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PURIFICATION OF MICROVILLUS MEMBRANE VESICLES FROM PIG SMALL INTESTINE BY ADSORPTION CHROMATOGRAPHY ON SEPHAROSE

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A simple, rapid method for the preparation of pure microvillus membrane vesicles from pig small intestine is described. The method is based on the ability of agarose beads to adsorb selectively the impurities, mainly basolateral membrane fragments, from a microvillus vesicle preparation isolated by hypotonic lysis, Mg^{2+} aggregation of contaminants and differential centrifugation.

The plasma membrane of the epithelial cells in the mammalian small intestine is composed of two morphologically quite different regions, the brush border and the basolateral region. The method of preparing microvillus membrane vesicles by precipitation of contaminants by divalent cations (Ca^{2+} or Mg^{2+}), originally described by Schmitz et al. [1] and modified by Kessler et al. [2] and by ourselves [3] allows quick production of large amounts of vesicles, but the preparation is to some extent contaminated, mostly with basolateral membrane fragments [3]. We have recently described a method to purify the preparation, based on the ability of the microvillus vesicles to adhere to an immunoadsorbent which has been prepared by the coupling of antibodies directed against brush border proteins to an insoluble matrix [4]. In this paper we describe a new, simple and fast method to achieve the final purification of a microvillus vesicle preparation, based on the ability of agarose beads to adsorb the impurities selectively.

The activity of aminopeptidase N (EC 3.4.11.2) and in some cases sucrase-isomaltase (EC 3.2.1.48) was used as markers for the microvillus membrane and 5'-nucleotidase (EC 3.1.3.5) and in some cases ($Na^+ + K^+$)-dependent ouabain-sensitive ATPase (EC 3.6.1.3) were used as markers for the basolateral membrane. All enzymes were assayed as previously described [3,4]. Protein was determined according to the method of Lowry et al. [5] using crystalline bovine albumin as standard. All reagents were obtained from standard commercial sources and were of analytical reagent grade.

Sepharose 4B was from Pharmacia, Uppsala, Sweden. Specimens for electron microscopy were prepared as previously described [4].

A microvillus membrane preparation was obtained from frozen inverted pig intestine by our modification of the method of Kessler et al. [3]. The preparation had the same specific activity of the marker enzymes, and the same morphology when thin sections were examined in the electron microscope, as previously described [3,4]. The preparation was washed and resuspended in 50 mM Tris-HCl (pH 7.4)/0.15 M NaCl [4] to a final protein concentration of 4.5 mg/ml (microvillus membrane preparation).

The suspension was chromatographed at 4°C on a column packed with Sepharose 4B and eluted with 50 mM Tris-HCl (pH 7.4)/0.15 M NaCl. The length of the column was always 15 cm but the

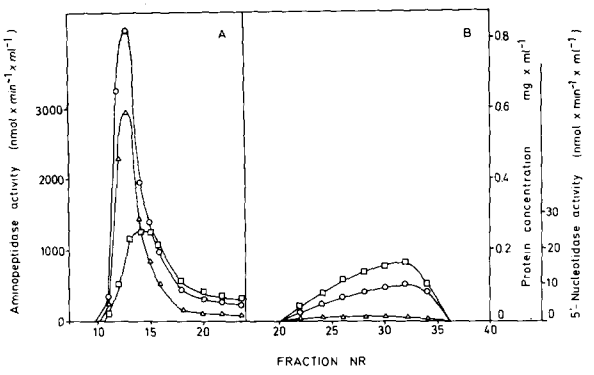

Fig. 1. Sepharose 4B chromatography elution profile. 2 ml of a microvillus membrane preparation were applied to the column (bed volume 60 ml), fractions of 2 ml were collected and assayed. ○———○, Protein concentration; △———△, aminopeptidase activity; □———□, 5′-nucleotidase activity. (A) Elution with 50 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, the elution was continued until fraction 100. (B) Elution of the adsorbed membranes with 2 mM Tris-HCl (pH 7.4). The fractions are renumbered from the buffer shift.

diameter varied according to the size of the preparation. The load on the column was 1 ml membrane suspension/30 ml settled gel and the flow rate was 2.5 ml/cm^2 per h. Fractions of the same volume as the sample applied were collected and assayed. The result of a typical experiment is shown in Fig. 1. The first fractions in the peak emerging from the column (Fig. 1A, fractions 12–14) have a high aminopeptidase activity compared to that of the 5′-nucleotidase, and constitute 40–60% of the total membrane protein applied to the column (unretarded membrane fraction). In the following fractions (retarded membrane fraction) the specific activity of the aminopeptidase decreases, whereas that of 5′-nucleotidase increases. Elution with at least 3 bed volumes of 50 mM Tris-HCl (pH 7.4)/0.15 M NaCl is necessary to wash the column completely free from retarded membranes. Membranes corresponding to 15–20% of the total protein applied to the column are adsorbed to the agarose beads and are not eluted with 50 mM Tris-HCl (pH 7.4)/0.15 M NaCl (adsorbed membrane fraction). This membrane fraction could be eluted by lowering the ionic strength of the buffer or by mechanical treatment. In Fig. 1B the elution with 2 mM Tris-HCl buffer (pH 7.4) is illustrated. Removal of the membranes by mechanical treatment without change of buffer is achieved by resuspending and stirring of the gel followed by rapid removal of the released membranes by filtration through sintret glass.

In routine purifications, the three first fractions which contain membranes could be collected and combined as unretarded membrane fraction and cleaning of the beads by elution with 2 mM Tris-HCl buffer (pH 7.4) (3 bed volumes) started thereafter. We have tested three different batches of Sepharose 4B and they all have the same properties.

A reproducible increase in aminopeptidase activity and a decrease in 5′-nucleotidase activity could be obtained in the unretarded membrane fraction. The ratio between the specific activity in the microvillus membrane preparation and that in the unretarded membrane fraction (defined as the effluent collected from the first membrane appearance, until a volume corresponding to 3-times the applied sample was accumulated) was for aminopeptidase 1.54 ± 0.04 and for 5′-nucleotidase 0.38 ± 0.05 (mean ± S.E. of ten experiments). There is still some 5′-nucleotidase present, but this enzyme is to some extent localized in the microvillus part of the membrane [4,6]. In two experiments we could not detect any ($Na^+ + K^+$)-dependent ouabain-sensitive ATPase in the unretarded membrane fraction, whereas sucrase-isomaltase was enriched to the same extent as the aminopeptidase. The vesicles of the unretarded membrane fraction are homogeneous according to density, as they all band in the 50% layer when centrifuged (Beckman SW 40.1 rotor, 35000 rpm, 2 h at 4°C) on a step gradient composed of 50, 40, 30, 20% sorbitol in 5 mM histidine-imidazole buffer (pH 7.5). When examined by electron microscopy, the unretarded membrane fraction consists almost entirely of uniform vesicles filled with fibrous material and overlaid with a fuzzy coat (Fig. 2A). We conclude that the unretarded membrane fraction consists of purified microvillus membrane vesicles.

The amount of retarded membranes varied from preparation to preparation. The specific activity of aminopeptidase in this fraction is always lower than in the original microvillus membrane preparation, whereas that of 5′-nucleotidase is higher.

The membrane fraction selectively adsorbed to the agarose beads consists mainly of basolateral membranes as judged from the activities of the marker enzymes. The ratio between the specific activity in the microvillus membrane preparation and the adsorbed membrane fraction was for aminopeptidase 0.19 ± 0.02 and for 5′-nucleotidase 2.31 ± 0.11 (mean ± S.E. of ten experiments). It was shown previously [4,6] that basolateral membranes contain minor amounts of the digestive hydrolases. In electron microscopy this fraction appears as empty or partly filled membrane structures, differing very much in size and with smooth surfaces. None of the dense microvillus membrane vesicles could be observed (Fig. 2B).

The polypeptide composition of the proteins in the unretarded membrane fraction and in the adsorbed membrane fraction also appears strikingly different. In Fig. 3 the result of a gel electrophoresis in SDS-10% polyacrylamide according to Laemmli [7] is shown. Prior to electrophoresis the samples were denatured by boiling for 5 min in 1% SDS and 2.5% 2-mercaptoethanol. After electrophoresis, the bands were stained for protein with Coomassie brilliant blue.

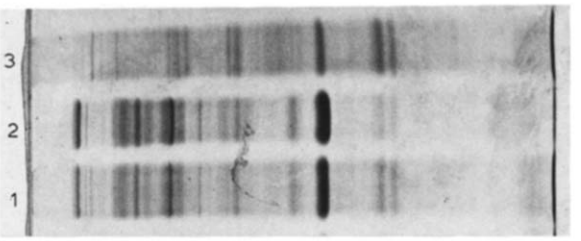

Fig. 3. SDS-polyacrylamide gel electrophoresis of fractions obtained by chromatography on Sepharose 4B of a microvillus membrane preparation. 110 μg of protein were applied to each lane. (1) Microvillus membrane preparation. (2) Unretarded membrane fraction, (3) Adsorbed membrane fraction. Left to right: top to bottom.

Upon rechromatography of the unretarded membrane fraction, no further purification can be obtained. Rechromatography of the adsorbed

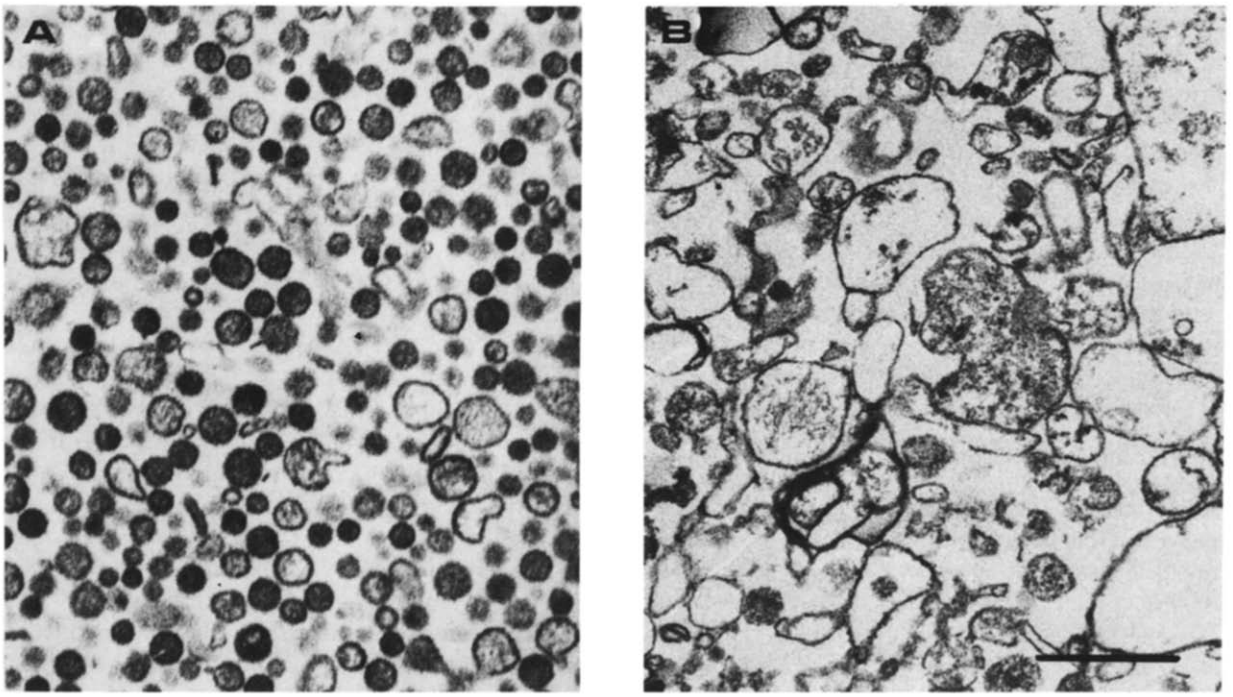

Fig. 2. Electron micrographs of fractions obtained by chromatography on Sepharose 4B of a microvillus membrane preparation. (A) Unretarded membrane fraction. (B) Adsorbed membrane fraction. The bar represents 0.5 μm.

membranes, released by mechanical treatment, results in readsorption. If a microvillus membrane preparation is chromatographed on a Sepharose 4B column, not cleaned of adsorbed membranes from a previous experiment, no separation occurs, indicating that all binding sites for adsorption are occupied.

The binding to the agarose beads is probably due to hydrophobic interactions, as elution of the adsorbed membranes could be obtained with buffer of low ionic strength. The adsorbed membranes are not released by elution with 50 mM Tris-HCl buffer (pH 7.4) containing 0.2 M lactose, 0.2 M galactose or 1 M NaCl. We suggest that hydrophobic areas of the membrane lipid bilayer are involved in the binding and only intact, right-side-out microvillus membrane vesicles, completely sheathed by the highly hydrophilic glycocalyx pass unimpeded through the column.

Other types of membrane, not shielded by a carbohydrate layer, are also adsorbed to the agarose beads. Endoplasmatic reticulum, although only present in very minute amounts in the microvillus membrane preparation [3] are also completely removed from the peak fraction. In one experiment we found no activity of the endoplasmatic reticulum marker enzyme, NADPH-cytochrome *c* reductase in the unretarded membrane fraction, whereas the adsorbed membrane fraction was enriched by a factor of 3 with this enzyme.

Compared to our previously reported method [4], based on immunoaffinity chromatography, the membrane fractions that adsorb to the agarose beads correspond to the pure nonadherent fraction, composed of membranes unable to adhere to the immunoadsorbent. The purified microvillus vesicles in the present study have a more uniform appearance in electron microscopy and show a higher increase in the specific activity of the aminopeptidase, compared to the immunoadsorbent purified vesicles. This improvement is probably due to removal of the retarded fraction of membranes with lower but still considerable activity of the hydrolases enable them to bind to the immunoadsorbent.

The method described in this paper is rapid and simple and has almost unlimited capacity. We have obtained pure microvillus membrane vesicles corresponding to 50 mg of protein by chromatography on a 7×15 cm column within 2.5 h. It seems likely that other types of membrane could be purified using this hitherto unknown property of agarose beads. In preliminary experiments we have shown that an endoplasmic reticulum preparation from pig intestine can be purified from contaminating microvillus membrane vesicles, using this principle.

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