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

1. ISOLATION AND IDENTIFICATION OF THE PEPTIDES RELEASED FROM CELL WALL PEPTIDOGLYCANS BY *STREPTOMYCES* L-3 ENZYME[1]

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SUMMARY Isolated cell walls from *Lactobacillus plantarum* ATCC 8014 were almost completely solubilized by L-3 enzyme from *Streptomyces sp.* Kinetic analyses of the peptide terminal groups liberated during enzymatic hydrolysis of the cell walls, coupled with analyses of total, NH_2-terminal and COOH-terminal amino acids in intact cell walls, indicate the existence of peptide cross linkages by which the α'-amino group of the DAP residue of one peptide unit is joined to the carboxyl group of the terminal D-alanine residue of a second. It was demonstrated by characterization of products formed by the L-3 enzyme from cell walls that a tripeptide, L-alanyl-D-(iso)glutaminyl-α,α'-diaminopimelic acid, and a tetrapeptide, L-alanyl-D-(iso)glutaminyl-α,α'-diaminopimelyl-D-alanine were the principal building blocks of the peptide portion of the cell wall peptidoglycans of *L. plantarum* ATCC 8014. Results also suggest that the carboxyl groups of α,α'-diaminopimelic acid were substituted with amide ammonia and that these amides were split by the action of the L-3 enzyme.

The probable structure of the peptidoglycan of the cell walls of *L. plantarum* ATCC 8014 and the sites of action of the L-3 enzyme on the walls are presented.

INTRODUCTION

Recently, there have been several reports on the general composition of the cell walls of lactobacilli (KANDLER and HUND, 1959; IKAWA and SNELL, 1960; IKAWA, 1961) and the overall amino acid and amino sugar constitutions of their peptidoglycans are now fairly well known. Teichoic acids, as major components of the walls of many Gram-positive bacteria, were first detected in trichloroacetic acid extracts of whole cells and isolated walls of *Lactobacillus arabinosus* (BADDILEY, BUCHANAN and GREENBERG, 1957; BADDILEY, BUCHANAN and CARSS, 1958).

1 A part of this work was read at the 40th Annual Meeting of the Japan Microbiological Society held at Nagoya, on March 31st, 1967.
Abbreviations used: DAP, α,α'-diaminopimelic acid; DNP-, 2,4-dinitrophenyl-.
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The discovery of these novel compounds was followed from the discovery of a new nucleotide which was first detected in extracts of *L. arabinosus* (BADDILEY and MATHIAS, 1954; BADDILEY *et al.*, 1956a) and later shown to be p^1-cytidine 5-(p^2-L-ribitol 1-pyrophosphate) (BADDILEY *et al.*, 1956b; BADDILEY, BUCHANAN and FAWCETT, 1959).

Thus extensive studies have been made on the chemical composition of the cell walls of lactobacilli. However the molecular structures of their peptidoglycans have only recently received attention when KANDLER and his coworkers partially identified the amino acid sequence of "murein" of various lactic acid bacteria (PLAPP, SCHLEIFER and KANDLER, 1967; PLAPP and KANDLER, 1967a, b; WEISS, *et al.*, 1967).

Their instructive studies are, however, criticizable, since their conclusions on the structures of cell wall peptidoglycans are based largely on identification of peptides isolated from partial acid hydrolyzates of the cell walls in unknown, but presumably very small yield.

The lytic enzyme produced by *Streptomyces sp.* (the L-3 enzyme, MORI *et al.*, 1960; MORI and KOTANI, 1962; KATO, STROMINGER and KOTANI, 1968), was found to exert a powerful lytic activity against cell walls of *Lactobacillus plantarum* ATCC 8014. This series of investigations is on the molecular structure of the cell wall peptidoglycan of this organism. The present paper reports the isolation and identification of the peptides released from *L. plantarum* cell walls by enzymatic hydrolysis with the L-3 enzyme.

MATERIALS AND METHODS

1. *Culture of L. plantarum*

L. plantarum ATCC 8014 was grown in a medium containing polypeptone, 20 g (Daigo Nutritional Chemicals, Osaka); yeast extract, 10 g (Daigo Nutritional Chemicals, Osaka); sodium acetate, 10 g; glucose, 20 g; potassium dihydrogen phosphate, 4.5 g; sodium hydroxide, 1.04 g; 5 ml of inorganic salt solution ($FeSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot 4H_2O$, 0.2 g; $MgSO_4 \cdot 7H_2O$, 4 g; and NaCl, 0.2 g in 100 ml of water) and water to a total volume of 1,000 ml (ARCHIBALD *et al.*, 1961). The medium was adjusted to pH 7.2 and autoclaved for 25 minutes at 121°C. Tubes containing 2 ml of medium were inoculated with a loopful of cells from a 16 hour slant (on medium with 1.5% agar) and incubated at 30°C for 16 hours. Flasks containing 80 ml of medium were then inoculated with 2 ml of liquid culture. After 16 hours incubation, these cultures were used to inoculate 8 liter medium in 10 liter glass vessels. The vessels were incubated at 30°C for 24–27 hours. The cells were harvested in a continuous refrigerated centrifuge (Model S-62, Tominaga Works, Tokyo) and washed three times with 1.8 liter portions of 0.15 M NaCl.

2. *Preparation of cell walls*

A 30 ml aliquot of the thick cell suspension (about 0.5 mg dry weight per ml of 0.15 M NaCl) was vigorously shaken by elliptical motion at 2,000 oscillations per minute with 30 g of glass beads (Glasperlen, Kat. Nr. 54140(2883), 0.11–0.12 mm in diameter, B. Braun Apparatebau, Melsungen, West Germany) in a 75 ml Duran flask in a Braun Mechanical Cell Homogenizer (Model MSK, B. Braun Apparatebau). Disruption of the cells was continued for 15 minutes while cooling the flask by passing a stream of CO_2 round it. Whole cells and glass beads were removed by centrifugation of the suspension at $300 \times g$ for 10 minutes. This low speed centrifugation was repeated 3 to 4 times until practically no whole cells were detected by the Gram stain in the supernatant fluid. The supernatant fluid was then centrifuged at $10{,}000 \times g$ for 30 minutes to precipitate the cell wall fraction. This was washed 10 times each with 900 ml portions of 0.001 M phosphate buffer, pH 7.0, and with 900 ml portions of water. About 22 g of a lyophilized preparation of crude cell walls were obtained from 140 g (dry weight) of cells. Ten grams of crude cell walls were digested with 125 mg of crystalline trypsin (Trypsillin, Mochida Pharmaceutical Co., Tokyo) for 1 hour at 37°C in a total of 200 ml of 0.01 M phosphate buffer, pH 7.0. The digestion was repeated twice more. The trypsinized cell walls were thoroughly washed with 0.01 M phosphate buffer and water and lyophilized. This purified cell wall preparation (7.8 g) was stored in a desiccator over silica gel until use.

3. *L-3 emzyme preparations*

Two kinds of partially purified preparation of the L-3 enzyme were used. One was obtained by Duolite C-10 column chromatography of the enzyme after concentration by precipitation with ammonium sulfate as described previously (MORI and KOTANI, 1962). This was used for large scale digestion for isolation of peptides. The other enzyme preparation, used for analyses of end groups liberated during digestion, was prepared as follows. Enzyme was precipitated from the supernatant of a culture of L-3 bacterium by 80% saturation with ammonium sulfate. It was dialyzed against 0.001 M phosphate buffer, pH 6.0, containing 0.5 M NaCl and applied on a DEAE-cellulose column equilibrated with the same buffer. The column was eluted with the same buffer and enzyme passed through unadsorbed, thus separating from brown colored materials. Fractions with lytic activity were combined and concentrated by ammonium sulfate.

The lytic activity of the preparation was assayed by following the rate of reduction in turbidity of a standard suspension of *L. plantarum* cell walls. To a 12×100 mm test tube in an ice bath were added 1.6 ml of a cell wall suspension (1 mg/ml) and 1.6 ml of 0.04 M phosphate buffer (pH 7.8) containing appropriate amounts of enzyme preparation. Appropriate control tubes were made by omitting either cell walls or enzyme. Tubes were incubated at 37°C and the change in optical density at 550 mμ was recorded at 5 minute intervals in a Shimadzu Bausch and Lomb Spectronic 20 Colorimeter (Shimadzu Seisakusho, Kyoto). One unit of lytic activity was defined as the amount of enzyme preparation required to reduce the optical density of the cell wall suspension by 35% in one hour.

4. *Analytical methods*

1) Total, NH_2-terminal and COOH-terminal amino acids

These were determined as described by GHUYSEN, TIPPER and STROMINGER (1966). The specimen of mono-DNP-DAP used as a reference in determination of DNP-amino acids by thin layer chromatography was prepared as follows.

To 10 ml of 0.1 M sodium borate solution containing 0.5 mmole of DAP (California Cooperation for Biochemical Research, Calif., U.S.A.; a mixture of 40 per cent L,L-isomer and 60 per cent meso-isomer) was added 0.5 mmole of 2,4,-dinitro-1-fluorobenzene (DNFB). The mixture was vigorously stirred for 1 hour at 40°C. After three extractions with 5 ml volume of ethyl ether to remove free dinitrofluorobenzene, the reaction mixture was acidified with 1.5 ml of concentrated HCl and then reextracted three times with 5 ml of ethyl ether to remove bis-DNP-DAP. The yellow mono-DNP-DAP which precipitated from the aqueous phase on acidification was collected on filter paper, and washed with a small amount of 1.5 N HCl. The preparation was dried *in vacuo* and stored in the dark. The purity and identity of the mono-DNP-DAP specimen was checked by one way ascending chromatography on Toyo Roshi No. 51A paper, using *n*-butanol-acetic acid-water (3:1:1, v/v) as solvent.

2) Determination of muramic acid and glucosamine by paper chromatography

Test secimens estimated to contain 20–80 mμmoles of hexosamines were hydrolyzed in 4 N HCl at 100°C for 8 hours. The hydrolyzates were dried *in vacuo* over NaOH and P_2O_5. The dried residues were dissolved in appropriate amounts of water and spotted on Toyo Roshi No. 51A paper (40×40 cm) with a reference mixture of amino sugars and amino acids. The procedures used for development of the paper and estimation of amino sugars and amino acids with ninhydrin spray reagent, were essentially those described by PRIMOSIGH *et al.* (1961).

3) Estimation of ammonia

Ammonia was estimated by a micromodification of the method of FAWCETT and SCOTT (1960) for determination of urea. For determination of total ammonia, a specimen, containing 20–60 mμ moles of ammonia, was dissolved in 40 μl of 4 N HCl, and hydrolyzed at 100°C for 8 hours. The hydrolyzate was dried *in vacuo* and the residue was dissolved in 100 μl of buffer containing 3 g of KH_2PO_4 and 14 g of Na_2HPO_4 per liter. To this solution were added in rapid succession 100 μl of sodium phenate reagent, 150 μl of 0.01% sodium nitroprusside and 150 μl of 0.02 N sodium hypochlorite. After standing the reaction mixture for 10 minutes at 37°C, the optical density at 630 mμ was measured. Isoglutamine, which was hydrolyzed in the same way as test specimens, was used as a standard. Free ammonia was determined on the non-hydrolyzed specimen. The amount of bound ammonia was calculated as the difference between the amounts of total and free ammonia.

4) Other assays

Hexosamines were determined by a modification

(GHUYSEN, TIPPER and STROMINGER, 1966) of the method of ROSEMAN and DAFFNER (1956). Hexose was determined by the Anthrone method (ASCHWELL, 1957). Phosphorus and reducing power were determined by the methods of LOWRY *et al.* (1954) and SOMOGYI (1952), respectively. Free amino groups were estimated as described by GHUYSEN, TIPPER and STROMINGER (1966). All these were microscale methods.

5) Determination of the optical configuration of alanine

The method of GHUYSEN, TIPPER and STROMINGER (1966) was followed with minor modifications in the quantity and concentration of the reagents employed.

6) Analysis of amino acid sequence on isolated peptides by Edman degradation

This was done by the procedures of KONIGSBERG and HILL (1962) and TIPPER, KATZ, STROMINGER and GHUYSEN (1967) with the modification described in a separate paper (KATO *et al.*, 1968).

7) Paper electrophoresis

Electrophoresis of isolated peptides was carried out on Toyo Roshi No. 514 paper (40×20 cm) at a potential gradient of 14 v/cm for 2.5 hours, using a Toyo Kagaku Sangyo (Osaka) power supply (Model III BA 8–2). The following buffers were employed: (1) 0.2 M pyridine-acetic acid, pH 5.0, and (2) formic acid-acetic acid-water (5:15:80, v/v), pH 1.9. Peptides and amino acids were located with ninhydrin reagent.

8) Spectrophotometry

A Hitachi Perkin-Elmer Spectrophotometer (Model 139 UV-VIS, Hitachi Ltd., Tokyo) with a quartz micro-cell of 300 μl capacity was used unless otherwise stated.

RESULTS

1. *Chemical composition of isolated cell walls from L. plantarum*

L. plantarum cell walls are known to contain two major polymers: one is the glucosyl ribitol phosphate polymer, ribitol teichoic acid, and the other is mucopeptide (ARMSTRONG *et al.*, 1958; IKAWA, 1961). Table 1 shows the contents of total and free amino groups, hexosamines, hexose and total phosphorus in the cell wall preparation used in this investigation before and after extraction with cold and hot trichloroacetic acid. Cold extraction was performed by suspending the cell walls in 10% (w/v) trichloroacetic acid at a concentration of 25 mg/ml and stirring the suspension in a cold room for 24 hours. The procedure was repeated until the extracts gave no appreciable turbidity on addition of 5 volumes of acetone. One quarter of the insoluble residue was then suspended in one quarter of the original volume of 10% (w/v) trichloroacetic acid and further extracted by heating at 60°C for 8 hours. The insoluble residues obtained by treatment with cold and hot trichloroacetic acid were thoroughly washed with water and lyophilized. Table 1 shows that the amounts of hexose and phosphorus, which may be from a ribitol teichoic acid polymer in the cell walls, decreased markedly after cold trichloroacetic acid treatment and practically disappeared on extraction with hot trichloroacetic acid. Tests with

TABLE 1 *General composition of L. plantarum (ATCC 8014) cell wall and change in composition on extraction with cold and hot 10 per cent (w/v) trichloroacetic acid*

Determination	mμmoles per mg (per cent) of cell wall		
	Untreated	Extracted with cold TCA	Extracted with hot TCA
Total NH_2-groups (as glutamic acid)	3030 (44.5)	5280 (87.7)	3700 (54.3)
Free NH_2-groups (as glutamic acid)	440 (6.5)	—[a]	—[a]
Hexosamines (as glucosamine·HCl)	700 (12.6)	960 (17.3)	760 (13.7)
Hexose (as glucose)	1320 (23.8)	36 (0.7)	7 (0.1)
Phosphorus	605 (1.9)	74 (0.2)	20 (0.1)

a Not estimated.

the glucostat® reagent (Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.) showed that most of the hexose was glucose. Amino acids and amino sugars in the hydrolyzate of the cell walls (with 4 N HCl at 100°C, for 8 hours) were analyzed by paper and thin layer chromatography and results are summarized in Table 2, with those for ammonia. The cell wall preparation contained alanine, glutamic acid, DAP, glucosamine, muramic acid and ammonia in the molar ratio of 1.30: 1.00: 0.99: 1.02: 0.98: 3.03. The amount of alanine in teichoic acid (0.37 mole/mole of total glutamic acid) was calculated from the amount of free alanine which was liberated from cell walls at alkaline pH in the dinitrophenylation procedure. DNP-alanine was extracted with ethyl ether before hydrolysis of the DNP-derivatives of the cell wall preparation, since ester-linked alanine residues of teichoic acids are known to be extremely labile to alkali treatment (ARMSTRONG *et al.*, 1958). About 80% of the component alanine (0.99 mole/mole of glutamic acid) was found to be the L-isomer by enzymatic assay with 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). DAP was shown to be the meso-isomer from the mobility of its bis-DNP-derivative on thin layer chromatography (solvent—*n*-butanol: water: 28% ammonia, 100: 100: 2, v/v; upper phase) and on paper chromatography (solvent—*t*-amyl alcohol saturated with 0.1 M phthalate buffer, pH 6.0) following the method of BRICAS, GHUYSEN and DEZÉLÉE (1967). The optical configuration of glutamic acid was not determined in this study because IKAWA and SNELL (1960) had found that glutamic acid in *L. plantarum* cell walls is all in the D-configuration.

Analyses of NH_2-terminal and COOH-terminal amino acids demonstrated that about three quarters (0.72 mole/mole of glutamic acid) of the α'-NH_2-groups of the DAP residues were free, indicating that one quarter (0.28 mole) of the α'-NH_2-groups of the DAP residues must be involved in cross linkage with the COOH groups of other amino acid residues. Approximately 0.3 mole equivalents of alanine and DAP residues may be concerned in the formation of cross bridges by peptide bond since no significant amount of COOH-terminal

TABLE 2 *Amino acids, amino sugars, ammonia and terminal groups in L. plantarum cell walls*

Determination	Total amino acids, amino sugars and ammonia		NH_2-terminal amino acids		COOH-terminal amino acids	
	mμmoles[a]	Molar ratio[b]	mμmoles	Molar ratio	mμmoles	Molar ratio
Total alanine[c]	415	1.30	0	0	trace	0
L-alanine	317	0.99				
D-alanine	98	0.31				
D-glutamic acid	320	1.00	0	0	0	0
meso-DAP	318	0.99	230[d]	0.72	trace	0
Glucosamine	327	1.02				
Muramic acid	315	0.98				
Ammonia	970	3.03				
Teichoic D-alanine[e]	118	0.37				

a As mμmoles per mg cell wall.
b Molar ratio to total glutamic acid.
c Total alanine in cell wall peptidoglycan.
d α'-mono-NH_2-DAP.
e Alkali-labile, ester-linked D-alanine in teichoic acid.

amino acid was detected and an excess of 0.3 mole of alanine over one mole of total glutamic acid was present.

2. *Lysis of L. plantarum cell walls with Streptomyces L-3 enzyme*

1) Analyses for end groups liberated by enzymatic hydrolysis of cell walls

Cell walls (32 mg) were suspended in 3.2 ml of 0.02 M phosphate buffer, pH 7.8, containing 2 units of L-3 enzyme and digested by incubating the mixture at 37°C for 96 hours. During digestion, aliquots were removed periodically and immediately inactivated by heating at 80°C for 15 min, for determination of the optical density, free amino groups, reducing groups and NH_2- and COOH-terminal amino acids. As shown in Fig. 1, lysis of the cell walls (expressed as per cent reduction in optical density) was accompanied by release of free amino groups.

During incubation for 96 hours, there was 90% reduction in optical density and the

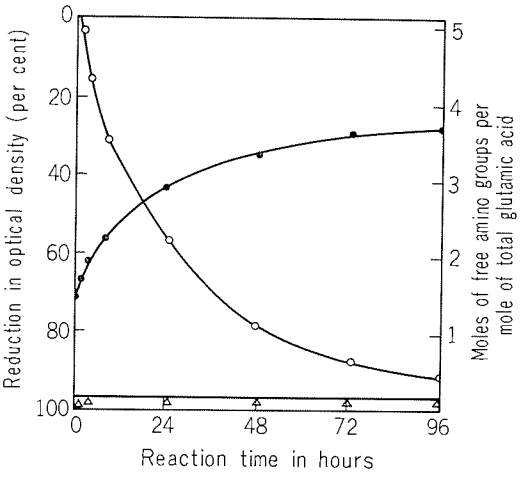

FIGURE 1 Reduction in optical density and liberation of free amino groups in a *L. plantarum* cell wall suspension incubated with L-3 enzyme.
○——○: Optical density
●——●: Free amino groups
△——△: Reducing power

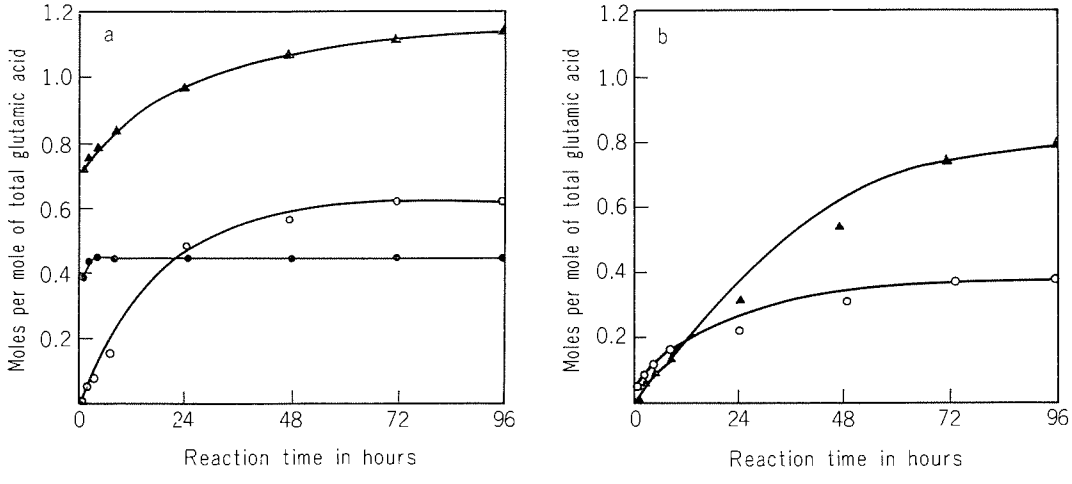

FIGURE 2 Kinetics of liberation of NH_2-terminal (a) and COOH-terminal (b) amino acids during lysis of *L. plantarum* cell walls with L-3 enzyme.
(a) ●——●: Free alanine, derived from ester-linked D-alanine in teichoic acid residues.
○——○: NH_2-terminal alanine ▲——▲: α'-NH_2-DAP
(b) ○——○: COOH-terminal alanine ▲——▲: COOH-terminal DAP

amount of free amino groups per mg cell walls increased from 420 to 1,200 mμmoles. No significant increase in reducing power was observed. These results suggest that the lysis of *L. plantarum* cell walls was exclusively due to the peptidase and/or amidase activity in the L-3 enzyme, and that neither muramidase nor glucosaminidase action were involved.

Terminal amino acid analyses[2] revealed that the increase in free amino groups was due to liberation of the amino groups of both alanine and DAP residues and the increase in these NH_2-terminal amino acids was accompanied by an increase in COOH-terminal alanine and DAP (Fig. 2). Fig. 2 also shows that the rate of release of α′-NH_2-DAP and COOH-terminal alanine was essentially parallel with the lysis and the two were essentially equivalent in molar amount (134 and 120 mμ moles, respectively, per mg cell walls) suggesting that the linkages between the α′-NH_2 groups of DAP and the COOH-groups of alanine were split by the L-3 enzyme. All the COOH-terminal alanine residues liberated were shown to be D-isomers by enzymatic assay. Significant increase of COOH-terminal DAP is presumed to be the result of cleavage of DAP amides by one of the enzymatic activities of the L-3 enzyme preparation employed (KATO, STROMINGER and KOTANI, 1968). This assumption was confirmed in a subsequent investigation, to be presented in detail elsewhere. Increase in NH_2-terminal alanine without concomitant increase in the amount of corresponding COOH-terminal groups of amino acids seems to be due to rupture of linkages between the amino groups of alanine and the COOH groups of muramic acid.

2) Large scale digestion

A specimen of 1.6 g of cell walls was incubated with 100 units of L-3 enzyme in total volume of 160 ml of 0.02 M phosphate buffer, pH 7.8, at 37°C for 98 hours. After digestion the following amounts of NH_2- and COOH-terminal amino acids were liberated by the enzymatic hydrolysis (as mμmoles of terminal amino acid liberated per mg cell walls): NH_2-terminal alanine, 184; α′-NH_2-DAP, 102; COOH-terminal alanine, 100; and COOH-terminal DAP, 232.

The digest was dialyzed with stirring against three 500 ml volumes of water for 2 hours each and finally against 1,000 ml of water for 24 hours at 4°C. Both dialyzable and non-dialyzable fractions were concentrated under reduced pressure to about 10 ml in a rotary evaporator (Miyamoto Riken, Model RE, Osaka) in a water bath at 42°C, and then lyophilized. A total of 1 g of non-dialyzable fraction was recovered from 1.6 g of cell walls.

3. *Isolation of peptides from the dialyzable fraction*

1) Gel filtration

The dialyzable fraction (300 mg) in 3 ml of water was submitted to gel filtration on Sephadex G-50 and G-25 (coarse, bead form, Pharmacia, Uppsala, Sweden) columns (2× 97 cm and 2.5×97 cm, respectively) connected in series. Elution was performed with water at a flow rate of 40 ml per hour, at 4°C (Fig. 3). Ten milliliter fractions were collected and assayed for free and total amino groups, total hexosamines and inorganic phosphorus. Fractions in tubes 19–45 inclusive, containing compounds with NH_2-groups, were pooled, and much of the inorganic phosphorus, originating from phosphate buffer in the reaction mixture for digestion of the cell walls, was eliminated. The pooled fractions were concentrated to 2.7 ml in a rotary evaporator.

2) Amberlite CG-120 column chromatography

The partially desalted diffusible fraction

2 As described in the discussion, the cell wall digest contained peptide, a DNP derivative of which was ether-soluble. Thus in determination of NH_2-terminal amino acids in this and following experiments, extraction of DNP-derivatives from the acidified digest with ether, which is usually done to remove DNP-amino acids, was omitted. However, a correction was made for free alanine derived from the alkali-labile alanine residues in the teichoic acid.

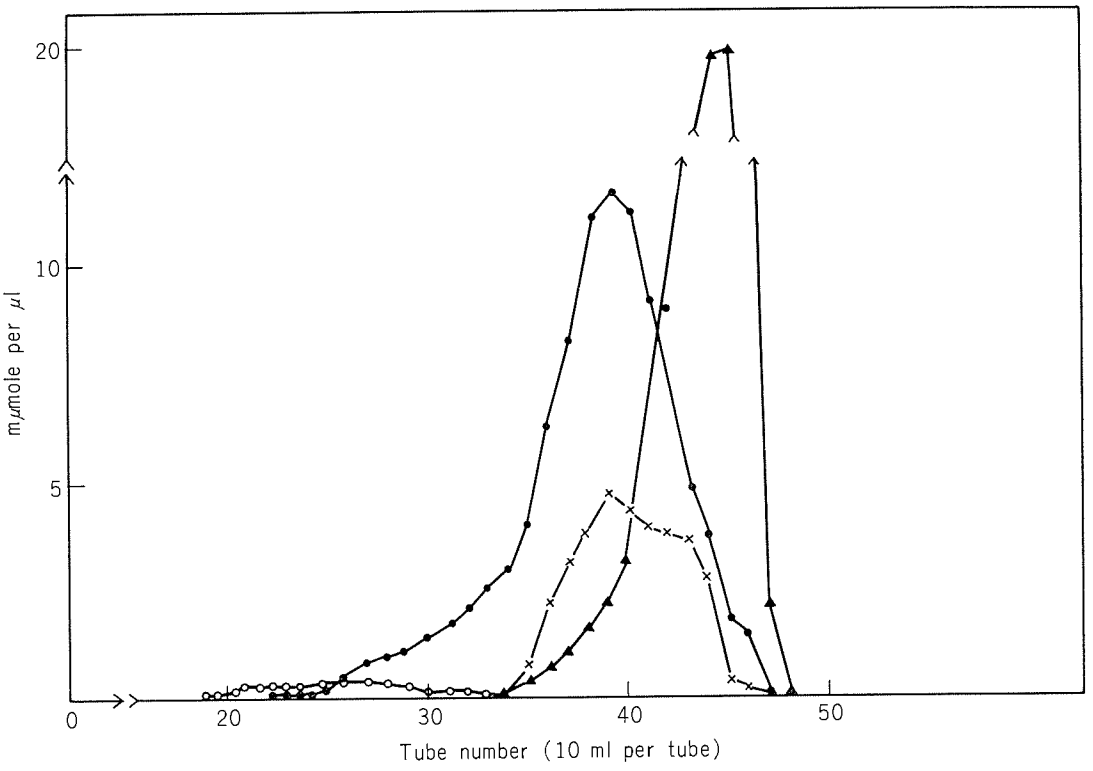

FIGURE 3 Gel filtration of the diffusible fraction from *L. plantarum* cell wall digest on Sephadex G-50 and G-25 columns connected in series.
Column size: G-50=2×97 cm, G-25=2.5×97 cm; V_0: 220 ml; Flow rate: 40 ml per hour.
●——●: Total amino groups ×——×: Free amino groups
○——○: Total hexosamines ▲——▲: Inorganic phosphorus

(2.7 ml) was acidified with 0.3 ml of 20% formic acid and chromatographed on a column (0.9 ×35 cm) of Amberlite CG-120 (19–22 μ particle size, Rohm and Haas, Pennsylvania, Pa., U.S.A.), previously equilibrated with 0.2 M pyridine-acetate buffer, pH 3.1. Elution was performed first with 500 ml of 0.2 M pyridine-acetate buffer, pH 3.1, then with a linear gradient with 350 ml of 0.2 M pyridine-acetate buffer, pH 3.1 in the mixing chamber and 350 ml of 2.0 M pyridine-acetate buffer, pH 5.0 in the reservoir. The flow rate was adjusted to 50 ml per hour by exerting pressure on the eluents with a micro metering pump (Yanagimoto Mfg., Kyoto). The temperature around the column was maintained at 50°C by circulating hot water through a water jacket round the column using an Ultra Thermostat Original Lauda (Type NB-D 8/17, Messgeräte Werk Lauda, West Germany). Fractions of 10 ml were collected and assayed for free and total amino groups and total hexosamines. Fig. 4 shows that peaks of amino groups were separated by elution with 0.2 M pyridine-acetate buffer, pH 3.1.

The fractions in each peak were combined separately and designated as F-1, F-2, F-3 and F-4 in order of their elution. Only F-1 contained hexosamines. Pyridine and acetic acid

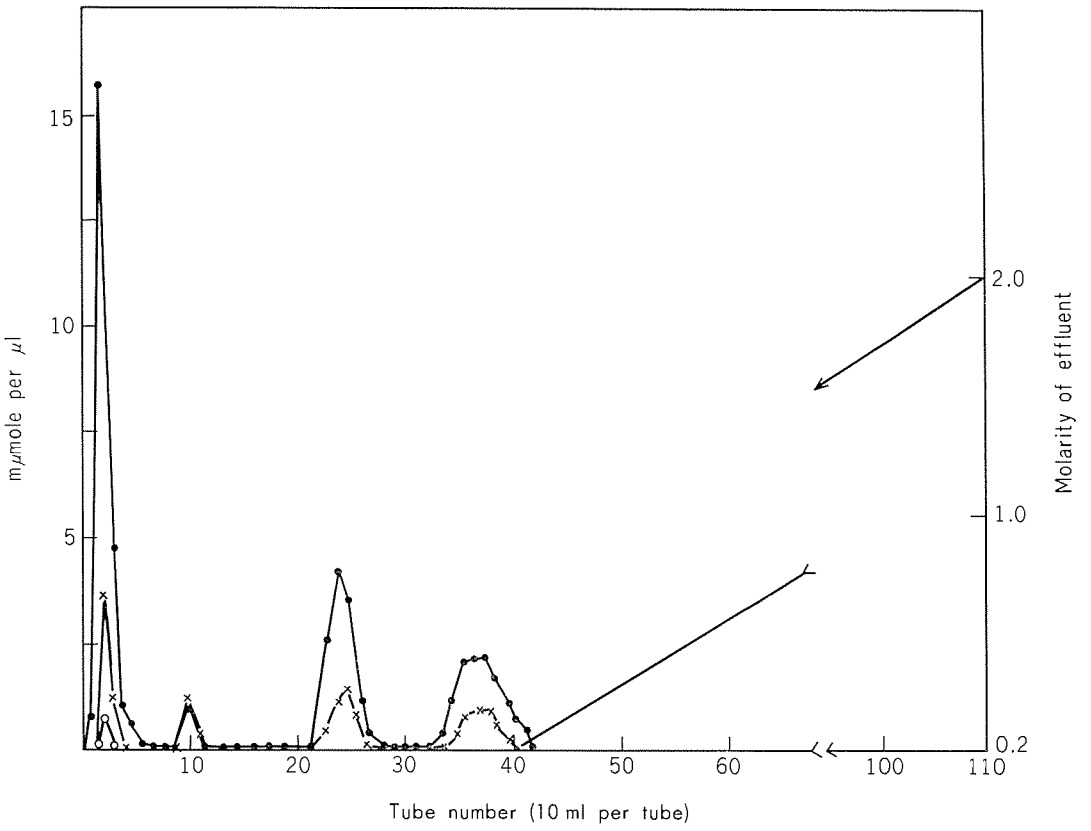

FIGURE 4 Separation of peptides from the dialyzable fraction of *L. plantarum* cell wall digest by chromatography on Amberlite CG-120.
Column size: 0.9×35 cm; Column temperature: 50°C; Flow rate: 50 ml per hour.
●——●: Total amino groups ×——×: Free amino groups ○——○: Total hexosamines

in the fractions were eliminated by repeated evaporating the material to dryness and redissoluting it in water. Finally each of the fractions was dissolved in 10 ml of water. No material containing amino groups or hexosamines was eluted by a linear gradient of increasing pH and concentration of pyridine-acetate buffer.

4. *Paper electrophoresis of the fractions from the Amberlite CG-120 column*

Their electrophoretic mobilities and homogeneities of the four fractions reacting with DNP isolated from the diffusible fraction as above, were examined. On paper electrophoresis at pH 1.9, all the fractions migrated toward the cathode as single spots (Fig. 5). At pH 5.0, F-1 migrated toward the anode as a single spot and F-2 remained at the origin, indicating that the former fraction had a slightly negative charge and the latter was uncharged at this pH. F-3 and F-4, separated into two spots, the major component remaining at the origin and the minor one migrating toward the anode (Fig. 6). F-3 and F-4 were then further fractionated by paper electrophoresis at

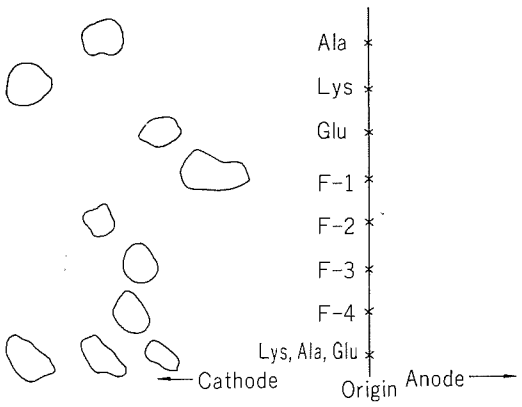

FIGURE 5 Electrophoretic mobilities of isolated peptides at pH 1.9

Buffer: formic acid-acetic acid-water (5: 15: 80, v/v), pH 1.9. Electrophoresis at a potential of 14 v/cm for 150 minutes.

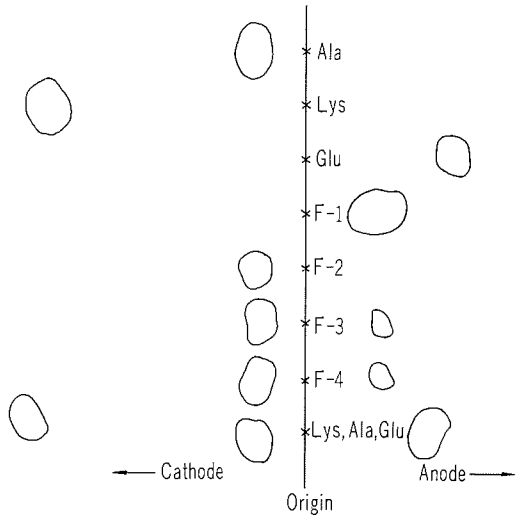

FIGURE 6 Electrophoretic mobilities of peptides isolated from the dialyzable fraction of *L. plantarum* cell wall digest, at pH 5.0.

Buffer: 0.2 M pyridine-acetate, pH 5.0.

pH 5.0 in the usual manner. In this way material remaining at the origin (F-3–1 and F-4–1, respectively) was separated from that migrating toward the anode (F-3–2 and F-4–2, respectively).

5. *Analyses of the fractions isolated from the cell wall digest by Amberlite CG-120 column chromatography and paper electrophoresis*

The results of analyses of total, NH_2-terminal and COOH-terminal amino acids, ammonia and hexosamines are summarized in Table 3, as moles per mole of total glutamic acid in the respective fractions. F-1 contained alanine, glutamic acid, DAP and hexosamine in the approximate molar ratio of 1.2: 1: 1: 1.1. Virtually all the DAP residues had free α'-NH_2-groups and none of the alanine residues was NH_2-terminal, indicating that hexosamine may be linked to the amino groups of alanine and there was no linkage between the peptide molecules through the amino groups of DAP. Hydrazinolysis showed the presence of both COOH-terminal alanine and COOH-terminal DAP (0.65 and 0.61 mole per mole of glutamic acid, respectively). Paper chromatographic analysis of the F-1 hydrolyzate (in 4 N HCl, at 100°C for 8 hours) showed that muramic acid but not glucosamine was present in this fraction. The results mentioned above suggest that this fraction may be a mixture of two sugar peptides, one with COOH-terminal alanine and the other with DAP as the COOH-terminal amino acid. F-1 was not purified further.

F-2, appeared to be free alanine, presumably originating from ester-linked alanine of the teichoic acid molecule. F-3–1 contained alanine, glutamic acid and DAP in the approximate molar ratio of 2: 1: 1. In this peptide, one mole of the alanine residues was NH_2-terminal, almost all the DAP residues had free α'-amino groups, and the remaining one mole of alanine residues was COOH-terminal. F-4–1 contained equimolar amounts of alanine, glutamic acid and DAP. Analyses of NH_2- and COOH-terminal amino acids revealed that one mole of alanine and α'-NH_2-DAP per mole of glutamic

TABLE 3 *Peptides and sugar peptide isolated from the L-3 enzyme digest of L. plantarum cell walls*

Fraction	Total amino acids			NH_2-terminal groups		COOH-terminal groups		Hexosamines	Ammonia	% of the peptide in cell walls
	Ala	Glu	DAP	Ala	α'-NH_2-DAP	Ala	DAP			
F-1	1.15	1.00	0.92	0	0.92	0.65	0.61	1.08	—	9.8
F-2	+	—	—	+	—	+	—	—	—	
F-3-1	2.10	1.00	1.06	1.09	1.09	1.06	trace	0	1.05	11.3
F-3-2	1.26	1.00	1.07	0.77	0.82	0.18	0.68	0	0	1.7
F-4-1	0.96	1.00	0.94	0.92	0.94	0	0.94	0	1.10	13.6
F-4-2	0.97	1.00	1.00	0.91	0.91	0	0.94	0	0	0.4

Data are expressed as molar ratios to glutamic acid.

acid were NH_2-terminal and virtually all the DAP residues were COOH-terminal[3]. Hydrolysis of F-3-1 and F-4-1 in 4 N HCl at 100°C for 8 hours yielded essentially one mole of ammonia per mole of glutamic acid. Both fractions were neutral on paper electrophoresis at pH 5.0. These findings strongly suggest that one of the carboxyl groups of the glutamic acid residue, which was not involved in a peptide linkage was substituted with amide ammonia. Substitution of amide ammonia on the α-carboxyl group of glutamic acid has already been reported by STROMINGER, GHUYSEN and their coworkers in the cell walls of several bacterial species (MUÑOZ *et al.*, 1966; TIPPER, STROMINGER and ENSIGN, 1967; TIPPER, KATZ STROMINGER and GHUYSEN, 1967; GHUYSEN *et al.*, 1967). F-3-2 which consisted of alanine, glutamic acid and DAP in the approximate ratio of 1.3 : 1 : 1, contained two kinds of COOH-terminal amino acid (0.18 mole of COOH-terminal alanine and 0.68 mole of COOH-terminal DAP), while virtually all the alanine and α'-NH_2 groups of DAP residues were NH_2-terminal. In agreement with the observed electrophoretic mobility, no ammonia was found in F-3-2. This fraction seems to be a mixture of alanyl-glutamyl-meso-DAP and alanyl-glutamyl-meso-diaminopimelyl-D-alanine. The analyses performed on F-4-2 indicate that the structure of this tripeptide corresponds to that of the peptide in F-4-1, in which the amide ammonia in the glutamic acid residues was eliminated. This conclusion seems consistent with the observation that F-4-2 was acidic on electrophoresis at pH 5.0.

Determination of the optical configuration of the alanine residues in these peptides by enzymatic assay indicated that practically all of the NH_2-terminal alanine was the L-isomer, and the COOH-terminal alanine was exclusively in the D-configuration.

6. *Edman degradation*

F-3-1, F-3-2, F-4-1 and F-4-2 were submitted to stepwise Edman degradation.

In all the fractions tested after one cycle of degradation, NH_2-terminal alanine had been quantitatively replaced by NH_2-terminal glutamic acid and concomitantly virtually all the α'-NH_2-terminal DAP had disappeared. The second cycle of reaction with phenyl-isothiocyanate did not release any amino acid as new NH_2-terminals (Table 4). No liberation of free ammonia during the cycles of Edman degradation from any of the peptides was

3 In the previous study on the enzymatic lysis of cell walls from *Corynebacterium diphtheriae* (KATO, STROMINGER and KOTANI, 1968) and in the subsequent studies in this series, it was found that if the carboxyl groups of the DAP residues were substituted with amide, these DAP residues were not recovered as COOH-terminal amino acid by hydrazinolysis.

TABLE 4 *Changes in NH_2-terminal amino acids in isolated peptides during Edman degradation*

Step	NH_2-terminal amino acids	F-3-1	F-3-2	F-4-1	F-4-2
0	Alanine	10.7	9.5	4.0	9.1
	Glutamic acid	0	0	0	0
	α'-NH_2-DAP	8.5	9.7	4.5	10.0
1	Alanine	0	0	0	0
	Glutamic acid	10.0	10.5	3.8	9.0
	α'-NH_2-DAP	0	0	0	0
2	Alanine	0	0	0	0
	Glutamic acid	0	0	0	0
	α'-NH_2-DAP	0	0	0	0

Data are expressed as mμmoles per 5-10 mμmoles of each peptide.

detectable. This may be due to the possible inhibitory effect of the reagent used in the degradation and/or the degradation products on color formation in the ammonia determination and further study on this is required.

7. Proposed structure of the peptides isolated from the dialyzable fraction of the cell wall digest

Fig. 7 gives the probable structures of peptides F-3-1, F-3-2, F-4-1 and F-4-2, and sugar peptide F-1 proposed from the analytical results described in the preceding paragraphs. It is uncertain which of the two carboxyl groups (α or γ) of glutamic acid is involved in the linkage with the amino groups of DAP.

It should be pointed out with regard to the proposed structure of peptide F-3-1, that one of the carboxyl groups of the glutamic acid residues was assumed to be amidated, but that instead one of the carboxyl groups of the DAP residues may be amidated, since the COOH-terminal amino acid was alanine, not DAP, in this peptide and no evidence that one of the carboxyl groups of DAP was not substituted was obtained by analysis by hydrazinolysis.

The relative amounts of the isolated peptides and sugar peptides to the total peptides in the cell walls were calculated on the basis of the analytical data. As shown in Table 3, about 40% of the wall peptides was recovered and the peptides in F-3-1 and F-4-1 (peptides A and B, respectively, in Fig. 7) were the major components.

DISCUSSION

Fig. 8 illustrates a structure proposed for the cell wall peptidoglycans of *L. plantarum* ATCC 8014 and the sites of action of L-3 enzyme in their degradation. The present data are compatible with the proposed scheme. Analyses of total, NH_2-terminal and COOH-terminal amino acids in intact cell walls showed that the α'-NH_2 groups of 0.28 mole of the DAP residues per mole of glutamic acid were not substituted and that there was 0.31 mole of D-alanine residue, in which the carboxyl groups were not free. This strongly suggests that about one quarter of the DAP is involved in cross linkages through α'-NH_2 groups to the carboxyl groups of D-alanine. Kinetic analysis of the peptide terminal groups released during digestion of *L. plantarum* cell walls with the *Streptomyces* L-3 enzyme showed that liberation of α'-NH_2-terminal DAP and COOH-terminal D-alanine increased in parallel until all the α'-NH_2 groups of the DAP re-

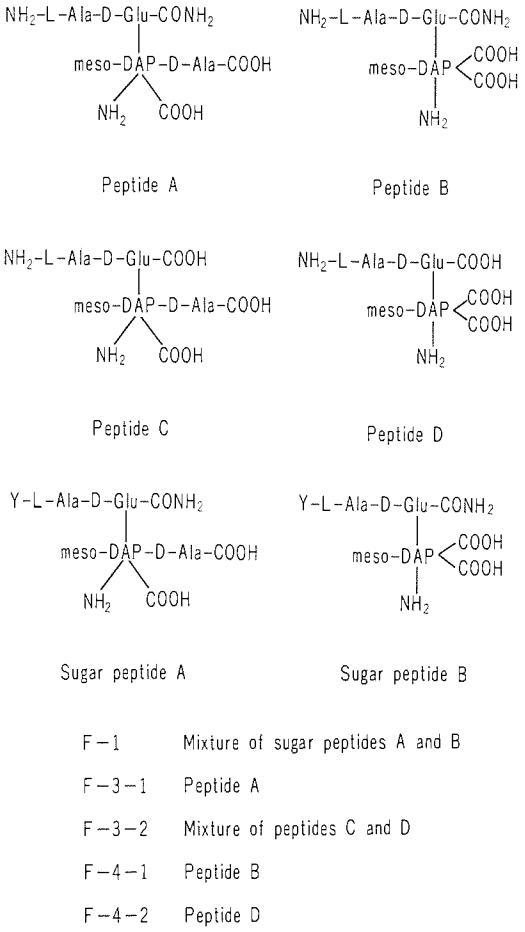

FIGURE 7 Probable structures of peptides and sugar peptides isolated from *L. plantarum* cell wall digests.

sidues had been released. This suggests that one site of action of the L-3 enzyme is D-alanyl-DAP linkages. The products of small molecular weight which were characterized in cell wall digests by the L-3 enzyme were compatible with this suggestion. NH_2-terminal alanine is presumably released by splitting the N-acetylmuramyl-L-alanine linkage, although the structure of the glycan portion of the cell walls has not yet been invesigated. However, breakage of this linkage by the L-3 enzyme was not complete, at least under the present experimental conditions, since the maximum release of NH_2-terminal alanine was less than 0.62 mole per mole of glutamic acid and sugar peptides with the structures shown in Fig. 7 were recovered in significant amounts from the cell wall digest.

Determination of ammonia liberated by acid hydrolysis of intact cell walls revealed that approximately 3 moles of amide ammonia per mole of glutamic acid were present. Evidence suggests that in native cell walls one mole of ammonia is attached to the (α)-carboxyl group of glutamic acid which is not involved in the peptide links and the remaining two moles are attached to the carboxyl groups of DAP. In at least one of the peptides (peptide B in Fig. 7) isolated from the cell wall digest the (α)-carboxyl group of glutamic acid was amidated. The presence of the peptides in which the glutamic acid residues were not amidated, on the other hand, may be explained by assuming that the carboxyl groups of some of the glutamic acid residues were not amidated in the intact walls themselves or that, due to enzymatic hydrolysis of the cell walls and/or during isolation of the peptides from the digests, some of the (iso)-glutamine residues in the peptidoglycan were deamidated. In this connection it is pertinent to mention that thin layer chromatography of the DNP-derivatives of the cell wall digest revealed an ether-soluble DNP compound differing from other DNP derivatives of free amino acids present in the walls and having a slightly higher Rf value than glutamic acid in the solvent (*n*-butanol: water: 28 per cent NH_4OH, 100: 100: 2, v/v; upper phase). Analysis of the hydrolyzate of this compound showed that it was peptide(s), consisting of alanine, glutamic acid and DAP. The DNP derivatives of peptides C and D, but not peptides A and B, were ether-soluble. These observations are interpreted by assuming that elimination of amide from the (iso-) glutaminyl residues in peptides makes their DNP derivatives ether-soluble. In this connection the work of MUÑOZ *et al.* (1966) would be cited, reporting that the DNP-derivative of isoglutaminyl-lysyl-alanine was poorly soluble

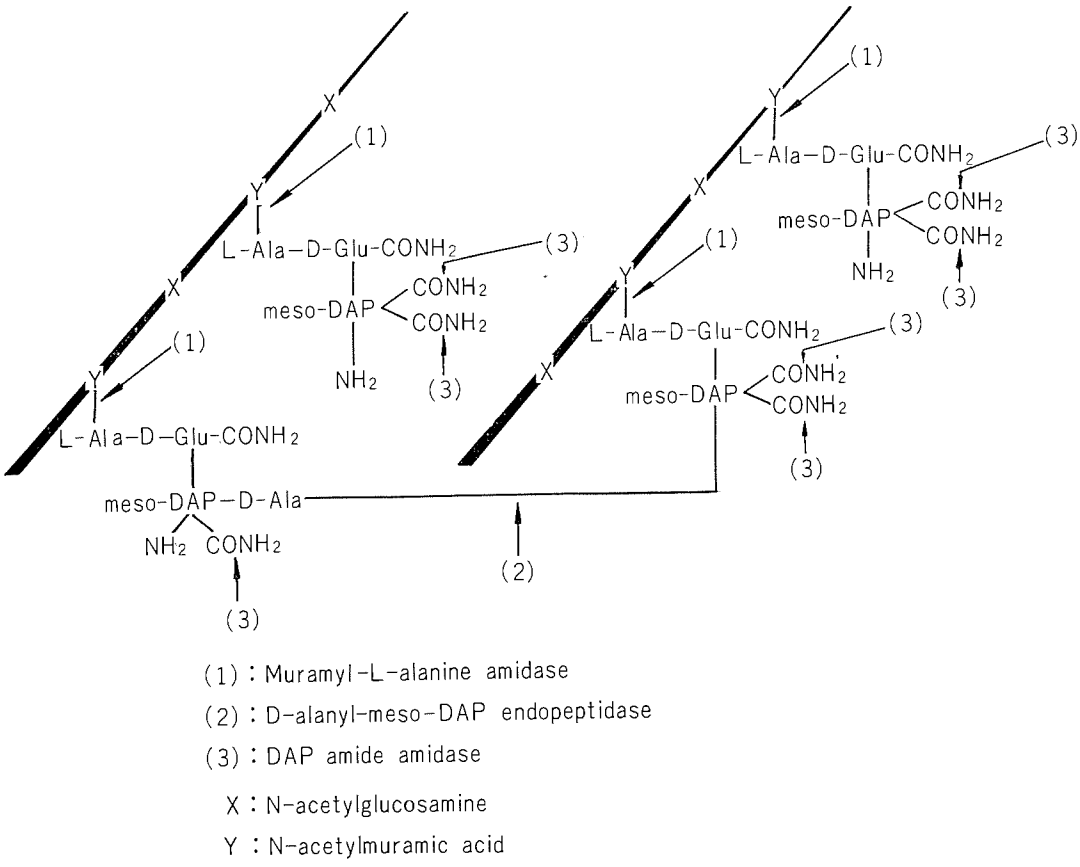

FIGURE 8 Proposed structure of *L. plantarum* cell wall peptidoglycans and sites of action of the *Streptomyces* L-3 enzyme.

in ether, but that of α- or γ-glutamyl-lysyl-alanine was soluble.

As regards amidation of the carboxyl groups of DAP, the work of KATO, STROMINGER and KOTANI (1968) should be mentioned. These authors determined the peptide terminal groups liberated from *C. diphtheriae* cell walls by digestion with L-3 enzyme and characterized the peptides isolated from the cell wall digest, they found that the lytic action of L-3 enzyme on the cell walls was mainly due to D-alanyl-DAP endopeptidase and N-acetylmuramyl-L-alanine amidase as it was with *L. plantarum* cell walls, and that the L-3 enzyme preparation exhibited another enzymic activity in deamidation of the amide(s) of DAP, namely DAP amide amidase activity. The L-3 enzyme exerts DAP amide amidase activity against both *L. plantarum* and *C. diphtheriae* walls, since no significant amount of COOH-terminal DAP was present in intact *L. plantarum* cell walls but by L-3 enzyme treatment the amount increased by about 0.4 mole per mole of glutamic acid without parallel liberation of any corresponding NH_2-terminal amino acids, and since most of the peptides isolated from the *L. plantarum* cell wall digest had COOH-terminal DAP. Recently, it was shown that the cell walls of *L. plantarum* ATCC 8014 were solubilized by an enzyme preparation (CM-1 enzyme) from

the culture supernatant of *Flavobacterium sp.* (L-11 bacterium, KOTANI *et al.*, 1959a, 1959b; KATO *et al.*, 1962, 1968) grown in a rich medium with vigorous aeration and that the only peptide terminal group liberated by digestion with this enzyme was NH_2-terminal L-alanine. Direct evidence for the cross linkage between D-alanine and DAP and for amidation of the carboxyl groups of DAP have also been obtained using CM-1 enzyme. Details of this study will be given in the following paper.

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