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Induced Secretion Expression of Salivary Apyrase from *Culex quinquefasciatus* in *Pichia pastoris*

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Salivary Apyrase(ATP diphosphohydrolase) from *Culex quinquefasciatus* was expressed in *Pichia pastoris*, which can lay foundation for further studying biological functions of *Culex quinquefasciatus* Apyrase in saliva.Biological information and RT-PCR technology were adopted for cloning and encoding mature peptide genome sequence of *Culex quinquefasciatus* Apyrase, it was cloned to downstream α-factor signal peptide sequence of *Pichia pastoris* constitutive secretion expression vector pGAPZα-A. pGAPZα-A-Apyrase recombinant secretion expression vector was constructed. *Pichia pastoris* GS115 competent cells were electroporated after *Bln* I linearization treatment on expression vector. Transformants underwent Zeocin resistance screening, colony PCR and SDS-PAGE analysis. pGAPZα-A-Apyrase/ GS115 engineering bacteria secreted and expressed Apyrase recombinant protein at expected size of 60 kD. It was fermented in shake flask for 72 h. The amount of protein expression achieved the highest level.The study proves that *Culex quinquefasciatus* Apyrase can achieve secretion expression in *Pichia pastoris*.

Key words: *Culex quinquefasciatus,* Apyrase,secretion expression, *Pichia pastoris*.

Culex quinquefasciatus is important medical insects mainly distributed in tropical and subtropical regions, can disseminate epidemic encephalitis B, Bancroftosis, West Nile fever and other arboviruses disease (Zhang,*et al*., 2005; Kumar, *et al*., 2008) as dissemination medium. The saliva component of saliva protein secreted by salivary glands which enters the host along with the blood sucking can cause local or systemic strong allergic inflammation (Rezza, 2012; Dong, *et al*., 2012) during blood-sucking of mosquitoes. The study confirmed that mosquito saliva component contains a variety of function pharmacologically active factors, has effects of anticoagulant, anti-inflammatory and modulating the host immune function on vertebrate hosts, which is convenient for promoting mosquito medium blood-sucking and disease spreading (Wu,*et al*., 2003; Leal *et al*., 2005). Salivary Apyrase (ATP diphospho-hydrolase) in mosquito has the function that ADP can be transformed into AMP and phosphate through hydrolysis during blood sucking, thereby inhibiting ADP-induced platelet aggregation. The apyrase is mainly enriched in the saliva of female mosquitoes (Komoszynski, *et al*., 1996; Marinotti, *et al*., 1996; Hamasaki, *et al*., 2009). Gene expression vector of *Pichia pastoris* constitutive *Culex quinquefasciatu*s salivary Apyrase was constructed in this study. salivary Apyrase was successfully secreted and expressed, thereby providing foundation for further discussing the biological function of *Culex quinquefasciatu*s Apyrase in saliva.

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MATERIALS AND METHODS

Experiment Materials

Mosquito, Vector, Strains and Culture Medium

Culex quinquefasciatus with the age of 3 to 5 days were conventionally bred in our lab. Breeding conditions: 25°C, relative humidity of 85%, light of 14h/d. pMD18-T vecctor was purchased from Dalian Takara Biotechnology Company. The host bacteria E.coli Top10F/(recAl, endAl) and *Pichia pastoris* GS115(his4) strains and pGAPZα-A expression vectors were purchased from Invitrogen Company. The bacteria culture medium was LB culture medium and low-salt LB culture medium. The yeast culture medium was YPD culture medium.

Major Reagents

Trizol was purchased from Invitrogen Company; Taq DNA polymerase, RT-PCR kit, RNA PCR Kit (AMV) Ver 3.0, T4-DNA ligase, restriction endonuclease, DNA molecular weight standard, protein molecular weight quasi-standard and agarose gel DNA extraction kit were purchased from Dalian Takara Biotechnology Company. The antibiotic Zeocin was produced of Invitrogen Company. Other conventional chemical reagents were domestic or imported packing AR.

EXPERIMENTAL

Bioinformatics Analysis of *Culex quinquefasciatus* Apyrase Protein

Conserved domain search analysis was conducted on NCBI site according to *Culex quinquefasciatus* salivary Apyrase amino acid sequence (GenBank accession no.AAV90659) recorded in GenBank. SignalP4.0 Server was utilized for on-line search on Apyrase protein, thereby determining the position of signal peptide sequence.

Extraction of *Culex quinquefasciatus* Total RNA and Apyrases Mature Protein Gene Amplification

Total RNA of *Culex quinquefasciatus* was extracted by referring to TRIzol (Invitrogen). A pair of primers were analyzed, designed and synthesized for cloning *Culex quinquefasciatus* Apyrase mature protein gene (79-1695 bp) according to Apyrase gene sequence (GenBank accession no. AY826087) recorded in GenBank and bioinformatics analysis. Xba I and Xho I restriction enzyme sites were respectively introduced on upstream and downstream are named as:Apy-F: 5' -CACTCGAGAAAAGAGAGGCTGAA GCTgataatatgcccgctgata agg -3'; Apy-R: 5'-GTTCTAGA TTAAGTACATGG TGAACCTGC TTTACATAC -' .

Primer and sequencing were finished by Shanghai Invitrogen Biotechnology Co., Ltd.The first chain of cDNA was synthesized according to manual of Takara RNA PCR Kit 3.0.The synthesized cDNA first chain was used as template, and Apy-F and Apy-R were used as primers for PCR amplification of the Apyrase mature protein gene fragment. Reaction conditions: it was denatured at 94°C for 2 min then entered cycle. The cycle parameters were 94° for 30 s, 58°C for 30 s and 72°C for 1.0 min. The number of cycles was 40. It was extended for 10min at 72°C after cycle. The target fragment was reclaimed by rubber cutting after the size of the electrophoresis identification amplification products was correct.

Construction and Identification of Cloning Vector

RT-PCR product was connected with pMD18-T after agarose gel electrophoresis recovery and purification. The cloning vector pMD18-T-apyrase was constructed. The E.coli DH5á was transformed and coated on LB solid culture medium containing 100mg/L AMP. It was cultured at 37°C overnight. The colonies were picked. PMD18-T sequencing primers were used for colony PCR identification. Positive clones were screened and sequenced (Shanghai Invitrogen Biotechnology Co., Ltd.). The clone vector with correct sequencing was named as pMD18-T-Apyrase.

Construction and Identification of pGAPZα-A-Apyrase Recombinant *Pichia pastoris* Expression Vector

The restriction endonuclease Xho I and Xba I were respectively used for double digestion construction of cloning vector pMD18-T/Apyrase and expression vector pGAPZα-A. Apyrase gene fragment and the linearized pGAPZα-A vector were respectively recycled. Under the action of T4- DNA ligase, the Apyrase gene fragment with complementary cohesive ends and pGAPZα-A linear vector were connected for constituting a recombinant expression vector pGAPZα-A-Apyrase.

The recombinant expression vector

transformed host bacteria E.coli Top10F' competent cells by CaCl2 method. The recombinant bacteria were screened on low-salt solid LB culture medium plates containing 25mg/L Zeocin. Plasmids were extracted, Xho I and Xba I were used for double digestion identification of recombinant vector. The screened positive clones were sent to Shanghai Invitrogen Biotechnology Co. , Ltd. for sequencing.

Construction and Screening of Engineering Bacteria GS115/pGAPZα-A-Apyrase

Pichia pastoris GS115 competent cells were prepared by referring to *Pichia pastoris* Expression Kit manual, and then 10μg expression vector pGAPZα-A-Apyrase underwent linearization by Bln I for electrotransformation of GS115 competent cells. Transformation parameters: Voltage of 1500V, capacity of 25μf and electric shock time of 10ms. Transformants were initially screened on YPD plates containing 300mg/L Zeocin. It was cultured for 2 to 3 days at 28°C to produce single colonies. Then pGAPZα-A vector universal primers pGAP Forward: 5′-GTCCCTATTTCAAT CA ATTGA A-3′and 3′AOX1 : 5′-GCAAATGGCA TTCTGA

CATCC-3′ were utilized for colony PCR screening of positive clones.Reaction condition:it was denatured at 94° for 2 min and then entered cycle. Cycle parameters: 94°C for 30s, 53° for 30s and 72°C for 1.0min. The cycle number was 40. It was extended for 10min at 72°C after cycle.

SDS-PAGE Analysis of Expression Products

Positive yeast single colonies were picked up and cultured in YPD liquid culture medium containing 100mg/L Zeocin at 300r/min for 30°C. Fermentation broth sample of 1ml were respectively sampled at every 0, 24, 48, 72 and 98h. The supernatant was obtained by centrifugation for SDS-PA GE. The target protein was identified to determine the best shake flask conditions.

RESULTS

Analysis of conserved domain with Apyrase from *Culex quinquefasciatus*

Conserved function domain search analysis was conducted on *Culex quinquefasciatus* Apyrase in the NCBI site. The result showed that the protein had a CD73, namely, ecto-5'-nucleotidase(eN) N end conservative Metallophosphatases (MPPs) structure domain and a 5'nucleotidase C end conserved structure domain (Fig.1). The main function of these structure domains was to hydrolyze AMP and generate important physiologically active substance adenosine hydrolysis.

Analysis of signal peptide with Apyrase from *Culex quinquefasciatus*

SignalP 4.0 Server was used for on-line search of derived protein sequence. It was discovered that a significant signal peptide was available at No. 1 to 70aa. The Apyrase signal peptide breakpoint was available at 26 to 27aa (Fig. 2)

Fig. 1. Analysis of conserved domain with Apyrase from *Culex quinquefasciatus*

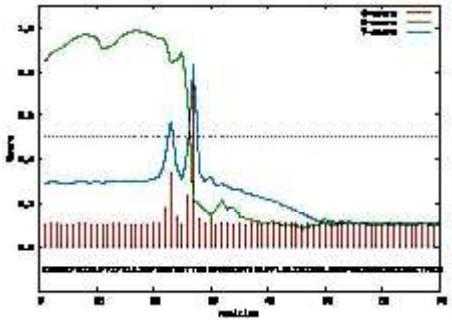

Fig. 2. Analysis of signal peptide with Apyrase from *Culex quinquefasciatus*

Cloning and Identification of Mature Protein Gene with Apyrase

Apyrase mature protein gene fragment sizes were respectively 1617 bp according to the *Culex quinquefasciatu*s Apyrase gene sequence recorded in GenBank (GenBank accession no. AY826087). The RT-PCR amplification results confirmed the fragment at expected size.PCR recovered product and cloning vector pMD18-T were connected to construct pMD18-T- apyrase. The fragment obtained by Xba I and Xho I enzyme digestion identification was consistent with the size of expected insert fragment. It was preliminarily proved that the cloning vector was successfully constructed (Fig.3). The sequencing results showed that the cloned Apyrase was completely consistent with the gene sequences published by GenBank.

Construction of pGAPZα-A-Apyrase *Pichia pastoris* **Secretory Expression Vector**

Xba I and Xho I was used for double digestion of PMD18-T-Apyrase, and Apyrase gene was reclaimed. Then, it was connected with pGAPZα-A vector fragment after double digestion and recycling by Xba I and Xho I. The expression vector pGAPZα-A/Apyrase was constructed. It was preliminarily proved by Xba I and Xho I enzyme digestion identification that *Pichia pastoris* secretory expression vector was successfully constructed (Fig.4). The sequencing results also prove that the open reading frame of Apyrase insertion position was proper.

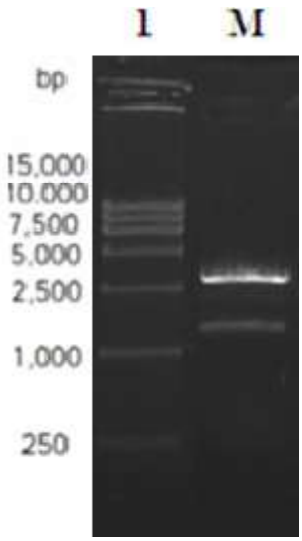

Fig.3. Digesting analysis of cloning vectors with *Xba* I and *Xho* I(M: DL15000 Marker; 1: pMD18-T-Apyrase digested with *Xba* I and *Xho* I)

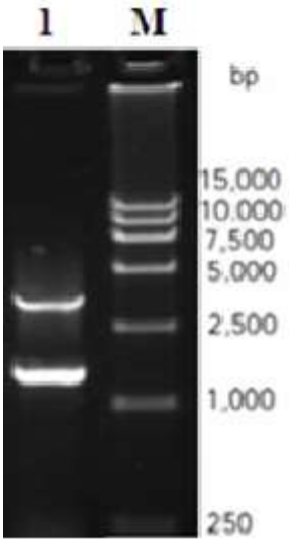

Fig. 4. Digesting analysis of cloning vectors with *Xba* I and *Xho* I(M: DL15000 Marker; 1: pGAPZα-A-Apyrase digested with *Xba* I and *Xho* I)

Screening of pGAPZα-A-Apyrase/GS115 Recombinant Engineering Bacteria

The electrotransformation was used for transforming GS115 competent cells after Bln I linearization on pGAPZα-A-Apyrase expression vector. The cells were picked up and cultured at 28° for 3 d to produce single colonies. The colony PCR was utilized for PCR screening of positive clones with pGAP Forward and 3'AOX as primer. PCR results were shown in Fig.5. Four of the five picked clones can be amplified into target band at expected size (the length of Apyrase gene fragment plus vector sheet was approximately 2010bp), thereby they were positive clones.

Secretion Expression of Recombinant Apyrase in *Pichia pastoris*

The culture medium supernatant of engineering bacteria after being cultured in shake flasks for 1-4d was analyzed. The SDS-PAGE analysis result showed that recombinant protein had a target band at approximately 60KD at 24h, and GS115 blank bacteria had no band in the part.

Apyrase expression amount was gradually improved with the extension of culture time. It had certain accumulation effect and reached the highest level at 72h (Fig.6).

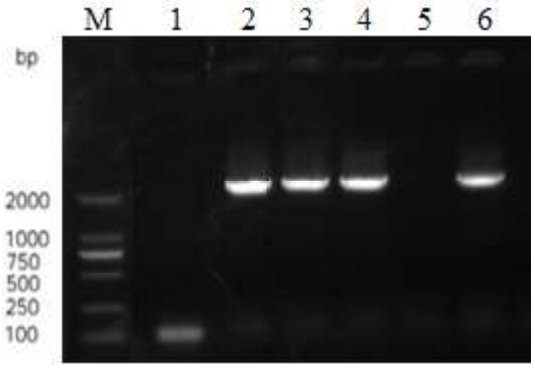

Fig. 5. Identification of positive clone of prokaryotic expression strain by colony PCR(M: DL2000 Marker; 1: GS115; 2-6: GS115/pGAPZα-A-Apyrase)

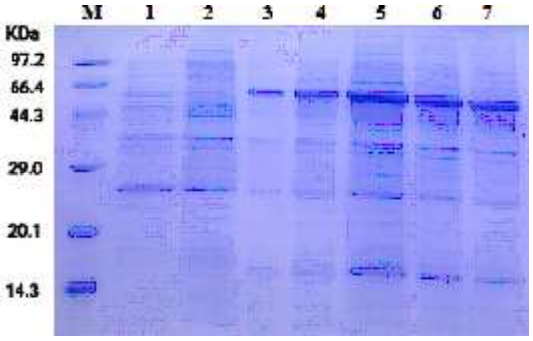

Fig. 6. SDS-PAGE analysis of expression of the Apyrase protein (1:The supernatant of GS115 for 120h; 2: The supernatant of pGAPZα-A/ GS115for 120h ;3-7: The supernatant of pGAPZα-A-Apyrase/ GS115 for 24, 48, 72, 96, 120h, respectively)

DISCUSSION

Mosquito salivary gland proteomics is the research focus in recent years. However, concrete condition of most protein molecules involved in the anticoagulation and disease propagation is unclear. The clarification of expression characteristics and functions of these protein components has significant significance for blocking the propagation of insect-borne diseases. The anti-platelet aggregation factor Apyrase is important factor of mosquito saliva, and can inhibit platelet aggregation by hydrolyzing ATP and ADP released from the blood platelet of the sucked hosts. The blood-sucking process can be smooth (Sun, *et al*.,2006).

Apyrase mature protein coding sequence was cloned from *Culex quinquefasciatus* in the study. Conserved function domain search analysis was conducted on Apyrase in NCBI site. The result showed that the protein has a CD73, namely, ecto-5'-nucleotidase(eN) N end conservative Metallophosphatases(MPPs) structure domain and a 5'-nucleotidase C end conserved structure domain. Apyrase protein sequence signal peptide on-line analysis was combined. This study adopted *Pichia pastoris* constitutive secretion expression vector for successfully expressing Apyrase mature protein gene (79-1695bp), thereby laying foundation for further studying biological functions of *Culex quinquefasciatus* Apyrase in saliva.

Pichia pastoris expression system, as an efficient expression system, has distinct advantages in the aspects of expression product processing, outside secretion, post-translational modification, glycosylation modification and the like. It not only overcomes the deficiency of bacterial expression system, but also has more advantage than wine yeast expression system. It can achieve high-density fermentation, and greatly improve the expression amount of the recombinant protein(Macauley-Patrick,*et al*., 2005;Cregg,*et al*., 2009).The constitutive secretion expression vector pGAPZα-A containing α-factor signal peptide sequence used in this study is adopted for expressing Apyrase. The expression exogenous target proteins are secreted into the fermentation broth, which is conducive to separation and purification, and production costs can be greatly reduced. Meanwhile, Apyrase expressed in the study also lays foundation for producing anti-platelet aggregation factor in genetic engineering and applying it in clinical purpose.

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