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GRAMICIDIN S-SYNTHETASE

PREPARATION OF THE MULTIENZYMIC COMPLEX WITH A HIGH SPECIFIC ACTIVITY

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Summary

A new purification procedure for the multienzyme of gramicidin S-synthetase has been developed. In vitro proteolysis with partial inactivation is suppressed by protease inhibitors EDTA, phenylmethylsulfonylfluoride, and fast preparation methods during initial separation steps.

Activity has only been assayed by the total reaction of gramicidin S-synthetase, not by partial reactions of amino acid activation. The assay has been improved by evaluation of inhibitory concentrations of buffers, salts, and the product gramicidin S. It has been demonstrated that the rate of peptide synthesis in extracts containing both enzymes of gramicidin S-synthetase depends on protein concentration in a second order function.

The multienzyme or heavy enzyme has been purified about 1400-fold to a specific activity of 24 nM/min per mg of protein, and the relation of this activity to the calculated in vivo activity is discussed.

Introduction

Gramicidin S, the decapeptide (D-Phe-Pro-Val-Orn-Leu)$_2$, is synthesized by two enzymes [1—3] of *Bacillus brevis* ATCC 9999 at the beginning of sporulation. These enzymes are called gramicidin S-synthetase and consists of a multienzyme, or heavy enzyme, of molecular weight of 280 000, and the phenylalanine-racemase, with a molecular weight of 100 000. The latter enzyme (EC 5.1.1.11) initiates peptide biosynthesis by transfer of thioester-activated

Abbreviations: TES, tris-(hydroxymethyl)-methyl-2-amino-ethane-sulfonic acid; Tricin, *N*-tris-(hydroxy-methyl)-methylglycine; HEPES, *N*-2'-hydroxy-ethyl-piperazine-2-ethane-sulfonic acid.

D-phenylalanine to a specific site of the multienzyme [4]. While the racemase has been purified extensively [5—7], only incomplete data have been available on the multienzyme. Some authors claim to have purified the enzyme to or almost to homogeneity [8—10], but exclude any data on specific activity and details of polyacrylamide gel electrophoretic characterization in different buffer systems and at different gel concentrations. Also it has to be pointed out that none of these investigators has presented evidence for a fully active enzyme, not partially degraded by proteolysis. This requires sodium dodecyl sulfate polyacrylamide gel electrophoresis combined with a second separation method in order to detect impurities or peptide fragments, together with subunit composition and stoichiometry.

We have improved in vitro activity determination by investigation of buffer-, salt-, and protein-concentration dependence of peptide synthesis. We report here a different procedure for the purification of the multienzyme, avoiding in vitro proteolytic degradation by use of protease inhibitors and exclusion of time consuming steps such as ultracentrifugation and Sephadex G-200 chromatography. The enzyme has been purified about 1400-fold to a specific activity of 24 nM/min per mg of protein. It is approximately 95% pure according to techniques of polyacrylamide gel electrophoresis; details of these investigations will be given in an accompanying report [11].

Methods and Materials

Growth of organism

B. brevis ATCC 9999 spores were prepared on potato agar [12] and grown in a glutamate minimal medium [13] in a 500 l-fermenter. Growth was stopped at a cell density of $4 \cdot 10^8$ cells per ml by temperature shift-down to 8°C by the addition of ice. Cells were collected by centrifugation at a rate of 200 l/h, frozen and stored in liquid nitrogen.

Alternatively cells were grown in a 120 l-fermenter in the same medium supplemented with 1% glycerol, but excluding vitamins. Cells were harvested half an hour after the end of logarithmic growth, frozen and stored at —20°C.

Preparation of crude extract

50 g of cells were thawed in a stream of cold air together with 10 ml of buffer B (50 mM tris(hydroxymethyl)-methyl-2-amino-ethane-sulfonic acid, pH 7.2, 10 mM dithiothreitol, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride). The paste was passed through a cooled French press at 15 000 lb/inch^2 into 240 ml buffer B. The suspension was homogenized by treatment with 2.5 ml 1 M $MgCl_2$ and 1 mg DNAase for 10 min at 4°C, and then centrifuged 5 min at 3500 × *g* and 10 min at 45 000 × *g*. The supernatant was adjusted to 55% saturation of ammonium sulfate by the addition of a saturated solution (pH 7.5, 0.1 mM EDTA). After 20 min at 4°C the precipitate was collected by centrifugation for 10 min at 45 000 × *g*, dissolved in 30 ml buffer P (20 mM sodium phosphate, pH 7.2, 5 mM dithiothreitol, 2 mM $MgCl_2$, 0.25 mM EDTA) and desalted on a Sephadex G 50 medium column (2.5 × 50 cm) equilibrated and eluted with buffer P. Alternatively buffers containing triethanolamine instead of phosphate or buffers without Mg^{2+} were used.

DEAE-cellulose chromatography

100 g DEAE-cellulose was equilibrated with 5 l of buffer P without dithiothreitol and EDTA. Desalted crude extract was applied and eluted with a linear potassium phosphate gradient (0.5 M) in buffer P containing only 2 mM of dithiothreitol at a flow rate of 120 ml/h. Active fractions were located by filter assay and conductivity measurement.

Sepharose 6B filtration

Active DEAE-fractions were precipitated with ammonium sulfate and applied in a volume of 6 ml buffer P to a Sepharose column (2.5 × 90 cm). Elution was performed with buffer P at a rate of 30 ml/h. Enzymes were assayed by addition of either phenylalanine racemase or multienzyme.

Hydroxyapatite chromatography

Active Sepharose-fractions were applied to a hypatite C-column (2 × 5 cm) equilibrated with buffer P. Elution was performed with 200 ml of a linear sodium phosphate gradient (0.25 M) at a rate of 100 ml/h. Enzyme activity was estimated with complementary phenylalanine racemase.

Sucrose gradient centrifugation

Hydroxyapatite purified enzyme was precipitated with twice its volume saturated ammonium sulfate solution, the precipitate dissolved in 1 ml buffer P and dialysed for 30 min against 500 ml buffer P. The sample was layered onto a linear sucrose gradient (10—30%, w/total v) in buffer P (32 ml). Centrifugation was carried out in a SW 27-rotor for 20 h at 0°C, and 27 000 rev./min.

Test of enzyme activity

Gramicidin S synthesis was estimated by the millipore filter assay of Gevers et al. [14]. Each assay contained 10 mM sodium-phosphate, pH 7.2, 5 mM ATP (pH 7 with NaOH), 25 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM of Phe, Pro, Val, Leu, 50 nM [^{3}H]Orn (specific activity 50 C/M), and 6.25 nM EDTA, in a volume of 0.2 ml. Incubation was carried out for 10 min at 39.5°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The gels contained 5% acrylamide and 0.28% *N,N'*-methylene-bis-acrylamide, and were run in 0.1 M Tris · HCl at pH 8.5 with 0.1% sodium dodecyl sulfate and 0.2% 2-mercapto-ethanol. Samples were treated with 0.2% sodium dodecyl sulfate and mercaptoethanol for 10 min at 50°C and applied directly in buffer P or triethanolamine-buffer onto the gel. Electrophoresis was carried out for 1 h at 1 mA/gel, than at 4 mA/gel. At the end of the run, gels were cut at the bromphenol blue position and stained according to Weber and Osborn [15]. Destaining was done by diffusion in 10% methanol/acetic acid/water (1 : 1 : 8, v/v).

Protein determination

Protein was determined either by a slightly modified method of Schaffner and Weissmann [16] or by absorbance measurement of polyacrylamide gels at 590 nm, using each time bovine serum albumin as a standard.

Preparation of phenylalanine racemase

Racemase as obtained from the Sepharose 6B column was used. This preparation contained approximately 2—5% enzyme.

Materials

Triethanolamine-hydrochloride was obtained from Boehringer, TES, tricin and HEPES from Serva, dithiothreitol from RSA. High specific activity [^{3}H]-L-ornithine (NEN) was diluted to the desired concentration. Column materials were DE 52 from Whatman and Hypatite C from Clarkson Chem. Co., Williamsport.

Results and Discussion

Effect of ion and buffer concentrations

Although optimal conditions for substrates have been evaluated previously [17,14], and ammonium sulfate and KCl have been excluded from assay mixtures [18,10], less attention has been given to buffer concentrations.

The results of this section refer to crude enzyme preparations only. Generally, we observe inhibition of peptide synthesis with increasing ionic strength. Purely cationic buffers such as Tris and triethanolamine behave like KCl (Fig. 1), whereas buffers such as tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES), *N*-2′-hydroxyethylpiperazine-2-ethane-sulfonic acid (HEPES), or *N*-tris-(hydroxymethyl)-methylglycine (Tricin), can be used to

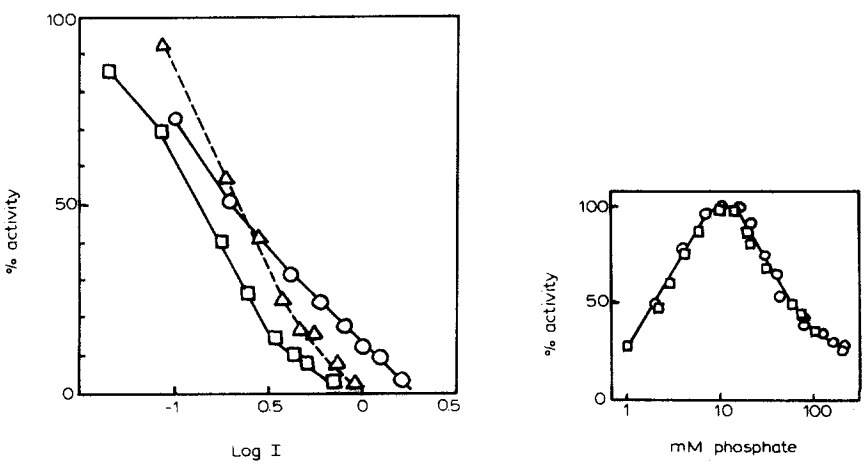


Fig. 1. Peptide-synthesizing activity in the crude extract as a function of the ionic strength of KCl (circles), Tris · HCl (squares), and triethanolamine/HCl (triangles). The ionic strength of buffers was calculated from p*k*-values (8.0 for Tris, 8.16 for triethanolamine), assuming dissociation constants to be independent of concentration. Assay conditions were as described in Methods, phosphate being replaced by 20 mM triethanolamine. Reactions were carried out without preincubation for 10 min. A typical experiment yielded 10 000 cpm gramicidin S, whereas 200 cpm [^{3}H] L-ornithine were detected as protein-bound in a control omitting the other amino acids.

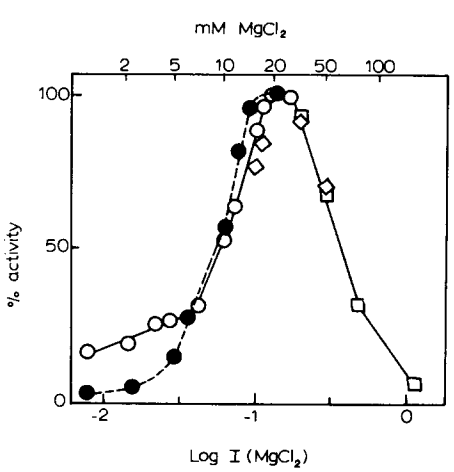
Fig. 2. Stimulation of peptide synthesis by phosphate (circles, K^+, squares Na^+, pH 7.2). Crude enzyme in 20 mM triethanolamine was used, the conditions were as described in Methods, the final assay volume being, however, 0.25 ml.

much higher concentrations (above 0.1 M) without significant effect (results not shown). The nature of this inhibition is not entirely determined by the chloride ion, since activity and ion concentration with Tris · HCl, triethanolamine · HCl, KCl, and $MgCl_2$ could not be correlated at ionic strength above 0.1 (evaluation not shown).

If the crude extract is stored in 20 mM triethanolamine, peptide synthesis may be stimulated by phosphate (Fig. 2). The stimulation is dependent on the concentration of Mg^{2+} (Fig. 3) or Mn^{2+} (results not shown). High Mg^{2+}/ATP ratios of 4—5 were required to saturate the system. At smaller ratios, added phosphate or pyrophospatase generated a stimulating effect (results not shown). The nature of these observations cannot be further discussed, since many still unknown ion equilibria are involved.

Effect of pH

Many pH-profiles for various buffers have been published [19—21]. We shall not discuss them, for the results are contradictory and need reevaluation. It can be noted that each buffer may have a different optimum for the incorporation of each amino acid into the peptide. This can be expected, since at least 18 different partial reactions are involved in the synthesis of gramicidin S. However, since very similar catalytic reactions such as the transfer of activated amino acids and peptides to thiols are involved, we would expect the overall rate to be



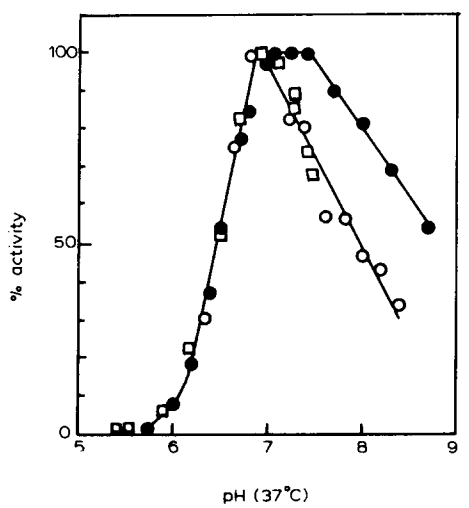


Fig. 3. Stimulation and inhibition of peptide synthesis by $MgCl_2$ in presence of 5 mM ATP and 20 mM phosphate. Stimulation by phosphate at low $MgCl_2$, and inhibition of synthesis at high salt represent new details (open circles, squares and lozenges represent three sets of experiments); while other data (full circles, no phosphate added) is comparable to previously published work. Crude extract was used, but DNAase treatment was omitted, and the ubffers did not contain Mg^{2+}-ions. These data can therefore not be related to Fig. 2, where stimulation is observed at higher $MgCl_2$- concentrations.

Fig. 4. Dependence of peptide synthesis on pH. Measurements were carried out with crude enzyme in 20 mM sodium phosphate, and 50 mM (squares) or 125 mM sodium phosphate (open circles), or HEPES buffer (full circles) to a final concentration of 0.12—0.25 M, were added. It was confirmed that the rate is not significantly dependent on HEPES-concentration under the conditions used. pH values were determined with a glass electrode at 25°C, and were corrected to 37°C.

related to (1) deprotonation of a thiol by some base, and (2) hydrolysis of thiol-bound substrates and intermediates. We expect the second limiting reaction to be strongly dependent on the nature of the buffer system employed. This is the interpretation of the pH-profile we have evaluated, using two different buffers at two different concentrations (Fig. 4). Apparently, deprotonation of thiols is related to some functional groups with a pk of 6.5, since the acidic side of the curve seems to be independent of buffer and buffer concentration, and Leung and Baxter [9] obtained a similar part of the curve by measuring pH-dependence of thioester-formation from activated amino acid in 0.2 M sodium cacodylate.

Product inhibition

It can be noted that production of antibiotic stops at concentrations as low as 10^{-6} M gramicidin S with partially purified enzyme [17]. We found that a crude system is completely inhibited at $2 \cdot 10^{-4}$ M gramicidin S, which corresponds approximately to the highest yields of preparative enzymatic synthesis of the antibiotic (Ref. 22, and Koischwitz and Kleinkauf, manuscript in preparation).

Protein concentration

It is well established that the biosynthesis of gramicidin S depends on two complementary and separable enzymes. So far no evidence for a complex of these enzymes in solution has been presented, indicating a small association constant under these conditions. In a crude extract, which contains equimolar amounts of synthetase and racemase [6], we would thus expect a second order relation between protein concentration and rate of gramicidin S formation, if no stable complex is formed:

$$dp/dt = k_1 e_1 e_2, \tag{1}$$

with $e_1 = e_2$ becomes

$$dp/dt = k_1 e^2 \tag{2}$$

(p stands for product, e for enzyme concentration). Indeed Tzeng et al. [23] noted a linear concentration dependence of specific activity of crude extracts. We have confirmed the function (2) over a wide range of concentrations (Fig. 5). At the present time, we ascribe deviations from this function to product inhibition.

It must be noted that other mechanisms involving dimerisation of the multienzyme, cannot be excluded, since product formation depending on the reaction

$$2\ E_1 \rightleftharpoons [E_1]_2 \tag{3}$$

could also account for the observed kinetics; combination of both equations (2) and (3) gives the similar second order relation (4):

$$dp/dt = k_1 e^2 + k_2 e^2 = (k_1 + k_2) e^2 \tag{4}$$

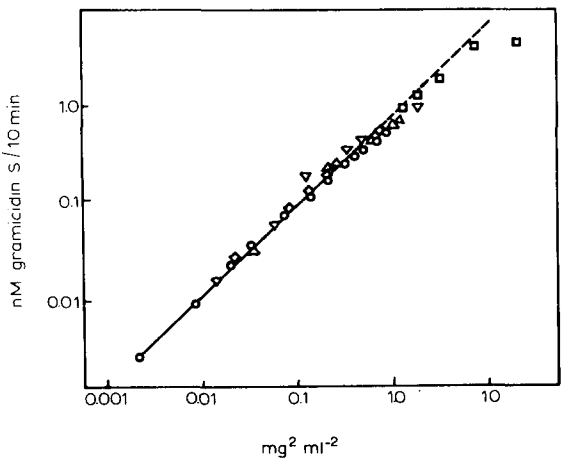


Fig. 5. Protein-concentration dependence of peptide synthesis in crude extract. As expected for a two-enzyme reaction with limiting enzyme concentrations, a second order relationship was confirmed. Rates were measured for 10 min without preincubation. Different symbols indicate 5 sets of experiments, squares show experiments in which L-ornithine concentrations was raised to 0.1 μM to eliminate substrate limitation.

Resolution of the type of enzyme interactions involved has to come from purified enzymes, but so far no evidence for a catalytic function of dimerisation of the multienzyme could be elucidated from concentration dependence data (results not shown). The possibility, however, cannot be excluded, since we do not know the rate-limiting step of the system.

Comment on in vitro protease action

Since the work of Gevers et al. [14], gramicidin S-synthetase has been assayed using amino acid dependent ATP-PP_i exchange. This method, however, which only detects aminoacyl adenylates, is by no means evidence for the complete functioning enzyme system. It has been noted that peptide synthesizing activity is rapidly lost during purification, while activation reactions still are measurable [10]. Since the work of Lee and Lipmann [24—26] it is known that tyrocidine synthesizing multienzymes can be split by an enzyme activity into subunits which catalyse amino acid activation. We have suggested that in vitro proteolytic modification may account for the differences observed in initial velocities at the ATP-PP_i exchange reaction [27,28] by several authors [6,9,14,29,30]. Although much evidence has been collected (Refs. 27, 28 and unpublished work) in favour of this interpretation, it has not been proved by isolation and observation of direct action of *B. brevis* proteases. In the following paper we present evidence that the active multienzyme contains no subunits [11].

Enzyme preparation

A new procedure has been worked out based on the work of Kleinkauf et al. [31], and Bredesen et al. [18]. For 50 g of cells, a buffer to cell ratio of 5 was used, and we finally selected 50 mM TES, since 20 mM triethanolamine could not prevent pH drop, while 50 mM triethanolamine already displayed an in-

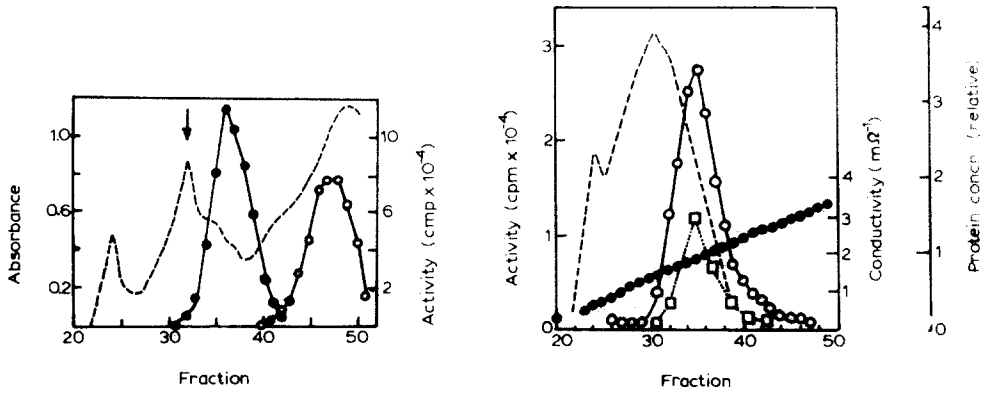


Fig. 6. Gel filtration of DEAE-purified extract on Sepharose 6B. Dashed line: absorbance at 280 nm; solid circles: gramicidin S formation with phenylalanine racemase (Fraction 46); open circles: gramicidin S formation with multienzyme (Fraction 35). In the preceding purification step, a KCl gradient had been used. When a phosphate gradient was used, the second protein peak (arrow) could be eliminated.

Fig. 7. Hydroxyapatite chromatography of Sepharose-purified multienzyme. Protein has been estimated by polyacrylamide gel electrophoresis and is given in relative units (dashed line): multienzyme content (squares) is correlated with activity (open circles), which has been determined using the complementary enzyme. Full circles show conductivity in mmho, which can be used for localisation of activity.

hibitory effect on peptide formation. To keep all thiols in reduced form, 10 mM dithiothreitol were used, which proved superior to 2-mercaptoethanol. During the first purification step 5 mM EDTA and 1 mM phenylmethylsulfonylfluoride were used to prevent protease action. It must be kept in mind that the protease content of cells is determined by time and conditions employed for freezing the cells.

Ultracentrifugation and streptomycin sulfate precipitation were omitted, and the 45 000 × *g* supernatant was treated directly with ammonium sulfate solution at constant pH. To remove the salt fast and quantitatively, since it is

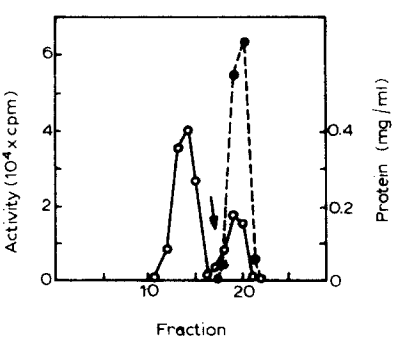


Fig. 8. Sucrose gradient centrifugation of hydroxyapatite-purified enzyme. In this experiment centrifugation was carried out in 10 mM triethanolamine, 5 mM $MgCl_2$, and 5 mM dithiothreitol for 18.5 h. Protein was estimated by polyacrylamide gel electrophoresis, and compared to bovine serum albumin as a standard (open circles). Activity could only be located in the second protein peak (full circles), background measurements in the complete gradient are not shown. Impurities were located in the left shoulder of the protein peak, as can be seen from protein-activity correlation (arrow).

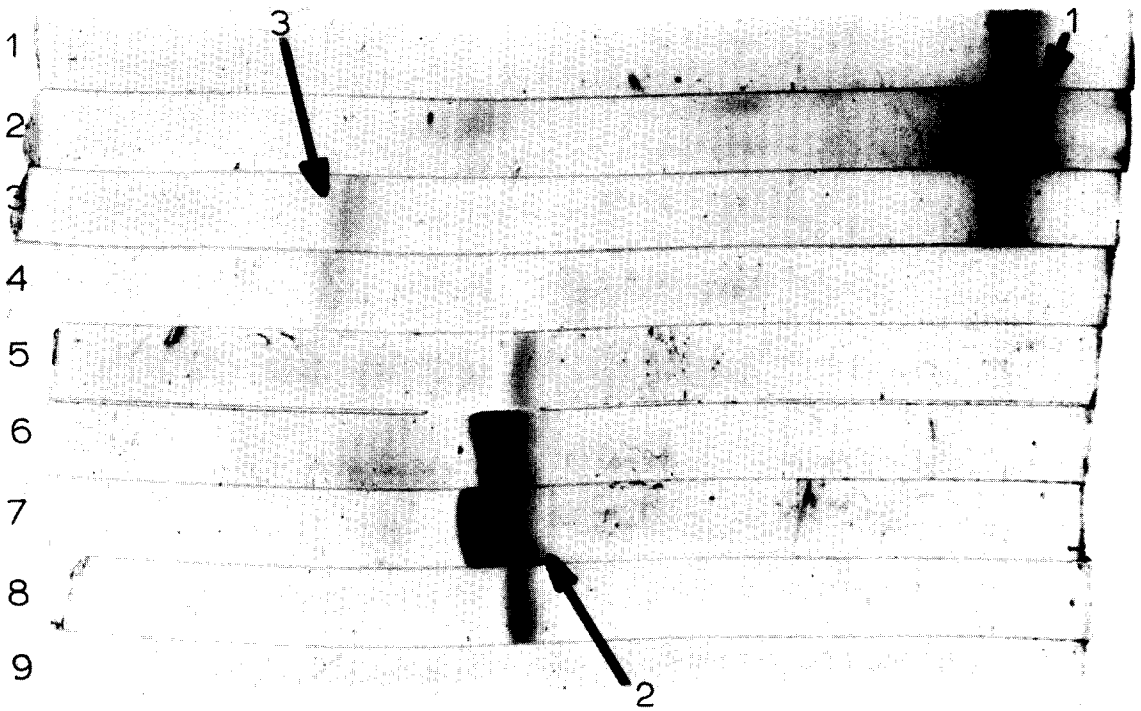


Fig. 9. Control of last purification step by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The procedure has been described in Methods. Gels contained 5% acrylamide. Protein 1 corresponds to the multienzyme; note that the band is broad and apparently split in gel 1. Work on evaluation of inhomogeneity is in progress. Protein 2 corresponds to the separated peak in Fig. 8, displaying subunits of 70 000 daltons. Protein 3 are traces of the impurity separated on hydroxyapatite (subunits of 35 000 daltons).

strong inhibitor of peptide synthesis, gel filtration was used. These preliminary steps were completed within 2 h.

The crude extract (about 1 g of protein) was fractioned on a DEAE-cellulose column using a linear potassium phosphate gradient (0.5 M). This gives a higher resolution than KCl, and a higher yield of activity, since inactivation has been observed in 0.5 M KCl. No significant separation of both enzyme fractions occurred. To resolve them, a Sepharose 6B column was used instead of Sephadex G-200, to obtain a higher flow rate (Fig. 6). By now the multienzyme contained only two major impurities: a protein of a molecular weight of 280 000 with subunits of 35 000 daltons, and a protein of a molecular weight of 420 000 with equal subunits of 70 000 daltons (unpublished observations). These could be separated by chromatography on hydroxyapatite (Fig. 7) and sucrose gradient centrifugation (Figs. 8 and 9).

Conclusion

The purification steps are summarized in Table I. To our knowledge only one comparable specific activity (18.9 nM/min per mg) has been reported by Bredesen et al. [18], but these results have not been substantiated by follow-

TABLE I

SUMMARY OF PURIFICATION STEPS

The synthesis of gramicidin S has been measured as described in Methods; the concentration of multienzyme was below 50 μg/ml in the assay, and sufficient racemase has been added to saturate the system. Specific activity is expressed in nM gramicidin S/min and mg of protein.

Purification step	Gramicidin S (cpm × 10^{-6})	Protein (mg)	Specific activity
Sephadex	240	1020	0.3
DEAE-cellulose	220	120	2.2
Sepharose 6B	30	6.3	5.9
Hydroxyapatite	24	2.1	14.2
Sucrose gradient	13.5	0.7	24.1

ing publications from this laboratory [10]. From estimations of in vivo activity we would expect a 10 to 100-fold higher specific activity. Work is in progress to avoid protein concentration and gel filtration after DEAE-chromatography, which cause a dramatic loss of activity. The homogeneity of the purified multienzyme has been checked by gel electrophoretic techniques (Fig. 9) [11], and is evident from protein-activity correlations in the last purification steps (Figs. 7 and 8). However, more details are needed in regard to low specific activity, which have to come from active site titrations.

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References

1 Kurahashi, K. (1974) Ann. Rev. Biochem. 43, 445—459
2 Lipmann, F. (1973) Acc. Chem. Res. 6, 361—367
3 Laland, S.G. and Zimmer, T.-L. (1973) Essays Biochem. 9, 31—57
4 Pass, L., Zimmer, T.-L. and Laland, S.G. (1974) Eur. J. Biochem. 47, 607—611
5 Yamada, M. and Kurahashi, K. (1969) J. Biochem. 66, 529—540
6 Kurahashi, K., Yamada, M., Mori, K., Fukikawa, K., Kambe, M., Imae, Y., Sato, E., Takahashi, H. and Sakamoto, Y. (1969) Cold Spring Harbor Symp. Quant. Biol. 34, 815—826
7 Vater, J. and Kleinkauf, H., (1976) Biochim. Biophys. Acta 429, 1052—1062
8 Gilhuus-Moe, C.C., Kristensen, T., Bredesen, J.E., Zimmer, T,-L. and Laland, S.G. (1970) FEBS Lett. 7, 287—290
9 Leung, D.C. and Baxter, R.M. (1972) Biochim. Biophys. Acta 279, 34—47
10 Zimmer, T.-L. and Laland, S.G. (1975) Methods Enzymol. 43, 567—579
11 Koischwitz, H. and Kleinkauf, H. (1976) Biochim. Biophys Acta 429, 1042—1051
12 Spizizen, J. (1958) Proc. Natl. Acad. Sci. U.S. 44, 1072—1078
13 Eikhom, T.S., Johnson, J., Laland, S.G. and Refsvik, T. (1963) Biochim. Biophys. Acta 76, 465—468
14 Gevers, W., Kleinkauf, H. and Lipmann, F.(1968) Proc. Natl. Acad. Sci. U.S. 60, 269—276
15 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412

15 Schaffner, W. and Weissmann, C. (1973) Anal. Biochem. 56, 502—514
17 Tomino, S., Yamada, M., Itoh, H. and Kurahashi, K. (1967) Biochemistry 6, 2552—2560
18 Bredesen, J.E., Berg, T.-L. Figenschou, K.J., Froholm, L.O. and Laland, S.G. (1968) Eur. J. Biochem. 5, 433—436
19 Spaeren, U., Froholm, L.O. and Laland, S.G. (1967) Biochem. J. 102, 586—592
20 Figenschou, K.J., Froholm, L.O. and Laland, S.G. (1967) Biochem. J. 105, 451—453
21 Bhagavan, N.V., Rao, P.M., Pollard, L.W. and Rao, R.K. (1966) Biochemistry 5, 3844—3850
22 Hamilton, B.K., Montgomery, J.P. and Wang, D.I.C. (1974) in Enzyme Engineering. II. (Wingard, L.B. and Pye, E.K., eds.) pp. 153—159 Plenum Press, New York
23 Tzeng, C.H., Thrasher, K.D., Montgomery, J.P., Hamilton, B.K. and Wang, D.I.C. (1975) Biotechn. Bioeng. 17, 143—151
24 Lee. S.G., Roskoski, Jr., R., Bauer, K. and Lipmann, F. (1973) Biochemistry 12, 398—405
25 Lee, S.G. and Lipmann, F. (1974) Proc. Natl. Acad. Sci. U.S. 71, 607—611
26 Lee, S.G. and Lipmann, F. (1975) Methods Enzymol. 43, 585—602
27 Kleinkauf, H. and Koischwitz, H. (1974) Lipmann-Symposium: Energy, Biosynthesis and Regulation in Molecular Biology (Richter, D., ed.) pp. 336—344 Walter de Gruyter Verlag
28 Koischwitz, H. and Kleinkauf, H. (1974) Abstr. Commun. 9th Meet. Fed. Europ. Biochem. Soc. Budapest 1974 p. 89, Elsevier, Amsterdam
29 Otani, S., Yamanoi, T. and Saito, Y. (1969) J. Biochem. 66, 445—543
30 Otani, S., Jr., Yamanoi, T. and Saito, Y. (1970) Biochim. Biophys. Acta 208, 496—508
31 Kleinkauf, H., Gevers, W. and Lipmann, F. (1969) Proc. Natl. Acad. Sci. U.S. 62, 226—233