STUDIES ON TWO ISOZYMES OF ACONITASE FROM Bacillus cereus T.

III. ENZYMATIC PROPERTIES.

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SUMMARY

The present communication describes a comparative study of some enzymatic properties of an early and a late aconitase (EC.4.2.1.3) present in Bacillus cereus T cells of 5 and 12 hr culture age, respectively. The activity of both enzymes increased linearly with increase in enzyme concentration. They demonstrated similar pH (7.5) and temperature (30 C) optima, but differed in their activation energy and affinity for substrate. Late aconitase had higher activation energy (16,100 cal) as compared to early aconitase (9,200 cal). Early aconitase showed a Km value of 100×10^{-4} M for sodium citrate and 33.3×10^{-4} M for isocitrate. Late aconitase exhibited 5 to 7 times greater affinity for citrate and isocitrate yielding Km values 14×10^{-4} M and 7×10^{-4} M, respectively. On the basis of available evidence, it is suggested that early and late aconitase present in 5 and 12 hr aged cells of Bacillus cereus T behave as isozymes, and may be designated as aconitase (EC.4.2.1.3) isozyme I and aconitase (EC.4.2.1.3) isozyme II, respectively. The significance of their plausible role during growth and sporulation has been discussed.

INTRODUCTION

It has been realized since long that tricarboxylic acid (TCA)***cycle plays a key role in sporulation, and in fact, mutants of Bacillus subtilis lacking in aconitase (EC.4.2.1.3), an enzyme of TCA cycle, were found to be asporogenic (1,2). Later, some studies in our laboratory indicated the possible existence of an early and a late aconitase in Bacillus cereus T cells of 5 and 12 hr culture age, respectively; which differed not only in electrophoretic mobilities in polyacrylamide gel (3) but also in their sensitivity

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***Other Abbreviations: APA, alpha-picolinic acid; EMP, Embden-Meyerhof-Parnas Pathway; EP, ethyl picolinate; DEAE-cellulose, diethyl-aminoethyl-cellulose; FAA, fluoroacetic acid; PHB, poly-hydroxy-butyrate.

to certain inhibitors of sporulation, such as EP and FAA (4,5,6). These observations were supported by Goldman and Spotts (1971) who, with the help of temperature-sensitive mutants, could provide genetic evidence for the presence of two aconitases in Bacillus subtilis (7). In order to provide direct evidence, we partially purified the early and late aconitase present in Bacillus cereus T (8) and separated them individually as well as in a mixture with the help of disc electrophoresis in polyacrylamide gel, and DEAE-cellulose and Sephadex G-100 gel column chromatography (9).

Normally in case of isozymes it is assumed that their catalytic sites will be identical. However, the differences in their properties such as pH, and temperature optima, substrate specificity and affinity to the substrate can be expected on the basis that isozymes may differ in their primary structure and conformation . Such differences in their properties will exist to suit their physiological role. Hence, in order to provide an impeccable evidence in favour of their isozymic nature, it was considered desirable to make a comparative study of some of the enzymatic properties of partially - purified early and late aconitase present in 5 and 12 hr aged cells of Bacillus cereus T. This communication describes such a study.

MATERIALS AND METHODS

All investigations were made on Bacillus cereus T, obtained from United States Department of Agriculture, WASHINGTON, D.C.; and subsequently maintained at this laboratory on nutrient agar slants. The organism was grown in the "glucose-yeast extract-minerals medium" (modified G medium) (10,11) by the "active culture technique" (12,13) at 30 ± 1 C on a gyrotary shaker (speed 160 rpm). All characterization studies were done on the partially (9 to 10-fold)purified preparations of early and late aconitase, obtained by harvesting and washing the cells of 5 and 12 hr culture age, respectively, preparing the cell free extracts, precipitating out the enzyme at 45 to 80 % ammonium sulphate saturation, dialysing in cold against phosphate buffer (0.01 M,pH 7.0), and finally fractionating them on DEAE-cellulose columns equipped with a linear NaCl gradient (from 0.2M to 0.6M); employing the procedures described previously (8,9,14).The DEAE-cellulose column peak fractions containing early and late aconitase activity were separately pooled together and used as the source of enzyme for characterization studies done under similar conditions.

Assay of Aconitase (EC.4.2.1.3) Activity: The enzyme was assayed by the method of Hanson et al.(1963b)(2). The reaction mixture (3 ml)contained 1.5 ml phosphate buffer (0.1M,pH 7.4), 0.5ml enzyme preparation, 0.5ml boiled cell free extract (as stimulant)and 0.3ml isocitric acid (0.01M)as substrate. 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 mμ, at 2 to 3 min intervals, for 10 min, against a blank using a Beckman DU Spectrophotometer with a hydrogen lamp. An optical density change of 0.001 per min under these conditions was defined as one unit of aconitase activity.

Materials: DL-isocitric acid lactone was purchased from Biochemicals Unit, V.P.Chest Institute, Delhi-7(India). Yeast extract (certified grade) was purchased from Difco (USA); calcium chloride (G.R.grade) and sodium citrate (G.R.grade) from E.Merck,West Germany; and DEAE-cellulose from W & R Balston Ltd.,England. All other chemicals used were of highest purity (preferable AR grade)available from British Drug House (India).

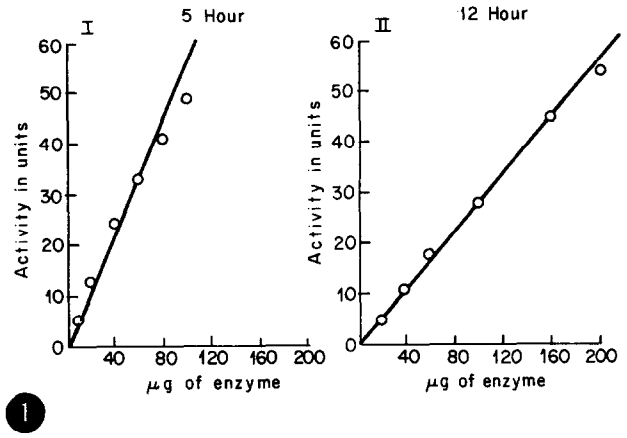
RESULTS

Effect of Enzyme Concentration: The effect of enzyme concentration on the rate of reaction as measured by standard assay procedure is presented in Fig.1. The rate of reaction is linear, in case of both early and late aconitase, with respect to enzyme concentration upto a concentration of 100 and 200 µg, respectively, under the assay conditions employed. Hence, enzyme concentration within this range was used in all the subsequent studies. It is also observed from Fig. 1 that same number of units of enzyme activity (60 units) are obtained by 100 µg of early and 200 µg of late aconitase, thus showing that the activity of early aconitase is twice that of late aconitase. It is possible that under the experimental conditions employed, the actual amount of aconitase protein present amongst the total cellular proteins at 5 hr culture age may be twice that in case of 12 hr age.

Effect of pH: To study the effect of pH on the rate of reaction catalyzed by early and late aconitase, following buffers were employed: 0.1M sodium phosphate buffer (pH 6.0 to 8.0) and 0.1M tris-HCl buffer (pH 8.5 and 9.0). The rest of the assay procedure was same as that for standard assay. It is revealed from Fig.2 that both enzymes are maximally active at a pH around 7.5, which is usually observed for aconitase from different sources (15,16, 17,18). However, late aconitase exhibits a sharper pH optimum (7.5) as compared to early aconitase which is maximally active in a rather broad pH range of 7.5 to 8.0 .

Effect of Temperature: It is observed that both enzymes are maximally active at a temperature around 30 C (Fig.3). As was the case with pH optimum studies, late aconitase exhibits a sharper temperature optimum at 30 C as compared to early aconitase which is maximally active in the temperature range of 30 to 35 C. Both enzymes show insignificant activities at temperatures beyond 45 C.

Table 1 summarises the Q_{10} values determined for the various temperature ranges, for both enzymes. The Q_{10} value for this enzyme catalyzed reaction is around or more than 2.0 near its optimum temperature (30 to 35 C). The Q_{10} values of much less than 1.0 encountered in the temperature range of 35 to 45 C for both enzymes are indicative of their inactivation at higher temperatures.



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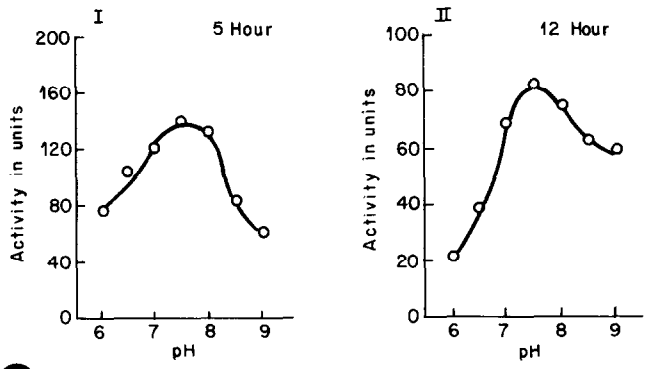

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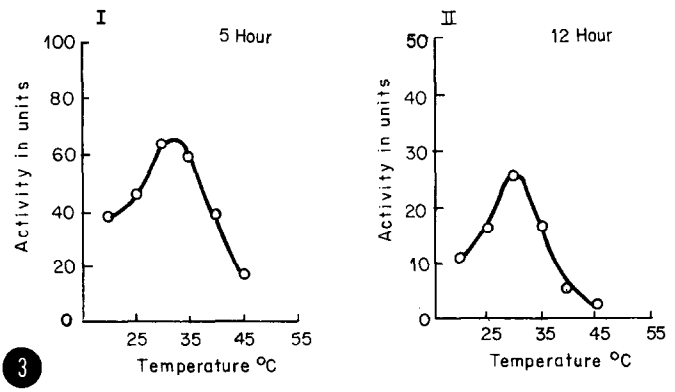

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Fig. 1 : Effect of enzyme concentration on the rate of reaction catalyzed by the early (5 hr) and late (12 hr) aconitase. Enzyme was added to 0.01 M isocitric acid solution used as substrate.

Fig. 2 : Effect of pH on the rate of reaction catalyzed by early (5 hr) and late (12 hr) aconitase, using 100 and 55/ug of enzyme protein respectively.

Fig. : 3: Effect of temperature on the rate of reaction catalyzed by early (5 hr) and late (12 hr) aconitase, using 100 and 55 /ug of enzyme protein , respectively.

Table 1. Temperature coefficient (Q_{10}) values of early and late aconitase.

S.No.	Temperature Range (C)	Early Aconitase	Late Aconitase
1.	20 - 30	1.65	2.32
2.	25 - 35	1.27	1.00
3.	30 - 40	0.60	0.21
4.	35 - 45	0.27	0.10

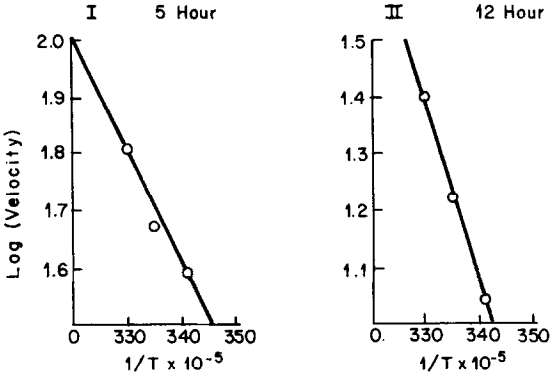
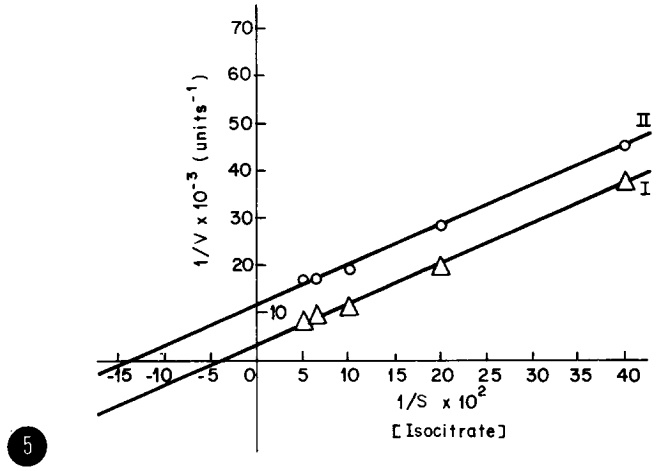


Fig. 4 : Arrhenius plot of log velocity vs. reciprocal of temperature (°K), for early (5 hr) and late (12 hr) aconitase.

The activation energy of early and late aconitase was calculated with the help of their respective Arrhenius plot (19) of log of enzyme activity against reciprocal of temperature (°K). Activation energy values of 9,200 and 16,100 cal were obtained for early and late aconitase, respectively, from their corresponding slopes (Fig.4). These values of the energy of activation for these enzymes are comparable to values obtained for aconitase (~ 13,000 cal) by earlier workers (20).

Substrate Specificity and Effect of Substrate Concentration: Since aconitase can convert both citric and isocitric acid into cis-aconitic acid, it was thought interesting to know if the two enzymes exhibit different affinity for these substrates.



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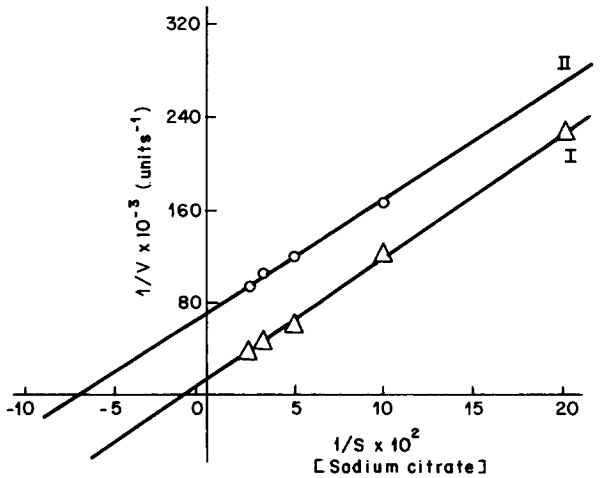

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Fig. 5 : Effect of isocitric acid concentration on the rate of reaction catalyzed by early (5 hr) and late (12 hr) aconitase, using 35 µg/ml of enzyme protein concentration. Symbols: I, early (5 hr)aconitase; II, late (12 hr) aconitase, Lineweaver-Burk plots.

Fig. 6 : Effect of sodium citrate concentration on the rate of reaction catalyzed by early (5 hr) and late (12 hr) aconitase, using 50 and 40 µg enzyme protein per ml, respectively. Symbols: I, early (5 hr)aconitase; II, late (12 hr) aconitase, Lineweaver-Burk plots.

The Lineweaver-Burk plots for both isocitric acid (Fig.5) and sodium citrate (Fig.6) were linear and permitted the calculation of the apparent Km values for these substrates in case of both enzymes. The Km values determined with isocitrate were 7×10^{-4}M and 33.3×10^{-4}M for late and early aconitase, respectively. The corresponding values for citrate were 14×10^{-4}M and 100×10^{-4}M for late and early aconitase, respectively.

Table – 2 – : Comparative properties of early and late aconitase isozymes present in Bacillus cereus T.

S.No.	Properties	Aconitase Isozyme I*	Aconitase Isozyme II**
1.	Sensitivity to Ethyl Picolinate	Less or Not sensitive	More Sensitive
2.	Purification level achieved	9 to 10 - fold	9 to 10 - fold
3.	Specific activity on purification	38,00 units/mg protein	2132 units/mg protein
4.	Inactivation	Rapid	Rapid
5.	DEAE-cellulose column adsorption:		
	i. Elution volume	183 ml	160 ml
	ii.NaCl concentration	265 mM	110 mM
	iii.Charge characteristics	More Anionic	Less Anionic
6.	Electrophoretic mobility in polyacrylamide gel	Less to anode	More to anode
7.	Sephadex G-100 gel column filtration:		
	i.Elution volume	18 ml	30 ml
	ii.Molecular weight	150,000 to 160,000	75,000 to 80,000
8.	pH optima	7.5 to 8.0	7.5
9.	Temperature optima	30 to 35 C	30 C
10.	Activation energy	9,200 cal	16,100 cal
11.	Km values:		
	i. For isocitric acid	33.3×10^{-4} M	7×10^{-4} M
	ii. For sodium citrate	100×10^{-4} M	14×10^{-4} M

* Early (5 hr) aconitase; Isozyme I
** Late (12 hr) aconitase; Isozyme II.

It is apparent from above that both enzymes have preferential affinity for isocitrate than for citrate, as is revealed from the fact that respective Km values for isocitrate, in case of both the enzymes, are 2 to 3 times less than those for citrate. Further, late aconitase has a much greater affinity for isocitrate in comparison to early aconitase, since it is evidently clear that the former has a Km value about 5 times less than that for the latter.

DISCUSSION

Since the enzyme preparations are only partially-(9 to 10-fold) purified, it would not be possible to attribute the low specific activity to the nature of late aconitase per se , despite of its lower slope of the curve (Fig.1). Both early and late aconitase showed almost identical pH optimum at 7.5, although the shapes of the curves were different (Fig.2). Early aconitase showed a broad pH optimum and had about60% activity at pH 6.0, whereas late aconitase was only one-fourth active at this pH.This may be due to the fact that early aconitase in the cell is operative when the pH of the culture is low (pH 5 to 7),whereas late aconitase is active only when the culture pH has already reached neutrality and is rather alkaline (pH 7 to 8).

Similar to pH optimum, the temperature optimum of both enzymes was in the same region (30 to 35 C); however, once again late aconitase exhibited a sharper temperature optimum (30 C)(Fig.3). It is difficult to attribute any physiological significance to this peculiarity. It may, however, be pointed out that the heat of activation (Fig.4) of early aconitase (9,200 cal) was much lower than that of late aconitase (16,100 cal). It is obvious, since at 12 hr culture age some of the enzymes present in the sporulating cells as well as the sporulating cells themselves acquire resistance to heat (21); and this acquisition of heat resistance is significantly influenced by the growth and sporulation temperature (22).

like nucleosidase(EC.3.2.2.1)

It is worthwhile to observe that during purification (8) and characterization studies total as well as specific activity of early aconitase was found to be considerably higher than those of late aconitase. It is possible that at early stage the enzyme has to be more active as lot of organic acids, viz.,pyruvate and acetate,are being utilized via TCA cycle, whereas late aconitase is mainly used for anabolic sequence (glyoxylate pathway). During late stages of sporulation, the reversible steps of EMP must be utilized for the synthesis of glycolytic intermediates (triose-phosphate, fructose 1,6-diphosphate) that are ultimately necessary for the de novo synthesis of spore membrane and hexosamine peptidoglycan (23,24). This seems quite logical as it is evident from Km studies (Fig.5 and 6) that late aconitase (Km, 7 x 10^{-4}M) has a much greater (about five times)affinity for isocitric acid than early aconitase (Km, 33.3 x 10^{-4}M). In addition, the PHB accumulated during transition period is also utilized for the synthesis of glycolytic intermediates. Hence, late aconitase may play its role through glyoxylate cycle as well during spore maturation. The foregoing discussion regarding the differences in the

characteristics of early and late aconitase depending upon their plausible physiological roles during growth and sporulation of Bacillus cereus T, is in line with the findings of Koen and Goodman (1969) who could separate a more anodal cytoplasmic aconitase from more cathodal mitochondrial one present in different organs of mouse; the tissue distribution studies revealed that spleen contained predominantly the cytoplasmic form, muscle the mitochondrial one, and other tissues varying proportions of each (18).

It is apparent from above and previous studies (8,9) that both early and late aconitase possess an almost similar pH and temperature optimum and are rapidly inactivated to the same extent. However, they differ markedly in their sensitivity to EP, elution pattern on DEAE-cellulose column, electrophoretic mobility in polyacrylamide gel, molecular weights, activation energy, substrate specificity and Km values. These observations strongly suggest that early and late aconitase present in Bacillus cereus T cells of 5 and 12 hr culture age, respectively; behave as isozymes and hence, on the basis of the recommendations of IUPAC-IUB Commission (1971) regarding the nomenclature of isozymes, the more anodal early (5 hr) aconitase may be designated as aconitase (EC.4.2.1.3)isozyme I, and the less anodal late (12 hr) aconitase as aconitase (EC.4.2.1.3)isozyme II. A summary of their characteristics is presented in Table 2.

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