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Full Length Research Paper

A novel and convenient method to immunize animals: Inclusion bodies from recombinant bacteria as antigen to directly immunize animals

Hanjiang Yang[#], Tingting Zhang[#], Kun Xu, Jie Lei, Ling Wang, Zhanwei Li, Zhiying Zhang*

College of Animal Science and Technology, Shaanxi Key Laboratory of Molecular Biology for Agriculture, Northwest A&F University, YangLing, Shaan'xi 712100, P. R. China.

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Purified recombinant protein has been routinely used to immunize rabbits to produce polyclonal antibodies. The process of the purification of recombinant proteins from bacterial inclusion bodies is both labor intensive and time consuming. To determine whether whole inclusion bodies can be used as antigen in polyclonal antibody production, we amplified *Gallus gallus* phospholipase A2 gene by RT-PCR, fused with glutathione-S-transferase (GST), and expressed recombinant *Escherichia coli* strain Rosseta (DE3) in the form of inclusion bodies. The inclusion bodies isolated from the bacteria cells were directly injected into rabbits and hens at 300 µg dose four times. Western blot was performed to detect antibodies in serum and in egg yolk, which revealed strong immunoreactions. The results suggested that using recombinant protein-containing inclusion bodies as antigen is a novel and convenient method to immunize animals for polyclonal antibody production.

Key words: Polyclonal antibodies, inclusion bodies, antigen, recombinant phospholipase A2.

INTRODUCTION

Inclusion bodies are commonly formed during over expression of heterologous genes in *Escherichia coli*, particularly of mammalian or viral origins (Carrio and Villaverde, 2002; Ventura and Villaverde, 2006). In actively producing recombinant *E. coli* cells, inclusion bodies are seen as refractile particles. Under electron microscopy, inclusion bodies appear rather amorphous (Carrio and Villaverde, 2005), but after detergent-based purification, scanning microscopy reveals them to be rod-shaped particles (Bowden et al., 1991; Ventura and Villaverde, 2006). Inclusion bodies can be isolated through centrifugation from the resulting cell extracts (Clark, 1998; Villaverde and Carrio, 2003). Several washing steps are then applied to remove membrane proteins and other contaminants, by using low concentrations of chaotropic agents, such as urea or guanidinium chloride and detergents like Triton X-100 or sodium dodecyl sulfate. An alternative method is gradient centrifugation which offers a more precise separation but is technically more complex (Villaverde and Carrio, 2003). The system in which foreign protein is expressed in bacteria, especially in *E. coli*, has been performed for close to forty years, now. One advantage of this system is the ability to produce protein in large quantities. *E. coli* grows at a very fast rate in comparison with mammalian cells, giving the opportunity to purify, analyze and use the expressed protein in a much shorter time. In addition, transformation of *E. coli* with the foreign DNA is easy and requires minimal amounts of DNA. Engineering protein using *E. coli* tends to be inexpensive. These reasons explain the popularity of bacterial system (Andersen and Krummen, 2002; Verma et al., 1998). There are a great number of researches using recombinant proteins as vaccines. Those studies show that recombinant protein as vaccine has a wonderful prospect (Dempster et al., 1996; Heraud et al., 2006; Lightowlers et al., 1996; Rand et al., 1989). Besides, recombinant proteins are often used to produce polyclonal antibodies (Chaivisuthangkura et al., 2006; Kapadia et al., 2003). But all of the recombinant antigens require an intricate

*Corresponding author. E-mail: zhangzhy@nwsuaf.edu.cn.

[#]Co-First Authors.

Abbreviations: PLA2, Phospholipase A2; **GST,** glutathione-S-transferase.

and elaborate purification process.

Phospholipase A2 (PLA2) is involved in the production of prostaglandins and leukotrienes, key mediators of the gut inflammatory response. Previous studies demonstrate that blocking the enzyme with anti-PLA2 antibodies (aPLA2) inhibits the inflammatory response, and thus may improve nutrient absorption and growth of animals. Anti-PLA2 antibody has been used as a feed additive to improve growth and feeding efficiency in animal industry. Anti-PLA2 antibodies as a feed additive improve growth 5 to 10% in broiler and pig (Cook, 2002; Danny and Hooge, 2006), and 25 to 30% in fish (Barry et al., 2008). Anti-PLA2 antibodies were mass-produced through injecting hens with purified PLA2 protein, collecting the antibody-rich eggs and preparing egg powder. In the production procedure, purification of PLA2 protein is painstaking in large scale.

In this study, we designed the experiments to examine the feasibility whether the recombinant protein-containing inclusion bodies could be used as antigens for polyclonal antibody production in rabbit and hen.

MATERIALS AND METHODS

Construction of expression vector

All DNA manipulation were performed essentially according to standard methods described previously (Sambrook and Russell, 2001). TRIzol Reagent (Invitrogen) and M-MLV (Takara Dalian China) were used for RNA extraction from chicken pancreas and reverse transcription reaction. According to the *Gallus gallus* PLA2 sequence from NCBI, accession: XM_415272, we designed a pair of primers to amplify PLA2 gene: PLA2 forward: 5'-GCGAATTCATGAAGACTCTTGGCGTGCT-3'; PLA2 reverse: 5'-GCAAGCTT ACTGCTGCAGTATTTCTTCT-3'.

*Eco*RⅠ and *Hin*dⅢ restriction enzyme recognition sites were incorporated into the primers to facilitate directional cloning into expression vector pGEX-4T-1 (GE health). The primers were synthesized by Biosune Int. Shanghai. A touchdown polymerase chain reaction (TD-PCR) condition was used to amplify the PLA2 gene following reverse transcription reaction with oligo $d(T)_{18}$. A 460 bp of PCR product was visualized with 1% agarose electrophoresis. The PCR product was digested with the restriction enzymes *Eco*RⅠ and *Hin*dⅢ. Following gel purification, the digested PCR product was ligated to the corresponding sites on the vector pGEX-4T-1. Ligated DNA was transformed into JM109 *E. coli* strain. The resulting vector was named as pGEX-PLA2. For the recombinant PLA2 gene expression, the pGEX-PLA2 was introduced into the expression host, Rosseta (DE3) *E. coli* strain.

Expression of PLA2 protein and inclusion body preparation from *E. coli*

The transformed *E. coli* cells were grown in 2 ml of Luria-Bertani broth containing ampicillin (100 µg/ml) at 37°C for 8 h, diluted with fresh broth (1:100), and when the cell density reached OD_{600} = 0.7, isopropyl-thio-β-D-galactoside (IPTG; 0.08mM concentration) was added for expression induction. After 4 h of shaking at 37°C, the bacteria cells were harvested by centrifugation at 10000 g for 5 min. The cell pellets were suspended in phosphate buffered saline (PBS), and suspension were subjected to sonication and the inclusion bodies were collected by centrifugation at 12000 g for 10 min. Inclusion bodies were washed twice with washing buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 1 M urea, pH 7.4), resuspended in PBS and stored in aliquots at -20°C until use.

The bacterial lysate and washed inclusion bodies were characterized by electrophoresis in 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE). Protein was visualized by staining with Coomassie brilliant blue. Protein concentration was measured by SDS-PAGE methods as previously described (Bradley et al., 2007; Casserly et al., 2000), using bovine serum albumin (Sigma) as a standard.

Rabbit immunization and blood samples collection

New Zealand white rabbits used in our experiment were purchased from Experimental Animal Centre (The Fourth Military Medical University, Xi'an). Two rabbits were injected subcutaneously. For the first immunization, inclusion body suspension containing 300 µg GST-PLA2 fusion protein in complete Freund's adjuvant (CFA) was administered. After 14, 21 and 28 days, 300 µg GST-PLA2 fusion protein in incomplete Freund's adjuvant (IFA) was administered.

Blood was sampled before first immunization as negative control and 3 days after last immunization. The serum samples were prepared as follow: Incubated at 37°C for 1 h, spin at 4000 g for 10 min, the supernatant was transferred in aliquots and stored at -20°C until use.

Hen immunization and yolk immunoglobulin (IgY) extraction

Seven Leghorn hens (18 weeks) were purchased at a local market. One hen was used as control. Six hens were immunized subcutaneously in the breast region with 300 µg GST-PLA2 fusion protein in CFA. After 14, 21 and 28 days, 300 µg of GST-PLA2 fusion protein in IFA was administered. Eggs were collected 3 days after the second immunization.

The extraction of IgY was modified according to the previous methods (Hansen et al., 1998; Moore and Sony, 1992). The yolk was separated from the white, and was disrupted by inserting a needle. The contents were allowed to drip through a nylon mesh into a measuring cylinder. The egg yolk was diluted 10 times with sterile water adjusted with acetic acid to a pH of 5, and was then incubated at 4°C for at least 6 h. The supernatant was slowly added with saturated ammonium sulfate to a concentration of 45% and stirred for 30 min. The IgY was precipitated by centrifugation at 10000 g for 10 min. The pellet was resuspended in 10 ml sterile water, was slowly added with saturated ammonium sulfate to a concentration of 40%, and then stirred for 30 min before the IgY was collected by centrifugation at 10000 g for 10 min. The precipitate was dissolved in 10 ml PBS.

Detection of antibodies against GST-PLA2

Serums and IgY solution were assayed for the presence of antibodies against GST-PLA2 antigen by Western blot. Inclusion bodies containing GST-PLA2 were run in 12% SDS-PAGE and were transferred onto polyvinylidene fluoride (PVDF) membrane. All serum samples were tested at a dilution of 1:500 and IgY solution samples at 1:1000. Goat anti-rabbit or anti-chicken IgG conjugated to horseradish peroxidase (HRP) (Boster Bio Int. Wuhan China) diluted 1:5000 was used as a secondary antibody. Protein was visualized by 3,3'-diaminobenzidine (DAB) kit (Boster Bio Int. Wuhan, China).

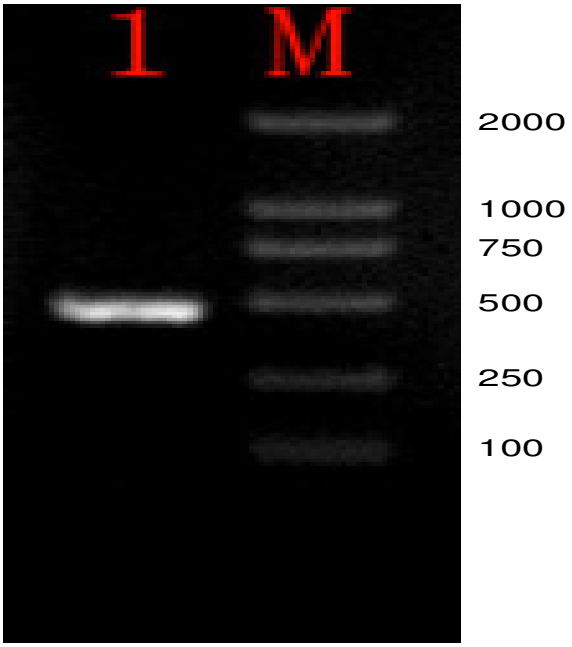

Figure 1. The PCR product was examined on 1% agarose gel, stained with ethidium bromide. Lane 1 is the PCR product and lane M is DL2000 marker.

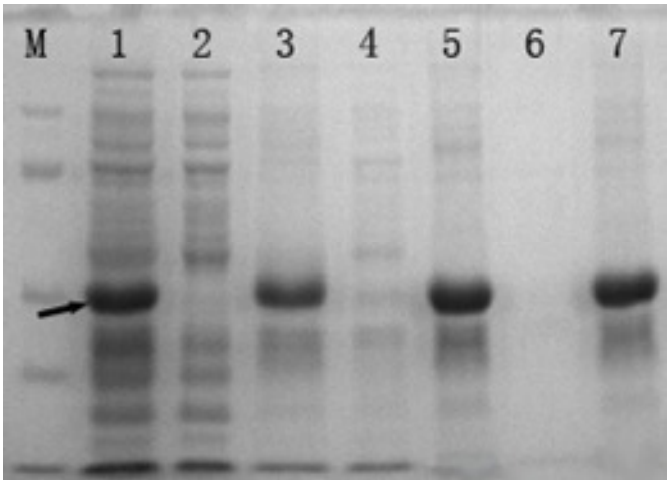

Figure 2. The GST-PLA2 recombinant protein was separated on 12% SDS-PAGE, stained with Coomassie brilliant blue, and marked by an arrow. Lane M is protein marker; lane 1 is whole cell lysate of bacteria; lane 2 is supernatant after sonication of cells; lane 3 is inclusion bodies; lane 4 is supernatant from the first washing solution of inclusion bodies and lane 5 is the first washed inclusion bodies, lane 6 and 7 are the supernatant and inclusion bodies from the second washing, respectively.

RESULTS

G. gallus PLA2 amplification and cloning

PLA2 is an enzyme that promotes the production of prostaglandins and leukotrienes, key mediators of the gut inflammatory response. Previous studies demonstrated that blocking the enzyme with anti-phospholipase antibody A2 inhibits the inflammatory response, and thus may improve nutrient absorption and growth of animals. We reasoned that active immunization of chicken with *G. gallus* PLA2 gene might improve hen performance on one hand, and the immunized hens could produce the anti-phospholipase antibody A2 in eggs in a large scale on the other hand. Thus, we choose the *G. gallus* PLA2 gene as a target gene to test the hypothesis.

To clone *G. gallus* PLA2 gene, a touchdown PCR condition was applied in this study, and the amplified fragment is about 460 bp (447 bp PLA2 cds plus two short flanking sequences) (Figure 1). The PCR product was digested with enzymes *Eco*R Ⅰ and *Hin*dⅢ, and inserted into the corresponding restriction sites of the expression vector pGEX-4T-1. The resultant vector was named pGEX-PLA2. The fidelity of the cloned PLA2 gene was confirmed by sequencing. The PLA2 gene was expressed as a fusion protein with glutathione-S-transferase (GST) tag, and the recombinant protein consists of 389 amino acids with a putative molecular weight of 44.59 Kd.

The recombinant PLA2 antigen preparation

The expression vector pGEX-PLA2 was firstly introduced into the *E. coli* strain Rosseta (DE3). After optimization of the expression conditions, we could not make the PLA2 recombinant protein to be expressed in a soluble form; instead the recombinant protein was largely expressed in the form of inclusion bodies. Following extensive washing process, the inclusion bodies were examined on 12% SDS-PAGE. As shown in Figure 2, the majority of proteins in inclusion bodies were PLA2 recombinant protein, which approximately accounted for 95% of the total protein of inclusion bodies. This prompted us to consider if we could use the whole inclusion bodies as antigen to immunize animals.

Using whole inclusion bodies as antigen to immunize rabbits

To test whether purified inclusion bodies from recombinant bacteria could induce animals to generate a specific immune response, we first purified the inclusion bodies and used them as antigens to immunize rabbits. Rabbits were injected subcutaneously with 300 μg inclusion bodies containing GST-PLA2 fusion proteins mixed with equal part of CFA. After 14, 21 and 28 days, 300 μg of the inclusion bodies prepared in IFA was applied to boost immunization, respectively. On day 31, the serum samples of immunized animals were collected for detection of anti-PLA2 antibody. As shown in Figure 3, a specific anti-PLA2 was detected from the immunized

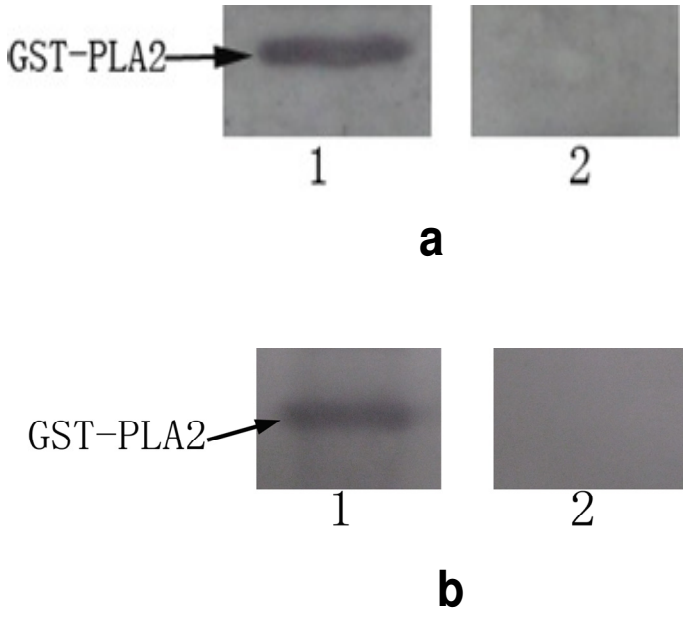

Figure 3. Western blot detection for the presence of serum antibody and IgY against the recombinant GST-PLA2 protein. (a) Lane 1 is the serum sampled 3 days late after the last immunization and Lane 2 is the serum sampled before first immunization. (b) Lane 1 is the IgY isolated from the treatment hens and Lane 2 is the IgY prepared from the control hens.

rabbits, suggesting that the inclusion bodies from recombinant bacteria could be used to generate polyclonal antibody in rabbit.

Using whole inclusion bodies of PLA2 as antigen to immune modulate endogenous PLA2 protein in hens

Here, we considered if the whole inclusion bodies, containing recombinant *G. Gallus*, PLA2 protein could stimulate the hen's immune system to generate specific anti-PLA2 antibody. We immunized hens with the purified inclusion body and the immunization protocol was applied as follows: 300 µg inclusion body solution containing GST-PLA2 fusion proteins mixed with equal amount of CFA was applied as the primary immunization. After 14, 21 and 28 days, 300 µg of the inclusion bodies prepared in IFA was applied to boost immunization, respectively. The egg samples were collected before primary immunization and 3 days after the last boosting immunization. As shown in Figure 3b, the anti-PLA2 antibody was detected from the eggs of the immunized hens, suggesting that the whole inclusion body prepared from recombinant bacteria could be used as antigen to stimulate hen immune system to generate a specific immune response. Furthermore, this result demonstrated that the recombinant GST fusion protein expressed in inclusion body of bacteria could be employed to immune modulate endogenous protein function in chicken.

DISCUSSION

Inclusion bodies are usually formed when recombinant protein is expressed at high levels (Bowden et al., 1991; Carrio and Villaverde, 2002; Ventura and Villaverde, 2006). In the process of recombinant protein for antigen or vaccine preparation, protein purification from inclusion bodies is always cumbersome, especially if the tag of the fusion protein is GST, MBP or other tag that need a functional structure. Yet, some researchers also use the whole bacteria cells as vaccine (Stubbs et al., 2001; Trott et al., 2008), and whole bacteria cell are injected into animals in order to get immunized or polyclonal antibody. This immunization approach could produce a lot of irrelevant immune responses. Therefore, we developed the new method of immunization-inclusion bodies as antigen. In comparison with other routines to immunize animals, the use of inclusion bodies isolated from bacteria is a convenient and cheap method to prepare antigen. Preparation of inclusion bodies is much easier than affinity purification of fusion protein, which is time-consuming and tedious. Inclusion bodies instead of whole-bacteria cells could enrich the interested protein at a higher level; meanwhile, immunization with inclusion bodies could reduce animal irrelevant immunoreactions. Recombinant proteins produced *in vitro* are under control, which avoids a lot of inherent problems for live-vector vaccines, such as the risk of heterologous DNA transfer to other micro-organism (Mercenier et al., 2001), unpredictable efficacy due to preexisting or acquired immunity to bacteria harboring the antigen, and the lack or low expression of vaccine antigen in the vaccinated host (Makela, 2000; Schodel, 1990).

Anti-PLA2 antibodies from egg yolk has been used as feeds additive to enhance growth and improve feed efficiency (Cook, 2002; Yang, 2003), which is produced by hens injected subcutaneously with purified PLA2. Purification of PLA2 protein from pancreas or recombinant bacteria is intricate and time-consuming. However, it is much easier to directly use recombinant PLA2 in the form of inclusion bodies. In summary, our results show that recombinant PLA2 expressed in *E. coli* and isolated in the form of inclusion bodies is low-cost and time-saving. The purified inclusion bodies could be used as antigen to produce IgY in a large scale in hens. The future work to examine the effect of anti-PLA2 antibody on endogenous PLA2 function will be warranted.

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