ISOLATION AND CHARACTERIZATION OF TWO DISACCHARIDE-PEPTIDES FROM LYSOZYME DIGESTS OF MICROCOCCUS LYSODEIKTICUS CELL WALLS

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Digestion of Micrococcus lysodeikticus cell walls with lysozyme yields a complex mixture of high and low molecular weight substances. In addition to the disaccharide (1) and tetrasaccharide (2, 3) isolated from the low molecular weight dialysable fraction of the digest by ion exchange chromatography, several glycopeptides were isolated in small quantities by paper chromatography and electrophoresis (4). Two of the glycopeptides were studied in some detail, and partial structures have been proposed for them (5).

In this communication we describe the isolation of two disaccharide-pentapeptides from lysozyme digests of M. lysodeikticus cell walls using gel filtration and ion exchange chromatography. The structure of these compounds has been established by the use of chemical and enzymatic techniques, and is also reported.

As starting material for this study we used the glycopeptide mixture which emerges as the first fraction (F_1) upon chromatography on a Dowex-1 acetate column of the dialysable material from lysozyme digests of M. lysodeikticus cell walls (2).

One gram of F_1 in 40 ml of 0.05 M ammonium acetate was incubated under toluene with 50 mg of lysozyme (hen's egg white, 2 x crystallized, Worthington) for 24 hours at 37^0. Twenty ml of the digest were applied to a Bio-Gel P-4 column (110 x 2.2 cm) which had been washed with water. Elution was carried out with water; fractions of 4.5 ml were collected at a rate of 50 ml/hr. Aliquots were analysed by the ninhydrin reaction (6) and by the modified Morgan-Elson assay (7) using a heating time of 35 minutes (1). The tubes containing mainly glycopeptides (effluent volume between 210 and 250 ml) were pooled and lyophilized, yielding 160 mg material (BG II).

Isolation of homogeneous glycopeptides from BG II was achieved by chromatography on Dowex-50 x 2 (Fig. 1).

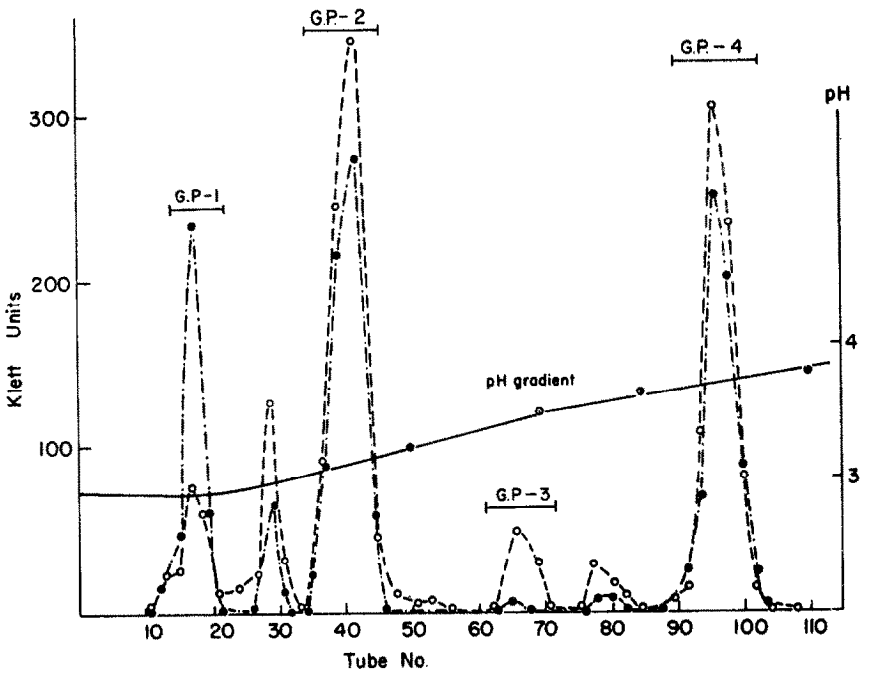

Fig. 1 A column (83 x 2.3 cm) of Dowex 50 x 2 (200-400 mesh) was equilibrated with pyridine-formic acid buffer (0.2 M in pyridine, pH 2.9). One g of BG II obtained from several Bio-Gel column experiments was dissolved in 5 ml water, brought to pH 2.2 with formic acid and added to the column. Elution was started with 200 ml of pyridine-formic acid buffer (0.2 M, pH 2.9). This was followed by a linear gradient of increasing pH and buffer concentration (mixing chamber - 1 l of the starting buffer; reservoir - 1 l of pyridine-formic acid buffer, 1 M in pyridine, pH 5.3). Fractions of 10.6 ml were collected at a rate of 60 ml/hr and assayed as before. Prior to analysis by the Morgan-Elson method, 0.2 ml aliquots were dried overnight over KOH. Morgan-Elson, •---• ; ninhydrin o -- o .

The major peaks, GP-2 and GP-4 from the Dowex-50 column were each pooled and lyophilized three times to remove traces of buffer, yielding 300 mg and 150 mg of material respectively. Small amounts of impurities were removed from GP-2 and GP-4 by rechromatography on Bio-Gel P-4.

The two glycopeptides migrated as single spots upon chromatography in two solvent systems, n-butanol-acetic acid-water (4:1:5) and n-butanol-pyridine-water (6:4:3), and on high voltage (50 v/cm) electrophoresis at three different pH values (Fig. 2).

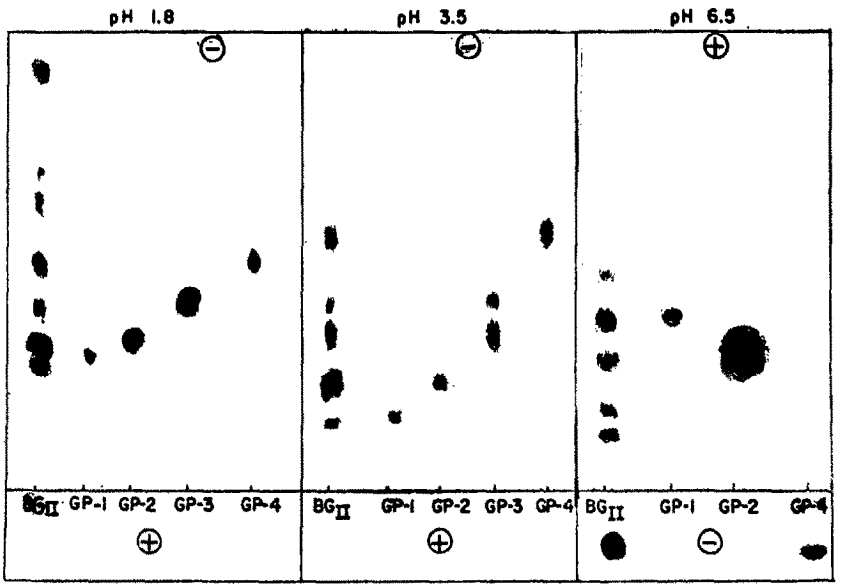

Fig. 2 Migration of glycopeptides on high voltage paper electrophoresis (1 hr, 50 v/cm) in pyridine-acetate buffers at different pH values. Spots revealed by ninhydrin.

The analytical data for GP-2 and GP-4 (Table I) show that both compounds have identical amino sugar, amino acid, and nitrogen contents and similar molecular weights They differ, however, in their acetyl content (Table I), in their migration on paper electrophoresis (Fig. 2) and in their pK values (Table II). These findings indicate that in GP-4 an amino group of one of the amino sugars is free.

Reduction with sodium borohydride followed by acid hydrolysis revealed that in both glycopeptides muramic acid is the terminal reducing group. From hydrazinolysis experiments (10) it was found that glycine and alanine were C-terminal. Dinitrophenylation studies showed that GP-2 contained ε-NH_2 of lysine as the only free amino group. The same free amino group was found in GP-4 but, in addition, DNP-muramic acid was identified by paper chromatography and electrophoresis of acid hydrolysates (2 N HCl, 2 hrs, 100^0) of DNP-GP-4. No DNP-muramic acid was detected in hydrolysates of DNP-GP-2.

TABLE I. Composition and molecular weights of GP-2 and GP-4 isolated from M. lysodeikticus cell walls

	GP-2		GP-4	
	μmole/mg	Ratio [e]	μmole/mg	Ratio [e]
Glucosamine [a]	0.90	1.01	0.87	0.96
Muramic acid	0.88	0.99	0.88	0.97
Alanine [b]	1.78	2.00	1.80	1.99
Glutamic acid	0.89	1.00	0.905	1.00
Glycine	0.91	1.02	0.85	0.94
Lysine	0.93	1.04	0.94	1.04
Acetate	1.89		1.32	
Molecular Weight Found [c]	864		1070	
Molecular Weight Calc. [d]	952		910	
Nitrogen Found [d]	10.6%		10.8%	
Nitrogen Calc.	11.7%		12.2%	

Materials dried at 50° in vacuo before analysis.

a. Assayed on the Beckman-Spinco amino acid analyser after hydrolysis in 2N HCl for two hours at 100°. The integration constants of glucosamine (17.2) and of muramic acid (12.5), in comparison with leucine (24.6) were obtained by analysis of acid hydrolysates (2 N HCl, 2 hr. 100°) of GlcNAc and MurNAc and therefore include correction for losses during hydrolysis.

b. Assayed on the amino acid analyser after hydrolysis (6 N HCl, 20 hrs, 100°).

c. Molecular weight determined from ultracentrifuge measurements by the Yphantis equilibrium method (8). Partial specific volume taken as $\bar{v} = 0.69$ (9).

d. For the calculation of nitrogen and M.W., it was assumed that both GP-2 and GP-4 are disaccharide-pentapeptides of the composition listed, with GP-4 having one of its amino groups non-acetylated.

e. Ratio calculated assuming glutamic acid as 1.00 .

TABLE II. Ionization constants of glycopeptides, obtained by potentiometric titration

GP-2 groups/mole	GP-2 pK	GP-4 groups/mole	GP-4 pK	Assignment
1	10.79	1	10.85	ε-NH_2 of lysine
0	-	0.9	7.8	NH_2 of amino sugar
1.9	3.58	1.9	3.43	COOH of peptide

The molar color yields of GP-2 in the Morgan-Elson reaction, both after 3 minutes and 35 minutes heating time, were identical with those of the disaccharide GlcNAc β(1→4) MurNAc (1). GP-4, on the other hand, gave the same molar color yields as the above compounds only after it was acetylated with acetic anhydride. GP-4 which was selectively acetylated on its amino group with the low pK (11) migrated on paper electrophoresis at pH 3.5 and 6.5 in the same manner as GP-2.

Treatment of GP-2 with the Streptomyces amidase (12), kindly sent to us by Dr. J.M. Ghuysen, released a peptide with N-terminal alanine and free ε-NH_2-lysine, and a disaccharide having the properties of GlcNAc β(1→4) MurNAc (1). The same disaccharide was released by the amidase from GP-4 only after acetylation of this compound. Furthermore, when GP-4 was acetylated with tritium labelled acetic anhydride, the disaccharide released from it by the amidase was radioactive. In the peptide released by amidase from acetylated GP-4, alanine was found to be N-terminal. The two pentapeptides released by the amidase, from GP-2 and acetylated GP-4, had the same migration on electrophoresis at pH 3.5 and 6.5.

The two glycopeptides were also investigated by nuclear magnetic resonance spectroscopy. Spectra were measured in D_2O with a Varian A-60 spectrometer, using solutions of the same concentration. The NMR spectra of the two glycopeptides were similar, the main difference being in the 2 ppm. region in which the acetyl methyl groups absorb. In the spectrum of GP-2, two sharp peaks, superimposed on broad peaks, were found in this region, whereas in the spectrum of GP-4, only one sharp peak was observed, the areas under these sharp peaks being in the ratio of 2:1 .

Partial acid hydrolysis (2 N HCl, 15 min, 100^0) of GP-2 gave a mixture of fragments from which three peptides were isolated by paper electrophoresis and paper chromatography:

(a) A pentapeptide of Ala, Lys, Glu, Gly with the molar ratios of 1.82; 0.91; 1.0, and 1.05 as determined on the amino acid analyser; in this peptide alanine was N-terminal. It had the same electrophoretic mobility at pH 6.5 and 3.5 as the pentapeptide released from GP-2 by the Streptomyces amidase.

(b) A dipeptide composed of Lys, Ala (1.1 : 1.0), which upon dinitrophenylation and hydrolysis yielded di-DNP-lysine. On incubation of acid hydrolysates of this dipeptide with D-amino acid oxidase (13), alanine was completely oxidized. L-Ala was not affected under these conditions. The peptide is therefore Lys-D-Ala-COOH.

(c) A tripeptide composed of Ala, Glu, Gly (1.2 : 1.0 : 0.9). Dinitrophenylation experiments revealed that alanine was N-terminal; this alanine belonged to the L-series

since it was not affected by D-amino acid oxidase. Upon hydrazinolysis, the α hydrazide of glutamic acid was obtained (14). It was identified by comparison with synthetic α and γ hydrazides of glutamic acid. This indicates that in the tripeptide, the α-COOH of glutamic acid is linked to glycine, and the γ-COOH is free.

Paper electrophoretic and paper chromatographic analyses of partial acid hydrolysates of GP-4 which had been previously N-acetylated (11), showed the same pattern of products as that of partial acid hydrolysates of GP-2.

Discussion

The structure proposed for GP-2 is given in Fig. 3. This compound is most likely identical with DSP II isolated by Ghuysen (5) although the structure presented here differs from the one given by him. The peptide sequence MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala is known to occur in nucleotide intermediates of bacterial cell wall biosynthesis (15) and has been established by chemical synthesis (16). It is also present in cell walls of S. aureus (17). GP-4 appears to possess the same structure as GP-2 with the exception of the acetyl group on muramic acid. Such a cell wall fragment has not been reported before, and its biological significance is not known.

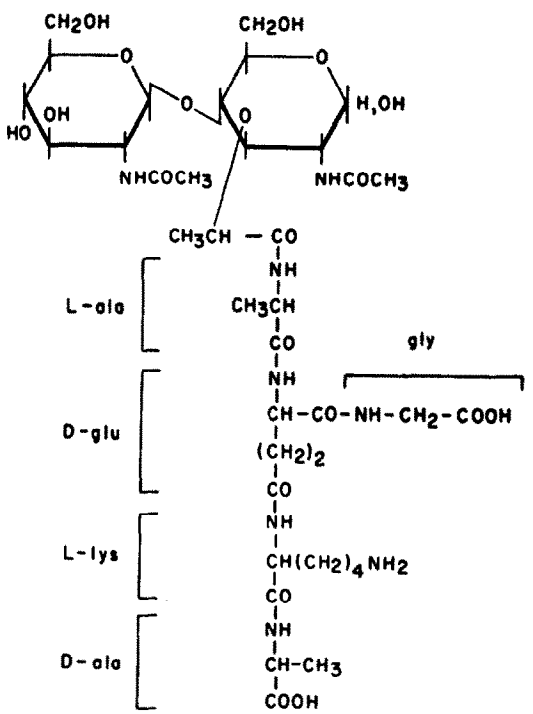

Fig. 3 The chemical structure of a disaccharide-pentapeptide (GP-2) isolated from M. lysodeikticus cell walls.

It is, however, pertinent to note that muramic acid residues with a free amino group have been previously detected by the DNP technique in lysozyme digests of M. lysodeikticus cell walls (18), and we have also found such residues in the intact walls.

Acknowledgements

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