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Refinement of the structures of cell-wall glucans of *Schizosaccharomyces pombe* by chemical modification and NMR spectroscopy

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Abstract—Alkali extraction and methylation analyses in the 1970s revealed that the cell walls of the yeast *Schizosaccharomyces pombe* contain a (1→3)-α-D-glucan, a (1→3)-β-D-glucan, a (1→6)-β-D-glucan, and a α-galactomannan. To refine the structures of these polysaccharides, cell-wall glucans of *S. pombe* were extracted, fractionated, and analyzed by NMR spectroscopy. *S. pombe* cells were treated with 3% NaOH, and alkali-soluble and insoluble fractions were prepared. The alkali-insoluble fraction was treated with 0.5 M acetic acid or Zymolyase 100 T to yield an alkali-insoluble, acetic acid-insoluble fraction, an alkali-insoluble, Zymolyase-insoluble fraction, and an alkali-insoluble, Zymolyase-soluble fraction. ^{13}C NMR and 2D-NMR spectra disclosed that the cell wall of *S. pombe* is composed of three types of glucans, specifically, a (1→3)-α-D-glucan, a (1→3)-β-D-glucan, which may either be linear or slightly branched, and a highly branched (1→6)-β-D-glucan, in addition to α-galactomannan. The highly branched (1→6)-β-D-glucan was identified by selective periodate degradation of side-chain glucose as a highly (1→3)-β-branched (1→6)-β-D-glucan with more branches than that of *Saccharomyces cerevisiae*. Flexibility of these polysaccharides in the cell wall was analyzed by ^{13}C NMR spectra in D_2O. The data collectively indicate that (1→3)-α- and (1→3)-β-D-glucans are rigid and contribute to the cell shape, while the highly branched (1→6)-β-D-glucan and α-galactomannan are flexible.

Keywords: Cell-wall glucan; *Schizosaccharomyces pombe*; NMR

1. Introduction

The cell wall protects yeast against lysis and determines the cell shape. Yeast cell walls account for 15–25% of the dry weight, and consist mainly of polysaccharides plus small amounts of proteins and lipids.[1] The components and structures of the cell walls differ between species. The differences in composition, secondary and tertiary structures of single chains, and their macromolecular assembly determining the physical properties of the polymer, are related to structural or physiological functions. Structures of cell-wall polysaccharides have been studied using various methodologies, including methylation analysis,[2–4] X-ray diffraction, and nuclear magnetic resonance (NMR) spectroscopy.[5,6] (1→3)-β-D-Glucans have received considerable attention due to their immunomodulatory properties, such as adjuvant, antimicrobial, antitumor, and radioprotective activities. In addition to studies on primary structures from various microbes, the ultrastructures and three-dimensional conformations of cell walls were analyzed.[7–9] The most

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highly characterized cell wall is that of *Saccharomyces cerevisiae*, which consists of branched (1→3)-β-D-glucan, (1→6)-β-D-glucan, chitin, and mannoproteins.[2,3] Two types of (1→3)-β-D-glucan in *S. cerevisiae* have been reported, specifically, alkali-soluble and alkali-insoluble.[1] The former may confer flexibility to the cell wall, while the latter plays a role in maintaining wall rigidity and shape.[1] ^{13}C cross polarization-magic angle spinning (CP/MAS) NMR spectroscopy data reveal that the (1→3)-β-D-glucan of *S. cerevisiae* forms a single or triple helix conformation in the native cell wall.[10,11] Extensive analyses of interchain interactions of these macromolecules disclose the following: (1→3)-β-D-glucan forms a framework structure on the inner surface of the wall, chitin and (1→6)-β-D-glucan are covalently linked to this structure by (1→3)-β-D-glucans, mannoproteins attach (1→6)-β-D-glucans via their glycosylphosphatidylinositol (GPI) glycan remnant or directly to (1→3)-β-D-glucans, and finally, these components form a chitin–(1→3)-β-D-glucan–(1→6)-β-D-glucan–mannoprotein connection, to constitute the cell wall.[12–16]

Alkali extraction and methylation analyses concluded in the 1970s showed that the characteristic features of the cell walls of fission yeast *S. pombe* include the presence of a (1→3)-α-D-glucan in addition to β-glucans, and connection of α-galactomannan to the cell-wall proteins.[4,17,18] Chitin synthase was additionally identified. Expression of this protein increases during sporulation, but during vegetative growth, chitin is not detected.[19] (1→3)-α-D-Glucan is highly localized in the septum.[20] Numerous studies on the physiological roles of (1→3)-α- and (1→3)-β-D-glucans in *S. pombe* strongly indicate that these compounds function to maintain cell shape.[21–24] Protoplasts and electron microscopy analyses disclose that the surfaces of regenerating protoplasts are covered with fibrous (1→3)-β-D-glucans, and α-galactomannan particles fill the crevices of the (1→3)-β-D-glucan framework.[25] In the present study, we refine the architecture of the yeast cell wall by re-examining cell wall polysaccharides of *S. pombe* using NMR spectroscopy, and specific chemical and enzymatic degradation experiments.

2. Results and discussion

2.1. Cell-wall extraction by alkali, followed by acetic acid

Cell-wall glucans were extracted from fission yeast *S. pombe* according to the method of Manners and Meyer (1977), and analyzed by chemical, biochemical, and physical methodologies. Initially, cells were treated with 3% NaOH at 4 °C for 6 days, followed by 0.5 M acetic acid at 90 °C seven times for 3 h each (Chart 1). We obtained alkali- and acid-insoluble glucan (fraction 1) and alkali-insoluble, acid-soluble glucan (fraction 2) fractions. The yields of each fraction are depicted in Chart 1 fractions were dissolved in Me_2SO-d_6. ^{13}C NMR spectra were measured, which are presented in Figure 1. Fraction 1 displayed three anomeric signals at 99.52, 102.74, and 103.87 ppm, and numerous signals between 90 and 60 ppm. However, assignment of fraction 1 was difficult (discussed later).

(1→3)-α-D-Glucan of *S. pombe* is reportedly solubilized by stronger alkali treatment.[4,6] Accordingly, fraction 1 was treated with 3% NaOH at 70 °C for 1 h, the resulting precipitate (fraction 3) collected, and ^{13}C NMR spectra measured. As shown in Figure 1c, the spectrum of fraction 3 contained six predominant signals at 102.82, 86.10, 76.24, 72.67, 68.30, and 60.80 ppm, attributable to highly purified (1→3)-β-D-glucan. In view of the finding that the spectrum contained only six signals that differed from branched (1→3)-β-D-glucans such as schizophyllan, it is strongly suggested that this glucan has no or only a few branches.[26]

The ^{13}C NMR spectrum of fraction 2 contained 12 predominant signals, as shown in Figure 1b. Signals at 103.87, 76.65, 76.12, 73.75, 70.13, and 61.04 ppm were attributable to β-linked nonreducing end glucose residues. Signals at 102.82, 87.41, 74.89, 72.26, 68.21, and 68.11 ppm were assigned to the branching point with *O*-substitutions at C-3 and C-6. These results suggest that fraction 2 is a highly branched (1→6)-β-D-glucan. The structure and conformation of fraction 2 are further discussed in later sections; 'Ultrastructure of highly branched β-glucan'.

2.2. Cell-wall extraction by alkali followed by Zymolyase digestion

Cell-wall glucans were extracted from the fission yeast, *S. pombe*, according to the method of Brown et al.,[27] and analyzed by chemical, biochemical, and physical methodologies. Alkali-insoluble, Zymolyase-insoluble fraction 4 and alkali-insoluble, Zymolyase-soluble fraction 5 were obtained by five times treatment with 3% NaOH at 70 °C for 1 h, followed by Zymolyase digestion at 37 °C for 16 h. The ^{13}C NMR spectrum of fraction 4 predominantly contained six signals at 99.49, 82.96, 71.85, 70.72, 69.43, and 60.30 ppm (Fig. 1d), identified as (1→3)-α-D-glucan.[28] These chemical shifts were indistinguishable from part of the signals observed in the spectrum of fraction 1. In a previous study, (1→3)-α-D-glucan of *S. pombe* was extracted with alkali and purified from the alkali-soluble fraction.[4,6] However, we obtained alkali-insoluble, Zymolyase-insoluble glucan from the alkali-insoluble fraction. The solubility of alkali-insoluble, Zymolyase-insoluble glucan obtained in this study was significantly different from the data of Kreger,[6] and Manners and Meyer.[4] A previous study showed that (1→3)-glucanase does not completely digest the (1→3)-β-D-glu-

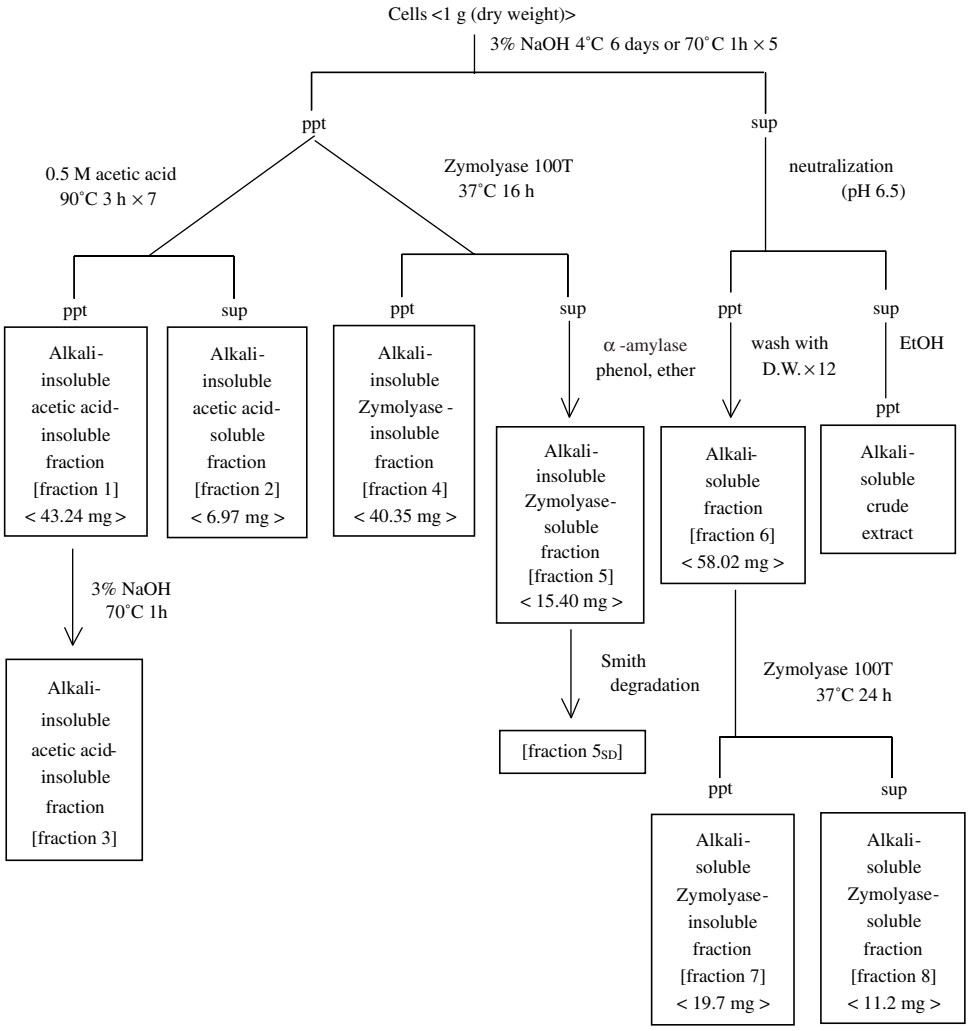

Chart 1. Fractionation of cell-wall polysaccharides of *S. pombe*.

can moiety in the intact wall, probably due to intimate association between (1→3)-α- and (1→3)-β-D-glucans.[29] In contrast, upon application of (1→3)-β-D-glucanase to sodium hydroxide-treated cells, we obtained alkali-insoluble (1→3)-α-D-glucan, suggesting complete degradation by (1→3)-β-D-glucanase. Significantly, cells were not treated with sodium hydroxide after β-glucanase digestion in this study. Light microscopy of alkali-insoluble, Zymolyase-insoluble glucan revealed that the cylindrical rod-like cell shape was maintained, despite digestion of (1→3)-β-D-g1ucan (Fig. 2). All the NMR spectra of fractions in this study were measured as solutions, using Me_2SO-d_6 as a solvent. In view of the data, it is evident that alkali-insoluble, Zymolyase-insoluble glucan retains its original architecture and is solubilized in solvent, although it is too large to be easily dissolved in sodium hydroxide. Mokl and agsl mutants with a substitution in (1→3)-α-D-glucan synthase and low glucan synthesis activity displayed a looser cell wall and round cell shape.[23,24] Data from this study strongly support a physiological role of (1→3)-α-D-glucan in *S. pombe*.

The alkali-insoluble, Zymolyase-soluble fraction of *S. cerevisiae* prepared by the same purification process consists of (1→6)-β-D-glucan.[27,30] Our results are consistent with these previous findings (Fig. 3a). However, the ^{13}C NMR spectrum of alkali-insoluble, Zymolyase-soluble glucan (fraction 5) from *S. pombe* contained 12 predominant signals, and was distinct from that of *S. cerevisiae* (Fig. 3b). Signals with chemical shifts at 103.73, 76.48, 75.88, 73.54, 69.90, and 60.83 ppm were assigned as nonreducing end residues. Signals at 102.65, 87.49, 74.70, 72.05, 68.02, and 67.89 ppm corresponded to the branching points of *O*-substitution at C-3 and C-6.

2.3. Ultrastructure of highly branched β-glucan

Notably, the ^{13}C NMR spectra of fraction 2 (alkali-insoluble, acid-soluble glucan) and fraction 5 (alkali-insoluble, Zymolyase-soluble glucan) were indistinguishable, suggesting similar primary structures. Gel chromatography data disclosed that the molecular size

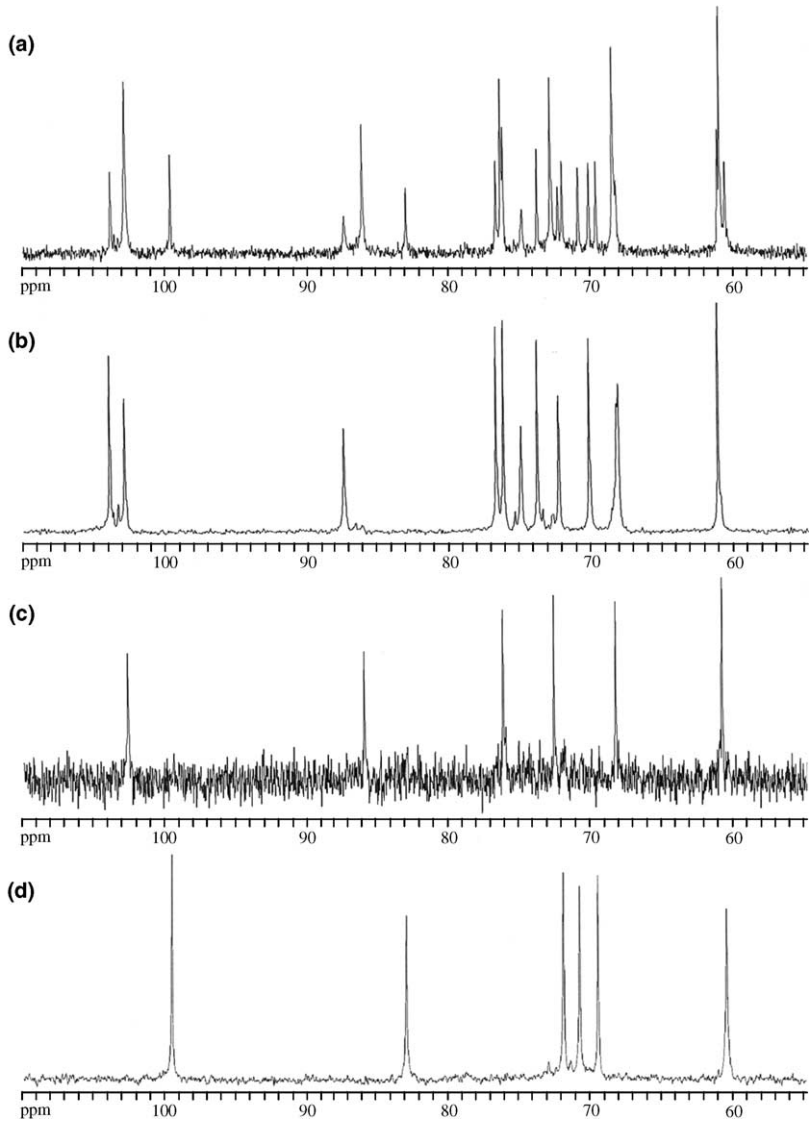

Figure 1. ^{13}C NMR spectra of isolated glucans. Cell-wall glucans were extracted from *S. pombe* with 3% NaOH and 0.5 M acetic acid or Zymolyase 100T. Each fraction was dissolved in Me_2SO-d_6, and ^{13}C NMR spectra were measured at 70 °C. Fraction 1 contained three anomeric signals and many signals between 90 and 60 ppm. Fraction 2 displayed 12 predominant signals attributable to β-linked nonreducing end glucose and the branching point with *O*-substitution at C-3 and C-6. Fraction 3 predominantly contained six signals attributable to highly purified (1→3)-β-D-glucan. Fraction 4 contained six signals attributable to highly purified (1→3)-α-D-glucan. (a) Fraction 1; (b) fraction 2; (c) fraction 3; (d) fraction 4.

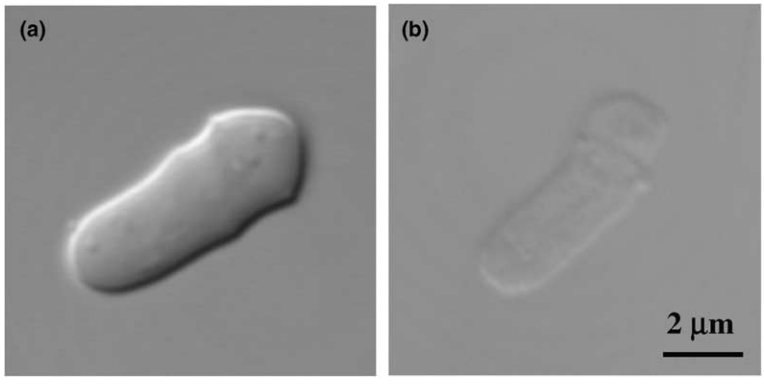

Figure 2. Light microscopy images of alkali-insoluble, Zymolyase-insoluble fraction. The cylindrical rod-like cell shape was maintained, despite digestion of (1→3)-β-D-glucan. The results suggest that fraction 4 functions in maintaining cell shape. (a) Intact cell; (b) fraction 4.

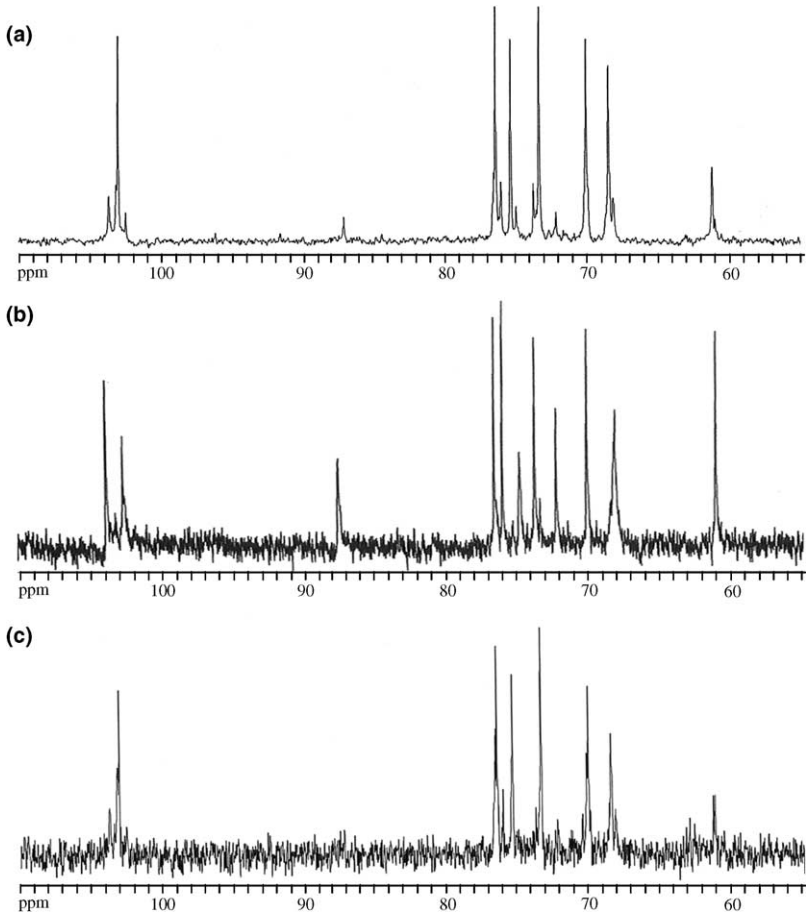

Figure 3. ^{13}C NMR spectra of alkali-insoluble, Zymolyase-soluble fraction and Smith degradation fraction. The alkali-insoluble, Zymolyase-soluble fraction of *S. cerevisiae* mainly contained six signals attributable to (1→6)-β-D-glucan. The same fraction of *S. pombe* (fraction 5) predominantly displayed 12 signals similar to fraction 2, assignable as highly branched (1→6)-β-D-glucan. Fraction 5_{SD} mainly contained six signals attributable to (1→6)-β-D-glucan. (a) Alkali-insoluble, Zymolyase-soluble fraction of *S. cerevisiae*; (b) fraction 5; (c) fraction 5_{SD}.

of fraction 2 was smaller than that of fraction 5 (Fig. 4). This difference may be due to partial hydrolysis during acid treatment. In addition, fraction 2 was obtained at a concentration of 6.97 mg/g dry cell, while the yield of fraction 5 was 15.40 mg/g dry cell. As yeast cell walls account for 15–25% of the dry weight, this value of fraction 5 corresponds to 6–10% of the cell wall. Our results suggest that the content of (1→6)-β-D-glucan in the cell wall is significantly higher than the previously published value (2%), and Zymolyase digestion is superior for use in a preparation of (1→6)-β-D-glucan of *S. pombe*.

The alkali-insoluble, Zymolyase-soluble fraction of *S. cerevisiae* consisted of (1→6)-β-D-glucan with a small number of (1→3)-β-branches. In view of the foregoing data, we hypothesize that alkali-insoluble, Zymolyase-soluble glucan of *S. pombe* contains more branches than that of *S. cerevisiae*. In a study by Manners and Meyer, the yields of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,4-di-methyl-*O*-D-glucose from fully methylated (1→6)-β-D-glucan of *S. pombe* were 43.9% and 42.7%, respectively,[4] indicating a highly branched structure. The ^{13}C NMR spectrum of fraction 5 in this study additionally

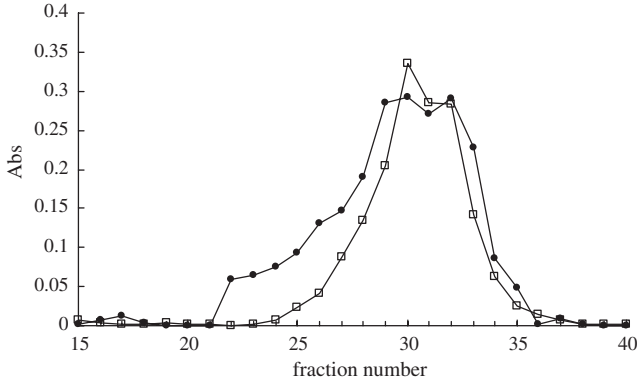

Figure 4. Molecular weight distribution of alkali-insoluble, acid-insoluble glucan and alkali-insoluble, Zymolyase-soluble glucan. Distribution of the molecular weights of fractions 2 and 5 was determined by gel chromatography. The molecular size of fraction 2 was smaller than that of fraction 5. (□) fraction 2; (●) fraction 5.

supports the absence of linear (1→6)-β-D-glucan. These findings collectively provide evidence of a highly branched structure. However, methylation analysis alone is not sufficient to completely identify the linkage of the main chain between (1→3)-β and (1→6)-β. To determine the main chain linkage, chemical modification, ^{1}H NMR and 2D-NMR experiments were performed. Fraction 5 was treated successively with sodium metaperiodate, sodium borohydride, and diluted sulfuric acid. The resulting macromolecular fraction (fraction 5_{SD}) displayed only six signals at 103.00, 76.37, 75.28, 73.21, 69.89, and 68.30 ppm (Fig. 3c), similar to the (1→6)-β-D-glucan of *S. cerevisiae*. The data confirm that fraction 5 is a highly branched (1→6)-β-D-glucan.

In ^{1}H NMR spectra, the anomeric signals of (1→3)-β-D-glucan and (1→6)-β-D-glucan appear at around 4.7–4.8 ppm and 4.5–4.6 ppm, respectively.[14,31] Additionally, the signals of branching points of (1→3)-β-branched (1→6)-β-D-glucan are observed at 4.55 ppm, and those of the nonreducing end at 4.73 ppm. Figure 5 represents the ^{1}H NMR spectrum of fraction 5 in D_2O at 70 °C. In the anomeric region of the spectrum, two resolved doublets were identified. The first at 4.737 ppm was assigned to the H-l proton of the side chain [(1→3)-β-glucopyranosyl unit], and the second at 4.553 ppm belonged to the H-l proton of the main chain (3,6-linked). The integrals of these doublets were almost equal, and accounted for the almost all of the main chain glucose was substituted. The ratio of branching points and side-chains was calculated as one to one. A third doublet at 4.519 and 4.538 ppm appeared as minor signals. We assume that this doublet belongs to linear (1→6)-β-linked glucopyranosyl residues. Anomeric protons were further assigned by 2D-NMR, using the GRASP-HMBC pulse sequence. The cross-peaks between proton and carbon signals directly represent the corresponding glycosidic linkage. The partial spectra of fraction 5 and the (1→6)-β-D-glucan segment of *Candida albicans* are depicted in Figure 6a and b, respectively. Both spectra contain similar cross-peaks, although they differ in intensity. In strong support of the above assumption, the signal at 4.7 ppm displayed a cross-peak around 85 ppm, and the signal at 4.55 ppm contained one at around 70 ppm.

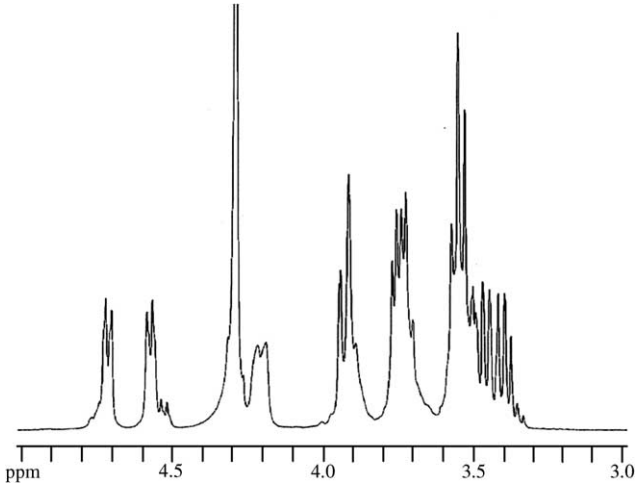

Figure 5. ^{1}H NMR spectrum of alkali-insoluble, Zymolyase-soluble glucan. Fraction 5 was dissolved in D_2O, and the ^{1}H NMR spectrum was measured at 70 °C. In the anomeric region of the ^{1}H NMR spectrum of fraction 5, there were two resolved doublets with almost equal integrals: the first was assigned as the H-l proton of the side-chain ((1→3)-β-D-glucopyranosyl unit), and the second to the main chain (3,6-linked). Minor signals belonging to linear (1→6)-β-linked glucopyranosyl residues were additionally present.

Molecular weights of fractions 5 and 5_{SD} were determined by MALDI-TOF-MS, and the structures compared. The molecular weight distributions are presented in Figure 7. Fraction 5 is distributed from 1500 to around 8000, while fraction 5_{SD} is from 1500 to around 5000. The molecular weight of fraction 5_{SD} was decreased to about 68% that of fraction 5 due to removal of the side-chain by Smith degradation, as calculated from the average molecular weights of both fractions (Fig. 7). We propose that *S. pombe* contains a highly branched (1→6)-β-D-glucan with β-monoglucosyl branches in almost every glucose residue of the main chain that form a comb-like structure (Fig. 8). The structure was not reported in *S cerevisiae* or *C. albicans*[3,32] and is thus unique in *S. pombe*.

2.4. Alkali-soluble fraction

Sodium hydroxide extractable glucan fractions were prepared by treatment with 3% NaOH at 4 °C for 6 days, on five extractions at 70 °C for 1 h followed by neutral ization. Fractions were collected from the final precipitate. Similar NMR spectra were obtained with both extractions, and the fraction was numbered as 6. The well-known antigenic compound, galactomannan, was solubilized by sodium hydroxide treatment, but recovered from the final supernatant after neutralization.[4] ^{13}C NMR spectra and GLC analysis of fraction 6 confirmed the presence of glucans without mannose or galactose residues (Fig. 9a). Signals were similar to those of glucans prepared from alkali-insoluble fractions (fraction 1) described above, namely, (1→3)-α-D-glucan, (1→3)-β-D-glucan, and highly branched (1→6)-β-D-glucan. Manners and Meyer suggested the presence of highly branched (1→3)-β-D-glucan due to the presence of 2,4-di-*O*-methyl glucose from the fully methylated fraction.[4] This type of linkage was not observed in the alkali-insoluble fraction. Fraction 6 was analyzed further. Digestion of fraction 6 by Zymolyase 100T resulted in water-insoluble (fraction 7) and soluble (fraction 8) fractions. The ^{13}C NMR spectrum of fraction 7 displayed six predominant signals at 99.50, 82.97, 71.84, 70.71, 69.40, and 60.27 ppm (Fig. 9b), characteris-

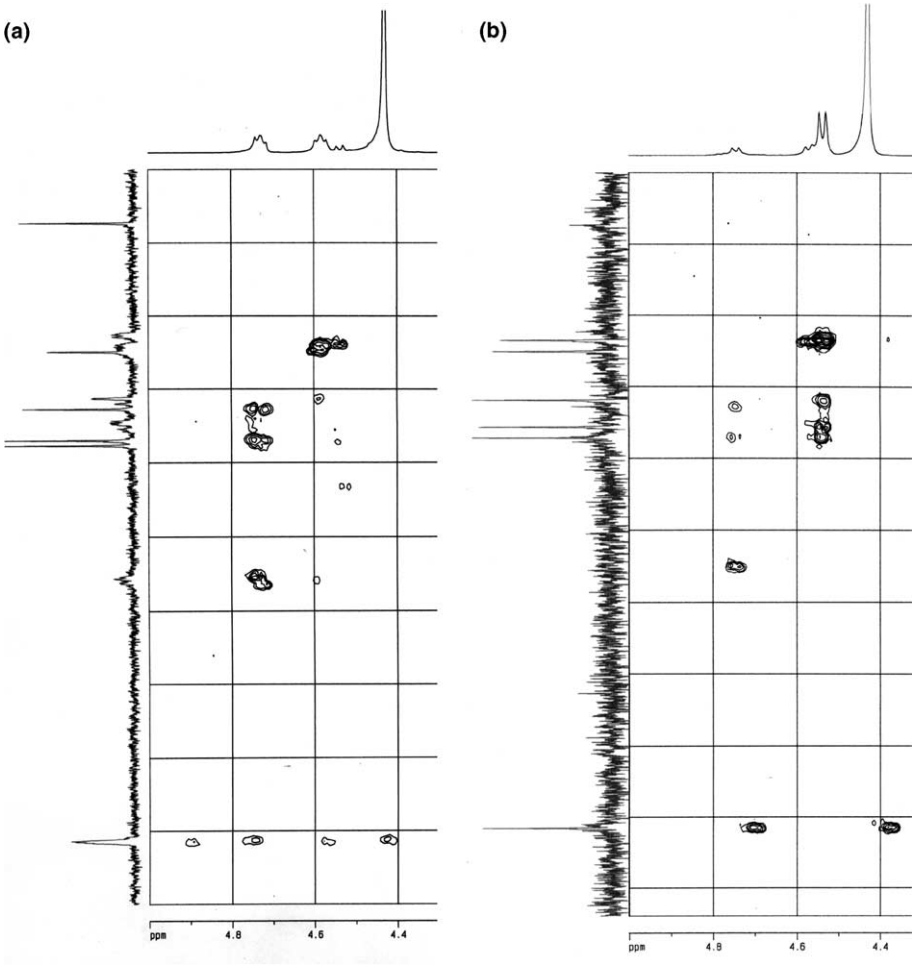

Figure 6. 2D-NMR spectra of alkali-insoluble, Zymolyase-soluble glucan and the (1→6)-β-D-glucan segment of *C. albicans*. Spectra of fraction 5 and the (1→6)-β-D-glucan segment of *C. albicans* displayed similar cross-peaks. (a) Fraction 5; (b) (1→6)-β-D-glucan segment of *C. albicans*.

tic of (1→3)-α-D-glucan. The ^{13}C NMR spectrum of fraction 8 was similar to that of highly branched (1→6)-β-D-glucan (fractions 2 and 5; Fig. 9c), and no signals were assigned to branched (1→3)-β-D-glucans.[26,33] These findings suggest that the alkali-soluble (1→3)-β-D-glucan is connected to highly branched (1→6)-β-D-glucan, but does not contain additional branches. Thus, the components of alkali-soluble fraction may include (1→3)-α-D-glucan, slightly branched (1→3)-β-D-glucan, and highly branched (1→6)-β-D-glucan.

2.5. ^{13}C NMR of whole cell

Figure 10a displays the ^{13}C NMR spectrum of defatted cells suspended in D_2O. Together with the signals attributable to protein components, several signals were detected between 60 and 105 ppm. To assign these signals, ^{13}C NMR spectra of several fractions were measured in D_2O. Significantly, many of the fractions prepared in this study (including fractions 3, 4, and 7) were insoluble in neutral aqueous solution. The spectrum of fraction 5 is shown in Figure 10b. Chemical shifts were similar to those obtained in Me_2SO-d_6. Many of the signals in the cell (at 102.32, 84.77, 75.40, 75.12, 73.87, 72.97, 72.63, 69.05, 68.73, 68.19, 60.28 ppm) were detectable in fraction 5, and thus assigned as branched (1→6)-β-D-glucan. α-Galactomannan, the major antigenic polysaccharide in *S. pombe*, was prepared by ethanol precipitation of a sodium hydroxide extract. Figure 10c shows the spectrum of α-galactomannan. Many signals were assignable in the cell. Major signals suggesting the absence of (1→3)-α- or (1→3)-β-D-glucans were assigned from the spectra of fraction 5 and α-galactomannan. This result is consistent with the observed water insolubility of these glucans. Transmission electron microscopy disclosed that α-galactomannan is located on the cell surface and connected to cell-wall proteins showing hair-like structure, supporting the possibility of high flexibility. Together with α-galactomannan, signals of fraction 5 were obtained. This finding suggests that the flexibility of fraction 5 is high under aqueous

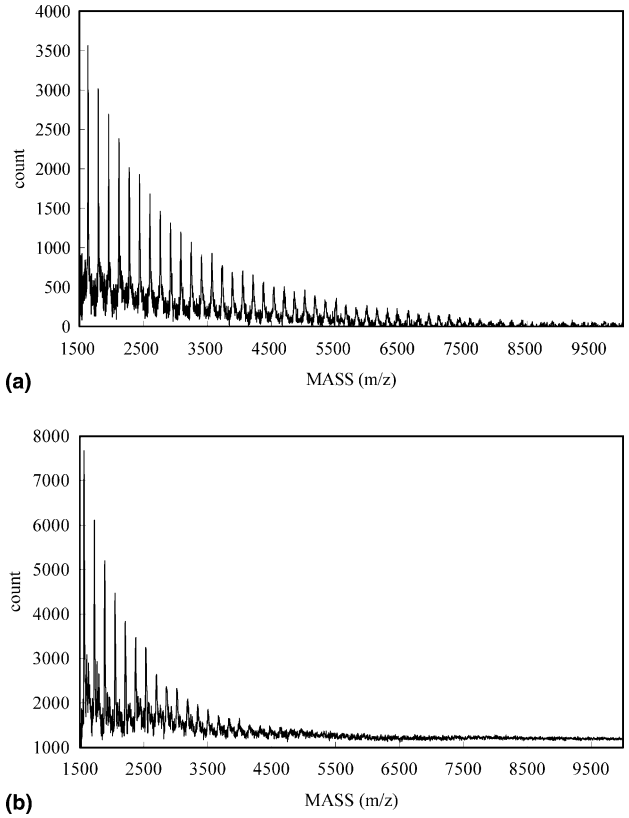

Figure 7. MALDI-TOF-MS spectra of the alkali-insoluble, Zymolyase-soluble fraction and its Smith degradation fraction. Molecular weights of fractions 5 and 5_{SD} were determined by MALDI-TOF-MS. The molecular weight of fraction 5_{SD} was decreased to 68% that of fraction 5, due to removal of the side-chain by Smith degradation. (a) Fraction 5; (b) fraction 5_{SD}.

physiological conditions. Thus, this component plays a role other than maintaining the rigidity of the cell wall. In *S. cerevisiae*, (1→6)-β-D-glucan molecules are located outside the skeletal framework, and connect the framework with a particular class of cell-wall proteins, specifically, glycosyl phosphatidylinositol-cell-wall proteins.[13,15,34,35] Previous results show that (1→6)-β-D-glucanase-released cell-wall proteins support this architecture.[36] In contrast, other investigators report that (1→6)-β-D-glucanase does not release cell-wall proteins in *S. pombe*.[37] This is explainable by the fact that the corresponding (1→6)-β-D-glucan in *S. pombe* cannot be digested by (1→6)-β-D-glucanase due to its higher branched structure. The signals of branched (1→6)-β-D-glucan shown in D_2O suggest that (1→6)-β-D-glucan of *S. pombe* is localized outside the skeletal framework, and connected to cell-wall proteins, such as *S. cerevisiae*. In contrast, none of the signals corresponding to (1→3)-α-D- or (1→3)-β-D-glucans are detected in ^{13}C NMR spectra in D_2O. This finding is consistent with the physical properties of these glucans, which are present in the cell wall as rigid and dense layers to function in determining and maintaining cell shape.

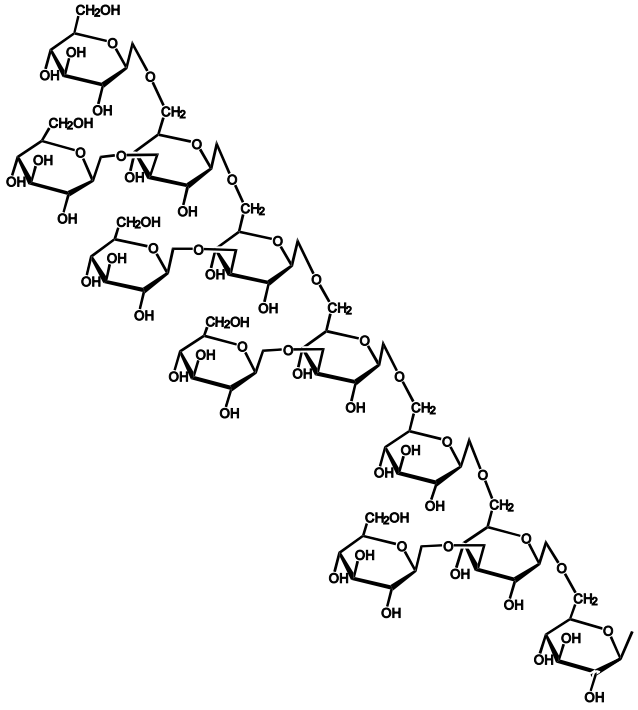

Figure 8. Image of the (1→6)-β-D-glucan structure. *S. pombe* contained a highly branched (1→6)-β-D-glucan with β-monoglucosyl branches in almost every glucose residue of the main chain, forming a comb-like structure.

3. Conclusions

Cell-wall polysaccharides were prepared by repeated extraction with sodium hydroxide, acetic acid, and/or Zymolyase digestion. The structures of these fractions were analyzed using ^{13}C NMR spectroscopy. Structural analysis disclosed that the cell wall of *S. pombe* is composed of three glucans, specifically, (1→3)-α-D-glucan, (1→3)-β-D-glucan, and highly branched (1→6)-β-D-glucan, in addition to α-galactomannan. The structure of highly branched (1→6)-β-D-glucan was confirmed by specific degradation of the side-chain glucose. Flexibility of these polysaccharides in the cell wall was analyzed by ^{13}C NMR spectra in D_2O. Our data indicate that (1→3)-α- and (1→3)-β-D-glucans are rigid, contributing to the cell shape, while highly branched (1→6)-β-D-glucan and α-galactomannan are flexible.

4. Experimental

4.1. Organisms and culture conditions

Wild-type *S. pombe* strain, L972 h^-, and *S. cerevisiae* (O-102) were used. Cells were cultured at 30 °C in YPD medium (1% yeast extract, 2% polypeptone, 2% glucose) until the stationary phase.

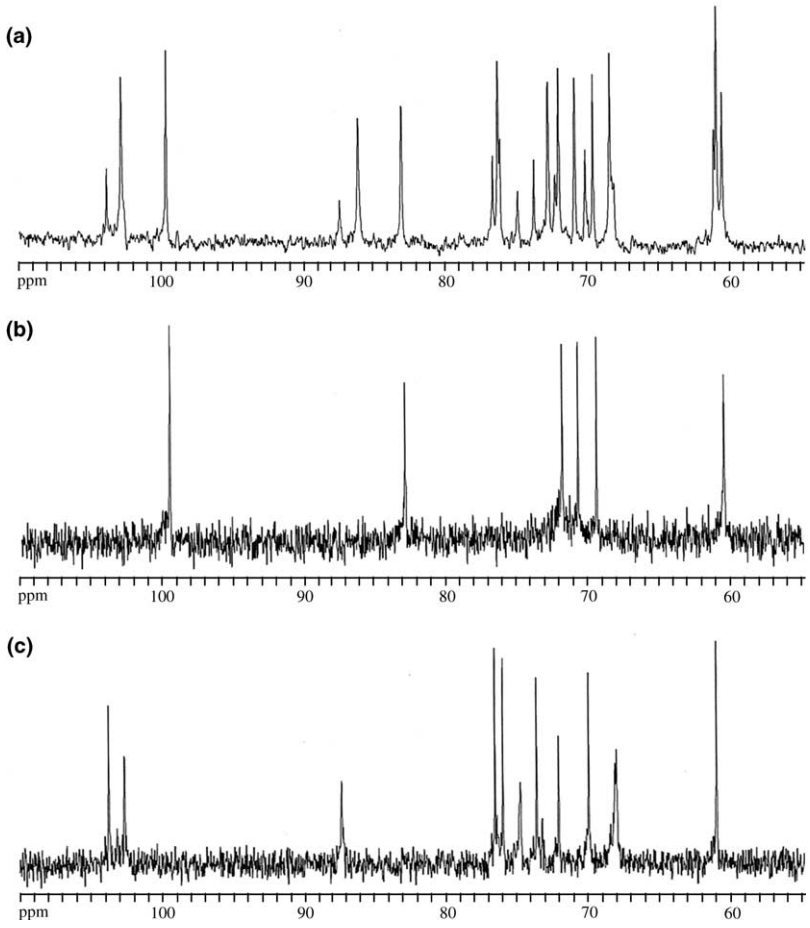

Figure 9. ^{13}C NMR spectra of alkali-soluble glucans. Fraction 6 was composed of glucans without mannose or galactose residues. These signals were similar to the alkali-insoluble fraction (fraction 1), assignable as (1→3)-α-D-glucan, (1→3)-β-D-glucan, and highly branched (1→6)-β-D-glucan. We did not detect highly branched (1→3)-β-D-glucan in this fraction. The ^{13}C NMR spectrum of fraction 7 displayed six predominant signals attributable to (1→3)-α-D-glucan. The ^{13}C NMR spectrum of fraction 8 was similar to that of highly branched (1→6)-β-D-glucan (fractions 2 and 5). (a) Fraction 6; (b) fraction 7; (c) fraction 8.

4.2. Isolation of cell-wall glucans (chart 1)

Alkali-insoluble, acetic acid-insoluble and alkali-soluble fractions were isolated as described previously.[4] Briefly, cells were treated with 3% NaOH under nitrogen at 4 °C for 6 days with continuous stirring. The supernatant solution was filtered through glass paper, and neutralized with acetic acid to pH 6.5. The precipitate was homogenized and washed twelve times with distilled water (DW) to produce an alkali-soluble fraction (fraction 6). Next, the supernatant was concentrated and precipitated with ethanol at 4 °C. The precipitate was re-dissolved in DW and dialyzed, followed by lyophilization to generate an alkali-soluble crude extract.

Alkali-insoluble fractions were treated seven times with 0.5 M acetic acid at 90 °C for 3 h. The resulting precipitate, an alkali-insoluble, acetic acid-insoluble fraction, was designated 'fraction 1'. The first three of above fractions were combined and treated with α-amylase (Wako Pure Chemical Industries, Ltd, Osaka), 0.5 M acetic acid, 10% trifluoroacetic acid, and dialyzed against DW. The macromolecular fraction was precipitated with ethanol to produce an alkali-insoluble, acetic acid-soluble fraction (fraction 2).

Alkali-insoluble, Zymolyase-insoluble and alkali-insoluble, Zymolyase-soluble fractions were isolated as described previously.[27] Briefly, cells were treated five times with 3% NaOH at 70 °C for 1 h while stirring continuously. The alkali-insoluble fraction was washed twice with 100 mM phosphate buffered saline (PBS) and twice with 10 mM PBS for neutralization, and digested for 16 h in 10 mM PBS with 1 mg/mL Zymolyase 100T (Seikagaku Corp., Tokyo) containing 0.01% of NaN_3 at 37 °C. The insoluble fraction was washed several times with DW, and lyophilized to produce an alkali-insoluble, Zymolyase-insoluble fraction (fraction 4). The supernatant solution was treated once with alpha-amylase (10 mg/mL) at room temperature for 2 h, and twice with phenol, followed by four extractions with ether. The aqueous solution was dialyzed against

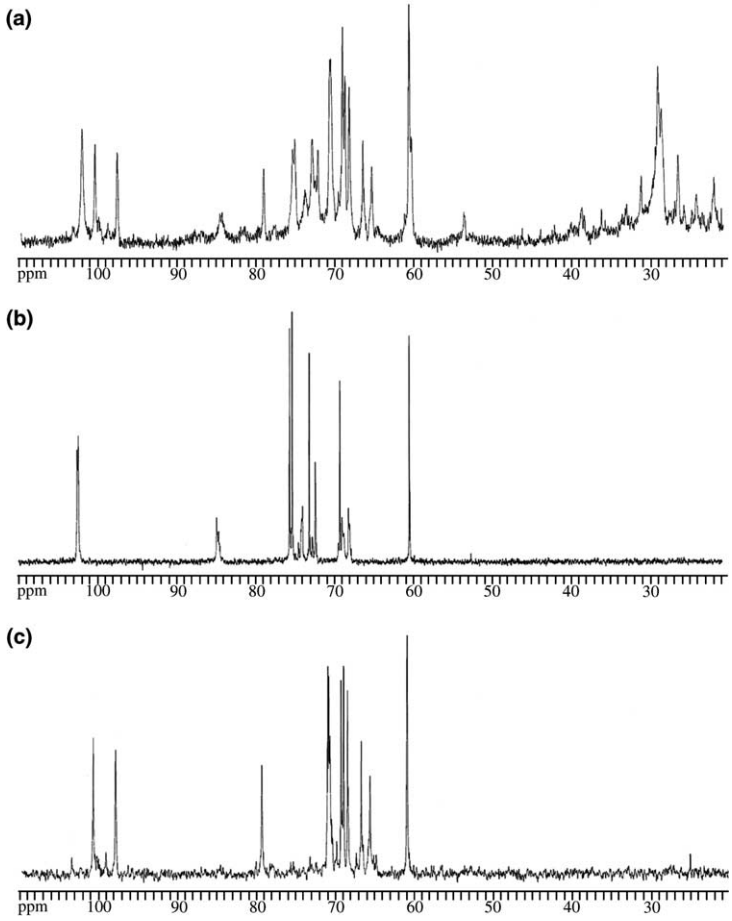

Figure 10. ^{13}C NMR spectra in D_2O. Defatted cells were dissolved in D_2O, and the ^{13}C NMR spectrum was measured at 70 °C. Together with the protein signals, several were assignable as fraction 5 and α-galactomannan in the whole cell. (a) Defatted cell; (b) fraction 5; (c) alkali-soluble crude extract.

DW for 4 h using Spectra-Por membranes with 6000–8000 mol. wt cut-off (Spectrum Medical Industries, Inc.), and lyophilized. Freeze-dried material was resuspended in 5–10 mL of DW, and re-dialyzed against DW for 30 h using 2000 mol. wt pore-size tubing. This solution was re-lyophilized to produce the alkali-insoluble, Zymolyase-soluble fraction (fraction 5).

4.3. Enzymatic fractionation of alkali-soluble glucans

Fraction 6 was treated with Zymolyase 100T (1 mg/mL) at 37 °C for 24 h. The resulting insoluble fraction was washed several times with DW, and lyophilized to produce fraction 7. The supernatant solution was treated in a similar way to fraction 5, generating fraction 8.

4.4. Alkali treatment of fraction 1

Fraction 1 was treated with 3% NaOH at 70 °C for 1 h to remove any remaining (1→3)-α-D-glucan. The insoluble fraction was neutralized, dialyzed against DW for two days and lyophilized, producing fraction 3.

4.5. Smith degradation

The alkali-insoluble, Zymolyase-soluble fraction was oxidized with 10 mM $NaIO_4$ at 4 °C for 24 h in the dark. Upon completion of the reaction, excess periodate was destroyed by adding ethylene glycol. The reaction mixture was dialyzed against tap water for two days, and DW for one day. The nondialyzable fraction was reduced with $NaBH_4$ at room temperature for 2 h. After acidification with acetic acid, neutralization, and dialysis, the oxidation product was hydrolyzed with 0.05 M H_2SO_4 at room temperature for 24 h. The reaction mixture was re-dialyzed against DW for two days, and lyophilized to produce fraction 5_{SD}.

4.6. ^{13}C NMR spectroscopy

For obtaining NMR spectra, microparticulate glucans and acetone-defatted cells were dissolved in Me_2SO-d_6 or D_2O. Proton-decoupled ^{13}C NMR and 2D-NMR spectra (HMBC pulse sequence) were recorded at

70 °C with Bruker AMX-400WB or DPX-400 instruments (Bruker Instruments Inc., Billerica, MA).

4.7. Carbohydrate analysis

For gel filtration, fractions 2 and 5 were dissolved in 0.3 M NaOH, applied to a SM450 column of Toyopearl HW65 (Tosoh, Tokyo), and washed with 0.3 M NaOH. The carbohydrate content of each fraction was determined using the phenol–H_2SO_4 method.

The component sugar of the alkali-soluble fraction was determined by capillary gas–liquid chromatography (Ohkura Riken Co., Ltd, Tokyo) of alditol acetate derivatives following complete hydrolysis with 2 M trifluoroacetic acid. A capillary column of fused silica (J & W Science, Inc., CA, 30 m×0.262 mm, liquid phase; DB-225, 0.25 μM) was used at 220 °C.

4.8. MALDI TOF MS

MALDI TOF MS analysis was conducted using a Perceptive Biosystems Voyager DP-RD equipped with delayed extraction and a nitrogen laser. All analyses were performed using α-cyano-4-hydroxy-cinnamic acid as the matrix.

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