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Rational immobilization of lipase by combining the structure analysis and unnatural amino acid insertion

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ABSTRACT

Improving the conventional covalent immobilization of enzyme and avoiding random covalent linkage to protect enzyme's active sites from unwanted covalent linkage at the mean time are the fundamental topics for enzyme immobilization. In this study, unnatural amino acid was introduced into a recombinant lipase and applied for the rational and smart covalent enzyme immobilization. In the first step, Tyr50, 137, 243, 274, and 355 of lipase were replaced with AzPhe unnatural amino acid based on the analysis of enzyme structure. Then, these novel recombinant lipases were coupled to support using strain-promoted azide–alkyne cycloaddition (SPAAC), respectively. Subsequently, both the effect of the immobilization site and the thermo-stability of immobilized lipases were also examined. The relative activities of the immobilized AzPhe-Lip243 and AzPhe-Lip274 were enhanced to 121.33% and 137.06%, respectively, presenting 6.0 and 6.8 fold higher than those of the lipase traditionally immobilized using glutaraldehyde (IM-Lip-GA). In addition, all the immobilized lipases presented better specific activity except for AzPhe-Lip355, whose immobilization site was close to its active site. The rational immobilized lipases also presented better thermo-stability than those by traditionally immobilization method (glutaraldehyde). To sum up, with the aid of protein structure analysis, unnatural amino acid can be rationally inserted into enzyme sequence to inform and direct the covalent enzyme immobilization. This method can be further developed for one-step enzyme purification and immobilization and applied to a broad scope of enzymes.

1. Introduction

As a bridge technology, enzyme immobilization enables free enzymes into industrial biocatalysis applications and is receiving attentions from researchers [1,2]. Among the immobilization methods, covalent immobilization is highly preferred because the tight covalent chemical linkage between enzyme and support can prevent the enzymes from leaking into aqueous media and contaminating products [3,4]. However, covalent linking reactions only occur for the amino acids with reactive groups such as Lys, Thr and Cys. Further, when using the cross-linkers include glutaraldehyde, *p*-benzoquinone and epoxy groups, linking reactions were always initiated randomly between the functional groups and the side chain groups of amino acids from enzyme. In general, the sites of amino acids are not preferred for covalent immobilization to protect protein structure burying or blocking active sites of enzymes (Fig. 1A). Moreover, increased steric hindrance may prevent substrate molecules from accessing the active sites of enzymes, leading to decreased activities of immobilized enzymes.

Although "traditional site-specific immobilization" of enzymes has been frequently reported, enzymes immobilization after chemical modifications are still dependent on amino acids with functional groups, such as Lys, Ser, and Cys etc. [5] The chemical modification only occurs to these amino acids which may not be suitable for the immobilization to keep the enzyme active site exposed to substrate. The unwanted linkages may take place near the enzyme active sites of enzymes and often resulted in decreased enzyme catalytic activity by 20%–80% [6–8]. Thus, this immobiliza-

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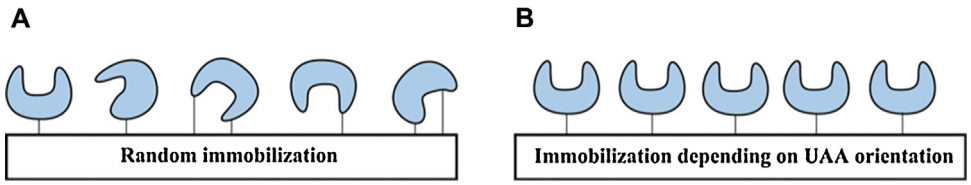

Fig. 1. Random covalent immobilization and rational and oriented immobilization depending on the UAA (A, Random covalent immobilization; B, Rational and oriented immobilization depending on the UAA).

tion method is really non-site-specific because the immobilization sites are generally chemical modified amino acids and not decided as the real requirement for catalysis and your determination. Another approach is using fusion proteins and tags for site-specific immobilization and has been reported by the Jaworsk's group [9], Distefano's group [10] and our group [11]. However, immobilization based on the fusion proteins or tags only applies to the C or N end of the enzyme protein peptide chain. In a nutshell, limited choices of available immobilization sites always lead to unfavorable covalent immobilization.

In this work, to avoid unwanted covalent linkage, the immobilization sites were rationally designed before covalently linking reactions (Fig. 1B). The selection of immobilization sites was based on structure analysis of the lipase from *Geobacillus sp*. Unnatural amino acid (UAA) was inserted into the desired site to replace the original native amino acid as designed. Afterwards, the recombinant lipases were coupled to support by using the bioorthogonal chemical reaction. Lastly, the activity and thermostability of the immobilized lipases were determined.

2. Experimentals

2.1. Materials

Plasmids such as pET24a(+), pET28a(+), pACYC184, and pBR322 were obtained from Invitrogen Corporation, USA; *Escherichia coli* DH5α and *E. coli* BL21(DE3) were obtained from Beijing Jinshijing Ltd., China. Plasmid pET28a-uox, encoding the lipase with a His6 tag at the C-terminal under the control of T7 promoter was stored in our laboratory. All of the plasmids were verified by DNA sequencing. The gene encoding lipase from *Geobacillus* sp. SBS-4S (GenBank: AB457187.1) [12] and other necessary genes were synthesized by Shanghai Jierui Biotechnology Ltd., China. Triblock copolymer poly(ethylene glycol)-blockpoly, (propyleneglycol)-block-poly (ethylene glycol) (P123), 1,3,5-Trimethylbenzene (TMB) and ammonium fluoride, was purchased from Sigma–Aldrich. Other chemicals were of analytical grade and provided by Sinopharm Chemical Reagent (Shanghai, Sinopharm). Deionized water with a resistance greater than 18 MQ was obtained from a Millipore-Q Plus water purifier.

2.2. Plasmids construction

2.2.1. Construction of pACYC184-2Mj*tRNA*$^{\mathbf{Tyr}}_{\mathbf{CUA}}$ *with the* lpp *promoter and* rrnC terminator

The gene encoding the *Mj*tRNA$^{Tyr}_{CUA}$ (Underlined) was synthesized and obtained from Shanghai Jierui Biotechnology Ltd. based on a previous as report [13]. The *lpp* promoter (Italic) and *rrnC* (Bold) terminator were added to the 5′- and 3′-end, respectively.

*CGAACGATCAAAAATAAGTGCCTTCCCATCAAAAAAATATTCTCAACAT AAAAAACTTTGTGTAATACTTGTAACGCTACATGGAGATTAACTCAATCTAG CCGG*CGGTAGTTCAGCAGGGCAGAACGGCGGACTCTAAATCCGCAT GGCGCTGGTTCAAATCCGGCCCGCCGGACCA**GAAATCATCCTTAGC GAAAGCTAAGGATTTTTTTTATCT**

The synthesized product was subsequently amplified by Polymerase Chain Reaction (PCR) via two pair of primers as the following. And the amplified DNA fragments were inserted between the HindIII and BamHI sites (Bold) of plasmid pACYC184, to generate the plasmid pACYC184-2*Mj*tRNA$^{Tyr}_{CUA}$.

2.2.2. Generation of the plasmid *pBR322*-Mj*TyrRS10*

To enhance heterogeneous expression of the gene encoding *Mj*TyrRS (Gene ID: 1451246) from *Methanococcus jannaschii* in *E. coli* BL21 (DE3), codon optimization was performed on this gene and the *glnS* promoter and terminator were added to the 5′ and 3′ end, respectively. Full DNA fragment was also synthesized and obtained from Jierui Biotechnology Ltd.

glnS promoter

CCGAGCTCCCGGGTCATCAATCATCCCCATAATCCTTGTTAGATTAT CAATTTTAAAAAACTAACAGTTGTCAGCCTGTCCCGCTTTAATATCAT ACGCCGTTATACGTTGTTTACGCTTTGAGGAATCCACG

glnS terminator

TTTTAAGTTTCGCTATGCCGGATGGGGCGTTTACGTCGCATCCGGC AAGGAACAGACAAACAGTTTCAAACGCTAAATTGCCTGATGCGCTAC GCTTATCAGGCCTACATGATCTCTGCAATATATTGAGTTTGCGTGCTT TTGTAGGCCGGATAAGGCGTTCACGCCGCATCCGGCAAGAAACAGC AAACAATCCAAAACGCCGCGTTCAGCGGCGTTTT

According to a previous report [14], DNA sequence of the MjTyrRSs mutant for recognizing AzPhe revealed that the major activity sites of the lipase included 31Ala, 32Tyr, 107Glu, 158Asp, and 159Ile. To enhance the recognization specificity of *Mj*TyrRS in the incorporation of AzPhe, Asp286 was replaced by Arg286 in addition to the above five mutations above. The fragments of the obtained *Mj*TyrRS mutants using SOE PCR and Hifi PCR under the control of the *E. coli* GlnRS promoter and terminator were digested by restriction enzymes BamHI and SalI, and inserted into the predigested plasmid pBR322 to afford plasmid pBR-TyrRS.

2.2.3. Generation of plasmid pET24a-lip_{TAG}

The protein sequence and structure of the lipase used in this work have been reported (http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdb_strview.cgi) (Fig. 2). The essential residues of active sites were Ser114k, Asp318 and His359, according to previous reports [12,15]. The images of protein structure were prepared by Accelry Discovery Studio 3.0 according to the crystal structure of lipase from *Geobacillus Sp*. Sbs-4s at 1.66 Å resolution (PDB code: 3AUK).

Before the mutation of lipase, we examined its protein sequence and structure and decided several mutation sites such as Lip-50, Lip-137, Lip-243, Lip-274, and Lip-355. The DNA sequence encoding the amino acids on these sites was replaced by ATG using SOE PCR, respectively. The genes of lipase amber^{50}, lipase amber^{137}, lipase amber^{243}, lipase amber^{274} and lipase amber^{355} mutants had a His6 tag at their C-terminal and their expressions were under the control of the T7 promoter and T7 terminator. These genes were excised with NdeI and HindIII and inserted into the predigested pET24a to generate plasmids pET24a-lip50_{TAG}, pET24a-lip137_{TAG}, pET24a-lip243_{TAG}, pET24a-lip274_{TAG}, and pET24a-lip355_{TAG}.