Enhancement of a protocol purifying T1 lipase through molecular approach

Che Haznie Ayu Che Hussian [1], **Raja Noor Zaliha Raja Abd. Rahman** [Corresp., 2], **Adam Leow Thean Chor** [3], **Abu Bakar Salleh** [4], **Mohd Shukuri Mohamad Ali** [3]

[1] Institute of Biosciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

[2] Enzyme and Microbial Technology Research, Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

[3] Enzyme and Microbial Technology Research, Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

[4] Enzyme and Microbial Technology Research, Department of Biochemistry, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

Corresponding Author: Raja Noor Zaliha Raja Abd. Rahman
Email address: rnzaliha@upm.edu.my

T1 Lipase is a thermostable secretary protein of *Geobacillus zalihae* strain previously expressed in a prokaryotic system and purified using three-step purification: affinity 1, affinity 2 and ion exchange chromatography (IEX). This approach is time consuming and offers low purity and recovery yield. In order to enhance the purification strategy of T1 lipase, affinity 2 was removed so that after affinity 1, the cleaved GST and matured T1 lipase could be directly separated through IEX. Therefore, a rational design of GST (Glutathione S-Transferase) isoelectric point (pI) was implemented by prediction using ExPASy software in order to enhance the differences of pI values between GST and matured T1 lipase. Site-directed mutagenesis at two locations flanking the downstream region of GST sequences (H215R and G213R) was successfully performed. Double point mutations changed the charge on GST from 6.10 to 6.53. The purified lipase from the new construct GSTm-T1 was successfully purified using two steps of purification with 6849 U/mg of lipase specific activity, 33% yield, and a 44-fold increase in purification. Hence, the increment of the pI values in the GST tag fusion T1 lipase resulted in a successful direct separation through ion exchange chromatography and lead to successful purification.

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Che Haznie Ayu Che Hussian [b], Raja Noor Zaliha Raja Abdul Rahman [a], Adam Leow Thean Chow [a], Abu Bakar Salleh [a], Mohd Shukuri Mohamad Ali [a]

C. H. A. C. Hussian (haznie_ayu@yahoo.com)

[b] *Institute of Biosciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia*

Tel: +6011 28830622

Corresponding author at present address:

R.N.Z.R.A. Rahman (rnzaliha@biotech.upm.edu.my)

[a] *Enzyme and Microbial Technology Research, Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia*.

Tel: +603 89471416 Fax : +603 89467590

A. T. C. Leow (adamleow@biotech.upm.edu.my)

[a] *Enzyme and Microbial Technology Research, Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia*

A. B. Salleh (abubakar@biotech.upm.edu.my)

[b] *Enzyme and Microbial Technology Research, Department of Biochemistry, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia*

M. S. M. Ali (shukuri@biotech.upm.edu.my)

[a] *Enzyme and Microbial Technology Research, Department of Biochemistry, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia*

Abstract

T1 Lipase is a thermostable secretary protein of *Geobacillus zalihae* strain previously expressed in a prokaryotic system and purified using three-step purification: affinity 1, affinity 2 and ion exchange chromatography (IEX). This approach is time consuming and offers low purity and recovery yield. In order to enhance the purification strategy of T1 lipase, affinity 2 was removed so that after affinity 1, the cleaved GST and matured T1 lipase could be directly separated through IEX. Therefore, a rational design of GST (Glutathione S-Transferase) isoelectric point (pI) was implemented by prediction using ExPASy software in order to enhance the differences of pI values between GST and matured T1 lipase. Site-directed mutagenesis at two locations flanking the downstream region of GST sequences (H215R and G213R) was successfully performed. Double point mutations changed the charge on GST from 6.10 to 6.53. The purified lipase from the new construct GSTm-T1 was successfully purified using two steps of purification with 6849 U/mg of lipase specific activity, 33% yield, and a 44-fold increase in purification. Hence, the increment of the pI values in the GST tag fusion T1 lipase resulted in a successful direct separation through ion exchange chromatography and lead to successful purification.

Keywords: *E.coli*; isoelectric point (pI); lipase; purifications; site-directed mutagenesis

Introduction

Lipases or acylglycerol hydrolases (E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of long chain triglycerides with the formation of diacylglycerides, monoglycerides, glycerols and free fatty acids at a lipid-water interface [7]. Lipases are considered prime players in most biotechnological application, as the flexibility of their functional properties and unique inherent specificity and stability have no doubt attracted attention as biocatalysts of the future [11]. Thermostable lipases demonstrate good demands in industrial applications because of their high conversion rate and stability at high temperature. They have huge potential in commercial applications, such as in the production of food additives (for flavor modification), fine chemicals (synthesis of esters), detergents (hydrolysis of fats), wastewater treatment chemicals (decomposition and removal of oil substances), cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fats in foods), leather (removal of lipids from animals skins) and medical

products (blood triglycerides assay) [8, 9, 12, 15]. A review of several findings for thermostable lipases has shown setbacks in obtaining lipase in a large scale due to the cost-intensive demand as compared to other commercial enzymes. Ideally, to fully utilize the beneficial properties of thermostable lipase, the overall production process and its commercialization should be less expensive and less time consuming. Consequently, the homogeneity and purity of these enzymes is fundamental to their overall applications as biocatalysts in industrial enterprises. Basically, most regular lipase purification strategies require at least three to five-step methods [4, 9, 17]. However, nowadays researchers have been working on molecular engineering as a toolbox to facilitate protein purification [6, 18]. Since the successful application of these enzymes requires their production on a large scale, commercializing them will require a novel and simplified approach towards their large-scale production and purification aimed at reducing cost of enzyme production. Therefore, a more efficient purification strategy must be established for future commercialization.

One novel lipase that has high potential to be marketed is T1 lipase, a thermoalkaliphilic enzyme secreted by *Geobacillus zalihae* strain T1. T1 lipase was previously overexpressed in pGEX4T1 vector in prokaryotic system, *Escherichia coli* BL21 (DE3) pLysS as GST tagged fusion T1 lipase and be captured at first step through an affinity chromatography. However, a problem was determined in the next step of separation when the digested T1 lipase and GST tag could be separated directly through IEX as the pI value were too close. Thus, another affinity step known as affinity 2 was used to captured digested GST tag while eluting lipase at flowthrough [14]. Nevertheless, there is still some GST tag eluting together with T1 lipase. To further achieve higher purity of T1 lipase, an additional step of anion exchange chromatography was reported with a substantial enzyme recovery and purity for crystallization [1].

The purification of lipase is not an easy task due to the complexity of proteins and their biological environment; therefore, separation and purification often account for a major proportion of commercialization and production costs. One useful purification technique used to separate proteins based on protein charges is ion exchange chromatography, an outstanding technique in protein purification either in the intermediate stage or during the polishing step. The isoelectric point of target proteins is initially determined followed by identification of suitable matrix either anion or cation exchanger. In this paper, the enhancement of a protocol aiming at purifying a

thermostable T1 lipase was developed in two steps: (i) affinity chromatography to purify the lipase fused to GST-tag out of *E.coli* lysate; and (ii) anion exchange chromatography to finally separate lipase and GST-T1 lipase. As the isoelectric points (pI) of GST and T1 lipase are close, performing anion chromatography directly after first affinity chromatography is initially not possible. To circumvent thus issue, site-directed mutagenesis on two residues was conducted to generate a GST-tag mutant (GSTm) with a higher pI. The resulting GSTm-tag could be used for the first affinity chromatography, then separated from T1 lipase by anion exchange chromatography.

Materials and methods

Bacterial strains and plasmids

Escherichia coli Top 10 and BL21 (DE3) pLysS cells harboring the recombinant pGEX/T1 of *Geobacillus zalihae* strain T1 (AY260764.2) were used for cloning and expression, respectively. The recombinant pGEX/T1 was used as a source for rational design.

Isoelectric point (pI) value computation

The computation of pI value of GST tag protein and mature T1 lipase was performed using ExPASy-compute pI/MW at http://www.expasy.ch/tools/pI-tool.html. Two point mutations H215R and G213R were conducted at C-terminal end of GST tag and the theoretical pI value computed using the above software.

Molecular modification by site-directed mutagenesis

Mutagenic primers (forward and reverse) were designed by referring to the point of mutation (bold and underlined) at C-terminal end of GST tag sequence. The designed mutagenic primers are, MutGST Forward: 5′-GCC ACG TTT GGT GGT **AGA** GAC **AGA** CCT CCA AAA TCG GAT-3′ and MutGST Reverse: 5′-ATC CGA TTT TGG AGG TCT GTC TCT ACC ACC AAA CGT GGC-3′. Site directed mutagenesis was performed using a Strategene QuikChange® Lightning kit according to manufacturer instructions. The recombinant mutagenic plasmid of positive transformant was confirmed by DNA sequencing before transformation into expression host *Escherichia coli* BL21 (DE3) pLysS.

Transformation into expression system

Once the sequencing results showed that the amino acids were successfully substituted, 5 µL of mutated vector was transferred into competent cells *E.coli* BL21 DE(3) pLySs (20µL) and incubated for 30 min on ice. The samples in the Eppendorf tubes were pulse heated in water bath at 42ºC for 90 seconds and immediately incubated at 37ºC for 1 h. Then, sample was spread on LB- Tributyrin agar containing ampicillin and chloramphenicol antibiotic and incubated at 37ºC for 1 day. Transformed colonies were screened on Triolein and Rhodamine agar plates.

Expression of GSTm-T1 lipase (mutated)

Mutagenic fusion lipase (GSTm-T1) expression was conducted in 200 mL LB containing ampicillin (50mg/mL) and chloramphenicol (35mg/mL) at 37ºC with an agitation rate of 200 rpm. The culture was induced with 0.025 mM of Isopropyl ß-D-thiogalactopyranosidase (IPTG) for 12 h and harvested at 10 000 rpm and 4ºC for 10 min and pellet stored in a freezer at -80ºC.

Cell lysis and clarification of lysate

The pellets were resuspended with 20mL of phosphate buffered saline (PBS) pH 8.0 containing 5 mM DTT and sonicated for 4 min (30 seconds on, 30 seconds off). Cell debris was removed by centrifugation at 12 000xg for 30 min. Pelleted cells debris was discarded and supernatant was taken as crude enzyme and filtered by using a 0.45 µm syringe filter.

Purification

Purifications of GSTm-T1 lipase and GST-T1 lipase (wild type) conducted as a comparison study. Crude enzyme (20 mL) was subjected to affinity chromatography column XK16/20 containing 5mL of Glutathione Sepharose Fast Flow from Amersham Bioscience. The 20% ethanol in resin firstly was removed by distilled water with a flow rate of 1 ml/min for 10 column volume (CV). Then resin was equilibrated with 3 to 5 CV of phosphate buffered saline (PBS) pH 7.4. The equilibrated column was attached to Fast-Performance Liquid Chromatography (FPLC) system (AKTA Explorer, GE Healthcare). Crude enzyme was loaded onto the column and operated by AKTA Explorer system at a flow rate of 1 ml/min. Fractions were collected and fusion lipases eluted with thrombin cleavage buffer pH8.0 (50 mM Tris-HCl), supplemented with 10 mM reduced glutathione.

Fusion lipase fractions from affinity chromatography were pooled and absorbance at 280 nm was read. Then, bovine thrombin was added into the pooled samples and incubated at 20ºC for 20 h prior to the cleavage of GST and matured T1 lipase. The incubated samples were analyzed by SDS PAGE to make sure GST and matured T1 lipase were cleaved and separated. The sample was then subjected to buffer exchange by using Sephadex G25. The digested fusion lipase was separated by injecting into Q sepharose FF with a flow rate of 0.5 mL/min. After sample injection, the flow-through fraction (5 CV) was collected using binding buffer as the eluent, followed by washing unbound proteins (5 CV) and eluted using salt gradient (20 CV) toward 25 mM Tris-HCl buffer (pH 8.5) containing 0.25 M NaCl. Lipase activity was checked for each fraction and protein bands were also analyzed by using SDS PAGE. The desired fractions were then pooled and protein contents were determined using Bradford assay (Bradford 1976).

Lipase assay

Lipase activity was assayed calorimetrically per Kwon and Rhee [13]. The culture filtrate (1 ml) was shaken with 2.5 mL of olive oil (70% oleate residues) emulsion (1:1, v/v) and 20 µL of 0.02 M $CaCl_2$ in a water bath shaker at an agitation rate of 200 rpm. The emulsion was prepared by mixing together an equal volume of olive oil (Bertoli, Italy) and 50 mM phosphate buffer with a magnetic stirrer for 10 min. The reaction mixture was shaken for 30 min at 50ºC. The enzyme reaction was stopped by adding 6N HCl (1 ml) and isooctane (5ml), followed by mixing using vortex mixer for 30s. The upper isooctane layer (4ml) containing the fatty acid was transferred to a test tube for analysis. Copper reagent (1ml) was added and again mixed with a vortex mixer for 30 s. The reagent was prepared by adjusting the 5% (w/v) solution of copper (II) acetate-1-hydrate to pH 6.1 with pyridine. The absorbance of the upper layer was read at 715 nm. Lipase activity was measured by measuring the amount of free fatty acids. One µmol of lipase activity was measured as the free fatty acids generated in 1 min.

SDS-PAGE analysis

SDS-PAGE (12 % running gel, 6 % stacking gel) was conducted according to the method of Laemli et al (1970). A broad range of protein standard (MBI Fermentas; St. Leon-Rot, Germany) was used as a molecular mass marker. Protein concentrations were determined according to the Bradford method, with bovine serum albumin as standard [3].

168 **Results**

169

170 **pI value computation**

171 The pI values of GST tag and matured T1 lipase were theoretically determined by using ExPASy tool at 6.10
172 (Fig.1a) and 6.02 (Fig. 1b), respectively, hindering their separation through anion exchange chromatography.
173 Therefore histidine at position 215 (H215) and glycine at position 213 (G213) of the amino acid were replaced with
174 arginine. The new pI value of GST tag after computing H215R and G213R is 6.53 (Fig. 1c), higher than the native
175 GST tag by 0.43

176

177 Site-directed mutagenesis at two amino acids was successfully performed using designed mutagenic primers with
178 melting temperature (Tm) of 78°C. Both of the 39 base pairs mutagenic primers (forward and reverse) contained
179 mutations and were annealed to the opposite strands of the pGEX4T1 plasmids. These mutagenic primers were
180 designed by locating the desired mutation sequence in the middle of the primer with 15 bases of correct sequence on
181 both sides. Sequencing results for the mutated GST tag were analyzed online at the SDSC Biology Workbench
182 website (http://workbench.sdsc.edu/update.html). As shown in Fig. 2, the two points of mutations were successfully
183 examined and no other changes in the GST sequence were found.

184

185 **Purifications**

186 The construct of GSTm was purified using two purification steps which were affinity chromatography and anion
187 exchange chromatography. Affinity chromatogram results showed that GSTm interacted with the ligand coupled to a
188 Glutathione Sepharose 4 Fast Flow and the elution was performed with 10 mM reduced glutathione as competitive
189 ligand with flow rate of 1 ml/min with a recovery of 82%. Abundance of proteins was detected at a flow through
190 with a reading 3000 mAU at 280 nm and no lipase activities were determined at flow through fractions,
191 demonstrating that GSTm were successfully bound to the resins after go through some of modification. Elution was
192 carried out with flow rate of 1 ml/min by the gradient with 20 CV (0-100%). The purified fusion lipase was eluted at
193 32% gradient, and one peak was obtained (Fig. 3a). The fractions of eluted fusion GST-T1 lipase were collected and
194 pooled, assayed, and subjected to SDS-PAGE analysis. Lipase activity and SDS-PAGE gave 138.42 U/mL and 63
195 kDa of fusion GST-T1 lipase molecular size respectively (Fig. 3b).

196

197 The separation of T1 lipase between modified GSTm and unmodified GST tag were examined. Digested GSTm and
198 GST tag from T1 lipases was subjected to anion exchange chromatography with flow rate 0.5 ml/min. Theoretically
199 GSTm and T1 lipase mixtures can be separated through anion exchanger as their pI value have been differentiated
200 (6.53 and 6.02, respectively) and raised by 0.43, otherwise not for unmodified GST tag as their pI values were close.
201 The chromatogram result for native GST in Fig. 4a has proven that GST and T1 lipase were not well separated as
202 two redundant peaks were obtained. SDS-PAGE analysis results revealed that T1 lipase was eluted with GST tags in
203 all fractions, starting from Peak 1 followed by Peak 2, while Peak 3 represented pure GST tag (23 kDa).

204

205 However, the chromatogram results for GSTm-T1 lipase in Fig. 5a show that three separated peaks were
206 successfully obtained and peak 1 was revealed by SDS PAGE as purified T1 lipase with 95.89 U/mL of pooled
207 lipase activity SDS-PAGE result in Fig. 5b it is evident that single band of purified T1 lipase was successfully
208 obtained as a purified enzymes, while the two bands were obtained for all fractions eluted at peak 2 and peak 3 were
209 purely GST tag with size 23 kDa.

210

211 From IEX results we noticed that experimentally, T1 lipase was eluted earlier instead of prediction that GST tag
212 should eluted earlier as the pI value of GST tag is higher compared to T1 lipase. Even though the prediction is not
213 same as experimentally but the GST tag and T1 lipase were separated after a pI value modification. Purity of T1
214 lipase pooled fractions was given in Table.1. The final yield of mature T1 lipase was 33%, with the later having a
215 specific activity of 6849 U/mg.

216

217 **Discussion**

218

219 **Alteration of value**

220 Emerging an efficient purification strategy by some of trials error is time-consuming. Therefore modelling of
221 biochemical properties by rational design is a promising approach to allow focused experimental testing [20]. The
222 major finding of this work is successful purification by enlarging the differences of the pI values between the GST
223 fusion tag and the matured lipase as obtained by site-directed mutagenesis, so that the two can be cleanly separated

in anion exchange step. The theoretical pI value of proteins can be calculated and can be used as a reference for choosing proper conditions for purification [5]. In details, we computed the GST tag and T1 lipase pI value based on protein amino acid compositions in the sequence data. However due to the close pI values of GST tag and T1 lipase by 0.08 differences, amino acid levels were changed to increase the pI value. Amino acids modification was only conducted on GST tag thus conformational structure of T1 lipase could be retained.

The GST tag expressed from the pGEX4T1 vectors is from *S. japonica* (Sj-GST) and it contains 218 amino acids. H215 and G213 were chosen as they located at the last eight amino acids in the Sj-GST domain (residues 211–218, GGGDHPPK) which not present in other mammalian GST structures and are unique for Sj-GST [21]. It forms an open hairpin loop. The unique sequence of GGG at the bottom of the loop just behaves as a better linker to provide different proteins to its C terminus and does not affect the folding of GST tag. The linker regions after the open hairpin loop have different conformations for different sequences and really flexible for any amino acids changes. In other cases the substitution of SjGST Glu26 for His increases its metal binding affinity and allows for the efficient purification of recombinant proteins using immobilized Ni affinity chromatography (Han et al 2010). The most relevant amino acids for changing the pI value of proteins is arginine (R) as pKa arginine's side chain (12.50) is higher compared to other six ionisable residues. It has been commonly reported that substitution of amino acid to arginine often leads to enhancement and lowers the pI value of proteins [22].

Purification of T1 lipase

Many separation techniques can be used in order to separate the protein mixture. Fast-performance liquid chromatography (AKTA Explorer) in combination with ion exchange chromatography is the most widely used method for industrial scaled-up purposes, as it can be used in either positive or negative capture modes. Affinity chromatography is frequently used as a first step for most protein purification studies. A previous purification strategy have been implemented using two steps of affinity chromatography [14] followed by ion exchange chromatography [1]. Second affinity chromatography with attachable three columns (GSTrap FF, Benzamidine, and GSTrap HP) is not relevant to use in large scale. Therefore, the elimination of second affinity chromatography step in this study greatly enhanced direct separation of GST tag and T1 lipase using ion exchange chromatography.

252

253 The prediction of GST tag to be separated through ion exchange chromatography after pI value modification was
254 experimentally confirmed by a Figure 5a. Three separated peaks were obtained and the SDS-PAGE profile revealed
255 that all fractions from Peak 1 are to be purified T1 lipase with molecular weight of 43 kDa (Fig.5b). Nevertheless, an
256 unmodified GST tag was unable to be separated on IEX, as redundant peaks were observed in Fig.4. The purity of
257 these pooled fractions is as given in Table 1.

258

259 Theoretically, pI value prediction shows an earlier elution of GST tag over matured lipase, but in this case matured
260 lipase came out first, followed by GST tag. In reality, simple pI computation does not take the 3D conformation into
261 account, so that the effects of intramolecular interactions between amino acids are ignored. If referred to a random
262 coil, the pI value only depends on a protein amino acid composition and can thus be computed from analytical or
263 sequence data. In contrast, in a native structure where interactions among amino acids stretches can occur, the
264 experimental pI may be subtly altered. Measuring a pI value with precision is not an easy task. A pI is a physio-
265 chemical parameter and its precise assessment might be quite precious in establishing the identity of a protein but a
266 number of experimental parameters might alter its value. One way to inquire is to determine the pI values by native
267 isoelectric focusing gel analysis, which could be compared with the denatured isoelectric focusing gel analysis. The
268 theoretical pI values should be close to the results of denatured isoelectric focusing gel analysis, while the results
269 from chromatography should be consistent with the results of native isoelectric focusing gel analysis.

270

271 The purity of the GSTm construct was compared to previous purification strategies (Table 2). From the previous
272 report, the final yield of mature T1 lipase after a two-step affinity and IEX was only 8.9% [2]. Higher yield recovery
273 of purified T1 lipase was obtained using this new purifying protocol at 33% recovery, thus skipping the affinity 2
274 steps, which are costly and time consuming when using three attached columns (GSTFF, GSTHP and Benzamidine)
275 at lower flowrate 0.25ml/min. Using the newly proposed strategy in this study, the purification steps were
276 successfully reduced from three steps to two steps. Final yield obtained in this strategy is 33% with high purity at44
277 purification fold, along with an acceptable yield and high fold obtained, indicating a high degree of T1 lipase purity.
278 Higher fold of purification is due to high resolution and high loading capacity of ion exchange chromatography
279 resins; therefore purification strategy of T1 lipase was successfully enhanced by using molecular modifications [16].

Conclusions

This study has demonstrated that via molecular modification, new separation and purification techniques were developed. The new constructs of pGEX4T1/GSTm-T1 with two purification step were developed by replacing affinity 2 with ion exchange chromatography. The construct was developed by rational design where double point's mutations at two location of GST tag downstream region by raised the pI value of GST tag. The incensement of pI value has improved the resolving between GST-tag and T1 lipase peak through anion exchange chromatography. Nevertheless, comparisons have been made on Aris (2014) purification strategy on time, yield and fold. The findings thus indicate that GSTm-T1 lipase appeared better with 44 purification fold in 5 h processing time than Aris (2014) with 19 h and 21 h taken to complete the purification process. Therefore, this construct has strong potential for use in scaling up purification of T1 lipase, as it was time saving while allowing for high levels of purification.

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Figure 1

Theoretical pI value of T1 lipase and GST tag before and after pI value was computed at Expasy tool website

(A) Theoretical pI value of T1 lipase. (B) Theoretical pI value of GST tag. (C) Theoretical pI value of GST tag after replacing two points of GST sequences. Glycine at position 213 replaced with Arginine (G213R) and Histidine at position 215 replaced with Arginine (H215R).

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

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(a) T1 lipase. 6.02/43584.71
1      PEFASLRAND APIVLLHGFT GWGREEMFGF KYWGGVRGDI EQWLNDNGYR
61     TYTLAVGPLS SNWDRACEAY AQLVGGTVDY GAAHAAKHGH ARFGRTYPGL
121    LPELKRGGRI HIIAHSQGGQ TARMLVSLLE NGSQEEREYA KAHNVSLSPL
181    FEGGHHFVLS VTTIATPHDG TTLVNMVDFT DRFFDLQKAV LEAAAVASNV
201    PYTSQVYDFK LDQWGLRRQP GESFDHYFER LKRSPVWTST DTARYDLSVS
241    GAEKLNQWVQ ASPNTYYLSF STERTYRGAL TGNHYPELGM NAFSAVVCAP
301    FLGSYRNPTL GIDDRWLEND GIVNTVSMNG PKRGSSDRIV PYDGTLKKGV
361    WNDMGTYNVD HLEIIGVDPN PSFDIRAFYL RLAEQLASLQ P

(b) Glutathione S-Transferase. Theoretical pI/MW : 6.10/26310.53
1      MSPILGYWKI KGLVQPTRLL LEYLEEKYEE HLYERDEGDK WRNKKFELGL
61     EFPNLPYYID GDVKLTQSMA IIRYIADKHN MLGGCPKERA EISMLEGAVL
121    DIRYGVSRIA YSKDFETLKV DFLSKLPEML KMFEDRLCHK TYLNGDHVTH
181    PDFMLYDALD VVLYMDPMCL DAFPKLVCFK KRIEAIPQID KYLKSSKYIA
201    WPLQGWQATF GGGDHPPKSD LVPRGS

c) Glutathione S-Transferase mutant. Theoretical pI/MW :
6.53/26310.53

1      MSPILGYWKI KGLVQPTRLL LEYLEEKYEE HLYERDEGDK WRNKKFELGL
61     EFPNLPYYID GDVKLTQSMA IIRYIADKHN MLGGCPKERA EISMLEGAVL
121    DIRYGVSRIA YSKDFETLKV DFLSKLPEML KMFEDRLCHK TYLNGDHVTH
181    PDFMLYDALD VVLYMDPMCL DAFPKLVCFK KRIEAIPQID KYLKSSKYIA
201    WPLQGWQATF GGRDRPPKSD LVPRGS
```

Figure 2

Sequence alignment result between GST and Mutated GST (Mut_C2_MutSeq_Rev_Reverse-C).

Two points of mutation were successfully created at position 213-GGC (glycine) changed to AGA (arginine) and position 215- CAT (histidine) changed to AGA (arginine).

```
GST                              ----------------------------ATGTCCCC-TATACTAGGTTA
Mut_C2_MutSeq_Rev__Reverse-C     AATTTTCACCACAGGAAACCAGTTTTTTCATGTCCCCCTATACTAGGTTA
                                                             ******** ************

GST                              TTGGAAAATTAA-GGGCCTTGTGCAACCCACTCGACTTCTTTTGGAATAT
Mut_C2_MutSeq_Rev__Reverse-C     TTGGAAAATTAAAGGGCCTTGTGCAACCCACTCGACTTCTTTTGGAATAT
                                 ************ ************************************

GST                              CTTGAAGAAAAATATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAA
Mut_C2_MutSeq_Rev__Reverse-C     CTTGAAGAAAAATATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAA
                                 ************************************************

GST                              ATGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTT
Mut_C2_MutSeq_Rev__Reverse-C     ATGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTT
                                 ************************************************

GST                              ATTATATTGATGGTGATGTTAAATTAACACAGTCTATGGCCATCATACGT
Mut_C2_MutSeq_Rev__Reverse-C     ATTATATTGATGGTGATGTTAAATTAACACAGTCTATGGCCATCATACGT
                                 ************************************************

GST                              TATATAGCTGACAAGCACAACATGTTGGGTGGTTGTCCAAAAGAGCGTGC
Mut_C2_MutSeq_Rev__Reverse-C     TATATAGCTGACAAGCACAACATGTTGGGTGGTTGTCCAAAAGAGCGTGC
                                 ************************************************

GST                              AGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAGATACGGTGTTT
Mut_C2_MutSeq_Rev__Reverse-C     AGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAGATACGGTGTTT
                                 ************************************************

GST                              CGAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTTGATTTTCTT
Mut_C2_MutSeq_Rev__Reverse-C     CGAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTTGATTTTCTT
                                 ************************************************

GST                              AGCAAGCTACCTGAAATGCTGAAAATGTTCGAAGATCGTTTATGTCATAA
Mut_C2_MutSeq_Rev__Reverse-C     AGCAAGCTACCTGAAATGCTGAAAATGTTCGAAGATCGTTTATGTCATAA
                                 ************************************************

GST                              AACATATTTAAATGGTGATCATGTAACCCATCCTGACTTCATGTTGTATG
Mut_C2_MutSeq_Rev__Reverse-C     AACATATTTAAATGGTGATCATGTAACCCATCCTGACTTCATGTTGTATG
                                 ************************************************

GST                              ACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGATGCGTTC
Mut_C2_MutSeq_Rev__Reverse-C     ACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGATGCGTTC
                                 ************************************************

GST                              CCAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGA
Mut_C2_MutSeq_Rev__Reverse-C     CCAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGA
                                 ************************************************

GST                              TAAGTACTTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAGGGCTGGC
Mut_C2_MutSeq_Rev__Reverse-C     TAAGTACTTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAGGGCTGGC
                                 ************************************************

GST                              AAGCCACGTTTGGTGGTGGCGACCATCCTCCAAAATCGGATCTGGTTCCG
Mut_C2_MutSeq_Rev__Reverse-C     AAGCCACGTTTGGTGGTAGAGACAGACCTCCAAAATCGGATCTGGTTCCG
                                 ***************** * ***   ************************

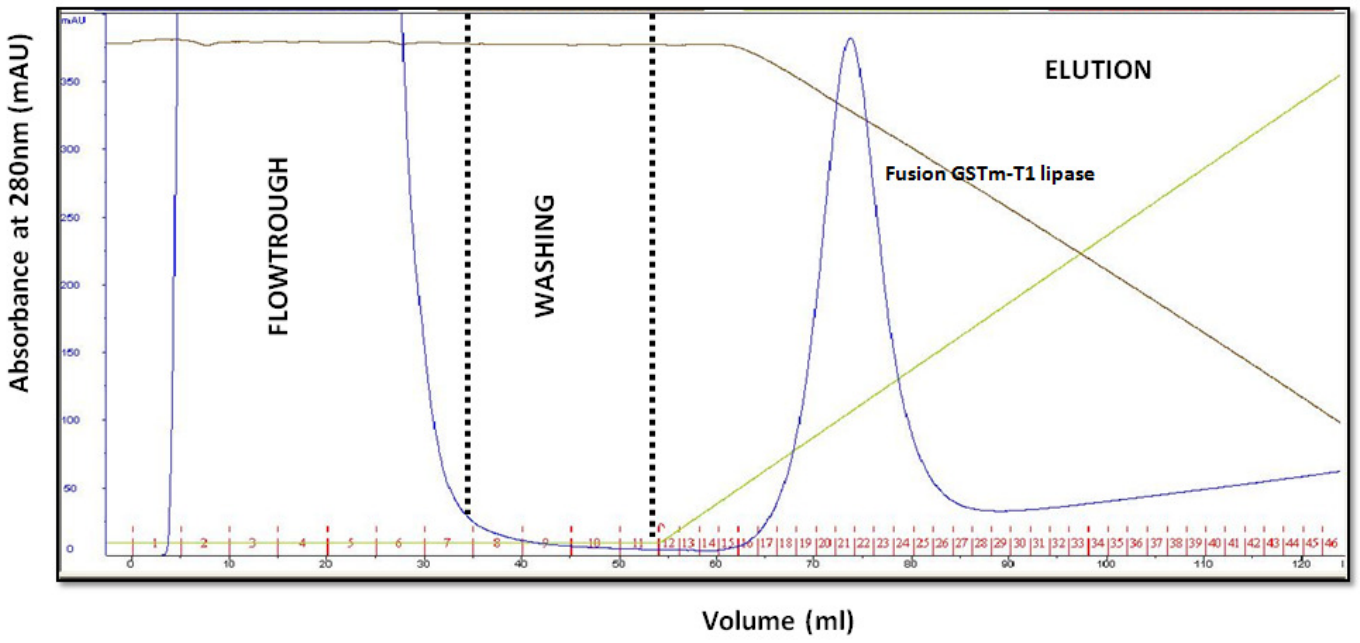
GST                              CGTGGATCC---------------------------------------
Mut_C2_MutSeq_Rev__Reverse-C     CGTGGATCCGCATCCCTACGCGCCAATGATGCACCGATTGTGCTTCTCCA
                                 *********
```

Figure 3

Chromatogram profile for affinity chromatography and SDS-PAGE analysis on eluted proteins.

(A) Chromatogram result of affinity chromatography (AKTA Explorer) for GSTm-T1 lipase; A single peak were obtained. (B) SDS-PAGE analysis results for the purification of fusion GST-T1 lipase. Lane M: standard protein marker; Lane 1: Crude enzymes; Lane 2-7: Purified fusion GST-T1 lipase (66 kDa)

(a)


Absorbance at 280nm (mAU)
FLOWTROUGH
WASHING
ELUTION
Fusion GSTm-T1 lipase
Volume (ml)

(b)

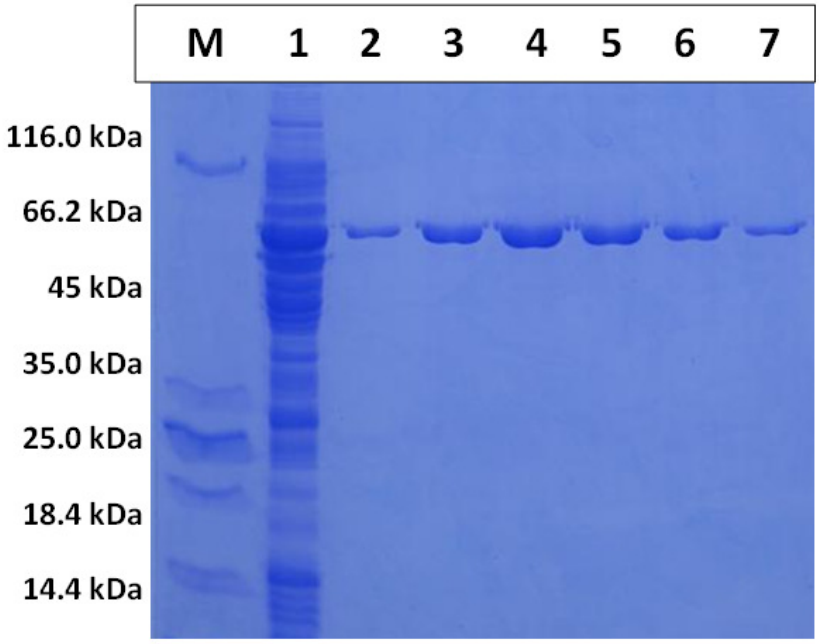
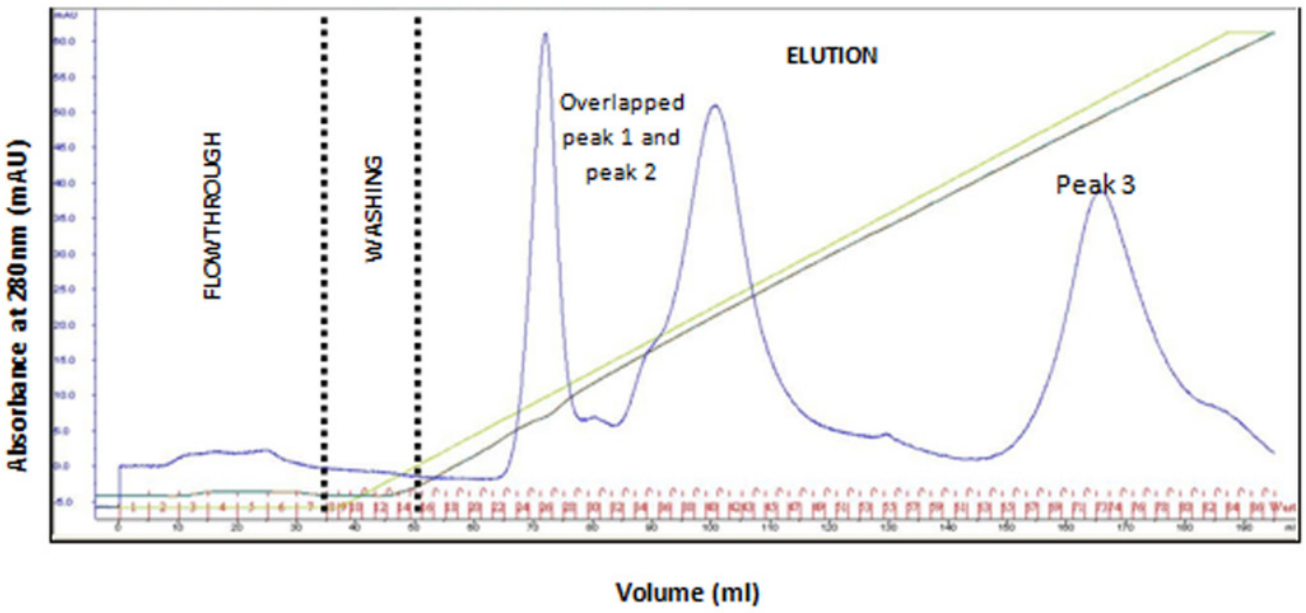

M
1
2
3
4
5
6
7
116.0 kDa
66.2 kDa
45 kDa
35.0 kDa
25.0 kDa
18.4 kDa
14.4 kDa

Figure 4

Chromatogram profile of ion exchange chromatography for GST-T1 lipase and SDS-PAGE analysis on eluted proteins

(A) Chromatogram result of ion exchange chromatography (AKTA) for native GST-T1 lipase; Overlapped peaks were obtained. (B) SDS-PAGE analysis results for the purification of a native T1 lipase. Lane M: standard protein marker; Lane 1-9: GST tag and T1 lipase eluted together at peak 1; Lane 10-12: GST tag and T1 lipase eluted together at peak 2 ; Lane 13 and 14: GST tag

(a)


Absorbance at 280nm (mAU)
FLOWTHROUGH
WASHING
ELUTION
Overlapped peak 1 and peak 2
Peak 3
Volume (ml)

(b)

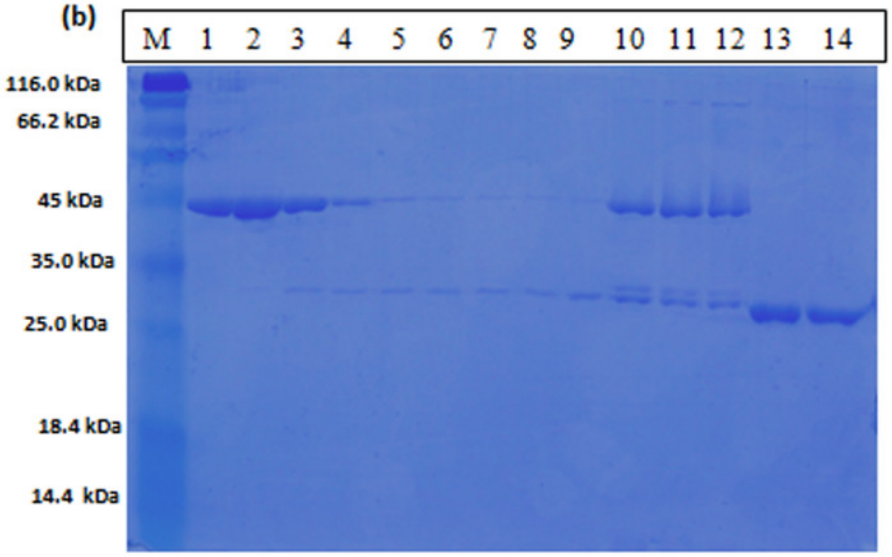
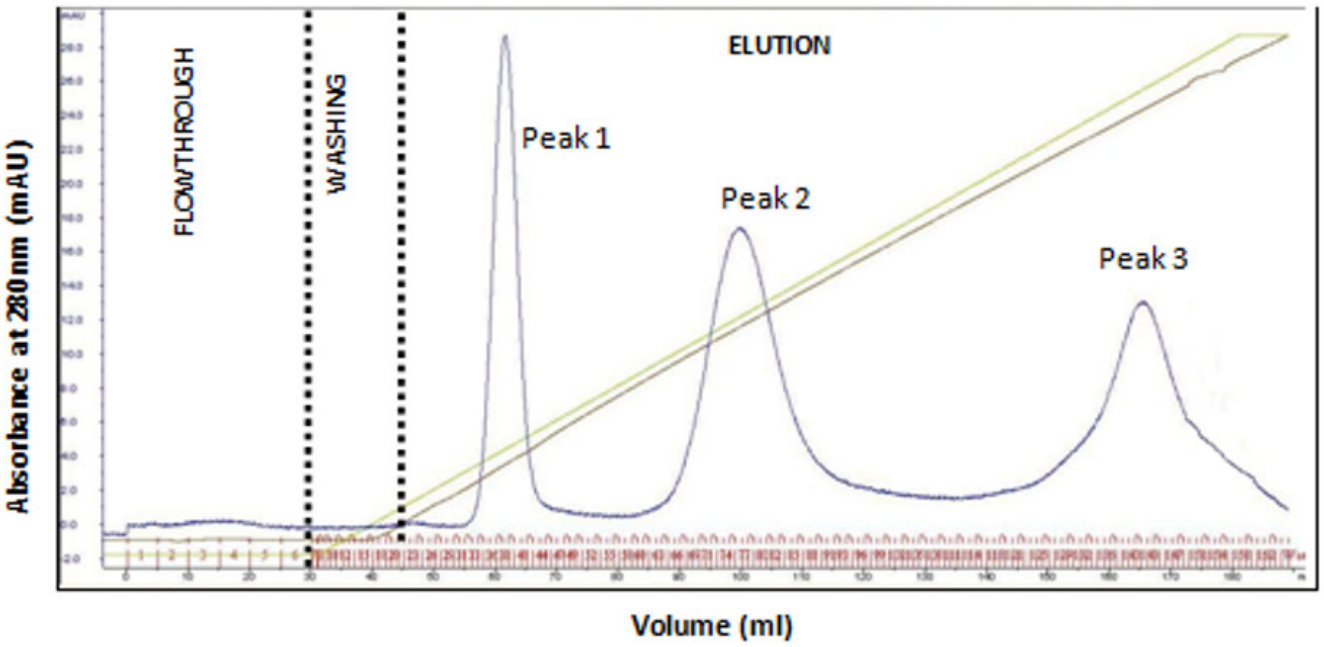

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
116.0 kDa
66.2 kDa
45 kDa
35.0 kDa
25.0 kDa
18.4 kDa
14.4 kDa

Figure 5

Chromatogram profile of ion exchange chromatography for GSTm-T1 lipase and SDS-PAGE analysis for eluted proteins.

(A) Chromatogram result of ion exchange chromatography for GSTm-T1 lipase; Three separated peaks were obtained. (B) SDS-PAGE analysis results for the purification T1 lipase from new construct (affinity chromatography and ion exchange chromatography). Lane M: standard protein marker; Lane 1: crude cell lysate ; Lane 2: purified fusion lipase (66 kDa); Lane 3: fusion lipase after thrombin cleavage at 16ºC; Lane 4; pooled purified matured T1 lipase at peak 1 (43 kDa); Lane 5: Some of T1 lipase and GST tag eluted in the same fractions at peak 2; Lane 6: GST tag at peak 3 (23 kDa).

(a)


FLOWTHROUGH
WASHING
ELUTION
Peak 1
Peak 2
Peak 3
Absorbance at 280nm (mAU)
Volume (ml)

(b)

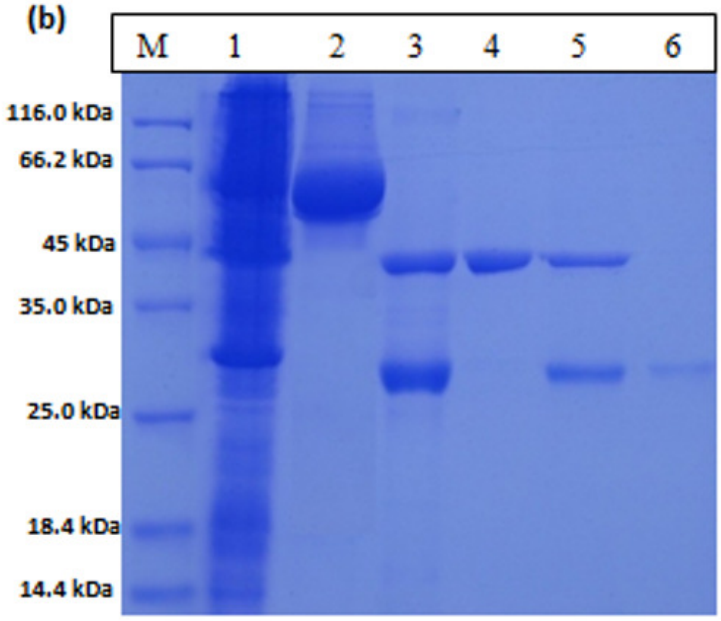

M
1
2
3
4
5
6
116.0 kDa
66.2 kDa
45 kDa
35.0 kDa
25.0 kDa
18.4 kDa
14.4 kDa

Table 1(on next page)

Purification of T1 lipase from a newly constructed GSTm-T1 lipase from *Escherichia coli* BL21 DE3 pLysS as an expression system.

Purification	Volume (mL)	Protein content (mg/mL)	Activity (U/mL)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
Crude	20	0.56	85.9	1718	153.4	100	1
Affinity	10.25	0.08	138.42	1418	1730.25	82	11
IEX	5.9	0.014	95.89	565	6849.28	33	44

1

Table 2(on next page)

Purification yield (%) and fold obtaining from previous purification strategies of T1 lipase.

Strategy	Resins	Time (h)	Yield (%)	Fold
GST-T1 lipase (Aris, *et al*., 2011)	Affinity 1: Glutathione Sepharose FF Affinity 2: Glutathione Sepharose HP Hi Trap Glutathione FF Hi Trap Benzamidine Ion Exchange Chromatography Q Sepharose FF	21	19.45	8.90
Construct 1 : GSTm-T1 lipase	Affinity 1: Glutathione Sepharose FF Ion exchange chromatography Q Sepharose FF	5	33	44

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