



## Journal Name

### COMMUNICATION

#### *Electronic Supporting Information (ESI).*

## Fabrication of heterogeneous biocatalyst tethering artificial prosthetic groups to obtain omega-3-fatty acids by selective hydrolysis of fish oils

S. Moreno-Pérez<sup>a</sup>, G. Fernández-Lorente<sup>b</sup>, O. Romero<sup>c</sup>, J.M. Guisán<sup>a\*</sup>, F. López-Gallego<sup>d\*</sup>

### Experimental section

#### Materials

Agarose 6BCL was purchased from Agarose Bead Technologies (Madrid, Spain). Cyanogen bromide activated Sepharose 4B (Ag-CB) and Octyl-Sepharose CL-4B (Ag-O) were purchased from General Electric Healthcare (Uppsala, Sweden). Agarose 6BCL activated with glyoxyl groups (Ag-G) was prepared as previously described<sup>1</sup>. DL-Dithiothreitol (DTT), Ethanolamine, Triton X-100, 1-Naphthalenethiol, Acetonitrile (AcN), Sodium borohydride (NaBH<sub>4</sub>), cis-5,8,11,14,17-Eicosapentaenoic acid, cis-4,7,10,13,16,19-Docosahexaenoic acid, *p*-nitrophenyl butyrate (p-NPB), Tris (diethylamino) phosphine, 5,5'-Dithiobis(2-nitrobenzoic acid) (Ellman's reagent), 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) were purchased from Sigma Chem. Co (St. Louis, USA). Molecular biology reagents were obtained from Bio-Rad. (Madrid, Spain). Alil metanotiosulfonato was purchased from Toronto chemical research (TRC) (Toronto, Canada). Plasmids encoding BTL2 and the different mutants utilized in this study were described in previous works<sup>2,3</sup>. Sardine Oil was purchased from BTSa, Biotecnologías Aplicadas, S.L. (Madrid, Spain).

#### Methods

##### Expression and purification of BTL2.

We used a small library of mono-cysteine mutants of BTL2, the mutants were previously described by our group<sup>2,3</sup> using the plasmid pT1-BTL2 described elsewhere<sup>4</sup> as template. The native lipase and the different mutants were expressed in *Escherichia coli* BL21(DE3) (laboratory stock). Freshly transformed bacteria were cultured in Luria Bertani (LB) medium supplemented with 150 µg/mL ampicillin as resistance marker. Preinocule (3ml) was cultured at 30°C during 18 h; such culture was used to inoculate 1 L of medium until it reached an optical density of 0.7 at 600 nm. The expression of recombinant BTL2 was induced by increasing the temperature up to 42°C for 20 h<sup>5</sup>. Then, cells were collected by centrifugation at 5524xg. Isolated cell pellets were suspended in 80 mL of 25 mM sodium phosphate, 0.05M sodium chloride, pH 7, and further sonicated (10 min, 30% amplitude, on ice). Then, lysate was centrifuged at 15344xg for 20 minutes to discard the cell debris and the membrane fraction, the supernatant containing the soluble fraction of protein was stored at 4°C for purification. Afterwards, BTL2 variants were purified from others proteins by interfacial activation using octyl agarose and the absorbed fraction was eluted with 0.2 % Triton X-100. All purification experiments were performed at 4°C.

##### Enzymatic Activity and Protein assays.

Generally, the lipase activity of BTL2 variants was assayed using as substrate *p*-nitrophenyl butyrate (p-NPB)<sup>6</sup>. This method was also used to monitor the immobilization process, using a spectrophotometer with a temperature-controlled cell and continuous magnetic stirring. Briefly, the activities of the lipase, both in its soluble and immobilized forms, were analyzed by measuring the increment of absorbance at 348 nm ( $\epsilon=5.150 \text{ M}^{-1}\text{cm}^{-1}$ ) produced by the release of *p*-nitrophenol by hydrolysis of 0.4 mM pNPB in 25 mM sodium phosphate buffer at pH 7 and 25 °C. One international activity unit (IU) was defined as µmols of hydrolyzed pNPB per minute.<sup>7</sup> The amount of protein was quantify by using micro BCA protein assay<sup>8</sup>

##### Immobilization of BTL2 on different carriers.

###### *Hydrophobic adsorption on Ag-O.*

Different BTL2 variants were immobilized on Ag-O by interfacial activation<sup>9</sup>. One gram of Ag-O was added to 10 mL of non purified 600 IU in 5 mM sodium phosphate buffer at pH 7 and 25°C under gentle stirring. A blank solution was prepared without adding

support to monitor the activity of the free protein under the immobilization conditions. Periodically, activities of suspensions and supernatants were withdrawn and then assayed by using the colorimetric assay with pNPB. Afterwards, immobilized preparations were washed extensively with distilled water and stored at 4 °C.

#### *Covalent immobilization on Ag-CB*

600 IU of pure native and mutants of BTL2 in 5mM sodium phosphate buffer pH 7 were incubated with 1 gram of Ag-CB for 20 min at 25°C under continuous gentle stirring. Periodically, samples from both suspensions and supernatants were withdrawn and then the activity of those samples assayed by using the pNPB assay. Once the immobilization was completed, 1g of the immobilized preparations were washed with 10 volumes of distilled water and then incubated with 10mL of 1 M ethanolamine at pH 8 for 2h<sup>10</sup>. Finally, the immobilized enzyme was filtered and washed with abundant water and stored at 4 °C.

#### *Covalent immobilization on Ag-G*

600 IU of pure native and mutants of BTL2 in 25mM bicarbonate buffer at pH 10 were incubated with 1 gram of Ag-G at 25°C and under continuous gentle stirring. Periodically, samples of suspensions and supernatants were withdrawn and then their activity was assayed by using the pNPB assay. Once the immobilization was completed, the immobilized preparation was reduced by adding solid NaBH<sub>4</sub> to reach a final concentration of 1mg/mL during 30 minutes at 25°C. Finally, the immobilized enzyme was filtered and abundantly washed with distilled water and stored at 4°C<sup>11</sup>.

#### **Solid-Phase Site-Selective Chemical Modification (SPSCM) of Monocysteine BTL2 Mutants.**

The immobilized preparation of each BTL2 variant was reduced in a 1:10 (w/v) ratio with a solution containing 5 mM DTT and 0.02% Triton X-100 at pH 8 and 25 °C for 30 min. Then, the derivatives were exhaustively washed, using vacuum filtration, with distilled water and finally equilibrated with 25 mM sodium phosphate at pH 8. For the naphthyl chemical modification, the reduced immobilized preparations were activated by thiol-exchange with a 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) solution prepared in 25 mM sodium phosphate and 0.02% Triton X-100 at pH 8 and 25 °C. This incubation was carried out with gentle stirring for 1 h. Following the activation step, the immobilized samples were washed with distilled water and finally equilibrated in 17.5 mM Tris-HCl buffer with 0.02% Triton X-100 and 60% of acetonitrile. At this point, the immobilized and activated enzymes were ready to tether with the Naphthalenethiol. In the case of the thiomethanesulfonic acid S-allyl ester, it is not required the cysteine activation with DTNB, then such compound was directly incubated with the reduced immobilized preparations. A solution of 1.5 mM 1-Naphthalenethiol was prepared in 17.5 mM Tris-HCl buffer with 0.02% Triton X-100 and 60% of acetonitrile at pH 8, while 1.5 mM of thiomethanesulfonic acid S-allyl ester was prepared with 50% of DMSO in 25 mM Tris-HCl buffer at pH 8 with 0.02% of Triton x-100. Each solution was incubated with the different immobilized BTL2 variants properly activated in a ratio 1:10 (w/v) for 2 h under gentle stirring at 25 °C. Finally, the BTL2 variants modified with the synthetic groups were exhaustively washed with distilled water and equilibrated with 25 mM Tris-HCl at pH 7. All samples were subsequently stored at 4 °C. Thiol groups were determined spectrophotometrically by the method described by Ellman<sup>12</sup> for quantifying free sulfhydryl group in solution before and after modification. The thiol concentration was calculated using the Beer-Lambert law, with a molar extinction coefficient for DTNB of 14150 M<sup>-1</sup>cm<sup>-1</sup> at 412 nm. Finally, 10mL 70mM CAPS buffer, 0.02% Tx-100, and 10mM Tris (diethylamino) phosphine at pH 9.5 and 25°C were incubated with 1 gram of the modified and immobilized proteins to transform the disulfide bonds into thioether bonds<sup>13</sup>. The suspension was gently stirred for 18 hours at 25°C and then the immobilized enzyme was filtered, washed with abundant water, and stored at 4 °C.

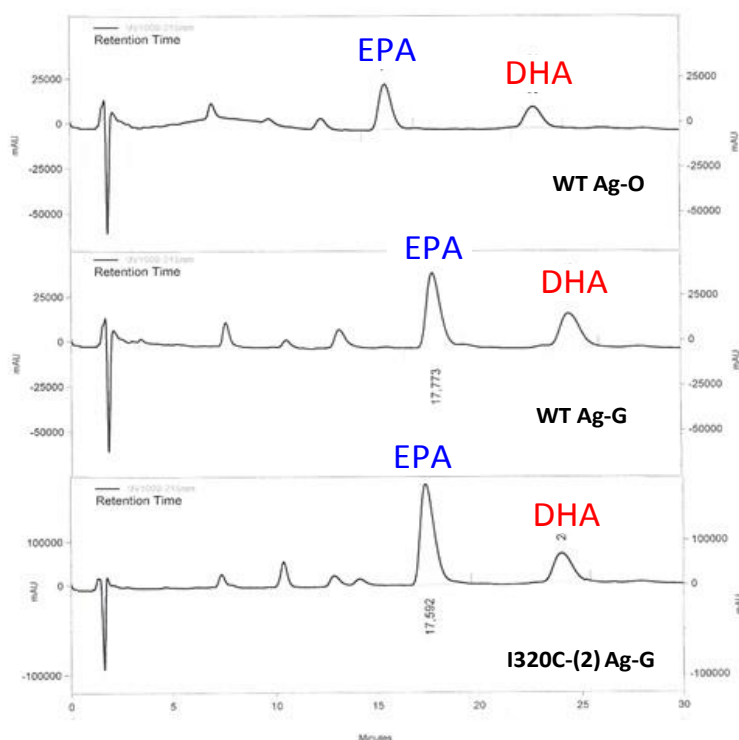
#### **Sardine oil hydrolysis catalyzed by immobilized BTL2.**

The omega-3 fatty acid composition of sardine oil from BTSA was 18% of EPA and 12% of DHA. The hydrolysis was performed at 25°C in an organic (4.5ml of cyclohexane and 0.5ml of sardine oil) and aqueous (5ml Tris-Cl buffer 10 mM pH 6) biphasic system as described Fernandez-Lorente et al<sup>14</sup>. The reaction was initiated by adding 0.1 g of different lipase derivatives. The concentration of free fatty acids was determined at different times by HPLC-UV method. All experiments were carried out at least in triplicate, and standard errors were never over 5%.

The determination of PUFAs was carried out by HPLC using an Spectra Physic SP 100 system equipped with a binary pump and a UV-detector (UV Spectra SP Physic 8450). A C18 column, (Ultrabase-C18, 250 x 4.6 mm, 5µm, Análisis Vínicos Spain) was used as stationary phase. We used the isocratic mobile phase Methanol:Water:Acetic acid (95:5:0.1) at 1 ml min<sup>-1</sup> flow. Pure commercial EPA and DHA were used as standards.

## Supporting Results

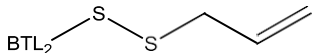
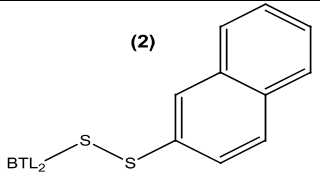
**Fig. S1. Chromatograms of sardine oil hydrolysis catalyzed by different immobilized BTL2 variants.** Retention time for EPA and DHA were 16.8-17.7min and 23.1-24.1min, respectively.



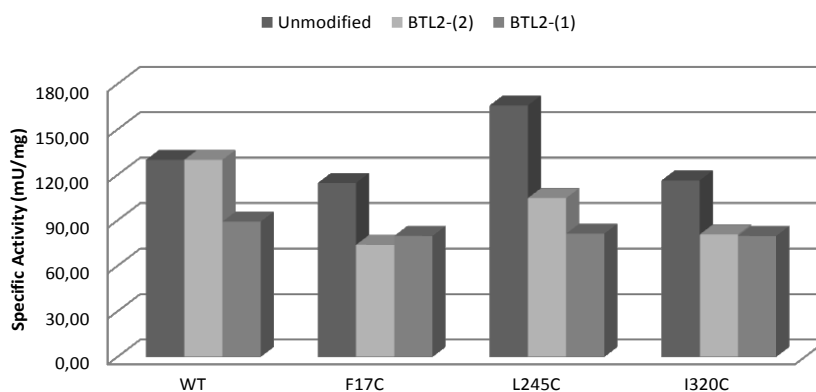
**Table S1. Immobilization yield and recovered activity (IU/g) after the immobilization of different lipases variants on different carriers.** Hydrolytic activity was determined towards pNPB. <sup>a</sup>Immobilization yield ( $\psi$ ) = (immobilized activity/offered activity) x 100. <sup>b</sup>The recovered activity is defined as the measured activity of the immobilized enzyme after immobilization and washing expressed as IU/g of wet support..

Biocatalyst variant	<sup>a</sup> Immobilization yield (%)				<sup>b</sup> Recovered activity IU/g			
	Wt	F17C	L245	I320C	Wt	F17C	L245	I320C
Ag-CB	52	64	62	53	313	386	373	320
Ag-O	47	67	60	47	289	400	360	282
Ag-G	63	62	43	46	380	374	261	277

**Table S2. Yields of chemical modification of different semi-synthetic BTL2 variants.** The modification yield was quantified by using Ellman's reagent. The yield of chemical modification was calculate as the moles of cysteines corresponding to the immobilized protein minus the moles of p-nitrothiobenzoic acid released after the reaction between the Ellman's reagent and the corresponding immobilized preparation.

Enzyme variant	(1) 	(2) 
<b>F17C</b>	100	96
<b>L245</b>	60	94
<b>I320C</b>	87	94

**Figure S2. Specific activity of different enzyme variants and their chemically modified counterparts immobilized on Ag-G and assayed with p-nitrophenyl butyrate as substrate.** Unmodified enzymes are samples that were not incubated with any compound, BTL2-(2) means immobilized enzymes incubated with naphthalenethiol and BTL2-(1) means immobilized enzymes incubated with allyl methanethiosulfonate.



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