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Synthesis, (1 → 3)-β-D-glucanase-binding ability, and phytoalexin-elicitor activity of a mixture of 3,4-epoxybutyl (1 → 3)-β-D-oligoglucosides

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Abstract—We describe a approach for the synthesis of a mixture of 3,4-epoxybutyl (1 → 3)-β-D-oligoglucosides. The particular (1 → 3)-β-D-glucan isolated from the cell walls of *Saccharomyces cerevisiae* was recovered from the aqueous medium as water-insoluble particles by the spray drying (GS) method, and it was characterized by FTIR spectroscopy. The acid-solubilized (1 → 3)-β-D-oligoglucosides were prepared by partial acid hydrolysis of glucan particles, which were qualitatively analyzed by fluorophore-assisted carbohydrate electrophoresis (FACE). The peracetylated 3-butenyl (1 → 3)-β-D-oligoglucosides were synthesized by treating peracetylated (1 → 3)-β-D-oligoglucosides with the 3-butenyl alcohols and a Lewis acid ($SnCl_4$) catalyst. Epoxidation of the peracetylated 3-butenyl oligoglucosides took place with *m*-chloroperoxybenzoic acid (*m*-CPBA). NaOMe in dry methanol was used for the deacetylation of the blocked derivatives, to give the 3,4-epoxybutyl (1 → 3)-β-D-oligoglucoside mixture in an overall yield of 21%. The sample was analyzed by positive-ion electrospray ionization mass spectrometry (ESIMS). In a 3,4-epoxybutyl (1 → 3)-β-D-oligoglucoside-binding (1 → 3)-β-D-glucanase assay, we found that the (1 → 3)-β-D-glucanase was obviously inactivated by the 3,4-epoxybutyl (1 → 3)-β-D-oligoglucosides. At the same time, we found the 3,4-epoxybutyl (1 → 3)-β-D-oligoglucoside mixture was more active as compared to the underivatized oligoglucoside mixture in eliciting phytoalexin accumulation in tobacco cotyledon tissue. Furthermore, it could be kept for a longer time than a (1 → 3)-β-D-oligoglucoside mixture, which indicated it is much more stable than (1 → 3)-β-D-oligoglucosides.

Keywords: 3,4-Epoxybutyl (1 → 3)-β-D-oligoglucosides; Synthesis; (1 → 3)-β-D-Glucanase-binding ability; Phytoalexin-elicitor activity

1. Introduction

Higher plants have the ability to initiate various defense reactions such as the production of phytoalexins, antimicrobial proteins, reactive oxygen species, and reinforcement of the cell wall when they are infected by pathogens such as fungi, bacteria, and viruses. If these reactions occur in a timely manner, the infection will not proceed further. However, if the defense reactions occur too late or are suppressed, the infection process will proceed successfully.[1] Thus, it is critically important for the plant to detect infecting pathogens effectively and deliver such information intracellularly/intercellularly to activate their defense machinery.

It is believed that the detection of pathogens is mediated by chemical substances secreted/generated by the pathogens. Various types of such compounds (elicitor molecules) including oligosaccharides, (glyco)proteins, (glyco)peptides, and lipids, have been shown to induce defense responses in plant cells, and their involvement in the detection of (potential) pathogens in plant has been discussed.[2–4] Oligosaccharides derived from fungal and plant cell-wall polysaccharides are one class of

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well-characterized elicitors that, in some cases, can induce defense responses at a very low concentration, for example, at nanomolar levels. At the same time, the elicitor activity of oligosaccharides is dependent on the structural properties of oligosaccharide molecules, such as the number of saccharide units, the length of the aglycon chain, and the molecule configuration.[5] However, the elicitor-active oligosaccharides can be hydrolyzed by endo- and exohydrolases from higher plants, and give elicitor-inactive oligosaccharide fragments.[6] Therefore, improving the stability of the elicitor-active oligosaccharides is the key to developing a viable biological pesticide from these oligosaccharides.

The use of epoxyalkyl glycosides as active-site-directed inhibitors has been invaluable in delineating the mechanism of action for a variety of hydrolases, for example, β-D-glucan endo- and exohydrolases.[7,8] The epoxyalkyl glycoside moiety targets the inhibitor to the substrate-binding site, and if the length of the alkyl chain is correct, the epoxide group is brought into the vicinity of the catalytic amino acids. Protonation of the epoxide oxygen opens the epoxide ring and results in the formation of a stable ester linkage between the inhibitor and the catalytic nucleophile. It has been well demonstrated[9] that the chain length of aglycon in the mechanism-based epoxide-bearing inhibitors have a significant effect on their activity.

With the aim of improving the stability of a mixture of elicitor-active oligosaccharides and studying the protein-binding ability to $(1\rightarrow3)$-β-D-glucanase, as well as phytoalexin-elicitor activity in tobacco cotyledon tissue, herein, we have synthesized a mixture of 3,4-epoxybutyl $(1\rightarrow3)$-β-D-oligoglucosides. These are analogues of oligoglucosides, where the 3,4-epoxybutyl functionality has been introduced at the reducing end of the glycon.

2. Results and discussion

2.1. Structural characterization of *Saccharomyces cerevisiae* glucan

The FTIR spectra of the glucan sample (Fig. 1) showed the typical spectral pattern of a $(1\rightarrow3)$-β-D-glucan, that is, it contained absorption bands arising from the *v*(CC) and the *v*(COC) stretching vibrations at 1159 cm^{-1}, two partially overlapped bands at 1078 and 1041 cm^{-1} attributable to ring and (C–OH) side group stretching, a band at 892 cm^{-1} assigned to the β-glycosidic (C_1–H) deformation mode, and the highest intensity of the *v* (OH) band at lower frequency (3425 cm^{-1}). The presence of amide I and amide II bands at 1642 and 1530 cm^{-1} accords with the residual protein content (1.6%) of the glucan.

2.2. Fluorophore-assisted carbohydrate electrophoresis (FACE) of the $(1\rightarrow3)$-β-D-oligoglucosides mixture

Polyacrylamide gel electrophoresis was employed for the analytical separation and identification of ANTS–derivatized $(1\rightarrow3)$-β-D-oligoglucosides. Saccharides of various lengths were derivatized, and the saccharide–ANTS adducts were separated on a 32% T, 2.4% C_{BIS} polyacrylamide gel at alkaline pH as shown in Figure 2. Lane 2 shows the migration of saccharide–ANTS adducts for a monosaccharide, a disaccharide, a trisaccharide, a tetrasaccharide, a pentasaccharide, a hexasaccharide, a heptasaccharide, and an octasaccharide, respectively. Saccharides of various lengths can easily be

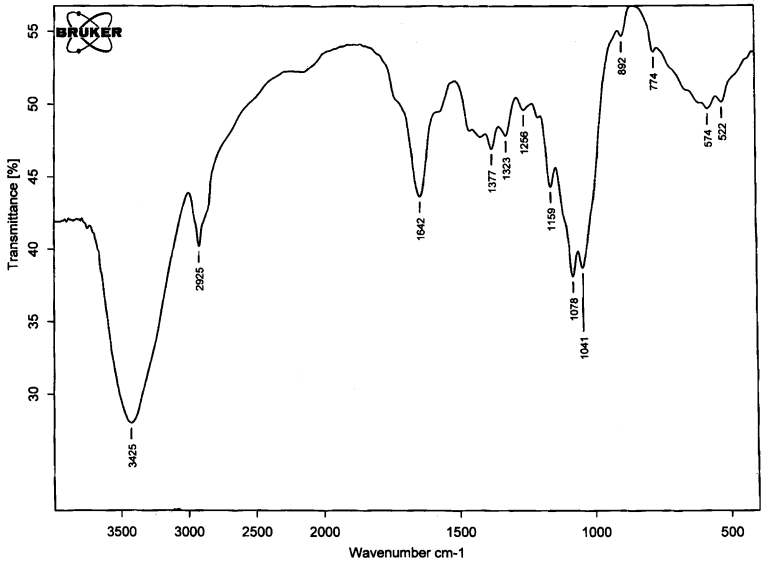


Figure 1. FTIR spectra (in KBr) of the particulate β-glucan sample dried by spraying (GS).

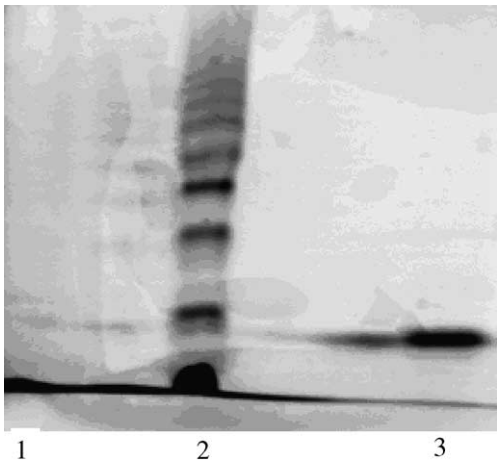
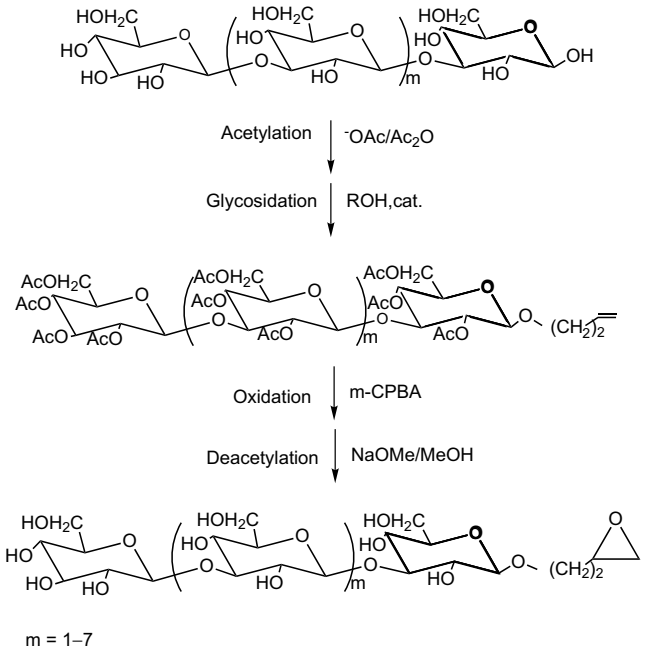


Figure 2. Separation of (1 → 3)-β-D-oligoglucoside–8-aminonaphthalene-1,3,6-trisulfonate (ANTS) adducts (1 μmol) of various lengths on a 32% T, 2.4% C_{BIS} polyacrylamide gel at alkaline pH. Lane 1: ANTS; Lane 2: octasaccharide, heptasaccharide, hexasaccharide, pentasaccharide, tetrasaccharide, trisaccharide, disaccharide, monosaccharide, and ANTS, from top to bottom, respectively; Lane 3: glucose–ANTS.

distinguished from one another because the ANTS adducts of the smaller saccharides have greater electrophoretic mobilities than those of the larger saccharides.

2.3. Synthesis and ESIMS of 3,4-epoxybutyl (1 → 3)-β-D-oligoglucosides mixture

The synthetic route to 3,4-epoxybutyl (1 → 3)-β-D-oligoglucosides is shown in Scheme 1. The (1 → 3)-β-D-oligoglucosides were acetylated with potassium acetate–acetic anhydride to maximize the yield of peracetylated



Scheme 1. Synthetic route for 3,4-epoxybutyl (1 → 3)-α-D-oligoglucosides.

(1 → 3)-β-D-oligoglucosides. The peracetylated (1 → 3)-β-D-oligoglucosides were treated with linear 3-butenyl alcohols and stannic chloride as the Lewis acid catalyst. The reaction of peracetylated 3-butenyl (1 → 3)-β-D-oligoglucosides with *m*-chloroperoxybenzoic acid (*m*-CPBA) in dichloromethane at room temperature gave the corresponding oxiranes. NaOMe in dry methanol at room temperature was used for the deacetylation of the blocked derivative, to give the corresponding 3,4-epoxybutyl (1 → 3)-β-D-oligoglucoside mixture in an overall yield of 21%.

The 3,4-epoxybutyl (1 → 3)-β-D-oligoglucosides mixture was analyzed by electrospray-ionization mass spectrometry (ESIMS) in the positive-ion mode, which is shown in Figure 3. The positive-ion spectrum showed the presence of the target oligosaccharides in the mass range between m/z 273 and 1083 corresponding to singly $[M + Na]^+$ charged ions of 3,4-epoxybutyl (1 → 3)-β-D-oligoglucosides with degrees of polymerization 1–6. At the same time, we thought that the 3,4-epoxybutyl (1 → 3)-β-D-oligoglucosides with degrees of polymerization 7–8 were not detected by ESIMS due to their small amounts in the sample.

2.4. Binding to (1 → 3)-β-D-glucanase and eliciting phytoalexin accumulation in tobacco cotyledon tissue bioassays of 3,4-epoxybutyl (1 → 3)-β-D-oligoglucosides

The results of these bioassays, shown in Table 1, demonstrated that the (1 → 3)-β-D-glucanase was obviously inactivated by 3,4-epoxybutyl (1 → 3)-β-D-oligoglucosides with $k_{app} = 6.68 \times 10^{-4}\,min^{-1}$. At the same time, we found that the 3,4-epoxybutyl (1 → 3)-β-D-oligoglucosides mixture was more active as compared to the (1 → 3)-β-D-oligoglucosides mixture in eliciting phytoalexin accumulation in tobacco cotyledon tissue, and it could be kept longer time than (1 → 3)-β-D-oligoglucosides, which indicated it is much more stable than the (1 → 3)-β-D-oligoglucosides.

3. Experimental section

3.1. General

IR spectra were recorded with an FTIR apparatus, and wavenumbers are reported in cm^{-1}. The ESIMS was carried out on a Micromass (Manchester, UK) Q-TOF2 hybrid tandem mass spectrometer. *S. cerevisiae* cell walls were purchased from the Anqi Company (Yichang, China). 8-Aminonaphthalene-1,3,6-trisulfonate (ANTS) and sodium cyanoborohydride were purchased from Sigma–Aldrich Chemical Company. (1 → 3)-β-D-Glucanase from *Helix pomatia* was purchased from Fluka Chemical Company. Solvents dichloromethane and 1,2-dichloroethane were distilled from P_2O_5.

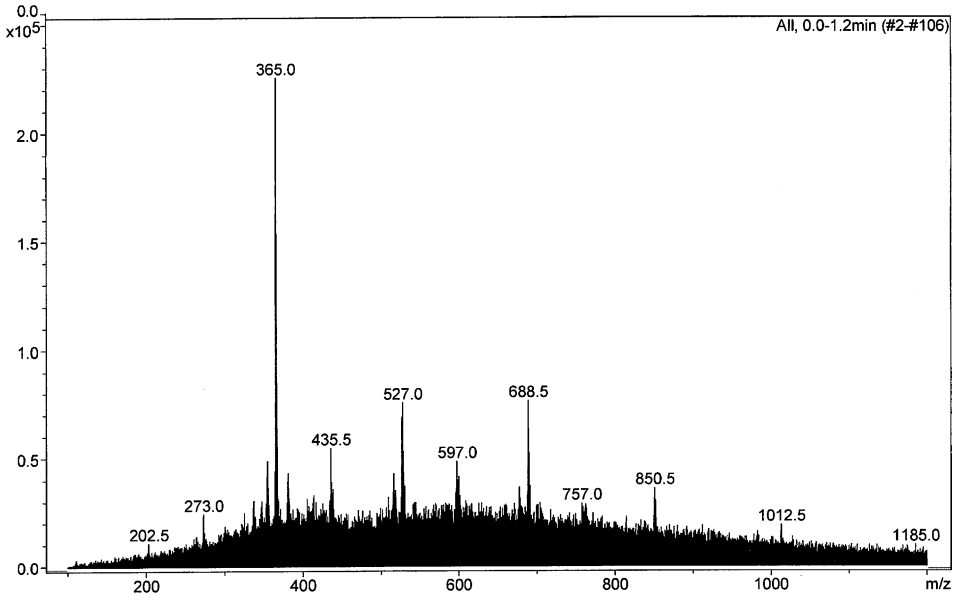


Figure 3. ESIMS of $[M + Na]^+$ adducts of the mixture of 3,4-epoxybutyl (1 → 3)-β-D-oligoglucoside.

Table 1. Binding and phytoalexin-elicitor activity of (1 → 3)-β-D-oligosaccharides mixture and its epoxyalkyl derivatives

Substance	k_{app} (min^{-1})	Biological activity (EC_{50}) (mM)		
		22 h	44 h	66 h
(1 → 3)-β-D-Oligosaccharides mixture		50	500	800
Epoxyalkyl derivatives mixture	6.68×10^{-4}	26	80	200

3.2. Plant material

Tobacco (*Nicotiana tabacum* cv Samsun NN) plants were grown from seed in a greenhouse under controlled conditions and used after two months of culture (eight leaves).

3.3. Preparation and isolation of the (1 → 3)-β-D-glucan

The water-insoluble (1 → 3)-β-D-glucan (particulate glucan) was obtained from *S. cerevisiae* cell walls by extraction with 6% NaOH at 60 °C for 4 h. Distilled water was added to the dispersion, and the insoluble part after stirring for 30 min was collected by centrifugation. The sediment was suspended in 3% NaOH and heated at 90 °C for 2 h. The insoluble material was recovered by centrifugation, washed three times with distilled water, and subsequently extracted twice with 4% phosphoric acid at room temperature for 2 h. The insoluble residue, representing the cell-wall (1 → 3)-β-D-glucan was separated by centrifugation, resuspended in distilled water, and decanted with water until neutral. The aqueous suspension was taken for the recovery of the particulate glucan using the technique of spray drying (GS). The value found was 13.5% for GS. The sample was characterized by FTIR spectroscopy.

3.4. Partial acid hydrolysis of glucan particles

To prepare hydrolysates, 50–100 mg of the glucan particles were suspended at a concentration of 5 mg/mL in 2 M TFA and heated at 85 °C for 110 min in 25-mL reaction flasks. The cooled samples were centrifuged at 160×*g* for 10 min at 25 °C. Supernatant fractions from 10 reaction flasks were pooled, and the residual TFA was removed by rotoevaporation at 35–40 °C in a silanized round-bottom flask. The sample was then lyophilized.

3.5. 8-Aminonaphthalene-1,3,6-trisulfonate (ANTS) labeling of oligosaccharides

One hundred nanomolar aliquots of the lyophilized oligosaccharides were tagged with 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) as described elsewhere.[10] The dried oligosaccharide sample was suspended in 5.0 mL of 0.2 M ANTS in 3:17 $HOAc/H_2O$ and freshly made 1.0 M sodium cyanoborohydride in Me_2SO and incubated at 37 °C for 16 h. The sample was dried under nitrogen at 45 °C, suspended in 50 mL of loading buffer (62.5 mM Tris–HCl, pH 6.8, containing 20% glycerol), and stored at −70 °C.

3.6. Electrophoresis of ANTS-labeled oligosaccharides

The electrophoretic method used was an adaptation of that previously reported.[10] The resolving gel was 32% acrylamide–2.4% bisacrylamide (Plus One Ready Sol

IEF 40; Pharmacia Biotech, Piscataway, NJ) in a $140 \times 160 \times 0.75$-mm glass cassette. For every 35 mL of resolving gel, 150 mL of 10% ammonium persulfate (A-6761; Sigma), and 15 mL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine {TEMED CAS Reg. no [17-1312-01] Pharmacia Biotech} were added. The stacking gel was 8% acrylamide–0.6% bisacrylamide containing 50 and 5 mL of ammonium persulfate and TEMED, respectively, for every 6 mL of stacking gel. The running buffer and the gel buffer were 0.025 M Tris base–0.192 M glycine (pH 8.4) and 0.42 M Tris base (pH 8.5), respectively. Electrophoresis was run at a constant current of 15 mA for 6 h in a cooled buffer system. For visualization of the ANTS-labeled oligosaccharides, the gel was removed from the glass cassette and placed onto the surface of a light box with UV illumination (300 nm).

3.7. Synthesis of 3,4-epoxybutyl (1 → 3)-β-D-oligoglucosides mixture

To boiling Ac_2O (20 mL) in a three-necked flask, 200 mg of KOAc was added. Then, 200 mg of (1 → 3)-β-D-oligoglucosides mixture was gradually added under vigorous stirring. The solution was kept for 1 h at 140 °C and then cooled to room temperature. The resulting peracetate (350 mg) and 30 mg of 3-buten-1-ol were added to 21 mL of dry 1,2-dichloroethane at 45 °C, followed by the addition of 58 mg of $SnCl_4$. The mixture was stirred for 5 h. After standard processing work-up, 190 mg of peracetylated 3-butenyl (1 → 3)-β-D-oligoglucoside mixture was obtained. In succession, to a solution of peracetylated 3-butenyl (1 → 3)-β-D-oligoglucosides in CH_2Cl_2 (7 mL), *m*-chloroperoxybenzoic acid (1.0 mmol) was added, and the suspension was stirred. When TLC showed that all starting compounds had been consumed, the reaction mixture was washed successively with 5% aq NaOH and H_2O, dried ($MgSO_4$), and filtered, and the filtrate was evaporated to dryness. The material thus obtained was stored in the dark at 4 °C. Prior to use, it was suspended in anhyd MeOH to a concentration of 100 mg/mL and deacetylated with an equal volume of 1 M NaOMe at room temperature for 60 min with continuous mixing. The same was then neutralized with 1 M HCl and filtered. The filtrate was evaporated to dryness under reduced pressure at 45 °C. The sample was analyzed by positive-ion electrospray ionization mass spectrometry (ESIMS) (see Fig. 3).

3.8. Assays for enzyme activity

The enzyme activity was measured by determining the number of reducing-sugar equivalents released on incubation of the enzyme with the corresponding substrates under the conditions stated. Assays were terminated by heating to 100 °C, and the release of reducing sugars was shown to be linear with respect to time and amount of enzyme used. The (1 → 3)-β-D-glucanase (40 μg/mL), BSA (0.8 mg/mL), glycerol 10% (v/v), and (3,4-epoxybutyl) oligoglucoside mixture (20 mM) were incubated at 18 °C in 40 mM NaOAc buffer at pH 5.0. The residual activity was determined at 100-min intervals.

3.9. Biological activity assays

Detached cotyledons from two-month-old greenhouse seedlings of tobacco were cut, and aliquots of β-glucooligosaccharide solutions (50 μL) were placed on the wounded areas. The cotyledons were incubated for 22, 44, and 66 h at 27 °C on moist filter paper in Petri dishes in the dark, respectively. Phytoalexin accumulation in the wound-droplet solutions was determined by measuring the absorbance (*A*) at 285 nm.

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