STUDIES ON TWO ISOZYMES OF ACONITASE FROM BACILLUS CEREUS T
I. PARTIAL PURIFICATION AND STABILITY

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SUMMARY: Earlier studies in our laboratory suggested the existence of an early and a late aconitase (EC.4.2.1.3) active at the 5 hr and 12 hr culture age, respectively. Present investigations indicate a 50% loss of early aconitase and complete loss of late aconitase in the presence of glucose and ethyl picolinate. Inhibition was not reversed by iron. The two aconitases were partially purified (9 to 10 fold) by ammonium sulfate precipitation and DEAE-cellulose column chromatography; the latter indicated that early aconitase is more anionic than late aconitase. Both enzymes are unstable and are rapidly inactivated at 30°C. At subzero temperatures, however, the two enzymes are more stable.

Enzymes of TCA§ cycle which remain inactive or absent during vegetative growth become functional during sporulation in aerobic bacilli (1). It has been realized for some time that the TCA cycle plays a key role in sporulation. Mutants of Bacillus subtilis lacking in aconitase (EC.4.2.1.3), an enzyme of TCA cycle, have been found to be asporogenic (2, 3). Some recent studies in our laboratory have indicated that aconitase may be involved at more than one stage of sporulation. It was observed that FAA, a typical inhibitor of aconitase, was effective in checking sporulation of Bacillus cereus T at two concentrations (4). Higher concentration of FAA inhibited the utilization of organic acids produced during growth. At lower concentration, the utilization of acid intermediates was normal, yet the sporulation was completely inhibited. It was proposed that possibly two isozymes of aconitase were involved: one "early aconitase" active during transition period, which is less sensitive to FAA inhibition than the second "late aconitase," whose role is not yet well defined. This was supported by the demonstration of two aconitase bands during

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§Abbreviations: TCA, tricarboxylic acid; FAA, fluoracetic acid; EP, ethyl picolinate; APA, alpha-picolinic acid; DEAE-cellulose, diethyl-aminoethyl-cellulose.

growth and sporulation of B. cereus T by polyacrylamide gel electrophoresis (5) One showed activity predominantly at 5 hr and the other at 12 hr. With the help of temperature-sensitive mutants, Goldman and Spotts have provided genetic evidence in support of the presence of two aconitases in B. subtilis (6).

Although these observations suggest the possible existence of two isozymes of aconitase in B. cereus T, direct evidence was lacking. The present communication describes a part of the separation, purification and characterization of the two activities.

METHODS:

Culture Conditions: B. cereus T was obtained from the USDA, Washington, D.C., and maintained on nutrient agar slants. The organism was grown on Glucose-yeast extract-minerals medium (modified G medium) (7, 8) by the active culture technique (9, 10) at 30°C on a gyrotary shaker (160 rpm). Routinely, three transfers of cells growing in logarithmic phase were made. Time of the last transfer is referred to as zero time, and subsequent hours before harvesting were used to define the age of the culture.

Preparation and Addition of Ethyl Picolinate (EP): Ethyl ester of alpha-picolinic acid was prepared in our laboratory by the method of Ulrich et al. with slight modifications (11, 12). An inhibitory concentration (3.7×10^{-2}M) of ethyl picolinate (7) was aseptically added to the culture at zero hour.

Preparation of Cell-Free Extract: The cells were harvested from a culture of desired age (5 hr or 12 hr) at 2000 rpm for 20 min in a Janetzky K-60 refrigerated centrifuge, washed twice with 50 ml each of cold phosphate buffer (0.05 M, pH 7.4), and suspended in 12 ml of cold buffer. The cells were disrupted in the Raytheon Sonic Oscillator (model DF 101) by applying current (0.95 and 1.10 amp, for 5 hr and 12 hr cells, respectively) for 15 min. The homogenate was centrifuged at 14,000 rpm for 40 min in a Sorvall (RC-2 model) centrifuge, and the supernatant used for enzyme purification. A part of the cell-free extract was boiled for 2 min, centrifuged, and the supernatant (boiled CFE) frozen for later use as stimulant for assay of aconitase activity in the purified enzyme preparations. All operations were performed at 0 to 4°C.

Ammonium Sulfate Fractionation: Nucleic acids from cell-free extract were precipitated by addition of $MnSO_4 \cdot H_2O$ to a final concentration of 10^{-2}M. Ammonium sulfate was then gradually added and dissolved to achieve 45% saturation. The solution was allowed to stand for 20 min, the resulting precipitate was centrifuged at 10,000 rpm for 10 min, and the residue dissolved in phosphate buffer to yield 0-45% ammonium sulfate fraction. Ammonium sulfate was gradually added to the supernatant and dissolved to achieve 80% saturation. The solution was again allowed to stand for 20 min and the precipitate was collected by centrifugation, as described above, and dissolved in the buffer to yield 45-80% ammonium sulfate fraction. The fractions were dialysed against 100 volumes of buffer for 5-6 hr with a change of buffer.

DEAE-cellulose Column Chromatography: The regenerated resin was packed in a glass column (1.5 x 30 cm) and equilibrated with elution buffer (phosphate buffer, 0.01 M, pH 7.0) using standard procedures (12). Eight ml of dialysed 45-80% ammonium sulfate fraction was slowly applied to the column and the latter washed with about 120 ml of elution buffer. A linear gradient from 0.2 M to 0.6 M NaCl in elution buffer was then applied to the column at a flow rate of 1 ml/min and fractions of suitable size collected. The aconitase activity and protein content were determined in the fractions, and the active fractions were pooled and used for characterization studies. Column chromatography was done at 8-10°C.

Assay of Aconitase (EC.4.2.1.3) Activity: The enzyme was assayed by the method of Hanson et al. (3). The reaction mixture (3 ml) contained 1.5 ml phosphate buffer (0.1 M, pH 7.4), 0.5 ml enzyme preparation (0.05 ml in case of cell-free extract) and 0.3 ml isocitric acid (0.01 M) as substrate. In case of ammonium sulfate and DEAE-cellulose column fractions, 0.5 ml boiled cell-free extract was also added as stimulant. The reaction was started by the addition of substrate, and the increase in optical density was measured in a 1 cm light path cuvette at 240 nm at 2 to 3 min intervals for 10 min against a blank, using a Beckman DU Spectrophotometer. An optical density change of 0.001/min under these conditions was defined as one unit of aconitase activity.

Determination of Protein Content: The protein content was estimated by the method of Lowry et al. (13) using bovine serum albumin as standard.

RESULTS AND DISCUSSION:

Ethyl Picolinate Sensitivity of Early and Late Aconitase (EC.4.2.1.3) in Presence and Absence of Glucose: It has been reported that APA inhibits sporulation by checking utilization of organic acids formed during growth, whereas EP inhibits sporulation but not utilization of organic acids (1). Further, APA, an inhibitor of sporulation known to suppress aconitase, was known to be effective only in the presence of glucose. It has been demonstrated in our laboratory that late (12 hr) aconitase was not detected in B. cereus T cells in the presence of EP; early (5 hr) aconitase was present (14). It was postulated that early aconitase is required mainly for utilization of acid intermediates formed during growth, whereas late aconitase plays a key role in sporulation. In the absence of glucose, acid intermediates are not formed; hence, early aconitase may not be present in the cells grown in the absence of glucose. It was considered that addition of EP or omission of glucose in the medium may selectively inhibit late or early aconitase, respectively, thus facilitating separation and purification of two aconitases. The results of such a study are presented in Table 1.

Contrary to expectation, both the omission of glucose and addition of EP inhibited the late aconitase activity more markedly than early aconitase. In the presence of glucose and EP, early aconitase activity was reduced to half, whereas late aconitase is virtually absent. Omission of glucose from the medium resulted in two-third reduction of early aconitase activity and about 90% reduction of late aconitase. Addition of Fe^{++} failed to reverse EP inhibition, thus indicating that inhibition is not due to removal of iron. Rather, enzyme levels were affected. Since activities of early aconitase in G^{+} + EP, and of late aconitase in G^{-} grown cells were low, these conditions were not of much help in their effective separation and purification. Cells grown on glucose were therefore used in these studies.

Partial Purification of Early and Late Aconitase: The results of a typical purification are presented in Table 2. A 9-10 fold purification was obtained by ammonium sulfate fractionation followed by DEAE-cellulose column chroma-

TABLE 1

EFFECT OF ETHYL PICOLINATE ON EARLY AND LATE ACONITASE (EC.4.2.1.3) ACTIVITY IN THE PRESENCE AND ABSENCE OF GLUCOSE

TREATMENT	ACTIVITY (units/ml)	PROTEIN (µg/ml)	SPECIFIC ACTIVITY
Early (5 hr) Aconitase Activity			
G^+ (control)	140	750	187
G^+ + EP	77	830	93
G^-	66	1060	62
G^- + EP	100	1200	83
G^+ + EP + $FeSO_4 \cdot 7H_2O$*	77	830	93
G^- + EP + $FeSO_4 \cdot 7H_2O$	113	1200	94
Late (12 hr) Aconitase Activity			
G^+ (control)	110	1510	73
G^+ + EP	3	850	3.5
G^-	5	600	8
G^- + EP	14	1060	13
G^+ + EP + $FeSO_4 \cdot 7H_2O$	11	850	13
G^- + EP + $FeSO_4 \cdot 7H_2O$	17	1060	16

Abbreviations: G^+, glucose present in the medium; G^-, glucose absent in the medium; EP, ethyl picolinate.

*$FeSO_4 \cdot 7H_2O$ concentration, 0.666×10^{-5} M.

tography. Most of the aconitase activity in each case was precipitated at 45-80% ammonium sulfate saturation. Fractionation into 45-60% and 60-80% saturation did not result in any further improvement in specific activity. At every purification step, specific activity of late aconitase was almost half that of early aconitase. It would not be reasonable to attribute the low specific activity to the nature of late aconitase per se in these partially purified preparations.

Representative DEAE-cellulose column elution patterns of early and late aconitase are presented in Figures 1(a) and 1(b). These enzymes eluted from the column at significantly different elution volumes, thus indicating that early and late aconitase are different proteins. Early aconitase seemed to

TABLE 2

PURIFICATION OF EARLY AND LATE ACONITASE (EC.4.2.1.3)

	STEP	VOLUME (ml)	ACTIVITY (units/ml)	TOTAL ACTIVITY (units)	PROTEIN (µg/ml)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (units/mg)	FOLD PURIFICATION	YIELD
Early (5 hr) Aconitase									
I	Cell Free Extract	30	866	25980	2000	60.0	433	1.0	100
II	0-45% Ammonium Sulfate Fraction	8	33	274	800	6.4	41	0.1	1
III	45-80% Ammonium Sulfate Fraction	12	888	10656	700	8.4	1270	3.0	41
IV	DEAE-cellulose Column Chromatography Fraction	7.6	133.2	1012.3	35	0.266	3800	9.0	3.8
Late (12 hr) Aconitase									
I	Cell Free Extract	30	444	13320	2200	66.0	201	1.0	100
II	0-45% Ammonium Sulfate Fraction	8	22	176	1500	12.0	15	0.075	1.3
III	45-80% Ammonium Sulfate Fraction	12	954	11448	1400	16.8	681	3.3	86
IV	DEAE-cellulose Column Chromatography Fraction	3.6	106.6	383.8	50	0.18	2132	10.6	2.8

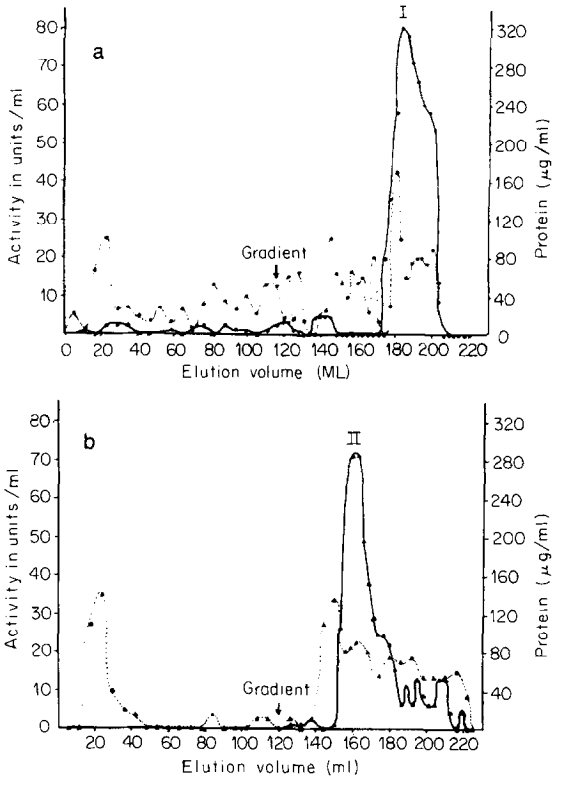

Figure 1. Elution on DEAE-cellulose column (1.5 x 20 cm) in 0.01 M phosphate buffer (pH 7.0), employing a linear NaCl gradient from 0.2 M to 0.6 M and collecting 3.8 ml fractions. Symbols: ————, aconitase activity; ---------, protein content. (a) Early aconitase (5 hr). (b) Late aconitase (12 hr).

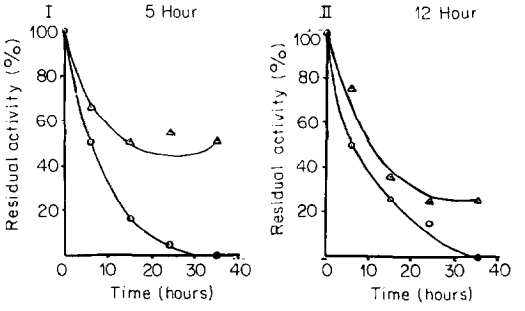

Figure 2. Percent residual activity of early (5 hr) and late (12 hr) aconitase as a function of time. Symbols: o, at room temperature, (30°C); Δ, at subzero temperature. 5 HOUR, early aconitase; 12 HOUR, late aconitase.

be more anionic and eluted in the form of a peak with a shoulder; maximum activity was obtained at 183 ml elution volume (Fig. 1(a)). In contrast, late aconitase is held on the column less tightly and the activity peak eluted at 160 ml.

Further purification of these enzymes to homogeneity is being attempted. It is necessary to perform all operations at 0 to 4°C and within 24 hr, however, to achieve 9 to 10 fold purification.

Stability of Early and Late Aconitase: Thermal stability of these enzymes was studied in phosphate buffer (0.01 M, pH 7.4) at room temperature (30°C) and at -5°C. The aliquots were drawn intermittently; residual activity was assayed and expressed as a percentage of the initial activity. It was observed that both enzymes were rapidly inactivated at room temperature (Figure 2), and were completely inactive within 30 hr. Early aconitase was more stable than late aconitase at subzero temperatures. The former was inactivated by 50% and the latter by 75% in 20 hr. Addition of iron and glutathione or ascorbate did not improve the stability. This rules out oxidative inactivation of these enzymes and the reason for high instability remains unknown. A protease may be present in the partially purified preparations which causes limited proteolysis of the enzymes at low temperatures. The difference in the residual activity may reflect the difference in the limit of proteolysis of two proteins. It is significant that Prestige et al. (15) observed a rapid increase in the activity of proteases with the development of refractile bodies in B. subtilis. It is difficult to rule out at this stage, however, that the stability pattern is not due to the presence of two enzyme proteins in each preparation and only one of them is rapidly inactivated at subzero temperature.

The present study provides support for the earlier findings suggesting the presence of an early and a late aconitase in the sporulating cells of B. cereus T. Isolation and partial purification of these proteins should enable us to further characterize the aconitases and show whether they are isozymes. Investigations to this effect are in progress.

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REFERENCES

1. Gollakota, K. G., and Halvorson, H. O. (1960) J. Bacteriol. 79, 1-8.
2. Hanson, R. S., Srinivasan, V. R., and Halvorson, H. O. (1963) J. Bacteriol. 85, 451-460.
3. Hanson, R. S., Srinivasan, V. R., and Halvorson, H. O. (1963) J. Bacteriol. 86, 45-50.

4. Rao, K. R., and Tilak, K. V. B. R. (1970) in Res. Bull. No. 2 (K. G. Gollakota, ed.) UPAU Press, Pantnagar, India, pp. 239-243.
5. Nanawati, G. C., and Sharma, D. (1970) in Res. Bull. No. 2 (K. G. Gollakota, ed.) UPAU Press, Pantnagar, India, pp. 244-248.
6. Goldman, R. C., and Spotts, C. R. (1971) Bacteriol. Proc. 135.
7. Gollakota, K. G. (ed.) (1970) Factors influencing dipicolinic acid synthesis and sporulation in bacilli, Res. Bull. No. 2, UPAU Press, Pantnagar, India.
8. Hanson, R. S. (1962) Ph.D. Thesis, Univ. Illinois, Urbana, Illinois.
9. Halvorson, H. O. (1957) J. Appl. Bacteriol. 20, 305-314.
10. Collier, R. E. (1957) in Spores (H. O. Halvorson, ed.) Amer. Inst. Biol. Sci., Washington, D. C., pp. 10-14.
11. Ulrich, A., Hang, and Hans, E. (1960) Chem. Ber. 93, 593-598. Cited, Chem. Abstr. 54, 153812.
12. Agrawal, P. K. (1972) Ph.D. Thesis, G. B. Pant Univ. Agr. & Technol., Pantnagar, India.
13. Lowry, O. H., Rosebrough, M. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
14. Jindal, R. C. (1971) M.Sc. Thesis, U.P. Agr. Univ., Pantnagar, India.
15. Prestige, L., Gage, V., and Spizzen, J. (1971) J. Bacteriol. 107, 815-823.