

# Human-milk proteins: analysis of casein and casein subunits by anion-exchange chromatography, gel electrophoresis, and specific staining methods<sup>1-3</sup>

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**ABSTRACT** Casein in human milk is believed to serve several biological functions in newborns. However, the content and subunit composition of human casein has so far received little attention. We recently developed a method to separate human-milk whey and casein by adjustment of whole human milk to pH 4.3 and addition of calcium followed by ultracentrifugation. In this study we analyzed and evaluated human casein prepared by different methods. We used fast protein liquid chromatography (FPLC) with an anion-exchange column (Mono-Q) and polyacrylamide gradient gel electrophoresis techniques to analyze the casein subunit composition. Total casein in human milk, as determined by the Kjeldahl method, varies during lactation; the casein content is ~20% of the total protein content in early lactation and 45% in late lactation. We found differences in both glycosylation and phosphorylation patterns of  $\kappa$ -caseins and  $\beta$ -caseins from premature and term milk samples. *Am J Clin Nutr* 1990;51:37-46

**KEY WORDS** Human-milk proteins, casein,  $\kappa$ -casein,  $\beta$ -casein, casein subunits, FPLC anion-exchange chromatography, gel electrophoresis

## Introduction

Human casein and its subunits represent the least understood and characterized class of proteins in human milk; for example, little is known with regard to micelle and submicelle assembly, variations in phosphorylation of  $\beta$ -casein or glycosylation of  $\kappa$ -casein, or the total amount of casein in human milk. In addition, the fundamental questions with regard to physiological functions of casein, casein subunits, and casein fragments need considerable further study (1). There are some reports that casein phosphopeptides influence the intestinal absorption of calcium (2-4) and that these peptides may have a regulatory role in the mineralization process of calcified tissues (2, 5). The complex carbohydrate composition (6-8) and functions of human  $\kappa$ -casein need to be investigated further. The discovery of casein fragments with opioid-like activities (9-12) or immunostimulatory properties (13-15) may unravel additional physiological functions of casein.

There is currently no method available that leads to a distinct separation of whey proteins and casein. Methods currently used were developed primarily to analyze bovine casein, which is in several aspects quite different from human casein. The differences in the content of soluble whey proteins in human and bovine milk as well as the marked differences in the content and in the casein subunits between the two species makes it unlikely that methods for analyzing bovine casein, such as adjustment

of milk to pH 4.6 followed by centrifugation (16), can easily be used for human milk. It is known that acid precipitation of human-milk casein leads to various amounts of coprecipitating whey proteins (17-20). A further problem is that literature data on the casein content are based either upon indirect methods determining the nitrogen content of whole milk and whey and calculating the amount of casein or upon gel electrophoresis of whole casein fractions. To use the indirect approach it is necessary to separate milk into fractions with either only whey proteins or caseins. Gel electrophoresis of whole casein to differentiate between  $\kappa$ - and  $\beta$ -casein does not seem appropriate, because the reported range in molecular weight for  $\kappa$ -casein is 25 000-40 000 (18, 21, 22) whereas the molecular weight of  $\beta$ -casein is ~24 000 (23). It is therefore likely that there are some overlapping bands of  $\beta$ - and  $\kappa$ -casein. When acid precipitation, sedimentation, and the indirect approach (whole-milk nitrogen - whey N = casein N) to analyze casein were com-

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pared, large variations within and between the methods were obtained (19). The apparent conclusion is that the three methods detect, at least in part, different proteins. Recently, we reported the separation and analysis of whey proteins in human milk after adjustment of the pH of whole milk to 4.3 and addition of calcium followed by ultracentrifugation (24).

In this paper we report the development of a method to analyze casein and casein subunits by anion-exchange chromatography. The resulting fractions were subjected to SDS-polyacrylamide gradient gel electrophoresis followed by specific staining to demonstrate the identity and purity of phosphorylated and glycosylated casein subunits. This method was applied to casein fractions from one mother during the lactation period to investigate changes in total casein content and in the glycosylation and phosphorylation patterns of the casein subunits.

## Materials and methods

### *Milk samples*

In this study we analyzed the casein preparations from the same human-milk samples for which we reported the separation and analysis of whey proteins (24). Human-milk samples were provided by healthy mothers at various stages of lactation. The protocol was approved by the Human Subjects Review Committee at the University of California, Davis. The samples were collected at the second nursing of the day by emptying one breast, gently shaking the individual milk samples, and taking an aliquot for analysis without prior freezing.

Preparation of human milk was done as described in detail in our previous paper (24). Briefly, either whole human milk (4 mL) or skim milk was used fresh to test different variables. Samples were centrifuged at low speed ( $10\,400 \times g$ ) at 4 °C with or without pH adjustment (to pH 4.6) or at high speed ( $189\,000 \times g$ ) at 4 or 20 °C with or without pH adjustment (to pH 4.6 or 4.3) with or without calcium (60 mmol  $\text{CaCl}_2/\text{L}$ ). After the final centrifugation step, caseins were lyophilized and fat and whey fractions were kept frozen at -20 °C until further analysis.

### *Anion-exchange fast protein liquid chromatography*

Whole casein samples were subjected to fast protein liquid chromatography (FPLC) on a Mono-Q HR 5/5 anion-exchange column (Pharmacia Fine Chemicals, Piscataway, NJ). After centrifugation of whole human milk or skim milk, the pellet was lyophilized and dissolved in 20 mmol ethanolamine/L (pH 9.5, 4 mg casein/0.5 mL). Solid urea was added to a final concentration of 6 mol/L. The samples were kept in a water-bath (25 °C) with occasional stirring for 0.5–1 h. Then, 500  $\mu\text{L}$  was injected onto the column and separated at room temperature by use of the following conditions. Buffer A, 20 mmol ethanolamine/L (pH 9.5) with 6 mol urea/L, and buffer B, buffer A containing 0.6 mol NaCl/L, were prepared fresh, filtered through 0.22  $\mu\text{m}$  filters (Millipore, Bedford, MA), and degassed before use. Gradient A used buffer A from 0 to 3 mL and a gradient of 0–100% buffer B from 3 to 30 mL in 27 min. Gradient B used buffer A from 0 to 3 mL and a gradient of 0–100% buffer B from 3 to 57 mL in 54 min. Gradient A was chosen to identify whey proteins in the casein fraction; gradient B was used to differentiate between the casein subunits,  $\beta$ - and  $\kappa$ -casein. The flow rate was 1 mL/min, the recorder setting was 0.5 cm/mL, and the pressure was 1.5 MPa. The column eluant was monitored continuously at 280 nm. Linearity of the ionic

strength gradient used was regularly checked by use of a conductivity meter (Barnstead Conductivity Bridge, Fisher Scientific, Pittsburgh, PA). The identity and purity of the eluted fractions were established by comparing the whole casein sample with the FPLC fractions by SDS polyacrylamide gradient gel electrophoresis (PAGE). To identify the elution positions for whey proteins in the same buffer system, 250  $\mu\text{L}$  whey was diluted with 250  $\mu\text{L}$  buffer and loaded onto the column.

### *Polyacrylamide gradient gel electrophoresis*

PAGE was performed in 3–27% or 10–20% polyacrylamide gels containing 1% SDS (precasted gels; Integrated Separation Systems, Newton, MA) as described (24) using the Hoefer Mighty Small Apparatus (Hoefer Scientific Instruments, San Francisco, CA). The lyophilized caseins (1 mg) were dissolved in 1000  $\mu\text{L}$  sample buffer (0.025 mol Tris-HCl/L, 0.1% SDS, pH 6.8, 0.25% bromophenol blue, 2.5%  $\beta$ -mercaptoethanol) and heated in boiling water for 1.5 min. Fifteen microliters of these solutions was loaded on the gel. Eluted FPLC anion-exchange-column fractions were concentrated by using microconcentrator tubes (Centricon tubes, Amicon, Danvers, MA). To 40  $\mu\text{L}$  of the concentrated fractions, 60  $\mu\text{L}$  sample buffer was added, the sample was heated, and 15  $\mu\text{L}$  was loaded on the gel. Coomassie Blue staining and destaining were performed as described (24). Ethyl Stains All (Gallard-Schlesinger, Carle Place, NY) was used to differentially stain pure proteins, phosphoproteins, and acidic proteins in the same gel (25).

To investigate the protein distribution of whole human milk by gel electrophoresis, 40  $\mu\text{L}$  milk (not delipidated) was diluted with 160  $\mu\text{L}$  sample buffer and heated in boiling water for 1.5 min, and 10  $\mu\text{L}$  was loaded onto the gel. The relative distribution of individual proteins after staining with Coomassie Blue was determined by densitometric scanning with a Hoefer GS 300 densitometer (Hoefer Scientific Instruments, San Francisco, CA) using a 585-nm-wavelength filter. The area under each peak was measured by integration.

### *Immunoelectrophoresis and immunodiffusion*

Immunoelectrophoresis and radial immunodiffusion of the whey fractions and the whey proteins in the supernatants after the casein pellet was washed were performed according to Laurell (26) and Mancini et al (27) as described (24).

### *Protein content*

The protein content was determined with a modified Kjeldahl method (28). The protein content in lyophilized casein was determined by multiplying the nitrogen content by 6.25. The protein content in whey fractions was determined by subtracting nonprotein N from whey protein N multiplied by 6.25.

### *Carbohydrate analysis*

Neutral sugars were quantified with anthrone reagent according to Dische (29) and with phenol reagent according to Dubois et al (30). Sialic acid was determined according to Svennerholm (31).

### *Phosphorus analysis*

The phosphorus content was determined according to the method of Eibl and Lands (32).

## Results

A distinct separation of whole human milk into fat, whey, and casein fractions by ultracentrifugation with-

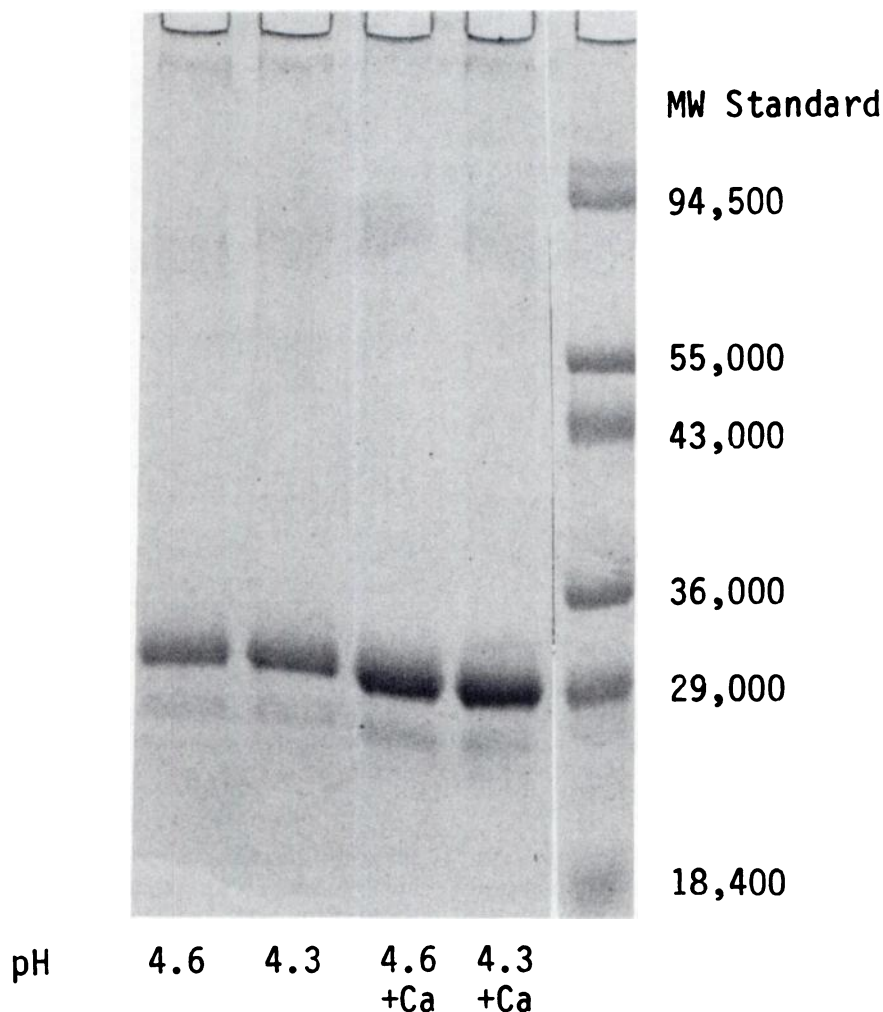


FIG 1. Gel electrophoresis of human-milk casein. Before centrifugation ( $189\,000 \times g$ ;  $4\text{ }^{\circ}\text{C}$ ; 1 h) whole human milk was treated as indicated at the bottom of the gel. Lyophilized casein ( $15\ \mu\text{g}$ ) diluted in  $0.025\ \text{mol Tris-HCl/L}$  (pH 6.1) containing 0.1% SDS, 0.25% bromophenol blue, and 2.5%  $\beta$ -mercaptoethanol was subjected to electrophoresis in 10–20% polyacrylamide gel and stained with Ethyl Stains All.

out prior pH adjustment is not possible (24). The casein pellet is not only very small but is highly contaminated by whey proteins (up to 50%, as could be shown by SDS-PAGE followed by densitometric determinations and immunoelectrophoresis and immunodiffusion of individual whey proteins). Adjustment of human milk to pH 4.6 before ultracentrifugation leads to a higher proportion of casein in the pellet. A further improvement in the recovery of casein in the pellet is obtained by adjusting the pH to 4.3 and, especially, by the addition of  $60\ \text{mmol Ca}^{2+}/\text{L}$  (Fig 1). This gel was stained with Ethyl Stains All, which has the advantage that it allows the investigator to specifically detect glycosylated casein subunits ( $\kappa$ -caseins) and phosphorylated caseins ( $\beta$ -caseins) because they stain different colors. A potential disadvantage of this staining method is a lower sensitivity, ie, small amounts of contaminating whey proteins are not visualized. The effect of pH and  $\text{Ca}^{2+}$  on whey contamination of casein pellets, however, was shown previously (24) by intentionally overloading the gels.

Casein subunits were separated with FPLC and an anion-exchange column (Fig 2). Elution positions for the  $\beta$ -caseins were identified by phosphate analysis and those for  $\kappa$ -caseins by carbohydrate assay (indicated in the figure). Because caseins occur in micellar form in human milk, it could be expected that the addition of  $\beta$ -mercaptoethanol or dithiothreitol (DTT) is necessary to cleave disulfide bonds that, in combination with urea, would allow the separation of casein subunits on this anion-exchange column. However, several experiments with different concentrations of  $\beta$ -mercaptoethanol or DTT (0.1% and 1%) added to the sample as well as to the running buffer did neither improve nor affect the separation of human casein. Therefore, in the subsequent experiments the caseins were separated in  $20\ \text{mmol ethanolamine/L}$  (pH 9.5) containing  $6\ \text{mol urea/L}$  but without further dissociating agents.

The effect of different NaCl concentrations (0.3, 0.6, and  $1.2\ \text{mol/L}$ ) in the gradient used to elute the caseins was also tested. Use of  $1.2\ \text{mol NaCl/L}$  in buffer B led to

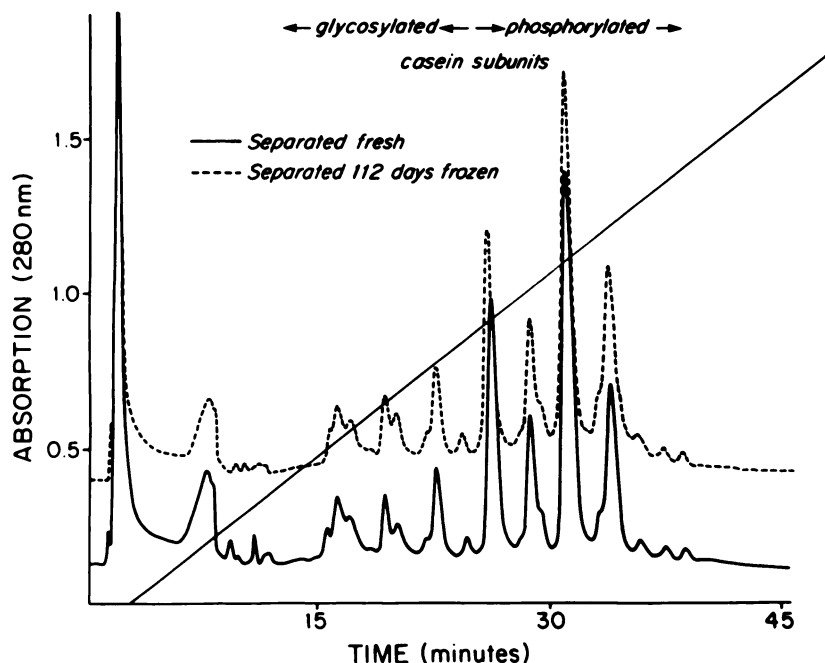


FIG 2. Anion-exchange chromatography of human-milk casein from one donor (day 112 of lactation) by FPLC. a: pH 4.6; b: pH 4.3. Lyophilized casein (4 mg) was dissolved in 0.5 mL ethanolamine (pH 9.5) with 6 mol urea/L added. Gradient B was used with NaCl from 0 to 0.6 mol/L.

a very fast analysis of the casein subunits (<20 min) with a satisfactory separation of the caseins. After the appropriate conditions to separate  $\kappa$ - and  $\beta$ -casein were selected, the casein fractions from the same human-milk samples from which we analyzed the whey fractions (24) were analyzed on the anion-exchange column with a 0–0.6 mol NaCl/L gradient that, compared with 0–1.2 mol NaCl/L, led to a delay in the elution of the different phosphorylated and glycosylated casein subunits, resulting in enhanced separation power.

The reproducibility of the FPLC system was shown by repeated injection of the sample on the same day and on subsequent days (data not shown). We also centrifuged whole human milk either fresh or after freezing the same whole-milk sample for 112 d (Fig 2). The chromatograms, which are superimposed on each other by changing the baseline, not only demonstrate the high reproducibility but also that there are no significant differences in the casein subunit pattern even after 16 wk storage.

As can be seen in Figure 3, adjustment of whole human milk to pH 4.3 instead of pH 4.6 before ultracentrifugation leads to more casein being precipitated but also to more whey proteins in the pellet. The presence of whey proteins in the casein fractions was verified by SDS-PAGE of the anion-exchange-column peaks and by comparing the elution pattern of the corresponding whey fraction from the column (using the running buffer for casein separation). Elution positions for the whey proteins were determined by running isolated whey proteins under these conditions. Besides  $\alpha$ -lactalbumin, which elutes between the glycosylated and phosphorylated caseins, all major whey proteins show a shorter re-

tention time than the caseins show. The addition of calcium improved the separation of soluble and insoluble proteins markedly. There was a further increase of caseins and a decrease of whey proteins in pellets of human milk that was adjusted to pH 4.3 instead of 4.6 (Fig 3).

The superior separation of whey proteins and casein after calcium was added to whole milk before centrifugation was used to isolate casein and whey in milk samples from one mother during the whole lactation period. The samples were subjected to FPLC and SDS-PAGE as well as to Kjeldahl analysis. As can be seen in Table 1, the casein content determined by densitometry varies between 22.5% and 45.8% of the total protein content during lactation. The increasing concentration of casein during lactation and the decrease in whey proteins leads to a continuous change in the ratio of whey proteins to casein from 77:23 on day 4 to 54:46 on day 35 of lactation (Fig 4). This changing ratio was confirmed by densitometric scanning after gel electrophoresis of whole human milk (Table 1).

The complexity of the human casein system was further investigated by SDS-PAGE of whole casein from different samples and of anion-exchange-chromatography fractions (Fig 5). Because there are no commercially available human casein standards, the identity of the eluted fractions was established by subjecting them to SDS-PAGE (Fig 6). The specific staining method using Ethyl Stains All led to the differentiation of glycosylated and phosphorylated caseins. Acidic proteins (sialic acid containing  $\kappa$ -casein) stain blue-green whereas phosphorylated proteins stain blue. As an example, Figure 5 shows the separation of a casein sample: fractions 4–12 were

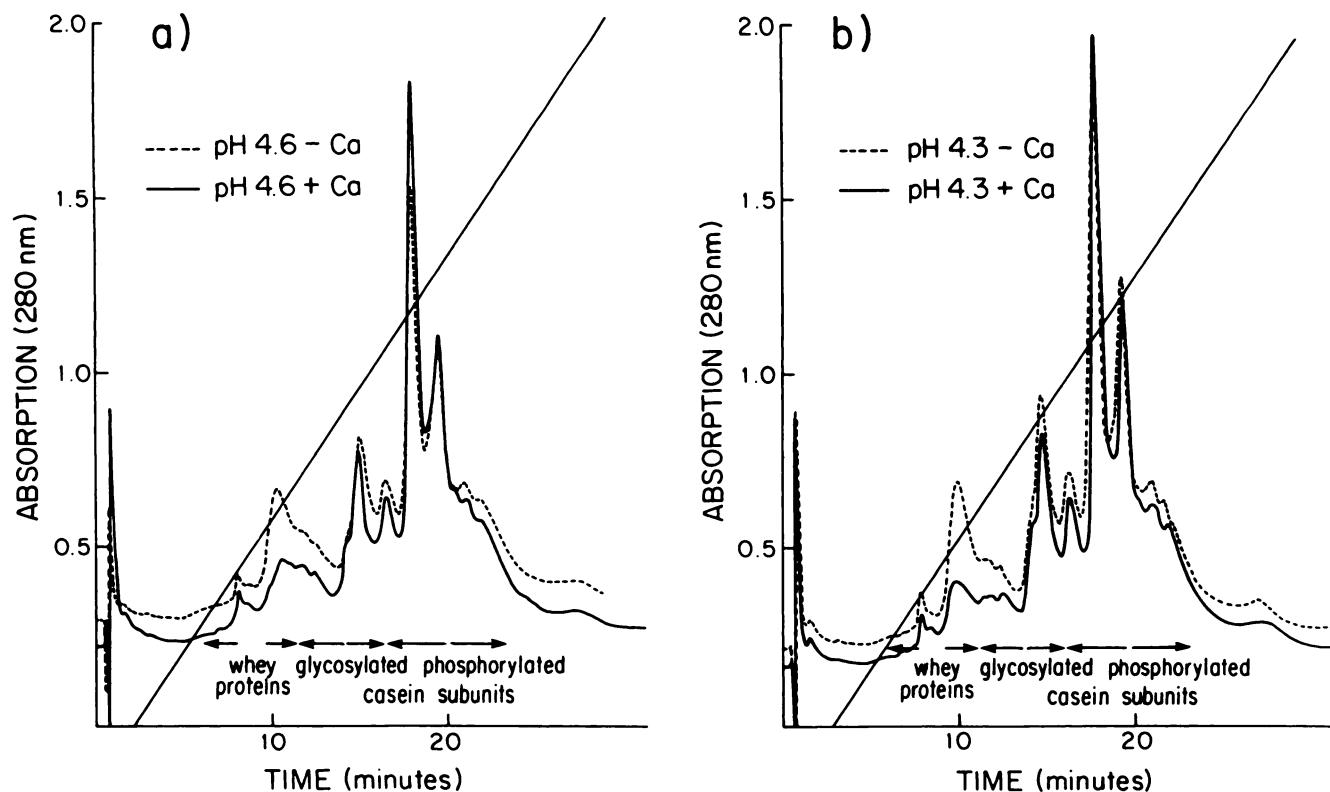


FIG 3. Anion-exchange chromatography of human-milk casein from one donor (day 6 of lactation) by FPLC. Whole human milk was adjusted to pH 4.6 or 4.3 with and without addition of 60 mmol  $\text{Ca}^{2+}$ /L followed by centrifugation. Lyophilized casein (4 mg) was dissolved in 0.5 mL ethanolamine (pH 9.5) with 6 mmol urea/L added. Casein separation was achieved using a NaCl gradient (0–0.6 mol/L) in 20 mmol ethanolamine/L (pH 9.5) containing 6 mol urea/L.

then subjected to an electrophoretic separation on SDS-PAGE. In Figure 6 there are several blue-green bands (fractions 6–8) in front of the blue bands (fractions 9–12), which is in agreement with carbohydrate and phosphate analysis:  $\kappa$ -caseins elute before the  $\beta$ -caseins with no cross-contamination in the fraction.

As can be seen in Figure 6 right lane, SDS-PAGE alone cannot be used for quantitation of  $\beta$ - or  $\kappa$ -casein or to investigate changes in the glycosylation or phosphorylation pattern because there are several overlapping bands. We therefore used FPLC anion-exchange chromatography to analyze potential changes in the casein subunit pattern occurring during lactation. Figure 7 shows a chromatographic comparison between preterm casein with an early and late casein sample from one mother. It is obvious that there is an increase in casein content during lactation mainly because of an increase of the glycosylated casein subunits. The lack of  $\kappa$ -casein is more pronounced in the premature casein sample than in the early milk sample.

## Discussion

The precipitation method we developed for separating human casein from whey proteins provides a rapid and convenient way to isolate casein quantitatively without

significant contamination by whey proteins. The lowering of pH to 4.3 and the addition of calcium apparently achieves an aggregation of the human casein subunits while whey proteins are excluded. Thus, this method provides advantages as compared with previously used methods that were based on pH adjustment to pH 4.6 or no pH adjustment. The increased specificity for the method of preparing whole casein also enhances the possibility of effectively separating casein subunits by column chromatography, as will be described below, because a significantly lower amount of whey proteins will obscure the chromatographic profile for the individual caseins.

The casein content in human milk has been believed to be  $\leq 30\%$  (19). However, previous studies on casein content in human milk were based on indirect methods. In addition, separation of casein and whey proteins was incomplete. In this study we showed by direct Kjeldahl analysis of casein pellets prepared under optimal conditions that there is marked variation in casein content during lactation. Analyzing the casein pellets from one mother throughout lactation shows 22.5–45.8% of the total protein to be casein (Table 1). This is in agreement with the gel electrophoretic separation of whole human milk and determination of the relative distribution of individual proteins by densitometric scanning. This

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