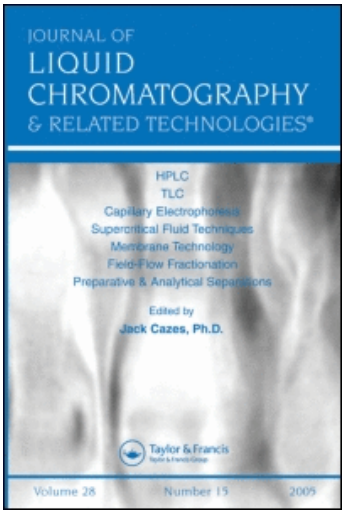

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Quantitative Analysis of Debranched Amylopectin by HPAEC-PAD with a Postcolumn Enzyme Reactor

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QUANTITATIVE ANALYSIS OF DEBRANCHED AMYLOPECTIN BY HPAEC-PAD WITH A POSTCOLUMN ENZYME REACTOR

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ABSTRACT

The chain-length distribution of debranched amylopectin was analyzed by using high performance anion-exchange chromatography with an amyloglucosidase (AMG) reactor and a pulsed amperometric detector (HPAEC-ENZ-PAD). The effects of reactor length, reactor temperature, and additional α-amylase reactor were studied. Results showed that a 2 mm i.d. x 23 mm AMG reactor at 25°C was sufficient to convert HPAEC-separated amylodextrins to glucose; the PAD response for the hydrolyzed amylodextrins of different chain-length was consistent, and quantitative results were achieved. With this enzymatic treatment, amylodextrin with DP up to 77 was detected for a debranched tapioca amylopectin sample. The chain-length distribution profile of debranched tapioca amylopectin showed three fractions with peak DPs 48 ± 1 (F1), 19 (F2a), and 12 (F2b).

INTRODUCTION

Branch chain-length distribution of amylopectin is one of the most important parameters in understanding the relationship between the chemical structure and the functional properties of starch. Many techniques have been applied for the study of branch chain-length distribution; a common one is gel-permeation chromatography (GPC). The total carbohydrate and reducing value of the collected fractions from GPC were determined by a phenol-sulfuric acid method[1] and a modified Park-Johnson procedure,[2] respectively. The whole protocol of the technique is time consuming, and both the phenol-sulfuric acid and the Park-Johnson methods involve hazardous reagents.

High performance size-exclusion chromatography with a refractive index detector (HPSEC-RI)[3-5] can also be used to determine the chain-length distribution of debranched amylopectin in a much shorter time than GPC. When a laser light scattering detector is used as a second detector with HPSEC and a RI detector (HPSEC-LS-RI),[6-9] the technique provides molecular weight distribution information without the need of column calibration.[10,11] One major weakness of the HPSEC technique is the limited resolution of the size-exclusion column.

The development of high performance anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD) provides a solution for this problem. The high separation power of the anion-exchange column allows amylodextrins to separate at individual homologous chain lengths, and the detector is selective and highly sensitive. By using the HPAEC-PAD, separation and detection of debranched amylopectin of normal maize starch up to DP 66 has been reported;[12] however, the detector response decreases as the chain-length of amylodextrin increases. Thus, the HPAEC-PAD chromatographic profile of debranched amylopectin does not provide a quantitative result.

To solve this problem, we decided to use an enzyme reactor to convert all the amylodextrins to glucose to achieve a consistent detection response. Amyloglucosidase (AMG) is a good choice for the purpose because it produces glucose exclusively, except for some residues, such as maltose and maltotriose, which are somewhat resistant to the enzyme hydrolysis. Larew and Johnson[13] have successfully demonstrated that the use of an AMG reactor resulted in quantitative conversion of HPAEC-separated maltooligosaccharides (G2 to G7) to glucose, which is then detected by PAD. In our studies, we used the HPAEC-PAD coupled with the AMG reactor to analyze debranched amylopectin. Because the debranched amylopectin sample contains amylodextrins with DP from 6 to over 70, the main challenges of this study

were to quantitatively convert all amylodextrins into glucose and maintain the chromatographic separation of each amylodextrin fraction. Effects of reactor length, reactor temperature, and additional α-amylase on the quantitative analysis of amylodextrin were investigated. Chain-length distributions of tapioca amylopectin obtained from the HPAEC-ENZ-PAD, HPSEC-RI, and GPC were compared.

EXPERIMENTAL

Chemicals

Amyloglucosidase (EC 3.2.1.3, from Rhizopus mold), α-amylase (EC 3.2.1.1, heat stable, from Bacillus licheniformis), soluble starch, normal maize starch, glucose, maltose, and glucose diagnostic kits (115-A) were purchased from Sigma Chemical Co. (St. Louis, MO). Acetic acid, citric acid, sodium acetate, sodium citrate, sodium hydroxide (5N), and sodium nitrate were purchased from Fisher Scientific (Fair Lawn, NJ). Nucleosil 300-10 and 1000-10 silica gels were purchased from Alltech (Deerfield, IL). Isoamylase (EC 3.2.1.68, crystal, from Pseudomonas amyloderamosa) and maltopentaose were purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Maltotriose, maltotetraose, maltohexaose, and maltoheptaose were purchased from Aldrich Chemical Co. (Milwukee, WI). Sodium azide was purchased from Eastman Organic Chemicals (Rochester, NY). Tapioca amylopectin was a gift from Dr. J.-F. Chen. Water (18 MΩ cm) used in all preparations was obtained from the Milli-Q Reagent Water System (Millipore, Bedford, MA).

Apparatus

Anion-exchange chromatography of debranched amylopectin employed a Dionex HPAEC-PAD system described previously.[12] An AI-450 software/interface (Model 1, Full-Control System, Dionex, Sunnyvale, CA) was used for instrument control and data processing.

Other apparatus used in the postcolumn enzyme reaction and column efficiency studies included: a metering pump (AA-72-S Precision Metering Pump, Eldex Laboratories, Napa, CA), a pneumatic pump (Postcolumn Pneumatic Controller, Dionex) equipped with a mixing tee and reaction coil, an isocratic pump (HP series 1050 HPLC pump, Hewlett Packard, Wilmington,

DE) equipped with an injection valve (Model 7125, Rheodyne, Cotati, CA) and a 20 μL sample loop, and a Visco Mixer (10 μL, The Lee Company, Westbrook, CT). A heating circulating bath (Lauda MS-3, Brinkmann, Westbury, NY) was used for column temperature control.

Sample Preparation

Isolation of amylopectin from normal maize starch has been described previously.[12] Amylopectin was debranched by isoamylase according to the procedures described by Jane et al.[14]

Preparation of Enzyme Reactors

Immobilizations of AMG and α-amylase were performed according to the procedures described by Emnéus and Gorton.[15,16] AMG was immobilized onto a Nucleosil 300-10 silica gel, and α-amylase was immobilized onto a Nucleosil 1000-10 silica gel. The coupling yields of the enzymes to the supporting silica gels were determined by measuring the enzyme contents in the supernatant before and after immobilization.[15] The enzyme activities (the amount of glucose released per min. by 10 mg of enzyme) before and after immobilization were determined by the following procedures: to a beaker containing 150 mL of 1% (W/V) soluble starch solution, 3 mL of citrate buffer (1 M, pH 4) was added. The solution was mixed by magnetic stirring. After a volume of immobilized gel with a known amount of enzyme or an enzyme solution with an equivalent amount of enzyme as that in the immobilized gel was added to the solution, an aliquot of the solution was transferred to a test tube every one to ten minutes. The enzyme activity was stopped by heating the transferred solution in a boiling water bath, and the solution was subjected for glucose analysis by using a glucose diagnostic kit. The enzyme reactors were prepared by vacuum slurry packing of the enzyme immobilized support into a 2 mm i.d. x 23 mm column unless otherwise noted. The efficiency of the enzyme reactor was evaluated according to Larew and Johnson[13] with some modifications. A debranched amylopectin sample was injected into a HP 1050 isocratic pump equipped with a 20 μL sample loop, and was carried by a stream of acetate buffer (0.05 M, pH 4.5) at a flow rate of 0.2 mL/min into an AMG reactor. The eluate from the reactor was then merged with a gradient from the Dionex gradient pump before entering the anion-exchange columns for separation. The gradient composed of eluent A (100 mM NaOH) and eluent B (100 mM NaOH and 500 mM NaOAc) with the following gradient profile: 0-2 min, 80% A and 20% B; 2-60 min, linear gradient to 100% B. The flow rate of the gradient was 0.5 mL/min at all time.

High Performance Anion-Exchange Chromatography with Postcolumn Enzyme Reactor and Pulsed Amperometric Detector (HPAEC-ENZ-PAD)

The HPAEC-ENZ-PAD setup was based on that described by Larew and Johnson[13] with modifications. All enzyme reactors were maintained at 25°C unless otherwise noted. Studies on pushing agents[12] show that sodium nitrate provides a better resolution than sodium acetate for amylodextrins analysis, therefore, sodium nitrate was chosen as the pushing agent in this study. The separation of a sample employed anion-exchange columns with a gradient composed of eluent A (100 mM NaOH) and eluent C (100 mM NaOH, 300 mM $NaNO_3$) with a flow rate of 0.5 mL/min.

The separation gradient for the system using one AMG reactor was as follows: 0-5 min, 94% A and 6% C; 5-10 min, linear gradient to 10% C; 10-150 min, linear gradient to 30% C; 150-200 min, linear gradient to 40% C; 200-220 min, linear gradient to 45% C. Separation gradient for the system using two AMG reactors was as follows: 0-5 min, 94% A and 6% C; 5-10 min, linear gradient to 10% C; 10-30 min, linear gradient to 13% C; 30-150 min, linear gradient to 30% C; 150-200 min, linear gradient to 40% C. The pH of the eluate from the anion-exchange column was adjusted to 4.5 by mixing with a citrate buffer (pH 4.1, 0.5 M, 0.25 mL/min).

In the study of the effect of additional α-amylase reactor, separation gradient was as follows: 0-5 min, 92% A and 8% C; 5-10 min, linear gradient to 12% C; 10-30 min, linear gradient to 15% C; 30-200 min, linear gradient to 40% C, and the eluate from the anion-exchange column was adjusted to pH 5 by a citrate buffer (pH 4.5, 0.5 M, 0.25 mL/min). The eluate from the enzyme reactor was adjusted to pH 12 by mixing with a 0.75 M sodium hydroxide solution. The total flow rate through the detector cell was 1.05 mL/min.

High-Performance Size-Exclusion Chromatography with Refractive Index Detector (HPSEC-RI)

Size-exclusion chromatography of debranched amylopectin employed an HP 1050 isocratic pump equipped with an injector with a 20 μL sample loop and a refractive index detector (HP 1047A, Hewlett Packard). The separation of the debranched amylopectin sample utilized a series of TSK-GEL PWXL size-exclusion columns (Tosohaas, Montgomeryville, PA) including a guard column and G4000 and G3000 separation columns. The mobile phase was deionized water at a flow rate of 0.5 mL/min. All columns were maintained at 60°C with a circulated water bath.

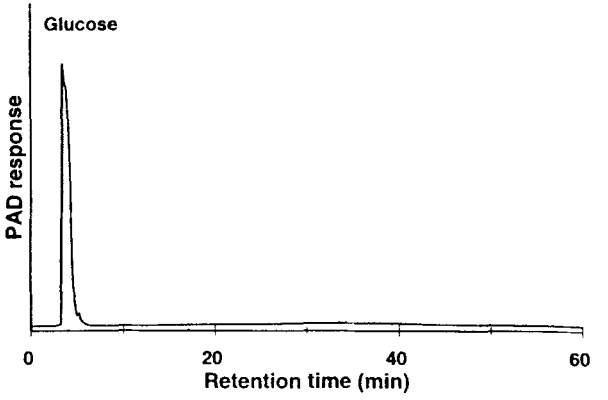

Figure 1. HPAEC chromatogram of debranched normal maize amylopectin (1.5 mg/mL) that passed through a 2 mm i.d. x 23 mm AMG reactor before the anion-exchange columns. Chromatographic conditions are described in 'Experimental'.

Gel-Permeation Chromatography (GPC)

Debranched amylopectin was analyzed by a Bio-Gel P-6 column (1.5 cm i.d. x 80 cm, Bio-Rad Laboratories, Richmond, CA)[14] followed by an anthrone sulfuric acid method for total carbohydrate analysis of each collected fraction.[17] The average branch chain-length was determined by analyzing the total carbohydrate (phenol-sulfuric acid method) and the reducing value (modified Park-Johnson method) of the three combined fractions at the peak.[14]

RESULTS AND DISCUSSIONS

Enzyme Reactors

The coupling yields of immobilizing α-amylase and AMG to one gram of the corresponding supporting silica gel were 16 mg and 243 mg, respectively. The activities of AMG before and after immobilization were 7.38±0.91 mg/min and 4.45±0.34 mg/min, respectively, and was 3.02±0.01 mg/min after the immobilized AMG reactor was kept at 4°C for eight months. Therefore, the enzyme activity was maintained at ca. 60% after immobilization, and the immobilized enzyme reactor remained ca. 68% of the reactor activity after a period of eight months. AMG is an exoenzyme that catalyzes the hydrolysis of

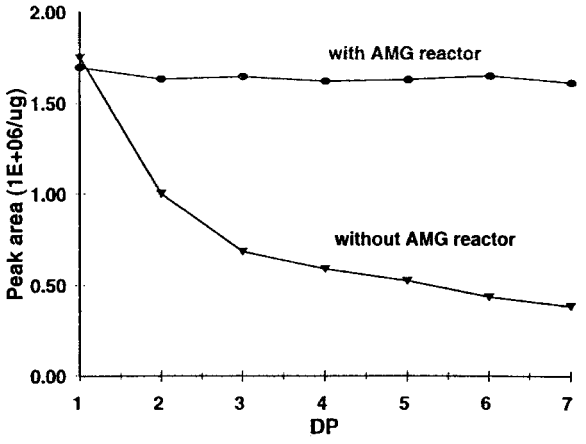

Figure 2. Sensitivity of PAD for unit mass of oligosaccharides with and without passing through an AMG reactor.

amylodextrin to glucose. The turnover rate of AMG increases as the molecular weight of oligosaccharides increases.[18] A study showed that the molar conversion of maltooligosaccharides to glucose by an immobilized AMG reactor reached 87-93% for G2 and G3 but up to 96% for G4-G7.[13] Because chain-lengths of amylopectin branches are at least 6 glucose units long,[8] it is likely that an AMG reactor can be sufficient in converting debranched amylopectin chains to glucose. The efficiency of the AMG reactor was evaluated by the injection of a 1.5 mg/mL debranched normal maize amylopectin. The result showed that glucose was the predominant product with a minor shoulder peak (peak area was less than 2% of the glucose peak) of maltose (Figure 1). The result confirmed that amylodextrins were effectively converted to glucose by the AMG reactor at 25°C.

Oligosaccharides and Debranched Tapioca Amylopectin Analyzed by HPAEC-ENZ-PAD

The HPAEC-ENZ-PAD system was first examined by injecting a standard oligosaccharide mixture (G1 to G7). All G1 to G7 had baseline separations and were quantitatively detected (Figure 2) after the in-line enzyme reactor

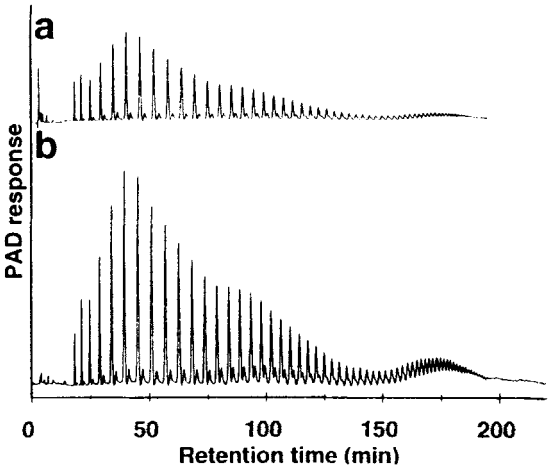

Figure 3. HPAEC chromatograms of 0.5 mg/mL debranched tapioca amylopectin (a) without and (b) with passing through a postcolumn AMG reactor. Chromatographic conditions are described in 'Experimental'.

treatment even though the enzymatic reaction time was less than 6 sec. The result was in good agreement with that reported by Larew and Johnson[13] in which enzyme reaction took place at 50°C. Figure 3 shows the analysis of debranched tapioca amylopectin by using the HPAEC-PAD with and without the AMG reactor. As expected, the PAD response for each amylodextrin was greatly enhanced by using the AMG reactor. On the basis of the AMG efficiency result shown in Figure 1 and that the concentration of each chromatographic fraction in this analysis was about 2% of that in the reactor efficiency study, we believe the amylodextrins were more than 98% converted to glucose.

Further studies on the effects of reactor length and reactor temperature showed that an increase in AMG reactor length from 23 mm (one AMG reactor) to 46 mm (two AMG reactors) or reactor temperature from 25°C to 50°C (optimum temperature for AMG) did not significantly change the chromatographic profile. The resulting normalized peak area plots of debranched tapioca amylopectin (Figure 4) showed that the detection of amylodextrin was to DP 77 when one AMG reactor was used but only to DP 68 when two AMG reactors were used. The decrease in detection sensitivity for

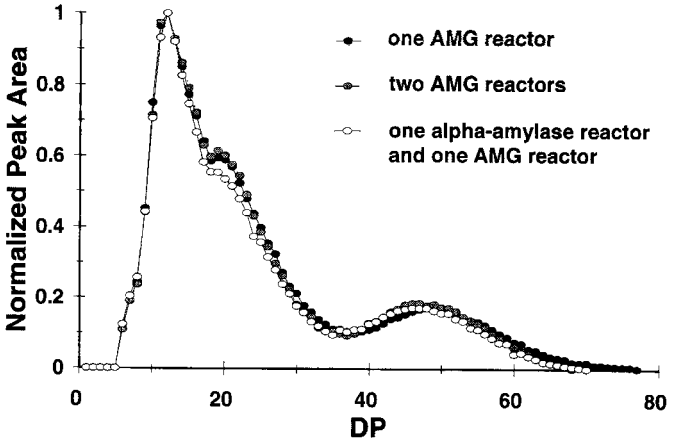

Figure 4. Normalized peak area plots for debranched tapioca amylopectin (0.5 mg/mL) analyzed by HPAEC-ENZ-PAD system with one AMG reactor, two AMG reactors, and a combination of an α-amylase reactor and an AMG reactor. Chromatographic conditions are described in 'Experimental'.

the latter was attributed to the diffusion effect introduced by the additional reactor. AMG has an optimum temperature at 50°C; however, it has been reported that a higher operating temperature shortens the lifetime of the AMG reactor.[19] The operation of the AMG reactor at 25°C instead of 50°C should prolong the life span of the enzyme reactor.

α-Amylase (B. licheniformis), an endo-enzyme that cleaves α-1,4-linkages within the starch molecule, can be used to increase the number of non-reducing ends of starch, thus, accelerating the AMG hydrolysis of starch. To investigate the effect of α-amylase on the hydrolysis of amylodextrin by an AMG reactor, an α-amylase reactor was connected in-line before the AMG reactor. α-Amylase and AMG have an optimum pH at 6 - 6.9[20] and pH 4.5,[21] respectively. To minimize the dilution and dispersion effects, we tried to select an appropriate working pH range for both enzyme reactors. In batch hydrolysis studies of soluble starch by a mixture of α-amylase and AMG at various pHs, the results showed that the enzyme mixture at pH 5 had the highest initial hydrolysis rate compared with that of the AMG alone at pH 4.5 and the enzyme mixture at pH 5.5. Therefore, pH 5 was selected for both the α-amylase and AMG reactors. The results showed that the efficiency of the conversion of

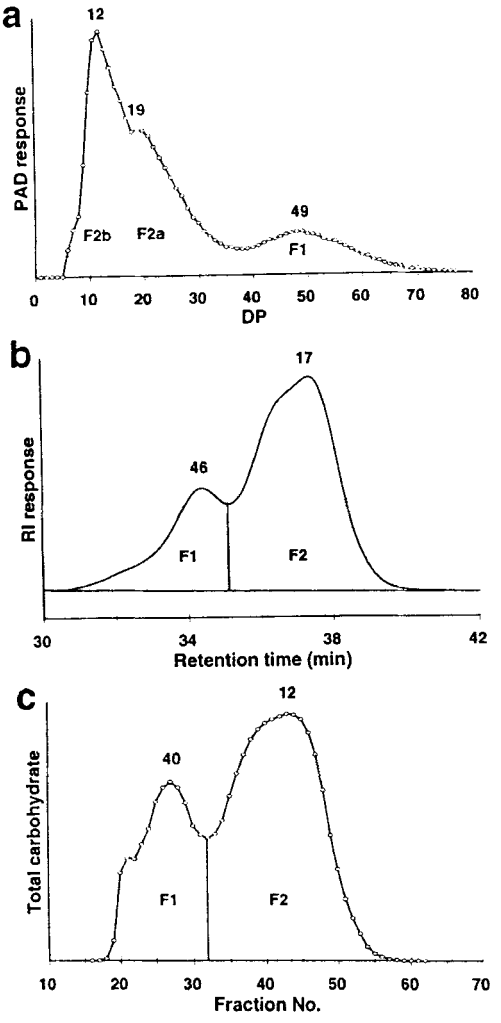

Figure 5. Chromatographic profiles of debranched tapioca amylopectin obtained by using (a) HPAEC-ENZ-PAD with one AMG reactor, (b) HPSEC-RI, and (c) GPC-total carbohydrate. Peak numbers indicate the degree of polymerization.

amylodextrin to glucose decreased when the combination of the α-amylase reactor and the AMG reactor was used (Figure 4). This difference may be attributed to the α-amylase hydrolyzing amylodextrin to short-chain oligosaccharides, in which maltose and maltotriose are more resistant to AMG hydrolysis.[13,22]

Table 1

Characterization of Branch Chain-Length Distribution of Tapioca Amylopectin by Using Different Chromatographic Techniques

Analytical Methods	Peak DP(% Peak Area) F1	F2a	F2	F2b
HPAEC-ENZ-PAD[a]	48 ± 1^b (21)	19^b(32)		12^b (47)
HPSEC-RI	46 ± 1^c (17)		16.0 ± 0.5^c (83)	
GPC	41 ± 3^b (32)		12.5 ± 0.5^b (68)	

[a] F1: DP 36-77, F2a: DP 19-35, F2b: DP 6-18.
[b] Three repetitions.
[c] Two repetitions.

Comparison of HPAEC-ENZ-PAD with HPSEC-RI and GPC for Debranched Amylopectin Analysis

After the in-line enzyme reactor treatment, the area (not the height) of each chromatographic peak corresponded to the total glucose concentration of the amylodextrin. Therefore, a plot of peak area against each DP represented a quantitative profile. Because there are no pure oligosaccharides with DP > 14 available as references, it is not possible to quantitatively calibrate each homologous peak. To further examine the effectiveness of HPAEC-ENZ-PAD for quantitative analysis of debranched amylopectins, we compared the HPAEC-ENZ-PAD profile of a debranched tapioca amylopectin with those from HPSEC-RI and GPC-total carbohydrate. Figure 5 shows the chromatographic profiles of debranched tapioca amylopectin obtained from these three techniques, and the chromatographic results are summarized in Table 1.

Because size-exclusion chromatography and anion-exchange chromatography have different separation mechanisms and different size-exclusion columns have different molecular size cut-offs, the profiles from the techniques varied. Figure 5a shows the HPAEC-ENZ-PAD chromatographic profile of debranched tapioca amylopectin in which the branch chain-length can be divided into three fractions with peak DPs of 48 ± 1 (F1), 19 (F2a), and 12 (F2b). HPAEC separates the amylodextrin mixture into individual homologous chain lengths; therefore, the DP of the peak can be conveniently determined by counting homologous peaks calibrated by using standard

references of G1 to G7. Figures 5b and 5c are the HPSEC-RI and GPC-total carbohydrate chromatographic profiles, respectively. Both HPSEC and GPC profiles of debranched tapioca amylopectin showed separated fractions F1 and F2. The peak DPs of the branch chain-length were 46 ± 1 (F1) and 16.0 ± 0.5 (F2) for HPSEC (calibrated by using pullulan standards) and were 40 ± 3 (F1) and 12.3 ± 0.5 (F2) for GPC (obtained by chemical analyses of the three fractions collected at the corresponding peak). Because HPSEC and GPC give broad and unresolved peaks, the DP at peak represents average DP of each arbitrary fraction. The close agreement in percentage areas of F1 and F2 between HPAEC-ENZ-PAD and HPSEC-RI (Table 1) provided further evidence in the quantitative analysis of the amylodextrin by using the HPAEC-ENZ-PAD. The source of discrepancy between the result of GPC-total carbohydrate and results of the other two is not clear.

CONCLUSION

The results of these studies indicated that the use of the AMG reactor alone at 25°C was sufficient to hydrolyze the HPAEC-separated amylodextrin. The use of extended reactor length did not improve the efficiency of the enzyme reactor but decreased the detection sensitivity of amylodextrin. HPAEC-ENZ-PAD provided excellent separation and quantitative detection for amylodextrin, and thus, the chain-length distribution was accurately determined by the HPAEC chromatogram and the result was reproducible.

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