Note

Effect of *N*-Acetyl Substitution at the Amino Sugar Residues on PL-1 Muramidase-catalyzed Hydrolysis of Cell Wall Peptidoglycan of *Lactobacillus casei*

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Various kinds of bacteriolytic enzymes which attack the different bonds of cell wall peptidoglycans have been reported, including muramidase, glucosaminidase, amidase and endopeptidase.¹⁾ Among them, hen's egg-white lysozyme (abbreviated as EWL), one of the muramidases, has been studied in the greatest detail. The activity of muramidase is known to be influenced by the presence or absence of acetyl groups on amino and hydroxyl groups at the amino sugar residues in the glycan component of cell wall peptidoglycan. Araki *et al.*^{2,3)} and Amano *et al.*⁴⁾ observed that an EWL-resistant strain of *Bacillus cereus* was not *N*-acetylated at most of its glucosamine residues in the cell wall peptidoglycan, and that the acetylation of unsubstituted glucosamine residues by acetic anhydride resulted in the increased solubilization of the peptidoglycan by EWL. Zipperle *et al.*⁵⁾ found that the cell walls of a strain of *Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis* contained high percentages of unsubstituted amino sugars, and unless acetylated were resistant to EWL and partially resistant to mutanolysin, a muramidase produced from *Streptomyces globisporus*.⁶⁾ On the other hand, Tipper *et al.*⁷⁾ showed that the original resistance of *Staphylococcus aureus* to EWL was ascribed to the occurrence of *O*-acetyl groups on the 6'-position of *N*-acetyl muramic acid. Yoshimoto and Tsuru⁸⁾ found that F2 enzyme, a muramidase produced from *Streptomyces griseus*, acted only on the peptidoglycans of *Staphylococcus aureus* and *Bacillus megaterium* IFO 12108, whose hexosamines were *N,O*-diacetylated, and concluded that the enzyme was an *N,O*-diacetylmuramidase.

In a previous study,⁹⁾ we isolated a bacteriolytic enzyme acting on the peptidoglycan of *Lactobacillus casei* ATCC 27092 from PL-1 phage-induced lysates of the cells, and tentatively concluded it to be an *N*-acetylmuramidase. In an attempt to study the substrate specificity with respect to

its amino sugar substitution, we have examined the effect of the presence or absence of acetyl groups at the amino sugar residues on PL-1 muramidase-catalyzed hydrolysis of cell wall peptidoglycan of *Lactobacillus casei*.

PL-1 muramidase was prepared as described in a previous paper.⁹⁾ The substrate cell wall peptidoglycan was prepared as described by Ishibashi *et al.*¹⁰⁾ D-Glucosamine and *N*-acetyl glucosamine were purchased from Nakarai-Kagaku Co. Muramic acid, *N*-acetyl muramic acid and crystalline EWL were from Sigma Chemical Co. Hydrolysis of *Lactobacillus casei* peptidoglycan with HCl yielded muramic acid, glucosamine, alanine (D- and L-), L-glutamic acid, L-lysine and L-aspartic acid in the approximate molar ratio of 1:1:2:1:1:1 (detailed data not shown). Total (*N*- and *O*-) and *O*-acetyl groups in the peptidoglycan were determined with a Shimadzu GC-7A gas chromatograph packed with Porapack Q using the method of Fromme and Beilharz.¹¹⁾ Propionic acid was used as an internal standard. The amount of the total (*N*- and *O*-) acetyl group was found to be 2.01 ± 0.13 mol, and that of the *O*-acetyl group 0.05 ± 0.04 mol in the repeating unit (MW=968) of the peptidoglycan. This indicates that neither muramic acid nor glucosamine moieties of the peptidoglycan were *O*-acetylated, but, rather, were largely *N*-acetylated.

The effect of some peptidoglycan-related hexosamines

FIG. 1. Effect of Some Peptidoglycan-related Hexosamines on PL-1 Muramidase Activity.

The 3 ml reaction mixture contained 10 mM Tris-HCl buffer (pH 7.2) and the cell wall peptidoglycan of *Lactobacillus casei* giving a final concentration of $OD_{600}=0.35$, and PL-1 muramidase, which had been pretreated with hexosamines of the indicated final concentrations at 37°C for 30 min. Enzyme activities are expressed as a percentage of the units for enzyme activity in the absence of added hexosamines (100%=6.0 units). ● = MurNAc; ○ = Mur; ■ = GlcNAc; □ = GlcN; ○ = control without hexosamine.

TABLE I. RELATIONSHIP BETWEEN THE ACETYLATION OF AMINO SUGAR RESIDUES IN *Lactobacillus casei* PEPTIDOGLYCANS AND SENSITIVITY TO PL-1 MURAMIDASE AND EWL

The 3 ml reaction mixture contained 10 mM Tris-HCl buffer (pH 7.2) and the cell wall peptidoglycans of *Lactobacillus casei* (modified in advance as shown in the table) giving a final concentration of $OD_{600}=0.35$, and PL-1 muramidase or EWL of 37 μ g of protein as determined as shown in the text.

R ₁	R ₂	Enzyme activity (units)	
			PL-1 Muramidase	EWL
	-COCH ₃	-H	8.9	1.8
	-H	-H	0.5	0.0
	-COCH ₃	-COCH ₃	0.7	0.5

on hydrolysis of the peptidoglycan of *Lactobacillus casei* by PL-1 muramidase was then investigated. The 3 ml reaction mixture containing the peptidoglycan suspensions gave an initial OD of about 0.35 at 600 nm. PL-1 muramidase (6.0 units) was pretreated with the indicated final concentrations of hexosamines at 37°C for 30 min, and 10 mM Tris-HCl buffer (pH 7.2). After measuring the OD for a zero time control, the reaction mixture was incubated at 37°C. At intervals, OD measurement was performed in the same manner using the buffer as a blank. One unit of enzyme activity was defined as the amount of enzyme that gave a decrease in OD of 0.001/min during the initial period of linear decrease: units = $1000 \times OD_{600}/\text{min}$. Among the hexosamines tested, *N*-acetyl muramic acid (MurNAc) showed the greatest inhibition of the enzyme activity (Fig. 1). The others resulted in only slight inhibition. *N*-Acetyl glucosamine (GlcNAc) showed stronger inhibition than did its unacetylated form (GlcN). These types of enzyme inhibition suggest the possibility that PL-1 muramidase may be an *N*-acetyl-muramidase.

The control peptidoglycan (2'-*N*-acetylated form) was then deacetylated with 0.2N HCl by the method of Fromme and Beilharz,¹¹⁾ and the sensitivity to PL-1 muramidase compared with that of the control *N*-acetylated one. As shown in Table I, the peptidoglycan samples with unsubstituted amino sugar residues were no more sensitive to PL-1 muramidase. Next the control peptidoglycan was 6'-*O*-acetylated by the method of Brumfitt¹²⁾ using pyridine-acetic anhydride, and the sensitivity of the 2',6'-diacetylated form thus prepared as above was examined. Again, the diacetylated form of peptidoglycan was essentially unaffected by PL-1 muramidase. Therefore, PL-1 muramidase prefers *N*-acetylated amino sugar polymers rather than both *N,O*-diacetylated and unsubstituted amino sugar substrates. For comparison, EWL was examined as a hydrolyzing agent of these modified peptidoglycans. EWL showed much lower activity on the control peptidoglycan with *N*-acetylated amino sugar residues than did PL-1 muramidase at the

same concentration (Table I). Both the unacetylated and *N,O*-diacetylated forms of peptidoglycans were essentially resistant to EWL. Furthermore, the cell walls of *Staphylococcus aureus*, whose amino sugar residues were *N,O*-diacetylated, were resistant to PL-1 muramidase, as was expected. PL-1 muramidase had little effect on either ethyleneglycol chitin or cells of *Micrococcus lysodeikticus* (*M. luteus*), which were used as a substrate of EWL. It is not clear for the present why this PL-1 enzyme attacks only slightly the cell walls of *M. luteus*. Many factors, such as the steric structure of the substrate, their degree of polymerization and others, as well as the amounts of the acetyl groups, may be concerned with the substrate specificity of the enzyme.

These results clearly confirm that PL-1 muramidase acts not as an *N,O*-diacetylmuramidase, as do *Chalaropsis* lysozyme Ch¹³⁾ and *Streptomyces* F2 enzyme,⁸⁾ but as an *N*-acetylmuramidase, acting exclusively on the peptidoglycan of *Lactobacillus casei* with *N*-acetylated amino sugar residues.

REFERENCES

- 1) D. Tsuru, *Hakko to Kogyo*, **41**, 180 (1983).
- 2) Y. Araki, T. Nakatani, H. Hayashi and E. Ito, *Biochem. Biophys. Res. Commun.*, **42**, 691 (1971).
- 3) Y. Araki, T. Nakatani, K. Nakamura and E. Ito, *J. Biol. Chem.*, **247**, 6312 (1972).
- 4) K. Amano, Y. Araki and E. Ito, *Eur. J. Biochem.*, **107**, 547 (1980).
- 5) G. F. Zipperle, Jr., J. W. Ezzell, Jr. and R. J. Doyle, *Can. J. Microbiol.*, **30**, 553 (1984).
- 6) K. Yokogawa, S. Kawata, T. Takemura and Y. Yoshimura, *Agric. Biol. Chem.*, **39**, 1533 (1975).
- 7) D. J. Tipper, J.-M. Ghuysen and J. L. Strominger, *Biochemistry*, **4**, 468 (1965).
- 8) T. Yoshimoto and D. Tsuru, *Kagaku to Seibutsu*, **12**, 33 (1974).
- 9) K. Watanabe, M. Hayashida, K. Ishibashi and Y.

- Nakashima, *J. Gen. Microbiol.*, **130**, 275 (1984). (1978).
- 10) K. Ishibashi, S. Takesue, K. Watanabe and K. Oishi, 12) W. Brumfitt, *Brit. J. Exptl. Pathol.*, **40**, 441 (1959).
J. Gen. Microbiol., **128**, 2251 (1982). 13) J. H. Hash and M. V. Rothlauf, *J. Biol. Chem.*, **242**,
11) I. Fromme and H. Beilharz, *Anal. Biochem.*, **84**, 347 5586 (1967).
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