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Mechanism of Interactions between Calcium and Viscous Polysaccharide from the Seeds of *Plantago asiatica* L.

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ABSTRACT: The present study aimed at investigating the mechanism of interactions between calcium and the psyllium polysaccharide. *Plantago asiatica* L. crude polysaccharide (PLCP) was subjected to ethylenediaminetetraacetic acid (EDTA) to yield calcium-depleted polysaccharide named PLCP-E. There was essentially no difference in the structure between PLCP-E and PLCP. However, PLCP-E exhibited a much lower apparent viscosity compared to that of PLCP. PLCP was treated with sodium hydroxide to deplete ferulic acid. The resultant material was named PLCP-FAS, which also exhibited lower viscosity. Adding Ca^{2+} could both increase apparent viscosity of PLCP-E and PLCP-FAS, but only PLCP-E could keep the high viscosity when dialysis was carried out to remove free Ca^{2+} in the solution. Thermal analysis showed that the thermal stability of the polysaccharide was reduced after EDTA chelation. Scanning electron microscopy (SEM) analysis showed that PLCP-E was flaky and curly aggregation, while PLCP was mostly filamentous in appearance. The results suggested that there are strong interactions between Ca^{2+} and the polysaccharide. The interactions contributed to the high viscosity, weak gelling property, and thermal stability of the polysaccharide.

KEYWORDS: *Plantago asiatica L., polysaccharide, calcium, apparent viscosity, thermal stability, morphology*

INTRODUCTION

The *Plantago* genus (also called psyllium) has 200 species widely distributed in the world and is used in folk medicine, functional food, and dietary supplemental products.[1,2] Many polysaccharides from the husk of seeds of the *Plantago* family were identified to be arabinoxylan.[3−7] These polysaccharides were used for a variety of drug delivery methods[8−11] and exhibited excellent bioactivities, including lowering plasma lipids,[12] immunomodulating,[13,14] delaying gastric emptying, and improving constipation by increasing stool weight.[15] The health benefits of the polysaccharide are highly related to its viscosity and gelling properties.[7,16−18]

There are several reports about the rheological characters of the polysaccharide. Alkaline-extracted polysaccharide from *Plantago ovata* Forsk showed weak gel properties, with the elastic modulus (G') exceeding the viscous modulus (G'') throughout the whole range of frequency tested.[19] Farahnaky et al. reported[20] that water-soluble polysaccharide also showed gelling properties. Concentration, temperature, and pH value could influence the rheology characters. Guo et al. reported[21] that psyllium polysaccharide could form a weak gel-like structure with a fibrous appearance, and Ca^{2+} had a significant influence on the gelling properties. The polysaccharide upon the addition of Ca^{2+} showed more resistance to temperature change. However, the polysaccharide was neutral,[3] and the authors did not give a detailed reason. Other researchers had found that psyllium could reduce Ca bioactivity.[22,23] Our preliminary studies also showed that there was enrichment of Ca during polysaccharide purification. These observations suggested that there would be some interactions between Ca and psyllium polysaccharide.

Therefore, the objective of this study is to investigate the interactions between Ca and psyllium polysaccharide. *Plantago asiatica* L. crude polysaccharide (PLCP) was prepared from the seeds of *P. asiatica* L. PLCP was treated first with ethylenediaminetetraacetic acid (EDTA) to deplete Ca and named as PLCP-E. Rheological properties of PLCP and PLCP-E were studied at different Ca^{2+} concentrations. Furthermore, ferulic-acid-depleted polysaccharide (PLCP-FAS) was obtained and subjected for rheological analysis. Scanning electron microscopy (SEM) was used in morphology observation for PLCP and PLCP-E. Thermal stability properties of PLCP and PLCP-E were also determined.

EXPERIMENTAL SECTION

Materials and Sample Preparation. The seeds of *P. asiatica* L. were purchased from Ji'an County, Jiangxi Province, China. PLCP was isolated and purified from the seeds of *P. asiatica* L. as described in our previous report.[4] Briefly, the seeds of *P. asiatica* L. (100 g) were defatted with ethanol (80%, v/v) and dried in the open air. They were extracted with boiling water (1000 mL) for 3 h. The aqueous extract was centrifugated, filtered, concentrated, and deproteined. The resulting aqueous solution was dialyzed and precipitated by ethanol at a final concentration of 80% for more than 12 h. After centrifugation, the precipitate was washed with anhydrous ethanol, dissolved in water, and lyophilized to yield crude polysaccharide PLCP (6.15 g). PLCP was subjected with 8.0% EDTA at pH 8 for 6 h to deplete Ca. Then, it was dialyzed against ultrapure water (18.2 MΩ) from a Milli-Q water purification system (Millipore, Bedford, MA) and freeze-dried to obtain PLCP-E. $CaCl_2$, NaCl, and monosaccharide standards (mannose, rhamnose, ribose, galactose, xylose, arabinose, fucose, and glucose) were obtained from Sigma Chemical Co. (St. Louis, MO). EDTA was

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from Caledon Laboratories, Ltd. (Georgetown, Canada). All other chemicals and reagents were of analytical grade.

Physicochemical and Structure Analysis. Mineral analysis was conducted at the Center of Analysis and Testing (Nanchang University, China), using inductively coupled plasma−atomic emission spectrometry (ICP−AES) (Optima 5300DV, Perkin-Elmer Corporation, Waltham, MA). The protein content was determined by a photometric assay[24] using bovine serum albumin as the standard. The uronic acid content was determined using the *m*-hydroxybiphenyl photometric procedure with D-glucuronic acid as the standard.[25] The total sugar content was determined using the phenol−sulfuric acid colorimetric method with xylose as the standard.[26,27] Polysaccharide was hydrolyzed with 2 M H_2SO_4 at 100 °C for 2 h and applied for monosaccharide compositions analysis using a high-performance anion-exchange chromatography system (Dionex, DX500, Sunnyvale, CA) coupled with a pulsed amperometric detector.[28]

Ferulic acid was extracted with 2.0 M NaOH according to the method by Nordkvist et al.[29] and determined by the high-performance liquid chromatography (HPLC) method. The ferulic-acid-depleted polysaccharide solution was collected, dialyzed, freeze-dried, and defined as PLCP-FAS.

Rheological Measurement. PLCP, PLCP-E, and PLCP-FAS were dissolved completely in ultrapure water (1.0%, w/v) at 55 °C with constant stirring for 2 h and cooled to room temperature. These were also carried out with PLCP, PLCP-E, and PLCP-FAS at concentrations of 0.1, 0.25, 0.5, and 2.0% (w/v) in ultrapure water and 1.0% in 0.001, 0.01, 0.1, and 1.0 M $CaCl_2$ solutions. The rheological properties of samples were measured with cone and plate (50 mm diameter with a gap of 0.047 mm) or parallel plate (50 mm diameter with a gap of 0.500 mm) geometry in an ARES rheometer (TA Instruments, New Castle, DE). Samples with low viscosity were measured with cone and plate geometry. The parallel plate was preferred to test sample solutions with high viscosity. The temperature was controlled by a SR5 Peltier Circulator (TA Instruments, New Castle, DE) at 25.0 °C.

PLCP, PLCP-E, or PLCP-FAS (2 mg/mL) were dissolved in water and added with $CaCl_2$ (0.5 M), respectively. They were kept under stirring at 55 °C for 20 h, then dialyzed, and lyophilized. The obtained samples were named as PLCP-Ca, PLCP-E-Ca, or PLCP-FAS-Ca, and they were carried out for rheological measurement.

Thermogravimetric Analysis (TGA). TGA was carried out with a simultaneous thermal analyzer TG/DTA Pyris Diamond (PE Instruments, Waltham, MA), under a nitrogen atmosphere at a flow rate of 100 mL/min with a 10 °C/min heating rate in the temperature range of ambient to 700 °C, using a platinum crucible. The activation energy (E_a) for the major decomposition stage was calculated by the Broido method[30]

$$\ln\left(\frac{1}{y}\right) = -\frac{E_a}{RT} + \text{constant}$$

where $y = (w_t - w_\infty)/(w_0 - w_\infty)$, w_t is the weight of the sample at any time, w_0 is the original weight of the polysaccharide, and w_∞ is the final weight of the polysaccharide. Plotting of the left-hand side of this equation against $1/T$ gives a slope, which gives the E_a value. Data were analyzed by the use of Muse Analysis software (version 3.5U, PE Instruments, Waltham, MA) and MS Excel 2010.

SEM Observation. Freeze-dried PLCP and PLCP-E samples were placed on the sample stage and coated with a thin layer of gold in a model IB-3 ion coater (Eiko Corp., Mito, Japan). Then, they were analyzed in a Quanta 200F SEM (FEI Company, Hillsboro, OR) and viewed at an accelerating voltage of 30 kV.

RESULTS

Mineral Analysis. The results showed sodium (Na), potassium (K), magnesium (Mg), and calcium (Ca) contents among the three samples from the seeds of *P. asiatica* L. (Table 1). The Ca content in the seeds was 1293.0 μg/g and increased to 5094.4 μg/g in water extract. The Ca content in PLCP was 11871.3 μg/g. Mg and Na demonstrated similar trends, as their contents increased from seeds to PLCP. However, their contents were much lower than that of Ca. In contrast, the content of K decreased obviously with the increasing purity of polysaccharide (from 8713.2 to 501.2 μg/g). These results suggested that there was probably some relationship between Ca and the polysaccharide.

Table 1. Mineral Composition of Different Samples from the Seeds of *P. asiastica* L.

	seeds (μg/g)[a]	water extract (μg/g)[a,b]	PLCP (μg/g)[a]
Na	162.0 ± 39.7	931.1 ± 162.2	1383.0 ± 208.4
K	8713.1 ± 843.0	44661.6 ± 499.1	501.2 ± 50.3
Mg	2306.2 ± 254.8	2979.6 ± 116.7	3360.3 ± 70.3
Ca	1923.0 ± 248.7	5094.4 ± 826.6	11871.3 ± 337.4

[a]Data were shown as the mean ± standard deviation (SD); $n = 3$. [b]Seeds were extracted with boiling water and precipitated by 80% ethanol to obtain water extract.

Effect of EDTA Chelation on the Structure of PLCP. EDTA, a strong chelator, was used to deplete Ca^{2+} ions and other minerals from PLCP to obtain PLCP-E. The content of calcium in the polysaccharide preparation reduced significantly after EDTA chelation, from 11 871.3 to 480.5 μg/g. Contents of Na, K, and Mg in PLCP-E were 8830, 160, and 59.23 μg/g, respectively. Monosaccharide analysis results (Table 2) showed that PLCP was composed of Rha (2.28%), Ara (20.65%), Xyl (72.17%), Glc (0.92%), and Gal (3.98%). There was 22.87% Ara and 72.23% Xyl in PLCP-E. There was no change of the ratio between Ara and Xyl before and after EDTA treatment. The results suggested that EDTA chelation had no effect on the structure of the polysaccharide.

Rheological Properties. *Rheological Properties of PLCP.* The flow behavior of PLCP at concentrations from 0.1 to 2.0% is shown in Figure 1a. The curves of PLCP solutions at the concentration higher than 0.50% showed shear thinning flow behavior, as the viscosity decreased with an increase of the shear rate. It was much more obvious when the polysaccharide concentration was higher than 1.0%. For lower concentrations, there was less change of the apparent viscosity with the increase of the shear rate.

Figure 1b shows the effect of Ca^{2+} on the apparent viscosity of PLCP. The viscosity of the polysaccharide increased significantly even when the concentration of Ca^{2+} was only 0.001 M. At the shear rate of 0.1 s^{-1}, the apparent viscosities were 18.7, 96.9, 136.7, and 217.3 Pa s for 0.001, 0.01, 0.1, and 1.0 M Ca^{2+}, respectively. All of these solutions showed shear thinning flow behavior as the viscosity decreased with an increasing shear rate. The above results suggested that Ca^{2+} could increase the solution viscosity of PLCP. The rheological properties of the polysaccharide under high concentrations of Ca^{2+} (0.01, 0.1, and 1.0 M for PLCP) near the shear rate of 100 s^{-1} were unusual. It was probably because the polysaccharide gel microstructure was broken under a high shear rate. The contribution of additional Ca^{2+} to PLCP solution was probably attributed to the interactions between Ca^{2+} and glucuronic acid (GlcA).

PLCP solution shows weak gel property from Figure 2a, because G' was always higher than G'' under detected frequency and both G' and G'' were frequency-dependent. Figure 2b shows that the weak gel property of PLCP-Ca was just equivalent to that of PLCP. It indicated that PLCP may not have a strong ability to bind free Ca^{2+} ions from solution.

Table 2. Contents of Sugar, Protein, Uronic Acid, and Monosaccharide Composition Analysis of PLCP and PLCP-E

				monosaccharide composition (%, w/w)[a]				
sample	sugar (%)[b]	protein (%)[b]	uronic acid (%)[b]	Rha	Ara	Xyl	Glc	Gal
PLCP	68.22[c]	1.45[c]	16.62[c]	2.28 ± 0.37	20.65 ± 0.21	72.17 ± 0.29	0.92 ± 1.30	3.98 ± 0.87
PLCP-E	69.19 ± 1.20	0.82 ± 0.10	11.83 ± 0.23	1.65 ± 0.48	22.87 ± 0.50	72.23 ± 0.05	nd[d]	3.24 ± 0.09

[a]Data are shown as the mean ± SD; n = 2. [b]Data are shown as the mean ± SD; n = 3. [c]Data are from Yin et al.[4] [d]nd = not determined.

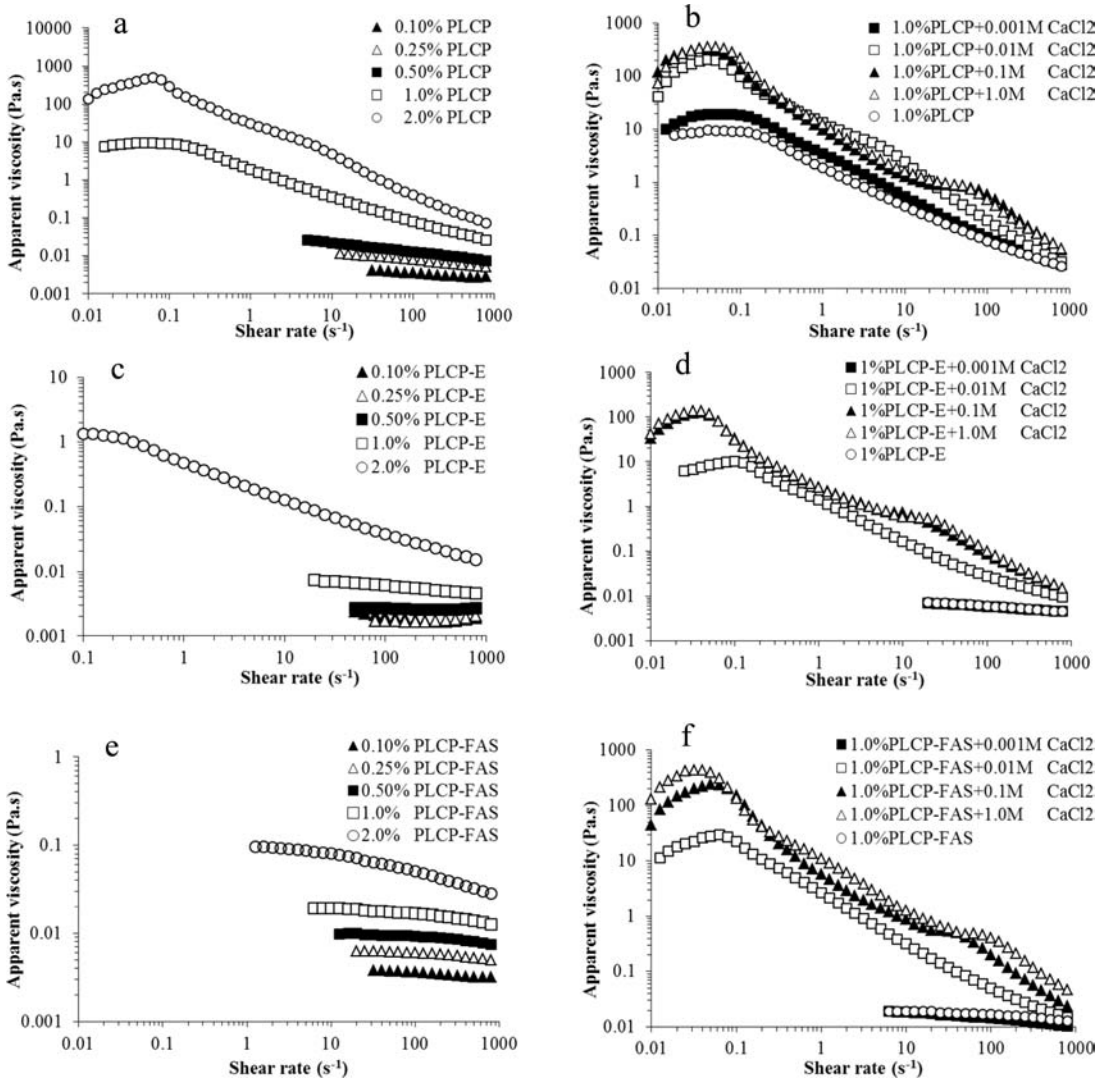

Figure 1. Apparent viscosity of (a and b) PLCP, (c and d) PLCP-E, and (e and f) PLCP-FAS, under different concentrations of polysaccharide (0.10, 0.25, 0.50, 1.0, or 2.0%, w/v) or $CaCl_2$ (0.001, 0.01, 0.1, or 1.M) at 25 °C.

Rheological Properties of PLCP-E. Panels c and d of Figure 1 show effects of the polysaccharide concentration and Ca^{2+} on the apparent viscosity of PLCP-E. In comparison to PLCP, the apparent viscosity of PLCP-E reduced significantly. For example, it was 291.9 Pa s for 2.0% PLCP at the shear rate of 1 s^{-1} at 25 °C, while it was only 1.3 Pa s for 2.0% PLCP-E at the same shear rate. Only 2.0% PLCP-E showed shear thinning flow behavior.

Upon the addition of Ca^{2+}, the apparent viscosity of PLCP-E increased significantly. It was 10.1 Pa s at the shear rate of 0.1 s^{-1} when Ca^{2+} was 0.01 M. The apparent viscosity increased to 33.1 Pa s when the Ca^{2+} concentration was increased to 0.1 M. However, there was little change for viscosity any more when the concentration of Ca^{2+} increased to 1.0 M. The gelling property of PLCP-E-Ca increased and was equivalent to that of PLCP (Figure 2c). It suggested that Ca-depleted polysaccharide PLCP-E had the ability of binding free Ca^{2+} ions from water.

Rheological Properties of PLCP-FAS. Previous studies reported that the high viscosity of water-extractable arabinoxylans could form gel under oxidative coupling of two ferulic acid residues by certain oxidizing agents.[31−33] However, Guo et al.[21] reported that Ca^{2+} had a significant influence on the gelling properties of the polysaccharide from psyllium, while the polysaccharide was neutral.[3] No oxidizing agent was added to the polysaccharide. The results suggested that interactions between Ca^{2+} and some possible organic acid probably contributed to the strong gel-forming properties of the polysaccharide. PLCP investigated herein was acidic polysaccharide.

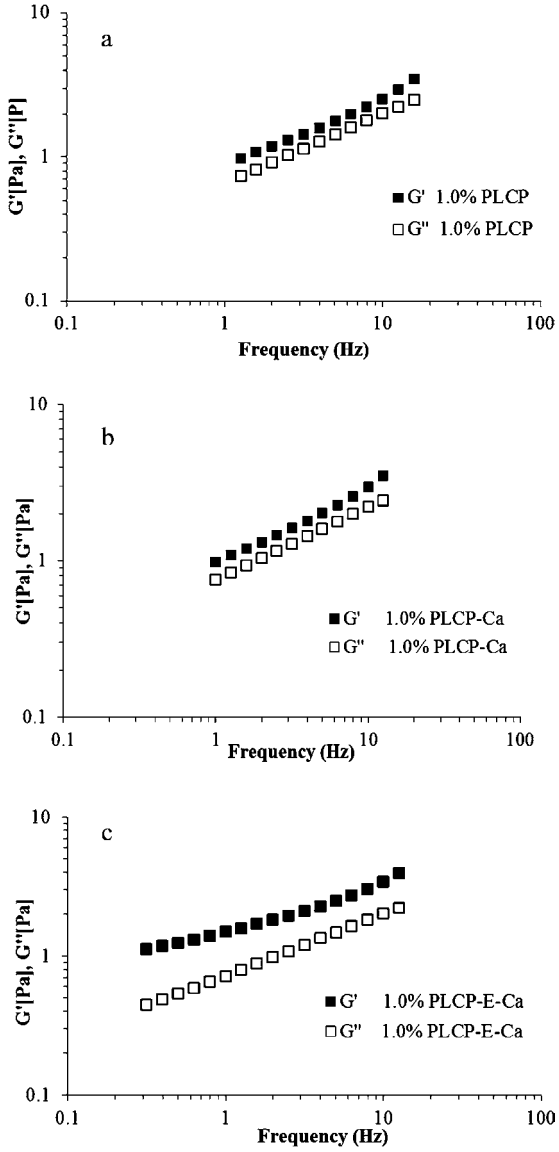

Figure 2. Mechanical spectra showing the variation of G' and G'' with a frequency of (a) 1.0% PLCP, (b) 1.0% PLCP-Ca, and (c) 1.0% PLCP-E-Ca, with 10% strain at 25 °C.

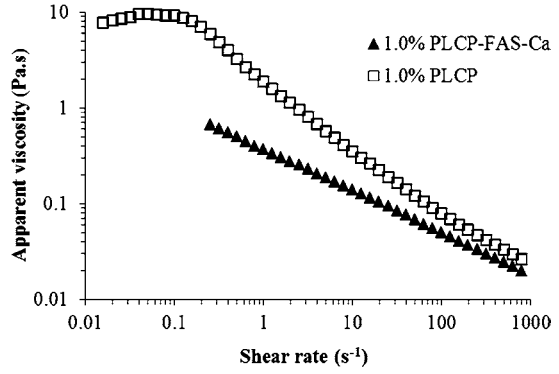

Figure 3. Apparent viscosity of 1.0% PLCP-FAS-Ca at 25 °C.

Therefore, whether Ca^{2+} interacted with uronic acid or another organic acid needed to be clarified, to better understand the interaction mechanism.

Ferulic acid was determined to be 0.61 mg/g in PLCP. It was found from Figure 1e that the apparent viscosity of the polysaccharide reduced significantly, when ferulic acid was depleted from PLCP. However, the lost viscosity can be regained when Ca^{2+} was added to PLCP-FAS solutions, as shown in Figure 1f. The higher the concentration of Ca^{2+}, the higher the apparent viscosity of the solution of the polysaccharide. However, the apparent viscosity of PLCP-FAS-Ca (Figure 3) was much lower than that of PLCP.

TGA. The thermal properties of the polysaccharide were achieved by TGA. Figure 4 shows the thermogravimetry (TG), differential thermogravimetry (DTG), and differential thermal analysis (DTA) curves of PLCP and PLCP-E in a N_2 atmosphere. For PLCP, there was only a decrease in weight (9.9%) from ambient temperature to 204.6 °C, which corresponded to water evaporation or desorption, as described by earlier studies.[34,35] The first major stage of decomposition was characterized by the initial decomposition temperature (IDT) of 204.6 °C and the final decomposition temperature (FDT) of 345.8 °C. It resulted in 45.2% weight loss, with a sharp DTG peak. In the second stage of decomposition, the IDT was 345.8 °C and the FDT was 677.5 °C. The weight loss in this stage was

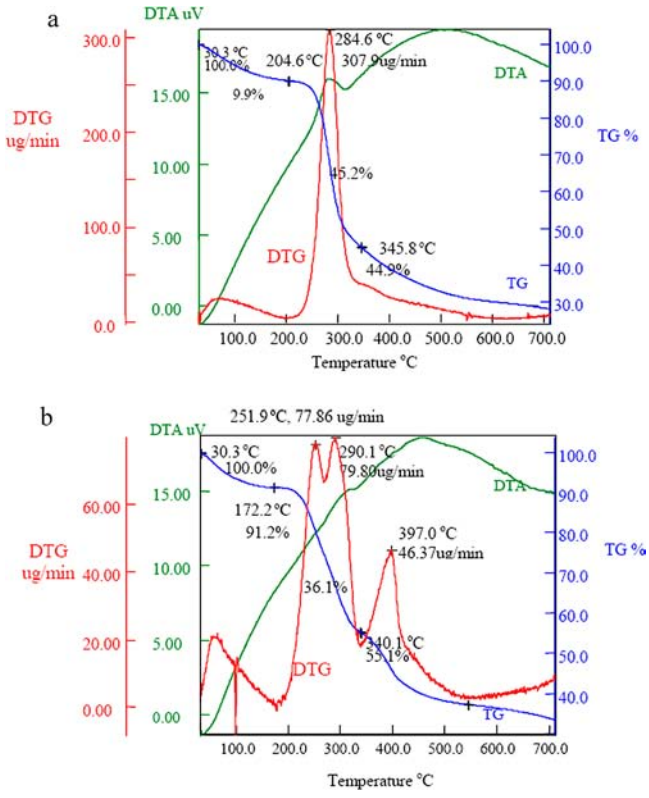

Figure 4. TGA results of (a) PLCP and (b) PLCP-E. They were carried out under a nitrogen atmosphere at a flow rate of 100 mL/min with a 10 °C/min heating rate in the temperature range of ambient to 700 °C using a platinum crucible.

Table 3. Activation Energies Calculated by the Broido Method[30] for Different Samples at Their First Decomposition Stage

sample	temperature range (°C)	activation energy, E_a (kJ mol^{-1})
PLCP	204−345	244.82
PLCP-E	170−340	196.34

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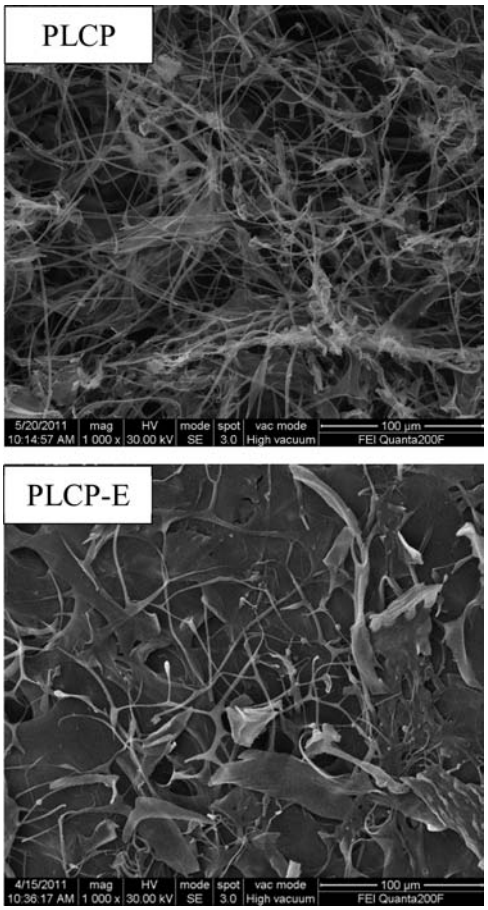


Figure 5. SEM of PLCP and PLCP-E (1000×), observed with a scanning electron microscope and viewed at an accelerating voltage of 30 kV. Both PLCP and PLCP-E were coated with a thin layer of gold before SEM observation.

approximately 16.1%; meanwhile, a wide exothermic enthalpy was detected. Shen et al.[36] reported that decomposition of xylans was a two-step process. The first step was associated with the scission of the glycosidic bond together with degradation of side-chain residues, while the second step corresponded to the breakage of the depolymerized fragments.

The effect of EDTA chelation on the thermal behavior of PLCP is shown in Figure 4b. The first major stage of decomposition began at 172.2 °C (IDT) and finished at 340.1 °C (FDT). The IDT of PLCP-E was lower than that of PLCP. There were two sharp peaks of DTG in this step. These features probably suggested that de-cross-linking took place during the EDTA chelation to PLCP.

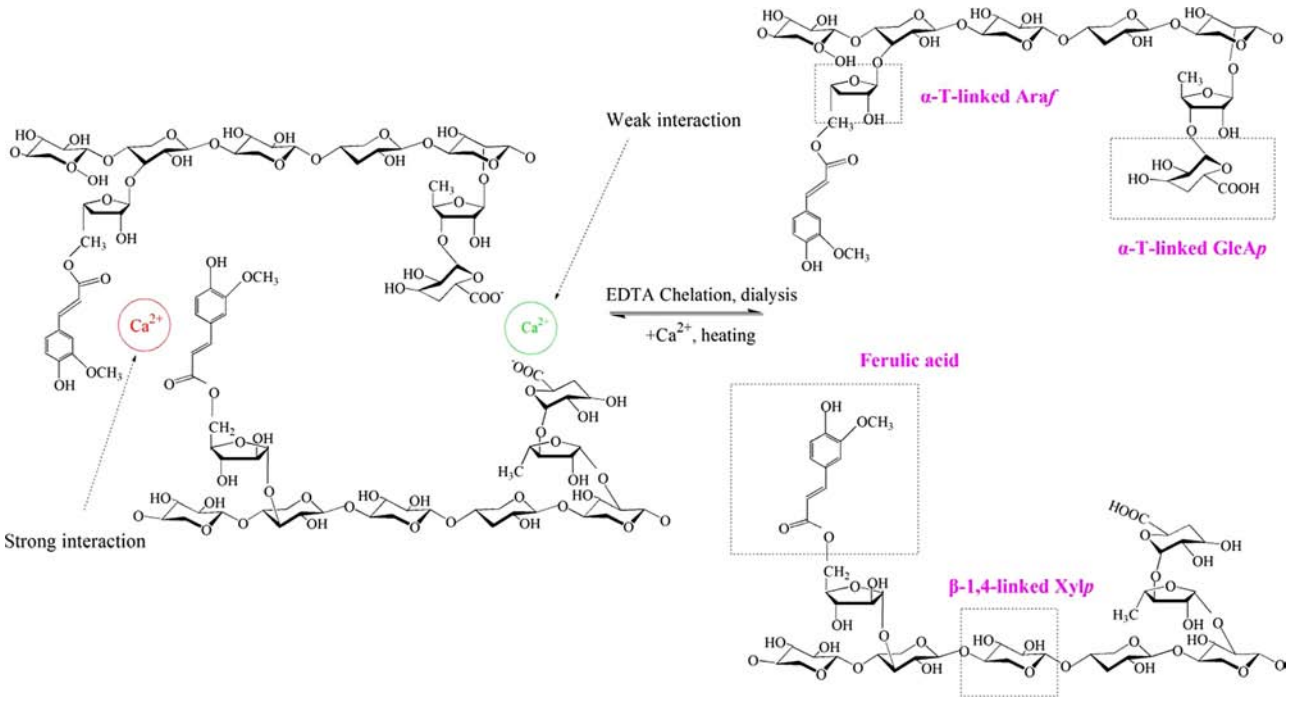
Thermal parameters E_a of the first decomposition stage of each sample, calculated from Brodio plots,[30] are presented in Table 3. Total E_a was 244.82 and 196.34 kJ mol^{-1} for PLCP and PLCP-E, respectively. These results confirmed that thermal stability of the polysaccharide was reduced after EDTA chelation.

SEM Observation. SEM micrographs of PLCP and PLCP-E powder are shown in Figure 5. The particles of PLCP were irregular in shape. Most were in linear style, and some were flaky curly aggregates. There were more random flaky curly aggregates in PLCP-E. The thickness of flakes was much more than that in PLCP. These observations suggested that EDTA chelation had an effect on the morphology of PLCP because of the removal of Ca. Ca may contribute to the filamentous appearance of the polysaccharide.

DISCUSSION

Ca^{2+} could increase the apparent viscosity of PLCP-FAS solution significantly. However, the viscosity of PLCP-FAS-Ca was much lower. It suggested that PLCP-FAS did not have a strong ability of binding free Ca^{2+} ions in the solution. At the

Scheme 1. Possible Coordination Relationship between Ca^{2+} and PLCP



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same time, the weak gelling property of PLCP-Ca was equivalent to that of PLCP. These results suggested that there were some ionic interactions between Ca^{2+} and GlcA. The low viscosity of PLCP-E and PLCP-FAS and high viscosity of PLCP suggested that there were ionic interactions between Ca^{2+} and ferulic acid. The weak gelling property of PLCP-E-Ca suggested that the interactions of Ca^{2+}−ferulic acid were much stronger than those of Ca^{2+}−GlcA. Ferulic acid was reported to be linked to O-5 of terminal arabinose residues in arabinoxylans.[33,37−39] In combination with structure information from our previous study,[4,6] a possible interaction between Ca and polysaccharide is proposed, as shown in Scheme 1. The chains of the polysaccharides were cross-linked to each other because of the ionic interactions of Ca^{2+}−ferulic acid and Ca^{2+}−GlcA interactions. Therefore, it showed high apparent viscosity, weak gel property, and good thermal stability. When EDTA was applied to deplete Ca^{2+}, the cross-linked chains disappeared. This was further confirmed by lower apparent viscosity and a poorer thermal stability of PLCP-E.

Our results may explain the effect of psyllium on reducing Ca bioavailability, which was reported by different research groups.[22,23,40] Polysaccharides from *Plantago* were arabinoxylans. They had a strong ability in binding Ca. If the binding ability was saturated during natural generation or the extraction process, it would not bind any exogenous Ca. However, it would absorb calcium *in vivo* when the binding ability was unsaturated. That lead to a decrease in Ca absorption. If the polysaccharide was hydrolyzed, its binding ability disappeared. Then, the inhibitory effects of the polysaccharide on mineral absorption were abolished.

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Notes

The authors declare no competing financial interest.

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