Electrophoretic Analysis (Tricine-SDS-PAGE) of Bovine Caseins

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SUMMARY. An alternative electrophoretic method that improves the separation, identification and relative quantification of bovine milk casein fractions and casein hydrolysates was developed. The method is based in the Tricine-SDS-PAGE technique while the relative quantification of milk proteins has been achieved by digital densitometry. The advantages of this procedure for milk proteins and milk protein hydrolysates are that bands are well separated and can be readily detected and quantified. In the new method, α_{s1}-, α_{s2}-, β- and κ-casein and low molecular weight peptides (casein degradation products with high electrophoretic mobility) that cannot be usually detected by other electrophoretic systems are visualized as sharp bands.

RESUMEN. "Un Método Electroforético mejorado destinado al Análisis de Caseína Bovina". Ha sido desarrollado un método electroforético alternativo que mejora la separación, identificación y cuantificación relativa de caseína bovina y de hidrolizados de caseína. El método se basa en la técnica de Tricina-SDS-PAGE, en tanto que la cuantificación relativa de las proteínas lácteas fue realizada por densitometría digital. Una de las ventajas de este procedimiento para proteínas de leche y de sus hidrolizados es que las bandas se encuentran bien separadas y pueden ser fácilmente detectadas y cuantificadas. En el nuevo método, α_{s1}-, α_{s2}-, β- y κ-caseína y los péptidos de bajo peso molecular (productos de degradación de la caseína con elevada movilidad electroforética), que no pueden ser habitualmente detectados por otros sistemas electroforéticos, son visualizados como bandas nítidas.

INTRODUCTION

Milk is a basic food for humans affording essential nutritional components (proteins, fats, carbohydrates, minerals and vitamins). Type and concentration of amino acids in cow milk proteins are very close to the amino acid requirements of humans.

Bovine milk contains 3.0-3.5% (w/v) protein [1]. Caseins are 2.4-2.8% of fluid milk and exist in milk as a micelle containing the four casein types: α_{s1}-, α_{s2}-, β- and κ-casein [2].

Electrophoresis has played an important role in the study of milk proteins and has been an integral part of research on the genetic variants of the major proteins components of milk. Indeed, the designation of caseins are derived from electrophoretic analysis and minor caseins components were discovered by electrophoresis [2].

Cow milk proteins are responsible for food allergies, most frequent in infants. The allergenic properties of native proteins can be reduced by enzymatic hydrolysis (cow milk hydrolysates are being widely used in the production of different infant formulas) [3]. Silver-stained PAGE can be used as a predictor of food allergen activity in cow milk hydrolysates [4].

The electrophoretic techniques currently used in the study of milk proteins (SDS-PAGE, alkaline urea PAGE, acid urea PAGE) [5] do not allow clear identification of the casein components. In the case of milk protein hydrolysates, only larger peptides can be adequately visualized by those techniques.

The development of a technique that allow the detection in a unique gel of casein fractions as well as low molecular weight peptides pre-

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sent in casein hydrolysates is necessary to provide a complete knowledge of the composition and distribution of the different milk proteins, in order to verify adulterations [2] and to stablish hydrolysis patterns.

In this article we report a method based in the Shägger and von Jagow´s [6] polyacrylamide tricine gel electrophoresis technique (Tricine-SDS-PAGE) that improves the isolation, identification and quantification of caseins and casein hydrolysates in a unique gel.

MATERIAL & METHODS
Urea polyacrylamide gel electrophoresis (Urea-PAGE)

Urea-PAGE was performed using a Mini-Protean III (Bio-Rad Laboratories, Watford, UK), according Andrews [7].

Tricine-SDS-PAGE
Gels preparation

Solutions for making gels were prepared according to Shägger & von Jagow [6]. The separating gel was 16.5% T, 3% C, the "spacer" gel was 10% T, 3% C, and the stacking gel was 4% T, 3 % C. All solutions were stored at 4 °C.

The electrophoresis was performed in a Mini-Protean III dual slab cell (Bio-Rad Laboratories, Watford, UK). The gel mixtures were gently poured in the casting modules. After filling, the separating gel (5 cm deep) was carefully overlaid with 1-2 mm deep layer of buthanol to allow a truly flat surface and protect the top of the gel mixture from atmospheric O_2. After polymerization, the alcohol was replaced by the "spacer" gel (1.2 cm deep). This procedure was repeated for the stacking gel (0.4 cm deep). After polymerization of the stacking gel, the wells were washed with cathodic buffer. In same cases only separating and stacking gels were performed.

Sample preparation

Casein solution was prepared by dissolving 1g casein in 100 mL of 100 mM Tris-HCl buffer (pH 8.0) and heating in a boiling bath for 20 min. The solution was filtrated without cooling and stored at 4 °C until to be used. Casein solutions must be discarded after 2 days.

Casein solutions were diluted by adding an equal volume of double-concentrated sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 0,4% bromophenol blue and 20% glycerol) and heated at 100 °C for 5 min, centrifuged and immediately stored at -20 °C

until analysis. Low molecular weight standards (Bio-Rad) were prepared in sample buffer.

Samples and standards (2.5-5 µl) were applied under the cathodic buffer using a 10 µl Hamilton micrometer syringe.

Run conditions

Electrophoresis was performed at room temperature using a voltage stepped procedure: voltage was kept constant (30 V) until the samples completely left the stacking gel and then the voltage was increased 15 V per min for 4 times. Voltage was maintained constant (90-100 V) until the tracking dye reached the bottom of the gel.

Fixing, staining and destaining

Immediately after ending electrophoresis, gels were removed from the plates and placed in a fixative solution containing 50% methanol and 10% acetic acid. After 30 min, the fixative solution was replaced by a staining solution containing 10% acetic acid and 0.1% Coomassie Brilliant Blue R-250 where gels were left for 30 min at 50 °C [8].

Destaining of gels were accomplished by heating in a microwave oven during short periods and making several changes of distilled water during the destaining procedure.

Gel scanning and densitogram performing

Gels were dried and scanned. Image analysis of each electrophoresis was performed by capturing the image of each gels with a PC scanner (UMAX Astra 600s) and saving it as a graphic files in TIFF format. Gels images were processed by means of a gel analysis software (Scion Image Beta 4.02 for Windows) [9] and the densitograms were then performed.

RESULTS AND DISCUSSION

Traditionally Urea-PAGE methods have been preferred for the study of milk proteins, owed to this dissociating agent prevents molecular aggregation [2]. Notwithstanding, Urea-PAGE gels afford a poor resolution (specially the α-caseins), the bands are rounded and often diffuse, specially in the α_{s2}-casein region. Moreover, κ-casein is not detectable (Figure 1a).

On the contrary, in the Tricine-SDS-PAGE method here reported the resolution was considerably improved with respect to Urea-PAGE methods, as well as SDS-PAGE and gradient PAGE methods. In the new method, α_{s2}-, α_{s1}-, β- and

β-CN

αs2-CN
αs1-CN

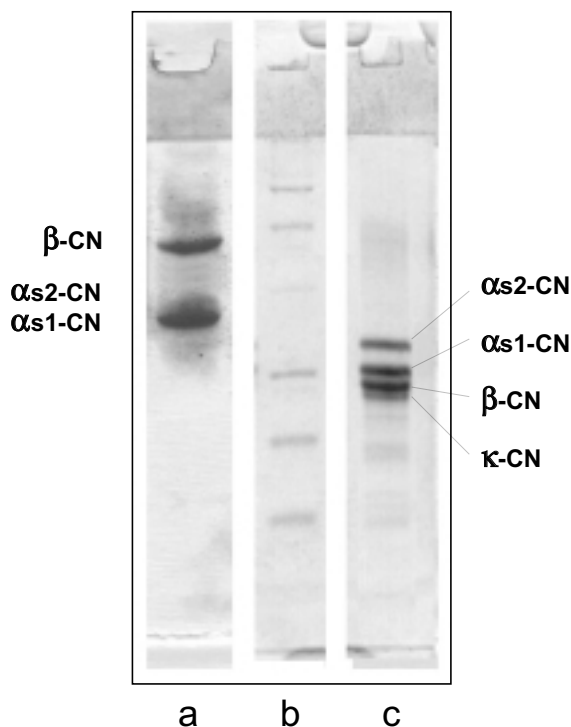**αs2-CN**

αs1-CN

β-CN

κ-CN

a   b   c

Figure 1. Electrophoretic patterns of bovine milk casein obtained by Urea-PAGE (lane a) and Tricine-SDS-PAGE (lane c). Lane b: Bio-Rad low molecular weight markers (lysozyme: 14.4 kDa, soybean trypsin inhibitor: 21.5 kDa, carbonic anhydrase bovine: 31.0 kDa, ovalbumin: 45.0 kDa, serum albumin bovine: 66.2 kDa, and phosphorylase B: 97.4 kDa). CN: casein.

Band Intensity

1 2

a

1 2 3
   4

b

Length of gel

Figure 2. Electrophoregrams and densitograms of Urea-PAGE (a) and Tricine-SDS-PAGE (b). In the upper densitogram (Urea-PAGE) 1 corresponds to β-casein while 2 includes α_{s1}- and α_{s2}-casein. In the lower densitogram (Tricine-SDS-PAGE) 1-4 correspond to α_{s2}-, α_{s1}-, β- and κ-casein, respectively.

κ-casein are visualized as sharp bands (Figure 1c).

By comparing Urea-PAGE and Tricine-SDS-PAGE densitograms (Figure 2) it can be seen that in the first case only two broad peaks appeared while in the second case four sharp bands corresponding unequivocally to the four casein types are present (included κ-casein, not detected by the other methods when whole casein is analyzed).

Figure 3 shows the electrophoretic patterns of casein fractions (lane 2) and casein enzymatic hydrolysates obtained after different hydrolysis times (lanes 3-6) using Tricine-SDS-PAGE gels composed by stacking, "spacer" and separating gels (with decreasing size pore). The advantages of this procedure for milk proteins and milk proteins hydrolysates are that bands are well separated and can be readily detected and quantified, even those of lower molecular weights (degradation products with higher electrophoretic mobility) that cannot be detected in a unique run by other electrophoretic systems.
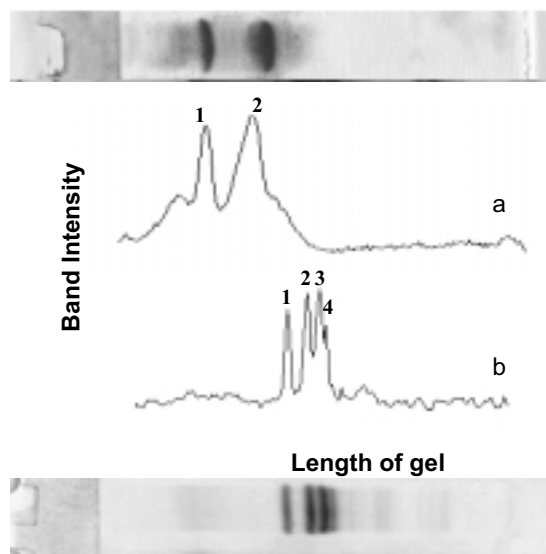

Stacking gel
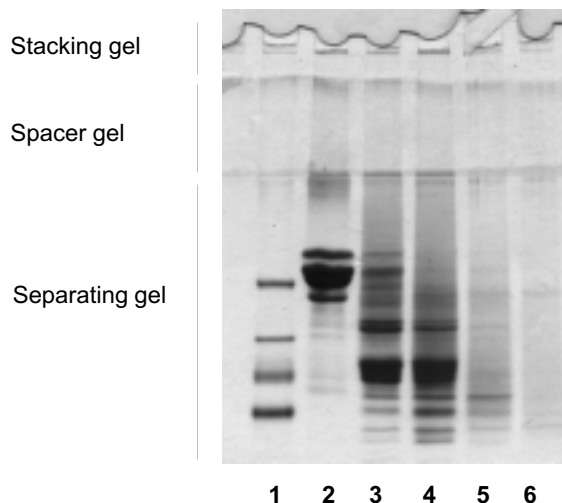
Spacer gel

Separating gel

1   2   3   4   5   6

Figure 3. Tricine-SDS-PAGE of casein fractions and casein hydrolysates. Lane 1: MW markers; lane 2: casein; lane 3-6: casein hydrolysates obtained after treatment with a plant endopeptidase along different times.

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