Purification and Characterization of Two Phage PBSX-induced Lytic Enzymes of *Bacillus subtilis* 168: An *N*-Acetylmuramoyl-L-alanine Amidase and an *N*-Acetylmuramidase

By J. BARRIE WARD,*† CAROL A. M. CURTIS, CHRISTOPHER TAYLOR AND ROGER S. BUXTON

Division of Microbiology, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

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After heat-induction of the defective phage PBSX in a *xhi-1479* mutant of *Bacillus subtilis* 168, the culture lysed rapidly even if the *lyt-2* mutation was present (which greatly reduces the amount of the bacterial autolysins). Two lytic enzymes, an *N*-acetylmuramoyl-L-alanine amidase and an endo-*N*-acetylmuramidase, were purified from the culture supernatant. The amidase was readily distinguished from the bacterial amidase by its low molecular weight. In addition, it was not inhibited by antibody directed against the bacterial enzyme. These results indicate that PBSX does not rely on the bacterial autolysins to accomplish lysis.

INTRODUCTION

When the defective bacteriophage PBSX carried by *Bacillus subtilis* 168 is induced from the prophage state, a lytic agent is produced. This results in lysis of the host bacteria and release of the progeny phage particles. Since PBSX is defective, not being capable of infectious growth, it was possible that the bacteriophage has lost the ability to produce its own lytic enzyme and makes use of the bacterial autolysins. In *B. subtilis*, these have been characterized as an *N*-acetylmuramoyl-L-alanine amidase and an endo- β -*N*-acetylglucosaminidase (Rogers, 1979; Herbold & Glaser, 1975; Taylor *et al.*, 1980). Alternatively, the bacterial autolysins may in fact be phage-coded. This situation would explain why PBSX and other related defective phages have been retained by *B. subtilis* during evolution.

In this paper we have attempted to examine this problem by characterizing the lytic enzyme produced upon induction of PBSX. The phage was induced in *B. subtilis* 168 carrying the *xhi-1479* mutation which is located in the prophage. This mutation results in the specific induction of PBSX when the growth temperature of the host organism is raised to 48 °C (Buxton, 1976). In this respect, the *xhi-1479* mutation is formally analogous to the *ci857* mutation of bacteriophage λ (Sussman & Jacob, 1962) and probably resides in the gene specifying the phage repressor. In order to minimize any possible contamination by the two *B. subtilis* autolysins, a strain carrying the *lyt-2* mutation was used. This mutation results in a 90–95% deficiency of both the autolytic enzymes (Fein & Rogers, 1976); thus, most of the lytic activity present in heat-treated cultures should be present as a consequence of the induction of PBSX. Moreover, the fact that this *xhi lyt* mutant undergoes heat-induced lysis (Buxton, 1980) makes it unlikely that lysis due to PBSX induction is mediated by the two bacterial autolysins.

† Present address: Department of Microbial Biochemistry, Glaxo Group Research Ltd, Greenford, Middx UB6 0HE, U.K.

METHODS

Organisms and cultural conditions. All the experiments on the isolation and characterization of the PBSX-induced lytic enzyme were performed with *Bacillus subtilis* strain RB1163 (*xhi-1479 lyt-2*). This was constructed using saturating concentrations of DNA isolated from strain RB1050 (*pyrX xhi-1479*) to transform strain FJ6 (*metC3 lyt-2*) (Fein & Rogers, 1976) to Met⁺ and screening these recombinants for inheritance of *xhi-1479* by testing for the absence of growth at 48 °C. *Bacillus subtilis* strain RB1163 is, therefore, heat-sensitive (*xhi*) and forms chains of unseparated bacilli (*lyt*).

Other organisms used were *B. subtilis* 168, *B. subtilis* W23, *Bacillus licheniformis* strains 94 and 6346, *Micrococcus luteus* NCTC 2665 and *Staphylococcus aureus* H.

Cultures for the induction of the PBSX lytic enzyme were grown at 35 °C in casein hydrolysate/yeast extract (CHY) medium. Otherwise, cultures were grown in tryptone/yeast extract (TYE) medium or casein hydrolysate/salts (CHSC) medium supplemented as required to meet auxotrophic requirements (Ward, 1974; Tynecka & Ward, 1975).

Measurements of lytic activity. In most cases lysis of walls was measured turbidimetrically by resuspending the walls in 25 mM-Tris/HCl buffer, pH 7.1, containing 10 mM-MgCl₂ at 37 °C and following the decrease in absorbance at 380 nm. One unit of lytic activity gave a decrease in absorbance of 0.001 min⁻¹ during the initial period of linear decrease. Alternatively, radioactive walls were resuspended in the above buffer and lysis was allowed to occur at 37 °C for periods of 10–30 min. After centrifuging (15 000 g for 5 min), the amount of soluble radioactivity in the supernatant was determined.

Preparation of walls and wall fractions. Walls treated with hot detergent (sodium dodecyl sulphate, SDS) to inactivate autolytic enzymes and extract associated proteins were prepared from *B. subtilis* 168, *B. subtilis* W23, *B. licheniformis* 6346, *M. luteus* NCTC 2665 and *S. aureus* H as described by Fein & Rogers (1976). Radioactive walls were prepared by the same method from *B. subtilis* 168 grown in CHSC medium containing *N*-[¹⁴C]acetylglucosamine: the purified walls were labelled with 1.63×10^5 d.p.m. ¹⁴C mg⁻¹. Soluble uncross-linked peptidoglycan was isolated from cultures of *B. licheniformis* 94 treated with benzylpenicillin (Tynecka & Ward, 1975). Disaccharide monomer (*N*-acetylglucosaminyl-*N*-acetylmuramoyl-L-alanyl-D-isoglutamyl-*meso*-diaminopimelic acid) and bis-disaccharide dimer were prepared from walls of *B. subtilis* 168 or *B. licheniformis* 94 after treatment with egg-white lysozyme (EC 3.2.1.17) (Ward, 1974). Purified peptidoglycan was isolated from the same walls after treatment with either 5% (w/v) trichloroacetic acid at 2 °C for 48 h or with 60% (w/v) hydrofluoric acid at -10 °C for 24 h to remove teichoic and teichuronic acids.

Antiserum to bacterial amidase. The bacterial *N*-acetylmuramoyl-L-alanine amidase was purified from *B. subtilis* 168 by the method of Herbold & Glaser (1975) and antisera to this purified protein were raised in rabbits. Antibody to the amidase was purified by ion-exchange chromatography on DEAE-cellulose and isoelectric focusing. Details of this purification procedure and the specificity of the isolated antibody will be described elsewhere.

Analytical methods. The release of free amino groups produced during lysis was assayed as described by Forsberg & Ward (1972). Dinitrophenylated (DNP) fragments of walls were hydrolysed in 4 M-HCl at 100 °C for either 4 h or 16 h. DNP-alanine was separated from the hydrolysate by thin-layer chromatography on silica gel plates (Merck silica gel 60) using chloroform/methanol/acetic acid (95:5:1, by vol.) as the solvent. The spots were eluted with methanol/1 M-NH₄OH (1:1, v/v) and the absorbance of the eluate was read at 360 nm; the molar absorption coefficient of DNP-alanine was taken as 15 000 l mol⁻¹ cm⁻¹ (Ghuysen *et al.*, 1966). To establish the configuration of the alanine released, the circular dichroism spectrum of the DNP-derivative (purified as described above and dissolved in 0.125 M-NaHCO₃) was compared with the circular dichroism spectra of DNP-alanine of known configuration using a Jasco J41C Spectropolarimeter. These measurements were performed by Dr S. R. Martin of this Institute.

The release of free reducing groups was assayed by the Thompson & Shockman (1968) modification of the method of Park & Johnson using *N*-acetylglucosamine as the standard. The reducing groups were further characterized after reduction with KB³H₄ and acid hydrolysis as described by Williamson & Ward (1979).

Protein was measured by the Lowry method using bovine serum albumin as standard. Radioactivity in walls or solubilized peptidoglycan was determined using a toluene-Biosolv scintillation fluid as described previously (Wyke & Ward, 1977).

RESULTS

Purification of the PBSX-induced lytic enzymes

Bacillus subtilis RB1163 was grown in broth at 35 °C until the culture reached an *A*₆₀₀ of approximately 0.5. It was then transferred to 48 °C where growth continued for a short period to reach an *A*₆₀₀ of 1.2–1.5 before lysis began. After 60–75 min incubation at the

Fig. 1. Separation by chromatography on Sephadex G-100 of the two PBSX-induced lytic enzymes of *B. subtilis* 168. Samples were assayed for lytic activity (●) and protein (▲). Fractions were pooled for enzyme I and enzyme II as shown. The void volume (V_0) and NaCl elution volume (V_{NaCl}) of the column are indicated by the arrows.

higher temperature, lysis was complete (A_{600} 0.02). Cell debris was removed by centrifuging the culture (16000 g, 4 °C for 10 min) and the supernatant was used for subsequent enzyme purification.

Initial experiments showed that the lytic enzyme could be absorbed from supernatants on to *B. subtilis* walls. However, all attempts to release the bound enzyme by treatment of the walls with high concentrations of LiCl or NaCl were unsuccessful. As a result the following purification procedure was developed.

The culture supernatant was passed successively through columns (20 × 1.5 cm) of DEAE-cellulose equilibrated with 50 mM-Tris/HCl, pH 7.1, and Amberlite CG-50(H⁺). The lytic activity was not absorbed by the DEAE-cellulose and was bound to the Amberlite. This column was washed with water until the A_{280} of the eluate had decreased to almost zero. Lytic activity was not detected in either the initial column eluate or in the water washes. The column was then eluted with 2 M-ammonium acetate and fractions (4 ml) were collected. Those containing the lytic activity were pooled. At this stage of the purification the yield of lytic activity appeared to drop substantially but this was probably due to the high concentration of salt present in the assay. The pooled fractions were concentrated by filtration through an Amicon ultrafilter using a UM-10 membrane. Attempts to concentrate the lytic activity by precipitation with (NH₄)₂SO₄ followed by dialysis to remove salt were not successful and resulted in considerable loss of activity. The non-diffusible fraction obtained by ultrafiltration contained all the lytic activity present in the pooled material and was chromatographed on a column (68 × 1.5 cm) of Sephadex G-100 eluted with 10 mM-morpholinopropanesulphonic acid (MOPS)/NaOH buffer, pH 6.5, containing 1 mM-MnCl₂. Assaying the fractions (2 ml) revealed the presence of two peaks of lytic activity (Fig. 1). The minor fraction (enzyme I) was of high molecular weight and was eluted close to the void volume of the column, whereas the major peak of lytic activity (enzyme II) was eluted just ahead of the salt. Again, appropriate fractions were pooled and the enzymes were concentrated by ultrafiltration. An interaction of several lytic enzymes with Sephadex results in anomalous behaviour by such enzymes (Williamson & Ward, 1981). A similar interaction might have been responsible for the elution profile of enzyme II; consequently, the concentrated enzyme II activity was re-chromatographed on a column (65 × 1.5 cm) of Biogel P-100 eluted with 0.1 M-MOPS/NaOH buffer, pH 6.5, containing 1 mM-MnCl₂. The enzyme was again eluted as a single peak of low molecular weight but in this case clearly ahead of the salt. Samples of the pooled enzymes were examined by SDS-polyacrylamide gel electrophoresis. Enzyme I was separated into several major and minor bands of protein, whereas enzyme II gave two bands

Table 1. *Purification of PBSX-induced lytic enzymes*

Fraction	Volume (ml)	Lytic activity		Protein		Specific activity [units (mg protein) ⁻¹]	Recovery (%)	Purification factor
		units ml ⁻¹	Total units	mg ml ⁻¹	Total mg			
Culture supernatant	1800	79.1	142 000	2.30	4140	34.4	100	1
DEAE-cellulose eluate	1800	78.3	141 000	1.00	1800	78.3	99.2	2.28
Amberlite CG-50 eluate (Amicon concentrated)	3.7	5 330	19 000	9.13	33.8	583	13.4	16.9
Sephadex G-100 eluate (Amicon concentrated)								
Enzyme I	1.8	3 800	6 800	0.71	1.28	5352	4.8	156
Enzyme II	3.3	20 150	66 500	0.68	2.24	29688	46.8	863

of protein, one of apparent molecular weight 30 000 and a smaller amount of molecular weight 14 500.

The results of a typical purification of the two enzymes are shown in Table 1. The final products had specific activities of 5.35×10^3 and 2.97×10^4 units (mg protein)⁻¹ for enzymes I and II, respectively, and represented approximately 50% of the lytic activity originally present.

Characterization and properties of the purified enzymes

Enzyme I

When assayed in 0.01 M-MOPS/NaOH buffer (pH 5.6–8.2) the lytic activity had a broad pH optimum over the range 7.0–8.0. Activity in 0.01 M-acetate buffer (pH 4.5–5.8) and 0.01 M-glycine/NaOH buffer (pH 8.0–9.4) was considerably lower. These assays did not take into account changes in ionic strength of the various buffers. Lytic activity was also influenced by ionic strength and when assayed in MOPS/NaOH buffer, pH 7.1, the enzyme showed maximum activity in the concentration range of 0.005–0.015 M buffer.

Lysis of *B. subtilis* walls was accompanied by an increase in the reducing power of the suspension but with little concomitant increase in the number of free amino groups present (Fig. 2a). Thus, lysis appeared to result from the action of a glycosidase and not from *N*-acetylmuramoyl-L-alanine amidase or endopeptidase activities. To determine the specificity of the glycosidase, samples of walls, and of the soluble products obtained after lysis was allowed to proceed for 6 h, were reduced with KB³H₄ to radioactively label the free reducing groups present. The reduced samples were then hydrolysed, and after chromatography of the hydrolysate on Dowex-50, the proportions of the various hexosamines and [³H]hexosaminols were determined (Table 2). The walls contained galactosamine from the glucosyl-*N*-acetyl-galactosamine phosphate polymer (Shibaev *et al.*, 1973) in addition to glucosamine and muramic acid from peptidoglycan. Glucosamine and galactosamine were not separated by the Dowex-50 column. After reduction of the walls, radioactivity was found chiefly as [³H]glucosaminol, and the average length of the glycan chains in the peptidoglycan was 46.7 disaccharides. This value was calculated from the ratio of free reducing groups present in muramic acid and glucosamine to the total amount of muramic acid present. Reduction of *B. subtilis* walls or the soluble products obtained after treatment with *Myxobacter* AL-1 amidase or lysozyme does not give free reducing groups of galactosamine (S. M. Fox & J. B. Ward, unpublished observations). After lysis with enzyme I for 6 h the total free reducing groups of muramic acid had increased approximately 30-fold whereas no significant change was found for glucosamine. At this time the average chain length of the glycan was reduced to 7.1 disaccharides. Thus, enzyme I is an endo-*N*-acetylmuramidase and the increase in free amino

Fig. 2. Lysis of *B. subtilis* walls by the purified enzymes produced after heat-induction of phage PBSX. Walls (1 mg ml^{-1}) were resuspended in 0.01 M-MOPS/NaOH buffer, pH 7.1, at 37°C and the decrease in turbidity was followed at 380 nm (■). Samples were assayed at intervals for reducing power (□) and *N*-terminal amino groups (●). (a) Enzyme I, the *N*-acetylmuramidase. (b) Enzyme II, the *N*-acetylmuramoyl-*L*-alanine amidase.

Table 2. Analysis of hexosamines obtained from reduced *B. subtilis* walls and after treatment with enzyme I

Sample	Hexosamine [$\mu\text{mol (mg wall)}^{-1}$]				Average glycan chain length (disaccharides)*
	Muramitol	Muramic acid	Glucosaminol	Glucosamine + Galactosamine	
Walls	0.95	310	5.70	434	46.7
Solubilized walls after treatment with enzyme I	30.20	230	6.40	382	7.1

* Calculated from the ratio of free reducing groups (as muramitol and glucosaminol) to the total amount of muramic acid present (muramic acid plus muramitol).

groups observed during lysis of the walls presumably arises from the activity of contaminating enzyme II (see below).

Enzyme II

Using identical assays to those described above for enzyme I, enzyme II had a pH optimum of 7.5 and maximum activity in $0.005\text{--}0.01 \text{ M-MOPS/NaOH}$ buffer, pH 7.5. Lytic activity was also influenced by divalent cations present in the assay mixture. Any metal ions present in the enzyme preparation could be removed by chromatography on Sephadex G-25 (eluted with 0.01 M-MOPS/NaOH buffer, pH 7.5). Addition of 1 mM Mn^{2+} or Mg^{2+} then stimulated activity 2- and 1.7-fold, respectively, while Ca^{2+} and Zn^{2+} were without effect. Lytic activity was completely inhibited by Hg^{2+} and Fe^{3+} , whereas addition of 1 mM-EDTA resulted in 25–50% inhibition. The purified enzyme was relatively stable and only a small loss of activity was observed on storage at 2°C for several weeks. The molecular weight of enzyme II was determined by comparison of the elution volumes on Biogel P-100 (eluted with 0.1 M-MOPS/NaOH , pH 7.0, containing 1 mM-MnCl_2) of cytochrome *c* (mol. wt 12380), trypsin inhibitor (mol. wt 21000), pepsin (mol. wt 34700), egg albumin (mol. wt 45000) and bovine serum albumin (mol. wt 66000) with that of the enzyme. An apparent molecular weight of 14800 was obtained.

Lysis of *B. subtilis* walls by enzyme II resulted in a large increase in the number of free amino groups present but no increase in free reducing groups (Fig. 2*b*). Dinitrophenylation and hydrolysis of the soluble products obtained after lysis for 6 h showed the free amino groups to be present as alanine. Further examination of the DNP-alanine by circular dichroism established it to be the L-isomer. Thus, enzyme II is an *N*-acetylmuramoyl-L-alanine amidase. It could be readily distinguished from the major autolysin of *B. subtilis*, which also is an *N*-acetylmuramoyl-L-alanine amidase, by molecular weight, that of the bacterial enzyme being 51 000 (Herbold & Glaser, 1975). Moreover, enzyme II was not inhibited by purified antibody which was raised against, and inactivated, the bacterial amidase (Taylor *et al.*, 1980).

Specificity of the enzymes

The purified enzyme II lysed walls of *B. subtilis* W23 and *B. licheniformis* strains 94 and 6346. In each case the activity of the preparation was similar to that observed with the walls of *B. subtilis* 168. In each of these organisms the basic structure of the peptidoglycan is the same although the nature of the secondary polymers present (the teichoic and teichuronic acids) show considerable differences.

The amidase had no demonstrable activity on the walls of *M. luteus* and *S. aureus* H, or on uncross-linked peptidoglycan, disaccharide monomer and bis-disaccharide dimer prepared from *B. licheniformis* 94 and *B. subtilis* 168. Enzyme I showed some slight activity against walls of *M. luteus* in MOPS/NaOH buffer, pH 7.1, whereas in sodium acetate buffer, pH 4.5, no activity could be detected. Walls of *M. luteus* have been used previously at pH 4.5–5.0 as a specific substrate for the endo- β -*N*-acetylglucosaminidase, the second autolysin of *B. subtilis* (Fan & Beckman, 1973; Taylor *et al.*, 1980).

On the basis of the above experiments the PBSX-induced amidase shows a requirement for cross-linked peptidoglycan of the type found in the walls of bacilli. The nature of the secondary polymers present in these walls appears to have only a marginal effect on lytic activity.

DISCUSSION

The results presented in this paper demonstrate that two previously unidentified autolytic enzymes, an endo-*N*-acetylmuramidase and an *N*-acetylmuramoyl-L-alanine amidase, are synthesized by *B. subtilis* 168 when the defective phage PBSX is induced by heating a *xhi-1479* mutant. The muramidase activity was not detected in uninduced bacteria although small amounts of bacterial amidase were found (Fein & Rogers, 1976). It therefore seems reasonable to conclude that the enzymes described in the present study are associated with the induction of this particular phage. This conclusion is strengthened by the fact that the *xhi-1479* mutation, which maps in the PBSX prophage, is specific for the induction of PBSX. A second phage, ϕ 105, is not induced (Buxton, 1976). The use of a strain carrying the *lyt-2* mutation minimized contamination by the two known bacterial autolysins. In fact, the results demonstrate that the two new autolytic enzymes are easily distinguishable from the bacterial enzymes. Thus, one is an endo-*N*-acetylmuramidase, a type of enzyme unknown in *B. subtilis* 168 although found in another strain of *B. subtilis* (Takahara *et al.*, 1974). The other, an *N*-acetylmuramoyl-L-alanine amidase, had an apparent molecular weight of 14 800, whereas that of the bacterial amidase is 51 000. Moreover, the new amidase was not inhibited by purified antibody raised against the bacterial amidase and, unlike the bacterial amidase (Herbold & Glaser, 1975), the nature of the secondary polymers present in the wall had little effect on lytic activity. These results eliminate the possibility that PBSX uses the known bacterial autolysins to accomplish phage-induced lysis of the bacterium. We cannot, however, be certain that the two new autolysins are phage-coded. By comparison with other bacteriophages, this conclusion seems likely, but since PBSX is defective, it still remains a

possibility that the genes specifying these enzymes lie elsewhere than in the prophage. In this case, their synthesis must be controlled by the phage. Only by the isolation and mapping of mutants affecting the autolysins, the equivalent of R mutants in phage λ (Campbell & Del Campillo-Campbell, 1963), can this question be answered. To date, information regarding the position of the structural genes coding for the bacterial enzymes has not been published (H. J. Rogers, personal communication).

As far as we are aware, there are no previous reports of two distinct autolysins being associated with phage induction. The significance of this observation remains unclear; one possibility is that the bacterial and phage-induced enzymes may have arisen by gene duplication. Perhaps faster lysis is accomplished with two enzymes having different specificities.

There seems to be no significance in the particular specificities of the two phage-induced autolysins. The range of enzyme specificities of phage-induced autolysins is just as large as that of the bacterial enzymes. For example, *E. coli* phage λ specifies an endopeptidase (Taylor, 1971), phage T2 an endo-*N*-acetylmuramidase (Primosigh *et al.*, 1961) and phage T7 an *N*-acetylmuramoyl-L-alanine amidase (Kleppe *et al.*, 1977).

The molecular weight of the phage-induced amidase was rather low compared with that of the bacterial amidase (14800 as against 51000). This may reflect the evolutionary size-limitation pressures imposed upon a phage genome, although large differences in molecular weight have been found in bacterial endo-muramidases isolated from two other strains of *B. subtilis* (13000 and 90000: Takahara *et al.*, 1974; Okada & Kitahata, 1973).

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