Extremophiles (2007) 11:781–788
DOI 10.1007/s00792-007-0096-8

ORIGINAL PAPER

Purification and characterization of a maltooligosaccharide-forming amylase that improves product selectivity in water-miscible organic solvents, from dimethylsulfoxide-tolerant *Brachybacterium* sp. strain LB25

Noriyuki Doukyu · Wataru Yamagishi · Hirokazu Kuwahara · Hiroyasu Ogino · Noritake Furuki

Received: 2 April 2007 / Accepted: 10 June 2007 / Published online: 10 July 2007

Abstract A bacterium that secretes maltooligosaccharide-forming amylase in a medium containing 12.5% (vol/vol) dimethylsulfoxide (DMSO) was isolated and identified as *Brachybacterium* sp. strain LB25. The amylase of the strain was purified from the culture supernatant, and its molecular mass was 60 kDa. The enzyme was stable at pH 7.0–8.5 and active at pH 6.0–7.5. The optimum temperature at pH 7.0 was 35°C in the presence of 5 mM $CaCl_2$. The enzyme hydrolyzed starch to produce maltotriose primarily. The enzyme was active in the presence of various organic solvents. Its yield and product selectivity of maltooligosaccharides in the presence of DMSO or ethanol were compared with those of the industrial maltotriose-forming amylase from *Microbacterium imperiale*. Both enzymes improved the production selectivity of maltotriose by the addition of DMSO or ethanol. However, the total maltooligosaccharide yield in the presence of the solvents was higher for LB25 amylase than for *M. imperiale* amylase.

Communicated by K. Horikoshi.

N. Doukyu (✉) · W. Yamagishi · N. Furuki
Department of Life Science, Faculty of Life Sciences,
Toyo University, 1-1-1 Izumino, Itakura-machi, Gunma, Japan
e-mail: doukyu@itakura.toyo.ac.jp

H. Kuwahara
Department of Biological Information,
Graduate School of Bioscience and Biotechnology,
Tokyo Institute of Technology, Nagatsuta-cho,
4259 Midori-ku, Yokohama, Japan

H. Ogino
Department of Chemical Engineering, Osaka Prefecture
University, 1-1 Gakuen-cho, Naka-ku, Sakai,
Osaka 599-8531, Japan

Keywords Amylase · Purification · Organic solvent · Dimethylsulfoxide · *Brachybacterium* · Maltooligosaccharide · Maltotriose

Introduction

Maltooligosaccharides have favorable properties, such as low sweetness, high water-holding capacity, prevention of sucrose crystallization, and an antistaling effect on bread (Park 1992). Microbial amylases that produce specific maltooligosaccharides have made it possible to produce syrups containing various maltooligosaccharides. Most α-amylases produce glucose or maltose as the major product from starch. However, amylases that specially produce maltooligosaccharides from starch have been reported (Ali et al. 2001; Kainuma et al. 1975; Kim et al. 1995; Kobayashi et al. 1992; Messaoud et al. 2004; Morgan and Priest 1981; Nagarajan et al. 2006; Robyt and Ackerman 1971; Satoh et al. 1997; Takasaki 1985; Takasaki et al. 1991a, b; Wako et al. 1978).

Several attempts to improve the product selectivity of cyclodextrin glucanotransferase (CGTase) by the addition of organic solvents have been reported (Blackwood and Bucke 2000; Doukyu et al. 2003; Lee and Kim 1991). On the other hand, there has been no report about the effect of organic solvents on maltooligosaccharide production by an amylase. Organic solvents often affect enzyme stability and activity (Antonini et al. 1981; Carrea 1984). Enzymes with high stability and activity in the presence of organic solvents would be useful for technological applications in which such solvents are used. Organic-solvent-tolerant microorganisms are useful in screening for extracellular enzymes in the presence of organic solvents. Organic-solvent-tolerant protease (Ogino et al. 1995), lipase (Ogino

et al. 1994), CGTase (Doukyu et al. 2003), halophilic α-amylase (Fukushima et al. 2005) and cholesterol oxidase (Doukyu and Aono 2001) have been found in organic-solvent-tolerant microorganisms. These microorganisms were screened using a medium overlaid with a water-immiscible organic solvent. Water-miscible organic solvents are often added to a reaction solution to improve the reaction rate of a hydrophobic compound or to shift the thermodynamic equilibrium toward synthesis. However, extracellular enzymes from water-miscible organic-solvent-tolerant microorganisms have not been studied. In the present study, we screened for a maltooligosaccharide-forming amylase that is active in the presence of water-miscible organic solvents and isolated a microorganism that produces an amylase in a medium containing dimethylsulfoxide (DMSO). Moreover, we purified the amylase to examine its enzymatic properties and the effect of organic solvents on its production of maltooligosaccharides.

Materials and methods

Strains and media

Organisms that produce maltooligosaccharide-forming amylase were screened with a medium consisting of 1% potato starch (Wako Pure Chemical Industries, Osaka, Japan), 1% Bacto Tryptone (Difco Laboratories, Detroit, MI, USA), 0.5% Bacto Yeast Extract (Difco), 1% NaCl, 10 mM $MgSO_4$, and 10–15% (vol/vol) DMSO or dimethylformamide (DMF). LBMg medium, consisting of 1% Bacto Tryptone (Difco), 0.5% Bacto Yeast Extract, 1% NaCl, and 10 mM $MgSO_4$, was used at times to culture isolates. When necessary, the medium was solidified with 1.5% (wt/vol) agar.

Isolation of an organism that produces a maltooligosaccharide-forming amylase on a medium containing DMSO or DMF

Soil samples were gathered from several locations in the Kanto area in Japan. A small amount of each soil sample was suspended in sterile 0.8% NaCl solution, and a portion of the suspension was spread on a screening medium agar plate. The plates were warmed at 30°C for 2–4 days. After incubation, 0.02% I_2 in 0.2% KI solution (about 10 ml) was poured onto the agar surface. Microorganisms that formed halos around their colonies were isolated by repeated single-colony isolation on the screening medium agar. The microorganisms were grown at 30°C for 48 h in the screening medium, and then the culture was centrifuged (6,000×*g*, 15 min, 4°C). The starch-hydrolyzing activity in the supernatants was measured by the iodine method described below. Maltooligosaccharide production by the culture supernatants was examined by TLC as described below. A microorganism that produced maltooligosaccharides from starch was selected.

Assay of starch-hydrolyzing activity

The starch-hydrolyzing activity was assayed with soluble starch as the substrate by measuring changes in iodine staining. A 20-μl portion of enzyme solution was added to 300 μl of a reaction mixture consisting of 0.5% soluble starch (Wako Pure Chemical Industries) and 100 mM sodium phosphate buffer (pH 7.0). This mixture was incubated at 30°C for 10 min. The reaction was stopped by the addition of 0.5 ml of 5 M acetic acid. Then, 0.5 ml of 0.02% I_2 in 0.2% KI solution was added to this mixture, and the absorbance of the final mixture was measured at 660 nm. One unit of enzyme activity was defined as the amount of enzyme that reduced the intensity of A_{660} of amylose-iodine complexes by 10% per minute under the conditions described.

In the other assay of starch-hydrolyzing activity, changes in the concentration of reducing sugars during incubation with starch were measured by the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). The reaction mixture was incubated at 30°C for 10 min. One unit of enzyme activity was defined as the amount of enzyme forming reducing sugars corresponding to 1 μmol of glucose per minute in the 3,5-dinitrosalicylic acid reaction.

TLC

After the enzymatic reaction described above with 0.5% soluble starch for 12 h, 1 μl of the reaction mixture was spotted onto a 0.2-mm-thick silica gel 60 plate (Merck, Darmstadt, Germany), and the sample was developed with a solvent mixture of acetic acid:ethyl acetate:water (2:2:1, by vol). A total of 0.5% glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were also used as substrate. The oligosaccharides were detected by spraying of 1% (wt/vol) orcinol and 50% (vol/vol) sulfuric acid on the TLC plate, which was then heated at 120°C for 10 min.

Amylase purification

Strain LB25 was grown at 30°C for 15 h in 3 l of LBMg medium containing 0.2% soluble starch, and the culture was centrifuged (6,000×*g*, 25 min, 4°C). Proteins in the supernatant were precipitated with $(NH_4)_2SO_4$ (70% saturation)

at 4°C overnight. The precipitate was recovered by centrifugation (15,000×*g*, 30 min, 4°C) and dissolved in 10 mM Tris–HCl (pH 8.0). This solution was dialyzed against the same buffer at 4°C and put on a column (2.5 × 8 cm) of DEAE-cellulose DE52 (Whatman, Maidstone, England) equilibrated with the Tris–HCl buffer. The column was washed with 150 ml of the Tris buffer and then eluted with a linear gradient of NaCl concentrations of 0–300 mM in 400 ml of the Tris buffer. The fractions with starch-hydrolyzing activity were pooled. The pooled solution was dialyzed against 10 mM sodium phosphate (pH 7.0) at 4°C and put on a column (1.5 × 6 cm) of hydroxyapatite (Nacalai Tesque, Kyoto, Japan) equilibrated with the sodium phosphate buffer. The column was washed with 20 ml of 10 mM sodium phosphate (pH 7.0) and then eluted with a linear gradient of sodium phosphate concentrations of 10–300 mM in 80 ml of the buffer. The enzyme solution was kept at 4°C until use.

Measurement of protein concentration

Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as the standard.

Zymograms

SDS-PAGE was done on a 12.5% (wt/vol) polyacrylamide gel by the method of Laemmli (1970) with an only modification of adding soluble starch to the gel. Soluble starch was added to the gel to a final concentration of 1% (wt/vol). After electrophoresis, the gel was washed with Tris–HCl buffer containing 25% (vol/vol) isopropanol. Then the gel was washed with 100 mM Tris–HCl buffer (pH 8.0) and incubated in the same buffer for 1 h at 30°C. The zone at which the starch was hydrolyzed was stained by soaking the gel in a solution of 0.2% I_2–2% (wt/vol) KI.

Effects of pH on enzyme activity and stability

The enzyme activity was assayed at 30°C at various pH levels with 100 mM CH_3COOH–CH_3COONa (pH 5.0–5.5), KH_2PO_4–Na_2HPO_4 (pH 5.5–7.5), Tris–HCl (pH 7.5–9.0), and Na_2CO_3–$NaHCO_3$ (pH 9.0–10.0). Enzyme stability was measured at 30°C after 1 h of incubation at each pH.

Measurement of maltooligosaccharides

A sample was analyzed with an NH_2-1251-N (4.6 × 250 mm) column (Senshu Science Co., Tokyo) attached to an HPLC apparatus. The column was eluted with acetonitrile:water (6:4, vol/vol) at a flow rate of 0.5 ml/min. The elution was monitored by a refractive index detector (RID-300, Japan Spectroscopic Co., Ltd.).

Nucleotide sequence accession number

The nucleotide sequence of the 16S rDNA gene has been deposited in the DDBJ/EMBL/GenBank databases under accession number AB257583.

Commercial amylase preparation

Maltotriose-forming amylase from *Microbacterium imperiale* is a product of Amano Enzyme, Inc. (Nagoya, Japan).

Results

Isolation of a microorganism producing maltooligosaccharide-forming amylase on a medium containing DMSO or DMF

We screened for microorganisms that produce starch-hydrolyzing enzymes on screening medium agar containing 10–15% (vol/vol) DMSO or DMF. These solvents were non-volatile and useful for several days of incubation. In the presence of DMSO, 480 strains formed halos around their colonies from about 400 soil samples. Of these strains, 81 had strong starch-hydrolyzing activity in the culture supernatant. Of the 81, only the culture supernatant of strain LB25, screened with a medium containing 12.5% (vol/vol) DMSO, showed predominant maltotriose formation by TLC analysis. Although we examined about 300 soil samples with a medium containing DMF, maltooligosaccharide-forming amylase was not found. DMF seemed to be more toxic than DMSO. The number of colonies grown on the medium containing 12.5% (vol/vol) DMF was about one-tenth that of 12.5% (vol/vol) DMSO.

Characterization and identification of strain LB25

We analyzed a partial sequence of the 16S rDNA gene sequence of LB25 as described by Lane et al. (1985). The 1,462 bp sequences showed significant similarity (99%) to the 16S rDNA sequences of *Brachybacterium* species such as *B.* sp. SKJH-25 (DDBJ/EMBL/GenBank accession no. AY741723), *B.* sp. R-23108 (no. AY786818), *B. paraconglomeratum* (no. AJ415377), and *B.* sp. R-23117 (no. AY786820). The microbiological and biochemical characteristics of strain LB25 were examined by methods reported for the classification of species of *Brachybacterium* (Heyrman et al. 2002). Cells of strain LB25 were rods measuring 0.8–1.0 × 1.5–2.0 μm and were Gram-positive,

nonmotile, and non-spore forming. The colonies were flat, smooth, circular, and white or pale yellow. Strain LB25 reduced nitrate and had positive catalase test results but negative oxidase and urease test results. Gelatin and starch were hydrolyzed. Mannose, mannitol, *N*-acetylglucosamine, gluconate, glucose, maltose, and arabinose were used, but phenyl acetate, citrate, adipiate, and *N*-capric acid were not. Strain LB25 grew at 30°C in the presence of up to 15% NaCl. Acid was produced from D-fructose, lactose, maltose, D-mannose, and sucrose. On the basis of these results, LB25 was shown to belong to the genus of *Brachybacterium* sp.

Purification of the amylase

The activity in the culture supernatant of LB25 grown on the screening medium was similar to that of LB medium without DMSO. Therefore, the amylase was purified from the culture supernatant of LB25 grown in LB medium. Table 1 summarizes the purification steps used. The starch-hydrolyzing activity was eluted from the DE52 column with NaCl at 250 mM and from the hydroxyapatite with sodium phosphate at 180 mM. In the end, amylase was purified 30-fold from the culture supernatant. The purified amylase had a specific activity of 62.9 U/mg of protein. The purified preparation gave a single band by SDS-PAGE (Fig. 1). The molecular mass was estimated to be 60 kDa. A 60-kDa protein from the culture supernatant was seen also on a zymogram.

Physicochemical properties of the amylase

The amylase was active at pH 5.5–8.0 and most active at pH 6.0–7.5 (Fig. 2). The enzyme was stable from pH 7.0–8.5 after incubation for 1 h at 30°C. The optimum temperature at pH 7.0 was 35°C (Fig. 3). The enzyme was stable at temperatures from 4 to 35°C. The enzyme retained 28% of its activity after incubation for 1 h at 40°C. In the presence of 5 mM $CaCl_2$, the enzyme still had 59% of its activity after incubation for 1 h at 40°C. However, the enzyme lost almost all activity after 1 h at 50°C.

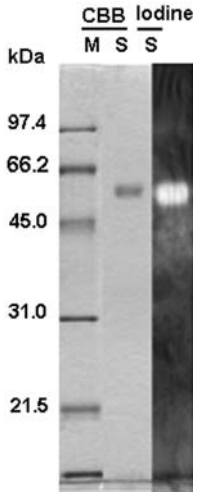

Fig. 1 SDS-PAGE of the amylase after final preparation obtained by hydroxyapatite column chromatography. Samples containing 0.2 μg of the amylase were applied on a SDS–12.5% (wt/vol) polyacrylamide–1% soluble starch gel. The gel was stained with Coomassie Brilliant Blue R-250 (*CBB*) or 0.2% I_2–2% (wt/vol) KI solution (Iodine). *Lane M* molecular size markers, *lane S* final preparation obtained by hydroxyapatite column chromatography

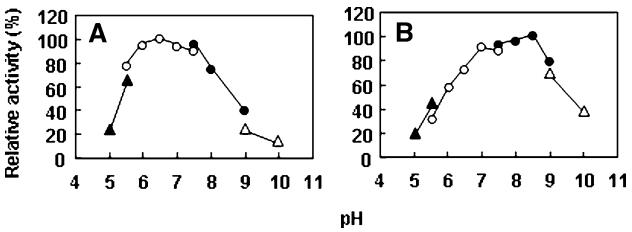

Fig. 2 Effects of pH on the amylase activity and stability. **a** Enzyme activity was assayed by the iodine method at 30°C under various pH conditions indicated in the figure. **b** The enzyme preparations were incubated at 30°C for 1 h under various pH conditions as indicated. The residual activity was examined by the iodine method at 30°C. The buffer systems (100 mM) used were CH_3COOH–CH_3COONa (pH 5.0–5.5) (*filled triangle*), KH_2PO_4–Na_2HPO_4 (pH 5.5–7.5) (*open circle*), Tris–HCl (pH 7.5–9.0) (*filled circle*) and Na_2CO_3–$NaHCO_3$ (pH 9.0–10.0) (*open triangle*)

To determine the effect of metal ions on the amylase activity, enzyme activity was measured at 30°C in the presence of various metal ions. At a concentration of

Table 1 Purification of the amylase from *Brachybacterium* sp. strain LB25

Step	Volume (ml)	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	2950	59.5	125	2.11	1	100
$(NH_4)_2SO_4$ precipitation[a]	52.0	19.3	120	6.24	3.0	96
DEAE-cellulose DE52	120	1.27	41.1	32.4	15	33
Hydroxyapatite	30.0	0.62	39.1	62.9	30	31

[a] The activity was measured by the iodine method

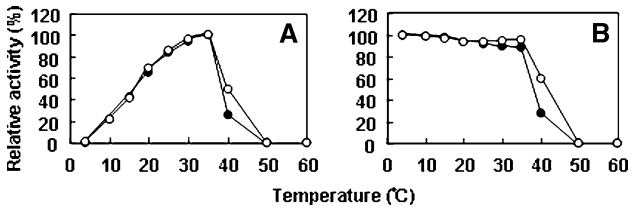

Fig. 3 Effects of temperature on the amylase activity and stability. **a** Enzyme activity was assayed by the iodine method at pH 7.0 at the temperatures indicated in the figure in the absence of $CaCl_2$ (*filled circle*) or in the presence of 5 mM $CaCl_2$ (*open circle*). **b** Enzyme dissolved in 100 mM phosphate buffer (pH 7.0) was incubated for 1 h at the temperatures indicated in the absence of $CaCl_2$ (*filled circle*) or in the presence of 5 mM $CaCl_2$ (*open circle*), and the relative activity was assayed by the iodine method at 30°C

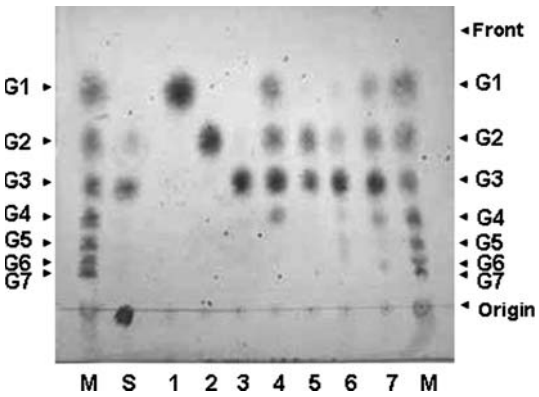

Fig. 4 Action patterns of the amylase on various substrates. The reaction products from soluble starch (*lane S*), glucose (*lane 1*), maltose (*lane 2*), maltotriose (*lane 3*), maltotetraose (*lane 4*), maltopentaose (*lane 5*), maltohexaose (*lane 6*) and maltoheptaose (*lane 7*) were analyzed by TLC. Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were used as the standards for starch-hydrolyzed products. *Lane M* represents a standard mixture of maltooligosaccharides ranging from glucose (*G1*) to maltoheptaose (*G7*)

1 mM, Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, and Fe^{2+} scarcely influenced the enzyme activity. However, the addition of 1 mM Ag^{+} reduced the activity to 48% of that without a metal ion.

Products from starch and various maltooligosaccharides

The reaction products from starch and various maltooligosaccharides were analyzed by TLC (Fig. 4). Starch, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were hydrolyzed mainly to maltotriose. Production of small amounts of glucose and maltose was observed. Maltose and maltotriose were not hydrolyzed. When maltotetraose, maltohexaose and maltoheptaose were used as substrates, the substrates not yet hydrolyzed were observed. These remaining substrates might be caused due to an insufficient reaction time.

Table 2 Effects of organic solvents on amylase activity

Solvent	Log P_{ow}	Relative activity[a] of the amylase from	
		LB25	*M. imperiale*
None	–	100[b]	100[b]
DMSO	–1.35	85.8 ± 3.9	74.7 ± 9.0
DMF	–1.04	78.7 ± 3.9	67.8 ± 3.8
Acetone	–0.26	68.5 ± 10.0	78.5 ± 7.9
Ethanol	–0.24	72.9 ± 6.3	72.0 ± 7.8
2-Propanol	0.28	69.2 ± 13.4	74.6 ± 3.0
Chloroform	1.9	82.6 ± 6.7	82.2 ± 1.2
Benzene	2.1	90.5 ± 8.5	86.7 ± 6.6
Toluene	2.6	91.6 ± 5.3	92.2 ± 9.1
Cyclohexane	3.4	94.2 ± 3.8	93.3 ± 4.8

[a] An organic solvent (40 µl) was added to 160 µl of enzyme solution (0.2 U/ml in 100 mM PIPES-NaOH buffer, pH 7.0) containing 0.5% (wt/vol) soluble starch. The mixture was shaken at 30°C for 10 min. The activity was measured by the DNS method

[b] The activity is relative to that in a control amylase solution not exposed to any organic solvent. Mean values and SD for three independent experiments are shown

Effects of organic solvents on enzyme activity

Table 2 shows the activity of the purified amylase of LB25 in the presence of various solvents and also the activity of commercially available maltotriose-producing amylase from *M. imperiale*. Maltotriose-producing amylase from *M. imperiale* is now produced on an industrial scale for the manufacture of high maltotriose-containing syrup (Yang and Liu 2004). Highly polar hydrophobic solvents with log P_{ow} values (less than 2) inactivate most enzymes through structural denaturation (Laane et al. 1987). In the presence of water-immiscible organic solvents, such as chloroform, benzene, toluene, or cyclohexane, both enzymes showed relatively high activity, 82–94% of that without any solvent. Both enzymes were partially inactivated by the addition of water-miscible organic solvents, such as DMF, acetone, ethanol, or 2-propanol. The LB25 enzyme showed relatively high activity in the presence of DMSO and DMF in comparison with *M. imperiale* amylase.

Effects of DMSO or ethanol on the yield and product selectivity of maltooligosaccharides

The effects of DMSO or ethanol on the yield and product selectivity of maltooligosaccharides by amylases from strain LB25 and *M. imperiale* are shown in Table 3. With both enzymes, glucose, maltose, maltotriose, and maltotetraose were produced from soluble starch, but no other maltooligosaccharides were detected. In the case of LB25

Table 3 Effects of DMSO or ethanol on yield and product selectivity of maltooligosaccharides

Amylase from	Solvent	Solvent-concn. (%)	Total yield[a] (%)	Product ratio[b] (%)			
				G1	G2	G3	G4
LB25	None	–	58.7 ± 0.7	2.3	14.7	83.0	0.0
	DMSO	5	56.0 ± 0.7	1.6	14.1	84.3	0.0
		10	56.8 ± 1.1	1.5	11.8	86.7	0.0
		20	54.4 ± 1.1	0.2	8.4	90.3	1.4
		30	48.9 ± 2.2	0.0	4.3	93.1	2.6
	Ethanol	5	57.5 ± 0.6	3.4	13.2	83.4	0.0
		10	57.6 ± 1.2	2.3	9.7	85.5	2.5
		20	52.9 ± 1.6	1.2	4.3	90.2	3.9
		30	27.8 ± 2.8	1.1	0.0	93.6	5.3
M. imperiale	None	–	45.2 ± 1.7	2.1	7.8	87.6	2.5
	DMSO	5	39.7 ± 2.7	0.0	6.0	92.5	1.5
		10	35.8 ± 3.6	0.0	3.1	95.3	1.6
		20	33.1 ± 3.7	0.0	0.0	98.5	1.5
		30	26.3 ± 3.1	0.0	0.0	99.2	0.8
	Ethanol	5	44.9 ± 1.5	0.9	7.1	90.2	1.8
		10	44.7 ± 1.5	0.8	6.3	91.1	1.8
		20	42.8 ± 2.8	0.0	4.9	93.0	2.1
		30	27.2 ± 2.5	0.0	4.3	94.5	1.2

Thirty microliters of the enzyme solution (200 U/ml) was added to the reaction mixture (300 μl) containing 1% (wt/vol) soluble starch and 100 mM sodium phosphate buffer (pH 7.0) in 0–30% (vol/vol) solvent

[a] Yields of maltooligosaccharides are shown as percentages of converted soluble starch after 6 h of incubation at 30°C. Mean values and SD for three independent experiments are shown

[b] Mean values for three independent experiments are shown. The values of SD were below 2.9

amylase, the total yields of maltooligosaccharides in 5–30% (vol/vol) DMSO or 5–20% (vol/vol) ethanol were 4.89–5.76 mg/ml (49–58% of the starting amount of starch). However, the yield was lowered to 2.78 mg/ml in the presence of 30% (vol/vol) ethanol. In the case of *M. imperiale* amylase, the total yields in 5–30% (vol/vol) DMSO or 5–20% (vol/vol) ethanol were 2.63–4.49 mg/ml (26–45% of the starting amount of starch). The total yields with LB25 amylase were about 1.2 to 1.9-fold higher than those of *M. imperiale*, except for that of 30% (vol/vol) ethanol. Importantly, the production of maltooligosaccharides by *M. imperiale* amylase was partially suppressed by the addition of DMSO. In both solvents, the product ratios of maltotriose with *M. imperiale* amylase were higher than those with LB25 amylase. However, the gross yields of maltotriose with *M. imperiale* amylase were lower than those of LB25 amylase. In both enzymes, the product ratios of glucose and maltose were decreased at the higher solvent concentration while the ratios of maltotriose were increased. The production of maltotetraose by LB25 amylase was improved by the addition of the solvents, although maltotetraose was not detected in the absence of organic solvents. This increase of maltotetraose by the addition of the solvents was not observed in the case of *M. imperiale* amylase.

The yield of maltotriose in the absence of the solvents by LB25 amylase was 4.87 mg/ml (49% of the starting amount of starch). On the other hand, the yields of maltotriose in the presence of 5–30% (vol/vol) DMSO or ethanol by LB25 amylase were 2.60–4.92 mg/ml (26–49% of the starting amount of starch). In the case of *M. imperiale* amylase, the yield of maltotriose in the absence of the solvents was 3.96 mg/ml (40% of the starting amount of starch). The yields of maltotriose in the presence of 5–30% (vol/vol) solvent were 2.57–4.07 mg/ml (26–41% of the starting amount of starch). Therefore, the yields of maltotriose by amylases from LB25 and *M. imperiale* were not improved by the addition of the solvents.

Discussion

The amylase from strain LB25 hydrolyzed starch to produce maltotriose primarily. Several amylases are known to specially produce maltotriose from starch; these have been found in *Streptomyces griseus* NA-468

(Wako et al. 1978), *Bacillus subtilis* (Takasaki 1985), *M. imperiale* (Takasaki et al. 1991a), *Natronococcus* sp. strain Ah-36 (Kobayashi et al. 1992), *Streptococcus bovis* 148 (Satoh et al. 1997), and *Thermobifida fusca* (Yang and Liu 2004). Optimum temperatures of the reported maltotriose-forming amylases are 40–60°C (Yang and Liu 2004). The optimum temperature (35°C) of the purified amylase from LB25 is remarkably different from those of the other reported maltotriose-forming amylases. On the basis of its 16S rDNA sequence as well as its morphological and physiological characteristics, strain LB25 was classified as belonging to *Brachybacterium* sp. No amylase from the genus *Brachybacterium* has been previously reported.

There have been several studies on improvement of product selectivity or increasing the yield of cyclodextrin production by CGTase in the presence of organic solvents (Blackwood and Bucke 2000; Doukyu et al. 2003; Lee and Kim 1991). However, there has been no report about the effect of organic solvents on maltooligosaccharide production by an amylase. In the present study, the product selectivity by amylases from LB25 and *M. imperiale* was improved by the addition of organic solvents. However, increasing the total yields of maltooligosaccharides and increasing the yield of major product maltotriose were not observed by the addition of organic solvents. As maltooligosaccharides are mostly used in food, ethanol is a possible solvent to use in the production of maltooligosaccharides. Addition of ethanol can also improve the overall process economics by reducing bacterial contamination in enzymatic processes. LB25 amylase showed relatively high activity in the presence of DMSO among the water-miscible solvents tested. Therefore, the maltooligosaccharide production in the presence of ethanol and DMSO was examined in this study. The product selectivity for maltotriose and maltotetraose by LB25 amylase was improved by the addition of ethanol or DMSO. Especially, maltotetraose was detected only in the presence of the solvents. TLC analysis showed that LB25 amylase hydrolyzed maltotetraose to maltotriose, maltose, and glucose. Therefore, the hydrolyzing activity for maltotetraose might be inhibited by the addition of the solvents. The difficulties of interpreting solvent effects on the product selectivity are immense. It is known that organic solvents cause conformational change of the enzyme and displacement of water molecules either on the surface or the catalytic site of the enzyme (Klibanov 1997). Therefore, changes in the infrastructure and hydration of the enzyme by organic solvents might explain the improved product selectivity.

LB25 amylase was active in the presence of various organic solvents. Little has been known about the activity of amylases in the presence of water-miscible organic solvents. The CGTase from *Bacillus macerans* has been extensively studied (Tonkova 1998). It was previously reported that the activity of the enzyme from *Bacillus macerans* in the presence of 20% (vol/vol) ethanol and 2-propanol was 20 and 15% relative to that without any solvent, respectively (Doukyu et al. 2003). The LB25 amylase was more active in these solvents than the enzyme from *Bacillus macerans*.

Our study showed that addition of an organic solvent could be used to improve the product selectivity of maltooligosaccharide-forming amylase. The total yield of maltotriose by LB25 amylase in the presence of ethanol was relatively higher than that of the industrial maltotriose-forming amylase from *M. imperiale*. Therefore, the LB25 amylase can be used for the economical enzymatic production of maltotriose from starch in the presence of ethanol.

Acknowledgments This work was supported in part by the Industrial Technology Research Grant Program in 2005 from New Energy and Industrial Technology Development Organization (NEDO) of Japan, the INOUE ENRYO Memorial Foundation for the Promotion of Sciences, and the Grant for the High Tech Research Center Program organized by Ministry of Education, Culture, Sports, Science and Technology of Japan since 2006.

References

Ali M, Mhiri S, Mezghani M, Bejar S (2001) Purification and sequence analysis of the atypical maltohexaose-forming α-amylase of the *B. stearothermophilus* US100. Enzyme Microb Technol 28:537–542

Antonini E, Carrea G, Cremonesi P (1981) Enzyme catalyzed reactions in water-organic solvent two-phase system. Enzyme Microb Technol 3:291–296

Blackwood A, Bucke C (2000) Addition of polar organic solvents can improve the product selectivity of cyclodextrin glycosyltransferase solvent effects on CGTase. Enzyme Microb Technol 27:704–708

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Carrea G (1984) Biocatalysis in water-organic solvent two phase systems. Trends Biotechnol 2:102–106

Doukyu N, Aono R (2001) Cloning, sequence analysis and expression of a gene encoding an organic solvent- and detergent-tolerant cholesterol oxidase of *Burkholdria cepacia* strain ST-200. Appl Microbiol Biotechnol 57:146–152

Doukyu N, Kuwahara H, Aono R (2003) Isolation of *Paenibacillus illinoisensis* that produces cyclodextrin glucanotransferase resistant to organic solvents. Biosci Biotechnol Biochem 67:334–340

Fukushima T, Mizuki T, Echigo A, Inoue A, Usami R (2005) Organic solvent tolerance of halophilic α-amylase from a Haloarchaeon, *Haloarcula* sp. strain S-1. Extremophile 9:85–89

Heyrman J, Balcaen A, Vos P, Schumann P, Swings J (2002) *Brachybacterium fresconis* sp. nov. and *Brachybacterium sacelli* sp. nov., isolated from deteriorated parts of a medieval wall painting of the chapel of Castle Herberstein (Austria). Int J Syst Evol Microbiol 52:1641–1646

Kainuma K, Wako K, Kobayashi S, Nogami A, Suzuki S (1975) Purification and some properties from *Aerobacter aerogenes*. Biochim Biophys Acta 410:333–346

Kim T, Gu B, Jeong J, Byun S, Shin Y (1995) Purification and characterization of a maltotetraose-forming alkaline (alpha)-amylase from an alkalophilic bacillus strain, GM8901. Appl Environ Microbiol 61:3105–3112

Klibanov A (1997) Why are enzymes less active in organic solvents than in water? Trends Biotechnol 15:97–101

Kobayashi T, Kanai H, Hayashi T, Akiba T, Akaboshi R, Horikoshi K (1992) Haloalkaliphilic maltotriose-forming α-amylase from the Archaebacterium *Natronococcus* sp. strain Ah-36. J Bacteriol 174:3439–3444

Laane C, Boeren S, Vos K, Veegar C (1987) Rules for optimization of biocatalysis in solvents. Biotechnol Bioeng 30:81–87

Laemmli U (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 224:680–685

Lane D, Pace B, Olsen G, Stahl D, Sogin M, Pace N (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. Proc Natl Acad Sci USA 82:6955–6959

Lee Y, Kim H (1991) Enhancement of enzymatic production of cyclodextrins by organic solvents. Enzyme Microb Technol 13:499–503

Messaoud E, Ali M, Elleuch N, Masmoudi N, Bejar S (2004) Purification and properties of a maltoheptaose- and maltohexaose-forming amylase produced by *Bacillus subtilis* US116. Enzyme Microb Technol 34:662–666

Miller GL (1959) Use of dinitro salicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428

Morgan F, Priest F (1981) Characterization of a thermostable α-amylase from *Bacillus licheniformis* NCIB 6346. J Appl Bacteriol 50:107–114

Nagarajan D, Rajagopalan G, Krishnan C (2006) Purification and characterization of a maltooligosaccharide-forming alpha-amylase from a new *Bacillus subtilis* KCC103. Appl Microbiol Biotechnol 73:591–597

Ogino H, Miyamoto K, Ishikawa H (1994) Organic-solvent-tolerant bacterium which secretes organic- solvent-stable lipolytic enzyme. Appl Environ Microbiol 64:1929–1932

Ogino H, Yasui K, Shinotani T, Ishihara T, Ishikawa H (1995) Organic solvent-tolerant bacterium which secretes an organic solvent-stable proteolytic enzyme. Appl Environ Microbiol 61:4258–4262

Park K (1992) Development of new carbohydrate materials. Food Sci Ind 25:73–82

Robyt J, Ackerman R (1971) Isolation, purification, and characterization of a maltotetraose-producing amylase from *Pseudomonas stutzeri*. Arch Biochem Biophys 145:105–114

Satoh E, Uchimura T, Kudo T, Komagata K (1997) Purification, characterization, and nucleotide sequence of an intracellular maltotriose-producing α-amylase from *Streptococcus bovis* 148. Appl Environ Microbiol 63:4941–4944

Takasaki Y (1985) An amylase producing maltotriose from *Bacillus subtilis*. Agric Biol Chem 49:1091–1097

Takasaki Y, Kitajima M, Tsuruta T, Nonoguchi M, Hayashi S, Imada K (1991a) Maltotriose-producing amylase from *Microbacterium imperiale*. Agric Biol Chem 55:687–692

Takasaki Y, Shinohara H, Tsuruhisa M, Hayashi S, Imada K (1991b) Maltotetraose-producing amylase from *Bacillus* sp. MG-4. Agric Biol Chem 55:1715–1720

Tonkova A (1998) Bacterial cyclodextrin glucanotransferase. Enzyme Microb Technol 22:678–686

Wako K, Takahashi S, Hashimoto S, Kanaeda J (1978) Studies on maltotriose- and maltose-forming amylases from *Streptomyces*. J Jpn Soc Starch Sci 25:155–161

Yang C, Liu W (2004) Purification and properties of a maltotriose-producing α-amylase from *Thermobifida fusca*. Enzyme Microb Technol 35:254–260