APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1990, p. 881–886
0099-2240/90/040881-06$02.00/0

Vol. 56, No. 4

Characterization of an *endo*-Acting Amylopullulanase from *Thermoanaerobacter* Strain B6A

BADAL C. SAHA,[1] RAPHAEL LAMED,[2] CHAN-YONG LEE,[2] SAROJ P. MATHUPALA,[2] AND J. GREGORY ZEIKUS[1,2,3]*

Michigan Biotechnology Institute, Lansing, Michigan 48909,[1] and Departments of Biochemistry[2] and Microbiology,[3] Michigan State University, East Lansing, Michigan 48824

Received 23 October 1989/Accepted 12 January 1990

A thermoanaerobe (*Thermoanaerobacter* sp.) grown in TYE-starch (0.5%) medium at 60°C produced both extra- and intracellular pullulanase (1.90 U/ml) and amylase (1.19 U/ml) activities. Both activities were produced at high levels on a variety of carbon sources. The temperature and pH optima for both pullulanase and amylase activities were 75°C and pH 5.0, respectively. Both the enzyme activities were stable up to 70°C (without substrate) and at pH 4.5 to 5.0. The half-lives of both enzyme activities were 5 h at 70°C and 45 min at 75°C. The enzyme activities did not show any metal ion activity, and both activities were inhibited by β- and γ-cyclodextrins but not by α-cyclodextrin. A single amylolytic pullulanase responsible for both activities was purified to homogeneity by DEAE–Sepharose CL-6B column chromatography, gel filtration using high-pressure liquid chromatography, and pullulan-Sepharose affinity chromatography. It was a 450,000-molecular-weight glycoprotein composed of two equivalent subunits. The pullulanase cleaved pullulan in α-1,6 linkages and produced multiple saccharides from cleavage of α-1,4 linkages in starch. The K_ms for pullulan and soluble starch were 0.43 and 0.37 mg/ml, respectively.

Pullulanase (pullulan-6-glucanohydrolase, EC 3.2.1.41) is a debranching enzyme that specifically cleaves α-1,6 linkages in pullulan, starch, amylopectin, and related polysaccharides (1). In recent years, pullulanase has gained importance in starch bioprocessing as a starch-debranching enzyme. It is generally used in combination with glucoamylase, fungal α-amylase, and β-amylase for the production of various sugar syrups because it improves saccharification rate and yield (16). Moreover, it has gained significant attention as a useful tool for structural studies of carbohydrates (30).

A pullulanase from a *Bacillus* sp. has been reported previously (16) as a suitable enzyme for use in the current starch saccharification process. However, the enzyme acts optimally at 60°C. There is a need for highly thermostable and thermoactive saccharifying amylases and debranching enzymes to run the starch saccharification process at a higher temperature. Thermoanaerobes have the potential for the production of thermoactive and thermostable enzymes (31) which can be cloned and overproduced in aerobic industrial hosts. Hyun and Zeikus demonstrated that the type strain of *Clostridium thermosulfurogenes* produces an extracellular β-amylase (6), whereas *Clostridium thermohydrosulfuricum* produces cell-bound pullulanase and glucogenic amylase activities, including an α-glucosidase (5, 7, 8) that works at or above 75°C. Several reports on novel thermostable pullulanases from thermophiles have appeared (12–14, 19–23). We have purified the pullulanase activity from *C. thermohydrosulfuricum* 39E and reported that the 136,000-molecular-weight monomeric glycoprotein cleaved α-1,6 linkages in pullulan and α-1,4 linkages in starch, and we have tentatively named it amylopullulanase (21). It is now clear from multiple studies that thermoanaerobes produce this kind of unique pullulanase activity (14, 19, 23).

A new organism, *Thermoanaerobacter* strain B6A, was isolated from a volcanic thermal spring habitat and reported to actively ferment hemicellulose above 60°C (28, 29). In this paper, we report on the physiological production and physicochemical and molecular properties of purified amylopullulanase from this new *Thermoanaerobacter* strain which are distinctly different from those of *C. thermohydrosulfuricum*.

MATERIALS AND METHODS

Organism and cultivation. *Thermoanaerobacter* strain B6A, a hemicellulose-fermenting thermophilic bacterium isolated from a geothermal site by Weimer et al. (29), was used. The organism was routinely grown at 60°C in 26-ml anaerobic pressure tubes (Bellco Glass, Inc., Vineland, N.J.) containing CM5 (29) or TYE (32) medium with 0.5% soluble starch and a N_2-CO_2 (95:5) gas headspace.

Preparation of crude enzyme. The organism was cultivated in several 1-liter round-bottom flasks that contained 500 ml of TYE medium with 0.5% soluble starch. Stationary phase cultures were centrifuged at 12,000 × *g* for 15 min. Cell suspensions were prepared by washing the cells twice with 50 mM acetate buffer (pH 6.0) with 5 mM $CaCl_2$ and then suspending the cells in the same buffer. The culture supernatant (specific activities, 0.21 and 0.12 U/mg of protein for pullulanase and amylase, respectively) was treated with $(NH_4)_2SO_4$ (80% saturation) and kept overnight at 4°C. The precipitate formed was collected by centrifugation (17,000 × *g*, 20 min), dissolved in acetate buffer (50 mM, pH 6.0), and dialyzed against the same buffer (4 liters) for 24 h at 4°C. The dialyzed enzyme preparation (specific activities, 7.91 and 4.43 U/mg of protein for pullulanase and amylase, respectively) was used as the extracellular crude enzyme preparation in these experiments.

Enzyme assay. Pullulanase and amylase activities were assayed by measuring the reducing sugar released from pullulan and soluble starch, respectively. The reaction mixture (1 ml) containing pullulan or soluble starch (1%, wt/vol), acetate buffer (50 mM, pH 5.0, 5 mM $CaCl_2$), and enzyme source was incubated at 60°C for 30 min. The reducing sugar was measured by the dinitrosalicylic acid method (3). One unit of pullulanase or amylase activity is defined as the

* Corresponding author.

TABLE 1. Comparison of pullulanase and amylase production by *Thermoanaerobacter* strain B6A in CM5- and TYE-starch media

Medium[a]	Growth (A_{660})	U of the following per ml			
		Pullulanase		Amylase	
		Total	Extracellular	Total	Extracellular
CM5	0.56	1.11	0.10	0.69	0.15
TYE	1.03	1.90	0.94	1.19	0.61

[a] Cells were cultivated in pressure tubes containing 10 ml of medium, pH 7.0, with 0.5% starch at 60°C without shaking.

amount of enzyme which produces 1 μmol of reducing sugar with glucose as the standard per min under the conditions described above.

Enzyme purification. The enzyme preparation obtained after ammonium sulfate treatment was applied to a DEAE–Sepharose CL-6B column (2.6 by 35 cm) preequilibrated with Tris hydrochloride buffer (50 mM, pH 7.4). The column was washed with the same buffer (2 liters) and was eluted with a gradient of NaCl (0 to 0.5 M) in the same buffer. The active pullulanase peak (eluted at 0.2 to 0.3 M NaCl) was pooled, concentrated, and dialyzed against 50 mM acetate buffer, pH 5.0. The dialyzed enzyme was concentrated by ultrafiltration (PM 10 membrane; Amicon Corp.) and subjected to gel filtration (Protein Pak 300 SW; Millipore Waters chromatography division, Milford, Mass.) using high-pressure liquid chromatography (HPLC). The column was equilibrated with acetate buffer (50 mM, pH 6.0). The active pullulanase peak was collected and subjected to pullulan-Sepharose affinity chromatography as described previously (21).

Other methods. Cell growth was determined by measuring the optical density of the culture broth at 660 nm. Protein was estimated by the method of Lowry et al. (11) with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (9). The molecular weight of native enzyme was estimated by gel filtration using HPLC, using the plot used by Andrews (2). For isoelectric point determination, a Servalyte Precote isoelectric focusing gel (pH 3 to 10) was used. Samples were run at a constant power of 1 W in an LKB Ultraphore isoelectric focusing apparatus, and the gel was stained with Serva Blue W. The K_m was determined by the Lineweaver-Burk method (10). The products of the enzymes on various substrates were analyzed by HPLC (21). An oligosaccharide analysis column (Aminex HPX-42A; Bio-Rad Laboratories, Richmond, Calif.) with a carbohydrate de-ashing system (Bio-Rad) was used. The column was maintained at 85°C, and elution of the sugars was performed with water. Trisaccharide (DP3) was further analyzed by using a Supelcosil LC-NH_2 column (Supelco, Inc., Bellefonte, Pa.) and acetonitrile-water (75:25) as the solvent as described by Nikolov et al. (15).

RESULTS

Production of pullulanase and amylase activities. The organism was grown overnight in simple CM5 medium and complex TYE medium with 0.5% starch. The results of pullulanase and amylase assays are shown in Table 1. The higher levels of pullulanase and amylase were produced in TYE medium; most of this activity was cell bound when CM5 medium was used. The addition of Trypticase (1%) (BBL Microbiology Systems) to CM5 medium increased

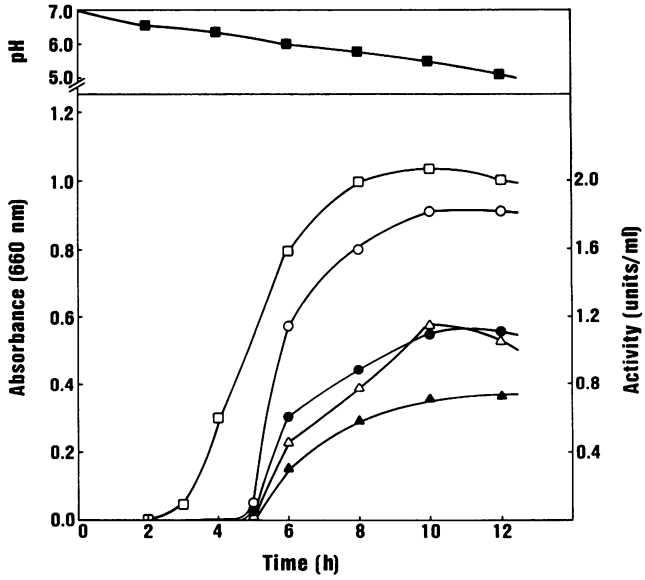

FIG. 1. Time course for growth and pullulanase and amylase production by *Thermoanaerobacter* strain B6A in TYE-starch (0.5%) medium. Symbols: □, cell growth; ○, total pullulanase activity; ●, extracellular pullulanase activity; △, total amylase activity; ▲, extracellular amylase activity; ■, pH.

amylolytic pullulanase production significantly (pullulanase, 1.79 U/ml; amylase, 1.23 U/ml), and the enzymes were more extracellular (pullulanase, 0.85 U/ml; amylase, 0.57 U/ml), as in TYE medium. A time course of growth and enzyme production in TYE-starch (0.5%) medium is shown in Fig. 1. Both enzyme activities were detected extracellularly from the beginning of enzyme production. The synthesis of the pullulanase and amylase activities was closely related to the growth phase of the organism. Longer-term incubation of the culture did not increase the amount of extracellular enzymes produced. The effect of the carbon source (0.5% each) on growth and enzyme production by *Thermoanaerobacter* strain B6A in TYE medium is shown in Table 2. The

TABLE 2. Effect of carbon source on growth and pullulanase and amylase production by *Thermoanaerobacter* strain B6A in TYE medium[a]

Carbon source (0.5%)	Growth (A_{660})	U of the following per ml			
		Pullulanase		Amylase	
		Total	Extracellular	Total	Extracellular
Soluble starch	1.09	1.26	0.73	0.91	0.65
Glycogen	1.08	1.41	1.13	0.98	0.82
Amylopectin	1.22	1.35	0.87	0.86	0.65
Pullulan	1.06	1.03	0.65	0.75	0.65
Maltotriose	0.91	0.73	0.45	0.67	0.38
Maltose	0.99	0.96	0.78	0.99	0.97
Glucose	0.91	0.19	0.19	0.37	0.34
α-Cyclodextrin	0.74	0.92	0.57	0.90	0.44
β-Cyclodextrin	0.67	1.12	1.13	0.97	0.81
Starch + maltose	1.09	1.25	0.63	1.11	0.63
Starch + glucose	1.09	0.28	0.10	0.36	0.19
Starch + α-cyclodextrin	0.92	1.14	0.70	0.94	0.53
Starch + β-cyclodextrin	0.57	0.89	0.53	0.81	0.64

[a] Cultures were grown in pressure tubes (10 ml of medium) at 60°C.

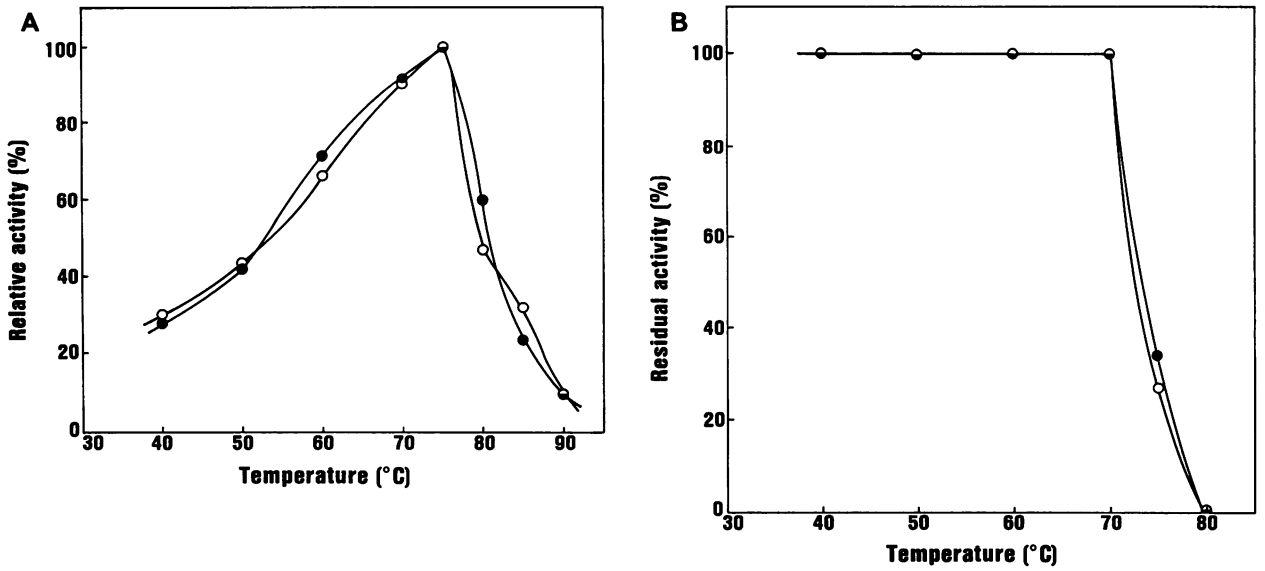

FIG. 2. Effect of temperature on *Thermoanaerobacter* strain B6A pullulanase and amylase activity and stability. (A) Thermoactivity. The enzyme activity was assayed by standard methods (30-min incubation). Symbols: ○, pullulanase activity; ●, amylase activity. (B) Thermal stability. The enzyme solution in acetate buffer (50 mM, pH 6.0) with 5 mM $CaCl_2$ was preincubated at various temperatures for 30 min, and then the residual activities were assayed. Symbols are as described for panel A.

organism produced both pullulanase and amylase activities with all the carbon sources tested, including lower levels produced on glucose. Although enzyme activity was constitutive, glucose repressed enzyme production on starch. The organism also grew well on maltodextrin DE 10, mannose, fructose, xylose, lactose, cellobiose, galactose, etc., and produced significant amounts of enzyme activities (data not shown). The ratio of pullulanase and amylase activities was almost the same for all the carbon sources used.

General characteristics of pullulanase and amylase activities. The results presented in Fig. 2A illustrate that both the pullulanase and amylase activities were active in a broad temperature range (that is, 40 to 90°C) and displayed optimum activity at 75°C. The effect of temperature on the heat stability of pullulanase and amylase in the absence of substrate is shown in Fig. 2B. Both the enzyme activities were entirely stable up to 70°C for the 60 min tested, but at 75°C these activities were lost rapidly. The half-lives of pullulanase and amylase activities were about 5 h (both cases) at 70°C and 45 min (both cases) at 75°C in the absence of a substrate (data not shown).

The pH activity and stability curves for pullulanase and amylase are shown in Fig. 3. The optimum pH for the activity of pullulanase and amylase was the same (5.0), and both the enzymes showed similar trends of activity over a wide pH range. Both pullulanase and amylase activities were fairly stable at pH 4.5 to 5.0. The cell-associated pullulanase and amylase activities also showed very similar activity and

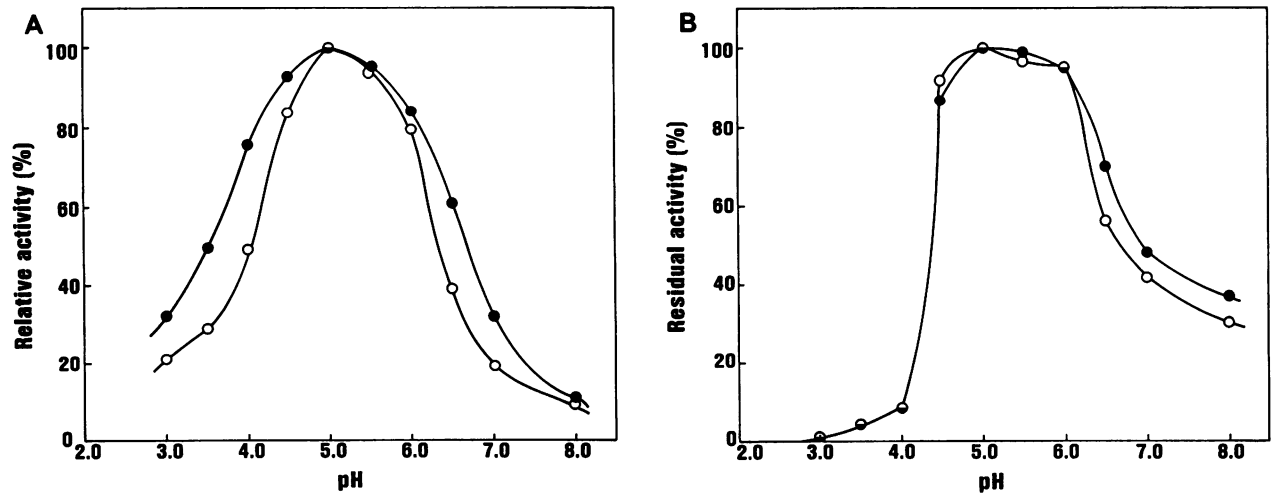

FIG. 3. Effect of pH on *Thermoanaerobacter* strain B6A pullulanase and amylase activity and stability. (A) pH activity. The activities of the enzymes were measured by the standard method by changing the buffer to obtain the desired pH. Symbols: ○, pullulanase activity; ●, amylase activity. (B) pH stability. The enzyme solution was incubated for 1 h at 60°C at various pHs. After adjustment of the pH, the residual activity was assayed by standard methods. Symbols are as described for panel A.

TABLE 3. Effect of various reagents on pullulanase and amylase activities of *Thermoanaerobacter* strain B6A

Reagent	Relative activity (%)[a] of:	
	Pullulanase	Amylase
Control	100	100
$CaCl_2$ (10 mM)	100	100
$MgCl_2$ (10 mM)	100	100
$MnCl_2$ (10 mM)	74	67
$CoCl_2$ (10 mM)	78	67
$ZnCl_2$ (10 mM)	2	16
$FeCl_3$ (10 mM)	10	17
p-Chloromercuribenzoate (0.02 mM)	86	100
Acarbose (20 μg)	96	93
α-Cyclodextrin (10 mM)	104	92
β-Cyclodextrin (10 mM)	32	58
γ-Cyclodextrin (10 mM)	48	80
N-Bromosuccinimide (0.1 mM)	0	0
EDTA (10 mM)	82	89
Dithiothreitol (10 mM)	104	93

[a] 100% activity corresponds to 1.20 U of pullulanase and 0.67 U of amylase under standard assay conditions.

stability curves with respect to pH and temperature. However, the cell-bound enzyme activities were more stable, and the half-life of the enzyme activities at 70°C was 66 h for both cases (data not shown).

The effect of ethanol on pullulanase and amylase activities and stability was tested. The enzyme preparation was treated at 60°C for 1 h with various concentrations of alcohol (0 to 20%), and the residual activity was determined. About 35% activity remained after the enzyme preparation was incubated for 1 h at 60°C in the presence of 20% alcohol (both cases). About 50% of both activities was destroyed by direct addition of 15% alcohol in the reaction mixture when tested under standard assay conditions.

The effects of sulfhydryl reagents, metal ions, and Schardinger dextrins on pullulanase and amylase activities were examined (Table 3). Both activities were not dependent on calcium ions. EDTA and *p*-chloromercuribenzoate did not significantly inhibit amylase or pullulanase activity. Although α-cyclodextrin had no effect on pullulanase and very little effect on amylase activities, β- and γ-cyclodextrins inhibited both activities. Both the enzymes were completely inhibited by *N*-bromosuccinimide.

Properties of purified amylopullulanase. The purified amylopullulanase displayed homogeneity on sodium dodecyl sulfate-polyacrylamide gel (10%) electrophoresis when stained by Coomassie brilliant blue R-250. It also showed a single band when stained with periodic acid-Schiff reagent, which indicates that the enzyme was glycoprotein in nature. The purified amylopullulanase had a specific activity of 215 U/mg of protein. The molecular weight of the native enzyme was estimated to be 450,000 by gel filtration and 220,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified enzyme migrated as a single protein band on isoelectric focusing and had a pI of about 4.5 by comparison with commercial standards.

The hydrolysis rate of various substrates by *Thermoanaerobacter* strain B6A amylopullulanase is given in Table 4. The purified enzyme degraded all the substrates tested. The K_ms for pullulan and soluble starch were 0.43 and 0.37 mg/ml, respectively.

The reaction products of *Thermoanaerobacter* strain B6A amylopullulanase on amylose, amylopectin, and pullulan at various times were analyzed in detail. The product formation

TABLE 4. Comparative saccharide hydrolysis rates for amylopullulanase from *Thermoanaerobacter* strain B6A

Substrate	Relative rate of hydrolysis (%)[a]
Pullulan	100
Amylopectin	55
Amylose	57
Soluble starch	57
Glycogen (oyster)	63
β-Limit dextrin from glycogen (oyster)	28

[a] Sufficient enzyme was added to cause a linear release of the product during the first 15 min of reaction. The substrate specificity is expressed as the percentage of initial hydrolysis velocity relative to that obtained with pullulan.

pattern of the purified enzyme on amylose and amylopectin indicates that the enzyme preparation contained an α-amylase-like activity which acted in an endofashion. The final pullulan hydrolysate contained only DP3 (100%), and amylose hydrolysate contained DP2 (44%), DP3 (33%), DP4 (17%), and higher saccharides (6%) (Fig. 4). The DP3 product in the case of pullulan hydrolysis was further analyzed by HPLC to determine whether the trisaccharide was maltotriose, panose, or isopanose. The column and solvent system used could separate maltotriose from panose and isopanose. Both panose and isopanose were coeluted in this system. Only maltotriose and no panose or isopanose was detected in the pullulan hydrolysates from various times. The final product of pullulan hydrolysate by the purified amylopullulanase is shown in Fig. 5.

DISCUSSION

Thermoanaerobacter strain B6A, which actively grows on and degrades starch, produces a novel amylopullulanase activity, and the physicochemical and molecular properties of this enzyme vary significantly in different species. Thus, amylopullulanase appears as a new class of amylase activity in microbes that actively grow by degrading starch. The enzyme is novel since it cleaves both α-1,6 and α-1,4 bonds in starch. We have previously shown that the enzyme is also formed in aerobic thermophilic *Bacillus* species and that a single protein is responsible for cleaving α-1,4 bonds in starch and α-1,6 bonds in pullulan (22). Hence, we suggest that this class of enzyme be named amylopullulanase to distinguish it from true pullulanases and α-amylase, which do not display these properties.

The cell-bound or extracellular location of amylopullulanase can depend on the nutritional and growth conditions used for *Thermoanaerobacter* strain B6A. The amylopullu-

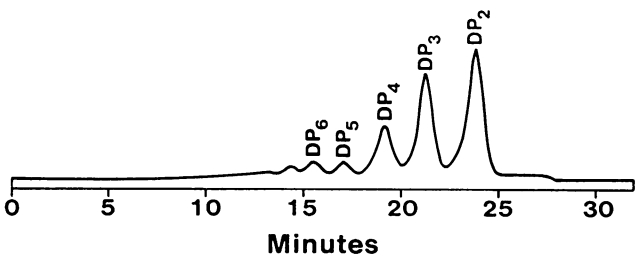

FIG. 4. HPLC analysis pattern of an amylose hydrolysate prepared by using the amylopullulanase purified from *Thermoanaerobacter* strain B6A. An oligosaccharide analysis column (Aminex HPX-42A) was used. The column was maintained at 85°C, and the elution of sugars was performed with distilled water.

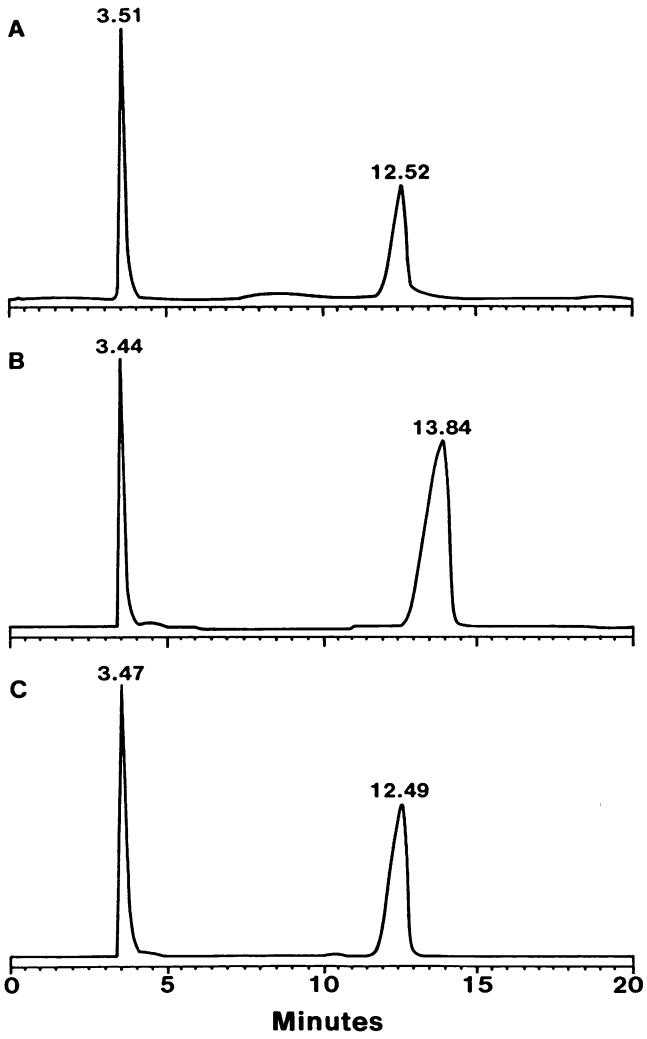

FIG. 5. HPLC analysis of pullulan hydrolysate prepared by using amylopullulanase purified from *Thermoanaerobacter* strain B6A. A Supelcosil LC-NH_2 column and an acetonitrile-water (75:25) solvent system were used at room temperature. (A) Maltotriose; (B) panose; (C) pullulan hydrolysate of purified pullulanase.

lanase of *Thermoanaerobacter* strain B6A was constitutive, unlike that reported for *C. thermohydrosulfuricum* 39E (8). Ohba and Ueda (17) described the influence of the nitrogen source on the production of intra- and extracellular pullulanases from *Aerobacter aerogenes*. They also found an inductive effector in the production of extracellular pullulanase by the same organism (18). Further studies are needed to find out why and what component(s) of Trypticase increased extracellular amylopullulanase production in *Thermoanaerobacter* strain B6A. The constitutive production of amylolytic enzymes as well as hemicellulases (28) by the organism indicates that the organism can produce a variety of extracellular enzymes.

The data indicate that the α-amylase- and pullulanase-like activities of *Thermoanaerobacter* strain B6A showed very similar characteristics in relation to temperature and pH activity optima and to enzyme inhibitors. When purified to homogeneity, the amylopullulanase displayed these same properties of activity and stability. The purified enzyme is a glycoprotein with a molecular weight of about 450,000. It is a dimer with a similar subunit molecular weight of about 220,000. In this respect, this enzyme differs significantly from the amylopullulanase of *C. thermohydrosulfuricum* 39E, which is a single monomeric protein with a molecular weight of about 136,500 (21). The temperature optimum and stability of *Thermoanaerobacter* strain B6A amylopullulanase are lower than the 90°C reported for the enzyme of *C. thermohydrosulfuricum* 39E (21).

Pullulan is a linear polymer molecule of maltotriose units linked by α-1,6 linkages. The amylopullulanase from *Thermoanaerobacter* strain B6A cleaved the α-1,6 bonds in pullulan and produced only maltotriose and not panose, isopanose, or a mixture of them. Thus, this amylopullulanase differs from enzymes like either the α-amylases from *Thermoactinomyces vulgaris* (25) and *Bacillus stearothermophilus* (26), which hydrolyze pullulan in α-1,4 linkages and produce panose, or the isopullulanase from *Aspergillus niger*, which cleaves α-1,4 linkages of pullulan to produce isopanose (24).

A number of reports on the interesting properties of thermostable amylopullulanases from thermoanaerobes have appeared. Coleman et al. (4) reported that the pullulanase activity obtained from *Thermoanaerobium brockii* and cloned into *Bacillus subtilis* hydrolyzed mostly α-1,4 and very few of the α-1,6 linkages in starch. Plant et al. (19) reported that the pullulanase activity from *Thermoanaerobium* strain Tok6-B1 possessed activity towards α-1,4 linkages in starch, amylopectin, and amylose, producing maltooligosaccharides (DP2 to DP4) as products. Melasniemi (13) suggested that the pullulanase and α-amylase activities of *C. thermohydrosulfuricum* are properties of the same protein. The denatured α-amylase-pullulanase was purified as two forms (I and II) from culture medium by using gel filtration in 6 M guanidine hydrochloride as the final step (14). Renatured α-amylase-pullulanases I and II had apparent molecular weights of 370,000 ± 85,000 and 330,000 ± 85,000, respectively. Both forms are dimers of two similar subunits with molecular weights of 190,000 ± 30,000 for enzyme I and 180,000 ± 30,000 for enzyme II.

Takasaki (27) reported on a pullulanase-amylase enzyme complex from *B. subtilis* which could hydrolyze α-1,4 linkages in amylose and amylopectin. Its molecular weight was estimated to be 450,000, which is the same as that of amylopullulanase in *Thermoanaerobacter* strain B6A. This enzyme produces a large amount of maltotriose, 50 to 55% from starch, whereas amylopullulanase from *Thermoanaerobacter* strain B6A makes maltose as the main product. The amylopullulanase from *Thermoanaerobacter* strain B6A, because of its unique product profile, may prove superior to commercially available saccharifying α-amylase for making specialty corn syrups.

LITERATURE CITED

1. **Abdullah, M., B. J. Catley, E. Y. C. Lee, J. Robyt, K. Wallenfels, and W. J. Whelan.** 1966. The mechanism of carbohydrate action. II. Pullulanase, an enzyme for the hydrolysis of α-1,6 bonds in amylaceous oligo- and polysaccharides. Cereal Chem. **43**:111–118.
2. **Andrews, P.** 1965. The gel filtration behavior of proteins related to their molecular weight. Biochem. J. **96**:595–606.
3. **Bernfeld, P.** 1955. Amylases alpha and beta. Methods Enzymol. **1**:149–150.
4. **Coleman, R. D., S.-S. Yang, and M. P. McAlister.** 1987. Cloning of the debranching-enzyme gene from *Thermoanaerobium brockii* into *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. **169**:4302–4307.
5. **Hyun, H. H., G.-J. Shen, and J. G. Zeikus.** 1985. Differential

amylosaccharide metabolism of *Clostridium thermosulfurogenes* and *Clostridium thermohydrosulfuricum*. J. Bacteriol. **164**:1153–1161.
6. **Hyun, H. H., and J. G. Zeikus.** 1985. General biochemical characterization of thermostable extracellular beta-amylase from *Clostridium thermosulfurogenes*. Appl. Environ. Microbiol. **49**:1162–1167.
7. **Hyun, H. H., and J. G. Zeikus.** 1985. General biochemical characterization of thermostable pullulanase and glucoamylase from *Clostridium thermohydrosulfuricum*. Appl. Environ. Microbiol. **49**:1168–1173.
8. **Hyun, H. H., and J. G. Zeikus.** 1985. Regulation and genetic enhancement of glucoamylase and pullulanase production in *Clostridium thermohydrosulfuricum*. J. Bacteriol. **164**:1146–1152.
9. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
10. **Lineweaver, H., and D. Burk.** 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56**:658–666.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. **Madi, E., G. Antranikian, K. Ohmiya, and G. Gottschalk.** 1987. Thermostable amylolytic enzymes from a new *Clostridium* isolate. Appl. Environ. Microbiol. **53**:1661–1667.
13. **Melasniemi, H.** 1987. Characterization of alpha-amylase and pullulanase activities of *Clostridium thermohydrosulfuricum*. Biochem. J. **246**:193–197.
14. **Melasniemi, H.** 1988. Purification and some properties of the extracellular alpha-amylase-pullulanase produced by *Clostridium thermohydrosulfuricum*. Biochem. J. **250**:813–818.
15. **Nikolov, Z. L., M. M. Meaghar, and P. J. Reilly.** 1985. High performance liquid chromatography of trisaccharides on amine-bonded silica columns. J. Chromatogr. **321**:393–399.
16. **Norman, B. E.** 1982. A novel debranching enzyme for application in the glucose syrup industry. Starch/Stärke **34**:340–346.
17. **Ohba, R., and S. Ueda.** 1980. Optimum medium components and culture conditions for the production of intra- and extracellular pullulanase by *Aerobacter aerogenes*. J. Fac. Agric. Kyushu Univ. **25**:135–151.
18. **Ohba, R., and S. Ueda.** 1982. An inductive effector in production of extracellular pullulanase by *Aerobacter aerogenes*. Agric. Biol. Chem. **46**:2425–2431.
19. **Plant, A. R., R. M. Clemens, R. M. Daniel, and H. W. Morgan.** 1987. Purification and preliminary characterization of an extracellular pullulanase from *Thermoanaerobium* Tok6-B1. Appl. Microbiol. Biotechnol. **26**:427–433.
20. **Plant, A. R., H. W. Morgan, and R. M. Daniel.** 1986. A highly stable pullulanase from *Thermus aquaticus* YT-1. Enzyme Microb. Technol. **8**:668–672.
21. **Saha, B. C., S. P. Mathupala, and J. G. Zeikus.** 1988. Purification and characterization of a highly thermostable novel pullulanase from *Clostridium thermohydrosulfuricum*. Biochem. J. **252**:343–348.
22. **Saha, B. C., G.-J. Shen, K. C. Srivastava, L. W. LeCureux, and J. G. Zeikus.** 1989. New thermostable α-amylase-like pullulanase from thermophilic *Bacillus* sp. 3183. Enzyme Microb. Technol. **11**:760–764.
23. **Saha, B. C., and J. G. Zeikus.** 1989. Novel highly thermostable pullulanase from thermophiles. Trends Biotechnol. **7**:234–239.
24. **Sakano, Y., M. Higuchi, and T. Kobayashi.** 1972. Pullulan 4-glucanohydrolase from *Aspergillus niger*. Arch. Biochem. Biophys. **153**:180–187.
25. **Shimizu, M., M. Kanno, M. Tamura, and M. Suekane.** 1978. Purification and some properties of a novel alpha-amylase produced by a strain of *Thermoactinomyces vulgaris*. Agric. Biol. Chem. **42**:1681–1688.
26. **Suzuki, Y., and T. Imai.** 1985. *Bacillus stearothermophilus* KP 1064 pullulan hydrolase. Its assignment to a unique type of maltogenic alpha-amylase but neither pullulanase nor isopullulanase. Appl. Microbiol. Biotechnol. **21**:20–26.
27. **Takasaki, Y.** 1987. Pullulanase-amylase complex enzyme from *Bacillus subtilis*. Agric. Biol. Chem. **51**:9–16.
28. **Weimer, P. J.** 1985. Thermophilic anaerobic fermentation of hemicellulose and hemicellulose-derived aldose sugars by *Thermoanaerobacter* strain B6A. Arch. Microbiol. **143**:130–136.
29. **Weimer, P. J., L. W. Wagner, S. Knowlton, and T. K. Ng.** 1984. Thermophilic anaerobic bacteria which ferment hemicellulose: characterization of organisms and identification of plasmids. Arch. Microbiol. **138**:31–36.
30. **Whelan, W. J.** 1971. Enzymic explorations of the structure of starch and glycogen. Biochem. J. **122**:609–622.
31. **Zeikus, J. G.** 1979. Thermophilic bacteria: ecology, physiology, and technology. Enzyme Microb. Technol. **1**:243–252.
32. **Zeikus, J. G., A. Ben-Bassat, and P. Hegge.** 1980. Microbiology of methanogenesis in thermal volcanic environments. J. Bacteriol. **143**:432–440.