Purification, M_r-value and subunit structure of a teichoic acid hydrolase from *Bacillus subtilis*

Wolfgang Kusser* and Franz Fiedler

Institut für Genetik und Mikrobiologie der Universität München, Maria-Ward-Straße 1a, 8000 München 19, FRG

Received 28 September 1982

A teichoic acid degrading enzyme (tiechoicase) was purified to apparent homogeneity from a water-soluble cell extract of sporulating *Bacillus subtilis* cells. A rapid test for the detection of teichoicase activity was developed. The purified teichoicase has an app. $M_r = 310000$. It consists of 4 identical subunits of $M_r = 78000$ each.

Teichoic acid hydrolase *Teichoicase* *Teichoic acid* *Bacterial cell surface* (Bacillus subtilis)

1. INTRODUCTION

Considerable progress has been made in unravelling the chemical structures, the biosynthesis, the location and, to some extent, the function of teichoic acids [1]. However, relatively little is known about how teichoic acids are degraded in nature [2]. The phosphodiester backbone of teichoic acids has proved to be resistant to nucleases and unspecific phosphodiesterase with the exception of an enzyme from *Aspergillus niger* [3]. Teichoic acid hydrolase activity has been shown under conditions of sporulation in *Bacillus subtilis* Marburg [4], termed teichoicase. Phosphate starvation induced teichoicase activity in *Bacillus subtilis* 168 [5,6].

We report here on the purification and partial characterization of the teichoicase from *Bacillus subtilis* Marburg induced under conditions of sporulation.

* This work is in partial fulfillment of requirements for a thesis by W.K. to the Faculty of Biology, University of Munich, FRG

A preliminary report of some of these results was presented at the ASM/DGHM meeting held in Darmstadt 17–20 March 1982

2. EXPERIMENTAL

2.1. *Organism and culture conditions*

For the preparation of the enzyme, *Bacillus subtilis* DSM 10 (ATCC 6051) (known as the Marburg strain and as the parent of the 168 strains) was grown for 20 h at 37°C under vivid aeration in nutrient sporulation medium [7]. For the preparation of teichoic acid as a substrate, *Bacillus subtilis* DSM 10 cells were labelled with ^{14}C by growing them at 37°C in rich medium [8], supplemented with [U-^{14}C]glucose (0.6 Ci/mol) and harvested at the end of the exponential growth phase.

2.2. *Chemicals*

Reagents for electrophoresis were purchased from Serva (Heidelberg). DEAE-Sephacel, CM-Sepharose CL-6B, concanavalin (con) A–Sepharose 4B and Sephacryl S-300 were obtained from Pharmacia (Uppsala). BIO-GEL HT (hydroxyapatite) came from Bio-Rad Labs. (Richmond CA).

2.3. *Preparation of teichoic acid*

Cell walls were prepared as in [9]. Teichoic acids were extracted from the cell walls with 0.1 M NaOH [10] and purified by affinity chromatography on con A–Sepharose [11].

2.4. *Assay of teichoicase*

Teichoicase was assayed at 37°C by incubating

Published by Elsevier Biomedical Press
001457593/82/0000—0000/$2.75

the enzyme with a ^{14}C-labelled substrate (α-glucosylated glycerol teichoic acid, containing 10–100 nmol phosphate) with spec. act. 0.5 mCi/mmol in 100 μl total of a 10 mM Tris–HCl, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.2 mM EDTA buffer (pH 8.5). After 10–60 min incubation, the assays were placed in ice and 10 μl of a 1% solution of chondroitin sulfate in 0.1 M $MgSO_4$ and 110 μl of 0.5% *N*-cetyl-*N,N,N*-trimethyl ammonium bromide (CTAB) were added. After 30 min at 0°C, polymeric teichoic acid could be sedimented by centrifugation (5 min, 10000 × *g*), whereas degraded teichoic acid remained in the supernatant. The radioactivity in an aliquot (110 μl) of the supernatant was determined.

Specific activity was expressed in terms of μmol phosphate liberated.mg protein^{-1}.h^{-1} at 37°C. Protein was measured as in [12]; phosphate was determined as in [13].

2.5. *Enzyme purification*

The subsequent steps were completed at 0–4°C. Cells were harvested and then washed and resuspended in 10 mM Tris–HCl, 10 mM $MgSO_4$, 0.2 mM EDTA buffer (pH 7.8) (buffer A). Crude cell extract was routinely prepared by disrupting the cells with glass beads and removing cell debris by centrifugation (48000 × *g*, 60 min). The supernatant was dialyzed twice against buffer A and subsequently passed over a DEAE–Sephacel column (2 × 30 cm), pre-equilibrated to buffer A. Bound material was eluted with 0.1 M NaCl followed by a linear 0.1–1 M NaCl gradient. Fractions containing teichoicase at 0.3 M NaCl were pooled and dialyzed twice against 5 mM Tris, 5 mM maleate, 5 mM $MgSO_4$, 0.2 mM EDTA buffer (pH 5.85). The dialyzed enzyme solution was applied to a CM-Sepharose column (1.6 × 16 cm), carefully equilibrated with CM-buffer. Bound material was eluted with a linear 0–1 M NaCl gradient. Fractions containing enzyme around 0.5 M NaCl were combined and loaded onto a Bio-Gel HT column (0.6 × 1 cm). Hydroxyapatite-bound teichoicase was desorbed from the column by elution with 0.3 M potassium phosphate (pH 7.0) (KP_i). Fractions containing enzyme were dialyzed against buffer A.

2.6. *Electrophoresis*

Sodium docecyl sulfate–polyacrylamide disc-gel electrophoresis was performed on slab gels as in [14].

2.7. *Native enzyme M_r-value*

The apparent M_r-value of teichoicase was determined on a Sephacryl S-300 column (2.5 × 150 cm) using a 10 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl buffer (pH 8.0). The following proteins were used as standards: bovine serum albumin (BSA); lactate dehydrogenase (LDH, hog muscle); catalase (beef liver); and ferritin (horse spleen).

3. RESULTS AND DISCUSSION

The enzyme assay in section 2 is based on the precipitation of polyanionic teichoic acid by the quaternary ammonium salt CTAB [15]. The test allows rapid screening for teichoicase activity. The liberation of CTAB-soluble reaction product was linear with enzyme concentration. The insoluble complex between non-degraded teichoic acid and CTAB cannot be formed however under conditions of high ionic strength (>0.4 M NaCl) or in the presence of neutral and anionic detergents like Triton X-100 and SDS.

The column chromatography steps of the purification procedure are summarized in fig. 1–3. The binding of the enzyme to CM-Sepharose requires that the dialyzed enzyme solution and the CM-Sepharose both attain a pH of 5.85. Since we found only binding of the enzyme to the column material with the CM-buffer in section 2 the attachment of the enzyme to CM-Sepharose might

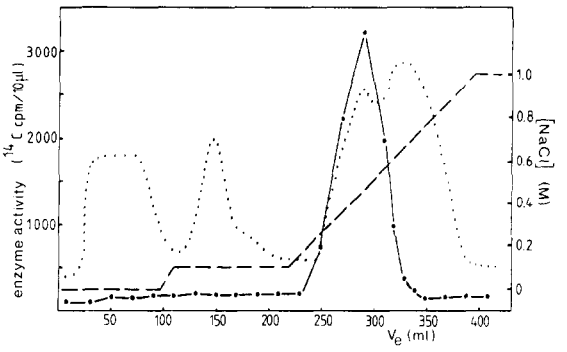

Fig. 1. Purification of teichoicase by DEAE-Sephacel chromatography. Enzyme activity (– ● –), A_{280} (····) and [NaCl] (-----) are plotted *vs* elution volume (V_e).

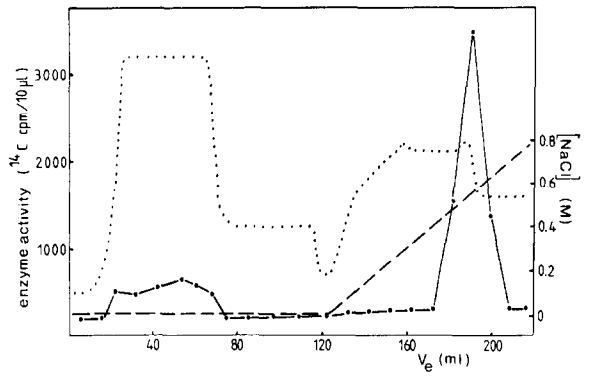

Fig. 2. Purification of teichoicase by CM-Sepharose CL-6B chromatography. Enzyme activity (– ● –), A_{280} (····) and [NaCl] (-----) are plotted *vs* elution volume (V_e).

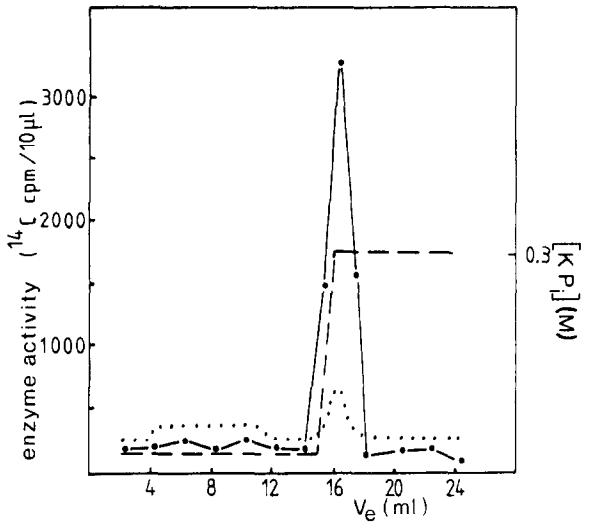

Fig. 3. Purification of teichoicase by Bio-Gel HT chromatography. Enzyme activity (– ● –), A_{280} (····) and [KP_i] (-----) are plotted *vs* elution volume (V_e).

be mediated by divalent ions present in the CM-buffer.

Binding of the enzyme to hydroxyapatite also occurs in the presence of NaCl, therefore dialysis of the enzyme from CM-Sepharose can be avoided. The desorption from hydroxyapatite was achieved with 0.3 M potassium phosphate but was not possible with 1 M NaCl or 50 mM $MgCl_2$, indicating a binding of the enzyme via the Ca^{2+}-moiety of hydroxyapatite [16].

Table 1

Purification scheme

Step	Protein (mg/ml)	Spec. act.	Purif. (-fold)	Yield (%)
1. Dialyzed, crude extract	1.45	1.0	1	100
2. DEAE-Sephacel column	0.67	2.9	3	77
3. CM-Sepharose CL-6B column	0.06	46	46	50
4. Bio-Gel HT column	0.03	143	143	35

The results of a typical enzyme purification are summarized in table 1. For enzymes which recognize polyanionic substrates, affinity chromatography methods have been successfully applied [17,18]. However, we found no binding of the teichoicase to blue–Sepharose, poly(U)–Sepharose and heparin–Sepharose. The relatively high yield (35%) of the conventional purification procedure used might be the result of two properties of the teichoicase:

(1) The enzyme is remarkably resistant to high temperature (boiling for 10 min causes 10% loss of activity);
(2) The enzyme is resistant to trypsin and endogenous proteases present in the crude extract.

Fig. 4 shows the molecular weight determination of the purified denatured teichoicase using a 6.5% slab gel. The result of $M_r = 78000$ was confirmed in gels containing 8% and 10% acrylamide.

As shown in fig. 5, the M_r of the native enzyme is 310000. The denatured enzyme yielded only one polypeptide of $M_r = 78000$. This implies a structure consisting of 4 identical subunits of $M_r = 78000$ each for the native teichoicase molecule. This finding was supported by studies using the bifunctional crosslinking agent dimethyl subimeridate [19]. After crosslinking the purified teichoicase two additional polypeptides, corresponding to the M_r of the dimer and the tetramer, were obtained by gel electrophoresis.

The purified enzyme may serve as a landmark for other teichoicases and as a tool for structural and biological studies concerning teichoic acids. A further characterization of this teichoicase in terms

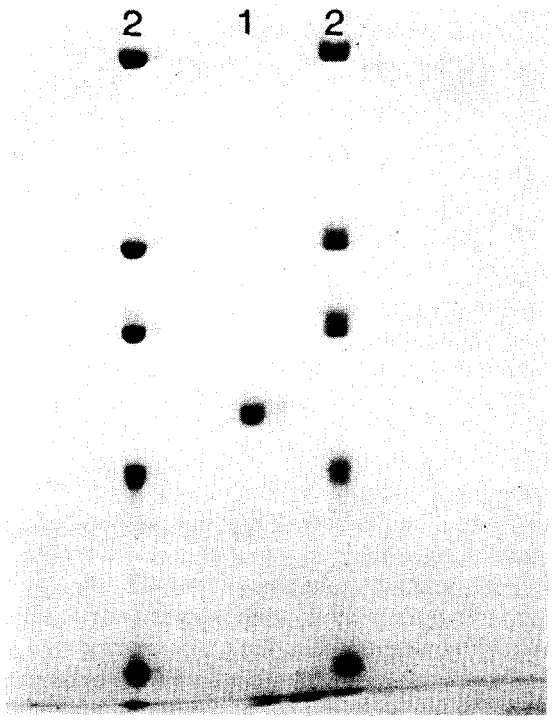

Fig. 4. Estimation of denaturated enzyme M_r value. Sodium dodecyl sulfate–polyacrylamide gel of purified teichoicase: (1) purified teichoicase (10 μg protein); (2) proteins with known M_r (from Bio-Rad Labs.), containing myosin (200000), β-galactosidase (116500), phosphorylase B (92500), bovine serum albumin (66200), and ovalbumin (45000). From the plot log M_r *vs* electrophoretic mobility of the protein standards, a M_r for denatured teichoicase of 78000 was calculated.

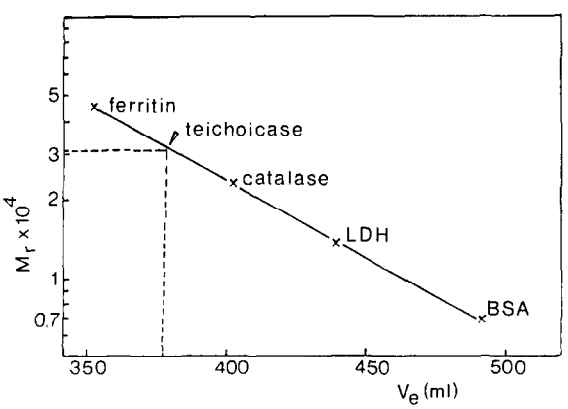

Fig. 5. Estimation of native enzyme M_r value. Gel chromatography on Sephacryl S-300 of the purified teichoicase: 0.5 mg purified teichoicase was simultaneously applied to the column with the protein standards mentioned in section 2. In the above plot, the app. M_r of native teichoicase was evaluated at 310000.

of substrate specificity, reaction product, reaction mechanism and some other biochemical parameters will be described elsewhere (in preparation).

ACKNOWLEDGEMENTS

The work was supported by the Deutsche Forschungsgemeinschaft. We are grateful to Brigitte Göser for excellent technical assistance.

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