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A novel α-glucosidase from the moss *Scopelophila cataractae*

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Scopelophila cataractae **is a rare moss that grows on copper-containing soils.** *S. cataractae* **protonema was grown on basal MS medium containing copper. A starch-degrading activity was detected in homogenates of the protonema, after successive extraction with phosphate buffer and buffer containing 3 M LiCl. Buffer-soluble extract (BS) and LiCl-soluble extract (LS) readily hydrolyzed amylopectin to liberate only glucose, which shows that α-glucosidase (EC 3.2.1.20) in BS and LS hydrolyzed amylopectin. The K_m value of BS for maltose was 0.427. The K_m value of BS for malto-oligosaccharide decreased with an increase in the molecular mass of the substrate. The value for maltohexaose was 0.106, which is about four-fold lower than that for maltose. BS was divided into two fractions of α-glucosidase (BS-1 and BS-2) by isoelectric focusing. The isoelectric points of these two enzymes were determined to be 4.36 (BS-1) and 5.25 (BS-2) by analytical gel electrofocusing. The two enzymes readily hydrolyzed malto-oligosaccharides. The two enzymes also hydrolyzed amylose, amylopectin and soluble starch at a rate similar to that with maltose. The two enzymes readily hydrolyzed panose to liberate glucose and maltose (1:1), and the K_m value of BS for panose was similar to that for maltotriose, whereas the enzymes hydrolyzed isomaltose only weakly. With regard to substrate specificity, the two enzymes in BS are novel α-glucosidases. The two enzymes also hydrolyzed β-limit dextrin, which has many α-1,6-glucosidic linkages near the non-reducing ends, more strongly than maltose, which shows that they do not need a debranching enzyme for starch digestion. The starch-degrading activity of BS was not inhibited by *p*-chloromercuribenzoic acid or α-amylase inhibitor. When amylopectin was treated with BS and LS in phosphate buffer, pH 6.0, glucose, but not glucose-1-phosphate, was detected, showing that the extracts did not contain phosphorylase but did contain an α-glucosidase. These results show that α-glucosidases should be capable of complete starch digestion by themselves in cells of *S. cataractae*.**

Keywords: α-glucosidase, *Scopelophila cataractae*, copper, moss

The enzyme α-glucosidase (EC 3.2.1.20) is ubiquitous in plants. The plant α-glucosidases readily hydrolyze malto-oligosaccharides (Matsui *et al.*, 1978; Chiba *et al.*, 1979; Yamasaki & Konno, 1991; Konishi *et al.*, 1992; Eksittikul *et al.*, 1993; Henson & Sun, 1995; Yamasaki *et al.*, 1996; 2005). Some authors have reported that reserve starch is hydrolyzed into glucose by the concerted action of α- and β-amylases, debranching enzyme and α-glucosidase (Briggs, 1978; Enari & Sopanen, 1986). On the other hand, α-glucosidases from barley (Sun & Henson, 1990), malt (Sissons & MacGregor, 1994), millet (Yamasaki *et al.*, 1996), and pea (Sun *et al.*, 1995) degrade intact starch granules by themselves. The enzyme from pea (*Pisum sativum*, cv Laxton's Progress No. 9) is located in the chloroplast and plays a role in the degradation of transitory starch (Sun *et al.*, 1995). Since these enzymes also hydrolyze intact starch granules only to a limited extent, *in vivo* starch digestion must occur through a concurrent action of the enzyme and some other starch-degrading enzymes.

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Abbreviations: BS, buffer-soluble extracts; HPLC, high-performance liquid chromatography; LS, LiCl-soluble extracts; NaOAc, sodium acetate; PCMB, *p*-chloromercuribenzoic acid.

On the other hand, we have found that the moss *Scopelophila cataractae* does not contain any starch-degrading enzymes except for α-glucosidase. Since *S. cataractae* was grown on basal MS medium without any organic compounds under light, the cells should photosynthetically produce transitory starch. However, the starch is thought to be degraded by the concurrent action of some starch-degrading enzymes. It is, therefore, of interest to investigate the properties of α-glucosidase in the moss. We describe here partial purification and properties of α-glucosidase from *S. cataractae*.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions. Mature capsules of *S. cataractae* were sterilized with 0.1% (w/v) benzalkonium chloride for 10 min and 2% (v/v) sodium hypochlorite for 5 min, and washed three times with sterilized water. The spores within the capsules were aseptically removed and grown on medium with one-tenth the concentration of Murashige and Skoog basal medium (1962) supplemented with 1% (w/v) agar. The protonema that developed from the aseptic spores were cultured on fresh medium at 22°C under constant irradiance of approx. 98 mmol m^{-2} s^{-1} and sub-cultured approx. every two months. Copper-treated cells were cultured by transferring a few pieces (approx. 0.1 g fresh mass) of protonema into 100-ml Erlenmeyer flasks containing 50 ml of the medium, with the addition of 0.2 mM $CuSO_4$ under the same environmental conditions. After two months of culture, the untreated control and copper-treated cells were carefully removed from the medium, briefly rinsed with 1 mM EDTA, washed with distilled water, and stored at –80°C until required.

Extraction of soluble protein and ionically bound cell wall protein. Frozen protonema were homogenized in 100 mM phosphate buffer, pH 7.0, containing 10 mM 2-mercaptoethanol using a Waring blender (PH91: MST Company, Tokyo, Japan) for 5 min at 0°C. All subsequent steps were conducted at 2 to 4°C and protein was extracted from the homogenate of protonema cells as described previously (Konno & Tsumuki, 1993). In brief, the homogenates were centrifuged at 8000 × *g* for 15 min, and the supernatant was dialyzed against 50 mM NaOAc buffer, pH 5.2, containing 10 mM NaCl and 10 mM 2-mercaptoethanol; this extract is referred to as "buffer-soluble" (BS) extract. The cell wall residue was washed, re-suspended in the same buffer containing 3 M LiCl, and stirred overnight. Following centrifugation, the supernatant was dialyzed against 50 mM NaOAc buffer, pH 5.2, containing 10 mM NaCl and 10 mM 2-mercaptoethanol; this extract is referred to as "LiCl-soluble" (LS) extract. Proteins in the buffer- and LiCl-soluble extracts were precipitated by the addition of solid $(NH_4)_2SO_4$ until 3.4 M was reached. The resulting precipitates were collected by centrifugation, re-suspended in 20 mM NaOAc buffer, pH 5.2, containing 10 mM NaCl and 10 mM 2-mercaptoethanol, and dialyzed against the same buffer.

The enzymes were concentrated using an Amicon ultrafiltration device (YM-10 membrane with 10 kDa cut-off; Amicon Corp, Lexington, MA, USA) and dialyzed against the same buffer.

Assay of enzyme activity. α-Glucosidase activity was determined as follows. The reaction mixture containing 1 mg of maltose and enzyme solution in 0.5 ml of 50 mM NaOAc buffer, pH 4.5, was incubated at 37°C for 60 min. After incubation, the reaction was stopped by boiling the mixture for 5 min. The amount of glucose formed was measured using glucose oxidase-peroxidase (Papadopoulos & Hess, 1960; Dahlqvist, 1961). In the substrate-specificity study, liberated glucose was measured similarly.

To determine the starch-degrading activity, the products liberated from soluble starch were measured according to the method of Somogyi (1952). One unit of α-glucosidase activity was defined as the amount of enzyme that liberated 1 mmol/h of glucose from maltose under the conditions described above.

To determine the phosphorylase activity, glucose-1-phosphate produced from amylopectin in 50 mM phosphate buffer, pH 6.0, was determined by HPLC as described in section on high-performance liquid chromatography.

Determination of protein. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Estimation of the isoelectric point. The isoelectric point of the enzyme was estimated as described (Awdeh *et al.*, 1968; Fawcett, 1968). After electrofocusing for 24 h at 80 V, the gel was cut into 3-mm sections and each section was crushed in 2 ml of deionized water, and the fractions were measured with regard to their enzyme activity and pH.

High-performance liquid chromatography. The products formed from the substrates treated with BS or LS were determined by HPLC using an Tosoh HPLC System 8020 (Tosoh Co., Tokyo).

For the determination of malto-oligosaccharides, chromatography was performed on a column (0.46 × 15 cm) of COSMOSIL 5NH2-MS eluted with 60% acetonitrile.

For the determination of glucose 1-phosphate, chromatography was performed on a COSMOSIL Packed Column 5 C_{18}-PAQ (0.46 × 25 cm); mobile phase, 20 mM phosphate buffer, pH 7.0; flow rate,

1.0 ml/min; temperature, 30°; detection, RI (Cosmosil Chromatogram Index 2003; Nacalai Tesque Inc, Kyoto, Japan).

RESULTS AND DISCUSSION

Partial purification of α-glucosidase from BS

BS was concentrated through a filter unit using centrifugation. The concentrate was subjected to isoelectric focusing (pH 3.5–10). After 24 h at 80 V, the gel was cut into 3-mm sections and each section was crushed in 2 ml of deionized water. The fractions were measured with regard to their maltose- and panose-hydrolyzing activities and pH (Fig. 1). The activity curve of each fraction for maltose was similar to that for panose. Consequently, two fractions (BS-1, pI=4.36: BS-2, pI=5.25) were obtained. When BS-1 was incubated with maltose, panose and amylopectin under various pH, the optimum pH for maltose-hydrolyzing activity (4.5) was identical to those for the panose- and amylopectin-hydrolyzing activities. Therefore, a single enzyme hydrolyzed maltose, panose and amylopectin. Similar results were also obtained for BS-2. After insoluble materials were removed by filtration, the enzymes were used as α-glucosidase preparations to determine enzymatic properties.

Two similar enzymes were obtained from buffer-soluble extract in untreated control cells (not shown).

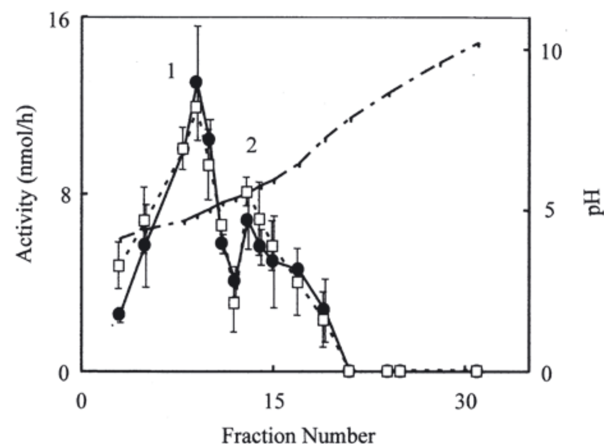

Figure 1. Isoelectric focusing of BS.
BS was subjected to isoelectric focusing (pH 3.5–10). After 24 h at 80 V, the gel was cut into 3-mm sections and each section was crushed in 2 ml of deionized water. The fractions were measured to determine maltose- and panose-hydrolyzing activities and pH. ●, Maltose-hydrolyzing activity; □, panose-hydrolyzing activity; -•-•-, pH; 1, BS-1; 2, BS-2. Values are means ± S.E. of three replicates.

Substrate specificity

Various substrates (1.7 mM) were incubated with BS-1 or BS-2 in NaOAc buffer, pH 4.5, at 37°C, and the relative rates of hydrolysis are shown in Table 1. The two enzymes readily hydrolyzed malto-oligosaccharides and polysaccharides. The two enzymes hydrolyzed amylose, amylopectin and soluble starch at a rate similar to maltose. The K_m value of α-glucosidases in BS for various substrates is also shown in Table 1. The K_m value for maltose is lower than those of α-glucosidases from other plants, such as sugar beet (Matsui *et al.*, 1978; Yamasaki & Konno, 1991), buckwheat (Chiba *et al.*, 1979), banana (Konishi *et al.*, 1992), rice seed (Eksittikul *et al.*, 1993), barley (Henson & Sun, 1995), and millet seed

Table 1. Substrate specificities of BS, BS-1 and BS-2.

Substrates (1.7 mM or 6 mg/ml) in 0.5 ml of 50 mM NaOAc buffer, pH 4.5, were incubated with BS, BS-1 or BS-2 at 37°C for 60 min. Liberated glucose was determined as described in Experimental. Values are means ± S.E. of three replicates.

	Hydrolysis			
	K_m (mM)	Relative rate (%)		
	BS	BS	BS-1	BS-2
Maltose[a]	0.446 ± 0.009	100	100	100
Nigerose[a]		12.3 ± 3.3	20.7	11.8
Kojibiose[a]		13.0 ± 2.0	17.8	11.7
Isomaltose[a]		10.1 ± 4.9	21.0	15.6
Panose[a]	0.297 ± 0.061	101.6 ± 2.9	102.3	107.2
Maltotriose[a]	0.183 ± 0.015	103.1 ± 6.4	118.3	106.1
Maltotetraose[a]	0.152 ± 0.006	142.6 ± 12.0	107.1	151.0
Maltohexaose[a]	0.122 ± 0.008	145.5 ± 12.8	110.1	159.4
Amylose EX-I[b]		145.5 ± 7.5	124.0	148.3
Amylopectin[b]		113.4 ± 13.1	79.5	105.9
Soluble starch[b]		144.5 ± 37.3	72.1	88.2
β-Limit dextrin[b]		227.9 ± 37.2	178.5	149.9

[a]1.7 mM; [b]6 mg/ml

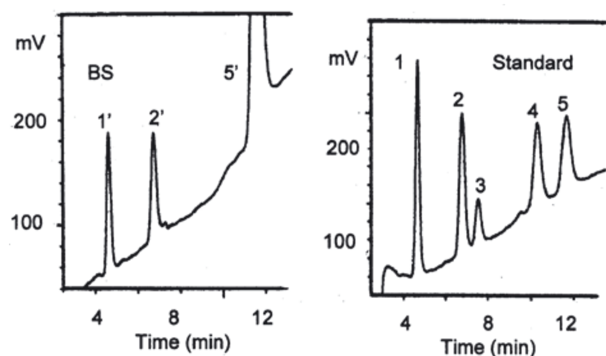

Figure 2. HPLC of products of panose digestion by BS.
BS was incubated with panose in 50 mM NaOAc buffer, pH 4.5, at 37ºC. After 60 min, products in the reaction mixture were determined by HPLC using a column (0.46 x 15 cm) of COSMOSIL 5NH2-MS eluted with 70% acetonitrile. 1, glucose; 2, maltose; 3, isomaltose; 4, maltotriose; 5, panose.

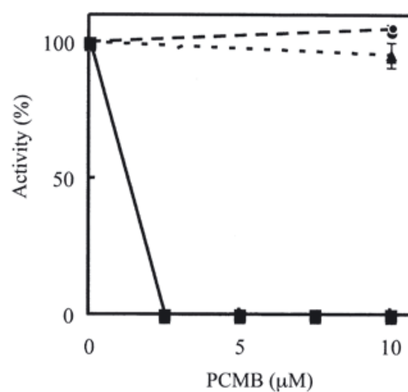

Figure 3. Effect of PCMB on starch-degrading enzymes.
BS was preincubated with PCMB at 37ºC. After 5 min, soluble starch was added and the mixture was reacted at 37ºC for 60 min. ●, BS; ▲, α-amylase from porcine pancreas; ■, β-amylase from germinating millet seeds (Yamasaki, 2003). Values are means ± S.E. of three replicates.

(Yamasaki *et al.*, 1996; 2005). The K_m value of α-glucosidases in BS for malto-oligosaccharide decreased with an increase in the molecular weight of the substrate. The value for maltohexaose is about four-fold lower than that for maltose, and several times lower than the values for maltohexaose of α-glucosidases from other plants, such as sugar beet (Matsui *et al.*, 1978; Yamasaki & Konno, 1991), buckwheat (Chiba *et al.*, 1979), banana (Konishi *et al.*, 1992), and millet seed (Yamasaki *et al.*, 1996; 2005). These results suggest that the α-glucosidases of BS may not need α- and β-amylases to degrade starch into oligosaccharide. Moreover, the two enzymes readily hydrolyzed panose and the K_m value of BS for panose was similar to that for maltotriose, whereas the enzymes only weakly hydrolyzed isomaltose. There are only a few reports on α-glucosidases which readily hydrolyze panose. α-Glucosidases from sugar-beet seeds (Yamasaki & Suzuki, 1980) and *Aspergillus awamori* (Yamasaki *et al.*, 1977) also readily hydrolyze panose. However, these enzymes readily hydrolyze isomaltose as well as panose. Therefore, the enzymes in BS are novel α-glucosidases and a malto-oligosaccharide larger than disaccharide will be needed at the reducing-end of the α-1,6-glucosidic bond to hydrolyze the substrate well. During the hydrolysis of panose, glucose and maltose (1:1) were detected as products by HPLC, which reflects hydrolysis of the α-1,6-glucosidic bond of panose by α-glucosidases of BS (Fig. 2). The two enzymes also hydrolyzed β-limit dextrin more readily than maltose. Since β-limit dextrin is a polysaccharide that remains intact after extensive hydrolysis (50–60%) of starch with β-amylase, this polysaccharide possesses many α-1,6-glucosidic linkages near the non-reducing ends. Therefore, the α-glucosidases in BS are not inhibited by any region in the starch molecule and the enzymes will not need any debranching enzyme for starch digestion. Therefore, it is assumed that the α-glucosidases in BS can completely degrade starch by themselves.

α- and *β*-amylase activities of BS

The starch-degrading activity of BS was not inhibited by PCMB even at a concentration of 100 mM, whereas β-amylase from millet was completely inhibited at 5 mM PCMB (Fig. 3). Since PCMB is a potent inhibitor of many β-amylases (Yamasaki, 2003), BS should not contain β-amylase. The starch-degrading activity of BS was also not inhibited with 100 mg α-amylase inhibitor, whereas α-amylase from porcine pancreas was strongly inhibited with 25 mg α-amylase inhibitor (Fig. 4).

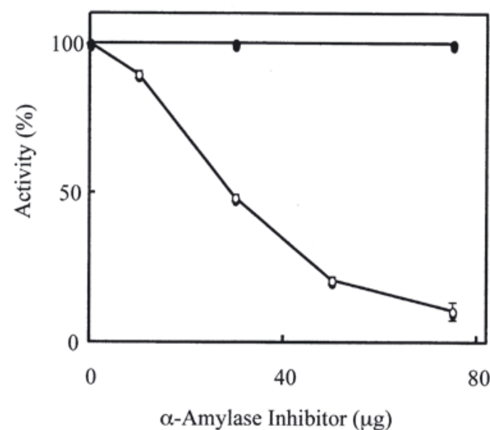

Figure 4. Effect of α-amylase inhibitor on starch-degrading enzymes.
BS was preincubated with α-amylase inhibitor at 37ºC. After 5 min, soluble starch was added and the mixture was reacted at 37ºC for 60 min. ●, BS; ○, α-amylase from porcine pancreas. Values are means ± S.E. of three replicates.

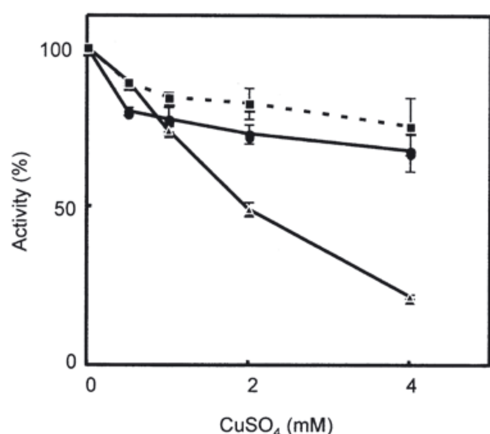

Figure 5. Effect of $CuSO_4$ on starch-degrading enzymes.
BS was preincubated with $CuSO_4$ at 37°C. After 5 min, soluble starch was added and the mixture was reacted at 37°C for 60 min. ●, BS; ▲, α-amylase (Yamasaki, 2003) purified from barley malt α-amylase VIII-A (Sigma); ■, α-glucosidase from millet seeds (Yamasaki *et al.*, 1996). Values are means ± S.E. of three replicates.

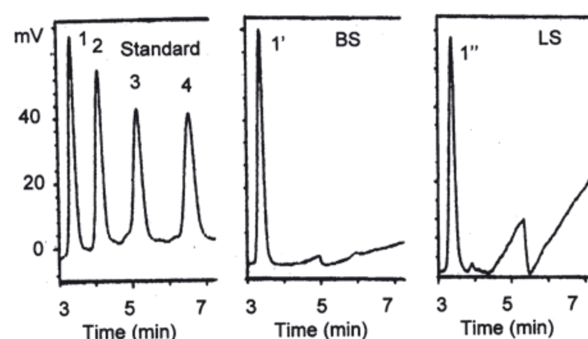

Figure 6. HPLC of products of amylopectin digestion by BS and LS.
BS and LS were incubated with amylopectin in 50 mM NaOAc buffer, pH 4.5, at 37°C. After 60 min, products in the reaction mixture were determined by HPLC using a column (0.46 × 15 cm) of COSMOSIL 5NH2-MS as described in the Experimental. 1, glucose; 2, maltose; 3, maltotriose; 4, maltotetraose.

Moreover, the starch-degrading activity of BS was weakly inhibited by $CuSO_4$, whereas malt α-amylase was strongly inhibited by $CuSO_4$ (Fig. 5). The degree of inhibition by $CuSO_4$ is similar to that of millet α-glucosidase. Therefore, BS should not contain α- or β-amylases, but may contain α-glucosidase. An experiment was carried out to clarify this point. When amylopectin was treated with BS or LS in NaOAc buffer, pH 5.3, at 37°C, glucose was detected by HPLC as the sole product (Fig. 6). Therefore, α- and β-amylases are not present and α-glucosidase must be the only enzyme to hydrolytically degrade starch in cells of *S. cataractae*, although many reports have demonstrated that α- and β-amylases play essential roles in starch digestion (Briggs, 1978; Enari & Sopanen, 1986; Scheidig *et al.*, 2002; Lu & Sharkey, 2004; Niittylä *et al.*, 2004; Sean *et al.*, 2004). This is supported by the finding that glucose was the sole product of amylopectin digestion with BS or LS. However, it has never been previously reported that a plant contains neither α-amylase nor β-amylase. Since the cells of *S. cataractae* were grown on basal MS medium containing copper without any organic compounds under light at 22°C for 2 months, the cells should photosynthetically produce transitory starch and α-glucosidase must be able to degrade the starch by itself. Since this moss grows slowly in nature, it may not need α-amylase, β-amylases or a debranching enzyme.

Phosphorylase of BS and LS

Amylopectin was treated with BS or LS in phosphate buffer, pH 6.0, to determine the phosphorylase activity. During the reaction, glucose-1-phosphate, which would be produced from amylopectin by phosphorylase, was not detected at all and glucose was detected as the sole product by HPLC (Fig. 7). Therefore, it is reasonable to conclude that starch is degraded by α-glucosidase alone in cells of *S. cataractae*.

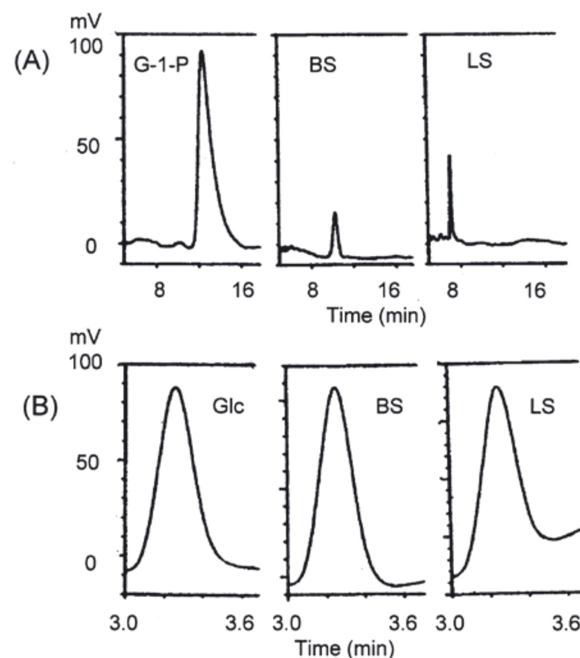

Figure 7. HPLC of products of amylopectin digestion by BS in the presence of phosphate.
BS and LS were incubated with amylopectin in 50 mM phosphate buffer, pH 6.0, at 37°C. After 60 min, products in the reaction mixture were determined by HPLC as described in the Experimental. (A) HPLC analysis to determine glucose-1-phosphate; G-1-P, glucose-1-phosphate; (B) HPLC analysis to determine malto-oligosaccharides; Glc, glucose.

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