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Purification and Properties of a Thermoactive Glucoamylase from *Clostridium thermosaccharolyticum*

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A bacterial glucoamylase was purified from the anaerobic thermophilic bacterium *Clostridium thermosaccharolyticum* and characterized. The enzyme, which was purified 63-fold, with a yield of 36%, consisted of a single subunit with an apparent molecular mass of 75 kDa. The purified enzyme was able to attack α-1,4- and α-1,6-glycosidic linkages in various α-glucans, liberating glucose with a β-anomeric configuration. The purified glucoamylase, which was optimally active at 70°C and pH 5.0, attacked preferentially polysaccharides such as starch, glycogen, amylopectin, and maltodextrin. The velocity of oligosaccharide hydrolysis decreased with a decrease in the size of the substrate. The K_m values for starch and maltose were 18 mg/ml and 20 mM, respectively. Enzyme activity was not significantly influenced by Ca^{2+}, EDTA, or α- or β-cyclodextrins.

In the last few years, a variety of anaerobic and aerobic bacteria have been investigated for their ability to produce thermoactive starch-hydrolyzing enzymes. The majority of these microbes secrete thermostable amylases and pullulanases (2–5, 21–23). Detailed biochemical investigations have been performed with enzymes from anaerobic bacteria belonging to the genera *Clostridium* and *Thermoanaerobium* (32, 34, 38). Besides these enzymes, glucoamylases, which are used for the production of glucose syrup, are among the most important industrial enzymes. Unlike amylases and pullulanases, glucoamylases are rare in prokaryotes. They are rather typical in fungi and are also produced by yeasts. Most glucoamylases are thermolabile and are active in the acidic range (35, 43). A few aerobic bacteria, such as *Bacillus stearothermophilus*, a *Flavobacterium* sp., and *Halobacterium sodomense*, have been reported to produce glucoamylases (7, 30, 40). Data from some reports have demonstrated the presence of glucoamylases in anaerobes such as *Clostridium acetobutylicum* and *Clostridium thermohydrosulfuricum* (13, 14, 20, 21), although detailed studies of the characteristics of the enzymes have not been conducted. However, detailed characterization appears to be the prerequisite for definite identification of an enzyme.

This paper describes, in detail, the purification and characterization of a thermoactive glucoamylase from an anaerobic bacterium. This enzyme, from *Clostridium thermosaccharolyticum*, is able to degrade α-1,4- as well as α-1,6-glycosidic linkages in various oligo- and polysaccharides, the only product formed being β-D-glucose.

MATERIALS AND METHODS

Organism and growth conditions. *C. thermosaccharolyticum* DSM 572 was obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. Growth experiments were conducted in 15-ml Hungate tubes containing 10-ml cultures. The strain was cultivated under strictly anaerobic conditions at 60°C in a complex medium containing 1% (wt/vol) soluble starch as described previously (28). For the preparation of large amounts of the enzyme, bacteria were cultivated in a 200-liter stainless steel fermentor. The cell-free supernatant was concentrated up to 35-fold with a 20-kDa-cutoff membrane (Sartocon II cross-flow filter system; Sartorius GmbH, Göttingen, Germany).

Preparation of cell extracts for enzyme localization. From 300 ml of culture broth (stationary growth phase) containing 3 g of cells of *C. thermosaccharolyticum*, cells were removed by centrifugation (10,000 × *g*, 20 min). The total amount of glucoamylase activity in the resulting supernatant was measured. The pellet (3 g of cells) was washed twice (in 0.1 M sodium acetate buffer, 1.35 mM EDTA [pH 5.0]) to remove glucoamylase possibly attached to the outside of the cells. Glucoamylase activity in the washing fluid was not determined. The washed cells were suspended in 4.4 ml of buffer (same as that used above) and disrupted in a French press (American Instruments Company, Inc., Silver Spring, Md.) at 80 MPa. The resulting sample was centrifuged (80,000 × *g*, 1 h); glucoamylase activities in the resulting supernatant and in the resuspended pellet were determined.

Enzyme assays. Glucoamylase was measured in sodium acetate buffer (0.1 M, pH 5.0) containing 1.35 mM EDTA and 2% (wt/vol) maltose. After incubation of the mixture (0.5 ml) at 65°C, samples were removed at various times and the reaction was stopped by boiling for 4 min. The denatured proteins were removed by centrifugation at 10,000 × *g* for 10 min. Glucose liberated by enzymatic action was measured enzymatically (8). One unit of glucoamylase catalyzes the liberation of 1 μmol of glucose per min under the specified conditions. Amylase and pullulanase were assayed by measuring the reducing sugars formed during incubation with starch (1% [wt/vol]) and pullulan (0.5% [wt/vol]), respectively (9). One unit of pullulanase or amylase catalyzes the liberation of 1 μmol of reducing sugars per min at 70°C with maltose as a standard. Protein was determined by the method of Bradford (11).

Purification of glucoamylase. All purification steps were carried out at 4°C under aerobic conditions. The concentrated supernatant was centrifuged at 100,000 × *g* for 1 h; the pellet was discarded. To the resulting supernatant, polyethylene glycol 400 was added to a final concentration of 20% (vol/vol), and the mixture was stirred at 0°C for 30 min. After centrifugation at 38,000 × *g* for 20 min, the concentration of polyethylene glycol 400 was increased to 35% (vol/vol), and

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the mixture was stirred for 20 min at 0°C and centrifuged at 38,000 × *g* for 20 min. The supernatant was discarded, and the pellet was suspended in 20 mM morpholinopropanesulfonic acid (MOPS) buffer (pH 7.0) containing 2 mM dithiothreitol (DTT). The sample (29.5 ml) was applied to a Q-Sepharose Fast Flow column (3 by 10 cm; Pharmacia, Uppsala, Sweden) equilibrated with 20 mM MOPS buffer (pH 7.0)–2 mM DTT. Fractions containing glucoamylase activity were eluted from the column at a rate of 1 ml/min with a linear gradient (50 to 250 mM) of NaCl (300-ml total volume). The pooled fractions were applied in portions of about 17 ml in four separate runs to a hydroxyapatite column (3 by 1 cm) filled with 1 g of hydroxyapatite (DNA grade; Bio-Rad, Richmond, Calif.) and equilibrated with 10 mM MOPS buffer (pH 7.0)–2 mM DTT. Fractions containing glucoamylase activity were eluted at a rate of 0.3 ml/min with a linear gradient (0 to 0.6 M) of KPO_4 (pH 7.0) (50-ml total volume). Fractions with high glucoamylase activity were pooled (71 ml) and concentrated to 24 ml in a Centriprep concentrator with a cutoff of 30 kDa. The concentrated sample was applied in 3-ml portions in eight separate runs to a Superdex 200 column (3 by 70 cm; Pharmacia) equilibrated with degassed and filtered 50 mM MOPS buffer (pH 7.0)–0.1 M NaCl–2 mM DTT. The gel filtration column was run at a rate of 0.5 ml/min and monitored for A_{280}. The high-pressure liquid chromatography (HPLC) equipment (Kontron, Eching, Germany) consisted of two HPLC pumps, a detector, and a Multiport. HPLC was controlled by a Kontron AT-Computer.

Electrophoretic techniques. Electrophoresis was carried out in 1-mm-thick polyacrylamide slab gels with the Biometra Minigel system (Biometra, Göttingen, Germany). Analytical gradient gel electrophoresis (5 to 20% polyacrylamide) was performed with Tris-glycine buffer (1) at a constant voltage of 100 V for 16 h at 4°C. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 12.5% gels was carried out as described by Laemmli (24). Samples were prepared after boiling for 5 min in a solution containing 1.5% (wt/vol) SDS and 3% (vol/vol) mercaptoethanol. Electrophoresis was performed at 4°C for 1.5 h at 15 mA. After an electrophoretic run, the proteins in the gels were detected by silver staining as described by Blum et al. (10). Isoelectric focusing with carrier ampholytes (pH 3 to 10; Pharmacia) was performed by the method of Schickle and Westermeier (36). Electrophoresis was performed at 10°C for 1.5 h at 1,000 V and 15 W (prefocusing, 0.5 h). A Pharmacia standard (Broad pI Calibration Kit 3-10) was used to calibrate the pH gradient of the gel. A titration curve was determined with carrier ampholytes (same as those used above) as described by Righetti and Gianezza (33) and modified by Westermeier (45). Prefocusing was done for 1 h at 10°C, 1,000 V, and 7 W. Focusing took another 20 min at 10°C, 600 V, and 5 W. One-centimeter pieces of the gel were cut and placed in 0.5 ml of distilled water. After 3 h, the pH gradient was measured. For detection of proteins, Coomassie hot staining was carried out (36).

Estimation of the Stokes radius and sedimentation coefficient. The molecular mass of the glucoamylase was determined as described by Siegel and Monty (37). The Stokes radius of the glucoamylase was determined by gel filtration chromatography with a Superose 12 column (1 by 30 cm; Pharmacia) equilibrated with 50 mM MOPS buffer (pH 7.0)–0.1 M NaCl–2 mM DTT. Standard proteins with known Stokes radii were RNase A (1.64 nm), chymotrypsinogen A (2.09 nm), aldolase (4.8 nm), and catalase (5.2 nm). The sedimentation coefficient of the glucoamylase was determined by sucrose gradient centrifugation as described by Martin and Ames (29). Standard proteins (0.4 mg each) were run separately: catalase ($s_{20,w}$ = 11.3), aldolase ($s_{20,w}$ = 7.35), serum bovine albumin ($s_{20,w}$ = 4.31), and ovalbumin ($s_{20,w}$ = 3.55). Glucoamylase (40 μg of protein) and standard proteins were layered separately onto an 8.5-ml linear sucrose gradient (10 to 30% [wt/vol]) (27), and centrifugation was carried out with a TH 641 Sorvall rotor at 177,000 × *g* for 16 h at 4°C (Sorvall OTD 55 B ultracentrifuge; Du Pont Instruments, Bad Nauheim, Germany).

Sugar analysis. The hydrolysis products of various carbohydrates were determined by HPLC with an Aminex Carbohydrate HPX-42A column at an operating temperature of 85°C (Bio-Rad). Sugars were eluted with degassed and filtered distilled water at a flow rate of 0.6 ml/min. The reaction mixture, consisting of 1.5 ml of 0.1 M sodium acetate (pH 5.0), 0.5% (wt/vol) substrate, 1.35 mM EDTA, and 10 μl of enzyme (0.24 U), was incubated at 65°C for different times. The sample was mixed with the anion exchanger (Serdolit MB) and centrifuged for 5 min at 5,000 × *g*. Thin-layer chromatography was also used to analyze the action of glucoamylase at a high maltose concentration (15% [wt/vol]) (18). Glycostaining was carried out with a glycan detection kit from Boehringer GmbH, Mannheim, Germany. The tested proteins were transferrin (4 μg), bovine serum albumin (4 μg), and glucoamylase (4 μg).

Optical rotation study. To study optical rotation, we used 0.1 M sodium acetate buffer (pH 5.0). In addition to the enzyme, 1% starch and 1.35 mM EDTA were added. The optical rotation was measured at 25°C in a Perkin-Elmer polarimeter (model 214) with the sodium line. When the optical rotation became constant, about 4.8 mg of sodium carbonate was added and the mutarotation of the solution was measured. The reference enzymes used were amylase from *Bacillus subtilis* (1.35 mM EDTA was replaced by 10 mM $CaCl_2$) and glucoamylase from *Aspergillus niger*.

Enzymes and chemicals. Pullulan from *Aureobasidium pullulans*, maltose, amylopectin, and glycogen were purchased from Sigma Chemical Co., St. Louis, Mo. Soluble starch was obtained from Fluka, Neu-Ulm, Germany. Biochemicals and enzymes were purchased from Boehringer. Chemicals for gel electrophoresis, polyethylene glycol 400, DTT, amylose, α- and β-cyclodextrins, and oligosaccharides (degree of polymerization, 2 to 7) were obtained from Serva, Heidelberg, Germany. All other chemicals were purchased from Merck, Darmstadt, Germany.

RESULTS

Growth experiments. As shown in Fig. 1, *C. thermosaccharolyticum* grew well on starch and reached the stationary growth phase after 10 h of cultivation at 60°C. At this stage, more than 80% of the starch was degraded. Growth was paralleled by the production of α-amylase and pullulanase; the activities of these enzymes amounted to totals of 800 and 350 U/liter after 10 h of cultivation, respectively. Glucoamylase activity could only be detected during the stationary growth phase, i.e., after 20 h of cultivation. Fifty percent of this enzyme activity was detected in the culture fluid. Cultivation of bacteria in continuous cultures was not paralleled by an increase in glucoamylase activity. This phenomenon, however, has not been observed for the production of α-amylase and pullulanase (4, 5).

Enzyme localization. The total glucoamylase activity in 300 ml of culture broth, after removal of the cells by centrifugation, was found to be 8.2 U. The glucoamylase activity

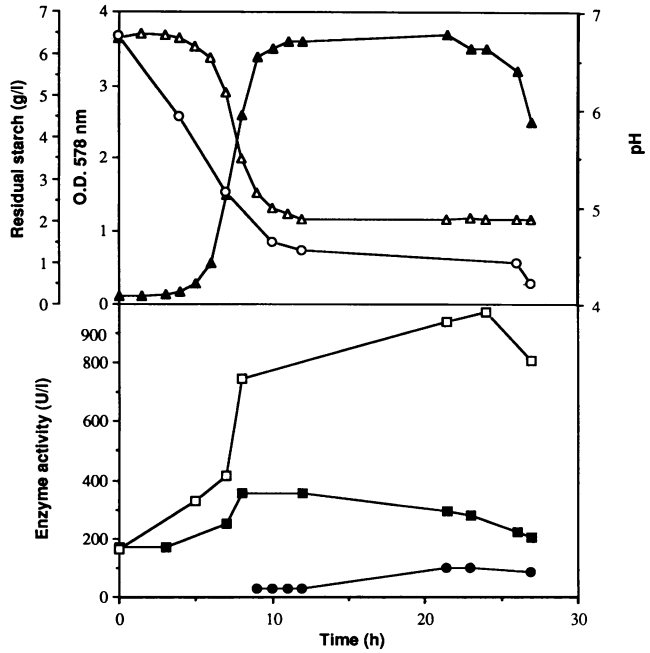

FIG. 1. Production of starch-degrading enzymes during growth of *C. thermosaccharolyticum* on 1% (wt/vol) starch at 60°C under anaerobic conditions. Samples (0.5 ml) were removed at various times, and the following parameters were measured: □, α-amylase activity; ■, pullulanase activity; ●, glucoamylase activity; ▲, optical density (O.D.) at 578 nm; △, pH; ○, residual starch. The amount of enzyme represents the sum of enzyme activities present on the cell surface and in the culture fluid.

measured in the supernatant obtained after disruption of 3 g of cells and ultracentrifugation of the sample was 2.4 U; the resulting pellet contained 7.4 U of glucoamylase activity. This means that 3 g of washed cells contained 9.8 U of glucoamylase activity, i.e., an amount similar to the total glucoamylase activity determined in culture broth devoid of bacteria, and indicates that about 50% of the glucoamylase activity present in a 300-ml culture containing 3 g of cells is extracellular.

Enzyme purification. After growth of *C. thermosaccharolyticum* on starch, the culture supernatant (200 liters) was concentrated to 5.7 liters; 1.1 liters (Table 1) was subjected to ultracentrifugation to remove precipitated compounds. By polyethylene glycol 400 precipitation, the specific activity of

TABLE 1. Purification of glucoamylase from *C. thermosaccharolyticum*[a]

Fraction	Vol (ml)	Activity (U)	Protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Supernatant	1,100	1,567	1,793	0.87	1.0	100
Ultra-centrifugation	1,000	1,500	651	2.3	2.6	96
Polyethylene glycol 400 precipitation	29.5	830	180	4.6	5.3	53
Q-Sepharose	70	761	41.3	18.4	21.1	49
Hydroxyapatite	71	881	38.6	22.8	26.2	56
Superdex 200	80	560	10.3	54.4	62.5	36

[a] After growth of the bacteria in 200-liter cultures for 24 h, the culture supernatant was concentrated to 5.7 liters; 1.1 liters was used for enzyme purification.

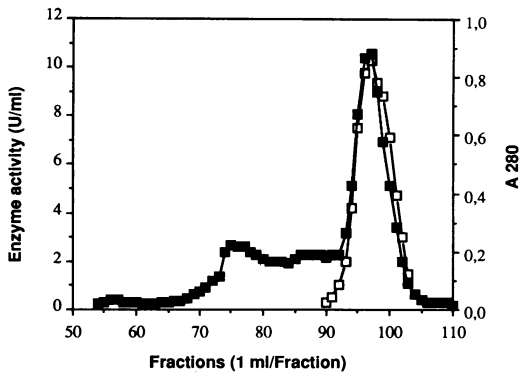

FIG. 2. Last step of purification of the glucoamylase from *C. thermosaccharolyticum* (gel filtration). For each run, 3 ml (37 U of glucoamylase) was applied to a Superdex 200 column (3 by 70 cm) and the enzyme was eluted at 0.5 ml/min with 50 mM MOPS buffer (pH 7.0)–0.1 M NaCl–2 mM DTT. Symbols: □, glucoamylase activity; ■, A_{280}.

glucoamylase in the pellet was raised from 2.3 to 4.6 U/mg. After this step, approximately 80% of pullulanase activity and 50% of α-amylase activity were separated. The dissolved pellet containing glucoamylase was subjected to anion-exchange chromatography. Glucoamylase was eluted from the column with 0.14 to 0.18 M NaCl. By this step, the enzyme was purified 21-fold and had a specific activity of 18.4 U/mg. Further purification was achieved by applying the pooled fractions to a hydroxyapatite column. Glucoamylase was eluted with 0.07 to 0.26 M potassium phosphate buffer (pH 7.0). This partially purified enzyme was free of α-amylase and pullulanase activities. Final purification of glucoamylase was achieved after gel permeation chromatography on a Superdex 200 column (Fig. 2). The purified enzyme had a specific activity of 54 U/mg and was purified 63-fold, with a yield of 36%. Table 1 summarizes the purification procedure.

Homogeneity, size, and isoelectric point of the enzyme. Upon subjection of glucoamylase to gel filtration chromatography on Superose 12 (Pharmacia FPLC-System), a single protein peak with enzymatic activity was eluted. Electrophoretic analysis of the purified enzyme by SDS-PAGE and silver staining revealed a single protein band (Fig. 3). Even overloading of the SDS-polyacrylamide gel with 30 μg of purified enzyme was not accompanied by the appearance of impurities.

By using gel filtration chromatography, sucrose gradient centrifugation, and SDS-PAGE, we calculated the apparent molecular mass of the glucoamylase to be 75 kDa. The enzyme was composed of a single subunit with a Stokes radius of 3.35 nm and a sedimentation coefficient of 5.45S. When the purified enzyme was analyzed by isoelectric focusing, at least five protein bands were observed. In a titration experiment, five protein lines were observed running parallel to each other, indicating that the purified protein was heterogeneous with regard to isoelectric point (Fig. 4). The points of intersection between the protein curves and a line drawn through the application point yield the isoelectric points of the protein lines. With the help of a calibration curve, the range of isoelectric points of the purified glucoamylase was determined to be 4.9 to 5.6. Using the glycan detection kit, we showed that glucoamylase was not glycosylated.

Effect of pH and temperature. The purified glucoamylase

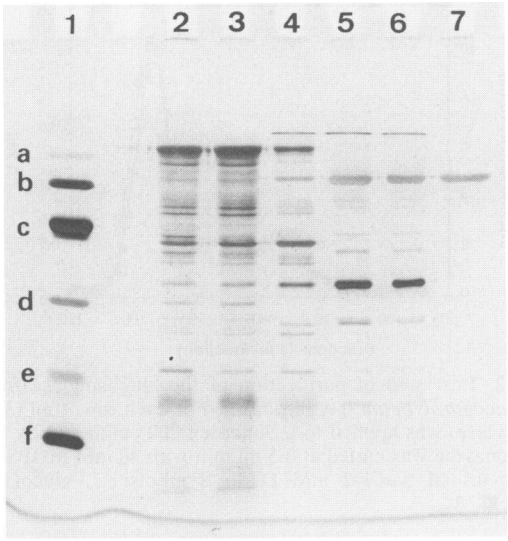

FIG. 3. SDS-PAGE of samples from different purification steps. Proteins (1 to 5 μg per lane) were detected by silver staining. Lanes: 1, standard proteins (a, phosphorylase *b*; b, albumin; c, ovalbumin; d, carbonic anhydrase; e, trypsin inhibitor; f, α-lactalbumin); 2, supernatant; 3, after ultracentrifugation; 4, after polyethylene glycol 400 precipitation; 5, after Q-Sepharose; 6, after hydroxyapatite; 7, after Superdex 200.

was active between pH 3.5 and pH 6.5 and showed optimal activity at pH 5.0. Glucoamylase activity was measurable between 30 and 80°C; optimal activity was found at 70°C. As shown in Fig. 5, enzyme activity decreased rapidly above

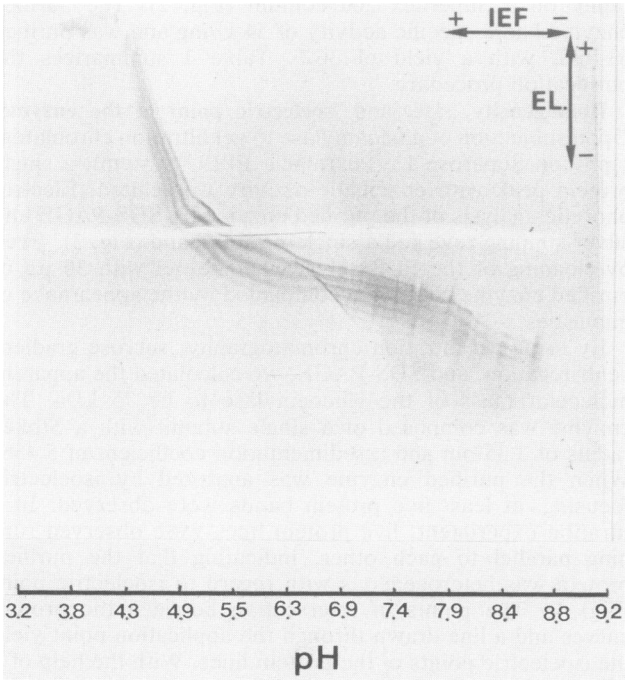

FIG. 4. Titration curves of glucoamylase from *C. thermosaccharolyticum*. The trench was filled with 50 μl (32 μg of protein). The arrows and the plus and minus symbols represent the direction and polarity of isoelectric focusing (IEF) and electrophoresis (EL.), respectively.

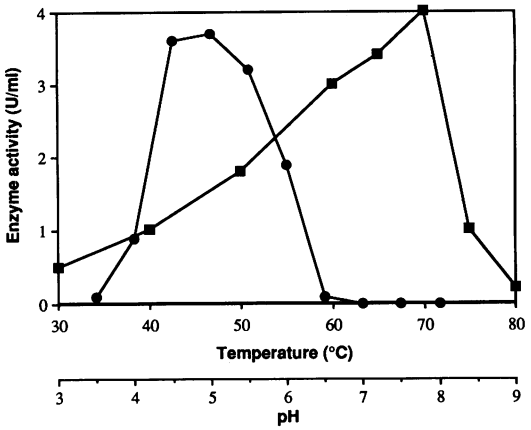

FIG. 5. Effect of temperature (■) and pH (●) on the glucoamylase activity of *C. thermosaccharolyticum*. Determination of the pH optimum was performed with a sample of purified enzyme in sodium acetate buffer (0.1 M)–1.35 mM EDTA–2% (wt/vol) maltose. For determination of the temperature optimum, the pH was adjusted to 5.0.

70°C. To investigate the effect of temperature on the stability of the enzyme, we incubated purified glucoamylase at 75°C under aerobic conditions at pH 5.0. After 1 h of incubation, only about 8% of the initial activity was detected. At 70°C, however, more than 90% of the enzyme activity was still present after 6 h of incubation. The thermal stability of glucoamylase was greatly enhanced in the presence of starch: In the presence of 3% (wt/vol) starch, about 80% of the enzyme activity was measurable after 1 h of incubation at 75°C.

Influence of metal ions and α- and β-cyclodextrins. Various metal ions, such as Zn^{2+}, Cu^{2+}, and Pb^{2+}, caused inhibition of the activity of the purified glucoamylase. As with other amylolytic enzymes from anaerobic bacteria, no metal ions were found to be required for enzymatic activity. Ca^{2+} and EDTA did not have an inhibitory effect up to concentrations of 4 and 10 mM, respectively. For α-cyclodextrins, no inhibition at concentrations up to 10 mM could be found; β-cyclodextrins showed slight inhibition at concentrations above 5 mM.

Substrate specificity. Analysis of the hydrolysis products of various α-glycans with HPLC clearly demonstrated that α-1,4- as well as α-1,6-glycosidic linkages were hydrolyzed by the purified enzyme. As shown in Table 2, various polysaccharides, such as starch, amylopectin, and amylose, were hydrolyzed rapidly, forming glucose as the sole product. Glycogen, a substrate more highly branched than the others, was also hydrolyzed, but at a slower rate (Table 2, data after 30 min of incubation). Pullulan, which is made up of maltotriose units joined by α-1,6-glycosidic linkages, was hardly attacked by the enzyme. After incubation for 4 h, only 20% glucose was formed. The enzyme was very active against oligosaccharides, as shown in Table 2. A clear correlation existed between the reaction velocity and the size of the substrate. After 10 min of incubation, the oligosaccharides maltotriose to maltoheptaose were completely hydrolyzed, whereas 87% of maltose was not attacked. The purified glucoamylase preferentially attacked α-1,4-glycosidic linkages rather than α-1,6-glycosidic linkages. This result was obvious when the rates of reaction with maltose and isomaltose were compared (Table 2). Incubation experiments with amylose (0% α-1,6-glycosidic linkages),

TABLE 2. Hydrolysis of different substrates by the action of the purified glucoamylase of *C. thermosaccharolyticum*[a]

Substrate	Degree of polymerization	% Hydrolysis at the following time (min): 0	1	5	10	30	60	120	180	240	300
Maltose	2	100	96	93	87	69	47	6			
	1	0	4	7	13	31	53	94			
Maltotriose	3	100	52	27	0	0	0	0			
	2	0	28	41	60	42	22	14			
	1	0	20	32	40	58	78	86			
Maltotetraose	4	100	61	9	0	0	0	0			
	3	0	27	33	10	0	0	0			
	2	0	1	19	37	36	22	10			
	1	0	10	38	53	64	78	90			
Maltopentaose	5	100	47	7	0	0	0	0			
	4	0	29	16	2	0	0	0			
	3	0	10	25	14	0	0	0			
	2	0	1	11	28	25	17	8			
	1	0	13	41	56	75	83	92			
Maltoheptaose	7	100	10	9	0	0	0	0			
	6	0	21	17	0	0	0	0			
	5	0	15	16	0	0	0	0			
	4	0	11	11	3	0	0	0			
	3	0	9	8	11	0	0	0			
	2	0	7	0	13	19	10	8			
	1	0	27	40	73	81	90	92			
Starch	n	100	86	74	64	36	23	15	11		5
	1	0	14	26	36	64	77	85	89		95
Amylose	n	100	98	88	71	44	39	30	0		
	1	0	2	12	29	55	61	70	100		
Maltodextrin	n	59	36	24	25	21	13	11	9		
	7	11	0	0	0	0	0	0	0		
	6	11	1	0	0	0	0	0	0		
	5	4	2	0	0	0	0	0	0		
	4	4	3	0	0	0	0	0	0		
	3	8	10	5	0	0	0	0	0		
	2	0	21	23	20	13	0	0	0		
	1	2	27	47	54	66	77	89	91		
Isomaltose	2	100	100	100	100	88	81	58		30	6
	1	0	0	0	0	12	19	42		70	94
Amylopectin	n	100	98	89	79	60	42	30	26	24	
	1	0	2	11	21	40	58	70	74	76	
Glycogen	n	100	100	83	71	68	55	41		29	21
	1	0	0	17	29	32	45	59		71	79
Pullulan	n	100	100	100	100	100	100	96		81	
	1	0	0	0	0	0	0	4		19	

[a] Incubation was performed with 0.1 M sodium acetate buffer (pH 5.0) containing 0.5% (wt/vol) substrate, 1.35 mM EDTA, and 0.24 U of purified enzyme. After incubation at 65°C, samples were removed and mixed with the anion exchanger (Serdolit MB) and the products were analyzed by HPLC. n, degree of polymerization above 7.

amylopectin (5% α-1,6-glycosidic linkages), glycogen (about 12% α-1,6-glycosidic linkages), and pullulan (about 30% α-1,6-glycosidic linkages) also showed that an increase in the proportion of α-1,6-glycosidic linkages was correlated with a decrease in the reaction rates. As shown in Table 2, the concentrations of glucose after 30 min of incubation with amylose, amylopectin, glycogen, and pullulan were 55, 40, 32, and 0%, respectively.

Mutarotation studies with the purified enzyme (Fig. 6) clearly showed an apparent shift of the optical rotation after base addition. It is hence evident that the action of this

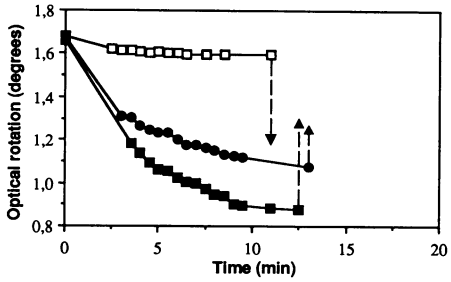

FIG. 6. Optical rotation study of glucoamylase from *C. thermosaccharolyticum* (●), α-amylase from *B. subtilis* (□), and glucoamylase from *A. niger* (■) in 1% (wt/vol) starch solution. The broken arrows indicate the amount of mutarotation in degrees after the addition of sodium carbonate.

enzyme liberated sugars that have a β-anomeric configuration. For these studies, α-amylase from *B. subtilis* and glucoamylase from *A. niger* were used as controls.

Further investigations were undertaken to detect a possibly existing reverse reaction catalyzed by the purified enzyme under the conditions used. Incubation of glucoamylase with 15% (wt/vol) maltose did not cause a measurable formation of reversion products, such as isomaltose, panose, or other branched oligosaccharides (data not shown). Elevation of the maltose concentration up to 30% (wt/vol), however, caused the formation of isomaltose.

Determination of K_m values showed that in the presence of starch and maltose, the purified glucoamylase displayed Michaelis-Menten kinetics. Data obtained from Lineweaver-Burk plots yielded K_m values of 20 mM for maltose and 18 mg/ml for starch (data not shown).

DISCUSSION

Analysis of the polysaccharide-degrading enzyme system of *C. thermosaccharolyticum* demonstrated that starch degradation by this microbe is carried out by the combined action of three different extracellular enzymes, namely, α-amylase, pullulanase, and glucoamylase. This degradation leads to a rapid and efficient conversion of highly branched polysaccharides into glucose, which can be easily fermented by this organism. To our knowledge, glucoamylase from *C. thermosaccharolyticum* is the first bacterial glucoamylase which has been purified and studied in detail. The presence of glucoamylase activity in the bacteria *Flavobacterium* sp., *C. acetobutylicum*, *C. thermosaccharolyticum* 39 E, and *H. sodomense* has been suggested. The *Flavobacterium* sp., however, is unable to grow on starch or amylacious compounds; it seems to produce cyclodextrin-degrading enzymes (7). The enzyme of *C. acetobutylicum* was first described as maltase (20) and later as glucoamylase (13, 14). The formation of glucose by the enzyme systems of *C. acetobutylicum*, *C. thermosaccharolyticum* 39 E, and *H. sodomense* (21, 30) is probably due to the action of more than one enzyme (maltase and α-amylase or α-amylase and pullulanase). Such a situation has already been described for the enzyme systems of various anaerobic bacteria (2, 4, 5, 23, 44). To obtain conclusive evidence, we must conduct further biochemical investigations with these microbes.

SDS-PAGE and silver staining demonstrated that the glucoamylase was purified to homogeneity. During separation along a pH gradient (isoelectric focusing), the same enzyme fraction showed multiple bands, most probably

isoenzymes. Up to now, we have not been successful in carrying out glucoamylase activity staining of the multiple bands after separation in a titration curve. It has been often documented that glucoamylases produced by fungi and yeasts, especially an *Aspergillus* sp., occur in multiple forms (25, 35, 41). There are several possible explanations for this multiplicity of bands of glucoamylases. They were reported to be caused by medium composition and culture conditions (6, 26). Some authors assumed that the purification procedures were responsible for the occurrence of multiple forms of glucoamylases (16). Pazur et al. (31) hypothesized that the two isoenzymes of *A. niger* may have identical protein components but that differences in the carbohydrate moieties (the enzymes are glycoproteins) are responsible for the observed differences in the sizes of these proteins. Hayashida and Yoshino (19) reported that multiple forms of glucoamylases were observed when native glucoamylase I (from *Aspergillus awamori* var. *kawachi*) was degraded stepwise with fungal acid protease and α-mannosidase. Polysaccharide-degrading cell-free cellulases of *Trichoderma reesei* exhibited heterogeneity when separated under conditions of isoelectric focusing (39). Posttranslational modification or phosphorylation (12, 15) could also be an explanation for the occurrence of multiple forms of the enzymes. In the case of glucoamylase from *C. thermosaccharolyticum*, variations in glycosylation as an explanation for the existence of isoforms can be excluded, as the enzyme is not a glycoprotein. Other possible explanations for the occurrence of multiple forms, e.g., desamidation, have not yet been investigated for this enzyme.

The purified glucoamylase had a temperature optimum of 70°C and a pH optimum of pH 5.0. The pH optimum was in agreement with those of other glucoamylases (pH 4.0 to 6.0). The temperature optimum of 70°C was higher than those of already-known glucoamylases. The specific activity of the enzyme was 54 U/mg, a value very similar to the specific activity of the glucoamylase from *Aspergillus saitoi* (32.6 U/mg) (42) but considerably lower than that of the enzyme from *A. niger* (523.5 U/mg) (17). In conclusion, the following properties make the glucoamylase from *C. thermosaccharolyticum* suitable for application in the starch industry: secretion of the enzyme into the medium, enzyme activity at a high temperature and a low pH, no requirement for metal ions, no inhibitory effect of cyclodextrins, and only a low level of reversion products at a high concentration of maltose.

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