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Note

Thin-layer chromatography using multiple development for analysis of reaction products of sucrases

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Sucrases, e.g. invertase (EC 3.2.1.26), amylosucrase (EC 2.4.1.4), dextransucrase (EC 2.4.1.5), inulosucrase (EC 2.4.1.9) and levansucrase (EC 2.4.1.10), produce various saccharides such as glucose, fructose, oligosaccharides and polysaccharides from sucrose [1]. In elucidating the reaction mechanism of sucrases and the action mechanism of their inhibitors, it is necessary to identify simultaneously all products of these enzymes from sucrose. The activities of sucrase have been determined by the determination of reducing sugars released [2], or polysaccharides synthesized from sucrose [3, 4]. However, only certain reaction products may be detected.

For the determination of the reaction products of sucrases, we have elaborated a new method of thin-layer chromatography (TLC) using multiple development.

EXPERIMENTAL

Chemicals

Mono- and oligosaccharides used in this study are listed in Table I, and were of reagent grade. Isomalto-oligosaccharides were prepared by partial hydrolysis of dextran T10 (Pharmacia, Uppsala, Sweden). Invertase from yeast (Wako, Osaka, Japan) and dextransucrase from *Streptococcus sanguis* ATCC 10558 [5] were used as the representative sucrases. [U-^{14}C]Sucrose (4.67 Ci/mol; 0.17 TBq/mol) was obtained from New England Nuclear (Boston, MA, U.S.A.).

Enzyme assay

Invertase (5 μl) was allowed to react with 10 m*M* [U-^{14}C]sucrose (1.25 Ci/mol; 46.25 GBq/mol; 175 000 cpm) in 10 μl of 0.1 *M* potassium phosphate buffer (pH

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TABLE I

R_F VALUES AND DETECTION LIMITS OBTAINED FOR VARIOUS SACCHARIDES BY TLC

TLC of the saccharides listed below was carried out using Avicel SF cellulose plates. These plates were developed for two successive ascents with solvent I (*n*-butanol–pyridine–acetic acid–water, 10:6:1:3, v/v), followed by another ascent with solvent II (phenol–1.5% ammonium hydroxide, 2:1, v/v). Sugars were detected by aniline–diphenylamine staining.

Compound	R_F value	Detection limit (μg)
D-Fructose	1.18	2.0
D-Glucose	1.00	1.0
Sucrose	0.89	1.0
Nigerose	0.83	1.0
Maltose	0.78	1.0
Isomaltose	0.65	1.0
Raffinose	0.59	1.0
Panose	0.55	1.0
Isomaltotriose	0.49	2.5
Isomaltotetraose	0.38	5.0
Isomaltopentaose	0.29	5.0

5.5). Dextransucrase (5 μl) was allowed to react with 10 m*M* [U-^{14}C]sucrose in 10 μl of 0.1 *M* potassium phosphate buffer (pH 6.0) in the absence or presence of 1% (w/v) isomaltose. After incubation at 37°C for 2–30 min, the mixture was heated at 100°C for 2 min to inactivate the enzyme, and the reaction products were analysed by TLC as described below.

Analytical procedure

Samples (in duplicate) of 10 μl were applied to Avicel SF plates layered with cellulose (20×20 cm; Asahi Chemical Industry, Tokyo, Japan). These plates were developed ascendingly with solvent I, which consisted of *n*-butanol–pyridine–acetic acid–water (10:6:1:3, v/v), for 4 h at 20°C, and then dried. They were returned to the solvent trough for repeated development with the same solvent. The plates were dried, and then developed ascendingly with solvent II (phenol–1.5% ammonium hydroxide, 2:1, v/v) for 4 h at 20°C and dried. From duplicate samples, one portion was stained with aniline–diphenylamine [6]. The corresponding portion containing saccharides was scraped from the plates, and the radioactivity in the saccharides was measured by scintillation spectrophotometry with the toluene scintillant.

RESULTS AND DISCUSSION

Fig. 1 shows thin-layer chromatograms obtained by development with solvent I, solvent II or both solvents. Solvent I enabled the separation of isomalto-oligosaccharides, but not of fructose from glucose (Fig. 1a). Solvent II separated only fructose from other saccharides (Fig. 1b). Using both solvent I and II, fructose, glucose, sucrose, maltose, panose and isomalto-oligosaccharides (degree of poly-

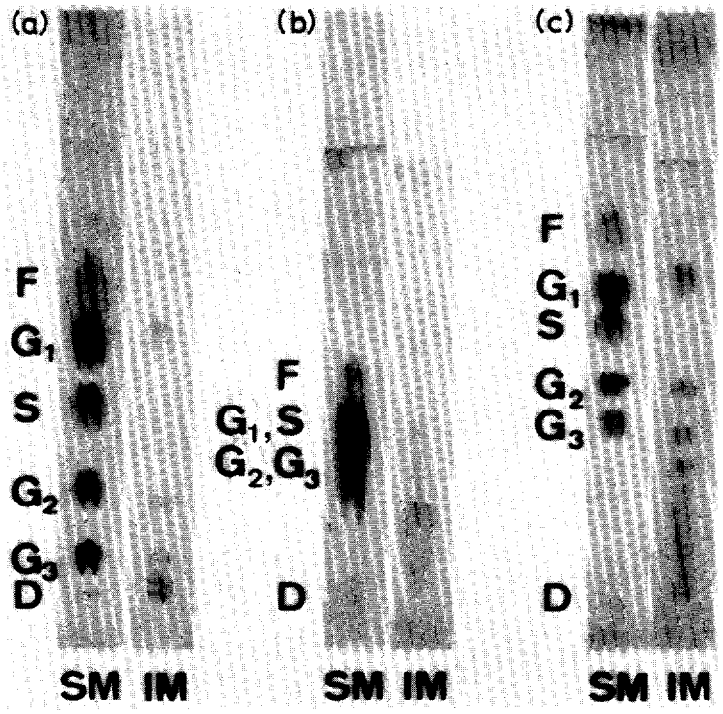

Fig. 1. Thin-layer chromatogram of various saccharides after ascending development on Avicel SF cellulose plate. Standard mixture (SM) consisted of fructose (F; 20 mg/ml), glucose (G_1; 10 mg/ml), sucrose (S; 10 mg/ml), isomaltose (G_2; 10 mg/ml), isomaltotriose (G_3; 10 mg/ml) and dextran (D; 10 mg/ml). Isomalto-oligosaccharide mixture (IM; 60 mg/ml) was prepared by partial hydrolysis of dextran T10. Equal volumes (10 μl) of each mixture were chromatographed with the following solvent system: (a) two ascending developments, in solvent I consisting of *n*-butanol-pyridine-acetic acid-water (10:6:1:3, v/v); (b) one ascending development in solvent II consisting of phenol-1.5% ammonium hydroxide (2:1, v/v); (c) two successive ascending developments in solvent I followed by one development in solvent II. Spots were visualized by spraying with aniline-diphenylamine.

merization, DP, 2–16) were separated (Fig. 1c). Isomalto-saccharides with a DP higher than 16 remained at the origin. Table I lists the R_F values for various saccharides analysed using two solvents.

Reaction products from [^{14}C]sucrose by invertase and dextransucrase were analysed by TLC using two solvents. The amount of fructose produced by invertase was the same as that of glucose (Fig. 2a). Dextransucrase produced fructose, glucose and glucan (DP > 16) from sucrose in the absence of isomaltose (Fig. 2b). This enzyme produced a large amount of isomalto-oligosaccharides, as well as fructose and glucan, from sucrose in the presence of isomaltose (Fig. 2c). The amount of fructose released by the enzyme was identical with the sum of glucose, isomalto-oligosaccharides and polysaccharides.

The TLC procedure using two solvents and multiple development is thus very useful for the quantitative fractionation of reaction products of various sucrases from sucrose. Moreover, many samples may be analysed simultaneously as well as inexpensively by this procedure.

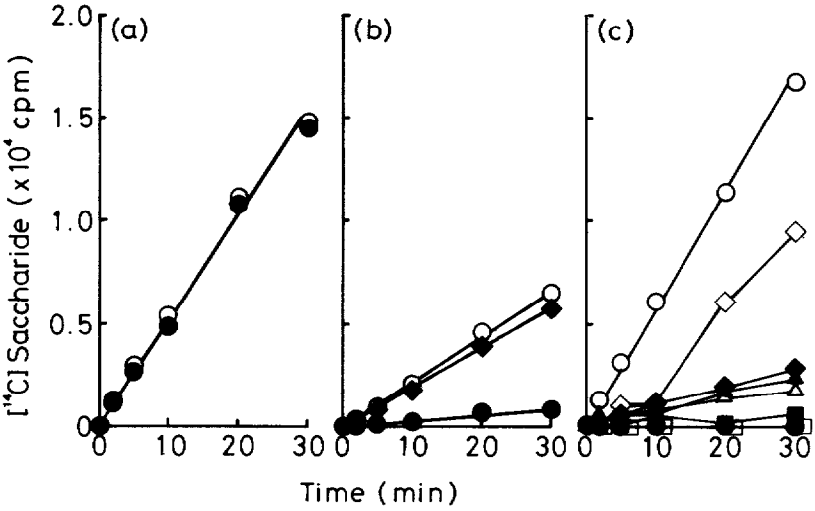

Fig. 2. Production of various saccharides from sucrose by invertase (a), dextransucrase (b) and dextran sucrase in the presence of isomaltose (c). Data points: ○ = fructose; ● = glucose; □ = isomaltose; ■ = isomaltotriose; △ = isomaltotetraose; ▲ = isomaltopentaose; ◇ = isomalto-oligosaccharides (DP 6-16); ◆ = dextran (DP > 16).

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