Molecular Cloning and Characterization of an Endogenous White Spot Syndrome Virus Gene from *Eriocheir Japonica Sinensis*

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Abstract: Based on expressed sequence tag(EST) and rapid amplification of cDNA ends(RACE) techniques,a WSSV gene designated as *EjsWSSV* was cloned and characterized from the Chinese mitten crab *Eriocheir japonica sinensis*. The full-length cDNA of *EjsWSSV* is 3 864 bp in size and contains an open reading frame of 3 732 bp which encodes a 1 243 amino acid polypeptide(138.25 kDa) with VWA domain. Sequence alignment,structure comparison and some bioinformatics analyses showed that *EjsWSSV* shared 100% similarity with WSSV genome ORF16 sequence. The bioinformatics analyses revealed that EjsWSSV had no putative signal peptide found and indicated that it was probably a non-secretory,alpha spiral and non-transmembrane protein. It was probably located in the cytoplasm. The PCR amplification results indicated that *EjsWSSV* was probably an endogenous virus gene of *E. j. sinensis*.

Key words: *Eriocheir japonica sinensis*,WSSV,RACE,cDNA cloning,sequence analyses

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中华绒螯蟹内源性白斑综合症病毒基因 *EjsWSSV* 的克隆与鉴定

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[摘要] 基于已获得的表达序列标签序列,应用 RACE 技术从中华绒螯蟹基因组中获得了一个白斑综合症病毒基因(*EjsWSSV*)的全长 cDNA 序列,全长为 3 864 bp 并包含一个 3 732 bp 的开放阅读框,编码一个 138.25 kDa 的含 1 243 个氨基酸的多肽,编码的氨基酸序列包含一个 VWA 结构域. 通过序列比对、序列结构比较和一些生物信息学预测分析,表明 EjsWSSV 蛋白序列与白斑综合症病毒基因组的 ORF16 具有高同源性,它不含有信号肽和跨膜区,为非分泌型和 α-螺旋型蛋白,可能定位于细胞质. 通过 PCR 检测验证,*EjsWSSV* 基因可能为内源性病毒基因.

[关键词] 中华绒螯蟹,白斑综合症病毒,cDNA 末端快速扩增,cDNA 克隆,序列分析

White spot syndrome virus(WSSV) is an enveloped,non-occluded and rod-shaped virus,which is one of the most devastating pathogens of shrimps and other species of crustacean[1]. The genome of WSSV has been sequenced on 3 different isolates with GenBank accession Nos. AF332093(305,107 bp),AF369029(292,967 bp),and AF440570(307,287 bp). Sequencing of 3 different isolates reveals that the circular dsDNA genome contains around 300 kb encoding about 184 putative open reading frames(ORFs)[2-4]. WSSV is at present the largest animal virus genome that has been entirely sequenced[3]. Since it was first detected in the 1990s in Taiwan,WSSV has quickly spread to the whole world and caused severe damage in the shrimp industry. Therefore,WSSV has attracted intensive investigations. The results revealed that it had a broad host range,infecting all cultured shrimps as well as other invertebrate aquatic organisms such as crabs,lobsters and crayfishes[5-10]. To date,

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more than 93 species of arthropods have been reported as hosts or carriers of WSSV either from culture facilities, the wild or experimental infection. Such as *Fenneropenaeus indicus*, *Marsupenaeus japonicas*, *Macrobrachium rosenbergii*, *Litopenaeus stylirostris*, *Litopenaeus vannamei*, *Penaeus monodon*, *Penaeus chinensis*, *Partelphusa hydrodomous* etc. [9,11-16]. The giant freshwater prawn, *M. rosenbergii* was reported to have a high tolerance to WSSV infections and known to act as an asymptomatic carrier for the virus [17]. The earlier growth stages of *M. rosenbergii* are more susceptible to WSSV infections compared to their sub-adult and adult stages wherein they are more tolerant to infection by this virus [16,18]. Because of the large size of the genome and the uniqueness of the proteins that the WSSV ORFs encode, WSSV has not yet been fully characterized [19].

According to the 8th animal viruses report of the international committee the taxonomy viruses(ICTV), WSSV was defined as the type species of the new virus family Nimaviridae, and it was not included in the Retroviridae [20]. Retrovirus group can be divided into exogenous virus and endogenous virus based on their biological characteristics. Exogenetic viruses are closely related with disease of animals and humans. The host can be infected directly from the environment. This kind of virus can be divided into acute and chronic virus according to the differences of their biological characteristics, infection process and genomes [21]. Animal cells infected by exogenous virus often cause cellular stress response. In most cases, cellular stress response resulted in expression level change of heat shock protein family members such as Hsp60, Hsp70 or Hsp90 genes, and these genes involved in against the infection of the virus [22]. Hsc70 expression was enhanced by WSSV infection at the early stage and peaked at 12h post-infection and decreased drastically at the late stage [23]. Endogenous retroviruses are sequences in the genome thought to be derived from ancient viral infections of germ cells of animals. Endogenous retroviruses may be a variant of a retrovirus which became permanently integrated with its host and is inherited from generation to generation as part of the genome of the host. Such viruses typically do not have an apparent infection. In an ordinary way, the sequences of endogenous viruses that integrated into the host's genome were dormant according to a study published in 2005, no HERVs capable of replication had been identified; Some studies had been done in vertebrates(eg. chicken, mouse, cat, baboon and sheep, etc.), and the results revealed their cells had the sequence of endogenous viruses, but these sequences usually did not harm the host cells [24-31].

In our previous work, we have attained many WSSV genes sequences from expression sequence tags cDNA library of *Eriocheir japonica sinensis* [32]. In the present study, we cloned the full-length cDNA of a WSSV gene (designated as *EjsWSSV*) from *E. j. sinensis*. This *EjsWSSV* cDNA will provide useful molecular information for further investigation on its role in disease of crabs. And it will provide the theory basis for epidemic prevention of invertebrate animal diseases.

1 Materials and Methods

1.1 Sampling

Tissue samples(about 30mg for each sample) were collected from *E. j. sinensis*(the megalopa stage, Stage M) breeding in an aquatic nursery. Samples were collected from different batches of *E. j. sinensis*. The samples were isolated and kept in different tubes: one crab individual per tube for a total of 20 tubes in the megalopa stage and the first crab stage(Stage J1) respectively. These fresh tissue samples were stabilized in RNA *later* RNA Stabilization Reagent(Qiagen, Germany), cut into tiny particles(less than 0.5mm thick) in separate tubes, kept overnight at 4℃, and finally stored at −20℃ before RNA isolation.

1.2 Total RNA isolation

Total RNA from samples was isolated separately using RNeasy Mini Kit(Qiagen, Germany), messenger RNA (mRNA) was purified from total RNA using the Oligotex mRNA Mini kit(Qiagen, Germany) and stored at −70℃. The RNA concentration and purity were assessed spectrophotometrically by measuring their absorbance at 260 nm and 280 nm in biophotometer(Eppendorf, Germany). RNA fragmentation state was evaluated by 1.2% agarose denatured gel electrophoresis.

1.3 Cloning of the full-length cDNA of *EjsWSSV* gene

An expressed sequence tag(EST,406 bp) fragment for *WSSV* gene was obtained from a subtractive hybridization cDNA Library of *E. j. sinensis* [32] . The cDNA from pleonal tissue of the first crab stage was used as "tester" and the cDNA from that of the megalopa stage as "driver" in the subtractive hybridization procedure,which was performed using the PCR-Select cDNA Subtraction Kit(Clontech,USA) . The EST fragment displayed 100% similarity to *WSSV* genes. The EST sequence was deposited in GenBank with the accession number GH985317. It was used for further cloning of the full-length cDNA of *EjsWSSV* by RACE(Rapid Amplification of cDNA Ends) using the SMART RACE cDNA Amplification Kit(Clontech,USA) according to the user's manual.

2 gene-specific primer sets,GSP1-WSSV5′ and GSP1-WSSV3′,GSP2-WSSV5′ and GSP2-WSSV3′(Table 1) ,were used to clone the 5′ and 3′ end of *EjsWSSV* cDNA. The 5′-RACE was carried out with 10 × Universal Primer A Mix(UPM) and GSP1-WSSV5′/GSP2-WSSV5′,while the 3′-RACE with UPM and GSP1-WSSV3′/GSP2-WSSV3′,respectively. The PCRs for RACE reactions were carried out respectively in a 50 μL reaction volume containing 5 μL of 10 × Advantage 2 PCR buffer,1 μL 50 × dNTP Mix(2.5 mmol/L) ,1 μL 10 × Universal Primer A Mix(10 μmol/L) ,40 μL PCR-grade water,1 μL 50 × Advantage 2 polymerase(Clontech,USA) ,1 μL gene-specific primer(10 μmol/L) and 1 μL RACE-Ready cDNA(from the megalopa stage crabs) . The PCR cycling parameters were as follows: 5 cycles of 94℃ for 30 s and 72℃ for 5 min; 5 cycles of 94℃ for 30 s,68℃ for 30 s and 72℃ for 5 min; At last 18 cycles of 94℃ for 30 s,65℃ for 30 s and 72℃ for 5 min for 5′-RACE, and 12 cycles of 94℃ for 30 s,65℃ for 30 s and 72℃ for 5 min for 3′-RACE respectively. The products were evaluated by 1.2% agarose denatured gel electrophoresis.

Table 1 Primers used for *EjsWSSV* cDNA amplification and confirmation

Primers name	Primers Sequence(5′ to 3′)
GSP1-WSSV5′	5′-CCACATTCAATCAAATCCATCAGC-3′
GSP1-WSSV3′	5′-CGAGACAAAAGAAAGGACGACAGT-3′
GSP2-WSSV5′	5′-GCACGCACAACAGGTCCACTT-3′
GSP2-WSSV3′	5′-GCTGCTGCTGCTGATACAGATACTC-3′
10 × Universal Primer A Mix	5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′ and 5′-CTAATACGACTCACTATAGGGC-3′
WSSVF	5′-TCCACCAGTCATTTAGTTTC-3′
WSSVR	5′-ATCCTGTAGTTTCCCTTGC-3′

A fragment of 411 bp was amplified by 3′-RACE technique from the 3′ end of *EjsWSSV* cDNA(from the megalopa stage crabs) . Stop codon TGA,canonical polyadenylation signal sequence AATAAA and a poly-(A) tail were found in the 3′ end of this fragment. A fragment of 3189 bp was amplified by 5′-RACE technique. The purified PCR products were cloned into the pGEM-T easy vectors(Promega,USA) ,and transformed into competent *Escherichia coli* cells DH5α(TaKaRa,Dalian) . Three of the positive clones were sequenced on an ABI PRISM 3730 Automated Sequencer using BigDye terminator v3.1(Applied Biosystems,USA) . The *EjsWSSV* sequence obtained after 5′- and 3′-RACE was assembled using DNA Star Lasergene 7.1 software to generate full length cDNA. A 3,864 bp nucleotide sequence representing the full-length cDNA sequence of *EjsWSSV* gene was obtained by cluster analysis of the above fragments and the EST sequence.

1.4 Reverse transcription polymerase chain reaction

In order to to confirm *EjsWSSV* gene whether is an endogenous virus gene or not. Tissue samples from different batches of *E. j. sinensis* were examined using reverse transcription polymerase chain reaction(RT-PCR) method. Primers used for RT-PCR were designed according to the corresponding cDNA sequences of *E. j. sinensis*(Table 1) . The RT-PCR was carried out in a 50 μL reaction volume containing 5 μL 10 × Ex PCR buffer,5 μL $MgCl_2$(25 mM) ,7 μL dNTP Mix(2.5mM each) ,1 μL WSSVF(10 μmol/L) and 1 μL WSSVR(10 μmol/L) , 29.5 μL PCR-grade water,1 μL PCR-Ready cDNA(from Stage M and Stage J1 crabs,respectively) ,and 0.5 μL Ex Taq(5U/μL) (TaKaRa,Dalian) . The PCR cycling parameters were as follows: initial denaturation in 94℃ for 5 min; 30 cycles of 94℃ for 30 s,50℃ for 30 s and 72℃ for 1 min; and at last,72℃ for 10 min. The products

were evaluated by 1.2% agarose denatured gel electrophoresis.

1.5 Sequence analysis of *EjsWSSV* gene

Similarity searching of amino acid sequences in GenBank database was conducted with BLASTp programs (http://www.ncbi.nlm.nih.gov/BLAST/) [33,34] at the National Center for Biotechnology Information(NCBI). The open reading frame(ORF) of *EjsWSSV* cDNA was determined using ORF Finder(www.ncbi.nlm.nih.gov/gorf/) while the putative signal peptide was predicted using SignalP(www.cbs.dtu.dk/services/SignalP) [35,36]. Potein physical and chemical properties were predicted using ProtParam tool(http://us.expasy.org/tools/protparam.html) [37] while hydrophobicity was respectively predicted using ProtScale tool(http://us.expasy.org/cgi-bin/protscale.pl) [37] and BioEdit tool of Tom Hall Company(http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Molecular mass and theoretical isoelectric point were predicted using compute pI/Mw tool(http://www.expasy.org/tools/pi_tool.html). Motif scan was respectively performed against databases of motifs(http://myhits.isb-sib.ch/cgi-bin/motif_scan) [38] and by PROSITE ScanProsite(http://www.expasy.org/prosite/) [39]. Domains analysis was performed by Simple Modular Architecture Research Tool(http://smart.embl-heidelberg.de/) [40,41]. The potential protein subcellular localization was predicted on the server(http://psort.ims.u-tokyo.ac.jp/form2.html). Transmembrane domain was respectively predicted using TMHMM Server v.2.0(http://www.cbs.dtu.dk/services/TMHMM/) and DAS-TMfilter server(http://mendel.imp.ac.at/sat/DAS/) [42]. Coils form was predicted using COILS Server(http://www.ch.embnet.org/software/COILS_form.html) [43]. Secondary structure was predicted using PORTER Server of University College Dublin(http://distill.ucd.ie/porter/) [44]. Fold Recognition was predicted using Phyre Version 0.2 webserver(http://www.sbg.bio.ic.ac.uk/~phyre/index.cgi) [45,46]. Additional assessments of domain structures were performed on ProSA-Web(https://prosa.services.came.sbg.ac.at/prosa.php) [47,48] and Verify 3D Structure Evaluation Server(http://nihserver.mbi.ucla.edu/Verify_3D/) [49,50].

2 Results and Discussion

2.1 Identification and analysis of *EjsWSSV* cDNA

The full-length *EjsWSSV* cDNA includes a 5′-terminal untranslated region(UTR) of 70 bp, a 3′-terminal UTR of 62 bp with a canonical polyadenylation signal sequence AATAAA, and contains a 3732 bp ORF encoding a 1243 amino acids protein polypeptide with a predicted molecular mass of 138.25 kDa and theoretical isoelectric point of 5.83. The A + T content and C + G content in the sequence are 58.02% and 41.98%, respectively(Fig. 1). The BLAST results of the nucleotide sequence and deduced amino acid sequence of *EjsWSSV* showed that the sequence obtained shared 99% similarity(nr: AF369029 and AF440570, aa: AAK77685 and NP_477987) with shrimp white spot syndrome virus. The complete cDNA sequence was deposited in the GenBank database under the accession number FJ483831.

Multiple alignments of *EjsWSSV* with WSSVs of other species showed high conservation, which indicated the Chinese mitten crabs had been infected with WSSV. To date, several species of crabs, lobsters and crayfishes have been reported as hosts or carriers of WSSV [9,16,18]. In our previous experiments, expressed sequence tags(from the suppression subtractive hybridization cDNA library of the Chinese mitten crab megalopa) homologous analysis showed that 90% ESTs fragments shared homology, while just one expressed sequence tag fragment from the first crab stage shared 100% similarity with the WSSV gene [32]. Sequence alignment showed that the expressed sequence tag fragment was an *ankyrin* gene, revealing that WSSV genome sequence had integrated into the Chinese mitten crab *E. j. sinensis* genome [51]. It indicated that *EjsWSSV* is probably an endogenous differential expression gene of *E. j. sinensis*. Other studies had shown that 100% of crabs, lobsters and crayfishes infected WSSV would die within days [12,52], while it never happened in our experiments. In most cases, animal cells infected by exogenous WSSV often cause expression level increasing of heat shock protein family members [22]. In our previous experiments, expression level of Hsp90 detected in the megalopa stage was lower than the first crab stage [53]. The

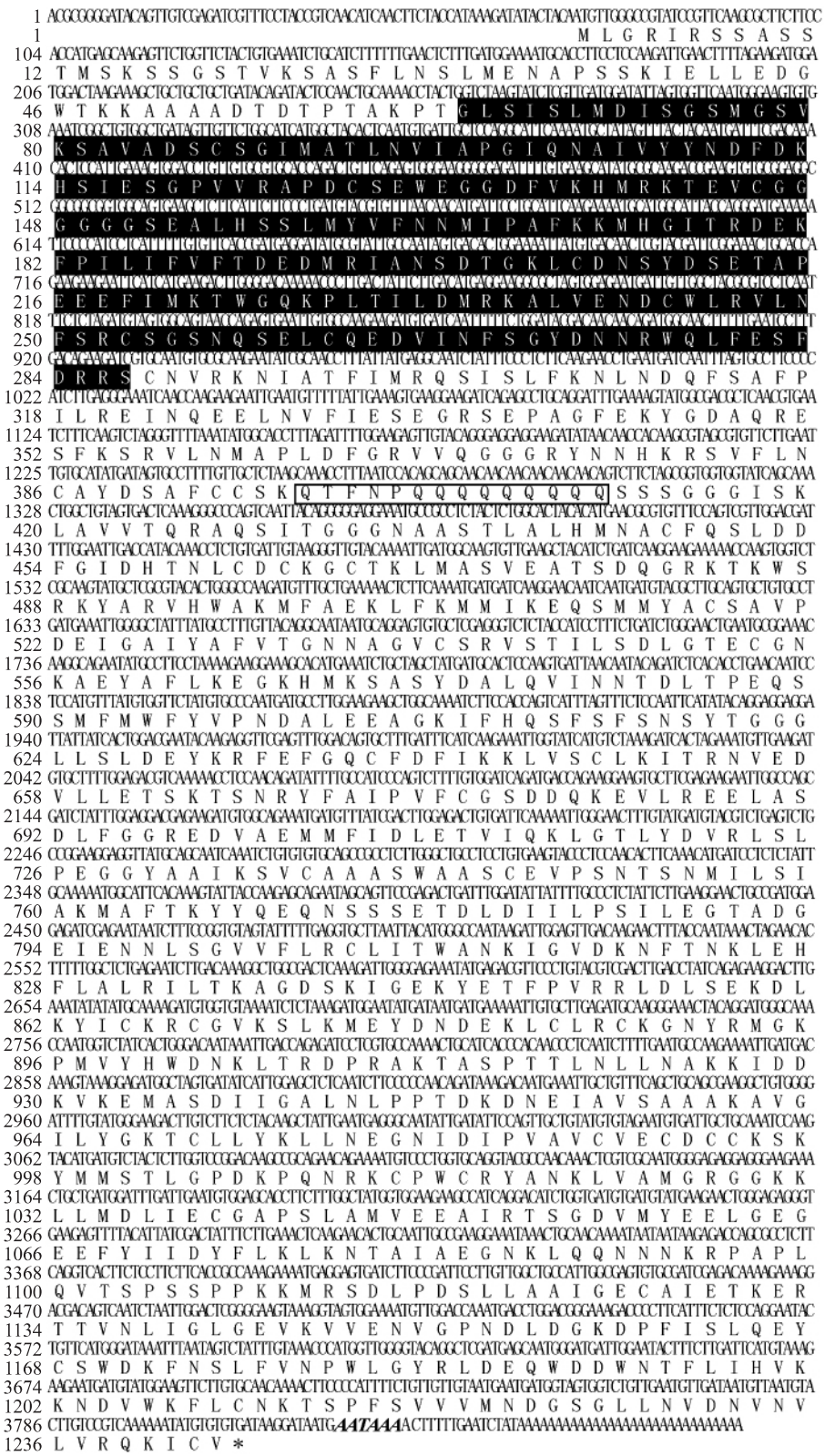

Fig. 1 Nucleotide and deduced amino acid sequences of *EjsWSSV* gene from *E. j. sinensis*

Von Willebrand factor type A(VWA) domain is shadowed. The box indicated a Gln-rich region. The putative polyadenylatio signal site is italic and bold. The stop codon is indicated by *

result also supports that *EjsWSSV* may be not from exogenous infection. In this work，a 3 864 bp nucleotide sequence representing the full-length cDNA sequence of *EjsWSSV* gene was obtained，which sharing 100% similarity with WSSV genome ORF16 sequence. But the sequence length of *EjsWSSV* gene is shorter than WSSV ORF16.

For virus, genomic deletions are frequently related with reduced infectious and decreased replication ability [54], reduction of capacity to fuse cells reduced [55] and viral virulence [56,57]. The experiment for WSSV infected shrimp found that the mortality of shrimp infected by the intact WSSV was higher than that of shrimp infected by DNA fragment-deleted WSSV, it indicated that this deletion was somehow lead to the reduction of virulence of the virus [58]. Therefore, if *EjsWSSV* is an endogenous virus gene, which retains only a partial sequence of the WSSV genome ORF16, the Chinese mitten crab does not produce white spot syndrome is easily explained. In addition, the RT-PCR was performed to amplify *EjsWSSV* gene from other tissue samples of *E. j. sinensis*. The results indicated that *EjsWSSV* gene was successfully amplified in the vast majority of the tissue samples of the Chinese mitten crabs(Fig. 2). Overall, these results revealed that *EjsWSSV* was probably an endogenous virus gene of *E. j. sinensis*.

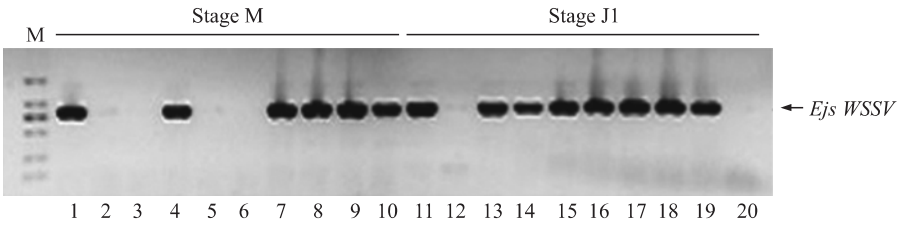

Fig. 2 Expression of *EjsWSSV* gene in different individual tissues during different molt stages of *E. j. sinensis*
Lane 1 – 10: the megalopa stage(Stage M); Lane 11 – 20: the first crab stage(Stage J1)
M: DL 2000 marker(TaKaRa) is shown on the left

2.2 Structural analysis of EjsWSSV

No signal sequence was identified in the transcript using the SignalP software, and it was probably a non-secretory protein. Through using COILS Server software, no typical coiled-coil domain was found in EjsWSSV. The result of EjsWSSV protein hydrophobicity analyses by ProtScale and BioEdit indicated that it contained hydrophobic area. Protein motifs were scanned from Myhits Motif Scan and PROSITE diagram. The Myhits Motif Scan result indicated EjsWSSV contained 11 protein kinase C(PKC) phosphorylation sites, 20 casein kinase Ⅱ(CK2) phosphorylation sites, 18 N-myristoylation sites, a domain rich in Glutamine and a domain rich in Serine. The PROSITE diagram predicted that EjsWSSV contained a domain rich in Glutamine(from 397 to 410 amino acid residues, QTFN-PQQQQQQQQQ), 15 protein kinase C(PKC) phosphorylation sites, 21 casein kinase Ⅱ(CK2) phosphorylation sites, 24 N-myristoylation sites, 2 casein kinase phosphorylation sites, 9 N-glycosylation sites, and 2 Amidation sites. Transmembrane topology prediction indicated that EjsWSSV was probably a non-transmembrane protein. The potential protein subcellular localization prediction indicated that the EjsWSSV was likely to locate in mitochondrion(34.8%). Endoplasmic reticulum membrane retention signals prediction indicated that XXRR-like(LGRI) motif was in the N-terminus and KKXX-like(QKIC) motif was in the C-terminus of EjsWSSV protein. Protein domains scanning from SMART diagram indicated that EjsWSSV had a vWF type domain(from 64 to 287 amino acid residues) which was proved wildly existing in various kinds of plasma proteins, integrins, collagens and extracellular proteins [59-61]. Numerous biological events such as cell adhesion, migration, homing, pattern formation, and signal transduction after interaction with a large array of ligands was participated by the proteins that incorporate a vWF type domain [62]. The result from EjsWSSV protein secondary structure prediction indicated that it was an alpha spiral protein. The three-dimensional structure analysis of EjsWSSV domains revealed that it contained some void regions adhered numerous nitrogen atoms, oxygen atoms, sulfur atoms and carbon atoms on the surface(Fig. 3). The Z-Score for overall model quality were −1.88 and local model quality values for parts of the residues were upon zero from ProSA-Web. The 3D-1D averaged score from Verify 3D Structure Evaluation Server were ranged generally from −0.45 ~ 0.68, of which only 29.74% were upon 0.2. The quality evaluation results of the predicted three-dimensional structure of EjsWSSV monomer indicated that it was of slightly poorer quality. The result may offer the positive reference for additional studies to determine the structure and function of *EjsWSSV* gene.

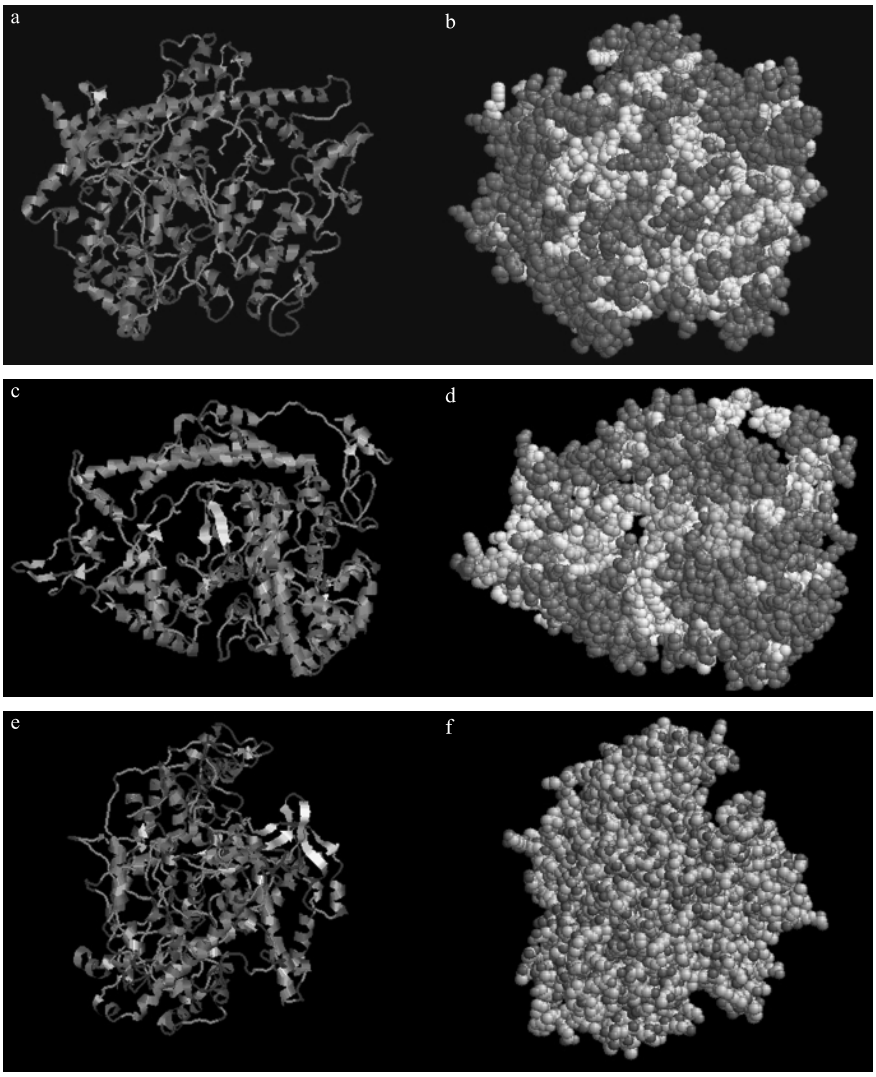

Fig. 3. Three-dimensional structure of EjsWSSV protein

a,c,e: Three-dimensional ribbons structure of EjsWSSV protein,a) anterior view,c) dorsal view,e) lateral view,α helices are shown in crimson,β-slices are shown in yellow,turns are shown in blue,others residues are shown in white. b,d,f: Three-dimensional spacefill structure of EjsWSSV protein,b) anterior view,d) dorsal view,f) lateral view; b,d: α helices are shown in crimson,β-slices are shown in yellow,turns are shown in blue,others residues are shown in white; f: Oxygen atoms are shown in red,nitrogen atoms are shown in baby blue,sulfur atoms are shown in yellow,carbon atoms are shown in grey

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