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Identification and purification of target protein using affinity resin bearing a photo-labile linker

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ABSTRACT

This Letter presents an effective method for the identification of target proteins of bioactive compounds such as drugs, natural products, and intrinsic ligands, using an affinity resin. The application of a photo-labile linker to an affinity resin enabled the selective elution of a target protein by irradiation for a short duration at 4 °C while leaving a large amount of non-specific binding protein on the resin. We have named this method the 'STEAP' method (selective target elution from affinity resins with photo-labile linker). Only a target protein that can bind the bioactive compound, the so-called 'active' protein, is eluted by the selective cleavage of the linker between the solid matrix and the target compound, and therefore, it is worth considering the potential of this method for the hyper-purification of proteins.

The identification of target proteins for bioactive compounds such as drugs, natural products and toxins is an essential component of the modern life sciences and biochemistry. Therefore, it is important to identify target proteins that specifically bind to such bioactive compounds and are vital for their biological responses. While affinity chromatography matrices bearing bioactive compounds are one of the major tools for achieving this goal,[1] it is often difficult to identify specific binding proteins because of a preponderance of non-specific binding proteins.[2] AffiGel™, an agarose derivative,[3] is now widely used for the preparation of affinity resins because of its high hydrophilicity, which reduces non-specific protein absorption; however, chemical modification of the resin to improve the affinity matrix has been limited by chemical instabilities that occur during synthetic conditions. However, a disadvantage of the use of synthetic solid materials is a large amount of non-specific protein absorption due to their hydrophobicity, even though they are stable enough for chemical modification of the resin.[2] We have reported a novel synthetic solid material for use as an affinity matrix, AquaFirmus™, which is composed of monomers that are hydrophilic enough to reduce non-specific absorption.[4] This solid material is chemically stable under ordinary synthetic conditions.[4] The chemical stability of this material enabled chemical modification to improve the affinity resin. Herein, we demonstrate that the combination of a photo-labile linker and the chemically stable solid material is widely effective for identification of target proteins; we have named this method the 'STEAP' method (selective target elution from affinity resins with photo-labile linker, Fig. 1a). Moreover, proteins obtained by the STEAP method have a high content of active conformer because only proteins that can bind to the ligand on the resin are selectively eluted from the affinity resin. Therefore, this method is also effective for the purification of an 'active' protein, for example, for the purification of therapeutic recombinant proteins or samples for crystallography studies.

It is known that *ortho*-nitrobenzylamine derivatives are rapidly cleaved by irradiation at 365 nm under mild conditions (Figs. S1 and S2) without additives and are widely used in solid synthesis methods.[5] This photo-labile linker was cleaved within 1 min in an aqueous solution (Fig. S3). We immobilized the photo-labile linker and a hydrophilic PEG spacer with an amino terminal for the immobilization of a bioactive compound on AquaFirmus™ (Fig. 1b). This PEG linker was used to reduce non-specific hydrophobic interactions between the hydrophobic photo-labile linker and compounds or captured proteins.[6] First, we immobilized the bioactive compound FK506 (**1**) and benzenesulfonamide (**2**) (Fig. 1b). FK506 is an immunosuppressive drug that targets a known FKBP12 (an FK506 binding protein) with a K_d of 0.4 nM.[7]

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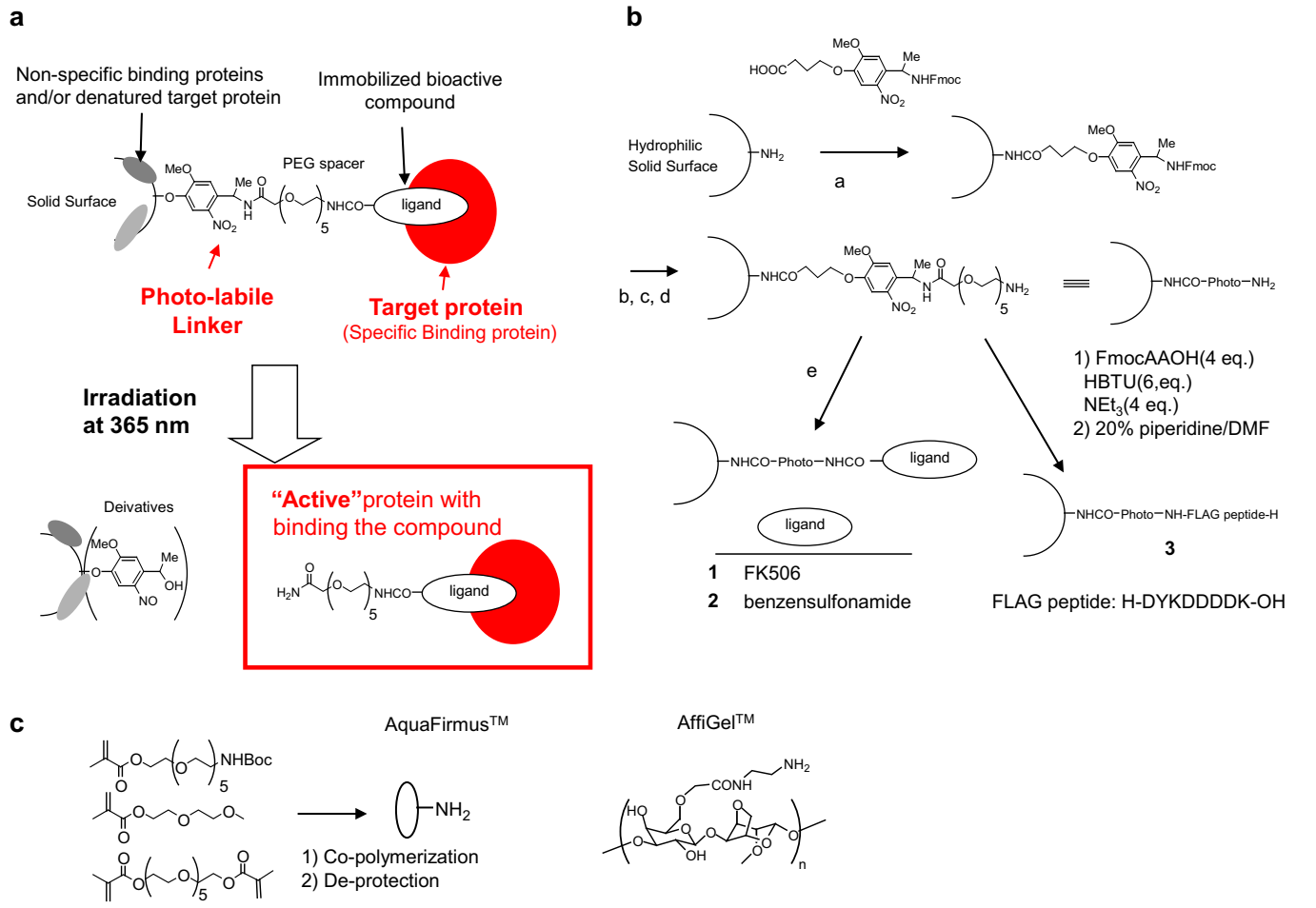

Figure 1. Schematic presentation and synthesis of affinity resins bearing a photo-labile linker. (a) An active protein that can bind to an immobilized ligand is selectively obtained by this method. (b) Affinity resins were prepared by standard solid phase method. The solid material consists of hydrophilic methacrylates and is chemically stable under the synthetic conditions and hydrophilic enough to reduce non-specific protein absorption. (c) Structure of AquaFirmus™ and AffiGel™. Reaction conditions; (a) EDC HCl, HOBt, NEt_3, (b) 20% piperidine/DMF, (c) $BocNH(CH_2CH_2O)_5CH_2COOH$, EDC HCl, HOBt, NEt_3, (d) 10% H_2O/TFA, (e) FK506-COOH or *p*-carboxyl-benzenesulfonamide, EDC HCl, HOBt, NEt_3.

The amount of FKBP12 eluted from the affinity resin 1 reached a maximum level after irradiation for 5–10 min at 365 nm at 4 °C (Fig. S2). Benzenesulfonamide and its derivatives are also well known as specific binding inhibitors of carbonic anhydrase type II (CAII) with a K_d of 0.32–1.25 μM.[8] Affinity resins (**1**, **2**) were mixed with a rat brain lysate in a simple buffer (0.25 M sucrose, 0.3 mM *N*,*N*-diethylthiocarbamate, 25 mM Tris (pH = 7.6)), and then washed and irradiated in the same buffer (Fig. 2). Consequently, the target proteins FKBP12 (Fig. 2a) and CAII (Fig. 2b) were successfully eluted from the resin while non-specific binding proteins were left behind. Only target proteins, FKBP12 and CAII, were obtained in the STEAP samples while much amount of FKBP12 and CAII remained. The specificity of the eluted proteins was confirmed by coexisting with FK506 or benzenesulfonamide derivative. These results indicated STEAP method was useful for identifications of target proteins because only target proteins were selectively eluted by cleavage of the photo-labile linker while non-specific absorbed proteins on surfaces of resins remained. There still were large amounts of target proteins on affinity resins after irradiation reaction. We thought that light did not reach some of photo-labile linker on surfaces of resins where light was shaded by solid surfaces. Next, an affinity matrix bearing the FLAG peptide (H-DYKDDDDK-OH[9]) was synthesized using the Fmoc strategy on the resins (3, Fig. 2c). As shown in Figure 2c, an anti-FLAG antibody was successfully isolated from a mixture of antibody and rat serum. Serum albumin was also eluted with the antibody in this study by an inherent property of this peptide-career protein.[10] We thought this protein was also a specific binding protein for the FLAG peptide because it did not bind to the resin in the presence of the FLAG peptide (the competition method). This example showed the potency of the STEAP method for the purification of antibodies based on their binding affinity with the antigen from crude serum or medium.

Next, we compared the binding of His6-tagged CAII with a benzenesulfonamide moiety when purified by the STEAP method and by the standard Ni column method to validate their active conformer content using Biacore analysis (Fig. 3). A STEAP sample was obtained by the method shown in Fig. 2a. After the binding experiment with the affinity resin, the flow through fraction of the STEAP sample was applied to the Ni column, and His6-tagged CAII was obtained by the standard method, that is, elution with an aqueous solution of 500 mM imidazole (Fig. 3a) after washing with 50, 100, and 200 mM imidazole solutions. SDS-polyacrylamide gel electrophoresis showed the amount of CAII in both samples was very similar (Fig. 3b). However, the Biacore analysis using a 4-caroboxyl-benzenesulfonamide immobilized CM5 chip

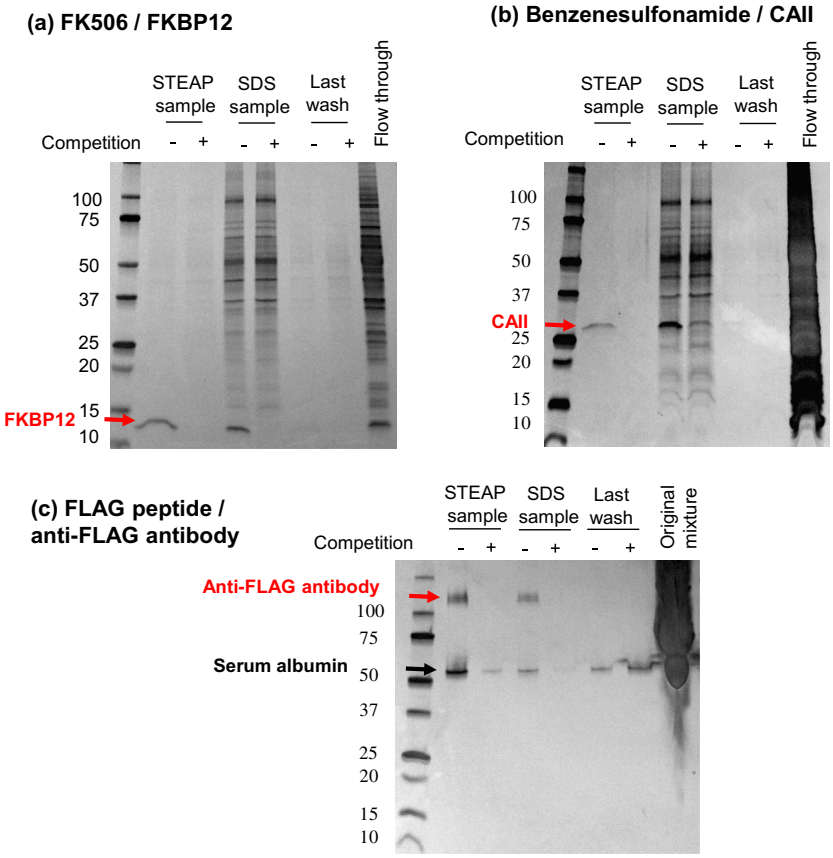

Figure 2. Selective elution of specific binding protein of FK506, benzenesulfonamide, and the FLAG peptide by STEAP method. (a) FKBP12, (b) carbonic anhydrate II (CAII), and (c) anti-FLAG antibody were successfully identified by the STEAP method.

demonstrated that the binding ability of the STEAP sample was almost 5 fold higher than for the Ni column sample (Fig. 3c). The amount of His6-tagged CAII with the ability to bind immobilized benzenesulfonamide is represented by the magnitude of the response units in the Biacore analysis. Therefore, this result indicated that the sample purified by the STEAP method included a high population of 'active' CAII compared to the sample purified by the Ni column method, even though their purities indicated by SDS-polyacrylamide gel electrophoresis analysis were indistinguishable. We believe isolation of protein based on ability to bind to a ligand is effective for the preparation of a pure active protein, for example, for the purification of protein medicines or samples for crystallization.

The biotin-avidin method is widely used for identification of a target protein. A biotinylated bioactive compound is first mixed with lysate to capture the target in the solution, then isolated from the lysate using an avidin or streptavidin column. The target protein is then eluted by the addition of a high concentration of biotin or SDS sample buffer, for instance, after washing with buffer. However, it is often difficult to identify the desired target protein because non-specific absorption proteins are eluted concurrently. We showed that the application of the STEAP method to the biotin-avidin method was also effective for identification of the target protein (Fig. 4). We synthesized a biotinylated FK506 derivative with the photo-labile linker moiety for this study (4), in which PEG300, a mixture of ethylene glycol units of various lengths (average MW. 300), was used as a spacer moiety to adjust the optimal dynamic distance between biotin and FK506. We first mixed 4 with rat brain lysate, and then captured it on the streptavidin column. The target protein was selectively eluted by irradiation after washing with lysate buffer (Fig. 4b). After elution by irradiation, the resulting resins were mixed with SDS sample buffer to elute the remaining proteins from the resin (Fig. 4b). A large amount of protein remained, including non-specific binding proteins to streptavidin resin and/or compound on the resin. As shown in Figure 4b, this novel method gave an excellent yield of the target protein. However, a large amount of FKBP12 remained on the resin even after irradiation. We thought the remaining FKBP12 on the resin was denatured on the resin by non-specific interactions, for example, with the surface of solid materials, after specific binding to FK506.

In conclusion, we showed a novel combination of a photo-labile linker and a chemically stable solid material for the identification of targets of bioactive compounds. Various alternative methods for the same purpose now exist. For example, the competition method is widely used to identify specific binding proteins among the proteins retained on an affinity resin. However, selective elution of a target protein by cleavage of the photo-labile linker is attractive for the biochemist because the application of the traditional method is often limited: for example, the competition method is not applicable to hydrophobic compounds due to their lack of solubility in aqueous solution. Moreover, the hyper-purification of proteins is also important because modern purification methods are usually based on the primary sequence, while the functionality of the protein depends on the three dimensional structure. We believe that the application of the STEAP method was effective not only for the identification of a target protein but also for protein purification.

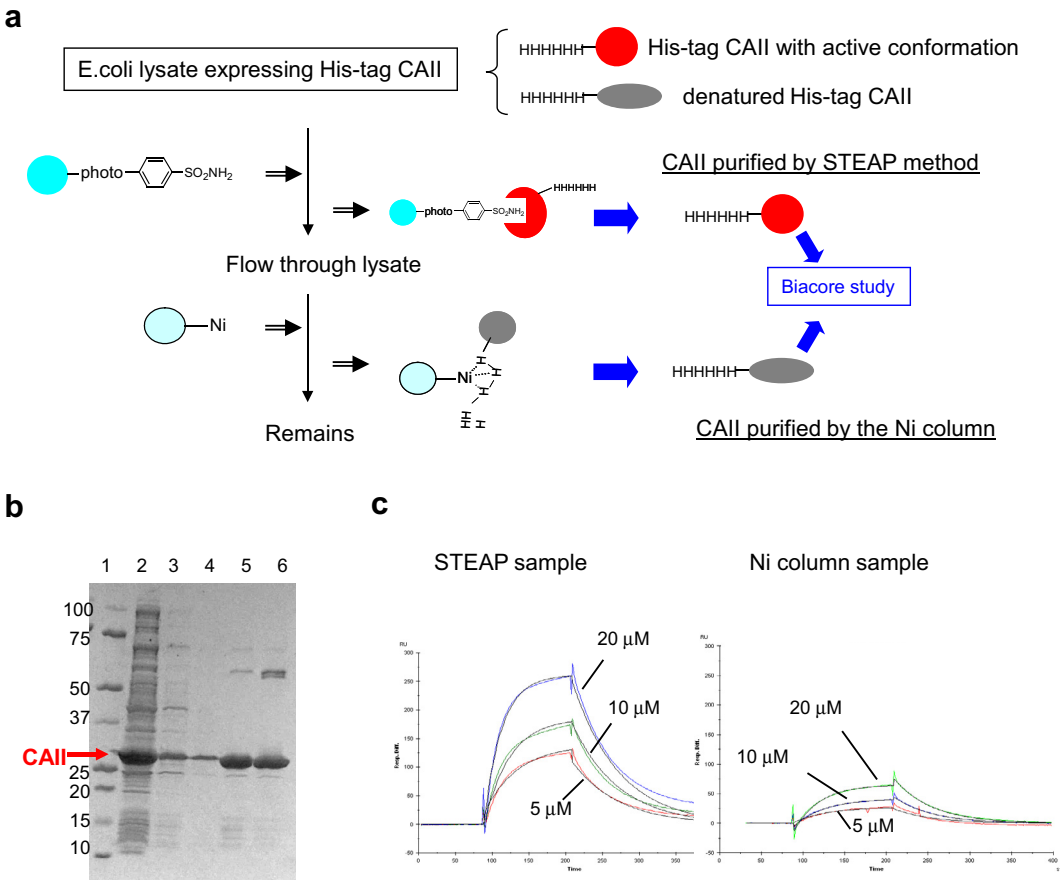

Figure 3. Comparison of the active content of His6-tagged CAII purified by the STEAP method and by the standard Ni column (Ni column sample) from a His6-tagged CAII expressing *Escherichia coli* lysate using Biacore analysis. (a) Protocol for preparation of the STEAP sample and the Ni column sample. (b) SDS-polyacrylamide gel electrophoresis results for each sample: 1; marker, 2; original lysate, 3; last wash before irradiation, 4; last wash before addition of 500 mM imidazole, 5; irradiation (STEAP) sample, 6; Ni column sample. (c) The resonance units of the Biacore analysis using benzensulfonamide-CM5 chips.

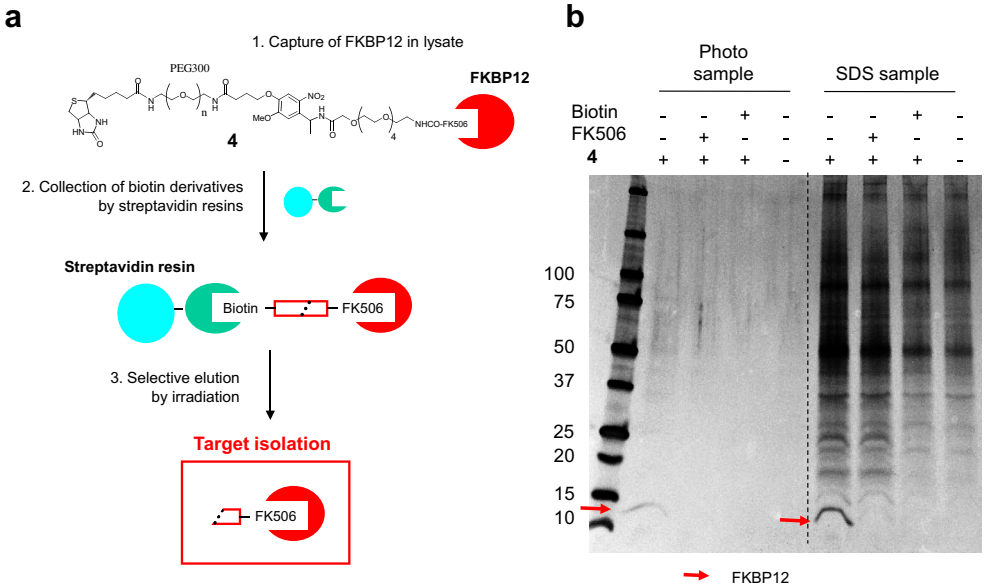

Figure 4. Combination of the STEAP method with the biotin-avidin method. (a) Protocol for the identification of FKBP12 by a combination of the STEAP method and the avidin method. (b) FKBP12 (red arrow) was selectively eluted by irradiation at 4 °C for 10 min, while proteins exhibiting non-specific absorption remained on the resin (eluted by SDS sample buffer). Photo sample; eluted by irradiation, SDS sample buffer; eluted by SDS sample buffer after irradiation reaction.

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Supplementary data

Supplementary data (experimental procedures, Figs. S1–S3) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.05.023.

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