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ISOLATION AND PURIFICATION OF D-ALANYL-*MESO*-2, 6-DIAMINOPIMELIC ACID ENDOPEPTIDASE OF *STREPTOMYCES* L-3 ENZYME USING SOLUBLE SUBSTRATES OF KNOWN CHEMICAL STRUCTURE FROM *LACTOBACILLUS PLANTARUM* CELL WALL DIGESTS[1]

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SUMMARY D-Alanyl-*meso*-2, 6-diaminopimelic acid (D-Alanyl-*meso*-A_2pm) endopeptidase was isolated and purified from a crude *Streptomyces* L-3 enzyme preparation by ion exchange chromatography and isoelectric focusing in a density gradient. During its purification, its hydrolytic activity was assayed on cell walls of *Lactobacillus plantarum* ATCC 8014 and soluble glycopeptides and peptides, of known chemical structures, prepared enzymatically from these cell walls. A fraction with an isoelectric point of pH 7.9 cleaved the bond between the carboxyl group of the D-alanine residue at the C-terminal in one peptide subunit and one of the two amino groups of the A_2pm residue in the neighboring peptide subunit. Unlike the crude enzyme, the endopeptidase in this fraction showed no *N*-acetylmuramyl-L-alanine amidase, A_2pm carboxyamide amidase or proteinase(s) activity and it was immunologically homogeneous.

INTRODUCTION

Enzymes which hydrolyze specific linkages in cell wall peptidoglycans under mild and controlled experimental condition are very useful in studies on the structures (subcellular or molecular) and functions of bacterial cell surface layers, and especially the biological and immunological properties of cell wall peptidoglycans. Thus there have been many studies on the isolation, properties and use of these enzymes (Ghuysen, Tipper and Stro-

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minger, 1966; Ghuysen 1968; Kato, 1975).

The L-3 enzyme of *Streptomyces* sp., isolated from a soil specimen by the authors (Mori et al., 1960; Mori and Kotani, 1962) specifically splits D-alanyl-*meso*-A_2pm linkages in cell wall peptidoglycans, in which the principal dibasic amino acid is A_2pm, from bacteria such as *Lactobacillus plantarum* ATCC 8014 (Matsuda et al., 1968a), *Mycobacterium bovis*, BCG Takeo (Kotani et al., 1968a, 1968b), *Corynebacterium diphtheriae* Park-Williams No. 8 (Kato et al., 1968), *Mycobacterium tuberculosis* H37Rv (Kotani et al., 1970) and *Listeria monocytogenes* NCTC 7973 (Kotani, Yanagida and Kato, unpublished). The L-3 enzyme seemed to have unique substrate specificity, although there are reports of the presence of D-alanyl-*meso*-A_2pm endopeptidase in autolyzates of *Escherichia coli* (Pelzer, 1963a, 1963b; Weidel and Pelzer, 1964) and in the culture filtrate of *Streptomyces albus* G grown in the medium supplemented with autoclaved cells of *Bacillus megaterium* KM (Bricas et al., 1967). Ghuysen and coworkers have studied the DD carboxypeptidase (KM endopeptidase) of *Streptomyces albus* G which hydrolyzes D-alanyl-*meso*-A_2pm linkages extensively over the past five years (Ghuysen et al., 1969; Ghuysen et al., 1970; Leyh-Bouille et al., 1970; Ghuysen et al., 1972; Ghuysen and Shockman, 1973).

Pure enzymes with clearly defined specificities are required for studies on peptidoglycans. Contaminating proteases in particular, may cause erroneous results in studies on bacterial cell surface layers, becuase they degrade protein components with special biological or immunological functions, such as the antiphagocytic M protein which is chief virulence factor and is essential to the cell walls of group A *Streptococcus pyogenes* (Namba et al., 1974, 1975).

Besides the D-alanyl-*meso*-A_2pm endopeptidase the L-3 enzyme preparation contains an *N*-acetylmuramyl-L-alanine amidase which cleaves the linkages between glycan and peptide moieties and a non-lytic amidase, which hydrolyzes amides on carboxyl groups of A_2pm residues (Matsuda et al., 1968a; Kato et al., 1968; Kotani et al., 1970). Protease activity on casein has also been detected in crude preparations of the L-3 enzyme. Separation of the L-3 endopeptidase by zone electrophoresis on a starch block has been described (Matsuda et al., 1968c). This paper is on the isolation of D-alanyl-*meso*-A_2pm endopeptidase in homogeneous form from a culture filtrate of *Streptomyces* sp. L-3 bacterium by ion exchange chromatography and isoelectric focusing in a density gradient.

MATERIALS AND METHODS

1. *Production of a crude L-3 enzyme preparation*

Streptomyces sp. L-3 bacterium was maintained by serial subculture on 0.1% Bacto peptone (Difco) slants supplemented with 0.025% $MgSO_4 \cdot 7H_2O$ and 0.025% K_2HPO_4, and stored at 4 C. A seed culture was grown for 7 to 10 days at 28 C, and three loopfuls of the culture were inoculated into a Roux's bottle containing about 400 ml of modified McCarty's medium. The L-3 bacterium was grown as a surface pellicle at 28 C for about one week (Mori et al., 1960).

About 30 liters of the L-3 bacterium culture were centrifuged at $10,000 \times g$ using a continuous flow rotor and the cells were discarded. All subsequent procedures were carried out at approximately 4 C. The dark-brown supernatant was adjusted to 30% saturation of ammonium sulfate with solid salt and left overnight. The mixture was centrifuged and the supernatant was adjusted to 80% saturation of ammonium sulfate with constant stirring and stood overnight. Then the precipitate was collected by filtration through filter paper with 2 cm of Celite 503 (John-Manville Products, N.Y., U.S.A.) as a filter aid. The resulting filter cake was suspended in cold water and filtered through a coarse sintered glass filter and the filter was washed with water. The darkbrown filtrate and washing fluid were combined and dialyzed against 0.01 M sodium phosphate buffer, pH 7.8. Insoluble material formed during dialysis was removed by centrifugation at $10,000 \times g$ for 15 min. The resulting solution contained about 70% of the lytic activity of the crude enzyme and showed 15-fold increase in specific activity per mg protein, as estimated to *C. diphtheriae* whole cells.

About 3 liters of this concentrated fluid were obtained from 150 liters of culture.

2. *Cell wall lytic enzymes other than the L-3 enzyme*

The following enzymes were used to isolate the soluble substrates required in assay of L-3 enzyme. Crude L-11 enzyme (Lot 45) from the culture supernatant of *Flavobacterium* sp. grown in 2% glucose-broth with aeration was obtained by 100% saturation of ammonium sulfate and generously supplied from Fuji Pilot Plant, Kyowa Fermentation Industries, Tokyo. It was partially purified by DEAE-cellulose and CM-cellulose column chromatographies, as will be reported elsewhere. The *Chalaropsis* enzyme (3688C-51-16) was a gift from Dr. P. A. Miller (Lederle Laboratories, American Cyanamide Co., N.Y., U.S.A.).

3. *Whole cells, cell walls and cell wall digests as substrates in L-3 enzyme assay*

C. diphtheriae whole cells and cell walls: Strain Park-Williams No. 8 was grown at 35 C with shaking in modified Taylor's medium for 48 hr. Then the cells were harvested by centrifugation, washed thoroughly with saline and deionized water and lyophilized. Cell walls were prepared by sonication of the cells with glass beads, and then digestion with pronase, as described previously (Matsubara, 1965).

L. plantarum cell walls: Cells of *L. plantarum* ATCC 8014 were disrupted using a Braun cell homogenizer. The crude cell wall fraction obtained by differential centrifugation was digested with crystalline trypsin as described previously (Matsuda et al., 1968a). Then the walls were treated with weak alkali to remove the alkali-labile D-alanine residue ester-linked to the teichoic acid in the walls. For this, lyophilized walls were suspended in 0.1 M sodium pyrophosphate, pH 9.3 at a concentration of 10 mg/ml, and stirred for 3 hr at 37 C. This treatment removed the teichoic acid D-alanine completely, as shown by estimating released alanine by thin layer silica gel chromatography (Ghuysen et al., 1966).

Digest of *L. plantarum* cell walls with the *Chalaropsis* enzyme as starting material for preparation of chemically defined substrates: Trypsinized cell walls (5 g) of *L. plantarum* were digested with 2.5 mg of the *Chalaropsis* enzyme in a final volume of 100 ml of 0.01 M acetate buffer, pH 4.7 at 37C for 7 hr. This treatment digested the cell walls almost completely. Then the mixture was centrifuged. The resulting supernatant contained about 1.4 moles of free reducing groups per mole of total glutamic acid residues.

Hydroxymethylation of the free amino groups of soluble, chemically defined substrates: Free amino groups were hydroxymethylated to reduce the background in estimation of the terminal groups liberated by the enzyme. Hydroxymethylation was carried out as described by Ghuysen et al. (1966).

4. *Enzyme assays*

Cell wall or whole cell lytic activity: Lytic activity was measured at 37 C by determining the rate of reduction of turbidity (optical density at 550 nm, OD_{550}) of a suspension of lyophilized cell walls or whole cells of *C. diphtheriae*. For this, 1.6 ml of suspensions of cell walls and whole cells (containing 1.6 mg of walls and 1.28 mg of cells, in dry wt.) were incubated with 0.8 ml of 0.02 M sodium phosphate buffer, pH 7.8, containing an appropriate amount of the sample of enzyme in a final volume of 3.2 ml and final molarity of 0.02 M in a 12 × 100 mm test tube in an ice bath. Turbidity was recorded at 5-min intervals using Shimadzu Bausch & Lomb Spectronic 20 Colorimeter (Shimadzu Seisakusho, Kyoto). One unit of lytic activity was defined as the amount causing 35% reduction in turbidity of the cell walls and 50% reduction in that of whole cells in one hour under these conditions. Controls without substrate or without enzyme were also set up.

Hydrolytic action on soluble substrates: Hydrolysis of soluble peptidoglycan subunits from *L. plantarum* cell walls was assayed by estimating liberation of amino groups from hydroxymethylated substrates. For this a sample of substrate containing 12 to 16 nmole equivalents of total glutamic acid residues was incubated with the sample of enzyme in 0.02 M sodium phosphate buffer, pH 7.8 at 37 C. Samples of the mixture were taken at intervals, dried in vacuo and incubated with 80 μl of a 0.8% (w/v) sodium borate solution and 10 μl of 0.1 M 2, 4-dinitrofluorobenzene (FDNB) at 60 C for 30 min. Then their absorbancy was measured at 420 nm. The amount of free amino groups liberated was expressed in moles per mole of total glutamic acid residues in the test substrate.

Caseinase activity: Caseinase activity was determined by the casein-Folin method, based on the description of Hagihara and Yonetani (1956). An appropriate amount of test sample was diluted with

water to 150 μl, and incubated with 300 μl of 1% casein solution (Hammarsten, E. Merck, West Germany) adjusted to pH 7.8 and 150 μl of 0.02 M phosphate buffer, pH 7.8 at 37 C for exactly 20 min. Then 500 μl of 0.11 M trichloroacetic acid containing 0.22 M sodium acetate and 0.33 M acetic acid were added to precipitate undigested casein. The mixture was centrifuged at $650 \times g$ for 10 min and 200 μl of the supernatant was mixed with 500 μl of 0.05 M sodium carbonate and 100 μl of Folin reagent, diluted with 2 volumes of water and incubated at 37C for 30 min. Then its absorbancy at 660 nm was measured with a Hitachi Perkin-Elmer Spectrophotometer (Model 139 UV-VIS, Hitachi Ltd., Tokyo). As a control, casein solution was incubated without enzyme. One unit of caseinase activity was defined as the amount causing 0.10 increase in OD_{660}. Using this procedure the absorbancy was linearly proportional to the activity over a range of 0.1 to 0.7 units.

5. *Isoelectric focusing in a density gradient*

Isoelectric focusing in a density gradient was carried out in an LKB column (LKB-Produkter AB, Broma, Sweden) of 110 ml capacity, as described by Vesterverg and Svensson (1966) and Vesterberg (1971). An almost linear gradient was prepared with 50% (w/v) sucrose and carrier ampholites (Ampholine pH 7.0 to 10.0, LKB-Produkter AB). The test sample (containing 1.8 to 10 mg protein) was placed on the column, and a potential of 700 v was applied for 43 hr at 1 C. Then fractions of 1.5 ml were collected from the bottom of the column and their pH value was rapidly determined at 1 C with a pH meter (Model PHM 26, Radiometer, Denmark). The enzyme activity of each fraction was estimated with *C. diphtheriae* whole cells and the protein concentration was estimated in terms of absorbancy at 280 nm.

6. *Immunological methods*

Preparation of anti-L-3 enzyme serum: A sample of 1.2 ml of the concentrated crude L-3 enzyme, containing 4 mg of protein and 38 lytic units on *C. diphtheriae* cell walls, was mixed with 0.8 ml of a solution of 100 mg of dehydrostreptomycin and 10,000 units of penicillin G in deionized water. The mixture was vigorously sonicated with 4 ml of Bacto-complete Freund adjuvant to make a water-in-oil type emulsion and 1 ml volumes were injected intramuscularly into two sites in back of Japanese domestic adult rabbits weighing 2 to 2.5 kg. Similar injections were given at weekly intervals for 2 weeks and one week after the third injection, the rabbits were injected intravenously with one ml of the same L-3 enzyme solution without the adjuvant. They were bled one week later. Immune serum specimens were added with 0.1% sodium azide as a preservative, and stored at 4 C.

Immunoelectrophoresis: This was performed as described by Ouchterlony (1968). Two wells (about 5 mm diameter) were cut out, as shown in Fig. 12, and filled with different test antigens. A potential of 6 v/cm was applied in barbital buffer (μ=0.05 and pH 6.8) at 4 C for 120 min. Then a trough (3×55 mm) was cut out between the two antigen wells and anti-L-3 crude enzyme immune rabbit serum was placed in it and immunodiffusion was allowed to proceed for 72 hr at room temperature in a moist chamber.

7. *Analytical procedures*

Free, total, N- and C-terminal amino acid: These were estimated by the method of Ghuysen et al. (1966). Total amino groups were determined after hydrolyzing in 4 N hydrochloric acid at 100C for 8 hr.

N-terminal and C-terminal amino acids were measured by thin layer chromatography by a slight modification of the method of Ghuysen et al. (1966). The mono-DNP-A_2pm specimen used as a reference in estimation of mono-amino-A_2pm was prepared as described by Jušić et al. (1963). To test the purity of a test mono-A_2pm-specimen, a sample was applied to Toyo Roshi filter paper (No. 51A) and chromatographed for 16 hr at room temperature by the descending technique in methanol-water-10 N hydrochloric acid-pyridine (80: 17.5: 2.5: 10, by vol). Then the paper was sprayed with ninhydrin reagent.

Amino acids and amino sugars: These were determined after hydrolysis with 6 N hydrochloric acid at 100 C for 12 to 14 hr, using a Hitachi Amino Acid Analyzer (Model KLA-3B). The column temperature was set at 45C to obtain good separation of muramic acid and glutamic acid. N-Terminal amino acids and amino sugars at the reducing end were determined by the dinitrophenylation technique (Ghuysen et al., 1966) and borohydride reduction method (Tipper and Strominger, 1966), respectively.

Ammonia: The ammonia content of hydrolyzates of test specimens was determined by the modification by Okuda and Fujii (1966) of the method of

Fawcett and Scott (1960). The microdiffusion method was used to determine ammonia in Edman degradation products for the reason described before (Kotani et al., 1970).

Edman degradation: The method of Tipper et al. (1967) was used to determine the amino acid sequence of N-terminal parts of test peptides.

Others: Total amino sugars were estimated by the method of Ghuysen et al. (1966) with *N*-acetylglucosamine as a standard. Free reducing groups were determined by the ferricyanide method of Park and Johnson (1949). Hexose and protein contents were estimated by the methods of Aschwell (1957) and Lowry et al. (1951), respectively. Protein was also measured as absorbancy at 280 nm.

RESULTS

1. *Preparation of chemically defined substrates for assay of the L-3 enzymes*

1) *L. plantarum* bisdisaccharide-peptide subunit dimer

a) Digestion of *L. plantarum* cell walls with the *Chalaropsis* enzyme and fractionation of the digest: Trypsinized cell walls (5 g) were incubated with 100 ml of 0.01 M acetate buffer, pH 4.7 containing 2.5 mg of the *Chalaropsis* enzyme (endo-*N*-acetyl- or endo-*N*, 6-*O*-diacetylmuramidase) for 7 hr at 37 C. During this treatment the cell walls became almost completely solubilized and turbidity decreased to nearly zero with liberation of 1.4 moles of reducing groups per mole of total glutamic acid residues in the walls. A sample of the digest, equivalent to 1.5 g of the walls, was applied to an ECTEOLA-cellulose (Cl^-) column (2.5 × 45 cm). The neutral and basic fractions eluted with water, consisting mainly of amino sugars and amino acids, were combined and filtered successively through columns of Sephadex G-50, G-50 and G-25 (1.9 × 97 cm, 1.9 × 97 cm and 2.5 × 97 cm, respectively) connected in series by capillary polyethylene tubes. The columns were eluted with water, and fractions of eluate were assayed for total hoxoses, free amino groups and reducing groups.

The fractions eluted immediately after the void volume contained almost all the hexose in the specimen applied and a small amount of amino sugars, and were essentially free from free amino and reducing groups (Fig. 1). Free amino and reducing groups were eluted in the medium and lower molecular weight fractions (IB-1 and IC-1, respectively). These two fractions were purified further by chromatography on a Dowex-1 (COO^- form) column (0.9 × 3.5 cm) and eluted with a linear gradient of 0 to 1.0 M acetic acid to remove acidic contaminants.

b) Chemical characterization of glycopeptides isolated from the *Chalaropsis* digest: The

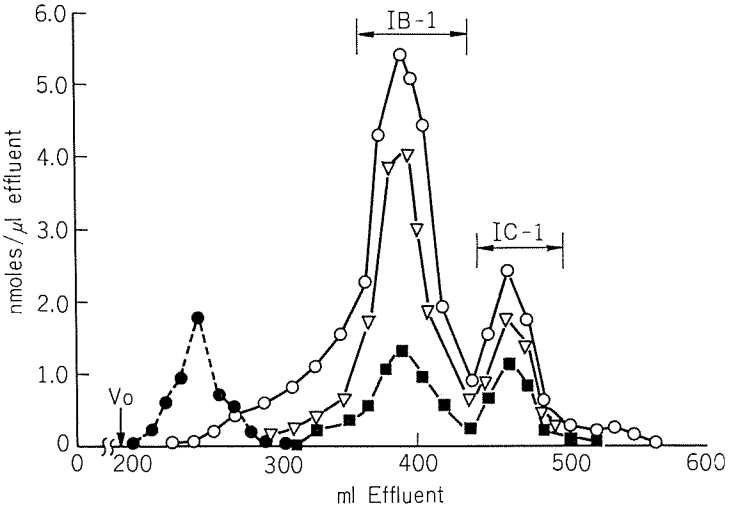

FIGURE 1. Gel filtration pattern of the ECTEOLA-cellulose H_2O fraction of the *Chalaropsis* enzyme digest of *L. plantarum* cell walls. The fraction eluted with water from the ECTEOLA-cellulose column was applied on columns of Sephadex G-50, G-50 and G-25 connected in series. The columns were eluted with water. Amino sugar (○——○), reducing groups (▽——▽), hexose (●·····●), free amino groups (■——■).

contents of amino sugars and amino acids in fractions IB-1 and IC-1, before and after dinitrophenylation or reduction with sodium borohydride are shown in Table 1 as molar ratios to total glutamic acid residues. Fraction IB-1 contained glucosamine, muramic acid, alanine, glutamic acid and A_2pm in a molar ratio of about 1: 1: 1.5: 1: 1. Half the A_2pm residue remained after dinitrophenylation, indicating that the peptide portion was a dimeric form linked through one of the amino groups of the A_2pm residue in one peptide subunit. The muramic acid disappeared on borohydride reduction, indicating the existence of a disaccharide unit with muramic acid at the reducing end. Thus fraction IB-1 was a bisdisaccharide-peptide subunit dimer, consisting of one disaccharide tetrapeptide N^{α}-(β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-*meso*-A_2pm-D-alanine and one disaccharide tripeptide N^{α}-(β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-*meso*-A_2pm (Fig. 2). Two moles of ammonia were obtained per mole of glutamic acid indicating that four of the five carboxyl groups of the glutamic acid and A_2pm residues were amidated.

Fraction IC-1 consisted of glucosamine, muramic acid, alanine, glutamic acid, A_2pm and ammonia in a molar ratio of about 1: 1: 1: 1: 1: 2. The A_2pm residue in this fraction, unlike that in fraction IB-1, disappeared almost completely on dinitrophenylation. All the muramic acid was reduced to muramicitol by treatment with borohydride showing that the glycan portion was a disaccharide with the

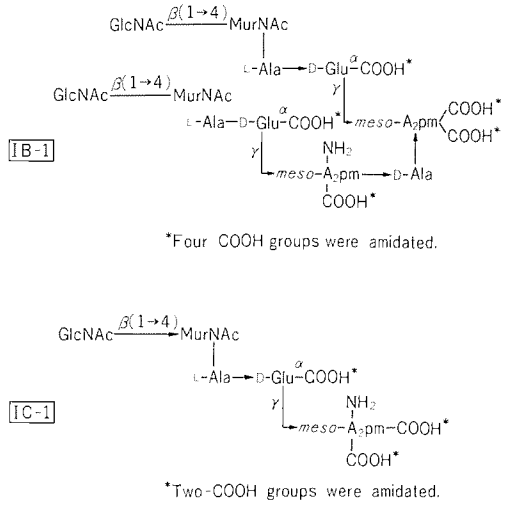

FIGURE 2. Proposed structures of glycopeptides isolated from the *Chalaropsis* enzyme digest of *L. plantarum* cell walls. GlcNAc: *N*-Acetylglucosamine, MurNAc: *N*-Acetylmuramic acid, A_2pm: 2, 6-Diaminopimelic acid.

TABLE 1. *Amino sugar and amino acid compositions of glycopeptides isolated from the Chalaropsis enzyme digest of L. plantarum cell walls before and after dinitrophenylation or borohydride reduction*

Fraction	Treatment[a]	GlcN	Mur	Ala	Glu	A_2pm	NH_3[b]
IB-1	1	0.88[d]	0.84[d]	1.56[d]	1.00	1.04[d]	1.93[d]
	2	0.73	0.75	1.53	1.00	0.57	ND[c]
	3	0.93	0	1.68	1.00	1.11	ND
IC-1	1	0.90	0.86	1.16	1.00	1.03	2.06
	2	0.80	0.71	1.12	1.00	0.13	ND
	3	0.80	0	1.20	1.00	1.05	ND

[a] 1: untreated, 2: dinitrophenylated, 3: reduced with sodium borohydride.

[b] Determined by the method of Okuda and Fujii.

[c] Not determined.

[d] Values are expressed as moles per mole of total glutamic acid residues. No corrections were made for loss during hydrolysis.

reducing muramic acid. Thus IC-1 is a disaccharide tripeptide monomer N^{α}-(β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-*meso*-A_2pm in which two of the three carboxyl groups of the A_2pm and glutamic acid residues are amidated (Fig. 2). The structures proposed in the preceding and following paragraphs were deduced with reference to previous findings (Matsuda et al., 1968a, 1968b).

2) *L. plantarum* peptide subunit monomers and dimer

a) Digestion of the *L. plantarum Chalaropsis* enzyme lysate with the L-11 enzyme and fractionation of the digest: The *Chalaropsis* enzyme lysate obtained from 3.5 g of *L. plantarum* cell walls was incubated with 350 U (estimated with *L. plantarum* cell walls) of the L-11 enzyme in 70 ml of 0.025 M barbital buffer, pH 8.5 for 72 hr at 37 C. During the digestion, about one mole of N-terminal alanine per mole of glutamic acid residues was liberated, indicating almost complete cleavage of the *N*-acetylmurmyl-L-alanine linkages between glycan and peptide portions.

Then the mixture was applied to a CM-cellulose (H^+ form) column (1.0×40 cm). The column was washed with water and then eluted with 0.5 M ammonia. The basic peptides eluted were chromatographed on an Amberlite CG-120 column (0.9×72 cm), as reported previously (Matsuda et al., 1968a). Five peak fractions (IIB-1, 2, 3, 4 and 5) were obtained on elution with a linear gradient of 0.2 M pyridine-acetate buffer, pH 3.1 to 2.0 M pyridine-acetate, pH 5.0, as shown in Fig. 3. The homogeneity of each fraction was examined by paper electrophoresis with 0.1 M pyridine-acetate buffer, pH 5.0 as solvent. Three fractions (IIB-3, 4 and 5) migrated towards the cathode as single spots (Fig. 4). The others, which were mixtures of neutral peptides, were not studied further.

b) Chemical characterization of peptides, IIB-3, IIB-4 and IIB-5: Table 2 summarizes the analytical results on the total, N-terminal and C-terminal amino acids and ammonia in the test peptides. No amino sugars were detected.

IIB-3: This peptide contained alanine,

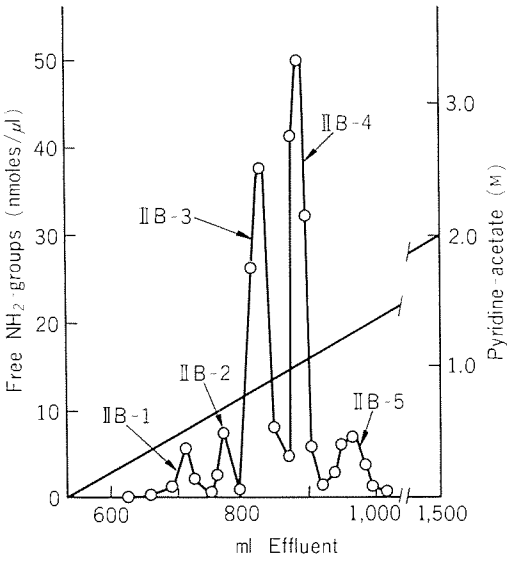

FIGURE 3. Fractionation of the L-11 enzyme digest of the *Chalaropsis* enzyme lysate of *L. plantarum* cell walls on an Amberlite CG-120 column. The column was eluted with 0.2 M pyridine-acetate buffer, pH 3.1 and a linear gradient of 0.2 to 2.0 M pyridine and of pH 3.1 to 5.0. Fractions of 10 ml were collected.

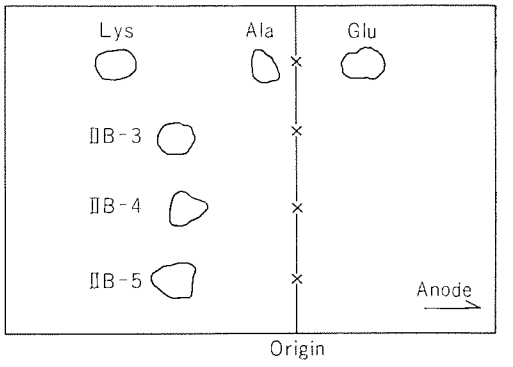

FIGURE 4. Paper electropherogram of peptide fractions isolated by Amberlite CG-120 column chromatography of the L-11 enzyme digest of the *Chalaropsis* enzyme lysate of *L. plantarum* cell walls. Electrophoresis was carried out in 0.1 M pyridine acetate buffer, pH 5.0 for 150 min at a potential of 14 v/cm.

TABLE 2. *Total, N-terminal and C-terminal amino acids and ammonia of peptide subunit monomers and dimer isolated from the L-11 enzyme digest of the Chalaropsis lysate of L. plantarum cell walls*

Fraction	Amino acid composition[a]			N-terminal amino acid[b]		C-terminal amino acid[b]		Ammonia
	Ala	Glu	A_2pm	Ala	mono-amino-A_2pm	Ala	A_2pm	
IIB-3	2.07	1.00	0.89	1.00	0.92	0.75	0	2.48[c] (2.95[a])
IIB-4	1.18	1.00	0.90	1.00	0.87	0	0	1.97 (1.74)
IIB-5	1.60	1.00	1.12	1.16	0.61	0	0	2.00 (2.10)

[a] By amino acid analyzer, [b] by thin layer chromatography and [c] by the method of Okuda and Fujii. Values are expressed as moles per mole of total glutamic acid residues.

glutamic acid, A_2pm and ammonia in a molar ratio of about 2: 1: 1: 3. One mole of either alanine or A_2pm residue was found to be N-terminal. Hydrazinolysis of the peptide showed that one mole alanine was the C-terminal amino acid. These data are compatible with the structure proposed in Fig. 5.

IIB-4: Approximately equimolar amounts of alanine, glutamic acid and A_2pm were found with one mole each of N-terminal alanine and mono-amino-A_2pm. No C-terminal amino acid was detected. The sequence of amino acids in this tripeptide was determined by Edman degradation. In the first cycle of degradation, N-terminal alanine disappeared and about one mole of glutamic acid was exposed as the new N-terminal amino acid with loss of mono-amino-A_2pm. The N-terminal glutamic acid disappeared during the second cycle of degradation, and a significant amount of ammonia was detected by microdiffusion. This suggests that the α-carboxyl group of the glutamic acid is combined with ammonia. A structure for peptide IIB-4 compatible with the analytic data is shown in Fig. 5.

IIB-5: The constituent amino acids of this peptide were alanine, glutamic acid and A_2pm in a molar ratio of about 1.5: 1: 1. About 2 moles of ammonia were present per mole of total glutamic acid. One mole of alanine and about 0.5 mole of A_2pm were located in the N-terminal. No C-terminal was detected. These analytical findings can be most reasonably explained by supposing that fraction IIB-5 is a peptide subunit dimer in which one tetrapeptide, L-alanyl-D-isoglutaminyl-*meso*-A_2pm-D-alanine and one tripeptide, L-alanyl-D-isoglutaminyl-*meso*-A_2pm are joined by a direct bond involving the C-terminal D-alanine residue of the tetrapeptide and the free amino

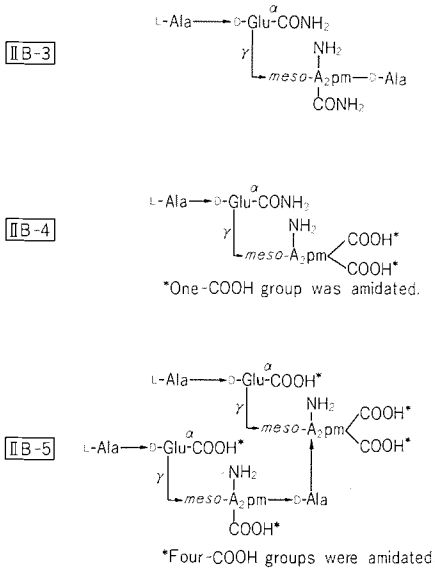

FIGURE 5. Proposed structures of peptide subunits isolated from the L-11 enzyme digest of the *Chalaropsis* lysate of *L. platarum* cell walls. GlcNAc: *N*-Acetylglucosamine, MurNAc: *N*-Acetylmuramic acid, A_2pm: 2, 6-Diaminopimelic acid.

group of the A_2pm residue of the tripeptide, as shown in Fig. 5. The absence of C-terminal A_2pm and the presence of 2 moles of ammonia per mole of total glutamic acid residues indicate that at least one carboxyl group of the A_2pm residue in the tripeptide was amidated.

2. *Partial purification of the crude L-3 enzyme*

1) Negative adsorption on a DEAE-cellulose column

A crude L-3 enzyme preparation (1 liter) was concentrated by dialysis against 20% (w/v) polyvinylpyrrolidone (mol wt 7,000,000 dalton, Nakarai Chemicals, Kyoto) for 96 hr. Then it was dialyzed against 0.05 M phosphate buffer, pH 8.0 at 4 C. A precipitate formed during dialysis was removed by centrifugation at 6,000 $\times g$ for 20 min and washed. The supernatnat and washing fluid were combined (total volume, 350 ml). This procedure increased the specific lytic activity per mg protein against *C. diphtheriae* cell walls 1.5 fold without any appreciable loss of enzyme activity.

A 2 ml sample of the concentrated enzyme (31.5 U/ml against *C. diphtheriae* walls) was applied to a DEAE-cellulose column (2.0 × 4.0 cm) equilibrated with 0.05 M phosphate buffer, pH 8.0. The column was washed with 150 ml of 0.05 M phosphate buffer, pH 8.0, and then eluted stepwise with 550 ml volumes of the same buffer containing 0.2 M, 0.4 M, 0.6 M and 0.8 M sodium chloride, respectively.

The elution profile is shown in Fig. 6. The cell wall lytic and caseinase activities were not adsorbed whereas much of inactive proteins and the bulk of the colored materials were. In this way nearly 90% of the lytic activity on *C. diphtheriae* walls was recovered and about half the inactive proteins were removed.

2) Analyses of terminal amino acids liberated from *L. plantarum* walls by the L-3 enzyme fraction from the DEAE-cellulose column (DEAE-cellulose fraction)

Alkali-treated cell walls (10 mg) of *L. plantarum* were incubated at 37 C with 2 U (assayed with *C. diphtheriae* walls) of the enzyme from DEAE-cellulose in one ml of 0.02 M phosphate buffer, pH 7.8. Samples of the reaction mixture were taken at intervals to measure reduction in OD_{550} and increase in free amino groups. The N-terminal and C-terminal amino acids were also determined.

As shown in Fig. 7, during the reaction the turbidity decreased nearly 95% with concomitant release of about 2 moles of free amino groups. As shown in Fig. 8, roughly equimolar amounts (0.5 moles per mole of glutamic acid residue) of mono-amino-A_2pm and C-terminal alanine were liberated, after incubation for 24 hr, one of the amino groups of all the A_2pm residues had become free. This suggests that the cross linkages between the C-terminal D-alanine residue of one peptide subunit and the mono-amino-A_2pm at the N-terminal of another in the test peptidoglycans were almost completely hydrolyzed. The liberations of about one mole of N-terminal alanine and 0.8 mole of C-terminal A_2pm indicated the cleavage of almost

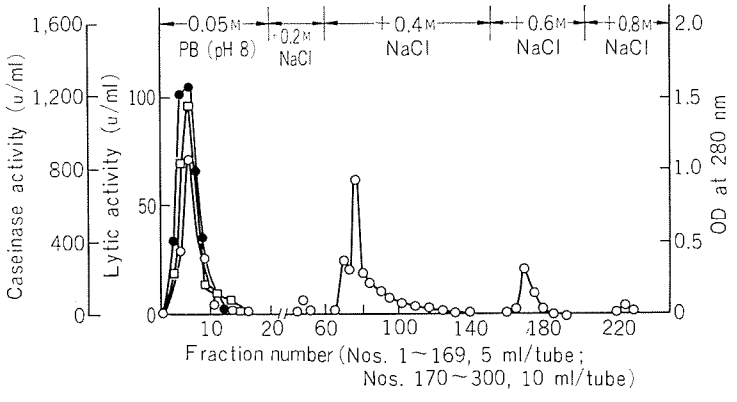

FIGURE 6. Column chromatography of the L-3 crude enzyme on DEAE-cellulose. Stepwise elution was performed by increasing concentrations of sodium chloride in 0.05 M phosphate buffer, pH 8.0. Lytic activity against *C. diphtheriae* whole cells (●——●), caseinolytic activity (□——□), absorbance at 280 nm (○——○).

all of the linkages between glycan and peptide moieties and the deamination of both carboxyl groups of almost all A_2pm residues. Thus D-alanyl-*meso*-A_2pm endopeptidase, *N*-acetylmuramyl-L-alanine amidase and A_2pm carboxyamide amidase, were all present in the enzyme from DEAE-cellulose.

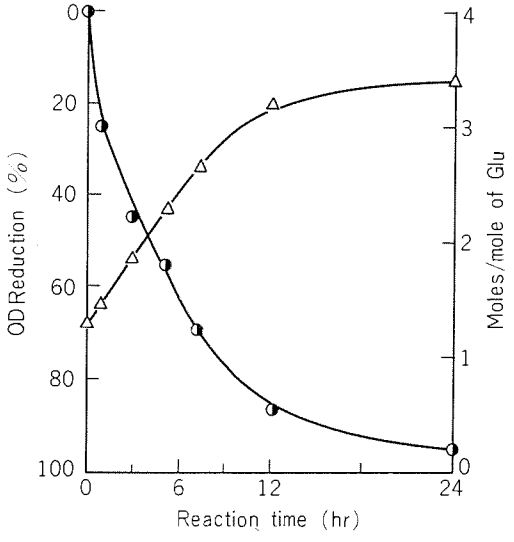

Figure 7. Digestion of *L. plantarum* cell walls with release of amino groups on incubation with the L-3 enzyme from DEAE-cellulose. Free amino groups (△——△), OD_{550} (◑——◑).

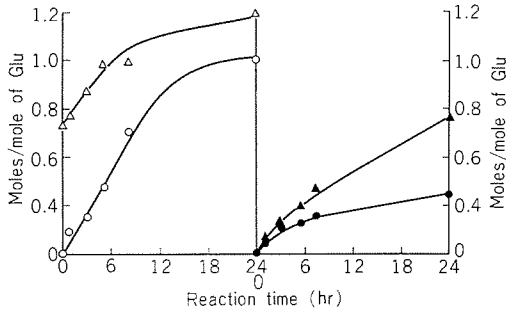

FIGURE 8. Liberations of N-terminal and C-terminal amino acids on digestion of *L. plantarum* cell walls with the L-3 enzyme from DEAE-cellulose. N-Terminal alanine (○——○), mono-amino-A_2pm (△——△), C-terminal alanine (●——●), C-terminal A_2pm (▲——▲).

3. *Separation of the* D-*alanyl-meso-*A_2*pm endopeptidase from amidases by chromatography*

The fractions of the L-3 enzyme from the DEAE-cellulose column with lytic activity (assayed with *C. diphtheriae* whole cells) were combined and concentrated by precipitation with 80% saturation of ammonium sulfate. The concentrated material was dissolved in water, and dialyzed first against water and then against 0.01 M phosphate buffer pH 7.0. A 15 ml sample of the resulting partially purified L-3 enzyme, containing 150 lytic units against *C. diphtheriae* cell walls and 6.35 mg of protein per ml, was adsorbed on a CM-cellulose column (3.0×21 cm) equilibrated with 0.01 M phosphate buffer, pH 7.0. The column was washed with the same buffer until the absorbance of the effluent at 280 nm became negligible, and then eluted with a linear gradient of 0 to 0.2 M sodium chloride in the same buffer, pH 7.0 at a flow rate of 60 ml per hr.

Figure 9 shows the elution profiles of lytic activity against *C. diphtheriae* whole cells, caseinase activity and absorbance at 280 nm. The fractions eluted with 0.01 M phosphate buffer, pH 7.0 (CM-cellulose Fraction A, tubes No. 5 to 20) had strong caseinolytic activity but very weak lytic activity. Two other fractions with strong lytic activity on whole cells of *C. diphtheriae* were separted by gradient elution with sodium chloride. One of these (Fraction B, tubes No. 60 to 80) had no significant caseinolytic activity and the other (Fraction C, tubes No. 86 to 120) had significant proteinase activity as well as lytic activity on whole cells.

1) Actions of each fraction from the CM-cellulose column on a peptide subunit dimer and a disaccharide tripeptide monomer

For this, the hydroxymethylated peptide subunit dimer (IIB-5, 16 nmoles equiv.) and disaccharide tripeptide monomer (IC-1, 12 nmoles equiv.) were incubated with 20 μl samples of each fraction in a total volume of 40 μl in 0.02 M phosphate buffer, pH 7.8. Figure 10 shows that Fractions A, B and C all released free amino groups from the peptide subunit dimer, but that only Fraction A hy-

drolyzed the disaccharide tripeptide monomer.

The disaccharide tripeptide monomer in which two of the carboxyl groups were amidated is a substrate for both *N*-acetylmuramyl-L-alanine amidase and A_2pm carboxyamide amidase. The peptide subunit dimer with four carboxyamide groups can be cleaved by the A_2pm carboxyamide amidase, but not by *N*-acetylmuramyl-L-alanine amidase. Thus the above results show that Fraction A contained all three enzyme activities present in the enzyme from DEAE-cellulose, whereas Fractions B and C contained D-alanyl-*meso*-A_2pm endopeptidase, but not A_2pm carboxyamide amidase.

2) Actions of the three fractions from the CM-cellulose on *L. plantarum* cell walls and a bisdisaccharide-peptide subunit dimer

Fractions A, B and C were each dialyzed against 0.02 M phosphate buffer, pH 7.8 and concentrated by ultrafiltration through a Diafilter G-10T (Nippon Vacuum Technique Co., Tokyo).

Table 3 shows the maximum yields of N-terminal and C-terminal amino acids liberated from the cell walls of *L. plantarum* by Fractions A, B and C, and estimated by incubating the alkali-treated cell walls (12 mg) with appropriate amounts of the fractions in 2.4 ml of 0.02 M phosphate buffer, pH 7.8. On incubation at 37 C for 48 hr under these conditions the turbidity of the cell wall suspension decreased by 70 to 90%.

a) Fraction A: An essentially equimolecular net increase in mono-amino-A_2pm and C-terminal alanine (about 0.5 moles, per mole of total glutamic acid) was observed, indicating almost complete hydrolysis of the interpeptide D-alanyl-*meso*-A_2pm bonds. The increases in N-terminal alanine (0.8 moles) and C-terminal A_2pm (0.3 moles), on the other hand, showed that Fraction A exerted *N*-acetylmuramyl-L-alanine amidase

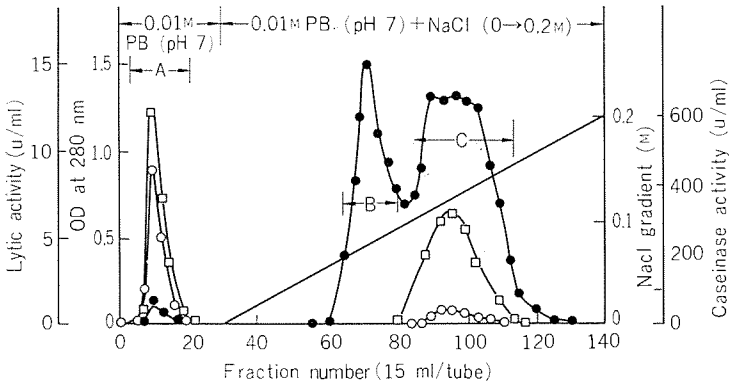

FIGURE 9. Chromatogram of the L-3 enzyme from DEAE-cellulose on a CM-cellulose column. Lytic activity against *C. diphtheriae* whole cells (●——●), caseinolytic activity (□——□), absorbance at 280 nm (○——○).

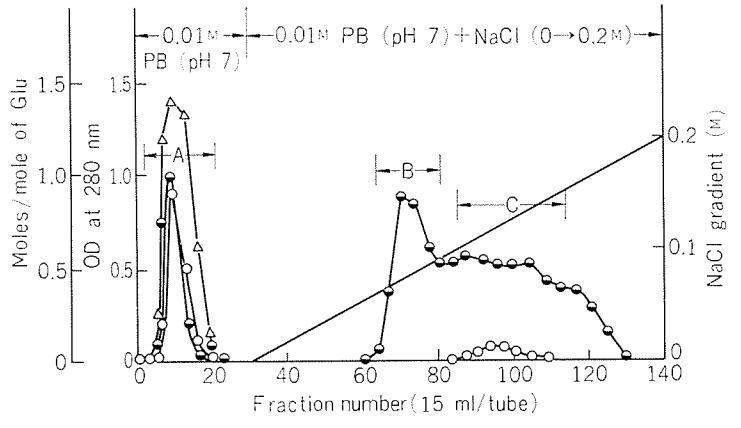

FIGURE 10. Actions of the L-3 enzyme from CM-cellulose on chemically defined, soluble substrates. Activities were determined in terms of liberation of free amino groups from hydroxymethylated specimens of disaccharide-peptide subunit monomer (di-carboxyamides) [IC-1] and peptide subunits dimer (tetra-carboxyamides) [IIB-5]. Disaccharide peptide subunit monomer (△——△), peptide subunit dimer (◒——◒), absorbance at 280nm (○——○).

and A_2pm carboxyamide amidase actions on *L. plantarum* cell walls.

b) Fractions B and C: Concomitant and practically equimolar liberations of mono-amino-A_2pm and C-terminal alanine were observed without release of either N-terminal alanine or C-terminal A_2pm (Table 3). Thus these two fractions seemed to contain only D-alanyl-*meso*-A_2pm endopeptidase. This conclusion is supported by the fact that these fractions liberated essentially equimolecular amounts of mono-amino-A_2pm and C-terminal alanine from a bisdisaccharide-peptide subunit dimer (IB-1), as shown in Table 4. Fraction C, however, was contaminated with caseinase(s) as described before.

4. *Further purification of the* D-*alanyl-meso-A_2pm endopeptidase by isoelectric focusing*

A 20 ml sample of concentrated Fraction B (containing 120 units of activity against *C. diphtheriae* cell walls and 1.8 mg of protein) was submitted to isoelectric focusing in a pH gradient from 7 to 10 for 43 hr. As shown in Fig. 11, two peaks of lytic activity on *C. diphtheriae* cells were obtained, with isoelectric points (pI) of 7.9 and 11.0, respectively. Studies with *L. plantarum* cell walls and casein as substrates showed that the fraction of pI 7.9 had strong cell wall lytic activity but no detectable caseinase activity and that of pI 11.0 had caseinase activity only.

Fraction C was also subjected to isoelectric focusing. As in the case of Fraction B, a frac-

TABLE 3. *Analyses for N-terminal and C-terminal amino acids liberated by hydrolysis of L. plantarum cell walls with the DEAE-cellulose and CM-cellulose fractions of the L-3 enzyme*

Enzyme preparation	Liberation in 24 hr[a]				Turbidity reduction %
	N-terminal[b]		C-terminal[b]		
	Ala	mono-amino-A_2pm	Ala	A_2pm	
DEAE-fraction	1.00	0.47	0.46	0.78	95
CM-fraction A	0.83	0.43	0.46	0.30	90
„ „ B	0	0.43	0.33	0	83
„ „ C	0	0.34	0.33	0	70

[a] The incubation time was 48 hr with the DEAE-cellulose fraction.

[b] Determined by thin layer chromatography. Values are expressed as moles per mole of total glutamic acid residues except those for turbidity reduction.

TABLE 4. *Actions of Fractions B and C on a bisdisaccharide-peptide subunit dimer (tetra-carboxyamide) [IB-1]*

Enzyme preparation	Liberation in 48 hr			
	N-terminal[a]		C-terminal[a]	
	Ala	mono-amino-A_2pm	Ala	A_2pm
CM-fraction B	0	0.50	0.40	0
„ „ C	0	0.45	0.36	0

[a] Determined by thin layer chromatography. Values are expressed as moles per mole of total glutamic acid residues.

tion of pI 7.9 was found to have cell wall lytic activity although this was very low, and a more basic fraction exhibited strong caseinase activity but no cell wall lytic activity.

It should be mentioned that the strong whole cell lytic activity of Fraction C and the more basic fractions separated from Fractions B and C is not due to lysis of the cell walls but could be due to digestion of cellular proteins.

1) Hydrolytic action of the pI 7.9 fraction on *L. plantarum* cell walls and a bisdisaccharide-peptide subunit dimer

The fractions of pI 7.9 (tubes No. 31 to 38 in Fig. 11) were combined and samples were incubated with *L. plantarum* cell walls and the bisdisaccharide-peptide subunit dimer in 0.02 M phosphate buffer, pH 7.8. Table 5 shows that

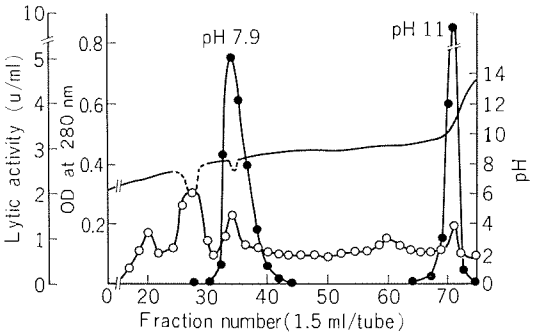

Figure 11. Fractionation of Fraction B of the L-3 enzyme by isoelectric focusing with Carrier Ampholite (gradient of pH 7 to 10). Fractions of 1.5 ml were collected. Lytic activity against *C. diphtheriae* whole cells (●——●), absorbance at 280 nm (○——○), pH (——).

nearly half the A_2pm and alanine residues of both *L. plantarum* walls and the dimer were liberated in parallel as N-terminal (as mono-amino-A_2pm) and C-terminal amino acids, respectively by the D-alanyl-*meso*-A_2pm endopeptidase action of the pI 7.9 fraction. No appreciable release of either N-terminal alanine or C-terminal A_2pm due to hydrolysis of the *N*-acetylmuramyl-L-alanine linkages or deamination of the A_2pm carboxyamide was observed.

2) Immunochemical homogeneity of the pI 7.9 fraction

This was examined by immunoelectrophoretic analysis, using antiserum from rabbits immunized with the crude L-3 enzyme preparation used as a starting material in the present study. As shown in Fig. 12, a crude enzyme preparation (DEAE-cellulose fraction) gave at least 9 precipitin arcs towards either the cathode or anode, but the pI 7.9 fraction gave only one precipitin line towards the cathode in barbital buffer, pH 8.6.

Thus this preparation of the L-3 endopeptidase of pI 7.9 which hydrolyzed the D-alanyl-*meso*-A_2pm interpeptide subunit cross-links in the cell wall of *L. plantarum* and *C. diphtheriae* was immunologically homogeneous.

DISCUSSION

There have been many reports on enzymes degrading peptidoglycans, as described in the

TABLE 5. *Actions of the pI 7.9 fraction from Fraction B on L. plantarum cell walls and a bisdisaccharide-peptide subunit dimer (tetra-carboxyamide) [IB-1]*

Substrate	Liberation in 48 hr			
	N-terminal[a]		C-terminal[a]	
	Ala	mono-amino-A_2pm	Ala	A_2pm
Cell walls	0	0.41	0.39	0
IB-1	0	0.46	0.46	0

[a] Determined by thin layer chromatography. Values are expressed as moles per mole of total glutamic acid residues.

Introduction, but only a few on the endopeptidase hydrolyzing D-alanyl-*meso*-A_2pm interpeptide bonds in cell wall peptidolgycans: D-Alanyl-*meso*-A_2pm endopeptidase activity like

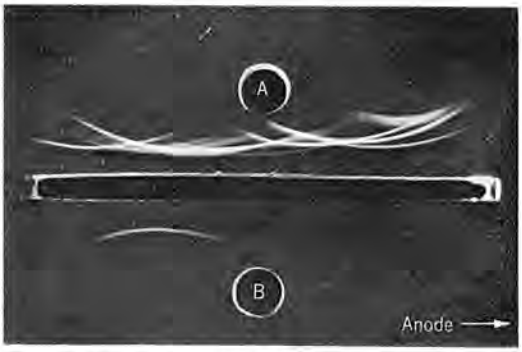

FIGURE 12. Immunological homogeneity of a D-alanyl-meso-A_2pm endopeptidase obtained from Fraction B by isoelectric focusing (immunoelectropherogram). Electrophoresis was carried out at 6 v/cm in barbital buffer, pH 8.0 and $\mu = 0.05$, for 120 min at 4 C. A, crude enzyme preparation; B, pI 7.9 fraction, D-alanyl-*meso*-A_2pm endopeptidase.

that in the *Streptomyces* L-3 enzyme had been found only in autolyzates of *E. coli* and the culture supernatant of *Streptomyces albus* G grown in the medium supplemented with autoclaved cells of *B. megaterium*.

The D-alanyl-*meso*-A_2pm endopeptidase of *Streptomyces albus* G, reported by Bricas et al. (1967), was only produced on addition of *B. megaterium* cells as an inducing agent (in *B. megaterium* cell walls, peptidoglycan interpeptide bonds are involved in C-terminal D-alanyl-*meso*-A_2pm linkages) and its production was variable. The L-3 endopeptidase reported here, on the contrary, was produced in fairly constant yield in the culture filtrate of the L-3 bacterium in the absence of inducing agents. The crude enzyme preparation used in this study was obtained from cultures of the L-3 bacterium grown as surface pellicles on the medium described before (Mori et al., 1960). However, we recently found that the L-3 enzyme was also produced when the L-3 bacterium was grown on a large scale under suitable conditions with aeration (unpublished observation).

Ghuysen et al. (1970) found that in the absence of an added inducer *Streptomyces albus* G secreted an endopeptidase with activity on cell walls with C-terminal D-alanyl-D-linkages between peptidoglycan subunits such as the walls of *B. megaterium*, *Corynebacteriun poinsettiae*, *Brevibacterium pusillum* and *Butyribacterium rettgeri*. This endopeptidase was first identified as soluble DD carboxypeptidase catalyzing hydrolysis of the C-terminal D-alanyl-D-alanine linkage of the UDP-precursor of peptidoglycan subunits. Later it was found to be identical with the enzyme named KM endopeptidase (Ghuysen et al., 1969), which splits CO-NH bonds in N^{α}-(D-Ala)-D-Lys and N^{α}-(D-Ala)-D-Orn as well as $N^{\alpha'}$-(D-Ala)-*meso*-A_2pm (Ghuysen et al., 1970; Leyh-Bouille et al. 1970). A particulate DD carboxypeptidase of *E. coli* exerts an endopeptidase action catalyzing the hydrolysis of the C-terminal D-alanyl-(D)-*meso*-A_2pm interpeptide bond (Bogdanovsky et al., 1969). More recently, it was reported that D-alanine carboxypeptidase IB obtained from *E. coli* membrane by LiCl extraction, and carboxypeptidase IC obtained from the supernatant of disintegrated cells both catalyzed an endopeptidase reaction on a bisdisaccharide-tetrapeptide dimer from *E. coli* cell walls, as well as showing carboxypeptidase activity (Tamura et al., 1976). Further work is required on whether the immunologically homogeneous D-alanyl-*meso*-A_2pm L-3 endopeptidase obtained in this study has similar activities or substrate specificities on C-terminal D-alanyl-D-linkages to the DD carboxypeptidase of *Streptomyces albus* G. In this connection, a preliminary study has revealed that the above L-3 endopeptidase exerted strong lytic action on the cell walls of *Corynebacterium insidiosum* NCPP 1110 and *C. poinsettiae* NCPP 177 (a gift from Dr. H. R. Perkins, Department of Microbiology, University of Liverpool) and caused incomplete, but definite lysis of the walls of *Eubacterium limosum* ATCC 10825

(*Butyribacterium rettgeri*, given by Dr. K. H. Schleifer, Institute of Botany and Microbiology, University of München) and *B. megaterium* (unpublished observation).

The purified L-3 endopeptidase obtained in this work has recently been used in studies on the chemical structures responsible for the immunoadjuvant activities of bacterial cell walls. Results showed that *N*-acetylmuramyl-L-alanyl D-isoglutaminyl-*meso*-A_2pm or *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-*meso*-A_2pm-D-alanine had definite adjuvancy, stimulating both increase in serum antibody levels and induction of delayed-type hypersensitivity to ovalbumin when administered to guinea pigs as a water-in-oil emulsion (Kotani et al., 1975). Recent results also showed that a digest of *L. plantarum* cell walls by the L-3 endopeptidase exhibits definite immunoadjuvancy, as described above, and elicits severe adjuvant arthritis in Lewis rats, unlike a digest of the same cell walls prepared by hydrolysis of the glycan moiety with Mutanolysin which lacked these two activities. However, digests of *L. plantarum* cell walls with the L-3 endopeptidase and Mutanolysin both showed definite adjuvant activity to stimulate humoral and cellular immune responses to ovalbumin in guinea pigs (to be published).

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