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High-Resolution Two-Dimensional Electrophoresis of Bovine Caseins[1]

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This work describes a high-resolution two-dimensional electrophoretic method for identification of bovine milk caseins. The isoelectric focusing separation was carried out in 100-μL capillary tubes using high field strengths to achieve enhanced resolution. Subsequent sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a minigel format, and the result of the combined techniques gave enhanced protein resolution while requiring substantially less time than conventional two-dimensional methods. The procedure readily distinguished various isoforms of caseins, as well as phosphorylated forms of the proteins. The method was also used to demonstrate the effect of a proteinase derived from a *Pseudomonas* species on milk proteins.

High-resolution two-dimensional electrophoresis is a phrase that has been rather loosely applied to separations involving isoelectric focusing followed by SDS-PAGE and was originally coined by O'Farrell (1975). More recently it has been shown that a significant increase in resolution of isoelectric focusing can be achieved through the use of very thin gels in combination with high field strengths (e.g., 500 V/cm) (Radola, 1980, 1984). The application of these technical improvements to two-dimensional electrophoresis holds promise for increased resolution with the bonus of greatly reduced time. For example, a typical 10 000 V-h separation at 500 V would require 20 h. In contrast, at 4000 V, that same separation could be accomplished in 2.5 h and would theoretically result in greater resolution. It is therefore desirable for purposes of clarity that the phrase "high resolution" be further defined or other terminology such as "high performance" be adopted. In this work isoelectric focusing separations were carried out with 300–400 V/cm field strengths in small-diameter tubes (approximately 1.0 mm). In addition, SDS-PAGE separations were performed in appropriately reduced size gels. The results presented here are in contrast to previous work with milk caseins employing much lower field strengths (e.g., 50 V/cm) (Trieu-Cuot and Gripon, 1981, 1982; Anderson et al., 1982; Marshall and Williams, 1988; Holt and Zeece, 1988) and yielding less resolution.

Bovine milk proteins represent a unique biological system with many functional and nutritional qualities. The principal proteins (α-, β- and κ-caseins) of bovine milk are predominantly found in a micelle complex that is responsible for the stability of the suspension and some of milk's unique functional properties (Kinsella 1984). The other major group of proteins (approximately 20% of total bovine milk protein) is the whey proteins. This class of proteins is composed of β-lactoglobulin and α-lactalbumin (Kinsella, 1984). A significant amount of heterogeneity exists among milk proteins (especially caseins), and the electrophoretic system presented here provides excellent resolution of individual proteins while reducing time required to perform the analyses.

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EXPERIMENTAL SECTION

Apparatus, Chemicals, and Reagents. Isoelectric focusing was performed using a modified Buchler disc gel electrophoresis apparatus with jacketed lower tank. The rubber grommets for holding 5-mm disc gel tubes were replaced with clear acrylic sections glued in place. Column tubing nipples (Catalog No. 90 035, Pharmacia Inc., Piscataway, NJ) were then fitted into threaded holes in the acrylic discs. These adapters held the isoelectric focusing gels, which were polymerized in 100-μL capillary tubes (Accufill 90 Micropet, volume accurate to ±0.5%; Clay-Adams Co., Parsippany, NJ) and allowed for easy placement and removal. These capillary tubes resulted in gels of 9.0 cm in length when filled to 100 μL. Isoelectric focusing was carried out on an ISCO (Lincoln, NE) Model 595 power supply capable of delivering 4000 V, 200 mA and limited to a maximum of 200 W total power output. The unit was programmed to change volts, amps, and watts in three steps with automatic crossover points (as well as end point) regulated by volt-hour values. Protection for the operator from the high voltages used was provided by the power supply's ground fault interrupt circuit and use of 5000-V rated cable for electrophoresis leads. The second-dimension separation (SDS-PAGE) was carried out in the minigel format (80 × 70 × 1.0 mm; Biorad Laboratories, Richmond CA) in constant-voltage mode.

Image analysis was performed with use of the Visage 110 (Bio Image, a Kodak Co., Ann Arbor, MI) machine vision image analyzer. The images were acquired by a solid-state CCD array 512 × 512, 8-bit camera. This camera provided 254 shades of gray and was equalized against a step wedge with 21 increments from 0 to 3.5 OD. The step wedge also allowed for pixel size determination and ranged from 0.177 mm/pixel for a 100-mm square image to 0.554 mm/pixel for a 254-mm square image. Analysis was performed with

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the Bio Image EQ 2-D electrophoresis pattern analysis software in a Sun 110/3 supermicro computer with a 141-Mb hard disk. This software performed image acquisition and pattern matching of paired images by triangulation of selected reference spots. Sample and reference images were superimposed onto a synthetic third image and the common spots identified. A data base was constructed that reported the location, intensity, area, and shape of each spot. Additionally, when two images were referenced, the relative position of each spot was referenced to each other. Each spot was presented as a regular ellipse when plotted with the Gould 6320 colorwriter.

All chemicals used were reagent grade or highest purity available. Special care was taken with urea and ampholytes used in this study. Urea-containing solutions were stored at −70 °C under nonalkaline pH conditions. Acrylamide and other stock solutions for electrophoresis were stored at 4 °C and discarded after a maximum of 60 days. Acrylamide, bisacrylamide, and urea were ultrapure grade purchased from Schwartz/Mann Biotech (Division of ICN, Cleveland, OH). Pharmalyte brand of ampholytes (pH 2.5–5.0 and pH 4.0–6.0), *N,N,N′,N′*-tetramethylethylenediamine (TEMED), 2-mercaptoethanol (MCE), dephosphorylated α- and β-casein standards, and octylphenoxypolyethoxyethanol (nonidet P40) were purchased from Sigma Chemical Co. (St. Louis, MO). Skim milk (pasteurized) was obtained from a local commercial supplier.

METHODS

Sample Preparation. Samples were prepared from skimmed milk, nonfat dry milk, or purified casein by mixing 2–5 mg of protein with distilled H_2O to a total volume of 0.4 mL and then added to a 1.5-mL microcentrifuge tube containing 0.57 g of urea, 0.2 mL of 10% nonidet P40, and 0.05 mL of MCE. Following this protocol, the total volume of the sample was approximately 1.0 mL. The closed tube was gently agitated in a 50 °C water bath until urea completely dissolved (2–3 min). The tubes were centrifuged at 11000*g* for 5 min and supernatants stored at −70 °C until isoelectric focusing. Samples were stable when stored under these conditions for up to 6 months as judged by the lack of alteration in the pattern of separation. Samples were thawed in a 25 °C water bath and ampholytes (5 μL/100 μL of sample) added just prior to isoelectric focusing. Final ampholyte concentration was 2% and consisted of a mixture of 60% pH 2.5–5.0 and 40% pH 4.0–6.0. The addition of ampholyte just prior to isoelectric focusing was desirable because the sample and its constituents (e.g., urea, ampholytes) were exposed to altered pH for only a brief period and it facilitated experimentation with mixtures of ampholytes to optimize resolution.

Isoelectric Focusing Gel Preparation. Isoelectric focusing gels contained the following: 4% acrylamide (bisacrylamide:acrylamide = 1:38), 9.0 M urea, 2.0% nonidet P40, and 2.0% ampholytes. Gels (12–16) were made by adding the following to a 16 × 150 mm glass test tube: 1.10 g of urea, 266 μL of 30% acrylamide stock solution, 400 μL of 10% nonidet P40, 100 μL of ampholytes (Pharmalytes consisting of a mixture composed of 60% pH 2.5–5.0 and 40% pH 4.0–6.0) and 394 μL of distilled H_2O. The tube containing this mixture was swirled in a 50 °C water bath for approximately 3 min to dissolve the contents, and vacuum was then applied to the tube for 5–10 min to degas the solution. Polymerization was initiated by addition of 2.0 μL of 10% ammonium persulfate and 1.4 μL of TEMED.

The capillary tubes were filled by first fitting them onto the end of a 0–200-μL mechanical pipetor (set to 100 μL) with a piece of plastic tubing attached to end of a disposable tip. The gel solution was drawn up through the capillary tube and the open end sealed by pushing it into a double layer of Parafilm secured to the base of a flat-bottomed test tube rack. The rack also served to hold the tubes in a vertical position. A 10-μL portion of overlay solution consisting of 9.0 M urea, 2% nonidet P40, and 2% ampholytes (same mixture as above) was carefully layered on top and allowed to stand for 1 h for polymerization of acrylamide. Tubes were then inspected, and any in which the height of polyacrylamide varied more than 1.0 mm from the 100-μL mark or which contained any defect (air bubbles) were discarded.

Isoelectric Focusing. Prefocusing of gels prior to sample addition was not found to affect resolution and was therefore eliminated. The overlay solution was removed, and samples (1–15 μL) were loaded, followed by 10 μL of fresh overlay solution. The remainder of the tube was then filled with freshly made and degassed 0.01 M NaOH that also served as the cathode solution. The gel tubes were secured in the upper chamber of the modified Buchler disc gel electrophoresis apparatus via the column nipples. The lower chamber (anode) was filled with 0.01 M H_3PO_4 to a height sufficient to cover the gels and maintained at 10 °C with a refrigerated circulating water bath. The isoelectric focusing separation was carried out with a programmable power supply to perform crossover and end point at selected volt-hour values as shown in Table I. At the end of the run tubes were removed from the apparatus and frozen at −20 °C until the SDS-PAGE separation.

Table I. Power Program for Isoelectric Focusing of Caseins

volts	milliamps	watts	volt-hours[a]
500	1.0	1.0	200
1500	2.0	2.0	800
3000	2.0	2.0	3000[b]

[a] This table summarizes the settings of volts, amps, watts, and volt-hours entered into the power supply's memory to perform the isoelectric focusing separation. Volt-hours represent the product of volts × time and were used by the program to determine automatic crossover points to the next higher voltage as well as end point. [b] Total volt-hours used in this separation was 4000.

SDS-PAGE. IEF gels were thawed (approximately 5 min) and removed from the capillary tube by attaching one end to a 1.0-mL plastic syringe with a suitable piece of plastic tubing or a tube gel ejector (Biorad Laboratories, Richmond, CA). The gel was pushed out with steady air pressure from the syringe into a small trough containing 5.0 mL of SDS equilibration buffer (consisting of 5% MCE, 0.067 M Tris-phosphate, pH 6.7, 1.0% SDS) and allowed to incubate for 5 min. Approximately 1 cm of the gel was removed from the acidic end and placed lengthwise on top of an SDS-containing slab gel (80 × 70 × 1.0 mm). The SDS-PAGE system was essentially that as described by Laemmli (1970) and consisted of a 5% stacking gel over a 14% separating gel. The IEF gel fit snugly into place on top of the slab gel, and securing it with melted agarose was not necessary. Two hundred microliters of equilibration buffer was added, and any bubbles trapped between IEF and SDS gels were removed. The SDS-PAGE was run at 200 V (constant voltage) for precisely 50 min, which allowed the dye front to just migrate off the bottom edge of the gel. The gels were stained in 0.1% coomassie blue R250 in 50% methanol, 10% acetic acid, for 2 h with constant agitation. The gels were destained in several changes of 7% acetic acid, 5% methanol overnight also with constant agitation.

Enzyme Preparation. A proteolytic strain of *Pseudomonas fluorescens*, which had been isolated from raw milk, was obtained from the culture collection in the Department of Food Science and Technology at the University of Nebraska. Identity of the bacterium was confirmed prior to use. An inoculum was prepared by adding a loopful of *P. fluorescens* to 10 mL of brain heart infusion broth (Difco) fortified with 20% sterile skim milk (BHIM) and incubating overnight without agitation at 22 °C. Ten milliliters of inoculum was aseptically added to 1.5 L of BHIM contained in a 2-L Erlenmeyer flask and incubated for 2 days at 22 °C. A magnetic stir bar was placed in the flask and rotated at about 60 rpm during the incubation period, providing constant mixing of the growth medium. A turbid solution resulted after 2 days of incubation.

Partial purification of *Pseudomonas* proteinase was performed essentially as described by Richardson (1981) and briefly outlined as follows. The incubation medium was centrifuged at 13000*g* for 20 min at 4 °C (all subsequent steps at 4 °C also) to remove suspended materials. The supernatant was adjusted to 50% ammonium sulfate by slow addition of solid and allowed to stand for 30 min before centrifugation (13000*g* for 15 min) to collect the pellet. The pellet was resuspended in 20–30 mL of 20 mM Tris-hydrochloride (pH 7.5, 10 mM $CaCl_2$) and dialyzed overnight vs 4 L of the same buffer. The solution was removed from dialysis and clarified by ultracentrifugation at 82000*g* for 1 h and then concentrated to 2–3 mL in an ultrafiltration cell (Amicon, Danvers, MA) with a 10000 molecular weight cut-off membrane (PM 10). Of the crude enzyme preparation, 1 mL was next chromatographed on a Sephadex G-75 column (1.5 × 40 cm) in 20 mM Tris-hydrochloride (pH 7.5, 10 mM $CaCl_2$) with a flow rate of 24 mL/h.

Proteinase activity of the isolated enzyme was determined with a modification of the procedure reported by Richardson and Te Whaiti (1978) using casein as the substrate. Activity was measured in a total volume of 2.0 mL containing 5 mg/mL casein, 100 mM KCl, 50 mM Tris-hydrochloride (pH 7.5), 2 mM $ZnSO_4$, and 10 mM $CaCl_2$. The assay mixture was incubated for 90 min at 37 °C, the reaction stopped by addition of 2.0 mL of 12% trichloroacetic acid (TCA), and the mixture then centrifuged for 5 min at 2000*g*. The activity of the enzyme was expressed as absorbance at 280 nm of TCA supernatants. One unit of activity was defined as 0.1 absorbance unit/90 min of incubation. Control for the assay consisted of reversing the order of addition so that TCA was added before enzyme.

Incubation of Skim Milk with *P. fluorescens* Proteinase. For this assay, 500 μL (18.85 mg) of skim milk was added to a tube containing 390 μL of distilled H_2O and 110 μL (10 μg) of G-75 purified *Pseudomonas* enzyme and the resulant mixture incubated at 37 °C for 90 min. At the end of the incubation, a sample was prepared for isoelectric focusing by adding a 0.1-mL aliquot (approximately 1.9 mg) directly to a tube containing 0.57 g of urea, 0.3 mL of distilled H_2O, 0.2 mL of 10% nonidet P40, and 50 μL of MCE and warmed briefly in a 50 °C water bath until the urea dissolved. Undissolved materials were removed by centrifugation at 11000*g* for 3 min, and the supernatant was stored at −70 °C until isoelectric focusing.

RESULTS AND DISCUSSION

Figure 1 shows the effect of increasing volt-hours on the separation of milk proteins from a nonfat dry milk (NFDM) source. Increasing total volt-hours from 2000 to 6000 (Figure 1a–c) resulted in increased resolution. The α_s class of proteins was separated into two groups in these gels based on charge. The most acidic group represented the major portion of this protein class and was composed of three spots. The other group of α_s-caseins was composed of approximately six species at 4000 V-h (Figure 1b) and seven species (five major and two minor) at 6000 V-h (Figure 1c). This group of less acidic caseins also exhibited slightly greater vertical mobility in the second-dimension separation. A total of 10 spots for the α_s class were resolved. β-Caseins were found to be composed of three species (two major and one minor) in these gels, and their resolution also increased as volt-hours increased. Four κ-caseins were observed in Figure 1a and their relative lateral separation increased significantly when the volt-hours were increased to 4000 (Figure 1b). At 6000 V-h (Figure 1c) only two species remained on the gel. β-Lactoglobulin was found to be composed of three species, with the most acidic being a very minor component. The most acidic β-lactoglobulin component may also be an artifact as suggested by Zadow and Hardham (1980). Only one

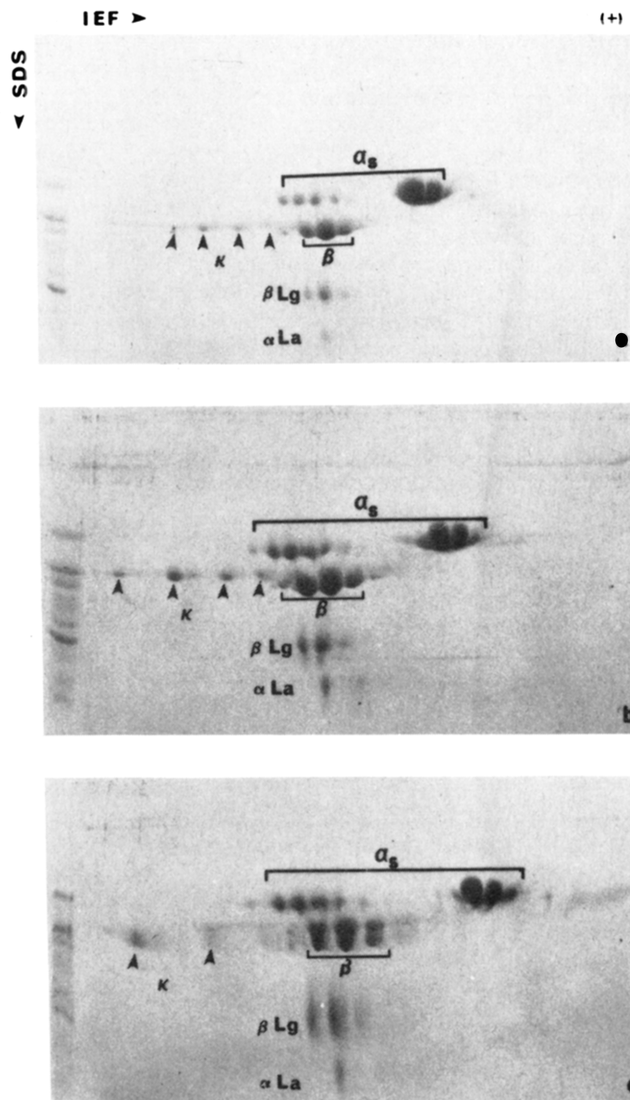

Figure 1. Effect of increasing volt-hours on two-dimensional separation of nonfat dry milk proteins. Isoelectric focusing was carried out as described in Methods for 2000 (a), 4000 (b), and 6000 (c) V-h with 50 μg of NFDM and then subjected to second-dimension SDS-PAGE on 14% gels. The (+) sign indicates position of the most acidic region in the isoelectric focusing gel. Proteins were labeled as follows: α_s, α_s-casein; β, β-casein; κ, κ-casein (arrowheads); β-Lg, β-lactoglobulin; α-Lg, α-lactalbumin. The lane at the left of each gel shows SDS-PAGE separation of 8 μg of skim milk.

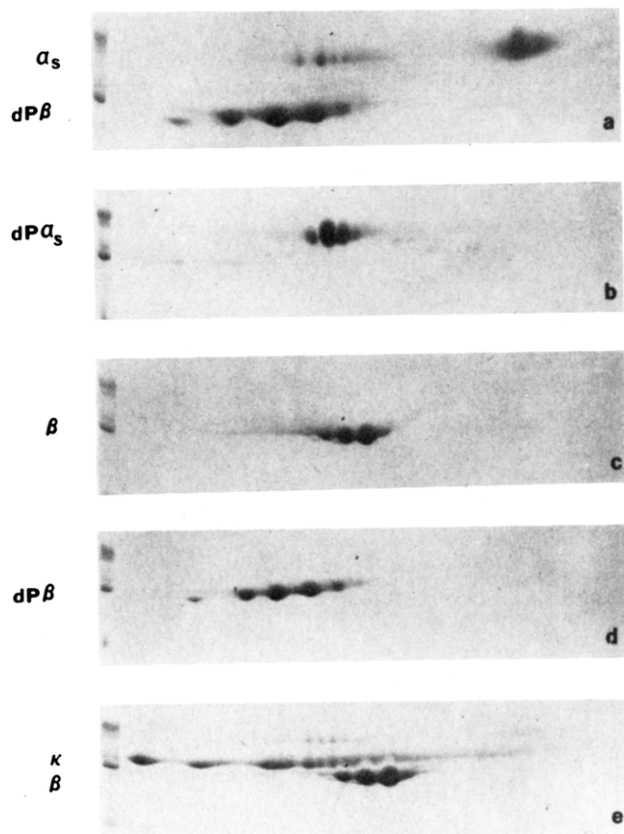

Figure 2. Two-dimensional electrophoretic separation of casein standards. Purified casein standards obtained from a commercial source were separated with two-dimensional electrophoresis as described in Methods for 4000 V-h. Panels contained the following: 13 μg of α_s-casein (α_s) and 13 μg of dephosphorylated β-casein (αPβ) (a); 13 μg dephosphorylated α_s-casein (dPα_s) (b); 13 μg of β-casein (β) (c); 13 μg of dephosphorylated β-casein (dPβ) (d); 16 μg of κ-casein (κ) and 13 μg of β-casein (β) (e). The lane at the left of each panel shows SDS-PAGE separation of 4 μg of skim milk.

spot was observed for α-lactalbumin in these separations. The separations conducted at 4000 V-h represent the best compromise between resolution of α_s- versus retention of κ-caseins and were therefore used in subsequent experiments.

Identification of α_s-, β-, and κ-casein proteins in these gels was accomplished with purified standards obtained from Sigma Chemical Co. Figure 2 shows the separation achieved at 4000 V-h for mixtures of these proteins. The patterns observed for dephosphorylated α_s- or β-caseins (Figure 2, parts b and d, respectively) were substantially different from their phosphorylated counterpart. Dephosphorylated α_s-caseins were observed to contain only three major components, and this group migrated to the midregion of the gel, reflecting its reduced negative charge (compare parts a and b of Figure 2). Similarly, three dephosphorylated β-casein isomers were found in a less acidic location, and at least one extra spot was observed (compare parts c and d of Figure 2). The extra spot found in Figure 2d migrated to a position identical with that of the least acidic species of phosphorylated β-casein (Figure 2c) and could have resulted from incomplete dephosphorylation. However, it was not possible to draw a conclusion from these results alone. κ-Casein's separation was less defined and consisted of at least four species migrating toward the more alkaline end of the gel and a horizontal streak in the acidic region.

The results reported here regarding the number of species of α_s- and β-caseins are not in agreement with those

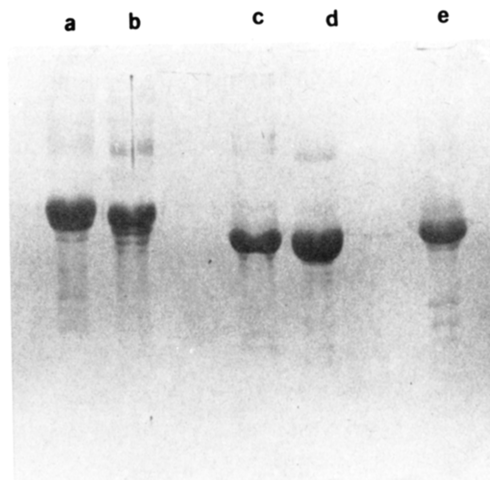

Figure 3. SDS-PAGE separation of casein standards. SDS-PAGE using 14% gel was carried out with purified casein standards obtained from a commerical source. Lanes a and b contain 10 μg of α_s-casein and dephosphorylated α_s-casein, respectively. Lanes c and d contain 10 μg of β-casein and dephosphorylated β-casein, respectively. Lane e contains 10 μg of κ-casein.

of Trieu-Cuot and Gripon (1981, 1982) who identified six α_s isomers by isoelectric focusing and resolved only two of these in two-dimensional electrophoresis. In addition these authors reported only one major isomer for β-casein. These differences may be due to the ampholyte pH range used (4.0–8.0) and/or lower field strengths employed in their separation.

The relative positions of α_s-, β- and κ-caseins in the second dimension were also determined by SDS-PAGE in one-dimensional gels as shown in Figure 3. Dephosphorylated α_s-caseins migrated slightly faster than their phosphorylated counterparts (compare lanes a and b, Figure 3). Similarly, dephosphorylated β-caseins had slightly faster mobility than the phosphorylated form (compare lanes c and d, Figure 3). The relative mobilities of α-, β-, and κ-casein shown in these figures are not in agreement with their known molecular weights reported as 23000, 23900, and 19000, respectively (Kinsella, 1984). If separation had occurred strictly on the basis of molecular weight as expected for SDS-PAGE, then the order of mobility would have been β, α_s, and κ. The results in Figure 3 suggest that the presence of phosphate in these caseins reduced the binding of SDS per unit mass, thus lowering the observed mobility. However, dephosphorylated caseins also did not migrate in agreement with their known molecular weights, suggesting that other factors influence SDS binding. The difference in migration for α_s-caseins in SDS-containing discontinuous electrophoresis has been observed previously and reviewed by Creamer and Richardson (1984). Altered SDS binding to α_s-caseins was proposed to be due to strongly acidic and negatively charged regions of this protein sequence. This property may also result in a larger hydrodynamic size, providing explanation for the higher M_r determined by SDS-PAGE and SDS-containing gel permation chromatography.

The two-dimensional electrophoretic protocol was used to examine the effects of a proteinase isolated from a species of *Pseudomonas* on milk proteins. A review by Fairbairn and Law (1986) reported that proteinases from *Pseudomonas* species may be responsible for several undesirable milk characteristics such as bitter off-flavors and gelation in ultra-high-temperature (UHT) processed milk. For this application 18.8 mg of skim milk was treated for

Table II. Quantitation of Individual Protein Constituents of Control and Proteinase-Treated Skim Milk Using Image Analysis of Two-Dimensional Electrophoresis Gels

spot no.	protein	location[a] X	location[a] Y	integrated intensity[b] control	integrated intensity[b] proteinase-treated
1	α_s	223	268	0.098 ± 0.042	0.095 ± 0.025
2	α_s	238	267	0.119 ± 0.004	0.084 ± 0.001
3	α_s	251	267	0.121 ± 0.001	0.065 ± 0.028
4	α_s	264	266	0.042 ± 0.020	
5	α_s	338	260	0.116 ± 0.036	
6	α_s	350	258	1.602 ± 0.164	1.168 ± 0.164
7	α_s	362	257	0.540 ± 0.032	0.423 ± 0.046
8	β	250	291	0.490 ± 0.018	0.206 ± 0.080
9	β	268	290	0.631 ± 0.113	0.182 ± 0.006
10	β	284	288	0.069 ± 0.002	
11	κ	115	286	0.019 ± 0.012	
12	κ	150	286	0.115 ± 0.020	
13	κ	189	284	0.020 ± 0.003	
14	κ	235	290	0.070 ± 0.046	
15	β-Lg	247	336	0.093 ± 0.004	0.057 ± 0.025
16	β-Lg	262	336	0.121 ± 0.033	0.063 ± 0.026

[a] Location of each spot center on 512 × 512 pixel array is given. The grid was designed such that abscissa values increased going from left to right and ordinate value increased going from top to bottom. [b] Values for integrated intensity were calculated as area (mm^2) × absorbance and represent an average (±SD) obtained from scanning each gel three times.

90 min at 37 °C with 10 μg (4.5 units) of proteinase isolated from *P. fluorescens* culture. The results of two-dimensional electrophoretic separation of aliquots of control (untreated) and proteinase-treated samples are shown here as the computer-generated image (Figure 4), and quantitative data for the proteins in those gels are listed in Table II. Table II contains the results for individual protein constituents expressed as integrated intensity (area × absorbance) and their location. Values for each peak's intensity, shape, and area have been omitted from Table II for reasons of brevity. Proteins in the graphic representation of the gels show only relative areas (no intensity input) and were recorded as ellipses by the computer program. Figure 4a shows the control that was incubated for 90 min but without enzyme. Figure 4b shows the proteinase-treated sample loaded at the same amount as in the control. Isoelectric focusing was performed for a total of 4000 V-h for both samples. Proteins in the spot list of Table II are correspondingly numbered within each protein class (i.e., α_s, β etc.) from left to right in Figure 4. There were 16 constituent proteins identified in the control (Figure 4a); however, only nine remained in the proteinase-treated sample. Almost all of the proteins were degraded to some extent by the proteinase treatment. α-Lactalbumin was not resolved well in these gels and was eliminated from the image analysis. A number of very faint spots were also not included in this analysis. The major α_s-caseins (spots 6 and 7, Table II), which are also the most acidic forms, were degraded approximately 25% by proteinase treatment under these conditions. The other species of α_s-caseins were degraded to varying degrees. Spot 1 in Table II showed little or no alteration. Spots 2 and 3 decreased approximately 29 and 46%, respectively, compared to the control, while spots 4 and 5 were completely degraded by proteinase treatment. Comparison of β-caseins (spots 8–10, Table II) showed that the proteinase degraded Spots 8 and 9 about 58 and 71%, respectively, and completely degraded spot 10. It is of interest to note that smaller molecular weight species corresponding to γ-caseins were not observed. One possible explanation is that γ-casein fragments have more alkaline isoelectric points and may not have entered the isoelectric focusing

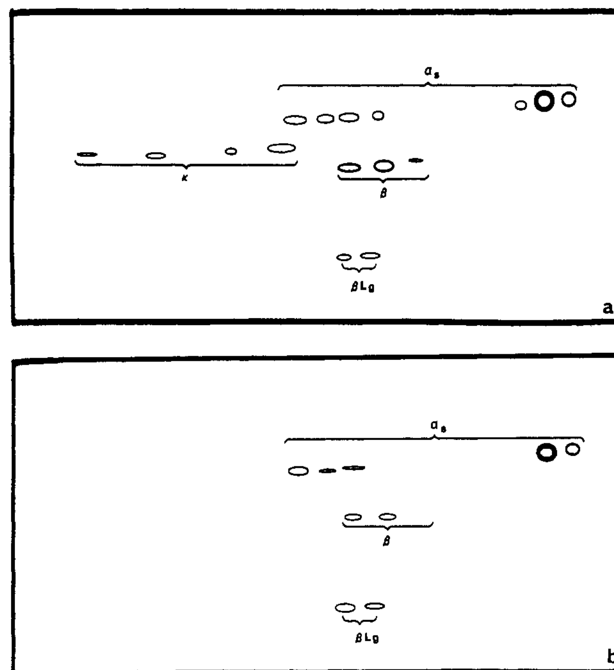

Figure 4. Image analysis of two-dimensional electrophoretic separation of *P. fluorescens* proteinase-treated skim milk. Two-dimensional electrophoretic separation of an aliquot of skim milk containing 40 μg of protein was carried out for 4000 V-h after incubation for 90 min at 37 °C without proteinase (a) or with proteinase (b) isolated from *P. fluorescens*. The ratio of enzyme to protein in (b) was approximately 1:1800 (w/w). Proteins are labeled as in Figure 1.

gel with the pH gradient used in these separations (i.e., 2.5–6.0). Marshall and Willams (1988) using two-dimensional electrophoresis in a study of cheese proteolysis found several γ-casein fragments in regions of the gel corresponding to pH higher than 6.0. An increase in small molecular weight fragments corresponding to proteose–peptone fraction also was not observed in these gels. Their absence may have been due to migration off the gel in either separation, or they may not have been fixed in the gel by the acetic acid concentration used in the stain. κ-Caseins were completely degraded by the proteinase treatment (Figure 4; Table II). β-Lactoglobulins (spots 15 and 16, Table II) were found to be degraded approximately 39 and 48%, respectively. The results concerning the effect of this proteinase on milk proteins are in agreement with a recent review of the effects of psychrotrophic bacterial proteinases on milk proteins by Fairbairn and Law (1986) who reported that proteinases from *Pseudomonas* species degraded caseins in the following order: $\kappa \gg \beta > \alpha$.

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Registry No. Proteinase, 9001-92-7.

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Incorporation of ^{14}C from [^{14}C]Phenylalanine into Condensed Tannin of Sorghum Grain

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A procedure is described for obtaining condensed tannin from sorghum [*Sorghum bicolor* (L.) Moench] seeds metabolically labeled from [^{14}C]phenylalanine. The [^{14}C]tannin should be useful in determining the metabolic fate of dietary condensed tannin.

The antinutritional effects of dietary polyphenols such as condensed tannins are usually ascribed to inhibition of digestion and absorption within the digestive tract (Cheeke and Shull, 1985). Some of these effects seem to require absorption of metabolically inhibitory polyphenols from the digestive tract, and preliminary evidence for such absorption was obtained by feeding sorghum tannin labeled with ^{125}I (Butler et al., 1986).

More definitive evidence on the question of absorption of dietary polyphenols from the digestive tract could be obtained with polyphenols metabolically labeled with ^{14}C. Condensed tannins such as those found in the grain of some cultivars of *Sorghum bicolor* (L.) Moench are polymeric flavonoids (Haslam, 1981). Phenylalanine is a metabolic precursor for 9 of the 15 carbon atoms of its flavonoid units (Haslam et al., 1977). Here we report a procedure for obtaining condensed tannins metabolically labeled from [^{14}C]phenylalanine supplied to developing panicles of sorghum cultivars whose mature seed is relatively rich in tannins. The [^{14}C]tannin should be useful in determining the metabolic fate of condensed tannin in foods and feedstuffs.

MATERIALS AND METHODS

Uniformly labeled [^{14}C]phenylalanine (specific activity 410 mCi/mmol) in 2% ethanol was obtained from ICN Biomedicals, Inc., Irvine, CA. Sorghum cultivars IS 8768 (primary panicles) and IS 6881 (panicles from tillers) were field-grown open pollinated plants from the Purdue Agronomy Farm, West Lafayette, IN. IS 8768 is a group II type with respect to tannin, and IS 6881 is a group III type (Price et al., 1978).

At 4–5 days after half-anthesis, panicles were cut, leaving stems at least 12 in. long, and were rapidly transported to the laboratory with the stems immersed in water. The stems were cut under water at approximately 45° angle (to increase the contact area) about 4 in. below the base of the panicle and were immediately inserted into 16 mm × 100 mm test tubes containing 5 μCi of [^{14}C]phenylalanine in 5 mL of water. The pancles in their test tubes were placed in a rack inside a plastic bag, and air was pulled through the bag and then through a trap containing 1 M KOH to trap respired CO_2. Under these conditions (room temperature, continuous air flow) the panicles imbibed the aqueous solution of [^{14}C]phenylalanine at an average rate of approximately 1 mL/h. When the level of liquid in the test tube diminished to near the cut surface of the stem, additional water, without phenylalanine, was added. When the panicles stopped imbibing water (usually 50–55 h; 30–50 mL of water was taken up per panicle), they were dried at room temperature for at least 2 days to permit tannin biosynthesis to go as far as possible toward completion. We have previously shown that polymerization of sorghum seed tannins occurs mainly during drying of the seed (Butler, 1982).

For extraction of the tannins, seeds and associated glumes (approximately 30 g from two dry panicles) were soaked for 12 h at room temperature in methanol containing 1 mM ascorbate and 1% (v/v) concentrated HCl, 15 mL/g of dried tissue. The solvent was removed by filtration, an equal volume of 1% HCl in methanol was added, and the tissue was homogenized on a Polytron homogenizer (Brinkmann Instruments). The acidic

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