Title	Structure of the Cell Walls of Lactobacillus Plantarum, ATCC 8014. II. Cross Linkage between D-Alanine and α, α' -Diaminopimelic Acid in the Cell Wall Peptidoglycans Studied with an L-11 Enzyme from Flavobacterium sp.
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Citation	Biken journal : journal of Research Institute for Microbial Diseases. 1968, 11(2), p. 127-138
Version Type	VoR
URL	https://doi.org/10.18910/82872
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BIKEN JOURNAL Vol. 11, 127-138, 1968

STRUCTURE OF THE CELL WALLS OF *LACTOBACILLUS PLANTARUM*, ATCC 8014

2. CROSS LINKAGE BETWEEN D-ALANINE AND α,α'-DIAMINOPIMELIC ACID IN THE CELL WALL PEPTIDOGLYCANS STUDIED WITH AN L-11 ENZYME FROM *FLAVOBACTERIUM SP.*[1]

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SUMMARY Cell walls from *Lactobacillus plantarum* ATCC 8014 were digested with an enzyme preparation from the supernatants of culture of *Flavobacterium sp.* grown with vigorous aeration in a rich medium. During digestion approximately one mole of NH_2-terminal alanine was liberated per mole of cell wall glutamic acid without any concomitant release of other terminal groups of peptides. Three kinds of peptide subunit were isolated from the enzymatic hydrolyzates of the cell walls: a tripeptide, L-alanyl-D-(iso)glutaminyl-meso-α,α'-diaminopimelic acid(DAP) diamide, a tetrapeptide, L-alanyl-D-(iso)glutaminyl-meso-DAP(monoamide)-D-alanine and a heptapeptide consisting of the above tripeptide and tetrapeptide connected by a cross bridge between the α'-amino group of DAP in the former and the carboxyl group of D-alanine in the latter.

L-3 enzyme from *Streptomyces sp.* was found to deamidate the DAP amide in these peptides and catalyzed hydrolysis of the D-alanyl-meso-DAP linkage in the heptapeptide. Direct evidence was thus obtained for D-alanyl-meso-DAP endopeptidase and meso-DAP-amide amidase activities in the *Streptomyces* L-3 enzyme.

INTRODUCTION

Previous work in this series (MATSUDA, KOTANI and KATO, 1968) demonstrated that a peptide portion of the cell wall peptidoglycan of *Lactobacillus plantarum* consists of the peptide subunits, L-alanyl-D-(iso)glutaminyl-meso-α, α'-DAP and L-alanyl-D-(iso)glutaminyl-meso-α,α'-diaminopimelyl-D-alanine. Results suggest that some of these subunits are interconnected

1 A part of this work was read at the 9th Annual Meeting of the Japanese Association for Oral Biology at Tokyo, on Oct. 1st, 1967.
This study was supported in part by a grant for Scientific Research from the Ministry of Education of Japan.

by D-alanyl-meso-DAP cross bridges. However, the L-3 enzyme from *Streptomyces* used in the previous study can hydrolyze all these possible cross bridges. Thus the presence of cross links was not proved definitely by isolation of dimeric or oligomeric compounds containing peptide subunits linked by D-alanyl-meso-DAP after digestion of the cell walls with L-3 enzyme.

During studies on production and concentration of *Flavobacterium* L-11 enzyme, which is another bacteriolytic enzyme studied in this laboratory (KOTANI *et al.*, 1959; KATO *et al.*, 1962; GHUYSEN *et al.*, 1966; KATO *et al.*, 1968a, 1968b), the enzyme preparation (CM-1 enzyme), isolated from the supernatant of cultures of *Flavobacterium* L-11 bacterium grown with vigorous aeration in a rich medium, was found to exert a powerful lytic activity on *L. plantarum* cell walls. Preliminary work suggested that the CM-1 enzyme lyzed the cell walls from *L. plantarum* through its muramyl-L-alanine amidase activity alone. Therefore, attempts were made to isolate dimers or oligomers of peptide subunits from the products of the action of the CM-1 enzyme on *L. plantarum* cell walls. This paper is on the isolation and identification of peptide subunits from the cell wall digests by the CM-1 enzyme and the possible action of the L-3 enzyme on isolated monomers and dimers of peptide subunits.

MATERIALS AND METHODS

Culture filtrates of *Flavobacterium* L-11 bacterium cultivated in a rich medium with vigorous aeration in a tank of 200 liter capacity were used as starting material. The CM-1 enzyme preparation used throughout this study was obtained by concentration of this with acetone and chromatography on DEAE and CM cellulose columns. Details of this preparation will be reported elsewhere, but it should be noted that the medium and culture conditions adopted for production of the enzyme are quite different from those used for the previous enzyme preparation. One unit of enzyme is defined as the amount of enzyme which causes a reduction in the optical density (at 550 mμ) of *L. plantarum* cell wall suspension (0.5 mg/ml final concentration) of 35 per cent on incubation in a total of 3.2 ml of 0.0125 M veronal buffer, pH 8.5, for 60 minutes at 37°C. The preparation used here had an activity of 1.5 units/mg. Other materials and methods were described in the preceding paper (Matsuda, Kotani and Kato, 1968).

RESULTS

1. *Digestion of L. plantarum cell walls with the Flavobacterium CM-1 enzyme*

1) Pilot experiment

Cell walls (16 mg) were digested with 2 units of CM-1 enzyme in 6.4 ml of 0.0125 M veronal buffer, pH 8.5, in a water bath at 37°C. On incubation for 72 hours, the turbidity of the cell wall suspension decreased by about 80 per cent with an increase in free amino groups from 420 to 725 mμmole per mg cell wall (Fig. 1). As shown in Fig. 2, terminal amino acid analyses revealed that this liberation of free amino groups was almost exclusively due to release of NH_2-terminal alanine (from 0 to 320 mμ moles per mg, or 1 mole per mole of cell wall glutamic acid). This figure also shows that the CM-1 enzyme did not release significant amounts of NH_2-terminal amino acids other than alanine or COOH-terminal amino acids from the walls in contrast to the *Streptomyces* L-3 enzyme which liberated mono(α')-NH_2-terminal DAP, COOH-terminal alanine and COOH-terminal DAP (see Fig. 2 in the preceding paper, MATSUDA, KOTANI and KATO, 1968). However, there was a small increase in α'-NH_2-terminal DAP from about 208 to 230 mμmoles/mg of cell walls. This increase only occurred within the first 180 minutes of incubation and there was no corresponding increase in COOH-terminal amino acid. Therefore, it is probably not the result of actual liberation of α'-amino groups of the DAP residues by the CM-1 enzyme, but may be explained by supposing that native α'-NH_2-terminal DAP residues are measured more accurately by the dinitrophenylation (DNP) method on peptidoglycans of reduced size.

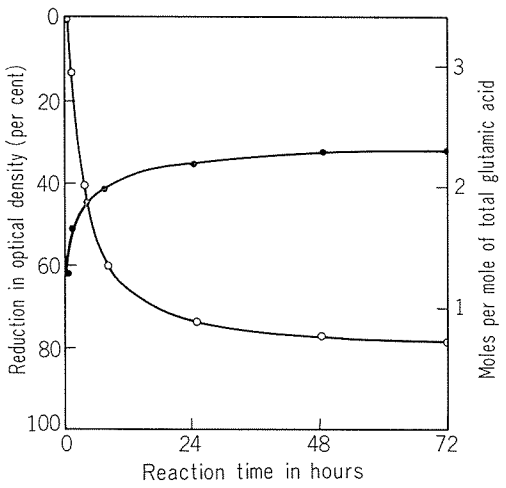


FIGURE 1 Lysis of *L. plantarum* cell walls with liberation of free amino groups by digestion with the *Flavobacterium* CM-1 enzyme.
○——○: Optical density
●——●: Free amino groups

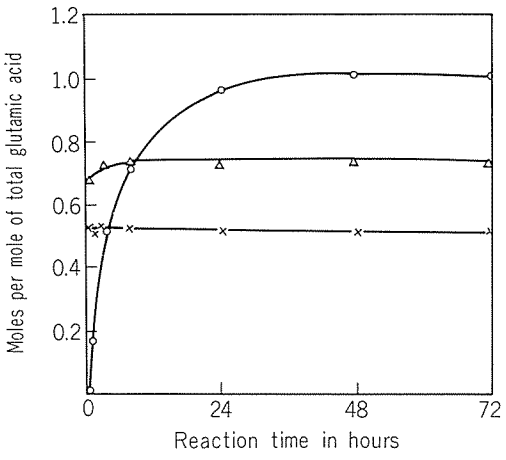


FIGURE 2 Kinetics of liberation of NH_2-terminal amino acids during lysis of *L. plantarum* cell walls with the CM-1 enzyme.
○——○: NH_2-terminal alanine
△——△: α'-NH_2-DAP
×——×: free alanine (derived from ester-linked alanine in teichoic acid residues).
There was no release of COOH-terminal amino acids other than teichoic acid D-alanine.

GHUYSEN *et al.* (1967) in a study on the cell wall peptidoglycan of *Streptococcus faecalis* noted an increase in the number of N^{ϵ}-terminal lysine residues by similar mechanism. The data seem compatible with the conclusion that lysis of *L. plantarum* cell walls by the CM-1 enzyme is due to enzymatic hydrolysis of muramyl-alanine linkages between the glycan and peptide portions of the cell walls, and does not involve significant breakage of interpeptide cross links.

2) Large scale digestion

A specimen (500 mg) of cell walls was digested with 30 units of CM-1 enzyme in 100 ml of 0.0125 M veronal buffer, pH 8.5, at 37°C. After incubation for 24 hours, another 30 units of the CM-1 enzyme were added and the mixture was incubated for 24 hours more. There was a 60% reduction in turbidity and an increase of 300 and 220 mμ moles of free amino groups and NH_2-terminal alanine per mg of cell walls, respectively.[2] The cell wall digests were centrifuged at 10,000×*g* for 30 minutes. The precipitate was washed three times with 25 ml portions of water. The supernatant and washings were combined and concentrated to about 3 ml in a rotary evaporator (Miyamoto Riken, Model RE, Osaka). Approximately 280 mg of soluble material were recovered from 500 mg cell walls.

2. *Isolation of peptides from the cell wall digest*

1) Gel filtration

This soluble material was fractionated on columns of Sephadex G-50 and G-25 (2×97 cm and 2.5×97 cm, respectively) connected in series. The V_0 value in these columns was found to be 220 ml with blue dextran-2000 (Pharmacia, Uppsala, Sweden). Fractions of 7 ml were collected (flow rate: 40 ml per hour) and submitted to chemical analyses. As illustrated in Fig. 3, a high molecular weight fraction (S-1 fraction) which contained almost all

2 Incomplete solubilization of the cell walls in this experiment was later shown to be due to inactivation of the CM-1 enzyme by chloroform added to the reaction mixture as a preservative.

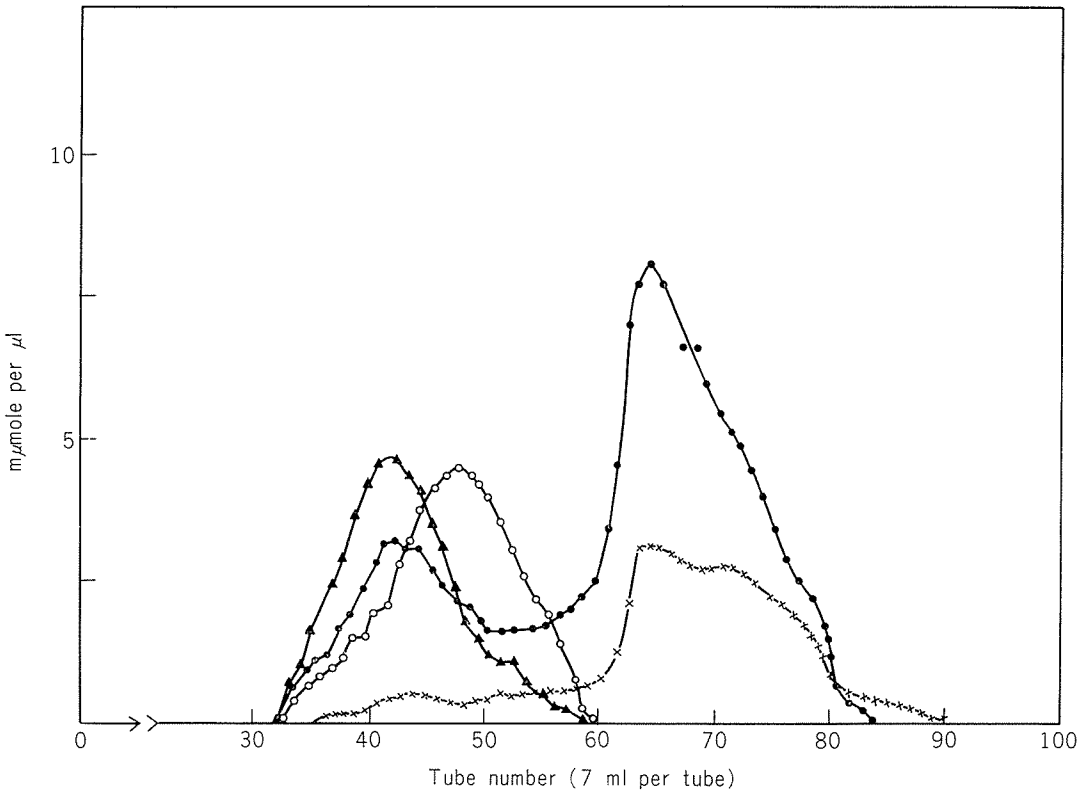


FIGURE 3 Gel filtration of *L. plantarum* cell wall digest with Sephadex G-50 and G-25 columns connected in series.

Column size: G-50=2×97 cm; G-25=2.5×97 cm; V_0: 220 ml; Flow rate: 40 ml per hour.

▲——▲: Organic phosphorus ●——●: Total amino groups

○——○: Total hexosamines ×——×: Free amino groups

of the hexosamines and organic phosphates in the digests was clearly separated from a small molecular weight fraction containing amino groups only. The latter fraction was divided into two portions, one in tubes 60 to 66 (S-2 fraction) and the other in tubes 67 to 85 (S-3 fraction). Each fraction was concentrated to 4.5 ml in a rotary evaporator.

2) Amberlite CG-120 column chromatography

These two small molecular weight fractions (4.5 ml each) were acidified by addition of 0.5 ml of 20% formic acid. With acidification, precipitates of 5, 5′-diethylbarbituric acid appeared. The precipitates were removed by centrifugation and the supernatants were fractionated by Amberlite CG-120 column chromatography as reported in the preceding paper (MATSUDA, KOTANI and KATO, 1968). Elution was performed first with 0.2 M pyridine acetate buffer, pH 3.1 and then by linear gradient elution with 300 ml of 0.2 M pyridine-acetate buffer, pH 3.1 in the mixing chamber and 300 ml of 2.0 M pyridine-acetate buffer, pH 5.0 in the reservoir. Fractions of 10 ml were collected and assayed for free and total amino groups. The S-2 and S-3 fractions showed essentially the same patterns of elution of materials reacting with DNFB, except that only S-3 fraction had peaks which were eluted

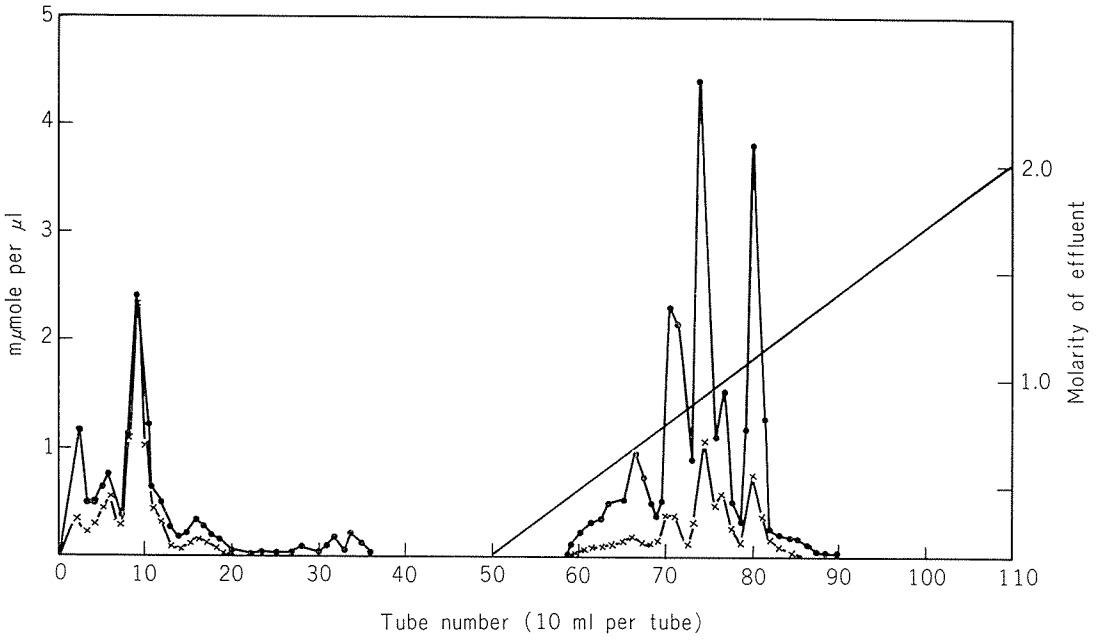


FIGURE 4 Fractionation of the small molecular weight fraction obtained by gel filtration of *L. plantarum* cell wall digest on Amberlite CG-120 column.
Column size: 0.9×35 cm; Column temperature: 50°C; Flow rate: 50 ml per hour.
The column was first eluted with 500 ml of 0.2 M pyridine-acetate buffer, pH 3.1 and then developed by linear gradient elution with solution of increasing pH and concentration of buffer.
×——×: Free amino groups ●——●: Total amino groups.

with 0.2 M pyridine-acetate buffer, pH 3.1. Fig. 4 shows the chromatographic pattern of the small molecular weight fraction of the cell wall digests, compiled from the data obtained in analyses of the S-2 and S-3 fractions.

Six distinct peaks of DNP-reactive materials are seen at tubes No. 2, 9, 70, 74, 76 and 80 and four small peaks at tubes No. 18, 32, 66 and 83. Fractions 2, 18, 32 and 83[3], however, were later shown to release no significant amount of amino acids by acid hydrolysis. Therefore, these fractions, with Fraction 66 which was very small, were not investigated further. It is interesting in this connection that on elution of the cell wall lyzate obtained with *Strepromyces* L-3 enzyme all of the DNP-reactive materials were eluted with 0.2 M buffer, pH 3.1, and using a linear gradient of increasing pH and molarity of pyridine-acetate buffer no further materials reacting with DNFB were obtained (MATSUDA, KOTANI and KATO, 1968).

3 It should be noted here that some DNP-reactive materials other than peptides or amino acids were sometimes eluted from the ion exchange resin.

3. *Paper electrophoresis of the peak fractions*

The electrophoretic mobilities on paper of the peak fractions as described above were examined at pH 1.9 and 5.0. On electrophoresis at pH 5.0 all except Fraction 9 migrated toward the cathode as single spots (Fig. 5). Their patterns were quite different from those of the peptides isolated from L-3 enzyme digests of the cell walls, indicating that the bulk of DNP-reactive materials from the CM-1

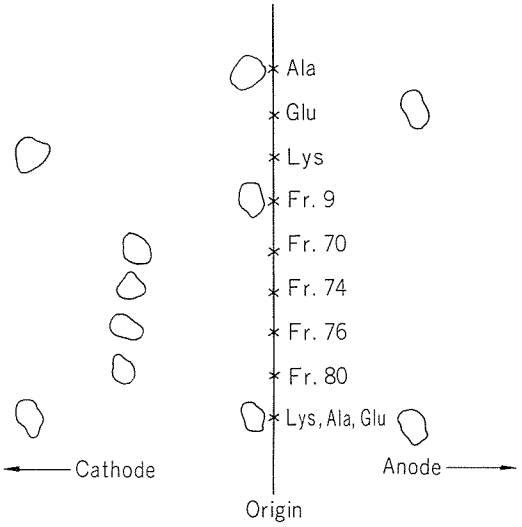


FIGURE 5 Electrophoretic mobilities of isolated peptides at pH 5.0.
Electrophoresis in 0.2 M pyridine-acetate buffer, pH 5.0, at 14 v/cm for 150 minutes.

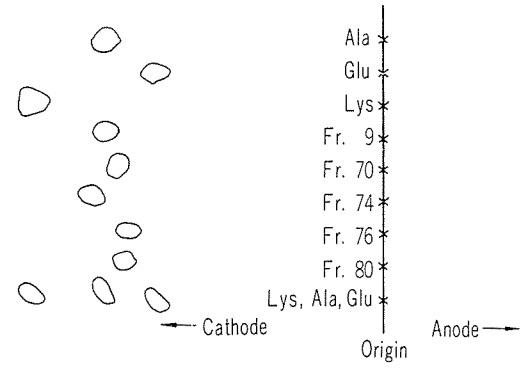


FIGURE 6 Electrophoretic mobilities of isolated peptides at pH 1.9.
Buffer: formic acid-acetic acid-water (5: 15: 80, v/v), pH 1.9.

enzyme digests were positively charged at this pH, in contrast to the neutral or negatively charged peptides obtained from the same cell wall preparation with L-3 enzyme. The material in Fraction 9 was neutral at this pH. All the peak fractions, including Fraction 9, migrated as single spots toward the cathode at pH 1.9 (Fig. 6).

4. *Identification of the peak fractions*

Table 1 shows results of analyses of the amino acids compositions, NH_2- and COOH-terminal amino acids and bound ammonia in Fractions 9, 70, 74, 76 and 80. Fraction 9 consisted of free D-alanine, the optical configuration of which was confirmed by enzymatic assay and it is probably liberated non-enzymatically from ester-linked alanine in teichoic acid residues. Fraction 70 contained alanine, glutamic acid and DAP in the approximate molar ratio of 1.5: 1.0: 1.1. NH_2-terminal amino acid analyses revealed the presence of 1.2 mole of alanine and 1.0 mole of α'-NH_2-DAP per mole of glutamic acid. After hydrazinolysis 0.4 mole of alanine per mole of glutamic acid as COOH-terminal amino acid was recovered. The results of peptide terminal analyses of Fraction 70 may be explained by supposing that this fraction is a mixture of two peptides with different COOH-terminal amino acids (see Fig. 7) although it gave a single spot on paper electrophoresis at both pH values. Approximately equimolar amounts of alanine, glutamic acid and DAP were found in Fraction 74. All the amino groups of alanine and the α'-NH_2 groups of the DAP residues were free, but no COOH-terminal amino acids were found. In both Fractions 76 and 80, the molar ratio of alanine : glutamic acid : DAP was approximately 1.5: 1.0: 1.0. One mole of alanine and 0.5 mole of α'-NH_2-DAP per mole of glutamic acid were NH_2-terminal amino acids in these peptides. No COOH-terminal amino acids were found. About half the α'-NH_2 groups of the DAP residues were not free and were probably involved in peptide bonds with the carboxyl groups of the alanine residues. This supports the idea that the peptides in these two fractions were in the dimeric form.

The presence of nearly 3.0 and 2.5 moles of ammonia per mole of glutamic acid in Fractions 70 and 74, and Fractions 76 and 80, respectively, strongly suggests that all the carboxyl groups not involved in peptide bonds of glutamic acid and of DAP in these peptides

TABLE 1 *Analyses of peptides isolated from L. plantarum cell wall digests with the Flavobacterium CM-1 enzyme*

Fraction	Total amino acids			NH_2-terminal amino acids		COOH-terminal amino acids		Ammonia	Yield (per cent of the peptides in cell wall)
	Ala	Glu	DAP	Ala	α'-NH_2-DAP	Ala	DAP		
9	+	−	−	+	−	+	−	−	
70	1.5	1.0	1.1	1.2	1.0	0.4	0	3.0	4.1
74	1.1	1.0	1.0	1.0	1.0	0	0	2.8	7.5
76	1.6	1.0	0.9	1.1	0.6	0	0	2.5	1.9
80	1.5	1.0	1.0	1.1	0.5	0	0	2.6	7.3

Data are expressed as mole per mole of glutamic acid.

were substituted by amide ammonia. This suggestion is supported by the finding that no COOH-terminal DAP was detected in these peptides. The findings also explain why electrophoresis showed that these peptides were positively charged at pH 5.0. Fig. 7 shows the probable structure of the peptides isolated from cell walls digested by the CM-1 enzyme, deduced from the findings described above. It is proposed that Fraction 70 is a mixture of the tetrapeptide, L-alanyl-D-(iso) glutaminyl- meso-DAP(monoamide)-D-alanine and the tripeptide, L-alanyl-D-(iso)glutaminyl-meso-DAP diamide, presumably in an approximate molar ratio of 1: 1, Fraction 74 is the tripeptide described above, and Fractions 76 and 80 are heptapeptides consisting of the above tripeptide and tetrapeptide with a cross bridge between the α'-amino group of DAP in the former and the carboxyl group of D-alanine in the latter. It is still uncertain why these two heptapeptide fractions separated from each other on Amberlite CG-120 column chromatography.

The relative amounts of the peptide fractions isolated to the cell wall peptides were calculated from the analytical data. Since solubilization of the cell walls was incomplete in the large scale digestion for the reason described in the foot note on page 129, the recovery was not high (approximately 20%). The tripeptide and the heptapeptide (a dimeric form) were found to be the major peptides.

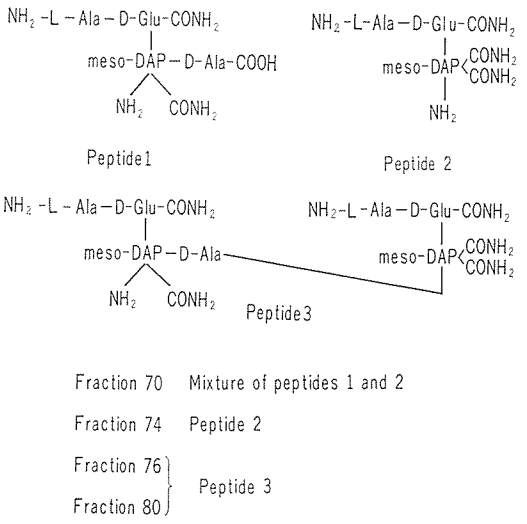


FIGURE 7 Probable structures of peptides isolated from *L. plantarum* cell wall digest with the *Flavobacterium* CM-1 enzyme.

5. *Action of the L-3 enzyme on the isolated peptides*

Aliquots of Fractions 70, 74, 76 and 80 containing about 300 mμmoles of the respective peptides were dried *in vacuo*. The residues were redissolved in 300 μl aliquots of 0.02 M phosphate buffer, pH 7.8, containing 0.5 unit of the L-3 enzyme. The solutions were incubated at 37°C for 24 hours. Aliquots were

TABLE 2 *Release of NH_2-terminal and COOH-terminal amino acids and decrease in amide ammonia during digestion of the isolated peptides by the L-3 enzyme*

Fraction	Increase in terminal amino acids				Decrease in amide ammonia
	NH_2-terminal amino acids		COOH-terminal amino acids		
	Ala	α'-NH_2-DAP	Ala	DAP	
70	0	0	0	0.5	2.1 (0.9[a])
74	0	0	0	1.1	2.0 (0.8)
76	0	0.3	0.4	0.5	1.7 (0.8)
80	0	0.6	0.4	0.6	1.5 (1.1)

Data are expressed as moles of increase in terminal amino acids or of decrease in amide ammonia per mole of glutamic acid.
a Amide ammonia (mole per mole of glutamic acid) remaining after digestion with the L-3 enzyme.

used to measure changes in NH_2-terminal and COOH-terminal amino acids, amino acid composition and bound ammonia before and after incubation of the peptides with the L-3 enzyme. The results are summarized in Table 2. In Fractions 70 and 74, 0.5 and 1.1 moles of COOH-terminal DAP residues per mole of glutamic acid, respectively, appeared without any significant increase in NH_2-terminal amino acids. In Fractions 76 and 80, equimolecular (approximately 0.5 mole per mole of glutamic acid) increases in α'-NH_2-terminal DAP and COOH-terminal D-alanine residues (the optical configuration of which was determined enzymatically) were found. In all four fractions tested, on treatment with the L-3 enzyme, their contents of bound ammonia decreased to approximately one mole per mole of glutamic acid.

A possible explanation for the parallel increase in α'-NH_2-terminal DAP and COOH-terminal D-alanine residues, in Fractions 76 and 80, is breakage of cross bridges in the peptides by the D-alanyl-meso-DAP endopeptidase action of the L-3 enzyme.

The appearance of the COOH-terminal DAP residues and decrease in content of bound ammonia in all the peptides during enzymatic hydrolysis are probably due to the DAP amide amidase action of the L-3 enzyme.

The change in the electrical charge of the test peptides from positive to neutral on digestion with the L-3 enzyme, as shown by their paper electrophoretic patterns at pH 5.0 presented in Fig. 8, further supports the idea that the amide(s) of the DAP residues of the peptides was deamidated.

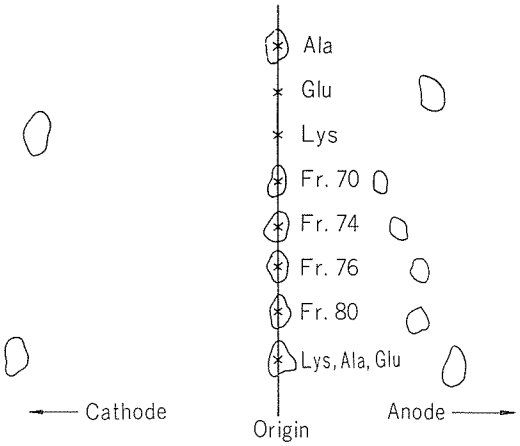
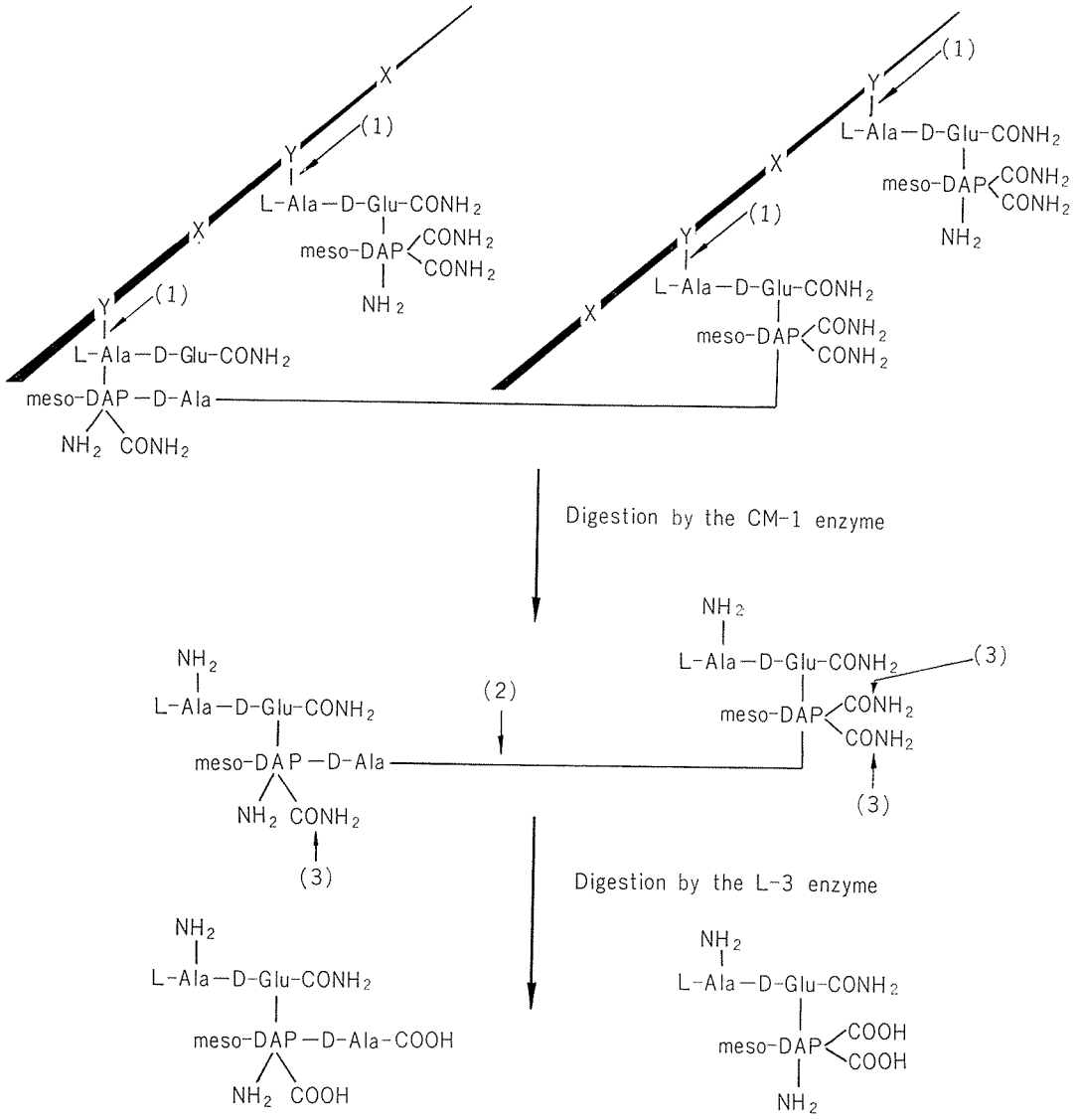


FIGURE 8 Electrophoretic mobilities of isolated peptides digested with the L-3 enzyme.
Electrophoresis was performed as described in Fig. 5.



(1): Muramyl-L-alanine amidase of the CM-1 enzyme

(2): D-alanyl-meso-DAP endopeptidase of the L-3 enzyme

(3): DAP amide amidase of the L-3 enzyme

FIGURE 9 Sequential degradation of *L. plantarum* cell walls with the CM-1 and L-3 enzymes.

DISCUSSION

Fig. 9 illustrates a scheme of sequential degradation of *L. plantarum* cell walls with the *Flavobacterium* CM-1 enzyme and *Streptomyces* L-3 enzyme based on the results in the present and preceding paper.

The CM-1 enzyme solubilizes *L. plantarum* cell walls by cleavage of muramic acid-L-alanine linkages through the action of its amidase, and degrades their peptidoglycans into glycan and peptide portions. The latter consists of peptide subunits, in which the carboxyl groups of the DAP residues are substituted with amide ammonia, cross-linked through D-alanyl-meso DAP linkages. This peptide portion is further hydrolyzed at the cross bridge by the L-3 enzyme, mainly liberating two basal peptides, L-alanyl-D-(iso)glutaminyl-DAP and L-alanyl-D-(iso)glutaminyl-DAP-D-alanine. The amide(s) of DAP residues in the peptide subunits is deamidated by hydrolysis by the L-3 enzyme. Similar sequential degradation of the peptidoglycan of *Corynebacterium diphtheriae* cell walls with the enzyme from *Myxobacterium* and the L-3 enzyme has recently been reported by KATO, STROMINGER and KOTANI (1968).

The existence of D-alanyl-meso-DAP cross bridges, connecting peptide subunits, has been demonstrated in the cell wall peptidoglycans of *Escherichia coli* (WEIDEL and PELZER, 1964), *C. diphtheriae* (KATO, STROMINGER and KOTANI, 1968) and *L. plantarum* (PLAPP, SCHLEIFER and KANDLER, 1967; WEISS, PLAPP und KANDLER, 1967). Several enzymes have been reported to cleave D-alanine-meso-DAP cross links: namely the endopeptidase of the autolytic system of *E. coli* (PELZER, 1963a, 1963b), the L-3 enzyme from *Streptomyces* (KATO, STROMINGER and KOTANI, 1968), and an enzyme from culture filtrates of *Streptomyces albus* G grown in a medium supplemented with autoclaved cells of *Bacillus megaterium* KM (BRICAS, GHUYSEN and DEZÉLÉE, 1967). The L-11 enzyme preparation from culture supernatants of *Flavobacterium sp.* grown in 0.1 per cent casamino acid medium as shallow layers for several days (KATO *et al.*, 1962), was found to exert a powerful lytic activity against cell walls from *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Micrococcus lysodeikticus*, but not those from *C. diphtheriae*, *L. plantarum*, *B. megaterium* or *Streptococcus pyogenes* (group A). Cultivation of the *Flavobacterium sp.* in a rich medium with vigorous aeration seems to broaden the lytic spectrum of the enzymes produced. Recently, some of these enzyme preparations were shown to solubilize efficiently cell walls from *C. diphtheriae* (strain Park-Williams No. 8), *Listeria monocytogenes* (serotype I, NCTC 7973) and *Streptococcus pyogenes* group A (type 12, SF 42) as well as *L. plantarum* cell walls (unpublished observation).

Analyses of the peptide terminal groups liberated during digestion and identification of the hydrolysis products indicated that the CM-1 enzyme from *Flavobacterium* solubilized cell walls from *C. diphtheriae* (unpublished observation) as well as *L. plantarum* by hydrolysis of N-acetylmuramyl-L-alanine linkages. Several enzymes with similar amidase activity have been described (as summarized by GHUYSEN, TIPPER and STROMINGER, 1966; STROMINGER and GHUYSEN, 1967). It is uncertain, however, whether the N-acetylmuramyl-L-alanine amidases in the various enzyme preparations lyse the cell walls by themselves. The N-acetylmuramyl-L-alanine amidase from *Streptomyces albus G* has been reported not to be lytic, but to act on peptidoglycans solubilized by prior treatment of the walls with other lytic enzymes (GHUYSEN, TIPPER and STROMINGER, 1966). It should be noted in this connection that the CM-1 enzyme preparation hydrolyzed the pentaglycine bridges in *S. aureus* cells walls in the same way as the L-11 enzyme preparation prepared by the former method (see KATO *et al.*, 1968a). But since there are no pentaglycine cross-links in *L. plantarum* cell walls, this endopeptidase activity of the CM-1 enzyme preparation does not affect *L. plantarum* cell walls.

A dimeric form of peptide subunit which was

still cross-linked through a D-alanyl-meso-DAP bridge was isolated after digestion of *L. plantarum* cell walls with CM-1 enzyme. Recently, PLAPP, SCHLEIFER and KANDLER (1967) and WEISS, PLAPP and SCHLEIFER (1967) proposed that *L. plantarum* cell walls contained a D-alanyl-meso-DAP linkage but they gave no direct evidence for the presence of such dimeric peptide subunits in the cell wall.

The present study also shows that the three COOH groups in glutamic acid and DAP residues per mole of monomeric peptide subunit which were not involved in peptide bonds were substituted with amide ammonia. The L-3 enzyme has been shown to liberate the amide ammonia of the DAP residues, leaving most of the glutamic acid residues substituted with amide.

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