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Enhanced Enzyme Stability Through Site-Directed Covalent Immobilization

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ABSTRACT

Breakthroughs in enzyme immobilization have enabled increased enzyme recovery and reusability, leading to significant decreases in the cost of enzyme use and fueling biocatalysis growth. However, current enzyme immobilization techniques suffer from leaching, enzyme stability, and recoverability and reusability issues. Moreover, these techniques lack the ability to control the orientation of the immobilized enzymes. To determine the impact of orientation on covalently immobilized enzyme activity and stability, we apply our PRECISE (Protein Residue-Explicit Covalent Immobilization for Stability Enhancement) system to a model enzyme, T4 lysozyme. The PRECISE system uses non-canonical amino acid incorporation and the Huisgen 1,3-dipolar cycloaddition "click" reaction to enable directed enzyme immobilization at rationally chosen residues throughout an enzyme. Unlike previous site-specific systems, the PRECISE system is a truly covalent immobilization method. Utilizing this system, enzymes immobilized at proximate and distant locations from the active site were tested for activity and stability under denaturing conditions. Our results demonstrate that orientation control of covalently immobilized enzymes can provide activity and stability benefits exceeding that of traditional random covalent immobilization techniques. PRECISE immobilized enzymes were 50 and 73% more active than randomly immobilized enzymes after harsh freeze–thaw and chemical denaturant treatments.

1. Introduction

According to Environmental Protection Agency statistics, the USA alone contains more than 13,500 chemical manufacturing facilities providing shipments valued at over $555 billion annually (EPA, 2011). Unfortunately, traditional chemical manufacturing processes often require the use of toxic reagents, strong acids and bases, and have high energy costs while suffering from low product specificity. These processes produce more than 1.5 million tons of air pollution and over 10 billion pounds of solid waste annually, much of it toxic (EPA, 2011).

A more environmentally friendly alternative to conventional processes is the use of biocatalysts, or enzymes, to catalyze reactions (Bornscheuer et al., 2012). Enzyme use eliminates the need for many toxic reagents, enables reactions to be performed in aqueous conditions at ambient temperatures, and reduces the need for purification while providing high chemo-, regio-, enantio-, and stereo-specificity (Sheldon, 2007). These advantages have fueled a 7.5% annual growth rate in the industrial enzyme biocatalysis market from 1995 to 2012 (240% total increase in market share) such that enzyme biocatalysis represented 10% of the global catalysis market in 2012 (Kirk et al., 2002; Milmo, 2012) However, enzymes have their own unique set of challenges including the following: enzyme production costs, enzyme stability, and enzyme recoverability and reusability (Wang et al., 2009).

Enzyme immobilization provides a simple and elegant solution to these challenges. It enables recovery and reuse of the biocatalysts, significantly driving down the biocatalyst cost over many reactions. Currently, a number of different enzyme immobilization techniques are being used in industry such as enzyme adsorption, entrapment, and cross-linking (Sheldon, 2007). A particularly attractive method is attachment/adsorption of the enzyme to the surface of a carrier which commonly results in enhanced enzyme stability (Alonso et al., 2005; Anwar et al., 2007; López-Gallego et al., 2005; Mozhaev et al., 1990; Qiu et al., 2005; Rocchietti et al., 2004). Stability enhancements have been shown after attachment

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to many different carriers, examples include magnetic beads (Qiu et al., 2005), mesoporous sol–gel glass (Wang et al., 2001), silica nanospheres (Wang and Caruso, 2004), Sepabeads (Rocchietti et al., 2004), agarose (Rocchietti et al., 2004), and protein-coated microcrystals (Kreiner and Parker, 2005). Many different conjugation chemistries have been used, relying on amine, epoxy, maleic anhydride, glyoxal, biotin/streptavidin, and many other reactive functional groups for enzyme immobilization (Leckband and Langer, 1991; López-Gallego et al., 2013; Maeda et al., 2008; Mateo et al., 2007a; Mazi et al., 2006; Tasso et al., 2009; Zhang et al., 2011). The chemical interactions used for surface attachment/adsorption may primarily be divided into three categories: Van der Waals-based adsorption, ionic adsorption, and covalent attachment (Sheldon, 2007).

Van der Waals enzyme adsorption techniques have been successfully used in the industry, but because these intermolecular forces are weak in comparison to ionic or covalent bonds, significant leaching of the enzyme from the support occurs over time (Zhao et al., 2006). Ionic adsorption improves retention over Van der Waals interactions, but leaching to a lesser extent is still common (Kirk and Christensen, 2002). Covalent immobilization is best suited for producing the strongest and longest lasting bonds between enzymes and surfaces; however it is most prone to undesired surface–enzyme interactions resulting in activity loss (Barbosa et al., 2014; Kim et al., 2006; Klibanov, 1979; Mateo et al., 2007b; Mozhaev et al., 1990). Traditionally, all of these techniques are limited in their inability to control the orientation at which the enzyme attaches/adsorbs to the surface to minimize undesired enzyme–surface interactions, maximize enzyme stability, and maximize active-site accessibility (Hernandez and Fernandez-Lafuente, 2011; Mateo et al., 2007b).

The development of technologies to enable site-specific enzyme immobilization at any desired residue on the protein would enable virtually all options. Prior modeling research has predicted that certain attachment sites can minimize undesired enzyme–surface interactions and optimize thermodynamic stability of immobilized proteins and enzymes (Friedel et al., 2007; Talasaz et al., 2006; Wei and Knotts, 2011; Zhuoyun et al., 2009). Moreover, careful immobilization orientation control can minimize the effects of steric hindrance of the enzyme active site post-immobilization and provide greater understanding of the enzyme–surface interactions.

Previous research demonstrating covalent site-specific immobilization techniques have suffered from significant limitations in potential immobilization locations, such as the N- or C-terminal regions (Smith et al., 2005; Tominaga et al., 2004). Other site-specific immobilization technologies have enabled immobilization at many potential residues, but rely on noncovalent techniques (Kalia et al., 2007; Lim et al., 2014; Seo et al., 2011). To our knowledge, no covalent site-specific immobilization method has been previously demonstrated enabling immobilization at residues throughout the enzyme while only requiring a single substitution or insertion. In this article, we seek to address the need of a covalent site-specific system enabling immobilization at potential residues throughout the enzyme to prevent enzyme leaching and also analyze the impact of immobilization orientation on enzyme stability.

Here, we report our PRECISE system's (Protein Residue-Explicit Covalent Immobilization for Stability Enhancement) (Smith et al., 2013) effective application for covalent, site-specific immobilization and increased stability of enzymes. This system facilitates easy, non-invasive enzyme recoverability and reusability, testing of enzyme activity at different immobilization sites, and comparisons of enzyme stability when attached at different orientations. These benefits are accomplished through the use of amber-codon-substitution to enable rationally directed non-canonical amino acid incorporation for site-specific enzyme immobilization using the covalent, biocompatible, and bioorthogonal Huisgen 1,3-dipolar cycloaddition reaction (Boyce and Bertozzi, 2011; Hong et al., 2010; Smith et al., 2013; Young et al., 2010). The results from these tests indicate that enzyme immobilization orientation plays a significant role in enzyme activity and stability and that meaningful stability enhancements above and beyond that provided by immobilization alone can be found through careful immobilization orientation control.

2. Materials and methods

2.1. Cloning

A T4 lysozyme variant (Plasmid 18111) with two mutations, C54T and C97A, but equivalent activity and stability to the wild-type lysozyme was acquired from Addgene (Cambridge, MA) (Matsumura and Matthews, 1989). This T4 lysozyme variant simplified cell-free protein synthesis (CFPS) because it eliminated the need to alter the environmental redox potential to promote disulfide bond formation (Matsumura and Matthews, 1989). As an additional benefit, the denaturation pathway of this variant has been well characterized (Peng and Li, 2008).

For protein purification purposes, a strep-tag (consisting of the eight amino acids Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) was added to the C-terminus of the cysteine-free T4 lysozyme using the Quikchange II mutagenesis protocol (Agilent Technologies, Santa Clara, CA). This T4 lysozyme is hereafter referred to as the "standard" lysozyme. Six sites were selected for modifications to enable non-canonical amino acid incorporation at each site and immobilization at that site. These sites were T21, K35, N53, L91, K135, and K162 and the Quikchange II mutagenesis protocol was used to substitute the amber stop codon at each location (Fig. 1A).

2.2. Cell-free protein synthesis

The *Escherichia coli* cell-free protein synthesis system is a protein production technology particularly valuable for its scalability (Zawada et al., 2011). Previously researchers have demonstrated equivalent protein production from the 100 μl to the 100 l scale (Zawada et al., 2011). Here, CFPS reactions for protein production were performed as described previously (Smith et al., 2013). The cell-free extract was prepared as described previously (Shrestha et al., 2014) and was produced from a BL21 Star™ (DE3) *E. coli* strain purchased from Invitrogen (Carlsbad, CA) harboring the pEVOL-pPrF plasmid, a kind gift from Peter Schultz (Scripps Research Institute). For cell-free reactions for lysozyme variants incorporating non-canonical amino acids, 2 mM p-propargyloxyphenylalanine (pPa) and 3 mg/ml pPa specific *Methanocaldococcus jannaschii* pPa tRNA-synthetase were additionally added (Smith et al., 2013). Post-cell free reaction, lysozyme was purified from the CFPS reaction using Strep-Tactin Superflow columns (IBA Life Sciences, Gottingen, Germany). The lysozyme variants were quantified as described below.

2.3. Protein yields

The yields for the total and soluble protein were calculated using liquid scintillation as described previously (Smith et al., 2013). Full-length protein production was verified by running sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by autoradiography as previously reported (Smith et al., 2013). In all reactions where the non-canonical amino acid was not added, all T4 lysozyme proteins were truncated and no full-length protein was observed (Fig. 1F). In contrast, where the non-canonical amino acid was added, full-length T4 lysozyme was produced (Fig. 1E). Once full-length protein production was verified, soluble proteins were recovered from the supernatant post-centrifugation at 13,000 × *g*