The non-enzymatic microbicidal activity of lysozymes

Klaus Düring[a,b,*], Petra Porsch[a,1], Andreas Mahn[a,1], Olaf Brinkmann[c,1], Werner Gieffers[c]

[a] *Federal Centre for Breeding Research on Cultivated Plants, Institute for Breeding Methods in Vegetables, Neuer Weg 22/23, D-06484 Quedlinburg, Germany*
[b] *MPB Cologne GmbH, Eupener Str. 161, D-50933 Köln, Germany*
[c] *Max-Planck-Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, D-50829 Köln, Germany*

Received 15 March 1999

Abstract T4 lysozyme was thought to destroy bacteria by its muramidase activity. However, we demonstrate here that amphipathic helix stretches in the C-terminus of T4 lysozyme mediate its bactericidal and fungistatic activities. In heat-denatured T4 lysozyme, the enzymatic activity is completely abolished but unexpectedly, the antimicrobial functions remain preserved. Small synthetic peptides corresponding to amphipathic C-terminal domains of T4 lysozyme show a microbicidal activity. Its membrane disturbing activity was directly demonstrated for bacterial, fungal and plant cells but not in a hemolysis assay. Comparable results were obtained with hen egg white lysozyme. This opens up many new opportunities for optimization of lysozymes as antimicrobial agents in various applications by protein engineering.

Key words: Lysozyme; Muramidase; Amphipathic helix; Non-enzymatic microbicidal activity; Membrane disturbing activity; Antimicrobial peptide

1. Introduction

T4 lysozyme is well known as an antibacterial protein with activity against Gram-positive and Gram-negative bacteria [1]. As murein, the substrate of T4 lysozyme, is unique to bacteria, T4 lysozyme was considered to affect only bacteria. Its bactericidal activity is hypothesized to reside in its muramidase activity, leading to degradation of the murein layer and reduction of the mechanical strength of the bacterial cell wall and eventually resulting in killing of bacteria by lysis [1]. A deep knowledge has been elaborated on structure-function relationships and the importance of individual amino acid residues for the muramidase activity of T4 lysozyme [2]. A large number of systematic mutants has been characterized for influences on the enzymatic muramidase activity which revealed a significant tolerance to many alterations [3].

Investigations on heat-denatured hen egg white lysozyme (HEWL) revealed an enhanced bactericidal activity towards Gram-negative bacteria of a partially unfolded, enzymatically inactive and more hydrophobic dimeric form. This activity is based on membrane insertion of the dimeric form and a subsequent membrane disruption. The bactericidal activity of denatured HEWL could be uncoupled from the enzymatic activity [4–7]. Furthermore, insertion of a hydrophobic pentapeptide into the C-terminus of HEWL resulted in the same effects [8].

In the course of analysing transgenic potato plants expressing T4 lysozyme as a new resistance factor, we could prove that the enhanced resistance to the phytopathogenic bacterium *Erwinia carotovora* results from the presence of the foreign protein [9]. Further extending the screening for an improved phytopathological performance of the transgenic potato plants, we discovered that T4 lysozyme effectively mediates an enhanced resistance also to phytopathogenic fungi including the non-chitin containing fungus *Phytophthora infestans* (Brinkmann, Düring, Gieffers, unpublished results). This prompted us to investigate other possible mechanisms of action of lysozymes than the well known enzymatic muramidase function. Here, we report on the identification of small peptide sequences in T4 lysozyme as well as in hen egg white lysozyme which mediate bactericidal and fungistatic activities. The membrane disturbing activity of these two lysozymes was proved in assays with bacteria, fungi and potato protoplasts.

2. Materials and methods

2.1. Cloning of genes encoding variant T4 lysozymes

An overproduction vector carrying a gene encoding T4 lysozyme bearing an additional N-terminal HIS-tag was available (pSR 8-23 [10]).

For introduction of the M6K mutation into T4 lysozyme, the plasmid pSR 8-59 was constructed in analogy to pSR 8-23 according to Düring [10], using a modified 5′ primer: GCGGATCCATCGAGGGTAGAATGAATATATTTGAAAAGTTAC

A native reference lysozyme lacking the N-terminal HIS-tag extension was obtained using another variant construct (pSR 8-61) designed for the TagZyme system II (Unizyme Laboratories, Denmark). Removal of the N-terminal tag was accomplished according to the manufacturer's protocol.

2.2. Overproduction of T4 lysozyme in Escherichia coli

All T4 lysozyme variants were overproduced and purified according to the method described by Düring [10].

2.3. Hen egg white lysozyme

Hen egg white lysozyme was purchased from Boehringer Mannheim (Germany).

2.4. Synthetic peptides

Synthetic peptides were purchased at a 99% purity level from Genosys (UK).

2.5. Heat denaturation of T4 lysozyme

T4 lysozyme was incubated for 10 min at 80°C. The precipitated heat-denatured protein was redissolved in 0.5×PBS/50% DMSO. Dissolution could not be achieved completely, part of the protein remained insoluble.

*Corresponding author. Fax: (49) (221) 4994893.
E-mail: k.during.mpb@online.de

[1] Present address: *MPB Cologne GmbH*, Eupener Str. 161, D-50933 Köln, Germany.

0014-5793/99/$20.00
PII: S0014-5793(99)00405-6

2.6. Viable count plating

E. coli strain MC1022 was grown in LB medium overnight at 37°C, diluted and regrown to 2×10^8 cells/ml. The bacterial culture was centrifuged, the pellet washed in 1×PBS and resuspended in 0.1×PBS. The concentration was adjusted to 2×10^7 cells/ml. A defined amount of T4 lysozyme or peptide, respectively, in a volume of 4 μl was added to 200 μl of the suspension of *E. coli*. After 1 h of incubation at 37°C, a 10^{-4} dilution was prepared and 100 μl suspension was plated. Colony forming units were counted after incubation of the plates overnight at 37°C.

Micrococcus lysodeikticus strain DSM 20030 was grown on LB medium and resuspended in 0.1×PBS. Viable count plating was performed as for *E. coli* except for the incubation temperature which was 30°C

2.7. Chitinase assay

Different amounts of lysozyme in a volume of 100 μl were incubated with 500 μl 0.2 N HEPES and 200 μl carboxymethyl chitin Remazol Brilliant violet 5R for 15 h at 37°C [11]. The reaction was stopped by the addition of 200 μl 0.2 M HCl and rapid cooling on ice. After pelleting uncleaved substrate by centrifugation, soluble dye was measured photometrically at 550 nm.

2.8. Measurement of the length of fungal germination tubes

Phytophthora nicotianae was used as the standard non-chitin containing test fungus and *Fusarium oxysporum* as chitin containing test fungus.

Sporangia formation of *P. nicotianae* (DSM strain 62704) was induced as described by Gooding and Lucas [12]. Sporangia were separated from the sporulation solution in a 20 μm sieve and resuspended in A1 buffer. Zoospore release occurred after chilling for 20 min at 4°C and the suspension was adjusted to 2.5×10^5 zoospores/ml. Lysozyme or peptide, respectively, was added to a final concentration of 0.5 μg/μl and the mixture was incubated for 20 h in the chamber slides in Lab-Tec Chamber Slides (Nunc, Germany) with eight wells at room temperature.

F. oxysporum was grown on potato dextrose agar (Difco) at 22°C until sporulation. A suspension of conidia was prepared in distilled water and adjusted to 2.5×10^5 spores/ml. Equal quantities of the conidia suspension were centrifuged and resuspended in A1 buffer (15 mM K_2HPO_4, 20 mM NaCl, pH 6.8) [13]. T4 lysozyme or peptide, respectively, was added to a concentration of 0.5 μg/μl. 16 μl of the mixture was filled into the chamber slides, each containing 500 μl water-agar, and incubated for 7 h at room temperature.

2.9. Isolation of potato protoplasts

Leaves of in vitro plants var. Désirée, 3–4 weeks after subcultivation, were cut into thin strips (circa 1 mm), incubated for 16 h at room temperature in 0.5 M glycine pH 5.7, containing 1.5 g/l cellulase and 0.75 g/l macerozyme R-10 (Serva, Heidelberg, Germany) and afterwards shaken for 5 min at 50 rpm. Protoplasts were filtered through a sterile nylon filter (pore size 60 μm) and the filtrate was centrifuged for 5 min at $50\times g$. The pellet was washed four times with 0.5 M glycine pH 5.7.

2.10. Sytox staining assays with potato protoplasts

Sytox green nucleic acid stain (Molecular Probes, USA) was added to the protoplasts to a final concentration of 1 μM. Living protoplasts (red background fluorescence of the chloroplasts) were counted under a fluorescence microscope (excitation 450–490 nm) using a Thoma chamber, before and several times after the addition of T4lys, HEWL, peptide A4, BSA (all in PBS) to a final concentration of 1 mg/ml or the equivalent volume of PBS, respectively.

2.11. LIVE/DEAD staining assay with bacteria

E. coli strain MC1022 was grown as for viable count plating and adjusted to 3.2×10^8 cells/ml in 0.1×PBS. SYTO 9 and propidium iodide (component A and B of the LIVE/DEAD BacLight bacterial viability kit, Molecular Probes, USA) were added to 300 μl bacteria to a final concentration of 3.34 μM and 20 μM, respectively. The bacterial suspension was divided into three parts of 100 μl each for the addition of 100 μg T4lys or HEWL, respectively, in 16 μl PBS or 16 μl PBS only as negative control. 2 h after addition of the lysozymes or PBS, bacteria were examined under a fluorescence microscope (excitation 450–490 nm).

2.12. Sytox staining assays with fungal spores

Fungal spores of *F. oxysporum* and *P. infestans* were stained with Sytox green nucleic acid stain according to the procedure of Reed et al. [14], except that the incubation time was extended to 3 h and the stain concentration was changed to 5 μM. HEWL or T4lys, respectively, were added to the suspension of fungal spores in Potato-Dextrose-Broth (PDB, Difco, USA) to a final concentration of 3 μg/μl. Bovine serum albumin (BSA) and distilled water were used as controls. The fluorescence of lysozyme-treated spores was compared to that of conidia of *F. oxysporum* or sporangia of *P. infestans*, respectively, incubated for 10 min in 70% ethanol and washed two times in PDB. The stained fungal spores were examined and photographed with a Zeiss Axiophot 2 fluorescence microscope (magnification 400×).

2.13. Hemolysis assay

40 g/l blood agar base (Difco, USA) was supplemented with 5% sterile defibrinated horse blood (bioMérieux, Germany) and poured into a 9 cm petri dish. Seven holes of 3 mm in diameter were punched into the agar and filled with 10 μl T4lys, T4lys(M6K), HEWL, peptide A4, α-hemolysin from *Staphylococcus aureus* (Sigma, Germany), BSA (each at a concentration of 1 mg/ml) and PBS. After 6 h, lysis was analyzed.

2.14. Computer analysis

A protein sequence analysis was performed with the GeneWorks Release 2.5 programme (Oxford Molecular Group, USA). Using the MacImdad 3D display programme (Molecular Applications Group, USA, V.5.0.1), α-helices of T4 lysozyme in the 2LZM entry of the Brookhaven Protein Databank were analyzed.

2.15. Statistical analysis

The results for the bactericidal activity are means of at least seven experiments with five replicates for each variable. The mean correlation between all experiments is $r=0.904$. Results for the fungistatic activity are means of four (*P. nicotianae*) or three (*F. oxysporum*) experiments, respectively, with 40 replicates for each variable. The mean correlation between all experiments is $r=0.925$ (*P. nicotianae*) and $r=0.758$ (*F. oxysporum*), respectively. Statistical analysis was made with the SPSS programme V7.5 (SPSS Software GmbH, Germany) using the Tukey-HSD test.

3. Results

3.1. T4 lysozyme fusion proteins

T4 lysozyme was mainly used as a fusion protein containing a N-terminal HIS-tag extension [10]. This fusion protein can be overproduced in fairly large amounts at a comparably low cost and, therefore, is a suitable component for a routine test system. In order to prove that this fusion protein is an appropriate substitute for native T4 lysozyme, we also produced a variant fusion protein which allows the precise cleavage of the N-terminal tag using the TagZyme system II (Unizyme Laboratories, Denmark). Direct comparison of the fusion protein with the tag and the processed native T4 lysozyme without the tag revealed that the HIS-tag containing fusion protein is about 2-fold more active than native T4 lysozyme without the tag (Table 1).

Concluding, the HIS-tag bearing the T4 lysozyme fusion protein was considered to be a suitable substitute for native T4 lysozyme. Therefore, all further experiments have been carried out with this readily accessible fusion protein.

3.2. The chitinase function of T4 lysozyme

Analysis for the presence of a chitinase function in T4 lysozyme using a colorimetric assay [11] revealed activity only at an acidic pH, whereas at neutral pH, no activity was measurable. A clear dependence of the enzyme activity on the concentration was observed (Fig. 1). The chitinase activity

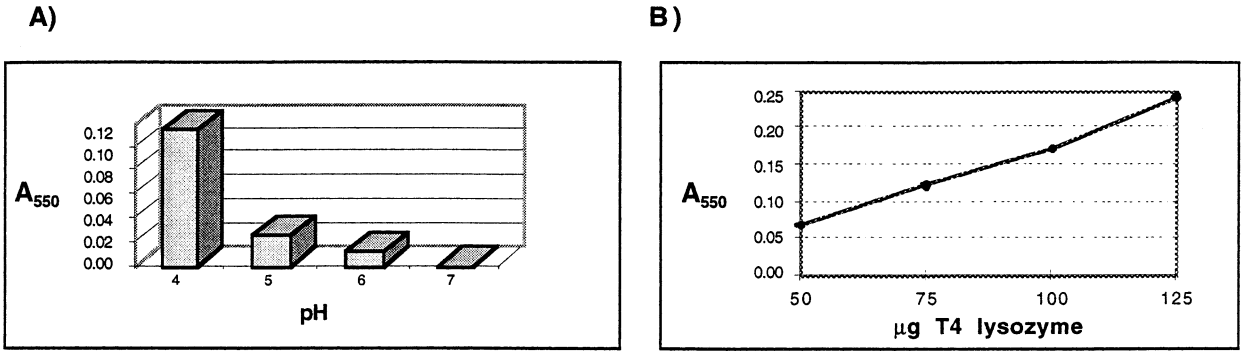

Fig. 1. Determination of the chitinase activity of T4 lysozyme using a dye labelling technique as described by Wirth and Wolf [11]. (A) pH-dependence and (B) concentration-dependence of the chitinase activity.

detected for T4 lysozyme at an acidic pH does not explain its activity towards non-chitin containing fungi such as *P. nicotianae*, as described below, and is probably not an important factor.

3.3. T4 lysozyme mutant M6K

For detailed antimicrobial activity studies, a mutant T4 lysozyme (M6K) with an amino acid exchange at position six from the hydrophobic methionine to the positively charged lysine was included. The gene encoding this mutant is part of an expression construct used for plant transformation. Especially, transgenic potato plants bearing this foreign gene have been intensely analyzed for resistance to a bacterial pathogen [9] and to fungal pathogens (Brinkmann, Düring, Gieffers, unpublished results). Derived from the results of these studies, we anticipated that besides the muramidase function there must be another molecular mechanism present in T4 lysozyme resulting in antimicrobial effects. For overproduction of this T4 lysozyme mutant as a HIS-tag fusion protein, we constructed an overproducer cassette analogous to the one for native T4 lysozyme (pSR 8-23) which was called pSR 8-59. In the following, the native fusion protein is referred to as 'T4lys' and the mutant fusion protein as 'T4lys(M6K)'.

3.4. Antimicrobial activity of T4 lysozyme

In a first instance, T4lys, heat-denatured T4lys and T4lys(M6K) were compared in the photometric muramidase assay and in the viable count plating assay after 1 h incubation of the bacterial suspension with T4 lysozyme. The former measures the enzymatic acitivity and the latter the bactericidal activity of a protein. Antimicrobial activity of T4 lysozyme was found to be independent of the enzymatic activity since heat-denatured T4lys is enzymatically inactive but retains its bactericidal activity. Furthermore, T4lys(M6K) showed no significant difference in enzymatic activity but an about 4-fold enhanced bactericidal activity (Fig. 2A). BSA was included as a negative control and persistence of the enzymatic inactivity due to heat-induced unfolding over the 1 h incubation time period has been assured (data not shown). Similar results were obtained using *M. lysodeikticus* as a Gram-positive test bacterium for both assays (data not shown). Consequently, the bactericidal function of T4 lysozyme must be caused by a different mechanism of action than murein cleavage.

Analogously, an assay system for measurement of the antifungal activity has been developed using a non-chitin containing plant pathogenic fungus, *P. nicotianae*, which can be sufficiently well handled in the laboratory. The length of growing fungal germination tubes was measured after a 20 h incubation period of the zoospores with T4 lysozyme. T4lys shows a clear and significant fungistatic activity. Again, heat-denatured T4lys fully retains this activity and, here, T4lys(M6K) is slightly more active (Fig. 2B). Similar results have been obtained with *F. oxysporum*, another plant pathogenic but chitin containing fungus (Fig. 2B). Concluding, the bactericidal as well as the fungistatic activity must be independent of the muramidase and chitinase activities.

3.5. Identification of amphipathic peptide stretches in T4 and hen egg white lysozyme

Using a 3D and protein sequence computer analysis, the α-helices in the C-terminal region of T4 lysozyme were identified

Table 1
Comparison of enzymatic and bactericidal activities of different recombinant lysozymes overproduced in *E. coli*

	Photometric lysis assay with *M. lysodeikticus*	Relative survival of *E. coli* MC1022 cells in 0.1×PBS	
	ΔA_{540} with 5 µg protein/ml	0.1 µg protein/µl	0.01 µg protein/µl
T4lys (pSR 8-23)	0.0278	0.14	n.d.
T4lys (M6K) (pSR 8-59)	0.0277	0	0.36
T4lys TagZyme (pSR 8-61)			
+ *tag*	0.0129	0.25	n.d.
− *tag*	0.0127	0.42	n.d.

The photometric lysis assay was performed as described [26]. The relative survival was assessed as described by viable count plating. n.d.: not determined.

in the 2LZM entry of the Brookhaven Protein Databank: amino acids 115–123 (termed α1), 126–134 (α2), 137–141 (α3) and 143–155 (α4). α2, α3 and α4 of T4 lysozyme possess amphipathic characteristics. The helical net representation predicts a clustering of the basic amino acids lysine and arginine at one side and the hydrophobic amino acids at the other side of α4. In general, a similar but less strict situation predicts for α2+α3 (data not shown). Peptide α4 forms a perfect positively-charged amphipathic α-helix containing several basic amino acid residues (Fig. 3) which allow interactions with the negatively-charged bacterial membrane components. Overall, the C-terminal part of T4 lysozyme from amino acid 115 on is structurally linked to the core domain by a hinge region which might confer sufficient flexibility to mediate membrane insertion of T4 lysozyme when coming into contact with a hydrophobic environment.

Two synthetic peptides, A23 (amino acids 126–141) and A4 (amino acids 143–155) were analyzed for enzymatic, antibacterial and antifungal activity. No enzymatic activity could be detected for these peptides as expected. Peptide A4 displayed a strong bactericidal and fungistatic activity whereas peptide A23 was only active towards fungi (Fig. 2). This means that the antimicrobial activity of T4 lysozyme resides in the C-terminal amphipathic region rather than in the muramidase function.

In parallel to this, we identified a β-sheet region of HEWL which also displays amphipathic characteristics (amino acids 98–112).

3.6. Membrane disturbing activity of T4lys and HEWL

Both lysozymes as well as peptide A4 have been used in LIVE/DEAD staining assays using SYTO 9 and propidium iodide for a direct proof of membrane disturbing activity. The green fluorescent nucleic acid stain SYTO 9 labels all bacteria. In contrast, the red fluorescent nucleic acid stain propidium iodide penetrates only bacteria with damaged membranes. Thus, bacteria with intact cell membranes stain fluorescent green, bacteria with compromized membranes stain fluorescent red. *E. coli* cells were treated with T4lys and HEWL. Whereas bacteria stained green and were highly mobile, 120 min after addition of PBS in the control experiment (Fig. 4A, left), HEWL-treated bacteria did not move and showed red

A) Bactericidal activity

	T4lys in 0.1xPBS	T4 lys mutant (M6K) in 0.1xPBS	peptide A4 in 0.1xPBS	heat-denatured T4lys (10´ 80°C) in 0.1xPBS/ 1% DMSO	T4lys in 0.1xPBS/ 1% DMSO	peptide A23 in 0.1xPBS/ 0.8% DMSO/ 0.006% Triton X-100	untreated controls in resp. buffers
concentration	0.1 μg/μl	0.01 μg/μl	0.1 μg/μl	0.1 μg/μl	0.1 μg/μl	0.1 μg/μl	0
relative survival of *E.coli* cells (mean value)	0.15	0.42	0.06	0.32	0.16	1.0	1.0
enzymatic activity	yes	yes	no	no	yes	no	/

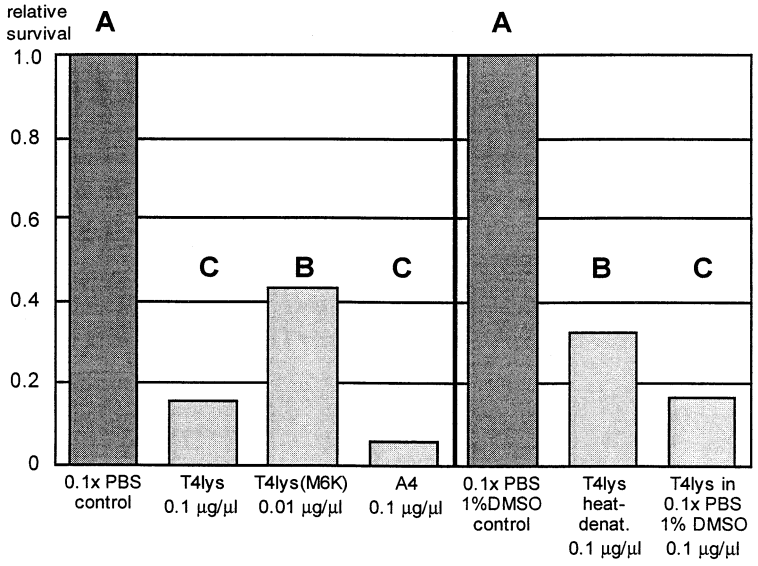

Fig. 2.

B) Fungistatic activity

	T4lys in buffer I	T4 lys mutant (M6K) in buffer I	peptide A4 in buffer I	heat-denatured T4lys (10´ 80°C) in buffer II	peptide A23 in buffer III	untreated controls in resp. buffers
concentration	0.5 µg/µl	0.5 µg/µl	0.5 µg/µl	0.5µg/µl	0.5 µg/µl	0
relative length of *P.nicotianae* germination tubes (mean value)	0.69	0.57	0.68	0.71	0.60	1.0
relative length of *F.oxysporum* germination tubes (mean value)	0.77	0.72	0.59	0.65	0.79	1.0
enzymatic activity	yes	yes	no	no	no	/

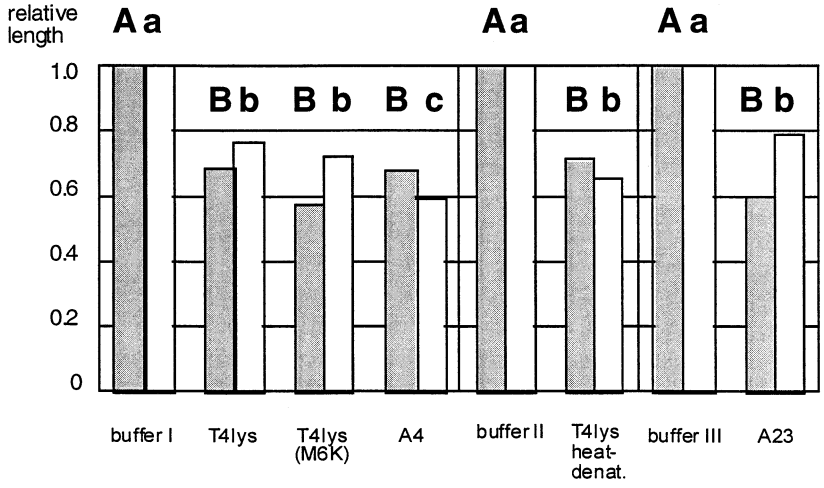

Fig. 2. The antimicrobial activity of T4 lysozyme and derived peptides. (A) The bactericidal activity measured as viable count plating of *E. coli* cells after 1 h incubation relative to untreated cells and (B) the fungistatic activity measured as the length of germination tubes of *P. nicotianae* zoospores after 20 h incubation and *F. oxysporum* conidia after 9 h incubation with T4 lysozyme or derived peptides relative to untreated zoospores. All experiments were evaluated relative to appropriate buffer controls. The enzymatic activity was determined using the standard photometric *M. lysodeikticus* turbidimetry assay [26]. Buffer I: 15 mM K_2HPO_4/20 mM NaCl/0.05×PBS; buffer II: 15 mM K_2HPO_4/20 mM NaCl/0.025×PBS/2.4% DMSO; buffer III: 15 mM K_2HPO_4/20 mM NaCl/0.025×PBS/1.9% DMSO/0.014% Triton X-100. Heat-denatured T4 lysozyme cannot be redissolved at 100% which leads to an experimental error underestimating its antimicrobial activity. Graphics: statistical evaluation of the significance. Columns with the same letters are not significantly different ($P = 0.05$) (B): upper case letters: *P. nicotianae*, lower case letters: *F. oxysporum*

staining of impaired bacterial cells (Fig. 4A, right). Treatment with T4lys resulted in agglomeration and red staining of all bacteria cells (Fig. 4A, center).

Similar results were obtained when conidia of different phytopathogenic fungi were tested in a comparable assay. T4 lysozyme as well as HEWL were assayed using the green fluorescent stain Sytox that is unable to penetrate intact cell membranes. Conidia displayed green staining after 3 h of lysozyme treatment (Fig. 4B, left). In addition, non-treated conidia started swelling and germinating whereas lysozyme-treated conidia did not swell and germinate (Fig. 4B, right).

Experiments incubating potato protoplasts with T4 lysozyme, peptide A4 and HEWL revealed a membrane disturbing activity also for plant cell membranes when compared to appropriate controls (Fig. 4C). On the other hand, in hemolysis assays, no activity of both lysozymes and peptide A4 on mammalian cells could be detected (Fig. 4D).

4. Discussion

The most likely mechanism for microbicidal activity of T4 lysozyme is membrane disruption as observed for several other antimicrobial peptides [15,16] rather than enzymatic degradation of the bacterial murein layer. Our results indicate that this mechanism seems to be much more widespread than thought before. The 2-fold enhanced bactericidal activity of the HIS-tag T4 lysozyme further strengthens this idea as well as the 4-fold enhanced activity of the mutant T4lys(M6K). A similar mutant T4lys(M6L) has been recently described by Lipscomb et al. [17] to be structurally significantly destabi-

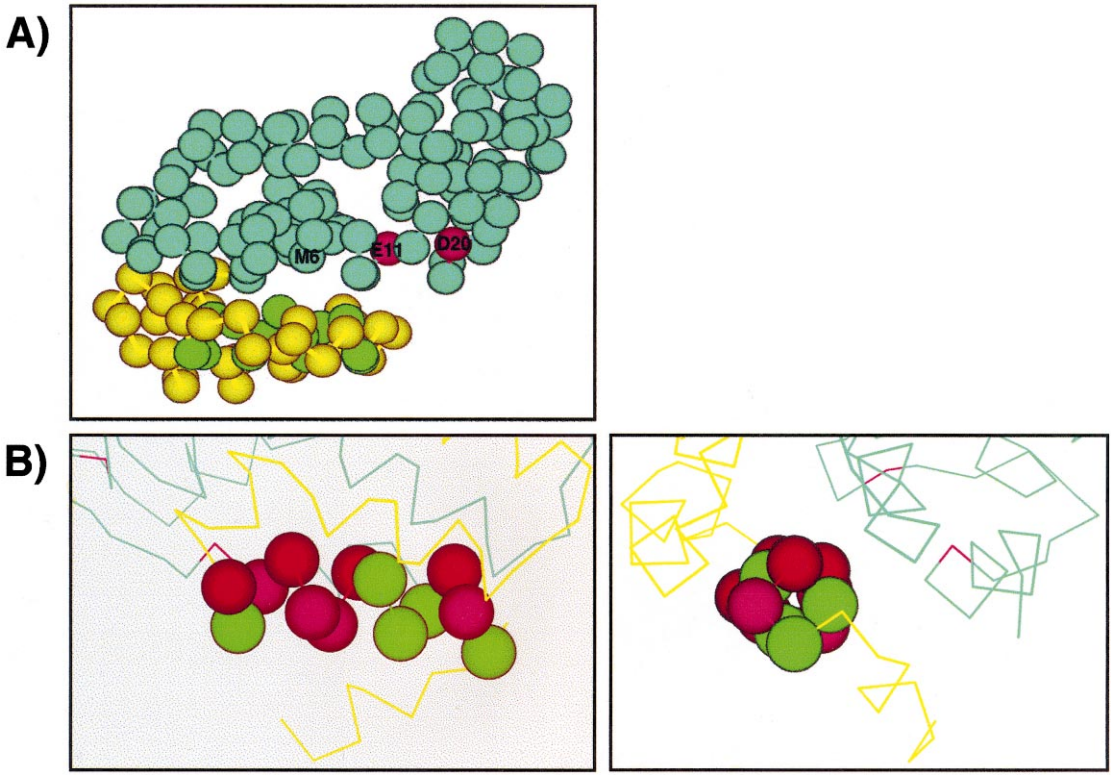

Fig. 3. 3D display of T4 lysozyme using the MacImdad 3D display programme (Molecular Applications Group, USA, V.5.0.1). (A) Overview demonstrating the location of the α4-helix. Cyan: main body; yellow (including green balls): C-terminus from amino acid 115 on; green: α4-helix (amino acids 143–155); magenta: the catalytically active amino acids 11 (Glu) and 20 (Asp). (B) The α4-helix (balls) in two rectangular magnified views. Red: hydrophobic amino acids (Pro-143, Ala-146, Val-149, Ile-150, Phe-153); green and magenta: hydrophilic amino acids; magenta: positively-charged basic amino acids (Arg-145, Lys-147, Arg-148, Arg-154).

lized. This can be assumed with a high probability also for the mutant T4lys(M6K) which we have analyzed here. Furthermore, T4lys(M6K) is significantly degraded in total protein extracts from potato tubers whereas native T4 lysozyme is remarkably stable under these conditions (Mahn and Düring, unpublished results) which suggests structural impairment. This can be explained by an opening of the active site cleft leaving the C-terminus more flexible which might allow a more efficient membrane interaction in the mutant.

Differences in the composition of bacterial and fungal cell membranes seem to be distinguished by the individual antimicrobial activities of the T4 lysozyme peptides A4 and A23 identified here. This assumption is strongly reinforced by the results of the fluorescence staining assays which clearly demonstrate a membrane perturbing activity of both T4 and HEWL, not only towards bacterial and fungal cells but also potato protoplasts. Obviously, mammalian cells are not destroyed by lysozyme amphipathic peptides.

Pellegrini et al. [18] published the detection of a bactericidal 15 amino acid peptide, generated by protease cleavage of HEWL, which is identical with the peptide sequence we already identified before. Recently, the same group demonstrated by electron microscopy that HEWL causes disintegration of the bacterial cytoplasm and disruption of the inner and outer membrane [19]. These effects can now be easily explained by the newly discovered antimicrobial mechanism of lysozymes described here. Summarising, these observations corroborate our findings.

The new results allow the development of optimized antimicrobial polypeptides based on T4 lysozyme with an enhanced activity and engineered stability for expression in transgenic plants or use as food, feed and pharmaceutical preservatives. Putatively, they can also be applied to a specific cancer cell growth inhibition, as recently this expected effect has been proved for the amphipathic lactoferricin peptide [20].

Hen eggs which are a common food contain high levels of HEWL. Concluding, exploitation of the membrane active mechanism for improvement of active agents based on T4 or HEWL should not pose any risk in terms of the biological safety, especially as no influence on mammalian cells could be revealed in hemolysis assays. HEWL becomes even more active after heat denaturion which is the commonly used structural status in food use and possesses a comparable membrane activity with eukaryotic cells as T4 lysozyme. Moreover, lactoferrin which is found in most exocrine secretions of mammals releases upon gastric cleavage by pepsin at an acidic pH an antimicrobially active peptide, lactoferricin [21]. A short peptide sequence of lactoferricins has recently been identified to mediate their antimicrobial activity [16,22,23]. The respective region of lactoferricin forms an amphipathic α-helix containing several basic amino acid residues. This allows interaction with the negatively charged membrane elements, i.e. lipopolysaccharide in Gram-negative and lipoteichoic acid in Gram-positive bacteria [24,25]. A similar situation can be expected for lysozymes as well.

Concluding, the detection of membrane activity due to the presence of amphipathic peptides in T4 and HEWL opens new ways to engineering those proteins as even more efficient

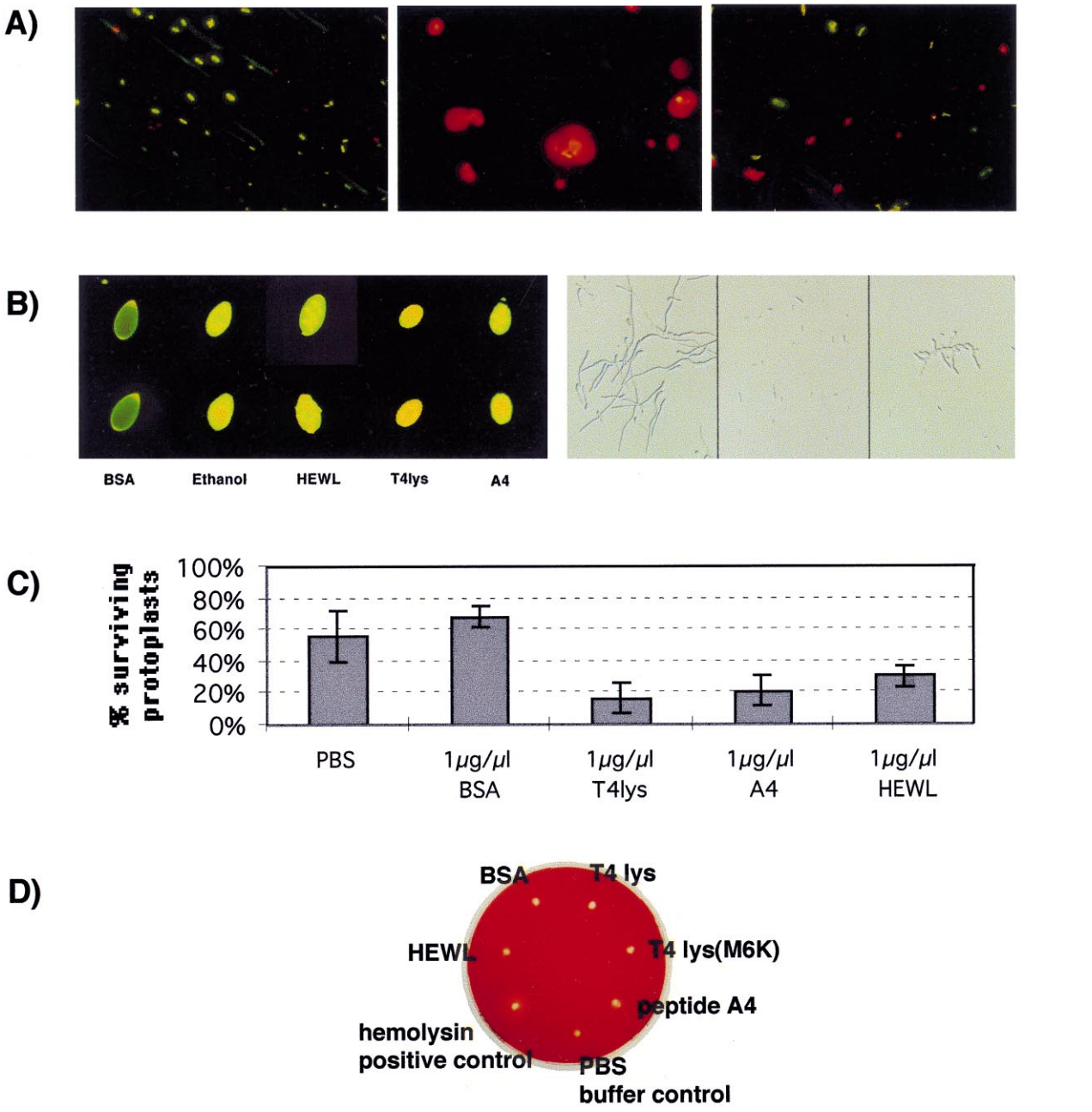

Fig. 4. Membrane disturbing activity of lysozymes. (A) On bacterial cells, left: PBS control, center: T4lys, right: HEWL; LIVE/DEAD staining of *E. coli* cells 120 min p.i. The final concentration of HEWL and T4lys was 1 mg/ml. Bacterial cells with damaged membranes fluoresce red because of staining with the membrane impermeable red fluorescent nucleic acid stain propidium iodide. Bacteria with intact membranes are stained with the membrane permeable green fluorescent nucleic acid stain SYTO 9. Since the photographic exposure time was 4 s, moving bacteria appear as stripes. (B) On sporangia of *P. infestans* (left) and on conidia of *F. oxysporum* (right): sporangia treated with BSA (control), 70% ethanol, HEWL, T4lys and peptide A4, respectively (left to right). Sytox-stained sporangia with damaged membranes display a bright fluorescence. *F. oxysporum*: germination after 24 h incubation with BSA (control, left), conidia with long germination tubes; T4lys or HEWL (center), no germination or peptide A4 (right), low germination rate and a different structure of hyphe. (C) On potato protoplasts. Percentage of protoplasts with an intact cell membrane after 20 min incubation with 1 mg/ml BSA, T4lys, peptide A4, HEWL and the equivalent volume of PBS. Columns represent the mean values of eight (PBS), two (BSA), seven (T4lys), two (A4) and three (HEWL) repetitions. (D) Control experiments for the putative activity on mammalian cells were conducted with the hemolysis assay.

antimicrobial agents by balancing the enhanced antimicrobial activity with sufficient and directed structural stability.

Acknowledgements: We thank Jacqueline Daehn and Ina Hempel for the excellent technical assistance and Inge Broer and Reinhard Töpfer for valuable comments on the manuscript.

References

[1] Tsugita, A. (1971) in: The Enzymes (Boyer, P.D., Ed.), Vol. V, pp. 344–411, Academic Press, New York, USA.
[2] Matthews, B.W. (1995) in: Adv. Protein Chemistry (Eisenberg, D.S. and Richards, F.M., Eds.), Vol. 46, pp. 249–278, Academic Press, San Diego, CA, USA.

[3] Renell, D., Bouvier, S.E., Hardy, L.W. and Poteete, A.R. (1991) J. Mol. Biol. 222, 67–87.
[4] Pellegrini, A., Thomas, U., von Fellenberg, R. and Wild, P. (1992) J. Appl. Bacteriol. 72, 180–187.
[5] Ibrahim, H.R., Higashiguchi, S., Juneja, L.R., Kim, M. and Yamamoto, T. (1996) J. Agric. Food Chem. 44, 1416–1423.
[6] Ibrahim, H.R., Higashiguchi, S., Koketsu, M., Juneja, L.R., Kim, M., Yamamoto, T., Sugimoto, Y. and Aoki, T. (1996) J. Agric. Food Chem. 44, 3799–3806.
[7] Ibrahim, H.R., Higashiguchi, S., Sugimoto, Y. and Aoki, T. (1997) J. Agric. Food Chem. 45, 89–94.
[8] Ibrahim, H.R., Yamada, M., Matsushita, K., Kobayashi, R. and Kato, A. (1994) J. Biol. Chem. 269, 5059–5063.
[9] Düring, K., Porsch, P., Fladung, M. and Lörz, H. (1993) Plant J. 3, 587–598.
[10] Düring, K. (1993) Protein Exp. Pur. 4, 412–416.
[11] Wirth, S.J. and Wolf, G.A. (1990) J. Microbiol. Methods 12, 197–205.
[12] Gooding, G.V. and Lucas, G.B. (1959) Phytopathology 49, 277–281.
[13] Woloshuk, C.P., Meulenhoff, J.S., Sela-Burlaage, M., van den Elzen, P.J.M. and Cornelissen, B.J.C. (1991) Plant Cell 3, 619–628.
[14] Reed, J.D., Edwards, D.L. and Gonzalez, C.F. (1997) Mol. Plant Microb. Int. 10, 537–549.
[15] Boman, H.G., Marsh, J. and Goode, J.A. (1994) in: Ciba Foundation Symposium, Vol. 186, John Wiley and Sons, Chichester.
[16] Odell, E.W., Sarra, R., Foxworthy, M., Chapple, D.S. and Evans, R.W. (1996) FEBS Lett. 382, 175–178.
[17] Lipscomb, L.A., Gassner, N.C., Snow, S.D., Eldridge, A.M., Baase, W.A., Drew, D.L. and Matthews, B.W. (1998) Protein Sci. 7, 765–773.
[18] Pellegrini, A., Thomas, U., Bramaz, N., Klauser, S., Hunziker, P. and von Fellenberg, R. (1997) J. Appl. Microbiol. 82, 372–378.
[19] Wild, P., Gabrieli, A., Schraner, E.M., Pellegrini, A., Thomas, U., Frederik, P.M., Stuart, M.C.A. and von Fellenberg, R. (1997) Microsc. Res. Tech. 39, 297–304.
[20] Yoo, Y.C., Watanabe, S., Watanabe, R., Hata, K., Shimazaki, K. and Azuma, I. (1997) Jpn. J. Cancer Res. 88, 184–190.
[21] Tomita, M., Takase, M., Bellamy, W. and Shimamura, S. (1994) Acta Paediatr. Jpn. 36, 585–591.
[22] Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K. and Tomita, M. (1992) Biochim. Biophys. Acta 1121, 130–136.
[23] Bellamy, W., Takase, M., Wakabayashi, H., Kawase, K. and Tomita, M. (1992) J. Appl. Bacteriol. 73, 472–479.
[24] Elass-Rochard, E., Roseanu, A., Legrand, D., Trif, M., Salmon, V., Motas, C., Montreuil, J. and Spik, G. (1995) Biochem. J. 312, 839–845.
[25] Hoek, K.S., Milne, J.M., Grieve, P.A., Dionysius, D.A. and Smith, R. (1997) Antimicrob. Agents Chemother. 41, 54–59.
[26] Audy, P., Trudel, J. and Asselin, P. (1988) Plant Sci. 58, 43–50.