RESULTS AND DISCUSSION

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3 RESULTS AND DISCUSSIONS

3.1 Choise of the enzyme source

The purpose of present investigation is to study various nucleolytic enzymes in moth beans (Phaseolus Aconitifolius). Moth beans are closely related to mung beans (Phaseolus vulgaris). Both are dicotyledons and belong to the same family. Mung bean nuclease I is very well characterized. It is a single-strand specific nucleade and is used in genetic studies.

During seed germination and growth of seedlings, protein synthesis is vigorous and it is expected that the enzyme in nucleic acid metabolism are also very active.

Considering all these factors, moth been seedlings were chosen as the source of enzymes.

3.2 Isolation and Purification

3.2.1 Crude extract

Crude extract of the Moth bean seedlings was prepared and partial purification of the enzymes was achieved as described in Methods and Materials. The results of a typical purification from 100 gms. of moth beans are presented in Table 1.

3.2.2 Heat treatment

The results show that the enzymes are thermostable and even after heat treatment at 70°C for 15 minutes the RNase and DNase activities were retained to about 99% and 87% of the original. Mung bean nuclease is also thermostable and heat treatment at 70°C resulted in 40 fold purification (66). In case of moth bean, however, there is a slight increase in specific activity.

3.2.3 Acetone treatment

Precipitation of enzyme proteins in plants with organic solvents is a necessary step during purification. It helps to remove the impurities like chlorophyll and phenolic bodies which interfere in the protein assay by Lowry's method (67). About six fold purification was achieved by acetone treatment. During purification of mung bean nuclease, chilled ethanol was used instead of chilled acetone (66). The precipitate obtained with 35%-55% ethanol concentration contained enzyme activities.

3.2.4 Precipitation with ammonium sulfate

To the enzyme solution obtained after acetone treatment, solid ammonium sulfate was added. The precipitate obtained between 40% to 80% saturation contained maximum activities. Fractional precipitation with ammonium sulfate was tried. Activities were

obtained in all fractions, 40% to 55%, 55% to 70% and 70% to 80% though with more or less specific activities. In order to avoid losses in enzyme activities, the fraction between 40% to 80% saturation was taken. In case of mung bean and wheat leaves the fractions obtained between 50% to 85% and 45% to 85% saturation respectively, contained the enzyme activities (66,70).

3.2.5 Dialysis

Dialysis was carried out against tris-Hcl buffer pH 7.2 which contained Zn^{2+}($1X10^{-3}M$) and thio glycerol ($1X10^{-3}M$). Significant amount of activity is lost if the dialysis is carried out in the absence of Zn^{2+} or thio glycerol or both.

3.2.6 DEAE-Cellulose Chromatography

The typical elution pattern obtained in the chromatography of dialysed enzyme on DEAE-Cellulose, is shown in figure 1.

RNase activity was resolved into three distinct peaks (RNase I, RNase II and RNase III), DNase activity in two peaks (DNase I and DNase II) whereas a single peak occurred for nuclease activity. The peaks corresponding to nuclease, RNase II and DNase II were further characterized. The purification achieved after DEAE-Cellulose chromatography for RNase was 64 fold, for DNase 88 fold and for nuclease RNase 69 fold and nuclease-DNase was 80 fold.

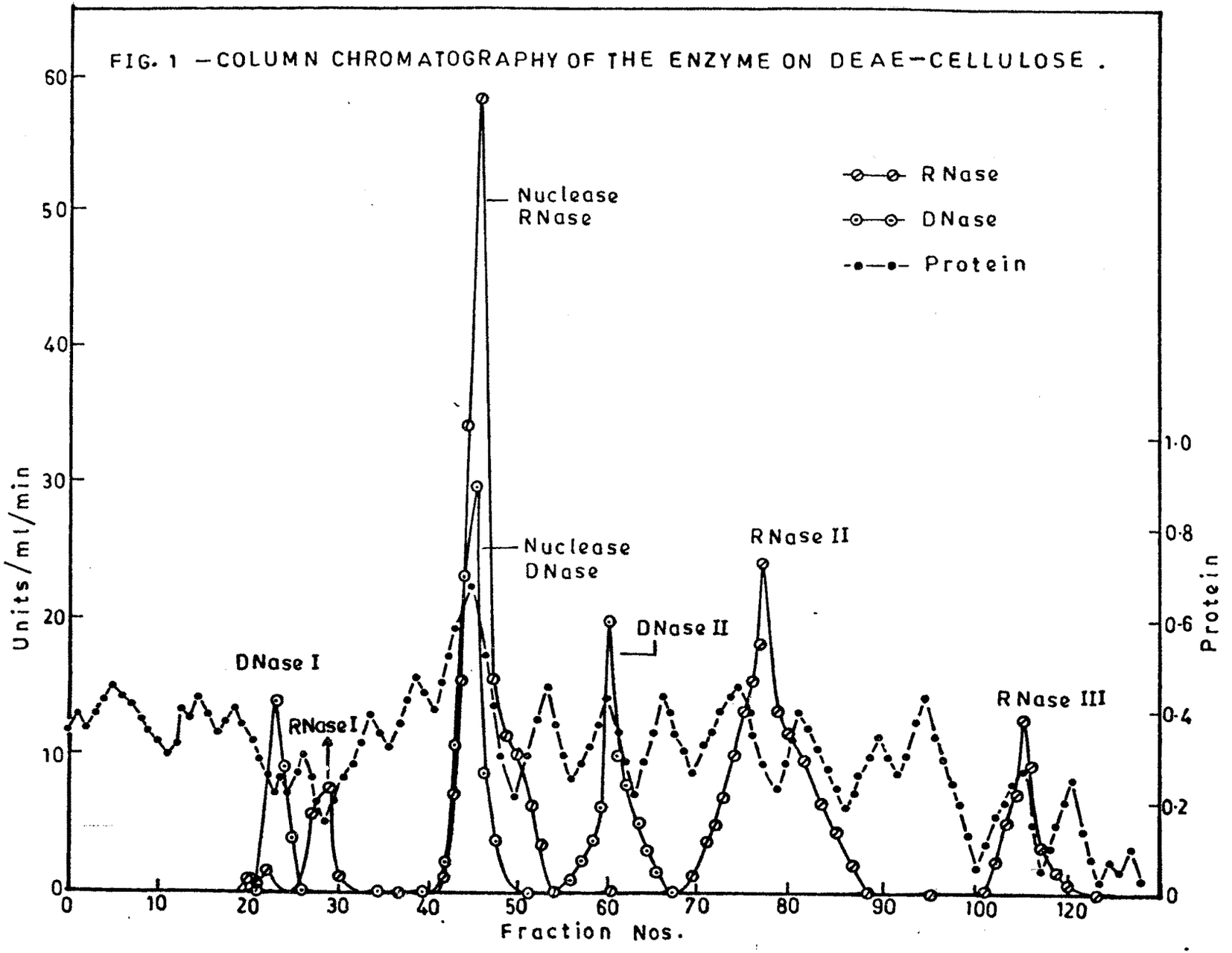
FIG. 1 —COLUMN CHROMATOGRAPHY OF THE ENZYME ON DEAE—CELLULOSE .
R Nase
DNase
Protein
Nuclease
RNase
Nuclease
DNase
DNase I
RNase I
DNase II
RNase II
RNase III
Units/ml/min
Protein
Fraction Nos.
0
10
20
30
40
50
60
70
80
90
100
120
0·2
0·4
0·6
0·8
1·0

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Mung bean enzymes were also subjected to DEAE-Cellulose chromatography however the enzymes, phosphomonoesterase, RNase and nuclease could not be separated into distinct peaks (68). Further separation of RNase and nuclease was tried using sephadex G-100 and hydroxyapatite (66,44).

In case of corn roots (36,77) and wheat leaves (70), purification was tried on Sephadex G-100. However no distinct separation of RNase and nuclease was obtained in both the cases. The enzymes from wheat leaves could not be separated even with DEAE-Sephadex A-25. The separation was achieved only with hydroxyapatite (70).

3.3 : Properties of the enzymes

3.3.1: Effect of pH on enzyme activity

The catalytic activity of the enzyme is markedly affected by environmental conditions, especially the pH of the aqueous media.

For determining effect of pH on enzyme activity, acetate buffer (pH 3.5 to 5.6) and Tris-HCl buffer (pH 7.2 to 9) were used, since both phosphate and citrate ions are inhibitory to nuclease activity; citrate or phosphate buffer are not employed to cover the pH range from 5.6 to 7.2.

The pH activity curves on RNase and Nuclease-RNase are shown in figure 2a, DNase and Nuclease-DNase shown in figure 2b and RNase and DNase shown in figure 2C.

The results are recorded in Table 2.

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Table 2 : Optimum pH

pH	Nuclease (Units/ml/min)			
	RNase	DNase	RNase	DNase
3.5	4	0.8	0.0	0.0
3.9	4.8	1.3	1.3	1.1
4.5	13.33	6.4	4	2.4
4.6	11	8.0	1.9	3.7
4.87	4.8	17.6	1.3	1.9
5.0	3.7	9.6	1.1	0.8
5.2	0.5	9.1	0.0	0.0
5.4	0.3	5.3	0.0	0.0
7.2	6.9	3.5	1.3	1.1
7.4	7.2	4.5	2.1	2.1
7.6	7.5	6.4	2.7	2.6
7.8	8.5	6.9	2.93	3.7
8.0	9.3	11.5	4.0	9.1
8.2	21.1	8.3	23.7	16.0
8.4	14.4	7.2	24.8	11.2
8.6	9.3	5.9	9.1	8.0
8.8	7.2	4.5	4.8	5.9
9.0	3.7	1.3	2.7	1.9
9.2	0.6	0.8	1.1	0.8

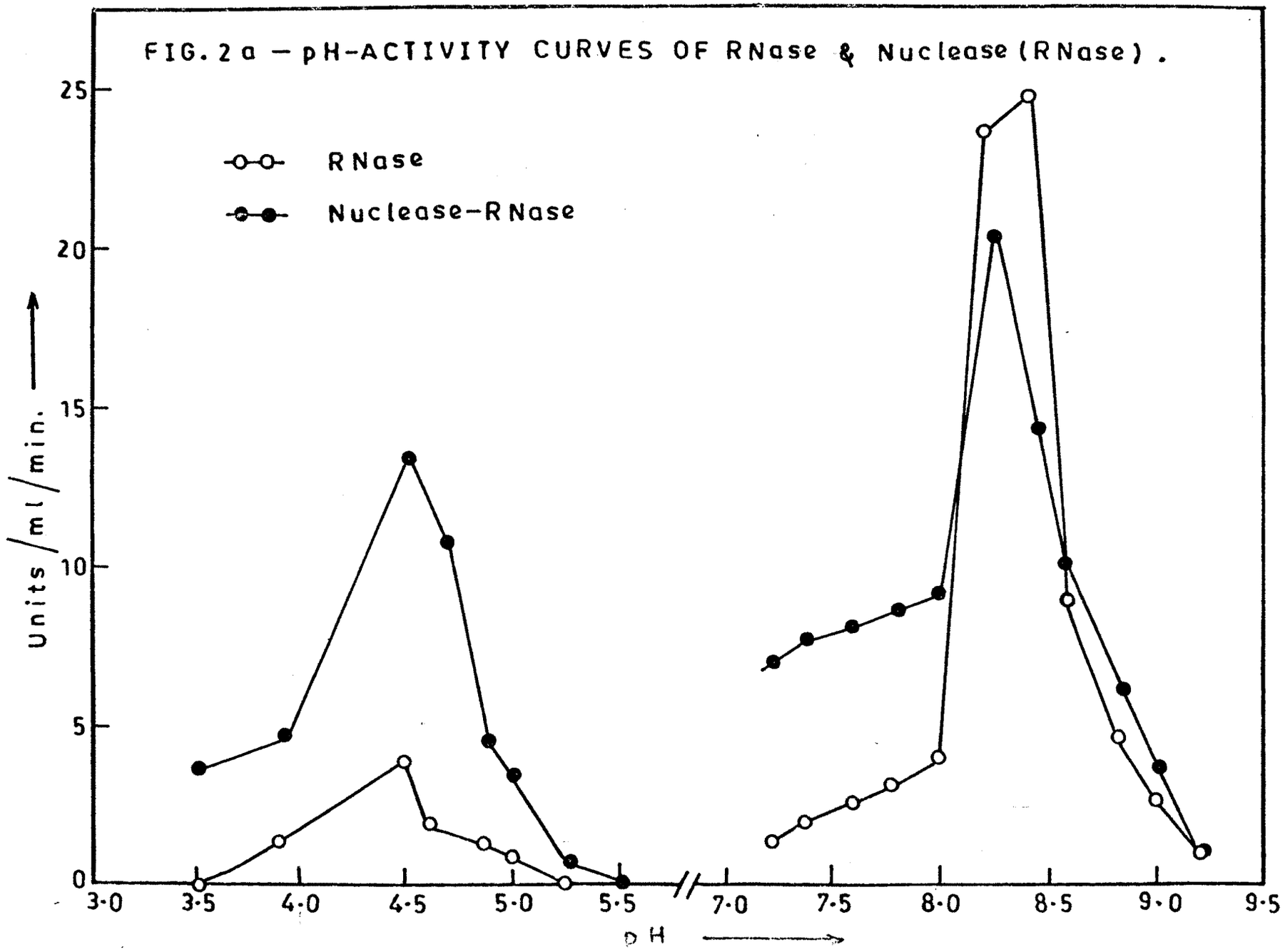
FIG. 2a – pH-ACTIVITY CURVES OF RNase & Nuclease (RNase).
RNase
Nuclease-RNase
Units /ml /min.
25
20
15
10
5
0
3·0
3·5
4·0
4·5
5·0
5·5
7·0
7·5
8·0
8·5
9·0
9·5
pH

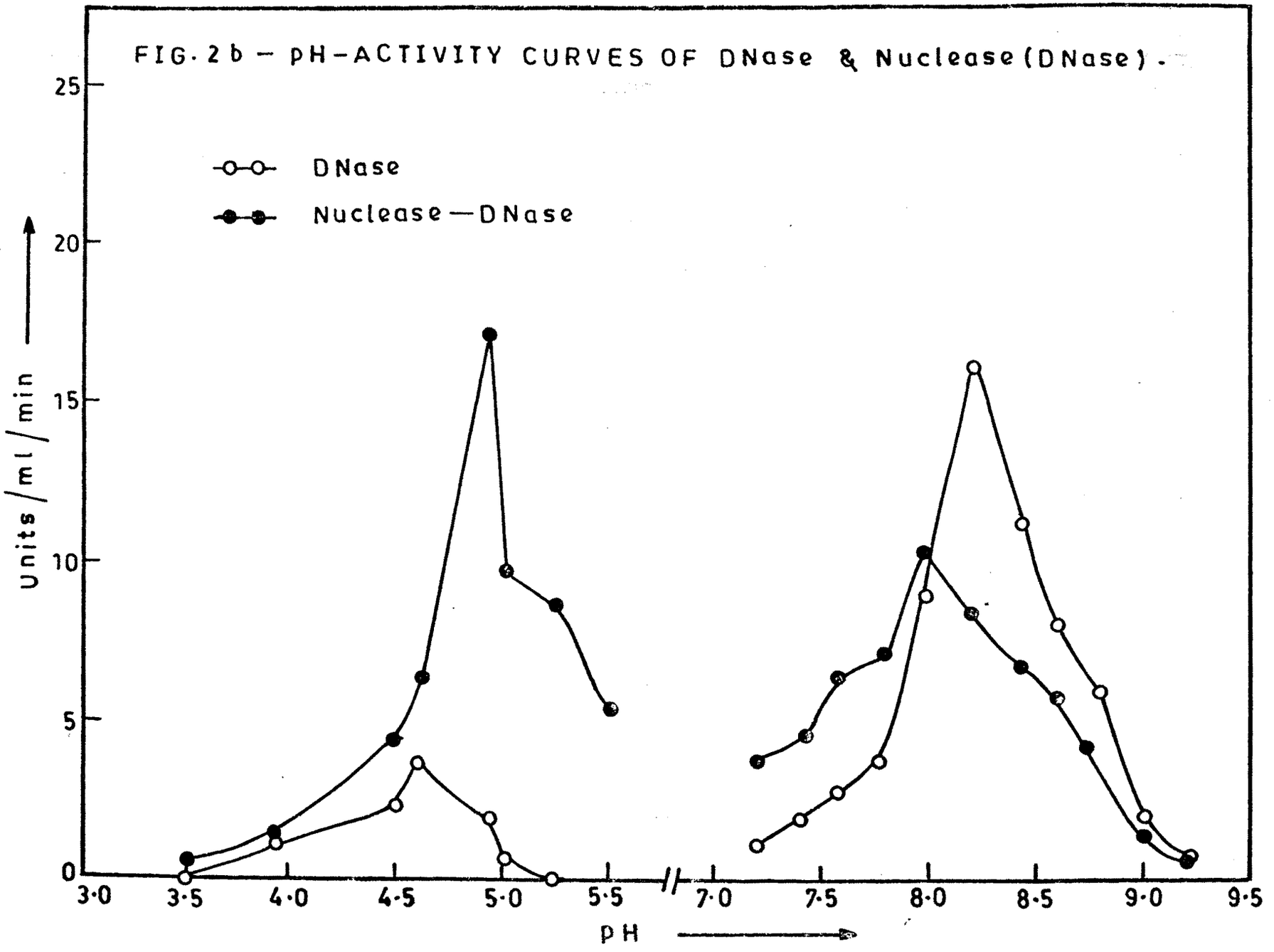
FIG. 2b – pH–ACTIVITY CURVES OF DNase & Nuclease (DNase).
DNase
Nuclease—DNase
Units /ml /min
25
20
15
10
5
0
3·0
3·5
4·0
4·5
5·0
5·5
7·0
7·5
8·0
8·5
9·0
9·5
pH

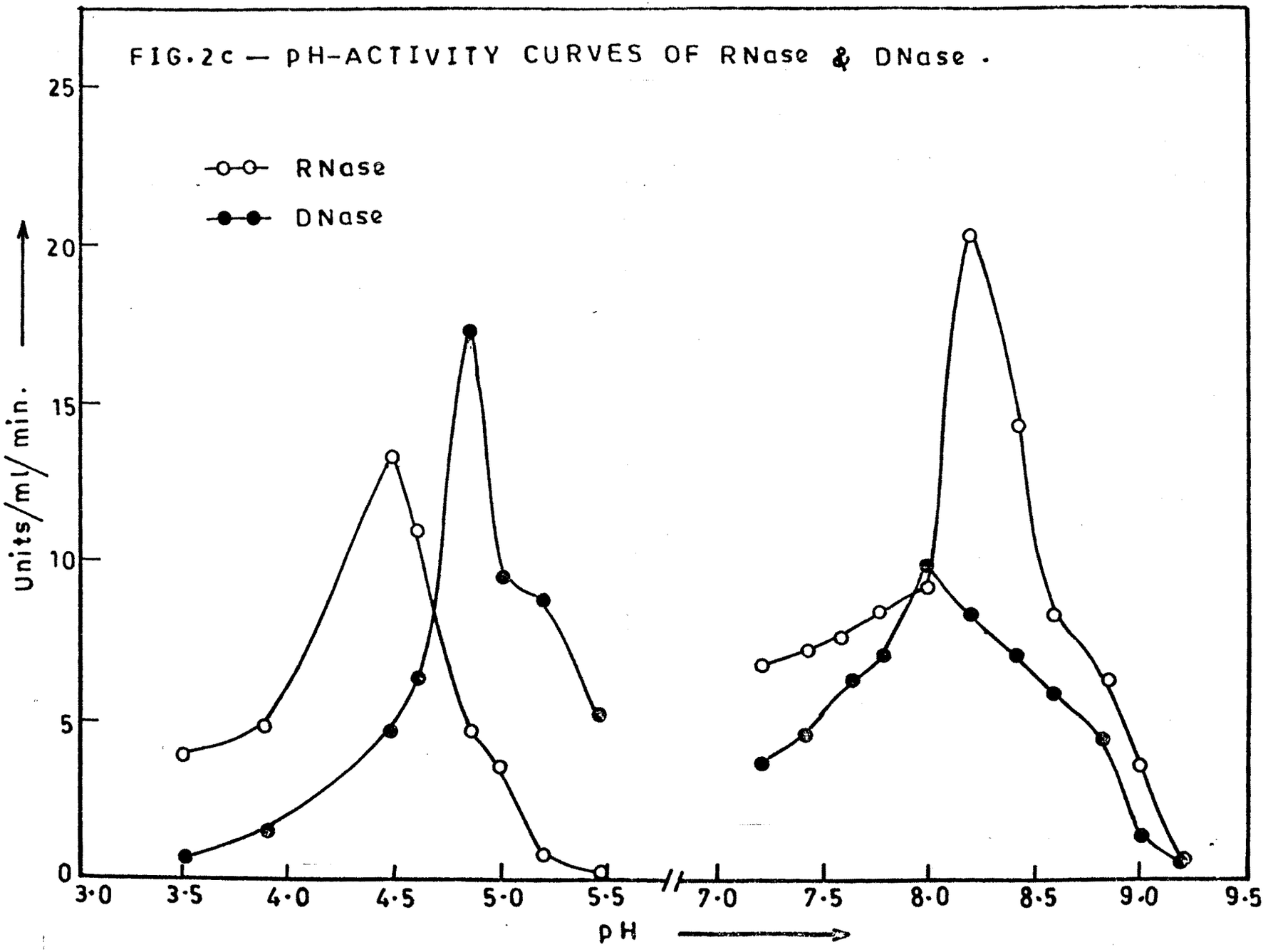
FIG.2c — pH-ACTIVITY CURVES OF RNase & DNase.
RNase
DNase
Units/ml/min.
25
20
15
10
5
0
3·0
3·5
4·0
4·5
5·0
5·5
7·0
7·5
8·0
8·5
9·0
9·5
pH

Moth bean RNase and DNase have two pH optima, one in acidic range at pH 4.5 to 4.87 and the other in alkaline range at pH 8.0 to 8.2.

Most of the RNases and Nucleases have either acid or alkaline pH optimum under standard conditions and can be classified accordingly.

Generally plant RNases have pH optimum in the acidic range. It varies with the plant source from pH 4.5 for rye grass RNase (15) to pH 6.7 for corn root RNase (36). RNase from Vicia faba roots is optimally active at pH 7.2 (64).

In General, plant nucleases have pH optimum either at acidic pH e.g. mung bean nuclease at pH 5.0 or they are active at or near about neutral pH e.g. Corn nuclease at pH 6.2 (36), Wheat leaf nuclease at pH 7.2-7.6 (70).

3.3.2 Effect of temperature on reaction rates

The catalytic activity of the enzyme is dependent upon temperature as in the case of ordinary chemical catalysis, but the activity is lost at the temperature above a certain limit due to denaturation of the enzyme.

The temperature activity curves of enzymes are shown in figure 3.

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Table 3 : Optimum Temperature

Temp	Nuclease		Units/ml/min	
	RNase	DNase	RNase	DNase
20	3.16	4.26	2.66	1.6
30	4	5.06	3.2	2.66
40	5.4	6.13	10.66	5.86
50	16.53	8.26	3.46	11.2
60	34.53	10.13	1.33	4.8
70	51.2	22.66	0.0	3.2
80	21.86	16.53	-	0.26
90	11.2	6.4	-	-
100	0.8	4	-	-

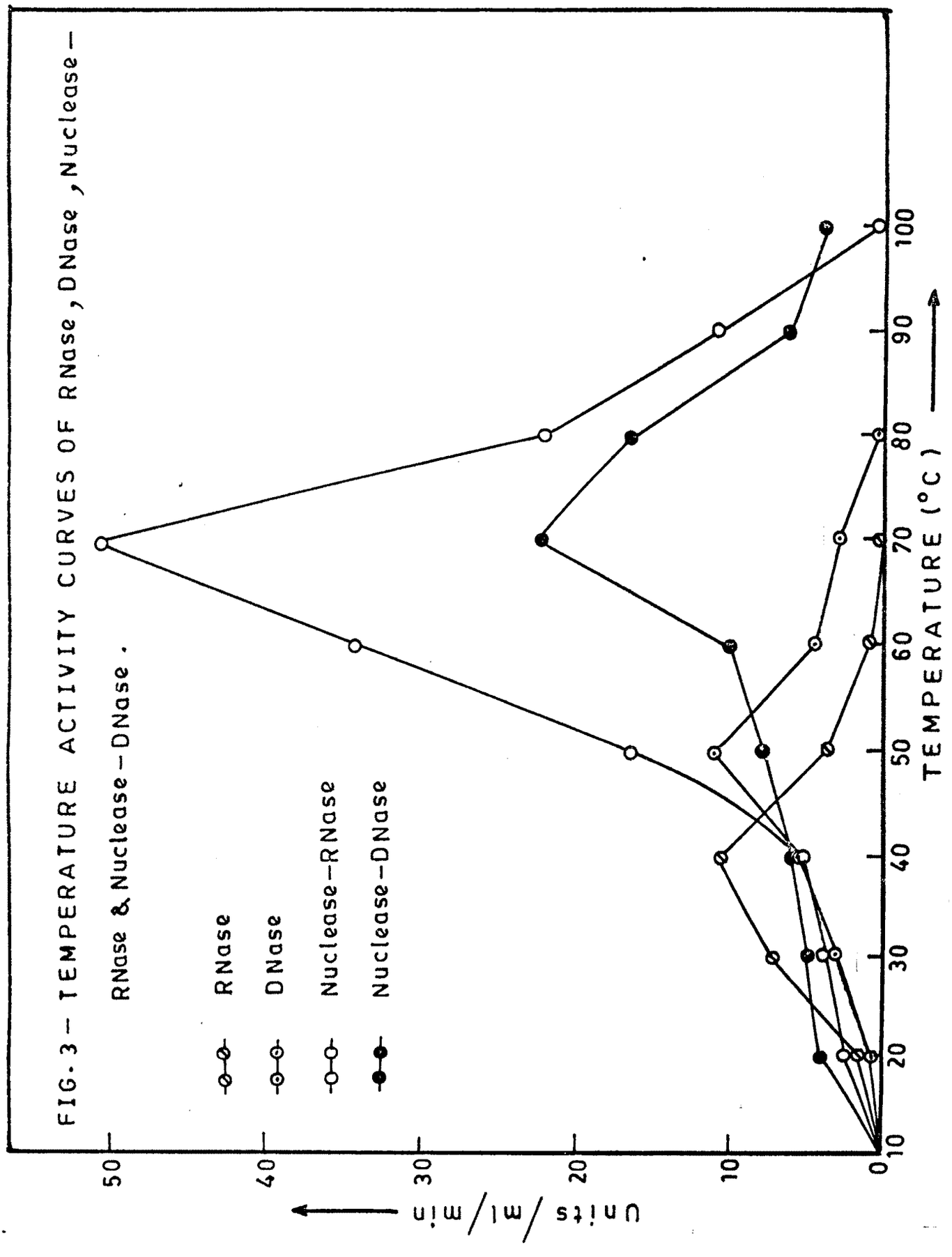
FIG. 3 – TEMPERATURE ACTIVITY CURVES OF RNase, DNase, Nuclease–
RNase & Nuclease–DNase.
RNase
DNase
Nuclease–RNase
Nuclease–DNase
Units / ml / min
TEMPERATURE (°C)
0
10
20
30
40
50
60
70
80
90
100

Results in Table 3 indicate that the reaction rate is increased with increase in temperature. In case of nuclease, the rate is maximum at 70°C and it considerably decreased at 100°C. RNase is optimally active at 40°C and DNase at 50°C.

The optimum temperature for nuclease-RNase and nuclease-DNase is the same. It indicates that both the activities are associated with the same protein.

3.3.3 Effect of Incubation period

Enzymes are incubated at various time intervals from 10 minute to 48 hours. General incubation period is 30 minute.

The results are recorded in Table 4.

Both RNA and DNA are polymeric substrates and contain several phosphodiester bonds susceptible to nucleolytic action. If the hydrolysis by nuclease at 24 hrs, is considered to be complete (100%). RNase from moth bean causes only 31.6% hydrolysis of RNA in 24 hours. Similarly moth bean DNase hydrolyzes DNA to 37.6% during the same period. Slow hydrolysis of the respective substrates by RNAse and DNase as compared to nuclease-RNase and nuclease-DNase suggests that RNases and DNases may be more specific towards the base content of the substrate.

Table 4 : Incubation period

Time in minute	Units/ml X 10^2			
	Nuclease			
	RNase	DNase	RNase	DNase
10	0.88	0.64	1.12	-
20	1.92	1.52	2.24	-
30	3.20	2.48	3.20	1.36
40	4.16	3.44	3.76	-
50	5.20	4.16	4.24	-
60	5.84	4.88	4.40	-
120	6.56	6.40	4.96	-
24 hrs	22.00	21.68	6.96	8.16
48 hrs	-			15.12
				* 1.20

* RNA is used as a substrate to confirm that this DNase is a true DNase and not a sugar nonspecific phosphodiesterase.

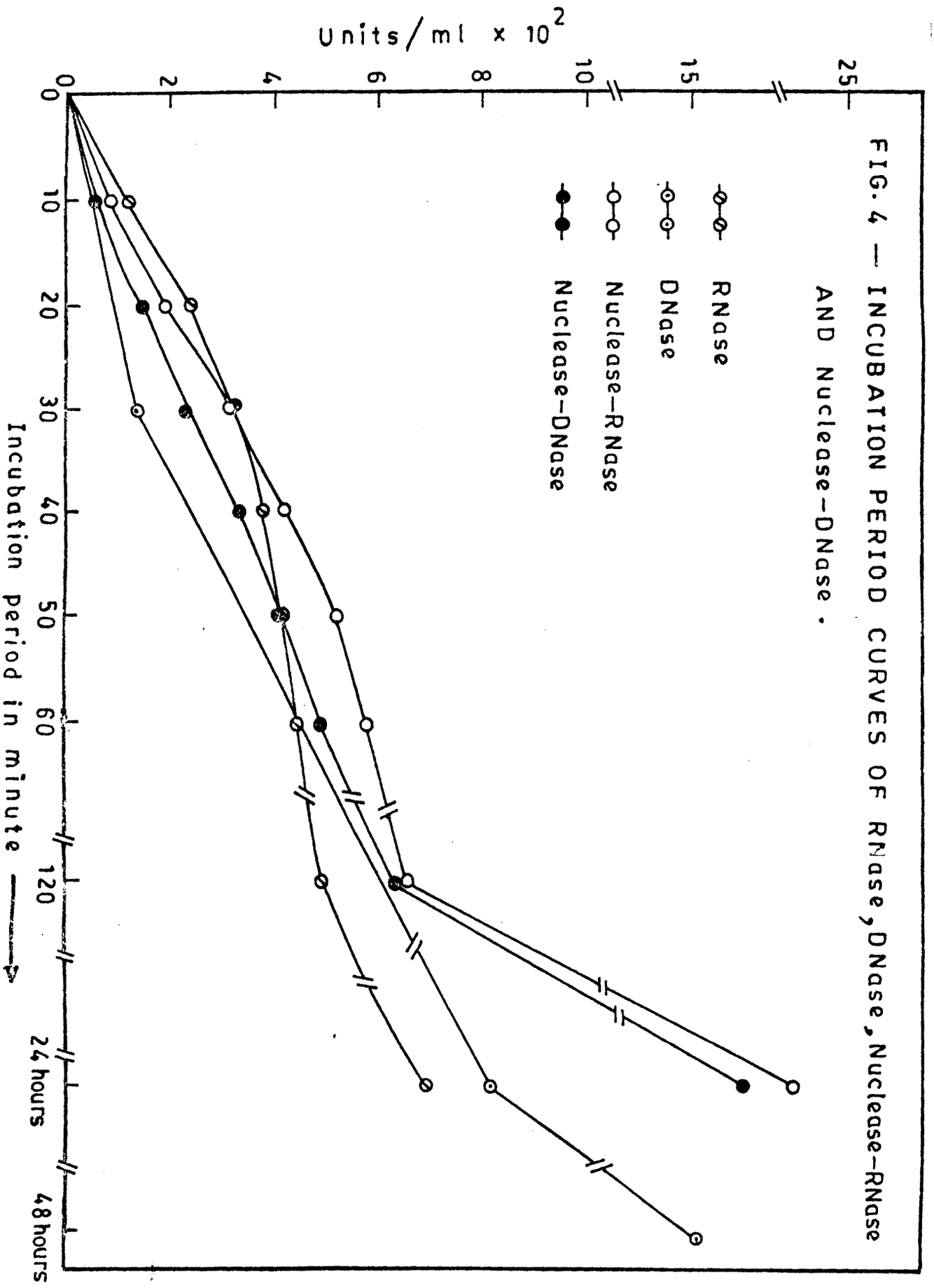

FIG. 4 — INCUBATION PERIOD CURVES OF RNase, DNase, Nuclease–RNase AND Nuclease–DNase.

Determination of base specificity of these enzymes will prove to be useful to confirm this aspect. In order to confirm that the DNase fraction is active only on DNA and is not a nonspecific phosphodiesterase acting both on RNA and DNA. The enzyme was incubated with RNA, it was observed that upto 24 hrs, RNA was not at all hydrolysed. However at 48 hrs there was slight hydrolysis of RNA by DNAse, showing that on prolonged incubation of the enzyme, it can hydrolyze RNA also, though to a very small extent.

3.3.4 Effect of Cations and Anions and other substances

The response of the enzymes to some cations, anions and other substances like EDTA and cysteine was studied. The results are recorded in Table No.5.

Nuclease is strongly inhibited by the cations Mg^{2+}, Ca^{2+} and Hg^{2+} and also by citrate and to some extent by phosphate. Citrate and phosphate buffers could not be used in the pH-activity profile study due to this reason.

Zn^{2+} and cysteine activate both nuclease-RNase and nuclease--DNase activities, Mn^{2+} also sctivates the nuclease and can substitute Zn^{2+}.

Table 5 : Effect of Cations and Anions and other substances

(Percentage activity remaining)

	Nuclease			
	RNase	DNase	RNase	DNase
Original	100	100	100	100
$MgCl_2$	0.0	11.1	31.4	27.2
$CaCl_2$	0.0	11.1	28.5	100
$MnCl_2$	142.8	200	31.4	36.4
$HgCl_2$	21.4	0.0	31.4	109.0
Zn-acetate	157.1	389.0	25.7	36.4
Citrate	0.0	0.0	77.0	0.0
phosphate	58.8	21.4	0.0	0.0
EDTA	35.7	33.3	100	309.0
Cysteine	214.7	188.8	14.3	63.6

EDTA is inhibitory to nuclease. The inhibition may be due to its chelation action.

Zn^{2+} specifically protected mung bean (45) and wheat (69) nucleases. Zn^{2+} inhibited the RNase activity but not the DNase activity of an Oat nuclease (12). Differential effect of zinc ions on moth bean nuclease-RNase (1.5 times) and nuclease-DNase (3.9 times) can be explained on this basis.

In case of moth bean RNase, all the cations studied inhibit the activity to about 70% to 75%. Phosphate and citrate are also inhibitory. Strong inhibition of RNase activity by cysteine suggest that the enzyme is not a sulfhydryl enzyme and does not require-SH groups for activity. The activity is not affected by EDTA. Similar observation is obtained with wheat leaf RNase (70).

Cu^{2+}, Ag^{2+}, Zn^{2+} ions are strong inhibitions of wheat germ RNase (73) whereas Ca^{2+}, Mn^{2+} and Mg^{2+} ions show less effect. Cu^{2+}, Hg^{2+},Zn^{2+} are inhibitors of tea leaf RNase (22) while Mg^{2+},Sn^{2+} and Fe^{2+} ions show the activating effect. Na^{+} and K^{+}ions are the activatiors of the corn leaf RNase (76) and Cu^{2+},Zn^{2+},Mn^{2+},Mg^{2+} are inhibitors.

Moth bean DNase is inhibited by Mg^{2+},Mn^{2+} and Zn^{2+}, but is not affected or slightly activated by Ca^{2+} and Hg^{2+}. Cysteine

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is also inhibitory, however EDTA causes three fold activation. These results shows that the DNase activity is distinct from nuclease-DNase and also from RNase.

3.4 Conclusion

The present investigation on the studies of nucleolytic enzymes from moth bean clearly indicate the presence of a Nuclease, a RNase, and a DNase, in moth bean seedlings.

DEAE Cellulose chromatography successfully separated these enzymes from each other. For convenience only the major peak of RNase and that of DNase was further characterised.

Few properties of these enzymes have been studied. All the three enzymes have two pH optima and therefore can not be classified either as acidic or alkaline enzyme. Nuclease is optimally active at 70°C, substantial activity occurs even at 80°C. Moth bean RNase and DNase have lower optimum temperature and reaction rate is zero at 50°C and 60°C respectively. Nuclease hydrolyzes both the substrates, RNA and heat denatured DNA completely in 24 hours, but RNase and DNase hydrolyse the respective substrate only partially. Slight hydrolysis of RNA by DNase is shown on prolonged incubation. Different cations, anions, cysteine and EDTA affect the three activities differentially.

All these observations suggest that Moth bean nuclease resemble mung bean nuclease I. Presence of DNase activity in moth bean seedling may be one of the few reports on plant DNase. However, it is essential to purify the enzyme extensively by using other ion exchangers or by gel filtration. Determination of molecular weights of all the three enzymes will also be helpful to confirm the presence of DNase in plant source.

Limited hydrolysis even after 24 hours by RNase and DNase suggest that these enzymes may be having specificity towards bases. Detailed studies to determine the base specificity are needed to confirm these observations which is beyond the time limits for M.Phil course.