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Twigged streptavidin polymer as a scaffold for protein assembly

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ABSTRACT

Protein assemblies are an emerging tool that is finding many biological and bioengineering applications. We here propose a method for the site-specific assembly of proteins on a twigged streptavidin (SA) polymer using streptavidin as a functional scaffold. SA was genetically appended with a G tag (sortase A recognition sequence) and a Y tag (HRP recognition sequence) on its N- and C-termini, respectively, to provide G-SA-Y. G-SA-Y was polymerized using HPR-mediated tyrosine coupling, then fluorescent proteins were immobilized on the polymer by biotin-SA affinity and sortase A-mediated ligation. Fluorescence measurements showed that the proteins were immobilized in close proximity to each other. Hydrolyzing enzymes were also functionally assembled on the G-SA-Y polymer. The site-specific assembly of proteins on twigged SA polymer may find new applications in various biological and bioengineering fields.

1. Introduction

Protein assemblies are an emerging tool in biology and bioengineering. The cellulosome is a natural protein assembly and comprises cellulases and hemicellulases assembled on microbial cell surfaces by cohesin-dockerin interactions (Carvalho et al., 2003). Cellulosomal enzymes act synergistically on substrates and efficiently deconstruct cellulosic or hemicellulosic materials into monosaccharides which can be used as a feedstock for microbial biorefineries. Artificial cellulosomes that exhibit enhanced hydrolysis of cellulose or hemicellulose have thus attracted attention (Mori et al., 2013; Sun et al., 2014). Artificial protein assemblies find utility in many additional applications, including as protein fibrils (Matsunaga et al., 2013), protein hydrogels (Ramirez et al., 2013), and as immobilized proteins (Steen Redeker et al., 2013). Moreover, thus artificial protein assemblies are potential scaffold to simply enhance the activity or ability of proteins.

Protein conjugation is one method for assembling proteins and allows the construction of new biomolecules whose characteristics reflect the functions of the constituent proteins. Proteins can be conjugated by chemical crosslinking, biomolecular interactions, and split-intein triggered protein splicing. Site-specific protein conjugation is desirable in order to prevent loss of function of the component proteins, but the most commonly used conjugation method, chemical crosslinking, generally results in random conjugation due to residue-specific reactivity. In contrast, enzymatic conjugation minimizes the loss of protein function and is thus one of the most promising approaches to site-specifically conjugate proteins. Many enzymes, and especially acyl-transfer enzymes, have been used to catalyze site-specific protein conjugation (Matsumoto et al., 2012a,b). For example, microbial transglutaminase (MTGase) catalyzes the acyl-transfer reaction to form a covalent bond between the γ-carboxylamide group of Glu residues and the ε-amino group of Lys residues. Since this unique reaction is catalyzed particularly efficiently between small peptide motifs (Tominaga et al., 2005), MTGase has been used to generate both various bioconjugates (Strop, 2014) and protein assemblies (Yang et al., 2009; Mori et al., 2013; Mariniello et al., 2014). Horseradish peroxidase (HRP) catalyzes the oxidation of a variety of phenolic moieties, including tyrosine, making HRP useful for conjugating proteins and generating assemblies (Minamihata et al., 2011, 2012). Another enzyme, staphylococcal transpeptidase sortase A (SrtA), recognizes the Leu-Pro-X-Thr-Gly sequence (LP tag), cleaves between the Thr and Gly residues, and subsequently links the carboxyl group of Thr to the amino group of N-terminal glycine oligomers (G tag) through a peptide bond. SrtA catalyzed-transpeptidation has been used for protein conjugation, including protein-protein, protein-lipid, and protein-bead ligation, and for

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assembling proteins (Tanaka et al., 2008; Wu et al., 2013; Cambria et al., 2015; Hata et al., 2015).

Streptavidin (SA) is a tetrameric protein with four biotin-binding pockets and is widely used in biotechnology to mediate conjugation. Using genetic techniques, SA has been appended to flexible peptide tags containing Tyr residues to generate Y tags. Y tags are a potential substrate for HRP-mediated tyrosine coupling and have been used for protein conjugation (Minamihata et al., 2011,2012). Assembled SA (hereinafter 'SA polymer') is a multiple SA conjugate network prepared by HRP-mediated tyrosine coupling and thus is a potential scaffold for the assembly of proteins driven by biotin-SA affinity. Site-specific SA conjugation using srt A-mediated modification has also been demonstrated (Matsumoto et al., 2012a,b). We previously showed that the LP tag can be genetically introduced onto the C-terminus of SA (SA-LP); the resulting complex was immobilized on biotin-coated particles to provide SA-LP immobilized particles capable of co-immobilizing proteins via biotin-SA affinity and SrtA-mediated ligation. Two enzymes were subsequently co-immobilized on the surface of the particles in a site-specific manner. Unlike general chemical modification, co-immobilization using the SrtA-based method does not disrupt the conformation of the enzymes and thus results in higher enzymatic activity.

Using a combination of the above approaches, we here demonstrate protein assembly on a "twigged" SA polymer functional scaffold. The SA monomer was constructed by genetically introducing a G tag (Srt A recognition sequence) and Y tag (HRP recognition sequence) at the N- and C-termini, respectively. The product, G-SA-Y, was polymerized using HRP-mediated tyrosine coupling. The resulting G-SA-Y polymer contains two types of conjugating sites: a G tag twig and biotin pockets. This potential scaffold can conjugate proteins through biotin-SA affinity and SrtA-mediated ligation with the LP tag-appended protein, and thus can be used to assemble proteins. We anticipated that these assembled proteins would be immobilized in close proximity to each other. Consequently, we explored the utility of the G-SA-Y polymer as a scaffold for protein assembly, as well as the proximity effect of assembled fluorescent proteins and assembled hydrolyzing enzymes.

2. Materials and methods

2.1. Materials

Avicel® PH-101 was purchased from Sigma-Aldrich (St. Louis, MO, USA). KOD-plus- DNA polymerase was purchased from TOYOBO (Osaka, Japan). pET22-b(+) and pCold IV vector, TALON metal affinity resin, a BCA protein assay kit, and *Escherichia coli* BL21(DE3) strain were purchased from Takara Bio Inc. (Shiga, Japan). All other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Gene expression and protein purification

Proteins (G-SA-Y; YFP-LP, yellow fluorescent protein with the LPETG sequence appended at the C-terminus; CFP-BAP, cyan fluorescent protein with a biotin acceptor peptide (GLNDIFEAQKIEWHE) appended at the C-terminus; EG-LP, endoglucanase Cel5A from *Thermobifida fusca* YX with the LPETG sequence appended at the C-terminus; CBH-BAP, exoglucanase Cel48A from *T. fusca* YX with a BAP tag appended at the C-terminus) were expressed and purified as follows. KOD plus DNA polymerase or KOD FX DNA polymerase were used for polymerase chain reaction (PCR). The gene encoding G-SA-Y was obtained using pColdI-Stav-LPETG as a template (Matsumoto et al., 2011) with 5′-aggtaataccatatgggcggcggcggctctatgaatcacaaagtgcatcatcatcatcatcatgccgaggccggcatcaccggc-3′ as the 5′ primer and 5′-atttacctatctagactaatagccgccgccgccggaggcggcggacggcttcac-3′ as the 3′ primer. The amplified fragment was subcloned into the *NdeI*/*XbaI* sites of the pColdIV vector to yield pColdIV_G-SA-Y, which was introduced into *E. coli* BL21(DE3). In a similar manner, the fluorescent genes encoding CFP-LPETG or YFP-BAP were obtained using the pEYFP-C1 or pECFP-C1 vectors (Takara Bio Inc., Shiga, Japan) as templates, respectively, using the following primers: 5′-ggagatatacatatggtgagcaagggcgaggagctgttcaccggg-3′ (fFP1) as the 5′ primer and 5′-gtgctcgagtgcggccgcgccaccagtttccggcagcttgtacagctcgtccatgccgagagtgat-3′ as the 3′ primer for CFP-LPETG, fFP1 as the 5′ primer and 5′-gattttctgagcctcgaagatgtcgttcagaccgccacccttgtacagctcgtccatgccgag-3′ as the 3′ primer for the first PCR of YFP-BAP, and fFP1 as the 5′ primer and 5′-gtgctcgagtgcggccgcttcgtgccattcgattttctgagcctcgaa-3′ (rBP) as the 3′ primer for the second PCR of YFP-BAP. The amplified fragments were subcloned into the *NdeI*/*NotI* sites of the pET22-b(+) vector to yield pET22_CFP-LPETG and pET22_YFP-BAP, which were introduced into *E. coli* BL21(DE3). The cellulase genes encoding EG-LP and CBH-BAP were obtained from the *T. fusca* YX genome as templates using the following primers: 5′-ggagatatacatatgctcaccgccacagtcaccaaagaatcctcg-3′ as the 5′ primer and 5′-ctcgagtgcggccgcgccaccagtttccggcagtgagccccgccaccggactggagcttgct-3′ as the 3′ primer for EG-LPETG, 5′-ggagatatacatatgtgctcggtggactacgacgactccaacgac-3′ as the 5′ primer and 5′-gattttctgagcctcgaagatgtcgttcagaccgccaccgggagctccggccccgaacagttcgtcgtg-3′ as the 3′ primer for the first PCR of CBH-BAP, and 5′-ggagatatacatatgtgctcggtggactacgacgactccaacgac-3′ as the 5′ primer and rBP as the 3′ primer for the second PCR of CBH-BAP. The amplified fragments were subcloned into the *NdeI*/*NotI* sites of the pET22-b(+) vector to yield pET22_EG-LPETG and pET22_CBH-BAP, which were introduced into *E. coli* BL21(DE3). Cells were grown in Luria-Bertani (LB) medium at 37 °C to an optical density (OD; 600 nm) of 0.5–0.7, then the cells were incubated for an additional 30 min at 25 °C (10 °C if pCold IV vector was used). Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM). After incubation for an additional 24 h at 25 °C (10 °C if pCold IV vector was used), the cells were harvested by centrifugation. The cell pellets were resuspended in 20 mM phosphate buffer (pH 8.0) containing 150 mM NaCl, then lysed using sonication. Each protein was purified from the soluble fraction using TALON metal affinity resin according to the manufacturer's protocol and then dialyzed against 50 mM phosphate buffer (pH 8.0) containing 150 mM NaCl. The concentration of each purified protein was determined using a BCA protein assay kit.

2.3. Streptavidin polymerization and protein assembly

The fluorescent proteins (or cellulases) were assembled on the G-SA-Y monomer by biotin-SA affinity; the transpeptidation reaction was performed in buffer (20 mM Tris–HCl, 150 mM NaCl, 0.5 mM $CaCl_2$) containing 5 μM SrtA, 12 μM CFP-LP (or EG-LP), and 12 μM YFP-BAP (or CBH-BAP) for 4 h at 37 °C (pH 7.5). G-SA-Y polymer was prepared in 10 mM phosphate buffer containing 150 mM NaCl by mixing G-SA-Y (20 μM), H_2O_2 (40 μM), and HRP (1 μM) at 37° C for 1 h. Therefore, the resulting G-SA-Y polymer was utilized as a scaffold for protein assembly.

2.4. Fluorescence measurements of the assembled fluorescent proteins

Fluorescence from the fluorescent proteins assembled on the G-SA-Y polymer was measured using an F-2700 spectrofluorophotometer (Hitachi, High-Tech Science Corporation, Tokyo, Japan). A continuous spectrum from 460 to 580 nm was monitored at an excitation wavelength of 440 nm. Fluorescence resonance energy transfer (FRET) was analyzed using a microplate reader (Wallac