ENZYMIC SYNTHESIS OF D-PHENYLALANYL-L-PROLYL-L-VALINE, A PEPTIDE SEQUENCE PRESENT IN GRAMICIDIN S*

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Gramicidin S is a cyclic deca-peptide produced by Bacillus brevis. Recently, much interest has developed in the study of the mechanism of peptide bond synthesis in the gramicidins (Winnick and Winnick, 1962, Mach et al., 1963, Eikhom et al., 1963) and a few reports have appeared on the syntheses of gramicidins by cell free systems (Uemura et al., 1963, Yukioka et al., 1963, Okuda et al., 1964 a, b,).

During the course of an investigation of the biosynthesis of gramicidin S, Kurahashi (1961) observed that incubation of L-phenylalanine and L-proline with cell free extracts fractionated with ammonium sulfate from Bacillus brevis Nagano, yielded a cyclic dipeptide, D-phenylalanyl-L-proline-diketopiperazine (DKP). The reaction was shown to require ATP and Mg^{++}, and not to be affected by the addition of nucleases or chloramphenicol.

Since the amino acid sequence of this peptide was the same as that found in gramicidin S and since it was well known that proline dipeptides easily cyclize to form piperazine, the formation of D-phenylalanyl-L-proline was considered to be the first peptidation leading to synthesis of gramicidin S. In order to investigate the above possibility, an attempt was made to elongate the peptide chain to form D-phenylalanyl-L-prolyl-L-valine.

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Bacillus brevis Nagano* which was supplied by Dr. S. Otani of Osaka City University was grown in 3% tryptone broth with vigorous aeration. Cells were harvested in the late logarithmic phase of growth and disrupted by sonication for 5 minutes in 0.005 M triethanolamine HCl (TEA) buffer (pH 8.0) containing 0.01 M $MgCl_2$ and 0.01 M β-mercaptoethanol. After removal of unbroken cells and cell debris by centrifugation, the supernatant was centrifuged at 10,500 x g for 1 hour. To the supernatant so obtained an equal volume of saturated solution of ammonium sulfate was added. The resulting precipitate was dissolved in 0.005 M TEA buffer (pH 8.0) which contained 0.01 M

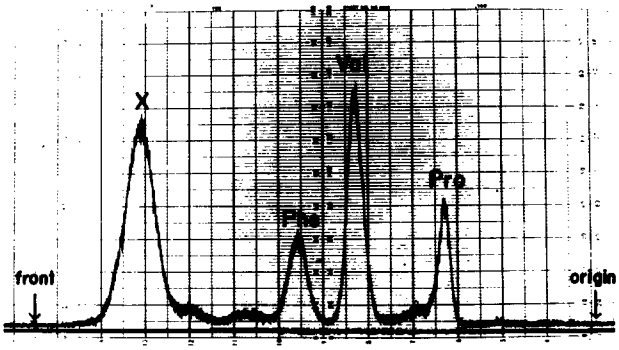

Fig. 1. Radio paper chromatogram of the reaction product. The incubation mixture contained 100 µmoles of TEA buffer (pH 8.0), 5 µmoles of ATP, 10 µmoles of $MgCl_2$, 5 µmoles of phosphocreatine, 200 µg of creatine kinase, 50 mµmoles of L-phenylalanine-U-C^{14} (Sp. Act., 10 µc/µmole), 50 mµmoles of L-proline-U-C^{14} (Sp. Act., 10 µc/µmole), 50 mµmoles of L-valine (Sp. Act., 10 µc/µmole), 10 µmoles of β-mercaptoethanol and 600 µl of dialyzed enzyme solution (protein concentration, 12 mg/ml) in 1.0 ml. The reaction mixture was incubated for 4 hours at 37° and heated in a boiling water bath for 1.5 minute at the end of incubation. After addition of 5 ml. of 50% methanol and removal of the resulting precipitate by centrifugation, the reaction mixture was fractionated by successive adsorption to and elution from columns of Dowex-50 (H^+-form) and Dowex-1 (OH^--form). Elutions from the columns were performed with 7.5 N NH_4OH in 50% methanol (from Dowex-50) and 2 N HCl (from Dowex-1). A radioactive fraction which adsorbed to both Dowex-50 and Dowex-1 columns was obtained. This fraction was concentrated, applied on Whatman No. 1 paper and the chromatogram was developed with n-butanol-glacial acetic acid-water (4 : 1 : 1 by volume).

* The antibiotic produced by this organism was proved to be identical with Gramicidin S (Kurahashi, 1964, Saito and Otani, 1964)

β-mercaptoethanol and was dialyzed against the same buffer for 2 hours.

The incubation mixture and isolation procedure of reaction product are described in the legend of Figure 1 which shows the radiochromatogram obtained by scanning with a windowless strip counter. Besides the three peaks which correspond to C^{14}-phenylalanine, C^{14}-valine and C^{14}-proline, a new peak for compound X was observed.

Compound X was eluted from the paper and hydrolyzed in 6 N HCl at 105° for 12 hours in a sealed evacuated tube. The hydrolyzate was chromatographed on paper with n-butanol-acetic acid-H_2O (4 : 1 : 5) solvent. Three radioactive peaks corresponding to phenylalanine, valine and proline were obtained and as shown in

Table I. Radio Active Amino Acids Found in Compound X

Before hydrolysis	
Compound X	136,900 c.p.m.
After hydrolysis	
phenylalanine	41,100 "
proline	50,300 "
valine	38,400 "

The three radioactive peaks were eluted with water from paper chromatograms, plated and counted at infinite thinness in a Nuclear-Chicago gas flow counter with thin window.

Table I; the total radioactivity of the three amino acids accounts for more than 95% of that of compound X. By the use of D- and L-amino acid oxidase, it was found that phenylalanine was the D-isomer and that proline and valine were L-isomers.

Compound X gave a single radioactive spot by paper chromatography in three different solvent systems and its Rf values were identical to those of synthetic D-phenylalanyl-L-prolyl-L-valine, e.g., Rf = 0.82 with n-butanol, acetic acid, water (4 : 1 : 1 by

volume), 0.70 with n-butanol saturated with water, and 0.62 with n-butanol saturated with 1 N-NH_4OH. The formation of Compound X was also observed when any two of the three labeled amino acids were replaced by C^{12} amino acids.

Compound X was dinitrophenylated according to the micro method of Sanger (1953). The Rf values of the DNP-Compound X were identical to those of DNP-D-phenylalanyl-L-prolyl-L-valine (0.73 with n-butanol saturated with water and 0.87 with n-butanol saturated with N-NH_4OH). Acid hydrolysis of DNP-Compound X with 6H-HCl for 12 hours at 105° produced only DNP-phenylalanine as radioactive DNP-derivative. (Rf 0.75 with n-butanol saturated with N-NH_4OH and 0.25 with 1.5 M-sodium phosphate buffer pH 6.7).

For further comfirmation, DNP-Compound X was cocrystallized repeatedly with DNP-D-phenylalanyl-L-prolyl-L-valine from acetone water. As shown in Table 2, the specific radioactivity of the DNP-

Table II. Specific Radioactivities of DNP-Compound X-C^{14} Cocrystallized with DNP-D-phenylalanyl-L-prolyl-L-valine

Recrystallization	Radioactivity (c.p.m.)	Absorbancy (O.D. x 10^3)	Specific radioactivity (c.p.m./O.D. x 10^3)
1st	798	793	1.01
2nd	844	860	0.96
3rd	570	541	1.05
4th	399	381	1.05
5th	645	610	1.03

After each crystallization, an aliquot was taken and its radioactivity and absorbancy at 360 mμ was determined by a Nuclea-Chicago gas flow counter and a Beckman spectrophotometer, respectively.

derivative was constant after successive recrystallizations. The results cited above were all favorable to the conclusion that Compound X was D-phenylalanyl-L-prolyl-L-valine.

The formation of Compound X was affected by none of inhibitors for protein syntheses, such as DNase, RNase, chloramphenicol and mitomycin C. The amount of Compound X synthesized was much decreased when L-leucine and L-ornithine were added to the incubation mixture. Under this condition, most of the radio activity was found in the gramicidin S fraction, which could be separated from Compound X by treatment with ion exchange resin. This indicates the possibility that Compound X is an intermediate in the synthesis of gramicidin S. However, no significant amount of radioactivity was detected in the gramicidin S fraction, when L-ornithine, L-leucine and C^{14}-Compound X were incubated with enzyme preparations. These observations suggest that the mechanism of peptidation in the synthesis of gramicidin S is not similar to that of either protein synthesis or glutathione synthesis, where free γ-glutamylcysteine serves as a substrate (Yanari, Snoke and Bloch, 1953).

Further studies on the peptidation in the synthesis of gramicidin are in progress.

We wish to express our gratitude to Dr. S. Sakakibara of this Institute for kindly supplying synthetic D-phenylalanyl-L-prolyl-L-valine, prepared by the Peptide Center of this Institute. The method of chemical synthesis for this peptide will shortly appear elsewhere.

REFERENCES

Eikhom, T., Jonsen, J., Laland, S. and Refsvik, T., Biochim. Biophys. Acta, 76, 465 (1963).

Kurahashi, K., Abstract, Vth Intern. Congr. Biochem., Moscow, 1961, Pergamon Press, Oxford, 1961, p.37.

Kurahashi, K., J. Biochem., (Tokyo) in press.

Mach. B., Reich, E. and Tatum, E. L., Proc. Nat. Acad. Sci., 50, 175 (1963).

Okuda, K., Uemura, I., Bodley, J. W. and Winnick, T., Biochemistry,

3, 100 (1964).

Okuda, K., Uemura, I., Bodley, J. W. and Winnick, T., Biochemistry, 3, 108 (1964).

Saito, Y. and Otani, S., J. Biochem., (Tokyo) in press.

Sanger, F. and Thompson, E. O. P., Biochem. J., 53, 353 (1953).

Uemura, I., Okuda, K. and Winnick, T., Biochemistry, 2, 719 (1963).

Winnick, R. E. and Winnick, T., Biochim. Biophys. Acta, 53, 461 (1961).

Yanari, S., Snoke, J. E. and Bloch, K., J. Biol. Chem., 201, 561 (1953).

Yukioka, M., Tsukamoto, Y., Saito, Y. and Otani, S., J. Japan. Biochem. Soc., (SEIKAGAKU), 35, 590 (1963).