Biochimica et Biophysica Acta, 514 (1978) 25—36

BBA 78192

SEPARATION OF THE PROTEINS OF BOVINE MILK-FAT GLOBULE MEMBRANE BY ELECTROFOCUSING

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(Received April 24th, 1978)

Summary

The proteins of milk-fat globule membrane have been separated by electrofocusing on both the analytical and preparative scale. Over forty separated proteins of the membrane can be identified after electrofocusing in the presence of urea, Triton X-100 and mercaptoethanol with apparent isoelectric points between pH 5.0 and 9.0. At least eight of these proteins appear to contain carbohydrate. After separation by electrofocusing the samples have been further analyzed by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate. Some of the proteins previously identified as single bands by electrophoresis in SDS are resolved into several components by electrofocusing. The major components of milk-fat globule membrane are a glycoprotein of 67 000 daltons, with an apparent isoelectric point of 5.55, and a protein of 155 000 daltons with an isoelectric point of 7.6. Partially purified fractions of the major proteins and glycoproteins can be obtained after preparative electrofocusing in flat-beds of Sephadex G-75.

Introduction

Fat globules in milk are coated with a membrane layer generally known as the milk-fat globule membrane. A substantial proportion of this membrane originates from the apical surface of mammary acinar cells during the process of triglyceride secretion [1—3]. The globule membrane may also be derived in part from membranes of the Golgi complex and associated secretory vesicles [2,4—6] and endoplasmic reticulum [3,7], although the contribution from these sources in the cow is probably low (refs. 3 and 7, but see refs. 4—6 for alternative viewpoints).

Previous analysis of fat globule membrane proteins by a number of electro-

Abbreviation: SDS, sodium dodecyl sulphate.

phoretic techniques has been reported [2,3,8—20] of which electrophoresis in sodium dodecyl sulfate (SDS) is the most commonly used [2,3,9—12,14—19]. Usually five to seven major Coomassie Blue positive bands and six components that stain with Schiff's reagent are observed after electrophoresis of the bovine membrane in SDS [2,3,9,11,12,14,16—19]. In the presence of SDS, however, proteins are mainly separated by virtue of differences in molecular size [21]. This limitation can be overcome by two-dimensional separations employing electrofocusing coupled to electrophoresis in SDS [22,23]. By this technique analysis of membrane samples from different cell-types and cellular structures confirms the lack of resolution obtained when electrophoresis in SDS is used exclusively.

There are no reports on the separation of bovine milk-fat globule membrane proteins by two-dimensional analysis of this type and only one preliminary study employing electrofocusing [24]. In this latter study, separation of several enzymes was achieved, although no attempts were made to analyze the separated proteins by electrophoresis in SDS, neither was a total analysis of all the membrane proteins attempted [24]. Analytical electrofocusing of buffalo fat globule membrane proteins isolated at several stages of lactation has also been reported [25].

The purpose of this present study was to attempt a total analysis of milk-fat globule membrane proteins using both electrofocusing and electrophoresis in SDS, and to investigate preparative electrofocusing as a means of separating the membrane components.

Materials and Methods

Materials

Urea was recrystallized from hot aqueous ethanol at least twice. To prevent build-up of isocyanate ion, the recrystallized urea was stored at 4°C in a vacuum dessicator and used within one week.

Ampholines and electrophoresis-grade acrylamide were obtained from Bio-Rad Laboratories. SDS, *N,N'*-methylene-bis-acrylamide, ammonium persulphate, *N,N,N',N'*-tetramethylethylenediamine, basic fuchsin, lysine, mercaptoethanol, phenylmethyl sulphonyl fluoride, ϵ-amino-*n*-caproic acid, aprotinin, Triton X-100 and Sephadex G-75 (superfine grade) were all obtained from Sigma Chemical Co. Scintillation-grade Triton X-100 was also obtained from Eastman Kodak.

Methods

(a) Preparation of membrane fractions. Fresh milk (4—6 l) from at least three cows was separated with a cream separator (De Laval, Model 100) and the cream washed twice with a total of 8 l Tris-buffered saline solution (10 mM Tris/140 mM NaCl, adjusted to pH 7.5 with HCl). The cream was then washed a third time with 4 l Tris-HCl buffer (10 mM, pH 7.5) and resuspended in the same buffer (100 ml). After cooling to 10°C the cream was churned in a Waring blendor until butter formed. The aqueous phase was filtered through several layers of cheesecloth to remove butter granules and designated Fraction 1. Fraction 1 was then centrifuged at 4°C (90 000 $\times g_{av}$, 60 min), and the trigly-

ceride that collected at the top of each tube discarded. The orange-brown membrane pellet obtained is referred to throughout this paper as Fraction 2 and the supernatant remaining after centrifugation Fraction 3.

(b) Preparation of samples for electrofocusing or SDS-polyacrylamide gel electrophoresis. Samples for analytical electrofocusing were prepared by taking Fractions 1, 2 or 3 (2—5 mg protein) and adding SDS at a ratio to protein of 1.3 : 1 (w/w) and mercaptoethanol (100 mM) in a final volume of 2.9 ml. Immediately after addition of both reagents the solutions were placed in boiling water for 2 min and then rapidly cooled to room temperature. The solutions were then made up to 5.0 ml by adding solid recrystallized urea (final concentration, 8 M) and Triton X-100 (final concentration 2%, v/v). To aid dissolution of the membrane the mixtures were exposed to ultrasound for 3—5 min at 0°C. For some experiments the steps involving the addition of SDS and heating were omitted, otherwise the procedure was as described above.

Samples for preparative electrofocusing were prepared by placing Fraction 2 (60—100 mg protein) with the following reagents (final concentrations in parentheses) in a final volume of 70 ml: urea (8 M), Triton X-100 (2%, v/v), lysine (5 mM), aprotinin (20 000 kallikrein inhibitor units), phenylmethyl sulphonyl fluoride (0.1 mM) and ϵ-amino-*n*-caproic acid (1 mM). The solutions were centrifuged at 4°C (90 000 $\times g_{av}$, 2 h) and the supernatants used as described in section (*d*).

Samples for electrophoresis in SDS were prepared as described by Laemmli [26].

(c) Analytical electrofocusing. Fractions 1, 2 and 3 were separated on the analytical scale by focusing in acrylamide gels (4.5%, w/v) cast in glass tubes (8.3 × 0.5 cm). The procedures were based on those of O'Farrell [22] and Ames and Nikaido [23]. The gels contained urea (8 M), Triton X-100 (2%, v/v) and glycerol (5%, v/v). Samples prepared as described in section (*b*) above were diluted 1 : 1 with a solution containing urea (8 M), mercaptoethanol (10 mM), glycerol (20%, v/v), Triton X-100 (2%, v/v) and ampholines (4%, w/v). Samples were focused for 5 h at the following voltages (100 V, 30 min; 200 V, 30 min; 350 V, 2 h; 500 V, 2 h). The gels were stained for protein with Coomassie Blue as described by Otavsky and Drysdale [27] and for carbohydrate with Schiff's reagent using the methods of Fairbanks et al. [28].

(d) Preparative electrofocusing. Membrane proteins were separated on a preparative scale in layers of Sephadex G-75 using the LKB Multiphor unit (LKB Instruments, Inc., Rockville, Md.). The protein samples were separated for 18 h at 5°C at constant power (8 W) with an upper limit of 1500 V using an LKB power supply (Model 2103). The gel bed was fractionated into 31 fractions and the separated proteins eluted from the Sephadex beads with distilled water at 0—5°C. Each fraction was made to 3.9 ml with distilled water and the pH of the solutions measured. The samples were then treated with ϵ-amino-*n*-caproic acid (0.1 ml, 20 mM) and dialyzed for 2 days against Tris-HCl solution (62.5 mM, pH 6.8) containing ϵ-amino-*n*-caproic acid (0.5 mM). Samples were prepared for electrophoresis and assayed for protein as described below.

Protein and glycoprotein material that had migrated into the paper strips at the anode was recovered by electrodialysis in the electrode buffer of Laemmli containing SDS (0.1%, w/v) [26].

(e) SDS-polyacrylamide gel electrophoresis. Samples of membrane protein were analyzed by SDS-electrophoresis [26] in slabs of polyacrylamide gel (0.15 × 10 × 14 cm) with a Hoefer vertical-slab gel electrophoresis unit (Model SE 500, Hoefer Scientific Instruments, San Francisco, Calif.). The concentrations of acrylamide for the stacking gels were 4% (w/v) and either 8 or 10% for the separating gels. Gels were stained for protein with Coomassie Blue and carbohydrate with Schiff reagent [28].

(f) Separation of membrane proteins in two dimensions. Fat globule membrane proteins were separated on an analytical scale in two dimensions employing electrofocusing in the first dimension and electrophoresis in SDS in second dimension. After separating the membrane proteins by analytical electrofocusing in acrylamide gels as described in section (*c*), the gels were pre-equilibrated in Tris-HCl buffer (0.125 M, pH 6.8) containing SDS (2%, w/v) and mercaptoethanol (100 mM) for 4 h at room temperature. The gels were then placed onto the separating gels (10%, w/v) of the Laemmli system [26] and sealed in position with hot agarose, as described by O'Farrell [22]. Electrophoresis in SDS-polyacrylamide gels was as described in section (*e*).

(g) Protein determination. Protein was assayed by the method of Lowry et al. [29] using bovine serum albumin as standard. Samples containing Triton X-100 were also assayed by Lowry's procedure after removing the Triton X-100 with iso-amylalcohol. This method will be published separately.

Results

Solubilization of membrane samples

As a first step in developing procedures for the separation of fat globule membrane proteins by electrofocusing it was necessary to develop methods for solubilizing the membrane proteins as completely as possible. Table I summarizes the results of treating two membrane fractions with a variety of disaggregating agents. In a first series of experiments the insoluble residue left after washing Fraction 1 according to the procedures of Jackson et al. [30] was used (Table I, section (a)). About 35% of the residue could be solubilized * with urea (8 M) alone, 60% with urea and mercaptoethanol and 85% with urea, mercaptoethanol and Triton X-100. The addition of SDS before adding Triton X-100 did not apparently lead to further solubilization (Table I, section (a)). Similar results were obtained with samples containing all the membrane proteins (Table I, section (b)). Exposure of the mixtures to ultrasound after addition of the reagents was required for 'complete solubilization'.

Analytical electrofocusing of milk-fat globule membrane

Fig. 1 compares the separation of Fractions 1, 2 and 3 after solubilization with SDS, mercaptoethanol, urea and Triton X-100. At least 40 separated components of Fractions 1 and 2 can be identified in gels stained with Coomassie Blue. Fraction 3 appears to be less complex. Most proteins appear to focus either over a narrow range of pH values between 5.3 and 5.7 or a broad range

* Operationally 'the solubilized fraction' in this paper is defined as that fraction which resists sedimentation after centrifugation of disaggregated membrane samples at 150 000 × g_{av} for 1 h.

TABLE I

SOLUBILIZATION OF MEMBRANE FRACTIONS WITH DISAGGEGATING AGENTS

Membrane fractions were suspended in Tris-HCl buffer (50 mM, pH 7.5) in a final volume of 5.0 ml at 37°C for 1 h with the disaggregating agents specified in the table. The mixtures were then centrifuged (150 000 × $g_{av.}$ 1 h) and the sediments resuspended in Tris-HCl buffer (50 mM, pH 7.5) and dialyzed against the same buffer for 36 h. The concentration of protein was determined in the dialyzed suspensions.

Membrane fraction	Disaggregating conditions	Amount protein sedimented during centrifugation		Amount membrane protein solubilized * (%)
		mg	(%)	
(a) Insoluble residue from procedures of Jackson et al. [30] further extracted with chloroform/-methanol [31].	No additions	10.6	100	0
	Urea (8 M)	6.74	63.6	36.4
	Urea (8 M) + mercaptoethanol (10 mM)	4.22	39.8	60.2
	Urea (8 M) + mercaptoethanol (10 mM) + Triton X-100 (10%, v/v)	1.56	14.7	85.3
	Urea (8 M) + mercaptoethanol (10 mM) + SDS : protein, 1 : 1, w/w) + Triton X-100 (10%, v/v)	1.20	11.3	88.7
(b) Insoluble residue after extracting washed fat globules with chloroform/-methanol [31]; total protein fraction	No additions	11.1	100	0
	Urea (8 M) + mercaptoethanol (10 mM) + Triton X-100 (10%, v/v)	1.62	14.6	85.4
	Urea (8 M) + mercaptoethanol (100 mM) + Triton X-100 (2%, v/v)	0.96	8.6	91.4
	Urea (8 M) + mercaptoethanol (100 mM) + Triton X-100 (2%, v/v) + sonication	0.31	2.8	97.2

* Determined by subtracting figures in adjacent column from 100.

of pH values from 6.4 to 8.0. Glycoproteins are present within both these ranges. Fig. 2 shows the Schiff-positive material identified after separating Fraction 2. At least eight components can be identified of which five focus as distinct bands within a pH range of 5.4—5.7. The glycoproteins focusing between pH 6.5 and 7.5 seemed to be microheterogeneous since they stained as three distinct peaks within a broader diffuse area of stained material. Similar results were obtained with Fraction 1, although Fraction 3 appeared to contain only three Schiff-positive components (identified by asterisks in Fig. 2).

The patterns shown in Figs. 1 and 2 were obtained with only minor variations (particularly at the cathodic end) with at least seven samples prepared at different times and with different batches of urea and ampholines. Omission of SDS from the samples had no overall effect on the pattern of Coomassie Blue-stained and Schiff-positive components identified. However, the bands identified by arrows in Fig. 1 were present in lower amounts on gels containing samples that had not been treated with SDS. Presumably in the absence of SDS these components are present in large micellar aggregates that do not penetrate the acrylamide gel during focusing.

Fractions 1 and 2 were also separated by two-dimensional electrophoresis which combined electrofocusing in acrylamide gels in the first dimension with electrophoresis in SDS in the second dimension. The pattern routinely obtained is shown in Fig. 3 and compared with the pattern of Coomassie Blue-stained

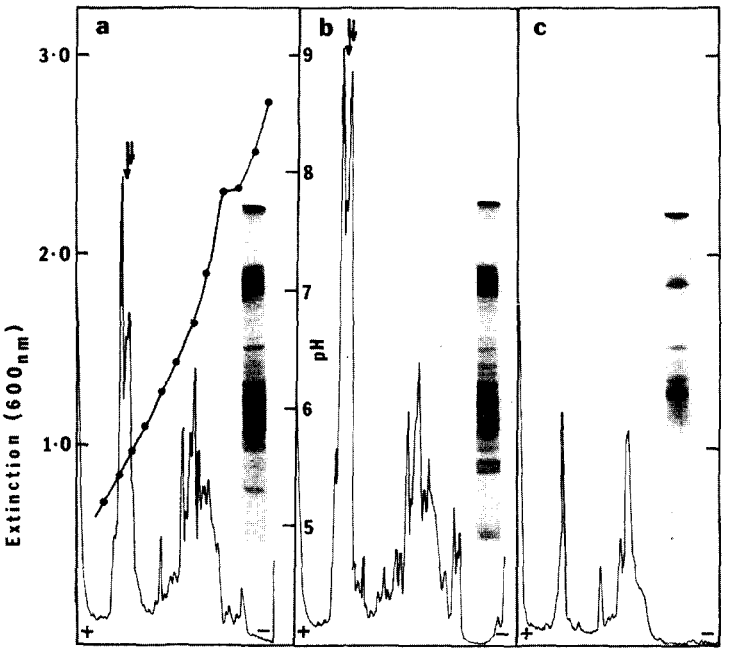

Fig. 1. Analytical electrofocusing of fat globule membrane proteins in polyacrylamide stained with Coomassie Blue. Ampholines (pH ranges 3—5, 5—7, and 3—10) were mixed in the ratio 2 : 2 : 1, (2%, w/v). (a), Fraction 1, 80 μg; (b), Fraction 2, 80μg; (c), Fraction 3, 40 μg; •———•, pH; ———, extinction 600 nm. Photographs of the gels are shown as insets with the anode at the top.

components obtained by one-dimensional SDS-electrophoresis. At least 12 components which are identified by letters in Fig. 3 were routinely detected in two dimensions, of which eight (identified by asterisks) also stained with the

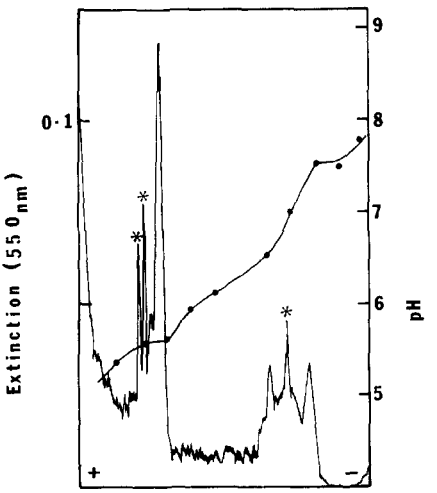

Fig. 2. Analytical electrofocusing of Fraction 2 proteins in polyacrylamide stained with the Schiff reagent. Ampholine concentration was as described in the legend to Fig. 1. 80μg protein was applied to the gel. •———•, pH; ———, extinction (550 nm). Components that were also present in Fraction 3 are identified by asterisks.

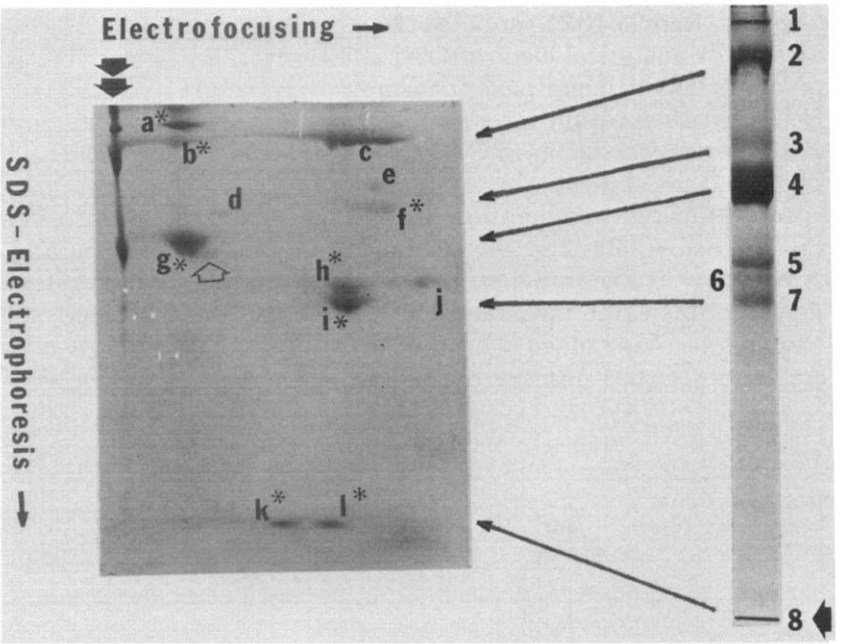

Fig. 3. Two-dimensional separation of Fraction 2 by electrofocusing and SDS electrophoresis. Electrofocusing in first dimension was as described in the legend to Fig. 1. 160 μg of protein was applied to the gel. Gel was stained with Coomassie Blue; Schiff-positive components are identified by asterisks. A sample of Fraction 2 separated by SDS electrophoresis is shown for comparison to the right of the two-dimensional separation. The bromophenol blue dye front is identified by a single black arrow.

Schiff reagent. Of the identified components, component j was present in the most variable quantity. Material that remained unseparated at the top of the first-dimension gel separated down the side of the second-dimension gel and is identified by a double arrow in Fig. 3. This material seems to contain most of the major proteins identified by SDS electrophoresis.

One-dimenisonal electrophoresis of fat globule membrane protein in SDS separates two major groups of polypeptides of molecular weight 155 000 and 67 000 (bands 3 and 12 of refs. 14, 19, bands 2 and 4 of Figs. 3 and 5 in this paper). The polypeptides of molecular weight 155 000 are resolved in two dimensions into at least two components (b and c of Fig. 3). Components b and c are probably composed of several proteins, however, since they consist of an almost continuous line within the pH range 5.0—8.0. The polypeptide of molecular weight 67 000 (component g), which is the major protein of the membrane, still appears as a single major component after two-dimensional separation, although there is evidence of a component of slightly lower molecular weight associated with this material (open arrow, Fig. 3).

Preparative electrofocusing of membrane proteins

The above preliminary experiments employing electrofocusing at the analytical level suggested the possibility of developing preparative procedures for the separation of individual membrane proteins. To test this possibility solubilized Fraction 2 proteins were separated by electrofocusing in beds of Sephadex

G-75 as described by Radola [32]. Initially attempts were made to duplicate the conditions used for analytical electrofocusing. However, the protein invariably focused as two peaks, a major peak focusing within the pH range 2.3—4.0 and a minor peak focusing within the range 4.0—5.0 (Fig. 4 (a)). Analysis of these fractions by electrophoresis in SDS revealed very little, if any, separation of the major proteins (not shown). Omission of both SDS and heat treatment during sample preparation led to separation of the membrane proteins into four major peaks (Fig. 4 (b)). Analysis of the fractionated Sephadex beds by SDS-electrophoresis is shown in Fig. 5. Separation of the major membrane proteins closely parallels that obtained on the analytical scale (Fig. 3) but with increased resolution. Components that stained with the Schiff reagent are identified by asterisks; several minor Schiff-positive bands of high molecular weight were

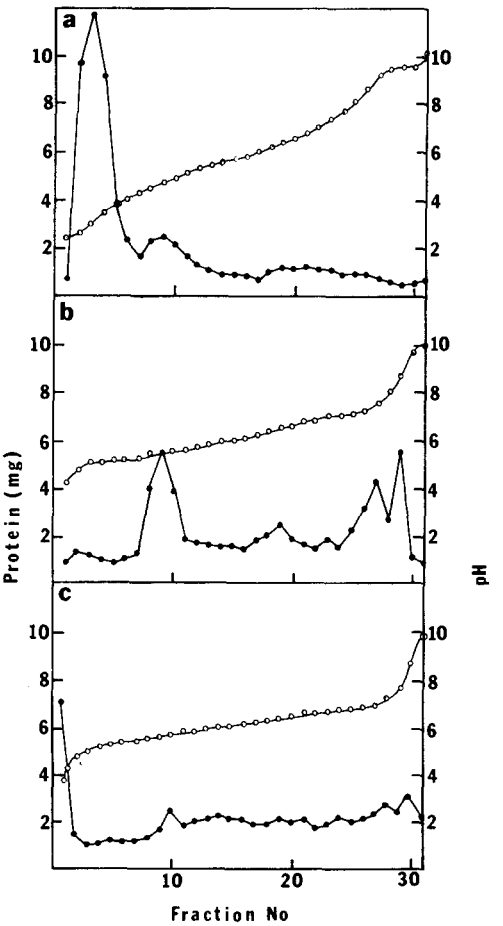

Fig. 4. Preparative electrofocusing of Fraction 2. (a), sample solubilized with SDS, (protein:SDS, 1 : 1.3, w/w) and mercaptoethanol (100 mM), heat treatment (2 min, 100°C) followed by urea (8 M) and Triton X-100 (2%, v/v), (b), sample solubilized as (a) except SDS and heat treament omitted. (c), sample solubilized in urea (8 M) and mercaptoethanol (100 mM). Fraction 2 (100 mg) was solubilized in each case. Ampholines (pH ranges 3—5, 5—7 and 3—10) were mixed in the ratio 1 : 2 : 1 (1%, w/v). ○———○, pH; ●———●, protein.

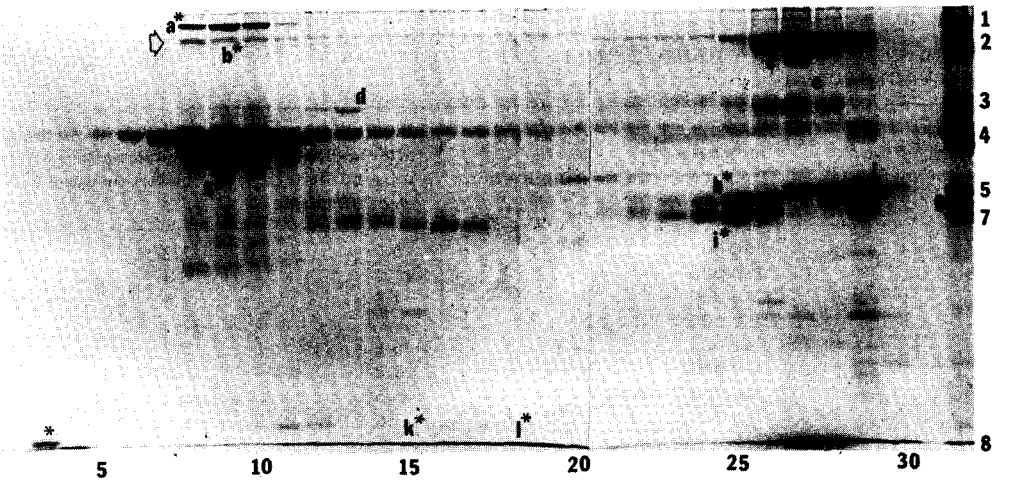

Fig. 5. SDS electrophoresis of sample of Fraction 2 separated by preparative electrofocusing. Analysis by SDS electrophoresis of samples obtained from the separation described in Fig. 4(b). Fraction numbers shown along the bottom of the photograph are directly comparable to those in Fig. 4(b). A sample of unseparated Fraction 2 is shown at the extreme right of the figure. Gels were stained with Coomassie Blue, components which were Schiff-positive are identified by asterisks.

present in fractions 8 and 9 (open arrow). All components are identified by the same letters as those in Fig. 3.

Recovery of total protein from the Sephadex beds after electrofocusing was about 70% of the amount applied. Some additional protein was recovered from the paper strips at the anode by electrodialysis. This material appeared to contain all the major membrane proteins and glycoproteins when analyzed by electrophoresis in SDS (not shown) and is probably composed of undissociated membrane or large micellar aggregates of membrane protein. A second possible reason for less than 100% recovery of protein from the Sephadex gel was the loss of protein by proteolysis during electrofocusing. This was investigated by combining equal aliquots of each fraction obtained after electrofocusing and using SDS electrophoresis to compare these combined fractions with the unfractionated membrane proteins. The amount of each protein present in the samples was estimated by measuring the areas of the peaks in densitometric scans of the stained gels after electrophoresis in SDS. Most noticeable was a relative decrease in the amount of material of molecular weights in excess of 150 000 that could be recovered after electrofocusing (Table II). It seems, therefore, that despite the addition of several protease inhibitors some protein is lost by proteolysis during electrofocusing.

The separation described in Figs. 4(b) and 5 was repeated several times employing different pH gradients to obtain optimal separation for each of the major membrane proteins. Apparent isoelectric points for the major proteins c and g (Figs. 3 and 5) were determined to be 7.6 and 5.55, respectively.

Omission of Triton X-100 from the samples led to inadequate separation (Fig. 4 (c)). The major polypeptide of molecular weight 67 000 (component g) was present in all the fractions from 8 to 31. Several of the other major components however, separated in a similar manner to that shown in Figs. 3 and 5.

TABLE II

ESTIMATION OF THE AMOUNT OF MEMBRANE PROTEIN IN SAMPLES BEFORE AND AFTER ELECTROFOCUSING

Protein (see Fig. 3 for numbering key)	Apparent molecular weight *	Fraction 2 before solubilization	Fraction 2 after addition of urea, but before electrofocusing	Combined samples after electrofocusing
1	>200 00	5.4 **	5.3	1.2
2	155 000	21.8	17.2	6.3
3	79 000	6.3	4.8	4.8
4	67 000	38.8	40.7	37.7
5	51 000	4.6	5.2	5.7
6	48 000	2.5	2.8	3.1
7	46 000	4.3	5.5	6.1
8	—	3.2	3.8	9.5

* Determined in 10% polyacrylamide gels.

** Figures are expressed as a % of the total amount of protein in the sample.

Discussion

Analytical or preparative electrofocusing of solubilized fat globule membrane (Figs. 1, 2 and 4) reveals a complex pattern of protein and glycoprotein bands that can be further resolved by SDS electrophoresis (Figs. 3 and 5). Several of the proteins originally identified as one component by SDS electrophoresis (e.g., band 3 of ref. 14; band 2 of Figs. 3 and 5 of this paper) can be resolved into several components by electrofocusing. Whether or not these separated components are different proteins or are variants of the same protein is currently under investigation. Some proteins, particularly components b and c of Figs. 3 and 5, appear as a continuous line after two-dimensional separation and may well be composed of a series of polymorphs differing, for instance, in lipid content or sialic acid in the case of the sialoglycoproteins. Fractions 8—10 and 26—29 of Fig. 5 were analyzed for phospholipid, cholesterol, and sphingolipids and appear on a qualitative basis to contain all the lipids of the unfractionated membrane (Vijay, I.K., unpublished observations). It is also possible that the heterogeneity observed has a genetic basis as is the case for the skim-milk proteins [33].

Several restrictions should be noted with the methods used in this present study. Firstly, complete solubilization of fat globule membrane proteins without the use of large excesses of SDS is difficult. Combinations of urea, mercaptoethanol and Triton X-100 appear to be effective in solubilizing the membrane proteins prior to electrofocusing (Table I). Some protein, however, remains in solution in aggregated form as evidenced by the retention of protein at the top of the acrylamide gels during analytical separations even if SDS is added (Figs. 1 and 3). Also, unseparated protein can be recovered from the electrode strips at the anode after preparative separations. The amount of completely solubilized protein analyzed in this present study is probably over 70% of the total membrane protein, but is certainly less than 100%. Further solubilization may, therefore, lead to additional protein bands after two-dimensional separation.

A second reservation with the separations achieved concerns the stimulation of proteolysis during electrofocusing. On the analytical scale proteolysis is probably minimal since the samples were pretreated by heating to 100°C in the presence of SDS and mercaptoethanol. This is generally assumed to minimize artifacts due to proteolytic breakdown. On the preparative scale however, some proteolytic breakdown was observed (Table II) despite the addition of the protease inhibitors phenylmethyl sulphonyl fluoride [34], ϵ-amino-*n*-caproic acid [35] and aprotinin [20]. Analytical and preparative separations, however, gave similar two-dimensional patterns (Figs. 3 and 5) and since proteolysis is minimized at the analytical level, this is also probably the case with preparative separations. Also components g, f, h, i, k and l (Fig. 3 and 5) are Schiff-positive, whereas component c, which is the major protein lost by proteolysis, does not stain with the Schiff reagent.

A third reservation with the techniques described concerns the use of urea for electrofocusing. Isocyanate, which accumulates in urea solutions, is known to alter the native charge on proteins by carbamylation of the free amino groups and can, therefore, lead to the formation of artifactual bands after electrofocusing [22]. The chance of this occurring was reduced as much as possible by using freshly recrystallized urea, and adding lysine to all samples separated at the preparative level [22].

Despite these above restrictions the methods described in this paper are a useful additional approach to the analysis and separation of fat globule membrane proteins. Further papers will be concerned with a more detailed analysis of the fractions obtained during this study.

Acknowledgements

The author wishes to thank Dr. Wayne J. Kuenzel for assistance with the photography. Dr. Inder K, Vijay for analyzing samples for lipid content and C.B. Tamplin for technical assistance. This work was funded in part by a grant from the National Science Foundation (Grant No. PCM 77-20167) and is scientific Article No. A-2434, Contribution No. 5461 of the Maryland Agricultural Experiment Station, Department of Dairy Science.

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