Appendix 1

Analytical Methods

1. Measurement of TCA-soluble peptide (Morrissey *et al.,* 1993)

Reagents

- 5% Trichloroacetic acid (TCA) (w/v)
- 1 mM Tyrosine

Method

1. Weight 3 g of sample and homogenize in 27 ml of 5% TCA for 3 min.
2. Keep on ice for 1 h, and centrifuge at 5,000 ×g for 5 min.
3. Measure tyrosine in the supernatant as an index of autolytic degradation products and express as μmol tyrosine/g sample.

2. Lowry's method for protein quantitation (with a slight modified Lowry *et al.,* 1951)

Reagents

- A: 2% Sodium carbonate in 0.1 N NaOH
- B: 0.5% $CuSO_4.5H_2O$ in 1% Sodium citrate
- C: 1N Folin Phenol reagent
- D: 1 ml Reagent B + 50 ml Reagent A (or similar ratio) (make up immediately before use)

Standard: Bovine serum albumin (BSA) at concentration of 1 mg/ml

Sample Volume: 200 μl

Method

1. Add 2 ml reagent D to each of the standards and unknown tubes. Vortex immediately.
2. Incubate precisely 10 min at room temperature.
3. Add 0.2 ml (200 μl) reagent C (previously diluted 1:1 with distilled water) and vortex immediately.
4. Incubate 30 min at room temperature. (Sample incubated longer than 60 min. Should be discarded).
5. Read absorbance at 750 nm.
6. Plot standard curve and calculate the unknown.

Standards

BSA: 1 mg/ml (used to determine protein concentration)

Tyrosine: 1mM (used to determine enzymatic activity)

Table Experimental set up for the Lowry's method

Tube number	Water (μl)	10 mg/ml BSA (μl)	Effective BSA concentration (mg/ml)
1	200	0	0
2	180	20	0.1
3	160	40	0.2
4	140	60	0.3
5	100	100	0.5
6	60	140	0.7
7	0	200	1.0

3. Bradford method for protein quantitation (with a slight modified Bradford, 1976)

Reagents

- The assay reagent is made by dissolving 100 mg of Coomassie blue G250 in 50 ml of 95% ethanol. The solution is then mixed with 100 ml of 85% phosphoric acid and made up to 1 lit with distilled water.

Standard: Bovine serum albumin (BSA) at concentration of 100 µg/ml

Sample Volume: 100 µl

Method

1. Pipette duplicate samples containing between 1 and 10 µg in a total volume of 100 µl into a test tube. If the appropriate sample concentration is unknown, assay a range of dilutions (1, 1/10, 1/100, 1/1000).
2. Add 1 ml of protein reagent to each tube, and mix gently, but throughtly. Measure the absorbance of each sample between 2 and 60 min after addition of the protein reagent.
3. Measure the A_{595} Value of the samples and standards against the reagent blank between 2 min and 1 h after mixing.

Table Experimental set up for the Bradford's method

Tube number	Water (μl)	10 mg/ml BSA (μl)	Effective BSA concentration (μg/ml)
1	100	0	0
2	90	10	10
3	80	20	20
4	60	40	40
5	40	60	60
6	20	80	80
7	0	100	100

4. Electrophoresis (SDS-PAGE) (Leamli, 1970)

Chemicals

- Protein molecular weight standards
- 30% Acrylamide-0.8% bis Acrylamide
- Sample buffer: Mix 30 ml of 10% of SDS, 10 ml of glycerol, 5 ml of β-Mercaptoethanol, 12.5 ml of 50 mM Tris-HCl, pH 6.8, and 5-10 mg Bromophenol blue (enough to give dark blue color to the solution). Bring the volume to 100 ml with distilled water. Divide into 1 ml aliquots, and stroed at –20°C.
- 2% (w/v) Ammonium persulfate
- 1% (w/v) SDS
- TEMED (N,N,N'N'-tetramethylenediamine)
- 0.5 M Tris-HCl, pH 6.8
- 1.5 M Tris-HCl, pH 8.8
- 0.1 M EDTA

- Electrode buffer: Dissolve 3 g of of Tris, 14.4 g of glycine and 1 g of SDS in distilled water. Adjust to pH 8.3. Add distilled water to 1 liter total volume.
- Staining solution: Dissolve 0.04 g of Coomassie blue R-250 in 100 ml of methanol. Add 15 ml of glacial acetic acid and 85 ml of distilled water.
- Destaining solution I: 50% methanol-7.5% glacial acetic acid
- Destatning solution II: 5% methanol-7.5% galcial acetic acid

Method

Pouring the separating gel:

1. Assemble the minigel apparatus according to the manufacturer's detailed instructions. Make sure that the glass and other components are rigorously clean and dry before assembly.
2. Mix the separating gel solution by adding, as defined in following Table.
3. Transfer the separating gel solution using a Pasture pipette to the center of sandwich is ~1.5 to 2 cm from the top of the shorter (front) glass plate.
4. Cover the top of the gel with a layer of isobutyl alcohol by gently squirting the isobutyl alcohol by gently squirting the isobutyl alcohol against the edge of one of the spacers. Allow the resolving gel to polymerize fully (usually 30-60 min).

Pouring the stacking gel:

1. Pour off completely the layer of isobutyl alcohol.
2. Prepare a 4% stacking gel solution by adding as defined in table.
3. Transfer stacking gel solution to tickle into the center of the sandwich along an edge of one of the spacers.

4. Insert a comb into the layer of stacking gel solution by placing one corner of the comb into the gel and slowly lowering the other corner in. Allow the stacking gel solution to polymerize 30 to 45 min at room temperature.

Reagents	10% running gel	4% stacking gel
30% Acrylamide-bis	1.167 ml	0.4 ml
1.5 M Tris-HCl buffer, pH 8.8	0.875 ml	-
0.5 M Tris-HCl buffer, pH 6.8	-	1.0 ml
1% SDS	0.35 ml	0.3 ml
Distilled water	0.758 ml	0.9 ml
0.1 EDTA	-	0.8 ml
2% Ammonium persulfate	0.35 ml	0.4 ml
TEMED	6 µl	5 µl

Sample preparation:

1. Weigh 3 g of sample and homogenize with 5% (w/v) SDS in a final volume of 30 ml.
2. Incubate the mixture at 85°C for 1 h.
3. Centrifuge at 3,500 xg for 5 min at ambient temperature and collect supernatant.

Loading the gel:

1. Dilute the protein to be 4:1 (v/v) with sample buffer in microcentrifuge tube and boil for 1 min at 100°C.
2. Remove the comb without tearing the edge of the polyacrylamide wells.
3. Fill the wells with electrode buffer.

4. Place the upper chamber over the sandwich and lock the upper buffer chamber to the sandwich. Pour electrode buffer into the lower buffer chamber. Place the sandwich attached to the upper buffer chamber into the lower chamber.
5. Fill the upper buffer chamber with electrode buffer so that the sample wells of the stacking gel are filled with buffer.
6. Use a 10-25 μl syringe with a flate-tipped needle, load the protein sample into the wells by carefully applying the sample as a thin layer at the bottom of the well.
7. Fill the remainder of the upper buffer chamber with additional electrode buffer.

Running the gel:

1. Connect the power supply to the anode and cathode of the gel apparatus and run at 50 V and 150 V.
2. After the bromophenol blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.

Disassembling the gel:

1. Remove the upper buffer chamber and the attached sandwich.
2. Orient the gel so that the order of the sample well is known, remove the sandwich from the upper buffer chamber, and lay the sandwich on a sheet of absorbent paper or paper towels. Carefully slide the spacers out from the edge of the sandwich along its entire length.
3. Insert a spatula between the glass plates at one corner where the spacer was, and gently pry the two plates apart.
4. Remove the gel from the lower plate. Place the plate with the gel attached into the shallow dish of fixing agent or dye and swishing the plate.

Staining the gel:

1. Place the gel in a small plastic box and cover with the staining solution. Agitate slowly for 1 h or more on a rotary rocker.
2. Pour off the staining solution and cover the gel with a solution of destaining solution I. Agitate slowly for ~15 min.
3. Pour off the destaining solution I and cover the destaining solution II. Discard destaining solution and replace with fresh solution. Repeat until the gel is clear except for the protein bands.

Appendix 2

Analysis of variance

Table 1-A Analysis of variance for autolytic degradation of mince of *P. macracanthus* at 50°C.

SV	DF	SS	MS	F
Treat	6	0.310	0.0516	100.38**
Error	7	0.0036	0.00051	
Total	13	0.313		

** = Significant at 1 % level

Table 2-A Analysis of variance for autolytic degradation of mince of *P. macracanthus* at 60°C.

SV	DF	SS	MS	F
Treat	6	191.79	31.97	7495.91**
Error	7	0.03	0.0043	
Total	13	191.82		

** = Significant at 1 % level

Table 3-A Analysis of variance for autolytic degradation of washed mince of *P. macracanthus* at 50°C.

SV	DF	SS	MS	F
Treat	6	0.259	0.0043	55.039**
Error	7	0.0055	0.00079	
Total	13	0.265		

** = Significant at 1 % level

Table 4-A Analysis of variance for autolytic degradation of washed mince of *P. macracanthus* at 60°C.

SV	DF	SS	MS	F
Treat	6	1.455	0.243	170.62**
Error	7	0.0095	0.0014	
Total	13	1.465		

** = Significant at 1 % level

Table 5-A Analysis of variance for autolytic degradation of mince of *P. tayenus* at 50°C.

SV	DF	SS	MS	F
Treat	6	0.713	0.119	147.20**
Error	7	0.0057	0.0008	
Total	13	0.719		

** = Significant at 1 % level

Table 6-A Analysis of variance for autolytic degradation of mince of *P. tayenus* at 60°C.

SV	DF	SS	MS	F
Treat	6	21.58	3.60	1353.72**
Error	7	0.0019	0.0027	
Total	13	21.60		

** = Significant at 1 % level

Table 7-A Analysis of variance for autolytic degradation of washed mince of *P. tayenus* at 50°C.

SV	DF	SS	MS	F
Treat	6	0.0093	0.015	38.029**
Error	7	0.0003	0.0004	
Total	13	0.096		

** = Significant at 1 % level

Table 8-A Analysis of variance for autolytic degradation of washed mince of *P. tayenus* at 60°C.

SV	DF	SS	MS	F
Treat	6	1.29	0.215	131.67**
Error	7	0.015	0.0016	
Total	13	1.30		

** = Significant at 1 % level

Table 9-A Analysis of variance for temperature profile of sarcoplasmic proteinase from *P. macracanthus* at pH 3.0.

SV	DF	SS	MS	F
Treat	9	434.23	48.25	33.73**
Error	10	14.30	1.43	
Total	19	448.53		

** = Significant at 1 % level

Table 10-A Analysis of variance for temperature profile of sarcoplasmic proteinase from *P. macracanthus* at pH 5.5.

SV	DF	SS	MS	F
Treat	9	1332.36	148.04	128.89**
Error	10	11.49	1.15	
Total	19	1343.84		

** = Significant at 1 % level

Table 11-A Analysis of variance for temperature profile of sarcoplasmic proteinase from *P. macracanthus* at pH 8.5.

SV	DF	SS	MS	F
Treat	9	8638.45	959.83	116.16**
Error	10	82.63	8.26	
Total	19	8721.08		

** = Significant at 1 % level

Table 12-A Analysis of variance for temperature profile of sarcoplasmic proteinase from *P. tayenus* at pH 3.0.

SV	DF	SS	MS	F
Treat	9	1146.31	127.37	108.27**
Error	10	11.76	1.18	
Total	19	1158.08		

** = Significant at 1 % level

Table 13-A Analysis of variance for temperature profile of sarcoplasmic proteinase from *P. tayenus* at pH 5.5.

SV	DF	SS	MS	F
Treat	9	2228.57	247.62	118.37**
Error	10	20.92	2.09	
Total	19	2249.49		

** = Significant at 1 % level

Table 14-A Analysis of variance for temperature profile of sarcoplasmic proteinase from *P. tayenus* at pH 8.5.

SV	DF	SS	MS	F
Treat	9	3924.59	436.07	642.13**
Error	10	6.79	0.679	
Total	19	3931.38		

** = Significant at 1 % level

Table 15-A Analysis of variance for pH profile of sarcoplasmic proteinase from *P. macracanthus* at temperature 60°C.

SV	DF	SS	MS	F
Treat	13	28058.60	2158.35	15.61**
Error	14	1936.05	138.29	
Total	27	29994.6		

** = Significant at 1 % level

Table 16-A Analysis of variance for pH profile of sarcoplasmic proteinase from *P. tayenus* at temperature 60°C.

SV	DF	SS	MS	F
Treat	13	2070.33	159.26	80.06**
Error	14	27.85	1.99	
Total	27	2098.18		

** = Significant at 1 % level

Table 17-A Analysis of variance for effect of inhibitors on sarcoplasmic proteinase from *P. macracanthus* at pH 6.5.

SV	DF	SS	MS	F
Treat	9	10532.60	1170.29	86.84**
Error	10	134.77	13.48	
Total	19	10667.40		

** = Significant at 1 % level

Table 18-A Analysis of variance for effect of inhibitors on sarcoplasmic proteinase activity from *P. macracanthus* at pH 8.5.

SV	DF	SS	MS	F
Treat	9	8474.10	941.57	86.28**
Error	10	109.13	10.91	
Total	19			

** = Significant at 1 % level

Table 19-A Analysis of variance for effect of inhibitors on sarcoplasmic proteinase from *P. tayenus* at pH 5.0.

SV	DF	SS	MS	F
Treat	9	9432.11	1048.01	29.15**
Error	10	359.56	35.96	
Total	19	9791.67		

** = Significant at 1 % level

Table 20-A Analysis of variance for effect of inhibitors on sarcoplasmic proteinase from *P. tayenus* at pH 8.5.

SV	DF	SS	MS	F
Treat	9	11042.0	1226.89	43.80**
Error	10	280.15	28.02	
Total	19	11322.2		

** = Significant at 1 % level

Table 21-A Analysis of variance for pH profile of purified proteinase from *P. macracanthus* at temperature 60°C.

SV	DF	SS	MS	F
Treat	5	631.54	126.31	6858.35**
Error	6	0.11	0.018	
Total	11	631.65		

** = Significant at 1 % level

Table 22-A Analysis of variance for temperature profile of purified proteinase from *P. macracanthus* at pH 8.5.

SV	DF	SS	MS	F
Treat	5	986.25	197.25	1093.11**
Error	6	1.08	0.18	
Total	11	987.34		

** = Significant at 1 % level

Table 23-A Analysis of variance for effect of inhibitor on purified proteinase activity from *P. macracanthus*.

SV	DF	SS	MS	F
Treat	6	13868.01	2311.34	1863.27**
Error	7	8.68	1.24	
Total	13	13876.70		

** = Significant at 1 % level

Table 24-A Analysis of variance for substrate specificitys of purified proteinase activity from *P. macracanthus*.

SV	DF	SS	MS	F
Treat	2	1076316.40	538158.22	91904.10**
Error	3	17.57	5.86	
Total	5	1076334.0		

** = Significant at 1 % level

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