Polym. Bull. (2010) 65:443–454
DOI 10.1007/s00289-009-0219-8

ORIGINAL PAPER

Chemical modification of polysaccharides by the use of intramolecular associations in polar organic solvents

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Received: 20 May 2009 / Revised: 2 November 2009 / Accepted: 21 November 2009 /

Abstract Precipitation from alcohol used against fractionation of polysaccharides was applied as an energy-efficient chemical modification with only a co-precipitation of polysaccharide and the modifying agent in polar organic solvents, and drying. Addition of ethanol caused a conformational change of polysaccharides with increasing intramolecular hydrogen bonding and a dense structure in close proximity to atoms, especially, in the presence of modifying agents. Extent of modification depended on the structure of polysaccharides and pH in polar organic solvents. The co-precipitates in polar organic solvents seem to act as a precursor while still maintaining the integrity of the modified polysaccharides. Eventually, a small amount of polar organic solvents (1.5 times volume to the weight of the mixtures) to disperse the reaction mixtures was enough to complete modification.

Keywords Intramolecular association · Modification · Polar organic solvents · Polysaccharides · Citric acid

Introduction

Generally, chemical reactions are performed by the dissolution of reacting substances to an appropriate solvent with aid of heat, pressure, and stirring. With regard to chemical modification of polysaccharides which is a useful means for altering their functions [1], especially, water-soluble ones, there are some challenges of increasing viscosity limited by their inability to solubilize at high

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Electronic supplementary material The online version of this article (doi:10.1007/s00289-009-0219-8) contains supplementary material, which is available to authorized users.

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concentrations, and strong stirring for efficient reactions. As well, water-insoluble polysaccharides have a preference to only a specific solvent to dissolve [2, 3]. Numerous modifications of polysaccharides have been shown and utilized as yet [1–3], although less concerned about the consumption of time and energy. Moreover, very little chemical modification of polysaccharides in polar organic solvents but not in non-aqueous solvents [4, 5] has been performed as yet. Fortunately, we discovered an innovative method in the modification of polysaccharides irrespective of their solubility and viscosity. The clue was that the bound amounts of citric acid to citrus pectin decreased without drying of precipitates from alcohol during the fractionation of the pectin in previous study [6].

Precipitation from alcohol is generally used for fractionation of polysaccharides. Addition of dehydrating agents such as ethanol promotes intramolecular associations through water competition [7, 8]. Our chemical modification utilized the conformational change of polysaccharide molecule's own motion in polar organic solvents due to increasing intramolecular hydrogen bonding without the use of energy. In the present study, the mechanism of chemical modification of polysaccharides in polar organic solvents was investigated using three different structures and compositions of polysaccharides.

Our goal in the present study was to overcome the limitations in chemical modification by low solubility and high viscosity of water-soluble polysaccharides and to provide a cost-effective modification available for various polysaccharides. Here we show novel chemical modification with only dehydration and drying.

Experimental

Materials

Citrus pectin (galacturonic acid, 50–70%; methyl group, 3–7%)and Chitosan (Type 50, deacetylation rate 80 mol/mol% minimum) were purchased from Wako Pure chemicals (Osaka, Japan). κ-Carrageenan (Type KC-200S) was a generous gift from Mitsubishi Corporation Food Tec Company. Citric acid was used as a modifying agent for chemical modification. All other chemicals were of reagent grade.

Modification of polysaccharides

Dried powder of polysaccharides was dissolved in distilled water (2%, w/v) containing various concentrations of citric acid at different pH values. Then ethanol was promptly added to the solution at final concentrations of 60% (v/v). Additional preparation consisting of dried powder was directly added to 60% ethanol solution (v/v) including citric acid at interested pHs. The mixtures were left for 1 h at room temperature. The precipitates were collected and dried by freeze-drying or air-drying at different temperatures, usually at 60 °C for 24 h. The dried reaction mixtures were dissolved and dialyzed against distilled water. Finally, the modified polysaccharides were obtained by the freeze-drying of dialyzed solutions.

Polysaccharides were modified with citric acid or sodium citrate salts in pastes. Dried powders of polysaccharides (2 g) and the modifying agents (2–10 mmol) were added and mixed in mortar. Then 60% ethanol solution (v/v) was added at 1.5 times volume to the weight of the mixtures and followed the same procedure described above.

Conductivity and pH measurements

The change in pH and conductivity of the polysaccharide solutions was recorded on a pH meter and conductivity meter (Horiba pH/cond meter D-54, Japan).

Determination of citric acid

Citric acid was measured with the Megazyme citric acid kit by enzyme assay. Five milligrams of polysaccharide were dissolved in 3 N HCl (0.5 mL) and heated at 105 °C for 16 h, and then neutralized by NaOH solutions. The solutions were dried with a rotary evaporator at 50 °C. The dried samples were resuspended in distilled water for citric acid determination.

Measurement of swelling

Dried samples were immersed into 60% ethanol solutions including 0.08 M citric acid at the concentrations of 1.0% (w/v) at different pHs, and then left for 3 h at room temperature. The bed volumes of swollen sample were determined. The measured condition was lack of citric acid precipitates in the presence of high ethanol concentrations which resulted in decreased solubility.

X-ray diffraction analysis

Measurements were made on a Philips PW3050 diffractometer. Monochromatized Cu Kα radiation generated at 40 kV and 30 mA was used. The samples were scanned through 2θ (diffraction angle) range of 5°–70°at $2° \times 2\theta$ per minute. A step interval of 0.002° was used.

FT-IR spectroscopy

FT-IR spectrum of polysaccharide was obtained at a resolution of 1 cm^{-1}. Sample was incorporated with KBr (spectroscopic grade) and pressed into a 3 mm pellet. The 256 scans were entered before Fourier transformation. Spectra were recorded in the transparent mode from 4000 to 400 cm^{-1}, using a Spectrum 2000 (PerkinElmer Ink., MA, USA).

Viscosity measurement

Viscosity measurements were performed by using a sine-wave viscometer (SV-10, A&D, Tokyo, Japan) with a water-jacket assembly (AX-SV-37, A&D). The vibration frequency was a constant 30 Hz. Pectin and κ-carrageenan were dissolved in distilled water at room temperature at the concentrations of 1.0% (w/v). Chitosan was dissolved in 5% (v/v) acetate solution at the same concentrations. The viscosities of polysaccharides were measured at 25 °C.

Results and discussion

The modification consists of precipitation from alcohol and drying. Clarification of the mechanism for this modification means a great deal to not only the establishment of the principal for chemical modification of polysaccharides in polar organic solvents but also advance of their application to various industrial fields, thus deepening the understanding of natural phenomena. First the change in pH by addition of ethanol to polysaccharide solution (1%, w/v) was investigated. Addition of ethanol led to a pH increase in acidic polysaccharides, especially, at the pH 5 solutions, except for chitosan (Fig. 1a). The acidic polysaccharides including pectin and κ-carrageenan have more charge at pH 7 than pH 5. Considering the fact that increase in charge led to increased hydration for pectic polysaccharides [9], the slow pH increase in neutral pH solutions was the lower extent of dehydration by ethanol from the hydrated polysaccharides. In the case of chitosan which exhibits basic properties (pK_a of chitosan is 6.3), the negligible change in pH by addition of ethanol in the pH 5 solutions was undoubtedly regarded as an increase in charge, compared to the pH 7 solutions [10].

Next the effects of ethanol addition to aqueous polysaccharide's solutions on changes in pH and conductivity were investigated in the presence of citric acid (Fig. 1b). Measurement of conductivity can detect the relative changes in the ionic substances concurrent with the change in pH [11]. In all the polysaccharides'

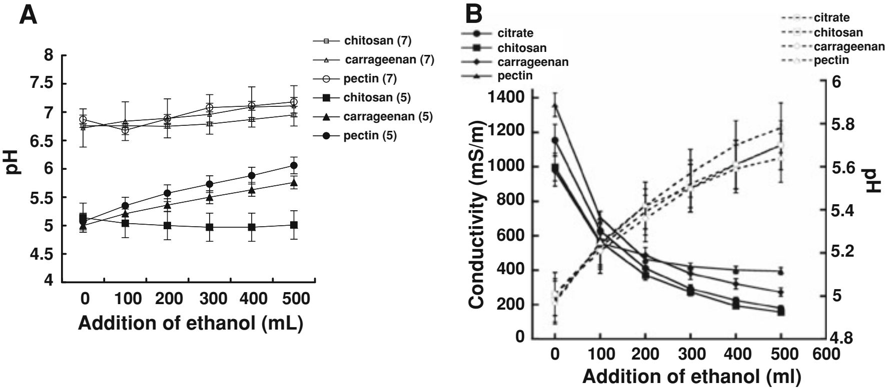


Fig. 1 Effects of ethanol on pH and/or conductivity in aqueous polysaccharide solutions in the presence and absence of citric acid. **a** Polysaccharides were dissolved with distilled water and adjusted to pH 5 or pH 7 at a final concentration of 1% (w/v) before ethanol addition. **b** Polysaccharides were prepared with the same procedure as **a** except in the presence of 0.1 M citric acid. Values are mean ± SEM of three independent experiments

solutions adjusted to pH 5, there was an increase in conductivity due to the increase in H^+ ions when compared with the sole citric acid. The observations imply that there could be an incorporation of citric acid into polysaccharide molecules with increasing ethanol concentrations.

Indeed, from the experiments by addition of dried powders of polysaccharides to polar organic solvents, the citric acid concentrations in supernatants decreased with increasing addition amounts of polysaccharides (Fig. 2a). In κ-carrageenan, the bound amounts of citric acid increased proportionally, however, these tended to decrease at the higher addition amounts in chitosan and pectin (Fig. 2b). Only κ-carrageenan had an increase in conductivity in accordance with the addition amounts (Fig. 2c). Thus, conductivity, that is to say, pH in alcohol precipitates of polysaccharides will influence the amounts of modifying agent incorporated into polysaccharide molecules. Beyond question, in chitosan (Fig. 3a) and κ-carrageenan (Fig. 3b), the bound amounts of citric acid decreased with increasing pH, while an optimum pH was at approximately pH 6.0 in pectin (Fig. 3c).

A favorable conformational change of polysaccharides in alcohol precipitates to react with the modifying agent by dehydration was examined with X-ray diffraction analysis. In the case of chitosan (Fig. 4), the alcohol precipitates at pH 7.0 exhibited

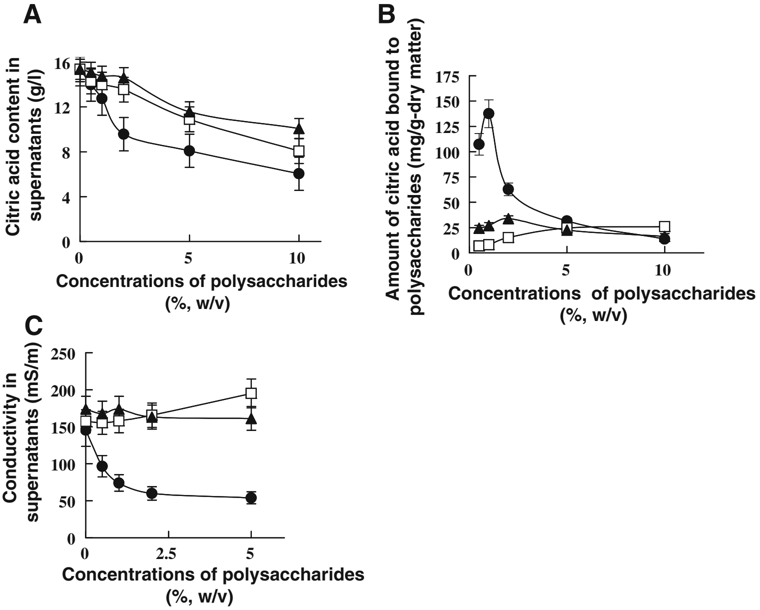


Fig. 2 Incorporation of citric acid into polysaccharides in ethanol solution. Dried powders of polysaccharides were added to 60% ethanol (v/v) including 0.08 M citric acid at pH 6.3 at various concentrations. Chitosan (*closed circles*), κ-carrageenan (*open squares*), citrus pectin (*closed triangles*). **a** Citric acid concentration in supernatants was measured after the addition of dried powders for an hour. **b** Alcohol precipitates were recovered, freeze-dried, and then dialyzed against distilled water. The dialyzed solutions were freeze-dried and the citric acid concentrations of the dried samples were measured after hydrolysis. **c** Conductivity was measured with supernatants obtained for determination of citric acid concentration. Values are mean ± SEM of three independent experiments

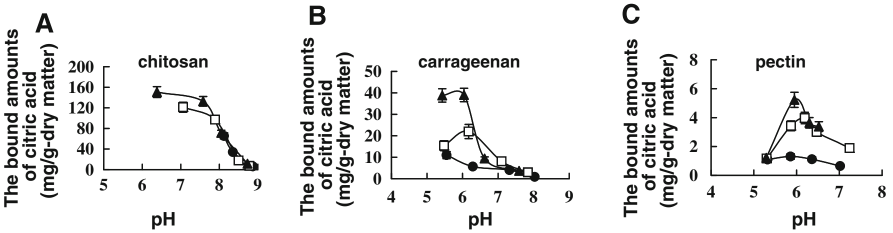


Fig. 3 Effect of pHs on incorporation of citric acid into polysaccharides in ethanol solution**.** Dried powders of polysaccharides were added to a final concentration of 5% (w/v) at different concentrations of citric acid and pHs. 0.05 M (*closed circles*), 0.08 M (*open squares*), 0.12 M (*closed triangles*). The bound amounts of citric acid to polysaccharides were measured in freeze-dried sample. **a** chitosan, **b** κ-carrageenan, **c** citrus pectin. Values are mean ± SEM of three independent experiments

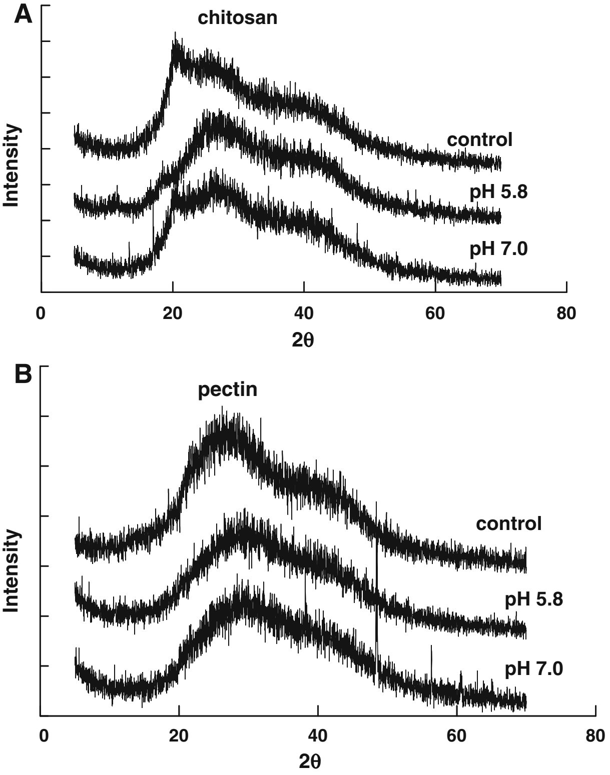
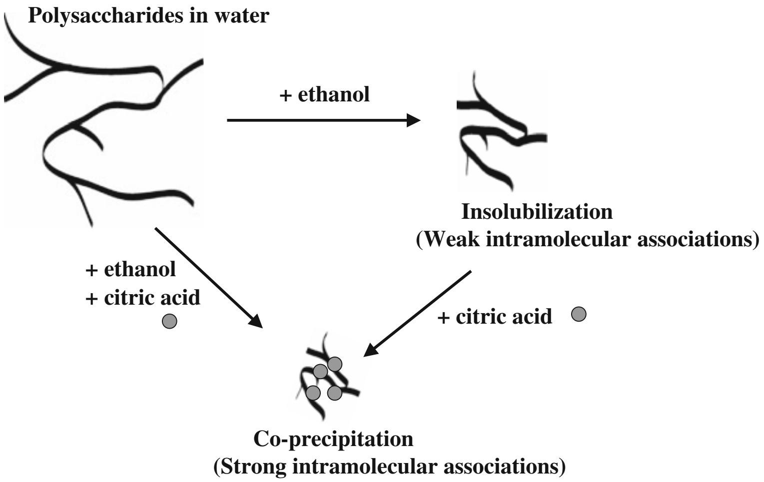


Fig. 4 X-ray diffraction analysis of polysaccharides. Dried powders of chitosan (**a**) and citrus pectin (**b**) were suspended in 60% ethanol including 0.12 M citric acid at pH 5.8, or pH 7.0 at a final concentration of 5% (w/v). The alcohol precipitates were collected and analyzed in the paste state. The experiments were repeated 3 times, with similar results

a broader peak with a significant shift to higher 2θ when compared with and without citric acid. The inclination was more obvious at pH 5.8. There was the same observation in pectin. The higher 2θ is, the closer lattice spacing of crystal is [12].



Scheme 1 Conformational transitions of polysaccharides in polar organic solvents

Thus, the alcohol precipitates of polysaccharides in the presence of citric acid would have a dense structure in close proximity to atoms. Indeed, organic acids exhibit an ability to strengthen the water–ethanol hydrogen-bonding structure [13]. The difference of 2θ shift between pectin and chitosan was regarded as the difference of their functional group's accessibility to the modifying agent. It is vital to be aware that incorporation of citric acid into polysaccharides increased as lattice spacing of crystal was closer. From our series of experiments, it can be deduced that polar organic solvents such as ethanol, caused polysaccharides to preferably change their conformation which was easily accessible to the modifying agent, reinforced by the fact that the modification was accomplished with only drying of alcohol precipitates (Scheme 1).

The effects of different drying and polar solvents on amounts of incorporating citric acid into polysaccharides were investigated. In pectin, the incorporated citric acid contents of drying at 30 °C for 48 h and 60 °C for 24 h were 150 and 270% toward freeze-drying, while these for methanol, 1-propanol, 2-propanol, and acetone were 105, 138, 143, and 170% toward ethanol, respectively. It can at least be said that polysaccharides were modified regardless of drying methods and kinds of polar organic solvents.

In view of the application of the modification to an industrially practical use, the need to minimize the use of polar organic solvents may present itself. To meet this potential demand, pectin was modified with citric acid or sodium citrate salts in the paste state (Table 1). The order of the reactivity with pectin was sodium dihydrogen > disodium hydrogen citrate > trisodium citrate > citric acid. The results of modification in the paste were consistent with those in the alcohol precipitates (Fig. 3c). Eventually, the modification was completed by dispersion of polysaccharides and the modifying agents into a small volume of polar organic solvent and then drying. Moreover, the concentrations of polar organic solvent for modification were lower in paste state than in alcohol precipitates which needed at

Table 1 Incorporation of citric acid into citrus pectin with chemical modification in the paste

Concentrations of modifying agents	The amount of citric acid bound to citrus pectin (mg/g dry matter)			
	Citric acid	Sodium dihydrogen citrate	Disodium hydrogen citrate	Trisodium citrate
2 mmol	ND	4.96 ± 0.52	4.90 ± 0.47	1.20 ± 0.15
5 mmol	1.65 ± 0.33	15.7 ± 0.85	10.1 ± 0.66	2.84 ± 0.22
10 mmol	1.60 ± 0.27	15.6 ± 0.74	6.85 ± 0.61	5.75 ± 0.36
pH of 0.2% modifying agents (w/v) in 60% ethanol (v/v)	3.3	4.3	6.7	9.0

The modifying agents were added to dried powder of pectin (2 g) at the concentrations from 2 to 10 mmol. 60% ethanol solution (v/v) was added at 1.5 times volume to the weight of the mixtures. The reaction mixtures were air-dried, and dialyzed, and then freeze-dried. Values are mean ± SEM of three independent experiments

least more than 50% (v/v). For instance, in the reaction with κ-carrageenan and sodium dihydrogen citrate the concentration of acetone was not less than 20% (v/v).

In order to confirm the presence of chemical modification, the polysaccharides modified with citric acid were analyzed by FT-IR (Fig. 5). In the modified pectin, the intensities of anion carboxylate and C=O vibration at 1743 and 1612 cm^{-1} [14], respectively, increased. A remarkable difference between the untreated and modified κ-carrageenans was not recognized, although the band at 1420 cm^{-1} attributed to free carboxyl stretching was observed as the weaker absorption in the modified κ-carrageenan. These features can be explained by the fact that citric acid was incorporated with polysaccharides. In the modified chitosan, the band at 1650 cm^{-1} due to amide I showed a shoulder shifted to the band at 1560 cm^{-1} due to the amide II component [15, 16]. This indicates that new molecules were produced with modification and that the number of NH groups involved in hydrogen bonds increased, as revealed by the increase in intensity of the amide II band. Additionally, the absorbance ratio A_{1655}/A_{3450} as an index of deacetylation degree [17] in the modified chitosan was higher than that in the unmodified chitosan. This indicates that the number of free amine groups in the modified chitosan was fewer as the result from the reaction between amine groups in chitosan and probably carboxyl groups in citric acid.

Table 2 shows the effects of citric acid on viscosities of the polysaccharides. The viscosity of the modified pectin was higher than that of the unmodified pectin and this result was consistent with our previous finding [6]. Analysis of molecular weight distribution by HPLC revealed that the modified pectin was polymerized with citric acid (Supplementary Fig. 1). Carrageenan and chitosan had a decrease in viscosity by the modification with citric acid. The decreased viscosity in the modified carrageenan did not depend on molecular size since the molecular distribution of the modified carrageenan was same as that of the unmodified carrageenan (Supplementary Fig. 1). Additionally, the 1% (w/v) gel strength of the modified carrageenan gel in the presence of 1 M KCl decreased compared to the

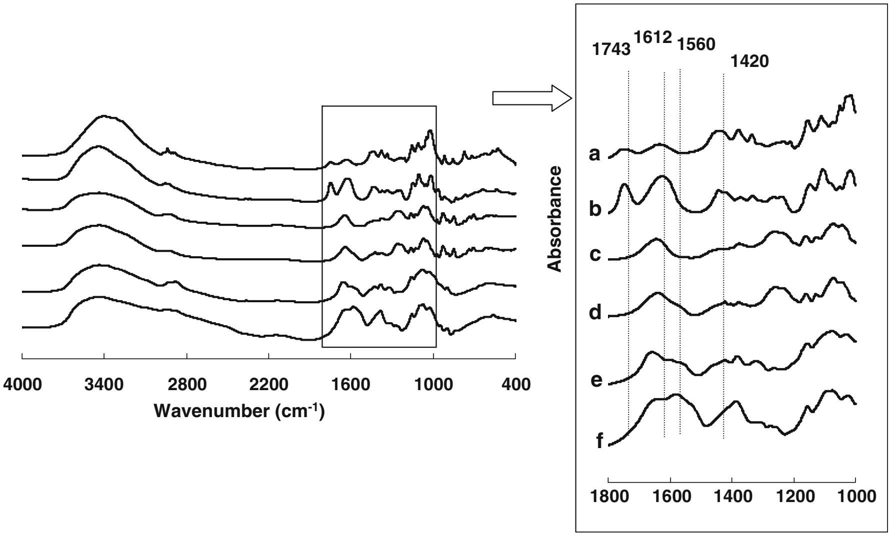


Fig. 5 Fourier transform infrared spectra of polysaccharides. The modified samples were prepared with 60% ethanol solution containing 0.2 M citric acid at pH 6. The experiments were repeated 3 times, with similar results. *a* unmodified pectin, *b* modified pectin, *c* unmodified carrageenan, *d* modified carrageenan, *e* unmodified chitosan, *f* modified chitosan

Table 2 Viscosities of different polysaccharides

	Pectin	Carrageenan	Chitosan
Unmodified	11.5 ± 0.9	44.2 ± 1.4	117 ± 13
Modified	47.5 ± 1.5	19.9 ± 1.4	61.3 ± 3.6

The modification was done with a final concentration of 5% polysaccharides (w/v) at pH 6.3 in 60% ethanol solution (v/v) containing 0.12 M citric acid. Polysaccharide solutions at a concentration of 1% (w/v) were prepared and measured. Values are mean ± SEM of three independent experiments

unmodified carrageenan (Supplementary Fig. 2). This indicates that citric acid incorporated into carrageenan molecules disordered conformational transition necessary for gelation. In chitosan dicarboxylic acid salts, the shear viscosity decreases when the deacetylation degree increases while the molecular weight remains practically unchanged [18]. Therefore, the decreased viscosity in the modified chitosan might be explained by the fact that the modified chitosan had a low deacetylation degree as obtained result from FT-IR analysis (Fig. 5). As previously seen in polysaccharides modified with citric acid, the effects on the physico-chemical properties of the modified products were different. It can at least be said that conformational transition of polysaccharides in polar organic solvents depended on the structure and the chemical composition which characterized intramolecular hydrogen bonding and reactivity to modifying agents. In respect of the reactivity of polysaccharides with modifying agents, we show that a favorable conformation of polysaccharides to react with the modifying agent could be estimated from the degree of swelling, depending on pH in polar organic solvents

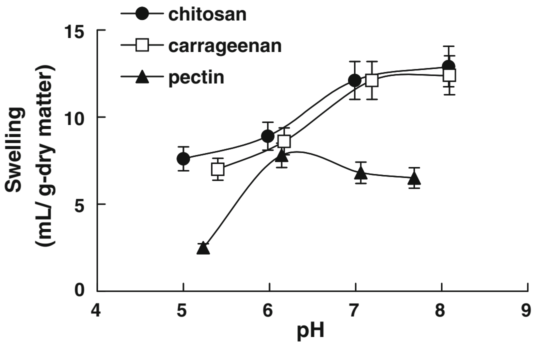


Fig. 6 Effect of pHs on swelling of polysaccharides in ethanol solution. Swelling was measured with 1% polysaccharide's solution (w/v) including 60% ethanol and 0.08 M citric acid at different pHs. Values are mean ± SEM of three independent experiments

(Fig. 6). It is worthwhile to note that the swelling behavior of pectin in polar organic solvents was in accord with the pH-dependent incorporation of citric acid into the pectin molecules and that in chitosan and κ-carrageenan, the higher swelling was, the less the bound amount was (Figs. 3, 6). At any rate, it is imperative for a better understanding of this modification to further study the physico-chemical properties of the modified products using different modifying agents.

Simply put, this modification displays hydration–dehydration. In the onset of seed germination, hydration–dehydration commences the physical changes in embryo growth [19]. The hydration–dehydration cycles increased in seed germination [20]. This implies that polysaccharides in seed could undergo a conformational change which might be somewhat effective to produce physical changes. Moreover, the binding of pectin and xyloglucan to cellulose microfibrils is considered as a significant phenomenon in the modeling of primary cell walls as well as in the process of cell assembly [21, 22]. However, very little information about how and when the binding starts has been acquired as yet. The constituent polysaccharides might bind during hydration–dehydration under a low moisture condition by restricting their molecules to move freely.

There are a number of conditions in which polysaccharides can be modified to alter their functions by use of this modification (Supplementary Figs. 3, 4). In a sense, the modification was a method through the use of conformational transition of polysaccharides in polar organic solvents. Therefore, molecules such as protein and nucleic acids which can possibly change their conformation in polar organic solvents could be utilized in this modification. Support for our idea can be found that human serum albumin microspheres formed by addition of acetone, incorporated rose bengal inside the protein molecules [23]. Finally, the cultivation of techniques by further study of factors affecting the conformational transitions of polysaccharides in polar organic solvents with NMR analysis will be expected to advance customized synthesis and selective modification of macromolecules.

Conclusion

Our finding reported here means that the method of precipitation from alcohol generally used for fractionation of polysaccharides could be instead adopted as a cost-effective modification utilizing conformational change of the modified molecule's own motion in polar organic solvents due to increasing intramolecular hydrogen bonding without the use of energy. Presumably, this modification of polysaccharides could be eligible for additional macromolecules such as protein and nucleic acids or in instances where coacervation of protein–polysaccharide complex which can be alcohol-precipitated as well. In a sense, our modification consisting of dehydration and drying could contribute a better understanding of natural biochemical reactions remaining to be clarified. We provide a novel modification for an achievement of Green Sustainable Chemistry as a global trend to conserve energy and biomass.

Acknowledgment We thank Shaun O'Brien for comments on the manuscript.

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