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GENERATION OF FORMIC ACID AND ETHANOLAMINE FROM SERINE IN BIOSYNTHESIS OF LINEAR GRAMICIDIN BY A CELL-FREE PREPARATION OF Bacillus brevis (ATCC 8185)

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A growing organism that produces antibiotic peptide was incubated with L-(U-^{14}C)serine for labeling linear gramicidin. Linear gramicidin was isolated by a simple chromatographic method from tyrothricin (mixture of linear gramicidin and tyrocidine) applied to a column of basic aluminum oxide. The hydrolysate of labeled linear gramicidin on thin layer chromatography showed that L-(U-^{14}C)serine was one of a precursor of ethanolamine moiety by autoradiography. L-(3-^{14}C)serine generated formic acid in the presence of tetrahydrofolic acid by an enzyme fraction prepared with ammonium sulfate, and further formed ethanolamine binding to the protein. Formylvaline was biosynthesized by it with tetrahydrofolic acid and ATP, and subsequently released from the protein.

Bacillus brevis (ATCC 8185) produces two kinds of antibiotic peptides, linear gramicidin and tyrocidine. The N-terminal of linear gramicidin is formylated L-valine and another terminal ethanolamine is peptidically bound to L-tryptophan. Sarges and Witkop clarified the formula of linear gramicidin A, **N-formyl-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-Trp-D-Leu-Trp-D-Leu-Trp-D-Leu-Trp-ethanolamine** (1). However, the source of ethanolamine and formate which seem involved in formylation of N-terminal, is yet unknown. This study shows that L-(U-^{14}C)serine was incorporated into linear gramicidin as ethanolamine moiety by growing cell culture but ^{14}C-ethanolamine itself was hardly incorporated. It was also found that L-(3-^{14}C)serine generated formic acid from a cell-free preparation with THFA and that ethanolamine was liberated by treatment with alkali from proteinous precipitate after extraction of formic acid, regardless of the presence or absence of ATP.

Abbreviations used are: LG, linear gramicidin; TEA, triethanolamine
TY, tyrocidine; THFA, tetrahydrofolic acid
TLC, thin layer chromatography
DTT, dithiothreitol

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The cell-free system for tyrocidine biosynthesis has been reported by several investigators (2,3). While Bauer et al. reported synthesis of linear gramicidin by a combination of biosynthetic and organic methods (4), Akers et al. showed synthesis of the initial peptide portion of linear gramicidin with cell-free extracts (5), although their studies made no attempt at clarification of the source of ethanolamine and formate.

Total synthesis of linear gramicidin was not obtained in a cell-free system in which the reaction mixture contained the constitutive amino acids, L-(U-^{14}C)serine, ethanolamine, sodium formate, THFA, ATP, CTP, $MgCl_2$, DTT and a protein fraction prepared with ammonium sulfate from cytosol. It was, however, observed that formylvaline was synthesized by this fraction incubated with L-(U-^{14}C)valine, sodium formate, ATP, THFA, $MgCl_2$ and DTT, as previously shown by Akashi et al., who reported enzyme-bound formylvaline biosynthesis as a presumable initiation complex of linear gramicidin biosynthesis (6,13,14). Formylvaline was, however, subsequently released from the enzyme fraction by prolongation of the reaction time.

MATERIALS AND METHODS

Characteristics of Cells Bacillus brevis (ATCC 8185) was cultured by the method of Fujikawa et al. (7) with some modification. Bacteria grown on a milk agar slant were transferred into a 500 ml flask containing 100 ml of milk-yeast extract medium and incubated at 33°C for 10-12 h on a New Brunswick gyratory shaker. Five milliliter aliquots of preculture were freshly transferred into six 500 ml flasks each containing 100 ml of peptone-meat medium for incubation. Foam was suppressed with a silicon emulsion (Antifoam AF). Cells were harvested at the late logarithmic phase of approx. 7 h after inoculation. The amount of LG and TY in a culture was determined colorimetrically (8). For determination of the peptides, the cells harvested from each 20 ml portions of the culture which were allowed to grow for further 1.5-2 h after harvest were separately precipitated by centrifugation at 10,000xg. Tyrocidine biosynthesis by a cell-free preparation showed that the enzyme activity was maximal at 7 h after inoculation, whereas the maximum production of antibiotic peptides in the culture required 8.5-9 h (2). In fact, cells harvested at the late logarithmic phase produced less antibiotics but had a strong activity to generate formic acid by the reaction with a cell-free preparation. Therefore, the cells that consistently achieve maximum production in the culture within 1.5-2 h after harvest were used for experiments in the cell-free system. There was some variability in the amount of peptides in each cell cultivation. For extraction of the enzyme protein, bacterial cells (7-8g) were harvested from 500 ml of the culture fluid.

Production of ^{14}C-labeled Peptides and Purification: Zero point five milliliter aliquots of the growing cell culture were collected into a small test-tube 7.5 h after inoculation. After adding separately 0.5 uCi each of L-(U-^{14}C)serine (0.03 nmoles), L-(3-^{14}C)serine (0.01 nmole), L-(U-^{14}C)valine (0.05 nmoles), L-(U-^{14}C)leucine (0.1 nmole), (U-^{14}C)glycine (0.02 nmoles), L-(methylene-^{14}C)tryptophan (0.01 nmole) and (2-^{14}C)ethan-1-ol-2-amine hydro-

chloride (0.01 nmole), each culture was incubated for 30 min at 33°C with shaking. The cells were then harvested by centrifugation. The pelleted cells were suspended in a 2% NaCl solution and repelleted by centrifugation. LG was finally purified by TLC, that is, labeled products extracted from the cell pellets with 2 ml of hot (60°C) ethyl alcohol for 2 min were centrifuged. The supernatant was applied to a column of basic aluminum oxide, and the effluent was evaporated. The residue finally obtained was dissolved in a small amount of ethyl alcohol and subjected to TLC with solvent system of ethylacetate:pyridine:water (12/3/1.5) and ethylacetate:pyridine:acetic acid:water (12/3/0.9/1.5). The location of commercial or biosynthetic peptides on TLC were visualized by the method of Pan and Dutcher (9). The effluent from basic aluminum oxide did not contain TY entirely, although neutral or acid aluminum oxide was incapable of absorbing both TY and LG. Autoradiographical identification of labeled LG also confirmed these characteristics of aluminum oxides on column chromatography.

Chemicals: The chemicals used were obtained from the following sources: lysozyme, ATP, CTP, gramicidin, tyrothricin, tetrahydrofolic acid, dithiothreitol, N-formyl-DL-valine and DNase from Sigma chemical Co.; tyrocidine-hydrochloride from ICN Pharm. Inc.; radioisotope compounds from Radiochemical Centre Amersham; precoated TLC plate, silica gel 60 F-254 (5x10 cm) and cellulose precoated (10x20 cm) from E. Merck, Darmstadt; Sephadex G-75 from pharmacia Fine Chem.; aluminum oxides (basic, neutral and acid) from Woelm Pharma; films Tri-X pan and RP Royal X-Omat from E. Kodak, Pandol a sensitization developer from Fuji Photo Film Co.

Enzyme Preparation: Similar methods to those reported by Fujikawa et al. (2,7), Akashi et al. (6,13) and Bauer et al. (4) were employed. Cells harvested were, however, immediately lysed with lysozyme. The protein prepared was never frozen but prepared fresh for each assay. The cells (7-8g) were suspended in 15 ml of TEA-HCl buffer (pH 7.7, 50 mM, containing 1mM DTT and 2 mM $MgCl_2$, Buffer B), and lysed with 10 mg of lysozyme at room temperature for 15 min. After hydrolysis of DNA with DNase, the lysate was centrifuged for 20 min at 15,000xg. Operations were carried out in the cold at 4°C, unless otherwise specified. The sediment obtained by centrifugation was discarded, and the supernatant fluid was precipitated between 35% and 60% saturation with solid ammonium sulfate. The precipitate obtained was dissolved in 1 ml of Buffer B and dialyzed against 2 l of the same buffer for 2 h and finally 2.5 ml of crude proteinous solution were prepared.

Partial Purification of Enzyme: Two milliliter (approx. 2.1g) portions of crude extract were subjected to Sephadex G-75 column chromatography (2x100 cm). The column was equilibrated and eluted with Buffer B. The fractions of 3.5 ml were collected. Three regions fractionated, that is, peak, shoulder and foot were concentrated by an Immersible Molecular Separator (Millipore Co.) to 0.4-0.5 ml each at 4°C. The protein concentration was determined by the biuret method with bovine serum albumin as a standard.

Assay for Generation of Formic Acid and Liberation of Ethanolamine Bound to the protein: The enzyme activity was determined by the following procedure. The standard reaction mixture tentatively disposed for biosynthesis of LG consisted of 25 umoles of TEA (pH adjusted to 7.7 by HCl), 1 umole each of ATP, CTP and $MgCl_2$, 0.5 uCi of L-(3-^{14}C)serine (0.01 nmole), 0.5 umoles each of L-valine, L-alanine, glycine, L-leucine, L-tryptophan, L-phenylalanine, sodium formate, ethanolamine hydrochloride and 2.8 mg of enzyme protein (dialyzed ammonium sulfate fraction) in a final volume of 0.25 ml. The protein fractionated by Sephadex G-75 (0.3-0.5 mg protein) was allowed to incubate in the same manner. The reaction was initiated by addition of 50 nmoles of THFA in TEA-HCl buffer, pH 7.7 and to stand for 10 min at 36°C. The reaction was stopped by heating the tube for 1 min in boiling water. Before precipitation of protein and products with chilled 5% trichloroacetic acid containing 3% NaCl, 0.2 mg

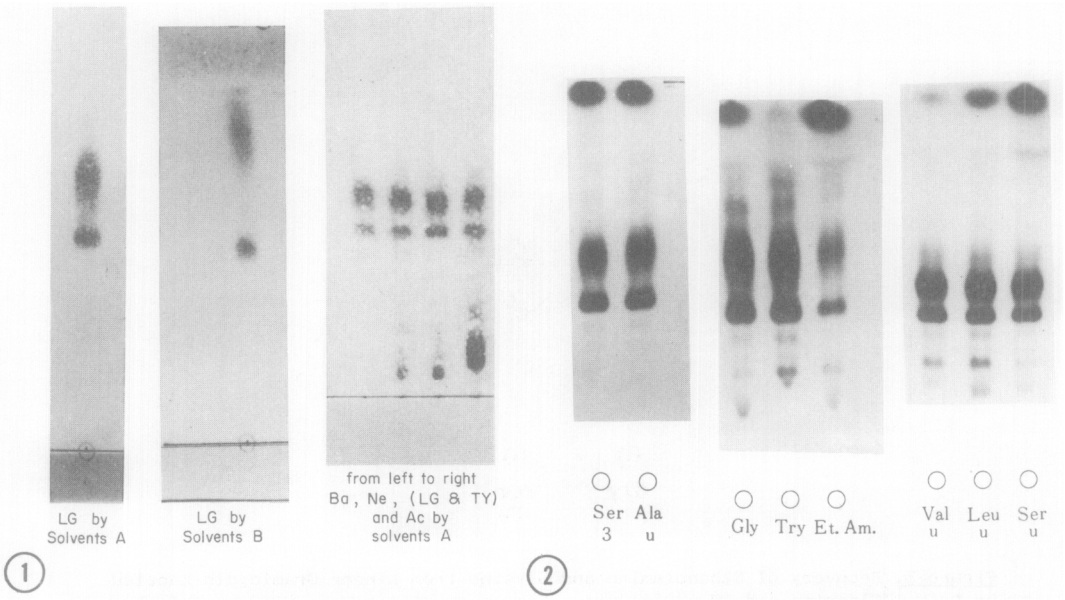


Figure 1. Behavior of Linear Gramicidin on Thin Layer Chromatography Commercial and isolated peptides were analyzed on a silica gel thin layer chromatography using solvents **A** ethylacetate:pyridine:water (12/3/1.5) and **B** ethylacetate:pyridine:acetic acid:water (12/3/0.9/1.5) and developed at 4°C. **LG** and **TY**: commercial specimens, **Ac**, **Ba** and **Ne**: isolated specimens obtained by extraction from cell pellets with ethyl alcohol and application of centrifuged supernatant to a column of acid, basic or neutral aluminum oxide, respectively. Each 10-20 ug was spotted on the plate. Visualization by Pan and Dutcher (9). **TY** hardly migrated from the origin with solvents **A** and **B**.

Figure 2. Autoradiogram of ^{14}C-labeled Linear Gramicidin Refer to **MATERIALS AND METHODS** for preparation of ^{14}C-labeled linear gramicidin. A small amount of culture fluid was incubated with each ^{14}C-labeled amino acid separately. Alcohol extractions from cell pellets were applied to a column of basic aluminum oxide. Abbreviations under origin show linear gramicidin labeled by each ^{14}C-amino acid. Labeled products obtained were analyzed on a silica gel thin layer chromatography using a solvent ethylacetate:pyridine:water (12/3/1.5) and developed at 4°C for preparation of autoradiogram (3-5 days exposure). Spot at the top of solvent is not identified.

of commercial gramicidin in 80% aqueous ethanol solution was added as a carrier. The supernatant fluid after centrifugation was discarded. The precipitate obtained was extracted with 2 ml of 99.5% ethanol alcohol at 60°C, and the extracted supernatant was subjected to column chromatography with aluminum oxides, neutral aluminum oxide initially followed by basic aluminum oxide (0.9x2 cm). Each column was washed with 1 ml of 99.5% ethanol. The radioactivity of the effluent (0.3 ml portions) was determined by a liquid scintillation counter with 5 ml of commercial scintillation cocktail. The remainder of eluate in vials was evaporated under reduced pressure in a desiccator containing P_2O_5, and their radioactivity were determined. The protein pellet after washing with ethanol was then alkalined by adding 0.5 ml of 0.2N KOH for 10 h at 33°C and neutralized with 0.93N of perchloric acid. After centrifugation, the aqueous phase obtained was concentrated for applying to a plate coated with cellulose. TLC was performed with systems of iso-propyl alcohol:acetic acid:water (20/1/6) and n-butyl alcohol:acetic acid:water (2/1/1). The position of labeled products was identified by autoradiogram.

Synthesis of Formylvaline and Its Release from the Enzyme by Prolonged Reaction: Synthesis of formylvaline was carried out in reaction mixtures containing L-(U-^{14}C)valine (0.5 uCi, 0.05 nmoles), 1 umole each of ATP, $MgCl_2$,

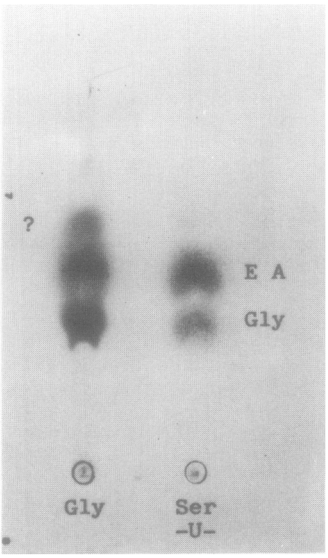


Figure 3. Recovery of Ethanolamine and Glycine from Linear Gramicidin Labeled by L-(U-^{14}C)serine and (U-^{14}C)glycine Linear gramicidin purified by thin layer chromatography was hydrolyzed and its lysate was applied to a silica gel plate which was developed with iso-propyl alcohol:formic acid:water (20/1/6) at room temperature and the autoradiogram was prepared thereby (one week exposure). **Gly** and **Ser(-U-)** under origin represent hydrolysates from linear gramicidin by each ^{14}C-amino acid.

sodium formate and 25 nmoles of THFA in 20 umoles of TEA (pH adjusted to 7.7 by HCl), with 2 mg of enzyme protein (dialyzed ammonium sulfate fraction), in a volume of 0.25 ml. Other constitutive amino acids of LG were excluded. The reaction was stopped by heating the tube for 1 min in boiling water after one hour intervals. After centrifugation at 1,500xg, the supernatant obtained was applied to a column of cation exchange, Dowex 50W x 8(H^+) 200-400 mesh (1x2.5 cm), and rinsed with 5 ml of water. The effluent was evaporated under reduced pressure in a desiccator containing P_2O_5. The glutinous residue was redissolved in 0.5 ml of ethyl alcohol and its radioactivity in 0.2 ml portions was determined with 5 ml of commercial scintillation cocktail, and a small aliquot was spotted on a silica gel plate for TLC which was developed with a solvent system of n-butyl alcohol:acetic acid:water (21/3.3/9) and of iso-propyl alcohol:water:25% ammonia water (20/2/1) and autoradiographed.

RESULTS

Recovery of Ethanolamine from Labeled Linear Gramicidin by L-(U-^{14}C)serine and Behavior of Linear Gramicidin: The commercial and biosynthetic LG showed twin spots on TLC which supported the dimer formation of LG reported by Veatch et al. and Fossel et al. in a nonpolar solvent such as ethylacetate (10,11,12). (Fig. 1,2). These two spots were scraped off the TLC-plate and the extracted ^{14}C-labeled LG was hydrolyzed with 6N HCl at 110°C for 12 h. The autoradiogram of the hydrolysate showed that the ethanolamine moiety was derived from the L-(U-^{14}C)serine residue and also from (U-^{14}C)glycine. (Fig. 3, Table 1).

Table 1. Uptake of ^{14}C-amino acids and Ethanolamine into Linear Gramicidin by Growing Cells

Amino acid	Radioactivity(CPM)	Amino acid	Radioactivity(CPM)
(U-^{14}C)glycine	33 x 10^3	L-(U-^{14}C)alanine	13.3 x 10^3
L-(U-^{14}C)valine	22.6	L-(- ^{14}C)leucine	24.1
L-(U-^{14}C)serine	23.3	L-(3-^{14}C)serine	12.6
L-(methylene-^{14}C)-tryptophan	34	(2-^{14}C)ethanol-amine	2.6

Refer to **MATERIALS AND METHODS** for the method of preparation of ^{14}C-labeled linear gramicidin. Labeled linear gramicidin was recovered from the twin spots on TLC after autoradiographic identification (Fig. 2). Radioactivity was determined by a liquid scintillation counter.

Generation of Formic Acid from L-(3-^{14}C)serine by a Cell-Free Preparation and Liberation of Ethanolamine by Alkali Treatment: The radioactivity from L-(3-^{14}C)serine in the effluent of both aluminum oxides chromatography completely disappeared on evaporation under reduced pressure and was hardly generated in the absence of THFA. The above results suggest that the radioactivity is formic acid, which is also supported by the fact that N-terminal valine is masked with formylation according to the formula structute of LG. Formation of formic acid was somewhat suppressed by ATP. (Table 2).

Table 2. Generation of Formic acid from L-(3-^{14}C)serine by an Ammonium Fraction and Its Gel Filtered Fractions

Fraction	Radioactivity (CPM)			
	with ATP		without ATP	
THFA	+	-	+	-
Ammonium sulfate (35-60%)	8,520	200	11,150	250
by Gel filtration				
"Peak"	830	---	920	---
"Shoulder"	9,020	200	15,350	300
"Foot"	460	---	550	---

Refer to **MATERIALS AND METHODS** for experimental procedures. Dialized ammonium sulfate fraction was applied to a Sephadex G-75 column (2x100 cm) which was equilibrated with Buffer B. The column was eluted with the same buffer and 3.5 ml fractions were collected. Three regions fractionated were termed a **peak** (fraction number 46-55), a **shoulder** (56-70) and a **foot** (71-80). L-(3-^{14}C)serine hardly generated ^{14}C-formic acid in the absence of THFA. Alcoholic extracts from the resultant precipitate were column chromatographed with both neutral and basic aluminum oxides for partial purification. Radioactivity from serine completely disappeared on evaporation under reduced pressure.

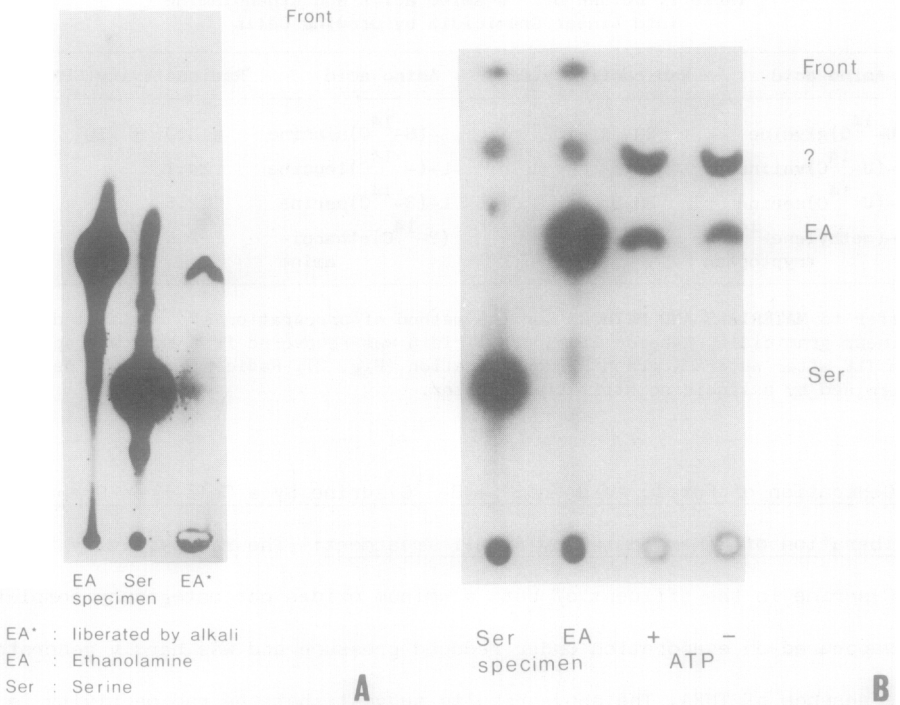


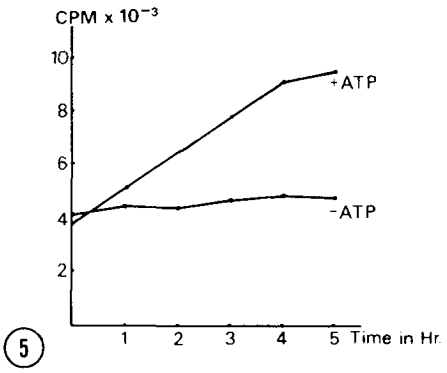
Figure 4. Liberation of ^{14}C-ethanolamine: L-(3-^{14}C)serine was Incubated with the Protein as a Precursor. In advance of alkaline liberation, the TCA-precipitate (refer to **MATERIALS AND METHODS**) was washed with alcohol for extraction of formic acid (**Table 2**). Liberated ^{14}C-ethanolamine (**Table 3**) was applied to a thin layer plate coated with cellulose which was developed with solvents **A** n-butyl alcohol:acetic acid:water (2/1/1) and **B** iso-propyl alcohol: formic acid:water (20/1/6). A film covered on TLC was exposed for a period required and developed. One of the products comigrated with an ethanolamine marker.

On the other hand, ethanolamine was liberated by KOH from the residual protein, as proven by autoradiographical identification. (Fig. 4, Table 3). The protein precipitated by ammonium sulfate was partially purified by Sephadex G-75 filtration. The shoulder fraction that is subsequent to the peak fraction had a specific activity to generate formic and to bind ethanolamine.

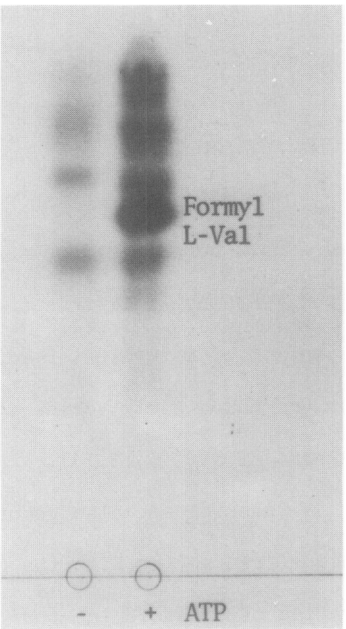
Table 3. Liberation of Ethanolamine by the Protein Alkalined

Fraction	Radioactivity (CPM)	
	with ATP	without ATP
Gel filtered "Shoulder"	2,700	2,600

Liberation of ethanolamine by KOH from the precipitate which extracted formic acid with alcohol as described in the text. The liberated product from **Table 2** (with THFA, "Shoulder") was identified by autoradiogram on TLC (**Fig. 4, B**) and then radioactivity of ^{14}C-ethanolamine located was determined.



(5)



(6)

Figure 5. Synthesis of Formylvaline and Its Release from the Protein by Prolonged Incubation The time course of synthesis was determined at 36°C. Refer to **MATERIALS AND METHODS** for ingredients synthesized. Constitutive amino acids of linear gramicidin except L-valine were omitted in the reaction. The supernatant by centrifugation was subjected to a column chromatography on Dowex 50W H^+ and radioactivity of the effluent was determined.

Figure 6. Autoradiogram of Released Formylvaline The glutinous solution finally condensed was redissolved in a small amount of ethyl alcohol and its aliquot was applied to a silica gel plate which was developed with a solvent n-butyl alcohol:acetic acid:water (21/3.3/9). In the absence of ATP in reaction mixture, formylvaline was not synthesized. The labeled product was identified by autoradiography using a sensitization developer.

Formylvaline Synthesis and Its Release from the Protein by Prolonged Incubation: Akashi et al. earlier reported enzyme-bound formylvaline synthesis and stepwise elongation of intermediate peptides of LG (6,13,14). Formylvaline was observed here to have been enzymatically synthesized with ATP and THFA, and subsequently released from the protein when other constitutive amino acids of LG except valine were excluded. Initiation of synthesis occurred by addition of THFA and incubation was stopped by heating test-tubes one by one at an interval of an hour. Formylvaline synthesized was gradually released into the reaction mixture in the course of 5 h, slowly resulting in liberation from the enzyme. (Fig. 5). The autoradiogram of TLC never indicated synthesis of free formylvaline in the absence of either ATP or THFA. (Fig. 6) The recovery of

valine residue from formylvaline obtained was carried out by hydrolysis with 1N HCl for 3 h at ambient temperature.

DISCUSSION

The present study showed that formic acid and terminative ethanolamine were both generated from serine, although an attempt at synthesizing linear gramicidin by a cell-free preparation failed. Formic acid, responsible for formylation of N-terminal L-valine, was formed in the presence of THFA, requiring enzyme-bound formylvaline synthesis via formyl-THFA as previously reported by Akashi et al. (6,13,14).

Bauer et al. also reported negative results in biosynthesis of ethanolamine from serine with the present strain (4), but the evidence showing derivation of ethanolamine from serine independent of ATP with a cell-free preparation was provided in the study presented. Formation of ethanolamine was, however, less efficient than that of formic acid perhaps because of conversion of ethanolamine to cytidine diphosphoethanolamine or phosphatidylethanolamine (15).

Synthesis of formylvaline and its release from the enzyme protein supported the initiation of linear gramicidin synthesis by formylation of the enzyme-bound N-terminal L-valine residue as reported by Akashi et al.. Formylvaline linked with enzyme-bound thiol group would likely be hydrolyzed on prolongated incubation. Prolonged incubation may induce dissociation of multienzymes as shown by Lee et al. (16,17). It appeared to suppress generation of formic acid in the presence of ATP, probably due to co-existence of some kinases in the protein, e.g., EC. 2,7,2,6 ATP : formate phosphotranseferase and EC. 6,3,4,3 formate : tetrahydrofolate ligase (ADP). Nascent formate from serine seemed to be metabolized steadily.

Dimer formation of linear gramicidin in a nonpolar organic solvent has already been reported by Veatch et al. and Fossel et al. (10,11,12). As shown by them, development of twin spots on TLC with a slovent system of ethylacetate:pyridine:water (12/3/1.5) and broad tailing of the upper spot by addition of acetic acid were consistently observed. For partial purification of linear

gramicidin by chromatography, application to a column of basic aluminum oxide proved to be the most helpful method where tyrocidine was entirely absorbed. On the other hand, acid and neutral aluminum oxides proved incapable of absorbing both peptides.

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