Colloids and Surfaces B: Biointerfaces 145 (2016) 854–861

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Biological preparation of highly effective immunomagnetic beads for the separation, concentration, and detection of pathogenic bacteria in milk

Min-Cheol Lim[a,b,1], Gwan-Hyung Lee[a,1,2], Duyen Thi Ngoc Huynh[a], Chae-Eun Hong[a], Soo-Yeon Park[a], Jong-Yun Jung[a], Cheon-Seok Park[a], Sungho Ko[c,*], Young-Rok Kim[a,*]

[a] Graduate School of Biotechnology & Department of Food Science and Biotechnology, College of Life Sciences, Kyung Hee University, Yongin, Republic of Korea
[b] Korea Food Research Institute, Seongnam, Republic of Korea
[c] Department of Biotechnology, CHA University, Seongnam, Republic of Korea

ARTICLE INFO

Article history:
Received 1 March 2016
Received in revised form 25 May 2016
Accepted 27 May 2016
Available online 2 June 2016

Keywords:
Amylose
Magnetic bead
Immunomagnetic separation
Protein G
Maltose binding protein
E. coli O157:H7
Food

ABSTRACT

We introduce a system for the efficient separation and concentration of pathogenic bacteria using biologically prepared immunomagnetic beads. Amylose magnetic beads (AMBs) were synthesized by an enzymatic reaction of amylosucrase from *Deinococcus geothermalis* (DGAS). The simple and rapid conjugation of AMBs and antibodies was achieved by the MBP-SPG fusion protein. MBP (maltose binding protein) binds to the surface of an AMB owing to its intrinsic affinity to the di-glucose in the AMB. SPG (streptococcal protein G) fused to the MBP has specific affinity to the Fc region of the antibody. Anti-*Escherichia coli* O157 antibodies were conjugated to the AMBs through a MBP-SPG linker without any physical and chemical treatments. The efficiency of separation and concentration of the target *E. coli* O157:H7 by the functionalized AMBs was revealed by plating counting, conventional polymerase chain reaction (PCR), and real-time RCR analysis. The immuno-AMBs effectively separated and concentrated the target bacteria from a commercial milk sample spiked with known number of bacteria, which was then analyzed by PCR to a detection limit of 10 CFU/mL. On the other hand, no PCR product was produced when milk was introduced directly to a PCR reaction. These results show that MBP-SPG is an effective linker and the resulting immuno-AMBs are capable of separating and concentrating the target bacteria from a food matrix.

1. Introduction

Immunomagnetic separation (IMS) is a widely used technique for the selective concentration and isolation of target cells [1,2], bacteria [3–5], viruses [6–10], and biological compounds [11–16] from a range of sample matrices [17,18]. The magnetic beads used in an IMS system are usually prepared by emulsion polymerization [19,20] and emulsion evaporation methods [21,22]. These methods are generally based on the blending of an organic polymer or inorganic material with superparamagnetic materials during emulsion production. The substances used for manufacturing the magnetic beads include natural polymers, such as cellulose [23] and chitosan [24]; organic polymers, such as polystyrene [25], polylactic-glycolic acid [26], and polyethylene glycol [27]; and inorganic materials, such as Au [28,29]. On the other hand, new magnetic beads have been in demand because of the limitations of emulsion-based preparation methods, such as the potential toxicity of volatile solvents, purity of isolated natural polymers, and high energy consumption.

The physical adsorption and covalent attachment of antibodies to the solid surface have been used widely to prepare a range of immunomagnetic beads and sensing components of biosensors. Conventional immobilization methods, however, have major problems, such as low capture efficiency caused by the random orientation of the immobilized antibodies and complicated chemical reactions [30,31]. Therefore, an effective way of immobilizing antibodies in a highly oriented manner is needed to maximize the binding capacity of the antibodies. The lengthy and complicated procedures for the chemical conjugation of antibodies to

* Corresponding authors.
E-mail addresses: shko7@cha.ac.kr (S. Ko), youngkim@khu.ac.kr (Y.-R. Kim).
[1] M.L and G.L contributed equally to this work.
[2] Present address: Daesang Food Co., Ltd., Icheon 17384, Republic of Korea.

http://dx.doi.org/10.1016/j.colsurfb.2016.05.077
0927-7765/

the surface are another hurdle to overcome. To solve the major problems associated with physical or chemical conjugation methods, bi-functional fusion proteins, such as GBP-ProA [32], GBP-ProG [33], and CBD-ProA [34], were developed and used as a cross-linker because of their simple preparation and high efficiency for the immobilization of antibodies to the surface of the sensor platform. These fusion proteins are comprised of two affinity moieties that bind specifically to the substrate of the sensor platform and the Fc region of the antibody.

Amylose, a natural polymer formed from glucose linked with a α-1,4 glycosidic bond, exists abundantly in nature and is biocompatible and hydrophilic. Pure amylose, however, is difficult to obtain because the effective separation of amylose from amylopectin in starch is quite complicated. A range of methods have been used for the separation of amylose from starch, including hot-water extraction [35], diluted alkaline solution [36], and phase separation [37]. These methods, however, show poor separation efficiency or require intricate processes. On the other hand, enzymatic polymerization using phosphorylase [38,39] and amylosucrase [40,41] is quite effective for the synthesis of pure amylose without a high energy treatment process. Phosphorylase uses glucose-1-phosphate (G-1-P) as the glucosyl donor to construct an α-1,4 glycosidic bond. In addition to the requirement of expensive G-1-P, this reaction also requires maltooligosaccharides as a glycosyl acceptor, which limits its applications to larger scale production. In contrast, amylosucrase produces amylose from sucrose without the need for a primer [42]. Recently, the enzymatic polymerization method using the amylosucrase for the synthesis of amylose complexes or microbeads with encapsulated guest molecules, such as carbon nanotubes, has been investigated to expand its applications to the field of functional materials [43].

This paper presents a novel method for the preparation of highly effective immuno-amylose magnetic beads (AMBs) using amylosucrase from *Deinococcus geothermalis* (DGAS), and the simple immobilization of oriented antibodies on the surface of the AMBs *via* the bi-functional fusion protein, maltose binding protein (MBP)-streptococcal protein G (SPG). MBP is associated with the uptake of maltose in *E. coli* and is used as an affinity tag for the recombinant protein, taking advantage of its intrinsic affinity to amylose. On the other hand, SPG is used to purify the antibodies owing to its specific binding affinity to the Fc region of the antibodies. Here, these two proteins were fused to simplify the antibody immobilization step with an improved orientation (Fig. 1). The MBP-SPG fusion protein enabled the oriented immobilization of antibodies on the surface of the AMBs without the need for complicated physical or chemical treatments. The efficiency of the developed immunomagnetic beads to separate and concentrate the target bacteria was evaluated. The captured target bacteria were released easily from the AMBs by the addition of an elution buffer containing maltose molecules, which was then analyzed by polymerase chain reaction (PCR). The efficiency of the system to separate the target bacteria in a real food matrix was also tested using whole milk.

2. Materials and methods

2.1. Materials

Iron oxide nanoparticles (hydrophilic nanoparticles, surface area 50–245 m^2/g, diameter < 50 nm), lecithin (lyophilized powder), lysozyme, and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Sucrose, phosphate buffer saline (PBS), and Tris–HCl were obtained from Noble Bio (Suwon, Korea). Tween-20 and glycerol were acquired from Daejung Chemicals & Metals (Siheung, Korea). Ampicillin was supplied by Biosesang (Seongnam, Korea). The Luria-Bertani (LB) broth was obtained from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Anti-*Escherichia coli* O157 monoclonal antibody (FITC conjugate) was purchased from Thermo Fisher Scientific Inc. (Cambridge, MA, USA). SYBR Lo-ROX One-Step Master mix was supplied by Bioline (Alexandria, NSW, Australia). All restriction enzymes were acquired from New England Biolabs (Ipswich, MA, USA). Ni-NTA Superflow resin was obtained from Qiagen (Valencia, CA, USA).

2.2. Preparation of amylose magnetic beads (AMBs)

The AMBs were prepared by enzymatic polymerization using DGAS, as described earlier [44]. Briefly, 1 mL of a reaction solution consisting of 50 mM Tris–HCl (pH 8.0), 500 mM sucrose, 1 mg of lecithin, 0.8 mg of iron oxide nanoparticles, and 500 units of DGAS was incubated at 40° C for 48 h, followed by washing 3 times with water and ethanol. The AMBs were then collected by a magnet and stored in 20% ethanol at 4° C until needed. The morphology, size, and electron-distributions of the prepared AMBs were analyzed by field emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM).

2.3. Construction, expression and purification of recombinant MBP-SPG fusion protein

To construct the MBP-SPG-His expression vector, the *spg-his* gene was amplified from the pET-22b(+)::*gbp/spg* vector [32] by PCR using a primer set (F: 5′-CCG GAA TTC GGT ACC ATT CAG TCT CCA TGG-3′; R: 5′-CGC GGA TCC CTT TCG GGC TTT GTT AGC AGC CGG ATC-3′). The amplified *spg-his* gene was purified and digested by *Eco*RI and *Bam*HI. The pMal-c2x vector was also treated using those two restriction enzymes. The digested PCR product was ligated into the pMal-c2x vector according to the manufacturer's instructions using T4 ligase (Fig. S1), and the resulting construct, pMal-c2x::*spg/his* vector, was transferred to *E. coli* DH5α by electroporation. *E. coli* DH5**α** harboring pMal-c2x::*spg/his* was screened by growing in selection media containing ampicillin followed by colony PCR using the same primer set described above. *E. coli* DH5α containing pMal-c2x::*spg/his* was cultured for the overexpression of MBP-SPG. A 100 mL sample of the cells was grown at 37 °C with constant shaking in LB medium containing ampicillin (100 μg/mL). After reaching an OD_{600} of 0.7–0.8, overexpression of the desired protein was induced with 0.1 mM IPTG and further incubated at 18 °C overnight. The cells were then pelleted by centrifugation at 3000*g* for 20 min at 4 °C and resuspended in 5 mL of a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) supplemented with 1 mg/mL lysozyme. The cells were disrupted by sonication (VC 750, Sonics & Materials Inc., Newtown, CT, USA) with a 25 s disruption period at 35 s intervals in an ice bath for 10 min at 225 W (duty cycle 50%). After centrifugation at 3000*g* for 20 min, the supernatant was loaded onto a column packed with Ni-NTA resin. The Ni-NTA column was washed with a washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), and the MBP-SPG proteins were eluted with an elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purified MBP-SPG was then identified and quantified by 10% SDS-PAGE (Fig. S2) and a Bradford assay, respectively.

2.4. Functionalization of AMBs with antibodies via the MBP-SPG linker

The recombinant MBP-SPG fusion protein was used as a cross-linker to immobilize the antibodies to the surface of the AMB through the specific affinity of MBP and SPG to amylose and the Fc region of the antibody, respectively. To immobilize the MBP-SPG onto the surface of the AMBs, a reaction solution containing