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Column liquid chromatographic determination of saccharides with a single calibration graph using post-column enzyme reactors and coulometric detection

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ABSTRACT

Immobilized enzymes were used as column reactors in a column liquid chromatographic system for the specific detection of the saccharides stachyose, raffinose and sucrose. Invertase and fructose dehydrogenase (FDH) were immobilized onto poly(vinyl alcohol) beads and porous glass beads, respectively. The oligosaccharides were separated on a cation-exchange resin column with water as the mobile phase. Invertase was capable of quantitatively hydrolysing the oligosaccharides to fructose, which reacts with the hexacyanoferrate(III) ion in the presence of FDH. The hexacyanoferrate(II) ion produced was monitored coulometrically. A single calibration graph for fructose based on the peak area was used to determine each oligosaccharide. The limits of detection for stachyose, raffinose and sucrose were 27, 5 and 2 ng (in a 50 μl sample), respectively.

INTRODUCTION

High-performance liquid chromatography (HPLC) is a useful technique for determining saccharides. Several HPLC methods have been reported, most of which make use of aminopropyl, C_{18} or ion-exchange stationary phases [1]. The detection methods used have been studied with the aim of improving the sensitivity; pulsed amperometric detection (PAD) has recently been used for the sensitive determination of saccharides in HPLC column effluents [2–5]. The sensitivity of PAD for individual members of a homologous series of oligosaccharides increases with increasing molecular mass. The immobilized glucoamylase reactor has been used as a post-column reactor for the determination of malto-oligosaccharides using a single calibration graph for glucose [6].

Invertase (β-D-fructofuranosyl fructohydrolase, EC 3.2.1.26) catalyses the hydrolysis of oligosaccharides with a terminal unsubstituted β-D-fructofuranosyl resid-

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ue to produce free D-fructose. An immobilized invertase reactor has been used for the flow-injection determination of sucrose [7,8]. Fructose dehydrogenase (FDH) [D-fructose:(acceptor) 5-oxidoreductase, EC 1.1.99.11] has been used for the specific determination of D-fructose in seminal plasma [9]. The enzyme was also immobilized and used for the amperometric flow-injection determination of fructose in foods [10,11].

In this work immobilized invertase and FDH reactors were used in tandem with cation-exchange chromatography and coulometric detection for the determination of oligosaccharides such as sucrose, raffinose and stachyose using a single calibration graph for fructose. Each saccharide was hydrolysed by the invertase and the fructose produced reacted with the hexacyanoferrate(III) ion in the immobilized FDH reactor as follows

$$\text{D-Fructose} + 2Fe(CN)_6^{3-} = \text{5-keto-D-fructose} + 2Fe(CN)_6^{4-}$$

The hexacyanoferrate(II) ion produced was monitored coulometrically.

EXPERIMENTAL

Chemicals

Invertase (from *Candida utilis*, 500 U mg^{-1}) was obtained from Sigma (St. Louis, MO, USA). FDH (from *Gluconobacter sp.*, 30 U mg^{-1} was obtained from Toyobo (Osaka, Japan). Fructose, sucrose, raffinose, stachyose, sodium gluconate and gluconic acid were from Nacalai Tesque (Kyoto, Japan). Poly(vinyl alcohol) beads (GS-520, 9 μm) were from Asahi Kasei (Tokyo, Japan). Long-chain amino-CPG (pore size, 50 nm; amount of amine, 83 μ equiv. g^{-1}; particle size, 200/400 mesh) was from Electro-Nucleonics (Fairfield, NJ, USA). The mobile phase was water and the reagent solution consisted of 0.2 *M* sodium gluconate–gluconic acid buffer (pH 5.0) and 40 m*M* potassium hexacyanoferrate(III). The counter electrode electrolyte was a potassium hexacyanoferrate(II)–potassium hexacyanoferrate(III)-potassium nitrate–potassium hydroxide solution with a concentration of 0.1 *M* of each component. All other chemicals were of analytical-reagent grade.

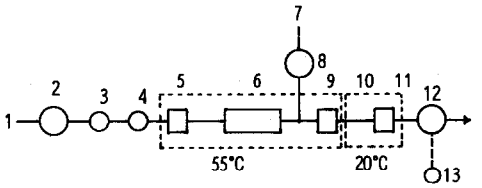

Fig. 1. Schematic diagram of a column liquid chromatographic system for the determination of stachyose, raffinose, sucrose and fructose. 1 = Mobile phase (water); 2 = pump (0.5 ml min^{-1}); 3 = damper; 4 = injector with 50-μl loop; 5 = guard column (TSKgel SCX, 5 μm, 3 cm × 7.8 mm); 6 = analytical column (TSKgel SCX, 5 μm, 60 cm × 7.8 mm); 7 = reagent solution (40 m*M* $K_3[Fe(CN)_6]$ in 0.2 *M* gluconate buffer (pH 5.0); 8 = pump (0.2 ml min^{-1}); 9 = immobilized invertase reactor (5 cm × 4 mm); 10 = cooling coil (1 m × 0.25 mm); 11 = immobilized fructose dehydrogenase reactor (5 cm × 4 mm); 12 = coulometric monitor; 13 = data processor.

Apparatus

A schematic diagram of the chromatographic configuration used in this work is shown in Fig. 1. The chromatographic system consisted of a mobile phase pump (L-6000, Hitachi, Tokyo, Japan), a pulse damper (LOD-1, Gasukuro Kogyo, Tokyo, Japan), an injection valve (7125, Rheodyne, Cotati, CA, USA) with a 50-μl loop, a guard column (30 × 7.8 mm I.D., stainless-steel), an analytical column (600 × 7.8 mm I.D., stainless-steel), a reagent solution pump (75613, Gaskuro Kogyo), two immobilized enzyme reactors (each 50 × 4 mm I.D., stainless-steel column), a coulometric monitor (655A-26, Hitachi) and a data processor (Chromatocorder II, System Instrument, Tokyo, Japan). The chromatographic columns were filled with TSKgel SCX (H^+ form) (5 μm) (Tosoh, Tokyo, Japan). The columns and the immobilized invertase reactor were kept at 55°C in a column oven and the cooling coil (1 m × 0.25 mm I.D., stainless-steel tube) and the immobilized FDH reactor were maintained at 20°C in a water-bath.

The mobile phase and the reagent solution were pumped at 0.5 and 0.2 ml min^{-1}, respectively, and mixed before entering the immobilized invertase reactor. The hydrolysis of the oligosaccharides took place in the reactor and D-fructose was produced. The fructose and hexacyanoferrate(III) reacted in the immobilized FDH reactor and the hexacyanoferrate(II) produced was monitored coulometrically at an electrolytic potential of 200 mV *versus* $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$. At the electrolytic potential, the electrolytic efficiency for hexacyanoferrate(II) reached 100%, and the background current was negligibly small.

Preparation of enzyme reactors

Invertase was immobilized onto the poly(vinyl alcohol) beads. The method of amination of the beads was similar to that used by Matsumoto *et al.* [12]. The attached amine was immobilized at a concentration of 3.2 mequiv. g^{-1} dry beads. The aminated beads were packed into a column (50 × 4 mm I.D.) by the slurry-packing method. Glutaraldehyde solution (4%) in 0.1 *M* sodium hydrogencarbonate solution was pumped through the column for 2 h at 0.2 ml min^{-1} and the column was washed with deaerated water for 15 min at 0.5 ml min^{-1}. Invertase solution [100 mg in 10 ml of 0.05 *M* phosphate buffer solution (pH 7.0)] was circulated through the column at 0.3 ml min^{-1} for 10 h at room temperature. Invertase was immobilized with a 70% yield.

FDH was immobilized onto the CPG column. FDH was dissolved at a concentration of 1 mg ml^{-1} in 0.1 *M* gluconate buffer solution (pH 5.0) and 0.1% Triton X-100. An aliquot (10 ml) of this solution was added to 0.8 g of the CPG support which was activated by glutaraldehyde and shaken for 3 h at 15°C. FDH was immobilized with a 10% yield. The immobilized FDH reactor was prepared by slurry-packing the FDH-linked support into a column (50 × 4 mm I.D.).

RESULTS AND DISCUSSION

Evaluation of enzyme reactors

The properties of immobilized FDH were first evaluated without using the guard and analytical columns nor the immobilized invertase reactor. The influence of pH on the enzymatic reaction was studied over the pH range 4.0–6.0. A standard

solution of D-fructose (50 μM) was injected onto the column and mixed with potassium ferrocyanate(III) solution buffered with 0.1 M gluconate at various pH values before the reaching reactor column. The optimum pH for the enzymatic reaction was about 5.0. The reactor was placed in a water-bath and the temperature was varied between 10 and 40°C. The reactor exhibited the highest activity at 20°C. The effect of the concentration of the hexacynoferrate(III) ion in the reactor was examined in the concentration range 5–20 mM. The peak height was constant at concentrations greater than 10 mM. The apparent Michaelis constant of the immobilized FDH for the hexacyanoferrate(III) ion was about 0.8 mM.

The peak height decreased linearly as the flow-rate was increased from 0.5 to 1.0 ml min^{-1}. With 10 mM hexacyanoferrate(III) in 0.1 M gluconate buffer solution (pH 5.0) at 20°C and a flow-rate of 0.7 ml min^{-1}, 100 μM D-fructose was converted by FDH with a 10% yield. The reactor was used for 8 h per day and was stored at 4°C in 0.1 M phosphate buffer solution (pH 7.0) when not in use. The activity remained at 80% of the initial value for 4 weeks. The plot of peak area against concentration was linear from 0.05 to 400 μM. Above 500 μM the plot began to curve. The apparent Michaelis constant for D-fructose was about 5.8 mM.

The immobilized invertase reactor was used in the flow injection analysis mode by omitting the guard and analytical columns to evaluate the efficiency of fructose production from each oligosaccharide at various temperatures. The reactor was placed in an oven and the temperature varied from 30 to 70°C in the presence of 10 mM hexacyanoferrate(III) in 0.1 M gluconate buffer solution (pH 5.0) at a flow-rate of 0.7 ml min^{-1}. The peak area of the liberated fructose was determined by injecting oligosaccharide standards (10 μM). The molar ratio of fructose produced from the oligosaccharide injected was used to calculate the reaction efficiency. An efficiency of 100% was obtained in the range 40–70°C for sucrose, 45–65°C for raffinose and 52–68°C for stachyose. A reaction temperature of 55°C was chosen for the immobilized invertase reactor. The efficiencies for sucrose, raffinose and stachyose were maintained at a constant value for 30, 15 and 8 days, respectively.

Separation of the saccharides

The separation of mixtures of fructose, sucrose, raffinose and stachyose was carried out by anion-exchange chromatography on a TSK gel SAX column (5 μm, 30 cm × 6 mm) with 0.1 M borate buffer (pH 7.5 at 70°C) or 0.1 M sodium hydroxide–0.2 M sodium acetate solution (at 50°C) as the mobile phase. The borate buffer interfered with the enzymatic reaction owing to the formation of complexes with the saccharides. The reproducibility of the analyses with the sodium hydroxide–sodium acetate mobile phase was poor as a result of the incomplete mixing of the mobile phase with the flow of the make-up solution. A cation-exchange resin column (TSK gel SCX, 5 μm, 600 × 7.8 mm I.D., H-form) was used in an attempt to separate the saccharides at 55°C with water as the mobile phase. A stable and reproducible chromatogram (peak areas) was obtained, as shown in Fig. 2. The peak area for stachyose, raffinose or sucrose was identical to that of fructose (19.3 μC for 10^{-9} mol of fructose). This made it possible to determine the saccharides using the single fructose calibration graph. As the peak area is independent of the peak dispersion which occurs within the guard and analytical columns and the immobilized enzyme reactors, and as it is also independent of the sample dilution, this method is valid only when

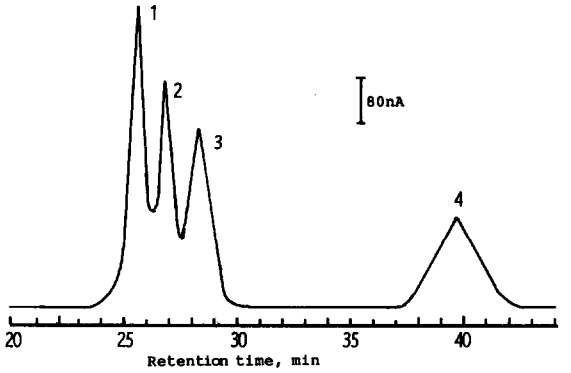

Fig. 2. Chromatogram of a standard mixture of 20 μM each of (1) stachyose, (2) raffinose, (3) sucrose and (4) fructose. Sample size 50 μl.

peak areas (coulomb) are used. The relative accuracy of the method is shown in Table I.

The peak area was plotted against the concentration of saccharides. The concentration ranges of linear response for fructose, sucrose, raffinose and stachyose were from 0.1 to 800 μM, 0.5 to 600 μM, 1.0 to 500 μM and 2.0 to 450 μM, respectively. The lower limit of the calibration graph for the oligosaccharides was governed by the efficiency of the hydrolysis. The slope of the graph was 0.965 ± 0.01 $\mu C\ \mu M^{-1}$ and the correlation coefficient was 0.9998 (21 points). The detection limits (signal-to-noise ratio 3) for fructose, sucrose, raffinose and stachyose were 0.08 (0.7), 0.1 (2), 0.3 (5) and 0.5 μM (27 ng in a 50-μl injection), respectively.

Application

This system was used to determine the amount of each saccharide found in a soybean extract. The sample was prepared by aqueous extraction according to the procedure of Kennedy *et al.* [13]. The sample was analysed under the same conditions as those described in the caption of Fig. 1. A single calibration graph for fructose was used to determine the amounts of fructose, sucrose raffinose and stachyose which

TABLE I

ACCURACY OF DETERMINATION OF THE SACCHARIDES IN THE STANDARD MIXTURE

Saccharide	Concentration in mixture (μM)	Apparent concentration (μM)[a]	Relative accuracy (%)
Fructose	200	201(2.8)[b]	0.5
Sucrose	200	199(2.1)	−0.5
Raffinose	100	101(2.0)	1.0
Stachyose	100	103(2.5)	3.0

[a] Calculated from the areas of the peaks using the calibration graph of fructose concentration *versus* fructose peak area.

[b] Values are means with the coefficient of variation (%) (n = 5) in parentheses.

TABLE II

RESULTS FOR FRUCTOSE, SUCROSE, RAFFINOSE AND STACHYOSE IN SOYBEAN EXTRACT

Saccharide	Saccharides found (%)[a]	
	Proposed method	Gas chromatography [14]
Fructose	0.50(3.5)[b]	0.48(5.4)[b]
Sucrose	5.87(2.4)	5.35(5.7)
Raffinose	0.87(3.8)	0.86(8.6)
Stachyose	3.88(2.2)	3.86(7.1)

[a] Percentage meal dry weight.
[b] Values are means with the coefficient of variation (%) (n = 5) in parentheses.

were present in the sample. The results obtained by this method and by gas chromatography [14] of the trimethylsilyl oxime derivatives of the saccharides are shown in Table II.

It is concluded that the immobilized invertase and FDH reactors are useful for the coulometric determination of sucrose, raffinose and stachyose using the single calibration graph for fructose based on peak area. This method is specific for oligosaccharides, but is not superior to anion-exchange chromatography with PAD [15] in terms of the time of analysis and limit of detection. It should be noted that the immobilized invertase reactor should be renewed every 8 days, and that this is a disadvantage of the proposed technique.

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