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Purification and Characterization of the Extracellular α-Amylase from *Clostridium acetobutylicum* ATCC 824

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The extracellular α-amylase (1,4-α-D-glucanglucanohydrolase; EC 3.2.1.1) from *Clostridium acetobutylicum* ATCC 824 was purified to homogeneity by anion-exchange chromatography (Mono Q) and gel filtration (Superose 12). The enzyme had an isoelectric point of 4.7 and a molecular weight of 84,000, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was a monomeric protein, the 19-amino-acid N terminus of which displayed 42% homology with the *Bacillus subtilis* saccharifying α-amylase. The amino acid composition of the enzyme showed a high number of acidic and hydrophobic residues and only one cysteine residue per mole. The activity of the α-amylase was not stimulated by calcium ions (or other metal ions) or inhibited by EDTA, although the enzyme contained seven calcium atoms per molecule. α-Amylase activity on soluble starch was optimal at pH 5.6 and 45°C. The α-amylase was stable at an acidic pH but very sensitive to thermal inactivation. It hydrolyzed soluble starch, with a K_m of 3.6 g · liter^{-1} and a K_{cat} of 122 mol of reducing sugars · s^{-1} · mol^{-1}. The α-amylase showed greater activity with high-molecular-weight substrates than with low-molecular-weight maltooligosaccharides, hydrolyzed glycogen and pullulan slowly, but did not hydrolyze dextran or cyclodextrins. The major end products of maltohexaose degradation were glucose, maltose, and maltotriose; maltotetraose and maltopentaose were formed as intermediate products. Twenty seven percent of the glucoamylase activity generally detected in the culture supernatant of *C. acetobutylicum* can be attributed to the α-amylase.

The anaerobic bacterium *Clostridium acetobutylicum* produces acetone, *n*-butanol, and ethanol from various carbohydrate substrates, including polysaccharides. Many studies concerning the purification and the characterization of its polysaccharide-hydrolyzing enzymes, i.e., inulinase (10, 23), cellulase (1, 19), and xylanase (18, 20, 21), have been published. Surprisingly, only a few reports are available on the amylases of *C. acetobutylicum*, although starch was the first industrial substrate used for the production of butanol and acetone (31). This microorganism produces both an α-amylase (14, 33) and a glucoamylase, originally referred to as a maltase (13, 14). These two enzymes can be partially adsorbed to cells (12), and their synthesis is generally induced by either starch or a product of starch degradation and repressed by glucose (6, 14).

The objective of this work was to purify to homogeneity the extracellular α-amylase from *C. acetobutylicum* ATCC 824 and to characterize its main biochemical properties.

MATERIALS AND METHODS

Organism and enzyme preparation. *C. acetobutylicum* ATCC 824 was obtained from the American Type Culture Collection (Rockville, Md.). For enzyme preparation, the strain was grown at 35°C, pH 4.8, and 200 rpm in a 2-liter SGI (Toulouse, France) fermentor with the synthetic medium previously described (36). In some experiments, glucose (50 g · liter^{-1}) was replaced with soluble starch (Prolabo, Paris, France) at the same concentration. Strict anaerobic culture techniques were used for medium preparation and cultivation. The fermentor was inoculated with a 1/45 volume of an early-log-phase culture of *C. acetobutylicum* ATCC 824 previously subcultured in the same medium. After 15 h of batch culturing, the fermentation broth was collected and centrifuged at 9,000 × *g* for 15 min at 4°C.

Enzyme assay. The routine enzyme assay used for α-amylase activity involved measuring the reducing sugars resulting from the enzymatic hydrolysis of soluble starch. A 0.15-ml enzyme sample was mixed with an equal volume of 2% soluble starch in 0.1 M sodium acetate buffer (pH 5.6), and the mixture was incubated at 30°C for 10 min. The amount of reducing sugars produced was determined by the dinitrosalicylic acid method (4), with maltose as the standard. One unit of amylase activity was defined as the amount of enzyme which liberated 1 μmol of reducing sugars, with maltose as the standard, per min under the specified conditions. Several other substrates were tested under the same conditions for relative amylase activity. For maltose, the glucose liberated was measured by the glucose oxidase-peroxidase method (3).

Protein concentration was estimated by the method of Lowry et al. (24) with bovine serum albumin as the standard.

Purification of the amylase. (i) Ultrafiltration. The supernatant fluid was concentrated to 50 ml, dialyzed against 10 liters of acetate buffer (20 mM, pH 5.4) with an ultrafiltration device (H1 P10, 10,000-Da cutoff; Amicon), and filtered through a 0.2-μm-pore-size membrane (Sartorius). Thirty two milliliters was recovered.

(ii) Column chromatography. Crude enzyme solution (8 ml, 108 U) was applied to a Mono Q column (column size, 1 by 10 cm) filled with a strong anion exchanger (Pharmacia, Uppsala, Sweden) previously equilibrated with sodium acetate buffer (20 mM, pH 5.4). Elution was done at a flow rate of 8 ml · min^{-1} with a linear NaCl gradient (0 to 0.30 M over 30 min). Fractions (8 ml) were collected, and their α-amylase activity was determined as described below. The amylase-positive fractions from the ion-exchange chromatography were concentrated by ultrafiltration through a Centriprep-10

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concentrator (10,000-Da cutoff; Amicon) to a final volume of 200 μl and applied to a Superose 12 gel (column size, 1 by 20 cm; Pharmacia). A bed volume of 100 mM sodium acetate buffer (pH 5.0) was used as an eluant at a flow rate of 0.3 ml · min^{-1}. Fractions (0.5 ml) were collected and tested for α-amylase activity.

Electrophoresis and molecular mass determination. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed in a Phast Gel gradient (10 to 15%) with the Pharmacia Phast system. Samples were previously heated at 100°C for 5 min in 2.5% SDS–5% 2-mercaptoethanol. Protein bands were stained with silver nitrate. Calibration proteins ranging in molecular weight from 14,400 to 94,000 (low-molecular-weight calibration kit; Pharmacia) were used for the estimation of the molecular weight of the purified α-amylase.

Isoelectric point determination. Analytical isoelectric focusing-PAGE was performed in a Phast Gel IEF 3-9 with the Pharmacia Phast system. Protein bands were stained with silver nitrate. The isoelectric point of the α-amylase was determined with broad-range standard protein markers (Pharmacia) ranging from pI 3 to 10.

Amino acid analysis. The amino acid composition of the purified enzyme was determined after hydrolysis of the enzyme with 6 N HCl at 155°C for 45 min by use of an Applied Biosystems automatic amino acid analyzer. Cysteine and cystine were determined as cysteic acid after oxidation with performic acid (26).

NH_2-terminal amino acid sequence. The NH_2-terminal amino acid sequence of the purified amylase was determined by stepwise Edman (9) degradation with a gas-phase sequencer (Applied Biosystems model 470A) and high-pressure liquid chromatography identification of the phenylthiohydantoin amino acids obtained from the sequencer (Applied Biosystems model 120A).

Measurement of optical rotation. The optical rotation of a hydrolysate of soluble starch produced by the purified amylase was determined by the method of Hyun and Zeikus (16). A reaction mixture consisting of 1% soluble starch in 0.1 M sodium acetate buffer (pH 5.6) and enzyme was incubated at room temperature. The optical rotation of the mixture was measured at various times in a Perkin Elmer model 241 polarimeter by use of the sodium line. The mutarotation of the hydrolysate was determined by adding about 5 mg of solid sodium carbonate per ml of mixture after the optical rotation had became approximately constant.

Effects of pH. The relative α-amylase activity was determined at several pHs (2.2 to 8.0) with citrate (50 mM) and acetate-phosphate (50 mM) buffers. The same buffers were used to determine pH stability. One volume of α-amylase dialyzed against water was diluted in an equal volume of 50 mM buffer, and the mixture was incubated for 1 h at 35°C. The samples were cooled on ice, and the residual activities were determined after threefold dilution of the samples in 100 mM acetate buffer (pH 5.6).

Effects of temperature on stability. Enzyme samples were incubated in acetate buffer (pH 5.6) at several temperatures between 30 and 60°C. They were cooled on ice after 1 h, and the residual activities were determined.

Effects of metal ions and other chemicals. Enzyme samples were incubated with 1 mM metal ions or 10 mM other reagents. Relative activities were measured by the dinitrosalicylic acid method. The activity of the enzyme assayed in acetate buffer alone was taken to be 100%. Metals were used as chloride salts, except for Ag^+ (nitrate), Cu^{2+} (sulfate), and Pb^{2+} (acetate). Standard curves for the dinitrosalicylic acid response of maltose were determined in the presence of each metal and each reagent.

Analysis of calcium. The calcium bound to the α-amylase was determined by atomic absorption at 422.7 nm with a Perkin Elmer 3030 spectrophotometer after dialysis of the purified enzyme against water of MilliQ quality (Millipore) for 24 h.

Kinetic determinations. The initial reaction rates of starch hydrolysis were measured by the dinitrosalicylic acid method at several starch concentrations (1.0, 2.5, 5.0, 7.5, 10.0, and 15.0 g · $liter^{-1}$). The reaction mixture contained 0.28 mg of protein extract per ml. The Michaelis constant (K_m) and the reaction rate at an infinite substrate concentration (V_{max}) were determined by the method described by Lineweaver and Burk (22). The turnover number (K_{cat}) of α-amylase on soluble starch was determined by the method described by Englard and Singer (11).

Analysis of hydrolysis products. The purified α-amylase was incubated at 30°C with maltohexaose (final concentration, 10 g · $liter^{-1}$) in 0.1 M acetate buffer (pH 5.6). Samples were removed at different times, and hydrolysis was stopped by heating the samples at 100°C for 3 min. Hydrolysis products were analyzed by high-pressure liquid chromatography on a μBONDAPAK C18 column (Waters) maintained at 50°C with water as an eluant at a flow rate of 1 ml · min^{-1}. The glucose present in the samples was determined as described above for the enzyme assay.

Chemicals and reagents. Soluble starch and glucose were obtained from Prolabo, Paris, France; amylose (150,000 Da), glycogen, and pullulan (50,000 Da) were obtained from Serva (Heidelberg, Federal Republic of Germany); maltose was obtained from Merck (Darmstadt, Federal Republic of Germany); maltooligosaccharides with a degree of polymerization of 3 to 7, dextran (70,300 Da), amylopectin, Schardinger dextrins, bovine serum albumin, glucose oxidase (type V-S), peroxidase, and *Bacillus* sp. α-amylase (type II-A) were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Enzyme production and purification. When *C. acetobutylicum* ATCC 824 was grown with glucose as the sole carbon source, amylase production began at the end of the exponential phase of growth at a glucose concentration of 35 g · $liter^{-1}$ to reach a maximum value at the beginning of the stationary phase of growth. The final concentration of amylase was similar to that in soluble starch-grown cultures: 1 U · ml^{-1} for glucose and 1.5 U · ml^{-1} for starch. Preliminary assays during purification of the amylase produced during growth on starch were made difficult by the adsorption of the enzyme onto the dextrins liberated during starch hydrolysis. As the concentration and the specific activity of the amylase produced on glucose were similar to those produced on starch, glucose was used as a substrate for the production of the amylase.

The crude enzyme sample obtained after concentration and dialysis against acetate buffer contained 13.6 U of amylase per ml. The amylase was first purified on an anion-exchange chromatography Mono Q column. The elution pattern (Fig. 1) showed a major peak of amylase activity. After gel permeation chromatography (Fig. 2), the amylase was purified to homogeneity, as seen by SDS-PAGE analysis (Fig. 3). A summary of the purification is given in Table 1. These procedures yielded a pure amylase with a specific activity of 57.7 U · mg of $protein^{-1}$, a purification factor of 22.2, and a reasonable yield of 23%.

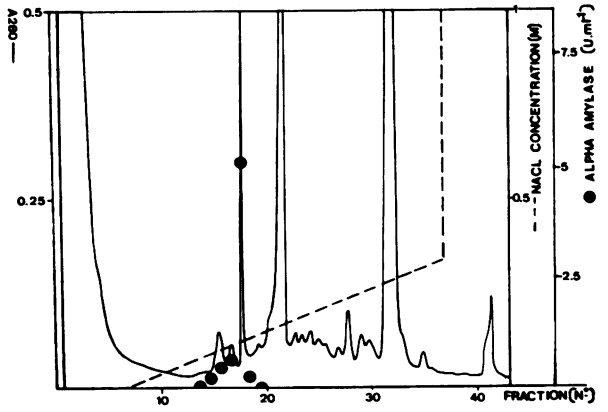

FIG. 1. Chromatography of the crude enzyme sample on an anion-exchange column. Experimental conditions are given in Materials and Methods. Symbols: —, A_{280}; ●, α-amylase activity; ----, NaCl gradient.

Molecular properties. Important differences were observed between the molecular weights determined by SDS-PAGE (84,000) and gel permeation chromatography (50,000). The molecular weight was significantly lower when estimated by gel filtration on a Superose 12 gel. However, when urea (6 M) was used, gel permeation chromatography yielded nearly the same value (83,000) as did SDS-PAGE. Furthermore, these results indicate that the amylase is a monomeric protein (Fig. 3).

The isoelectric point of this enzyme was found to be 4.7 by PAGE-isoelectric focusing.

An amino acid composition analysis of the amylase revealed that the enzyme was rich in both acidic and hydrophobic amino acids. Like the *Bacillus subtilis* saccharifying α-amylase, the *C. acetobutylicum* amylase contained a relatively large number of hydroxyamino acid residues and only one cysteine per mole (Table 2). This enzyme differed from the *Bacillus licheniformis* liquefying α-amylase in these last two respects.

A comparison of the NH_2-terminal amino acid sequence of the *C. acetobutylicum* amylase with that of the *B. subtilis* saccharifying α-amylase revealed 42% homology (Fig. 4). This homology only appeared after the 7th amino acid.

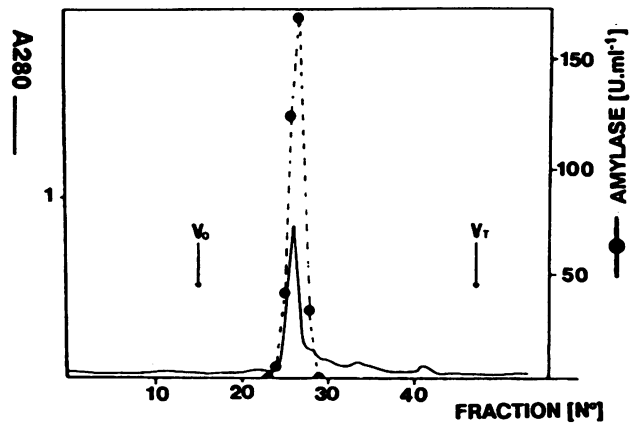

FIG. 2. Purification of the amylase from anion-exchange chromatography by Superose 12 gel filtration. Experimental conditions are given in Materials and Methods. Symbols: —, A_{280}; ●, α-amylase activity. V_O, Dead volume; V_T, total volume.

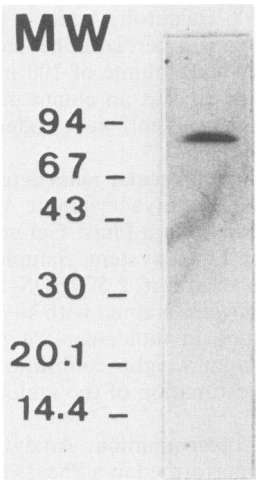

FIG. 3. SDS-PAGE of the amylase and determination of molecular weight. SDS-PAGE was carried out as described in Materials and Methods. The positions of molecular weight markers (MW, in thousands) are indicated.

Optical rotation. The optical rotation of the hydrolysate was measured during hydrolysis of soluble starch. From the mutarotation study with *Bacillus amyloliquefaciens* α-amylase as the control, the downward shift of optical rotation on the addition of sodium carbonate to the starch hydrolysate indicated that the hydrolysis products had an α-anomeric configuration.

Physicochemical properties. The α-amylase displayed optimal activity at pH 5.6 (Fig. 5A) and showed broad pH stability over the range from 3.0 to 5.5 (Fig. 5B).

The α-amylase showed optimal activity at 45°C (Fig. 6A). The Arrhenius law was found to be followed between 25 and 40°C, and an activation energy of 26.3 kJ · mol^{-1} was calculated. The α-amylase was very sensitive to temperature inactivation, losing part of its activity even after 1 h at 30°C (Fig. 6B).

Catalytic properties. The substrate specificity of the α-amylase was studied with maltooligosaccharides, polysaccharides, and cyclodextrins (10 g · $liter^{-1}$), and relative activities are listed in Table 3. The α-amylase activity increased from maltotriose to maltoheptaose. Amylopectin and amylose were the best substrates. Glycogen and pullulan were hydrolyzed at a lower rate, while α-amylase hydrolyzed neither dextran nor cyclodextrins.

The purified α-amylase produced oligosaccharides with a degree of polymerization of 1 to 5 from maltohexaose after 10 min of hydrolysis (Fig. 7). The amount of maltopentaose

TABLE 1. Purification of the extracellular amylase of *C. acetobutylicum* ATCC 824[a]

Purification step	Total activity (U)	Amt of protein (mg)	Sp act (U · mg^{-1})	Yield (%)	Purification (fold)
Culture supernatant	817.2	314.4	2.60	100	1.0
Concentration, dialysis, and filtration	433.2	66.6	6.5	53	2.5
Anion-exchange chromatography	216	4.16	51.9	26.4	20
Gel filtration	189.2	3.28	57.7	23.2	22.2

[a] Purification was started with 1.5 liters of culture supernatant. The values given for the anion-exchange chromatography step resulted from four runs.

TABLE 2. Amino acid composition of the *C. acetobutylicum* amylase

Amino acid	No. of residues/molecule of:		
	C. acetobutylicum amylase	*B. subtilis* saccharifying α-amylase[a]	*B. licheniformis* liquefying α-amylase[b]
Asx	120	99	62
Glx	63	51	45
Ser	74	54	26
Gly	47	51	45
His	16	16	24
Arg	14	24	22
Thr	47	45	27
Ala	62	50	35
Pro	22	23	15
Tyr	30	27	30
Val	45	32	32
Met	14	9	7
Ile	26	34	20
Leu	47	36	28
Phe	23	20	20
Lys	30	30	28
Cys	1[c]	1	0
Trp	ND[d]	14	17

[a] From Yang et al. (43).
[b] From Yuuki et al. (44).
[c] Determined as cysteic acid after perchloric oxidation.
[d] ND, Not determined.

gradually decreased after 1 h of hydrolysis, while the amounts of maltotetraose and maltotriose decreased after 2 h. The major end products of hydrolysis were maltose, maltotriose, and glucose, in that decreasing order, after 180 min of hydrolysis, indicating an endo-type mechanism of action. While the enzyme was not active on maltose, glucose was liberated from maltohexaose (Fig. 7) and starch. On starch, 1 U of purified α-amylase possessed a glucoamylase activity of 27 mU.

The α-amylase showed Michaelis-type kinetics when hydrolyzing soluble starch. As calculated from Lineweaver-Burk plots, the apparent K_m and V_{max} values at 30°C were 3.6 mg · ml^{-1} and 87.5 U · mg of $protein^{-1}$ (equivalent to a K_{cat} of 122 mol of reducing sugars · s^{-1} · mol of α-$amylase^{-1}$), respectively.

The following metal ions had no effect on the activity of the α-amylase: Ca^{2+}, Mg^{2+}, and Ba^{2+}. On the other hand,

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      1                   5                  10
I.    Glu - Leu - Arg - Glu - Asn - Thr - Lys - Asp - Gly - Val -
II.   Leu - Thr - Ala - Pro - Ser - Ile - Lys - Ser - Gly - Thr -

      11                  15
I.    Met - Leu - His - Ala - Phe - Asp - Trp - Ser - Phe -
II.   Ile - Leu - His - Ala - Trp - Asn - Trp - Ser - Phe -
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FIG. 4. Comparison of NH_2-terminal amino acid sequences of the α-amylase from *C. acetobutylicum* (I) and the α-amylase from *B. subtilis* (II). Data for II were from Yang et al. (43).

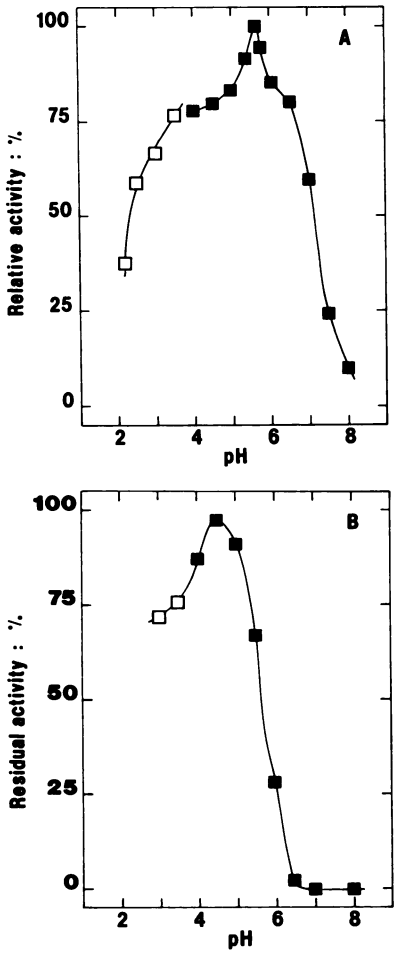

FIG. 5. Effect of pH on the α-amylase. Shown are relative activity (A) and stability (B) in 50 mM citrate buffer (□) and acetate-phosphate buffer (■). α-Amylase was used at 0.3 U · ml^{-1}. For details, see Materials and Methods.

several metal ions (Fe^{3+}, Fe^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, and Pb^{2+}) at a final concentration of 1 mM had a slight inhibitory effect (less than 35%) on the enzyme, while sulfhydryl oxidant metals (Cu^{2+}, Ag^{+}, and Hg^{2+}) totally inhibited enzyme activity. The enzyme activity was affected by sulfhydryl reagents such as parachloromercuribenzoic acid (PCMB) (total inhibition at a concentration of 1 mM) and, to a lesser extent, iodoacetic acid (10% inhibition at a concentration of 10 mM). The inhibition by PCMB and Ag^{+} could be reversed by the addition of 10 mM dithiothreitol.

At a final concentration of 10 mM, EDTA and *O*-phenanthroline had no effect on the α-amylase, although calcium analysis after extensive dialysis revealed that the enzyme contained seven calcium atoms per mole. 2-Mercaptoethanol and dithiothreitol activated the enzyme (relative activity, 140%). α-Cyclodextrin at a concentration of 10 mM did not influence the activity of the α-amylase, but slight inhibition was observed in the presence of 10 mM β-cyclodextrin (relative activity, 86%).

DISCUSSION

C. acetobutylicum ATCC 824 produced similar levels of α-amylase whether growing on glucose or on starch. This

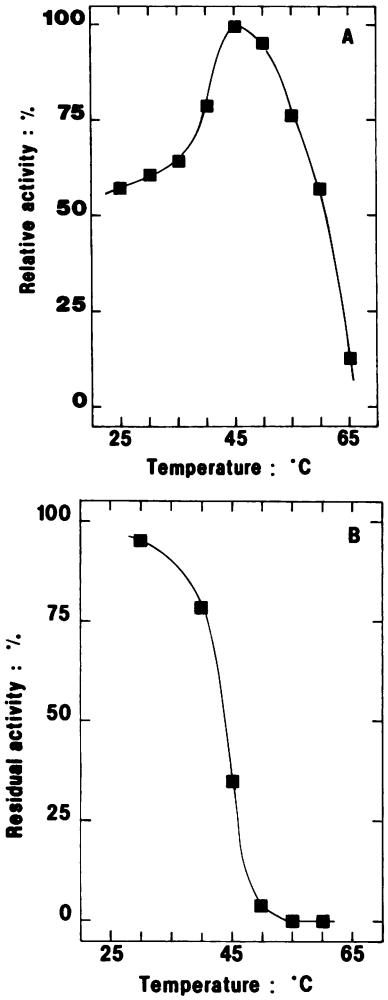

FIG. 6. Effect of temperature at pH 5.6 on the α-amylase. Shown are relative activity (A) and stability (B). α-Amylase was used at 0.35 U · ml^{-1}. For details, see Materials and Methods.

result is in agreement with that of Ensley et al. (12) but differs from those of other researchers who reported catabolite repression of α-amylase synthesis (6, 14, 33). From the supernatant of a glucose-grown culture, the α-amylase of *C. acetobutylicum* was purified to homogeneity by anion-exchange chromatography and gel filtration. The purified α-amylase had an acidic isoelectric point of 4.7. This value is not in agreement with the value of 6.5 given by Scott and Hedrick (34). It must be considered that the value of 6.5 was an approximation which was determined from a study of α-amylase adsorption on charcoal and lignin. The discrepancy observed between the molecular weights determined by SDS-PAGE and gel filtration with a polysaccharidic gel has already been reported for different amylases (5, 7, 8, 27, 30, 39). This anomalous behavior can be explained in terms of interactions between the amylase and the agarose gel. Under such conditions, enzyme elution was delayed and molecular weight was underestimated. Confirmation of this effect was obtained by gel filtration in 6 M urea, which revealed a molecular weight (83,000) consistent with that obtained by SDS-PAGE (84,000).

TABLE 3. Relative activities for the hydrolysis of various substrates for the purified α-amylase[a]

Substrate	Relative activity (%)
Soluble starch	100.0
Maltotriose	6.2
Maltotetraose	11.6
Maltopentaose	14.5
Maltohexaose	26.3
Maltoheptaose	28.4
Amylose (potato)	209.4
Amylopectin (potato)	114.3
Pullulan	13.6
Glycogen	22.3

[a] No activity was obtained with maltose, *p*-nitrophenyl-α-D-glucopyranoside, dextran, or cyclodextrins (α, β, and γ).

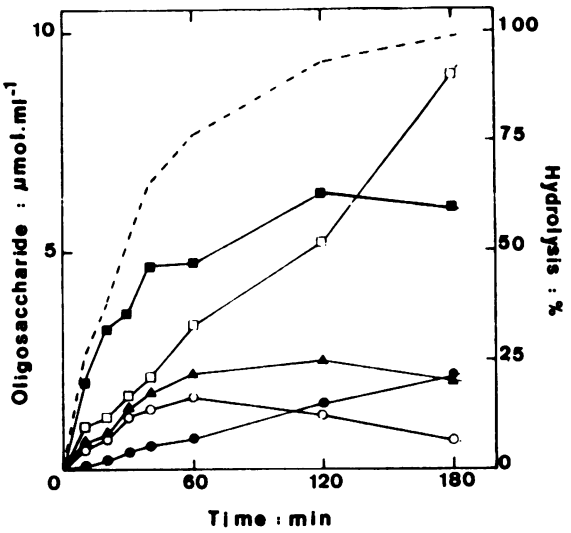

FIG. 7. Time course of maltooligosaccharide formation from maltohexaose by the purified α-amylase. The activity of the enzyme sample was about 0.4 U · ml^{-1}. Experimental conditions are described in Materials and Methods. Symbols: ●, glucose; □, maltose; ■, maltotriose; ▲, maltotetraose; ○, maltopentaose; ----, percent hydrolysis of maltohexaose.

In common with several bacterial and mold α-amylases (37, 44), *C. acetobutylicum* α-amylase was rich in both acidic and hydrophobic amino acids. The α-amylase also contained a relatively large amount of hydroxyamino acids and a small amount of arginine and histidine, like the saccharifying α-amylase of *B. subtilis* (37). A comparison of the NH_2-terminal amino acid sequences of the α-amylases of *C. acetobutylicum* and different *Bacillus* strains revealed homology with the saccharifying α-amylase of *B. subtilis* (42%) but not with the *B. licheniformis* liquefying α-amylase (17, 29, 43). As suggested by this analysis and confirmed by the hydrolysis of maltohexaose, the *C. acetobutylicum* α-amylase was related to the saccharifying α-amylases.

The pH for optimal α-amylase activity was within the range of values reported for most bacterial (5, 6, 25, 28, 38) and yeast (7, 8) α-amylases. However, it differs from the value of 4.8 given by Hockenhull and Herbert (14) for the same enzyme. A remarkable feature of the *C. acetobutylicum* ATCC 824 α-amylase is its stability at an acidic pH. The same pH stability profile has been observed for the α-amylase of *Bacillus acidocaldarius* (5). The stability of the *C.*

acetobutylicum α-amylase at an acidic pH will be particularly important for the efficient conversion of starch to solvent, as it has been shown that a culture pH below 5.5, with an optimum at pH 4.4, is essential for solvent production (35). The optimal temperatures for the activity and stability of the α-amylase were quite low. The α-amylase of *C. acetobutylicum* ATCC 824 was thermally sensitive as compared with other bacterial α-amylases (5, 25, 27, 32).

The activity of the *C. acetobutylicum* α-amylase was not stimulated by calcium ions (or other metal ions) or inhibited by EDTA, although the enzyme contained seven calcium atoms per mole. Such resistance to a chelating agent of calcium metalloamylase has already been reported for the *B. subtilis* saccharifying α-amylase (15). Among the five calcium atoms that the latter enzyme contained, one was strongly bound and essential for catalytic activity (42). PCMB, sulfhydryl oxidant metals and, to a lesser extent, iodoacetate inhibited the enzyme. It was shown for the taka-amylase A and for the *B. subtilis* saccharifying α-amylase that the calcium atom strongly bound to the enzyme could protect the sulfhydryl group from the action of the sulfhydryl reagents iodoacetate and DTNB but not PCMB (40, 41). An identical interaction between calcium and the sulfhydryl group of the single cysteine could be postulated for the α-amylase of *C. acetobutylicum* because of the close similarity of the enzymes properties.

Among the cyclodextrins tested, only β-cyclodextrin inhibited the α-amylase. Such inhibition was reported in the literature for other α-amylases and determined to be competitive (2, 8).

It is difficult to compare the apparent K_m of 3.6 g · liter^{-1} for soluble starch with the K_m of 2.08 g · liter^{-1} given by Hockenhull and Herbert (14), since the latter value was not determined from the initial rate of hydrolysis. The K_ms found in the literature vary from 0.35 to 4.3 g · liter^{-1}, depending on the α-amylase source and the starch origin and treatment (5, 7, 8, 25).

The rate of α-amylase hydrolysis increased with the degree of polymerization of the homologous maltooligosaccharide series from maltotriose up to amylose. A low activity was detected on pullulan, probably due to hydrolysis of α-1-4 glucan bonds rather than a debranching activity. The analysis of the maltohexaose hydrolysis products provided more information about the mechanism of action of the α-amylase. The results were consistent with the action of an endoenzyme which released oligosaccharides with a polymerization degree of 1 to 5. The simultaneous liberation of all the maltooligosaccharides indicated a random attack of the substrate chain by the α-amylase. Maltotriose, maltose, and glucose were the end products of maltohexaose hydrolysis. Another experiment with starch confirmed this result and revealed that short-chain dextrins were formed as hydrolysis intermediates (data not shown). The same results have been obtained with an α-amylase of *Clostridium butyricum* (38). On the other hand, Scott and Hedrick (33) reported only maltose and maltotriose as end products of starch hydrolysis by the *C. acetobutylicum* α-amylase. Furthermore, the production of glucose by the α-amylase was consistent with the activity on maltotriose and pullulan. It would seem that an oligomeric chain containing a minimum of three glucose units is required for α-amylase catalytic activity to be operative.

C. acetobutylicum also produces a glucoamylase as a starch-hydrolyzing enzyme, with a ratio of glucoamylase activity to α-amylase activity of 1:10 (data not shown). The fact that the α-amylase possesses some glucoamylase activity (2.7%) indicates that more than 27% of the glucoamylase activity classically estimated in the fermentation broth is due to the α-amylase.

Further work on the regulation of the production of the α-amylase needs to be undertaken before yields can be improved by genetic manipulation. The direct conversion of raw starch material to acetone and butanol would then be possible at a rate similar to that observed for the conversion of glucose.

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