Optimization of torpedo scad fish (*Megalaspis cordyla)* viscera protease extraction and purification

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ABSTRACT

Protease comprises the class of enzymes that are most used worldwide, accounting for 60% of the world total enzyme production. This study was carried out to determine the proteolytic activity of protease extracted from torpedo scad fish (*Megalaspis cordyla*) viscera, and to determine the optimum pH and ammonium sulphate concentration for the extraction and purification of the extracted visceral protease. The combination of optimum condition for pH and ammonium sulphate concentration was optimized using the Response Surface Methodology (RSM). The crude enzyme extracts are then precipitated in ammonium sulphate followed by dialysis and gel filtration chromatography where the proteolytic activity was studied. The concentration of ammonium sulphate used ranged from 40-80% and pH used for extraction was between 6-9. The enzyme showed the highest proteolytic activity at pH 9 and 80% ammonium sulphate concentration matching with RSM suggested condition. The proteolytic activity obtained under this condition was 445U. The total activity of the crude extract, ammonium sulphate precipitation, and dialyzed proteases were 820, 680 and 425U, respectively. The viscera contained 15.87% protein. Thus, the optimum condition for extraction and purification of protease from torpedo scad fish viscera were at pH 9 with 80% ammonium sulphate concentration, respectively resulting in high proteolytic activity.

Keywords*:* *Megalaspis cordyla*, torpedo scad, viscera, protease, ammonium sulphate, response surface methodology

INTRODUCTION

Torpedo scad (*Megalaspis cordyla*) is one of the famous fish species from the Carangidae family. The annual catches of torpedo scad in Malaysia are estimated at 22 000 tonnes, making up 15–20% of the total pelagic catch [1]. Approximately 75% of the fish supply is for human consumption in which 30% are processed into canned food, fish fillets, chips, and protein products while 25% is utilized for fish oil production [2]. It has been stated that for each tonne of fish eaten, an equal volume of fish material is discarded as waste [3]. Fish waste such as viscera, skin, bones and air bladder can be converted into useable products, however, most of them are used to produce feeds and fertilizers [4] whereas they can also be a valuable source of enzymes [5].

The most important proteolytic enzymes in fish viscera and other aquatic invertebrates are aspartic and serine protease as well as pepsin [6]. Studies involving the extraction of protease from fish viscera include pepsins from pectoral rattail (*Coryphaenoides pectoralis*) [6], trypsin from *Catla catla* [7], digestive protease from *Labeo rohita* [8] and alkaline proteases from thornback ray (*Raja clavata*) [9]. Proteases from fish viscera could be used in industrial applications, therefore recovery from viscera might be a partial solution to the wastage problem [10].

Response Surface Methodology (RSM) is a collection of mathematical and statistical technique used for modeling and analyzing problem [11]. It is an effective tool for optimizing processes whereby information can be obtained within the shortest time and the number of experiments required can be reduced [12]. In this study, the extraction condition and concentration of ammonium sulphate were optimized using RSM. The objectives of this study are 1) to determine the optimum extraction pH and optimum concentration of ammonium sulphate for the purification of protease from torpedo scad viscera using RSM and 2) to determine the proteolytic activity of the extracted and purified proteases.

EXPERIMENTAL

Materials

The viscera were provided by a local fish supplier in Shah Alam, Selangor, Malaysia. Fish samples were selected within the range of 23-27 cm in length. The range was categorized as Grade B fish. All chemicals used are of analytical grade purchased from Sigma Aldrich, USA.

Sample preparation

An amount of 100g viscera was first washed thoroughly with distilled water and then cut into small pieces of 0.5-1cm. This was followed by homogenizing in a blender (Model: Panasonic MX-SM1031S) for 30 seconds in 100 mL buffer (phosphate buffer, pH 7.5 and Tris-HCl) at a ratio of 1:1 of viscera to buffer. Then the mixture was centrifuged using a microcentrifuge

instrument (Model: Eppendorf 5418) at 10 000rpm for 15 minutes. The supernatant collected was known as the crude extract. The crude extract was then purified in ammonium sulphate (X_2) ranging from 40-80%. The pH (X_1) used for extraction was between 6-9.

Determination of protein content

The protein content of the viscera was analyzed by the Kjeldahl method according to the Association of Official Analytical Chemists [13].

Optimization of protease extraction and purification

Optimization was carried out by RSM using the Design Expert version 11.1.1 software, (Stat-ease Inc., Minneapolis, Minn., USA). The effect of independent variables; pH, (X_1) and ammonium sulphate, % (X_2) were analyzed. The coded and uncoded levels are presented in Table 1. Central composite design (CCD) (three levels) and the quadratic model was used to design the experiment. The central composite design (CCD) was chosen because it is an ideal solution for fitting a second-order response surface model and it provides excellent prediction capability near the center of the design space [14]. Thirteen runs, including one axial point, one fractional factorial points, and five central points were randomly performed according to CCD (Table 2).

Table 1: Independent variables with coded and uncoded levels for optimization of the proteolytic activity of protease in torpedo scad viscera

Independent Variable	Symbol	Coded		
		-1	0	+1
pH	X_1	6	7.5	9
Ammonium sulphate, (%)	X_2	40	60	80

Purification by ammonium sulphate precipitation

10mL of liquid crude protease extracted in different buffer solutions was added with ammonium sulphate up to a degree of saturation. The process was performed at 4°C followed by incubation in ice for at least 2h to allow for precipitation and then centrifuged at 10,000rpm at 4°C for 15 minutes. After centrifugation, the pellet was collected and subjected to diafiltration for desalting using a diafiltrator (Model: AKTA flux, US).

Gel filtration column chromatography

After diafiltration, gel filtration was performed in Bio-Gel P-100 column. Initially, the column was washed with 20mM tris-buffer (pH 7.5). The elution was then carried out at a flow rate of 20 mL/h and a fraction of 10mL each was collected for obtaining the elution profile. The absorbance of each fraction was measured at 280nm using spectrophotometer (Model: Aquamate Plus UV/VIS, US). The fraction with the highest absorbance was pulled and freeze-dried (Model: C-GEN BIOTECH, India).

Determination of protein concentration

The protein concentration of the protease was determined based on the Lowry method [15]. Absorbance was measured at 750nm and bovine serum albumin (BSA) was used as a standard ranging from 0-100 mg/L.

Determination of proteolytic activity, specific activity, and purification fold

Proteolytic activity was determined by using a caseinolytic activity assay according to the method of Ketnawa et al., [16]. A 5 mL of 0.65% casein solution (0.65mg in 100mL of 50mM potassium phosphate buffer, pH 7.5) was incubated for 5 min. at 37°C and then a 1mL of enzyme sample was added and the mixture was further incubated for another 10 min at the same temperature. Then, the reaction was terminated by adding 5mL of 5% trichloroacetic acid and let to cool to room temperature.

Finally, 0.5M of Folin-ciocalteau reagent was added and the absorbance was measured at 660nm. The tyrosine standard curve ranging from 0.2-1mLwas plotted. One caseinolytic unit is defined as the number of enzymes needed to produce an increment of 0.01 absorbance unit per minute at the assayed condition. Proteolytic activity was calculated as follows:

$$\frac{Ea-Eb}{Es} \times 50 \times \frac{11}{10} \times DF$$

Where;
Ea = absorbance of sample, Eb = absorbance of blank,
Es = absorbance of standard, DF = dilution factor

The specific activity, purification fold and percent protease recovery were calculated according to Normah and Nur Anati [17] by the following formula:

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme activity (U/mL)}}{\text{Total protein (mg/mL)}}$$

$$\text{Purification fold} = \frac{\text{Specific activity of a purified enzyme}}{\text{The specific activity of a crude enzyme}}$$

$$\text{\% Protease recovery} = \frac{\text{Total activity} \times 100}{\text{Total activity of a crude extract}}$$

RESULTS AND DISCUSSION

The protein content of torpedo scad fish viscera

The viscera contained 15.87% protein which falls within the protein range of other fish such as mackerel, pollock, and cod from 13-18% as stated in the previous study [18]. High protein content in the viscera is essential for obtaining high proteolytic activity of the extracted

protease. Fish viscera is an important source of protein and other components, such as polyunsaturated fatty acids, phospholipids, soluble vitamins and bioactive compounds [19].

Optimization of the extraction and purification condition

Central composite design (CCD) was used to optimize the extraction condition (pH) and purification in ammonium sulphate for torpedo scad fish visceral protease by monitoring the proteolytic activity as the response. Thirteen combinations of independent variables were measured (Table 2). Highest proteolytic activity was achieved at pH 7.5, ammonium sulphate concentration of 88.78% (w/v) with the proteolytic activity of 745U.

However, through RSM, the suggested optimum condition was at pH 9 and 80% ammonium sulphate concentration. Previous studies showed that the optimum condition of protease was achieved at pH 8 and with ammonium sulphate at 50% saturation [10]. According to Vannabun et al., [20] the visceral enzyme highest activity falls in the range of pH 8.0-12.0. The visceral protease of fish such as *Mytella charruana* that showed a trypsin-like activity was stable in a wide pH range (3.0-9.0) and protease activity of 339.5U mg^{-1} [21] whereas study on *Tilapia nilotica* protease showed optimal activity at pH 8 where the proteolytic activity value obtained was 428U mg^{-1} [4].

Table 2: Actual levels of independent variables used in the optimization of torpedo scad (*Megalaspis cordyla*) fish visceral protease extraction and purification with proteolytic activity as the response

Run	X_1 = pH	X_2 = Ammonium sulphate concentration (%)	R_1 = Proteolytic Activity (U)
1	5.4	60	210
2	7.5	60	255
3	9	40	245
4	9.6	60	460
5	6	40	160
6	7.5	88.28	745
7	6	80	295
8	7.5	60	255
9	7.5	60	255
10	7.5	60	255
11	7.5	60	255
12	7.5	31.72	320
13	9	80	445

The independent and dependent variables were analyzed to obtain a regression equation that could predict the response within the given range. The regression equation for proteolytic activity which is the response variable (y) was derived using the regression coefficient of quadratic and interaction terms to fit a response surface model. The response surface equation model obtained through a response surface model, RSM is shown below:

Actual equation:

Proteolytic activity = +24.096 + (0.332*pH) – (0.632*ammonium sulphate concentration) + (0.008*pH*ammonium sulphate concentration) + (0.040*pH^2) + (0.006*ammonium sulphate $concentration^2$)

$y = + 24.096 + (0.332*A) – (0.632*B) + (0.008*AB) + (0.040*A^2) + (0.006*B^2)$

Coded equation:

$y= + 15.97 + (2.10*A) + (2.91*B) + (0.2290*AB) + (0.0910*A^2) + (2.40*B^2)$

Where;
y, A, and B are proteolytic activity, pH, and ammonium sulphate concentration, respectively.

The analysis of variance (ANOVA) results is shown in Table 3. The quadratic model adequately represented the experimental data with a high coefficient of determination value of R^2 = 0.8323 while the predicted R^2 = 0.6759 and the adjusted R^2 = 0.7125. Thus, the regression model is significant with $p< 0.005$. The regression model has well defined if the R^2 value is higher than 0.80 [22]. A small value of R^2 indicates a poor relevance of the dependent variables in the model. Thus, the model can fit well with the actual data when R^2 approaches unity of the experiment.

Table 3: Analysis of variance (ANOVA) for optimization of torpedo scad fish (*Megalaspis cordyla*) visceral protease.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	143.51	5	28.70	6.95	0.0122	significant
A-pH	35.11	1	35.11	8.50	0.0225	
B-Ammonium Sulphate Concentration	67.69	1	67.69	16.39	0.0049	
AB	0.2098	1	0.2098	0.0508	0.8281	
A^2	0.0576	1	0.0576	0.0139	0.9093	
B^2	40.14	1	40.14	9.72	0.0169	
Residual	28.91	7	4.13			
Lack of Fit	28.91	3	9.64	1.40	0.2709	Not significant
Pure Error	0.3816	4	0.0365			
Cor Total	172.42	12				

R^2 = 0.8323, Predicted R^2 = 0.6759, Adjusted R^2 = 0.7125
A=pH, B=ammonium sulphate concentration (%)

The p-values less than 0.05 indicates that model terms are significant. In this case, A, B, B^2 values of these quadratic coefficients are significant model terms. Besides, the fitness of the model was evaluated through the lack of fit test. The p-values for the lack of fit test was not significant ($p>0.05$) indicating that the model was fit for predicting the optimization of torpedo scad fish visceral protease where in this experiment the lack of fit obtained is

0.2709 which is not significant model terms. A good fit means that the generated models adequately explained the data variation.

The effect of the independent variable data on the optimization of torpedo scad fish visceral protease can also be visualized through a three-dimensional response surface graph which is based on the quadratic model variables of pH and ammonium sulphate concentration (Figure 1). The graph shows at ammonium sulphate concentration of 60%, the proteolytic activity starts to increase and the highest proteolytic activity was recovered at 80% ammonium sulphate concentration.

According to Rawdkuen et al., [23] increasing in the ammonium sulphate concentration resulted in an increase in protease recovery in the interphase, especially with high salt content. Similarly, the proteolytic activity increases with an increase in pH value where the activity starts to increase from pH 7.2 up to pH 9. Bhaskar et al., [7] also stated that increasing pH does affect the activity of the protease of Catla (*Catla catla*). Studies by Harivaindaran and Tajul [5] stated that at the optimum pH, surface charge distributions and even conformations remain unchanged and enzymes could bind the substrate properly. Salwanee et al., [24] stated that changes of pH throughout the process might significantly disturb the efficiency of the enzyme capability where pH can also affect enzyme function. Changes in pH can affect these residues and make it difficult for substrates to bind [5].

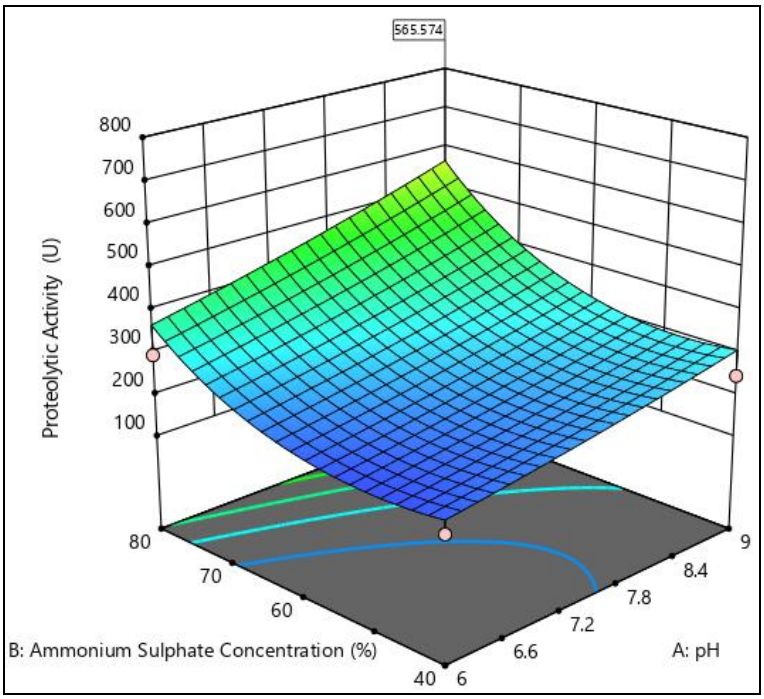

Figure 1: Response surface graph for proteolytic activity of torpedo scad visceral protease as a function of A: pH and B: ammonium sulphate concentration

The enzyme from tuna (*Euthynnus affinis*) viscera was gradually active between pH 7 and 9, with an optimum pH at pH 9 [24]. The optimum pH of *S. scrofa* proteases was similar to that of tambaqui fish (*Colossoma macropomum*) proteases [25] and striped seabream

(*Lithognathus mormyrus*) proteases [6]. From those studies it shows that crude enzyme extract is highly stable over a broad pH range, maintaining its original activity between pH 6 and 9 in 80% ammonium sulphate [26]. The results suggest that the viscera of torpedo scad would be a potential source of alkaline proteases for certain industrial applications that require high alkaline conditions similar to study by Younes et al., [26], who discovered that the alkaline proteases from the red scorpion fish (*Scorpaena scrofa*) viscera were active and stable in alkaline solution at pH range of 5 -12 with optimum pH at 10 and temperature 55°C, respectively using casein as a substrate.

Proteolytic activity increase with ammonium sulphate concentration and pH. This result is in agreement with the finding by Khaled et al., [27] who studied alkaline protease activity and obtained the highest activity at pH 8 to 10. The differences in pH stability are influenced by different molecular properties and enzyme conformation [27]. The loss of enzyme activity at pH values outside optimum pH is probably due to protein conformational changes caused by the repulsion of charges [20].

Verification of the optimum condition of torpedo scad fish visceral protease

Verification was conducted under the optimum condition for three runs to compare the predicted and the experimental values of the independent variables pH and ammonium sulphate concentration. The condition analyzed were experimental values at pH 9 and ammonium sulphate concentration 80% against the predicted values pH 7.5 and ammonium sulphate concentration 60%. The results obtained show that there was no significant difference ($p > 0.05$) between the experimental and predicted value (Table 4).

Therefore, the verifications of the model were accepted. To verify the model, RSM suggested the optimum levels of independent variables based on the desirability. The desirability value close to 1.0, indicates that the suggested conditions are suitable to achieve the highest proteolytic activity [28]. The model desirability value was 0.889 (Figure 2). Therefore, the verifications of the model were confirmed and fit for the study. This indicates the model used is valid for predicting the optimum condition of torpedo scad (*Megalaspis cordyla*) fish visceral protease. Thus, the optimum conditions selected was pH 9 and ammonium sulphate concentration of 80%. At this condition, the proteolytic activity obtained was 445 U.

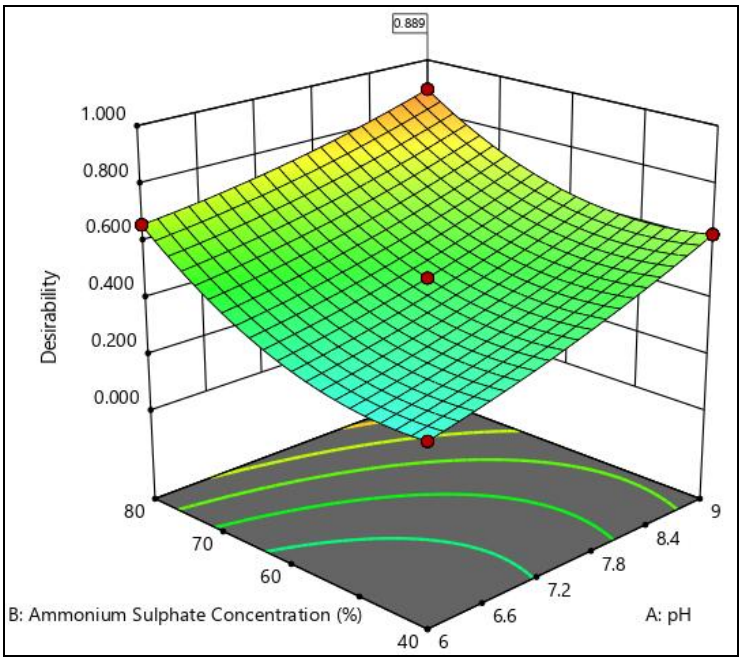

Figure 2: Response surface graph for the desirability value for proteolytic activity for torpedo scad fish visceral protease

Table 4: Verification of proteolytic activity of optimum condition for torpedo scad (*Megalaspis cordyla*) fish visceral protease.

X_1	X_2	Proteolytic Activity	
pH	Ammonium sulphate concentration	Predicted	Experimental
9	80%	565.57 ± 0.00[a]	445.00 ± 0.00[a]

Purification of torpedo scad fish visceral protease

Total activity in each stage decreased from 820U in the crude extract to 410U after the enzyme was subjected to various stages of purification with gel filtration chromatography showing the lowest total activity (Table 5). This result is in agreement with the previous study [10] where the total activity of crude extract from Monterey sardine (*Sardinops sagax caeruleus*) viscera decreased from 945 to 198U after undergoing the gel filtration process.

Studies by El–Beltagy et al., [4] showed a decreased value for total activity of bolti fish viscera (*Tilapia nilotica*) where the activity of the crude extract was 1960U and decreased to 238U when crude extract was then subjected to ammonium sulphate precipitation (30–90%), dialysis and gel filtration. This was due to the homogeneity of the protease during purification steps, the results of ammonium sulphate purification and dialysis value to be gradually decreasing due to the undergone processes.

Table 5: Proteolytic activity of torpedo scad (*Megalaspis cordyla*) fish visceral protease at each purification stage

Purification stage	Volume (ml) per 100g	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold	Recovery (%)
Crude extract	83.5	174.51	820	4.69	1	100
Ammonium sulphate precipitation	71.21	107.41	680	6.33	1.35	82.92
Dialysis	60	141.7	425	3.00	1.56	51.83
Gel filtration chromatograpy	30	170.8	410	2.41	1.95	50.00

The specific activity and purification folds were 6.33U/mg and 1.35 respectively, with 80% ammonium sulphate precipitation. The purification fold of torpedo scad fish visceral at each purification stage increased from 1 to 1.95. During the purification of alkaline protease from pyloric caeca of pirarucu, the enzyme showed maximum activity at pH 9.0 whereby 80% of its maximum activity was observed in the pH range 8.0–10.0 [29] where the yield was 35.4% when ammonium sulphate was at a saturation degree of 80%.

In most studies, the range for variables of protease extraction being used to obtain optimum proteolytic activity was between 6–9 for pH while for the ammonium sulphate concentration used was in the range of 40 - 80% [21, 4]. Most researchers reported that the pH between 8.0 and 10.0 was suitable for enzyme activities of fish species such as Giant Amazonian fish pirarucu [29], zebra blenny [25] and Tunisian barbell [30].

CONCLUSIONS

The optimum pH and ammonium sulphate concentration for the extraction and purification of torpedo scad fish visceral protease determined in the study ~~were~~ was at pH 9 and 80% ammonium sulphate concentration, respectively with proteolytic activity value of 445U. Upon purification, the highest activity was observed in the crude extract, followed by ammonium sulphate precipitation, dialyze protease and gel filtration with activity values of 820, 680, 425, and 410U, respectively.

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