Protein Expression and Purification 75 (2011) 225–229

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Immunoaffinity purification of polyepitope proteins against *Plasmodium falciparum* with chicken IgY specific to their C-terminal epitope tag

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ARTICLE INFO

Article history:
Received 1 August 2010
and in revised form 6 October 2010
Available online 19 October 2010

Keywords:
Immunoaffinity chromatography
Chimeric protein purification
IgY antibody
Polyepitope vaccine
Plasmodium falciparum

ABSTRACT

Developing a polyepitope vaccine, a chimeric protein which contains diverse types of antigenic epitopes, is a promising strategy to prevent malaria. Previously, we had constructed a library of polyeptitope chimeric genes against *Plasmodium falciparum* without any protein tags. In an attempt to develop an efficient and universal procedure for purification of polyepitope chimeric proteins, we assembled an immunoaffinity chromatography (IAC) column with affinity-purified specific polyclonal IgY (mpIgY) antibodies that recognized the same C-terminal epitope tag of chimeric proteins in the library. A single-step and universal protocol was established and successfully applied for the purification of chimeric proteins. Using this protocol, chimeric proteins were specifically purified from an *Escherichia coli* expression system, and the purity and authenticity were verified by gel electrophoresis and Western blot analysis. Moreover, the comparison between this IAC method and the conventional chromatography, using two anion exchange columns followed by a step of gel filtration, showed that the new method was more efficient with an 8-fold greater yield. The results suggest that this IAC method will be an efficient approach for the purifications of polyepitope vaccine candidates against *P. falciparum* in our future study, and also be valuable for other similar applications.

Introduction

Malaria is one of the most severe infectious diseases in the world, and there is a pressing need for a powerful vaccine to protect the endemic population [1]. In recent years, much effort has been directed to the development of a subunit vaccine or polyepitope vaccine against the asexual stage of *Plasmodium falciparum*. In our previous studies, a technology named "epitope shuffling" had been well established, and a library of polyepitope chimeric genes had been constructed, from which candidate proteins against *P. falciparum* could be screened and identified [2,3]. To evaluate immunological function of a protein vaccine, purification is the first step. Though several protein tags (e.g. 6 × His, MBP and GST etc.) are commonly used to facilitate the purification of recombinant protein, they must be cut off to meet the clinical trial standard of vaccine production. On the other hand, it is uncertain to what extent the tags would affect the function of recombinant protein [4]. Besides, the purification procedure of conventional chromatography is usually unique for a given protein [5]. All these facts make purification of vaccine proteins without tags a very laborious and time-consuming step [6], especially in our study, since there would be a batch of polyepitope proteins screened from the library and then, needed to be immunologically identified.

Immunoaffinity chromatography (IAC) is a kind of affinity chromatographic approach using immobilized antibodies as affinity ligands which are specific to either an antigen, an epitope tag or a group of structurally related antigens [7,8]. Most of the reported IAC columns use monoclonal antibodies (mAbs), among them, Burgess et al. have successfully isolated core RNA polymerases from many bacterial species by a single IAC column [9,10]. However, high-cost and low-output properties of the mAbs limit their application. In contrast, monospecific chicken IgY antibodies (mpIgY) are low-cost, high-output and easier process [11–13], which encourage us to use it in an IAC method.

In this study, we developed an immunoaffinity column based on affinity-purified polyclonal IgY (mpIgY) antibodies specific to the same C-terminal epitope tag of all polyepitope proteins, and successfully purified chimeric proteins from the polyepitope library (as mentioned above). The comparison between this immunoaffinity chromatography with mpIgY and conventional chromatography indicated the promising advantages of the former approach both in easy manipulation and high product yield.

Materials and methods

Materials

LB medium: 10 g tryptone (OXOID), 5 g yeast extract (OXOID), 10 g NaCl in 1 L H_2O; *Coupling buffer:* 0.2 M $NaHCO_3$, pH 8.3,

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doi:10.1016/j.pep.2010.10.003

0.5 M NaCl; *Acetate buffer:* 0.1 M sodium acetate, pH 4, 0.5 M NaCl; *Blocking agent:* 0.5 M ethanolamine, pH 8.3, 0.5 M NaCl; *Binding buffer:* 50 mM phosphate buffer saline, pH 7.4, 50 mM NaCl; *Lysis buffer A:* 300 μg lysozyme per ml and 1% PMSF (in binding buffer); *Elution buffer A:* 0.1 M glycine, pH 2.7, 0.5 M NaCl; *Elution buffer B:* 0.1 M glycine, 2 M urea, pH 2.3, 0.1 M NaCl; *Neutralization solution:* 1 M Tris–HCl, pH 9.0; *Buffer A:* 25 mM Tris–HCl, pH 8.0, 50 mM NaCl; *Buffer B:* 25 mM Tris–HCl, pH 8.0, 1 M NaCl; *Lysis buffer B:* 300 μg lysozyme per ml and 1% PMSF (in Buffer A); *PBS:* 43 mM Na_2HPO_4, 9.8 mM KH_2PO_4, 82 mM NaCl; 1 × *SDS sample buffer:* 62.5 mM Tris–HCl, pH 6.8, 3% SDS, 10% glycerol, 0.6 M 2-mercaptoethanol, 0.0125% (w/v) bromphenol blue. All buffers were cleared by filtration through a 0.22 μm filter.

Two polypeptides comprised in C-terminal epitope tag

A: KNVIKCTGESQTGNTGGGQAGNTGGGQAGN, B: AGNTVGDQA GSTGGSPQGSTGASQPGSSEP were synthesized by the peptide-synthesis facility of the Chinese Academy of Army Medical Science, Beijing, using solid phase strategies in a multiple-peptide synthesizer (Abimed AMS 422; Langenfeld, Germany), purified by reverse-phase high performance liquid chromatography and stored at −20 °C.

Construction of vaccine candidate library

The library of polyepitope chimeric genes was constructed by the method as described in [2], and cloned into vector pET30a without any protein tags for purification (e.g.: 6 × His-tag) (unpublished data). There is a unique C-terminal epitope tag consisted of 57 amino acids contained in all chimeric proteins in the library, comes from Serine-Repeat Antigen (SERA) of *P. falciparum* [14] with a sequence of KNVIKCTGESQTGNTGGGQAGNTGGGQAGNTVGDQ AGSTGGSPQGSTGASQPGSSEP.

Construction, expression and purification of recombinant GST-C protein

After C-terminal epitope gene was successfully cloned into vector pGEX4T-1, the recombinant GST-C protein was expressed in *Escherichia coli* (BL21) cells and affinity purified as described in [15].

Immunological techniques

Anti-GST-C IgY antibodies production

Two Leghorn chickens (about 1.8 kg each) were subcutaneously injected with 0.3 mg of the purified GST-C protein with complete Freund's adjuvant (v:v) in the neck. After 3 and 5 weeks, two booster injections of 0.2 mg of GST-C with incomplete Freund's adjuvant (v:v) was injected i.m. in the leg. From 1 week after the second injection to 10 days after the last injection, eggs were collected and total IgY antibodies were partially purified [16].

Preparation of NHS-polypeptides immunoaffinity column

A HiTrap NHS-activated HP 1-ml column (GE Healthcare, USA), a preactivated set for covalent binding of ligands containing primary amines, was used according to the manufacturer's recommendations. Briefly, 10 mg of polypeptides (A:B = 1:1) was dissolved in 1 ml coupling buffer. HiTrap NHS-activated HP 1-ml column was thoroughly activated by addition of 0.1 mM HCl solution of pH 4.0 at 4 °C. Then, immediately the polypeptide solution (1 ml at 10 mg/ml) was added into the column and incubated at room temperature for 0.5 h. Finally, after the matrix was washed and blocked by acetate buffer and block agent, immunoaffinity column NHS-polypeptides was obtained. Coupling yield was calculated by the equation: $[(10\text{-}C_{ft})/10]^{*}100\%$, C_{ft} represents protein concentration of flowthrough which was quantitatively determined by BCA™ protein assay kit (Pierce) with bovine serum albumin as a protein standard.

Affinity purification of anti-polypeptides monospecific polyclonal IgY antibodies (mpIgY)

Crude IgY antibodies isolated using precipitation by caprylic acid, containing monospecific anti-polypeptide polyclonal IgY antibodies (mpIgY), were brought to 40% saturated ammonium sulfate solution, stirred for 30 min and then centrifuged at 10,000 rpm for 30 min at 4 °C. Then, the pellet was dissolved in 30 ml binding buffer. After being filtrated through a 0.45 μm membrane, the total IgY solution was applied to NHS-polypeptides column that has been previously equilibrated with binding buffer at a flow rate of 1 ml/min, and the column was washed with an excess of binding buffer. Finally, mpIgY antibodies were eluted by elution buffer A. Fractions (1 ml) were collected (with addition of 150 μl of neutralization solution), and the immunoreactivity of mpIgY, compared with that of total IgY was analyzed by ELISA [17] with recombinant GST-C protein. Afterward, the column was regenerated with 10 ml of buffer A. Anti-peptides mpIgY antibodies were pooled, quantitated by BCA™ protein assay kit (PEIRCE) with bovine serum albumin as a protein standard and dialyzed against Coupling Buffer overnight, then preserved at −20 °C until use.

Affinity purifications of recombinant GST-C protein and chimeric proteins from the library

NHS-msIgY immunoaffinity column (IAC) was prepared as described above. To develop a practical protocol for purification, the IAC column was first applied to purify recombinant GST-C protein. Briefly, GST-C-expressing *E. coli* was cultured in 5 ml of LB medium at 37 °C with 220 rpm for 3 h and induced by 1 mM IPTG for 3 h at 37 °C, then harvested at 4 °C with 5000 rpm for 10 min. Afterward, the *E. coli* cells were resuspended in 0.8 ml of lysis buffer A, sonicated to break up the cells. The lysate was centrifuged at

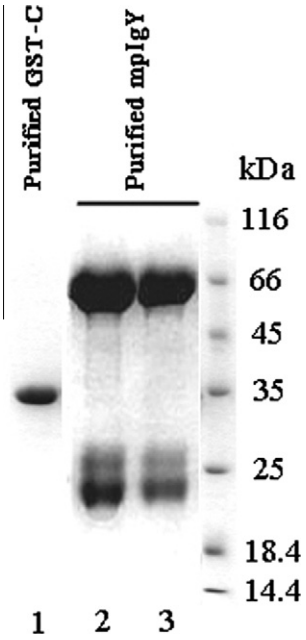

Fig. 1. SDS–PAGE analysis of the purified GST-C recombinant protein and ant-polypeptides mpIgY. Lane 1: purified GST-C protein; lane 2 and 3: purified mpIgY.