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Note

Purification of phlorizin by column chromatography on Sephadex LH-20 with aqueous propan-2-ol

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In several mammals, including man, the brush-border membrane of the small intestine exhibits a β-glucosidase activity capable of hydrolysing phlorizin* to phloretin and glucose[1]. This "phlorizin hydrolase" activity is associated with the lactase (neutral β-galactosidase) enzyme of the small intestinal mucosa[2], and has been shown to hydrolyse a wide range of glucosylceramides and lactosylceramides occurring in milk[3]. The high cost of pure glycosylceramides, and their low solubility in detergent-free aqueous solutions, precludes their regular use for the measurement of phlorizin hydrolase activity and so phlorizin is usually the substrate employed.

During our studies on phlorizin hydrolase it became apparent that the free glucose and phloretin content of some batches of phlorizin interfered, presumably by product inhibition, with the kinetic analysis of enzymatic activity. As recrystallization from aqueous ethanol gave no marked increase in purity, column chromatography on Sephadex LH-20 with aqueous solutions of propan-2-ol was investigated and found to be a suitable purification method.

MATERIALS AND METHODS

Phlorizin, phloretin (phlorizin aglucon), phloroglucinol (1,3,5-trihydroxybenzene), 2,4,6-trihydroxybenzoic acid and 3-(4-hydroxyphenyl)propionic acid were purchased from commercial suppliers. D-[U-^{14}C]Glucose was obtained from the Radiochemical Centre (Amersham, Great Britain). A bed of Sephadex LH-20 (Pharmacia, Uppsala, Sweden), 33 × 2.5 cm, was packed in a Pharmacia SR25/45 column in accordance with the manufacturers instructions. Preliminary experiments indicated that a mixture of redistilled propan-2-ol (35–50%) and water was suitable as eluent. The column was eluted upwards with a peristaltic pump at a flow-rate of 0.4 ml/min and 7.5 ml fractions were collected. Fluctuations in temperature were observed to affect markedly the elution volume of each compound so the entire operation was performed in a cold room at 4°, and care was taken to protect the column and collected samples from strong light. Standard marker solutions of

* Phlorizin = 4,6-dihydroxy-2-(β-D-glucosido)-β-(*p*-hydroxyphenyl)propiophenone; also known as phlorhizin, phloridzin or phlorrhizen.

phlorizin and its related component molecules were made at a concentration of 1 mg/ml in a mixture of propan-2-ol and water corresponding to the column eluent. All samples were labelled with *ca.* 10^5 cpm of [^{14}C]glucose before being loaded onto the column. Aliquots (0.5 ml) of each eluted fraction were mixed with 10 ml Aquasure (NEN Chemicals, Dreieich, G.F.R.) and the ^{14}C-content measured in a liquid scintillation counter. The elution of aromatic compounds off the column was followed by measuring the UV absorption of the eluate at 220 nm. Each peak was identified by comparison of its elution volume and UV spectrum with those of standard marker compounds.

The free glucose content of phlorizin before and after chromatography was measured at 365 nm with the glucose–fructose UV test kit produced by Boehringer (Mannheim, G.F.R.).

RESULTS AND DISCUSSION

Chromatography on Sephadex LH-20 using propan-2-ol[4] or acetone[5] as solvent has been shown to be an effective method for the separation of aromatic and cyclic compounds by reversible adsorption. Our initial experiments with Sephadex LH-20 and solvent mixtures of 35–50% propanol-2-ol and water indicated that phlorizin retention was inversely related to propan-2-ol concentration whereas glucose always eluted in the void volume. In the solvent mixture finally chosen, 38% propan-2-ol in water, an excellent separation of phlorizin from glucose and phloretin (peaks E, A and F respectively in Fig. 1) was achieved, and in addition the system

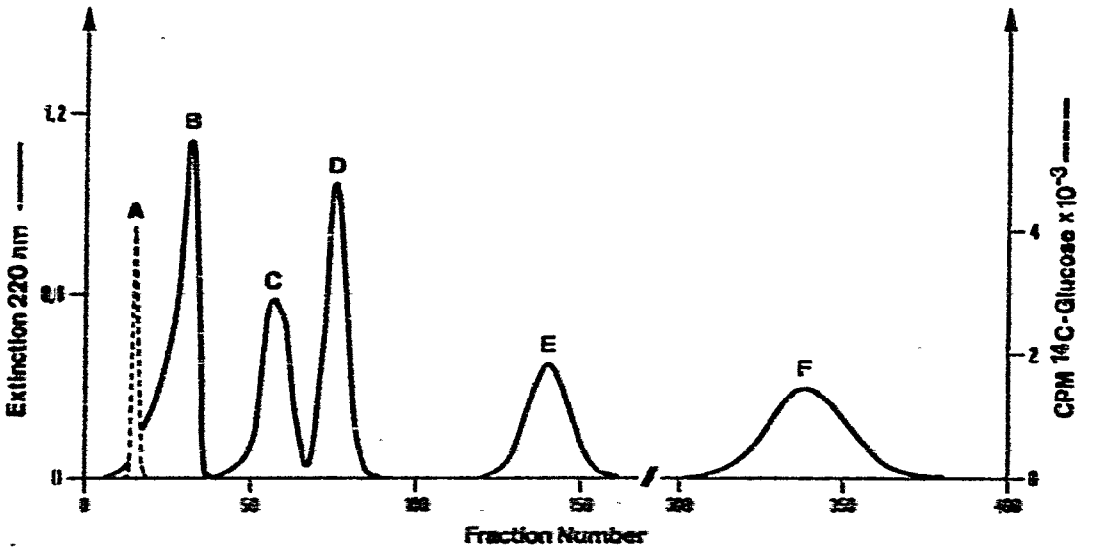

Fig. 1. The chromatographic separation on Sephadex LH-20 of a mixture of 1 mg glucose containing 10^5 cpm [^{14}C]glucose (A), 0.5 mg 2,4,6-trihydroxybenzoic acid (B), 1 mg 3-(4-hydroxyphenyl)-propionic acid (C), 1 mg phloroglucin (D), 1 mg phlorizin (E) and 1 mg phloretin (F). The column, 33 × 2.5 cm, was eluted upwards at 4° with 3 l of 38% propan-2-ol in water at a flow-rate of 0.4 ml/min. Fractions of 7.5 ml volume were collected every 18.8 min. The elution profile was measured at 220 nm; the broken line represents radioactivity.

resolved 2,4,6-trihydroxybenzoic acid, 3-(4-hydroxyphenyl)propionic acid and phloroglucin (peaks B, C and D respectively in Fig. 1). With higher concentrations of propan-2-ol the phlorizin eluted earlier but too close to other components to be of use as a preparative method, although still suitable for analytical work.

For the routine purification of phlorizin the column was loaded with 100 mg phlorizin (containing 0.1–9% free glucose as an impurity) in 1.5 ml of solvent and eluted with 3 l of 38% propan-2-ol at 4°. Those eluate fractions containing phlorizin were pooled and lyophilized to give a dry phlorizin powder in which there was no detectable free glucose. The same column has been used continuously for more than twenty such runs over a period of 6 months without any alteration in the elution profile.

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