Protein Modification

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Capture and Recycling of Sortase A through Site-Specific Labeling with Lithocholic Acid

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Abstract: *Enzyme-mediated protein modification often requires large amounts of biocatalyst, adding significant costs to the process and limiting industrial applications. Herein, we demonstrate a scalable and straightforward strategy for the efficient capture and recycling of enzymes using a small-molecule affinity tag. A proline variant of an evolved sortase A (SrtA 7M) was N-terminally labeled with lithocholic acid (LA)—an inexpensive bile acid that exhibits strong binding to β-cyclodextrin (βCD). Capture and recycling of the LA-Pro-SrtA 7M conjugate was achieved using βCD-modified sepharose resin. The LA-Pro-SrtA 7M conjugate retained full enzymatic activity, even after multiple rounds of recycling.*

Enzyme-catalyzed bioconjugation has gained increasing interest because of the ability of biomolecular catalysts to recognize and modify unique peptide tags with high specificity under mild conditions.[1] Compelling examples include the use of formylglycine generating enzyme,[1,2] Sfp phosphopantetheinyl transferase mediated labeling,[3] the modification of glutamine side-chains with transglutaminase,[4] and the conjugation of carboxylic acid derivatives using lipoic acid ligase.[5] In addition to these, sortase A (SrtA) is perhaps the most extensively studied and widely used enzyme for site-specific protein modification in academic labs.[6–8] SrtA recognizes the conserved pentapeptide sequence LPXTG and catalyzes, through a reactive cysteine, the transpeptidation of an oligoglycine derivative to the C-terminal site of the threonine residue. Despite tremendous effort in improving SrtA activity through directed evolution,[9] amounts exceeding equimolar stoichiometries are often needed for efficient conversion.[10,11] Purification of the modified protein products from the enzyme mixtures is therefore challenging, and this has limited the use of enzymes like SrtA for large-scale bioconjugation reactions.[12] It is, furthermore, difficult to recycle these enzymes, adding significant costs to the overall process. As such, methods for the selective capture and subsequent reuse of enzymes are in high demand.

Recently, the immobilization of SrtA was demonstrated through the covalent attachment of its lysine residues to a carbohydrate resin.[12] This approach allowed successful recycling, but required 2.5 times more enzyme relative to soluble SrtA to achieve comparable conversions. According to the authors, this decreased activity was likely caused by partial blocking of the active sites as a result of randomly oriented immobilization. An alternative strategy would be to capture the enzyme through specific and reversible non-covalent interactions after the reaction was complete. In particular, this would allow separate recycling of the enzyme and the resin material, which is valuable because of their very different operational lifetimes. Additionally, soluble enzymes are necessary to catalyze reactions on heterogeneous substrates,[13–16] and can facilitate the modification of larger protein substrates that would otherwise be impeded by inefficient diffusion through the polymer support. Most noncovalent immobilization strategies, however, suffer from either leaching as a result of low binding affinity or slow release and poor recovery as a result of binding that is too strong. Immobilized metal ion affinity chromatography (IMAC) is extensively used to purify proteins expressed with a His_6-tag, but can only be applied to one species in a mixture. Applications involving multiple substrates and enzymes would clearly benefit from the availability of additional, complementary techniques that could isolate enzymes for recycling purposes while still allowing IMAC purification of the reaction products.

Herein, we present the efficient capture and recycling of enzymes using an affinity tag that is complementary to Ni-NTA-His_6 techniques (NTA = nitrilotriacetic acid). Specifically, an evolved heptamutant of SrtA with improved enzymatic activity[9] and Ca^{2+} independence[17] (SrtA 7M)[18] was expressed with an N-terminal proline residue (Pro-SrtA 7M) to allow site-specific labeling with lithocholic acid (LA) through oxidative coupling. Recovery and recycling of the modified enzyme (LA-Pro-SrtA 7M) was achieved by noncovalent binding to sepharose resin functionalized with β-cyclodextrin (βCD; Figure 1). The enzyme conjugate retained full catalytic activity and was successfully recycled over four cycles. The presented procedure is inexpensive, rapid, scalable, and likely applicable to many different enzymes of interest.

Resin-immobilized versions of βCD have been used to purify proteins labeled with fluorophores[19] and lipid chains.[20] However, most βCD guests have binding affinities[21] in the order of 10^3–$10^5\ M^{-1}$, which are too weak for efficient capture and separation unless multivalent coordination can be exploited.[22] In our hands, common βCD binders, such as adamantane and azo compounds,[23] could be used for chromatographic separation, but proved insufficient for

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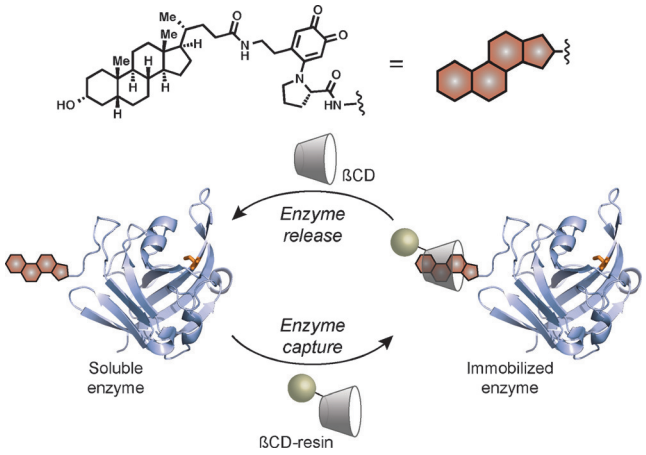

Figure 1. General scheme for the selective capture of LA-modified enzymes using βCD-functionalized sepharose resin. The noncovalently immobilized enzyme is released from the resin by competitive binding with βCD.

complete resin capture. Lithocholic acid (LA) stands out as one of the strongest monovalent binders to βCD with a reported affinity of about $10^6\,\text{M}^{-1}$.[24] Although this binding affinity could be influenced by attached proteins, we identified LA as an ideal affinity handle for the quantitative capture of modified proteins. However, the extremely low solubility of LA in water (0.05 μM at 25 °C[25]) has complicated direct bioconjugation, and so far only very few examples of synthetically attaching LA to proteins have been demonstrated.[26–28] In these studies, green fluorescent protein derivatives were C-terminally modified through expressed protein ligation—a technique that is not directly transferable to many proteins.

To install LA groups on proteins site-specifically, we explored two modification strategies recently reported by our group. The first involved N-terminal modification using 2-pyridinecarboxaldehyde (2PCA) derivatives,[29] a high yielding method that enables conjugation to a wide variety of substrates. Lithocholic acid was therefore synthesized with a 2PCA functionality through the carboxylic acid moiety (Figure 2a,b; see also the Supporting Information for details), but due to the low solubility of LA in water, we initially observed no reaction when treating RNase A (50 μM) or SrtA 7M (50 μM) with LA-2PCA (5 mM) in the presence of 10% MeOH. Notably, the addition of βCD (10 mM) to coordinate the LA-2PCA reagent provided sufficient solubility to allow the efficient labeling of both RNase A (72%) and SrtA 7M (71%), as shown in Figure 2a,b. Following the reaction, spin filtration (MWCO 10 kDa) was used to remove remaining LA-2PCA and excess βCD. However, the requirement for MeOH as a cosolvent is undesirable for large-scale reactions and can potentially affect protein folding. Also, the imidazolidinone product is somewhat prone to hydrolysis as temperatures increase.[29]

The second strategy involved N-terminal modification with *o*-aminophenols[30] in the presence of $K_3Fe(CN)_6$ as a mild oxidant. This reaction is particularly efficient for the modification of N-terminal proline groups, occurs in less than 30 min without leading to protein oxidation byproducts, and affords an irreversible linkage to the protein. SrtA 7M was therefore expressed with an N-terminal proline residue (Pro-SrtA 7M). To avoid coupling to the catalytic site cysteine, Pro-SrtA 7M was quantitatively protected with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman's reagent), as shown in Figure 2c. Once again, excess βCD (10 mM) was required for labeling of DTNB-protected Pro-SrtA 7M with *o*-aminophenol-functionalized lithocholic acid (LA-AP) in the presence of $K_3Fe(CN)_6$. Thus, the low amount of *o*-aminophenol reagent necessary (only about 10 equiv) makes this approach ideal for the conjugation of substrates with limited water solubility without the use of cosolvents. By screening reaction conditions, we found that 10 μM Pro-SrtA 7M, 100 μM LA-AP, and 250 μM $K_3Fe(CN)_6$ at pH 7.0 was optimal (see Figure S1 in the Supporting Information). Spin filtration (MWCO 10 kDa) following the reaction removed the remaining LA-AP reagent and excess βCD. Finally, deprotection of the cysteine with tris(2-carboxyethyl)phosphine (TCEP; 2 mM) liberated the lithocholic acid-SrtA 7M conjugate (LA-Pro-SrtA 7M) in good yield (68%; Figure 2c).

To capture and recover LA-modified SrtA 7M selectively, we functionalized sepharose resin with βCD[23] (see the Supporting Information for details). As a demonstration of the concept, we purposefully used a 56:44 mixture of unmodified to LA-modified Pro-SrtA 7M, even though higher levels of modification were achievable (see above). By addition of this mixture to the resin, followed by washing with 50 mM phosphate buffer, 59% of the total protein eluate was isolated as determined by UV/Vis absorption spectroscopy (Figure 3a; "Ft" and "w" series). ESI-TOF LC–MS analysis confirmed this to be unmodified Pro-SrtA 7M, corresponding well with the expected 56% (Figure 3b). Next, the retained LA-Pro-SrtA 7M was separately eluted from the resin through competitive binding with βCD-containing buffer (10 mM; Figure 3a,b, "e" series). Similar separation was demonstrated for RNase A modified using LA-2PCA (Figure S2). The amount of individual protein recovered from the resin was between 90% (for SrtA) and 100% (for RNase A; Figures S2c, S3c). In all cases, clean separation was obtained, resulting in the complete isolation of the LA conjugate and the recovery of pure unmodified protein.

When using this technique, it should be appreciated that LA represents a large hydrophobic group on the protein surface. This addition could cause some proteins to aggregate or misfold, although we have not observed this to date. SrtA has a number of hydrophobic residues in its active site, and yet does not lose catalytic ability upon modification (see below). As an additional test of LA compatibility with proteins bearing hydrophobic patches, the LA-2PCA reagent was used to modify a T3 ice-structuring protein (ISP) with an AKT N-terminal sequence (AKT-ISP).[31] Full conversion to a single LA-bioconjugation product was observed (Figure S4), with no evident loss in solubility. As a result of the small size of this protein (7.3 kDa), the LA group is a significant contributor to the mass, yet it does not appear to interact with the hydrophobic residues on the protein surface. It is also likely that one equivalent of the βCD remains complexed to the protein after the excess has been removed by spin filtration,

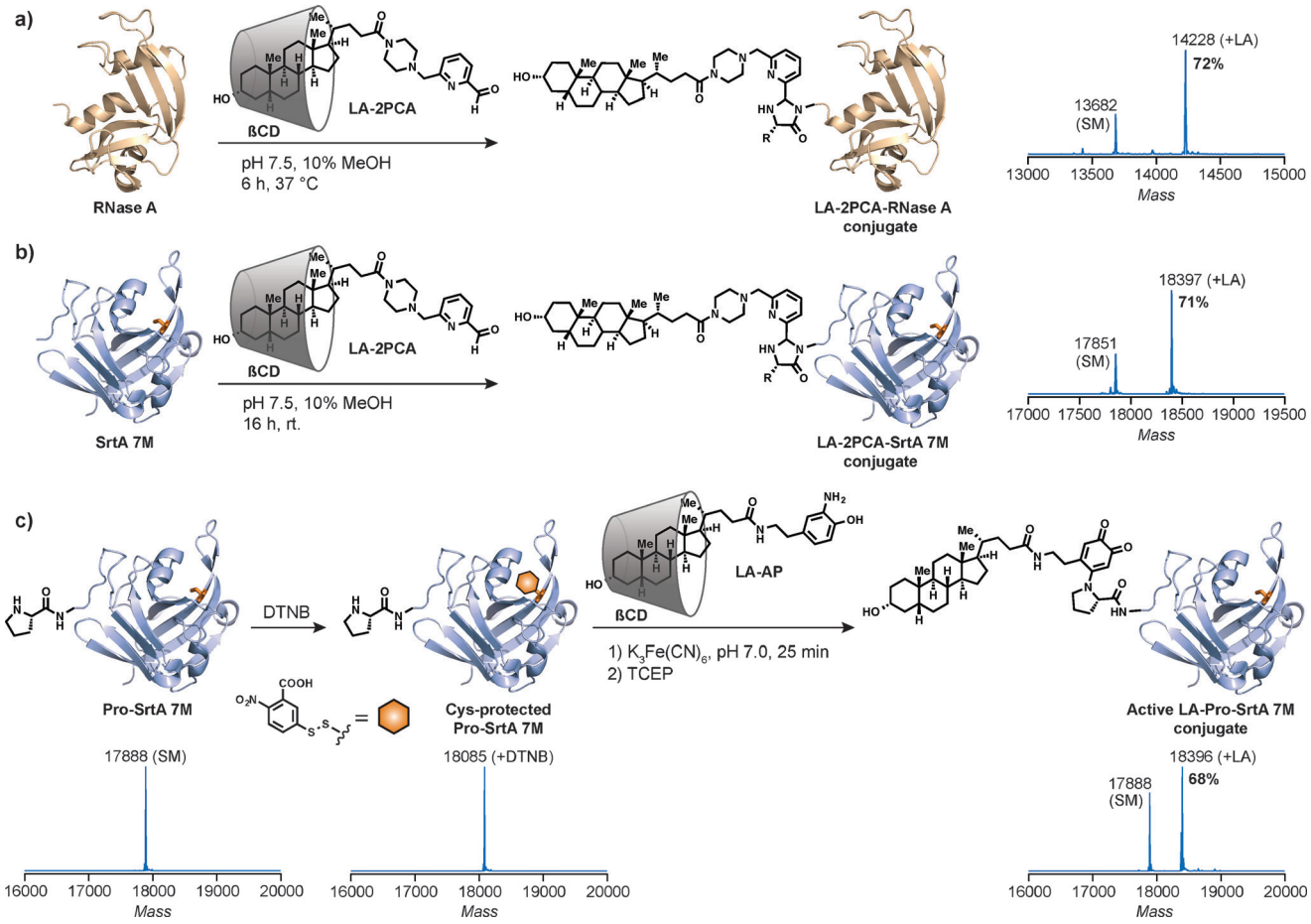

Figure 2. Site-specific labeling of RNase A, SrtA 7M, and Pro-SrtA 7M with LA. a) Labeling of RNase A (Lys N terminus; 13682 Da) with LA-2PCA (5 mM) in the presence of βCD (10 mM) and MeOH (10%) at 37°C for 6 h. The LA-2PCA-RNase A conjugate (14228 Da) was formed in 72% yield, as determined by ESI-TOF LC–MS. b) Labeling of SrtA 7M (Met N terminus; 17851 Da) under similar conditions as for RNase A, except reacted for 16 h at room temperature, yielded the LA-2PCA-SrtA 7M conjugate (18397 Da) with 71% conversion. c) N-terminal oxidative coupling with LA. The catalytic site cysteine (orange amino acid) of Pro-SrtA 7M (17888 Da) was protected using Ellman's reagent (DTNB; orange hexagon) with full conversion. Then N-terminal oxidative coupling with LA-AP (100 μM) was performed using 250 μM $K_3Fe(CN)_6$ in the presence of βCD (10 mM) at pH 7.0. TCEP treatment (2 mM) resulted in the enzymatically active LA-Pro-SrtA 7M conjugate (18396 Da) with 68% conversion. The mass units in all mass spectra in (a–c) are given in Daltons.

thus assisting solubility. Separation of a mixture of unmodified and LA-modified AKT-ISP with βCD-resin led to equivalent results to those observed for Pro-SrtA 7M and RNase A (Figure S4d).

Having the pure LA-Pro-SrtA 7M in hand, we compared the ability of the SrtA mutants to catalyze peptide ligation. The N terminus of SrtA is spatially distant from the catalytic site; thus its modification was not expected to interfere with the enzymatic activity. Indeed, Figure 3c shows that the activity of LA-Pro-SrtA 7M is retained and is comparable to both SrtA 7M and Pro-SrtA 7M. For this peptide substrate, equimolar amounts of the SrtA mutants were required to obtain satisfactory conversion at 37°C.

A useful strategy to purify C-terminally sortagged proteins from unreacted starting material is to install a His_6 tag on the C-terminal side of the LPXTG recognition sequence.[32–35] LA binding to βCD is orthogonal to Ni-NTA-His_6 coordination and we therefore envisioned that a combination of the two methods would enable purification of the sortagged product as well as SrtA recovery. To test this, we expressed a variant of the E2#23 FN3 monobody that binds the human estrogen receptor[36] with a C-terminal LPETGG-His_6 sequence (Figure 4a,b). The additional glycine residue between LPETG and the His_6 tag was introduced to optimize ligation.[37] The oligoglycine nucleophile, GGGGK containing a fluorophore (GGGG-K(HiLyte Fluor 488)-NH_2), was chosen to label the monobody fluorescently. After partial labeling (31% remaining starting material (SM) as determined by ESI-TOF LC–MS; Figure 4c), the reaction mixture was loaded onto the βCD-modified resin to capture the LA-Pro-SrtA 7M. The resin was washed with buffer to elute the labeled and unlabeled monobody, which was then directly loaded onto Ni-NTA resin to retain the starting material selectively and allow isolation of the labeled product (Figure 4d,e). Finally, the pure LA-Pro-SrtA 7M was recovered from the βCD-resin by elution with βCD-containing buffer (Figure 4d,f).

The selective recovery of LA-Pro-SrtA 7M from a protein mixture potentially allows enzyme recycling, thereby substantially decreasing the overall cost of its use. To evaluate

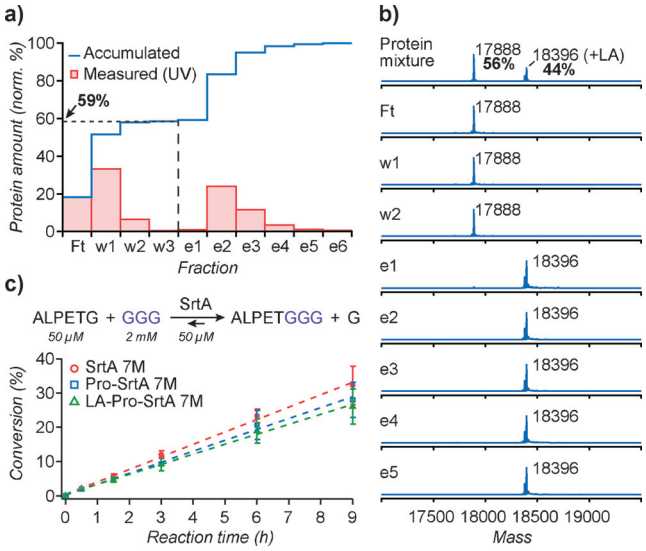

Figure 3. Recovery of LA-Pro-SrtA 7M and evaluation of its enzymatic activity. a) Representative separation of unmodified (56% by LC–MS) and LA-modified (44% by LC–MS) Pro-SrtA 7M using 0.5 mL of βCD-resin (swollen and drained volume). Flow-through (Ft) and washes (w1–w3) correspond to 59% of the total protein eluate; this correlates well with the expected 56% from LC-MS analysis. The remaining protein (41%) was isocratically eluted (e1–e6) with excess βCD, correlating well with the expected 44% (by LC–MS) LA-Pro-SrtA 7M. The amount of protein in each fraction (0.5 mL) was determined by UV/Vis absorption spectroscopy (280 nm). b) LC–MS analysis of the initial protein mixture and the fractions from the separation experiment (a) confirmed efficient isolation of the unmodified and LA-modified enzymes. Pro-SrtA 7M: 17888 Da; LA-Pro-SrtA 7M: 18396 Da. c) Comparison of the catalytic activity of SrtA derivatives. The assay involved the enzyme-mediated peptide ligation of ALPETG (50 μM) and GGG (2 mM) at 37°C using different SrtA variants (50 μM). Comparing the reaction conversions by LC–MS using SrtA 7M (red), Pro-SrtA 7M (blue), and purified LA-Pro-SrtA 7M (green) confirmed retained activity of the LA-Pro-SrtA 7M conjugate. Error bars represent the standard deviation of three separate reactions.

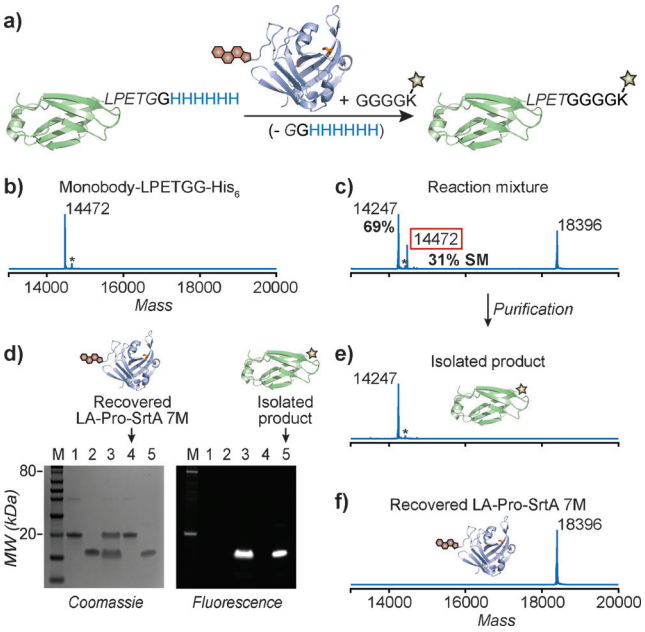

Figure 4. Multicomponent protein separation using orthogonal affinity pairs. a) The monobody-LPETGG-His_6 (50 μM) was labeled with an oligoglycine fluorophore (2 mM) using purified LA-Pro-SrtA 7M (25 μM). b) LC–MS analysis of monobody-LPETGG-His_6 is shown. The asterisk (*) indicates an impurity in the starting material. c) LC–MS analysis of a quenched reaction mixture showed 31% remaining starting material (SM; 14472 Da), 69% conversion to the labeled product (14247 Da), and the LA-Pro-SrtA 7M (18396 Da). d) SDS-PAGE (12%) analysis is shown with Coomassie staining and fluorescence imaging for the separation using orthogonal affinity pairs. Lane M = molecular weight marker; lane 1 = purified LA-Pro-SrtA 7M; lane 2 = monobody-LPETGG-His_6; lane 3 = quenched reaction mixture (similar to (c)); lane 4 = recovered LA-Pro-SrtA 7M from the reaction mixture (similar to (f)); lane 5 = isolated monobody product from the reaction mixture (similar to (e)). e) LC–MS analysis after sequential βCD-resin and Ni-NTA-resin purification of the reaction mixture showed pure isolated product. f) LC–MS analysis of pure LA-Pro-SrtA 7M recovered from the reaction mixture using βCD-resin.

this possibility, we compared the labeling of monobody-LPETGG-His_6 using the same sample of LA-Pro-SrtA 7M recovered over four cycles (Figure 5). Each ligation was performed using 10 μM LA-Pro-SrtA 7M. To compare enzyme activity properly, the reactions were stopped identically after 30 min, resulting in 51–58% remaining starting material (Figure 5b). After each reaction the LA-Pro-SrtA 7M was recovered using βCD-resin as previously described (typically with about 90% recovery). The material was concentrated by ultracentrifugation (with about 67% recovery), quantified by UV/Vis absorption spectroscopy, and added to a fresh batch of the reaction substrates. Importantly, Figure 5a reveals fully retained enzymatic activity over all four cycles. Additionally, for all reactions the labeled product was successfully isolated using the same βCD-resin material throughout all rounds of recycling.

In this work, the affinity-based recovery of LA-Pro-SrtA 7M was demonstrated in combination with Ni-NTA purification, enabling the separation of all reaction components as well as enzyme recycling. The monovalent LA-βCD binding affinity is ideally suited for the selective capture and controllable release of protein bioconjugates with near-quantitative recovery. In addition to eliminating the steric issues of covalent immobilization, the noncovalent capture of SrtA allows the separate reuse of the resin material for extended periods, far exceeding the lifetime of most enzymes. The recycling procedure should be amenable to many proteins other than SrtA, RNase A, and AKT-ISP. Although our studies did not require the use of extended linkers, these could potentially prove useful in some cases.

The overall generality of the method will likely be dictated by the bioconjugation method that is used to install the LA group. Depending on the protein of interest and the specific application, the LA moiety can be attached using 2PCA-based conjugation, oxidative coupling, or any additional site-specific bioconjugation technique. Of these, the two N-terminal functionalization strategies described herein are straightforward and can be applied to both small-scale academic research and large-scale industrial applications as long as the protein N terminus is accessible. In current efforts, we are expanding the concept to include other matrix materials and other protein-modifying enzymes.

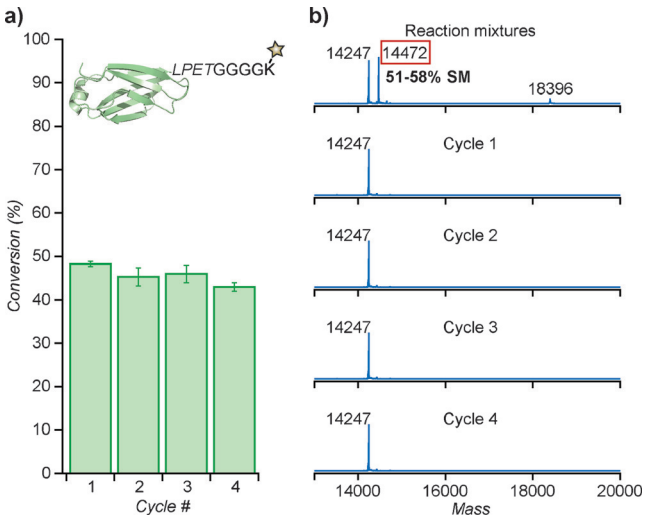

Figure 5. Recycling of LA-Pro-SrtA 7M and repeated isolation of reaction products. a) The labeling of monobody-LPETGG-His_6 (50 μM) is shown using repeatedly recovered LA-Pro-SrtA 7M (10 μM), revealing fully retained enzymatic activity. Error bars represent the standard deviation of three separate reactions. b) A representative LC–MS analysis is shown for a quenched reaction mixture (51–58% remaining starting material in all analyzed reactions). LC–MS data indicate the purity of the labeled monobody product (14247 Da) after reaction with recycled LA-Pro-SrtA 7M, βCD-resin capture, and Ni-NTA resin purification. The same βCD-resin was used throughout all rounds of enzyme recycling.

Acknowledgements

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