Two-dimensional paper chromatography interspersed with reaction on the paper*

Many situations arise in which it is desired to examine the effect of a reagent or enzyme on the individual constituents of a chromatographically separable mixture. The use of more or less specific spray or dip reagents is commonplace for both paper and thin-layer chromatography. Endless variations are possible, as in the use by FRENCH AND WILD of a phosphorylase–glucose-1-phosphate spray to reveal specific primers for phosphorylase[1]. The present communication deals with a modification of the FRENCH AND WILD technique. In this modification, the mixture is applied near one corner of a large sheet of paper, as for two-dimensional chromatography. Following irrigation in one dimension, the area containing the separated or partially separated constituents is treated with a specific reagent or enzyme, and reaction is allowed to occur on the paper. Finally the paper is dried and subjected to chromatographic irrigation in a direction at right angles to the original direction. The rationale of the method, as applied to structure analysis of ε-dextrin, is illustrated in Fig. 1a.

We here report application of this technique to enzyme action on starch oligosaccharides. The method is particularly useful to rapidly survey enzyme action on individual members of homologous series. In some of the following examples, we have also used it as a convenient method for identifying and characterizing constituents of mixtures.

Experimental

Chromatography has been carried out on Whatman No. 1 paper at room temperature[2,3] or Whatman 3 MM paper at about 80°[4]. The latter was washed with water and ethanol to remove interfering impurities. Irrigating solvents employed for separation of starch oligosaccharides have been mainly 80 % *n*-PrOH, and *n*-BuOH–pyridine–water (6:4:3 or 6:4:4, v/v)[3,4]. Multiple ascent (2–6 times) is particularly effective in resolving higher starch oligosaccharides in the range up to 15 D-glucose units. The mixture of oligosaccharides is applied in a single spot near the corner of a square or rectangular sheet of paper, dried, and subjected to irrigation in the first direction. After the desired amount of irrigation, the paper is dried and the area containing the resolved components is sprayed with an appropriate reagent or enzyme solution. With reactions that require more than a few minutes, the damp paper may be suspended in a damp chamber to prevent it from drying out. No serious difficulties have been encountered from diffusion during the damp reaction, although overspraying leads to "bleeding" and distortion of the finished chromatogram. After the reaction has gone for the desired length of time, the paper is dried and again subjected to irrigation in a direction at right angles to the original direction.

Revelation of spots may be made by any suitable method, for example, the silver nitrate–alkali method[2] which is very effective for reducing sugars. Results may be improved by substituting sodium carbonate for sodium hydroxide, and dipping the

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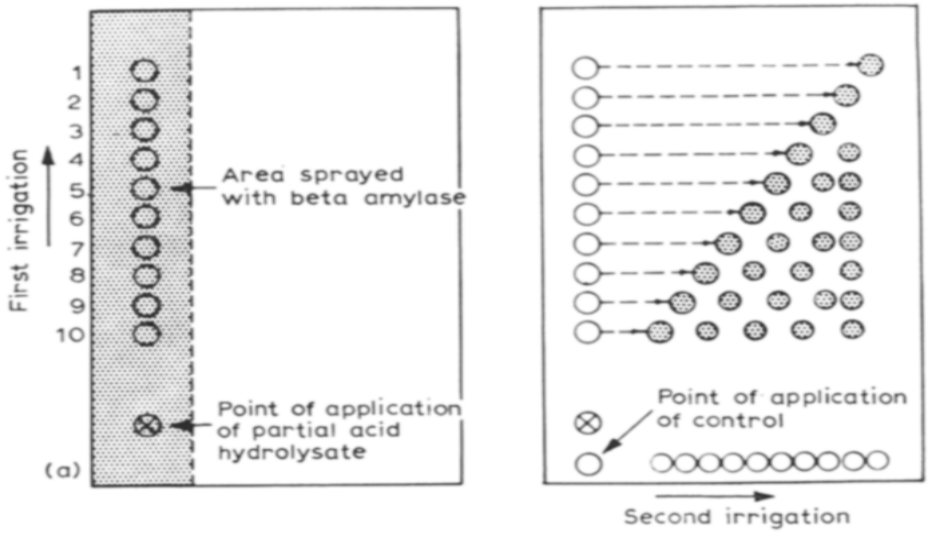
papers into the reagents rather than spraying. Following adequate revelation of the carbohydrate zone, further darkening is prevented by briefly treating with photographic fixer (Kodak F-24) and water washing.

Example 1. Action of beta amylase on partial acid hydrolysis products of epsilon dextrin. Crystalline ε-dextrin[5] (1.35 mg) was partly hydrolyzed by heating in a sealed tube at 100° with 0.15 ml of 0.1 *N* HCl for 60 min. It was neutralized with pyridine and applied near one corner of a paper. After four ascents with *n*-BuOH–



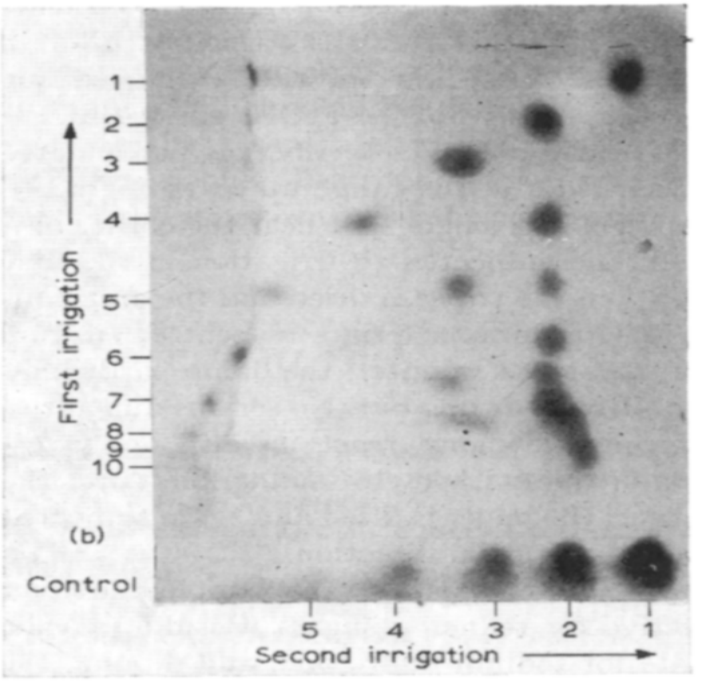


Fig. 1. Action of β-amylase on products of partial acid hydrolysis of ε-dextrin. Fig. 1 a is the schematic representation, and Fig. 1 b is the actual experimental result. The original sample was applied at ⊗. Overspraying with β-amylase solution in the lower left-hand part of Fig. 1 b has resulted in some distortion of the chromatogram in the G_8–G_{10} region. A control was applied at ○ just prior to rirrigation in the second dimension. The numbers 1–10 indicate G_1–G_{10}.

pyridine–water (6:4:4) the paper was dried and sprayed with a solution of β-amylase (Worthington Biochemical Corp., Freehold, N.J.; 0.01 ml of suspended crystals per ml of water). After standing 60 min in a damp atmosphere, the paper was dried and again subjected to chromatography (2 ascents) in the perpendicular direction. After drying, the reducing sugars were revealed using the silver dip method. Fig. 1a represents the rationale in schematic form and Fig. 1b the actual experimental result.

The enzyme β-amylase is very specific for linear starch chains[6]. Therefore, susceptibility to β-amylase is convincing evidence that the oligosaccharides (and hence the parent cyclic molecule) must contain only α-1 → 4-D-glucosidic links. Moreover, because β-amylase is very specific in removing maltose from the non-reducing terminus of starch chains, from the intermediate and final products one can easily tell how many D-glucose units are present in each individual oligosaccharide even though these may not be clearly resolved on the one-dimensional chromatogram. For example, from maltopentaose (G_5) one can clearly see unchanged G_5, as well as G_3 and G_2 in at least approximately equal amounts. In the range G_8–G_{12} where the one-dimensional resolution is impaired after enzyme reaction and perpendicular chromatography there is an obvious pattern of "even" and "odd" oligosaccharides produced by partial action of beta amylase.

The finished chromatogram clearly shows that the original series of starch oligosaccharides terminated very sharply with G_{10}, and hence the ε-dextrin must have contained 10 D-glucose units. This result could not have been obtained by conventional one- or two-dimensional chromatography inasmuch as the unchanged ε-dextrin interfered seriously with the resolution of oligosaccharides in the critical range of 8–12 glucose units.

Example 2. Radioactive oligosaccharides formed during photosynthesis in the presence of $^{14}CO_2$. The formation of radioactive oligosaccharides during photosynthesis in $^{14}CO_2$ was brought to our attention by BASSHAM AND CALVIN[7], who kindly furnished radioactive algal oligosaccharides. Action of β-amylase on the isolated individuals indicated that they were starch oligosaccharides[8]. Similar compounds were produced by photosynthesis in soybean leaves with $^{14}CO_2$ as described by FISHER[9]. The deionized, neutral leaf extracts containing radioactive oligosaccharides were subjected to chromatography in one dimension, sprayed as in Example 1 with β-amylase and again subjected to chromatography in the perpendicular direction. The resulting chromatogram was subjected to radioautography (Fig. 2). As in Example 1, susceptibility to β-amylase confirmed that the original radioactive compounds are linear starch oligosaccharides. Moreover, by counting the radioactivity in the individual maltose and glucose spots, it was possible to infer the distribution of radioactivity in the oligosaccharides. In this experiment, quantities of material too small for ordinary chemical work were identified by their behavior with a specific enzyme, and some indication was obtained regarding the distribution of radioactivity in the individual compounds.

Example 3. Starch oligosaccharides produced by action of malt α-amylase on waxy maize starch. This mixture was thought to consist of linear as well as branched components. The mixture was subjected to chromatography as in Example 1, treated with pullulanase[10,11] (an enzyme specific for α-1 → 6-D-glucosidic links), and again subjected to chromatography in the perpendicular direction (Fig. 3). Linear oligo-

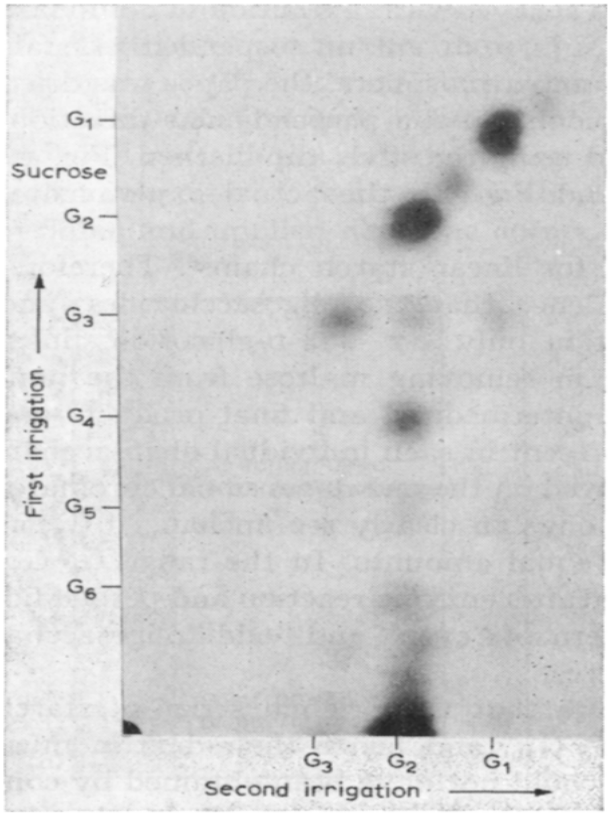
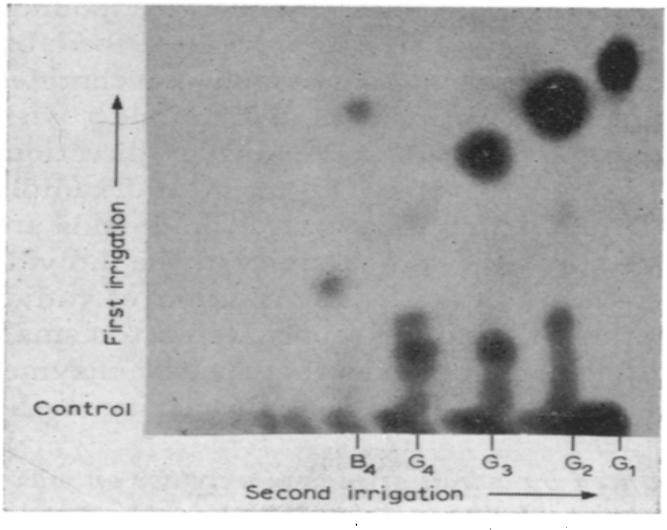


Fig. 2. Identification of radioactive starch oligosaccharides by action of β-amylase. Solvents: first irrigation, 2 ascents in BuOH–pyridine–H_2O (6:4:4); second irrigation, one ascent in the same solvent.



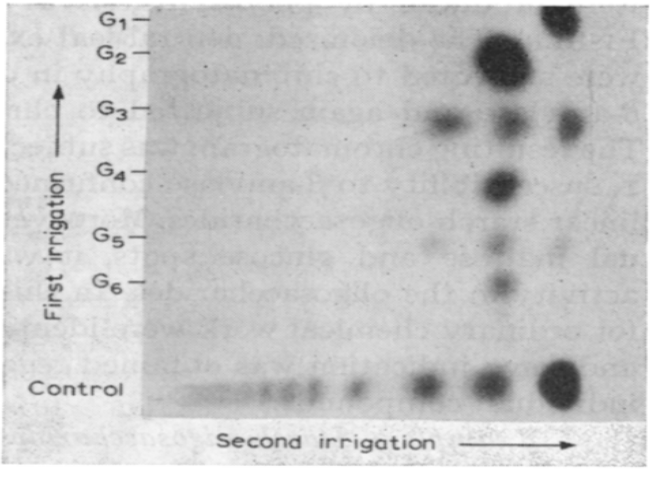


Fig. 3. Action of pullulanase on malt enzyme dextrins.

Fig. 4. Action of β-amylase on products of malt enzyme acting upon maltohexaose.

saccharides were not cleaved by pullulanase; oligosaccharides containing 1 → 6 links could be identified by their cleavage products. Unexpectedly, maltose gave a tetrasaccharide indicating that a condensation reaction had occurred[12].

Example 4. Starch oligosaccharides produced by action of a malt enzyme on maltohexaose. A malt α-amylase sample was prepared from barley malt by heat treatment and glycogen precipitation[13]. The preparation was suspected to contain a transferase in addition to an α-amylase. Hence, products of its action on maltohexaose might contain α-1 → 6-linked oligosaccharides, which would be at least partly resistant to β-amylase. The oligosaccharide mixture was treated as in Example 1, and the results are presented in Fig. 4. All oligosaccharides in the sample were degraded to give maltose (even-membered oligosaccharides) or maltose plus glucose and maltotriose (odd-membered oligosaccharides). Thus the original sample contained only glucose and linear α-1 → 4-linked oligosaccharides, and there cannot have been any appreciable 1 → 4; 1 → 6 transferase activity in the malt enzyme preparation.

Example 5. Branched oligosaccharides produced by action of salivary amylase on glycogen. Shellfish glycogen (purchased from Sigma Chemical Co., St. Louis, Mo.) was extensively treated with crystalline salivary amylase[14] to give products as shown in the one-dimensional chromatogram (Fig. 5a). Chromatographically visible products include glucose, maltose, singly branched oligosaccharides (B_4–B_7) and putative doubly-branched oligosaccharides (BB)[8,15]. The mixture of products was applied to the corner of a paper as in Example 1, separated in one dimension (3 ascents), dried,

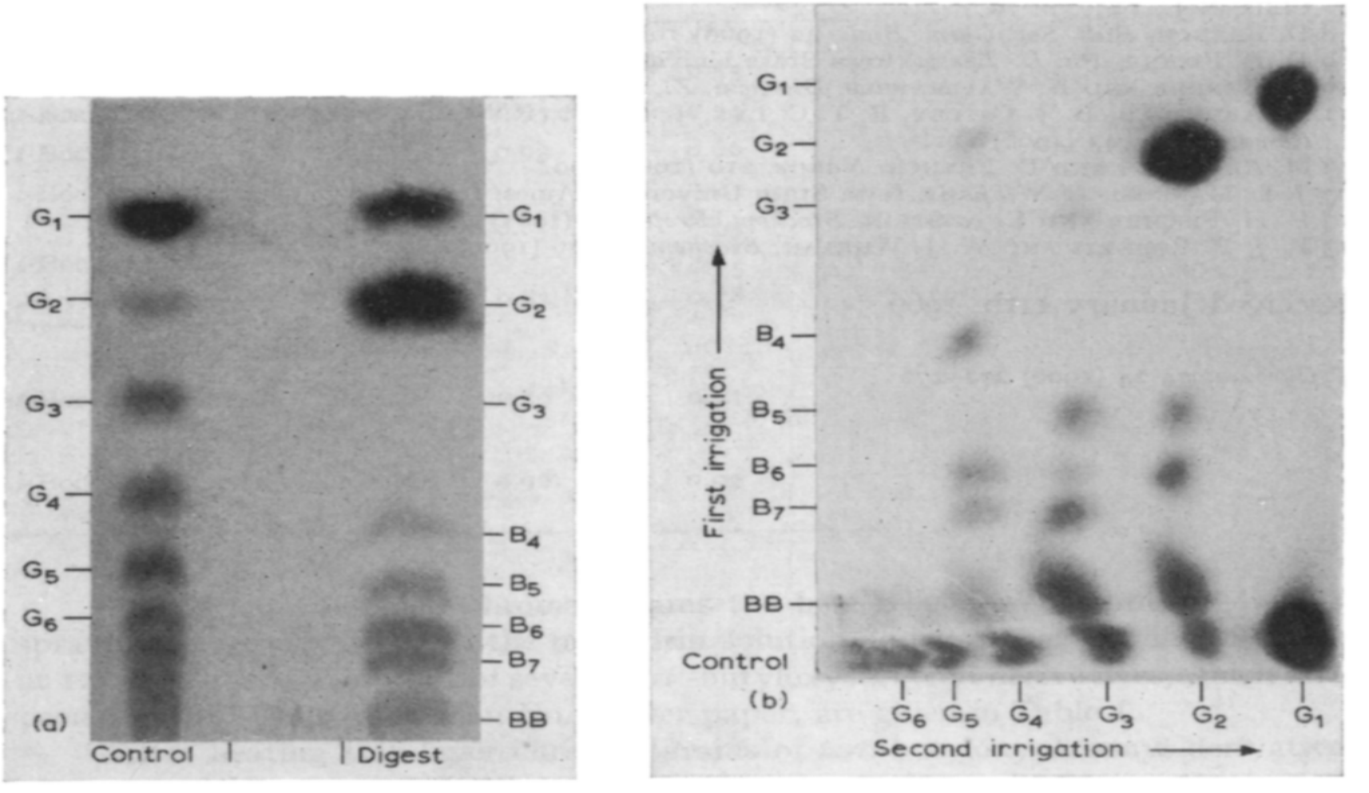


Fig. 5. (a) One-dimensional chromatogram of products of salivary amylase action on shellfish glycogen. B_4–B_7: singly-branched oligosaccharides; BB: doubly-branched oligosaccharides. Control contains only linear oligosaccharides. (b) Two-dimensional chromatogram showing products of pullulanase action on the sample of Fig. 5a.

and sprayed with a solution of pullulanase. After standing overnight in a damp atmosphere, the paper was dried and submitted to chromatography in the perpendicular direction (2 ascents). The results (Fig. 5b) indicating the susceptibility of the salivary amylase branched limit dextrins to pullulanase action as follows. All the singly and doubly branched oligosaccharides, except B_4 and a trace component of B_5, are degraded to form linear oligosaccharides, mainly maltose, maltotriose and maltotetraose. The doubly-branched oligosaccharides produce in addition small amounts of higher oligosaccharides in the range G_5–G_8 and possibly higher (unresolved in this chromatogram).

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