peptides, including glycylglycine, could also be detected after starch-gel electrophoresis in 1 *M* acetic acid at 40 V/cm, although the zones were up to 5 mm wide after ½ h.

I wish to thank Mr. M. FRENKEL for technical assistance.

*Division of Protein Chemistry, CSIRO,* R. L. DARSKUS
*Parkville (Melbourne), Victoria 3052 (Australia)*

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Received November 5th, 1970



CHROM. 5148

## Two dimensional paper chromatography for the detection of oxidation of maltose by glucose oxidase

The present report describes a novel paper chromatographic procedure for studying the action of glucose oxidase on glucosyl oligosaccharides. With this technique, it has been shown conclusively that maltose is oxidized to maltobionic acid by glucose oxidase. Other methods for following glucose oxidase action are based on oxygen uptake[1,2] or hydrogen peroxide production[3,4] and are not suitable for use with oligosaccharides since the primary carbohydrate products cannot be identified. Further, since glucose oxidase preparations may contain contaminating glucosidase and transferase activities[4], conversion of oligosaccharides to glucose followed by oxidation of glucose may occur and may be interpreted erroneously as indicative of oligosaccharide oxidation[5]. By separating the products of glucose oxidase action on maltose on paper chromatograms, then utilizing a specific glucosidase for hydrolyzing the products directly on the chromatogram, and finally separating the products of the glucosidase reaction by chromatography in a second direction, it has been shown that maltobionic acid is the carbohydrate product of glucose oxidase action on maltose.

*Materials and methods*

The maltose used in all experiments was prepared from amylose via β-amylase and was free of glucose[6]. Reference maltobionic acid was synthesized by oxidation of maltose with bromine[7]. The maltobionic acid thus produced was purified by a paper chromatographic procedure using the *n*-butanol–pyridine–water solvent system to remove unoxidized maltose. Glucose and gluconic acid were obtained from commercial sources. Glucose oxidase (EC 1.1.3.4), obtained from Miles Laboratories, was purified according to the procedure described by PAZUR AND KLEPPE[8]. This glucose oxidase preparation did not contain any contaminating α-glucosidase activity. Glucoamylase (α-1,4-glucan glucohydrolase, EC 3.2.1.3) was prepared according to the methods described by PAZUR AND ANDO[9].

Paper chromatography was carried out at room temperature using Whatman No. 1 filter paper. The two solvent systems employed in this study were *n*-butanol–pyridine–water, 6:4:3 by volume, and isobutyric acid–1 *N* $NH_4OH$, 10:6 by volume.

A typical incubation mixture for checking the activity of glucose oxidase on maltose contained 10 mg of maltose dissolved in 80 $\mu$l of 0.05 *M* sodium acetate buffer, pH 5.0, to which 20 $\mu$l of a solution of glucose oxidase (5.6 mg/ml) were added. A crystal of thymol was placed in the final solution to prevent bacterial contamination. After 24 h of incubation at room temperature, a 5 $\mu$l aliquot of the incubation mixture was placed on a 9 × 9-in. paper chromatogram and subjected to a single ascent in the *n*-butanol–pyridine–water solvent system followed by a second ascent in the same direction in an isobutyric acid–1 *N* $NH_4OH$ solvent system. The region of the chromatogram containing the maltose and maltobionic acid was then sprayed with a solution of glucoamylase (0.5 mg/ml). This area of the chromatogram was maintained in a moist condition for approximately 2 h by spraying the chromatogram every 15 min with the glucoamylase solution. The chromatogram was allowed to dry and

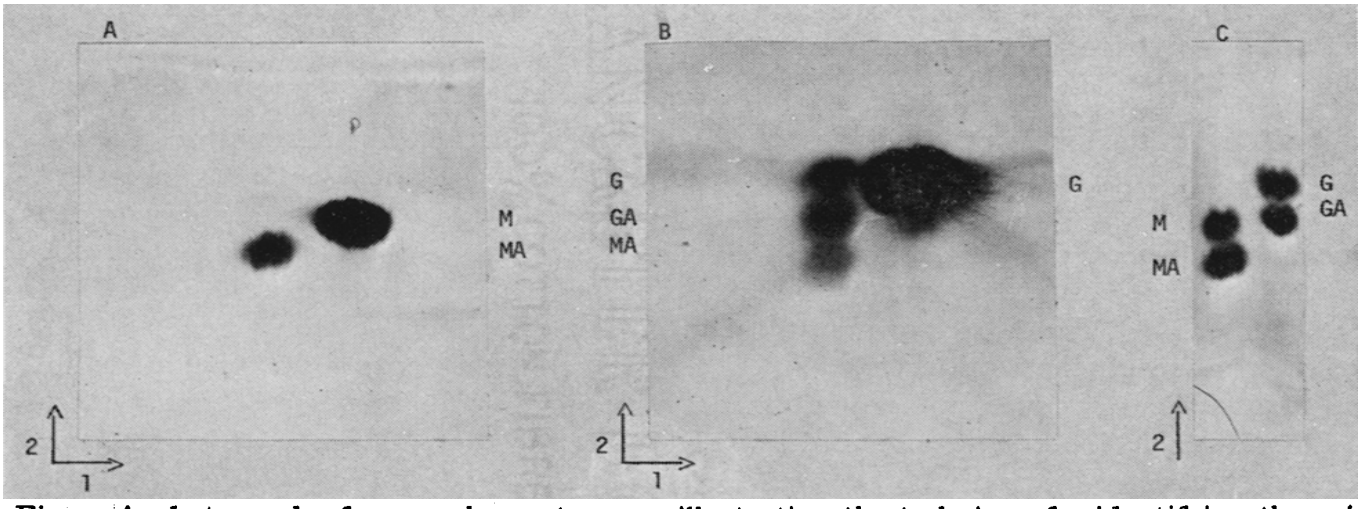


Fig. 1. A photograph of paper chromatograms illustrating the technique for identifying the primary carbohydrate product present in an incubation mixture of maltose and glucose oxidase. Part A of the figure illustrates the mobility of the carbohydrate components found in a 24-h digest. Part B shows the new carbohydrates that appear when the chromatogram is treated with glucoamylase before development in the second direction. Part C illustrates mobility in the second direction of pertinent reference compounds. Abbreviations are: G, glucose; GA, gluconic acid; M, maltose, and MA, maltobionic acid.

then subjected to two ascents in the second direction in the isobutyric acid–1 *N* $NH_4OH$ solvent system. The compounds were located on the chromatograms by a silver nitrate procedure[10] (see Fig. 1).

The acidic product obtained from oxidation of maltose with glucose oxidase was isolated by placing the total content of the final incubation mixture in a band along the base line of a chromatogram. The chromatogram was developed by 2 ascents in the *n*-butanol–pyridine–water solvent system. After detection by the silver nitrate procedure, two carbohydrate components were observed. One cochromatographed with maltose and the other, the product of the oxidation, cochromatographed with gluconic and maltobionic acids. The material cochromatographing with authentic

gluconic acid and maltobionic acid was eluted with water from the chromatogram. An aliquot of this material was placed as a single spot on a chromatogram. Glucoamylase (0.5 mg/ml) was spotted at the same location, and this area of the chromatogram was again maintained in a moist condition for 1 h by repeated applications of the glucoamylase solution. After spotting the appropriate reference compounds at different locations along the origin, the chromatogram was subjected to two passes in the isobutyric acid–1 *N* $NH_4OH$ solvent system. The compounds present on the chromatogram were again located by the silver nitrate procedure (see Fig. 2).

*Results and discussion*

The information presented in Figs. 1 and 2 clearly illustrates that glucose oxidase oxidizes maltose to maltobionic acid. The chromatograms reproduced in Fig. 1 illustrate that the product of the glucose oxidase reaction has the same $R_F$ value as maltobionic acid and a lower $R_F$ value than maltose or gluconic acid. Fig. 1B illustrates that the product is hydrolyzed by glucoamylase indicating an $\alpha$-glucosidic linkage. In addition, the products of the glucoamylase action on the compound co-chromatographed with glucose and gluconic acid. Enzymatic hydrolysis of maltobionic

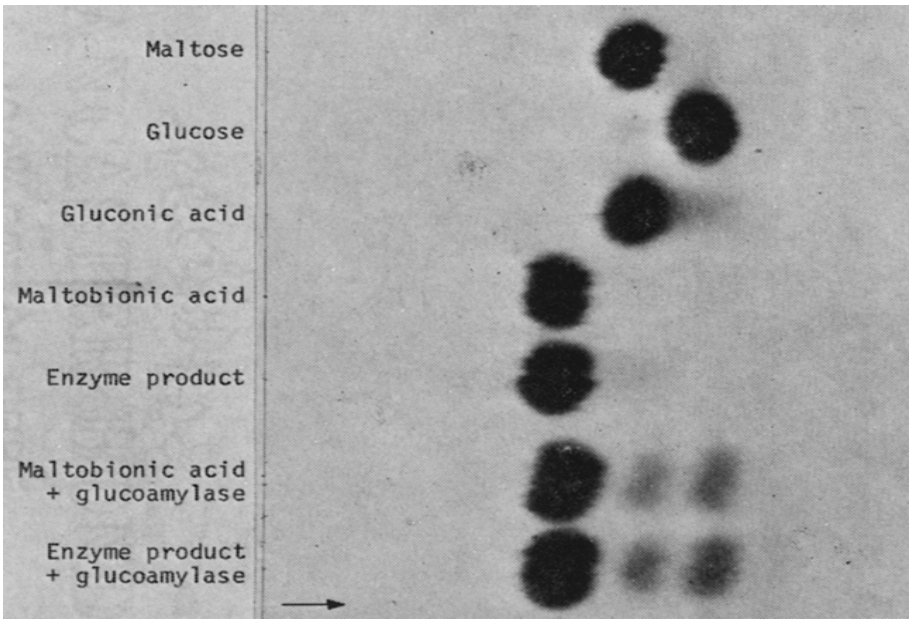


Fig. 2. A photograph of a paper chromatogram comparing both the relative mobilities of maltose, glucose, gluconic acid, maltobionic acid, and enzyme product, and the effect of glucoamylase on maltobionic acid and the enzyme product. Enzyme product refers to the product isolated from the maltose–glucose oxidase incubation mixture.

acid by glucoamylase has been demonstrated in an earlier report from this laboratory[11]. Utilizing this property of glucoamylase, further evidence demonstrating that maltobionic acid is the oxidation product of maltose is presented in Fig. 2. The chromatogram reproduced in this figure shows the mobility of different compounds that may be found in the assay mixture as well as the product of the glucose oxidase reaction. Also presented are results following treatment of the enzymatically produced maltobionic acid and chemically synthesized maltobionic acid with glucoamylase directly

on the chromatogram. In both cases, glucose and gluconic acid are the products of the hydrolase activity. The data in Fig. 2 also show that the rates of hydrolysis of maltobionic acid and the glucose oxidase product are very similar.

Thus, that maltobionic acid is the product of the action of glucose oxidase on maltose has been demonstrated by both cochromatography and by enzymatically characterizing maltobionic acid by direct application of glucoamylase to the chromatograms. The solvent systems selected for this study effected appropriate separation of the products, resulting in the reaction. The *n*-butanol–pyridine–water solvent system allowed the separation of unoxidized maltose from the possible oxidation products, maltobionic acid, and gluconic acid. Separation of maltobionic acid from gluconic acid was achieved using the isobutyric acid–1 *N* $NH_4OH$ solvent system. The inclusion of the enzymatic hydrolysis step directly on the chromatogram aided in the final characterization of the small amount of maltobionic acid present in the reaction mixture.

This work was supported in part by grants from the National Institutes of Health (AM-10822), Bethesda, Md. and the Corn Refiners Association, Washington, D.C.

*Department of Biochemistry,*
*Pennsylvania State University,*
*University Park, Pa. 16802 (U.S.A.)*

H. R. KNULL*
J. H. PAZUR

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Received October 19th, 1970

* Present address: Department of Biochemistry, Michigan State University, East Lansing, Mich. 48823, U.S.A.