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General Biochemical Characterization of Thermostable Extracellular β-Amylase from *Clostridium thermosulfurogenes*

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***Clostridium thermosulfurogenes*, an anaerobic bacterium which ferments starch into ethanol at 62°C, produced an active extracellular amylase and contained intracellular glucoamylase but not pullulanase activity. The extracellular amylase was purified 2.4-fold, and its general physicochemical and catalytic properties were examined. The extracellular amylase was characterized as a β-amylase (1,4-α-D-glucan maltohydrolase) based on demonstration of exocleavage activity and the production of maltose with a β-anomeric configuration from starch. The β-amylase activity was stable and optimally active at 80 and 75°C, respectively. The pH optimum for activity and the pH stability range was 5.5 to 6 and 3.5 to 6.5, respectively. The apparent $[S]_{0.5V}$ and V_{max} for β-amylase activity on starch was 1 mg/ml and 60 U/mg of protein. Similar to described β-amylase, the enzyme was inhibited by *p*-chloromercuribenzoate, Cu^{2+}, and Hg^{2+}; however, α- and β-cyclodextrins were not competitive inhibitors. The β-amylase was active and stable in the presence of air or 10% (vol/vol) ethanol. The β-amylase and glucoamylase activities enabled the organism to actively ferment raw starch in the absence of significant pullulanase or α-amylase activity.**

Food and beverage industries employ β-amylase (EC 3.2.1.2) to convert starch into maltose solutions (1, 7). High value is placed on extreme thermostability and thermoactivity of amylases for use in the bioprocessing of starch (2, 4, 13). Nonetheless, most industrial amylases are produced by organisms that grow at moderate temperatures and which, except for α-amylase, contain enzymes that lack extreme thermal attributes.

β-Amylase hydrolyzes the α-1,4-glucosidic linkages in an exo-fashion from the nonreducing end of starch-type substrates and produces both maltose in the β-anomeric configuration and β-limit dextrins from starch (6, 7, 18). β-Amylase is present in many higher plants, and attempts have been made to obtain the enzyme for microorganisms which may produce a more active, thermostable, and extracellular enzyme. However, the described microbial β-amylases are not active or thermostable enough to substitute for plant enzymes; therefore, the plant β-amylases usually have been used for maltose production, although they are expensive and unstable (1, 5–7, 8, 17, 21, 23, 24).

Only a few enzymes currently of interest to industry have been isolated and characterized from thermoanaerobes, in spite of their potential industrial utility associated with higher activity and thermostability (25). The thermostable and active enzymes characterized to date from thermoanaerobes include endoglucanase of *Clostridium thermocellum* (15, 16), alcohol dehydrogenase of *Thermoanaerobium brockii* and *C. thermohydrosulfuricum* (12); and the polygalacturonate hydrolase of *C. thermosulfurogenes* (19, 20).

Essentially, nothing is known about the biochemical attributes of thermophilic bacteria that actively ferment raw starch to ethanol at greater than 60°C. Nonetheless, thermoanerobic bacteria can often process faster metabolic rates and more thermostable enzymes than mesophilic microorganisms (25). We initiated comparative studies of amylolytic enzymes produced by diverse anerobic species. *C. thermosulfurogenes* ferments a wide variety of saccharides, including pectin and starch (19). We established that both *C. thermohydrosulfuricum* and *C. thermosulfurogenes* actively produce ethanol from starch (10). The purpose of this study is to describe the major amylolytic enzymes associated with growth of *C. thermosulfurogenes* on starch and to characterize the extracellular β-amylase it produces. We report here that this extracellular β-amylase displays unique thermoactive and thermostable characteristics.

MATERIALS AND METHODS

Chemicals. All chemicals used were reagent grade and were obtained from either Mallinckrodt (Paris, Ky.) or Sigma Chemical Co. (St. Louis, Mo.). Trypticase and yeast extract were obtained from Difco Laboratories (Detroit Mich.). Hexokinase, glucose-6-phosphatase dehydrogenase, *Bacillus* α-amylase, fungal glucoamylase, and other biochemicals were obtained from Sigma. All gases were obtained from Matheson Scientific, Inc. (Joliet Ill.) and were purified and made free of oxygen by passage over heated (370°C) copper filings.

Organism and cultivation. *C. thermosulfurogenes* 4B was isolated from an algal-bacterial mat in Octopus Spring, Yellowstone National Park (19), and it has been deposited in both the Deutsche Sammlung von Mikroorganismen (DSM 2229) and the American Type Culture Collection, Rockville, Md. (ATCC 33743). Stringent anaerobic culture techniques (26) were employed for medium preparation and cultivation. The organism was routinely grown at 60°C in 26-ml anaerobic pressure tubes (Bellco Glass, Inc., Vineland, N.J.) containing 10 ml of TYE medium (26) with 0.5% glucose or soluble starch and an N_2-CO_2 (95:5) gas head space. Culture medium was autoclaved for 45 min to ensure destruction of the extremely heat-resistant spores of thermoanaerobes (11).

For determination of starch hydrolysis reactions on petri dishes, the organism was streaked onto plates of TYE medium that contained 1.0% soluble starch and 3.0% purified agar (Difco) in an anaerobic chamber (Coy Products, Ann Arbor, Mich.). The plates were placed into an anoxic paint can (W. R. Brown Division Intermatic, Spring Grove,

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Ill.) under nitrogen and incubated at 60°C for 4 days. The plates were removed from the paint can and flooded with iodine solution (1% I_2 and 2% KI in water), and hydrolysis zones were visually observed.

Preparation of enzyme. Washed cells and culture supernatant were prepared as the amylase sources by cultivating the strain in serum vials that contained 50 ml of TYE medium with 0.5% soluble starch. Stationary phase cultures were then centrifuged at 12,000 × *g* for 10 min. Cell suspensions were prepared by washing twice with 20 mM sodium acetate buffer (pH 6.0) and then suspending the cells in buffer. For preparation of concentrated extracellular amylase, large-scale growth was performed in a 14-liter fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) that contained 10 liters of LPBB medium (27) with 1.0% maltose–0.02% yeast extract under N_2-CO_2 (95:5). The culture supernatant was obtained from cultures grown at 60°C until the late exponential phase with a DuPont (Wilmington, Del.) KSB continuous-flow centrifuge system. The supernatant (10 liters) was concentrated first by precipitating proteins with two volumes of cold ethanol (20 liters) and filtering through charcoal powder to collect the precipitate. The precipitate then was suspended in 300 ml of 20 mM sodium acetate buffer (pH 6.0), and ammonium sulfate was added to 70%. The precipitated proteins were centrifuged at 15,000 × *g* and then suspended in 100 ml of the buffer described above. This suspension was centrifuged again to remove insoluble materials and then precipitated with two volumes of cold acetone. This last precipitate was suspended in 100 ml of the same buffer, and then 100 ml of the enzyme solution was dialyzed against 4 liters of double-distilled water for 24 h. The specific activity of this supernatant amylase solution was 60 U mg of protein and was purified 2.4-fold.

Cells were mass cultured and harvested for preparations of cell extracts by the same procedures as described above, except that TYE medium supplemented with 0.5% soluble starch was employed. Cell extracts were prepared aerobically by suspending 3 g of wet cell paste in 12.5 ml of 20 mM sodium acetate buffer (pH 6.0). This suspension was treated at 37°C for 1 h with 5 mg of lysozyme from chicken egg white and disrupted by passage through a French pressure cell at 20,000 lb/in^2. The supernatant was collected by centrifugation at 30,000 × *g* for 30 min at 4°C. Protein concentration was determined by the method described by Lowry et al. (13).

Enzyme assays. Amylase or β-amylase activity was assayed by measuring the reducing sugar released from the reaction on starch. The reaction mixtures contained 1 ml of 10% soluble starch, 1 ml of 0.5 M sodium acetate buffer (pH 6.0), and 3 ml of enzyme solution which was appropriately diluted with water. The reaction was stopped by cooling on ice after incubation for 30 min at 60°C.

Reducing power was measured by the dinitrosalicylic acid method (3). For assay of pullulanase activity, the reaction mixture consisted of 0.5 ml of 2% pullulan in 0.2 M sodium acetate buffer (pH 6.0) and 0.5 ml of enzyme solution. After incubation at 60°C for 30 min, the reaction was stopped by cooling on ice and by adding 4 ml of 3,5-dinitrosalicylic acid.

One unit of amylase, β-amylase, or pullulanase is defined as the amount of enzyme that produced 1 μmol of reducing sugar as a glucose standard per min under the conditions described above. The activities measured were linear for at least 2 h.

Glucoamylase activity was assayed by incubating 1 ml of reaction mixture that contained 1% soluble starch, 0.1 M sodium acetate buffer (pH 4.8), and the appropriately diluted enzyme solution at 60°C for 30 min. The reaction was stopped immediately by cooling on ice and then by boiling in a steam bath for 10 min. These reaction mixtures were centrifuged to remove the insoluble precipitates, and then released glucose was analyzed by the hexokinase and glucose-6-phosphate dehydrogenase method (2). One unit of glucoamylase is defined as the amount of enzyme that produced 1 μmol of glucose per min under the assay conditions.

Qualitative and quantitative analysis of starch hydrolysis products. Enzyme (12 U) was placed into 20 ml of 0.1 M sodium acetate buffer (pH 6), containing 1% soluble starch, and incubated at 65°C. Samples were withdrawn at intervals, and the reducing sugar and glucose contents were analyzed as described above.

Maltose was identified in the starch hydrolysates by high-pressure liquid chromatography. The separation system consisted of a Perkin-Elmer series B liquid chromatograph equipped with a Sigma 10 data station (Perkin Elmer Corp., Norwalk, Conn.), a refractive index detector (Laboratory Data Control, Riviera Beach, Fla.), and an oligosaccharide analysis column (300 by 7.8 mm; Aminex HPX-42A; Bio-Rad Laboratories, Richmond, Calif.) fitted with a micro-guard precolumn (40 by 4.6 mm; packed with Aminex HPX-85C; Bio-rad). Starch hydrolysate samples were centrifuged at 5000 × *g* and loaded onto the column (50 μl).

Determination of reducing value and blue value curve. The reaction mixture contained enzyme and a 0.25% amylose solution in 0.1 M sodium acetate buffer (pH 6.0) and was incubated at 65°C. Samples (0.5 ml) were withdrawn at 5-min intervals and were placed in tubes that contained 2 ml of 0.1 M HCl for determination of the blue value. After mixing, 0.5 ml samples were withdrawn and added to 5 ml of iodine solution (0.05 g of iodine and 0.5 g of potassium iodide per liter). The absorbance then was measured at 620 nm in a spectrophotometer with water-iodine used as a blank. The blue value was calculated by dividing the absorbance of the digest with that of the substrate-iodine blank and multiplying by 100. Another 0.5-ml sample of the reaction mixture was withdrawn for determination of the reducing value. The reducing power was analyzed by the colorimetric copper method described by Nelson (12). Total carbohydrate was assayed by the phenol-sulfuric acid method (4).

Optical rotation study. A reaction mixture (5 ml), consisting of a 1% starch solution in 0.1 M sodium acetate buffer (pH 6.0) and enzyme, was added to a 10-cm cell. Optical rotation was measured at room temperature in a Perkin Elmer model 214 polarimeter by using the sodium line. When the optical rotation became approximately constant, about 20 mg of solid sodium carbonate was added, and the mutarotation of the mixture was measured.

Enzyme characterization. Enzyme inhibition and kinetic studies were performed under the standard assay conditions described above. The stability studies employed enzyme solution (about 40 U/ml) in 0.1 M sodium acetate buffer (pH 6.0) under the described conditions described above.

RESULTS

Location and type of amylase activities. In preliminary experiments, large reddish-colored zones against a blue background were detected around colonies grown on starch-agar plates stained with iodine solution. This result implied that *C. thermosulfurogenes* produced amylases that were not capable of completely hydrolyzing starch.

Results in Table 1 demonstrate the cellular location and types of amylase activities in *C. thermosulfurogenes*. The organism produced extracellular amylase, the activity of which will be classified below as that of a β-amylase, but not extracellular pullulanase or glucoamylase in the exponential growth phase. In addition, cell-bound glucoamylase and amylase but not pullulanase activity were detected. Neither amylase nor glucoamylase activity was inhibited by aerobic assay conditions.

High-pressure liquid chromatographic analysis of starch hydrolysates formed by extracellular amylase revealed that only maltose was produced as a detectable product, in addition to high-molecular-weight oligosaccharides (i.e., limit dextrins). In spite of prolonged incubation (Fig. 1), reducing sugars but not glucose were detected by quantitative analysis of starch hydrolysates. Enzymatic hydrolysis of maltotriose and maltotetraose hydrolysis were also studied. Only maltose (8 mg/ml) was produced as a result of the incubation of 1% maltotetroase in 0.1 M sodium acetate buffer with 12 U of the enzyme at 65°C for 3 h. Under these same conditions, enzymatic hydrolysis of maltotriose (1%) yielded both glucose (0.4 mg/ml) and maltose.

Figure 2 shows the release of reducing power and reduction of iodine staining capacity by action of the enzyme on amylose. The slow decrease of the blue value against the reducing value in comparison with that of the α-amylase control indicates that the enzyme is an exo-acting amylase. From the mutarotation study (Fig. 3), which employed α-amylase as the control, the upward shift of optical rotation on the addition of base to the starch hydrolysate formed by the *C. thermosulfurogenes* enzyme indicates that the hydrolysis products have a β-anomeric configuration. The data presented above provide evidence that the extracellular amylase activity isolated from *C. thermosulfurogenes* was that of β-amylase.

Physicochemical properties of β-amylase. The effect of temperature on the activity of β-amylase is illustrated in Fig. 4A. The optimum temperature for the enzyme activity was 75°C, and 85% of maximal activity was measured at 80°C. When Arhenius plots of specific activity versus temperature were determined (data not shown), Q_{10} values of 1.7 were calculated for β-amylase.

The effect of temperature on the stability of β-amylase in the absence of substrate is shown in Fig. 4B. The enzyme was completely stable up to 70°C, and 83% of the original activity was retained after treatment at 75°C for 1 h. Fig. 4C shows that the enzyme was almost completely destroyed within 20 min at 80°C in the absence of starch, but addition of starch to the enzyme solution greatly enhanced the heat stability in proportion to the concentration of starch. The enzyme was entirely stable at 80°C in the presence of 5% starch.

TABLE 1. Amylolytic enzyme activities and cellular location in *C. thermosulfurogenes*[a]

Enzyme	Assay condition	Activity in the following locations:		
		Supernatant (U/ml)	Washed cells (U/ml)	Cell extracts (U/mg of protein)
Amylase	Aerobic	5.5	0.07	0.26
	Anaerobic	5.5	0.07	0.26
Pullulanase	Aerobic	<0.01	<0.01	<0.01
	Anaerobic	<0.01	<0.01	<0.01
Glucoamylase	Aerobic	<0.01	0.02	0.07
	Anaerobic	<0.01	0.02	0.07

[a] *C. thermosulfurogenes* was grown to the late exponential phase on TYE medium that contained 0.5% soluble starch as the carbon source. Anaerobic assay conditions were established by the addition of dithiothreitol (2 mM) and gassing with N_2 gas during preparation of the enzyme solution and all reactants.

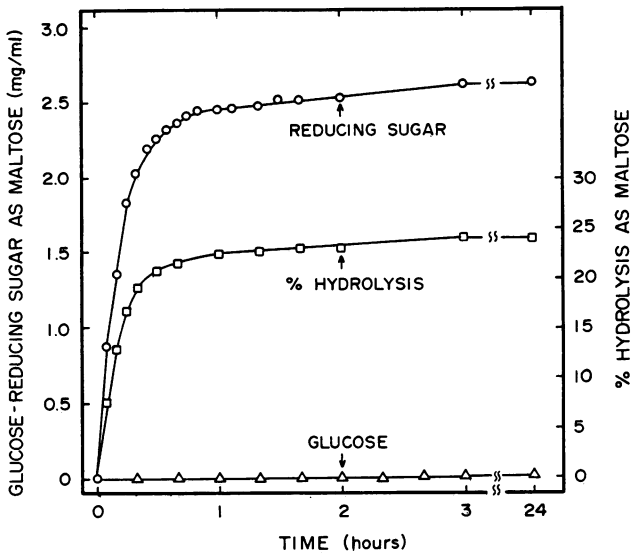

FIG. 1. Starch hydrolysis time course by *C. thermosulfurogenes* extracellular amylase. A 1% starch solution (20 ml) in 0.1 M acetate buffer (pH 6.0) was incubated with 12 U of enzyme at 65°C.

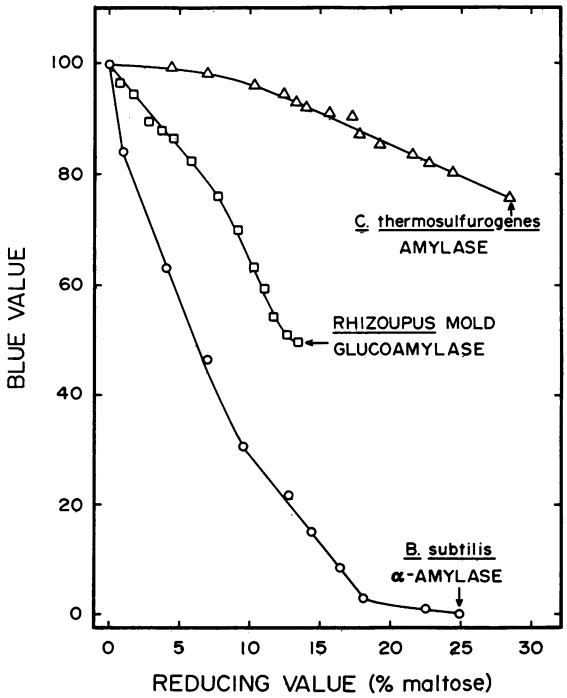

FIG. 2. Reducing value-blue value curve on amylose for *C. thermosulfurogenes* extracellular amylase, *Rhizopus* mold glucoamylase, and *Bacillus subtilis* α-amylase. The reaction was performed in serum vials that contained 20 ml of 0.25% amylose in 0.1 M acetate buffer (pH 6.0) and the respective enzyme (3 U each), and at 65°C for *C. thermosulfurogenes* amylase and 50°C for the other two.

Figure 5 shows the effect of pH on β-amylase activity and stability. The optimum activity was observed at pH 5.5 to 6.0, and the activity dropped gradually at more alkaline or acidic pH values. Greater than 43% of maximal activity was detected between pH values of 2.5 and 9.0. The enzyme was stable in the pH range of 3.5 to 6.5, but it was unstable below pH 3.0 and above pH 7.5.

The effects of sulfhydryl reagents, metal ions, and Schardinger dextrins (α- and β-cyclodextrin) on β-amylase activity were examined (Table 2). β-Amylase activity was strongly inhibited by Cu^{2+}, Hg^{2+}, and *p*-chloromercuribenzoate, but weakly inhibited by hydrogen peroxide, 5,5'-dithiobis-2-nitrobenzoic acid, *N*-ethylmaleimide, iodoacetic acid, and Zn^{2+}. The inhibitions could be prevented and restored by the addition of dithiothreitol (10 mM). The Schardinger dextrins did not inhibit enzyme activity and were not hydrolyzed by β-amylase. Under the assay condition used, the enzyme activity was not affected by the addition of 1 to 30 mM of calcium ion.

The effects of ethanol on the enzyme activity and stability were also performed. β-amylase activity was not affected in the presence of 3% (vol/vol) ethanol, but it dropped slowly in proportion to the concentration of ethanol at concentrations greater than 3% (vol/vol). In the presence of 10% (vol/vol) ethanol, 65% of maximal activity was observed. This effect is attributed to the precipitation of the starch substrate by ethanol because β-amylase was completely stable at 65°C for 1 h in the presence of 10% (vol/vol) ethanol before activity analysis.

Kinetic properties of β-amylase. The apparent kinetic constants were determined for β-amylase on starch versus amylose by determination of product formation rate versus time curves. The dependence of the rate of enzymatic hydrolysis on the substrate concentration followed Michaelis-Menten kinetics, and linear relationships of I/V versus $I/[S]$ were obtained. The apparent $[S]_{0.5V}$ and V_{max} values as determined from the double reciprocal slot for starch versus amylose were 1 mg/ml and 60 U/mg of protein versus 1.67 mg/ml and 30 U /mg of protein, respectively.

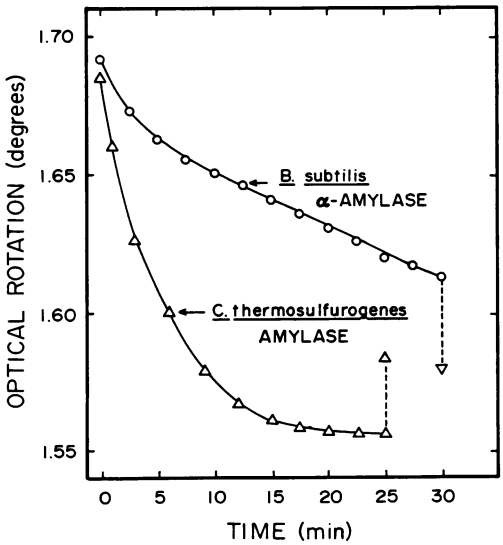

FIG. 3. Optical rotation of the action of *C. thermosulfurogenes* extracellular amylase and *B. subtilis* α-amylase with starch. The dashed lines indicate the optical rotation after addition of sodium carbonate to the digests.

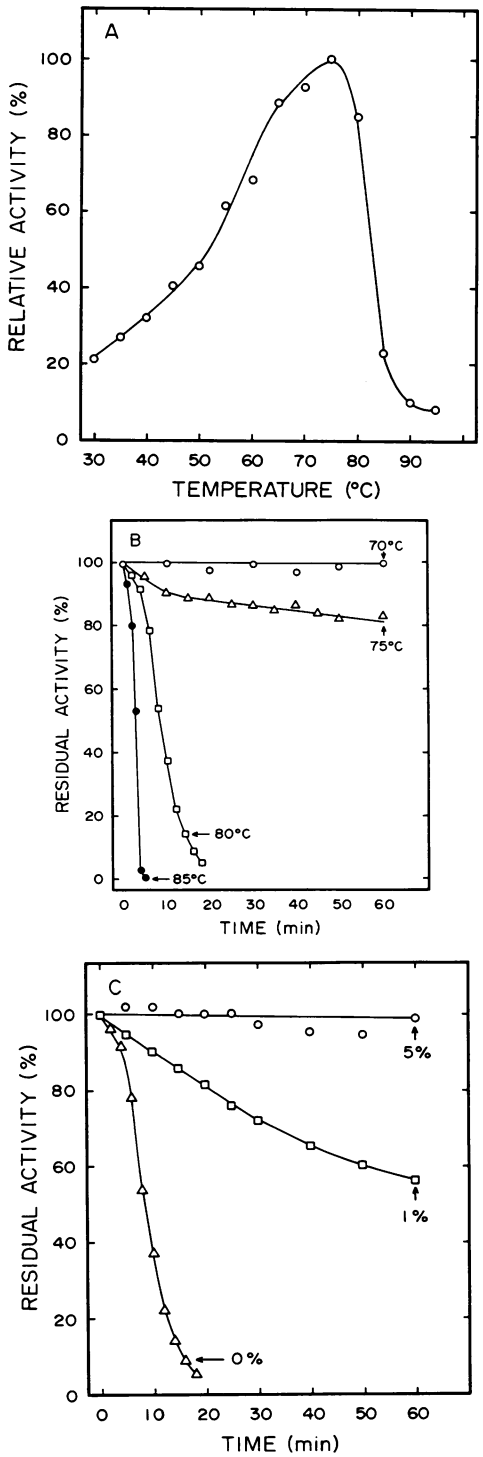

FIG. 4. Effects of temperature on activity (A) and stability of *C. thermosulfurogenes* β-amylase in the absence (B) or presence (C) of starch. (A) Temperature profile of β-amylase activity (100% activity corresponds to 57 U/mg of protein). (B) Thermal stability of β-amylase. The enzyme solutions (0.31 mg of protein per ml) in 0.1 M acetate buffer (pH 6.0) were incubated at the indicated temperatures. The residual activity was assayed under the standard conditions, with 100% activity corresponding to 24.4 U/mg of protein. (C) Effects of starch on thermal stability of β-amylase. Experiments were performed as described above for (B), except for the addition of the indicated amount of starch.

DISCUSSION

In general, these data established that *C. thermosulfurogenes* produces both cell-bound glucoamylase and extracellular β-amylase activities. The β-amylase is produced in high yield as a primary metabolite during growth on starch. As expected for enzymes produced by an extreme thermophile (25), the β-amylase possessed a high thermal stability. Moreover, if the β-amylase of *C. thermosulfurogenes* can be produced inexpensively, then it may be of industrial utility in brewing and starch processing because of its unique physicochemical properties.

It is of interest that active degradation of raw starch at 60°C by *C. thermosulfurogenes* is achieved by β-amylase and glucoamylase activities. Starch degradation mechanisms by mesophilic or thermophilic bacteria are generally associated with active α-amylase production (12). This is not the case for raw starch transformation by *C. thermosulfurogenes*. However, other obligate thermoanaerobes may show great diversity in the specific biochemical mechanisms employed for starch degradation. In this regard, we showed that *C. thermohydrosulfuricum* completely hydrolyzes starch, but as a consequence of producing a very active glucoamylase and pullulanase (19).

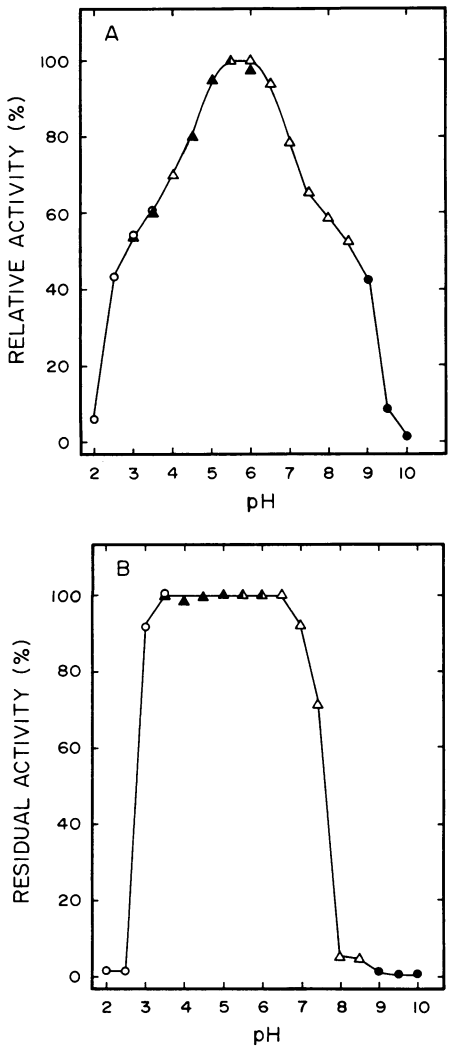

FIG. 5. Dependence of *C. thermosulfurogenes* β-amylase activity and stability on pH (100% activity corresponds to 24.4 U/mg of protein). (A) pH dependence of enzyme activity. Enzyme activities were assayed with 50 mM glycine hydrochloride (○), sodium acetate (▲), sodium phosphate (△), and glycine sodium buffer (●). (B) pH stability of the enzyme. The enzyme solutions (0.62 mg of protein per ml), in the same buffers as described above, were incubated at 60°C for 1 h, and the residual activities were assayed under the standard conditions.

TABLE 2. Effects of sulfhydryl reagents, metal ions, and Schardinger dextrins on β-amylase activity

Reagent added (concn [mM])	Percent activity remaining[a]
None	100
Hydrogen peroxide	
(1)	98
(5)	86
5,5′-Dithiobis-2-nitrobenzoic acid (0.2)	96
N-Ethylmaleimide	
(1)	93
(5)	54
Iodoacetate	
(1)	100
(5)	52
p-Chloromercuribenzoic acid	
(0.02)	0.2
0.1	0.0
$CuCl_2$ (1)	1.8
$HgCl_2$ (1)	0.0
$ZnCl_2$	
(1)	93
(10)	73
$CaCl_2$	
(5)	100
(30)	100
α-Cyclodextrin (10)	100
β-Cyclodextrin (10)	100

[a] 100% activity corresponds to 24.4 U/mg of protein under standard assay conditions.

The extracellular amylase was classified as having β-amylase activity based on previously established criteria (6, 7, 18, 24). The action of this activity on starch yielded only maltose as a detectable product besides high-molecular-weight limit dextrin, but not glucose or other low-molecular-weight maltosaccharides. Maltotetraose hydrolysis yielded only maltose, and maltotriose yielded glucose and maltose. The β-amylase was inactive against pullulan, sucrose, cellobiose, trehalose, Schardinger dextrins, and maltose. A large release of reducing power with relatively little reduction of the iodine-staining capacity on hydrolysis of amylose and the upward mutarotation of starch digest on the addition of alkali indicate that the enzyme acts in an exo-fashion and produces maltose with the β-anomeric configuration. In these respects, the extracellular amylase of *C. thermosulfurogenes* was similar in the action patterns of plant and other microbial β-amylases (6, 18).

To our knowledge, the β-amylase of *C. thermosulfurogenes* represents the first extremely thermostable β-amylase that has been reported. This enzyme displays a high maximum temperature (75°C) for enzyme activity and extreme thermostability (i.e., it is stable up to 80°C). Although attempts have been made to identify more active and thermostable microbial β-amylases, those produced by *Bacillus* species (5, 8, 21–24) and *Streptomyces* species (21) have optimal activity at lower than 50°C and are not more thermostable (<55°C) than plant β-amylases (18). Recently,

Obi and Odibo (17) have reported that *Thermoactinomyces* sp. no. 2 β-amylase was optimally active at 60°C and fairly stable at 60°C.

The β-amylase of *C. thermosulfurogenes* may have industrial application because of its novel features. The enzyme is extracellular, a primary metabolite, and active and stable at high temperatures. This β-amylase is also active and stable at a wide range of pHs. Because the enzyme has optimal activity (pH 5.5 to 6.0) and stability (pH 3.5 to 6.5) in the acidic range, it is distinguished from other β-amylases that display optimal activity and stability around neutral pHs (7, 17, 18). The β-amylase may have novel applications for the production of maltose syrups in conjunction with the pullulanase of *C. thermohydrosulfuricum* (9) because both enzymes are active at nearly the same pH and temperature range.

β-Amylases from higher plants and microorganisms are sulfhydryl enzymes and are inactivated by sulfhydryl reagents or oxidation (7, 17, 18). The present data indicate that *C. thermosulfurogenes* β-amylase is also inactivated by sulfhydryl reagents (i.e., *p*-chloromercuribenzoate and *N*-ethylmaleimide). However, this inhibition could be prevented and reversed by the addition of a reducing agent (dithiothreitol). Also, metal ions (e.g., Cu^{2+}, Hg^{2+} and Fe^{2+}) inhibited β-amylase. However, Schardinger dextrins, which notably are competitive inhibitors of other β-amylases (7, 18), did not inhibit the β-amylase of *C. thermosulfurogenes*.

β-Amylase activity and stability were little affected by ethanol, although growth of this species was very sensitive (i.e., no growth in the presence of 2% ethanol; unpublished data). The organism and its β-amylase may have potential for future industrial applications in ethanol production from starch as well as in the brewing of alcoholic beverages. Finally, the enhancement of β-amylase productivity through mutation of this organism or genetic recombination techniques will advance its potential for future industrial applications. An invention report covering these unique thermostable β-amylase has been forwarded to the U.S. Patent Office.

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