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Purification of Plasma Membranes From Rat Mammary Gland by a Density Perturbation Procedure

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A highly purified plasma membrane fraction was obtained from a microsomal subfraction of rat mammary gland after treatment with digitonin to increase its density. The purified membranes were enriched 70-fold overall in 5′-nucleotidase with essentially no contamination from glactosyltransferase, succinate-INT reductase, or NADPH-cytochrome c reductase. The membranes were also highly enriched in sialoglycoproteins.

Key words: rat mammary gland; plasma membranes, purification of mammary glands: Golgi vesicles; subfraction of microsomal vesicles, density perturbation

INTRODUCTION

Isolation of plasma membranes from mammalian tissues is a difficult and laborious task (1). The greatest amount of work in this area has been performed on rat liver, from which membrane sheets can be isolated after homogenization (2). Varying degrees of membrane purification have been claimed for these preparations, based on examination of marker enzymes and morphological criteria (3). The quality of preparation appears to be influenced by a number of factors inherent in the preparation techniques. Plasma membrane isolation from mammary tissue is further complicated by the distensibility of the tissue arising from the large quantities of connective tissue present (4). This factor makes disruption of the cells virtually impossible by the conventional techniques used with liver. Therefore most efforts to prepare mammary plasma membranes have met with limited success, although Keenan et al. (5) have reported isolation of low yields of plasma membranes from bovine mammary gland. We report here a procedure which yields highly purified plasma membranes from lactating mammary gland involving density perturbation and subfractionation of microsomal vesicles.

METHODS

Isolation of Plasma Membrane Fraction

The normal lactating mammary gland was excised from female Fisher 344 rats (14 days postpartum) and placed in ice cold buffered sucrose (0.25 M sucrose-20 mM Tris, pH 7.4). All subsequent steps were performed at 4°C unless otherwise indicated. The gland was cut into small pieces with scissors, washed, minced with scissors, diluted to 50–70 ml/10 gm tissue with buffered sucrose and homogenized with a Sorvall Omni-mixer in a

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50 ml bucket (2 bursts of 30 sec each, power setting 5). The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 2,500 rpm (755 g) for 10 min in a Sorvall SS-34 head. The supernatant was centrifuged at 10,000 rpm (17,300 g) for 10 min. The second supernatant was centrifuged at 27,000 rpm (96,000 g) for 75 min in a Beckman SW 27 rotor to yield a pellet designed P-3, which was adjusted to 40% (w/w) buffered sucrose and a final volume of 8.0 ml. Ten ml of 36%, 32%, and 0.9% sucrose (20 mM in Tris, pH 7.4) were layered onto the sample, and it was centrifuged at 27,000 rpm (96,000 g) for 4 hr to equilibrate the microsomal vesicles by flotation to their buoyant density. The band at the 0.9–32% interface (designated F_1) was removed with a bent needle, resuspended in 38 ml of 10 mM Tris pH 7.4 and centrifuged 27,000 rpm for 60 min in a Beckman SW 27 rotor to remove sucrose.

Digitonin Shift Purification

The F_1 fraction was suspended in buffered sucrose (pH 7.4) and divided into 2 aliquots. The control fraction was incubated at 23°C for 15 min, while the other fraction made to 0.03% in digitonin and incubated at 23°C for 15 min. Both fractions were then made to 40% sucrose, and 36, 32, and 0.9% sucrose solutions layered over them. The digitonin fraction had 0.03% digitonin incorporated into all sucrose solutions in the gradient. The 2 gradients were centrifuged 27,000 rpm for 4 hr. The untreated sample had 1 band at the 0.9–32% sucrose interface, which was designated F_1F_1. The digitonin gradient had bands at the 0.9–32% interface (designated F_1DF_1) and the 36–40% interface (designated F_1DF_3). All bands were removed, diluted with 10 mM Tris pH 7.4 and centrifuged at 27,000 rpm × 60 min.

Analytical Procedures

5′-Nucleotidase was assayed by a modification of the method of Ipata (6, 7). The sample was incubated for 15 min at 37°C in 2.85 ml of 0.10% sodium deoxycholate in 50 mM Tris pH 8.0 and assayed by the addition of reagents to give 4.5 units of adenosine deaminase and 0.1 mM AMP in a final volume of 3.0 ml. Galactosyl transferase was assayed by a modification of the incorporation method of Fitzgerald et al. (8) using N-acetyl glucosamine as galactosyl acceptor and 1 mM AMP to inhibit nonspecific hydrolases. Succinate dehydrogenase (succinate 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium reductase) was assayed as described by Morré (9). Protein was determined by the method of Lowry et al. (10) and NADPH cytochrome C reductase by the method of Ragnotti et al. (11). Dodecyl sulfate acrylamide gel electrophoresis was performed on 7.0% gels at 6 mA/gel for 10 hr. Gels were stained for protein and carbohydrate by the method of Fairbanks et al. (12).

RESULTS

Figure 1 shows a flow sheet for the purification of plasma membranes from normal lactating rat mammary gland. The procedure using a Sorvall Omni-Mixer for the disruption of mammary tissue, followed by differential centrifugation and flotation of the microsomes on a sucrose gradient permits isolation of a partially purified plasma membrane fraction (F_1) containing the plasma membrane markers 5′-nucleotidase and Na^+, K^+-ATPase, which copurify during the procedure (13). These enzymes have been previously shown to occur predominatly in the plasmalemma of mammary cells (4), as they do in liver (3). Contamination of these membranes was suggested by the extensive

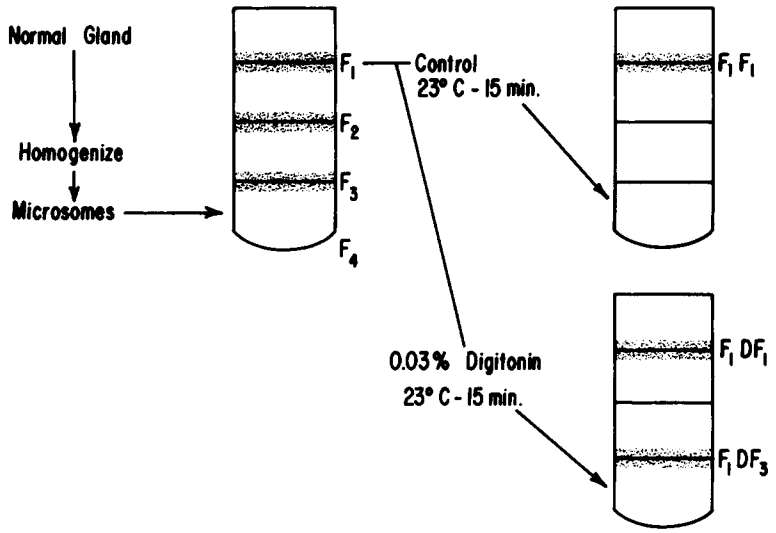

Fig. 1. Flow diagram for plasma membrane purification.

amounts of galactosyltransferase activity present in the membrane fraction (13). This enzyme appears to be concentrated primarily in the Golgi apparatus of mammary gland (4). Since the homogenization procedure causes extensive disruption of the Golgi, it was presumed that Golgi vesicles were segregating with plasma membrane vesicles during centrifugation. Therefore attempts have been made to separate the plasma membrane and Golgi markers in order to achieve further purification of the plasma membranes. To separate these activities, a density perturbation procedure was developed in which digitonin was used to alter the buoyant densities of the membrane vesicles. Digitonin complexes with cholesterol, a major constituent of plasma membranes, and has been shown previously to alter the density distributions of membrane enzymes observed by analytical gradient centrifugation of treated rat liver membranes (14). Untreated F_1 fractions subjected to a repeat flotation procedure recentrifuge to give a single band at its original position ($F_1 F_1$). The digitonin-treated sample showed 2 bands after flotation, one of which ($F_1 DF_1$) occurred at the same position as on the original gradient. The second band was shifted to a higher density and occurred at the 36–40% sucrose interface ($F_1 DF_3$). When these 3 fractions from a typical experiment were assayed for the enzyme activities (Table I), it was found that $F_1 DF_3$ showed a marked enhancement of 5′-nucleotidase activity (70-fold overall). This fraction showed no detectable galactosyltransferase activity, although there is high uncertainty in the values of this fraction due to the presence of high nucleotide pyrophosphatase activity, an enzyme which is known to occur in plasma membranes (15). By contrast the $F_1 DF_1$ fraction showed a marked decrease of 5′-nucleotidase and a substantial increase in galactosyltransferase. Since digitonin treatment alone (DF_1) gives relatively minor enhancement of the enzyme activities and since re-centrifugation without digitonin does not cause any significant changes, it can be assumed that the enzyme markers are segregating after digitonin treatment because they are present on different vesicle types. No significant amounts of succinate-INT reductase or NADPH cytochrome c reductase were found in the purified plasma membranes.

TABLE I. Enzyme-Specific Activities for Fractions Obtained From a Typical Experiment During Plasma Membrane Purification From Normal Lactating Rat Mammary Gland Tissue

	5′-Nucleotidase		Galactosyl transferase	
	Specific activity	Relative specific activity	Specific activity	Relative specific activity
	(μmoles/hr/mg protein)		(μmoles/hr/mg protein)	
Homogenate	5.0	1.0	0.1	1.0
P-3	15.8	3.2		
F_1	131.0	26.2	2.4	24
DF_1	146.0	29.2	3.6	36
F_1F_1	133	26.6	3.4	34
D_1DF_1	54.8	11.0	4.9	49
F_1DF_3	352	70.4	< .01	0

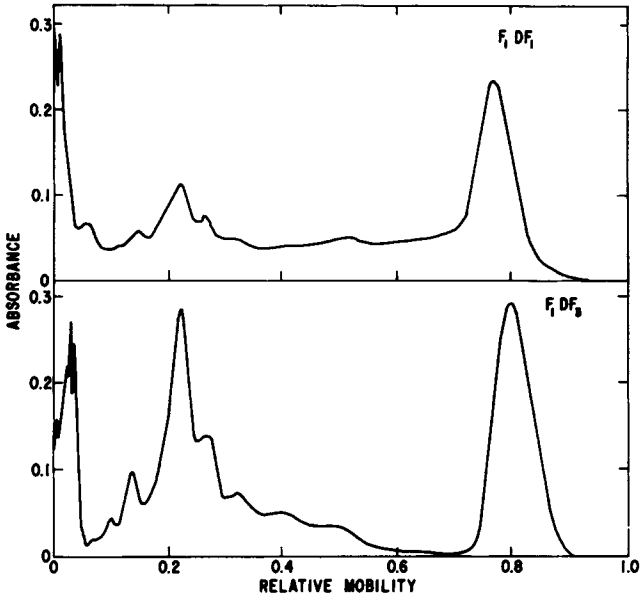

Fig. 2. Glycoprotein patterns for purified plasma membrane (F_1DF_3) and Golgi-enriched fractions (F_1DF_1) obtained from digitonin-treated microsomal subfraction.

The glycoproteins of the purified fractions were examined by dodecyl sulfate acrylamide gel electrophoresis. The patterns for F_1, F_1F_1, F_1DF_1, and F_1DF_3 are qualitatively the same, but the greatest concentration of glycoprotein is obtained in the most purified plasma membranes, and the lowest concentration is in the Golgi fraction (Fig. 2), which still appears to contain some plasma membrane based on its 5′-nucleotidase activity.

DISCUSSION

These results clearly indicate that the digitonin shift method can be used for purification of plasma membranes of mammary gland. The preparative value of the technique is suggested by the fact that > 90% and > 70% of the 5′-nucleotidase and galactosyltransferase activities, respectively, are recovered from the gradients after digitonin treatment.

Recoveries in the earlier stages of the preparative procedure are less impressive. About 65% of the 5′-nucleotidase is recovered in the microsomal fraction and is distributed predominantly in F_1 and F_2 fractions on flotation in a discontinuous sucrose gradient (13). However, rehomogenization of the nuclear and mitochondrial pellets or additional centrifugation of the microsomal supernatant yields additional microsomal material showing the same behavior as that isolated with the F_1 and F_2 fractions. These results indicate that the membrane fractions described here are representative of the population of secretory epithelial cells which predominate in this tissue rather than being derived from a selected minor population of cells.

The distribution of sialoglycoproteins, as localized by periodate-Schiff strain, agrees with our earlier findings that purified Golgi contain little sialoglycoprotein (13) and indicate that sialoglycoprotein is a useful marker for the plasma membrane in mammary gland.

The question of whether galactosyl transferase occurs as a surface membrane enzyme in mammary gland (16, 17) cannot be answered conclusively from these experiments, although they clearly show that part of the mammary cell plasma membrane does not contain galactosyltransferase as it is isolated. Differences observed between F_1 and F_2 fractions suggest a heterogeneous population of plasma membrane vesicles. This is not unexpected from a tissue sample in which the cell membrane domains must be differentiated according to localization (apical, lateral, basal) and function. The exact localization of the purified membranes described here is unknown, but immunological localization methods should aid in establishing their identity.

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