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Impact of Cabbage Pectin–Protein Complex on Microbial β-Glucuronidase Activity

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ABSTRACT: We previously demonstrated that pectin–protein complex (PPC) isolated from white cabbage adsorbs the β-glucuronidase (βG) enzyme of *E. coli*. Concurrently, we discovered a significant increase in βG activity in the presence of PPC. The aim of this study is to identify the structural components of PPC that are responsible for βG adsorption and activation. PPC was isolated from white cabbage using a saline solution containing hydrochloric acid (pH 1.5) at 37 °C for 4 h. PPC proteins were precipitated by aqueous 10% (m/v) trichloroacetic acid to yield the pectin–protein fractions PPC_1 and PPC_2. PPC was digested using 1,4-α-D-galacturonase, yielding the PPC_6 fraction. Partial acid hydrolysis of PPC revealed the galacturonan fraction, PPC_3, to be the core of the macromolecule. The purified PPC_4 and PPC_5 fractions were isolated from PPC by ion-exchange chromatography on DEAE-cellulose. βG activity and its adsorption in the PPC fractions were studied *in vitro*. Crystalline cellulose was used as a control. This study found that the PPC_3 fraction (the galacturonan core) does not adsorb βG and does not affect its activity. The adsorption of βG in the PPC samples is inversely proportional to the degree of methyl esterification of its carbohydrate component. The PPC_4 and PPC_5 fractions adsorb the highest proportion of βG (51.2% and 54%, respectively). The stimulation of βG enzyme activity is directly proportional to the protein content of the PPC sample. The PPC and PPC_1 samples have the greatest ability to increase βG activity (57.6% and 52.1%, respectively).

KEYWORDS: *white cabbage (Brassica oleracea), pectin–protein complex, β-glucuronidase of E.coli, adsorption, activity*

INTRODUCTION

Consumption of foods rich in dietary fiber reduces the risk of cancer.[1,2] The mechanism of the anticancer activity of water-insoluble dietary fiber is well studied and is associated with their ability to adsorb estrogens, which reduces the level of estrogens in the blood and interrupts the enterohepatic circulation of estrogen metabolites.[3−5] However, the anticancer mechanism of water-soluble dietary fiber is insufficiently studied. Previously, we identified two *in vitro* properties of vegetable pectins that could potentially affect the mechanism of enterohepatic circulation of estrogens. It was shown that pectin–protein complexes (PPCs) that were isolated from sweet pepper and white cabbage have an affinity for estrogens.[6] It was also shown that vegetable PPCs have an affinity for the *E. coli* β-glucuronidase (βG).[7] A model explaining the anticancer effect of vegetables pectins was proposed. According to this model, vegetable pectins adsorb estrogens and microbial βG in the human gastrointestinal tract and accelerate their excretion from the body, thus interrupting the enterohepatic circulation of sex hormones.[7]

Additionally, we have shown that vegetable PPCs not only adsorb *E. coli* βG but also increase enzyme activity; this fact does not quite conform to the proposed model. Similar *in vivo* results were obtained earlier by other authors. Cellulose, hemicellulose, apple pectin, and citrus pectin also increase the activity of microbial βG in the large intestine and animal and human feces.[8−11] This increase in enzymatic activity may contribute to the activation of carcinogens. More data concerning the interaction of vegetable PPCs with microbial βG are needed to resolve this contradiction.

Pectins belong to a class of high molecular weight polysaccharides that are associated with the cell wall and intercellular regions of plants. Pectins are widely used as gelling and stabilizing agents in foods. They have irregular block sugar chains and contain various macromolecular segments that have linear and ramified regions.[12] The linear regions consist of α-1,4-D-galacturonan chains and are the backbone of all pectins. The degree of methyl esterification (DM) divides pectins into high methoxylated (HM) and low methoxylated (LM) pectins, which are characterized by DMs above 50% and below 50%, respectively. The ramified regions contain various heteroglycanogalacturonan segments, such as rhamnogalacturonan I, rhamnogalacturonan II, xylogalacturonan, and apiogalacturonan.[13] There is evidence in the literature to suggest that pectic polysaccharides may also be cross-linked to hemicelluloses, phenolic compounds, and wall proteins.[14] Chemical data also support the existence of pectin–arabinogalactan protein linkages in extracts from hops [15] and a covalent association between the RG1 and the extensin protein from the cell walls of cotton suspension cultures.[16] Commercial pectins may have a protein content of approximately 1.5% to 3%.[17]

The purpose of this study is to identify the structural components of vegetable PPCs responsible for their interactions with microbial βG and activation of this enzyme. The focus of this study was cabbage PPC, which has an affinity for *E. coli* βG and increases its activity.

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Table 1. Chemical Characteristics of PPCs and Its Fragments

fraction	yield, %	protein[c,e]	OMe[c]	DM[d]	GalA[c]	Rha[c]	Ara[c]	Xyl[c]	Man[c]	Glc[c]	Gal[c]
PPC	0.2[a]	22.4	4.0	71	34.2	3.3	11.9	1.5	0.4	1.9	9.7
PPC_1	58.0[b]	27.7	2.0	35	22.5	2.5	7.0	1.4	0.6	0.7	6.4
PPC_2	38.0[b]	1.6	4.9	46	64.9	2.8	11.3	2.2	0.1	0.4	11.2
PPC_3	11.3[b]	2.2	0.4	3	79.8	0.3	1.6	0.0	0.0	0.0	0.0
PPC_4	43.8[b]	6.7	1.3	20	39.7	2.9	10.6	1.7	0.0	0.8	12.0
PPC_5	18.0[b]	20.9	1.6	28	34.1	2.0	5.4	1.1	0.0	0.7	5.1
PPC_6	45.3[b]	4.2	5.2	62	50.5	2.2	13.7	2.9	0.1	0.4	7.0

[a]Yield of the plant raw material. [b]Yield of the PPCs. [c]Data are calculated as weight %. [d]Data are given in molar %. [e]Protein content of the fractions was determined using the Bradford assay.

MATERIALS AND METHODS

Reagents. Enzyme (βG): β-glucuronidase Type IX-A from *Escherichia coli* (Sigma). Substrate: 10 mM aqueous solution of phenolphthalein-β-D-glucuronide (Sigma). Standard: 0.03 M solution of phenolphthalein in 70% (v/v) ethanol. Incubation solution: 0.1 M phosphate buffer, pH 7.0, and 0.1 mM EDTA. Alkaline mixture: 200 mL of glycine buffer, 0.2 M, pH 11, added to 50 mL of 0.5 N NaOH. Sorbents: microcrystalline cellulose (Lachema Chemapol, Czech Republic), PPC isolated from white cabbage, and PPC fractions (PPC_{1-6}).

Equipment. A Vivaspin 500 concentrator (Sartorius AG, Germany) with a pore diameter of 300 kDa and a Microplate PowerWave 200 spectrophotometer (Bio-Tek Instruments, USA) were used.

Isolation of PPC from White Cabbage. Macromolecular PPC (>300 kDa) was extracted from cabbage (*Brassica oleracea*) using a saline solution containing hydrochloric acid (pH 1.5) at 37 °C for 4 h to simulate a gastric environment. PPC extraction was performed according to a previously described method.[18] The output and chemical characteristics of the isolated fractions are shown in Table 1.

General Analytical Methods. The glucuronic acid contents of the fractions were determined using 3,5-dimethylphenol in the presence of concentrated sulfuric acid [19] with a D-galacturonic acid standard (Sigma-Aldrich). The protein contents of the fractions were determined using the Bradford assay [20] with a BSA standard (MP Biomedicals). The amount of methyl ester groups was determined as previously described[21] using absorption at 412 nm with a methanol standard. Spectra were measured on an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, England).

The solutions were concentrated on a rotary evaporator under reduced pressure at 40−45 °C and centrifuged at 5000−11000 rpm for 10−20 min. Samples were lyophilized using a VirTis lyophilizer (USA) with a constant vacuum of <10 mTorr at −65 °C.

Monosaccharide Analysis. The samples (3−5 mg) were completely hydrolyzed with 1 mL of 2 M TFA containing *myo*-inositol (1 mg/mL) at 100 °C for 5 h. The mixture of neutral monosaccharides was converted to alditol acetates[22] and identified by gas−liquid chromatography (GLC) on a Varian 450-GC chromatograph (Varian BV, The Netherlands) equipped with a flame ionization detector using a VF_5 ms capillary column (Varian), as previously described.[23]

Removal of Protein by TCA. PPC (506.7 mg) was dissolved in 100 mL of water. The solution was cooled in a water−ice bath at 4 °C for 10 min, after which 100 mL of aqueous 20% (m/v) TCA was added. After stirring thoroughly for 15 min at 4 °C, the mixture was centrifuged at 4000 rpm at 4 °C for 15 min. The resulting pellet was mixed with water, dialyzed, and lyophilized to yield the PPC_1 fraction (258.9 mg). The supernatant was concentrated and precipitated with a 4-fold volume of 96% ethanol. The precipitate was separated by centrifugation, dissolved in water, dialyzed, and lyophilized to yield the PPC_2 fraction (190.0 mg) (Table 1).

Ion-Exchange Chromatography of PPC on DEAE-Cellulose. PPC (80 mg) was dissolved in 3 mL of 0.01 M NaCl and fractionated on a DEAE-cellulose OH^--form column (34.5 cm × 2.2 cm). Fractions were eluted consecutively with 0.01, 0.1, 0.2, 0.3, and 0.4 M NaCl at a flow rate of 1 mL/min. Fractions were analyzed by the phenol−sulfuric acid test for carbohydrates.[24] Fractions corresponding to separate peaks were combined, concentrated, dialyzed, and lyophilized. Four polysaccharide fractions were obtained as follows (Table 1): PPC_4 was eluted with 0.1 M NaCl (35 mg); PPC_5 was eluted with 0.2 M NaCl (14.4 mg); and two minor fractions were eluted with 0.01 M NaCl (4.9 mg) and 0.3 M NaCl (5.0 mg).

Partial Acidic Hydrolysis of PPC. PPC (443 mg) was heated in 1 M TFA (88.6 mL) at 100 °C for 5 h. The insoluble residual material was separated by centrifugation (11 000 rpm for 20 min) and discarded. TFA was removed by evaporation; the supernatant was precipitated with a 4-fold volume of 96% ethanol. The precipitate was separated by centrifugation, dissolved in water, dialyzed, and lyophilized to produce the galacturonan fraction (PPC_3) (50.1 mg) (Table 1).

Enzymatic Digestion of PPC. PPC (265 mg) was dissolved in water (20 mL). Then 0.5 mL of an aqueous pectinase solution (16 mg, activity 690 U/mg, EC 3.2.1.15, Sigma, Germany) was added, and the mixture was incubated at 37 °C for 5 h. The digestion was controlled according to Nelson[25] to estimate the reducing sugar quantities. The pectinase was inactivated by boiling, and the insoluble residual material was removed by centrifugation. The supernatant was concentrated and precipitated using a 4-fold volume of 96% ethanol. The precipitate was separated by centrifugation, dissolved in water, dialyzed, and lyophilized to yield the polysaccharide fraction PPC_6 (120 mg) (Table 1). The alcoholic supernatant was concentrated, creating the mono/oligosaccharide fraction. The monosaccharide composition of this fraction was determined using paper chromatography (Filtrak FN-12, butanol−pyridine−water, 6:4:3). The sugars were detected using aniline hydrogen phthalate followed by heating at 105 °C.

Determination of βG Activity. Enzyme activity was measured according to a previously described method.[7] βG (1 mg) was dissolved in 0.5 mL of phosphate buffer. The mixture was incubated in a glass container with a flat bottom with constant shaking for 1 h at room temperature. In each well of a microplate, 75 μL of the incubation mixture and substrate was added. The reaction was performed at room temperature for 1 h and was stopped by adding 150 μL of the alkaline mixture. The optical density of the colored product was measured using a microplate spectrophotometer at a wavelength of 540 nm. Under these conditions, 1 mg of βG showed an activity equal to 192.0 ± 4.7 Sigma units (mean ± SD; n = 34).

Measuring the Effect of PPCs on βG Activity. Each sample was analyzed in a series of 4−6 experiments. Each experiment analyzed samples in 6−8 replications (N = 34). PPC fractions were added to an enzyme solution at a final concentration of 0.1%. Further procedures were conducted as described above. βG activity in the presence of PPC fractions was expressed as a percentage of the activity of pure enzyme.

Measuring the Adsorption of βG onto PPCs. Each sample was analyzed in a series of four experiments. Each experiment analyzed samples in triplicate (N = 12). Incubation of the enzyme with the PPC fractions was conducted as described above. Then, the incubation mixture was transferred to the concentrator and centrifuged for 30 min at 12 000 rpm at room temperature. The solution that passed through the membrane of the concentrator was used for the determination of βG activity as described above.

Statistical Analysis. The STATISTICA 6.0 software package was used for statistical analysis. Data are presented as the mean ± standard deviation. Factorial ANOVAs were also performed.

RESULTS

Purification and Degradation of PPC. The PPC sample was isolated from commonly used white cabbage using conditions that simulated a gastric environment (37 °C for 4 h without pepsin).[18] Chemical analysis of the samples showed that they contain both carbohydrate and protein components (Table 1). A complete acidic hydrolysis of the extracted PPC with 2 M aqueous TFA at 100 °C for 5 h identified the following monosaccharides in the hydrolysate of the carbohydrate portion: GalA, Rha, Ara, Gal, Glc, Xyl, and Man. These sugars constitute the sugar chains of the PPC macromolecule (Table 1).

Treatment of PPC with aqueous TCA showed that the polysaccharide−protein fraction PPC_1 contained considerable amounts of protein (~30%), and the polysaccharide fraction PPC_2 contained protein in trace amounts (Table 1). The polysaccharide fraction PPC_2 was shown to contain mainly GalA residues and had a DM below 50%. In addition, the sugar chains of PPC_2 were shown to contain Gal, Ara, and Rha in the same quantities as PPC (Table 1). These sugars make up the macromolecule carbohydrate chains and are typical components of pectins.

The isolated PPC was subjected to ion-exchange chromatography on a DEAE-cellulose column, resulting in two main fractions. The PPC_4 fraction, which was eluted with 0.1 M NaCl, was found to consist mainly of GalA, Gal, Ara, and Rha residues in the same quantities as the PPC. Most of the protein component was found in the PPC_5 fraction, which was eluted with 0.2 M NaCl (Table 1).

The isolated PPC was subjected to hydrolysis with 1 M TFA to afford the galacturonan fraction, PPC_3, which contained ~80% galacturonic acid. The neutral monosaccharides Rha and Ara were detected at trace levels of less than 2% (Table 1). These data show that galacturonan constitutes the pectin backbone in the PPC. The high positive specific rotation of the galacturonan fraction PPC_3, $[\alpha]^{20}_D$ +180.9 (*c* 0.16; water), suggests the alpha-configuration of the glycoside linkages in the backbone of galacturonan.

An insignificant cleavage of the PPC pectin carbohydrate chain was observed during pectinase treatment. Pectinase digestion of the isolated PPC yielded some free GalA and oligogalacturonides. Free GalA was detected using paper chromatography of the supernatant after digestion (data not shown). The residual material resistant to 1,4-α-D-polygalacturonase activity was designated as the polysaccharide PPC_6 fraction, which contained protein in low amounts. The PPC_6 sugar chain was shown to consist primarily of GalA, Ara, and Gal residues (Table 1). These data demonstrate that the PPC_6 fraction contains fragments of branched rhamnogalacturonan from the white cabbage pectin.

Impact of PPC Components on βG Activity. These data indicate that cellulose and PPCs increase βG activity to varying degrees ($F_{7,264}$ = 102.59; p < 0.00001; η^2 = 0.74) (Figure 1).

The greatest stimulation of βG activity (more than 50%) was achieved in the presence of the PPC and the PPC_1 fraction. Cellulose and the PPC_3, PPC_4, and PPC_5 fractions have moderate stimulatory effects on βG activity: the enzyme activity was increased by ~20% in their presence (post hoc comparisons, Tukey's test, p < 0.00001). The PPC and PPC_1 fraction had a stronger influence on βG activity than cellulose and the PPC_3, PPC_4, and PPC_5 fractions (post hoc comparisons, Tukey's test, p < 0.00001) (Figure 1). The PPC_2 and PPC_6 fractions did not exhibit a change in βG activity (post hoc comparisons, Tukey's test, p > 1.0).

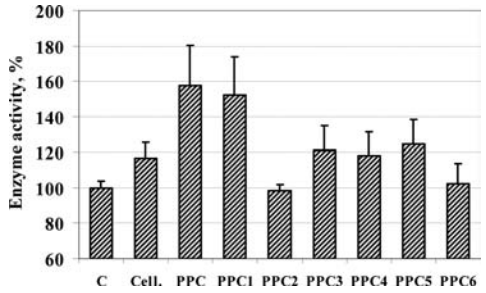

Figure 1. Impact of cabbage PPCs on the βG activity. C, control; error bars represent SD.

According to one-way ANOVA, only the protein contents of the PPC samples have a significant influence on the βG activity ($F_{1,5}$ = 33.70; p < 0.002; η^2 = 0.87) among all structural components of cabbage PPC. Samples containing more protein more strongly stimulated βG activity (Figure 2).

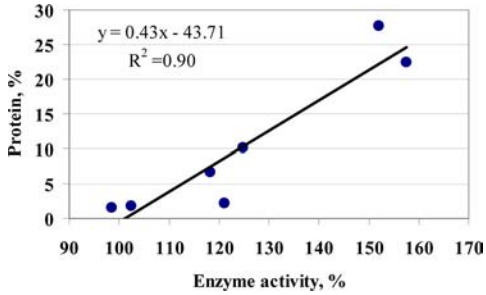

Figure 2. Analysis of relationship between the content of protein in the samples of PPCs and their ability to increase the activity of βG.

Adsorption of βG onto PPCs. All of the PPCs studied here adsorb βG in varying degrees ($F_{7,88}$ = 105.37; p < 0.00001; η^2 = 0.90) (Figure 3). Minimal affinities are observed in the PPC_2 and PPC_6 fractions. Cellulose has a higher affinity for βG than the PPC_2 and PPC_6 fractions (post hoc comparisons, Tukey's test, p < 0.0001). The PPC, PPC_1, and PPC_3 fractions adsorbed βG more effectively than cellulose (post hoc comparisons, Tukey's test, p < 0.0001). The PPC_4 and PPC_5 fractions had the maximal affinity for βG (post hoc comparisons, Tukey's test, p < 0.0001) (Figure 3).

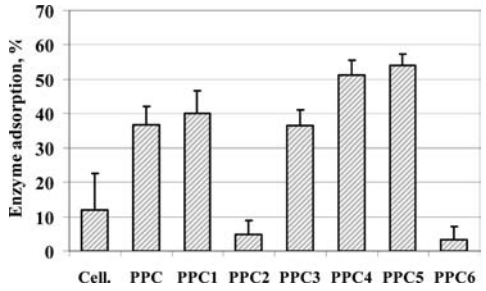

Figure 3. Adsorption of βG on the cellulose and cabbage PPCs. Error bars represent SD.

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Adsorption of βG in the PPC fractions did not depend on the protein content of the sample ($F_{1,5}$ = 1.15; p = 0.332) but significantly depended on the DM of its carbohydrate component ($F_{1,4}$ = 8.84; p < 0.041; η^2 = 0.69). There is an inverse relationship between the content of methoxy groups in the sample and its affinity for βG (Figure 4).

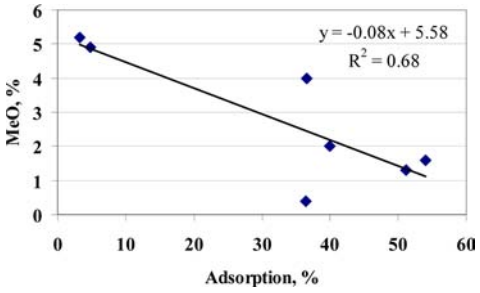

Figure 4. Dependence of PPCs' affinity to βG on the degree of their methoxylation.

DISCUSSION

To evaluate the physiological impact of food-derived pectins, we used a fluid that simulates the gastric environment to extract pectins from vegetables. Pectin fractions extracted from white cabbage using a saline solution containing hydrochloric acid at 37 °C with and without pepsin were reported to have protein contents of 9.3% and 26.2%, respectively.[6] The present study demonstrates that the PPCs of white cabbage contain pectin with a monosaccharide composition (Table 1) similar to pectin previously isolated by sequential aqueous ionic solutions and pure water extraction and heating to 50−100 °C.[26]

In this study, we tested the biological activity of cabbage PPC fractions, which allowed us to study in detail the role of separate PPC components in the stimulation of βG enzymatic activity and the adsorption of this microbial enzyme.

To separate protein components, free proteins and pectin PPCs were fractionated using TCA precipitation in cold water and ion-exchange chromatography. The PPC_1 and PPC_5 fractions contained considerable amounts of protein (20−30%). The PPC_2 and PPC_3 fractions had low protein contents (2−7%). These data demonstrate the difficulty of separating protein from the pectin chains. The protein cannot be separated from the pectin by chromatography. These data suggest that PPCs isolated from white cabbage contain a mixture of pectin polysaccharides and protein−pectin complexes that appeared to be physically associated. At the same time the covalent linkage between polysaccharide and protein chains should be proved in more detail in future investigations. Acidic hydrolysis of the PPCs yielded a fraction containing the polysaccharide fragment of the pectin backbone (the galacturonan fraction PPC_3). Hydrolysis using 1,4-α-D-polygalacturonase created the polysaccharide fragment (PPC_6) that contains the branched region of the pectin macromolecule.

These results show that βG activity increased by approximately 20% in the presence of cellulose and the PPC_3, PPC_4, and PPC_5 fractions and more than 50% in the presence of the PPC and PPC_1 fraction. A direct relationship between protein content in the PPC fractions and βG activity indicates that white cabbage PPC preparations contain a protein that increases βG activity. However, the adsorption of this enzyme is mainly dependent on the DM of the polysaccharide component of the PPC. Pectin is known to be a porous supramolecular complex. Previous studies[27] have shown that the pore size of this supramolecular complex is inversely proportional to the DM of macromolecules. Microbial βG functions as a high molecular weight tetramer (290 kDa), causing its adsorption to be possible only with PPCs with low DMs and sufficiently large pore sizes.

These findings suggest that the ability of cabbage PPCs to increase the activity and adsorption of βG is dependent on different structural components. βG activity is increased by the presence of the PPC protein components, whereas adsorption is dependent upon low-methoxylated pectin side chains. We have not studied these phenomena *in vivo*; however, given the properties of the enteric environment, it is possible to predict the structural changes and physiological activities of cabbage PPC in the human digestive tract. The proteolytic enzymes of the digestive tract can break down the protein components of PPC.[18] Therefore, we can assume that cabbage PPCs in the large intestine are analogous to the low-protein content fractions. According to our data, these PPC derivatives lose the ability to increase the βG activity but maintain the ability to adsorb this enzyme and interrupt the enterohepatic circulation of estrogens and accelerate their excretion from the organism. In other words, cabbage PPC should be converted to its low-protein form under the influence of enteric factors, raising its cancer-protective properties.

In summary, this study has shown that the galacturonan core of cabbage pectin does not adsorb βG and does not affect its activity. The affinity of cabbage PPCs to the *E. coli* βG enzyme is inversely proportional to the DM of the polysaccharide component of the PPC. Samples of PPC_4 and PPC_5 adsorbed the highest proportions of βG (51.2% and 54%, respectively), whereas the ability to enhance βG activity mainly depended on the protein components of the PPC. The PPC and PPC_1 fraction had the greatest ability to increase βG activity (by 57.6% and 52.1%, respectively). Further studies are needed to identify the type of protein−pectin linkages and to characterize the nature of the pectin chains of the PPC_1 and PPC_5 fractions. Isolation and characterization of the protein components are also needed to ascertain their roles in the biological activity of PPC.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

βG, β-glucuronidase; PPC_{1-6}, fractions of pectin−protein complex isolated from white cabbage; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; GalA, galacturonic acid; Rha, rhamnose; Ara, arabinose; Gal, galactose; Glc, glucose; Xyl, xylose; Man, mannose; OMe, methyl ester groups; DM, degree of methyl esterification, ratio of methylesterified galacturonic acid groups to total galacturonic acid groups

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on March 11, 2013, with errors to Table 5. The corrected version was reposted on March 15, 2013.