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Modified *Listeria* Bacteriophage Lysin Genes (*ply*) Allow Efficient Overexpression and One-Step Purification of Biochemically Active Fusion Proteins

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***Listeria* bacteriophage lytic enzymes are useful for in vitro applications such as rapid, gentle cell disruption, and they provide new approaches as selective antimicrobial agents for destruction of *Listeria monocytogenes* in contaminated foods. We describe here the amino-terminal modification of three cloned *Listeria* phage lysin genes (*ply*), resulting in fusion proteins with a 12-amino-acid leader containing six consecutive histidine residues. The recombinant enzymes retain their native specific activity and can be efficiently overproduced in *Escherichia coli*. By one-step metal chelate affinity chromatography, active lysins could be purified to more than 90% homogeneity.**

Phage-encoded lysins, or endolysins, are highly active enzymes hydrolyzing bacterial cell walls. They are late gene products, appearing at the end of the lytic cycle of bacteriophage multiplication, and they enable the release of progeny phage. We have previously cloned and sequenced the endolysin genes from *Listeria* bacteriophages A118, A500, and A511, encoding L-alanoyl-D-glutamate peptidases Ply118 (30.8 kDa) and Ply500 (33.4 kDa) and the *N*-acetylmuramoyl-L-alanine amidase Ply511 (36.5 kDa), respectively (11). These enzymes enable the rapid and gentle, yet specific, lysis of *Listeria* cells in vitro (10). Moreover, their specificity and strong activity render them potentially useful antimicrobial agents for elimination of the opportunistic pathogen *Listeria monocytogenes* (4) from foods and food-grade ingredients. However, these applications require the availability of large amounts of purified, active enzymes.

This study was conducted to investigate the feasibility of modifying the endolysin proteins by fusion with an amino-terminal purification tag, without decreasing or destroying enzymatic activity. Also, it was desirable to enable efficient overexpression in *Escherichia coli*, with subsequent easy, one-step purification.

Organisms and cloning procedures. *L. monocytogenes* WSLC 1001 (Weihenstephan *Listeria* Collection) was maintained and grown (at 30°C in tryptose medium) as previously described (8). *E. coli* JM109 [K-12 *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q} ZΔM15)] and W3110 (1) were passaged in standard Luria-Bertani medium (12) at 37°C. For selection of plasmid-bearing cells, ampicillin was added at 100 μg ml^{-1} to liquid or solid medium.

The vector pQE-30 (Qiagen) was selected for expression of endolysin gene fusions. A strong phage T5 promoter enables transcription of cloned genes, which is regulated by a *lacI* repressor-binding site between the promoter and multiple cloning site. Using a similar vector (pQE-8) in the host *E. coli* M15, Düring (3) found a tightly regulated system for overexpression of the T4 muramidase. Usually, leaky expression from genes cloned in M15 is minimized by the presence of a second plasmid (pREP4), which overproduces the LacI repressor. In contrast, the host strain used in this study (JM109) features the *lacI*^{q} genotype (15), which itself provides elevated levels of repressor molecules. Also, in contrast to the associated holins, *Listeria* bacteriophage endolysins were found to be nontoxic for *E. coli* (11, 17). Transcription of cloned genes can be induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to the growth medium. The plasmid also provides a 36-nucleotide 5′ end leader upstream of the multiple cloning site, resulting in fusion of a hexahistidine-containing 12-amino-acid peptide to the N-terminal ends of synthesized proteins. Directional, in-frame insertion of *Listeria* phage *ply* genes (*ply*118, GenBank no. X85008; *ply*500, GenBank no. X85009; and *ply*511, GenBank no. X85010) (11) into the *Bam*HI-*Sal*I sites directly downstream of the hexa-His box in pQE-30 (Fig. 1) was possible after PCR amplification of the genes with the following primers (*Bam*HI-*Sal*I sites are underlined; start and stop codons are shown in boldface letters): PLY118-Bam (36-mer), 5′-TCTAGG ATCC**ATG**ACAAGTTATTATTATAGTAGAAG-3′; PLY118-Sal (36-mer), 5′-AAGTGTCGAC**CTA**AATCTTTTTAACAAA CTTCGTGT-3′; PLY500-Bam (33-mer), 5′-TCTAGGATCC **ATG**GCATTAACAGAGGCATGGCT-3′; PLY500-Sal (36-mer), 5′-AAGTGTCGAC**TTA**TTTTAAGAAGTATTCTGC TGTGT-3′; PLY511-Bam (36-mer), 5′-TCTAGGATCC**ATG** GTAAAATATACCGTAGAGAACAA-3′; and PLY511-Sal (36-mer), 5′-AAGTGTCGAC**TTA**TTTTTTGATAACTGCT CCTGTAC-3′.

Thirty nanograms each of purified DNAs from phage A118, A500, or A511 (9) was used as a template for amplification of the *ply* genes in the PCR, which was carried out according to standard protocols supplied by the enzymes' manufacturer (Boehringer). Reaction products were purified by using ion-exchange microcentrifuge spin columns (QIAquick; Qiagen), digested with *Bam*HI and *Sal*I (Boehringer), and purified by agarose gel electrophoresis (12). DNA fragments were then ligated (T4 ligase; Boehringer) into pQE-30, which had been predigested with *Bam*HI-*Sal*I. Ligation reaction mixtures were desalted and electrotransformed into competent *E. coli* JM109

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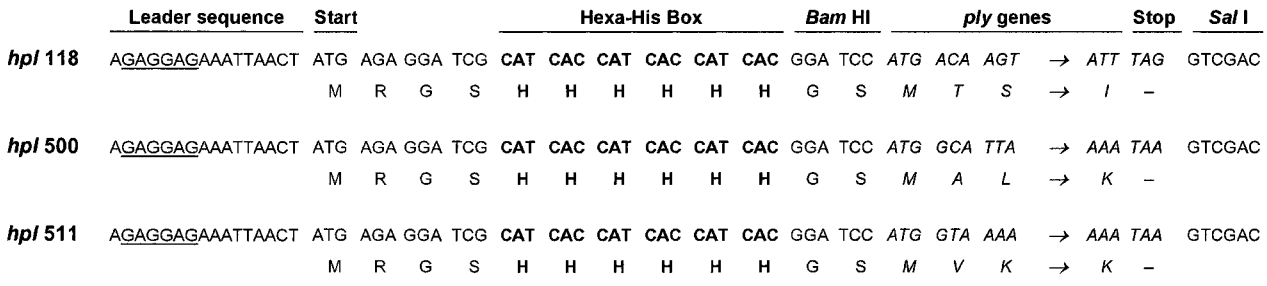

FIG. 1. Partial nucleotide sequences and corresponding amino acid sequences of the modified endolysin genes *hpl118*, *hpl500*, and *hpl511* cloned into vector pQE-30. Genes were fused at their 5′ ends to a 36-nucleotide sequence, which encodes a 12-amino-acid peptide with six consecutive histidine residues. For efficient transcription, a phage T5 promoter is present upstream of the partly shown leader sequence. The synthetic ribosome binding site is underlined.

cells, which were then plated onto antibiotic-containing agar plates and incubated at 37°C for 20 h. Colonies which expressed a lytic phenotype for *Listeria* cells upon induction with IPTG were identified as described earlier (11). Plasmids from clones expressing either one of the modified, functional endolysins were analyzed by restriction enzyme digestion and nucleotide sequencing of the 5′ ends of the inserts. The primer used here (21-mer; 5′-AATAGATTCAATTGTGAGCGG-3′) is complementary to a pQE-30 region 50 nucleotides upstream from the ATG start codon of the hexa-His leader sequence. Plasmids specifying the modified endolysins were designated pHPL118, pHPL500, and pHPL511.

Overexpression of gene products. Plasmid-bearing *E. coli* was grown overnight in a volume of 50 ml. Then, 500 ml of prewarmed broth was inoculated with 20 ml of the overnight culture and further incubated (with vigorous shaking) until the optical density at 600 nm reached 0.5 to 0.6. Then, IPTG was added to a final concentration of 0.5 mM. In preliminary experiments, the time course of expression was determined by taking samples (1 ml) every 30 min. Cells were pelleted by centrifugation, resuspended in sodium dodecyl sulfate (SDS) sample buffer (12), and lysed by boiling for 5 min. This was followed by analysis of the total cellular protein content by SDS-polyacrylamide gel electrophoresis (PAGE; ExcelGel, 8 to 18%; Pharmacia). Protein bands were stained with Coomassie blue R-350 (Pharmacia), and a laser-densitometrical analysis of the lanes was performed as previously described (16). The newly synthesized proteins appeared 30 min after induction with IPTG and increased to a maximum after 4 h. The maximum yield of recombinant hexa-His enzyme was 18 to 20% of total cellular protein. This is very similar to the values obtained for native Ply118 and Ply500 proteins and represents a substantial improvement for the phage 511 lysin gene, since synthesis of the native protein from its own translation initiation signals yielded only 6 to 8% recombinant enzyme (11).

Constitutive expression of endolysin. To determine whether it would be possible to obtain constitutive lysin gene expression without IPTG induction, pHPL118 was transformed into *E. coli* W3110. This strain has a near-wild-type phenotype (it lacks the *lacI*^{q} mutation) and cannot efficiently control the strong T5 promoter on pQE-30. In contrast to the JM109 derivatives, whose growth rate was severely impaired after induction, W3110(pHPL118) grew very well (in the absence of IPTG). The Hpl118 concentration in cells of a late-log-phase culture was approximately 5 to 8% of total cellular proteins, as determined by SDS-PAGE. However, additional induction with 0.5 mM IPTG did not further increase synthesis of recombinant protein. Therefore, JM109 was used as the expression host in further experiments.

Lysin purification by metal chelate affinity chromatography. For preparative purification, cells were harvested by centrifugation 4 h after induction (10,000 × *g*, 10 min, 4°C). Pellets were resuspended in buffer A (protein-binding and wash buffer; 50 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole [pH 8.0]) at 4 ml/g (cell paste wet weight). After a single passage through a French pressure cell (SLM Aminco) at 100 MPa, debris was removed by centrifugation (50,000 × *g*, 2 h, 4°C). Endolysin-containing supernatants were sterilized by filtration (0.22-μm-pore size cellulose acetate membrane; Sartorius) and stored at −20°C.

The His-tagged lysins were purified from crude cell extracts under native conditions. An XK 16/20 column (Pharmacia) was packed with 15 ml of nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen). This affinity matrix is a Sepharose support coupled to Ni-NTA ligands, a chelating adsorbent for tight but reversible binding of proteins containing neighboring histidine residues (5, 6). It was important to apply the crude cell lysate very slowly to the column (5 mg of total protein per ml of resin), at approximately 4 column volumes per hour. Unbound protein was washed from the column by using buffer A, until the baseline (A_{280}) was stable. Because we observed that some other proteins (from *E. coli*) could associate with either the tagged proteins or the Ni-NTA resin, increasing the NaCl concentration to 500 mM was necessary. This resulted in higher purity of the endolysin-containing fractions. Binding the hexa-His-modified proteins to the resin in the presence of low levels of imidazole (5 mM) was found to further decrease the background. The elution buffer (buffer B) additionally contained 100 mM imidazole, which excludes the histidine residues from the binding sites on the Ni-NTA resin. Figure 2A demonstrates that the endolysins could be eluted in a single peak. None of the secondary peaks contained measurable enzymatic activity. Active fractions were desalted by ultrafiltration (Centriprep-10; Amicon) and resuspended in 20 mM Tris Cl–50 mM NaCl, pH 8.0. For determination of enzyme purity, samples were analyzed by SDS-PAGE as described above. The proteins appeared as almost homogeneous bands (more than 90% pure) in SDS-PAGE gels (Fig. 2B). The few additional bands (especially in lane D) could be contaminating proteins from *E. coli* (with high affinity to the Ni^{2+} resin) or incompletely translated forms of the tagged endolysin proteins. The observed molecular sizes of the fusion proteins were in good agreement with the predicted values of 32.3 kDa (Hpl118), 37.9 kDa (Hpl511), and 34.8 kDa (Hpl500).

The modification of the phage endolysin genes resulted in the addition of 12 amino acids to the gene products, a slightly increased molecular mass (increase of 1,399 Da), and an almost identical pI (increase of 0.04).

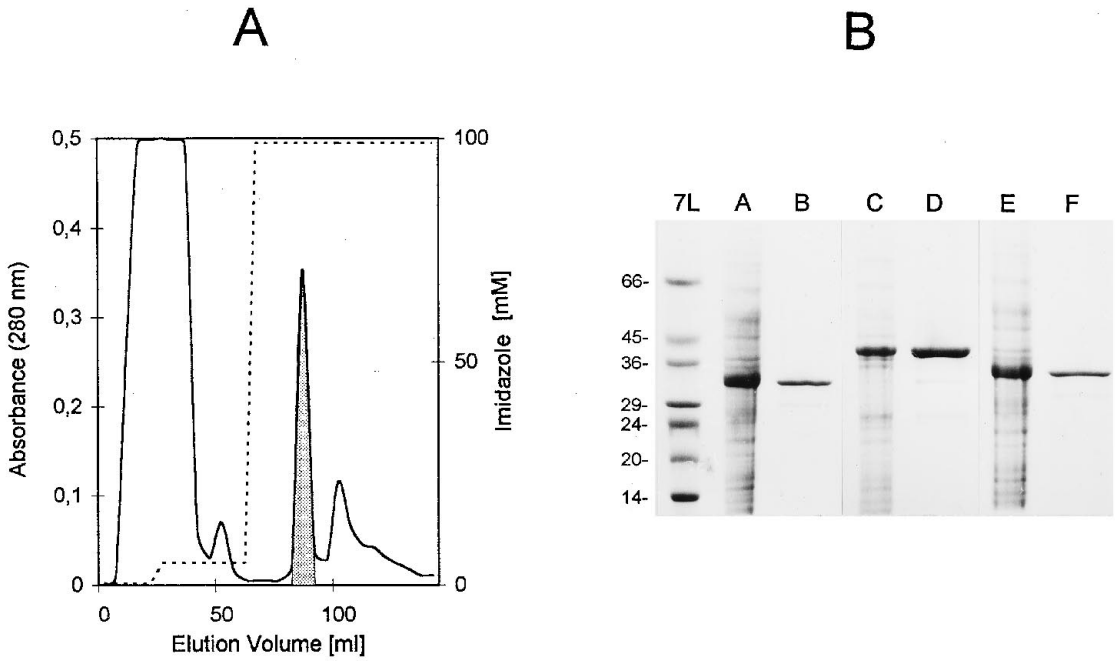

FIG. 2. Purification of His-tagged endolysins. (A) Elution profile of Hpl118 (left *y* axis), purified directly from crude cell extracts, obtained by using Ni-NTA resin and a stepped imidazole gradient (right *y* axis). The shaded peak represents the active enzyme. The profiles for Hpl500 and Hpl511 were highly similar (results not shown). (B) SDS-PAGE of proteins before and after purification. Lanes: 7L, molecular mass marker (indicated in kilodaltons); A, cell extract of JM109(pHPL118); B, purified Hpl118; C, cell extract of JM109(pHPL511); D, purified Hpl511; E, cell extract of JM109(pHPL500); F, purified Hpl500.

Determination of lytic activity. One unit of endolysin activity is defined as the amount of endolysin necessary to decrease the optical density at 600 nm by 0.01/min, at pH 8.0 and 25°C in a volume of 1 ml, when heat-killed, washed cells of *L. monocytogenes* WSLC 1001 are used as a substrate (10). For every assay, (optical density at 600 nm, ≈1.5 to 2.0), 100 μl of endolysin solution was added to 900 μl of a cell suspension, and the optical density was monitored over time. In the crude extracts, the enzyme activity in preparations of all three modified endolysins was 50 to 60 U/ml. After purification and buffer exchange, enzyme preparations were adjusted to contain approximately 50 U/ml. At an enzyme concentration of approximately 5 U/ml, the decrease in optical density was very similar for the three different enzymes (Fig. 3). After 10 to 15 min, the cell suspensions appeared almost clear. Table 1 shows the efficiency of the purification procedures for the native Ply118 lysin and the His-tagged Hpl118 enzyme (results for Hpl500 and Hpl511 were very similar). The metal chelate affinity chromatography employed here enables rapid, one-step purification of recombinant lysins with high yields. The amino-terminal fusion with the leader peptide had no negative effect on the enzymatic activity of the modified enzymes versus the native proteins Ply118, Ply500, and Ply511 (11), since the specific activity remained unchanged (Table 1).

To verify that the purified endolysins are free from contaminating DNases, RNases, and proteases, endolysin samples (5 μl) were mixed with the following standards in a total volume of 10 μl: bacteriophage A511 DNA (0.5 μg), rRNA (2.0 μg, from *Brevibacterium linens*), and bovine serum albumin (100 μg). As a control, water was added instead of lysin fractions. Following incubation for 30 min at 37°C, DNA and RNA samples were evaluated by agarose gel electrophoresis (12). Bovine serum albumin was analyzed by SDS-PAGE as described above. Endolysin preparations were found to be substantially free from nucleases and proteases, since no degradation of DNA, RNA, or bovine serum albumin could be detected (results not shown).

Conclusions. We have constructed bacteriophage endolysin fusion proteins which could be overexpressed and purified with high efficiency. Our data indicate that the proteins retain their full specific activity, with no loss due to the N-terminal modification.

These enzymes are useful tools for *Listeria* cell lysis in vitro, with subsequent purification of nucleic acids or proteins. Purity

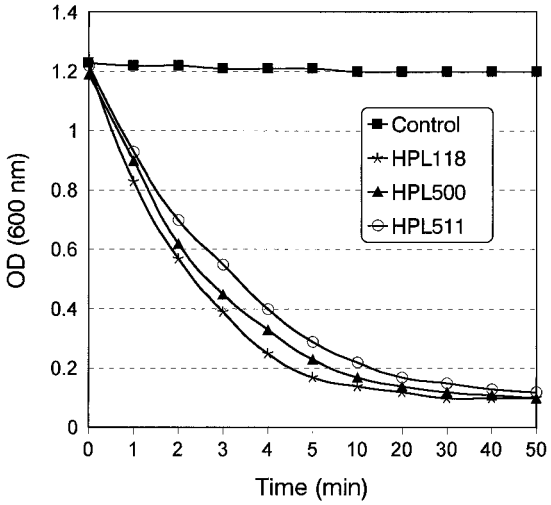

FIG. 3. Lysis of *L. monocytogenes* WSLC 1001 cells by the modified phage endolysins. Cells were mixed with 100 μl (approximately 5 U) of the purified hexa-His enzymes. The subsequent decrease in optical densities (OD) of the cell suspensions was determined photometrically at 600 nm. No lysin was added to the control.

TABLE 1. Comparison of purification efficiencies for the native and N-terminally modified *Listeria* bacteriophage A118 endolysins

Enzyme	Grade	Total protein (mg/ml)	Activity (U/ml)	Sp act (U/mg)	Yield (% recombinant protein)	Purity (% of total protein)
Ply118	Crude cell extract	14.9	59	4	100	18–20
	After chromatography	0.5	13	27	22	~80
Hpl118	Crude cell extract	12.0	60	5	100	18–20
	After chromatography	1.9	52	28	87	>90

and the absence of nucleases and proteases are especially important for practical applications which may require longer incubations, such as multilocus enzyme electrophoresis (2) or pulsed-field gel electrophoresis (7).

Large-scale purification of Hpl118 is presently being carried out in this laboratory, and preliminary results regarding the experimental use of this recombinant endolysin to combat *L. monocytogenes* in soft cheeses and other dairy products look very promising (13). Moreover, the modified lysins are being used for the development of a new procedure for rapid and specific detection of *Listeria* cells; the procedure is based on the selective release of ATP from intact bacterial cells, with subsequent detection with firefly luciferase (14).

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