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THE MODE OF HYDROLYSIS OF A GLYCAN PORTION OF *MICROCOCCUS LYSODEIKTICUS* CELL WALLS BY ENDO-*N*-ACETYLGLUCOSAMINIDASE OR ENDO-*N*-ACETYLMURAMIDASE ISOLATED FROM CRUDE BARLEY β-AMYLASE[1]

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SUMMARY *Micrococcus lysodeikticus* cell walls were digested with a pI 6.8 endo-*N*-acetylglucosaminidase or a pI 9.5 endo-*N*-acetylmuramidase. The digests were further treated with a *N*-acetylmuramyl-L-alanine amidase of *Flavobacterium* L-11 enzyme to remove the peptide portion. The products were fractionated by gel filtration and ion-exchange chromatography, and the glycan portion of fractions were analyzed for their average amino sugar chain lengths.

The following results were obtained. 1. The glycan portion of the main products in the pI 6.8 enzyme digest consisted of (-*N*-acetylmuramic acid-*N*-acetylglucosamine-)$_{2-3}$. 2. The glycan moiety of the pI 9.5 enzyme digest was mainly composed of (-*N*-acetylglucosamine-*N*-acetylmuramic acid-)$_{3-4}$. 3. The glycosidic linkages around the muramic acid 6-phosphate residues which linked to a special structure through a phosphodiester bond were rather refractory to the glycosidase action of both pI 6.8 and 9.5 enzymes.

INTRODUCTION

A previous paper (Iwata et al., 1972) described the existence in crude β-amylase from barley of two glycosidase, pI 6.8 endo-*N*-acetylglucosaminidase and pI 9.5 endo-*N*-acetylmuramidase, which are active against a glycan portion of the cell walls from *Micrococcus lysodeikticus* and some other bacteria. General enzyme properties of these two endo-*N*-acetylhexosaminidase, including non-identity with the β-amylase itself and hydrolytic activity on bacterial cell walls, isolated peptidoglycans and other polysaccharides, were also described.

The purpose of this paper is to clarify the the mode of enzyme action of the two enzymes on bacterial peptidoglycans by analysing how and to what extent the glycan portion of the

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M. lysodeikticus cell wall is degraded by digestion with the enzyme in question, with respect to the possible interfering effect on the enzyme action of a special structure, 2-amino-2-deoxymannouronic acid-glucose polymer (Perkins, 1963; Hase and Matsushima, 1970) linked with a mannouronic acid residue to the glycan moiety.

MATERIALS AND METHODS

1. *M. lysodeikticus cell walls, the pI 6.8 endo-N-acetylglucosamidase and pI 9.5 endo-N-acetylmuramidase*

Preparation of these materials was described in a previous paper (Iwata et al., 1972), and one unit of lytic activity was defined as the amount of enzyme required to reduce the OD_{550} of the assay mixture by 50% in 30 min of incubation under the standard conditions described (Iwata et al., 1972). The amino acid and amino sugar composition of the cell wall served as a substrate in this study was shown in Table 1.

TABLE 1. *Amino sugar and amino acid content of M. lysodeikticus cell walls*[a]

Amino sugar and amino acid	nmoles/mg	Molar ratio
Muramic acid 6-phosphate	13.7	0.021
Glucosamine 6-phosphate	10.0	0.016
Muramic acid	358	0.60
Glutamic acid	641	1.00
Glycine	571	0.89
Alanine	1343	2.09
Glucosamine	528	0.82
Lysine	629	0.98

[a] Data are not corrected for the destruction during acid hydrolysis.

2. *Flavobacterium L-11 enzyme*

Crude enzyme preparation (Lot No. D-24) was supplied by Kyowa Fermentation Industry (Tokyo).

3. *Chemical and analytical methods*

Free amino groups were determined by the DNP method and total hexosamines were determined by the Morgan-Elson method as described by Ghuysen et al. (1966). Reducing sugar and hexoses were estimated by the ferricyanid method (Park and Johnson, 1942) and the anthron method (Ashwell, 1957), respectively. Amino acids and amino sugars were analysed with a Hitachi amino acid analyser (Model KLA-3B, Hitachi Ltd. Co., Tokyo) after hydrolysis in 6 N HCl at 100 C for 14 hr. N-Terminal amino acid was determined as DNP amino acid by thin layer chromatography as described by Ghuysen et al. (1966).

The average chain length of a glycan portion of the intact cell walls and their enzymic degraded fragments were measured by the method described by Kato et al. (1976).

RESULTS

1. *Analyses of cell wall lysates obtained by pI 6.8 endo-N-acetylglucosaminidase digestion*

1) Fractionation of the lysate

a) Digestion of cell wall with the pI 6.8 enzyme: Cell walls (1,054 mg) were suspended in 50 ml of sodium acetate buffer (pH 4.0, ionic strength 0.01), containing 17 U of pI 6.8 enzyme and digested by incubating the mixture at 37 C for 48 hr. During digestion, aliquots were removed periodically and immediately inactivated by heating at 100 C for 5 min, for determination of free amino groups and reducing groups. Determination of the optical density (OD_{550}) was made at 10 to 30 min intervals with a Shimadzu Bausch & Lomb spectronic 20 colorimeter (Shimadzu Seisakusho, Kyoto). As shown in Fig. 1, lysis of the cell walls (expressed as percent reduction in optical density) was accompanied by release of reducing groups. No significant increase in free amino groups was observed. During incubation for 48 hr, there was a 98% reduction in optical density and the net increase of reducing groups per mg of cell walls amounted to 176 nmoles (0.27 moles per mole of glutamic acid).

After heating at 100 C for 5 min, the digestion products were centrifuged at 10,800 $\times g$ for 30 min. The insoluble residue was 2.05 mg in dry weight (0.2% of the starting ma-

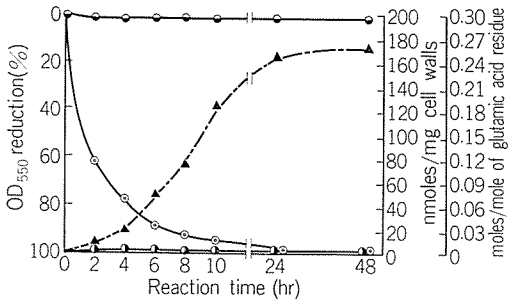

FIGURE 1. Reduction in optical density and liberation of reducing power in a *M. lysodeikticus* cell wall suspension incubated with pI 6.8 enzyme. Optical density (◎——◎), control of optical density (◒——◒), reducing power (▲——▲), free amino groups (◑——◑).

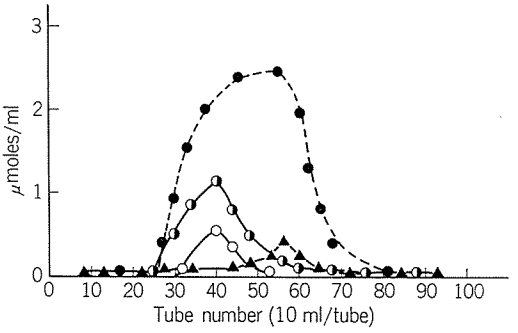

FIGURE 2. Gel filtration of pI 6.8 enzyme lysate of *M. lysodeikticus* cell walls on columns of Sephadex G-50, G-50 and G-25 connected in series. Free amino groups (◑——◑), hexose (○——○), reducing power (▲——▲), total amino sugars (●——●).

terial). The supernatant was concentrated under reduced pressure to about 10 ml in a rotary evaporator (the pI 6.8 enzyme digest).

b) Gel filtration of the enzyme digest: The concentrate of the digestion product (631.3 mg equivalent to cell walls) was submitted to gel filtration on Sephadex G-50, G-50 and G-25 (coarse, bead form) columns (2×97 cm, 2×97 cm and 2.5×97 cm, respectively) connected in series. Elution was performed with water at a flow rate of 30 ml per hr, at 4 C. Ten milliliter fractions were collected and assayed for free amino groups, total amino sugars, reducing groups and hexoses (Fig. 2). This experiment was undertaken to fractionate the degradation products by the pI 6.8 enzyme according to the chain length of a glycan portion, and to determine the distribution of each fragment, but the presence of an intact peptide portion heavily interfered an orderly separation of the degraded glycan fragments of the different chain length.

c) L-11 enzyme digestion of the pI 6.8 enzyme lysate: The contents in tube Nos. 25–75 inclusive (in Fig. 2) were pooled and concentrated to about 2 ml in a rotary evaporator. The concentrate of the pI 6.8 enzyme digest was incubated with 100 mg of the L-11 enzyme (180 U in terms of the lytic activity against *M. lysodeikticus* cell walls) in 100 ml of 0.012 M barbital buffer, pH 8.5, at 37 C for 120 hr. Sodium azide was added as a preservative at a final concentration of 0.2%. During the incubation, 0.39 moles of amino-terminal alanine was liberated per mole of glutamic acid residue, but no increase in reducing power was observed.

d) Column chromatography of the L-11 enzyme digest of the pI 6.8 enzyme lysate on Dowex-50×2 (H^+): The reaction mixture described above was concentrated to about 5 ml and adjusted with diluted formic acid to pH 2.2. The concentrate was fractionated on a column of Dowex-50×2 (200–400 mesh, H^+ form, 3×42 cm) preequilibrated with 0.2 M pyridine-formate buffer, pH 2.9. The column was eluted at first with 500 ml of 0.2 M pyridine-formate buffer, pH 2.9, and then with a linear gradient of both pH and buffer concentration, with 1,000 ml of 0.2 M pyridine-formate buffer, pH 2.9 in the mixing vessel and 1,000 ml of 1 M pyridine-formate buffer, pH 5.3 in the reservoir. As shown in Fig. 3, almost all of the glycan fragments which were split off from the peptide portion by the action of *N*-acetylmuramyl-L-alanine amidase and endopeptidase present in the L-11 enzyme was eluted with the first buffer. The peptide

portion liberated by the L-11 enzyme from the glycan portion was then adsorbed on the column and eluted by the buffer of about pH 4 and 4.5.

e) Gel filtration of the glycan fragments unadsorbed on a Dowex-50 column: Tube Nos. 2–30 inclusive in Fig. 3 were pooled and concentrated to about 3 ml. This was applied on the Sephadex column described above and eluted with water. The elution pattern shows that three well-separated peaks were obtained (Fig. 4). The first (A I, Nos. 27–35) and the second peak (A II, tube Nos. 36–45) have not only amino sugars but also hexose derived from the special structure. The last peak (A III, tube Nos. 46–75) has only amino sugars. No significant free amino group were detected on all the tubes. After representative fractions were assayed by amino acid analyser (the results are described in the next section and in Table 2), all the contents in tube Nos. 27–75 were combined and concentrated to about 5 ml, and applied on Dowex-1×8 column (200–400 mesh, OH^- form, 2.2×39 cm).

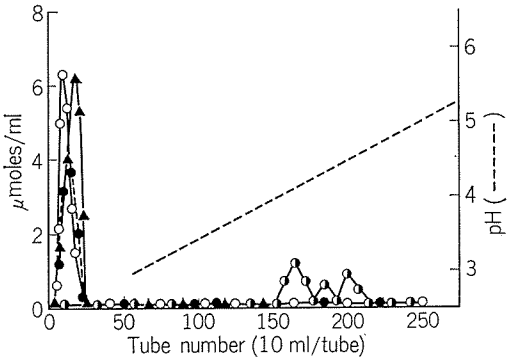

FIGURE 3. Dowex-50×8 column chromatography of an L-11 enzyme digest of the solubilized materials from *M. lysodeikticus* cell walls treated with pI 6.8 enzyme. Free amino groups (◑——◑), hexose (○——○), reducing power (▲——▲), total amino sugars (●——●).

f) Column chromatography of the glycan fragments with or without the special structure unadsorbed on a Dowex-50 with a Dowex-1×8 columns: The column was eluted at first with 500 ml water and then with a linear gradient of salt concentration by mixing 1,000 ml of water and 1,000 ml of 5 M acetic acid.

TABLE 2. *Analyses of the fractions obtained by gel filtration of the fraction unadsorbed on Dowex-50 from a pI 6.8 and L-11 enzyme digest (Fig. 4) with special reference to the chain length of their glycan parts*[a]

Fraction	Representative tube No.	$\overline{CL}$[b]	Muramic acid 6-phosphate[c]	Glucosamine 6-phosphate[c]	Hexose	Muramic acid	Glucosamine	Distribution (%)[e]
A I (tube Nos. 27–35)	31	10	21.4(0.04)[d]	35 (0.06)	800(1.45)	262	550	6
A II (tube Nos. 36–45)	40	11	48.0(0.08)	2.5(0.004)	1250(1.96)	359	638	6.5
A III (tube Nos. 46–74)	47	8.4	0	0	200(0.20)	830	1025	87.6
	50	5.2	0	0	100(0.11)	612	877	
	52	4.6	0	0	100(0.11)	790	890	
	55	3	0	0	100(0.11)	273	317	
	57	2.6	0	0	90(1.61)	178	223	

[a] Figures are nmoles/ml, except $\overline{CL}$ and %.
[b] $\overline{CL}$: average chain length.
[c] Data are not corrected for destruction on acid hydrolysis.
[d] Molar ratio to glutamic acid.
[e] Relative value to the total amino sugars.

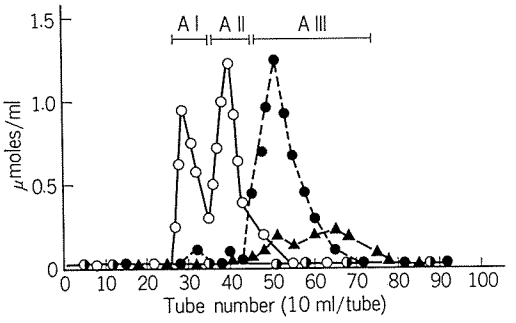

FIGURE 4. Gel filtration by connecting columns of Sephadex G-50, G-50 and G-25 of the fraction unadsorbed on a Dowex-50 from a pI 6.8 and L-11 enzyme digest. Free amino groups (◑——◑), hexose (○——○), reducing power (▲——▲), total amino sugars (●——●).

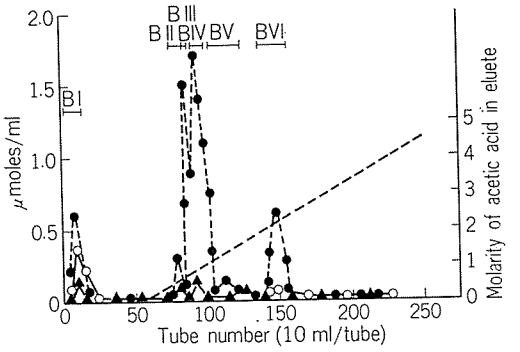

FIGURE 5. Ion exchange chromatography by a Dowex-1×8 column of the fraction unadsorbed on Dowex-50 from a pI 6.8 and L-11 enzyme digest. Hexose (○——○), reducing power (▲——▲), total amino sugars (●——●).

Six peak fractions (B I–B VI) were obtained as shown in Fig. 5. Hexose indicative of the presence of the special structure was detected only in fractions B I and B VI

2) Chemical analyses, especially analyses of the chain length of a glycan part, of representative fractions obtained from the pI 6.8 enzyme digest of *M. lysodeikticus* walls

Representative fractions separated by gel filtration (Fig. 4) or by Dowex-1 chromatography (Fig. 5) of the unadsorbed fraction on Dowex-50 were assayed for the amino acid and amino sugar composition, and average chain length. Table 2 indicates that the chain length of a glycan part in the degradation product (Fractions A I, A II and A III in Fig. 4) were distributed widely from about 3 to 11 calculated as a mono amino sugar unit. It was difficult to determine the chain length of the main part. The content of each representative tube of the Fraction A I, A II and A III was reduced by sodium borohydride and the resulting amino sugar alcohol was proved to be only glucosaminitol.

The chain length of the representatives of Fraction B I to B VI separated by Dowex-1 column chromatography (Fig. 5) is shown in Table 3. The chain length was relatively widely distributed from 3.5 to 12, but the main part (Fraction B IV represented by tube No. 92) was found to have the average chain length of 4. The proportion of each fraction was estimated in terms of total amino sugar value. All of the amino sugar alcohol formed by borohydride reduction of the representatives from B I-VI were proved to be glucosaminitol.

It is noteworthy that muramic acid 6-phosphate and/or glucosamine 6-phosphate was detected only in hexose rich fractions. Fraction A II (representative tube No. 40) obtained by gel filtration (Fig. 4) and Fraction B VI (pool of tube Nos. 140–160 in Fig. 5) of the Dowex-1×8 column chromatography, contained a larger amount of muramic acid 6-phosphate than glucosamine 6-phosphate. But reverse relationship was seen in Fraction A I in the gel filtration fraction, and Fraction B I contained only glucosamine 6-phosphate. The chain length of glycan fragments (Fraction A I and A II in Fig. 4), and Fraction B VI in Fig. 5 containing muramic acid 6-phosphate, irrespective of the presence or absence of glucosamine 6-phosphate, tended to be longer than that (Fraction B I in Fig. 5) of glycan fragments containing solely glucosamine 6-phosphate, of which chain length was rather short and only 3.5.

TABLE 3. *Analyses of the fractions separated by Dowex-1 column chromatography of the fraction unadsorbed on Dowex-50 from a pI 6.8 and L-11 enzyme digest (Fig. 5) with special reference to the chain length of their glycan parts*[a]

Fraction	Representative tube No.	$\overline{CL}$[b]	Muramic acid 6-phosphate[c]	Glucosamine 6-phosphate[c]	Hexose	Muramic acid	Glucosamine	Distribution (%)[e]
B I (tube Nos. 4–20)	10	3.5	0	46 (0.08)[d]	800 (1.40)	597	570	12
B II (tube Nos. 75–84)	pool	4.4	0	0	0	1031	1240	5
B III (tube Nos. 85–90)	87	5.2	0	0	0	31	21	16
B IV (tube Nos. 91–100)	92	4.0	0	0	0	522	584	48
B V (tube Nos. 101–120)	pool	ND[f]	—	—	—	—	—	11
B VI (tube Nos. 140–160)	pool	12.0	622 (0.56)	61 (0.05)	1000 (0.90)	845	1112	7

[a,b,c,d,e] See the corresponding legend in Table 2.
[f] Not determined.

2. *Analyses of cell wall lysate obtained by pI 9.5 endo-N-acetylmuramidase digestion*

1) Fractionation of the lysate

a) Digestion of cell wall with the pI 9.5 enzyme: Cell walls (661 mg) were suspended in 30 ml of sodium acetate buffer (pH 4.0, ionic strength 0.01), containing 4.2 U of the pI 9.5 enzyme and incubated at 37 C for 48 hr. After the maximum OD_{550} reduction (98%) and release of reducing groups (196 nmoles per mg cell walls) were attained, the enzyme was inactivated by heating for 5 min at 100 C. The reaction mixture was then adjusted to pH 8.5 with barbital buffer and the L-11 enzyme (184 U) was added. The final concentration of the buffer was 0.0125 M and the total volume of the mixture was 120 ml. As shown in Fig. 6, 1.5 moles of free amino groups (among them, 0.5 mole belong to amino-terminal alanine) were liberated per mole of the glutamic acid residue after a further 72 hr incubation. No increase in reducing power was observed. The digestion product was heated for 2 min at 100 C, concentrated and the concentrate was centrifuged at $10,800 \times gf$ or 30 min.

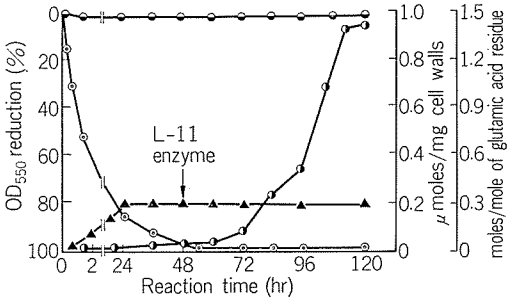

FIGURE 6. Reduction in optical density and liberation of reducing power of free amino groups in a *M. lysodeikticus* cell wall suspension incubated with pI 9.5 enzyme and L-11 enzyme. Optical density (◎——◎), control of optical density (◒——◒), reducing power (▲——▲), free amino groups (◑——◑).

b) Fractionation of the cell wall lysate: The supernatant from the above enzyme digest was submitted to gel filtration on Sephadex G-50, G-50 and G-25 columns connected in series as described above. Elution was performed with water at a flow rate of 50 ml per hour. No clearcut separation of material was attained, however. Therefore, all of the fractions were collected, concentrated and ap-

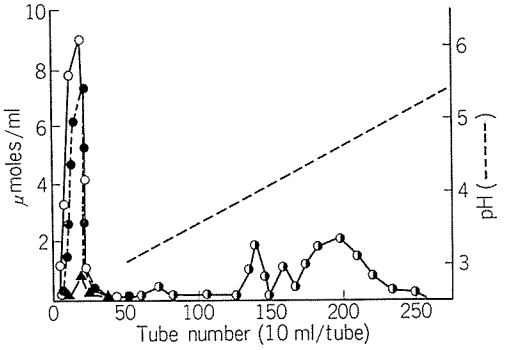

FIGURE 7. Dowex-50×8 column chromatography of *M. lysodeikticus* cell wall digests treated with pI 9.5 and L-11 enzymes. Free amino groups (◑——◑), hexose (○——○), reducing power (▲——▲), total amino sugars (●——●).

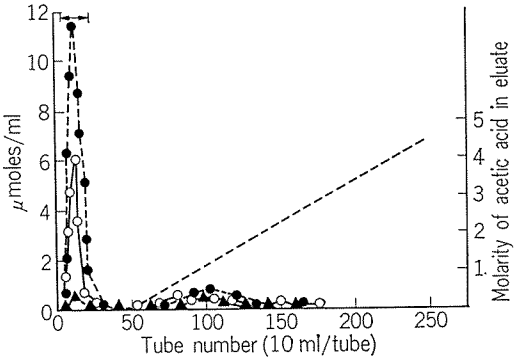

FIGURE 8. Ion exchange chromatography by a Dowex-1×8 column of the fraction unadsorbed on Dowex-50 from a pI 6.8 and L-11 enzyme digest. Hexose (○——○), reducing power (▲——▲), total amino sugars (●——●).

plied to a similar column of Dowex-50×2 (H^+ form, 2.2×40 cm) as described above. As shown in Fig. 7, almost all of the material containing amino sugars and hexose was unadsorbed but the material containing a free amino groups was adsorbed. The peptide fractions, on the other hand, were eluted at high pH values.

The unadsorbed fraction was concentrated and applied to a similar column of Dowex-1×8 as mentioned above. As shown in Fig. 8, the elution pattern is different from that in Fig. 5, and almost all the amino sugars and hexoses were unadsorbed on this column (a small amount of these components adsorbed on the column were eluted with 2 M acetic acid). The first pooled fraction was concentrated, and submitted for gel filtration on Sephadex G-50, G-50 and G-25 columns connected in series. As shown in Fig. 9, the first and the second peaks (Fraction C I and II, respectively) were hexose rich and the last peak (C III) was amino sugar rich.

2) Chemical analyses of representative fractions obtained from the pI 9.5 enzyme digestion, with special reference to the chain length of a glycan part

Chemical analyses of three fractions (C I, C II and C III) obtained by gel filtration was

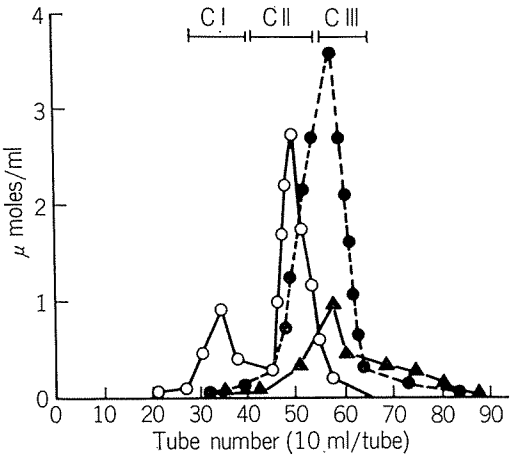

FIGURE 9. Gel filtration by connecting columns of Sephadex G-50, G-50 and G-25 of the fraction unadsorbed on Dowex-50 and Dowex-1 from a pI 9.5 and L-11 enzyme digest of *M. lysodeikticus* cell wall. Hexose (○——○), reducing power (▲——▲), total amino sugars (●——●).

summarized in Table 4. The chain length of a glycan part of Fraction C III (a representative tube No. 58) was 7.4, but the chain length of the glycan moiety of Fraction C II and C I, especially the former, which contained glucose, muramic acid 6-phosphate and glucosamine 6-phosphate, were found to be longer.

TABLE 4. *Analyses of the fractions from gel filtration of the fraction unadsorbed on Dowex-50 and Dowex-1 columns from a pI 9.5 and L-11 enzyme digest of M. lysodeikticus cell wall (Fig. 9)*[a]

Fraction	Representative tube No.	$\overline{CL}$[b]	Muramic acid 6-phosphate[c]	Glucosamine 6-phosphate[c]	Hexose	Muramic acid	Glucosamine	Distribution (%)[e]
C I (tube Nos. 28-40)	34	9	9.3 (0.27)[d]	17 (0.50)	720 (21.18)	12	34	4
C II (tube Nos. 41-54)	50	20.2	344 (0.42)	258 (0.32)	2700 (3.31)	734	815	31
C III (tube Nos. 55-65)	58	7.4	0	0	200 (0.13)	745	1493	65

[a,b,c,d,e] See the corresponding legend in Table 2.

The result indicates that these glycan parts linked to the special structure are refractory to the pI 9.5 enzyme action. Analyses of the reduced representative samples of all of Fractions C I, C II and C III are only muramicitol in the sugar alcohol analyses. The reduced specimen of representative of Fraction C III (tube No. 58) gave glucosaminitol (about one third of muramicitol) in addition to muramicitol. Both of the hexose-rich Fractions C I and C II contained not only muramic acid 6-phosphate but glucosamine 6-phosphate were also found, unlike the pI 6.8 enzyme digest.

DISCUSSION

There are a number of peptidoglycan degrading enzymes of glycosidase type of animal-, plant- and bacterial origins. As far as the mode of hydrolytic action has been clarified, most of them are endo-*N*-acetylmuramidase active against the β-1,4 linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine, and only a fraction can split by an endo-*N*-acetylglucosaminidase action the linkage of *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramic acid as discussed in a previous paper (Iwata et al., 1972). However, there is only very limited, information on the following questions: (1) how many theoretically hydrolysable bonds a test enzyme could efficiently split, and (2) how a non-peptidoglycan moiety (a so-called special structure), the majority of which seemed to link with some muramic acid residue in the peptidoglycan through a phosphodiester bond, could influence the susceptibility of a linking part of a peptidoglycan to the test enzyme. Even in the extensive study of Sharon et al. (1966) on the chicken egg white lysozyme digests of *M. lysodeikticus* walls, where a GlcNAc-MurNAc disaccharide and a $(GlcNAc\text{-}MurNAc)_2$ tetrasaccharide were shown to account for 13% and 2.5% of the dialysable portion respectively, there were no analyses on the above question of the non-dialysable portion holding about two thirds of the total digest.

In this study, the pI 6.8 endo-*N*-acetylglucosaminidase, one of the cell wall lytic enzymes present in a crude barely β-amylase was shown to split the glycan portion of *M. lysodeikticus* cell walls mainly into tetrasaccharide, $(MurNAc\text{-}GlcNAc)_2$, with a small amount of $(MurNAc\text{-}GlcNAc)_{3\text{-}4}$. It also turned out that the glycan portion where the special structure linked was less susceptible to the pI 6.8 enzyme, giving longer fragments such as $(MurNAc\text{-}GlcNAc)_{5\text{-}6}$.

The main product in the pI 9.5 endo-*N*-acetylmuramidase digest, on the other hand, was found to be $(GlcNAc\text{-}MurNAc)_{3\text{-}4}$. However, very big fragments, the chain length of which was estimated as 20, was obtained from the glycan portion linked with the special structure, suggesting that the pI 9.5 endo-*N*-

acetylmuramidase was more heavily interfered by the presence of the special structure than the endo-*N*-acetylglucosaminidase.

However, there is some reservation in the above conclusion: egg white lysozyme had a transglycosidase activity which brought elongation of the once degraded glycan chain, while it hydrolysed β-1,4-*N*-acetylmuramyl-*N*-acetylglucosamine bonds (Chipman et al., 1968; Pollock and Sharon, 1970). The possibility that both pI 6.8 and pI 9.5 enzymes had a similar transglycosidase activity could not be excluded at present.

It should be added here that a part of the glycan fragments obtained from the pI 9.5 endo-*N*-acetylmuramidase digest gave glucosaminitol in addition to muramicitol by sodium borohydride reduction. A large amount of the glycan fragments were used for reduction, since the color yield of muramicitol in ninhydrin reaction in amino acid analyser was low as 35% of that of glucosaminitol. This may derive from the glucosamine residue which localized at the reducing end in a native cell wall peptidoglycan, but not newly formed by the pI 9.5 enzyme digestion.

In the present study, an amino sugar phosphate different from muramic acid 6-phosphate was found in hexose-rich fractions as shown in Tables 3 and 4. This amino sugar phosphate was isolated from *M. lysodeikticus* cell walls in a pure state identified as a glucosamine 6-phosphate in a separate study (Kato et al., 1972). Although muramic acid 6-phosphate has been known to derive from linking points between the peptidoglycan and the special structure which are bound by the phosphodiester bond (Liu and Gotschlich, 1967), the presence of glucosamine 6-phosphate in bacterial cell wall has rarely been reported except the paper of Archibald and Stafford (1972) who found glucosamine 6-phosphate in the special structure of *Staphylococcus lactis* cell walls. The role of glucosamine 6-phosphate in the structure or function of bacterial cell walls remains to be elucidated, in view of the fact that this amino sugar phosphate has been shown to be rather widely distributed in various bacterial cell walls (Kato et al., 1972).

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