A novel glycerophosphodiesterase from *Bacillus pumilus*

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A novel glycerophosphodiesterase activity was detected in extracts from phosphate-starved *Bacillus pumilus* DSM27 cells. The enzyme had a substrate specificity for glycerophosphodiester bonds and the reaction product formed with partially purified enzyme was *(sn)*-glycero-3-phosphate. Purified cell wall teichoic acid of the polyglycerophosphate type, as well as deacylated, unsubstituted lipoteichoic acid of the polyglycerophosphate type, di(glycerophospho)glycerol (deacylated cardiolipin) and mono(glycerophospho)glycerol (deacylated phosphatidylglycerol) served as substrates for the enzyme. Their native counterparts, however, cell wall-bound polyglycerophosphate, lipoteichoic acid (D-alanine substituted and dealanylated), cardiolipin and phosphatidylglycerol were poor or no substrates, respectively. Enzyme activity was inhibited by purified cell walls and by heparin. The enzyme was partially purified using a column of Heparin–Sepharose.

Teichoic acid *Teichoicase* *Lipoteichoic acid* *Phospholipid* *Phosphate starvation*

1. INTRODUCTION

A teichoic acid degrading enzyme (teichoicase) was described and purified from *Bacillus subtilis* Marburg [1–3]. This enzyme splits cell wall-bound as well as isolated purified teichoic acid with apparently the same reaction velocity [3]. Its occurrence is restricted to *Bacillus subtilis* Marburg strains and it shows a substrate specificity for the endogenous α-glucosylated glycerol teichoic acid from *B. subtilis* Marburg cell walls [3]. Using purified polyglycerophosphate and the deacylated derivatives of lipoteichoic acid, cardiolipin and phosphatidylglycerol as substrates, we were able to demonstrate a new enzyme activity in *Bacillus pumilus* DSM27 which split the glycerophosphodiester bonds of these substrates. Important properties and a partial purification of this enzyme are described in this report.

2. EXPERIMENTAL

2.1. *Organism and culture conditions*

Bacillus pumilus DSM27, obtained from the Deutsche Sammlung für Mikroorganismen (Göttingen) was used here. Phosphate-starved cultures were grown in the medium in [4] which contained 0.25 mM inorganic phosphate. For the growth of *B. pumilus* this medium was supplemented with 0.05 μg/ml biotin. For phosphate-supplied control cultures, the phosphate content of this medium was raised to 2.5 mM as in [4]. Cultures were grown in parallel at 37°C under vigorous aeration and cells were harvested 5 h after phosphate was exhausted in the phosphate-starved culture.

2.2. *Chemicals*

Heparin, sodium salt (162000 IE/g) was obtained from Serva (Heidelberg). Heparin–Sepharose CL-6B and DEAE–Sephacel were purchased from Pharmacia (Freiburg). Bio-Gel P-6 was obtained from Bio-Rad Laboratories (Munich). *(sn)*-

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Glycero-3-phosphate dehydrogenase from rabbit muscle, L-α-phosphatidyl-DL-glycerol from egg lecithin and cardiolipin from bovine heart were obtained from Sigma (Munich). Alkaline phosphatase from calf intestine (grade II) came from Boehringer (Mannheim).

2.3. *Preparation of substrates*

Tryptically digested cell walls were prepared as in [5]. Murein sacculi from *Escherichia coli* B were obtained by treatment of whole cells with hot 4% (w/v) aqueous sodium dodecyl sulfate [6]; to remove the detergent, the sacuuli were washed 6 times with double-distilled water. Cell wall teichoic acids were routinely extracted from cell walls with 5% (w/v) aqueous trichloroacetic acid (0.2 ml/mg of cell walls) in the cold (24 h, 0°C). In addition, solubilization of teichoic acid from cell walls of *B. pumilus* was also achieved by lysozyme treatment of cell walls (0.1 mg lysozyme/mg cell wall/ml 0.01 M Tris–HCl, pH 7.5, for 16 h at 37°C). Solubilized teichoic acids were purified by chromatography on DEAE-Sephacel as in [7] and finally dialysed against double-distilled water. In contrast to this standard procedure, the cell wall teichoic acid from *Bacillus subtilis* DSM10 was isolated and purified as in [8]. Lipoteichoic acids were isolated by phenol extraction of disrupted cells [9] and further treated at pH 4.0 [10]. Purification was achieved by hydrophobic interaction chromatography ([11,12], in preparation). By this procedure, highly purified, D-alanine substituted lipoteichoic acids were obtained. Lipoteichoic acid with an ethylenediol phosphate terminus was prepared as in [13]. Purified lipoteichoic acid samples, cardiolipin and phosphatidylglycerol were deacylated by alkaline hydrolysis essentially as in [14], except that 0.1 M NaOH was used instead of 0.2 M NaOH. The lipoteichoic acid preparation from *Staphylococcus aureus* H contained phosphorus, glycerol and alanine in a molar ratio of 1.0:1.0:0.50 (by vol.). The lipoteichoic acid preparation from *B. pumilus* contained phosphorus, glycerol, alanine, glucosamine and glucose in a molar ratio of 1.0:1.0:0.57:0.23:0.14 (by vol.).

2.4. *Enzyme assays*

Glycerophosphodiesterase activity (GPDase activity) was routinely assayed with purified cell wall teichoic acid from *B. pumilus* DSM27 as a substrate. Enzyme activity was detected and quantified by the determination of liberated glycerol in the heat-denatured enzyme assay at the end of the incubation period. Enzyme solution (0.05–0.3 ml dialyzed crude extract or partially purified enzyme) was incubated in a total of 0.5 ml 10 mM Tris–HCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM EDTA buffer, pH 8.5 (buffer B) with teichoic acid (equivalent to 0.5 μmol phosphate) as a substrate. Incubation was done at 37°C for 30–360 min. To assay partially purified GPDase, 0.1 units alkaline phosphatase were added. After the incubation period, the assays were heated for 10 min in a boiling water bath and subsequently centrifuged (5 min, $8000 \times g$); 0.4 ml of the supernatant were removed and its glycerol content was determined enzymatically as in [15]. Activity was expressed in terms of μmol glycerol liberated · mg protein^{-1} · h^{-1} at 37°C. Phosphomonoesterase activity was measured with *p*-nitrophenylphosphate as a substrate at 25°C in a 0.1 M Tris–HCl, 10 mM $MgCl_2$ buffer, pH 8.0. Activity was expressed in terms of μmol *p*-nitrophenol formed · mg protein^{-1} · min^{-1} at 25°C.

2.5. *Preparation and partial purification of glycerophosphodiesterase*

The subsequent steps were done at 0–4°C. Cells were harvested and then washed and resuspended in buffer B. Crude cell-free extract was routinely prepared by disrupting the cells with glass beads and removing cell debris by centrifugation ($48\,000 \times g$, 30 min). The supernatant was dialysed against buffer B and used as an enzyme source. For a partial purification of GPDase, an aliquot of the crude cell extract (4 ml, 2 mg protein/ml) was passed through a column of Heparin–Sepharose CL-6B (1 × 3 cm) previously equilibrated to buffer B. Bound material was eluted first with 0.09 M NaCl in buffer B, followed by a step of 0.5 M NaCl in buffer B. GPDase activity which eluted with 0.5 M NaCl was collected and dialysed against buffer B.

2.6. *Analytical methods*

Protein was measured as in [16]. Phosphate was determined as in [17]. The uronic acid content of cell walls was estimated as in [18]. *(sn)*-Glycero-3-phosphate was quantitated with (*sn*)-glycero-3-phosphate dehydrogenase as in [19]. The

alanine content of lipoteichoic acids was estimated in hydrolysed samples (2 N HCl, 100°C, 3 h) using an automatic amino acid analyzer. Teichoic acid monomers other than glycerol were detected by gas-liquid chromatography as in [7].

3. RESULTS AND DISCUSSION

With soluble purified cell wall teichoic acid from *B. pumilus* DSM27 as a substrate, the glycerophosphodiesterase (GPDase) activity was detected in cell-free extracts from phosphate-starved *B. pumilus* DSM27 cells; 80% of the enzyme activity in the crude extract could be sedimented by ultracentrifugation (160 000 × *g*, 30 min, Beckman Airfuge), indicating that enzyme activity occurs in a high -M_r complex. The enzyme activity and the phosphate and uronic acid content of cell walls of phosphate-starved cells were compared with the corresponding values obtained from a phosphate-supplied control culture (table 1). As can be seen, the switch from teichoic acid to teichuronic acid biosynthesis under conditions of phosphate starvation as is established for several gram-positive bacteria [20], is also indicated for *B. pumilus* DSM27.

The pH-dependence of the GPDase activity was determined between pH 5.0 and 9.0. The standard enzyme assay was used, except that 10 mM Tris–maleate instead of 10 mM Tris–HCl was used as a buffer between pH 5.0 and 7.0. Optimal activity of the enzyme was found at pH 8.5. Under the conditions tested, the liberation of glycerol from cell wall teichoic acid was linear with time until 20% of the substrate was degraded. To determine the substrate specificity of the GPDase, various substrates were tested with dialysed crude cell-free extract as the enzyme source. In contrast to the purified cell wall teichoic acid from *B. pumilus* DSM27, the chain of which was characterized as a polyglycerophosphate [3], α-glucosylated glycerol teichoic acid from *B. subtilis* DSM10 was not degraded by the enzyme. This indicated the structural requirement of the enzyme for an unsubstituted teichoic acid chain. When tested with an unsubstituted ribitol teichoic acid chain from *S. aureus* 52A2 [21], no formation of ribitol was detected. From the teichoic acid from *S. griseus* DSM20236, which contains glycerophosphate as substituent of a polyribitol phosphate chain ([22,23], unpublished) no glycerol was liberated upon incubation with GPDase. Thus GPDase activity seemed to exhibit a structural requirement for phosphodiester bonds between adjacent glycerol units. Consequently, GPDase activity was tested towards such substrates (table 2). Substrates supposed to be products of metabolic turnover were degraded by the enzyme, whereas their native counterparts were very poor substrates (cell wall-bound teichoic acid) or no substrates at all (lipoteichoic acid, cardiolipin and phosphatidylglycerol). GPDase activity was not affected by the presence of 0.1% Triton X-100 in the standard enzyme assay. Also in this environment the fatty acid-containing substrates were not degraded by the GPDase. The enzyme activity might be related to the phosphate supply of the phosphate-starved cell (table 1). The protection of native substrates however seems useful in view of their potential physiological functions. Presumably because of its glycosidic substituents, the dealanylated, deacylated lipoteichoic acid from *B. pumilus* DSM27 was not degraded by the GPDase. The failure of the enzyme to degrade dealanylated, deacylated lipoteichoic acid from *S. aureus* H with

Table 1

Comparison of phosphate-starved and phosphate-supplied *B. pumilus* DSM27 cultures

	Phosphate content (μmol/mg cell walls, dry wt)	Uronic acid content (μmol/mg cell walls, dry wt)	Glycerophosphodiesterase ($\mu mol \cdot mg^{-1} \cdot h^{-1}$)
Phosphate-starved	0.46	0.78	0.25
Phosphate-supplied	1.20	n.d.	0.04

n.d., not detectable

Table 2

Substrate specificity of glycerophosphodiesterase

Substrate tested	Glycerol formed (nmol)
Polyglycerophosphate (solubilized from *B. pumilus* DSM27 cell walls by trichloroacetic acid)	58
Polyglycerophosphate (solubilized from *B. pumilus* DSM27 cell walls by lysozyme)	60
Polyglycerophosphate (covalently attached to *B. subtilis* DSM27 cell walls)	8
Deacylated, dealanylated lipoteichoic acid from *S. aureus* H (unsubstituted polyglycerophosphate chain)	52
Deacylated, dealanylated lipoteichoic acid from *B. subtilis* DSM27 (polyglycerophosphate chain partially substituted by glucose and glucosamine[a])	5
Gro-P-Gro-P-Gro (deacylated cardiolipin)	75
Gro-P-Gro (deacylated L-α-phosphatidyl-DL-glycerol)	90
Deacylated, dealanylated lipoteichoic acid from *S. aureus* H with an ethylenediol phosphate terminus	n.d.
Cardiolipin	n.d.
L-α-Phosphatidyl-DL-glycerol	n.d.
Lipoteichoic acid from *B. pumilus* DSM27 (with or without D-alanine substitution)	n.d.
Lipoteichoic acid from *S. aureus* H (with or without D-alanine substitution)	n.d.

n.d., not detectable; Gro, glycerol; P, phosphate
[a]Unpublished results

an ethylenediol phosphate terminus points to a strict requirement for glycerophosphate and furthermore to an exomechanism of the reaction catalysed by GPDase.

Tryptically digested cell walls from various gram-positive bacteria as well as murein sacculi prepared from *E. coli* B showed an inhibitory effect on GPDase activity as measured in the standard enzyme test (60% inhibition by 0.5 mg/ml). The polyanion heparin was also found to be an inhibitor of the enzyme (70% inhibition by 20 μg/ml heparin in the standard enzyme test). Consequent-

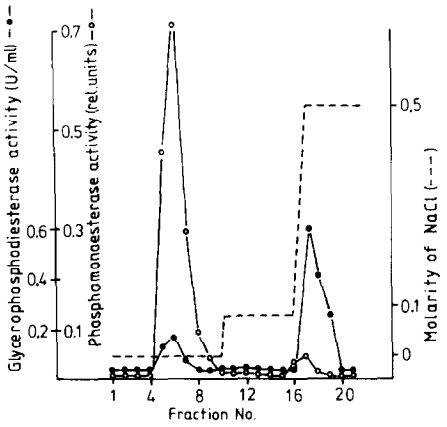

Fig.1. Chromatography of crude cell-free extract on Heparin-Sepharose CL-6B. (—●—) Glycerophosphodiesterase activity, (—○—) phosphomonoesterase activity, (– – –) molarity of NaCl in buffer B.

ly, a column of Heparin-Sepharose CL-6B could be used for a partial purification of the GPDase. This column chromatography step is shown in fig.1. The enzyme was purified 20-fold by this procedure. The bulk of the high phosphomonoesterase activity of the crude cell-free extract (22 units/mg protein) could be separated from the GPDase activity and was recovered in the flow-through fraction of the column. The small amount of phosphomonoesterase activity which remained bound to Heparin-Sepharose is most probably not an intrinsic activity of the GPDase, because the elution patterns of the two enzyme activities were not superimposed (fig.1). As shown in table 3, the partially purified enzyme (fraction 18 and 19 in fig.1) allowed the determination of the reaction product of the GPDase. Initially a phosphate-containing reaction product was liberated from the glycerophosphodiester backbone of the teichoic acid. It could be converted to free glycerol by alkaline phosphatase, which acted with the same efficiency either simultaneously with the GPDase or independently in an incubation step after heat denaturation of the GPDase activity. Thus the enzyme acted in the same manner as the teichoicase from *B. subtilis* Marburg [3], in contrast however to the phosphodiesterase isolated from *Aspergillus niger* [24] which initially liberates free glycerol and consequently needs a simultaneous action of a phosphomonoesterase for the degradation of

Table 3

Reaction product of GPDase and mode of its liberation

Partially purified GPDase[a] assayed with	Reaction product (nmol) detected as	
	(sn)-Glycero-3-phosphate	Glycerol
Polyglycerophosphate (from *B. pumilus* DSM27 cell walls) plus 0.1 unit alkaline phosphate during incubation	n.d.	80
Polyglycerophosphate (from *B. pumilus* DSM27 cell walls) incubation with 0.1 unit alkaline phosphatase after heat denaturation of GPDase	n.d.	82
Polyglycerophosphate (from *B. pumilus* DSM27 cell walls)	50	27[b]

[a]Fraction 18 and 19 in fig.1
[b]Traces of phosphomonoesterase were present (see fig.1)
n.d., not detectable

polyglycerophosphate. In the experiment without the addition of alkaline phosphatase, the nature of the reaction product could be determined. It was found that it could be quantitatively oxidized by the stereospecific *(sn)*-glycero-3-phosphate dehydrogenase. This strongly suggests that GPDase liberates *(sn)*-glycero-3-phosphate units from polyglycerophosphate. The same result was obtained when deacylated lipoteichoic acid from *S. aureus* H was used as a substrate for the enzyme. This was not expected, because cell wall teichoic acid and lipoteichoic acid are considered to be of opposite stereochemistry with regard to C-2 of their glycerophosphate units [14]. There are, however, several possibilities to explain this finding:

(i) The GPDase was only partially purified and consequently the participation of more than one enzyme species cannot be ruled out;
(ii) Strand breaks in the lipoteichoic acid backbone might have occurred during preparation, allowing degradation from sites stereochemically identical to cell wall teichoic acid;
(iii) The GPDase might cleave cell wall teichoic acid and lipoteichoic acid at C-1 and C-3 of their glycerophosphate units, respectively.

The type of enzyme described here might be of importance for the teichoic acid and lipid turnover of the cell. Lipoteichoic acid turnover has been reported for several gram-positive organisms [25–27]. The role of cell wall teichoic acid as phosphate donor was also discussed [4]. In this context, a further investigation concerning the induction of GPDase activity under various physiological conditions and an examination of its occurrence among other gram-positive bacteria seem useful. A comparison with phosphodiesterase activities described in *Bacillus* [28,29] will be done when highly purified GPDase has been prepared.

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