BIKEN JOURNAL Vol. 11, 1-12, 1968

STUDIES ON THE MODE OF ACTION OF *FLAVOBACTERIUM* L-11 ENZYME ON THE CELL WALLS OF *STAPHYLOCOCCUS AUREUS* STRAIN COPENHAGEN. IDENTIFICATION OF ISOLATED CELL WALL PEPTIDES[1]

KEIJIRO KATO, TAKANORI HIRATA, YŌJI MURAYAMA, HIDEKAZU SUGINAKA and SHOZO KOTANI

Department of Microbiology, Osaka University Dental School, Osaka

(Received December 27, 1967)

SUMMARY 1. Isolated cell walls from *Staphylococcus aureus* strain Copenhagen were solubilized by the L-11 enzyme from *Flavobacterium*. The digests were fractionated by Sephadex gel filtration into a higher molecular weight fraction, rich in hexosamines and organic phosphates, and a lower molecular weight fraction, containing only free amino groups.

2. Seven peptides were isolated from the lower molecular weight fraction by column chromatography on Amberlite CG-120. The amino acid composition, NH_2-terminal and COOH-terminal amino acids of these peptides were analyzed. Their migrations on paper electrophoresis and the mobilities of their dinitrophenylated derivatives on thin layer chromatography were determined. Their amino acid sequences were also analyzed by Edman degradation.

3. These analyses showed that, six of the seven peptides were tetraglycine, triglycine, diglycine and N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ε}-$(glycyl)_{5,2,1}$-L-lysyl-D-alanine, respectively. The structure of the remaining peptide, which contained hexosamines in addition to amino acids, has not yet been determined.

4. The results obtained provide additional evidence that *S. aureus* cell walls are solubilized by *Flavobacterium* L-11 enzyme by cleavage of the linkages of pentaglycine cross-bridges to the D-alanyl terminus of the basal tetrapeptide, L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine, those of glycyl-glycine in the interpeptide bridges, and the amide links between the polysaccharide and peptide moieties in the peptidoglycan.

[1] A part of this work was presented at the 40th Annual Meeting of the Japan Microbiological Society (March 30–31, 1967, at Nagoya), the Annual Meeting of the Society Japan Agriculture Chemistry (March 31–April 4, 1967, at Tokyo), and the 141st Monthly Meeting of Members of the Research Institute for Microbial Diseases, Osaka University (June 27, 1967).

INTRODUCTION

In an earlier paper (KATO *et al.*, 1962) of this series, it was reported that a *Flavobacterium sp.* produced a powerful lytic agent which was active against *Staphylococcus aureus* in the culture supernatant and that lysis of isolated cell walls of *S. aureus* strain Newman 1 was accompanied by the release of ninhydrin positive materials. Analysis by paper chromatography demonstrated that the dialyzable portion of the lysates consisted mainly of two kinds of peptides, one of which contained alanine, glutamic acid, lysine and glycine and the other only glycine. A subsequent study by kinetic analyses of end groups liberated during digestion of cell walls from *S. aureus* strain Copenhagen showed that NH_2-terminal glycine and alanine were released with COOH-terminal glycine and alanine by the L-11 enzyme. It was tentatively concluded that the enzyme catalyzes the hydrolysis of linkages of muramyl-L-alanine, glycyl-glycine and D-alanyl-glycine in *S. aureus* walls (KATO and STROMINGER, 1968).

This conclusion was confirmed in the present study by isolation of peptide fragments derived from the cell wall peptidoglycan digested with the L-11 enzyme and by determination of their chemical structure.

MATERIALS AND METHODS

1. *S. aureus cell walls*

A specimen of cell walls was prepared from *S. aureus* strain Copenhagen as described in a previous paper (SUGINAKA *et al.*, 1967) with some minor modifications. Cells were grown with aeration in nutrient broth supplemented with 0.1 per cent yeast extract (Daigo Nutritional Chemicals, Osaka) and 0.5 per cent glucose, harvested after 18 hours cultivation at 37°C, and washed with deionized water. The washed cells were suspended in an appropriate amount of 1 M NaCl solution and disrupted mechanically in a Braun Mechanical Cell Homogenizer (Model MSK, B. Braun Apparatebau, Melsungen, West Germany) with glass beads of 0.11–0.12 mm diameter (Glasperlen, Kat. Nr. 54140 (2883), B. Braun Apparatebau). Further purification was accomplished by digesting the deposit containing cell walls from 180 g (dry weight) of disrupted cells with 9 mg of pronase (Kaken Chemicals, Tokyo) for 48 hours at 37°C. The digestion was repeated three more times.

2. *L-11 enzyme*

An enzyme preparation was isolated from the culture supernatants of *Flavobacterium sp.* grown in 0.1 per cent casamino acid medium for several days at 30°C, and purified by chromatography on a hydroxylapatite column, as described previously (KATO *et al.*, 1962). The enzyme solution used in this study was a combination of the fractions eluted from the column with 0.05 M potassium phosphate buffer, pH 6.8. The cell wall lytic activity, assayed by determining the reduction in optical density of a suspension of *S. aureus* cell wall under standard conditions (KATO *et al.*, 1962), was found to be 10 units per ml.

3. *Analytical methods*

Determinations of total, NH_2-terminal, COOH-terminal and free amino acids, total hexosamines and configuration of alanine isomers were carried out as described by GHUYSEN, TIPPER and STROMINGER (1966). For the estimation of total and terminal amino acids, materials were hydrolyzed in 4 N HCl at 100°C for 8 hours. Hydrazinolysis for the determination of COOH-terminal amino acid was performed by incubating specimens with redistilled anhydrous hydrazine at 100°C for 6 hours. Total and inorganic phosphates, reducing sugars and ammonia were estimated by the methods of LOWRY *et al.* (1954), PARK and JOHNSON (1949) and FAWCETT and SCOTT (1960), respectively, all on a microscale.

4. *Gel filtration and column chromatography*

For gel filtration Sephadex G-50 and G-25 (bead form, 100–300 μ, Pharmacia, Uppsala, Sweden) were used in columns connected in series. Amberlite CG-120 (Rhom and Haas, Pennsylvania, Pa., U.S.A.) of 19–22 μ particle size, used for separation of peptides, was purchased from Yanagimoto Manufacturing Co. (Kyoto). The Amberlite column (0.9×30 cm) was kept warm by circulating water at 50°C through an outer jacket fitted with an ULTRA Thermostat (Type NB-D8/17, Messgeräte-Werk Lauda, West Germany). The flow rate of solvents was adjusted to 40 ml per hour with a

micrometer pump of a double-sealed type (Yanagimoto Co., Kyoto).

5. *Thin layer chromatography*

1) Amino acids: Dinitrophenylated derivatives were determined by quantitative thin layer chromatography on plates of silica gel (Kieselgel G nach Stahl für die Dünnschichtchromatographie, E. Merck AG., Darmstadt, West Germany) as described by GHUYSEN, TIPPER and STROMINGER (1966). The following solvents were used. Solvent 1: *n*-butanol–1 per cent ammonium hydroxide (1 : 1, upper phase), 2: chloroform–methyl alcohol–glacial acetic acid (85 : 14 : 1), 3: benzyl alcohol–methyl alcohol–chloroform–water–ammonium hydroxide (30 : 30 : 30 : 6 : 2), and 4: *tert*-amyl alcohol–chloroform–methyl alcohol–water–glacial acetic acid (30 : 30 : 30 : 20 : 3, organic phase). Ether-soluble DNP-amino acids were developed with solvent 1 for 2 hours at room temperature. Then the solvent on the plate was removed by drying with an air stream and the dried plate was further developed with solvent 2 for 45 minutes at 2°C. The ether-insoluble DNP-amino acids were developed with solvent 3 for 2 hours at room temperature.

2) Peptides: Solvent 1 and 4 was used for development of DNP-derivatives of test peptides, to determine their homogeneity and mobility. Dinitrophenylated derivatives of authentic peptides were run on the same plate.

6. *Paper electrophoresis*

An apparatus of the horizontal type (power supply: Model III B A·8–2, Toyo Kagaku Sangyo Co., Osaka) was used. Specimens were applied to No. 514 Toyo-filter paper (34.5 cm in length) and run at a potential gradient of 14 volt/cm in 0.1 M pyridine–acetic acid buffer, pH 5.0, and/or 80 per cent formic acid–acetic acid–water (5 : 15 : 80), pH 1.9, at 2°C for 90 to 250 minutes. As references lysine, alanine (or glycine) and glutamic acid were run at pH 5.0 and glycine, diglycine, triglycine and tetraglycine were run at pH 1.9.

7. *Edman degradation*

The amino acid sequences of the isolated peptides were analyzed by Edman degradation following the descriptions of KONIGSBERG and HILL (1962) and of TIPPER *et al.* (1967), with some modifications: cyclization of the phenylthiocarbamyl derivative of a peptide was effected by incubation with 4 N HCl, instead of anhydrous trifluoroacetic acid as in the original methods, for 6 hours at room temperature, and ethylacetate was used for extraction of the 2-anilino-5-thiazolinone derivatives of NH_2-terminal amino acids. Before and after each cycle of the degradation, the NH_2-terminal and amino acid compositions of test peptides were determined as DNP-derivatives by thin layer chromatography.

RESULTS

1. *Preparation of cell wall digests by L-11 enzyme*

Isolated cell walls (200 mg) from *S. aureus* strain Copenhagen were incubated with L-11 enzyme (200 units of cell wall lytic activity) in a total volume of 80 ml of 0.0125 M potas-

TABLE 1 *Liberation of NH_2-terminal and COOH-terminal amino acid during lysis of S. aureus cell walls by L-11 enzyme*

Incubation time (hrs)	Free amino acid	NH_2-terminal amino acids		COOH-terminal amino acids	
	Ala	Gly	Ala	Gly	Ala
0	0.11	0.15	0.41	0.10	0.24
1	0.11	0.60	0.47	0.36	0.54
3	0.13	0.89	0.48	0.51	0.71
7	0.12	1.03	0.50	0.73	0.77
24	0.13	1.14	0.48	0.82	0.81
48	0.13	1.25	0.52	0.87	0.82
Increase in 48 hrs	0.02	1.10	0.11	0.77	0.58

The data are expressed as mole per mole of total glutamic acid.

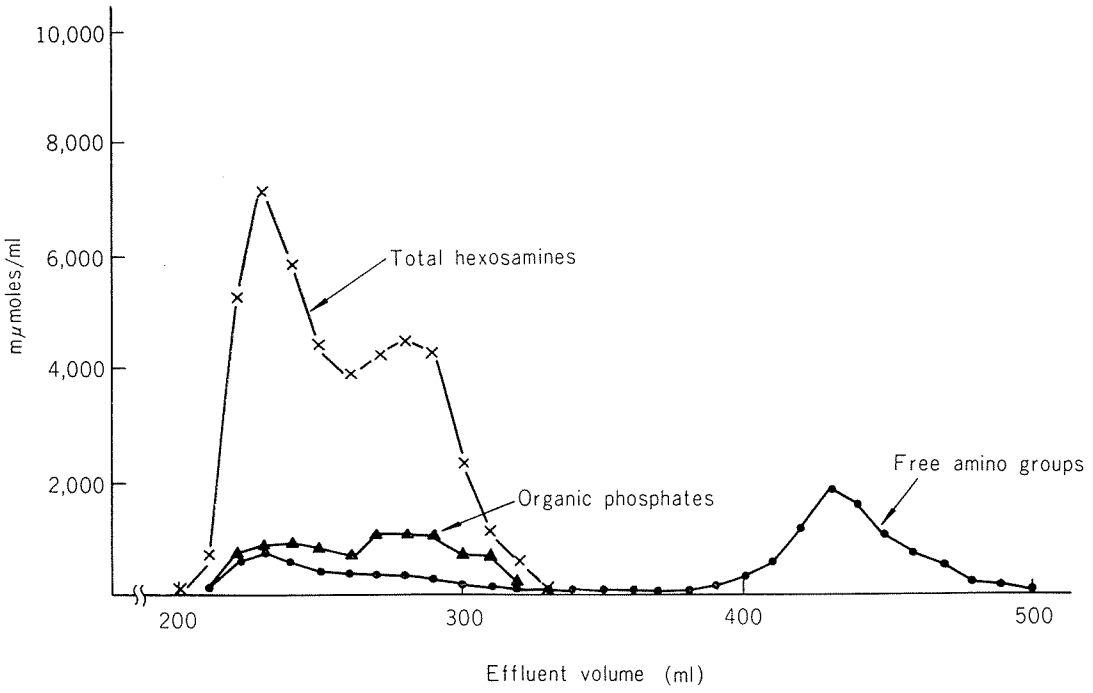

FIGURE 1 Gel filtration of the L-11 enzyme digests of *S. aureus* cell walls with Sephadex G-50 and G-25 columns connected in series.

sium phosphate buffer, pH 6.8, at 37°C. A small amount of chloroform was added as a preservative.

After 48 hours incubation, aliquots of 60 μl and 100 μl of the reaction mixture were analyzed for NH_2-terminal and COOH-terminal amino acid residues, respectively. As shown in Table 1, 1.1 mole of glycine and 0.1 mole of alanine were liberated as NH_2-terminal amino acids, per mole of cell wall glutamic acid. This release of NH_2-terminal amino acids was accompanied by liberation of 0.77 mole of COOH-terminal glycine and 0.58 mole of COOH-terminal alanine.[1]

The lysate of walls which had been incubated for 48 hours and showed about 83 per cent reduction in optical density, was centrifuged at 16,000 $\times g$ for 60 minutes. The supernatant was separated from insoluble material (41 mg dry weight). The supernatant and washings of the residue were combined and concentrated to about 2 ml under reduced pressure in a rotary evaporator (Miyamoto Riken Ind. Co., Osaka).

2. *Gel filtration of cell wall lysate*

The concentrated cell wall lysate thus obtained was applied to a Sephadex G-50 column (1.9 $\times$ 96 cm) connected with a Sephadex

[1] On hydrazinolysis of the cell wall digests, an unidentified compound with an Rf value of its DNP-derivative similar to that of the lysine derivative, was detected in addition of alanine and glycine. This compound constituted about 0.2 mole equivalent as lysine per mole of glutamic acid.

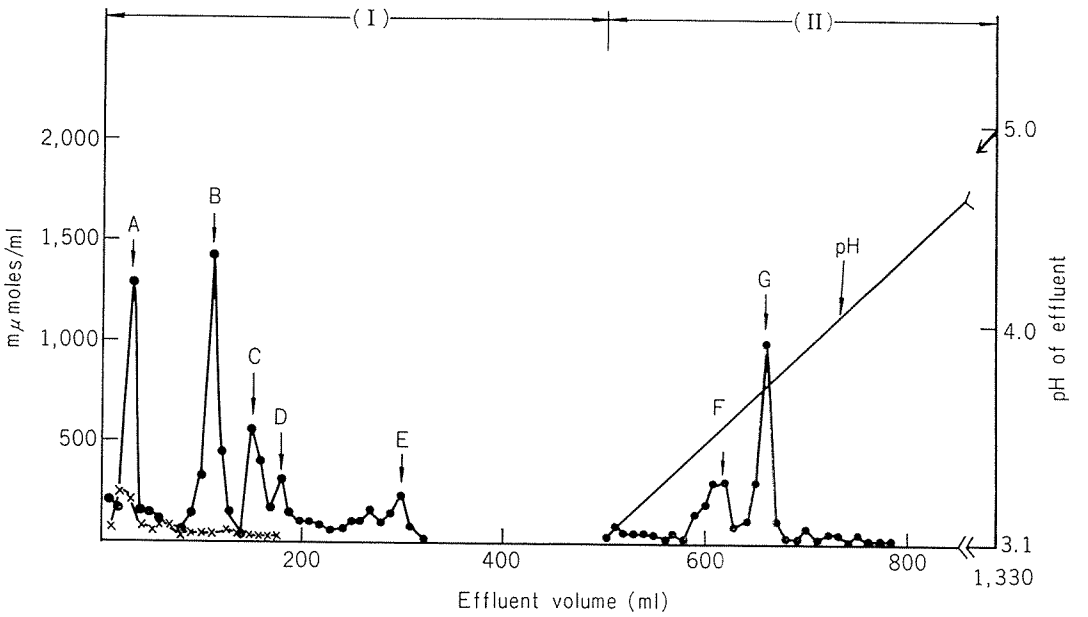

FIGURE 2 Fractionation of the lower molecular weight fraction isolated by Sephadex gel filtration of the L-11 enzyme digests of *S. aureus* cell walls on an Amberlite CG-120 column.
-●-●-: Free amino groups, -×-×-: Total hexosamines (I): 0.2 M pyridine-acetic acid buffer, pH 3.1, (II): A linear gradient of increasing pH (3.1–5.0) and concentration (0.2–2.0 M) of pyridine

G-25 column (2.5×96 cm) through a polyethylene capillary tube. The columns were developed with deionized water at a flow rate of 40 ml per hour and fractions of 10 ml were collected. Aliquots of each fraction were analyzed for free amino groups, total hexosamines and total and inorganic phosphates.

As shown in Fig. 1, two hexosamine peaks, both of which contained organic phosphates and free amino groups, were eluted near V_0 (225 ml), and these peaks were followed by a peak of free amino groups. No significant amount of hexosamines or organic phosphates was present in this third peak.

Although the higher molecular weight fractions (tubes 20–30 inclusive) were not investigated extensively in this study, they were combined and further fractionated on an ECTEOLA-cellulose column by the method of GHUYSEN, TIPPER and STROMINGER (1965). One peptide fraction was eluted with water, and the other two hexosamine-rich fractions were eluted with a linear gradient of increasing LiCl (pH 3.1) concentration from 0 to 1 M. The peptide with no organic phosphates was thought to be peptidoglycan and the other, containing organic phosphates, to be a teichoic acid-peptidoglycan complex.

The lower molecular weight fractions (tubes 37–49 inclusive) from the Sephadex columns were pooled, concentrated in a rotary evaporator and applied to an Amberlite CG-120 columns, as described in the following section.

3. *Separation of peptides on an Amberlite CG-120 column*

The cation exchange resin, Amberlite CG-120, was washed successively with 4 N HCl,

2 N NaOH, and deionized water. The column of the washed resin was then equilibrated with 0.2 M pyridine-acetic acid buffer, pH 3.1 (SCHROEDER *et al.*, 1962) by washing it with 200 ml of the buffer. To this column, a concentrated solution of the lower molecular weight fractions was applied, after acidification by addition of formic acid to a final concentration of 2 per cent. Elution was started with 500 ml of 0.2 M pyridine-acetic acid buffer, pH 3.1. This was followed by a linear gradient of increasing pH and concentration of buffer, as indicated in Fig. 2 (mixing chamber, 400 ml of the starting buffer; reservoir, 400 ml of 2.0 M pyridine–acetic acid buffer, pH 5.0).

Fractions of 10 ml were collected and aliquots were analyzed for free amino groups and total hexosamines. Five DNP-reactive peaks (Fractions A—E) were eluted with 0.2 M pyridine-acetic acid buffer, pH 3.1, and two additional DNP-reactive peaks (Fractions F and G) were obtained by elution with a linear gradient of increasing pH and concentration of buffer. Hexosamines were detected only in Fraction A.

4. *Analyses of DNP-reactive fractions separated by chromatography on Amberlite CG-120*

1) Paper electrophoresis: The electrophoretic mobilities of the fractions were examined at pH 5.0 and 1.9, as described in the method section.

At pH 5.0, Fractions B, C and D remained near the origin ($R_{Ala}=1.0$), indicating that these materials were almost uncharged at this pH. Fractions E, F and G, on the other hand, moved toward the cathode to 2.6, 2.9 and 3.2 cm from the origin, respectively. Thus these peptides were positively charged. The distances of migration of lysine and alanine on the same paper were 5.0 and 0.9 cm, respectively, toward the cathode, and that of glutamic acid was 1.7 cm toward the anode.

At pH 1.9, Fractions B—G all moved toward the cathode. The mobilities of Fractions B, C and D were almost the same as those of tetraglycine (distance of migration from the origin, 6.6 cm), triglycine (7.2 cm) and diglycine (8.0 cm), respectively.

All fractions except A gave a single spot on electrophoresis at both pH values. Fraction A, however, was not shown to be homogeneous.

2) Chemical analyses: Table 2 summarizes the results of analyses of the NH_2-terminal, COOH-terminal, and total amino acids and ammonia in the fractions. The neutral fractions, B, C and D, contained glycine only. The molar ratios of COOH-terminal glycine to total glycine indicate that Fractions B, C and D were tetra-, tri- and di-glycine, respectively. The lower values than the theoretical for NH_2-terminal glycine in Fractions B and C might be due to difficulty in determining NH_2-terminal glycine in long glycine peptides, since similar results were obtained with authentic glycine peptides.

Fractions E, F and G were all composed of alanine, glutamic acid, lysine and glycine. The molar ratios of alanine, glutamic acid and lysine were essentially the same (approximately 2: 1: 1) in these fractions, but the glycine contents differed. Fractions E, F and G contained (4.0), 2.1 and 1.1 moles of glycine per mole of glutamic acid, respectively. In all fractions one of the two moles of alanine was found to be an NH_2-terminal residue and the other to be a COOH-terminal. Glycine was also an NH_2-terminal residue in each of the fractions. Ammonia determination showed that Fractions E, F and G contained 0.65, 0.9 and 1.09 moles of ammonia per mole of glutamic acid. The presence of ammonia in these fractions seems to be consistent with the fact that the peptides migrated as basic materials on paper electrophoresis.

Fraction A, unlike the other fractions, contained hexosamine in addition to alanine, glutamic acid, lysine and glycine. End group analysis showed that this fraction contained two kinds of COOH-terminal amino acid, alanine (0.5 mole per mole of glutamic acid) and glycine (0.7 mole). This finding suggests that Fraction A was not homogeneous, as revealed also by examination by paper electro-

TABLE 2 *Analyses for total, NH_2-terminal, COOH-terminal amino acids and ammonia in peptides isolated from the L-11 enzyme lysate of S. aureus cell walls*

Peptide	Amino acid, hexosamine and ammonia							NH_2-terminal amino acids			COOH-terminal amino acids			Percentage of peptide moiety in the cell walls
	Glu	Gly	Ala	(L-Ala per cent)[1]	Lys	Hex	NH_3	Gly	Ala	(L-Ala per cent)[2]	Gly	Ala	(L-Ala per cent)[3]	
A	1.0	2.0	2.0		1.1	0.5		0.6			0.7	0.5		4.5
B		4.0						0.7			1.0			16.1
C		3.0						0.7			0.9			6.2
D		2.0						0.9			1.0			1.8
E	1.0	(4.0)[4]	1.8	(56.5)	1.0		0.65	0.4	0.7	(—)[5]		0.7	(—)[5]	5.7
F	1.0	2.1	2.0	(50.0)	1.1		0.90	0.5	0.9	(100)		0.7	(0)	4.6
G	1.0	1.1	2.1	(53.8)	1.3		1.09	0.8	1.0	(100)		0.9	(0)	6.7
													(Total)	45.6

The data are expressed as mole of total glutamic acid.
[1] L-alanine / total alanine.
[2] L-alanine / NH_2-terminal alanine.
[3] L-alanine / COOH-terminal alanine.
[4] This value may be underestimated (see the result of Edman degradation).
[5] Not determined.

phoresis.

3) Determination of the optical configuration of alanine in Fractions F and G: Assays with glutamate-pyruvate transaminase and D-amino acid oxidase have shown that about half the total alanines and essentially all the NH_2-terminal alanines were the L-isomer, while the COOH-terminal alanines were almost exclusively the D-isomer.

4) Analysis by thin layer chromatography: The neutral peptides, B, C and D were treated with fluorodinitrobenzene and the DNP-derivatives were spotted on a silica gel plate, with the DNP-derivatives of authentic specimens of glycine peptides. The plate was developed three times (for 2 hours each) with solvent 1. The DNP-derivatives of peptides B, C and D, had essentially the same mobilities as those of tetra-, tri- and di-glycine, respectively.

5) Relative amounts of isolated peptides in the cell wall peptide: The recovery of each of the isolated peptides, A to G, was calculated on the basis of analytical data described in the foregoing paragraphs. These are presented in Table 2, in terms of the percentage of the cell wall peptide. The peptide B, tetraglycine, seems to be the main component.

5. *Edman degradation of isolated peptides*

Three glycine peptides (Fractions B, C and D) and three basic peptides (Fractions E, F and G) were subjected to three to four cycles of degradation. An aliquot of the test peptide, containing about 100 mμmoles of free amino groups was dried and mixed with 40 μl of N-ethylmorpholine buffer, pH 8.6 (KONIGSBERG and HILL, 1962) and with 0.5 μl of phenylisothiocyanate. The mixture was incubated for 2.5 hours at 37°C, diluted with 40 μl of deionized water, and then extracted three times with 150 μl of ethylether. The residual water phase was dried *in vacuo* over H_2SO_4, redissolved in 80 μl of 4 N HCl and kept at room temperature for 6 hours. The 2-anilino-5-thiazolinone derivative of the NH_2-terminal amino acid was removed by repeated extraction with 150 μl volumes of ethylacetate. The residual water phase was dried, redissolved in an appropriate amount of deionized water and used for determination of NH_2-terminal

TABLE 3 *Results of Edman degradation analysis of peptides isolated from the L-11 enzyme lysate of S. aureus cell walls*

Peptide	Step	Amino acid composition mμmoles				NH_2-terminal amino acid mμmoles				
		Glu	Gly	Ala	Lys	Glu	Gly	Ala	Lys	ε·NH_2-Lys
B	0		251.0				66.7			
	1		156.4				60.9			
	2		114.6				64.8			
	3		51.3				62.9			
	4		2.6				0			
C	0		169.0				70.2			
	1		110.4				77.7			
	2		58.1				72.5			
	3		7.8				0			
D	0		77.6				51.8			
	1		46.6				52.2			
	2		4.8				0			
E*	0	27.8	102.2	40.9	26.4		7.7	13.2		
	1	30.4	97.7	27.6	28.9	6.9	7.7			
	2		68.9	28.5	22.6		8.6			
	3		46.7	28.6	23.6		8.6			
	4		28.9	23.8	18.0		8.0			
F	0	32.0	61.0	59.0	33.0		23.0	26.0		
	1	29.0	30.0	31.0	32.0	30.0	26.0			
	2			27.0			4.0			16.9
	3			8.0						
G	0	24.0	23.0	44.0	27.0		22.0	23.0		
	1	33.0		26.0		20.0				16.4
	2			7.0						
	3			4.0						

* The reason for the low recovery of NH_2-terminal amino acids in peptide E is not known at present.

residue and of amino acids.

The results of Edman degradation are summarized in Table 3. In the glycine peptides (Fractions B, C and D), all the new NH_2-terminal residues which appeared on repeated cycles of degradation were glycine. The appearance of new NH_2-terminal glycine in Fractions B, C and D ceased after the third, second and first cycles of degradation, respectively. These findings are in good agreement with those of analyses given in the foregoing sections, and confirm the conclusion that the peptides in Fractions B, C and D are tetra-, tri- and di-glycine, respectively.

In peptide F with two moles of glycine per mole of glutamic acid, after the first cycle of degradation, the NH_2-terminal alanine and glycine were replaced by glycine and glutamic acid. In parallel with the changes in NH_2-terminal residues, one mole each of the constituent alanine and glycine disappeared per mole of glutamic acid. After the second cycle of reaction with phenylisothiocyanate, approximately equal amounts of NH_2-terminal glutamic acid and glycine were liberated and ε-NH_2-lysine appeared. No amino acids were

recovered after the third cycle of degradation. After the first cycle of reaction of peptide G with phenylisothiocyanate, NH_2-terminal alanine and glycine were replaced by NH_2-terminal glutamic acid and ε-NH_2-lysine in approximately equivalent amounts. Analysis indicated that in peptide E too, L-alanine is the first amino acid in the peptide sequence, glutamic acid is the second, and the ε-amino group of lysine is substituted by a glycine peptide. The fact that even after the fourth cycle of degradation, the glycine residue was present as a constituent and NH_2-terminal amino acid, strongly suggests that the glycine peptide which substitutes the ε-amino group of lysine is pentaglycine. This deduction, however, seems inconsistent with the results of analysis for amino acid composition, indicating the presence of only four moles of the glycine residues per mole of glutamic acid in peptide E. A plausible explanation for this discrepancy would be that the glycine content was underestimated because all the constituent amino acids were estimated on the same quantity of test specimen in spite of a disproportionately high content of the glycine residues. A small amount of the material available made it impossible to re-examine its amino acid composition, using different quantities of the specimen appropriate to estimate the contents of glycine and other amino acids, respectively.

Fig. 3 shows the probable structures for peptides F, G and E, proposed on the basis of analytical data obtained in this study.

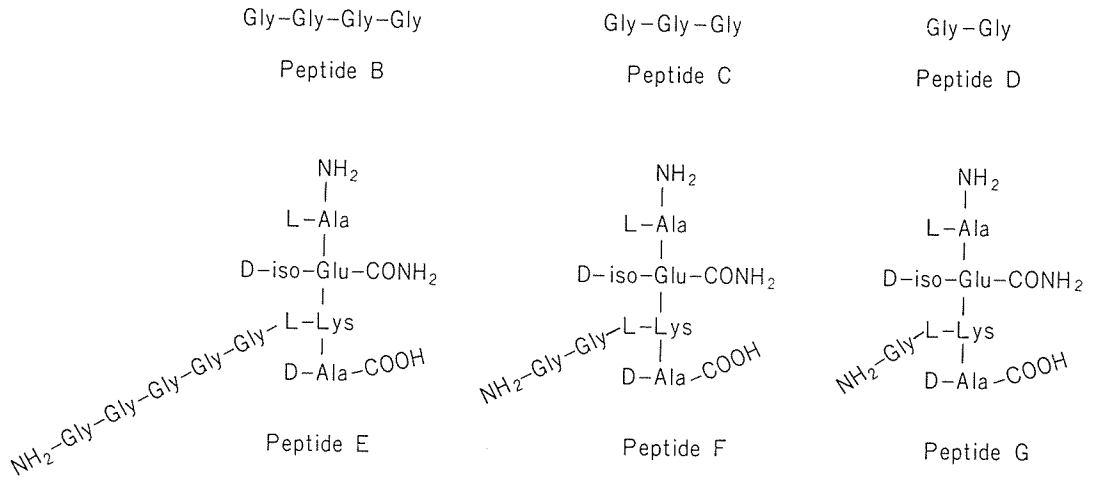

FIGURE 3 Proposed structures of peptides isolated from L-11 enzyme digests of *S. aureus* cell walls.

DISCUSSION

As summarized by GHUYSEN, TIPPER and STROMINGER (1966) and STROMINGER and GHUYSEN (1967), there are several staphylolytic enzymes, some of which are glycosidase type while others are amidase and/or peptidase type. Kinetic analyses of NH_2-terminal and COOH-terminal amino acids liberated from *S. aureus* cell walls by *Flavobacterium* L-11 have indicated that this enzyme belongs to the latter type and that the linkages attacked are acetylmuramyl-L-alanine, D-alanyl-glycine and glycyl-glycine. This has been confirmed by characterization of the products of hydrolysis of *S. aureus* cell walls by L-11 enzyme, as

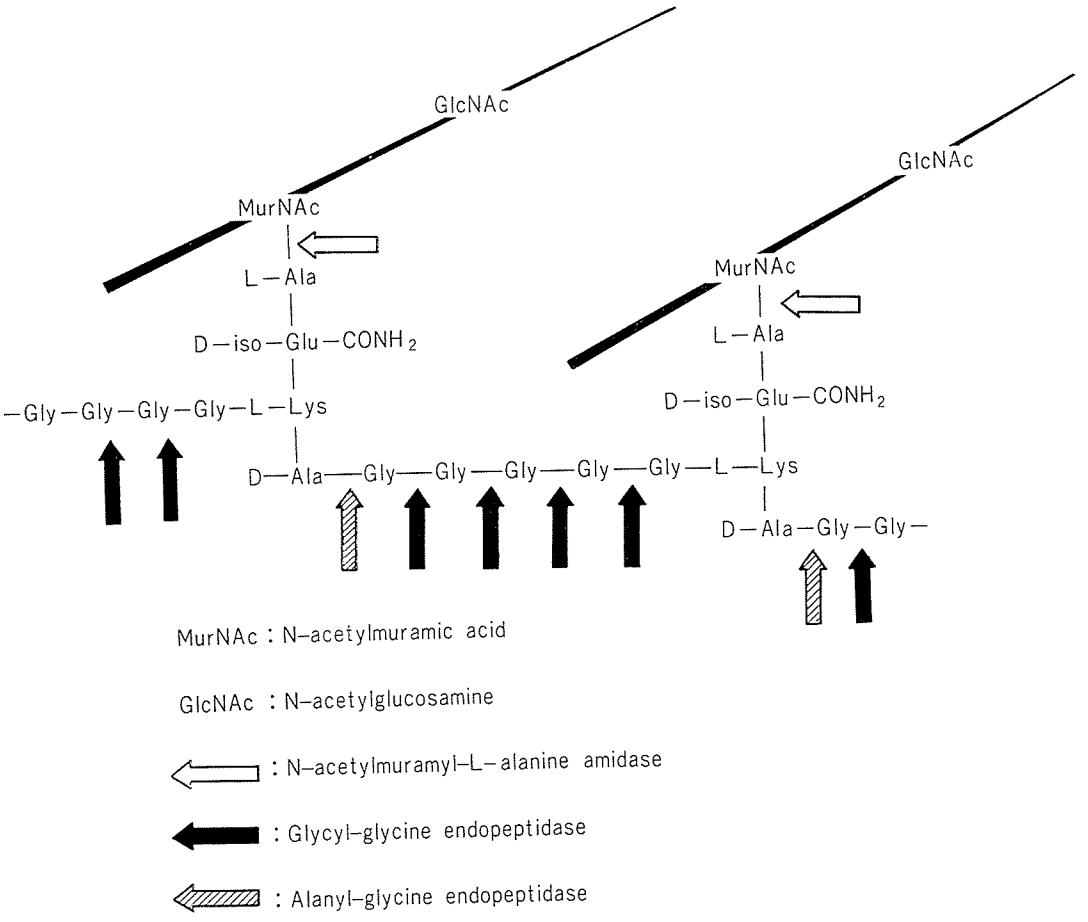

FIGURE 4 Proposed points of attack of L-11 enzyme on the peptidoglycan of *S. aureus* cell walls on the basis of the structure presented by TIPPER, STROMINGER and ENSIGN (1967).

presented in this paper.

Of the other staphylolytic peptidases hitherto reported, the enzyme from *Myxobacterium* acts in essentially the same manner to the L-11 enzyme (ENSIGN and WOLFE, 1966; TIPPER, STROMINGER and ENSIGN, 1967; JARVIS and STROMINGER, 1967), and the SA-endopeptidase from *Streptomyces albus* G also splits the D-alanyl-glycine linkage (PETIT, MUÑOZ and GHUYSEN, 1966; GHUYSEN *et al.*, 1965a, b, 1966). On the other hand, the peptidases of lysostaphin (BROWDER *et al.*, 1965; TIPPER *et al.*, 1967) and of ALE from *S. epidermidis* (SUGINAKA *et al.*, 1967, 1968) hydrolyze glycyl-glycine links in pentaglycine cross-bridges, but do not attack the D-alanyl-glycine linkage. All seven peptides and one sugar-peptide isolated from the ALE digests of *S. aureus* have glycine as NH_2-terminal and COOH-terminal residues, but have no COOH-alanine, unlike the peptides isolated from the cell wall digests by the L-11 enzyme.

The acetylmuramyl-L-alanine amidase activity of the particular specimen of L-11

enzyme used in this study was rather weak, as illustrated by liberation of only 0.1 mole of NH_2-terminal alanine per mole of glutamic acid. It should be mentioned here that in a previous study approximately 0.4 mole of NH_2-terminal L-alanine was liberated during digestion of *S. aureus* walls with another specimen of L-11 enzyme. According to the descriptions of GHUYSEN, TIPPER and STROMINGER (1966) and STROMINGER and GHUYSEN (1967), the hydrolysis of the acetylmuramyl-L-alanine linkage is slow and is not complete until long after solubilization of cell walls by the action of other enzymes, such as bridge splitting enzymes. The possibility, therefore, can not be excluded that much of the acetylmuramyl-L-alanine amidase is lost during purification procedures, when the enzyme activity of the products at each step was checked only by determining the activity producing cell wall lysis, and also that the acetylmuramyl-L-alanine amidase activity varies from one enzyme specimen to another.

Recent studies have indicated that the conditions of cultivation of *Flavobacterium sp.* strongly influences the production of acetylmuramyl-L-alanine amidase. An enzyme preparation obtained from culture supernatants of *Flavobacterium sp.* grown in a rich medium in a tank with ample air has been shown to hydrolyze the same cell wall specimen as that used in the present study, liberating NH_2-terminal alanine (0.78 mole per mole of glutamic acid) and glycine (1.43 mole), and COOH-terminal alanine (0.89 mole) and glycine (0.78 mole). Thus, it has been demonstrated that the specimen of cell walls used in this study contains substrate for acetylmuramyl-L-alanine amidase, and the production of this enzyme seems to be greatly influenced by culture conditions.

In an attempt to separate the entities responsible for the actions of acetylmuramyl-L-alanine amidase and D-alanyl-glycine and glycyl-glycine endopeptidases from the crude enzyme preparation, one preparation was obtained which was only active toward pentaglycine cross-bridges at both D-alanyl-glycine and glycyl-glycine linkages, but had no amidase activity. A kinetic study of the lysis of *S. aureus* walls by this enzyme specimen, named L-11 endopeptidase, showed the liberation of 1.00 mole of NH_2-terminal glycine per mole of glutamic acid, along with the release of 0.59 and 0.45 mole of COOH-terminal glycine and alanine, respectively. Details of this study will be published elsewhere.

The probable structures of six peptides isolated from the cell wall digest by the L-11 enzyme are shown in Fig. 3. From the structure of a peptide moiety of the cell wall peptidoglycan of *S. aureus* strain Copenhagen proposed by STROMINGER, GHUYSEN and their coworkers (for instance, TIPPER, STROMINGER and ENSIGN, 1967), the points of attack of the L-11 enzyme on *S. aureus* walls should be as illustrated in Fig. 4. An attempt has also been made to infer at what linkages the pentaglycine cross-bridge is cleaved by the L-11 enzyme on the basis of the relative proportions of isolated peptides and sugar-peptide, but no conclusion has yet been reached.

REFERENCES

BROWDER, H. P., ZYGMUNT, W. A., YOUNG, J. R. and TAVORMINA, P. A. (1965). Lysostaphin: Enzymatic mode of action. *Biochem. Biophys. Res. Commun.* **19**, 383–389.

ENSIGN, J. C. and WOLFE, R. S. (1966). Characterization of a small proteolytic enzyme which lyses bacterial cell walls. *J. Bacteriol.* **91**, 524–534.

FAWCETT, J. K. and SCOTT, J. E. (1960). A rapid and precise method for determination of urea. *J. Clin. Pathol.* **13**, 156–159.

GHUYSEN, J.-M., TIPPER, D. J. and STROMINGER, J. L. (1965 a). Structure of the cell wall of *Staphylococcus aureus*, strain Copenhagen. IV. The teichoic acid-glycopeptide complex. *Biochemistry* **4**, 474–485.

GHUYSEN, J.-M., TIPPER, D. J., BIRGE, C. H. and STROMINGER, J. L. (1965 b). Structure of the cell wall of *Staphylococcus aureus*, strain Copen-

hagen. VI. The soluble glycopeptide and its sequential degradation by peptidases. *Biochemistry* **4**, 2245–2254.

GHUYSEN, J.-M., PETIT, J.-F., MUÑOZ, E. and KATO, K. (1966). Peptide crosslinks in bacterial cell walls studied with bacteriolytic enzymes. *Federation Proc.* **25**, 410.

GHUYSEN, J.-M., TIPPER, D. J. and STROMINGER, J. L. (1966). Enzymes that degrade bacterial cell walls. In *Methods in Enzymology* (edited by NEUFELD, E. F. and GINSBURG, V.), Vol. VIII: Complex Carbohydrates, pp. 685–699. Academic Press, New York.

JARVIS, D. and STROMINGER, J. L. (1967). Structure of the cell wall of *Staphylococcus aureus*. VIII. Structure and chemical synthesis of the basic peptides released by the *Myxobacterium* enzyme. *Biochemistry* **6**, 2591–2598.

KATO, K., KOTANI, S., MATSUBARA, T., KOGAMI, J., HASHIMOTO, S., CHIMORI, M. and KAZEKAWA, I. (1962). Lysis of *Staphylococcus aureus* cell walls by a lytic enzyme purified from culture supernatants of *Flavobacterium species*. *Biken J.* **5**, 155–179.

KATO, K. and STROMINGER, J. L. (1968). Structure of the cell wall of *Staphylococcus aureus*. IX. Mechanism of hydrolysis by the L-11 enzyme. *Biochemistry* (in press).

KONIGSBERG, W. and HILL, R. J. (1962). The structure of human hemoglobin. III. The sequence of amino acids in the tryptic peptides of the α-chain. *J. Biol. Chem.* **237**, 2547–2561.

LOWRY, O. H., ROBERTS, N. R., LEINER, K. Y., WU, M.-L. and FARR, A. L. (1954). The quantitative histochemistry of brain. I. Chemical methods. *J. Biol. Chem.* **207**, 1–17.

PARK, J. T. and JOHNSON, M. J. (1949). A submicrodetermination of glucose. *J. Biol. Chem.* **181**, 149–151.

PETIT, J.-F., MUÑOZ, E. and GHUYSEN, J.-M. (1966). Peptide cross-links in bacterial cell wall peptidoglycans studied with specific endopeptidase from *Streptomyces albus* G. *Biochemistry* **5**, 2764–2776.

SCHROEDER, W. A., JONES, R. T., CORMICK, J. and MCCALLA, K. (1962). Chromatographic separation on ion exchange resins. *Anal. Chem.* **34**, 1570–1575.

STROMINGER, J. L. and GHUYSEN, J.-M. (1966). Mechanisms of enzymatic bacteriolysis. *Science* **156**, 213–221.

SUGINAKA, H., KASHIBA, S., AMANO, T., KOTANI, S. and IMANISHI, T. (1967). Purification and properties of a staphylolytic factor produced by a strain of *Staphylococcus epidermidis*. *Biken J.* **10**, 109–120.

SUGINAKA, H., KOTANI, S., KATO, K., KASHIBA, S. and AMANO, T. (1968). Action of a staphylolytic enzyme (ALE) produced by a strain of *Staphylococcus epidermidis*. *Biken J.* **11** ,15–26.

TIPPER, D. J., KATZ, W., STROMINGER, J. L. and GHUYSEN, J.-M. (1967). Substituents on the α-carboxyl group of D-glutamic acid in peptidoglycan of several bacterial cell walls. (1967). *Biochemistry* **6**, 921–929.

TIPPER, D. J., STROMINGER, J. L. and ENSIGN, J. C. (1967). Structure of the cell wall of *Staphylococcus aureus*, strain Copenhagen. VII. Mode of action of the bacteriolytic peptidase from *Myxobacter* and the isolation of intact cell] wall polysaccharides. *Biochemistry* **6**, 906–920.