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Immunochemical Detection of Formylated γ_2-Casein in Cheese

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An immunochemical approach has been developed to detect the use of formaldehyde as a bacteriostatic agent in dairy products. A synthetic peptide, reproducing the first five amino acid residues of the γ_2-casein sequence, was formylated to generate the novel haptenic structure, already well-recognized in formaldehyde-treated milk and arising out of molecular rearrangement after the addition of formaldehyde to the alpha-amino group of the histidine residue at the N terminus of γ_2-casein. A polyclonal antibodies preparation produced against the formylated peptide adduct proved to be a highly specific analytical tool for detecting the formylated adduct of γ_2-casein in formaldehyde-treated milk. Polyclonal antibodies obtained against the unmodified peptide were able to detect selectively residual native γ_2-casein in ripened cheese.

KEYWORDS: Formaldehyde; food additives; antipeptide antibodies; γ_2-casein; cheese ripening

INTRODUCTION

Formaldehyde is a highly reactive chemical that readily binds to proteins (*1*) and nucleic acids (*2*). As a result, the administration of formaldehyde to experimental animals by inhalation was found responsible for severe functional alterations, including the development of cancer (*3*). The well-documented relationship between environmental exposure to formaldehyde and cancerogenicity in animals led to the notion that formaldehyde is a probable carcinogenic hazard to humans (*4*).

Consequently, questions have been since raised on the use of formaldehyde as a food additive in the dairy industry and its potential toxicity and likely cancerogenicity by ingestion. Formaldehyde and its precursor, hexamethylenetetramine, (additives known as E 240 and E 239, respectively) added to milk in cheese-making competently prevent blowing and cracking in hard cheese during ripening. What is known as "late blowing" arises from the germination of Clostridium spores contained in milk, causing the development of holes and eyes inside the cheese (*5*). It is important to specify that formaldehyde is a normal metabolite in mammalian systems and that it is naturally present in various foodstuffs (fruits and vegetables, in the order of parts per million) (*6*). On the basis of this evidence and on results from studies on short-term toxicity and long-term carcinogenicity following formaldehyde ingestion in amounts required for it to function as food additive, no dietary risk factor could be ascribed to the consumption of formaldehyde-treated foods (*7*).

However, according to registration of Grana Padano cheese as a Protected Designation of Origin (PDO) product (*8*), treating milk with formaldehyde is not part of standard production regulation, although previously accepted in past years (*9*). Apart from health risks connected with formaldehyde ingestion, the addition of formaldehyde to milk is also an indication of inferior microbiological quality of the raw milk used and is therefore below the required minimum for a PDO cheese.

To ascertain full compliance of a Grana Padano cheese lot with PDO registration, analytical methods able to detect the improper use of formaldehyde-treated milk were developed. In cheese produced from milk treated with 25−30 ppm formaldehyde, the required amount for preventing "late blowing", it was observed that over 80% added formaldehyde was mainly associated with casein and linked to γ2-casein particularly, whereas no free formaldehyde was detectable after a few months of ripening (*10*). The amount of formaldehyde-modified γ2-casein, as determined by isoelectric focusing, was used to quantify formaldehyde added to milk in Grana Padano cheese production (*11*). Spinacine, generated by formaldehyde in action with the N-terminal histidine residue of γ2-casein, was identified as the principal reaction product (*12*). In consequence, an HPLC-based method for spinacine determination was proposed to evaluate bound formaldehyde in cheese and other dairy products (*13*).

The aim of this work was to prepare new immunochemical reagents for detecting formylated adducts of γ2-casein in cheese from formaldehyde-treated milk. The peptide HKEMPGGC, containing the N terminal region of γ2-casein, and its formylated adduct were employed as immunogens to obtain polyclonal antibodies at predetermined specificity toward the γ-casein site

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mainly involved in the formylation reaction and the same site as it appears after formaldehyde addition, respectively.

MATERIALS AND METHODS

Synthetic Peptides and Production of the Antisera. The peptide HKEMPGGC was chemically synthesized and HPLC purified by Primm (Milano, Italy). A peptide sample (20 mg) was dissolved in 2 mL of 20 mM sodium phosphate buffer pH 7.5 containing 2 mM formaldehyde and incubated at 37 °C. After an 8 h incubation, the reaction mixture was dried down in a Speed-Vac centrifuge (Savant, Farmingdale, NY). The solid residue was dissolved in 2 mL of 0.1% trifluoroacetic acid, loaded on a pre-packed Sep-Pak C_{18} cartridge (Waters, Milford, MA), and desalted by washing with 10 mL of 0.1% trifluoroacetic acid. The peptide fraction was then eluted with 5 mL of 70% acetonitrile. The chemical structure of both the synthetic peptide and its formylated adduct was verified by MALDI-TOF mass spectrometry. In addition to the signal at m/z 858.791, found in the unmodified peptide spectrum and consistent with the expected peptide mass, the formaldehyde-treated peptide gave a signal at m/z 870.809, corresponding to a 12 mass increase with respect to the synthetic peptide. Native and formaldehyde-treated peptide were linked to ovoalbumin through the sulfhydryl group of their C-terminal cysteine residue, according to the coupling procedure described by Mattson et al. (*14*). Each ovoalbumin conjugate was used to immunize two rabbits: the two antisera raised against the unmodified peptide were called C22 and D22, while those raised against the formylated peptide mixture were indicated as Y25 and Z25. Finally, the antisera were filtered on 0.45-μm (Millipore, Bedford, MA), split up in aliquots of 1 mL, and stored at −20 °C.

Preparation of Bovine γ-Casein Fraction. Bovine β-casein was obtained by fractionating whole casein from skimmed raw bovine milk on a Q-Sepharose Fast Flow anion exchanger (Pharmacia, Uppsala, Sweden), as already reported (*15*). A 100-mg aliquot of electrophoretic pure β-casein was dissolved in 2 mL of 50 mM NH_4HCO_3, pH 8.5, and 10 μL of 5 U/mL plasmin suspension (EC. 3.4.21.7, Boehringer Mannheim GmbH, Mannheim, Germany) was added. After a16 h incubation at 37 °C, the reaction mixture was heated at 100 °C for 5 min to inactivate plasmin, and then the pH was slowly brought to 4.6 with HCl under pH meter control. The precipitate was recovered by centrifugation at 4500 rpm for 10 min, dissolved in 4 mL of the running buffer (50 mM sodium formate pH 4.0, 4.5 M urea), and applied on a column (16 × 200 mm) containing 40 mL of S−Sepharose Fast Flow cation exchanger (Pharmacia, Uppsala, Sweden) equilibrated at room temperature at a flow rate of 1 mL/min in the running buffer. After column washing with 50 mL of the running buffer, elution was carried out by a 200-mL linear NaCl gradient from 0 to 0.30 M for 3.5 h. Fractions of 2 mL were collected with a FRAC-100 collector (Pharmacia, Uppsala, Sweden), and absorbance of the eluate was determined at 280 nm using an Uvicord S II detector linked to a two channel REC-482 recorder (Pharmacia, Uppsala, Sweden). A 10-μL aliquot of each absorbing chromatographic fraction was analyzed by isoelectric focusing onto a PhastSystem apparatus (Pharmacia, Uppsala, Sweden), according to the procedure reported by Moio et al. (*16*). Fractions containing γ-casein, as resulting from the electrophoresis analysis, were pooled, extensively dialyzed at 4 °C against 50 mM NH_4HCO_3, pH 8.5, and freeze-dried.

Formylation of Bovine γ-Casein Fraction. Formylation reaction was performed incubating γ-casein samples (2 mg/mL) with 2 mM formaldehyde in 20 mM sodium phosphate buffer, pH 7.5 at 37 °C for 8 h. The reaction mixture was desalted by means of a pre-packed Sep-Pak C18 cartridge, equilibrated in 0.1% trifluoroacetic acid. Protein samples were eluted in 0.1% trifluoroacetic acid in 70% acetonitrile and dried down in an evaporating centrifuge Speed-Vac. An aliquot of the protein sample was analyzed by electrospray mass spectrometry (ES/MS). Another aliquot was incubated with Asp-N endoprotease, (EC 3.4.24.33), using an enzyme-to-substrate ratio of 1:100 w/w, in 100 mM NH_4HCO_3, 10% acetonitrile, pH 8.0 at 37 °C for overnight and analyzed by MALDI-TOF mass spectrometry.

ES/MS Analysis. ES/MS spectra were recorded on a Platform single-quadrupole instrument, equipped with an electrospray ion source (Micromass, Manchester, U.K.). Samples were injected directly into ion source (kept at 80 °C) via a loop injection at a flow rate of 10 μL/min. The spectra were scanned from 500 to 1800 m/z at 10 s/scan, acquired and elaborated using Mass-Linx software (Micromass). Mass scale calibration was performed by a separate injection of myoglobin (16951.5 Da). Mass values are reported as average masses.

MALDI-TOF Analysis. Peptide mixtures (100 fmol) mixed with α-cyano-4-hydroxycynnammic acid (10 mg/mL in 50% acetonitrile) were deposited onto MALDI sample probe and dried under ambient conditions. All mass spectra were generated on a MALDI-TOF mass spectrometer Voyager DE PRO (Applied Biosystems), operating in positive reflectron mode. Mass spectra were externally calibrated using the monoisotopic peaks at m/z 5730.60 and 2865.80 Da/z generated by the singly and doubly charged ions of insulin. All mass values are reported as monoisotopic masses.

Grana Padano Cheese Samples Preparation. Grana Padano cheese at different ages was supplied by the Consorzio per la Tutela del Formaggio Grana Padano (Desenzano del Garda, Brescia, Italy) within a previous research program. The other Grana Padano cheese samples were obtained from commercial sources and stored at −20 °C for several years until analysis. All cheese samples were grated, freeze-dried, and defatted with diethyl ether in a Soxhlet apparatus. For electrophoresis and immunoblotting analysis, a 20-mg aliquot from the dry residue was dissolved in 0.2 mL of 9 M urea, 143 mM 2-mercaptoethanol (1% v/v) and 5 μL of the resulting solution was loaded on the gel.

Cheese Samples Formylation. A 100-mg aliquot of the cheese sample, defatted as above indicated, was dissolved in 1 mL of sodium citrate 0.1 M pH 8 containing formaldehyde. Four different concentrations of formaldehyde were used: 0.26, 2.6, 26, and 260 ppm. After an overnight incubation at 37 °C, 1 mL of 9 M urea, 143 mM 2-mercaptoethanol was added to the reaction mixture, and 10 μL of the resulting solution was loaded on the gel. A blank reaction was carried out by incubating the cheese sample in similar conditions but in absence of formaldehyde.

Electrophoresis and Immunoblotting. Isoelectric focusing was currently carried out on polyacrylamide gel (thickness, 0.75 mm) (*17*), otherwise on ultrathin-layer polyacrylamide gel (thickness, 0.25 mm), prepared as described in the Official Journal of the European Communities. No. 1081/96 (*18*), as specified (i.e., the experiment reported in **Figure 4**). The pH gradient in the range 2.5−6.5 was obtained by mixing Ampholine (Pharmacia) 2.5−5.0, 4.5−5.4, and 4.0−6.5 in the ratio 1.6:1.4:1 (v/v/v). The gels were stained with Coomassie Brilliant Blue G-250 according to the procedure described by Blakesley and Boezi (*19*). After gel electrophoresis, the proteins were transferred from the gels onto nitrocellulose paper by capillary diffusion. The procedure was basically that described by Chianese et al. (*20*), but the blocking solution was replaced with PBS containing heat-inactivated horse serum at 10% in volume. Immunostaining was carried out using the developed antipeptide antisera as primary antibodies and horseradish peroxidase-labeled goat anti-rabbit IgG polyclonal antibodies as secondary reagent (BioRad, Hercules, CA). For bands immunostaining, the nitrocellulose paper was placed in 10 mM TrisCl pH 7.5 (approximately 10 mL for a 15-cm × 15-cm membrane) containing 0.5 mg/mL 3,3′-diaminobenzidine (Fluka, Buchs, Switzerland) and 0.06% (w/v) $NiCl_2 6H_2O$ (Fluka, Buchs, Switzerland). To start the reaction, 20 μL of 30% H_2O_2 were added. Incubation was performed at room temperature with agitation until the bands became dark (1/5 min). To stop the reaction, H_2O_2 was washed off with PBS.

RESULTS AND DISCUSSION

Design of the Peptide Antigens. To direct antibody response toward the novel haptenic structure appearing on γ2-casein exposed to formaldehyde, formylated peptide HKEMPGGC was chosen as the suitable antigen. The HKEMP sequence stretch reproduces the N-terminal sequence of γ2-casein, containing the histidine residue found to be a very reactive site toward free aldehydes (*11*). The synthetic peptide HKEMPGGC was used as the control antigen to produce antibodies possibly targeting native γ2-casein. The C-terminal cysteine residue

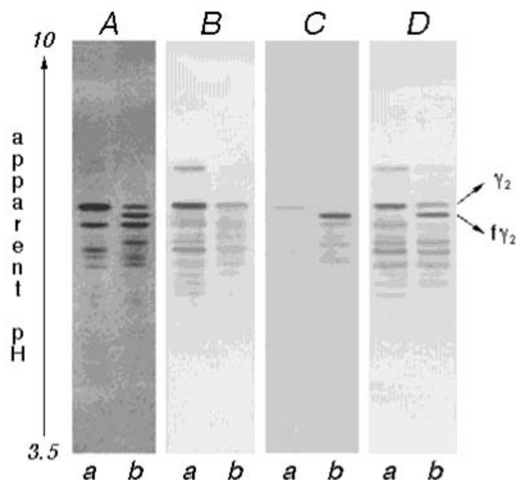

Figure 1. The isoelectric profiles of both the native FPLC-purifiedγ-casein fraction (lanes *a*) and that of the formylated γ-casein fraction (lanes *b*) were stained by G-250 Coomassie Brilliant Blue (panel A) or transferred onto nitrocellulose and immunochemically stained using the C22 (panel B), Y25 (panel C), or Z25 (panel D) antiserum as primary reagent. The γ_2-casein band is shortly indicated as γ_2 and its formylated adduct as fγ_2.

provided the sulfhydryl group required by the procedure chosen to link the correctly oriented peptide to a protein carrier (*14*) to improve peptide immunogenicity. The two glycine residues were also included in the C-terminal region of the peptide to form a spacer arm between the native or formylated N-terminus γ2-casein sequence and the carrier protein in the coupled products.

Binding Specificity of the Polyclonal Antibodies Raised against the Peptide Antigens. The peptide HKEMPGGC and its formylated form were prepared. Two separate antisera were obtained from each antigen, according to the procedures reported in Materials and Methods. The two antisera produced against the peptide P were named C22 and D22, those against the formylated adduct of the peptide were named Y25 and Z25. The binding specificity of the antipeptide antibodies contained in each antiserum was assayed toward the putative protein substrates by immunoblotting and results are reported in **Figure 1**. The native FPLC-purifiedγ-casein fraction from a plasmin hydrolyzate of bovine β-casein was indeed a mixture of γ_1-, γ_2-, and γ_3-casein, corresponding to the β-casein fragments 29−209, 106−209, and 108−209, respectively (*21*), as recognized by evaluating the electrophoretic migration of the three major bands in the lane *a* of the panel A and confirmed by ES/MS determination (not shown). Formylation of this sample mainly affected the γ_2-casein fraction, as previously reported by Restani et al. (*11*). In fact, by incubating the γ-casein fraction with formaldehyde, the intensity of the γ_2-casein electrophoretic band was reduced to about one-half of its initial value (cf. lanes *a* and *b* of the panel A) and partly transferred into a novel band, migrating just near the native γ_2-casein band, but clearly distinct from it.

By ES/MS analysis of the reaction mixture, a novel signal was found having a molecular mass of 11 835.68 ± 0.60 Da, besides that assigned to the native γ_2-casein, detected at a molecular mass of 11 823.56 ± 0.59 Da. The difference of 12 Da, found between the molecular mass of these two species, can be explained by the addition of one formaldehyde molecule to the N-terminal histidine residue of γ_2-casein and the consequent molecular rearrangement of the primary adduct, thus leading to the spinacine residue formation, as already reported (*12*). To confirm that only the N-terminus of γ_2-casein had been modified by formaldehyde, the reaction mixture was digested with Asp-N endoprotease and analyzed by MALDI-TOF mass spectrometry. Apart from the signal at *m/z* 2706.37, corresponding to the peptide 1−23 of γ_2-casein, a signal at *m/z* 2718.34 was detected, accounting for the formylated and rearranged form of the peptide 1−23 of γ_2-casein, whereas single signals at *m/z* 6239.40 and 2909.59 were obtained, relative to peptides 24−78 and 79−104 of γ_2-casein, respectively, thus showing that the other regions of γ_2-casein were unaffected by formaldehyde treatment.

The C22 and D22 antisera directed against the unmodified synthetic peptide showed quite similar reactivity. Therefore, the immunoblotting results obtained by using C22 only are reported (**Figure 1**, panel B). Among the major bands nonspecifically stained along the γ-casein fraction profile, only the one corresponding to γ_2-casein was recognized by these antisera (cf. lane *a* of the panels A and B), though γ_2-casein subfraction showed some heterogeneity that was most likely due to the use of quite drastic hydrolysis conditions used to prepare the γ-casein fraction from β-casein (high enzyme/protein ratio, prolonged time of incubation). The C22 and D22 immunodetection pattern of the formylated γ-casein sample was quite similar to that of the native γ_2-casein (cfr. lane *b* and lane *a* of the panel B) and no additional band was recognized. However, the overall immunoreactivity detected along the lane *b* was not as much as that found along the lane *a*, thus confirming the reduction of N-terminus unmodified γ_2-casein in the formylated γ-casein sample.

Interestingly, the Y25 and Z25 antisera showed some difference in their reactivity spectra, even though they were obtained in response to the same immunogen agent. As shown in panel C, the Y25 antiserum was not able to recognize any protein band within the γ-casein fraction (lane *a*), whereas it selectively stained the novel band migrating close to γ_2-casein and previously recognized as the formylated γ_2-casein (lane *b*). On the contrary, the Z25 antiserum was reactive toward each molecular species recognized by both C22 and Y25 antisera (panel D). Most likely, differences between the binding specificities exploited by Y25 and Z25 antisera are due to the individual character of the immunological response in different subjects, as both the relative preimmune sera were nonreactive toward both native and modified γ-casein isoelectric profiles (results not shown). The Z25 reactivity may be explained by assuming that the immunological response was focused on the peptide sequence as well as on its N-terminus addition product, whereas the Y25 response was raised mostly against the formylated adduct. Nevertheless, the Z25 antiserum proved to be a very useful tool in staining electrophoretic bands of samples containing both formylated and native γ_2-casein, as shown next.

Formylation of a 12-month aged Grana Padano Cheese Sample. Antisera performance in recognizing formylated protein species in a ripened cheese is shown in **Figure 2**. A defatted 12-month old Grana Padano cheese sample was incubated at 37 °C at increasing concentration of formaldehyde from 0.26 to 260 ppm and subsequently proteins were separated by isoelectric focusing. The resulting profiles were stained by Blue Coomassie Brilliant Blue G-250 (panel A) or immunoblotted and stained by the C22 (panel B), Y25 (panel C), and Z25 antiserum (panel C). Notwithstanding that a ripened cheese sample actually contains a very complex mixture of caseins and relative degradation products (panel A, lane 1), the antisera proved to be extremely selective in detecting their own protein targets. In detail, the C22 antiserum was able to recognize only the native γ_2-casein band along the electrophoretic profile of the cheese sample (lane 1). Furthermore, its intensity gradually decreased at increasing concentrations of formaldehyde added to cheese sample (lanes from 2 to 5), thus accounting for the

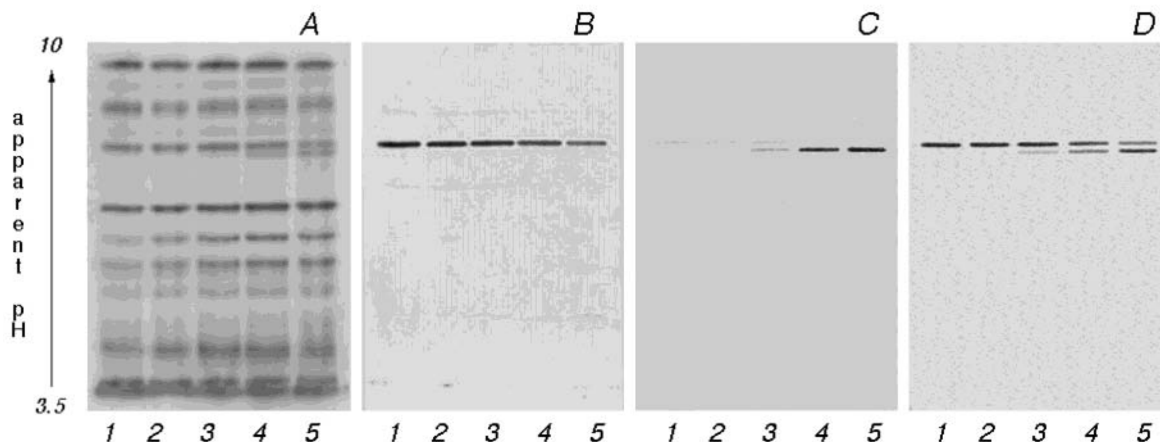

Figure 2. Isoelectric focusing and immunoblotting prints of the protein fraction of a 12-month old Grana Padano cheese sample (lane 1) incubated with increasing concentrations of formaldehyde (lanes from 2 to 5: 0.26, 2.6, 26, 260 ppm). Profiles were stained by G-250 Coomassie Brilliant Blue (panel A) or transferred onto nitrocellulose and immunochemically stained using the C22 (panel B), Y25 (panel C), or Z25 (panel D) antiserum as primary reagent.

conversion of γ_2-casein into the antibody nonreactive formylated product. Coherently, the Y25 antiserum detected increasing amounts of formylated γ_2-casein in the lanes from 2 to 5, whereas it did not recognize any band in untreated cheese sample (lane 1). The Z25 antiserum, which had previously proven to be able to detect both the native and formylated protein species, confirmed the progressive decrease of γ_2-casein and the corresponding production of the adduct in the cheese samples incubated with increased levels of formaldehyde. Interestingly, reaction mixtures were reanalyzed after a 3-month storage at −20 °C and the amounts of the addition product found in samples analyzed in lanes 2 to 5 were quite similar to that previously detected in the freshly prepared sample of lane 5, thus indicating that free formaldehyde keeps on acting in cheese even at −20 °C (results not shown).

To act as an antimicrobial agent, formaldehyde must be added to milk up to a final concentration of 25−30 ppm (v/v) (*5*). Assuming a cheese yield of about 10% (w/v), residual 2−3 ppm (w/w) formaldehyde should be retained at least in a hard cheese, such as Grana Padano. Indeed, Resmini et al. (*10*) showed that formaldehyde content in Grana Padano cheese from formaldehyde-treated milk was actually much higher than that expected (about 40−50 ppm) because of the stable linkage of formaldehyde to caseins. Dose-dependence analysis reported in **Figure 2** shows that the antisera sensitivity is appropriate to follow protein adduct formation at the formaldehyde concentration currently used in cheese making. On the other hand, the addition of higher amounts of formaldehyde gives rise to insoluble protein products, most probably resulting from protein cross-linking. In fact, a cheese sample incubated with formaldehyde at a final concentration of 2600 ppm formed a precipitate during reaction and a very poor protein amount could be recovered from the sample, even by suspending the precipitate in urea (9 M) so that no further analysis of the sample could be performed.

It is worthy to note that the peptide used as immunogen to produce the C22 and D22 antisera reproduced only five amino acid residues of the N-terminus γ_2-casein, whereas the same sequence carrying formylated N-terminal His residue was used to produce the Y25 antiserum. Nevertheless, extremely specific immune response was developed in both cases because no cross-reaction was revealed even in a 12-month aged cheese sample, being actually a complex mixture of close-related protein species. Moreover, the formation of different antibody reactive adducts in a formaldehyde-treated 12-month aged Grana Padano cheese sample could be excluded.

Polyclonal Antibodies Reactivity toward the Whole Protein Fraction of Grana Padano Cheese Samples during Ripening. A marker of Grana Padano cheese ripening has been proposed by Restani et al. (*22*) consisting of γ_2-casein modified by reactive aldehydes coming from microbial metabolism. To test the possibility that this aldehyde-modified γ_2-casein occurring in advanced cheese maturation could interfere in antibody recognition of formaldehyde-modified γ_2-casein, antisera responsiveness was assayed toward the whole protein fraction of Grana Padano cheese throughout ripening (**Figure 3**). Cheese samples from 1 day to 20 months were analyzed by isoelectric focusing and profiles were stained by Coomassie Brilliant Blue G-250 (panel A) or immunostained by the Y25 (panel B), the Z25 (panel C), and the C22 antiserum (not shown). The same analysis was carried out using the same cheese samples preincubated with 26 ppm of formaldehyde in a parallel experiment to label bands relative to γ_2-casein and its formylated adduct (results not shown). As shown in panel B, the Y25 antiserum effectively detected one band along the electrophoretic profile of the 20 month-aged cheese sample, at the same mobility of the formylated adduct of γ_2-casein, as resulted by comparing the immunostained profile with those relative to formaldehyde-treated Grana Padano cheese samples. In the same sample, a similar band was also recognized by the Z25 antiserum, shown in panel C. In agreement with the results reported above and the C22 reactivity, the other band displayed by the Z25 antiserum in all of the samples, at intensity increasing with ripening time, was attributed to native γ_2-casein.

On the whole, the results confirmed the occurrence of aldehyde-modified γ_2-casein during ripening, having electrophoretic mobility similar to that of formylated γ_2-casein, as already reported (*22*). Our finding that both the formylated and the aldehyde-modified γ_2-casein are undistinguishable by use of the advanced tool of antipeptide antibodies reduces the possibility of their use in the development of ELISA tests for detecting formaldehyde-treated milk in aged cheese. This analytical strategy can instead be appropriately applied to assay fresh milk and cheese samples from the early period of maturation because there is no interference of antibody reactive species as in cheese ripened over 12 months.

Polyclonal Antibodies Reactivity toward the Protein Fraction of Grana Padano Cheese Samples. To analyze the situation of commercial cheeses found on the market, the antisera were used to assay the protein fractions from a number of Grana Padano cheese samples. First of all, the ripening age

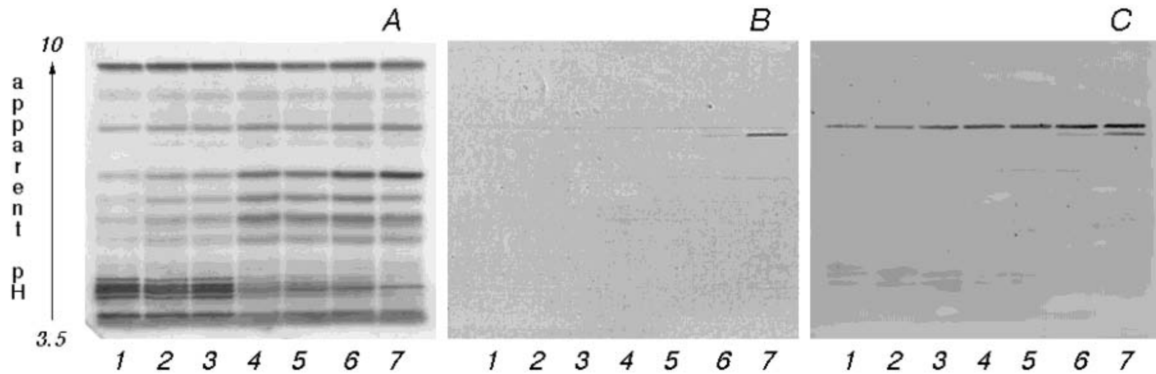

Figure 3. Isoelectric focusing and immunoblotting prints of the protein fraction of Grana Padano cheese samples at different times of ripening (lanes 1–7: 1 day, 10 days, 21 days, 6 months, 8 months, 12 months, 20 months). The profiles were stained by G-250 Coomassie Brilliant Blue (panel A) or transferred onto nitrocellulose and immunochemically stained using the Y25 (panel B) or Z25 (panel C) antiserum as primary reagent.

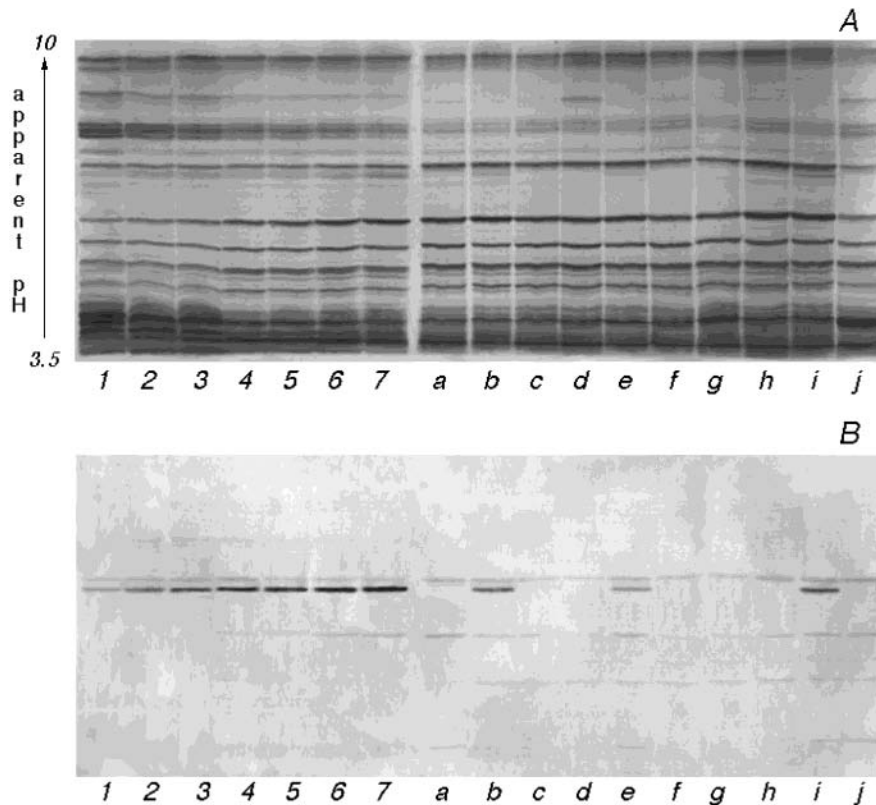

Figure 4. Isoelectric focusing on ultrathin-layer polyacrylamide gel and immunoblotting prints of the protein fraction of Grana Padano cheese samples from commercial sources at different age (in months) (lanes *a*, *b* = 15; lanes *c*, *d*, *e* = 9; lanes *f*, *g* = 6; lanes *h*, *i* = 12; lane *j* = 3). The protein fraction of Grana Padano cheese at different times of ripening (from 1 day to 20 months, as in **Figure 3**) incubated with 26 ppm formaldehyde was used as reference (lanes 1–7). The profiles were stained by G-250 Coomassie Brilliant Blue (panel A) or transferred onto nitrocellulose and immunochemically stained using the Y25 antiserum (panel B) as primary reagent.

of the samples was evaluated according to a previously reported method, based on the ELISA determination of the residual native β-casein (*23*). To avoid detection of antibody reactive aldehyde-modified γ_2-casein arising late in maturation, 10 samples aged up to 15 months were selected for the analysis reported in **Figure 4**. The isoelectric protein profiles stained by Coomassie Brilliant Blue G-250 (panel A) compared with those obtained by immunostaining with the Y25 antiserum (panel B) are shown. Samples were divided into five different groups according to their ripening age: 15 months (lanes *a*, *b*), 9 months (lanes *c*, *d*, *e*), 6 months (lanes *f*, *g*); 12 months (lanes *h*, *i*), 3 months (lane *j*). Grana Padano cheese samples from 1 day to 20 months old (lanes from 1 to 7) preincubated with 26 ppm of formaldehyde were used as reference samples. One band at the height of formylated γ_2-casein was immunodetected along the electrophoretic profiles of three cheese samples. It is important to underline that in each group there was at least one Y25 negative sample. Hence, eventual detection of any antibody cross-reacting species linked to the ripening was effectively excluded, and the Y25 positive band found in the three cheese samples could be reasonably assigned to the formylated adduct of γ_2-casein, thus indicating that the Y25 positive cheeses had been produced from formaldehyde-treated milk.

A weak signal was recorded at the level of the native γ_2-casein both in cheese and reference samples in addition to that of the formylated γ_2-casein. This does not seem to agree with the above-stated findings concerning the antibody specificity. Appearance of the signal is likely due to the use of "ultrathin-layer gels" (0.25 mm thick), instead of "normal gel" (0.75 mm thick), to improve protein separation and blotting.

It must be noted that densitometric analysis of the electrophoretic profiles of these cheese samples according to the method proposed by Restani et al. (*22*) can be misleading with respect to the evaluation of cheese ripening time. In fact, the ratio between γ_2-casein modified by aldehydes coming from microbial metabolism and native γ_2-casein was indicated as a reliable marker of cheese ripening. However, the microbial aldehyde γ_2-casein adduct is electrophoretically indistinguishable from the formaldehyde γ_2-casein adduct, as shown above. Consequently, owing to the presence of a modified γ_2-casein having a slightly lower pI than normal γ_2-casein, cheese from formaldehyde-treated milk should be even more valuable than one at the same ripening time but from genuine milk. For example, the adulterated cheese sample run in lane *i* of **Figure 4**, actually ripened for 12 months, as determined independently by γ_2-casein fraction electrophoretic analysis, should be classified as a high-quality 24-month aged cheese sample, according to the method described by Restani et al. (*22*).

On the whole, this study proved that properly modified peptides, mimicking novel haptenic structures raised by exposing proteins to xenobiotic agents can be used advantageously as model substrates in novel applications of antipeptide antibody technology for the evaluation of protein quality in foods.

The Y25 antiserum, directed against the formylated synthetic peptide reproducing the N terminus of formaldehyde γ_2-casein adduct, was able to detect the parent chemically modified γ_2-casein, but not the unmodified γ_2-casein, even in complex protein mixtures such as cheese containing parent β-, γ_2-, and γ_3-casein.

The C22 antiserum, directed against the synthetic peptide reproducing the N-terminal sequence of γ_2-casein, had proven to be a valuable analytical tool to specifically detect γ_2-casein, so that it can be exploitated to discriminate within the protein fraction of cheese samples at advanced times of ripening.

Work is in progress to develop ELISA tests to detect formaldehyde treatments in milk through the immunochemical determination of formaldehyde γ_2-casein adduct, provided that plasmin hydrolysis of milk proteins is previously performed to convert all of β-casein into γ-fraction, followed by incubation to allow free formaldehyde addition to γ_2-casein. Unfortunately, the method cannot be directly applied to any cheese analysis, owing to the formation of microbiological aldehyde adducts electrophoretically and immunologically indistinguishable from formaldehyde γ_2-casein adducts in ripened cheese.

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