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Note

Post-column oligosaccharide dehydrogenase reactor for coulometric detection of malto-oligosaccharides in a liquid chromatographic system

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Malto-oligosaccharides (MOSs) can be separated successfully on cation- [1–3] and anion-exchange [4] resins and chemically bonded stationary phases with amino [5] or carbamoyl [6] groups. MOSs, which bear a free formyl group, can be detected with high sensitivity and selectivity with the aid of electrochemical detectors [3–5].

An immobilized enzyme reactor is suitable for use in the post-column reaction system because of its high selectivity [7]. By coupling liquid chromatography (LC) with electrochemical detection to the immobilized enzyme reactor, further selectivity can be achieved. An immobilized glucoamylase reactor has been used in a post-column reaction system for the determination of MOSs with pulsed amperometric detection [8].

Oligosaccharide dehydrogenase (ODH) catalyses the oxidation of the glucose unit of MOSs, producing a glucono-lactone end [9]. The reaction catalysed by the enzyme is [10]

$$(\text{glucose})_n + \text{A} = (\text{glucose})_{n-1}\text{-glucono-}\delta\text{-lactone} + \text{AH}_2$$

where $n \geqslant 1$, A is a hydrogen acceptor such as phenazine methosulphate, 1-methoxyphenazine methosulphate, 1-acetamidephenazine methosulphate, Meldola blue, phenylindophenol or 2,6-dichlorophenylindophenol and AH_2 is the reduced form of the acceptor.

This paper describes a post-column immobilized ODH reactor system coupled to anion-exchange chromatography and coulometric detection for the determination of MOSs up to maltoheptaose. The MOSs from the separation column are mixed with 1-methoxyphenazine methosulphate (MPS) solution and oxidized in an immobilized

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ODH reactor. MPS is reduced to $MPSH_2$ according to the amount of MOSs present in the solution. The $MPSH_2$ is monitored by coulometric detection.

EXPERIMENTAL

Reagents

D-Glucose (G_1), D-maltose (G_2), maltotriose (G_3), maltotetraose (G_4), maltopentaose (G_5), maltohexaose (G_6) and maltoheptaose (G_7) were obtained from Nakalai Tesque (Kyoto, Japan), 1-methoxyphenazine methosulphate (MPS) from Dojindo (Kumamoto, Japan) and oligosaccharide dehydrogenase (ODH, from *Staphylococcus* sp., 130 U/mg protein) from Toyo Jozo (Tokyo, Japan). Poly(vinyl alcohol) beads (9 μm, GS-520) were purchased from Asahi Kasei (Tokyo, Japan). TSK gel SAX (5 μm) (Tosoh, Tokyo, Japan) was packed into a stainless-steel column (30 cm × 5.8 mm I.D.). The counter electrode electrolyte was potassium hexacyanoferrate(II)–potassium hexacyanoferrate(III)–potassium nitrate–potassium hydroxide solution with a concentration of 0.1 *M* of each component.

The mobile phases were 0.2 *M* sodium acetate–0.1 *M* sodium hydroxide solution and 0.3 *M* sodium acetate–0.1 *M* sodium hydroxide solution. The mobile phases were deaerated. All other reagents were of analytical-reagent grade.

Immobilization of ODH

The method for the preparation of the aminated poly(vinyl alcohol) beads was similar to that of Matsumoto *et al.* [11]. The beads were packed into a stainless-steel column (5 cm × 4 mm I.D.) by the slurry-packing method. Glutaraldehyde solution (4.0%) in 0.1 *M* sodium hydrogencarbonate was pumped through the column for 2 h at 0.3 ml/min and then the column was washed with deaerated water for 30 min at 0.5 ml/min. The enzyme solution [5 mg in 10 ml of 0.05 *M* phosphate buffer (pH 7.0)] was circulated through the column at 0.3 ml/min for 4 h at room temperature. The enzyme solution was kept at about 4°C throughout the procedure.

LC system

The liquid chromatographic system is shown in Fig. 1. It consisted of a mobile phase pump (Model 655; Hitachi, Tokyo, Japan), a pulse damper (Model LOD-1; Gasukuro Kogyo, Tokyo, Japan), an injector with a 50-μl loop (Model SVI-5U7;

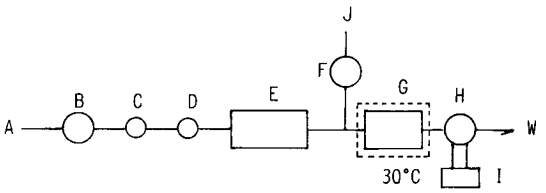

Fig. 1. Flow diagram of liquid chromatographic system for coulometric detection of malto-oligosaccharides with an immobilized enzyme column reactor. A = Mobile phase (0.1 *M* sodium hydroxide–0.2 *M* sodium acetate or 0.1 *M* sodium hydroxide–0.3 *M* sodium acetate, 0.8 ml/min); B = LC pump; C = damper; D = injector with 50-μl loop; E = analytical column (30 cm × 5.8 mm I.D., TSK gel SAX, 5 μm); F = reagent pump; G = reactor (5 cm × 4 mm I.D.); H = coulometric monitor; I = data processor; J = reagent solution (25 m*M* MPS in 0.5 *M* acetic acid, 0.2 ml/min); W = waste.

Sanuki Kogyo, Tokyo, Japan), a separation column containing TSK gel SAX (5 μm) (30 cm × 5.8 mm I.D.), a double plunger reagent pump (Model DMX-2200; Sanuki Kogyo), the column reactor (5 cm × 4 mm I.D.) packed with the immobilized enzyme, a coulometric monitor (Model 655A-26; Hitachi) and a data processor (Chromatocorder II; System Instruments, Tokyo, Japan). The column reactor was kept at 30°C.

The mobile phase and deaerated reagent solution consisting of 0.5 *M* acetic acid containing 25 m*M* MPS were pumped at 0.8 and 0.2 ml/min, respectively, and mixed before the column reactor. Enzymatic reaction proceeded in the reactor and the $MPSH_2$ produced was monitored coulometrically at an electrolytic potential of 0.6 V *vs.* $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$. At the electrolytic potential, the electrolytic efficiency for $MPSH_2$ reached 100% and the background current was moderately small (0.5 μA).

RESULTS AND DISCUSSION

Properties of immobilized ODH column reactor

To evaluate the immobilized enzyme reactor, G_5 was selected as a model substrate and the experiments were carried out in the flow-injection mode by omitting the separation column.

The influence of pH on the enzyme activity was studied over the range 7.0–9.0 using sodium acetate (0.3 *M*) and acetic acid. As shown in Fig. 2, the optimum pH was about 8.0. The electrolytic efficiency for $MPSH_2$ was 100% in the pH range 7.0–9.0.

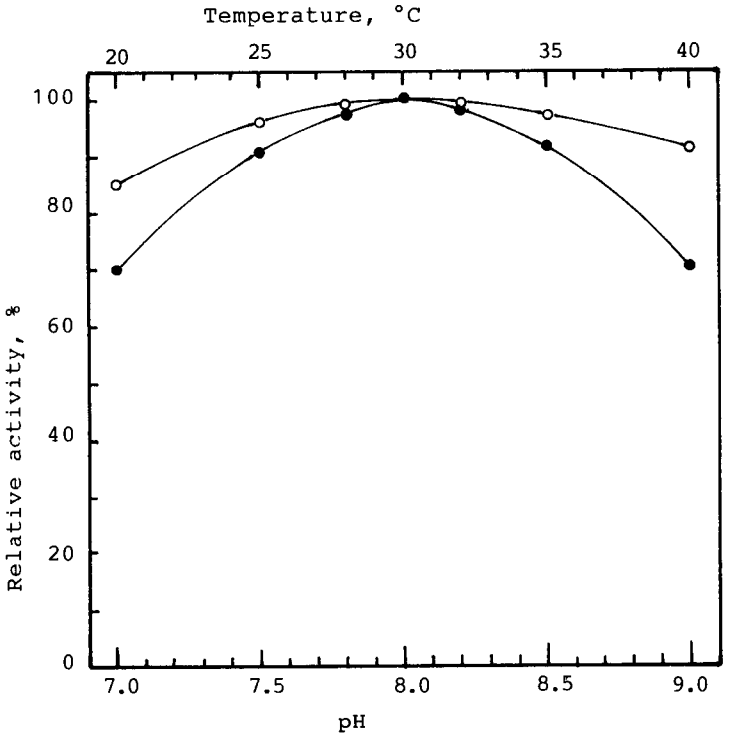

Fig. 2. Effects of (○) pH and (●) temperature on the activity of the immobilized enzyme.

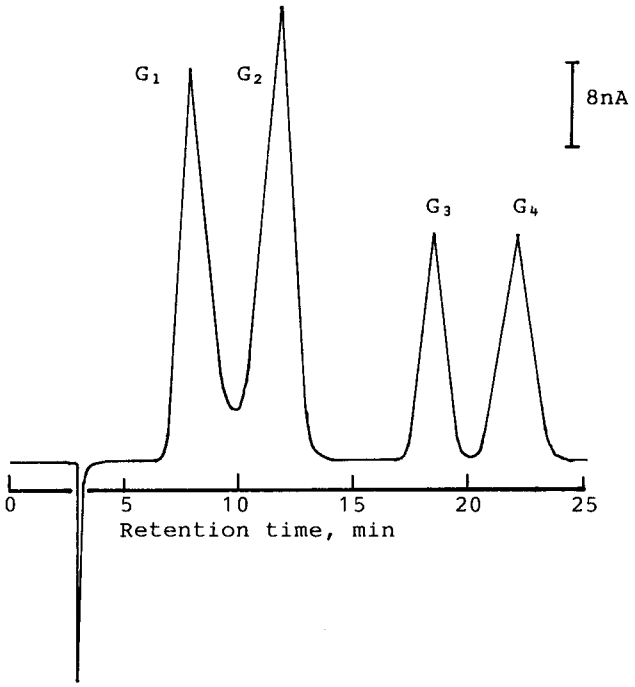

Fig. 3. Chromatogram of a standard mixture of glucose (G_1), maltose (G_2), maltotriose (G_3) and maltotetraose (G_4) (each 500 pmol). A TSK gel SAX column (30 cm × 5.8 mm I.D.) was used at room temperature with 0.1 *M* sodium hydroxide–0.2 *M* sodium acetate as the mobile phase at a flow-rate of 0.8 ml/min.

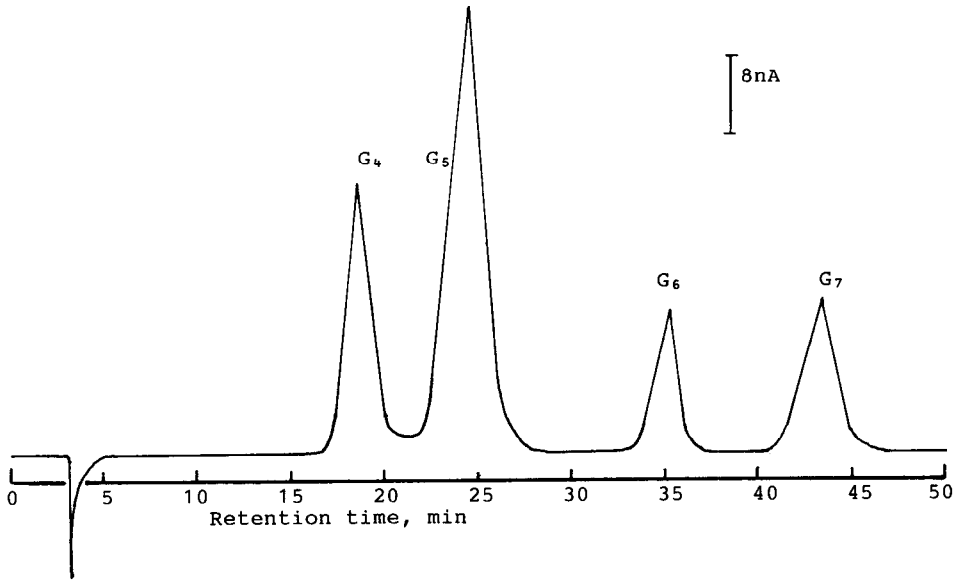

Fig. 4. Chromatogram of a standard mixture of maltotetraose (G_4), maltopentaose (G_5), maltohexaose (G_6) and maltoheptaose (G_7) (each 500 pmol). Mobile phase, 0.1 *M* sodium hydroxide–0.3 *M* sodium acetate at a flow-rate of 0.8 ml/min.

The activity in buffer solutions such as Tris–HCl, tricine–NaOH, bicine–NaOH and phosphate at pH 8.0 was identical with that in sodium acetate–acetic acid. In the buffers containing borate the activity was about one tenth of that in sodium acetate–acetic acid. The activity of the immobilized enzyme at various temperatures was examined. The maximum activity was observed at 30°C, as shown in Fig. 2.

The optimum concentration of MPS was investigated. The response increased linearly with increasing concentration of MPS and became constant at above 5 m*M* for 1 m*M* G_5 at a flow-rate of 1.0 ml/min. Considering the stoichimetry between MOSs and MPS (1:1), it will be safe to use five times the level of the MOS concentration. The respone decreased linearly as the flow-rate increased from 0.5 to 2.0 ml/min. To confirm the operational stability of the reactor, it was used for 10 h in a day and stored at 4°C in 0.1 *M* phosphate buffer (pH 7.0) when not in use. The reactor retained more than 80% of its original activity after 1 month. Storage stability was also examined. The enzyme was lyophilized and stored at −20°C in the presence of glycine (0.1 g/g of the immobilized enzyme). The activity was 95% of the initial value after 2 months.

ODH catalyses the oxidation of MOSs, other oligosaccharides and monosaccharides. The substrate specificity toward saccharides was examined. The immobilized enzyme was active towards G_1 (relative activity 100), G_2 (112), G_3 (70), G_4 (71), G_5 (59), G_6 (65), G_7 (53), xylose (39), galactose (93), mannose (34) and lactose (105). With 0.3 *M* sodium acetate–acetic acid (pH 8.0) and 5 m*M* MPS at a flow-rate of 1.0 ml/min at 30°C, 1 m*M* D-glucose (50 μl) was oxidized to a lactone in 24% yield.

Separation of MOSs by LC

As the immobilized ODH was deactivated in aqueous acetonitrile (30%, v/v) or methanol (3%, v/v) and borate was undesirable for the enzymatic reaction, an anion-exchange column with sodium acetate solution as mobile phase was used in an attempt to separate the MOSs at room temperature. Separation of mixtures of G_1, G_2, G_3 and G_4 and of G_4, G_5, G_6 and G_7 was effected on a TSK gel SAX column with 0.2 *M* sodium acetate–0.1 *M* sodium hydroxide and 0.3 *M* sodium acetate–0.1 *M* sodium hydroxide, respectively, as mobile phase as shown in Figs. 3 and 4. An effective separation of individual members from G_1 to G_7 was achieved using gradient elution, accomplished by maintaining the sodium hydroxide concentration at 0.1 *M* and increasing the sodium acetate concentration from 0.01 to 0.6 *M* in 20 min. Gradient elution caused serious problems with regard to the operational stability of the immobilized enzyme. The immobilized enzyme was almost completely deactivated after repeating the gradient ten times. The separation parameters for the MOSs are listed in Tables I and II. The flow-rate of the mobile phase was kept constant at 0.8

TABLE I

SEPARATION PARAMETERS FOR GLUCOSE, MALTOSE, MALTOTRIOSE AND MALTOTETRAOSE

Saccharide	Abbreviation	Capacity factor	Resolution	Separation factor
Glucose	G_1	1.66	1.36	1.81
Maltose	G_2	3.00	2.47	1.72
Maltotriose	G_3	5.16	1.37	1.24
Maltotetraose	G_4	6.38		

TABLE II

SEPARATION PARAMETERS FOR MALTOTETRAOSE, MALTOPENTAOSE, MALTOHEXAOSE AND MALTOHEPTAOSE

Saccharide	Abbreviation	Capacity factor	Resolution	Separation factor
Maltotetraose	G_4	4.85		
Maltopentaose	G_5	6.72	1.62	1.39
Maltohexaose	G_6	10.08	3.08	1.50
Maltoheptaose	G_7	12.67	2.37	1.26

ml/min. The flow-rate of the reagent solution influenced the band broadening and the conversion efficiencies. An increase in the flow-rate of the reagent solution from 0.2 to 0.5 ml/min resulted in a decreased response due to a lower residence time in the column reactor and hence in a lower conversion efficiency. At lower flow-rates, from 0.05 to 0.1 ml/min, serious problems arose with respect to reproducibility because of incomplete mixing of the reagent solution with the mobile phase. The flow-rate of the reagent solution was set to 0.2 ml/min. At this flow-rate, the contribution of the post-column reactor system to band broadening was less than 25% of the peak width. Concentrations of 25 m*M* MPS and 0.5 *M* acetic acid in the reagent solution were chosen, resulting in an MPS concentration of 5 m*M* and pH 8 in the column reactor.

The peak areas were plotted against the concentration of the MOSs. Calibration graphs were prepared for G_1, G_2, G_3, G_4, G_5, G_6 and G_7, covering the range 0.01–1 m*M*. The ratio of peak areas for G_1, G_2, G_3, G_4, G_5, G_6 and G_7 was 100:127:50:87:163:40:49. Detection limits (signal-to-noise ratio = 3) for G_1, G_2, G_3, G_4, G_5, G_6 and G_7 were 0.5 (4.5), 0.5 (8.6), 0.7 (18), 0.7 (23), 0.4 (17), 2.0 (99) and 3.0 μM (173 ng per 50-μl injection), respectively. The detection limit (0.5 μM) for G_1 by this method is equal to that reported previously for glucose using anion-exchange chromatography with pulsed amperometric detection [12].

The immobilized ODH column reactor is concluded to be useful for the coulometric detection of trace amount of MOSs. MOSs have been determined amperometrically by anion-exchange chromatography using immobilized glucoamylase post-column [8]. The detection limit for G_7 was 6.8 μM. This method is more sensitive (3.0 μM).

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