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Structural Similarities of the Staphylococcin-Like Peptide Pep-5 to the Peptide Antibiotic Nisin

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The staphylococcin-like peptide Pep-5 was shown to be a complex mixture of closely related and strongly basic peptides. Five peptides were purified by high-pressure liquid chromatography on reversed-phase and gel filtration columns and further characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and amino acid analysis. Four peptides have molecular weights of ca. 3,500, whereas one is of double size. All contain the thioether amino acid lanthionine and a large number of lysine residues per molecule. The amino terminus of the main active peptide is blocked; the carboxy-terminal end is formed by a lysine residue. The data obtained for Pep-5 suggest striking structural similarities to the peptide antibiotics nisin and subtilin.

The staphylococcin-like peptide Pep-5 produced by *Staphylococcus epidermidis* 5 is a small and strongly basic peptide with a bactericidal action on members of the family *Micrococcaceae* (12). It has been shown to cause a rapid efflux of low-M_r compounds such as K^+, amino acids, and ATP from the cytoplasm of sensitive cells (14). Therefore, the cytoplasmic membrane appears to be the primary target of its action. As a consequence of the efflux of substrates, cofactors, and phosphorylated nucleosides, total inhibition of biosynthesis was observed after the addition of Pep-5 to growing cells (13). In early investigations on the structure of Pep-5 (12), a high content of hydrophobic amino acids was found in hydrolysates along with a large number of lysine and arginine residues, resulting in an isoelectric point of the peptide of about 10.5. Furthermore, two ninhydrin-positive substances were detected which could not be identified. These substances were supposed to contain the sulfur found in elementary analysis of Pep-5, which could not be attributed to the amino acids identified after acidic hydrolysis. These results prompted the more detailed investigations on the structure of Pep-5 reported in this paper. It is shown that Pep-5 can be fractionated into several closely related peptides with different specific activities. All peptides contain the rare amino acid lanthionine, which suggests similarities of Pep-5 to the known peptide antibiotics nisin and subtilin (3, 6). These are produced by group N streptococci and *Bacillus subtilis*, respectively, and strongly resemble Pep-5 with respect to their molecular properties (9) and mode of action (11).

MATERIALS AND METHODS

Strains. The Pep-5-producing strain *S. epidermidis* 5 and the indicator strain *S. cohnii* 22 were maintained on blood agar plates and subcultured weekly. Production and purification of Pep-5 as well as the activity tests were performed as described previously (13).

Analytical methods. (i) HPLC. For high-pressure liquid chromatography (HPLC) investigations a Gilson gradient system was used, including a Microsorb C-18 (particle size, 5 μm; Rainin Instrument Co., Inc., Woburn, Mass.) or a Lichrosorb C-18 (particle size, 5 μm; Bischoff Leonberg, Federal Republic of Germany) column. A precolumn (25 by 3.5 mm) filled with the same resin was included in the circuit. For gel filtration a Bio-Sil TSK 125 (300 by 7.5 mm; Bio-Rad Laboratories, Richmond, Calif.) column was used. Pep-5 peptides were separated on a reversed-phase (RP) C-18 column with acetonitrile gradients in 0.2% trifluoroacetic acid at a flow rate of 1 ml/min. The *o*-phthaldialdehyde (OPA)-derivatized amino acids were separated on the same columns with a methanol–3% tetrahydrofuran (by volume) gradient in 50 mM Na_3PO_4 (pH 6.5). The flow rate was kept at 1.3 ml/min. Chromatography of dansylated amino acids was performed basically by the method of Kaneda et al. (8), except that an RP C-18 column was used instead of an RP C-8 resin and the amino acids were detected by absorbance at 280 nm instead of fluorescence intensity.

(ii) Amino acid analysis. The amino acid analysis was performed by conventional ion-exchange chromatography and OPA derivatization. The OPA reagent was kept as a stock solution of 56 mg of OPA dissolved in 1 ml of methanol and 10 μl of 2-mercaptoethanol. This stock solution was stored at 4°C and diluted 1:10 with sample buffer (0.4 M Na_3BO_3 [pH 9.5]) for use. This dilution was freshly prepared daily. Standard amino acids were dissolved in 0.1 N HCl and peptide hydrolysates in distilled water. Portions containing 20 nmol of each amino acid and tyrosine as internal standard were subjected to derivatization by mixing 1:9 (vol/vol) with OPA reagent. A 20-μl portion was injected onto the column after a 60-s derivatization period.

The dansylation of Pep-5 was performed essentially by the method of Gray as described by Allen (1). Dansylated amino acids were separated on polyamide thin-layer sheets as well as on the HPLC RP C-18 column.

(iii) SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Weber et al. (15) in 0.8-mm horizontal slab gels. The final concentrations were 20% polyacrylamide, 0.5% diallyltardardiamide, 0.1% SDS in the gel, and 2% SDS in the sample buffer. Gels were stained with Coomassie blue.

(iv) Enzymatic procedures. Carboxypeptidase digestions of Pep-5 were conducted as described by Allen (1). Digestion was stopped by addition of the samples to equal volumes of 1 N acetic acid; the solutions were then dried under vacuum and dissolved in OPA derivatization buffer. Portions corre-

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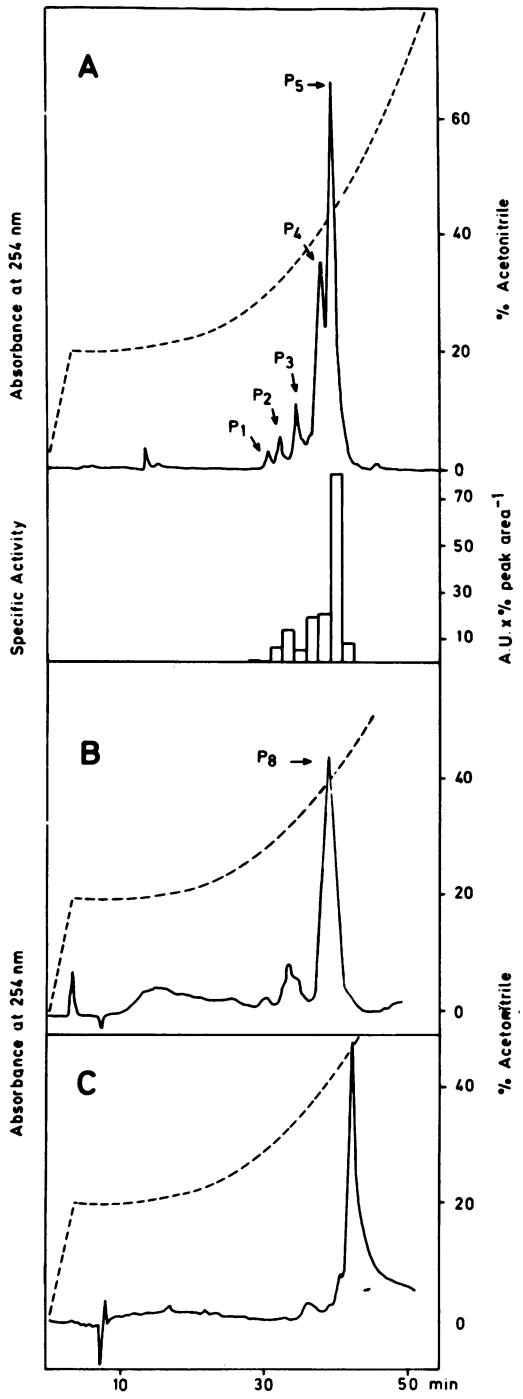

FIG. 1. Analysis of Pep-5 (A, B) and nisin (C) on RP C-18 HPLC. Lyophylized peptides (100 to 200 μg) were dissolved in 20 μl of 0.2% trifluoroacetic acid, injected, and eluted with an acetonitrile gradient (– – – –). Elution profile of a Pep-5 preparation with (A) and without (B) the peptide of M_r 7,000. The activity of Pep-5 is expressed in arbitrary units (AU) as defined previously (13); the specific activity is referred to the proportionate peak area of the corresponding fraction (AU × % peak area^{-1}).

sponding to 20 nmol of internal standard were analyzed by HPLC. For D-amino acid oxidation, 2 U of D-amino acid oxidase were dissolved in 1 ml of $NaPO_4$ buffer (100 mM, pH 8.3), 0.5 mg of catalase was added, and the enzymes were dialyzed against the same buffer. A 500-μl portion of the dialysate was incubated with 500 μl of hydrolysate (100 nmol of amino acids) at 25°C. Samples taken after 1, 5, and 20 h were handled as described above.

Reagents. Amino acids including DL- and *meso*-lanthionine and dansylated amino acid standards, dansyl chloride, OPA, carboxypeptidases A and B, and D-amino acid oxidase were obtained from Sigma, Munich, Federal Republic of Germany. Chemicals for SDS-PAGE were purchased from Bio-Rad, Munich, Federal Republic of Germany. Solvents for HPLC and all basic chemicals (p.A. grade) were from E. Merck AG, Darmstadt, Federal Republic of Germany. Nisin was obtained from Koch & Light through Paesel, Frankfurt, Federal Republic of Germany.

RESULTS

Separation of Pep-5 by HPLC. It was observed with all Pep-5 enrichments performed so far that the producing strain *S. epidermidis* 5 excreted, along with Pep-5, various amounts of a peptide with a higher M_r (13). These two peptides could hardly be separated by conventional liquid chromatographic methods or PAGE without detergent. Two bands, however, were obtained in SDS-PAGE (12). Therefore, different Pep-5 preparations were subjected to RP C-18 HPLC. Figure 1 shows the elution profile of two different Pep-5 preparations containing less than 2% of the larger peptide (Fig. 1B) and an amount of almost 40% of this peptide (Fig. 1A), respectively. It can be seen that apart from some minor peaks, most of the Pep-5 material elutes in two prominent peaks, peaks 4 and 5 (designated P4 and P5), at about 35% acetonitrile. From the corresponding activity plot (Fig. 1A), it becomes obvious that the highest specific activity is associated with P5, which is the only prominent peak in those Pep-5 preparations lacking the higher-M_r peptide (Fig. 1B). Furthermore, it can be seen that several minor peaks occur which show a much lower specific activity than the main active peptide in P5.

The larger-M_r band peptide elutes in P4 (Fig. 1A). This peak is consequently absent in Fig. 1B, showing the elution profile of a Pep-5 preparation which lacks the larger-M_r peptide. These results were confirmed by SDS-PAGE (Fig. 2, lanes 6, 7, 9, and 10). SDS-PAGE also demonstrated that the less active peptides all migrated in the same position as the highly active peptide (Fig. 2, lanes 3 through 5). Nisin, with an M_r of 3,500, shows an R_f-value identical to that of the P5 fraction of Pep-5 and behaves similarly on the HPLC RP C-18 column (Fig. 1C). By comparison with nisin (Fig. 2, lane 11), similar M_r values must be calculated for Pep-5, e.g., 3,500 for the different low-M_r peptides, including the highly active one, and 7,000 for the large-M_r peptide. These values are confirmed, although they are somewhat lower (3,000 and 6,200, respectively) by comparison with M_r standard peptides (Fig. 2, lanes 1, 2, 8, and 12). It must be stated that SDS-PAGE is not very reliable for M_r determination in this M_r range.

As the active peptide of M_r 3,500 and the peptide of M_r 7,000 could only be enriched but not purified by RP C-18 chromatography, HPLC gel filtration was conducted on a Bio-Sil TSK 125 column. With 30% acetonitrile–0.1% trifluoroacetic acid as eluant, the two peptides could be further separated as shown (Fig. 2, lanes 9 and 10; Fig. 3). The question whether the peptide of M_r 7,000 is completely inactive or has only a low specific activity cannot be settled entirely, since under all chromatographic conditions used there was still some overlapping with the active peptide. Separation by SDS-PAGE and elution of the bands from the

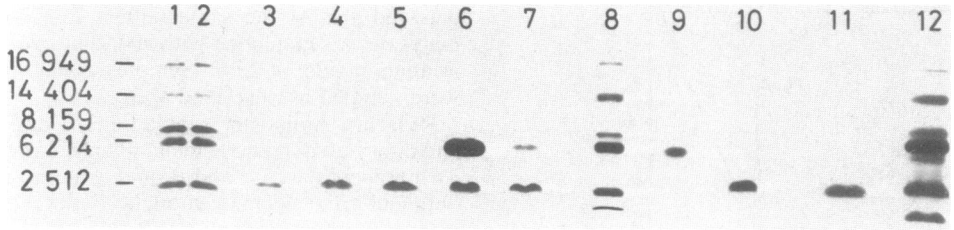

FIG. 2. SDS-PAGE of Pep-5 peptides in a 20% acrylamide gel. Lanes 1, 2, 8, and 12 contain M_r standard peptides of the M_r values indicated (standard mixture no. 1; Merck, 15124). P1 (lane 3), P2 (lane 4), P3 (lane 5), P4 (lane 6), and 20% P5 (by volume) (lane 7), all corresponding to Fig. 1A, as well as P6 (lane 9) and P7 (lane 10), corresponding to Fig. 2, were evaporated to dryness and taken up in 20 μl of sample buffer. Lane 11 contains 20 μg of nisin.

gel with 70% CH_3OH (pH 2) yielded the completely inactive peptide of M_r 7,000. However, it cannot be excluded that inactivation occurs under these conditions, as only about 10% of the activity of the active peptide of M_r 3,500 was recovered.

Recently, Nishio et al. (10) found that the peptide antibiotic subtilin, which is closely related to nisin, is synthesized via precursor peptides with higher M_rs, which are processed to the active peptide of M_r 3,500 in several steps. To test whether the peptide of M_r 7,000 is a Pep-5 precursor, the appearance of active Pep-5 and of the peptide of M_r 7,000 in the course of a growth curve was recorded by SDS-PAGE and RP C-18 HPLC. Bactericidal activity was first detectable after 4 h of growth and reached its highest titers at the end of the log phase. The corresponding bands at M_r 3,500 in SDS-PAGE and P5 in RP C-18 HPLC, respectively, were first found after 8 h of growth of *S. epidermidis* in 100-fold-concentrated medium supernatants. In those cases, when the peptide of M_r 7,000 was not present after 18 to 22 h—the usual harvesting time for Pep-5—it could not be found during the whole course of the growth curve (48 h). In cases when the large peptide was excreted, it was first detected after 10 h of growth, i.e., somewhat later than the peptide of M_r 3,500. The amounts of the larger peptide increased during the log phase simultaneously with the peptide of M_r 3,500. At the end of the stationary phase, both peptides disappeared along with the bactericidal activity, most probably as a result of protease excretion by *S. epidermidis* 5. These findings are not in favor of a Pep-5 precursor role for the peptide of M_r 7,000.

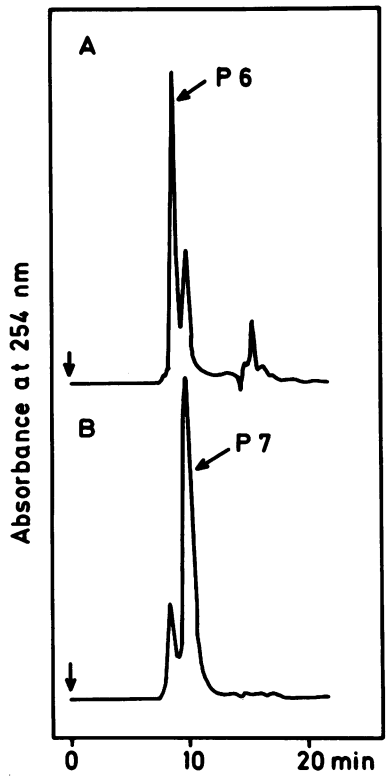

FIG. 3. HPLC gel filtration of Pep-5 peptides on a Bio-Sil TSK 125 column. P4 (A) and P5 (B), corresponding to Fig. 1A, were concentrated to 20 μl and injected onto a column equilibrated with 30% acetonitrile (by volume)–0.1% trifluoroacetic acid (by volume).

Amino acid analysis of Pep-5 peptides. To more closely characterize the peptides separated on HPLC, the amino acid compositions of peaks 1 through 7 (Fig. 1 and 3) were determined and are compiled in Table 1. The number of residues ascribed to one molecule of peptide is based on the assumption that phenylalanine and isoleucine, which were found in hydrolysates in the lowest and almost equal amounts, are represented in the peptide with one residue per molecule. On this basis, molecular weights of about 3,300 to 3,475 for the small peptides (P1 to P3, P5, and P7) can be calculated. As the amino acid analysis obtained for the large peptide (P4 and P6) was identical with the one for P5, two residues of phenylalanine and isoleucine per molecule were assumed, yielding a molecular weight of 6,716 for this peptide. These values are in good agreement with the values obtained by SDS-PAGE. Furthermore, Table 1 shows that the peptides differ only in the number of alanine, aspartate-

TABLE 1. Amino acid composition of Pep-5 peptides (P1 through P7)[a]

Amino acid	No. of residues in:						
	P1	P2	P3	P4	P5	P6	P7
Alanine	3	3	4	8	4	8	4
Arginine	2	2	2	4	2	4	2
Aspartate-asparagine	1.5	1.5	1	2	1	2	1
Glutamate-glutamine	2	2	2	4	2	4	2
Glycine	3	4	3	6	3	6	3
Isoleucine	1	1	1	2	1	2	1
Lanthionine	3	3	3	6	3	3	3
Leucine	2	2	2	4	2	4	2
Lysine	7	6	6	12	6	12	6
Phenylalanine	1	1	1	2	1	2	1
Proline	0	0.5	0.5	2	1	2	1
Serine	0	1	0	0	0	0	0
Valine	2	2	2	4	2	4	2

[a] P1 through P7 correspond to peaks 1 through 7 (Fig. 1A, 3A, and B). Calculated M_rs for P1 through P7 are, respectively, 3,366.5, 3,375, 3,300.5, 6,716, 3,349, 6,716, and 3,349.

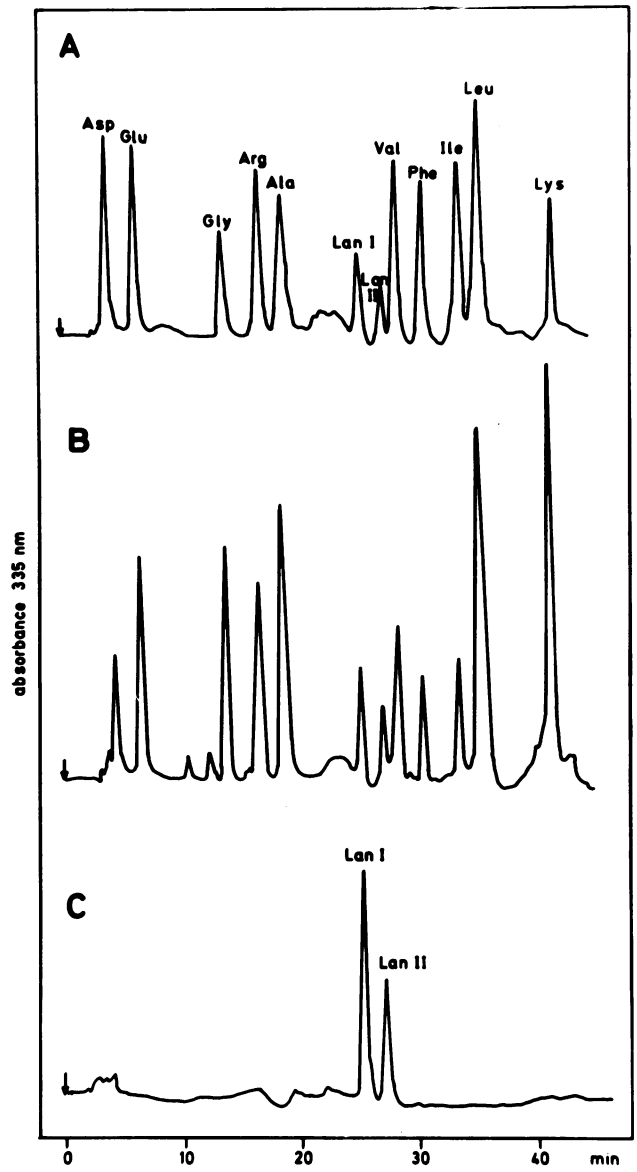

FIG. 4. Amino acid analysis of a Pep-5 peptide obtained after RP C-18 HPLC, corresponding to P8 (Fig. 1B). The amino acids were derivatized with OPA as described in the text and chromatographed with a methanol gradient (buffer A: 50 mM $NaPO_4$ [pH 6.5]; buffer B: methanol–3% tetrahydrofuran). (A) Chromatogram of an amino acid standard mixture containing 5.52 nmol of each amino acid; (B) acidic hydrolysate of 5 nmol of Pep-5 corresponding to P8 (Fig. 1B); (C) 2 nmol of DL- and *meso*-lanthionine (Sigma).

asparagine, glycine, lysine, and proline residues. Serine is present only in P3. Arginine, glutamate-glutamine, isoleucine, lanthionine, leucine, phenylalanine, and valine are conserved in all peptides.

Incubation of Pep-5 hydrolysates with D-amino acid oxidase did not change the quantitative composition of the amino acid pattern listed in Table 1, except for lanthionine (see below). This indicates that all amino acids are in the L-configuration.

Furthermore, pyruvate and α-ketobutyrate were not detected with L-lactate dehydrogenase in hydrolysates. Both derive from dehydroalanine and dehydrobutyrine, respectively, under the conditions of acidic peptide hydrolysis (4). Dehydroalanine and dehydrobutyrine are present in the peptide antibiotics nisin and subtilin (9) but are obviously lacking in Pep-5.

Identification of lanthionine. The presence of lanthionine in Pep-5 hydrolysates was shown by conventional amino acid analysis on ion-exchange resins by using three different elution programs and by derivatization of amino acids with OPA and subsequent HPLC on an RP C-18 column. In all cases the previously unidentified peaks in Pep-5 hydrolysates (13) coeluted with DL- and *meso*-lanthionine standards.

Figure 4 shows the HPLC of OPA-derivatized amino acids. Standard mixtures of DL- and *meso*-lanthionine and lanthionine in Pep-5 hydrolysates always eluted in two peaks, which represent *meso*-lanthionine (peak I) and DL-lanthionine (peak II). This assignment is most likely because (i) the peak area distribution is about 1.5:1 (peak I/peak II); a racemization of lanthionine via an unknown mechanism into 60% *meso*-lanthionine and 40% DL-lanthionine is reported (6); and (ii) peak I disappeared almost completely on incubation with D- and with L-amino acid oxidase, whereas peak II was hardly reduced in either case.

The presence of lanthionine in Pep-5 also explains the occurrence of 3% sulfur (by weight) in Pep-5 elementary analysis (12). Three molecules of lanthionine corresponding to three sulfur atoms in Pep-5 would account for 2.9% sulfur (by weight), calculated on the basis of an M_r of 3,350 for Pep-5.

Investigations on the C and N termini of Pep-5. When the purified main active component of Pep-5 was subjected to automated Edman degradation, no amino acid phenylthiohydantoin derivative could be detected after the first cycle, indicating that the N terminus of Pep-5 was blocked. This was verified by dansylation of the different Pep-5 peptides and subsequent chromatography of the dansyl derivatives on silica gel thin-layer plates as well as on an HPLC RP C-18 column after acidic hydrolysis. Carboxypeptidase B liberated lysine in amounts corresponding to the amount of Pep-5 added, proving that lysine is the C-terminal amino acid. Simultaneous digestion with carboxypeptidases A and B and separate digestion with carboxypeptidase Y did not yield further information about the C-terminal sequence. The reason remains unclear, but it seems possible that a lanthionine residue is close to the C terminus and prevents further enzymic sequencing.

DISCUSSION

The antistaphylococcal agent Pep-5 is shown not to be a homogenous peptide but rather a complex mixture of very closely related peptides only differing in the amount of residues of some amino acids in the molecule. These differences are obviously also the reason for the different specific activities of these peptides. Furthermore, a peptide showing twice the molecular weight of the peptide with the highest activity but an identical amino acid composition was detected. The M_rs found in this study, 3,500 and 7,000 for the active and inactive peptide, respectively, differ considerably from those obtained by SDS-PAGE in gel tubes and gel filtration on Sephadex G-50 in earlier studies on Pep-5 (6,000 and 18,000, respectively [12]). The values reported here are more reliable, as they are in good agreement with the values calculated on the basis of the amino acid composition. The exact molecular weights cannot be given before the total elucidation of the structure of Pep-5. These studies are in progress now.

The structural features of Pep-5 reported here show a strong similarity to the peptide antibiotics nisin (produced by

group N streptococci) and subtilin (produced by *Bacillus subtilis*): (i) they are mixtures of peptides with one predominant and most active component, (ii) their M_rs are about 3,500, (iii) they are strongly basic peptides, (iv) lysine is the C-terminal amino acid, and (v) they contain the thioether amino acid lanthionine, which is scarcely encountered in bacterial metabolism. These striking similarities in structure should result in a common mode of action. That this is indeed the case at least for nisin and Pep-5, is reported in an accompanying paper (11). On the other hand, there are some differences. Nisin and subtilin contain four intramolecular rings, formed by one molecule of lanthionine and three molecules of β-methyllanthionine. In contrast, Pep-5 hydrolysates only contain three lanthionine residues.

It has not yet been proven that lanthionine forms the same intramolecular ring structures in Pep-5 as in nisin, but it is tempting to speculate about this, especially with respect to the mechanism of posttranslational synthesis of lanthionine (7). Another difference is the apparent absence of the α, β-unsaturated amino acids dehydroalanine and dehydrobutyrine (α-methyldehydroalanine) in Pep-5, whereas both are present in nisin (4–6) and subtilin (2).

Despite these differences and the unsolved questions concerning the structure of Pep-5, it seems justified to classify it with nisin and subtilin into a group of large peptide antibiotics. In contrast to small peptide antibiotics, a ribosomal mechanism and posttranslational modification of precursor peptides have been shown to be involved in the biosynthesis of active nisin and subtilin (7, 9). The fact that these antibiotics are produced by bacteria such as *Bacillus*, *Streptococcus*, and *Staphylococcus* spp., which are taxonomically not closely related, may point to a common role of the precursors in the cell cycles of gram-positive bacteria.

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