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Stable, Inducible Thermoacidophilic α-Amylase from *Bacillus acidocaldarius*

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Bacillus acidocaldarius Agnano 101 produces an inducible thermoacidophilic α-amylase. The enzyme production occurs during the stationary phase of growth in the presence of compounds with α-1,4-glucosidic linkages. The enzymatic activity is both present in the culture medium and associated with the cells; the enzymes purified from both sources show identical molecular and catalytic properties. The purified amylase has a single polypeptide chain of molecular weight 68,000 and behaves like an α-amylase with affinity constants for starch and related substances of 0.8 to 0.9 mg/ml. The pH and temperature optima for activity are 3.5 and 75°C, respectively. The amylase is stable at acidic pH (below 4.5). Its thermal stability is strictly dependent upon protein concentration; the half-life at 60°C of the amylase in a 70-μg/ml solution is about 5 days.

Many investigators have compared enzymes from thermophilic and mesophilic organisms to determine physicochemical differences responsible for thermophily. There is little evidence supporting any explanation of thermophily; however, it has been definitely proven that enzymes from obligate thermophiles are intrinsically thermostable (22). A good deal of attention has been devoted to thermostable amylases (12, 16, 17, 20, 23, 26, 27), which are of both scientific interest and considerable industrial importance (10, 13); in particular, stable thermoacidophilic amylases might be ideally suited for some industrial processes (23). De Rosa et al. (5) recently reported the presence of bacteria in several springs, in the volcanic area of Agnano near Naples, ranging in temperature from 58 to 80°C and in pH from 1.7 to 2.7; we screened the microorganisms isolated from these springs for their capacity to produce extracellular amylases. *Bacillus acidocaldarius* Agnano 101 synthesizes a stable, inducible thermoacidophilic α-amylase. This paper is concerned with the biosynthesis, purification, and preliminary characterization of the enzyme.

MATERIALS AND METHODS

Chemicals. Rabbit liver glycogen, potato starch amylopectin, and amylose were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. From the same source were also obtained acrylamide, bisacrylamide, and α-amylase-free barley β-amylase. Bovine serum albumin, egg albumin, chymotrypsinogen, cytochrome *c*, and *B. subtilis* α-amylase were from Sigma Chemical Co., St. Louis, Mo. Hydroxyapatite (Bio-Gel HTP) and AG 501x8 were purchased from Bio-Rad Laboratories, Richmond, Calif. Thin-layer chromatography plates of cellulose F were obtained from E. Merck AG, Darmstadt, Germany. The other chemicals were of the analytical grade commercially available.

Organism and media. *B. acidocaldarius*, an acidophilic thermophile with optimal temperature growth of 60°C at pH 3.5, was originally isolated from hot springs in Agnano, near Naples (5). To isolate a strain producing extracellular amylase, a selective medium was used containing (per liter) the following basal salts: $(NH_4)_2SO_4$, 2.5 g; $MgSO_4 \cdot 7H_2O$, 0.20 g; KH_2PO_4, 3.0 g; and $CaCl_2 \cdot 6H_2O$, 0.25 g. The medium was supplemented (per liter) with: starch (Connaught, Toronto, Canada), 10 g; yeast extract (Difco), 5 g; and purified agar (Difco), 20 g. The petri plates, prepared at pH 4 as described by Darland and Brock (3), were incubated for 24 h at 60°C, and amylase-producing colonies were detected by flooding with a dilute iodine solution (9). Each isolate was transferred to a standard liquid medium containing basal salts, 5 g of yeast extract per liter, and 5 g of starch per liter. The pH was adjusted to 3.5 with 1 N H_2SO_4, and the culture was incubated at 60°C for 48 h. Finally, a strain coded 101, giving higher yields of amylase activity, was selected for further studies. Stock cultures were maintained on agar-starch slants at room temperature and renewed at monthly intervals. The growth media used for experiments of amylase induction contained basal salts, 5 g of yeast extract per liter, and the inducer at the concentration indicated; the pH was adjusted to 3.5 with 1 N H_2SO_4. The cultures were incubated for 48 h, without shaking, at 60°C in 500-ml conical flasks containing 50 ml of medium.

Enzyme production. Starter cultures were inoculated with cells from an agar-starch slant and were grown without shaking at 60°C for 18 h in 1-liter conical flasks containing 100 ml of the standard medium. The inocula were transferred to 2-liter conical flasks containing 500 ml of standard medium and grown at 60°C in an oven. Alternatively, 3 liters of a starter culture was used to inoculate a 30-liter fermentor (Terzano) with paddle agitation and an air stream (500 ml/min per liter of culture medium). At various time intervals, samples of the culture were analyzed for growth, by turbidimetric measurements at 640 nm, and enzyme production. The cells were harvested at the maximum enzyme level (40 to 48 h for nonstirred systems and 35 to 40 h for fermentors) either by centrifugation at 20,000 × *g* or by continuous-flow centrifugation in an Alfa-Laval centrifuge (model LAB 102B-20). The cells were washed twice with a basal salts solution adjusted to pH 3.5 with HCl and stored at −20°C.

Purification of the enzyme from culture supernatant. The liquor (28 liters) was concentrated to $^1/_{10}$ of its original volume by evaporation under vacuum at 60°C; it was then dialyzed against 0.02 M $MgCl_2$ adjusted to pH 3.5 with HCl. Further concentration of the dialysate was achieved by adding solid ammonium sulfate up to 70% saturation. The precipitate was collected by centrifugation at 20,000 × *g*, suspended in 15 ml of 0.02 M $MgCl_2$ (pH 3.5), and dialyzed against the same solution. Ammonium sulfate treatment of the unconcentrated culture supernatants gave low recoveries of amylase activity in the precipitate. The dialyzed solution (22 ml) was added to 2.5 ml of 0.1 M potassium phosphate buffer (pH 5.5) and then applied to a hydroxyapatite column (2.5 by 35 cm) equilibrated previously with 0.01 M potassium phosphate buffer-0.01 M $MgCl_2$ (pH 5.5). The column was washed with the same buffer until no more material absorbing at 280 nm was eluted. The amylase activity was then eluted by applying a linear gradient (400 ml of 0.01 M potassium phosphate buffer-0.01 M $MgCl_2$ [pH 5.5] and 400 ml of 0.31 M potassium phosphate buffer-0.01 M $MgCl_2$ [pH 5.5]). Fractions (8 ml each) were collected at a flow rate of 32 ml/h. Those containing amylase activity were pooled and concentrated by ultrafiltration on an Amicon stirring cell equipped wtih a PM-10 Diaflo membrane. Finally, concentrated enzyme was dialyzed against 0.02 M glycine-hydrochloride buffer–0.01 M $MgCl_2$ (pH 3.5).

Purification of the bound enzyme. Wet cells (98 g) were suspended in 200 ml of a basal salts solution containing 2 M guanidine-hydrochloride (pH 4.0), and stirred occasionally for 30 min at 60°C. The suspension was then cooled to 4°C and centrifuged for 10 min at 34,000 × *g*. The guanidine-hydrochloride treatment was repeated three times for each batch of cells, and the three supernatants were pooled and dialyzed against 0.1 M $MgCl_2$ (pH 3.5). The dialysate, clarified by centrifugation, was fractionated with solid ammonium sulfate. The precipitate from the 20 to 70% saturated salt, collected by centrifugation, was dissolved in 0.02 M $MgCl_2$ (pH 3.5) and dialyzed against the same solution. The dialysate was then chromatographed on hydroxyapatite as described above. Unless otherwise indicated, all of the purification steps can be carried out either at 4°C or at room temperature without any significant difference in enzyme recovery.

Rapid purification techniques, such as adsorption on solid substrates (11, 21) or interaction with immobilized protein inhibitors (1), were also used. Adsorption on solid starch or glycogen was at 0 or 20°C. One milliliter of 80% ethanol and 10 mg of solid adsorbent were added to 1 ml of 0.02 M $MgCl_2$ (pH 3.5) containing 60 U of amylase. The suspension was stirred for 10 min and then centrifuged for 10 min at 1,000 × *g*. The supernatant was dialyzed against 0.02 M $MgCl_2$ (pH 3.5) and then assayed for amylase activity.

Different amounts (0.3 to 20 μg) of protein inhibitors from wheat kernel (1) were incubated for 10 min at 40 or 60°C with 0.1 U of amylase either in 0.02 M glycine-hydrochloride buffer–0.02 M $MgCl_2$ (pH 3.5) or in 0.01 M potassium phosphate buffer-0.02 M $MgCl_2$ (pH 5.5), in a final volume of 0.9 ml. Starch was then added (0.1 ml of a 1.5% solution), and enzyme activity was assayed as described below.

Protein and enzyme determination. Protein concentration was determined by the Lowry method as modified by Hartree (8), with bovine serum albumin as a standard. The amylase activity was assayed by the Nelson colorimetric method as described by Robyt and Whelan (19). Unless otherwise stated, the reaction mixture for *B. acidocaldarius* amylase activity contained, in a final volume of 1 ml, 0.02 M glycine-hydrochloride buffer (pH 3.5)–0.02 M $MgCl_2$, 1.5 mg of starch, and 0.01 to 0.2 U of amylase. The reaction was started by the addition of the enzyme and was carried out at 75°C for 10 min. One unit of amylase is the amount of enzyme that produces 1 μmol of maltose in 1 min at 75°C under our experimental conditions. For routine assays, the iodine-staining method was used (19).

B. subtilis α-amylase and barley β-amylase were assayed by the same procedure, except that the incubation temperature was 37°C and the buffers used for the reaction mixtures were 0.05 M potassium phosphate buffer (pH 6.0)-0.01 M NaCl-0.01 mM $CaCl_2$, and 0.05 M sodium acetate buffer (pH 4.8), respectively.

Disc-gel electrophoresis. Polyacrylamide gel electrophoresis in tris(hydroxymethyl)aminomethane-glycine buffer (pH 8.5) was performed with standard 7.5% acrylamide gels as described by Davis (4). Polyacrylamide gel electrophoresis at acidic pH (4.5) was carried out as described by Reisfeld et al. (18).

Molecular weight studies. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate and 2-mercaptoethanol was carried out as described by Weber and Osborn (24). Filtration was on a Sephadex G-100 column (1.5 by 62 cm) equilibrated with 0.02 M potassium phosphate buffer-0.01 M $MgCl_2$ (pH 5.0). Reference proteins for both electrophoresis and gel filtration were bovine serum albmin, chymotrypsinogen, and cytochrome *c*. Sedimentation in a sucrose gradient (5 to 20%, wt/vol) was performed at 40,000 rpm for 20 h at 4°C in a Beckman-Spinco L2-65B preparative ultracentrifuge equipped with a SW40 swinging-bucket rotor (14); bovine serum albumin and egg albumin were used as internal markers.

Action pattern and products of hydrolysis. The

action pattern of *B. acidocaldarius* amylase was checked by the method of Drummond et al. (6), with oxidized amylose as a substrate. Amylose was partially oxidized with sodium periodate as described previously (2).

The products of enzymatic hydrolysis were analyzed by multiple ascending chromatography on cellulose thin-layer plates. Amylase (0.5 U) was incubated at 75°C with 50 mg of starch in 0.02 M glycine-hydrochloride buffer (pH 3.5)–0.02 M $MgCl_2$ (final volume, 5 ml). Samples were taken at various times, and the enzymatic reactions were stopped by treatment with ice. The digest samples and the standard mixture (1% glucose, maltose, maltotriose, and maltotetraose in the same glycine-hydrochloride buffer) were desalted rapidly at 4°C in batches on an AG 501x8 mixed-bed resin. The standard mixture (10 μl) and each digest (50 μl) were spotted on a thin-layer cellulose plate and subjected to ascending chromatography at 50°C in the solvent system *n*-propanol-water (70:30, vol/vol). After two ascents, the chromatogram was dried at room temperature and the products were detected with an alkaline silver oxide reagent (15).

RESULTS

Effect of different carbon sources on amylase production. The effect of different carbon sources on amylase production was tested in the basal salts solution supplemented with 0.5% yeast extract and 0.5% of a carbon source. The total amylase activity of the whole culture (liquid medium plus cells) after 48 h of growth, concomitant with the maximum enzyme production, was assayed (Table 1). The effect of varying the concentration of some inducers (maltose, starch, and glycogen) on the enzyme levels was studied. The amylase production increased linearly with the inducer concentration, reaching a maximum level at 0.3 to 0.5%; for higher inducer concentrations, a slow decrease of the total amylase activity was observed. No significant variation of cell growth occurred when the type or the concentration of inducer was varied. In the presence of an inducer, the amylase activity appeared at the end of the logarithmic growth phase and accumulated during the stationary phase. Total enzymatic activity reached the maximum level concomitant with the beginning of cell autolysis, and then slowly decreased. Only 5 to 10% of the enzyme was produced during the logarithmic phase of growth. Similar results were obtained with stirred fermentors, where an even lower percentage (0.2 to 0.5) of the enzyme was produced in the logarithmic phase.

Distribution of amylase activity in *B. acidocaldarius* cultures. When culture growth was interrupted at the stationary phase, amylase activity was found both in the culture medium and associated with the cells. The ratio of soluble/bound enzyme was dependent upon growth conditions and culture age. After 48 h of growth in unshaken conical flasks, corresponding to the time of maximum enzyme production, most (80 to 90%) of the amylase activity was found in the culture supernatant, whereas cultures grown in a stirred fermentor contained, at the maximum enzyme level, only 40 to 60% of the enzymatic activity in the supernatant. Moreover, the ratio of soluble/bound enzyme increased with the culture age. In unstirred cultures this ratio increased from 1, after 24 h of growth, to about 4, after 48 h of growth. In stirred cultures a similar behavior was observed, but the ratio usually only approximated 2.

In both cases, in the interval indicated above, the cultures were in the stationary phase of growth, with a biomass of about 3 mg of wet cells per ml; in this time period, the total enzyme production increased from 0.07 to 0.15 U/ml.

Cell-associated amylase recovery. Cell-associated amylase was extracted with 2 M guanidine-hydrochloride. When cells harvested at the maximum enzyme production were employed, the amylase activity recovered was 70 to 75% of the total; older cultures, indeed, usually gave lower recoveries. The extraction conditions were critical in regard to guanidine concentration, pH, and temperature. Guanidine-

TABLE 1. *Effect of different carbon sources on the production of amylase by B. acidocaldarius*[a]

Carbon source added	Relative activity (%)
No addition	0[b]
Glycerol	0[b]
Glucose	0[b]
Lactose	0[b]
Sucrose	0[b]
Trehalose	0[b]
Cellobiose	0[b]
Maltose	87
Maltotriose	50
Maltotetraose	55
Glycogen	100
Starch	87

[a] Cultures were grown at 60°C in basal salts medium supplemented with 0.5% yeast extract and 0.5% of the carbon source indicated. The amylase activity, concomitant with maximum enzyme production, was assayed on the whole culture (liquid medium plus cells) after 48 h. The amylase activity observed in the presence of glycogen was taken as 100; absolute values of activity were 0.16 U/ml, with a biomass of 3 mg of wet cells per ml.

[b] No amylase production was observed even after 96 h of growth.

hydrochloride concentrations higher than 4 M or pH values higher than 4.5 caused complete denaturation of the enzyme; at guanidine-hydrochloride molarities lower than 1.5 or at temperatures lower than 30°C, enzyme release did not exceed 10 to 15%. Attempts to solubilize cell-associated enzyme with 0.1% surfactants, such as Tween 80 or sodium dodecyl sulfate, at 20 and 60°C were not successful. These compounds, at the concentration used, did not interfere with the enzymatic assay.

Enzyme purification. *B. acidocaldarius* amylase was purified from both culture supernatants and cells (Table 2). The enzymes from both sources showed identical molecular and catalytic properties. The enzyme showed high affinity for hydroxyapatite at low phosphate concentrations and was eluted at 0.12 to 0.18 M phosphate. The presence of 1 M KCl in the buffer did not affect the eluting molarity of phosphate. The amylase preparations were homogeneous according to both alkaline and acidic polyacrylamide gel electrophoresis. Purified enzyme preparations showed an ultraviolet absorption spectrum (Cary 14 spectrophotometer) typical for proteins, with a maximum at 278 nm. The $E^{1\%}_{1cm}$ at this wavelength was 9.7.

The amylase, under the experimental conditions tested, neither adsorbed on solid starch or glycogen, nor was inhibited by albumin amylase inhibitors from wheat kernel.

Molecular weight studies. *B. acidocaldarius* amylase and bovine serum albumin exhibited identical patterns upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis and sucrose gradient sedimentation. It appears that, within the standard error of the methods used, the molecular weight of the *B. acidocaldarius* amylase is identical to that of bovine serum albumin, i.e., 68,000. Moreover, according to polyacrylamide gel electrophoresis in 2-mercaptoethanol-sodium dodecyl sulfate, the amylase consists of a single polypeptide chain. The *B. acidocaldarius* amylase was retarded by Sephadex matrices, showing an elution volume corresponding to an apparent molecular weight of 57,000.

Parameters influencing amylase activity and stability. Purified *B. acidocaldarius* amylase was tested at 75°C at different pH values. As shown in Fig. 1a, the pH optimum for the enzymatic activity was about 3.5. In the same figure, the pH activity profile of *B. subtilis* α-amylase is given for comparison. When the activity of *B. acidocaldarius* amylase was tested at 60°C at different pH values, a similar pH activity profile was observed.

At pH 3.5, the enzyme exhibited maximal activity at a temperature close to 75°C; a sharp drop in the activity occurred above 80°C (Fig. 1b). The time course reaction at 75°C was linear at least for 20 min; at 60°C the linearity continued for a much longer time. The stability of *B. acidocaldarius* amylase at various pH values was studied by preincubating the enzyme at 20°C for 90 min and for 24 h at different pH values before the enzymatic assay. A 90-min preincubation at pH values in the range 1.2 to 6.5 did not affect enzymatic activity; a dramatic drop in the activity was observed after 24 h of incubation at pH values higher than 4.5. The effect of temperature on amylase stability was studied by preincubating the enzyme at 75°C and pH 3.5 and then measuring the residual activity at various intervals. In the absence of the substrate, enzyme stability was strongly dependent upon the enzyme concentration in the incubation mixture (Fig. 2); low amylase concentrations could also be partially protected from thermal denaturation by the presence of another protein, such as bovine serum albumin. Comparable results were obtained by

TABLE 2. *Purification of α-amylase from B. acidocaldarius*

Purification step	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Enzyme of the supernatant[a]					
Culture filtrate	1,330[b]	230[b]	5.8	1	100
Concentration by evaporation	1,180	232	5.1	0.9	88.7
Concentration by $(NH_4)_2SO_4$	905	30.8	29.4	5.1	68.0
Hydroxyapatite and ultrafiltration	830	3.23	257.0	44.3	62.4
Cell-associated enzyme[a]					
Guanidine extraction	1,365	163.1	8.4	1	100
$(NH_4)_2SO_4$ cut (20 to 70% saturation)	1,050	19.5	53.8	6.4	76.9
Hydroxyapatite and ultrafiltration	917	3.84	238.8	28.4	67.2

[a] The amylase units in the whole culture (liquid medium plus cells) were about 3,300. After centrifugation, 28 liters of supernatant and 98 g of wet cells were obtained.

[b] Protein and amylase activity at this step were determined on samples dialyzed against 0.02 M $MgCl_2$ adjusted to pH 3.5 with HCl.

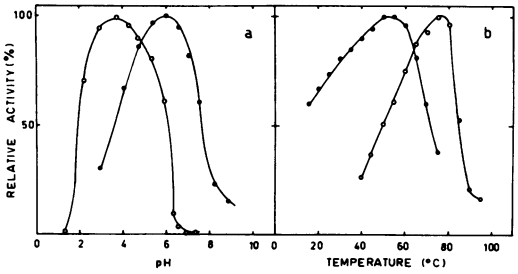

FIG. 1. *Effect of pH (a) and temperature (b) on the activity of B. acidocaldarius amylase* (○). *The pH and temperature activity profiles for the B. subtilis amylase* (●) *are given for comparison. Maximal activity for both amylases has been taken as 100.*

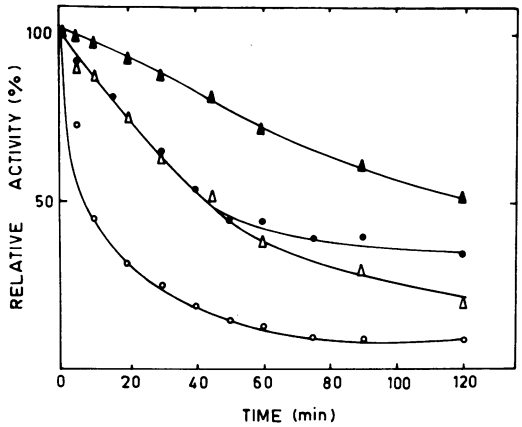

FIG. 2. *Thermal stability of B. acidocaldarius amylase at 75°C as a function of time. Symbols:* ○, *4 μg of amylase per ml in the incubation mixture;* ●, *4 μg of amylase per ml plus 70 μg of bovine serum albumin per ml;* △, *73 μg of amylase per ml;* ▲, *73 μg of amylase per ml plus 460 μg of bovine serum albumin per ml.*

preincubating at 60°C, but at such a temperature higher residual activities were observed: the half-life of *B. acidocaldarius* amylase in a 70-μg/ml solution (15 U) was about 5 days, whereas in a 10-fold diluted solution it was only 30 to 40 min. Exhaustive dialysis of *B. acidocaldarius* amylase against distilled water caused only a partial loss of activity (about 35%). The enzymatic activity was fully restored, dose dependently, by the addition of either Ca^{2+} or Mg^{2+}. Maximal activity was observed at 0.01 to 0.025 M ion concentrations.

Determination of the amylolytic pattern. *B. acidocaldarius* amylase was able to hydrolyze both native and oxidized amylose, although the latter at a lower rate since it usually occurs because of partial steric hindrance by oxidized glucosidic residues. In contrast, barley β-amylase, used as a control, was able to hydrolyze native amylose, but showed poor degrading activity on the oxidized substrate (Fig. 3). These results, showing that the *B. acidocaldarius* enzyme has a typical endoamylolytic nature, were supported by results of the thin-layer chromatographic analysis of the products of starch hydrolysis. After 10 to 20 min of hydrolysis, maltotetraose was the main product followed, in lesser amounts, by maltopentose, maltose, maltotriose, and glucose, in the order given. Maltotriose, maltose, and glucose accumulated after 1 h of hydrolysis.

Kinetic parameters and substrate specificity. *B. acidocaldarius* amylase was able to hydrolyze starch, amylose, amylopectin, and glycogen, whereas no degradation of maltose or *O*-(carboxymethyl)-cellulose was observed. The affinities of the enzyme for starch, amylose, and amylopectin were not significantly different. As calculated from Lineweaver-Burk plots, the K_m values for these three substrates were 0.8 to 0.9 mg/ml; a slightly higher value (1.25 mg/ml) was found for glycogen. However, the V_{max} values for the four substrates were of the same order of magnitude (about 35 μmol of maltose per min per mg of protein). The kinetic parameters reported above were determined with a partially purified amylase preparation showing a specific activity of 34.1 U/mg.

DISCUSSION

In culture media supplied with oligo- or polysaccharides having α-1,4-glucosidic linkages (Table 1), *B. acidocaldarius* Agnano 101 actively produced an extracellular α-amylase

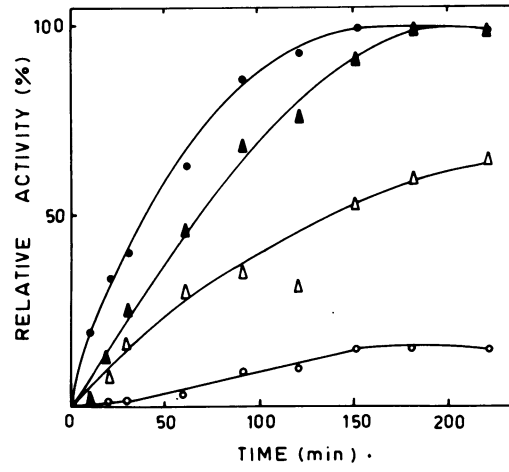

FIG. 3. *Hydrolysis of native* (▲) *and oxidized* (△) *amylose by B. acidocaldarius amylase as a function of time. Hydrolysis of native* (●) *and oxidized* (○) *amylose by barley β-amylase is given for comparison. Maximal activity of the two amylases with native amylose has been taken as 100.*

during the stationary phase of growth. Similar results have been reported by Welker and Campbell (25) for *B. stearothermophilus*, which produces a partially constitutive amylase. We have not been able to assay any appreciable activity in original or concentrated media of *B. acidocaldarius* cultures grown in the absence of an appropriate inducer. As has been observed recently for an α-amylase from *B. amyloliquefaciens* (7), α-amylase from *B. acidocaldarius* partly accumulates in the culture medium in a soluble form and partly is associated with the cells. The cell-associated enzyme is not released by salt or detergent extraction but only by treatment with 2 M guanidine-hydrochloride under conditions conducive for growth. These results might indicate that, between the enzyme and cell, polar interactions are prevailing in the growing conditions when the enzyme is slowly released; however, further studies are needed for a better understanding of the enzyme-cell interactions. The amount of enzyme solubilized by treatment with guanidine-hydrochloride never did exceed that measured for the starting cells, suggesting that the cell-associated enzyme is present in an active form available to the substrate. Several physical parameters (temperature, volume, agitation, air exchange) influenced both the yields of amylase production and the ratio of soluble/cell-associated enzyme. However, since all of these factors are virtually interdependent, it is quite impossible to study systematically the contribution of every single factor. Compared with other amylase purifications, the choice of suitable techniques for purifying the *B. acidocaldarius* amylase was unusually difficult. Since the enzyme did not adsorb on insoluble substrates and was not inhibited by wheat albumin amylase inhibitors, the bioaffinity techniques described as general methods for α-amylase purification (1, 11, 21) were not suitable. Chromatography on molecular sieves (Sephadex, Bio-Gel) or ionic exchangers (modified Sephadex or cellulose) generally suffered from low recoveries and poor purifications. In contrast, the enzyme exhibited a high affinity for hydroxyapatite, at a low phosphate concentration, and was recovered in good yields (75 to 95%) from this adsorbent. This result might be ascribed to the fact that the enzyme underwent little dilution during the hydroxyapatite chromatography and/or stabilization by the calcium of the matrix. Other evidence supporting both possibilities has been obtained: (i) a marked effect of the protein concentration on the amylase thermal stability in the absence of starch (Fig. 2), and (ii) stimulation by Ca^{2+} (and Mg^{2+}) of the activity of water-dialyzed enzyme.

Several structural and kinetic properties (molecular weight, number of polypeptide chains, dependence upon divalent cations, affinity constants, substrate specificity) are common to most α-amylases (23). In these regards the *B. acidocaldarius* enzyme behaved as a typical endoamylase consisting of a single polypeptide chain wtih a molecular weight of 68,000; its activity was stimulated by calcium and magnesium ions, and the affinity constants for the substrates were of the same order of magnitude as those usually reported for α-amylases. Moreover, the *B. acidocaldarius* amylase did not show any of the peculiar molecular properties described for the thermophilic α-amylases from *B. stearothermophilus* (12) and *B. licheniformis* (20); however, the peculiar features of the former enzyme have been questioned (16, 17, 26). In contrast to the similarity of the molecular aspects, a large variability has been observed for the catalytic properties of the α-amylases. Optimal temperature and pH ranges for both activity and stability of amylases vary remarkably, reaching extreme values for acid-stable or thermostable enzymes; however, no other example of α-amylase exhibiting, at the same time, such features of acidophily and thermophily has been reported so far.

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