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Tamavidin, a versatile affinity tag for protein purification and immobilization☆

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ABSTRACT

Tamavidin 2 is a fungal avidin-like protein that binds biotin with high affinity and is highly produced in soluble form in *Escherichia coli*. By contrast, widely used biotin-binding proteins avidin and streptavidin are rarely produced in soluble form in *E. coli*. In this study, we describe an efficient system for one-step purification and immobilization of recombinant proteins using tamavidin 2 as an affinity tag. A bacterial sialyltransferase and soybean agglutinin were fused to tamavidin 2 and expressed in *E. coli* and tobacco BY-2 cells, respectively. High-level expressions of the fusion proteins were detected (80 mg l^{-1} *E. coli* culture for bacterial sialyltransferase–tamavidin 2 and 2 mg l^{-1} BY-2 cell culture for soybean agglutinin–tamavidin 2). To immobilize and purify the fusion proteins, biotinylated magnetic microbeads were incubated with the soluble extract from each recombinant host producing the fusion protein and then washed thoroughly. As the result, both fusion proteins were immobilized tightly on the microbeads without substantial loss of activity and simultaneously highly purified (90–95% purity) on the microbeads. Biotin with a longer linker contributed to higher affinity between the fusion protein and biotin. These results suggest that tamavidin fusion technology is a powerful tool for production, purification, and immobilization of recombinant proteins.

1. Introduction

Affinity tags are efficient tools for protein purification (Waugh, 2005; Arnau et al., 2006) and, in some cases, for protein immobilization. For example, the hexahistidine tag (Smith et al., 1988), the most commonly used affinity tag, is genetically fused to the N- or C-terminus of a protein of interest, and the fusion protein is produced in an appropriate expression system and then purified by immobilized metal ion affinity chromatography. However, the interaction between the hexahistidine tag and nickel ions is not so high, and therefore this tag is not suitable for single-step purification of a protein with high purity from crude protein extracts of complex organisms like animals or plants; in such systems, background binding of nonspecific protein sources is high, and therefore rigorous washing is required. There are many other tags for biotechnological applications, including FLAG, T7-tag, glutathione *S*-transferase, and maltose-binding protein (Waugh, 2005; Arnau et al., 2006). These tags vary in the specificity and affinity of their corresponding affinity matrix. However, like the hexahistidine tag, they are generally not suitable for single-step purification with high purity and are even less suitable for immobilization because of the relatively low binding affinity. Therefore, affinity tags that bind strongly to the affinity matrix, and thereby are suitable for single-step purification and immobilization, are highly sought after. Such tags are effective especially in the case of preparing an immobilized enzyme.

Avidin, a tetrameric glycoprotein from egg white, binds biotin (vitamin H) with remarkably high affinity; the interaction between avidin and biotin is the strongest known noncovalent bond between two biomolecules (Green, 1990). Streptavidin, an avidin-like protein from *Streptomyces avidinii*, also binds biotin strongly (Green, 1990). Consequently, (strept)avidin/biotin systems have been exploited for various biotechnology applications (Airenne et al., 1999b). Avidin and streptavidin have been suggested as potential affinity tags for protein purification (Airenne et al., 1999a; Sano and Cantor, 2000) and for protein immobilization (Walsh and Swaisgood, 1994; Clare et al., 2001). Although the *Escherichia coli* expression system is inexpensive and easy to work with, many (strept)avidin fusion proteins have been expressed in insect cells (Karp et al., 1996; Airenne et al., 1999a,b) or mammalian cells (Shin et al., 1997; Ng et al., 2002; Boado et al., 2008). One of the reason is that in *E. coli* (strept)avidin and their fusion proteins are expressed as insoluble inclusion bodies in most cases (Sano and Cantor, 1991; Sano et al., 1992; Kipriyanov et al., 1996; Li et al., 1999), and renaturation and tedious downstream processing are required for the

☆ *Note*: Nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB531014 for *ST-S-TM2*, AB531015 for *ST-M-TM2*, AB531016 for *ST-L-TM2*, and AB531017 for *SBA-S-TM2*.

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preparation of active proteins. Even when proteins are expressed in soluble form, the expression levels are very low (Airenne and Kulomaa, 1995; Clare et al., 2001). These difficulties have restricted the practical use of (strept)avidin fusion proteins. Therefore, biotin-binding proteins that can be efficiently produced in *E. coli* are highly desired.

Recently, we reported that tamavidins, fungal avidin-like biotin-binding proteins, are produced at high levels in soluble form in *E. coli* (Takakura et al., 2009). In particular, tamavidin 2, like avidin and streptavidin, binds biotin strongly, and it shows high thermal stability and low nonspecific binding. Here, we report a strategy for efficient one-step purification and immobilization of recombinant proteins using tamavidin 2 as an affinity tag. A bacterial sialyltransferase and soybean agglutinin were used as fusion partners for expression in *E. coli* and tobacco BY-2 cells, respectively. The fusion proteins were simultaneously purified and immobilized on biotin-bearing magnetic microbeads. The proteins were found to be tightly immobilized and highly pure. The parameters affecting biotin-binding of the fusion proteins were also discussed.

2. Materials and methods

2.1. Construction of sialyltransferase–tamavidin 2 fusion gene

For amplification of the sialyltransferase (ST) part of the fusion gene, a set of primers ST-N1-PciI and ST-link (Table 1) were used for PCR. As a template, the N1C0 clone (without a signal peptide) of β-galactoside α2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224 (Tsukamoto et al., 2008) was used. For amplification of the tamavidin 2 (TM2) part with three kinds of linker (S, Gly-Gly-Gly-Gly-Ser-Gly; M, (Gly-Gly-Gly-Gly-Ser) × 3; L, (Gly-Gly-Gly-Gly-Ser) × 5), we used three sets of primers: ST-linkTM2 (for *ST-S-TM2*)/TM2-3′BamHI, ST-3 × linkTM2 (for *ST-M-TM2*)/TM2-3′BamHI, and ST-5 × linkTM2 (for *ST-L-TM2*)/TM2-3′BamHI (Table 1). A cDNA for tamavidin 2 (Takakura et al., 2009) was used as a template. The PCR products of the sialyltransferase part and the tamavidin 2 part with the linkers (there is an overlapping DNA region in the linker part that allows joining the two PCR products) were purified by using QIAEX II (QIAGEN Inc.), and used as templates for the second PCR with ST-N1-PciI and TM2-3′BamHI as primers (Table 1). Each PCR product (*ST-S-TM2*, *ST-M-TM2*, or *ST-M-TM2*) was digested with *Pci*I and *Bam*HI and recloned between the *Nco*I and *Bam*HI sites of expression vector pTrc99A (Amersham Biosciences, Piscataway, NJ) to give *ST-S-TM2*/pTrc99A, *ST-M-TM2*/pTrc99A, or *ST-L-TM2*/pTrc99A. After verification of the sequence, the expression cassettes were introduced into *E. coli* strain BL21. Nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB531014 for *ST-S-TM2*, AB531015 for *ST-M-TM2*, and AB531016 for *ST-L-TM2*.

2.2. Construction of soybean agglutinin–tamavidin 2 fusion gene

For amplification of the soybean agglutinin (SBA) part with a linker, a set of primers, SBA-5′XbaI and SBA-linkTM2-R (Table 1), were used for PCR. The open reading frame (with a region for a signal peptide) of the gene for SBA (Vodkin et al., 1983) amplified from soybean genomic DNA was used as the template. For amplification of the tamavidin 2 part with a linker, a set of primers SBA-linkTM2-F/TM2-3′SacI (Table 1) were used. The amplified PCR products were gel purified (there is an overlapping DNA region in the linker part that allows joining the two PCR products) and used as templates for the second PCR, with SBA-5′XbaI and TM2-3′SacI as primers. The PCR product (*SBA-S-TM2*) was digested with *Xba*I and *Sac*I and recloned between the *Xba*I and *Sac*I sites of a binary vector pSB24 for plant transformation (Komari et al., 1996) to yield 35S-*SBA-S-TM2*/pSB24 (cauliflower mosaic virus 35S RNA gene promoter-driven soybean agglutinin–tamavidin 2 fusion gene). After verification of the sequence, the resultant plasmids were transferred to pSB4U and introduced into tobacco suspension—cultured BY-2 cells as described (Hiei et al., 1994). Nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession number AB531017 for *SBA-S-TM2*.

2.3. Expression of tamavidin 2 fusion proteins

The sialyltransferase–tamavidin 2 fusion proteins were expressed in *E. coli* according to the method of Takakura et al. (2009). The culture was performed in Luria–Bertani broth containing ampicillin and 1 mM (final concentration) isopropyl β-D-1-thiogalactopyranoside at 30 °C for 5 h. The bacterial pellet was collected by centrifugation. Tobacco BY-2 cells harboring 35S-*SBA-S-TM2*/pSB24 were cultured in liquid medium containing MS salts supplemented with 1 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 0.2 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 30 g l^{-1} sucrose at 25 °C for 7 days in the dark. The BY-2 cells were collected by centrifugation. The total soluble proteins were extracted in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–KOH (pH 7.4) from each host cells (*E. coli* or BY-2) by sonication, and cell debris and insoluble proteins were removed by centrifugation. Then, the supernatant was used for further analyses.

2.4. SDS-PAGE and immunoblot analyses

The protein concentration was determined by a Bradford dye reagent (Bradford, 1976) with bovine serum albumin (BSA) as a standard. The total soluble protein samples from *E. coli* and tobacco cells were separated by SDS-PAGE (Laemmli, 1970) on 12.5% gel and stained with Coomassie Brilliant Blue (CBB) (Sigma, St. Louis, MO). For immunoblot analysis, after the SDS-PAGE, the protein samples were blotted onto a polyvinylidine fluoride (PVDF) membrane. The membrane was incubated in Tris-buffered saline (TBS) containing 0.5% of skim milk with tamavidin 2 antibody (Takakura et al., 2009) (1:1000 dilution) over night. Goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad Laboratories, Inc., Hercules, CA) (1:1000 dilution) was used as the second antibody. Alkaline phosphatase substrate kit II (Vector Laboratories, Inc., Burlingame, CA) was used to visualize immunoreactive molecules. The expressed fusion proteins were quantified by a densitometer with the recombinant tamavidin 2 expressed in *E. coli* (Takakura et al., 2009) as a calibration standard. The molecular mass of the proteins separated by SDS-PAGE was estimated based on the mobility of the low-molecular-weight electrophoresis calibration sample from Amersham Biosciences.

2.5. Estimation of subunit association of the fusion proteins

The protein samples were dissolved in 1 × SDS gel-loading buffer (Sambrook et al., 1989) without reducing agent at room temperature (no heating) for 10 min, separated by SDS-PAGE on 10% gel, and stained with CBB. Alternatively, after the non-heating SDS-PAGE, the proteins were blotted onto a PVDF membrane. The membrane was blocked with TBS containing 3% of BSA and 0.2% of Tween 20 overnight. Then, the membrane was incubated in TBS containing 3% of BSA with biotinylated horseradish peroxidase (HRP) (Vector Laboratories, Inc.) (1:1000 dilution) at room temperature for 1 h. ECL™ Detection Reagents (Amersham Biosciences) was used to visualize the biotin-binding fusion proteins. The molecular mass of the proteins separated by the non-heating SDS-PAGE was estimated based