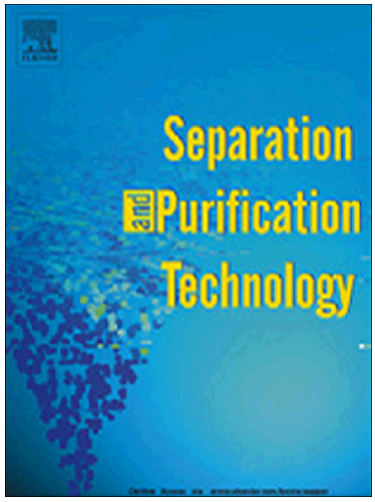

Three-phase Partitioning and Proteins Hydrolysis Patterns of Alkaline Proteases Derived from Fish Viscera

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Three-phase Partitioning and Proteins Hydrolysis Patterns of Alkaline Proteases Derived from Fish Viscera

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Abstract

In this study, the recovery alkaline proteases from farmed giant catfish viscera were isolated by using three-phase partitioning (TPP). Factors affecting partitioning efficiency such as salts, solvent types, pH, and incubation temperatures were studied. Furthermore, the application of extracted alkaline proteases on proteins hydrolysis was also determined. The system consisted of crude enzyme extract: *t*-butanol 1: 0.5 (w/v), 50% sodium citrate, pH 8.0 with incubation temperature of 25ºC provided the highest enzyme recovery (220%). The smear protein bands with molecular weight of 20, 24, and 215 kDa of TPP fractions appeared on the protein stained gel. Two major clear zones (24 and 130 kDa) in the interphase were observed on casein-substrate gel electrophoresis. Extracted alkaline proteases showed relatively high effective in protein hydrolysis. As a result, TPP provided high enzyme recovery and could be applied to other enzymes. The obtained alkaline proteases can be further applied in preparation of protein hydrolysates.

Keywords: Alkaline proteases, Fish viscera, Protein hydrolysate, Three-phase partitioning, Trypsin

1. Introduction

In recent years, the trend has been developed rapid, efficient, economical and scalable approach for separation and purification of enzymes [1] and [2]. Three-phase partitioning (TPP), a one-step enzyme purification approach, is carried out by mixing salts i.e. ammonium sulfate, potassium phosphate, and sodium citrate and organic solvents i.e. *t*-butanol, 2-butanol, 1-propanol, and 2-propanol to obtain organic phase, interfacial precipitate and aqueous phase [3]. TPP employs collective operation of principles involved in numerous techniques like salting out, isoionic precipitation, cosolvent precipitation, osmolytic, and

kosmotropic precipitation of proteins [1]. In addition, the addition of organic solvent in the presence of salt pushes the protein out of the solution to form an interfacial precipitate layer between the lower aqueous and upper organic layers. TPP is a concentrating or dewatering step and some enzymes have enhanced catalytic activities in these conditions within short periods of time (about 1 h) [4]. TPP has been widely used to separate and purify various enzymes such as *Aspergillus oryzae* [4], protease/amylase inhibitor from wheat germ [5] and ragi (*Eleusine coracana*) [6], phospholipase D from *Dacus carota* [7], α-galactosidase form tomato [8], and pepino (*Solanum muricatum*) [9], invertase from baker's yeast [10], and tomato [11], proteases from papaya peels [12], *Calotropis procera* latex [13] and giant catfish viscera [14], inulinase from *Aspergillus niger* [15]. Rawdkuen et al. [14] showed the best condition for separating of alkaline proteases from farmed giant catfish viscera that was consisted of the crude enzyme extract to *t*-butanol ratio of 1.0: 0.5 in the presence of 50% $(NH_4)_2SO_4$. Besides, TPP was used to isolate trypsin inhibitors from navy bean, red kidney bean and adzuki by mixing the crude extract with solid ammonium sulfate (30% saturation, w/v) and *t*-butanol with the ratio of 1: 1 (v/v) [16].

With an increase in fish processing, a large amount of internal organs and by-products will be generated. The viscera in some types of fish account for approximately 5-10% of the entire weight of the fish; the viscera percentage tends to increase with the fish body weight [14]. The most important proteases in the viscera of fish and aquatic invertebrates are aspartic protease (pepsin) and serine proteases (trypsin, chymotrypsin, collagenase, and elastase) [17]. Proteases are by far the most studied enzymes for industrial bio-processing. Almost half of all industrial enzymes are proteases, mostly used in the detergent, leather, and food industries [18]. Recently, the use of alkaline proteases from marine digestive organs, especially trypsin, has increased remarkably since they are both stable and active under harsh conditions, such as at temperatures of 50-60ºC, high pHs, and in the presence of surfactants or oxidizing

agents [18]. The enzymes recovered from fish viscera have also been successfully used as seafood processing aids including the acceleration of fish sauce fermentation [19], extraction of carotenoprotein [20], and production of protein hydrolysates [21], [22], [23], [24] and [25]. Alkaline proteases or trypsin have been used to produce protein hydrolysates and the obtained hydrolysates showed bioactive properties such as antioxidant and angiotensin I converting enzyme (ACE) inhibitory properties [21], [22], [23] and [25]. Crude enzyme extract from sardine (*Sardina pilchardus*) viscera was used to produce protein hydrolysates from heads and viscera of sardinelle compared with Alcalase and provided the potent ACE inhibitory peptides with IC_{50} 1.2 mg/ml [25]. Alkaline proteases from pyloric caeca extract of three fish species including brownstripe red snapper (*Lutjanus vitta*), bigeye snapper (*Priacanthus tayenus*) and threadfin bream (*Nemipterus marginatus*) have been used to produce gelatin hydrolysates from the skin of brownstripe red snapper possessing antioxidative activities [22]. Tuna frame protein and salmon by-products were hydrolyzed by using trypsin compared to other proteases like Alcalase, Neutrase, Flavourzyme, Protamex, pepsin, papain, and, α-chymotrypsin and providing antihypertensive effect [26] and [27].

The effect of different salts, organic solvents, pHs, and incubation temperatures on TPP has never been prevalently investigated. Previous reports indicate that significant selectivity in precipitation can be obtained by varying the concentration of ammonium sulfate and the aqueous phase-to-*t*-butanol ratio [14]. Therefore, the present work was involved in amelioration of alkaline proteases recovery from farmed giant catfish and different parameters required were optimized, and finally the enzyme was used for protein hydrolysis in comparison with commercial bovine trypsin against egg white, whey protein concentrates, and soy protein isolates.

2. Materials and Methods

2.1. Chemicals and raw materials

2-Methyl-2-propanol (*t*-butanol; $C_4H_{10}O$), 1-Butanol (*n*-butanol; $C_4H_{10}O$), 1-Propanol (*n*-Propanol; C_3H_8O), 2-Propanol (Isopropanol; C_3H_8O) were purchased from Panreac (Barcelona, Spain). Ammonium sulfate ($(NH_4)_2SO_4$), potassium phosphate (K_2HPO_4), sodium citrate ($C_6H_5Na_3O_7.2H_2O$) were purchased from Univar (Ajax Finechem, Australia). Trypsin from bovine pancreas (CAS NO. 9002-07-07, 3312.2 U/mg), trichloroacetic acid (TCA), hydrochloric acid, sodium hydroxide, tris-(hydroxymethyl)-aminomethane and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), and casein were obtained from Fluka (Buchs, Switzerland). Beta-mercaptoethanol (β-ME) and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Crude enzyme extract preparation

Viscera of farmed giant catfish were obtained from Charun Farm, Chiang Rai, Thailand. Pooled viscera were immediately frozen and stored at -20ºC until used. The frozen viscera were thawed using running tap water (26-28°C) until the core temperature reached (-2±2°C). The sample was cut into small pieces and homogenized for 2 min with extraction buffer (10 mM Tris-HCl pH 8.0, containing 10 mM $CaCl_2$) in the ratio of 1: 5 (w/v). The mixture was centrifuged at 10,000×*g* for 10 min at 4ºC. The pellet was discarded and the supernatant was collected and referred to as "crude enzyme extract" (CE). Protein content and protease activity in CE were measured.

2.3. Alkaline proteases partitioning

2.3.1 Effect of salts on proteases partitioning

In this study used the optimum condition and method according to Rawdkuen et al. [14] that using crude enzyme extract to *t*-butanol in ratio of 1.0: 0.5 in the presence salt of 50% (w/v). The effect of salts on alkaline proteases partitioning was carried out by using different salts; $(NH_4)_2SO_4$, K_2HPO_4, and $Na_3C_6H_5O_7$ at the concentration of 50% (w/v). The salts were added to CE in 50 mL centrifuge tubes. The solutions in the tubes were mixed vigorously to dissolve the salt for 5 min, followed by adding *t*-butanol in the ratio of 1: 0.5 (v/v) (ratio of crude extract to *t*-butanol). The mixture was vortexed for 1 min, and shaken at 90 rpm at ambient temperature (27-33ºC) for 60 min. Then, the mixtures were then centrifuged at 5,000×*g* for 10 min at 4ºC. Three-phase formed and were collected separately. The lower aqueous layer was collected by piercing and the organic phase (upper phase) was removed carefully with a pasteur pipette. All fractions was measured for volume and recorded. Afterwards, the interfacial precipitate containing alkaline proteases was dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ by the ratio 1: 1 (w/v) and dialyzed in distilled water for 12 h at 4ºC. A change in distilled water at 3 h intervals was required for efficient removal of salt and *t*-butanol bound to the protein aggregates. The dialyzed interfacial precipitate and lower aqueous phases were measured for volume, analyzed for alkaline proteases activity, and protein content. The best condition which resulted in maximum recovery was chosen for further study. All the experiments were performed in triplicates.

2.3.2 Effect of solvents on proteases partitioning

The effect of solvent types on alkaline proteases partitioning was studied by adding various organic solvents (*t*-butanol, *1*-butanol, *1*-propanol and *2*-propanol) in the ratio of 1.0: 0.5 (v/v) (ratio of crude enzyme extract to organic solvents) to the condition that provided the maximum proteases recovery from previous step. After that, collecting each phase and

investigating protein content and protease activity as mentioned above. The best condition which resulted in the highest recovery was chosen for further study.

2.3.3 Effect of pHs on proteases partitioning

The effect of pHs on alkaline protease partitioning was also investigated. After mixing crude enzyme extract and selected salt from previous step, the pH of the system was adjusted to each pH values (6, 7, 8, 9, and 10) by addition of 2M HCl or NaOH. Next, the selected organic solvent was added to the mixture with the ratios of 1: 0.5 (v/v). Partitioning for this step were done and determined as previously described. After that, collecting each phase and investigating protein content and protease activity as mentioned before. The optimum pH value providing the highest protease recovery was chosen for further study.

2.3.4 Effect of incubation temperatures on proteases partitioning

The best condition that provided the highest recovery from previous step was used to study the effect of incubation temperatures. The effect of temperatures on alkaline proteases partitioning was investigated by incubating the mixtures at in an incubator shaker at 4, 25, 37, and 45ºC at 90 rpm for 1 h. Then, the mixtures were then centrifuged at 5,000×*g* for 10 min at 4ºC. Partitioning for this step was done and determined as previous described. After that, collecting each phase and investigating protein content and protease activity as mentioned before. The optimum temperature providing the highest protease recovery was chosen for further investigation.

2.4. Characterization of isolated alkaline proteases

2.4.1 Proteolytic activity and protein content determinations

Proteolytic activity was determined by using caseinolytic activity assay according to the method of Rawdkuen et al. [13] with a slight modification. An enzyme sample of 500 µL was mixed with 500 µL of 1% (w/v) casein in 0.10 M Tris-HCl pH 8.0. The mixture was incubated at 37ºC for 10 min. After that, the reaction was stopped by adding 500 µL of 5% trichloroacetic acid. After centrifugation at 12,000×*g* for 5 min, the absorbance of the supernatant was measured at 280 nm. One caseinolytic unit is defined as the amount of enzymes needed to produce an increment of 0.01 absorbance unit per minute at the assayed condition.

Bradford method [28] was used for determination of protein concentration and bovine serum albumin (BSA) was used as a protein standard.

2.4.2 Protein pattern and substrate gel electrophoresis

The protein pattern of the extracted proteases was evaluated by using SDS-PAGE according to the method of Laemmli [29]. The protein solutions were mixed at a 1: 1 (v/v) ratio with the sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol). The samples (8 µg and 2 µg for protein and activity staining, respectively) were loaded onto a 4% stacking gel and a 15% separation gel. The samples were subjected to a constant current of 15 mA/gel. After electrophoresis, the gel was stained overnight with a solution of 0.1% (w/v) Coomassie Brilliant Blue R-250 in 45% (v/v) methanol and 10 % (v/v) acetic acid. The gels were then destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 15 min before being washed and dried.

The protein separated on SDS-PAGE was verified for proteolytic activity by using casein-substrate gel electrophoresis according to the method of Garcia-Carreno et al. [30]. The gel was immersed in 50 ml of 2% (w/v) casein in 100 mM of a Tris-HCl buffer at pH 8.0, followed by constant agitation at 4ºC for 45 min. The reaction was initiated by

incubating the gel at 37ºC for 10 min. The treated gel was then stained and destained as described above. The development of a clear band on the dark background indicated the caseinolytic activity of the enzymes. The gels were fixed and stained with Coomassie Blue R-250. Development of clear zones on blue background indicated proteolytic activity.

2.5. Application of proteases on proteins hydrolysis

Selected proteins (whey protein concentrates, soy protein isolates, and egg white) were solubilized in distilled water at 10 mg/ml and homogenized at 3000 rpm for 3 min (T18 ULTRA-TURRAX® IKA, Japan)**.** Suspensions of the proteins (10 mg/ml) were hydrolyzed under controlled hydrolysis conditions using extracted alkaline proteases, trypsin (from bovine pancrease, Merck, Germany). The reaction was started by incubating the mixture of proteins with selected enzymes at different concentrations at pH 8.0, at 37ºC for 10 min. The enzymatic reactions were stopped by heating the mixture at 90ºC for 3 min. The supernatant obtained was used for subsequent studies. Pattern of proteins generated was determined by SDS-PAGE using 15% (for soy protein isolates and egg white protein) and 20% (for whey protein concentrates) separating gel and 4% stacking gel as mentioned above.

2.6. Statistical analysis

The data was subjected to analysis of variance (ANOVA). A means comparison was carried out by Duncan's multiple range tests. The analysis was performed by using an SPSS package (SPSS 11.0 for window, SPSS Inc, Chicago, IL).

3. Results and Discussion

3.1. Alkaline proteases partitioning

3.1.1 Effect of salts on proteases partitioning

Apart from salt concentration, type of salts used in TPP should be concerned. In generally, ammonium sulfate was used in almost of TPP system [4], [9], [11], [14] and [15]. However, in this study tried to use different types such as sodium citrate, potassium phosphate for selecting the most suitable salt that provide the maximum recovery of alkaline proteases from farmed giant catfish viscera. Hence, effect of salts on partitioning of alkaline protease was investigated by using different salts in the concentration of 50% (w/v) and CE:*t*-butanol 1: 0.5 (%v/v). The system composition derived from the research of Rawdkuen et al. [14] that provided the best recovery (163%) of protease from the viscera of farmed giant catfish.

From other previous studies, no evidence was found that other salts were superior or even equal to ammonium sulfate. However, in this study, sodium citrate has been found to work best in most of the cases. Effect of different salts on the degree of purification and activity recovery of alkaline proteases from farmed giant catfish viscera was shown in Fig. 1A. The results showed that using sodium citrate provided the highest recovery (142%) with purification (4.65-fold) (Table 1) followed by ammonium sulfate (134%, 3-fold) and potassium phosphate (109%, 5.5-fold) (Fig. 1A). It indicated that ionic strength alone is not a major player in this technique [1]. Besides, dissolution of sodium citrate in CE gave the pH value of 7.83 that it may alter the charge of the solute or it may alter the conformation of enzyme leading to more precipitation out at interphase and increasing higher activity.

Dhananjay et al. [4] found that the optimum TPP condition for α-galactosidase was the use of 30% ammonium sulfate along with 1: 1 ratio of *t*-butanol to aqueous crude extract provided the recovery around 99%. From the previous works showed that ammonium sulfate were superior to other salts [4], [9], [11], [14] and [15]. This might be due to the Hofmeister series, which is a classification of ions based on their salting-out ability [31] and [32].

3.1.2 Effect of solvents on proteases partitioning

Of all the solvents tried *t*-butanol gave the best results with 4.2-fold purification and 142% activity recovery (Fig. 1B). Although *2*-butanol is less miscible in water than *t*-butanol, it gave 4.5-fold purification with 116% recovery. Meanwhile, comparing to *t*-butanol and *1*-butanol, *1*-propanol provided the same proteases purity but lower recovery (95%). Whereas, *2*-propanol gave higher purification (6-fold), the recovery activity was much lower (86%). It appears that both higher deactivations of the enzyme as well as lower interfacial precipitates are responsible for the low activity yield [4]. The organic solvents provided the best condition for α-galactosidase from fermented media of *Aspergillus oryzae* [4]. The results showed *t*-butanol gave the 12 purification fold and 92% activity recovery while *1*-butanol, *1*-propanol and *2*-propanol gave 69%, 40% and 35% recovery, respectively.

TPP so far has been dependent on *t*-butanol and ammonium sulfate, a C_4 alcohol which is a differentiating solvent and ammonium sulfate as effective kosmotropic agent. It has been reported that, it might not be necessary to use *t*-butanol always, other C3, C4 and cosolvents may also serve because they too act as a differentiating cosolvents [1]. *t*-Butanol binds to the precipitated proteins, thereby increasing their buoyancy and causing the precipitates to float above the denser aqueous salt layer [9]. Pigments, lipids or hydrophobic materials are concentrated in the upper phase while the polar components like remaining proteins, saccharides and cell debris are enriched in the lower phase [1] and [2]. *t*-butanol appears to be kosmotropic and crowding agent at room temperature, resulted in "alkaline proteases-*t*-butanol co-precipitate" which float above denser aqueous salt layer. 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 [1]. In this TPP based protocol for protein separation, *t*-butanol has been found to consistently perform better than

all other organic solvents. Besides, *t*-butanol can be practically used either at room temperature, or even at higher temperature.

Hence, *t*-Butanol was chosen as the organic cosolvent for partitioning of alkaline protease from farmed giant catfish viscera in TPP due to generally reported to give best results when used as ratio 1: 0.5 v/v (CE:*t*-butanol).

3.1.3 Effect of pHs on proteases partitioning

The pHs of the system is also a very important parameter in TPP. Enzymes show different behavior in TPP systems depending on the pH of the system. The efficiency of the salting out of proteins will also depend on the net charge of the proteins which is highly pH dependent [9]. Proteins tend to precipitate most readily at their isoelectric point (pI) [10]. Electrostatic forces and binding of salts anion (i.e. SO_4^{2-}, HPO_4^{2-}, and $C_3H_5O(COO)_3^{3-}$) to cationic protein molecules, which promote macromolecular contraction and conformational shrinkage, are the main causes of the strong salt anion-pH dependency in salting out [11]. Proteins tend to precipitate most readily at their pI (isoelectric point). Below the pI, proteins are positively charged and quantitatively precipitated out by TPP. On the other hand, negatively charged proteins are more soluble and not easily precipitated [1]. Hence, the effect of pH on the partitioning of alkaline proteases was investigated. In the case of crude extracts containing proteins with divergent isoelectric points, five different pHs, ranging between 6.0 and 10.0 was studied.

Alkaline proteases had partitioned selectively to the interphase of 50% (w/v) sodium citrate with 1: 0.5 ratio of crude extract to *t*-butanol (w/v). At pH 8.0 gave a maximum 155% activity recovery of alkaline proteases (Fig. 2A). However, the maximum purity of 17 and 23-fold of proteases was obtained from system pH 9 and 10, respectively. This reason might because of the electrostatic component of the reactions when citrate anion ($C_3H_5O(COO)_3^{3-}$)

binding to cationic proteins leading to the partition of most contaminant proteins into aqueous bottom phase above isoelectric point and showed as the total protein were very low at pH 9 and 10. The increased recovery at pH 8 could be due to the better conformational stability of alkaline proteases towards *t*-butanol at this pH. Below the pI, the protein is positively charged because of H^+ titration of side chains and is quantitatively precipitated out by TPP. As is seen from Fig. 2A, alkaline proteases precipitate selectively at pH 8, leaving most of contaminant proteins in the aqueous bottom phase as shown that relatively low total protein and high purity. The obtained results indicated that alkaline proteases precipitated into interfacial phase most readily at its pI and was soluble above its pI value [1].

TPP systems usually sharply change around the isoionic point of each protein because of the electrostatic component of the reactions when sulfate anion binding to cationic proteins is involved [8]. Çalci et al. [8] reported that at pH 4.5 and 50% of ammonium sulfate saturation with 1: 1 ratio of crude extract to *t*-butanol gave 4.3-fold purification with 80% activity yield of α-galactosidase from tomato and increased from unmodified pH (6.0) nearly two times. Whereas at pH 5.25 gave a maximum 6.2-fold and 127% activity recovery of α-galactosidase from pepino (*Solanum muricatum*) fruit that more than those obtained from unmodified pH of 6.0 for three times [10]. It can be seen that the optimized conditions of pH 4.0, the invertase from Baker's yeast (*Saccharomyces cerevisiae*) was purified to 15-fold with 363% activity recovery whilst unmodified pH 5.0 provided 8.5-fold and 350% [9]. Invertase from tomato precipitate selectively at pH 4.5 providing 8.6 purification fold with 190% activity recovery. The purification fold and recovery values for invertase were 0.5-fold and 3.54% at pH 3.0 and 1.9-fold and 33% at pH 5.5, respectively [11]. It has been observed that, precipitation of invertase by TPP below pH 4.0 and above pH 5.0 the invertase became part of interfacial precipitate with other contaminating proteins. Therefore, the selective pH should be carefully investigated to enhance the purification fold and recovery of target

enzymes. Proteins are damaged by moderate or severe pH conditions, e.g., acidic pHs or below, exposure before addition of sulfate or *t*-butanol may denature them. They are likely to partition out in a denaturated state but may lose activity [33].

3.1.4 Effect of incubation temperatures on proteases partitioning

The effects of varying the process parameters for alkaline proteases were analyzed to determine the best conditions with the maximum recovery. Apart from pH, temperature for this operation is important since the use of low temperatures in solvent or salt precipitation dissipates the heat generated, ensuring minimal protein denaturation [34]. Hence, the incubation temperature at 4, 25, 37, and 45ºC were investigated instead of ambient temperature and the effect was shown in Fig. 2B. The temperature of 25°C was found to be the best for achieving greatest recovery (220%). Lowering the temperature from ambient temperature (27-33ºC) to 25ºC led to increase in the activity recovery by 64% whilst the purification was not significantly different ($P>0.05$). The increased flexibility of the molecule probably is mostly responsible for higher activity observed with TPP treated alkaline proteases [5]. This shows that decrease in temperature affects partitioning of all contaminant proteins into aqueous bottom phase. In this case at 25ºC seems to be a better option for carrying out TPP. At 4ºC provided the recovery as same as ambient temperature. This might be due to lower temperature giving the low movement of particle solvent, but at 25ºC increased the conformation of enzyme into active form. Enzyme activity recovery was decreased at higher temperature (147%, 37ºC; 131%, 45 ºC) in comparison to ambient temperature (154%, 31ºC) but increased significantly in the purification fold (25 and 18-fold) of the enzyme in the precipitate. This may be due to the combined effect of sodium citrate and *t*-butanol along with temperature coming into play, which decreases the selectivity of extraction, thus reducing activity yield [35].

Thus, TPP, at least in some cases results in enhancing the activity of the enzymes. Hence any purification fold obtained using TPP may result from two factors (a) actual purification i.e. removal of contaminating proteins (b) activation of the enzyme molecules [5] and [7]. Although the requirement of low temperatures in TPP has not been clearly reported, it has been suggested in new systems [1]. In TPP of phospholipase D from *Dacus carota*, lowering the temperature from 25 to 4ºC led to a decrease in the activity yield by 13% but no change in the specific activity of the enzyme in the precipitate [7]. Dogan et al. [36] also reported improved yields and purification of exo-polygalacturonase using TPP at 25ºC rather than 37ºC. At 25ºC gave a 25.5% recovery of with a 6.7-fold purification, only 8.8% recovery and 0.7-fold purification was obtained at 37ºC [36]. However, Rajeeva et al. [34] showed that there was no significance in recovery and purification fold of laccase by TPP at 5ºC compare to 35ºC. At 5ºC gave a 55% yield of with a 4.2-fold purification, whereas a 53% recovery and 4.1-fold purification was obtained at 35ºC [34]. Moreover, Kumar et al. [15] reported that the purification fold and recovery values for inulinase were 6.8-fold and 91% at 25ºC and decrease to 5.3-fold and 81% at 20ºC and to 3.1-fold and 65% at 37ºC, respectively.

The overall partitioning parameters of alkaline proteases from giant catfish viscera were summarized in Table 1. Form the study of effect of salts, we found that the best recovery of 142% was obtained from using 30% (w/v) sodium sulfate. Besides, organic solvent like *t*-butanol showed the highest recovery (142%) over others when used as 1.0: 0.5 (v/v) ratio of crude enzyme extract to *t*-butanol. At pH 8.0 provided the maximum recovery of 154%. Furthermore, the recovery of alkaline proteases was very high (220%) at the incubation temperature of 25ºC. By using this TPP technique and studied the factors, the results showed that the TPP system comprised of 50% (w/v) sodium citrate with 1.0: 0.5 (v/v) ratio of crude enzyme extract to *t*-butanol at pH 8.0 and incubation temperature of 25ºC

provided the highest recovery (219%). Hence, this system was used to produce the alkaline proteases for application in protein hydrolysis.

3.2. Characterization of isolated alkaline proteases

3.2.1 Protein patterns of TPP fractions

Protein patterns of the crude enzyme extract and their fractions from the TPP are shown in Fig. 3. Since the protein content in all fraction very low, protein patterns might not be appeared clearly. However, there were smear of protein bands. These results show slightly different protein patterns between the crude enzyme extract and its fractions (at both the interphase and the bottom phase). Four smear protein bands with molecular weights of 215, 130, 94, and < 18.3 kDa were found in the CE. When the crude extract was partitioned with the TPP, some of the major protein bands disappeared; moreover, a number of protein bands with different MWs compared to the crude extract were present on the gel. The protein band with a MW of 39.2, 28, and 24 kDa was present in the interphase of the system tested (I1-I4). The protein band of 24 kDa found in all fractions from the interphase agreed well with the reported molecular mass of protease from fish viscera that was approximately varied 24-28 kDa according to their source and the applied method [14], [23] and [37]. The protein patterns of the fractions obtained from the bottom phase (B1-B4) were significantly different when compared to those of the crude enzyme extract and the interphase. This result suggests that TPP could move other contaminant proteins to the bottom phase.

3.2.2 Activity staining of TPP fractions

Separated proteins by using gel electrophoresis were further subjected to determine enzymes activity on substrates gel electrophoresis by using casein. The results activity staining of TPP fractions and CE are also in Fig. 3. A clear zone on the dark background was

identified as the activity of proteases against its substrate (casein) on the polyacrylamide gel. Clear zone at the MW of 120, 39.2, 24, and below 18.2 kDa appeared on the polyacrylamide gel. These proteins provided the hydrolytic activity on the casein after gel electrophoresis. Interestingly, there was no clear zone appeared in all fractions from aqueous bottom phase refers to the efficiency of TPP can remove almost contaminant proteins to the bottom phase and push the target enzyme precipitated to the inter-phase. The apparent MWs of trypsin-like enzyme were estimated to be 48, 23 and 23 kDa for skipjack, tongol and yellowfin tuna spleen, respectively [18]. MWs of trypsin-like enzymes from pyloric caeca brownstripe red snapper were 20, 24-29, 45 and 97 kDa; bigeye snapper were 17, 20, 22, 45 and 97 kDa; and threadfin bream were 20, 22, 36 and 45 kDa [21]. The alkaline protease that recovered from giant catfish viscera were approximately as 25 kDa [14].

3.3. Application of alkaline proteases on proteins hydrolysis

The peptide mapping of proteins (soy protein isolates, egg white protein, and whey protein concentrates) treated with the extracted alkaline proteases were compared to those obtained with commercial bovine pancrease trypsin (initial activity 250 units) (Fig. 4). The major components in the protein hydrolysate from soy protein isolates had a MW around 215,120, and 80 kDa, while those main components in egg white protein were around 75 and 65 kDa and those in whey protein concentrates were approximately 17.5 and 15 kDa. Hydrolytic patterns of the key components of all proteins were clearly observed when increasing the units of the extracted alkaline proteases in the system. Compared to commercial trypsin, the extract alkaline proteases showed higher efficacy in hydrolysis of all protein isolate, except egg white protein. The soy protein isolates were more resistant to commercial trypsin than extracted alkaline protease (Fig. 4). As seen from the figure the major protein bands of soy protein isolates were absolutely disappeared at the level of 80 unit of extracted alkaline protease whereas no change in protein pattern when the levels of

commercial trypsin were increased. It could be due to trypsin inhibitors that presence in soy proteins strongly inhibits trypsin activity [38]. Disappeared of major components in the egg white protein was observed when the addition levels were 125 and 80 units for extract alkaline proteases and commercial trypsin, respectively. Besides, only 10 units of extracted alkaline proteases hydrolyzed all major protein components in whey protein concentrates when compared to 20 unit of commercial trypsin. It showed that alkaline proteases from farmed giant catfish viscera produced higher protein degradation than that observed with commercial trypsin indicating a higher specific activity of the alkaline proteases towards all protein tested.

From hydrolytic patterns, the alkaline proteases extracted from farmed giant catfish viscera can further be used for other food proteins to produce protein hydrolysate with bio-fuctional properties such as antioxidant and ACE inhibitory peptides. Alkaline proteases such as trypsin, chymotrysin, Alcalase or pancreatin have been used to hydrolyze proteins from Ostrich, egg white possess hydrolysates with antioxidant activities [39], whey protein concentrates generated ACE inhibitory action [40] and dipeptidyl peptidase-IV inhibitory peptides [41], and isolated soy protein produce the hydrolysates with ACE inhibitory activity [42] and [43].

4. Conclusion

The single step of three-phase partitioning, by using sodium citrate (50%, w/v) with 1.0: 0.5 (v/v) (crude enzyme extract to *t*-butanol), pH 8.0 at incubation temperature of 25ºC gave the maximum recovery of 220% with 6-fold purification. TPP is rapid, economical technique and scalability is convenient. The process described here has the potential to carry out as purification protocol for fish viscera enzymes or others. The obtained enzyme showed the high effectiveness in protein hydrolysate production when compared to commercial

trypsin. Finally, the enzymes could be applied in food, detergent, neutraceutical, and pharmacological industries.

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References

[1] C. Dennison, R. Lovrien, Three phase partitioning: poncentration and purification of proteins, Pro. Exp. Pur. 11 (1997) 149-161.

[2] I. Roy, M.N. Gupta, Three-Phase Affinity Partitioning of Proteins, Anal. Biochem. 300 (2002) 11-14.

[3] I. Roy, A. Sharma, M.N. Gupta, Recovery of biological activity in reversibly inactivated proteins by three phase partitioning, Enz. Mic. Tech. 37 (2005) 113-120.

[4] S.K. Dhananjay, V.H. Mulimani, Three-phase partitioning of α-galactosidase from fermented media of *Aspergillus oryzae* and comparison with conventional purification techniques, J.Ind. Micro.Biotech. 36 (2009) 123-128.

[5] A. Sharma, M.N. Gupta, Three phase partitioning as a large-scale separation method for purification of a wheat germ bifunctional protease/amylase inhibitor, Pro. Biochem. 37 (2001) 193-196.

[6] L. Saxena, B.K. Iyer, L. Ananthanarayan, Three phase partitioning as a novel method for purification of ragi (*Eleusine coracana*) bifunctional amylase/protease inhibitor, Pro. Biochem. 42 (2007) 491-495.

[7] S. Sharma, M.N. Gupta, Purification of phospholipase D from *Dacus carota* by three-phase partitioning and its characterization, Pro. Exp. Pur. 21, (2001) 310-316.

[8] E. Çalci, T. Demir, E. Biçak Çelem, S. Önal, Purification of tomato α-galactosidase by three-phase partitioning and its characterization, Sep. Pur. Tech. 70 (2009) 123-127.

[9] A. Şen, M. Eryılmaz, H. Bayraktar, S. Önal, Purification of α-galactosidase from pepino (*Solanum muricatum*) by three-phase partitioning, Sep. Pur. Tech. 83 (2011) 130-136.

[10] E. Akardere, B. Özer, E.B. Çelem, S. Önal, Three-phase partitioning of invertase from Baker's yeast, Sep. Pur. Tech. 72 (2010) 335-339.

[11] B. Özer, E. Akardere, E.B. Çelem, S. Önal, Three-phase partitioning as a rapid and efficient method for purification of invertase from tomato, Biochem. Eng. J. 50 (2010) 110-115.

[12] P. Chaiwut, P. Pintathong, S. Rawdkuen, Extraction and three-phase partitioning behavior of proteases from papaya peels, Pro. Biochem. 45 (2010) 1172-1175.

[13] S. Rawdkuen, P. Chaiwut, P. Pintathong, S. Benjakul, Three-phase partitioning of protease from *Calotropis procera* latex, Biochem. Eng. J. 50 (2010) 145-149.

[14] S. Rawdkuen, A. Vanabun, S. Benjakul, Recovery of proteases from the viscera of farmed giant catfish (*Pangasianodon gigas*) by three-phase partitioning, Pro. Biochem. 47 (2012) 2566-2569.

[15] V. Vinoth Kumar, M.P. Premkumar, V.K. Sathyaselvabala, S. Dineshkirupha, J. Nandagopal, S. Sivanesan, *Aspergillus niger* exo-inulinase purification by three phase partitioning, Eng. Life Sci. 11 (2011) 607-614.

[16] R.K. Wati, T. Theppakorn, S. Benjakul, S. Rawdkuen, Three-phase partitioning of trypsin inhibitor from legume seeds, Pro. Biochem. 44 (2009) 1307-1314.

[17] S. Klomklao, S. Benjakul, B.K. Simpson, Seafood Enzymes: Biochemical properties and their impact on quality. In Simpson, B. K. (Eds.), Food Biochemistry and Food Processing. Wiley-Blackwell, USA, 2012, pp. 271-273.

[18] S. Klomklao, S. Benjakul, W. Visessanguan, B.K. Simpson, H. Kishimura, Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems, Pro. Biochem. 40 (2005) 3061-3067.

[19] S. Klomklao, H. Kishimura, Y. Nonami, S. Benjakul, Biochemical properties of two isoforms of trypsin purified from the intestine of skipjack tuna (*Katsuwonus pelamis*), Food Chem. 115 (2009) 155-162.

[20] S. Klomklao, S. Benjakul, W. Visessanguan, H. Kishimura, B. K. Simpson, Effects of the addition of spleen of skipjack tuna (*Katsuwonus pelamis*) on the liquefaction and characteristics of fish sauce made from sardine (*Sardinella gibbosa*), Food Chem. 98 (2006), 440-452.

[21] S. Khantaphant, S. Benjakul, Comparative study on the proteases from fish pyloric caeca and the use for production of gelatin hydrolysate with antioxidative activity, Comp. Biochem. Phys. Part B: Biochem. Mol. Bio. 151 (2008) 410-419.

[22] S. Khantaphant, S. Benjakul, H. Kishimura, Antioxidative and ACE inhibitory activities of protein hydrolysates from the muscle of brownstripe red snapper prepared using pyloric caeca and commercial proteases, Pro. Biochem. 46 (2011) 318-327.

[23] S. Klomklao, H. Kishimura, S. Benjakul, Use of viscera extract from hybrid catfish (*Clarias macrocephalus*×*Clarias gariepinus*) for the production of protein hydrolysate from toothed ponyfish (*Gazza minuta*) muscle, Food Chem. 136 (2013), 1006-1012.

[24] S. Ketnawa, S. Benjakul, T.C. Ling, O. Martínez-Alvarez, S. Rawdkuen, Enhanced recovery of alkaline protease from fish viscera by phase partitioning and its application, Chem. Cent. J. 7 (2013) 79-88.

[25] A. Bougatef, N. Nedjar-Arroume, R. Ravallec-Plé, Y. Leroy, D. Guillochon, A. Barkia, M. Nasri, Angiotensin I-converting enzyme (ACE) inhibitory activities of sardinelle (*Sardinella aurita*) by-products protein hydrolysates obtained by treatment with microbial and visceral fish serine proteases, Food Chem. 111 (2008) 350-356.

[26] S.H. Lee, Z.J. Qian, S.K. Kim, A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats, Food Chem. 118 (2010) 96-102.

[27] C.B. Ahn, Y.J. Jeon, Y.T. Kim, J.Y. Je, Angiotensin I converting enzyme (ACE) inhibitory peptides from salmon byproduct protein hydrolysate by Alcalase hydrolysis, Pro. Biochem. 47 (2012) 2240-2245.

[28] M.M. Bradford, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.

[29] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970), 680-685.

[30] F.L. Garcia-carreno, L.E. Dimes, N.F. Haard, Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors, Anal. Biochem. 214 (1993) 65-69.

[31] J. Huddleston, A. Veide, K. Kohlez, J. Flanagan, S.O. Enfors, A. Lyddiatt, The molecular basis of partitioning in ATPS: Review, Trend Biotechnol. 9 (1991) 381-388.

[32] S. Roe, Protein purification techniques: A Practical Approach. Second ed., UK: Oxford University Press. (2000) 11-15.

[33] R.N. Pike, C. Dennison, Protein fractionation by three-phase partitioning in aqueous/t-butanol mixtures, Biotech. Bioeng. 33 (1989) 221-228.

[34] S. Rajeeva, S.S. Lele, Three-phase partitioning for concentration and purification of laccase produced by submerged cultures of *Ganoderma sp.* WR-1. Biochem. Eng. J. 54 (2011) 103-110.

[35] A.V. Narayan, M.C. Madhusudhan, K.S.M.S. Raghavarao, Extraction and Purification of *Ipomoea Peroxidase* Employing Three-phase Partitioning, App. Biochem. Biotech. 151 (2008), 263-272

[36] N. Dogan, C. Tari, Characterization of three-phase partitioned exo-polygalacturonase from *Aspergillus sojae* with unique properties, Biochem. Eng. J. 39 (2008) 43-50.

[37] A.C.V. Freitas-Júnior, H.M.S. Costa, M.Y. Icimoto, I.Y. Hirata, M. Marcondes, L.B. Carvalho Jr, V. Oliveira, R. S. Bezerra, Giant Amazonian fish pirarucu (*Arapaima gigas*): Its viscera as a source of thermostable trypsin, Food Chem. 133 (2012) 1596-1602

[38] E. Pisulewska, P.M. Pisulewski, Trypsin inhibitor activity of legume seeds (peas, chickling vetch, lentils, and soya beans) as affected by the technique of harvest, Ani. Feed Sci. Tech. 86 (2000), 261-265

[39] H. Tanzadehpanah, A. Asoodeh, J. Chamani, An antioxidant peptide derived from ostrich (*Struthio camelus*) egg white protein hydrolysates, Food Res. Inter. 49 (2012) 105-111.

[40] I.M.P.L.V.O. Ferreira, O. Pinho, M.V. Mota, P. Tavares, A. Pereira, M.P. Gonçalves, D. Torres, C.Rocha, J.A. Teixeira, Preparation of ingredients containing an ACE-inhibitory peptide by tryptic hydrolysis of whey protein concentrates, Int. Dairy J. 17 (2007), 481-487

[41] S.T. Silveira, D. Martínez-Maqueda, I. Recio, I.B. Hernández-Ledesma, Dipeptidyl peptidase-IV inhibitory peptides generated by tryptic hydrolysis of a whey protein concentrate rich in β-lactoglobulin, Food Chem. 141 (2013) 1072-1077.

[42] J. Wu, X. Ding, Characterization of inhibition and stability of soy-protein-derived angiotensin I-converting enzyme inhibitory peptides, Food Res. Int. 35 (2002) 367-375.

[43] W.D. Chiang, M.J. Tsou, Z.Y. Tsai, T.C. Tsai, Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor, Food Chem. 98 (2006) 725-732.

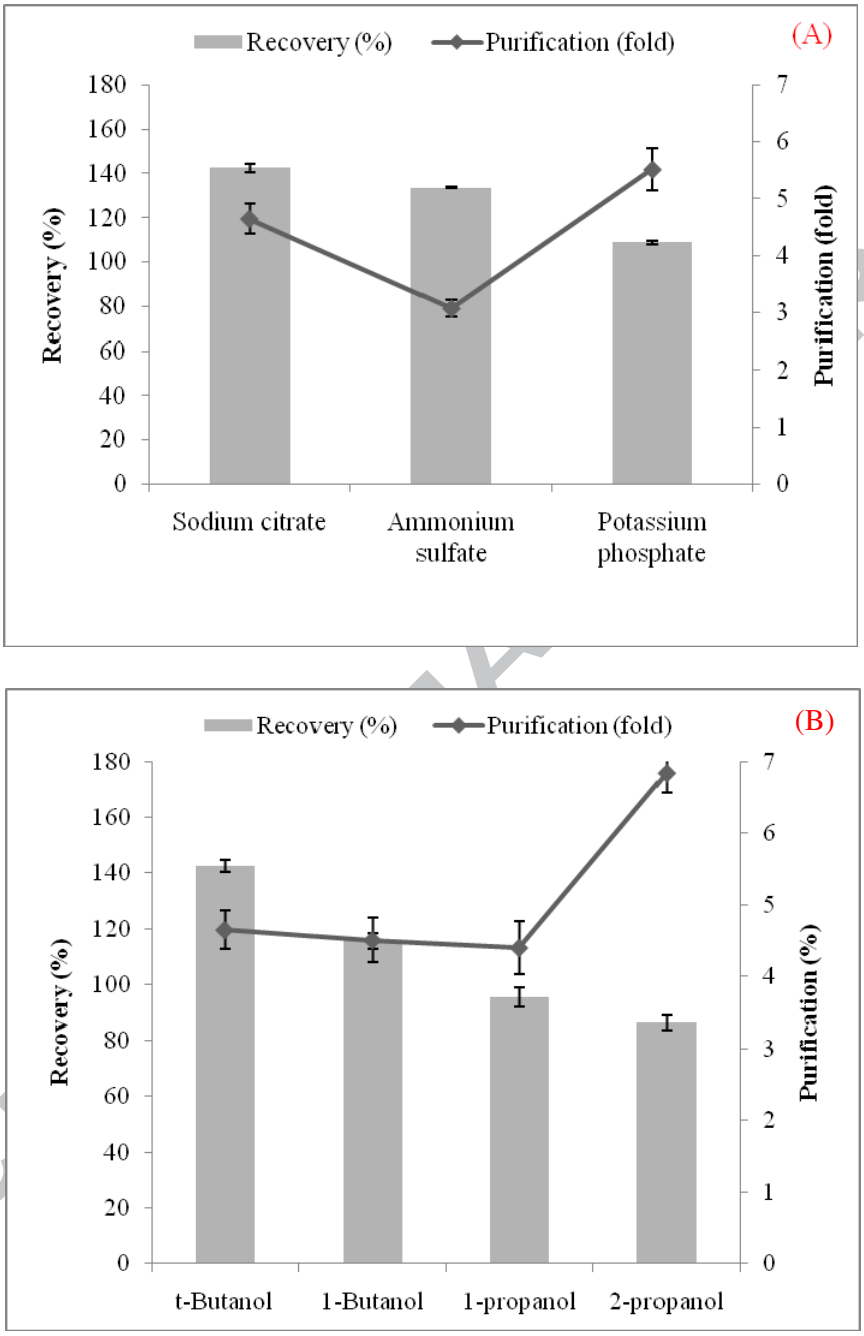
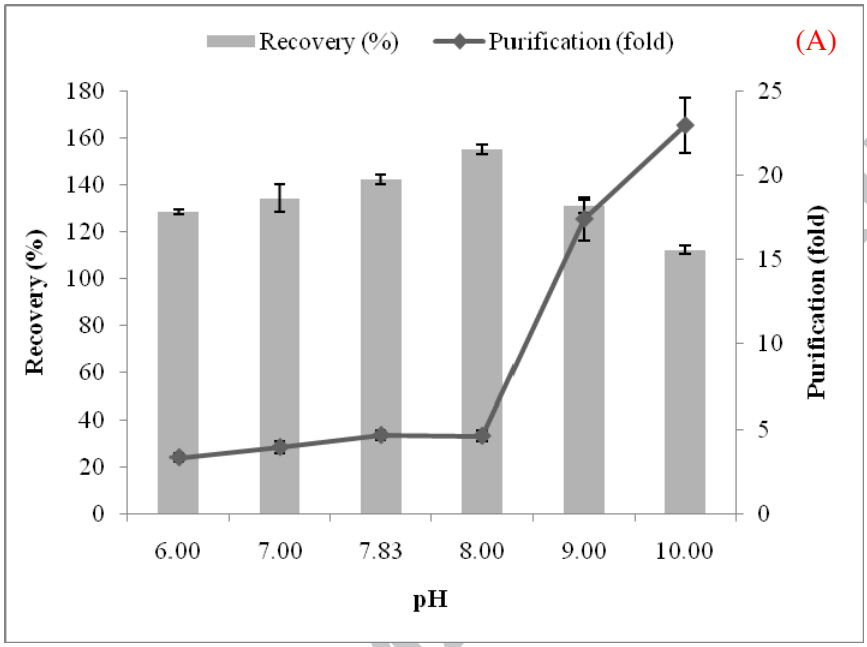


Figure 1. Effect of different salts (A) and organic solvents (B) on the degree of purification and activity recovery of alkaline proteases from farmed giant catfish viscera [50 (%, w/v) each salt was added to the crude enzyme extract followed by addition of organic solvents in a ratio of 1: 0.5 (w/v) (crude enzyme extract to *t*-butanol)] [50 (%, w/v) sodium citrate was added to the crude enzyme extract followed by addition of organic solvents in a ratio of 1: 0.5 (w/v) (crude enzyme extract to solvent)].



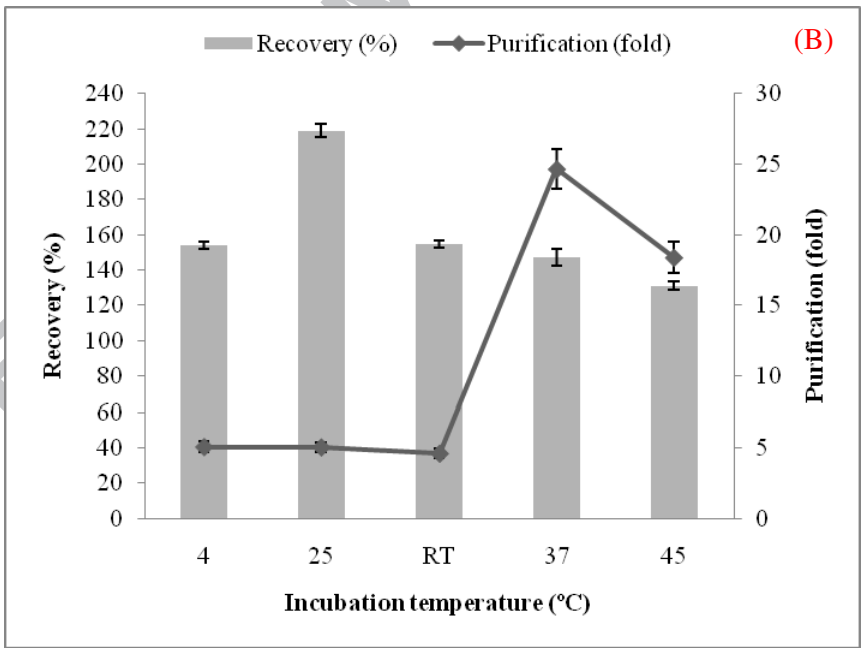


Figure 2. Effect of pHs (A) and incubation temperatures (B) on the degree of purification and activity recovery of alkaline proteases from farmed giant catfish viscera [50 (%, w/v) sodium citrate was added to the crude enzyme extract then pH of the mixture was adjusted to different pH values (6-10) and incubated at different temperatures, RT stands for ambient temperature (27-33ºC)].

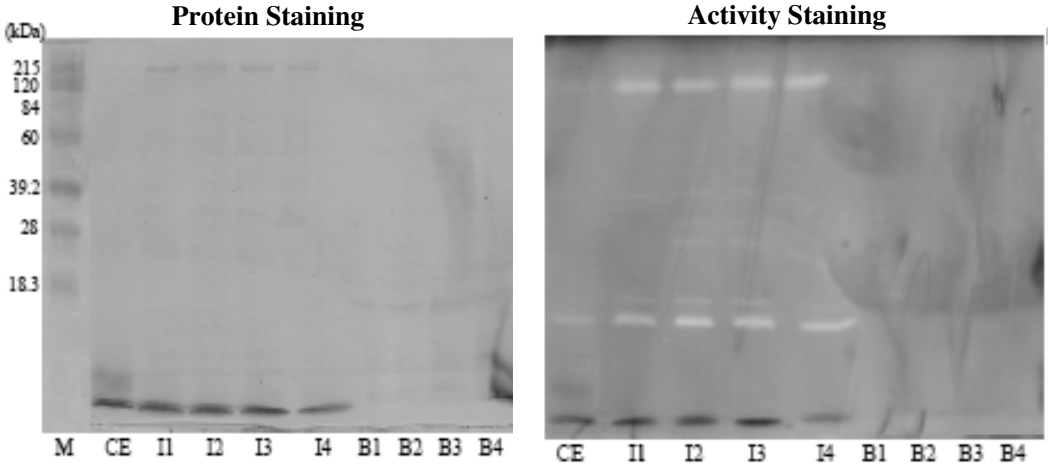


Figure 3. Protein patterns and activity staining performed using 4% stacking gel and 15% separating gel of alkaline protease from farmed giant catfish viscera [50 (%, w/v)sodium citrate and 1: 0.5 (w/v) (crude enzyme extract to *t*-butanol); 8 and 2 μg protein were loaded into the gel for protein patterns and activity staining, respectively.] M: molecular weight protein makers; CE: crude enzyme extract; I: interphase; B: bottom phase; 1-4: fractions that provides the best recovery from each factor studied.

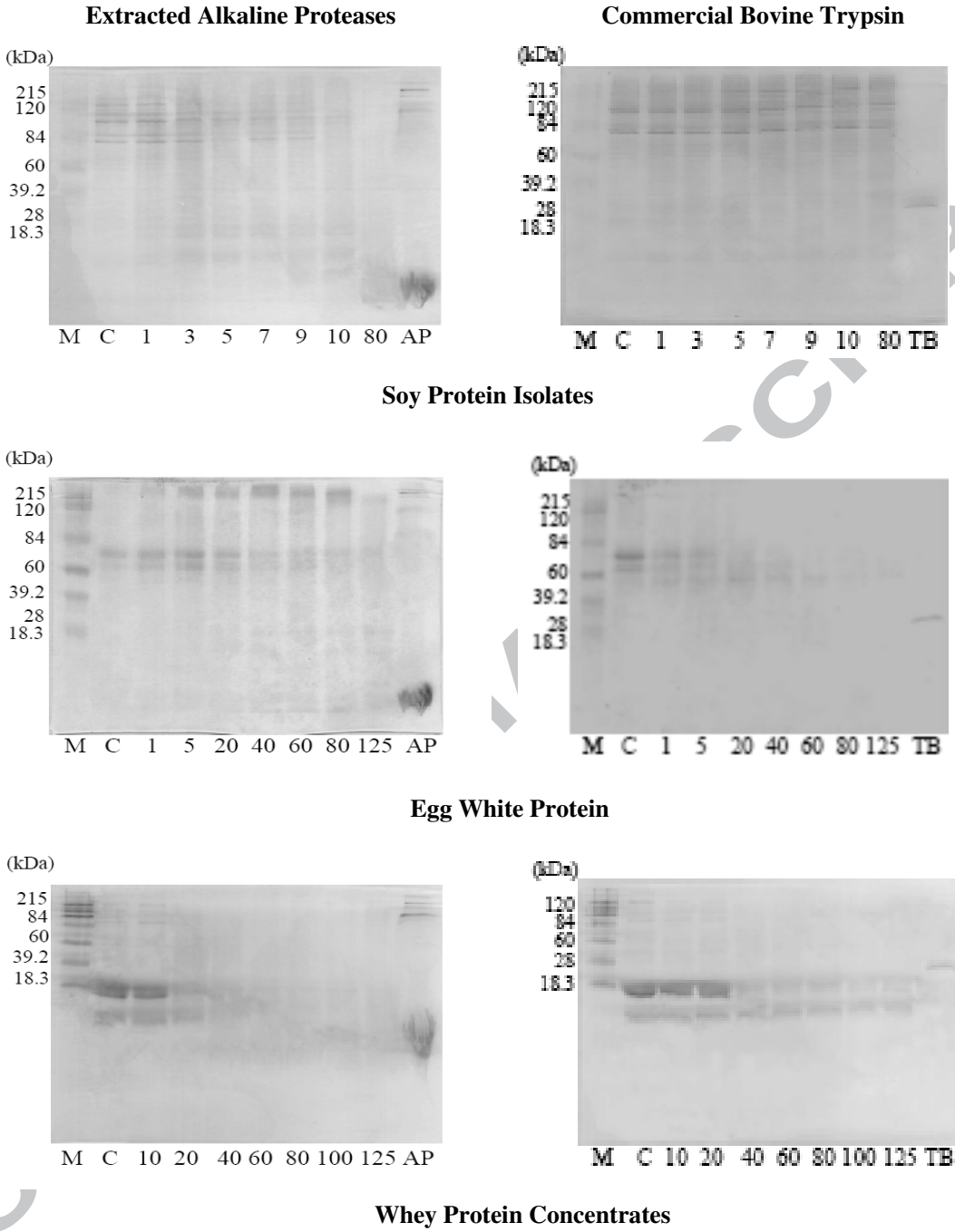


Figure 4. Peptide mapping of soy protein isolates, egg white protein and whey protein concentrates treated with the extracted alkaline proteases from the interphase of TPP 50% sodium citrate adjust pH to 8.0 followed by *t*-butanol in the ratio of 1.0: 0.5 (v/v) crude enzyme to *t*-butanol at incubation temperature of 25ºC (AP) and commercial trypsin from bovine pancrease (TB) [4% stacking gel and 15% separating gel SDS-PAGE for soy protein isolates and egg white protein and 20% separating gel SDS-PAGE for whey protein

concentrates]; M: molecular weight protein makers; C: control; numbers refer to the unit of enzyme used (initial enzyme 250 units).

Table 1
Three-phase partitioning of alkaline proteases from the viscera of farmed giant catfish.

Factors	Total activity (U)	Total protein (mg)	SA (U/mg)	PF (fold)	Recovery (%)
Crude enzyme extract	2753.00±2.61	88.68±5.77	31.14±2.30	1.00	100
Salt: Sodium citrate[a]	3921.64±2.13[a]	28.18±1.07[a]	144.45±6.02[b]	4.65±0.27[a]	142.46±2.04[c]
Solvent: *t*-Butanol	3921.64±2.13[a]	28.18±1.07[a]	144.45±6.02[b]	4.65±0.27[a]	142.46±2.04[c]
pH: 8	3788.43±1.60[b]	26.53±1.29[a]	142.82±0.95[b]	4.60±0.33[a]	154.94±1.98[b]
Temperature: 25 ºC	3575.58±2.61[c]	22.86±0.76[b]	156.42±0.64[a]	5.04±0.35[a]	219.04±4.03[a]

[a] The sodium citrate (50%, w/v) was added to the CE and then pH was adjusted to pH 8.0. This was followed by addition of *t*-butanol in a ratio of 1: 0.5 (v/v) (crude extract: *t*-butanol). Each experiment was carried out in triplicate. Means with different superscripts within the same column are statistically different (P<0.05).

Highlights

► Three-phase partitioning gave the max. recovery of 220% with 6-fold purification.

►The best condition is 50% Na-citrate, 1:0.5 crude extract: *t*-butanol, pH 8.0 at 25ºC.

► Effectiveness in protein hydrolysis of the enzyme is better than commercial trypsin.

► Three-phase partitioning is rapid, economical, and scalability technique.