An Endo-(1→6)- β -D-glucanase of *Flavobacterium* M64 Hydrolyzing the Octasaccharide Repeating Unit of Succinoglycan to Two Tetrasaccharides

Jun-ichi ABE, Akinori AMEMURA and Tokuya HARADA*

*Institute of Scientific and Industrial Research,
Osaka University, Suita, Osaka 565, Japan*

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An endo-(1→6)- β -D-glucanase capable of hydrolyzing octasaccharide to two tetrasaccharides was isolated from cells of *Flavobacterium* M64. The octasaccharide represents the repeating unit of succinoglycan (SG-D). One tetrasaccharide was composed of D-glucose, succinic acid and pyruvic acid (4:1:1, molar ratio), and the other was composed of D-glucose and D-galactose (3:1, molar ratio). This enzyme hydrolyzed the (1→6)- β -D-glucosidic linkage adjacent to the (1→6)-linked β -D-glucose residue in the octasaccharide repeating unit of succinoglycan and also hydrolyzed the octasaccharide repeating units of similar polysaccharides produced by many strains of *Agrobacterium* and *Rhizobium* species.

Succinoglycan, an extracellular acidic polysaccharide produced by *Alcaligenes faecalis* var. *myxogenes* 10C3, consists of D-glucose, D-galactose, succinic acid and pyruvic acid.^{1~3)} Its polysaccharide moiety consists of (1→3)-, (1→4)-, (1→6)- and (1→4, 1→6)-linked β -D-glucose residues and (1→3)-linked β -D-galactose residues.^{4,5)} Recently many strains of *Agrobacterium* and a strain of *Rhizobium meliloti* were also shown to form a water-soluble, succinoglycan-like polysaccharide.^{3,6~8)} The structure of *Rhizobium* polysaccharide was determined by Jansson *et al.*⁷⁾

Succinoglycan is resistant to several known β -D-glucanases.⁹⁾ We found that *Flavobacterium* M64, isolated from soil, produces an extracellular β -D-glycanase (succinoglycan depolymerase) capable of depolymerizing the polymer to its repeating unit (SG-D) with the degree of polymerization of 8, when grown on a medium containing succinoglycan as the sole carbon source.^{8~11)} The enzyme also hydrolyzed the similar polysaccharides produced by all 9 strains of *Agrobacterium* species, suggesting the simi-

larity in the mode of glycosidic linkages of these polysaccharides with that of succinoglycan.³⁾

It seemed probable that *Flavobacterium* M64 also produces an intracellular glycanase(s) and/or glycosidase(s) that can degrade the repeating unit of succinoglycan (SG-D) produced by the extracellular enzyme because this organism can grow on succinoglycan as the sole source of carbon. This paper describes the purification of an intracellular enzyme of *Flavobacterium* M64 capable of hydrolyzing SG-D, the isolation of the hydrolysis products, and the usefulness of this enzyme for structural analyses of the succinoglycan-type polysaccharides. Studies on the structure of succinoglycan elucidated by fragmentation with this enzyme was reported.¹¹⁾

MATERIALS AND METHODS

Materials. Gentiobiose, cellobiose and carboxymethyl cellulose were purchased from Nakarai Chemical Co. (Kyoto). Laminarabiose was isolated by partial hydrolysis of curdlan, followed by chromatography of the products on a charcoal column. Curdlan¹²⁾ and succinoglycan⁴⁾

* To whom reprint requests should be sent.

were prepared as reported previously from the culture broths of *Alcaligenes faecalis* var. *myxogenes* 10C3K and 10C3, respectively. Water-soluble polysaccharides of *Agrobacterium* species were prepared as reported previously.³⁾ Luteose was prepared from lutean, which is synthesized by *Penicillium aculeatum* var. *apiculatum* No. 2281.¹³⁾ The octasaccharide (SG-D) which represents the repeating unit of the succinoglycan was isolated by hydrolysis of succinoglycan with succinoglycan depolymerase of *Flavobacterium* M64.¹⁰⁾

Culture. *Flavobacterium* M64, isolated from soil,⁹⁾ was used as the enzyme source. The synthetic medium used for culture contained (per 100 ml): 0.5 g of succinoglycan, 0.15 g of $(\text{NH}_4)_2\text{HPO}_4$, 0.1 g of KH_2PO_4 , 0.05 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 ml of mixed salt solution. This salt solution contained (per liter) NaCl 10 g, CaCl_2 10 g, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 10 g, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 10 g, $\text{ZnCl}_2 \cdot 7\text{H}_2\text{O}$ 70 mg, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 50 mg, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 20 mg and H_3BO_3 10 mg. The medium was adjusted to pH 7.2. Conical flasks containing 95 ml of medium were inoculated with 5 ml of a seed culture which had been grown in the same medium. The medium was shaken reciprocally at 30°C for 24 hr.

Enzyme assay. Enzyme activity was assayed at 40°C. The reaction mixture contained 50 mM sodium acetate buffer (pH 5.8), 0.2% substrate and enzyme in a final volume of 1 ml. Increase in reducing power was determined either with glucose oxidase or by the method of Nelson,¹⁴⁾ with glucose as a standard. One unit of enzyme activity is defined as the amount of enzyme which liberates 1 μmol of aldehyde group as glucose per hr.

When *p*-nitrophenyl- β -D-glucoside was used as substrate, the reaction mixture contained 5 mM substrate, 50 mM sodium acetate buffer (pH 5.8) and the enzyme in a final volume of 1 ml. The reaction was stopped by adding 3 ml of 0.1 M ammonium buffer (pH 10). Released *p*-nitrophenol was determined by measuring the absorption at 400 nm with a Hitachi 124 UV-VIS spectrophotometer. One enzyme unit is defined as the amount releasing 1 μmol of *p*-nitrophenol per hr.

Protein was measured by the method of Lowry *et al.*¹⁵⁾ using bovine serum albumin as a standard. The amounts of protein in effluents from columns were determined from the absorbance at 280 nm, measured with a Hitachi 124 UV-VIS spectrophotometer.

Preparation of deacylated polysaccharide. The deacylated polysaccharide was obtained by hydrolyzing a 0.1% solution of polysaccharide in 10 mM potassium hydroxide for 5 hr at 20°C under nitrogen as described by Sloneker and Jeanes.¹⁶⁾ The solution was neutralized with 0.1 N hydrochloric acid, dialyzed against water and lyophilized.

Preparation of desuccinylated octasaccharide (SG-D) of succinoglycan. SG-D isolated from succinoglycan was desuccinylated by the method described above.¹⁶⁾ The

solution was neutralized with 0.1 N hydrochloric acid, concentrated to a small volume and desalted by passage through a column of Sephadex G-15. The effluent containing oligosaccharide was concentrated by evaporation *in vacuo* and lyophilized.

Digestion of SG-D with endo- β -D-glucanase. SG-D or desuccinylated SG-D (500 μg) was digested with the intracellular endo- β -D-glucanase (0.5 unit) for 16 hr at 30°C in 0.5 ml of 50 mM sodium acetate buffer (pH 5.8), with one drop of toluene to prevent microbial growth. The mixture was then boiled for 1 min and passed through a column of Amberlite IR-120 (H^+ resin). The effluent was evaporated *in vacuo*, and applied to Toyo-Roshi No. 50 paper.

Successive digestion of polysaccharides with succinoglycan depolymerase and endo- β -D-glucanase. The deacylated polysaccharide (1 mg) was dissolved in 200 μl of 0.1 M sodium acetate buffer (pH 5.8) and incubated with 100 μl of succinoglycan depolymerase (2 units) for 5 hr at 40°C. The mixture was then boiled for 1 min, mixed with 100 μl of intracellular β -D-glucanase (2 units) and incubated for another 5 hr. The mixture was then boiled again for 1 min and passed through a column of Amberlite IR-120 (H^+ resin). The effluent was evaporated *in vacuo*, applied to Toyo-Roshi No. 50 paper, and subjected to paper chromatography.

Paper chromatography. Paper chromatography was carried out by the descending method using Toyo-Roshi No. 50 paper with a solvent system of 6:4:3 (v/v) 1-butanol-pyridine-water. Sugars were detected on paper chromatograms with silver nitrate reagent.

Isolation of desuccinylated oligosaccharide I and oligosaccharide II. The desuccinylated SG-D (50 mg) was extensively digested with endo- β -D-glucanase (40 units) for 24 hr at 30°C in 1.5 ml of 50 mM sodium acetate buffer, with a few drops of toluene to prevent microbial growth. After incubation, the mixture was boiled for 1 min and centrifuged to remove denatured protein.

The supernatant was passed through an Amberlite IR-120 (H^+ resin) column and the effluent was concentrated to a small volume, applied to Toyo-Roshi No. 50 paper and developed with 6:4:3 (v/v) 1-butanol-pyridine-water to separate the two oligosaccharide products. The products were extracted with water from appropriate sections of the paper and concentrated to dryness by vacuum evaporation. The fast and slow migrating oligosaccharides (yields; 23.5 mg and 19.8 mg, respectively) were termed desuccinylated oligosaccharide I and oligosaccharide II, respectively.

Determination of the degree of polymerization. Desuccinylated oligosaccharide I and oligosaccharide II (200 μg each) were reduced with 0.5 ml of 2 M sodium

borohydride at room temperature overnight and treated with Amberlite IR-120 (H^+ resin). Boric acid was removed by co-distillation with methanol. The product was hydrolyzed in a sealed tube with 0.5 ml of 2 N hydrochloric acid for 2 hr at $100^\circ C$ under nitrogen, and the hydrolysate was evaporated to dryness to remove hydrochloric acid. The residue was acetylated and the ratio of D-glucose to hexitol originating from the reducing end residue was assayed by GLC using a Shimadzu GC7A gas chromatograph with a column (3 mm $\times$ 2 m) of 1.5% Silicone OV-17 on Shimalite W at $190^\circ C$. The degree of polymerization was expressed as the ratio obtained on gas chromatogram plus one.

Determination of sugars. Sugars were quantitatively analyzed by GLC as the alditol acetates with a column of 3% ECNSS-M on Gas-chrom Q as described previously.³⁾

Determination of pyruvic acid. Pyruvic acid was determined by the method of Koepsell and Sharpe.¹⁷⁾

Determination of succinic acid. Succinic acid was determined by the method of McComb and McCready.¹⁸⁾

RESULTS

Purification of endo- β -D-glucanase

An intracellular endo- β -D-glucanase acting on the octasaccharide repeating unit of succinoglycan (SG-D) was purified as follows: Ten liters of culture of *Flavobacterium* M64 was centrifuged at $20,000 \times g$ for 20 min. The cells (approx. 26 g dry weight) were washed

twice with 800 ml of 10 mM sodium phosphate buffer, pH 6.5, suspended in 400 ml of the same buffer and subjected to sonic oscillation for 30 min at 20 Kcycles in a Kaijo Denki ultrasonic disintegrator. The sonicate was then centrifuged for 30 min at $27,000 \times g$.

The crude extract was brought to 40% saturation of ammonium sulfate and the resulting precipitate was removed by centrifugation. Further ammonium sulfate was added to the supernatant to give 80% saturation. The resulting precipitate was collected by centrifugation, dissolved in 100 ml of 10 mM sodium phosphate buffer, pH 6.5, and dialyzed against 5 liters of the same buffer for 24 hr.

The dialyzed material was applied to a CM-cellulose column (3.5 $\times$ 12 cm) equilibrated with 10 mM sodium phosphate buffer, pH 6.5. The column was washed with 140 ml of sodium phosphate buffer and then eluted with 800 ml of a linear gradient of 0 ~ 0.4 M sodium chloride in sodium phosphate buffer and then 200 ml of 0.4 M sodium chloride in the same buffer (Fig. 1). Three peaks with activity on SG-D were eluted, and fractions in the main eluted with 0.25 M sodium chloride (fractions 114 ~ 150), were pooled and concentrated to 6.3 ml by ultrafiltration (Amicon Co.). The activity for luteose is also shown in Fig. 1.

FIG. 1. Elution of Enzyme from a CM-cellulose Column.

—, activity for SG-D; ---, activity for luteose; ····, absorbance at 280 nm; - · - · -, concentration of sodium chloride.

Fractions of 5 ml were collected. Pooled fractions are indicated by a solid bar.

FIG. 2. Elution of Enzyme from a Sephadex G-150 Column.

—, activity for SG-D; ---, activity for luteose; ····, absorbance at 280 nm.

Fractions of 3 ml were collected. Pooled fractions are indicated by a solid bar.

TABLE I. ENZYME PURIFICATION

Step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)
1. Crude extract	726	4530	426	0.094
2. $(\text{NH}_4)_2\text{SO}_4$, 40~80% satn	113	716	388	0.54
3. CM-cellulose	6.3	31.0	127	4.10
4. Sephadex G-150	3.0	5.63	74.5	13.2

The concentrated protein (or enzyme) was applied to a Sephadex G-150 (superfine) column (2.2×88 cm) equilibrated with 40 mM sodium acetate buffer (pH 5.8), and the enzyme was eluted from the column with the same buffer (Fig. 2). The enzyme activity to SG-D showed a single peak. The active fraction for luteose gave two. The enzyme in the first peak had no activity to SG-D and seemed to be the $(1 \rightarrow 6)$ - β -D-glucosidase as reported by Sano *et al.*,¹⁹⁾ since it hydrolyzed gentiobiose rapidly and hydrolyzed luteose exolytically. The second protein peak coincided with the activity peak on SG-D. Fractions 59~67 were pooled and concentrated to 3.0 ml by ultrafiltration.

The results of a typical purification are shown in Table I. In this way endo- β -D-glucanase with activity towards SG-D was

purified 140-fold with a yield of about 5.6 mg of protein from 10 liters of culture.

Action of endo- β -D-glucanase on SG-D

SG-D was digested with the endo- β -D-glucanase and the products were examined by paper chromatography. As shown in Fig. 3, only one spot was detected. However, a second faster migrating spot was also found when desuccinylated SG-D was used as the substrate (Fig. 3). The fast migrating spot and the slow migrating spot from desuccinylated SG-D were termed desuccinylated oligosaccharide I and oligosaccharide II, respectively. The spot of the native oligosaccharide I was located in the same position as that of oligosaccharide II due to the presence of succinic acid residue in oligosaccharide I. The β -D-glucanase preparation was not homogeneous on polyacrylamide

FIG. 3. Paper Chromatogram of Products Formed by Incubation of Desuccinylated SG-D (B) and Native SG-D (C) with Endo- β -D-glucanase.

A, standard mixtures of glucose and gentiobiose.

gel electrophoresis, but since it depolymerized SG-D to yield defined two products, it was used for further studies on the fine structure of succinoglycan.

Substrate specificity of endo- β -D-glucanase

The actions of the purified endo- β -D-glucanase on various substrates were examined and compared with those of crude extracts (Table II). The results indicated that this enzyme has a high activity for SG-D. It has no activity on succinoglycan, the native polymer of SG-D. Most of the β -D-glycanase and β -D-glycosidase activities in crude extracts, such as succinoglycan degrading activity, and (1 $\rightarrow$ 4)- β -D-glucanase, (1 $\rightarrow$ 6)- β -D-glucosidase and (1 $\rightarrow$ 4)- β -D-glucosidase activities, were removed during the purification process. However, the activity on luteose, which is exclusively (1 $\rightarrow$ 6)-linked β -D-glucan, was still high in the purified preparation, and the peak of this activity coincided with that of activity for SG-D in the elution profile from Sephadex G-150 (Fig. 2).

TABLE II. SUBSTRATE SPECIFICITY OF PURIFIED ENDO- β -D-GLUCANASE AND CRUDE EXTRACT

Substrate	Activity (units/mg)	
	Purified	Crude
SG-D	13.2	0.1
Succinoglycan	0.2	0.1
Curdlan	0.0	0.0
Carboxymethyl cellulose	0.6	0.1
Luteose	15.8	0.5
Laminarabiose	0.0	0.0
Cellobiose	0.0	0.1
Gentiobiose	4.8	3.1
<i>p</i> -Nitrophenyl- β -D-glucoside	0.2	0.4

Digestion of water-soluble polysaccharides of *Agrobacterium* species

Previously we found that the water-soluble, extracellular polysaccharides from 9 strains of *Agrobacterium* species contained the same constituents as succinoglycan and that their proportions were similar to those of succinoglycan although the contents of acyl groups varied in different polysaccharides.³⁾ To confirm the similarity between succinoglycan and the polysaccharides from *Agrobacterium* species we tested whether these compounds could be hydrolyzed by succinoglycan depolymerase, which is an extracellular enzyme of *Flavobacterium* M64 with high specificity to succinoglycan. Paper chromatography revealed that all the polysaccharides were hydrolyzed, giving one spot of reducing material near the origin.³⁾

We also used the endo- β -D-glucanase to compare structure of these polysaccharides. The deacylated polysaccharides were digested with succinoglycan depolymerase and then with endo- β -D-glucanase and the digests were subjected to paper chromatography. As shown in Fig. 4 all the polysaccharides were hydrolyzed, giving two spots of oligosaccharides which had same *R_f* values to those of desuccinylated oligosaccharide I and oligosaccharide II, respectively. Thus, the polysaccharides from *Agrobacterium* species were confirmed to have similar structure to suc-

FIG. 4. Paper Chromatogram of Products Formed by Successive Digestion of Deacylated Water-Soluble Polysaccharides with Succinoglycan Depolymerase and Endo- β -D-glucanase.

A-J: deacylated polysaccharides of *Ag. radiobacter* IFO 12607, IFO 12664, IFO 12665, IFO 13127, IFO 13256, IFO 13532, IFO 13533, *Ag. rhizogenes* IFO 13259, *Ag. tumefaciens* IFO 3058, and *Al. faecalis* var. *myxogenes* 10C3; K: standard mixture of glucose and gentiobiose.

cinoglycan, so far as the action of the present enzyme was concerned.

Products after endo- β -D-glucanase digestion of desuccinylated SG-D

Desuccinylated oligosaccharide I and oligosaccharide II, the products obtained by the endo- β -D-glucanase digestion of desuccinylated SG-D, were isolated as described in the MATERIALS AND METHODS. The two oligosaccharides were both homogeneous on a

paper chromatogram (6:4:3 (v/v) 1-butanol-pyridine-water) and on a paper electrophoretic chromatogram (1:1:48 (v/v) pyridine-acetic acid-water). The components and degrees of polymerization of these oligosaccharides were shown in Table III. SG-D was composed of D-glucose, D-galactose, pyruvic acid and succinic acid in a molar ratio of 7:1:1:1. Desuccinylated oligosaccharide I was composed of D-glucose and pyruvic acid in a molar ratio of 4:1. Oligosaccharide II was composed of D-glucose and D-galactose in a molar ratio of 3:1. Since the reducing end of SG-D is formed with D-galactose residue,¹¹⁾ oligosaccharide II, which contains D-galactose, is located at the reducing end of SG-D. The degree of polymerization of both desuccinylated oligosaccharide I and oligosaccharide II was 4. That of SG-D was previously determined to be 8.¹¹⁾

DISCUSSION

Alcaligenes faecalis var. *myxogenes* 10C3, isolated from soil by Harada and Yoshimura,²⁰⁾ produces a water-soluble polysaccharide containing succinic acid (succinoglycan).²⁾ The occurrence of succinic acid in polysaccharides is rather rare.²¹⁻²⁴⁾ However, recently³⁾ we showed that many strains of *Agrobacterium* also produce a polysaccharide containing succinic acid and moreover that the sugar composition and linkage pattern of these polysaccharides are similar to those of succinoglycan. Extracellular specific β -D-glucanase of *Flavobacterium* M64, designated

TABLE III. COMPONENTS AND DEGREE OF POLYMERIZATION OF SG-D, DESUCCINYLATED OLIGOSACCHARIDE I AND OLIGOSACCHARIDE II

	D-Glucose	D-Galactose	Pyruvic acid	Succinic acid	D.P.
	(mol ratio)				
SG-D	7.2	1.0	1.1	0.9	8 ^a
Desuccinylated oligosaccharide I	4.1	0	1.0	0	4
Oligosaccharide II	2.9	1.0	0	0	4

^a Determined previously.¹¹⁾

FIG. 5. Fragmentation of Succinoglycan by the Two β -Glycanase.

○, glucose; ●, galactose.

Linkages a and b are hydrolyzed by succinoglycan depolymerase and the endo- β -D-glucanase.

succinoglycan depolymerase, was useful for comparing the structures of these polysaccharides.³⁾ This enzyme depolymerized succinoglycan and the polysaccharides from 9 strains of *Agrobacterium* to the repeating units. The intracellular endo- β -D-glucanase of *Flavobacterium* M64 described in the present paper was another useful enzyme for examining the structures of succinoglycan and the related polysaccharides. This enzyme hydrolyzes the octasaccharide repeating unit of succinoglycan (SG-D) and of the polysaccharides of *Agrobacterium* to give two oligosaccharides (Fig. 4).

The finding of these enzymes prompted us to examine the fine structure of succinoglycan. Now the structure has been determined by analyzing the products produced by hydrolysis with these enzymes.¹¹⁾ Fragmentation of succinoglycan by the enzymes is shown in Fig. 5.

Linkages a and b are hydrolyzed by succinoglycan depolymerase and the endo- β -D-glucanase of *Flavobacterium*, respectively. Linkage b was not hydrolyzed until linkage a (Table II) is cleaved, possibly owing to steric hindrance resulting from the branched structure. Structural studies on succinoglycan showed that the endo- β -D-glucanase hydrolyzes one of the contiguous (1→6)- β -D-glucosidic linkages in SG-D.¹¹⁾ This enzyme preparation can also hydrolyze (1→6)- β -D-glucosidic linkages in luteose to the same extent (Table II).

There are several reports on enzymes capable of hydrolyzing (1→6)- β -D-glucosidic linkages. We have reported a (1→6)- β -D-glucosidase of *Flavobacterium*,¹⁹⁾ which does not attack the repeating unit of succinoglycan. Exo-(1→3)- β -D-glucanase of yeasts was shown to exhibit exo-(1→6)- β -D-glucanase activity.²⁵⁾ This enzyme would probably not have any activity on the repeating unit. Endo-(1→6)- β -D-glucanases of *Bacillus circulans*,²⁶⁾ *Gibberella fujikuroi*²⁷⁾ and *Rhizopus chinensis*²⁸⁾ have also been characterized. Although there is, so far, no definite evidence that the activities of our enzyme preparation against luteose and SG-D are due to the same enzyme, the enzyme acting on SG-D seems to be an endo-(1→6)- β -D-glucanase, because it hydrolyzes the (1→6)- β -D-glucosidic linkage adjacent to the (1→6)-linked β -D-glucose residue in SG-D. We have not examined whether endo-(1→6)- β -D-glucanase of *Bacillus*, *Gibberella* or *Rhizopus* hydrolyzes (1→6)- β -D-glucosidic linkages in SG-D. An endo-(1→6)- β -D-glucanase from a strain of fungi imperfecti kindly supplied by Prof. S. Nagasaki showed activity against SG-D less than one two thousandth of that against luteose (unpublished data). Also, an extracellular endo-(1→6)- β -D-glucanase⁸⁾ of *Flavobacterium* M64 induced with luteose hardly attacks SG-D, its activity against SG-D being less than 1/40 of that against luteose (unpublished data). Thus the endo- β -D-glucanase may be a specific (1→6)- β -D-glucanase capable of hydrolyzing SG-D and the repeating unit of polysaccharides from *Agrobacterium*.

The endo- β -D-glucanase was used in combination with succinoglycan depolymerase to study the structures of complicated polysaccharides. Not only extracellular polysaccharides from many strains of *Agrobacterium*, but also a polysaccharide from *Rhizobium meliloti* U27 was hydrolyzed successively to two tetrasaccharides.⁸⁾ Thus these polysaccharides are identical to succinoglycan from *Alcaligenes faecalis* var. *myxogenes* 10C3, irrespective of their modes of acylation.^{7,8,11)}

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