1] Abdelal A.T.H., Kennedy E.H., Ahearn D.G., Purification and characterization of a neutral protease from *Saccharomycopsis lipolytica*, J. Bacteriol. 130 (1977) 1125–1129.

[2] Andrews A.T., The composition, structure and origin of proteose-peptone component 5 of bovine milk, Eur. J. Biochem. 90 (1978) 59–65.

[3] Andrews A.T., The composition, structure and origin of proteose-peptone component 8F of bovine milk, Eur. J. Biochem. 90 (1978) 67–71.

[4] Baykov A.A., Evtushenko O.A., Avaeva S.M., A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase based enzyme immunoassays, Anal. Biochem. 171 (1988) 266–270.

[5] Benfeldt C., Sørensen J., Petersen T.E., Inhibitory activity against plasmin, trypsin, and elastase in rennet whey and in cheese fortified with whey protein, J. Dairy Sci. 81 (1998) 615–620.

[6] Benfeldt C., Sørensen J., Ellegård K.H., Petersen T.E., Heat treatment of cheese milk: effect on plasmin activity and proteolysis during cheese ripening, Int. Dairy J. 7 (1997) 723–731.

[7] Bradford M.M., A rapid and sensitive method for quantitation of microgram quantities of proteins utilizing the principle of protein dye binding, Anal. Biochem. 72 (1976) 248–254.

[8] Caessens P.W.J.R., Gruppen H., Visser S., van Aken G.A., Voragen A.G.J., Plasmin hydrolysis of β-casein: foaming and emulsifying properties of the fractionated hydrolysate, J. Agric. Food Chem. 45 (1997) 2935–2941.

[9] de Noni I., Pellegrino L., Resmini P., Ferranti P., About presence of free phosphoserine in ripened cheese and in enzymatic hydrolysate of casein, Nahrung 41 (1997) 268–273.

[10] Fajardo-Lira C.E., Nielsen S.S., Effect of psychrotrophic microorganisms on the plasmin system in milk, J. Dairy Sci. 81 (1998) 901–908.

[11] Galabova D., Tuleva B., Balasheva M., Phosphatase activity during growth of *Yarrowia lipolytica*, FEMS Microbiol. Lett. 109 (1993) 45–48.

[12] Guillou H., Miranda G., Pelissier J.-P., Hydrolysis of β-casein by gastric proteases, Int. J. Peptide Protein Res. 37 (1991) 494–501.

[13] Jolivet P., Queiroz-Claret C., Bergeron E., Meunier J.-C., Purification and characterization of a type 2A protein phosphatase from *Yarrowia lipolytica* grown on a phosphate deficient medium, C. R. Acad. Sci. Paris 320 (1997) 441–449.

[14] Jolivet P., Queiroz-Claret C., Bergeron E., Meunier J.-C., Characterization of an exocellular protein phosphatase with dual substrate specificiy from the yeast *Yarrowia lipolytica*, Int. J. Biochem. Cell Biol. 30 (1998) 783–796.

[15] Kyriakidis S.M., Sakellaris G., Sotiroudis T.G., Is protein phosphorylation a control mechanism for the degradation of caseins by lactic acid bacteria? The detection of an extracellular acid phosphatase activity, Lett. Appl. Microbiol. 16 (1993) 295–298.

[16] Lorenzen P.C., Reimerdes E.H., Enzymatic dephosphorylation of caseins and creaming behaviour of o/w emulsions stabilized with dephosphorylated casein fractions, Nahrung 36 (1992) 595–599.

[17] Lorient D., Linden G., Dephosphorylation of bovine casein by milk alkaline phosphatase, J. Dairy Res. 43 (1976) 19–26.

[18] Macedo I.Q., Faro C.J., Pires E.M., Caseinolytic specificity of cardosin, an aspartic protease from the cardoon *Cynara cardunculus* L.: action on bovine α_s- and β-casein and comparison with chymosin, J. Agric. Food Chem. 44 (1996) 42–47.

[19] Macedo I.Q., Jolivet P., Meunier J.-C., Capillary electrophoresis of caseino-peptides: influence of applied voltage and column temperature, Biotechnol. Tech. 13 (1999) 647–651.

[20] Macedo I.Q., Jolivet P., Meunier J.-C., Peptide mapping of β-casein by capillary zone electrophoresis, Biotechnol. Tech. 13 (1999) 657–659.

[21] Marcos A., Esteban M.A., Leon F., Salguero J., Electrophoretic pattern of european cheeses: comparison and quantitation, J. Dairy Sci. 62 (1979) 892–900.

[22] McGowan C.H., Cohen P., Protein phosphatase 2C from rabbit skeletal muscle and liver: an Mg^{2+}-dependent enzyme, Methods Enzymol. 159 (1988) 416–426.

[23] Ogrydziak D.M., Scharf S.J., Alkaline extracellular protease produced by *Saccharomycopsis lipolytica* CX 161-1B, J. Gen. Microbiol. 128 (1982) 1225–1234.

[24] Ollikainen P., Nyberg K., A study of plasmin activity during ripening of Swiss-type cheese, Milchwissenschaft 43 (1988) 497–499.

[25] Pellegrino L., Battelli G., Resmini P., Ferranti P., Barone F., Addeo F., Effects of heat load gradient occurring in moulding on characterization and ripening of Grana Padano, Lait 77 (1997) 217–228.

[26] Queiroz-Claret C., Meunier J.C., Staining technique for phosphatases in polyacrylamide gels, Anal. Biochem. 209 (1993) 228–231.

[27] Queiroz-Claret C., Jolivet P., Delérable C., Bergeron E., Meunier J.-C., Gel-immobilized protein phosphatase 2A from *Yarrowia lipolytica* dephosphorylates phosvitin and modifies its functional properties, J. Agric. Food Chem. 45 (1997) 2899–2906.

[28] Ribadeau-Dumas B., Brignon G., Grosclaude F., Mercier J.C., Structure primaire de la caséine β bovine. Séquence complète, Eur. J. Biochem. 25 (1972) 505–514.

[29] Sundararajan T.A., Sarma P.S., Action of proteolytic enzymes on dephosphorized casein, Enzymology 20 (1958) 57–61.

[30] Tobe S., Takami T., Ikeda S., Mitsugi K., Production and some enzymatic properties of alkaline proteinase of *Candida lipolytica*, Agric. Biol. Chem. 40 (1976) 1087–1092.

[31] Usui H., Kinohara N., Yoshikawa K., Imazu M., Amaoka T., Takeda M., Phosphoprotein phosphatases in human erythrocyte cytosol, J. Biol. Chem. 258 (1983) 10455–10463.

[32] van Hekken D.L., Strange E.D., Rheological properties and microstructure of dephosphorylated whole casein rennet gels, J. Dairy Sci. 77 (1994) 907–916.

[33] van Hekken D.L., Thompson M.P., Application of PhastSystem to the resolution of bovine milk proteins on urea-polyacrylamide gel electrophoresis, J. Dairy Sci. 75 (1992) 1204–1210.

[34] Visser S., Noorman H.J., Slangen C.J., Rollema H.S., Action of plasmin on bovine β-casein in a membrane reactor, J. Dairy Res. 56 (1989) 323–333.

[35] Yamada T., Ogrydziak D.M., Extracellular acid proteases produced by *Saccharomycopsis lipolytica*, J. Bacteriol. 154 (1983) 23–31.

[36] Yamauchi K., Yoneda Y., Effect of dephosphorylation of casein on its coagulation and proteolysis by chymosin, Agric. Biol. Chem. 42 (1978) 1031–1035.

[37] Yoshikawa M., Tamaki M., Sugimoto E., Chiba H., Effect of dephosphorylation on the self-association and the precipitation of β-casein, Agric. Biol. Chem. 38 (1974) 2051–2052.