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Research Article

Identification and characterization of a highly thermostable bacteriophage lysozyme

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Abstract. *Pseudomonas aeruginosa* bacteriophage ϕKMV is a T7-like lytic phage. Liquid chromatography-mass spectrometry of the structural proteins revealed gene product 36 (gp36) as part of the ϕKMV phage particle. The presence of a lysozyme domain in the C terminal of this protein (gp36C) was verified by turbidimetric assays on chloroform-treated *P. aeruginosa* PAO1 and *Escherichia coli* WK6 cells. The molecular mass (20,884 Da) and pI (6.4) of recombinant gp36C were determined, as were the optimal enzymatic conditions (pH 6.0 in 16.7 mM phosphate buffer) and activity (4800 U/mg). Recombinant gp36C is a highly thermostable lysozyme, retaining 26% of its activity after 2 h at 100 °C and 21% after autoclaving. This thermostability could prove an interesting characteristic for food conservation technology.

Key words. Phage; phage infection; lysozyme; recombinant expression; ϕKMV; mass spectrometry; thermostable.

The availability of novel lysozymes or other lytic enzymes (lysins) is of great interest for numerous applications in biotechnology, conservation processes and dairy processing [1–3]. Many of these applications have focused on the use of hen egg-white lysozyme (HEWL) to achieve bacterial decay, while only a few lysins are commercially available [4–6]. Specific properties as e.g. host range and activity have driven the search for HEWL derivatives and new lysins. Lysins are very diverse in their biochemical activity, ranging from glycosylase (muramidase; E.C. 3.2.1.17), transglycosylase and amidase to endopeptidase in the process of peptidoglycan disruption.
Bacteriophages provide a source of such proteins and were first used for preparing lysates containing intracellular enzymes, e.g. L-lysine decarboxylase and L-arginine decarboxylase from T2-infected *Escherichia coli* cells [7]. Lysins are classified based on evolutionary relationships into chicken-type (C), goose-type (G), virus-type (V), chalaropsis-type (CH) and the recently established invertebrate-type (I) [8, 9]. Most phage lysins belong to the V-type (e.g. gp *e* of T4), while some correspond to the G- or CH-type (e.g. gp16 of T7 and CPL1 lysozyme of phage CP-1, respectively). Phage lysozymes with transglycosylase activity, like the phage λ lysin, are placed under a sixth category, the λ-type lysins. In this atypical transglycosylase activity, the energy released during hydrolysis of the β-1,4 glycosidic bond is conserved by the formation of 1,6 anhydrous muramic acid allowing only local degradation of the peptidoglycan layer during infection [10].
In view of potential applications of phage lysins, a number of lysins were isolated as a native product or from a recombinant source, and purified by chromatography [11, 12]. With the completion of over 200 genome sequences of phages infecting different bacterial hosts, a great number of new lytic proteins were identified. Bacteriophage ϕKMV is a strongly lytic, T7-like bacteriophage infecting *Pseudomonas aeruginosa*. Its genome

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sequence revealed a gene organization similar to *E. coli* phage T7, totalling 48 predicted open reading frames (ORFs), 11 of which show sequence homology to T7 proteins [13]. The complete genome sequence of Podoviridae member SP6 (infecting *Salmonella typhimurium*) showed that SP6 is the closest relative of ϕKMV to date in terms of phylogenetic analysis and genome organization [14, 15]. In this report we analyse the C-terminal end of gp36, a putative internal virion protein of ϕKMV, which shows similarity to T4 lysozyme gp *e* (fig. 1). The presence of this C-terminal lysozyme domain in gp36 strongly suggests that it is part of the injection needle, involved in phage infection. This is a specific feature of ϕKMV and SP6, compared to other T7-like phages that have a transglycosylase domain at the N terminal of an internal virion protein such as gp16 of phage T7 [16, 17].

Materials and methods

Mass spectrometry identification

Bacteriophage particles from ϕKMV were purified by two consecutive CsCl gradient centrifugations, disrupted by three freeze-thawing cycles and loaded on a 12% acrylamide (BioRad, Hercules, Calif., USA) SDS-PAGE gel for one-dimensional separation [18]. Protein bands were visualized by either silver staining [19] or Coomassie (Simply Blue Safestain; Invitrogen, Groningen, The Netherlands), excised and digested with trypsin prior to LC-MS/MS analysis on an LCQ Classic (ThermoFinnigan, San Jose, Calif.) equipped with a nano-LC column switching system as described in Dumont et al. [20]. All MS/MS data were searched against the GenBank non-redundant protein database, using SEQUEST (ThermoFinnigan), identifying the significant peptides [cross correlation (xC) > 2.5 for ion charge (Z) = 2; xC > 3.5 for Z = 3], and MASCOT (Matrix Sciences, London, UK).

Cloning, protein expression and purification

Purified DNA from ϕKMV [13] was used as template to amplify the coding sequence of gp36C (M_{737}–E_{898}) in a standard PCR reaction (*Pfu* polymerase, Stratagene, La Jolla, Calif., USA) using primers KMVgp36B-F and KMVgp36-R (table 1). Subsequently, the PCR product (504 bp) was digested with *Bgl*II and cloned into the *Bam*HI site of *E. coli* expression vector pQE-EC. This vector was derived from pQE-16 (Qiagen, Hilden, Germany), in a procedure similar to the construction of pQE-EN [21], replacing the dihydrofolate reductase coding sequence by a synthetic cassette. The cassette was assembled from ETAG oligonucleotides (table 1). Consequently, pQE-EC contains *Bam*HI and *Bgl*II insertion sites upstream of coding sequence of the E-tag (Amersham Biosciences, Uppsala, Sweden) and the His_6-tag sequence. Verification of the vector sequence and identification of a pQE-EC clone containing the 36C ORF was done by automated DNA sequencing on an ABI 377 Sequencer using BigDye chemistry (Applied Biosystems, Foster City, Calif., USA) and primers pQEf and pQEr (table 1).

The fusion protein gp36C-E-His_6 (named gp36C) was overexpressed in *E. coli* (XL1 Blue MRF′; Stratagene) after induction for 4 h with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) of exponentially growing cells at 37 °C. Lysate preparation (Qiagen) and protein purification (Ni-NTA agarose beads; Qiagen) were performed as suggested by the manufacturer, while protein solubility was tested by loading equal amounts of total cell lysate and soluble and insoluble protein fractions of the lysate on SDS-PAGE. The addition of HEWL for cell lysis was replaced by an extra cycle of freeze-thawing (–80 °C, 25 °C) and sonication. An LMW marker (Amersham Biosciences) was used to compare protein band

Table 1. Oligonucleotides used in this study.

KMVgp36B-F	5′-GTGAGATCTATGCTGAAGCAGG ACGTGT
KMVgp36-R	5′-TATAGATCTCTCCGCTGTTGCGG
ETAGf3	5′-GATCCAGATCTGGTGCGCCGGTG
ETAGf4	5′-CCTTATCCAGACCCGCTGGAACCGCGTG
ETAGr3	5′-GATCCACGCGGTTCCAGCGGTGC
ETAGr4	5′-TGGATAAGGCACCGGCGCACCAGATCTG
pQEf	5′-GCGGATAACAATTTCAC
pQEr	5′-CTGGATCTATCAACAG

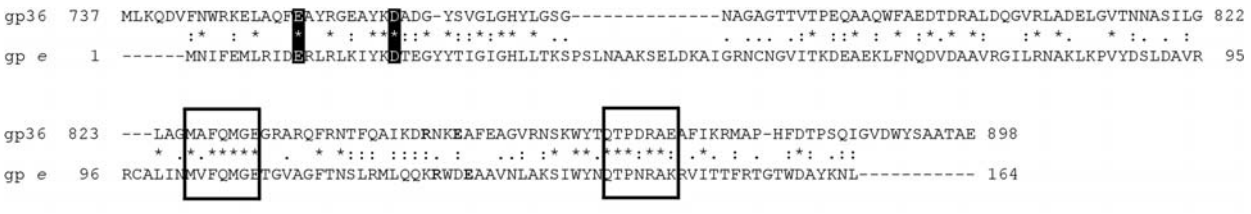

Figure 1. Alignment of ϕKMV gp36C and gp *e* of bacteriophage T4. Comparison of gp36C and gp *e* shows conserved (*) and positive (:/.) AA matches. The ClustalW alignment indicates conservation in both essential catalytic residues E_{753} and D_{762} for the glycosylase reaction (black). Two regions of quite extended sequence identity in the two enzymes are boxed. Based on the three-dimensional structure of the T4 enzyme [KUROKI], these regions are close to the active site of the enzyme, strongly suggesting the structural relationship of the gp36 C terminal and T4 lysozyme. R_{848} and E_{851} (and corresponding residues Rxxx and Exxx in T4 gp *e*) are shown in bold, in view of their essential nature and potential peptidoglycan peptide binding [26].

sizes and yields, after electrophoresis and Coomassie blue staining. Protein amounts were determined digitally using Un-Scan-it gel v5.1 software (Silk Scientific Corporation, Orem, Utah, USA).

Isoelectric focusing was performed exactly as described in Gebruers et al. [22] by comparison with a commercial standard [IFL; Pharmacia Biotech (Uppsala, Sweden) calibration kit, pI 3.5–9.3]. Accurate determination of protein molecular mass was performed by SELDI-TOF analysis (courtesy of B. Landuyt, Laboratory of Experimental Oncology, University of Leuven) [23].

Determination of lysozyme activity

Substrates for lysozyme activity measurement consisted of either commercially available lyophilized *Micrococcus lysodeikticus* (ATCC4698) cells (Fluka, Buchs, Switzerland) or *P. aeruginosa* (PAO1) and *E. coli* cells (WK6) treated with $CHCl_3$ to remove the outer cell membrane [24]. Luria-Bertani medium (50 ml) containing exponentially growing cells (±0.6 at OD_{600}) was centrifuged (4000 g, 15 min, 4 °C) and decanted. The cell pellet was suspended in chloroform-saturated 0.05 M Tris buffer (pH 7.7). After gently shaking for 45 min at room temperature, protoplasts were collected (4000 g, 15 min, 4 °C), washed and suspended in 10 mM phosphate buffer (pH 6, 7 and 8) or acetate buffer (pH 4 and 5) at an OD_{600} between 0.6 and 1.0 [24]. Protein samples of 30 µl were added to aliquots of 270 µl cell suspension in honeycomb plate wells and measured in a Bioscreen C Microbiology reader (Labsystems Oy, Helsinki, Finland) at room temperature at 5-min intervals over 3 h. Each sample was prepared in triplicate to measure the drop in turbidity (OD_{600}). All samples were plotted relative to negative controls and compared to HEWL 70,000 U/mg (Sigma, St. Louis, Mo., USA).

Quantification of enzymatic activity

The definition of 1 unit (U) lysozyme activity corresponds to a linear descent in OD_{600} of 0.001 per min [25]. To pinpoint the region of linear descent for each curve, we calculated the maximal determination coefficient (R^2) for an increasing sample size (n = number of measurements in time). This maximized R^2 value ensures the most reliable fit and hence the most reliable linear regression. The slope of this curve can be used to calculate the decrease in OD_{600} per unit of time, the activity of which can be calculated based on the amount of lysozyme added in a volume of 1 ml of cell suspension. This assay and calculation were performed in triplicate. Subsequently, curves were analysed for incremental amounts of lysozyme (m) to determine the curves showing a linear correlation between the drop in optical density and the amount of enzyme (see Results). Curves with a linear correlation between these parameters were retained for the overall enzyme activity calculation. Since all measurements were performed in triplicate, a complexity of [3 × n × m] was obtained, allowing calculation of standard deviations.

Results and discussion

Identification of gp36 as a structural protein

The in silico analysis of gp36 and genome organization of the *ϕ*KMV genes hinted that this protein is part of the mature phage particle and contains a lysozyme domain at the C-terminal end [13]. To prove that this protein is indeed a structural protein, mature phage particles were dismantled and proteins were fractionated by SDS-PAGE (fig. 2A). The 94-kDa protein band was analysed by LC-MS/MS after tryptic digestion, showing a total of 28 sig-

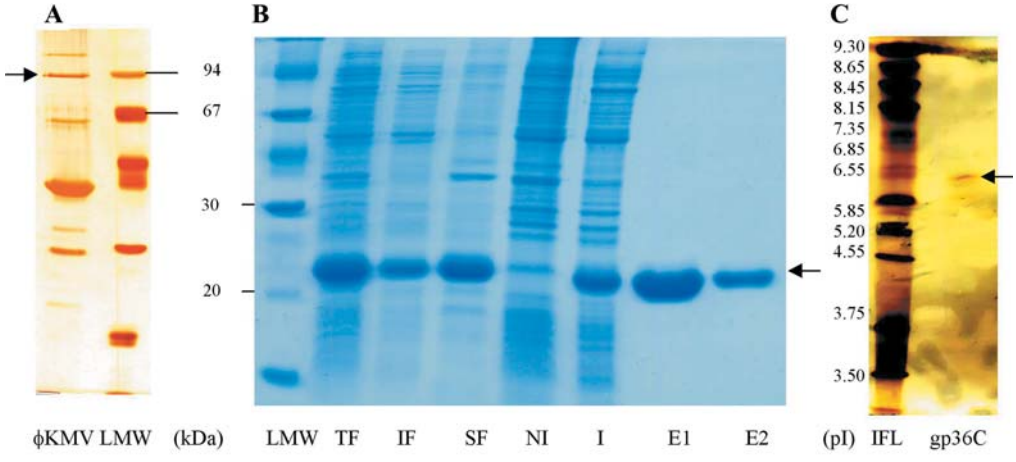

Figure 2. Protein gels involved in the identification and characterization of gp36. (*A*) SDS-PAGE (12%) analysis of phage structural proteins derived from disrupted phage particles. The protein band around 94 kDa (arrow) was shown to contain gp36 by LC-MS/MS analysis. (*B*) Protein expression and purification of gp36C: lanes 2–4 contain the total (TF), insoluble (IF) and soluble (SF) lysate fraction, respectively. Lanes 5–8 contain the total cell fraction of non-induced (NI) and induced (I) samples, followed by the first and second Ni^{2+} elution fraction (E1, E2). (*C*) Isoelectric focusing of gp36C (arrow).

nificant gp36 peptides. Identified peptides originated from throughout the entire predicted gp36 sequence, thus corroborating the gene annotation described previously [13]. We analysed all peptide bands using Sequest to determine the amino acid (AA) coverage based on significantly scoring peptides. An AA coverage of 315 AA (of 898 AA) or 35.1% was achieved, positively identifying gp36.

Protein expression of the lysozyme domain gp36C

In silico analysis of ϕKMV gp36 revealed the presence of a C-terminal phage-lysozyme domain, comparable to that of T4 gp *e*, since both catalytic residues (E_{753} and D_{762}) and possible peptidoglycan-recognizing residues (R_{848} and E_{851}) [26] are present within the predicted pFAM domain (PF00959) [27]. Furthermore, two identical AA regions between gp36 and gp *e* are present, close to the active site in the three-dimensional structure of the T4 lysozyme (fig. 1).

Usually, lytic enzymes active during phage infection have transglycosylase activity as opposed to the more destructive glycosylases active during the lysis phase of phage replication [10]. The in silico analysis suggests that the C terminal of gp36 bears lysozyme activity, while peptidase or amidase activity seem unlikely.

The functional domain (gp36C) was tentatively defined as the region between M_{737} and E_{898} (162 AA) and includes all essential residues for lysozyme activity (fig. 1). Overexpression of this product in *E. coli* provided sufficient soluble protein (gp36C) for a one-step purification, yielding 0.7 µg of purified protein per millilitre of *E. coli* expression culture. Purity of the recombinant gp36 (over 90%) was evaluated by SDS-PAGE (fig. 2B) and confirmed by SELDI-TOF analysis, which showed no discernible secondary peaks (unpublished data) and accurately determined the molecular mass (20,884 Da). The pI of gp36C was determined by isoelectric focusing (fig. 2C). A value of 6.4 is rather acidic, compared to the pI (around 9) of other phage lysozymes (e.g. from T7 and T4), predicted by Protparam (ExPASy, SIB).

Biochemical characterization

Buffer pH and ionic strength (I) are major parameters in the activity of phage lysins [24, 28, 29]. Both parameters were tested simultaneously, varying I between 0.04 and 0.12 and the pH between 4 and 8. The results of these analyses for both gp36C and HEWL are displayed in table 2. Both parameters have a strong influence on lysozyme activity. For gp36C, the highest activity was obtained at pH 6 and I = 0.1, corresponding to a 16.7 mM potassium phosphate concentration, while HEWL peaked at 13.3 mM potassium phosphate and pH 6. This is different from the phage lysins involved in cell lysis, which have optimal activities between pH 7 and 8, but is comparable to other tail-associated lysozyme domains [30, 31]. The variation in pH optimum can be important for application purposes, where pH is often a critical factor.

Bacterial sensitivity

Bacterial sensitivity to gp36C was tested on lyophilized *M. lysodeikticus* and chloroform-treated *E. coli* (WK6) and *P. aeruginosa* (PAO1). Though *M. lysodeikticus* is a widely used test substrate for lysozyme assays, gp36C showed no visible lysozyme activity towards this Gram-positive bacterium. Adding equal amounts of gp36C (13.3 µg/ml) to WK6 and PAO1 (brought to the same OD_{600}) gave strong lytic activity compared to negative controls, and showed that the gp36 protein fragment is twice as effective towards PAO1 compared to WK6 at optimal conditions. One should note that lysins of phages with Gram-negative hosts often have low affinity to the peptidoglycan of *M. lysodeikticus*. Phage P22 lysozyme has a twenty-fold higher affinity towards *S. typhimurium* – its natural host – than to *M. lysodeikticus*, while phage λ lysozyme shows no activity towards *M. lysodeikticus* [29, 32]. Conversely, enterobacteriophage T4 lysozyme is able to degrade the *M. lysodeikticus* peptidoglycan, though it is 136 times more effective on *E. coli* peptidoglycan [30]. Better alignment of the substrate to the active domain of the lysin may be the reason why T4 lysozyme shows a much higher affinity towards *E. coli* [26]. The

Table 2. Influence of pH and ionic strength (I) on the lytic activity of gp36C and HEWL.

	I						I				
pH	0.04	0.06	0.08	*0.10*	0.12	pH	0.04	0.06	*0.08*	0.10	0.12
4	0	0	0	0	0	4	0	0	0	0	0
5	0	3	4	2	5	5	0	19	10	24	23
6	8	51	71	*100*	86	*6*	3	52	*100*	59	62
7	6	12	13	55	24	7	19	16	29	41	28
8	3	7	5	5	9	8	15	15	31	40	37

Rows indicate the tested pH range, while columns display the different ionic strengths tested on gp36C (left) and HEWL (right). Relative activity (in percentage) towards chloroform-treated PAO1 is calculated relative to the maximal values for both gp36C (I = 0.1, pH 6, 14.6 µg/ml) and HEWL (I = 0.08, pH 6, 10 µg/ml).

activity of gp36C towards both PAO1 and WK6 can be explained by the high similarity in peptide composition [33]. Alternatively, limited muramidase activity on Gram-positive bacteria may not be observed as a decrease in turbidity because of the multiple-layered structure in Gram-positive bacteria.

Enzymatic activity of gp36C and HEWL

We calculated the activity of gp36C and HEWL on chloroform-treated PAO1 cells at the optimal conditions described above. These values are approximately 4800 U/mg and 6800 U/mg for gp36C and HEWL, respectively, with a standard deviation of about 500 U/mg (table 3). We minimized and quantified errors (standard deviations) during activity calculations (e.g. errors occurring during dilution of lysozyme) by multiple measurements with incremental amounts of enzyme. The variation within the linear domain (table 3) indicates the importance of taking into account different concentrations of enzyme. Gp36C can be stored at 4 °C for over a month without loss of activity.

Table 3. Lytic activity of gp36C and HEWL.

	µg	ΔOD_{600}/min	U/mg
gp36C	5.3	0.0258	1455
	2.7	0.02539	2864
	1.3	0.02084	4702
	0.66	0.01193	5383
	0.53	0.00909	5125
	0.44	0.00599	4054
	0.35	0.00564	4775
	0.18	0.00426	7216
4808 ± 503 U/mg			
HEWL	5	0.0175	1050
	1	0.01568	4704
	0.5	0.01077	6460
	0.4	0.00943	7074
	0.3	0.0073	7303
	0.2	0.0041	6147
	0.1	0.00243	7284
	0.05	0.00271	16264
6854 ± 522 U/mg			

Enzymatic activity (in U/mg) of gp36C and HEWL were calculated according to Landuyt et al. [25] for each dilution of added enzyme (5.3 µg of gp36C and 5 µg of HEWL). Based on these activities, we defined the region with a linear correlation between activity and added enzyme (between the dotted lines) and calculated the overall activity (in italics) on the values within this region.

Determination of thermostability

Phage lysins are not known to be thermostable. The T4 tail-associated lysozyme retains only a minor fraction of its activity after a 5-min treatment at 65 °C [31], whereas the T4 lysozyme is completely inactivated by 5 min at 75 °C [30]. Initial experiments showed that gp36C is resistant to temperatures up to 100 °C for 5 min. We therefore gradually increased the time of heat treatment of gp36C and HEWL at 100 °C up to 2 h. Residual activity of the proteins was analysed at optimal conditions in the Bioscreen Microbiology Reader at room temperature with PAO1 as substrate. HEWL is completely inactivated after 1 h at 100 °C. gp36C still has over 50% activity after a similar treatment. Furthermore, after 2 h at 100 °C, 26% residual activity is left. Autoclaving for 20 min at 120 °C and 2 bar still leaves 21% of activity (fig. 3).

Such high thermostability has not yet been described for any phage lysin. Although the thermostability of HEWL

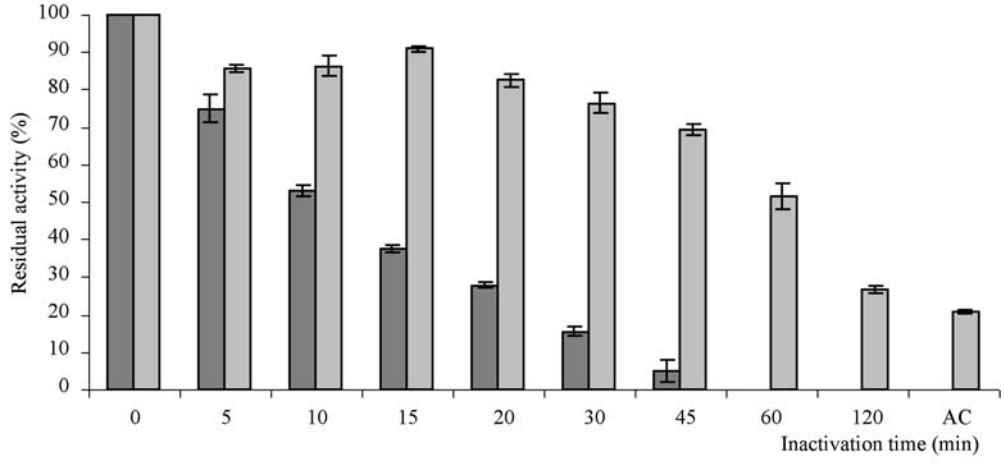

Figure 3. Thermostability of gp36C versus HEWL. Lysozyme samples of gp36C and HEWL (10 µg/ml) heated at 100 °C for an increasing time (horizontal axis; 0–120 min) or autoclaved (AC: 120 °C; 20 min, 2 bar). Samples were subsequently placed on ice before measuring their residual lytic activity at room temperature. The activity of gp36C (pale-grey bars) and HEWL (dark-grey bars) is indicated relative to untreated samples (as percentages). The indicated bars are an average of six measurements, compared to negative controls (without lysozyme).

has been attributed to its compact structure and the presence of four disulphide bonds [34], gp36C does not contain any cysteine residues. Most likely, gp36C has a strong refolding capability after heat treatment. This may be related to the refolding process required during infection. Studies on gp16 of phage T7 (the counterpart of ϕKMV gp36) have shown that this large protein (1318 AA, including the lysozyme domain) has to unfold to pass through the head-tail connection during infection [35].

Conclusion

We have identified gp36 of ϕKMV as a structural protein of the mature phage particle and have successfully cloned, recombinantly expressed and purified the C-terminal end of this protein (M_{737}–E_{898}) in the new expression vector pQE-EC, with a final yield of 0.7 µg/ml and a purity of more than 90% after affinity purification in immobilized Ni^{2+} ions.

The (trans)glycosylase activity of recombinant gp36C was shown for Gram-negative bacterial strains *E. coli* WK6 and *P. aeruginosa* PAO1 and optimized for pH and ionic strength, obtaining an activity of 4800 U/mg.

A unique feature of this protein is its capacity to withstand prolonged heat treatment, making it an interesting candidate for evaluation as a component in food conservation 'hurdle technologies' against Gram-negative bacteria.

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1 Dabora R. L. and Cooney C. L. (1990) Intracellular lytic enzyme systems and their use for disruption of *Escherichia coli*. Adv. Biochem. Eng. Biotechnol. **43:** 11–30
2 Masschalck B., Van Houdt R., Van Haver E. G. R. and Michiels C. W. (2001) Inactivation of Gram-negative bacteria by lysozyme, denatured lysozyme, and lysozyme-derived peptides under high hydrostatic pressure. Appl. Environ. Microbiol. **67:** 339–344
3 De Ruyter P. G. G. A., Kuipers O. P. and De Vos W. M. (1996) Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. **62:** 3662–3667
4 Browder H. P., Zygmunt W. A., Young J. R. and Tavormina P. A. (1965) Lysostaphin: enzymatic mode of action. Biochem. Biophys. Res. Commun. **19:** 383–388
5 Yokogawa K., Kawata S., Takesmura T. and Yoshimura Y. (1975) Purification and properties of lytic enzymes from *Streptomyces globisporus* 1829. Agric. Biol. Chem. **39:** 1533–1543
6 Siegel J. L., Hurst S. F., Liberman E. S., Coleman S. E. and Bleiweis A. S. (1981) Mutanolysin-induced spheroplasts of *Streptococcus mutans* are true protoplasts. Infect. Immun. **31:** 808–815
7 Sher I. H. and Mallette M. F. (1954) Purification and study of L-lysin decarboxylase from *Escherichia coli* B. Arch. Biochem. **53:** 354–369
8 Fastrez J. (1996) Phage lysozymes. In: Lysozymes: Model Enzymes in Biochemistry and Biology, pp. 35–64, Jollès P. (ed.), Birkhauser, Basel
9 Bachali S., Bailly X., Jollès J., Jollès P. and Deutsch J. S. (2004) The lysozyme of the starfish *Asterias rubens*. Eur. J. Biochem. **271:** 237–242
10 Newcombe J., Cartwright K., Dyer S. and McFadden J. (1998) Penetration of the bacterial cell wall: a family of lytic transglycosylases in bacteriophages and conjugative plasmids. Mol. Microbiol. **30:** 453–457
11 Garcia P., Mendez E., Garcia E., Ronda C. and Lopez R. (1984) Biochemical characterization of a murein hydrolase induced by bacteriophage Dp-1 in *Streptococcus pneumoniae*: comparative study between bacteriophage-associated lysin and the host amidase. J. Bacteriol. **159:** 793–796
12 Loessner M. J., Schneider A. and Scherer S. (1996) Modified *Listeria* bacteriophage lysin genes (ply) allow efficient overexpression and one-step purification of biochemically active fusion proteins. Appl. Environ. Microbiol. **61:** 1150–1152
13 Lavigne R., Burkal'tseva M. V., Robben J., Sykilinda N. N., Kurochkina L. P., Grymonprez B. et al. (2003) The genome of bacteriophage ϕKMV, a T7-like virus infecting *Pseudomonas aeruginosa.* Virology **312:** 49–59
14 Dobbins A. T., George M. Jr, Basham D. A., Ford M. E., Houtz J. M., Pedulla M. L. et al. (2004) Complete genomic sequence of the virulent *Salmonella* bacteriophage SP6. J. Bacteriol. **186:** 1933–1944
15 Scholl D., Kieleczawa J., Kemp P., Rush J., Richardson C. C., Merril C. et al. (2004) Genomic analysis of bacteriophages SP6 and K1-5, an estranged subgroup of the T7 supergroup. J. Mol. Biol. **335:** 1151–1171
16 Moak M. and Molineux I. J. (2000) Role of the Gp16 lytic transglycosylase motif in bacteriophage T7 virions at the initiation of infection. Mol. Microbiol. **37:** 345–355
17 Moak M. and Molineux I. J. (2004) Peptidoglycan hydrolytic activities associated with bacteriophage virions. Mol. Microbiol. **51:** 1169–1183
18 Sambrook J. and Russell D. W. (2001) SDS-polyacrylamide gel electrophoresis of proteins. In: Molecular Cloning: A Laboratory Manual, vol. 3, 3rd edn, pp. A8.40–A8.51, Argentine J. (ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.
19 Shevchenko A., Wilm M., Vorm O. and Mann M. (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal. Chem. **86:** 850–858
20 Dumont D., Noben J. P., Raus J., Stinissen P. and Robben J. (2004) Proteomic analysis of cerebrospinal fluid from multiple sclerosis patients. Proteomics **4:** 2117–2124
21 Hertveldt K., Dechassa M. L., Robben J. and Volckaert G. (2003) Identification of Gal80p-interacting proteins by *Saccharomyces cerevisiae* whole genome phage display. Gene **307:** 141–149
22 Gebruers K., Debyser W., Goesaert H., Proost P., Van Damme J. and Delcour J. A. (2001) *Triticum aestivum* L. endoxylanase inhibitor (TAXI) consists of two inhibitors, TAXI I and TAXI II, with different specificities. Biochem. J. **353:** 239–244
23 Landuyt B., Jansen J., Wildiers H., Goethals L., De Boeck G., Highley M. et al. (2003) Immuno affinity purification combined with mass spectrometry detection for the monitoring of VEGF isoforms in patient tumor tissue. J. Sept. Sci. **26:** 1–6
24 Jensen H. B. and Kleppe K. (1972) Studies on T4 lysozyme: affinity for chitin and the use of chitin in the purification of the enzyme. Eur. J. Biochem. **26:** 305–312
25 Cheng X., Zhang X., Pflugrath J. W. and Studier F. W. (1994) The structure of bacteriophage T7 lysozyme, a zinc amidase

and an inhibitor of T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **91:** 4034–4038
26 Grütter M. G. and Matthews B. W. (1982) Amino acid substitutions far from the active site of bacteriophage T4 lysozyme reduce catalytic activity and suggest that the C-terminal lobe of the enzyme participates in substrate binding. J. Mol. Biol. **54:** 525–535
27 Bateman A., Coin L., Durbin R., Finn R. D., Hollich1 V., Griffiths-Jones S. et al. (2004) The Pfam protein families database. Nucleic Acids Res. **32:** D138–D141
28 Tsugita A. and Inouye M. (1968) Purification of bacteriophage T4 lysozyme. J. Biol. Chem. **243:** 391–397
29 Rao G. R. K. and Burma D. P. (1971) Purification and properties of phage P22-induced lysozyme. J. Biol. Chem. **246:** 6474–6479
30 Szewczyk B. and Skorko R. (1981) Lysozyme activity of bacteriophage T4 ghosts. Biochim. Biophys. Acta **662:** 131–137
31 Nakagawa H., Arisaka F. and Ishii S. (1985) Isolation and characterization of the bacteriophage T4 tail-associated lysozyme. J. Virol. **54:** 460–466
32 Black L. W. and Hogness D. S. (1969) The lysozyme of bacteriophage λ. I. Purification and molecular weight. J. Biol. Chem. **244:** 1968–1975
33 Heilmann H. D. (1973) On the peptidoglycan of the cell walls of *Pseudomonas aeruginosa*. Eur. J. Biochem. **43:** 35–38
34 Masschalck B. and Michiels C. W. (2003) Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. Crit. Rev. Microbiol. **29:** 191–214
35 Molineux I. J. (2001) No syringes please, ejection of phage T7 DNA from the virion is enzyme driven. Mol. Microbiol. **40:** 1–8