C H A P T E R - II

MATERIAL AND METHODS

C H A P T E R - II

MATERIAL AND METHODS

As discussed in the first chapter Rana cyanophlyctis the skipper frog was used for the present investigation. The frog is seasonal breeder and shows prebreeding sexually quiescence condition from October to December, growth period (prebreeding) from January to May, and reproductive period (breeding) from June to September (Saidapur, 1986; Devanath and Kanakaraj, 1988).

The adult female frogs were collected from the ponds, surrounding the area of Kasaba Bawada, Kolhapur, where they were available in plenty.

During March and April, in prebreeding condition the adult female frogs (about 45) were captured and were immediately anaesthesized and ovaries were removed in chilled petridishes.

Enzyme Isolation :

(Yabusaki and Ballou, 1981)

Chemicals used -

1. O.01 M Tris-HCL buffer of pH-8
2. Ammonium sulfate (Analar grade).

Method of Isolation -

i) The chilled ovaries were immediately weighed and were suspended in the minimum volume of 0.01 M Tris-HCl buffer of pH-8.

ii) Ovaries were homogenized with Teflon homogenizer at 15000 rpm speed giving 8 strokes.

iii) The homogenate was transferred into the sterile chilled beaker.

iv) With constant stirring ammonium sulfate was added till the saturation of the homogenate (About 40% ammonium sulfate was required).

v) The beakers were covered with aluminium foil and stored at $10^{o}C$ for 24 hrs.

vi) Then by centrifugation the precipitate was separated.

vii) The precipitate was resuspended in the 0.01 M Tris-HCl buffer of pH - 8.0 and dialysed in dialysis membrane which retained the molecules above 10,000 Dalton molecular weight against 0.01 M Tris-HCl buffer of pH - 8.0 with two to three changes of buffer during 24 hrs.

viii) The precipitate was dissolved in respective buffers for the use of kinetic studies.

The methods used for enzyme assay studies :

Chemicals required :

i) Sodium acetate ($NaC_2H_3O_2.3H_2O$)

ii) Acetic acid ($C_2H_4O_2$)

iii) P-nitrophenyl phosphate

iv) Na-β-glycerophosphate

v) Glucose-6-phosphate

vi) Adenosine 5'-Triphosphate (ATP)

vii) Copper sulfate ($CuSO_4 5H_2O$)

viii) Iodoacetate ($C_2H_3IO_2$)

ix) N-ethylinmaleinmide ($C_6H_7NO_2$)

x) Sodium tartrate ($C_4H_4O_6Na_2.2H_2O$)

xi) Trisodium citrate ($NaO_2CCH_2C(OH)(CO_2Na)CH_2CO_2Na.2H_2O$).

xii) Phenol (C_6H_5OH)

xiii) Ethylene glycol tetracetic acid (EGTA)

xiv) Ethylene diamine tetracetic acid (EDTA)

xv) p-Chloromercurybenzoic acid

xvi) Magnesium sulfate ($MgSO_4.7H_2O$)

xvii) Manganese sulfate ($MnSO_4\ H_2O$)

xviii) Ammonium sulfate [$(NH_4)_2SO_4$]

xix) Citric acid ($C_6H_8O_7.H_2O$)

xx) Sodium hydroxide (NaOH)

xxi) Sodium carbonate (Na_2CO_3)

xxii) Tris-HCl buffer [0.01 M pH 8.00]

xxiii) Ethanol (C_2H_5OH)

xxiv) Methanol (Ch_3OH)

xxv) Triton X-100

xxvi) Formalin [HCHO]

xxvii) Acetone [$(CH_3)_2CO$]

xxviii) Tartarate [NaO_2 $C.CH_2$ (OH).CH(OH).CO_2.Na.$2H_2O$]

xxix) Glycerol [$CH_2OH.CHOH.CH_2OH$]

xxx) Sodium fluoride (NaF)

The double (glass) distilled ion free distilled water was used for all the experiments.

The enzyme assay was carried out using 0.2 M Acetate buffer of respective pH containing the substrate of respective Km of the Enzymes I, II and III, using 0.1 ml solution of isolated enzyme sufficiently diluted and with known amount of proteins.

Acetate Buffer (Dawson et. al.,1978)

Sodium acetate - acetic acid buffer solutions, pH approximately 3.25-5.6.

x-Sodium acetate trihydrate ($CH_3COONa.3H_2O$. Molecular weight 136.09) 0.2 M (27.22 grams in 1000 ml of ion free distilled water).

y-0.2 M acetic acid (12 ml diluted to 1000 ml with ion free distilled water).

The varied pH were prepared as per the following table :

No.	pH	x in ml	y in ml
1	3.25	6.00	94.00
2	3.5	8.00	92.00
3	3.7	10.00	90.00
4	3.8	12.00	88.00
5	4.0	18.00	82.00
6	4.2	26.50	73.50
7.	4.4	37.00	63.00
8	4.6	49.00	51.00
9	4.8	59.00	41.00
10	5.0	70.00	30.00
11	5.2	79.00	21.00
12	5.4	86.00	14.00
13	5.6	91.00	9.00

Assay of Acid Phosphatase

Acetate buffer of respective pHs are used for the preparations of substrate buffer. For kinetic studies respective Km solutions of p-nitrophenyl-phosphates were prepared and accordingly used.

		Blank	Sample	Control
i)	Buffer of respective pH.	1.2 ml	0.1 ml	0.1 ml
ii)	Substrate buffer (with respective pH and appropriate Km of substrate	-	1.00 ml	1.00 ml

Equilibriate at 30°C for 10 minutes

iii)	Enzyme sample (with known portein amount)	-	0.1 ml	-

Incubate for 30 minutes at 30°C.

Stop the reaction with 4 ml of 0.1 N NaoH

Add 0.1 ml of enzyme sample in control tube and use it for reading. Read at 400 mμ on Spectronic 20 (Boush and Lomb Spectrophotometer)

Units of enzyme activities were calculated as per Linhardt and Walter (1965) with acetate buffer (Mest er, et. al., 1985) and expressed as per mg protein activity for all the kinetic studies.

$$\frac{\text{O.D. X 2.76 X dil.}}{\text{mg proteins from given sample}} = \text{Acid phosphatase activity in p-nitrophenol units/mg proteins}$$

Modifiers

The concentrations of modifiers used are in final dilutions of the assay system.

Acid phosphatase activity is expressed as units/mg of protein determined. Proteins were estimated from isolated enzyme samples according to Lowry et al.,(1951).

i) Lowry's A - 2 % Sodium carbonate in 0.1 N NaOH

ii) Lowry's b - 0.5 % Copper sulfate in 1 % Sodium tartarate with pH 7.00

iii) Lowry's C - 50 ml of Lawry's A + 1 ml of Lowry's B Mix at the time of use.

iv) Folin-Ciocalten - Phenol reagent.

100 gm - Sodium tungstate - $Na_2WO_4.2H_2O$

25 gm - Sodium molybdate - $Na_2MoO_4.\ 2H_2O$

700 ml - Distilled water.

100 ml - Concentrated hydrochloric acid (HCl)

50 ml - 85 % phosphoric acid H_3PO_4.

Reflux above mixture for 10 hrs in all glass apparatus.

To the refluxed mixture add

150 gm - Lithium sulfate [$Li_2SO_4.H_2O$]

50 ml - Double distilled water

5 - drops of bromine water.

Boil this mixture to remove excess of bromine.

Dilute this to 1 N acid for use.

Assay of total proteins.

	sample	Blank
a.	1 ml of sample diluted	1 ml of double distilled water
b.	5 ml of freshly prepared Lowry's C	5 ml of freshly prepared Lowry's C
	Mix well and keep at room temperature for 10 minutes	
c.	Add 0.5 ml of 1 N. Folin-Ciocalteuphenol reagent.	Add 0.5 ml of 1 N Folin-Ciocalteuphenol reagent.

Using the blank read the optical density of the sample at 750 nm. Using standard graph of bovine serum albumin the amount of protein can be calculated.

Results of various kinetic studies were analysed statistically.

The statistical calculations had been performed according to routine formulae given in text book of statistics used for biological works (Fisher, 1936; Aggarwal, 1990).

The following abbreviations and formulae were used :

x - independent variable

n - number of observations

Formulae

1. $\bar{x}$ = Arithmetic mean of X

$$\bar{x} = \frac{x}{n}$$

2. SD - Standard deviation.

$$SD = \sqrt{\frac{(\bar{x} - x)^2}{n-1}}$$

3. SE - Standard error for a mean of n variables

$$SE = \frac{SD}{n}$$

4. Student's t - test

$$t = \frac{\bar{x}_1 - \bar{x}_2}{0(E_1 - E_2)}$$

where

$\bar{x}_1 - \bar{x}_2$ = Mean difference of two variables.

$0\ SE_1 - SE_2$ = Error of the mean difference

$$= \sqrt{\frac{SD_1^2}{n_1} + \frac{SD_2^2}{n_2}}$$

5. df = Degree of freedom for a difference of two means of n_1 and n_2 variables

$df = n_1 + n_2 - 2$

6. Significance - The probability P of obtaining 't' value at least as the calculated for a given number of df, is found in Fisher's table (Fisher, 1936). The P-values are signified according to the following conventions :

P = > 0.05 The difference is said to be insignificant

P = ≮ 0.05 The difference is said to be almost signnificant.

P = < 0.01 The difference is said to be significant.

P = <0.001 The difference is said to be highly significant.

***