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Purification of the constituent enzyme fractions of mycobacillin synthetase

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The final purification of the three-fraction enzyme complex mycobacillin synthetase was done by hydroxyapatite column chromatography and sucrose-density-gradient centrifugation; each of the fractions obtained migrates as a single component in SDS/polyacrylamide-gel electrophoresis and gel electrofocusing. The M_r of the enzyme fractions A, B and C by gel filtration is 260000, 190000 and 105000, and that by SDS/polyacrylamide-gel electrophoresis is 252000, 198000 and 108000 respectively. None of the enzyme fractions appears to possess subunit structure.

INTRODUCTION

Extensive purification of the multifunctional enzyme complexes responsible for peptide-antibiotic synthesis have been reported for gramicidin S, tyrocidine, bacitracin etc. Gramicidin S synthetase consists of two complementary enzymes, light enzyme (M_r 100000) and heavy enzyme (M_r 280000) (Koischwitz & Kleinkauf, 1976*b*; Kleinkauf *et al.*, 1969). The light enzyme was purified almost to a pure state by chromatography on DEAE-cellulose amino-free D-phenylalanine–Sepharose and carboxyl-free D-phenylalanine–Sepharose (Kanda *et al.*, 1978). The heavy enzyme was purified almost to the pure state by chromatography on L-ornithine–Sepharose, DEAE-cellulose and Ultrogel filtration (Hori *et al.*, 1978). However, the methods of purification of the two enzyme fractions have been modified differently in different laboratories (Koischwitz & Kleinkauf, 1976*a*; Vater & Kleinkauf, 1976). Purification of the tyrocidine-synthesizing system yields three complementary enzymes, light (M_r 100000), intermediate (M_r 230000) and heavy (M_r 460000). The intermediate and heavy enzymes have been isolated in 90% -pure form, whereas the light enzyme was obtained essentially in pure form (Lee *et al.*, 1973). Leupeptin acid synthetase (E_2) was partially purified by Sephadex G-200 gel filtration and DEAE-cellulose column chromatography, and its M_r is about 260000 (Suzukake *et al.*, 1979). The enzyme complex of bacitracin synthetase, which can be resolved into three complementary enzymes (Frøyshov, 1974), named A (M_r 200000), B (M_r 210000) and C (M_r 360000) (Ishihara *et al.*, 1975), has been highly purified by Ultrogel filtration and hydroxyapatite chromatography after fractionation with streptomycin sulphate and $(NH_4)_2SO_4$ (Roland *et al.*, 1977).

Mycobacillin (Majumdar & Bose, 1958) is a cyclic tridecapeptide antibiotic produced by *Bacillus subtilis* B_3 (Majumdar & Bose, 1960; Banerjee & Bose, 1963). The partial purification and fractionation of the mycobacillin-synthesizing enzyme system by gel filtration on Sephadex G-200 revealed that the enzyme complex involved in the synthesis consists of three fractions (A, B and C) (Ghosh *et al.*, 1983).

The present paper described the isolation, homogeneity and monomeric or multimeric nature of the constituent enzyme fractions of mycobacillin synthetase.

MATERIALS AND METHODS

Chemicals and radiochemicals

Column materials (Sephadex G-200, DEAE-cellulose, hydroxyapatite) and the calibration proteins (β-glucuronidase, bovine liver catalase, yeast alcohol dehydrogenase, yeast hexokinase) used for M_r determination were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). U-^{14}C-labelled *Chlorella*-protein hydrolysate (sp. radioactivity 42 mCi/mg-atom of C) were purchased from Bhabha Atomic Research Centre (Trombay, India). Other chemicals used in the experiment were obtained from commercial sources.

Growth of organism

A mycobacillin-producing strain of *Bacillus subtilis* B_3 (Majumdar & Bose, 1958) was grown for 15–16 h at 30±1 °C with vigorous shaking in a fresh nutrient broth (Sengupta & Bose, 1971) supplemented with 1% glucose. The cells were collected by centrifugation and washed with 0.9% NaCl and 5 mM-$MgCl_2$.

Purification of the enzyme

The initial steps for the purification, i.e. preparation of 20000 *g* supernatant, precipitation with $(NH_4)_2SO_4$, column chromatography on Sephadex G-200 and DEAE-cellulose, were identical with those used previously for the purification of mycobacillin synthetase (Ghosh *et al.*, 1983).

Chromatography on hydroxyapatite

All three enzyme fractions A, B and C recovered from DEAE-cellulose were concentrated separately, dissolved in a minimum volume of 0.02 M-sodium phosphate buffer, pH 7.2, containing 0.25 mM-EDTA, 5 mM-$MgCl_2$ and 1 mM-dithiothreitol, dialysed against 0.02 M-sodium phosphate buffer, pH 7.2, containing 0.25 mM-EDTA, 5 mM-$MgCl_2$ and 1 mM-dithiothreitol and then applied to the hydroxyapatite column (2 cm × 6 cm) pre-equilibrated with the same buffer. The column was washed with 48 ml of the above buffer, and then stepwise elution was performed with 0.05 M-, 0.08 M-, 0.10 M-, 0.12 M-, 0.14 M- and 0.18 M-phosphate buffer containing the other ingredients at a rate of 16 ml/h. Each of the fractions was monitored by A_{280} and also by enzyme

activity in terms of mycobacillin formation in the presence of complementary enzyme fractions.

Sucrose-density-gradient centrifugation

Hydroxyapatite-purified enzyme fractions were concentrated, dialysed separately against 500 ml of buffer B (50 mM-Tris/HCl, pH 7.8, containing 0.25 mM-EDTA, 10 mM-$MgCl_2$ and 1 mM-dithiothreitol) to about 1 ml and subjected to sucrose density gradient centrifugation. A discontinuous sucrose gradient was prepared by layering in succession 2 ml each of 22.5%, 20%, 17.5%, 15%, 12.5% and 10% (w/v) sucrose in buffer B over a 40% (w/v) sucrose layer in buffer B as the cushion. Each of the enzyme fractions was separately applied over the above gradient and were then centrifuged for 8 h at 40000 rev./min in a Beckman SW40 rotor at 0 °C. Fractions (0.4 ml) were collected and tested for enzyme activity with complementary enzyme fractions. All the subsequent studies were performed with these enzyme fractions.

Polyacrylamide-disc-gel electrophoresis

Gels containing 5% (w/v) acrylamide and 0.28% *NN'*-methylenebisacrylamide were polymerized in gel tubes to which specified enzyme fractions in buffer were applied. Electrophoresis was carried out in a glycine/Tris buffer system containing 1.44% (w/v) glycine and 0.3% (w/v) Tris (pH 9.4), at 4 °C and 2 mA/tube until the tracking dye reached the separation gel and at 4 mA/tube thereafter, and stained for protein with Coomassie Brilliant Blue R 250 and destained in 5% (v/v) methanol/7.5% (v/v) acetic acid (Ornstein, 1964; Davis, 1964).

For SDS/polyacrylamide-gel electrophoresis, gels were polymerized as usual with 0.2% SDS, and the running buffer used contained 0.2% SDS and 0.2% 2-mercaptoethanol. The enzyme samples were also treated with 0.2% SDS and mercaptoethanol, usually for 15 min at 50 °C, and then applied to the gel. The gels were run as above and then stained and destained as usual (Weber & Osborn, 1969).

Analytical isoelectric focusing

Isoelectric focusing was performed in a Sephadex IEF gel slab containing Pharmalite (Pharmacia Fine Chemicals), and it was prepared according to the instructions of the manufacturer. The electrolyte solution contained 0.1 M-NaOH at the cathode and 40 mM-aspartic acid at the anode. The carrier Pharmalite solution in the pH range 3.0–10.0 was used. The pH gradient (3.0–10.0) was established by running the gel for 2 h at 500 V before application of the sample. The samples were applied on the surface of the gel, and focusing was then carried out at 1000 V for 3 h. After the run the focused proteins were blotted in nitrocellulose paper and fixed in a solution of 10% (w/v) trichloroacetic acid and 5% (w/v) sulphosalicylic acid overnight. Staining and destaining were done by the modified method of Wrigly (1971).

Measurement of mycobacillin synthetase activity

The incubation mixture and the method of isolation of radioactive mycobacillin were the same as described by Ghosh *et al.* (1983), except that the enzyme fraction to be tested was used in 50 μg amounts in the presence of sufficient amounts of the complementary enzyme fractions to saturate the system.

Determination of M_r

By gel filtration. The M_r of the enzyme fractions was determined by gel filtration (Reiland, 1971) on Sephadex G-200 (2.5 cm × 70 cm). Elution was performed with buffer B and the column was calibrated with β-glucuronidase (M_r 280000), bovine liver catalase (248000), yeast alcohol dehydrogenase (150000) and yeast hexokinase (96000). The distribution coefficient ($K_{av.}$) was determined from the relation $K_{av.} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the protein, V_0 the void volume and V_t the total volume of the gel bed.

By SDS/polyacrylamide-gel electrophoresis. SDS/polyacrylamide-gel electrophoresis as described by Weber & Osborn (1969) was used for estimation of M_r. Thyroglobulin (M_r 330000), ferritin (220000, 18000), rabbit phosphorylase *b* (94000), bovine serum albumin (66000), catalase (58000) and lactate dehydrogenase (36000) were used as the standard marker proteins.

Determination of protein concentration

This was done by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

RESULTS

Purification of the three enzyme fractions of mycobacillin synthetase on hydroxyapatite column

The protein content and enzyme-activity profiles of the three enzyme fractions as eluted by phosphate buffer are shown in Fig. 1, which shows that the fractions eluted by 0.10–0.14 M-phosphate buffer contained enzyme fraction A, those by 0.08–0.12 M-phosphate enzyme fraction B, and by 0.05–0.08 M-phosphate enzyme fraction C. The protein peaks and the enzyme-activity peaks coincided for all three fractions. However, there were some other minor protein peaks (not shown in the Figure), which did not contain any enzyme activity.

Purification of the three enzyme fractions of mycobacillin synthetase by sucrose-density-gradient centrifugation

All the peak fractions corresponding to each of the three enzyme fractions A, B and C chromatographed separated on hydroxyapatite columns were pooled together, layered on discontinuous sucrose gradients and centrifuged. The sedimentation patterns show that under these conditions the peak activity of enzyme fraction A sedimented at 20%, of fraction B at 17.5% and of fraction C at 12.5% sucrose (results not shown).

SDS/polyacrylamide-gel electrophoresis of mycobacillin synthetase

The homogeneity of the mycobacillin synthetase preparation, tested by polyacrylamide-gel electrophoresis at pH 9.4, is shown in Fig. 2. It appears that each of the enzyme fractions (A, B and C), finally purified by sucrose-density-gradient centrifugation, migrates as a sharp single band on native disc-gel electrophoresis. Each of the enzyme fractions on a 0.2%-SDS/polyacrylamide gel also appears to have a single band which could not be dissociated into subunits, even by more extensive treatment of the enzyme fraction with 2% SDS and 1% 2-mercaptoethanol for 60 min.

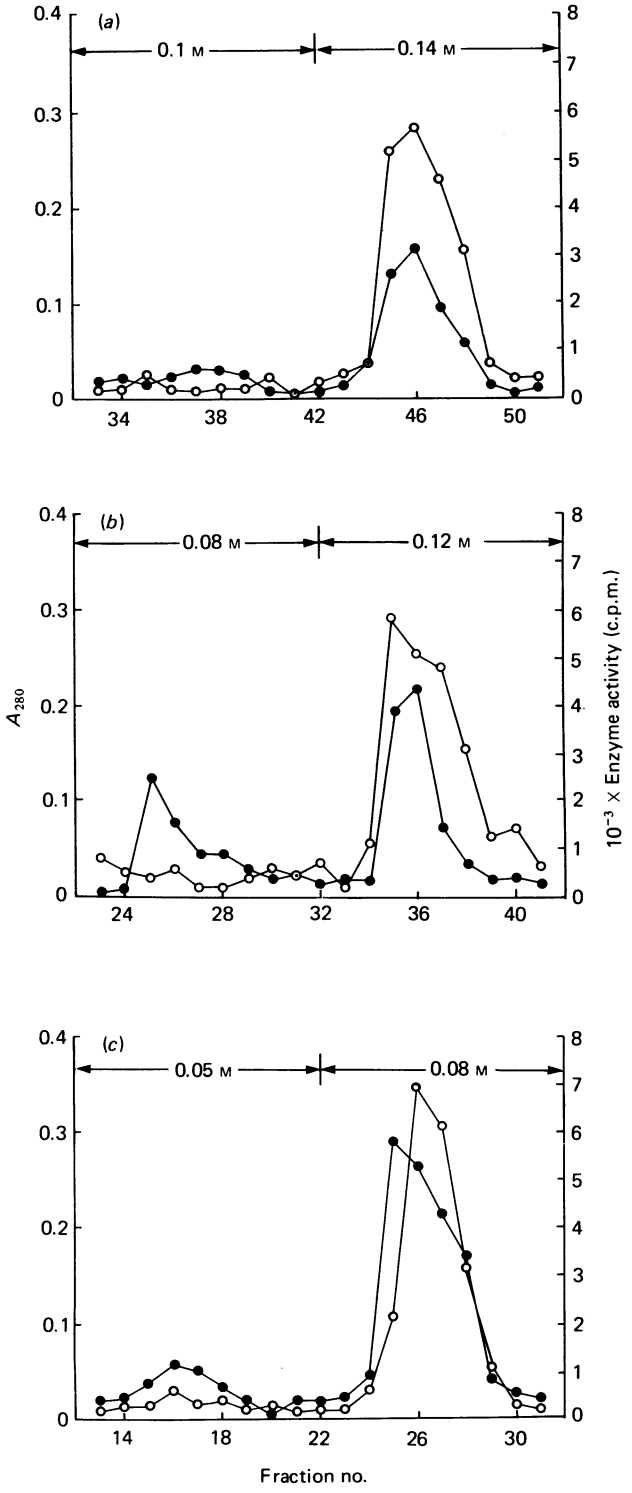

Fig. 1. Adsorption chromatography on hydroxyapatite of the components A (*a*), B (*b*) and C (*c*) of mycobacillin synthetase

The DEAE-cellulose peak fractions were pooled, dialysed and separately applied to hydroxyapatite columns (2 cm × 6 cm). After washing with 48 ml of phosphate buffer the column was eluted stepwise with increasing concentrations of phosphate buffer as shown. Each collected fraction (4 ml) was monitored both for A_{280} (●) and by assay of enzyme activity (○) in the presence of complementary enzyme fractions plus mycobacillin constituent amino acids.

Fig. 2. Polyacrylamide-disc-gel electrophoresis of the purified enzyme fractions of mycobacillin synthetase

About 50 μl of each of the fractions obtained after sucrose-density-gradient purification was applied to polyacrylamide gel (5%). The electrophoresis was carried out at 4 mA/gel for 4 h with Tris/glycine buffer.

Isoelectric focusing of mycobacillin synthetase

The homogeneity of each of the three fractions of mycobacillin synthetase was determined by isoelectric focusing on Sephadex gels, by using Pharmalite in the range pH 3.0–10.0. Each of the fractions gave a single band (results not shown). The pI values were 6.0, 5.8 and 6.0 for fractions A, B and C respectively.

M_r of the three fractions of mycobacillin synthetase

By gel filtration on Sephadex G-200. Each of the fractions obtained from sucrose-density-gradient centrifugation was chromatographed on the column along with marker proteins. The distribution coefficient calculated from the elution pattern was plotted against $\log M_r$ of the marker proteins (Fig. 3*a*), which shows M_r values of fractions A, B and C of 260000, 190000 and 105000 respectively.

By SDS/polyacrylamide-gel electrophoresis. Each of the fractions obtained from the sucrose density gradient was run on SDS/polyacrylamide gel along with marker proteins. The relative mobility of the proteins were plotted against $\log M_r$ of the marker proteins (Fig. 3*b*), which shows M_r values of fractions A, B and C of 252000, 198000 and 108000 respectively.

DISCUSSION

In continuation of our previous work, when we reported the partial purification and fractionation of the

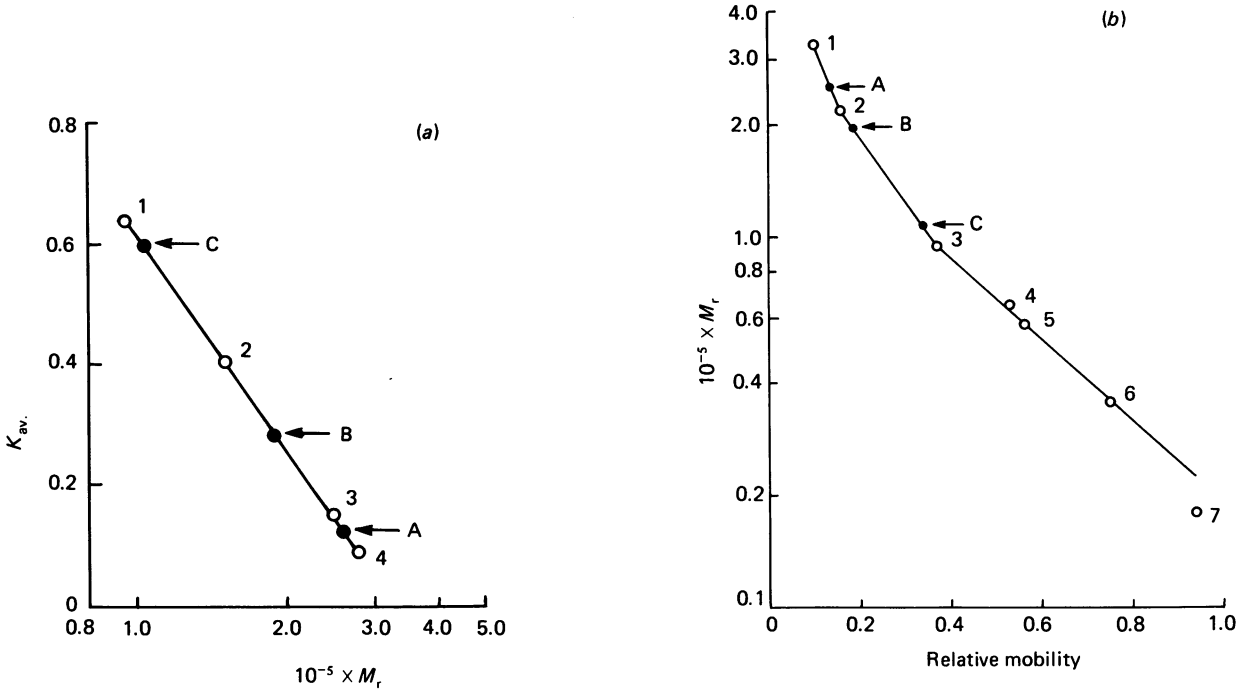

Fig. 3. Estimation of the M_r of enzyme fractions by (*a*) Sephadex G-200 gel filtration and (*b*) SDS/polyacrylamide-gel electrophoresis

(*a*) Mycobacillin synthetase and marker proteins were applied to the Sephadex G-200 column (2.5 cm × 70 cm) pre-equilibrated with buffer B. The distribution coefficients ($K_{av.}$) for the markers and the enzyme were calculated, and the linear correlation between $K_{av.}$ and log M_r for globular proteins was used for M_r estimations. The marker proteins were: 1, hexokinase; 2, alcohol dehydrogenase; 3, catalase; 4, β-glucuronidase. (*b*) SDS/polyacrylamide-gel electrophoresis was performed on 5%-acrylamide gels. The marker proteins were: 1, thyroglobulin; 2, ferritin (half); 3, phosphorylase *b*; 4, bovine serum albumin; 5, catalase; 6, lactate dehydrogenase; 7, ferritin. Their mobilities were plotted against the logarithm of their M_r.

Table 1. Summary of purification of mycobacillin synthetase from *Bacillus subtilis* B_3

The synthesis of mycobacillin was measured as described in the Materials and methods section. The enzyme fractions to be tested were used in 50 μg amounts in the presence of sufficient amounts of complementary enzyme fractions to saturate the system. The data below refer to the enzyme protein obtained from 30 g wet wt. of cells (6.5 litres of culture).

Purification step	Protein (mg)	10^{-4} × Total activity (c.p.m.)	10^{-4} × Specific activity (c.p.m./mg of protein)	Yield (%)	Purification (fold)
20000 *g* supernatant	1010	141	0.14	100	—
Streptomycin sulphate fraction	981	140	0.14	99	—
30–55%-sat.-$(NH_4)_2SO_4$ fraction	245	123	0.50	87	3.6
Sephadex G-200					
(i) Fraction A	14.2	46	3.50	32	25.0
(ii) Fraction B	32.5	100.1	3.08	71	22.0
(iii) Fraction C	18.0	45.3	2.52	32	16.5
DEAE-cellulose					
(i) Fraction A	3.5	19.6	5.60	14	40.0
(ii) Fraction B	5.0	25.5	5.10	18	36.0
(iii) Fraction C	4.0	26.88	6.72	19	47.0
Hydroxyapatite					
(i) Fraction A	1.2	12.0	10.08	9	72.0
(ii) Fraction B	2.2	23.1	10.50	16	75.0
(iii) Fraction C	1.5	20.16	13.44	14	96.0
Sucrose gradient					
(i) Fraction A	0.5	8.8	17.64	6	126
(ii) Fraction B	1.1	21.3	19.40	15	139
(iii) Fraction C	0.9	13.6	15.12	9	106

three-fraction enzyme complex mycobacillin synthetase (Ghosh *et al.*, 1983), the present work describes the final purification of the enzyme, involving two more purification steps, i.e. hydroxyapatite column chromatography and sucrose-density-gradient centrifugation, giving ultimately 126-, 139- and 106-fold purification of the three enzyme fractions A, B and C respectively (Table 1). It is noteworthy that the highest enzymic activity was obtained, like that for gramicidin S, in the purest preparation (Koischwitz & Kleinkauf, 1976*a*; Christiansen *et al.*, 1977), the purification step being monitored in both cases by synthesizing capacity. With bacitracin synthetase (Roland *et al.*, 1977) and tyrocidine (Lee *et al.*, 1973), the most purified preparation, monitored by amino acid activation, lost the ability to synthesize the antibiotic.

The purified enzyme fractions when tested by polyacrylamide-gel electrophoresis and by isoelectric focusing proved to be homogeneous (Fig. 2). The pI values for enzyme fractions A, B and C were 6.0, 5.8 and 6.0 respectively. The M_r values of the purified preparation were determined, by Sephadex G-200 gel filtration, to be 260000, 190000 and 105000 for fractions A, B and C respectively (Fig. 3*a*).

Since each of the fractions of mycobacillin synthetase is a multifunctional enzyme containing a number of catalytic sites for the synthesis of polypeptide fragments in each case, attempts were therefore made to separate them into functional domains by SDS/polyacrylamide-gel electrophoresis, which showed that each of the enzyme fractions migrated as a single band. The M_r of each fraction was then determined by SDS/polyacrylamide-gel electrophoresis, and was 252000, 198000 and 108000 for enzyme fractions A, B and C respectively (Fig. 3*b*). The M_r values of each of the fractions by gel filtration and that by SDS/polyacrylamide-gel electrophoresis are in fair agreement, which shows that each of the fractions is a monomeric protein, being a single polypeptide chain. It may be recalled that gramicidin S synthetase consists of two fractions, light (M_r 100000) and heavy (M_r 280000), which cannot be dissociated into subunits by SDS/polyacrylamide-gel electrophoresis (Koischwitz & Kleinkauf, 1976*b*; Hori *et al.*, 1982; Vater & Kleinkauf, 1976). On the other hand, of the three fractions, light (M_r 100000), intermediate (M_r 230000) and heavy (M_r 460000), of tyrocidine synthetase, the last two fractions have been reported to be dissociated into subunits on SDS/polyacrylamide-gel electrophoresis (Lee *et al.*, 1973).

Thus the three constituent enzyme fractions of mycobacilin synthetase appear to be homogeneous, monomeric and high-M_r proteins.

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