MULTIPLE FORMS OF *BACILLUS SUBTILIS* α-AMYLASE*

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The calcium- and zinc-requiring aggregation of *Bacillus subtilis* α -amylase is pH dependent. Enzyme aggregates form at pH 7.0 and 9.0 in the presence of 1 m*M* Ca^{2+} but not at pH 5.0. In the presence of zinc, enzyme aggregates form at pH 5.0 and pH 7.0. In the presence of calcium all forms show the same relative activity, but zinc aggregates at pH 7.0 show a higher relative activity.

INTRODUCTION

We reported previously the behavior of *Bacillus subtilis* α-amylase in SDS gel electrophoresis, in the absence of added calcium or zinc (1). We also reported a molecular weight of 48,000 daltons without added calcium or zinc, or after removal of zinc by chelation with EDTA. The existence of multiple forms of this enzyme in the presence of zinc has been very well documented (2, 3, 4); however, since most other divalent cations do not affect α-amylase, the effect of calcium without zinc on the multiple forms has not been extensively studied because of the calcium requirement for activity (5). In this study we describe the effect of calcium without zinc on the association of *B. subtilis* α-amylase into multiple forms.

MATERIAL AND METHODS

Bacillus subtilis α-amylase was purchased from Sigma Chemicals and was homogeneous as determined by acrylamide gel electrophoresis and by ultracentrifugation as previously described (1). The enzyme was dialyzed against 10 m*M* sodium acetate buffer, pH 5.0, for three days at 4 C with a continuous change in buffer and then lyophilized. Enzyme solutions were prepared in 0.2 *M* acetate buffer, pH 5.0, and 0.1 *M* phosphate buffer, pH 7.0 and pH 9.0. Calcium chloride and zinc chloride were reagentgrade chemicals. α-Amylase enzyme activity was determined by the use of Azure Amylose (6) purchased from Calbiochem. All activity determinations were made with identical protein concentrations. Sedimentation velocity experiments were completed with a Spinco Model E ultracentrifuge at 59,780 rpm at 20 C; no correction for the Johnson Ogston effect was made. In all determinations the enzyme solution was dialyzed against the appropriate buffer with 1.0 *M* KCl for 24-28 hr (1).

RESULTS AND DISCUSSION

Calcium is an activator and stabilizer for α-amylase from many sources (2). Table 1 shows the results of incubation of *B. subtilis* α-amylase with calcium and zinc at pH 5.0, 7.0 and 9.0 and activity measurements at pH 5.0 and 7.0. We found the highest relative activity when the enzyme assay was conducted at pH 5.0 even though the enzyme protein used for the assay was dialyzed at pH 7.0 and 9.0 respectively. The activity is relative to the α -amylase activity without calcium and we have designated the native enzyme as the preparation without added calcium or zinc after dialysis against sodium acetate buffer. Atomic absorption indicated that calcium and zinc levels were less than 0.1 g atom/mole (48,000 daltons).

TABLE 1. *Enzyme activation by calcium and zinc ions*

Additive (10^{-3} *M*)	Assay pH	Relative Activity
Ca^{2+}, pH 5.0	5.0	3
	7.0	2
Ca^{2+}, pH 7.0	5.0	3
	7.0	2
Ca^{2+}, pH 9.0	5.0	3
	7.0	2
Zn^{2+}, pH 5.0	5.0	1.5
	7.0	1.0
Zn^{2+}, pH 7.0	5.0	3
	7.0	2
EDTA, pH 11.8	5.0	0
	7.0	0
None, pH 5.0	5.0	1
	7.0	1

*Journal Article 3981 of the Oklahoma Agricultural Experiment Station.

TABLE 2. *Calcium and zinc effects on the sedimentation coefficient*

$c = 10^{-3}$ *M*	Observed Sedimentation Coefficient Values		
Ca^{2+}	(pH 5.0) 4.44 S MW $\simeq$ 48.5 $\times$ 10^3	(pH 7.0) 6.50 S MW $\simeq$ 96 $\times$ 10^3	(pH 9.0) 5.30 S MW $\simeq$ 72 $\times$ 10^3
Zn^{2+}	(pH 5.0) 5.49 S MW $\simeq$ 72 $\times$ 10^3	(pH 7.0) 7.46 S MW $\geqslant$ 96 $\times$ 10^3	(pH 9.0) —[a]

[a]At this pH zinc precipitated from solution.

Table 2 shows the effects of calcium and zinc on the sedimentation coefficient of *B. subtilis* α-amylase. Calcium without added zinc causes the enzyme to exist in multiple forms. Interestingly, it is customary to crystallize the enzyme in the presence of Ca^{2+} , and Kakiuchi *et al.* (3) always maintained their enzyme preparation in calcium acetate buffer for determining the sedimentation coefficient. This would explain why they obtained high sedimentation coefficient values even though there was no zinc present. We find a pH dependence of the sedimentation coefficient in the presence of calcium. Calcium, which is required for enzymatic activity, causes association of α-amylase into multiple forms; Robyt and Ackerman (4) also observed Zn^{2+} -induced associations.

A single symmetrical peak in the ultracentrifuge was observed in the presence of calcium at pH 5.0; the sedimentation coefficient was not affected by increasing the Ca^{2+} concentration. However, at pH 7.0 the enzyme in the presence of calcium showed an increase in the sedimentation coefficient. In the presence of zinc at pH 5.0 and 7.0 the sedimentation coefficients were 5.5 S and 7.5 S respectively. Since zinc hydroxide precipitates at pH 9.0, we were unable to determine a sedimentation coefficient value for the enzyme at this pH. We have also found the association of α-amylase in the presence of calcium and zinc to be reversible. That is, when we dialyzed the zinc- or calcium-treated enzyme in buffer without calcium or zinc, or with 1 m*M* EDTA, we obtained a sedimentation coefficient of 4.4 S, which corresponds to a molecular weight of 48,000 daltons.

These results indicate that calcium can cause *B. subtilis* α-amylase to associate and that the increased activity in the presence of zinc may be due to the associated properties. Even though calcium caused aggregation, the relative activity was not different for the aggregate forms when activity was measured at pH 5.0. Because of the differences that exist with zinc, one might raise the question of the total importance of zinc as a stabilizer for *B. subtilis* α-amylase.

ACKNOWLEDGMENT

This research was supported in part by NIH Grant AM 13489.

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