



Partial depolymerization of pectin by a photochemical reaction

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ABSTRACT

Complex heterogeneous polysaccharides that comprise pectin were partially depolymerized by a photochemical reaction using ultraviolet light in the presence of titanium dioxide catalyst. In a period of 6 h at pH 7, this UV/TiO₂ process decreased the average molecular weight of pectin from 400 kDa to 200 kDa. The characterization of the partially depolymerized pectin, which was fractionated by size-exclusion chromatography, was performed by ¹H NMR spectroscopy, and the spectra obtained showed that the resulting oligosaccharides and polysaccharides maintained the intact core structure of pectin. The monosaccharide content and depolymerization profile were determined by high-performance anion-exchange chromatography coupled with pulsed amperometric detection. This controlled photochemical depolymerization technique might be useful for preparation of pectin oligosaccharides as an ingredient in food and pharmaceutical products.

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1. Introduction

Pectins are complex heterogeneous polysaccharides found in the primary cell wall of most plants. The dominant saccharide component of pectin is galacturonic acid (GalA), with varying proportions of additional neutral sugars consisting primarily of galactose, arabinose, rhamnose, and xylose. Three major pectic polysaccharides (homogalacturonan, rhamnogalacturonan-I, and rhamnogalacturonan-II) are thought to occur in all primary cell walls and all contain GalA linked at the O-1 and the O-4 position.¹

Pectin serves as a thickening, gelling, and stabilizing polymer in pharmaceutical and food products and has positive effects on human health with multiple biomedical uses. Specific structural elements within pectin are responsible for its biological activity and gelling properties.^{2,3} Oligosaccharides derived from pectin have been found to have various biological activities,^{3–5} such as immuno-modulating,^{6–11} anti-ulcer,¹² anti-cancer,¹³ and anti-inflammatory activity.¹⁴ Pectic oligosaccharides (POS) have an important reputation as prebiotic agents;^{15,16} therefore, the characterization of POS is currently of great interest.

Generally, the preparation of POS is accomplished by either controlled chemical or enzymatic depolymerization processes.¹⁷ Chemical methods, relying on acids such as sulfuric acid, trifluoroacetic acid, hydrochloric acid, and methanolic hydrochloric acid,

are commonly used in the preparation of POS. Acid-catalyzed hydrolysis can result in the cleavage of different glycosidic linkages resulting in a variety of different POS preparations.¹⁸ Another disadvantage of acid-catalyzed hydrolysis is the concomitant decomposition of sugars to furfuraldehydes and other side products, which decrease the yield and result in toxic components.¹⁹ Hydrolysis using ammonium hydroxide results in the conversion of some of the ester groups into amide groups, producing amidated methoxyl pectins.²⁰ Oxidative depolymerization of pectin using hydrogen peroxide with copper(II) has also been recently reported.²¹ Enzymatic depolymerization methods require the use of different types of enzymes and can result in microbial contamination of POS preparations.¹⁹ Moreover, acidic, oxidative, and enzymatic treatments are not easy to control, resulting in products of variable chain lengths that can result in the loss of the methyl ester groups within pectin.

In previous studies we reported the depolymerization of sodium alginate, an acidic polysaccharide structurally similar to pectin, using an environmentally friendly procedure, a photochemical reaction relying on titanium oxide catalyst.²² The controlled depolymerization of sodium alginate was successfully accomplished using a UV/TiO₂ reaction at pH 7 for 3 h. To our knowledge, no photochemical method has ever been reported for the controlled depolymerization of pectin.

In the present paper, we apply the same photochemical depolymerization strategy to obtain POS. This depolymerization reaction was studied at different pH values and for reaction times, and the

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structures of the resulting products were characterized using ^1H NMR spectroscopy. The POS product was also fractionated, and the chromatographic profile and the content of GalA and neutral sugars were determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD).

2. Results and discussion

2.1. Depolymerization of pectin by a photochemical UV/titanium dioxide process

The average molecular weight (M_r) of the product of photochemical depolymerization of pectin could be controlled by varying pH and reaction time. The effect of pH on the depolymerization reaction was investigated at pH 4 (adjusted with 10 mM hydrochloric acid), at pH 7 (adjusted with 10 mM ammonium hydroxide), and at pH 10 (adjusted with 100 mM ammonium hydroxide). Each solution was exposed to low-pressure mercury UV light for a period of 0, 1, 3, 6, and 10 h. Replicate sample solutions ($n = 6$) were analyzed to assess the precision of the method for the determination of M_r .

The viscosity of the sample solution was decreased after 1 h of photolysis, indicating that depolymerization of the pectin sample in an aqueous suspension of TiO_2 had taken place. The M_r of samples at each pH and time point was determined by high-performance size-exclusion chromatography (HPSEC). The M_r of pectin decreased at all pH values. A pH value of 10 afforded the most rapid degradation of pectin with 71.2% decrease in M_r (Table 1). The M_r of pectin dramatically decreased during the first 6 h of photolysis and then only slowly decreased after an additional 4 h of photolysis. The same trend was observed at all pH values examined. At pH 7 the sample showed the slowest rate of depolymerization, affording a ~50% decrease in M_r after 6 h, but giving an M_r value that was very close to the value observed at pH 4 in 1 h. However, at pH 4, the sample solution became turbid and could not be easily filtered, while the result from ^1H NMR spectra demonstrated the decomposition of pectin at pH 10 (described in the next section). Thus, the optimal conditions for a photochemical reaction to obtain fractionated pectin were established as exposure to the UV light in the presence of TiO_2 for 6 h at pH 7. HPSEC chromatograms of the pH 7 samples also showed three other peaks corresponding to depolymerized products of $M_r < 100$ kDa (20, 11, and 5 kDa with 2%, 8%, and 6% yield, respectively) (Fig. 1). The heterogeneous molar mass distribution of the depolymerized pectin suggests a random breakdown process. The R.S.D. of M_r showed the satisfactory repeatability of the method (<10%).

2.2. Characterization of pectin and partially depolymerized products by ^1H NMR spectroscopy

The ^1H NMR spectrum of the original pectin sample is shown in Figure 2 A. The peaks of the components of this pectin sample were assigned based on previously reported values.^{23–26} The large singlet signal at 3.8 ppm is assigned to the methyl signal of the

Table 1

The average molecular weight (kDa) of depolymerized pectin prepared at different pH and reaction time values (mean \pm SD, $n = 6$)^a

Reaction time (h)	pH 4	pH 7	pH 10
0	424 \pm 5	424 \pm 5	424 \pm 5
1	215 \pm 18	338 \pm 17	122 \pm 13
3	142 \pm 24	284 \pm 13	53 \pm 4
6	131 \pm 20	208 \pm 12	36 \pm 1
10	105 \pm 5	126 \pm 5	25 \pm 1

^a All data shown are average molecular weight values estimated using defined dextran and dextran sulfate molecular weight standards.

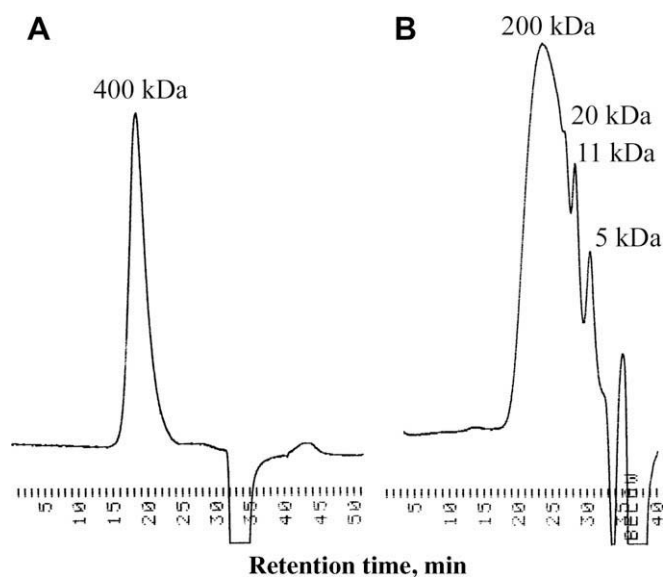


Figure 1. HPSEC chromatograms of pectin samples. (A) Intact pectin and (B) depolymerized pectin products after 6 h reaction at pH 7. HPLC conditions: column, Asahipak 510 HQ (7.6 mm i.d. \times 300 mm); mobile phase, 10 mM NH_4HCO_3 at a flow rate of 0.3 mL/min; detection, refractive index.

COOCH_3 ester. ^1H NMR spectra of the partially depolymerized pectin samples, obtained from the photochemical reaction after 6 h at pH 4, 7, and 10 are shown in Figure 1B–D. At the pH values of 4 and 7, the signals corresponding to galacturonate were observed in the ^1H NMR spectra (Fig. 2B and C), indicating that while the glycosidic linkages had been hydrolyzed, the sugar ring units maintained their structures. Moreover, the methyl ester groups remained, indicating that these products were essentially low-molecular-weight pectins. Depolymerization at pH 10 resulted in de-esterification of galacturonate, and no signal corresponding to the methyl group of the COOCH_3 ester was observed. Based on these results, depolymerization at pH 7 was studied in greater detail. The ^1H NMR spectra of pectin after photochemical reaction for reaction times of 1–16 h at pH 7 were examined (Fig. 3). Each signal changed slightly in both breadth and intensity as the M_r and the distribution of pectin chain lengths changed. The signals for depolymerized pectin samples, however, showed the same chemical shift values as observed for the intact pectin sample, indicating that the chemical structures were identical. These results suggest that photochemical degradation occurs through the random breakage of the glycosidic bonds in the pectic polysaccharide with no concomitant change in the primary structure of the polymer.

The pectin depolymerization for 6 h at pH 7 was fractionated on HPSEC (data not shown), affording three fractions that were recovered in 88% overall yield. Each fraction was characterized by analysis by ^1H NMR spectroscopy, and the signals of all products were verified as pectin (Fig. 4). However, the relative areas of signals assigned to the methyl groups of the COOCH_3 ester in fractions 2 and 3 (Fig. 4C and D) were smaller than observed in fraction 1 (Fig. 4B). Moreover, in the ^1H NMR spectrum of fraction 3, new signals appeared that were clearly assignable to the H-2, H-3, H-4, and H-5 of galactose (Fig. 4D).

The content of galacturonic acid and neutral sugars of each fraction was next quantitatively analyzed by HPAEC–PAD (Table 2). The monosaccharide content of each fraction was different from that of the original pectin. All fractions were rich in galacturonate as the predominant peak, and peaks corresponding to the methyl ester were observed in each fraction. Interestingly, the amount of galacturonic acid decreased with decreasing M_r by ~15%, which is comparable to the ~15% reduction in the integration of the methyl signal

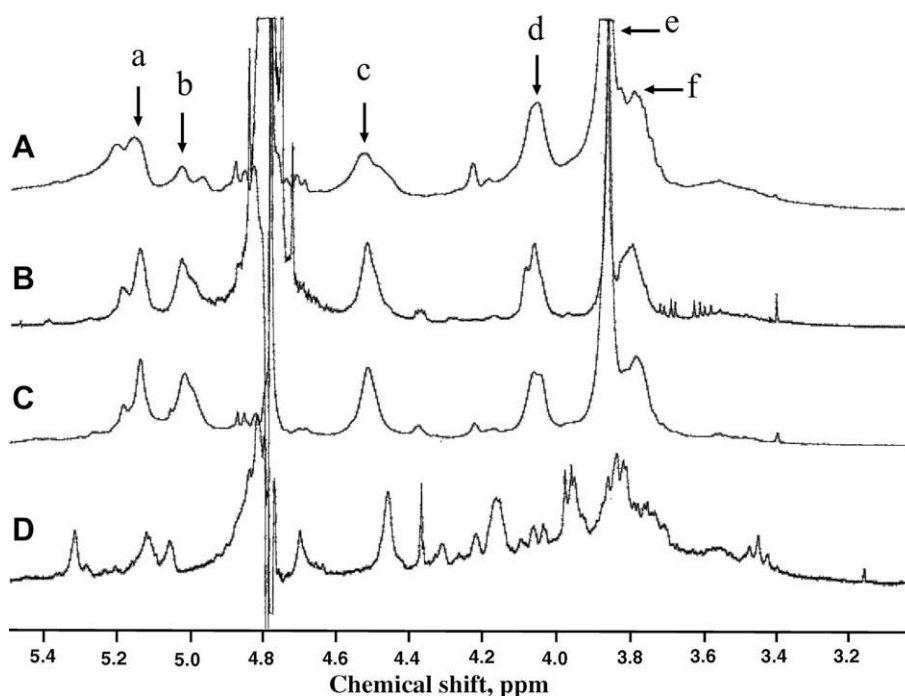


Figure 2. ^1H NMR spectra of pectin samples. (A) Intact pectin; (B) product after 6 h reaction at pH 4; (C) product after 6 h reaction at pH 7; (D) product after 6 h reaction at pH 10. Signals were assigned as: (a) H-1; (b) H-5; (c) H-4; (d) H-3 of galacturonic acid residues; (e) methyl group of COOCH_3 ; and (f) H-2 of galacturonic acid residues.

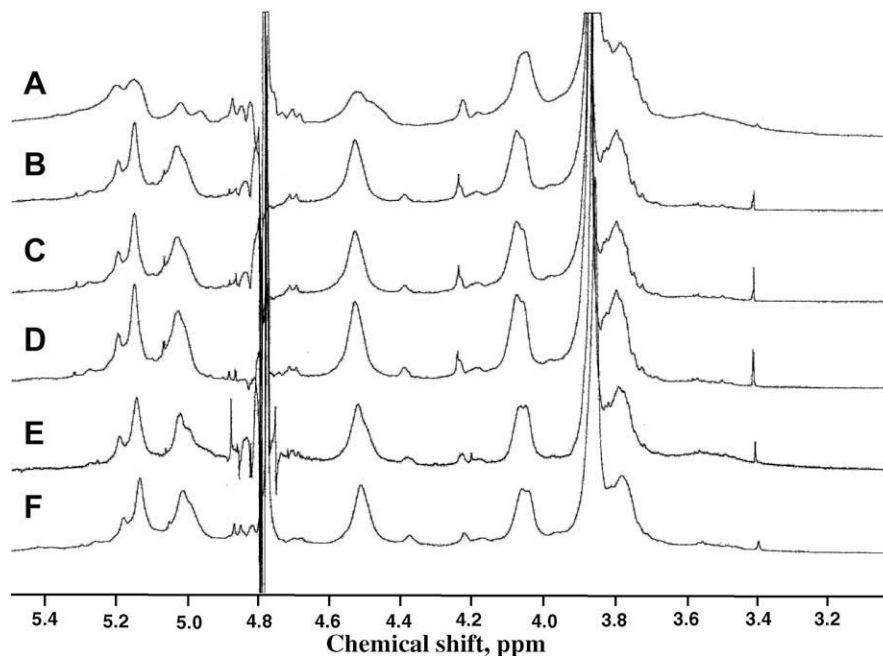


Figure 3. ^1H NMR spectra of pectin samples. (A) Intact pectin; (B) product after 1 h reaction at pH 7; (C) product after 3 h reaction at pH 7; (D) product after 6 h reaction at pH 7; (E) product after 10 h reaction at pH 7; and (F) product after 16 h reaction at pH 7.

of the COOCH_3 ester group and to the $\sim 15\%$ increase observed in galactose content. This indicated that breakdown of pectin is associated with the specific photochemical breakdown of GalA that releases of a random series of heterogeneous oligomers.

2.3. The chromatographic profile of pectin oligosaccharides derived by photolysis

The depolymerized pectin obtained from photolysis was next examined by HPAEC chromatography on a PA-200 column with

PAD. The calibration curve between the degree of polymerization (DP) and retention time was plotted using oligogalacturonic acid (OGA) standards: D-galacturonic monohydrate, digalacturonic, and trigalacturonic acid (DP 1–3). Since OGA standards above 3 DP units are not commercially available, polygalacturonic acid was also degraded and used for calibration curve plotting and compared with a chromatogram of depolymerized pectin. The elution order of POS increased with the increasing degree of polymerization, and there was a good correlation ($y = 4.02x + 21.96$ with $R^2 = 0.997$) between the DP and the retention time (t_R) values of the POS.

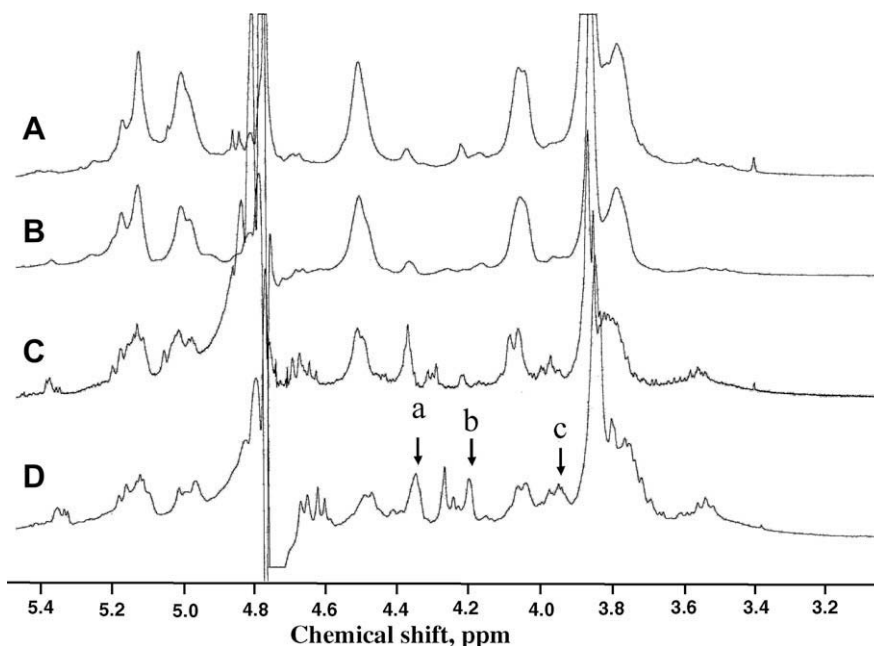


Figure 4. ^1H NMR spectra of photochemically sized fractions of depolymerized pectin. (A) Low-molecular-weight depolymerized pectin prepared at pH 7 after 6 h photochemical reaction; (B) Fraction 1 (highest M_r); (C) fraction 2 (intermediate M_r); and (D) fraction 3 (lowest M_r). Signals were assigned as: (a) H-4; (b) H-5; and (c) H-2 and H-3 of galactose.

Table 2

The % yield, the amount of galacturonic acid and neutral sugars (mol %), and the average molecular weight (kDa) of the degraded pectin in fractionated pectin using UV/TiO₂ photolytic process at pH 7 for 6 h and analyzed by HPAEC-PAD^a

Product	% Yield ($n = 3$)	Content (mol %) ($n = 3$)					GalA
		Rha	Ara	Gal	Glc	Xyl	
Intact	5.9	5.4	10.6	0.6	0.4	77	
Fraction 1	5.8	7.1	11.3	1.3	0.5	74.1	
Fraction 2	2.7	5.2	18.0	1.0	0.6	72.4	
Fraction 3	2.1	8.8	26.9	0.9	0	61.3	

^a Rha, rhamnose; Ara, arabinose; Gal, galactose; Glc, glucose; Xyl, xylose; GalA, galacturonic acid.

The chromatogram of POS derived from pectin is shown in Figure 4. The DP of oligomers of higher molecular weight was based on extrapolation. The POS obtained in photolytically depolymerized pectin could be well separated up to a DP of 18 units. Larger polymers were not soluble in the acetate eluent used. A peak corresponding to galacturonic acid was also observed in this profile. The retention times of several peaks in the POS chromatogram (Fig. 5) were shifted from those in the OGA standard curve chromatogram. Different levels of methyl esterification of oligogalacturonic acids in the POS preparations can be used to explain these differences in retention times.²⁷ These results again confirm that the methyl ester groups of galacturonic acid remain even after the photolytic reaction.

3. Conclusions

The UV/TiO₂ photolytic process applied for 6 h decreases the average molecular weight of pectin from 400 to 200 kDa. The molecular size of the depolymerized product could be controlled by the exposure time to the UV light with high per cent yield and good reproducibility. ^1H NMR spectra have shown that the POS maintained the intact structure of pectin. The monosaccha-

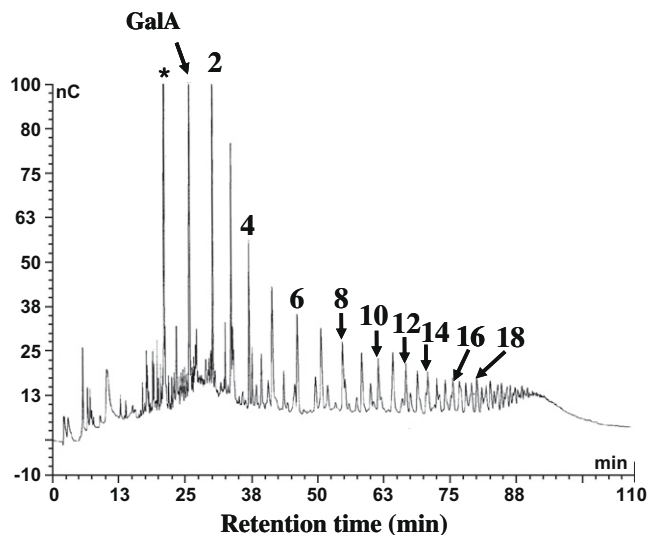


Figure 5. Depolymerization profile of pectin sample using UV/TiO₂ photolytic process at pH 7 for 6 h. The numbers above the peaks correspond to degree of polymerization (DP). GalA, galacturonic acid; *, represents an unidentified contaminant.

ride content of each POS product differed from intact pectin. While the main component in all fractions was galacturonic acid, the content of galacturonic acid decreased with decreasing M_r , suggesting that the depolymerization of pectin occurred through the loss of the consecutive sequence of galacturonic acid residues. In addition free galacturonic acid monosaccharide was observed in each POS. The DP profile of the oligosaccharide product implied that the oligomers with DP 2–18 could be prepared without the loss of the methoxy group. This proposed procedure might represent a convenient and reliable method for a routine production of POS for the food and pharmaceutical industries.

4. Experimental

4.1. Chemicals

Commercial citrus pectin (DM 6%) having an M_r of ~ 400 kDa, titanium dioxide (anatase type, particle size average, 50 μm), and monosaccharide standards were purchased from Wako Pure Chemical Co. (Osaka, Japan). The pectin sample was further purified from low-molecular-weight contaminants by dialysis against Milli-Q water in a Spectrapor[®] dialysis tube (molecular weight cut off, 500 Da) for three days at 4 °C. The sample was then freeze dried prior to use. All other reagents were of analytical reagent grade.

4.2. Photochemical reaction apparatus

The photochemical reaction experiment device (Sen Lights Corporation, Osaka, Japan) consists of a VG1500 reaction tank with five inlets, a UV light source (low-pressure mercury lamp HL400B-8, 400 W), a power source (HB400P-1), and a lamp jacket (quartz glass JW-2Q). The apparatus was connected with a water circulating system to cool the lamp.

4.3. Degradation of pectin by photochemical reaction

Pectin solution (10 mg/mL) was prepared in distilled water. An aliquot (1 mL) of the solution was transferred to six tubes of a 6 mL-screw-capped test tube (borosilicate glass), 1 mg of titanium dioxide (TiO_2) particles was added separately, and a cap was closed loosely. The sample tube was then placed in the photochemical reaction tank and exposed to UV light. The pectin was depolymerized at room temperature, and the light exposure time was varied between 1 h and 10 h. Six replicates ($n=6$) of sample solutions were analyzed at the same exposure time. A mechanical stirrer was used in addition to a magnetic stirrer to ensure the dissolution of air into the solution. After the reaction, the sample was centrifuged at 1500g for 5 min, the supernatant was filtered through a 0.45- μm membrane filter to eliminate all of the TiO_2 , and the product solution was dialyzed and freeze dried.

Pectin sample solutions with different pH values were prepared to study the influence of pH on the degradation. An aqueous 1% (w/v) pectin solution at pH 4 was made by adjusting the pH with 10 mM hydrochloric acid using a mechanical stirrer. Pectin with pH 7 was made by adjusting with 10 mM ammonium hydroxide. Pectin with pH 10 was made by adjusting with 100 mM ammonium hydroxide.

4.4. Estimation of the average molecular weight

The average molecular weights of the pectin samples were estimated by an HPSEC system that consisted of a Hitachi L-600 pump (Hitachi Co., Japan), a Rheodyne 7725i loop injector (USA), and a YRD-880 refractive index detector (Shimamura Instruments Co., Japan). The column used was an Asahipak 510 HQ column (7.6 mm, i.d. \times 300 mm) (Shodex Co. Ltd, Tokyo) and eluted with 10 mM NH_4HCO_3 at a flow rate of 0.3 mL/min. The calibration curve for molecular weight estimation was performed using T-series dextran standards (Shodex Co. Ltd, Tokyo). The samples were estimated in triplicate.

4.5. Fractionation of depolymerized pectin

The depolymerized pectin was dissolved in water and fractionated on an HPSEC system as described above. The columns used were a series of two Asahipak 510 HQ columns. Elution was carried out at a flow rate of 0.3 mL/min with 10 mM NH_4HCO_3 . The frac-

tions collected were desalted using dialysis membranes (cut off 500 Da) and freeze dried.

4.6. Structural analysis by ^1H NMR spectroscopy

Samples were kept in a desiccator over phosphorus pentoxide in vacuo overnight at room temperature. Each of the thoroughly dried samples was then dissolved in 0.5 mL of D_2O (99.96%), centrifuged at 2000g for 15 min, and then transferred to an NMR tube (5.0 mm o.d. \times 25 cm; Wilmad Glass Co. (Buena, NJ)). NMR experiments were performed on a JNM-500A spectrometer equipped with a 5-mm field-gradient tunable probe with standard JEOL software at 60 °C for all experiments on 500- μL samples. The HOD signal was suppressed by presaturation for 1.5 s for a 1D spectrum.

4.7. Determination of monosaccharide contents by HPAEC-PAD

The fractions of pectin (1.0 mg/mL each) decomposed by photolysis was hydrolyzed by 2.5 M trifluoroacetic acid (TFA) at 100 °C for 6 h. The hydrolyzed samples were then filtered (0.20- μm membrane filter) and injected (10 μL) on a CarboPac PA-1 guard column (25 mm \times 4 mm i.d.) attached to a CarboPac PA-1 anion-exchange column (100 mm \times 4 mm i.d.) (Dionex, Sunnyvale, CA, USA). A HPAEC-PAD separation was performed with an ICS-3000 Dionex chromatography system (Sunnyvale, CA, USA) equipped with an ED-3000 electrochemical detector, a SP gradient pump with degasser, and a Rheodyne injector with a thermal compartment. Data were collected and analyzed on computers equipped with Chromeleon 6.80 Sp2 Build 2212 software (Dionex Corp., Sunnyvale, USA). Neutral monosaccharides derived from the fractions were eluted isocratically with 10 mM sodium hydroxide for 25 min, followed by a linear gradient of 0–150 mM NaOAc in 100 mM NaOH for 20 min to elute acidic monosaccharides. Before each injection, a column re-equilibration program was run for 15 min with 100 mM NaOH, followed by 10 min with 10 mM NaOH. The eluent flow rate was constantly kept at 1.0 mL/min. The monosaccharide content was calculated based on the peak area response of known amounts of monosaccharide standards. The samples were determined in triplicate.

4.8. Degree of depolymerization profile by HPAEC-PAD

The two samples of pectin depolymerized by UV/ TiO_2 process at pH 7 for 6 h were prepared in an aqueous solution and injected on a CarboPac PA-200 guard column (25 mm \times 4 mm i.d.) attached to a CarboPac PA-200 anion-exchange column (100 mm \times 4 mm i.d.). Neutral sugars were eluted linear gradient of 50–100 mM NaOH for 10 min, followed by a linear gradient of 0.3–0.7 M NaOAc in 100 mM NaOH for 50 min to elute the oligosaccharides. Peak identification was based on retention times.

Polygalacturonic acid standard solution was hydrolyzed by 0.5 M TFA at 100 °C for 2 h and then analyzed on a CarboPac PA-200 as described above. The calibration curve between the degree of polymerization (DP) and retention time was plotted using oligogalacturonic acid (OGA) standards: D-galacturonic monohydrate, digalacturonic, and trigalacturonic acid (DP 1–3), and the depolymerized products of polygalacturonic acid (DP >3).

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