Purification and Properties of Autolytic Endo- β -*N*-acetylglucosaminidase and the *N*-Acetylmuramyl-L-alanine Amidase from *Bacillus subtilis* Strain 168

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β -*N*-Acetylglucosaminidase has been purified from the walls of *Bacillus subtilis* 168 and compared with the other known autolysin, *N*-acetylmuramyl-L-alanine amidase (amidase). The β -*N*-acetylglucosaminidase was a dimer in LiCl buffers with a sub-unit molecular weight of 90000 and a pH optimum of about 5.0. It was very sensitive to proteolytic enzymes and was critically activated by 0.1 to 0.2 M-LiCl. It was insoluble in concentrations of LiCl lower than 0.05 to 0.1 M. It was less strongly bound to walls than was the amidase, which was a monomer of molecular weight 30000 to 40000 in LiCl buffers. The β -*N*-acetylglucosaminidase is an endo-enzyme and showed no exo-activity. Lysozyme-like enzyme (muramidase) activity was undetectable in the wall extracts examined.

INTRODUCTION

Bacillus subtilis 168, and many other strains of the organism growing in the vegetative phase, produce two enzymes that hydrolyse bonds in the wall peptidoglycan, and can thus cause bacterial autolysis (Young, 1966; Brown & Young, 1970). Their specificities are (a) for the bonds between the L-alanyl and *N*-acetylmuramyl residues – this enzyme is called the *N*-acetylmuramyl-L-alanine amidase (amidase), and (b) for those between the *N*-acetylglucosaminyl and the *N*-acetylmuramyl residues in the glycan chains, an enzyme called endo- β -*N*-acetylglucosaminidase. Other strains of *B. subtilis* (Takahara *et al.*, 1974; Okada & Itahata, 1973) additionally produce extracellular muramidases, that is enzymes with the same specificity as lysozyme, which attack the (1→4)- β -*N*-acetylmuramyl-*N*-acetylglucosaminyl bonds. An enzyme with this specificity has not been found in *B. subtilis* 168.

The amidase was first purified by Herbold & Glaser (1975). The purification and properties of the β -*N*-acetylglucosaminidase have not hitherto been described although a brief note on the subject has appeared (Taylor *et al.*, 1980).

METHODS

Micro-organism and culture conditions. *Bacillus subtilis* 168 *trpC* was maintained on nutrient agar plates (Luria & Burroughs, 1957). Batches of 40 litres of tryptone/yeast extract medium, consisting of tryptone (Difco) 10 g, yeast extract (Difco) 10 g, K_2HPO_4 5 g, H_2O to 1 litre adjusted to pH 7.2, supplemented with 0.5% glucose, contained in a 50 litre fermenter, were inoculated with 1 litre of culture in the same medium incubated at 35 °C to give an A_{675} of about 1.0. A stream of sterile air was blown through the medium. Growth was at 35 °C and was allowed to proceed until an A_{675} of about 2.0 was reached. The culture was then rapidly cooled to 4 °C and the cells, collected in the bowl of a Sharples centrifuge as a paste, were stored at –20 °C.

Preparation of walls from *B. subtilis* containing autolysin. Batches of paste equivalent to about 50 g dry weight of bacteria were brought to 0 °C, diluted with 25 mM-Tris/HCl buffer, pH 7.2, to contain 30 mg dry weight ml⁻¹,

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washed twice by centrifugation and passed twice through a French pressure cell working at 1406 kg cm^{-2} and 0 to 2°C . The walls were recovered by centrifugation at 15000 g and were washed twice with distilled H_2O . The washed walls were freeze-dried.

Extraction of enzymes from B. subtilis walls. The freeze-dried walls (5 g) were extracted by stirring with 300 ml 2 M-LiCl in 25 mM-Tris/HCl , $\text{pH } 7.2$, at 0°C for 1 h , they were removed by centrifugation and the extraction was repeated twice more.

Preparation of B. subtilis and Micrococcus luteus substrate walls (SDS-walls). Batches of freeze dried cells (50 to 100 g dry weight) were suspended in water (about 5 g cells to 200 ml), mixed with an equal volume of ballotini no. 12 beads and shaken at as near 0°C as possible in the cooled pot of a disintegrator (Merkeshlager *et al.*, 1957). The treatment of the walls with SDS and subsequent purification were as previously described (Fein & Rogers, 1976). Samples of all batches of walls were hydrolysed by 1 M-HCl and examined by standard techniques for amino acid content (Rogers *et al.*, 1980). The final wall preparations were kept at -20°C in the freeze-dried form for use as substrates in the enzyme assays.

Radioactively labelled *B. subtilis* SDS-walls ($7.48 \times 10^4 \text{ d.p.m. mg}^{-1}$) were prepared by the same method, but *N*-acetyl[$1\text{-}^{14}\text{C}$]glucosamine (1.1 kBq ml^{-1} ; specific activity $3.7 \text{ kBq } \mu\text{mol}^{-1}$) was included in CHY medium (Hughes, 1968). Radioactively labelled *M. luteus* walls ($3.07 \times 10^4 \text{ d.p.m. mg}^{-1}$) were prepared by including [$3\text{-}^{14}\text{C}$]glucose ($14.8 \text{ kBq mol}^{-1}$; specific activity $2.66 \text{ kBq } \mu\text{mol}^{-1}$) in CHY medium.

Preparation of soluble glycan from SDS-walls. Radioactively labelled *B. subtilis* walls were suspended in Tris/HCl buffer, $\text{pH } 9.0$, at $20 \text{ mg dry weight ml}^{-1}$ and mycobacterial AL-1 amidase (Ensign & Wolfe, 1966) was added. After 24 h incubation at 37°C , any insoluble material was removed by centrifugation. The supernatant was dried *in vacuo* and then dissolved in $0.1 \text{ M-pyridine/acetate}$ buffer, $\text{pH } 5.1$. This solution was chromatographed on a column of DE-52 (volume 50 ml). The peptides were removed by washing the column with the same buffer. The glycans were eluted by a linear gradient of pyridine/acetate (0.1 to 5 M), $\text{pH } 5.1$, at 1.0 M . The pooled samples containing the glycan were dried *in vacuo*, redissolved in H_2O and redried. This was repeated three times to remove pyridine/acetate. The solid was finally dissolved in water and freeze-dried. Analysis of the product after hydrolysis for 4 h with 4 M-HCl at 100°C showed it to contain glucosamine, muramic acid, the peptidoglycan amino acids, galactosamine and phosphorus in the molar proportions of $1.0:1.18:0.1:0.07:0.2$.

Lipoteichoic acid. The method of Fischer *et al.* (1980) was used to prepare alanine ester-containing material which contained phosphorus, glycerol, glucose and ester-linked D-alanine in the molecular proportions of $1:0.86:0.07:0.24$. It was found particularly important to ensure the complete removal of Triton X-100 from the preparation since this relieved the inhibitory action on the enzyme.

Endo- β -N-acetylglucosaminidase estimation. (i) Unlabelled *M. luteus* SDS-walls were suspended in $50 \text{ mM-succinic acid/NaOH}$ buffer, $\text{pH } 5.5$, at $10 \text{ mg dry weight ml}^{-1}$. This suspension was given a brief burst of ultrasonic vibration to obtain an even suspension. Suspension ($50 \mu\text{l}$) was mixed with 0.65 ml 50 mM-succinate buffer, $\text{pH } 5.5$, and the mixture cooled to 0°C . The enzyme extract ($100 \mu\text{l}$), also at 0°C , was then added. It contained 2 M-LiCl so that the final concentration of this salt was 0.25 M , and this final concentration was used in all assays. The mixture was allowed to stand for 15 min at 0°C during which time the β -N-acetylglucosaminidase bound to the walls. The wall and the enzyme were then deposited by centrifugation at 0°C and resuspended in 0.65 ml of the succinate buffer, $\text{pH } 5.5$, containing 0.25 M-LiCl at 37°C . The reduction in A_{450} during incubation at 37°C was then measured. One unit of enzyme activity represents a fall in A_{450} of 0.001 min^{-1} when the initial value is 1.00 .

(ii) The solubilization of radioactively labelled *M. luteus* walls was also used to estimate enzyme activity. A suspension of walls ($10 \text{ mg dry weight ml}^{-1}$) was diluted in $50 \text{ mM-succinic acid/NaOH}$ buffer, $\text{pH } 5.5$, to give 1.0 mg ml^{-1} and warmed to 37°C . An equal volume of the enzyme extract at 37°C adjusted to contain 0.5 M-LiCl was added and the mixture incubated for 1 h at 37°C . The walls were then removed by centrifugation and the radioactivity in samples of the supernatant estimated. If the enzyme activity was too small to allow sufficient dilution of the extract to give 0.5 M-LiCl , the buffered wall suspension at 0°C was mixed with a suitable volume of enzyme extract diluted so that the final mixture contained 0.25 M-LiCl . This mixture was stored at 0°C for 15 min . The walls with adsorbed enzyme were then washed with cold buffer and resuspended so that the final concentrations were: walls 0.5 mg ml^{-1} , $\text{LiCl } 0.25 \text{ M}$ and buffer (succinic acid/NaOH, $\text{pH } 5.5$) 25 mM . This mixture was incubated for 1 h at 37°C and treated as before. In both methods results were expressed as the proportion of the radioactively labelled walls rendered soluble.

N-Acetylmuramyl-L-alanine amidase (amidase) assay. SDS-walls from *B. subtilis* 168 were suspended in $25 \text{ mM-sodium tetraborate}$ adjusted to $\text{pH } 9.5$ with 0.1 M-NaOH and the mixture was given a brief ultrasonic vibration to obtain an even suspension. Sufficient 1.0 M-MgCl_2 solution was added to give a final concentration of 20 mM , the solution was warmed to 37°C and $60 \mu\text{l}$ of the enzyme extract added to 1.94 ml suspension. A_{450} was measured during continued incubation at 37°C . One unit of enzyme activity represents a fall in A_{450} of 0.001 min^{-1} when the initial value of the suspension is 1.00 . On occasion, radioactive *B. subtilis* 168 SDS-walls were used. The radioactivity in the supernatant was then measured and results expressed as for β -N-acetylglucosaminidase.

Measurement of specific bond hydrolysis in wall and glycan preparations. The action of β -*N*-acetylglucosaminidase upon (1 $\rightarrow$ 4)- β -*N*-acetylglucosaminyl bonds in a number of SDS-wall and other preparations was determined. The substrate preparations were first suspended in 1 mM-KOH and treated with NaBH₄ (50 mM, 16 h at 0 °C) to reduce existing free C-1 *N*-acetylglucosaminyl groups. The suspensions were then centrifuged and washed three times with H₂O. Purified enzyme preparations were allowed to act on them under the conditions described when SDS-walls were used as substrate. The hydrolysates were raised above pH 9.0 with 2 M-KOH and treated with KB³H₄ (20 mM; 370 kBq μ mol⁻¹) for 16 h at 0 °C. They were then neutralized with 1.0 M-acetic acid, evaporated *in vacuo* to dryness and hydrolysed with 4 M-HCl at 100 °C for 4 h in sealed ampoules. After removing HCl, they were dissolved in 0.1 M-pyridine/acetate buffer, pH 2.8, applied to small columns of Dowex H⁺ equilibrated with the same buffer, and the [³H]glucosaminol was eluted with 0.13 M-pyridine/acetate, pH 3.8.

Adsorption on, and elution from, *M. luteus* SDS-walls. The 2 M-LiCl extract of the *B. subtilis* walls containing the autolysins was diluted to a final concentration of 0.25 M-LiCl in 25 mM-Tris/HCl, pH 7.2. About 300 ml at 0 to 4 °C was stirred with 1 g *M. luteus* SDS-walls for 20 min; whilst maintained at this temperature, the walls were removed by centrifugation and 0.5 g more added to the supernatant solution. Again, the mixture was vigorously stirred for 20 min at 0 °C to 4 °C. The walls were again deposited by centrifugation and the total amount washed twice with 500 ml 0.25 M-LiCl in 25 mM-Tris/HCl, pH 7.2. They were then extracted three times each with 40 ml 2 M-LiCl in the same buffer. The final extract and washings were pooled and concentrated by ultrafiltration through an Amicon UM10 filter.

Chromatography on chitin. Columns (12 ml) of freshly prepared pulverized chitin (Jensen & Kleppe, 1972) were washed with 0.25 M-LiCl in 25 mM-Tris/HCl, pH 7.2, until the eluate was free of material absorbing at 280 nm. The enzyme preparation, also in 0.25 M-LiCl in 25 mM-Tris/HCl buffer, pH 7.2, was then loaded on the column. Enzyme activity was eluted with a linear gradient (0.25 to 5.0 M) of LiCl in 2.5 mM-Tris/HCl, pH 7.2.

Chromatography on Biogel A. The column had a bed volume of 377 ml. The void volume of such a column determined by the use of blue dextran 200 was 110 ml. It was equilibrated with 2 M-LiCl in 25 mM-Tris/HCl buffer, pH 7.2, and after application of the enzyme in 2 M-LiCl it was eluted with LiCl buffer of the same composition.

Purification of the amidase. The supernatants from *M. luteus* SDS-wall preparations remaining from the purification of the β -*N*-acetylglucosaminidase were pooled and concentrated by ultrafiltration through an Amicon PM30 membrane. Traces of DNAase and RNAase (1 μ g ml⁻¹ of each) were added and the solution was dialysed, first against 1 M-LiCl and then against 0.2 M-KCl in 0.1 M-phosphate buffer, pH 7.5. Thereafter the purification was as described by Herbold & Glaser (1975).

SDS-PAGE. Enzyme preparations (20 to 40 μ g protein) were subjected to SDS-PAGE according to Laemmli (1970) using 13% (w/v) acrylamide. Standard (10 μ g protein) of hen-egg lysozyme, trypsin inhibitor, ovalbumin and bovine serum albumin were run alongside the samples.

Radioactive determinations. Scintillation counting was used as previously described (Rogers *et al.*, 1983). Samples up to 100 μ l were added directly to the scintillator.

RESULTS

Purification of β -*N*-acetylglucosaminidase. Table 1 shows the steps in the purification with the yields achieved. A large number of batches have been made and latterly the selective adsorption on SDS-walls of *M. luteus* was omitted, since recovery of the enzyme was poor in two batches. Chromatography using Biogel A was then repeated but the risk of contamination of the final product with amidase was increased. For chromatography on chitin, it was found to be important to start with freshly prepared material, otherwise up to 50% of the enzyme passed through the column on the solvent front. Concentration by ultrafiltration through Amicon filters UM10 and PM10 from time to time gave considerable losses of enzyme for unknown reasons. It

Table 1. *Purification of β -N-acetylglucosaminidase*

Procedure	Total enzyme activity (units)	Total protein (mg)	Specific activity [units (mg protein) ⁻¹]	Yield (%)
2 M-LiCl extract of walls	55000	158	349	100
Binding and elution from <i>M. luteus</i> walls	44500	7.02	6340	80
Chromatography on chitin	30260	1.12	26900	55
Chromatography on Biogel A	16500	0.23	71500	30

Fig. 1. Immunodiffusion of β -*N*-acetylglucosaminidase. The central well contained 10 μ l antiserum from a rabbit immunized with purified β -*N*-acetylglucosaminidase (specific activity 21 300 units mg^{-1}). The outer wells contained 10 μ l 2 M-LiCl extract of native walls from *Bacillus subtilis* 168.

was found to be important to keep the salt concentration throughout all procedures above 0.1 M, since below this concentration enzyme precipitated and activity was irreversibly lost. The specific activity of the final preparations varied from 21 000 to 72 000 units $(\text{mg protein})^{-1}$. All of the preparations gave either a single band or a single strong band and another very weak one, after electrophoresis of 20 to 40 μ g protein in SDS on 13% crosslinked polyacrylamide gels and staining with Coomassie blue. Antiserum against one of the active purified preparations also gave a single very strong precipitin band, along with another very faint one, on Ouchterlony plates containing the crude initial LiCl extracts in the wells (Fig. 1).

Molecular weights of the enzymes. From the behaviour in SDS-PAGE, the molecular weight of the β -*N*-acetylglucosaminidase was 90 000. Chromatography on a Biogel A column using 2 M-LiCl in 25 mM-Tris/HCl buffer, pH 7.2, containing 10 mM-MgCl₂, suggested a molecular weight of 190 000. The amidase was eluted in a position corresponding to 30 000 to 40 000 (Fig. 2). Since the value by gel-chromatography for β -*N*-acetylglucosaminidase was about twice that found by SDS-PAGE, it seems probable that in aqueous buffers this enzyme exists as a dimer, consisting of two subunits of 90 000. The amidase on the other hand appears to exist as a monomer of 30 000 to 40 000 by gel chromatography, which is of the same order as the value of 50 000 (Herbold & Glaser, 1975) derived by SDS-PAGE.

pH optima of the enzymes. The amidase had its greatest activity at pH 8.6 in a tetraborate/boric acid buffer (20 ml 0.25 M-sodium tetraborate + 11.21 ml 1 M-boric acid). The optimum was however subject to the nature of the buffer in that phosphate buffers indicated a value nearer to 7.5. All the buffers tested had the same ionic strength. The β -*N*-acetylglucosaminidase showed its greatest activity at pH 4.5 to 5.5 using either 50 mM-acetic acid/acetate or succinate/NaOH buffers and *M. luteus* walls as substrate. However, the nature of the buffers had considerable effects on activity at higher pH; with *B. subtilis* SDS-walls the pH optimum was about 4.5 and the activity was much less affected by the nature of the buffer.

Substrate specificity of β -*N*-acetylglucosaminidase. Table 2 shows the turnover number of the purified enzyme acting on different substrates. The presence or absence of teichoic acid in walls of *B. subtilis* 168 had very little effect on the activity of the enzyme. Indeed, the glycan isolated from the peptidoglycan in these walls was also an excellent substrate. In no test was any muramyl C-1 reducing group liberated. The walls of *M. luteus* and *B. licheniformis* were also good substrates but those of *B. subtilis* W23 were not. Chitin was not significantly hydrolysed. The

Fig. 2. Molecular weight determinants of β -*N*-acetylglucosaminidase and amidase. The column used was of Biogel A, 1.6 cm in diameter and with a void volume of 130.2 ml. The enzymes were dissolved in 2 M-LiCl in 25 mM-Tris/HCl buffer, pH 7.2, containing 10 mM-MgCl₂ and applied to the column: they were eluted with the same buffer. The marker proteins were chromatographed under the same conditions. The enzyme activity in the fractions was measured by the absorbance method already described whilst the marker proteins were detected by their absorption at 280 nm. TI, trypsin inhibitor; OA, ovalbumin; BSA, bovine serum albumin; RMA, rabbit muscle aldolase; BC, beef liver catalase; V_c , elution volume for protein; V_0 , elution volume for dextran blue, i.e. void volume.

Table 2. Turnover number of β -*N*-acetylglucosaminidase acting on various substrates

The turnover number is expressed as nmol free C-1 of *N*-acetylglucosamine liberated per nmol enzyme.

	Substrate	Turnover number
SDS-walls	<i>B. subtilis</i> 168	6.2
	<i>B. subtilis</i> W23	1.7
	<i>M. luteus</i> 2665	4.9
	<i>B. licheniformis</i> M4-1	5.0
Prepared from <i>B. subtilis</i> 168 <i>trpC</i> SDS-walls	Teichoic acid removed*	9.6
	Teichuronic acid walls†	7.3
	Isolated glycan	6.6
	Procion red conjugated walls	0.1
	<i>M. luteus</i> SDS-walls conjugated with procion red	0.75
	Lobster chitin	0.08

* Removed by the Hughes & Tanner (1968) method.

† Cell walls prepared from bacteria grown under phosphate limitation in a chemostat.

coupling of procion red to either *B. subtilis* 168 or *M. luteus* walls (Forsberg & Rogers, 1971) rendered them resistant to the action of the enzyme.

Effect of Mg^{2+} and Li^+ concentrations. The amidase was activated by low concentrations of Mg^{2+} , whereas β -*N*-acetylglucosaminidase was only very slightly activated by low concentrations of the ion and inhibited by higher ones. The latter enzyme was, however, very strongly activated by LiCl. Maximum activity was obtained near 0.2 M (Fig. 3) with *M. luteus* walls as substrate but with *B. subtilis* walls the equally sharp optimum activity at 0.1 M. The cation content of the substrate wall preparations was not examined.

Absence of exo- β -*N*-acetylglucosaminidase activity. Exo- β -*N*-acetylglucosaminidase activity (Ortiz *et al.*, 1972) was tested using β -*p*-nitrophenyl-2-acetamido-2-deoxyglucose (2 mM) as substrate in 0.1 M-sodium phosphate buffer, pH 5.9. Three preparations of purified β -*N*-acetylglucosaminidase from *B. subtilis* were tested. A known exo- β -*N*-acetylglucosaminidase preparation from *Aspergillus niger* was included as a positive control. There was no evidence of hydrolysis of the artificial substrate by any of the *B. subtilis* preparations and the exo-enzyme

Fig. 3

Fig. 4

Fig. 3. Effect of ion concentration upon enzyme activity. Enzyme activity was estimated after adsorption to radioactively labelled walls (see Methods). ■, Li^+ with β -*N*-acetylglucosaminidase acting on *M. luteus* walls; ▲, Mg^{2+} with amidase acting on *B. subtilis* walls; ○, Mg^{2+} with β -*N*-acetylglucosaminidase acting on *B. subtilis* walls; ●, Mg^{2+} with β -*N*-acetylglucosaminidase acting on *M. luteus* walls.

Fig. 4. Inhibition of β -*N*-acetylglucosaminidase by lipoteichoic acid isolated from *B. subtilis* 168. □, Native lipoteichoic acid; ▲, lipoteichoic acid treated with 0.1 M- NH_4OH to remove the ester-linked D-alanine; ■, deacylated lipoteichoic acid.

had no effect on the peptidoglycan of *M. luteus*, which was rapidly hydrolysed by the *B. subtilis* preparations.

Inhibition by lipoteichoic acid and membrane lipids. Lipoteichoic acid isolated from *B. subtilis* 168 strongly inhibited the action of β -*N*-acetylglucosaminidase (Fig. 4). A final concentration of 5 nmol ml^{-1} gave more than 95% inhibition which was not modified by removal of ester-linked alanine from the compound but was destroyed almost entirely by deacylation.

Although not as effective as lipoteichoic acid, cardiolipin and phosphatidyl glycerol isolated from *B. subtilis* 168 were also potent inhibitors of β -*N*-acetylglucosaminidase activity. Commercially obtained (Sigma) samples of cardiolipin, phosphatidyl glycerol and phosphatidic acid were somewhat stronger inhibitors than the homologous samples. Neither phosphatidyl ethanolamine nor phosphatidyl choline on the other hand had any effect when tested at concentrations up to 100 nmol ml^{-1} . No form of lipoteichoic acid at concentrations up to 80 nmol ml^{-1} inhibited the action of the amidase.

Action of trypsin on the enzymes. The β -*N*-acetylglucosaminidase was very sensitive to the action of proteolytic enzymes. For example, 15 min incubation at 37 °C with 10 μg trypsin ml^{-1} pH 7.2 in 50 mM-Tris/HCl led to 70% inactivation. The amidase was inhibited only 20 to 30% by similar treatment.

Binding of β -*N*-acetylglucosaminidase to wall preparations. Table 3 shows that enzyme binding was extensive to three out of four of the preparations and nearly complete with homologous walls. The exception was walls from *B. subtilis* strain W23, which were also not rapidly hydrolysed (Table 2). In view of the binding by walls the pattern of wall breakdown was investigated further by the method reported for the amidase (Herbold & Glaser, 1975). To do this, 15 mg of SDS-walls from *B. subtilis* 168 were suspended in 50 mM-succinic acid/NaOH buffer, pH 5.5, containing 0.25 M-LiCl at 0 °C. Purified β -*N*-acetylglucosaminidase (450 units) was added and the mixture kept at 0 °C for 15 min. The walls were deposited by centrifugation,

Table 3. *Binding of endo- β -N-acetylglucosaminidase to wall preparations*

Purified enzyme from *B. subtilis* 168 (10 μ l, 50 units) was mixed with 1.95 ml 50 mM-succinate/NaOH buffer, at pH 5.5, containing 0.25 M-LiCl at 0 °C and 0.5 mg SDS-wall preparations which had first been reduced with NaBH₄. This mixture stood at 0 °C for 15 min. It was then centrifuged and the enzyme activity present in the supernatant fluid determined in the standard absorbance assay. The rate of reduction of A₃₈₀ of the wall suspension with adsorbed enzyme incubated in the Li⁺ buffer, pH 5.5, was also measured at 37 °C.

Source of wall	Adsorbed activity (units)	Soluble activity (units)	Percentage adsorbed
<i>M. luteus</i>	41.6	6.7	86
<i>B. subtilis</i> W23	24	27	47
<i>B. licheniformis</i> 6346	28	10.6	73
<i>B. subtilis</i> 168	45.0	0.28	99

washed twice with the suspension buffer and mixed with 6 mg of SDS-walls of *B. subtilis* labelled with *N*-acetyl[1-¹⁴C]glucosamine suspended in a buffer of the same composition as used previously. The mixture of walls was incubated at 35 °C and the A₃₈₀ measured at intervals. Also at these times, samples were taken, heated at 100 °C for 5 min and reduced with KB³H₄ to give *N*-acetyl[³H]glucosaminol from free C-1 *N*-acetylglucosamine groups. This was then separated from the non-reduced [1-¹⁴C]glucosamine present in the acid hydrolysates of the samples using electrophoresis on paper in 50 mM-borate buffer, pH 8.5. The ³H/¹⁴C ratio of the glucosaminol was measured. If the walls to which the enzyme was bound in the first place had been the only ones hydrolysed this ratio would theoretically be infinite since no ¹⁴C-labelled material should be present. After 3 min the ratio was found to be about 1.0 and it rose during the course of the 200 min incubation to between 4 and 5. These results show that a small amount of enzyme distributes rapidly to the ¹⁴C-labelled walls and that there is little more redistribution thereafter.

DISCUSSION

Isolated purified β -*N*-acetylglucosaminidase from *B. subtilis* 168 is an endo enzyme, hydrolysing only internal (1 $\rightarrow$ 4)- β -*N*-acetylglucosaminyl bonds in peptidoglycan and had none of the activity of the known (Ortiz *et al.*, 1972) exo-enzyme. It can attack almost equally well the polymer in walls of *M. luteus*, *B. subtilis* and *B. licheniformis*, or the isolated glycans almost free of attached peptide, but it does not hydrolyse chitin at a significant rate. Its properties are in striking contrast to those of the other known autolytic enzyme in *B. subtilis*, the amidase (Herbold & Glaser, 1975). The latter is a relatively small monomeric enzyme of molecular weight 30 000 to 50 000 whereas the β -*N*-acetylglucosaminidase exists in aqueous buffer as a dimer with a molecular weight of about 190 000. The regulation of the activity of the enzymes is also different, in that the glycanase is solubilized and activated by low concentrations of Li⁺ and is strongly inhibited by acylated lipoteichoic acid, and by some membrane phospholipids, including phosphatidyl glycerol, which predominates in the membranes of *B. subtilis*. The amidase is not inhibited by these compounds but is strongly activated by Mg²⁺.

The degree of alanyl substitution on the lipoteichoic acid did not seem to be important for inhibition of the activity of β -*N*-acetylglucosaminidase as it is for blocking autolysins of *Staphylococcus aureus* (Fisher *et al.*, 1981). The pH optima of the two *B. subtilis* enzymes are different and the glycanase is very much more sensitive to proteolysis than the amidase. The latter enzyme is very strongly bound to cell walls whereas the β -*N*-acetylglucosaminidase is somewhat less strongly bound in that small amounts can migrate within a wall population.

These contrasting properties would seem likely to have important effects on the functions of the two enzymes. For example, the molecular weight of the amidase is similar to that of enzymes commonly exported from Gram-positive bacteria (Pollock, 1962) whereas the β -*N*-acetylglucosaminidase is likely to be too large to enter the wall (Hughes *et al.*, 1975; Williamson & Ward, 1981) and might be expected to be trapped at its inner face. The very critical activation of the

glycanase by rather high concentrations of inorganic ions compared with the amidase also fitted the former for activity at the inner margin of the wall rather than within its matrix where most of the cations might be expected to be immobilized by fixed negative charges. If so, it would be understandable that its action should be carefully regulated. In the region between the membrane and the wall the new peptidoglycan strands are fed out from the membrane to the inner surface of the wall. Massive hydrolysis of the glycan chains by the glycanase would be disastrous for the organism. The strong inhibition by membrane lipid and lipoteichoic acid would ensure only minimal hydrolysis as the glycan strands pass into the wall, where the large enzyme cannot follow. The amidase, on the other hand, being small and adhering very strongly to peptidoglycan (Herbold & Glaser, 1975) would be expected to pass through the wall, possibly in a relatively inactive state due to the unfavourable cationic environment and its requirement for relatively high Mg^{2+} concentrations. It might be best able to act at the outer surface (Rogers *et al.*, 1974) where some evidence has suggested that it is indeed available (Fein & Rogers, 1976). There it could liberate wall turnover products by hydrolysing amide bonds attached to the shorter chains in the heterogeneous population of glycans.

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