ABSTRACT

A two-dimensional electrophoretic technique for the analysis of bovine milk proteins was developed. The first dimension separation was by isoelectric focusing in the presence of a nonionic detergent (Nonidet NP-40) and urea and utilized a modified pH gradient from pH 3 to 10. Isoelectric separation was followed by discontinuous, dissociating PAGE using a 14% acrylamide concentration for the resolving gel. This procedure is able to separate the major bovine milk caseins into classes, and to resolve at least some of the genetic variants of these classes. β-Lactoglobulin was also resolved into two bands differing in isoelectric point. The technique was used to examine the effects of high speed centrifugation on the distribution of casein proteins in raw skim milk.

INTRODUCTION

Electrophoresis is an important tool for the protein chemist and biochemist. In its many forms, electrophoretic techniques can serve to characterize molecular architecture, determine homogeneity, and quantitate proteins. Under appropriate conditions, subtle differences in molecular properties such as those due to posttranslational modifications or genetic variation within protein classes, can be determined. Electrophoresis has allowed chemists to study changes in proteins induced by processing treatments (7, 8, 10, 12, 13). It is not surprising that electrophoresis serves as one technique for positive identification of specific milk proteins (13).

One successful electrophoretic technique is known as isoelectric focusing (IEF). As the name implies, IEF separates proteins on the basis of their isoelectric point (pI). The pI is an inherent characteristic of a particular protein species and serves as a useful parameter for study when evaluating the nature of a protein molecule. The actual chemistry of IEF has been covered admirably in several reviews (2, 4). New advances in ampholytes (the amphoteric species that form a pH gradient in the presence of a strong electric field), in gel matrices, and in electrical power sources have made IEF a tool that is rapidly finding many applications in dairy protein studies.

Polyacrylamide gel electrophoresis is another powerful technique for the analysis of proteins. Separation by PAGE is based on overall protein charge and molecular size. This technique has also been reviewed in considerable detail by several authors, and the literature is replete with examples of the successful application of PAGE to the analysis of milk proteins (5, 8, 10, 11, 13). A modification of PAGE known as SDS-PAGE uses the anionic detergent SDS to minimize the influence of protein charge in the PAGE separation, allowing estimation of protein molecular weight (5, 6, 13).

Although both IEF and SDS-PAGE are extremely useful in themselves, the two techniques can be combined into a two-dimensional (2-D) system that greatly increases the amount of available information beyond that obtained from use of the two separate systems. Successful 2-D electrophoresis was developed by O'Farrell in 1975 for the analysis of proteins from Escherichia coli cell extracts (3). This report describes a modification of IEF/SDS-PAGE 2-D electrophoresis that is specifically designed for the analysis of milk proteins.
MATERIALS AND METHODS

Reagents

All reagents were purchased at the highest possible purity and used without further purification. Urea (28,361-4) was obtained from Aldrich Fine Chemicals (Milwaukee, WI). Ampholytes (Pharmalytes®, P-1522 and 1647 EDTA, (E-9884), 2-mercaptoethanol (MCE, M-6250), 2-[N-morpholine] ethanesulfonic acid (MES, M-8250), Nonidet NP-40® (N-6507), SDS (L-4509), N,N,N',N'-tetramethylethylenediamine (TEMED, T-8133), and tris(hydroxymethyl)-aminomethane (Tris, T-1503) were purchased from Sigma Chemical Company (St. Louis, MO). Electrophoretic grade acrylamide (X-5521), N,N-methylenebisacrylamide (Bis, 8383) and ammonium peroxydisulfate (11151) were from Eastman Kodak (Rochester, NY). Agarose (SeaPrep, 50302) was purchased from FMC Corporation (Rockland, ME). Other reagents were obtained from various suppliers. All water used was distilled and deionized (ddH2O). Adjustments in pH were made at room temperature.

Stock Solutions

Composition of stock solutions is described.

Solution A: 30% acrylamide, .8% (wt/vol) Bis in ddH2O;

Solution B: .2 M EDTA in ddH2O, pH 7.0;

Solution C: 1.5 M Tris in ddH2O, adjusted to pH 8.9 with 6.0 M HCl;

Solution D: 1.0 M MES in ddH2O, adjusted to pH 6.5 with 6.0 N NaOH;

Solution E: 20% (wt/vol) SDS in ddH2O; and

Solution F: 10% (vol/vol) Nonidet NP-40 in ddH2O.

Tank buffer concentrate was .2 M Tris, 1.536 M glycine, .8% SDS in ddH2O.

This solution was diluted 1:8 prior to use. Tracking dye consisted of .5 ml solution E, 2.5 ml solution D, 5.0 g sucrose, .1 ml of a .4% solution of bromphenol blue, and 20 ml ddH2O.

Gel Preparation- Isoelectric Focusing

Ten milliliters of a 5% acrylamide, 9.0 M urea, 2% ampholyte, 2% Nonidet NP-40 solution were required to make 12 2.0 × 100 mm rod gels for the first dimension separation. This was made by mixing 5.5 g urea, 1.5 ml solution A, 2.0 ml solution F, 1.85 ml ddH2O, and .45 ml ampholytes (usual mixture was 90% pH 3 to 10 ampholyte and 10% pH 2.5 to 5 ampholyte). The resulting solution was warmed under running tap water to dissolve the urea and then degassed (>10 min) under vacuum. After degassing .008 ml TEMED and .012 ml of freshly made 10% (wt/vol) aqueous ammonium peroxydisulfate was added to initiate polymerization. Glass tubing 2.0 × 150 mm was sealed on one end with Parafilm, and gel mixture was added with the aid of a Pasteur pipette, taking care that no air bubbles were formed during the process. The gel mixture was overlaid with 10 μl ddH2O and allowed to polymerize for 30 min. Gels were used the same day they were cast due to the tendency of urea to decompose.

Gel Preparation- Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Thirty milliliters of SDS-PAGE gel solution were required to make two, 110 × 150 × 2 mm slab gels. Most often the gels used were 12% acrylamide and were made by mixing 12 ml solution A, 6.0 ml solution C, 11.25 ml ddH2O, .15 ml solution E, .3 ml solution B, .02 ml TEMED, and .3 ml of 10% (wt/vol) ammonium peroxydisulfate. Gels were overlaid with .5 ml ddH2O until polymerized. Polymerization was generally complete within 30 min.

Sample Preparation

Protein solutions (.4 ml) containing between 2.5 and 5.0 mg protein/ml were added to .57 g urea, .2 ml solution F, .05 ml ampholyte mixture, and .05 ml 14.32 M MCE. The sample mixture was gently warmed with running tap water to dissolve the urea, then centrifuged at 4000 × g for 10 min to remove any insoluble material. Failure to centrifuge samples resulted in streaking in the second dimension, obscuring minor proteins. Samples were stored at −70°C until analysis. When needed, the samples were allowed to thaw at room temperature for about 10 min before loading onto the IEF gels.

The SDS-PAGE molecular weight standards or samples were prepared by mixing 2.0 mg of protein solution (usually nonfat dry milk), 10 mg agarose, .25 ml tracking dye, .1 ml 14.32 M
MCE, and ddH$_2$O to 2.0 ml. This mixture was heated in a boiling water bath and cast into a gel rod for use in the second dimension. After the mixture solidified, the rod was cut into pieces approximately 10 mm long and cemented into place on the surface of the SDS-PAGE gel (see Sample Application-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis).

**Running Conditions-Isoelectric Focusing**

After polymerization, the IEF gels were overlaid with a solution of 9.5 M urea, 2% (vol/vol) Nonidet NP-40, 2% (vol/vol) ampholyte, 5% (vol/vol) MCE and allowed to stand approximately 1 h. This overlay buffer was replaced with fresh overlay solution and the gels were prefocused for 15 min at 200 V, 15 min at 300 V, and 30 min at 400 V (constant voltage), applied by an ISCO model 595 power supply (Lincoln, NE). Electrode solutions were .02 N NaOH (exhaustively degassed before use), and .01 M H$_3$PO$_4$. Both solutions were chilled to 4°C before use. Focusing was performed in a disc gel electrophoresis apparatus (Buchler Instruments, Fort Lee, NJ) maintained at 4°C with chilled water. After prefocusing, the NaOH was removed from the upper chamber. The overlay solution was removed with the aid of a Pasteur pipette. The gels were loaded with sample, typically 3.0 to 5.0 µl/gel. The sample was then overlaid with 10 µl of a solution containing 9 M urea, and 1% (vol/vol) ampholyte mixture. After replacement of the NaOH with fresh solution, the samples were focused for 10,000 volt-hours at 500 V (constant voltage).

At the end of the focusing run, samples were frozen in the glass tube to facilitate removal. Samples could be stored frozen (−70°C) at this point for up to 6 mo without serious loss of resolution. Before second dimension separation, samples were thawed (approximately 10 min) and removed from the glass tubes by gentle pressure from a pipette bulb. A few rod gels were stained in Coomassie blue to determine effectiveness of IEF separation. Most IEF separation gels were equilibrated for 30 min in 10 ml of an SDS sample buffer (10% (vol/vol) glycerol, 5% (vol/vol) MCE, 2.3% (wt/vol) SDS, .0625 M Tris-HCl, pH 6.8) before loading to the SDS-PAGE gel.

**Sample Application-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**

Two to 3 ml of a 1% (wt/vol) agarose solution was made up in the SDS sample buffer. This material was melted in a boiling water bath and poured onto the upper surface of the previously prepared SDS-PAGE gel. The equilibrated IEF rod gel was forced into the agarose and cemented into place on the surface of the slab gel as the agarose cooled. (The IEF gel is sandwiched between glass plates in the space normally used for a stacking gel in SDS-PAGE procedures.) Molecular weight standards were also fixed to the gel in a similar manner.

**Running Conditions-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**

The second dimension was run at room temperature in an Aquabogue Model 100 electrophoresis tank (Aquabogue, NY) at 50 mA (constant power) for 2 to 3 h or until the tracking dye had completely eluted from the gel.

**Visualization**

Gels were stained in .1% (wt/vol) Coomassie blue in methanol:water:acetic acid (5:4:1) for about 7 h and destained in methanol:water:acetic acid (7.5:87.5:5) for > 5 h. A few gels were stained using the BioRad Silver staining kit (BioRad Laboratories, Richmond, CA).

**RESULTS AND DISCUSSION**

Two-dimensional electrophoresis has been successfully applied to several food systems, including cheese (10) and whey (8). However, the system described here does not require gradient gels, or urea in the second dimension, and does allow for estimation of molecular weights and isoelectric points of the analyzed proteins. By reducing IEF and SDS-PAGE preparation to a simple formula approach, reproducible results could be expected from even the novice technician.

The resolving power of the 2-D separation is apparent in Figure 1. The major proteins of whole milk do not vary widely in pI (between pH 4 and 5). By use of a modified gradient, the pH region of interest is flattened, spreading the proteins over a wider area. Similarly, the caseins do not vary greatly in molecular weight,
yet the separation by isoelectric point in the first dimension allows the SDS-PAGE separation to resolve adequately the caseins into distinct areas corresponding to the $\alpha_S$-casein and $\beta$-casein fractions. The identity of proteins was determined by isoelectric point and molecular weights according to the properties of the specific proteins reported by Whitney et al. (13) and the analysis of purified protein standards.

This technique is a sensitive way to visualize the effects of various separation treatments, for example centrifugation, on the protein fractions of milk. In Figure 2, whole milk was centrifuged at 190,000 $\times$ g for 1 h, then separated according to the described protocol. The area corresponding to the $\alpha_S$-caseins is much smaller than in Figure 1, indicating that the centrifugation removed a large portion of these caseins. However, the $\beta$-caseins were unchanged.

The molecular weight of the various casein fractions has been determined precisely by analysis of the genome responsible for production of these proteins in vivo (13). In SDS-PAGE the caseins should elute in the order of (from top to bottom) $\beta$-casein (24,000 daltons), $\alpha_S$-caseins (23,236 daltons), $\gamma$-caseins (20,500 daltons), and $\kappa$-casein (19,000 daltons). However, using purified protein standards, $\alpha_S$-casein migrated with an apparent molecular weight greater than that of $\beta$-casein using SDS-PAGE. This order of electrophoretic mobility has also been reported by other workers (8, 10). It has been postulated that aberrant mobility of glycoproteins in SDS-

Figure 1. Two-dimensional electrophoresis of 31 $\mu$g whole milk protein. Lane on far right, 50 $\mu$g $\alpha_S$-casein depleted whole milk. The plus indicates the direction of the anode in the respective electrophoretic separations. Labels on Figure indicate the following components: BSA = Bovine serum albumin, $\alpha_S$-CN = $\alpha_S$-caseins, $\beta$-CN = $\beta$-caseins, $\beta$-LG = $\beta$-lactoglobulin, and $\alpha$-LA = $\alpha$-lactalbumin.
PAGE is due to a reduction in the ratio of SDS bound to the molecule. The lower SDS binding results in a decreased charge per unit mass, and is translated into decreased mobility (hence, a higher apparent molecular weight) in the PAGE separation (6). However, neither \( \alpha \)-casein nor \( \beta \)-casein contain significant carbohydrate residues. These two caseins do differ in the amount of phosphorylation; \( \alpha \)-casein has 8 to 12 ester-phosphate linkages per molecule and \( \beta \)-casein has only 5 similar residues (11). Creemer and Richardson have shown that dephosphorylation of \( \alpha \)-casein does not significantly change the mobility of this molecule during SDS-PAGE (1). These authors report that \( \alpha \)-casein has a larger hydrodynamic size due to the large number of negative amino acid residues found in the internal peptide (residues 43 to 78) of this casein. The larger hydrodynamic size would result in a slower migration of the protein in SDS-PAGE.

More difficult to rectify is the apparent discrepancy between the pI of the various proteins reported by other studies [e.g., see (8, 10, 13)]. In particular, relative pI of \( \alpha \)-lactalbumin has been reported as pH 4.2 to 4.5 (13) and 5.15 (8). In the present work, the pI assigned to \( \alpha \)-lactalbumin would suggest the pI to be nearer the more alkaline value, since it appears in vertical proximity to \( \beta \)-lactoglobulin, which has an established pI of 5.3 (13). It is possible that the shift in apparent pI is an artifact of the particular ampholyte system employed. However, with repeated analysis of milk proteins using many different ampholyte combinations (results not shown), the position of \( \alpha \)-lactalbumin is always in the same relative location, indicating the pI of 5.15 reported by Shimazaki and coworkers (8) may be correct.

Many milk proteins seemingly homologous to SDS-PAGE analysis actually contain a number of genetic variants that differ slightly in

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Figure 2. Two-dimensional electrophoresis of 40 \( \mu \)g whole milk proteins. Sample was centrifuged at 190,000 \( \times g \) for 1 h before electrophoresis. The plus indicates the direction of the anode in the respective electrophoretic separations. Labels on Figure indicate the following components: BSA = Bovine serum albumin, \( \alpha_s \)-CN = \( \alpha_s \)-caseins, \( \beta \)-CN = \( \beta \)-caseins, \( \beta \)-LG = \( \beta \)-lactoglobulin, and \( \alpha \)-LA = \( \alpha \)-lactalbumin.

isoelectric point (13). This phenomenon is putatively referred to as microheterogeneity. The microheterogeneity of caseins is apparent in Figure 1 where at least three different αs-caseins and two β-caseins can be seen. When the protein load of the same sample is increased as in Figure 3, the area around the caseins becomes difficult to interpret. The many spots may be due to further genetic variants, processing treatments, or proteolysis fragments (e.g., the proteose-peptone fraction). The 2-D procedure has sufficient resolving ability to be useful to scientists studying this interesting area of protein biochemistry.

In addition, the 2-D procedure described here lends itself to further manipulations such as electroblotting (Western blotting) and immunochemical identification of proteins. With the advent of more useful methods of image analysis, the 2-D technique could possibly be used to detect the presence of specific types of milk and provide clues to the effects of specific proteins on cheese yield for example.

Several items must be controlled to successfully use and interpret the 2-D patterns. Purity of reagents is most critical. Some problems with the polymerization of IEF gels were encountered until highly purified acrylamide was used. The presence of acrylic acid in the acrylamide causes shifts in the pH gradient and is a problem in making the system reproducible (2). Contaminants or degradation products of urea, particularly cyanate, cause the modification of proteins, also complicating analysis (9). Cyanate will result in carbamylation of lysine residues, shifting the apparent pI of the protein in the acidic direction. In addition, ampholytes have a limited shelf life of about 6 mo at 4°C. Molecular weight estimation should be made with caution in SDS-PAGE gels, since many proteins do not exhibit ideal behavior in the presence of SDS (6).

Figure 3. Two-dimensional electrophoresis of 128 μg whole milk protein. The plus indicates the direction of the anode in the respective electrophoretic separations. Labels on Figure indicate the following components: BSA = Bovine serum albumin, αs-CN = αs-caseins, β-CN = β-caseins, β-LG = β-lactoglobulin, and α-LA = α-lactalbumin.
The two-dimensional electrophoretic separation system proposed by O'Farrell (3) has been specifically modified to allow the analysis of bovine milk proteins and should find many applications in the study of processing on this menstruum. Additional studies in our laboratory are proceeding to further enhance resolution and sensitivity and to examine differences produced in various protein fractions by acid precipitation, bacterial fermentation, acetone-ether extraction and proteolysis by purified enzymes.

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