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Note

A post-column co-immobilized galactose oxidase/peroxidase reactor for fluorometric detection of saccharides in a liquid chromatographic system

NOBUTOSHI KIBA*, KAZUYOSHI SHITARA and MOTOHISA FURUSAWA
Department of Chemistry, Faculty of Engineering, Yamanashi University, Takeda 4-3-11, Kofu 400 (Japan)
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Liquid chromatographic determination of trace amounts of saccharides typically needs derivatization to improve the detectability due to their low UV-absorptivities. Pre-column derivatization of saccharides, followed by reversed-phase chromatography is a sensitive method[1–4]. However, quantitation is one of the problems associated with the methods.

Post-column derivatization has been employed for the quantitative analysis of saccharides and a number of different fluorescent reagents have been examined for reducing saccharides[5–10]. However, very few fluorescent reagents are known for non-reducing saccharides[11,12].

Galactose oxidase (D-galactose:oxygen oxidoreductase, E.C. 1.1.3.9) (Gal-OD) catalyzes the oxidation of galactose by molecular oxygen. Oligosaccharides containing galactose such as raffinose and stachyose are oxidized much more rapidly than galactose itself. Gal-OD has been used as a reagent for the determination of galactose[13–16]. It has also been immobilized on glass beads and used as a reactor for the determination of galactose in flow injection analysis[17–19].

This paper describes the use of immobilized enzyme as a column reactor in a liquid chromatographic system for the specific detection of stachyose, raffinose, melibiose and galactose. Gal-OD and peroxidase (doner:hydrogen peroxide oxidoreductase, E.C. 1.11.1.7) (POD) were co-immobilized onto hydrophilic vinyl polymer beads. The saccharides were separated on a cation-exchange resin column with water as the mobile phase. In the column reactor, the saccharides were converted into hydrogen peroxide by Gal-OD, which reacts with *p*-hydroxyphenyl acetate in the presence of POD, and the fluorophore produced was detected. The fluorophore (6,6′-dihydroxy-3,3′-biphenyldiacetate) is formed in the following reaction sequence:

$$\text{D-Galactose} + O_2 \xrightarrow{\text{Gal-OD}} \text{D-galatohexodialdose} + H_2O_2$$
$$H_2O_2 + p\text{-hydroxyphenylacetate} \xrightarrow{\text{POD}} H_2O + \text{fluorophore}$$

This method is sensitive and highly specific for the detection of stachyose, raffinose, melibiose and galactose.

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EXPERIMENTAL

Reagents

D-galactose, D-melibiose, α-lactose, D-raffinose and stachyose tetrahydrate were obtained from Nakarai Tesque (Kyoto, Japan), galactose oxidase (from Dactylium sp., 28 U/mg of solid) and peroxidase (from horseradish, 266 U/mg) from Toyobo (Osaka, Japan). TSK gel Toyopearl HW-55S (20 − 40 μm, hydrophilic vinyl polymer beads) was obtained from Tosoh (Tokyo, Japan).

The purity of the *p*-hydroxyphenylacetic acid (HPA) had a large influence on the background signal. HPA was purified as follows. A 10-g amount was dissolved in 50 ml of aceton–benzene (10:3, v/v). The solution was passed through a column (10 cm × 1 cm I.D.) of activated alumina (*ca.* 200 mesh). The eluate was added dropwise to a mixture of *n*-hexane–benzene (5:5, v/v)(50 ml). The solution was left overnight at room temperature. The precipitated HPA was filtered off and dried under reduced pressure. The purified HPA (0.23 g) was dissolved in water (30 ml), adjusted to pH 7.5 with sodium hydroxide and then diluted in phosphate buffer (0.1 *M*, pH 7.5) to 100 ml. The solution was made up freshly every week. Stock solutions (0.1 *M*) of saccharides were prepared in water and allowed to mutarotate to anomeric equilibrium before use. All other chemicals were of analytical reagent grade.

Selection of support

Hydrophilic vinyl polymer beads (TSK gel Toyopearl HW-55S, 20 − 40 μm), polystyrene beads (Bio-Beads SX-8, 55 ± 20 μm) and glass beads (CPG-10, 500A, 55 ± 20 μm) were examined as supports for covalent attachment of the enzymes. Each support was aminoalkylated by several methods[20–22] and the amounts of amino group attached were measured by Kjeldahl method[23]: 0.6, 0.8 and 0.05 mequiv. per g of dry vinyl polymer beads, polystyrene beads and glass beads. The amounts grafted onto the glass beads were too little to co-immobilize the enzymes. The column reactor (50 mm × 4.6 mm I.D.) which was packed with the enzymes–polystyrene conjugates gave a pronounced peak tailing because of the adsorption of the fluorophore. The vinyl polymer beads gave a sharp peak and were chosen as the support.

Preparation of enzyme column reactor

The method of epoxy activation and amination of the vinyl polymer beads was similar to that of Matsumoto *et al.*[20]. The aminated polymer beads were packed into a stainless-steel column (50 mm × 4.6 mm I.D.) by the slurry-packing method. Glutaraldehyde solution (2.5%) in phosphate buffer (0.1 *M*, pH 7.0) was pumped through the column for 1 h at 0.3 ml/min and then the column was washed with deaerated water for 20 min at 0.5 ml/min. The Gal-OD solution (5 mg in 10 ml of phosphate buffer (0.05 *M*, pH 7.0) was circulated through the column for 1 h at 0.2 ml/min at room temperature and then, after addition of POD (5 mg) to the solution, the circulation was allowed to continue for 4 h. During the immobilization procedure the solution was kept at below 4°C.

System

The system is shown schematically in Fig.1. The apparatus consisted of a mobile phase pump, Hitachi 655A, an injector with an 100-μl loop, Rheodyne 7125, a

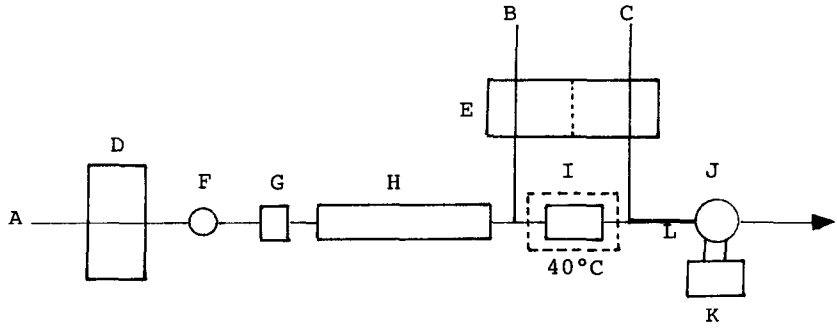

Fig. 1. A liquid chromatographic system for fluorometric detection of saccharides with an immobilized enzyme column reactor. A = Mobile phase (water, 0.5 ml/min); B = reagent solution (15 m*M* *p*-hydroxyphenylacetic acid, pH 7.5, 0.5 ml/min); C = buffer solution (0.3 *M* glycine-sodiumchloride/sodium hydroxide, pH 10.8, 0.25 ml/min); D = LC pump; E = reagent pump (double-plunger type); F = injector with 100-μl loop; G = guard column (50 mm × 6 mm); H = analytical column (60 cm × 7.8 mm); I = reactor (50 mm × 4.6 mm); J = fluorescence spectrophotometer; K = recorder; L = mixing tube (50 cm × 0.5 mm).

guard column of Shodex Ionpak KS800P (50 mm × 6 mm I.D.), a separation column (600 mm × 7.8 mm) of TSK gel SCX (5 μm), a reagent pump (double-plunger type), Kyowa Seimitsu KHU-W-52, a PTFE mixing coil (50 cm × 0.5 mm I.D.), a fluorescence spectrophotometer with a flow cell (18 μl), Hitachi 650-10s, and a strip-chart recorder, TOA FBR 251A.

The sample solution (100 μl) was injected onto the separation column with water as the mobile phase (0.5 ml/min). The temperature of the separation column was ambient. The HPA solution (15 m*M*) was added to the eluate at flow-rate of 0.5 ml/min. The mixture then passed into the column reactor, which was thermostatted at 40°C. Enzymatic reactions of saccharides and of hydrogen peroxide proceeded in the reactor, and the fluorophore produced was mixed with the buffer (0.3 *M* glycine–sodium chloride/sodium hydroxide, pH 10.8) (0.25 ml/min) in a mixing tube and was monitored at 410 and 330 nm.

RESULTS AND DISCUSSION

Experiments were conducted to optimize the reaction conditions, without the guard and separation columns. The influence of pH on the enzymatic reaction was studied over the range 6.0–8.0. A standard solution of stachyose (0.1 m*M*) was injected and mixed with HPA solution buffered with 0.1 *M* phosphate of various pH values before the reactor and then the stream emerging from the reactor was mixed with glycine sodium chloride/sodium hydroxide buffer (pH 10.8). The optimum pH for the enzymatic reactions was about 7.5. The reactor was placed in a water-bath, the temperature was varied between 30 and 50°C and a standard solution of 0.1 m*M* stachyose was injected at each temperature. The reactor exhibited the highest activity at 40°C. The effect of the concentration of HPA was examined in the range of 1–10 m*M*. The peak height was constant above 6 m*M*. Michaelis constants of Gal-OD for galactose, melibiose, raffinose and stachyose are 0.3, 0.05, 0.03, and 0.02 *M* respectively[24]. Below the concentration of 0.1 m*M* saccharides the rate of the enzymatic

reaction (peak height) is directly proportional to the concentration. The peak height decreased linearly with increasing flow-rate from 0.8 to 1.5 ml/min. Lower flow-rates were preferable to higher ones for analytical sensitivity, but the peak broadening at lower flow-rates was undesirable. The fluorescence of the fluorophore emerging from the reactor was measured at pH from 9.0 to 12.0, with glycine–sodium chloride/sodium hydroxide buffer. The fluorescence was optimal at pH 10.8. Under the conditions of 7.5 m*M* HPA in phosphate buffer at pH 7.5 and 40°C the relative peak heights for stachyose, raffinose, melibiose, galactose and lactose were 240, 228, 88, 100 and 1, respectively. Under the same conditions, 100 μM stachyose was converted by Gal-OD into hydrogen peroxide in only 1.0% yield. By using this enzyme reactor, it was difficult to detect trace amounts of α-lactose. There were no peaks for D-glucose, D-mannose, D-fructose, D-tagatose, D-lyxose, D-ribose, D-xylose, maltose and sucrose.

Separation of mixtures of galactose, melibiose, raffinose and stachyose into their components was effected by anion-exchange chromatography on a column (TSK gel SAX, 5 μm, 300 mm × 6 mm I.D.) with 0.1 *M* borate pH 7.5 as the mobile phase at 70°C. However, the reproducibility of the chromatogram obtained was poor. A cation-exchange resin column (TSK gel SCX, 5 μm, 600 mm × 7.8 mm I.D.) was used in an attempt to separate the saccharides with water as the mobile phase at room temperature. A stable chromatogram was obtained, as shown in Fig.2. The separation of stachyose and raffinose was incomplete. The ratio of peak heights for stachyose, raffinose, melibiose and galactose was 445:336:182:100. In this liquid chromatographic system the band width of each peak was dominated by the separation

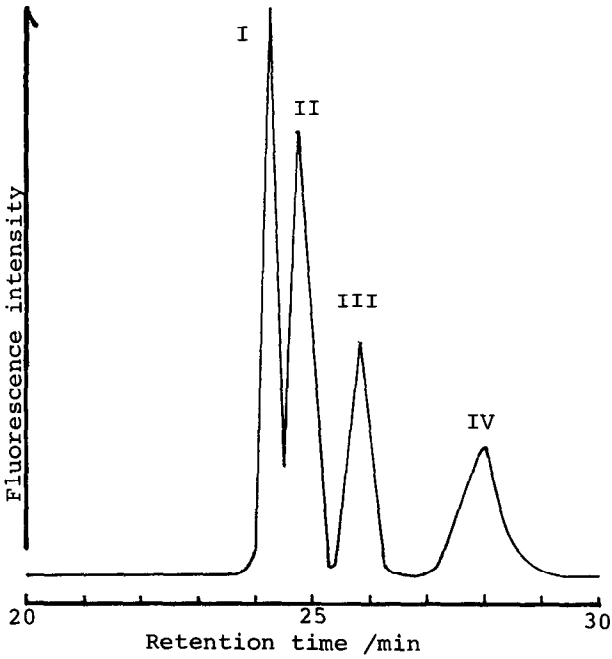

Fig. 2. Chromatogram of a standard solution: 0.01 m*M* each of stachyose (I), raffinose (II), melibiose (III) and galactose (IV).

column and the contribution of the post-column reaction system to the peak broadening was less than 40% in peak width. In the post-column reaction system, the peak broadening was due predominantly to the addition of HPA solution. Increasing the concentration of the HPA-solution and decreasing the flow-rate of the solution caused serious problems in reproducibility because of incomplete mixing of the solution with the mobile phase (water).

The peak height was plotted against the concentrations of the saccharides. The calibration graphs were linear from 100 to 5 μM for stachyose and raffinose and 100 to 10 μM for melibiose and galactose. The detection limits were 1.0, 1.0, 2.0 and 5.0 μM for stachyose, raffinose, melibiose and galactose, respectively.

The column reactor was used for 8 h in a day and stored at 4°C in 0.1 M phosphate buffer pH 7.0 when not in use. It retained more than 60% of its original activity after 6 weeks.

In conclusion, the co-immobilized Gal-OD/POD reactor is useful for the fluorometric detection of trace amounts of stachyose and raffinose. Stachyose and raffinose have been detected fluorometrically by anion-exchange chromatography using post-column derivatization with taurine-periodate[12]. The detection limits were 3 μM for each. This method is more sensitive (1 μM) and specific for the oligosaccharides.

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