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Note

Screening, Purification and Characterization of a Prokaryotic Isoprimeverose-producing Oligoxyloglucan Hydrolase from *Oerskovia* sp. Y1

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Abstract: Isoprimeverose-producing oligoxyloglucan hydrolase (IPase; EC 3.2.1.120) is a unique β-glycosidase that cleaves xyloglucan oligosaccharides at the non-reducing end, producing isoprimeverose. Here, we describe the first reported identification and characterization of a prokaryotic IPase. We purified an IPase with a molecular mass of 105 kDa from the culture supernatant of an Actinomycetes species, *Oerskovia* sp. Y1, and characterized its pH and thermal stability. The enzyme was stable between pH 3.5 and 7.5, and its optimum pH was 4.5. We also found that it was stable at temperatures up to 45°C, and the optimal temperature for enzyme activity was 55°C. The K_m value for XXXG (the letters G and X refer to an unbranched Glc residue and an α-D-Xyl*p*-(1→6)-β-D-Glc*p* segment, respectively) was determined to be 0.7 mM, and the specific activity was 85 U per mg protein. HPLC analysis revealed that IPase cleaves XXXG to X and XXG, then cleaves XXG to X and XG, and finally cleaves XG to X and G. Transglycosylation activity was also clearly evident; HPLC analysis revealed that the enzyme could transfer isoprimeverose to XXXG to produce XXXXG.

Key words: isoprimeverose-producing oligoxyloglucan hydrolase, xyloglucan, isoprimeverose

Xyloglucan is a major hemicellulose polysaccharide of primary plant cell walls. It consists of a backbone chain of β-1,4-linked D-Glc*p* residues (β-1,4-glucan) and various branching side-chain structures, such as α-D-Xyl*p*-(1→6)-, β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-, and α-D-Xyl*p*-(1→6)-. The branching patterns depend on the plant species. Naming of xyloglucan side-chain structures is simplified using a one-letter nomenclature[1]; for example, the letters G, X and L refer to an unbranched Glc residue, an α-D-Xyl*p*-(1→6)-β-D-Glc*p* segment and a β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* segment, respectively.

In plant cell walls, xyloglucan associates with cellulose microfibrils *via* hydrogen bonds, forming a cellulose-xyloglucan network that must be partially disassembled for cell expansion and development.[2] Thus, xyloglucan metabolism is thought to be important in cell definition, cell expansion, and regulation of plant growth and development. In growing plant-cell walls, xyloglucan oligosaccharides can accelerate cell elongation.[3] Determining the fine structure of xyloglucans and elucidating the relationship between their structure and physiological function are important goals. Toward this end, xyloglucan-active enzymes are used as tools for analyzing the fine structure of xyloglucan oligosaccharides and for preparation of various xyloglucan structures.

Isoprimeverose-producing oligoxyloglucan hydrolase (IPase; EC 3.2.1.120) was first identified in 1985 as a unique enzyme of *Aspergillus oryza*.[4] This enzyme cleaves xyloglucan oligosaccharides to remove an isoprimeverose residue (α-D-Xyl*p*-(1→6)-D-Glc*p*) from the non-reducing end. IPase has been used in the analysis of xyloglucan structures and in the preparation of various xyloglucan oligosaccharides[5,6] and isoprimeverose. Since isoprimeverose enhances the growth of *Lactobacillus pentosus* (unpublished), which has been reported to have an isoprimeverose transporter,[7,8] isoprimeverose might be useful as a prebiotics material. However, only a few characterizations of this enzyme have been reported. Some fungal IPases have been identified but only minimally characterized. This account describes the first reported identification and characterization of a prokaryotic IPase.

First, we screened soil from Tsukuba, Japan, for a novel IPase-producing microorganism. We used a selection medium consisting of 0.2% KH_2PO_4, 0.05% $MgSO_4$, 0.1% $(NH_4)_2SO_4$, 0.01% $CaCl_2$ and 0.5% XXXG (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); the xyloglucan oligosaccharide, XXXG, was the sole carbon source for the culture. The soil was diluted in water and spread onto a plate containing selection medium with 1.5% agar, and the plate was incubated at 30°C. Individual colonies were removed from the selection plate and placed in 96-deep wells containing the selection medium. After incubation with shaking at 30°C for 1 week, the cultures were centrifuged, and the supernatant fractions were analyzed by normal-phase HPLC on an Amide 80 column (4.6×250 mm; Tosoh Co., Tokyo, Japan).

Using the above process, we identified an Actinomycetes species that accumulated isoprimeverose in the culture medium (Fig. 1), suggesting that it secreted IPase into the culture supernatant. An analysis of the 16S rRNA of this organism indicated that it belonged to the genus Oerskovia, and it was designated *Oerskovia* sp. Y1. Although IPase cleaves XXXG to produce not only isoprimeverose but also glucose, the HPLC profile of the

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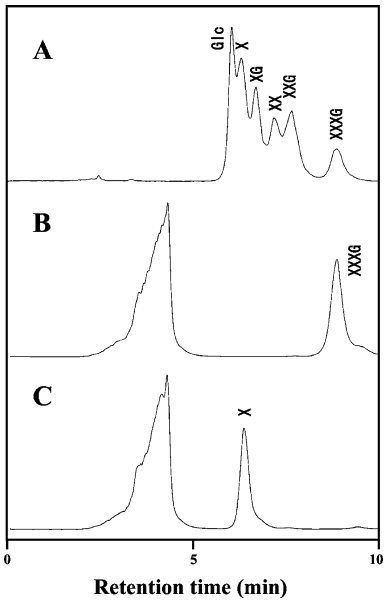

Fig. 1. HPLC analysis of *Oerskovia* sp. Y1 culture supernatant.

Oerskovia sp. Y1 was cultivated in selection medium at 30°C for 1 week. After the culture was centrifuged, the supernatant fraction was loaded onto a TSKgel Amide 80 column (4.6×250 mm). The column was isocratically eluted with 55% acetonitrile at a flow rate of 0.8 mL/min. A, xyloglucan oligosaccharide markers; B, selection medium; C, *Oerskovia* sp. Y1 culture supernatant.

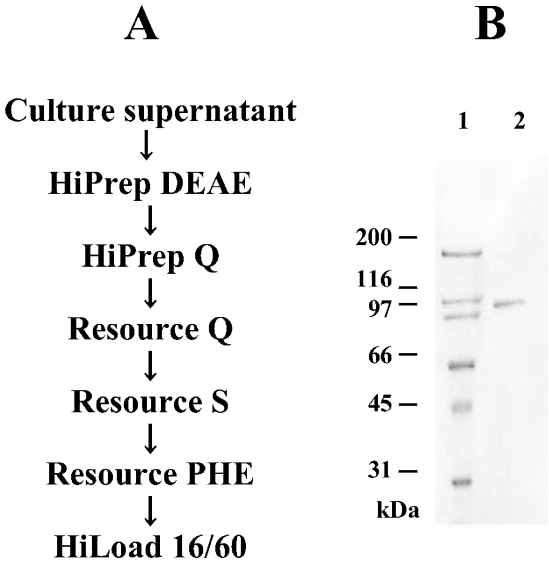

Fig. 2. Purification of IPase from *Oerskovia* sp. Y1.

A, IPase was purified from *Oerskovia* sp. Y1 by serial liquid chromatography. B, The purified IPase was subjected to SDS-PAGE. Lane 1, molecular weight markers; lane 2, purified IPase.

Oerskovia sp. Y1 culture supernatant contained no additional peaks for sugars such as glucose or xylose. Most likely, the *Oerskovia* sp. Y1 cells utilized the glucose, but not the isoprimeverose, as a carbon source, with the result that isoprimeverose accumulated in the culture medium. Our observations indicate that *Oerskovia* sp. Y1 might be useful for preparation of isoprimeverose.

We purified the IPase activity from the *Oerskovia* sp. Y1 culture supernatant using the procedure summarized in Fig. 2A. Cells were cultured in 1.8 L of culture medium (0.8% Bacto-peptone, 0.2% KH_2PO_4, 0.05% $MgSO_4$, 0.05% yeast extract and 1% of a xyloglucan oligosaccharide mixture containing XXXG, XXLG, XLXG and XLLG) at 30°C for 1 week. This xyloglucan oligosaccharide mixture was obtained by *Geotrichum* xyloglucan endoglucanase[9]-catalyzed digestion of tamarind seed xyloglucan (Glyloid; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). The cells were pelleted, and the supernatant was concentrated by ultrafiltration.

The concentrated supernatant was diluted with buffer A (25 mM imidazole-HCl buffer, pH 7.4) and loaded onto a HiPrep 16/10 DEAE FF anion-exchange chromatography column (Amersham Biosciences) equilibrated with buffer A. The column was eluted with a linear gradient of 0–0.5 M NaCl (total, 400 mL). Each fraction was assayed for XXXG hydrolysis activity by measuring the production of reducing sugars from a reduced XXXG substrate (XXXGol), which was produced by borohydride reduction[10] of the reducing end of XXXG. The active fractions (0.16–0.18 M NaCl) were pooled, dialyzed against buffer A, loaded onto a HiPrep 16/10 Q XL anion-exchange column (Amersham Biosciences) equilibrated with buffer A, and then eluted with a linear gradient of 0–0.5 M NaCl (total, 300 mL). The active fractions (0.32–0.37 M NaCl) from the HiPrep Q column were dialyzed against buffer A and loaded onto a Resource Q 1 mL anion-exchange column (Amersham Biosciences) equilibrated with buffer A. The column was eluted with a linear gradient of 0–0.5 M NaCl (total, 30 mL).

The active fractions (0.28–0.34 M NaCl) from the Resource Q column were dialyzed against buffer B (50 mM sodium acetate buffer, pH 5.5), loaded onto a Resource S 1 mL cation-exchange column (Amersham Biosciences) equilibrated with buffer B, and eluted with a linear gradient 0–0.5 M of NaCl (total, 30 mL). $(NH_4)_2SO_4$ was added to the active fractions (0.11–0.16 M NaCl) from the Resource S column to a final concentration of 0.75 M, and the fractions were then loaded onto a Resource PHE 1 mL hydrophobic interaction chromatography column (Amersham Biosciences) equilibrated with buffer A containing 0.75 M $(NH_4)_2SO_4$. The column was eluted with a linear gradient of 0.75–0 M $(NH_4)_2SO_4$ in buffer A (total, 30 mL). The active fractions (0.18–0.07 M $(NH_4)_2SO_4$) were pooled, concentrated to 1 mL by ultrafiltration, and loaded onto a HiLoad 16/60 Superdex 200 pg column (Amersham Biosciences) in buffer A. The active fractions (retention volume, 95–110 mL) were pooled, concentrated by ultrafiltration, and subjected to SDS-PAGE. SDS-PAGE analysis of the purified enzyme revealed a single band of 105 kDa (Fig. 2B). About 200 μg of purified IPase was obtained from 1.8 L culture.

We next analyzed the effects of pH and temperature on the purified IPase. The optimum pH was 4.5, and the optimum temperature was 55°C. The pH stability was assessed at 35°C using McIlvaine buffer solutions (0.2 M Na_2HPO_4 and 0.1 M citric acid) at various pHs. IPase was stable between pH 3.5 and 7.5. Thermostability was assessed by incubating the enzyme at various temperatures for 20 min. More than 90% of the enzyme activity was retained at 45°C.

The enzyme activity of the purified IPase was analyzed using various concentrations of XXXGol (0.18–4.6 mM) in 50 mM sodium acetate buffer (pH 4.5) at 50°C. At substrate concentrations>1.5 mM, apparent substrate inhibition

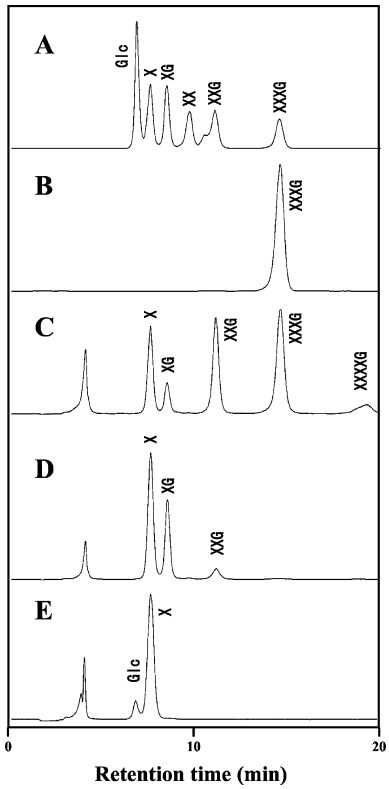

Fig. 3. HPLC analysis of products of IPase digestion of XXXG.

XXXG (1 mg/mL) was incubated with purified *Oerskovia* sp. Y 1 IPase (7 μg/mL) in 50 mM sodium acetate buffer (pH 4.5) at 45° C. After various incubation periods, 10 μL of reaction products were loaded onto a TSKgel Amide 80 column, and the column was isocratically eluted with 60% acetonitrile at a flow rate of 0.8 mL/min. A, xyloglucan oligosaccharide markers; B, XXXG; C–E, incubation for 10 min, 30 min and 4 h, respectively.

was observed (data not shown). The data obtained using 0 −1.2 mM XXXGol were used in calculating kinetic constants; K_m was found to be 0.7 mM, and the specific activity was found to be 85 U per mg protein. One unit was defined as the amount of enzyme necessary to release 1 μmol of glucose equivalents as reducing sugars from XXXGol per min.

To examine the mode of enzyme activity, the purified IPase was incubated with XXXG, and the resulting products were analyzed by normal-phase HPLC on a TSKgel Amide 80 column (4.6×250 mm). The enzyme cleaved XXXG to produce X and Glc (Fig. 3E), and XG and XXG were observed as partial digestion products (Fig. 3C and D). Peaks for XX and XXX were not observed (Fig. 3). These results indicate that this enzyme acts in the "exo" mode at the non-reducing end of the substrate to produce an isoprimeverose residue. IPase cleaves XXXG to X and XXG, then cleaves the resulting XXG to X and XG, and then cleaves the resulting XG to X and Glc. This mode of action is identical to that of *Aspergillus oryza* IPase.[4)]

Although a previous report about IPase from *Aspergillus oryza* described only the hydrolyzing activity,[4)] our data demonstrate that IPase also has transglycosylation activity. As shown in Fig. 3C, a peak of higher molecular weight than that of XXXG was observed. Based on MALDI-TOF-MS analysis, this peak appears to represent XXXXG, which could arise from IPase transfer of X (donor) to XXXG (acceptor) to produce XXXXG.

This study constitutes the first known identification and characterization of a prokaryotic IPase. Although IPase has been used as a tool for xyloglucan structure analysis and for preparing xyloglucan oligosaccharides, limited information about fungal IPases is available. To our knowledge, no IPase genes have previously been cloned, and no prokaryotic IPase has previously been identified.

Our data demonstrate for the first time that a prokaryotic species, *Oerskovia* sp. Y1, produces an IPase. We are currently cloning the gene for this IPase and developing an *Escherichia coli* system for its heterologous expression. This expression system offers the advantage that *E. coli* host cells do not produce endogenous xyloglucan-active enzymes that might otherwise act as detrimental contaminants in fine structure analysis and preparation of xyloglucan oligosaccharides.

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原核生物由来初のイソプリメベロース生成酵素の探索，精製および性状解析

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イソプリメベロース生成酵素 (IPase: EC 3.2.1.120) は，キシログルカンオリゴ糖の非還元性末端からイソプリメベロース単位で切断する加水分解酵素である．これまでに原核生物由来の IPase は報告例がなかったが，本研究では原核生物由来で初の IPase を単離し，性状解析を行った．まず，IPase 産生菌をスクリーニングした結果，放線菌 *Oerskovia* sp. Y1 株が単離された．そこで，培養上清中より精製を行い，分子量 105 kDa の IPase を単離した．本酵素の性状解析を行ったところ，至適 pH および至適温度は，それぞれ pH 4.5 と 55°C で，pH 3.5 から pH 7.5 の間および 45°C まで安定であることが明らかになった．また，K_m 値は 0.7mM で比活性は 85 ユニット/mg 酵素であった．HPLC による解析の結果，本酵素はキシログルカンオリゴ糖 XXXG (G は側鎖のないグルコース残基を，X はキシロース側鎖をもつグルコース残基を表す) を X と XXG に，XXG を X と XG に，最終的に XG をイソプリメベロースとグルコースに分解することが明らかとなった．また，転移活性も有しており，X を XXXG に転移して XXXXG を産生することも明らかになった．