A RAPID METHOD FOR THE PREPARATION OF PURE HEAVY ENZYME OF GRAMICIDIN S SYNTHETASE

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1. Introduction

Gramicidin S synthetase consists of the light enzyme (mol. wt 100 000) and the heavy enzyme (mol. wt 280 000) which both have been isolated in essentially pure form [1–3].

The present report describes a rapid, simple and reproducible procedure for the isolation of pure heavy enzyme in high yield. Evidence is also presented that the heavy enzyme is not split into subunits by standard methods suggesting that the enzyme contains only one polypeptide chain or that several polypeptide chains may be covalently linked by bonds other than disulphide bridges.

2. Methods

2.1. *Growth of organism*

Bacillus brevis ATCC 9999 was cultivated as described previously [1].

2.2. *ATP–[^{32}P]PP$_i$ exchange reactions*

The ATP–[^{32}P]PP$_i$ exchange reaction was carried out according to Zimmer and Laland [1] in a total incubation volume of 0.1 ml. The radioactivity in ATP was determined according to the methods of Simlot and Pfaender [4].

2.3. *Estimation of gramicidin S synthesis*

Gramicidin synthesis was estimated by the Millipore filter assay of Gevers et al. [5]. The optimum buffer conditions described by Koischwitz et al. [2] was used. Each assay contained 10 mM sodium phosphate, pH 7.2, 5 mM ATP, 25 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM of L-Phe, L-Pro, L-Val, L-Leu and 0.05 mM of L-[^{14}C]Orn (20 Ci/mol) and 6.25 mM EDTA in vol. 0.2 ml and incubated for 10 min at 37°C. The amount of heavy enzyme was about 4 μg and sufficient light enzyme was added to saturate the system. Specific activity is expressed as nmol gramicidin S/min/mg.

2.4. *Polyacrylamide gel electrophoresis*

The discontinuous Tris–Glycine buffer (pH 8–9) system of Kedinger et al. [6] was used.

In addition, gels containing 5% acrylamide, 0.28% *N,N'*-methylene-bisacrylamide, 0.04 M sodium phosphate buffer (pH 7.0) polymerized with Temed and persulphate were used. Gels were run in the above buffer at 2 mA/gel for 15 min, then at 5 mA/gel for about 4 h, stained in 0.2% Coomassie Brilliant Blue 250 at 65°C for 30 min and destained in methanol/acetic acid/H_2O (2:1:7).

Sodium dodecyl sulphate–mercaptoethanol polyacrylamide gel (5%) electrophoresis was carried out in phosphate and Tris–glycine buffer as described by Koischwitz and Kleinkauf [7]. The enzyme was pretreated for 10 min at 50°C with 0.2% sodium dodecyl sulphate/mercaptoethanol.

All gels were scanned in 600 nm with a Gilford instrument.

2.5. *Treatment of heavy enzyme with $NaBH_4$*

The method described by Glazer and Smith was used [8].

Abbreviations: TEMED, *N,N,N',N'*-tetramethylenethylendiamine; TEA, Triethanolamine; PMSF, Phenylmethylsulphonylfluoride; DTT, Dithiothreitol

2.6. *Estimation of protein*

Protein was estimated by the method described by Schaffner and Weissmann [9]. Bovine serum albumin was used as standard.

2.7. *Isolation of heavy enzyme of gramicidin S synthetase*

2.7.1. 55% $(NH_4)_2SO_4$ fraction

Washed cells (40 g wet wt) were suspended in a solution containing 2.4 mg/ml lysozyme, 50 mM TEA–HCl (pH 7.4), 10 mM DTT, 5 mM EDTA, 1 mM PMSF and 20% glycerol, 5 ml being used/g wet cells. The mixture was incubated for 30 min at 30°C and the lysate was rapidly chilled to 0°C. Incubation was continued at 0°C for 15 min after addition of $CaCl_2$ and DNAase to final concentrations of 6 mM and 0.4 μg/ml, respectively. All subsequent purification procedures were carried out at +4°C. The suspension was centrifuged at 48 000 × *g* for 15 min and streptomycin sulphate added to a final concentration of 0.5% (w/v). After standing for 30 min, the precipitated material was removed by centrifugation, and solid ammonium sulphate was added to 55% saturation. The precipitated proteins were collected by centrifugation and resuspended in 20 ml buffer A (50 mM potassium phosphate buffer, pH 7.5), 2 mM DTT, 0.25 mM EDTA and 20% glycerol to give a protein concentration of approx. 10 mg/ml. The ammonium sulphate fraction when kept frozen at −20°C retains its gramicidin S synthesizing activity.

2.7.2. Fractionation on Ultrogel AcA34

The column (4 × 80 cm) was equilibrated with buffer B (20 mM TEA–HCl, (pH 7.2), 2 mM DTT, 0.25 mM EDTA, 20% glycerol) containing 0.1 M KCl. 10 ml of the 55% ammonium sulphate fraction (10 mg/ml) was applied and elution carried out with the equilibrating buffer at a flowrate of 20 ml/h. Fractions of 10 ml were collected and the D-Phe- and L-Orn-dependent ATP–$[^{32}P]PP_i$ exchange activities were measured. The light and heavy enzyme fractions were separately pooled.

2.7.3. Further purification of the heavy enzyme fraction on DEAE-Sephadex A-25

The heavy enzyme fraction (10 mg protein) from Ultrogel AcA34 was applied to a DEAE-Sephadex A25 column (2.5 × 25 cm) and equilibrated with buffer B containing 0.1 M KCl. After washing with 40 ml buffer B containing 0.2 M KCl, elution was carried out with a linear gradient from 0.2–0.5 M KCl in buffer B (600 ml) at a flowrate of 40 ml/h. Fractions of 10 ml were collected and their L-Orn-dependent ATP–$[^{32}P]PP_i$ exchange activity measured. The fractions containing the heavy enzyme were pooled (about 45 ml), protein concentration 0.08 mg/ml.

2.7.4. Concentration and desalting of the heavy enzyme fraction using spheroidal hydroxyapatite

The heavy enzyme fraction (45 ml) from the ion-exchange column was applied to a minimum sized column of spheroidal hydroxyapatite (1.25 × 5 cm) equilibrated with 20 mM potassium phosphate buffer (pH 6.8) containing 2 mM DTT and 20% glycerol. The column was then washed with 2 column volumes of the equilibrating buffer and subsequently eluted with 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM DTT, 0.25 mM EDTA and 20% glycerol. Fractions, 0.5 ml, were collected. The heavy enzyme emerged as a sharp band at the eluant front. A 10-fold concentration of the solution was achieved. The adsorption characteristics of the spheroidal hydroxyapatite was found to vary from one batch to another, requiring adjustment in the phosphate concentrations for application and elution.

2.8. *Preparation of light enzyme*

The light enzyme obtained after fractionation of Ultrogel AcA34 was chromatographed on DEAE-Sephadex A-25 in buffer B (KCl-gradient 0.1–0.35 M). Light enzyme fractions were desalted and concentrated on a column of spheroidal hydroxyapatite.

3. Materials

Ultrogel AcA34 was obtained from LKB and DEAE-Sephadex A-25 from Pharmacia. Spheroidal hydroxyapatite was purchased from BDH Chemicals Ltd and L-[U-^{14}C]ornithine (420 Ci/mol) from New England Nuclear.

4. Results and discussion

In the present method, the cells are disrupted by lysozyme digestion. The success of this enzyme in

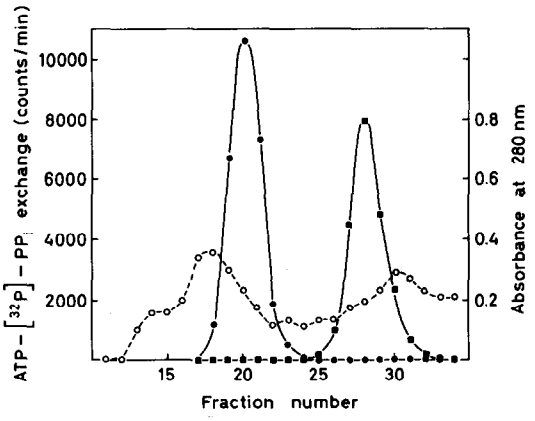

Fig.1. Fractionation of the 55% $(NH_4)_2SO_4$ fraction (100 mg protein) on a Ultrogel AcA34 column (4 × 80 cm). For details see Methods. (○——○) Absorbance at 280 nm. Position of heavy enzyme (●——●) and light enzyme (■——■) determined as described in Methods.

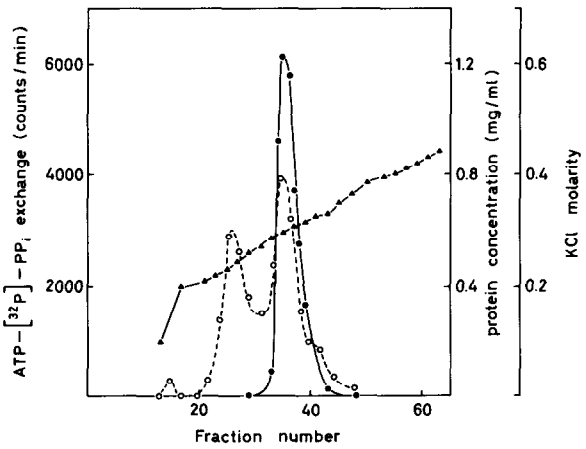

Fig.2. Fractionation of the heavy enzyme fraction (10 mg protein) obtained from Ultrogel AcA34 on DEAE-Sephadex A 25 column (2.5 × 25 cm). For details see Methods, (○——○) mg/ml of protein, (▶——▶) KCl concentration (●——●) position of heavy enzyme.

lysing *B. brevis* cells depends on the growth conditions of the cells.

It is seen from fig.1 that fractionation of the 55% $(NH_4)_2SO_4$ fraction on Ultrogel AcA34 separates completely the light and heavy enzyme. The heavy enzyme fraction obtained was applied directly on a column of DEAE-Sephadex A25 and eluted with a linear gradient of buffered KCl (fig.2). DEAE-Sephadex A25 was found to be superior to other DEAE adsorbents such as DE cellulose 52, DEAE-Bio-Gel A in removing contaminating protein. The purified heavy enzyme was desalted and concentrated by using spheroidal hydroxyapatite. The purity of the enzyme was examined by polyacrylamide gel electrophoresis in phosphate buffer (see fig.3) and in Tris–glycine buffer (not shown) and only one

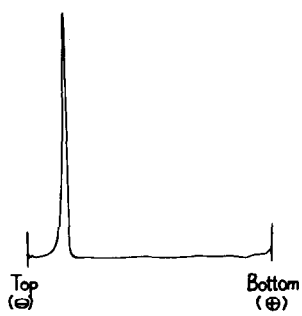

Fig.3. Polyacrylamide gel scan of electrophoresis of heavy enzyme in phosphate buffer pH 7.0. The gel was cut at the point of the bromphenol blue marker.

Table 1
Purification of the heavy enzyme

Fraction	Protein (mg)	Gramicidin S synthesis	
		Total activity (counts/10 min)	Specific activity (nmol/min/mg protein)
55% $(NH_4)_2SO_4$	83	36×10^6	0.7
Ultrogel AcA34	10	32×10^6	5.0
DEAE-A25	3.6	27×10^6	12.1
Spheroidal hydroxyapatite	2.3	32×10^6	23.0

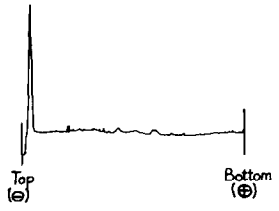

Fig.4. Polyacrylamide gel scan of electrophoresis of heavy enzyme on sodium dodecyl sulphate–Tris buffer. The gel was cut at the point of the bromphenol blue marker.

band was detected. We did not detect the presence of dimers, as reported by others [2]. It is seen from table 1 that the yield of heavy enzyme is 2.3 mg from 20 g bacteria. The specific activity of the heavy enzyme in the presence of saturating amounts of the light enzyme is 23 nmol gramicidin S/min/mg protein. It is seen from table 1 that the yield of heavy enzyme (based on gramicidin S synthesis) from the 55% $(NH_4)_2SO_4$ fraction is about 84%. In a recent report the amount of heavy enzyme from 50 g bacteria having about the same specific activity was 0.7 mg [2]. Thus, the present method gives about 8 times the amount of enzyme and is much less time consuming. The whole purification procedure takes about 2 days.

In an attempt to split the heavy enzyme into subunits, the enzyme was treated with SDS and mercaptoethanol. When run in SDS–polyacrylamide gel electrophoresis, no splitting into subunits was observed (fig.4). The minor irregularities of the base line could represent the proteolytic degradation products observed by others [10] which are normally strongly associated with the native heavy enzyme. As an alternative method of breaking disulfide bridges, the enzyme was treated with borohydride and then run in SDS–polyacrylamide gel electrophoresis. The heavy enzyme remained unchanged.

The results show that the heavy enzyme consists of one polypeptide chain or that if it contains several, they must be covalently linked. These results are in agreement with that of other workers [2].

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