STUDIES ON TWO ISOZYMES OF ACONITASE FROM Bacillus cereus T.

II. FURTHER EVIDENCE ON TWO DISTINCT ACTIVITIES.

P.K.AGRAWAL* , G.K.GARG** AND K.G.GOLLAKOTA.

Department of Biochemistry, College of Basic Sciences & Humanities, G.B.Pant University of Agriculture & Technology, PANTNAGAR-263145(U.P.) INDIA.

Received March 4,1976

SUMMARY

In our previous communication, we had reported the existence of an early and a late aconitase in Bacillus cereus T, active during 5 and 12 hr age of culture, respectively. This report was based on their partial purification and stability pattern studies. Present investigations deal with their specific separation by DEAE-cellulose column chromatography, polyacrylamide gel electrophoresis and Sephadex G-100 gel column chromatography, both individually and in mixture. On DEAE-cellulose column chromatography , early and late aconitase (EC.4.2.1.3) are eluted at 183 ml (265 mM NaCl concontration) and 160 ml (110 mM NaCl concentration)elution volume indicating former to be more anionic. In contrast to this, on polyacrylamide gel electrophoresis, early aconitase moves slower towards anode as compared to late aconitase, indicating former to be less anionic than later. The discrepancy has been ascribed to the molecular sieving effect of polyacrylamide, since Sephadex G-100 gel column chromatography has suggested the molecular weight of early aconitase (160,000) to be twice that of late aconitase. Our attempts to demonstrate that early aconitase was a dimer could not succeed.

INTRODUCTION

Importance of tricarboxylic acid (TCA)*** cycle during sporulation in bacilli was elucidated mainly with the studies on aconitase (EC.4.2.1.3.). The inhibitors of aconitase activity e.g.APA, FAA were found to be very potent inhibitors of sporulation (1,2,3). Further, the mutants of bacilli lacking in aconitase were found to be asporogenic(4,5). Earlier studies in our laboratory indicated the probable presence of an early and a late aconitase in Bacillus cereus T, at culture age of 5 and 12 hr,respectively,

* Present Address: Biochemistry Division, National Sugar Institute, KANPUR-208017 (U.P.) INDIA.

**Present Address: Biochemical Laboratories, Department of Chemistry, University of Illinois, URBANA,Illinois 61801.U.S.A.

***Other Abbreviations: APA, alpha-picolinic acid; DEAE-cellulose, diethyl-aminoethyl-cellulose; EP, ethyl picolinate; FAA, fluoroacetic acid.

which differed in their sensitivity to several inhibitors of sporulation, viz.,APA, FAA, EP etc.(1,2,6). Polyacrylamide gel electrophoresis of cell free extracts of sporulating cultures of Bacillus cereus T demonstrated two aconitase bands;one of them showing activity predominantly at 5 hr and the other at 12 hr culture age (7).

In our previous communication (8), we had reported the partial purification and stability characteristics of early and late aconitase present in Bacillus cereus T. Since both the enzyme preparations could not be fractionated to a considerably high degree of purity, the fact that both of them got eluted from DEAE-cellulose column at different elution volumes may merely be an experimental artefact.To eliminate this possibility, the two enzyme activities obtained from the same sporulating culture were specifically separated by subjecting them, both individually as well as in mixture, to DEAE-cellulose column chromatography, polyacrylamide gel electrophoresis and Sephadex G-100 gel column chromatography.

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)(9,10)by the "active culture technique"(11,12) at 30 ± 1 C on a gyrotary shaker (speed 160 rpm). All other cultural conditions and methods for preparation of cell free extracts, partial purification of early and late aconitase by ammonium sulphate fractionation, assay of aconitase activity and protein content have been reported previously (8,13). For specific separation of early and late aconitase, 5 and 12 hr aged cells were obtained from the same experiment and both the enzymes from their respective 45 - 80 per cent ammonium sulphate fractions were used for column chromatography on DEAE-cellulose.

DEAE-cellulose Column Chromatography: The resin was regenerated, packed upto a height of 20 cm in a glass column (1.5 x 30 cm), and equilibriated with "elution buffer" (phosphate buffer, 0.01M, pH 7.0) using standard procedures (13). Three identical columns of DEAE-cellulose were prepared in the usual manner. 8 ml samples each of early and late aconitase preparation (containing 5 and 10 mg protein, respectively) were loaded on two of the three columns separately. To the third column, a mixture of the 4 ml each of early and late aconitase preparation (containing 2.5 and 5 mg protein, respectively) was loaded. All the three columns were then run, one after other, in the same way and under identical conditions. 3 ml fractions were collected at 8 to 10 C and analysed for aconitase activity and protein content. The peak fractions were used for polyacrylamide gel electrophoresis and Sephadex G-100 gel column chromatography.

Polyacrylamide Gel Electrophoresis: Disc electrophoresis in polyacrylamide gels was conducted (13)according to the procedure of Davis (1964)(14) except that riboflavin was used instead of ammoniumpersulphate to catalyze the polymerization of the gels (15). Tris-glycine buffer, pH 8.6, served as the electrode buffer tray buffer and bromophenol blue as the 'tracking dye'. Early and late aconitase peak fractions from DEAE-cellulose columns were taken and concentrated against sucrose for 4 - 8 hrs at 5 C. The protein, 500 µg each of early and late aconitase, in 7M urea, was layered on the spacer gel under the buffer. Both enzyme preparations were applied, in duplicate, separately as well as in mixture. A current of 4 mA per tube was applied for about 2 hr at 5 C, until tracking dye moved to the bottom of the gel columns. The gels were cut at the centre of the tracking dye, stained for aconitase detection, and photographed. For aconitase detection, gels were incubated for 5 min in benzidine solution(2g in 10 ml of glacial acetic acid). Immediately thereafter, the gels were dipped in 1 per cent hydrogen peroxide for one minute and rinsed with running tap water. Aconitase bands were stained greenish blue which turned brown when fixed in 7 per cent acetic acid (7).

Sephadex G-100 Gel Column Chromatography: The molecular weights of the two aconitases were estimated by the gel filtration experiments according to Andrews (1965)(16). Sephadex G-100 was allowed to swell for 6 - 7 hrs in 0.05 M phosphate buffer (pH 7.0)containing 0.1 M KCl, on a water bath. The gel was deaerated under suction & packed to a column size 1 x 60 cm. The column was calibrated with 2 mg each of five reference proteins, viz.,cytochrome c, lysozyme, pepsin, aldolase and catalase. The fractions were collected at a flow rate of 9 ml per hour and the protein was determined by measuring the optical density at 240 mµ . Sucrose concentrated, DEAE-cellulose column peak fractions of early and late aconitase were analysed on this calibrated column, in turn. A 0.2 ml sample, in 20 per cent sucrose, was loaded on the column, the fractions were collected and aconitase activity determined. After the elution patterns of the two enzymes was ascertained separately, a 0.1 ml sample each of early and late aconitase was taken in 20 per cent sucrose, mixed well together, and loaded on the column. The fractions were collected and aconitase activity determined in the same way. A 0.05 M phosphate buffer (pH 7.0) containing 0.1 M KCl was used as elution buffer and all operations were done at 8 to 10 C.

Assay of Aconitase (EC.4.2.1.3) Activity: The enzyme was assayed by the method of Hanson et al.(1963b)(5). The reaction mixture (3 ml) contained 1.5 ml phosphate buffer (0.1 M,pH 7.4),0.5 ml enzyme preparation, 0.5 ml boiled cell free extract (as stimulant)and 0.3 ml isocitric acid (0.01 M) 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 nm, at 2 to 3 min intervals, for 10 min against a blank using a Beckman DU Spectrophotometer with hydrogen lamp. An optical density change of 0.001 per min under these conditions was defined as one unit of aconitase activity.

MATERIALS: Catalase, Cytochrome c and DL-isocitric acid lactone were purchased from Biochemicals Unit,V.P.Chest Institute, Delhi(India). Yeast extract (certified grade) was procured from Difco (U.S.A.); pepsin from E.Merck, West Germany; aldolase and lysozyme from Sigma Chemical Co.,U.S.A.; DEAE-cellulose from W & R Balston Ltd., England; and Sephadex G-100 from Pharmacia, Uppsala, Sweden. NaCl concentration in fractions was determined by the method and Chloride Testing Kit kindly provided by Sigma Chemical Co., U.S.A.All other chemicals used were of the highest purity (preferably Analar grade) available with B.D.H.(India).

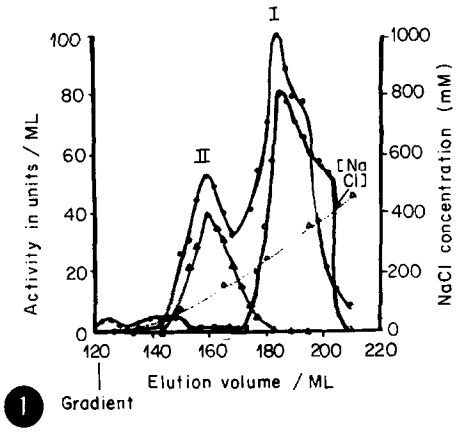
RESULTS AND DISCUSSION

The isozymes have been separated depending upon the net charge, size & shape, molecular weight of protein, any many other enzymatic properties. Electro-

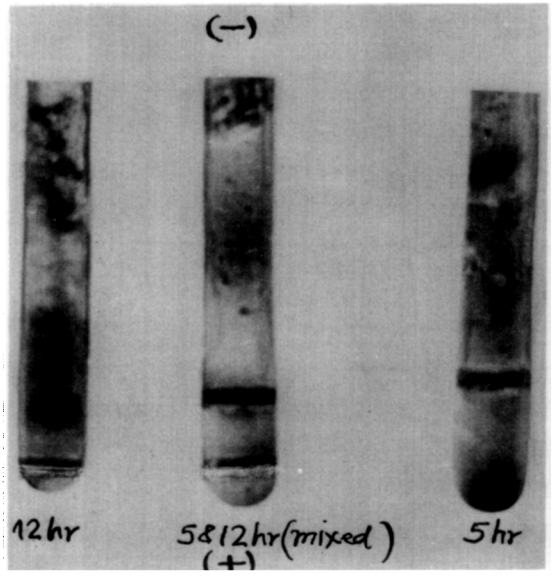
phoresis has been extensively used to separate isozymes on the basis of their electrophoretic mobilities (17,18). Several media such as agar gel, starch gel, polyacrylamide gel, cellulose acetate paper, cellulose powders have been used for electrophoresis and immunoelectrophoresis of isozymes. Column chromatography using DEAE-cellulose, DEAE-Sephadex, CM-cellulose etc. has been employed in isozyme separation depending upon the net charge over them. Gel filtration technique using Sephadex, Bio-gel etc. has also been employed to separate them on the basis of their molecular weights, size & shape of the protein molecules. Considering all the facts stated above, it was presumed that fractionation behaviour of early and late aconitase on DEAE-cellulose and Sephadex G-100 gel columns and electrophoretic mobility pattern on polyacrylamide gel (when subjected to these techniques individually and in mixture) would provide definite information to indicate if they were , in fact, same or different enzyme species.

Fractionation on DEAE-cellulose Column: The elution patterns of early and late aconitase, applied separately and in mixture, have been illustrated in Fig.1. It would be noted that when they are loaded on the column separately, early and late aconitase yield a single peak each (I and II, respectively) at 183 and 160 ml elution volume, respectively, as was observed in case of their partial purification (8). These elution volumes correspond to 265 and 110 mM concentration on the NaCl linear gradient. Incidentally, Fig. 1 also depicts two distinct peaks corresponding to the separate peaks for early and late aconitase; which are obtained on plotting the enzyme activities present in the eluate from the third column on which the mixture of early and late aconitase was loaded. It is apparent from these observations that early aconitase is held more tightly on the DEAE-cellulose column and, henceforth, elutes out at higher NaCl concentration (265 mM) as compared to the late aconitase which is held less tightly and elutes at lower (110 mM)NaCl concentration. This supports our earlier observation indicating early aconitase to be more anionic(8).

Separation on Polyacrylamide Gel Columns: The results with DEAE-cellulose clearly indicated that the two enzymes differed in their ionic charge on the molecule. Further attempts were made to distinguish them with the help of polyacrylamide gel electrophoresis. Disc electrophoresis of the two aconitases purified by DEAE-cellulose column chromatography revealed a single aconitase band each for both enzymes, when applied separately. It also exhibited two corresponding bands in case of gels on which the mixture of early and late aconitase was applied. It was observed that early aconitase moved slower towards anode as compared to late aconitase, suggesting that the former was less anioic than later (Fig.2).



1



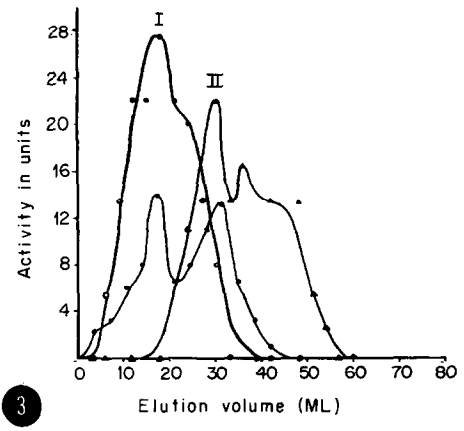
2

Fig.1 : Fractionation of early (5 hr) and late (12 hr) aconitase, separately and in mixture, on DEAE-cellulose columns (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 at 120 ml (↓)elution volume and collecting 3 ml fractions. Symbols: o—o ,early aconitase activity in fractions collected from the first column; ▲—▲ , late aconitase activity in fractions collected from the second column; ●—● , aconitase activity in fractions collected from the third column loaded with the mixture of early and late aconitase; Δ·····Δ , NaCl concentration in the effluent.

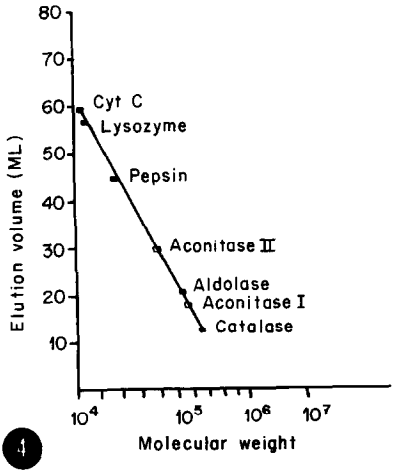
Fig.2 : Polyacrylamide gel electrophoresis of DEAE-cellulose column purified fractions of early (5 hr) and late (12 hr) aconitase, indicating migration from top (cathode) to bottom (anode). Symbols: 5 hr, early aconitase; 12 hr, late aconitase; mixed, early and late aconitase applied in mixture.

It may be noted that the conclusions reached with DEAE-cellulose column chromatography were otherwise. In that case early aconitase had eluted from the column at higher NaCl concentration indicating it to be more anioic than late aconitase. The discrepancy in case of polyacrylamide gel electrophoresis results suggesting early aconitase to be less anioic can be attributed to the molecular sieving effect of the polyacrylamide (19). It is possible that early aconitase is having higher molecular weight than late aconitase and this may retard its movement in the polyacrylamide gel. Therefore, molecular weights of both enzymes were determined using Sephadex G-100 gel column chromatography.

Molecular Weight Determination on Sephadex G-100 Gel Column: It is obvious from Fig.3 that



3



4

Fig.3 : Fractionation of DEAE-cellulose column purified early (5 hr) and late (12 hr) aconitase separately and in mixture, on Sephadex G-100 gel column (1.5 x 60 cm)in 0.05 M phosphate buffer (pH 7.0) containing 0.1 M KCl and collecting 3 ml fractions. Symbols: ○ , early aconitase activity in fractions collected from the column; ▲ , late aconitase activity in fractions; ● ,aconitase activity of the fractions collected after loading the column with both early and late aconitase, in mixture.

Fig.4 : Calibration curve for determination of molecular weights of early (5 hr) and late aconitase (12 hr) by gel filtration on Sephadex G-100 column.Plot of elution volume against molecular weights of proteins on a semi-logarithmic graph paper.Symbols: ACONITASE I, early (5 hr) aconitase; ACONITASE II, late (12 hr) aconitase.

both aconitases are eluted from the column at different elution volumes. Whether loaded on the column individually or in mixture, almost similar elution patterns were are obtained. The early aconitase activity elutes in the form of a peak at 18 ml elution volume whereas late aconitase activity comes out of column to form a peak with shoulder at an elution volume of 30 ml. Further, two corresponding peaks are observed in the case of elution pattern obtained when the mixture of both enzymes is applied on the column. The corresponding molecular weights for early and late aconitase calculated on the basis of the calibration curve(Fig.4) appear to be in the range of 150,000 - 160,000 and 75,000 - 80,000; respectively.

It is clear from the present studies that early and late aconitase differ in their charge and molecular weight, thus providing additional evidence in favour of their distinct activities. However, it does not necessarily mean that these two enzymes are products of two different genes. The molecular weight

of early aconitase was approximately twice that of late aconitase. Based on this, a dimer - monomer relationship can not be ruled out, however, our attempts to establish this could not succeed.

Retardation of early aconitase on DEAE-cellulose and polyacrylamide may be attributed to its high molecular weight. On DEAE-cellulose the total charges on this molecule will be approximately twice that of late aconitase, hence it will bind more tightly. However, if unit charges on molecules should remain same, if not less, then both molecules should show similar mobility on electrophoresis. The slow movement of early aconitase on polyacrylamide gel electrophoresis should be attributed to the molecular sieving effect of the gel and high molecular weight of the enzyme.

It may be noted that Nanawati and Sharma (1970) showed two bands of aconitase both in cell free extracts prepared from 5 and 12 hr aged cells (7). They observed that only one band out of two was maximally active at 5 hr whereas the other was predominantly active at 12 hr. Since in the present studies the DEAE-cellulose column was monitored only by following aconitase activity, it is expected that only one of the two enzyme species will be detected; whereas in case of polyacrylamide gel electrophoresis, the benzidine stain will detect all iron-containing-proteins.

Further, Kandala (1972) has shown that it is mostly iron level in the cell which controls the aconitase activity (20). However, exact mechanism in this regard will be difficult to predict. Evidently, if it is assumed that only iron was regulating the activity of aconitase, and both early and late aconitases were present, it is difficult to explain how iron selectively chooses one enzyme species at a time and not the other. Probably, the intracellular environment of the cell at 5 and 12 hr culture age is such that only one of the two enzymes present at a time achieves an active conformation.

The evidence obtained in the present studies appears sufficient to establish that early aconitase active at 5 hr culture age is different from late aconitase active at 12 hr culture age. Further investigations to characterize them more specifically have been made and would be reported elsewhere.

ACKNOWLEDGEMENTS

We are grateful to the Indian Council of Agricultural Research, New Delhi for the award of Senior Research Fellowship to one of us (PKA) during the period of investigation.

REFERENCES

1. Gollakota,K.G.and Halvorson,H.O.(1960).J.Bacteriol.,79: 1-8.
2. Rao,K.R.and Tilak, K.V.B.R.(1970).pp.239 - 244.In:Gollakota,K.G.
3. Sharma, M.K.and Narayan, R.(1970).pp.231 - 238.In:Gollakota,K.G.
4. Hanson, R.S.;Srinivasan,V.R.and Halvorson,H.O.(1963a).J.Bacteriol.,85:451-460.
5. Hanson, R.S.;Srinivasan,V.R.and Halvorson,H.O.(1963b).J.Bacteriol.,86:45-50.
6. Jindal,R.C.(1971).M.Sc.Thesis.U.P.Agricultural University,Pantnagar(India).
7. Nanawati, G.C.and Sharma, D.(1970).pp.19,244-248.In:Gollakota, K.G.
8. Agrawal,P.K.;Garg,G.K.and Gollakota,K.G.(1975).Biochem.Biophys.Res. Commun.,67:2, 645.
9. Gollakota, K.G.(1970). (ed.)Factors influencing dipicolinic acid synthesis and sporulation in bacilli.Res.Bull.No.2.U.P. Agricultural University Press,Pantnagar(India).
10. Hanson, R.S.(1962).Ph.D.Thesis.University of Illinois,Urbana,Ill.,U.S.A.
11. Collier,R.E.(1957).In:Spores (ed.)H,O,Halvorson,Am.Inst.Biol.Sci.,Washington, D.C.,p.10-14.
12. Halvorson, H.Orin.(1957).J.Appl.Bacteriol.,20:305-314.
13. Agrawal,P.K.(1972).Ph.D.Thesis,G.B.Pant University of Agriculture & Technology, Pantnagar(India).
14. Davis,B.J.(1964).Ann.N.Y.Acad.Sci.,121:404-427.
15. Fantes, K.H.and Furminger,I.G.S.(1967).Nature, 215: 750-751.
16. Andrews,P.(1965).Biochem.J.96:595-606.
17. Markert,C.L.(1968).Ann.N.Y.Acad.Sci.,151:14-40.
18. Markert, C.L.and Møller,F.C.(1959).Proc.Natl.Acad.Sci.,45:753-763.
19. Work,T.S.and Work,E.(1969).(eds.)Laboratory techniques in biochemistry and molecular biology.Elsevier Pub.Co.,Amsterdam, North Holland.
20. Kandala,J.C.(1972).M.Sc.Thesis.G.B.Pant University of Agriculture & Technology Pantnagar(India).

****