ENZYMATIC DEACETYLATION OF N-ACETYLGLUCOSAMINE RESIDUES IN PEPTIDOGLYCAN FROM *BACILLUS CEREUS* CELL WALLS

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Summary Studies on the enzymatic mechanism of the formation of the N-unsubstituted glucosamine residues in *Bacillus cereus* cell wall peptidoglycan resulted in finding of an enzyme which hydrolyzes acetamido groups of N-acetylglucosamine residues. The enzyme appears to be specific for undegraded peptidoglycan and is distinguishable from N-acetylglucosamine-6-phosphate deacetylase. It shows a pH optimum of 7.0 and requires Co^{++} for its full activity.

Some strains of *Bacillus cereus* have been found to contain N-unsubstituted glucosamine residues in their cell wall peptidoglycan (1, 2). The known pathway of the enzymatic synthesis of bacterial cell wall peptidoglycan suggests that removal of the N-acetyl groups from the N-acetylglucosamine residues may take place following the formation of the polysaccharide backbone (3). The present communication describes the occurrence in *B. cereus* cells of an enzyme that catalyzes deacetylation of the N-acetylglucosamine residues in peptidoglycan. This enzyme is distinguishable from N-acetylglucosamine-6-phosphate deacetylase, which is an only well-investigated enzyme hydrolyzing the acetamido groups of N-acetylamino sugar derivatives (4, 5).

Materials and Methods *Bacillus cereus* strains AHU 1030, AHU 1355, AHU 1356, and T were grown as described previously (2). Cells from 2 liters of the cultures were harvested at their half maximum growth, and suspended in 30 ml of 0.02 M Tris-Cl, pH

7.2. The suspension was treated in a 10 Kc sonic oscillator for 7 minutes. The homogenate was centrifuged at 5,000 rpm for 15 minutes and the supernatant was further centrifuged at 13,000 rpm for 20 minutes. The precipitate, resuspended in 0.02 M Tris-Cl, pH 7.2, was designated the membrane fraction. The supernatant was used as enzyme either directly or after purification.

The substrates for deacetylase were prepared in the following manner. HCl-treated cell walls (20 mg), which were prepared as described previously from _B. cereus_ AHU 1356 and contained 0.28 µmole/mg of 2,6-diaminopimelic acid with a free amino group in addition to 0.48 µmole/mg of N-unsubstituted glucosamine (2), were treated for 12 hours at 4^{o} in a suspension containing 100 mg of $NaHCO_3$ and either acetic anhydride-^{14}C (10^8 cpm, 44 µmoles) or acetic anhydride-^{3}H (2×10^9 cpm, 68 µmoles) in 1.5 ml of water. Then, 100 µl of unlabeled acetic anhydride was added and the treatment was continued for further 24 hours. The labeled product was washed repeatedly with water and lyophilized. In order to determine the position of the radioactive substituents, a sample of HCl-cell wall-N-acetyl-^{14}C (5×10^5 cpm, 1.17 mg) was treated with muramyl L-alanine amidase from _Flavobacterium_ L-11 enzyme (6), giving a dialyzable fraction which represented the peptide fraction. The nondialyzable fraction was then digested with lysozyme and again dialyzed. The resulting oligosaccharides in the dialyzable fraction were separated by paper chromatography in butanol-acetic acid-water (4:1:5). The radioactivities recovered in these fractions were as follows: peptide, 22 %; disaccharide, 22 %; tetrasaccharide, 14 %; higher oligosaccharides, 20 %. On β-N-acetylglucosaminidase treatment followed by paper chromatography, the disaccharide fraction gave a single radioactive product which coincided with N-acetylglucosamine. This result shows that the bound radioactivity was shared between the N-acetylglucosamine residues and the peptide moiety in an approximate ratio of 2:1 as expected from the free amino group content of the original HCl-treated cell walls. Glycolchitin-acetyl-^{3}H was similarly prepared from glycolchitin which had been deacetylated with alkali. N-Acetylglucosamine-6-phosphate-acetyl-^{14}C was prepared according to the method of Brown (7). N-Acetylglucosamine-acetyl-^{14}C was obtained from this preparation by alkaline phosphatase hydrolysis. N-Acetylglucosaminyl-β(1-4)-N-acetylmuramic acid-acetyl-^{3}H was prepared by treating glucosaminyl-β(1-4)-muramic acid with acetic anhydride-^{3}H as described above.

For the assay of peptidoglycan deacetylase, a reaction mixture, containing HCl-cell wall-acetyl-^{3}H (20,000 cpm, 50 μg), 1 mM $CoCl_2$, 40 mM Tris-Cl, pH 7.2, and enzyme in a final volume of 150 μl, was incubated at 37° for 2 hours. After addition of 20 μl of 0.2 N HCl and 5 μl of 1 M acetic acid, the mixture was extracted three times with 0.5 ml of ethyl acetate, and the ethyl acetate phase combined was measured in Bray's scintillator for radioactivity in a liquid scintillation counter. The reaction mixture for the assay of N-acetylglucosamine-6-phosphate deacetylase contained N-acetylglucosamine-6-phosphate-acetyl-^{14}C (7,000 cpm, 5mμmoles), 3 mM $MnCl_2$, 40 mM Tris-Cl, pH 7.8, and enzyme in a final volume of 50 μl. After incubation at 37° for 30 minutes, 100 μl of water was added to each tube. The extraction of acetic acid and the measurement of radioactivity were carried out as described above. Other materials and methods were the same as those described previously (2).

<u>Results and Discussion</u> As shown in Table 1, deacetylation of

Table 1. Deacetylase Activity in Various Strains of _B. cereus_

Strain*	Fraction	Deacetylase activity	
		Peptidoglycan	N-Acetylglucosamine-6-phosphate
		cpm x 10^{-6}	cpm x 10^{-6}
AHU 1030	Supernatant	0.16	4.3
	Membrane	0.01	0.01
AHU 1355	Supernatant	2.57	8.7
	Membrane	0.88	0.01
AHU 1356	Supernatant	2.11	9.5
	Membrane	1.31	0.01
T	Supernatant	2.55	4.9
	Membrane	0.33	0.01

Assay of deacetylase activity was carried out as described in the text with HCl-cell wall-acetyl-^{3}H or N-acetylglucosamine-6-phosphate-acetyl-^{14}C as substrate. The values are expressed in radioactivity released per fraction from cells from 2 liters of culture.

* Cell wall peptidoglycans from these strains contain the following amounts of N-unsubstituted glucosamine residues as expressed in μmoles per mg of cells (2). AHU 1030, 0.06; AHU 1355, 0.26; AHU 1356, 0.26; T, 0.26.

peptidoglycan was demonstrated both with the membrane fraction and with the supernatant fraction as enzyme, whereas N-acetylglucosamine-6-phosphate deacetylase activity was found almost exclusively in the supernatant fraction. In contrast to the uniform occurrence of the latter activity in every strain of _B. cereus_, the former activity was present in a considerable strength only in the strains with walls rich in the N-unsubstituted glucosamine residues. This result suggests that the deacetylation of peptidoglycan is catalyzed by an enzyme different from N-acetylglucosamine-6-phosphate deacetylase. The two enzymes, peptidoglycan deacetylase and N-acetylglucosamine-6-phosphate deacetylase, were separated from each other by gel filtration as shown in Fig. 1. The former enzyme showed a maximum activity at pH 7 and in the presence of 2 mM Co^{++}, while the latter enzyme required the presence of Mn^{++} and its pH optimum was about 9.

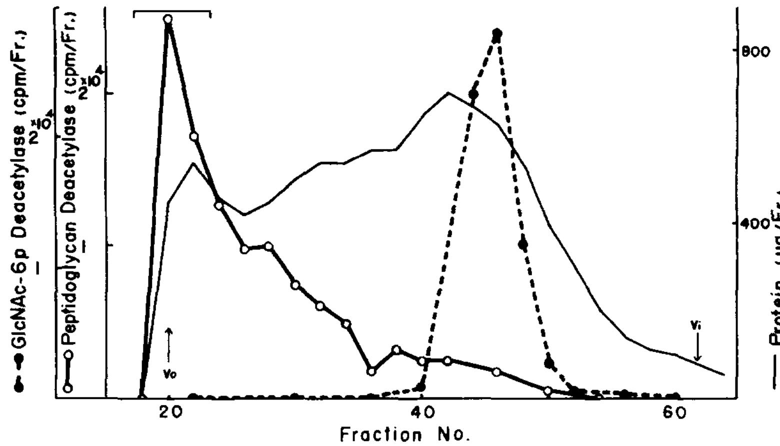

Fig. 1. Sepharose 6B column chromatography of deacetylases. The supernatant fraction (100 ml) from B. cereus AHU 1355 was treated with 20 ml of 1 % protamine sulfate, pH 7, and centrifuged. The supernatant was brought to 30 % saturation with ammonium sulfate. After centrifugation, the supernatant was brought to 57 % saturation by further addition of ammonium sulfate. The precipitate collected was dissolved in and dialyzed against 5 mM Tris-Cl, pH 7.2. A portion (1 ml) of the dialyzed solution (22 ml) was applied to a column of Sepharose 6B (0.8 x 54 cm). Flow rate was 1.4 ml per hour. The effluent was collected in 0.35 ml fractions. Enzyme activities are expressed in cpm per fraction, and protein concentration, in μg per fraction. The fractions indicated were pooled and used as peptidoglycan deacetylase.

The specificity of peptidoglycan deacetylase for substrates was studied using the partially purified enzyme (Table 2). This enzyme appears to be specific for the substrate with a high molecular weight. It was virtually inactive on lysozyme digest of peptidoglycan and on di- and monosaccharides. The low activity observed with N-acetylglucosamine-6-phosphate seems to be ascribable to contaminating N-acetylglucosamine-6-phosphate deacetylase. It may be noted that glycolchitin also was deacetylated although the rate was low. After prolonged incubation with this enzyme, liberation of the labeled groups from glycolchitin was 6.4 %, while that from peptidoglycan reached 36 %. This enzyme appeared to attack the glycan fraction more slowly

Table 2. Substrate Specificity of Peptidoglycan Deacetylase

Substrate	cpm
Cell wall-^{3}H (9,900 cpm, 27 µg)	935
Cell wall-^{3}H, digested with lysozyme (9,900 cpm, 27 µg)	20
Cell wall-^{3}H, digested with L-alanine amidase, nondialyzable fraction (10,950 cpm)	320
Cell wall-^{3}H, digested with L-alanine amidase, dialyzable fraction (3,960 cpm)	0
Glycolchitin-^{3}H (51,500 cpm, 186 µg)	170
N-Acetylglucosaminyl-β(1-4)-N-acetylmuramic acid-^{3}H (2,930 cpm, 0.34 mµmole)	0
N-Acetylglucosamine-^{14}C (10,240 cpm, 1 mµmole)	0
N-Acetylglucosamine-6-P-^{14}C (9,060 cpm, 0.9 mµmole)	170

Assay of enzymatic deacetylation was carried out under the conditions described in the text using the substrates indicated and 20 µl of peptidoglycan deacetylase purified by Sepharose chromatography as shown in Fig. 1.

than intact peptidoglycan. A likely explanation for this result is that peptidoglycan, after removal of its peptide moiety, may be easily degraded by a lysozyme-like enzyme involved in the enzyme preparation. Another possibility to be considered is that the deacetylase hydrolyzes not only the acetamido groups in the glycan moiety but also those in the peptide moiety.

The acetamido groups subjected to the attack of deacetylase were identified in the following experiments: Peptidoglycan, fully N-acetylated with unlabeled acetic anhydride, was incubated with deacetylase, and the free amino groups formed were again acetylated by the treatment with acetic anhydride-^{14}C. The labeled product was digested with lysozyme either directly or after removal of the peptide moiety in terms of muramyl L-alanine amidase treatment followed by dialysis, and the resulting oligosaccharide fraction was separated by dialysis and

Table 3. Degradation of Deacetylase Reaction Product after Acetic Anhydride-^{14}C Treatment

Experiment 1	cpm		
Total bound radioactivity	55,800		
Peptide fraction released on L-alanine amidase treatment	3,720		
Nondialyzable fraction from lysozyme digestion	8,400		
Dialyzable fraction from lysozyme digestion			
Total amount	44,000		
Disaccharide	24,000		
Tetrasaccharide	8,000		
Experiment 2	**0 hour**	**4 hours**	**24 hours**
Dialyzable fraction from lysozyme digestion	cpm	cpm	cpm
Total amount	12,300	38,300	52,500
Oligosaccharide fraction	6,700	28,400	45,200

Experiment 1. HCl-treated cell walls of B. cereus AHU 1356 were N-acetylated with unlabeled acetic anhydride as described previously (2). 4.4 mg of the N-acetylated walls was incubated in a reaction mixture, containing 0.55 ml of Sepharose-treated enzyme, 40 mM TES, pH 6.88, and 0.7 mM $CoCl_2$ in a final volume of 5 ml, for 4 hours at 37°. Then, the mixture was centrifuged at 13,000 rpm for 45 minutes, and the sediment was treated for 40 hours at 0° in a solution containing 50 mg of $NaHCO_3$, acetic anhydride-^{14}C (1.3×10^7 cpm, 11 µmoles), and 0.5 ml of water. The treatment was continued for further 36 hours following addition of 33 µl of acetic anhydride. The sediment from the mixture was successively digested with muramyl L-alanine amidase and lysozyme as described in the text. Paper chromatography of the dialyzable fraction from the lysozyme digestion was carried out in butanol-acetic acid-water (4:1:5).
Experiment 2. Conditions for deacetylase reaction and subsequent treatment with acetic anhydride-^{14}C were the same as those in Experiment 1 except that the enzyme reaction was terminated at the indicated time. The labeled product was dialyzed against water, and the nondialyzable fraction was digested with lysozyme. The lysozyme digest was dialyzed, and the dialyzable fraction was separated by paper chromatography.

paper chromatography. As shown in Table 3, only a small part of the bound radioactivity was present in the peptide fraction, and the majority of the radioactivity was found in the oligosaccharide fraction. The main radioactive fragment, which coincided with N-acetylglucosaminyl-β(1-4)-N-acetylmuramic acid, was hydrolyzed with β-N-acetylglucosaminidase, giving N-acetylglucosamine as the sole radioactive product on paper chromatograms in butanol-acetic acid-water (4:1:5) and in butanol-pyridine-acetic acid-water (6:4:0.3:3). The yield of the radioactive oligosaccharide fraction was dependent on the time of incubation with deacetylase.

The above result indicates that the present enzyme hydrolyzes the acetamido groups of the N-acetylglucosamine residues in peptidoglycan, leaving those of the N-acetylmuramic acid residues and probably those of the peptide moiety intact. The details of this investigation will be described elsewhere.

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