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SHORT COMMUNICATION

STUDY OF THE STRUCTURE OF *MICROCOCCUS LYSODEIKTICUS* CELL WALLS WITH *FLAVOBACTERIUM* L-11 ENZYME[1]

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In spite of extensive investigations on the cell walls of *Micrococcus lysodeikticus*, the structure of their peptidoglycan is still uncertain. Accordingly, the structure of the cell walls of this organism was studied using the L-11 enzyme from *Flavobacterium sp.*

Cells of *M. lysodeikticus* strain NCTC 2665 were grown on nutrient agar media, pH 7.2, supplemented with glucose (0.5%) and yeast extract (0.1%) and harvested after 48 hours cultivation at 37°C. The washed cells were disrupted by sonic oscillation with glass beads (100–150 mesh) in the cold and a cell wall fraction was separated by repeated differential centrifugation. Further purification was achieved by incubation of the fraction with trypsin. This cell wall preparation contained as major components muramic acid, glucosamine, alanine, glutamic acid, lysine, glycine and glucose in the molar ratio of 1.1: 1.1: 2.5: 1.0: 1.3: 1.5: 0.25. These major components constituted 79% of the total cell walls. Measurement of NH_2-terminal, and COOH-terminal groups revealed that 60% of the lysine residues had a free ε-amino group, 60% of the glycine and 3% of the alanine were at NH_2-terminals, and 4% of the alanine had a COOH-terminal (Table 1). Determinations of total, NH_2-terminal and COOH-terminal amino acids were carried out by the methods of GHUYSEN, TIPPER and STROMINGER (1966). The contents of muramic acid and glucosamine were estimated by quantitative paper chromatography by the method of PRIMOSIGH *et al.* (1961). Glucose was determined with a glucostat® (Worthington Biochemical Corp., New Jersey, U.S.A.).

Cell walls (2,200 mg) were suspended in 220 ml of 0.0125 M phosphate buffer, pH 6.8, containing 1,375 units of the *Flavobacterium* L-11 enzyme which had been partially purified by hydroxylapatite column chromatography (KATO *et al.*, 1962). A small amount of chloroform was added as a preservative. The reaction mixture was incubated with constant stirring for 72 hours at 37°C.

During lysis of the cell walls, free amino groups were released and reached a maximum level equivalent to only 0.15 mole/mole of the glutamic acid in the walls. There was no

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TABLE 1 *Chemical Constitution of the Cell Walls of M. lysodeikticus strain NCTC 2665*

Analyses	Ala	Glu	Lys	Gly	Mur	GlcN	Glc
Total							
mμmoles/mg cell walls	1640	665	855	1008	732	725	169
Molar ratio	2.5	1.0	1.3	1.5	1.1	1.1	0.25
NH_2-terminal amino acids							
mμmoles/mg cell walls	53(3)*		502(59)*				
COOH-terminal amino acids							
mμmoles/mg cell walls	66(4)*			602(60)*			

* Per cent of respective amino acids. Mur : muramic acid, GlcN : glucosamine, Glc : glucose

significant liberation of reducing groups (determined by the method of NELSON, 1944). Analyses by dinitrophenylation and hydrazinolysis demonstrated that 0.1 mole/mole of total glutamic acid of NH_2-terminal alanine, equivalent to two thirds of the free amino groups released, was liberated with concomitant liberation of 0.05 mole of COOH-terminal alanine. The low liberation of end groups during digestion, despite nearly 90% reduction in optical density, suggests that in the cell wall peptidoglycan of *M. lysodeikticus*, there are few linkages which are susceptible to the action of L-11 enzyme, but that these susceptible linkages are essential in maintenance of the structures of the cell wall. It should be added in this connection that in a separate experiment, a similar preparation of L-11 enzyme was shown to liberate about 1.1 mole of free amino groups/mole of glutamic acid from the cell walls of *Staphylococcus aureus* strain Copenhagen.

The cell wall lysate obtained after 72 hours incubation was centrifuged at $10,000 \times g$ for 40 minutes to separate a small amount of insoluble residue. The supernatant and washings of the residue were combined and concentrated to about 40 ml. The concentrate was dialyzed through Visking tubing against three changes of a total of 550 ml of water for 2, 2 and 12 hours, respectively. The diffusible dialyzates were combined and lyophilized. The lysate in the tubing was further dialyzed against repeated changes of water, and the thoroughly dialyzed material was then lyophilized. Its dry weight was 1950 mg representing 88.5% of the starting material. The amount of dialyzable substances recovered from the lysate, therefore, would be less than 11.5% of the original cell walls. This finding seems consistent with the low liberation of end groups by L-11 enzyme, indicating that the cleavages of the peptidoglycan structure by L-11 enzyme were critical for solubilization of the cell walls, but were not extensive.

In an attempt to isolate possible repeating units from the basal structure of the walls, the lyophilized dialyzable materials were fractionated in the following manner. A specimen of 215 mg of material from 985 mg of cell walls, containing a large amount of phosphates originating from the phosphate buffer in the reaction mixture, was dissolved in about 2 ml of water and applied to a column (2×96 cm) of Sephadex G-10 (40–120 μ in size). Fractions (5 ml) eluted with water were analyzed for free amino groups, total hexosamines and inorganic phosphates. Hexosamines and inorganic phosphates were determined by the methods of GHUYSEN, TIPPER and STROMINGER (1966) and of LOWRY *et al.* (1954), respectively. Although separation of materials with free amino groups and hexosamines from inorganic phosphates was not complete, the fractions (effluent volume, 55–75 ml) which contained least contamination with inorganic phosphates were pooled, concentrated, and submitted to ion-exchange chromatography on an Amberlite

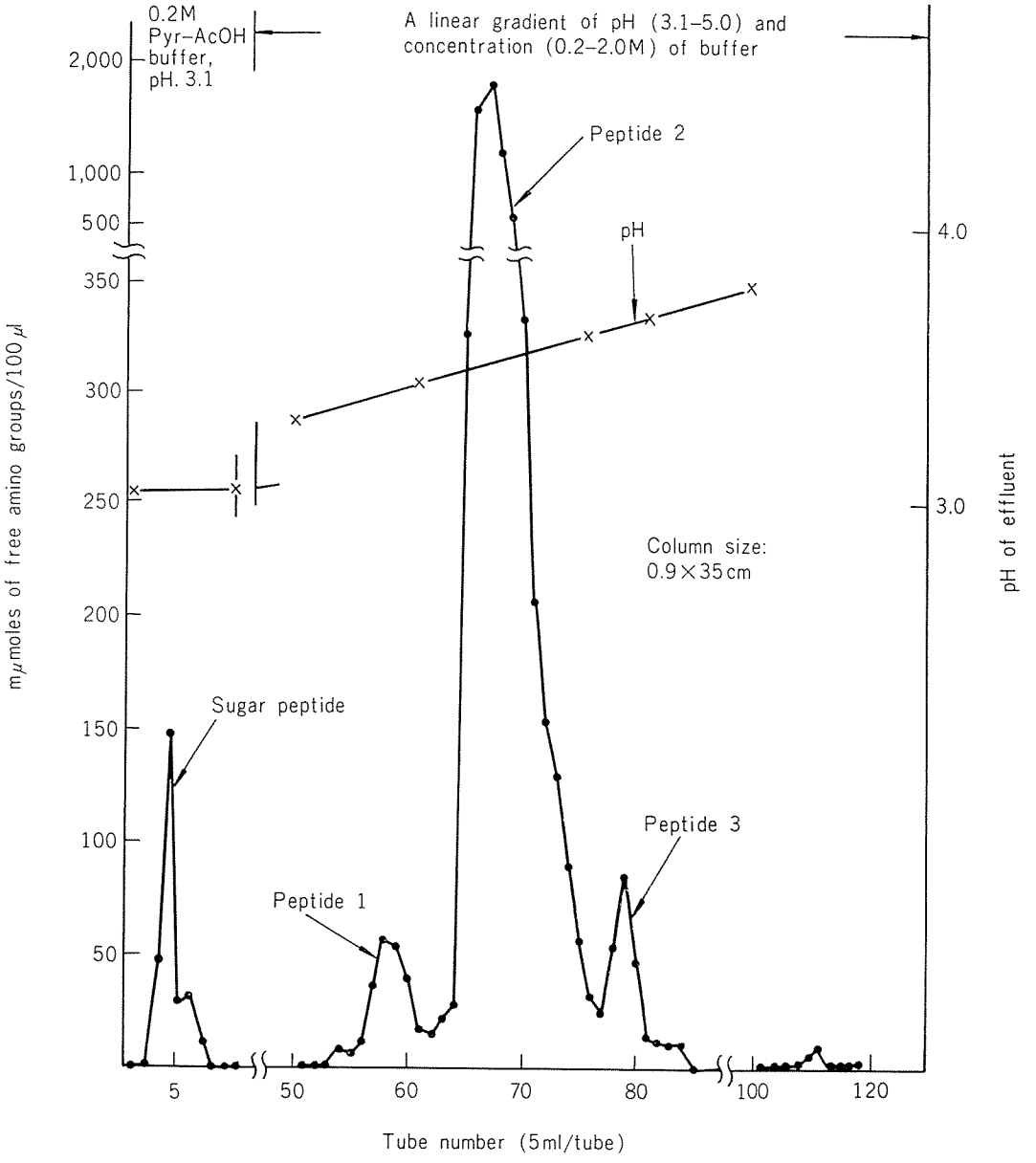

FIGURE 1 Fractionation of a dialyzable fraction of *M. lysodeikticus* cell walls on an Amberlite column CG-120 after digestion by *Flavobacterium* L-11 enzyme

CG-120 column (0.9×35 cm). The method of column chromatography was as described in a separate paper (KATO *et al.*, 1968).

The elution pattern of free amino groups (DNP-reactive materials) is illustrated in Fig. 1. The first peak, which came straight through the column, contained hexosamines in addition to amino acids. This fraction was not homogenous, and was not studied further. The remainder of the DNP-reactive materials were slowly eluted with a linear gradient of increasing pH and concentration of pyridine-acetic acid buffer. The materials in tubes 51–61, 62–76 and 77–85 were pooled in separate tubes. Each fraction was concentrated and purified by rechromatography on an Amberlite CG-120 column.

Determinations for total, NH_2-terminal and COOH-terminal amino acids of the materials in these three peaks (peptides 1, 2 and 3, respectively) revealed that each contained alanine, glutamic acid, lysine and glycine, but in different molar ratios, as summarized in Table 2. In each of the peptides, about one mole of alanine/mole of glutamic acid was NH_2-terminal, practically all the glycine was COOH-terminal, and almost all the ε-amino groups of the lysine were free. About one third of the three alanine residues in peptide 1 and one-half of the two alanine residues in peptide

TABLE 2 *Total, NH_2-terminal and COOH-terminal amino acids of peptides isolated from the L-11 enzyme lysate of M. lysodeikticus cell walls*

Peptide	Amino acid composition				NH_2-terminal amino acids		COOH-terminal amino acids		
	Glu	Gly	Ala	Lys	Ala	ε-NH_2-Lys	Gly	Ala	Lys
1	1.00	0.99	2.70	1.05	0.98	0.96	1.06	0.88	
2	1.00	1.09	1.97	1.25	0.87	0.93	1.00	0.76	
3	1.00	1.04	1.15	1.06	0.89	0.97	1.18		1.06

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

TABLE 3 *Edman degradation of peptides isolated from the L-11 enzyme lysate of M. lysodeikticus cell walls*

Peptide	Step of degradation	Amino acid composition mμmoles				NH_2-terminal amino acids mμmoles			
		Glu	Gly	Ala	Lys	Glu	Gly	Ala	ε-NH_2-Lys
1	0	22.8	24.3	66.0	25.8			25.2	21.0
	1	22.1	18.9	37.5	12.3	16.2			10.5
	2		19.2				13.2		1.5
2	0	77.4	87.0	168.0	99.0			58.5	91.5
	1	83.1	73.5	102.0	57.0	52.8			8.1
	2		48.0				54.0		1.9
3	0	30.9	31.8	37.2	39.3			26.9	45.7
	1	27.6	26.7	15.3	18.9	15.9			6.5
	2		22.5				19.8		1.9
L-Ala-Gly-Gly	0		132.0	49.6				54.0	
	1		132.0				54.7		
	2		53.4				60.2		

The data were not corrected for recovery.

2 were COOH-terminal. Peptide 3, which consisted of alanine, glutamic acid, lysine and glycine in the approximate ratio of 1: 1: 1: 1, differed from the other two peptides in that lysine, but not alanine, was the COOH-terminal residue.

Edman degradation of these peptides was carried out to determine their amino acid sequence, using an authentic specimen of L-alanyl-glycyl-glycine as a reference. The method described in a separate paper (KATO *et al.*, 1968) was followed. The accuracy of determinations was not very great, especially with regard to ε-NH_2-lysine, because of the small amounts of materials available, but the following points will be apparent from the data presented in Table 3. After the first cycle of reaction with the Edman reagent, in each of the three peptides, all the NH_2-terminal alanine disappeared, the free ε-amino groups of the lysine decreased markedly, and the new NH_2-terminal group which appeared was glutamic acid. After the second cycle of degradation, this NH_2-terminal glutamic acid disappeared and NH_2-terminal glycine appeared, indicating that glutamic acid is linked through its α-carboxyl group to glycine. The probable structures of peptides 1, 2 and 3 which are compatible with the data presented in the foregoing paragraphs are presented in Fig. 2.

Peptide 1

Ala–Glu–Lys–Ala–Ala
α
Gly

Peptide 2

Ala–Glu–Lys–Ala
α
Gly

Peptide 3

Ala–Glu–Lys
α
Gly

Figure 2 Proposed structures of peptides isolated from the L-11 enzyme lysate of *M. lysodeikticus* cell walls.

The non-dialyzable fraction from the cell wall lysate was further fractionated by column chromatography on ECTEOLA-cellulose and DEAE-cellulose (borate-form). In this way 8 fractions, differing in chemical composition, terminal groups and susceptibility to hydrolysis with egg white lysozyme, were obtained. Detailed examination on these fractions has not yet been performed, but a compound containing muramic acid, glucosamine, alanine, glutamic acid, lysine, glycine and glucose in the molar ratio of 0.1: 0.1: 2.1: 1.0: 1.1: 1.0: 0.03 was isolated by elution with 0.01 M $Na_2B_4O_7$ from a DEAE-cellulose column to which had been applied the fraction of the non-dialyzable fraction eluted with water from the ECTEOLA-cellulose column. Analyses of terminal groups showed that about 16% of the alanine residues were NH_2-terminal, one-half of the lysine residues has free ε-amino groups and 8% of the alanine and all the glycine were COOH-terminal. These analytical data suggest that this compound may be a polymer of pentapeptide subunits, such as peptide 2 in Fig. 2, attached to a few disaccharide units of muramic acid and glucosamine per mole of glutamic acid. This polymer constituted only 5.5% of the non-dialyzable fraction and its structure has not yet been fully elucidated. However, the fact that a compound such as this could be isolated from cell walls after hydrolysis of only relatively restricted linkages by L-11 enzyme lends support to the idea that there is extensive cross linking between the basal peptide subunits in the cell walls of *M. lysodeikticus*.

It has been demonstrated by MIRELMAN and SHARON (1966, 1967) that the repeating unit in the cell wall peptidoglycan of *M. lysodeikticus* is a disaccharide-pentapeptide with the structure GlcNAc β(1→4) MurNAc-L-Ala-D-γ-Glu(α-Gly)-L-Lys-D-Ala. Peptide 2 isolated in the present study as the major peptide seems to correspond to the peptide moiety of the basal disaccharide-pentapeptide unit.

There have been several investigations in-

dicating the existence of cross linkages between the repeating subunits. D-alanyl-N^{ε}-lysine linkages connecting the basal peptides were claimed by GHUYSEN (1961), SALTON (1961), TSAI, WHITAKER and JURÁŠEK (1965) and others. A dimer and oligomers of pentapeptides were isolated by KATZ and STROMINGER (1967). Using a different method of approach, PERKINS and others (PERKINS and ROGERS, 1959; CZERKAWSKI, PERKINS and ROGERS, 1963) concluded that there was probably extensive cross linking in the cell walls of *M. lysodeikticus*. In a recent study, SCLEIFER and KANDLER (1967) have proposed a new type of cross linkage, on the basis of analyses of partial acid hydrolyzates of the "murein" of *M. lysodeikticus* and of the finding (LEYH-BOUILLE *et al.*, 1966) that almost half the muramic acid is not substituted by peptides. Isolation of a polymer of pentapeptide subunits with a small amount of disaccharide units from the cell wall digest seems consistent with the conclusion of SCHLEIFER and KANDLER that the neighbouring basal peptides are interconnected by identical pentapeptide subunits through the ε-amino group of lysine on the one peptide and the carboxyl group of D-alanine on the other peptide.

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