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Extraction of Chymotrypsin from Red Perch (*Sebastes marinus*) Intestine Using Reverse Micelles: Optimization of the Backward Extraction Step

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Abstract

Fish processing waste can be used to produce valuable by-products such as chymotrypsin which has applications in the food, leather, chemical and clinical industries. In this study, a reverse micelles system of AOT/isooctane was used to extract chymotrypsin from crude aqueous extract of red perch intestine. The effects of pH and KCl concentration of the backward extraction step on the total volume (TV), volume ratio (VR), total activity (TA), enzyme activity (A_E), specific activity (SA), purification fold (PF), protein concentration (Cp) and recovery yield (RY) were studied. Changing the pH from 6.5 to 8.5 and the KCl concentration from 0.5 to 2.0 M during the backward extraction step had no effects on the TV or VR. Increasing the pH from 6.5 to 7.5 increased A_E, SA, Cp, PF and RY by up to 47.06%, 30.0%, 27.0%, 26.9% and 18.47%, respectively but they all then declined with further increases in the pH. Similar trends were observed when the KCl concentration was increased from 0.5 to 1.5 M. The decreases in these parameters were due to the denaturation of protein under high pH. The highest A_E, Cp and RY were achieved with pH 7.5 and 1.0 M KCl concentration while the highest SA and PF were achieved with pH 7.5 and 1.5 M KCl concentration. Addition of isobutyl alcohol in the backward extraction step increased the TV, A_E, TA, Cp, SA, PF and RY by 13.6%, 336.4%, 342.6%, 81.1%, 146.4%, 146.2% and 345.8%, respectively. Alcohol reduced the interfacial resistance for the reverse micelles and, thus, destroyed the reverse micelles structure. The values of A_E, TA, SA, PF and RY obtained with reverse micelles methods were much higher (2.3 fold) than those obtained with the ammonium sulphate method.

Keywords: Fish waste; Chymotrypsin; Extraction; Purification; Ultrafiltration; Fractionation; Microemulsions; Enzyme activity; Protein concentration; Recovery yield

Introduction

Currently, fish waste is an approved substance for disposal at sea and the Canadian fish industry is dumping all fish waste into the sea because there is no economical way of utilizing the waste off shore and it is costly to transport the large amount of fish waste to meal plants or land-based waste disposal systems [1,2]. The decomposition of large volumes of wastes lowers the level of dissolved oxygen in the water and generates toxic by-products [3,4]. However, fish waste is a valuable resource and can be used to produce value added products such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), enzymes (pepsin, trypsin, chymotrypsin), collagen and oil [5-7].

Chymotrypsin has wide applications in food, leather processing, chemical and clinical industries. Industrially, chymotrypsin is produced from fresh cattle or swine pancreas and is commonly made in either a tablet form for oral consumption or as a liquid for injection. The price of chymotrypsin is related to the cost of raw material and the purity of the products. Using fish waste, rather than fresh cattle or swine pancreas, could dramatically lower the cost of chymotrypsin production. Chymotrypsin can be extracted from fish waste using a reverse micelles method. Reverse micelles are thermodynamically stable molecules that can extract large biomolecules like proteins through electrostatic interaction that attracts soluble proteins into the inner layer of the reverse micelles [8,9]. They form amphiphilic structures in polar organic media which can be used to extract large amounts of proteins in the aqueous phase without denaturation.

The reverse micelles extraction process is divided into two steps: forward extraction and back extraction. During the forward extraction step, the aqueous and organic phases are separately prepared and homogenized. After transfer of protein from the aqueous phase to the organic phase, the phases are separated by centrifugation and the protein is measured [8-10]. In the forward extraction step, selection of surfactants and pH play significant roles in protein stabilization. Sodium di-2-ethylhexyl sulfosuccinate (AOT) is the most common surfactant used in chymotrypsin purification [8-11]. pH influences ionic molecular interactions in solution and therefore, influences the efficiency of extraction by reverse micelles [8,10]. In the backward extraction step, protein is transferred from reverse micelles to the aqueous solutions. This step is usually very slow and salt is added into the aqueous phase to assist the process [9]. However, increasing chloride ion concentration will decrease chymotrypsin yield by competing with chymotrypsin in the extraction process and the effect is particularly significant at low ionic strength [8,11]. According to Goto et al. [12] and Hu and Gulari [9], the limitations of the backward extraction step are due to the difficulty in separating proteins from AOT reverse micellar phase and the excessive time involved in the process.

Objectives

The aim of this study was to optimize the backward extraction step of the reverse micelles method while purifying chymotrypsin from fish processing waste. The specific objectives were: (a) to study the effect of pH (6.5, 7.0, 7.5, 8.0 and 8.5) and salt concentration (0.5, 1.0, 1.5 and 2.0 M) and alcohol addition in the backward extraction step of the reverse micelles method on the enzyme activity (A_E), protein concentration (Cp), specific activity (SA), purification fold (PF) and recov-

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ery yield (RY)and (b) compare the effectiveness of the reverse micelles method to that of the ammonium sulphate precipitation method.

Materials and Methods

Chemicals and reagents

Tris, HCl, $CaCl_2$, NaCl, ammonium sulphate, N-benzoyl-L-tyrosine ethyl ester (BTEE) and the release, methyl alcohol and n-butyl alcohol were obtained from Sigma-Aldrich, Oakville, Ontario, Canada. AOT, isooctane (2,2,4-trimethylpentane), chymotrypsin, isobutyl alcohol and BSA (bovine serum albumin) were obtained from Fisher scientific, Ottawa, Ontario, Canada. Reagents used included 0.05M Tris-HCl buffer (0.01 M $CaCl_2$, pH 7.5), 15% v/v isobutyl alcohol, 10 µM BTEE.

Sample collection and preparation

The fish, red perch (*Sebastes marinus*), used in the experiment were collected from Clearwater Seafood's Ltd., Halifax, Nova Scotia, Canada. The intestines were separated from fish, washed with cold water and isotonic saline solution to remove the blood in the tissue according to the procedure described by Chong et al. [13] and Boeris et al. [14]. The fish intestine was chopped into small pieces (1 cm^3), weighed, marked and stored at -20°C.

Experimental design

Tables 1 and 2 show the experimental parameters investigated in this study and their levels. In the forward step pH of 8.0 and KCl concentration of 1.5M was applied [10]. In the backward extraction step, the effect of pH (6.5, 7.0, 7.5, 8.0 and 8.5) and four KCl concentration (0.5, 1, 1.5 and 2 M) were studied. After optimization of the backward step, the effect of alcohol addition (15% v/v) on chymotrypsin recovery yield was studied. Finally, both the reverse micelles extraction method and the ammonium sulphate extraction method were compared on the basis of enzyme activity, specific activity purification fold and recovery yield.

Factors	Parameters
Forward Extraction	
AOT	20 mM
pH	7.0
Time	30 min
Temperature	4˚C
Backward Extraction	
Salt concentration	0.5, 1.0, 1.5 and 2 M
pH	6.5, 7.0, 7.5, 8.0, 8.5
Time	1 hr
Temperature	4˚C

No. of replicates = 3
No. of runs = 60

Table 1: Optimization of the backward extraction (changing pH and KCl concentration).

Factors	Parameters
Forward Extraction	
AOT	20 mM
pH	7.0
Time	30 min
Temperature	4˚C
Backward Extraction	
Salt concentration	Optimum
pH	Optimum
Isobutyl alcohol	15% v/v alcohol or 15% v/v distilled water
Time	1 hr
Temperature	4˚C

No. of replicates=3
Total no. of runs= 6

Table 2: Effect of alcohol addition during backward extraction.

Crude enzyme extraction

The extraction procedure (Figure 1) described by Heu et al. [15] and Castillo-Yáñeza et al. [16] was followed. The prepared frozen samples were thawed at 4°C overnight. A 50 g (wet basis) sample of fish gut was mixed with 150 mL isotonic saline solution and homogenized using a laboratory homogenizer (Polytron PT1035, Brinkmann Instruments, Toronto, Ontario, Canada) for 5 min, then incubated for 8 hr at 4°C to activate the chymotrypsinogen in the samples. After incubation, the sample was centrifuged at 20,000 g at 4°C for 30 min (MP4R, International Equipment Company, Needham, Massachusetts), then filtered and defatted with 50 mL CCl_4. The supernatant was considered a crude enzyme extract. The volume at each step was measured and the activity and concentration of crude enzyme were determined.

Ammonium sulphate extraction

The procedure (Figure 1) described by Kunitz [17] was followed. Ammonium sulphate was slowly added to the crude extract to reach 35% saturation with continuous stirring. The mixture was stirred for a further 30 minutes at 4°C and then centrifuged at 20,000 g for 15 min (MP4R, International Equipment Company, Needham, Massachusetts). The supernatant was collected and the saturation was then adjusted to 70% by addition of ammonium sulphate. After 30 min, the suspension was centrifuged at 20,000 g for 15 min. The pellets collected from the 35% and 70% saturation steps contained precipitated enzymes. Dialysis was performed on the pellets using Tris-HCl buffer. The enzyme concentration, activity and yield were then determined.

Reverse micelles extraction

The process of extracting chymotrypsin from fish waste by the reverse micelles method is shown in Figure 1. The forward extraction step was carried out usingan AOT concentration of 20 mM and at a pH of 7.0. In the forward extraction step, crude enzyme was mixed with AOT organic solution and stirring at 300 rpm using magnetic stirrer for 30 minutes and then separating the two phases by centrifugation. Aqueous solutions containing different KCl concentrations (0.5, 1, 1.5 and 2M) were prepared. Tris buffer was used as a stripping solution in the backward transfer and the pH of the solution was adjusted to 6.5, 7.0, 7.5, 8.0 or 8.5 as recommended by Hu and Gulari [9]. Equal volumes of aqueous solution and organic solution were mixed in the tube and 15% v/v isobutyl alcohol was added to the reversed micelles phase. Then the two phase systems were mixed for one hour in a beaker placed on a magnetic stirrer (Canlab NO. S8290, Atlanta GA, Georgia, USA). The mixture was then centrifuged for 15 min at 4000 rpm (MP4R, International Equipment Company, Needham, Massachusetts) in order to separate into two phases [9,12]. The enzyme activity and yield were determined. During the backward extraction step the salt concentration and pH were changed and the same procedure was followed.

Effect of alcohol

After the optimal conditions for the backward extraction were determined, the effect of alcohol addition was studied. Distilled water was used as a control. The same volumes (1.5 mL) of distilled water and isobutyl alcohol were added to the aqueous phase during backward extraction.

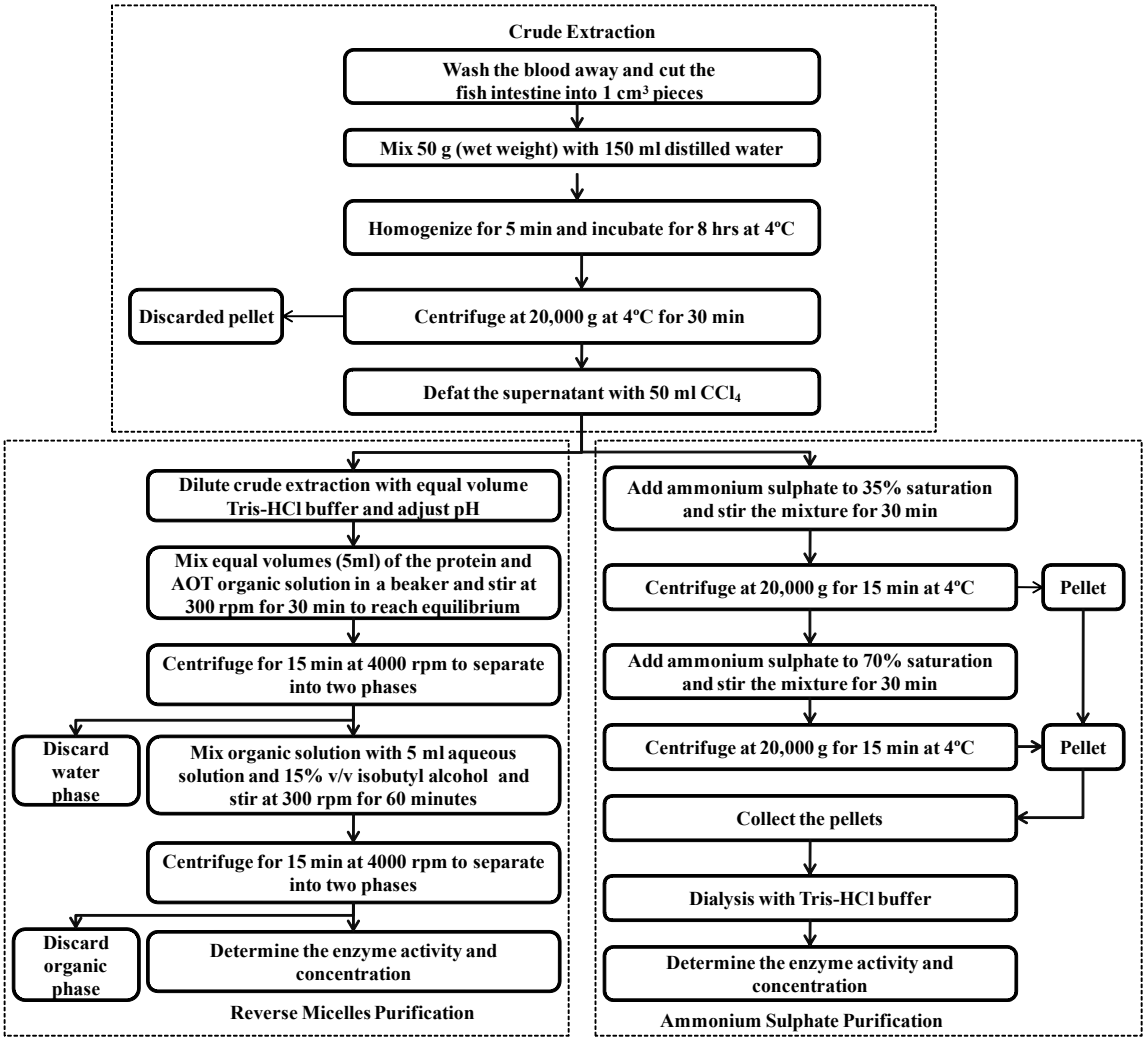

Figure 1: The extraction procedure of chymotrypsin from fish waste.

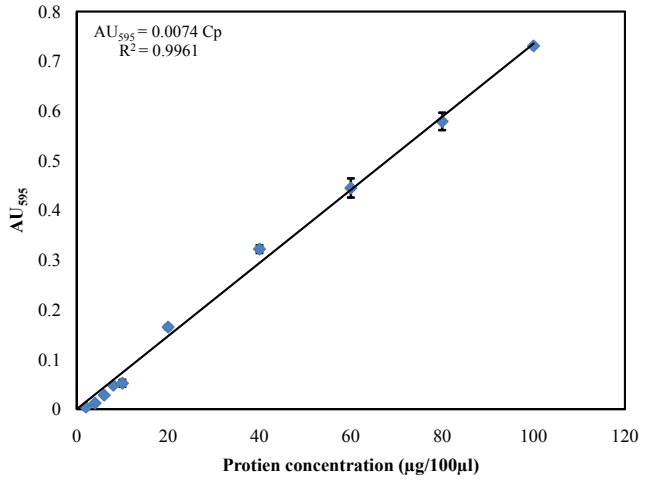

Figure 2: The standard curve for protein concentration.

Determination of protein concentration

The Bradford method was used for the determination of protein concentration according to the procedures described by Yang et al. [18] and Castillo-Yáñeza et al. [16]. Two standard curves were developed using a series of concentrations of bovine serum albumin (BSA): the standard curve (assay range 10-150 µg/mL) and a micro standard curve (assay range 1-10 µg/mL). The following solutions were prepared: (a) 0.1 g of BSA was dissolved in 10 mL of Tris-HCl buffer at room temperature (b) the stock BSA solution was diluted to span the 100- 1,500 µg/mL range and (c) BSA solution in the range of 100-900 g/mL was diluted ten times more for the micro standard curve. The two standard curves are combined together as shown in Figure 2.

10 µL of each standard was mixed with 5 mL of Bradford reagent. Each sample was allowed to incubate at room temperature for 10 minutes and the absorbance of each standard was measured at 595 nm against a blank that was composed of 10 µL of buffer and 5 mL of Bradford reagent. 0.1 mL diluted sample (concentration between 5 to 100 µg/L) was mixed with 5 mL Bradford reagent, incubated for 5

min and then the absorbance was measured at 595 nm [19]. The result was compared with the standard curve to determine the sample protein concentration.

Determination of enzyme activity

The activity of chymotrypsin was defined as the change of absorbance measured at 256 nm in one minute caused by the addition of 1 mL of chymotrypsin protein solution [20]. The substrate used in the experiment was benzoyl- tyrosine ethyl ester (BTEE). The p-nitroaniline was cleaved by BTEE and the release of N-benzoyl-L-tyrosine was followed by recording the increase in the absorbance every min for 5 min at 256 nm [13]. 1.5 mL Tris-HCl buffer (0.08 M tris, pH 7.8, 0.1M $CaCl_2$), 1.4 mL of 0.00107 M BTEE and 0.1 mL test enzyme solution were placed into cuvettes. The enzyme activity was calculated as follows.

$$A_E = \frac{\Delta U_{256} \times (3) \times (Df)}{(0.964) \times (0.10)} \quad (1)$$

Where:

A_E : Enzyme activity (Units/mL Enzyme)

ΔU_{256} : The change of the absorbance at the wave length 256 nm per minute

3 : Volume of reaction mixture (mL)

Df : Dilution factor

0.964 : Millimolar extinction coefficient of BTEE at 256 nm

0.10 : Volume of test enzyme solution used in assay (mL)

Determination of total activity (TA)

The total activity is defined as the change in the absorbance value per min for the total chymotrypsin extracted from the entire sample of red perch intestine. The total activity was calculated as follows.

$$TA = \frac{\Delta U_{256} \times (3) \times (Df)}{(0.964) \times (0.10)} \times \text{Total volume volume of crude extraction} \quad (2)$$

Determination of specific activity (SA)

Specific activity is defined as the ability of 1 mg enzyme to hydrolysis BTEE in one min at a pH of 7.5 and a temperature of 25°C. 0.1 mL enzyme solution was added into cuvettes and the change in absorbance was measured at 256 nm every half minute for 5 minutes. The specific activity was calculated using the following equation.

$$S_A = \frac{\text{Units / mL Enzyme}}{\text{mg protein / mL Enzyme}} \quad (3)$$

Where:

S_A: Specific activity (Units/mg Protein)

Determination of purification fold (PF)

Purification fold is used to evaluate the increase in purity of the enzyme after the purification step. It was calculated using the following equation:

$$PF = \frac{\text{Units / mg Purified protein}}{\text{Units / mg Crude protein}} \quad (4)$$

Determination of recovery yield (RY)

Recovery yield is defined as the ratio of total refined enzyme activity and total crude enzyme activity. Recovery represents the chymotrypsin activity remaining in the purification process. When combined with specific activity, it can show the effectiveness of a purification method.

$$RY = \frac{\text{Units / mL Purified protein}}{\text{Units / mL Crude protein}} \times 100\% \quad (5)$$

Statistical analysis

The data for solution volume protein concentration and activity

Extraction step	TV (mL)	AE (Unit/mL)	TA (Unit)	Cp (µg/mL)	SA (Unit/mg)	PF (-)	RY (%)
After Homogenizing*	183	0.80	146.40	4486.20	0.18	-	-
AfterCentrifuging	144	0.94	135.36	1975.80	0.48	2.69	92.70
AfterDilution							
and pH adjustment	288	0.47	135.36	987.90	0.48	-	100.00

*Sample size: 50 g

Table 3: Crude protein extraction parameters.

Parameter	Purified chymotrypsin
A_E (Unit/mL)	0.22±0.01
TA (Unit)	1.10±0.05
Cp (µg/mL)	41.64±1.16
SA (Unit/mg)	5.31±0.19
PF (-)	11.10±0.39
RY (%)	46.72±2.23

*Sample size: 5 mL

Table 4: Extraction parameters after ammonium sulphate precipitation.

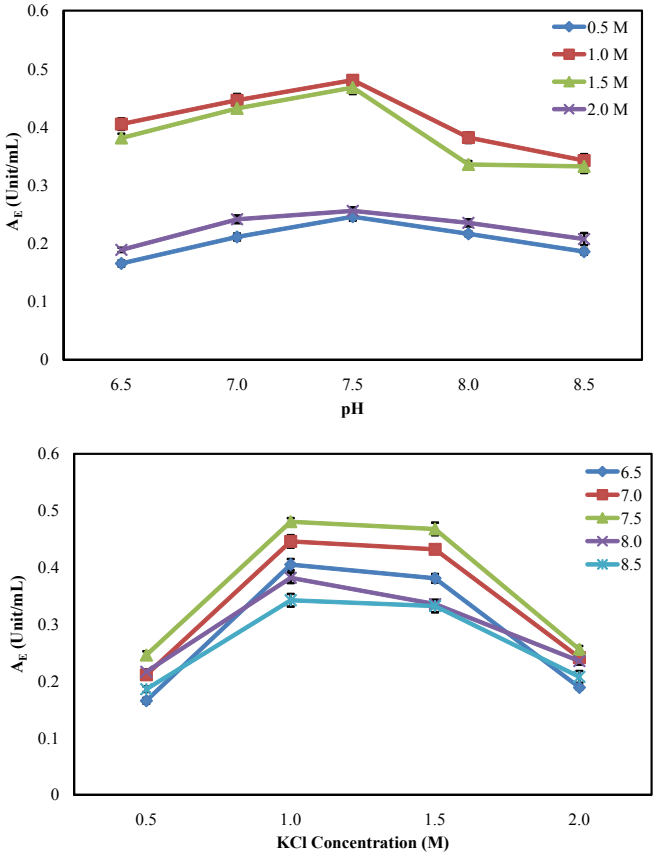

Figure 3: Effects of pH and KCl concentration on the enzyme activity.

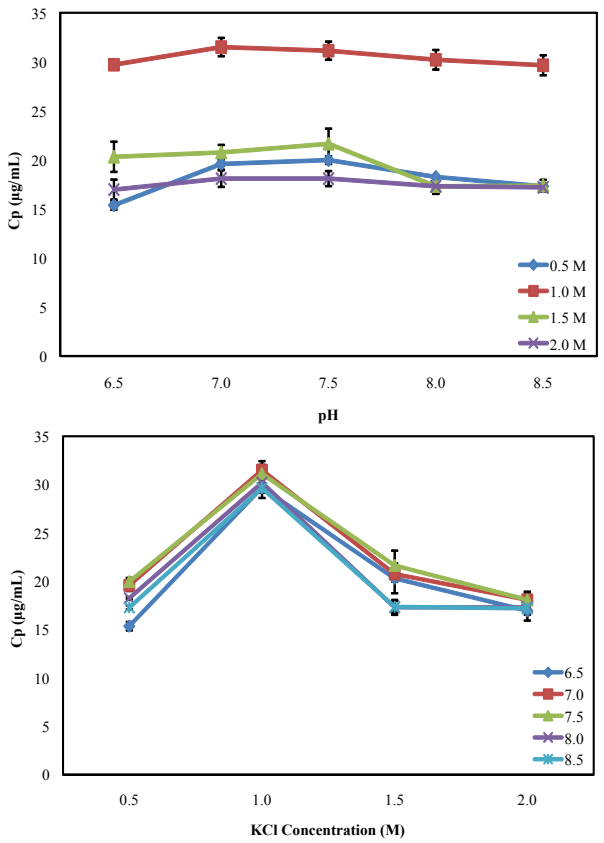

Figure 4: Effects of pH and KCl concentration on the protein concentration.

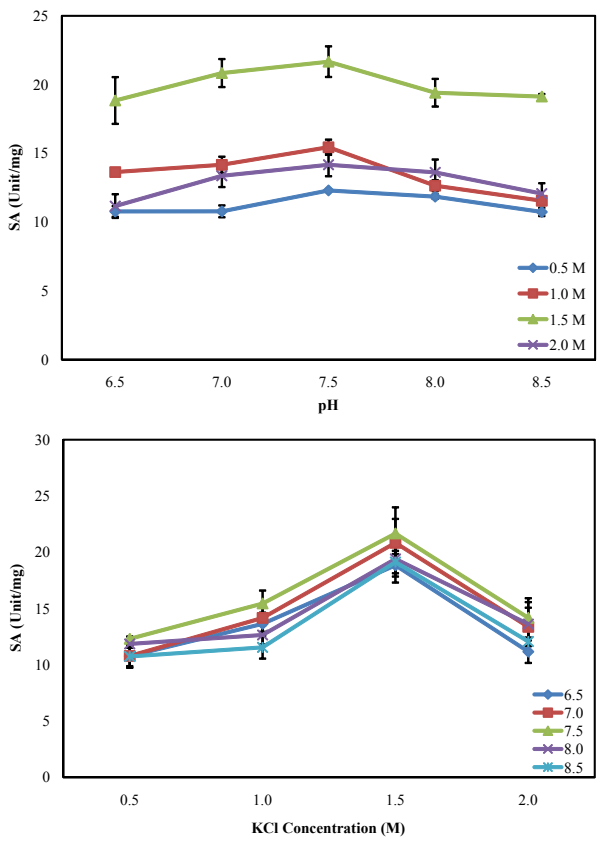

Figure 5: Effects of pH and KCl concentration on the specific activity.

were collected and total activity, recovery yield, specific activity and standard errors were calculated. The α-level was chosen as 0.05. All the statistical analysis of data was conducted using Minitab statistics software (Ver 15.1.10, Minitab Inc) to examine the coefficient data with a two-way analysis of variance (ANOVA) to determine the significant effects of single and two parameters on the results.

Results

Crude extraction

Crude protein was extracted from the intestine (50 g) of red perch and the total volume (TV) was measured after homogenization, centrifugation and dilution. The enzyme activity (A_E), total activity (TA), specific activity (SA), protein concentration (Cp), purification fold (PF) and recovery yield (RY) were determined as shown in Table 3. After centrifugation, the TV, TA and Cp decreased from 183 to 144 mL (21.32%), from 146.40 to 135.36 U (7.54%) and from 4486.2 to 1975.8 µg/mL (55.96%), while the A_E and SA increased from 0.80 to 0.94 U/mL (17.50%) and from 0.18 to 0.48 U/mg (169.1%), respectively. The purification fold and the recovery yield were 2.69 and 92.7%, respectively.

Ammonium sulphate extraction

The crude extract was purified using the ammonium sulphate precipitation method. The A_E, TA, Cp, SA, PF and RY were determined (Table 4). The A_E, TA and Cp values of the purified enzyme were lower than those of the crude enzyme. The A_E, TA and Cp decreased from 0.47 to 0.22 Unit/mL (53.3%), from 2.37 to 1.10 Unit/mL (53.3%) and from 987.90 to 41.64 µg/mL (95.8%), respectively. On the other hand, the SA and PF of purified enzyme were much higher than those of crude enzyme. The SA increased from 0.48 to 5.31 Unit/mg (1012%) and the PF increased from 2.69 to 11.1 (313.64%). The final yield was 46.72%.

Reverse micelles extraction

The effects of salt concentration and pH on the backward extraction parameters (A_E, Cp, SA, PF and RY) were studied. Four levels of KCl salt concentrations (0.5, 1.0, 1.5 and 2.0 M) and five levels of pH (6.5, 7.0, 7.5, 8.0 and 8.5) were investigated. The results are shown in Figures 3-7. The analysis of variance performed on the data (Table 5) indicated that salt concentration and pH were highly significant at the 0.0001 level. The interaction between the salt concentration and pH was highly significant at the 0.0001 level. The highest values for the backward extraction parameters are shown in Table 6.

The volumes for water phase and organic phase remained constant regardless of the pH level and KCl concentration used. The TV was 11.5 mL and the VR was 0.77. The A_E increased initially when the pH and KCl were increased and then decreased with further increases in pH and KCl concentration (Figure 3). The maximum A_E value (0.48 unit/mL) was observed at pH 7.5 and KCl concentration of 1.0 M. The Cp showed similar trend with the maximum value (31.13 µg/mL) observed at pH 7.5 and KCl concentration of 1.0 M (Figure 4). The SA and PF showed similar behaviours with maximum values (21.67 unit/mg and 45.23) observed at pH of 7.5 and KCl concentration of 1.5 M (Figures 5 and 6). The RY had a maximum value (102.24%) at pH of 7.5 and KCl concentration of 1.0 M. These results showed that the optimum condition for the backward extraction step of chymotrypsin is at a pH of 7.5 and KCl concentration of 1.0 M and 1.5 M.

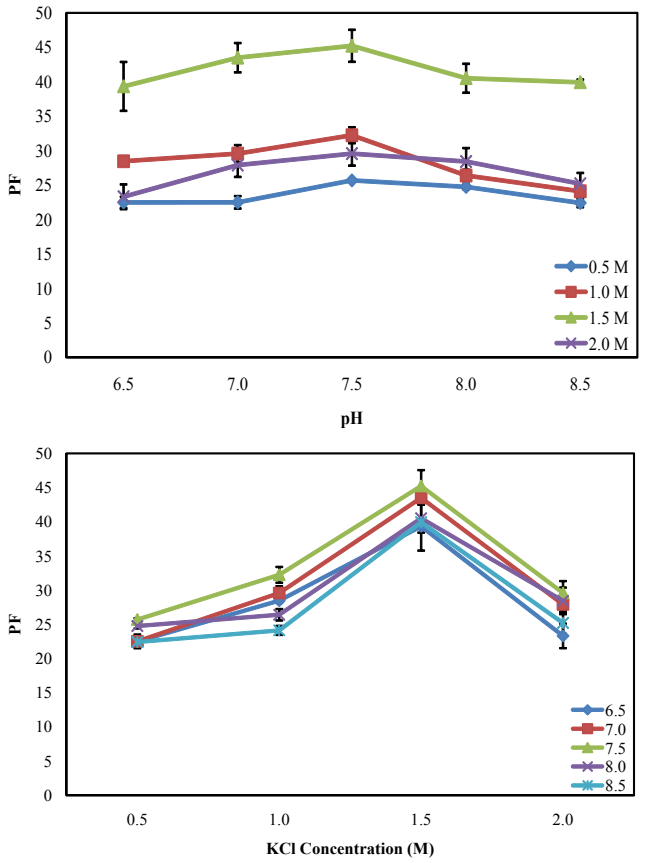

Figure 6: Effects of pH and KCl concentrations on the purification fold.

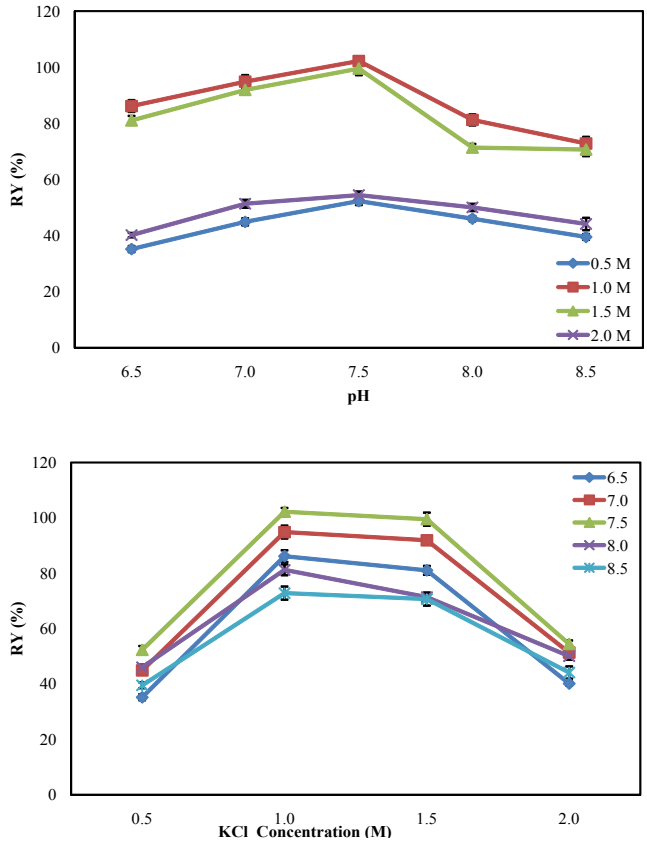

Figure 7: Effects of pH and KCl concentration on the recovery yield.

Alcohol effect

In order to determine the effect of alcohol in the backward extraction step, an experiment was carried out at the optimal conditions for forward (AOT 20 mM, and pH 7.0) and backward (KCl 1.0 M and pH 7.5) extraction in which isobutyl alcohol was added in the backward extraction step. Another experiment without alcohol was used as a control. The TV, AE, TA, Cp, PF and RY obtained form both experiments are shown in Table 7. When alcohol was added in the backward extraction step, the TV, A_E, TA, Cp, SA, PF and RY increased from 4.4 to 5.0 mL (13.6%), from 0.11 to 0.48 Unit/mL. (336.4%), from 0.54 to 2.39 U (342.6%), from 17.60 to 31.87 µg/mL (81.1%), from 6.08 to 14.98 Unit/mg (146.4%), from 12.70 to 31.27 (146.2%) and from 22.62% to 100.85% (345.8%), respectively

Comparing RM with AS

The reverse micelles (RM) method was compared to the ammonium sulphate (AS) method on the basis of their AE, TA Cp, SA, PF and RY (Table 8). The value of extraction parameters obtained with RM were much higher than those obtained with AS. The A_E, TA, and RY obtained with RM were 2 fold higher than those obtained with the AS method, while the SA and PF were 3 fold higher than those obtained with AS.

Discussion

Extraction profiles

After homogenization and centrifugation, the A_E, TA, Cp and RY

Parameters	Source	DF	SS	MS	F	P
A_E	Total	107	0.74882			
	pH	5	0.1533	0.03066	52.87	0.0001
	AOT	5	0.50872	0.10174	175.43	0.0001
	pH-AOT	25	0.04504	0.0018	3.11	0.0001
	Error	72	0.04176	0.00058		
Cp	Total	107	2496.22			
	pH	5	299.76	59.952	72.25	0.0001
	AOT	5	1930.26	386.051	465.26	0.0001
	pH-AOT	25	206.46	8.258	9.95	0.0001
	Error	72	59.74	0.83		
SA	Total	107	340.295			
	pH	5	105.573	21.1027	19.04	0.0001
	AOT	5	133.499	26.6998	24.09	0.0001
	pH-AOT	25	21.482	0.8593	0.78	0.758
	Error	72	79.801	1.1083		
PF	Total	59	3268.06			
	pH	4	213.18	53.294	21.89	0.0001
	AOT	3	2871.22	957.074	393.15	0.0001
	pH-AOT	12	86.28	7.19	2.95	0.0050
	Error	40	97.38	2.434		
RY	Total	59	28097.9			
	pH	4	3278	819.49	291.02	0.0001
	AOT	3	23562.5	7854.18	2789.23	0.0001
	pH-AOT	12	1144.7	95.39	33.88	0.0001
	Error	40	112.6	2.82		

pH = Mean effect of pH
AOT = Mean effect of AOT
pH-AOT=interaction between pH and AOT

Table 5: Analysis of variance for the various parameters.

Parameters	pH	KCl	Optimum values
A_E (Unit/mL)	7.5	1.0 M	0.48±0.06
Cp (µg/mL)	7.5	1.0 M	31.13±0.93
SA (Unit/mg)	7.5	1.5 M	21.67±1.11
PF(-)	7.5	1.5 M	45.23±2.32
RY (%)	7.5	1.0 M	102.24±1.35

*Sample size: 5 mL

Table 6: The optimum values of chymotrypsin purification parameters.

Parameters	Without alcohol	With alcohol
A_E (Unit/mL)	0.11±0.01	0.48±0.01
TA (Unit)	0.54±0.05	2.39±0.05
Cp (µg/mL)	17.60±5.48	31.87±1.31
SA (Unit/mg)	6.08±0.19	14.98±0.49
PF	12.70±0.39	31.27±1.02
RY (%)	22.62±2.23	100.85±2.23

Forward extraction: AOT concentration 20 mM, pH7.0
Backward extraction: KCl concentration 1.5 M, pH7.5
*Sample size: 5 mL

Table 7: Effect of alcohol addition on chymotrypsin purification parameters.

Parameters	AS	RM
A_E (Unit/mL)	0.22±0.01	0.48±0.01
TA (Unit)	1.10±0.05	2.39±0.05
Cp (µg/mL)	41.64±5.48	31.87±1.31
SA (Unit/mg)	5.31±0.19	14.98±0.49
PF	11.08±0.39	31.27±1.02
RY (%)	46.72±2.23	100.85±2.23

Forward extraction: AOT concentration 20 mM, pH7.0
Backward extraction: KCl concentration 1.5 M, pH7.5
*Sample size: 5 mL

Table 8: Optimum values of the AS and RM purification parameters.

decreased while the SA and PF increased which indicated that a portion of chymotrypsin was lost during the crude extraction process but the remaining portion was concentrated during the extraction.

Reverse micelles method

During the backward extraction step, salt was required to break the reverse micelles structure in the organic phase to release chymotrypsin into the aqueous phase. However, high salt concentrations will denaturate proteins [21]. The pH on the other hand affects the net charge of protein molecules and the electrostatic interaction force between the chymotrypsin and the surfactant. This in turn influences the extraction efficiency of the process [9,21-23]. In the present study, the effects of pH (6.5, 7.0, 7.5, 8.0 and 8.5) and KCl concentration (0.5, 1.0, 1.5, and 2.0 M) were investigated in order to determine the optimum conditions for the backward extraction step.

During the backward extraction step, the volumes for the organic and aqueous phases remained constant regardless of the pH and KCl concentration used. Thus, the total volume for each of the five pH levels and the four KCl concentrations was 11.5 mL and the VR of the two phases remained constant at 0.77. Several researchers reported similar results [9,12,21,22,24].

It has been reported that changes in the structure of proteins and the low rate of extraction are the main problems with the backward extraction step. Changing the pH and the concentration of salt in the aqueous phase are the most common methods applied to improve A_E, Cp, SA, PF and RY of the backward extraction [24-27]. In the study, A_E, Cp, SA, PF and RY reached their maximum values at pH 7.5 and KCl concentration of 1.5 M.

Goto et al. [12] reported that during the backward extraction of chymotrypsin using 200 mM AOT concentration, an increase in A_E (from 1 to 6 Unit/mL) was observed when the pH was increased from 4.0 to 7.5 which was followed by a decrease (to 0.5 Unit/mL) with further increases in the pH (to 11.0). When the positive net charge on the chymotrypsin surface decreased, the electrostatic interaction between the protein and the negative AOT molecular head became weaker and chymotrypsin was released into the aqueous phase [12].

When the pH of the aqueous phase approaches the isoelectric point (*pI*) of proteins, the Cp increases because of the electrostatic interaction between the protein and AOT reverse micelles is weakened and more protein molecules are released from the reverse micelles into the aqueous phase. However, further increases in the pH result in decreasing Cp because of protein denaturation. Ono et al. [21] reported that when the pH was increased during the extraction of haemoglobin using dioleyl phosphoric acid (DOLPA), the backward extraction rate dramatically increased reaching 90% at a pH of 8.0 and then declined with further increases in the pH. Similar results had been observed by Goto et al. [12].

RY is one of the most important parameters used in evaluating the extraction process. Generally, the RY of the backward extraction is relatively lower than the forward extraction due to the strong interaction between the protein and reverse micelles [9,11,12,21,23,24,28]. Several researchers reported similar effects of pH in the backward extraction on RY [12,21,23,28]. Hebbar et al. [28] reported that during the extraction of bromelain from pineapple waste, RY increased from 68 to 100% when the aqueous phase pH was increased from 3.9 to 4.2 and then decreased with further increases in pH. Goto et al. [12] reported a 100% RY of chymotrypsin using AOT-DOLPA at pH of 7.0.The reason that RY was over 100% in this study could be due to the presence of impurities with chymotrypsin.

Chang et al. [23] stated that the KCl concentration had significant effects on the radius of reverse micelles and changing the salt concentration affected A_E, Cp, SA, PF and RY. They reported that when KCl concentration was increased from 0.2 to 0.8 M, the radius of reverse micelles decreased from 62 to 41 Å which in turn decreased protein solubility and was responsible for the release of protein to the aqueous phase. High salt concentration resulted in unstable reverse micelle structures and led to increases in the A_E, Cp, SA, PF and RY. Hebbar et al. [28] reported that during the backward extraction step of extracting bromelain from pineapple waste, the A_E increased when the KBr concentration was increased from 0.25 to 0.50 M and then decreased when the KBr concentration further increased from 0.50 to 0.75 M. Hatton et al. [29] and Dekker et al. [25] also found that a high ionic strength in the aqueous phase was not good for protein extraction in the backward extraction step. Hong et al. [24] extracted BSA, carbon anhydrase and β-lactoglubulin from AOT reverse micelles using low ionic strength conditions in the aqueous phase (0.1M KCl) and found that high ionic strength could result in protein denaturation. High ionic strength was considered to be a salt concentration >1 M. High salt concentrations can destabilize reverse micelles and release target proteins back to the aqueous phase and as such increase SA. Hebbar et al. [28] reported that when the aqueous phase pH was increased from 3.9 to 4.2 during the backward extraction of bromelain from pineapple waste, SA and PF increased from 22.6 to 56.15 CDU/mg and from 2.1 to 5.3, respectively.

Then, they decreased to 25 CDU/mg and 2.4 when the pH was further increased to 4.5. They also reported that when the KBr concentration increased from 0.25 to 0.50 M, SA increased from 20 to 56 CDU/mg and then decreased to 23 CDU/mg and PF increased from 2.1 to 5.6 and then decreased to 2.4 when the KBr concentration was further increased to 0.75 M. Hu and Gulari [9] reported a RY of 67.6% during the extraction of α-chymotrypsin using sodium bis (2-ethyIhexyI) phosphate (NaDEHP) with a $CaCl_2$ concentration of 0.1 M. Hentsh et al. [11] reported a 100% RY during the backward extraction of chymotrypsin using 1.0 M KCl at pH of 8.0. In this study, SA and PF decreased when KCl concentration increased above 1.5 M, a higher concentration than that reported by others.

The optimum pH and KCl concentration that gave the highest A_E, Cp, SA, PF and RY for the backward extraction are shown in Table 4. The highest A_E, Cp and RY were reached at the pH 7.5 and KCl concentration of 1.0 M while the highest SA and PF were reached at the pH 7.5 and KCl of concentration 1.5 M. Since RY has been considered the most important parameter in evaluation of the extraction process, pH of 7.5 and KCl concentration of 1.0 M were chosen as the optimum backward extraction conditions. The optimal pH applied in the backward extractions (pH 7.5) is higher than that in forward extraction (pH 7.0). Ono et al. [21] reported pH 6.5 and 8.0 for the forward and backward extraction during the extraction steps of haemoglobin using DOLPA. Hentsch et al. [11] used pH 5.0 and 8.0 for forward and backward extraction steps during the extraction of chymotrypsin using AOT. Goto et al. [12] found the optimal pH conditions for extraction of chymotrypsin using AOT-DOLPA mixed reverse micelle system to be pH 6.8 for forward extraction and pH 7.0 for backward extraction.

Effect of alcohol on backward extraction

Adding alcohol in the backward extraction step increased TV, A_E, TA, Cp, SA, PF and RY. Hu and Gulari [9], Goto et al. [12], Ono et al. [21], Paradkar and Dordick [22] and Hong et al. [24] reported that a clear phase was quickly obtained in the presence of alcohol after stopping the stirring process and with the addition of 10-20%(v/v) alcohol in the backward extraction step, the protein transfer from reverse micelles was 10 times faster than in the absence of alcohol. Paradker and Dordick (1993) added 10%(v/v) ethyl acetate in the backward extraction step and noticed significant increases in A_E, TA and RY. Goto et al. [12] reported that without the addition of alcohol, 24 hours were required to obtain equilibrium in the back-extraction step and the extraction time was reduced to less than 2 hours by adding 10% (v/v) isobutyl alcohol. Hong et al (2000) reported that adding 10-15% isopropanol in the backward extraction step resulted in 100% extraction of pepsin and 70% extraction of chymotrypsin. Hu and Gulari [9] reported that only 10-20% RY were obtained using the NaDEHP reverse micelle system without the addition of alcohol in the backward extraction but 98% of active cytochrome-c and 67% of active chymotrypsin were recovered from the aqueous phase in the presence of alcohol.

Goto et al. [12] studied the effect of alcohol type on the RY and relative activity of recovered chymotrypsin. Their results showed that the RY of the backward extraction with isopropyl alcohol, isobutyl alcohol, isoamyl alcohol, n-hexyl alcohol, n-octyl alcohol, n-decanol and oleyl alcohol were 93.8%, 97.1%, 90.7%, 84.5%, 67.4%, 59.9%and 37.1%, respectively. The relative activity (recovered specific activity/original specific activity) for recovered chymotrypsin in the backward extraction using isopropyl alcohol, isobutyl alcohol, isoamyl alcohol, n-hexyl alcohol, n-octyl alcohol, n-decanol and oleyl alcohol were 0.03, 1.00, 0.74, 0.68, 0.24, 0.10 and 0.55 respectively.

Ono et al. [21] studied the effect of alcohol type and concentration in the backward extraction step using methanol, ethanol, isopropyl alcohol (IPA) and n-propyl alcohol (nPA) at concentrations of 0-30% (v/v). When the IPA and nPA were added in the back-extraction step, the hemoglobin recovery rate increased significantly from 0 to 60% when alcohol concentration was increased from 5 to 10% (v/v) and then decreased with further increases in the alcohol concentration. The hemoglobin recovery rate dramatically increased from 0 to 70% (v/v) when the ethanol concentration was increased from 10 to 15% (v/v) and then decreased when the concentration was further increased. The recovered hemoglobin rate dramatically increased from 0 to 70% (v/v) when the methanol concentration in the back extraction was increased from 20 to 30% (v/v). Alcohol can reduce the interfacial resistance for the reverse micelles because it promotes the fusion/fission of reverse micelles which destabilizes the structure.

Conclusions

Changing the pH from 6.5 to 8.5 and the KCl concentration from 0.5 to 2.0 M during backward extraction step (with addition of alcohol) had no effects on the total volume (TV) or the volume ratio (VR). The TV for all samples was 6.5 mL and the VR was 0.77. The reverse micelles emulsion structure was destroyed in the presence of alcohol. Increasing the pH from 6.5 to 7.5 increased the enzyme activity (A_E) by up to 47.06%, protein concentration (Cp) by up to 30.0%, specific activity (SA) by up to 27.0%, purification factor (PF) by up to26.9% and recovery yield (RY) by up to 18.47% and they all then declined with further increases in the pH. Similarly, increases in the KCl concentration from 0.5 to 1.0 M increased A_E by up to 192.9%, Cp by up to 93.2% and RY by up to 50.97% and they all then decreased with further increases in the KCl concentration. SA and PF continued to increase up to 93.3% when the KCl concentration increases from 0.5 to 1.5 M and then decreased with further increases in the KCl concentration. The decreases in A_E, Cp, SA, PF and RY were due to the denaturation of protein under a relatively high pH and the ionic strength caused by high pH and KCl concentration in the backward extraction step. The highest A_E, Cp and RY were achieved with pH of 7.5 and 1.0 M KCl concentration. The highest SA and PF were achieved with pH of 7.5 and 1.5 M KCl concentration. The optimal conditions for the backward extraction step was pH 7.5, KCl concentration 1.0 M. Addition of alcohol in the backward extraction step increased TV by 13.6%, A_E by 336.4%, TA by 342.6%,Cp by 81.1%, SA by 146.4%, PF by 146.2% and RY by 345.8% because alcohol reduced the interfacial resistance for the reverse micelles. The values of A_E, TA, SA, PF and RY obtained with reverse micelles methods were much higher (2.3 fold) than those obtained with the AS method.

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