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Isolation and characterization of α-amylase derived from starch-grown *Clostridium acetobutylicum* ATCC 824

Bassam A. Annous[1] and Hans P. Blaschek

Department of Food Science, University of Illinois, Urbana, Illinois 61801, USA

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SUMMARY

An extracellular α-amylase was purified to homogeneity from the culture supernatant of *Clostridium acetobutylicum* ATCC 824 grown in synthetic medium containing starch by using a combination of ammonium sulfate fractionation, anion exchange chromatography and HPLC-gel filtration. The molecular weight of the 160-fold purified α-amylase was determined by SDS-PAGE to be 61 kDa. HPLC analysis of end-products of enzyme activity on various substrates indicated that the enzyme acted specifically in an endo-fashion on the α-1,4-glucosidic linkages. Enzyme activity was optimal over a pH range of 4.5–5.0 and temperature of 55 °C, but was rapidly inactivated at higher temperatures. Addition of calcium chloride (2–5 mM) increased α-amylase activity by ca. 20%, while the addition of 19 μg ml^{-1} of acarbose (a differential inhibitor of amylases) resulted in 50% inhibition. The V_{max} and K_m of α-amylase were 2.17 mg min^{-1} and 3.28 mg ml^{-1} on amylose, and 1.67 mg min^{-1} and 1.73 mg ml^{-1} on soluble starch, respectively.

INTRODUCTION

Starch, a major component of agricultural crops, is an important substrate for production of microbial end products and amylolytic enzymes. Results from various laboratories suggested that amylolytic enzyme biosynthesis in *C. acetobutylicum* is subject to catabolite repression by glucose and induction by starch [1,2,5,9,11,14,19]. Although Paquet et al. [24] reported a 50% increase in α-amylase activity when *C. acetobutylicum* ATCC 824 was grown on starch relative to when grown on glucose, they concluded that the activities were similar. Furthermore, they used glucose as substrate for production of α-amylase during their purification and characterization study. Since our earlier work suggested that the amylolytic activity produced by *C. acetobutylicum* ATCC 824 on starch was 8.3-fold higher than that produced on glucose and was primarily extracellular [1,2], the cell-free supernatant of a starch-grown culture was used as the enzyme source. The objective of this study was to purify to homogeneity and characterize the amylolytic enzymes produced by *C. acetobutylicum* ATCC 824 when this microorganism is grown in chemically-defined medium containing starch.

Correspondence to: H.P. Blaschek, Department of Food Science, University of Illinois, Urbana, Illinois 61801, USA.

[1]Present address: Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824, USA.

MATERIALS AND METHODS

Culture maintenance, inoculum development and experimental media

C. acetobutylicum ATCC 824 was obtained from the American Type Culture Collection (Rockville, MD, USA). Culture maintenance and inoculation of chemically defined P2 medium containing 2% (w/v) soluble starch was carried out as previously described [1].

Analytical procedures and chemical reagents

Amylolytic activity was determined by monitoring an increase in reducing sugars using the dinitrosalicylic acid (DNS) method [1]. Protein concentration was determined by Lowry [25]. All experimental results represent an average of at least three separate determinations. The chemicals used were of analytical reagent grade quality.

Purification of extracellular amylolytic enzymes

A late exponential phase P2 medium (8-L) culture of *C. acetobutylicum* ATCC 824 was centrifuged at 10 400 × *g* for 20 min at 2 °C and solid ammonium sulfate was added to the cell-free supernatant to give 40% saturation. After 1 h of stirring, this solution was centrifuged, the pellet was discarded and ammonium sulfate was added to 90% saturation. The pellet from this treatment was dissolved in 220 ml of 50 mM sodium acetate buffer (pH 6) and dialyzed against the same buffer for 48 h at 2 °C. The sample was then concentrated to 20 ml using CentriCell.60 (30 000 MW) centrifugal ultrafiltration units (Polysciences Inc., Warrington, PA, USA).

The concentrated sample was applied to a DEAE-sephadex A-50 anion-exchange column (35 × 2.6 cm) equi-

librated with a 50 mM sodium acetate buffer (pH 6) at 2 °C, and eluted at a flow rate of 4.2 ml h^{-1} using a linear gradient of sodium chloride (0–0.25 M) developed in 600 ml of the equilibration buffer. Fractions (4 ml) exhibiting amylolytic activity were pooled, dialyzed against the same buffer for 48 h at 2 °C, concentrated to 4 ml using CentriCell.60 units and applied to a 10 × 300 mm Superose 6 gel-filtration column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ, USA) equilibrated with 25 mM Bis-Tris propane buffer (pH 6.9) and 100 mM potassium chloride. Elution was carried out with the equilibration buffer at a flow rate of 0.25 ml min^{-1} and 0.25-ml fractions were collected. Single protein peak fractions demonstrating amylolytic activity were pooled, dialyzed against buffer A (sodium acetate, 50 mM; calcium chloride, 20 mM, pH 5) for 48 h at 2 °C and concentrated to 50 ml using the CentriCell.60 units.

Preparation of crude enzyme

C. acetobutylicum ATCC 824 was grown in 500 ml of P2 medium containing 20 g of soluble starch per liter until late exponential phase. Following centrifugation, the cells were then suspended in 50 ml of buffer A and subsequently disrupted by French Pressure cell treatment (18000 p.s.i., SLM Aminco, Urbana, IL, USA). The sample was then centrifuged and the lysed cell pellet was washed once with 50 ml of buffer A. After centrifugation, the supernatant was pooled and concentrated (ca. 35-fold) using CentriCell.60 units. The concentrated sample which served as the crude enzyme preparation was then dialyzed against buffer A for 48 h at 2 °C.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [18]. Samples were run at 20 mA for 8 h at 2 °C in 11% polyacrylamide-slab gels using the Sturdier SE 400, 16-cm single slab unit (Hoefer Scientific Instruments, San Francisco, CA, USA).

Amylolytic activity was localized on SDS-PAGE after electrophoresis by repeatedly rinsing the gels with distilled and deionized water at room temperature to remove SDS. The gels were then immersed in amylolytic enzyme buffer (100 mM sodium acetate, 20 mM $CaCl_2$, pH 5) containing 1% (w/v) soluble starch for 30 min at 37 °C, rinsed with the same buffer without the soluble starch for 10 min, stained with iodine solution (iodine, 0.3%; potassium iodide, 3%) for 5 min and rinsed with distilled and deionized water. Amylolytic activity was visualized as a clearing in a dark-blue background.

Protein bands were visualized following silver staining [13] using Sigma silver stain kit #AG-25 (Sigma Chemical Company, St Louis, MO, USA). The gels were scanned for the determination of the molecular weight of the purified protein using a Beckman DU-40 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA) equipped with a gel scanner. High (29–205 kDa) and low (14.2–66 kDa) molecular weight protein standards obtained from Sigma (Kit #s MW-SDS-200 and MW-SDS-70L, respectively) were used as the reference standards.

Chromatography of hydrolysis products of amylolytic activity

The amylolytic enzyme assay was carried out in a 1-ml reaction mixture (10 mM sodium acetate, 3 mM calcium chloride, pH 5) containing 5 mg of the appropriate carbohydrate source and 1.6 U of purified enzyme at 37 °C for 30 min. The sample was then placed in boiling water for 10 min to inactivate the enzyme, cooled on ice and diluted twice with distilled and deionized water. When the carbohydrate source was amylose, maltose, pullulan or soluble starch, the incubation period was 10, 30, 120 or 360 min, respectively. The end-products of hydrolysis of various substrates were then determined using a Dionex (Dionex Corporation, Sunnyvale, CA, USA) HPLC equipped with Dionex pulsed amperometric detector and Carbopac P1 (4 × 250 mm) carbohydrate column equilibrated with sodium hydroxide solution (150 mM) and compared to known standards.

Characterization of amylolytic enzymes

Unless otherwise indicated, quantitative amylolytic enzyme assays were carried out in a 1-ml buffer A reaction mixture containing 10 mg soluble starch and 0.5 U of purified enzyme for 30 min at 37 °C. Amylolytic activity was reported as % relative activity (% of the highest activity obtained).

pH optimum. The desired pH of the enzyme sample was obtained by adjusting the pH of buffer A containing soluble starch to the appropriate value with either 10 M NaOH or concentrated acetic acid prior to mixing with the enzyme.

Effect of temperature on activity and stability. The effect of temperature on amylolytic activity and stability was evaluated between 20 and 100 °C. Amylolytic enzyme samples were incubated at the appropriate temperature for 5–30 min and cooled on ice for 5 min prior to the determination of residual activity.

Calcium ion. Enzyme samples were dialyzed against buffer A without calcium chloride for 24 h at 2 °C. The effect of calcium ions on amylolytic activity was examined by varying the calcium chloride concentration of the soluble starch solution prior to the addition of the enzyme solution.

Inhibition studies. α-amylase (50 μl) was mixed with an equal volume of inhibitor solution in buffer B (sodium acetate, 50 mM; calcium chloride, 3 mM; pH 5) to give a final acarbose concentration of 0–1 mg ml^{-1}. The mixture was pre-incubated for 15 min at 37 °C prior to the addition of the soluble starch solution to a final concentration of 10 mg.

Kinetics of α-amylase

The K_m and V_{max} of the purified *C. acetobutylicum* extracellular α-amylase was determined using soluble starch and amylose. The reaction mixture contained 50 μl purified α-amylase and 950 μl of buffer B containing 0–20 mg of either amylose or soluble starch. V_{max} was reported in mg of reducing sugars (determined as maltose) produced per

min under the reaction conditions specified and K_m was reported as mg of substrate used per ml.

RESULTS

Enzyme purification

The purification of extracelular α-amylase from *C. acetobutylicum* ATCC 824 is outlined in Table 1. The α-amylase demonstrated one protein peak following HPLC-gel filtration. The pooled fraction showed a 158-fold purification with a yield of 22% (Table 1).

The third ultrafiltration fraction (160-fold purification; Table 1) showed a single band with a molecular weight of 61 kDa on an SDS-PAGE (Fig 1, lane 3), which via activity staining demonstrated amylolytic activity (Fig. 2, lane 5). Although the anion-exchange fraction showed multiple protein bands on SDS-PAGE (Fig. 1, lane 2), only one band demonstrated activity when stained for amylolytic activity (Fig. 2, lane 1). Furthermore, activity staining demonstrated the presence of a single band in the *C. acetobutylicum* ATCC 824 crude enzyme preparation (Fig. 2, lane 2). These results suggest that only one type of amylolytic enzyme is being produced by *C. acetobutylicum* when grown on starch as the carbohydrate source. This enzyme was identified as α-amylase.

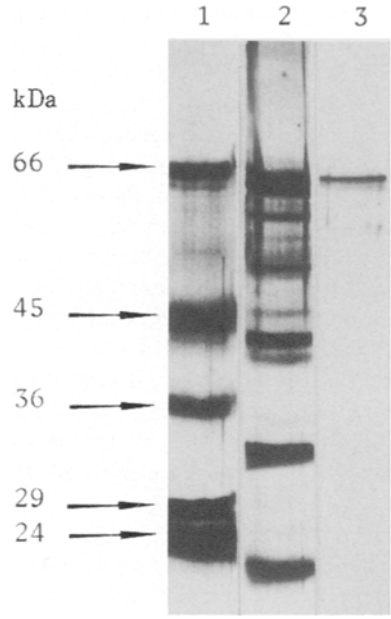

Fig. 1. SDS-PAGE electrophoretogram of protein from *C. acetobutylicum* ATCC 824. Lane 1: Low molecular weight marker: bovine albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa. Lane 2: 2nd ultrafiltration sample. Lane 3: 3rd ultrafiltration sample.

Substrate specificity and mode of action of α-amylase

The relative activity of the purified α-amylase on various substrates is shown in Table 2. The activity of α-amylase increased with an increase in the degree of polymerization of the substrate. α-Amylase displayed the highest activity on amylose and soluble starch and no activity on isomaltose, isomaltotriose, panose and pullulan, suggesting that this enzyme is not able to hydrolyze α-1,6-glucosidic linkages. Although enzyme activity on cyclodextrins was very low (up to 1.3%) relative to when soluble starch was the substrate, this activity indicates that this enzyme acts in an endo-fashion, since these substrates are not hydrolyzed by an exo-enzyme [4,7].

Hydrolysis products resulting from the action of purified *C. acetobutylicum* α-amylase on various substrates is shown in Table 2. Although maltose was slightly hydrolyzed to glucose, and upon extended incubation (2 h) the amount of glucose released was increased by 3-fold (data not shown), the reaction proceeded slowly. The hydrolysis of maltotriose by α-amylase resulted in almost equimolar amounts of

TABLE 1

Purification of the extracellular α-amylase from *C. acetobutylicum* ATCC 824

Purification step	Volume (ml)	Activity (U ml^{-1})	Protein (mg ml^{-1})	Specific activity (U mg^{-1} protein)	Total activity[a] (U)	Yield[b] (%)	Purification[c] (fold)
Culture supernatant	8000	0.9	0.31	2.9	7120	100	1
Ammonium sulfate fractionation	200	13.6	0.25	54.3	2988	42	19
1st ultrafiltration	20	137.7	2.18	63.1	2753	39	22
DEAE-sephadex A-50 chromatography	85	20.5	0.07	290.9	1731	24	101
2nd ultrafiltration	4	413.7	1.2	320.7	1655	23	112
HPLC-superose 6 gel-filtration	250	6.3	0.01	452.9	1585	22	158
3rd ultrafiltration	50	31.3	0.07	459.7	1563	22	160

[a]One unit of activity is defined as the amount of enzyme producing 1 μmol reducing sugar per min under the reaction conditions specified. [b]Based on total activity. [c]Based on specific activity.

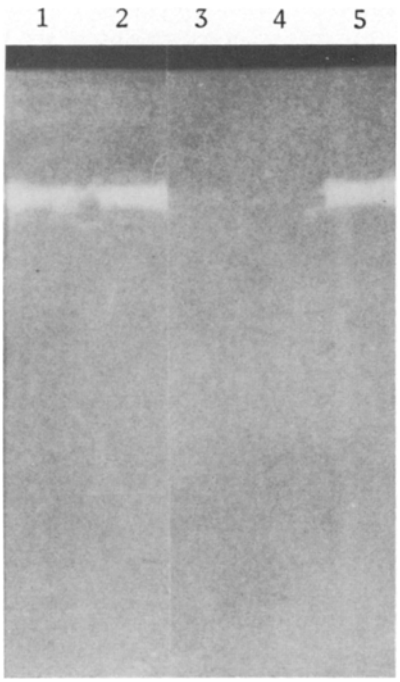

Fig. 2. SDS-PAGE activity stained proteins from *C. acetobutylicum* ATCC 824. Lane 1: 2nd ultrafiltration sample. Lane 2: Crude enzyme from *C. acetobutylicum* ATCC 824. Lane 3: Low molecular weight marker. Lane 4: High molecular weight marker: myosin, 205 kDa; bovine albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa. Lane 5: 3rd ultrafiltration sample.

glucose (0.67 μmol) and maltose (0.76 μmol). The % hydrolysis of amylose was ca. 92% (Table 2), consistent with the observation that hydrolysis by α-amylase increases with an increase in the linear chain length of the substrate in agreement with the relative activity values (see above). The hydrolysis of amylopectin by α-amylase was ca. 10-fold less than that of amylose (Table 2). This may be due to the highly branched α-1,6-glucosidic linkages of this carbohydrate [11]. The relative activity and rate of hydrolysis of α-amylase (Table 2) suggests that maltose, maltotriose and amylopectin are poor substrates for this enzyme in agreement with prior observations [6,10,24,30].

The initial products of amylose and soluble starch digestion by α-amylase following 10 min of incubation included traces of maltose, a small amount of maltotriose and larger amounts of higher oligosaccharides with maltotetraose and maltopentaose being the dominant products (data not shown). An increase in the incubation period to 30 min resulted in the formation of glucose and an increase in the levels of malto-oligosaccharides (maltose through maltohexaose; Table 2). Further incubation resulted in a decrease in the concentration of malto-oligosaccharides higher than maltotriose, with a corresponding increase of glucose, maltose and maltotriose in agreement with Paquet et al. [24].

Characterization of α-amylase

Optimum pH. α-amylase displayed the highest activity over a narrow range of pH (4.5–5.0) with a pH optimum at 4.8.

TABLE 2

Relative activities and hydrolysis products of *C. acetobutylicum* α-amylase on various substrates following 30 min incubation at 37 °C

Substrate (5 mg)	Relative activity[a] (%)	Hydrolysis products (mg ml^{-1})[b]						Total[c] (mg)	Hydrolysis[d] (%)
		G1	G2	G3	G4	G5	G6		
Maltose	0.1	0.02						0.02	0.4
Maltotriose	2.6	0.12	0.26					0.38	7.6
Maltotetraose	4.5	0.56	0.94	1.05				2.55	51.0
Maltopentaose	12.9	0.26	1.59	1.32	0.46			3.63	72.6
Maltohexaose	16.1	0.18	1.47	1.75	0.77	0.02		4.19	83.8
Amylose	129.9	0.04	0.58	1.20	1.03	1.31	0.42	4.58	91.6
Starch	100.0	0.04	0.43	1.02	0.51	1.25	0.42	3.67	73.4
Amylopectin	2.4	0.02	0.06	0.15	0.21	0.03	0.01	0.48	9.6

[a]% of activity obtained on soluble starch (considered as 100%) as determined by the DNS method. % relative activities on cyclodextrins (α, β and γ) were 0.1, 0.2 and 1.3%, respectively. No activity was detected on iosomaltose, panose, isomaltotriose or pullulan.
[b]Hydrolysis products higher than G6 released from amylose and soluble starch are not included. Products of cyclodextrin digestion were traces (less than 0.01 mg ml^{-1}) of G1 through G4. Isomaltose, isomaltotriose, panose and pullulan were not hydrolyzed by the purified α-amylase. G1, Glucose; G2, Maltose; G3, Maltotriose; G4, Maltotetraose; G5, Maltopentaose; G6, Maltohexaose.
[c]Sum of hydrolysis products produced by the enzyme activity.
[d]% hydrolysis = [total (mg)/total substrate used (5 mg)] × 100.

Effect of temperature. α-amylase displayed maximum activity between 50 to 60 °C with an optimum at 55 °C, but was rapidly inactivated at higher temperatures. An activation energy of 30.2 kJ mol^{-1} was calculated using the Arrhenius equation [28] which is similar to the value of 26.3 kJ mol^{-1} reported by Paquet et al. [24].

Effect of calcium. The addition of 2–5 mM of calcium ions (added in the form of calcium chloride) increased the α-amylase activity by ca. 20% (Fig. 3). A further addition of calcium chloride resulted in a 22% decrease in α-amylase activity.

Inhibition studies. Acarbose is a differential inhibitor of amylolytic enzymes such as glucoamylase, glucosyltransferase and α-amylase [8,21,29]. Fifty percent inhibition of *C. acetobutylicum* α-amylase by acarbose occurred at a concentration of 19 μg ml^{-1}. This is in good agreement with the value of 20 μg ml^{-1} reported for 50% inhibition of *Filobasidium capsuligenum* α-amylase.

Kinetics of α-amylase

The kinetics for α-amylase followed the Michaelis–Menten equation [28] on either amylose or soluble starch. The V_{max} and K_m of the α-amylase, calculated from Lineweaver–Burk plot, were 2.17 mg min^{-1} and 3.28 mg ml^{-1} for amylose and 1.67 mg min^{-1} and 1.73 mg ml^{-1} for soluble starch, respectively.

DISCUSSION

Paquet et al. [24] purified α-amylase from the supernatant of a glucose-grown *C. acetobutylicum* ATCC 824 culture. However, amylolytic enzyme biosynthesis in *C. acetobutylicum* ATCC 824 is subject to catabolite repression by glucose [1,2,5,9,11,14,19]. Previous work in our laboratory demonstrated that α-amylase is produced at a low basal or un-induced level and is primarily intracellular when *C. acetobutylicum* ATCC 824 is grown on glucose [1,2]. Since the specific amylolytic activity produced on starch was ca. 8-fold higher than that produced on glucose and the enzyme is primarily extracellular [1,2], the cell-free supernatant of a starch-grown *C. acetobutylicum* culture was used for the production of α-amylase.

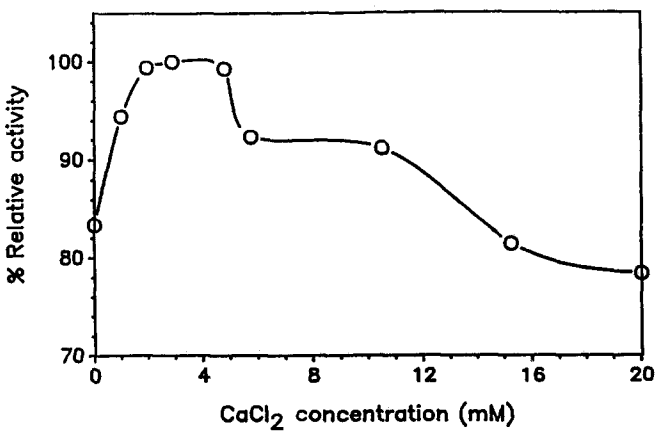

Fig. 3. Effect of calcium chloride on the relative activity of *C. acetobutylicum* ATCC 824 purified α-amylase when incubated at pH 5.0 and 37 °C.

Madi et al. [20] reported ultrastructural changes in the cell envelope of *Clostridium* sp. strain EM1 when grown on starch that allowed for continual secretion of amylases into the growth medium. They indicated that such changes were not apparent when this microorganism was grown on glucose. These observations suggest that there may be different processing systems for the secretion of clostridial amylase when the micororganism is grown on these two carbohydrates. These variations may also explain the observed differences in molecular weights of the *C. acetobutylicum* α-amylase recovered from a starch-grown (see below) versus a glucose-grown culture [24].

The % relative activity and pattern of products released by α-amylase activity on various substrates (Table 2) indicated that *C. acetobutylicum* α-amylase has endo-splitting activity much like that of an α-amylase which hydrolyses internal α-1,4-glucosidic bonds randomly and produces a mixture of malto-oligosaccharides [4,6,22,23,24,27,32]. Greater amounts of hydrolysis products were released following α-amylase action on amylose (Table 2) than that released from starch. This can be attributed to the highly branched α-1,6-glycosidic linkages associated with the amylopectin fraction of starch when compared to the linear α-1,4-linkages making up amylose. These results are in agreement with previous reports which indicated that maltose and maltotriose are poor substrates for α-amylase and that the rate of hydrolysis of amylopectin is slow [6,10,24,30].

The molecular weight of *C. acetobutylicum* α-amylase, as determined by SDS-PAGE, was 61 kDa (Fig. 1, lane 3), which differs considerably from the value of 84 kDa reported by Paquet et al. [24]. However, the value reported herein is in close agreement with the estimate of 61–68 kDa obtained by SDS-PAGE for the α-amylase derived from a starch-grown culture of different strains of *Bacillus* [23], *C. thermosulfurogenes* DSM 3896 [12] and different strains of yeast (7,32). The molecular weights of microbial α-amylases differ considerably, ranging from 22.5 to 100 kDa [10]. HPLC-gel filtration provided a lower estimate for the molecular weight of *C. acetobutylicum* ATCC 824 α-amylase (49 kDa, [24]). The variation observed between the molecular weights determined by SDS-PAGE and gel filtration has already been reported for different amylases [8,23,24].

The pH optimum of 4.8 for the *C. acetobutylicum* α-amylase is similar to that reported by Hockenhull and Herbert [14], although it differs from the value of 4.0 reported by Ingle and Erickson [15] and the value of 5.6 reported by Paquet et al. [24]. Although Ingle and Erickson [15] did not give any details on experimental conditions, Paquet et al. [24] used somewhat different buffering and assay conditions than employed in this study. The pH optimum reported herein is within the range of most microbial amylases [7,10,12,16,17,20,32].

The temperature optimum of 55 °C for *C. acetobutylicum*

ATCC 824 α-amylase differs from the value of 45 °C reported by Paquet et al. [24], but is similar to the value of 50 °C reported for the same enzyme [15] and the value of 60 °C reported for other clostridial species [12,30]. Although calcium ions increase the stability of most α-amylases at higher temperatures (see below), Paquet et al. [24] did not include this metal ion in their enzyme stability assays.

Although some microbial α-amylases are considered atypical in terms of their dependence on calcium, most are calcium metallo-enzymes having a minimum of one atom of this metal per molecule of enzyme which is required for catalytic activity [10]. While the addition of calcium has no effect on the activity of some microbial α-amylases [17,26,30], it did increase the stability of these enzymes at higher temperatures and extremes in pH. Asther and Meunier [3] reported that extensive dialysis of α-amylase from *B. licheniformis* in the absence of added calcium led to total enzyme inactivation. Although Paquet et al. [24] indicated that *C. acetobutylicum* ATCC 824 α-amylase contained seven calcium atoms per mole, they concluded that this metal ion does not stimulate α-amylase activity. The presence of tightly bound calcium atoms may explain the 83% residual activity of *C. acetobutylicum* ATCC 824 α-amylase in the absence of added calcium (Fig. 3). Furthermore, the lack of added calcium in P2 medium may explain the 58% loss of activity during ammonium sulfate precipitation (Table 1). The inhibition of α-amylase activity in the presence of more than 5 mM calcium in the reaction mixture (Fig. 3) is in agreement with other results which indicated that levels above the optimum can lead to a decrease in α-amylase activity [16,17].

Although the K_m value of 1.73 mg ml^{-1} for α-amylase on soluble starch compares favorably to the value of 2.08 mg ml^{-1} reported by Hockenhull and Herbert [16], it differs from the value of 3.6 mg ml^{-1} reported by Paquet et al. [24]. The K_m values found in the literature for different microbial α-amylases typically range between 0.35–2.7 mg ml^{-1} [14,20,32].

Although analysis of the kinetics of product formation from soluble starch by either the crude enzyme preparation or the purified extracellular α-amylase in the presence of increasing concentrations of acarbose (0–0.5 mg ml^{-1}) showed a decrease in all hydrolysis products, there continued to be formation of glucose at 0.5 mg ml^{-1} acarbose. Since glucoamylase is inhibited at this level, this result suggests that glucose is being produced by the action of α-amylase, and further supports the data which suggest that *C. acetobutylicum* ATCC 824 produces only α-amylase when grown in starch-based P2 medium. *C. acetobutylicum* ATCC 824 α-amylase has been reported to produce glucose from the cleavage of starch [24,31]. These results help to explain the previous reports which suggested the production of glucoamylase and α-amylase by *C. acetobutylicum* when grown on starch [5,9,11,14].

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