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Cell-free synthesis of the dipeptide antibiotic bacilysin

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SUMMARY

Bacilysin, a dipeptide antibiotic produced by *Bacillus subtilis* A14, was synthesized by a cell-free extract of the producing organism from its constituent amino acids, L-alanine and L-anticapsin. The synthesis required ATP and Mg^{2+} and was optimal at pH 8.1. The same extract also synthesized L-alanyl-L-alanine. The synthesis of bacilysin was not inhibited by chloramphenicol, DNase or RNase.

INTRODUCTION

Bacilysin is a dipeptide antibiotic with the structure of L-alanyl-(2,3-epoxycyclohexanone-4)-L-alanine (Fig. 1) which is produced by *Bacillus subtilis* A14 [13]. It is identical to bacillin [2,5] and tetaine [6]. *B. subtilis* A14 also produces anticapsin, the C-terminal amino acid of the bacilysin molecule, and di- and oligopeptides of L-alanine in the fermentation medium [13]. Bacilysin causes lysis of *Staphylococcus aureus* [1] and its action is antagonized by dipeptides [8], as well as D(+)-glucosamine and *N*-acetylglucosamine [14]. Its antibacterial activity against *S. aureus* depends on the transport of bacilysin into *S. aureus* cells and its hydrolysis in the cell to anticapsin which subsequently inhibits glucosamine synthetase [9]. Biosynthesis of bacilysin has been studied using growing cells of *B. subtilis* and was found to be independent of ribosomal protein synthesis [12]. Also shown was the incorporation of DL-[^{14}C]alanine and [1,6-*ring*-$^{14}C_2$]shikimic acid into bacilysin by growing cells. In the experiments described below, we report the first cell-free synthesis of bacilysin.

METHODS

Preparation of B. subtilis A14 spores

A freeze-dried culture of *B. subtilis* A14 (obtained from E.P. Abraham via R.H. Doi) was grown on nutrient agar (Difco) overnight at 37°C. The cells were washed from a slant with 10 ml of sterile 0.85% NaCl and inoculated into 300 ml of

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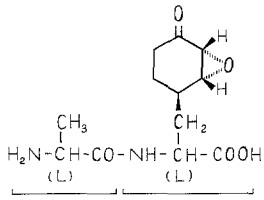

Fig. 1. Structure of bacilysin (L-alanyl-(2,3-epoxycyclohexanone-4)-L-alanine). The moiety on the left is L-alanine and that on the right is L-anticapsin.

SCM medium [4] in a 2.8-l Fernbach flask. Incubation was for 4 days at 37°C with shaking at 120 rpm (1-inch stroke), during which time the population sporulated. The spores were harvested by centrifugation (3000 × *g*, 4°C, 20 min), washed and resuspended in water to give 3.2×10^8 spores per ml (microscopic count) and kept at −70°C.

Growth of B. subtilis A14

A seed culture was prepared from the spores in the chemically defined medium (PA) of Perry and Abraham [11] supplemented with 14 mM L-alanine and 0.005% vitamin-free casamino acids (Difco). The frozen spore suspension was thawed at 37°C, heat-activated at 80°C for 15 min, and 0.1 ml was inoculated into each 40 ml of seed medium. The culture was shaken at 28°C for 18 h. PA medium contains (g/l); KH_2PO_4 (1.0), KCl (0.5), $MgSO_4 \cdot 7H_2O$ (0.5), monosodium glutamate · H_2O (4.0), sucrose (10), ferric citrate (0.15) and trace elements. Ferric citrate was prepared as a filter-sterilized solution of $FeCl_3 \cdot 6H_2O$ (2 mg/ml) and tri-sodium citrate · H_2O (20 mg/ml). The trace elements were composed of (mg/l); $ZnSO_4 \cdot 7H_2O$ (0.1), $CoCl_2 \cdot 6H_2O$ (0.1), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.1), $MnCl_2 \cdot 4H_2O$ (1.0) and $CuSO_4 \cdot 5H_2O$ (0.1); 1 ml of this solution was used for every 1 l of medium. Magnesium sulfate and sucrose were autoclaved together, separate from the rest of the medium. The pH of the medium was adjusted to 7.0. Two milliliters of the seed culture (150 Klett units) were inoculated into 40 ml of PA medium in a 250-ml Erlenmeyer flask. The culture was incubated at 27–30°C on a shaker (200 rpm). Samples were withdrawn at intervals for determination of growth, pH and antibacterial activity. *B. subtilis* was also grown in 80 ml medium in a 500-ml flask, 500 ml medium in a 2.8-l flask, and 10 l medium in a 16-l fermenter with similar results. Growth was estimated using a Klett-Summerson colorimeter with a red filter.

Preservation of S. aureus cell suspension

Stock cultures of the bacilysin assay organism *S. aureus* ATCC 9144 were prepared by growing the organism in 40 ml of the ATCC medium 117 (Micrococcus medium) (without agar) in a 500-ml flask at 37°C for 24 h. Medium 117 contains (g/l); peptone (5), yeast extract (3), beef extract (1.5), glucose (1.0); pH adjusted to 7.4. The cells were centrifuged (3400 × *g*, 4°C, 10 min), washed once with ice-cold sterile M/15 potassium phosphate buffer, pH 7.0, and resuspended in the same buffer containing 15% glycerol. One-milliliter aliquots of the suspension (2×10^9 CFU/ml) were distributed into small vials and stored at −70°C.

Assay of antibiotic activity

The antibacterial activity of bacilysin in culture supernatant fluids and that produced by cell-free extracts was assayed against *S. aureus* by a disc-agar diffusion method. The mecium of Mah et al. [10] was modified to allow reproducible growth of *S. aureus* on plates in 18–20 h. The medium contained (g/l): $Na_2HPO_4 \cdot 2H_2O$ (3.3), KH_2PO_4 (1.0), NaCl (1.0), $MgSO_4 \cdot 7H_2O$ (0.7), $FeSO_4 \cdot 7H_2O$ (0.01), tri-sodium citrate · $2H_2O$ (0.5), sodium glutamate · H_2O (2.4), glucose (10), amino acid mixture (L-arginine, L-cysteine, glycine, L-histidine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-threonine, L-tryptophan, L-tyrosine and L-valine (0.025 each), yeast extract (0.05), and agar (10). The pH was adjusted to 7.1 and the medium autoclaved at 121°C for 15 min. A frozen suspension of *S. aureus* was thawed at 37°C and 1 ml was used to seed each 100 ml of top agar. Four milliliters of inoculated top agar were poured onto 6 ml of the same solidified base agar which was not seeded. Twenty-microliter samples were applied onto antibiotic assay discs (diameter 1/4 inch, Schleicher & Schuell, NH) and the discs were laid on the agar plates. The plates were incubated at 37°C for 18–20 h. Since pure bacilysin was not available, an arbi-

trary bacilysin activity unit was defined such that the antibacterial activity of a standard culture supernatant fluid was designated 200 units/ml. The standard preparation was stored at −20°C and used to construct standard curves for each bioassay. Anticapsin at concentrations used in the experiments did not produce inhibition zones.

Preparation of cell-free extract

Cells of *B. subtilis* A14 in the exponential phase (i.e., after ca. 8-h fermentation) were harvested by centrifugation (3000 × g, 4°C, 20 min). The pellet was washed twice with 1 vol. of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM KCl and 10 mM $MgSO_4$ and stored at −20°C. The cells were lysed by incubating 0.5 g thawed wet cells in 4 ml of the above buffer containing 8 mg lysozyme (Sigma, L-6876) at 0°C for 10–20 min, and then at 30°C for 30 min with continuous gentle stirring. The lysate was centrifuged (10000 × *g*, 4°C, 30 min) and the supernatant fluid was used as the enzyme source. Generally, 5–7 mg protein per ml was obtained. For some experiments, DNase I (type III, Sigma) and RNase (type I-AS, Sigma) were added to the lysate which was further incubated for 30 min at 30°C prior to centrifugation. The supernatant fluid was applied to a pre-packed Sephadex G-25 column (PD-10, Pharmacia), equilibrated with 50 mM Tris-HCl buffer (pH 7.4) and eluted with the same buffer. Fractions which eluted at the void volume were used as the enzyme source.

Bacilysin synthetase assay

The reaction mixture (developed during the course of this work) contained the following in a volume of 0.5 ml (unless otherwise stated): 50 mM Tris-HCl buffer (pH 8.4), 10 mM KCl, 10 mM $MgSO_4$, 4 mM ATP, 0.14 mM L-alanine, 80 μg anticapsin, 25 μg chloramphenicol and 0.1 ml of cell-free extract; the pH was 8.1. The reaction was started by adding the cell-free extract to the rest of the mixture and incubating for 5 min at 30°C. The reaction was terminated by immersing the reaction tube in a boiling water bath for 2 min. The precipitate was removed by centrifugation and the supernatant fluid used for bioassay. For TLC analysis, the sample was deproteinized and desalted with 70% acetone.

Incorporation of L-[^{14}C]alanine

The 0.2-ml reaction mixture used contained those reagents described above (bacilysin synthetase assay) plus 8 mM phospho*enol*pyruvate, 0.05 mg pyruvate kinase, 45 μM $FeSO_4$, 2.8 mM ascorbic acid, 1 mM shikimic acid and 0.15 ml cell-free extract. This mixture was preincubated in a 30°C water bath for 5 min and the reaction initiated by adding L-[^{14}C]alanine (0.6 μmol, 0.1 μCi, New England Nuclear). At various times up to 4 h, the reaction was terminated by adding 0.5 ml acetone. The precipitate was removed by centrifugation and the clear supernatant fluid concentrated in vacuo to 50 μl. Chloramphenicol was removed by two extractions, each with 2 vol. of ethyl acetate followed by two extractions with 2 vol. of ether. The aqueous fraction was concentrated to dryness and reconstituted in water. (In later experiments, chloramphenicol was not extracted since it migrated with the solvent front and did not interfere with bioautography of bacilysin.) The concentrates were chromatographed on cellulose TLC sheets (see below). After staining with the ninhydrin reagent, ninhydrin-positive spots were cut out, placed in scintillation counting vials, 3 ml of water was added and the cellulose pieces were soaked for 20–30 min. The cellulose coating was dislodged by gentle shaking. Ten milliliters of Hydrofluor (National Diagnostics) were added to yield stable gels on vigorous shaking for counting of radioactivity in a Beckman LS-230 Liquid Scintillation Counter.

Thin-layer chromatography

TLC was carried out at room temperature on cellulose sheets (20 × 20 cm, Eastman Kodak Co.). Samples were prepared by treating culture broths or cell-free reaction mixtures with acetone at a final concentration of 70% (v/v); clear supernatant fluids were obtained by centrifugation. The supernatant fluids were concentrated to dryness in vacuo and reconstituted in water prior to application onto TLC plates. The TLC plates were first developed with a mixture consisting of 110 ml of the upper

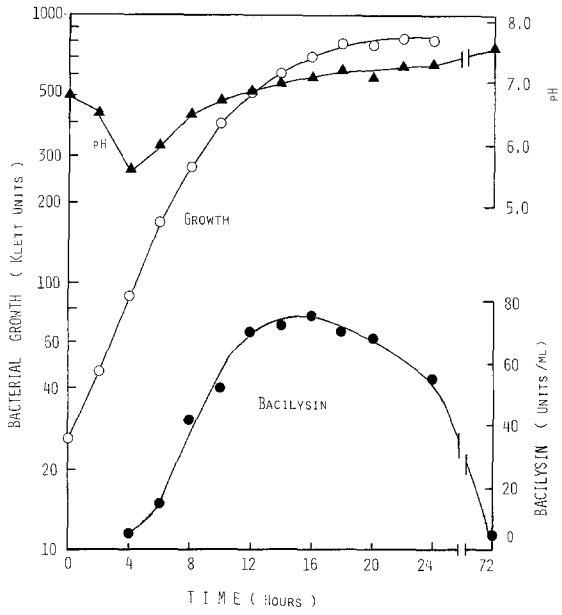

Fig. 2. Growth of *B. subtilis* A14, production of bacilysin and pH changes. ○: growth, ●: bacilysin production, and ▲: pH.

layer of butan-1-ol/acetic acid/water (4:1:4) and 10 ml of acetone. After air-drying the plates were developed in the second dimension with a mixture of 110 ml of the upper layer of butan-1-ol/concentrated ammonium hydroxide/water (4:1:4), 10 ml of acetone and 10 ml of water. The plates were sprayed with ethanolic ninhydrin (0.2% v/v) containing 2,4,6-collidine (0.2% v/v) and heated at 105–110°C for 5–10 min to visualize amino acids and peptides. Bacilysin was located by bioautography. The well-dried (6–16 h) plate was placed in contact for 15 min with a bacilysin assay agar plate seeded with *S. aureus*. The plate was peeled off and the assay plate was incubated for 18–20 h at 37°C to develop inhibition zones. Other TLC plates used included ITLC-SA plates (Gelman Instruments, Ann Arbor, MI) and Baker Flex Silica gel I-B and Si-C_{18} plates (J.T. Baker, Phillipsburg, NJ).

Estimation of protein

Protein in cell extracts was estimated by the method of Bradford [3] using bovine serum albumin as standard.

Buffer solutions

Sodium acetate/acetic acid buffer (50 mM, pH 4.0–6.5), sodium phosphate buffer (20 mM, pH 6.0–8.0), Tris-HCl buffer (50 mM, pH 7.1–9.0), and glycine-NaOH buffer (50 mM, pH 8.5–10.5) were used.

RESULTS

Growth of B. subtilis A14 and production of bacilysin

Fig. 2 shows that bacilysin was synthesized in PA medium towards the end of the exponential phase and reached maximum titer as the cells entered the early stationary phase. This was followed by a gradual decrease in the titer. The pH dropped to 5.6 in the mid-exponential phase and returned to near 7 as the growth rate dropped. Generally, cells were harvested when the pH value returned to 6.8–7.0. Fermentation in PA medium produced antibiotic activity which gave a single inhibition spot co-migrating with the authentic bacilysin on the following TLC plates (solvent, R_F values): Kodak cellulose sheet (butan-1-ol/acetic acid/water = 4:1:4, 0.63; butan-1-ol/ammonium hydroxide/water = 8:1:8, 0.50; acetonitrile/propan-2-ol/acetic acid/water = 70:10:0.1:20, 0.61), ITLC-SA plate (butan-1-ol/acetic acid/water = 4:1:4, 0.60), Baker silica gel I-B plate (butan-1-ol/acetic acid/water = 4:1:4, 0.49), Baker Si-C_{18} plate (acetonitrile/propan-2-ol/water = 70:10:20, 0.46) and two-dimensionally developed cellulose sheets (see Methods and Fig. 3). In SCM medium, used for sporulation of *B. subtilis* A14, growth was rapid but little to no antibiotic was produced. The medium used for production of bacillin [14] did not support good growth and no antibiotic was detected.

Cell-free incorporation of L-[^{14}C]alanine

Early cell-free studies were done in the presence of 1 mM dithiothreitol (DTT) at pH 7.4 followed by one-dimensional TLC. TLC plates were developed with butan-1-ol/acetic acid/water (4:1:4). The substrate used was L-[^{14}C]alanine alone or together with shikimic acid. A major part of the radioactivity was found in the L-alanyl-L-alanine zone and the intensity of radioactivity in this case increased with time during a 4-h incubation period. However, the

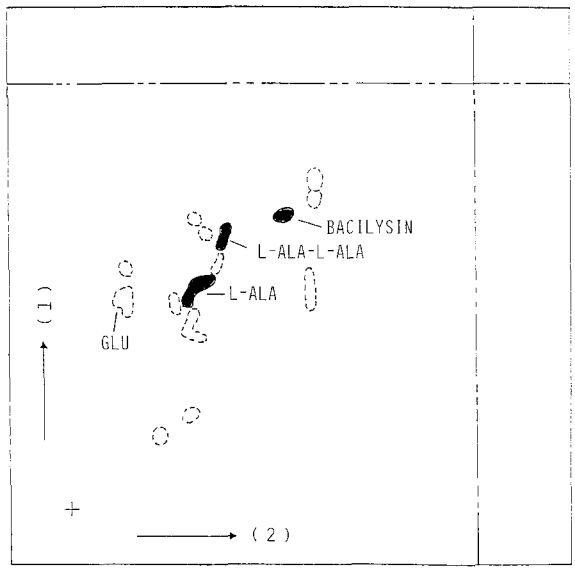

Fig. 3. Compounds present in a cell-free reaction mixture. A cell-free reaction mixture incubated with L-[^{14}C]alanine at 30°C for 4 h, treated with acetone, concentrated and separated on a cellulose sheet by two-dimensional TLC. + shows the origin. — - - — indicates the solvent front. Spots stained with ninhydrin are shown. Shaded spots were radioactive: bacilysin (50 cpm); L-Ala-L-Ala (530 cpm); L-Ala (1920 cpm).

incorporation of radioactivity into the bacilysin zone, which was close to the L-alanyl-L-alanine zone on the one-dimensional TLC plate, was marginal. At that time, it was found that the antibacterial activity of bacilysin was significantly reduced in the presence of 1 mM DTT, indicating that the epoxide group of the anticapsin moiety of bacilysin had opened in the presence of DTT, resulting in a low incorporation of radioactivity into intact bacilysin. DTT was therefore excluded from the reaction mixture. Further, to clearly separate bacilysin and L-alanyl-L-alanine, two-dimensional TLC was employed. TLC plates were run in triplicate; one was used for bioautography to locate bacilysin and the other two were stained with ninhydrin; spots were cut out for counting of radioactivity. Two-dimensional TLC allowed the separation of bacilysin, L-alanyl-L-alanine, L-alanine, glutamic acid, and L-alanyl-L-tyrosine (Fig. 3). Nine to ten other ninhydrin-positive compounds were revealed on the plate, but these were not identified. Among all the ninhydrin-positive spots, radioactivity was only found in bacilysin, L-alanyl-L-alanine and L-alanine (the radioactive substrate). When the reaction was terminated immediately after addition of cell-free extract, radioactivity was located in the L-alanine zone and was not found in bacilysin or L-alanyl-L-alanine. Although the incorporation of radioactivity into bacilysin was low compared to L-alanyl-L-alanine, this was the first observation of the cell-free synthesis of bacilysin.

Cell-free synthesis of bacilysin

Since our interest was in the biosynthesis of bacilysin, we developed a bioassay method to detect bacilysin. Use of a thin double-layer agar plate and a medium modified from Mah et al. [10] with *S. aureus* as the indicator organism increased sensitivity of the assay and yielded clear inhibition zones. This method allowed the detection of bacilysin in a 40-fold-diluted culture supernatant fluid with sharp inhibition zones. The linear relationship be-

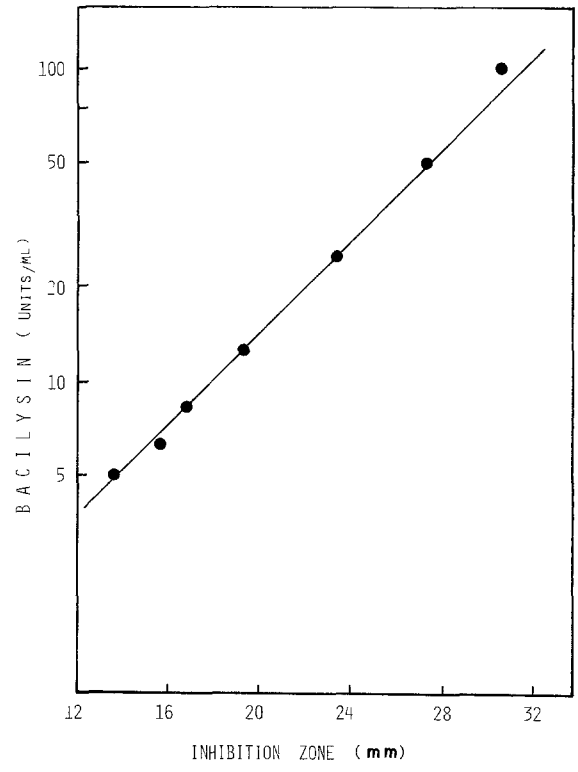

Fig. 4. Relationship between bacilysin concentration and diameter of inhibition zone.

Table 1

Requirements for cell-free synthesis of bacilysin

Components	Bacilysin (units/ml)
All factors added	26
less ATP	4
less ATP, PEP, PK[a]	5
less PEP, PK	26
less Mg^{2+}	6
less Fe^{2+}	28
less ascorbic acid	27
Cell-free extract alone	<4

[a] PEP, phospho*enol*pyruvate; PK, pyruvate kinase.

tween bacilysin concentration and size of inhibition zone is shown in Fig. 4. On plates made from nutrient agar (Difco) and Lab-Lemco agar (Oxoid), the sensitivity of the bioassay was low, probably due to reversal of antibacterial activity by peptides

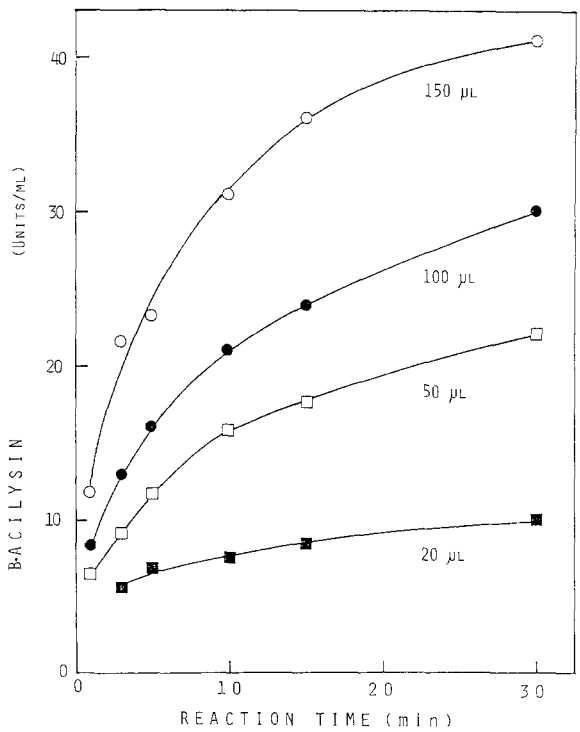

Fig. 5. Time course of cell-free synthesis of bacilysin and effect of the amount of cell-free extract on bacilysin synthesis. ■: 20 μl, □: 50 μl, ●: 100 μl, and ○: 150 μl of cell-free extract. Total volume was 0.5 ml.

in the media. Preliminary experiments showed that bacilysin biosynthetic activity was in the soluble fraction of the cell lysate, only little activity being found in the particulate fraction. Synthesis of bacilysin by the soluble portion of the extract from its constituent amino acids, L-alanine and L-anticapsin, increased with time in a non-linear fashion (Fig. 5). Inhibitory activity detected at zero time was due to bacilysin carried over from the fermentation into the cell-free extract. Boiled cell-free extract, alone or with the reaction mixture, also showed a similar level of antibacterial activity (4–5 units of bacilysin per ml). Production was dependent on the amount of cell-free extract added to the reaction mixture. ATP and Mg^{2+} were found to be essential for the cell-free synthesis of bacilysin, but an energy-generating system consisting of phosph*enol*pyruvate and pyruvate kinase was not required (Table 1). The addition of Fe^{2+} to the reaction mixture did not affect the production of bacilysin. The optimal concentrations of ATP and Mg^{2+} were found to be 4 mM ATP and 12 mM Mg^{2+} (data not shown). The optimum pH for cell-free synthesis was 8.1 (Fig. 6). In the glycine-NaOH buffer, bacilysin synthesis was somewhat inhibited. Tris-HCl buffer at pH 8.1 was chosen for the standard assay.

Effect of concentrations of anticapsin and L-alanine

Cell-free synthesis of bacilysin was dependent on the concentration of anticapsin (Fig. 7). The optimum concentration of anticapsin was found to be 160 μg/ml, and above this concentration, production was decreased. We cannot give a molar concentration since the anticapsin was of unknown purity. No dependence on L-alanine was observed using the crude cell-free extract, presumably because it had a high L-alanine content. However, with a cell-free extract prepared by passage through a Sephadex G-25 column to decrease the concentration of the amino acid, a partial dependence of bacilysin synthesis on L-alanine was observed (Fig. 8). Production of bacilysin increased with an increase in concentration of L-alanine to 0.15 mM, and then reached a plateau. Bacilysin production at the zero concentration of L-alanine was evidently due to residual L-alanine in the extract. More recent experi-

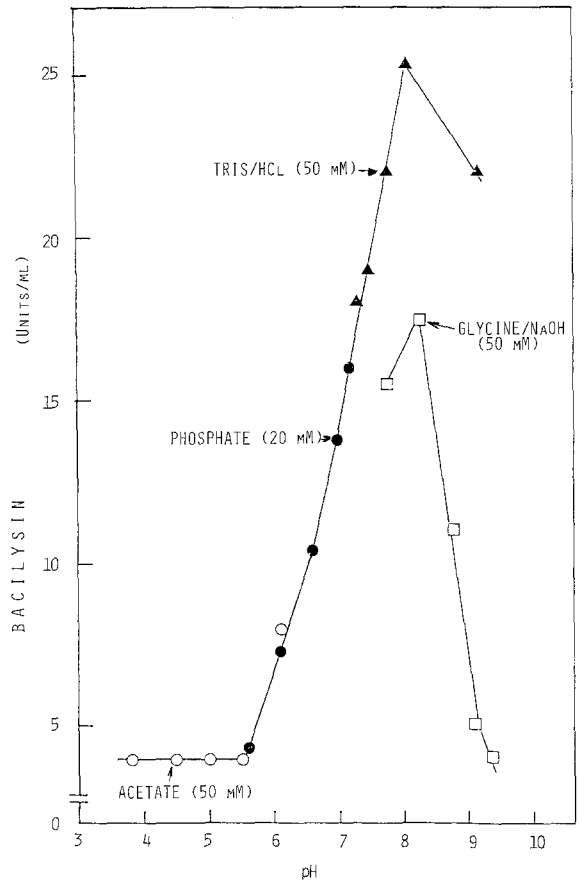

Fig. 6. Effect of pH on cell-free synthesis of bacilysin. ○: 50 mM sodium acetate-acetic buffer, ●: 20 mM sodium phosphate buffer, ▲: 50 mM Tris-HCl buffer, and □: 50 mM glycine-NaOH buffer.

ments, using a cell-free extract from which L-alanine was more extensively removed, have shown no detectable production of bacilysin in the absence of either exogenous L-alanine or anticapsin. With boiled extract, or when the reaction was terminated at zero time (i.e., immediately on addition of cell-free extract), no bacilysin was detectable. Treatment of the cell lysate with RNase or DNase or addition of chloramphenicol to the assay mixture did not inhibit bacilysin production. Boiling the enzyme destroyed its activity. To eliminate the possibility of alanine being used for protein synthesis, chloramphenicol was routinely added to the reaction mixture. When *N*-acetylglucosamine was included in the assay agar, antibacterial activity pro-

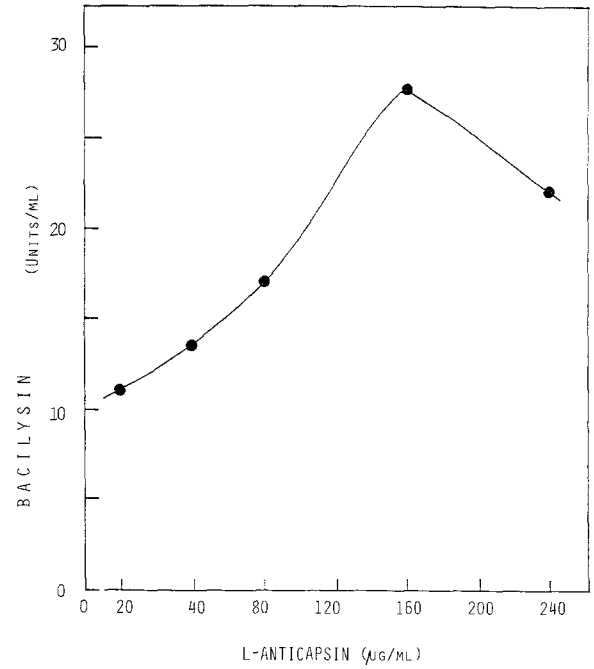

Fig. 7. Effect of anticapsin concentration on the cell-free synthesis of bacilysin.

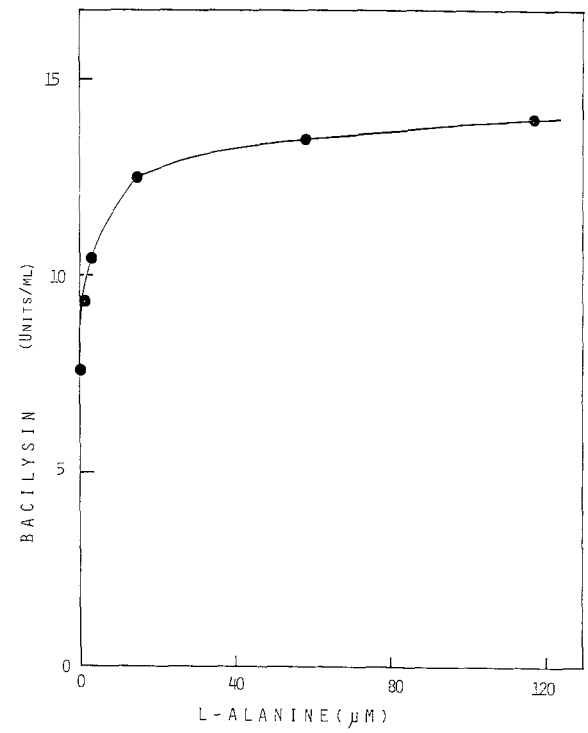

Fig. 8. Effect of L-alanine concentration on cell-free synthesis of bacilysin. 20 μl of cell-free extract (passed through a Sephadex G-25 column) was used in a 0.1-ml reaction volume.

duced by the cell-free extract was markedly reduced. We thus conclude that bacilysin is synthesized by a peptide synthetase similar to that used for biosynthesis of many peptide antibiotics [7]. For further characterization of bacilysin synthetase, purification of the enzyme will be required.

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REFERENCES

1 Abraham, E.P., D. Callow and K. Gilliver. 1946. Adaptation of *Staphylococcus aureus* to growth in the presence of certain antibiotics. Nature (Lond.) 158: 818–821.

2 Atsumi, K., R. Oiwa and S. Omura. 1975. Production of bacillin by *Bacillus* sp. strain No. KM-208 and its identity with tetaine (bacilysin). J. Antibiot. 28: 77–78.

3 Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

4 Doi, R.H. and R.T. Igarashi. 1964. Ribonucleic acids of *Bacillus subtilis* spores and sporulating cells. J. Bacteriol. 87: 323–328.

5 Foster, J.W. and H.B. Woodruff. 1946. Bacillin, a new antibiotic substance from a soil isolate of *Bacillus subtilis*. J. Bacteriol. 51: 363–369.

6 Kaminski, K. and T. Sokolowska. 1973. The probable identity of bacilysin and tetaine. J. Antibiot. 26: 184–185.

7 Katz, E. and A.L. Demain. 1977. The peptide antibiotics of *Bacillus:* Chemistry, biogenesis and possible functions. Bacteriol. Rev. 41: 449–474.

8 Kenig, M. and E.P. Abraham. 1976. Antimicrobial activities and antagonists of bacilysin and anticapsin. J. Gen. Microbiol. 94: 37–45.

9 Kenig, M., E. Vandamme and E.P. Abraham. 1976. The mode of action of bacilysin and anticapsin and biochemical properties of bacilysin-resistant mutants. J. Gen. Microbiol. 94: 46–54.

10 Mah, R.A., D.Y.C. Fung and S.A. Morse. 1967. Nutritional requirements of *Staphylococcus aureus* S-6. Appl. Microbiol. 15: 866–870.

11 Perry, D. and E.P. Abraham. 1979. Transport and metabolism of bacilysin and other peptides by *Staphylococcus aureus*. J. Gen. Microbiol. 115: 213–221.

12 Roscoe, J. and E.P. Abraham. 1966. Experiments relating to the biosynthesis of bacilysin. Biochem. J. 99: 793–800.

13 Walker, J.E. and E.P. Abraham. 1970. The structure of bacilysin and other products of *Bacillus subtilis*. Biochem. J. 118: 563–570.

14 Walton, R.B. and E.L. Rickes. 1962. Reversal of the antibiotic, bacillin, by N-acetylglucosamine. J. Bacteriol. 84: 1148–1151.