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1 **Partitioning and recovery of proteinase from tuna spleen**
2 **by aqueous two-phase systems**

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1 **Abstract**

2 Partitioning of spleen proteinase from yellowfin tuna (*Thunnus albacores*) in
3 an aqueous two-phase system (ATPS) was investigated. Phase compositions including
4 PEG molecular mass and concentration as well as types and concentration of salts
5 affected the protein partitioning. ATPS comprising PEG1000 (15% w/w) and
6 magnesium sulfate (20% w/w) provided the best condition for the maximum
7 partitioning of the proteinase into the top phase and gave a highest specific activity
8 (47.0 units/ μ g protein) and purification fold (6.61). The yield of 69.0% was obtained.
9 Under the same ATPS condition used, the partitioning of proteinase of splenic extract
10 from three tuna species involving skipjack tuna, yellowfin tuna and tongol tuna were
11 compared. The purity of splenic extract from all tuna species increased after ATPS
12 process. Among all species tested, yellowfin tuna showed the highest purification fold,
13 followed by tongol tuna and skipjack tuna, respectively. SDS-substrate gel
14 electrophoresis revealed that the band intensity of major proteinase in ATPS fraction
15 from all tuna species slightly increased with the concomitant decrease in band
16 intensity of other contaminating proteins, indicating the greater specific activity of
17 splenic extract. Therefore, ATPS was an effective method for partitioning and
18 recovery of proteinases from tuna spleen.

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20 *Keywords:* Aqueous two-phase system; Proteinase; Purification; Spleen; Tuna

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1 **1. Introduction**

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3 Tuna processing industry, especially canning, has become increasingly
4 important as an income generator for Thailand. In terms of volume, Thailand is the
5 world's largest exporter of canned tuna, for over 20 million cans annually during the
6 past 5 years [1,2]. Large volumes of raw tuna go through the canning process, by
7 which about two-third of the whole fish are utilized. As a consequence, processing
8 wastes from the tuna canning industry are generated and estimated at 450,000 metric
9 tons annually [1]. More than 200,000 metric tons of tuna viscera and offal can be
10 collected and used mostly for animal feed.

11 Fish viscera is a potential source for recovering enzymes such as proteinases
12 that may have some unique properties for industrial applications; e.g. in the detergent,
13 food, pharmaceutical, leather and silk industries [3-7]. The use of alkaline proteinases
14 has increased remarkably since they are both stable and active under harsh conditions
15 such as at temperatures of 50 to 60°C, high pHs and in the presence of surfactants or
16 oxidizing agents [7,8].

17 Trypsins have been characterized thoroughly based on their physicochemical
18 and enzymatic properties from the intestine of crayfish [9,10], dogfish [11], mackerel
19 [12] and capelin [13]. Bezerra et al. [14] partially purified trypsin from pyloric caeca
20 of tambaqui (*Colossoma macropomum*) and found that the enzyme had an optimal pH
21 of 9.5. Byun et al. [15] purified and characterized serine proteinases from pyloric
22 caeca of tuna (*Thunnus thynnus*). Recently, Klomklao et al. [2] reported that major
23 proteinases in spleen of three tuna species including skipjack tuna, yellowfin tuna and
24 tongol tuna were trypsin-like serine proteinases. Among the spleen from three species,
25 that from yellowfin tuna showed the highest activity.

1 Partitioning in an aqueous two-phase system (ATPS) is a selective method
2 used for biomolecule purification [16]. Aqueous two-phase system is generally
3 formed by mixing two or more incompatible polymers in aqueous condition. Phase
4 separation occurs over certain concentrations of phase components. Alternatively,
5 polymer and salt can also be used to generate an aqueous two-phase [16]. Among the
6 polymer/salt systems, polyethylene glycol (PEG)/potassium phosphate and
7 PEG/magnesium sulphate are most frequently used [17-19]. Generally, the
8 biomolecule partition coefficient, K , defined as the ratio of the biomolecule
9 concentration in the top phase to that in the bottom phase, was used to quantify the
10 biomolecule partition behaviour. If the partition coefficients (or ratios) of two
11 substances differ by a factor of 10 or more, their separation can be satisfactorily
12 carried out [20]. When a single component must be extracted from a mixture, phase
13 system compositions are often manipulated in such a manner that the component
14 partitions into one of the phases, while the other components of the mixture partition
15 into the other phase [20]. Additionally, the surface charge of biological materials is
16 one of the most significant factors affecting the separation by use of partitioning [21].
17 Molecular weight, shape, hydrophobicity and specific binding sites of biological
18 materials also affect the partition profiles. Electrical interaction and repulsion between
19 charged aqueous phase systems and the proteins affect the partitioning of system [21].

20 ATPS have found application in the industrial scale purification of proteins
21 from biomass [22]. The use of ATPS in downstream processing has been focused on
22 the extraction, separation and concentration of various biomolecules including
23 xylanases [23], amylase [24], anyloglucosidase [25], amino acid [26], etc. However,
24 sometimes it is used as a potential primary purification technique to reduce the bulk of
25 the processing stream, if not the only step to be followed by more selective final

1 purification steps such as chromatography, electrophoresis, etc [27]. Industries desire
2 procedures which are less time consuming and give high enzyme yields with
3 considerable purity. In this regard, partitioning in ATPS provides a powerful method
4 for separating and purifying mixtures of proteins [23-25]. ATPS also offers many
5 advantages including low process time, low energy consumption and biocompatible
6 environment to the biomolecule due to the presence of large amounts of water in the
7 extraction systems [22]. Furthermore, ATPS can remove contaminants such as nucleic
8 acids and undesirable proteins. Hence, ATPS has been recognized as an efficient and
9 economical downstream processing method due to the ease and lower cost [22, 28].
10 Our objective was to investigate the feasibility of utilizing ATPS for partitioning and
11 recovery of proteinase from tuna spleen.

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1 **2. Materials and methods**

2 *2.1 Chemicals*

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

13

14 *2.2 Fish Sample preparation*

15 Internal organs from three species of tuna including skipjack tuna
16 (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacares*) and tongol tuna (*Thunnus*
17 *tonggol*) were obtained from Chotiwat Industrial Co. (Thailand) Ltd., Songkhla.
18 Those samples (5 kg) were packed in the polyethylene bag, kept in ice and transported
19 to the Department of Food Technology, Prince of Songkla University, Hat Yai,
20 Thailand within 30 min. Pooled internal organs were then excised and separated into
21 individual organs. Only spleen was collected, immediately frozen and stored at -20°C
22 until used.

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1 2.3 Preparation of spleen extract

2 Frozen spleens were thawed using a running water (26-28°C) until the core
3 temperature reached -2 to 0°C. The samples were cut into pieces with a thickness of
4 1-1.5 cm. Samples were ground into powder in the liquid nitrogen using a National
5 Model MX-T2GN blender (Taipei, Taiwan) according to the method of Simpson and
6 Haard [29] as modified by Garcia-Carreno et al. [30].

7 Spleen powder was suspended in the distilled water at a ratio of 1:9 (w/v) and
8 stirred continuously at 4°C for 15 min. The suspension was centrifuged for 15 min at
9 4°C at 5,000×g using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA) to
10 remove the tissue debris. The supernatant was collected and referred to as “tuna
11 spleen extract”.

12

13 2.4 Enzyme assay

14 Proteinase activity of spleen extract from each tuna was determined using
15 casein as a substrate according to the method of An et al. [31] with a slight
16 modification. To initiate the reaction, 200 µl diluted spleen extract (500 folds) was
17 added into assay mixtures containing 2 mg of casein, 200 µl of distilled water and 625
18 µl of assay buffer (0.1 M glycine-NaOH, pH 9.0). The mixture was incubated at 55°C
19 for precisely 15 min. Enzymatic reaction was terminated by adding 200 µl of 50%
20 (w/v) trichloroacetic acid (TCA). Unhydrolyzed protein substrate was allowed to
21 precipitate for 15 min at 4°C, followed by centrifuging at 7,000×g for 10 min. The
22 oligopeptide content in the supernatant was determined by the Lowry assay [32] using
23 tyrosine as a standard. One unit of activity was defined as that releasing 1 nmole of

1 tyrosine per min (nmol/Tyr/min). A blank was run in the same manner, except the
2 enzyme was added after addition of 50 % TCA (w/v).

3

4 *2.5 Preparation of aqueous two phase systems*

5 ATPS were prepared in a 10-ml centrifuge tubes by adding the appropriate
6 amount of PEG, salts and tuna spleen extract. To study the effect of salts on
7 partitioning the proteinase from tuna spleen extract using ATPS, different salts
8 including NaH_2PO_4 , K_2HPO_4 , MgSO_4 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, $(\text{NH}_4)_2\text{SO}_4$ and Na_2SO_4 at
9 different concentrations (15, 20 and 25% w/w) were mixed with 20% PEG1000 in
10 aqueous system. Distilled water was used to adjust the system to obtain the final
11 weight of 5 g. The mixtures were mixed continuously for 3 min using a Vortex mixer
12 (Vortex-genie2, G-560E, USA). Phase separation was achieved by centrifugation for
13 5 min at $2000\times g$. Top phase was carefully separated using a pasteur pipette and the
14 interface of each tube was discarded. Volumes of the separated phases were measured.
15 Aliquots from each phase were taken for enzyme assay and protein determination.

16 Purification factor (PF), defined as the ratio of specific proteinase activity
17 (SA) of each phase to the initial specific proteinase activity of crude extract was
18 calculated. Partition coefficient (K_E or K_P), the ratio of enzyme activity or protein
19 concentration in the top phase to that in the bottom phase was also calculated. The
20 volume ratio (V_R) defined as the ratio of volume in the top phase to that in the bottom
21 phase was recorded.

22 To study the effect of the concentrations (10, 15, 20 and 25% w/w) of
23 PEG1000 and PEG4000 on partitioning of proteinase in tuna spleen extract, MgSO_4 at
24 a level of 20% was used in the system. Partitioning was performed as previously

1 described. All experiments were run in duplicate. The ATPS rendering the most
2 effective partitioning was chosen. Phase with high specific activity, was dialyzed
3 against 10 volumes of 50 mM Tris-HCl, pH 7.5 for 18 h with 3 changes of buffer in
4 the first 3 h and 5 changes in the last 15 h.

5

6 *2.6 Sodium dodecyl sulfate-gel electrophoresis*

7 SDS-PAGE was performed according to the method of Laemmli [33]. Protein
8 solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample treatment buffer
9 (0.125M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% β -mercaptoethanol) and
10 boiled for 3 min. The samples (20 μ g) were loaded on the gel made of 4% stacking
11 and 10% separating gels and subjected to electrophoresis at a constant current of 15
12 mA per gel using a Mini-Protean II Cell apparatus. After electrophoresis, the gels
13 were stained with 0.2% Coomassie brilliant blue R-250 in 45% methanol and 10%
14 acetic acid and destained with 30% methanol and 10% acetic acid.

15

16 *2.7 Activity staining*

17 Spleen extract and selected phase with high specific proteolytic activity
18 obtained from ATPS were separated on SDS-PAGE, followed by activity staining
19 according to the method of Garcia-Carreño et al. [30]. The samples were mixed with
20 sample buffer (0.125M Tris-HCl, pH 6.8 containing 20% (v/v) glycerol, 10% β -
21 mercaptoethanol) at a ratio of 1:1 (v/v). Two μ g of proteins were loaded into the gel
22 made of 4% stacking and 12% separating gels. The proteins were subjected to
23 electrophoresis at a constant current of 15 mA per gel by a Mini-Protean II Cell
24 apparatus. After electrophoresis, gels were immersed in 100 ml of 2% casein (w/v) in

1 50 mM Tris-HCl buffer, pH 7.5 for 1 h with constant agitation at 0°C to allow the
2 substrate to penetrate into the gels. The gels were then transferred to 2% casein (w/v)
3 in 0.1 M glycine-NaOH, pH 9.0 and incubated at 55°C for 15 min with constant
4 agitation to develop the activity zone. The gels were fixed and stained with 0.125%
5 Coomassie blue R-250 in 45% ethanol and 10% acetic acid and destained in 30%
6 methanol and 10% acetic acid. Development of clear zones on blue background
7 indicated proteolytic activity.

8

9 *2.8 Protein determination*

10 Protein concentration was measured by the method of Bradford [34] using
11 bovine serum albumin as a standard.

12

13 **3. Results and discussion**

14 *3.1 Effect of salts on the proteinase partitioning in ATPS*

15 The partitioning of spleen proteinase from yellowfin tuna was carried out in
16 several biphasis system of 20% PEG1000 with different salts, NaH₂PO₄, K₂HPO₄,
17 MgSO₄, Na₃C₆H₅O₇, (NH₄)₂SO₄ and Na₂SO₄ at different concentrations (Table 1).
18 With either PEG1000 or salts alone, no phase separation was observed (data not
19 shown) indicating that the combination of both PEG and salt was necessary for
20 partitioning process. After phase separation, two phases were obtained, PEG-rich top
21 phase and salt-rich lower phase. For all ATPS studied, the proteinase was partitioned
22 predominantly in the PEG-rich top phase, principally those with hydrophobic
23 characteristics [20]. However, the recovery of proteinase from the opposite phase
24 (lower phase) was relatively low. In general, negatively charged proteins prefer the

1 upper phase in PEG/salt systems, while positively charged proteins normally partition
2 selectively to the bottom phase [35]. Hence, most of spleen proteinases partitioned in
3 top phase might be negatively charged. The maximum SA and PF of proteinase
4 obtained in PEG1000/salt systems depended on the medium composition (Table 1). A
5 phase system containing 20% PEG1000 and 20% MgSO_4 gave the highest SA (24.5
6 units/ μg protein) and PF (3.4 fold). Pan and Li [36] reported that the use of 25% (w/v)
7 PEG1500 and 20-25% (w/v) NaH_2PO_4 was effective in concentrating and purifying
8 the β -xylosidase. The system containing 20% MgSO_4 was selected for further study
9 on the effect of PEG concentration on proteinase partitioning.

10 The distribution of the proteins in ATPS is characterized by partition
11 coefficient K . K values for proteinase and protein partitioning are reported as K_E and
12 K_P , respectively. From the result, ATPS with magnesium sulfate at a concentration of
13 20% showed the lowest K_P (0.32) indicating that it caused a shift of contaminant
14 proteins, nucleic acid and other undesirable components to the lower phase. Thus, the
15 extraction conditions employed resulted in the enrichment of specific proteinase
16 activity, which was due to the differential partitioning of the desired proteinase and
17 contaminating enzymes and proteins to the opposite phases. Table 1 also showed that
18 increasing salt concentration resulted in a less activity recovery. Loss in activity might
19 be due to the denaturation of proteinases caused by “salting out” effect [2]. Isable and
20 Otero [35] found that the presence of high concentrations of salt in the reaction
21 medium greatly decreased both the yield and the selectivity towards the trisaccharide
22 from lactose. Pan and Li [36] also reported that increasing NaH_2PO_4 concentration
23 resulted in a less activity recovery as well as a poorer specific activity. Therefore, type
24 of salt and concentration used were critical for yellowfin tuna spleen proteinase
25 recovery or partitioning in ATPS.

1

2 *3.2 Effect of PEG molecular weight on the proteinase partitioning in ATPS*

3 Proteinase partitioning in ATPS with varying concentration of PEG and 20%
4 MgSO₄ is shown in Table 2. Partitioning of proteinase in PEG/ MgSO₄ system was
5 strongly dependent on the molecular weight of the PEG. With PEG1000, all
6 proteinases partitioned into the top phase ($K_E > 1$). Conversely, most proteinases were
7 partitioned into the lower phase ($K_E < 1$) in ATPS containing PEG4000. Thus, K_E
8 values depended on the PEG molecular weight. The K_P increased when PEG with
9 higher molecular weight was used. However, Reh et al. [20] reported that most
10 proteins were partitioned to the top phase in phase systems with low molecular weight
11 PEG. The influence of the molecular mass of PEG on protein partitioning can be
12 explained on the basis of Flory Huggins theory for polymers in solution [20]. A
13 preferential interaction between the PEG molecule and the protein domain decreased
14 when the molecular mass of PEG increased due to its exclusion from the protein
15 domain [20]. This might lead to the movement of proteinase to the salt lower phase.
16 Additionally, use of the higher molecular weight PEG gave a lower yield of
17 proteinase recovered, compared with the lower molecular weight PEG (Table 2).
18 Fernandez Lahore et al. [37] reported that the use of high molecular weight PEG is
19 unsuitable for purification purpose. Among all ATPS tested, system comprising 15%
20 PEG 1000 and 20% MgSO₄ partitioned the proteinase to the top PEG-rich phase and
21 undesired protein to the bottom salt phase most effectively. Under the optimal
22 conditions, 69% of the enzyme was recovered in the top phase, providing
23 approximately 6.6 folds of purification for spleen proteinase of yellowfin tuna.

24 *3.3 Recovery of spleen proteinase from other tuna species*

1 The 15%PEG-20%MgSO₄ ATPS was used to partition spleen proteinase from
2 three tuna species (Table 3). With ATPS partitioning, higher folds of purification
3 were obtained for splenic extract of yellowfin tuna, compared with those of tongol
4 tuna and skipjack tuna. The recovery yields were of 69.2, 73.6 and 82.5 for yellowfin
5 tuna, skipjack tuna and tongol tuna, respectively (Table 3). Different protein
6 compositions among three species possibly affected the partitioning of proteinase in
7 ATPS used. It is also speculated that differences in the level of purification fold after
8 ATPS process among tuna species might be related to different physicochemical and
9 enzyme properties. Most of the methods reported for proteinase purification from fish
10 digestive organs involved several steps, including ammonium sulfate precipitation,
11 size-exclusion and ion-exchange chromatography [14,15], hydrophobic interaction
12 chromatography [5] and affinity chromatography [29, 38]. In view of their
13 characteristics, these multi-step methods result in high cost and time consuming
14 purification process. Thus, ATPS could be an efficient method for the recovery of
15 proteinase from tuna spleen due to the ease and lower cost.

16

17 *3.4 Protein pattern and activity staining of spleen proteinase from three tuna species* 18 *partitioned with ATPS*

19 The purity of the enzyme from three tuna species after ATPS process was
20 analyzed by SDS-PAGE (Fig. 1). Crude splenic extract contained a variety of proteins
21 with different molecular weight. However, a large number of contaminating proteins
22 were removed after partitioning with ATPS, particularly proteins with higher or lower
23 MW. As a result, a higher purity of interested proteinase was obtained. When the
24 proteins or enzymes to be separated differ significantly in their structural properties

1 from others, partitioning can be carried out successfully. The partitioning by ATPS
2 becomes more complicated when those differences are minor [21].

3 The proteinases in tuna splenic extract and fraction obtained from ATPS were
4 identified by SDS-substrate polyacrylamide gels (Fig. 2). The apparent MWs of the
5 major activity bands were estimated to be 48, 23 and 23 kDa for skipjack, tongol and
6 yellowfin tuna, respectively. However, minor activity bands were observed with
7 apparent MW of 32 and 21 kDa for tongol and 31 and 21 kDa for yellowfin,
8 respectively. The results indicated the differences in the major proteinases in splenic
9 extract among all tuna species tested. Generally, activity bands of skipjack were
10 different from those of other two species. Slightly greater band intensity in ATPS
11 fraction was observed, suggesting the higher specific activity of proteinase loaded into
12 the gel.

13

14 **4. Conclusion**

15 Aqueous two-phase system was demonstrated to be an efficient primary
16 purification step for the tuna spleen proteinase. The best condition of ATPS for
17 partitioning proteinase from tuna spleen was the PEG1000 (15%)-MgSO₄ (20%)
18 system. Scaling up two-phase partition could form part of future industrial
19 purification protocols for recovery of tuna spleen proteinase.

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1 Table 1 Effect of phase composition in PEG 1000-Salt ATPS on partitioning of spleen
 2 proteinase from yellowfin tuna.

Phase composition (% w/w)	V _R	K _P	K _E	SA	PF	Yield (%)
20% PEG1000 - 15% NaH ₂ PO ₄	3.23	1.55	8.15	12.71	1.76	69.7
20% PEG1000 - 20% NaH ₂ PO ₄	1.31	1.29	33.59	13.37	1.85	57.4
20% PEG1000 - 25% NaH ₂ PO ₄	0.92	0.99	26.11	12.40	1.71	33.3
20% PEG1000 - 15% K ₂ HPO ₄	1.07	1.55	23.54	13.79	1.91	76.7
20% PEG1000 - 20% K ₂ HPO ₄	0.91	1.25	35.89	14.46	2.00	64.1
20% PEG1000 - 25% K ₂ HPO ₄	0.74	0.78	22.89	12.42	1.72	40.0
20% PEG1000 - 15% MgSO ₄	2.46	0.60	1.11	16.58	2.29	83.6
20% PEG1000 - 20% MgSO ₄	1.33	0.32	1.77	24.55	3.40	71.7
20% PEG1000 - 25% MgSO ₄	1.13	0.53	2.14	13.50	1.87	55.5
20% PEG1000 - 15% Na ₃ C ₆ H ₅ O ₇	1.42	1.09	15.44	11.32	1.57	72.2
20% PEG1000 - 20% Na ₃ C ₆ H ₅ O ₇	1.17	1.05	11.79	12.19	1.69	64.1
20% PEG1000 - 25% Na ₃ C ₆ H ₅ O ₇	0.71	0.81	22.44	13.03	1.80	52.0
20% PEG1000 - 15% (NH ₄) ₂ SO ₄	1.28	1.55	22.49	11.28	1.56	73.2
20% PEG1000 - 20% (NH ₄) ₂ SO ₄	0.95	1.73	42.60	13.36	1.85	53.3
20% PEG1000 - 25% (NH ₄) ₂ SO ₄	0.89	2.16	62.47	16.41	2.27	66.6
20% PEG1000 - 15% Na ₂ SO ₄	1.04	1.57	22.12	17.28	2.39	59.7
20% PEG1000 - 20% Na ₂ SO ₄	0.84	2.10	24.49	12.14	1.68	44.7
20% PEG1000 - 25% Na ₂ SO ₄	0.70	1.43	21.26	15.14	2.09	35.4

3 V_{B_R} : Volume ratio (Upper/Lower)

4 K_{B_P} : Partition coefficient of protein

5 K_E : Partition of proteinases in the upper phase

6 SP : Specific activity (U/mg Protein)

7 PF : Purification factor

8 Yield : Recovery yield

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1 Table 2. Effect of PEG molecular mass and concentration in a PEG-MgSO₄ ATPS on
 2 partitioning of spleen proteinase from yellowfin tuna.

Phase composition (% w/w)	V _R	K _P	K _E	SA	PF	Yield (%)
10% PEG1000 - 20% MgSO ₄	-	-	-	-	-	-
15% PEG1000 - 20% MgSO ₄	1.03	0.14	1.94	46.87	6.61	69.0
20% PEG1000 - 20% MgSO ₄	1.45	0.31	1.82	23.66	3.34	71.8
25% PEG1000 - 20% MgSO ₄	1.87	0.51	2.13	16.04	2.26	68.3
10% PEG4000 - 20% MgSO ₄	0.73	0.35	0.56	11.14	1.57	37.8
15% PEG4000 - 20% MgSO ₄	1.05	0.45	0.57	9.14	1.29	38.3
20% PEG4000 - 20% MgSO ₄	1.54	0.60	0.74	7.00	0.99	36.2
25% PEG4000 - 20% MgSO ₄	1.90	1.36	0.07	0.16	0.02	1.3

3 -No phase separation

4 Abbreviation: See Table 1 footnote.

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1 Table 3 Purification of spleen proteinase from three tuna species using 15%PEG1000
 2 – 20%MgSO₄ ATPS

Fraction	Total activity Units	Total protein μg	Specific activity Units/μg prtein	Purification fold	Yield (%)
Yellowfin tuna	10898.14	1551.77	7.02	1.00	100.0
ATPS*	7543.86	162.21	46.51	6.62	69.2
Skipjack tuna	10588.54	1801.04	5.88	1.00	100.0
ATPS*	7787.42	379.70	20.51	3.49	73.6
Tongol tuna	10032.25	1889.59	5.31	1.00	100.0
ATPS*	8276.36	429.67	19.26	3.63	82.5

3 *15%PEG1000 – 20%MgSO₄ ATPS

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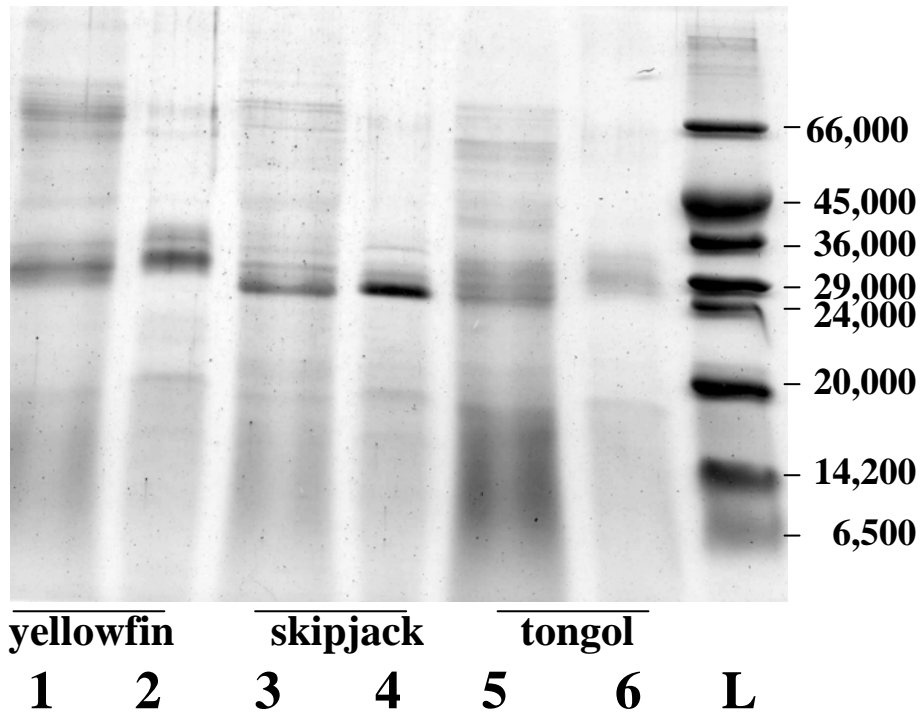


Fig. 1

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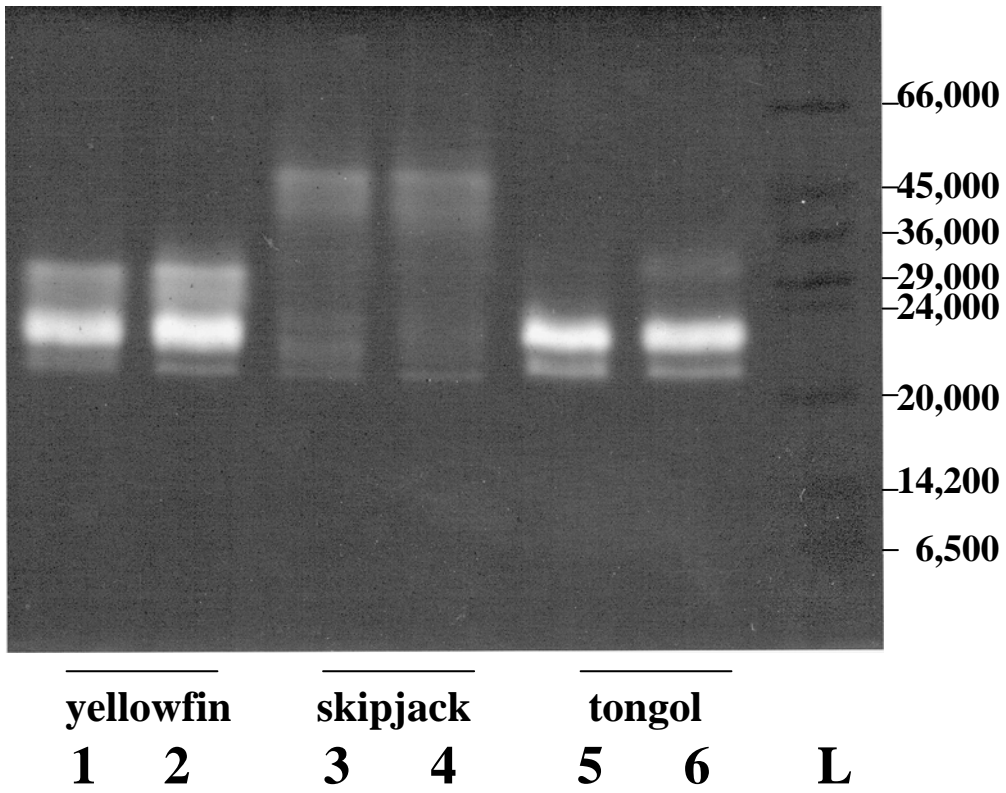


Fig. 2

1 **Figure Legends**

2 Figure 1. SDS-PAGE of spleen extracts and ATPS fraction from different tuna

3 species. L, Low-molecular-weight standard; lane 1,3,5, spleen extract; lane

4 2,4,6, 15% PEG1000-20% MgSO₄ ATPS fraction.

5 Figure 2. Activity staining of spleen extracts and ATPS fraction from different tuna

6 species. L, Low-molecular-weight standard; lane 1,3,5, spleen extract; lane

7 2,4,6, 15%PEG1000-20%MgSO₄ ATPS fraction.

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