Biochem. J. (1970) **118**, 557–561
Printed in Great Britain

Isolation of Bacilysin and a New Amino Acid from Culture Filtrates of *Bacillus subtilis*

By J. E. WALKER* and E. P. ABRAHAM
Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, U.K.

(*Received* 22 *December* 1969)

1. Bacilysin, a labile dipeptide antibiotic that lyses growing staphylococci, was isolated from culture fluids of *Bacillus subtilis* by a process giving higher yields than those previously obtained. 2. The process involves adsorption on a cation-exchange resin and elution with aqueous trimethylamine, separation from neutral amino acids and glutamic acid by chromatography on DEAE-Sephadex at pH 8.7 and separation from other neutral peptides by chromatography in aqueous propan-2-ol on Sephadex G-25. 3. A new amino acid, which is chemically related to bacilysin, was isolated from the fraction containing neutral amino acids. 4. Two substances that yield alanine on hydrolysis, in addition to bacilysin, were obtained from the neutral peptide fraction.

The isolation of bacilysin in low yield from culture filtrates of a strain of *Bacillus subtilis* (A 14) was described by Rogers, Newton & Abraham (1965*a*). The product obtained gave L-alanine and L-tyrosine on hydrolysis but did not contain a tyrosine residue. A possible type of structure for it, containing a labile epoxide group, was suggested by Rogers, Lomakina & Abraham (1965*b*), but the material available was insufficient for a definitive structure to be determined. To facilitate the production of bacilysin in amounts adequate for further chemical and biological studies attempts were made to devise a more efficient purification process. The present paper describes such a process, which led to the isolation of bacilysin and also a chemically related new amino acid.

METHODS

General

Measurement of bacterial growth. This was made by measurements of the opacity of cultures suitably diluted in tubes (1.5cm diam.) in a Spekker absorptiometer (Hilger and Watts Ltd.) with a neutral-grey filter. An extinction value of 1.0 corresponded to a cell dry weight of 0.41–0.45mg/ml.

Assay of antibacterial activity. The method of assay and unit activity were as described by Roscoe & Abraham (1966). Before assay of bacilysin in aqueous propan-2-ol the latter was first removed by extraction with 2vol. of ether. For semi-quantitative estimates of bacilysin in aqueous propan-2-ol 25–100μl of each solution was pipetted on to Whatman antibiotic assay paper discs (13mm diam.). The discs were dried rapidly *in vacuo* (10min), placed on plates seeded with *Staphylococcus aureus* (N.C.T.C. 6571) and wetted with 30μl of water before incubation.

Paper chromatography and electrophoresis. These were carried out as described by Rogers *et al.* (1965*a*). Substances were eluted from paper with water in an apparatus similar to that described by Lockhart & Abraham (1954).

Bioautography. For the location of bacilysin on paper after electrophoresis, chromatography, or both, bioautographs were prepared as described by Roscoe & Abraham (1966).

Production of bacilysin

Preparation of a spore suspension of B. subtilis *A*14. The freeze-dried organism was grown overnight in Oxoid broth at 37°C. The resulting culture was streaked on to slopes in 28ml bottles of Oxoid nutrient agar and the latter were incubated at 37°C overnight. The organisms were washed from a slope with sterile water into 80ml of 'S' broth (Pollock, 1953) in a 500ml Erlenmeyer flask. The flask was placed on a rotary shaker (5cm throw, 160–170rev./min) at 35°C for 40h and then heated for 1h in a water bath at 60°C. The spores were centrifuged (2000*g* for 20min), washed once with water (25ml) and finally resuspended in water (20ml) and kept at 4°C. Storage of this spore suspension for up to 6 months had no detectable effect on the bacilysin titres of cultures derived from it.

Production medium. A basal medium was prepared containing (g/l): KH_2PO_4 (1), $MgSO_4$, $7H_2O$ (0.5), KCl (0.5), monosodium glutamate monohydrate (4.0). The production medium contained (ml/l): basal medium (954), ferric citrate solution (5) [$FeCl_3$,$6H_2O$ (20mg/ml) and trisodium citrate monohydrate (20mg/ml)] and an oligodynamic solution (1) (Pollock & Kramer, 1958).

* Present address: School of Pharmacy, University of Wisconsin, Madison, 53706, U.S.A.

The pH was adjusted to 7.0 with 5M-NaOH and the solution was autoclaved at 15lb/in^2 for 20min. The precipitate that formed was not removed. To this sterilized solution was added a solution of sucrose (40ml, 342g/l) that had been sterilized separately.

Growth of B. subtilis *A*14. For the production of bacilysin the organism was grown in 5 litre whirling flasks, fitted with an air-exchange head, of the type described by Mitchell (1949). The flasks rotated at 200rev./min and were enclosed in a box in which the temperature was thermostatically controlled at 33–34°C.

A slope of Oxoid nutrient agar was inoculated with 0.25ml of the spore suspension of *B. subtilis* A14 and incubated for 24h at 37°C. The organisms were washed from the slope with 0.15M-NaCl and the suspension was pipetted into 1 litre of production medium in a whirling flask. Usually four cultures at a time were set up in this way. The cultures were harvested after about 17h, when the pH (which fell earlier to 6.0) had risen to a value (about 6.9) at which it remained constant for several hours. The cells were removed by centrifugation at 4°C (2200*g* for 30min) and the supernatants were assayed for antibacterial activity. The supernatants, containing 20–35 units/ml, were stored in polythene bottles at −20°C until 12.5 litres of fluid had been obtained. They were then melted and combined for use in the first stage of the purification process.

Purification of bacilysin

Desalting of culture supernatant. The culture supernatant (12.5 litres, 20 units/ml) was pumped through a column (40cm×7cm diam.) of Zeo-Karb 225 (SRC 5, 14–52 mesh, H^+ form) at 40ml/min with the aid of a Sigmamotor Model TS6 pump (Sigmamotor Inc., Middleport, N.Y., U.S.A.) fitted with an Allspeed regulator (Allspeeds Ltd., Accrington, Lancs., U.K.). The resin was then extruded into a 5 litre polypropylene beaker and washed six times with water (1 litre), the supernatant being removed each time by decantation. The washed resin was suspended in water (about 500ml), the mixture stirred vigorously and M-trimethylamine (about 520ml) added slowly until the pH of the supernatant was 9.2. The resin was filtered off on a sintered-glass filter (27cm diam., no. 1 porosity). It was then washed twice by resuspension in water (300ml) and filtration. The filtrates were combined in a 5 litre polypropylene beaker and damp-dry CM-Sephadex C-25 (H^+ form) (about 5g) was added with stirring until the pH was 6.5–7.0. The CM-Sephadex was removed by filtration through Whatman no. 1 paper and the filtrate (3.6 litres) was concentrated at 20°C in a rotary evaporator, a few drops of pentan-1-ol being added to prevent frothing. The concentrate (about 900ml) was freeze-dried, giving a yellow-brown solid (5.6g, 45 units/mg).

Chromatography on DEAE-Sephadex A-25. This was carried out at 4°C. The solid obtained as described above was dissolved in 5ml of *N*-ethylmorpholine–acetate buffer (0.3M with respect to *N*-ethylmorpholine), pH8.7. The viscous brown solution was added to a column (80cm× 2.5cm diam.) of DEAE-Sephadex A-25 that had been equilibrated with the buffer. The solution was allowed to enter the column (2h) and was washed in with 5ml of buffer. Buffer was then pumped through the column with an LKB Recychrom peristaltic pump at 2ml/min and fractions were collected at 7.5min intervals. A sample from each fraction was assayed for antibacterial activity after adjustment of the pH to 7.0 and suitable dilution with 0.1M-potassium phosphate buffer, pH7.0. Samples of ninhydrin-positive fractions were subjected to paper chromatography in butan-1-ol–acetic acid–water (4:1:4, by vol.) and in propan-2-ol–water (7:3, v/v). Fractions 35–39, 40–63 and 64–105 were pooled separately. Amino acids and peptides in the three pooled fractions were separated from the buffer by adsorption on Zeo-Karb 225 (H^+ form) (41, 50 and 300g respectively) and elution by stirring with M-trimethylamine until the pH rose to 9.2. Trimethylamine and the remaining *N*-ethylmorpholine were removed by stirring the eluates with CM-Sephadex C-25 (H^+ form) until the pH of the supernatant was 7.0. The CM-Sephadex was removed by filtration and washed with water. Each combined filtrate and washing was concentrated at 20°C in a rotary evaporator and freeze-dried. The resulting solids from fractions 35–39, 40–63 and 64–103 weighed 321mg, 680mg and 353mg respectively and their activities were 106 units/mg, 147 units/mg and 68 units/mg respectively. The overall recovery of bacilysin was 77% and of this 63% was in fractions 40–63. In fractions 35–39 bacilysin was mixed with a second ninhydrin-positive substance (AA1) and in fractions 40–63 with two further ninhydrin-positive substances (P2 and P3). Neither substance AA1 nor substances P2 and P3 appeared to be any of the common amino acids when subjected to paper chromatography and electrophoresis.

In a second experiment under similar conditions with material from the original Zeo-Karb eluate (4.6g, 42 units/mg) the overall yield of bacilysin was 60%. Of this 77% (550mg) was obtained free from amino acids and with an activity of 162 units/mg.

Chromatography on Sephadex G-25 *in aqueous propan*-2-*ol.* This was carried out at 4°C. Freeze-dried material obtained after chromatography on DEAE-Sephadex (550mg, 162 units/mg) was dissolved in 0.6ml of water, and 1.4ml of propan-2-ol was added. A slight precipitate that formed was removed by centrifugation (2000*g* for 10min) and was found to have no detectable antibacterial activity. The supernatant was added to a column of Sephadex G-25 (120cm×1.2cm diam.) packed in propan-2-ol–water (7:3, v/v). This solvent mixture was pumped through the column at 6ml/h with an LKB Miniflow all-glass pump. Fractions were collected at 30min intervals and screened for antibacterial activity and the presence of ninhydrin-positive substances. Ninhydrin-positive fractions were analysed by paper chromatography in butan-1-ol–acetic acid–water (4:1:4, by vol.) and in propan-2-ol–water (7:3, v/v). The bacilysin-containing fractions (44–72) were pooled and the propan-2-ol was removed by extraction with 2vol. of ether. The aqueous phase was freed from ether by a stream of air and freeze-dried to give a white powder (260mg, 230 units/mg). The recovery of activity was 65%.

Gel filtration on Sephadex G-10. The bacilysin preparation from the preceding stage (150mg) was dissolved in 0.5ml of water and the pale-yellow solution was applied to a column (90cm×1.8cm diam.) of Sephadex G-10. Water was pumped through the column at 6ml/h with an LKB Miniflow pump and 2ml fractions were collected. The effluent from the column was monitored with a

Uvicord recorder. A band of material that absorbed u.v. light and that gave a yellow solid when its solution was freeze-dried emerged from the column before bacilysin. The bacilysin-containing fractions were freeze-dried giving a white solid (120mg, 260 units/mg). The recovery of antibiotic was 90%.

Purification of substances AA1, P2 and P3

Substance AA1. Fractions 30–34 from the column of DEAE-Sephadex contained compound AA1 mixed with other amino acids but no bacilysin. The buffer was removed from these pooled fractions by the procedure used with the bacilysin-containing fractions. The resulting freeze-dried material (250mg) was rechromatographed on a column (80cm×2.5cm diam.) of DEAE-Sephadex A-25 in 0.3M-*N*-ethylmorpholine–acetate buffer, pH8.7, under conditions similar to those described for the purification of bacilysin. Substance AA1 was eluted in fractions 26–40 and known neutral amino acids in fractions 17–25. Buffer was removed from fractions 26–40 as described above and the solution was freeze-dried. The product (51mg) gave a single major ninhydrin-positive spot when chromatographed in butan-1-ol–acetic acid–water (4:1:4, by vol.) ($R_{bacilysin}$ 0.75) or in propan-2-ol–water (7:3, v/v) ($R_{bacilysin}$ 1.0) and was contaminated with only traces of other ninhydrin-positive materials. The latter were removed by gel filtration through a column (80cm×1.8cm diam.) of Sephadex G-10 as described above for bacilysin, 1.5ml fractions being collected every 20min. Substance AA1 emerged in fractions 11–14, which were pooled and freeze-dried (41mg).

Substances P2 and P3. During the purification of bacilysin by chromatography on Sephadex G-25 in aqueous propan-2-ol two uncharacterized ninhydrin-positive substances emerged from the column after bacilysin. Substance P2 was in fractions 74–93 and substance P3 in fractions 120–140. The pooled fractions were freeze-dried, yielding 21mg of substance P2 and 54mg of substance P3. Each yielded only a single ninhydrin-positive spot when chromatographed on paper in butan-1-ol–acetic acid–water (4:1:4, by vol.) and in propan-2-ol–water (7:3, v/v). In the former solvent system substances P2 and P3 showed $R_{bacilysin}$ values 1.0 and 0.34 respectively and in the latter 0.84 and 0.52 respectively.

RESULTS AND DISCUSSION

Isolation of bacilysin

In attempts to devise a satisfactory process for the isolation of the labile neutral dipeptide, bacilysin, three factors were taken into consideration: first, the desirability of using a simple chemically defined culture medium that would provide the minimum contamination with other amino acids and peptides; secondly, the advantage of harvesting cultures immediately after bacilysin production had reached its peak, to avoid contamination with products of the subsequent autolysis of *B. subtilis*; thirdly, the need to avoid buffers and solvents that would inactivate the antibiotic by a nucleophilic attack on its epoxide grouping.

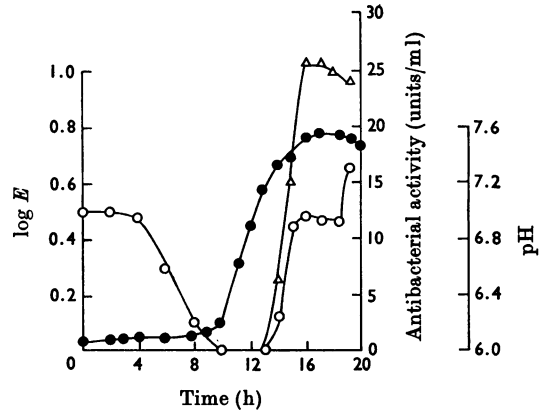

Fig. 1. Growth of *B. subtilis* A14 and production of bacilysin in a chemically defined medium. For details see the Methods section. ●, Extinction of culture; ○, pH; △, antibacterial activity.

The chemically defined medium used here for the production of bacilysin was similar to that of Rogers *et al.* (1965*a*) except that it contained sucrose in place of glucose and sodium glutamate in place of ammonium acetate as a source of nitrogen. The average yields of antibiotic were about 1.5 times those obtained by Rogers *et al.* (1965*a*) and comparable with those obtained by Roscoe & Abraham (1966) in a medium containing ammonium acetate but buffer with high concentrations of phosphate. The latter would have complicated the desalting of culture supernatants on a preparative scale.

The growth of *B. subtilis* A14 under the conditions used is shown in Fig. 1. Determination of the optimum time for harvesting the cultures was facilitated by a characteristic change in the pH of the culture. This fell from 7.0 to 6.0 during the first stages of growth and then rose, with bacilysin production, to reach a plateau at about 7.0, when the titre of antibiotic was maximal. Cultures were normally harvested as soon as the pH plateau had clearly been reached.

In the process of purification described by Rogers *et al.* (1965*a*) bacilysin was eluted from a strong cation-exchange resin with aqueous pyridine and chromatographed in pyridine–acetate buffers. The volatility of these eluting agents facilitated their subsequent removal, but it was later concluded that their ability to react with the epoxide group of bacilysin was responsible for low yields in some of the procedures used. In the process used here bacilysin that had been adsorbed from culture supernatant on to Zeo-Karb 225 (H^+ form) was

eluted with aqueous trimethylamine. Since it was necessary to raise the pH to 9.2 for complete elution the adsorption was apparently not due exclusively to electrostatic forces. However, lowering of the pH to 7, at which bacilysin was more stable, could be accomplished without loss, or the addition of anions to the solution, by the use of CM-Sephadex C-25 (H^+ form). In this desalting procedure, which was also used after the second stage of the purification process to free bacilysin from *N*-ethylmorpholine–acetate buffer, the recovery of antibiotic was at least 90%.

Freeze-dried preparations of crude bacilysin obtained from culture supernatants after adsorption on and elution from Zeo-Karb 225 were analysed on paper by electrophoresis (pH 1.8 and 4.5) followed in each case by chromatography in butan-1-ol–acetic acid–water (4:1:4, by vol.) and propan-2-ol–water (7:3, v/v) in a perpendicular direction. Spots were stained with ninhydrin and bacilysin was located on duplicate papers by bioautography. Major spots were observed corresponding to bacilysin, alanine and glutamic acid, smaller ones to glycine, valine, leucine/isoleucine, and just detectable ones to phenylalanine and tyrosine. In addition, spots were detected due to three uncharacterized substances, which showed no net charge when subjected to electrophoresis on paper at pH 4.5. One (substance AA 1) showed $R_{\text{bacilysin}}$ 0.75 in butan-1-ol–acetic acid–water but was not resolved from bacilysin in propan-2-ol–water. On electrophoresis at pH 1.8 it migrated 0.89 times as far as bacilysin towards the cathode. The second (substance *P* 2) showed $R_{\text{bacilysin}}$ 0.84 in propan-2-ol-water but was not resolved from bacilysin in butan-1-ol–acetic acid–water. The third (substance *P* 3) showed $R_{\text{bacilysin}}$ 0.34 and 0.52 and R_{Gly} 1.12 and 1.0 in butan-1-ol–acetic acid–water and aqueous propan-2-ol respectively.

The second stage of the purification of bacilysin was designed to separate the antibiotic from the known amino acids and uncharacterized substances with which it was contaminated. The method used was based on that of Carnegie (1961*a*,*b*) and depends on the fact that the pK_a of the amino group of the *N*-terminal residue of a peptide with α-linkages is significantly lower than that of the amino group of an α-amino acid (about 9.6). The pK_a of the amino group of bacilysin at 22°C is 8.2 (Rogers *et al.* 1965*b*). Thus it was to be expected that the retardation of bacilysin on a column of anion-exchange material would be significantly greater at pH 8.7 (when the antibiotic would have a net negative charge) than that of the common amino acids. The result obtained by chromatography of crude bacilysin on DEAE-Sephadex A-25 in 0.3M-*N*-ethylmorpholine–acetate buffer, pH 8.7, is shown in Fig. 2. Alanine, valine, leucine/isoleucine and (later) tyrosine emerged from the column before any bacilysin. The fractions con-

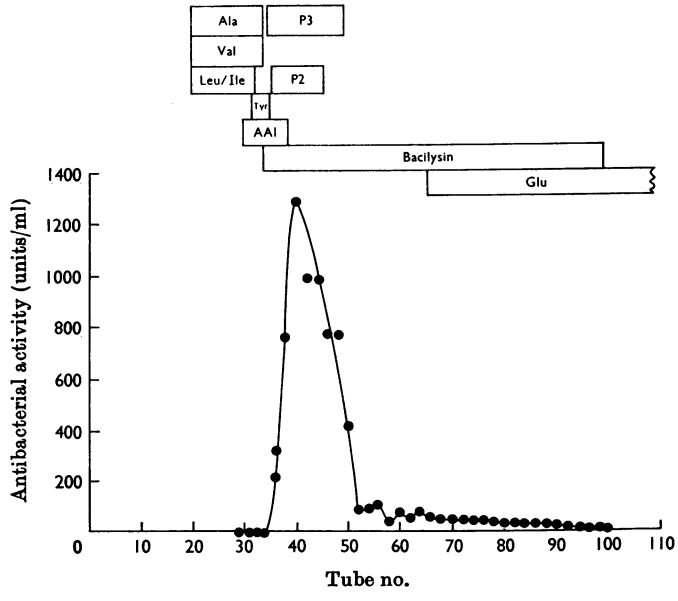

Fig. 2. Chromatography of crude bacilysin on DEAE-Sephadex A-25 in 0.3M-*N*-ethylmorpholine–acetate buffer, pH 8.7. For details see the Methods section.

Table 1. *Behaviour of bacilysin and other substances on paper chromatography and electrophoresis*

Paper chromatography was in butan-1-ol–acetic acid–water (4:1:4, by vol.) (BAW) and in propan-2-ol–water (7:3, v/v) (PW). Electrophoresis at pH1.8 was for 40min at 60V/cm and at pH4.5 for 20min at 60V/cm. Substances with no net charge at pH4.5 were carried 1.5cm towards the cathode by endosmosis.

Substance	R_{Tyr}		Migration towards cathode (cm)	
	In BAW	In PW	At pH1.8	At pH4.5
Bacilysin	1.0	0.95	18	1.5
AA1	0.75	0.8	16	1.5
P2	1.0	0.95	23	1.5
P3	0.34	0.5	17	1.5

taining bacilysin overlapped with some of the fractions containing substance AA1. The major portion of the bacilysin peak was free of substance AA1 but mixed with substances P2 and P3. Glutamic acid emerged later and overlapped only with a long tail with low activity following the main antibiotic peak.

The results of paper chromatography suggested that bacilysin might be separated from substances P2 and P3 by chromatography on a column of Sephadex G-25 in propan-2-ol–water (7:3, v/v). On chromatography under these conditions bacilysin, substance P2 and substance P3 emerged successively from the column in the order expected from their behaviour on paper (see the Methods section). The bacilysin contained a smaller amount of pigmented material whose origin and nature were not determined, but which could be separated by gel filtration on Sephadex G-10, when it emerged before bacilysin from the column.

Bacilysin was isolated from the culture supernatant by the procedures described here in an overall yield of 33%, which represented a threefold improvement on the recovery obtained by Rogers *et al.* (1965*a*). The loss of antibiotic during chromatography on Sephadex G-25 in propan-2-ol–water was decreased from 50 to 35% by running the column at 4°C. When subjected to electrophoresis and chromatography in the systems used (Table 1) purified bacilysin behaved as a single ninhydrin-positive substance. The specific activity assigned to it (260 units/mg) was somewhat higher than the value of 208 units/mg obtained by Rogers *et al.* (1965*a*). However, the assays were probably not reliable to within less than ±15%.

Substances AA1, P2 and P3

The elution of substance AA1 before bacilysin from a column of DEAE-Sephadex at pH8.7 indicated that the pK_a of the amino group of the former was higher than that of the amino group of the latter. The finding that substance AA1 migrated less far than bacilysin towards the cathode on electrophoresis at pH1.8 (Table 1) could be accounted for if the pK_a of the carboxyl group were lower than that of the carboxyl group of bacilysin, even if substance AA1 were a smaller molecule. On treatment with 6M-hydrochloric acid at 105°C for 17h substance AA1 yielded an amino acid that behaved like tyrosine on paper chromatography. These facts together suggested that substance AA1 is a neutral amino acid related to the tyrosine-yielding fragment of bacilysin.

The elution of substances P2 and P3 with bacilysin from a DEAE-Sephadex column at pH8.7 suggested that these are peptides. The fact that substance P2 migrated further than bacilysin towards the cathode on paper electrophoresis at pH1.8, but substance P3 less far, could then be accounted for if the molecular weights of substances P2 and P3 were lower and higher respectively than that of the antibiotic.

The structures of bacilysin and substances AA1, P2 and P3 are discussed by Walker & Abraham (1970).

We are grateful to Miss Lin Peacham, Miss Sylvia Main and Mr T. J. Beesley for expert technical assistance and to the Medical Research Council for financial assistance.

REFERENCES

Carnegie, P. R. (1961*a*). *Nature, Lond.*, **192**, 658.
Carnegie, P. R. (1961*b*). *Biochem. J.* **78**, 687.
Lockhart, I. M. & Abraham, E. P. (1954). *Biochem. J.* **58**, 633.
Mitchell, P. (1949). *Nature, Lond.*, **164**, 846.
Pollock, M. R. (1953). *J. gen. Microbiol.* **8**, 186.
Pollock, M. R. & Kramer, M. (1958). *Biochem. J.* **70**, 665.
Rogers, H. J., Lomakina, N. & Abraham, E. P. (1965*b*). *Biochem. J.* **97**, 579.
Rogers, H. J., Newton, G. G. F. & Abraham, E. P. (1965*a*). *Biochem. J.* **97**, 573.
Roscoe, J. & Abraham, E. P. (1966). *Biochem. J.* **99**, 793.
Walker, J. E. & Abraham, E. P. (1970). *Biochem. J.* **118**, 563.