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AN EXO-β-*N*-ACETYLGLUCOSAMINIDASE FROM *BACILLUS SUBTILIS* B; CHARACTERIZATION

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SUMMARY

The highly purified exo-β-*N*-acetylglucosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) of *Bacillus subtilis* B has a pH optimum of 5.9 and is most stable at 8.5. Its isoelectric point appears to be pH 3.8 and it has a mol. wt of about 75 000. The results of studies on its ability to attack a wide range of synthetic and natural substrates are given and kinetic data for the hydrolysis of a number of these reported. The K_m and V values for *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside and *O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-dexoy-D-glucose are, respectively, 0.15 and 0.018 mM and 14.50 and 32.57 μmoles/min per mg. The rate of hydrolysis of chitin oligosaccharides decreases with increasing molecular size. Neither *O*-[2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl]-(1→4)-2-acetamido-2-deoxy-D-glucose nor 2-acetamido-2-deoxy-β-D-galactosides, are attacked and from these results and those of inhibition studies it is concluded that the enzyme is entirely specific for substrates with non-reducing *N*-acetylglucosamine end groups. In the light of its substrate specificity and other evidence the possibility that this enzyme has a role in cell wall metabolism is discussed.

INTRODUCTION

β-*N*-Acetylglucosaminidases (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) have been described from a wide variety of sources. In many cases there is a lack of evidence as to their specificity but it has become clear recently that both endo- and exoglucosaminidases exist. Enzymes of the former type have been described from a number of bacterial sources: *Staphylococcus aureus* (Tipper[1]; Korman *et al.*[2]), *Staphylococcus epidermis* (Schindler and Schuhardt[3]) *Streptococcus pyogenes* (Barkulis *et al.*[4]) and from *Staphylococcus aureus* M18. The latter has been purified and causes lysis of *Micrococcus lysodeikticus* cells but does not

have any activity against *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucose (Wadström and Hisatsune[5,6]).

Descriptions of exoglucosaminidases (EC 3.2.1.30) are much more numerous. Enzymes hydrolysing *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucoside or other aryl or alkyl 2-acetamido-2-deoxy-β-D-glucosides have been identified in all major groups of organism (Walker[7]; Leaback[8]). In bacteria this enzyme occurs in a wide variety of types (Berkeley *et al.*[9]). It has been partially purified from *Diplococcus pneumoniae* (Hughes and Jeanloz[10]), a Group A *Streptococcus* (Ginsberg *et al.*[11]) and from *Escherichia coli* (Maass *et al.*[12]) but in no case has a very pure enzyme been obtained and characterized with a wide range of substrates.

The preparation of a highly purified β-*N*-acetylglucosaminidase from *Bacillus subtilis* B is described in a preceding paper (Ortiz *et al.*[13]) and the work reported here includes characterization studies with several substrates both natural and synthetic and is part of a study of the β-*N*-acetylglucosaminidase of one strain of *Bacillus subtilis* aimed at establishing the role of this enzyme particularly in connection with its possible involvement in cell wall metabolism.

Three possible roles in bacterial cell wall growth have been proposed for enzymes which hydrolyse mucopeptide; separating newly formed daughter cells, opening the mucopeptide net to allow insertion of new material and remodelling of the wall (Forsberg and Rogers[14]). The results presented here allow a consideration of these possibilities with respect to this enzyme and also of the particular suggestion of Fan *et al.*[15] that *Bacillus subtilis* glycosidases may be involved in end wall synthesis.

MATERIALS AND METHODS

The strain of *Bacillus subtilis* used, the culture medium, growth conditions and purification procedure are described in a previous paper (Ortiz *et al.*[13]).

Enzyme preparation

The highly purified product of the last stage of the purification procedure was used for most of the work described below but sometimes the product of earlier stages in the procedure was used. In these instances the preparation purity is indicated by reference to the stage in the purification procedure from which it was taken.

Substrates

The synthetic substrates *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucoside, *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-galactoside, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucoside, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactoside were purchased from Koch-Light Laboratories Ltd, Colnbrook, Bucks. We wish to thank Dr Y. Matshushima, Osaka University College of Science, Toyonaka, for gifts of methyl-2-acetamido-2-deoxy-α-D-glucoside ($[\alpha]_D + 125°$) and methyl-2-acetamido-2-deoxy-β-D-glucoside ($[\alpha]_D - 39°$), Dr D. Fukushima, Montifiore Hospital and Medical Center, New York, for cyclohexyl α- and β-2-acetamido-2-deoxy-D-glucosides and steroid α- and β-2-acetamido-2-deoxy-D-glucosides.

We also wish to thank Dr R. C. Hughes, National Institute for Medical Research, Mill Hill for gifts of α_1-acid glycoprotein and three of its derivatives produced

by treatment with neuraminidase alone, with neuraminidase and β-galactosidase or with neuraminidase, β-galactosidase and β-*N*-acetylglucosaminidase (Hughes and Jeanloz[10,16]).

Natural oligosaccharide substrates were prepared in this laboratory. The chitin oligosaccharides were prepared as described by Berkeley *et al.*[17].

The bacterial cell wall disaccharide, *O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose was prepared by a modification of the method of Wadström and Hisatsune[6]. Apart from one batch of cell walls kindly given by Dr T. Wadström, National Bacteriological Laboratory, Stockholm, *Micrococcus lysodeikticus* dried cells were purchased from Sigma Chemical Co., 12 Lettice Street, London, S.W. 6. The yield of cell walls from the latter source was about 250 mg from 6 g. 600 mg of cell walls were digested with 2 mg of lysozyme (Sigma, 3 times crystallized egg white lysozyme) in 120 ml 0.02 M sodium phosphate buffer pH 6.5, at 37 °C until the fall in $E^{1\,cm}_{675\ nm}$ ceased. The digest was then dialysed and the diffusate dried in a rotary evaporater. The product was redissolved in 10 ml distilled water and applied to a Sephadex G-25 superfine column (185 cm × 2 cm) and eluted with 0.05 M acetic acid. The eluate was monitored using the modified Morgan–Elson reaction of Sharon *et al.*[18]. After material in the void volume, one sharp peak was found which corresponded to the elution position of the disaccharide *N*,*N'*-diacetylchitobiose. The material in this peak was freeze dried, yield 5 mg.

The cell wall disaccharide, *O*-[2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose was prepared in a similar way but using purified *Staphylococcus aureus* endo-β-*N*-acetylglucosaminidase (Wadström and Hisatsune[6]), generously given by Dr T. Wadström, in place of lysozyme.

The cell wall preparation from *Micrococcus lysodeikticus* used as substrate was obtained as described above. That from *Bacillus subtilis* B was prepared according to the method of Ortîz, J. M., Berkeley, R. C. W. and Brewer, S. J. (unpublished).

Enzyme assays

Using *p*-nitrophenyl compounds according to Oritz, *et al.*[13]. Unless otherwise stated the assay conditions used were as follows.

Using 4-methylumbelliferyl compounds, enzyme solutions (0.1 ml) were incubated with 0.25 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucose or 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactose in 0.1 M sodium phosphate buffer, pH 5.9, at 37 °C. After 10 min the reaction was stopped by the addition of 3 ml of 1 M sodium glycinate buffer, pH 10.2, (Sorensen[20]) and the 4-methylumbelliferone measured fluorimetrically (Leaback and Walker[21]).

Using oligohexosamines, enzyme solutions (0.25 ml) in 20m M sodium phosphate buffer, pH 5.9, were incubated with up to 4 mM solutions of substrate (0.25 ml) at 37 °C for 30 min in the case of di- and trisaccharides and for 45 min in the case of tetra- and pentasaccharides. *N*-Acetylhexosamine released was measured by the method of Reissig *et al.*[22].

Effect of pH

Enzyme activity. The pH optimum for the enzyme was determined by varying the buffer under otherwise standard assay conditions using both *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucose and 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-

glucose. The buffers used were 0.1 M sodium phosphate and 0.1 M citric acid–disodium phosphate (McIlvaine[23]).

Enzyme stability. 25 μl of enzyme in 50 mM Tris buffer, pH 8.0, were mixed with 0.5 ml of one of the buffers listed below and incubated at 25 °C for 1 h. After this period 0.25-ml samples were withdrawn and incubated with 0.5 ml of 2 mM *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucose in 0.1 M sodium phosphate, pH 5.9. After appropriate periods the reaction was stopped in the usual way. As a control, enzyme in 50 mM Tris buffer, pH 8.0, was kept for 1 h at 4 °C. The buffers used were 0.2 M citric acid–disodium phosphate (McIlvaine[23]), 0.1 M Tris–HCl (Gomori[24]), 0.2 M sodium borate (Clark and Lubs[19]), and 0.1 M sodium glycinate (Sorensen[20]).

Isoelectric focusing

Eluate from Sephadex G-200 was dialysed against 50 mM Tris buffer, pH 8.0, and concentrated by ultrafiltration. 1 ml of concentrated enzyme was subjected to isoelectric focusing according to the method of Vesterberg[25]. Carrier ampholytes were used in a final concentration of 1% to establish the pH gradient (3–10). The run was carried out at 4 °C for 48 h in a 110-ml column using a sucrose gradient. The final potential was 600 V. 3-ml fractions were collected when the column was drained and these were assayed for protein and enzyme activity.

Molecular weight

Approximate values were obtained for enzyme preparations from various stages of the purification procedure using the method described by Andrews[26]. A column of Sephadex G-100 (145 cm × 1 cm) equilibrated in 0.1 M Tris buffer, pH 8.0, containing 3 M NaCl was used.

Estimates were also obtained from results of centrifugation experiments in sucrose gradients according to the method of Martin and Ames[27]. Crude sedimentable enzyme and Sephadex G-200 eluate both in 0.1 M Tris buffer, pH 8.0, containing 2 M NaCl were separately layered with bovine serum albumin as the standard, on sucrose gradients (5–20%, w/v) prepared in the same buffer. After centrifuging at 14 000 × *g* for 4 h the tubes were pierced, the contents fractionated and protein and enzyme activity in the fractions assayed.

Using purified enzyme, estimates were obtained by sodium dodecyl sulfate gel electrophoresis as described by Weber and Osborn[28].

In addition to bovine serum albumin (Koch-Light) the proteins used as standards were: human γ-globulin (Koch-Light), α-chymotrypsinogen A, ovalbumin grade V, pepsin 1:60 000 and phosphorylase *a* (Sigma).

Inhibitors and Activators

Non-competitive. Solutions (0.1 ml) of the compounds tested were added to 0.9 ml enzyme solution in 0.2 M sodium succinate, pH 5.9 (Gomori[24]) to give the final concentrations shown in Table II. After 10–20 min at 37 °C, 2 mM *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucose in 0.2 M sodium succinate, pH 5.9, was added and incubation at 37 °C continued for 30 min when the reaction was stopped in the normal way. The chemicals used were obtained from B.D.H. Ltd, Poole and were, where possible, Analar grade.

Competitive. Solutions (0.5 ml) of potential competitive inhibitors were added

to a final concentration of 1 mM, except in the case of the lactones when the final concentration was 10 μM, to 0.5 ml of a range of dilutions of *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucose and 0.25 ml enzyme added. All solutions were made in 0.1 M sodium phosphate buffer, pH 5.9. After 10 min incubation at 37 °C the reaction was stopped as described above. *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, *N*-acetyl-D-mannosamine and D-glucosamine·HCl were obtained from B.D.H.; *N*-acetylmuramic acid and muramic acid from Sigma, and 2-acetamido-2-deoxy-D-glucono-1,5-lactone and 2-acetamido-2-deoxy-D-galactono-1,5-lactone from Koch-Light, D-quinovosamine·HCl was generously given by Dr J. Brimacombe, University of Dundee and was acetylated by the method described by Kuhn *et al.*[29]. Because of the small quantity available, a pilot experiment was performed on D-glucosamine·HCl at 1/60 the scale used by Kuhn *et al.*[29]. Difficulty was encountered in dissolving 5 mg of D-glucosamine·HCl in a sufficiently small quantity of water to allow subsequent successful acetylation. This problem was overcome by dissolving the crystalline material in 0.2 ml of water and freeze-drying it in the reaction flask. The residue could then more easily be dissolved in about 20 μl of water.

Protein determination

The method of Warburg and Christian[30] was used.

RESULTS AND DISCUSSION

Effect of pH

Preliminary studies with crude culture filtrate indicated an optimum pH of 5.9. This was confirmed with purified enzyme using both *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucose and 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucose as substrates, the optima obtained being 5.9–6.0 (Fig. 1). This pH optimum lies between the values obtained for most other bacteria; *Diplococcus pneumonia*, pH 5.3 (Hughes and Jeanloz[10]); Group A *Streptococcus*, 5.3 (Ginsberg *et al.*[11]); *Streptococcus mitis*, 4.5–5.5 (Wadström, T., personal communication) and that of *Escherichia coli* whose optimum was pH 7.5 (Maass *et al.*[12]) Woolen *et al.*[42] were unable to detect this enzyme in several bacteria in which it has subsequently been shown to occur (Berkeley *et al.*[9]) and it seems possible that this was due to the use of too low a pH in their assay system.

The effect of pH on enzyme stability is shown in Fig. 2. This β-*N*-acetylglucosaminidase is most stable at about pH 8.5, lability rapidly increasing with increasing acidity or alkalinity.

Effect of substrate concentration

Plots of the initial velocity of hydrolysis of *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucose by a purified enzyme preparation gave a curve asymtotic to a horizontal straight line. There was no evidence of excess substrate inhibition with *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucose or *N,N'*-diacetylchitobiose up to concentrations of 1 mM. However, with *O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose, excess substrate inhibition occurred at concentrations above 0.2 mM (Fig. 3). In contrast, although

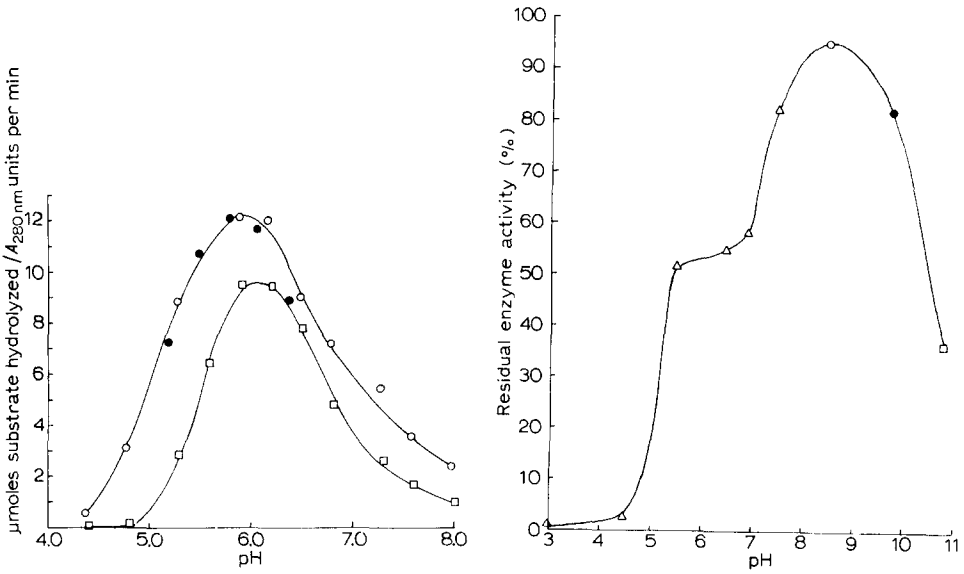

Fig. 1. Effect of pH on enzyme activity. The experimental details are given in the Materials and and Methods section. The buffers used were: 0.1 M citric acid–disodium phosphate (○—○) and 0.1 M sodium phosphate (●—●) for *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucose hydrolysis and 0.1 M sodium phosphate (□—□) for 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucose hydrolysis.

Fig. 2. Effect of pH on enzyme stability. The experimental details are given in the Materials and Methods section. The residual enzyme activity at each pH is expressed as a percentage of the activity of a control sample in 50 mM Tris buffer, pH 8.0, held at 4 °C. Citric acid–disodium phosphate (△—△), Tris–HCl (○—○), sodium borate (●—●) and sodium glycinate (□—□) buffers were used.

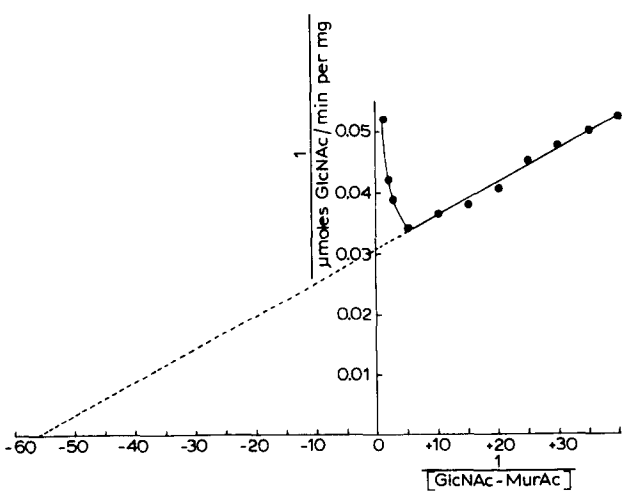

Fig. 3. Effect of variation in substrate concentration on hydrolysis of *O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose. (GlcNAc-MurAc) The assays were carried out under standard conditions except that the substrate concentration was varied.

Reys and Byrde[32] found no excess substrate inhibition with *N,N'*-diacetylchitobiose up to 0.5 mM, *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucose inhibited the *Sclerotinia* enzyme at concentrations of 0.4 mM and above.

The V and K_m values obtained from double reciprocal plots (Lineweaver and Burk[31]) for a range of synthetic and natural substrates are given in Table I. The K_m values for all substrates except *O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose, which is exceptionally low, are of the same order of magnitude and agree with the value found for the *Diplococcus pneumonia* enzyme in being one order lower than that of several enzymes from animal sources (Hughes and Jeanloz[10]) and of the *Sclerotinia fructigena* β-*N*-acetylglucosaminidase[32].

Hydrolysis of other substrates

No detectable hydrolysis of either *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-galactose or 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactose occurred with purified enzyme, although there is just detectable β-*N*-acetylgalactosaminidase activity in the crude enzyme preparation. The results for other potential substrates which were either not available in sufficient quantities or were insufficiently pure for kinetic studies are summarized below:

Methyl-2-acetamido-2-deoxy-β-D-glucose, cyclohexyl-2-acetamido-2-deoxy-β-D-glucose, 17-keto-5α-androstan-3β-yl-2-acetamido-2-deoxy-β-D-glucose, 17β-hydroxy-5β-androstan-3α-yl-2-acetamido-2-deoxy-β-D-glucose, testosterone-2-acetamido-2-deoxy-β-D-glucose, 3β-hydroxy-5α-pregnan-20α-yl-2-acetamido-2-deoxy-β-D-glucose, α_1-acid glycoprotein treated with neuraminidase and galactosidase and *Micrococcus lysodeikticus* cells were all hydrolysed with the release of *N*-acetylglucosamine but in the case of the latter there was no evidence of cell lysis. 3β-Hydroxy-5α-pregnan-20 α-yl-2-acetamido-2-deoxy-α-D-glucose, cyclohexyl-2-acetamido-2-deoxy-α-D-glucose and α_1-acid glycoprotein treated with neuraminidase, galactosidase and β-*N*-acetylglucosaminidase did not release any *N*-acetylglucosamine. With methyl-α-2-acetamido-2-deoxy-α-D-glucose a small amount of *N*-acetylglucosamine was released but this is probably attributable to the impurity of the preparation (Matsushima, Y., personal communication).

TABLE I

K_m AND V VALUES FOR THE ACTION OF *Bacillus subtilis* B β-*N*-ACETYLGLUCOSAMINIDASE ON A RANGE OF SYNTHETIC AND NATURAL SUBSTRATES

The values were obtained from reciprocal plots (Lineweaver and Burk[31]). Data for these were obtained by varying the substrate concentration under otherwise standard assay conditions.

Substrate	K_m *(mM)*	V *(μmoles/min per mg)*
p-Nitrophenyl-2-acetamido-2-deoxy-β-D-glucose	0.15 ± 0.004	14.50 ± 0.04
4-Methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucose	0.11 ± 0.005	5.26 ± 0.21
O-2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose	0.018 ± 0.001	32.57 ± 0.63
N,N'-Diacetylchitobiose	0.31 ± 0.04	1.22 ± 0.15
N,N',N'-Triacetylchitotriose	0.39 ± 0.04	1.31 ± 0.11
N,N',N',N'-Tetraacetylchitotetraose	0.38 ± 0.05	1.05 ± 0.13
N,N',N',N',N'-Pentaacetylchitopentaose	0.35 ± 0.05	0.71 ± 0.09

TABLE II

THE EFFECT OF VARIOUS COMPOUNDS ON *Bacillus subtilis* B β-*N*-ACETYLGLUCOSAMINIDASE ACTIVITY

Solutions of the compounds were added to enzyme preparations in 0.2 M sodium succinate, pH 5.9, to give the final concentration shown. After 10–20 min the activity of the preparations was assayed in the usual way except that a 2 mM solution of *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucose in 0.2 M sodium succinate buffer was used.

Compound	*% Inhibition*			
	0.01 mM	*0.1 mM*	*2 mM*	*10 mM*
$HgCl_2$	19	—	97	—
CdCl	3	—	41	59
$ZnSO_4$	0	—	57	78
$AgNO_3$	30	—	100	—
$Co(NO_3)_2$	—	—	—	37
$Ca(NO_3)_2$	—	—	—	8
$MgSO_4$	—	—	—	7
PCMB	—	16	—	—
EDTA	1 mM and 20 mM caused about 10% stimulation			

Activators and inhibitors

Crude enzyme preparation was inhibited by neither 0.05 M sodium acetate nor by the addition, to a final concentration of 10 mM, of each of the disaccharides cellobiose, lactose, maltose, salicin and sucrose. The effect of certain detergents has been reported previously (Ortiz *et al.*[13]).

Of the substances listed in Table II tested for inhibition and activation, the cations Hg^{2+} and Ag^{+} were the most potent inhibitors and EDTA caused slight stimulation. The effects of various cations on this enzyme are similar to those described for the *Diplococcus*[10] and group A *Streptococcus*[11] enzymes. The metal ions Mg^{2+} and Ca^{2+} were slightly inhibitory and Hg^{2+}, Ag^{+}, Zn^{2+} and Cd^{2+} markedly so at 2 or 10 mM, but at 0.01 mM the effect of the last two ions was substantially reduced.

N-Acetylglucosamine and 2-acetamido-2-deoxy-D-glucono-1,5-lactone are effective competitive inhibitors whereas *N*-acetylgalactosamine inhibits only slightly. 2-Acetamido-2-deoxy-D-galactono-1,5-lactone (Figs 4 and 5 and Table III), however, like *N*-acetylmuramic acid, muramic acid, *N*-acetylmannosamine, *N*-acetylquinovosamine and glucosamine does not cause any inhibition.

Isoelectric focusing

Crude culture completely lost its activity when incubated overnight at 4 °C with ampholine, pH 4.0. However, using the purified enzyme with a much higher specific activity it was possible to just detect activity in the fractions corresponding to the peak of material absorbing at 280 nm. It therefore seems that the isoelectric point (pI) is about 3.8. This value is similar to that obtained by Reys and Byrde[32] for the β-*N*-acetylglucosaminidase of *Sclerotinia fructigena* but is one pH unit lower than the pI estimates of Li and Li[33] and Wadström, T., personal communication, for the enzyme from jack bean meal and *Streptococcus mitis*, respectively. It is also lower than all the values obtained by Sandhoff[34] for the multiple peaks from mammalian sources.

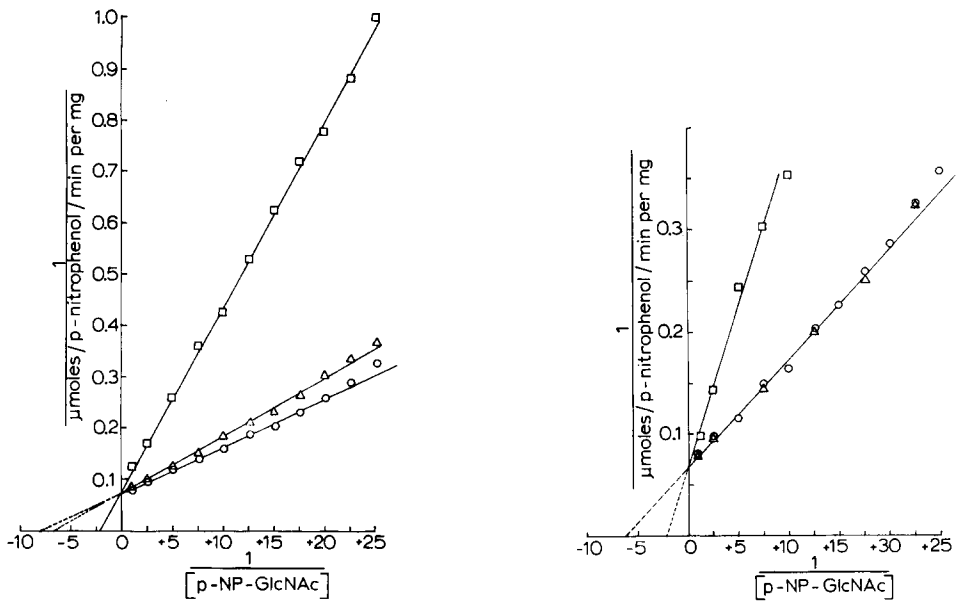

Fig. 4. Effect of *N*-acetylglucosamine and *N*-acetylgalactosamine on hydrolysis of *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucose (*p*NPGlcNAc) by *Bacillus subtilis* B β-*N*-acetylglucosaminidase. The standard assay conditions were used except that the substrate concentration was varied and that to one series of substrate dilutions *N*-acetylglucosamine (□—□) and to another *N*-acetylgalactosamine (△—△) were added, both to a final concentration of 1 mM. A third series had no additions (○—○).

Fig. 5. Effect of 2-acetamido-2-deoxy-D-glucono-1,5-lactone and 2-acetamido-2-deoxy-D-galactono-1,5-lactone on hydrolysis of *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucose by *Bacillus subtilis* B β-*N*-acetylglucosaminidase. The standard assay conditions were used except that the substrate concentration was varied and that to one series of substrate dilutions 2-acetamido-2-deoxy-D-glucono-1,5-lactone (□—□) and to another 2-acetamido-2-deoxy-D-galactono-1,5-lactone (△—△) were added, both to a final concentration of 10 μM. The lactone solutions were absolutely fresh (see Leaback[41]). A third series of substrate dilutions had no additions (○—○).

TABLE III

INHIBITION OF *Bacillus subtilis* B β-*N*-ACETYLGLUCOSAMINIDASE BY VARIOUS COMPETITIVE INHIBITORS

Solutions of *N*-acetylglucosamine and *N*-acetylgalactosamine to a final concentration of 1 mM and absolutely fresh solutions of 2-acetamido-2-deoxy-D-glucono-1,5-lactone to a final concentration of 10 μM were added to a range of dilutions of *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucose in 0.1 M sodium phosphate buffer, pH 5.9. After incubation at 37 °C for 10 min the reactions were stopped in the usual way.

Inhibitor	K_i *(mM)*
N-Acetylglucosamine	0.36
N-Acetylgalactosamine	3.98
2-Acetamido-2-deoxy-D-glucono-1,5-lactone	$3.38 \cdot 10^{-3}$

Molecular weight

The molecular weight as determined by Sephadex G-100 chromatography and sodium dodecyl sulfate electrophoresis is about 75 000 and 74 000, respectively. The results obtained from centrifugation on sucrose gradients were rather higher; about 91 000. The estimates are rather smaller than those for two fungal enzymes (Mega *et al.*[35], Reys and Byrde[32]) and the jack bean meal enzyme (Li and Li[33]) whose molecular weights were estimated to be 140 000–146 000, 141 000 and 100 000, respectively, but are very similar to the values of 65 000 and 72 000 obtained by Wadström, T., personal communication, for the *Streptococcus mitis* enzyme.

Concluding remarks

There is some confusion over the nomenclature of enzymes which hydrolyse hexosaminides. It has recently been demonstrated by Nord and Wadström[36] that, according to their source, 'lysozymes' may be either chitinases (EC 3.2.1.14) or endo-β-*N*-acetylmuramidases (true lysozymes, EC 3.2.1.17). A third type of enzyme, endo-β-*N*-acetylglucosaminidase (Wadström and Hisatsune[6]), probably falls into neither category since it hydrolyses *Micrococcus lysodeikticus* cells much more effectively than it does chitin.

The naming of the complementary enzymes attacking the hydrolysis products of such endoenzymes is also often uncritical. In the case of insect and fungal enzymes, even though they have only been characterised using *N*,*N*′-diacetyl chitobiose and aryl 2-acetamido-2-deoxy-D-glucosides, it is to be expected that they are indeed chitobiases. The same is not true of enzymes from plant, animal and bacterial sources. It is therefore important to characterize such enzymes using a sufficiently wide range of substrates.

The exo-hydrolytic nature of *Bacillus subtilis* B enzyme is indicated by its ability to hydrolyse alkyl and aryl 2-acetamido-2-deoxy-β-D-glucosides and disaccharides, in contrast to the endo-enzymes, and also by the fall in rate of hydrolysis of higher oligosaccharides. The enzyme does not attack *O*-[2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl]-(1→4)-2-acetamido-2-deoxy-D-glucose and, as judged by the V and K_m data, its primary substrate is *O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose. It can therefore be regarded as an exo-β-*N*-acetylglucosaminidase.

This enzyme, like others so far isolated from bacteria but unlike those from all other sources except brain (Frohwein and Gatt[37]), is completely specific for the C-4 configuration of the non-reducing sugar residue. The presence of an *O*-lactyl group at C-3 of the reducing sugar residue completely abolishes enzyme activity as was also found for pig epididymal enzyme, by Tipper and Strominger[38]. Potential substrates with other non-reducing groups were not available but, on the assumption that, like *N*-acetylgalactosamine and *N*-acetylmuramic acid, the sugar would not be a competitive inhibitor if the glycoside containing it was not hydrolysed, we have tested a range of possible compounds in this respect. The results indicate that the enzyme has an absolute specificity for the configuration of *N*-acetylglucosamine.

The failure to hydrolyse 2-acetamido-2-deoxy-α-D-glucosides is in accord with the results for other β-*N*-acetylglucosaminidases which are entirely specific for the β-glycosidic linkage (Walker[7]). The enzyme will, however, tolerate considerable vari-

ation in the nature but not the size of the aglycone. It is interesting to note the enhanced rate of hydrolysis of *O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose over *N*,*N*′-diacetylchitobiose which indicates the stimulatory effect that the presence of an *O*-lactyl substituent at C-3 of the aglycone has on the cleavage of the glycosidic linkage. A cell wall fragment has been shown to be the most efficient inducer of this enzyme (Ortiz, J. M., Berkeley, R. C. W. and Brewer, S. J., unpublished) and we are now investigating the chemical nature of the inducer and the possibility that even high molecular weight substrates are readily hydrolysed where there is a *N*-acetylmuramyl residue adjacent to the non-reducing terminal *N*-acetylglucosaminyl group.

The exohydrolytic nature of the *Bacillus subtilis* B β-*N*-acetylglucosamidase seems to rule out any possibility that it is involved during growth either in mucopeptide net opening or in daughter cell separation. Similarly, in view of the enzymes specificity, the very restricted alterations it could cause to wall mucopeptide suggest that it may not be involved in wall remodelling either. Furthermore, a mutant apparently lacking the enzyme has been isolated in this laboratory. This appears to grow and sporulate normally in a complex medium. Thus it seems that essential involvement of the enzyme in cell wall growth can be excluded and, since we have been unable to detect endoglycosidase activity in *B. subtilis* B, also that any function for glycosidases in wall growth can be ruled out, at least in this strain. It is difficult to reconcile this with the demonstration by Fan and Beckman[39] that in a mutant of *B. subtilis* βAO with a diminished growth rate paralleled by lowered autolytic activity, the addition of either autolytic amidase or egg-white lysozyme enhanced this rate. Thus the question arises as to whether, although amidase is the most prominent autolysin of the genus *Bacillus*[14], interchangeability of these enzymes according to growth conditions could occur in an analogous way to that demonstrated for wall polymers[40]. Another possibility is, as has been suggested for *Sclerotinia fructigena* β-*N*-acetylglucosaminidase, that it may scavenge wall debris but even in this it would be severely limited because of its inability to cleave compounds with non-reducing *N*-acetylmuramic acid residues. However, we now have evidence of exomuramidase activity in *B. subtilis* B cultures and, depending on its substrate requirements, such an enzyme could, in combination with exo-glucosaminidase, completely hydrolyse the polysaccharide backbone of mucopeptide.

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