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Aqueous two-phase partitioning of liver proteinase from albacore tuna (*Thunnus alalunga*): Application to starry triggerfish (*Abalistes stellaris*) muscle hydrolysis

Pakteera Sripokar[a], Manat Chaijan[b], Soottawat Benjakul[c], Asami Yoshida[d], and Sappasith Klomklao[e]

[a]Biotechnology Program, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung, Thailand; [b]Department of Food Technology, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat, Thailand; [c]Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, Thailand; [d]Graduate School of Fisheries Science and Environmental Studies, Nagasaki University, Nagasaki, Japan; [e]Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus, Phatthalung, Thailand

ABSTRACT

The potential of aqueous two-phase system (ATPS) for the purification and recovery of proteinase from albacore tuna (*Thunnus alalunga*) liver was explored. Influence of phase compositions such as type of phase forming salts, PEG molecular weight, concentration of salt and PEG, pH of the system, and NaCl addition on partitioning of proteinase was investigated. ATPS comprising PEG1000 (25%, w/w) and NaH_2PO_4 (20%, w/w) at pH 7.0 provided the best condition for the maximum partitioning of proteinase into the top phase and gave the highest purification factor (5.58-fold) and specific activity (20.65 unit/mg protein). The yield of 89.99% was obtained. The addition of NaCl up to a final concentration of 6% (w/w) decreased the degree of purification and enzyme recovery of proteinase. Based on electrophoresis and activity staining, the fractionated proteinases had the MW 21, 24, 30, and 34 kDa. The effect of fractionated proteinases on starry triggerfish (*Abalistes stellaris*) muscle hydrolysis was also studied. Fractionated proteinases were able to hydrolyze triggerfish muscle in a dose-dependent manner. Overall, results demonstrated the feasibility of ATPS for the recovery and purification of proteinase without the need for multiple steps, and the obtained proteinase can be further in preparation of protein hydrolysate.

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Aqueous two-phase system; Proteinase; Partitioning; Viscera; Hydrolysis

Introduction

Albacore tuna (*Thunnus alalunga*) is an important raw material used for the production of canned tuna in Thailand.[1] Large volumes of raw tuna go through the canning process, by which about two-thirds of whole fish is utilized.[2] During canned tuna manufacturing, high amounts of viscera are generated.

Fish viscera is known to be a rich source of proteinases that have high activity over a wide range of pH and temperature conditions[3] and exhibit high catalytic activity at relatively low concentration.[3] Proteinases have been widely used in food, medical-phamaceutical, cosmetic, and other industries. Fish proteinases have been used for preparation of protein isolate[4] extraction of collagen and gelatin.[5] Nowadays, the efficient and economical downstream processes for the

CONTACT Sappasith Klomklao sappasith@tsu.ac.th Department of Food Science and Technology, Faculty of Technology and Community Department, Thaksin University, Phatthalung Campus, Phatthalung, 93210, Thailand.

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partitioning and purification of biomolecules that give high yield and high purity of the product have been demanded by industries.

The powerful and versatile aqueous two-phase system (ATPS) has been employed as an efficient tool in several biotechnology processes for the partitioning of biomolecules like proteins, enzymes, nucleic acids, animal, plant, and microbial cells.[6] ATPS forms readily upon mixing aqueous solution of two hydrophobic polymers, or of a polymer and salt, above a certain threshold concentration.[6] Proteins are partitioned between the two phases with a partition coefficient that can be modified by changing the experimental conditions of the medium such as pH, salts, and ionic strength, among others.[7] The basis of separation is the selective distribution of a given biomolecule between the phases, depending on the characteristics of the phase system, properties of the biomolecule, and the interaction between them.[6] ATPS has several advantages such as low processing time and energy consumption, high capacity and yield, biocompatibility, easy to scale up, and non-toxicity.[6] ATPS has been successfully used for partitioning and recovery of various proteinases such as trypsin,[8] tuna spleen proteinase,[2] tuna stomach protease,[9] bromelain,[10] and protease from *Caltropis procera* latex.[11] Therefore, the objective of this study was to determine the optimal conditions for partitioning and separating proteinase from albacore tuna liver by ATPS. The other aims of this were to apply the fractionated enzyme for starry triggerfish muscle protein hydrolysis.

Materials and methods

Chemicals

Polyethylene glycol (PEG) 1000 and 4000 were obtained from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Sodium caseinate, β-mercaptoethanol (βME), L-tyrosine, wide range molecular weight markers, and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). Trichloroacetic acid, tris (hydroxymethyl) aminomethane, and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The salts and other chemicals with the analytical grade were procured from Merck (Darmstadt, Germany).

Preparation of crude proteinase extract

Internal organs from albacore tuna (*Thunnus alalunga*) were obtained from Tropical Canning (Thailand) Public Co. Ltd., Hat Yai, Songkhla. Pooled internal organs were then excised and separated into individual organs. Only the liver was collected, immediately frozen and stored at -20°C until used. Frozen livers were thawed using running water (26–28°C) until the core temperature reached -2 to 0°C. The samples were cut into pieces with a thickness of 1–1.5 cm and homogenized into powder in three volumes of acetone at -20°C for 30 min according to the method of Klomklao et al.[12]

To prepare the liver extract, the liver powder was suspended in 50 mM Na-phosphate buffer, pH 7.0 containing 0.2% Brij 35 at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The suspension was centrifuged for 30 min at 4°C at 5,000×g to remove the tissue debris. The supernatant was collected and referred to as "liver extract."

Preparation of aqueous two-phase system

ATPS was prepared in 10-ml centrifuge tubes by adding the different amounts of PEG and salts together with liver extract according to the method of Klomklao et al.[2]

Effect of salts on proteinases partitioning

To study the effect of salts on partitioning of the proteinases from liver extract using ATPS, different salts including NaH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, K_2HPO_4, $Na_3C_6H_5O_7$, and Na_2SO_4 at different concentrations (15, 20, and 25%, w/w) were mixed with 20% PEG1000 in aqueous system. Distilled water was used to adjust the system to obtain the final weight of 5 g. The mixtures were mixed continuously for 3 min using a Vortex mixer (Vortexgenic 2, G-560E, USA). Phase separation was achieved by centrifugation for 5 min at 5,000×g. Top phase was carefully separated using a pasteur pipette, and the interface of each tube was discarded. Volumes of the separated phases were measured. Aliquots from each phase were taken for enzyme assay and protein determination.

The specific activity of stomach proteinase in the aqueous two-phase system was defined as

$$\mathrm{SA} = \frac{\text{proteinase activity}}{\text{protein concentration}}\ (\text{Unit/mg protein}); \tag{1}$$

The purification factor as

$$\mathrm{PF} = \frac{\mathrm{SA_e}}{\mathrm{SA_i}} \tag{2}$$

where SA_e is the SA of each phase, and SA_i is the initial SA of crude extract. The partition coefficient of protein concentration was defined as:

$$\mathrm{K_P} = \frac{\mathrm{C_T}}{\mathrm{C_B}} \tag{3}$$

where C_T and C_B are concentrations of protein in top and bottom phase, respectively. The partition coefficient of proteinase activity was defined as:

$$\mathrm{K_E} = \frac{\mathrm{E_T}}{\mathrm{E_B}} \tag{4}$$

where E_T and E_B are proteinase activity in top and bottom phase, respectively.

The volume ratio is:

$$\mathrm{V_R} = \frac{\mathrm{V_T}}{\mathrm{V_B}} \tag{5}$$

where V_T and V_B are top and bottom phase volume, respectively, and the protease activity recovery yield was defined as:

$$\mathrm{Yield}(\%) = \frac{\mathrm{A_T}}{\mathrm{A_i}} \times 100 \tag{6}$$

where A_T is total proteinase activity in top phase, and A_i is the initial proteinase activity of crude extract. Based on purity and recovery yield, the appropriate salt in ATPS rendering the most effective partitioning was selected for further study.

Effect of molecular weight and concentration of PEG on proteinases partitioning

To study the effect of the concentrations (10%, 15%, 20%, 25%, and 30%, w/w) of PEG1000 and PEG4000 on partitioning of proteinase in tuna liver extract, NaH_2PO_4 at a level of 20% was used in the system.[2] Partitioning was performed as previously described. All experiments were run in duplicate. Based on purity and recovery yield, the ATPS rendering the most effective partitioning was chosen for further study.

Effect of pH on the proteinases partitioning

ATPS containing PEG1000 (25%, w/w) and NaH_2PO_4 (20%, w/w) was used for study on the effect of pH on liver extract proteinase partitioning. The original pH of the system was measured and then

adjusted to 3.0, 5.0, 7.0, 9.0, and 11.0 with 1 M HCl or 1 M NaOH. Partitioning was performed as previously described. The system pH showing the highest purity and recovery yield was selected for further study.

Effect of NaCl on the proteinases partitioning

The phase system containing 25% PEG1000 and 20% NaH_2PO_4 at pH 7.0 was chosen for study on the effect of NaCl on proteinase partitioning. Adjustment of salt content in the system was made by addition of NaCl (solid form) into the system to obtain concentrations of 0%, 2%, 4%, and 6% (w/w). Partitioning was performed as previously described. The ATPS rendering the most effective partitioning was chosen. Phase with high specific activity was dialyzed against 10 volumes of 50 mM Tris-HCl (pH 7.5) for 18 h with three changes of buffer in the first 3 h and five changes in the last 15 h. ATPS fraction with highest purity and yield was used for hydrolysis study.

Enzyme assay and protein determination

Proteinase activity of liver extract was measured using casein-TCA-Lowry assay.[13,14] To initiate the reaction, 200 µL of liver extract was added into assay mixtures containing 200 µL of 2% (w/v) casein, 200 µL of distilled water, and 625 µL of assay buffer (0.1 M glycine-NaOH, pH 8.5). The mixture was incubated at 55°C for precisely 15 min. The enzymatic reaction was terminated by adding 200 µL of 50% (w/v) trichloroacetic acid (TCA). The reaction mixture was centrifuged at 7,500×g for 10 min at room temperature. The oligopeptide content in the supernatant was determined by the Lowry assay[14] using tyrosine as a standard. One unit of activity was defined as that releasing 1 mmol of tyrosine per min (mmol Tyr/min). A blank was run in the same manner, except the enzyme was added after 50% TCA (w/v) addition. Protein concentration was measured by the method of Bradford[15] using bovine serum albumin as a standard.

Characterization of recovered proteinase

SDS-polyacrylamide gel electrophoresis and activity staining

SDS-PAGE was performed according to the method of Laemmli.[16] Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer (0.5 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% βME) and boiled for 3 min. The samples (20 µg) were loaded on the gel made of 4% stacking and 12% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II cell apparatus (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gels were stained with 0.2% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid and destained with 30% methanol and 10% acetic acid.

Liver extract and selected phase with high SA and yield obtained from ATPS were separated on SDS-PAGE, followed by activity staining according to the method of Klomklao et al.[2] The samples were mixed with sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS, 20% (v/v) glycerol, and 10% βME) at a ratio of 1:1 (v/v). Two µg of proteins were loaded into the gel made of 4% stacking and 12% separating gels. The proteins were subjected to electrophoresis at a constant current of 15 mA per gel by a Mini-Protean II Cell apparatus (Bio-Rad, Hercules, CA, USA). After electrophoresis, gels were immersed in 100 mL of 2% (w/v) casein in 50 mM Tris-HCl buffer, pH 7.5 for 1 h with constant agitation at 0°C to allow the substrate to penetrate into the gels. The gels were then transferred to 2% (w/v) casein in 0.1 M glycine-NaOH, pH. 8.5, and incubated at 55°C for 15 min with constant agitation to develop the activity zone. The gels were fixed and stained with 0.125% Coomassie blue R-250 in 45% ethanol and 10% acetic acid and destained in 30% methanol and 10% acetic acid. Development of clear zones on blue background indicated proteolytic activity.

Hydrolysis of starry triggerfish muscle by fractionated proteinase

Starry triggerfish (*Abalistes stellaris*) with the length of 10 cm were purchased from the dock in Trang, Thailand. The fish, off-loaded approximately 18–24 h after capture, were placed on ice at a

fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Science and Technology, Thaksin University, Phatthalung, within 2 h. Upon arrival, the fish were filleted, and the ordinary muscle was collected and ground to uniformity. A portion of mince (500 g) was placed in a polyethylene bag and stored at -20°C until used.

Fractionated proteinase (0, 25, 50, 75, and 100 unit) was added to the reaction mixture containing 3 g starry triggerfish mince and 6 ml of 0.1 M glycine-NaOH, pH 8.5. The hydrolysis was conducted by incubating the mixture at 55°C for 0, 15, 30, 60, and 120 min. The control was performed by incubating the reaction mixture at 55°C for 120 min without the addition of fractionated enzyme. At hydrolysis time designated, 1 ml of sample was taken and mixed with 1 ml of 1% SDS solution (85° C) before placing in a water bath at 85°C for 15 min to inactivate proteinase. The degree of hydrolysis of protein hydrolysate was analyzed according to the method of Benjakul and Morrissey.[17]

To monitor the protein pattern, another lot of sample was added with hot 5% SDS solution (85°C) to terminate the reaction and solubilize total protein. All samples was subjected to SDS-PAGE.[16] The degree of hydrolysis (DH) of protein hydrolysate was analyzed according to the method of Benjakul and Morrissey.[17] The samples (125 µl) were mixed throughly with 2.0 mL of 0.2125 M phosphate buffer, pH 8.2, and 1.0 mL of 0.01% TNBS solution. The mixtures were then placed in a water bath at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulfite. The mixtures were cooled at ambient temperature for 15 min. The absorbance was measured at 420 nm, and α-amino acid content was calculated and expressed in terms of *L*-leucine. DH was calculated as follows:

$$\mathrm{DH} = [(L_t - L_0)/(L_{max} - L_0)] \times 100$$

where L_t is the amount of α-amino acid released at time t. L_0 is the amount of α-amino acid in original starry triggerfish muscle. L_{max} is the total amount of α-amino acid in original starry triggerfish muscle obtained after acid hydrolysis with 6 N HCl at 100°C for 24 h.

Statistical analysis

A completely randomized design was used throughout this study. Data were subjected to analysis of variance (ANOVA), and and mean comparison was carried out using Duncan's Multiple Range Test.[18] Statistical analysis was performed using the Statistical Pacleage for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

Results and discussion

Use of ATPS for partitioning of proteinase

Effect of salts on the proteinase partitioning in ATPS

Table 1 shows the effects of type and concentration of salts on the partitioning and recovery of proteinase from liver of albacore tuna. The proteinase partitioning was assayed in several biphasis systems of 20% PEG1000 containing different salts, including NaH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, K_2HPO_4, $Na_3C_6H_5O_7$, and Na_2SO_4 at various concentrations. After phase separation, two phases were obtained. The upper phase becomes PEG rich, and the lower phase becomes salt rich. However, no phase separation was observed in the system containing 20% PEG1000-15% NaH_2PO_4. Raghavarao et al.[19] reported that two phases are formed when the polymer concentration is in the range of 8–16% (w/w) and salt concentration must be as high as 10% (w/w). The addition of salts to the aqueous PEG solution led to an arrangement of ordered water molecules around PEG molecule due to their water structure breaking effect.[12] For all ATPS studied, the proteinase was partitioned predominantly in the PEG-rich top phase, principally those with hydrophobic characteristics.[20] In general, negatively charged proteins prefer the upper phase in PEG-salt systems, while positively charged proteins normally partition selectively to the bottom phase.[20] Hence,

Table 1. Effect of phase composition in PEG1000-salt ATPS on partitioning of liver proteinase from albacore tuna.

Phase composition (%, w/w)	V_R	K_P	K_E	SA	PF	Yield
20% PEG1000-15% NaH_2PO_4	–	–	–	–	–	–
20% PEG1000-20% NaH_2PO_4	1.83	0.53	7.01	8.56	2.31	68.75
20% PEG1000-25% NaH_2PO_4	0.83	1.14	7.56	3.00	0.81	41.84
20% PEG1000-15% $(NH_4)_2SO_4$	1.07	1.02	5.70	3.72	1.00	46.49
20% PEG1000-20% $(NH_4)_2SO_4$	0.90	1.00	3.47	3.03	0.82	37.74
20% PEG1000-25% $(NH_4)_2SO_4$	0.64	1.08	4.97	3.12	0.84	39.83
20% PEG1000-15% $MgSO_4$	2.45	0.65	0.37	3.61	0.98	41.32
20% PEG1000-20% $MgSO_4$	1.36	0.79	0.44	2.67	0.72	31.65
20% PEG1000-25% $MgSO_4$	0.92	0.90	0.53	1.39	0.37	16.50
20% PEG1000-15% K_2HPO_4	0.97	1.09	1.98	4.10	1.11	64.12
20% PEG1000-20% K_2HPO_4	0.57	1.04	2.10	3.23	0.87	52.30
20% PEG1000-25% K_2HPO_4	0.67	1.08	4.25	3.09	0.83	50.97
20% PEG1000-15% $Na_3C_6H_5O_7$	1.09	0.75	3.27	3.68	0.99	48.21
20% PEG1000-20% $Na_3C_6H_5O_7$	0.86	0.96	3.34	2.49	0.67	38.52
20% PEG1000-25% $Na_3C_6H_5O_7$	0.72	0.86	1.13	1.62	0.44	22.53
20% PEG1000-15% Na_2SO_4	1.08	1.47	1.08	3.74	1.01	47.85
20% PEG1000-20% Na_2SO_4	0.85	1.31	0.81	2.41	0.65	35.87
20% PEG1000-25% Na_2SO_4	0.67	1.42	0.87	1.74	0.47	30.10

(-) No phase separation.

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein; K_E: partition coefficient of proteinases; SA: specific activity (U/(g protein) in the upper phase; PF: purification factor in the upper phase; Yield: recovery yield in the upper phase.

liver proteinase partitioned in the top phase might be negatively charged. SA, PF, and % yield of proteinase obtained from PEG1000-salt systems depended on types of salt used. System of composition 20% PEG1000 and 20% NaH_2PO_4 showed the highest SA (8.56 units/μg protein), PF (2.31-fold) and yield (68.75%) indicating that this system has the best capacity of separating proteinase from liver of albacore tuna. Therefore, the system containing 20% NaH_2PO_4 was selected for further study on the effect of PEG concentration on proteinase partitioning and recovery. Senphan and Benjakul[21] reported that ATPS comprising PEG1000 (15% w/w) and $MgSO_4$ (25% w/w) provided the best condition for the maximal partitioning of proteases from hepatopancreas of Pacific white shrimp into the top phase and gave the highest PF (8.6-fold) and yield (65.5%). Protease from *Calotropis procera* latex was separated in the top PEG-rich phase in ATPS composed of 12% PEG4000 and 17% $MgSO_4$.[22] Ketnawa et al.[23] found that the best ATPS condition for protease partitioning from viscera extract of Giant catfish (*Pangasianodon gigas*) was 15% PEG2000-15% $Na_3C_6H_5O_7$ with 1% (w/w) NaCl, which increased the purity by 3.33-fold and recovery yield (64.18%).

The distribution of the proteins in ATPS is characterized by partition coefficient *K*. *K* values for proteinase and protein partitioning are reported as K_E and K_P, respectively. From the results, the lowest K_P (0.53) and the highest K_E (7.56) were found in the system of 20% PEG1000 - 20% NaH_2PO_4 and 20% PEG1000 - 25% NaH_2PO_4, respectively. Generally, the lowest K_P indicates a shift of contaminant proteins, nucleic acid, and other undersirable components to the lower phase. For K_E, high K_E value indicates that only proteinase from crude extract was partitioned more to the top phase. Hence, the extraction conditions employed resulted in the enrichment of specific proteinase activity, which was due to the differential partitioning of the desired proteinase and contaminating enzymes and proteins to the opposite phases. Johansson[24] reported that the partition of a protein is influenced by the presence of salts. This effect increases with the net charge of protein. It has been found that the efficiency of the salts in promoting phase separation reflects the lyotropic series (a classification of ions based upon salting-out or salting-in ability).[22] Their effectiveness is mainly determined by the nature of the anion. Multicharged anions being the most effective are ordered of SO_4^{2-} > HPO_4^{2-} > acetate > Cl^-, whereas the order of cations is usually given as $(NH_4)^+$ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} [22].

Table 1 also showed that increasing salt concentration resulted in less activity recovery. Loss in activity might be due to the denaturation of proteinases causes by "salting out" effect.[25] Isable and

Otero[26] found that the presence of high concentrations of salt in the reaction medium greatly decreased both the yield and the selectivity toward the trisaccharide from lactose. Pan and Li[27] also reported that increasing NaH_2PO_4 concentration resulted in less activity recovery as well as poorer specific activity. Therefore, the type of salt and concentration used were critical for albacore tuna liver proteinase recovery or partitioning in ATPS.

Effect of PEG molecular weight on the proteinase partitioning in ATPS

Proteinase partitioning using ATPS with varying concentration of PEG and 20% NaH_2PO_4 is depicted in Table 2. No phase separation was observed in the 10% PEG1000 with 20% NaH_2PO_4. The V_R of the system ranged from 0.53 to 1.51. Generally, an increase in the PEG molecular mass reduces free volume by increasing the chain length of the PEG polymer, resulting in partitioning of the biomolecules to the bottom phase. The increase in polymer weight causes the reduction of free volume of the top phase, so the partition of biomolecules in the salt-rich bottom phase decreases the partitioning coefficient.[7] With PEG1000 and 4000, all proteinases partitioned into the top phase (K_E >1). However, use of the lower molecular weight PEG gave a higher K_E, compared with the higher molecular weight. Thus, K_E values depended on the PEG molecular weight. Tubio et al.[6] suggested that for ATPS formed by PEG of low molecular weight (600–3350 kDa), the protein transfer to the top phase is enthalpically driven mainly due to a strong interaction between PEG and the protein. PEG of the highest molecular weight (PEG8000) excludes the protein from the top phase driven by an entropically unfavorable term.[7] For K_P value, when PEG with higher molecular weight was used, the K_P increased. The lowest K_P (0.44) was observed in ATPS composed of 25% PEG1000 and 20% NaH_2PO_4. High in K_P values indicating most of proteins were more partitioning to the top phase, while the high in K_E implying only the target enzyme was partitioned to the top phase.[22] The highest SA (17.03 units/mg protein) and PF (4.60-fold) of proteinase was obtained in 25% PEG1000 and 20% NaH_2PO_4 systems. Therefore, PEG1000 was a suitable polymer for partitioning of proteinase in albacore tuna liver as indicated by the higher SA and PF than PEG with higher molecular weight. This was possibly due to the fact that interfacial tension is lower when molecular weight of PEG is lower.[25] A preferential interaction between PEG molecule and protein domain decreased when the molecular weight of PEG increased because of its exclusion from the protein domain.[7] Moreover, the surface charge of biological materials is one of the most significant factors affecting the separation by using partitioning. Molecular weight, shape, and specific binding sites of biological materials also affect the partition profiles. Electrical interaction and repulsion between charged aqueous phase systems and the proteins affect the partitioning of system.[2] Among all ATPS tested, system comprising 25% PEG1000 and 20% NaH_2PO_4 partitioned the proteinase to the top PEG-rich phase and undesired protein to the bottom salt phase most effectively.

Table 2. Effect of PEG molecular mass and concentration in a PEG-NaH_2PO_4 ATPS on partitioning of liver proteinase from albacore tuna.

Phase composition (%, w/w)	V_R	K_P	K_E	SA	PF	Yield
10% PEG1000-20% NaH_2PO_4	–	–	–	–	–	–
15% PEG1000-20% NaH_2PO_4	1.04	0.87	1.97	7.45	2.01	70.88
20% PEG1000-20% NaH_2PO_4	1.05	0.77	3.93	9.34	2.52	66.62
25% PEG1000-20% NaH_2PO_4	1.18	0.44	8.88	17.03	4.60	73.70
30% PEG1000-20% NaH_2PO_4	1.51	0.45	2.27	12.21	3.30	53.28
10% PEG4000-20% NaH_2PO_4	0.53	0.73	1.05	4.32	1.17	16.52
15% PEG4000-20% NaH_2PO_4	0.77	0.89	1.14	6.75	1.82	28.30
20% PEG4000-20% NaH_2PO_4	0.87	0.98	1.64	4.91	1.33	21.15
25% PEG4000-20% NaH_2PO_4	1.25	1.34	1.66	5.07	1.37	25.95
30% PEG4000-20% NaH_2PO_4	1.40	1.67	1.01	3.30	0.46	20.13

(-) No phase separation.

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein; K_E: partition coefficient of proteinases; SA: specific activity (U/(g protein) in the upper phase; PF: purification factor in the upper phase; Yield: recovery yield in the upper phase.

Effect of pH on the proteinase partitioning in ATPS

The influence of pH on the partitioning of proteinase from liver of albacore tuna was investigated using the ATPS composition of 25% PEG1000-20% NaH_2PO_4 that provided the highest proteinase recovery. The pH of the ATPS was adjusted to 3.0, 5.0, 7.0, 9.0, and 11.0 in comparison to the control system (without pH adjustment). In general, the pH had influenced on protein partitioning, either by changing the charge of the solute or by altering the ratio of the charged species present. Negatively charged proteins prefer the upper PEG-rich phase, and positively charged proteins partition to the lower salt phase. Therefore, as the pH increases above the isoelectric point (pI) of a protein, it becomes negatively charged, its interaction with PEG becomes stronger, and the partition coefficient increases.[22] A higher PF was found when the system pH increased up to 7.0 (Fig. 1). The highest PF (5.58-fold) and recovery yield (89.99%) were obtained in the system pH of 7.0. However, the decrease in the recovery and PF was observed when the pH of system of 9 and 11 was used. Most of the biomolecules, especially proteins and enzymes, are stable at neutral pH that is favorable condition to conduct the ATPS partitioning. Enzyme stability slightly reduced in the acidic area, but it was dramatically lost at pH above 9.0.[22]

Effect of NaCl on the proteinase partitioning in ATPS

Partitioning of proteinase from liver of albacore tuna in the presence of NaCl was also studied in the system of 25% PEG1000-20% NaH_2PO_4 at pH 7.0 (Fig. 2). In general, addition of NaCl to the ATPS

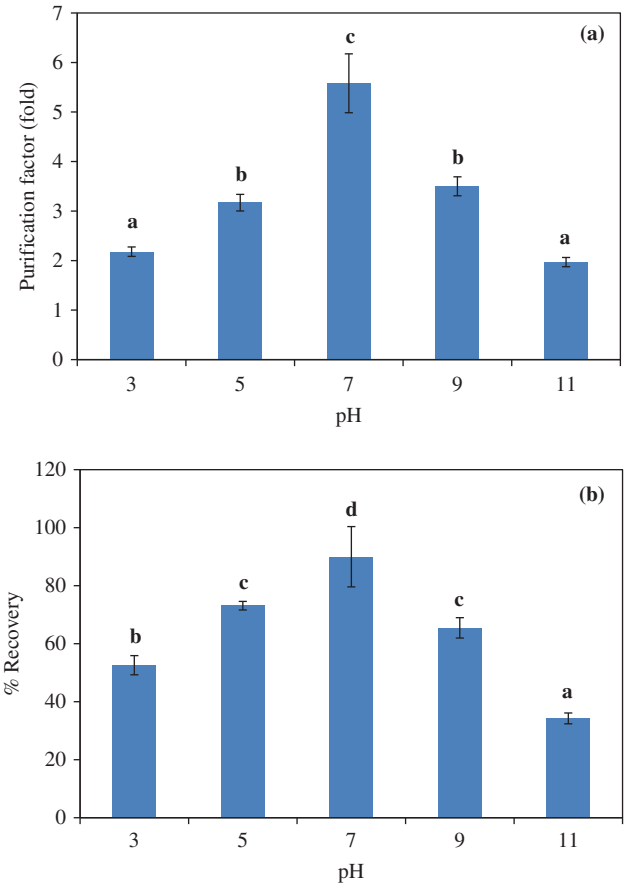

Figure 1. Effect of system pH on the purification factor (a) and proteinase recovery (b) of liver proteinase partitioning in 25% PEG1000-20% NaH_2PO_4 ATPS. Bars represented the standard deviation from triplicate determinations. Different letters within the same parameter indicate the significant differences ($P<0.05$).

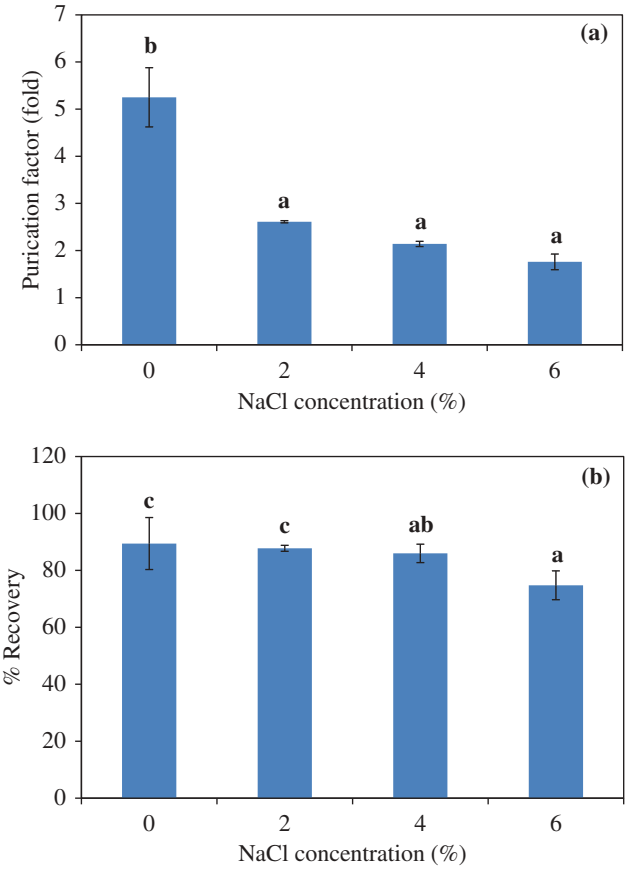

Figure 2. Effect of NaCl concentration on the purification factor (a) and proteinase recovery (b) of liver proteinase partitioning in 25% PEG1000-20% NaH_2PO_4 ATPS at pH 7.0. Bars represented the standard deviation from triplicate determinations. Different letters within the same parameter indicate the significant differences ($P<0.05$).

results in an increase in the hydrophobic difference due to generation of an electrical potential difference between two phases. The result showed that purification factor (PF) and yield decreased with increasing NaCl concentration (Fig. 2). The PF of proteinase decreased from 5.25 (no NaCl addition) to 1.76 (6%, w/w). Higher concentration of NaCl showed a significantly negative effect on partitioning and yield of the enzyme, probably due to protein denaturation and precipitation at high concentration of this salt.[28] These results were in agreement with Ketnawa et al.[23] who studied the PF of alkaline protease from fish viscera in 15% PEG2000-15% $Na_3C_6H_5O_7$ ATPS, using NaCl at different concentrations, ranging from 1% (w/w) to 7% (w/w). The PF of alkaline protease was significantly decreased from 15.34 at 1% (w/w) NaCl to 10.67 at 7% (w/w) NaCl.

Characterization of recovered proteinase

Protein pattern and activity staining of proteinase from albacore tuna liver partitioned with ATPS

SDS-PAGE pattern of proteinase obtained from the partial purification using the ATPS process is shown in Fig. 3. Crude liver extract contained a variety of proteins of different molecular weight. However, a large number of contaminating proteins were removed after partitioning with ATPS, particularly proteins with higher or lower molecular weight. As a result, a higher purity of interested

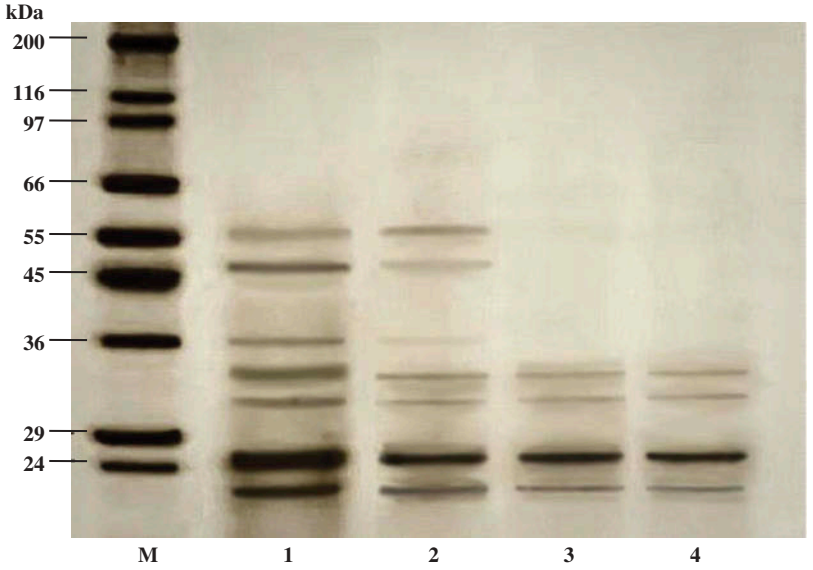

Figure 3. SDS-PAGE of liver extract and ATPS fraction from albacore tuna. M, molecular weight standard; lane 1, liver extract; lane 2, 20% PEG1000-20% NaH_2PO_4 ATPS fraction; lane 3, 25% PEG1000-20% NaH_2PO_4 ATPS fraction; lane 4, 25% PEG1000-20% NaH_2PO_4, pH 7.0 ATPS fraction.

proteinase was obtained. When the proteins or enzymes to be separated differ significantly in their structural properties from others, partitioning can be carried out successfully.[29]

Activity staining of proteinase in liver extract and various fractions obtained from ATPS process were analyzed on SDS-substrate gel (Fig. 4). The presence of the clear zone suggested that it is the proteinase that can be hydrolyzed casein in the gel. The band intensity with apparent MW of 21, 24, 30, and 34 kDa slightly increased after the ATPS process, suggesting the higher specific activity of proteinase loaded into the gel. Molecular weights of trypsin-like enzymes from pyloric ceca brown-stripe 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.[30]

Hydrolysis of starry triggerfish muscle using fractionated proteinases

When starry triggerfish muscle was hydrolyzed using fractionated proteinase (0, 25, 50 75, and 100 unit), a rapid hydrolysis was found within the first 15–30 min, followed by a slower hydrolysis rate up to 120 min (Fig. 5). The fast hydrolysis in the initial phase indicated that a large number of peptide bonds were hydrolyzed.[31] Thereafter, the hydrolysis rate was decreased, mainly due to a decrease in available hydrolysis sites, enzyme autodigestion and/or product inhibition.[30] At the same time of hydrolysis, higher DH was observed with higher activity level of fractionated proteinase used. The results of this study revealed that the degree of hydrolysis increased with increasing hydrolysis time. Also, the degradation of protein increased by increasing the enzyme concentration. Naveena et al.[31] reported that when using protease from *Cucumis trigonus* in buffalo meat samples, the increase in proteolysis can be correlated with significantly higher protein solubility. Senphan and Benjakul[21] found that proteinases recovered from hepatopancreases using the combined partitioning systems could be used for gelatin hydrolysis.

The proteolytic degradation pattern of starry triggerfish muscle protein analyzed by SDS-PAGE revealed that among all proteins, myosin heavy chain (MHC) was susceptible to hydrolysis, followed by actin (Fig. 6). The band intensity of MHC decreased with increasing fractionated liver proteinase concentration up to 50 unit. Total disappearance of MHC was observed when enzyme at concentration of 75 and 100 unit was used. For actin, no hydrolytic degradation was observed when starry triggerfish

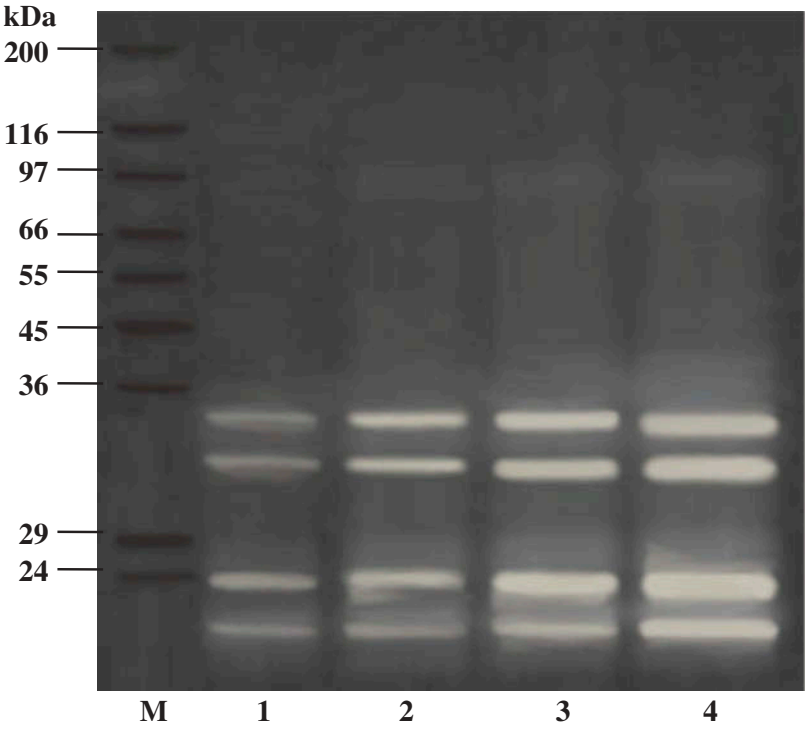

Figure 4. Activity staining of liver extract and ATPS fraction from albacore tuna. M, molecular weight standard; lane 1, liver extract; lane 2, 20% PEG1000-20% NaH_2PO_4 ATPS fraction; lane 3, 25% PEG1000-20% NaH_2PO_4 ATPS fraction; lane 4, 25% PEG1000-20% NaH_2PO_4, pH 7.0 ATPS fraction.

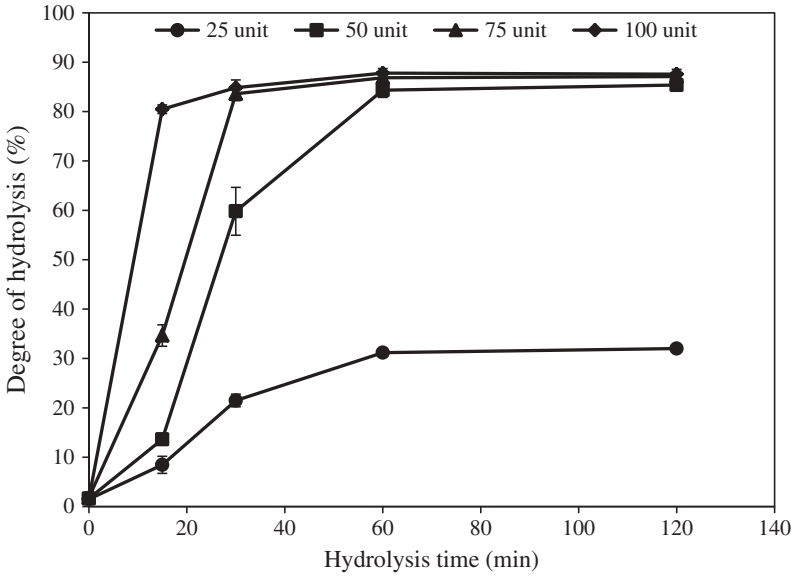

Figure 5. Degree of hydrolysis (DH) of starry triggerfish muscle during hydrolysis with liver enzyme from ATPS fraction (top phase of system 25% PEG1000-20% NaH_2PO_4, pH 7.0). The hydrolytic reaction was performed at pH 8.5, 55°C. Bars represented the standard deviation from triplicate determinations.

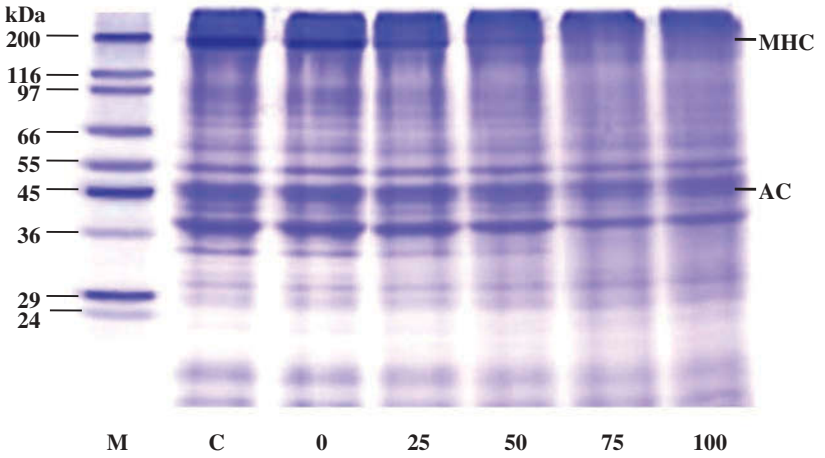

Figure 6. SDS-PAGE patterns of starry triggerfish muscle with liver enzyme from ATPS fraction (top phase of system 25% PEG1000-20% NaH_2PO_4, pH 7.0). The hydrolytic reaction was performed at pH 8.5, 55°C. Numbers designate the enzyme level (unit). M: molecular weight standard; C: control MHC: myosin heavy chain; AC: actin.

muscle was incubated in the presence of 25 and 50 unit fractionated proteinases from albacore tuna liver. However, the degradation increased as the enzyme concentration increased. Also, the degradation rate was lower than that of MHC. Therefore, the rate of hydrolysis was dependent on the amount of enzyme added. From the result, it was noted that autolysis of sample (without fractionated proteinase addition) occurred to some extent during incubation at 55°C. From the results, proteinases recovered from liver of albacore tuna using ATPS could be an alternative potential aid for production of fish protein hydrolysate, in which the cost of enzyme could be reduced.

Conclusion

ATPS can be effectively used to recover and purify proteinase from albacore tuna liver. ATPS with 25% PEG1000-20% NaH_2PO_4, pH 7.0 provided the best enzyme recovery and purity. NaCl concentration had no effect on partitioning of the target enzyme. Based on the protein degradation of starry triggerfish muscle, it is suggested that the fractionated proteinase from albacore tuna liver could be used for protein hydrolysate production.

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