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Immobilized Peptide Asparaginyl Ligases Enhance Stability and Facilitate Macrocyclization and Site-specific Ligation

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Abstract

The recently discovered peptide asparaginyl ligases (PALs) from cyclotide-producing plants are efficient and versatile tools for protein and peptide engineering. Here we report immobilization of two glycosylated PALs, butelase-1 and VyPAL2, using three different attachment methods and their applications for peptide engineering. We compared immobilization indirectly via noncovalent affinity capture using NeutrAvidin or concanavalin A agarose beads, or directly via covalent coupling of free amines on the enzyme surface with the N-hydroxysuccinimide (NHS) ester attached on agarose beads. The catalytic efficiency of immobilized PALs correlated with the distance between the biocatalysts and the solid supports, and in turn, the mobility of enzymes and the accessibility of substrates. Compared to their soluble counterparts, the site separations of immobilized PALs retain higher activity after prolonged storage and confer reusability for over 100 runs with less than 10% activity loss. We also showed that the cyclization and ligation of peptides and proteins with varying shapes and sizes can be accelerated by providing higher concentration of reusable immobilized PALs. These advantages could be exploited for large-scale industrial applications and nanodevices.

Introduction

Peptidyl asparaginyl ligases (PALs) are Asn-specific peptide ligases.¹⁻² They belong to the C13 subfamily of cysteine protease represented by asparaginyl endopeptidases (AEPs).³ Compared to

AEPs which are hydrolases, PALs reverse the enzymatic direction of AEPs and strongly promote aminolysis to catalyze peptide-bond formation.

First discovered from cyclotide-producing plants, PALs are processing enzymes responsible for the post-translational macrocyclization of the linear mature domains of cyclotides.^{1, 4} PALs catalyze the breakage of peptide bond after Asn, as well as Asp at lower pH, and result in the formation of an S-acyl intermediate and release of a peptide tail. The S-acyl intermediate undergoes S-to-N acyl shift to form a new peptide bond via nucleophilic attack by the N-terminal amine of an incoming peptide (Scheme 1). The recognition signal at the C-terminus of the target peptide/protein is generally Asx-Xaa-Yaa tripeptide where Asx must be present to bind with S1 pocket. The length of recognition signal could be longer or shorter than a tripeptide depending on a particular PAL. The most potent PAL known so far is butelase-1 with reported catalytic efficiency of $1,340,000 \text{ M}^{-1}\text{s}^{-1}$. The advantages of broad substrate tolerance, high catalytic efficiency, and traceless ligations of PALs and thus can be used in diverse applications and in combination with other ligation strategies.⁵⁻⁶

Scheme 1. PAL-mediated ligation

In the catalytic center of PALs, S-acyl intermediate are stabilized and prone to nucleophilic attack by an incoming amine rather than water molecules. The conserved S1-pocket-forming residues and the catalytic dyad Cys-His are labeled with butelase-1 numbering.

PALs and AEPs are expressed as proenzymes, generally consisting a 10-kDa pro-domain, an active 32-kDa core domain formed by six β -strands surrounded by five α -helices, and a 15-kDa C-terminal cap domain formed by six tightly-bound helices. Their biosynthetic processing is similar, involving an autolytic activation in the acidic subcellular compartments such as lysosomes and lytic vacuoles.⁷⁻¹⁰ A major structural change after the acidic activation is the cleavage and dissociation of the C-terminal cap domain, and which exposes the catalytic site in the core domain. Thus, both AEPs and PALs display intrinsic protease activity at acidic pH, generally around pH 4 for auto-activation. At near neutral to basic pH, some AEPs display weak ligase activity, and could religate the cap and core domains.¹¹⁻¹²

The pH-dependent and weak ligation activities of plant AEPs provide contrasting features to distinguish AEPs from PALs. In general, PALs display consistent and potent ligase activity from pH ranging from 4.5 to 7.5. Currently, only a handful of such PALs have been identified. They include the prototypic PAL butelase-1,¹ as well subsequent discoveries such as OaAEP1b^{4/3/4},¹³ HeAEP3¹⁴ and VyPAL2.² Interestingly, our laboratory has recently shown that the enzymatic directionality of PALs and AEPs is likely determined by the subtle sequence variations near the catalytic S1 site, and which exerts profound influence on the stability and orientation of the S-acyl intermediate and the accessibility of water or amine nucleophiles.²

Immobilizing enzymes on solid supports has a long history with a primary goal of lowering enzyme consumption by repetitively using the same batch of enzymes.¹⁵⁻¹⁶ In addition, site-separation of solid-phase immobilization reduce aggregation, leading to increased stability and activity of biocatalysts,¹⁷⁻¹⁸ and simplify the purification by avoiding contamination of products by enzymes. Proteins can also be purified and immobilized under one-pot conditions to facilitate follow-up applications.¹⁹ Consequently, immobilized biocatalysts have been developed for industrial uses to a billion-scale market, such as immobilized lactase in food industry and immobilized lipase in biodiesel production.²⁰ Compared with conventional industrial processes using chemical catalysts, immobilized enzymes are economically attractive and environmentally friendly.

There are three main-stream immobilization technologies, including attachment to carriers either or non-covalently, physical entrapment, and self-crosslinking.^{16, 21-22} For biocatalysts such as PALs with an exposed substrate-binding surface for biomolecule-based substrates, strategies based on attachment to hydrophilic porous resins by either covalent-binding and affinity-binding methods would

appear direct, convenient, and feasible to facilitate their performance in aqueous conditions.²³⁻²⁴ The natural functional groups such as surface glycans and free amines on N-terminus and lysine side chains have been exploited to achieve multipoint immobilization, and the methods should not affect the viability of the catalytic Cys.

Herein, we report and compare three different methods of immobilizing two highly efficient peptide ligases, butelase-1 and VyPAL2. The immobilization yield and activity of each immobilization method have been quantitated and compared. We showed that the immobilized ligases are stable, reusable and highly efficient in mediating macrocyclization and site-specific ligation reactions.

Results and Discussion

Preparation of active butelase-1 and VyPAL2

We used two different sources of PALs, the naturally-occurring and activated butelase-1 isolated from plant¹ and the insect-cell expressed VyPAL2 zymogen which requires an acid-induced step to be activated.² Butelase-1 used in this study was extracted from fresh plant tissues of *Clitoria ternatea* and purified *via* anion-exchange and size-exclusion chromatography as previously described.²⁵

Recombinant VyPAL2 was expressed in the proenzyme form by baculovirus expression system²⁶ in a secretory pathways using insect cells.² The activated forms of VyPAL2 were obtained by an acid-induced auto-activation at pH 4.5 and purified by size-exclusion chromatography using a sodium citrate buffer at pH 4. Both butelase-1 and expressed VyPAL2 zymogen were glycosylated, and their glycosylated forms appear as bold bands that are larger than the calculated protein weights in SDS-PAGE (Supplement Figure S1).

Non-covalent Immobilization of active PALs

Butelase-1 are glycosylated at N94 and N286 with bulky heterogeneous glycans based on our preliminary studies, and which results in an increase of additional mass of ~ 6 kDa. The recombinant VyPAL2 is glycosylated at N102, N145 and N237 with small glycans, and which results in an additional increased mass of ~3 kDa (Supplement Figure S2B). Thus, the lectin-beads would serve as an obvious first choice and the most direct method to immobilize these two glycosylated PALs *via* affinity attachment.²⁷⁻²⁹ ConA is one of the most commonly and widely used plant lectins.³⁰ ConA-attachment is reversible, allowing the recovery of glycoenzymes using elution buffers containing mannosyl and glucosyl monosaccharides³¹ (Figure 1A). For insoluble support, we used 6% crosslinked agarose beads as they are highly porous, hydrophilic, stable, inert to chemical and

physical modifications, and their relatively large pore sizes allowing free diffusion of compounds <4000 kDa.³²

A Non-covalent immobilization via Lectin-glycan affinity binding

B Non-covalent immobilization via NeutrAvidin-biotin affinity binding

C Covalent immobilization via direct coupling NHS-ester to amine of PALs

Figure 1. Immobilization of PALs, butelase-1 and VyPAL2, by non-covalent affinity binding or covalent attachment. (A) Affinity binding of glycosylated PALs with concanavalin A (ConA) agarose beads to give ConA-PAL **1** and ConA-Vy2 **2**. (B) Affinity binding of biotinylated PALs with NeutrAvidin agarose beads to give NA-Bu1(b) **3** and NA-Vy2(b) **4**. Biotinylated PALs were prepared by coupling of succinimidyl-6-(biotinamido)-6-hexanoate (NHS-LC-biotin) to the amino groups of PALs presented on N-terminus and Lys side chain. (C) Covalent attachment of PALs with active NHS-ester on agarose beads to give agarose-Bu1 **5** and agarose-Vy2 **6**. The distance between enzymes and the agarose beads are calculated by the sizes of the spacer moieties and the pre-coupled affinity binding ligands.

Affinity binding of glycoenzymes with ConA beads was performed by mixing 1 mg of freshly prepared ligases and 1 mL beads that were pre-equilibrated with a pH 6.5 ConA-reaction buffer and gently shake at 4 °C for 3 h. The low enzyme loading of 1 mg/mL (equivalent to ~27 µM of ligase) and the gentle shaking could facilitate diffusion of solutes.^{16, 23} Beads were washed with ConA reaction buffer after binding. In the tested condition, butelase-1 immobilized on ConA-beads gave ConA-Bu1 **1** in 39% yield as 61% of enzymes remained in the solution. To compare the efficiency of different immobilization methods, we used a fixed enzyme-to-beads ratio at the beginning of immobilization, rather than pursuing 100% yield by using more beads. In contrast, ConA-bound VyPAL2 was

dissociated from beads quickly after a few rounds of washing (Supplement Figure S3). Consequently, we excluded the ConA-Vy2 **2** in all subsequent experiments.

The observed difference of ConA affinity to butelase-1 and VyPAL2 can be attributed to their glycosylated forms. Plant-derived butelase-1 contains the complex high-mannose *N*-glycans that bind to ConA with high affinity.³³⁻³⁴ In contrast, the insect cell-expressed VyPAL2 contains simple *N*-glycans that binds to ConA with low affinity.³⁵ In the crystal structure of VyPAL2, confirmed glycans are not bigger than tri-saccharides.² In addition, ConA-immobilized PALs are not suitable to catalyze reactions containing either soluble sugars or glycoproteins that may bind to ConA and exchange with the immobilized PALs.

For comparison, we experimented a second non-covalent immobilization by exploiting the exceptionally high binding between biotin and avidin.³⁶⁻³⁷ The avidin-biotin binding is considered practically irreversible with dissociation constants in the range of 10^{-15} M.³⁸ To eliminate non-specific lectin binding, we used a deglycosylated form of avidin, NeutrAvidin (NA), which retains the strong affinity binding of amine-linked biotins as the glycosylated avidin (Figure 1B).

This method requires modifying some of the primary amines of PALs with biotins. The sequences of both active butelase-1 and VyPAL2 contain multiple Lys residues, which are not located close to the catalytic site or substrate binding surface (Supplement figure S2C). Thus, immobilization of PALs involving Lys-NH₂ would not be expected to hinder the catalytic site of PALs.

To biotinylate the lysine side chains, we used succinimidyl-6-(biotinamido)-6-hexanoate (NHS-LC-biotin) for biotinylation of active butelase-1 and VyPAL2. The coupling reaction of N-hydroxy succinimide ester (NHS-ester) to primary amines on the ligases is generally performed in basic conditions with pH ranging from 7.2 to 9.0. Since active PALs, regardless of whether they are plant-produced or insect-cell expressed, are less thermally stable in basic conditions (Supplement Figure S4), we performed the biotinylation at pH 7.4 at 4 °C to minimize degradation of the ligases (Supplement Fig. S6). Under pH 7.4, both N-terminal amine and Lys-NH₂ at multiple sites are accessible to NHS-coupling.³⁹ Affinity binding of the biotinylated butelase-1, Bu1(b), and biotinylated VyPAL2, Vy2(b) with NA beads was performed at pH 6.5 at 4 °C for 3 h. After immobilization, beads were washed with chilled pH 6.5 reaction buffer. This method resulted in 49% and 45% immobilization yield to give NA-Bu1(b) **3** and NA-Vy2(b) **4**, respectively.

Covalent Immobilization of active PALs by direct coupling

The covalent approach confers an irreversible stable immobilization. We selected a well-established covalent immobilization method by coupling the primary amines on the N-terminus or Lys-side chains of PALs to an NHS-ester (Figure 1C).⁴⁰⁻⁴¹ Similar to the previously described biotinylation of ligases with NHS-LC-biotin, the direct immobilization on NHS-activated agarose beads were performed at pH 7.4 at 4 °C overnight to give agarose-Bu1 **5** and agarose-Vy2 **6**. The beads were then washed with chilled pH 6.5 reaction buffer. Our results showed that this method directly immobilized active butelase-1 and VyPAL2 with 83% and 81% yield, respectively.

Activity of Immobilized PALs

The activity of immobilized PALs was determined by comparing the initial reaction rate catalyzed by immobilized PALs with the rate catalyzed by their soluble counterparts. This parameter is also called expressed activity or recovered activity.⁴² The ligase activity of free butelase-1 or VyPAL2 was measured by the macrocyclization of a model peptide substrate KN14-GL, KLGTSPGRLRYAGN-GL **7**, sequence derived from a natural cysteine-rich peptide bleogen pB1⁴³ with a C-terminal PAL-recognition signal tripeptide NGL to give the end-to-end cyclic product cKN14 **8** (Supplement Figure S6). We used a substrate concentration of 0.2 mM to maximize the reaction rate because this concentration is much higher than the known Michaelis constant K_M of butelase-1 and VyPAL2. Reaction was quenched after 5 min and the amount of cKN14 produced in each reaction was measured by RP-HPLC. The standard curves of reaction rate against the concentrations of free enzymes were plotted to calculate the turnover rate (Supplement Figure S7). The effective concentration of immobilized PALs were determined by interpolating the measured reaction rates of immobilized PALs on the standard curve.

Table 1 summaries our results which show that non-covalent attachment of ConA-Bu1 **1** and the NA-linked biotinylated enzymes NA-Bu1(b) **3** and NA-Vy2(b) **4** retained 50% and 20-30% activity of their soluble enzymes, respectively, while the covalent attachment of agarose-Bu1 **5** and agarose-Vy2 **6** retained about 5% activity of their soluble enzymes. Direct attachment to agarose beads via a four-atomic spacer that was calculated to be about 1 nm only in agarose-Bu1 and agarose-Vy2 is perhaps too short (Figure 1). In contrast, ConA-Bu1 has a spacer longer than 8 nm (ConA tetramer + glycan)⁴⁴ and the NeutrAvidin-immobilized NA-linked biotinylated enzymes have a spacer approximately 8 nm long (NeutrAvidin tetramer + NHS-LC-biotin).⁴⁵ The correlation between the activity of immobilized PALs with the distance between the enzymes and solid supports suggested that short spacer may reduce enzyme mobility and accessibility of substrates. To improve the activity of immobilized enzymes *via* direct attachment method, a longer spacer needs to be exploited.

Table 1. Summary of immobilization yield and effective concentration of immobilized PALs

		PAL Loading		Effective	
		Obs. Conc	Yield	Conc	Ratio
		(μ M)	(%)	(μ M)	(%)
Non-covalent					
ConA-Bu1	1	10.5	39	4.6	44
NA-Bu1(b)	3	13.2	49	3.3	25
NA-Vy2(b)	4	12.1	45	2.8	23
Covalent					
Agarose-Bu1	5	22.4	83	1.1	5
Agarose-Vy2	6	21.8	81	0.6	3

Expected maximal PAL concentration on beads is 1 mg/mL $\approx$ 27 μ M.

Obs. Conc = observed protein loading of PAL on beads.

Yield = Observed concentration/expected maximal concentration.

Ratio = V(immobilized enzyme) / V(free enzyme) = Effective Conc./Obs. Conc.

ConA = concanavalin A.

NA = NeutrAvidin.

(b) = biotin.

Immobilized PALs display high operational stability and prolonged storage stability

Solid-phase immobilization of PALs would minimize self-aggregation and auto-proteolysis, and in turn, enhanced stability. To show the operational stability and reusability, we reused each immobilized PAL 100 times and analyzed their efficacy in the cyclization of linear peptide KN14-GL **7**. In each run, we used the same batch of immobilized-PAL agarose beads, and the reaction mixture was analyzed using C18 reversed-phase HPLC (Figure 2A). Figure 2B summarizes our product analysis of five immobilized PALs, all of which showed that >90% catalytic activity was retained after 100 runs.

Figure 2. Operational stability of immobilized PALs. (A) RP-HPLC monitoring of cyclization of KN14-GL into cKN14 by five immobilized PALs **1**, **3-6** in 100 repeated reactions. For each experiment, 100 μ L of reaction mixture (pH 6.5) containing 0.1 mM KN14-GL was given. Amount of beads used was adjusted according to the effective concentration of each type to give the molar ratio of effective enzyme:substrate = 1:350 ~ 1:600. Reaction was conducted at room temperature for 3-5 min. (B) Summary of operational stability of five immobilized PALs **1**, **3-6**.

To show the prolonged shelf-life of immobilized PALs stored at 4 $^{\circ}$ C, we monitored their ligase activity every week for a period of two months in cyclizing the peptide substrate GN14-HV (GISTKSIPPISYRN-HV, **9**) or GN14-SLAN (GISTKSIPPISYRN-SLAN, **10**) to yield cGN14 **11** by MALDI-TOF mass spectrometry (Supplement Figure S8). Figure 3 shows that immobilized PALs are more stable than their soluble counterparts in prolonged storage. All five immobilized PALs, retained >90% activity after nine weeks. In contrast, butelase-1 or VyPAL2 lost about 30% activity after a two-month storage under the same storage conditions.

Figure 3. Stability of five immobilized PALs **1**, **3-6**, butelase-1 and VyPAL2 after storing in 4 °C for 1, 29 and 64 days.

It is worthwhile to note that addition of a reducing reagent, such as Tris(2-carboxyethyl)phosphine (TCEP), dithiothreitol (DTT) or β -mercaptoethanol (β -ME), is crucial for keeping the catalytic Cys of active PALs in the reduced form. Both soluble and immobilized PALs stored in a non-reducing buffer lost activity in two weeks due to the oxidation of the catalytic cysteinyl sulfhydryl. Once the sulfhydryl is oxidized leading to inactivation, the ligase activity can be restored, sometimes but not always, after treating with buffers containing one or more reducing reagents. We also observed that immobilized butelase-1 are slightly more stable than immobilized VyPAL2, suggesting that plant-derived PALs may benefit from higher-level of glycosylation which enhances their molecular stability from proteolytic degradation.

Applications of immobilized PALs for ligation reactions

The reusability of immobilized PALs allows us to use a much higher enzyme concentration than their soluble counterpart to accelerate the catalytic ligation reactions. In the following five examples, we used NeutrAvidin-immobilized NA-Bu1(b) **3** and NA-Vy2(b) **4** to showcase this advantage of immobilized PALs for cyclization and ligation, as well as their uses in a continuous flow system.

The first example was a cyclization reaction of a SFTI-substrate containing a sterically hindered Pro at the P2 position, which causes a slower ligation reaction than those substrates with a less

hindered amino acid occupying the same P2 position.⁴⁶⁻⁴⁷ Figure 4A shows that butelase-1-mediated cyclization of a 14-residue disulfide-containing peptide SFTI analog, GRCTKSIPPICFPN-HV **12**, is 50% complete to yield the cyclic SFTI **13** after 30 min (Supplement Figure S9). In contrast, increasing the effective concentration of the NeutrAvidin-immobilized butelase-1 NA-Bu1(b) **3** five-fold resulted in accelerating the cyclization ligation to complete within 10 min.

A Cyclization of peptide with Pro at P2 position

B Cyclization of naturally-cyclic protein

Figure 4. Macrocyclization of peptides and proteins by NA-Bu1(b) **3** at pH 6.5 at room temperature for 10 min with gentle shaking. (A) Cyclization of SFTI(D/N)-HV **12** (0.1 mM, calc. mass 1767.9 Da, obs. Mass 1767.9 Da) into SFTI(D/N) **13** (calc. mass 1513.8 Da, obs. Mass 1513.3 Da) by NA-Bu1(b) **3** to give 95% crude yield (*) of cyclic SFTI(D/N) **13** as determined by MALDI-TOF MS. (B) Cyclization of the folded linear bacteriocin precursor AS-48K **14** (50 μ M, calc mass 7783.5 Da, obs. Mass 7779.1 Da) by NA-Bu1(b) **3** to give cyclic AS-48 **15** (calc. mass 7145.1 Da, obs. Mass 7148.1 Da) with 83% yield as determined by UHPLC.

In the second example, we compared the soluble and NeutrAvidin-immobilized butelase 1 for cyclizing a 70-residue protein, circular bacteriocin AS-48. This circular bacteriocin is a highly sought-after food preservative produced by the lactic bacteria for its ability to kill broad-spectrum of microorganisms.⁴⁸⁻⁴⁹ AS-48 is the second largest naturally-occurring head-to-tail macrocycles known and it does not contain a cysteine in its sequence to be exploited by the thia-zip cyclization developed by our laboratory 20 years ago.⁵⁰ We used the free butelase-1 to cyclize the folded AS-48K **14**, which contains an N-terminal VV dipeptide and C-terminal NHVKKK hexapeptide sequence for butelase-1

recognition.⁵¹ Using an enzyme:substrate ratio of 1:100 at 37 °C, the reaction was complete in 1 h whereas increasing effective concentration of NA-Bu1(b) five-fold of the free butelase-1 accelerated the completion of cyclization in 10 min in a 83% isolated yield of cyclic AS-48 **15** (Figure 4B).

The third example is PAL-mediated cyclooligomerization of peptides. This reaction involves both oligomerization and head-to-tail cyclization of the nascent oligomers. Using this approach we demonstrated the formation of bioactive cyclo-oligomeric peptides using a simple peptidyl monomer as building block.⁴⁷ The cyclooligomerization of RV7 (RLYRNHV, **16**), using the NeutrAvidin-immobilized butelase NA-Bu1(b) in an enzyme:substrate ratio of 1:100, completed within 40 min to yield 83% cyclodimer **c17** and 8% cyclotrimer **c18** of RLYRN (Figure 5). In contrast, the reaction using butelase-1 with an enzyme:substrate ratio of 1:500, in which effective concentration of the soluble form was five-fold lower the immobilized form, did not complete after 4 h (Supplement Figure S10).

Figure 5. Cyclooligomerization of peptide RV7 **16** (0.2 mM, calc mass 956.5 Da, obs. Mass 957.7 Da) by NA-Bu1(b) **3** to give 85% cyclodimer **c17** (calc mass 1404.8 Da, obs. Mass 1405.9 Da) and 8% cyclotrimer **c18** (calc mass 2107.2 Da, obs. Mass 2109.3 Da) in 40 min.

In the last two examples, we used the PAL-mediated intermolecular ligation in a continuous-flow system. Unlike cyclization reactions which have the advantage of high effective concentrations, intermolecular ligations would require both high concentrations of substrates and enzymes, and thus immobilized PALs can be reused in high concentration to overcome this limitation. Using a self-packed column (internal diameter 4 mm) with NA-Vy2(b) **4** beads, we performed peptide ligation of Ac-RYANGI **19** (10 µM) with a synthetic fluorescent peptide GLAK(FAM)RG **20** (100 µM) under different flow rate from 0.05 to 0.5 mL/min (Figure 6A). At flow rate of 0.05 mL/min, we observed completed ligation reaction to yield Ac-RYANGLAK(FAM)RG **21**. Finally, we used this packed-bed column of NA-Vy2(b) to label a 193-residue recombinant protein, anti-Her2 DARPin9_26-NGL⁵² **22**

with GLAK(FAM)RG **20**. A reaction containing 1 μ M DARPin9_26-NGL and 5 μ M GLAK(FAM)RG afforded 78% yield of DARPin9_26-NGLAK(FAM)RG **23** with a flow rate of 20 μ L/min (Figure 6B). The unreacted peptides were readily removed from ligation products by dialysis or centrifugal filters with molecular weight cut off >3 kDa.

A Continuous-flow peptide ligation

B Continuous-flow protein labeling

Figure 6. Continuous-flow peptide and protein ligation by NA-Vy2(b) **4**. (A) Ligation of Ac-RYANGI **19** (calc mass 735.4 Da, obs. Mass 734.3 Da) and GLAK(FAM)RG **20** (calc mass 958.7 Da, obs. Mass 959.6 Da) in 1:10 molar ratio by NA-Vy2(b) **4** to yield ligation product Ac-RYANG(LAK(FAM))RG **21** (calc mass 1506.0 Da, obs. Mass 1507.0 Da). (B) C-terminal fluorescent labeling of recombinant protein DARPin9_26-NGL **22** (calc. mass 19968 Da, obs. mass 19950 Da) by GLAK(FAM)RG **20** in 1:5 molar ratio to give fluorescent protein DARPin9_26-NGLAK(FAM)RG **23** (calc. mass 20728 Da, obs. mass 20703 Da). Reaction products were analyzed by MALDI-TOF MS in the positive-ion linear-mode and the crude yield was calculated by peak area.

Conclusions

In this report, we compare different methods to immobilize naturally-occurring butelase-1 and recombinantly expressed asparaginyl ligases VyPAL2. Immobilization of PALs overcomes the limitations of soluble enzymes, which include aggregation and autolysis into less active forms, albeit at a very slow rate at near neutral pH. The major advantages of immobilization on a solid support provide site separation and psedodilution to prevent trans-autolytic degradation and enhance stability.

We confirmed these major advantages of the immobilized ligases: reusable >100 runs with undiminished enzymatic activity, enhanced stability and prolonged shelf-life, and simpler downstream purification process. More importantly, site-separation of immobilized enzymes permits the use of high enzyme concentrations to accelerate ligation reactions to complete in minutes, such as cyclization, cyclooligomerization and ligation reactions either under one-pot conditions or in a continuous flow-reactor. These advantages bode well in reducing amount of ligases, scale-up use for industrial scale and adaptation for nanodevices.

Material and methods

Enzyme, beads and substrates

Butelase-1 was extracted from plant material of *Clitoria ternatea* grown in the local herb garden. After a few rounds of size-exclusion and anion exchange chromatography on HPLC (Shimadzu) as described in previously protocol,²⁵ purified butelase-1 was obtained and store in a pH 6.0 buffer containing 20 mM sodium phosphate, 0.15 M NaCl, 5 mM β -mercaptoethanol (β -ME) and 20% sucrose at 4 °C or -80 °C. Recombinant VyPAL2 was expressed using Bac-to-Bac® Baculovirus system (Thermo Fisher Scientific) in Sf9 insect cells via a secretory pathway governed by the N-terminal GP64 signal peptide as previously described.² Expressed proenzymes were purified by Nickel-affinity binding on HisTrap Excel column (GE Healthcare), ion-exchange chromatography on HiTrap Q column (GE Healthcare) and size exclusion chromatography on HiLoad Superdex 75 column (GE Healthcare) using a NGC-FPLC System (Bio-Rad). Acid-induced autoactivation was performed at pH 4.5 at 4 °C overnight in the presence of 1 mM Dithiothreitol (DTT) and 0.5 mM N-lauroylsarcosine. Activated enzymes with molecular weight about 35 kDa was purified again by size-exclusion chromatography with a pH 4.0 citrate buffer. Purified active enzymes was stored in a pH 6.5 buffer containing 20 mM sodium phosphate, 0.1 M NaCl, 5 mM β -ME and 20% sucrose at 4 °C or -80 °C.

All beads are from commercial sources. Pierce™ NHS-Activated Agarose beads (Thermo Fisher Scientific) have protein loading of 1-20 mg protein per mL. Pierce™ NeutrAvidin™ Agarose beads (Thermo Fisher Scientific) have protein loading of >8 mg biotinylated protein per mL. Concanavalin A (ConA) Agarose beads (G-Biosciences) have protein loading of 15-30 mg ConA per mL.

All peptide substrates used in the activity assays, including KN14-GL, GN14-HV, GN14-GL, GN14-SLAN, SFTI(D/N)-HV, RV7, and GLAK(FAM)RG (FAM, Fluorescein amidite) were synthesized

by Fmoc chemistry on a Liberty-1 microwave synthesizer (CEM) using the protocol described before.⁴⁷ The protein substrate AS-48K was also synthesized chemically. Purified AS-48K was dissolved in 8 M urea and underwent refolding by dialysis.⁵¹ The protein substrate DARPin9_26-NGL (sequence in Supplement Table S1) was cloned into pET28a(+) vector with N-terminal His6-TEV-GLGSG sequence and C-terminal GSGSNGL tail. Recombinant expression was done in Shuffle® T7 *E. coli* (New England Biolabs) after a 24 h induction by 0.1 mM IPTG at 16 °C. Soluble proteins were extracted from cell lysate and purified by Ni-NTA affinity chromatography on a HisTrap HP 5 mL column (GE Life Sciences) and ion-exchange chromatography on a HiTrap Q 5 mL column (GE Life Sciences) using a NGC-FPLC System (Bio-Rad).

Thermostability of PALs

1 µg enzyme was mixed with 8x SYPRO orange fluorescent dye (Thermo Fisher Scientific) and diluted to a final volume of 25 µL with a series of buffers (50 mM sodium phosphate, 0.1 M NaCl, 5 mM β-ME) with pH ranged from 5 to 8 in a 96-well plate. pH buffers are ThermoFluor Assay was conducted in a Real-Time PCR Detection System (Bio-Rad) with temperature increased from 25 to 85 °C. The melting temperature was calculated by plotting the change of RFU per degree against temperature.

Reactions mediated by soluble and immobilized PALs

All reactions by soluble or immobilized PALs were conducted using a phosphate reaction buffer (20 mM sodium phosphate, pH 6.5, 0.1 M NaCl, 1 mM DTT) with the exception of ConA-Bu1, of which the ConA-reaction buffer contained additional 5 mM CaCl₂ and 5 mM MgCl₂. The reaction pH was kept at 6.5 to maximize the enzyme activity. The reducing reagent DTT was added freshly before use. Reactions were performed at room temperature without heating to prevent degradation of reused enzymes. Reaction mixtures were analyzed by MALDI-TOF mass spectrometry or reversed-phase (RP) HPLC.

Immobilization on NHS-activated agarose beads

Enzyme solutions were prepared with cold PBS (pH 7.4) to the concentration of 10 µM and added to NHS-Activated Agarose Dry Resin (75 mg requires 1 mL solution) and the preparation was shake slowly at 4 °C for 3 h. The mixture was then loaded into a chilled spin column and the flow through was collected. Beads were wash with 2x bead-volume of wash buffer (20 mM sodium phosphate, 1 mM DTT, 5% glycerol, pH 6.0 or 6.5) and the flow through was collected after binding and washing. Excessive NHS groups were blocked by soaking beads in quenching buffer (1 M Tris-HCl, pH 7.4) for

1 h with gentle shaking at 4 °C. Beads were washed again with reaction buffer, followed by the addition of 2x bead-volume of reaction buffer with 20% ethanol and keeping the slurry at 4 °C.

Biotinylation and Immobilization on NeutrAvidin agarose beads

Enzyme solutions were prepared with cold PBS (pH 7.4) containing 5 mM β -ME to the concentration of 10 μ M and mixed with 20-fold molar equivalent of Ezlink® NHS-LC-biotin (succinimidyl-6-(biotinamido)-6-hexanoate, spacer length ~2.2 nm, Thermo Fisher Scientific) dissolved in DMF (stock concentration 10 mM). Biotinylation was conducted 4 °C overnight. Excessive NHS-LC-biotin was removed by buffer exchange with PBS (pH 7.4) using Vivaspin 10 kDa MWCO centrifuge concentrators (Sartorius, Germany). After buffer exchange, activity of biotinylated enzymes was compared with the untreated enzymes to show no activity loss. NA agarose beads was equilibrated with pH 6.5 reaction buffer. A mixture of 0.2 mL equilibrated beads with 1 mL biotinylated enzymes (5 μ M) was prepared by gentle shaking at 4 °C for 3 h, followed by loading into a chilled spin column and collection of flow through. Beads were washed with 20x bead-volume of reaction buffer, and kept as a slurry at 4 °C in the presence of 2x bead-volume of reaction buffer with 5 mM β -ME and 20% ethanol.

Immobilization on concanavalin A agarose beads

ConA beads were equilibrated with 10 bead-volume of cold equilibration buffer (1 M NaCl, 5 mM MgCl_2 , 5 mM CaCl_2 , pH 7.2, Mg ions were used here to substitute Mn ions).⁵³ Enzyme solutions were prepared with ConA reaction buffer to the concentration of 5 μ M. One milliliter of enzyme solution was mixed with 1 mL of equilibrated ConA beads with slowly shaking at 4 °C for 3 h. The mixture was loaded into a chilled spin column and the flow through was collected. Beads were wash with 20x bead-volume of ConA reaction buffer and kept as a slurry at 4 °C in 2x bead-volume of ConA reaction buffer with 20% ethanol. Activity of immobilized enzymes stored with or without ethanol showed no significant difference.

Determination of immobilization yield

The concentration of unbound proteins in the flow through after binding and washing were determined using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) by measuring the UV absorbance at 280 nm. For the flow through after washing, a concentration step using centrifugal filter (Vivaspin 10 kDa MWCO, Sartorius) was performed to bring up the protein concentration readable by spectrophotometer which has sensitivity threshold of 0.008 at 280 nm.

Determination of activity of immobilized PALs

Free butelase-1 and VyPAL2 were prepared in stock concentrations ranged from 1 to 8 μM . In each reaction, 1 μL enzyme stock was added to 100 μL 0.2 mM KN14-GL so the final enzyme concentrations ranged from 10 to 80 nM. After 5 min incubation at room temperature, the reaction was quenched by adding 0.5% TFA to bring down the pH to 2 and all the reaction solution was injected into analytical RP-HPLC (Aries-C18, 150x4.6 mm, 3 μ , Shimadzu). The amount of cKN14 was calculated by the peak area at 220 nm in the HPLC profile. The initial reaction rate V was then calculated by increase of cKN14 concentration per second. A standard curve of reaction rate against enzyme concentration was plotted for each PAL, which reflects the turnover rate of free enzymes in the tested system. For the ease of calculation, the units of enzyme concentration and the corresponding reaction rate were converted based on the stock concentrations of free enzymes, as (μM) and ($\mu\text{M/s}$), respectively. Activity of immobilized PALs were examined using 10 μL beads in a 1 mL system with the same experimental setting. 100 μL out of 1 mL reaction solution was injected into RP-HPLC for product quantification. The reaction rate of each immobilized PAL was also converted into ($\mu\text{M/s}$) and compared with the stand curve to calculate the actual effective concentration. The activity ratio was calculated by the rate of immobilized PALs against rate of soluble PALs.

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SUPPORTING INFORMATION

Schemes, gel images, amino acid sequences, structures, thermal stability data, mass spectrometry and HPLC profiles were included.

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