

The molecular characterization of a cyclophilin A from Chinese mitten crab *Eriocheir sinensis* and the antifungal activity of its recombinant protein

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Abstract

Background: Cyclophilin A (CypA), a receptor for the immunosuppressive agent cyclosporin A (CsA), is a *cis-trans* peptidyl-prolyl isomerase (PPIase) which accelerates the *cis-trans* isomerization of prolyl-peptide bonds and interacts with a variety of proteins to regulate their activities.

Results: The full-length cDNA of crab *Eriocheir sinensis* CypA (*EsCypA*) was cloned by EST and RACE technique. The complete sequence of *EsCypA* cDNA contained a 5' untranslated region (UTR) of 50 bp, a 3' UTR of 233 bp with a polyA tail, and an open reading frame (ORF) of 495 bp encoding a polypeptide of 164 amino acids with the predicted molecular weight of 17.36 kDa. The deduced amino acid sequence of *EsCypA* contained two highly conserved signature sequences of peptidyl-prolyl *cis-trans* isomerase and a pro-isomerase domain. The mRNA transcripts of *EsCypA* were detectable in all the examined tissues, including haemocytes, gill, hepatopancreas, gonad, muscle and heart, with higher expression level in hepatopancreas and gonad. No significant difference in the relative mRNA expression level of *EsCypA* was observed during the whole course of bacteria challenge, whereas it was up-regulated during fungi challenge. The purified recombinant protein r*EsCypA* exhibited a significant PPIase activity and an antifungal activity.

Conclusions: All these results indicated that it was a typical CypA member and potentially involved in the innate immune responses of crab.

Keywords: antifungal activity, cyclophilin A, *Eriocheir sinensis*, immune response, PPIase activity.

INTRODUCTION

Cis-trans isomerization of the Xaa-Pro (Xaa stands for unspecified amino acid) bond is an important step in protein folding and a critical determinant of protein structure (Shen and Bax, 2010). Peptidyl-prolyl *cis-trans* isomerases (PPIases, EC 5.2.1.8) contain three unrelated subfamilies, including the cyclophilins (Cyps), FK binding proteins (FKBPs) and parvulins (Schiene-Fischer et al. 2012). According to their ability to bind the potent immunosuppressive drugs, the first two subfamilies are collectively known as immunophilins (Kang et al. 2008; Gauwerky et al. 2009). The Cyps was first identified from human as a cytosolic binding protein for the immunosuppressive drug cyclosporin A (CsA) (Handschumacher et al. 1984). Subsequently, various Cyps have been found in diverse organisms ranging from bacteria to human, and they are usually grouped based on their size and target location (Rutherford and Zuker, 1994). Five isoforms have been reported in yeast Cyps family,

including CypA (Cyp1) of 17 kDa cytosolic protein, CypB (Cyp2) of 20 kDa secretory protein, CypC (Cyp3) of 18 kDa mitochondrial protein, CypD of 22.8 kDa endoplasmic reticulum protein, and Cyp4 of 33.4 kDa secretory protein (Frigerio and Pelham, 1993). Similarly, five members of Cyps family have also been identified from mammalian, CypA of 18 kDa soluble cytosolic proteins, CypB of 22 kDa proteins with signal sequences for ER, CypC of 23 kDa proteins with endoplasmic reticulum signal sequences, CypD of 22 kDa proteins with putative mitochondrial signal sequences, and Cyp40 of 40 kDa proteins with low affinities to CsA (Gething and Sambrook, 1992).

Among all the Cyp members, CypA acts as a ubiquitously distributed intracellular protein and carries out a wide range of functions, including protein folding, receptor complex stabilization, apoptosis participation, and receptor signaling through its PPIase activity (Brazin et al. 2002; Lin and Lechleiter, 2002). CypA is also considered as a host cell receptor for the potent immunosuppressive drug CsA (Mikol et al. 1993). The immunosuppressive activity of CsA is mediated via Cyp-dependent calcineurin (Coto and Tavera, 2009), and calcineurin also plays a key role in initiating T lymphocyte activation by dephosphorylating the nuclear factor of activated T-cells (NFAT) (Li et al. 2012), which subsequently translocates from the cytoplasm to the nucleus. NFAT in nucleus is required for the induction of transcription of cytokines, such as Interleukin-2 (IL2), IL3, IL4, and interferon- γ (INF γ) (Yarilina et al. 2011). CypA-CsA complex can bind intracellular proteins and plays important roles in immune system of mammals (Angelucci et al. 2011). Recent studies revealed that CypA could activate innate immune system and inhibit the infectivity of wild type HIV-1 via interacting with HIV-1 gag protein (Towers et al. 2003; Sayah et al. 2004; Berthoux et al. 2005). Additionally, CypA was also suggested to play important roles in innate immune system of marine invertebrate. For examples, *PmCypA* from shrimp *Penaeus monodon* (Qiu et al. 2009), *CfCypA* from scallop *Chlamys farreri* (Song et al. 2009), and *VpCypA2* from clams *Venerupis philippinarum* (Chen et al. 2011) were reported to be involved in the immune responses to pathogen infection. However, the activity of CypA protein and the detailed mechanism in invertebrates are still not quite clear.

The Chinese mitten crab *Eriocheir sinensis* is one of the most important aquaculture species in South-East Asia. With the development of intensive culture and environmental deterioration, various diseases caused by bacteria, viruses and rickettsia-like organisms had frequently occurred in cultured *E. sinensis* populations, which resulted in enormous losses to this aquaculture industry (Gai et al. 2009). A better understanding of the immune defence mechanisms of crab is necessary for the health management of crab farming. The main objectives of this study were (1) to clone the full-length cDNA of CypA from *E. sinensis*, (2) to investigate the tissue distribution of *EsCypA* transcript, and its temporal profile after microbe challenge, and (3) to validate the possible activity of recombinant *EsCypA* protein.

MATERIALS AND METHODS

Crabs, microbe challenge and haemocyte collection

Healthy *E. sinensis* were collected from a local farm in Qingdao, China, and acclimated for two weeks before processing. Approximately two hundred crabs were employed for microbe challenge experiment, and they were divided into three groups. Seventy crabs in the first group were kept in tanks containing live *Vibrio anguillarum* strain M3 at final concentration of 8×10^6 CFU mL⁻¹ and employed as bacteria challenge group. Other seventy crabs were transferred to the fungi-containing tanks with live *Pichia pastoris* strain GS115 at final concentration 8×10^6 CFU mL⁻¹, and employed as fungi challenge group. Five individuals were randomly sampled at 1.5, 3, 6, 12, 24 and 48 hrs post challenge. The rest thirty unchallenged crabs were kept in the fresh seawater as blank group, and five individuals were randomly sampled at 0 hrs. The haemolymph was collected from cheliped using a syringe with an equal volume of anticoagulant (27 mmol L⁻¹ sodium citrate, 336 mmol L⁻¹ NaCl, 115 mmol L⁻¹ glucose, 9 mmol L⁻¹ EDTA, pH 7.0), and centrifuged at 800 g, at 4°C for 10 min to harvest the haemocytes for RNA preparation. Hepatopancreas, muscle, gill, heart and gonad from five untreated crabs were collected to determine the tissue distribution of *EsCypA* mRNA transcript. Total RNA was immediately extracted using RNAiso plus reagent (TaKaRa, Japan) according to the manufacture's protocol.

EST analysis and cloning the full-length *EsCypA* cDNA

One EST (CMCES_A_5626) of 473 bp from the cDNA library (Gai et al. 2009) was homologous to CypAs identified previously, and it was selected for further cloning of CypA cDNA from *E. sinensis* (designated as *EsCypA*). The clone corresponding to this EST was selected for completely sequencing with T7 promoter primer (Table 1). Two gene-specific primers, *Es-CypA-Race-R1* and *Es-CypA-Race-R2* (Table 1) were designed based on the certain EST to clone the 5' sequence of *EsCypA* cDNA by rapid amplification of cDNA ends (RACE) technology. A pair of gene-specific primers *Es-CypA-cDNA-F* and *Es-CypA-cDNA-R* (Table 1) was designed to amplify and verify the cDNA sequences. All PCR amplification was performed in a PCR Thermal Cycler (Takara, Japan). And the PCR products were gel-purified and cloned into the pMD18-T simple vector (Takara, Japan). After being transformed into the competent cells *Escherichia coli* strain Top10 (TianGen, China), the positive recombinants were identified through anti-Amp selection and PCR screening with M13-47 and RV-M primers (Table 1). Three of the positive clones were sequenced on a PRISM 3730 automated sequencer (ABI, USA). The sequencing results were verified and subjected to cluster analysis.

Table 1. Oligonucleotide primers used in the experiments.

Primer	Sequence (5'-3')	Information
<i>Es-CypA-Race-R1</i>	CATTGCCAGCAGTGAAGTCG	Gene specific primer for RACE
<i>Es-CypA-Race-R2</i>	CGAGAACCGAACGCCTTGA	Gene specific primer for RACE
Adaptor-oligo (dG)	GGCCACGCGTGCAGTAGTACG ₁₀ HN	Anchor primer for RACE
Adaptor-oligo (dT)	GGCCACGCGTGCAGTAGTACT ₁₈ VN	Olido(dT) for cDNA synthesizing
<i>Es-CypA-cDNA-F</i>	CGTCTACCTCCTCCACTCCA	Gene specific primer for sequence identification
<i>Es-CypA-cDNA-R</i>	ATCACGCAAACGAATCTCAT	Gene specific primer for sequence identification
<i>Es-CypA-qRT-F</i>	TTCACCCTTCGTCACTGG	Gene specific primer for real-time PCR
<i>Es-CypA-qRT-R</i>	CCCTCAACAACACTCCCGA	Gene specific primer for real-time PCR
<i>Es-Actin-qRT-F</i>	GCATCCACGAGACCACTTACA	Internal control for real-time PCR
<i>Es-Actin-qRT-R</i>	CTCTGCTTGCTGATCCACATC	Internal control for real-time PCR
<i>Es-CypA-recombinant-F</i>	ATGGGCAACCCTCAGGTCTTC	Gene specific primer for recombination
<i>Es-CypA-recombinant-R</i>	CAACTGGCCAGAGTTGGCAAT	Gene specific primer for recombination
T7 promoter	GTAATACGACTCACTATAGGGC	Vector primer for sequencing
T7 terminator	TGCTAGTTATTGCTCAGCGG	Vector primer for sequencing
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	Vector primer for sequencing
RV-M	GAGCGGATAACAATTTACACAGG	Vector primer for sequencing

Sequence analysis, multiple sequences alignment and phylogenetic analysis

The searches for nucleotide and protein sequence similarities were conducted with BLAST algorithm at the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.gov/blast>). The deduced amino acid sequence of *EsCypA* was analyzed with the Expert Protein Analysis System (Expasy, <http://www.expasy.org/>). SignalP 3.0 program was utilized to predict the presence and location of signal peptide (<http://www.cbs.dtu.dk/services/SignalP/>). The protein motif features were predicted by Simple Modular Architecture Research Tool (SMART) version 7.0 (<http://smart.embl-heidelberg.de/>). Multiple sequence alignment of *EsCypA* and other reported Cyps was performed with ClustalW multiple alignment program (<http://www.ebi.ac.uk/Tools/clustalw2/>) and multiple alignment show program (<http://www.bioinformatics.org/sms/>). An unrooted phylogenetic tree was constructed based on the deduced amino acid sequences of *EsCypA* and other reported Cyps by the neighbour-joining (NJ) algorithm using the MEGA 5.05 software (<http://www.megasoftware.net>). To derive the confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times. The amino acid sequences information for homologous and phylogenetic analysis was shown in Table 2.

Table 2. Species and accession number of CypAs and CypBs used in the homologous and phylogenetic analysis.

Species	Ac. No. of CypA	Ac. No. of CypB
<i>Argopecten irradians</i>	ABM92916	
<i>Chlamys farreri</i>	AAR11779	
<i>Danio rerio</i>	AAQ91263	NP_998184
<i>Homo sapiens</i>	AAH05982	CAG33110
<i>Ictalurus punctatus</i>	AAY86951	
<i>Ictalurus punctatus</i>		ABO15710
<i>Mus musculus</i>	NP_032933	NP_035279
<i>Pan troglodytes</i>	ABB77876	
<i>Penaeus monodon</i>	ABV90639	
<i>Rattus norvegicus</i>	NP_058797	AAH61971
<i>Xenopus laevis</i>		NP_001080505

Tissue distribution of *EsCypA* mRNA and its temporal expression in haemocytes after microbechallenge

The mRNA transcripts of *EsCypA* in various tissues, including haemocytes, hepatopancreas, muscle, gill, heart and gonad of five untreated crabs, and its temporal expression in haemocytes of crabs challenged with *V. anguillarum* or *P. pastoris* were determined by real-time PCR. Total RNA from different tissues was isolated according to the protocol of RNAiso plus reagent (TaKaRa, Japan). The first-strand synthesis was carried out based on Promega M-MLV RT Usage information using the DNaseI (RQ1, Promega, USA)-treated total RNA as template and adaptor primer-oligo (dT) (Table 1) as primer. The reaction mixtures were incubated at 42°C for 1 hr, terminated by heating at 95°C for 5 min, and subsequently stored at -80°C. Real-time PCR was performed with the SYBR premix Ex Taq II (Takara, Japan) on an ABI 7300 Real-Time Detection System (ABI, USA) to investigate the mRNA expression of *EsCypA*. The information of all primers used in this assay was shown in Table 1. The expression of *EsCypA* was normalized to the expression of β -actin gene for each sample. The comparative *Ct* method ($2^{-\Delta\Delta Ct}$ method) was used to analyze the expression level of *EsCypA*. All data was given in terms of relative mRNA expression level as means \pm S.D. (n = 5) and subjected to one-way analysis of variance (one-way ANOVA) followed by an unpaired, two-tailed student's *t*-test. Differences were considered significant at $p < 0.05$.

Recombination, expression and purification of *EsCypA* in *E. coli*

The cDNA fragment encoding the mature peptide of *EsCypA* was amplified with two gene specific primers, *Es-CypA-recombinant-F* and *Es-CypA-recombinant-R* (Table 1), and then ligated to the expression vector pEASY-E1 (TransGen, China). The recombinant plasmid pEASY-E1/*EsCypA* was isolated via E.Z.N.A. Plasmid Mini Kit (Omega, USA) and transformed into *E. coli* strain BL21 (DE3) (Novagen, USA). The positive transformants *E. coli* BL21 (DE3)/pEASY-E1/*EsCypA* were incubated in LB medium containing 100 mg mL⁻¹ ampicillin at 37°C with shaking at 220 rpm. When the culture mediums reached OD₆₀₀ of 0.5-0.7, the cells were incubated for 4 additional hrs with the induction of IPTG at the final concentration of 1 mmol L⁻¹. The recombinant protein (designed as r*EsCypA*) was purified by a Ni²⁺ chelating sepharose column under denatured condition (8 mol L⁻¹ urea), and refolded in gradient urea-TBS glycerol buffer (50 mmol L⁻¹ Tris-HCl, 50 mmol L⁻¹ NaCl, 5% glycerol, 2 mmol L⁻¹ reduced glutathione, 0.2 mmol L⁻¹ oxidized glutathione, a gradient urea concentration of 6, 4, 3, 2, 0 mol L⁻¹ urea in each gradient, pH = 8.0, each gradient at 4°C for 12 hrs). The resultant protein was separated by reducing 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and visualized with coomassie brilliant blue R-250.

PPIase activity analysis of recombinant *EsCypA* protein

PPIase activity was determined by an assay with α -chymotrypsin (Sigma, USA) that utilized *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma, USA) in 88% *trans*- and 12% *cis*- configuration as the substrate in TBS buffer (50 mmol L⁻¹ Tris-HCl, 50 mmol L⁻¹ NaCl, 5%, pH = 8.0) (Lee et al. 2007). The *trans*- form of the substrate is cleavable by α -chymotrypsin, which releases *p*-nitroanilide, while the *cis*- form cannot be cleaved. The *p*-nitroanilide was quantified spectrophotometrically at 405 nm, and PPIase activity was measured by the *cis-trans* isomerization of the proline alanine peptide bond in the peptide. For each assay, 45 μ L of TBS buffer and 5 μ L of 100 μ g mL⁻¹ α -chymotrypsin were equilibrated in a well of the assay plate. Typically, 50 μ L of 100 μ g mL⁻¹ r*EsCypA* was then added, followed by 50 μ L of 5 mmol L⁻¹ *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrate. Absorbance at 405 nm of each well was determined every 30 seconds by a precision micro plate reader (BioTek, USA). TBS buffer and 100 μ g mL⁻¹ BSA were employed as blank control and negative control group, respectively. The PPIase activity is simply qualitatively represented by the change of OD₄₀₅.

Analysis of antifungal activity of recombinant *EsCypA* protein

Antifungal activity of the recombinant r*EsCypA* was assessed by a radial diffusion assay with the *P. pastoris* strain GS115 as substrate (Zhao et al. 2007a; Zhao et al. 2007b). The fungi concentration in medium was adjusted to 0.2 absorbance unit at 600 nm and poured onto 90 mm plates. Wells (diameter approximately 7.5 mm) were cut into freshly poured plates after the solidification of the agar. For radial diffusion assays, 100 μ L of a gradient concentration of r*EsCypA* was pipetted into individual wells and the agar plates were incubated at 28°C. After 48 hrs incubation, the radius of the clearing zone was measured. TBS buffer, 500 μ g mL⁻¹ of zeocin (Invitrogen, USA), and a gradient concentration of BSA were employed as blank control, positive control and negative groups, respectively.

Nucleotide sequence

The complete cDNA sequence of *EsCypA* was deposited in GenBank under the accession number GQ240290.

RESULTS

Molecular features of *EsCypA* sequence

Sequencing of *E. sinensis* cDNA library yielded 7535 EST sequences, among which one EST CMCES_A_5626 was homologous to previously identified CypA. A 777 bp nucleotide sequence was obtained by overlapping the certain EST with the amplified fragments. It represented the complete cDNA sequence of *EsCypA*, and was deposited in GenBank under the accession number GQ240290. The complete sequence of *EsCypA* cDNA contained a 5' untranslated region (UTR) of 50 bp, a 3' UTR of 233 bp with a polyA tail, and an open reading frame (ORF) of 495 bp encoding a polypeptide of 164 amino acids with the predicted molecular weight of 17.36 kDa and theoretical isoelectric point of 8.482 (Figure 1). There was no availability of a signal peptide revealed by SignalP program analysis. Two highly conserved signature sequences of peptidyl-prolyl *cis-trans* isomerase (¹⁹RIVMELRADVVPKTAENF³⁶ and ⁴⁸YKGCAFHRVIPNFMCGG⁶⁵) and a pro-isomerase domain (from Q⁴ to L¹⁶⁴) was identified in the amino acid sequence of *EsCypA* (Figure 1).

Multiple sequences alignment and the phylogeny of *EsCypA*

The deduced amino acid sequence of *EsCypA* shared significant homology with other reported CypAs, such as 85.9% identity with *P. monodon*, 81.7% with *C. farreri*, and 81.0% with both *Ictalurus punctatus* and *Argopecten irradians* (Figure 2). Fourteen amino acid residues critical for the fundamental structure and function of the CypA PPIase activity, including R⁵⁵, I⁵⁷, F⁶⁰, M⁶¹, Q⁶³, G⁷², A¹⁰¹, N¹⁰², A¹⁰³, Q¹¹¹, F¹¹³, W¹²¹, L¹²² and H¹²⁶ (Tu et al. 2003), were all conserved in *EsCypA* (Figure 2). A phylogenetic tree was constructed based on the amino acid sequences of 16 Cyps by the NJ method (Figure 3). *EsCypA* was first clustered with CypA from *P. monodon*, and then clustered with CypAs from *Danio rerio* and *I. punctatus*.

Tissue distribution of *EsCypA* mRNA

The mRNA expression level of *EsCypA* in different tissues of *E. sinensis* was quantified by real-time PCR with the β -actin gene as an internal control. For both *EsCypA* and β -actin genes, only one peak was observed at the corresponding melting temperature in the dissociation curve analysis, indicating that the PCR was specifically amplified (data not shown). The mRNA transcripts of *EsCypA* were detectable in all the examined tissues, including haemocytes, gill, hepatopancreas, gonad, muscle and heart (Figure 4). The expression level observed in muscle, heart, gill, hepatopancreas and gonad was 3.11-fold, 1.39-fold, 2.37-fold, 24.91-fold and 9.88-fold higher than that in haemocytes, respectively.

The temporal expression of *EsCypA* mRNA after microbe challenge

The temporal expression of *EsCypA* in haemocytes of *E. sinensis* after microbe challenge was detected via real-time PCR with β -actin gene as an internal control. No significant change in the relative expression level of *EsCypA* mRNA was observed during the whole course of bacteria challenge (Figure 5a). However, the relative expression level of *EsCypA* mRNA was up-regulated to 4.57-fold at 12 hrs ($p < 0.05$) and 2.61-fold at 24 hrs ($p < 0.05$) post *P. pastoris* challenge, and then down to the normal level at 48 hrs (Figure 5b).

The PPIase activity of r*EsCypA*

The r*EsCypA* protein was purified from the IPTG induced whole cell lysate and a signal sharp band was obtained with the exact molecular weight (Figure 6). Its PPIase activity was determined according to the described protocol. The r*EsCypA* is an active PPIase that can catalyze the *cis*- form of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide to *trans*- form. The *trans*- form of the substrate can be cleavable by α -chymotrypsin, which releases *p*-nitroanilide, while the *cis*- form cannot be cleaved. The *p*-nitroanilide was quantified spectrophotometrically at 405 nm. The PPIase activity is simply qualitatively represented by the change of OD₄₀₅, and the results were compared with the spontaneous isomerization that occurs in the absence of r*EsCypA* in negative control and blank groups (Figure 7).

The antifungal activity of r*EsCypA*

Radial diffusion assay was employed to evaluate the antifungal activity of r*EsCypA* against *P. pastoris* (Zhao et al. 2007a; Zhao et al. 2007b). Based on the radius of the antifungal zone, r*EsCypA* was found to harbour remarkable *in vitro* inhibitive effect on fungi *P. pastoris* at the concentration of 1024 $\mu\text{g mL}^{-1}$, 512 $\mu\text{g mL}^{-1}$ and 256 $\mu\text{g mL}^{-1}$, respectively, compared with the negative control groups (Table 3).

Table 3. The inhibit fungal activity of the r*EsCypA*.

Conc. ($\mu\text{g mL}^{-1}$)	r <i>EsCypA</i> (mm)			BSA (mm)		
1	7.17	±	0.29	7.67	±	0.76
2	8.00	±	0.87	7.83	±	0.76
4	7.33	±	0.58	7.67	±	0.76
8	7.83	±	0.58	7.67	±	0.76
16	7.33	±	0.29	7.33	±	0.29
32	7.17	±	0.29	7.33	±	0.58
64	7.83	±	0.76	7.50	±	0.87
128	7.67	±	0.76	8.17	±	0.29
256*	12.33	±	1.04	8.00	±	0.50
512*	14.00	±	0.50	7.83	±	0.76
1024*	17.83	±	0.29	7.50	±	0.50

*Each values is shown as mean \pm S.D. (n = 5), and concentration value with asterisk are significantly different ($p < 0.05$).

DISCUSSION

The first CypA, also called Cyp protein, was discovered in 1984 (Handschumacher et al. 1984) and its amino acid sequence was subsequently chartered in 1989 (Fischer et al. 1989; Takahashi et al. 1989). From then on, the CypAs have been identified in a wide range of species from prokaryotes and single cellular eukaryotes to higher plants and animals (Ribeiro et al. 2005). In the present study, the full length cDNA of a CypA was identified from Chinese mitten crab *E. sinensis*. The calculated molecular weight of *EsCypA* was of 17.36 kDa, which was consistent with the standard value of CypA proteins. Multiple sequence alignment revealed that two Cyp-type peptidyl-prolyl *cis-trans* isomerase signature sequences existed in the deduced amino acid of *EsCypA*. They were located at sites 19-36 (RIVMELRADVVPKTAENF) and 48-65 (YKGCFAHRVIPNFMCCGG) (Figure 1). Fourteen amino acid residues critical for the fundamental structure and function of the CypA PPIase activity, including R⁵⁵, I⁵⁷, F⁶⁰, M⁶¹, Q⁶³, G⁷², A¹⁰¹, N¹⁰², A¹⁰³, Q¹¹¹, F¹¹³, W¹²¹, L¹²² and H¹²⁶ (Tu et al. 2003), were all conserved in *EsCypA*. Among them, the residues R⁵⁵, Q⁶³, and N¹⁰² composed of the enzyme active site, and the residues I⁵⁷, A¹⁰¹, F¹¹³, W¹²¹, and L¹²² constituted the binding site for CsA, while the other residues F⁶⁰, M⁶¹, G⁷², A¹⁰³, Q¹¹¹ and H¹²⁶ were involved in the biological functions of CypA (Eisenmesser et al. 2002; Dornan et al. 2003; Howard et al. 2003; Piotukh et al. 2005; Mark and Nilsson, 2007). Similarity analysis revealed that the deduced amino acid sequence of *EsCypA* shared significant homology with other reported CypA, such as 85.9% identity with *P. monodon*, 81.7% with *C. farreri*, and 81.0% with both *I. punctatus* and *A. irradians*. These results indicated that *EsCypA* protein was a PPIase and could act as an intracellular receptor for immunosuppressant drug CsA.

EsCypA mRNA transcript was found universally distributed in all the tested tissues, including haemolymph, gill, hepatopancreas, gonad, muscle and heart (Figure 4), which was also consistent with the opinion that Cyps were ubiquitous in animal tissues (Galat, 1993). The ubiquity of *EsCypA* transcripts in different tissues indicated that it could be potentially involved in some important physiological processes and played important roles in the basal metabolism of crab cells. In previous reports, the expression level of CypA was found to be highest expressed in hepatopancreas and haemolymph of shrimp *P. monodon* (Qiu et al. 2009), while in gonad and gill of scallop *C. farreri* (Song et al. 2009). In the present study, *EsCypA* was higher expressed in gonad, and it was suspected that *EsCypA* might be involved in the maturation of gonad for its molecular chaperones functions such as PPIase activity. The higher expression level of *EsCypA* in hepatopancreas also suggested that CypA might play an important role in the immune defence system of crab, as hepatopancreas was regarded as the main immune center in crustaceans and many immune-related genes were high expressed in this tissue (Gai et al. 2009; Lu et al. 2012). Haemocytes were demonstrated to play important roles in the immune response against the invasive pathogens in invertebrates, and they were always selected as candidate for investigating the immune response in crabs (Mu et al. 2009). The temporal expression of *EsCypA* mRNA in haemolymph of *E. sinensis* after microbe challenge was detected by real-time PCR, and its relative expression level exhibited no significant changes during the whole course of *V. anguillarum* challenge, while it was up-regulated after the challenge of fungi *P. pastoris*. In previous reports, the temporal expression profiles of CypA in various species were different for the tissue and stimulators. For example, the mRNA expression of *PmCypA* in the hepatopancreas of shrimp *P. monodon* was up-regulated after the stimulation of lipopolysaccharide (Qiu et al. 2009). The expression level of *CfCypA* in *C. farreri* was almost unchanged in haemocytes, but up-regulated in gonad post injection of *V. anguillarum* (Song et al. 2009). After challenged by *L. anguillarum*, the relative expression level of two CypA in *V. philippinarum*, *VpCypA1* and *VpCypA2*, was opposite. *VpCypA1* mRNA was down-regulated, while *VpCypA2* mRNA in haemocytes was significantly up-regulated (Chen et al. 2011). These results collectively indicated that CypAs from marine invertebrate were not only a ubiquitous protein, but also an inducible protein and involved in the immune defence. However, the mRNA expression profiles of these CypAs were different from each other, indicating that CypA was a multifunctional molecule, and might be involved in different immune response in different species. Moreover, the specific response of *EsCypA* transcripts against fungi stimulation indicated the crab CypA might act as an antifungal protein.

The activities of *EsCypA* were further determined by examining its recombinant protein expressed in *E. coli*. Compared with the blank and control groups, the purified protein displayed significant PPIase activity and it could catalyze the *cis-trans* inter-conversion of Ala-Pro bond in the substrate. In the anti-fungi assay, *rEsCypA* was found to harbor remarkable *in vitro* inhibitive effect on fungi *P. pastoris* at the concentration of 1024 $\mu\text{g mL}^{-1}$, 512 $\mu\text{g mL}^{-1}$ and 256 $\mu\text{g mL}^{-1}$, respectively. Similarly, the CypA isolated previously from Chinese cabbage *B. campestris* could inhibit the growth of several fungal pathogens (Lee et al. 2007). Moreover, some other proteins with PPIase activity also exhibited

antifungal activity, such as FKBP from *B. campestris* (Park et al. 2007), Ess1 from *Candida albicans* (Li et al. 2005) and so on. These results indicated that the antifungal activity of rEsCypA might lie on its PPlase activity. However, compared with the authentic antimicrobial peptide (Zhao et al. 2007a; Zhao et al. 2007b), the inhibitive concentration of rEsCypA was rather high and the protein could not be so concentrated apparently in the normal physiological condition of crab. It was suspected EsCypA was mainly involved in immune defence via its PPlase activity, while its antifungal activity was of adjuvant.

In conclusion, the full-length cDNA encoding CypA was identified from Chinese mitten crab *E. sinensis*. It was constitutively expressed in all the tested tissues, and up-regulated in haemocytes after challenged by fungi. The purified rEsCypA protein showed significant PPlase activity to catalyze the *cis-trans* inter-conversion of Ala-Pro bond in the substrate and antifungal activity against *P. pastoris*. All these results indicated that it was a typical CypA member and was potentially involved in the innate immune responses of crabs.

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Figures

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1  ATTCCAAGGCGTTCGGTTCCTCGTCTACCTCCTCCACTCCAGCAGCTCACCATGGGCAAC
1                                     M G N
60 CCTCAGGTCTTCTTCGACATCACTGCCAATGGGCGGCCCGTCGGCAGGATTGTCATGGAG
4  P Q V F F D I T A N G R P V G R I V M E
120 CTGCGTGCTGATGTTGTACCCAAGACTGCTGAGAACTCCCGTGCCCTGTGCACTGGGGAG
24  L R A D V V P K T A E N F R A L C T G E
180 AAGGGCTTCGGCTACAAGGGTTGCGCCTTCCACCGTGTGATCCCCAACTTCATGTGTCAA
44  K G F G Y K G C A F H R V I P N F M C Q
240 GGTGGCGACTTCACTGCTGGCAATGGCACAGGTGGGAAGTCCATCTATGGCTCGAAGTTC
64  G G D F T A G N G T G G K S I Y G S K F
300 GCCGATGAAAACCTCACCTTCGTCACACTGGCCCTGGCATTCTGTCCATGGCCAATGCT
84  A D E N F T L R H T G P G I L S M A N A
360 GGACCCAACACCAATGGTTCAGTTCTTCTGTGTACTGTCAAGACTGCCTGGCTGGAT
104 G P N T N G S Q F F L C T V K T A W L D
420 GGCAAGCACGTTGTCTTCGGGAGTGTGTTGAGGGCATGGATGTTGTTCCGCGAGTTGAG
124 G K H V V F G S V V E G M D V V R Q V E
480 GGATACGGTACACCTAATGGCAGCACCTCCGCCAAGATTGTTATTGCCAACTCTGGCCAG
144 G Y G T P N G S T S A K I V I A N S G Q
540 TTGTAACATTCTACCACGCACTTGAAAGAACCATAGCAGCAGTCATTGCATTGTTTAGT
145  L *
600 GTAGGAACATGTATGTAACTTGAATAAAGGTTAATTTTCATATTGCTTTATGAGATTTCGT
660 TTGCGTGATTTTCATTGTATTGAATGTTGACGCCAGGCTGATATTCAGTGTTAGTCGTCTA
720 CTCACTTTTCATCAATAAATCATGAAGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAA

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Fig. 1 Nucleotide and deduced amino acid sequences of *EsCypA*. The nucleotides are numbered along the left margin. The start and codon was marked in bold, the asterisk indicated the stop codon and the classical polyadenylation signal in the 3'-UTR is double underlined. The signature sequence of peptidyl-prolyl *cis-trans* isomerase is shadowed. The conserved amino acids residues are underlined.

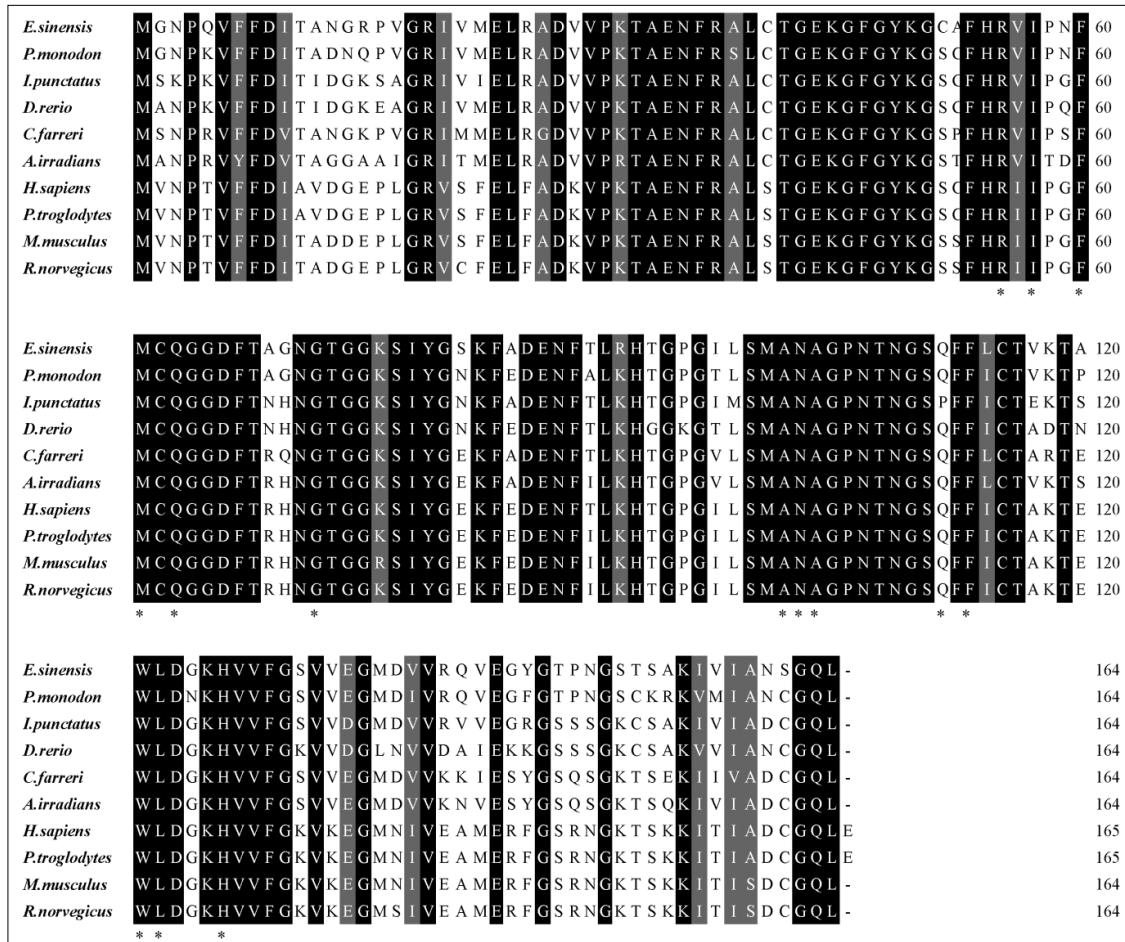


Fig. 2 Multiple alignment of EsCypA with other CypAs deposited in GenBank. The black shadow region indicated positions where all sequences share the same amino acid residue. Gaps are indicated by dashes to improve the alignment. The asterisk indicated the conserved amino acids residues.

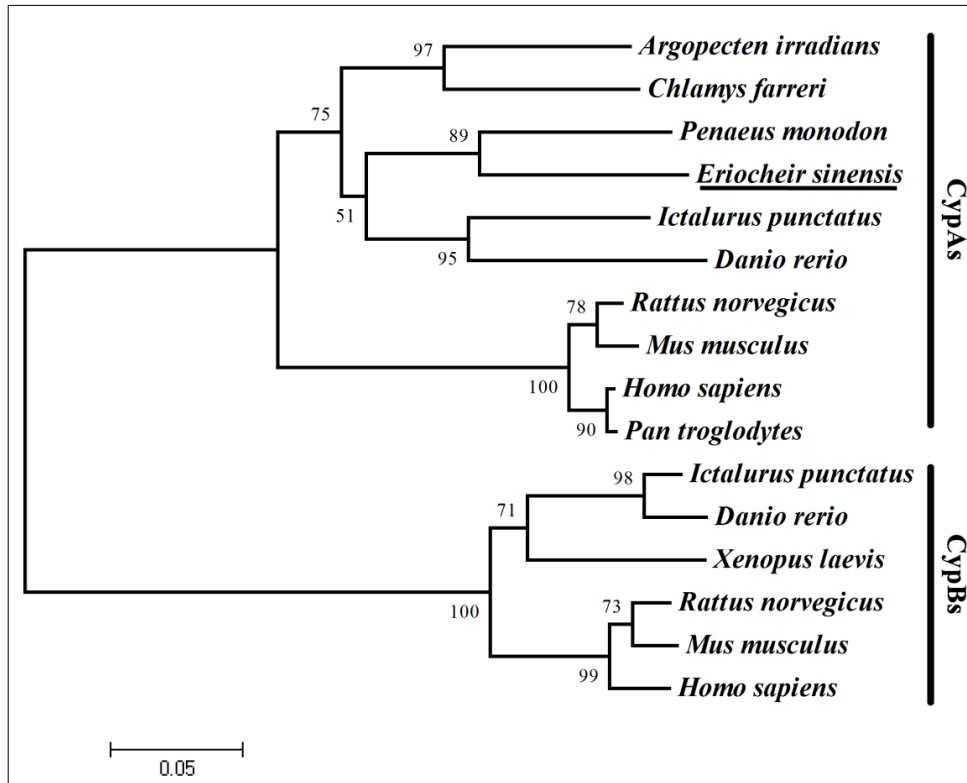


Fig. 3 Consensus neighbour-joining tree based on the sequences of CypAs and CypBs from different animals. The numbers at the forks indicated the bootstrap value.

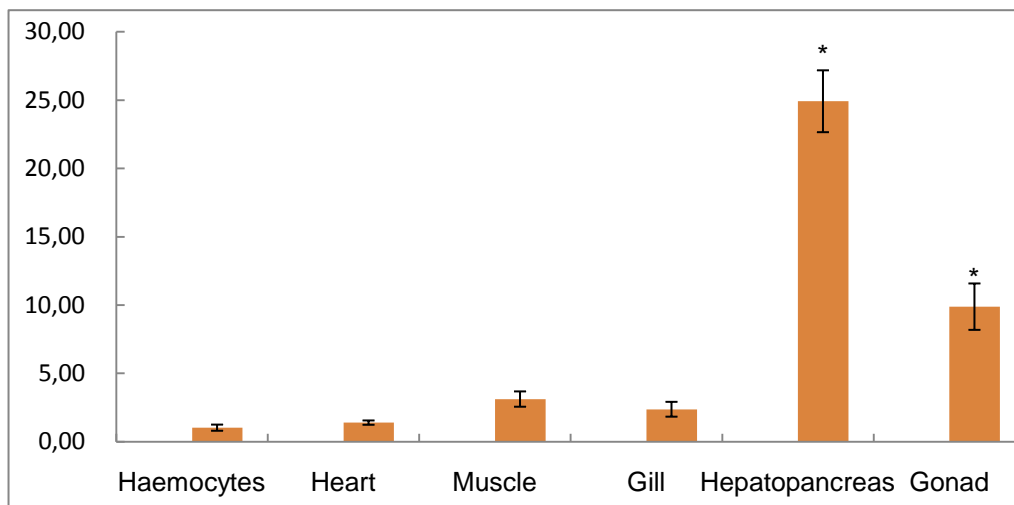


Fig. 4 Tissue distribution of the *EsCypA* mRNA transcript measured by real-time PCR. β -actin gene was used as an internal control to calibrate the cDNA template for all the samples. *EsCypA* mRNA transcript level in hepatopancreas, haemocytes, gill, gonad, muscle and heart of adult crabs is normalized to that in haemocytes. Vertical bars represent mean \pm S.D. ($n = 5$), and bars with asterisk were significantly different ($p < 0.05$).

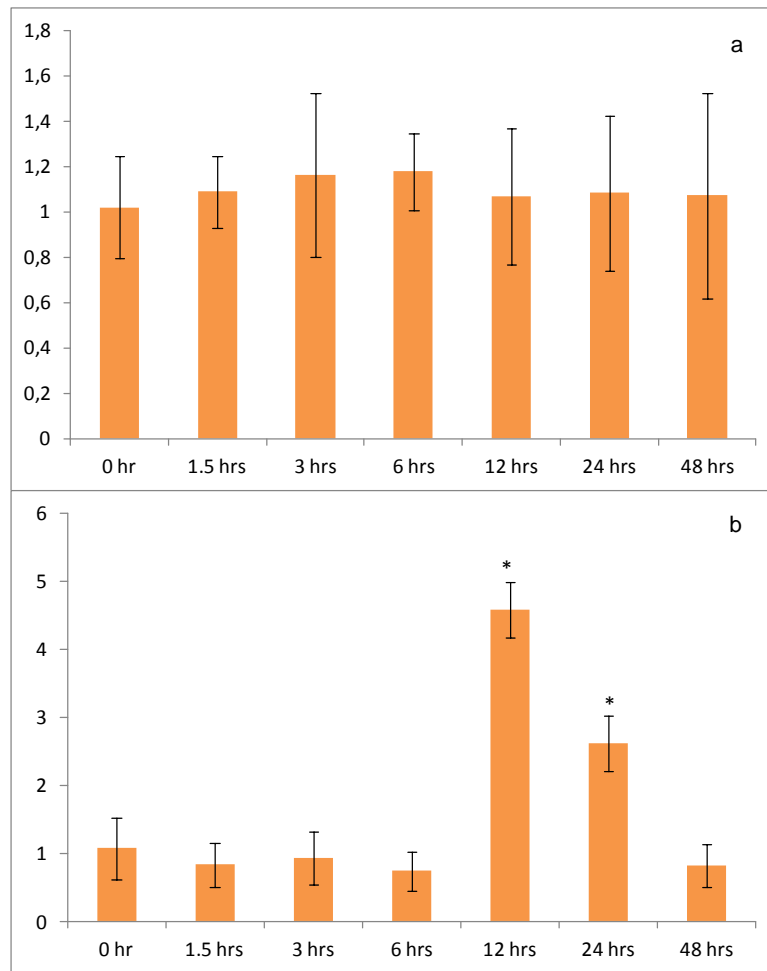


Fig. 5 Temporal expression of *EsCypA* mRNA detected by real-time PCR in crab haemocytes after microbe challenge (a) *V. anguillarum* and (b) *P. pastoris* for 1.5, 3, 6, 12, 24 and 48 hrs. β -actin gene was used as an internal control to calibrate the cDNA template for all the samples. Each values is shown as mean \pm S.D. (n = 5), and bars with asterisk are significantly different ($p < 0.05$).

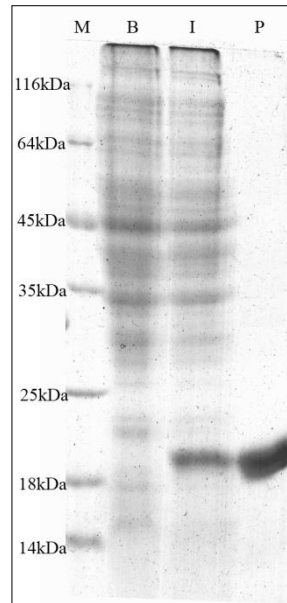


Fig. 6 SDS-PAGE analysis of the rEsCypA in *E. coli* BI21 (DE3). Line M was the unstained protein marker SM0431 (MBI, USA). Line B was the supernatant of non-induced bacteria lysate. Line I was the supernatant of IPTG-induced bacteria lysate. Line P was the purified rEsCypA.

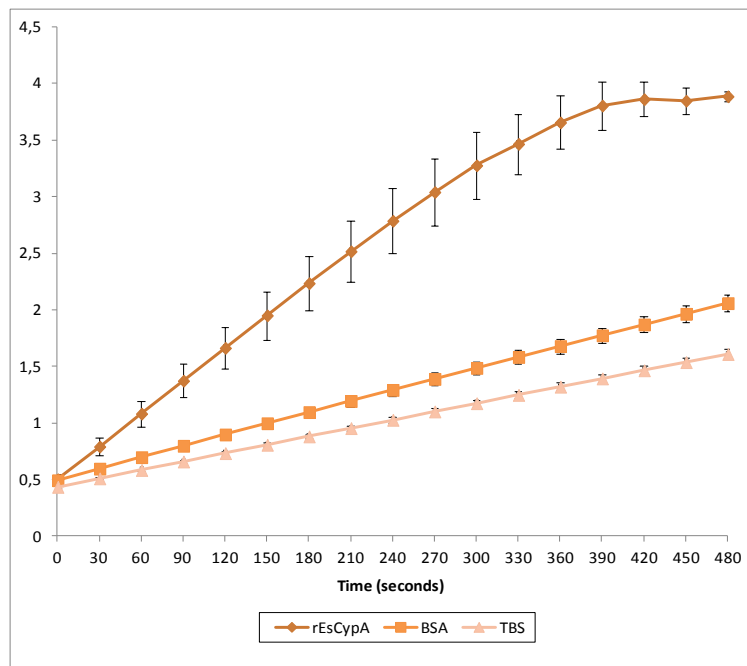


Fig. 7 The PPLase activity of the rEsCypA. PPLase activity was qualitatively represented by the change of OD₄₀₅, which was quantified spectrophotometrically every 30 sec. TBS buffer and BSA protein were employed as blank and negative control.