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One-Tube Preparation of Magnetic Nanoparticles Specifically Binding to Antibodies for Efficient Foodborne Pathogen Detection

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Abstract

In food processing and production, microbiological control is essential. However, complex food matrices and low microorganisms levels require effective sample pretreatment prior to a detection method. Although conventional immunomagnetic bead preparation is time-consuming and environmentally hazardous, immunomagnetic separation is a potential pretreatment technique. This study introduced a streamlined, eco-friendly one-tube 'Mix-and-Match' pretreatment using ProAx1-Fe3O4, a recombinant adapter protein that facilitates regioselective antibody attachment to magnetic nanoparticles. Western Blot and SDS-PAGE were used to confirm the expression of the protein. The nanoparticles coated with ProAx1-Fe3O4 exhibited enhanced stability and antibody binding. They successfully captured *Salmonella* when used with PCR for confirmation, providing a powerful pretreatment approach for identifying foodborne bacteria.

Keywords: Antibody, antibody functionalized magnetic nanoparticles, immobilized antibodies, immunomagnetic separation, magnetic nanoparticles, one-tube preparation.

1. Introduction

Foodborne poisoning, a major public health concern, has a global impact similar to that of HIV/AIDS, malaria, and tuberculosis (Havelaar *et al.*, 2015). The primary causes of food poisoning are pathogenic microorganisms or their toxins (Soto Varela *et al.*, 2016) (Bintsis and Thomas, 2017) (Marrez *et al.*, 2019). Rapid and sensitive detection methods are essential for the detection of these pathogens. Traditionally, cultivating microorganisms has been the most common method (Hassoun *et al.*, 2023), but it is time-consuming (taking five to six days) and requires strict safety protocols (Rodríguez *et al.*, 2018). To overcome these limitations, polymerase chain reaction (PCR) techniques, including standard PCR and Real-time PCR, have been developed to reduce detection time to 12-24 hours and offer high specificity (Hyeon and Deng, 2017) (Zueter and Harun, 2018). However, PCR requires sample enrichment to avoid false positives, which complicates the detection process due to the complexity of food matrices and the low density of pathogenic bacteria.

Immunomagnetic separation (IMS) has emerged as a simple pretreatment method for target bacteria separation and enrichment, providing good quality samples for PCR with high specificity (Hsu *et al.*, 2014). IMS involves capturing target microorganisms with antibody-coated magnetic nanoparticles (MPs) and separating them from the food matrix using magnetism. However, conventional methods for immobilizing antibodies onto MPs often result in protein denaturation or degradation, which reduces their effectiveness. Randomly immobilized antibodies are less effective at binding antigens than orientationally immobilized antibodies (Lu *et al.*, 1996). Therefore, orienting target molecules on the surface of nanoparticles is a key strategy for improving the performance of magnetic particles (Quinn *et al.*, 1999) (Lee *et al.*, 2013) (Gan *et al.*, 2023).

This study developed a novel recombinant adapter protein, ProAx1-Fe_3O_4 to facilitate antibody conjugation onto MP surfaces. ProAx1-Fe3O4 is a dual-binding protein with one domain specifically binds to ferromagnetic nanoparticles and another recognizes the fragment crystallizable (Fc) region of most immunoglobulins (IgGs). This approach allows the fragment antigen-binding (Fab) regions of IgGs to remain on the MPs surface, improving the effectiveness of antibody binding compared to random immobilization. By fusing the magnetite-binding peptide with protein A, this one-tube preparation method for antibody-specific magnetic nanoparticles offers a rapid, efficient, and straightforward 'Mix-and-Match' pretreatment for immunomagnetic particles (Fig. 1).

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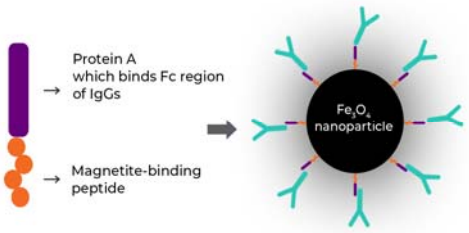

Figure 1. Schematic of 'Mix-and-Match' pretreatment immunomagnetic particles immobilizing antibodies using ProAx1-Fe3O4 binding protein.

2. Materials and Method

2.1. Construction of recombinant pET-proAx1-Fe_3O_4 binding

A DNA fragment encoding for the domain A of protein A was amplified from pET22b-proA plasmid (Tran-Nguyen *et al*., 2021) by the overlap extension PCR method with specific primers including F-*Nde*I, R1, R2, and R-*Hind*III to generate the ProAx1-Fe_3O_4 binding encoding gene (Table 1). The overlapped reverse primers containing Fe_3O_4 binding peptide encoding gene in which the two Fe_3O_4 binding peptides (HYIDFRW and TVNFKLY) (You *et al*., 2016) were linked by a GGSG linker. PCR product and plasmid pET22b were digested with the same enzymes *Nde*I and *Hind*III (Thermo Scientific) and ligated by T4 ligase. After that, the recombinant plasmid was chemically transformed into competent *E. coli* DH5a cells and screened with Luria-Bertani (LB-Amp) containing Ampicillin agar plate. The cloning results were experimentally confirmed by PCR colony method using T7 promoter and R-*Hind*III. Plasmid from positive colonies was extracted and sequenced by (PhuSa Biochem Ltd., Vietnam)

Table 1 List of primers used in this study

Primer's name	Primer sequence (5'-3')	Primer pair	Amplicon (bp)
F-*Nde*I	catatggacaacaaattcaacaaagaac		
R1	ccagcgaaaatcaatataatggtcgacggagctcg	F-*Nde*I + R1	261
R2	ttcacggtgccgctgccgccccagcgaaaatcaat	F-*Nde*I + R2	281
R-*Hind*III	aagcttatacagtttaaagttcacggtgccgct	F-*Nde*I + R-*Hind*III	300
T7 promoter	cgaaattaatacgactcactatagg	T7 promoter + R-*Hind*III	391

Restriction enzymes indicate by underlined letters.

2.2. Expression and confirmation of recombinant ProAx1-Fe_3O_4 binding protein

The pET-proAx1-Fe_3O_4 binding plasmid was transformed into *E. coli* BL21(DE3) chemically competent cells and performed expression with optimum conditions, i.e. the concentration of 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG), shaking cultured at 25°C, speed of 250 revolutions per minute for 6 hours. The cell pellets were collected and disrupted by sonication on ice to collect proteins in total, supernatant, and pellet fractions, respectively. The protein fractions were analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and confirmed by Western Blot and probed with 6xHis antibody (Santa Cruz) and goat anti-mouse IgG-HRP antibody (Proteintech).

2.3. Evaluation the MPs binding ability of ProAx1-Fe_3O_4 binding protein

The synthesis and properties of magnetic nanoparticles were described in (Thanh *et al*., 2019). Briefly, superparamagnetic nanoparticles were synthesized in the size range of 10-30 nm and were characterized using Transmission Electron Microscope (TEM) and X-Ray diffraction (XRD) to assert their structure and properties. The ProAx1- Fe_3O_4 binding in the total protein fraction was mixed with 25 µL of magnetic particles at 4 °C in 45 minutes for testing binding capability. The particles were collected by the magnetic bar and were washed with phosphate-buffered saline (PBS) solution thrice. Then, the particles were re-washed in 100 µL of PBS and well-vortexed for 20 minutes to test durability. Finally, the solution after incubation with nanoparticles, wash solution, and nanoparticles after washing were collected, treated with loading buffer 6X, and heated at 100 °C for 10 minutes. The binding ability was tested by SDS-PAGE with silver-stained. Non-purified ProAx1- Fe_3O_4 binding and magnetic beads were loaded onto the gel as controls.

2.4. Antibody capturing

The ProAx1-Fe_3O_4 binding-coated magnetic beads (volume of 100 µL) obtained from the previous experiment were mixed with the antibodies that match with the pathogen of interest at 4 °C and for the binding time of 45 minutes to evaluate antibody binding ability. In this study, the *Salmonella* O antiserum Poly A (OMA) (BD Difco) was used. The nanoparticles were magnetically collected and washed twice with PBS. A protein uncoated nanoparticles sample was also performed as a control. After that, all samples were prepared and analyzed by SDS-PAGE with silver staining.

2.5. Testing the ability to detect Salmonella enterica ser. Enteritidis on culture samples

The *Salmonella enterica* ser. Enteritidis (*Salmonella* Enteritidis) used for detection ability testing of MPs in this study was kindly provided by National Agro-Forestry-Fisheries Quality Assurance Department Branch (NAFIQAD) 4. In brief, the *Salmonella* Enteritidis suspension cultured overnight in 37 °C was centrifuged and discarded supernatant. The pellet then was washed thrice and resuspended in 1 mL PBS. After that, the suspension was divided into two samples: (1) was mixed with the MPs conjugated with the polyvalent anti-OMA antibodies, and (2) was mixed with the MPs. Both of them were incubated in room temperature, 30 minutes. Subsequently, the MPs were collected using a magnetic bar and then washed three times with PBS. The MPs were resuspended in 50 µL double-distilled water (ddH_2O) and then heated in 95 °C, 10 minutes to release the microorganisms. Supernatant was separated by using a magnetic bar and used as samples to perform PCR method to detect *Salmonella* Enteritidis with the specific primers for the *inv*A gene.

3. Results and Discussion

3.1. Characterization of magnetic nanoparticles

Figure 2A represented the XRD pattern of the synthesized magnetic nanoparticles based on co-

precipitation method. As shown in Figure 2A, the crystalline nature of synthesized magnetic nanoparticles showed six recognizably different peaks at (220), (311), (400), (511), and (440), which are consistent with the expected composition of Fe_3O_4. The relative intensity and position of diffraction peaks for the pattern mentioned was matched with the database in JCPDS file (No. 01-075-1373) for bulk Fe_3O_4. To examine the size of the nanoparticles, we also performed TEM images of Fe_3O_4 nanoparticles. Figure 2B shows TEM images of the synthesized magnetic nanoparticles, which have an average size of 30 nm. Notably, the Fe3O4 nanoparticles are cubic or octahedral in shape and are monodisperse, which enhances their potential for biomedical applications.

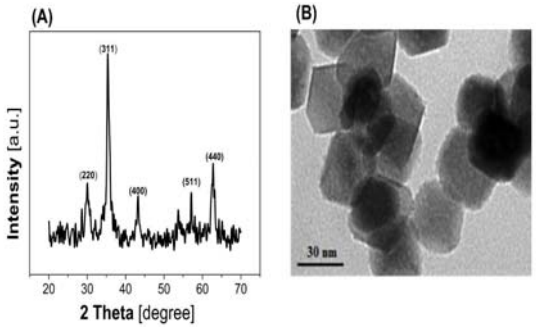

Figure 2. Characterization of synthesized magnetic nanoparticles. (A) XRD patterns and (B) TEM image of the nanoparticles.

3.2. Cloning and expression of ProAx1-Fe_3O_4 binding

Amplicons at 353 bp were obtained by PCR reaction using T7pro and R-*Hind*III primer. Negative control did not have any band, which indicated that there was no contamination in the PCR reaction. Results from sequenced data confirmed the amplified product was identical with published sequences from GenBank and the proAx1-Fe_3O_4 binding gene was cloned in the frame (data not shown). This experimental result showed that the recombinant plasmid was successfully constructed.

The recombinant protein ProAx1-Fe_3O_4 binding was induced from *E. coli* BL21(DE3)/pETT22b-proAx1-Fe_3O_4 binding for expression with a molecular weight of approximately 14.4 kilo Dalton (kDa) that was confirmed by SDS-PAGE analysis. Figure 4 shows an overexpressed protein band at approximately 14.4 kDa in the total protein fraction (Fig. 4A, lane 3). No band was observed in the negative control (Fig. 3A, lanes 1-2), confirming that the protein band in lane 3 corresponds to ProAx1-Fe3O4 binding. Additionally, an overexpressed protein band was present in the supernatant fraction (Fig. 4A, lane 4) but not in the pellet fraction (Fig. 3A, lane 5), indicating that the target protein was solubly expressed. This was further confirmed by Western Blot analysis, which detected the protein in the total and supernatant fractions using an anti-His antibody (Fig. 3B, lanes 3-4). Therefore, the ProAx1-Fe3O4 binding protein with a 6xHis tag was successfully expressed.

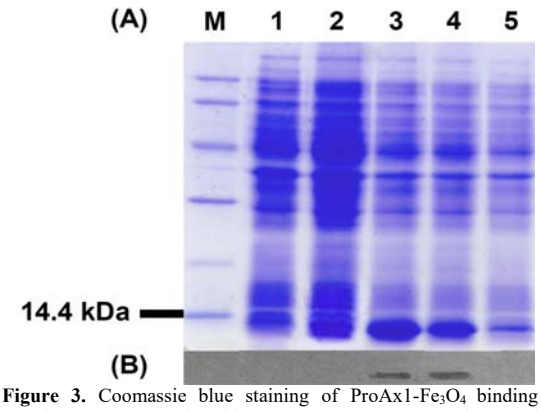

Figure 3. Coomassie blue staining of ProAx1-Fe_3O_4 binding protein expression analyzed by SDS-PAGE on 12.5% gel (A) and confirmed by Western blot probed with 6xHis (B). M, Low range weight protein marker, 97–14.4 kDa; 1, *E. coli* BL21(DE3)/pET22b (+ITPG); 2, *E. coli* BL21(DE3)/pET22b proAx1-Fe_3O_4 binding (-IPTG); 3-5, *E. coli* BL21(DE3)/pET22b proAx1-Fe_3O_4 binding (+IPTG); 3, total protein fraction; 4, supernatant protein fraction; 5, pellet protein fraction.

3.3. Evaluation the binding ability to magnetic nanoparticles

Practically, MPs size is bigger than the pore size of 15% polyacrylamide gel, so the MPs cannot migrate into SDS-PAGE gel. As shown in Figure 4, a single protein band (lane 9) at approximately 14.4 kDa presented in the particle sample after washing and vortexing, equal to the molecular weight of ProAx1-Fe_3O_4 binding (Fig. 4, lane 2), whereas no band was detected in lane 1. After mixing, it can be seen that the majority of proteins were bound to particles, and a minor residual ones remained in the supernatant after removing the particles (Fig. 4, lane 3-8). This demonstrated that ProAx1-Fe_3O_4 binding protein was able to bind to MPs strongly and their conjugate was stable under normal and harsh washing conditions (vortex for 20 minutes). Only one single band of ProAx1-Fe_3O_4 binding protein in lane 9 revealed this protein bound specifically to MPs with ease, which suggested that this protein can be used directly after expression without many steps of purification process in large scale.

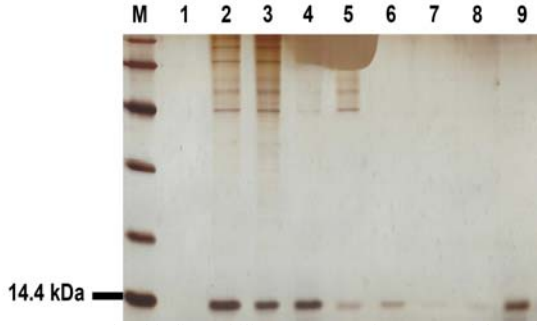

Figure 4. The binding ability to magnetic nanoparticles of ProAx1-Fe_3O_4 binding. M, Low range weight protein marker 97–14.4 kDa; 1, uncoated particles; 2, ProAx1-Fe_3O_4 binding in the total protein fraction after expression; 3, protein and particles before mixing; 4, particles after mixing; 5, supernatant fraction after mixing and removing particles; 6-7, wash fractions; 8, wash and vortex fraction; 9, particles after washing and vortexing.

3.4. Antibody capturing assessment

After testing the binding ability to MPs, the recombinant protein was continued to assess the antibody capturing ability. MPs were subjected to the gel but not shown any protein band (Fig. 5A, lane 1 and Fig. 5B, lane 1). The molecular weight of antibody fragments is approximately 25 kDa and 50 kDa (Fig. 5A, lane 2, and Fig. 5B, lane 3). They formed distinct bands compared to the molecular weight of ProAx1-Fe_3O_4 binding on the gel (Fig. 5B, lane 4 and 6). In the SDS-PAGE gel, there were a ProAx1-Fe_3O_4 binding band and two antibody fragment bands in particles after the washing step (Fig. 5B, lane 9), while there was no visible band in wash fractions (Fig. 5B, lane 7-8). This indicated the antibodies were successfully captured. Figure 5A also showed that after mixing with antibodies without the support of ProAx1-Fe_3O_4 binding protein, the particles were unable to capture antibodies. Consequently, all the amount of antibodies remained in the supernatant (Fig. 5A, lanes 3-5). It can be seen that a negligible amount of antibody appeared after the first washing step (Fig. 5A, lane 6), but it was totally removed after the second step (Fig. 5A, lane 7), which is explained as unspecific bonds between particles and antibodies. After two washing steps, there was no visible band in lane 8; hence, the uncoated particles cannot bind to antibodies (Fig 4A, lane 1, and lane 8). It could also be inferred that the ProAx1-Fe_3O_4 binding protein was not only able to capture antibodies but also retained the ability to strongly bind to particles under harsh washing conditions.

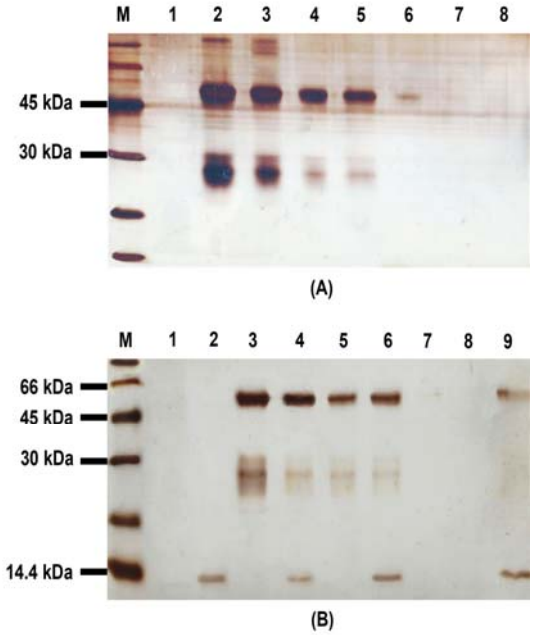

Figure 5. Antibody capturing assessment of uncoated MPs (A) and of ProAx1-Fe_3O_4 binding coated MPs (B). M, Low range weight protein marker, 97–14.4 kDa; A: 1, uncoated particles; 2, anti-OMA; 3, anti-OMA+particle before mixing; 4, particles after mixing; 5, supernatant after mixing; 6-7, wash fractions; 8, particles after wash; B: 1, uncoated particles; 2, coated particles; 3, anti-OMA; 4, anti-OMA+coated particle before mixing; 5, supernatant after mixing; 6, particles after mixing; 7-8, wash fractions; 9, particles after wash.

Previously, to attach specific antibodies to magnetic nanoparticles, chemical functional groups such as -NH_2, -Carbonyldiimidazole were widely used to immobilize protein A on the surface of MPs (Huynh *et al.*, 2020) (Ta *et al.*, 2016). The binding process of these groups is under investigation; the -Carbonyldiimidazole functional group can only react in organic solvents and is easily hydrolyzed in water (Ta *et al.*, 2016). The process of binding these functional groups is also time-consuming. In contrast, the interaction between Fe_3O_4 binding peptide and MPs (Fe_3O_4) was mainly formed via electrostatic interaction between iron ions with hydroxyl and carbonyl radicals (Li *et al.*, 2019). Therefore, under strong denaturing agents and harsh conditions such as SDS and high temperature (100 °C), the bound was easily broken. This is convenient for the control and regeneration of MPs. Moreover, protein A is a protein encoded by the *spa* gene with five IgG binding domains (Jansson *et al.*, 1998), which capture Fc region of IgG thereby leaving the Fab regions of IgG flexible when conjugate onto MPs surface. By selecting the strongest A domain, the ProAx1-Fe_3O_4 binding-coated MPs is possible to bind most antibodies from mammalian species, and notably IgGs, thus leading to act as a model 'Mix-and-Match' immune-magnetic, used for a variety of microorganisms.

3.5. Detection of Salmonella from culture media by PCR-coupled immune-magnetic beads

The results of PCR product electrophoresis on agarose gel in Figure 6 showed that the amplified product from magnetic conjugated anti-OMA antibody (Fig. 6, lane 2) appeared in a band between 200 bp and 400 bp, corresponding to the expected fragment of 285 bp for *Salmonella* gene (Fig. 6, lane 1). Negative control did not have any band (Fig. 6, lane 3). These results indicated that the immune-magnetic separation could be well fit for most polymerase chain reactions for *Salmonella* detection from the culture sample, creating a platform for the application of microorganisms testing in food samples.

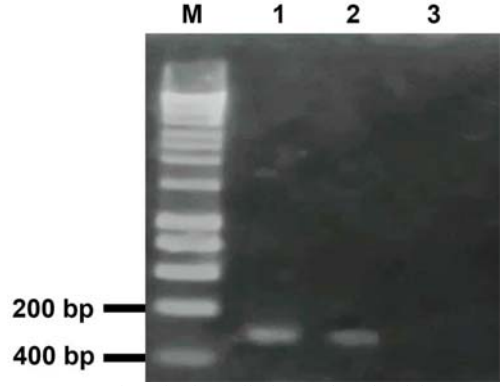

Figure 6. Detection of Salmonella from culture broth by PCR-coupled immune-magnetic beads. M, DNA ladder 1 kilobase; 1, invA gene (positive control); 2, MPs conjugated with anti-Salmonella OMA; 3, MPs.

Furthermore, IMS is an effective method for purifying sample systems from food matrices without easily being clogged by large particles (Ersahin *et al.*, 2012) or limited compatibility with large sample volumes (Garrido-Maestu *et al.*, 2018). Based on IMS technology, Merck's real-time PCR Assurance Genetic Detection System captures only intact bacteria in the pre-enrichment fluid. As a result, unlike other real-time PCR procedures (total DNA extraction from pre-enrichment fluid), the Assurance Genetic Detection System procedure has a false-positive

rate of almost 0% in comparison with traditional methods (Feldsine *et al.*, 2010). Our laboratory-made 'Mix-and-Match' ProAx1-Fe_3O_4 binding-coated MPs exhibited a broad forthcoming in the field of foodborne bacteria detection. To our best knowledge, this was the first of its kind of 'Mix-and-Match' MPs being prepared. In further experiments, the MPs can be tested for their ability to capture and detect any poisoning microorganisms in food samples.

4. Conclusion

The PCR method is an efficient way to detect pathogens in food by corporating with IMS technology as a pretreatment step in the whole process. In this study, we demonstrated a proof-of-concept for 'Mix-and-Match' MPs by designing, cloning, and expressing the recombinant protein ProAx1-Fe_3O_4 binding, and performed a specific antibody capturing experiment of ProAx1-Fe_3O_4 binding-coated magnetic beads. Here, we represented a bi-functional protein (ProAx1-Fe_3O_4 binding) that could bind to MPs with high affinity and capture antibodies to generate protein-coated MPs for specific detection of microorganisms in the food matrix. These beads underwent a couple of stability tests, antibody binding capability, and microorganism isolation. Our ProAx1-Fe_3O_4 binding-coated MPs may find potential applications for bacterial detection and disease diagnosis with high efficiency and accuracy.

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Declaration of Competing Interest

The authors declare that they have no conflict of interests.

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