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ELECTROCHEMICAL DETECTION OF REDUCING CARBOHYDRATES PRODUCED BY THE TRANSFERASE ACTION OF YEAST DEBRANCHING ENZYME ON MALTOSACCHARIDES

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ABSTRACT

A sensitive method for the detection of maltosaccharides up to maltoheptaose is based on an electrochemical detector using bis(1,10-phenanthroline)-copper(II) in the post column reaction after h.p.l.c. on an amino-bonded column. This method has been used for the analysis of the maltosyl and maltotriosyl transferase action of the yeast debranching enzyme with maltosaccharides as the substrates. The smallest donor substrate for maltosyl transfer was maltotetraose, and maltopentaose, maltohexaose, and maltoheptaose were donor substrates for both maltosyl and maltotriosyl transfers. Maltosyl residues were transferred in preference to maltotriosyl residues from maltopentaose, but maltotriosyl residues were transferred preferentially from maltohexaose and maltoheptaose. Maltotriose is an acceptor but not a donor of maltosyl and maltotriosyl transfers.

INTRODUCTION

Two types of yeast debranching enzyme systems involved in the degradation of glycogen and amylopectin have been found, namely, (*a*) direct debranching by isoamylase (EC 3.2.1.68)[1,2], which hydrolyses the branch point directly to release the linear α-(1→4)-linked exterior dextrin, and (*b*) an indirect debranching system that consists of two discrete enzymic activities, oligo-1,4→1,4-glucan transferase (EC 2.4.1.25) and amylo-1,6-glucosidase (EC 3.2.1.33)[3–6]. The oligo-1,4→1,4-glucan transferase transfers a maltotriose segment from the outer branches of glycogen phosphorylase limit-dextrin, exposing the (1→6)-linked α-D-glucosyl residue involved in the branch linkage. The amylo-1,6-glucosidase then removes this residue, producing a new chain that is susceptible to further action by phosphorylase.

The action of oligo-1,4→1,4-glucan transferase on maltosaccharides was shown by Lee *et al.*[4] to involve the transfer of a maltosyl segment from maltotetraose and maltopentaose, and both maltosyl and maltotriosyl segments from maltohexaose. However, these findings were not confirmed quantitatively.

Recently, a novel method for the detection of reducing carbohydrates, using

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h.p.l.c. with an electrochemical detector, has been developed[7,8]. This method is highly sensitive and can detect pmol of glucose by using bis(1,10-phenanthroline)copper(II) (CBP) as a mediator.

We now report on the application of this method in the analysis of the products obtained by the action of transferase on various maltosaccharides.

EXPERIMENTAL

Maltosaccharides. — D-Glucose–maltoheptaose (Nakarai Chemicals) were purified to >99.8% by h.p.l.c. on a column (19 × 150 mm) of BONDAPAK C18 (Waters).

D-[^{14}C]Glucose and [^{14}C]maltose were obtained from the Radiochemical Centre, Amersham. [^{14}C]Maltotriose (G–G–[^{14}C]G) was prepared from cyclomaltohexaose and D-[^{14}C]glucose[9].

Bis(1,10-phenanthroline)copper(II) (CBP). — An ethanolic solution of *o*-phenanthroline (0.02 mol in 30 mL) was added to a warm aqueous solution of copper(II) sulfate (0.01 mol in 30 mL). The product crystallised on cooling and was collected, washed with water and ethanol, and dried at 60° under vacuum[10].

H.p.l.c. — Sugars (5 μL, containing <500 ng) were separated on a column (3.9 × 150 mm) of μBONDASPHERE (5 μm, NH_2-100 Å, Waters) with acetonitrile–water (60:40) at 0.3 mL/min.

The reagent solution, containing mM CBP in 0.1M disodium hydrogenphosphate (pH 11.0 adjusted with 2M NaOH) as the supporting electrolyte, was delivered at 0.6 mL/min and mixed with the column eluate. A PTFE tube (5 m × 0.5 mm i.d.) was used as the reaction coil, which was kept at 98 ±0.1° in a Temperature Control System (Waters). The mixture was passed through a cooling coil (PTFE tube, 50 cm × 0.5 mm i.d.) and dipped in water before reaching the electrochemical detector (ECD M460, Waters). For quantitative analysis, 5 μL of the sample, which was diluted with water containing D-glucose as internal standard, was injected.

Preparation of debranching enzyme. — *Saccharomyces cerevisiae* D-346[11] (ATCC 56960) was grown on glucose medium (peptone, 1%; yeast extract, 1%; D-glucose, 3%) and the cells were harvested by centrifugation. The cells were lysed with Zymolyase 100T (Kirin Brewery Co.), and the debranching enzyme was purified by the method of Lee *et al.*[4] with slight modifications involving column chromatography on DEAE-cellulose, DEAE-Sephadex A-50, and hydroxyapatite (Bio Rad), and gel filtration using Bio-gel P-300 (Bio Rad).

The amylo-1,6-glucosidase activity was measured by the release of D-glucose from glycogen phosphorylase limit-dextrin[4]; 1 unit of activity represents the amount of the enzyme that catalyses the release of 1 μmol of D-glucose per min.

The purified enzyme (~1 mg) obtained from 200 g of yeast cell (wet weight) corresponded to an overall yield of 10%, and the specific activity of 6.8 units/mg was an ~1,000-fold increase over the lysate. The purified enzyme migrated as a single band in polyacrylamide gel electrophoresis, using a gel concentration of 7.5%[12].

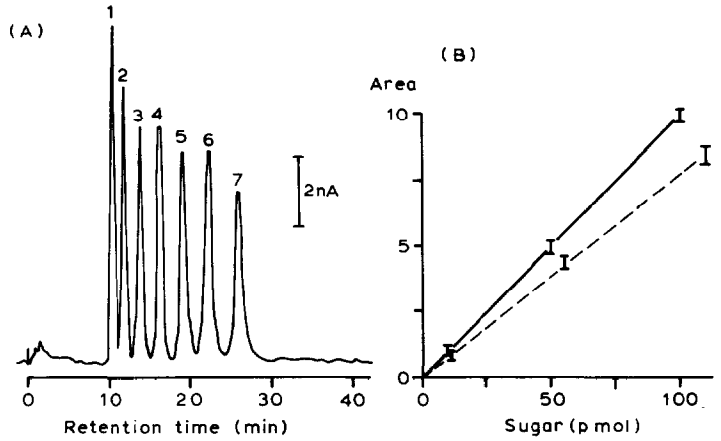

Fig. 1. A, H.p.l.c. (see Experimental) of an authentic mixture of reducing sugars: peaks 1, glucose (50 pmol); 2, maltose (50), 3, maltotriose (50); 4, maltotetraose (55); 5, maltopentaose (55); 6, maltohexaose (60); 7, maltoheptaose (55). B, Calibration curves for D-glucose (——) and maltoheptaose (– – –).

RESULTS

H.p.l.c. of maltosaccharides. — Fig. 1A shows a typical chromatogram for the series D-glucose–maltoheptaose (50 pmol of each; 5 μL of solution injected). The rate of reduction of CBP diminished with increasing size of the maltosaccharide. The sensitivity of maltoheptaose corresponded to ~80% of that of D-glucose. Fig. 1B shows the calibration curve for D-glucose and maltoheptaose, which was almost linear in the range 5–100 pmol.

Analysis of the products formed by the action of oligo-1,4→1,4-glucan transferase on maltosaccharides. — Each maltosacchride (5mM) was incubated at 30° with the enzyme (0.003 unit of amylo-1,6-glucosidase) in 10mM phosphate buffer (pH 6.5).

The enzyme did not act on maltose and maltotriose during 24 h.

Fig. 2 shows the chromatograms of products in the early stages of reactions when each maltosaccharide was incubated with the enzyme. For maltotetraose, maltose and maltohexaose were detected after incubation for 10 h, indicative of the transfer of maltosyl units (Fig. 2a). For maltopentaose, maltohexaose, and maltoheptaose, the products formed by the transfer of maltosyl and maltotriosyl units appeared in the early stages of the reactions (Figs. 2b–d).

Products that were one glucose residue larger or smaller than the substrate did not appear in the early stages of the reactions, indicating that a glucosyl transfer did not occur.

The quantitative data are shown in Table I. The transferase acts on maltotetraose to give equal amounts of maltose and maltohexaose, indicative of a maltosyl transer. The rate of transfer from maltotetraose was ~5% of that for maltopentaose. Maltose, maltotriose, maltoheptaose, and malto-octaose had been

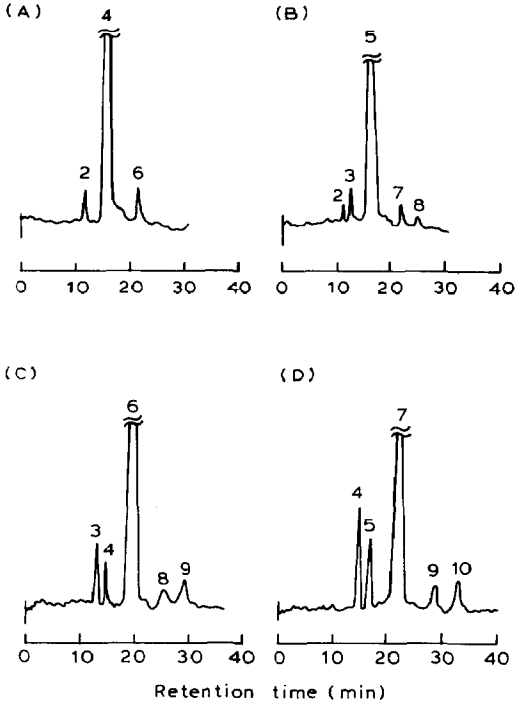

Fig. 2. H.p.l.c. of the products from the action of the transferase on maltosaccharides. Each maltosaccharide (5mM) was incubated in 20mM phosphate buffer (pH 6.5) with the enzyme (0.003 unit of amylo-1,6-glucosidase) at 30° for 1 h (10 h for maltotetraose). The reaction was terminated by adding 1 vol. of acetonitrile, followed by centrifugation at 10,000 r.p.m. The supernatant solution was diluted with water and 5 μL was used for h.p.l.c. Substrates: (A) maltotetraose, (B) maltopentaose, (C) maltohexaose, (D) maltoheptaose; peaks 2–10 indicate maltose–maltodecaose.

formed from maltopentaose after incubation for 1 h. The amount of maltotriose produced from maltopentaose was equivalent to that of maltoheptaose, and maltose was detected in amounts equal to that of malto-octaose. The numerical results in Table I indicate the rate of transfer of maltosyl units to be twice that for maltotriosyl units. Maltotriose, maltotetraose, malto-octaose, and maltononaose had been formed from maltohexaose after incubation for 1 h, indicative of the transfer of both maltosyl and maltotriosyl units, and a similar result was obtained with maltoheptaose.

For maltohexaose and maltoheptaose, the transfer of maltotriosyl units preponderated over maltosyl units. The relative rates of transferase actions were calculated from the sum of the maltosyl and maltotriosyl units transferred (Table I). The higher maltosaccharides are superior substrates.

Acceptor substrates for the transferase. — Maltotetraose was found to be the smallest donor substrate. Maltose and maltotriose, which were not donor substrates, were tested as acceptor substrates. When maltopentaose (4 mg/mL) and each radioactive sugar (D-[^{14}C]glucose, [^{14}C]maltose, or [^{14}C]maltotriose, 1–3

TABLE I

TRANSFERASE ACTION ON VARIOUS MALTOSACCHARIDES[a]

Substrate	*Products (nmol)*[b]									*Relative rate*[c] *(nmol.min^{-1})*
	G2	*G3*	*G4*	*G5*	*G6*	*G7*	*G8*[d]	*G9*[d]	*G10*[d]	
G4	11				12					4.6
G5	6.3	13				12	6.5			80.4
G6		22	13				15	21		146
G7			32	20				21	29	217

[a]Each substrate (1 μmol, maltotetraose–maltoheptaose) was incubated in 20mM phosphate buffer (pH 6.5) with the enzyme (0.004 unit of amylo-1,6-glucosidase for maltopentaose–maltoheptaose, 0.04 unit for maltotetraose) at 30° for 1 h (total volume of 100 μL). The reaction was stopped by the addition of 1 vol. of acetonitrile, and a solution of D-glucose was added as internal reference. H.p.l.c. was performed as described in Fig. 2. The values are expressed as nmol/h of maltosaccharide transferred. [b]G2, maltose; G3, maltotriose; *etc.* [c]The relative rate was expressed as the sum of maltosyl and maltotriosyl transfers per unit of amylo-1,6-glucosidase. [d]These values were determined by using the correction factors of 1.03, 1.07, and 1.12 for each maltosaccharide as G7.

mg/mL) were incubated with the transferase, p.c. showed that D-glucose and maltose could not accept any maltosyl or maltotriosyl residues from maltopentaose, even after incubation for 24 h. With [^{14}C]maltotriose, radioactive maltopentaose and maltohexaose appeared after incubation for 1 h, indicative of the transfer of maltosyl and maltotriosyl units from maltopentaose to radioactive maltotriose (Fig. 3).

DISCUSSION

There are two types of yeast debranching enzymes which act on glycogen, namely, (*a*) isoamylase [amylopectin 6-glucanohydrolase (EC 3.2.1.9)[1,2]], which acts directly on the α-(1→6) branch linkages of glycogen to release a (1→4)-α-D-glucosyl chain, and (*b*) amylo-1,6-glucosidase/oligo-1,4→1,4-glucan transferase[3–6]. The main difference in debranching action between types (*a*) and (*b*) is the release of oligosaccharide in the former or D-glucose as the sole low-molecular-weight carbohydrate in the latter. Moreover, only type (*b*) has glucan transferase action. The purified debranching enzyme used in this study possesses maltosyl and maltotriosyl transferase activity for maltosaccharide and amylo-1,6-glucosidase activity for glycogen phosphorylase limit-dextrin; therefore, the enzyme belongs to amylo-1,6-glucosidase/oligo-1,4→1,4-glucan transferase group as reported by Lee *et al.*[3,4]. The purified enzyme could not hydrolyse maltose and *p*-nitrophenyl α-D-glucopyranoside (a substrate for α-D-glucosidase), and there was no increase in reducing power during transfer action on maltosaccharides. Therefore, the enzyme preparation was free from α-D-glucosidase (maltase).

In order to clarify the specificity of the transferase, carefully purified maltosaccharides were used as substrates. It is possible that a maltosaccharide produced

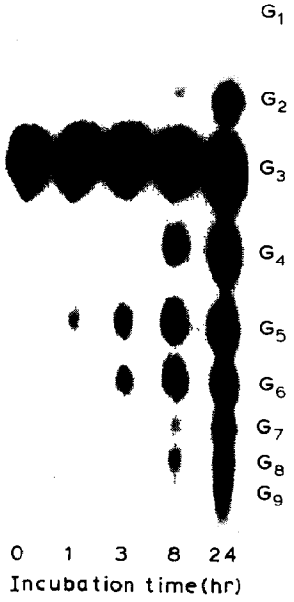

Fig. 3. Autoradiogram of maltosaccharides produced by the action of the transferase on a mixture of maltopentaose and [^{14}C]maltotriose. The mixture of maltopentaose (1.25 mg) and [^{14}C]maltotriose (G–G–[^{14}C]G, 0.75 mg, 6×10^4 c.p.m.) was incubated with the enzyme (0.06 unit of amylo-1,6-glucosidase) at 30° in 10mM phosphate buffer (pH 6.0; total volume, 290 μL). At the time indicated, a portion (15–20 μL) of the mixture was subjected to descending p.c. [3 developments, TOYOROSHI No. 51 paper, 1-propanol–water (7:3), room temperature]. Fuji X-ray film was exposed to the paper chromatogram for 2 weeks. G1–G9, glucose–maltononaose.

by a transfer reaction could also be a substrate for the enzyme. However, when <10% of the substrate had been consumed, no maltosaccharides arising from the product could be detected. Thus, the purified enzyme had a transferase activity towards both maltosyl and maltotriosyl residues, with the higher maltosaccharide being the superior substrate. Maltotriose was not a donor but an acceptor for both maltosyl and maltotriosyl units.

Although the transfer activity on maltoheptaose was the highest of the maltosaccharides tested, it was ~20% of that for glycogen phosphorylase limit-dextrin (Table I).

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REFERENCES

1 B. MARUO AND T. KOBAYASHI, *Nature (London)*, 167 (1951) 606–607.
2 G. N. BATHGATE AND D. J. MANNERS, *Biochem. J.*, 107 (1968) 443–445.

3 E. Y. C. LEE, L. D. NIELSEN, AND E. H. FISCHER, *Arch. Biochem. Biophys.*, 121 (1967) 245–246.
4 E. Y. C. LEE, J. H. CARTER, L. D. NIELSEN, AND E. H. FISCHER, *Biochemistry*, 9 (1970) 2347–2355.
5 E. Y. C. LEE AND J. H. CARTER, *Arch. Biochem. Biophys.*, 154 (1973) 636–641.
6 E. Y. C. LEE AND J. H. CARTER, *FEBS Lett.*, 32 (1973) 78–80.
7 N. WATANABE AND M. INOUE, *Anal. Chem.*, 55 (1983) 1016–1019.
8 N. WATANABE, *J. Chromatogr.*, 330 (1985) 333–338.
9 J. H. PAZUR AND T. ANDO, *Methods Carbohydr. Chem.*, 1 (1961) 285–288.
10 B. J. HATHAWAY, I. M. PROCTER, R. C. SLADE, AND A. A. G. TOMLISON, *J. Chem. Soc., A*, (1969) 2219–2224.
11 S. TABATA, T. IDE, Y. UMEMURA, AND K. TORII, *Biochim. Biophys. Acta*, 797 (1984) 231–238.
12 J. L. HEDRICK AND A. J. SMITH, *Arch. Biochem. Biophys.*, 126 (1968) 155–164.