

Analysis of the Human Casein Phosphoproteome by 2-D Electrophoresis and MALDI-TOF/TOF MS Reveals New Phosphoforms

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Mammalian breast milk contains an array of proteins and other nutrients essential for the development of the newborn. In human milk, the caseins (α_{S1} , β and κ) are a major class of proteins; however, the dynamic range of concentrations in which the various isoforms of each casein exist presents challenges in their characterization. To study human milk casein phosphoforms, we applied traditional two-dimensional polyacrylamide gel electrophoretic (2-DE) separation combined with matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) tandem mass spectroscopic analysis. The abundant β -casein was resolved as a train of 6 spots differing in phosphorylation level with 0–5 phosphates attached. To study the less abundant α_{S1} -casein, a cysteine-tagging enrichment treatment was used prior to 2-DE. A train of 9 spots with $4.4 < pI < 5.3$ were identified as α_{S1} -casein. This included five previously uncharacterized phosphoforms with up to 8 phosphate groups located in two serine-rich tryptic phosphopeptides ($^{27}L-R^{51}$, $^{69}N-K^{98}$) consistent with α -caseins from various ruminant species. MS/MS analysis of the phosphopeptides released by tryptic digestion enabled identification of the residue-specific order of phosphorylation among the 6 β -casein and 9 α_{S1} -casein phosphoforms. Deamidation of N^{47} of α_{S1} -casein was also a feature of the MS analysis. This study represents the first comprehensive analysis of the human casein phosphoproteome and reveals a much higher level of phosphorylation than previously recognized. It also highlights the advantages of 2-DE for examining the global pattern of protein phosphoforms and the limitations of attempting to estimate phosphorylation site occupancies from “bottom-up” studies.

Keywords: α_{S1} -casein • β -casein • human milk • phosphorylation • deamidation

Introduction

Caseins are phosphoproteins responsible for the formation of micelles, which allow the stabilization of high levels of calcium phosphate in milk. As well as providing an important source of nutrition, they are also the source of a range of bioactive phosphopeptides of interest for their endogenous activities and potential applications in the food, nutraceutical and pharmaceutical industries.^{1–4} The bovine α - and β -caseins have been widely used as model phosphoproteins in the development of proteomic techniques to examine global phosphorylation patterns.^{5–9} Bovine β -casein exists as a single phosphoform, containing 5 phosphate groups, while bovine α_{S1} -casein is predominantly a single form with 8 phosphates.¹⁰ In contrast, studies on the milks of other mammals have revealed more variable phosphorylation of component caseins.^{11–14} Human β -casein has been shown to be variably modified with

0–5 phosphates.¹⁵ Human α_{S1} -casein has also been reported to be hypophosphorylated with only 0–2 phosphates.¹⁶ More recently, evidence for an additional 2 phosphorylation sites on human α_{S1} -casein has been presented.¹⁷

While bottom-up LC-MS/MS approaches have gained wide support for high-throughput proteomic studies, they do have limitations when it comes to phosphorylated proteins. This has been largely attributed to poor ionization efficiency of phosphorylated peptides and/or the substoichiometric nature of protein phosphorylation resulting in relatively low levels of phosphorylated peptides compared to their unphosphorylated counterparts.¹⁸ As a result, they are less likely to be selected for MS/MS and the presence of phosphorylated forms of proteins can be easily missed. To counter this limitation, a number of phosphopeptide enrichment strategies have been developed in recent years to improve detection of phosphorylated proteins, but the distinct, overlapping coverage of different methods indicates no single method can give complete coverage of the phosphoproteome.¹⁹

Although not suited to high-throughput strategies, a strength of 2-D electrophoresis (2-DE) is the ability to qualitatively visualize the global protein content/population in a sample.

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This approach has previously been successful in the study of casein phosphorylation (or protein phosphorylation in general), where variable modification appeared as a train of spots with similar MW and differing pI .²⁰ In this report, we utilized 2-DE and MALDI-TOF/TOF to examine the distribution of α - and β -casein phosphoforms in human milk.

Experimental Section

Reagents and Samples. Human milk samples were obtained from 6 donors at various stages of lactation in Brisbane, Australia and stored at -80°C prior to analysis. *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (EZ-link biotin-HPDP), Immunopure immobilized avidin and D-Salt polyacrylamide 6000 desalting columns were obtained from Pierce (Rockford, IL). Immobililine Drystrips (24 cm) and IPG buffer (both pH 4–7) were purchased from Amersham Biosciences (Sydney, NSW, Australia). Proteomics grade modified porcine trypsin was obtained from Sigma-Aldrich (Sydney, NSW, Australia). All other reagents were of at least analytical reagent grade.

Enrichment. Milk samples were separated as previously described.²¹ Briefly, milk samples (4 mL) were removed from -80°C storage and placed in an ice bath prior to a pH adjustment to 4.3 with 56 μL of 1 M HCl to allow precipitation of caseins. After 1 h, the acidified milk samples were diluted 1:1 with 120 mM CaCl_2 and vortexed gently for 20 min.

Samples were centrifuged at 40 000g for 1 h at 4°C , and the milk fat, whey and casein fractions collected separately. The casein pellet was washed three times and dissolved in a reducing buffer consisting of 8 M urea, 4% CHAPS and 300 mM DTT buffered to pH 8 with 80 mM NH_4HCO_3 .

After incubation for 1 h at room temperature (RT), the reduced casein solution was passed through a D-salt column to remove DTT. Basically, a 10 mL polyacrylamide column was equilibrated with 50 mL of 2 M urea and 0.1% CHAPS solution prior to addition of the casein solution (1 mL). Ten discrete 1 mL washes with the same buffer were collected. The caseins which eluted in the void volume were identified by their milky appearance, presumably due to the persistence of micellar structures. Biotin-HPDP (100 μL of a 4 mM solution in DMF) was added to the first milky fraction and allowed to incubate at RT for 1 h. A second D-salt polyacrylamide column was used to separate unbound biotin-HPDP from biotinylated protein using the same method.

Immobilized avidin gel (500 μL) was washed with 4×1 mL aliquots of 2 M urea, 0.1% CHAPS and then the proteins in the first milky fraction (1 mL) from the second D-salt column were added and allowed to incubate for 1 h at RT. The supernatant was removed following centrifugation of the gel (2500g for 2 min). The avidin gel was thoroughly washed with 5×1.5 mL aliquots of 2 M urea, 0.1% CHAPS to remove nonbound proteins. Bound proteins were cleaved from the gel beads during a 20 min incubation with 500 μL of solubilization buffer comprising 8 M urea, 4% CHAPS, 0.001% bromophenol blue and 100 mM DTT.

2-D Electrophoresis. Solubilization buffer (390 μL) was used to dissolve 60 μL of whole human milk. A total of 450 μL of either the whole milk or enriched casein in solubilization buffer (as prepared above) was used to rehydrate 24 cm pH 4–7 IPG strips over a period of 8 h. Isoelectric focusing was performed on an Ettan IPGphor with voltage program 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 98 400 Vh with post-run voltage set at 100 V.

IPG strip equilibration and electrophoresis procedures were performed as described previously.²² IPG strips were reduced during a 10 min exposure to equilibration buffer (6 M urea/2% SDS/20% glycerol/0.375 M Tris-HCl, pH 8.8) containing 15 mg/mL DTT, then alkylated during a 20 min exposure to equilibration buffer containing 20 mg/mL iodoacetamide. Polyacrylamide gels (25.5 $\times$ 20 cm; 14%, 29:1 acrylamide/bis) were crowned with the IPG strips and set in 0.5% agarose containing 0.001% bromophenol blue. Electrophoresis occurred in an Ettan DaltSix apparatus at 10°C with current program 5 mA per gel for 2 h, 20 mA per gel for 14 h. Gels were stained with colloidal Coomassie brilliant blue G-250 (CBB). Scanned gel images were obtained using an Amersham Pharmacia flatbed scanner. Gel spot pI values were calculated upon their position assuming a linear gradient between the IPG strip holder electrodes for the pH range of the IPG strips.

In-Gel Digestion. Spots from 2-DE gels selected for further analysis were sampled using a ~ 2 mm diameter punch and gel pieces transferred to microfuge tubes. After rinsing with distilled water and undergoing multiple destaining washes in 40 mM NH_4HCO_3 /50% acetonitrile, the gel pieces were dehydrated in 100% acetonitrile. After removing the acetonitrile, 20 μL (200 ng) of modified porcine trypsin in 40 mM NH_4HCO_3 , pH 8, was delivered to each sample and incubated for 16 h at 37°C . After incubation, the trypsin solutions were collected and the remaining peptides were extracted from the gel pieces by incubating them in 5% formic acid/50% acetonitrile for 60 min prior to drying in a vacuum centrifuge. Samples were stored at 4°C prior to analysis.

MALDI-TOF/TOF Analyses. MALDI-TOF/TOF experiments were conducted on a Model 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). Tryptic digests were redissolved in 0.1% TFA/50% acetonitrile and mixed 1:1 with matrix consisting of 20 mg/mL DHB, 50% acetonitrile, 1% phosphoric acid and 5 mM NH_4HPO_4 prior to spotting on a sample plate. The sample plate was externally calibrated using the Mass Standards kit for the 4700 (Applied Biosystems, Foster City, CA) and each spectrum analyzed was additionally internally calibrated using trypsin, CBB or peaks matched to theoretical tryptic digests of human α_{s1} -casein (P47710) or β -casein (P05814) generated with PeptideMass (<http://expasy.org/tools/peptide-mass.html>) where applicable. MALDI-TOF spectra were acquired automatically in reflector positive operating mode with source voltage 20 kV and Grid1 voltage 12 kV, mass range 500–5000 Da, focus mass 1500 Da, collecting 4000 shots with a random edge bias laser pattern. MALDI-TOF/TOF spectra were acquired automatically with source voltage 8 kV and Grid1 voltage 6.8 kV, precursor mass window set to relative 30 fwhm, collecting 20 000 shots with acceptance criteria >60 base peak intensity to counter the uneven crystallization pattern produced with DHB.

Database Searching. After acquisition, spectra were handled using Data Explorer version 4.6 (build 111). Prior to PMF database searching, mass spectra were baseline corrected, Gaussian smoothed with filter width 5, and internally calibrated as above. Peaks with $S/N \geq 15$ were selected and deisotoped before monoisotopic peak lists were submitted to Aldente (<http://expasy.org/tools/aldente>) for searching the UniProtKB/Swiss-Prot database with nonstandard search parameters: *Homo sapiens* (predefined Taxon), $4 < pI < 7$, tryptic digestion with up to 2 missed cleavages, carbamidomethylation as a fixed modification, methionine oxidation and phosphorylation on serine or threonine as variable modifications.

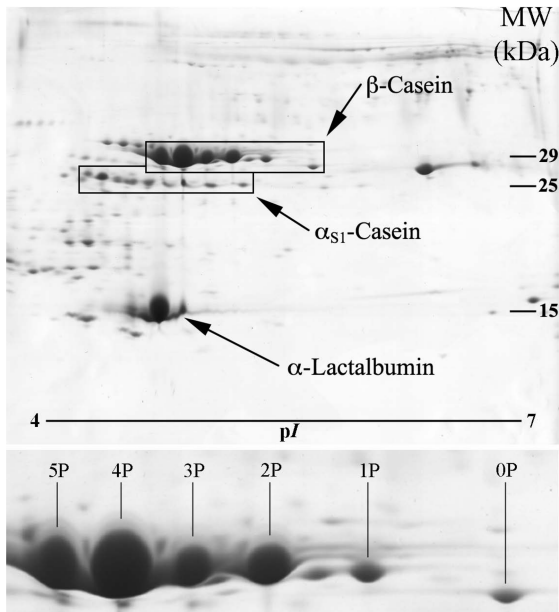
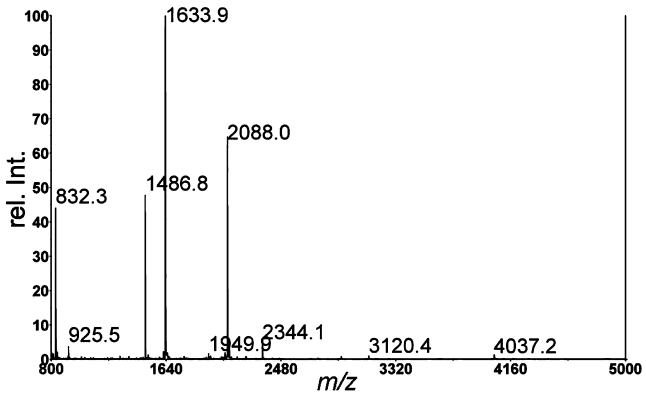


Figure 1. 2-D gel of whole human milk. Sixty microliters of milk (~600 μ g of protein) was solubilized as described in the Experimental Section. The first dimension was IEF on 24 cm pH 4–7 IPG strips and the second dimension was SDS-PAGE on 14% gels (25.5 $\times$ 20 cm). The major proteins are labeled and MW is indicated on the right-hand side. Lower panel shows the β -casein spots described in the text.

To confirm the assignment of phosphorylation sites, MS/MS data were smoothed, filtered and deisotoped as above. The peak lists were submitted to MS-Product (<http://prospector.ucsf.edu/>) along with the relevant putative fragment sequences using nonstandard search parameters: cysteines modified by carbamidomethylation, neutral loss H_3PO_4 and fragment tolerance 0.2 Da. MS/MS spectra were also manually inspected for ions that confirmed or contradicted the presence of phosphates (or neutral loss of phosphate) at the indicated sites.

Results

Analysis of β -Casein Phosphorylation. With the use of a top-down approach, the global protein complement in human milk was visualized via 2-DE. Figure 1 illustrates the typical separation achieved following analysis of a whole milk sample from a single donor 2 months postpartum. Milk samples from 5 other donors showed similar but not identical patterns. The spots corresponding to the casein phosphoforms described below were present in all the samples. The main features observed on the gel were spots representing β -casein, α_{S1} -casein and α -lactalbumin. Peptide mass fingerprinting of in-gel tryptic digests confirmed the presence of β -casein in the six spots indicated spanning $4.9 < pI < 5.8$ with MW ~29 kDa, and we sought to confirm whether these corresponded to the six phosphoforms previously reported.¹⁵ Phosphorylated peptides in β -casein tryptic digests were initially detected following MALDI-TOF MS experiments employing the matrix 2,5-dihydroxybenzoic acid with additives optimized for the detection of multiply phosphorylated peptides.^{23,24} The phosphorylated region of β -casein occurs in the N-terminal tryptic peptide indicated in Figure 2. Although we did not obtain full sequence coverage, the phosphorylation of this peptide alone was sufficient to explain all the phosphoforms of β -casein on the gel. As illustrated in Figure 3, the N-terminal β -casein tryptic



Exp (Da)	Theo (Da)	MC	Position	Sequence
2087.98	2087.99	1	16–33	RETIESLSSESSEESITEYK
2344.11	2344.15	2	16–35	RETIESLSSESSEESITEYKQK
1949.85	1949.86	1	39–54	VKHEDQQQGEDEHQDK
925.47	925.50	1	104–111	AKDTVYTK
1486.77	1486.79	0	120–132	SPTIPFFDPQIPK
1633.94	1633.94	0	176–189	VLPIPQQVVPYPQR
4037.19	4037.26	0	190–226	AVPVQALLNQELLNPTHQ IYPVTQPLAPVHNPIISV

1 *mkvlilac*lv *alala***RETIE** * **SLSSSEESIT** 30
31 **EYKQK**vek**VK** **HEDQQQGEDE** **HQDK**iypsfq 60
61 *pqpli*yfpve *pipyg*flpq*n* *ilpla*qpavv 90
91 *lpvp*qpeime *vpk***AKDTVYT** **K**grvmpvl**K**S 120
121 **PTIPFFDPQI** **PK**ltdlenlh *lpl*pllqplm 150
151 *qqvp*qpipqt *lalpp*qplws *vpqpk***VLPIP** 180
181 **QQVVPYPQRA** **VPVQALLNQ** **ELLNPTHQI** 210
211 **YPVTQPLAPV** **HNPIISV** 226

Figure 2. Identification of the unphosphorylated (0P) β -casein tryptic digest showing MALDI-TOF MS spectrum, peptides matched by Aldente, missed cleavages (MC) and sequence coverage. Asterisks indicate phosphorylation sites. Bold asterisks denote primary phosphorylation sites. Sequence coverage (51%) is indicated with bold upper case text, and signal peptide with italicized lower case. The peak at 832.3 Da is from CBB.

peptide ^{16}R – K^{33} increased in mass by 80 Da between consecutive β -casein spots from 2088 Da in the 0P (unphosphorylated) β -casein spot to 2488 Da in the 5P (penta-phosphorylated) spot. The intensity of the ion, relative to the main peak at m/z 1633.9, decreased with increasing phosphorylation level consistent with an inverse relation between phosphorylation level and ionization efficiency.

MALDI-TOF/TOF analysis of the respective peptides was used to examine the phosphorylation sites of the phosphoforms (Figure 4). For the 1P form (Figure 4B) an almost complete b-ion series was identified in the spectrum with the b8 fragment (916.6 Da) indicating no phosphorylation up to that point. However, the ions assigned as b11 and b12 ions showed an increase in mass of 80 Da compared to the unphosphorylated peptide in Figure 4A, consistent with phosphorylation of the serine in position 9 or 10 of the peptide (residue S^{24} or S^{25} of the protein). The presence of a nonmodified y9 (1085.5 Da)

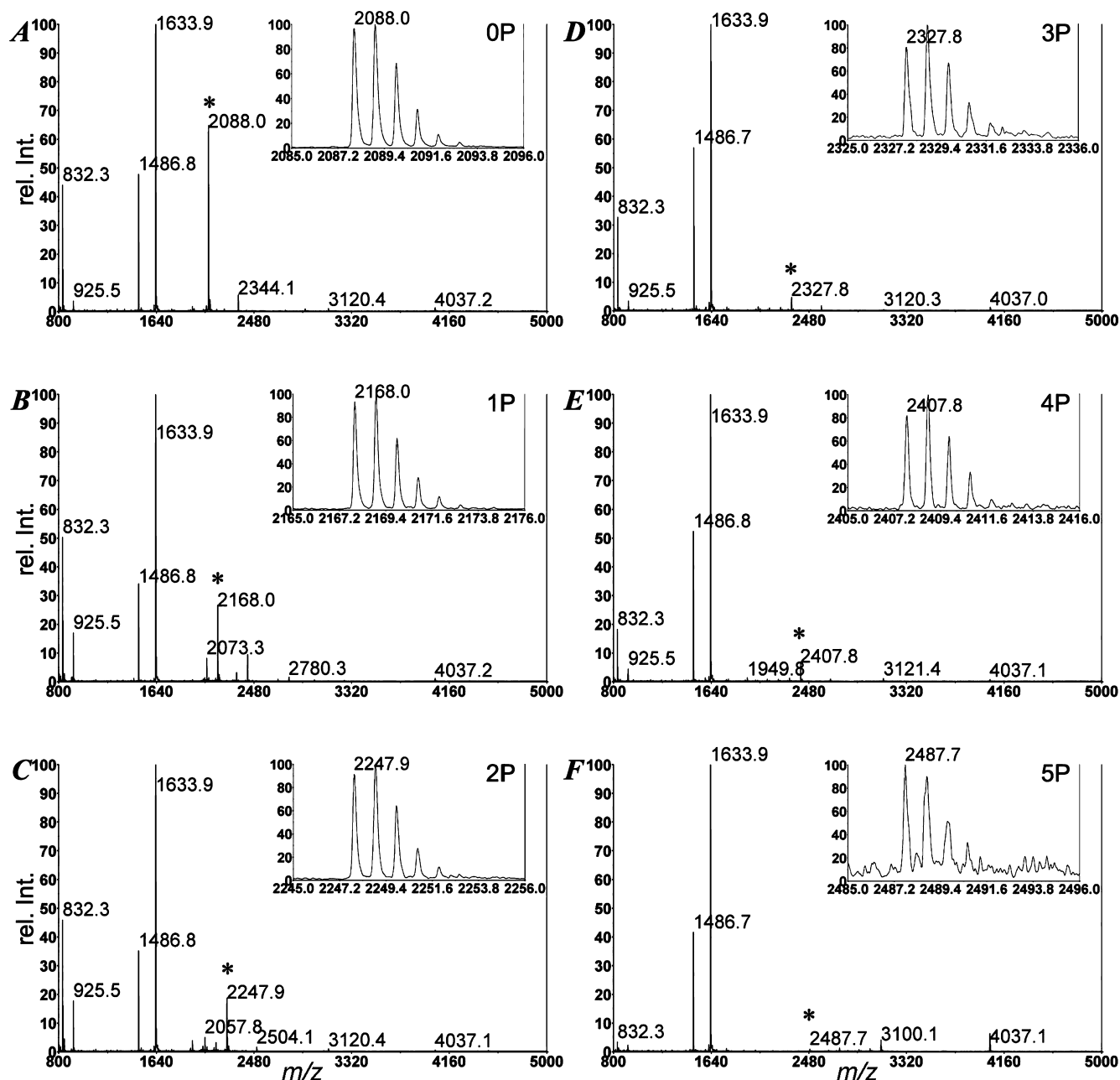


Figure 3. MALDI-TOF MS of β -casein tryptic digests. Phosphoforms with 0–5 phosphates (0P–5P) are shown in panels A–F, respectively. Asterisks indicate the tryptic peptide $^{16}\text{RETIESLSSEESITEYK}^{33}$ with up to 5 phosphates which is shown in the insets.

indicated phosphorylation at peptide residue 9 (S^{24}) rather than residue 10. We did not observe ions at 1083.5 or 1170.5 Da, corresponding to phosphorylated b9 or b10 respectively, but there were ions at 985.6 Da ($\text{b9-H}_3\text{PO}_4$) and 1072.6 Da ($\text{b10-H}_3\text{PO}_4$) indicating some loss of phosphate during MS/MS. The assignment of these ions as dephosphorylated arising from loss of phosphate from a phosphoserine at residue 9, rather than dehydrated arising from loss of H_2O from an unphosphorylated serine at residue 9, was supported by the lack of prominent dehydrated ions in the spectrum of the unphosphorylated peptide in Figure 4A. In the 2P form (Figure 4C), the same b8 (916.6 Da) and b9- H_3PO_4 (985.6 Da) ions present in the MS/MS spectrum of the 1P form were present in the spectrum of the 2248 Da precursor, whereas b10- H_3PO_4 (1152.6 Da) and y9 (1165.6 Da) were 80 Da higher indicating that the phosphorylated residues in this case are S^{24} and S^{25} . In the 3P form

(Figure 4D), ions from b4-b7 and y9 had the same mass as the 2P form. An increase in mass of 80 Da for b9- H_3PO_4 (1065.6 Da) and b10- H_3PO_4 (1232.7 Da) as well as a new ion at 898.6 Da ($\text{b8-H}_3\text{PO}_4$), indicated that residue 8 of the peptide (S^{23}) was phosphorylated along with S^{24} and S^{25} . The MS/MS spectrum of the 4P form (Figure 4E) contained a nonmodified b5 (629.3 Da) together with an increase in mass of 80 Da for b8- H_3PO_4 (978.6 Da). A new ion consistent with b6- H_3PO_4 (698.4 Da) indicated an additional phosphorylation on residue 6 (S^{21}). Finally fragmentation of the 5P form (Figure 4F) produced b4 (580.3 Da) and b5 (709.3) ions with an increase in mass of 80 Da as well as new ions at 369.1 Da ($\text{b3-H}_3\text{PO}_4$) and 482.3 Da ($\text{b4-H}_3\text{PO}_4$), all consistent with the presence of a fifth phosphorylation site on residue 3 of the peptide (T^{18}). Taken together, the ions present in spectra of all the phosphoforms allowed the unambiguous assignment of the order of

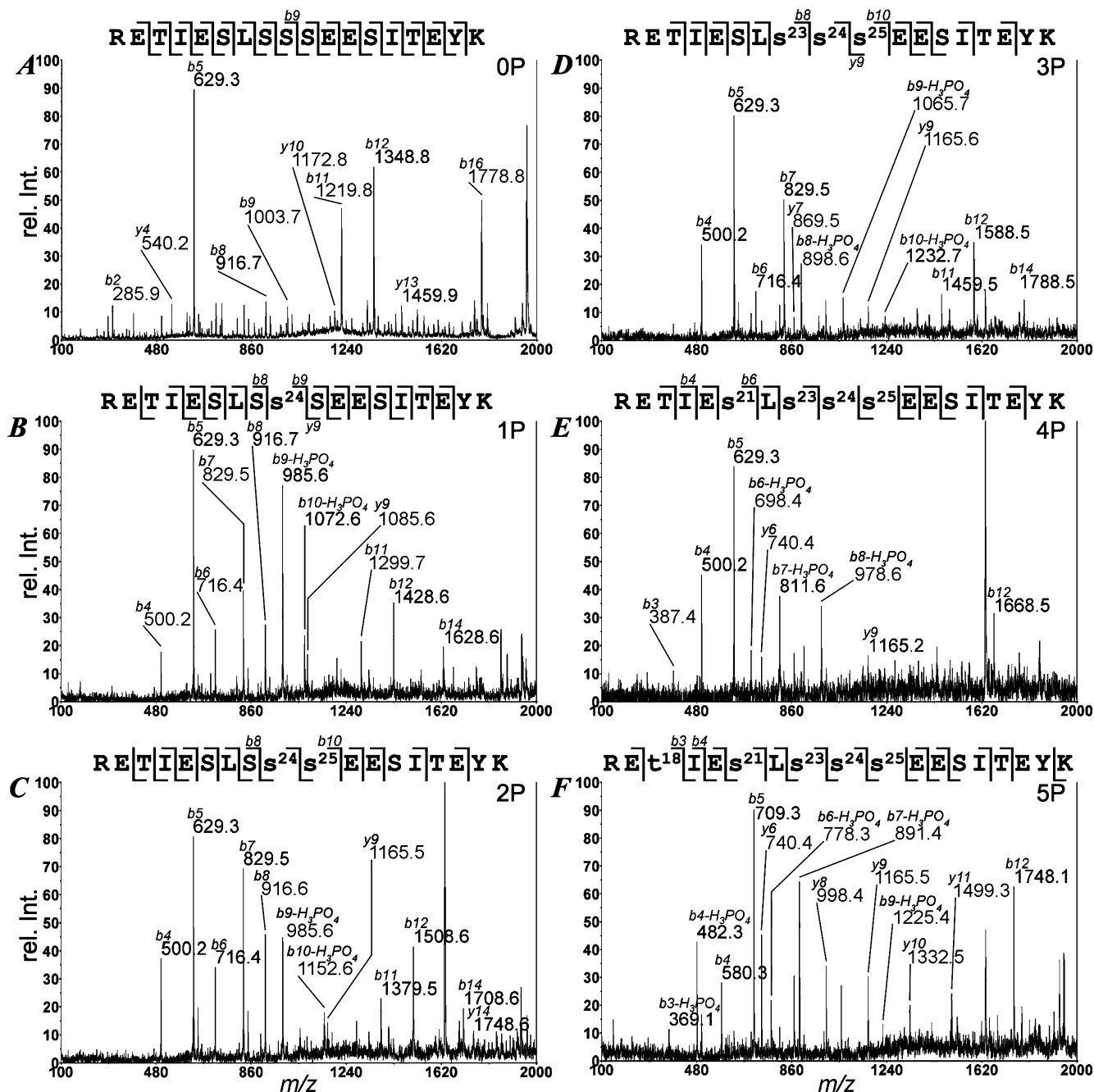


Figure 4. MALDI-TOF/TOF MS of β -casein phosphopeptides. Panels A–F show the peptide, $^{16}\text{RETIESLS}[\text{S}^{24}]\text{S}^{25}\text{EESITEYK}^{33}$, with 0–5 phosphates, respectively. Fragment ions and sequence ladders showing the phosphorylated residues are indicated in each panel.

phosphorylation as S²⁴, followed by S²⁵, followed by S²³, followed by S²¹ and finally T¹⁸, consistent with published data.¹⁵

α_{S1} -Casein Phosphorylation. Interrogation of the UniProtKB/Swiss-Prot database with PMF data from the train of spots beneath β -casein with $4.4 < \text{pI} < 5.3$ revealed their identities as α_{S1} -casein; however, due to poor sequence coverage and signal strength, it was not possible to speculate upon their structural differences. As shown in Figure 1, β -casein spots resolve in close proximity to α_{S1} -caseins following 2-DE, and due to their high concentration, the resolution of α_{S1} -casein isoforms are affected. To reduce the relative amounts of β -casein present in the sample, ultracentrifugation and cysteine-tagging enrichment strategies were implemented prior to typical 2-DE separation and analysis. The results from this approach are shown in Figure 5, where the exclusion of the

major interfering proteins resulted in a greatly improved visualization of α_{S1} -casein compared to the separation achieved using whole milk. Three distinct trains of 9 spots were observed with $4.4 < \text{pI} < 5.3$ and different apparent molecular weights. The major train of spots resolved at the same molecular weight as α_{S1} -casein on the whole milk 2-DE gel at 25 kDa. Spots within the upper train appeared at similar pI values to the main train; however, spots in the lower train were shifted to the right, indicating an increase in pI.

The increased resolution of α_{S1} -casein isoforms potentiated their further examination via MALDI-TOF and MALDI-TOF/TOF experiments. After tryptic digestion of the spots following pretreatments and 2-DE separation, MALDI-TOF analyses were conducted and querying the UniProtKB/Swiss-Prot database using Aldente confirmed the identity of all spots as being α_{S1} -

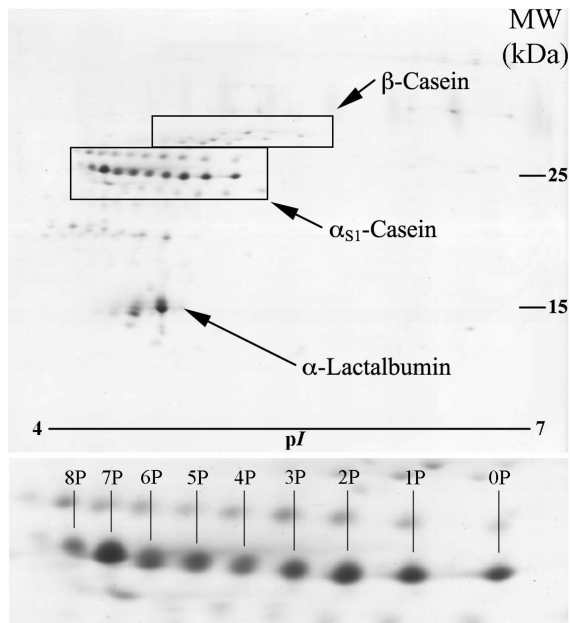
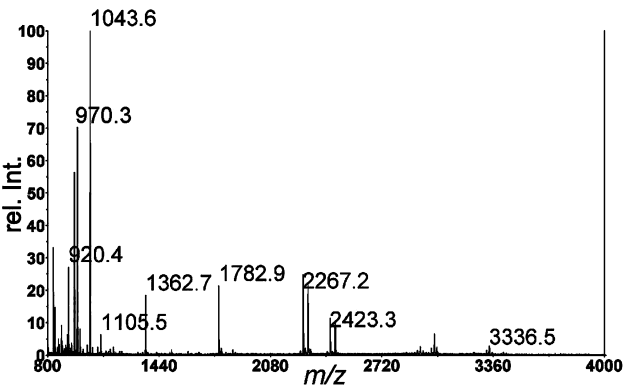


Figure 5. 2-D gel of the enriched casein sample. The first dimension was IEF on a 24 cm pH 4–7 IPG strip loaded with the protein recovered from 450 μ L of avidin beads. The second dimension was SDS-PAGE on 14% gels (25.5 $\times$ 20 cm). The major proteins are labeled and MW is indicated on the right-hand side. The lower panel shows the α_{S1} -casein spots described in the text.

casein. Figure 6 illustrates a typical MALDI spectrum along with the list of matched peptides. The typical sequence coverage achieved is illustrated for the unphosphorylated α_{S1} -casein, appearing at the highest pI of the major train observed with 2-DE. The tryptic peptide in the region $^{126}\text{M}-\text{K}^{178}$ was not observed in MALDI experiments for any of the α_{S1} -casein spots, consistent with other studies.¹⁷ Among the peptides matched in PMF analyses of the various α_{S1} -casein spots were those containing the serine-rich putative phosphodomains, $^{27}\text{LQNPSSESSEPIPLESR}^{42}$, $^{27}\text{LQNPSSESSEPIPLESREEYMNGMNR}^{51}$ and $^{69}\text{NESTQNCVVAEPEKMESSISSSSSEMSLSK}^{98}$. The MS spectrum from each spot in the major train was examined for these peptides and their phosphorylated forms (Figure 7). While $^{27}\text{L}-\text{R}^{42}$ was prominent in the unphosphorylated form of α_{S1} -casein, $^{27}\text{L}-\text{R}^{51}$ was more prominent in the phosphorylated forms. The isotopic profile of the $^{27}\text{L}-\text{R}^{51}$ peptide was not as expected. The intensity of the monoisotopic peak was much lower, relative to the ^{13}C peak, than the theoretical distribution predicted (see Supplementary Figure S1 in Supporting Information). This was indicative of partial deamidation of the peptide and is considered further below. Discrete increases of 80 Da to each peptide indicated additional phosphorylations and $^{27}\text{L}-\text{R}^{51}$ had a maximum of three phosphorylations, while $^{69}\text{N}-\text{K}^{98}$ was modified with a maximum of five phosphorylations. The degree of phosphorylation on α_{S1} -casein varied from 0P (unphosphorylated) to 8P (octo-phosphorylated) and the two phosphodomains appeared to be independently phosphorylated. Gel spots corresponding to phosphoforms 1P to 6P were comprised of proteins phosphorylated to differing degrees in each phosphodomain, but with the same total number of phosphorylations. The exception was a persistent low level of unphosphorylated $^{27}\text{L}-\text{R}^{51}$, probably a result of either minor loss of labile phosphate during MS or the comigration of deamidated protein with one less phosphate. Unfortunately, these peaks were too



Exp (Da)	Theo (Da)	MC	Position	Sequence
1043.60	1043.60	1	19-26	LPLRYPER
1782.88	1782.89	0	27-42	LQNPSSESSEPIPLESR
1362.68	1362.69	2	58-68	EKQTDEIKDTR
1105.54	1105.55	1	60-68	QTDEIKDTR
3336.55	3336.44	1	69-98	NESTQNCVVAEPEKMESSISS SSSEMSLSK
970.39	970.39	0	99-105	CAEQFCR
2267.15	2267.15	0	106-124	LNEYNQLQLQAAHAQEQR
2423.26	2423.25	1	106-125	LNEYNQLQLQAAHAQEQIRR
920.42	920.43	0	179-185	NNVMQLQW

1 *mrlliltclv* *avalarpkLP* **LRYPER***LQNP* 30
31 ***S***ESSEPIPLE* ***S***Reeymngmn* *rqrnilr***EKQ** 60
61 **TDEIKDTRNE** **STQNCVVAEP** **EKMESSI****SSS** 90
91 ***SEEMSLSKCA** **EQFCRLNEYN** **QLQLQAAHAQ** 120
121 **EQIRR***mnens* *hqvvpfqqln* *qlaaypyavw* 150
151 *yypqimqyvp* *fppfsdisnp* *tahenyek***NN** 180
181 **VMLQW** 185

Figure 6. Identification of 0P α_{S1} -casein tryptic digest showing MALDI-TOF MS spectrum, peptides matched by Aldente and sequence coverage. Asterisks indicate phosphorylation sites. Bold asterisks denote primary phosphorylation sites. Sequence coverage (54%) is indicated with bold upper case text, and signal peptide with italicized lower case.

low in intensity for reliable interpretation of the isotopic pattern. Upon reaching maximum phosphorylation occupancy on $^{69}\text{N}-\text{K}^{98}$ in the 7P phosphoform, the eighth phosphorylation was shown to be located in $^{27}\text{L}-\text{R}^{51}$. The sequence coverage for α_{S1} -casein was not complete, so the possibility of phosphorylation at other sites exists. However, the sites identified above were sufficient to explain all the phosphoforms observed on the gel.

MALDI-TOF/TOF analysis was conducted on the N-terminal α_{S1} -casein peptide(s) $^{27}\text{L}-\text{R}^{42}$ and $^{27}\text{L}-\text{R}^{51}$ to determine the phosphorylation sites (Figure 8). In Figure 8B, the MS/MS spectrum of the monophosphorylated $^{27}\text{L}-\text{R}^{51}$ peptide is shown. The presence of y10 (1332.5 Da) and y11 (1499.5 Da) ions indicated the presence of a phosphoserine at position 15 in the peptide, corresponding to phosphorylation of the intact protein at S41. All other ions observed were consistent with the above assignment. A similar set of y-ions were observed for the doubly phosphorylated peptide $^{27}\text{L}-\text{R}^{51}$ in Figure 8C.

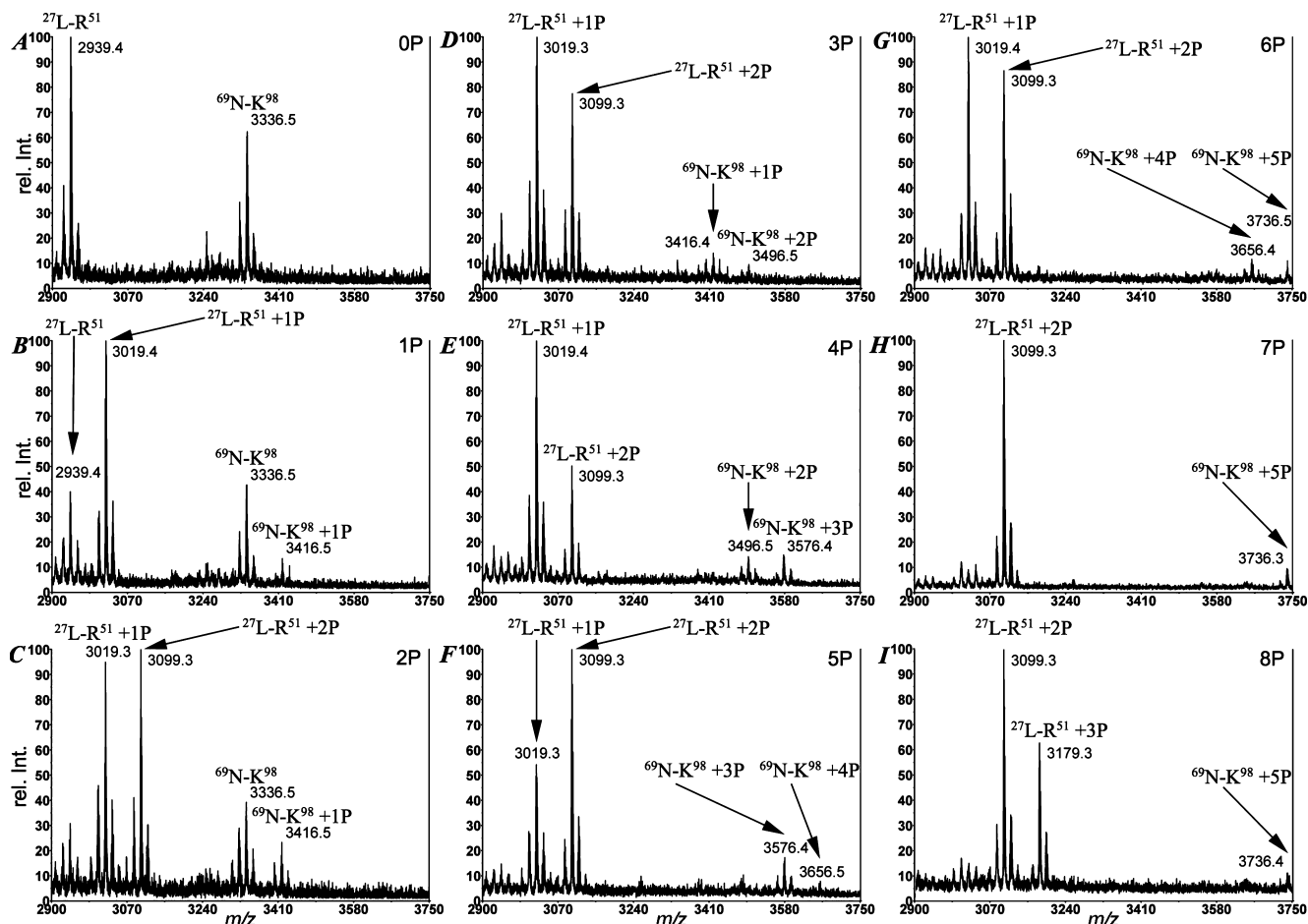


Figure 7. MALDI-TOF MS of α_{S1} -casein tryptic digests. The distribution of phosphate groups between the two phosphorylation domains corresponding to peptides, ^{27} LQNPSESSEPIPLESREEYMNGmNR⁵¹ and ^{69} NESTQNCVVAEPEKmESSISSSEEmsLSK⁹⁸, for α_{S1} -casein phosphoforms with 0–8 phosphates are shown in panels A–I, respectively. Lower case “m” indicates an oxidized methionine residue.

The y10 (1332.4 Da) and y11 (1499.4 Da) ions confirmed the phosphorylation on S41, while y18 (2264.8 Da) and y19 (2431.8 Da) distinguished S³³ as the location of a second phosphorylation site. The spectrum of the triply phosphorylated peptide in Figure 8D showed the same sequential y18 (2264.8 Da) and y19 (2431.9 Da) ions as the doubly phosphorylated form whereas the y22 ion (2825.1 Da) showed an increase of 80 Da allowing the assignment of the third phosphorylation site to S³¹.

The peptides selected for the MS/MS analysis shown in Figure 8 panels B–D (^{27} L–R⁵¹ with varying phosphorylation) all showed the altered isotopic profile noted above and in Supplementary Figure S1 in Supporting Information. The profile is indicative of partial deamidation producing aspartate from asparagine or glutamate from glutamine with a concomitant increase in mass of 1 Da. The apparent distortion of the isotopic profile arises from the overlap of the isotopic envelopes of the normal and the deamidated forms. The broad precursor mass window (used to maximize the signal intensity) resulted in both peptides being selected and the MS/MS spectra were dominated by the more abundant form. A closer look at fragment ion masses in Figure 8 showed that y4 was present at the expected mass (493.2 Da), whereas y5 (608.2 Da) and larger y-ions were 1 Da higher than expected. In panels B and C, N⁴⁷ is bracketed by y4 (493.2 Da) and y5 (608.2 Da) ions; however, confirmation of the deamidated residue's location in

panel D relied upon y4 (493.2 Da) and y7 (918.2 Da) fragment ions. Inset spectra in Figure 8 panels B–D show the isotopic profile of the y7 ion at 918.2 Da (expected 917.36) indicating deamidation in all 3 samples.

Discussion

In the initial separation of human milk with 2-DE, resolution of β -casein phosphoforms was clearly achieved. With the use of standard in-gel tryptic digestion and MALDI-TOF/TOF techniques, the location of phosphorylation sites and their order of modification among all phosphoforms confirmed the results obtained by Greenberg et al., with primary serines occupied prior to secondary serines, and last, a primary threonine.¹⁵

Having demonstrated the ability of MALDI-TOF/TOF to give sequence coverage on a peptide with up to 5 phosphorylations, we sought to analyze less abundant caseins present in human milk. To decrease the relative concentrations of α -lactalbumin and β -casein and successfully visualize α_{S1} -casein isoforms, we employed an enrichment strategy prior to 2-DE analysis. With the use of published methodology, caseins were precipitated from whole milk at low pH with calcium added prior to ultracentrifugation and electrophoresis.²¹ Whereas relatively large amounts of the cysteine-containing whey protein, α -lactalbumin, were present in the whole milk sample, following

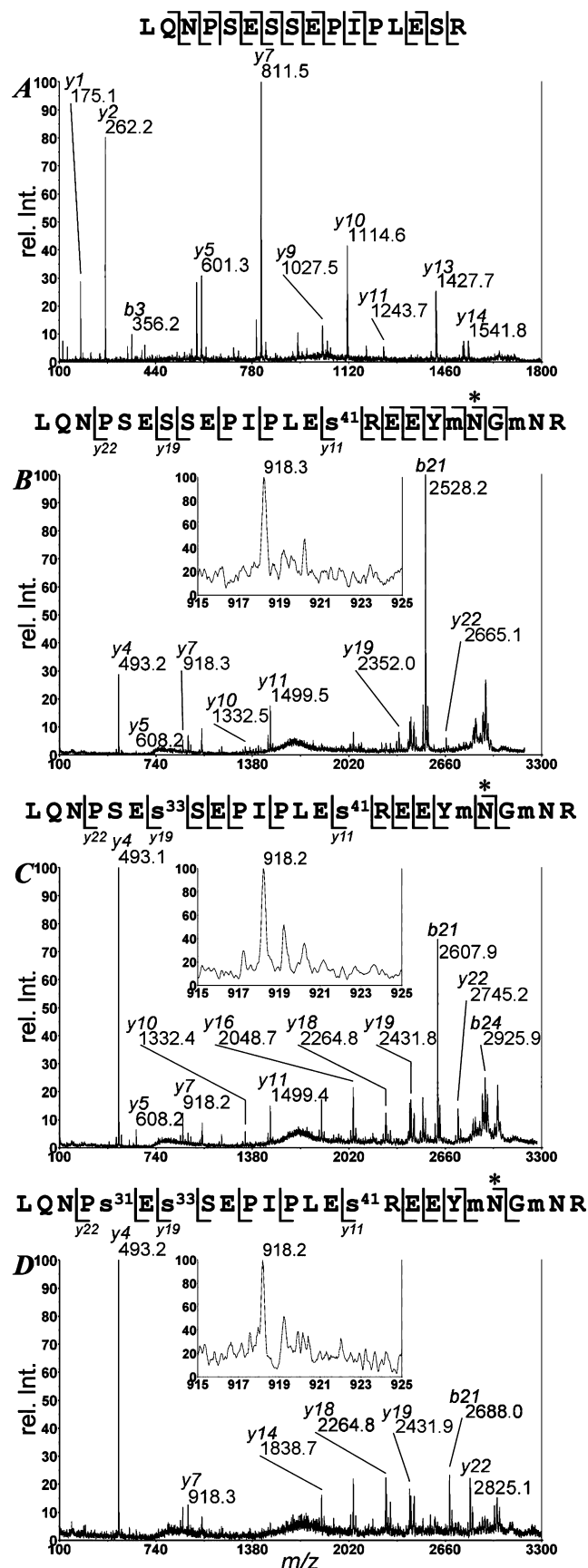


Figure 8. MALDI-TOF/TOF MS spectra showing sequence assignment of the α_{s1} -casein tryptic phosphopeptide, $^{27}\text{LQNPSESEPIPLESR}^{42}(\text{EEYMNGmNR})^{51}$, with 0–3 phosphates in panels A–D, respectively.

this procedure, most of it was removed. α_{s1} -Casein constitutes approximately 5% of the total protein content in mature human milk; however, it is spread out over a large number of spots on a 2-D gel with varying concentration. Still present in the sample following this treatment, however, were significant levels of the various β -casein isoforms in close proximity to α_{s1} -casein spots. Human β -casein lacks cysteine residues, whereas α_{s1} -casein contains three, and this difference was exploited through the use of a cysteine-tagging enrichment procedure utilized in similar circumstances in the study of bovine milk caseins.²² Following these treatments, a greatly improved resolution was achieved, allowing a more thorough characterization of the less abundant α_{s1} -casein components than previously reported.

Following the enrichment procedures, the most prominent feature of 2-DE analyses was a train of 9 α_{s1} -casein spots with pI values varying from 4.4 to 5.3. Alongside the darkest train were faint trains of 9 spots appearing above and below the major α_{s1} -spots with similar pI values and characteristic spacing on the 2-D gel. Peptide mass fingerprinting of these spots confirmed their identities as also being α_{s1} -casein (data not shown); however, the key structural differences allowing their separation from one another on a 2-D gel are still under investigation. While the central and upper trains of α_{s1} -casein closely mirror each other in terms of pI spacing, spots in the corresponding lower molecular weight train appear at higher pI . Given the similar patterns and number of spots observed via 2-DE, it is likely that both phosphorylated regions in the major form of the α_{s1} -casein are retained in the minor forms. A number of splice variants of human α_{s1} -casein are known, and the change in pI and apparent molecular weight of the lower train of spots is consistent with one of these (P47710-3). Characterization of the higher molecular weight train is ongoing.

In this study, we detected α_{s1} -casein from a single donor's milk sample and identified the numerous phosphoforms of α_{s1} -casein present via 2-DE and MALDI-TOF/TOF. Although analysis of only a single donor's milk sample is reported in this article, the gel spot patterns described were consistent among numerous milk samples from other donors. The primary phosphoacceptor sites designated by the mammary gland casein kinase are serines or threonines two residues on the N-terminal side of glutamate or aspartate residues (S/T)X(E/D), and secondary phosphoacceptor sites are serines or threonines two residues on the N-terminal side of phosphorylated serines (S/T)XpS.^{25,26} As a result, there are some phosphorylation sites that cannot be occupied prior to others. Secondary phosphorylation sites are present in both of the α_{s1} -casein phosphopeptides, $^{27}\text{LQNPSESEPIPLESR}^{42}(\text{EEYMNGmNR})^{51}$ and $^{69}\text{NESTQNCVVAEPEKMESSISSSEEMSLSK}^{98}$; however, each also has more than one primary phosphorylation site. Therefore, the order of phosphorylation among putative phosphorylation sites on each of the peptides could not be predicted solely upon the mechanism of the casein kinase specificity described above.

Initial MALDI-TOF experiments provided enough data to speculate upon the order of phosphorylation among the potential phosphorylation sites on α_{s1} -casein, and it was found that the two regions were independently phosphorylated. The doubly phosphorylated α_{s1} -casein spot excised from the 2-D gel, for example, comprised proteins with both $2\text{P-}^{27}\text{L-K}^{51}/\text{OP-}^{69}\text{N-K}^{98}$ and $1\text{P-}^{27}\text{L-K}^{51}/1\text{P-}^{69}\text{N-K}^{98}$. Upon the basis of the published understanding of the mammary gland casein kinase and our MALDI data, Table 1 shows an overview of the predominant phosphorylation pattern among α_{s1} -casein isoforms.

Table 1. The phosphorylation pattern of human α_{S1} -casein isoforms

α_{S1} ^a	relative abundance ^b	L-R ^c	N-K ^d	phosphorylated sites
0	9.3 $\pm$ 2.1%	0	0	
1	12.9 $\pm$ 2.3%	1	0	41
		0	1	90 or 91
2	14.7 $\pm$ 1.5%	1	1	41 and (90 or 91)
		2	0	33 and 41
3	10.2 $\pm$ 0.8%	1	2	41, 90 and 91
		2	1	33, 41 and (90 or 91)
4	9.8 $\pm$ 0.8%	1	3	41, (88 or 89), 90 and 91
		2	2	33, 41, 90 and 91
5	12.0 $\pm$ 1.5%	1	4	41, 88, 89, 90 and 91
		2	3	33, 41, (88 or 89), 90 and 91
6	11.5 $\pm$ 1.5%	1	5	41, 86, 88, 89, 90, 91
		2	4	33, 41, 88, 89, 905 and 91
7	15.4 $\pm$ 2.6%	2	5	33, 41, 86, 88, 89, 90, 91
8	4.2 $\pm$ 1.2%	3	5	31, 33, 41, 86, 88, 89, 90 and 91

^a Number of phosphates on the intact protein. ^b Percentage of total α_{S1} -casein determined using Imagemaster 2-D Platinum software (mean $\pm$ standard deviation of triplicate gels). ^c Number of phosphates on the peptide ²⁷LQNPSESSEPIPLESR⁴²(EEYMNGMNR⁵¹). ^d Number of phosphates on the peptide ⁶⁹NESTQNCVVAEPEKMESSISSSSEEMSLSK⁹⁸.

In the case of the unphosphorylated form of α_{S1} -casein (appearing at the most basic end of the train of spots), the signal from the unphosphorylated peptide ²⁷L-R⁴² with no missed cleavage was the most intense peptide covering that region of the sequences. In contrast, analyses of spots containing the mono-, di-, and triphosphorylated forms of that peptide indicated that the longer peptide ²⁷L-R⁵¹ was more abundant. Previous studies have shown that trypsin activity can be restricted by nearby phosphorylation.^{27,28} This hinted at the possibility that the first phosphorylation occurred on S⁴¹, possibly inhibiting the action of trypsin at the adjacent arginine residue and resulting in a greater proportion of peptides with a missed cleavage at this position. As illustrated in Figure 8, MALDI-TOF/TOF experiments conducted on ²⁷L-R^{42/51} with 0–3 phosphorylations bolstered this theory, revealing the distinct order of phosphorylation among the phosphoforms present in tryptic digests from the various α_{S1} -casein spots as being S⁴¹ > S³³ > S³¹. In the case of this tryptic phosphopeptide, both primary phosphoacceptor sites S⁴¹ and S³³ were occupied before the secondary site.

It is widely recognized that phosphopeptides can be difficult to observe via MS, and even more difficult to obtain useful information from in MS/MS experiments. Because of its relatively large size (>3000 Da), multiple phosphorylations and variably oxidized methionines, we were unable to unambiguously characterize the order or precise location of phosphorylated residues on the larger phosphopeptide, ⁶⁹NESTQNCVVAEPEKMESSISSSSEEMSLSK⁹⁸ using MALDI-TOF/TOF. The peptide ⁸³M-K⁹⁸, which presumably would have been more useful in MS/MS experiments, was observed only occasionally in the unphosphorylated form (data not shown). It may be possible that phosphorylation can have effects on trypsin cleavage even 8 or 9 residues downstream of the cleavage site. Nevertheless, the phosphorylation pattern can be deduced in part from the sequence and results from the other peptide. S⁹⁰ and S⁹¹ would need to be phosphorylated first to generate the secondary sites at S⁸⁸ and S⁸⁹. The last phosphorylation would almost certainly be S⁸⁶ as it must be preceded by S⁹⁰ and then S⁸⁸.

Except in the case of the cow (*Bos taurus*), α_{S1} -caseins from the milk of other mammals are known to be hypophosphorylated, with maximum phosphorylation site occupation varying from 6 to 12 phosphates per molecule. In a study of Haflinger mares' milk, 2-DE analysis revealed the presence of two distinct trains of spots with 4.4 < pI < 6.3 attributed to α_{S1} -casein, mirroring the pattern observed for human milk observed in this study.¹¹ It was proposed that the isoelectric variants were due to differing degrees of phosphorylation; however, the structural differences between the two trains were not examined experimentally or discussed.

Evidence of deamidation was present in all α_{S1} -casein spots. This was demonstrated in TOF/TOF analyses of ²⁷LQNPSESSEPIPLESREEYMNGMNR⁵¹ revealing evidence of deamidation at asparagine⁴⁷. As the deamidated residue increases the acidity of the peptide, the gel spot attributed to the 1P isoform of α_{S1} -casein therefore consisted of both nondeamidated 1P α_{S1} -casein and deamidated 0P α_{S1} -casein. We therefore hypothesize that the presence of a faint 10th spot at the acidic end of the α_{S1} -casein train is due to the deamidated 8P isoform of α_{S1} -casein.

Deamidation has recently also been shown to be a natural feature in β -casein from mares' milk,²⁰ and therefore, deamidation could conceivably be a common feature of caseins from other species, and many other proteins in milk. The most common sites for deamidation within protein sequences in order of abundance are NG > NS > NA, occurring on the asparagine.²⁹ Both the deamidation occurring in equine β -casein and the human α_{S1} -casein described in this paper are located at an NG site. Compared to all other studied species, only the human α_{S1} -casein has an NG site; however, many NS sites appear within the sequences of α_{S1} -casein from other species. It is possible that these sites could be deamidated; however, in bovine milk which is perhaps the best studied, α_{S1} -casein contains three such NS sites and deamidation at these sites has never been reported. However, given the level of spontaneous deamidation observed at the NG site with equine β -casein incubated at 37 °C,²⁰ it is likely that some of the deamidation we observed in α_{S1} -casein could have occurred during the trypsin digest. This is supported by the presence of deamidation in the α_{S1} -casein 0P gel spot.

In previous studies on human α_{S1} -casein, the common approach was to utilize "bottom-up" separation strategies prior to characterization of milk components. Reliance on this approach results in predictions of a sample's properties based on an unrepresentative portion of that sample. When the milk samples were digested prior to their characterization, important information about the relative quantities of related components was lost. Initial work by Sorensen et al. concluded that the isoforms of α_{S1} -casein in human milk are limited to those with 0, 1 or 2 phosphates, all of these being located in a serine-rich tryptic peptide with features highly conserved among the N-termini of homologous mammalian proteins.¹⁶ The second serine-rich domain, which is conserved among many other mammalian species (represented by the peptide ⁶⁹N-K⁹⁸ in this study), was not found to be modified in the human milk samples analyzed, and it was concluded that this was a key difference between human and other α_{S1} -caseins.

However, in another study focusing on the potentially bioactive milk-derived peptides present in human milk, intact proteins, assigned identities as α_{S1} -casein, were detected via mass spectrometry indicating modification with 9 or 10 phosphates.³⁰ The justification for the assignment of these phos-

phorylation states was based on the fact that peaks with masses consistent with α_{S1} -casein and at molecular weights of +80 Da (and multiples thereof) were detected. As determined from the primary structure of α_{S1} -casein, only 9 possible phosphorylation sites can be identified as fulfilling the required (S/T)X(E/D/pS) phosphorylation motif. Only 4 peptides detected in the milk sample studied included those assigned with identities of the α_{S1} -casein peptide $^{61}\text{T}-\text{C}^{99}$ with 2–5 phosphates, presumably present due to the action of plasmin. Unfortunately, no supporting evidence was provided for these assignments. As the samples in that study were obtained in the first week after parturition, the differences between their results and ours may reflect the stage of lactation.

Recent work by Kjeldsen et al. uncovered the presence of a single phosphorylation S^{90} on the second serine-rich domain conserved among other mammalian species, in disagreement with Sorensen et al.'s key finding on the exclusivity of phosphorylation in a single domain.¹⁷ While the authors acknowledged a number of limitations in the ability to measure phosphorylation site occupancy, they provided estimates for the 3 sites, S^{33} , S^{41} and S^{90} (7, 20 and 27%, respectively). Our results suggest these values were grossly underestimated. For example, S^{41} appears to be phosphorylated in the 2P–8P forms and partially in the 1P form. From the relative abundances of the phosphoforms in Table 1, it is apparent that the occupancy would be between 80 and 90%, not the 20% estimated previously. Clearly, there is a distinct advantage in being able to array the global pattern of phosphoforms on a 2-D gel and assess the phosphopeptides and phosphorylation sites of each form individually rather than speculate on what forms may be present based on the subfraction of phosphopeptides identified during LC-MS(MS) procedures.

Casein micelles are supramolecular structures composed predominantly of caseins and calcium phosphate, and while studied for over 50 years, the structural details have still to be fully elucidated.^{31–33} The driving forces in micelle assembly are hydrophobic interactions between the caseins and electrostatic interactions between the phosphoserine clusters and calcium phosphate. In the broader biochemical context, the results presented here raise some interesting questions. For instance, how do mammary epithelial cells manage to synthesize the same protein in so many different phosphorylation states and what role do the differently phosphorylated forms play in casein micelle assembly and structure? It is also important to recognize that casein micelles are not just a source of amino acids and calcium phosphate for infant nutrition, but also a source of bioactive peptides that are released during digestion and can potentiate behavioral, gastrointestinal, hormonal, immunological, neurological and nutritional responses.^{1,34} It is apparent that the repertoire of casein phosphopeptides produced during milk digestion will be very different for breast-fed infants compared to those fed formula or cow's milk. Whether or not this could have any impact on infant nutrition and development is unclear, but given the many bioactivities ascribed to peptides, particularly phosphopeptides, derived from milk proteins, it would appear to warrant further investigation.

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Supporting Information Available: Spectral evidence for deamidation of α_{S1} -casein. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Figure S1. Spectral evidence for deamidation of α_{S1} -casein: MALDI-TOF MS of α_{S1} -casein tryptic digest highlighting the peptide $^{27}\text{LQNPSESSEPIPLESREEYmNGmNR}^{51}$ and showing the experimental isotopic distribution (left inset) compared to the theoretical isotopic distribution (right inset). Partial deamidation, $\text{N}^{47} > \text{D}^{47}$, with an increase in mass of 1 Da, results in overlapping isotopic envelopes for the native and deamidated forms of the peptide.

