Partition of Pepsinogen from the Stomach of Red Perch (Sebastes marinus) by Aqueous Two Phase Systems: Effects of the Salt Type and Concentration

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Abstract
An important acidic protease, pepsin, is synthesized and secreted in the gastric membrane in an inactive state called pepsinogen (PG) and has applications in the food and manufacturing industries, collagen extraction, gelatin extraction and in regulating digestibility. Fish processing waste can be used to produce commercially valuable by-products such as pepsinogen. In the present study, the purification of pepsinogen from the stomach of red perch using aqueous two phase systems (ATPS) formed by polyethylene glycol (PEG) and salt at 4°C was optimized. The effects of salt type (MgSO4, (NH4)2SO4, Na2C2H6O7 and K2HPO4) and concentration (6, 7, 8, 9, 10, 11, 12, 13, 15, 17, 19%) on the partitioning of PG were studied and parameters including total volume (TV), volume ratio (VR), enzyme activity (AE), protein content (CP), specific activity (SA), partition coefficient (KP), purification fold (PF) and recovery yield (RY) were evaluated. Salt type and salt concentration had significant effects on each parameter. MgSO4, (NH4)2SO4, Na2C2H6O7 and K2HPO4 each showed different effects at different salt concentrations (9, 12, 12 and 10%, respectively) to form biphasic systems. TV and VR decreased with increased salt concentration since salt formed hydrogen bonds with water molecules and created a more compact and ordered water structure. AE, CP, SA, PF and RY showed a maximum increase with intermediate salt concentration, while KP had the opposite pattern. The highest TV and AE values were obtained at 12% (NH4)2SO4 while the highest SA and PF values were obtained at 12% MgSO4. The highest TV and CP values were obtained at 12 and 15% Na2C2H6O7, respectively. (NH4)2SO4 at 15% concentration gave the highest RY (71.7%) and was selected as the optimum salt type and concentration. Thus, 15% (NH4)2SO4 - 18% PEG 1500 was the optimal ATPS combination and presented the best partition. The values of SA and PF and RY obtained with ATPS method were two fold higher than those obtained with the ammonium sulphate fractionation (ASF) method.

Keywords: Fish waste; Pepsinogen; Xtraction; Purification; Fractionation; Enzyme activity; Protein concentration; Recovery yield

Introduction
The increasing consumption of fish has led to a thriving fish processing industry worldwide. A large fraction (30–80%) of processed fish (flesh, heads, bones, fins, skin, tails and viscera) is generated as waste which is usually disposed of in landfills or poured directly into the sea resulting in high disposal cost and causing environmental problems. This conventional disposal of fish waste underscores the need for a reasonable utilization approach and effective recovery of valuable ingredients from this waste. Fish waste can be utilized as animal feed ingredients [1] and organic fertilizers [2] and for the recovery of valuable biomolecules such as collagen [3–6], α-3 fatty acids [7], trypsin [8,9], chymotrypsin [10-12] and elastase [13]. Among these valuable products, pepsin is one of the most abundant and useful biomolecules that can be recovered from fish viscera.

Pepsin is an important acidic protease widely applied in the hydrolysis of proteins in the food and manufacturing industries [14], collagen extraction [15-17], gelatin extraction [18], cheese making [19] and regulating digestibility [20]. Recovering pepsins from fish viscera significantly may reduce the capital costs of enzyme production and the cost of disposal of fish wastes.

Pepsin and itszymogen, pepsinogen (PG), were widely purified from several fish species including arctic fish capelin (Mallotus villosus) [21], rainbow trout (Salmo gairdneri) [22], Atlantic cod (Gadus morhua) [23], bolti fish (Tilapia nilotica) [2], Antarctic rock cod (Trematomus bernacchii) [24], sea bream (Sparuslates Houttuyn) [25], African coelacanth (Latimeria chalumnae) [26], Mandarin fish (Siniperca chuatsi) [27], smooth hound (Mustelus mustelus) [28], orange-spotted grouper (Epinephelus coioides) [29], albacore tuna (Thunnus alalunga) [30] and European eel (Anguilla anguilla) [31]. Several conventional purification techniques such as ammonium sulfate fractionation (ASF), gel filtration chromatography (GFC) and ion exchange chromatography (IEC) are frequently used. Although conventional purification methods can give good enzyme purity, they are complex, time-consuming and expensive. Therefore, an innovative, efficient and economical method for the purification of pepsin and PG that gives both high yield and high purity is needed. Recently, aqueous two phase systems (ATPS) has been established as an effective pathway to purify proteins. However, its efficacy and feasibility for pepsin and PG purification is not well investigated.

Objectives
The aim of this study was to evaluate the efficacy of the ATPS purification method using polyethylene glycol (PEG)-salt combinations...
for purifying pepsinogen (PG) from fish processing waste. The specific objectives were: (a) to study the effect of salt type (MgSO₄, (NH₄)₂SO₄, Na₂C₂H₃O₂, K₂HPO₄) and concentration (6-19%) on the total volume (V₉), volume ratio (V₅), enzyme activity (Aₚ), protein concentration (CP) specific activity (SA), purification fold (PF), partition coefficient (Kₚ) and recover yield (RY) and (b) to compare the effectiveness of the ATPS and ASF methods in purifying PG on the basis of SA, PF and RY.

Materials and Methods

Chemicals and reagents

Polyethylene glycol (PEG1000) was obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Hemoglobin, bovine serum albumin, trichloroacetic acid (TCA) and Coomassie Brilliant Blue G-250 were purchased from Fisher Scientific Company (Ottawa, Ontario, Canada). Analytical grade salts of (NH₄)₂SO₄, MgSO₄, Na₂C₂H₃O₂, K₂HPO₄, KPO₄ and NaSO₄ were procured from Fisher Scientific Company (Ottawa, Ontario, Canada). Reagents (50 mM sodium phosphate buffer, 100 mM phosphate-citrate buffer) were prepared as described by Ruzin [32].

Sample collection and preparation

Red perch (Sebastes marinus) was collected from the Fisherman’s Market (607 Bedford Highway, Halifax, Nova Scotia, Canada) and packed in polyethylene bag and transported in ice to the Biotechnology Laboratory, Department of Process Engineering and Applied Science, Dalhousie University, Halifax. The fish guts were opened and the individual organs were separated. The stomachs were collected and the undigested food in the stomach was removed. The stomach mucosa was rinsed with cold distilled water, then immediately frozen and stored at -20°C until used in the experiments. This was done to minimize autolysis of enzymes.

Crude pepsinogen extraction

Frozen stomachs (35 g) were thawed using running water (4°C) until the core temperature reached -2 to 0°C. The samples were cut into pieces of 1.0-1.5 cm and homogenized in four volumes of 50 mM sodium phosphate buffer having a pH of 7.0. The homogenate was centrifuged in a refrigerated high speed table top centrifuge (IEC Centra-MP4R, International Equipment Company, Needham, Massachusetts, USA) at 15,000 g and 4°C for 20 min to remove the tissue debris. The supernatant was collected and referred to as crude PG (crude PG). Crude extract was divided and stored in 4 ml vials at -20°C.

Pepsinogen purification by ATPS

The ATPS procedure used to purify pepsinogen (PG) is shown in Figure 1. Four salt types (MgSO₄, (NH₄)₂SO₄, Na₂C₂H₃O₂ and K₂HPO₄) and 11 salt concentration (6, 7, 8, 9, 10, 11, 12, 13, 15, 17, 19 %w/w) were evaluated in order to select the best salt and the optimum salt concentration. PEG with a molecular weight of 1000 and a concentration of 18% (w/w) was used in these experiments. ATPS were prepared in 15 ml centrifuge tubes by mixing PEG, salts and crude extract according to the methods described by Klomkla and Nalilanonn [33,34]. ATPS were initially prepared at room temperature but the extracted PG had poor stability and showed a rapid decrease in activity at the room temperature. Therefore, all the purification steps were performed at 4°C to reduce the autolysis or self-digestion of enzyme that resulted in drop of activity. The solid salt was selected from (NH₄)₂SO₄, Na₂C₂H₃O₂, MgSO₄ and K₂HPO₄ and 50% stock of 18% PEG 1000 solutions were weighted and mixed to achieve the designated concentrations (6-19%) in an aqueous system at room temperature. Although attempts were made to use buffers, it was difficult to maintain the pH of 7.0 for all salts in the bottom phase. Therefore, distilled water was used to adjust the system to obtain the final weight of 9 g (pH range in the top phase: 5.8-6.2 for MgSO₄, 6.1-6.3 for (NH₄)₂SO₄, 8.2-8.9 for Na₂C₂H₃O₂, 9.2-9.5 for K₂HPO₄). The mixtures were mixed using a vortex mixer (Fisher G-560 Vortex Genie 2, G-560, Fisher Scientific, Ottawa, Ontario) until the salt was completely solubilized and then stored at 4°C. Attempts were also made to use NaSO₄ and KPO₄ in ATPS. However, at 4°C, NaSO₄ failed to form an ATPS at concentrations of 9-19% due to low solubility and high viscosity. KPO₄ did form an ATPS within this salt concentration range and temperature but gave very low RY (<10%), which may be attributed to the destruction and denaturation of PG at the very basic pH created by KPO₄. Therefore, NaSO₄ and KPO₄ were eliminated as salts used in PG purification with ATPS.

Approximately 1 g crude PG extract (thawed overnight) was added into the cold salt and PEG mixture and mixed by inversion several times. The cold mixture was transported in ice for centrifugation. After centrifugation for 5 min at 2000 g and 4°C (IEC Centra-MP4R, International Equipment Company, Needham, Massachusetts, USA), the phases were separated. For each tube, the top phase (polymer phase) and bottom phase (salt phase) were carefully separated using a pre-chilled pipette and the interface (≤0.05ml) was discarded. Volumes of the separated phases were measured using a 10 ml graduated cylinder. According to Imelio and Nalilanon [34,30] about 60-95% PG partitioned into the top phase. Aliquots of the top phase were taken for determination of enzyme activity while aliquots of both phases were taken for determination of protein content. Based on purity and yield, the salt type and concentration which gave the highest RY was selected as the most effective purification salt.

Pepsinogen purification by ammonium sulphate fractionation (ASF)

The ASF procedure used to purify PG is shown in Figure 2. Approximately 4 g PG crude extract were used for ASF purification. Ammonium sulfate powder was added very carefully and slowly and the precipitates in the saturation range of 20–60% were collected. The solution was brought to 20% saturation first and centrifuged in a refrigerated centrifuge (IEC Centra-MP4R, International Equipment Company, Needham, Massachusetts, USA) at 10,000 g for 15 min. The supernatant was collected and the volume was measured. Then the supernatant was brought to 60% saturation and centrifuged in the same way. After centrifugation, the precipitate was collected. The purified protease was dialyzed against 50 mM sodium phosphate buffer with pH 7.0 overnight during which time the buffer was changed three times. The enzymes were stored at 4°C for assay and comparison with ATPS.

Protease assay

The protease assay for the ATPS and ASF methods are different as the two phases in ATPS account for more parameters in the assay process. For the ATPS method, the protease assay parameters determined were: total volume (TV), volume ratio (VR), enzyme activity (A_E), protein content (C_P), specific activity (SA), partition coefficient (K_P), purification fold (PF) and recovery yield (RY). For the ATPS method, only A_E, C_P, SA, PF and RY were assessed. For the ATPS method, only A_E, C_P, SA, PF and RY were assessed. For the ATPS method, only A_E, C_P, SA, PF and RY were assessed. For the ATPS method, only A_E, C_P, SA, PF and RY were assessed. For the ATPS method, only A_E, C_P, SA, PF and RY were assessed. For the ATPS method, only A_E, C_P, SA, PF and RY were assessed.

Determination of total enzyme activity (A_E)

Aliquots of the PEG-rich top phase of the ATPS were taken for the determination of A_E. The activity of PG was determined as described by Anson and Mirsky [37] with a minor modification. Crude or purified PG (0.5 ml) was added to 2.5 ml of 2% (w/v) hemoglobin in a phosphate-citrate buffer. The reaction was conducted at a pH of 2.5 and a temperature of 37°C for 10 min. To terminate the enzymatic reaction, 5 ml of 5% (w/v) trichloroacetic acid (TCA) were added. The nonhydrolyzed substrates were filtered and removed. The clear filtrate was collected and the absorbance was measured at 280 nm using a spectrophotometer (Ultrrospec 1100-pro, Amersham Pharmacia Biotech, Piscataway, NJ, USA). One unit was defined as an increase of 1.0 in absorbance at 280 nm per minute at pH 2.5 and 37°C. A blank was conducted in a similar way and protease was added into the reaction mixture after the addition of 5% (w/v) TCA. The activity was assayed in triplicate. Aliquots from ASF purification were assayed in a similar way.

Determination of apecific enzyme activity (SA)

The SA of recovered proteases in the top PEG phase was determined in units/mg protein as follows [30]:

\[
SA = \frac{A_E}{C_P} \quad (\text{units/mg protein})
\]

where:

- A_E is the enzyme activity in the top phase (U)
- C_P is the protein content in the top phase (mg)

Determination of protein content (C_P)

Aliquots of both phases were taken for determination of C_P according to the method described by Bradford [35] using BSA as a standard. BSA solutions (0.1 ml) with concentrations of 0, 100, 200, 400, 600 and 800 µg/ml were prepared in test tubes by mixing stock BSA solution (1 mg/ml) with enzyme buffer. The samples to be tested for protein (0.1 ml) were pipetted (0.1 ml) into the similar test tube. Each tube containing BSA solutions and protein samples were made up to 5 ml with Bradford reagent and mixed using a vortex mixer. The color reaction was conducted at room temperature for 5 min. The absorbance was measured at 595 nm. A standard curve (Figure 3) of absorbance vs protein concentration was plotted. A blank was conducted and triplicates were performed. PEG mixed with the protein samples consistently caused a small reduction in absorbance. According to Barbosa [36], this effect can be reduced if the PEG concentration is diluted below 10% (w/w). Therefore, this dilution was made for all samples and the dilution factor was taken into account in the calculation of the original C_P.

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where:

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- C_P is the protein content in the top phase (mg)
Determination of purification fold (PF)

The PF (also called purification factor) of PG in the top phase was defined as follows [30]:

\[
P_F = \frac{S_A^T}{S_A^C} \tag{2}
\]

where:

- \( S_A^T \) is the SA of the purified top phase in ATPS (U/mg)
- \( S_A^C \) is the SA of the crude PG extract (U/mg)

Determination of partition coefficient (Kp)

The Kp of recovered proteases for the ATPS was defined as follows [30]:

\[
K_p = \frac{C_T}{C_B} \tag{3}
\]

where:

- \( C_T \) is the CP in the top phase (mg)
- \( C_B \) is the CP in the bottom phase (mg)

Determination of volume ratio (VR)

The VR of recovered proteases for the ATPS was defined as follows [30]:

\[
V_R = \frac{V_T}{V_B} \tag{4}
\]

where:

- \( V_T \) is the volume of the top phase (ml)
- \( V_B \) is the volume of the bottom phase (ml)

Determination of total volume (TV)

The TV of recovered proteases in the ATPS was defined as follows [30]:

\[
T_V = V_T + V_B \tag{5}
\]

where:

- \( V_T \) is the volume of the top phase (ml)
- \( V_B \) is the volume of the bottom phase (ml)

Determination of recovery yield (RY)

The protease RY was calculated using the ratio of protease activities as follows [30]:

\[
R_Y = \frac{A_T}{A_C} \times 100 \tag{6}
\]

where:

- \( A_T \) is \( A_T \) in the top phase (U)
- \( A_C \) is the \( A_C \) of the crude PG extract (U)

Statistical analysis

Analysis of variance (ANOVA) was performed on the data. Duncan multiple test was also performed on the data to determine the differences among the levels of the salt types and concentrations. All the statistical analysis of data was conducted using Minitab statistics software 16 (Minitab Inc., State College, PA, USA).

Results

Crude pepsinogen extraction

The results of the extraction of crude PG from the stomach (35 g) of red perch are summarized in Table 1. During the extraction process, the volumes of crude extract first decreased from 175 to 121 ml after centrifugation due to the removal of the mucosa residues and then increased to 152 ml after dialysis because of the absorption of water. AE and CP decreased from 2154 to 1655 U and from 3871 to 1595 mg, respectively. SA and PF increased from 0.56 to 1.04 U/mg and from 1.00 to 1.90, respectively. RY decreased from 100.0 to 86.6%.

Purification by ATPS

The effects of MgSO4, (NH4)2SO4, Na3C6H5O7 and K2HPO4 on ATPS purification using 18% PEG 1000 are presented in Table 2. MgSO4, (NH4)2SO4, Na3C6H5O7 and K2HPO4 started to form two phases at the salt concentrations of 9, 12, 12 and 10%, respectively. They were also able to form two phases up to 19%. Although higher salt concentrations could form ATPS, a low RY was achieved in preliminary experiments. Therefore, 19% was selected as an upper level for purification by ATPS.

The analysis of variance performed on the data (Table 3), indicated that the effects of salt type and salt concentration were significant at the 0.0001 level. A significant interaction between the salt type and salt concentration was also observed at the 0.0001 level.

Total volume (TV)

The effects of MgSO4, (NH4)2SO4, Na3C6H5O7 and K2HPO4 on TV are shown in Figure 4a. TV decreased with increased salt concentration. TV initially decreased by 5.28 and 7.56%, when the concentrations of MgSO4 and K2HPO4 salts were increased from 9 to 19% and then decreased by 5.12 and 5.13% when the concentrations of (NH4)2SO4 and Na3C6H5O7 salts were increased from 12 to 19%, respectively. (NH4)2SO4 at 12% concentration gave the highest TV value (8.93 ml) while K2HPO4 at 19% concentration gave the lowest TV value (8.19 ml).

Volume ratio (VR)

The effects of MgSO4, (NH4)2SO4, Na3C6H5O7 and K2HPO4 on VR are shown in Figure 4b. All VR sharply decreased first with increased salt concentration (up to 13%) and then decreased more gradually with further increases in salt concentration. VR of MgSO4 decreased by 70.03% when its concentration was increased from 9 to 12% and then decreased by another 6.63% when its concentration was further increased from 12 to 19%. The VR of (NH4)2SO4 decreased by 69.24% when its concentration was increased from 12 to 19% and then decreased by another 16.55% when its concentration was further increased.

<table>
<thead>
<tr>
<th>Extraction step</th>
<th>Total extract volume (ml)</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg protein)</th>
<th>Purification fold</th>
<th>Recovery yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After homogenation</td>
<td>175</td>
<td>2154</td>
<td>3871</td>
<td>0.56</td>
<td>1.00</td>
<td>100.0</td>
</tr>
<tr>
<td>After centrifugation</td>
<td>121</td>
<td>1969</td>
<td>2022</td>
<td>0.97</td>
<td>1.75</td>
<td>91.41</td>
</tr>
<tr>
<td>After dialysis</td>
<td>152</td>
<td>1655</td>
<td>1595</td>
<td>1.04</td>
<td>1.90</td>
<td>86.55</td>
</tr>
</tbody>
</table>

Sample size = 35 g

Table 1: PG extraction parameters from red perch.
from 13 to 19%. The \( V_\text{S} \) of Na\(_2\)C\(_6\)H\(_5\)O\(_7\) decreased by 62.88% when its concentration was increased from 12 to 13% and then decreased by another 17.04% when its concentration was further increased from 13 to 19%. The \( V_\text{S} \) of K\(_2\)HPO\(_4\) decreased by 56.42% when its concentration was increased from 12 to 15% and then decreased by another 17.65% when its concentration was further increased from 12 to 19%. This resulted in total reductions in \( V_\text{S} \) of 80.00%. The highest total CP was given by K\(_2\)HPO\(_4\) at the concentration of 12%.

### Protein content (\( C_p \))

The effects of MgSO\(_4\), (NH\(_4\))\(_2\)SO\(_4\), Na\(_2\)C\(_6\)H\(_5\)O\(_7\), and K\(_2\)HPO\(_4\) on \( C_p \) are shown in Figure 5. The \( C_p \) in both phases (as well as total \( C_p \)) showed a maximum at intermediate salt concentration.

In the top phase, the \( C_p \) of MgSO\(_4\) increased by 3.23% when the salt concentration was increased from 9 to 10% and then decreased by another 45.83% when the salt concentration was further increased from 10 to 19%. The \( C_p \) of (NH\(_4\))\(_2\)SO\(_4\) and Na\(_2\)C\(_6\)H\(_5\)O\(_7\) increased by 4.07% and 3.25% when the salt concentrations were increased from 12 to 15% and then decreased by 10.61% and 6.30% when the salt concentrations were further increased from 15 to 19%, respectively. The \( C_p \) of K\(_2\)HPO\(_4\) increased by 4.35% when the salt concentration was increased from 10 to 11% and then decreased by 21.53% when the salt concentration further increased from 11 to 19%. The highest \( C_p \) in the top phase was given by K\(_2\)HPO\(_4\) at the concentration of 12%.

In the bottom phase, the \( C_p \) of MgSO\(_4\) increased by 7.58% when the salt concentration was increased from 9 to 10% and then decreased by 59.15% when the salt concentration was further increased from 10 to 19%. The \( C_p \) of (NH\(_4\))\(_2\)SO\(_4\) and Na\(_2\)C\(_6\)H\(_5\)O\(_7\) increased by 18.75 and 12.28% when the salt concentrations were increased from 12 to 15% and then decreased by 39.47% when the salt concentrations were further increased from 11 to 19%. The highest \( C_p \) in the bottom phase was given by MgSO\(_4\) at the concentration of 12%.

### Table 2: Effects of MgSO\(_4\) concentration on partition of 1 g PG using 18% PEG 1000.

<table>
<thead>
<tr>
<th>Salt Type</th>
<th>Salt Concentration (% w/w)</th>
<th>Volume (ml)</th>
<th>( V_\text{S} ) (U)</th>
<th>( A_p ) (U)</th>
<th>( C_p ) (mg)</th>
<th>( K_p )</th>
<th>PF</th>
<th>RY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO(_4)</td>
<td>9</td>
<td>6.7±0.01</td>
<td>9.9±0.01</td>
<td>8.7±0.02</td>
<td>3.47±0.02</td>
<td>6.56±0.12</td>
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<td></td>
<td>10</td>
<td>5.6±0.01</td>
<td>2.7±0.01</td>
<td>8.6±0.02</td>
<td>2.14±0.01</td>
<td>6.80±0.16</td>
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<tr>
<td></td>
<td>11</td>
<td>5.0±0.01</td>
<td>3.4±0.01</td>
<td>8.5±0.01</td>
<td>1.45±0.01</td>
<td>6.75±0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>4.3±0.01</td>
<td>4.1±0.01</td>
<td>8.4±0.02</td>
<td>1.04±0.01</td>
<td>6.42±0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>3.9±0.01</td>
<td>4.4±0.01</td>
<td>8.4±0.02</td>
<td>0.88±0.01</td>
<td>5.99±0.27</td>
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<tr>
<td></td>
<td>14</td>
<td>3.8±0.01</td>
<td>4.5±0.01</td>
<td>8.3±0.03</td>
<td>0.84±0.01</td>
<td>5.32±0.19</td>
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<td></td>
<td>15</td>
<td>3.7±0.01</td>
<td>4.6±0.01</td>
<td>8.2±0.01</td>
<td>0.82±0.00</td>
<td>4.82±0.10</td>
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Top phase: PEG phase
Bottom phase: salt phase

Table 2: Effects of MgSO\(_4\) concentration on partition of 1 g PG using 18% PEG 1000.

The effects of MgSO₄, (NH₄)₂SO₄, Na₃C₆H₅O₇ and K₂HPO₄ on total enzyme activity (AE) are shown in Figure 6a. AE initially increased and then decreased with increased salt concentration. The AE of MgSO₄ increased by 3.66% when the salt concentration was increased from 9 to 10% and then decreased by 50.29% when the salt concentration was further increased from 10 to 11%. The AE of (NH₄)₂SO₄ and Na₃C₆H₅O₇ increased by 10.94 and 6.06%, when the salt concentrations were increased from 12 to 15% and then decreased by 28.68 and 25.19% when the salt concentrations were further increased from 15 to 19%, respectively. The AE of K₂HPO₄ increased by 6.89% when the salt concentration was increased from 10 to 11% and then decreased by 34% when the salt concentration was further increased from 11 to 19%. The highest AE was achieved with (NH₄)₂SO₄ at the concentration of 15%.

Specific enzyme activity (SA)

The effects of MgSO₄, (NH₄)₂SO₄, Na₃C₆H₅O₇ and K₂HPO₄ on specific enzyme activity (SA) are shown in Figure 6b. All SA initially increased and then decreased with increased salt concentration. The SA of MgSO₄ increased by 0.85% when the salt concentration was increased from 9 to 11% and then decreased by 8.58% when the salt concentration was further increased by 88.89%. The SA of (NH₄)₂SO₄ and Na₃C₆H₅O₇ increased by 6.60 and 2.70% when the salt concentration was increased from 12 to 15% and then decreased by 20.18 and 20.39% when the salt concentration was further increased from 15% to 19%, respectively. SA of K₂HPO₄ increased by 3.27% when the salt concentration increased from 10 to 11% and then decreased by 20.25% when the salt concentration was further increased by 11 to 19%. The highest SA was achieved with MgSO₄ at the concentration of 12%.

Partition coefficient (Kp)

The effects of MgSO₄, (NH₄)₂SO₄, Na₃C₆H₅O₇ and K₂HPO₄ on partition coefficient (Kp) are shown in Figure 7a. The Kp of (NH₄)₂SO₄, Na₃C₆H₅O₇, K₂HPO₄ and MgSO₄ showed minimum at intermediate salt concentrations. The Kp of MgSO₄ decreased by 4.29% when the salt concentration was increased from 9 to 11% and then increased by 35.82% when the salt concentration was further increased from 11 to 19%. The Kp of (NH₄)₂SO₄ and Na₃C₆H₅O₇ decreased by 12.45 and 8.10% when the salt concentration was increased from 12 to 15% and then decreased by 20.18 and 20.39% when the salt concentration was further increased from 15% to 19%, respectively.
concentrations were increased from 12 to 15% and then increased by 47.77 and 46.10% when the salt concentrations were further increased by 15 to 19%. The K_P of K_2HPO_4 decreased by 13.30% when the salt concentration was increased from 10 to 12% and then increased from 9.97 to 15.2% when the salt concentration was further increased from 12 to 19%. The highest K_P was achieved with K_2HPO_4 at the concentration of 19%.

Purification fold (PF)

The effects of MgSO_4, (NH_4)_2SO_4, Na_3C_6H_5O_7 and K_2HPO_4 on PF are shown in Figure 7b. All PF increased initially and then decreased with increased salt concentration. The PF of MgSO_4 increased by 0.74% when the salt concentration was increased from 9 to 11% and then decreased by 8.61% when the salt concentration was further increased from 11 to 19%. The PF of (NH_4)_2SO_4 and Na_3C_6H_5O_7 increased by 6.60 and 2.82% when salt concentrations were increased from 12 to 15% and then decreased by 20.24 and 19.86% when the salt concentrations were further increased from 15 to 19%, respectively. The PF of K_2HPO_4 increased by 12.59% when the salt concentration was increased from 10 to 11% and then decreased by 15.79% when the salt concentration was further increased from 11 to 19%. The highest PF was achieved with MgSO_4 at the concentration of 12%.

Recovery yield (RY)

The effects of MgSO_4, (NH_4)_2SO_4, Na_3C_6H_5O_7 and K_2HPO_4 on RY are shown in Figure 7c. All RY increased initially and then decreased with increased salt concentration. The RY of MgSO_4 increased by 3.65% when the salt concentration was increased from 9 to 10% and then decreased by 50.32% when the salt concentration was further increased from 10 to 19%. The RY of (NH_4)_2SO_4 and Na_3C_6H_5O_7 increased by 10.82 and 6.01%, respectively when the salt concentrations were increased from 12 to 15% and then decreased by 28.59 and 24.93% when the salt concentrations were further increased from 15 to 19%, respectively. The RY of K_2HPO_4 increased by 6.72% when the salt concentration was increased from 10 to 11% and then decreased by 32.93% when the salt concentration was further increased from 11 to 19%. The highest RY was achieved with (NH_4)_2SO_4 at the concentration of 15%.

Comparing ATPS and ASF methods

The values of A_E, CP, SA, PF and RY were 30.66 ± 1.84 U, 12.0 ± 0.32 mg, 2.55 ± 0.14 U/mg, 2.46 ± 0.14 and 70.4 ± 4.23% for ASF and 37.67 ± 0.38, 6.98 ± 0.12, 5.40 ± 0.09, 5.20 ± 0.08 and 86.6 ± 0.88 for ATPS, respectively. The comparison between the ATPS and the ASF methods shown in Table 4. All the parameters were significantly different between the two methods at the 0.05 level.

Discussion

Extraction of crude pepsinogen

The same PG extract and crude PG were used in the ATPS and ASF purification methods. A_E, CP and RY decreased while SA and PF increased.
increased during PG extraction. This indicated that some of the PG was lost but the portion remaining was concentrated, resulting in a higher purity. The higher purity was due to the removal of proteins and small molecular peptides. Lower RY was due to the destruction of enzyme structure and denaturation of PG caused by homogenization, centrifugation and dialysis.

Effects of salt type and concentration in ATPS

The results showed that different initial salt concentrations were required for the formation of the two phases. These differences may be attributed to differences among ionic strengths of the different salts. Shang et al. [38] observed similar results and suggested that the required salt concentration was controlled by the ionic radius of the negative ions. According to Huddleston et al. [39], formation of a biphasic system is based on the balancing of enthalpic and entropic effects involved in the aqueous hydration of the solutes. The enthalpic effect is repulsion, driven by the total energy of a thermodynamic system. The entropic effect is a thermodynamic force resulting from the change in density of the aqueous solution caused by salt type and/or salt concentration. Salt is a more charge-polarized compound that is capable of hydrogen bonding with water molecules than PEG (which is more hydrophobic than the salt). As salt concentration increase, more salts migrate to the aqueous PEG phase and break its original water structure. Water molecules will undergo a rearrangement and surround the salt to form a more ordered water layer [41]. Therefore, a more compact water structure with a smaller volume of PEG molecules will be formed Nalinanon et al. [30].

There were obvious similarities in the relationships of A50 and RY (Figures 6a and 7c) and SA and PF (Figures 6b and 7b). Because purified PG was derived from the same stomach samples, the A50 of the crude PG had a constant value in all experiments. RY was calculated from the ratio of A50 after purification to the value of A50 crude extract. Thus, RY only depended on A50 from purified PG and produced very similar trends. The same reasoning applied for the relationships of PF and SA. Nalinanon et al. [30], Spelzini et al. [42] and Imelio et al. [34] reported that PG partitioned predominantly in the PEG-rich top phase but was negligible in the bottom phase. In the present study, A50 was measured into all top phases whereas in the bottom phase A50 was only measured in the ATPS of MgSO4 and no activity was found. A50 in the bottom phase was not measured in the ATPS of the other three salts. These results are consistent with those of Nalinanon et al. [30].

To form two phases, a critical salt concentration (dependent on the temperature) must be met and higher salt concentration was required at low temperatures. The critical concentrations for ATPS formation at 4°C were found to be 9, 12, 12 and 10% (w/w) for MgSO4, (NH4)2SO4, Na3C6H5O7 and K2HPO4, respectively.

The salt type had a significant effect on TV and VR. The TV and VR decreased with increased salt concentration. Similar patterns were found by Nalinanon et al. [30]. The decrease in TV may be attributed to the change in density of the aqueous solution caused by salt type and/or salt concentration. Salt is a more charge-polarized compound that is capable of hydrogen bonding with water molecules than PEG (which is more hydrophobic than the salt). As salt concentration increase, more salts migrate to the aqueous PEG phase and break its original water structure. Water molecules will undergo a rearrangement and surround the salt to form a more ordered water layer [41]. Therefore, a more compact water structure with a smaller volume of PEG molecules will be formed Nalinanon et al. [30].

The mechanism driving PG partition is not well-understood. One possible explanation is that the phase to which the protein partitioned is determined by its hydrophobic properties and net charge. Protein tends to go to the PEG-rich top phase because its hydrophobic groups have electrostatic interactions with the oxygen molecules in the PEG chains Nalinanon et al. [30]. Another possible explanation is that negatively charged protein tends to go to the PEG-rich phase while positively charged protein tends to go to the salt-rich phase (Klomklao et al. [33]; Del-Val and Otero [43]; Yang et al. [44]). According to Bandmann et al. [45], better partitioning of protein can be achieved in the top phase as their negative charge increases. Their results showed that with an isolectric point (pl) of 1-1.5, PG was negatively charged in all partition systems with a neutral or a slightly basic pH environment and was, therefore, only found in the PEG-rich top phase. Johansson et al. [46] reported that the partitioning of proteins is influenced by the presence of salts and the effect is enhanced with increases in the net charge of the protein. The partition patterns are determined by the balancing of salting-in and salting-out effects in the top phase. In a study by Arakawa and Timasheff [47], it was postulated that the salting-in effect occurred at low concentrations where salts stabilize proteins and other polyelectrolytes through nonspecific electrostatic interactions in the top phase which are dependent on the ionic strength of the medium and favor partition of PG and proteins. Salting in has a stronger effect than salting-out. At high concentrations, however, salts exert specific effects on proteins resulting in the destabilization or denaturation of proteins and a reduction in solubility, causing a salting out effect. They also noted that salting-in has a stronger effect than salting-out.

The results of A50 indicate that the efficacy of the salts and the
preferential hydration of protein follow the lyotropic series, a classification of ions based upon salt-ins or salt-out ability, where a stronger hydration favors PG partition in the top phase due to an increase in the solubility of PG (Huddleston et al. [39]; Rawdkuen et al. [48]). According to, Carbonnaux et al. [49] and PrimerDigital [50], anions are more effective than cations for salt-ins. The most effective multi-charged anions are $SO_4^{2-}$ > $HPO_4^{2-}$ > $C_6H_5O_7^{3-}$ > $C_4H_4O_6^{2-}$, regardless of the cations used whereas the most effective cations or cation combination are $Li^+$ > $Na^+$ > $K^+$ > $NH_4^+$ > $Mg^{2+}$ > $Ca^{2+}$.

Higher hydration and interaction means more enzymes in the top phase. Based on this, the partition effect of the four salts used in present study should follow the sequence ($NH_4)_2SO_4 > MgSO_4 > K_2HPO_4 > Na_3C_6H_5O_7$, which was consistent with AE and RY results obtained in the present study. For instance, at a concentration of 13%, AE was ($NH_4)_2SO_4 > 5.99 U for MgSO_4 > 4.13 U for K_2HPO_4 > 3.72 U for Na_3C_6H_5O_7.

Chaiwut et al. [51] and Rawdkuen et al. [48] used the same four salts to partition protease from Calotropis procera latex and reported similar results. However, the C_p in the top phase did not follow this sequence. This may be due to the hydrophobic properties of other proteins present as impurities. Those proteins may have a weaker interaction with the anion and did not follow lyotropic series.

The partition coefficient of the enzyme (K_p) and the partition coefficient of the protein (K_o) are used to characterize the distribution of enzyme and protein in the ATPS. The K_p and K_o refer to the ratio of the enzyme activities and the ratio of protein contents in the top and bottom phases, respectively. When the enzyme is present in both phases, K_p can be calculated as with trypsin [32] and protease [47]. In the present study, the K_p could not be calculated because PG was not present in the bottom phase. Alternatively, K_p can be used to characterize the partition. For PG, a low K_p usually gives a high PF and implies a higher purity of the interested enzyme [29,32,47] as was the case with $K_2HPO_4$, MgSO_4 and ($NH_4)_2SO_4$. For example, MgSO_4 with a K_p, 1 gave a high P_f (>6.2), $K_2HPO_4$ with a higher K_p (>9), gave a low P_f (<1.2 PF<1.5). Higher K_p implies that more impurities of proteins and peptides were partitioned in the top phase. However, Na C_6H_5O_7 gave a similar K_p to ($NH_4)_2SO_4 but produced a much lower PF, which indicated a high C_p but low A_p in the top phase. This may be attributed to the salt properties such as ionic charge and strength. For instance, C_6H_5O_7^-$, which carries more charges, may have stronger effect (anionic denaturation) that reduces the enzyme stability. Moreover, the weak interaction between C_6H_5O_7^- and PG as predicted by the lyotropic series also offers explanations for this effect.

The RY was considered to be the most important parameter in the present study. The results showed that all the salt types gave a PF range of 1.17-6.26, much lower than that achieved with chromatography. The ATPS method is only the first step (PF of 1-10) in PG partition; further steps such as chromatography (PF can be 10-400 at proper conditions) must be followed for higher purity. Although MgSO_4 at 11% gave the highest enzyme productivity, ($NH_4)_2SO_4 at 15%, giving the highest RY, was chosen as the best salt type and salt concentration.

### Comparing ATPS and ASF methods

The ammonium sulfate fractionation (ASF) was performed using the saturation range selected by Bougatfel et al. [28] and Feng et al. [29]. Using similar saturations for PG purification, the ASF method gave a SA of ~0.88-3.0 U; PF of ~1.1-3.7 and RY of ~64-75% [28,26,25,52]. Present data for SA, PF and RY were in these ranges. ATPS and ASF are based on different separation principles. ATPS employed selective partitioning of the protein of interest in one aqueous phase while other proteins remained in the other phase. In contrast, ASF purified the protein of interest by selective precipitation of protein within one saturation range based on protein solubility while other proteins remained in the solution. The A_p, C_p, SA, PF and RY obtained from the ATPS and ASF methods were significantly different from each other. The ATPS method gave a higher A_p than that of the ASF method while the ASF method gave a higher C_p than that of the ATPS method. The ASF method was found to be less selective to protein separation than ATPS method and therefore it provided more proteins as impurities mixed with PG. The ATPS method gave much higher SA, PF and RY compared to those of the ASF method. Therefore, ATPS method showed better partition and higher effectiveness than ASF method.

### Conclusions

The partition of PG from red perch using ATPS at 4°C was investigated. The effects of the salt type (MgSO_4, ($NH_4)_2SO_4, Na C_6H_5O_7, K_2HPO_4) and concentration (6, 7, 8, 9, 10, 11, 12, 13, 15, 17, 19%) on the total volume (V_t), volume ratio (V_r), specific activity (SA), purification fold (PF), partition coefficient (K_p) and recovery yield (RY) on the purification of chymotrypsin from red perch were studied. The salt type and salt concentration had significant effects on all these parameters. MgSO_4, ($NH_4)_2SO_4, Na C_6H_5O_7, and K_2HPO_4 required different minimum salt concentrations (9, 12, 12 and 10%, respectively) to form biphasic systems. The results showed that the TV decreased with increased salt concentration with all salts. All V_t sharply decreased first with increased salt concentration (up to 13%) and then slowly decreased with further increases in salt concentration. ($NH_4)_2SO_4 at 12% concentration gave the highest TV value while K_2HPO_4 at 19% concentration gave the lowest TV value. The A_p, C_p, SA, PF and RY of the top phase are shown in Table 4.

### Table 4: ASF and ATPS comparison.

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<th>Purification Method</th>
<th>A_p (U)</th>
<th>C_p (mg)</th>
<th>SA (U/mg)</th>
<th>PF</th>
<th>RY (%)</th>
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<tr>
<td>ASF</td>
<td>30.66 ± 1.84</td>
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<td>ATPS</td>
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<td>P-value*</td>
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* P-values were obtained from the t-test by comparing A_p, C_p, SA, PF and RY of the two methods, respectively.
SA, PF and RY initially increased with increased salt concentration and then decreased, while the Kc had an opposite pattern. The salt partition effects were determined by the balancing of salting-in and salting-out effects. Low salt concentrations favoured salting-in and stabilized the PG while high salt concentrations produced salting-out and caused an adverse partition effect. The salt efficacy and preferential hydration followed the lyotropic series and higher interaction gave a higher A and RY. A low Kc usually gave a high purity of PG while the skin of brownstripe red snapper (lutjanus vitta). Food Chemistry 93: 475-484.


