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Characteristics and Application of Lipase from Asian Seabass Liver Fractionated Using Aqueous Two-phase Partition Technique for Defatting Fish Skin before Collagen Extraction

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Abstract

Lipase, from crude extract of fish viscera including liver, is highly contaminated with other hydrolytic enzymes such as proteases that would cause severe degradation of native collagen during defatting. Asian seabass liver lipase was therefore subjected to fractionation with the aqueous two-phase system (ATPS) to remove proteases from lipase. ATPS was carried out using various salts and polyethylene glycol (PEG) having various molecular weights at different concentrations. The concentration of 20% ammonium sulfate (w/v) and 50% PEG-6000 (w/w) could reduce protease by 85%. Lipase-rich fraction showed a specific activity of 68 U/mg protein, purification fold of 9, selectivity of 167, and yield of 48%. When pulse electric field (PEF) was treated Asian seabass fish skin was defatted, and the acid-soluble collagen was extracted and characterized. The ATPS fractionated lipase could remove more than 92% of lipid contents. Polyunsaturated and monounsaturated fatty acids were mainly eliminated. The extracted collagen from defatted skin showed typical type Ⅰ collagen with negligible degradation. FTIR spectra substantiated the presence of amide groups in resulting skin collagen. Thus, the fractionation of Asian seabass liver crude extract with ATPS could significantly remove the contaminated proteases. The obtained fraction could be used to defat Asian seabass skin without drastic damage to the extracted native collagen.

1. Introduction

Collagen is one of the highly prevalent structural proteins in animals and is crucial for the formation of the structural components of cells and tissues. Collagen is involved in cellular and tissue-level functions including tissue repair, immunological response, cellular communication, and migration (Meyer, 2019). Recently, collagen peptides with a low molecular weight (3-6 KDa) known as hydrolyzed collagen (HC) have become popular (Benjakul et al., 2017). Under specific incubation temperatures, enzymes can catalyze the production of these substances in carefully regulated acidic or alkaline conditions (Nilsuwan et al., 2021). Primarily, collagen or HC derived from bovine or porcine sources have religious and ethical constraints (Wang, 2021). As a result, marine collagen from skin, scales, and bones can be used to overcome such problems. Fish HCs have been documented to have numerous bioactive properties, especially for skin nourishment and bone strengthening (Chotphruethipong et al., 2021).

Asian seabass (*Lates calcarifer*) skin has been proven as the potential collagenous precursor for collagen and HC production (Leon-Lopez et al., 2019; Sinthusamran et al., 2013). However, its skin is rich in lipids or fat, which could be oxidized during HC preparation this phenomenon leads to fishy flavor and order in the finished products (Yingchutrakul et al., 2022). Recently, lipids located in Asian seabass skin were removed by hydrolysis via lipase-assisted treatment (Sae-Leaw & Benjakul, 2018). Lipase is an enzyme classified as a hydrolase, which catalyzes the hydrolysis of ester bonds in triacylglycerols (Baloch et al., 2021). Lipases possess promising practices within the food industry from eliminating unwanted fatty acids to structural modification of important fatty acids including healthy polyunsaturated fatty acids (Chandra et al., 2020). Lipase is mainly extracted from microbial culture. However due to food safety concerns, lipases from microbial sources cannot be directly applied in the food sector (Hasan et al., 2006), Nevertheless, lipase extracted from animal sources including fish viscera can be another promising means to replace microbial lipase. Fish viscera generally contain proteases, which could be co-extracted with lipase. The contaminated proteases are problematic for applications since they can hydrolyze proteins including native collagens. This might cause a loss in some peptides during defatting pretreatment. Therefore, the potential fractionation of lipase from protease is required.

An aqueous two-phase system (ATPS) is an important fractionation method that separates biomolecules, mainly proteins and enzymes. It is developed either with two different polymers including dextran and polyethylene glycol (PEG) or one polymer and one salt such as PEG with varying molecular weights and different salts (Raja et al., 2012). The two immiscible phases develop a system that fractionates the protein either in the top or in the bottom phase depending upon the ATPS phase components and the fractionating biomolecule (Yau et al., 2015). Lipases from different origins such as hepatopancreas of *Litopenaeus vannamei*, yeasts, fungi and bacteria have successfully been fractionated using ATPS developed by different polymers and salts (Anvari, 2015; Carvalho et al., 2017; Dobreva et al., 2019; Show et al., 2012). An aqueous two-phase system (ATPS) could be implemented to reduce the protease activity from the extracted crude extract rich in lipase to avoid the unwanted hydrolysis of the collagen by proteases.

Recently, Chotphruethipong et al. (2019b) used porcine lipase in combination with vacuum impregnation for lipid removal from Asian seabass skin. Nonetheless, HC produced from skin pretreatment with porcine lipase is not acceptable for the Muslim community. Thus, fish viscera lipase could be used to accomplish skin defatting without the aforementioned limitations. However, there is no available data regarding the utilization of protease-free lipase from fish viscera for extracting fat from fish skin. The objective of this study was to partially purify lipase from the liver of Asian seabass using ATPS and to apply lipase for the removal of fat with the aid of vacuum impregnation. Also, collagen was extracted from defatted skin and further characterized.

2. Materials and Methods

2.1 Chemicals

All chemicals were supplied in analytical grade. Polyethylene glycols (PEG1000-PEG20000), ammonium sulfate and other salts, Nile blue A, and *p*-nitrophenyl palmitate (*p*-NPP) were acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Preparation of Asian Seabass Liver Crude Powder

Three batches of Asian seabass viscera (2 kg/batch) were procured from a fish market at Hat Yai, Songkhla, Thailand. The samples were placed in zip lock bags and kept on ice (viscera/ice = 1:2). The viscera were freshly dissected, and the livers were separated. All adipose tissues surrounding the liver were removed. Liver samples were sliced into small pieces, added with liquid nitrogen, and blended with a blender. The blended samples were further defatted with chilled acetone at 1:3 (w/v). The blend was then homogenized at 15,000 rpm for 5 min using an ultra-turrax (IKA, Labortechnik, Selangor, Malaysia) and then stirred at 300 rpm and 4˚C for 40 min using an overhead stirrer (IKA-RW20, Werke Staufen, Germany). The homogenate was vacuum filtered with Whatman paper No. 4 and the residue was dried in air overnight. The dried samples named liver powder (LP) were placed in sealed bags at -40 ˚C until further use.

2.3 Extraction of Liver Crude Lipase

The buffer used for Asian seabass liver crude lipase (ASL-CL) was 25 mM Tris–HCl, pH 8.0 including 1 mM $CaCl_2$. To extract ASL-CL, LP was mixed with extraction buffer at a ratio of 1:9 (w/v) and thoroughly stirred at 300 rpm with an overhead stirrer (IKA-RW20, Werke Staufen, Germany) at 4˚C for 40 min. The samples were then subjected to centrifugation at 10,000 × g and 4˚C for 30 min. The supernatant was vacuum filtered with Whatman paper No. 4 to eliminate the remaining fat fragments. The filtrate or ASL-CL was kept in ice for further processing.

2.4 Aqueous Two-phase System (ATPS)

2.4.1 Effect of Various Salts on Lipase Fractionation

Different salts including ammonium sulfate, sodium citrate, sodium sulfate and magnesium sulfate were used in combination with 15% (w/w) PEG-4000. Briefly, 15% PEG-4000 was mixed with equal volume

(v/v) of different salts at various concentrations comprising 10%, 15%, 20% and 25% (w/v). The PEG-4000 and salt mixtures were then added with 20% (v/v) ASL-CL. The mixture was vortexed vigorously at highspeed vortex using Multi Speed Vortex (Biosan MSV-3500, Riga, Latvia) for 10 min to properly mix the two phases and ASL-CL. Thereafter, the samples were centrifuged at 7,000 × g for 10 min at 4˚C to achieve a proper phase separation using a centrifuge (Model 5427R, Eppendorf, Germany). Both top and bottom phases were cautiously separated with a Pasteur pipette and determined for lipase activity and protein concentration in comparison with ASL-CL. Salt showing the highest efficacy in phase separation and rendering the highest lipase activity on the top or bottom phase was selected for further investigations.

During fractionation, all the fractions were examined for lipase activity, total protein concentration, lipase partition co-efficient (Ke), protein partition coefficient (Kp), specific activity (SA), purification fold (PF), volume ratio (V_R), yield and selectivity (S) with equations below:

$$Ke = A_T/A_B \quad (1)$$

where A_T and A_B represent the total units of lipase in the top and bottom phases respectively.

$$Kp = P_T/P_B \quad (2)$$

where P_T represents the protein concentration in the top phase and P_B represents the protein concentration in the bottom phase.

$$\text{SA (lipase unit/mg protein)} = \text{lipase activity/protein content} \quad (3)$$

$$PF = SA_T/SA_I \quad (4)$$

where SA_T represents the specific activity on the top phase, while SA_I denotes the initial specific activity.

$$\text{Yield (\%)} = (A_T/A_i) \times 100 \quad (5)$$

where A_T and A_i represent the total lipase activity in the top phase and the initial activity in ASL-CL respectively.

$$V_R\,(v/v) = V_T/V_B \quad (6)$$

where V_T : volume of top phase, V_B: volume of bottom phase.

$$\text{Selectivity (S; \%)} = Ke/Kp = A_T/A_B \times P_B/P_T \quad (7)$$

where Ke/Kp or A_T/A_B is the lipase activity ratio in the top and bottom phases and Kp or P_B/P_T is the protein ratio in the bottom and top phases.

2.4.2 Effect of Various PEG on Lipase Fractionation

Different PEGs (PEG-1,000, PEG-2,000, PEG-4,000, PEG-6,000, PEG-8,000 and PEG-20,000) were investigated for fractionation of lipase in ASL-CL to separate the contaminated enzymes, mainly proteases. Ammonium sulfate (25%; w/v) was mixed with the same volume of PEG with different molecular weights at varying concentrations (15%, 20%, 30%, 40%, and 50%) (w/w). Two phases (1:1, v/v) were thoroughly mixed for 10 min at 3000 rpm using a vortex. Then, the phases were separated as mentioned in section 2.4.1 and determined for lipase activity and protein concentration in comparison with the fraction showing higher lipase activity in the previous section. ATPS developed by 50% PEG-6000 and 20% ammonium sulfate showed higher specific activity, purification fold and selectivity than others. Therefore, this ATPS was selected for further studies.

Ammonium sulfate (AS) at varying concentrations (15%, 20%, 25%, 30%, 40% and 50%) was used in combination with 50% PEG-6000. ATPSs were prepared as described above. The densities of the phase components i.e., PEG-6000 (50%; w/w) and AS at different concentrations were recorded. Lipase activities in the respective phases were also determined. AS concentration from this section showing higher lipase activity and yield was selected for further experiment.

Based on the primary ATPS, the lipase was highly enriched in the PEG-6000 phase. The secondary ATPS was performed using the method reported by Patchimpet et al. (2021). Briefly, the PEG-6000 phase was blended with distilled water at a 1:1 ratio. The mixture was incubated at 50 ˚C for 25 min, then centrifuged at 5500 × g at 25 ˚C. The pellets in the upper water-rich phase were collected and dissolved by mixing with 10 mL of tris-HCL buffer (pH 8) and subjected to dialysis against 1 L of tris-HCL buffer (25 mM, pH 8) overnight with changing the dialysis buffer every 4 h.

2.5 Assays and Characterization of Enzymes

2.5.1 Lipase Activity

Lipase activity was assayed using *p*-nitrophenyl palmitate (*p*NPP) as substrate. Solution A was 16.6 mM *p*NPP in isopropanol and solution B was 0.4% (w/v) gum Arabic, 0.6% (w/v) triton X-100 in 50 mM tris HCl buffer (pH 7.5). To conduct the hydrolytic reaction, 100 µL of solution A was mixed with 2.8 mL of solution B and incubated in a water bath at 37 ˚C for 5 min. Subsequently, 100 µL of the sample was included in the substrate solution and kept at 37 ˚C for 10 min. The absorbance of the mixture was measured at a wavelength of 410 nm. A standard curve of nitrophenol (1–10 mM) was prepared. One unit was described as the enzyme needed to release 1 mg of nitrophenol/min under the specified assay conditions (Baloch et al., 2021; Jaeger & Kovacic, 2014).

2.5.2 Protease Activity

Protease activities were measured in the samples after optimized fractionation of lipase. To determine the protease activity, the method described by Rawdkuen et al. (2012) was followed, which involved using casein as a substrate. The substrate solution was 0.65% casein, which was subsequently equilibrated in a water bath at 37 ˚C for 5 min. Afterwards, 100 µL of the selected fraction was added, mixed well and then incubated at 37 ˚C for 10 min. Five mL of 0.1 M trichloroacetic acid solution was added to stop the reaction and the solution was kept at 37 ˚C for 30 min. 0.45 µm polyether sulfone syringe filters were used to filter the samples and then 2 mL of filtrate was combined with 5 mL of 0.5 M sodium carbonate and 1 mL of Folin's reagent. After incubation at 37 ˚C for 30 min, 2 mL of samples were filtered with 0.45 µm polyether sulfone syringe filters, and the absorbance was measured at 660 nm. The protease activity was calculated using the tyrosine standard curve (0.01-1.1 mM). One unit of protease activity was the enzyme that released oligopeptides or free amino acids at 1 µmol tyrosine equivalent/min under the specified assay conditions.

2.5.3 Protein Determination

Total protein contents were determined with bovine serum as a standard, following the method of Lowry et al. (1951).

2.6 Use of Asian Seabass Lipase for Defatting of Skin Prior to Collagen Extraction

2.6.1 Skin Preparation and PEF Treatment

The frozen Asian seabass skin was obtained from the King-fisher Holdings Co., Ltd., Songkhla, Thailand. The frozen skin was cut into pieces (2 × 2 cm^2) using a sewing machine, followed by pretreatment with 0.10 M NaOH as described by Benjakul et al. (2017).

PEF-assisted pretreatment of the skin was performed following the conditions guided by Chotphruethipong et al. (2019a). Alkaline-treated samples (50g) were wrapped in cheesecloth and placed in a chamber (3.5 × 10 × 12 cm). A gap of 3.3 cm between the electrode plates inserted into the chamber was maintained. After adding the samples, 300 mL of chilled distilled water was added into the chamber and PEF equipped with a high voltage (15kV, 100A) supply system (PEF LAB-400W, Febix Intl Inc., Chiang Mai, Thailand) was applied. To monitor the electric voltage and current, a digital oscilloscope (UTD2052CEX, UNT, Dongguan) was connected to the PEF. A mercury-filled glass thermometer (Guangdong, China) was used to measure the temperature together with a UT-P03 probe (600MHz, 10). The temperature of the chamber was 25 ± 5 ˚C by keeping it in an iced-chilled water bath. The pretreatment with PEF was performed at an optimized electric field (24 kV/cm), 1080 number of pulses (n) with 0.10 pulse width (τ) and 20 ms repetition time (Δt) as previously reported (Chotphruethipong et al., 2019a). PEF-treated skins were then swollen by soaking in 50 mM citric acid solution for 2 h at a skin-to-citric acid ratio of 1:10 (w/v) as previously described by Benjakul et al. (2017).

2.6.2 Defatting of Asian Seabass Skin Using Lipase in Conjunction with Vacuum Impregnation

Defatting of PEF-treated skin was performed in a 5-L chamber equipped with a vacuum pump (VE 125 N, Zhejiang Value Mechanical & Electrical Products Co., Ltd., Wenling, Zhejiang, China) and vacuum meter (PG2, NUOVA FIMA, Novara, Italy) to examine the vacuum pressure. PEF-treated skins (200 g) were moved into the chamber, followed by the addition of 2 L of lipase solution either ASL-CL or the selected ATPS fraction in 10 mM phosphate buffer (pH 8) containing lipase at 50 U/ g dry weight of skin. The samples were allowed to be retained in the chamber for 5 min, followed by vacuum pressure for 20 min and then the pressure was released for 10 min. These vacuum impregnation processes (VI) were repeated for four cycles with a total of 140 min duration. Control was also prepared by adding skins in the chamber with 2 L of 10 mM phosphate buffer (pH 8) without lipase and subjected to VI as mentioned above. All treated skins were analyzed.

2.6.3 Analysis

2.6.3.1 Lipid Content

The Bligh and Dyer method was employed to extract the lipids from both the defatted and control skin samples (Bligh & Dyer, 1959). Lipid contents were then computed and reported as % lipid reduction.

2.6.3.2 Fatty Acid Profile of Remaining Lipid in Skin

Methyl ester (FAME) profiling of extracted lipids from treated skin was done following the AOAC method (1994). The fatty acid profiles were examined using a GC-FID instrument (7890B, Agilent, Santa Clara, CA, USA) with a capillary column (Agilent J&W CP-Sil 88, 100 m×0.25 mm i.d.×0.2 µm film thickness). The fatty acid contents were estimated and expressed as mg fatty acids/ 100 g of dried skin.

2.6.3.3 Lipid Distribution of Skin

The lipid distribution of skin was studied to visualize the defatting efficacy of skin. Skin samples both defatted and control were cut into (0.2×0.5×0.2 cm^3) pieces and then suspended for 10 min in Nile blue A solution (0.1%, v/ v). Thereafter, a confocal laser scanning microscope (CLSM) (Model LSM 800; ZEISS, Jena, Germany) using the fluorescence mode having

excitation (533 nm) and the emission (630 nm) wavelength with 20x magnification was employed to visualize the lipid distributed in the skin samples.

2.7 Extraction of Collagen from Skin Defatted by Liver Lipase

The method described by Nilsuwan et al. (2022) was used to extract acid-soluble collagen (ASC). The skins defatted by the selected ATPS fraction with the lowest protease or by ASL-CL were placed in 0.5 M acetic acid solution at 1:50 ratio (w/v) and homogenized at 9800 rpm for 3 min at 4 ˚C. The homogenate was stirred continuously at 4 ˚C for 48 h. Afterwards, the samples were filtered using a layer of cheesecloth, and the filtrates were adjusted to pH 7.5 and added with 2.6 M NaCl. The solution was stirred at 4 ˚C for 1 h. The pellets were collected by centrifuging for 5 min at 10,000 × g and 4 ˚C. The obtained pellets were then resuspended in distilled water at a ratio of 1:50 (w/v) and homogenized at 5000 rpm for 5 min, followed by stirring at 4 ˚C for 30 min. After 30 min, the samples were centrifuged at 10,000 × g, 5 min, and 4 ˚C. After washing the samples for three cycles with distilled water, the resulting pellets were retrieved and subjected to freeze-drying (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). Both collagens were then analyzed.

2.7.2 Analysis

2.7.2.1 SDS PAGE Pattern

SDS-PAGE was run using 4% stacking gel and 7.5% separating gel reported by Laemmli (Laemmli, 1970). After separation gels were stained and destained, respectively. Protein bands were calculated using high molecular weight markers.

2.7.2.2 FTIR Spectrum

Collagens were well milled with KBr powder (200 mg) and then the spectra were recorded by FTIR spectrometer (Equinox 55, Bruker Co., Ettlingen, Deutschland). Spectra of the mid-infrared region (4000–400 cm^{-1}) were obtained.

2.8 Statistical Analysis

CRD (completely randomized design) was employed. All experiments and analyses were conducted in triplicate. The data were analyzed using analysis of variance (ANOVA). Duncan's multiple range test was used for mean comparison, while t-tests were utilized for pair comparison.

3. Results and Discussion

3.1 Fractionation of Lipase from Asian Seabass Liver Using ATPS

3.1.1 Effect of Various Salt on Lipase Fractionation

The principle of ATPS lies in the formation of two distinct, highly aqueous phases that can be composed of either two polymers or a polymer and salt (Esquena, 2023; Patchimpet et al., 2021). The immiscibility and the aqueous nature of the separated phases make ATPS a very suitable technique to separate the desired biomolecules with minimal loss and degradation (Raguraman et al., 2023). The net charge on the biomolecule is one of the important factors in the ATPS system that allows the biomolecule to retain in the upper polymer-rich phase. For ATPS developed by polymer and salt, the salt would absorb the maximum amount of water causing exclusion and subsequently, two aqueous phases appeared. Since biomolecules mainly proteins and enzymes are negatively charged at a pH higher than their isoelectric points when they are added in an ATPS prepared by polymer and salt, biomolecules would tend to migrate towards the polymer-rich phase (Singla & Sit, 2023). The lipases have relatively lower isoelectric points (pH 5.0-8.0) (Binhayeeding et al., 2020), while the system had pH 8.0. This would facilitate the lipase migration toward the upper PEG-rich phase. Various salts including ammonium sulfate, sodium citrate, sodium sulfate and magnesium sulfate were used to develop ATPS by mixing these salts at various concentrations with 40% of PEG-4000 (w/w). Ammonium sulfate (20%) showed a higher specific activity of 56.8 U/mg protein with a yield of 5.4, and the highest selectivity (127.4%) and purification fold of 5.5 was also obtained ($p < 0.05$). The efficacy of ammonium sulfate was followed by the top ATPS phase generated by Na-citrate (25%; w/v) having a SA of 48.7 U/mg (**Table 1**). Also, ammonium sulfate showed high Ke (1.1) and Kp of 0.9. The results revealed that a better phase separation occurred with an increase in the salt concentration. Various salts at lower concentrations do not facilitate phase separations (Singla & Sit, 2022). For instance, 10% and 15% (w/v) of various salts such as sodium citrate, sodium sulfate and magnesium sulfate could not facilitate the phase separation system and no separated phases were observed ($p > 0.05$). However, at higher concentrations such as 20% and 25% (w/v), all the salts facilitated the creation of two phases in the presence of PEG-4000 (40%, w/w). Better phase separation in ATPS has been attained with higher molecular weight polymers and at higher amounts of salt, due to the volume exclusion that facilitates the biomolecule separation (Singla & Sit, 2022). Nonetheless, at higher salt concentrations the ionic strength of the salt phase is very high. As a result, the biomolecule was migrated to the top phase (Babu et al., 2008; Neves et al., 2019; Raja et al., 2012). Higher

Table 1. ATPS for seabass liver lipase partitioning as influenced by various types and amounts of salts in the presence of PEG-4000

Phase components (%, w/w)		SA		PF		S	Ke	Kp	Y (%)
	V_R	T	B	T	B				
15% PEG-4000/10% AS	0.2 ± 0.0**e**	49.1 ± 5.0**b**	48.8 ± 3.0**d**	4.7 ± 0.6**b**	4.7 ± 0.7**a**	99.4 ± 2.0**b**	1.0 ± 0.0**c**	1.0 ± 0.1**a**	4.8 ± 0.0**b**
15% PEG-4000/15% AS	0.2 ± 0.0**e**	57.2± 4.2**a**	45.2 ± 2.5**d**	4.7 ± 0.2**b**	4.3 ± 0.3**b**	74.2 ± 4.0**c**	1.3 ± 0.3**b**	0.9 ± 0.1**a**	4.2 ± 0.6**c**
15% PEG-4000/20% AS	0.7 ± 0.0**a**	56 .8± 6.2**a**	47.8 ± 4.6**d**	5.5 ± 0.0**a**	4.6 ± 0.4**a**	127.4 ± .0**a**	1.1 ± 0.0**c**	0.9 ± 0.0**a**	5.4 ± 0.0**a**
15% PEG-4000/25% AS	0.4 ± 0.0**c**	29.7 ± 4.4**c**	22.0 ± 2.0**f**	3.1 ± 0.2**c**	2.1 ± 0.6**d**	69.8 ± 3.0**d**	1.2 ± 0.4**b**	0.8 ± 0.0**b**	5.2 ± 0.5**a**
15% PEG-4000/10% SC	-	-	-	-	-	-	-	-	-
15% PEG-4000/15% SC	-	-	-	-	-	-	-	-	-
15% PEG-4000/20% SC	0.3 ± 0.0**d**	14.5 ± 1.1**f**	47 .0± 5.0**d**	1.4 ± 0.0**e**	4.7 ± 0.7**a**	31.8 ± 3.0**f**	0.1 ± 0.0**e**	0.5 ± 0.0**c**	1.4 ± 0.1**e**
15% PEG-4000/25% SC	0.1 ± 0.0**f**	48.7 ± 2.1**b**	30.8 ± 3.1**e**	4.8 ± 0.0**b**	2.9 ± 0.1**c**	63.1 ± 5.0**d**	0.1 ± 0.0**e**	0.6 ± 0.0c	2.8 ± 0.2**d**
15% PEG-4000/10% SS	-	-	-	-	-	-	-	-	-
15% PEG-4000/15% SS	-	-	-	-	-	-	-	-	-
15% PEG-4000/20% SS	0.3 ± 0.0**d**	18.2 ± 0.8**e**	66.1 ± 5.8**c**	2.1 ± 0.0**d**	1.4 ± 0.1**e**	32.7 ± 3.5**f**	0.3 ± 0.0**d**	0.1 ± 0.0**e**	2.3 ± 0.0**d**
15% PEG-4000/25% SS	0.3 ± 0.0**d**	13.9 ± 0.8**f**	101.8 ± 5.2**a**	1.7 ± 0.2**e**	2.1 ± 0.1**d**	58.8 ± 4.7**e**	0.3 ± 0.0**d**	0.2 ± 0.0**e**	1.1 ± 0.0**f**
15% PEG-4000/10% MS	-	-	-	-	-	-	-	-	-
15% PEG-4000/15% MS	-	-	-	-	-	-	-	-	-
15% PEG-4000/20% MS	0.3 ± 0.0**d**	21.3 ± 1.5**d**	80.4 ± 4.5**b**	3.1 ± 0.4**c**	1.3 ± 0.0**e**	9.7 ± 0.5**h**	2.7 ± 0.3**a**	0.3 ± 0.0**d**	1.4 ± 0.3**e**
15% PEG-4000/25% MS	0.5 ± 0.0**b**	22.1 ± 2.3**d**	102 .2± 7.0**a**	3.3 ± 0.3**c**	1.8 ± 0.1**d**	15.2 ± 0.9**g**	2.8 ± 0.0**a**	0.4 ± 0.0**d**	1.2 ± 0.0**e**

AS= Ammonium Sulfate, SC = Sodium citrate, SS = Sodium sulfate, MS = Magnesium sulfate
V_R = Top and bottom phases volume ratio (v/v)
SA = Specific activity (U/mg protein)
PF = Purification fold
S = Selectivity (%)
Ke = Enzyme co-efficient
Kp = Protein co-efficient
Y= Yield of lipase activity recovered (%)
Different lowercase letters in the same column indicate significant differences ($p<0.05$).

molecular weight polymers can cause a reduced biomolecule concentration in the polymer-rich upper phase (Anvari, 2015). Hence low molecular weight PEG-4000 (40%; w/w) in the initial screening was preferred. Since Ke and *Kp* were similar in the selected ATPS fraction (**Table 1**), further optimization was still required.

3.1.2 Effect of PEG at Various Molecular Weights on Lipase Fractionation

Polymer molecular weight is an impactful factor for the separation of a biomolecule in ATPS (Singla & Sit, 2023). Different molecular weights PEG (4000-20000) was used to develop the ATPS system with (20%) ammonium sulfate selected from section 3.1.1. An equal volume of 20% (w/v) ammonium sulfate was added to the PEG 4000-20000 mixture. The fractionation of lipase was affected by both molecular weight and concentrations of PEG used. For ATPS development, polymers with low molecular weights (1000 and 2000) did not facilitate phase separation (data not shown). Singla & Sit et al. (2023) and Babu et al. (2008) also declared that no phase partition occurred when low molecular weight PEGs containing PEG-1000 and PEG-2000 were used.

In general PEG-6000 (40%; w/w) and ammonium sulfate (20%; w/v) showed the desired phase separation as witnessed by a high yield of 48% and selectivity of 167.2% (**Table 2**). When compared to other PEG at varying concentrations PEG-6000 (50%, w/w) and ammonium sulfate (20%, w/v) showed the highest purification fold of 9.1 A noticeable difference in Ke and Kp values were also witnessed (**Table 2**). Similarly, PEG-6000 and PEG-8000 showed higher specific activity and recovery when *Enterococcus faecium* MTCC5695 lipase was fractionated via ATPS (Ramakrishnan et al., 2016), whereas for the porcine lipase separation, PEG-4000 and PEG-6000 showed better recovery and specific activity (de Carvalho Batista Muniz et al., 2022).

The determination of the phase density and viscosity of ATPS is essential to follow the behavior of these aqueous phase systems. (Grilo et al., 2016; McQueen & Lai, 2019). The relative density of the PEG-6000-rich upper phase and that of ammonium sulfate as the bottom phase is depicted in Figure 1a. After vigorous mixing, the lipase fraction (top phase) collected at 50% (w/w) PEG-6000 and 20% (w/v) ammonium sulfate showed higher lipase activity 246.8 U/mL ($p < 0.05$) as well as a higher density of 1.2 g/mL (**Figure 1a**), whereas the ammonium sulfate-rich bottom phase of the same fraction showed minimum lipase activity (131 U/mL) (**Figure 1b**). The results suggested that most lipases were localized in the top phase. Further, an increase in the salt concentration lowered the lipase activity in the top phase (**Figure 1 a, b**). Although salts facilitate a better biomolecule separation at a certain high concentration, the excessive presence of the salt in the ATPS alters the phase tendencies, thus disturbing the optimum separation of the biomolecules (Raja et al., 2012). Additionally, the water structure breaking effect was also observed at higher salt concentrations. Salt at higher concentrations especially at 40% and 50% (w/w) decreased the density of the PEG-rich phase (**Figure 1 a, b)** (Kuepethkaew et al., 2017). This might favor the migration of lipase to the salt-rich bottom phase. In conclusion, lipases located in the top phase became less.

3.2 Protease Activity in the Selected Lipase Fraction

Commercial enzymes including porcine pancreatic lipase have been implemented to eliminate the fat

Table 2. ATPS for seabass liver lipase partitioning as influenced by various types and amounts of PEG in the presence of 20% ammonium sulfate

Phase components (%, w/w)		SA		PF					
Primary ATPS	V_R	T	B	T	B	**S**	**Ke**	**Kp**	**Y (%)**
PEG-4000									
15%PEG+20%AS	-	-	-	-	-	-	-	-	-
20%PEG+20%AS	-	-	-	-	-	-	-	-	-
30% PEG+20%AS	0.5 ± 0.0**d**	42.6 ± 4**d**	63.7 ± 4 **b**	4.1 ± 0.6**e**	6.1 ± 0.2**b**	66.8 ± 3**f**	0.7 ± 0.01**e**	1.1 ± 0.01**b**	34.8 ± 2.0**c**
40% PEG+20%AS	0.7 ± 0.0**c**	42.8 ± 2**d**	6 ± 4**f**	4.2 ± 0.2**e**	5.8 ± 0.4**b**	49.3 ± 3**g**	0.6 ± 0.02**e**	1.3 ± 0.08**b**	29.2 ± 4.0**c**
50% PEG+20%AS	0.8 ± 0.0**c**	67.8 ± 3**a**	43.7 ± 5**d**	5.8 ± 0.5**d**	4.1 ± 0.2c	787.9 ± 5**a**	0.9 ± 0.00**d**	1.2 ± 0.03**b**	36.1 ± 4.0**c**
PEG-6000									
15%PEG+20%AS	0.3 ± 0.0**e**	20.2 ± 1**e**	57.8 ± 3**b**	2.4 ± 0.1**f**	5.8 ± 0.2**b**	35 .0 ± 5**h**	0.5 ± 0.01**e**	1.6 ± 0.10**a**	12.7 ± 1.5**e**
20%PEG+20%AS	0.5 ± 0.0**d**	41.7 ± 3**d**	52.5 ± 6**c**	4.4 ± 0.4**e**	5.0 ± 0.2**c**	79 .3 ± 4**e**	0.8 ± 0.04**d**	0.9 ± 0.08**b**	28.0 ± 2.0**c**
30% PEG+20%AS	0.7 ± 0.0**c**	60.1 ± 2**b**	47.7 ± 3**c**	5.0 ± 0.5**d**	5.7 ± 0.4**b**	69.5 ± 2**f**	0.8 ± 0.07**d**	1.6 ± 0.12**a**	35.6 ± 4.5**c**
40% PEG+20%AS	0.3 ± 0.0**e**	65.2 ± 3**b**	29.7 ± 2**e**	5.8 ± 1.0**d**	3.4 ± 0.2**d**	105.3 ± 8**d**	1.1 ± 0.00**c**	0.3 ± 0.00**c**	48.0 ± 5.6**b**
50% PEG+20% AS	0.2 ± 0.0**f**	68.2 ± 5**b**	35.9 ± 1**d**	9.1 ± 1.0**a**	2.8 ± 0.5**d**	167.2 ± 6**b**	1.5 ± 0.6**c**	0.9 ± 0.01**b**	28.2 ± 3.8**c**
PEG-8000									
15%PEG+20%AS	1.0 ± 0.0**b**	72.1 ± 6**a**	47.8 ± 3**c**	6.9 ± 0.8**c**	5.0 ± 0.2**c**	13.7 ± 2**i**	0.1 ± 0.00**g**	1.1 ± 0.09**b**	12.6 ± 2.0**e**
20%PEG+20%AS	0.5 ± 0.0**d**	51.9 ± 2**c**	64.8 ± 5**b**	5.0 ± 0.4**d**	6.2 ± 0.3**b**	80.1 ± 3**e**	0.4 ± 0.04**f**	0.5 ± 0.05**c**	16.3 ± 1.6**d**
30% PEG+20%AS	0.1 ± 0.0**f**	47.0 ± 3**c**	40.6 ± 6**d**	3.9 ± 0.7**e**	4.5 ± 0.3**c**	115.7 ± 4**d**	0.1 ± 0.01**g**	0.1 ± 0.03**d**	0.9 ± 0.0**g**
40% PEG+20%AS	0.2 ± 0.0**f**	20.4 ± 1**e**	39.7 ± 3**d**	8.4 ± 0.5**b**	3.3 ± 0.4**d**	51.3 ± 5**g**	0.1 ± 0.00**g**	0.2 ± 0.07**d**	2.6 ± 0.04**f**
50% PEG+20% AS	1.3 ± 0.0**a**	8.6 ± 6**f**	27.0 ± 2**e**	7.0 ± 03**c**	2.8 ± 0.2**d**	126.1 ± 2**c**	1.2 ± 0.00**c**	0.9 ± 0.08**b**	20.0 ± 1.6**d**
PEG-20000									
15%PEG+20%AS	1.4 ± 0.0**a**	28.3 ± 4**d**	46.4 ± 4**d**	2.7 ± 0.4**f**	4.4 ± 0.6**c**	68.7 ± 2**f**	1.1 ± 0.00**c**	0.5 ± 0.05**c**	49.0 ± 2.6**b**
20%PEG+20%AS	1.0 ± 0.0**b**	33.6 ± 3**d**	85.4 ± 4**a**	3.3 ± 0.6**f**	8.2 ± 0.9**a**	52.2 ± 5**g**	1.4 ± 0.00**c**	0.4 ± 0.03**c**	35.2 ± 3.6**c**
30% PEG+20%AS	0.5 ± 0.0**e**	28.4 ± 6**d**	42.3 ± 5**d**	2.7 ± 0.2**f**	4.0 ± 0.3**c**	84.8 ± 6**e**	4.4 ±0.50**a**	0.6 ± 0.04**c**	59.5 ± 6.6**a**
40% PEG+20%AS	0.3 ± 0.0**e**	61. ± 5**b**	51.1 ± 2**c**	5.9 ± 0.5**d**	4.9 ± 0.2**c**	67.3 ± 3**f**	2.7 ± 0.70**b**	1.1 ± 0.70**b**	52.0 ± 3.5**a**
50% PEG+20% AS	1.4 ± 0.0**a**	22.2 ± 5**e**	51.8 ± 3**c**	2.2 ± 0.1**f**	5.2 ± 0.6**b**	68.6 ± 7**f**	1.1 ± 0.00**c**	0.5 ± 0.03**c**	48.9 ± 6.0**b**

V_R = Top and bottom phases volume ratio (v/v)
SA = Specific activity (U/mg protein)
PF = Purification fold
S = Selectivity (%)
Ke = Enzyme co-efficient
Kp = Protein co-efficient
Y= Yield of lipase activity recovered (%)
Different lowercase letters in the same column indicate significant differences ($p < 0.05$).

contents of fish skin before the HC extraction (Chotphruethipong et al., 2019a; Nilsuwan et al., 2021; Sae-Leaw & Benjakul, 2018). The porcine biomolecule products are not acceptable for many religious groups and ethnicities, mainly the Muslim community. Therefore, the collagen extracted after defatting the fish skins with porcine lipases could not attract consumers or the market. Generally, fish viscera have high protease and lipase activities (Prasertsan & Prachumratana, 2008). Various fish livers have been intensively studied for lipase extraction (Mardina et al., 2018; Sae-Leaw & Benjakul, 2018; Sae-leaw & Benjakul, 2017). However, the crude extract from fish viscera always contained high protease activities (Ketnawa et al., 2014; Prasertsan & Prachumratana, 2008; Rawdkuen et al., 2012; Senphan & Benjakul, 2014). This would negatively affect the quality of the extracted HC via degradation. Therefore, ATPS was developed to fractionate lipase from Asian seabass fish liver and separate it from the protease. A reduction of 85% ($p < 0.05$) in protease activity was observed when the ASL-CL was subjected to ATPS fractionation with PEG-6000 (50%; w/w) and ammonium sulfate (20%; w/v) (**Figure 2a**). Simultaneously, lipase/protease activity in ATPS fraction was increased by 582%, compared to that found in ASL-CL (**Figure 2b**). The results revealed that an appropriate ATPS could effectively fractionate lipase into the PEG phase, which simultaneously removed protease into the bottom phase. The top fraction rich in lipase was further fractionated using distilled water and subjected to heat separation. At their cloud points, polymers tend to aggregate into macroscopic aqueous spheres or droplets and prefer to migrate in the bottom phase. Since the cloud-point of PEG-6000 at 50% (w/w) is lower than 50 ˚C (Sürücü et al., 2022), and the optimum temperature of ASL-CL is 50 ˚C (Sae-Leaw & Benjakul, 2018). Therefore, incubation at 50 ˚C facilitated the separation of the lipase in the top phase mainly containing water (Patchimpet et al., 2021). After fractionation lipase migrated into the water phase (top phase) and dialyzed. The dialysate had a specific activity of 60.2 U/mg protein, a yield of 20.3, and a purification fold of 12.5 (data not shown). The decrease in specific activity and yield was likely a result of lipase loss that occurred during the secondary ATPS fractionation and dialysis steps.

3.3 Use of Selected Lipase in Conjunction with Vacuum Impregnation for Defatting Asian Seabass Skin

3.3.1 Impact on Lipid Contents in Conjunction with Vacuum Impregnation (VI)

The effect of PEF on defatting of Asian seabass skin was thoroughly studied elsewhere (Chotphruethipong et al., 2019). It was observed that the PEF could enhance

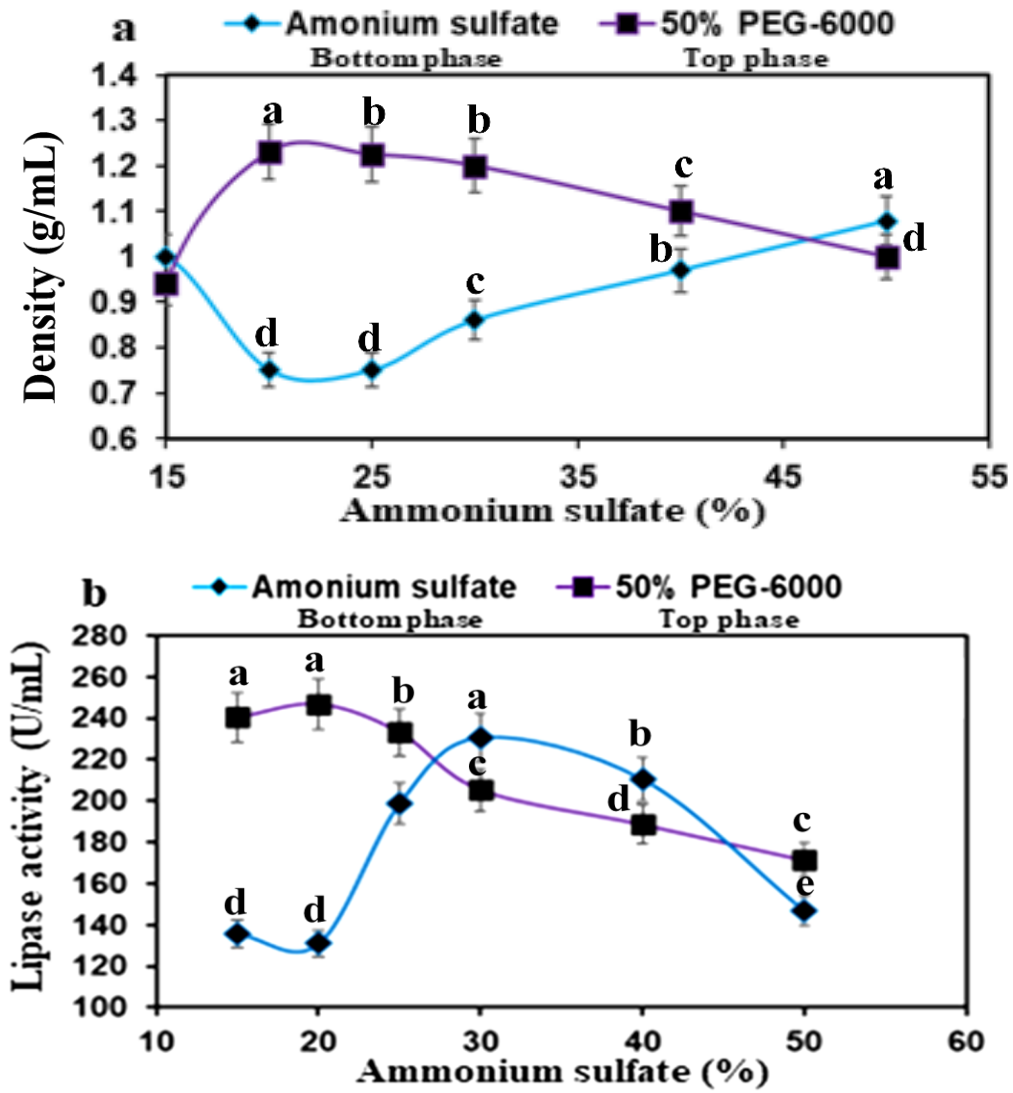

Figure 1. The change in density of the top polymer-rich and bottom salt-rich phases after ATPS formation **(a)**, and their effects on lipase activity **(b),** as affected by ammonium sulfate at various concentrations. Bars represent standard deviation (n=3) and different lowercase letters show a significant difference ($p<0.05$) in density and lipase activity the top and bottom phases.

the porosity of the skin and facilitate the penetration of lipase into the skin. Lipase could hydrolyze the lipid effectively as shown by the almost disappearance of oil droplets stained by Nile blue A, while the untreated skin had droplets at high density. Nevertheless, only PEF treatment could not reduce the lipid from the skin (Chotphruethipong et al., 2019a, 2019b). Therefore, the effect of the selected lipase fraction from ATPS (50% PEG-6000; w/w and 20% ammonium sulfate; w/v) and ASL-CL in conjunction with VI compared to the control (without lipase) is shown in Figure 3. The PEF-treated skin defatted with the selected ATPS fraction showed 92.6% lipid reduction, followed by that defatted using ASL-CL (77.5% of lip reduction) ($p < 0.05$) (**Figure 3**). Nevertheless, the samples treated with only VI without lipase showed 80% lipid retained compared to that found in the initial Asian seabass skin (**Figure 3**). Some lipids could be leached out during VI via the physical release of lipids from the skin. However, its efficacy in defatting was much less than the use of lipases together with VI. It was noted that the removal of the air in PEF-treated skin could facilitate the migration of lipase during the release of the vacuum. Lipases in ASL-CL and the selected ATPS fraction could enter into the widened pores of the skin and accelerate the hydrolysis of lipids (Derossi et al., 2013). VI along with PEF pretreatment loosened the fish skin matrix, which ultimately exceeded the transmission of lipase potentially into the skin or other tissues. Lipases could hydrolyze ester bonds, resulting in the release of free fatty acids and glycerol from the skin, which could then be removed, leading to a decrease in the amount of fat retained in the treated fish skin (Figure 3).

3.4.1 Impact on the Fatty Acid Profile of Remaining Lipids

The lipase from the selected ATPS fraction could remove most fatty acids, especially monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids

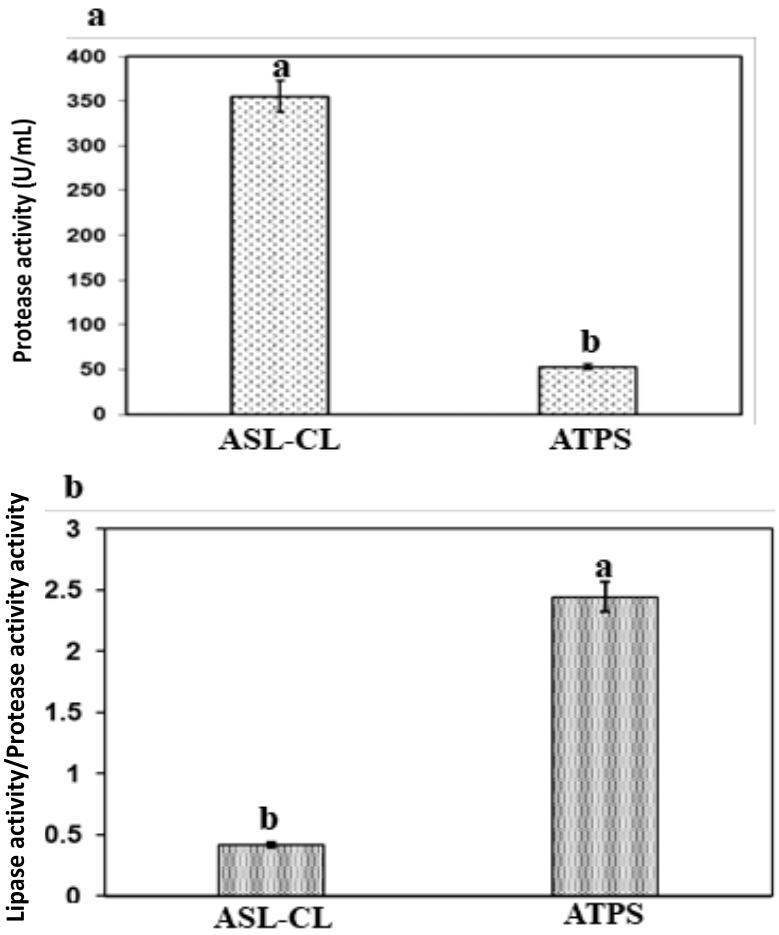

Figure 2. Protease activity in crude extract (ASL-CL) and the selected ATPS fraction (ATPS) (a) and lipase/protease activity ratio (b). Different lowercase letters on the bars denote significance difference ($p < 0.05$). Bars represent standard deviation (n=3).

(PUFAs). Saturated fatty acids (SFAs) were also removed. SFAs, MUFAs and PUFAs were removed by the lipase in the selected ATPS fraction by 68%, 95.7% and 85%, respectively. Skin defatted with ASL-CL showed reductions of SFAs, MUFAs and PUFAs by 56.2%, 86.5% and 77.7%, respectively. Nonetheless, VI without any lipase did not significantly affect the fatty acid profile of lipids in Asian seabass skin ($p > 0.05$) (**Table 3**). Based on fatty acid profile of Asian seabass skin lipids, fish skin defatted with ATPS-based fractionated lipase had the drastic decrease in most fatty acids ($p < 0.05$). The concentration of the major fatty acids in the non-treated sample including C14:0, C16:0, and C22:6 remained unchanged ($p < 0.05$) (**Table 3**). Fatty acids from Asian seabass fish skin lipids majorly contained polyunsaturated fatty acids. Similar patterns of the residual fatty acid profile were observed when seabass skin was defatted with porcine lipase assisted by a pulse electric field and vacuum impregnation (Chotphruethipong et al., 2019a, 2019b). However, some of the short-chain fatty acids such as C4:0-C10:0 and some polyunsaturated fatty acids were decreased more when treated with lipase fractionated by ATPS than reported by using porcine lipase in conjunction with VI.

3.4.2 Impact on Lipid Distribution of Asian Seabass Skin

The distribution of lipids in the skin samples treated with PEF + VI + lipase from ASL-CL or lipase fractionated by the selected ATPS is illustrated in Figure **4**. Fish skin defatted with Asian seabass liver lipase fractionated via ATPS showed the lowest distribution of lipids after the skins were stained with Nile blue. On the other hand, samples treated with VI alone had a similar pattern of lipid distribution, compared to the initial fish skin (**Figure.4a. b**). The lower lipid distributions of the samples were in line with the high efficacy in lipid reduction (**Figure 3**). It was noted that VI-ATPS showed a massive reduction of the lipid, followed by VI-ASL-CL. This was probably due to the higher purity of lipases in the ATPS fraction, allowing lipase to function more potentially as witnessed by lower lipid distribution in the skin.

Such a massive lipid removal was due to VI which allows the lipase to enter into the pores of PEF-treated fish skin (Chotphruethipong et al., 2019b). However, the lower performance of ASL-CL might have been due to the presence of proteases in the crude extract which might cleave the lipases, thus inactivating lipase. This resulted in lower efficiency in defatting Asian seabass skin (Lasher et al., 2019).

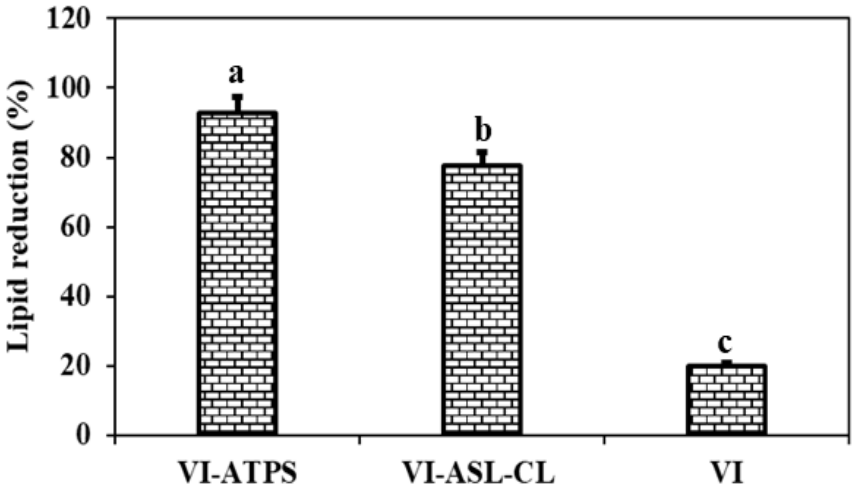

Figure 3. Effect of Asian seabass liver lipase in conjunction with vacuum impregnation on lipid reduction of Asian sea bass skin pretreated with the pulsed electric field. VI-ATPS: the lipase fractionated with ATPS, VI-ASL-CL: Asian seabass liver crude lipase, VI: vacuum impregnation without lipase (n=3). One cycle of the VI process took 20 min, followed by restoration to atmospheric pressure for 10 min with a total operation cycle of total 140 min. Different lowercase letters on the bars denote significance difference ($p < 0.05$). Bars represent standard deviation (n=3).

Table 3. Fatty acid profiles of lipids selected from Asian seabass skin before and after defatting with different processes.

Fatty acid mg/100g sample (dry wt.)	Non-treated	VI	VI-ASL-CL	VI-ATPS
C12:0	28.7 ± 1.6**d**	17 ± 0.9**c**	15.7 ± 1.2**b**	12 ± 0.2**a**
C14:0	107.5 ± 3.3**d**	90.4 ± 0.6**c**	32.4 ± 8.0**b**	16.2 ± 0.6**a**
C14:1	7.4 ± 1.5	Na	Na	Na
C15:0	27 ± 2.7**d**	11.7 ± 0.6**c**	9.5 ± 0.65**b**	9.1 ± 0.1**a**
C16:0	262.1 ± 15**d**	198.5 ± 2.1**c**	147.5 ± 19.4**d**	117.3 ± 1.5**b**
C16:1	153.4 ± 5.4**d**	89 ± 4.5**c**	46.1 ± 2.1**b**	7.7 ± 0.1**a**
C17:0	36.0 ± 2.4**d**	16.7 ± 0.1**c**	14.2 ± 1.2**b**	8.8 ± 0.0**a**
C17:1 cis 1	16.5 ± 1.0**d**	9.3 ± 0.9**c**	7.2 ± 0.4**b**	5.4 ± 0.4**a**
C18:1 trans 9	8.7 ± 0.5**b**	6.7 ± 0.5**a**	6.2 ± 0.2**a**	Na
C18:1 cis 9	383.3 ± 11.2**a**	353.3 ± 15.2**b**	Na	Na
C20:0	36.9 ± 0.7**c**	33 ± 5.0**b**	12.7 ± 0.5**a**	8.3 ± 1.0**a**
C18:3 cis 6,9,12 gamma	21.7 ± 0.5**d**	12.6 ± 3.0**c**	11.6 ± 0**b**	10.6 ± 0.6**a**
C20:1 cis 11	29.5 ± 1.0**d**	10.4 ± 1.6**c**	9.6 ± 0.68**b**	7.5± 0.0**a**
C18:3 cis 9,12,15 alpha	64 ± 1.8**d**	53.4 ± 2.6 **b**	11.2 ± 2.0**a**	Na
C21:0	24.8 ± 4.0**d**	17.7 ± 2.0**c**	11.7 ± 1.0 **b**	7 ± 0.4 **a**
C20:2 cis 11,14	30 ± 1.8**a**	24.08**b**	Na	Na
C20:3 cis 8,11,14	15 ± 1.7**c**	11.4 ± 0.6**b**	9.8 ± 1.2**a**	Na
C22:1 cis 13	7.9c ± 0.5**d**	7.7 ± 0.2**d**	7.2 ± 0**b**	5.8 ± 1.0**a**
C20:3 cis 11,14,17	7.3b ± 0.8**b**	4.9 ± 0.7**a**	Na	Na
C20:4 cis 5,8,11,14	5.5 ± 0.0**a**	Na	Na	Na
C23:0	28.1 ± 0.5**a**	26.1 ± 0.8**a**	Na	Na
C22:2 cis 13,16	16.7 ± 0.8**d**	11.3 ± 0.5**c**	10.5 ± 1.0**b**	7.2 ± 1.5**a**
C24:0	12.2 ± 0.3**d**	7.5 ± 0.4**c**	3.1 ± 0.2**b**	5.5 ± 0.2**a**
C20:5 cis 5,8,11,14,17	63.8 ± 4.5**d**	42.8 ± 2.2**c**	18.9 ± 1.3**b**	8.5 ± 0**a**
C24:1 cis 15	13 ± 1.2**c**	9.5 ± 0.0**b**	5.8 ± 0.2**a**	Na
C22:6 cis 4,710,13,16,19	214.7 ± 10.5**d**	160.8 ± 2.8**c**	54.6 ± 5.0**b**	33.6 ± 0.8a
SFA	563.3 ± 20.5**a**	416.8 ± 30.2**b**	246.3 ± 20.4**c**	176.3 ± 13.3**d**
MUFA	611.0 ± 28.8**a**	525.5 ± 21.3**b**	82.1 ±5.8**c**	26.4 ± 1.5**d**
PUFA	438.7 ± 35.8**a**	333.2 ± 18.8**b**	97.72 ±8.2**c**	65.7 ±2.3**d**

Values are expressed as mean ± SD (n = 3). SCL, Asian seabass crude lipase; VI, vacuum impregnation without lipase; VI-ASL-CL, vacuum impregnation with SCL, VI-ATPS, vacuum impregnation with lipase fractionated by ATPS.
SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid.
Different lowercase letters in the same row indicate significant differences ($p < 0.05$).

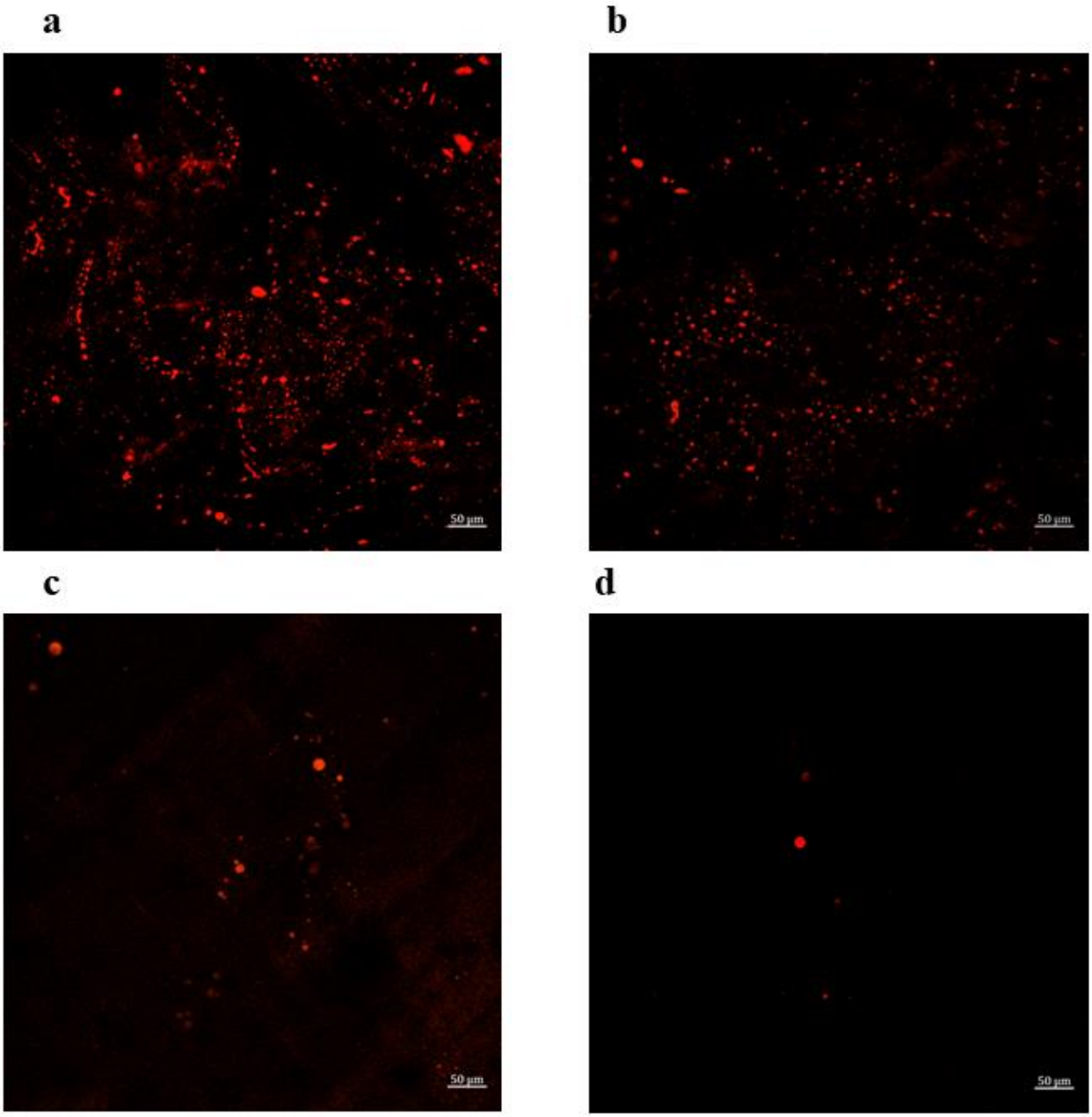

Figure 4. Confocal laser microscopic images of lipid distribution of Asian seabass skin before defatting (a) and after defatting using VI (b), VI-ASL-CL (c) and VI-ATPS (d) magnification: 20x.

3.5 Characteristics of Acid-soluble Collagen (ASC) from Defatted Asian Seabass Skin

3.5.1 Protein Patterns

Protein patterns of ASC extracted from Asian seabass skin defatted with lipase fraction by the selected ATPS were analyzed in comparison with ASC extracted from non-treated skin. The major components of ASC were *β*-chains, which are the dimer of *α*-chains. ASC consisted of 2 α-chains namely α_1- and α_2-chains. The ASC also comprised a trimer of α-chains, called γ-chain. The protein pattern of the collagen from skin defatted with ATPS fraction containing lipase was similar to that of ASC from non-treated skin (**Figure 5**). However, band intensities of all protein patterns in ASC prepared from defatted skin were slightly lower than that of ASC from non-treated skin. This might have occurred due to the slight degradation caused by the remaining 15% of proteases present in the selected ATPS fraction. The results suggested that defatting using liver lipase in ATPS fraction had no remarkable effect on the protein profile of ASC. The ratio of α1:α2-chain band intensity at 2:1 indicated that both ASC were composed of type 1 collagen (Nilsuwan et al., 2022). Similar α_1:α_2-chains ratios were also documented in ASC from various fish skin such as bighead carp (Cruz-López et al., 2021), striped catfish (Singh et al., 2011), and salmon fish (Nilsuwan et al., 2022). Therefore, lipase from fish liver fractionated using ATPS under optimal conditions could not degrade the collagen located in the defatted fish skin matrix.

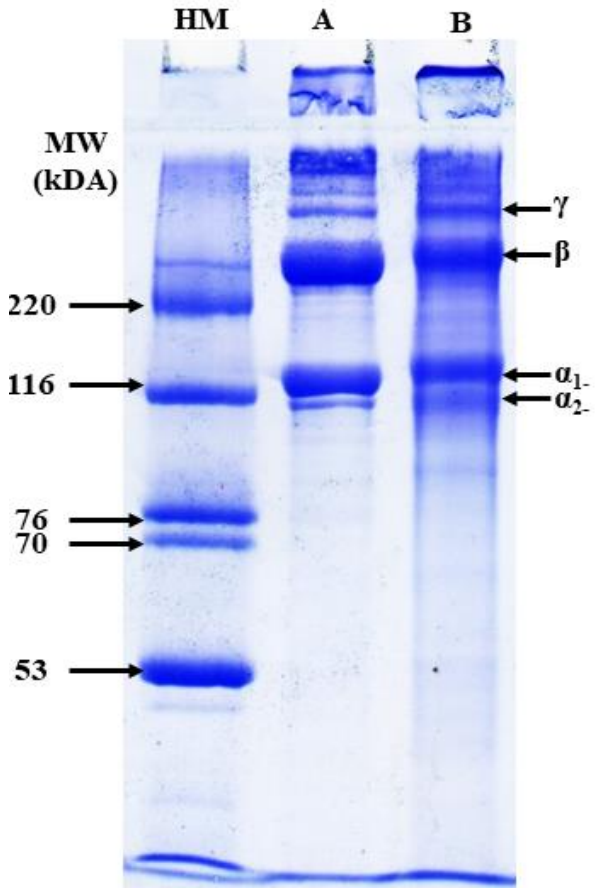

Figure 5. Protein pattern of acid-soluble collagen extracted from Asian seabass skin without (**A**) and with defatting by Asian seabass liver lipase fractionated with ATPS in conjunction with vacuum impregnation (**B**). **HM:** High molecular weight marker**;** γ, β, α_1 and α_2 denote trimer, dimer, α_1 and α_2 chains, respectively.

3.5.2 Fourier Transform Infrared (FTIR) Spectrum

FTIR spectra of ASC commonly showed characteristic peaks such as amide A, B, I, II and III (Ali et al., 2018). The peak representing the N-H stretching vibration was observed in the wavenumber of 3292–3296 cm^{-1}, whereas the CH_2 stretching vibration (amide B) was found at the wavenumber of 3292–3307 cm^{-1} (Liu et al., 2012). The amide A and amide B peaks for Asian seabass skin defatted with ATPS fractionated lipase and the non-treated skin were found at the range of 3200-3300 cm^{-1} and 2800-2900 cm^{-1}, respectively (**Figure 6**). The typical collagen spectra peaks for amide I, II and III were located at 1600–1700, 1500–1600, and 1200–1300 cm^{-1}, respectively (Ali et al., 2018). Likewise, the amide I peak of ASC prepared from the skin defatted with ATPS fractionated lipase was observed at wavenumber ranging from 1600-1700 cm^{-1}, while amide II was observed at 1500-1600 cm^{-1}. Amide III was observed at 1300-1400 cm^{-1}. Additionally, the amide I peak corresponds to the stretching vibration of the C=O located in the polypeptide backbone (Ali et al., 2018), whereas amide II corresponds to the N−H bending (Singh et al., 2011). Amide III shows the collective signals of N−H deformation and C−N stretching which are the characteristics of intermolecular interactions taking place in collagen structure (Sinthusamran et al., 2013). The native collagen having hydrogen bonds consisted of an amide III region at the wavelength range of 1200–1300 cm^{-1}. The ratio of the peak amplitude of amide III and the peak at 1450 cm^{-1} was close to 1, suggesting that ASC had a triple-helical structure (Nilsuwan et al., 2022). In the present study, this ratio was 1.1 for both collagens extracted from ATPS fraction defatted and non-treated Asian seabass skin, suggesting no drastic change in native structure caused by protease (**Figure 6**). Moreover, the ester carbonyl functional group of lipids was generally found at 1741-1746 cm^{-1} wavenumber (Sae-Leaw & Benjakul, 2018), Nevertheless, this group was not found in the FTIR spectra of the ASC extracted from Asian seabass skin defatted with the lipase fractionated through ATPS (**Figure 6**).

From the protein pattern and FTIR spectra of ASC obtained from Asian seabass skin after defatting with the Asian seabass liver lipase fractionated with ATPS, it was substantiated that the protease retained at a minimal level exhibited minimal impact on collagen structure (**Figures 2, 5 and 6**). Therefore, the native collagen structure after defatting showed intact protein patterns and the major FTIR-associated spectra of the native collagen.

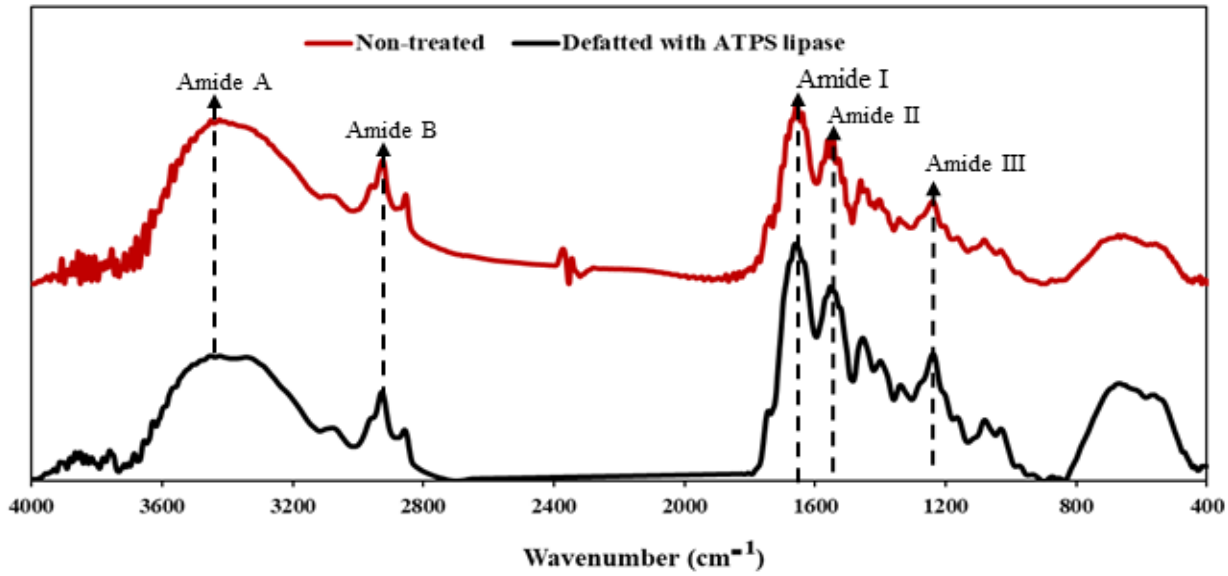

Figure 6. FTIR spectra of acid-soluble collagen extracted from Asian seabass skin with and without defatting by Asian seabass liver lipase fractionated with ATPS in conjunction with vacuum impregnation.

4. Conclusion

An aqueous two-phase system (ATPS) was efficiently optimized to fraction lipase from the liver of Asian seabass. Different salt and PEG at varying amounts were used. Among all salts and PEG, ammonium sulfate (20%, w/v) and PEG-600 (50%, w/w) at equal quantity were effective in fractionation of lipase with high purification fold and yield, while markedly eliminating proteases from the crude extract. Before extracting collagen, the above-mentioned fraction was used to remove the fat from Asian seabass skin. Most of the lipids were removed from the skin by 92%. Acid soluble collagen (ASC) showed native molecular characteristics to that extracted from non-treated skin. However, the remaining proteases should be further inactivated or removed to avoid undesirable effects, especially proteolysis. As a consequence, Asian seabass liver lipase could be used more widely and effectively.

Ethical Statement

Ethical approval is not required for this study.

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Author Contribution

K.A.B: *Formal Analysis, Methodology, Visualization and Writing -original draft,* U.P: *Formal Analysis, Investigation, Methodology, Visualization and Writing - original draft,* A.F.Y.: *Conceptualization, Data Curation, Writing -review and editing,* S.B.: *Conceptualization, Supervision , Methodology, Data Curation, Writing - review and editing*

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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