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Immobilization of proteins onto microbeads using a DNA binding tag for enzymatic assays

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A novel DNA-binding protein tag, scCro-tag, which is a single-chain derivative of the bacteriophage lambda Cro repressor, has been developed to immobilize proteins of interest (POI) on a solid support through binding O_R consensus DNA (ORC) that is tightly bound by the scCro protein. The scCro-tag successfully bound a transglutaminase 2 (TGase 2) substrate and manganese peroxidase (MnP) to microbeads via scaffolding DNA. The resulting protein-coated microbeads can be utilized for functional analysis of the enzymatic activity using flow cytometry. The quantity of bead-bound proteins can be enhanced by increasing the number of ORCs. In addition, proteins with the scCro-tag that were synthesized using a cell-free protein synthesis system were also immobilized onto the beads, thus indicating that this bead-based system would be applicable to high-throughput analysis of various enzymatic activities.

[**Key words:** scCro; OR consensus DNA; Transglutaminase 2; Manganese peroxidase; Flow cytometry; Cell-free protein synthesis]

Compared to a free enzyme reaction system, immobilized enzyme bioprocessing provides several advantages, including easier reuse of the biocatalyst, reactor operation, product separation, as well as a wider choice of reactor systems (1). Immobilized enzyme bioprocessing has been applied to industrial production of multiple products such as sugars, amino acids, and pharmaceuticals (1). In addition, the immobilized enzymes are also used for diagnostics and biosensors (1).

Bioaffinity binding is a well-known technique used for immobilization of enzymes. This strategy exploits the selectivity of bioaffinity interactions and is highly specific for both the identity of binding partners and the location on the molecules at which binding occurs (2).

A polyhistidine-tag, which comprises of at least 6 consecutive histidine resides, binds to metal–chelators such as Ni-nitrilotriacetic acid and is widely used in protein purification. In addition, enzymes can be tethered to a surface such as a microtiter plate using this tag while retaining their biological activities (3,4). However, the binding affinity between the tag and the metal-chelator is relatively weak (K_d approximately around 1–10 μM) (2); therefore, it is not well suited for repetitive use with unchanged immobilized enzymes. Proteins can also be immobilized onto a solid support using protein–protein interactions such as an antibody–antigen interaction. Ramachandran et al. (5,6) have developed a protein microarray system that uses a GST tag and an anti-GST antibody. However, this strategy requires expensive antibodies and laborious steps to attach the antibodies to a solid support.

Streptavidin (SA) is a homotetramer with molecular weight of approximately 56,000 from the bacterium *Streptomyces avidinii* that binds up to four biotin molecules with K_d = around 10^{-14} M (7). It is a widely used tool to immobilize proteins of interest (POI) onto various solid supports, including microtiter plates, microarrays, and microbeads (8,9). In this strategy, the biotinylation of POI requires either chemical steps or concurrent expression with birA in *Escherichia coli* (10). Due to the large size of streptavidin, there is potential for steric crowding of the immobilized POI, leading to restricted physical arrangements of POI.

DNA binding motifs have attracted much attention as potential tags for site-specific binding onto various solid supports (11–13) because DNA is more stable than proteins, and its chemical synthesis is relatively straightforward. Here, we have generated a DNA-binding tag developed from a single-chain derivative of the bacteriophage lambda Cro repressor (14). It has relatively low molecular weight (15,000), and it binds O_R consensus DNA (ORC) as a 1:1 complex with high affinity (K_d of approximately 1.4×10^{-12} M) (14,15). Five amino acid residues (Q27, S28, A29, N31 and K32) placed in the N-terminal domain are important for this specific binding (16). In addition, scCro was adaptable to mutagenesis and many variants possessing different DNA-binding properties were isolated by phage display (15–17). Particularly, a scCro variant C12:404 binds ORas11, which is a ORC mutant, with K_d of 5.7×10^{-9} M (15,16).

In order to develop a novel immobilizing system using the scCro, we attempted to apply for efficient evaluation system for the activities of two enzymes: transglutaminase (TGase: EC. 2.3.2.13) and manganese peroxidase (MnP: EC. 1.11.1.13). TGase catalyzes isopeptide formation between the glutamine-residue and the lysine-

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residues in proteins (18). Since primary amine is also available instead of the lysine-residue, measurement of the incorporation of the labeled amine is typically used for evaluation of the activity (19). MnP catalyzes the oxidation of Mn^{2+} to Mn^{3+} by utilizing hydrogen peroxide (20,21). The produced Mn^{3+} and organic acid can form a chelate complex, which acts as a diffusible charge-transfer mediator, allowing for the oxidation of various phenolic substrates (22). We immobilized a tag-fused peptide sequence (mouse TGase 2 substrate) onto microbeads using DNA with an ORC sequence for the flow cytometric assay of TGase (Fig. 1A). In addition, MnP was also immobilized onto the microbeads in a similar manner, followed by flow cytometric assay (Fig. 1B). To further improve the efficiency of immobilization, adjusting the amount of ORC sequences present regulated the quantity of molecules bound to the microbeads. Finally, the application for high-throughput screening based on this system is discussed.

MATERIALS AND METHODS

Plasmids All plasmids used in this study are listed in Table S1. All PCR products were obtained using PrimeSTAR HS DNA Polymerase (Takara, Otsu, Japan), Tks Gflex DNA Polymerase (Takara) or *Pyrobest* DNA polymerase (Takara). Detailed procedures for plasmid construction are described as follows.

scCro containing a peptide linker of 8 hydrophilic residues (scCro8) (14) was obtained from Integrated DNA Technologies (Coralville) and supplied as pIDTSMART-AMP-scCro1 containing scCro8 with N-terminal polyhistidine and hemagglutinin (HA) tags, and C-terminal FLAG tag. For the addition of peptide Q or K to the C-terminus of scCro, scCro-Fw-IFC with scCro-Rv Gln-IFC or scCro-Rv Lys-IFC (Table S2) were used as primers using pIDTSMART-AMP-scCro1 as the template. Linearized vector was prepared with PRSET-Fw-IFC and PRSET-Rv-IFC (Table S2) using pRSETb (Life Technologies) as the template. scCro8-peptide Q or scCro8-peptide K was cloned into the linearized pRSETb with an In-Fusion Cloning Kit (Clontech, Mountain View, CA, USA). The resulting plasmids contain scCro8 with N-terminal polyhistidine and HA tags and C-terminal FLAG tag and peptide Q or peptide K (pRSETb scCro-peptideQ or pRSETb scCro-peptideK). To remove the FLAG tag and peptide K region from pRSETb scCro-peptideK, phosphorylated oligonucleotides scCro-peptide K-IFC-Fw and scCro-peptide K-IFC-Rv (Table S2) were used as primers for inverse PCR, using pRSETb scCro-peptide K as the template. The linear DNA was treated with DNA Ligation mix (Mighty Mix, Takara) (pRSETb scCro).

scCro8 fragments were amplified with primers PRSET-IFC-scCro-F and PRSET-IFC-scCro-R (Table S2) using pRSETb scCro as the template (scCro fragment 1). scCro8-peptide Q fragments were amplified with pRSETb scCro-peptideQ as the template and the same primers (scCro-peptideQ fragment 1). Linearized vector was prepared with primers PET-IFC-F and PET-IFC-R (Table S2) using pET22b as the template. scCro fragment 1 or scCro-peptideQ fragment 1 were cloned into the linearized vector using an In-Fusion Cloning Kit (Clontech) (pET22-scCro or pET22-scCro-peptideQ).

The scCro8 fragment was amplified with primers scCro-fusion-Fw and MS-S-Rv (Table S2) with pRSETb scCro as the template (scCro fragment 2). Linearized vector was prepared with primers IV-Fw and MnP-in-Rv (Table S2) using pET23b-MnP-HA, which contains MnP isoenzyme 2 with C-terminal HA and polyhistidine tags (23), as the template. scCro fragment 2 was cloned into the linearized vector with an In-Fusion Cloning Kit. The resulting plasmid contains scCro8 with C-terminal FLAG and polyhistidine tags (pET23b-scCro-His).

The scCro fragment for *in vitro* expression of MnP-scCro8 was amplified with primers MS-S-Fw and MS-S-Rv (Table S2) using pRSETb scCro as the template (scCro fragment 3). MS-M-Fw and MS-M-Rv (Table S2) were used as primers using pET23b-MnP-HA (23) as the template to generate the MnP fragment. Linearized vector was prepared with primers IV-Fw and IV-Rv (Table S2) using pET23b-MnP-HA (23) as the template. The MnP fragment and scCro fragment 3 were cloned into the linearized vector simultaneously with an In-Fusion Cloning Kit. The resulting plasmid MnP with an N-terminal T7 tag and C-terminal HA, scCro, FLAG, and polyhistidine tags (pET23b-MnP-scCro-His).

Mouse TGase 2 cDNA was obtained through PCR using a liver cDNA library (24). An *Eco*RI and *Hin*dIII site was added to the 5′ and 3′ end of TGase 2 cDNA, respectively. The cDNA fragment was then inserted into the *Eco*RI and *Hin*dIII sites of the pET24 vector (Novagen, Madison, WI, USA), which was modified to contain a polyhistidine tag at the N-terminus (pET24d mouse TG2) (Fig. S1).

To construct a fragment containing a single ORC, ORC-Fw and ORC-Rv-2 (Table S2) were used as primers with pUC18 as the template. After *Dpn* I treatment, the fragment was phosphorylated using T4 Polynucleotide Kinase (Takara). The fragment was treated with DNA Ligation mix (Mighty Mix) (pUC18-ORC). To construct a fragment containing two ORCs, which are separated by 13 bp of intermediate sequence, 5′ phosphorylated PUC18-2ORC-13bp-Fw and 5′ phosphorylated PUC18-2ORC-13bp-Rv (Table S2) were used as primers using pUC18 as the template. The template was digested with *Dpn* I, separated using gel electrophoresis, recovered using a FastGene Gel/PCR Extraction Kit (Nippon Genetics Co., Ltd., Tokyo, Japan), and the fragment was treated with DNA Ligation mix (Mighty Mix) (pUC18-2ORC).

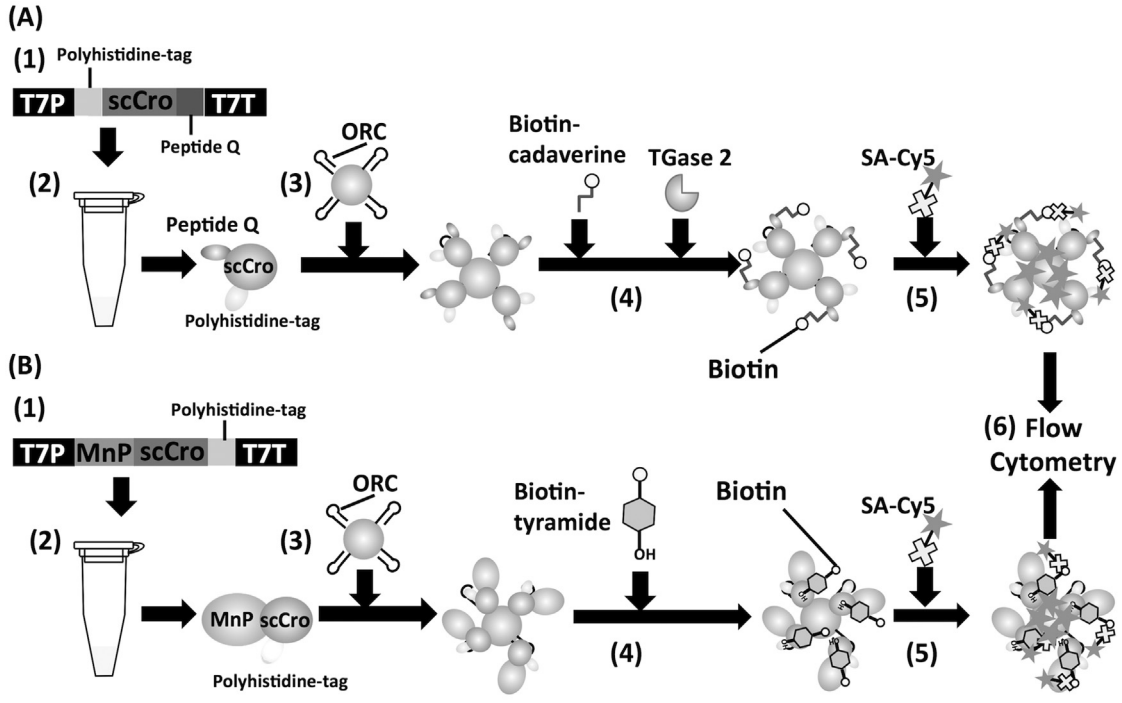

FIG. 1. Overview of the *in vitro* assay system on microbeads using ORC/scCro-tag for analysis of TGase 2 activity (A) or MnP activity (B). 1, Construction of a full-length template containing a T7 promoter (T7P), a T7 terminator (T7T), a ribosome binding site, a protein of interest (POI) region, and a single chain Cro (scCro) tag. 2, Synthesis of scCro-tagged proteins using a cell-free protein synthesis system. 3, Immobilization of the fusion proteins onto beads using O_R consensus DNA (ORC). 4, Enzymatic reaction on microbeads. The complexes are labeled with biotin by the enzymatic activity. 5, Labeling with Cy5 conjugated streptavidin (SA-Cy5). 6, Flow cytometry for activity quantification.