Universe Scientific Publishing

ORIGINAL ARTICLE

Prokaryotic Expression of SGEG1 Protein from Artemia Diapause Cysts

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Abstract: Artemia is a small marine animal survived in extreme environments. Its diapause cysts can maintain long-term vitality in dry and anoxic environment and develop into larvae under suitable condition. Artemia passed through the extreme environment by its dormancy state. The shell gland-specifically expresses gene (SGEG) is specifically expressed in secretory cells of shell gland of oviparous Artemias ovisac and SGEG peptide is an important componenProkaryotic Expression of SGEG1 Protein from Artemia Diapause Cystst of diapause cysts shell protein. In this study, the SGEG1 gene sequence of deduced mature peptide was cloned into the prokaryotic expression vector pET-28a (+) with 6×His tag. A recombinant plasmid containing pET-28a (+) -SGEG1 was successfully constructed and transformed into E. coli BL21(DE3). The fusion expression of recombinant SGEG1 (rSGEG1) protein was induced. Meanwhile, the induced IPTG concentration, induction time of rSGEG1 and the method of protein electrophoresis were optimized. The recombinant SGEG1 peptide with a molecular weight of about 10 K Da was highly expressed,and the fusion protein mainly existed in the form of inclusion body, which supported a foundation for the preparation of SGEG1 protein in vitro.

Keywords: Artemia; SGEG1; Prokaryotic Expression

Introduction

Artemia, also known as brine shrimp or brine shrimp, belongs to arthropod phylum and is a small marine animal that can survive extremely harsh environment in dormant state. Artemia reproduction has two modes of reproduction, namely, oviparous and oviparous. Oviposition refers to the fact that the offspring have hatched into nauplii when they are produced from the mother, while oviparous refers to the fact that the offspring are produced from the mother in the form of eggs. When the external environmental conditions are poor, Artemia oviparous produces resting eggs, which can be produced under the conditions of long-term hypoxia, dehydration, extreme temperature, gamma-ray, etc. still maintains high vitality and hatching rate[1-4]. The nauplius of Artemia resting eggs after hatching is a living bait[5] widely used in the aquaculture process of fish, shrimp and crab, etc., and has important economic value.

The outer layer of Artemia resting eggs is a complex non-cellular egg shell, which forms a special stress-resistant mechanism together with factors such as p26 and Artemin in its cells. Secretory gland specific expression gene (Shell Gland-Specific Expressed Gene, SGEG) is a gene specifically expressed only on secretory cells of egg sac secretory gland of Artemia in the oviparous pathway, and is Artemia resting eggs shell protein

An important part of the 55 is to participate in resting eggs's anti-adversity mechanism for extreme environments[6]. The SGEG1 gene is one of the members of the Artemia SGEG geneticists. Its cDNA has a full length of 450bp and has an 345bp open reading frame. The deduced polypeptide consists of 33 amino acid signal peptides and 81 amino acid mature peptides, which are of great significance to the highly ordered assembly of Artemia egg shells and their

doi: 10.18686/fsa.v1i2.1317

participation in protecting dormant embryos from various extreme environmental stresses[7,8].

In this study, the mature peptide sequence of Artemia SGEG1 gene was cloned into prokaryotic expression vector pET-28a(+) 60 with 6×His tag, and protein induction and in vitro expression were carried out to optimize the optimal induction conditions[9,10] and electrophoretic detection methods[11,12] of SGEG1 recombinant protein. The high-efficiency expression and in vitro preparation of the target protein are realized.

1. Materials and methods

1.1 Materials and instruments

Taq DNA polymerase, DNA molecular weight standard, protein molecular weight standard, kanamycin, peptone, yeast powder, 65.40% acrylamide -methylene acrylamide solution (29:1), urea, isopropyl -β-D- thiogalactoside (IPTG), egg white electrophoresis buffer, DNA "fragment recovery kit, gel gel cutting recovery kit, plasmid small amount extraction kit, etc. are purchased from Bioengineering (Shanghai) Co., Ltd. T4 DNA ligase, Nco I and Xho I were purchased from Takara. Plasmids of pET-28a(+), E.coli DH5α and e.coli bl21 (de3) are preserved in this laboratory. Gel imaging system (Hangzhou baocheng biotechnology co., ltd)

1.2 Experimental methods

1.2.1 pET-28a(+)-SGEG1 construction of prokaryotic expression vector

According to the cDNA sequence of SGEG1 gene in GenBank (gene sequence number EU683079.1) and the polyclonal site sequence of prokaryotic expression vector pET28a(+), a PCR amplification primer with an enzymatic cleavage site is designed: upstream primer SGEG1F :5-CATG CCATGG GGAAGAAAAGAAAGATAGCGGA-3 (underlined by Nco I "enzymatic cleavage site); downstream primer SGEG1R: 5-CCG CTC GAG CagGTCTCTCCCCTGGTCTT-3 (underlined by Xho I cleavage site) to amplify SGEG1 mature peptide sequence. Meanwhile, a nucleic acid sequence encoding 6 histidine is introduced into the 3 end of the downstream primer immediately adjacent to the stop codon to express a recombinant protein consisting of 91 amino acids. Artemia parthenogenetica (Artemia parthenogenetica) is used to extract RNA and synthesize cDNA. " Using PCR " Methods: The mature peptide sequence of SGEG1 was amplified. PCR reaction conditions: 94℃ pre-denaturation 5 min; 94℃ denaturation 30 s, 55℃ annealing 30 s, 72 c extension 1min, 35 cycles; 72℃ extension 10 min. The PCR product was detected and analyzed by 1.2% agarose gel electrophoresis and purified by DNA recovery kit for later use.

The SGEG1 purified product and the pET-28a(+) vector were cleaved with Nco I and Xho I respectively. After purification of the cleaved product, the T4 DNA ligase 16 was ligated overnight and transformed to "E.coli DH5α. Positive clones were selected for sequencing to determine the correctness of the sequence and reading frame of the target fragments.

1.2.2 Recombinant SGEG1 induced expression of protein

E.coli BL21 (DE3) containing pET-28a(+)-SGEG recombinant plasmid is inoculated into LB liquid medium containing kanamycin (final concentration is 30 μg/ml), 37 C, 200rpm culture overnight, then 1 :50 ratio is expanded to OD 600 = 0.4-0.6, and IPTG "is added to induce expression of the target protein. After inducing the expression of bacterial liquid, 4℃, 5000 rpm centrifugation 10 min, collecting bacterial sediment. Under the action of lysozyme, 37℃ for 30 min ; Then turn to 30s and 37"℃ in liquid nitrogen water bath 2min is repeatedly frozen and thawed 6 times, and Triton-X-100 (final concentration 2%) is added to shake 30min at room temperature interval to completely crush thalli. Centrifuge to collect supernatant and precipitate respectively, and SDS-PAGE protein electrophoresis to detect the expression and existing form of the target protein.

1.2.3 Determine the optimal iptg concentration for protein induction

When the bacterial liquid of the expression bacteria is cultured to OD 600 = 0.4-0.6, the same amount is split into 4 bottles, and IPTG is respectively added to the bottles to reach final concentrations of 0mM, 0.4mM, 0.8mM, 1mM, 37℃ induced culture 4h, 4℃ and 5000rpm centrifugation for 10min to collect bacterial precipitates. SDS-PAGE electrophoresis analysis to determine the best induction concentration.

1.2.4 Determine the optimal induction time for protein expression

When the bacterial liquid of the expression bacteria is cultured to OD 600 = 0.4-0.6, the same amount of the bacterial liquid is split into 3 bottles, and the induction culture with the final concentration of 1 mM IPTG 37℃ is added. The bacterial liquid is taken at 0h, 2h and 4h respectively for SDS-PAGE detection to determine the optimal induction time.

1.2.5 Determination of optimal electrophoresis conditions for proteins

The recombinantly expressed SGEG1 protein is a small molecular polypeptide. Four different protein electrophoresis methods are used to optimize the separation effect of the target protein. The samples were placed in 15% SDS-PAGE (5% concentrated gel ,15% separation gel) and 15%SDS-PAGE (including 6M urea), 16% tricine SDS-page (6% concentrated gum, 16% separation gum) and (containing 6M urea) protein electrophoresis system for protein separation. Among them, 15%SDS-PAGE protein electrophoresis uses constant current 15mA, 2h electrophoresis. The Tricine SDS-PAGE electrophoresis buffer solution adopts anode and cathode electrophoresis buffer systems, and is firstly constant in voltage 30V and 30min electrophoresis, after the sample enters the separation gel, the voltage rises to constant voltage 200V and electrophoresis 2h. After electrophoresis, coomassie brilliant blue R-was dyed, decolorized and photographed by gel imaging system.

2. Materials and methods

2.1 pET-28a(+)-SGEG1 construction of prokaryotic expression vector

Artemia SGEG1 polypeptide consists of signal peptide and mature peptide. in this study, the mature peptide gene sequence of Artemia SGEG1 gene was cloned into prokaryotic expression vector pET-28a(+) with 6×His tag for protein expression (Figure 1A). First, the mature peptide fragment of SGEG1 was amplified by PCR using Artemia cDNA as template, and a single specific band (Figure 1B-1) was obtained at about 270bp. Subsequently, the pET-28a(+) plasmid was used to insert the mature peptide sequence of SGEG1 directionally, and the recombinant expression vector pET-28a(+)-SGEG1 was constructed and transformed into E.coli. Using PCR method, the transformant was identified. The results showed that the target gene was successfully amplified into the transformant. After sequencing the transformant plasmid DNA, it was proved that it was consistent with the expected gene sequence, indicating the successful construction of recombinant expression vector (Figure 1B-2).

The SGEG1 polypeptide recombinantly expressed in vitro contains mature peptide and histidine tag, forming a small molecular polypeptide composed of 91 amino acid group. the deduced protein has a molecular weight of 10.537 KDa and an isoelectric point of 5.69 (Figure 1c).

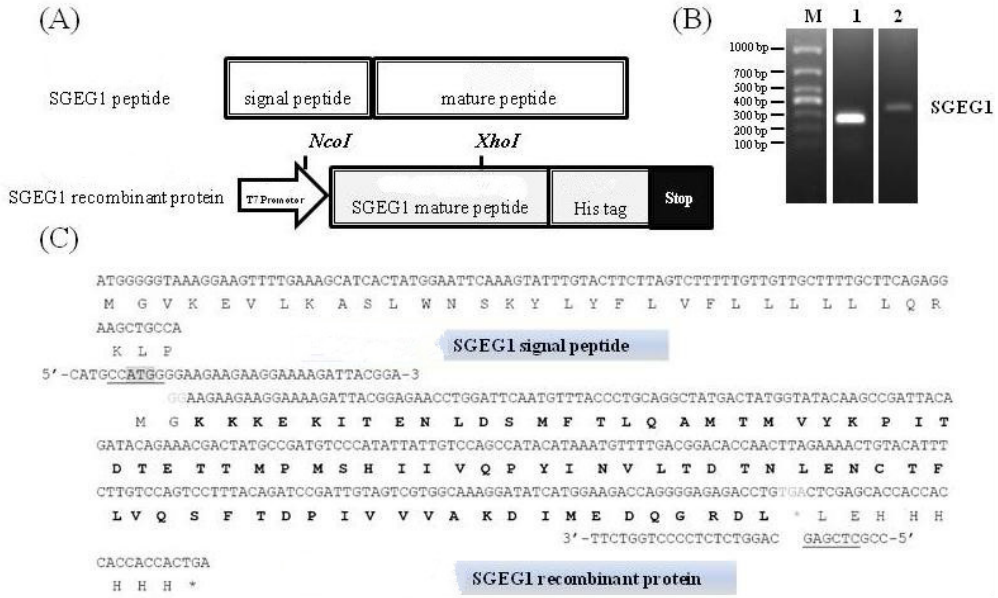

Figure 1. Construction of prokaryotic expression vector of recombinant SGEG1 protein.

(A)The prokaryotic expression strategy of SGEG1 recombinant protein. (B)PCR product detection of SGEG1: M:

DNA molecular weight standard; 1: PCR product of SGEG1; 2: PCR product of pET-28a(+)-SGEG1 transformation.(C) Amino acid sequence of SGEG1 recombinant protein.

2.2 Recombinant SGEG1 induced expression and detection of protein

The recombinant pET-28a(+)-SGEG1 plasmid was transformed into BL21(DE3), induced by IPTG, and the collected product was detected by SDS-PAGE electrophoresis. The results showed that, compared with the thallus not induced by IPTG, the thallus induced by IPTG showed an obvious band at about 10KDa, which was consistent with the expected size of SGEG1 recombinant protein, indicating that the constructed pET-28a(+)-SGEG1 recombinant plasmid has been successfully expressed in Escherichia coli. At the same time, the soluble analysis of recombinant protein showed that there was also a 10KDa size protein band in the thallus sediment after Escherichia coli was broken, which indicated that the recombinant protein SGEG1 mainly exists in the form of inclusion bodies (Figure 2).

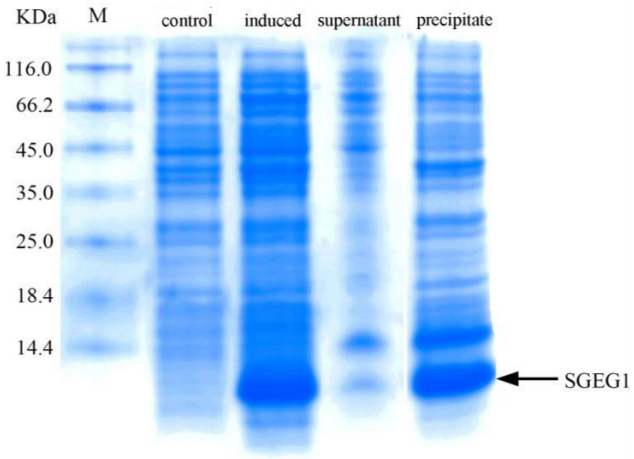

Figure 2. Induced expression and soluble analysis of recombinant SGEG1 protein.

2.3 SGEG1 optimization of expression conditions of recombinant proteins

2.3.1 Determination of optimal induction concentration

The induced expression of IPTG with concentrations of 0 mM, 0.4 mM, 0.8 mM and 1mM was detected and analyzed respectively. The results of protein electrophoresis showed that when the concentration of IPTG was 0.4 mM, the expression of SGEG1 was relatively low. With the increasing concentration of IPTG, the expression of SGEG1 protein showed an upward trend (Figure 3). Therefore, IPTG with a final concentration of 1mM was selected to induce expression.

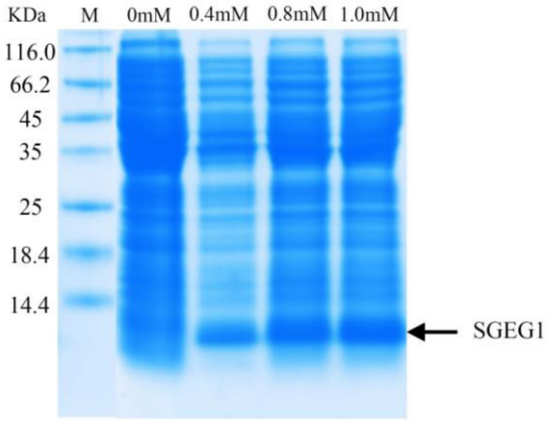

Figure 3. Effects of IPTG in different concentration to the quantity of recombinant SGEG1 protein.

2.3.2 Determination of optimal induction time

The BL21(DE3) expression strain containing pET-28a(+)-SGEG1 was cultured to logarithmic growth phase, and IPTG with final concentration of 1mM was used to induce electrophoresis detection after 0h, 2h and 4h, respectively. The results showed that with the increase of induction time of IPTG, the expression of SGEG1 recombinant protein gradually increased (Figure 4). Therefore, the optimal expression condition of SGEG1 recombinant protein is 1mM

IPTG Induce 4h. To sum up, the optimal expression conditions of SGEG1 recombinant protein are as follows: Inducing when the bacterial liquid concentration OD is 600 = 0.4-0.6; The final concentration of IPTG was 1mM and 4h induced at 37℃.

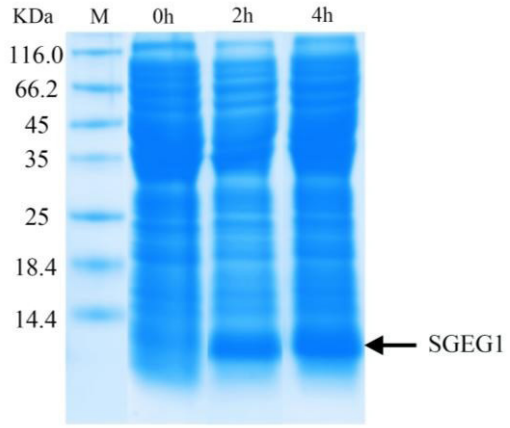

Figure 4. Effects of different induction time to the quantity of recombinant SGEG1 protein

2.4 Recombinant SGEG1 optimization of electrophoresis conditions for proteins

The recombinant expressed SGEG1 protein has a molecular weight of about 10.5kDa and belongs to a small molecular peptide. The commonly used protein electrophoresis is difficult to realize the expected separation effect. In this study, 15%SDS-PAGE, 15%SDS-PAGE (including urea), 16%Tricine-SDS-PAGE and 16%Tricine-SDS-PAGE (including urea) protein electrophoresis were used to separate thalli proteins respectively. By comparing the electrophoresis results of the four proteins, the most suitable electrophoresis and detection method for separating SGEG1 protein was selected.

Figure 5 electrophoresis results show that the 15%SDS-PAGE electrophoresis method can well separate protein bands and strips above 20kDa belt boundary is clear and the effect is ideal. The bands below 20kDa are clustered in fuzzy clusters, and the separation effect of small molecular bands is not ideal. Secondly, the 15%SDS-PAGE (containing urea) electrophoresis band showed deviation, and the protein band was dispersed, which failed to separate the protein well. Thirdly, 16% Tricine-SDS-page electrophoresis was carried out by using tricine instead of glycine and zwitterion buffer. Electrophoresis results show that the method can effectively separate proteins with different molecular weights in the sample, and the bands are clear, and the effect is obviously improved compared with the first two. However, the heat generated during electrophoresis is high and the electrophoresis speed is slow. Finally, 16%Tricine-SDS-PAGE (containing urea) electrophoresis results show that proteins of different size fragments can be separated well and clear border bands appear in small molecule protein band regions, which proves that this method has the best separation effect on small molecule peptides. At the same time, under the same electrophoresis time, the separation speed of this method is faster than 16%Tricine-SDS-PAGE, which is the optimal electrophoresis and detection method for recombinant SGEG1 protein.

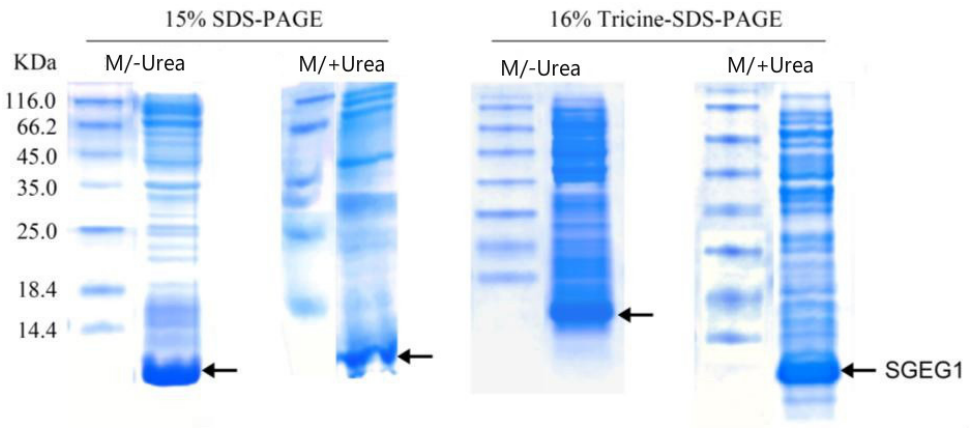

Figure 5. Comparison of separation effects of different protein electrophoresis.

3. Discussion

SGEG, a specific expression protein of Artemias shell gland, is an important component of resting eggss shell protein. It plays an important role in the formation of Artemia's shell and participates in the anti-stress dormancy mechanism of Artemia in extreme environment. The SGEG gene was discovered by Liu Yulei[7] *et al.* It is presumed to be related to the formation of Artemia resting eggs shell. Daili[1] *et al.* used RNAi technology to study and found that the resting eggs anterior cortex produced by the mother Artemia that silenced the SGEG1 gene disappeared and the thickness of the cortex was only half that of the normal resting eggs. They speculated that SGEG1 was dispersedly expressed in the anterior cortex and cortex and participated in the construction of Artemia resting eggs egg shell.

In this study, pET-28A (+)-SGEG1 prokaryotic expression vector was successfully constructed by using Artemia cDNA as template, using PET prokaryotic expression vector and using Nco I and Xho I restriction enzymes. At the same time, SGEG1 recombinant protein was obtained through induced expression of E.coli BL21 (DE3) strain, and it was proved that it mainly existed in inclusion body form, which provided basic data for efficient acquisition of SGEG1 recombinant protein and laid a foundation for later purification and related functional research.

At the same time, in order to efficiently express SGEG1 recombinant protein, prokaryotic expression induction conditions were optimized. IPTG inducer is expensive and has certain toxicity to bacteria. Prolonging induction time can increase protein expression, but it may also degrade egg white and cause bacterial aging, which will react on protein expression. In this experiment, the concentration and induction time of IPTG induced by SGEG1 recombinant protein were explored, and the optimal expression conditions of SGEG1 recombinant protein were determined as follows: OD 600 = 0.4-0.6, the final concentration of IPTG was 1mM, and the induction time was 4h at 37℃.

Polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used to separate proteins. Common Tris- glycine -SDS-PAGE is mostly used to separate proteins with molecular weight of 10-200kDa .When the molecular weight is less than 10kDa ",the separation effect is poor. The molecular weight of SGEG1 recombinant protein deduced in this study is 10.537kDa, and the commonly used SDS-PAGE is not very suitable. Tricine-SDS-PAGE is commonly used to separate small molecular peptides with molecular weight of 1-10kDa. Tricine exists in cationic form and can be used as trailing ion, so that small molecular peptides can form as narrow a band[13] "as possible in concentrated gel. At the same time, adding urea to protein electrophoresis can obviously improve the separation effect of small molecular peptides and increase the resolution[13,14]. At the same time, by improving the conventional SDS-PAGE and Tricine-SDS-PAGE electrophoresis and adding urea to the separated gel of electrophoresis, the pore size of the gel can be changed, small molecular polypeptides can be effectively compressed, and tailing phenomenon can be weakened. In this study, the comparison of four protein electrophoresis methods provides good technical support for the efficient separation of small molecular peptides.

The successful preparation of recombinant expression protein SGEG1 of Artemia laid a foundation for further research on the function of Artemia resting eggs egg shell protein and the diapause and stress resistance mechanism of Artemia, and provided a new idea for the development of new biological materials with UV isolation, corrosion resistance and high temperature resistance.

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