SUPPORTING INFORMATION

# Site-Specific Modification of *Candida Antarctica* Lipase B via Residue-Specific Incorporation of a Non-Canonical Amino Acid

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TITLE RUNNING HEAD Site-Specific Modification of CalB

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## Materials

Primers were purchased from Biolegio (Malden, the Netherlands) and dNTP mix from Invitrogen. Restriction enzymes were from New England Biolabs and Promega. Natural amino acids, chloroamphenicol, lysozyme, PMSF, TritonX100, imidazole and mPEG were obtained from Sigma Aldrich. Ampicillin and IPTG were purchased from MP Biomedicals, and thiamine and glucose from Merck. Triflic anhydride, sodium azide sodium hydride and propargyl bromide were purchased from Acros. Alkynyl dansyl and azide functionalized Merrifield resin were kindly provided by R. Brinkhuis and J. Opsteen respectively.

## Construction of CalB plasmids

The gene encoding CalB was isolated from the yeast *Pseudozyma aphidis*, strain 70725 (DSM, the Netherlands) by colony PCR. Forward (*CATATG*AAGCTACTCTCTCTGACCG) and reverse (*AGATCT*TAGGGGGTGACGATGC) primers were derived from the beginning and the end of the lipase B gene from *Candida Antarctica* respectively as determined by Uppenberg *et al*.[1] Restriction enzyme recognition sites, *Nde*I in the forward primer and *Bgl*II in the reverse primer are shown in italics. For colony PCR, cells of an overnight culture (50 µl) were pelleted and treated with a zymolase solution (1 mg/ml) containing 1 M sorbitol, 10 mM Tris pH 8.0 for 1 hr at 37 ºC. Cells were again pelleted by centrifugation at 5000 rpm. PCR was conducted on a PE Applied Biosystem 9700 thermal cycler under the following conditions: 95 ºC for 1 min followed by 25 cycles of (95 ºC for 30 s, 55 ºC for 30s, 68 ºC for 120s) and finally 68 ºC for 3 min. The reaction

mixture (50 µl) contained 200 pmol of each primer, Advantage cDNA polymerase mix (1 µl, Clontech), dNTP mix (0.2 mM), the cell lysis pellet and 10x reaction buffer (5 µl). The PCR amplified fragment was purified using QIAQuick PCR purification kit (Qiagen) and was cloned into the *Nde*I/*Bam*HI of the pET15b expression vector (Novagen).

The mature CalB gene (lacking signal and propeptide coding sequences) was cloned into pET22b vector (Novagen) for periplasmic expression. pET22b contains a pelB signal sequence and a $His_6$-tag. The CalB gene was amplified from the plasmid containing the complete CalB gene in pET15b described above by PCR using the primers, AGTA*CCATGG*GACTACCTTCCGGTTCGGACC and AGTA*CTCGAG*GGGGGTGACGATGCCG. Restriction sites, *Nco*I and *Xho*I are shown in italics. After purification the PCR fragment was cloned into the *Nco*I and *Xho*I sites of pET22b, thus creating pET22b-CalB.

The sequence of this lipase contains 8 nucleotides different to the original CalB gene as reported by Uppenberg *et al.* (1994, *Structure 2*, 293-308.) Only two of these mutations lead to amino acid changes which are Thr (82)→Ala and Ala (114) →Thr, respectively.

**In silico engineering of the CalB structure**

The protein structure was derived from the pdb file '1tca'. Methionine and glycine were added to the N-terminus using the "WHAT IF & Yasara Twinset" software developed by Krieger *et al*. (2002, *Proteins 47*, 393-402).

**Synthesis of azidohomoalanine**

$TfN_3$ was prepared by dropwise adding triflic anhydride (8.8 ml, 52 mmol) to $NaN_3$ (6.8 g, 104 mmol) in a mixture of $H_2O$ (20 mL) and $CH_2Cl_2$ (20 mL), pre-cooled at 0 °C. The reaction mixture was stirred at 0 °C for 2-3 hours. Saturated $NaHCO_3$ was added at room temperature until no more $CO_2$ was liberated. The aqueous phase was extracted twice with $CH_2Cl_2$ (25 mL). $CH_2Cl_2$ fractions were combined and washed with saturated $NaHCO_3$.

*tert*-butyloxycarbonyl (Boc)-protected L-diaminobutyric acid (10 mmol, Bachem) was dissolved in $H_2O$ (37.5 mL) followed by addition of MeOH (125 mL), $Et_3N$ (4.18 mL) and $ZnCl_2$ (10 mg, 1 mol%). A freshly prepared solution of $TfN_3$ (37.5 mL) was added at once. The reaction mixture was stirred overnight at room temperature. Methanol was evaporated under vacuum and $H_2O$ (50 mL) was added. The pH was lowered till 6 by adding HCl (1 M). The aqueous solution was extracted with EtOAc (2 times 50 mL), keeping the pH at 6. Subsequently, pH was adjusted to 2 by adding $KHSO_4$ (1 M) and the product was extracted with EtOAc (3 times 70 mL). The extract was dried by adding $MgSO_4$. EtOAc was evaporated under vacuum giving Boc-protected azidohomoalanine as an oil. HCl (2 M) in EtOAc was added to remove the Boc group. The precipitate was filtered and dried in an exsiccator giving a white powder in quantitative yield. $^1$H-NMR (400 MHz, DMSO): δ=8.39 (s, 2H), 3.89 (t, *J*=6.4 Hz, 1H), 3.52 (m, 2H), 1.99 (m, 2H). $^{13}$C-NMR (75 MHz, $D_2O$): δ=171.3, 50.6, 46.6, 28.5. FTIR-ATR: *v*=2982, 2088 ($N_3$), 1735, 1449, 1245, 1160 $cm^{-1}$.

**Production of azido-functionalized CalB**

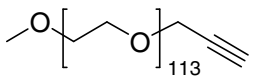
*E. coli* B834 (DE3) pLysS cells (Novagen) were transformed with pET22b-CalB plasmid. 2x YT medium (100 mL) was inoculated with a single colony and incubated overnight at 37 ºC. The overnight culture was diluted 10-fold into M9 minimal medium (1 L) supplemented with all 20 natural amino acids (40 mg/L each), glucose (0.4%), $MgSO_4$ (1 mM), thiamine (0.0005%), ampicillin (200 mg/L) and chloramphenicol (50 mg/L). The bacteria culture was incubated at 37 ºC. Upon reaching an $OD_{600}$ of 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to allow synthesis of T7 polymerase in presence of methionine. Protein expression was induced for 15 minutes at 37 ºC. The culture was sedimented by centrifugation for 10 minutes (6500 g) at 4 ºC and washed twice in cold NaCl (0.9%). Cells were resuspended in M9 minimal medium (1 L) containing 19 amino acids (40 mg/L, no methionine), glucose, $MgSO_4$, thiamine, ampicillin and chloramphenicol. After incubation for 10 minutes at 37 ºC, methionine (40 mg/L) was added to a 50 mL aliquot. 900 mL was supplemented with azidohomoalanine (40 mg/L). Then, protein expression was induced by adding IPTG (1 mM) followed by incubation overnight at 25 ˚C. Cells were harvested by centrifugation for 10 minutes (6500 g) at 4 ºC.

The $His_6$-tagged protein was purified by $Ni^{2+}$ NTA affinity chromatography under native conditions (Qiagen, Hilden, Germany). The bacteria pellet was lysed in lysis buffer (30 mL of 50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH8.0) containing

Triton-X100 (0.1%), PMSF (1 mM) and lysozyme (1 mg/mL). After sonication the lysate was cleared by centrifugation at 4 °C (13000 g, 30 minutes). The protein was allowed to bind to $Ni^{2+}$ NTA beads (1.5 mL, Qiagen) for 2 hours at 4 °C. Upon loading, the column was washed twice with wash buffer (20 volumes of 50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0). The protein was eluted in elution buffer (20 mL of 50 mM $NaH_2PO_4$, 300 mM NaCl, 250 mM imidazole, pH 8.0).

Elution fractions containing CalB were dialysed against 50 mM $NaH_2PO_4$, 150 mM NaCl, pH 7.0 (Spectra, MWCO 6-8,000) and concentrated afterwards (Amicon® Ultra-15 Centrifugal Filter Device 10,000 NMWL). Further purification was performed by size exclusion chromatography using a Superdex 75 PC 10/300 column on an ÄKTA FPLC™. Protein concentration was determined by measuring the absorption at 280 nm on a Nanodrop ND-1000 spectrometer. Approximately 2 mg purified protein was obtained from a 1 L bacteria culture.

**Synthesis of alkynynl-PEG(5000)**




α-methoxy poly(ethylene glycol) (mPEG, $M_n$ = 5000 g/mol; 2.5 g, 0.50 mmol) was dissolved in toluene (30 mL) and concentrated under reduced pressure for three times, in order to remove possibly present water by means of co-evaporation. The mPEG was then dissolved in anhydrous THF (80 mL) under an argon atmosphere and cooled to 0 °C. NaH (60 mg of a 60% dispersion in

mineral oil, 1.5 mmol) was added and the mixture was stirred at 0 °C for 15 min. Subsequently, the mixture was warmed to room temperature and propargyl bromide (140 μL of an 80% solution in toluene, 1.26 mmol) was added. The mixture was allowed to stir at room temperature for 18 hours, after which water (10 mL) was added to quench the remaining NaH. Next, the THF was largely removed by evaporation under reduced pressure and the residue was taken up in $CH_2Cl_2$ (100 mL). After washing the mixture with water (100 mL), the organic phase was dried over anhydrous $Na_2SO_4$ and concentrated under reduced pressure until approximately 10 mL of liquid remained. Upon precipitation in $Et_2O$ (500 mL) the desired product was obtained as a white solid (2.15 g, 85%). $^1$H-NMR (300 MHz, $CDCl_3$): δ=4.21 (d, *J* = 2.3 Hz, 2H), 3.64 (br s, 452H), 3.38 (s, 3H), 2.44 (t, *J* = 2.3 Hz, 1H).

**Click reaction between AHA-CalB and alkynyl-dansyl or alkynyl-PEG(5000)**

$Cu^I$-catalyzed cycloaddition between AHA-CalB and alkynyl-dansyl was performed in 25 or 50 mM phosphate buffer (pH 7-8). A stock solution of $CuSO_4$ and sodium ascorbate in water (10 mM each) was prepared and pre-mixed with bathophenanthroline sulfonated sodium salt (10 mM, GFS Chemicals) which acts as $Cu^I$ ligand. Alkyne-functionalized dansyl was dissolved in THF to a final concentration of 9 mM. AHA-CalB (1.875 nmol) was reacted overnight at room temperature in the dark with alkynyl-dansyl (20 equivalents) and $CuSO_4$/ascorbate/ligand (30 equivalents) in 50 μL reaction volume. Next day, reactions were dialysed against buffer (five times 8 volumes of 50 mM $NaH_2PO_4$, 150 mM NaCl, pH 7.0) to remove click reagents and unreacted dansyl using

Microcon® Centrifugal Filter Units (10,000 kDa, Millipore). As a negative control Met-CalB was added to the reaction mixture instead of AHA-CalB.

Similar conditions were used for the click reaction between AHA-CalB and alkyne-PEG(5000). PEG(5000) (20 equivalents) was added to AHA-CalB (1.875 nmol) or Met-CalB together with $CuSO_4$, ascorbate and ligand (30 equivalents each). Reaction samples were incubated at room temperature for 1-3 days and analysed by SDS-PAGE. Before measuring enzyme activity of PEGylated CalB, azide-functionalized Merrifield resin (1.5 mg) was added to enable the removal of non-reacted PEG(5000) as much as possible. An additional amount of $CuSO_4$, ascorbate and ligand was added as well. Overnight incubation and dialysis against buffer was followed by size-exclusion chromatography on a Superdex 75 PC 2.3/30 column.

**Enzyme activity assay**

Lipase activity was analysed by the hydrolysis of *para*-nitrophenol butyrate (*p*-NPB, Sigma). The reaction mixture (50 μL, pH 7) was composed of 50 mM $NaH_2PO_4$, 150 mM NaCl, enzyme (100 nM), isopropanol (5%), Triton (0.1%) and *p*-NPB (1 mM). The production of *para*-nitrophenol was monitored for 5-6 minutes at 25 °C by measuring absorbance at 405 nm in a UV-Visible spectrometer (Varian). The slope of the curve was taken as a measure of hydrolytic activity.

**SDS-page analysis**

Protein samples were analysed by electrophoresis on 12 % (w/v) polyacrylamide gels followed by Coomassie staining. To detect coupling of fluorescent alkynyl-dansyl to AHA-CalB, the gel was placed onto a UV light box prior to staining.

**Mass characterization**

Incorporation of azidohomoalanine into CalB and coupling of the alkyne-functionalized dansyl to AHA-CalB was detected by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The protein was treated with trypsin in advance. Sequencing-grade modified trypsin (0.5 μg, Promega) was added to AHA-CalB or Met-CalB (approximately 6 μg) in 50 mM $NH_4HCO_3$, pH 8.0. After incubation overnight at 37 °C, the tryptic digest was analysed on a Bruker Biflex III spectrometer with α-cyanohydroxycinnamic acid (Sigma) as matrix.

The whole protein was analysed by electron-spray ionization time-of-flight (ESI-TOF) on a JEOL AccuTOF. CalB (~4-10 μM) in formic acid (0.1-1 %) was injected. Deconvoluted spectra could be obtained using Magtran software.