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Expression, characterization, and antimicrobial ability of T4 lysozyme from methylotrophic yeast *Hansenula polymorpha* A16

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Lysozyme is an enzyme that is essential for protection against bacterial infections. In this study, a T4 lysozyme gene was cloned into the yeast expression vector pPIC9K under the control of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase promoter (pGAP). A *Hansenula polymorpha*-derived ribosomal DNA (rDNA)-targeting element was inserted into the expression vector and was critical for stable DNA integration into the *H. polymorpha* chromosome. Recombinant T4 lysozyme was successfully expressed in the yeast *H. polymorpha* A16; 0.49 g L^{-1} secreted recombinant T4 lysozyme was obtained 72 h after incubation in culture broth that had an initial pH of 6.0. Recombinant T4 lysozyme showed lytic activity against the cell walls of the gram positive bacteria, *Micrococcus lysodeikticus*, and the gram negative bacteria *Xanthomonas campestris* pv. *malvacearum* and *Xanthomonas oryzae* pv. *oryzae*. The zone of inhibition assay was used to evaluate antimicrobial activity. Mass spectrometry showed the N-terminal sequence of recombinant T4 lysozyme was identical to that of the native enzyme. SDS-PAGE indicated that the molecular mass of recombinant T4 lysozyme was 18.7 kD which corresponds to a monomer of the native enzyme. SDS-PAGE without 0.2 mol L^{-1} dithiothreitol treatment detected two bands (15 and 31 kD) suggesting that some recombinant T4 lysozyme formed inter- and intra-molecular disulfide bonds which resulted in loss of enzyme activity.

T4 lysozyme, antimicrobial activity, *Hansenula polymorpha*, pGAP, rDNA

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Lysozymes are enzymes that are naturally present in prokaryotes and eukaryotes, including bacteria [1], plants [2], and animals [3]. They possess antimicrobial activity, inhibit tumor growth and angiogenesis, and may have therapeutic value in antitumor drug development [4]. Furthermore, cloned human, bovine, microbial [5], and hen egg-white [6,7] lysozymes have been effectively used for *in situ* bio-preservation of foodstuffs.

The phage T4 lysozyme gene (GenBank accession No. AF158101) has been cloned and expressed in *Escherichia coli* and its structure and function has been extensively studied [8]. The monomeric lysozyme protein is 164 amino acid residues in length and has a molecular weight of 18.7 kD. The antibacterial activity of T4 lysozyme involves hydrolysis of the bacterial cell wall peptidoglycan murein, and disruption of the bacterial cell membrane [9]. Recent reports showed that T4 lysozyme also has antifungal activity [10].

The phage T4 lysozyme gene has been expressed in various systems [10,11]; however, process efficiency and net yield of active protein are low. Previous studies indicate that *E. coli* transformed with plasmid containing page T4 gene expresses active T4 lysozyme at levels up to 2% of the

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cellular protein after induction with isopropyl-beta-D-thiogalactoside [11]. These recoveries are not high enough for practical applications.

Hansenula polymorpha is a safe reliable expression system that has been successfully developed for commercial-scale recombinant protein production [12–14]. Several *H. polymorpha*-derived products, including Hepatitis B vaccines and insulin, have been approved, marketed, and used in many countries [15]. In this study, we chose the methylotrophic yeast *H. polymorpha*, as the host organism for recombinant T4 lysozyme expression. T4 lysozyme was inserted into the pPIC9K expression vector driven by the *Pichia pastoris* pGAP promoter (GenBank accession No. U62648.1). *H. polymorpha* derived-ribosomal DNA (rDNA) (GenBank accession No. AF467695) was cloned into the transformation vector to serve as a target sequence for homologous recombination [16]. Recombinant T4 lysozyme was secreted in large quantities (0.49 g L^{-1}) and its antimicrobial activity was characterized.

1 Materials and methods

1.1 Strains, vectors, and reagents

Yeast *H. polymorpha* A16 was kindly provided by Dr. Li Ying, Professor of China Agriculture University (Beijing, China). *Micrococcus lysodeikticus* (*M. lysodeikticus*), *Xanthomonas campestris* pv. *malvacearum* (*X. c. malvacearum*), and *Xanthomonas oryzae* pv. *oryzae* (*X. o. oryzae*) were obtained from China General Microbiological Culture Collection Center (Beijing, China). The plasmid pT-T4 was constructed in our laboratory. DNA primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Plasmid pPIC9K was purchased from Invitrogen Corp. (Carlsbad, CA, USA). PCR kits, T4 DNA ligase, and all restriction enzymes were obtained from Clonetech (Mountain View, CA, USA). DNA labeling kits were purchased from Roche Applied Science (Indianapolis, IN, USA). All chemicals, unless otherwise stated, were obtained from Sinopharm Chemical Reagent Beijing Co., Ltd. (Beijing, China).

1.2 Construction of expression plasmids

Bacterial transformations were carried out using *E. coli* DH5α, and plasmid DNA was isolated and purified using DNA kits (BioTeKe Corporation, Beijing, China).

The *P. pastoris* pGAP promoter (485 bp) was PCR amplified from the genome with the following primers: F-G, 5′-TAGAGCTCTTTTTTGTAGAAATGTCTTGGTGT-3′; R-G, 5′-TAGGATCCTGTGTTTTGATAGTTGTTCAATTG-3′ (*Sac* I and *Bam*H I sites are underlined), and inserted into the *Sac* I and *Bam*H I sites of the *P. pastoris* vector pPIC9K to yield the intermediate vector p9K-G. The T4 lysozyme gene was isolated from plasmid pT-T4 with *Eco*R I and *Not* I and ligated into the *Eco*R I/*Not* I sites of p9K-G to generate the yeast expression plasmid p9K-G-T4. A 2.4 kb rDNA fragment was PCR amplified from the genome of *H. polymorpha* with the primers: F-rD, 5′-AAGCATGCTTGCCATAGGCTAGTAATCC-3′; R-rD, 5′-AACATATGGAGACAGGTTAGTTTTACCC-3′ (*Sph* I and *Nde* I are underlined), and cloned into the *Nde* I and *Sph* I sites of p9K-G-T4 to generate the yeast expression plasmid p9K-G-T4-rD. All DNA segments were confirmed at each step and upon completion by restriction digestion and DNA sequencing.

1.3 Transformation of *H. polymorpha* and detection of transformant copy numbers

H. polymorpha A16 transformation was performed according to the method described by Liu *et al*. [17]. Prior to transformation, p9K-G-T4 and p9K-G-T4-rD were linearized with *Xba* I and purified (DNA Purification Kit; BioTeKe). Electrocompetent *H. polymorpha* cells were transformed with the linearized plasmids by electroporation using a Micropulser (200 Ω, 25 μF, 1.5 kV, BTX ECM630, USA), immediately suspended in 1 mL non-selective YPDS media (1% yeast extract, 2% peptone, 2% dextrose, 1.0 mol L^{-1} sorbitol), and plated on selective media (YPDS+G418, 0.3 mg mL^{-1}). Plates were incubated at 37°C for 2–4 d.

Ten randomly picked transformants were screened by PCR using T4 lysozyme primers: F5, 5′-TAGAATTCATGAACATCTTCGAGAAGTTGAG-3′; R3, 5′-TGCGGCCGCTTACAAGTTCTTGTAAGCGTCCC-3′. To select multicopy integrants, 100 randomly picked transformants were plated on YPD media (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) containing increasing concentrations of G418 (0.5, 1, 2 mg mL^{-1}) at 37°C. A randomly selected transformant designated H5 was further characterized by Southern blot analysis. 10 μg H5 genome DNA was digested with *Bgl* II overnight and separated on a 0.8% agarose gel. The DNA was blotted onto an Amersham Hybond™ membrane (GE Healthcare, USA) and probed with a digoxigenin labeled T4 lysozyme PCR fragment. Hybridization, washing, and chemiluminescent detection were performed with the DIG DNA Labeling Kit (Roche, USA).

1.4 Determination of optimum pH and time of culture conditions

Single H5 transformant colonies were picked from a YPD plate containing 2 mg mL^{-1} G418, inoculated into 10 mL BMGY (1% yeast extract, 2% peptone, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol medium; pH values: 5.8, 6.0, 6.2, 6.4, 6.6, 7.0, 7.2, adjusted using 100 mmol L^{-1} potassium phosphate buffer) in 250 mL flasks, and cultured at 37°C with vigorous shaking (180–200 r min^{-1}). Samples

were taken at various time intervals (24, 48, 72 h) to investigate the effects of pH and incubation time on cell growth and recombinant T4 lysozyme expression. Cell densities were determined spectrophotometrically at an absorbance of 660 nm (A_{660}). The cells and culture supernatants were separated by centrifugation at 11000×*g* and 1°C for 1 min. 10 μL crude supernatant from each sample was treated with 0.2 mol L^{-1} dithiothreitol (DTT) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to a previously described method using 15% (v/v) polyacrylamide gels. Protein was detected with Coomassie brilliant blue R-250 staining.

1.5 Scale-up expression and purification of T4 lysozyme

The selected H5 clone was inoculated in 5 mL BMGY medium at 37°C in a shaking 200 r min^{-1} incubator overnight and transferred into 500 mL BMGY at 37°C with vigorous shaking (200 r min^{-1}) for 72 h. The supernatant was collected by centrifugation at 4500×*g* for 10 min and concentrated with 60% ammonium sulfate. The precipitate was collected by centrifugation at 12000×*g* for 30 min, re-suspended in 10 mL 50 mmol L^{-1} sodium phosphate buffer pH 7.2, and dialyzed against 2 L of 50 mmol L^{-1} pH 7.2 sodium phosphate buffer using a 12000–14000 MWCO membrane (GreenBird Co., Ltd., Shanghai, China) for 5 h at room temperature with three changes of dialysis buffer. After the last buffer change, protein dialysis was continued at 4°C overnight to ensure thorough exchange of buffering salts. Recombinant T4 lysozyme protein content was determined with a White/Ultraviolet transilluminator (UVP LLC, USA); 0.25 mg mL^{-1} hen egg-white lysozyme was used as the standard. 10 μL of hen egg-white lysozyme and 10 μL of crude supernatant were analyzed by SDS-PAGE and stained with Coomassie brilliant blue R-250 (Amersco, USA). Proteins on SDS-PAGE gels were analyzed with LabWork 3.0 software (UVP LLC). Recombinant T4 lysozyme protein content in the supernatant was estimated based on the hen egg-white lysozyme solution.

1.6 Disulfide bonds and N-terminal sequence analysis

To assess protein disulfide bond formation, 10 μL lysozyme supernatant was treated with or without 0.2 mol L^{-1} DTT. The samples were boiled for 10 min and analyzed by SDS-PAGE as previously described. To determine the N-terminal amino acid sequence, purified recombinant T4 lysozyme was subjected to SDS-PAGE. The band containing the protein of interest was excised from the polyacrylamide gel and analyzed by mass spectrometry (this service was performed by Beijing Protein Institute, China).

1.7 Enzymatic action on *M. lysodeikticus*, *X. c. malvacearum* and *X. o. oryzae* cell walls

The enzymatic activity of H5 recombinant T4 lysozyme was compared with that of hen egg-white lysozyme using *M. lysodeikticus* (gram-positive) cell wall as the substrate. To prepare the substrate, a single *M. lysodeikticus* colony was selected from a LB (10 g L^{-1} bacto-tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, 1.2% agar) plate and inoculated into 5 mL LB liquid medium in a 150 mL flask at 37°C with overnight vigorous shaking (200 r min^{-1}). The cell pellet was collected by centrifugation at 5000×*g* for 5 min at room temperature, re-suspended in 500 mL 0.05 mol L^{-1} Tris-HCl (pH 7.2) buffer, and separated again by centrifugation. The final cell pellet was re-suspended in the same buffer, frozen immediately at −80°C for 30 min, and freeze-dried (BYK Co., Ltd., Beijing, China) at 15 Pa and −50°C for 30 h. 100 mg of freeze-dried *M. lysodeikticus* powder was suspended in 200 mL of 0.05 mol L^{-1} Tris-HCl, pH 7.2 for use in the assay.

BSA (negative control; Roche), recombinant T4 lysozyme, and hen egg-white lysozyme solutions were diluted in 0.05 mol L^{-1} Tris-HCl (pH 7.2) to concentrations of 0.0, 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, 5×10^{-3}, 1×10^{-2}, 5×10^{-2}, 0.1, 0.5, and 1.0 mg mL^{-1}. 0.1 mL of prepared lysozyme solution and 0.9 mL substrate (0.5 g L^{-1} *M. lysodeikticus*) were added to a 1.4 mL plastic cuvette, covered with Parafilm, gently inverted several times to mix, and incubated at 37°C for 1 h. The turbidity change of the *M. lysodeikticus* suspension at A_{350} measured with a spectrophotometer (Purkinje General Instrument Co. Ltd., Beijing, China) provided an estimate of lysozyme enzymatic activity. A decrease in the 350 nm absorbance (A_{350}) of the mixture was caused by the lysis of *M. lysodeikticus* (1). *X. c. malvacearum* and *X. o. oryzae* cell wall lysis were measured by the same method.

1.8 Lysozyme inhibition of bacteria

A single *M. lysodeikticus* colony was selected from a LB plate and inoculated in 5 mL LB liquid medium in a 150 mL flask at 37°C with shaking (200 r min^{-1}) overnight. The LB agar was boiled (100°C) to melt, cooled to 45°C, and maintained in a 45°C water bath. The *M. lysodeikticus* LB culture was diluted 1000 folds and 1 mL was transferred into 149 mL LB agar in a 250 mL flask. After mixing the flask with vigorous shaking in the water bath, the inoculated agar was poured into a petri dish (20 mL). The plate was left undisturbed until the agar solidified. Three wells (5 mm in diameter) from each plate were punched out with an aseptic hole punch. 50 μL 10 mg mL^{-1} recombinant T4 lysozyme, BSA (negative control; Roche), and hen egg-white lysozyme (positive control) were pipetted into separate wells. After incubating the cultures at 37°C for 3 d, zones of inhibition (diameters of the clear areas) were measured with a ruler (mm). The diameter of the well was excluded.

Growth inhibition for *X. c. malvacearum* and *X. o. oryzae* was assessed using the same method, except the culture medium for *X. c. malvacearum* was SOB (20 g L^{-1} bacto-tryptone, 5 g L^{-1} bacto-yeast extract, 0.5 g L^{-1} NaCl) and the culture medium for *X. a. oryzae* was PSA (10 g L^{-1} bacto-tryptone, 10 g L^{-1} sucrose, 1 g L^{-1} L-glutamate).

2 Results and discussion

2.1 Construction of recombinant vectors p9K-G-T4 and p9K-G-T4-rD

Two yeast expression plasmids, p9K-G-T4 and p9K-G-T4-rD, were successfully constructed (Figure 1). The *P. pastoris*-derived pGAP promoter was used to drive T4 lysozyme expression in *H. polymorpha* in both plasmids. rDNA from *H. polymorpha* was introduced to p9K-G-T4-rD to test rDNA function on integrated homologous integration in *H. polymorpha* (16). Final DNA sequencing of the pGAP promoter, T4 lysozyme gene, and rDNA confirmed that they matched the GenBank database. The orientation of the cloned DNA fragments, and the structures of the intermediate vectors and the final expression constructs were verified by restriction endonuclease cleavage (data not shown).

2.2 *H. polymorpha* A16 transformation

rDNA is a site-specific integration element [16] and is the site of stable DNA integration in the yeast chromosome. We analyzed the transformation efficiency of the expression vector containing the 2.4-kb segment of *H. polymorpha* rDNA (partial 25S, a complete 5S, and the non-transcribed spacer region between 25S and 18S rDNA) [16]. p9K-G-T4 and p9K-G-T4-rD were linearized with *Xba* I and transformed into *H. polymorpha* A16 cells by electroporation. No transformants were obtained from plasmid p9K-G-T4. However, the transformation frequency of p9K-G-T4-rD was 100–1000 transformants/μg DNA. Ten of these transformants were randomly selected and screened using PCR analysis with T4 primer pairs; all contained the T4 lysozyme gene (data not shown). One hundred transformants were taken at random and screened on YPDS plates in the presence of different concentrations of G418. None of the transformants exhibited obvious growth inhibition when treated with 0.5 g mL^{-1} G418, the transformants grew more slowly as the concentration of G418 was increased, and 70% transformants survived in the presence of 2 mg mL^{-1} G418 (data not shown). These results suggest that the transformants had multiple copies of the T4 lysozyme gene which allowed them to resist G418 selection pressure [13]. To estimate the copy-number of the T4 lysozyme gene in the transformants we selected a single colony, H5 from a 2 mg mL^{-1} G418 plate. H5 genomic DNA was extracted, digested with *Bgl* II restriction enzyme, which did not cut within T4 lysozyme gene (cDNA), and subjected to Southern blot analysis. Two bands (11 and 9 kb) were detected when blots were hybridized with the T4 lysozyme gene (495 bp) DNA probe (Figure 2).

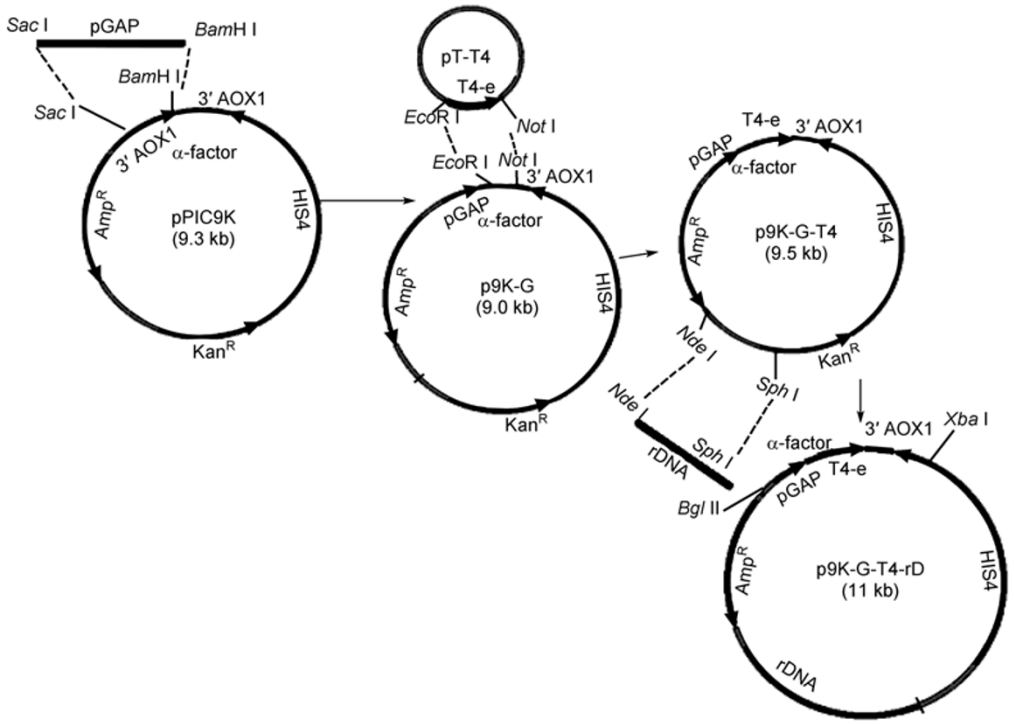

Figure 1 Construction of *H. polymorpha* T4-lysozyme expression system. pGAP, *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase promoter; Amp^R, aminobenzylpenicillin-resistant gene; Kan^R, kanamycin-resistant gene; rDNA, ribosomal DNA.

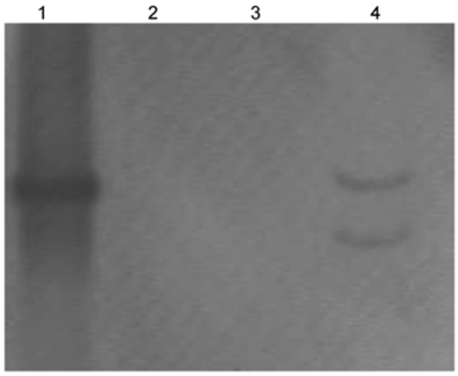

Figure 2 H5 transformant copy number was evaluated by Southern blot analysis. Lane 1, plasmid DNA p9k-G-T4-rD digested with Bglll (positive control); Lane 2, blank; Lane 3, *H. polymorpha* A16 genomic DNA (5 μg); Lane 4, recombinant H5 genomic DNA (5 μg).

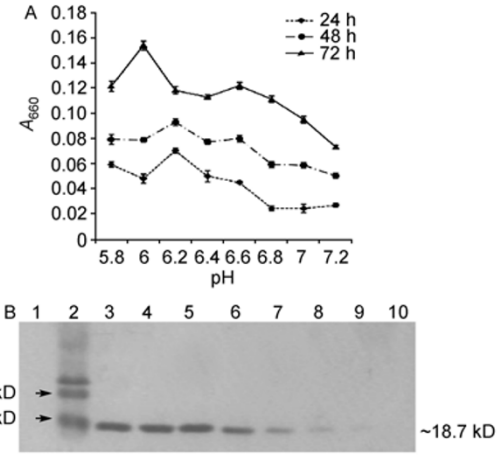

Figure 3 Specific growth rate of transformants at 37°C in media at various pH values (A) and SDS-PAGE analysis of recombinant T4 lysozyme proteins expressed in media at various pH values (B). A, The culture media were collected at 24, 48, and 72 h. The growth rate was analyzed at A_{660}. Values are means of three replicates±SD. B, The cells were sampled at 72 h incubation. 10 μL crude supernatant from each sample was loaded. Lane 1, *H. polymorpha* A16 supernatant (negative control); Lane 2, standard protein marker; Lanes 3–10, recombinant H5 supernatant sampled at pH 5.8, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2. Typical results from three similar experiments.

Our results suggest that rDNA is required for DNA integration in *H. polymorpha* and is important for high-copy-number integration. These data are in accordance with other studies that show rDNA promotes efficient integration and transformation in *H. polymorpha*, and that 2–30 copies can be integrated into rDNA loci [17].

2.3 Effects of pH and culture length on protein expression

To identify the optimal pH for growth of the transformants, H5 was inoculated into media at various pH values (Figure 3A).

Culture media were collected at 24, 48, and 72 h intervals. The growth of transformant yeast cultures was measured by absorbance at a wavelength of 660 nm (A_{660}). The pH of the culture media had a significant effect on the growth rate of the yeast. The optimal pH range for growth of *H. polymorpha* transformants was between 5.8 and 6.2, and growth rates successively declined as the pH of the media increased above 6.2. To identify the optimal time of harvest, recombinant T4 lysozyme expression was measured as a function of time. Recombinant T4 lysozyme protein was extracted from the cultures at different time points, treated with 0.2 mol L^{-1} DTT, and analyzed by SDS-PAGE (Figure 3B). The absorbance of cultures at 660 nm did not increase after 72 h incubation, which showed that the cells had reached confluence.

No expression of recombinant T4 lysozyme protein was observed at 24 and 48 h (data not shown). A 19 kD protein band corresponding to T4 lysozyme protein (18.7 kD) was observed in the 72 h culture. Based on these data, for subsequent experiments, we chose pH 6 as optimal for the yeast growth medium and an incubation time of 72 h as an optimal harvest time.

2.4 Production and characterization of recombinant T4 lysozyme

Recombinant T4 lysozyme protein was expressed in *H. polymorpha* and purified by dialysis. The N-terminal sequence of the SDS-PAGE band was analyzed by mass spectrometry and was identical to that of native T4 lysozyme (MNCFEMLR). When compared with various known concentrations of hen egg-white lysozyme, the yield of recombinant T4 lysozyme was 0.49 g L^{-1} culture medium. These observations confirm that foreign T4 lysozyme protein can be expressed in *H. polymorpha* under the control of the *P. pastoris* pGAP promoter. Our data are in accordance with previous reports which show that pGAP is useful for large-scale constitutive expression of many heterologous proteins in *P. pastoris* [18–20].

2.5 Antimicrobial activity of recombinant T4 lysozyme

The lytic activity of T4 lysozyme against the gram-positive bacteria *M. lysodeikticus* was determined by its enzymatic action on the cell wall [1]. A decrease in the absorbance of *M. lysodeikticus* cell wall suspensions (substrate solution) at 350 nm indicated an increase in recombinant T4 lysozyme concentration (Figure 4A).

The results show that recombinant T4 and hen egg-white lysozymes hydrolyzed the *M. lysodeikticus* cell wall, and indicate that recombinant T4 lysozyme possesses muramidase activity that can damage the peptidoglycan layers in bacterial cell walls [9]. However, the enzymatic activity of recombinant T4 lysozyme on the *M. lysodeikticus* cell wall was lower than that of hen egg-white. At 0.05 mg mL^{-1} protein, the *M. lysodeikticus* A_{350} for hen egg-white and recombinant T4 lysozymes were 0.50 and 1.53, respectively; at 1 mg mL^{-1}, the A_{350} for recombinant T4 lysozyme was

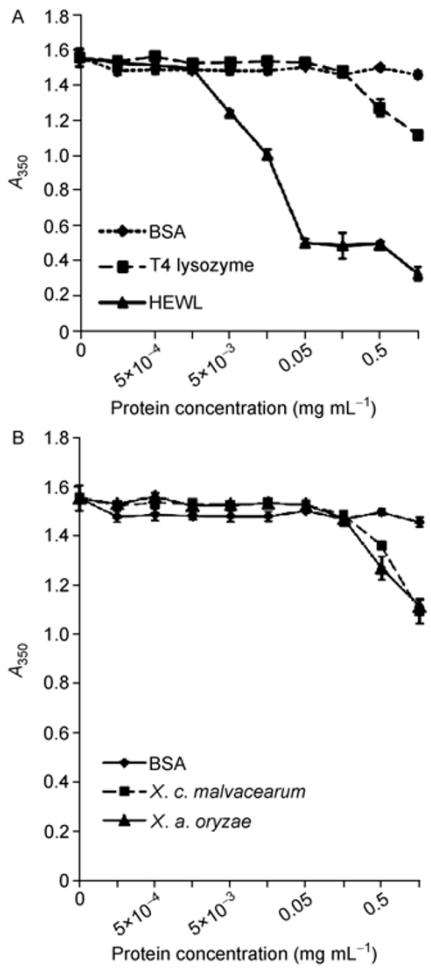

Figure 4 Hydrolysis of *M. lysodeikticus* cell wall by different concentrations of recombinant T4 phage lysozyme and hen egg-white lysozyme (HEWL; 0.0, 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, 5×10^{-3}, 1×10^{-2}, 5×10^{-2}, 0.1, 0.5, 1.0 mg mL^{-1}) (A) and hydrolysis of *X. c. malvacearum* and *X. a. oryzae* cell walls by different concentrations of recombinant T4 phage lysozyme (0.0, 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, 5×10^{-3}, 1×10^{-2}, 5×10^{-2}, 0.1, 0.5, 1.0 mg mL^{-1}) (B). A, BSA was the negative control. Cell turbidity was determined spectrophotometrically at A_{350}. Values are means of three replicates±SD. B, BSA was the negative control. The cell turbidity was determined spectrophotometrically at A_{350}. Values are means of three replicates±SD.

1.11. In contrast to these data, previous reports showed that phage T4 lysozyme had higher lytic activity than that of hen egg-white [1]. We found that recombinant T4 lysozyme also lysed the gram negative bacteria, *X. c. malvacearum* and *X. a. oryzae*. Based on these results, we propose that recombinant T4 lysozyme disrupts or alters the outer membrane layers of gram negative bacteria and causes them to form osmotically fragile rods [21].

The antimicrobial activity of recombinant T4 lysozyme was confirmed by measuring zones of inhibition on bacterial culture agar plates. As shown in Table 1, recombinant T4 lysozyme inhibited the growth of *X. c. malvacearum* and *X. a. oryzae* with inhibition zone diameters of (11±1) and (19.3±0.58) mm, respectively. However, recombinant T4 lysozyme did not inhibit *M. lysodeikticus* growth even though it exhibited the ability to lyse the cell wall. In addition, the inhibition assay indicated that recombinant T4 lysozyme has lower antibacterial ability than hen egg-white (*M. lysodeikticus*, (34.7±0.58) mm; *X. c. malvacearum*, (35±1) mm; *X. a. oryzae*, (21±1) mm). We also used *E. coli* (gram-negative) to measure recombinant T4 lysozyme bactericidal activity. Our results show that recombinant T4 and hen egg-white lysozymes had no effects on the growth of *E. coli* (Table 1). These data are in contrast to reports showing native T4 lysozyme degradation of the periplasmic peptidoglycan layer of *E. coli* (9).

Disulfide bond formation of recombinant T4 lysozyme was analyzed by DTT treatment. DTT is commonly used to reduce disulfide bonds in proteins [22]. Concentrated recombinant T4 lysozyme was treated with or without DTT and analyzed by SDS-PAGE (Figure 5). In the presence of DTT (Figure 5, Lane 2), a single band was produced with a molecular weight of 18.7 kD, which is equivalent to the molecular weight of native T4 lysozyme. Two bands were observed without DTT treatment (Figure 5, Lane 1). The major band in the untreated sample was approximately 15 kD. A minor band had a molecular weight of 31 kD. These data indicate that most recombinant T4 lysozyme forms intramolecular disulfide bonds, and some forms intermolecular disulfide bonds.

We also overexpressed T4 lysozyme in *E. coli*. However, after T4 transformation, the yield was very low as most of

Table 1 Zones of inhibition (mm)[a)]

	T4 lysozyme	HEWL
E. coli	0	0
M. lysodeikticus	0	34.7±0.58
X. c. malvacearum	11±1	35±1
X. a. oryzae	19.3±0.58	21±1

a) Comparison of the bactericidal activities of recombinant T4 phage lysozyme and hen egg-white lysozyme (HEWL) based on the average diameter (mm) of the zones of inhibition. 10 mg mL^{-1} protein was used for the assay. Values are means of three replicates±SD.

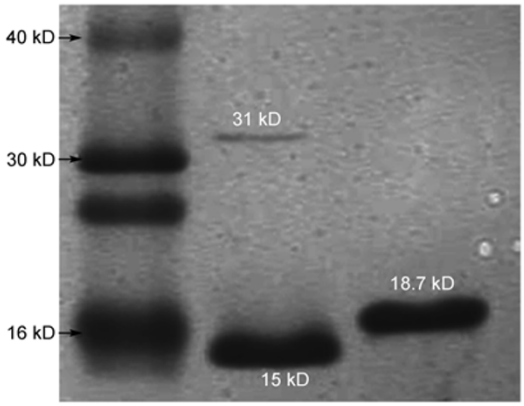

Figure 5 SDS-PAGE analysis of T4 disulfide bonds. Lane 1, standard protein weight; Lane 2, T4 lysozyme supernatant without DTT treatment; Lane 3, T4 lysozyme supernatant with DTT treatment.

the *E. coli* was hydrolyzed (data not shown). Functional native T4 lysozyme has two cysteines, but it does not form disulfide bonds [8,22]. By contrast, recombinant T4 lysozyme produced from *H. polymorpha* was unable to degrade the peptidoglycan in *E. coli* probably because it possessed incorrectly formed disulfide bonds which affected protein activity [23]. Future studies may involve altering one of the T4 phage lysozyme cysteines to prevent the formation of these disulfide bonds.

3 Conclusion

The *H. polymorpha* system has been widely used for heterologous protein production. In this study, we used the *H. polymorpha* expression system to produce T4 phage lysozyme. The protein was driven by the *P. pastoris* pGAP promoter. We produced a large quantity of recombinant T4 lysozyme (0.49 g L^{-1} culture broth supernatant) in a relatively short period of time (72 h), as compared with the *E. coli* protein expression system. The *H. polymorpha*-derived rDNA-targeting element was found to be essential for overexpressing recombinant T4 lysozyme in *H. polymorpha*. Without the rDNA-targeting element, the foreign T4 lysozyme could not be integrated into the *H. polymorpha* genome. Recombinant T4 lysozyme inhibited certain species of gram positive and gram negative bacteria. However, it exhibited lower enzyme activity than hen egg-white lysozyme. Investigations of the protein structure of recombinant T4 lysozyme indicated that it formed disulfide bonds which resulted in the loss of lysozyme activity. Further experiments are needed to develop strategies to prevent erroneous disulfide bond formation in recombinant T4 lysozyme in the *H. polymorpha* expression system.

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