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ACTION OF A STAPHYLOLYTIC ENZYME (ALE) OF A STRAIN OF *STAPHYLOCOCCUS EPIDERMIDIS*[1]

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SUMMARY The mode of action of a staphylolytic enzyme, named ALE, isolated from culture filtrates of *Staphylococcus epidermidis*, strain EP-Kl has been investigated. Kinetic analyses of NH_2-terminal and COOH-terminal amino acids liberated by digestion of isolated cell walls from *Staphylococcus aureus*, strain Copenhagen with ALE showed that approximately 1 mole of NH_2-terminal L-alanine and equimolecular amounts (0.7 mole each) of NH_2-terminal and COOH-terminal glycine per mole of glutamic acid in the walls were released during enzymatic hydrolysis of the walls. Seven peptides, the structures of which have been found to be N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ε}-$(glycyl)_{0-4}$-L-lysyl-D-alanyl-$(glycine)_{1-3}$, respectively, were isolated by Amberlite CG-120 column chromatography from a small molecular weight fraction separated by gel filtration after digestion of the cell walls by ALE. A nonapeptide with the structure N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ε}-$(glycyl)_3$-L-lysyl-D-alanyl-$(glycine)_2$ was found to be the main component, constituting nearly 40 per cent to the total peptides in the cell walls.

It was concluded from these results that ALE exerts its lytic action on *S. aureus* cell walls through the activities of its N-acetylmuramyl-L-alanine amidase and glycylglycine endopeptidase.

INTRODUCTION

In the study described in a previous paper (SUGINAKA *et al.*, 1967), a strain of *Staphylococcus epidermidis* named EP-K1, was isolated which produced in culture supernatants a powerful lytic factor active against the cell walls from *Staphylococcus aureus* and *Micro-*

1 Parts of this work were read at the 40th Annual Meeting of the Japan Bacteriological Society at Nagoya (March 30-31, 1967), and at the 141st Monthly Meeting of Members of the Research Institute for Microbial Diseases, Osaka University (June 27, 1967).

coccus lysodeikticus. The active principle in culture supernatants of the EP-K1 strain grown in Trypticase Soy Broth with aeration was highly purified by precipitation with ammonium sulfate and acetone, and by DEAE-cellulose column fractionation. The properties of the purified, active principle (ALE) were investigated, and it was shown that ALE is an enzyme (or enzymes) which exerts its lytic action against susceptible cell walls through its amidase and/or endopeptidase activity.

The present paper reports further studies on the action of ALE on *S. aureus* cell walls.

MATERIALS AND METHODS

1. *Cell wall preparation of S. aureus*

A cell wall preparation was obtained by disruption of the cells of *S. aureus*, strain Copenhagen grown with aeration in nutrient broth supplemented with 0.5 per cent glucose for 18 hours at 37°C. Cells were disrupted in a Braun Mechanical Cell Homogenizer (Model MSK, B. Braun Apparatebau, Melsungen, West Germany) as described in a separate report (KATO *et al.*, 1968a).

2. *Preparation of the staphylolytic enzyme, ALE*

The enzyme preparation used in this work was concentrated and purified from culture supernatants of *S. epidermidis*, strain EP-K1. The lytic activity of the enzyme preparation was measured by the reduction in optical density of a suspension of isolated cell walls of *S. aureus*, strain Copenhagen under standard conditions. Details of the method of purification of ALE and of measurement of lyitc activity were described previously (SUGINAKA *et al.*, 1967).

3. *Analytical procedures*

For estimation of hexosamines, free amino groups, and total, NH_2-terminal and COOH-terminal amino acids, the methods of GHUYSEN, TIPPER and STROMINGER (1966) were followed with minor modifications. Hydrazinolysis was carried out by incubation of dried test specimens with redistilled, anhydrous hydrazine for 6 hours at 100°C instead for 16 hours at 60°C as in the original report. The optical configuration of alanine was also determined enzymatically following the method of GHUYSEN, TIPPER and STROMINGER (1966), using glutamate-pyruvate transaminase (C.F. Boehringer and Soehne GmbH, Mannheim, West Germany) and D-amino acid oxidase prepared from pig kidney by the method of MASSEY, PALMER and BENNETT (1961). Ammonia was estimated by a micromodification of the method of FAWCETT and SCOTT (1960) with isoglutamine as standard, and total and inorganic phosphates by the method of LOWRY *et al.* (1954) on a micro scale. Color densities in these determinations were measured in the Hitachi Perkin-Elmer Spectrophotometer (Model 139 UV-VIS, Hitachi Ltd., Tokyo) at the indicated wave length, using microcells with a light path of 1 cm and capacity of 400 μl.

4. *Edman degradation procedure*

Specimens containing approximately 150 mμmoles of peptide were dried *in vacuo* over H_2SO_4 and redissolved in 40 μl of N-ethylmorpholine buffer of KONIGSBERG and HILL (1962). One μl of phenylisothiocyanate (Tokyo Kasei Organic Chemicals, Tokyo) was added. The mixture was incubated for 2.5 hours at 37°C with stirring and then mixed with 40 μl of water. Excess reagent was extracted with ether (3×200 μl). The water phase, containing the PTC-derivative of the peptide, was dried over H_2SO_4 and the residue was dissolved in 80 μl of 4 N HCl. After standing for 6 hours at room temperature to allow cyclization, the 3-phenyl-2-thiohydantoin derivative(s) of the NH_2-terminal amino acid(s) was removed by extraction with ethylacetate (3×200 μl). The water phase was dried over H_2SO_4. The residue dissolved in an appropriate amount of water was then analyzed for total and NH_2-terminal amino acids with 2,4-dinitrofluorobenzene.

RESULTS

1. *Determination of NH_2-terminal and COOH-terminal amino acids liberated from S. aureus cell walls during lysis with ALE*

Cell walls (420 mg) of *S. aureus* which had been treated in 0.01 M pyrophosphate buffer, pH 9.0, at 40°C for 4 hours, and then thoroughly washed with water, to remove the ester-linked D-alanine of their teichoic acid[1], were mixed under cooling in ice-water with ALE

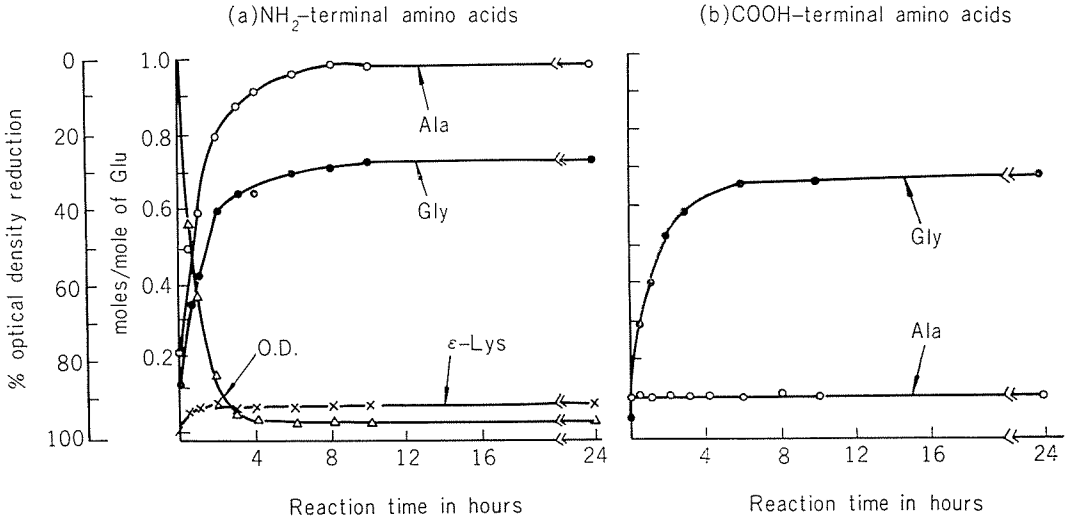

FIGURE 1 NH_2-terminal and COOH-terminal amino acids liberated from *S. aureus* cell walls by ALE.

(210 cell wall lytic units) in a total volume of 84 ml of 0.0125 M phosphate buffer containing 0.1 M NaCl, pH 6.8. After removal of an aliquot of 500 μl as a 0 time specimen, the reaction mixture was incubated with constant mixing by magnetic stirrer, at 37°C for 24 hours. Aliquots of 500 μl of the reaction mixture were removed at intervals for determination of optical density and liberated NH_2-terminal and COOH-terminal amino acids. All specimens were heated immediately after withdrawal at 100°C for two minutes to stop the enzymic activity of ALE.

As shown in Fig. 1 (a), NH_2-terminal alanine and glycine were released during lysis of the cell walls (as expressed in terms of per cent optical density reduction) and reached a maximum level equivalent to 1.0 and 0.7 mole/mole of total glutamic acid in the walls, respectively. No significant release of the ε-NH_2 groups of lysine occurred. Fig. 1(b) shows that the increase in NH_2-terminal alanine and glycine described above was accompanied by liberation of COOH-terminal glycine, in parallel to release of NH_2-terminal glycine, amounting to a maximum of 0.7 mole/mole of glutamic acid.

2. *Configuration of the NH_2-terminal alanine liberated by the action of ALE*

An aliquot of the cell wall digest taken after 24 hours incubation, was divided into two parts. One was treated with 2,4-dinitrofluorobenzen and the other was left untreated. Both the untreated and dinitrophenylated digests were then hydrolyzed in 4 N HCl at 100°C for 8 hours, and then their L- and D-alanine contents were measured. It can be seen from Table 1 that there was no measurable difference in D-alanine content between the cell wall digests hydrolyzed with and without prior dinitrophenylation, while the L-alanine susceptible to glutamate-pyruvate transaminase disappeared when the ALE digests were dinitrophenylated prior to hydrolysis. These results show that the NH_2-terminal alanine liberated by digestion of the walls with ALE was almost exclusively L-alanine.

[1] The preparation of alkali-treated cell walls used in the present study was shown to have the following amino acid composition (mμmoles/mg): glutamic acid 450, alanine 900 (L-form 425, D-form 475), lysine 570 and glycine 2220. The ammonia content was 590 mμmoles/mg.

TABLE 1 *Analysis of optical configuration of NH_2-terminal alanine liberated from the cell walls of S. aureus by ALE*

Test material	Contents in ALE digests	
	L-alanine*1	D-alanine*2
	mole/mole of glutamic acid	
Hydrolyzed without prior dinitrophenylation	0.98	1.06
Hydrolyzed after dinitrophenylation	trace	0.96

*1 Susceptible to glutamate-pyruvate transaminase.
*2 Susceptible to D-amino acid oxidase.

3. *Isolation of a fraction containing small peptides from the cell wall digests*

1) Fractionation of the ALE digests by gel filtration

The main part of the cell wall digest after 24 hours incubation in the experiment mentioned above (74 ml, equivalent to 370 mg walls) was centrifuged at $10,000 \times g$ for 40 minutes. The supernatant was separated from a small amount of insoluble residue (8.9 mg dry weight) which was washed once with 20 ml of water. The supernatant and washing water were combined and reduced in volume to about 2 ml in a rotary evaporator (Miyamoto Riken, Tokyo) at 40°C.

The concentrated solution was applied to a column (4.9 $cm^2 \times 95$ cm) of Sephadex G-50 (bead form, coarse 100–300 μ, Pharmacia, Uppsala, Sweden) connected in series with Sephadex G-25 (bead form, coarse 100–300 μ) column (3.1 $cm^2 \times 92$ cm). The columns were then developed with water at a flow rate of 50 ml/hour. Fractions of 10 ml were collected. They were analyzed for free amino groups, total hexosamines (measured on specimens after hydrolysis in 3 N HCl at 100°C for 4 hours) and total and inorganic phosphates.

The results of the analyses are presented in Fig. 2. Almost all the material containing hexosamines and organic phosphates with a small amount of free amino groups was eluted in the fractions from 230 ml to 360 ml (the void volume in these columns, measured by exclusion of blue dextran-2000, Pharmacia, Uppsala, Sweden, was 230 ml). This first peak was followed by a second peak with free amino groups, but containing very little hexosamines and organic phosphates. The third peak with only inorganic phosphates was derived from the phosphate buffer used in the reaction mixture for digestion of cell walls.

The fractions from 370 ml to 540 ml, corresponding to the second peak, were pooled and reduced in volume in a rotary evaporator. The concentrated solution (a peptide fraction) was then desalted by filtration through a column (4.9 $cm^2 \times 45$ cm) of Bio-Gel P-2 (200–400 mesh, BIO·RAD Laboratories, Richmond, Calif., U.S.A.) and again concentrated to about 1 ml in an evaporator. Fractions of the first peak from 230 ml to 360 ml of the effluent were pooled and lyophilized. This fraction, which was thought to contain the glycan and teichoic acid portions of the cell walls, was not investigated further.

2) Fractionation of the peptide fraction on Amberlite CG-120

The peptide fraction separated by gel filtration was further fractionated by chromatography on a column (0.6 $cm^2 \times 33$ cm) of Amberlite CG-120 (Rhom and Haas, Pennsylvania, Pa., U.S.A.) which was repeatedly washed alternately with 2 N NaOH and 4 N HCl, then treated with 2.0 M pyridinium-acetic acid, pH 5.0, and finally equilibrated with 0.2 M pyridinium-acetic acid, pH 3.1. The desalted concentrated solution was applied to the column as described above, after adjusting the pH to 3.1 by addition of formic acid. Elution was carried out first with 500 ml of 0.2 M pyridin-

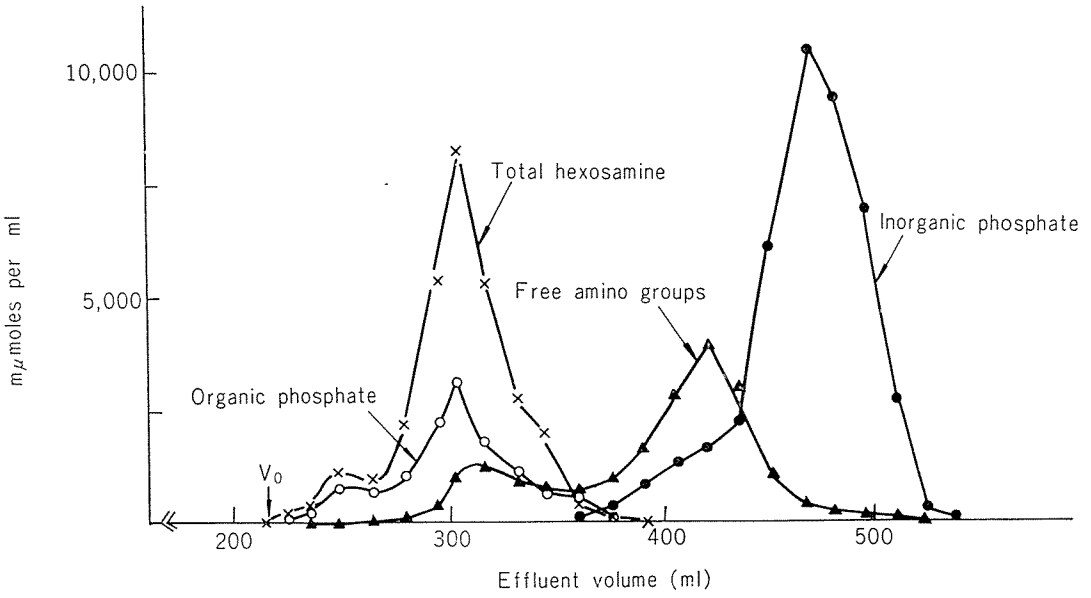

FIGURE 2 Gel filtration of *S. aureus* cell wall digest on columns of Sephadex G-50 and G-25 connected in series.

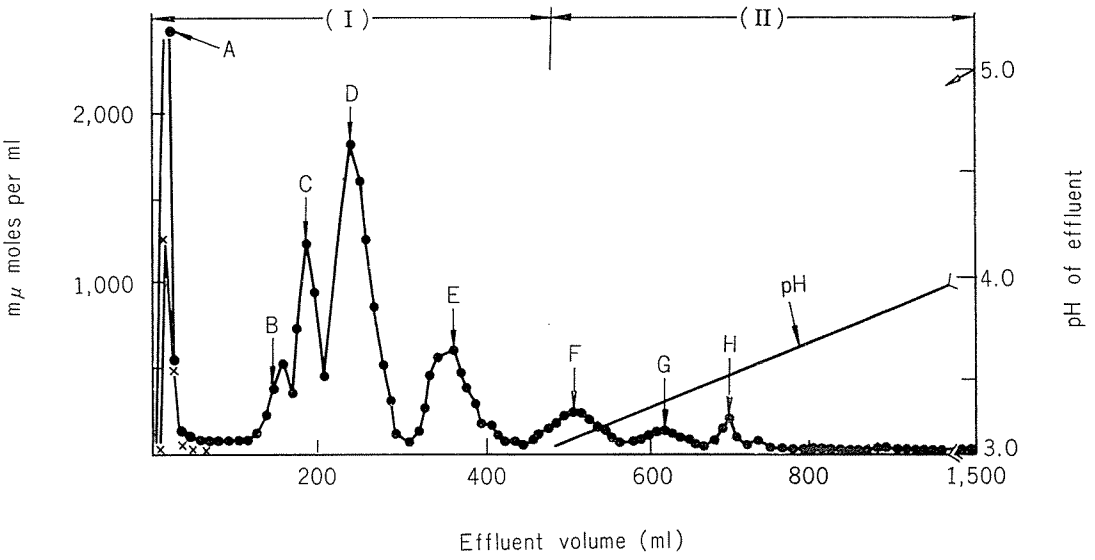

Figure 3 Fractionation of the low molecular weight fraction separated by gel filtration on Amberlite CG-120.
●——●: Free amino groups; ×——×: Total hexosamines.
(I): 0.2 M pyridinium-acetic acid buffer, pH 3.1;
(II): Linear gradient of increasing pH (3.1–5.0) and concentration (0.2–2.0 M) of buffer.

ium-acetic acid, pH 3.1, and then with a linear gradient between 0.2 M pyridinium-acetic acid, pH 3.1, and 2.0 M pyridinium-acetic acid, pH 5.0 (500 ml, each), at a flow rate of 50 ml/hour adjusted by a micro meter pump (Yanagimoto Co., Kyoto), as indicated in Fig. 3. The temperature during elution was maintained by circulating hot water at 50°C through a jacket around the column fitted with an ULTRA Thermostat Original Lauda (Type NB-D8/17, Messgeräte-Werk Lauda, West Germany). Fractions of 10 ml were collected and aliquots of these were analyzed for free amino groups and total hexosamines.

As illustrated in Fig. 3, five peaks (A, B, C, D and E) with free amino groups were eluted with 0.2 M buffer and three further peaks (F, G and H) with free amino groups were separated by elution with a linear gradient of increasing pyridinium-acetic acid concentration and increasing pH. Of these eight peaks, only A was shown to contain hexosamines.

4. *Characterization of peak fractions A to H*

1) Examination by paper electrophoresis

To determine the homogeneities and electrical charges of the peak fractions, their migration on paper elctrophoresis was examined at pH 5.0 (0.1 M pyridinium-acetic acid) and at pH 1.9 (80 per cent formic acid-acetic acid-water, 5: 15: 80). After electrophoresis at 14 volts/cm for 2 hours, spots were located by spraying the paper with 0.1 per cent ninhydrin in ethyl alcohol and then heating the paper at 60°C for 10 minutes.

Peak A was a mixture of two components of different electrophoretic mobilities, but the other seven peak fractions all migrated as single spots towards the cathode at both test pH values, as illustrated in Fig. 4.

2) Chemical composition

Quantitative chemical analyses showed that fractions B to H were peptides all consisting of alanine, glutamic acid, lysine and glycine in the molar ratio of 2: 1: 1: 7–1, respectively, and that all of them contained one mole of

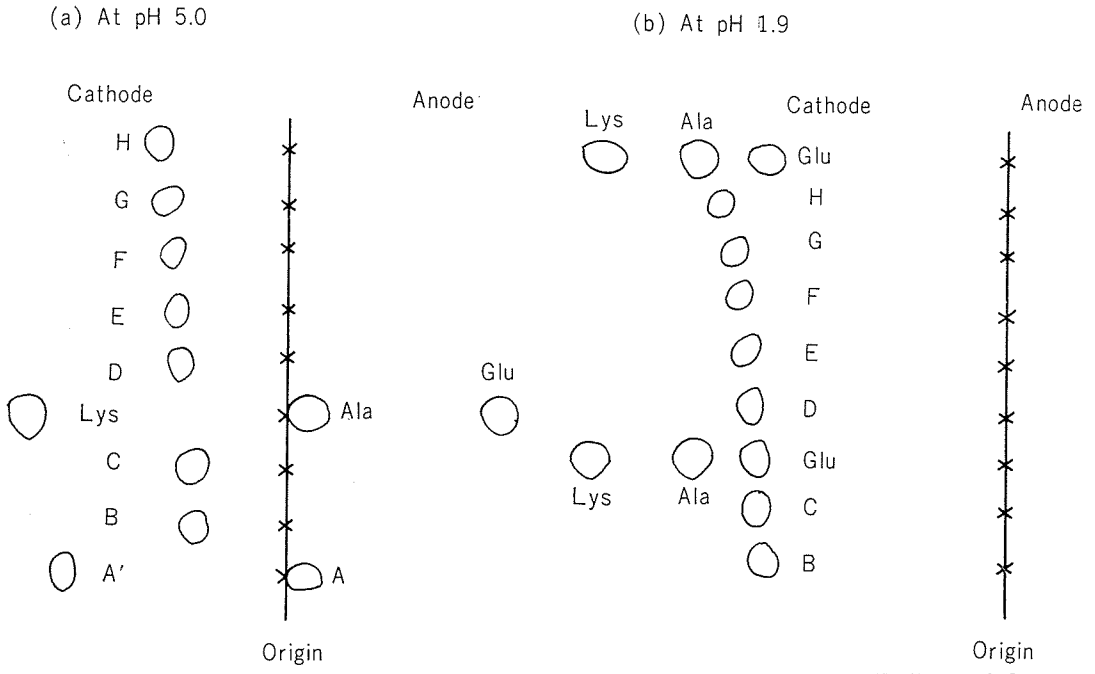

FIGURE 4 Electrophoretic pattern of peptides and sugar-peptide isolated from the ALE digest of *S. aureus* cell walls.

TABLE 2 *Summary of quantitative analyses of peptides and sugar-peptide isolated from ALE digest of S. aureus cell walls*

Fraction	Amino acids, hexosamine and ammonia							NH_2-terminal amino acids				COOH-terminal amino acids	Proportion (per cent) to cell wall peptides
	Glu	Gly	Ala	(L-isomer[1], D-isomer[2])	Lys	NH_3	HexN[3]	Gly	Ala	(L-isomer[1], D-isomer[2])	ε-Lys	Gly	
A	1.0	5.0	2.1	(1.0, 1.0)	1.2	0.9	2.1	0.6	—	(— , -)	—	0.9	5.4
B	1.0	7.1	2.1	(1.1, 1.0)	1.1	1.2	—	0.7	1.1	(1.1, 0)	—	1.2	5.6
C	1.0	6.1	2.1	(0.9, 1.0)	1.0	0.7	—	0.6	0.8	(0.7, 0)	—	1.2	19.0
D	1.0	4.9	2.1	(1.2, 0.8)	1.0	0.9	—	0.6	0.9	(1.1, 0)	—	1.1	38.0
E	1.0	4.0	2.0	(1.0, 1.0)	1.1	1.0	—	0.7	1.1	(0.8, 0)	—	1.1	14.0
F	1.0	3.0	2.1	(0.9, 1.0)	1.2	1.0	—	0.6	0.9	(0.7, 0)	—	1.0	5.4
G	1.0	2.1	2.4	(1.1, 1.1)	1.0	1.0	—	0.5	1.2	(1.2, 0)	—	1.2	1.9
H	1.0	0.9	2.2	(1.0, 1.0)	0.9	1.0	—	—	0.8	(0.8, 0)	1.0	0.9	0.9

The data are expressed as mole per mole of total glutamic acid. (Total) 90.2

1 Assayed with glutamate-pyruvate transaminase.

2 Assayed with D-amino acid oxidase.

3 Hexosamine.

In peptides containing more than three moles of the glycine residues per mole of glutamic acid, different and appropriate quantities of test specimens were used in determination of the content of glycine and those of other amino acids, respectively.

ammonia per mole of glutamic acid (Table 2). It was also shown by enzymatic assay that one of the two moles of alanine was the L-form and the other was D-alanine. In each of these peptides, one mole of alanine/mole of glutamic acid was NH_2-terminal, and these NH_2-terminal alanines were shown to be exclusively the L-form, by analysis similar to that of the ALE digests shown in Table 1. All the peptides, except H, had an additional NH_2-terminal group of glycine. However, the molar ratio of NH_2-terminal glycine to glutamic acid was less than one. These low values might be due to difficulty in determining NH_2-terminal glycine linked to the ε-amino groups of lysine. The peptide H with no NH_2-terminal glycine was found to contain lysine, the ε-amino groups of which were free. As regards COOH-terminal amino acids, it was shown by hydrazinolysis that all peptides had about one mole of COOH-terminal glycine/mole of glutamic acid. Although fraction A was not electrophoretically homogeneous, it gave an analytical result suggesting that the main component was a disaccharide-nonapeptide.

The relative amounts of the seven peptides (B to H) and one sugar-peptide (A) to total peptides in the whole walls were calculated from the analytical figures. Although it should be noted that this calculation was not very accurate because of inherent errors, it was found that the nonapeptide, peptide D, containing 5 moles of glycine/mole of glutamic acid was the main component, and that about 90 per cent of the wall peptides were recovered in the Amberlite CG-120 fraction.

3) Edman degradation of peptides B to H

To analyze the structure of the NH_2-terminal portion of peptides B to H, and to determine the distribution of glycine in the molecular structure of peptides containing more than 2 moles of glycine/mole of glutamic acid, the peptides were subjected to Edman degradation. The results are summarized in Table 3.

Although accurate quantitative results were not obtained, partly because of the limited

TABLE 3 *Analyses by Edman degradation*

Peptide	Cycle of degradation	NH_2-terminal amino acids mμmoles/appropriate amount of material				Amino acid composition			
		Glu	Gly	Ala	ε-Lys	Glu	Gly	Ala	Lys
H	0	—	—	22	12	19	15	31	14
	1	16	—	—	—	9	7	10	—
	2	—	—	—	—	—	8	14	—
G	0	—	21	34	—	32	56	68	26
	1	27	—	—	12	32	42	38	26
	2	—	—	—	—	—	16	19	—
F	0	—	27	30	—	34	89	65	32
	1	18	25	—	—	33	64	40	28
	2	—	—	—	14	—	27	31	26
	3	—	—	—	—	—	13	14	—
E	0	—	34	34	—	35	144	61	30
	1	35	30	—	—	45	136	52	31
	2	—	—	—	—	—	56	32	—
	3	—	—	—	—	—	26	27	—
D	0	—	32	29	—	43	192	70	38
	1	36	32	—	—	48	140	57	32
	2	—	15	—	—	—	105	37	15
	3	—	—	—	—	—	35	15	—
C	0	—	46	25	—	42	222	62	40
	1	24	28	—	—	36	140	47	32
	2	—	20	—	10	—	134	35	36
	3	—	11	—	—	—	66	24	11
	4	—	—	—	—	—	18	11	—
B	0	—	30	36	ND[1]	37	242	ND	32
	1	16	20	—	ND	24	139	19	20
	2	—	18	—	ND	—	30	10	—
	3	—	7	—	ND	—	33	8	—
	4	—	—	—	ND	—	19	6	—

[1] Lost by accident.

amounts of test specimens available, the following points are apparent from the data given in the Table.

In all the peptides tested, in the first cycle of degradation, NH_2-terminal alanine was replaced by NH_2-terminal glutamic acid. This NH_2-terminal glutamic acid then disappeared during the second cycle of degradation. The results indicate that the amino groups of glutamic acid are linked to carboxyl groups of NH_2-terminal alanine in all the peptides. In peptide H with one mole of glycine as COOH-terminal amino acid, ε-NH_2-terminal lysine was removed with NH_2-terminal alanine by coupling with phenylisothiocyanate and cyclization by HCl treatment. Peptide G contained two moles of glycine/mole of glutamic acid, one mole as NH_2-terminal and the other as COOH-terminal amino acid. When it was submitted to Edman degradation, both the

NH_2-terminal alanine and glycine disappeared in the first cycle and the newly developed ε-NH_2-terminal lysine and glutamic acid were released during the second cycle of degradation. In peptide F with three moles of glycine/mole of glutamic acid, the NH_2-terminal glycine did not decrease in the first degradation but disappeared in the second cycle, simultaneously freeing the ε-amino groups of lysine. This ε-NH_2-terminal lysine was removed in the next treatment with phenylisothiocyanate. The molar ratio of glycine to alanine was approximately one after the disappearance of NH_2-terminal glycine, namely liberation of two moles of glycine. Similar results were obtained with the octapeptide E, except that the peptide obtained in the second cycle of degradation had approximately two moles of glycine/

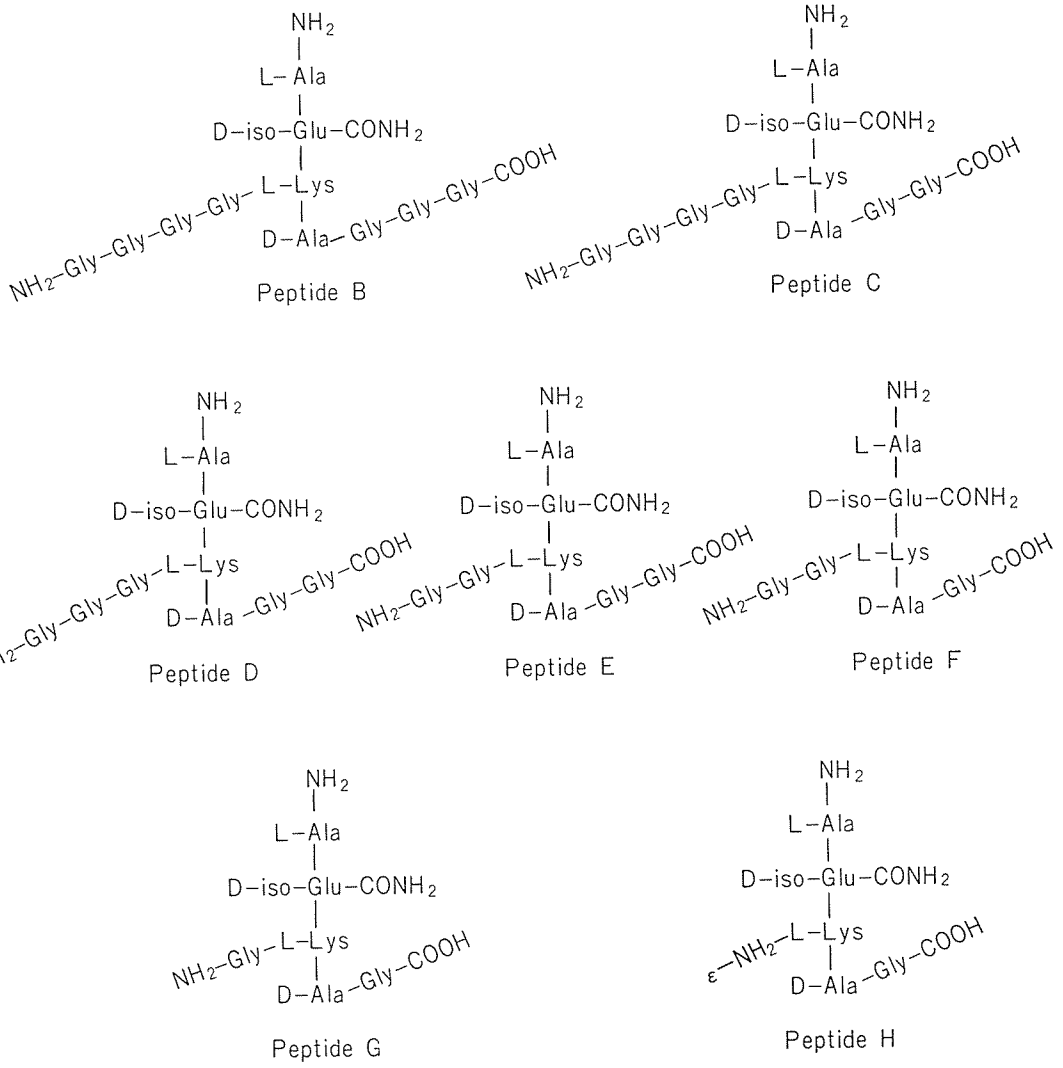

FIGURE 5 Structures proposed for seven peptides (B to H) isolated from the ALE digest of *S. aureus*.

mole of alanine. These results indicate that the chains with NH_2-terminal glycine in peptides F and E are two glycine residues long. By similar reasoning, the behaviour of NH_2-terminal glycine and the changes in the molar ratio of glycine to alanine during successive Edman degradation of peptides D, C and B are most reasonably explained by the assumption that these peptides respectively have $(glycyl)_3$, $(glycyl)_4$ and $(glycyl)_4$ chains linked to the ε-amino groups of lysine. However, the behaviour of lysine in the degradation in peptides E to B is difficult to be explained and may be because glutamic acid is linked to lysine through the γ-carboxyl group. Further studies are necessary on this.

Fig. 5 illustrates the structures of peptides B to H deduced from the results of the analyses presented in Table 2 and those of the Edman degradation experiment described in the foregoing paragraph. The electrophoretic mobilities of the peptides seem consistent with the proposed structures. The optical configurations of glutamic acid and lysine, the location of the ammonia in the peptide molecule and the type of peptide linkage between glutamic acid and lysine were not studied since the amounts of the peptides available were very limited, but these were deduced on the basis of observations of TIPPER, KATZ, STROMINGER and GHUYSEN (1967).

DISCUSSION

The mode of action of a staphylolytic enzyme (ALE) isolated from culture supernatants of *S. epidermidis*, strain EP-K1 was studied by analyses of end groups liberated during digestion and by characterization of the products of its action on isolated cell walls from *S. aureus*, strain Copenhagen. It was demonstrated that ALE has a lytic effect on *S. aureus* walls through the actions of its N-acetylmuramyl-L-alanine amidase and glycyl-glycine endopeptidase, as illustrated in Fig. 6.

A kinetic study of end groups liberated by the action of ALE indicated that both N-acetylmuramyl-L-alanine linkages connecting glycan to peptide and pentaglycine cross bridges between the neighboring basal tetrapeptides, L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine, are cleaved by the action of ALE and hydrolysis of the former precedes cleavage of the latter. End group analyses showed that pentaglycine cross bridges are exclusively cleaved by ALE at -glycyl-glycyl- linkages, but not at linkages to the D-alanine terminus of the basal tetrapeptide, since no measurable amount of COOH-terminal alanine was released during ALE digestion of the walls.

Examination of peptides isolated from the ALE digest of the walls confirmed the conclusions drawn from analyses of liberated end groups: peptides with various structures were isolated from the ALE digests and these, together with one sugar-peptide, amounted to about 90 per cent of the total peptides in the cell walls used for digestion. Further, in all peptides and sugar-peptide the only COOH-terminal amino acid detected was glycine, and there was no alanine.

As reported in separate papers (KATO *et al.*, 1968a, b), the L-11 enzyme from a *Flavobacterium sp.*, which is another staphylolytic enzyme which has been extensively investigated in our laboratory, liberates COOH-terminal alanine as well as COOH-terminal glycine. Three peptides and one sugar-peptide, with D-alanine as COOH-terminal amino acid as well as di-, tri- and tetra-glycine peptides were isolated from digests of cell walls by the L-11 enzyme. These results indicate that the L-11 enzyme has D-alanyl-glycine endopeptidase activity unlike ALE, in addition to the activities of N-acetylmuramyl-L-alanine amidase and glycyl-glycine endopeptidase of ALE.

STROMINGER and GHUYSEN (GHUYSEN, TIPPER and STROMINGER, 1966; STROMINGER and GHUYSEN, 1967) summarized the types of linkage split by cell wall lytic enzymes with fairly well established modes of action. Of the

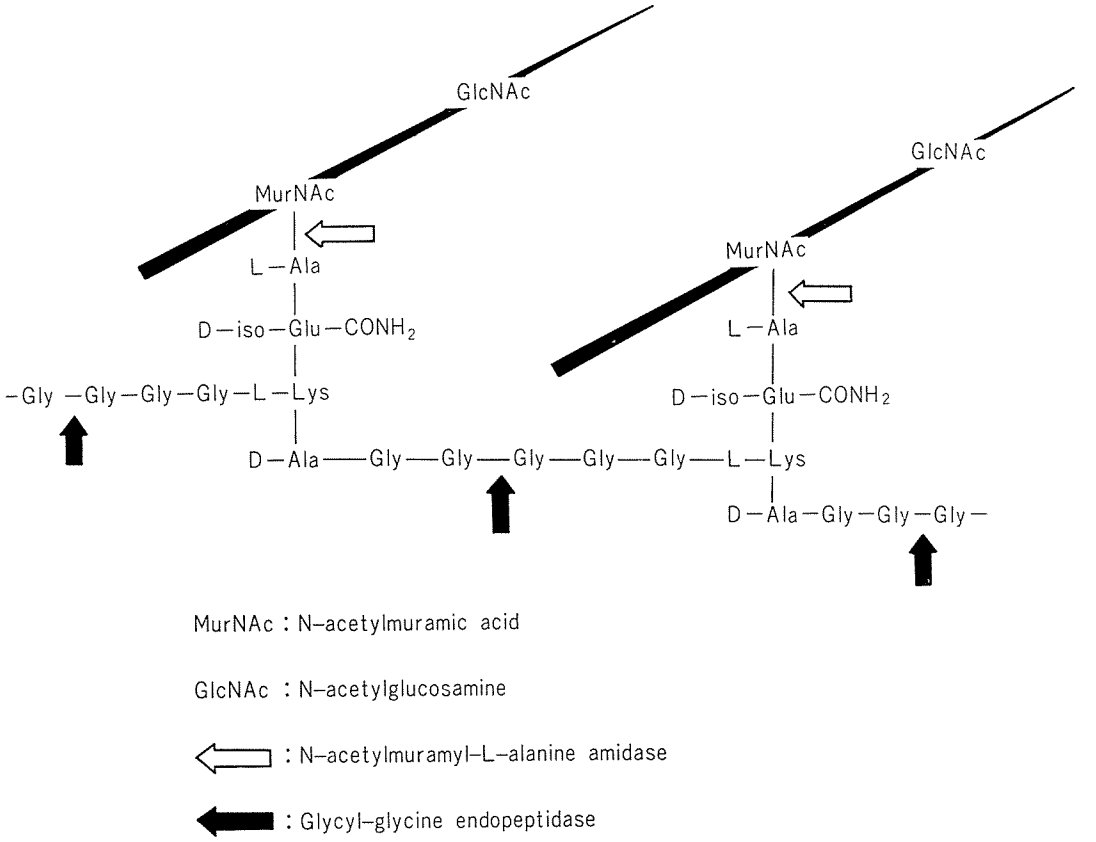

FIGURE 6 Proposed attack points of ALE on the peptidoglycan of *S. aureus* cell walls on the basis of the structure presented by TIPPER, STROMINGER and ENSIGN (1967).

staphylolytic enzymes which exert their lytic action by amidase and/or endopeptidase activities, the enzyme from *Myxobacterium* seems to act on similar linkages to the L-11 enzyme. The SA endopeptidase and nonlytic endopeptidase from *Streptomyces albus* G have been reported to exhibit D-alanyl-glycine endopeptidase activity, and D-alanyl-glycine and glycyl-glycine endopeptidase activities, respectively, against *S. aureus* walls. In the table presented by STROMINGER and GHUYSEN, the linkages split by lysostaphin were noted to be N-acetylmuramyl-L-alanine, glycyl-glycine and N-acetylglucosaminyl-N-acetylmuramic acid. There seems to be a distinct similarity in the modes of staphylolytic action of ALE and lysostaphin, although no evidence has been obtained indicating that ALE attacks the glycan portion of *S. aureus* cell walls.

The exact point of attack of ALE on the pentaglycine cross bridge is not clear at present. Peptide D, in which three of the five glycine molecules are attached to the ε-amino groups of lysine, and the remaining two molecules are linked to carboxyl groups of D-alanine, as triglycine- and diglycine-peptides, respectively, was the main component in the peptide fraction isolated from the ALE digest. This suggests that the linkage between the second and third glycines in the cross bridge may be mainly hydrolyzed by ALE. The peptide isolated from digests of *S. aureus* cell

walls solubilized by lysostaphin in about 50 per cent of the theoretical yield, had the structure N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ε}-(glycyl)$_4$-L-lysyl-D-alanyl-glycine (TIPPER, KATZ, STROM- INGER and GHUYSEN, 1967). It should also be noted that ALE digests, unlike the L-11 enzyme digests, did not contain peptides consisting of glycine only.

There are no reports on the type of linkages attacked by staphylolytic enzymes of *Pseudomonas aeruginosa* (ZYSKIND and PATTEE, 1965; LACHE, ZYSKIND and HEARN, 1966; BURKE and PATTEE, 1967) or of the phage-infected *S. aureus*, phage type 80 (DOUGHTY and MANN, 1967), except that these enzymes act as amidases and/or endopeptidases.

It is unknown whether the activities of N-acetylmuramyl-L-alanine amidase and glycylglycine endopeptidase found in the preparation of ALE used in the present study are due to two separable enzyme proteins. Fractionation and purification of the enzyme will be needed to answer this question.

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