Analytica Chimica Acta 859 (2015) 66–71

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Development of an efficient signal amplification strategy for label-free enzyme immunoassay using two site-specific biotinylated recombinant proteins

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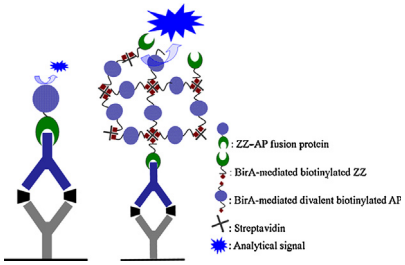
HIGHLIGHTS

- An efficient signal amplification strategy for label-free EIA is proposed.
- Divalent biotinylated AP and monovalent biotinylated ZZ were prepared via Avitag–BirA system.
- The above site-specific biotinylated fusion proteins form complex via SA–biotin interaction.
- The mechanism relies on the ZZ–Avi-B/SA/AP–(Avi-B)$_2$ complex.
- The analytical signals are enhanced (32-fold) by the proposed strategy.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 27 September 2014
Received in revised form 4 December 2014
Accepted 9 December 2014
Available online 12 December 2014

Keywords:
Alkaline phosphatase
Label-free
Enzyme immunoassay
Site-specific enzymatic biotinylation
Signal amplification
ZZ domain

ABSTRACT

Constructing a recombinant protein between a reporter enzyme and a detector protein to produce a homogeneous immunological reagent is advantageous over random chemical conjugation. However, the approach hardly recombines multiple enzymes in a difunctional fusion protein, which results in insufficient amplification of the enzymatic signal, thereby limiting its application in further enhancement of analytical signal. In this study, two site-specific biotinylated recombinant proteins, namely, divalent biotinylated alkaline phosphatase (AP) and monovalent biotinylated ZZ domain, were produced by employing the Avitag–BirA system. Through the high streptavidin (SA)–biotin interaction, the divalent biotinylated APs were clustered in the SA–biotin complex and then incorporated with the biotinylated ZZ. This incorporation results in the formation of a functional macromolecule that involves numerous APs, thereby enhancing the enzymatic signal, and in the production of several ZZ molecules for the interaction with immunoglobulin G (IgG) antibody. The advantage of this signal amplification strategy is demonstrated through ELISA, in which the analytical signal was substantially enhanced, with a 32-fold increase in the detection sensitivity compared with the ZZ–AP fusion protein approach. The proposed immunoassay without chemical modification can be an alternative strategy to enhance the analytical signals in various applications involving immunosensors and diagnostic chips, given that the label-free IgG antibody is suitable for the ZZ protein.

Abbreviations: AP, alkaline phosphatase; EIA, enzyme immunoassay; IgG, immunoglobulin G; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; SA, streptavidin; SABC, streptavidin–biotin complex.

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http://dx.doi.org/10.1016/j.aca.2014.12.020
0003-2670/

1. Introduction

Enzyme immunoassays (EIAs) are widely used for the qualitative and quantitative analyses of trace substances, in which conjugations between reporter enzymes and antibodies are required [1,2]. The traditional coupling technique is performed through chemical modification at the functional side groups (*e.g.*, amine, carboxyl, and thiol) of proteins for the conjugations [3–7]. However, due to lack of the definite-site specificity, the random chemical conjugation method produces heterogeneous modifications in both regiochemistry and stoichiometry. Hence, those heterogeneous conjugations often negatively affect the EIA outcome by decreasing specificity, sensitivity, and stability [8–10]. To overcome the disadvantages of chemical modification, the genetic fusion approach provides an alternative strategy for immunoreagents with a defined structure and uniform-specific conjugation; moreover, the parental intrinsic activities are mostly well preserved in their fusion proteins [11–13]. Some studies have reported the production of recombinant proteins between reporter enzymes (*e.g.*, luciferase, alkaline phosphatase (AP), and glucose oxidase) and genetic engineering antibodies (*e.g.*, single-chain Fv antibody fragment and ScFv), and the feasibility and advantages of the applications of these recombinant proteins in the EIA are also discussed [9,11,12,14,15]. In addition, other antibody-binding proteins, such as the well-known protein A (staphylococcal protein A, SpA) with the ZZ domain (engineered IgG-binding domain from the B-domain of protein A [16,17]), are also recombined with reporter enzymes to detect immunological analytes [10,18–20]. However, despite significant advancements, these difunctional fusion proteins between reporter enzymes and detector molecules without multiple reporters cause insufficient amplification of the enzymatic signal, thereby limiting their applications for further enhancement of analytical signals.

A 15-amino acid sequence, called Avitag, can be biotinylated by *Escherichia coli* biotin ligase (BirA), which covalently attaches a biotin molecule to the lysine residue of the Avitag, *in vivo* or *in vitro* [21,22]. The site-specific biotinylation of Avitag is advantageous over random chemical conjugation for the preparation of biotinylated proteins. To date, BirA-mediated biotinylation of Avitag fusion protein has been used in protein purification, immobilization, and detection [23–26]. Here, a novel and nonchemical modification strategy suitable for the enhancement of the enzymatic signal of the EIA is proposed. In this method, two recombinant proteins (double-Avitag-fused AP and single-Avitag-fused ZZ domain) are prepared in the *E. coli* system, and the site-specific biotinylation of both recombinant proteins are fulfilled with the BirA ligase *in vitro*. Based on the principle of streptavidin–biotin complex (SABC) technique [27,28], the divalent biotinylated APs are firstly clustered in SA–biotin AP complex, and then the biotinylated ZZs are assembled to the surface of the SA–biotin complex, resulting in a functional macromolecule involves numerous APs for the enhancement of enzymatic signal and some ZZ molecules for interaction with the immunoglobulin G (IgG) antibody. A schematic illustration of the proposed approach is shown in Fig. 1. Based on this idea, we also designed an example, and the feasibility and advantages of this novel analytical signal amplification method were comparatively characterized in an ELISA with our previously reported ZZ–AP fusion protein [20].

2. Experimental

2.1. Materials

Restriction enzymes and DNA ligase needed for gene fusion were purchased from Takara (China). BirA ligase was from Genecopoeia (USA). D-biotin, streptavidin (SA, Mr = 60 kD), and chicken IgY were purchased from BBI (Canada), streptavidin conjugated horseradish peroxidase (SA-HRP) was from Boster (China). 2-(4-Hydroxyphenylazo) benzoic acid (HABA) was purchased from Aldrich (USA). Rabbit anti-IgY and goat anti-IgY were purchased from Jackson (USA). HiTrap Chelating HP column was product of Amersham Biosciences (USA). Polystyrene ELISA plate and SA pre-coated 96-well microplate was from Nunc (Denmark). Micro BCA (bicinchoninic acid) protein assay kit was purchased from Sangon Biotech (China). Tetramethylbenzidine (TMB) substrate kit (for immunostaining and for ELISA) was from Beyotime (China), and *p*-nitrophenyl phosphate (pNPP) was from Sigma (USA). All reagents used were of analytical grade unless otherwise stated.

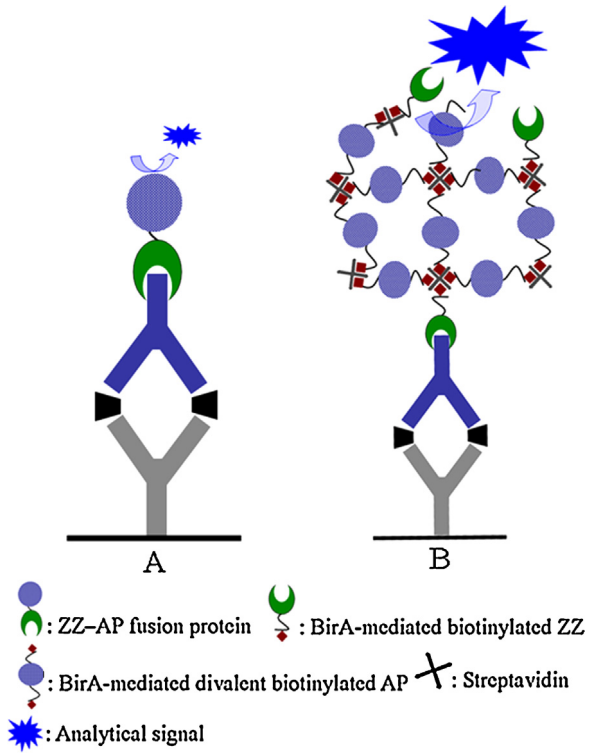

Fig. 1. Schematic illustrations of ZZ–AP fusion protein (A), BirA-mediated, site-specific biotinylated AP–$(Avitag)_2$ and ZZ–Avitag resulted in SA–biotin complex (B) for the amplification of analytical signals.

2.2. Instrumentation

Protein was purified on an ÄKTA FPLC system (Amersham, Sweden) equipped with a HiTrap Chelating HP column (1 mL, 5 mL; GE Healthcare, USA). A Thermo Multiskan MK3 Micro-plate reader (Thermo Scientific, USA) was used for microplate colorimetric analysis. A Shimadzu UV-3100 spectrophotometer (Shimadzu, Japan) was used for cuvette colorimetric analysis.

2.3. Double-Avitag-fused AP

To generate the divalent biotinylated AP, a two-step gene manipulation was performed to construct the vector for the AP–$(Avitag)_2$ recombinants. First, the DNA fragment that contains the twofold coding sequence of the Avitag and $(G_4S)_3$ linker was designed: Avitag–$(G_4S)_3$-MCS-$(G_4S)_3$–Avitag (multiple cloning site, MCS). The double-stranded DNA fragment was synthesized using a chemical method (consigned to Sangon Biotechnology Co., China) and then inserted into the *Eco*RI/*Hin*dIII sites of the pGreen–S