# A NOVEL GLYCOSIDASE, AN ENDO-GLUCOSAMINIDASE ACTIVE ON THE CELL WALL PEPTIDOGLYCAN WITH *N*-UNSUBSTITUTED GLUCOSAMINE RESIDUES

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## 1. Introduction

The cell walls of *Bacillus cereus* AHU 1356, which are known to be resistant to lysozyme because of the occurrence of *N*-unsubstituted glucosamine residues in their peptidoglycan components [1,2], were indicated to be also resistant to several other cell-wall lytic glycosidases, whereas they were readily hydrolyzed by the same enzymes when tested after *N*-acetylation. In contrast, the crude cell-wall autolysate of this strain was found to release reducing groups from both the intact and the *N*-acetylated cell wall preparations to the same extent. This paper reports the occurrence in this autolysate of a novel glycosidase, an endo-glucosaminidase which hydrolyzes the glycosidic linkages of *N*-unsubstituted glucosamine in the cell-wall peptidoglycan.

## 2. Materials and methods

### 2.1. *Bacteria and cell walls*

The strain, culture conditions and methods for preparations of cell walls and peptidoglycan of *B cereus* AHU 1356 were the same as those in [1].

### 2.2. *Preparation of autolytic enzyme*

*B. cereus* AHU 1356 cells harvested from 1 l culture at the late exponential phase were suspended in 35 ml 20 mM Tris–Cl (pH 7 2) and treated at 0°C in a 10 kHz sonic oscillator for 5 min. The homogenate was centrifuged at 2200 × *g* for 5 min, and the supernatant was further centrifuged at 20 000 × *g* for 25 min. The resulting precipitate (1.2 g wet wt) was resuspended in 20 ml of 20 mM Tris–Cl (pH 7.2) and pooled to be used as the crude cell-wall fraction. This fraction (240 ml) was incubated with a small amount of toluene at 37°C for 7 h, and then centrifuged at 20 000 × *g* for 25 min. A fraction precipitated from the supernatant between 35% and 75% $(NH_4)_2SO_4$ saturation was collected and used as the autolytic enzyme preparation (3 6 ml, 36 mg protein) after dialysis against 20 mM Tris–Cl (pH 7.2).

*Abbreviations* GlcN, glucosamine, GlcNAc, *N*-acetylglucosamine, Mur, muramic acid, MurAc, *N*-acetylmuramic acid

### 2.3. *Assay of enzyme activity*

The reaction mixture contained 1.2 mg purified cell walls, 20 mM Tris–Cl (pH 8.2) and the enzyme preparation in total vol. 2 ml. After incubation for various time intervals at 37°C, aliquots were withdrawn and measured for decrease in turbidity (cell-wall lytic activity) [1], for reducing-group liberation (glycosidase activity) [1] and for amino-group liberation by the dinitrophenylation method (*N*-acetylmuramyl-L-alanine amidase and peptidase activities) [3].

### 2.4 *Separation of products from digestion of peptidoglycan with autolytic enzyme*

The cell-wall peptidoglycan (35 mg) was incubated under toluene at 37°C with the autolytic enzyme preparation (750 μl, 7.5 mg protein) in total vol. 75 ml. After 65 h, 600 μl enzyme preparation ( 6 mg protein) was supplemented and the mixture was further incubated for 30 h The lysate was concen-



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trated to 5 ml in a rotary evaporator and dialyzed 5 times against 200 ml water for 12 h. The dialysates were pooled, concentrated to 500 μl and applied to a column of Sephadex G-15 (1 × 45 cm). The column was eluted with water and 500 μl fractions were collected. Reducing material, emerging in a single broad peak at fractions 28–55, was pooled, lyophilized and subjected to preparative paper chromatography in 1-butanol/acetic acid/water (4/1/5) (solvent A), giving three bands of ninhydrin-positive reducing materials with $R_{GlcNAc-MurAc}$ values of 0.74 (fraction I), 0.31 (II) and 0 (III). Fractions I and II were purified successively by paper electrophoresis in pyridine/acetic acid/water (35/5/960, pH 5.8) (buffer A) and by paper chromatography in 1-butanol/pyridine/acetic acid/water (6/4/0.3/3) (solvent B) and in 1-butanol/acetic acid/water (2/1/1). The materials purified from fractions I and II were denoted as compounds I and II, respectively.

Other materials and methods were as in [1].

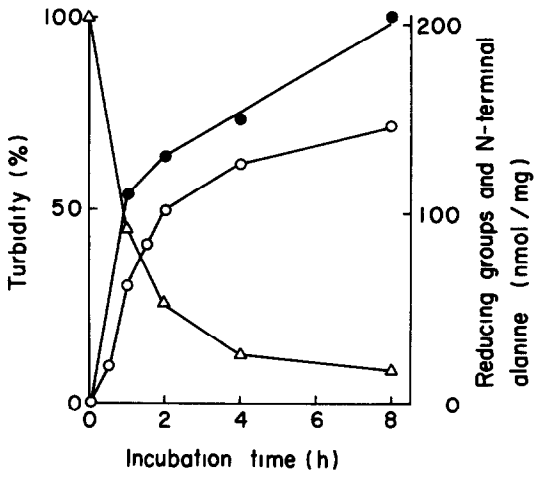


Fig.1. Liberation of reducing groups and N-terminal alanine from *B. cereus* AHU 1356 cell walls during incubation with the autolytic enzyme preparation. Purified cell walls (1.2 mg) were incubated at 37°C with 40 μl enzyme preparation (400 μg protein). (△) Turbidity expressed as a percentage of initial value, (○) liberation of reducing groups expressed as nmol/mg cell walls; (●) liberation of N-terminal alanine expressed as nmol/mg cell walls.

## 3. Results and discussion

Figure 1 shows the time course of digestion of *B cereus* AHU 1356 cell walls with the autolytic enzyme preparation. Reducing groups were liberated in parallel with decrease in turbidity, and N-terminal alanine was also liberated, but liberation of other N-terminal amino acids was not detected. This result suggests the occurrence in the autolytic enzyme preparation of a unique glycosidase active on the cell-wall peptidoglycan with *N*-unsubstituted glucosamine residues.

To study the mode of the glycosidase action, the cell-wall peptidoglycan (21 μmol disaccharide units)

Table 1
The mobility of compounds I and II on paper chromatography and paper electrophoresis

| Compound | $R_{GlcNAc-MurAc}$ in solvents | | $M_{GlcNAc-MurAc}$ |
|---|---|---|---|
| | A | B | |
| GlcNAc | 1.00 | 2 04 | 0 |
| GlcN–Mur | 0.40 | 0 36 | –1.35 |
| GlcNAc–MurAc | 1.00 | 1.00 | 1 00 |
| $(GlcNAc–MurAc)_2$ | 0.48 | 0 21 | 1 39 |
| Compound I, MurAc–GlcN | 0.74 | 0.50 | 0 |
| Compound I after *N*-acetylation | 1.00 | 0.98 | 0.94 |
| Compound II, $(MurAc–GlcN)_2$ | 0.31 | 0.09 | 0 |
| Compound II after *N*-acetylation | 0.60 | 0.31 | 1.34 |

$R_{GlcNAc-MurAc}$, mobility on paper chromatography relative to GlcNAc–MurAc, $M_{GlcNAc-MurAc}$, mobility to anode on paper electrophoresis in buffer A relative to GlcNAc–MurAc

was exhaustively digested with the autolytic enzyme preparation, and oligosaccharides in the dialyzable fraction (6.5 μmol reducing groups) of the lysate (13 μmol reducing groups) were separated as in section 2. Three ninhydrin-positive reducing materials (fraction I, 3.2 μmol, II, 1.4 μmol; III, 1.1 μmol) were obtained by paper chromatography in solvent A. Purification of fractions I and II, respectively, yielded compounds I (1.8 μmol) and II (0.8 μmol), which gave single spots on paper chromatography in several solvents and paper electrophoresis. The mobility of compounds I and II and their *N*-acetylation products is shown in table 1.

Compound I was characterized as MurAc–β(1–4)-GlcN from the already known structure of the cell-wall peptidoglycan of this strain, from the paper-chromatographic and paper-electrophoretic mobilities and from the following results of analysis. Analysis by an amino-acid analyzer after acid hydrolysis (4 N HCl, 100°C, 4 h) gave muramic acid and glucosamine in an equimolar proportion. Reduction with $NaBH_4$ followed by acid hydrolysis revealed the glucosamine component to be all at the reducing end. Either intact or *N*-acetylated compound I was completely insensitive to exo-β-*N*-acetylglucosaminidase. Dinitrophenylation followed by acid hydrolysis gave *N*-(2,4-dinitrophenyl)-glucosamine in an amount corresponding to 1 mol/mol.

Hydrolysis of compound II yielded the disaccharide GlcN–Mur, muramic acid and glucosamine in a molar ratio of 1 1 1, whereas hydrolysis of this compound after reduction with $NaBH_4$ gave the disaccharide, muramic acid and glucosaminitol in a molar ratio of 1 1 1. As a glycosidic linkage of *N*-unsubstituted glucosamine is resistant to acid hydrolysis, the above evidence is consistent with the structure MurAc–GlcN–MurAc–GlcN for compound II. The *N*-unsubstitution at the glucosamine residues was supported by analysis of the dinitrophenylation product. In addition, compound II was cleaved to compound I on prolonged incubation with excess of the autolytic enzyme preparation.

On the other hand, major saccharide fragments in the lysate of the *N*-acetylated peptidoglycan were isolated and characterized as MurAc–GlcNAc and MurAc–GlcNAc–MurAc-GlcNAc in procedures similar to those above, indicating that the enzyme preparation also possesses an endo-*N*-acetylglucosaminidase activity. However, it is unknown whether or not a single enzyme is responsible for both the endo-glucosaminidase and endo-*N*-acetylglucosaminidase activities.

The yields of compounds I and II accounted for the majority of the disaccharide units of dialyzable saccharide fragments in the peptidoglycan lysate. In addition, other small saccharide fragments, such as monosaccharides, GlcN–MurAc and GlcN–MurAc–GlcN–MurAc, were not found in the dialyzable fraction. Therefore, the endo-glucosaminidase seems to be responsible for autolytic digestion of the glycan chain of the cell-wall peptidoglycan in this strain.

The autolytic enzyme preparation could not hydrolyze glycol chitosan, glycol chitin or colloidal chitin. This fact indicates that the endo-glucosaminidase of this strain differs from microbial chitosanase, capable of hydrolyzing the glycosidic linkages of polysaccharides with free amino groups [4–6] Studies on the separation and characterization of the glycosidase(s) and amidase in the autolytic enzyme preparation are in progress.

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