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Research Article

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Expression and purification of 3-1E gene from *Eimeria acervulina* in *Pichia pastoris*

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ABSTRACT

Coccidiosis is a kind of disease in chickens, which has a serious economic impact on poultry production worldwide. In this experiment, the 3-1E gene from Eimeria acervulina was amplificated by RT-PCR. The target gene was linked to pPICZαA vector, then electrotransformed into Pichia pastoris. The secreted recombinant protein expression was induced by methanol, and the recombinant 3-1E protein was obtained every24hours post induced, then it was identified by SDS-PAGE and Western Blot. The protein was purified using the His-tagged protein purification kit and its concentration was determined by Bradford method using a protein quantitation kit. This study laid a foundation for the vaccine production and the evaluation of vaccine efficacy.

Keywords: *Eimeria acervulina*, expression, 3-1E, *Pichia pastoris*, chicken

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INTRODUCTION

Avian coccidiosis is an intestinal protozoan disease caused by *Eimeria*, which results in annual economic losses for the worldwide poultry industry[1].At present, drug prevention and treatment are the main methods for the control of coccidiosis in chickens. The use of coccidiostats are subject to tight restrictions because chickens will produce drug residues and withdrawal period in the course of treatment[2]. Inoculation with anticoccidial vaccines is an effective alternative to drugs for coccidiosis control[3].

Recombinant vaccine against coccidiosis become the focus of research in the prevention and control of coccidiosis, so the research of the main antigenic gene is very important[4]. The life history of Chicken coccidian is complex. The antigenicity and antigenic composition are not same at different developmental stages.

E. acervulina is one of the most prevalent Eimeria species in chicken. So far, few genes of this coccidian have been reported and tested for their immunogenicity[5, 6]. 3-1E, which is expressed in *E. acervulina* sporozoites and merozoites, is a kind of surface membrane protein. Its antigenicity is good and gene sequences are highly conserved. In recent years, this gene has been expressed in *Escherichia coli*[7, 8].

Pichia pastoris has been developed into a highly successful expression system for the production of heterologous proteins. The expression of any foreign gene in*P. pastoris* comprises three principal steps: (a) insertion of the gene into an expression vector; (b) introduction of the expression vector into the *P. pastoris* host; and (c) examination of potential strains for the expression of the foreign gene[9, 10]. The Pichia expression system is better than other eukaryotic expression systems because it is efficient, simple, cheap, and its expression level is higher.

In the present study, recombinant 3-1E protein was expressed using *P. pastoris*, and purified using the His-tagged protein purification kit. Its concentration was determined by Bradford method using a protein quantitation kit. This

work laid a foundation for the vaccine research.

EXPERIMENTAL SECTION

Parasites

Beijing strain *E. acervulina* was maintained at the College of Animal Science and Technology, Beijing University of Agriculture, China. It was passaged every three months in susceptible chickens.

Vectors

The expression vector is pPICZαA plasmid and the *Pichia pastoris* host is GS115 strain. They were conserved in our laboratory.

Main reagents

Media components include yeast extract, tryptone, yeast nitrogen base and phosphate buffer. Restriction enzyme was purchased from NEB (USA) and T4 ligase was purchased from TaKaRa (China). His tag monoclonal antibody was purchased from Abcam (UK). Goat-antimouse IgG was purchased from CWBIO (China).

Primer design

According to *E. acervulina* 3-1E gene sequence reported by GenBank (AY660553.1), the primer was designed using Primer 5.0 biological software(Table 1).

Table 1 3-1E gene specific primers

	Gene sequence
The upstream primer	5'CGGAATTCATGGGTGAAGAGGCTGAT
The downstream primer	5'AAGCGGCCGCGGTACAGGTACTCAGC

Amplification of 3-1E gene

A count of 2×10^7 fresh *E. acervulina* oocysts were washed three times by centrifugation with 0.1 M PBS (pH 7.2). According to the manufacturer's protocol, total RNA from purified *E. acervulina* oocysts was isolated with TRIZOL Reagent (Invitrogen, USA). Then the total RNA was reverse transcribed into cDNA, which was as template for PCR. The PCR reaction procedure was as follows: pre-denaturation at 95 ºC for 5 min, 30 cycles of amplification (30s at 94 ºC, 30s at 55 ºC, and 30s at 72 ºC), and followed by extending for 10 min at 72 ºC. The results of PCR were analyzed by electrophoresis in a 1% (w/v) agarose gel. The target gene was recycled using the DNA gel extraction kit.

Construction of the recombinant expression plasmid

The recovered target gene was digested with EcoRI and NotI, then ligated into pPICZαA vector previously digested with the same enzyme， as described[11, 12]. The recombinant expression plasmid was transformed into Escherichia coli strain JM109, then cultured for 2h at 37ºC. The recombinant plasmid was identified by PCR, double enzyme digestion and gene sequencing.

Expression of the recombinant 3-1E protein

The 3-1E gene was expressed in *P. pastoris* according to the manufacturer's manual. Briefly, pPICZαA-3-1E was linearized by BstXI single enzyme digestion and then electroporated into *P. pastoris* strain GS115.The positive yeast was added to the BMGY culture medium(2.0% peptone, 1.0% yeast extract, 1.3% YNB, 4.0×10^{-5}% biotin,100 mM potassium phosphate (pH 6.0) and 1.0% glycerol), and cultured at 30℃ in a shaking incubator (200 rpm) for about 18h until OD_{600} = 6. The cells were harvested by centrifuging at 3500rpm for 10 min and resuspended in BMMY culture medium(the same as BMGY but with glycerol replaced by methanol) for inducing the expression of recombinant 3-1E protein at the same condition. The methanol was added to the culture to a final concentration of 1% every 24 h for induction. The samples were collected every 24h for 4 days and analyzed by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[13] and Western blot[14].

Purification of the recombinant 3-1E protein

After induced for 72 hours, the supernatant of BMMY medium was harvested by centrifugation, then concentrated by ultrafiltration tube (Millipore, USA). The recombinant 3-1E protein was purified using the His-tagged protein purification kit (CWBIO, China) according to its instruction manual. Finally, the eluent was collected (a total of eight column volume). The result of purification was identified by SDS-PAGE and its concentration was determined by the Bradford method[15]using a protein quantitation kit (Aidlab, China).

RESULTS

The results of PCR amplification

The result of PCR amplification was tested by 1% agarose gel electrophoresis. There is a bright band near 500bp, which is consistent with the size of 3-1E gene(Fig.1).

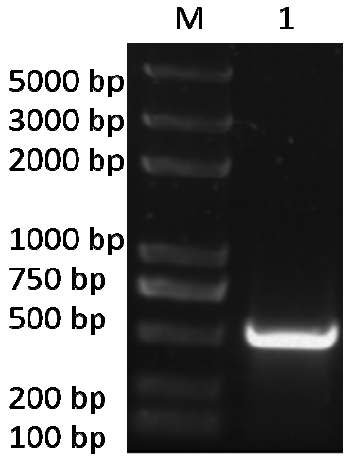

Fig.1The result of PCR amplification. M:Marker; 1:3-1E gene

Identification of the recombinant expression vector

The 3-1E gene and pPICZαA vector were connected by T4 ligase (Fig.2). After antibiotic screening, the recombinant expression vector was identified by double enzyme digestion(Fig.3) and DNA sequencing (Fig.4).There are two bright bands: the target gene is near 500bp and the other is the expression vector.

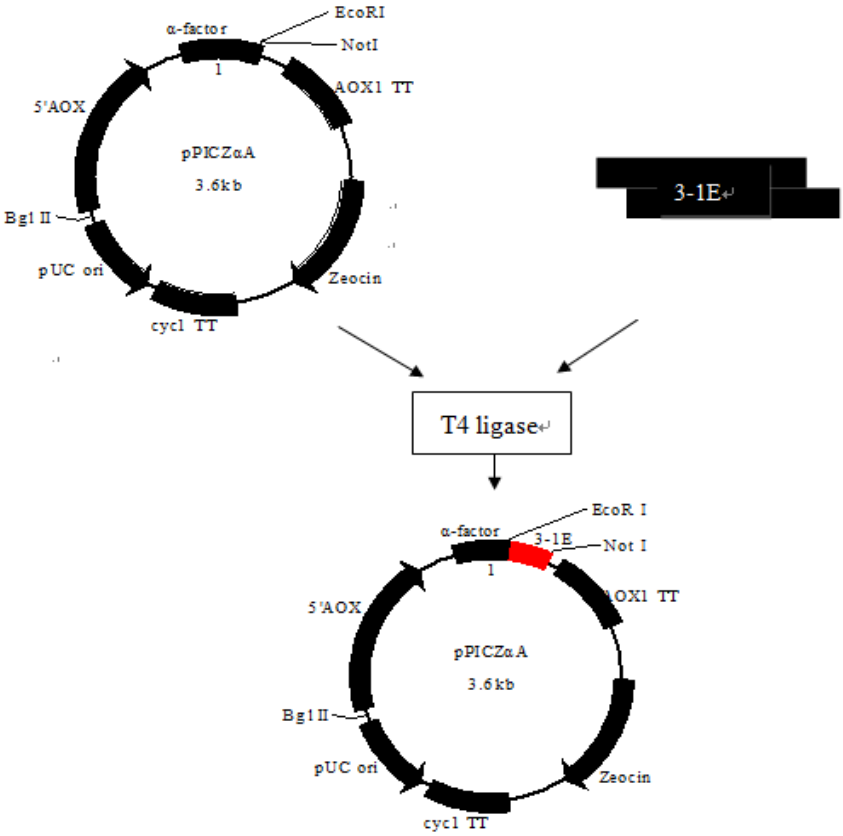

Fig.2 Construction of recombinant eukaryotic expression plasmid pPICZαA/3-1E

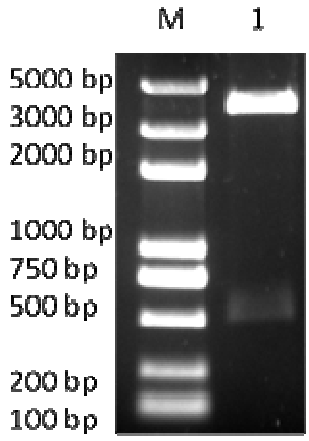

Fig.3The result of the double digestion. M:Marker;1:the recombinant vector

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Query  7    ATGGGTGAAGAGGCTGATACTCAGGCGTGGGATACCTCAGTGAAGGAATGGCTCGTGGAT  66
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGGGTGAAGAGGCTGATACTCAGGCGTGGGATACCTCAGTGAAGGAATGGCTCGTGGAT  60

Query  67   ACGGGGAAGGTATACGCCGGCGGCATTGCTAGCATTGCAGATGGGTGCCGCCTGTTTGGC  126
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   ACGGGGAAGGTATACGCCGGCGGCATTGCTAGCATTGCAGATGGGTGCCGCCTGTTTGGC  120

Query  127  GCTGCAATAGACAATGGGGAGGATGCGTGGAGTCAGTTGGTGAAGACAGGATATCAGATT  186
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GCTGCAATAGACAATGGGGAGGATGCGTGGAGTCAGTTGGTGAAGACAGGATATCAGATT  180

Query  187  GAAGTGCTTCAAGAGGACGGCTCCTCAACTCAAGAGGACTGCGATGAAGCGGAAACCCTG  246
            |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  181  GAAGTGCTTCAAGAGGACGGCTCTTCAACTCAAGAGGACTGCGATGAAGCGGAAACCCTG  240

Query  247  CGGCAAGCAATTGTTGACGGCCGTGCCCCAAACGGTGTTTATATTGGAGGAGTTAAATAT  306
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CGGCAAGCAATTGTTGACGGCCGTGCCCCAAACGGTGTTTATATTGGAGGAGTTAAATAT  300

Query  307  AAACTCGCAGAAGTTAAACGTGATTTCACCTATAACGACCAGAACTACGACGTGGCGATT  366
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  AAACTCGCAGAAGTTAAACGTGATTTCACCTATAACGACCAGAACTACGACGTGGCGATT  360

Query  367  TTGGGGAAGAACAAGGGTGGCGGTTTCCTGATTAAGACTCCGAACGACAATGTGGTGATT  426
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TTGGGGAAGAACAAGGGTGGCGGTTTCCTGATTAAGACTCCGAACGACAATGTGGTGATT  420

Query  427  GCTCTTTATGACGAGGAGAAGGAGCAGAACAAAGCAGATGCGCTGACAACGGCACTTGCC  486
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GCTCTTTATGACGAGGAGAAGGAGCAGAACAAAGCAGATGCGCTGACAACGGCACTTGCC  480

Query  487  TTCGCTGAGTACCTGTAC  504
            ||||||||||||||||||
Sbjct  481  TTCGCTGAGTACCTGTAC  498
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Fig.4 The result of the sequencing comparison

Electrotransformation and the screening of positive strains

The recombinant expression vector was electro transfected into GS115 *P. pastoris*, then they were cultured on Yeast Extract Peptone Dextrose (YPD) solid culture medium containing Zeocin (100 μg/ml) and the positive strains were identified by PCR using AOX consensus primer(Fig.5).

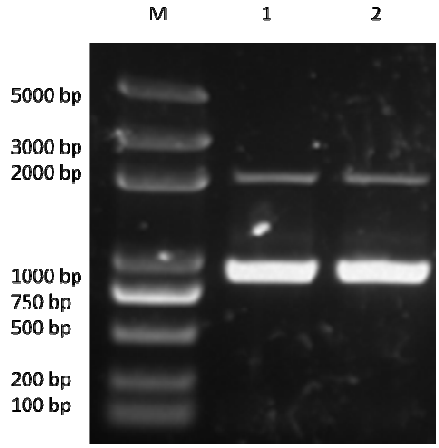

Fig.5 The result of the recombinant yeast PCR. M:Marker;1,2:Recombinant yeast

Expression and identification of the recombinant 3-1E protein

The expected molecular weight of recombinant 3-1E protein is 21kD and the electrophoresis results were about 26KDa. This result may be caused by the His-tag of the recombinant protein. The BMMY culture medium supernatant was collected every 24h for 4 days. The recombinant protein was tested and analyzed by SDS-PAGE electrophoresis and Western Blot after precipitation by TCA. The results showed that the amount of recombinant 3-1E protein was largest when induced for 3 days. There is no target band in the BMMY culture medium supernatant of GS115 electrotransformed with empty plasmid. It is suggested that the aim protein was secreted by positive yeast strain induced by methanol(Fig.6). Western Blot analysis showed a band near 26KDa, suggesting that the protein expressed by *P. pastoris* is recombinant 3-1E protein(Fig.7)

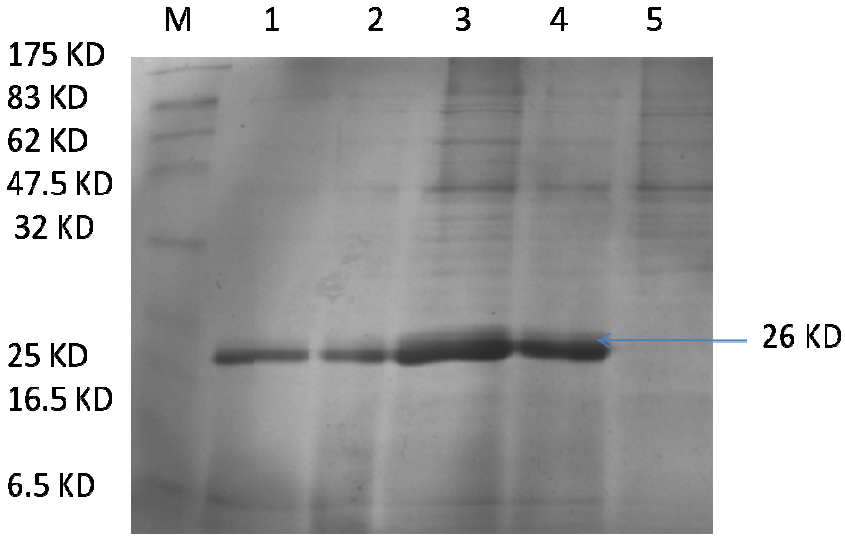

Fig.6 Identification of recombinant protein. M: Marker;1-4:BMMY culture medium supernatant induced for 1d,2d,3d,4d;5:control

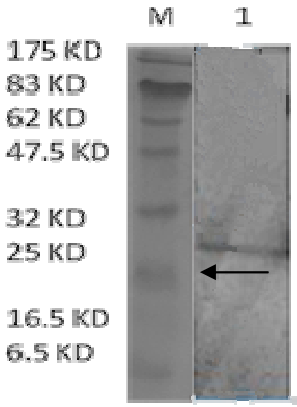

Fig.7 Western Bolt analysis. M:Marker;1:recombinant 3-1E protein

Purification of the recombinant 3-1E protein

The eluent of the His-tagged protein was collected. The result of the purification was assessed by SDS-PAGE. After Coomassie blue staining, it showed a band near 26KDa in the fourth column volume without contaminating proteins (Fig.8).

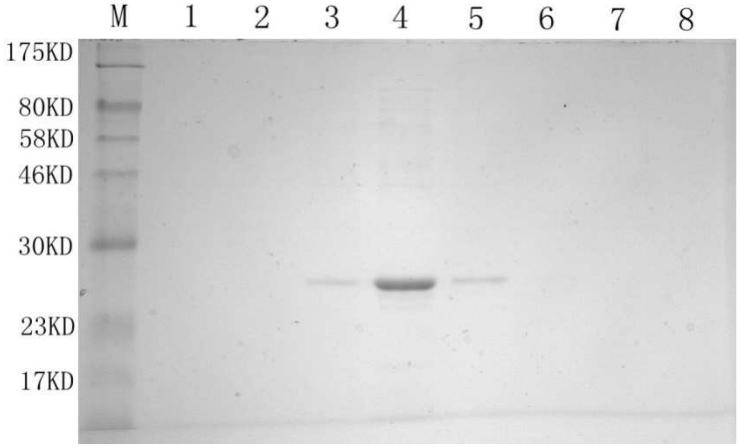

Fig.8The result of the purification was identified by SDS-PAGE. M: marker, 1-8: 1st column volume of the eluent- 8th column volume of the eluent

DISCUSSION

There are many kinds of heterologous gene expression system, including the *Escherichia coli* expression system, baculovirus expression system, vaccinia virus expression system and *P. pastoris* expression system. Among these systems, *P. pastoris* expression system has great advantages in the expression of antigen. It was able to perform many of the post translational modifications of higher eukaryotes and secrete high levels of heterologous proteins into the supernatant under the control of AOX1 promoter induced by methanol[16, 17].

In this study, we found that the size of recombinant 3-1E protein was approximately 26KDa, which was larger than the predicted size of 21KDa. This is probably the result of protein glycosylation, and the his-tag can also affect the protein molecules. The addition of $(His)_6$ tag has been reported not to affect secretion, folding or function of recombinant protein[18].

As shown in Fig.6, the amount of the recombinant 3-1E protein expression gradually increased with the induction time increasing. However, the amount of the protein decreased after the expression reached a high level. It is also reported that the expression level can be improved when *P. pastoris* is grown in a fermentor in which much higher cell densities can be achieved and parameters like aeration, pH, and feed rate canbe exactly controlled[19].

In this experiment, we constructed a secreted *P. pastoris* expression system, which is convenient for protein purification and also can avoid the degradation of target protein under the action of intracellular protease. But the secreted protein soluble in the supernatant of culture medium, so that the initial concentration of target protein is lower. In order to obtain a higher concentration of the target protein, it is very necessary to use some concentration methods. Affinity chromatography and ion exchange chromatography are the two methods commonly used in protein purification. Due to its high efficiency in purifying proteins with different tags, affinity chromatography is widely used in protein engineering[12]. In this study, the recombinant 3-1E protein was purified using the His-tagged protein purification kit (CWBIO, China). The target protein could be attached without any contamination (Fig.8).

CONCLUSION

In this study, we successfully expressed the recombinant 3-1E protein and laid the foundation for the subsequent vaccine production. The effect of the recombinant 3-1E protein on the *E. acervulina* infection should be researched for further study.

Acknowledgment

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