Rotofor® Fractionation of Intestinal Brush Border Membrane Proteins

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Abstract

Organic substrates (sugars, amino acids, carboxylic acids, and neurotransmitters) are actively transported into eukaryotic cells by Na^+ cotransport.[1] The Na^+-dependent glucose transporter is an integral membrane protein located in the apical membrane of the intestinal epithelium. This protein transports glucose against its concentration gradient by utilizing the energy of the downhill Na+ gradient across the membrane. Once glucose enters the cell, it diffuses across the basolateral membrane, passively down its concentration gradient. A defect in the co-transporter is the origin of the congenital glucose-galactose malabsorbtion syndrome.[2]

Previous work has shown that the Na^+/glucose transporter is a transmembrane protein of approximately 70–75 kD in its native form.3 It contains glucose and Na+ binding sites. The protein undergoes a conformational change upon Na+ binding, which exposes the sugar binding site. It has a turnover rate of 5 sec at 22 °C, similar to other energy driven pumps. The gene which encodes this protein has been cloned, sequenced, and functionally expressed in *xenopus* oocytes[3] and COS-7 cells.[4] Antibody prepared against a synthetic peptide derived from the primary sequence of the cloned transporter DNA was used for western blot analysis of the Na^+/glucose co-transport protein purified in the Rotofor cell.

The Na^+/glucose transport protein has eluded attempts at purification for many years, partly because of the difficulties inherent in the purification of low abundance hydrophobic membrane proteins (<0.2% of the total membrane protein). Liquid phase preparative isoelectric focusing in the Rotofor cell solved the challenging problem of fractionating brush border transmembrane proteins. A single 3 hour Rotofor run resulted in a 5-fold increase in specific activity of pooled active fractions over the crude unpurified supernatant, allowing enrichment of Na^+/glucose co-transport function and immunoreactivity.

Methods

Epithelial cells were obtained from the intestines of adult male rabbits. The cells were homogenized and centrifuged. Apical membrane vesicles were obtained by precipitating the supernatant fluid with calcium.[5]

SOLUBILIZATION OF RABBIT INTESTINAL BRUSH BORDER MEMBRANES

Brush border membrane vesicles (BBMVs), 4 mg protein/ml, were solubilized in 10% CHAPS (w/v) and 6 mg/ml sonicated lipids (80% asolectin, 20% cholesterol by weight) in 150 mM NaCl, 10 mM D-glucose, 10 mM proline, 1 mM DTT, 1 mM EDTA, 10% glycerol, 10 mM HEPES-Tris, pH 7.4. Solubilization was carried out with constant stirring at 4 °C for 1 hour. The material was centrifuged for 1 hour at 48,000 rpm. The supernatant was collected for further fractionation and reconstitution.

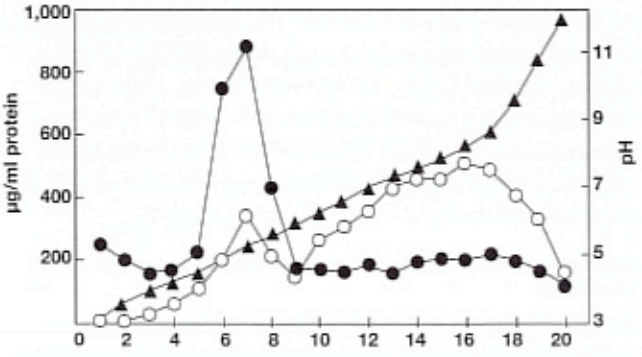

Fig. 1. Protein and pH distributions in Rotofor fractions. pH was measured immediately after collecting fractions and is shown above as (▲). On the plot of protein concentration, closed circles (●) are A_{280}. The open circles (○) are modified Lowry protein concentrations.

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LIQUID PHASE ISOELECTRIC FOCUSING

Thirty-three mg (~8 ml) of BBMVs were solubilized as described. The supernatant fluid was desalted using Bio-Gel® P-6DG gel and diluted to a final volume of 55.0 ml containing 3% CHAPS (w/v), 1 mM DTT, 10 mM glucose, and 0.5 ml Bio-Lyte® ampholytes, pH range 3–10. This solution was loaded into the Rotofor cell without further treatment. Focusing was carried out at 12 watts constant power for 3 hours at 4 °C. The initial conditions were 800 V and 15 mA. At equilibrium the values were 2,000 V and 6.0 mA. Twenty fractions were harvested and their pH values measured. Protein concentration was determined by a modified Lowry procedure.[6] See Figure 1.

RECONSTITUTION OF ROTOFOR PURIFIED SOLUBILIZED BBMVS

After measurement of pH and protein concentration, samples were pooled into five groups of four fractions each for reconstitution. Solubilized protein fractions were adjusted to a final concentration of 150 mM NaCl, 10 mM proline, 10 mM glucose, 1 mM DTT, 1 mM EDTA, 1.2 mg/ml sonicated lipids (80% asolectin, 20% cholesterol by weight), 2% CHAPS, 10% glycerol, 0.1 mM MgSO4, 0.1 mM $CaCl_2$, 10 mM HEPES-Tris, pH 7.4. Bio-Beads® SM-2 beads (for removal of CHAPS) were added to the solubilized protein at 0.1 g beads/ml protein solution. Samples were incubated at 4 °C with gentle mixing for 16 hours.

After incubation, the beads were removed from the sample solutions by filtration through a polystyrene frit placed in a 10.0 ml Econo-Column® column. The reconstituted liposomes were diluted 3-fold with 150 mM KCl, 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 mM HEPES-Tris, pH 7.4, and centrifuged for 1 hour at 55,000 rpm in a 60 Ti rotor. Reconstituted liposomes were washed twice in the above solution. Protein incorporation into liposomes and recovered transport activity were ~10% of starting material.

MARKER ENZYME ASSAYS OF BRUSH BORDER MEMBRANE PROTEINS

Two standard brush border marker enzymes, gamma-glutamyl transpeptidase and alkaline phosphatase, solubilized and focused as described above, were assayed for biological activity. See Figure 2.

ASSAY FOR BIOLOGICAL ACTIVITY OF RECONSTITUTED NA^+/GLUCOSE TRANSPORTER

After reconstitution, transport activity was measured as the ability of liposomes to support uptake of ^{3}H-glucose in the presence of Na^+ versus K^+ (in each case K^+ was used as a negative control to account for passive glucose uptake and nonspecific binding). Samples were incubated in the presence of radiolabeled glucose, Na^+, or K^+. The reactions were quenched with ice cold KCl, liposomes were filtered, and the filtrate counted.[7] See Figure 3.

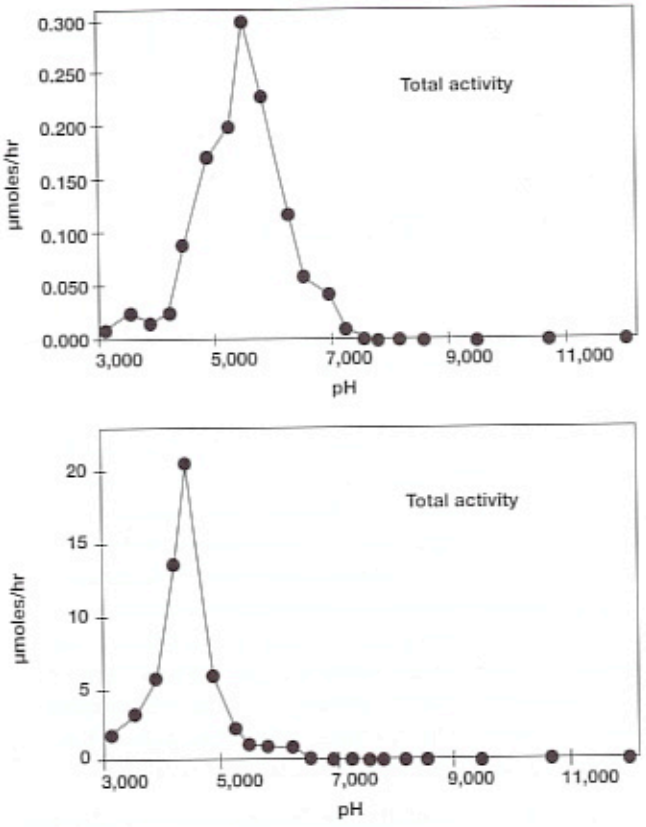

Fig. 2. Marker enzyme assays of brush border membrane proteins. A. Gamma-glutamyl transpeptidase. Activity was measured using L-gamma-glutamyl-p-nitroanilide as substrate. Reaction was initiated by adding 50 µl of each fraction, and initial rates were monitored by absorbance of the product at 410 nm. The pI for this enzyme was 5.3. B. Alkaline phosphatase activity was measured using para-nitrophenyl-phosphate as substrate. Reaction was initiated by adding 50 µl of each fraction, and initial rates were monitored by absorbance of the product at 410 nm. The pI for this enzyme was 4.5.

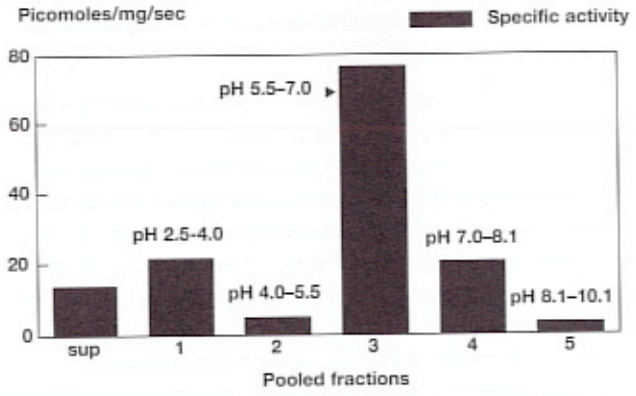

Fig. 3. Reconstitution and activity of focused proteins. After measurement of the pH and protein, fractions were pooled into five groups of four fractions each for reconstitution. After reconstitution, transport activity was measured as the ability of liposomes to support uptake of ^{3}H-glucose in the presence of Na^+ versus K^+. The graph shows a 5-fold increase in specific activity of pooled Rotofor fractions 9–12 of the initial solubilized supernatant (sup).

SDS-PAGE ANALYSIS OF PROTEIN FRACTIONS

Polyacrylamide gels were run by the method of Laemmli, with the separating gel 8% acrylamide. Gels were stained with silver or Coomassie® brilliant blue. See Figure 4.

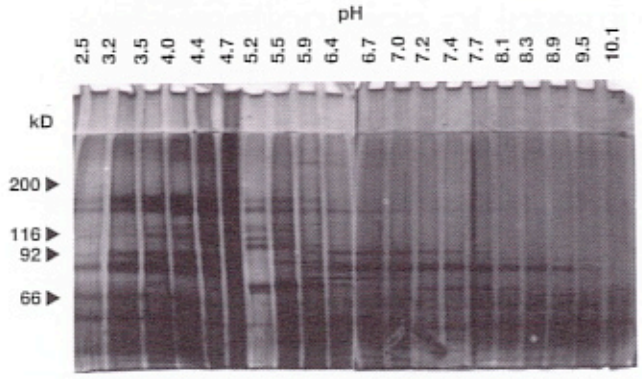

Fig. 4. Silver stained polyacrylamide gel of Rotofor fractions.

WESTERN BLOT ANALYSIS OF POLYACRYLAMIDE GELS

Gels were electrophoretically blotted onto nitrocellulose for 1 hour at 4 °C. Primary antibody was prepared in rabbits against synthetic peptides conjugated to keyhole lympet hemocyanin (KLH),[8] and purified on immobilized protein A. Second antibody detection was with goat anti-rabbit antibody conjugated to HRP. Development was with diaminobenzidine and H_2O_2. See Figure 5.

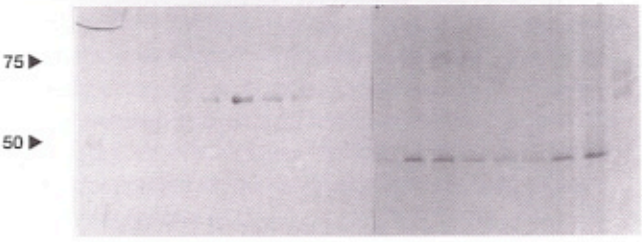

Fig. 5. Western blot of brush border membrane Rotofor fractions. Only low pH fractions show specific antibody recognition. High pH fractions are recognized by goat anti-rabbit IgG and are not inhibited by peptide (data not shown).

Results and Conclusion

Isoelectric focusing in the Rotofor cell was used for the purification of the Na^+/glucose co-transporter from rabbit epithelia. Two assay methods were used for localizing the transporter following purification in the Rotofor cell. One was the traditional method of solubilization and functional reconstitution into liposomes. The other method was western blot, using antibodies raised against synthetic peptides derived from the primary sequence of the cloned transporter DNA. Two marker enzymes were easily purified as well.

Results show that the transporter can be solubilized, purified, and functionally reconstituted into asolectin/cholesterol liposomes. It was also shown that anti-peptide antibodies specifically recognize a protein of ~70 kD with a pI of ~5.2 from the solubilized brush border membrane, and that antibody recognition correlates with glucose transport function.

It is the aim of this research to locate the active sites of the co-transporter protein and probe the conformational states through site directed mutagenesis of the primary DNA sequence. The next phase of this research will combine the use of Rotofor purification, SDS-preparative electrophoresis, reversed phase HPLC, and sequence analysis in order to elucidate the factors required for both sugar and Na^+ binding.

The Rotofor cell has proven itself indispensable as an integral method for purification of detergent solubilized membrane proteins on a preparative scale.

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