ORIGINAL ARTICLE

Strep-tag II fusion technology for the modification and immobilization of lipase B from *Candida antarctica* (CALB)

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Abstract Fusion tags – amino acid sequences that are genetically coded to be expressed as attached moieties to a protein – have the potential to enhance the activity of native enzyme, enable specific purification of the enzyme, and promote simple and efficient immobilization of enzymes onto material supports. In this work, we demonstrate the effect of a Strep-tag II fusion tag on the properties of free and immobilized lipase B from *Candida antarctica* (CALB). The gene encoding the mature portion of CALB was codon-optimized and cloned in pASG-IBA2 plasmid for expression in *E. coli*. Purified recombinant Strep-tag II CALB was immobilized to Strep-Tactin based support through affinity binding, and the immobilized and free Strep-tag II CALB were compared to a commercial CALB. Following modification, the enzyme could be selectively purified from culture media with no observable non-specific binding. The catalytic efficiency of the purified fusion-tagged enzyme was significantly greater than that of the commercial CALB in its free form. Immobilization of the fusion-tagged enzyme to Strep-Tactin modified crosslinked agarose support yielded a catalytically active enzyme; however, the k_{cat} of the immobilized enzyme was significantly reduced compared to the free tagged enzyme. This work indicates that a C-terminus Strep-tag II fusion tag may be employed to improve the catalytic efficiency of free CALB, but may not be suitable for immobilized applications that employ binding of the enzyme to a Strep-Tactin-modified support.

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1. Introduction

Lipases (triacylglycerol acyl-hydrolases) belong to class of hydrolase enzymes that act on carboxylic ester bonds. They play an essential physiological role in lipid digestion by breaking down complex triglycerides (esters derived from glycerol and three fatty acids) into simpler forms such as diglycerides, monoglycerides, fatty acids and glycerol. In addition to their natural role of hydrolyzing ester bonds, lipases can catalyze a broad spectrum of synthesis reactions (esterification and transesterification) under thermodynamically favored conditions of low water activity [1]. Production of industry relevant compounds using lipases confers several benefits over chemical routes such as milder conditions, enhanced specificity resulting

in fewer side products, high selectivity, environmentally friendlier, low-energy demanding operation and easier downstream processing leading to reduction in overall operation costs [2]. High versatility of lipases owing to their wide substrate acceptance, selectivity, stability in many organic solvents, ability to catalyze reactions without the need for expensive co-factors, convenient production and activity in a wide range of conditions makes them highly attractive for potential applications in food, dairy, pharmaceutical, agrochemical, biofuels, oleochemical, detergent, cosmetics, paper, and textile industries [3,4]. Lipase B from yeast *Candida antarctica* (CALB) represents one of the most widely used lipases in industrial biotransformation applications over other lipases due to its excellent features such as broad substrate specificity, high stereoselectivity in aqueous as well as organic media, enantio preference and high thermostability, [5]. These unique properties have led researchers to focus on further enhancing the enzyme to improve the kinetics and stability of the enzyme through immobilization and genetic modification techniques [6–8].

Fusion tags – amino acid sequences that are genetically coded to be expressed as attached moieties to a protein – have the potential to enhance the activity of native enzyme, enable specific purification of the enzyme, and promote simple and efficient immobilization of enzymes onto material supports [9–11]. Commonly used fusion tags include the polyhistidine tag (His-tag), FLAG-tag, Strep-tag, Streptavidin Binding Peptide, GST-tag, Calmodulin-tag, and S-tag, and Carbohydrate Binding Modules (CBMs) [12]. Several factors govern the type of fusion tag chosen for enzyme modification including solubility, activity, specificity, application, binding affinity, and cost.

With respect to CALB, several fusion tags have been evaluated as a means to improve the properties of the enzyme. Larsen et al., have demonstrated the purification of polyhistidine tagged recombinant CALB from different *E. coli* strains by Immobilized Metal Affinity Chromatography (IMAC) [13]. However, they also detected several other proteins in the purified protein samples that were not completely removed by the IMAC purification procedure. Naturally occurring histidine rich regions in host proteins may result in this non-specific protein binding during IMAC purification [14]. CALB fusion with large protein affinity tags such as Glutathione-S-transferase (GST), Cellulose Binding Domain (CBD) and Maltose Binding Protein (MBP) has been observed to improve the enzyme solubility in *E. coli*, but can negatively affect the activity of the recombinant enzyme [8,15,16]. This suggests that large size fusion tags can lead to conformational changes in the recombinant enzyme thereby interfering with its substrate accessibility [15]. In addition, large fusion tags may also put heavy metabolic burden on the host organism during overproduction of the recombinant protein [11]. Therefore, it is essential to explore various other fusion tags to improve the production and immobilization of catalytically active CALB.

In this research work, we evaluate the effects of Strep-tag II fusion tag on the properties of free and immobilized lipase B from *Candida antarctica*. Strep-tag II is a short synthetic peptide comprising eight amino acids (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys). This sequence can be fused to recombinant proteins at the N- or C-terminus and displays intrinsic affinity toward Strep-Tactin – an engineered form of streptavidin [17,18]. Strep-tag II was selected due to its various inherent features that are desirable for successful enzyme modification and immobilization. Strep-tag II affinity purification relies on

highly specific interaction between Strep-tag II and Strep-Tactin resin, thereby minimizing non-specific interaction with other host proteins and enabling the recombinant protein to be obtained in highly pure form [19,20]. The tag allows the elution of recombinant protein under gentle, physiological conditions, thus making it suitable for the production of biologically active proteins [21]. Strep-tag II is a small peptide tag with neutral pI that is expected to have limited influence on protein folding or function, and thus does not require removal from the recombinant protein after purification. The tag exhibits a dissociation constant (K_D) of ∼1 μM toward Strep-Tactin resin, which is desirable for efficient specific attachment to a support without leaching which can occur when a protein is hydrophobically adsorbed to a support [20,22,23]. In addition, Strep-Tactin resins are robust and their regeneration and reuse can be attained several times for purification, which is useful in immobilization application where it is desirable to recover and reuse a support [20,24].

2. Materials and methods

2.1. Materials

pASG-IBA2 Star Gate Acceptor Vector, Strep-Tactin Superflow (high capacity; 6% crosslinked agarose; 60–160 μm) resin and Strep-tag protein purification buffer set were purchased from IBA Life Sciences. p-nitrophenyl butyrate (p-NPB) was from Sigma. Oligonucleotides were from Integrated DNA Technologies. Fast digest restriction endonucleases (Esp3I, Hind III, NdeI, Xho I) and T4 DNA ligase enzymes were from Thermo Fisher Scientific. Q5 Hot Start High-Fidelity DNA Polymerase, Deoxynucleotide (dNTP) Solution Mix, Shrimp Alkaline phosphatase, NEB® 5-alpha Competent *E. coli* (Subcloning Efficiency) and NEB® Express Competent *E. coli* (High Efficiency) cells were purchased from New England Biolabs. Ampicillin was from G-Biosciences. Anhydrotetracycline hydrochloride, 4-morpholineethanesulfonic (MES) acid monohydrate and MES sodium salt were purchased from Acros Organics. Blue-Clean Protein Stain was purchased from IBI Scientific. Coomassie G-250 (Bradford) dye and bovine serum albumin were purchased from Thermo Scientific. 12% precast polyacrylamide gels for use with Mini-PROTEAN Electrophoresis Cells, Poly-Prep Chromatography Columns (2 ml bed volume and 10 ml reservoir), Precision Plus Protein All Blue Standards and Precision Plus Protein Dual Color Standards were from Bio-Rad. Commercial CALB was from Chiral Vision (CV-CALBY).

2.2. Plasmid construction

CALB gene was custom-synthesized by GeneScript (USA) based on the amino acid sequence of mature portion of original CALB gene from *C. antarctica* (LF 058, GenBank accession No.: Z30645.1) and was codon-optimized by taking into account the codon usage of the *E. coli* B strain. This gene was amplified by Polymerase Chain Reaction (PCR) using two phosphorothioate protected primers: Forward Primer - 5′-AGC GCG TCT CCA ATG CTG CCG TCT GGT AGC G*A-3′ and Reverse Primer - 5′AGC GCG TCT CCT CCC CGG CGT CAC GAT ACC A*G-3′ where * indicates a phosphorothioate bond between the last two nucleotides. The

amplified PCR product and the pASG-IBA2 empty vector were digested with Esp3I restriction endonuclease. Esp3I 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 then ligated together using T4 DNA ligase to generate a pASG-IBA2-CALB construct. Sanger DNA Sequencing (performed at the Iowa State University DNA Facility) was employed to confirm the sequence.

2.3. Culture media

For cultivation of the recombinant *E. coli* strain, the following sterilized culture media were used: Luria–Bertani (LB; 1% tryptone, 0.5% yeast extract, 1% NaCl) for plasmid extraction and Terrific Broth (TB; 1.2% tryptone, 2.4% yeast extract, 0.5% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) for expression of Strep-tag II CALB. Ampicillin (Ap) was used as a selection antibiotic at 100 μg/mL.

2.4. Expression of the recombinant Strep-tag II CALB

A sample (60 μL) of an overnight growth culture of NEB® Express *E. coli* harboring pASG-CALB was inoculated into 6 mL of fresh TB medium supplemented with 100 μg/mL Ap. The cells were cultivated aerobically at 37 °C until reaching an O.D. of 0.5–0.6 at 550 nm. Anhydrotetracycline hydrochloride was added to give a final concentration of 200 ng/mL, and the cultivation continued at 20 °C for 24 h. The culture was centrifuged to separate the supernatant and the cell pellet. The cell pellet was re-suspended in 0.6 mL of Buffer P (100 mM Tris/HCl pH 8.0, 500 mM sucrose and 1 mM EDTA), disrupted by sonication on ice using an ultrasonic processor (QSonica Sonicators Part No. Q55), and centrifuged at $14000 \times g$ for 10 min. The resulting supernatant containing cellular soluble proteins was recovered. The precipitate containing cellular insoluble proteins was re-suspended in 0.6 mL of Buffer P. The obtained fractions (i.e., extracellular fraction, intracellular fraction – soluble and intracellular fraction - insoluble) were used for further analysis for the expression of recombinant lipase by SDS-PAGE (same proportion of all three fractions was loaded on the gel) and lipase enzyme assay. Expression was performed in duplicate independent experiments to verify expression using the stated methods.

2.5. Specific activity

The specific activity of unpurified Strep-tag II CALB during expression was determined in all the three fractions using p-nitrophenyl butyrate (p-NPB) as the substrate at a final concentration of 0.5 mM in 10 mM MES buffer (pH 7.0). For this, 2 μg/mL of protein for the extracellular fraction, 1.7 μg/mL of protein for the intracellular –soluble fraction and 4 μg/mL of protein of the intracellular – insoluble fraction were tested. The progress of the hydrolysis was monitored, spectrophotometrically, at 400 nm at 37 °C for 15 min. Specific activity was expressed relative to the protein content as determined using the Bradford method.

2.6. Purification of the recombinant Strep-tag II CALB from E. coli culture

The recombinant NEB® Express *E. coli* strain harboring pASG-CALB was grown in Terrific Broth (TB) media containing ampicillin (100 μg/mL) for overnight. Then, 1% inoculum was transferred into a fresh TB medium. At an OD_{550} of 0.5–0.6, lipase expression was induced by adding anhydrotetracycline hydrochloride at final concentration of 200 ng/mL. The induced culture was grown at 20 °C for 24 h, and the culture media was separated from cells by centrifugation and then concentrated using a centrifugal filtration device (Centricon® Plus-70 Centrifugal Filter Units, molecular weight cut off 10 kDa, EMD Millipore). Concentrated culture media was loaded onto Strep-Tactin packed column to purify the Strep-tag II CALB. Elution fractions were evaluated for lipase detection using 12% precast polyacrylamide gels for sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE). Elution fractions containing purified Strep-tag II CALB were pooled and Bradford protein estimation was conducted on pooled eluate using bovine serum albumin (BSA) as a standard. Purified Strep-tag II CALB was further concentrated using centrifugal filtration device (Corning® Spin-X® UF 20 mL Centrifugal Concentrator, molecular weight cut off 10 kDa). This was also performed to facilitate desalting by removal of D-desthiobiotin and EDTA from the purified lipase sample. Concentrated and desalted purified Strep-tag II CALB was stored separately in 10 mM MES Buffer (pH 7.0) and Buffer W (100 mM Tris/HCl pH 8.0, 150 mM NaCl and 1 mM EDTA) at −20 °C until further use.

2.7. Immobilization of Strep-tag II CALB using strep-tactin resin

Enzyme loading was assessed using 40 μL of 50% suspension of Strep-Tactin resin (beads). The beads were centrifuged at $5000 \times g$ for 2 min and the supernatant was removed using a syringe needle. Beads were washed with 2 volumes of Buffer W (100 mM Tris/HCl pH 8.0, 150 mM NaCl and 1 mM EDTA) and the buffer was removed after centrifugation using a syringe needle. 25 μL of seven different concentrations (0.1, 0.2, 0.4, 0.8, 3, 6 and 12 μg/μL) of purified Strep-tag II CALB was applied to wash 20 μL beads. The samples were incubated on ice for 30 min and supernatant containing the unbound fraction of purified Strep-tag II CALB was recovered using centrifugation as described previously. Bradford protein estimation was done on supernatant to determine the amount of unbound Strep-tag II CALB. This amount was subtracted from the initial amount applied to the beads to calculate the amount of Strep-tag II CALB bound to the beads. The percentage of bound Strep-tag II CALB was plotted against the initial enzyme concentration applied to the beads.

2.8. Enzyme kinetics

Purified commercial CALB, free Strep-tag II CALB and immobilized Strep-tag II CALB (prepared as described above using 6 μg/μL of purified enzyme) samples were tested for activity using 0.1–2 mM p-NPB at 37 °C in 10 mM MES buf-

fer (pH 7.0) under constant shaking (Biotek Synergy H4; medium speed). Immobilized Strep-tag II CALB samples were prepared as described above using 6 μg/μL of purified enzyme. Absorbance values were read at 400 nm excitation for 15 min (Biotek Synergy H4). A standard curve of p-nitrophenol (extinction coefficient ∼ 7700 M^{-1} cm^{-1}) was used to determine substrate conversion and subsequent rates. The Michaelis constant (K_m) and maximum velocity (V_{max}) were extrapolated from nonlinear regression of a plot of velocity versus substrate concentration using Michaelis–Menten enzyme kinetics (Graph Pad Prism Software, U.S.A.). V_{max} was divided by the enzyme concentration to obtain the apparent turnover number (k_{cat}).

2.9. Enzyme stability

Commercial lipase (∼0.44 mg/mL), Strep-tag II CALB (∼0.44 mg/mL), and immobilized Strep-tag II CALB (∼3.07 mg/mL) in 10 mM MES buffer (pH 7.0) were incubated at 37 °C under static conditions. At 3, 6, 9, and 25 h, samples were pulled, diluted, and tested for specific activity as described previously. Activity retention was expressed as a fraction of retained activity relative to the initial activity of each sample.

2.10. Statistical analysis

To determine statistical differences at $p < 0.05$, curve fitting and one-way analysis of variance (ANOVA) with Tukey's pairwise comparison were performed using Prism software (Graph Pad, USA).

3. Results and discussion

3.1. Plasmid construction

Fig. 1 shows the structure and confirmed sequence of the construct. The developed plasmid contains the mature portion of the desired CALB gene with an OmpA signal sequence at its N-terminus and Strep-tag II at its C-terminus. The OmpA sequence was introduced in order to facilitate periplasmic secretion of the recombinant lipase that confers several advantages, such as simplicity of purification, avoidance of protease attack and a better chance of correct protein folding with the formation of the disulfide bridges in the oxidative environment of the periplasm [6,25].

3.2. Expression of Strep-tag II CALB in E. coli

Fig. 2A shows the expression of Strep-tag II CALB in NEB express E. coli grown in TB medium and induced with anhydrotetracycline hydrochloride at 20 °C for 24 h. It can be seen that target protein was expressed not only in the soluble fraction but also in the extracellular fraction (culture medium). This was not surprising as previous research suggests that, although a periplasmic signal sequence directs CALB toward the periplasm, the enzyme may also appear in the culture supernatant [6]. This phenomenon is expected to occur when the amount of the accumulated overexpressed protein in E. coli surpasses the threshold amount that can be retained

inside the periplasm, resulting in escape of the enzyme from the periplasm into the culture medium with the rupturing of the outer periplasmic membrane. This extracellular expression is useful to facilitate simplicity of recombinant protein purification due to no requirement for outer-membrane disruption to recover target proteins, thereby, avoiding intracellular proteolysis by periplasmic proteases as well as due to the presence of fewer contaminating proteins in the extracellular culture medium [25].

The expression of Strep-tag II CALB protein was further confirmed by determining the lipase activity in all the three fractions from NEB express E. coli cells. Fig. 2B indicates that all fractions exhibit lipase activity thereby confirming the expression of catalytically active target protein. While the highest activity is found in the intracellular – insoluble fraction, this fraction was not employed for further experiments due to complexity of isolation and the presence of inclusion bodies. Because of the high activity and reduced complexity of processing, the extracellular fraction (culture medium) was employed for subsequent purification of the recombinant lipase.

3.3. Purification of Strep-tag II CALB from E. coli

Strep-tag II CALB protein was purified from the extracellular fraction (culture medium) of the recombinant NEB Express E. coli cells using Strep-Tactin affinity chromatography. The use of Strep-Tactin column was effective in enabling recovery of the target protein in pure form that gave the expected single band at ca 33 kDa when using SDS-PAGE (Fig. 3). Moreover, purification reveals that the binding is highly specific with no observable non-specific binding of other proteins in the expression media. Following purification, a 73-fold enhancement in specific activity was observed.

3.4. Immobilization of Strep-tag II CALB on Strep-Tactin resin

After obtaining Strep-tag II CALB in purified and catalytically active form, purified lipase was immobilized on Strep-Tactin-modified crosslinked agarose using Strep-tag II-Strep-Tactin affinity interaction. In order to determine the optimal condition for the immobilization of purified Strep-tag II CALB to Strep-Tactin resin, the binding ability of Strep-Tactin resin was studied. When different concentrations of purified Strep-tag II CALB were applied to 20 μL of resin, no significant amount of protein (< 20% of the initial amount applied) was detected in the supernatant under the described conditions – suggesting almost complete enzyme (ranging from > 80% to 100% of the initial applied amount) binding at every concentration evaluated (Fig. 4). For subsequent immobilization experiments, 25 μL of 6 μg/μL stock concentration of Strep-tag II CALB was used with 20 μL of Strep-Tactin beads.

3.5. Enzyme kinetics

Immobilized Strep-tag II CALB, free Strep-tag II CALB, and commercial CALB were evaluated to determine the effect of modification on enzyme kinetics. As seen in Table 1, CALB modified with the fusion tag resulted in a K_m of 0.18 mM and a k_{cat} of 70.7 s^{-1}. While the K_m was not significantly different from the commercial CALB, the k_{cat} was significantly

A) pASG-IBA2-CALB-Strep-tag II

B)

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ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTAGCGCAG
 M  K  K  T  A  I  A  I  A  V  A  L  A  G  F  A  T  V  A  Q
|—→ OmpA signal sequence

GCCGCAATGCTGCCGTCTGGTAGCGACCCGGCGTTTTCCCAACCGAAAAGCGTGCTGGAT
 A  A  M  L  P  S  G  S  D  P  A  F  S  Q  P  K  S  V  L  D
     |    |—→ mature CALB

GCGGGCCTGACGTGCCAAGGTGCCTCTCCGAGTAGCGTGTCGAAACCGATCCTGCTGGTG
 A  G  L  T  C  Q  G  A  S  P  S  S  V  S  K  P  I  L  L  V

CCGGGCACCGGCACCACCGGTCCGCAGAGCTTTGATTCGAATTGGATTCCGCTGAGCACC
 P  G  T  G  T  T  G  P  Q  S  F  D  S  N  W  I  P  L  S  T

CAGCTGGGCTATACCCCGTGCTGGATTAGCCCGCCGCCGTTTATGCTGAATGATACCCAG
 Q  L  G  Y  T  P  C  W  I  S  P  P  P  F  M  L  N  D  T  Q

GTGAACACCGAATATATGGTGAACGCGATTACCGCCCTGTATGCGGGCTCGGGCAATAAC
 V  N  T  E  Y  M  V  N  A  I  T  A  L  Y  A  G  S  G  N  N

AAACTGCCGGTGCTGACCTGGTCGCAGGGCGGCCTGGTGGCGCAGTGGGGCCTGACCTTT
 K  L  P  V  L  T  W  S  Q  G  G  L  V  A  Q  W  G  L  T  F

TTCCCGAGCATCCGCTCGAAAGTGGATCGTCTGATGGCGTTTGCCCCGGATTATAAAGGC
 F  P  S  I  R  S  K  V  D  R  L  M  A  F  A  P  D  Y  K  G

ACCGTGCTGGCGGGCCCGCTGGATGCGCTGGCCGTGTCGGCACCGTCGGTGTGGCAGCAG
 T  V  L  A  G  P  L  D  A  L  A  V  S  A  P  S  V  W  Q  Q

ACCACCGGCTCGGCACTGACCACCGCCCTGCGCAATGCCGGCGGCCTGACCCAGATTGTG
 T  T  G  S  A  L  T  T  A  L  R  N  A  G  G  L  T  Q  I  V

CCGACCACCAACCTGTATAGCGCGACCGATGAAATCGTGCAGCCGCAAGTGAGCAATTCG
 P  T  T  N  L  Y  S  A  T  D  E  I  V  Q  P  Q  V  S  N  S

CCGCTGGATAGCTCGTACCTGTTTAATGGCAAAAATGTGCAGGCCCAGGCGGTGTGTGGT
 P  L  D  S  S  Y  L  F  N  G  K  N  V  Q  A  Q  A  V  C  G

CCGCTGTTTGTGATCGATCATGCGGGCAGCCTGACCTCGCAGTTTAGCTATGTGGTGGGC
 P  L  F  V  I  D  H  A  G  S  L  T  S  Q  F  S  Y  V  V  G

CGCTCGGCACTGCGTAGCACCACCGGCCAGGCCCGTAGCGCGGATTATGGCATTACCGAT
 R  S  A  L  R  S  T  T  G  Q  A  R  S  A  D  Y  G  I  T  D

TGCAATCCGCTGCCGGCGAACGATCTGACCCCCGGAACAGAAAGTGGCGGCGGCGGCACTG
 C  N  P  L  P  A  N  D  L  T  P  E  Q  K  V  A  A  A  L

CTGGCGCCGGCGGCGGCGGCAATCGTGGCGGGCCCGAAACAGAACTGCGAACCGGATCTG
 L  A  P  A  A  A  A  I  V  A  G  P  K  Q  N  C  E  P  D  L

ATGCCGTATGCCCGCCCGTTCGCCGTGGGTAAACGCACCTGCTCTGGTATCGTGACGCCG
 M  P  Y  A  R  P  F  A  V  G  K  R  T  C  S  G  I  V  T  P|

GGGAGCGCGCTTGGAGCCACCCGCAGTTCGAAAAATAA
 G  S  A  W  S  H  P  Q  F  E  K  -
       |—→ Strep-tag II  |
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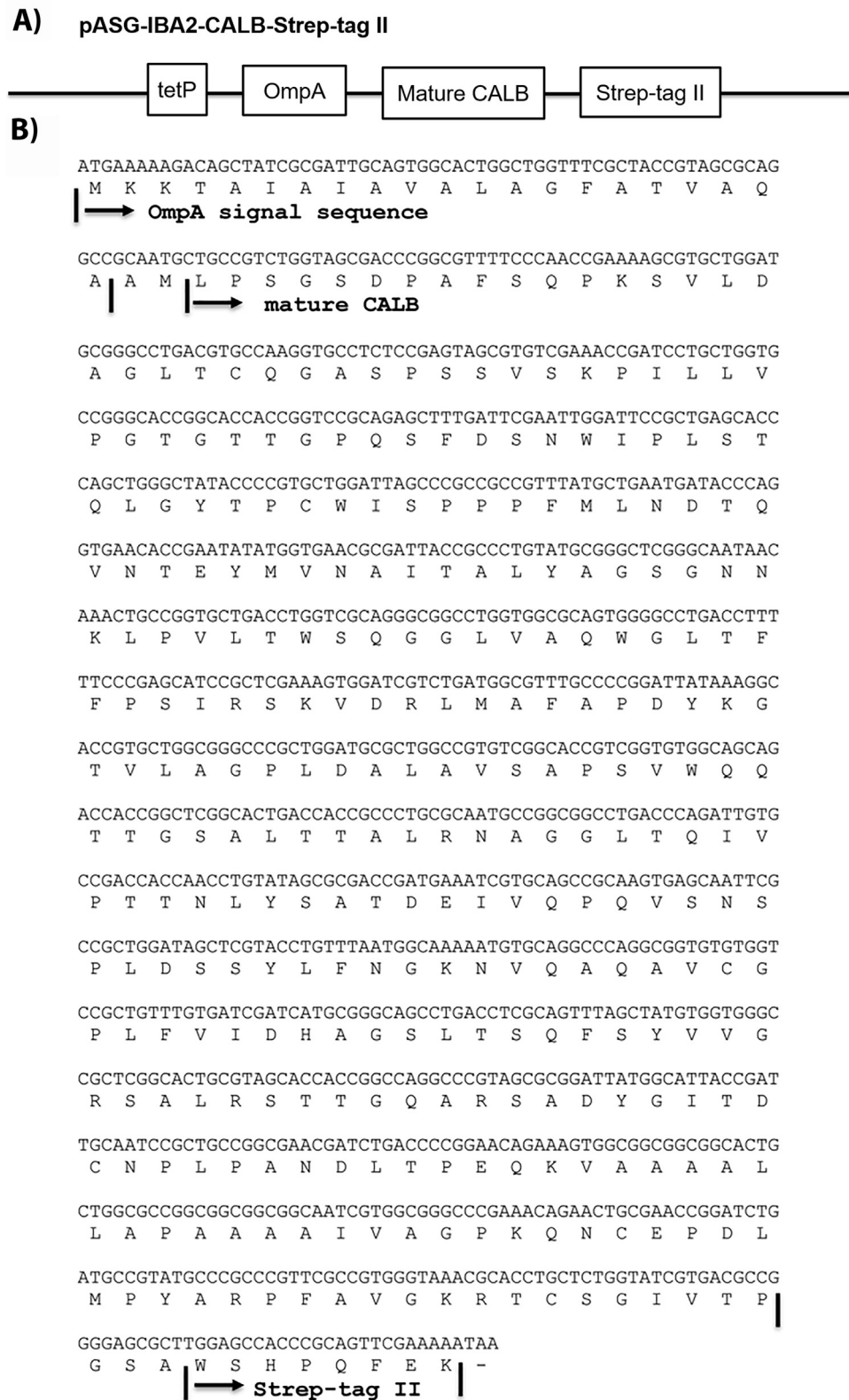
Fig. 1 Structure of Strep-tag II CALB-expressing plasmid. (A) Schematic diagram of the expression cassette of pASG-IBA2-CALB-Strep-tag II. Only the part from the tet promoter (tetP) to Strep-tag II is shown. Esp3I restriction sites were used for cloning, however, these sites are not retained after cloning. (B) Nucleotide sequences of OmpA signal-mature CALB gene-Strep-tag II with the corresponding amino acid sequence.
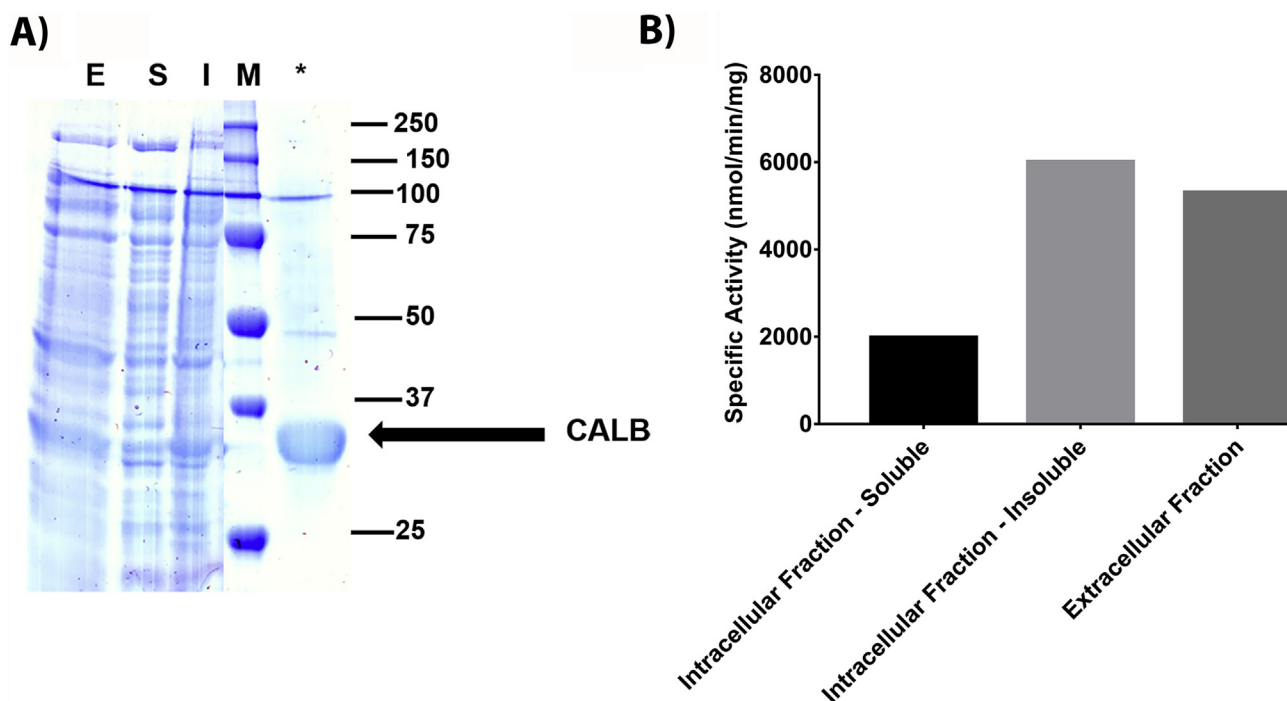
A)

B)

Fig. 2 Analysis of Strep-tag II CALB expression in recombinant NEB (New England Biolabs) Express *E. coli* cells. Recombinant NEB express *E. coli* cells harboring pASG-IBA2-CALB-Strep tag II plasmid were grown in Terrific Broth media and induced with anhydrotetracycline (at final concentration of 200 ng/ml) at 20 °C for 24 h. (A) SDS-PAGE analysis for expression of Strep-tag II CALB was done on different fractions – extracellular fraction (E), intracellular soluble fraction (S) and intracellular insoluble fraction (I) on a 12% polyacrylamide gel along with the molecular size marker (lane M) and commercial CALB (lane *). Protein bands were visualized with Blue-Clean Protein Stain. The arrows indicate the position of CALB (MW $\sim$ 33 kDa) on the gels. (B) Same fractions were also tested for lipase activity using 0.5 mM p-nitrophenyl butyrate at 37 °C in 10 mM MES buffer (pH 7.0) for 15 min. Protein estimation was done using Bradford method with bovine serum albumin as a standard. Enzyme activity values were converted to specific activity values. Representative specific activity shown is an average from triplicate repeated measurements of each sample.
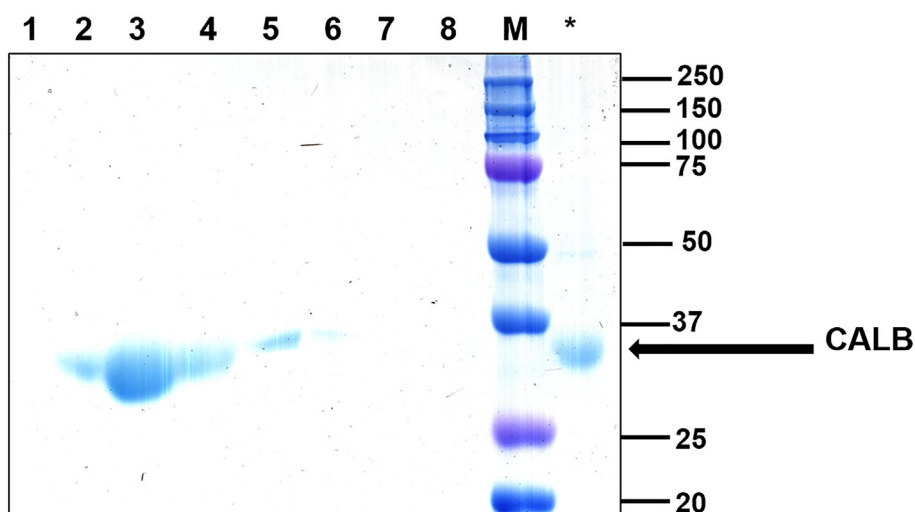

Fig. 3 Purification of Strep-tag II CALB from recombinant NEB Express *E. coli* cells culture media. Recombinant NEB express *E. coli* cells harboring pASG-CALB-Strep tag II plasmid were grown in Terrific Broth media with induction by anhydrotetracycline (at final concentration of 200 ng/ml) at 20 °C for 24 h. Extracellular fraction (culture medium) was collected for purification of Strep-tag II CALB using Strep Tactin affinity chromatography. Elution fractions collected during purification were analyzed by SDS-PAGE on a 12% polyacrylamide gel along with the molecular size marker (lane M) and commercial CALB (lane *). Lanes # 1–8 – Elution Fractions 1–8.
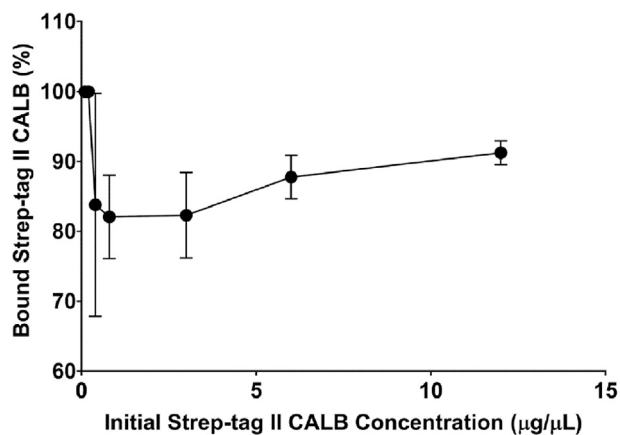
Fig. 4 Immobilization of different concentrations of purified Strep-tag II CALB on 20 μL of Strep-Tactin Superflow (high capacity) resin in Buffer W on ice. Values represent an average ± standard deviation from triplicate independent measurements of each sample.

Table 1 Apparent kinetic parameters of commercial CALB, free and immobilized Strep-tag II CALB at 37 °C/pH 7.0. Values represent average ± standard deviation of n = 3 measurements.

Enzyme source	K_m (mM)	k_{cat} (s^{-1})
Commercial CALB	0.21 ± 0.17[a]	25.7 ± 4.56[a]
Free Strep-tag II CALB	0.18 ± 0.06[a]	70.7 ± 11.3[b]
Immobilized Strep-tag II CALB	0.27 ± 0.15[a]	6.53 ± 0.767[c]

Different superscript letters indicating significant differences at p < 0.05.

greater. This result suggests that the introduction of a Strep-tag II fusion tag on the C-terminus of terminus of CALB can yield an enzyme with a higher apparent turnover number than that of commercial variant. When immobilized, the apparent turnover number (k_{cat}) of Strep-tag II lipase decreased to 6.5 s^{-1} while the change in the apparent K_m was not statistically different. Given that the K_m of the enzyme is not altered, the decrease in the k_{cat} of immobilized lipase can

be potentially attributed to: (i) immobilization leading to an alteration in enzyme conformation due to surface interaction between the enzyme and the resin or (ii) protein-protein interactions on the surface of material support resulting in structural changes through lateral contacts with neighboring enzyme molecules [26,27]. Given that the enzyme can be recovered from the support in an active confirmation following attachment (as evidenced during purification) changes in the structure of the enzyme in the immobilized form may be slow or reversible.

3.6. Enzyme stability

As modification as well as immobilization can have an effect on enzyme stability, the storage stabilities of commercial, free Strep-tag II CALB and immobilized Strep-tag II CALB were evaluated at 37 °C/pH 7.0 across 25 h. Fig. 5 shows that there was significant drop in activity retention for all the samples within first three hours of incubation. It has been observed that when the free enzyme is stored at −20 °C under the same buffer conditions, it retains complete activity (data not shown). This suggests that temperature plays a critical role in influencing the stability of enzyme [28]. Immobilization has been employed to enhance the stability of native CALB with significant improvements in stability being demonstrated [7,28–31]. However, compared to these other forms of lipase immobilization, attachment of the enzyme through affinity interaction with Strep-Tactin resin did not promote stability. This could be because immobilization using Strep-tag II and Strep-Tactin affinity interaction may not be strong enough to impose physical restrictions and rigidity on the immobilized enzyme, which can be achieved when the enzyme is immobilized in hydrophobic porous materials or by multi-point attachment. The single point affinity attachment to the Strep-Tactin surface may provide the enzyme freedom for molecular motion and conformational changes leading to unfolding during incubation—mirroring the case of free fusion-tagged and commercial enzymes [27,28].

4. Conclusion

In summary, we have described the extracellular production and immobilization of a C-terminus Strep-tag II CALB from
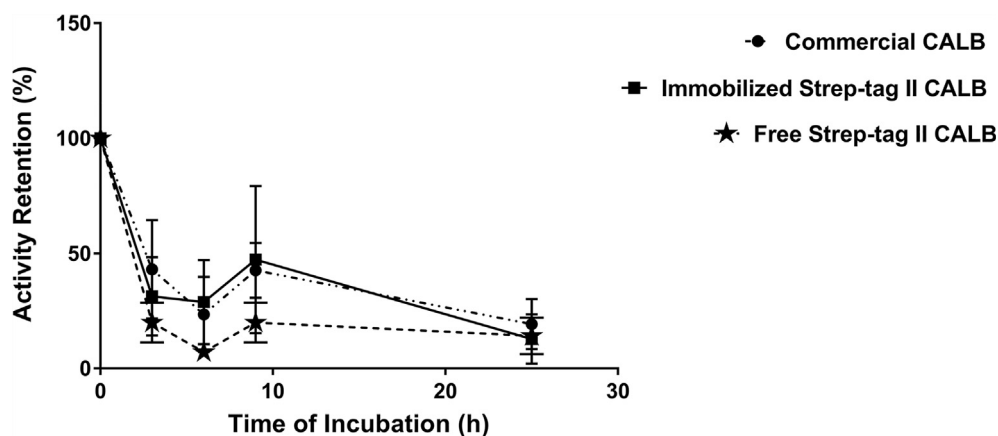

Fig. 5 Stability of activity of commercial CALB, free and immobilized Strep-tag II CALB at 37 °C in 10 mM MES buffer (pH 7.0) using p-nitrophenyl butyrate as the substrate. Values represent average ± standard deviation of n = 3 independent determinations.

a recombinant strain of *E. coli*. Recombinant Strep-tag II CALB can be produced in the extracellular fraction during expression, and the enzyme can be specifically recovered from this fraction in a pure and catalytically active form. CALB modified with the Strep-tag II fusion displays a significantly greater turnover number that that of a commercial CALB (Chiral Vision) at 37 °C/pH 7.0. The recombinant enzyme can be immobilized on a Strep-Tactin modified crosslinked agarose support and retained enzymatic activity after immobilization. However, the k_{cat} is significantly reduced following immobilization. Neither addition of the fusion tag nor immobilization improved the stability of the enzyme when compared to the commercial variant. Therefore, CALB with a Strep-tag II fusion tag, produced as described, may be beneficial for the specific isolation of the enzyme while retaining catalytic efficiency. Immobilization of the enzyme using a Strep-Tactin support may not be beneficial, though alternative methods (e.g. adsorption, covalent attachment, and entrapment) of the modified enzyme need to be explored.

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