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Doctor Forum

TAT Improves *in vitro* Transportation of Fortilin Through Midgut and into Hemocytes of White Shrimp *Litopenaeus vannamei*

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Abstract Fortilin is a multifunctional protein implicated in many important cellular processes. Since injection of Pm-fortilin reduces shrimp mortality caused by white spot syndrome virus (WSSV), there is potential application of fortilin in shrimp culture. In the present study, in order to improve trans-membrane transportation efficiency, the protein transduction domain of the transactivator of transcription (TAT) peptide was fused to fortilin. The *Pichia pastoris* yeast expression system, which is widely accepted in animal feeds, was used for production of recombinant fusion protein. Green fluorescence protein (GFP) was selected as a reporter because of its intrinsic visible fluorescence. The fortilin, TAT and GFP fusion protein were constructed. Their trans-membrane transportation efficiency and effects on immune response of shrimp were analyzed *in vitro*. Results showed that TAT peptide improved *in vitro* uptake of fortilin into the hemocytes and midgut of *Litopenaeus vannamei*. The phenoloxidase (PO) activity of hemocytes incubated with GFP-Fortilin or GFP-Fortilin-TAT was significantly increased compared with that in the control without expressed fortilin. The PO activity of hemocytes incubated with 200 $\mu g\,mL^{-1}$ GFP-Fortilin-TAT was significantly higher than that in the group with the same concentration of GFP-Fortilin. Hemocytes incubated with GFP-Fortilin-TAT at all concentrations showed significantly higher nitric oxide synthase (NOS) activity than those in the control or in the GFP-Fortilin treatment. The present *in vitro* study indicated that TAT fusion protein improved the immune effect of fortilin.

Key words shrimp; *Litopenaeus vannamei*; TAT; fortilin; immunity

1 Introduction

Fortilin, also called translationally controlled tumor protein (TCTP), is ubiquitously expressed in all eukaryotic organisms (Sanchez *et al*., 1997; Bommer and Thiele, 2004). This protein is proved to perform several functions including tubulin-binding (Gachet *et al*., 1999), calcium-binding (Xu *et al*., 1999) and anti-apoptotic activities (Li *et al*., 2001). It is involved in many important cellular processes, such as cell growth, cell cycle and the protection of cells against various stress conditions and apoptosis (Bommer and Thiele, 2004).

To date, some fortilin genes have been identified in shrimp including *Penaeus monodon*, *Litopenaeus vannamei*, and *Marsupenaeus japonicus* (Bangrak *et al*., 2004; Wang *et al*., 2008; Chen *et al*., 2009). It is noteworthy that fortilin of shrimp is shown to have similar Ca^{2+}-binding and anti-apoptotic properties (Bangrak *et al*., 2004; Graidist *et al*., 2006). Furthermore, fortilin is reported to be involved in the antiviral response. Bangrak *et al*. (2004) reported that the fortilin gene was slightly up-regulated during the early stages of virus infection, and the expression level decreased when shrimp showed the mortality characteristics. Similar results have been reported by Rojtinnakorn *et al*. (2002) in the shrimp infected with white spot syndrome virus (WSSV) by expressed sequence tag (EST) approach. Moreover, in the white spot syndrome virus resistant shrimp (*M. japonicus*), the fortilin gene is one of the most abundant genes in the subtracted library (He *et al*., 2005). Results from these studies suggest that fortilin plays a critical role in the defense process during viral infection (Graidist *et al*., 2006).

Subsequent researches showed that injecting WSSV-infected shrimps with recombinant Pm-fortilin protein resulted in 80%–100% survival and low WSSV detection (Tonganunt *et al*., 2008). However, intramuscular injection is costly and laborious. More effective and practical methods to deliver protein such as oral administration would be highly desirable in shrimp culture. In previous studies, the recombinant Pm-fortilin (300 $mg\,kg^{-1}$) mixed with commercial pellets was fed to shrimp, which was subsequently challenged with WSSV. Compared to the control group, survival in dietary Pm-fortilin group in-

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creased by 10%. Nevertheless, dietary fortilin supplements still had low survival (10%) (Tonganunt *et al*., 2008). Reasons for the low oral bioavailability of fortilin could be enzymatic degradation and poor penetration of the intestinal membrane (Morishita and Peppas, 2006).

It has been shown that the transactivator of transcription (TAT) peptide from human immunodeficiency virus 1 (HIV-1) is capable of mediating heterologous proteins across biological membranes into nearly all eukaryotic cells (Green and Loewenstein, 1988; Fawell *et al*., 1994; Wagstaff and Jans, 2006). The transportation process was called 'protein transduction' (Frankel and Pabo, 1988). Besides the application in cultured cells, TAT peptide-mediated transcellular protein transduction also occurs in living animals *in vivo* (Schwarze and Ho, 1999). Protein transduction domains (PTDs) of TAT is a highly basic region (residues 49-57, containing 6 Arg and 2 Lys residues), which is important for transduction ability. The 11-amino acid TAT peptide YGRKKRRQRRR is sufficient for intracellular transduction and subcellular localization (Vives *et al*., 1997).

This study is to investigate *in vitro* whether TAT peptide is capable of mediating fortilin across the membranes of gut cells and hemocytes in white shrimp *L. vannamei*. The immune responses of hemocytes will be studied as well. Green fluorescence protein (GFP) is a good reporter protein and fluorescence marker molecule, and it allows the direct visualization of the subcellular localization of fusion proteins in living cells (Cubitt *et al*., 1995). So in the present study GFP was selected as an intracellular reporter with its intrinsic green fluorescence. Therefore, TAT and GFP were fused to fortilin gene in this study.

2 Materials and Methods

2.1 Microorganism Strains and Media

E. coli DH5a was used as cloning host for vector storage and amplification. *E. coli* was grown in Lauria-Bertani (LB) containing 1% tryptone, 0.5% yeast extract and 1% NaCl. *P. pastoris* strain X-33 was grown in YPD medium containing 2% peptone, 1% yeast extract and 1% glucose. *P. pastoris* transformants containing pGAPZαA vector were selected on YPD containing 100 μg mL^{-1} Zeocin.

Restriction endonucleases, T4 DNA ligase, Taq DNA polymerase and primers were purchased from Takara Biotech Co. Ltd. (Dalian, P. R. China). All other chemicals used were of the highest grade commercially available.

2.2 Expression of GFP-Fortilin-TAT and GFP-Fortilin Fusion Protein

The fortilin coding sequence (GenBank accession no. DQ231062) was amplified from shrimp (*L. vannamei*) hepatopancreas cDNA with Fortilin-F and Fortilin-R as primers (Table 1). The GFP coding sequence was created by PCR using the vector pTracer-CMV2 (Invitrogen, USA) as a template with GFP-F and GFP-R as primers (Table 1). Then, the combinations (GFP-Fortilin-TAT) of GFP, fortilin and TAT genes were amplified by overlap PCR approach (Warrens *et al*., 1997). Firstly, two hybrid primers GF-F and GF-R were used. They were designed from Fortilin and GFP sequences to generate fragment that would have overlapping sequence. Meanwhile, the TAT sequence was fused in 3' point of fortilin using the primer FT-R (Table 1). Each of the first stage products was tapped with a short sequence derived from the other. Then the two products were partially annealed when they were mixed, and participated in the second-stage PCR to produce the final fusion gene using the two flanking primers GFT-F and GFT-R with *EcoR* I and *Xba* I restriction sites (Table 1 and Fig.1). The GFP-Fortilin fusion gene was amplified by a similar PCR procedure except for using primer FX-R instead of FT-R and GFT-R.

The purified PCR fragments were cloned into the *EcoR* I/*Xba* I sites of the pGAPZαA vector. Sequencing analysis was to verify whether the target genes were correctly inserted in the right reading frame. The construct was linearized and integrated into the yeast *P. pastoris* X-33 by electroporation under the selection of Zeocin. Subsequently, the transformants were obtained through Zeocin screening and genomic DNA amplification. The expressed proteins were identified by SDS-PAGE and characterized by matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS).

Table 1 The sequences of primers used in this experiment

Primers	Nucleotide sequence (5′→3′)	Features
Fortilin-F	ATGAAGGTCTTCAAGGACATGCTCACAGGT	
Fortilin-R	TTATAGCTTCTCCTCTGTTAGACCGTATTTTGG	
GFP-F	ATGGCTAGCAAAGGAGAAGAACTTTTCAC	
GFP-R	ATCCATGCCATGTGTAATCCCAGCAGCAGT	
GF-R	ATGTCCTTGAAGACCTTCATATCCATGCCATGTGTAATCCCAGCAGCAGT	Underline: reverse complemented sequence of 5′end of Fortilin
GF-F	GGATTACACATGGCATGGATATGAAGGTCTTCAAGGACATGCTCACAGGT	Underline: 3′end of GFP
FT-R	TCA**TCTTCGTCGCTGTCTCCGCTTCTTCCTGCCATA**TAGCTTCTCCTCTGTTAGACCGT	Bold: TAT sequence
GFT-F	*CCG*GAATTCATGGCTAGCAAAGGAGAAGAACTTTTCAC	Italic: protection base; Underline: *EcoR* I site
GFT-R	*GC*TCTAGATCATCTTCGTCGCTGTCTCCGCTTCTTC	Italic: protection base; Underline: *Xba* I site
FX-R	*GC*TCTAGATTATAGCTTCTCCTCTGTTAGACCGTATTTTGG	Italic: protection base; Underline: *Xba* I site

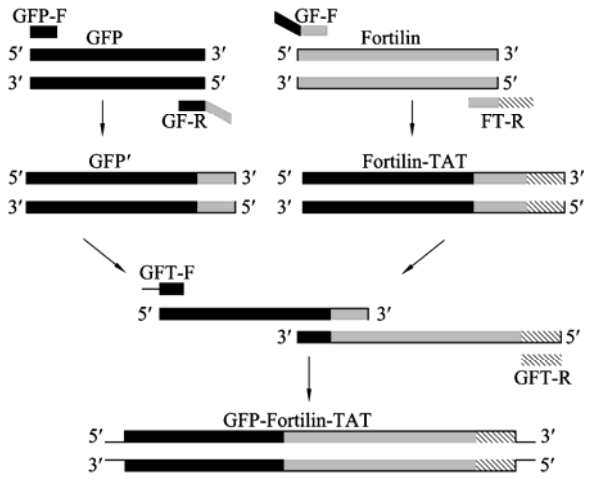

Fig.1 Schematic representation of fusion gene cloning through overlap PCR.

Then the culture supernatants of recombinant yeasts were lyophilized for the following experiment. The culture supernatants from the host yeast harboring an empty pGAPZαA vector were also prepared.

2.3 Experimental Animals

White shrimp *L. vannamei* individuals with 10–12cm initial body length were obtained from Qingdao Baorong Aquatic Product Technological Company (Qingdao, P. R. China). The animals were maintained in 100 L aquaria supplied with a constant flow of circulating seawater. Water temperature ranged from 25 to 28℃, salinity was 32, and dissolved oxygen was not less than 7.0 mg L^{-1}.

2.4 Primary Hemocyte Culture

Hemolymph of white shrimps was withdrawn from the ventral sinus using a 25-gauge needle and 1-mL syringe containing an equal volume of anticoagulant solution (10 mmolL^{-1} EDTA Na_2, 450 mmolL^{-1} NaCl, 10 mmolL^{-1} KCl, 10 mmolL^{-1} HEPES, pH 7.3, osmolality adjusted with glucose to 850 mOsm kg^{-1}) (Vargas and Guzman, 1993).

The basal hemocyte culture medium was Leibovitz's L-15 (Gibco BRL) at double strength and supplemented with 20% fetal bovine serum (FBS), 100 UI mL^{-1} penicillin, and 100 μgmL^{-1} streptomycin. Medium was adjusted to pH 7.3 with 1 molL^{-1} hydrochloric acid and 1 molL^{-1} sodium hydroxide, and then sterilized by passing through 0.22 μm pore filters.

Hemolymph was then centrifuged at 400×g for 10 min to separate hemocytes, which were resuspended in culture media after washed with culture media twice. Then the number of hemocytes was counted followed by inoculating into 96-well microplates at about 1×10^6 cells per well and then incubated at 28℃. The hemocytes were observed daily with an inverted phase-contrast microscope (Nikon TS100), and the culture medium was changed daily.

2.5 *In vitro* Intracellular Transduction Assays

Intracellular transduction assays were performed in 6-well plates (Costar). First, the sterile coverslips were placed in the well (one coverslip per well), and then hemocytes at 1×10^5 were cultured on the coverslips. After incubation for about 24 h to allow attachments of hemocytes to form a monolayer, hemocytes were treated with 1 mg mL^{-1} GFP-Fortilin or GFP-Fortilin-TAT for 0.5 h, 3 h, 6 h, and 24 h, respectively. After rinsing twice with phosphate buffer, the intracellular transduction was observed under a fluorescence microscope (Nikon eclipse 50i, Pixera pro 150ES).

2.6 Activities of Phenoloxidase (PO) and Nitric Oxide Synthase (NOS)

Hemocytes were incubated in 96-well microplates at 28℃ for 24 h. Then three concentrations (100, 200 and 500 μg mL^{-1}) of GFP-Fortilin, GFP-Fortilin-TAT or the culture supernatants from the yeast harboring an empty vector were added in the media respectively and the hemocytes were incubated for another 24 h. Each concentration was tested with four replicates. The incubated hemocytes were then homogenized with a sonicator on ice (Sonic, Vibra Cell, USA) for 8 s at 40% amplitude and centrifuged at 5000 rmin^{-1} for 10 min. Supernatants were collected to analyze the activities of phenoloxidase and nitric oxide synthase. Hemocytes incubated in the same amount of L-15 medium were used as control.

Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) following the procedures of Hernández *et al*. (1996). In brief, 50 μL of sample was placed in a 96-well plate and preincubated with 50 μL of trypsin (0.1 mgmL^{-1} in CAC buffer: 10 mmolL^{-1} sodium cacodylate, 10 mmolL^{-1} $CaCl_2$, pH 7.0) for 10 min at 25℃, and then 50 μL of L-DOPA (3 mg mL^{-1} in CAC buffer) was added. The reaction was allowed to proceed for 10 min at 25℃. After that, the absorbance was measured at 492 nm using a microplate reader (Model Multiskan Spectrum, Thermo, MA, Waltham, USA). One unit of enzyme activity is defined as an increase in absorbance of 0.001 per min per mL sample.

Activity of NOS was determined by its ability to convert L-arginine to nitric oxide (NO) using a NOS Kit (Nanjing Jiancheng Bioengineering Institute, P. R. China). The assay was performed following the manufacturer's introduction. One unit NOS activity is defined as: 1 mL of sample produces 1 nmol of NO per min. Specific activity is reported as units per mL cell culture supernatants.

2.7 Transportation Across the Midgut *in vitro*

The entire midgut, posterior to the digestive gland and anterior to the hindgut, was removed from shrimps. The intestinal contents of the excised midgut were flushed with physiological saline using a syringe. The cleaned midgut was turned over by a blunt-pointed needle, and then both ends were ligated with cotton threads, immersed in the physiological saline. The composition of a physiological saline was based primarily on the ionic

composition and osmotic pressure of the shrimp serum (Ahearn, 1974; Chu, 1986). The saline contained (in $mmolL^{-1}$) NaCl, 460; KC1, 11; $CaCl_2$, 13; $MgSO_4.7H_2O$, 13; mannitol, 50; and HEPES 5; with an osmolality of 850 $mOsmkg^{-1}$. The saline was titrated to a pH of 7.5 with a few drops of 1 $molL^{-1}$ NaOH solution.

Intra-gut transduction assays were performed in 12-well plates (Costar). The ligated midguts were immersed in 4 $mgmL^{-1}$ GFP-Fortilin and GFP-Fortilin-TAT, respectively, for 1 h at 25℃. Meanwhile, the ligated midguts immersed in the culture supernatants from the yeast harboring an empty vector were set as the control. Each treatment was conducted with three replicates. After incubation, the intestines were rinsed twice with physiological saline, and observed under fluorescence microscope (Nikon eclipse 50i, Pixera pro 150ES).

2.8 Statistical Analysis

All data were subjected to one-way analysis of variance (one-way ANOVA) using SPSS 13.0 for Windows. Differences between the means were tested by Tukey's test. The level of significance was chosen at $P<0.05$ and the results were presented as means ± S.E. (standard error).

3 Results

3.1 Expression of Fusion Proteins in *P. pastoris*

PCR amplification of the Fortilin gene yielded a 507 bp DNA (Fig.2A) fragment with the expected sequence, and a single band of the expected 708 bp corresponding to the expected size of GFP gene was obtained (Fig.2B). In a second round of amplification, the two products were used as templates to generate the fusion gene GFP-Fortilin (1229 bp) and GFP-Fortilin-TAT (1262 bp) (Figs.2C and D).

The fusion gene was inserted into the vector pGAPZaA. When the DNA sequences of the construct was confirmed and in frame with pGAPZaA, the plasmid containing the fusion gene was transformed into X-33 cells by selection for resistance to Zeocin. SDS-PAGE analysis (Fig.3A) revealed that the fusion protein secreted into supernatant. Gel-Pro analysis from SDS-PAGE results estimated that the molecular masses of GFP-Fortilin and GFP-Fortilin-TAT were 56.7 kDa and 57.4 kDa, respectively. Further confirmation was done using MALDI-TOF analysis. Furthermore, there was no degradation in lyophilized culture supernatants (Fig.3B).

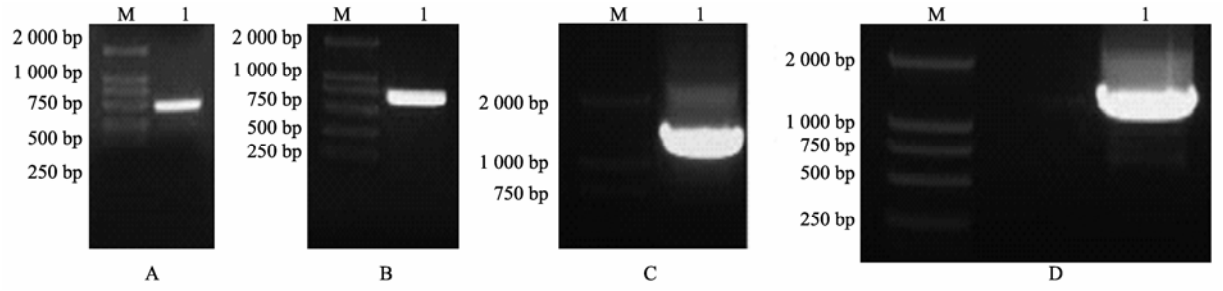

Fig.2 PCR amplification of target genes. M: DNA Marker (DL2000); A: Fortilin; B: GFP; C: GFP-Fotilin; D: GFP-Fotilin-TAT.

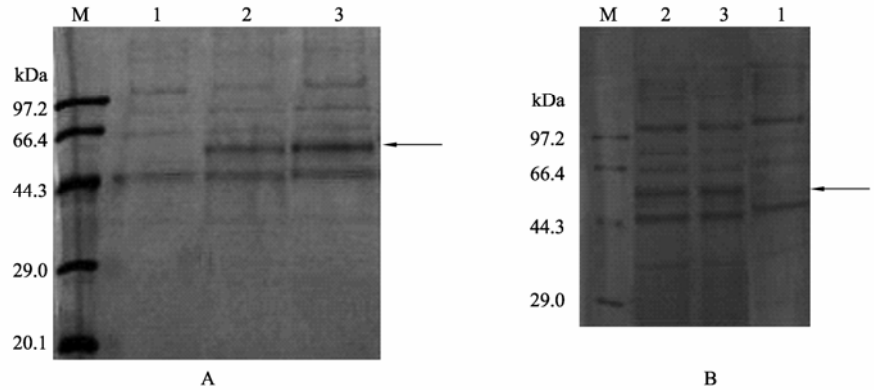

Fig.3 SDS-PAGE analysis of fusion protein expression in *P. pastoris*. The culture supernatants before (A) and after (B) lyophilization. Lane M: protein molecular weight markers; Lane 1: supernatant from the culture of X-33/pGAPZαA; Lane 2: supernatant from the culture of X-33/pGAPZαA-GFP-Fortilin; Lane 3: supernatant from the culture of X-33/pGAPZαA-GFP-Fortilin-TAT. Fusion proteins were marked by arrows.

3.2 Intracellular Transduction of GFP-Fortilin-TAT in Cultured Primary Hemocytes

The GFP-Fortilin and GFP-Fortilin-TAT fusion protein were applied to assess their intracellular transduction capacity on cultured primary shrimp hemocytes. When hemocytes were treated with GFP-Fortilin-TAT (Fig.4D), intracellular green fluorescence was detected starting at

30 min, increasing progressively till 24 h. Hemocytes treated with GFP-Fortilin only exhibited indistinct cell outlines, and no clear fluorescence was detected (Fig.4B).

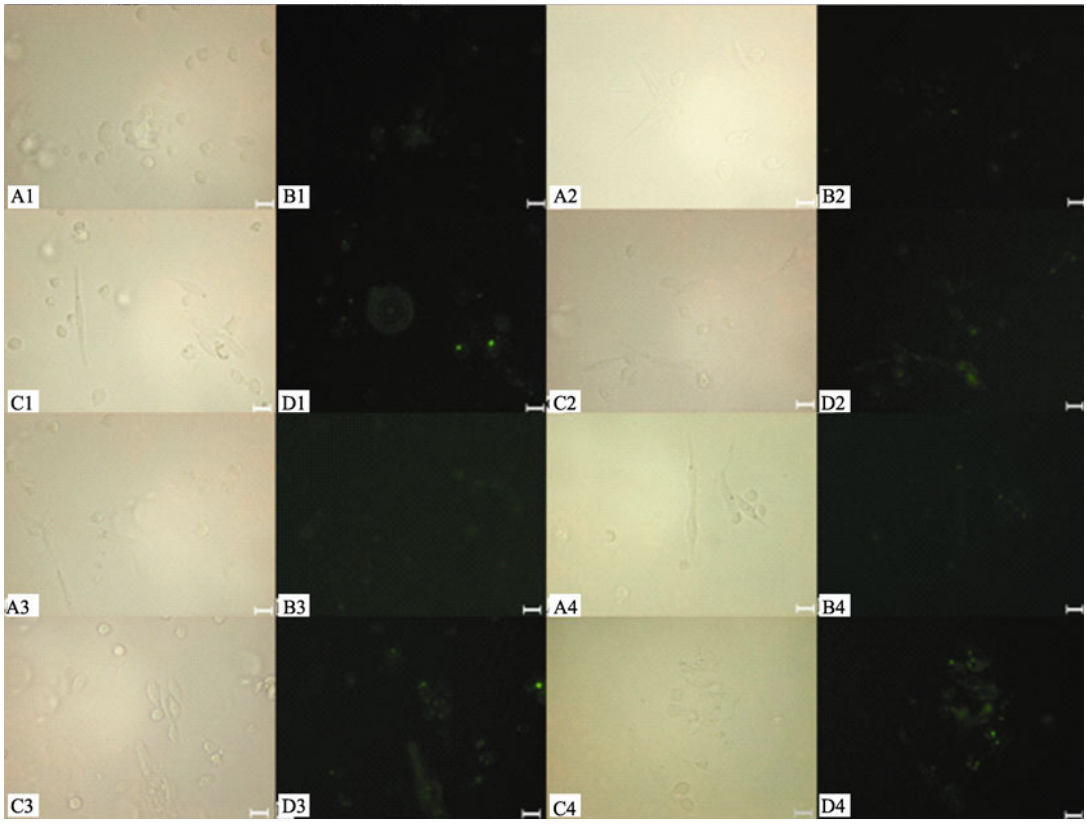

Fig.4 Fluorescence microscopy analysis of GFP-Fortilin-TAT transportation to hemocytes of *L. vannamei*. A and B: GFP-Fortilin group; C and D: GFP-Fortilin-TAT group; the incubation time of 1, 2, 3 and 4 was 0.5 h, 3 h, 6 h and 24 h, respectively. Results are images in bright field (A/C) and corresponding images in fluorescence mode (B/D). Scale bar = 5 μm.

3.3 Activities of Phenoloxidase (PO) and Nitric Oxide Synthase (NOS)

The PO activity of hemocytes incubated with GFP-Fortilin or GFP-Fortilin-TAT was significantly higher than that in both the control and empty vector control (P<0.05) (Fig.5). The significant highest PO activity among all the treatments was found in the group incubated with 200 μg mL^{-1} GFP-Fortilin-TAT (P<0.05). There were no significant differences in PO activity among the other treatments (P>0.05).

There was no significant difference in NOS activity of hemocytes between the control, empty vector control and those incubated with GFP-Fortilin at any concentration (P>0.05) (Fig.6). Meanwhile, hemocytes treated with GFP-Fortilin-TAT at all concentrations showed significantly higher NOS activity than those in the control and GFP-Fortilin treatment (P<0.05). There was no significant difference in NOS activity among the three GFP-Fortilin-TAT treatments (P>0.05).

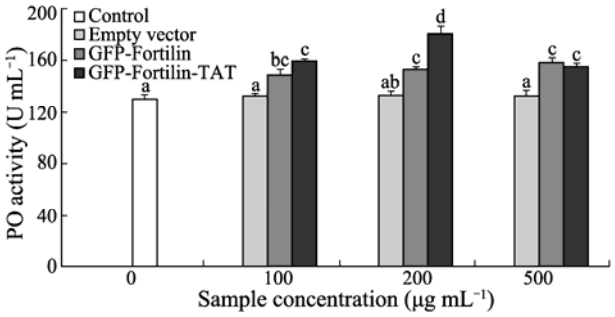

Fig.5 Phenoloxidase (PO) activity in hemocytes of *L. vannamei* incubated with different concentrations of lyophilized culture supernatants of recombinant yeasts. Values are expressed as mean ± SE (n=4). Bars with different superscripts are significantly different (P<0.05) among treatments.

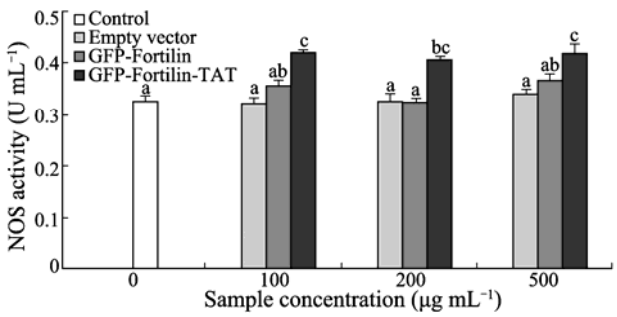

Fig.6 Nitric oxide synthase (NOS) activity of hemocytes of *L. vannamei* incubated with different concentrations of lyophilized culture supernatants of recombinant yeasts. Values are expressed as mean ± SE (n=4). Bars with different superscripts are significantly different (P<0.05) among treatments.

3.4 Intra-Midgut Delivery of GFP-Fortilin-TAT *in vitro*

The midgut of *L. vannamei* is a straight tube extending from the posterior border of the cephalothorax to the end of the abdomen. To make the experiment results comparable, the whole midgut was used for each experiment. The midgut was generously supplied with chromatophores along most of its length, so there were dark spots as shown in Fig.7.

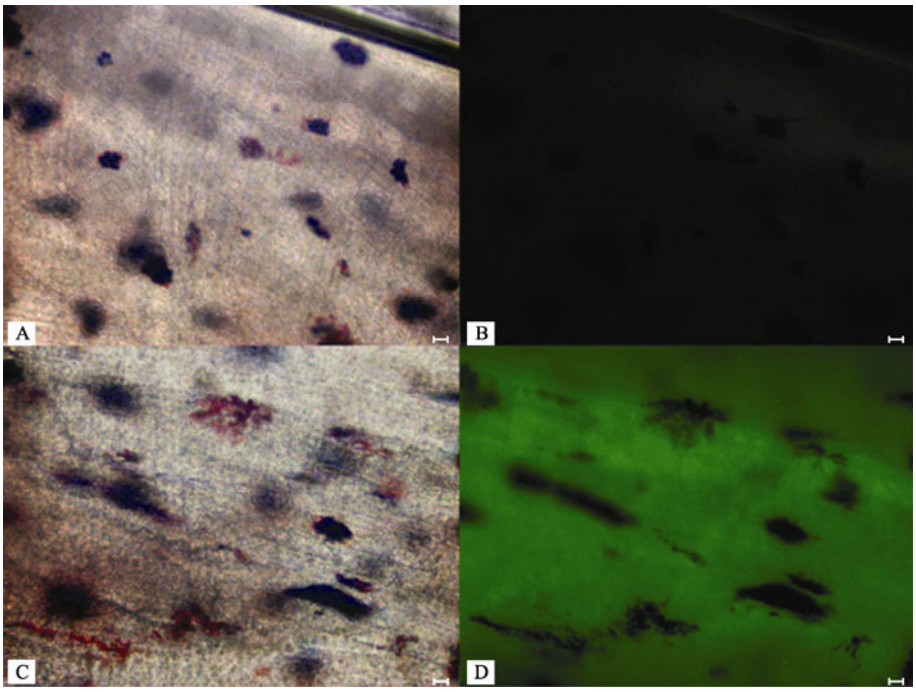

Fig.7 Fluorescence microscopy analysis of the delivery of GFP-Fortilin or GFP-Fortilin-TAT to midgut of *L. vannamei in vitro*. A, B: GFP-Fortilin group; C, D: GFP-Fortilin-TAT group; Shown are observed fluorescence microscopy by imaging in bright field (A, C) and corresponding image in fluorescence mode (B, D). Scale bar = 20μm.

As shown in Fig.7, the midgut incubated with the GFP-Fortilin-TAT presented bright green fluorescence, but no clear fluorescence was detected in the GFP-Fortilin-treated midgut. That is to say TAT mediated fusion protein through the intestinal membrane effectively.

4 Discussion

As mentioned above, fortilin is a multifunctional protein involved in important cellular activities (Bommer and Thiele, 2004). In addition, shrimp fortilin may interfere with viral infection by inhibiting viral replication (Tonganunt *et al*., 2008). However, the low efficiency of oral administration limits the application of fortilin in shrimp culture, which has been a common challenge for oral administration of proteins and peptides (Park *et al*., 2011). The prevalence of diseases, especially viral diseases, has caused heavy losses to the world shrimp farming industry. How to apply bioactive proteins and peptides with potential antivirus property in an efficient and practical way needs further study. The oral administration through feeding is undoubtedly a good choice. However, this method is limited as the proteins or peptides are sensitive to digestive enzymes and are difficult to penetrate through the biomembrane barriers. Three approaches for the oral delivery of protein are: (1) modification of the physicochemical properties of macromolecules; (2) addition of novel function to macromolecules; or (3) use of improved delivery carriers (Morishita and Peppas, 2006). The present study focused on improving transportation efficiency of fortilin protein using cell-penetrating peptides (CPPs), and attempted to seek for a feasible method for its application in feeds.

During the last decade, it has been reported that the CPPs including arginine-rich peptides (*e.g*., HIV-1 TAT and oligoarginine) and amphipathic peptides (*e.g*., penetratin) (Joliot and Prochiantz, 2004; Kamei *et al*., 2009) were capable of delivering large molecules across cellular membranes. The TAT peptide is one commonly used and widely studied CPP. Hence, TAT peptide was selected to investigate whether it could improve the penetrating efficiency of fortilin-fused protein. The results showed that the TAT peptide can deliver fortilin into primary shrimp hemocytes (Fig.4D). Moreover, GFP-Fortilin-TAT showed a successful TAT-mediated penetration through the intestinal membrane (Fig.7D). Based on these observations, it was speculated that TAT could promote transportation and absorption of TAT-fused protein in shrimp. This result might open the way to the application of TAT-mediated transcellular protein transduction in shrimp.

The proPO-activating system is a very important component of innate immune system, which is an efficient nonself-recognition cascade in crustaceans. It can be triggered by minute amounts of lipopolysaccharides, peptidoglycans and β-l, 3-glucans (Cerenius and Söderhäll, 2004; Ai *et al*., 2008). In shrimp, PO is reported to be

involved in anti-bacterial and anti-viral immune responses (Dziarski, 2004; Evelyne, 2003). As shown in Fig.5, the PO activity of hemocytes incubated with GFP-Fortilin or GFP-Fortilin-TAT was significantly higher than that in the control without expressed Fortilin. The PO activity of hemocytes incubated with 100 and 200 μg mL^{-1} GFP-Fortilin-TAT was higher than that in the group with the same concentration of GFP-Fortilin. However, the 500 μg mL^{-1} GFP-Fortilin-TAT group had a lower PO activity than the GFP-Fortilin group (500μg mL^{-1}). It clearly showed that both GFP-Fortilin and GFP-Fortilin-TAT improved the PO activity in shrimp hemocytes. Furthermore, this effect was enhanced by TAT peptide.

It was found that the expression of NOS in hemocytes, hepatopancreas and nerve changed rapidly and dynamically in response to injection of lipopolysaccharide and poly I:C (Yao *et al*., 2010). NOS activity in hemocytes might relate to the resistance ability of shrimps to WSSV infection (Jiang *et al*., 2006). In the present study, the GFP-Fortilin had no significant effect on the NOS activity. However, hemocytes incubated with GFP-Fortilin-TAT at different concentrations showed significantly higher NOS activity than those in the control and GFP-Fortilin treatments (Fig.6). The results from PO and NOS indicated that the presence of TAT fusion protein improved the immune effect of fortilin. The successful expression and application of the fusion protein of TAT and fortilin *in vitro* shows an encouraging prospect of using it in health management and disease control in shrimp aquaculture. Recombinant proteins can be added in shrimp feeds as an additive. Of course, further study is needed to demonstrate this effect *in vivo*.

Yeast can transfer plasmids encoding foreign proteins to the host cells and can be administered orally. This convenient and inexpensive delivery way can make the application of fortilin more simple and acceptable (Cregg *et al*., 2000). This system allows the production of fortilin at an acceptable cost in the shrimp industry. As the first step towards the large-scale production of fortilin for application in shrimp feeds, an expression system for fortilin in the yeast *P. pastoris* by using vector pGAPZαA was developed. As the lyophilized culture supernatants were directly used in this study, other proteins expressed by yeasts might contribute to the final results even though an empty vector treatment was used as control. In order to confirm the function and further study the mechanism of TAT in improving fortilin transportation across the membrane, purification of GFP-Fortilin-TAT from the yeast expression proteins will be conducted in our next study.

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