APPENDICES

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APPENDIX- I

PREPARATION OF CRUDE HOMOGENATE

(Sadasivam and Manickam, 2008)

Collect the visceral organ and head and tail wastes from the selected fish and clean with tap water 3 times followed with distilled water. Then cut the tissues into small pieces and weigh. Homogenize the pieces with 0.02M phosphate buffered saline (PBS pH 7.4) using a homogenizer. Centrifuge homogenate at 10,000 rpm for 10 minutes at 4°C and collect the supernatant. This is the crude homogenate of the enzyme.

APPENDIX- II

AMMONIUM SULPHATE FRACTIONATION OF PROTEASE

(Nigam and Ayyagari 2007)

PRINCIPLE

Ammonium sulphate is particularly a useful salt for the fractional precipitation of proteins. The precipitation is based on the principle of salting out. Ammonium sulphate is available in a highly purified form, has great solubility allowing for significant changes in the ionic strength and is also inexpensive. Changes in ammonium sulphate concentration of a solution can be brought about either by adding the solid substance or by adding a solution of known saturation, generally a fully saturated solution (100%) solution.

MATERIALS

Ammonium sulphate

PROCEDURE

1. Ammonium sulphate precipitation (0-100%) of the crude homogenate is done. For 100 ml of the crude homogenate add the following amounts of ammonium sulphate at 4°C.

10% - 5.6g
20% - 5.7g
30% - 5.9g
40% - 6.2g
50% - 6.3g
60% - 6.6g
70% - 6.9g
80% - 7.2g
90% - 7.7g
100% - 7.9g

2. After the salt has dissolved, continue stirring for 30 minutes to allow equilibrium to be reached between dissolved and aggregated proteins.
3. Centrifuge at 10,000 rpm for 10 minutes at 4°C.
4. Decant and save the supernatant.
5. Dissolve the precipitate in PBS buffer.

APPENDIX III

ACETONE PRECIPITATION OF PROTEASE

PRINCIPLE

The solubility of a protein depends on, among other things, the dielectric constant of the solution. Organic solvents with small dielectric constants, e.g. acetone and methanol, discourage the dispersion of protein molecules. Thus, the solubility of proteins can be lowered and precipitation can be induced by

lowering the effective dielectric constant of the media. This is commonly achieved by adding a water-soluble solvent such as acetone to an aqueous solution of protein. Acetone has the advantage that it is relatively inexpensive and available in a pure form.

REAGENTS

Cold (-20°C) acetone

PROCEDURE

1. Cool the required volume of acetone to -20°C.
2. Place protein sample in acetone-compatible tube.
3. Add four times the sample volume of cold (-20°C) acetone to the tube.
4. Vortex tube and incubate for 60 minutes at -20°C.
5. Centrifuge 10 minutes at 13,000-15,000 × *g*.
6. Decant and properly dispose of the supernatant, being careful not to dislodge the protein pellet
7. Allow the acetone to evaporate from the uncapped tube at room temperature for 30 minutes .
8. Add buffer appropriate for the downstream process and vortex thoroughly to dissolve protein pellet.

APPENDIX IV

ETHANOL PRECIPITATION OF PROTEASE

PRINCIPLE

The solubility of protein depends on, among other things, the dielectric constant of the solution. In general, solvent molecules with large dielectric constants, e.g. water and dimethylsulphoxide, can stabilize the interaction between themselves and protein molecules and favor the dissolution of protein. Organic solvents with small dielectric constants, e.g. acetone and ethanol, discourage the dispersion of protein molecules. Thus, the solubility of proteins

can be lowered and precipitation can be induced by lowering the effective dielectric constant of the media. This is commonly achieved by adding a water-soluble solvent such as ethanol to an aqueous solution of protein. Ethanol has the advantage that it is relatively inexpensive and available in a pure form.

REAGENTS

Cold ethanol

PROCEDURE

1. Add to 1 volume of protein solution 2 volumes of cold ethanol ranging from 10 to 100%. Mix and keep atleast 60 minutes at -20°C.
2. Spin 15 minutes 4°C in microcentrifuge at maximum speed (15000 rpm). carefully discharge supernatant and retain the pellet.
3. Wash pellet with 90% cold ethanol (keep at -20°C). Vortex and repellet samples 5 min at full speed.
4. Dry samples under vaccum (speed vac) or dry air to eliminate any ethanol residue.

APPENDIX-V
PURIFICATION OF PROTEASE BY DIALYSIS
(Nigam and Ayyagari 2007)

PRINCIPLE

Dialysis is commonly used to remove salts from proteins. The presence of salts in protein interferes in many ways. Special semi-permeable membranes called dialysis bags have the property to allow compounds with small molecular weights to pass through them while those with high molecular weight like proteins are held back

MATERIALS

- Dialysis bag
- Magnetic bar and stirrer motors

- 2% Sodium bicarbonate
- 0.05 % EDTA
- 20% ethanol
- 0.1% sodium azide

PROCEDURE

1. Select a dialysis bag of suitable diameter and cut into the required length.
2. Submerge the bag in to a solution of 2% Sodium bicarbonate and 0.05% EDTA and boil for 10 minutes. Discard the solution and boil for 10 minutes in distilled water. Repeat once more.
3. Rinse the dialysis bag with distilled water or buffer prior to use.
4. Seal the bag at one end with a double knot.
5. Add the solution to be dialyzed into the dialysis bag.
6. Place the bag in a large volume of buffer and agitate with a magnetic bar and stirrer motor.
7. Allow to reach equilibrium greater than 3 hours at 4^0C.

APPENDIX VI

PURIFICATION OF PROTEASE BY GEL FILTRATION CHROMATOGRAPHY

(Nigam and Ayyagari 2007)

PRINCIPLE

Gel filtration can be used to separate proteins based on differences in their molecular size, or to desalt proteins.

MATERIALS

1.0.2M phosphate buffered saline (pH 7.5)

2. Sephadex G -100 column materials:

Suspend 2g of Sephadex G 100 in 25 ml of PBS buffer of pH 7.5 in a beaker. Allow it to imbibe water and swell for 1 hour. After that discard the floating materials in the supernatant and using a glass rod made the particles into slurry. Pour the slurry in to a 10 cm column plated with glass wool at the bottom. Wash the column with 50ml of buffer.

PROCEDURE

1. Wash the column with PBS buffer pH 7.8 and equilibrate with the same buffer and adjust the flow rate.
2. Load 3 ml of dialyzed sample into the column.
3. As the sample percolates the column, collect 1.5 or 2 ml fractions continuously by adding the buffer.
4. Check the presence of protein in each fraction by measuring the absorbance at 280 nm.
5. Collect the fractions till the absorbance at 280 nm reach zero.
6. Calculate the amount of protein and express as mg/ml of protein.

APPENDIX VII

ESTIMATION OF PROTEIN BY LOWRY'S METHOD

(Lowry *et al.*, 1951)

PRINCIPLE

The blue colour developed by the reduction of the phosphomolybdic–phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured by the Lowry's method.

REAGENTS

Reagent A: 2 % Sodium carbonate in 0.1 N Sodium hydroxide

Reagent B: 0.5% Copper suphate in 1% Potassium sodium tartrate

Reagent C: Alkaline copper solution: Mix 50 ml A and 1 ml B prior to use.

Reagent D: Folin Ciocalteau reagent

Protein Stock solution: Weigh accurately 50 mg of bovine serum albumin (fraction V) and dissolve in distilled water and make up to 50 ml in a standard flask.

Working standard solution: Dilute 5ml of stock solution in 50 ml with distilled water in a standard flask.1 ml of this solution contains 200µ protein.

PROCEDURE

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard solutions into a series of test tubes.
2. Pipette out 0.2 ml of the sample extract in two other test tubes.
3. Make up the volumes to 1.0 ml in all the test tubes. A tube with 1 ml of water serves as the blank.
4. Add 5 ml of reagent C to each tube including the blank. Mix well and allow standing for 10 minutes.
5. Add 0.5 ml of reagent D, mix well and incubate at room temperature in the dark for 30 minutes to develop the blue colour.
6. Take the readings at 660 nm.
7. Draw the standard graph and calculate the amount of protein in the sample.

APPENDIX VIII

ASSAY OF PROTEASE

(Anson, 1938; Folin and Ciocalteau, 1929)

PRINCIPLE

Protease hydrolyses casein in the presence of water to give amino acids.

Casein + Water $\xrightarrow{\text{protease}}$ Amino acids.

CONDITIONS: T=37 °C, pH =7.5, A660 nm, light path=1 cm

METHOD: Colorimetric

REAGENTS

A) 50 mM Potassium phosphate buffer, pH 7.5 at 37°C.

Prepare 200 ml in deionized water using Potassium phosphate, dibasic, trihydrate. Adjust to pH 7.5 at 7.5 at 37°C with 1M HCl.

B) 0.65% (w/v) Casein solution (Casein).

Prepare 125 ml in reagent 1 using Casein, heat gently (do not boil) to 80-90°C for 10 minutes with stirring. Adjust the pH 7.5 at 37°C, if necessary, with either 1M NaOH or 1M HCl.

C) 110 mM Trichloroacetic acid reagent (TCA)

Dilute 9ml of Trichloroacetic acid, 6.1 N, approximately 100% (w/v), to 500 ml with deionized water

D) Folin-Ciocalteau Phenol Reagent (F-C)

Dilute 10 ml Folin Ciocalteau Phenol Reagent to 40 ml with deionized water.

E) 500 mM Sodium carbonate solution

Prepare 500 ml in deionized water using anhydrous sodium carbonate.

F) 10 mM Sodium acetate buffer with 5mM calcium acetate, pH 7.5 at 37°C.

(Enzyme diluent)

Prepare 100 ml in deionized water using sodium acetate, trihydrate and calcium acetate. Adjust the pH 7.5 at 37°C with 0.1 M Acetic acid or 0.1 M NaOH.

G) 1.1 mM L-Tyrosine Standard solution.

Prepare 100ml in deionized water using L-tyrosine. Heat gently (do not boil) until tyrosine dissolves and cool to room temperature.

Prepare a solution containing 0.1-0.2 unit/ml of protease in cold reagent F.

i) Crude extract.

ii) Ammonium sulphate precipitated samples.

iii) Ammonium sulphate precipitated samples + dialysed samples.

iv) Ammonium sulphate precipitated samples + Sephadex G-50 run samples

v) Acetone precipitated samples

vi) Acetone precipitated samples + dialysed samples

vii) Acetone precipitated samples + Sephadex G-50 run samples

viii) Ethanol precipitated samples

ix) Ethanol precipitated samples +dialysed samples

x) Ethanol precipitated samples + Sephadex G-50 run samples.

PROCEDURE

Pipette the following reagents into suitable vials (in milliliters)

	Test	Blank
Reagent B (Casein)	5.00	5.00
Equilibrate to 37°C. Then add:		
Reagent G (Enzyme Solution)	1.00	------
Mix by swirling and incubate at 37 °C for exactly 10 minutes. Then add:		
Reagent C (TCA)	5.00	5.00
Reagent G (Enzyme Solution)	------	1.00

Mix by swirling and incubate at 37 °C for about 30 minutes. Filter through Whatman 50 filter paper or a 0.45 μm filter and use the filtrate in color development

COLOUR DEVELOPMENT

Standard Curve:

Prepare a standard curve by pipetting the following reagents into suitable vials (in milliliters):

	Std 1	Std 2	Std 3	Std 4	Blank
Reagent G (Std Soln)	0.05	0.10	0.20	0.40	0.00
Deionized Water	1.95	1.90	1.80	1.60	2.00
Reagent E (Na2CO3)	5.00	5.00	5.00	5.00	5.00
Reagent D (F-C)	1.00	1.00	1.00	1.00	1.00

COLOUR DEVELOPMENT

Sample:

Pipette the following reagents into 4-dram vials (in milliliters):

	Test	Blank
Test Filtrate	2.00	------
Blank Filtrate	------	2.00
Reagent E (Na2CO3)	5.00	5.00
Reagent D (F-C)	1.00	1.00

Mix by swirling and incubate at 37 ^{o} C for 30 minutes. Remove the vials and allow them to cool to room temperature. Filter through a 0.45 μm filter immediately prior to reading. Read the absorbance at 660 nm for each of the vials in suitable cuvettes.

CALCULATIONS

Standard Curve

DA_{660nm} Standard = A_{660}nm Standard - A_{660}nm Standard Blank

Plot the $D\mu A_{660}$nm Standard Vs μmoles of Tyrosine.

Sample Determination

DA_{660}nm Sample = D A_{660}nm Test - DA_{660}nm Sample Blank

Determined the μmoles of Tyrosine equivalents liberated using the standard curve.

$$\text{Units/ml enzyme} = \frac{(\mu \text{ mole tyrosine equivalents released})\ (11)}{(1)\ (10)\ (2)}$$

11 = Total volume (in milliliters) of assay

10 = Time of assay (in minutes) as per the unit definition

1 = Volume of enzyme (in milliliter) of enzyme used

2 = Volume (in milliliters) used in colorimetric determination

$$\text{Units/mg solid} = \frac{\text{Units / ml enzyme}}{\text{mg solid / ml enzyme}}$$

UNIT DEFINITION

One unit will hydrolyze casein to produce color equivalent to 1.0 µmole (181 µg) of tyrosine per minute at pH 7.5 at 37 ^{o} C (colour by Folin & Ciocalteau reagent).

FINAL ASSAY CONCENTRATION

In a 6.00 ml reaction mix, the final concentrations are 42 mM potassium phosphate, 0.54% (w/v) casein, 1.7 mM sodium acetate, 0.8 mM calcium acetate, and 0.1 - 0.2 unit protease.

SPECIFIC ACTIVITY OF PROTEASE

$$\text{Specific activity} = \frac{\text{Total activity (U/ml enzyme)}}{\text{Protein content (mg/ml enzyme)}}$$

PREPARATION OF PHOSPHATE BUFFER

(Sadasivam and Manickam, 2008)

STOCK SOLUTIONS

A: 0. 2M solution of monobasic sodium phosphate (27.8g of NaH_2PO_4. $2H_2O$ in 1 L)

B: 0.2M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1L)

x ml of A, y ml of B , diluted to a total of 200ml.

x	Y	pH
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	84.0	7.5
13.0	87.0	7.6
10.5	89.5	7.7
8.5	91.5	7.8
7.0	93.0	7.9
5.3	94.7	8.0