SYNTHESIS OF ANTIBIOTICS BY ENZYMES FROM ALTERED GROWTH CONDITIONS BY *BACILLUS LICHENIFORMIS*

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1. Introduction

Bacitracins are a group of a number of polypeptide antibiotics produced by *Bacillus licheniformis* [1, 2]. Bacitracin A (fig. 1), the major component, is highly active as an antibiotic, whereas bacitracin F is inactive. Bacitracin B appear to have the same structure as A with replacement of ring isoleucine by valine [3]. The structure of other bacitracins is not known, but D and E yield also valine and C yields glycine as well [2]. Bacitracin A can be synthesized by enzymes isolated from cells of *B. licheniformis* [4] similar to the synthesis of gramicidin S and tyrocidine [5]. It has been reported that this organism produces yet another group of antibiotics known as licheniformins [6] and that the production of one or the other group depends upon the C/N ratio in the medium [7]. We have also observed that if the fermentation conditions are not properly controlled, the production of bacitracin is hindered, although there is no appreciable change in the total amount of antibiotics produced. This led to the present study to see if the enzymes synthesizing bacitracin A are the same or missing from the fermentations producing little or no bacitracin A.

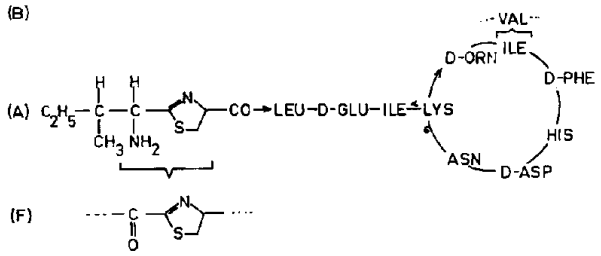

Fig. 1. Bacitracins A, B and F. B and F are similar to A except for the variations shown.

2. Materials and methods

2.1. *Analytical procedures*

Protein determination [8], antibiotic assay [9], sucrose density gradient centrifugation [10], selectron filter retention test for [^{14}C]amino acid incorporation and amino acid dependent ATP-$^{32}PP_i$ exchange measurements were as described earlier [4]. When the amino acid dependent ATP-$^{32}PP_i$ exchange on a single amino acid was measured, the incubation mixture was modified by replacement of cysteine and other bacitracin amino acids by that particular amino acid (1.5 mM).

2.2. *Growth of Bacillus and preparation of cells*

B. licheniformis (ATCC 10716) was grown on the medium of Cornell and Snoke [11] under slightly different conditions. Fermentation A used 9 g/ℓ and Fermentation B 12 g/ℓ of L-glutamic acid. The growth conditions for Fermentation B were the same as for previously described Fermentation A [4] Fermentation C contained also 12 g/ℓ of L-glutamic acid in a 1000 liter fermentor, but with an increased air flow (400 ℓ/min), thereby completing the fermentation in 17 hr as compared to 4–5 days for A and B. The pH change was from 7.5–9.0. This particular fermentation was carried out for us by E. Merck,

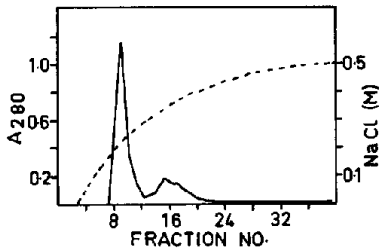

Fig. 2. DEAE-Cellulose chromatography of PPT II from fermentation C. 10 ml fractions were collected. NaCl (---); A_{280} (——).

Darmstadt, Germany. The cells from the three fermentations were treated as described by Pfaender et al. [4] and stored at −80°C.

2.3. *Synthesis of antibiotics*

The enzymes were incubated with an equal volume of the incubation mixture [4] at 37°C for 10 min. In case of enzymes from fermentation C, a mixture of all 20 L-amino acids (1.5 mM each) was used. The product was lyophilized, extracted with methanol and chromatographed on precoated silica gel thin layer plates (Kieselgel F 254, Merck, Germany) using *n*-butanol:acetic acid:water (4:1:2) as the developer. After drying, various bands were scraped off, extracted twice with methanol and twice with methanol:water (1:1) mixture. The solvents were removed thoroughly from the extract and the aqueous solution of the residue was used for antibiotic assay. A duplicate, which was immediately frozen, was the control and the difference between the two was used as a indication of the synthesis of antibiotics.

2.4. *Disc gel electrophoresis*

The gels (7.5% acrylamide, pH 7.5) were prepared as described by Maurer [12] and the electrophoresis was performed at 4°C (4 mA/tube; 90 min) in Tris–barbiturate buffer, pH 7.5. 10 µl of enzyme in buffer A [4] diluted with an equal volume of 40% sucrose solution were applied to the gel. After the run, the gels were frozen and extruded out by gentle warming. One set of the gel was stained with 1% Amido black and washed with 7% acetic acid. Bands (5 mm wide) were sliced from the other set, macerated with a glass rod in 0.35 ml of buffer A, diluted with 0.5 ml of the buffer, centrifuged (2000 *g*, 5 min) and the supernatant decanted. This procedure was repeated once more and 0.4 ml of the combined supernatant was used for the selectron filter retention test.

3. Results and discussions

3.1. *Preparation of enzymes from cells*

Enzyme preparations from cells of Fermentation A have been described [4]. Cells from Fermentation B were treated exactly as from A. DEAE-cellulose chromatography as before yielded two active enzymes I and II (their positions were similar to the positions of enzyme I and II of Fermentation A) as tested by selectron filter retention test and ATP-$^{32}PP_i$ exchange measurements. Cells from Fermentation C were disrupted by a modified method of Roskoski et al. [13]. A hundred grams of wet cells were suspended in 100 ml of 0.025 M sodium phosphate buffer, pH 7.5, containing 0.75 mM EDTA; 1 mM $MgCl_2$; 10 mM 2-mercaptoethanol and lysed with a mixture of 160 mg lysozyme (Sigma Chemicals, USA), 400 µg DNAase I and 100 µg RNAase A (both from Boehringer, Mannheim, Germany) at 30°C for 20 min. It was immediately cooled, diluted with 100 ml of phosphate buffer, pH 7.5, containing 1 M NaCl and 10 mM $MgCl_2$ and then centrifuged (27 000 *g*, 40 min). The supernatant was decanted and the residue lysed once more. Solid, cold ammonium sulfate was then added to the cooled combined supernatant to 55% saturation. It was centrifuged (27 000 *g*, 40 min) after standing overnight in the cold. The precipitate, PPT I, was dissolved in buffer A ($A_{280} \simeq 3$), reprecipitated by 55% ammonium sulfate saturation and centrifuged after 4 hr. This precipitate, PPT II, was further used for DEAE-cellulose chromatography, whereas the rest of the protein from the supernatant was precipitated down by 90% ammonium sulfate saturation. This precipitate is referred to as 90% PPT II. DEAE-cellulose chromatography of PPT II in a NaCl gradient (0–0.5 M) in buffer A (fig. 2) gave 2 peaks, but only peak 2 had the ATP-$^{32}PP_i$ exchange activity dependent on each of ten bacitracin amino acids, and was active.

3.2. *Synthesis of antibiotics by isolated enzymes*

The enzyme preparations from three fermentations obtained after DEAE-cellulose chromatography on in-

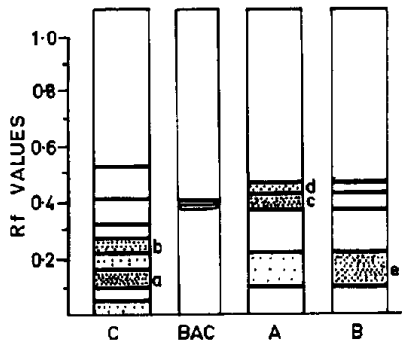

Fig. 3. Thin layer chromatography of products. A, B and C denote the fermentations. Enzymes (A, 1000 μg; B, 185 μg; C, 250 μg) from cells after DEAE-Cellulose chromatography were incubated, products chromatographed and zones scraped off. After extraction assayed for antibiotic. For details see Materials and methods. Zones cut for assay, ☐; Antibiotic activity, ▨; BAC, bacitracin. Slow moving is A and other F. Bacitracin equivalent activities in zones: a) 2.35 μg; b) 0.60 μg; c) 5.1 μg; d) 6.0 μg; e) 3.2 μg.

cubation showed the presence of bands upon TLC, which were antibiotically active (fig. 3). Results of the enzyme activity from Fermentation A were redrawn from Pfaender et al. [4] for comparison. Bacitracin A was synthesized by enzymes isolated

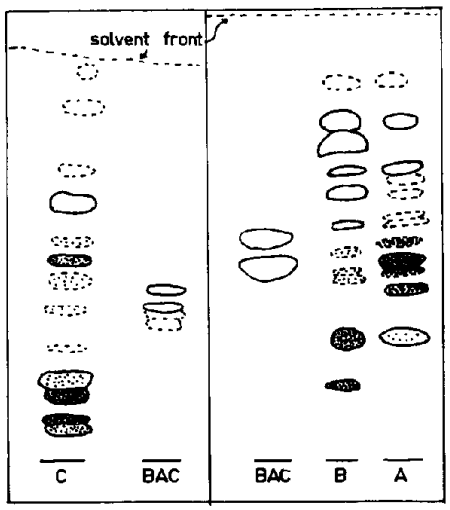

Fig. 4. Thin layer chromatography of culture broths of *B. licheniformis*. A, B and C denote the fermentations. Plates were sprayed with Chlor–Toluidine reagent for peptide identifications. BAC is bacitracin. Broken line enclosures indicate weakly stained spots and others are strongly stained. Shaded area and its density show the antibiotic activity.

Table 1
ATP-$^{32}P_i$ exchange in presence of amino acids.

Amino acids	nmoles ATP/mg protein		
	Fermentation B		Fermentation C
	Enzyme I (10)[a]	Enzyme II (18)[a]	Fraction 15[a]
L-Ile	47.5	217.3	28.2
L-Cys	57.7	162.6	29.0
L-Leu	49.0	389.8	21.2
L-Glu	24.5	88.5	6.3
L-Lys	41.0	115.4	28.3
L-Orn	39.7	46.7	9.4
L-Phe	40.6	160.6	10.5
L-His	52.5	59.7	11.4
L-Asp	33.6	36.1	6.6
L-Asn	37.7	84.8	19.5
L-Val	–	–	27.0

[a] This denotes the fraction from the DEAE-cellulose chromatography. For details about the exchange measurements, see Materials and methods.

from Fermentation A, but not from B or C. Although enzymes from Fermentations B and C produced primarily low *Rf*-value antibiotics, the one from C gave two highly and two weakly active bands as compared to one strongly active band in the case of B. These differences in the activities of the enzymes isolated from three different fermentations were in agreement with the activity of the microorganism itself. The culture broths from these fermentations were chromatographed on silica gel thin layer plates and the peptides identified by spraying with Chlor-Toluidine reagent of Greig and Leaback [14]. The results are shown in fig. 4. The culture broth from Fermentation A showed a strong band at the position of bacitracin A and F, but very faint in case of fermentations B and C. In the low *Rf*-value region, the number of peptide bands are greater in Fermentation C, smaller in B and least in A. When these bands were extracted and assayed for antibiotic, bands from fermentation C and B showed faint activity, but high activity from A in bacitracin A band. Fermentation C had two bands in low *Rf*-value region with strong antibiotic activities.

Table 2
Multiplicity of the enzymes from Fermentation B.

Enzymes	Disc gel electrophoresis		Density gradient centrifugation	
	(a) cpm/mg/ 30 min	(b) mol. wt.	(a) cpm/mg/ 30 min	(b) mol. wt.
I	111 200	200 000	672	197 000
	3 070	100 000		
	141 600	63 000	546	47 000
II	–	178 000	9 485	317 000
			10 430	252 000
			9 835	51 000
I + II	25 060	200 000	1 582	206 000
	141 000	159 000		
	–	141 000	1 864	125 000
	45 560	70 800	1 241	100 000

(a): Selectron filter retention tests; (b) molecular weights of the corresponding fractions. Marker proteins used for electrophoresis were catalase, alcohol dehydrogenase, bovine serum albumin and isocitrate dehydrogenase. For details see text.

3.3. *Amino acid dependent ATP-$^{32}PP_i$ exchange*

Enzymes I and II of Fermentation B catalysed the ATP-$^{32}PP_i$ exchange dependent on each of the 10 bacitracin amino acids (table 1). Enzyme II was much more active than enzyme I. The rate of exchange was nearly the same for all amino acids (except L-glutamic acid) in enzyme I, but varying in enzyme II. This would perhaps also indicate the presence of the ligase activity in enzyme II. The enzyme isolated from Fermentation C also had the exchange activities dependent on these amino acids, but comparatively lower. This low activity could be due to the different procedure of cell disruption used in this case – yielding perhaps larger amounts of inactive protein. Of further interest was the equally high exchange activity dependent on valine. There was no doubt that part of the activity was due to contaminating ligase activity. On the one hand, the valine containing bacitracins in a commercial sample appeared to run closely behind bacitracin A in TLC. On the other hand one finds [^{14}C]valine incorporation in the low *Rf*-value antibiotically active bands of fig. 3.

3.4. *Multiplicity of enzymes*

Sucrose density gradient centrifugation of enzymes I and II from Fermentation B, yielded a number of fractions of molecular weight ranging from 47 000–317 000 which gave [^{14}C]amino acid incorporation as tested by selectron filter retention test (table 2). Similar multiple bands with such activities were also observed upon disc gel electrophoresis of these enzymes, but with molecular weight range between 63 000–200 000 (table 2). This property of the multiplicity of these enzymes is similar to those from Fermentation A [4]. Since there is no direct relationship between the molecular weight and [^{14}C]amino acid incorporation, this would suggest a fast equilibrium between the various molecular entities of the enzyme complex.

3.5. *Miscellaneous*

Since for gramicidin S and tyrocidine, the biosynthesis occurred through a chain elongation mechanism [15], experiments were conducted to see its applicability to the present system. Using [^{14}C]leucine as the starting amino acid, the increase of the enzyme bound radioactivity for the enzyme from Fermentation C on sequential addition of other amino acids (fig. 1) was found to be upto cysteine (in certain cases upto leucine) as tested by the filter disc method of Mans and Novelli [16]. However in this procedure if the step 'heating at 90°C for 30 min' was omitted and a mixture of enzymes PPT II, PPT I, and 90% PPT II was used, the increase in radioactivity continued up until the addition of lysine. After this there was no regular pattern. These results are not conclusive, but only point to the differences from those of tyrocidine or gramicidin S.

4. Conclusion

From the present study it is clear that for the maximum synthesis of bacitracin by *Bacillus licheniformis* (ATCC 10716) the conditions, especially the amount of carbon source, are very critical. Changing these conditions does not alter the synthesis of antibiotics, but only the type of antibiotics. From the behaviour of the enzymes isolated under different fermentation conditions, it appears that the enzyme complex syn-

thesizing the various antibiotics is the same. Their differential activities when produced under different fermentational conditions could be assigned to some missing or additional factor/factors in these preparations.

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References

[1] Craig, L.C., Weisiger, J.R., Hausmann, W. and Harfenist, E.J. (1952) J. Biol. Chem. 199, 259.
[2] Newton, G.G.F. and Abraham, E.P. (1953) Biochem. J. 53, 597.
[3] Lockhart, I.M. and Abraham, E.P. (1954) Biochem. J. 58, 633.
[4] Pfaender, P., Specht, D., Heinrich, G., Schwarz, E., Kuhnle, E. and Simlot, M.M. (1973) FEBS Letters 32, 100.
[5] Lipmann, F. (1971) Science 173, 875.
[6] Callow, R.K. and Work, T.S. (1952) Biochem. J. 51, 558.
[7] Hills, G.M., Belton, F.C. and Blatchley, E.D. (1949) Brit. J. Exptl. Pathol. 30, 427.
[8] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265.
[9] Hoff, D.A., Bennett, R.E. and Stanley, A.R. (1947) Science 106, 551.
[10] Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372.
[11] Cornell, N. and Snoke, J.E. (1964) Biochim. Biophys. Acta 91, 533.
[12] Maurer, H.R. (1968) Disk-Elektrophorese pp. 42–43, Walter de Gruyter and Co. Berlin.
[13] Roskoski, Jr., R., Gevers, W., Kleinkauf, H. and Lipmann, F. (1970) Biochemistry 9, 4839.
[14] Greig, C.G. and Leaback, D.H. (1960) Nature 188, 310.
[15] Kleinkauf, H., Roskoski, Jr., R. and Lipmann, F. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2069.
[16] Mans, R.J. and Novelli, G.D. (1961) Arch. Biochem. Biophys. 94, 48.