Appl Microbiol Biotechnol (1989) 31:32–37

Applied and Microbiology Biotechnology

A relationship between efficiency of isomaltosaccharide hydrolysis and thermostability of six *Bacillus* oligo-1,6-glucosidases*

Yuzuru Suzuki and Kazumi Oishi

Department of Agricultural Chemistry, Kyoto Prefectural University, Shimogamo, Sakyo-ku, Kyoto 606, Japan

Summary. A *p*-nitrophenyl-α-D-glucopyranosidase from *Bacillus thermoamyloliquefaciens* KP 1071 capable of growing at 30°–66° C was assigned to an oligo-1,6-glucosidase (dextrin 6-α-D-glucanohydrolase, EC 3.2.1.10). The enzyme was compared with its homologous counterparts from *B. cereus* NY-14, *B. cereus* ATCC 7064 (each mesophile), *B. coagulans* ATCC 7050 (facultative thermophile), *B. thermoglucosidasius* KP 1006 (DSM 2542, obligate thermophile) and *B. flavocaldarius* KP 1228 (extreme thermophile) in thermostability and kinetic parameters at suboptimal temperatures for isomaltosaccharides (2–6 glucose units). This analysis showed that the efficiency of each isomaltosaccharide hydrolysis changes in a convex manner with increasing thermostability on the transition, NY-14 → ATCC 7064 → ATCC 7050 → KP 1071 → KP 1006 → KP 1228 enzymes, with a maximum at KP 1071 or ATCC 7050 enzyme.

Introduction

Bacillus thermoamyloliquefaciens KP 1071, which grows at 30°–66° C, can release a series of thermostable enzymes responsible for complete hydrolysis of amylopectin, amylose, pullulan and cyclodextrins (Suzuki et al. 1987c). Of these, two distinct α-amylases were purified and characterized as, respectively, a maltotriogenic endo-acting α-amylase I (mol. wt., 78000) and a maltogenic exo-acting α-amylase II (mol. wt. 115000) comprising two identical subunits (Suzuki et al. 1987b). α-Amylase II acts on cyclodextrins, and on pullulan to yield mainly panose. The enzyme II, unlike the other, hydrolyses α-1,6-bonds of amylopectin, but both enzymes hardly act on α-1,6-bonds of panose and isomaltose. The cleavage of these bonds could be effected by oligo-1,6-glucosidase (dextrin 6-α-D-glucanohydrolase, EC 3.2.1.10). In a previous study, a *p*-nitrophenyl-α-D-glucopyranosidase of *B. thermoamyloliquefaciens* KP 1071 was purified and shown to be molecularly similar to oligo-1,6-glucosidase from *B. thermoglucosidasius* KP 1006 (DSM 2542, obligate thermophile) (Suzuki et al. 1987d). This paper describes the assignment of the KP 1071 enzyme to an oligo-1,6-glucosidase and its comparison with five *Bacillus* counterparts in thermostability and efficiency in isomaltosaccharide hydrolysis.

Materials and methods

p-Nitrophenyl-α-D-glucopyranosidase was assayed spectrophotometrically at 60° C in a mixture (1 ml) containing 33.3 m*M* phosphate (pH 6.8, K salts), 2 m*M* *p*-nitrophenyl-α-D-glucopyranoside and 0.024 unit enzyme (Suzuki et al. 1976). One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of the substrate for 1 min. Glucose and reducing power were determined with, respectively, a glucose oxidase/peroxidase reagent and 3,5-dinitrosalicylic acid (Trinder 1969; Bernfeld 1955). Hydrolysis products of saccharides were analysed by paper chromatography with *n*-butanol/pyridine/water (6:4:3, volume/volume) and by HPLC on a Jasco Finepak SIL NH_2 column (Japan Spectroscopic Co., Tokyo) with acetonitrile/water (6:4) (Suzuki et al. 1979, 1987b; Welker and Campbell 1963).

Bacillus thermoamyloliquefaciens KP 1071 *p*-nitrophenyl-α-D-glucopyranosidase (629 units/mg protein) was purified to homogeneity (Suzuki et al. 1987d). α-Limit dextrins 1 and 2 (each with about ten glucose units on average) were prepared from potato amylopectin with respective α-amylases from human saliva and *B. subtilis* (Larner and McNickle 1955). Isomaltosaccharides were prepared by acid hydrolysis of dextran (Suzuki et al. 1979).

* Presented at the Annual Meeting of the Agricultural Chemical Society of Japan, Kyoto, April 1, 1986

Offprint requests to: Y. Suzuki

Results

Hydrolysis of oligo- and polysaccharides by *B. thermoamyloliquefaciens* KP 1071 *p*-nitrophenyl-α-D-glucopyranosidase was tested by incubating at 60° C for 10 min each mixture (0.2 ml) containing 33.3 m*M* phosphate (pH 6.8), 0.29 unit enzyme and each substrate. After mixing with 0.2 ml of 2 *M* TRIS-HCl buffer (pH 7.4), the mixture was assayed for glucose and reducing power. The enzyme released glucose from phenyl-α-D-glucoside, panose, isomaltose, palatinose, kojibiose, nigerose, sucrose and methyl-α-D-glucoside (each 10 m*M*). The respective degrees of hydrolysis were 29%, 25%, 9.0%, 1.8%, 1.5%, 0.2%, 0.2% and 0.2% (cf. 100% with 2 m*M* *p*-nitrophenyl-α-D-glucopyranoside). The enzyme hydrolysed α-limit dextrin 1 (1%) to yield glucose. However, neither glucose nor reducing power was produced from soluble starch, amylose (mol. wt., 2900), dextran (mol. wt., 190000), dextrin (each α-glucan purchased from Nacarai Tesque, Kyoto), α-limit dextrin 2 (each 1%), maltose, maltotriose, turanose, lactose, raffinose, cellobiose, melezitose, trehalose, phenyl-α-maltoside (each 10 m*M*) or *p*-nitrophenyl glycosides (each 2 m*M*) presented in Table 3, but only from α-D-glucopyranoside.

The enzyme (0.051 unit) was incubated for 2 min at 60° C in 33.3 m*M* phosphate (pH 6.8, 40 µl) with 10 m*M* panose or each of the isomaltosaccharides (2–6 glucose units). The mixture was heated for 5 min at 98° C and applied to paper chromatography. This demonstrated only two products in the individual digests of sugars, except for isomaltose. One was glucose, the sole product of isomaltose. The other was maltose from panose, or the isomaltosaccharide one glucose unit less than the added substrate. The degradation pattern of panose suggests the cleavage of the non-reducing terminal α-1,6-bond of each isomaltosaccharide. The Michaelis constants (K_m) and molecular activities [k_0, maximal hydrolysis velocity (V) divided by enzyme molar concentration] are given in Table 1.

Isomaltotetraose (10 m*M*) was incubated at 60° C for 0, 5, 30, 60 or 120 min with 0.25 unit enzyme in 0.2 ml of 33.3 m*M* phosphate buffer (pH 6.8). The mixture was heated at 98° C for 5 min at each interval and analysed by HPLC. This showed glucose increase throughout a 2 h reaction with a continuous fall in isomaltotetraose level. Isomaltotriose increased for the first 30 min of the reaction and then decreased. Isomaltose appeared faintly after 5 min reaction, accumulating until the end of incubation. These findings indicate exo-hydrolysis of isomaltotetraose by successively removing single glucose residues from the non-reducing end.

Table 1. The K_m, k_0 and k_0/K_m values of *Bacillus thermoamyloliquefaciens* KP 1071 *p*-nitrophenyl-α-D-glucopyranosidase for various substrates[a]

Substrates	K_m (m*M*)	k_0 (s^{-1})	k_0/K_m ($s^{-1} \cdot mM^{-1}$)
p-Nitrophenyl-α-D-glucopyranoside	0.46	780[b]	1700
Phenyl-α-D-glucopyranoside	0.78	258[b]	331
Palatinose	44	62[b]	1.4
α-Limit dextrin sample 1	31	132[c]	4.3
Isomaltose	5.9	98[d]	17
Panose	2.5	315[d]	126
Isomaltotriose	3.7	308[d]	83
Isomaltotetraose	4.2	325[d]	77
Isomaltopentaose	13	307[d]	24
Isomaltohexaose	40	370[d]	9.3

[a] The initial rates (v) of *p*-nitrophenol release were measured over 0.1–2 m*M* *p*-nitrophenyl-α-D-glucopyranoside levels ([*S*]) using 0.029 unit enzyme/ml assay medium. The v values of glucose formation were determined over 0.5%–1% α-limit dextrin, or 1–4 m*M* phenyl-α-D-glucoside or isomaltose or panose, or 1–10 m*M* palatinose or each of the isomaltosaccharides ([*S*]), with 0.147–0.896 unit enzyme/0.2 ml assay medium. The K_m and maximum initial hydrolysis velocity (V) were determined by plotting $1/v$ vs $1/[S]$ or $[S]/v$ vs $[S]$ (Lineweaver and Burk 1934; Dixon and Webb 1964). The k_0 was calculated from the V and mol. wt. (=60000) (Suzuki et al. 1987d)
[b] As glucosidic linkage hydrolysis
[c] As glucose formation
[d] As non-reducing terminal α-1,6-bond hydrolysis

Heavy metal ions, SH-reagents, amines and carbohydrates are compared in Tables 2 and 3 for their degrees of inhibition of *p*-nitrophenyl-α-D-glucopyranosidase activity.

Discussion

There is no doubt that *B. thermoamyloliquefaciens* KP 1071 *p*-nitrophenyl-α-D-glucopyranosidase corresponds to an oligo-1,6-glucosidase, since it preferentially hydrolyses non-reducing terminal α-1,6-bonds of panose, low molecular weight isomaltosaccharides and α-limit dextrin of many naturally-occurring sugars tested, the action on isomaltotetraose is of exo-type, and the activity greatly depends on the chain length of isomaltosaccharide supplied (Tables 1 and 4) (Larner 1955; Bailey and Roberton 1962; Walker and Builder 1967). Although the enzyme is weakly active on nigerose, sucrose, kojibiose and methyl-α-D-glucoside, this is not a common aspect of *Bacillus* oligo-1,6-glucosidases. All these sugars are at-

Table 2. Effects of heavy metal ions, ethylenediaminetetraacetate (EDTA), and SH-reagents on *B. thermoamyloliquefaciens* KP 1071 *p*-nitrophenyl-α-D-glucopyranosidase activity[a]

Additions	Final concentration (m*M*)	Inhibition of activity (%)
None	—	0
Hg^{2+}	2	100
Cd^{2+}	2	100
Cu^{2+}	2	100
Pb^{2+}	2	100
Ni^{2+}	2	97
Mn^{2+}	2	91
Co^{2+}	2	89
Ca^{2+}	2	81
Zn^{2+}	2	41
Sn^{2+}	2	12
Mg^{2+}	2	9
Ba^{2+}	2	8
Sr^{2+}	2	8
EDTA	2	1
p-Chloromercuribenzoate (*p*CMB)	0.050	0
5,5′-Dithio-bis(2-nitrobenzoate)	0.050	0

[a] The activity was assayed as in Materials and methods, except that the reaction mixture contained 5 m*M* borate (pH 7.5) as buffer and heavy metal ion or EDTA. The enzyme (0.028 unit) was exposed at 60° C for 20 min to 55.3 μ*M* SH-reagent in 0.9 ml of 29.4 m*M* phosphate (pH 6.8) before addition of 0.1 ml of 20 m*M* substrate

tacked by the *B. flavocaldarius* KP 1228 (extreme thermophile) enzyme, but never by the *B. cereus* ATCC 7064 (mesophile) enzyme (Suzuki et al. 1982, 1987a). The *B. thermoglucosidasius* KP 1006 enzyme acts on all these saccharides except sucrose (Suzuki et al. 1976, 1979). Of these, only sucrose and turanose are hydrolysed by the *B. coagulans* ATCC 7050 (facultative thermophile) enzyme (Suzuki and Tomura 1986). The *B. amyloliquefaciens* ATCC 23844 and alkalophilic *Bacillus* sp. NCIB 11203 (each mesophile) enzymes resemble the *B. cereus* ATCC 7064 enzyme in a lack of activity for turanose, sucrose or methyl-α-D-glucoside (Fogarty et al. 1985; Kelly et al. 1987). The activity for turanose distinguishes *B. cereus* NY-14 (mesophile) enzyme from the ATCC 7064 one (Yoshigi et al. 1985). Unlike extreme thermophile enzymes, all these extra activities are far lower than those for isomaltose.

Bacillus thermoamyloliquefaciens KP 1071 oligo-1,6-glucosidase is similar to its equivalent from *B. thermoglucosidasius* KP 1006 rather than those from *B. cereus* ATCC 7064, *B. coagulans* ATCC 7050 and *B. flavocaldarius* KP 1228 in the light of the entire spectra of inhibition degrees caused by heavy metal ions, amines and carbohydrates (Tables 2 and 3) (Suzuki et al. 1976, 1982, 1987a; Suzuki and Tomura 1986). This is consistent with their behaviours for the following specific antagonists. Benzylamine competitively inhibits the *B. flavocaldarius* enzyme, but not all other enzymes. *p*-Nitrophenyl-α-D-xylopyranoside commonly acts on the *B. thermoamyloliquefaciens*, *B. thermoglucosidasius* and *B. flavocaldarius* enzymes as a potent competitive inhibitor, while it only slightly affects the *B. cereus* and *B. coagulans* enzymes. This enzyme is non-competitively blocked by phenyl-α-maltoside and *p*-chloromercuribenzoate without significant effect on other enzymes. *p*-Nitrophenyl-α-D-galactopyranoside and *p*-nitrophenyl-α-D-mannopyranoside function as non-competitive inhibitors for the *B. cereus* enzyme, but for none of the others.

Oligo-1,6-glucosidases from *B. cereus* NY-14, *B. cereus* ATCC 7064, *B. coagulans* ATCC 7050, *B. thermoamyloliquefaciens* KP 1071, *B. thermoglucosidasius* KP 1006 and *B. flavocaldarius* KP 1228 are compared in Table 4 for their thermostability and kinetic parameters for isomaltosaccharides at suboptimal temperatures. From values of k_0/K_m, it is apparent that the efficiency of isomaltosaccharide hydrolysis changes in a convex manner with increasing thermostability on the transition, NY-14 → ATCC 7064 → ATCC 7050 → KP 1071 → KP 1006 → KP 1228 enzymes, with a maximum at the KP 1071 or ATCC 7050 enzymes. This is produced by the convex type of k_0 change with the trend of K_m increase against thermostability, except for isomaltose to which the k_0 change predominantly contributes. The rate of K_m increase tends to be greatly enhanced with sugar chain elongation. The *B. flavocaldarius* enzyme is the worst in terms of kinetic parameters, as often observed with other extreme thermophile enzymes along with low substrate specificity (König et al. 1982; Stetter 1986).

Protein flexibility enables enzymes to bind substrate in a specific orientation for catalysis (Getzoff et al. 1987; Creighton 1983). However, six lines of evidence suggest that, above six, *Bacillus* oligo-1,6-glucosidases would reduce conformational flexibility around the active center as well as in the whole molecule with increasing thermostability, affecting their binding substrate and catalysis. (1) There is a strong correlation between their tolerance against heat and denaturants (Suzuki et al. 1987d). (2) The enzymes tend to weaken the affinity for each isomaltosaccharide except isomaltose against thermostability, and this tendency is apt to be stimulated with sugar

Table 3. Effects of amines and sugars on *B. thermoamyloliquefaciens* KP 1071 *p*-nitrophenyl-α-D-glucopyranosidase activity[a]

Additions	Final concentration (m*M*)	Inhibition of activity (%)	K_i[b] (m*M*)	Type of inhibition
None	—	0		
TRIS	5	73	0.37	Competitive
Histidine	5	16	91	Non-competitive
NH_4Cl	5	10		
Aniline	5	9		
Glycine	5	1		
Glucose	10	55	2.6	Competitive
Glucono-δ-lactone	10	46	12	Non-competitive
Glucosamine	10	20		
Melezitose	10	12		
Phenyl-α-D-glucoside	10	10		
Gluconate	10	4		
Mannose	10	3		
Cellobiose	10	3		
D-Xylose	10	3		
Turanose	10	3		
Lactose	10	2		
p-Nitrophenol	2	100		
p-Nitrophenyl-α-D-xylopyranoside	2	43		
p-Nitrophenyl-α-D-mannopyranoside	2	7		
p-Nitrophenyl-β-D-glucopyranoside	2	4		
p-Nitrophenyl-β-D-xylopyranoside	2	4		
p-Nitrophenyl-β-D-galactopyranoside	2	2		
Other compounds[c]		0		

[a] The activity was measured as in Materials and methods, except that the reaction mixture contained one of the inhibitors
[b] The inhibition constants were determined by the methods of Lineweaver and Burk (1934) and of Dixon (1953)
[c] Benzylamine, hydrazine hydrate, serine (each 5 m*M*), trehalose, fructose, mannitol, sucrose, raffinose, maltose, maltotriose, methyl-α-D-glucoside, phenyl-α-maltoside, isomaltose (each 10 m*M*), *p*-nitrophenyl-α-D-galactopyranoside and *p*-nitrophenyl-β-L-fucopyranoside (each 2 m*M*)

Table 4. A comparison of oligo-1,6-glucosidases from *B. cereus* NY-14 (I), *B. cereus* ATCC 7064 (II), *B. coagulans* ATCC 7050 (III), *B. thermoamyloliquefaciens* KP 1071 (IV), *B. thermoglucosidasius* KP 1006 (V) and *B. flavocaldarius* (VI) in thermostability (T_m) and in K_m, k_0 and k_0/K_m values for isomaltosaccharides[a]

Enzymes	T_m[b] (°C)	K_m (m*M*) for					k_0 (s^{-1}) for					k_0/K_m ($s^{-1} \cdot mM^{-1}$) for				
		I2[c]	I3[c]	I4[c]	I5[c]	I6[c]	I2[c]	I3[c]	I4[c]	I5[c]	I6[c]	I2[c]	I3[c]	I4[c]	I5[c]	I6[c]
I	38	5.3	2.1				17	36				3.2	17			
II	48	6.2	3.0	2.8	4.6	11	37	139	114	86	84	6.0	47	41	19	7.6
III	60	3.0	4.4	7.4	13	15	399	567	512	288	235	133	129	69	22	16
IV	70	5.9	3.7	4.2	13	40	98	308	325	307	370	17	83	77	24	9.3
V	73	3.3	11	13	21		134	376	422	302		41	34	32	14	
VI[d]	91	2.6	12	14	23	32	8.5	30	27	20	17	3.3	2.5	1.9	0.9	0.5

[a] The K_m and k_0 values are quoted from Table 1 and from published reports (Suzuki et al. 1976, 1979, 1982, 1987a; Suzuki and Tomura 1986; Yoshigi et al. 1985). The values at pH 6.8 were determined at 21° C for enzyme I, at 35° C for enzyme II, at 50° C for enzyme III, at 60° C for enzymes IV and V, and at 80° C for enzyme VI, at which their activities were 72%, 78%, 61%, 57%, 54% and 87% of the respective optima at 21° C, 41° C, 62° C, 74° C, 75° C and 85° C
[b] Thermostability is expressed as the temperature (T_m) for 50% inactivation of enzyme in 10 min at pH 6.8. Data are quoted from published reports (Suzuki et al. 1987d; Yoshigi et al. 1985)
[c] Denotes isomaltose, isomaltotriose, isomaltotetraose, isomaltopentaose and isomaltohexaose, respectively
[d] k_0 and k_0/K_m expressed as those for each subunit of enzyme VI (Suzuki et al. 1987a)

chain elongation (Table 4). (3) The catalysis of isomaltosaccharide hydrolysis tends to decrease in increasing order of thermostability, *B. coagulans* or *B. thermoamyloliquefaciens* → *B. thermoglucosidasius* → *B. flavocaldarius* enzymes, despite their elevating suboptimal temperatures of

activity (Table 4). (4) The five enzymes except the *B. cereus* NY-14 enzyme tend to raise the activation energy of *p*-nitrophenyl-α-D-glucopyranoside hydrolysis against thermostability (Suzuki et al. 1976, 1982, 1987a, 1987d: Suzuki and Tomura 1986). (5) The *B. thermoglucosidasius* enzyme resists proteolysis much more strongly than the *B. cereus* ATCC 7064 enzyme (Suzuki and Imai 1982). (6) There is no evidence that each *Bacillus* species produces multiple types of oligo-1,6-glucosidase at any growth temperature. It may be plausible according to Arrhenius' law that the catalysis of isomaltosaccharide hydrolysis increases in elevating order of suboptimal temperature, *B. cereus* NY-14 → *B. cereus* ATCC 7064 → *B. coagulans* or *B. thermoamyloliquefaciens* enzymes (Table 4). Thus, the convex change in efficiency of isomaltosaccharide hydrolysis as six *Bacillus* enzymes increase thermostability (Table 4) would result from an alteration in balance between the positive effect of increasing suboptimal temperature on catalysis and the negative effect of reducing conformational flexibility on catalysis and substrate binding. The rise in efficiency depends mainly on the contribution by the first effect rather than the second, while the second predominates over the first with fall in efficiency.

In connection with this investigation, we proposed previously that the enhanced thermostability of *Bacillus* oligo-1,6-glucosidases could be gained by increasing the frequency of proline occurrence at β-turns (presumably their second sites) and the total of hydrophobic residues, whereby the polypeptide chain may be more densely packed with more strengthened hydrophobic interactions, resulting in the molecule being tighter as a whole (Suzuki et al. 1987d). This appears to be given strong support by Matthews et al. (1987), who very recently increased thermostability of bacteriophage T4 by the replacement of alanine with proline at one of the β-turns so as to decrease backbone entropy of unfolding.

References

Bailey RW, Roberton Am (1962) Carbohydrases of a rumen strain of *Lactobacillus bifidus*. Biochem J 82:272–277

Bernfeld P (1955) Amylases, α and β. Methods Enzymol 1:149–158

Creighton TE (1983) Proteins. Structure and molecular properties. Freeman and Company, New York, pp 380–396, 426–427

Dixon M (1953) The determination of enzyme inhibitor constant. Biochem J 55:170–171

Dixon M, Webb EC (1964) Enzymes, 2nd edn. Academic Press, New York, pp 69–79

Fogarty WM, Kelly CT, Kadam SK (1985) Separation and characterization of an α-glucosidase and maltase from *Bacillus amyloliquefaciens*. Can J Microbiol 31:670–674

Getzoff ED, Geysen HM, Rodda SJ, Alexander H, Tainer JA, Lerner RA (1987) Mechanism of antibody binding to a protein. Science 235:1191–1196

Kelly CT, Brennan PA, Fogarty WM (1987) Resolution of the extracellular α-glucosidase system of *Bacillus* sp. NCIB 11203. Biotechnol Lett 9:125–130

König H, Skorko R, Zilling W, Reiter W (1982) Glycogen in thermoacidophilic archaebacteria of the genera *Sulfolobus, Thermoproteus, Desulfurococcus* and *Thermococcus*. Arch Microbiol 132:297–303

Larner J (1955) Hydrolysis of isomaltotriose by oligo-1,6-glucosidase. J Am Chem Soc 77:6385–6386

Larner J, McNickle CM (1955) Gastrointestinal digestion of starch. I. The action of oligo-1,6-glucosidase on branched saccharides. J Biol Chem 215:723–736

Lineweaver H, Burk D (1934) The determination of enzyme dissociation constants. J Am Chem Soc 56:658–666

Matthews BW, Nicholson H, Becktel WJ (1987) Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. Proc Natl Acad Sci USA 84:6663–6667

Stetter KO (1986) Diversity of extremely thermophilic archaebacteria. In: Brock TD (ed) Thermophiles; general, molecular and applied microbiology. Wiley, New York, pp 39–74

Suzuki Y, Imai T (1982) Abnormally high tolerance against proteolysis of an exo-oligo-1,6-glucosidase from a thermophile *Bacillus thermoglucosidius* KP 1006, compared with its mesophilic counterpart from *Bacillus cereus* ATCC 7064. Biochim Biophys Acta 705:124–126

Suzuki Y, Tomura Y (1986) Purification and characterization of *Bacillus coagulans* oligo-1,6-glucosidase. Eur J Biochem 158:77–83

Suzuki Y, Yuki T, Kishigami T, Abe S (1976) Purification and properties of extracellular α-glucosidase of a thermophile, *Bacillus thermoglucosidius* KP 1006. Biochim Biophys Acta 445:386–397

Suzuki Y, Ueda Y, Nakamura N, Abe S (1979) Hydrolysis of low molecular weight isomaltosaccharides by a *p*-nitrophenyl-α-D-glucopyranosidehydrolyzing α-glucosidase from a thermophile, *Bacillus thermoglucosidius* KP 1006. Biochim Biophys Acta 566:62–66

Suzuki Y, Aoki R, Hayashi H (1982) Assignment of a *p*-nitrophenyl-α-D-glucopyranoside-hydrolyzing α-glucosidase of *Bacillus cereus* ATCC 7064 to an exo-oligo-1,6-glucosidase. Biochim Biophys Acta 704:476–483

Suzuki Y, Fujii H, Uemura H, Suzuki M (1987a) Purification and characterization of extremely thermostable exo-oligo-1,6-glucosidase from a caldoactive *Bacillus* sp. KP 1228. Starch/Stärke 39:17–23

Suzuki Y, Nagayama T, Nakano H, Oishi (1987b) Purification and characterization of a maltotriogenic α-amylase I and a maltogenic α-amylase II capable of cleaving α-1,6-bonds in amylopectin. Starch/Stärke 39:211–214, 246–252

Suzuki Y, Nagayama T, Oishi K (1987c) Hydrolysis of α-glucans and pullulan by a thermostable enzyme mixture from facultative thermophile *Bacillus thermoamyloliquefaciens* KP 1071. In: The Agricultural Chemical Society of Japan (ed. Tokyo): Abstracts of the Annual Meeting, p 22

Suzuki Y, Oishi K, Nakano H, Nagayama T (1987d) A strong correlation between the increase in number of proline residues and the rise in thermostability of five *Bacillus* oligo-1,6-glucosidases. Appl Microbiol Biotechnol 26:546–551

Trinder P (1969) Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann Clin Biochem 6:24–27

Walker GJ, Builder JE (1967) Metabolism of the reserve polysaccharide of *Streptococcus mitis:* properties of α-(1→6)-glucosidase, its separation from transglucosylase, and the action of the two enzymes on branched oligosaccharides. Biochem J 105:937–942

Welker NE, Campbell LK (1963) Effect of carbon sources on formation of α-amylase by *Bacillus stearothermophilus.* J Bacteriol 86:681–686

Yoshigi N, Chikano T, Kamimura M (1985) Purification and properties of α-glucosidase II from *Bacillus* cereus NY-14. J Jpn Soc Starch Sci 32:280–286

Received September 8, 1988/Accepted December 19, 1988