

Application of PhastSystem® to the Resolution of Bovine Milk Proteins on Urea-Polyacrylamide Gel Electrophoresis¹

ABSTRACT

Optimal conditions were established for alkaline urea-PAGE using modified precast, ultrathin gradient gels on the automated PhastSystem®. Profiles of milk proteins showed that the caseins and whey proteins resolved extremely well. Major bands were observed for α_{s1} -casein and β -casein, and α_{s2} -casein appeared as a well-resolved doublet. In contrast, κ -casein separated from other caseins as a faint doublet, and purified κ -casein appeared as one major and one minor band. Whey proteins (serum albumin, α -lactalbumin, β -lactoglobulin) separated into broad bands resolved from each other and from the caseins. Partially (40%) dephosphorylated whole casein showed multiple bands for α_{s1} -casein and β -casein at different levels of phosphorylation. Separation of genetic phenotypes was observed for β -lactoglobulin A and B; α_{s1} -casein A, B, and C; and β -casein A, B, and C. Electrophoretic patterns of milk proteins extracted from cheese samples varied among the different types of cheeses. Our modified procedure provides researchers with a rapid technique to separate both caseins and whey proteins on the same urea gel according to their charge to mass ratios. (Key words: casein, PhastSystem®, urea-electrophoresis, whey proteins)

Abbreviation key: BSA = bovine serum albumin; CN = casein, used with α_{s1} -, α_{s2} -, β -, and κ -; LA = lactalbumin, LG = lactoglobulin; and Rm = relative migration.

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¹Mention of brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

INTRODUCTION

Polyacrylamide gel electrophoresis is an excellent research tool for the identification of milk proteins because proteins can be separated according to size (1) or charge to mass ratio (8, 9). The four caseins that make up whole casein (CN) have similar molecular weights (19,000 to 25,230) and distinct differences in their amino acid sequences and their degree of phosphorylation (1, 5, 8 to 9, and 10 to 13 phosphate groups per molecule for κ -CN, β -CN, α_{s1} -CN, and α_{s2} -CN, respectively) (3). Alkaline PAGE uses this difference in charge to mass ratio to resolve the individual caseins. Incorporation of urea into the samples and the use of 7% acrylamide slab gels permit the separation of most casein phenotypes (7, 9, 10), detect the removal of phosphate groups from individual caseins (2), and monitor the proteolysis of casein in aging cheeses (4, 5). Electrophoretic separation of whey proteins usually requires native (without urea) alkaline PAGE (7, 9, 10) because the use of urea results in smeared, unresolved whey proteins (7) and poor resolution of α -lactalbumin (LA) from α_{s1} -CN and β -lactoglobulin (LG) from β -CN (10).

Slab gels are large (12 × 15 × .5 cm) and usually require many hours to separate the proteins and to stain and destain protein bands. Recent developments in automated electrophoretic equipment and the commercial availability of precast, ultrathin, miniature gels allow for the resolving, staining, and destaining of proteins within a few hours. The PhastSystem® (Pharmacia, Uppsala, Sweden) has been used for SDS-PAGE (6), isoelectric focusing (13) of caseins, and identification of dephosphorylated caseins (12).

The goal of this study was to select urea-PAGE conditions for the PhastSystem® that would resolve caseins according to charge to mass ratio and permit the simultaneous resolution of casein and whey proteins.

MATERIALS AND METHODS

Materials

The materials and their sources used in this study were as follows: α -LA and bovine serum albumin (BSA) from Sigma Chemical Company (St. Louis, MO); β -LG from Pentex Biochemical (Kankakee, IL); 2-mercaptoethanol, Phast[®] buffer strips, and PhastGels[®] from Pharmacia LKB Biotechnol. Inc. (Piscataway, NJ); and potato acid phosphatase (59 units/ml at 25°C, pH 4.8) from Calbiochem Corp. (La Jolla, CA). Samples obtained from within the Eastern Regional Research Center included Mozzarella cheese from Michael Tunick; rennet κ -CN from Edyth Malin; and β -LG A and B and caseins containing α_{s1} -CN A, B, and C and β -CN A, B, and C from Harold Farrell, Jr. processed American, Danish Blue, Cheddar, creamed cottage, and Ricotta cheeses were purchased locally.

Sample Sources

Skim milk, prepared from pooled raw milk from a commercial herd, was dialyzed extensively against deionized, distilled water at 4°C to remove lactose and salts and then lyophilized. Sodium caseinate was prepared as follows: skim milk was acid-precipitated at pH 4.6 and 20°C using 1N HCl and centrifuged at 6000 \times g for 15 min at 20°C. The precipitate was dissolved in water adjusted to pH 7.0 with 1 N NaOH. The acid-precipitating, centrifuging, and dissolving steps were repeated. The casein solution was dialyzed overnight and lyophilized.

Partially dephosphorylated whole casein was obtained according to Van Hekken and Strange (11). An aqueous solution containing 2.5 mg of whole casein/ml and .065 units of potato acid phosphatase/ml at pH 6.5 was incubated in a shaking water bath (37°C, 150 rpm) for 60 min. The solution was heated at 80°C for 5 min to inactivate the enzyme; dialyzed for 36 h in 4°C distilled, deionized water; and lyophilized.

The procedure of Zittle and Custer (14), involving sulfuric acid and ammonium sulfate precipitation and ethanol purification, was used to extract κ -CN from wet acid casein. The protein was dialyzed for 5 d and lyophilized.

Cheese samples (5 g) were chopped finely with a knife except for cottage and Ricotta, the soft curds of which did not require mechanical disruption. All cheeses were then extracted three times with acetone (100 ml) to remove fat and water. The wet cheese SNF were rinsed once in diethyl ether and air dried.

Urea-PAGE

Urea-PAGE with the PhastSystem[®] was based on Pharmacia's native PAGE separation technique (file number 120) as modified for caseins by Van Hekken et al. (12) and described in detail herein. All of the gels available through Pharmacia were tested, and the best resolution of milk proteins was obtained on the 8 to 25% gradient gels. Prior to use, native miniature (50 \times 43 \times 45 mm) gels were modified by soaking for 15 min in 6.6 M urea, .112 M Tris, .112 M acetate buffer at pH 6.4 and air dried for 3 to 5 min. Native buffer strips containing .88 M L-alanine and .25 M Tris at pH 8.8 were used. Samples (8 to 100 μ g/ μ l) were dissolved in the urea-Tris-acetate buffer with 10% 2-mercaptoethanol and .025% bromophenol blue. Approximately 1 μ l of sample was deposited in each lane. Electrophoretic conditions programmed into the PhastSystem[®] were 300 V, 7.5 mA, 2.5 W, at 15°C for 10 Vh (volthour). Samples were applied at 5 mA until 2 Vh were attained, and then the setting was returned to initial values. Gels were run for 110 accumulated Vh (approximately 75 min). Running at V and mA settings lower than suggested by Pharmacia was required to prevent α -LA and α_{s1} -CN from migrating too close to the leading edge. Protein bands were stained for 8 to 10 min at 50°C in .25% Coomassie brilliant blue R dissolved in 30:10:60 methanol:acetic acid: water (PhastSystem[®] development technique file number 200). The gels were destained in 30:10:60 methanol:acetic acid:water until the background was clear. Relative migration (Rm) rates were calculated as described by Thompson (10) and were based on a minimum of five runs. The distance from the origin to the slower band of the α_{s2} -CN doublet was given a value of 1.00. The migration distances of all other protein bands were measured relative to this standard.

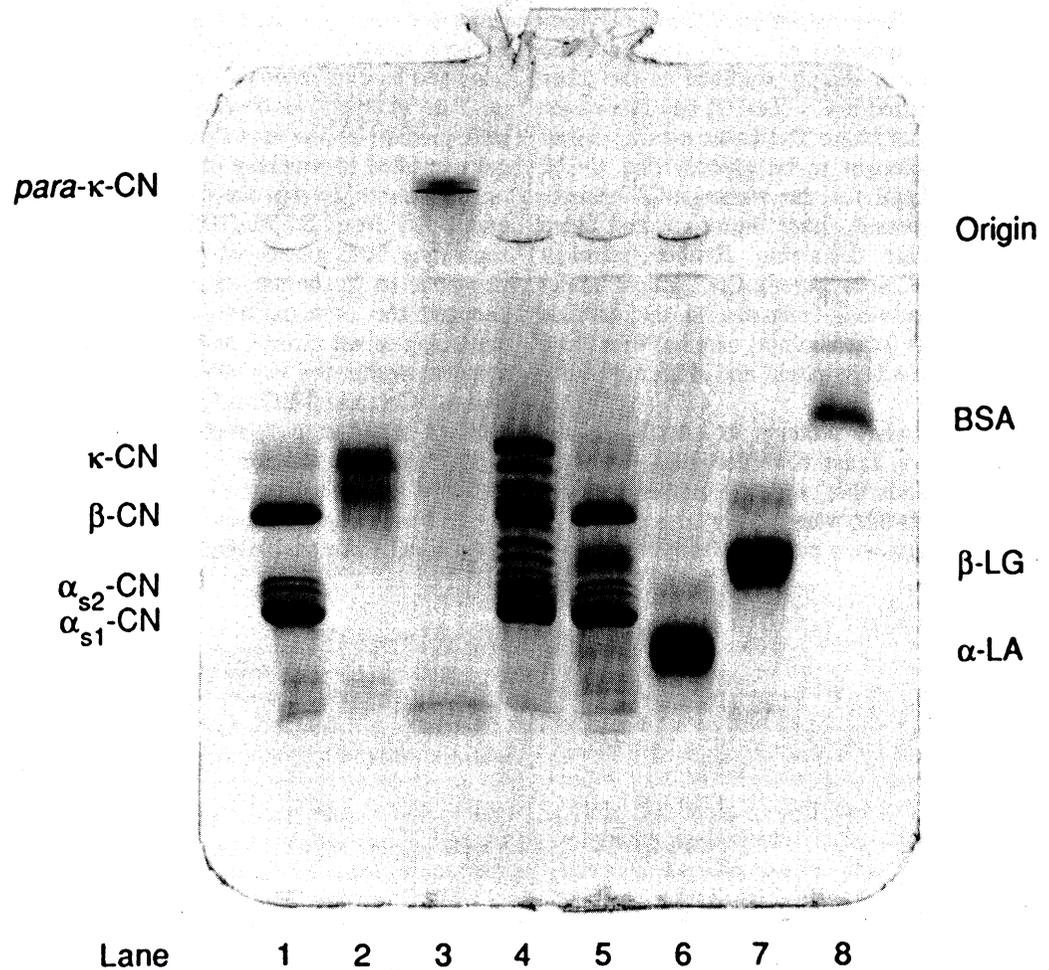


Figure 1. Urea-PAGE profile of bovine caseins and whey proteins using 8 to 25% gradient gel and the PhastSystem[®]. Samples in lanes are 1) whole casein (CN), 2) κ -CN, 3) *para*- κ -CN, 4) 40% dephosphorylated whole casein, 5) skim milk, 6) α -lactalbumin (α -LA), 7) β -lactoglobulin (β -LG), and 8) bovine serum albumin (BSA).

RESULTS AND DISCUSSION

Electrophoresis

Urea-PAGE using large slab gels has disadvantages in the lengthy running time required for the resolution of caseins and in the wide variety in procedures used among different laboratories to obtain profiles (8, 9). Use of the PhastSystem[®] significantly decreased the time and quantity of materials required to obtain quality profiles and allows for the electrophoretic conditions to be programmed in advance

and automatically carried out. The availability of standardized and prepared gels and buffer strips reduced variation because of methodology. This should encourage direct comparisons of gel profiles among laboratories.

Casein and Whey Profiles

Caseins were the best resolved of all the milk proteins when examined on urea-PAGE (Figure 1). The profiles for whole casein and skim milk (lanes 1 and 5, respectively) had α -CN and β -CN bands typical of urea-PAGE (9,

10, 12). In whole casein and skim milk profiles, κ -CN appeared as a very faint doublet that was not as sharply resolved as the other caseins. Purified κ -CN (lane 2) had one major and one minor band. The faster moving minor band was thought to be glycosylated κ -CN. The only band for the rennet κ -CN sample (lane 3) appeared above the origin and faded rapidly during destaining. Rennet treatment converts κ -CN to *para*- κ -CN, which, under alkaline conditions, migrates to the cathode (4). Various other minor caseins were also noted in the whole casein and skim milk samples.

Purified whey proteins, α -LA, β -LG, and BSA (lanes 6, 7, and 8, respectively), migrated in broad bands that, although not as sharp as the casein bands, were easily identified. The whey proteins were purchased as purified pro-

teins, and each contained minor contaminants. As seen in skim milk (lane 5), the whey proteins and caseins resolved well from each other. Past attempts to observe both groups of milk proteins on the urea-PAGE slab gels typically resulted in streaking of whey proteins (7) or the inability to separate α -LA from α_{s1} -CN and β -LG from β -CN (10). Our success in separating both groups of proteins may have been due to 1) the use of urea, which disassociated the proteins and separated proteins according to net charge, and the presence of a gradient within the gel, which resolved α -LA and α_{s1} -CN, and β -LG and β -CN by molecular sieving; and due to 2) rapid separation times that minimized diffusion of the sample in the gel.

Resolution according to charge to mass ratio has been the major reason for using urea-

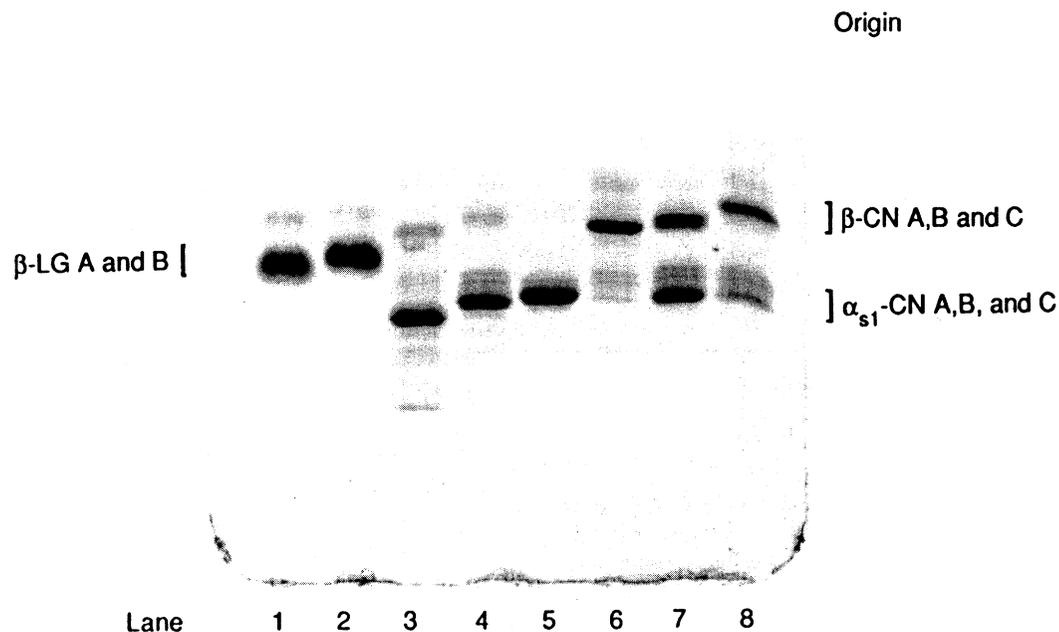


Figure 2. Urea-PAGE profile of bovine whey proteins and caseins using 8 to 25% gradient gel and the PhastSystem[®]. Samples in lanes contain 1) β -lactoglobulin (β -LG) A, 2) β -LG B, 3) α_{s1} -casein (α_{s1} -CN) A, 4) α_{s1} -CN B, 5) α_{s1} -CN C, 6) β -casein (β -CN) A, 7) whole casein containing β -CN A/B, and 8) β -CN C. Variant milk proteins, in order of migration rates (highest first), resulted in the following order: A, B, and C.

TABLE 1. The relative migration values of milk proteins separated on ultrathin (polyacrylamide) and slab (starch and polyacrylamide) gels.

	Ultrathin polyacrylamide	Slab ¹	
		Starch	Polyacrylamide
α_{s1} -Casein A	1.15	1.18	1.22
α_{s1} -Casein B	1.10	1.10	1.13-1.14
α_{s1} -Casein C	1.07	1.07	1.10
α_{s2} -Casein	1.03	1.04	1.03
	1.00	1.00	1.00
β -Casein A	.79	.80	.65
β -Casein B	.74	.76	.61
β -Casein C	.70	.70	.54
κ -Casein	.65		
	.59		
α -Lactalbumin B	1.21-1.31		
β -Lactoglobulin A	.88-.99		
β -Lactoglobulin B	.85-.96		
Bovine serum albumin	.47-.53		

¹From Thompson (10).

PAGE. The dephosphorylation of whole and individual caseins has been monitored by urea-PAGE (2, 11, 12) because the removal of negatively charged phosphate groups significantly slowed the migration rate of the protein. Our 40% dephosphorylated whole casein (Figure 1, lane 4) showed four bands migrating slower than native β -CN and four bands migrating slower than native α_{s2} -CN. This was similar to the multiple bands reported for dephosphorylated α_{s1} -CN, β -CN (2, 12), and whole casein (12).

Urea-PAGE has also been used to identify milk protein phenotypes (7, 9, 10), which may vary from one another by as little as 1 amino acid substitution or by as much as the deletion of 13 amino acids. Samples containing known phenotypes of β -LG, α_{s1} -CN, and β -CN were run on the PhastSystem[®] and had good resolution (Figure 2). In side by side comparisons, differences in migration rates were seen for β -LG A and B (lane 1 and 2); α_{s1} -CN A, B, and C (lanes 3, 4, and 5); and β -CN A, A/B, and C (lanes 6, 7, and 8).

The Rm values calculated from the milk proteins profiles obtained with the PhastSystem[®] were similar to values published by Thompson (10) (Table 1). Milk proteins, in order of migration rates (with highest first), resulted in the following order: α -LA, α_{s1} -CN, α_{s2} -CN, β -LG, β -CN, κ -CN, and BSA. This

order was different from native (without urea) alkaline PAGE profiles in which β -LG migrated faster than α -LA (9). The urea-PAGE order was also different from the SDS-PAGE order, which separated according to size (molecular weight) and had α -LA and β -LG migrating well ahead of the casein cluster of α_{s1} -CN, α_{s2} -CN, β -CN, and κ -CN (2, 6).

An automated electrophoretic system, which separates both caseins and whey proteins on the same gel according to charge to mass ratio, provides researchers with a rapid technique for examining and evaluating milk proteins.

Cheese Profiles

Caseins and whey proteins present in the cheese extracts were clearly resolved for processed American, Blue, Cheddar, creamed cottage, Mozzarella, and Ricotta cheeses (Figure 3, lanes 2 to 7, respectively).

The very faint κ -CN doublet detected in whole CN and skim milk was noted in the acid cheese samples (cream cottage and Ricotta) (lanes 1, 8, 5, and 7, respectively). Neither κ -CN nor *para*- κ -CN was detected in the rennet cheese extracts. The κ -CN was not expected because rennet converts the casein to *para*- κ -CN. The absence of the *para*- κ -CN band was probably due to its loss during destaining.

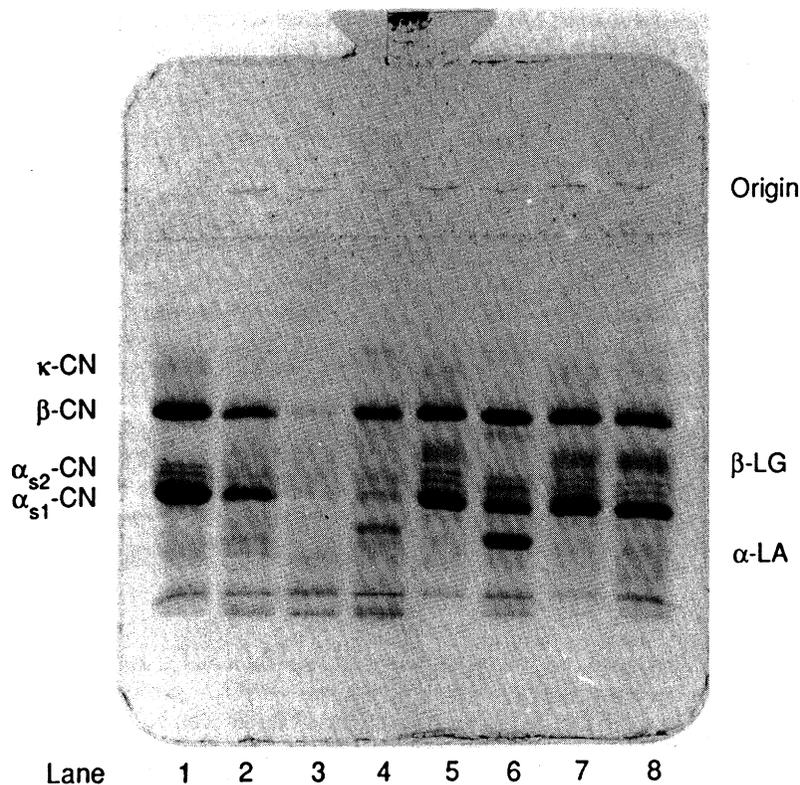


Figure 3. Urea-PAGE profile of bovine milk proteins and commercial cheeses using 8 to 25% gradient gel and the PhastSystem[®]. Samples in lanes are 1) whole casein, 2) processed American, 3) Danish blue, 4) Cheddar, 5) creamed cottage, 6) Mozzarella, 7) Ricotta, and 8) skim milk. CN = Casein, LA = lactalbumin, and LG = lactoglobulin.

The cheese extract profiles were similar to published patterns (5). The native α_{s1} -CN band was still prominent in the processed American, creamed cottage, Mozzarella, and Ricotta cheeses. Extensive and partial degradation of α_{s1} -CN was noted in the Blue and Cheddar cheese profiles, respectively (lanes 3 and 4). New zones that migrated faster than native α_{s1} -CN were present in processed American, Blue, Cheddar, and Mozzarella profiles. The native β -CN bands changed very little in all cheese profiles with one exception. Extensive degradation of β -CN was noted in the Blue cheese profile (lane 3). Alkaline urea-PAGE has been used to examine proteolysis of α_{s1} -CN and β -CN following chymosin treatment (4) and subsequent aging of cheeses (4, 5). The review by Grappin et al. (4) reported that extensive degradation of α_{s1} -CN early in cheese ripening resulted in many fragments

that migrated faster than α_{s1} -CN and between the native α_{s1} -CN and β -CN zones and that were eventually completely hydrolyzed. He also reported that only 40% of the native β -CN undergoes proteolysis during cheese ripening, and only internally mold-ripened cheeses have had extensive hydrolysis of the β -CN.

The whey protein, β -LG, was present in the creamed cottage and Ricotta cheeses and skim milk profiles (lanes 5, 7, and 8), and a very faint β -LG zone was noted in the American processed cheese (lane 2). The faint α -LA zone seen in the skim milk sample had a migration rate similar to some of the α_{s1} -CN fragments, which made identification difficult. The large amounts of casein present in the sample of Ricotta cheese were not unexpected (lane 7). Ricotta is traditionally prepared from cheese whey by precipitation of the proteins with heat, but, in the United States, much

Ricotta is manufactured from whole milk. The presence of whey proteins is expected in products that contain added milk powders in the form of dressings, such as in creamed cottage cheese, and in products that blend in whey powder during processing, such as pasteurized process American cheese. The presence of whey proteins in cheeses may have also resulted from certain manufacturing techniques; for example, semi-hard cheeses made from UF milk had 18.5% of the total protein as whey proteins (4). Because these undenatured whey proteins are resistant to proteolysis during aging, their presence in cheese could be used as a marker to detect the use of UF milk in cheese making.

The cheese samples examined here had distinct patterns for casein, and the profiles were similar to published findings (5). This suggests that urea-PAGE with the PhastSystem® could be adapted for cheese studies.

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