Biocatalysis and Agricultural Biotechnology 13 (2018) 265–271

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Fusion of carbohydrate binding module to mutant alkaline phosphatase for immobilization on cellulose

Sangita Singh[a], Troy Hinkley[b], Sam R. Nugen[b], Joey N. Talbert[a,*]

[a] Department of Food Science and Human Nutrition, Iowa State University, Ames, IA, United States
[b] Department of Food Science, Cornell University, Ithaca, NY, United States

A B S T R A C T

Immobilized alkaline phosphatase (AP) has the potential to be utilized in biotechnology applications including molecular cloning, inhibitor screening, and the production of phosphorylated compounds. Traditional immobilization methods are limited by specificity and reproducibility, and can require multiple steps to modify the support or the enzyme. In this study, a double mutant alkaline phosphatase (D153G/D330N AP*) was expressed with a carbohydrate binding module (CBM 2a) fused to the N- or C-terminal to enable immobilization of the enzyme to cellulose microparticles. The modified enzyme was characterized in both free and immobilized states. Immobilization was achievable with a maximum loading of 0.33 μmole/g of cellulose for the N-tagged enzyme (CBM-AP*) and 0.26 μmole/g of cellulose for the C-tagged enzyme (AP*-CBM). Fusion of the CBM tag to either the N- or C-terminal resulted in catalytically active enzymes, with modification of the C-terminal retaining the highest catalytic efficiency (52%) relative to the unmodified mutant. The immobilized conjugates retained 83.7% and 80% catalytic efficiency for N-terminal and C-terminal tagged AP*, respectively, when compared to their free enzyme counterparts, and could be washed ten times without a significant loss in catalytic activity. These results suggest that immobilized CBM-tagged alkaline phosphatase may be a viable form for the pragmatic utilization of the enzyme in biotechnology applications.

1. Introduction

The immobilization of enzymes onto material supports has been studied as a means to increase the stability and activity of enzymes, promote recovery and reusability, as well as reduce the operational cost of enzymes (Mateo et al., 2007). While a number of strategies for enzyme immobilization are utilized, many of these strategies are limited by the specificity of the attachment or require extensive modification of the material or the enzyme. Proteins and peptides having a specific affinity for a material such as natural and synthetic polymers, metals, and minerals have been employed to immobilize enzymes, directly, to a support (Lu et al., 2012; Naal et al., 2002; Lee and Swaisgood, 1998). This immobilization is accomplished by genetically fusing a sequence encoding the binding protein to the gene encoding for the enzyme of interest. When expressed, the resulting fusion tagged-enzyme is capable of binding to the desired material. The advantages of fusion-tag immobilization directly to materials include reproducible orientation of the bound enzyme, specific binding, and no chemical modification of the enzyme or the support.

Alkaline phosphatase (AP; EC 3.1.3.1), a hydrolase that catalyzes the hydrolysis of phosphoric esters, is widely used in a conjugated form as an indicator enzyme in enzyme-linked immunosorbent assays and western blots (Alissandratos and Halling, 2012). The enzyme is also employed in free and immobilized forms in molecular biology to remove phosphate groups from DNA and RNA to prevent re-ligation and facilitate labeling (Zubriene et al., 2002). Moreover, alkaline phosphatase has been utilized in immobilized forms to screen for inhibitors of the enzyme, enable the production of phosphorylated compounds, and promote bone formation (Wang et al., 2013; Osathanon et al., 2009; Babich et al., 2013). Mammalian and bacterial alkaline phosphatases are used in these applied technologies, with mammalian alkaline phosphatase being employed in applications that require higher turnover or stability at higher pH values, while bacterial alkaline phosphatase is utilized when the application requires higher thermostability. In its native form, AP is dimeric and contains two zinc ions and one magnesium ion per monomer. Mutation of the active site residue Asp 153 to glycine has produced mutants with increased activity and decreased magnesium affinity (Dealwis et al., 1995). Similarly, a double mutant version of *E. coli* (D153G/D330N) alkaline phosphatase has been developed that combines the beneficial properties of

* Corresponding author.
E-mail address: jotalber@iastate.edu (J.N. Talbert).

https://doi.org/10.1016/j.bcab.2018.01.003
Received 8 November 2017; Received in revised form 14 December 2017; Accepted 4 January 2018
Available online 06 January 2018
1878-8181/

Table 1
List of primers used for cloning of AP* and its variants in pET 20b (+).

Gene	Restriction site for cloning	Forward primer	Reverse primer
AP*	NcoI-XhoI	SG-003 TAAGCACCATGGCTCGTACACCGGAAATGCCGG	SG-010 TAAGCACCATGGCTACACCGGAAATGCCGG
AP*-CBM_{Cex}	NcoI-XhoI	SG003 TAAGCACC**ATGGCT**CGTACACCGGAAATGCCGG	SG005 TGCTTACTCGAGACCAACGGTACACGGGGTGCC
CBM_{CenA}-AP*	NcoI-XhoI	SG-12- TAAGCACCATGGCTATGTCCACGCGCCGTACTG	SG-13 TGCTTACTCGAGTTTCAATCCTAAGGCAGC

mammalian and bacterial alkaline phosphatase—displaying a turnover similar to that of bovine alkaline phosphatase along with the thermostability of *E. coli* alkaline phosphatase (Muller et al., 2001). This double mutant can also be expressed in *E. coli* using standard techniques, which is beneficial for laboratory and commercial applications.

Cellulosic materials including films, filter membranes, particles, and fibers have been applied as immobilization matrices for a number of biomolecules due to the abundance of the raw material, biocompatibility, susceptibility to modification, and ease of fabrication. Alkaline phosphatase has been immobilized to cellulosic materials by covalent attachment, adsorption (Greenwood et al., 1994) and ionic binding (Khan and Garnier, 2013; Tzanavaras and Themelis, 2002; Cao et al., 2015; Zubriene et al., 2002). Other materials such as chitosan have also been used to immobilize AP as an effort to improve its properties (Jafary et al., 2016). Though these methods are effective, immobilization methods that are more direct, specific, and reproducible would be desirable. Carbohydrate binding modules (CBMs) are a class of proteins that have an affinity for cellulose. Depending on the sequence of the CBM, a reversible or irreversible attachment to cellulose can be achieved. Proteins that are members of CBM 2 bind to cellulose in an apparently irreversibly manner (Tomme et al., 1994; Greenwood et al., 1994; Boraston et al., 2004). Due to this property, CBM 2 fusion tags have been applied to immobilize enzymes, including alkaline phosphatase, for industrial applications (Greenwood et al., 1994; Myung et al., 2011). While alkaline phosphatase has been fused with CBM tags to promote purification of the enzyme as well as to enable screening of CBMs for the development of cellulose-degrading enzymes, there have been no studies characterizing the effects of CBM tags on the properties of the free and immobilized enzyme (Greenwood et al., 1989; Kim et al., 2013). In this article, we explore the use of CBM tags for the immobilization of a double mutant AP to cellulosic materials, and describe the resulting properties of the immobilized conjugates.

2. Materials and methods

2.1. Materials

pET 20b(+) was a kind gift from Professor Robert S. Haltiwanger at Complex Carbohydrate Research Center, The University of Georgia. Hispur Ni-NTA (Nickel-nitrilotriacetic acid) superflow agarose, diethanolamine, imidazole, Bugbuster 10× protein extraction reagent, p-nitrophenyl phosphate (p-NPP), Coomassie (Bradford) dye, casein, Whatman paper 1001-090, and 1-Step™ NBT/BCIP (nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolyphosphate) substrate solution were purchased from ThermoFisher Scientific. Fast digest restriction endonucleases (*NcoI*, *XhoI*) and T4 DNA ligase enzymes were also from ThermoFisher Scientific. Q5 Hot start high-fidelity DNA polymerase, deoxynucleotide (dNTP) solution mix, shrimp alkaline phosphatase, NEB® 5-alpha competent *E. coli* (Subcloning Efficiency), and T7 Express lysY/I^q competent *E. coli* (high efficiency) were purchased from New England BioLabs. Oligonucleotides were synthesized in DNA Facility at Iowa State University. 10% precast polyacrylamide gels for use with Mini-PROTEAN electrophoresis cells, econo-column, 1.5 × 10 cm, glass chromatography column (maximum volume 18 mL), and precision plus protein dual Color Standards were from Bio-Rad. Isopropyl-β-D-thiogalactoside (IPTG) was from IBI Scientific, Commercial *E. coli* AP (P 5931), phenylmethylsulfonyl fluoride (PMSF) and microcrystalline cellulose (Avicel; 50 μm) were purchased from Sigma.

2.2. Plasmid construction

Plasmids used in the study were synthesized by Invitrogen (USA). The following amino acid mutations (D153G/D330N) were introduced to the *phoA* gene of *E. coli* strain RM191F (GenBank accession nos. M29664.1). A double mutant (D135G/D330N) of AP (referred as AP*) was used in this study due to the similar k_{cat} relative to mammalian AP. All genes were codon optimized and synthesized by Invitrogen. Two CBM genes were fused to AP* and synthesized in a pMK- vector—AP*-CBM_{Cex} (AP* with CBM of exoglucanase (Cex) from *Cellulomonas fimi* at C-terminus) and CBM_{CenA}-AP* (AP* with CBM of endoglucanase A (CenA) from *Cellulomonas fimi* at N-terminus). AP* was cloned from Pmk- AP*-CBM_{Cex} plasmid. CBM_{Cex} and CBM_{CenA} belong to the family of CBM 2 with GenBank accession nos. M15824.1 and M15823.1, respectively.

The genes were amplified by polymerase chain reaction (PCR) using primers listed in Table 1. The amplified PCR products along with an empty pET-20b(+) vector were digested with restriction endonucleases *NcoI* and *XhoI*. The digested PCR product and vector were further digested with *DpnI* and Shrimp Alkaline Phosphatase, respectively. The digested PCR product and vector from these reactions were ligated together using T4 DNA ligase and transformed into NEB 5-alpha cells to generate pET-20b(+)-AP*, pET-20b(+)-CBM_{CenA}-AP*, and pET-20b (+)-AP*-CBM_{Cex} plasmids. The sequences of the generated constructs were confirmed using Sanger DNA sequencing performed at the Iowa State University DNA Facility. Following sequence and restriction digestion confirmation, the resulting plasmids were transformed into *E. coli* expression strain T7 Express lysY/I^q.

2.3. Expression of AP* and CBM-fused AP*

All inoculations and *E coli* cell growth were performed in sterilized culture media of LB (Luria–Bertani 1% tryptone, 0.5% yeast extract, 1% NaCl). Ampicillin (100 μg/mL) was used as a selection antibiotic. An overnight culture was started from a fresh plate streaked with cells expressing target enzyme at 37 °C. The next day 10 mL of an overnight culture was diluted into 1000 mL of LB media and grown for 5 h at 37 °C until reaching an OD of 0.7. For slow induction, prior to induction, cells were chilled at 4 °C for 30 min. Subsequently, cells were induced with a sterile filtered solution of IPTG (200 μM), and then grown another 18 h at 20 °C, shaking at 150 rpm. Cells (1 L) were harvested by centrifuging for 45 min at 5000 rpm in Sorvall RC 3B Plus centrifuge. Medium and cell lysate were analyzed for protein expression as well as activity using SDS-PAGE and pNPP assay, respectively.

2.4. Paper-based assay for CBM -fused AP*

Induced cells expressing AP* and CBM-fused AP* in the growth media were also analyzed for expression using a paper-based assay. Strips (3 cm × 1 cm) of Whatman filter paper were pre-incubated with 5% casein (to prevent non-specific binding of proteins) for 20 min at