AN ENZYME FROM *BACILLUS SUBTILIS* B WITH EXO-β-*N*-ACETYLMURAMIDASE ACTIVITY

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1. Introduction

Lysozyme (endo-β-*N*-acetylmuramidase, EC 3.2.1.17) and endo-β-*N*-acetylglucosaminidase (EC 3.2.1.-) attack the polysaccharide moiety of the bacterial cell wall peptidoglycan to form the disaccharides *O*-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (GlcNAc–MurAc) and *O*-[2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-glucopyranosyl]-(1→4)-2-acetamido-2-deoxy-D-glucose (MurAc–GlcNAc) respectively. Exo-β-*N*-acetylglucosaminidase (EC 3.2.1.30) is widely distributed [1] and has been obtained in a highly purified state from *Bacillus subtilis* B [2]. Neither this enzyme [3] nor those from *Streptococcus mitis* (Wadstron, T., personal communication) and from pig epididymis [4] hydrolyse MurAc–GlcNAc.

We have been unable to detect any endoglycosidase activity in cultures of *B. subtilis* B (Brewer and Berkeley, unpublished results) and therefore scope for degradation or alteration of the polysaccharide moiety of the peptidoglycan in this strain would be very limited if there were no additional exo-hexosaminidase to act in conjunction with the exo-glucosaminidase. The presence of an enzyme with exo-muramidase activity was therefore sought and found [3]. In this paper we present evidence for the existence of this new enzyme.

2. Methods

B. subtilis B was grown overnight at 37°C by shaking in a 1 litre Erlenmeyer flask with about 200 ml of FEMMS medium containing 1% peptone and 1% succinate [5]. The bacteria were removed by centrifugation at 10 000 *g* for 20 min at 4°C and solid ammonium sulphate added to the cell-free supernatant to give a final concentration of 85% (w/v). After standing overnight the resulting precipitate was recovered by centrifugation at 18 000 *g* for 20 min at 4°C and dissolved in 15 ml of 20 mM sodium phosphate buffer, pH 5.9 for the oligosaccharide experiments and in 4 ml 50 mM Tris–HCl, pH 8.2 containing 2 M NaCl for those with fluorogenic substrates. For the latter determinations the resultant solution was also clarified by centrifugation at 18 000 *g*.

GlcNAc–MurAc and MurAc–GlcNAc were prepared as described earlier [3]. 4-Methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranose (MeU–GlcNAc) and 2-acetamido-2-deoxy-D-glucono-1,5-lactone were purchased from Koch-Light Ltd., Colnbrook, Bucks. and 4-methylumbelliferyl-2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranose (MeU–MurAc) synthesised by a similar method to that used by Jeanloz et al. [6] for other glycosides of this sugar. All other reagents were purchased from BDH Ltd., Poole, Dorset and were, where possible, AR grade.

Assays of activity against the two disaccharides were carried out as described [3] but, in order to conserve MeU–MurAc, assays using fluorogenic substrates

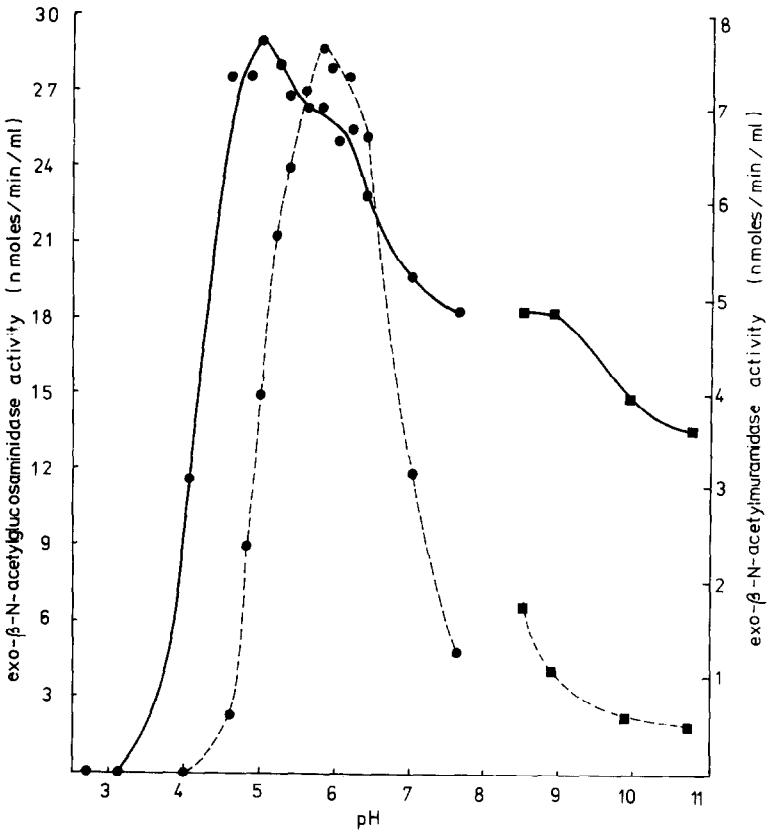

Fig. 1. Effect of pH on exo-β-*N*-acetylglucosaminidase (----) and exo-β-*N*-acetylmuramidase (——) activities. The experiments were carried out using fluorogenic substrates as described in the Methods section except that the buffers used were 0.1 M citric acid in 0.2 M disodium hydrogen phosphate (•) and 0.1 M glycine in 0.1 M NaOH (•).

were done using 0.1 ml of substrate and 10 μl of enzyme and the reactions stopped with 4 ml of 0.25 M glycine in 0.2 M NaOH buffer, pH 10.3. The reproducibility of this method is as good as that using larger volumes but tests of accuracy showed that the smaller scale method gave results lower than the method used previously.

3. Results and discussion

Incubation of crude enzyme preparation with MurAc–GlcNAc and GlcNAc–MurAc resulted in the liberation of free hexosamine in both cases and therefore the enzyme preparation must contain some exo-muramidase activity. It has been shown previously [8] that under the culture conditions used *B. subtilis* B produces an exo-β-*N*-acetylglucosaminidase but this did not attack MurAc–GlcNAc [3]. Also, as shown in fig. 1, the pH optimum of crude preparations of this enzyme (5.9–6.0) differs from that of the exo-muramidase activity (5.0–5.2). Nevertheless attempts were made to inhibit the exo-muramidase activity with saturating concentrations, with respect to exo-β-*N*-acetylglucosaminidase activity, of a partially purified specific antiserum against the purified exo-β-*N*-acetylglucosaminidase. No inhibition of the hydrolysis of MurAc–GlcNAc and a slight enhancement of activity against MeU–MurAc was observed whereas that against GlcNAc–MurAc and MeU–GlcNAc was reduced (table 1).

The possibility remained that the muramidase activity was due to a different exo-β-*N*-acetylglucosaminidase of broader specificity than that previously

Table 1
Effects of antiserum against purified exo-β-*N*-acetylglucosaminidase on the hydrolysis of GlcNAc–MurAc and MurAc–GlcNAc.

Substrate	Activity (% of control)
GlcNAc–MurAc	28.9
MurAc–GlcNAc	111.4
MeU–GlcNAc	32.1
	32.9
MeU–MurAc	108.0
	107.5

Enzyme preparation was incubated with substrate (2 mM final concentration) in the presence and absence of antiserum and activity determined as described in the Methods section. The results are expressed as percentages of the activity in controls without antiserum.

purified. We therefore examined the effect of GlcNAc and 2-acetamido-2-deoxy-D-glucono-1,5-lactone, known inhibitors of exo-glucosaminidase, and of MurAc, a potential inhibitor of exo-muramidase. The first two had no effect on the muramidase activity which was, however, substantially reduced by MurAc (table 2).

It is concluded that the exo-muramidase activity is distinct from the exo-glucosaminidase previously isolated and characterized [2, 3] and that the muramidase activity is probably not due to another broad specificity glucosaminidase but to an exo-β-*N*-acetylmuramidase. This is the first report of any enzyme with such a specificity.

It has previously been suggested that, in the absence of endoglycosidases, the combined action of exo-glucosaminidase and exo-muramidase could completely hydrolyse the cell wall aminopolysaccharide chain [3]. In the light of the recent report [7] that the chain lengths of the peptidoglycan polysaccharide moiety of *B. subtilis* 168, *B. licheniformis* 6346 and two derivatives and *S. aureus* Copenhagen are about 100–200 disaccharide units long and assuming the same to be true of *B. subtilis* B this now seems unlikely and the suggestion must be reconsidered.

Table 2
Effect of various inhibitors on exo-β-*N*-acetylmuramidase and exo-β-*N*-acetylglucosaminidase.

Inhibitor (final concn.)	Activity (% of control) Substrate MeU–MurAc	MeU–GlcNAc
MurAc	76.9	95.1
(1 mM)	75.9	100.5
GlcNAc	101.9	46.1
(1 mM)	104.9	51.2
Lactone	101.3	22.8
(50 μM)	96.5	24.6

Enzyme preparation was incubated in duplicate with the inhibitor to the final concentrations shown and the activity determined as described in the Methods section. The results are expressed as percentages of the activity in controls without inhibitor.

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