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Application of capillary zone electrophoresis to the characterisation of the human milk protein profile and its evolution throughout lactation

M.A. Manso, M. Miguel, R. López-Fandiño *

Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

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Abstract

This work describes the use of capillary zone electrophoresis for the characterisation of human milk proteins. The major proteins were identified following different strategies, such as the treatment with enzymes for selective protein modification. Using this method we studied the proteins in human milk from different donors throughout lactation. Qualitative and quantitative differences in the composition of the individual proteins were observed. The different β-casein phosphoforms were separated and quantified. The average proportion of the 0P:1P:2P:3P:4P:5P was, approximately, 3:6:9:4:10:2. The evolution of the ratio of the different β-casein phosphoforms during lactation is reported.

Keywords: Human milk; Lactation; Caseins; Whey proteins; Capillary zone electrophoresis

1. Introduction

Breast feeding by a healthy mother is the feeding standard from birth to 6 months in healthy term infants [1]. Many of the beneficial properties of human milk are inherent to the protein fraction. Breast-milk contains a wide variety of proteins that contribute to its unique qualities by providing adequate nutrition, while simultaneously aiding in the defence against infection and facilitating optimal development of important physiologic functions in newborns. It is recognised that the various proteins can have different nutritional roles for the infant or provide biological activities ranging from antimicrobial effects to immunostimulatory functions [2,3]. However, the precise protein requirement remains a controversial issue. Furthermore, the composition of human milk varies in each individual and over the course of lactation as a function of the needs of the infant over time [4], so that an exhaustive investigation of the quantitative changes in human milk during lactation is still required for a better knowledge of the lactogenic response. These points are important in the design of better infant formulas providing nutrients as close as possible to human milk.

Several analytical techniques have been applied to the study of human protein components. In most cases, milk is previously fractionated by a combination of precipitation and different chromatographic techniques, such as anion-exchange (Mono Q, DEAE-cellulose), gel filtration (Sephadex), affinity chromatography (thiol-Sepharose) and hydroxyapatite chromatography, and the fractions are subsequently characterised by polyacrylamide gel electrophoresis (PAGE) [5–9]. In addition, the quantification of some individual human milk proteins, such as lactoferrin and α-lactalbumin, has been performed by sodium dodecyl sulfate (SDS)-PAGE [10] and HPLC [11]. Immunological techniques, such as microparticle-enchanced nephelometric immunoassays, have been developed for nine human milk proteins, using the corresponding reference standard curves [12]. However, a simple, rapid, sensitive and quantitative method for the simultaneous analysis of human milk proteins directly from milk is still lacking.

A capillary zone electrophoresis method (CZE), originally developed for the analysis of bovine milk [13,14], demonstrated a good resolution of the individual proteins based on their charge to mass ratio, allowing their identification and quantification. This method has found a number of successful applications in the control of the quality of different dairy products [15,16], the analysis of the genetic polymorphism of milk from different species [17] and the determination of the whey protein to total protein ratio in milk [18,19]. Although capillary electrophoresis offers a high resolving power, high sensitivity and selectivity, easy quantification and the advantage of its low sample

* Corresponding author. Tel.: +34 91 5622900; fax: +34 91 5644853.
E-mail address: rosina@ifi.csic.es (R. López-Fandiño).

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and solvent consumption, to date, no capillary electrophoresis method has been used for the analysis of human milk proteins. In this work, we report a study of human milk protein composition and its evolution throughout lactation by CZE.

2. Experimental

2.1. Samples

Human milk samples were obtained from five volunteer lactating women. The samples were collected by manual expression once a week, from the 1st to the 18th week of lactation, after nursing. Milk was skimmed and kept at −20 °C until used. Nitrogen contents were determined by the Kjeldahl method and total protein contents were calculated by using a conversion factor of 6.38 [20].

When required, isoelectric casein was obtained by adjusting the pH to 4.6 with 1 M HCl and by centrifugation at 10,000 × *g*, 10 min, 10 °C. The casein pellet was washed twice with double distilled water. Both casein and whey fractions were subsequently lyophilised. Casein was reconstituted in 0.1 M Tris–HCl buffer pH 7.0, in a proportion as to double the original concentration in milk.

Human milk protein standards, α-lactalbumin (α-La), lactoferrin (Lf), lysozyme (Lz) and secretory immunoglobulin A (sIgA) were purchased from Sigma (St. Louis, MO, USA).

2.2. Enzyme treatments

Human skim milk and casein solutions were incubated with 8.3×10^{-3} units/ml of plasmin from bovine plasma (EC 3.4.21.7, Sigma) and 2.4×10^{-3} units/ml of rennet (containing 85% chymosin, EC 3.4.23.4, and 15% pepsin, EC 3.4.23.1; Chr. Hansen, Copenhagen, Denmark), at 37 °C for 10, 60 and 180 min [21]. Hydrolyses were also conducted with 0.36 units/ml of potato acid phosphatase (EC 3.1.3.2.; Sigma) at 37 °C for 1, 3 and 6 h and with 5.0×10^{-2} units/ml of neuraminidase from *Clostridium perfringens* (EC 3.2.1.18; Boehringer; Mannheim, Germany), at 37 °C for 1, 6 and 24 h [22]. All reactions were terminated by addition of the CZE sample buffer and the samples were analysed immediately.

2.3. Capillary zone electrophoresis

CZE was carried out using a Beckman P/ACE MDQ instrument (Beckman Instruments, Fullerton, CA, USA). Milk proteins were separated in an OV-1701-OH-deactivated fused-silica capillary column (BGB Analytik Vertrieb, Schlossböckelheim, Germany) of 50 μm i.d. and 60 cm total length (50 cm effective length). Separations were performed following the method described in [13], with some modifications. Briefly, electrophoresis was run at 45 °C, with a linear voltage gradient from 0 to 30 kV in 3 min followed by constant voltage at 30 kV for 42 min with normal polarity (ground at the detector side). Before each separation the capillary was flushed in the reversed direction with the electrophoresis buffer for 6 min. Absorbance was monitored on the column at 214 nm. Corrected areas of the peaks were determined by using the MDQ software, in order to take into account the possible variations in protein migration.

The electrophoresis buffer (pH 3.2 ± 0.1) was 0.19 M citric acid and 20 mM sodium citrate in 6 M urea, containing 0.05% methylhydroxyethylcellulose (MHEC 30000, Serva, Heidelberg, Germany). The urea solution was previously passed over a mixed-bed ion-exchange resin (AG 501-X8, Bio-Rad, Hercules, CA, USA) until the conductivity was lower than 2 μS/cm. The buffer was filtered through a 0.45 μm polyvinylidene fluoride (PVDF) filter (Millipore, Bedford, MA, USA).

The sample buffer (pH 8.6 ± 0.1) consisted of 167 mM Tris (Sigma), 42 mM 3-morpholinopropanesulfonic acid (Sigma–Aldrich, Steinheim, Germany), 67 mM EDTA (Merck, Darmstad, Germany), 17 mM D,L-dithiothreitol (DTT) (Sigma) in 10 M urea, containing 0.083% MHEC. The sample was mixed 1:2 with the sample buffer. After waiting for 45 min sample solutions were injected for 20 s at 0.5 psi (3.4 kPa).

3. Results and discussion

3.1. Protein identification and characterisation

As shown in Fig. 1a, CZE allowed the separation of the main human milk proteins, providing a well-defined separation pattern, with well-resolved peaks. In Fig. 1e, the CZE electropherogram of a bovine UHT skim milk sample is also shown for comparison [14]. Bovine milk contains some proteins not found in human milk, for example β-lactoglobulin (β-Lg) and α_{S2}-casein. Other protein components are similar, but present at different concentrations [23].

Several strategies were used to identify the main proteins. The major whey proteins, Lz, Lf and α-La, were identified by using human protein standards (Fig. 1b–d). Lz, a very basic protein, was only detected in some of the individual samples, while sIgA, whose standard showed two major subunits with a very short migration time (results not shown), could not be found in the electropherograms of the human milk samples. Human milk contains very low amounts of α_{S1}-casein (α_{S1}-CN) and κ-casein (κ-CN), with β-casein (β-CN) being the major casein. In order to identify the individual caseins and their post-translational modifications, milk and casein samples, at several points throughout lactation, were treated with different enzymes and analysed after various hydrolysis times.

It is well known that, while bovine β-CN is found as a single phosphorylated form containing 5P groups, human β-CN occurs as multiphosphorylated forms [24]. Dephosphorylation with acid phosphatase allowed the identification of the main casein peaks as six β-CN forms, containing from 0P to 5P, thus showing that the CZE method provides a very high resolution of proteins that differ in just one phosphate residue. Under our electrophoretic conditions, with a coated capillary at acidic pH, proteins migrate according to their charge to mass ratio, so that the most electropositive proteins migrate faster. The elimination of a phosphate group attached to a Ser residue of a protein increases the positive charge and, therefore, the protein presents a shorter migration time. As illustrated in Fig. 2, after hydrolysis with acid phosphatase for 6 h, the phosphorylated forms