ENZYMES OF *BACILLUS LICHENIFORMIS* IN THE BIOSYNTHESIS OF BACITRACIN A*

P. PFAENDER, D. SPECHT, G. HEINRICH, E. SCHWARZ, E. KUHNLE and M.M. SIMLOT

Institut für Biologische Chemie und Ernährungswissenschaft, Universität Hohenheim, D-7 Stuttgart 70, West Germany

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1. Introduction

In contrast to the biosynthesis of proteins by ribosomes, cyclic polypeptides gramicidin S and tyrocidin are synthesized by enzymes. Lipmann [1] has reviewed the work done on this aspect. A mechanism for the synthesis has been postulated by Kleinkauf et al. [2], which involves continuous transpeptidation and transthiolation. Laland et al. [3] have indicated that the model of enzyme 1 of gramicidin S synthetase would possess 18 or 19 catalytic functions. Another of the polypeptide antibiotics, for which evidence has been obtained to show its non-ribosomal biosynthesis, is Bacitracin A [4, 5]. Bacitracin A, obtained from *B. licheniformis*, has a unique structure (fig. 1), in that it comprises of a cyclic chain, a straight chain, 4 D-amino acids and a thiazoline ring formed between isoleucine and cysteine [6]. Thus enzymes synthesizing such a complex structure may contain many more active sites with more varied functions than those of gramicidin S or tyrocidin. With this in view, therefore, in the present study non-ribosomal synthesis of bacitracin A has been confirmed and the enzyme fractions synthesizing it have been obtained.

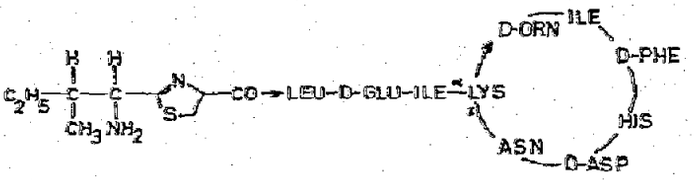

Fig. 1. Bacitracin A.

2. Materials and methods

2.1. *Analytical methods*

Analytical methods used were those of Lowry et al. [7] for protein, of Schneider [8] for DNA and RNA and of Munro and Fleck [9] for total lipids. The antibiotic activity was determined by measuring inhibition zones against *Micrococcus flavus* as described by Hoff et al. [10] using bacitracin (Serva Feinbiochemica, Germany) as a standard.

2.2. *Growth of bacillus and preparation of cells*

Bacillus licheniformis (ATCC 10716) was grown in the medium of Cornell and Snoke [11], but contained 9 g/ℓ of L-glutamic acid. The inoculum was prepared by shaking the microorganism in 15 ml of medium at 37° for 48 hr and then transferring it to a 10 ℓ glass fermentor (New Brunswick) with stirrer at 100 rpm and airflow at 800 ml/min or to a 100 ℓ stainless steel fermentor (Marubishi Lab. Equipment Co., Tokyo) with airflow of 20 ℓ/min. When the pH of the medium reached between 9.0 and 9.15 (antibiotic content 97 μg/ml), the cells were harvested. The cells were then washed twice with distilled water and three times with 0.01 M Sørensen's sodium phosphate

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buffer, pH 7, starved at 30° with three changes of buffer after every 30 min and stored in either a 0.3 M phosphate-salt medium [5] or a buffer A containing 10 mM $MgCl_2$; 0.25 mM EDTA; 10 mM 2-mercaptoethanol, in 0.025 M sodium phosphate buffer, pH 7.5.

2.3. *Preparation of [^{35}S]bacitracin*

The bacillus was grown (37°, 70 hr) in 500 ml of medium containing 8 mCi of [^{35}S]sodium sulfate. After centrifugation, the culture broth was adjusted to pH 7 with acetic acid and lyophilised. Crude [^{35}S]bacitracin from this was prepared by extracting the aqueous solution with an equal volume of water saturated n-butanol. Further purification was done by chromatography on a CM-cellulose column [12], after the removal of butanol. The specific activity (0.08 mCi/mmole) and the antibiotic activity (5 units/mg) were, however, low and the preparation contained large amount of salts.

2.4. *Preparation of subcellular fractions*

These were prepared with slight modification of the procedure of Shimura et al. [5]. 20 g wet cells of *B. licheniformis*, suspended in 5 ml of phosphate-salt medium, were disrupted (15 × 2 min) with a sonic oscillator (Branson Sonifier). A stream of moist nitrogen was directed at the horn during sonication. The resultant sonicate was centrifuged at 2000 *g* to yield a heavy precipitate and then at 10,000 *g* giving a light precipitate and the supernatant. The light precipitate was purified by centrifuging (3 × 10,000 *g*) in phosphate-salt medium. This precipitate was, henceforth, called 10,000 *g* particles.

2.5. *ATP–$^{32}PP_i$ exchange measurements*

The amino acid dependent exchange was measured according to Sand et al. [13]. The incubation mixture of 0.4 ml, final pH 7.3, contained 3.3 mM ATP; 5 mM $MgCl_2$; 5 mM KF; 1.5 mM dithiothreitol; 0.5 mM cysteine; 1.2 mM [^{32}P]sodium pyrophosphate (1.37 mCi/mmole) and 1.5 mM each of other 9 bacitracin amino acids and 0.2 ml of enzyme solution in buffer A, and incubated at 37° for 10 min. Blanks were prepared in the same manner, but frozen after 20 min at 0°.

2.6. *Assay for bacitracin synthesis*

The synthesis of bacitracin was measured by incorporation of radioactive amino acids in bacitracin and its retention by selectron filters (0.45 μm, dia. 24 mm, Carl Schleicher & Schull, Germany). The incubation mixture of 0.1 ml, pH 7.3, contained 6.6 mM ATP; 10 mM cysteine; 3 mM each of other 9 amino acids (all L-form); 0.16 M Tris-HCl buffer and 50 nCi [U-^{14}C]protein hydrolysate (Amersham Buchler, Germany). 0.1 ml of enzyme solution in buffer A was added in cold and incubated (37°, 10 min). Blanks were prepared as above, but were frozen immediately. Blanks and the incubated mixtures were filtered through selectron filters in the cold, washed with buffer A (2 × 1 ml) and once with water (1 ml). The filter discs were dried at 50° for 20 min and counted in 5 ml of 5% diphenyloxazol in toluene in a Beckman scintillation counter LS150. To check further, the incubated mixture was chromatographed on TLC-plates Silica gel F_{254} (Merck, Germany) using n-butanol:acetic acid:water (4:1:2, v/v) as the developer. After the development, the areas corresponding to bacitracins were scraped off, extracted with methanol (2 × 1 ml) and then with water (2 × 0.5 ml). The pooled extract was dried under vacuum and lyophilised with a small amount of water to remove any trace of methanol. The residue was taken in water and the antibiotic activity determined.

2.7. *Sucrose density gradient centrifugation*

The procedure of Martin and Ames [14] was followed. 60 μl of enzyme solution was carefully layered on 5 ml of buffer A having a linear sucrose gradient from 5% to 20% and then centrifuged (204,000 *g* × 5 hr; 3°) in an Omega II ultracentrifuge (Christ, Osterode, Germany). The marker enzymes used were lysozyme, catalase and urease and assayed according to Shugar [15], Bergmeyer et al. [16] and Sumner and Hand [17], respectively. Two drop fractions were collected from the bottom of the tube by puncturing and each fraction was assayed for the activity by selectron filter retention test.

3. Results and discussion

3.1. *Bacitracin synthesis by subcellular fractions*

Of the three fractions, only the 10,000 *g* particles (DNA 26%; RNA 39%; protein 25% and total lipids 10%) synthesized bacitracin as shown in table 1. The synthesis was not affected by the blockers of

Table 1
Effect of various reagents on the synthesis of bacitracin by 10,000 g particles.

Conditions	(mg/ml)	bac.[a] (ng)	Conditions	(μg/ml)	bac.[a] (ng)
Whole system[a]			Whole system plus		
			ADP	5	350
Whole system plus					
RNAase	0.6	450	AMP+ADP	5 each	300
DNAase	0.6	375	AMP	5	300
Puromycin	0.1	400	Substitution of		
p-Fluoro phenylalanine	0.1	375	L-glu/orn/phe/asp		
			by their D-form		
Lysozyme	0.1	450	singly or all	3	0
			Whole system minus		
Trypsin	0.6	350			
EDTA	0.4	475	ATP		0
DOC	0.4	300	any one of the		
[^{35}S]sodium sulfate	4.5 μCi	400[b]	amino acids		0–300

[a] Whole system contained 100 mg wet particles, 0.1 M phosphate-sal medium, pH 7; 10 mM ATP; 0.16 mM $MgSO_4$; 6 mM each of 10 amino acids; vol. 1.5 ml. Incubated at 37°, 30 min. Add 2 ml cold methanol, centrifuge and supernatant lyophilised and dissolved in 1 ml water. Aliquots were taken for TLC and CM-cellulose chromatography and then antibiotic assay.
[b] [^{35}S]Bacitracin could not be found either by selectron filter retention test or TLC. Incubation was for 3 hr at 37°.

ribosomal protein synthesis – RNAase, DNAase, puromycin or *p*-fluorophenylalanine or by enzymes – lysozyme or trypsin. However, there was a drop in the synthesis in presence of deoxycholate, perhaps due to its detergent effect. As expected, omission of ATP or any one of ten amino acids did not produce bacitracin. Substitution of L-amino acids by D-glutamic, D-phenylalanine, D-ornithine and/or D-aspartic also did not synthesize bacitracin in appreciable amount. This inhibition by D-amino acids may be similar to that of gramicidin S synthesis [18]. AMP also reduced the rate, probably due to a competition between ATP and AMP and PP_i. In the presence of [^{35}S]sodium sulfate, there was a synthesis, but of unlabelled bacitracin. Synthesis of labelled bacitracin would have indicated a conversion of sulfate to cysteine followed by its incorporation into bacitracin. Since only intact cells are capable of this conversion, their absence from this preparation was concluded. Bacitracin synthesis is, therefore, evidently due to enzymes present.

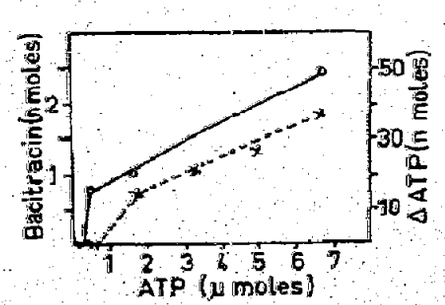

Fig. 2. ATP requirement of bacitracin synthesis by 10,000 *g* particles. Bacitracin synthesized (o—o—o); ATP consumed (x--x--x). For details see results and discussion.

3.2. *ATP requirement for bacitracin synthesis*

100 mg of 10,000 *g* particles were incubated in the presence of 0.1 to 7 μmoles of ATP in final volume of 2 ml. To an aliquot of 0.2 ml, an equal amount of methanol was added, centrifuged, the clear supernatant lyophilized and ATP was determined [19]. Concomitantly, synthesis of bacitracin was also measured by inhibition test after TLC. Fig. 2 shows that below 0.3 μmole ATP, no bacitracin was synthesized implying insufficient amount of ATP. However, a linear increase in bacitracin synthesis with ATP concentration was found. From the data, it could be calculated that 2 moles ATP are required for the formation of 1 peptide bond.

3.3. *Retention of bacitracin by selectron filters*

[^{35}S]Bacitracin in aqueous solution (1 μmole in 1 ml) was, on filtration, retained by selectron filter.

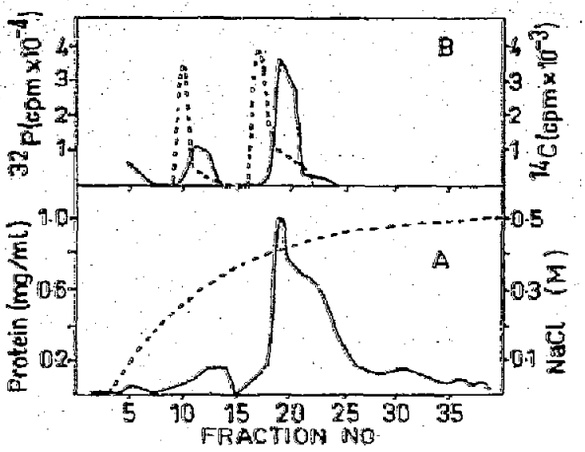

Fig. 3. Purification of enzyme on DEAE-cellulose column. Fractions of 10.5 ml were collected. A) Protein content (——); NaCl (----). B) Selectron filter retention test (----); ATP–$^{32}PP_i$ exchange in the presence of all bacitracin amino acids (——). For details see text.

When the retained bacitracin was eluted with 70% ethanol and the filtrate and the eluate were tested for bacitracin by TLC, it was found in both. This indicated an overload. A smaller amount (0.1 μmole) when used showed only about 5% of bacitracin in the filtrate.

3.4. *Preparation of the enzymes*

20 g wet cells were disrupted in buffer A with 10 × 1 min pulses from the sonifier under cooling. The brown suspension was centrifuged (100,000 *g* × 15 hr, 2°) and the pale yellow supernatant and top fluffy layer were decanted. After recentrifugation (100,000 *g* × 3 hr), the fluffy layer was removed and the supernatant brought to 1% streptomycin sulfate with its 10% solution in buffer A within 5 min and under slow stirring. The precipitate was centrifuged (20,000 *g* × 10 min) within 15 min in the cold. Dry, cold ammonium sulfate (final conc. 55% saturation) was added slowly to a constantly stirred supernatant during a 1 hr period. After standing overnight, the yellowish precipitate was collected by centrifugation (20,000 *g* × 20 min), dissolved in minimum amount of buffer A and chromatographed on a DEAE-cellulose (Cellex D) column (1.9 × 40 cm) using an exponential gradient of NaCl (0–0.5 M) in buffer A. Each fraction was tested for protein, absorbance at 280 and 260 nm, selectron filter retention test and ATP–$^{32}PP_i$ exchange in presence of 10 amino acids. Some of the

Table 2
Synthesis of antibiotics by enzyme fractions I and II.

Fractions	R_f values on TLC			
	0.16	0.28	0.43	0.46
	(μg bacitracin)			
I (55 μg)		1.65	0.7	–
II (1 mg)	2.0	6.6	6.0	7.5
I + II (16 μg)	1.0	1.0	0.8	–

After incubation, areas corresponding to the R_f-values were extracted and assayed for antibiotic activity. For details see text.

results are shown in fig. 3. The shift in the peak position in fig. 3B is not clear, but it appeared that the fractions between 10–12 and 17–21 synthesize bacitracin. The fractions from these peaks were, henceforth, referred to as enzyme fraction I and enzyme fraction II, respectively. The enzyme fraction/fractions were precipitated by 55% saturation with ammonium sulfate and centrifuged. The precipitate was redissolved in buffer A for further studies or resuspended in a small amount of the supernatant, quickly frozen and stored at –80°. A rechromatography of the enzyme fraction II on DEAE-cellulose with the same buffer and gradient, split it giving both enzyme fractions I and II, whereas no such split was obtained for enzyme fraction I on rechromatography.

To check for the actual synthesis of bacitracin by these fractions, the incubated mixture was chromatographed on TLC-plates. The results are shown in table 2. Enzyme fractions I and II both showed the synthesis of bacitracin A (R_f value 0.43) as well of antibiotic with higher R_f value. Besides these, the antibiotics of lower R_f values were also found to be present, which might mean the presence of other enzymes. Combination of the two fractions did not enhance the rate of bacitracin synthesis as compared to the individual rates. Inclusion of the low R_f value [^{14}C]amino acids (lysine, histidine, glutamic and asparagine) in the incubation mixture, resulted in the synthesis of labelled bacitracin, which could be identified on TLC by radioscanning, thereby meaning an incorporation of these amino acids into bacitracin.

3.5. *Sucrose density gradient centrifugation*

Centrifugation of enzyme fractions I and II gave results shown in table 3. The data are shown only for

those fractions that gave a positive selectron filter retention test. It appears that there are a number of enzymes between molecular weight of 200,000 to 350,000. However, it was interesting to observe that the enzyme fractions further dissociated down to a minimum molecular weight of 50,000 upon storing in the cold for 1 day. These results along with those of rechromatography of enzyme fraction II would indicate the existence of enzyme in different forms that may be associating or dissociating under conditions of incubations, since each of these molecular entities appears to independently incorporate amino acids into bacitracin.

Table 3
Sucrose density gradient centrifugation of enzyme fractions I and II.

Fractions	(a) (cpm/mg/ 30 min	(b) Mol. Wt.	After 1 day storage in cold (cpm/mg/ 30 min)[a]	Mol. Wt.[b]
I	3,550	237,000	12,600	161,000
			20,350	105,000
			10,600	50,000
II	700	246,000	50,700	161,000
	6,000	189,000		
I + II	2,840	348,000		
	4,080	199,000		

[a] Selectron filter retention test.
[b] Molecular weight of the corresponding fractions. For details see text.

Acknowledgements

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