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High-performance liquid chromatography for the assessment of glucoamylase, immobilized on metallic membranes

H. J. WANG* and R. L. THOMAS
223 Poole Agricultural Center, Department of Food Science, Clemson University, Clemson, SC 29634-0371 (U.S.A.)

ABSTRACT

High-performance size-exclusion chromatography was used to quantitate the amount of glucoamylase (GA) immobilized on various membrane materials. The amount of GA immobilized ranged from 0.39 to 1.90 g/ft^2 of membrane. A Dextro-Pak C_{18} plastic cartridge in a radial compression module was used to evaluate the reaction products of the immobilized GA, using 1.0% dextrin as a substrate. The low-molecular-weight oligo-saccharides (degree of polymerization 1–10) in 24.0 and 1.0% dextrin and in the membrane permeates were identified and quantitated in 25 min. High-performance liquid chromatography analyses were demonstrated to be easier and more sensitive than conventional protein assays and colorimetric sugar analyses.

INTRODUCTION

Immobilization techniques have been introduced into the enzyme industries to increase product quality and reduce cost [1,2]. Various protein assays were used to estimate the amount of enzyme adsorbed on the carriers. The Lowry method [3] was used to determine the amount of glucoamylase (GA) immobilized on silanized aluminium oxide [4] and on polyvinylpyrrolidone gel [5] for the saccharification of starch and the digestion of cassava solution. Bio-Rad protein assay [6] was used by McKamy [7] for the determination of GA adsorbed on a metallic membrane for the hydrolysis of dextrin. However, these protein assays could only approximate the amount of enzyme actually immobilized. Therefore, high-performance size-exclusion chromatography (HPSEC) was used for the determination of the specific amount of GA immobilized on a metallic membrane with simultaneous estimation of the molecular weight of GA.

In addition, due to the increasing interest regarding the individual sugar level of starch hydrolyzates, high-performance liquid chromatography (HPLC) techniques have also become essential methods for the determination of sugar concentrations instead of conventional colorimetric assays, which can only measure total carbohydrate. A radially compressed C_{18} cartridge, having the capability of resolving oligomers up to a degree of polymerization (DP) of 14, was used for the investigation of partially hydrolyzed starch [8]. Recently, a Waters Dextro-Pak plastic C_{18} cartridge

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was used to analyze oligosaccharides of DP 1–10 in hydrolyzed starch [9], corn syrup solids [10], and maltodextrins [10,11]. Separation of the saccharides was based on the chain length [12].

EXPERIMENTAL

Materials

Glucoamylase from Rhizopus mold (mixture of *exo*-1,4-D-glucosidase, EC 3.2.1.3) with an activity of 12 000 units/g was purchased from Sigma (St. Louis, MO, U.S.A.). Type I dextrin was also purchased from Sigma. Maltose, maltotriose, and β-D-glucose were obtained from Fisher Scientific (Dallas, TX, U.S.A.).

The metallic membrane system, the membrane coating materials {designated WD1, WD2, WD1 + WD2, F1, ZOSS (hydrous zirconium oxide) and ZOPA [hydrous zirconium oxide poly(acrylic acid)]} and the membrane-coating techniques were provided by DuPont Separation Systems (Seneca, SC, U.S.A.). WD1, WD2, WD1 + WD2 and F1 were coated on porous, stainless-steel tubes 5 ft. × 1.25 in. I.D. (152 cm × 3.18 cm I.D.). The composition of these membrane materials is proprietary for test materials of DuPont Separation Systems [11]. ZOSS and ZOPA were coated on 2 ft. × 0.625 in. I.D. (61 cm × 1.59 cm I.D.) porous, stainless-steel tubes. Each stainless-steel tube was installed in a sintered, stainless-steel shell [13]. The ZOPA membrane was selected for dextrin hydrolysis by immobilized GA.

Methods

The immobilization of GA was carried out according to the procedures of McKamy [7]. A 10-l volume of 0.1% (w/v) GA in 0.05 *M* sodium acetate buffer at pH 5.0 was recirculated through each membrane material which had been coated on a porous, stainless-steel tube. The maximum GA immobilization on the membrane was achieved after 20 min at room temperature. The amount of GA immobilized on the membrane was determined from the decrease of its concentration in the buffer solution by the HPSEC method. A 10-l volume of distilled water, adjusted to pH 5.0 was recirculated through the membrane to remove not-immobilized GA. Finally, a 10-l volume of 1.0% dextrin in distilled water, adjusted to pH 5.0, was recirculated through the ZOPA membrane to the feed tank at 300 p.s.i. pressure. The permeates (dextrin hydrolyzates) were collected at various times.

The GA concentration was determined with a Beckman TSK 3000SW column (300 × 7.5 mm), connected to a TSK precolumn (75 × 7.5 mm). The mobile phase was a 0.1 *M* potassium phosphate buffer (pII 6.0), which had been filtered through a 0.45-μm filter and degassed under vacuum. GA standard was prepared by dissolving GA in the phosphate buffer to yield a 1.0 mg/ml solution. Each GA sample was passed through a 0.45-μm filter before HPSEC analysis. A 20-μl sample of GA solution was eluted at a flow-rate of 0.5 ml/min and detected with a Waters Model 440 absorbance detector at 280 nm. The GA concentration was calculated with reference to peak-height standard curves, which were integrated with a Waters Model 740 data module, attenuated at 128×. The GA concentration was also determined by the Bio-Rad protein assay [6] using GA as a standard.

Low-molecular-weight (DP 1–10) oligosaccharides were resolved by HPLC using a 10 cm × 8 mm I.D. C_{18} cartridge installed in a rubber sleeve wihtin a RCM-100

radial compression module which was described by Fallick and Rausch [14]. The rubber sleeve is surrounded by a hydraulic fluid (glycerine), pressure is generated by moving three lever-driven pistons into the glycerine. The pressure is transmitted through the flexible sleeve to the wall of the cartridge, as well as to the particles of the packed bed. A modified inlet connector was used to fit the Guard-Pak precolumn inserts directly into the Radial-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.). HPLC-grade water as the mobile phase was filtered through a 0.22-μm filter and degassed under vacuum. Sugar standards for DP 1, 2 and 3 were prepared from 1.0 mg/ml glucose, maltose and maltotriose, respectively. Standard solutions were used to determine the retention time and concentration. The sugars were passed through a 0.45-μm filter prior to injection. The concentration of each saccharide (DP 4–10) was determined relative to a glucose standard [9]. A 15-ml volume of the dextrin hydrolyzate was eluted with water at a flow-rate of 1.0 ml/min and detected with a Waters Model 410 differential refractive index detector.

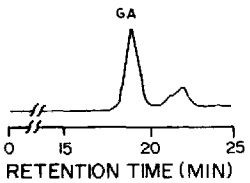


Fig. 1. Chromatogram of 1.0 ml/mg GA solution.

RESULTS AND DISCUSSION

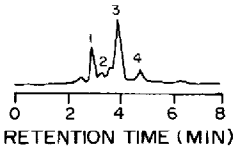
A chromatogram for the analysis of GA is shown in Fig. 1. The first peak is ascribed to GA and the other peaks represents protein contaminants in the enzyme preparation. These contaminants can be detected by conventional colorimetric protein assays. Therefore, separation and quantitation of GA by HPSEC allowed the quantitation of GA alone.

The amount of GA immobilized on various membrane materials is shown in Table I. The variations among membrane materials were no doubt caused by the

TABLE I

QUANTITATION OF GA IMMOBILIZED ON VARIOUS MEMBRANE MATERIALS BY MEANS OF HPSEC AND BIO-RAD PROTEIN ASSAY

Membrane materials	Glucoamylase (g/ft.^{2} of membrane)	
	HPSEC	Bio-Rad
WD1	1.90	6.97
WD2	1.74	6.23
ZOSS	1.29	4.84
Bare tube	1.21	4.23
ZOPA	1.19	4.11
F1	0.76	3.10
WD1 + WD2	0.39	1.36



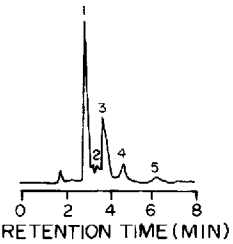


Fig. 2. Chromatogram of low-molecular-weight oligosaccharides present in membrane permeate without GA treatment (MPWOG). 1 = Glucose; 2 = maltose; 3 = maltotriose; 4 = DP 4.

Fig. 3. Chromatogram of low-molecular-weight oligosaccharides present in membrane permeate with immobilized GA treatment (MPWG). 1 = Glucose; 2 = maltose; 3 = maltotriose; 4 = DP 4; 5 = DP 5.

characteristics of the membranes. Comparisons were made between the HPSEC method and the Bio-Rad protein assay. The amount of GA determined by Bio-Rad protein assay was about 2.5 times higher than that from HPSEC. This may be expected because of the variations in binding ability of the dye to different proteins and the detection of all of the proteins in the enzyme preparation. Also, the contaminant protein may be bound more than GA. This indicates an advantage of using HPSEC for the specific quantitation of GA.

A 1.0% dextrin solution was used for the comparisons of low-molecular-weight oligosaccharides in the membrane permeates with and without immobilized GA treatment (MPWG and MPWOG, respectively). Figs. 2 and 3 illustrate the differences of the oligomers between MPWOG and MPWG, respectively, after a 10-min membrane operation. Table II shows the HPLC analyses of the low-molecular-weight oligosaccharides in various dextrin samples. The content of low-molecular-weight oligosaccharides in MPWOG was significantly ($p < 0.05$) lower than that in the 1.0% dextrin feed, indicating that the ZOPA membrane rejected a significant ($p < 0.05$) amount of low-molecular-weight oligosaccharides from a dextrin solution. The

TABLE II

COMPOSITION (mg/ml) OF DEXTRIN SAMPLES AND MEMBRANE PERMEATES DETERMINED BY HPLC

	24.0% Dextrin	1.0% Dextrin	MPWOG[d]	MPWG[e]
Glucose	1.019	0.0520[a]	0.0254[b]	0.0936[c]
Maltose	0.744	0.0393[b]	0.0100[a]	0.0123[a]
Maltotriose	4.110	0.1687[b]	0.0925[a]	0.0855[a]
DP 4	0.711	0.0022[b]	0.0005[a]	0.0005[a]
DP 5	0.332	0.0095	–	–
DP 6	0.201	0.0005	–	–
DP 7	0.170	0.0003	–	–
DP 8	0.120	–	–	–
DP 9	0.097	–	–	–
DP 10	0.084	–	–	–

[a,b,c] For each constituent, values with the same letter are not significantly ($p > 0.05$) different.
[d] MPWOG = Membrane permeate without GA treatment.
[e] MPWG = Membrane permeate with immobilized GA treatment.

glucose content in MPWG was signficantly higher ($p < 0.05$) than that in MPWOG and in 1.0% dextrin solution. This demonstrates the technical feasibility of immobilizing GA on a ZOPA membrane. This may be of benefit in continuous production of glucose syrups of higher purity, since many of the low-molecular-weight oligosaccharides are unable to pass through the membrane.

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