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Preparation of carboxylic acid-bearing polysaccharide nanofiber made from euglenoid β-1,3-glucans

Motonari Shibakami[a,*], Gen Tsubouchi[a], Makoto Nakamura[b], Masahiro Hayashi[c]

[a] Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Central 6th, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan
[b] Industrial Technology Center of Wakayama Prefecture, Ogura 60, Wakayama, Wakayama 649-6261, Japan
[c] Department of Marine Biology and Environmental Science, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki, Miyazaki 889-2192, Japan

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ABSTRACT

This paper introduces a new strategy for creating surface modified polysaccharide nanofibers. To demonstrate proof of principle, the synthesis, structure, and self-assembly behavior of a carboxylic acid-bearing polysaccharide made from paramylon (β-1,3-glucan) and succinic anhydride were investigated. Examination by a combination of NMR, FT-IR, and SEC-MALLS confirmed that successful preparation of the desired succinylated paramylon without significant depolymerization. NMR, SEC-MALLS, visible absorption and CD spectroscopic analyses indicated that the paramylon derivative forms the triplex structure in solutions. SEM observation revealed that succinylated paramylon forms a nanofiber that has carboxylic acid on the surface.

1. Introduction

Woody biomass continues to be of broad interest because of its potential use as biomaterials featuring reproducibility and environmental friendliness. Polysaccharide nanofibers are of special interest in this regard because of their high mechanical stiffness (Bhatnagar & Sain, 2005; Isogai, Saito, & Fukuzumi, 2011; Iwatake, Nogi, & Yano, 2008). Many polysaccharide nanofibers, including cellulose nanofibers, have been prepared by fibrillating natural materials using a top-down approach such as mechanical treatment (Bhatnagar & Sain, 2005; Chakraborty, Sain, & Kortschot, 2005; Paakko et al., 2007; Taniguchi & Okamura, 1998; Zhao, Feng, & Gao, 2007). Since these nanofibers are composed of natural materials, there is no significant difference in their chemical nature if they are the same type of biomass. Innovative applications such as drug delivery systems and stimuli-responsive polymers may be envisaged if the chemical nature of the nanofibers is changed. A promising approach to changing the chemistry of the nanofibers is to chemically modify their surface while keeping the fiber structure intact. This approach has been successfully applied to the synthesis of a limited number of target compounds such as 2,2,6,6-tetramethylpiper-idine-1-oxyl (TEMPO)-oxidized cellulose nanofibers (Isogai & Kato, 1998).

Our efforts for creating surface modified polysaccharide nanofibers have focused sharply on using paramylon, a naturally occurring linear β-1,3-glucan produced by euglenoid alga, as a nanofiber component (Booy, Chanzy, & Boudet, 1981; Clarke & Stone, 1960; Kobayashi, Kimura, Togawa, Wada, & Kuga, 2010; Kreger & Meeuse, 1952; Shibakami, Tsubouchi, Nakamura, & Hayashi, 2013). Although β-1,3-glucans are structurally related to cellulose (β-1,4-glucan), a slight linkage difference between two glucose units results in a large structural difference; β-1,3- and β-1,4-glucans form a triple-helix structure and a sheet structure, respectively (Chuah, Sarko, Deslandes, & Marchessault, 1983; Clarke & Stone, 1960; Numata et al., 2006; Ogawa, Tsurugi, & Watanabe, 1972; Saito, Ohki, & Sasaki, 1979; Saito, Ohki, Takasuka, & Sasaki, 1977; Yanaki, Kojima, & Norisuye, 1981; Zhang, Li, Zhou, Zhang, & Chen, 2002; Zhang et al., 2001; Zhang, Li, & Zhang, 2010). Of particular interest is that a single-strand β-1,3-glucan self-assembles to form a triplex when the solvent conditions are changed (Numata et al., 2006; Wang, Zhang, Zhang, & Ding, 2009; Xu, Wang, Cai, & Zhang, 2010; Yanaki et al., 1981; Zhang et al., 2002). This inherent self-assembling ability suggests that it is feasible to use a bottom-up approach as a means of preparing nanofibers

* Corresponding author. Tel.: +81 29 861 4547; fax: +81 29 861 4547.
E-mail address: moto.shibakami@aist.go.jp (M. Shibakami).

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made from β-1,3-glucans. Previous structural studies have shown that not only linear β-1,3-glucans but also branched glucans with side chains on the O(6) primary hydroxyl position form a triplex structure (Saito et al., 1979; Saito, Ohki, Takasuka, et al., 1977; Zhang et al., 2002, 2001, 2010). Furthermore, X-ray studies have shown that only the secondary hydroxyls of the glucose unit contribute to the triplex formation (Bluhm & Sarko, 1977; Chuah et al., 1983). Thus, our working hypothesis has been that, if the primary hydroxyls of paramylon are preferentially modified while the secondary hydroxyls remain almost intact, paramylon derivatives can self-assemble to form a triplex.

In the work reported here, our primary aim was to demonstrate the feasibility of creating surface modified nanofibers made from the paramylon triplex by using a combination of chemical modification and subsequent self-assembly. Potential advantages of this strategy are twofold. First, since there are many chemical reactions that are applicable to single-strand β-1,3-glucans dissolved in solvents, this strategy may allow us to prepare a wide variety of functional nanofibers. Second, the triplex-based nanofibers may exhibit higher thermal and mechanical stability than single-strand polymer-based ones.

2. Experimental

2.1. General methods

All chemicals and reagents are commercially available and were used without further purification. Paramylon particles were obtained from *Euglena gracilis* in accordance with a previously reported method (Shibakami, Sohma, & Hayashi, 2012). ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE 500 spectrometer using 1,4-dioxane (10 μL) as an internal standard. A quantitative ^{13}C NMR spectrum was performed by means of the inverse gated decoupling method. Fourier transform infrared (FT-IR) spectra were recorded using a JASCO FT/IR-480ST spectrophotometer equipped with an attenuated total reflectance accessory (ATR Pro 400-S, ZnSe prism, JASCO). The spectra were recorded with a resolution of 4 cm^{-1}.

2.2. Synthesis of succinylated paramylon

Into a 200-mL round-bottomed flask containing 60 mL of absolute *N*,*N*-dimethylacetamide (DMAc) were placed 1.00 g (6.17 mmol, glucose unit) of paramylon and 784 mg (18.49 mmol) of lithium chloride (LiCl) under a nitrogen atmosphere. The mixture was heated at 120 °C for 1 h, and the resulting homogeneous solution was left to cool to ambient temperature. To this solution were then added 616 mg (6.16 mmol) of succinic anhydride and 1.29 mL (9.26 mmol) of triethylamine (NEt_3). After stirring for 3 h at room temperature, 50 mL of acetone was added to the mixture. The resulting white precipitate was thoroughly washed with acetone to obtain the desired product, i.e., a free acid form. Next, before it had completely dried, the product was poured into 100 mL of a 0.067-mol/L disodium hydrogenphosphate aqueous solution, and the mixture was mechanically stirred until it became homogeneous (ca. 1 h). Addition of acetone (80 mL) gave a white precipitate. The precipitation procedure was repeated in triplicate. Then the solution was dialyzed against water for 72 h using a dialysis membrane (Viskase) made from regenerated cellulose (molecular weight cut off of 14,000) to create a transparent solution. Freeze-drying overnight resulted in the formation of a white cottony solid referred to as a "sodium salt form" (502 mg). The ^{13}C NMR of the sodium salt form (12.8 mg in 1.0 mL of D_2O, pH 8.2) was δ 31.0 (O(C=O)CH_2CH_2COOH), 32.5 (O(C=O)CH_2CH_2COOH), 61.3 (C6), 68.7 (C4), 73.7 (C2), 76.2 (C5), 84.8 (C3), 103.1 (C1), 176.2 (O(C=O)CH_2CH_2COOH), 181.6 (O(C=O)CH_2CH_2COOH). The ^{13}C NMR of paramylon (10 mg in 1.0 mL of D_2O containing 1.0 mol/L NaOH) was δ 62.0 (C6), 69.5 (C4), 74.5 (C2), 77.4 (C5), 87.6 (C3), and 104.3 (C1).

2.3. Determination of degree of substitution

The degree of substitution (DS), which is the average number of functional groups attached to the glucose unit, was determined by the neutral titration method. In essence, ca. 100 mg of succinylated paramylon (salt form) and 4.0 mL of a NaOH aqueous solution (0.25 mol/L) were placed in a 20-mL conical flask, and the mixture was mechanically stirred at ambient temperature overnight. The resulting homogeneous solution was titrated with a HCl aqueous solution (0.25 mol/L) using phenolphthalein as an indicator. This measurement was repeated in triplicate.

2.4. Size exclusion chromatography

The weight-average molecular weights (M_w) of paramylon and succinylated paramylon (acid form) were determined by using size-exclusion chromatography with multiangle laser light scattering (SEC-MALLS). The SEC-MALLS measurements were carried out on a DAWN HELEOS II multiangle laser photometer (Wyatt Technology) and an Optilab T-rEX refractive index detector (Wyatt Technology) equipped with a gel permeation chromatography column (Shodex KF-807L) (eluent 10 mmol/L LiBr in DMSO/DMF 75/25 (v/v), 1.0 mL/min, 50 °C) and a gel filtration chromatography column (Shodex SB-804HQ) (eluent 50 mmol/L NaCl aqueous solution, 0.5 mL/min, 40 °C) for paramylon and succinylated paramylon, respectively. All of the polysaccharide solutions were purified using a 1.0-μm filter. The injection volume was 100 μL with a concentration of 1.06 and 2.20 mg/mL for paramylon and succinylated paramylon, respectively.

2.5. Visible absorption spectroscopy

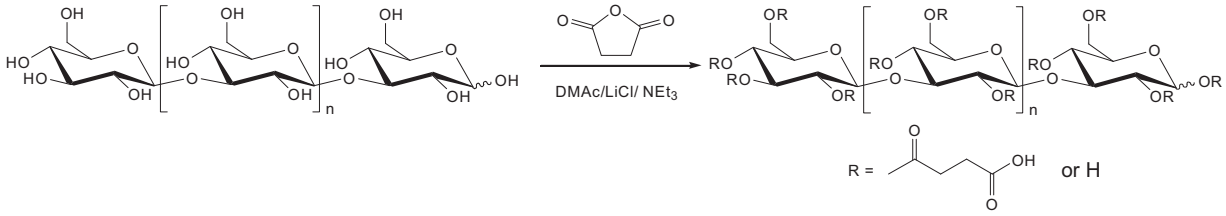
A Congo red (CR) aqueous solution (0.032 mmol/L) was prepared by dissolving CR powder in water. An aqueous solution containing succinylated paramylon (10 mg/mL) was obtained by dissolving 10 mg of succinylated paramylon (salt form) in 1.0 mL of water. Equal volumes (0.65 mL) of the CR solution and the succinylated paramylon solution were well mixed. The visible absorption spectra of the mixture were measured with a Shimadzu UV-2500 spectrophotometer at various pH values upon incremental additions of 2 μL of a 1.0-mol/L HCl aqueous solution at room temperature. The visible absorption spectra of the CR solution (0.016 mol/L) in the 3.5–6.0 pH range were also measured as a control.

2.6. Circular dichroism spectroscopy

The samples used for circular dichroism (CD) spectroscopy were prepared in a similar way as those for the visible absorption spectroscopic measurements. In brief, after equal volumes (1.75 mL) of the CR solution and the paramylon solution were mixed, the CD spectra of the mixture were measured using a JASCO J-820 spectropolarimeter upon incremental additions of 6–18 μL of a 1.0-mol/L HCl aqueous solution at room temperature.

2.7. Scanning electron microscopy

Four types of solutions for nanofiber preparation were prepared. (i) 11 mg of succinylated paramylon (salt form) and 1.0 mL of water were mixed together in a 10-mL flask. The mixture was then mechanically stirred until it became homogeneous. Then the solution was diluted to 1/10 with water to create a 1.1-mg/mL solution



Scheme 1. Synthetic approach used for preparing succinylated paramylon.

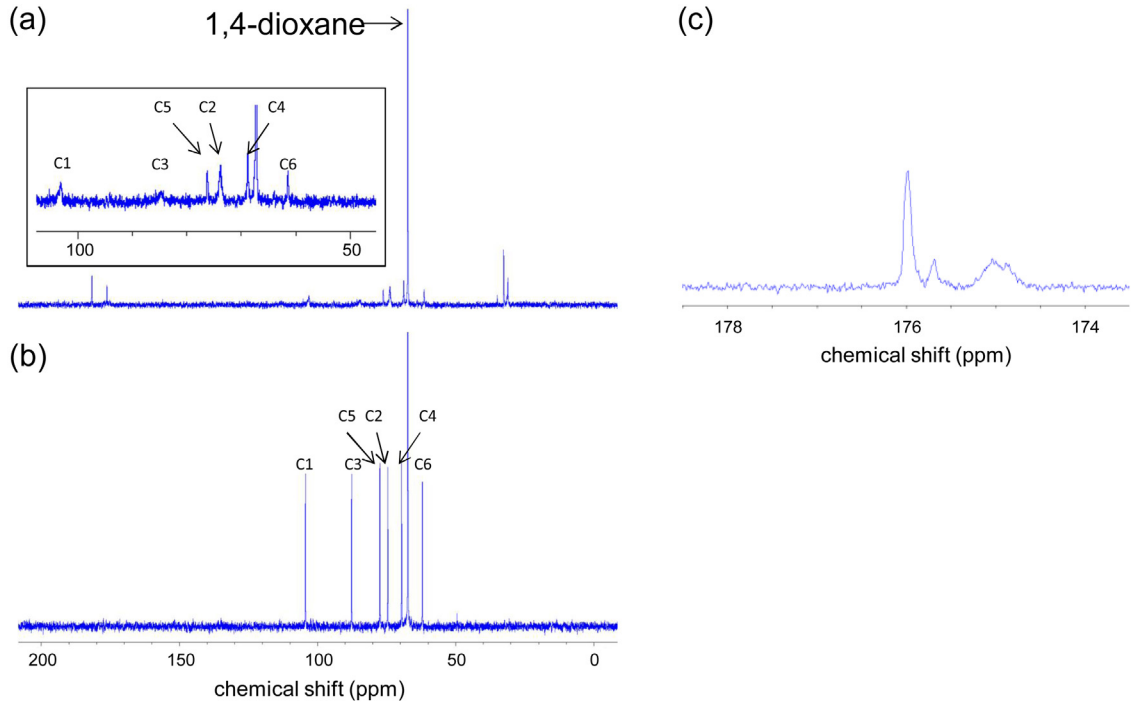


Fig. 1. ^{13}C NMR spectra of (a) succinylated paramylon; inset shows magnification, (b) paramylon, and (c) succinylated paramylon (quantitative mode spectrum).

(pH 7.5). (ii) A clear solution made from succinylated paramylon (salt form, 23 mg) and water (2.0 mL) was dialyzed against water overnight. It was then diluted to 1/10 with water to create a ca. 1.15-mg/mL solution (pH 5.2). (iii) 1.3 μL of a 0.25-mol/L HCl aqueous solution was added to the pH-5.2 solution to create a ca. 1.15-mg/mL solution (pH 4.0). (iv) 2.0 μL of a 1.0-mol/L HCl aqueous solution was added to the pH-5.2 solution to create a ca. 1.15-mg/mL solution (pH 2.5). Each prepared solution was then quickly frozen with liquid nitrogen, followed by drying under reduced pressure to create a cottony solid. The freeze-dried samples were fixed on a microscope metal stage using carbon conductive double-sided tape. Microscopic observations were carried out under high vacuum using a 2-kV accelerating voltage and a JEOL JSM-6060 scanning electron microscope.

3. Results and discussion

3.1. Synthesis of succinylated paramylon

To demonstrate the feasibility of preparing functional group-bearing nanofibers, we first attempted to synthesize a carboxylic acid-bearing one made from paramylon and succinic anhydride. The reasons for choosing carboxylic acid as the functional group introduced to paramylon are threefold. First, carboxylic acid makes the polysaccharide water soluble. Second, since carboxylic acid can carry an anionic charge, it promotes dispersion in aqueous solutions by creating electrostatic repulsion between carboxylate anions. Third, carboxylic acid can connect other functional groups with paramylon through chemical bonds. The synthesis of succinylated paramylon (acid form) was conducted in a manner similar to previously reported synthetic schemes for other succinylated polysaccharides (Scheme 1) (Shigemasa et al., 1999; Sun & Sun, 2002; Yoshimura, Matsuo, & Fujioka, 2006). The molar ratio of a glucose unit of paramylon and succinic anhydride was set as low as 1/1 to enable succinic anhydride to preferentially react with the primary hydroxyl group. We also prepared sodium salt in succinylated paramylon to facilitate preparation of homogeneous aqueous solutions containing this polysaccharide. It is likely that three times repeat of reprecipitation method for purifying the synthesized polymer as well as intensive dialysis using a membrane with molecular weight cut off of 14,000 result in the approximately 50% yield.

3.2. Structure of succinylated paramylon

To confirm successful preparation of the succinylated paramylon and to explore its structure in detail, we carried out NMR spectroscopic, DS determination, FT-IR spectroscopic, and size-exclusion chromatographic measurements.

3.2.1. NMR analysis of succinylated paramylon

As shown in Fig. 1(a), the appearance of ^{13}C signals at 31.0, 32.5, 176.2 and 181.6 ppm indicates successful esterification; the signals in the lower and higher magnetic fields are ascribed to the

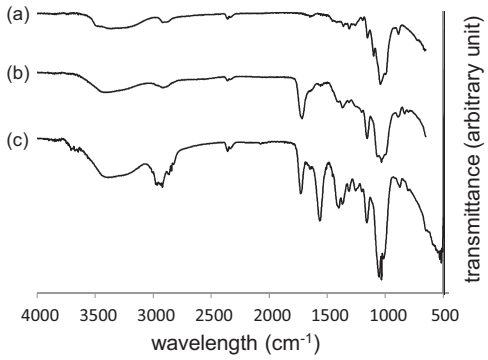


Fig. 2. FT-IR spectra of (a) paramylon, (b) acid-form succinylated paramylon, and (c) salt-form succinylated paramylon.

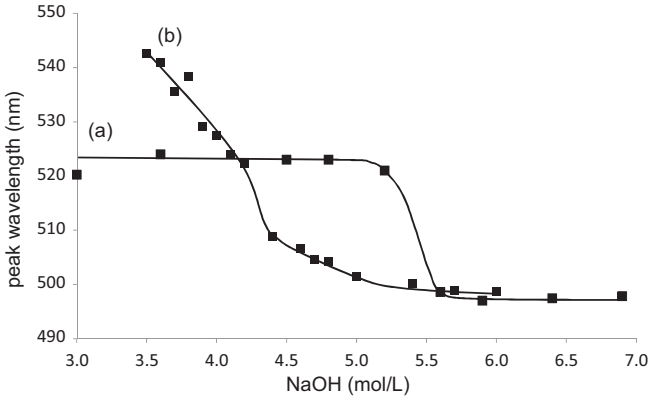


Fig. 3. Dependence of peak wavelength of absorption maximum of Congo red on pH in (a) presence and (b) absence of succinylated paramylon.

succinyl ester carbonyl and methylene carbon atoms, respectively. Fig. 1(b) shows the spectrum of paramylon in a 1.0-mol/L NaOH aqueous solution, which assumes a randomly coiled conformation. Comparison of these spectra showed that the intensities of all the signals due to the glucose unit of succinylated paramylon were smaller than those of paramylon. Heterogeneous distribution of the succinate groups within three hydroxyls of one glucose unit may contribute to the intensity suppression of the C1–C6 signals of succinylated paramylon. A closer examination of the succinylated paramylon spectrum revealed that the intensities of the C1 and C3 signals were lower and broader than those of C2, C4, C5, and C6. If we consider that similar selective suppression is observed in helical β-1,3-glucans, the present results suggest that the molecular motion of succinylated paramylon around a β-1,3-bond is restricted by the interstrand interaction (Bryce, Mckinnon, Morris, Rees, & Thom, 1975; Chien & Wise, 1975; Saito, Ohki, & Sasaki, 1977; Smith, Jennings, & Deslauriers, 1975). A plausible conformation that provides the polysaccharide with such a restriction is the triplex structure (Saito, Ohki, et al., 1977).

The acyl carbonyl carbon signals have been used as a sensitive NMR probe for monitoring its substitution position on a glucose residue (Tezuka, 1993; Tezuka, Imai, Oshima, & Ito, 1991; Tezuka & Tsuchiya, 1995). Fig. 1(c) shows a quantitative ^{13}C NMR spectrum of succinylated paramylon (salt form, 44.0 mg in 0.8 mL of D_2O). The succinic ester carbonyl signals were apparently resolved into three peaks reflecting the substitution position on the glucose residue. Relative integral values of the three peaks at 176.0, 175.7, and 175.0, which are assigned to C6, C4, and C2, respectively, were 4.6, 1.0, and 3.6, respectively. If we consider the total DS value of 0.46, these integral values indicate that partial DS values of C6, C4, and C2 were 0.23, 0.05, and 0.18, respectively. Thus, the quantitative ^{13}C NMR measurement indicates that although the primary hydroxyls are not preferentially modified, many secondary ones remain intact.

3.2.2. FT-IR analysis of succinylated paramylon

As is shown in Fig. 2(a) and (b), the succinylated paramylon (acid form) had a strong absorption at 1713 cm^{-1} while paramylon did not have any absorption around 1700 cm^{-1}. Thus, comparison of these spectra also confirmed successful introduction of succinic acid into paramylon. The FT-IR spectrum of the salt form solid shows strong absorption bands at 1718 and 1555 cm^{-1} (Fig. 2(c)). The carboxylic acid was apparently transformed into a carboxylate anion due to treating the acid form with a disodium hydrogenphosphate aqueous solution.

3.2.3. Molecular weight determination of succinylated paramylon by size exclusion chromatography

The M_w of the succinylated paramylon determined by SEC-MALLS was 6.644×10^5. Since the mobile phase used for the SEC-MALLS measurement of the succinylated paramylon was a 50-mmol/L NaCl aqueous solution that promotes the triplex formation of paramylon, it is likely that this value is the M_w of the triplex of succinylated paramylon. If so, the M_w of a single-strand polysaccharide polymer is one-third the M_w value (i.e., 2.215×10^5). That this value is comparable to or a bit smaller than that of paramylon (2.959×10^5) indicates that significant depolymerization did not occur during the reaction.

3.3. Conformation of succinylated paramylon

As described in Section 1, the triplex-based nanofibers are expected to have higher thermal and mechanical stability than the single-strand polymer-based one. The NMR and SEC-MALLS results, which were described in Sections 3.2.1 and 3.2.3, respectively, suggested that the β-1,3-glucan derivative preserves the helix forming ability inherent to β-1,3-glucans. To determine whether this is true, we measured the visible absorption and CD spectra of CR in the presence and absence of succinylated paramylon.

3.3.1. Visible absorption spectroscopic analysis of succinylated paramylon

Since electrostatic repulsion between carboxylate anions may disturb association of the single-strand polymers, we assumed that protonation of carboxylate anion is required for the transition from the single-strand structure to the triplex structure if the polysaccharide preserves the helix forming ability. To confirm this, we examined the pH-dependence of the peak wavelength of the CR absorption maximum. It has been reported that if β-1,3-glucan helices form an inclusion complex with CR, the peak wavelength of the absorption maximum of the CR shifts (Ikeda & Shishido, 2005; Ogawa et al., 1972; Xu et al., 2010). As such, the visible absorption measurement has been a facile method for confirming the presence of helical conformation. As is apparent in Fig. 3, the peak wavelength of the absorption maximum started to shift from 498 to 521 nm at a pH of around 5.2–5.6 in the presence of succinylated paramylon while it gradually increased in the absence of polysaccharide as the pH was reduced. The present results indicate that polysaccharide changes its conformation from a randomly coiled structure to a helical structure that can accommodate CR molecules by protonation of the carboxylate anion at a pH of around 5.2–5.6.

3.3.2. CD spectroscopic analysis of succinylated paramylon

To confirm the hypothesis that protonation of the carboxylate anion induce the triplex formation, we measured the CD spectra of CR in the presence and absence of polysaccharide with decreasing pH value. Previous studies reported that the appearance of an induced CD signal for optically inactive molecules in the presence of

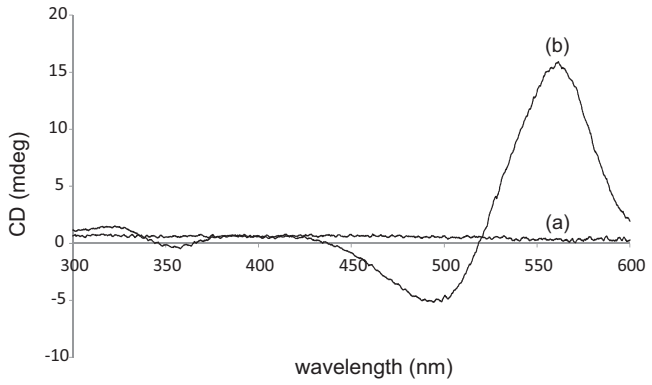


Fig. 4. Dependence of CD signal of Congo red on pH in presence of succinylated paramylon: (a) 6.2 and (b) 5.1 pH.

β-1,3-glucans indicates the formation of a helical structure because the optically inactive dye molecules inside the one-dimensional helical cavity gain twisted molecular arrangement (Numata et al., 2006; Ogawa et al., 1972). In the absence of succinylated paramylon, CR did not show an induced CD signal (data not shown). In the presence of succinylated paramylon, it did not show an induced CD signal as the pH was reduced from 6.4 to 5.9 (Fig. 4(a) shows the spectrum at a pH of 6.2). An induced CD signal did appear when it was reduced to 5.1 in the presence of succinylated paramylon (Fig. 4(b)). These results indicate that succinylated paramylon starts to form a helical structure in the 5.1–5.9 pH range. It is also noteworthy that this pH range is comparable to that for the peak wavelength of the absorption maximum change. Thus, both the visible absorption and CD spectroscopic analyses indicate that succinylated paramylon has the helix forming ability.

3.3.3. Coexistence of succinylated paramylon with triplex and single-strand conformation

The results of ^{13}C NMR measurement of succinylated paramylon suggested that this polysaccharide assumes a triplex conformation at a pH of 8.2. At this pH, both the visible absorption and CD spectra indicated that the polysaccharide assumes a randomly coiled conformation. This apparent contradiction can be explained by the coexistence of two types of polysaccharides: a succinylated paramylon that forms the triplex and a polysaccharide with CR that assumes a single-strand conformation. It is likely that the former stably exists as a charged self-associated polysaccharide triplex in water and the creation of the latter is due to the shortness of chain. The results presented here provide evidence not only that the helix forming ability is preserved, but also the possibility that dispersion of the salt form solid in water yields the triplex.

3.4. Formation of succinylated paramylon nanofiber

3.4.1. Scanning electron microscopic analysis of nanofiber

Freeze-drying has been a simple preparation method of cellulose aerogels (i.e., a three-dimensional fibrous network) (Jin, Nishiyama, Wada, & Kuga, 2004). To see if succinylated paramylon can create the nanofiber using this method, we performed scanning electron microscopic observation of a freeze-dried sample made from a dilute aqueous solution of the salt form solid with a concentration of 1.1 mg/mL (pH 7.5). As is evident in Fig. 5(a), this sample apparently contained a three-dimensional network made from fibers with an average width of 201 ± 73 nm (measured by a digital image measuring software "MicroAnalyzer" (Nihon Poladigital, K.K)). This apparent result confirmed that succinylated paramylon forms a nanofiber in an aqueous solution. Based on a previous X-ray diffraction study on the crystal structure of paramylon (Chuah et al., 1983), the presumed width of the triplex is approximately 2 nm Significant difference in width between the observed nanofiber and the triplex implies that the freeze-drying process induces the aggregation of the triplex.

3.4.2. pH dependence of nanofiber morphology

Another concern was whether or not carboxylic acid groups are exposed on the nanfiber's surface. To confirm this, we carried out scanning electron microscopic analyses on solids made from aqueous solutions containing succinylated paramylon with different pH values. A freeze-drying process of a pH-5.2 solution gave a three-dimensional network made of nanofibers (Fig. 5(b)), which is analogous to that obtained at pH 7.5. When the pH was

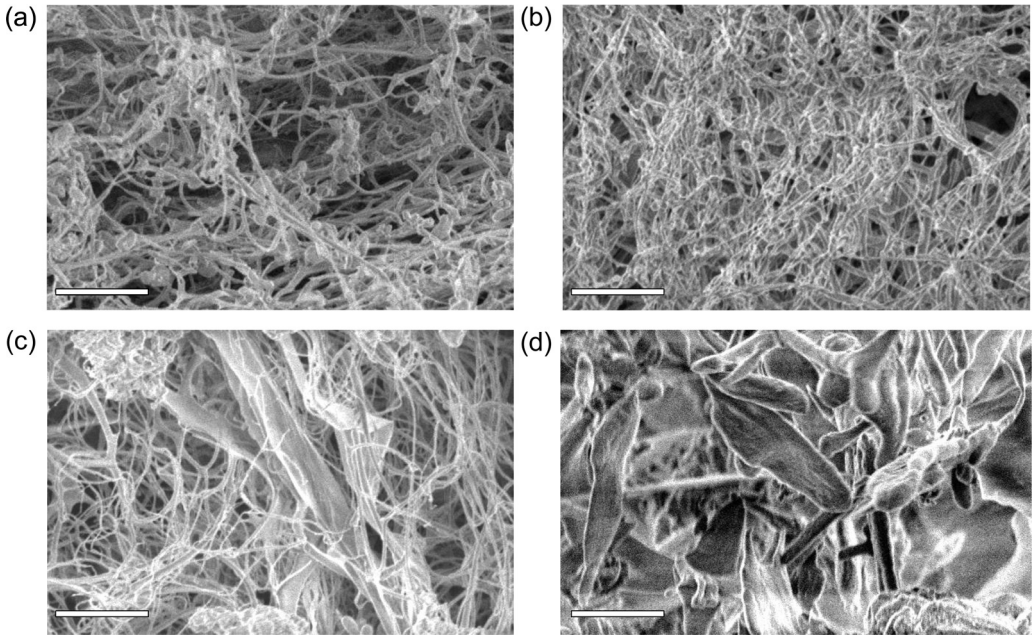


Fig. 5. Scanning electron microscopic images of solids made from solution containing succinylated paramylon prepared at pH of (a) 7.5, (b) 5.2, (c) 4.0, and (d) 2.5. Scale bar represents 5 μm.

reduced to 4.0, sheet-like structures emerged as well (Fig. 5(c)). A further decrease in pH to 2.5 resulted in the disappearance of the nanofiber; instead, amorphous lumps appeared (Fig. 5(d)). Such pH dependence implies that carboxylic acid groups are exposed on the surface of the nanofiber; in the higher pH range, the electrostatic repulsion between carboxylate anions led to good dispersion of the fibers, while interfibrillar hydrogen bonds resulted in a decrease in the number of independent fibers and the disappearance of the fibrous structures in the lower pH range.

4. Conclusion

We have demonstrated the feasibility of chemically modified β-1,3-glucans serving as a component of a functionalized nanofiber. Although direct microscopic evidence was not obtained in this study, it is likely that the nanofiber is composed of the triplex if we consider that succinylated paramylon has the helix forming ability. Nanofiber formation mechanism still remains to be elucidated. One plausible explanation is that the sodium salt form solid contains the nanofiber that is created by dialysis and subsequent freeze-drying. An alternative possibility is that association of the triplex induced by the freeze-drying process results in the nanofiber formation. Comparison of our approach with the top-down approach frequently used for preparing cellulose nanofibers shows that it is characterized by the feasibility of choosing a variety of functional groups. A similar water soluble paramylon derivative was prepared by the TEMPO oxidation method (Tamura, Wada, & Isogai, 2009). While regioselective oxidization is successfully achieved by this method, significant depolymerization occurs on the main chains during the oxidation. Features of our preparation method include circumvention of significant depolymerization, which becomes apparent by contrast with the TEMPO oxidation method.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.05.026.

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