

Full Length Research Paper

Codon optimization, constitutive expression and antimicrobial characterization of hen egg white lysozyme (HEWL) in *Pichia pastoris*

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Accepted 22 June, 2012

Fusarium oxysporum (*F. oxysporum*) and *Verticillium dahlia* (*V. dahlia*) causes severe cotton disease in China and other cotton-producing countries. Hen egg white lysozyme (HEWL) has antimicrobial properties. In this study, a codon-optimized HEWL gene was synthesized and cloned into the yeast expression vector, pPIC9K, under the control of the *Pichia pastoris* (*P. pastoris*) glyceraldehyde-3-phosphate dehydrogenase promoter (pGAP). Results showed that codon-optimized HEWL (oHEWL) was constitutively expressed. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) indicated that the molecular weight of recombinant HEWL (roHEWL) was 14 kDa which corresponds to the standard HEWL. The expression of the roHEWL reached to 54 mg/L. Activity of the roHEWL was 1680 U/mL. The optimum pH for roHEWL was from 6.0 to 7.0, and the optimum temperature was 55°C. *In vitro* antimicrobial activity assay revealed that roHEWL can lyse cell walls of the gram positive bacteria, *Micrococcus lysodeikticus* (*M. lysodeikticus*). *In vivo* studies showed that it inhibits plant fungi, *F. oxysporum* and *V. dahlia*. roHEWL anti-fungal properties might be useful for future genetically engineered cotton plant resistance against pathogenic fungal disease.

Key words: Hen egg white lysozyme (HEWL), antimicrobial activity, glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter, codon optimization, constitutive expression.

INTRODUCTION

Lysozyme is an antimicrobial enzyme that is found in a wide variety of organisms, including bacteria (Tsugita et al., 1968), plants (Wang et al., 2005) and animals (Merlini et al., 2005). Among those lysozymes, hen egg white lysozyme (HEWL) has been extensively studied, as HEWL is also used as model proteins for protein folding and aggregation (Voets et al., 2010; Xie et al., 2011). Inducible expression of HEWL has been studied in yeast *Pichia pastoris* (Liu et al., 2003; Masuda et al., 2005). The inducible promoter, AOX1 promoter (pAOX1),

derived from the alcohol oxidase (AOX) gene, has been used to express foreign genes in *P. pastoris* (Cereghino et al., 2000; Woo et al., 2002). pAOX1 allows *P. pastoris* to use methanol as a carbon resource. The AOX1 promoter is induced by methanol, and repressed by glucose and glycerol. However, methanol is extremely toxic. It is a volatile, colorless and flammable liquid which is very difficult to remove during the downstream protein production.

Since Waterham et al. (1997) successfully cloned the glyceraldehyde-3-phosphate dehydrogenase promoter (pGAP) from *P. pastoris*, pGAP has been widely utilized for expression of various heterologous proteins in *P. pastoris*. A number of heterologous proteins have been produced under the control of pGAP (Delroisse et al., 2005; Zhang et al., 2007; Qiao et al., 2010) and because no methanol is needed, the hazard and cost associated with methanol is eliminated. It is an attractive alternative

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Abbreviations: roHEWL, Recombinant codon optimized hen egg white lysozyme; pGAP, glyceraldehyde-3-phosphate dehydrogenase promoter.

expression system. Constitutive expression of HEWL has not been reported. In order to study HEWL anti-plant fungal property, we successfully expressed codon-optimized HEWL in *P. pastoris* under the pGAP. *In vitro* antimicrobial activity assay showed that roHEWL is resistant to *Micrococcus lysodeikticus*. *In vivo* assay revealed that roHEWL is against plant fungi, *Fusarium oxysporum* and *Verticillium dahliae*. Trudel et al. (1992, 1995) also reported anti-*M. lysodeikticus* activity of the transgenic tobacco plant. On the basis of these results, we propose that over expression of codon-optimized HEWL in future transgenic plant might provide a promising approach for anti-bacterial and anti-fungal pathogens.

MATERIALS AND METHODS

Strains, vectors, and reagents

Yeast, *P. pastoris* GS115 and Plasmid pPIC9K were bought from Invitrogen (Carlsbad, CA, USA). Plasmid pGAP9K and *M. lysodeikticus* were reserved in our laboratory. *F. oxysporum* and *V. dahliae* were kindly provided by Dr. Jian GuiLiang, professor of the Plant Protection Institute at the Chinese Academy of Agricultural Sciences (Beijing, China). T4 DNA ligase and all other restriction enzymes used were purchased from TaKaRa Biotechnology Company Limited (DaLian, China). Standard HEWL, snailase and G418 were purchased from BioDee BioTech Company Limited (Beijing, China).

Construction of expression plasmid

HEWL full-length DNA (390 bp) (GenBank accession no. NP_990612.1) was codon-optimized for *P. pastoris* expression using Invitrogen Gene Optimizer® design platform. An *Xho*I restriction site and a yeast KEX2 signal cleavage sequences (GAGAAAAGA) were added in front of the start codon AAG. Optimized codon was synthesized and assembled by Invitrogen (Carlsbad, CA, USA). The plasmid pGAP common forward primer (9k5':5'-CGGTTACTCCGACTTGGAGGGTG-3') and co-HEWL reverse primer (hel3':5'-GCGGCCGCTTACAATCTACAACCTCTGATCC-3', *Not*I site underlined) were synthesized by Sangon Biotech Company Limited (Shanghai, China). The Codon optimized HEWL was cloned into *Xho*I and *Not*I yeast expression vector, pGAP9K, to yield pGAP9K-hel.

Transformation of *P. pastoris* and transformants detection

P. pastoris GS115 transformation was processed according to the method described by Invitrogen protocol. Prior to transformation, pGAP9K-hel was linearized with *Sa*I. After electroporation, the samples were immediately suspended in 1 mL sorbitol solution, and then co-cultivated with 1 mL non-selective yeast peptone dextrose (YPD) media at 28°C for 1 h. Yeast cells were plated on selective RDB media (1 M sorbitol, 2% dextrose, 1.34% YNB, 0.005% biotin, 0.005% L-glutamic acid, 0.005% L-methionine, 0.005% L-lysine, 0.005% L-leucine, 0.005% L-isoleucine, 2% agar, 0.5 mg/mL G418) and incubated at 28°C for 2 to 3 days. 60 transformants were randomly picked and screened by polymerase chain reaction (PCR) using designed primers, 9k5' and hel3'. PCR positive transformants were inoculated and thoroughly mixed in 20 µL sorbitol solution with snailase in Eppendorf tube, ice incubation for 30 min, and then cell

lysis at 37°C water bath for 15min. Supernatant was collected after centrifugation (11000 × g, 10 min) and analyzed by SDS-PAGE.

Recombinant protein detection by SDS

The selected clone was initially inoculated in 50 mL BMGY medium at 28°C for 72 h, and then transferred in 50 L yeast fermenter for large scale expression. The supernatant was collected by centrifugation at 5000 × g for 10 min at 4°C, and filtered by a 50 kD hollow fiber cartridge device (No. MOF-503, MoTimo Company Limited, Tianjin, China). The collected solution was dialyzed again with a 10 kD nano-filtration membrane (GE, USA). 700 mL collection was diluted with 6.3 L water, mixed thoroughly and dialyzed with the 10 kD nano-filtration membrane. The dialysis process was repeated 5 times. The final product was freeze-dried and stored at 4°C. Purified roHEWL was analyzed by SDS-PAGE. The intensity of stain in the bands is measured and recorded by a densitometer. The data was analyzed by UVP Labwork 3.0. Purity of roHEWL was estimated based on the UVP Labwork 3.0 analysis.

Determination the enzymatic activity, effects of pH and temperature for roHEWL activity assay

The enzymatic activity of roHEWL was estimated by measuring the clearing of turbidity of *M. lysodeikticus* suspension (substrate solution) at OD₆₅₀ (Muraki et al., 1988). The rate of absorbance change at 650 nm per min was defined as 1 U of roHEWL enzymatic activity. Freeze-dried *M. lysodeikticus* powders were suspended in 0.01 M phosphate buffer, (pH 6.2) and diluted to 0.6 to 1.0 of OD₆₅₀. To a 96 well plate, 0.1 mL of purified roHEWL (0.1 mg/ml) solution was added to 0.1 mL prepared substrate (*M. Lysodeikticus*) for each well. The samples were then measured at 650 nm (OD₆₅₀) immediately. After 1 min reaction time, a decrease in the absorbance at 650 nm (OD₆₅₀) of the mixture, caused by the lysis of *M. lysodeikticus*, was measured again. To find the optimal pH and temperature for roHEWL activity, respectively, the roHEWL was tested at various pH and temperature conditions, respectively, using this method.

Lysozyme inhibition of bacteria

A single *M. lysodeikticus* colony was inoculated in 3 ml LB liquid medium at 37°C with shaking (200 rpm) overnight. The LB agar was boiled (100°C) to melt, cooled to 50°C, and mixed with diluted *M. lysodeikticus* LB culture (diluted by 10000 folds). The mixture was poured into a petri dish. After the agar solidified, five wells (5 mm in diameter) from each plate were punched out with an aseptic hole punch, and 50 µL of roHEWL supernatant was pipetted into the wells. After incubating the cultures at 37°C for 24 h, zones of inhibition (clear area diameters) were measured by a ruler (mm).

Antifungal activity assay

Phytopathogenic fungi species, *F. oxysporum* and *V. dahliae*, were inoculated on potato dextrose agar (PDA) plate and incubated at 25°C for ten days. Fresh PDA was prepared and autoclaved. Before agar solidified, purified roHEWL was added to the PDA at a concentration of 5, 2.5, 1.25, 0.625 and 0.3125 mg/mL. The roHEWL-agar solution was mixed thoroughly to ensure even distribution and then decanted into petri dishes. A circle disc (5 mm in diameter) from previously prepared inoculated plate, with actively growing fungi culture, was punched out and placed on the center of the roHEWL-agar plate. Fungi were incubated at 25°C for 10 days in the dark. Fungal growth (colony diameter) was measured, and

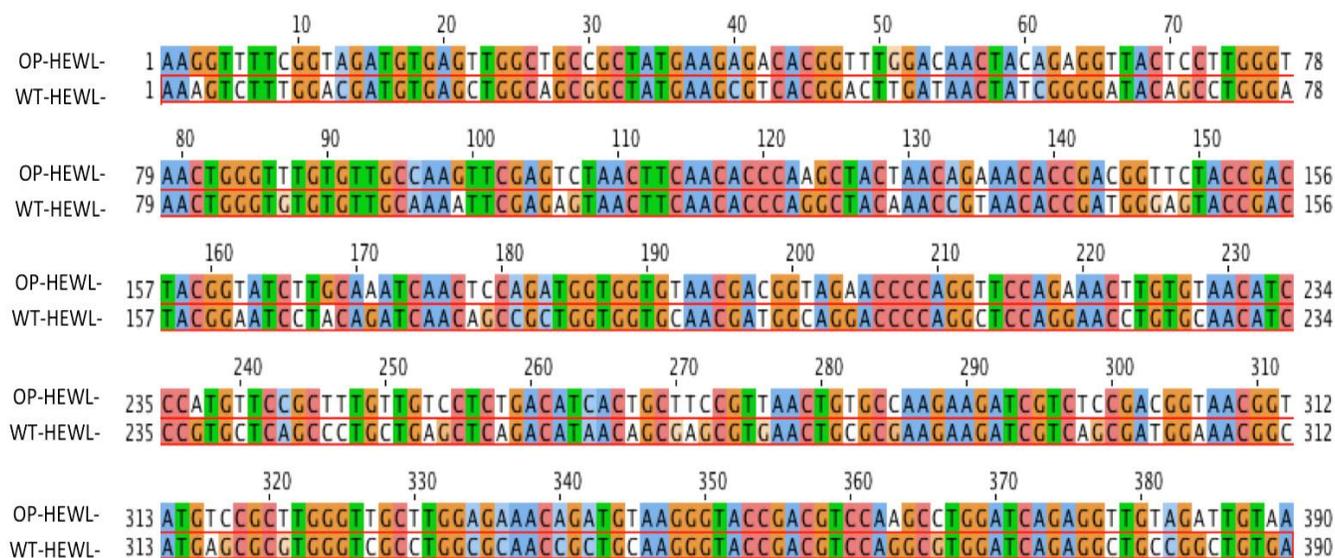


Figure 1. Comparison of the optimized HEWL gene sequence (OP-HEWL) with sequence (WT-HEWL) from Genbank (accession no. NP_990612.1).

percentage inhibition calculated according to the formula:

$$\text{Percentage inhibition} = (C - T) / C \times 100\%$$

Where, C = Colony diameter (mm) of the control, T = colony diameter (mm) of the treated plate.

RESULTS

Construction of expression vector pGAP9K-hel

The HEWL nucleotide sequence was optimized by Invitrogen Gene Optimizer[®] design platform. Codon substitutions were based on a usage frequency known to be highly expressed in *P. pastoris*. Optimized GC content was adjusted from 57 to 45% (Figure 1). The amino acid sequence between the two proteins remained the same. The synthesized gene fragment was inserted into *Xho* I/*Not* I sites of pGAP9K to produce pGAP9K-hel, and the optimized HEWL was confirmed by DNA sequencing. The final expression constructed was verified by restriction endonuclease cleavage (data not shown).

P. pastoris GS115 transformation, roHEWL expression and purification, SDS-PAGE analysis.

Hundreds of colonies were grown on RDB selective plates. Randomly selected transformants were screened using PCR analysis with 9K5' and hel3' primer pairs. A 600 bp fragment, with designed primers from PCR result suggested the presence of insert optimized HEWL gene fragment (data not shown). The selected transformant was inoculated in RDB plate containing 0.5 mg/mL G418

at 28°C for 2 to 3 days, and then transferred to 50 L yeast fermenter for large scale expression. Broth supernatant was collected by centrifugation and purified by dialysis. The purified roHEWL was analyzed by SDS-PAGE. A band with the expected molecular weight (14 kD) (Figure 2) was observed. The protein content of the roHEWL was analyzed by the SDS-PAGE gel, compared with the commercial HEWL (98% purity). Comparing with the standard HEWL 200 mg/L, the yield 54 mg/L of roHEWL was determined by densitometer, and analyzed by UVP Labwork 3.0. However, the lane roHEWL lane also showed several other bands, which indicates that the purification process needs more improvement.

Determination the enzymatic activity, effects of pH and temperature for roHEWL activity assay

The roHEWL activity was determined up to 1680 U/mL according to the method described by Muraki (1998). In order to find the optimal pH and temperature of roHEWL, the roHEWL activity was tested at various pH and temperature conditions, respectively (Figures 3 and 4). The highest enzymatic activity was determined from pH 6.0 to 7.0, which is similar to the standard HEWL. The activity successively declined, as the pH of the medium increased above pH 7.0. Under alkaline conditions, the enzymatic activity of roHEWL was slightly higher than the standard HEWL (Figure 3). The impact of temperature on the roHEWL activity is shown in Figure 4. roHEWL showed enzymatic activity at the temperature range of 15 to 75°C, which is similar to the standard HEWL. The optimal temperature of roHEWL activity was 55°C. When the temperature exceeded 75°C, both recombinant and

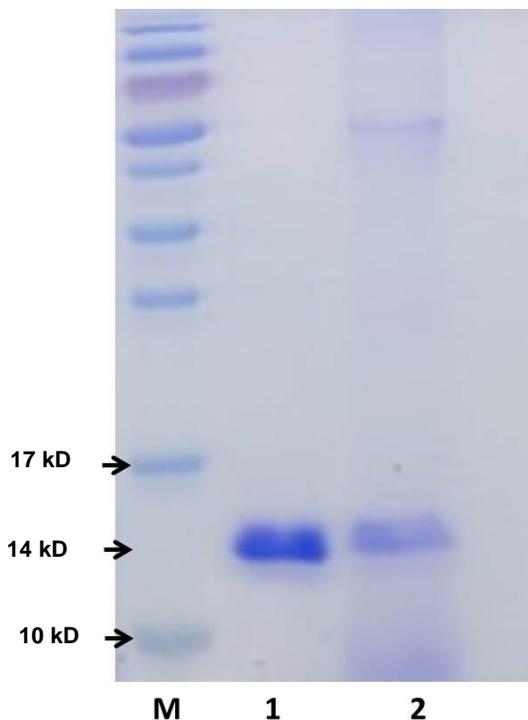


Figure 2. SDS-PAGE analysis of purified roHEWL.

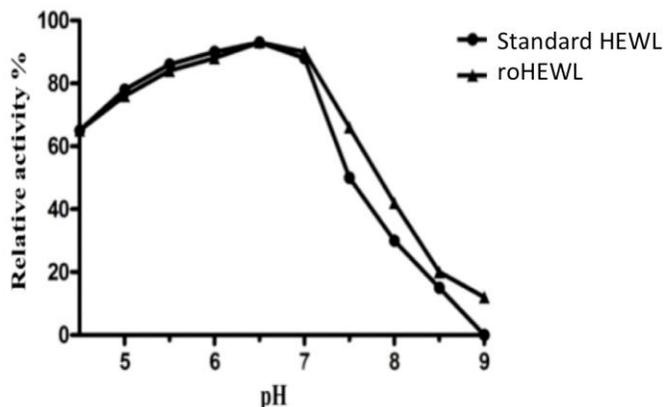


Figure 3. Effect of pH on the roHEWL stability.

standard HEWL lost bacteriolytic activity completely (Figure 4).

Antimicrobial activity of roHEWL

The lytic activity of roHEWL against *M. lysodeikticus* was tested with the zone of inhibition method. The result showed that the roHEWL can form a clear zone of inhibition (Figure 5). Comparing with the standard HEWL (0.1 mg/mL), purified roHEWL showed smaller zone of inhibition. Since some other proteins contaminated with

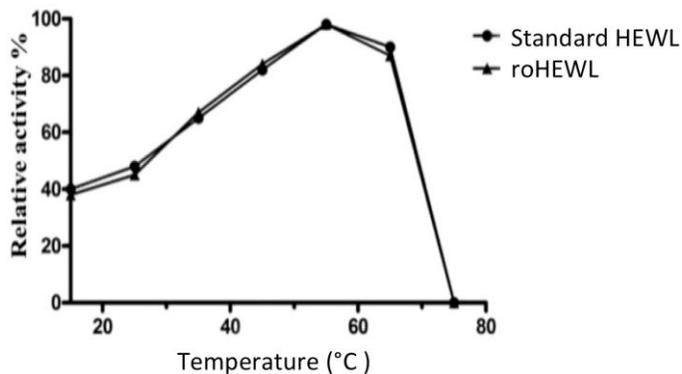


Figure 4. Effect of temperature on the roHEWL stability.

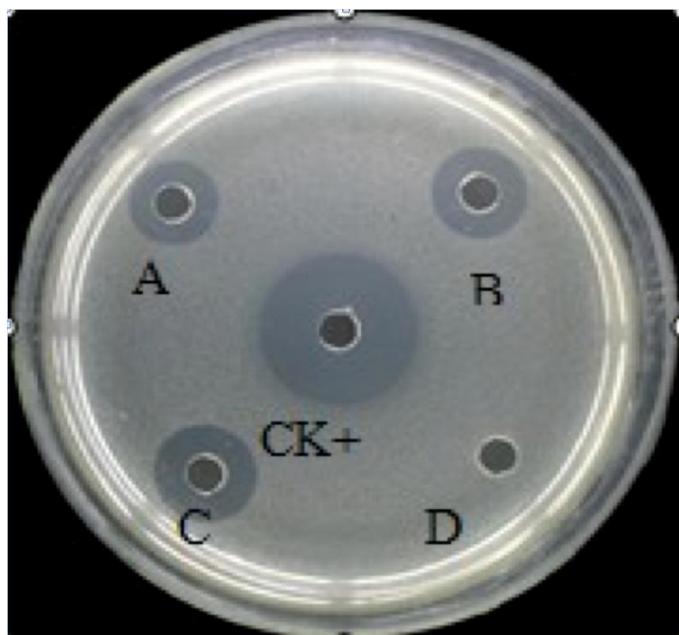


Figure 5. Lytic activity of the roHEWL against *M. lysodeikticus*. CK+, the native HEWL (0.1 mg/mL); A to C, the purified roHEWL (0.1 mg/mL); D, broth supernatant from negative control.

roHEWL (Figure 2), the purification process needs to be further improved. The optimization of fermentation conditions and purification might increase the expression of roHEWL levels.

Inhibition growth of pathogenic fungi

Compared with the negative control, the petri dish added with the roHEWL showed significantly smaller fungal colony (Figures 6 and 7). During the entire observation period (cultivation up to 8 days), with the increasing of the roHEWL concentrations, the effect of *F. oxysporum* inhibition also increased (Figure 6). In the early stage of incubation, the highest inhibitory effect was observed.

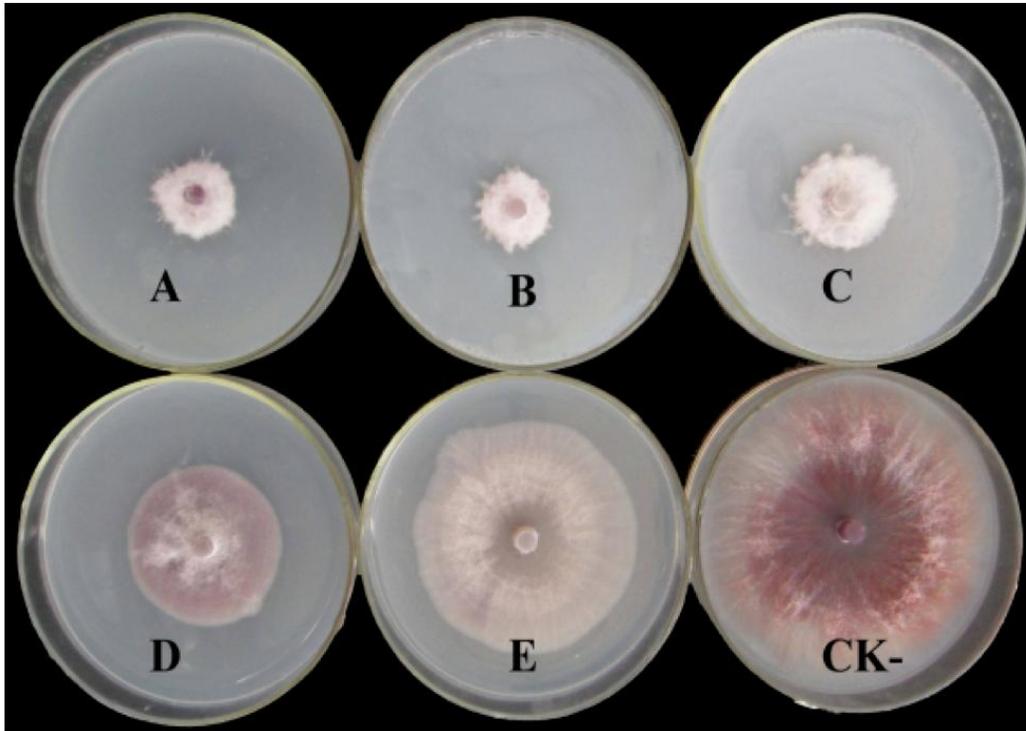


Figure 6. roHEWL inhibits the growth of *F.oxysporum* (cultured up to 8 days) A, B, C, D, and E: roHEWL concentrations were 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL, and 0.3125 mg/mL, respectively, CK- : negative control (phosphate buffer, pH6.2).

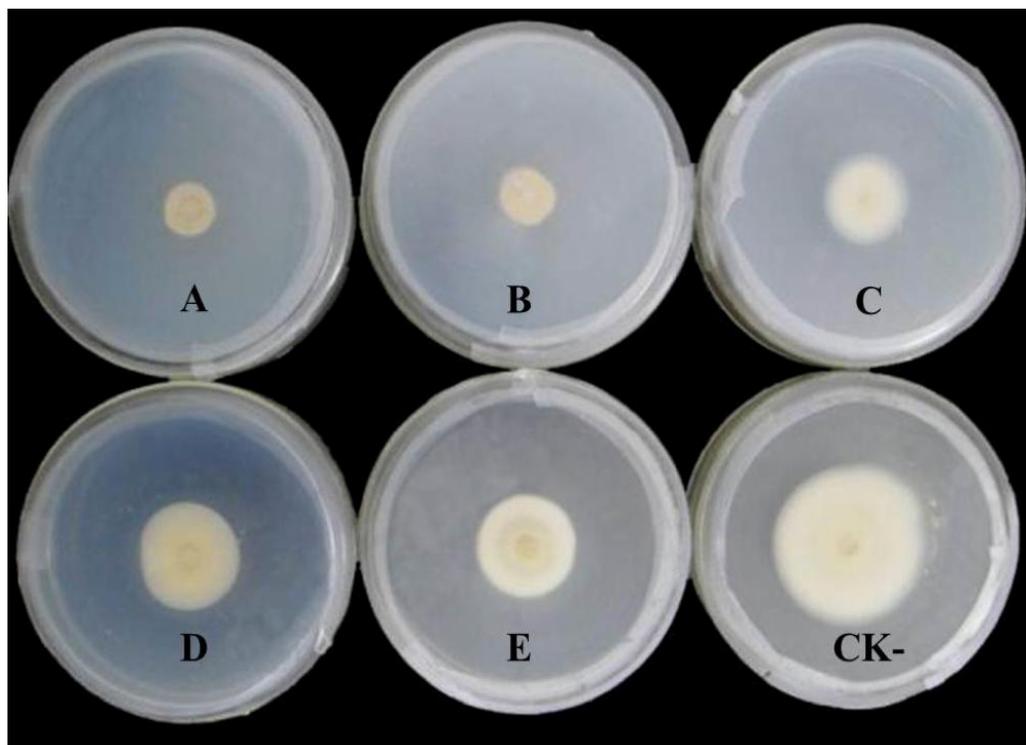


Figure 7. roHEWL inhibits the growth of *V. dahliae* (Cultured up to 10 days) A, B, C, D, and E: roHEWL concentrations were 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL, and 0.3125 mg/mL, respectively, CK- : negative control (phosphate buffer, pH 6.2).

Table 1. Different concentrations of roHEWL inhibit the growth of cotton *F. oxysporum*.

Test day	5 mg/mL		2.5 mg/mL		1.25 mg/mL		0.625 mg/mL		0.3125 mg/mL		CK
	CD	Rate	CD	Rate	CD	Rate	CD	Rate	CD	Rate	CD
2	5.1±0.35	84.9	5.4±0.52	84.2	6.5±0.53	80.9	11.1±0.83	67.3	16.9±0.64	50.4	34.0±0.53
4	8.1±0.64	83.7	8.4±0.52	83.2	9.8±0.46	80.5	17.9±0.83	64.2	28.4±0.52	43.2	50.0±0.93
6	12.3±0.89	83.3	12.4±0.74	83.1	14.1±0.99	80.7	26.0±0.76	64.5	40.3±0.89	45.1	73.3±0.71
8	22.0±0.53	73.7	20.9±0.83	75.1	24.8±0.71	70.5	40.6±0.93	51.5	57.3±1.04	31.6	83.8±1.04

Each experiment was repeated three times, values are means of three replicates ± SD. The fungal inhibition rate is represented by %, fungal growth (colony diameter) was measured, and percentage inhibition calculated according to the formula: Percentage inhibition = (C - T) / C × 100%. Here, C = colony diameter (mm) of the control; T = colony diameter (mm) of the treated plate and CD = colony diameter (mm).

Table 2. Different concentrations of roHEWL inhibit the growth of cotton *V. dahlia*.

Test day	5 mg/mL		2.5 mg/mL		1.25 mg/mL		0.625 mg/mL		0.3125 mg/mL		CK
	CD	Rate	CD	Rate	CD	Rate	CD	Rate	CD	Rate	CD
2	4.4±0.74	58.8	4.8±0.46	55.3	5.5±0.53	48.3	6.1±0.64	42.3	6.4±0.52	40.0	10.6±0.74
4	5.9±0.64	66.9	6.6±0.74	62.7	7.5±0.76	57.8	9.4±0.92	47.2	10.4±0.74	41.5	17.8±0.71
6	7.1±0.64	72.3	8.5±0.76	67.0	9.6±0.74	62.6	14.3±0.71	44.7	16.0±0.76	37.9	25.8±0.71
8	8.6±0.74	75.0	9.0±0.93	73.9	11.9±0.83	65.6	19.1±0.83	44.6	20.8±0.89	39.9	34.5±0.76
10	10.5±0.76	75.7	12.0±0.76	72.2	17.5±0.53	59.4	25.1±0.83	41.7	25.9±0.83	40.0	43.1±0.99

Each experiment was repeated three times, values are means of three replicates ± SD. The fungal inhibition rate is represented by %, fungal growth (colony diameter) was measured, and percentage inhibition calculated according to the formula: Percentage inhibition = (C - T) / C × 100%. Here, C = colony diameter (mm) of the control, T = colony diameter (mm) of the treated plate and CD = colony diameter (mm).

The anti-*F. oxysporum* activity was 84.9% after 2 days period at 5 mg/mL of roHEWL (Table 1). With the increase of cultivation period, the inhibitory effect remained stable. Comparing with the early antifungal effect in the same concentration, the inhibition rate decreased slightly after 8 days incubation, suggesting that roHEWL might partially be degraded, or *F. oxysporum* lysozyme gained some resistance. Antifungal activity of *V. dahliae* is similar to *F. oxysporum*, except slight difference (Table 2). For example, with the increase of the culture time, high concentration of roHEWL (5 mg/mL) showed higher antifungal activity (Figure 7); the activity was also stable.

After 10 days incubation, the inhibition rate reached 75.7%. Thus, high concentration of roHEWL showed consistent suppression of *V. dahliae*, while low roHEWL concentrations (1.25 and 0.625 mg/mL) resulted in decreasing inhibition of *V. dahliae*.

DISCUSSION

The wild type HEWL gene was cloned into pPIC9K under the control of the pGAP promoter in our previous project. However, for some unknown reasons, wild type gene was not expressed (data

not shown). In this study, codon optimization of the HEWL gene for *P. pastoris* was constitutively expressed. It suggests that different hosts have different codon preference. Under normal circumstances, optimization of codons for *P. pastoris* can increase protein expression level by 2 to 10 times (Gustafsson et al., 2004). SDS-PAGE and *in vitro* activity assay results showed that the roHEWL was expressed in mature form (14 kDa). The pH, temperature condition and biological activity of roHEWL are found similar to the natural HEWL lysozyme. The foreign gene was driven by a strong promoter that does not require methanol induction. However, the yield

(54 mg/L) is still low. The fermentation conditions could be further optimized to improve the target protein yield.

Like T4 lysozyme, HEWL can attack peptidoglycans and the glycosidic bond which link the sugars of cell wall polysaccharides (Jolles and Jolles, 1984). Zone of inhibition test suggests that the roHEWL has antibacterial property which is able to inhibit the growth of *M. lysodeikticus* by destroying bacterial cell wall. Tobacco with over expressed HEWL was active against *Micrococcus luteus* cells (Trudel et al., 1992, 1995). Fungal cell wall is different from the bacteria. Its main component is chitin rather than the peptidoglycan.

The growth of two pathogenic fungi, *F. oxysporum* and *V. dahlia*, was inhibited on the roHEWL plate showing roHEWL antifungal activity. It suggests that, in addition to bacteriolytic activity, other molecular mechanisms can inhibit the growth of pathogenic fungi. In addition to its amphiphilic α -helix domain to inhibit fungi, it may also have some chitinase activity (Lundblad et al., 1979). In fact, the chitin molecule also contains β -1,4 glycosidic bonds which are the two N-acetylglucosamine, through β -1,4 glycosidic bonds. The over expression of T4 lysozyme into tall fescue resulted in resistance to fungal diseases of the gray leaf spot (*Magnaporthe grisea*) and brown patch (*Rhizoctonia solani*) (Dong et al., 2008). We intend to introduce HEWL gene into cotton plant in the future, to enhance its potential antifungal role.

ACKNOWLEDGEMENTS

This work was supported by the National High-tech Research and Development Program of China (863 Program: 2007AA02Z111), National Technology for the 11th Five-year Plan of China (2006BAD31B01-04 and 2008BAK41B02-3), National Biotechnology Development Plan (2008ZX08005-004) and (2009ZX08005-004B) and the Researcher Foundation of the Chinese Academy of Agricultural Sciences.

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