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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Listeria bacteriophage peptidoglycan hydrolases feature high thermoresistance and reveal increased activity after divalent metal cation substitution

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Abstract The ability of the bacteriophage-encoded peptidoglycan hydrolases (endolysins) to destroy Gram-positive bacteria from without makes these enzymes promising antimicrobials. Recombinant endolysins from *Listeria monocytogenes* phages have been shown to rapidly lyse and kill the pathogen in all environments. To determine optimum conditions regarding application of recombinant *Listeria* phage endolysins in food or production equipments, properties of different *Listeria* endolysins were studied. Optimum NaCl concentration for the amidase HPL511 was 200 nM and 300 mM for the peptidases HPL118, HPL500, and HPLP35. Unlike most other peptidoglycan hydrolases, all four enzymes exhibited highest activity at elevated pH values at around pH 8–9. Lytic activity was abolished by EDTA and could be restored by supplementation with various divalent metal cations, indicating their role in catalytic function. While substitution of the native Zn²⁺ by Ca²⁺ or Mn²⁺ was most effective in case of HPL118, HPL500, and HPLP35, supplementation with Co²⁺ and Mn²⁺ resulted in an approximately 5-fold increase in HPL511 activity. Interestingly, the glutamate peptidases feature a conserved SxHxxGxAxD zinc-binding motif, which is not present in the amidases, although they also require centrally located divalent metals for activity.

The endolysins HPL118, HPL511, and HPLP35 revealed a surprisingly high thermostability, with up to 35% activity remaining after 30 min incubation at 90°C. The available data suggest that denaturation at elevated temperatures is reversible and may be followed by rapid refolding into a functional state.

Keywords Peptidoglycan hydrolase · Lytic enzyme · *Listeria* · Bacteriophage · Food safety

Introduction

Endolysins are bacteriophage-encoded murein hydrolases, which are produced inside an infected bacterial host cell at the end of the phage lytic multiplication cycle. Endolysin-mediated cell wall degradation results in disruption of the cell wall and release of the phage progeny. From within the bacterial cytoplasm, the enzymes gain access to the peptidoglycan with the aid of holins, hydrophobic proteins that oligomerize and form pores in the membrane, allowing the endolysins to pass through (reviewed in Young et al. 2000; Bernhardt et al. 2002). Based on the bonds targeted within the peptidoglycan network, endolysins can be classified into different categories (reviewed in Loessner 2005): (1) glucosaminidases, (2) muramidases, also known as “lysozymes,” (3) transglycosylases, which all cleave within the aminosugar backbone, (4) *N*-acetylmuramoyl-L-alanine amidases, and (5) endopeptidases, both of which hydrolyze bonds in the crosslinking peptide stems. Due to the absence of an outer membrane in Gram-positive bacteria, endolysins can also lyse these cells from without and are receiving increasing attention as alternative antimicrobials with potential applications in food, biotechnol-

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ogy, and medicine (Hagens and Loessner 2007; Fischetti 2005; Loessner et al. 1995a; Loessner 2005; Hermoso et al. 2007; Fischetti 2010).

Listeria monocytogenes is an invasive and intracellular pathogen, transmitted to humans via contaminated food. Among risk groups such as neonates, elderly, pregnant women, and immunocompromised individuals, it can cause severe infections with potentially fatal outcome (Vazquez-Boland et al. 2001). Although the incidence of the disease known as listeriosis is relatively low compared to other food-borne pathogens, mortality rates of more than 30% make *Listeria* an important concern in the food industry.

Listeria cell wall peptidoglycan belongs to the A1 γ type, featuring directly crosslinked peptidoglycan via meso-diaminopimelic acid (Schleifer and Kandler 1972). Cell wall-associated carbohydrates, in particular teichoic acids, are responsible for most of the antigenic variability within the genus, with five major serovar groups being distinguished (Fiedler et al. 1984; Fiedler and Ruhland 1987).

Endolysins from *Listeria* phages have been shown to be highly active against these bacteria from without (Loessner et al. 1995a,b, 1996). Two different enzymatic specificities of *Listeria* phage endolysins have been reported: *N*-acetylmuramoyl-L-alanine amidases and L-alanoyl-D-glutamate peptidases. The latter was first identified in Ply118 and Ply500 (Loessner et al. 1995b) and has recently also been reported for a *Bacillus subtilis* enzyme (Fukushima et al. 2007) and *Escherichia coli* phage T5 endolysin (Mikoulińska et al. 2009). Like most known endolysins, the *Listeria* phage enzymes show a modular organization: an N-terminal enzymatically active domain (EAD) is linked to a C-terminal cell wall binding domain (CBD), which directs the enzyme to a cell wall-associated ligand (Korndoerfer et al. 2006; Loessner et al. 2002; Schmelcher et al. 2010). The CBD domains of the peptidases Ply118 and Ply500 feature complementary binding properties, where CBD118 binds to *Listeria* cells of serovars 1/2 and 3, and CBD500 recognizes those of serovars 4–6 (Loessner et al. 2002). However, Ply118 was shown to also be able to lyse cells outside the CBD recognition pattern (Schmelcher et al. 2011). Ply511 is an *N*-acetylmuramoyl-L-alanine amidase from the virulent broad host range phage A511, recognizing and lysing strains from all serovars (Loessner et al. 1995b; Schmelcher et al. 2010). The PlyP35 peptidase endolysin of phage P35 (Dorscht et al. 2009) and its CBD were shown to recognize a majority of serovar 1/2 and 3 strains and some serovar 4–6 strains (Schmelcher et al. 2010, 2011). PlyPSA and Ply500 contain Zn²⁺ as an integral component of the catalytic domain (Korndoerfer et al. 2006; 2008).

Listeria phage endolysins are useful reagents in molecular biology (Loessner et al. 1996), and their CBDs can be used for development of novel analytical procedures

(Loessner et al. 2002; Hagens and Loessner 2007; Kretzer et al. 2007; Schmelcher et al. 2010). They also have potential as antimicrobials in food and have been cloned and modified for secretion in lactic acid bacteria (Gaeng et al. 2000; Turner et al. 2006). To further develop the various endolysin-based applications, better knowledge of their properties is desirable. Therefore, this study aimed to biochemically characterize Ply118, Ply500, Ply511, and the novel enzyme PlyP35 and study the effects of ionic strength, pH, divalent metal cations, and various temperatures on lytic activity and enzyme stability.

Materials and methods

Bacteria, culture conditions, plasmids, and phages

E. coli strain JM109 (NEB) was used for cloning of the *plyP35* gene and production of the His-tagged recombinant endolysin HPLP35. Strains JM109 pHPL118, JM109 pHPL500, and JM109 pHPL511 (Loessner et al. 1996) served for overexpression of genes encoding HPL118, HPL500, and HPL511, respectively. *E. coli* was cultivated in Luria–Bertani (LB) medium at 37°C, supplemented with 100 μ g/ml ampicillin as needed. *L. monocytogenes* strains WSLC 1001 (serovar 1/2 c) and WSLC 1042 (serovar 4 b) were grown in tryptose broth at 30°C. Phage P35 DNA (Dorscht et al. 2009) served as template for PCR amplification of the *plyP35* gene. Plasmid pQE-30 (Qiagen) was used for construction of pHPLP35.

Cloning of *plyP35*

The *plyP35* gene was amplified by PCR with High Fidelity Taq Polymerase (Roche), using primers *plyP35_BamHI_F* (5'-ATCAGGATCCTTATTTCTTGATGTCAAACATGTAACG-3') and *plyP35_SalI_R* (5'-ATCAGTCGACTTATTTCTTGATGTCAAACATGTAACG-3'), thereby introducing TAA as stop codon and adding *Bam*HI and *Sal*I restriction sites (underlined) to the 5' and 3' ends of the products, respectively. After endonuclease digestion, the DNA fragment was inserted into *Bam*HI/*Sal*I sites of pQE-30, the resulting plasmid pHPLP35 introduced into *E. coli* JM109 and the construct verified by nucleotide sequencing (GATC, Konstanz, Germany).

In silico analyses

The software AlignX (Vector NTI; Invitrogen) was used for creating multiple amino acid sequences alignments, employing the ClustalW algorithm (Thompson et al. 1994).

Production and purification of endolysins

This was performed essentially as previously described (Loessner et al. 1996; Schmelcher et al. 2011). In brief, *E. coli* strains harboring the desired constructs were grown in modified LB medium (15 g/l tryptose, 8 g/l yeast extract, and 5 g/l NaCl) with ampicillin, to an OD_{600nm} of 0.5, when gene expression and protein synthesis were induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside. After 4 h incubation at 35°C with shaking, cells were harvested by centrifugation, resuspended in 20 ml buffer A (50 mM Na₂HPO₄, 500 mM NaCl, 5 mM imidazole, 0.1 % Tween 20, pH 8.0) per 1 l of culture, and cells disrupted by two passages through a French Press 20K cell (SLM Aminco) at 100 MPa. Debris was removed by centrifugation and the supernatant filtered (0.2 μM PES membrane, Millipore). His-tagged proteins were purified from the supernatant by affinity chromatography, using Ni-NTA Superflow resin (Qiagen). Immobilized protein was eluted using buffer B (50 mM Na₂HPO₄, 500 mM NaCl, 250 mM imidazole, 0.1% Tween 20, pH 8.0), dialyzed (50 mM NaH₂PO₄, 100 mM NaCl, 0.005% to 0.1% Tween 20, pH 8.0), filtered (0.2 μM PES membrane, Millipore), and stored at –20°C in 50% (v/v) glycerol. Purity and concentration of the purified enzyme samples was analyzed by gel electrophoresis and spectrophotometry (NanoDrop ND-1000).

Preparation of *Listeria* cells and purified cell walls as assay substrate

L. monocytogenes strains WSLC 1001 and WSLC 1042 were grown to an OD_{600nm} of approximately 0.8, harvested by centrifugation, washed, and resuspended in deionized water at approximately 50-fold concentration. Aliquots were stored at –80°C until use.

Preparation of purified cell walls was carried out as described earlier (Wendlinger et al. 1996). *Listeria* cells in the late log phase were disrupted by use of a French press (40 K cell, SLM Aminco), and the remaining intact cells were removed by centrifugation at 1,400×g for 5 min. Raw cell walls were then harvested at 15,000×g for 30 min, washed with water, and resuspended in 7.0 ml SM buffer (Sambrook et al. 1989) per 1 g pellet. Nucleic acids were removed by incubation with 0.1 mg DNase and 0.1 mg RNase (Sigma) per gram cell walls for 4 h, and proteins were degraded by Proteinase K (Sigma) digestion (0.1 mg/g pellet for 3 h). The preparations were then boiled at 100°C in 4% (w/v) sodium dodecyl sulfate (SDS) for 30 min, washed with water at least three times, and resuspended in deionized water. The purified cell walls were stored in aliquots at –80°C until use.

Photometric lysis assay

Lytic activity of purified recombinant endolysins was determined by photometric lysis assays as described previously (Loessner et al. 1995a, 1996). The assays were carried out using polystyrene cuvettes (Greiner) in a total volume of 1 ml, in 10 mM Tris, 150 mM NaCl (300 mM in case of HPLP35), pH 8.0, at 30°C (the “standard conditions”). These conditions were modified for some experiments, as detailed below. Substrate was added to an initial OD_{600nm} of approximately 1.0 in case of intact *Listeria* cells and 0.2–0.3 with purified cell walls. Enzymes were added to the suspension at concentrations ranging from 2 to 5 μg/ml (approximately 50–150 nM) in a volume of 15 μl, and the OD_{600nm} was recorded for up to 10 min. As negative control, buffer was added. In tests using various pH values, these were measured directly in the cuvettes at the beginning and the end of each assay.

All experiments were carried out in triplicate, and data processing was performed as described before (Korndoerfer et al. 2006). Lysis data were normalized and corrected by subtraction of the corresponding control values [corrected value = normalized value + (1 – normalized control value)]. The resulting curves were fitted to the sigmoid function $f = y_0 + a / \{1 + \exp[-(x - x_0)/b]\}^c$, employing Sigma-Plot 9.0 (Systat Software, Inc.). The steepest slope of the function was used to calculate lytic activity. Activities of all four recombinant endolysins were determined under standard conditions, and 1 U was defined as the amount of enzyme that decreases the OD_{600nm} of *Listeria* cells in suspension by 0.01/min (Loessner et al. 1995a, 1996).

Biochemical characterization of endolysins

In order to determine the effect of pH on lytic activity, lysis assays were performed at different pH values between 3.8 and 10.1, with intact *Listeria* cells as substrate. Six different buffer systems (Good et al. 1966) at a concentration of 20 mM were used to cover the entire pH range: phosphate, citrate, MOPS, Tris, Tricine, and CHES (Sigma). The influence of salt concentration (i.e., ionic strength) on lytic activity was determined using various NaCl concentrations from 0 mM to 1.0 M. To assess the role of the divalent metal cations Mn²⁺, Mg²⁺, Ca²⁺, Cu²⁺, Co²⁺, Ni²⁺, and Zn²⁺ for enzyme activity, purified cell walls served as substrate. Both enzymes and purified cell walls were incubated with 250 mM EDTA for 30 min to sequester and remove any residual metal ions. Subsequently, enzymes were dialyzed against assay buffer, and cell walls were washed with deionized, highly purified water (Milli-Q, Millipore). Lysis of EDTA-treated cell walls by endolysins was monitored with and without addition of divalent metal ions at different

concentrations (1 and 10 mM). However, Zn^{2+} could not be examined at 10 mM, due to precipitation effects. Lytic activities were compared to those recorded without EDTA treatment and cation substitution.

In order to test temperature stability of enzymatic activity, aliquots of the endolysins were incubated at different temperatures (40°C, 50°C, 60°C, 70°C, 80°C, and 90°C) for 5, 10, and 30 min, followed by cooling on ice for 10 min. Lytic activity on intact cells under standard conditions was then determined and compared to controls using non-heat treated enzymes.

Results

Expression, purification, and lytic activity of recombinant *Listeria* phage endolysins

Listeria phage endolysins HPL118, HPL500, HPL511, and HPLP35 were produced in *E. coli* and purified by immobilized metal ion adsorption chromatography. Following dialysis and concentration, the proteins appeared as almost homogeneous bands (more than 90% pure) on SDS polyacrylamide gel electrophoresis gels (data not shown), with concentrations between 2.0 and 9.5 mg/ml (Table 1). A linear relationship between enzyme concentration and the steepest slope of the lysis curve was observed in photometric lysis assays using intact *Listeria* cells as substrate. The results for HPL500 are shown in Fig. 1, and lytic activities of all four enzymes used here are listed in Table 1.

Endolysins show similar ionic strength requirements

Lytic activities of all enzymes were determined at various NaCl concentrations up to 1,000 mM. Results were similar for peptidases HPL118 (Fig. 2a) and HPL 500 (Fig. 2b), which both showed optimum activity at 300 mM NaCl. The HPL511 amidase exhibited a slightly lower optimum (200–250 mM), while its activity decreased to roughly 70% at 300 mM salt (Fig. 2c). At lower ionic strength, however, HPL511 was more active than the peptidases, displaying 50% activity at 100 mM NaCl, compared to 25% (HPL118) and 12% (HPL500). The peptidase HPLP35 showed best activity at 300 mM NaCl, albeit with a narrower range

compared to HPL118 and HPL500. It retained 50% lysis activity at 200 and 400 mM, and <5% at 100 mM and below (Fig. 2d). None of the four enzymes caused visible cell lysis and concomitant reduction of turbidity at 1,000 mM NaCl.

Influence of pH and buffer systems on lytic activity

Endolysin activity was determined in a pH range of 3.8–10.1, and optima for all enzymes were found to be at pH 8.0 and higher (Fig. 3). HPL118 and HPL500 displayed best activity in a range from pH 9 to 10 (Fig. 3a, b), whereas HPL511 and HPLP35 were most active from pH 8 to 9 (Fig. 3b, c). At more acidic conditions, activity of all tested enzymes dropped significantly. At neutral pH, enzymes exhibited between 30% (HPL500) and 70% (HPL511) activity, which dropped below 10% at pH<5.5. This decrease was most abrupt in case of HPL511, dropping from 70% at pH 7.0 to <20% at pH 6.4. Figure 3 also illustrates the effect of the different buffer systems used on lytic activity. Although the enzymes showed the same trends regarding their pH optima in different buffers, comparison of specific data obtained at similar pH values in the various buffers revealed considerable differences. For instance, the activity of HPL118 and HPL500 at pH 6.6 in citrate buffer was higher than at pH 6.4 and 7.0 in MOPS buffer. For all assays, pH was shown to be stable throughout the duration of the measurements (data not shown).

Removal of divalent metals and complementation by heterologous ions

Determination of the EAD500 structure disclosed the presence of a Zn^{2+} ion in the catalytic center of Ply500 (Korndoerfer et al. 2008), which is in perfect agreement with the zinc binding motif $SxHxxGxAxD$ present in Ply118 (McCafferty et al. 1997). Figure 4 reveals the high conservation of this motif in sequence alignments of glutamate peptidases from different *Listeria* phages, including PlyP35 (Schmelcher et al. 2010). This suggested a general requirement of metal cations in the catalytic domain of these enzymes. In order to further investigate the role of divalent metals on lytic activity, both the enzymes and the

Table 1 *Listeria* bacteriophage endolysin preparations used

Enzyme	Concentration (mg/ml)	Purity (% of total protein)	Lytic activity (U/mg)	Substrate cells used for lysis assays
HPL118	3.3	>90	9,370	WSLC 1001
HPL500	3.8	>90	25,259	WSLC 1042
HPL511	9.5	>90	22,871	WSLC 1001
HPLP35	2.0	>90	2,016	WSLC 1001

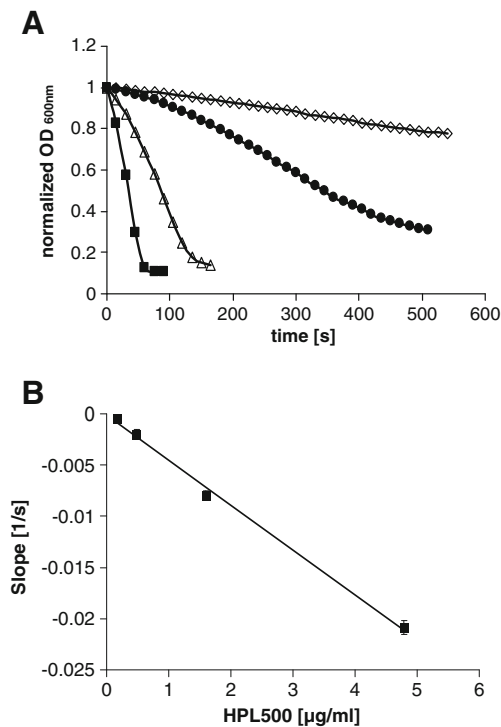


Fig. 1 Linear relationship of HPL500 concentration used in a photometric lysis assay and steepest slope of the cell lysis curve. **a** Decrease in OD_{600nm} of a suspension of WSLC 1042 cells after addition of 0.2 µg/ml (diamonds), 0.5 µg/ml (circles), 1.6 µg/ml (triangles), and 4.8 µg/ml (squares) of HPL500 (at $t=0$). All curves were normalized to an initial OD_{600nm} of 1.0, and the control (addition of buffer instead of enzyme) was subtracted. All assays were carried out in triplicate and curves represent mean values. **b** Steepest slopes of the lysis curves from **a** plotted against HPL500 concentration. The linear regression line has an R^2 of 0.9965. For all further lysis assays performed in this work, HPL500 was used at a concentration of 2.87 µg/ml

cell wall substrates were treated with 250 mM EDTA to sequester and remove residual divalent metals. This treatment drastically reduced or abolished activity of HPL118, HPL500, and HPL511, and HPLP35 (Table 2). We then used different divalent metal cations (Mn^{2+} , Mg^{2+} , Ca^{2+} , Cu^{2+} , Co^{2+} , Ni^{2+} , and Zn^{2+} , at 1–10 mM) to supplement the EDTA-treated enzymes. We found Mn^{2+} , Mg^{2+} , Ca^{2+} , and Co^{2+} to be most effective in reconstituting the endolysin peptidoglycan hydrolase activity. HPL118 regained full activity after addition of Ca^{2+} (10 mM) or Co^{2+} and up to 80% in the case of Mg^{2+} . Surprisingly, 1 mM Mn^{2+} caused a more than 2.4-fold boost in activity compared to the control. With HPL500, the addition of 10 mM Ca^{2+} resulted in close to 90% activity, while Mn^{2+} , Mg^{2+} , and Co^{2+} reconstituted activity to at least 50%. HPL511 regained its full activity with 10 mM Mg^{2+} or Ca^{2+} , and a more than 5-fold increase in activity compared to the non-EDTA treated control was observed following addition of 1 mM Mn^{2+} or Co^{2+} . For HPLP35, Ca^{2+} at a

concentration of 10 mM appeared to be most effective, whereas Mn^{2+} , Mg^{2+} , and Co^{2+} only partly reconstituted enzyme activity. Generally speaking, Ca^{2+} and Mg^{2+} were more effective when added at 10 mM, whereas Mn^{2+} and Co^{2+} enabled best enzyme function at 1 mM concentration. In contrast, substitution with Cu^{2+} , Ni^{2+} , and Zn^{2+} was less effective or even inhibitory for endolysin activity.

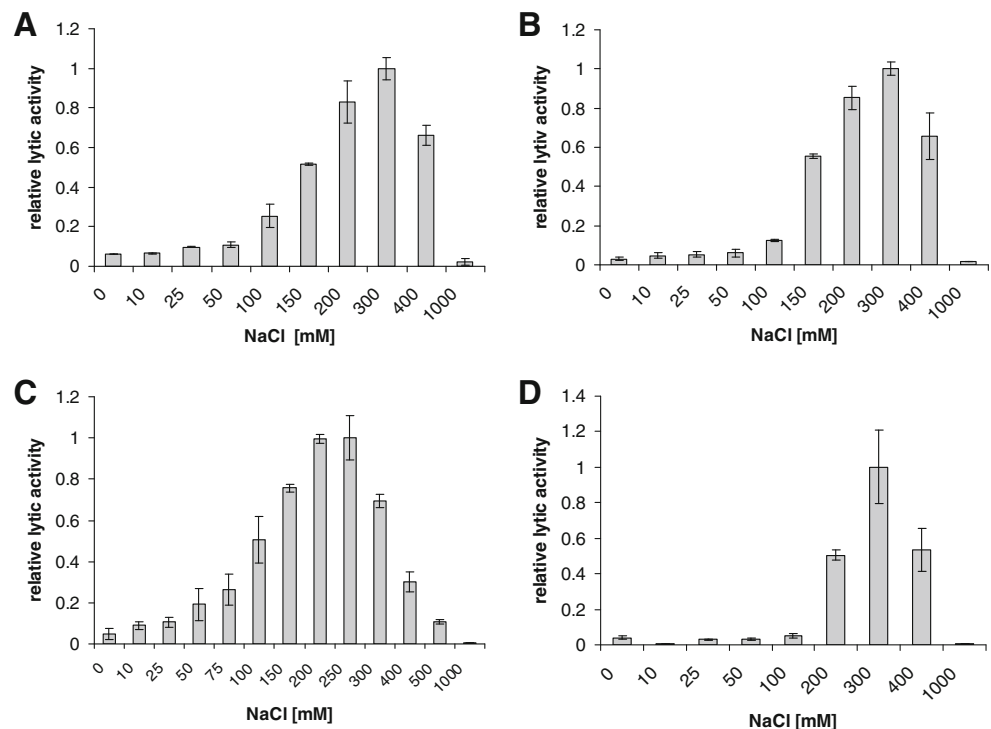
HPL118, HPL511, and HPLP35 exhibit high temperature resistance

Temperature stability (defined as remaining enzyme activity after a heating step, not as activity at elevated temperature) of the four endolysins was determined following incubating the enzymes at temperatures of up to 90°C for maximum 30 min, and monitoring lytic activity by the standard photometric assay using intact *Listeria* substrate cells. Surprisingly, HPL118, HPL511, and HPLP35 revealed high temperature stability. After heating to 50°C for up to 30 min, HPL118 retained more than 90% of its activity (Fig. 5a). From 60°C upwards and increasing treatment time, activity was gradually reduced. After 5 min at 90°C, the enzyme still showed 40% activity compared to the non-heat-treated control. Only after 30 min at 90°C, lytic activity dropped below 10% of the control. The HPL511 amidase exhibited even higher stability (Fig. 5c). Even though incubation at 50°C reduced activity by approximately 25%, this remained unchanged after incubation at up to 70°C. At higher temperatures and an incubation time of 30 min, its activity gradually decreased. However, the enzyme still retained more than 60% of its lytic activity following 5 min at 90°C. Similarly, HPLP35 showed a rapid decrease in leftover activity following treatment at 40°C (approximately 90%) and 50°C (35%), independent of the heating time (Fig. 5d). However, using 50°C and 90°C as incubation temperatures, the enzyme retained activity. Even after 30 min at 90°C, it was able to quickly lyse substrate cells. Only boiling at 100°C was able to completely inactivate HPLP35 (data not shown). In contrast, HPL500 revealed only weak heat stability (Fig. 5b). While at 40°C lytic activity was not affected, treatment at 50°C reduced activity to approximately 60%, and temperatures of 60°C or higher completely inactivated the enzyme.

Discussion

We report the effects of different factors on lytic activity of four different *Listeria* bacteriophage peptidoglycan hydrolases, including three L-alanoyl-D-glutamate peptidases (HPL118, HPL500, and HPLP35) and one N-acetylmuramoyl-L-alanine amidase (HPL511). Lytic activities of the

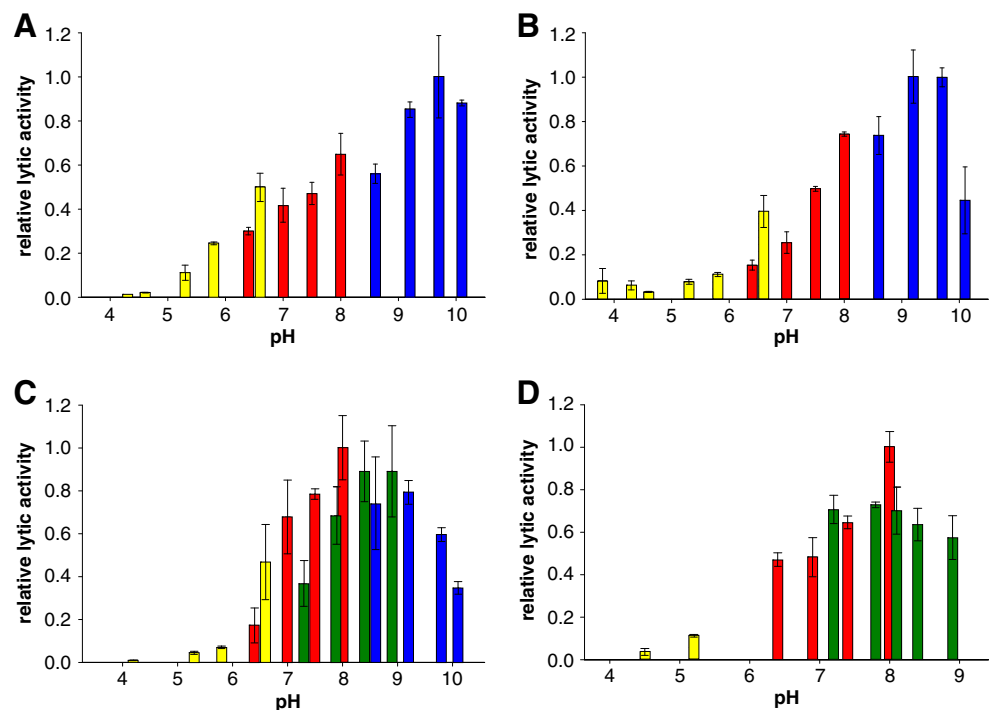
Fig. 2 Influence of NaCl concentration on lytic activity of HPL118 (a), HPL500 (b), HPL511 (c), and HPLP35 (d) against *Listeria* cells in suspension



many different phage-encoded and bacterial cell wall lytic enzymes investigated to date range from 10^2 to 10^8 “units” per milligram (Vasala et al. 1995; Loessner et al. 1995a, 2001; Nelson et al. 2001; Shida et al. 2001; Loeffler et al. 2003; Yoong et al. 2004; Fukushima et al. 2007; Kyomuhendo et al. 2007). However, different assay types used and arbitrary unit definition makes comparison

difficult or impossible. Under the conditions employed here, concentrations as small as 2–5 $\mu\text{g}/\text{ml}$ (approximately 100 nmol) of purified endolysin added to *Listeria* cells in suspension resulted in an instantaneous and rapid decrease in $\text{OD}_{600\text{nm}}$ of the substrate cells, reaching a baseline within minutes or sometimes seconds. This highly efficient enzymatic lysis not only offers advantages for laboratory

Fig. 3 Effect of pH and different buffer systems on lytic activity of HPL118 (a), HPL500 (b), HPL511 (c), and HPLP35 (d) against *Listeria* cells in suspension. Results of following buffers are shown, covering a pH range of 3.8–10.1: citrate (yellow bars), MOPS (red bars), Tricine (green bars), and CHES (blue bars)



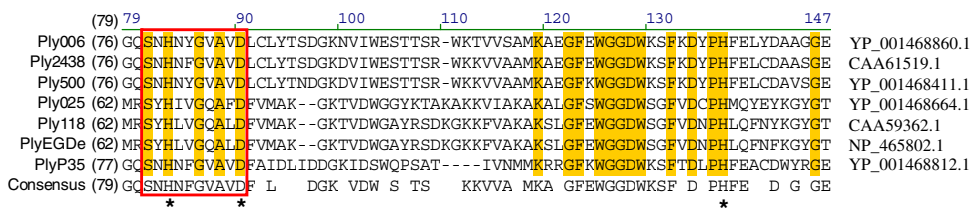


Fig. 4 Amino acid sequence alignment of the zinc binding central regions of peptidase endolysins from different *Listeria* phages. Residues conserved in all seven enzymes are highlighted in yellow. The zinc binding motif SxHxxGxAxD is boxed in red, and residues

required for coordination of the central Zn²⁺ ion in Ply500 are marked by asterisks (see “Discussion”). Database accession numbers of all proteins are indicated on the right

applications (Loessner et al. 1995a) but also demonstrates the potential of these enzymes as novel antibacterial food additives (Hagens and Loessner 2007).

The optimum ionic strength (200–300 mM NaCl) corresponds quite well to the salt concentration found in many food products associated with a high risk for *Listeria* contamination, such as soft-ripened cheeses and meat products. Our data are also in line with findings on other phage or bacterial cell wall hydrolases (Vasala et al. 1995; Loeffler et al. 2003; Cheng and Fischetti 2006; Yoong et al. 2006; Becker et al. 2008), although some enzymes were shown to be more active at physiological conditions (approximately 130 mM NaCl) or even in the absence of NaCl (Yoong et al. 2004; Fukushima et al. 2007). Interestingly, the best binding of the CBDs of Ply118 and Ply500 to the *Listeria* cell wall occurs at lower ionic strength (100 mM) (Loessner et al. 2002). This indicates that the optima for binding (CBD) and enzymatic function (EAD) are different, and both contribute to the overall

activity of the enzymes. The somewhat lower NaCl concentration favored by the amidase HPL511 may also be due to the lower binding affinity of its CBD to the cell wall compared to the CBDs of the other three endolysins (Schmelcher et al. 2010). As CBD binding is required for full lytic activity and is based on ionic interactions between the binding domain and *Listeria* cell wall carbohydrates (Eugster et al., submitted for publication), higher binding affinity supports activity at higher ionic strength (Loessner et al. 2002; Cheng and Fischetti 2006; Schmelcher et al. 2010).

With respect to optimum pH, the data obtained for HPL118 and HPL500 in this study were slightly different from those reported earlier for their CBDs (Loessner et al. 2002). We found that the pH optima of HPL118 and HPL500 match well with predicted isoelectric points (pI) of 9.91 and 9.56, respectively. For HPL511 and HPLP35, the predicted pI/s are in the same range (9.81 and 9.89, respectively), which in this case is higher than the

Table 2 Effect of different divalent metal cations on lytic activity of *Listeria* phage endolysins

	HPL118		HPL500		HPL511		HPLP35	
Control ^a	100.0±1.1%		100.0±3.0%		100.0±1.6%		100.0±18.1%	
EDTA ^b	40.4±4.2%		7.7±0.1%		0.8±0.1%		<0.5%	
Cation ^c	1 mM	10 mM	1 mM	10 mM	1 mM	10 mM	1 mM	10 mM
Mn ²⁺	242.3±5.2%	207.3±11.7%	59.7±1.2%	75.0±3.2%	548.1±22.2%	371.0±5.4%	26.7±21.4%	15.6±2.1%
Mg ²⁺	68.4±2.1%	80.4±3.0%	24.6±0.0%	51.1±9.0%	18.3±1.4%	133.6±19.2%	6.3±2.0%	8.4±1.7%
Ca ²⁺	62.7±0.6%	104.6±3.6%	47.6±3.5%	87.4±1.3%	70.3±8.5%	108.3±22.5%	13.5±2.1%	105.1±75.4%
Cu ²⁺	2.4±0.3%	2.6±0.3%	0.6±0.1%	0.9±0.1%	0.9±0.4%	3.2±1.7%	<0.5%	<0.5%
Co ²⁺	99.6±2.3%	103.2±0.2%	68.5±1.5%	65.4±1.8%	526.5±8.6%	278.1±15.6%	22.4±21.3%	11.0±1.1%
Ni ²⁺	17.5±0.8%	24.3±0.1%	4.9±0.1%	7.1±0.1%	6.5±0.3%	10.7±0.5%	<0.5%	<0.5%
Zn ²⁺	9.9±1.1%	n/d	6.8±0.5%	n/d	4.4±0.2%	n/d	<0.5%	n/d

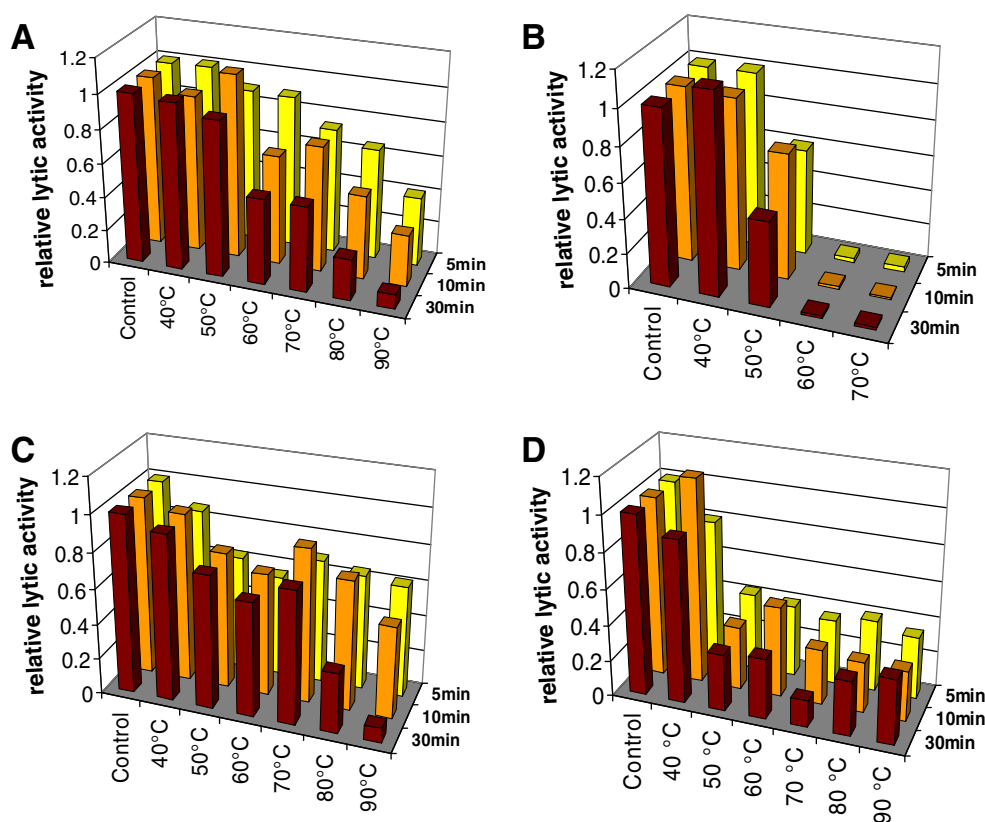
Cell walls of *Listeria monocytogenes* WSLC 1042 were used for HPL500, those of strain WSLC 1001 for all other enzymes. Activities are expressed as percentage in relation to the non-treated enzyme controls. Same enzyme concentration was used before and after EDTA treatment n/d not determined (see text)

^a Lytic activity under standard conditions before EDTA treatment of cell walls and enzyme (see text)

^b Lytic activity of EDTA treated and dialyzed enzyme against EDTA-treated cell walls under standard conditions

^c Lytic activities of EDTA treated and dialyzed enzyme against EDTA-treated cell walls supplemented with different divalent metal ions at two different concentrations

Fig. 5 Temperature stability of HPL118 (a), HPL500 (b), HPL511 (c), and HPLP35 (d). The enzymes were incubated at different temperatures for 5, 10, or 30 min, cooled down on ice, and then photometric lysis assays were performed under standard conditions. Lytic activity values are expressed relative to the control (non-heat treated enzyme)



experimentally determined pH optima, suggesting that these enzymes require a positive net charge for full activity. In general, pH optima above 8.0 seem uncommon for cell wall hydrolases. In fact, most other phage and bacterial lysins studied require neutral or slightly acidic conditions, with activity rapidly decreasing above pH 7.5 or 8 (Vasala et al. 1995; Morita et al. 2001; Loeffler et al. 2003; Pritchard et al. 2004; Yoong et al. 2004; Cheng and Fischetti 2006; Donovan et al. 2006; Fukushima et al. 2007; Sugahara et al. 2007). However, another L-alanoyl-D-glutamate peptidase from *E. coli* phage T5 revealed a pH profile similar to the enzymes reported here, with highest activity at pH 8.5 (Mikouliniskaia et al. 2009). It is interesting to note that these properties correspond well to the optimum range for *Listeria*, which also grows best at a pH of around 8.0 (Vanderzant and Splittstoesser 1992). At this point, it is not clear whether the endolysins would be fully active in complex food matrices such as cheese, which often feature low pH values. However, on surface ripened soft cheeses, which are particularly prone to *Listeria* contamination, growth of the pathogens is paralleled by an increase in pH during ripening from about 4.5 to above 8.0 (Farber and Peterkin 1991; Guenther and Loessner 2011), which also supports activity of the *Listeria* phage endolysins.

The fact that EDTA treatment reduces or abolishes endolysin function, which can be reconstituted by supplementation with several divalent metal cations, confirms

their crucial role for catalytic activity. The differences in inactivation observed by EDTA treatment might be explained by different affinities of the metal ions to their coordination sites or varying accessibility within the proteins. The zinc binding site SxHxxGxAxD was first identified in the *Enterococcus* dipeptidase VanX, which cleaves the terminal D-Ala–D-Ala bond in the peptidoglycan peptide stem and was shown to contain a central Zn²⁺ required for catalytic function (McCafferty et al. 1997). In Ply500, the Zn²⁺ ion is also coordinated by His and Asp residues within an identical motif, in addition to another His at position 133 (Korndoerfer et al. 2008). The latter is located within a unique region highly conserved among *Listeria* phage peptidases, featuring the consensus sequence KxxGFxWGGDWxxFxDxPH (Fig. 4). These similarities suggest an identical catalytic mechanism for these enzymes, employing Zn²⁺ as integral component required for function. Corresponding sites are also present in the murine sonic hedgehog protein Shh-N and carboxypeptidase Ddp from *Streptomyces albus* (Murzin 1996). Even though EAD500 is a peptidase and its sequence differs from the EADPSA amidase (Korndoerfer et al. 2006; Schmelcher et al. 2010), the latter also features a catalytic zinc, which is, however, positioned by two distinct His residues. Considering that Zn²⁺ obviously assumes a crucial role in catalytic function of these enzymes, it was surprising to find that Ca²⁺ and Mn²⁺ were more effective in restoring lytic activity after

removal of the Zn^{2+} . Similar observations regarding the effects of divalent metals on activity of cell wall hydrolases were reported earlier (Vasala et al. 1995; Shida et al. 2001; Pritchard et al. 2004; Donovan et al. 2006; Sugahara et al. 2007; Mikoulinskaia et al. 2009; Garcia et al. 2010). For aminopeptidase A, a zinc metalloendopeptidase that can be reactivated by Ca^{2+} or Mn^{2+} after exposure to chelating agents (Danielsen et al. 1980), it was recently shown that the Ca^{2+} ion increases substrate affinity by binding to two aspartate residues of the enzyme and interacting with an acidic side chain of the substrate (Claperon et al. 2008). We believe that this might also be the case for the peptidases described here and the interaction of the catalytic domain with the D-glutamic acid residue of the peptidoglycan peptide stem.

An entirely unexpected finding was the unusual temperature stability of HPL511, HPL118, and HPLP35, mostly because there is no obvious selection pressure for phages infecting psychrotrophic bacteria such as *Listeria* to evolve and maintain heat-stable proteins. Peptidoglycan hydrolases may still function after heating up to 60°C (Vasala et al. 1995; Fischetti 2005; Yoong et al. 2006; Schmitz et al. 2011); however, lytic activity after treatment at 90°C has not been reported before. Possible reasons for this phenomenon may include occurrence of cysteine residues (Cys), which are able to stabilize proteins through formation of disulfide (cystine) bonds. However, this mechanism is unlikely to be responsible for the effects seen here, since HPL118, HPL511, and HPLP35 feature only one or two Cys residues each, whereas the least thermostable enzyme HPL500 contains four Cys residues (data not shown). SalG, a goose-type lysozyme from salmon fish, also revealed high heat stability and does not contain Cys, unlike other less heat stable lysozymes of the same type (Kyomuhendo et al. 2007). This suggests that not disulfide bonds, but unfolding of the protein with increasing temperatures, followed by rapid reactivation due to instant refolding upon cooling may reconstitute enzyme activity after prolonged high temperature exposure. The latter assumption receives support from a study on thermoresistant lytic structural proteins of *Pseudomonas aeruginosa* phages (Lavigne et al. 2004; Briers et al. 2008). It was found that Gp36C, a part of the putative injection needle of phage Φ KMV, retained 50% and 21% of its activity after 1-h incubation at 100°C or 121°C, respectively (Lavigne et al. 2004) and also lacks Cys residues. Further investigations revealed that the enzyme starts to unfold at 50°C and, therefore, features only low thermodynamic stability. However, it shows high kinetic stability, i.e., resistance to irreversible denaturation, which allows the protein to refold at appropriate temperatures (Briers et al. 2006). With respect to the enzymes studied here, available evidence suggests that HPL118 and HPL511 have similar properties. Regarding HPLP35,

however, the prominent drop in activity after incubation at 40–50°C suggests a significant degree of irreversible denaturation. Here, it is possible that the CBD is more prone to irreversible inactivation than the EAD, which would reflect and explain the observed reduction in lytic activity of the enzyme to 30–40%.

Thermoresistance of phage endolysins can be considered a very useful property regarding their potential application as antimicrobials in food products that undergo heat treatment. The potential of using purified recombinant *Listeria* phage endolysins in food was recently assessed in a pilot study and yielded promising results. Application of HPL118, HPL500, or HPL511 to leaves of iceberg lettuce artificially contaminated with *L. monocytogenes* reduced viable counts of the pathogen by up to 2.4 logs after storage for 6 days at two different temperatures (6°C and 12°C) (Guenther et al., unpublished data). In general, the extraordinary lytic specificity of bacteriophage endolysins represents an important advantage since they specifically act on only their target cells, leaving the endogenous and often desired background flora untouched. Furthermore, as they cleave bonds in highly conserved portions of the peptidoglycan, formation of resistance is unlikely and has not yet been reported (Loessner 2005; Fischetti 2005; Low et al. 2005). These favorable properties, in conjunction with the high stability and robustness of the lysins studied here, offer promising perspectives for their application in foods and associated production and packaging environments.

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