Biochimica et Biophysica Acta, 307 (1973) 133–140

BBA 76291

PROTEOLYTIC DIGESTION OF PROTEINS OF THE MILK FAT GLOBULE MEMBRANE*

DAVID KOBYLKA** and KERMIT L. CARRAWAY

Department of Biochemistry, Oklahoma State University, Stillwater, Okla. 74074 (U.S.A.)

(Received September 5th, 1972)

(Revised manuscript received January 15th, 1973)

SUMMARY

Proteolytic digestion of milk fat globules with trypsin or Pronase results in cleavage of all of the major associated membrane proteins, as determined by acrylamide gel electrophoresis in sodium dodecyl sulfate. The concentration dependence of the digestion of the membrane proteins was essentially the same for the intact fat globules and their isolated membranes. These results indicate that the membrane surrounding the fat globule does not represent a significant permeability barrier to proteolytic enzymes and suggest that the membrane does not exist in an intact form on the globule.

INTRODUCTION

Milk fat secretion has been presumed to occur by a process which involves pinching off the plasma membrane of the mammary secretory cell to yield a fat globule which is surrounded by plasma membrane as it leaves the cell[1]. In support of this hypothesis Keenan *et al.*[2] have shown similarities between the milk fat globule membrane and plasma membranes from mammary tissue. Further support comes from the demonstration of considerable quantities of plasma membrane marker enzymes in the milk fat globule membrane[3,4], although the presence of enzymes from other cellular membranes has been indicated[5]. Patton and Trams[3] have suggested that milk fat globules can be used for studying the "sidedness" of the plasma membrane of the mammary cell. This would require that the fat globule be surrounded by an intact membrane completely enveloping the globule. Recent studies by Wooding[6] have questioned the intactness of the milk fat globule membrane. Evidence from electron microscopy during the process of secretion suggests that a dual membrane is present at the time of secretion. The outer membrane, which is apparently identical to the secretory cell plasma membrane, fragments

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*, *N'*-tetraacetic acid.

* Publication No. 2559 of the Oklahoma Agricultural Experiment Station.

** Present Address: Department of Biochemistry, St. Louis University, School of Medicine, St. Louis, Mo. (U.S.A.).

and sloughs off of the fat globule after secretion occurs. This leaves only "blebs" of the cell plasma membrane still associated with an apparently structureless "secondary membrane".

In order to examine the nature of the membrane of the fat globule we have used proteolytic digestion as a tool to monitor the accessibility of the membrane proteins. The proteins have been fractionated by polyacrylamide electrophoresis in sodium dodecyl sulfate[7,8]. If there is a structured membrane surrounding the milk fat globule which represents a true permeability barrier, one would expect that some of the proteins of this membrane would be inaccessible to protease because of the asymmetric distribution of protein components relative to the permeability barrier[9]. This was not observed. The effects of protease treatment on milk fat globule membrane ATPase and 5'-nucleotidase were also examined because of the suggestion by Patton and Trams[3] that these enzymes are asymmetrically oriented in the membrane of the fat globule.

MATERIALS AND METHODS

All enzymes and substrates for enzyme assays were obtained from Sigma. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed as previously described[7,8]. Protein was determined by the method of Lowry *et al.*[10].

Preparation of cream and milk fat globule membranes from chilled and unchilled samples

Fresh raw milk as a composite sample was obtained from three Holstein cows. The sample was divided in half, one portion being chilled immediately to 4 °C (chilled sample) and the other being maintained at 25 °C (unchilled sample). The cream from the chilled sample was prepared by centrifuging the chilled raw milk at $5000 \times g$ for 15 min at 4 °C, removing the solid floating cream and suspending it in buffered sucrose at 25 °C. The centrifugation and washing procedure was repeated two additional times. After the initial centrifugation and each washing step, an aliquot of cream was immediately solubilized by making the mixture 2% in sodium dodecyl sulfate and 0.2% in mercaptoethanol and heating at 100 °C for 5 min. The sample was further incubated overnight at room temperature under nitrogen. The extraction mixture was centrifuged at $35000 \times g$ for 30 min at 25 °C. Aliquots of the supernatant were taken from beneath the buttercream layer and dialyzed against 40% methanol. The dialysates were then prepared for electrophoresis as usual. The unchilled sample was treated similarly except that the cream from it was washed with buffer at 37 °C and centrifuged at 25 °C.

Milk fat globule membrane samples were prepared as previously described[7].

Enzyme assays of washed cream and isolated milk fat globule membranes

The assay of 5'-nucleotidase was performed by a modification of the method of Widnell and Unkeless[11] in a volume of 0.6 ml containing 100 mM Tris–HCl (pH 8.5), 10 mM 5'-AMP, and 10 mM $MgCl_2$ *plus* 0.050 ml of 33% cream (2–3 mg/ml protein) or 0.005 ml of milk fat globule membrane preparation (20–30 mg/ml protein). After incubation at 37 °C for 20 min, the reaction was stopped by the addition of 1.4 ml of a solution containing 1 part 10% ascorbic acid and 6 parts

0.42% ammonium molybdate in 0.5 M H_2SO_4 (mixed fresh daily). After incubation at 45 °C for 20 min and extraction into 2 ml of reagent grade isoamyl alcohol the absorbance was determined at 795 nm.

The assays of Mg^{2+}- and Ca^{2+}-ATPases were performed by the method of Cha *et al.*[12]. Again the reaction volume was 0.6 ml and contained 0.05 ml of 33% cream or 0.005 ml of milk fat globule membrane preparation. The Mg^{2+}-ATPase mixture also contained 120 mM KCl, 0.5 mM EGTA (ethyleneglycol-bis-(β-aminoethyl ether)-*N*, *N'*-tetraacetic acid), 30 mM histidine–imidazole buffer (pH 7.0), 5 mM $MgCl_2$ and 2 mM ATP. The Ca^{2+}-ATPase assay mixture contained 120 mM KCl, 0.5 mM $CaCl_2$, 30 mM histidine–imidazole buffer (pH 7.0), 5 mM $MgCl_2$ and 2 mM ATP. The samples were incubated, quenched, extracted and read at 795 nm exactly as in the assay for 5'-nucleotidase. It was necessary to have a non-enzyme blank for each time to be assayed in the case of the ATPases, since non-enzymatic hydrolysis of ATP is quite significant.

The assay of xanthine oxidase was performed according to the procedure of Zittle *et al.*[13]. To a tube was added 1 ml of 0.5 M phosphate buffer (pH 7.5), 0.2 ml of 0.05 M xanthine and enough distilled water to make the total volume 3.2 ml including the 0.05 ml of 33% cream or 0.005 ml of milk fat globule membrane preparation. The solution was purged of oxygen by bubbling nitrogen through it for 5 min. At this time the aliquot of washed cream or milk fat globule membrane was added and the bubbling of nitrogen was continued for an additional 5 min. Finally, 0.2 ml of 0.05 M triphenyltetrazolium chloride in 0.01 M phosphate buffer (pH 7.5) was added and the reaction continued for 20 min. The reaction was stopped by the addition of 5.0 ml of glacial acetic acid to each tube, and bubbling of the nitrogen was stopped. To each tube was added 4.0 ml of toluene. The tubes were stoppered and shaken vigorously. The red color extracted into the toluene was diluted, when necessary, with more toluene and the absorbance read at 485 nm.

Digestion of washed cream and isolated milk fat globule membrane with trypsin and Pronase

To 10 ml of washed cream or milk fat globule membrane (1 mg/ml protein) in 0.25 M sucrose– 0.1M imidazole–0.002 M $MgCl_2$ (pH 7.0) was added trypsin in 0.155 M NaCl adjusted to pH 3.0 to a final concentration of 1–100 μg/ml. Digestion was allowed to proceed for 1 h at room temperature. To the mixture was added 2.5 ml of 10% dodecyl sulfate and 0.025 ml mercaptoethanol. The suspension was then heated to 100 °C for 5 min and incubated overnight at room temperature under nitrogen[14]. The cream samples were centrifuged for 30 min at $35000 \times g$ and 25 °C. Supernatants were removed, dialyzed overnight against 40% methanol and lyophilized before electrophoresis. Dodecyl sulfate-solubilized milk fat globule membrane samples were treated in the same manner as the supernatants from the cream samples.

Pronase digestion[14] was performed at 37 °C in an identical manner except that both the incubation buffer and the buffer used to dissolve the pronase were 17.3 mM Tris–130 mM NaCl–3.6 mM KCl–1.6 mM $CaCl_2$–1.2 mM $MgSO_4$ (pH 7.6).

Samples to be assayed for enzyme activity after trypsin digestion were treated

with a 3-fold excess of trypsin inhibitor (ovomucoid) before assaying for the appropriate enzyme.

Phospholipase A assays of the pronase preparations were performed as described by Scherphof *et al.*[15], except that lecithin was used as a substrate instead of egg yolk.

RESULTS

Preparation of cream and milk fat globule membrane

In order to examine the integrity of the membrane of the milk fat globule, it is desirable to use milk samples which are as fresh as possible. In our previous studies cream from chilled milk was used for electrophoretic analyses[7]. However, Brunner[16] has indicated that there are differences in the yields of milk fat globule membrane protein from chilled and unchilled samples of milk. Therefore we examined the proteins of cream which had been separated and washed at two different temperatures. The results are shown in Fig. 1 as protein patterns of the cream immediately after separation from the milk and after 1, 2 or 3 washes. Only minor variations are noted as a result of the temperature difference. Components III and IV appear to be relatively less prominent in cream washed at the higher temperatures, suggesting that they may be subject to removal from the milk fat globule membrane. The present results are essentially in agreement with the recent studies of Anderson *et al.*[17], who found only minor changes in protein components of fat globule membrane fractions of aged, chilled milk by detergent gel electrophoresis. In the current work proteolytic digestion studies were performed on cream separated and washed at both temperatures in order to reduce the chance of artifacts.

Proteolysis of milk fat globules and their membranes

To investigate the organization of the proteins of the milk fat globule membranes, washed cream and milk fat globule membrane samples were subjected to

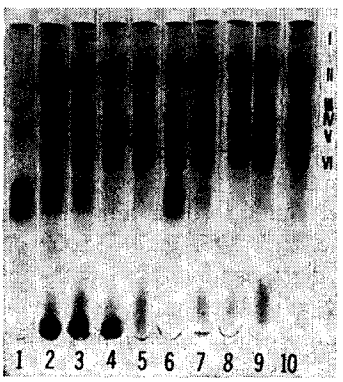

Fig. 1. Acrylamide gel electrophoresis patterns of dodecyl sulfate-extracted chilled and unchilled cream from fresh raw milk. Gel 1 is extract of whole raw milk. Gels 2, 3, 4, and 5 represent patterns obtained from dodecyl sulfate extraction of chilled cream after 0, 1, 2, and 3 washes, respectively. Gels 6, 7, 8 and 9 are the patterns obtained from the corresponding samples of unchilled cream. 10, milk fat globule membrane control. Proteins are numbered as previously described[7].

digestion with trypsin. Figs 2 and 3 show the effects of this treatment on the proteins and glycoproteins of the membrane. There are essentially no differences between the effects on cream and those on the isolated milk fat globule membranes. Components I and V are cleaved and lost from the patterns at the lowest trypsin concentrations. Components IV and VI are slightly more resistant, but are almost completely lost at trypsin concentrations of 25 μg/ml. The major glycoproteins and component III all show a marked resistance to cleavage even at the highest enzyme concentrations. Component II shows quite interesting behavior. It undergoes a concentration-dependent cleavage to give a polypeptide of slightly lower molecular weight. This latter polypeptide is also degraded at higher trypsin concentrations. It is interesting to note that a faint band appears in many milk fat globule membrane and cream preparations corresponding to the component II degradation product. This would suggest a slight action of proteolytic enzymes in the milk upon the cream components.

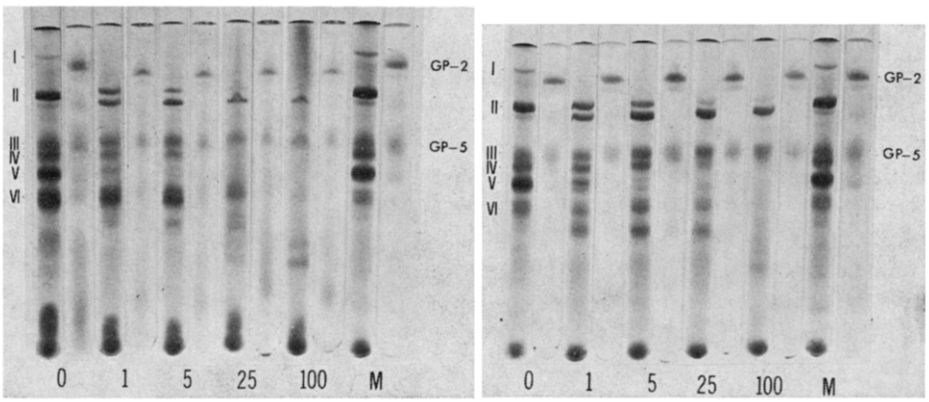

Fig. 2. Concentration dependence of trypsin digestion of cream. Samples were digested 1 h with appropriate trypsin concentrations and solubilized in dodecyl sulfate. Acrylamide gels are arranged in order of increasing trypsin concentrations (left to right) with alternating gels stained for protein (coomassie blue) and carbohydrate (periodate–Schiff). Numbers below gel pairs are trypsin concentrations (μg/ml). M, milk-fat globule membrane control; GP, glycoprotein.

Fig. 3. Concentration dependence of trypsin digestion of milk fat globule membrane. Details are given in Fig. 2.

The resistance of some of the membrane polypeptides, particularly glycoproteins, suggests that they may be inherently resistant to proteolysis. That this is not true is shown by digestion with Pronase. With either cream or milk fat globule membrane pronase at 5 μg/ml cleaves all of the polypeptides, including the glycoproteins, to such an extent that they are no longer detectable by electrophoresis. Therefore it appears that there are no polypeptides which are resistant to digestion. The greater degree of cleavage by Pronase can be explained by its lack of specificity which allows it to attack at more sites along the polypeptide chain and by the presence of phospholipase A activity in the Pronase, which could disrupt the membrane structure by cleavage of phospholipids.

Although all of the trypsin and Pronase experiments described above were

performed on samples from chilled cream, identical experiments have been performed with unchilled cream samples with essentially no differences in the results.

Milk fat globule membrane enzymes

Patton and Trams[3] have suggested that the ATPase and 5′-nucleotidase are localized at the inner and outer surfaces of the milk fat globule membranes, respectively, based on changes in activities observed as a result of disruption of

TABLE I

ENZYME ACTIVITIES OF CREAM AND MILK FAT GLOBULE MEMBRANE

The enzyme activity assays are given in Materials and Methods. Values are averages for three individual preparations for nucleotidase and ATPases and for two preparations for xanthine oxidase. Values are expressed as nmoles/min per mg protein of phosphate released or μmoles/min per mg protein of triphenyltetrazolium chloride reduced.

Enzyme	*Source*	*Activity*
5′-Nucleotidase	Cream	340
	Milk fat globule membrane	290
Mg^{2+}-ATPase	Cream	13
	Milk fat globule membrane	15
Ca^{2+}-ATPase	Cream	7
	Milk fat globule membrane	12
Xanthine oxidase	Cream	7
	Milk fat globule membrane	10

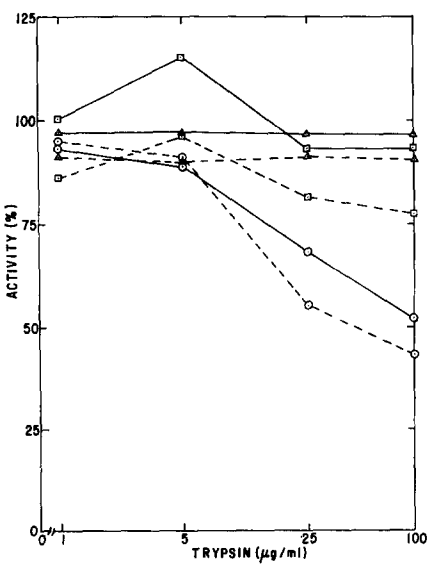

Fig. 4. Effect of trypsin digestion on membrane-associated enzymes of cream and milk fat globule membrane. Experimental details are given in Materials and Methods. ⊙, ATPase; ⊡ 5′-nucleotidase; △, xanthine oxidase. ——, cream; – – –, milk fat globule membrane.

the fat globule to remove the membranes. Therefore the effects of proteolysis on these enzymes have been investigated. Table I shows the activities of 5′-nucleotidase, ATPase and xanthine oxidase for washed cream and milk fat globule membrane samples. The ATPase activities were measured in the presence of Mg^{2+} and K^{+}, but not Na^{+}. In addition measurements were performed in the presence of Ca^{2+} to determine if Ca^{2+}-ATPase were present. The Ca^{2+} actually caused an inhibition of the Mg^{2+}-ATPase activity. Slight activations of the ATPase and xanthine oxidase were noted in comparing milk fat globule membrane with cream samples. These effects are similar to those noted by Patton and Trams[3] for Mg^{2+}-ATPase. The 5′-nucleotidase showed a decreased specific activity in the milk fat globule membrane compared to the cream. This was most likely due to an inactivating effect of the freezing and thawing procedure used to prepare the membranes.

Fig. 4 shows the effects of trypsinization on the milk fat globule membrane enzymes. Cream and milk fat globule membrane samples show qualitatively similar behavior, although there are some quantitative differences between the two types of samples. Only the ATPase is strongly inactivated over the protease concentration range used. Nucleotidase is slightly activated in cream samples at low trypsin concentrations.

DISCUSSION

The hypothesis that the milk fat globule membrane is derived primarily from the plasma membrane is supported by evidence from a number of studies of milk fat globule membrane composition, including its enzymes[3,4], lipids[2], proteins[2], and glycoproteins[7]. Patton and Trams[3] have suggested that the milk fat globule membrane can be used as a tool for studies of the organization of the components of the plasma membrane of mammary cells. In practice such studies require that the membrane surrounding the fat globule be intact so that the inner and outer surface of the membrane can be differentiated. The current proteolytic digestion studies do not support the premise that this membrane is intact. None of the proteins of the membrane appear to be inaccessible to proteolysis, and the digestion of cream yields essentially the same result as the digestion of milk fat globule membrane, which is fragmented by the isolation procedure. These observations are contrary to the findings with erythrocytes, in which most of the proteins of the intact cell membrane are resistant to proteolysis, while those of the isolated membrane are quite susceptible[14,18]. These results suggest that all of the polypeptides are accessible to the trypsin in the intact cream globule, unless, of course, the membrane is being degraded during the course of digestion such that additional protein components become accessible during digestion. This latter possibility is difficult to rule out, but experiments with red blood cells suggest that the membrane permeability barrier is stable during proteolysis under the conditions used. This supposition appears quite reasonable for any membrane consisting of a lipid bilayer with limited amounts of protein within the bilayer and at its exterior surface.

The proteolysis results with cream samples would therefore suggest one of three possible structures for the milk fat globule membrane; (1) the membrane is unstructured, *i.e.* shows no "sidedness", (2) the membrane structure has all protein components outside the permeability barrier and (3) the membrane is broken

rather than continuous and is freely permeable to protease. The last is more consistent with the observations of Wooding[6] using electron microscopy. The failure of the membrane to act as a permeability barrier to K^+ (ref. 4) also suggests a broken membrane, as does the ability of the ATP to penetrate the membrane. The ATPase activities of the cream samples do not indicate a strong barrier to ATP. By comparison the erythrocyte or resealed ghost, which excludes ATP, shows quite low ATPase activity when assayed with external substrate[19]. The increased ATPase activities observed by Patton and Trams[3] may well reflect an activation of the enzyme resulting from altered interactions within the membrane rather than an exposure of the inner membrane surface to the external medium. Although their postulated arrangement of the enzymes relative to the membrane surfaces may be correct, the rationale behind these assignments is less easily justified. It would appear that the use of the membrane of the fat globule as a model for an intact plasma membrane must be exercised with great caution. Changes in activities of membrane-bound enzymes may reflect many factors other than accessibility of the enzyme to the incubation medium.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the National Institutes of Health (GM 16,870), American Cancer Society (P-563, BC 77A and IN-99B) and by the Oklahoma Agricultural Experiment Station. We wish to thank Mr John Huggins for assistance with the phospholipase assays.

REFERENCES

1 Patton, S. and Fowkes, F. M. (1967) *J. Theor. Biol.* 15, 274–281
2 Keenan, T. W., Morre, D. J., Olson, D. E., Yunghans, W. N. and Patton, S., (1970) *J. Cell. Biol.* 44, 80–93
3 Patton, S. and Trams, E. G. (1971) *FEBS Lett.* 14, 230–232
4 Dowben, R. M., Brunner, J. R. and Philipott, D. E. (1967) *Biochim. Biophys. Acta* 135, 1–10
5 Martel-Pradal, M. B. and Got, R. (1972) *FEBS Lett.* 21, 220–222
6 Wooding, F. B. P. (1971) *J. Ultrastruct. Res.* 37, 388–400
7 Kobylka, D. and Carraway, K. L. (1972) *Biochim. Biophys. Acta* 288, 282–295
8 Kobylka, D., Khettry, A., Shin, B. C. and Carraway, K. L. (1972) *Arch. Biochem. Biophys.* 148, 475–487
9 Hendler, R. W. (1971) *Physiol. Rev.* 51, 66–97
10 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
11 Widnell, C. C. and Unkeless, J. C. (1968) *Proc. Natl. Acad. Sci. U.S.* 61, 1050–1057
12 Cha, Y. N., Shin, B. C. and Lee, K. S. (1971) *J. Gen. Physiol.* 57, 202–215
13 Zittle, C. A., Dellamonica, E. S., Custer, J. H. and Rudd, R. K. (1956) *J. Dairy Sci.* 39, 522–527
14 Triplett, R. B. and Carraway, K. L. (1972) *Biochemistry* 11, 2897–2903
15 Scherphof, G. L., Scarpa, A. and Van Toorenenbergen, A. (1972), *Biochim. Biophys. Acta* 270, 226–240
16 Brunner, J. R. (1969) in *Structural and Functional Aspects of Lipoproteins in Living Systems* (Tria, E. and Scanu, A. M., eds), pp. 545–578, Academic Press, New York
17 Anderson, M., Cheeseman, G. C., Knight, D. J. and Shipe, W. F. (1972) *J. Dairy Res.* 39, 95–105
18 Bender, W. W., Garan, H. and Berg, H. C. (1971) *J. Mol. Biol.* 58, 783–797
19 Hanahan, D. J. and Ekholm, J. (1972) *Biochim. Biophys. Acta* 255, 413–419