Use of TPP and ATPS for partitioning and recovery of lipase from Pacific white shrimp (*Litopenaeus vannamei*) hepatopancreas

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Abstract Lipase recovery from Pacific white shrimp hepatopancreas using a three-phase partitioning (TPP) system in combination with an aqueous two-phase system (ATPS) was studied. TPP system was formed with a simultaneous addition of salt directly to crude extract (CE) followed by an organic solvent addition. The various process parameters required for efficient purification of lipase were optimized. The best lipase yield (87.41%) and purification fold (PF) (3.49-fold) were obtained in the interphase of TPP system, which consisted of the CE to *t*-butanol ratio of 1:1 (v/v) in the presence of 50% (w/v) (NH₄)₂SO₄. Subsequently, TPP fraction was subjected to ATPS. Effects of phase compositions including PEG molecular weight and concentration, types and concentration of salts, NaCl addition and system pH on lipase partitioning were investigated. With the application of 25% (w/w) PEG1000 and 15% (w/w) MgSO₄, at pH 5.0 was found most appropriate since high lipase PF (5.19-fold) and yield (78.46%) in top phase were obtained. The partitioned enzyme exhibited optimal activity

at pH 8.0 and 55 °C and was stable at a temperature range of 0–40 °C and a pH range of 7–10. The partitioned lipase showed high tolerance in the presence of ethanol and methanol. Hence, the combined partitioning systems, TPP–ATPS, were found to be an attractive technique for the recovery and partial purification of lipase from Pacific white shrimp hepatopancreas.

Keywords Lipase · TPP · Isolation · Characterization · Hepatopancreas

Introduction

Shrimp has high demand in many countries including Japan, the United States and Europe because of their delicacy (Senphan and Benjakul 2014). Thailand is the world's leading shrimp farming country and has become the top supplier of farmed shrimp. Pacific white shrimp (*Litopenaeus vannamei*) is an important commercial species primarily cultured in Thailand and accounts for 90% of the global aquaculture shrimp production (Senphan and Benjakul 2014). By the year 2012, the total amount of frozen Pacific white shrimp and products was 550,147 metric tons and the products were mainly exported to the United States and Japan (Senphan and Benjakul 2014). Among all shrimp products, there is currently an increasing demand for whole shrimp without hepatopancreas. The hepatopancreas is removed using a vacuum sucking machine and can serve as the potential source of enzymes such as proteases (Senphan and Benjakul 2014) and lipase (Kuepethkaew et al. 2017; Perez et al. 2011).

Three-phase partitioning (TPP) is a modest and relatively recent bioseparation technique which involves the addition of a salt to the aqueous suspension of the feed or crude extract followed by an addition of an organic solvent

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(Senphan and Benjakul 2014). In less than an hour, three phases are formed. The upper solvent phase containing pigments, lipids, and hydrophobic materials is separated from the lower aqueous phase containing proteins, saccharides and cell debris by an intermediate layer. This protein-rich middle layer generally contains desired enzymes or proteins (Rawdkuen et al. 2012). TPP is simple, inexpensive, scalable and a rapid procedure that works at room temperature in comparison to conventional separation and purification processes. TPP has been successfully employed in the purification of industrial important enzymes like protease (Senphan and Benjakul 2014), α -galactosidase (Dhananjay and Mulimani 2008), invertase (Dhananjay and Mulimani 2008).

Partitioning using aqueous two-phase system (ATPS) has proved to be a valuable tool for separating and purifying mixtures of biomolecules via extraction as it is a mild technique. The systems can form a gentle environment for enzyme and other biologically active proteins. Therefore, the extraction in the system has the advantages of high capacity, high activity yields and easy to scale up. ATPS form readily upon mixing aqueous solutions of two hydrophilic polymers, or of a polymer and salt, above a certain threshold concentrations (Klomklao et al. 2005). ATPS has been applied for partitioning and recovery of various enzymes such as protease (Senphan and Benjakul 2014) and lipase (Zhou et al. 2013).

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are a class of enzymes, termed as carboxyl esterases, that catalyze the hydrolysis and synthesis to form esters from the glycerol and the long chain fatty acids at the lipid–water interface. During hydrolysis, lipases pick acyl group from glycerides forming lipase-acyl complex, which then transfers its acyl group to O–H group of water. Lipases have become increasingly important in the last two decades for industrial applications in the food, detergent, pharmaceutical, bioenergy and biodiesel industries (Aryee et al. 2007). Different strategies for lipases purification methods in general are time-consuming, low scalability and cost effectiveness, and sometimes may offer an unsatisfied purification yield of desired product (Gupta et al. 2004). The objective of this study was to demonstrate the use of combined partitioning systems, TPP and ATPS, for partitioning and recovery of lipase from the hepatopancreas of Pacific white shrimp. The biochemical properties of the partitioned enzyme were also studied.

Materials and methods

Chemicals

Acetone, tris (hydroxymethyl) aminomethane, Brij35, Triton X-100, gum arabic, polyethylene glycol (PEG) 1000

and PEG4000 were obtained from Merck (Darmstadt, Germany). Bovine serum albumin, wide range molecular weight markers, Coomassie Brilliant Blue R-250 and *p*-nitrophenyl palmitate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS) was obtained from Fluka (Buchs, Switzerland). *N,N,N',N'*-tetramethylethylenediamine (TEMED) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). The salts and other chemicals with the analytical grade were procured from Merck (Darmstadt, Germany).

Preparation of crude extract

Fresh hepatopancreas of Pacific white shrimp was collected from the Sea wealth frozen food Co. Ltd., Songkhla. Samples were placed in polyethylene bags and imbedded in polystyrene boxes containing ice with an ice/sample ratio of 2:1 (w/w) during transportation for approximately 2 h. Upon arrival, the samples were homogenized into powder in three volume of acetone at -20°C for 30 min according to the method of Klomklao et al. (2005). The homogenate was filtered in vacuo on Whatman No. 4 filter paper. The residue obtained was then homogenized in two volumes of acetone at -20°C for 30 min, and then the residue was left at room temperature until dried and free of acetone odor.

To prepare the crude extract, the defatted hepatopancreas powder was suspended in 50 mM Tris–HCl, pH 7.0 containing 0.2% (v/v) Brij35 at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min (Kuepethkaew et al. 2017). The suspension was centrifuged for 30 min at 4°C at $5000\times g$ to remove the tissue debris. The supernatant was collected and referred to as “crude extract, CE”.

Three-phase partitioning (TPP) system

Effect of salts on lipases partitioning

TPP was carried out as described by Roy and Gupta (2002). TPP containing different salts, ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$, dipotassium hydrogen phosphate (K_2HPO_4) and trisodium citrate $(\text{Na}_3\text{C}_6\text{H}_5\text{O}_7)$, at different concentrations (30, 40, 50 and 60%) (w/w) was investigated by using the ratio of crude extract to *t*-butanol of 1.0:0.5 (v/v). The mixture was mixed thoroughly and then allowed to stand for 60 min before subjecting to centrifuge at $7500\times g$ for 20 min at 4°C to facilitate the separation of phases. The lower aqueous layer and the interfacial phase were collected and dialyzed against 50 volume of distilled water overnight at 4°C with 4 changes of distilled water. After dialysis, the samples were determined for lipase activity and total protein content. Yield, specific activity (SA) and purification factor (PF) were calculated as follows:

$$\text{Yield (\%)} = \frac{A_t}{A_i} \times 100$$

where A_t is total lipase activity in the lipase rich phase and A_i is the initial lipase activity of the crude extract or fraction before being partitioned.

$$\text{SA (unit/mg protein)} = \frac{\text{lipase activity}}{\text{protein concentration}}$$

$$\text{PF} = \frac{\text{SA}_t}{\text{SA}_i}$$

where SA_t is the SA of the lipase rich phase and SA_i is the initial SA of the crude extract or fraction before being partitioned. The system providing the highest lipase recovery and purity was chosen for further study.

Effect of organic solvents on lipase partitioning

The effect of organic solvents on partitioning of lipase was studied. Firstly, CE was added with different organic solvents (1-propanol, 2-propanol, 1-butanol and *t*-butanol) at different ratios (1.0:0.5, 1.0:1.0 and 1.0:1.5 (v/v)). Thereafter, ammonium sulphate at 50% saturation was added into the mixture. Partitioning was performed as described previously. The CE/organic solvent ratio yielding the highest enzyme recovery and purity was selected. The selected TPP fraction was used for ATPS.

Aqueous two-phase system (ATPS)

ATPS was prepared in 10-mL centrifuge tubes by adding the different amounts of PEG and salts together with the selected TPP fraction according the method of Klomklao et al. (2005).

Effect of salts on lipase partitioning in TPP fraction

To study the effect of salts on lipase partitioning in TPP fraction, ATPS containing different salts, disodium hydrogen phosphate (Na_2HPO_4), dipotassium hydrogen phosphate (K_2HPO_4), trisodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$), ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$), sodium sulfate (Na_2SO_4) and magnesium sulfate (MgSO_4), at different concentrations (10, 15, 20 and 25%, w/w) with 20% PEG1000 were used. 1 mL of TPP fraction was added into the system. Distilled water was used to adjust the system to obtain the final weight of 5 g. The mixtures were mixed continuously for 3 min using a vortex mixer (G506E, Scientific Industries, USA). Phase separation was achieved by centrifugation for 10 min at $5000\times g$. Top phase was carefully separated using a Pasteur pipette and the interface of each tube was discarded. Volumes of the separated phase, top and bottom phases, were measured. Aliquots from each

phase were taken for lipase assay and protein determination. Yield, SA and PF were then calculated.

Additionally partition coefficient (K_p) and volume ratio (V_R) were also calculated using the following equations:

$$K_p = \frac{C_T}{C_B}$$

where C_T and C_B are concentrations of protein in top phase and bottom phase, respectively.

$$V_R = \frac{V_T}{V_B}$$

where V_T and V_B are the volume of top and bottom phase, respectively.

Based on purity and yield, the appropriate salt in ATPS rendering the most effective partitioning was selected for further study.

Effect of MW and concentration of PEG on lipase partitioning in TPP fraction

Effect of PEG1000 and PEG4000 at different concentrations (15, 20, 25 and 30%, w/w) on partitioning of lipase in TPP fraction was investigated in the presence of salt with the type and concentration showing the highest yield and purity. Partitioning was performed as previously described. ATPS giving the most effective partitioning of lipase was chosen for further study.

Effect of pH on lipase partitioning in TPP fraction

The effect of pHs on lipase partitioning was also studied. After mixing CE, selected salt and selected PEG from the previous step, the original pH of the system was measured and then the pH of system was adjusted to 3, 5, 7, 9 and 11 with 6 N HCl or 6 N NaOH. Partitioning for this step was done and determined as previously described.

Effect of NaCl addition on lipase partitioning in TPP fraction

The best condition that provided the highest recovery from the previous step was used to study the effect of NaCl addition on partitioning of lipase in TPP fraction. Adjustment of salt content in the system was made by addition of NaCl (solid form) into the system to obtain concentration of 0, 2, 4 and 6% (w/w). The ATPS fraction with the highest purity and yield was used for characterization study.

Lipase activity assay and protein determination

Lipase activity was determined by the *p*-NPP (*p*-nitrophenyl palmitate) method as described previously by

Karadzic et al. (2006) and Kuepethkaew et al. (2017) with some modifications. The substrate solution was prepared by dissolving 30 mg *p*-NPP in 10 mL of propane-2-ol. The lipase assay was carried out at 37 °C by direct mixing in cuvette of 100 µL of an appropriately diluted enzyme with 810 µL of preincubated 50 mM phosphate buffer (pH 8.0) and 90 µL of substrate solution. The amount of liberated *p*-nitrophenol was determined at 410 nm during the first 5 min of reaction. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of *p*-nitrophenol per minute under the assay conditions.

Protein concentration was measured by the Bradford method using BSA as a protein standard (Bradford 1976).

Characterization of recovered lipases

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of fractionated lipases was performed according to the method of Laemmli (1970). CE, TPP and ATPS fractions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample treatment buffers (0.5 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol) and boiled for 3 min. The samples (20 µg) were loaded on the gel made of 4% stacking and 12% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protein II Cell apparatus. After electrophoresis, the gels were stained with 0.2% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid and destained with 30% methanol and 10% acetic acid.

pH and temperature profiles

The activity of lipase from Pacific white shrimp hepatopancreas after ATPS process was measured within the pH range of 6.0–10.0. Different buffers were used for different pH conditions: 0.2 M McIlvaine's buffer (0.2 M sodium phosphate–0.1 M sodium citrate) for pH 6.0–8.0, 50 mM Tris–HCl for pH 8.0–9.0 and 0.1 M glycine–NaOH for pH 9.0–10.0. The activity was assayed at 37 °C for 5 min. For the temperature profile study, the activity was assayed at different temperatures (20, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 80 °C) for 5 min at pH 8.0.

Thermal and pH stability

ATPS fraction was incubated at various temperatures (0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 °C) for 30, 60 and 120 min, followed by cooling in iced water. The residual activity was assayed using *p*-NPP as a substrate and reported as the relative activity (%) compared with the

original activity. The effect of pH on enzyme stability was evaluated by measuring the residual enzyme activity after incubation at various pHs (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) for 30, 60 and 120 min at room temperature. The residual activity was assayed with *p*-NPP as previously described.

Effect of organic solvents

The effect of methanol and ethanol at a concentration of 2.5, 5, 10, 15, 25, 35, 45 and 50% (v/v) on the lipase activity was investigated by incubating the fractionated lipase for 30, 60 and 120 min at room temperature. The residual enzyme activity was measured by the spectrophotometric method using *p*-NPP as substrate.

Effect of sodium deoxycholate (NaDC)

The effect of varying concentrations of NaDC on activity of the lipase was studied by incubating the ATPS fraction with the NaDC at room temperature (28–30 °C) for 30 min. The remaining activity was assayed with *p*-NPP.

Statistical analysis

A completely randomized design was used throughout this study. All data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range test (Steel and Torrie 1980). Statistical analysis was performed using a SPSS package (SPSS 11.5 for windows, SPSS Inc., Chicago, IL, USA).

Results and discussion

Use of TPP for lipase partitioning

Effect of salts with different types and concentrations

Distribution behavior of biomolecules in TPP system is a very complex phenomenon due to many factors which affect the separation of proteins in TPP system. In general, salt and organic solvent type and their concentrations can precipitate protein at the interphase of TPP system (Rawdkuen et al. 2012). Hence, the effect of various process parameters on partitioning of lipase from Pacific white shrimp hepatopancreas was analyzed to determine the best purification condition for TPP.

The type of salt and its concentration were an important parameters for effective partitioning of lipases to the middle phase of the system (Table 1). 50% (NH₄)₂SO₄ produced the best results with 2.46-fold purification and 63.51% activity recovery. The other salts gave lower fold

Table 1 TPP for partitioning of lipase from hepatopancreas of Pacific white shrimp as affected by salts with different types and concentrations and organic solvents with different types and ratios

Phase composition	Total protein (mg)	Total activity ($\times 10^3$ unit)	SA (unit/mg protein)	PF	Yield (%)
<i>Effect of salts</i>					
CE	828.37 $\pm$ 2.63 ^k	40.86 $\pm$ 0.79 ^h	49.33 $\pm$ 0.95 ^{bcd}	1.00 $\pm$ 0.02 ^{bcd}	100.00 $\pm$ 1.92 ^h
30%(NH ₄) ₂ SO ₄	326.34 $\pm$ 2.57 ^g	13.85 $\pm$ 4.61 ^{cdef}	42.44 $\pm$ 14.13 ^{abcd}	0.86 $\pm$ 0.28 ^{abcd}	33.89 $\pm$ 11.29 ^{cdef}
40%(NH ₄) ₂ SO ₄	350.40 $\pm$ 4.11 ⁱ	15.37 $\pm$ 6.29 ^{ef}	43.87 $\pm$ 17.95 ^{abcd}	0.89 $\pm$ 0.36 ^{abcd}	37.63 $\pm$ 15.39 ^{ef}
50%(NH ₄) ₂ SO ₄	213.73 $\pm$ 1.36 ^d	25.95 $\pm$ 0.45 ^g	121.41 $\pm$ 2.11 ^c	2.46 $\pm$ 0.04 ^c	63.51 $\pm$ 1.10 ^g
60%(NH ₄) ₂ SO ₄	291.22 $\pm$ 1.62 ^f	14.54 $\pm$ 1.64 ^{def}	49.93 $\pm$ 5.63 ^{bcd}	1.01 $\pm$ 0.12 ^{bcd}	35.58 $\pm$ 4.01 ^{def}
30%K ₂ HPO ₄	215.23 $\pm$ 2.05 ^d	7.85 $\pm$ 3.37 ^{abcd}	36.45 $\pm$ 15.69 ^{abc}	0.74 $\pm$ 0.32 ^{abc}	19.20 $\pm$ 8.26 ^{abcd}
40%K ₂ HPO ₄	338.92 $\pm$ 5.53 ^h	20.19 $\pm$ 3.73 ^{fg}	59.56 $\pm$ 10.99 ^{cd}	1.21 $\pm$ 0.22 ^{cd}	49.40 $\pm$ 9.12 ^{fg}
50%K ₂ HPO ₄	359.87 $\pm$ 14.06 ^j	9.26 $\pm$ 1.03 ^{abcde}	25.74 $\pm$ 2.87 ^{ab}	0.52 $\pm$ 0.06 ^{ab}	22.67 $\pm$ 2.53 ^{abcde}
60%K ₂ HPO ₄	197.56 $\pm$ 6.28 ^c	6.18 $\pm$ 1.06 ^{ab}	31.27 $\pm$ 5.37 ^{ab}	0.63 $\pm$ 0.11 ^{ab}	15.12 $\pm$ 2.60 ^{ab}
30%Na ₃ C ₆ H ₅ O ₇	287.60 $\pm$ 5.94 ^f	13.23 $\pm$ 8.50 ^{bcd}	45.99 $\pm$ 29.56 ^{abcd}	0.93 $\pm$ 0.60 ^{abcd}	32.37 $\pm$ 20.80 ^{bcd}
40%Na ₃ C ₆ H ₅ O ₇	186.57 $\pm$ 4.12 ^b	12.61 $\pm$ 3.79 ^{bcd}	67.59 $\pm$ 20.32 ^d	1.37 $\pm$ 0.41 ^d	30.86 $\pm$ 9.27 ^{bcd}
50%Na ₃ C ₆ H ₅ O ₇	150.61 $\pm$ 2.50 ^a	7.14 $\pm$ 1.87 ^{abc}	47.42 $\pm$ 12.44 ^{abcd}	0.96 $\pm$ 0.25 ^{abcd}	17.48 $\pm$ 4.58 ^{abc}
60%Na ₃ C ₆ H ₅ O ₇	226.21 $\pm$ 1.66 ^e	4.68 $\pm$ 3.44 ^a	20.68 $\pm$ 15.21 ^a	0.42 $\pm$ 0.31 ^a	11.45 $\pm$ 8.42 ^a
<i>Effect of organic solvents</i>					
CE	837.87 $\pm$ 6.12 ^g	40.44 $\pm$ 1.74 ^h	48.27 $\pm$ 2.07 ^a	1.00 $\pm$ 0.04 ^a	100.00 $\pm$ 4.29 ^g
1-propanol, 1.0:0.5	239.03 $\pm$ 0.04 ^{cde}	17.97 $\pm$ 2.45 ^{bc}	75.20 $\pm$ 10.26 ^{bc}	1.56 $\pm$ 0.21 ^{bc}	44.45 $\pm$ 6.07 ^{bc}
1-propanol, 1.0:1.0	220.94 $\pm$ 30.00 ^{cd}	15.96 $\pm$ 0.91 ^b	72.26 $\pm$ 4.41 ^{bc}	1.50 $\pm$ 0.86 ^{bc}	39.47 $\pm$ 2.26 ^b
1-propanol, 1.0:1.5	163.56 $\pm$ 3.30 ^{ab}	12.77 $\pm$ 1.09 ^a	78.06 $\pm$ 6.70 ^{bc}	1.62 $\pm$ 0.14 ^{bc}	31.57 $\pm$ 2.71 ^a
2-propanol, 1.0:0.5	196.79 $\pm$ 7.13 ^{bc}	15.10 $\pm$ 0.49 ^{ab}	76.49 $\pm$ 2.50 ^{bc}	1.58 $\pm$ 0.52 ^{bc}	37.22 $\pm$ 1.22 ^{ab}
2-propanol, 1.0:1.0	210.12 $\pm$ 4.25 ^{cd}	21.18 $\pm$ 0.80 ^d	100.80 $\pm$ 3.81 ^d	2.09 $\pm$ 0.08 ^d	52.37 $\pm$ 1.98 ^d
2-propanol, 1.0:1.5	127.75 $\pm$ 12.71 ^a	12.49 $\pm$ 0.83 ^a	97.79 $\pm$ 6.52 ^d	2.03 $\pm$ 0.14 ^d	30.89 $\pm$ 2.06 ^a
1-butanol, 1.0:0.5	282.03 $\pm$ 15.89 ^{ef}	14.95 $\pm$ 3.31 ^{ab}	53.02 $\pm$ 11.79 ^a	1.10 $\pm$ 0.24 ^a	36.98 $\pm$ 8.17 ^{ab}
1-butanol, 1.0:1.0	249.40 $\pm$ 10.77 ^{def}	16.79 $\pm$ 0.26 ^{bc}	67.30 $\pm$ 1.06 ^b	1.39 $\pm$ 0.02 ^b	41.51 $\pm$ 0.65 ^{bc}
1-butanol, 1.0:1.5	231.77 $\pm$ 5.08 ^{cd}	19.26 $\pm$ 1.38 ^{cd}	83.10 $\pm$ 5.95 ^c	1.72 $\pm$ 0.12 ^c	47.62 $\pm$ 3.41 ^{cd}
<i>t</i> -butanol, 1.0:0.5	226.39 $\pm$ 3.94 ^{cd}	26.43 $\pm$ 0.53 ^f	116.75 $\pm$ 2.36 ^e	2.42 $\pm$ 0.05 ^e	65.36 $\pm$ 1.32 ^e
<i>t</i> -butanol, 1.0:1.0	209.84 $\pm$ 2.60 ^{cd}	35.35 $\pm$ 2.87 ^g	168.46 $\pm$ 13.70 ^f	3.49 $\pm$ 0.28 ^f	87.41 $\pm$ 7.11 ^f
<i>t</i> -butanol, 1.0:1.5	288.66 $\pm$ 82.53 ^f	33.11 $\pm$ 1.31 ^g	114.72 $\pm$ 4.02 ^e	2.38 $\pm$ 0.09 ^e	81.87 $\pm$ 3.23 ^f

SA specific activity (unit/mg protein), PF purification factor, Yield recovery yield

The different letters in the same column denote the significant difference ($p < 0.05$)

purification and activity recovery values. At concentrations of (NH₄)₂SO₄ greater than 50% (w/v), the yield decreased significantly due to the precipitation of protein and may be possibly due to its irreversible denaturation. (NH₄)₂SO₄ concentration plays a major role in TPP as it is responsible for protein–protein interaction and precipitation (Dhananjay and Mulimani 2009). It causes protein precipitation by salting out mechanism. Protein solubility is affected by ionic strength of the solution which subsequently depends on salt concentration. On addition of sufficient salt protein precipitation occurs. This is known as the salting out effect. At higher salt concentration, water molecules are attracted by salt ions resulting in stronger protein–protein interactions and the protein molecules coagulate through hydrophobic interactions (Senphan and Benjakul 2014). Various salt types were tested in TPP system, but (NH₄)₂SO₄ was often provided the most efficient on protein

partitioning (Roy and Gupta 2002). Rawdkuen et al. (2012) have also found that the 50% (w/w) (NH₄)₂SO₄ is the best for the partitioning of proteases from the viscera of farmed giant catfish (*Pangasianodon gigas*) with TPP. The principle of sulphate ion for salting out protein has been viewed in five different ways namely, ionic strength effects, kosmotropy, cavity surface tension and enhancement osmotic stressor (dehydration), exclusion crowing agent and binding of sulphate ion to cationic sites of protein (Dhananjay and Mulimani 2009). As maximum purity and recovery were achieved at 50% (NH₄)₂SO₄ concentration, it was chosen as optimum value for the next set of experiments.

Effect of organic solvents with different types and ratios

In this TPP based protocol for protein separation, *t*-butanol has found to be consistently better than all organic solvents

with 65–87% activity recovery (Table 1). It is believed that because of its size and branched structure, *t*-butanol does not easily permeate inside the folded protein molecules and hence does not cause denaturation (Rawdkuen et al. 2012). A ratio of 1.0:1.0 of *t*-butanol to crude volume produced the best results with 3.49-fold purification and 87.41% enzyme activity recovery when compared with other ratios ($p < 0.05$). Further increase from 1.0:1.0 volume led to a decrease in activity recovery and purification fold values. The results suggested that the type and volume of organic solvent and concentration ammonium sulphate in the TPP system played a key role in partitioning the target lipases. The extract to *t*-butanol ratio of 1.0:1.0 in the presence of $(\text{NH}_4)_2\text{SO}_4$ at 50% saturation was the optimal condition for protease partitioning from hepatopancreas of Pacific white shrimp (Senphan and Benjakul 2014). Dhananjay and Mulimani (2009) reported that when crude α -galactosidase from *Aspergillus oryzae* was saturated to 30% $(\text{NH}_4)_2\text{SO}_4$ (w/v) in the presence of *t*-butanol at a ratio of 1.0:1.0 (v/v), 43.96% recovery with a PF of 1.7-fold was achieved. From the results, CE/*t*-butanol ratio 1.0:1.0 was selected as the optimal ratio for partitioning of lipase from CE.

Use of ATPS for partitioning of lipase

Effect of salts with different types and concentrations

In order to identify a suitable salt for the partitioning of Pacific white shrimp lipase, TPP fraction was carried out using several biphasis systems of 20% PEG1000 with different salts, Na_2HPO_4 , K_2HPO_4 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, $(\text{NH}_4)_2\text{SO}_4$, Na_2SO_4 and MgSO_4 at different concentrations (10, 15, 20 and 25%) (w/w) (Table 2). After phase separation, two phases were obtained. Those included a PEG-rich top phase and a salt-rich lower phase. For all ATPS studied, systems were demonstrated to be able to partition lipase from Pacific white shrimp hepatopancreas and the lipase were partitioned predominantly in the PEG-rich top phase, principally those with hydrophobic characteristics (Klomklao et al. 2005). However, the recovery of lipase from the opposite phase (lower phase) was relative low. Therefore, we used the other data of SA, PF and yield in the top phase to show the partitioning of lipase in different ATPS in this paper.

Changes in the phase system were due to the different salts used that changed the way in which the proteins partitioned. In general, negatively charged proteins prefer the upper phase in PEG-salt systems, while positively charged proteins normally partition selectively to the bottom phase (Barbosa et al. 2011). Hence, most hepatopancreas lipase partitioned in the top phase might be negatively charged.

The V_R of the assayed systems generally decreased when salt concentration increased. The addition of salts to

the aqueous PEG solution led to an arrangement of ordered water molecules around the PEG molecules. This was due to their water structure breaking effects (Rawdkuen et al. 2012). The formation of a water layer around the cation resulted in a more compact structure with a minor volume of PEG molecules. This led to the decrease in volume of the PEG-rich top phase. Table 2 also showed that SA, PF and yield of lipase obtained from PEG1000-salt systems depended in the types of salts used. The phase systems containing MgSO_4 generally showed the superior partitioning efficiency to those containing other salts. A phase system containing 20% PEG1000 and 15% MgSO_4 gave the highest SA (188.70 units/mg protein). Fraction obtained had PF of 3.91 and 1.12-fold, compared with CE and TPP fraction, respectively. Klomklao et al. (2005) reported that the use of 20% (w/w) PEG1000 and 20% (w/w) MgSO_4 was effective in concentrating and purifying the proteinase from yellowfin tuna spleen. Chaiwut et al. (2010) also found that the highest SA and PF in partitioning protease from *Calotropis procera* latex was obtained by using 12% (w/w) PEG4000 and 17% (w/w) MgSO_4 . It has been found that the efficiency of the salts in promoting phase separation reflects the lyotropic series (a classification of ions based upon salting-out or salting-in ability) (Klomklao et al. 2005). Their effectiveness is mainly determined by the nature of the anion. Multi-charged anions being the most effective are ordered of $\text{SO}_4^{2-} > \text{HPO}_4^{2-} > \text{acetate} > \text{Cl}^-$, whereas the order of cation is usually given as $(\text{NH}_4)^+ > \text{K}^+ > \text{Na}^+ > \text{Li}^+ > \text{Mg}^{2+} > \text{Ca}^{2+}$ (Senphan and Benjakul 2014). Thus, the extraction conditions employed determined the partitioning efficacy of the desires to the top phase and partition the contaminating enzyme and protein to the opposite phase. Due to the highest yield and SA, ATPS with 15% MgSO_4 was selected for the further experiment on the effect of PEG on lipase partitioning.

Effect of PEG with different MW and concentrations

The PEG molecular weight has an important role on the partition behavior of protein (Zhou et al. 2013). Partitioning of lipase from TPP fraction using ATPS containing PEG with varying MW (PEG1000 and PEG4000) and concentration (15, 20, 25 and 30%) (w/w) in the presence of 15% MgSO_4 is shown in Table 3. In general, PEG with higher MW tended to yield the lipase with the lower K_p , SA, PF and yield. Therefore, PEG1000 was a suitable polymer for partitioning of lipase in TPP fraction as indicated by the high PF than PEG with higher MW. Also, it was observed that volume ratio (V_R) decreased with an increase in molecular weight of PEG. When the same PEG was used, V_R , K_p , SA, PF and yield varied, depending upon level of PEG. ATPS of 25% PEG1000-15% MgSO_4

Table 2 ATPS for partitioning of lipase from hepatopancreas of Pacific white shrimp as affected by salts with different types and concentrations

Phase composition (% w/w)	V_R	K_p	SA	PF		Yield (%)	
				CE	TPP	CE	TPP
20% PEG 1000 + 10% Na_2HPO_4	1.39 ± 0.08 ⁿ	3.10 ± 0.03 ^{bc}	120.57 ± 3.98 ^{fg}	2.49 ± 0.08 ^{fg}	0.72 ± 0.02 ^{fg}	69.70 ± 3.14 ^{ghij}	79.75 ± 3.50 ^{ghij}
20% PEG 1000 + 15% Na_2HPO_4	0.86 ± 0.07 ^{ijkl}	3.71 ± 0.58 ^c	58.34 ± 2.08 ^{ab}	1.21 ± 0.05 ^{ab}	0.35 ± 0.01 ^{ab}	51.14 ± 9.68 ^{bc}	58.51 ± 11.08 ^{bc}
20% PEG 1000 + 20% Na_2HPO_4	0.75 ± 0.03 ^{ghijkl}	7.51 ± 0.44 ^b	138.83 ± 4.89 ⁱ	2.88 ± 0.10 ⁱ	0.83 ± 0.03 ⁱ	50.28 ± 1.16 ^{abc}	57.53 ± 1.32 ^{abc}
20% PEG 1000 + 25% Na_2HPO_4	0.66 ± 0.01 ^{efghij}	5.64 ± 0.52 ^{de}	105.68 ± 3.83 ^{de}	2.19 ± 0.08 ^{de}	0.63 ± 0.03 ^{de}	47.52 ± 7.42 ^{ab}	54.37 ± 8.49 ^{ab}
20% PEG 1000 + 10% K_2HPO_4	0.89 ± 0.03 ^{kl}	5.91 ± 0.29 ^{ef}	80.74 ± 2.88 ^c	1.67 ± 0.06 ^c	0.48 ± 0.02 ^c	73.74 ± 1.96 ^{ij}	84.37 ± 2.24 ^{ij}
20% PEG 1000 + 15% K_2HPO_4	0.79 ± 0.04 ^{hijk}	6.74 ± 0.15 ^{fgh}	59.96 ± 0.32 ^{ab}	1.24 ± 0.01 ^{ab}	0.36 ± 0.01 ^{ab}	65.24 ± 3.27 ^{fgh}	74.64 ± 3.74 ^{fgh}
20% PEG 1000 + 20% K_2HPO_4	0.62 ± 0.01 ^{defghi}	7.48 ± 0.11 ^b	51.99 ± 1.63 ^a	1.08 ± 0.03 ^a	0.31 ± 0.01 ^a	52.29 ± 8.38 ^{bc}	59.82 ± 9.59 ^{bc}
20% PEG 1000 + 25% K_2HPO_4	0.49 ± 0.04 ^{abcde}	6.30 ± 0.30 ^{efg}	55.73 ± 2.75 ^{ab}	1.16 ± 0.06 ^{ab}	0.33 ± 0.02 ^{ab}	43.16 ± 6.92 ^a	49.37 ± 7.91 ^a
20% PEG 1000 + 10% $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$	1.01 ± 0.02 ^{lm}	6.91 ± 0.04 ^{gh}	65.17 ± 2.09 ^d	1.35 ± 0.04 ^b	0.39 ± 0.01 ^b	71.38 ± 4.63 ^{hij}	81.67 ± 5.29 ^{hij}
20% PEG 1000 + 15% $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$	0.76 ± 0.04 ^{ghijk}	8.37 ± 0.26 ⁱ	62.54 ± 2.17 ^b	1.30 ± 0.04 ^b	0.37 ± 0.01 ^b	66.88 ± 2.02 ^{fghi}	76.52 ± 2.30 ^{fghi}
20% PEG 1000 + 20% $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$	0.68 ± 0.05 ^{efghijk}	8.68 ± 0.61 ⁱ	65.49 ± 0.99 ^b	1.36 ± 0.01 ^b	0.39 ± 0.01 ^b	64.51 ± 6.72 ^{fgh}	73.80 ± 7.69 ^{fgh}
20% PEG 1000 + 25% $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$	0.54 ± 0.01 ^{bcdef}	9.77 ± 0.34 ^j	56.71 ± 1.05 ^{ab}	1.18 ± 0.02 ^{ab}	0.34 ± 0.01 ^{ab}	52.32 ± 2.76 ^{bc}	59.86 ± 3.17 ^{bc}
20% PEG 1000 + 10% $(\text{NH}_4)_2\text{SO}_4$	0.67 ± 0.02 ^{efghij}	12.75 ± 0.66 ^k	109.98 ± 2.99 ^{de}	2.28 ± 0.07 ^{de}	0.66 ± 0.02 ^{de}	71.14 ± 3.13 ^{hij}	81.39 ± 3.58 ^{hij}
20% PEG 1000 + 15% $(\text{NH}_4)_2\text{SO}_4$	0.55 ± 0.01 ^{bdefg}	9.97 ± 0.36 ^j	113.54 ± 4.31 ^{ef}	2.35 ± 0.09 ^{ef}	0.68 ± 0.03 ^{ef}	66.74 ± 2.97 ^{fghi}	76.35 ± 3.40 ^{fghi}
20% PEG 1000 + 20% $(\text{NH}_4)_2\text{SO}_4$	0.35 ± 0.04 ^{ab}	9.91 ± 0.34 ^j	108.11 ± 13.65 ^{de}	2.24 ± 0.28 ^{de}	0.64 ± 0.08 ^{de}	60.32 ± 5.57 ^{df}	69.01 ± 6.37 ^{df}
20% PEG 1000 + 25% $(\text{NH}_4)_2\text{SO}_4$	0.29 ± 0.02 ^a	17.91 ± 0.49 ^m	110.17 ± 2.50 ^{de}	2.28 ± 0.53 ^{de}	0.66 ± 0.02 ^{de}	47.73 ± 3.64 ^{ab}	54.61 ± 4.17 ^{ab}
20% PEG 1000 + 10% Na_2SO_4	0.80 ± 0.03 ^{hijk}	14.81 ± 0.42 ^j	100.55 ± 6.59 ^d	2.09 ± 0.13 ^d	0.60 ± 0.04 ^d	57.03 ± 2.41 ^{cd}	65.24 ± 2.76 ^{cd}
20% PEG 1000 + 15% Na_2SO_4	0.67 ± 0.01 ^{efghij}	2.82 ± 0.28 ^b	133.22 ± 7.97 ^{hi}	2.76 ± 0.46 ^{hi}	0.79 ± 0.05 ^{hi}	62.63 ± 3.71 ^{dfe}	71.64 ± 4.24 ^{dfe}
20% PEG 1000 + 20% Na_2SO_4	0.40 ± 0.03 ^{abc}	1.70 ± 0.45 ^a	176.79 ± 6.64 ^{kl}	3.66 ± 0.14 ^{kl}	1.05 ± 0.04 ^{kl}	64.25 ± 2.27 ^{fgh}	73.50 ± 2.59 ^{fgh}
20% PEG 1000 + 25% Na_2SO_4	0.44 ± 0.04 ^{abcd}	4.81 ± 0.71 ^d	127.67 ± 0.80 ^{gh}	2.65 ± 0.02 ^{gh}	0.76 ± 0.01 ^{gh}	52.19 ± 1.45 ^{bc}	59.71 ± 1.65 ^{bc}
20% PEG 1000 + 10% MgSO_4	1.08 ± 0.38 ^m	2.01 ± 0.02 ^a	160.94 ± 2.25 ^j	3.33 ± 0.04 ^j	0.96 ± 0.01 ^j	74.51 ± 2.59 ^j	85.24 ± 2.96 ^j
20% PEG 1000 + 15% MgSO_4	0.82 ± 0.01 ^{ijkl}	4.88 ± 0.19 ^d	188.70 ± 6.12 ^m	3.91 ± 0.13 ^m	1.12 ± 0.03 ^m	75.60 ± 2.81 ^j	86.49 ± 3.22 ^j
20% PEG 1000 + 20% MgSO_4	0.73 ± 0.15 ^{fghijk}	12.19 ± 0.38 ^k	185.26 ± 3.08 ^{lm}	3.84 ± 0.07 ^{lm}	1.10 ± 0.02 ^{lm}	64.40 ± 4.90 ^{fgh}	73.67 ± 5.61 ^{fgh}
20% PEG 1000 + 25% MgSO_4	0.60 ± 0.01 ^{cdefgh}	6.58 ± 0.23 ^{fg}	168.06 ± 23.63 ^{jk}	3.48 ± 0.49 ^{jk}	1.00 ± 0.14 ^{jk}	50.49 ± 5.02 ^{bc}	57.77 ± 5.74 ^{bc}

V_R volume ratio (upper/lower), K_p partitioning coefficient of protein in the upper phase, SA specific activity (unit/mg protein), PF purification factor, Yield recovery yield. The different letters in the same column denote the significant difference ($p < 0.05$)

Table 3 ATPS for partitioning of lipase from hepatopancreas of Pacific white shrimp as affected by PEG with different molecular weight and concentrations

Phase composition (% w/w)	V_R	K_p	SA	PF		Yield (%)	
				CE	TPP	CE	TPP
15% PEG 1000 + 15% MgSO ₄	0.98 ± 0.06 ^b	5.51 ± 0.06 ^e	120.94 ± 6.37 ^a	2.51 ± 0.13 ^a	0.72 ± 0.04 ^a	66.06 ± 4.62 ^{ab}	75.59 ± 5.28 ^{ab}
20% PEG 1000 + 15% MgSO ₄	1.19 ± 0.04 ^{cd}	3.88 ± 0.07 ^c	187.33 ± 9.31 ^c	3.88 ± 0.19 ^c	1.11 ± 0.06 ^c	75.41 ± 3.28 ^{bc}	86.28 ± 3.75 ^{bc}
25% PEG 1000 + 15% MgSO ₄	1.29 ± 0.03 ^d	4.08 ± 0.04 ^d	246.15 ± 6.50 ^e	5.10 ± 0.13 ^e	1.46 ± 0.04 ^e	77.52 ± 6.40 ^c	88.69 ± 7.32 ^c
30% PEG 1000 + 15% MgSO ₄	1.58 ± 0.03 ^e	6.98 ± 0.19 ^f	233.03 ± 3.33 ^d	4.83 ± 0.07 ^d	1.38 ± 0.02 ^d	72.65 ± 2.79 ^{bc}	83.13 ± 3.20 ^{bc}
15% PEG 4000 + 15% MgSO ₄	0.70 ± 0.01 ^a	0.84 ± 0.04 ^a	135.93 ± 5.90 ^b	2.82 ± 0.12 ^b	0.81 ± 0.03 ^b	60.91 ± 8.72 ^{ab}	69.69 ± 9.98 ^a
20% PEG 4000 + 15% MgSO ₄	0.80 ± 0.01 ^a	0.96 ± 0.03 ^a	142.30 ± 2.73 ^b	2.94 ± 0.05 ^b	0.84 ± 0.02 ^b	69.08 ± 12.58 ^{abc}	79.02 ± 14.40 ^{abc}
25% PEG 4000 + 15% MgSO ₄	1.13 ± 0.12 ^c	1.58 ± 0.04 ^b	146.02 ± 4.03 ^b	3.03 ± 0.08 ^b	0.87 ± 0.02 ^b	73.02 ± 2.64 ^{bc}	83.54 ± 3.03 ^{bc}
30% PEG 4000 + 15% MgSO ₄	1.24 ± 0.01 ^{cd}	0.95 ± 0.07 ^a	226.28 ± 14.16 ^d	4.69 ± 0.29 ^d	1.34 ± 0.08 ^d	70.85 ± 3.57 ^{abc}	81.06 ± 4.09 ^{abc}

See legend of Table 2

The different letters in the same column denote the significant difference ($p < 0.05$)

rendered the highest PF (1.46-fold) with the yield of 88.69% for partitioning of lipase from TPP fraction ($p < 0.05$). The influence of the MW of PEG on protein partitioning can be explained on the basis of Flory–Huggins theory for polymer. For ATPS formed by PEG of low MW (600–3350 kDa) the proteins transfer to the top phase in enthalpically driven, mainly due to a strong interaction between PEG and the protein (Gupta et al. 2004). Reh et al. (2002) reported that the most proteins were partitioned to the top phase in phase systems with the low molecular weight PEG. A preferential interaction between PEG molecule and the protein domain decreased when the molecular mass of PEG increased due to its exclusion from the protein domain (Reh et al. 2002). It can be observed from Table 3 that among various phase compositions of PEG and MgSO₄, PEG1000/MgSO₄ system of 25/15 (%) indicated highest activity recovery (77.52%) and PF (5.10-fold). Therefore, this system composition was chosen for the further study.

Effect of pH

System pH is a key factor that influences the partitioning of lipase due to its change in pH. It has been reported that the system pH will modify the partitioning of the targeted protein and alter the concentration of the phase-forming polymer because the polymer loses/gains charge form functional groups, which cause repulsion/attraction and material expansion/contraction (Saravanan et al. 2008). Based on the results, a system composed of 25% PEG1000 and 15% MgSO₄ was selected for the study of lipase partitioning from Pacific white shrimp using different initial pH values (pH 3, 5, 7, 9 and 11), which was accomplished by adding appropriate amounts of hydrochloric acid (HCl) and sodium hydroxide (NaOH) solution (Fig. 1a). The maximum values for lipase production in the top phase, PF (5.21-fold) and yield (90.02%) were obtained at a pH of approximately 5. The increase in pH from 5.0 to 11.0 reduced the activity recovery from 90.02 to 40.17% and the PF also decreased in this pH range. The reduction in these results may be explained by Albersson equation. According to the Albersson equation (Albersson 1971):

$$\ln K = \ln K_0 + F\Delta\psi Z_p/RT$$

Protein partition is driven by two effects: the electrostatic component ($F\Delta\psi Z_p/RT$) determined by the electrical protein charge, and a hydrophobic component (K_0) which has a maximal effect when the pH medium is near the isoelectric pH. When pH increased from 3.0 to 7.0, the hydrophobic component is greater than the electrostatic forces, therefore the PEG has tends to interacts with the protein which results in the increase of the partition coefficient and PF. Barbosa et al. (2011) reported that a system

Fig. 1 ATPS for partitioning of lipase from hepatopancreas of Pacific white shrimp as affected by pH (a) and NaCl (b). Bars represent the standard deviation from triplicate determinations

composed of 20% (w/v) PEG8000, 18% (w/v) potassium phosphate, 6% (w/v) NaCl, pH 6.0 at 4 °C could be used to selectively purify lipase from *Bacillus* sp. ITP-001 in submerged fermentation. As a result, pH 5.0 was proved to be optimum pH for ATPS of lipase and was selected for the subsequent experiments.

Effect of NaCl addition

In order to study the influence of NaCl addition (0, 2, 4 and 6%, w/w) on lipase partitioning in the selected phase system (25% PEG1000 and 15% MgSO₄), experiments was performed at pH 5, and the results are presented in Fig. 1b. The addition of neutral salts is known to generate an electrical potential difference between the two phases, thus affecting the protein partitioning in ATPS (Barbosa et al. 2011). The presence of NaCl in ATPS alters partition coefficient because of the differential distribution of the salt ions between the phases. The hydrophobic ions force the partitioning of their counter ions to the more hydrophobic phase (Raja et al. 2011). From the result, NaCl concentration (2–6%, w/w) in the PEG-MgSO₄ system had adverse effect on lipase partition. The result showed that PF decreased with NaCl addition. The PF of lipase decreased from 5.19-fold (no NaCl addition) to 1.48-

fold (6%, w/w NaCl). The recovery yield has practically decreased with increasing NaCl concentration. Occurrence of this effect may be due to a decrease in protein solubility as a result of the addition of NaCl (Naganagouda and Mulimani 2008). Moreover, higher concentration of NaCl showed a significantly negative effect on yield of the enzyme, probably due to protein denaturation and precipitation at high concentration of this salt.

Protein pattern of lipase from hepatopancreas partitioned with combined partitioning system

In the present study, the SDS-PAGE pattern of ATPS fractions obtained from the system of 25% PEG1000-15% MgSO₄, pH 5 is depicted in Fig. 2. Crude extract contained a variety of proteins with different molecular weight. A large number of contaminating proteins were removed after partitioning with TPP, particularly proteins with higher MW. After ATPS was used, proteins with MW 36, 33 and 32 kDa were eliminated. As a result, a higher purity of lipases was obtained. When the proteins or enzymes differ significantly in their structural properties from others, partitioning can be carried out successfully (Rosa et al. 2009). The realization of recovery of lipase from crude extract in a partially form was confirmed by SDS-PAGE analyses with molecules weight of about 45, 34 and 24 kDa. The SDS-PAGE analysis revealed a substantial level of purification of lipase from Pacific white shrimp hepatopancreas. Therefore, the application of TPP system in combination with ATPS in this study could be used as a partial purification step of the lipase from hepatopancreas of Pacific white shrimp.

Effect of temperature and pH

The lipase activity was tested at temperatures ranging from 20 to 80 °C using *p*-NPP as substrate (Fig. 3a). The optimum temperature of fractionated lipase from hepatopancreas of Pacific white shrimp was 55 °C, which is higher than the values reported for other fish lipases. For instance, the reported optimum temperature of lipase from sardine and cod were 37 and 25 °C, which tributyrin and *p*-nitrophenyl myristate were used as substrate, respectively (Mukundan et al. 1985; Smichi et al. 2013). The difference in temperature optima might be due to several factors including the varying mechanical properties of the homologous lipases, the different substrate used for measurements, since different substrates do exhibit temperature activity differences with enzymes (Aryee et al. 2007). A sharp decrease in activity was observed at temperatures above 60 °C. The decrease in activity at high temperature was possibly due to the thermal denaturation of the enzyme (Smichi et al. 2013).

Fig. 2 SDS-PAGE patterns of CE, TPP and ATPS fractions from hepatopancreas of Pacific white shrimp. *M* molecular weight standard, *CE* crude lipase extract, *TPP* TPP fraction (The middle phase of TPP containing CE: *t*-butanol (1:1) and 50% $(\text{NH}_4)_2\text{SO}_4$); *ATPS* ATPS fraction containing 25% PEG1000-15% MgSO_4 at pH 5.0

The maximal lipase activity was obtained at pH 8.0 (Fig. 3b). This activity was reduced drastically above pH 8.0. This trend was similar to finding reported for other fish lipases such as grey mullet (*Mugil cephalus*) (Smichi et al. 2013), Indian major carp (*Labeo rohita*) (Nayak and Swain 2003) and cod (*Gadus morhua*) (Gjellesvik et al. 1992).

The thermal stability of fractionated lipase from Pacific white shrimp hepatopancreas is depicted in Fig. 3c. The enzyme was stable when incubated at temperatures up to 40 °C for 30–120 min. Nevertheless, the sharp decrease in activity was noticeable at temperatures above 50 °C. Generally, the stability of lipase decreased with increasing the heating time. A heating time of 120 min caused the highest loss of activity at every temperature above 40 °C used (Smichi et al. 2013). Enzymes are inactivated at high temperature due to the partial unfolding of the enzyme molecule. Aryee et al. (2007) reported that lipase from grey mullet viscera was active within the temperature range of 20–60 °C. Zarai et al. (2012) found that thermal stability of the marine snail digestive lipase retained about 85% of its maximum activity after incubation for 30 min at 50 °C and when the enzyme was incubated at 65 °C, it was completely inactive.

The fractionated lipase was stable in the pH range of 7.0–10.0 with the exposure time of 30–120 min (Fig. 3d). With an extended incubation time, lipase activity was lost

to a greater extent. At pH value below 7.0, the stability of the fractionated lipase decreased sharply. The grey mullet lipase was stable between pH 7.0 and 10.0 during incubation at 25 °C for 30 min (Aryee et al. 2007). Both lipase and phospholipase from grey mullet viscera were found to be mostly stable in alkaline pH value ranging from 7.5 to 9.0. (Smichi et al. 2013). Zarai et al. (2012) reported that marine snail digestive lipase was found to be most stable at higher pH values and maintained 100% of its activity after 30 min of incubation at pH 10.0. At acidic pHs, it is suggested that conformational changes in the enzyme affected the proper binding of the enzyme to the substrate (Mukundan et al. 1985). Since tolerance in the alkaline range is observed, this can be considered as a potential candidate for application in processes that are conducted in the alkaline range such as detergent applications (Zarai et al. 2012).

Effect of organic solvents on enzyme activity

The stability of fractionated lipase from Pacific white shrimp hepatopancreas was studied in ethanol and methanol by the measuring the residual activity versus time (30, 60 and 120 min) (Fig. 3e, f). The fractionated lipase showed high stability in the presence of ethanol and methanol, remaining more than 85% of original activity at 50% methanol and ethanol after incubation for 60 min at room temperature. With an extended incubation time, lipase activity was lost to a greater extent. At the same level of organic solvent, methanol showed relative lipase activity than ethanol. This suggested that the stability of enzyme in the presence of organic solvents depended on both the enzyme and the nature of the organic solvent being used. It is well known fact that the biocatalytic activity of enzymes was greatly affected by the nature of organic solvents. As the organic solvent may change the parent conformation of the lipase by disturbing the hydrogen bonding and hydrophobic interactions, which leads to affect the activity and stability of the enzymes (Karra-Châabouni et al. 2008). This result was in agreement with Castro-Ochoa et al. (2005) and Ungcharoenwiwat and Kittikuna (2015).

Because of industrial application, especially lipase-catalyzed biodiesel production, organic solvent tolerance is a very important factor. Biodiesel production is generally carried out by methylation of various oils, it is extremely important for potential enzyme to be stable in methanol. Therefore, the fractionated lipase from Pacific white shrimp hepatopancreas can be used as biocatalyst for biodiesel production. Enzyme stability in the presence of organic solvents is a highly desirable characteristic for enzymatic biodiesel production.

Fig. 3 Temperature (a) and pH (b) profiles and thermal (c), pH (d), ethanol (e) and methanol (f) stability of fractionated lipase from hepatopancreas of Pacific white shrimp. Bars represent the standard deviation from triplicate determinations

Effect of NaDC on enzyme activity

The effect of NaDC on the activity of the fractionated lipase was determined by incubating the enzyme at different concentrations (0–11 mM) for 30 min at room temperature. After incubation, the activity of fractionated lipase decreased gradually with increasing NaDC concentration (data not shown). A residual lipase activity (54.31%) was detected when the enzyme solution was incubated in NaDC at a concentration of 11 mM. Smichi et al. (2013) reported that grey mullet digestive lipase maintained 30% of its initial activity in the presence of NaDC concentration as high as 8 mM when olive oil was used as substrate. The NaDC have been shown to inhibit hydrolysis of triacylglycerols in concentration below their critical micellar concentration. This inhibition is attributed to electrostatic repulsions between the negatively charged surfactant and the lipase (Zarai et al. 2012).

Conclusion

This paper reported on the use of combined phase partitioning systems for recovery of lipase from Pacific white shrimp hepatopancreas, which is described for the first time. TPP with CE/*t*-butanol ratio of 1.0:1.0 in the presence of 50% (w/v) $(\text{NH}_4)_2\text{SO}_4$ was firstly implemented. ATPS containing 25% PEG1000-15% MgSO_4 at pH 5.0 was applied as the last step. The highest PF (5.19-fold) with 78.46% yield were obtained. Optimum temperature and pH values of the enzyme were found as 55 °C and pH 8.0, respectively. Lipase had high activity at a range of 0–40 °C and pH 7.0–10.0. As a result of these, Pacific white shrimp hepatopancreas is a good source of lipase. With necessary optimization, TPP system in combination with ATPS is possible and useful for purification of lipase. Biochemical properties of the enzyme have shown that the enzyme has the potential to be used for various applications.

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