Isolation and Identification of β-Casein A1-4P and β-Casein A2-4P in Commercial Caseinates

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Caseinate contained two modified β-casein (β-CN) fractions that together represented from 5 to 27% of the total β-CN depending on the type of caseinate analyzed (sodium, calcium, or potassium). Mass spectroscopy showed that the modified β-CN fractions had molecular weights of 23 940 ± 3 and 23 904 ± 2, ≈80 (or the mass of one phosphate group) less than that of the native β-CN fractions found in milk, β-CN A1-5P (24 028) and β-CN A2-5P (23 988). ^{31}P NMR verified mass spectroscopy results showing that the modified fractions contained four instead of five phosphorylated serine residues. Molecular weight differences between the modified and unmodified fractions also indicated that the dephosphorylation was a result of enzyme, acid, or alkali hydrolysis and not alkali hydrolysis that proceeds through β-elimination. The two modified fractions identified as β-CN A1-4P and β-CN A2-4P are probably present in caseinate as a result of the dephosphorylation of the main β-CN gene products β-CN A1-5P and β-CN A2-5P, respectively.

Keywords: *Caseinate; β-casein; dephosphorylation; phosphatase*

INTRODUCTION

β-Casein (β-CN) is a phosphoprotein found in bovine milk with an approximate molecular weight of 24 000. Three genetic variants account for >95% of all β-CN found in common breeds of cattle and include β-CN A1-5P, β-CN A2-5P, and β-CN B-5P (Swaisgood, 1992). Current nomenclature for CN consists of naming the CN family first followed by the genetic variant and then the number of phosphorylated residues (Eigel et al., 1984).

The common genetic variants of β-CN contain five phosphorylated serine residues, Ser 15, 17−19, and 35, which are clustered near the amino terminus (Swaisgood, 1992) and have a large impact on the functional properties of β-CN. An example is the calcium sensitivity of β-CN. Though β-CN precipitates from solution in the presence of 8.6 mM calcium at 30 °C (Parker and Dalgleish, 1981), dephosphorylated β-CN does not (Yoshikawa et al., 1974). Dephosphorylation of β-CN also results in a softer curd during cheese making (Yun et al., 1982). A review (West, 1986) of the structural and functional properties of phosphorylated serine residues in CN indicates their technological and biological importance.

Milk contains both alkaline and acid phosphatases, which are phosphomonoesterases capable of dephosphorylating phosphoserine residues. Alkaline phosphatase is a dimer with a molecular weight of ≈160 000−190 000 and contains four to five Zn atoms per molecule (Andrews, 1992). Its concentration in milk varies as much as 40-fold during the lactation period and among individual cows (Haab and Smith, 1956). Several compounds in milk such as inorganic phosphate, lactose, and whey proteins act as alkaline phosphatase inhibitors (Lorient and Linden, 1976) and prevent dephosphorylation of CN. Two studies (Zittle and Bingham, 1959; Lorient and Linden, 1976) indicate that the pH optimum for dephosphorylation of isolated CN in buffered solutions is between 6 and 7.

Acid phosphatase has a molecular weight of ≈42 000 and has a pH optimum of 4.9 using *p*-nitrophenyl phosphate as the substrate (Andrews and Pallavicini, 1973). Acid phosphatase is more heat stable than alkaline phosphatase and survives pasteurization treatments (Andrews, 1974). There are several reviews (Jenness and Patton, 1959; Kitchen, 1985; Andrews, 1992) that cover the characteristics of acid and alkaline phosphatases.

Dephosphorylation of CN also can occur as a result of alkaline hydrolysis (Manson, 1973; Manson and Carolan, 1980). Simple alkaline hydrolysis results in the release of phosphate and produces serine. Alkaline hydrolysis of phosphorylated serine residues can also proceed through a β-elimination reaction producing dehydroalanine residues capable of intermolecular or intramolecular reaction with lysine to form lysinoalanine (Lorient, 1979; Hasegawa et al., 1981; de Koning and van Rooijen, 1982). The optimum pH for the reaction is ≈12 (Creamer and Matheson, 1977; Friedman et al., 1981), but lysinoalanine will form at pH 5 with a severe heat treatment (120 °C) (Sternberg and Kim, 1977). A review (de Koning and van Rooijen, 1982) of the lysinoalanine content in milk and milk products indicates that both sodium and calcium caseinates contain from 0 to 1560 and from 0 to 6800 mg of lysinoalanine/kg of protein, respectively.

Casein isolation on an industrial level is done by isoelectric precipitation followed by neutralization with alkali [NaOH, $Ca(OH)_2$, KOH] to produce caseinates that are incorporated into a wide variety of food products (Mulvihill, 1992). During different steps in caseinate manufacture there is the potential for changing CN functionality through enzyme, acid, or alkali dephosphorylation. The enzymatic dephosphorylation

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could occur since several phosphatase inhibitors such as lactose, whey proteins, and inorganic phosphates are removed during caseinate manufacture.

Chromatographic analysis of β-CN isolated from commercial caseinate showed a major unidentified peak. The objective of this research was to isolate and characterize the unknown peak. Research showed that the unknown peak contained β-CN A^1-4P and β-CN A^2-4P, dephosphorylated derivatives of the gene products β-CN A^1-5P and β-CN A^2-5P.

MATERIALS AND METHODS

Materials. Caseinate samples were obtained from New Zealand Milk Products Inc. (Santa Rosa, CA) or from DMV International (Fraser, NY). Urea, NaCl, β-mercaptoethanol, Triton X-100, trichloroacetic acid, methanol, acetic acid, glycerol, deuterium oxide, Spectra/Por 6 dialysis tubing (1000 molecular weight cutoff), Whatman No. 4 filter paper, Whatman 113V filter paper, 3 cm^3 syringes, and 8 in. 5 mm inner diameter NMR tubes were obtained from Fisher Scientific (Fair Lawn, NJ). Chymosin (Chymax) was donated by Chris Hansen's Laboratories (Milwaukee, WI). Bis-Tris-propane was obtained from Sigma Chemical Co. (St. Louis, MO). A Mono Q HR 5/5 anion exchange column, an XK 50/60 column, DEAE Sepharose FF, carrier ampholytes (Pharmalyte), IEF Phastgels 4-6.5, Coomassie blue stain (PhastGel Blue R), and sample applicators (12/0.3 μL) were obtained from Pharmacia Biotechnology (Uppsala, Sweden). Chelex 100 resin (200−400 mesh) was obtained from Bio-Rad Laboratories (Hercules, CA). Filters (0.2 and 0.8 μm) used to filter buffer solutions and 10 000 molecular weight cutoff membranes for ultrafiltration were from Millipore (Bedford, MA). Filters (0.2 μm) for sample filtration were obtained from Chrom Tech Inc. (Apple Valley, MN). Centricon concentrators (3000 molecular weight cutoff) were from Amicon (Beverly, MA).

Isolation of β-CN. β-Casein was isolated from commercial caseinate samples using a previously described method (Ward and Bastian, 1996). The isolated β-CN was ≈95% pure by chromatographic analysis. Specific genetic variants of β-CN were isolated and used as controls in several of the experiments. The following protocol was used for isolating β-CN A^1-5P and β-CN A^2-5P. Holstein cows from the University of Minnesota dairy herd were phenotyped according to the method of Bovenhuis and Verstege (1989) as modified by Hollar (1992). Milk from cows homozygous for β-CN A^1-5P and β-CN A^2-5P was collected during the morning milking and immediately cooled to 4 °C and transported back to the laboratory. The whole milk was centrifuged (7520*g*) for 15 min. The skim milk fraction was collected by filtering through Whatman No. 4 filter paper. Isoelectric precipitation of CN was done at 30 °C and pH 4.6. The precipitate was collected by filtering through Whatman 113V filter paper. The precipitate was resuspended in distilled water and precipitated two more times. The washed precipitate was resuspended in distilled water and neutralized with NaOH. The solids concentration was adjusted to 3% using a refractometer (Fisher Scientific). The 3% sodium caseinate solution was used for β-CN isolation as described (Ward and Bastian, 1996). Both β-CN A^1-5P and β-CN A^2-5P were isolated in this manner.

FPLC. Ion exchange chromatography (FPLC, Pharmacia Biotechnology) was used to analyze caseinate samples according to the method of Davies and Law (1987). Buffer 1 contained 0.005 M bis-Tris-propane and 3.3 M urea, adjusted to pH 7.0 with HCl. Buffer 2 was identical to buffer 1 except 1 M NaCl was added. Buffers were filtered through 0.8 and 0.2 μm filters and degassed before using. Samples were dissolved in buffer 1 and filtered. Caseinate samples were reduced by adding 0.1% β-mercaptoethanol. A 500 μL sample loop was used for applying the sample to the column. The flow rate was 1 mL/min. Absorbance was monitored at 280 nm.

Protein Sequencing. A FRAC-200 (Pharmacia Biotechnology) fraction collector connected to the FPLC system was used to collect the modified β-CN fraction for sequencing. Samples were dialyzed using 1000 molecular weight cutoff dialysis tubing and then concentrated using a 3000 molecular weight cutoff concentrator. An ABI 477 protein sequencer (Applied Biosystem Inc., Foster, CA) was used to do N-terminal amino acid sequencing.

Isoelectric Focusing (IEF). IEF followed the method of Bovenhuis and Verstege (1989) as modified by Hollar (1992). PhastGels (IEF 4−6.5) were incubated overnight in an 8 M urea/1% Triton X-100 solution. After incubation, the gels were soaked in an 8 M urea solution containing 0.8% Triton X-100 and 16% carrier ampholytes for 15 min and then allowed to dry for 5 min. Samples were dissolved into an 8 M urea solution containing 3% β-mercaptoethanol and allowed to sit for 15 min. Sample application, running, Coomassie blue staining, and destaining were done using a PhastSystem (Pharmacia Biotechnology).

Mass Spectroscopy. Electrospray ionization mass spectra (ESI-MS) were acquired using a PE SCIEX API III triple-quadrupole mass spectrometer (Norwalk, CT). Samples of β-casein containing 2−20 mg of protein/mL (pH 6.8) were prepared by dialyzing collected fractions against deionized water for 24 h and then sprayed at 2−5 μL/min using a Harvard Apparatus Model 22 syringe pump (South Natick, MA). β-CN A^2-5P was used as a control.

Phosphorus-31 Nuclear Magnetic Resonance (^{31}P NMR). ^{31}P NMR on a Varian Unity 300 MHz spectrometer (Varian, Palo Alto, CA) operating at 121 MHz was used to identify the number of phosphorylated residues in the protein samples according to the method of Humphrey and Jolley (1982) with slight modification. To obtain a sufficient amount of modified β-CN, an XK 50/60 column packed with DEAE-Sepharose FF was used. Buffers A and B were the same as used by Davies and Law (1987). Approximately 5 g of protein was dissolved in buffer A (60 mL) and then loaded onto the column. Pure Buffer A was used to flush the column before the run was started. The gradient used consisted of 0% B at 3 min, 10% B at 30 min, 40% B at 220 min, 100% B at 221 min, 100% B at 281 min, 0% B at 282 min, and 0% B at 341 min (flow rate = 5 mL/min). The column was then cleaned and equilibrated before another run was started. Fractions were collected every 15 min and then dialyzed and concentrated using an ultrafiltration Minitan-S II system (Millipore, Bedford, MA) with a 10 000 molecular weight cutoff polysulfone membrane (Millipore). Fractions containing the modified β-CN were identified using isoelectric focusing. The β-CN was collected by isoelectric precipitation. The precipitate was resuspended in deuterium and the pH adjusted to 6.7. Chelex resin was added to chelate any divalent cations and the pH readjusted to read 6.7−6.9. Final deuterium concentration was at least 30%. Sample concentration ranged from 6 to 8%. Samples were filtered directly into the NMR tubes using a 3 cm^3 syringe and 0.2 μm syringe filter. Scans (1024) with a repetition delay of 2 s were recorded with 16K data points using a spectral width of 1360 Hz and an acquisition time of 6 s. Data were collected at ambient temperature.

RESULTS AND DISCUSSION

Identification of a Modified β-CN. According to the chromatographic method (Davies and Law, 1987) used to separate CN, all main genetic variants of β-CN (β-CN A^1-5P, β-CN A^2-5P, and β-CN B-5P) elute at 20 min as a single peak. Figure 1A contains a chromatogram of commercial calcium caseinate. κ-Casein eluted as a series of peaks from 14 to 18 min. Varying degrees of glycosylation result in several κ-CN peaks. β-Casein eluted as a single peak at 21 min. α_{s2}-Casein eluted from 26 to 31 min. α_{s1}-Casein eluted from 31 to 37 min. Figure 1B contains a chromatogram of β-CN isolated from commercial calcium caseinate using the method (Ward and Bastian, 1996) described earlier. β-Casein isolated from calcium caseinate showed one main β-CN peak at 20 min. A much smaller peak eluted right before the β-CN at 19 min.

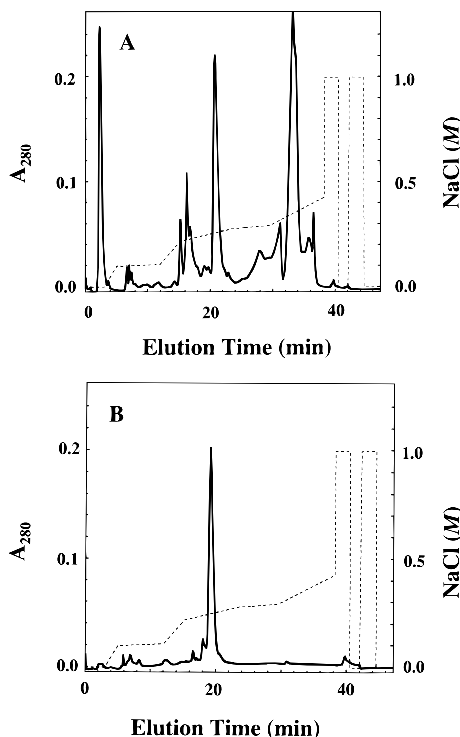


Figure 1. Calcium caseinate (A) and β-CN isolated from calcium caseinate (B). Absorbance at 280 nm (—) and the salt gradient (- - -) are shown.

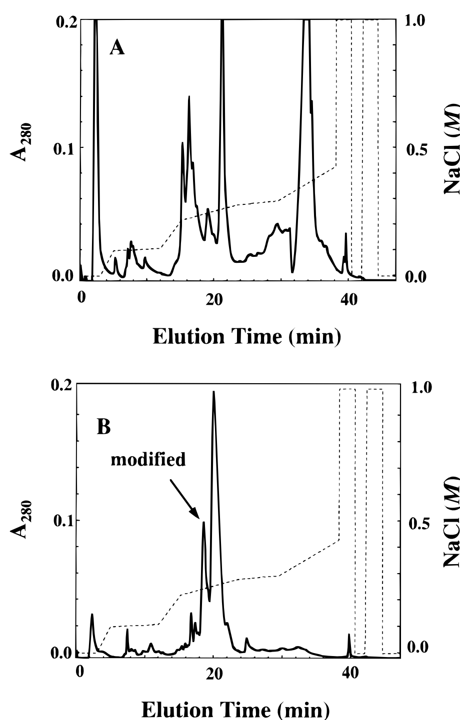


Figure 2. Sodium caseinate (A) and β-CN isolated from sodium caseinate (B). Absorbance at 280 nm (—) and the salt gradient (- - -) are shown.

Commercial sodium caseinate showed differences in elution profile. Figure 2A contains a chromatogram of sodium caseinate. The main CN fractions eluted at approximately the same time. The difference in elution profiles that influences the β-CN fraction is the peak that occurs right before the β-CN peak at 19 min. Figure 2B contains a chromatogram of β-CN isolated from the sodium caseinate. The main peak at 20 min corresponds to β-CN. There is also a significant peak that occurs right before the β-CN. Though this peak was observed in commercial calcium caseinate, it was at a much lower level. Since the method for isolating β-CN relied upon the unique characteristics of β-CN, the peak was considered to be a modified β-CN. The earlier elution time would indicate that the modification decreased the overall negative charge of β-CN, allowing it to release earlier from the anion exchange resin.

Table 1. Percent Modified β-CN Found in Several Commercial Caseinates

sample	% modified
roller-dried sodium caseinate[a]	18.4
spray-dried sodium caseinate[b]	14.5
sprai-dried sodium caseinate[a]	27.3
spray-dried potassium caseinate[a]	22.6
spray-dried calcium caseinate[b]	5.5
agglomerated calcium caseinate[a]	11.0
spray-dried calcium caseinate[a]	11.3
instant calcium caseinate[a]	14.3

[a] Caseinate obtained from DMV International. [b] Caseinate obtained from New Zealand Milk Products Inc.

To positively identify the peak at 19 min as a β-CN, the peak was collected and partially sequenced. Sequencing showed that the amino terminus (first 14 residues) was identical to β-CN, suggesting that the peak at 19 min was a modified β-CN. Thus, the calcium caseinate contained very little modified β-CN compared with the sodium caseinate sample. Several different caseinate samples were analyzed for the modified β-CN to determine the extent to which β-CN is modified in caseinates. Table 1 summarizes the results obtained from analyzing several different types and sources of caseinate. The percent modified β-CN was determined by integrating the modified β-CN peak area, dividing by the total β-CN peak area (β-CN + modified β-CN), and multiplying by 100. All samples analyzed contained at least 5% modified β-CN, and in some samples as much as 27% of the β-CN was in the modified form. As a general trend, sodium caseinate contained more modified β-CN than the calcium caseinate.

IEF. IEF separates the three main genetic variants of β-CN according to differences in their isoelectric point. A charge modification would change the isoelectric point and result in a different band. IEF was used to separate the three main genetic variants of β-CN in milk and compare them to the modified fraction. An example of an IEF gel in Figure 3 shows the three common variants of β-CN (β-CN A^1-5P, β-CN A^2-5P, and β-CN B-5P). Bulk herd milk (lanes 1 and 12) contained all three genetic variants. Other lanes (2−11) of individual cows showed that three cows (lanes 2, 3, and 6) produced only β-CN A^1-5P, three cows (lanes 5, 7, and 10) produced only β-CN A^2-5P, and four cows (lanes 4, 8, 9, and 11) produced both β-CN A^1-5P and β-CN A^2-5P. None of the cows tested produced β-CN B-5P. Cow phenotype is summarized at the bottom of Figure 3.

β-Casein isolated from sodium caseinate containing both the modified fraction and native β-CN showed four different bands (Figure 4A). The locations of β-CN A^1-5P and β-CN A^2-5P are labeled. The modified fraction corresponding to the collected modified peak (Figure 2B) is shown in Figure 4B (lane 2) and is compared to sodium caseinate (lane 1) and calcium caseinate (lane 2). The modified fraction contained two proteins. One protein migrated between β-CN A^1-5P

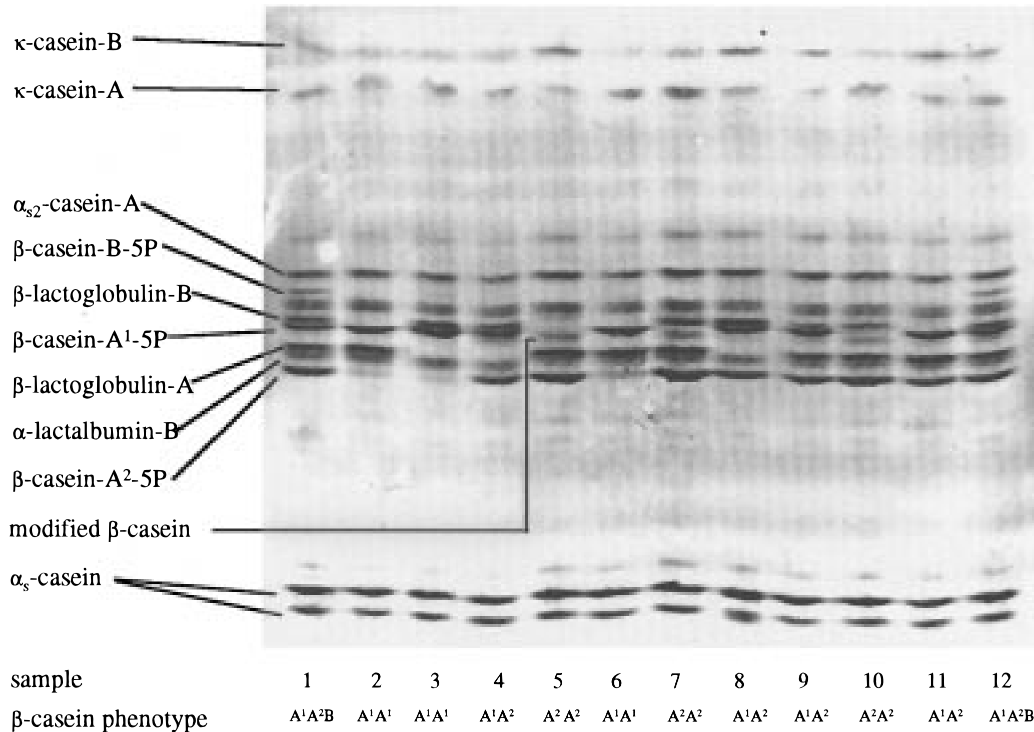


Figure 3. β-Casein phenotype determined for bulk herd milk (lanes 1 and 12) and milk collected from individual cows (lanes 2−11).

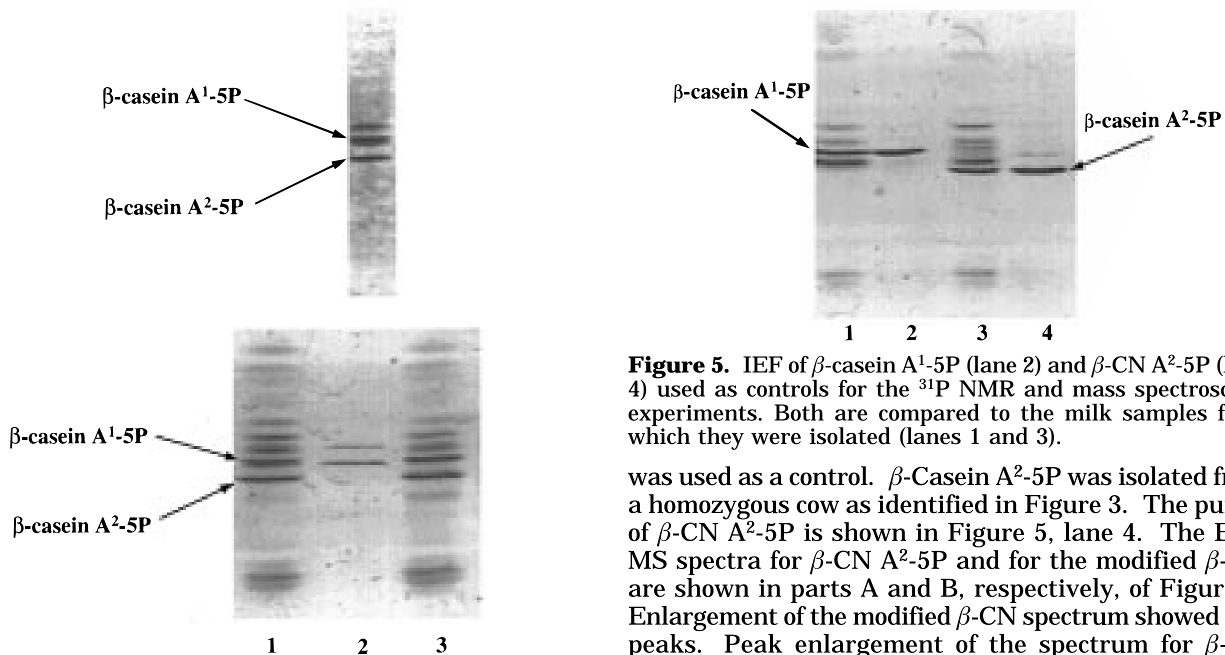


Figure 4. β-Casein isolated from sodium caseinate (A, top). The modified fraction was collected and is shown in lane 2 (B, bottom) compared to sodium and calcium caseinate in lanes 1 and 3.

Figure 5. IEF of β-casein A1-5P (lane 2) and β-CN A2-5P (lane 4) used as controls for the ^{31}P NMR and mass spectroscopy experiments. Both are compared to the milk samples from which they were isolated (lanes 1 and 3).

and β-CN A2-5P, and another band migrated above β-CN A1-5P. From Figure 4 it was apparent that there were at least two different proteins in the modified fraction. One of the modified proteins occurred in the whole milk samples. IEF results of whole milk samples (Figure 3) showed a band that corresponded to the modified band between β-CN A1-5P and β-CN A2-5P. This band is identified in lane 5 as a modified β-CN, but it also can be seen clearly in lanes 7 and 10.

ESI-MS. ESI-MS was used to determine the molecular weights of the modified proteins. β-Casein A2-5P was used as a control. β-Casein A2-5P was isolated from a homozygous cow as identified in Figure 3. The purity of β-CN A2-5P is shown in Figure 5, lane 4. The ESI-MS spectra for β-CN A2-5P and for the modified β-CN are shown in parts A and B, respectively, of Figure 6. Enlargement of the modified β-CN spectrum showed two peaks. Peak enlargement of the spectrum for β-CN A2-5P still showed only one well-resolved peak. Figure 7 shows an enlargement of the two peaks that are present in the modified β-CN sample corresponding to the two proteins in the modified fraction (Figure 4).

Well-resolved peaks were used to calculate the mass of each compound and obtain an average molecular weight and standard deviation for each protein. Table 2 summarizes these results. A value of $23\,986 \pm 5$ was obtained for the control, β-CN A2-5P. This was close to the literature value for β-CN A2-5P of 23 988 (Swaisgood, 1992). For the modified β-CN, results showed that the two proteins had molecular weights of $23\,940 \pm 3$ and $23\,904 \pm 2$. The difference between these two modified proteins was ≈40. This is the same difference as found between the common genetic variants β-CN

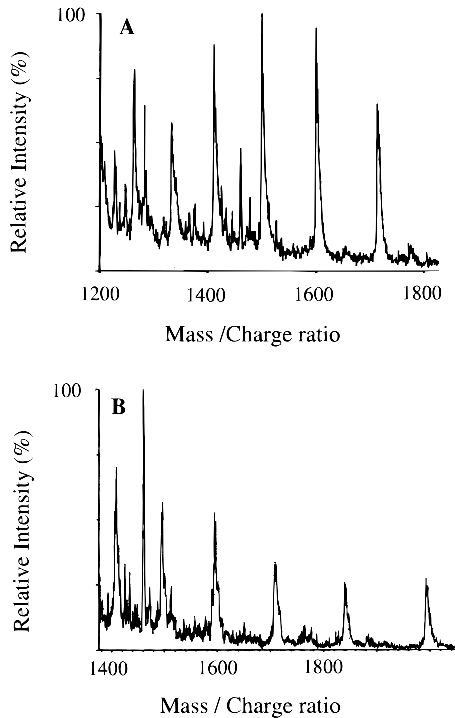


Figure 6. Mass spectra for β-CN A^2-5P (A) and for the modified fraction (B). Proteins were ionized by electrospray.

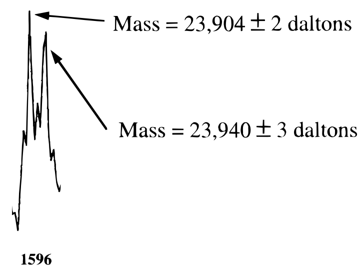


Figure 7. Enlarged peak from mass spectroscopy of the modified fraction.

Table 2. Summary of Molecular Weight Determinations

protein	theoretical	experimental
β-casein A^1-5P	24028	
β-casein A^2-5P	23988	23986 ± 5
β-casein B-5P	24097	
monodephosphorylated		
β-casein A^1-4P	23949	23940 ± 3
β-casein A^2-4P	23909	23904 ± 2

A^1-5P (24 028) and β-CN A^2-5P (23 988). This information provided evidence that the isolated, modified β-CN fractions, which had molecular weights of 23 940 and 23 904, were derived from the genetic variants β-CN A^1-5P and β-CN A^2-5P. The difference between β-CN A^2-5P (MW = 23 986) and one of the modified proteins (MW = 23 904) was 82. Using the published molecular weight for β-CN A^1-5P (MW = 24 028), the difference between this genetic variant and the second modified protein (MW = 23 940) was 88. We noted that these molecular weight differences were, within the experimental error of ESI-MS, the same as losing a phosphate group (MW = 80), so we decided to quantify the number of phosphorylated serine residues in each of these proteins using ^{31}P NMR.

^{31}P NMR. β-Casein A^1-5P was used a control for the ^{31}P NMR experiments. Figure 5 shows the purity of this preparation. The ^{31}P NMR spectrum obtained for β-CN

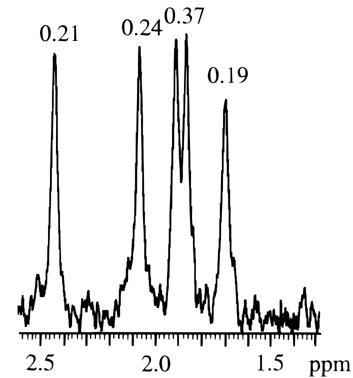


Figure 8. ^{31}P NMR spectrum obtained for β-CN A^1-5P.

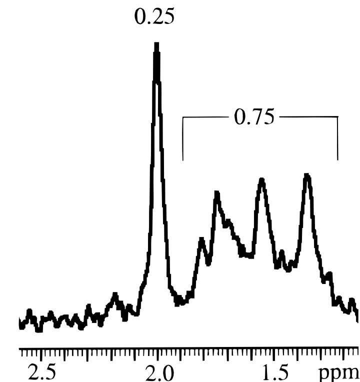
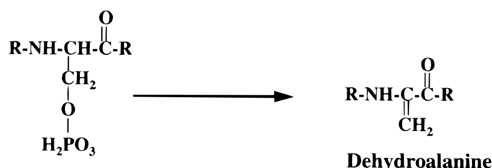


Figure 9. ^{31}P NMR spectrum obtained for the modified β-CN fraction.

A^1-5P showed five phosphorylated residues (Figure 8). Each peak has been assigned to a phosphorylated residue (Humphrey and Jolley, 1982). Peak areas were integrated, and the ratio of each peaks' area to the total integrated area was calculated. The two peaks that do not show baseline separation were integrated together. The total integrated area was 1.0. The spectrum for the modified sample is shown in Figure 9. One sharp peak was observed at 2.0 ppm and had an integrated area of 0.25. The other peaks from 1.2 to 1.8 ppm were grouped since these peaks were not separated by baseline and the entire area was integrated. The integrated area was 0.75, or 3 times the peak area of the single peak. This gives a ratio of 3:1 or a total number of four phosphorylated residues instead of five. One reason the peak separation was not as good in the modified β-CN may be the result of more than one protein since the sample contained modified β-CN A^1-5P and modified β-CN A^2-5P. Chemical shifts also occur as a result of trace minerals, and samples should contain $<2.5 \times 10^{-7}$ M minerals (Humphrey and Jolley, 1982). This makes it very difficult to prepare samples. Because of the chemical shifts that occurred in the modified preparation, it is difficult to clearly determine the residue that is dephosphorylated. According to phosphoserine residue assignment (Humphrey and Jolley, 1982), phosphoserine 35 has the largest chemical shift. The largest chemical shift in the β-CN A^1-5P was ≈2.5 ppm. The other residues showed a smaller chemical shift and were grouped between 1.6 and 2.2 ppm. The modified fraction showed four peaks grouped together between 1.2 and 2.2 ppm showing a slight shift from the β-CN A^1-5P. These results suggest that phosphoserine residue 35 is dephosphorylated, but because of the chemical shifts that occur, some doubt still exists. The MS-ESI



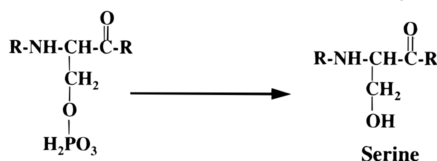


Figure 10. Mechanisms of β-CN dephosphorylation.

and ^{31}P NMR results both indicated that the modification was a result of the loss of a phosphate group.

There are two classes of mechanisms for the dephosphorylation of β-CN. One mechanism is alkali hydrolysis that proceeds through a β-elimination reaction and the production of a dehydroalanine residue (Manson, 1973; Manson and Carolan, 1980). The other mechanism is through enzyme, alkali, or acid hydrolysis that results in the dephosphorylation and release of the phosphate group but the serine residue is preserved (Jenness and Patton, 1959). These two classes of mechanisms are reviewed in Figure 10. A single dephosphorylation that proceeds through β-elimination would result in a modified β-CN with a molecular weight less than that of the unmodified β-CN by 97. If the reaction proceeded through an enzyme, alkali, or acid dephosphorylation, the molecular weight of the modified β-CN would be reduced by 80. According to the ESI-MS data a difference of 82 was calculated for β-CN A^2-5P. We concluded that the dephosphorylation was a result of enzyme, alkali, or acid hydrolysis and not a result of dehydroalanine formation. We do not have data to suggest whether enzyme, alkali, or acid hydrolysis predominates; a combination of these may be occurring during caseinate manufacture. The lack of mechanistic information gives direction on future work in this area.

Research (West and Dalgleish, 1976) done with a β-CN phosphopeptide containing the four closely grouped phosphates showed that one residue was more susceptible to dephosphorylation than other phosphorylated residues even though all residues were eventually hydrolyzed. Davies and Law (1987) also found that casein contained a protein with slightly less mobility than β-CN and suggested that it might be a partly dephosphorylated form of β-CN. Our results also indicate that phosphorylated residues on β-CN are susceptible to hydrolysis, and the dephosphorylated products are found in caseinate. The extent to which this occurs in laboratory preparations for scientific study is not known, but it is possible that some β-CN is dephosphorylated during bench-top preparations. Dephosphorylation would lead to altered functional properties such as calcium binding, association, and precipitation characteristics.

Conclusions. Both β-CN A^1-4P and β-CN A^2-4P were found in caseinate as monodephosphorylated derivatives of β-CN A^1-5P and β-CN A^2-5P. The amount of dephosphorylated β-CN in caseinate ranged from 5 to 27% depending on the sample that was analyzed. The dephosphorylation is probably a result of phosphatase activity, acid, or alkali hydrolysis and not a result of alkaline hydrolysis proceeding through β-elimination.

ABBREVIATIONS USED

CN, casein; IEF, isoelectric focusing; ESI-MS, electrospray ionization mass spectroscopy; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance.

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