Protein C-Terminal Tyrosine Conjugation via Recyclable Immobilized BmTYR

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Tyrosine-terminated protein — Immobilized enzyme

Modified protein — Small molecules

Magnet
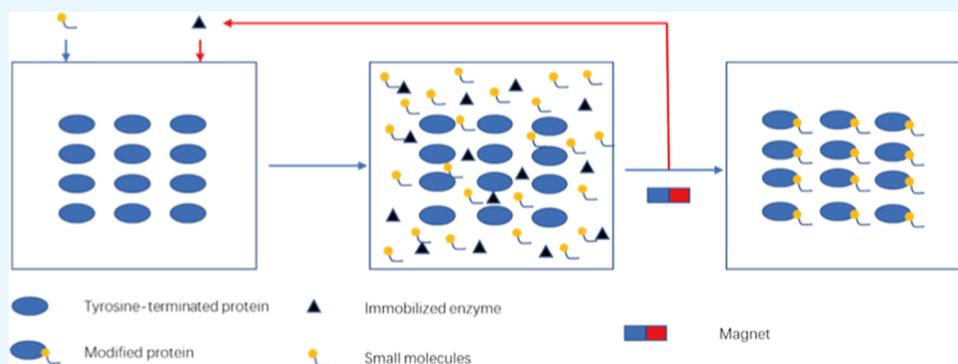
ABSTRACT: Protein modification plays an essential role in biological and pharmaceutical research. Due to the ordinary selectivity and inevitable damage to proteins of chemical synthetic methods, increased efforts were focused on biocatalysts which exhibited high regioselectivity and mild reaction conditions. However, separation of the biocatalysts and modified proteins remained a problem, especially when scaling up. Here, we developed a simple method for site-specific protein modification with a recyclable biocatalyst. The immobilizing tyrosinase (BmTYR) on magnetic beads can oxidize C-terminal tyrosine residues of the target protein to o-quinone, followed by the spontaneous addition of different nucleophiles (e.g., aniline derivatives), resulting in a C-terminal modified protein. Compared to the homogeneous biocatalytic system reported before, this heterogeneous system leads to an easier separation. Furthermore, the solid-phase biocatalyst can be regenerated during separation, providing reusability and lower costs.

INTRODUCTION

Proteins are attractive medicine and diagnostic tools, but their applications usually require the installation of additional, non-genetically encoded functional groups.[1,2] Covalent modification of proteins while retaining their natural functions has always been the goal of chemical biology research.[3,4] Unfortunately, it is currently difficult to target chemically different functional groups in protein sequences, resulting in heterogeneous product mixtures with diverse modified states.[5] Early biomolecule couplings usually used heterobifunctional cross-linking agents, such as maleimide linkers commonly used in antibody−drug conjugates with endogenous nucleophilic amino acid residues such as cysteine or lysine.[6] However, small molecules targeting at amino acid residues often have poor selectivity, non-specific labeling, and loss of protein function.[7−10] Therefore, the development of protein modification methods with high selectivity and conversion rate has become a hot spot in protein drug development, molecular diagnosis, and chemical biology.

In recent years, with the development of chemical biology, suitable bioorthogonal reactions for protein modification have been exploited. The click chemistry reaction developed by Sharpless' group created a new era of efficient bioorthogonal reactions.[11] However, the protein should be installed with the reactive sites for click chemistry reactions (functional groups such as azide and alkyne) before, via a high-cost and low-efficiency genetic engineering.[12−15] Other residues have also been targeted, such as histidine,[16] serine,[17] tryptophan,[18] methionine,[19] and tyrosine[20,21] with the selective reaction. Although these methods reached higher chemoselectivity, targeting an amino acid at specific site is still impossible, which could affect protein function significantly. Thus, small molecule mediated protein modification still had certain obstacles to deal with.
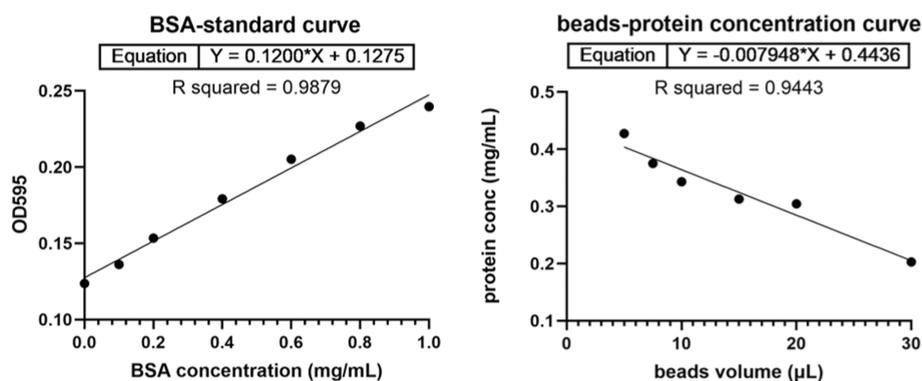
Enzyme-catalyzed protein modification is an emerging method in the past decades. Compared with traditional chemical modification, enzyme-catalyzed reactions usually occur under physiological conditions and will not cause

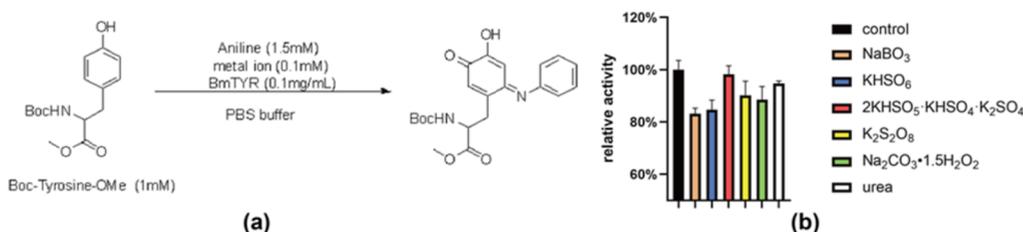**Figure 1.** Evaluation of MB capability of Bm-TYR. A BSA standard curve was built to evaluate the MB capability. The slope of protein concentration to bead volume revealed the capability.

Figure 2. (a) Model reaction with variable additives. (b) The effect of different auxiliaries, control group (no auxiliary added) was set as 100% relative activity.

protein denaturation. The high selectivity of enzymes for substrates naturally leads to a high degree of homogeneity of the reaction products, which exactly meets the requirements of protein modification.[22] As a natural and efficient catalyst, the enzyme has a fast reaction rate and can complete the modification of the target protein in a brief time. Take sortase A, a transpeptidase that has been deeply studied in recent years, as an example. It can transfer the target small molecule to the specific peptide tag of the protein through the cleavage and recombination of specific peptides to realize the small-molecule modification of the protein.[23,24] Engineering variants of transpeptidase developed in recent years have increased the reaction rate and reduced the dependence on calcium ions.[25] In addition to sortase A, other enzymes that catalyze protein modification reactions were developed, such as tyrosinase,[26] biotin ligase,[27] transglutaminase,[28] and so on.

Although enzymes overcame many problems in the protein modification, there are still new issues that should be faced with. For instance, contamination of products with enzymes makes it problematic in application.[29] To conquer these issues, immobilization of enzymes was introduced into this field, which has more advantages regarding homogeneous reaction and overcoming to the lack of reusability.[30,31] In application, the simplicity of immobilization, the extent of enzyme reusability, and ease in support separation are the essential factors that are usually considered. Among all immobilization matrices, the magnetic nanoparticles have unique place regarding to their distinguished high specific surface area and biocompatibility.[32] Functionalized magnetic nanoparticles have been studied as a promising matrix for the immobilization of enzyme, but most of them were covalent formations by the reactive chemical reagents, which might be harmful to the activity of the enzyme.[33,34] Since the 15 amino acid streptavidin-binding peptide (nano-tag)[35] could bind with high affinity to the streptavidin protein-coated magnetic beads,

introducing this nano-tag into the biocatalytic enzymes might be suitable for the immobilizing method to improve the efficacy of separation. As a proof of concept, we chose it for the immobilization of tyrosinase, which oxidizes tyrosine to o-diquinone[36] and then followed by the addition with the nucleophiles to produce covalently linked products spontaneously. We demonstrated that this tag system can be efficiently used for the immobilization with enzymatic activity maintained. Furthermore, compared with the enzyme in solutions, the immobilized enzyme gave a cleaned product without the enzyme involved. After simple separation, the immobilized enzyme can be recycled without obvious activity loss.

■ RESULTS AND DISCUSSION

This study began with immobilization of BmTYR (*Bacillus megaterium* tyrosinase, PDB: 3NM8) onto a solid phase. When constructing the BmTYR plasmid, a nano-tag was installed onto the C-terminal (after the His-tag) in advance. Although the His-tag did have the ability to immobilize protein, the binding affinity was not strong enough. However, the nano-tag had a $K_d = 4$ nM, which tightly bonded to the solid phase. Furthermore, nano-tag had a higher selectivity than His-tag to avoid non-specific binding since there could be fewer sequences that contain 15 specific amino acids. As mentioned before, BmTYR fused with nano-tag was incubated with magnetic beads (MB) for 5 min. To determine the capability of MB, a BmTYR solution with known concentration was treated with different volumes of MB. After immobilization, the concentration of BmTYR dropped linearly to the volume of MB. Bradford method was applied to determine the remaining enzyme concentration, and the capability was calculated to be 7.95 mg/mL MB (Figure 1).

As a metalloenzyme, BmTYR has a protogenetic cofactor of Cu(II). When binding to Cu(II), tyrosinase is activated and

able to oxidize tyrosine with oxygen. Previous studies focused on the kinetic data of the enzyme.[36] We further investigate the enzymatic activity with different cofactors and oxidants. A set of model reactions were performed at 1 mM N-Boc tyrosine methyl ester, 1.5 mM aniline, 10 μM BmTYR, and 100 μM different cofactors (Figure S1a). Since BmTYR was overexpressed and purified, followed by EDTA treatment to remove possible metal ions, metal ions were added in appo-protein . However, only Cu(II) ions showed the activity. Considering that multimer formation and oxygen activation were important in tyrosinase activity,[37] some auxiliaries such as oxidants and urea (promoting dimerization) were examined (Figure 2b).

Soluble oxidants which were reported to have potential to oxidize tyrosine were applied.[38−40] However, to our disappointment, no notable change of the activity occurred between them. Thus, Cu(II) as a cofactor without auxiliaries was chosen as the best reaction condition.

The rate of the reaction was investigated with different concentrations of N-Boc tyrosine methyl ester, and the kinetic constants were calibrated using Lineweaver−Burk plots (Figure S2). The V_{max}, K_m, and K_{cat} are given in Table 1.

Table 1. Kinetic Constants of Free and Immobilized BmTYR

kinetic constants	free BmTYR	immobilized BmTYR
V_{max} (mmol/min)	0.220	0.789
K_m (mM)	3.55	15.9
K_{cat} (min^{-1})	1.63×10^3	5.85×10^3

Here, we noticed that the high concentration of N-Boc tyrosine methyl ester and aniline resulted in protein precipitation from enzyme in solution, leading to lower conversion. Meanwhile, the immobilized enzyme could tolerate on the high concentration of substrates (Figure S1b,c). Compared with the free enzyme, the K_m value was increased for the immobilized enzyme to show the decreased affinity for its substrate. However, the increased K_{cat} revealed that the turnover rate of the enzyme increased after immobilization.
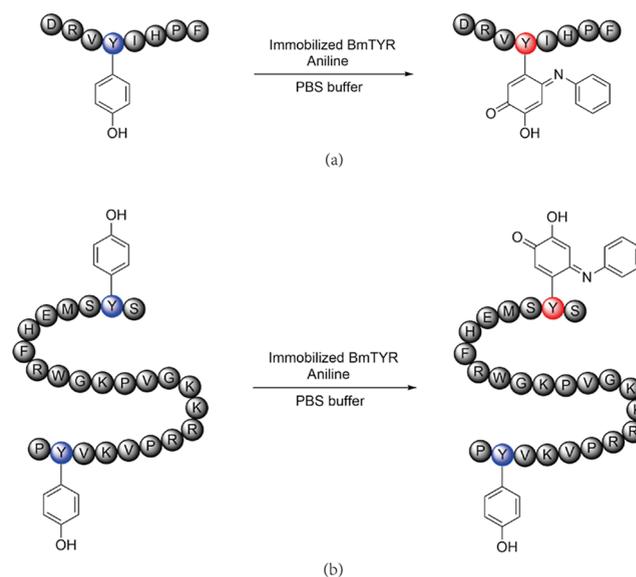
In previous studies, the nucleophiles such as aniline derivatives and cysteine can undergo nucleophilic addition to the quinone intermediate, producing a coupled molecule. Herein, we explored more kinds of potential nucleophiles which may react with the quinone intermediate. The low nucleophilic reactivity of different alcohols resulted in no reaction, and phenol exhibited the conversion slightly (Figure S3). Based on the reactivity of all substrates we tested, aniline was chosen to undergo detailed investigation (Table 2). Steric hindrance will prevent nucleophilic addition, resulting in a dramatic drop of conversion, such as 2,6-diethylaniline (entry 4, 37%) and 2,6-diisopropylaniline (entry 5, 35%). To our satisfaction, para-substitutions tolerated the different substitutions, which could link with the other functional groups.

On the next stage, we used peptides to study whether a protein-like molecule is suitable in this reaction condition. One endogenous peptide, angiotensin II with one tyrosine residue near N-terminal, was chosen to undergo oxidative addition with the aniline (Scheme 1a). When treated with 1.5 equiv of the aniline, angiotensin II can be quasi-fully oxidized and added by the aniline, forming a M + 105 Da product with over 90% conversion (determined by HRMS) (Table 3, entries 1a and 1b). As the chromatograms and mass spectra showed, only one notable peak was observed with a MW matched with the

Table 2. Conversion of Different Aniline Derivatives with Different Steric Hindrance and Electron Density

Entry	Compounds	Conversion%
1		>95
2		>95
3		56
4		37
5		35
6		10
7		>95
8		83

Scheme 1. Peptides Modified with Aniline Using Immobilized BmTYR[a]

[a](a) Angiotensin II modification at 1.5 equiv aniline. (b) Cosyntropin modification at 1.5/10 equiv aniline.

Table 3. Peptide Modification Data

number	observed m/z	calculated m/z	predicted MW
1a[a]	523.7754	523.7746	1046.2
1b[a]	577.3012	577.2853	1151.3
2a[b]	587.5239	587.5241	2933.5
2b[b]	608.5394	608.5284	3038.6

[a]1a and 1b refer to angiotensin II and angiotensin II-aniline, whose z = 2. [b]2a and 2b refer to cosyntropin and cosyntropin-aniline, whose z = 5.

desired product (Figure S4). This result proved that under the condition we developed, the peptide with tyrosine residue can be selectively oxidized and coupled with the aniline, without undesired byproducts. Then, cosyntropin with two tyrosine residues was tested with different aniline equivalents (1.5 and 10) (Scheme 1b). The results showed that only one product with $M + 105$ Da was obtained under different concentrations of the aniline with over 90% conversion (Table 3, entries 2a and 2b, Figures S5 and S6). To further investigate whether it can generate two different mono-modified products and which exact modified position was, LC−MS/MS was applied to determine that the modification was near the C-terminal. As shown in Figure 3, the adduct had a base peak of $m/z =$
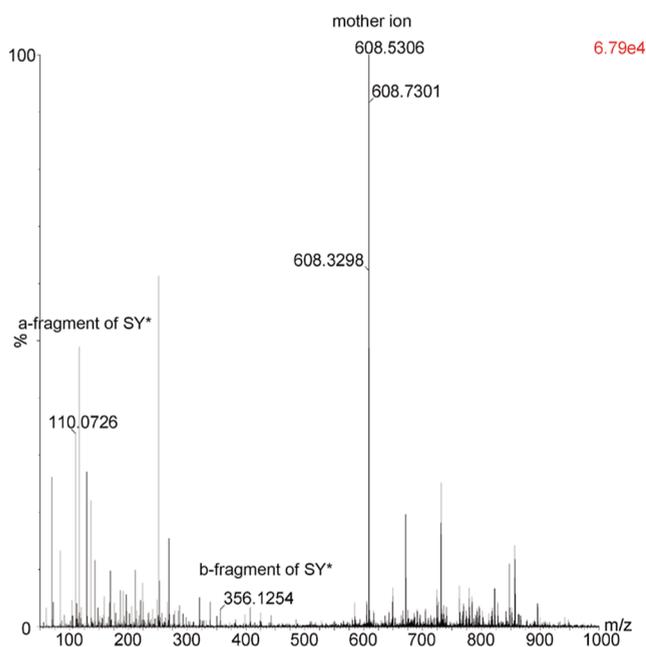

Figure 3. MS2 spectrum of modified angiotensin II.

608.5302 ($z = 5$) in the MS1 spectrum. It was chosen to be analyzed in the MS2 spectrum. The a-fragment ($m/z = 110.0726$, calculated 110.0479) and b-fragment ($m/z = 356.1254$, calculated 356.1241) of SY* (the first and second amino acid residue on the sequence of cosyntropin) were found in the spectrum, which proved that the modification happened at the tyrosine residue near the C-terminal.

Based on the commonly accepted "Keil rule" on trypsin that it cannot digest lysine or arginine followed by a proline,[41] an assumption came up that BmTYR had a similar activity: Since BmTYR has a limited active pocket, a proline residue may cause an obvious steric hindrance which would prevent the nearby tyrosine residue from entering the active site of the enzyme.

Based on the modifications of small molecules and peptides, we constructed a GFP protein with C-terminal tyrosine (GFP-Y). In our assumption, the original tyrosine residues in the sequence could not be oxidized since they might not be exposed to the active pocket of BmTYR. Only the flexible C-terminal tyrosine had the chance to get in BmTYR and be oxidized. Verification began with 3 equiv of aniline, together with immobilized BmTYR and GFP-Y (Scheme 2a). Compared to the original GFP-Y, GFP-Y* had an increase of 102 Da (Table 4, entries 1 and 2, Figures S7 and S8),
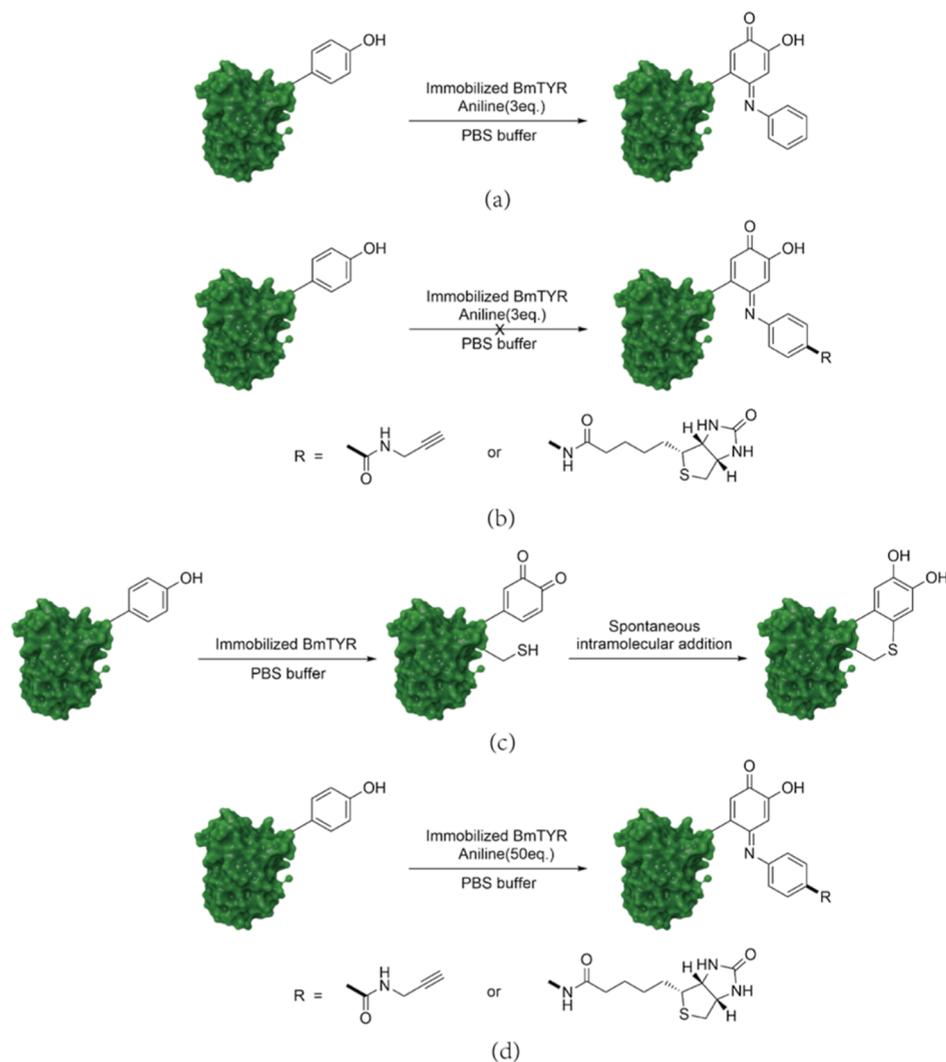
indicating that a single modification happened (MW of aniline = 93). The conversion was still higher than 90%. This result proved that immobilized BmTYR could modify the protein with special small molecules in a mild condition. By the digestion of GFP-Y and GFP-Y* with trypsin, only one modified tyrosine residue could be found with the high-resolution mass spectrum. Unreacted GFP-Y after digestion gave a sequence of DHMVLLEFVTAAGITHGMDELY, while GFP-Y* gave DHMVLLEFVTAAGITHGMDELY*. The different retention time and m/z value between the two peptides proved our assumption. This experiment confirmed that only the C-terminal tyrosine residue can be oxidized and coupled with aniline (Figure S9).

Considering the functional group of biotin or alkyne is a useful tool in chemical biology, we decided to install the aniline derivatives with the two functional groups onto the GFP-Y protein. When 3 equiv of aniline with biotin or alkyne group was applied in this reaction for 2 h (Scheme 2b), the peak of the target product (MW = 28,039 for aniline-biotin adduct, while MW = 27,893 for aniline-yne adduct) by MS was weak (Figure S10), and a similar strong peak of the byproduct with a molecular weight of 27,707−27,709 was obtained (Table 4, entry 3). After treatment with higher ratio of BmTYR, the reaction gave a similar result. Through comparison of MS of an original GFP-Y protein and a GFP-Y protein treated with immobilized BmTYR but without the aniline (Figure S11), the molecular weight of the modified GFP-Y was increased at 13, which indicated that GFP-Y can be directly oxidized by BmTYR. After BmTYR oxidized GFP-Y, aniline was added in the mixture. The resulting product was the same before. This indicated that the product of GFP-Y treated with only immobilized BmTYR was not a o-diquinone intermediate. On comparison with the original sequence of GFP-Y, we speculated the intramolecular addition happened after the oxidation (Scheme 2c). By increasing the ratio of aniline derivatives to 50 (Scheme 2d), the desired modification was observed with a conversion >90% (Table 4, entries 4 and 5, Figure S12).

Given that immobilized BmTYR is a heterogeneous catalyst, it can be added or removed from the reaction mixture. We reasoned that it should be possible to recycle immobilized BmTYR. We conducted catalyst-recycling experiments wherein immobilized BmTYR was removed from a completed modification reaction with a magnet, rinsed with PBS buffer, and reused directly in the next modification reaction. As shown in Figure 4, when the same immobilized BmTYR was used for five cycles, the conversions of small molecules or peptides were almost the same. However, when modifying proteins, the conversion rate decreased a little after five cycles. These experiments demonstrated the ease of recycling and the consistent enzymatic activity for immobilized BmTYR is possible.

◼ CONCLUSIONS

In this work, a simple and efficient method for the modification of protein with tyrosine residue was developed by using immobilized tyrosinase on magnetic beads. The easily prepared immobilized tyrosinase can selectively oxidize the tyrosine residue, reacting with an aniline derivative, producing an adduct of small molecules with target protein. After spontaneous addition of small molecules, the solid-phase biocatalyst can be separated with a magnetic device. The

Scheme 2. GFP-Y Protein Modified with Aniline Derivatives Using Immobilized BmTYR[a]

[a](a) GFP-Y modification with aniline. (b) GFP-Y modification with aniline-yne or aniline-biotin at 3 equiv. (c) GFP-Y treated with only immobilized BmTYR resulted in intramolecular addition. (d) GFP-Y modification with aniline-yne or aniline-biotin at 50 equiv.

Table 4. Protein Modification Data

name	deconvoluted MW
GFP-Y	27,694
GFP-Y*	27,796
GFP-Y[o][a]	27,707
GFP-yne	27,893
GFP-biotin	28,039

[a]This refers to the byproduct.

immobilized BmTYR can be regenerated simply by washing with buffer after magnetic separation.

Compared to chemical reagents, enzymes could provide a way without off-targeted modification of proteins. Immobilized enzymes further give a simple method to avoid a mixture of biocatalysts with the desired products, which cost high for purification. The heterogeneous biocatalyst is stable and can be reused more than five times with maintained conversion, compared with the relative homogeneous biocatalyst. Given the advantages of heterogeneous biocatalysts, we anticipate that the preparation for antibody−drug conjugates would benefit from this catalytic system. Further development is also

expected to offer an access to industrial applications since the immobilized enzyme platform is expandable to different enzymatic reactions.

METHODS

UV−Vis Measurements. A NanoDrop 1000 (Thermo) was used to quantify the concentrations of DNA plasmid samples and for measuring protein concentrations of samples in Bradford assays.

HPLC-UV Analysis. The small-molecule-level evaluation used HPLC to determine conversion. HPLC analysis was performed using an Agilent 1200 series liquid chromatography system. The UV spectrum was recorded on an Agilent G1316A VWD with 254 nm. For mobile phases, the system utilized pure water (Wahaha) with 0.1% (v/v) formic acid (solvent A) and HPLC-grade MeOH (solvent B). Before submission to analysis, each sample was added with 100 μL of MeOH and centrifuged for 5 min at 15,000 rpm. The supernatant was passed through a 0.22 μm filter. Samples of 10 μL were injected into the system. The sample was separated on an Agilent ZORBAX SB-C18 (4.6 mm × 150 mm) analytical
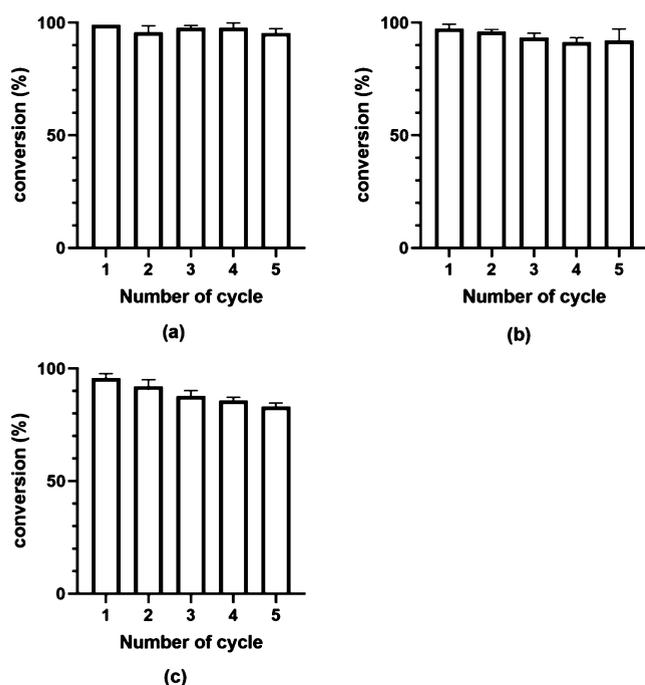
Figure 4. Consecutive reaction conversions in the oxidative addition with aniline catalyzed by immobilized BmTYR. (a) N-Boc-tyrosine-OMe as a substrate, (b) angiotensin II as a substrate, and (c) GFP-Y protein as a substrate (for details of reaction conditions, see the Methods section).

column with a flow rate of 0.80 mL/min and a gradient listed in the Supporting Information (Table S1)

UPLC-HRMS for Peptide and Protein Analysis. The peptide and protein analysis was performed with a Waters ACQUITY UPLC H-Class system (Waters Corporation, Milford, MA, USA) coupled with a Waters Synapt G2-Si quadrupole time-of-flight mass spectrometer (Q/TOF-MS, Waters Corporation, Milford, MA, USA). A Waters ACQUITY HSS T3 column (2.1 mm × 100 mm, 1.7 μm) was used for chromatography separation. Mobile phase A was water, and mobile phase B was ACN/MeOH (9:1, v/v), both containing 0.1% (v/v) formic acid. The flow rate was 0.4 mL/min. The gradient elution started with 5% B and maintained for 0.5 min, linearly increased to 95% B in 3 min and maintained for 1 min, and returned to the initial gradient ratio for 1 min equilibrium. The ion source was electron spray ionization (ESI), and the condition for the ionization mode was positive electrospray. The desolvation gas (nitrogen) flow rate was 750 L/h, and the temperature was 350 °C. The source temperature was 120 °C. The capillary voltage was 3.0 kV. The low collision energy (CE) was set as 2 eV for trap CE and 2 eV for transfer CE. The high CEs were set as a trap CE ramp of 4–18 eV and a transfer CE of 10–35 eV. Mass spectra were analyzed and deconvoluted with MassLynx V4.1 (Waters Corporation, Milford, MA, USA).

Expression and Purification of BmTYR-Nano-tag and GFP-Y. A 10 mL overnight culture of BL21 (DE3) *Escherichia coli* in LB medium was inoculated into a 2 L Erlenmeyer flask with 1 L of sterile LB with a final concentration of 100 μg/mL kanamycin. The culture was incubated at 37 °C, 220 rpm, and cell density was monitored until the OD600 value was between 0.6 and 0.8. The cells were then induced by adding IPTG to a final concentration of 0.5 mM and subsequently incubated for

another 12 h at 30 °C. The cells were then harvested, and the cell pellet was thawed on ice in lysis buffer (500 mM NaCl, 20 mM imidazole, and 20 mM PBS, pH 7.5) and PMSF (0.1 mM), and cells were lysed by sonication for 15 min [4 s on, 6 s off, 50% amplitude, with a SCI-ENTZ ultrasonic cell disrupter]. The cellular debris was removed with centrifugation (17,000g, 15 min, 4 °C), and the supernatant was applied to a Ni-NTA gel column. The column was washed three times with 20 mL of lysis buffer (lysis buffer without PMSF), and fractions were collected. The protein was then eluted using a gradient of different imidazole elution buffer (500 mM NaCl, 20–200 mM imidazole, and 20 mM PBS, pH 7.4). All fractions were analyzed using SDS-PAGE, and the tyrosinase-containing fractions were collected. The buffer was exchanged and concentrated for protein storage buffer (20 mM PBS) using a spin concentrator (30 KDa MWCO, 15 mL, Amicon Ultra). The protein solutions were stored at −80 °C.

EDTA Processing. The protein solution was dialyzed against 1 mM EDTA solution for 1 h under 4 °C. To remove excess EDTA, the solution was dialyzed to PBS buffer for another day. After dialysis, the protein solution was concentrated again to about 1 mg/mL.

Magnetic Bead Capability Test. BSA powder was dissolved and diluted to a final concentration of 1.0 mg/mL. In a 96-well plate, a set of different concentrations of BSA (100 μL) was built from 0.0 to 1.0 mg/mL. In each case, 3 μL of Bradford solution was added, and the mixture was vortexed for 15 s. A microplate reader (Spark 20M, Tecan) was used to read the OD595 value and calculate the standard curve. To determine the protein capability of magnetic beads, different volumes of magnetic beads were added to the same sample of BmTYR protein solution (100 μL). After incubation for 5 min, beads were separated using a magnetic separator. Each sample was then converted to a 96-well microplate with a volume of 100 μL. Bradford solution (3 μL) was added, and the mixture was vortexed for 15 s. A microplate reader was used to read the OD595 value, and the relative protein concentrations can be calculated from the BSA standard curve. Note that the protein concentration should be calibrated with the bead volume added. A curve of protein concentration after immobilization in solution to beads volume can be calculated.

Metal Cofactor Test. BmTYR-immobilized beads (10 μL) were added to an Eppendorf tube and diluted to 100 μL with PBS buffer (50 mM, pH = 7.4). Sequentially, 1 μL of Boc-tyrosine-OMe (100 mM), 1 μL of aniline (150 mM), and 1 μL of cofactor (10 mM, bivalent, and with chloride salt) were added; vortexed for 15 s; and reacted for 10 min. After reaction, beads were separated with a magnetic separator, and 100 μL of cold methanol was added to the solution. HPLC analysis was applied. The product area was recorded and normalized where the group with no cofactor was set as 100% relative activity.

Auxiliary Test. BmTYR-immobilized beads (10 μL) were added to an Eppendorf tube and diluted to 100 μL with PBS buffer (50 mM, pH = 7.4). Sequentially, 1 μL of Boc-tyrosine-OMe (100 mM), 1 μL of aniline (150 mM), and 1 μL of oxidant (100 mM, use immediately after dissolved in solution) were added; vortexed for 15 s; and reacted for 10 min. After the reaction, the beads were separated with a magnetic separator, and 100 μL of cold methanol was added to the solution. HPLC analysis was applied. The product area was recorded and normalized where the group with no auxiliary was set as 100% relative activity.

Aniline Derivative Conversion Test. BmTYR-immobilized beads (10 μL) were added to an Eppendorf tube and diluted to 100 μL with PBS buffer (50 mM, pH = 7.4). Sequentially, 1 μL of Boc-tyrosine-OMe (100 mM), 1 μL of aniline derivatives (150 mM), and 1 μL of cofactor (10 mM) were added; vortexed for 15 s; and reacted for 30 min. After the reaction, the beads were separated with a magnetic separator, and 100 μL of cold methanol was added to the solution. HPLC analysis was applied to determine conversion.

Enzyme Kinetics of BmTYR. For both free and immobilized BmTYR (135 nM each), enzyme kinetic parameters such as K_m, V_{max}, and catalytic efficiency were calculated with various concentrations (0.5−5 mM) of the substrate. The Lineweaver−Burk plot was used to study the kinetic parameters.

Peptide Conversion Test. BmTYR-immobilized beads (5 μL) were added to an Eppendorf tube and diluted to 900 μL with PBS buffer (50 mM, pH = 7.4). Sequentially, 10 μL of peptide (1 mg/mL in water), 1 μL of aniline (15 mM for angiotensin and 5 mM/30 mM for cosyntropin), 1 μL of cofactor (10 mM) were added; vortexed for 15 s; and reacted for 1 h. After the reaction, the beads were separated with a magnetic separator, and 100 μL of cold methanol was added to the solution. HRMS analysis was applied to determine conversion by comparing the peaks of the substrate and product. LC-MSMS was performed using the same samples.

GFP-Y Conversion Test. GFP-Y stock solution was prepared at a concentration of 1.5 mg/mL and diluted 10 times with PBS buffer (50 mM, pH = 7.4). Namely, 10 μL of GFP-Y stock solution was added in 90 μL of PBS buffer. Sequentially, 10 μL of BmTYR-immobilized beads, 1 μL of aniline or aniline derivatives (1.5 mM for 3 equiv and 25 mM for 50 equiv), and 1 μL of cofactor (10 mM) were added; vortexed for 15 s; and reacted for 1 h. After the reaction, the beads were separated with a magnetic separator. Protein solutions were diluted 5−10 times for LC−MS analysis. HRMS analysis was applied to determine conversion by comparing the peaks of the substrate and product.

Biocatalyst Regeneration. Once the immobilized BmTYR was applied in the reactions mentioned above, it can be removed with a magnetic device. The regeneration was operated by adding fresh PBS to wash the beads. Generally, the volume ratio of PBS washing buffer to beads was 10:1. The washing procedure was repeated 2−3 times. After removal of PBS buffer, the beads were ready to use for the next reaction.

ASSOCIATED CONTENT

SI **Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c05794.

> Additional experimental details, materials, and methods, including mass spectrum data (PDF)

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Notes

The authors declare no competing financial interest.

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