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Inclusion bodies of recombinant Epstein–Barr virus capsid antigen p18 as potential immobilized antigens in enzyme immunoassays for detection of nasopharyngeal carcinoma

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ABSTRACT

Background: Development of indirect enzyme-linked immunosorbent assays (ELISAs) often utilizes synthetic peptides or recombinant proteins from *Escherichia coli* as immobilized antigens. Because inclusion bodies (IBs) formed during recombinant protein expression in *E. coli* are commonly thought as misfolded aggregates, only refolded proteins from IBs are used to develop new or in-house diagnostic assays. However, the promising utilities of IBs as nanomaterials and immobilized enzymes as shown in recent studies have led us to explore the potential use of IBs of recombinant Epstein–Barr virus viral capsid antigen p18 (VCA p18) as immobilized antigens in ELISAs for serologic detection of nasopharyngeal carcinoma (NPC).
Methods: Thioredoxin fusion VCA p18 (VCA-Trx) and IBs of VCA p18 without fusion tags (VCA-IBs) were purified from *E. coli*. The diagnostic performances of IgG/VCA-IBs, IgG/VCA-Denat-IBs (using VCA-IBs coated in 8 mol/l urea), IgG/VCA-Trx, and IgG/VCA-Peptide assays were compared by screening 100 NPC case-control pairs.
Results: The IgG/VCA-Denat-IBs assay showed the best area under the receiver operating characteristic curve (AUC: 0.802; $p < 0.05$), while the AUCs for the IgG/VCA-IBs, IgG/VCA-Trx, and IgG/VCA-Peptide assays were comparable (AUC: 0.740, 0.727, and 0.741, respectively).
Conclusion: We improved the diagnostic performance of the ELISA significantly using IBs of recombinant VCA p18.

1. Introduction

One of the keys to improve the diagnostic performance of an indirect enzyme-linked immunosorbent assay (ELISA) is by optimizing the source of an immobilized antigen. The common sources of antigens are synthetic peptides and recombinant proteins from *Escherichia coli*, yeast, insect, and mammalian cells. *E. coli* is preferred over eukaryotic cells as a recombinant protein expression host because *E. coli* systems are relatively easier, cheaper and quicker to use [1]. During recombinant protein expression, however, *E. coli* deposits some of the target polypeptides as aggregates which have been termed inclusion bodies (IBs). IBs are commonly thought to hinder bioprocess productivity owing to the notion that they contained only misfolded polypeptides. In many cases, only small portions of recombinant proteins were expressed as soluble proteins. In concordance with the conventional view of associating protein conformation and functional quality with its solubility [2], only refolded proteins from IBs are used as antigens. The hurdles to obtain soluble or refolded antigens may delay the development of new or in-house diagnostic assays.

Recent studies on IBs have led to a new paradigm for the cellular factories producing IBs and their non-classical nature, suggesting that IBs have great potential in industrial applications of enzymes and nanomaterials [2–6]. One such application is tissue engineering which makes use of the bio-adhesiveness, wettability, size, geometry and mechanical stability of IBs as inert nanomaterials to assist surface colonization of mammalian cells without developing any cytotoxicity [3,6]. The application of IBs as naturally immobilized enzymes in biocatalysis has also been investigated [3]. The ability of IBs to disintegrate and release functional proteins has also been explored as nanopills for effective protein–drug delivery systems [4].

Epstein–Barr virus (EBV) is an important causative agent of nasopharyngeal carcinoma (NPC) [7,8], in which elevated levels of circulating antibodies against EBV antigens are suggestive of EBV reactivation and/or tumor malignancy [9]. Anti-EBV IgG and IgA antibodies scored high positive detection rates for NPC patients with active disease in contrast with healthy EBV carriers [10,11]. In particular, viral capsid antigen p18 (VCA p18; also known as capsid protein VP26), an immunogenic structural protein of EBV, has been consistently shown to induce strong IgA and IgG antibody responses in NPC patients [12,13]. Because of the desirable

Abbreviations: BFRF3, *Bam*HI F rightward reading frame 3; IBs, inclusion bodies; IMAC, immobilized metal ion affinity chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; IV, index value; NPC, nasopharyngeal carcinoma; PBST, phosphate-buffered saline-Tween-20; PP, percentage positivity; SEM, scanning electron microscope; Trx, thioredoxin; VCA, viral capsid antigen.

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properties of IBs as described above, here, we explored the use of IBs as an alternative source of antigens in indirect ELISAs for detection of a disease. As a proof-of-concept, we used IBs of VCA p18 as immobilized antigens to detect NPC. By comparing the ELISAs using different forms of VCA p18 including IBs without fusion tags (VCA-IBs), VCA-IBs in 8 mol/l urea (VCA-Denat-IBs), Trx fusion protein (VCA-Trx), and synthetic peptide (VCA-Peptide of a commercial ELISA kit), we found that both VCA-IBs and VCA-Denat-IBs could be useful in detection of NPC.

2. Materials and methods

2.1. Study subjects

Ethical approval of this study was given by the Medical Ethics Committee of University Malaya Medical Center. Volunteers were enrolled over 3.5 years from the hospital from the year 2007 to 2011. Patients with biopsy-proven NPC were recruited at the otorhinolaryngology clinic of the hospital during their follow-up appointments. They were in remission. On the other hand, normal subjects were recruited during blood donation campaigns organized by the hospital. A survey was done to obtain their family history and individual information, i.e., age, gender, national identification, ethnicity and cancer staging (if applicable). Their blood samples were collected with written consents. Using individual matching based on age (within 5 years), gender and ethnicity, 100 case–control pairs were chosen. The subjects were of Malaysian Chinese ethnicity without any mixed parentage because the sample sizes for the NPC cases of other ethnic groups were small. EBV infection status of the chosen NPC and normal subjects were unknown at the time of blood sample collection.

2.2. Protein expression and purification

2.2.1. Plasmids

pYES2.1 plasmid containing full-length *BFRF3* (*Bam*HI F rightward reading frame 3, 531 bp; EBV strain B95-8, GenBank: V01555) that encodes VCA p18 was provided by Prof. Sam Choon Kook. *BFRF3* was subcloned into pET102/D-TOPO (Invitrogen) using forward primer 5′-CACCATGGCACGCCGGCTGCCCAA-3′ (5′-CACC allowed TOPO directional cloning), and reverse primer 5′-CTTATCGTCATCGTCCTGTTTCTTACG TGC-3′ (5′-CTTATCGTCATCGTC added an enterokinase cleavage site to the upstream of C-terminal tags). The target protein produced using pET102 (VCA-Trx) possesses an N-terminal His-patch Trx tag and C-terminal V5 and His_6 tags. His-patch Trx is a mutated Trx which contains a metal binding domain that allows immobilized metal ion affinity chromatography (IMAC) purification [14].

To construct a plasmid encoding VCA p18 without fusion tags, pEC8 was first constructed by removing the Trx coding sequence of a self-ligated pET102 (not shown). pEC8 contains an *Eco*RI and a *Hind*III sites that allow directional insertion of a DNA fragment. Forward primer 5′-TATGAATTCATGGCACGCCGGCTGCCCAA-3′ and reverse primer 5′-TCGAAGCTTTCACTGTTTCTTACGTGC-3′ (cut sites underlined) were used to amplify *BFRF3* and then inserted into pEC8.

2.2.2. Auto-induction

Auto-induction of BL21 Star (DE3) (Invitrogen) expressing VCA-Trx was done as described previously [15]. The starter culture was prepared using MDG minimum media containing 100 μg/ml ampicillin. ZYM-5052 complex media (100 ml) containing 100 μg/ml ampicillin was inoculated with the starter culture and incubated at 37 °C overnight with shaking (250 rpm).

2.2.3. IMAC

VCA-Trx was purified using cobalt chelate membrane adsorbers as described previously [15]. A wet cell pellet of about 0.4 g from the auto-induced culture was resuspended in 15 ml of native purification buffer (50 mmol/l NaH_2PO_4, 0.5 mol/l NaCl, pH 8.0). The suspension was sonicated at 250 W for a total period of 8 min (cycles of 10 s pulses and 10 s pauses), and centrifuged at 12,000 ×*g* and 4 °C for 1 h. The supernatant was loaded into the membrane adsorbers. The membrane adsorbers were washed with 10 bed volumes of native wash buffer (20 mmol/l sodium phosphate, 0.5 mol/l NaCl, 10 mmol/l imidazole, pH 8.0). Elution was done using native elution buffers (20 mmol/l sodium phosphate, 0.5 mol/l NaCl, 250 mmol/l imidazole, pH 8.0).

2.2.4. IPTG (isopropyl-β-D-thiogalactopyranoside) induction

In order to obtain non-classical IBs, IPTG induction of BL21 Star (DE3) expressing VCA p18 without fusion tags was done under suboptimal culture conditions [5]. Fresh LB broth (50 ml) containing 100 μg/ml ampicillin was inoculated with the starter culture and incubated at 25 °C with shaking (160 rpm) until the cell growth reached mid-log phase. IPTG was added to a final concentration of 0.8 mmol/l. The culture was incubated at 25 °C overnight with shaking (160 rpm).

2.2.5. IB purification

To examine the solubility of VCA p18 without fusion tags, a wet cell pellet of about 0.2 g was resuspended in 20 ml of 10 mmol/l Tris–HCl containing 1% (v/v) Triton X-100, 1 mmol/l EDTA, and 10 mmol/l β-mercaptoethanol, pH 8, and then incubated for 1 h with shaking. The lysate was centrifuged at 9500 ×*g* and 4 °C for 10 min. The gentle cell lysis/pellet wash procedures were repeated 5 times.

VCA-IBs were purified from the IPTG-induced culture using 2 wash cycles. Each wash cycle consists of 2 sonication/wash steps. For the first sonication/wash step, a wet cell pellet of about 0.2 g was resuspended in 20 ml of 10 mmol/l Tris–HCl containing 1% (w/v) sodium deoxycholate, pH 8.0. The suspension was sonicated at 200 W for a total period of 4 min (cycles of 2 s pulses and 2 s pauses) (Omniruptor 4000 ultrasonic homogenizer; Omni International), and centrifuged at 9500 ×*g* and 4 °C for 10 min. The supernatant was discarded. For the second sonication/wash step, the same procedures were repeated using 20 ml of 10 mmol/l Tris–HCl containing 1% (v/v) Triton X-100, pH 8.0. The purified IBs (50 μl) were resuspended in 10 ml of 10 mmol/l Tris–HCl buffer, pH 8.0.

2.2.6. SDS-PAGE

In addition to routine SDS-PAGE analysis, the concentrations of the purified proteins were determined by densitometric analysis on the SDS-PAGE gel using Image Lab 5.2.1 (Bio-Rad).

2.2.7. Western blot

The purified proteins were identified using Western blot. VCA-Trx was detected using an alkaline phosphatase (AP)-conjugated anti-V5 antibody (Invitrogen) at 1:5000. Both VCA-Trx and VCA-IBs were detected using an anti-VCA p18 polyclonal antibody (Virostat) as the primary antibody at 1:2000 and rabbit anti-goat IgG Reserve AP antibody (KPL) or rabbit anti-goat horseradish peroxidase (HRP) antibody (Santa Cruz Biotechnology) as the secondary antibody at 1:10,000 and 1:5000, respectively.

2.2.8. Scanning electron microscope (SEM)

For those samples visualized using a variable-pressure SEM (SU3500 SEM; Hitachi), the samples were prepared by placing a drop of the IB suspension onto a 0.1% (w/v) gelatin-coated coverslip in a humidified chamber for 3 h or overnight. The IB-coated coverslips were gently washed in distilled water for 5 times, 5 min each and air-dried. The samples were mounted onto metal stubs with an electrically conductive double sided adhesive tape and then sputter-coated with gold. For those samples visualized using a field-emission SEM (Quanta 250 FEG SEM; FEI), uncoated coverslips were used. No wash step was done.