MATERIALS AND METHODS

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2.1 : Plant Material

Moth bean seeds (Phaseolus Aconitifolius)

2.2 : Chemicals and Biochemicals

a) Yeast RNA was obtained from BDH chemicals Ltd. England, (Concentration 2 mg/ml).

b) Heat denatured buffalo liver DNA was prepared according to chevrier N, and F. Sarhan (70), (Concentration 2 mg/ml)

DNA was prepared according to Ronald W. Davis et.al. (78) using chloroform : Alcohol (3:1) as a deprotenizing agent. DNA was precipitated by using chilled absolute alcohol.

Heat Denaturation of DNA

Denatured DNA was prepared by heating the native DNA to 80^{o}C for 10 minutes followed by chilling on ice (70).

c) Uranyl acetate was obtained from BDH chemicals Ltd. England (Laboratory Reagent).
Uranyl acetate reagent - 0.25% uranyl acetate in 2.5% trichloroacetic acid.

d) DEAE-Cellulose : DE-52 Microgranular from whatman.

e) Buffers : i) Buffer A : Acetate buffer pH 4.8, 100 mM which contains $Zn^{2+}(1X10^{-3}M)$ and Thioglycerol $(1X10^{-3}M)$

ii) Buffer B : Tris-Hcl buffer pH 7.2, 100 mM which contains $zn^{2+}(1X10^{-3}M)$ and Thioglycerol $(1X10^{-3}M)$

f) Thioglycerol : (3-Mercapto 1,2-propanediol) from serva.

2.3 : Enzyme assays

The enzyme activity was determined by measuring uranyl acetate reagent soluble, uv positive material in the supernatant of reaction mixture in a Hitachi 330 model uv - spectrophotometer.

i) Determination of RNase activity :

One ml incubation mixture contains 0.25 ml RNA (2mg/ml) + 0.25 ml buffer, (Buffer used in assay media contains $Zn^{2+}(1X10^{-3}M)$ and Thioglycerol $(1X10^{-3}M)$) + 0.25 ml distilled water and 0.25 ml enzyme. The mixture was incubated at $37^{o}C$ for 30 minutes. The reaction was stopped by adding one ml uranyl acetate reagent. The mixture was allowed to stand at $4^{o}C$ for 10 minutes. The contents were centrifuged at 3000 rpm for 10 minutes and the supernatant was suitably diluted. The absorbance was measured at 260 nm against a control.

ii) Determination of DNase activity :

DNase activity was measured using the same procedure except that buffalo liver denatured DNA was used as a substrate.

iii) Definition of Enzyme Unit :

One enzyme unit is defined as that amount of enzyme which cause an increase in absorbance at 260 hm of 0.01 optical density under standard experimental conditions.

2.4 : Protein Determination :

The protein content was determined according to Lowry et.al (67) using Bovine Serum Albumin as a standard.

2.5 : Enzyme Purification

Step 1 : Preparation of extract :

100 gms. seeds of Moth bean (Phaseolus Aconitifolius) soaked in warm water for 4 hours after washing them with alcohol. The water drained off and the seeds were wrapped in two layers of cheese cloth and kept in dark. Every day water was sprinkled on the seeds. The seedlings (7-8 cms. long) were removed from the roots and cotyledons and were homogenized with equal volume of 0.14M saline containing $Zn^{2+}(1X10^{-3}M)$ and thioglycerol ($1X10^{-3}M$). After homogenization, the insoluble material was filtered off. The cloudly filtrate was then centrifuged at 8000 rpm for 20 minutes and precipitate discarded.

Step 2 : Heat Treatment :

The clear crude extract was heated at 70°C in a water bath for 15 min. and immersed in ice bath. The solution was centrifuged at 8000 rpm for 15 minutes. Precipitate was discarded and the supernatant was further purified.

Step 3 : Acetone treatment :

The above extract was chilled at 4°C. One volume of enzyme was mixed with five volumes of chilled acetone. The precipitate was collected by centrifugation at 5000 rpm for 10 minutes and it was dissolved in buffer A.

Step 4 : Ammonium sulfate Fractionation :

The solution from the previous step was brouht to 40% saturation. After allowing it to stand for one hour, the precipitate was removed by centrifugation and discarded. The supernatant was brought to 80% saturation with solid ammonium sulfate. This mixture was kept at 4°C for 4 hour. The mixture was centrifuged and the supernatant discarded. The precipitate was dissolved in buffer A.

Step 5 : Dialysis :

a) The above clear solution was dialysed overnight against two liters of buffer A. The enzyme is stable at pH 4.8 for two months or more and can be used for further purification.

b) If the enzyme is immediately applied to DEAE - Cellulose column it is dialysed against buffer B or the previously dialysed enzyme against buffer A was again dialysed against buffer B to bring the pH to 7.2.

Step 6 : DEAE - Cellulose Chromatography :

A column (1.8X28 cm) was packed with DE-52 cellulose (Whatman) and equillibrated with buffer B. The enzyme which was dialysed against buffer B was applied to the column and washed with 100 ml buffer B. Elution was performed with a 650 ml linear gradient from 0.05 to 0.4 M Nacl in buffer B. The resulting protein activity profile is shown in figure 1.

Nuclease fraction eluted with 0.1 M Nacl in buffer B. DNase I eluted with 0.05 M Nacl and DNase II eluted with 0.15 M Nacl. RNase I eluted with 0.05 M Nacl, RNase II eluted with 0.15 M Nacl and RNase III eluted with 0.3 M Nacl. Peaks 5 and 6 were of only RNase activity and were free from DNase activity.

For further studies, the eluted fractions of appropriate enzymes i.e. only RNase (F 70 - F 88), only DNase (F 52 - F 66) and Nuclease (F 40 - F 48) were pooled together separately.

2.6 : Factors affecting enzyme activity :

2.6.1 : Effect of temperature on enzyme activity :

Determination of the optimum temperature for enzymes i.e. RNase, DNase and Nuclease, appropriate substrates such as RNA or DNA

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(0.25 ml), buffer B (0.25 ml), distilled water (0.25 ml) was mixed with 0.25 ml enzyme and incubated at series of temperatures ranging from 20^{o} to $100^{o}C$ for 30 minutes.

2.6.2 : Effect of pH on enzyme activity

For determination of optimum pH, acetate buffer (pH 3.5 to 5.5) and Tris-Hcl buffer (pH 7.2 to 9.2) were used $Zn^{2+}(1X10^{-3}M)$ and Thioglycerol ($1X10^{-3}M$) was added to each buffer.

The incubation mixture was prepared as described in assay system except 0.25 ml buffer of different pH was added.

2.6.3 : Effect of Incubation period :

For studying the effect of incubation period the incubation mixture of RNase, DNase and Nuclease was incubated for various time intervals i.e. from 10 minutes to 24 hours at $37^{o}C$.

2.6.4 : Effect of Cations, Anions and Other Substances :

The effect of cations, anions and other substances was studied by adding $1X10^{-3}M$ of cations/anions to the reaction mixture. i.e. 0.9 ml enzyme was treated with 0.1 ml of 0.01 M cations/anions. 0.25 ml of this treated enzymes was added to the reaction mixture. Reaction was stopped by adding uranyl acetate reagent and absorbance at 260 nm was taken.