STUDIES ON THE NUCLEIC ACID HYDROLYSING ENZYMES OF *ASPERGILLUS CANDIDUS* M16a

THE
THESIS SUBMITTED TO THE UNIVERSITY OF MYSORE
FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

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MARCH, 1983

D E C L A R A T I O N

I hereby declare that this thesis entitled "STUDIES ON THE NUCLEIC ACID HYDROLYSING ENZYMES OF ASPERGILLUS CANDIDUS M16a" submitted to the University of Mysore for the award of Degree of "DOCTOR OF PHILOSOPHY" is the result of the work carried out by me under the guidance of Dr.M.R. Raghavendra Rao, Deputy Director and Head, Discipline of Biochemistry and Applied Nutrition, Central Food Technological Research Institute, Mysore during the period February 1980-November 1982. I further declare that the results of this work have not been previously submitted for any other degree or fellowship.

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CERTIFICATE

I hereby certify that the thesis on "Studies on the Nucleic Acid Hydrolysing Enzymes of Aspergillus candidus M16a" submitted by Sri A.A.MOHAMMAD KUNHI for the degree of DOCTOR OF PHILOSOPHY ~~of~~ to the UNIVERSITY OF MYSORE is the result of research work carried out by him in the Discipline of Microbiology and Fermentation Technology, Central Food Technological Research Institute, Mysore under my guidance during the period 1980-1982.

M.R. Raghavendra Rao 14/3/83
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ACKNOWLEDGEMENTS

It is with great pleasure that I express my sincere and heartfelt thanks to Dr.M.R. Raghavendra Rao, Deputy Director and Head, Discipline of Biochemistry and Applied Nutrition, CFTRI, Mysore for his invaluable guidance and constant encouragement throughout the course of this investigation.

I wish to express my sincere thanks to Dr.V.Sreenivasa Murthy, Project Co-Ordinator, Discipline of Microbiology and Fermentation Technology, Mr.Syed Younus Ahmed, former Project Coordinator, Discipline of Fermentation Technology, Dr.K.R. Sreekantiah, Scientist, MFT Discipline, CFTRI, Mysore for their encouragement and keen interest during the course of this work.

I am extremely thankful to Dr.Richard Joseph, Scientist, MFT Discipline, CFTRI, Mysore for his useful suggestions and constant help throughout this study.

My sincere thanks are also due to a number of my colleagues, especially to Dr.S.C.Basappa, Dr.M.S.Prasad, Dr.K.Venkataramu, Dr.B.K.Lonsane, Mr.M.G.Byndoor, Miss T.Lalitha, Mr.B.C.Shenoy and Mr.L.C.Katwa for their help.

I am grateful to the Director, CFTRI, Mysore for the facilities provided and for permission to submit the results of the work in the form of Thesis.

I also wish to thank my wife Shameem for her constant encouragement.

(A.A. MOHAMMAD KUNHI)

C O N T E N T S

LIST OF TABLES

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LIST OF TABLES (contd.)

LIST OF FIGURES

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LIST OF ABBREVIATIONS

α	- Alpha
A	- Adenosine
β	- Beta
BSA	- Bovine Serum Albumin
C	- Cytidine
CDA	- Czapeck-Dox Agar
CDS	- Czapeck-Dox Solution
CF	- Culture Filtrate
CFTRI	- Central Food Technological Research Institute
CM-cellulose	- Carboxymethyl cellulose
cm	- Centimetre/s
°C	- degree centigrade
dia	- diameter
DEAE-	- Diethyl aminoethyl-
DFP	- Di-isopropylfluorophosphate
DNase	- Deoxyribonuclease
EDTA	- Ethylene Diamine Tetra Acetic Acid
e.g.	- for example (exempli gratia)
et c.	- et cetera
Fig/s.	- Figure/s
G	- guanosine
gm	- gram/s
hr/s	- hour/s
I	- Inosine
i.e.	- that is (id est)

LIST OF ABBREVIATIONS (contd.)

Kg.	- Kilogram
L/l	- Litre
µ	- Micrometer
M	- Molar
MBE	- Moldy Bran Extract
µg	- Microgram
MFT	- Microbiology & Fermentation Technology
mg	- milligram/s
ml	- millilitre
mM	- millimolar
mm	- millimeter
min.	- minute/s
MRBE	- Moldy rice bran extract
NBS	- N-bromosuccinimide
nm	- Nanometer
O.D.	- Optical Density
%	- Per cent
PCMB	- Para-chloromercuribenzoate
PDase	- Phosphodiesterase
PMase	- Phosphomonoesterase
psi	- Pounds per square inch
RNase	- Ribonuclease
rpm	- Revolutions per minute
U	- Uridine
Ψ	- Pseudouridine

LIST OF ABBREVIATIONS (contd.)

Viz.	- Namely (Videlicet)
V/V	- Volume/Volume
W/V	- Weight/Volume
Wt.	- Weight
W/W	- Weight/Weight

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S Y N O P S I S

SYNOPSIS

The ribonucleases (RNases) are perhaps the most extensively and widely studied enzymes. Historically, they are unique, having served as model systems for understanding various aspects of an enzyme. A great deal of basic data on RNases are available that concern amino acid sequence, chemical synthesis, structure-function relationship etc. There is, however, not much significant information on the application of these enzyme nor any data available pertaining to their commercial exploitation. The present work is, to a fair degree, aimed at collecting data on the RNase with a possibility for its commercial utilization. The major part of the present investigation was carried out on the RNase produced by Aspergillus candidus which has, hitherto, not been reported to produce extracellular RNases. An important application envisaged in the present work was for the reduction of nucleic acid content in single cell proteins (SCP). So far, only the bovine pancreatic RNase was shown to be capable of reducing nucleic acid in SCP and hence an economical source was to be sought in a microorganism. A. candidus RNase was therefore studied from this viewpoint also.

The thesis is presented in four parts. The first part comprises introduction and literature survey. In the introduction the relevance of the present investigation is highlighted, indicating particularly that extracellular RNase of A. candidus has, hitherto, not been characterised nor its use in the reduction of nucleic acid content of single cell protein reported. Further, an inexpensive microbial process for the production of RNase for this purpose has apparently not been worked out yet. Literature survey includes a brief account of the historical developments in the field of enzymology and the present status of industrial enzymology followed by a brief survey of literature on nucleolytic enzymes, with emphasis on RNases. A short review on single cell protein with reference to its use as human food and the various methods adopted for reduction of nucleic acids in SCP is also included in this part.

The second part, Materials and Methods, presents details of experimental procedure, materials and equipments employed in the present work.

The third part, Results and Discussion, is divided into four chapters. In the first chapter the results of screening, selection, and identification of the potent fungal strain and studies on optimization of various environmental and nutritional parameters are given.

Studies on the crude enzyme preparations from A. candidus M16a form the subject of second chapter. The third chapter gives details of the purification and properties of RNase. Fourth chapter deals with the application of RNase in the reduction of nucleic acids in SCP and the recovery of the products of enzyme hydrolysis. The results are discussed in each chapter.

In the fourth part, the Summary and Conclusions are presented.

The experimental data pertaining to part three are summarised under the following heads:

Screening of fungal strains:-

A total of sixty strains of fungi belonging to the genera Aspergillus, Penicillium, Fusarium, Trichoderma, Trichothecium, Botryodiploidea, Gibberella, Sporotrichum, Rhizopus, Glomeralla and Rhizoctonia were isolated or obtained from various culture collections for screening their ability to produce RNase. Among them 5 isolates designated as Isolate M16a, A. terreus, A. ochraceous, A. carbonarius and A. oryzae showed good RNase activity of which the maximum was shown by Isolate M16a. The taxonomical studies on this strain revealed that it belonged to Aspergillus candidus.

Optimization of nutritional and environmental parameters:-

Various nutritional and physical parameters were optimised for the maximal production of RNase by A. candidus M16a. Starch was found to be the best carbon source followed by maltose and glucose. Among six nitrogenous salts, ammonium nitrate gave the highest yield. Out of eight organic nitrogen sources used, casein was the best followed by peptone. The yield of enzyme with peptone, a organic nitrogen source was about 10 times more than that with the best inorganic nitrogen source, NH_4NO_3. The optimum ratio of ammonium nitrate to starch was 1:7.5. The optimum ratio of C and N sources was found to be 1.125% starch and 0.15% ammonium nitrate. Trace metals and vitamins did not show any significant effect. Supplementing the medium with rice bran at 0.1% and sodium glycerophosphate at 0.01% levels almost doubled the enzyme yield whereas RNA did not show any significant effect.

Out of a number of chemically undefined media containing cheaper raw materials tested 10% rice bran extract without any fortification gave good enzyme yield. Ragi flour (5%), wheat bran extract (10%), wheat bran extract fortified with peanut meal were also promotory of enzyme production.

Solid state fermentation:-

Wheat bran, rice bran, or ragi husk with or without fortification with trace minerals were used individually and in combinations for solid state fermentation by A. candidus M16a. Rice bran alone was found to give maximum yield. Distilled water, tap water, and 0.2 N HCl with or without trace minerals were tested as moistening substrates at different levels and moistening with tap water to about 50% moisture level was found to be the best.

Environmental parameters:-

The medium containing glucose, peptone and minerals at pH values ranging from 2.0-11.0 were tested and the maximum enzyme yields were obtained at pH 6.3 and 9.6, while the biomass produced was not significantly different at the pH range of 5.0 to 10.0. The enzymes produced at both the pH values were similar on the criteria of pH and temperature optima. A volume to surface ratio of 75 ml medium in 500 ml Erlenmeyer flask, shaken on a rotary shaker at 230 rpm, was found to be optimal. Ambient temperature ranging from 24-27°C and incubation period of 80-85 hrs were most suitable for solid state fermentation.

Studies on the crude enzyme:-

Using enzymes prepared by solid state and submerged fermentations it was noted that pH 4.5, temperature 55°C, 0.25% RNA concentration and incubation period of 30 min were optimum, while addition of EDTA to the reaction mixture was not necessary. Based on these an assay procedure was devised.

The crude enzyme was most stable at pH range 5.0 to 6.0. Heat denaturation pattern of the crude enzyme was very interesting. The samples preincubated at 40°C or below did not show any loss of activity, at 50° and 60°C there was a 50% or more loss of activity, and, unexpectedly, at higher temperatures viz. 70° or higher the loss of activity was about 25%. Proteolytic activity of the crude enzyme was found to be the cause of this abnormal heat inactivation pattern. The proteolytic activity was maximum at 50°C. The involvement of proteolytic activity was further evidenced by the normal pattern obtained after inactivating the protease by di-isopropyl fluorophosphate (DFP) and also by the addition of bovine serum albumin (BSA) to the crude enzyme.

The heat inactivation pattern of moldy rice bran extract (MRBE) was different from that of culture filtrate from

glucose-peptone-mineral medium. 5 min incubation at 80°C resulted in about 95% loss of activity as against 25% loss with enzyme from submerged culture. Addition of EDTA at 0.4-0.5% level was found to be effective to a considerable extent in protecting the enzyme indicating the role of metallic contaminants present in MBE in the enhanced inactivation of the enzyme by heat. The enzyme preparation with added EDTA exhibited a pattern similar to that of the enzyme prepared by submerged cultivation.

Other enzymes in the crude preparations:

Submerged culture filtrate and MBE were tested for the presence of other enzymes. Both showed DNase activity against native as well as heat denatured DNA. Non-specific phosphodiesterase activity was present in both the preparations. Nucleotidase activity against 3'- and 5'- AMP was about 3 times more in MBE as compared to that in submerged culture filtrate.

The presence of proteolytic enzymes in crude preparations is of critical concern as the preparation is intended to be used in reducing nucleic acid content in SCP. Therefore studies were carried out to establish the nature of the proteolytic enzyme present in the crude preparations. The protease showed optimum activity at 50°C in the pH range 6.0-7.0. It retained 25%

activity when heated at 80°C and pH 5.6 for 5 min, but at higher pH values the loss of activity was about 90%. It was inhibited completely by DFP indicating the involvement of serine in the active site. It has been found to be the major contaminating protein at various steps of RNase purification.

Purification of RNase:-

RNase from MRBE was purified to about 100 folds by using six steps: precipitation by ethanol between 30 and 50%, gel filtration on Sephadex G-100 column, adsorption on DEAE-cellulose and batch wise desorption with sodium chloride solutions (twice), and finally fractionation on a DEAE-Sephadex A-50 column. The yield of pure enzyme was about 16%. The purified enzyme was found to be homogeneous by polyacrylamide disc-gel electrophoresis at pH 4.3 and 8.1.

Properties of the purified RNase:-

The purified enzyme was found to be active on yeast RNA optimally at pH 4.5 and between 55 and 60°C. It was heat stable at pH 5.5 to 6.0 in potassium phosphate buffer. At 55°C after 60 min incubation it retained about 98% activity, while more than 50% activity was retained at 60°C even after 60 min incubation. At 70°C and 95°C the retention of activity after 30 min incubation was 38% and 6% respectively.

Heavy metal salts such as $CuCl_2$, $Pb(NO_3)_2$, $FeCl_3$, $HgCl_2$ and $ZnCl_2$ were inhibitory to the enzyme at 2mM. Among group specific reagents N'-bromosuccinimide and para-hydroxymercuribenzoate were 100% and 65% inhibitory at 1mM, respectively. Di-isopropylfluorophosphate, malathion, sodium azide as well as photooxidation with rose bengal and methylene blue did not show any effect indicating that serine and histidine are not involved in the active site. Iodoacetamide and EDTA showed slight improvement in the activity.

The molecular weight of the RNase as determined by gel filtration on Sephadex-G-100 was 26,000.

Km value for RNA was 3.64 mg/ml.

The rate of hydrolysis of different polynucleotides was in the order: Poly U $>$ Yeast RNA $\gg$ Poly C $>$ Poly A. The enzyme did not degrade poly G and showed only very insignificant activity towards DNA. The enzyme indicated absence of activity against bis-para-nitrophenyl phosphate. No strict base specificity was exhibited by the enzyme, although the rate of release of mononucleotides from yeast RNA was in the order: AMP $>$ UMP $\gg$ CMP $\ggg$ GMP. The enzyme forms from RNA 2',3'-cyclic nucleotides as intermediates, the final products being 3'-mononucleotides of adenine, uracil and cytosine. A mixture

of mono- and oligo nucleotides of guanine is the other product. From paper chromatographic evidence the enzyme was found to act endonucleolytically.

Application of the RNase in the reduction of nucleic acid content in SCP:-

The enzyme was employed in reducing the nucleic acid content of Saccharomyces and Candida yeast cells. For the maximal removal of nucleic acids from the yeast biomass, parameters such as pretreatment of the cells, pH, temperature and enzyme-cell-ratio were studied. The yeast strains used were S. cerevisiae, C. lipolytica, C. tropicalis (one strain each) and C. utilis (2 strains). The pre-treatments for improving the cell permeability to the enzyme indicated that heat treatment was effective only in the case of C. utilis and C. tropicalis strains, whereas chloroform treatment was effective in all the strains. The concomitant loss of protein from the cell pellet was high in the case of chloroform treated cells. However, a heating process subsequent to the chloroform treatment improved the protein retention considerably leading to a marginal loss of only about 10-13% which occured after heat treatment also. Chloroform treatment for 6 hrs followed by slight heating (65°C) to evaporate the chloroform was ideal. Increase in pH of the cell suspension of S. cerevisiae and C. utilis showed an

increased leakage of nucleic acids. However, it was indicated that the enzyme action was optimum at pH 4.5-5.0. Optimum temperature for the enzymatic reduction of nucleic acids from yeast cells was 55°C while the optimum enzyme to cell ratio was 1:5,000 (W/W).

DFP treated MBE having no protease activity was most efficient with 85% reduction of nucleic acids while with purified RNase it was about 75-80%. Only 10% loss of protein was observed in both these cases. Heat treated MBE with partially inactivated protease and non-treated MBE were also effective in the reduction of nucleic acids. However, the protein loss was as high as 25 and 42%, respectively.

Fractionation and recovery of nucleotides:-

The nucleotides produced by the hydrolysis of RNA from yeast cells by RNase were fractionated by ion-exchange column chromatography on Dowex-1 (Cl^- form) and eluted in 4 fractions. The fractions contained CMP, AMP, UMP and GMP and were eluted in that order. The recovery of each fraction was 100 per cent.

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PART I

INTRODUCTION AND REVIEW OF LITERATURE

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1. NUCLEIC ACIDS

The nucleic acids, DNA and RNA are the normal constituents of all cells; plant, animal and microbial. The RNA and DNA content of microorganisms shows much variation. RNA content varies from 0.3-51% in the case of bacteria, 2.7-11% in the case of yeasts and 0.7-28% in the case of molds (Ogata, 1963). The DNA content has been reported to vary from 0.37 to 4.5% in bacteria, 0.03-0.52% in yeasts and 0.15%-3.3% in fungi (Tomita, 1976).

Discovered by Miescher in 1868, this group of compounds was named nucleic acids in 1889 by Altmann. Subsequently the base components purines and pyrimidines and later the pentoses present were identified as ribose in yeast nucleic acid and deoxyribose in thymus nucleic acid (Levene, 1930; Levene and Jacobs, 1909). This was followed by classification of nucleic acids into ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) depending upon the type of pentose moiety.

Chemistry of nucleic acids:

Nucleic acids consist of purine and pyrimidine bases, ribose or deoxyribose and esterified phosphoric acid. The organic components in RNA are the purines, adenine and guanine and the pyrimidines cytosine and uracil; the sugar present is D-ribose. DNA on the other hand contains adenine and guanine as purines, but cytosine and thymine as pyrimidines and the pentose is 2-deoxy-D-ribose; in rare cases 5-methyl

cytosine or 5-hydroxymethyl cytosine is present, as in some bacteriophages. Three purine derivatives hypoxanthine, xanthine and uric acid, also occur in nature. A number of other "minor bases" mostly methylated ones are also found in some nucleic acids as t-RNA.

Nucleosides:- A nucleoside is a N-glycosylated (ribofunanosyl) purine (at 9) or pyrimidine (at 1): the carbohydrate moiety is either D-ribose or 2-deoxy-D-ribose. Thus adenine with ribose forms the nucleoside adenosine, guanine forms guanosine, cytosine forms cytidine and uracil forms uridine. The ribonucleoside of hypoxanthine is called inosine. The nucleosides formed from 2-deoxyribose are known as deoxyribonucleosides - deoxyadenosine, deoxyguanosine, deoxycytidine, deoxythymidine and so on.

Nucleotides:- Nucleotides are phosphoric esters of the nucleosides. The one derived from ribonucleosides are usually called ribonucleotides and those derived from deoxyribonucleosides are referred to as deoxyribonucleotides. As there are three free hydroxyl groups in the sugar ring of a ribonucleoside, three ribonucleoside monophosphates can be formed. Adenosine for example, can give rise to three monophosphates, adenosine 5'-phosphate (5'-AMP), adenosine 3'-phosphate (3'-AMP) and adenosine 2'-phosphate (2'-AMP). Similarly guanosine, cytidine and uridine can give rise to three guanosine monophosphates (guanylic acids), three cytidine monophosphates (cytidylic acids) and three uridine mono-phosphates (uridylic acids) respectively. Adenosine 5'-

phosphate (5'-AMP) on deamination gives rise to inosine 5'-phosphate (5'-IMP), a ribonucleotide containing hypoxanthine.

The ribonucleoside 5'-phosphates may be further phosphorylated at position 5' to yield 5'-di-and-tri- phosphates. For example, adenosine 5'-phosphate (AMP) yields adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP). 5'-tetraphosphates of adenosine and guanosine have also been reported. In the same way other ribonucleoside 5'-phosphates yield such di- and tri-phosphates as GDP, CDP, UDP, GTP, CTP and UTP.

Hydrolysis of RNA can give rise to ribonucleoside 2', 3'-cyclic monophosphates as well as 3',5'-cyclic monophosphates (This will be discussed again under the Section on the action of enzyme on RNA).

Deoxyribonucleic acids:

It is present mainly in the nucleus of the eukaryotic cells; mitochondria and chloroplasts also contain DNA. The prokatyotes do not contain a nucleus and hence the DNA exists "bare" in the cytoplasm, probably attached to the plasma membrane. Depending upon the conformation of the molecule DNA can be grouped into various types such as linear-single stranded, linear duplex, cyclic-single stranded, cyclic duplex etc. (Adams et al., 1976). Linear DNA occurs naturally and is also a product of the degradation of cyclic DNA. Single-stranded DNA, both in linear and cyclic

forms, is found in some viruses, and can be formed artificially by denaturing double-stranded (duplex) DNA (Adams et al., 1976).

The monomeric units of DNA are deoxyribonucleotides containing the bases adenine, cytosine, guanine and thymine. However, many DNAs have been reported to contain other bases such as 5-methylcytosine (wheat germ DNA) and 5-hydroxy methylcytosine (bacteriophages T_2, T_4 and T_6). Wide variations in the molar proportions of bases in DNAs from different species are observed (Schildkraut et al., 1962; Kit, 1963). However, the sum of the purines is always equal to the sum of the pyrimidines. Adenine and thymine are present always in equimolar amounts: so are guanine and cytosine. The equivalence of A and T and of G and C is of the utmost importance in relation to the formation of the DNA helix.

The DNA molecules are of enormous size (10^6 to 10^{10} daltons) and are difficult to isolate without shearing them into smaller fragments (10^6-10^7) (Adams et al., 1976). The classical and revolutionary paper by Watson and Crick first elucidated their double helical structure with complementary base pairing (A with T and G with C with 2 and 3 hydrogen bonds respectively) (Watson and Crick, 1953). There are other structures proposed recently (Tunis-Schneider and Maestre, 1970; Gupta et al., 1980; Sasisekharan, 1981; Sasisekharan and Brahmachari, 1981; Zimmerman, 1982). It is outside the scope of the present review to discuss the genetic, structural and biosynthetic aspects.

Ribonucleic acids:

Types:- The living cell from any source contains mainly three kinds of RNA, viz. r-RNA or ribosomal RNA, t-RNA or transfer RNA and m-RNA or messenger RNA. r-RNA and m-RNA exist as complexes with proteins, but t-RNA exists in a free form. The major portion of RNA, about 75-80 per cent is contained in the cytoplasmic particles known as ribosomes. r-RNA which is of high molecular weight and metabolically stable falls into 2 main categories. In Esch. coli the 50S and 30S ribosomal units contain RNAs of mol. wts. 1.1×10^6 (23 S RNA) and 0.6×10^6 (16 S RNA) respectively. The two RNAs contain different base ratios and differ in base sequences. Similarly, the 70S and 80S ribosomes from mammalian cells also yield two r-RNA components corresponding to the 30-40 S and 50-60 S ribosomal units (Markham, 1957).

Transfer RNA (t-RNA) is the next most abundant type, i.e. 10-15 per cent. They have much lower molecular weight (23000-28000) and particle size, 4S. Many different t-RNAs exist, each being specific for an amino acid (Markham, 1957; Rich and Bandary, 1976).

The remaining 5 per cent or less is RNA with a base composition corresponding very closely to that of DNA. It is sometimes referred to as DNA-like RNA and includes 'messenger' RNA (m-RNA). The molecular weight of m-RNA shows wide variations, 0.5×10^6 or higher.

Base composition: The four constituent bases are not distributed in the RNA molecule in equimolar amounts as it was believed to be earlier. The amounts vary from one RNA to another (Adams et al., 1976). Pseudouridine (Ψ) and the methylated bases (the so called minor or modified bases) are found in RNA (Hall, 1970). They are more abundant in the t-RNA fraction. Most r-RNAs have relatively high levels of guanine plus cytosine (usually 50-60 per cent) regardless of the base composition of the cellular DNA (Markham, 1957). On the other hand, the m-RNA class as a whole appears to have a guanine plus cytosine composition more closely corresponding to that of the cellular DNA. In some RNAs such as those in reovirus and wound tumour virus (and also in t-RNA) there is close equivalence between A and U and between C and G.

The relative molar proportions of bases in different RNAs from different sources as compiled by Adams et al.(1976) are shown in Table 1.

The nucleotides are linked by phosphodiester bonds connecting C-5'-OH in one nucleotide with C-3'-OH of the next nucleotide. A polynucleotide chain is built up by the same process. A segment of a polynucleotide chain is given in Fig. 1.

Secondary structure: The secondary and tertiary structure of RNA is not so well defined eventhough it has been partially established (Stent, 1963; Tattersall et al., 1973).

Table - 1

Molar proportions of bases (as moles per 100 moles nucleotide) in RNAs from various sources

Source	Type	Adenine	Guanine	Cytosine	Uracil	Uracil as Ψ	Uracil as Methyl bases
Human (HeLa)	18S r-RNA	21.0	29.6	27.5	21.7	-	-
	28S r-RNA	16.0	35.6	31.7	16.5	-	-
	5S r-RNA	18.3	30.3	29.0	22.3	-	-
	m-RNA (bulk)	32.5	21.0	21.6	24.8	-	-
Yeast	m-RNA	28.6	21.2	21.7	28.7	-	-
	r-RNA	25.6	29.0	18.6	26.8	-	-
	t-RNA	19.4	26.6	25.1	20.1	4.6	3.1
	Bulk RNA	25.7	27.0	20.6	26.7	-	-
E. coli	16S r-RNA	24.8	31.0	22.7	21.5	-	-
	23S r-RNA	25.4	33.5	21.5	19.6	-	-
	m-RNA (bulk)	25.1	27.1	24.1	23.7	-	-
	t-RNA	18.3	30.3	30.3	15.9	2.4	2.2

Ref. Adams et al, (1976)

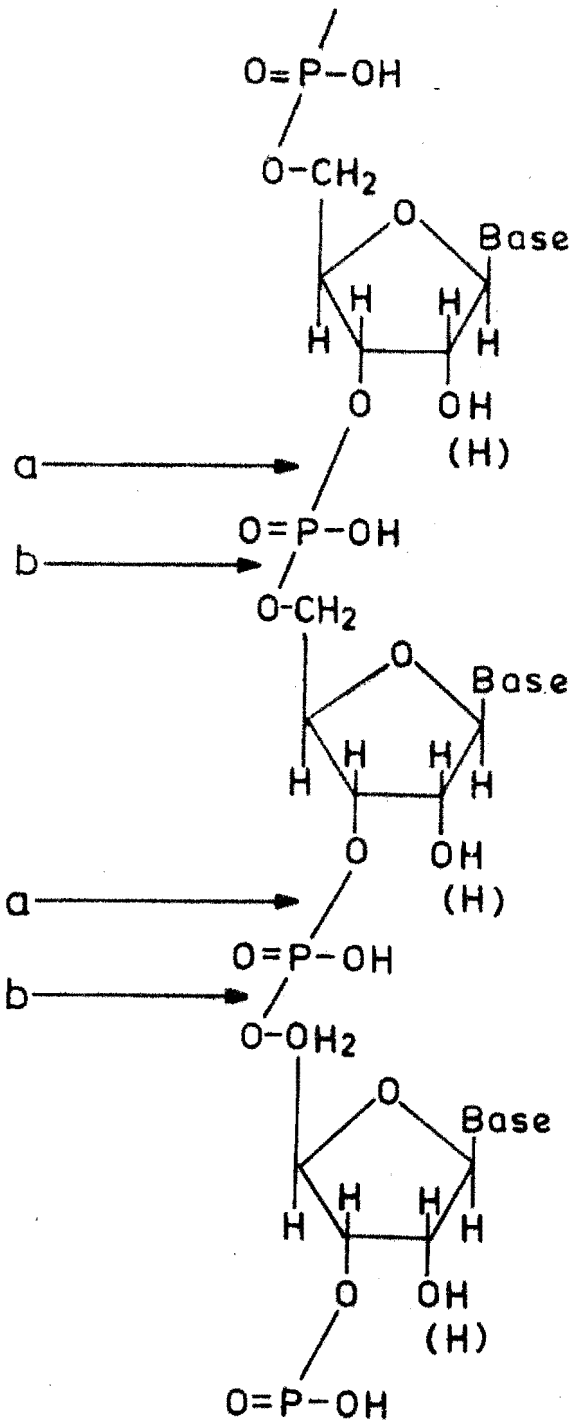

Fig. 1. A Segment of a Polynucleotide Chain showing the phosphodiester linkages. 5'-P-forming nucleases, RNases and DNases split linkage 'a', while 3'-P-forming nucleases, RNases and DNases split linkage 'b'.

Base-pairing in certain regions of the RNA chain is established by their behaviour, in solutions of high ionic strength, of contracting and displaying relatively low intrinsic viscosities and high sedimentation rates. This suggests the existence of base pairing in certain regions of the RNA chain and has been shown to occur in some of the biosynthetic polyribonucleotides (Grunberg-Manago, 1956; Hilmoe, 1959; Ochoa, 1960).

When equimolar concentrations of poly(A) and poly(U) are mixed in dilute aqueous solution they form a complex known as poly(A):poly(U) in which the adenine moieties of one strand are linked by hydrogen bonds to the uracils of the complementary strand (Hershey et al., 1963; Wu and Taylor, 1971). The X-ray diffraction pattern of this complex indicates a double helical structure, as in DNA, with 10 base pairs per turn of helix, the pitch of which is 3.4 nm. This helical formation causes a depression in absorbance at 260 nm by 34 per cent below the value for the sum of the two constituents, which is called 'hypochromicity' (Wu and Taylor, 1971).

The helical complex behaves like DNA in many ways. It shows the phenomenon of 'molecular melting' or 'helix coil transition'. When it is heated in 0.15 M NaCl at neutral pH, the absorbance at 260 nm rises sharply (the hyperchromic effect) by 34 per cent at a temperature of about 60°C, the so called melting temperature Tm. This is due to the separation of the two chains. When it is cooled the helix is reformed (Doty, 1961).

A similar phenomenon is observed, though to a less extent, when solutions of r-RNA or RNA from certain viruses such as TMV are heated. The RNA chain folds back on itself in a number of places so that pairs of bases may come together and become linked by hydrogen bonds, e.g. between adenine and uracil and between guanine and cytosine. As the apposition of the chains in this way may not be exactly complementary, looping out of non-bonding residues takes place. Such loops could be stabilised through 'base-stacking' interactions. The RNA molecule is therefore thought to consist of a polynucleotide chain containing short imperfect helical regions in which base pairing occurs between 40 and 70 per cent of the nucleotides being involved in such helix formation. Data obtained from extensive studies on the secondary structure of t-RNA have led to workers in this field to suggest a generalised 'clover-leaf' arrangement for the secondary structure of t-RNAs from various sources (Tinoco et al., 1971). Secondary structures for r-RNAs and m-RNAs are also proposed.

2. NUCLEOLYTIC ENZYMES

Enzymes catalysing the degradation of nucleic acids are ubiquitous in nature; present in all biological systems. Some of them are specific for RNA (ribonucleases), others for DNA (deoxyribonucleases) and still others can hydrolyse both RNA and DNA (non-specific nucleases). The nucleolytic enzymes are otherwise called phosphodiesterases in general terminology; phosphomonoesterases on the other hand liberate

inorganic phosphate from a nucleotide (polynucleotides also), sugar phosphate or other monophosphate esters of organic alcohols. Other related enzymes are phosphorylases, polynucleotide phosphorylase and pyrophosphorylase, which are capable of degrading RNA, but their function *in vivo* is not known with certainty (Grunberg-Manago, 1963).

This review will be mostly confined to the nucleolytic enzymes viz. RNases, DNases and other nucleases with emphasis on RNases. A number of excellent and comprehensive reviews and monographs are available on these topics. A few of them are Josefsson and Lagerstedt (1962); Lehman (1963); Cantoni and Davies (1966); Laskowski Sr. (1967); Razzell (1967); de Garilhe (1967); Barnard (1969); Egami and Nakamura (1969); Richards and Wyekoff (1971); Adams *et al.* (1976); Ogata *et al.* (1976).

2.1 Classification and Nomenclature of Nucleolytic Enzymes:

The three main features of nuclease action that have been generally used as a basis for the classification are: 1) substrate specificity, 2) mode of attack, and 3) mode of phosphodiester bond cleavage.

(a) Substrate specificity:- On this criterion nucleases can be divided mainly into three groups *viz.*, a) ribonucleases (RNases), the enzymes that act specifically on ribonucleic acid (RNA), b) deoxyribonucleases (DNases) acting on deoxyribonucleic acid (DNA), and c) non-specific nucleases which can act both on RNA and DNA.

(b) Mode of attack:- Nucleases are divided mainly into two categories based on this property viz., (1) endonucleases which attack the polynucleotides at points within the polymer and produce mostly oligonucleotides, and (2) exonucleases which act on the polymer stepwise from one end of the chain forming mononucleotides. However, a few enzymes appear to act as both endo- and exonucleases - e.g. micrococcal nuclease (deMeuron-Landott and deGarilhe, 1964).

(c) Mode of phosphodiester bond cleavage:- Unlike other biopolymers such as proteins and carbohydrates, polynucleotides can give products bearing i) 5'-phosphate groups by hydrolysis of the bond between the 3'-OH and the phosphate group, or ii) 3'-phosphoryl end groups by hydrolysis of the bond between the 5'-OH and the phosphate group. The former one is usually called as 5'-P-forming nuclease and the latter as 3'-P-forming nuclease. The latter can be further divided into 2 groups based on whether the degradation is by nucleotidyl transfer [ribonucleate nucleotido-2'-transferase (cyclizing)] or it is by hydrolysis [ribonucleate (deoxyribonucleate)-3'-nucleotido hydrolase]. In the former case the product may be 2',3'-cyclic nucleotide ~~or without~~ or a 3'-nucleotide with [the intermediate formation of 2',3'-cyclic nucleotide. (~~cyclizing~~). In the latter case the products, 3'-nucleotides will be directly released from the polynucleotide chain by hydrolysis without the formation of an intermediate cyclic nucleotides. Fig. (1) and (2) represent the different modes of phosphodiester bond cleavage.

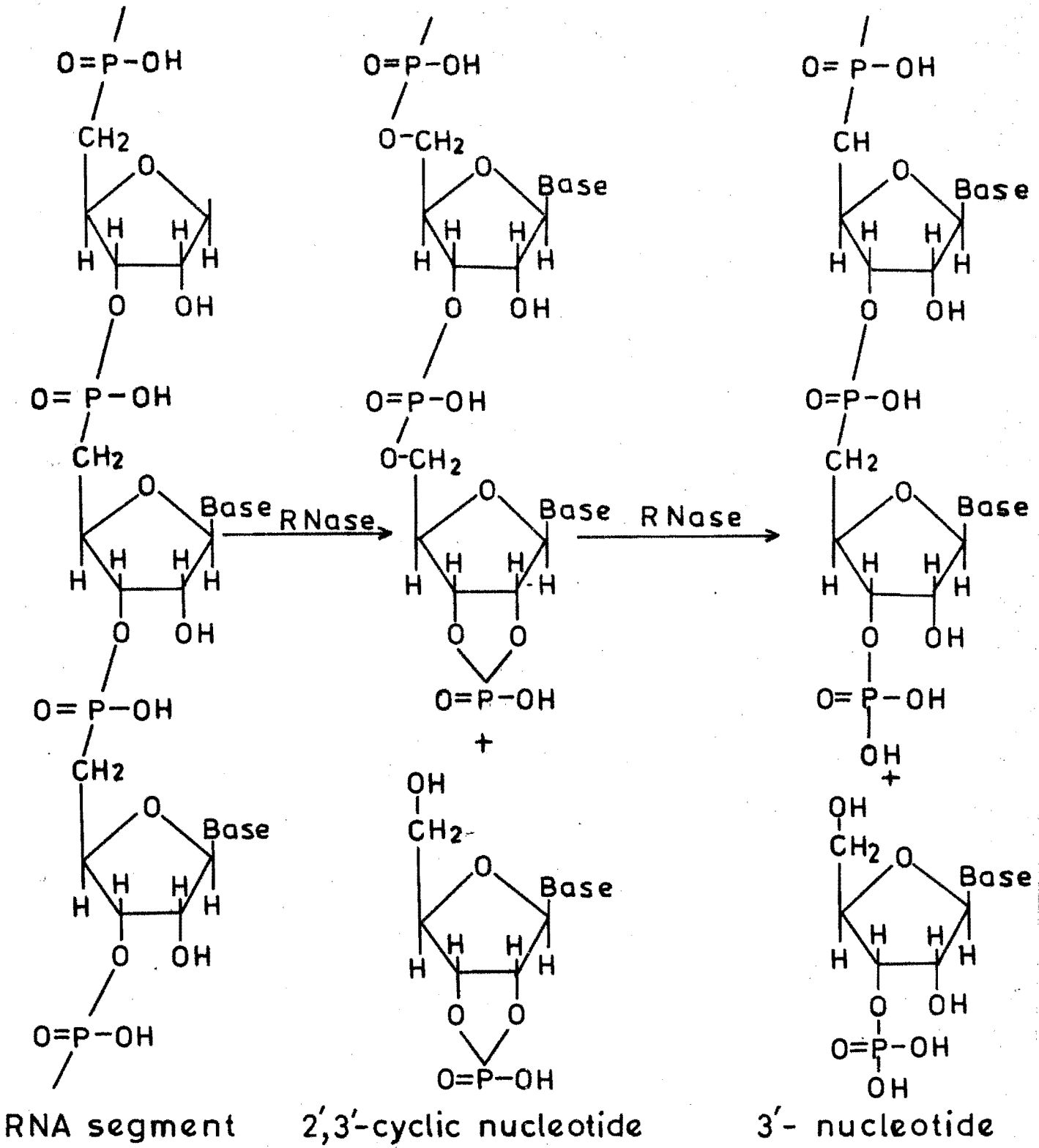

Fig. 2. The action of a 3'-P-forming, cyclizing RNase on RNA, showing the intermediate formation of cyclic nucleotides.

Additional criteria are specificity towards secondary structure of the substrate (e.g. RNase H is specific for RNA:DNA hybrids), specificity towards a particular base [guanine-specific (e.g. the mechanism of action of RNase T_1, a guanyloribonuclease is diagramatically represented in Fig. 3), purine-specific, pyrimidine-specific or non-specific], direction of attack by exonuclease (3'---> 5' or 5'---> 3'), mode of existence (extracellular, intracellular, particulate or soluble), effect of certain physical or chemical factors (e.g. ATP-inhibited RNases, temperature-tolerant and EDTA-sensitive P_1 type nucleases, etc.), acid-nuclease or alkaline nuclease on the basis of optimum pH etc.

In the standard systematic classification of enzymes (1972), all well-known nucleolytic enzymes are classified in EC 3.1.4, phosphodiester hydrolases. RNases forming 3'-phosphomono- and oligonucleotides via 2',3'-cyclic phosphates were formerly classified in EC 2.7.7, nucleotidyl transferases. They are now reclassified as follows: pancreatic RNase (EC 2.7.7.16 ---> 3.1.4.22), RNase T_2 (EC 2.7.7.17 ---> 3.1.4.23), RNase T_1 (EC 2.7.7.26 ---> 3.1.4.8) etc.

In the following paragraphs the occurrence and some of the important enzymatic properties and other salient features of some of the well-studied nucleolytic enzymes, namely, DNases and non-sugar specific nucleases will be briefly reviewed. RNases will be separately described in

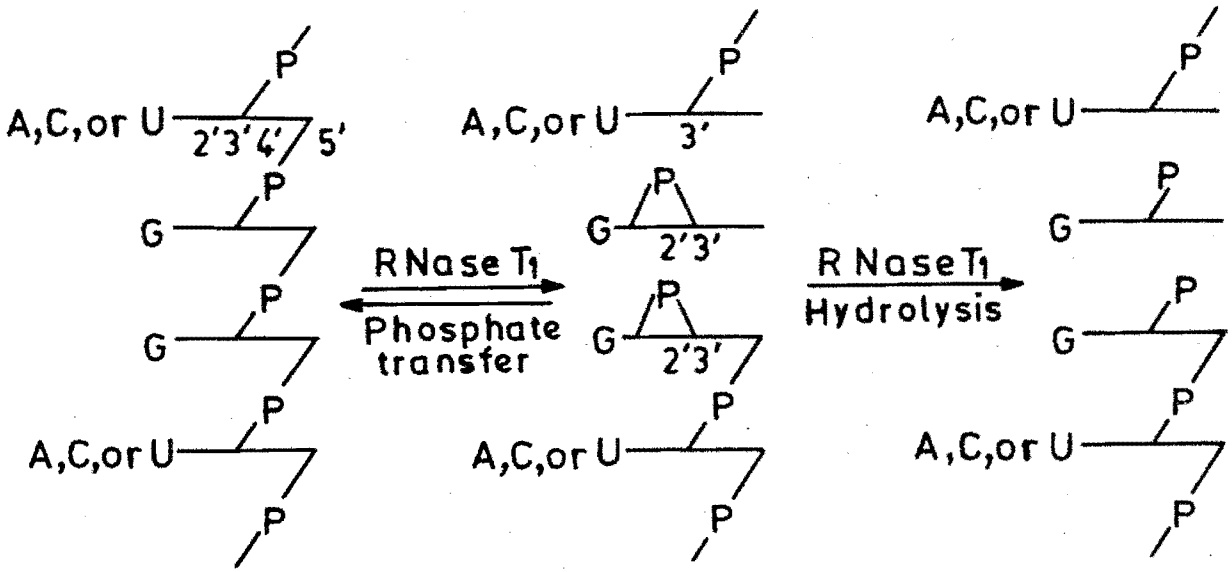

Fig. 3. Mechanism of action of RNase T_1, a guanylo-ribonuclease on RNA.

some detail in a separate section. In all cases, the products formed by the enzyme action are taken as a major criterion for the classification.

2.2 Sugar non-specific nucleases:

A number of sugar non-specific nucleases have been reported in literature from various sources (Kuninaka, 1976; Adams et al., 1976). However, the best studied ones from microbial sources will be dealt with here briefly. Mention will be made of well studied nucleases from non-microbial sources also. These nucleases may be divided into 5'-P-forming and 3'-P-forming nucleases. The former may be further classified as mainly 5'-phospho-oligonucleotide forming and 5'-phospho-mononucleotide forming.

2.2.1 5'-oligonucleotide forming enzymes: Nuclease O from A. oryzae mycelia (Uozumi et al., 1968), an endonuclease from conidia of Neurospora crassa (Linn and Lehman, 1966; de Garilhe, 1967) an extracellular nuclease from a hybrid yeast (S. fragilis X S. dobzhanskii) (Nakao et al., 1968) nucleases from S. aureus (Yoneda, 1964; Suhara and Yoneda, 1973) Azotobacter agilis (Egami and Nakamura, 1969; DeGarilhe, 1967) and Serratia marcescens (Nestle and Roberts, 1969) and a nuclease isolated from mycelia of N. crassa (Takahashi and Uchida, 1977) are some of the well-studied 5'-oligonucleotide forming nucleases. Most of them degrade heat-denatured DNA and RNA faster than native DNA except that from S. marcescens. An endonuclease isolated from culture filtrate of Acrocylindrium sp. preferred native DNA (Suhara, 1973; Suhara and Yoneda, 1973).

Similar endonucleases have also been reported in silk worm (Funagawa et al., 1975), chick pancreas (Eley and Roth, 1966), rat liver (Curtis et al., 1966), etc.

Generally 5'-P-forming nucleases are activated by Mg^{2+} and/or Mn^{2+}.

5'-P-forming nucleases without phosphomonoesterase activity include nuclease S_1 from 'Takadiastase', a digestive enzyme preparation from A. oryzae (Ando, 1966), an extracellular nuclease from A. quercinus (Ohta and Ueda, 1968) an exonuclease from Streptomyces sp. No.41 (Sugimoto et al., 1964) nuclease P_{p-2} from Physarum polycephalum (Hiramaru et al., 1969), an exonuclease from Acrocylindrium sp. (Suhara, 1974) and nuclease TT_1 from Thermus thermophilus (Takahashi, 1980) among others.

Snake venom phosphodiesterase (EC 3.1.4.1, phosphodiesterase I) (Laskowski Sr., 1971; DeGarilhe, 1967) and rat liver oligonucleotidase (EC.3.1.4.19) (Futai and Mizuno, 1967) are considered as typical 5'-P-forming exonucleases of non-microbial origin.

2.2.2 5'-mononucleotide forming nuclease with phospho,-mono-and-diesterase activities: The presence of nucleases have been reported in several moulds and plants which are capable of hydrolysing not only phosphodiester linkages in RNA and DNA but also phosphomonoester linkages in mono- and oligonucleotides terminated by 3'-phosphate. These enzymes are

generally inhibited or inactivated by EDTA and usually hydrolyse heat-denatured DNA faster than native DNA.

Among them nuclease P_1 from Penicillium citrinum has been extensively studied (Kuninaka, 1976). This enzyme has been used industrially in the production of flavour nucleotides in Japan. Nuclease P_1 is characterised by its heat-stability and EDTA sensitivity. Similar enzymes are also reported in a number of other species of Penicillium such as P. expansum, P. notatum, P. steckii, P. meleagrinum etc. (Fujimoto et al., 1977c). Nucleases very similar to nuclease P_1 have been also isolated from Monascus purpureus (Saruno et al., 1964; Soeda et al., 1968) and Phoma cucurbitacearum (Tone and Ozaki, 1968).

An extracellular nuclease purified from Micrococcus sodonensis hydrolysed DNA and RNA into 5'-mononucleotides which were further degraded into nucleosides and Pi (Berry and Campbell, 1967).

3'-P-forming nucleases are not commonly seen in micro-organisms. They have been, however, reported in a few cases. Nuclease from Staphylococcus aureus (Micrococcal nuclease EC.3.1.3.7) (DeGarilhe, 1967; Cantoni et al., 1966; Adams et al., 1976; Anfinsen et al., 1971) is the most extensively studied 3'-forming nuclease from a microbial source. Among other sources containing similar enzymes are Bacillus subtilis Marburg strain (Nakai et al., 1965), B. subtilis SB 19 strain (Kerr et al., 1965), Lactobacillus

acidophilus (Fiers and Khorana, 1963) and Phyrarum polycephalum (nuclease P_{p-1}) (Uchida and Egami, 1971).

A number of plant sources such as wheat seedlings (Hansen and Fairley, 1969), mung bean (Mikulski and Laskowski Sr., 1973), potato tubers (Nomura et al., 1971; Suno et al., 1973), ginkgo nuts (Hara et al., 1969 and 1970), tobacco (Oleson et al., 1974) and corn (Wilson, 1968) also contain similar enzymes.

Spleen endonuclease (EC 3.1.4.7) and phosphodiesterase II (EC 3.1.4.18) isolated from bovine spleen are the best studied 3'-P-forming nucleases from non-microbial sources (DeGarilhe, 1967; Adams et al., 1976).

2.3 DNases:

DNases can be classified mainly into two groups based on the products formed: a) 5'-P-nucleotide forming DNases, and b) 3'-P-nucleotide forming DNases. The latter are rarely seen in microorganisms.

A number of microorganisms were shown to elaborate 5'-P-forming DNases. DNase K_2 from A. oryzae (Kato and Ekeda, 1968; Kato et al., 1969), DNase Rh isolated from 'Gluczyme', a product of R. niveus (Kurano et al., 1971), streptodornase from Streptococcus sp. (deGarilhe, 1967; Laskowsky Sr. 1967), DNases I and II from E. coli K_{12} (Weissbach and Korn, 1963), endonuclease I (Okazaki and Sugimoto, 1967; Lehman, 1971), endonuclease II or nickase (Ando, 1973) and UV-endonuclease

or endonuclease V (Shimizu et al., 1971; Minton et al., 1975) also from E. coli, DNase from B. subtilis Marburg strain (Shibano and Kumano, 1979) etc. are some of the well-studied DNases which form 5'-oligonucleotides from DNA (preferably native or double-stranded DNA). Recently three isoenzymes of DNase from Physarum polycephalum have been separated by Waterborg and Kuyper (1980).

5'-oligonucleotide forming DNase have also been isolated from various non-microbial sources such as bovine pancrease (DNase I) (Khorana, 1961; Laskowski Sr., 1971; Moore, 1981), human duodenal juice (Tsubota et al., 1977) and chick embryo (Shimabayashi et al., 1980).

All these DNases are characterised by their requirement of Mg^{2+}/and Mn^{2+} for their activity.

Another group of 5'-oligonucleotide-forming DNases are dependent on ATP for their activity, besides Mg^{2+} and Mn^{2+}. Such DNases were reported in E. coli, Micrococcus lysodeikticus, Diplococcus pneumoniae, B. laterosporks, Mycobacterium smegmatis, Haemophilus influenzae etc. (Takagi, 1971; Anai et al., 1975).

Typical 3'-P-forming DNases have been reported in calf thymus, bovine spleen and pig spleen (DeGarilhe, 1967). Type II DNases were also found in other tissues such as parotid gland, adrenal medulla, myocardium, chick liver, rat liver nuclei, neurons, rabbit leucocytes and genital secretions (Laskowsky, 1967). An acid DNase isolated from pupae of

Bombyx mori (silk worm) hydrolysed native and denatured DNA to form 3'-phospho-mono-and oligonucleotides (Koga and Akune, 1972). DNase II type enzyme was reported in human urine which acted optimally at pH 5.1-5.3 and formed 3'-phospho-oligonucleotides from native and heat denatured DNA (Marai et al., 1980). Yanagawa et al. (1977) purified DNase A and A-1 from Achatina fulica (an agate snail) which hydrolysed poly(dA), DNA and poly(dT) to form 3'-phospho-di-and tri-nucleotides at pH 5.0. As already mentioned, microbial 3'-P-forming DNases are not common but have been found in Micrococcus luteus (Lehman, 1971) and A. oryzae (DNase K_1) (Kato and Ikeda, 1968).

2.3.1 Restriction endonucleases are enzymes which catalyse endonucleolytic cleavage of one of the duplex strands of DNA, followed by cleavage of a second strand to give double-stranded fragments with sticky ends. They are highly specific endonucleases degrading foreign DNA from phages or cells of different strains. The enzymes require Mg^{2+} for activity. Some of them require ATP or both ATP and S-adenosyl methionine, in addition to Mg^{2+} (Lehman, 1971; Adams et al., 1976; Nathans and Smith, 1975). There are three classes or types of restriction enzymes.

(a) Class I restriction enzymes (Endlich and Linn, 1981): These are multifunctional complex proteins which cleave unmodified DNA in the presence of S-adenosyl-L-methionine (SAM), ATP and Mg^{2+} (Meselson and Yuan, 1968; Linn and Arber, 1968). These proteins have methylase and ATPase activities

and have two or more non-identical subunits, with molecular weight around 400,000 or more (Boyer, 1971; Arber and Linn, 1969). Examples of this class of restriction nucleases are Eco B, Eco K, etc. These enzymes have methylases and ATPases also. The enzymes bind to DNA at a specific site in the presence of S-adenosyl-methionine (Yuan et al., 1975). Only if both strands of DNA are not methylated the restriction mode of action occurs. Cleavage occurs at sites situated away from the binding site and at two stages. After this enzyme ceases to be a nuclease it becomes a vigorous ATPase.

(b) Class II restriction enzymes (Wells et al., 1981): These are simpler enzymes which require only Mg^{2+} for activity. They have smaller molecular weights ranging from 20,000 to 100,000 (Smith, 1979). Some of them have 2identical subunits. They do not possess either methylase or ATPase activity. They recognize a specific site on the DNA and if this is unmodified cleavage occurs at this site. Eco RI, Hpa II, Bcu I, Hind II, Eco RII are a few examples of this group of restriction endonucleases (Smith and Wilcox, 1970; Betlach et al., 1976).

(c) Class III restriction enzymes: The endonucleases of Haemophilus influenzae serotype f and of prophages P_1 and P_{15} were originally grouped with the type I systems due to their complex properties, but they are now considered to belong to a third distinct group (Kauc and Pickarowicz, 1978). These enzymes are actually of intermediate complexity,

comprising of two subunits with molecular weights in the range of 250,000. These enzymes like type I require Mg^{2+}, SAM and ATP. However, the type III endonucleases, for which ATP is strictly required, do not exhibit any measurable ATP hydrolysis.

Very recently a number of restriction nucleases have been isolated from H. influenzae and Bacillus strains (Sixiang et al., 1980). They are Hin S_1I, Hin S_2I, Endo R 211, Endo R 226, Endo R 64, Endo R 67. Endo R 67 was reported be capable of digesting modified DNA also.

3. RIBONUCLEASES (MICROBIAL)

The presence of a large number of enzymes capable of degrading RNA to varying degrees by different modes have been reported from various sources ranging from bacteria to mammals. It is intended to give here an account of only a few well studied RNases with emphasis on microbial enzymes; a very brief passing mention will be made of non-microbial RNases. For convenience these RNases are grouped on the basis of the products formed, the mode of action (i.e. cyclizing and non-cyclizing) and the base specificity.

3.1 RNases from non-microbial sources

A number of RNases have been isolated and purified from various animal and plant sources. Pancreatic RNase or RNase I (E.C. 3.1.4.22) is, apparently, one of the most extensively studied enzymes (Laskowski Sr. 1967; Khorana, 1961;

Anfinsen and White, 1961; Richards and Wyekolf, 1971). This is a very small protein, molecular weight 13,700, is stable over a wide range of pH, and is remarkably heat stable in acid solution. It is optimally active at pH 7.0-8.2 and at 60°C. Its amino acid sequence, active site and mechanism of action have been intensively investigated (Stein, 1964; Richards and Wyekoff, 1971). It is a cyclizing, pyrimidine-specific endoribonuclease. Very recently, two RNases have been purified from human urine and are reported to be pyrimidine-specifc with a preference to cytosine (Iwama et al., 1981).

A number of RNases have been observed in rat liver (Roth, 1954; Reid and Nodes, 1959). Two of them are endo-nucleases forming 3'-monophosphates. Another, an alkaline RNase I forms 5'-monophosphates from RNA optimally at pH 7.5 in the presence of Mg^{2+} (Neu and Heppel, 1964).

RNase H which specifically degrades the RNA strand of DNA-RNA hybrid to acid soluble products with 5'-monophosphate ends has been purified from calf thymus tissue (Stavrinopoulos and Chargaff, 1973; Haberkern and Cantoni, 1973), rat liver (Roewekamp and Sekeris, 1974), chick embryo cells (Keller and Crouch, 1972), human KB cells (Keller and Crouch, 1972) etc.

Many RNases have been purified from a number of plant sources. Among them RNases from tobacco and pea leaves (Reddi, 1958; Reddi, 1966; Schuster et al., 1959), garlic

(Carlsson and Frick, 1964), spinach (Tuve and Anfinsen, 1960), soy bean (Merola and Davies, 1962), potato tuber (Bjork, 1965), cucumber seedlings (Kado, 1968), corn, seed and root (Wilson, 1963; Wilson, 1967) form 3'-P-forming endoribonucleases. An RNase from rye grass degrades RNA to give 2',3'-cyclic nucleotides as final products (Egami et al., 1964).

5'-P-forming RNases have been isolated from barley rootlet (Nakagiri et al., 1968), mung bean sprouts (Ishii et al., 1967). Besides these cultured plants were found to be very promising sources of these enzymes. Cultured cells of Vinca rosea showed 30 times as much 5'-P-forming RNase as the parent plant on a dry cell weight basis (Ukita et al., 1973). Formation of two forms of RNase H by cultured cells of carrot root has been reported recently by Sawai et al. (1979). Three RNase fractions, RNases RB-1, RB-2 and RB-3 with molecular weights 6,200, 35,000 and 14,500 respectively were isolated from rice bran (Yokoyama et al., 1982). All these RNases were found to be purine-specific and preferred adenine. RNase RB-3 was purified to homogeniety, was optimally active at pH 5.0 and was inhibited by Cu^{2+}. It was fairly heat stable, 75% activity remained after 16 min heating at 100°C.

3.2 Microbial Ribonucleases

3.2.1 3'-nucleotide forming RNases (cyclizing)

3.2.1.1 Guanine-specific RNases (EC 3.1.4.8): A number of RNases have been reported to be specific for guanylic acid residue of the RNA chain. Some of them are listed in table 2 and some of them are described below.

RNase T_1, one of the most extensively studied RNases, is a guanyloribonuclease, isolated from "Takadiastase" with a pH optimum of pH 7.3-7.5, it is highly stable at neutral and acidic pH values. Heating it at 100°C for 10 min (pH 6.0) did not result in any loss of activity. Zn^{2+} cause strong inhibition and Mg^{2+}, Ca^{2+} and Mn^{2+} are also inhibitory. Its chemical nature and the structure function relationships have been extensively studied (Egami and Nakamura, 1969; DeGarilhe, 1967; Cantoni et al., 1966; Uchida and Egami, 1971). RNase T_1 splits inosinic acid bonds and xanthylic acid bonds in deaminated RNA under appropriate conditions. The former can be cleaved almost exclusively (Sato-Asano and Fujii, 1960).

RNase N_1 was isolated from the culture filtrate of Neurospora crassa strain 74A-T32-M12. It acts optimally at pH 7.0 and is strongly inhibited by Hg^{2+}; however, it is not inhibited by Zn^{++} unlike RNase T_1. It is quite stable in neutral or acid media at 37°. Although not as heat stable as RNase T_1, it is resistant to heating at 80°C for

Table - 2

Guanyloribonucleases (EC.3.1.4.8)

Name	Source	Reference
	Fungal:	
RNase T_1	Aspergillus oryzae	(Sato & Egami, 1957; Egami & Nakamura, 1969)
RNase N_1	Neurospora crassa	(Takai et al., 1966)
RNase N_3	N. crassa (Intracellular)	(Takai et al., 1966)
RNase U_1	Ustilago sphaerogena	(Arima et al., 1968)
RNase F_1	Fusarium moniliforme	(Omori et al., 1972)
RNase Ch	Choloropsis sp.	(Fletcher & Nash, 1972)
RNase	Ustilago zeae	(Yanagida et al., 1964)
RNase P_{p-1}	Physarum polycephalum	(Uchida & Egami, 1971; Hiramaru et al., 1969)
RNase	Acrocylindrium sp.	(Suhara et al., 1972)
RNase	Stysanus stemonitis	(Ohmura et al., 1964)
RNase	Mucor genevensis	(Rushizky et al., 1964)
	Actinomycetes:	
RNase St.	Streptomyces erythreus	(Yoshida et al., 1971)
RNase	S. albogriseolus	(Yoneda, 1964)
RNase	S. aureoverticillatus	(Tatarskaya et al., 1966)
RNase	Actinomyces aureoverticillatus	(Uchida & Egami, 1971)
RNase	Actinomyces	(Tatarskaya, 1964)
	Bacterial:	
RNase	Bacillus pumilus	(Rushizky et al., 1964; Nakao and Ogata, 1963)

2 min in acidic media (pH 2-4), but unstable at alkaline pH (Egami and Nakamura, 1969, Takai et al., 1966). It is used for synthesising oligonucleotides (Koike et al., 1971). RNase N_3 is an intracellular enzyme purified from another strain of N. crassa and is specific for guanine (Takai et al., 1966).

RNase U_1. This was one of the RNases isolated from Ustilago sphaerogena (a smut fungus) and most active at pH 8.0-8.5. It was inhibited by Zn^{2+}, Cu^{2+} and Ag^{2+}. RNA was used as the sole source of carbon in the medium for the production of this extracellular enzyme (Glitz and Dekker, 1964; Arima et al., 1968). An extracellular guanylonbonuclease was produced by U. zeae in large amounts when RNA was used as the sole source of phosphorus. Even poly-U was found to enhance the enzyme yield (Yanagida et al., 1964).

RNase St. from Streptomyces erythreus was another heat-stable guaniloribonuclease which acted optimally at pH 7.3-7.4 (Yoshida et al., 1971). RNases from S. albogriseolus (Yoneda, 1964) and Actinomyces aureoverticillatus (Uchida and Egami, 1971) were guanyloribonucleases which acted optimally at pH 7.0-8.5 and 7.6 respectively.

Physarum polycephalum RNase P_{p-1} (Uchida and Egami, 1971 and Hiramaru, et al., 1969) Acrocyclindrium sp. NM2 RNases I and II (Suhara et al., 1972) RNase Ch from Chaloropsis sp. (Fletcher and Hash, 1972) RNase F_1 from Fusarium moniliforme (Omori et al., 1972), RNases from Mucor genevensis

(Rushizky et al., 1964), Stysanus stemonitis, an imperfect fungus (Ohmura et al., 1964) Streptomyces aureoverticillatus (Tatarskaya et al., 1966), Actinomyces (Tatarskaya, 1964), Bacillus pumilus (Rushizky et al., 1964; Nakao and Ogata, 1963) are other examples of guaniloribonucleases produced by microorganisms.

Recently this property of guanine-specificity has been exploited in purification of some of these guanilo-ribonucleases by affinity chromatography. Guanylyl (-2',5'-) guanosine coupled to aminohexyl-Sepharose-4B was success-fully used as affinity adsorbant for purifying guanyloRNases such as RNase N_1, RNase F_1, RNase T_1 and RNase St. (Ishiwata and Yoshida, 1978).

3.2.1.2 Purine-specific RNases: RNases U_2 and U_3 (Egami and Nakamura, 1969; Uchida and Egami, 1971) were most active at pH 4.5 and there was no loss of activity on heating at 80°C and pH 6.9 for 4 min. They were inhibited by $CuSO_4$ and $AgNO_3$. They were specific for internucleotide bonds between 3'-purine nucleotides in RNA. They formed purine nucleoside 2',3'-phosphates which were slowly hydrolysed to 3'-purine nucleotides. Double-stranded RNA was resistant to these enzymes. RNase U_2 has been found to be quite useful for the synthesis of oligonucleotides such as adenylyl -3'-5'-nucleosides, adenylyl (-3'-5'-)-guanosine 2':3'-cyclic phosphate and oligoadenylic acids (Koike et al., 1971a) RNase U_2 has been recently separated into 2 fractions (RNase U_2-A and U_2-B) by an improved purification method which

included affinity chromatography with 5'-adenylate-amino-hexyl-sepharose 4B. RNase U_2-B showed lower specific activity (Uchida and Shibata, 1981).

3.2.1.3 Base Non-specific RNases (EC 3.1.4.23, RNase-II) are widely distributed in microorganisms and plants.

3.2.1.3.1 RNase T_2 from A. oryzae (DeGarilhe, 1967; Cantoni et al., 1966; Uchida and Egami, 1971; Egami and Nakamura, 1969) is one of the most extensively studied base non-specific RNases. This enzyme is optimally active at pH 4.5 and was stable at pH 6.0 and 80°C for 5 min. It was found to be very sensitive to Cu^{2+}. Eventhough it showed a preference for adenylic acid bonds no absolute base specificity was observed. Recently multiple forms of this enzyme were reported by Kanaya and Uchida (1981b). By a new method of purification which involved 4 steps including affinity chromatography on 5'-adenylate-aminohexyl-Sepharose 4B the yield of the enzyme was improved 4-fold. Two fractions, a major fraction with mol. wt of 80,000 (RNase T_2-L) and a minor fraction (with mol. wt. of 36,000) were separated. RNase T_2-L was further fractionated by repeated gel-filtration to five forms, RNase T_2-L_{1-5}. The heterogeneity was found to be due to varying galactose content.

RNase M (Irie, 1967, Irie, 1969) and Ms (Ohgi and Irie, 1975) are RNases isolated from 'Molsin' a commercial product of A. saitoi. RNase-M and Ms have mol. wts of ca 36,000 and 12,000 respectively. They are optimally active

at pH 4.0 and 4.5 respectively and at temperature 50°C. RNase-M releases 3'-nucleotides in the order A > U > G > C whereas Ms yields GMP > AMP > CMP > UMP. RNase Ms is stable at 70°C for 5 min from pH 2-10.

RNase Rh (Tomoyeda et al., 1969; Komiyama and Irie, 1971, 1972 and 1974). This is another T_2-type RNase isolated from a commercial amylase preparation, 'Gluczyme' from Rhizopus niveus. It is optimally active at pH 5.0 and temperature 50-50°C, and is inhibited by Zn^{2+}, Cu^{2+} and Hg^{2+}. In this case the final products were found to be 2',3'-cyclic nucleotides and were released in the order A > G > C > U.

RNase from Azotobacter agilis (Shio et al., 1966) and Proteus mirabilis (Centre and Bahal, 1968) were also have been shown to form 2',3'-cyclic phosphate as final products.

3.2.1.3.2 ATP-Sensitive RNases: RNases inhibited by ATP have been reported from various sources. They were base-non-specific RNases and formed either 3'-nucleotides via 2',3'-cyclic nucleotides or 2,3'-cyclic nucleotides as final products. An extracellular RNase in B. subtilis Marburg strain degraded RNA to 2',3'-cyclic phosphates (Nakai et al., 1965). Another ATP-sensitive RNase was present in B. subtilis K which acted on RNA optimally at pH 5.5-5.7 and formed 2',3'-cyclic nucleotides in the order of C > U > A > G (Yamasaki et al., 1970). A number of ATP-sensitive RNases present in various species of Bacillus have been classified into 2 types: (1) with pH optimum 5.7 and sensitive to 5mM EDTA (e.g. RNases from

B. subtilis and B. liquefaciens) and (2) with pH optimum 6.8 and partially inhibited by 5mM EDTA (e.g. RNases from B. cereus, B. cereus var. mycoides, and B. thuringiensis (Yamasaki et al., 1973). Recently an ATP-sensitive RNase, RNase F_1 was isolated from the fruit body of Flammulina velutipes which was optimally active at pH 5.5 and EDTA-insensitive (Kurosawa et al., 1980).

Besides these enzymes a number of other RNases which are base non-specific have been reported by several authors from various sources. The base non-specific RNases from microbial sources are listed in Table 3.

3.2.1.4 3'-P-forming-non-cyclizing RNases

Only a few such RNases have been reported so far. An RNase from baker's yeast degrades RNA into 3'-mononucleotides. This does not split 2',3'-cyclic phosphates. It is considered to be a 3'-P-nucleotide forming, non-cyclizing RNase which forms 3'-nucleotides directly from RNA without the formation of 2':3'-cyclic phosphates as intermediates (Ohtaka et al., 1963). Ustilago RNase U_4 (Egami and Nakamura, 1969; Uchida and Egami, 1971) is also suspected to be a non-cyclizing 3'-P-forming RNase.

Existence of pyrimidine-specific RNases in microorganisms has not been confirmed, although an RNase from Thiobacillus thioparus was found to split RNA preferentially at 3'-position of a pyrimidine residue, but cyclization has not yet been demonstrated (Egami and Nakamura, 1969; Uchida and Egami, 1971).

Table - 3

Microbial Base-non-specific RNases

Name	Source	Reference
	Fungi:	
RNases	Penicillium spp.	(Fujimoto et al., 1977)
RNase T_2	Aspergillus oryzae	(Uchida, 1966)
RNase M	A. saitoi	(Irie, 1967)
RNase M_s	A. saitoi	(Ohgi & Irie, 1975)
RNase Rh	Rhizopus niveus	(Tomoyeda et al., 1969)
RNases I & II	Trichoderma koningii	(Harada & Irie, 1969)
RNases P_{p-2} & P_{p-3}	Physarum polycephalum	(Hiramaru et al., 1969)
RNase N_2	Nuerospora crassa	(Takai et al., 1966)
RNase	Monascus purpureus	(Soeda et al., 1973)
RNase	A. niger NRC-A-1-233	(Horitsu et al., 1974)
RNase L	Aspergillus sp.	(Tomoyeda et al., 1977)
RNase	Trichothecium roseum	(Ohmura et al., 1964)
RNase	Monascus pilosus	(Rushizky et al., 1964)
RNase	Polystictus hirsutus	(Ohmura et al., 1964)
RNase	Lenzites tenuis	(Rushizky et al., 1964)
RNase	Absidia butleri	(Ohmura et al., 1964).

contd...

Table - 3 (contd.)

Name	Source	Reference
	Yeasts:	
RNase CL	Candida lipolytica	(Imada et al., 1972, 1975)
RNase	Rhodotorula glutinis	(Ogata et al., 1971; Nakao & Ogata, 1963)
	Bacteria:	
RNase	Azotobacter agilis	(Shiio et al., 1966)
RNase I	Escherichia coli	(Anraku & Mizuno, 1967)
RNase II	Proteus mirabilis	(Centre & Bahal, 1968)
RNase	Salmonella typhimurium	(Chakraburtty & Burma, 1968)
RNase I	Lactobacillus plantarum	(Logan & Singer, 1968)
RNase	Bacillus amylo-liquefaciens	(Cantoni et al., 1966)
RNase	B. cereus	(Rushizky et al., 1964)
	Protozoa:	
RNase	Tetrahymena pyriformis	(Lazarus & Scherbaum, 1967)

3.2.2 Microbial 5'-P-forming RNases

Many microorganisms produce RNases capable of degrading RNA into 5'-mono or/and oligonucleotides. 5'-P-forming RNases from microbial sources are listed in Table 4, a few examples are discussed below.

About 300 strains of moulds were tested for their RNase activity by Hasegawa et al., (1964) and strains belonging to Pellicularia, Rhizoctonia and Phaeoisariopsis were found to degrade RNA into 5'-nucleotides. Culture filtrates of Pellicularia sp. H-11 hydrolysed RNA and formed 5'-mononucleotides in about 74% yield. The enzyme was inhibited by EDTA-and the activity could be restored by addition of Zn^{2+}.

Similar enzymes were also obtained from Physarum polycephalum (RNase P_{p-4}) (Hiramaru et al., 1969; Uchida and Egami, 1971), Lactobacillus casei (Keir et al., 1964; DeGarilhe, 1967), L. plantarum (Logan and Singer, 1968; Uchida and Egami, 1971). RNase P_{p-4} is an endonuclease which preferably breaks the bonds next to purine bases, whereas RNase from L. casei acts both endo-and exo-nucleolytically and that from L. plantarum acts exonucleolytically. Enzyme isolated from Bacillus AU_2 strain (Jacobson and Rodwell, 1972) possesses 5'-nucleotidase activity in addition to RNase. Hence, the end-products of RNA hydrolysing by this enzyme are nucleosides and Pi.

Table - 4

Microbial 5'-P-Forming RNases

Name	Source	Reference
	Fungi:	
RNase	Pellicularia sp.H-11	(Hasegawa et al., 1964; Fujimura et al., 1964)
RNase P_{p-4}	Physarum polycephalum	(Hiramaru et al., 1969; Uchida & Egami, 1971)
RNase	Lentinus edodes	(Mouri et al., 1968)
RNase	Tricholoma matsutake	(Mouri et al., 1968)
RNase	Psalliota bisporus	(Mouri et al., 1968)
RNase	Ustilago maydis	(Banks, 1974)
RNase	Pichia	(Sugimori, 1964)
RNase	Rhodotorula glutinis	(Nakao et al., 1963)
	Bacteria:	
RNase II	Escherichia coli	(Davidson, 1969; Uchida & Egami, 1971)
RNase III	E. coli	(Nestle & Roberts, 1969)
RNase V	E. coli	(Kuwano et al., 1969; Uchida & Egami, 1971)
RNase H	E. coli	(Bekower et al., 1973; Leis et al., 1973)
RNase	Lactobacillus casei	(Keir et al., 1964; DeGarilhe, 1967)
RNase II	L. plantarum	(Logan & Singer, 1968; Uchida & Egami, 1971)
RNA phosphodiesterase	Bacillus AU2	(Jacobson & Rodwell, 1972)

E. coli has been found to be the source of various types of 5'-nucleotide forming enzymes. RNase II (EC 3.1.4.20, exoribonuclease) requires the presence of both a monovalent cations (K^+ or NH_4^+) and a divalent cation (Mg^{++} or Mn^{++}) for its activity. The enzyme progressively liberates 5'-mononucleotides from a polyribonucleotide chain until a small oligonucleotide (n = 2 to 4) which is resistant to the enzyme, is formed (DeGarilhe, 1967; Uchida and Egami, 1971; Spahr, 1964; Nossal and Singer, 1968). RNase III (EC 3.1.4.24, endoribonuclease III) is a highly specific enzyme for RNA:RNA duplex. This acts endonucleolytically to form 5'-phosphate and 3'-hydroxyl terminii (Robertson and Dunn, 1975). RNase V is specific for m-RNA and acts exonucleolytically to form 5'-mononucleotides (Uchida and Egami, 1971). RNase H (EC 3.1.4.34, hybrid nuclease) is specific for the RNA strand of a RNA:DNA hybrid. The degradation products of this enzyme were identified to be oligonucleotides with 3'-hydroxyl and 5'-phosphate terminii (Leis et al., 1973; Bukower et al., 1973). RNase H activity has been reported from a number of other sources also. It is present in U. maydis (Tashiro et al., 1976), yeast (Wyers et al., 1976a and 1976b) and viruses (Wu et al., 1974).

Enzymes degrading RNA into 5'-nucleotides have been reported in culture filtrates or cell extracts of a number of microorganisms, though they have not been purified. Some of them are listed below:

Fungi: Acrycylindrium sp. (Suhara and Yoneda, 1973; Suhara, 1974); Neurospora Crassa (Linn and Lehman, 1965); Fusarium roseum, F. solani, Gliomastrix convoluta, Helminthosporium sigmoideum var. irregulare, Verticillium niveostratosum, Anixiella retienlispora, Chaetomidium japonicum, Glomerella singulata, N. sitophila, Ophiobolus miyabeanus, Ophiostoma ulmi, Sordaria fumicola, Aspergillus elegans, A. flavipes, A. melleus, A. nidulans, A. quercinus, A. sulphureus, A. ustus. (Egami and Nakamura, 1969).

Streptomyces: Str. aureus (Nakao and Ogata, 1963a), Str. coelicolor, Str. albogriseolus, Str. gougerotti, Str. griseoflavus, Str. griseus, Str. purpurascens, Str. ruber, Str. viridochromogenes (Egami and Nakamura, 1969).

Bacteria: Bacillus brevis, B. subtilis, Pseudomonas aeruginosa (Kakunuma et al., 1962).

It has been reported that most of the enzymes which degrade RNA to produce 5'-nucleotides can also hydrolyse DNA (Nakao and Ogata, 1963). For example, some of these micro-organisms, N. crassa, Str. aureus, Acrocylindrium sp. A. quercinus etc. which are dealt with under section on nucleases, have been shown to have non-sugar specific 5'-P-forming nucleases.

3.2.3 2'-P-forming RNases

The final product of RNA hydrolysis by an RNase is normally 5',3' or 2',3'-cyclic mono- or oligo-nucleotides. But very rarely 2'-nucleotides have been found

to be final products. Macleod and Huang (1967) have claimed to have purified an RNase which formed nucleoside 2'-phosphates from RNA. The RNase isolated by Adachi et al., (1964) formed a mixture of 2'- and 3'-phosphates as final products.

RNases from millet, milo, maize and soy bean sprouts have been purified and formed 2'-mono-nucleotides from RNA via nucleoside 2',3'-cyclic phosphates (Kataya et al., 1969). However, 2'-P-forming RNases have not been reported in microorganisms.

RNase C (2'-O-methyl RNase):- An RNase has been isolated from Anacystis nidulans which cleaved phosphodiester linkage in RNA which is methylated in the 2'-hydroxy-position of ribose. This was active against a naturally occurring 2'-O-methyl RNA isolated from rhabdosomes and against chemically 2'-O-methylated RNA. This did not hydrolyse normal yeast RNA appreciably nor did it degrade DNA (Norten and Roth, 1967).

3.2.4 General Considerations

After reviewing different types of nucleolytic enzymes, a few workers have come out with certain generalisations regarding the properties of certain groups of these enzymes (Barnard, 1969; Egami and Nakamura, 1969). Some of the important general characteristics are given below:

3.2.4.1 Specificity for sugar. The enzymes have some common modes of action. (i) Cyclizing PDEs attack only ribose

containing polynucleotides, i.e. only RNA but the other phosphodiesterases may be a) ribose-specific or b) sugar non-specific or c) deoxyribose specific; (ii) Cyclizing RNases are, in general, independent of divalent cations when compared to non-cyclizing RNases which usually require cations such as Mg^{2+} or Ca^{2+} or a substitute; (iii) Most, but not all, of the known RNA-phosphodiesterases that are non-cyclizing are 5'-phosphate formers.

3.2.4.2 Mode of action. Apparently strict exonucleases are usually sugar non-specific. But an exo-RNase has been purified from several sources requiring Mg^{++} and forming 5'-phosphates (Anderson and Heppel, 1960; Lazarus and Sporn, 1967).

3.2.4.3 Base specificity. Enzymes which are base specific have certain characteristics with regard to products, bond attacked and mol. size. (i) All the enzymes which showed base-specificity are 3'-monoester formers; (ii) RNases which specifically attack the bonds next to pyrimidine nucleotides have not been found in microorganisms. One exception to this seems to be the Thiobacillus thioparus RNase (Egami and Nakamura, 1969; Uchida and Egami, 1971); (iii) Almost all the species investigated have intracellular RNase activity and they generally have no specificity for bases; (iv) Guanyloribonucleases (i.e. those which are specific for guanine nucleotide) are extracellular enzymes released from bacteria and fungi (Table 2). Guanyloribonucleases have optimum pH around neutrality. They are generally heat-stable

and have a mol. wt. of about 10000-13000. Zn^{2+} and Cu^{2+} are generally highly inhibitory, whereas SH-reagents and EDTA have no effect. Among extracellular RNases, of course, non-specific enzymes have been found. However, recently RNases were found which were specific for purine nucleotides besides others in the culture filtrates of Ustilago sphaerogena and Pleospora sp. (Ribonucleases specific for purines). These generally have pH optimum on the acidic side; (v) All RNases are comparatively small molecules. The non-specific ones have a molecular weight of about 30,000-40,000, while base-specific RNases have one of 11,000-13,000.

3.2.5 Methods of Assay:

Several methods of assay of RNase have been used based on different principles. They will be briefly outlined:

(i) Determination of solubilised mono-or/and oligonucleotides: After the RNase action, the residual RNA is precipitated with HCl (Dubos and Thompson, 1938), glacial acetic acid (Kunitz, 1940), perchloric acid (Schneider and Hogeboom, 1952), ethanol with HCl (Roth and Milstein, 1952), uranyl acetate with trichloroacetic acid (Holden and Pirie, 1955), glacial acetic acid tertiary butanol (Dickman et al., 1956), glacial acetic acid with ethanol (Schucher and Hokin, 1954) or uranyl acetate with perchloric acid (Anfinsen et al., 1954). The soluble nucleotides in the supernatant fluid is, generally, estimated by spectrometry or radioactivity.

(ii) Manometric method: The release of acidic groups resulting from hydrolysis of RNA in bicarbonate buffer can be determined from the liberation of CO_2 (Rusch, 1944).

(iii) Use of radioactive substrate: A very sensitive method by which as little as 0.001 µg of RNase can be detected was used by Roth and Milstein (1952) employing P^{32}-labelled RNA and measuring radioactivity in the filtrate after acid precipitation of residual RNA.

(iv) Turbidimetric method: After RNase reaction the reaction mixture is acidified, the turbidity determined and the residual RNA computed from a standard graph (McCarty, 1946). In another variant of this method, the turbidity due to the bovine serum albumin-RNA complex is measured to follow the extent of degradation of RNA (Houck, 1957, 1958). By this method 1-50 mµg of RNase can be determined.

(v) Use of 2',3'-cyclic nucleotide as substrate: A spectrophotometric method using 2',3'-cyclic nucleoside phosphate depends upon the difference in absorption between that of the substrates and the corresponding nucleoside-3'-phosphates (Richards, 1955).

(vi) Titrimetric methods: Titrimetric methods in which a constant pH is maintained during hydrolysis by the addition of alkali are very useful, especially in kinetic studies using RNA (Edelhoch and Coleman, 1956; Kalnitsky et al., 1959) as well as synthetic polynucleotides as

substrates (Davis and Allen, 1955; Kalmitsky et al., 1959; Anfinsen et al., 1955).

However, it appears that the method of Anfinsen et al., (1954) in which the uranyl reagent (uranyl acetate in perchloric acid solution) is employed to precipitate the residual RNA and the soluble products in the supernant is determined spectrophotometrically (260 nm) seems to be the most widely used procedure for RNase assay.

In the present study also an assay procedure was standardised based on the same principle.

3.3 Applications of Nucleolytic Enzymes

Nucleolytic enzymes have found various uses in both research and industry. The most spectacular and perhaps revolutionary use of a special class of endonucleases, the restriction endonucleases is in genetic engineering which has fostered great hopes and fears and is being used in industry (Sinsheimer, 1977; Curtiss, 1976). Some of the nucleolytic enzymes have proved efficient tools in deciphering the base sequences in nucleic acid chains. Others have been used in the preparative chemistry for the synthesis of cyclic nucleotides, oligonucleotides and such compounds. A few of them have been used for the production of flavour nucleotides. Some others have been shown to be of use in reducing the nucleic acid content of single cell proteins.

3.3.1 Chromosome mapping and genetic engineering:

Restriction endonucleases belonging to class II (described earlier) can be used as a first step in the sequencing of DNA or in the isolation of specific genes because of their ability to make relatively few specific cuts (Danna and Nathans, 1971; Danna et al., 1973). However, their most important uses so far has been in the field of chromosome mapping. Thus the small chromosome of the tumour virus SV 40 (Simian Virus 40) is cleaved by each restriction enzyme into a small number of small pieces which are readily separated by electrophoresis in gels of polyacrylamide or agarose (Nathans and Smith, 1975; Sugisaki and Takanami, 1973; Sharp et al., 1973). The order of the fragments in the genome can be determined by different techniques such as by analysis of partial digests or by successive cleavage by multiple restriction endonucleases (Nathans and Smith, 1975; Danna et al., 1973). The knowledge of the chromosome map thus gained may be used to compare closely related organisms and to localise various functions to describe regions of the chromosome. For example, it has been shown that early viral functions are coded for by the continuous fragments A, H, I and B or the Hind II/Hind III restriction map of SV 40 (Khoury et al., 1973). Similarly the origin and direction of replication of several viral and plasmid chromosomes have been discovered in particular by using Eco RI (Fareed et al., 1972; Lovett et al., 1974).

As cleavage by restriction enzymes is staggered, this leads to formation of fragments with sticky ends (i.e. terminii with overlapping complimentary sequences) which can be rejoined by DNA ligase (Goulian, 1971). Moreover, fragments from different genomes can be joined together to form hybrid genomes and a number of potential uses such as coupling of mammalian genes to bacteriophage or plasmid chromosomes etc. could be envisaged. The cloned hybrids can be used to study the control of gene expression.

This area of recombinent DNA technology has been a most intensively investigated one now. It opens up a real possibility of producing the very difficultly available human hormones, antibodies, gene therapy for metabolic diseases, etc. A number of restriction enzymes have been employed which permitted the cleavage of DNA at a small number of reproducible sites (Arber, 1974; Nathans and Smith, 1975). Eco RI, Hind III, Hae II, Hpa II, Hha I, Bam HI, Sal I, Pst I etc. are the restriction endonucleases commonly employed for this purpose (Sinsheimer, 1977). Techniques have been developed for splicing together DNA segments in any combination (Sinsheimer, 1977). These clustered or recombined DNA carrying a known message can be employed as a unit structure to provide a 'vehicle' to which other DNAs may be attached for replication or integration and this could be introduced, intact, into viable cells (e.g. E. coli) (Mandel and Higa, 1970; Cohen et al., 1972). The transformed cells can be grown for the amplification of the components of the complex

genomes. The recombinant DNA technology achieves a potential to create new genetic combinations advantageous for specific human purpose. However, this remains a controversial topic as it may lead to hazardous consequences. This aspect has been extensively discussed in the famous NIH guideline (Federal Register, 1976) and also by Curtiss (1976).

3.3.2 Nucleotide sequence analysis:

That nucleotide sequences in RNA might be determined by applying the specific cleavages induced by RNase A (pancreatic), RNase T_1 and RNase T_2 was suggested by Egami (1958) and Sato-Asano and Egami (1960). However, RNase T_2 was not found to be satisfactory for this purpose as it is less specific.

RNase T_1 splits inosinic acid bonds and xanthylic acid bonds in deaminated RNA at a far greater rate for the former than for the latter and in appropriate conditions the former can be cleaved almost exclusively (Sato-Asano and Fujii, 1960). This property has been exploited successfully by several workers for the sequence analysis in RNA, especially in highly purified specific t-RNAs. The brilliant research of Holley and his associates in 1965 resulted in the first elucidation of the complete nucleotide sequence of an RNA, alanine-specific yeast t-RNA (Holley et al., 1965). It involved the identification of the small fragments formed by digestion of the RNA with pancreatic RNase and RNase T_1, followed by determination of the structures of successively

larger fragments obtained by partial RNase T_1 digestion, until the complete sequence of the RNA was established. Similarly, many other specific RNases have been employed in nucleotide sequence analysis studies by many other workers; for example the use of RNase U_2 for sequence analysis of longer oligonucleotides formed by RNase T_1 digestion of RNA (Takemura et al., 1969; Cory and Marcker, 1970) and for specifically cleaving the adenylate linkages in RNA (Uchida et al., 1970). Josse et al., (1961) have used Staphylococcal nuclease for the same purpose.

Laskowski Sr. has reviewed a number of DNases from different sources and has detailed the application of them in elucidating the structure of nucleic acids (Laskowski Sr., 1967). Lehman et al., (1965) have described the properties of three nucleases viz. E. coli exonuclease I, E. coli exonuclease III, and N. crassa endonuclease and have suggested their possible uses in the study of nucleic acid structure.

3.3.3 Nearest-neighbour frequencies in RNA:

Miura (1964) determined the nearest-neighbour frequencies in several RNAs as follows:

(i) Determination of guanylic acid produced by RNase T_1 digestion. Thus the ratio GpGp (guanylyl residues adjacent to other guanylyl residues/total guanylyl residues) may be obtained.

$$.....(Gp)_nGp------ ..... Gp + nGp\ (n-1)$$

(ii) Determination of guanylic acid produced by the simultaneous action of RNase I-A and RNase T_1 on RNA. Thus $g_g + g_{u+c}$ may be obtained, where g_{u+c} may be deduced as the difference of the amount of guanylic acid produced in both cases. The ratio g_a = guanylyl residues adjacent to adenylyl residues/total guanylyl residues may be deduced as $g_a = 1 - (g_g + g_{u+c})$.

(iii) Similarly a_{u+c}, u_{u+c}, C_{u+c}, a_g, u_g, C_g, a_a, U_a and C may be calculated. Here it should be resistant to both enzymes in the simultaneous digestion by both the enzymes.

3.3.4 Determination of terminal sequences in RNA: RNase T_1 has the advantage of producing larger oligonucleotides fragments from RNA than does RNase I-A. This characteristic may be useful for the determination of terminal sequence in a particular RNA. Terminal sequence of various s-RNAs (t-RNAs) has been determined by a number of workers by using s-RNAs charged with C^{14}-labelled amino acid mixtures and digested with RNase T_1 (Asano and Egami, 1961; Berg et al., 1962; Ishida and Miura, 1963; Smith and Herbert, 1963).

3.3.5 Preparative chemistry

(i) Preparation of nucleoside-2',3'-cyclic phosphates:

During the hydrolysis of the secondary phosphate ester bonds of 3'-GMP in RNA by RNase T_1 guanosine 2',3'-cyclic phosphate is produced as an intermediate. Similarly during the digestion of deaminated RNA, two new compounds, inosine

2',3'-cyclic phosphate and xanthosine 2',3'-cyclic phosphate are produced (Sato-Asano et al., 1959).

(ii) Synthesis of oligonucleotides:

Enzymatic synthesis of oligo- and polynucleotides are considered to be more efficient than chemical synthesis.

Generally cyclizing RNases catalyse two-step reactions in the degradation of RNA. The first step of the reaction, transesterification, is reversible and is generally faster than the second step, hydrolysis of the intermediate products, cyclic phosphates (Uchida and Egami, 1971). These cyclizing RNases have been used for the synthesis of oligonucleotides with 3',5'-phosphodiester linkages from nucleoside 2',3'-cyclic phosphate and an appropriate phosphate acceptor, by reverse transesterification (Kossel and Seliger, 1975). This approach was first reported by Heppel et al., (1955) with RNase A which showed pyrimidine specificity. Sato-Asano (1960) prepared oligo- and polyguanilic acids from guanosine-2',3'-cyclic phosphates by using RNase T_1. 12 triribonucleoside diphosphates having guanosine residue at the 5'-end were synthesised by Sekia et al., (1968) using RNase T_1. Mohr and Thach (1969) could prepare 32 different oligonucleotides of chain length 2 to 8 by the application of RNase T_1. Koike et al., (1969) found that RNase N_1 was a better tool than RNase T_1 for the synthesis of oligoguanylic acids. Some authors reported the synthesis of oligoadenylic acids, adenyl (3',5'-) guanosine, adenylyl (3'-5') uridine etc. by

using RNase U_2 (Koike et al., 1971a). Eight diribonucleoside monophosphates (UpU, CpU, ApU, GpU, UpC, CpC, ApC and GpC) were synthesised by Sato et al., (1969) using RNase from B. subtilis. Oligo-ApGp and other oligonucleotides such as ApGpU, IpApApC etc. were prepared by Koike et al., (1971b) by using RNase N_1. RNases U_2,N_1 and a non-specific RNase from B. subtilis were used by Uchida and Funayama-Machida (1977) for the synthesis of dinucleotides and trinucleotides.

3.3.6 For purification of DNA:

Traces of RNA in DNA preparations are completely eliminated by the simultaneous action of pancreatic RNase and RNase T_1. Non-specific RNases would be better for this purpose (Saito and Miura, 1963).

3.3.7 The production of Flavour nucleotides:

Kuninaka, Sakaguchi and others in 1959 showed for the first time that an enzyme of P. citrinum could hydrolyse RNA to form 5'-nucleotides and also that 5'-GMP was an active flavour component. Later a number of other organisms were found to elaborate 5'-nucleotide forming nucleases as already mentioned. However, at present only 5'-P-forming nucleases from P. citrinum and Str. aureus are being employed for the industrial production of 5'-nucleotides (Ogata et al., 1976).

3.3.8 Reducing the nucleic acid content in Single Cell Proteins

Single Cell Proteins (SCP) are a promising means of enhancing protein supplies both for feed and food (Matels and Tannenbaum, 1968; Tannenbaum and Wang, 1975). Their nutritional quality has been shown to be good (Kihlberg, 1972; Lovland et al., 1976; Chen and Peppler, 1978) and in general, are considered to be safe for both humans and animals from the view points of toxicity, pathogenicity and carcinogenicity (Garattini et al., 1979). However, their high nucleic acid content is a disadvantage, since it may cause uricaemia on high uptake which may lead to gout, kidney stone and other complications if they have to be used for food purposes.

Several methods have been developed for the reduction of nucleic acid content in SCP, which will be briefly reviewed later in Chapter 4. One of the methods tried for this purpose was exogenous application of bovine pancreatic RNase A on heat-shocked Candida yeast cells (Castro et al., 1971). The cell suspension was treated with the enzyme which led to a decrease in the nucleic acid content from the initial 7.5-9.0% to 1-2% without any significant concomitant loss of protein.

SCOPE AND OBJECTIVES OF THE INVESTIGATION

SCOPE AND OBJECTIVES OF THE INVESTIGATION

Reducing the nucleic acid content is a very important step in the processing of single cell proteins for human consumption. Castro et al., (1971) showed that bovine pancreatic RNase A could be effectively used in the reduction of nucleic acid content of Candida yeast cells. However, the prohibitive cost and the limited availability of this enzyme restricts its use in a commercial scale.

With the objective of isolating a microorganism producing RNase abundantly, a preliminary screening was carried out using a number of fungal cultures. A potent fungal isolate was selected for further studies. This strain was identified to be Aspergillus candidus. Apparently, there is no report on this species of Aspergillus as an extracellular RNase producer.

There is not much information in literature regarding the nutritional and cultural conditions influencing the production of RNases, probably due to the fact that most of the microbial enzymes studied were not intended to be applied industrially. Hence, in the present case it was thought that a detailed study on the optimization of various nutritional and environmental conditions was necessary.

As we were dealing with a hitherto unknown RNase, it was necessary to standardize conditions for an assay method. Hence, some studies were made with crude enzymes. Studies

on the crude enzyme preparation was also necessitated by its intended use in the reduction of nucleic acids in yeast cells. Since this is a first report of A. candidus producing an RNase, the enzyme was purified to homogeneity and the various characteristics of the enzyme studied and compared with the reported RNases. It was also planned to carry out a fairly detailed study on the application of the fungal RNase enzyme for the reduction of nucleic acid content of yeast cells. Most of the reports on the reduction of nucleic acid content in yeast cells pertain to strains of Candida yeast. Relatively, there is no useful information available on the reduction of nucleic acid content of Saccharomyces which is yet another important source of single cell protein. Hence, it was intended to emphasize a study of reduction of nucleic acid content in Saccharomyces cerevisiae cells in the present work by using fungal RNase preparation. For comparison cells of Candida spp. were also studied. The studies envisaged development of an integrated process wherein yeast could be processed by using fungal RNase leading to the reduction of its nucleic acid content on one hand and the recovery of the yeast RNA degradation products on the other.

The results of the different aspects studied are presented in four chapters under PART III of this thesis.

PART II

MATERIALS AND METHODS

I. MATERIALS

A. CHEMICALS:

The following chemicals used in this study were purchased from the respective suppliers:

DNA (calf thymus), poly A, poly G, poly C, poly U, all the nucleotides, all the protein standards, Sephadex G-100, DEAE-Sephadex A-50, DEAE-Cellulose, acrylamide, Bis-acrylamide, TEMED, Coomassie Brilliant Blue, PCMB, iodoacetamide and DFP were purchased from Sigma Chemical Co. St. Louis, Mo, USA.

All the vitamins, carbohydrates (except cellulose), mannitol, glycerol, sodium β-glycerophosphate, ammonium persulphate and glutathione were obtained from B.D.H.Ltd., England, UK.

EDTA, Methylene Blue, Rose Bengal, Peptone, Casein and Phenol reagent were procured from Glaxo Laboratories (India) Ltd., Bombay, India.

Diphenylamine and orcinol used were supplied by May and Baker Ltd., Dagenham, England, UK.

Yeast extract and lithium chloride were procured from Oxoid Ltd., England, UK and Sojuzchimexport, Moscow, USSR, respectively.

Chloroform and toluene were obtained from Purex Laboratories (India) Pvt. Ltd., Bangalore, India.

Malathione and parathione were supplied by Infestation Control and Pesticides Discipline, C.F.T.R.I., Mysore, India.

Rice bran, wheat bran, ragi husk, malted ragi flour were obtained from the Rice and Pulse Technology Discipline, C.F.T.R.I., Mysore, India.

B. MICROBIOLOGICAL:

i) Microorganisms:- The following microorganisms used in this study were obtained from the respective sources:

Aspergillus candidus ICP, A. fischeri ICP, A. flavipes ICP, A. flavus ICP, A. fumigatus ICP, A. nidulans ICP, A. niger ICP, A. ochraceus, A. oryzae ICP, A. ruber ICP, A. ustus ICP, and A. versicolor ICP were obtained from Infestation Control and Pesticides Discipline, C.F.T.R.I., Mysore, India.

Rhizoctonia solani Coffee isolate was obtained from Department of Applied Botany, University of Mysore, Mysore, India.

Botryodiploidea sp. K_1GM, Botryodiploidea sp. K_4Io, Curvularia sublata, Fusarium moniliforme 1, Gibberella fujikuroi and Glomerella sp. were obtained from Department of Botany, University of Saugar, Sagar, India.

Aspergillus sp. cel., A. candidus M16a, A. carbonarius, A. flavus 1 and 2, A. niger, A. ochraceus, A. oryzae No.1, A. terreus, A. terreus 6365, Aspergillus spp. No.1 to 5, Fusarium sp., Penicillium sp. 6, Sporotrichum pruinosum,

Trichoderma spp. No.1 and 2, Trichothecium and many other unidentified cultures were either isolated from various sources such as soil, decaying plant materials, contaminated agar plates etc., or taken from the culture collection of the Microbiology and Fermentation Technology Discipline, of this Institute.

The following yeast strains used in this study were from the sources indicated in parentheses: Saccharomyces cerevisiae (Microbiology and Fermentation Technology Discipline culture collection), Candida tropicalis NCIM 3119, C. lipolytica NCIM 3229, C. utilis NCIM 3336, C. utilis NCIM 3055 (National Collection of Industrial Microorganism, National Chemical Laboratory, Pune, India).

ii) Media used and their composition: The different media used in this study are given below. The figures represent the concentration of the components in g/L unless otherwise stated.

1. Potatodextrose-Agar (PDA)

Potato infusion from	200
Dextrose	20
Agar	20
pH	5.5

2. Glucose-peptone-minerals (Kuninaka et al., 1959)

Glucose	50.0
Peptone	5.0
K_2HPO_4	0.5
KH_2PO_4	0.5
$CaCl_2.2H_2O$	0.4
$MgSO_4.7H_2O$	0.4
pH	5.6

3. Czepeck's solution Agar

Sucrose	30
Sodium nitrate	3
Dipotassium phosphate	1
Magnesium sulfate	0.5
Potassium chloride	0.5
Ferrous sulphate	0.1
Agar	15

4. Malt-Extract-Agar

Malt extract	20
Peptone	1
Dextrose	20
Agar	20

5. MY-20 Agar

Peptone	5
Yeast extract	3
Malt extract	3
Glucose	200
Agar	20

6. Basal medium

Carbon source	equivalent of 3% glucose
Nitrogen source	500 mg N per litre
K_2HPO_4	0.5
KH_2PO_4	0.5
$MgSO_4.7H_2O$	0.4
$CaCl_2.2H_2O$	0.4

7. Trace-mineral composition (Chahal & Gray, 1969)

	mg/ml
$FeCl_3$	100
H_3BO_3	114
$(NH_4)_6Mo_7O_{24}.4H_2O$	480
$CuSO_4.5H_2O$	780
$ZnSO_4.7H_2O$	16.72
$MnCl_2$	144

One ml was added to one liter medium.

8. Vitamin solution composition

	mg/ml
Thiamine	100
Riboflavin	50
Pyridoxin	50
Calcium pantothenate	200
p-aminobenzoic acid	50
Nitotinamide	200
Choline	200
Inositol	400
Folic acid	4

1 ml was added to one liter medium.

9. Bran semi-solid media

i)	Rice bran	1 kg
	Tap water	800 ml
ii)	Wheat bran	1 kg
	Tap water	800 ml
iii)	Ragi husk	1 kg
	Tap water	800 ml

10. Formulated medium for A. candidus M16a

Starch	18.75
NH_4NO_3	2.50
K_2HPO_4	0.50
KH_2PO_4	0.50
$CaCl_2.2H_2O$	0.40
$MgSO_4.7H_2O$	0.40

11. Medium for yeast biomass production

Glucose	10.0 (autoclaved separately)
$NH_4H_2PO_4$	5.0
KH_2PO_4	0.7
$MgSO_4.7H_2O$	0.5
$CaCl_2.2H_2O$	0.1
Yeast extract	2.0
pH before autoclaving	4.5

II. METHODS

A. MICROBIOLOGICAL

(i) Isolation, screening, identification and culturing of fungal strains:

The fungal cultures were either procured from various culture collections as indicated in MATERIALS Section or isolated in the laboratory from various sources such as soil, decaying organic matters and contaminated agar plates.

For isolation samples were suspended in sterile distilled water, diluted and plated out on potato-dextrose agar medium (Medium 1). Colonies that developed on the plates were picked up and purified by repeated plating or by hyphal tip-isolation.

The cultures were maintained on the same medium by bimonthly transfers, growing for one week and storing at 5°C.

For identification of the selected fungal strain the procedures and criteria described by Raper and Fennel (1965) and Onions et al., (1981) were followed. The media used for this purpose were Czepeck's solution agar (CSA, Medium 3), Malt extract agar (MEA, Medium 4) and MY-20 agar (Medium 5).

In all submerged fermentation studies 50 ml medium in 500 ml Erlenmeyer flasks was autoclaved at 15 psi for 20 min and, after cooling, inoculated with a loopful of spores or mycelia (in the case of screening studies) or one ml of spore suspension in the case of A. candidus M16a (50 ml suspension prepared in distilled water from 10 PDA slants) unless otherwise mentioned. After 5 days growth on a rotary shaker (230 rpm) at 30°C the culture filtrate (CF) was collected by filtration and tested for enzyme activity. Biomass was determined gravimetrically by weighing mycelia after drying to constant weight at 80°C.

Surface culture on bran media was carried out either in flasks or trays. Details of the procedures followed are given under individual experiments in "Results and Discussion" Section.

(ii) Production of yeast Biomass:

For the production of yeast biomass Medium No.11 was used. 500 ml conical flasks containing 150 ml medium were incubated on a rotary shaker (230 rpm) at ambient temperature (24-28°C) for 16-24 hrs. The cells were harvested by centrifugation at 6000 g and were washed by resuspending in distilled water and centrifuging.

B. ENZYME ASSAYS

Ribonuclease:- McDonald's (1955) method with a slight modification as given below was followed for screening the culture filtrates for RNase activity. To 1 ml of RNA solution (6.0 mg/ml) in 0.1 M acetate buffer (pH 5.2), 1 ml crude enzyme (CF) was added and incubated at 25°C for 15 min. The reaction was stopped by adding 2 ml of uranyl reagent (0.25% uranyl acetate in 2.5% perchloric acid) (MacFadyen, 1934), kept for 30 min at 25°C and centrifuged. The supernatant solution was diluted appropriately and the absorbance at 260 nm was determined in a Carl-Zeiss spectrophotometer. The enzyme activity was expressed as absorbance (A_{260}).

The standard assay procedure:- The determination of RNase from A. candidus M16a was performed as follows: The reaction mixture containing 0.5 ml enzyme, 0.5 ml of 1% RNA solution and 1 ml of 0.2 M sodium acetate buffer (pH 4.5) was incubated for 30 min. at 55°C. The reaction was stopped by the addition of 2 ml of uranyl reagent kept at 0°C for 30 min

and centrifuged off (5000 g for 5 min) the precipitate. The supernatant was diluted 40 times (0.1 to 4.0 ml) and absorbance at 260 nm was measured.

An increase in absorbance of 0.1 multiplied by the enzyme dilution factor was defined as one unit of enzyme per ml. Specific activity was expressed as unit activity per mg protein.

Deoxyribonuclease (DNase):- Procedure of Fujimoto et al., (1974) with a slight modification was adopted. Calf thymus DNA (Sigma) was used as the substrate both in native and heat denatured forms. Heat denatured DNA was prepared according to the procedure of Woodward (1944). The reaction mixture contained 0.2 ml of 0.2 M acetate buffer (pH 4.5), 0.2 ml of DNA solution (5 mg/ml) and 0.1 ml of appropriately diluted enzyme solution. The reaction mixture was incubated for 30 min at 55°C. The reaction was stopped by the addition of 1 ml of uranyl reagent, kept at 0°C for 20 min and the precipitate was removed by centrifugation. The supernatant was diluted 3-fold and absorbance was measured at 260 nm in a Carl-Zeiss spectrophotometer.

An increase in O.D. of 0.1 multiplied by the enzyme dilution factor is defined as one unit of the enzyme activity per ml under the above assay conditions.

Non-specific phosphodiesterase:- A modified procedure of Koerner and Sinsheimer (1957) was adopted. The reaction mixture containing 1.0 ml of bis-p-nitrophenylphosphate solution (0.4 mg/ml), 0.2 ml of 1 M acetate buffer (pH 5.5) and 0.3 ml

of appropriately diluted enzyme was incubated at 37°C for 3 hrs. The reaction was stopped by the addition of 1.5 ml of M NH_3. Absorbance at 410 nm was determined in a Spekol spectrophotometer.

Phosphomonoesterases:- Both 3'- and 5'-phosphomonoesterase (nucleotidase) activities were determined by a modified procedure of Fujimoto et al., (1974). The reaction mixture of 0.5 ml volume contained 0.2 ml of the substrate (3'- or 5'-adenosinemonophosphate, 10 mM), 0.2 ml of 0.1 M acetate buffer (pH 5.2) and 0.1 of the enzyme. The mixture was incubated at 37°C for 15 min and the reaction was stopped by the addition of 4 ml of 6% perchloric acid. The liberated inorganic phosphate (Pi) was estimated by Tausky and Shorr's method (Tausky and Shorr, 1953).

Protease assay was carried out according to a modified procedure of Anson (1937, 1938) using either BSA or casein as substrate. The reaction mixture containing 1 ml of the substrate (10 mg/ml) dissolved in 0.2 M phosphate buffer (pH 7.0) and 1 ml enzyme solution (appropriately diluted) was incubated at 55°C for 30 minutes. The reaction was stopped by the addition of 3 ml of 5% trichloroacetic acid. This was allowed to stand at room temperature for 30 minutes and filtered.

To 1 ml of the filtrate was added 2 ml of 0.5 N NaOH and 0.5 ml of phenol reagent (diluted 1:2). The colour developed was measured at 660 nm in a Spekol spectrophotometer

between 5 and 20 minutes after adding the reagent when the colour intensity was stable. Tyrosine (in µg) released were calculated by extrapolation from a standard curve. One unit of enzyme activity is defined as that amount of enzyme which releases one µg of tyrosine under the above assay conditions. Specific activity is the number of units per milligram of enzyme protein.

C. CHROMATOGRAPHIC TECHNIQUES

(i) Gel permeation chromatography:- Sephadex G-100 having medium mesh size was prepared according to standard procedure (Fischer, 1969) and was packed on a column of 2 cm x 95 cm to a height of 85 cm. The column was washed well and equilibrated with 0.05 M potassium phosphate buffer (pH 5.7) containing 0.5 M sodium chloride. The void volume was determined by Blue dextran-2000.

(ii) Ion-exchange chromatography:- DEAE-cellulose was treated with 0.5 N HCl and 0.5 N NaOH alternatively according to standard procedure (Peterson and Sobers, 1962) and equilibrated in 0.005 M potassium phosphate buffer (pH 5.6). The centrifuged wet cake was used for adsorption and batchwise elution of the enzyme.

The ion-exchange gel DEAE-Sephadex A-50 was prepared according to standard procedure. The gel was packed in a column (0.9 x 13 cm), equilibrated with 0.005 M phosphate buffer (pH 5.6). This was used for fractionation of the enzyme by gradient elution with NaCl solution.

The hydrolysis products of nucleic acid in yeast cells were analysed by fractionation on Dowex-1x8 (Cl^- form, 200-400 mesh) according to the method of Cohn (1950).

Each fraction was concentrated by perevaporation and was analysed by paper chromatography, TLC and spectrophotometry as follows:

(iii) Paper chromatography:- was carried out by descending method. The following solvent systems were employed: (1) Isobutyric acid : glacial acetic acid : 1 N NH_4OH (10:1:5); (2) Isopropanol : Conc. NH_4OH : Water (6:3:2); (3) Isobutyric acid : 0.5 N NH_4OH (10:6); and (4) Saturated solution of $(NH_4)_2SO_4$: Isopropanol : 1 M sodium acetate (80:2:20).

In all the cases Whatman 3 MM papers were used and were developed for 16-20 hrs. The spots were detected under UV light at 254 nm and marked.

Standard 3',5' and 2',3'-cyclic mono-nucleotides were also spotted on the paper as reference.

The spots were cut out and eluted with suitable solvents and spectral characters were studied for confirming the base.

(iv) TLC:- Thin layer chromatography were carried out by using either silica gel G layers according to the method of Scheig et al., (1963) or polyethylenimine cellulose (PEI cellulose) layers according to the method of Randerath and Randerath (1965).

D. POLYACRYLAMIDE DISC-GEL ELECTROPHORESIS (PAGE)

Disc-gel electrophoresis was carried out according to the method of Davis (Davis, 1964) using Toshniwal Electrophoresis apparatus at two pH values 4.3 (using β-alanine-acetic acid buffer) and pH 8.1 (using Tris-glyline buffer). 7.5% polyacrylamide gel was used. Samples containing 25-100 µg protein were loaded and the gels were run at a current of 3 mA/tube till the marker dye moved to the bottom of the gel.

The gels were stained with Coomassie Brilliant Blue and destained by Isopropanol-acetic water. The dye was prepared by dissolving 0.5 gm of Coomassie Brilliant Blue powder in a solution containing 181.6 ml of 50% methanol and 18.4 ml of glacial acetic acid. The destaining solution contained 5% isopropanol and 7.5% acetic acid in distilled water. The gels after destaining were scanned using a gel scanner and then stored in 7.5% acetic acid.

E. CHEMICAL AND OTHER ESTIMATIONS

a) Dry weight determination of yeast biomass:- The turbidity of cell suspensions containing different amounts of cells was measured at 600 nm. Cell suspensions of known turbidity were centrifuged and the dry weights of the pellets were determined by drying to constant weight at 80°C. A calibration curve was prepared by plotting O.D. vs. dry weight.

b) Spectrophotometry:- Absorption spectrum of the isolated compounds of the RNA hydrolysed products were compared

with those of standard nucleotides at different pH values. Measurements of A_{250}/A_{260}, A_{280}/A_{260} and A_{290}/A_{260} were also made and compared with the values of standard nucleotides.

c) Nucleic acid estimation:- The total nucleic acid in the supernatant after treatments were determined by measuring the absorbance at 260 nm and extrapolating from a calibration curve using RNA.

The total nucleic acids in the cells also was determined spectrophotometrically as above after extracting it in 0.5 N perchloric acid at 70°C for 20 min (Ohta et al., 1971). Calibration curve for this purpose was prepared by plotting O.D. vs. RNA concentration (prepared in 0.5 N perchloric acid).

d) Protein determination:- Protein in the enzyme samples and supernatants of different yeast cell treatments was estimated by a modified Lowry's method (Chaykin, 1966; Lowry et al., 1951).

The protein content of yeast cells was determined by biuret method as described by Stickland (1951) with slight modification as mentioned below. Yeast suspensions upto 5 mg dry weight were measured out into small test tubes, water added to make each sample upto 3.3 ml and each treated with 0.6 ml 20% (W/V) sodium hydroxide by heating for 10 min in a boiling water bath. After cooling, 0.1 ml of 25% (W/V) cupric sulphate solution was added to the above solution. The copper hydroxide precipitate formed was broken with a glass rod and this was centrifuged off at 3,500 rpm for 15 min.

The colour intensity of the supernatant was determined at 550 nm in Spectronic 21 spectrophotometer. The amount of protein was read from a calibration curve prepared for O.D. *vs.* mg protein using BSA as standard.

PART III

RESULTS AND DISCUSSION

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CHAPTER - 1

SCREENING AND SELECTION OF FUNGAL STRAINS AND A STUDY OF CULTURAL CONDITIONS

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SECTION I

SCREENING AND SELECTION OF FUNGAL STRAINS FOR RNase PRODUCTION

Extracellular enzymes are preferred for industrial production because of easy extractability and purification. Among the microbial sources fungi appear to be most suitable. Hence, most of the industrial enzymes such as amylases, pectinases, proteases and cellulases are produced from fungal strains.

RNases are present in all biological systems. These enzymes have been reported to be produced extracellularly by a wide variety of microorganisms viz. bacteria, actinomycetes, yeasts and fungi (Barnard, 1969; Egami and Nakamura, 1969; Kuninaka, 1976).

In the present study sixty different fungal strains belonging to various genera as given in the "Materials and Methods" Section were examined for their ability to produce extracellular RNase. Of these nearly 50% belonged to the genus Aspergillus. On the basis of RNase activity in the crude filtrate they were grouped into a) good producers, b) moderate producers and c) poor producers (Table 5). Of the 37 strains which showed RNase activity, 11 were good producers, isolate M16a, A. terreus 6365, and A. ochraceous being the best. Others which showed fairly good activity were A. carbonarius, Penicillium sp., Isolate M16b and Isolate M7a.

Table - 5

RNase Production by fungal cultures

Potency of the organisms	Cultures*
1. Good producers (more than 1.0 O.D. unit RNase/ml)	Isolate M16a (2.54) Aspergillus terreus 6365 (2.32) A. ochraceous (1.81) A. carbonarius (1.52) Penicillium sp. (1.26) Isolate M16b (1.26) Isolate M7a (1.25) Isolate M6b (1.20) A. oryzae No.1 (1.14) Isolate M14a (1.14) Isolate M6d (1.07) Gibberella fujikuroi (1.00)
2. Moderate producers (Less than 1.0 O.D. unit RNase/ml)	Fusarium moniliforme II (0.98) A. flavipes (0.93) A. flavus (0.92) A. oryzae 1048 (0.88) Trichothecium sp. (0.85) Sporotrichum pruinosum (0.84) Fusarium moniliforme I (0.78) Isolate M6a (0.74) A. fumigatus (0.72) A. niger (0.72) A. oryzae No.3 (0.70) Isolate M6c (0.70) Trichoderma sp. (0.67) Aspergillus sp. (0.66) Rhizopus nigricans (0.58) A. ruber (0.56) Fusarium sp. (0.51)
3. Poor producers (Less than 0.5 O.D. unit RNase/ml)	A. oryzae No.2 (0.44) Botryodiploidea sp. K_4IO (0.36) Glomerella sp. (0.28) A. nidulans (0.27) A. versicolor (0.27) Cont. RNA 2 (0.10) Botryodiploidea sp. (0.09) A. ochraceous No.1 (0.07) A. ochraceous No.2 (0.05)

* RNase activity expressed as absorbance at 260 nm is given in parenthesis.

Identification of Isolate M16a:-

The various criteria described by Onions et al., (1981) and Raper and Fennel (1965) were followed for determining the taxonomical position of the selected strain, Isolate M16a. Growth characteristics and morphological features were studied by growing the culture on CSA, MEA and MY 20A media. The salient features observed are recorded in Table 6. The observed characteristics of isolate M16a were similar to those of Aspergillus candidus. This culture will henceforth be referred to as Aspergillus candidus M16a.

Isolate M16a was chosen for further studies because it gave the highest yield of enzyme in the screening but also because the species A. candidus to which this strain was found to belong by taxonomic studies has not been reported earlier as a producer of RNase.

Table - 6

Morphological and cultural characteristics of Isolate M16a

1.	Colony diameters after 2 weeks in:	
	a) CSA	- 2.5 cm to 2.9 cm
	b) MEA	- 3.1 cm to 3.8 cm
	c) MY 20 A	- 4.5 cm to 5.0 cm
2.	Morphology when grown on CSA:	
	a) Colonies	- Slow growing, thin, with vegetative mycelium largely submerged and surface growth consisting only of fruiting structures, white turning, slightly yellowish cream in age, sclerotia formed which are yellowish brown initially turning black in age; Riverse of colonies very pale yellow or ivory coloured; no exudate, no distinct odour.
	b) Conidial heads	- White, globose when young, splitting in age. Diameters of young large heads ranged from 110 µ to 200 µ. Small heads measured 35-75 µ in diameter.
	c) Conidiophores	- 525-675 µ long, 6.0-10 µ diameter; smooth non-septate and colourless.
	d) Vesicles	- globose, 15-24 µ in diameter, fertile over the whole surface.
	e) Sterigmata	- Colourless, wedge shaped, in two series, primaries ranged in dimension from 5.0 to 6.6 µ by 1.6 to 3.0 µ. Secondaries - 5 to 6 µ by 1.5 to 2.5 µ.
	f) Conidia	- Subglobose, measuring 3.3-4.0 µ in diameter, thin walled, colourless and smooth.
	g) Sclerotia	- White at first, turning yellowish brown and then to black. 700-750 µ in diameter.
3.	Nomenclature:	- Close relationship to Aspergillus candidus.

SECTION II

INFLUENCE OF NUTRITIONAL AND ENVIRONMENTAL CONDITIONS ON RNase PRODUCTION

A. SUBMERGED FERMENTATION

Media constituents influencing the production of RNase:- It is well-known that the various constituents of the cultivation medium could influence the physiology of an organism and as a result, the production of metabolites and enzymes by it (Davies, 1963). In the present study the influence of different nutritional and environmental factors on the growth and the yield of RNase by the culture, A. candidus M16a was studied with the objective of optimizing the conditions.

Effect of carbon sources:- The different carbon sources tested for their ability to support the growth and the enzyme yield are shown in Table 7. Except cellulose all the other 10 carbon sources supported the growth of the organism. However, the biomass formed was significantly lower in the case of inulin and lactose when compared to others. Starch, mannose and maltose were the best carbon sources as far as the growth of the organism was concerned. No apparent growth was observed in the case of cellulose. The highest enzyme yield was obtained when the organism was grown on starch followed by maltose, glucose and raffinose. No enzyme was detected in the culture filtrates on lactose and cellulose. Glycerol and mannitol, the two sugar alcohols tested, showed only a little enzyme production.

Table - 7

Effect of different carbon sources on the RNase production

Sl. No.	Carbon source	Concentration used (g/lit.)	pH of the culture broth (Final)	Biomass (dry wt mg/50 ml)	RNase activity (Units/ml)
1	Glucose	30.0	5.3	234	11.2
2	Starch	27.0	5.6	359	24.8
3	Maltose	30.0	5.3	303	12.5
4	Lactose	30.0	5.0	27	0
5	Sucrose	30.0	4.7	187	7.0
6	Cellulose	27.0	5.2	1144*	0
7	Mannitol	30.0	6.2	165	3.6
8	Mannose	30.0	5.5	318	9.5
9	Inulin	30.0	4.8	30	7.0
10	Raffinose	30.0	5.5	201	10.0
11	Glycerol	30.0	5.8	204	2.0

*biomass + residual substrate.

NH_4NO_3 (0.05%) served as the N-source in the basal medium (Medium No.6). Carbon sources were autoclaved separately. The pH of the media was adjusted to 5.2. The culture was grown for 5 days on a rotary shaker (230 rpm) at 30°C.

It is interesting to observe that the addition of starch had a significant influence on the enzyme production. With other carbon sources such as glucose, maltose and raffinose the enzyme production was about 50% of that with starch. Even though mannitol and glycerol supported good growth the enzyme yield was very low. The biomass formation and the enzyme yield, however, did not show any positive correlation.

Effect of nitrogen sources on the production of the enzyme:- The data on the effect of various inorganic and organic nitrogen sources on the RNase production by A. candidus M16a are given in Table 8. It could be seen that NH_4NO_3 was the best inorganic N source. Next to this was $(NH_4)_2HPO_4$. Ammonium salts of organic acids were very beneficial with ammonium citrate being the best. Sodium and potassium nitrates were poor sources of N. Sodium nitrite supported fairly good growth but did not influence the production of any significant amount of the enzyme. Among the complex nitrogen sources tested, casein and peptone were found to be the best N sources.

A remarkable feature that could be observed was that the complex organic nitrogen sources such as casein and peptone showed a very significant effect of enhanced enzyme production when compared to inorganic sources. The enzyme yield in these two cases were about 12-14 times more than that with NH_4NO_3.

Table - 8

Effect of various N sources on RNase production

Nitrogen source*	Final pH	Biomass mg/50 ml	RNase activity units/ml
Sodium nitrate ($NaNO_3$)	6.20	187.5	1.30
Potassium nitrate (KNO_3)	5.95	127.0	1.65
Ammonium nitrate (NH_4NO_3)	5.55	410.0	14.80
Ammonium chloride (NH_4Cl)	2.50	88.0	3.00
Ammonium sulfate $(NH_4)_2SO_4$	2.80	80.0	3.40
Ammonium phosphate dibasic $(NH_4)_2HPO_4$	2.60	142.5	5.80
Sodium nitrite ($NaNO_2$)	7.00	115.5	0.70
Urea	5.25	121.5	0.80
Ammonium citrate	3.50	424.0	69.00
Ammonium oxalate	3.20	185.0	12.20
Ammonium tartrate	3.85	175.5	13.50
Ammonium acetate	5.00	281.0	7.10
Glycine	6.10	199.0	2.35
Casein	5.65	477.5	200.00
Peptone	5.50	465.5	176.0

*Added to the basal medium (No.6 containing 3% glucose) to give 50 mg N/100 ml.

Effect of different concentrations of NH_4NO_3 as the nitrogen source:- The concentration of the nitrogen source, NH_4NO_3 was varied from 0.025% to 0.5% in a medium containing 2.5% starch as the carbon source. Other constituents were the mineral salts (Medium 6).

The results are presented in Table 9. It could be observed that an increase in concentration of NH_4NO_3 from 0.025% to 0.20% showed an increase in the enzyme yield from 20 units to 29 units/ml. Further increase in the nitrogen source did not show any change in the enzyme production though at 0.5% level the enzyme yield showed a slight drop. 0.2% NH_4NO_3 was found to be the optimal concentration.

Effect of different concentration of starch:- Using a medium containing 0.2% NH_4NO_3 and the minerals (as in Medium 6) the level of starch was varied from 0.25% to 10% in order to determine the optimum concentration. A steady increase in enzyme production was observed from 6.6 units to 21.0 units/ml. when the starch concentration was altered from 0.25% to 1.5% (Table 10). A steady decrease in pH of the culture filtrate from 6.0 to 5.4 also was observed. Further increase in the starch concentration showed a tendency to lower the enzyme yield and also pH.

Effect of different concentrations of the carbon and the nitrogen sources at constant ratio:

Keeping the ratio of NH_4NO_3 to starch (2:15) constant, their concentrations were varied (0.05% to 0.4% for NH_4NO_3

Table - 9

Effect of different concentrations of NH_4NO_3

Sl. No.	NH_4NO_3 concentration (%)	pH	Biomass mg/50 ml	RNase units/ml
1	0.025	5.50	220	20.0
2	0.050	5.45	275	21.5
3	0.100	5.30	356	22.5
4	0.150	5.10	414	25.0
5	0.200	5.10	396	29.0
6	0.300	5.10	425	29.0
7	0.400	5.00	398	29.0
8	0.500	5.60	380	28.0

Starch 2.5%. Mineral composition was as in the previous table.

Table - 10

Effect of different concentration of starch

Sl. No.	Starch concentration (%)	Final pH	Biomass mg/50 ml	RNase units/ml
1	0.25	6.0	48	6.6
2	0.50	5.7	106	11.3
3	0.75	5.6	152	13.6
4	1.00	5.5	171	17.2
5	1.50	5.4	216	21.0
6	2.00	5.4	217	17.5
7	2.50	5.4	218	13.0
8	5.00	4.8	324*	11.0
9	10.00	4.6	687*	11.0

NH_4NO_3 - 0.2%

* biomass + residual starch

and 0.375% to 3.0% for starch). Other ingredients in the medium were minerals (Medium 6).

It could be seen (Table 11) that the enzyme yield increased from 10 units to 16.0 units/ml when the concentration of both N and C increased upto 0.2% NH_4NO_3 and 1.5% starch. At higher levels not only was there no further increase, but also it tended to decrease the enzyme production. However, a steady increase in biomass production was observed with increase in those media ingredients.

Effect of trace elements:

The effect of certain trace elements such as Zn, Cu, Fe, Mo, B and Mn on the enzyme production of A. candidus M16a was studied. The medium contained 0.2% NH_4NO_3, 1.5% starch and minerals as in Medium 6. The concentration of the different metallic salts tested were the same as that used by Chahal and Gray (1969) (Medium 7) for fungal cultures. The data on the effect of these trace minerals are presented in Table 12. It could be observed that the elimination of $ZnSO_4$, $FeCl_3$ and $MnCl_2$ from the medium slightly lowered the enzyme formation indicating their requirement for higher enzyme yield. Elimination of ammonium molybdate, on the other hand, showed an increase in the enzyme yield indirectly showing the inhibitory effect of this compound. The trace minerals also showed some effect on the growth of the organism. Inclusion of all the six trace elements promoted

Table - 11

Effect of different concentration of NH_4NO_3 and starch keeping their ratio constant

Sl. No.	Concentration of NH_4NO_3 (%)	Starch concentration (%)	pH	Biomass mg/~~ml~~ 50 ml	RNase units/ml
1	0.05	0.375	5.7	72	10.0
2	0.10	0.750	5.4	112	14.0
3	0.15	1.125	5.4	151	16.6
4	0.20	1.500	5.3	163	16.0
5	0.25	1.875	5.2	251	16.6
6	0.30	2.250	5.4	283	15.6
7	0.40	3.000	5.1	268	13.8

Other media constituents were as in the previous cases.

Table - 12

Effect of trace elements

Sl. No.	Trace elements omitted	pH	Biomass mg/50 ml	RNase units/ml
1	Zinc sulphate	4.5	145	13.6
2	Copper sulphate	4.5	272	22.8
3	Ferric chloride	4.8	224	11.2
4	Ammonium molybdate	4.5	210	28.4
5	Boric acid	4.5	196	16.0
6	Manganous chloride	4.6	168	14.4
7	All minerals added	4.5	214	18.4
8	Control with no trace minerals	5.0	157	21.6

The concentration of the various trace elements added were as given by Chahal and Gray (1969).

good growth. $CuSO_4$ showed a slight inhibitory effect on the growth whereas $ZnSO_4$ and $MnCl_2$ seemed beneficial.

Effect of certain vitamins on the growth and the RNase production:

It is known that the presence or absence of certain vitamins shows an effect on the growth and the production of different enzymes by microorganisms (Davies, 1963). Hence, the effect of some of the vitamins viz., thiamine, riboflavin, pyridoxin, Ca-pantothenate, p-aminobenzoic acid (PABA), nicotinamide, choline, inositol and folic acid was tested. The results are given in Table 13.

The overall effect of the addition of all these vitamins was not significant although elimination of individual vitamins showed some effect. Omission of pyridoxin, PABA and choline showed a decreased enzyme yield indicating their promotory effect. On the other hand, elimination of inositol caused an increased enzyme formation indicating its inhibitory effect.

Incorporation of all the vitamins resulted in increased growth of the organism when compared to the control. It could also be noticed that the growth decreased in the absence of PABA and riboflavin as compared to the one with all the vitamins. However, growth and enzyme yield did not show any correlation.

Table - 13

Effect of certain vitamins on RNase production

Sl. No.	Vitamin omitted	pH	Biomass mg/50 ml	RNase units/ml
1	Thiamine	5.6	205	24.0
2	Riboflavin	5.7	159	24.0
3	Pyridoxin	5.6	179	14.0
4	Ca-pantothenate	5.5	178	23.0
5	p-amino benzoic acid	4.9	152	17.0
6	Nicotinamide	5.6	199	21.4
7	Choline	5.5	200	18.4
8	Inositol	5.3	211	35.0
9	Folic acid	5.3	187	20.4
10	None	5.3	211	26.0
11	All	4.7	161	24.4

Vitamin concentration was as in Medium 8 .

Studies on the use of cheap and complex substrates for the RNase production:

For the development of an economically feasible process production cost is an important criterion which in turn depends to a very significant extent on the cost and availability of the raw material. Hence, various cereal-based by-products as well as cheaper cereal flours were tested for their ability to support the growth and production of RNase by A. candidus M16a. These materials were used in liquid medium in submerged culture and also some of them were tested in surface culture method.

Effect of different cereal-based substrates:- The various substances tested were corn flour, ragi flour, hot water extracts of ragi husk, wheat bran and rice bran. They were incorporated into the medium either individually or in various combinations and also with or without fortification with minerals or groundnut meal. The different combinations and concentrations used and the results of the experiments are given in Table 14.

It could be noticed that when these substrates were used individually, rice bran extract (10%), malted and dehusked ragi flour (5%) and wheat bran extract (10%) promoted the enzyme formation considerably; 169, 130 and 100 units/ml respectively. A combination of 5% rice bran extract and 5% wheat bran showed an activity of 136 units/ml. Enzyme

Table - 14

Effect of complex media on enzyme formation by submerged fermentation

Sl. No.	Media composition	Final pH	Biomass (mg/50ml)	RNase activity (units/ml)
1	Corn flour (5%)	5.4	976*	21.6
2	Malted and dehusked Ragi flour (5%)	6.1	695*	130.0
3	Corn flour (2.5%) + malted and dehusked Ragi flour (2.5%)	6.2	813*	92.8
4	Ragi husk extract (10% in tap water)	7.0	403	68.0
5	Corn flour (5%) + casein (0.3%) + Minerals[a]	7.2	951*	30.0
6	Wheat bran extract (10%)	8.2	478	100.0
7	Rice bran extract (10%)	6.6	499	169.0
8	Wheat bran extract (10%) + Peanut meal (5%)	8.5	1728*	102.0

* biomass + residual substrate.

50 ml of the medium in 500 ml flasks were autoclaved at 15 psi for 20 min, cooled, inoculated with 1 ml of spore suspension, incubated at 30°C for 5 days on a rotary shaker (230 rpm) and harvested the culture filtrate by filtering through Whatman No.1 or No.41 filter papers. Biomass was dried at 80°C (along with the residual substrate, if any) and weighed. RNase assay was carried out by using appropriately diluted culture filtrates following the standardised procedure.

[a] Minerals added was of the following composition:

K_2HPO_4	0.05%
KH_2PO_4	0.05%
$MgSO_4.7H_2O$	0.04%

yields were rather low in the case of corn flour (5%). There was more than 3-fold increase in yield (67.8 units/ml) when corn flour (5%) medium was fortified with minerals. However, fortification with 0.3% casein had no effect. Even the beneficial effect of minerals was suppressed by the addition of casein, i.e. from 68 units to 30 units/ml. Peanut meal (5%) had a similar effect on rice bran extract medium (i.e. from 169 units to 64 units). But in the case of wheat bran extract, however, no effect was observed by the addition of peanut meal. Slight improvement in enzyme yield was observed when defatted peanut powder (5%) was added to the wheat bran extract (10%) medium (i.e. from 100 to 125 units/ml).

The growth of the organism in all these media was found to be considerably high, when compared to the synthetic media which were described earlier.

Effect of different concentration of rice bran extract:- To determine the optimum level of rice bran extract (RBE) as the sole source of nutrients for the production of RNase different concentrations such as 5, 10 and 20% were tested. The results are presented in Table 15. 10% extract of rice bran was found to be optimal as there was no further increase in the enzyme yield with an increase in substrate concentration. From 5% to 10% there was exact doubling in the enzyme yield showing the linear dose effect.

Table - 15

Effect of different concentration of rice bran extract on enzyme production

Sl. No.	Concentration of rice bran extract* (%)	Final pH	Biomass (mg/50 ml)	RNase activity (units/ml)
1	5	6.80	462	92.0
2	10	6.80	781	185.0
3	20	6.95	1054	180.0

* RBE was the sole source of nutrients. 5, 10 and 20 gm rice bran were mixed with 100 ml tap water, boiled for 15 min and filtered through cheese cloth. The rest of the procedure was as before.

Effect of addition of malted and dehusked ragi flour to 5% RBE media:- As already mentioned RBE and ragi flour showed the highest enzyme yield when used separately. In order to find out whether there was any additive effect when these substrates were used in combination, different amounts (0 to 7.5%) of ragi flour was added to the medium containing 5% RBE. The results are tabulated in Table 16.

5% RBE alone showed 104 units/ml activity whereas 5% ragi flour alone showed 78 units/ml, when the ragi flour was added at levels from 1% to 7.5% a steady increase in enzyme yield was observed, i.e. 120, 140, 145, 150, 152 and 171 units/ml when the ragi flour concentrations were 1.0, 2.0, 3.0, 4.0, 5.0 and 7.5% respectively.

Effect of different levels of RBE in the medium containing 2% ragi flour:- This experiment was similar to the earlier one. But in this case the levels of RBE were varied keeping ragi flour concentration constant. The results are given in Table 17.

There was a steady increase in enzyme yield when the RBE concentration was increased. However, the net effect was nil, i.e. effect of combination of 10% RBE with 2% ragi flour was same as that with 10% RBE alone. These two experiments indicate that there was no synergistic or even additive effect with the combination of these two substrates.

Table - 16

Effect of different concentration of malted and desusked Ragi flour

Sl. No.	Ragi flour added* (%)	pH	Biomass (mg/50 ml)	RNase activity (units/ml)
1	0	6.2	486	104.0
2	1.0	6.8	653	120.0
3	2.0	6.4	813	140.0
4	3.0	6.2	928	145.0
5	4.0	6.2	1076	150.0
6	5.0	6.2	1151	152.0
7	7.5	6.2	1464	171.0
8	5.0	6.0	690	78.0

* Culture media (Sl.Nos. 1 to 7) contain 5% rice bran extract.

Table - 17

Effect of addition of rice bran extract to ragi flour medium

Sl. No.	Concentration of rice bran extract* (%)	pH	Biomass (mg/50 ml)	RNase activity (units/ml)
1	0	6.3	405	50.0
2	2.5	6.3	406	80.0
3	5.0	6.4	541	106.8
4	7.5	6.4	648	176.0
5	10.0	6.4	735	184.0
6	10.0	6.8	484	184.0

* The medium (Sl.Nos.1-5) contained 2% malted dehusked ragi flour.

Effect of surface to volume ratio in shake flask experiment:- In submerged fermentation in shake flasks the surface to volume ratio is an important criterion as far as oxygen transfer efficiency is concerned. Hence, to determine the optimum volume of the medium in a vessel of given size, the quantity of medium in a 500 ml capacity Erlenmeyer flask was varied from 25 ml to 200 ml. The medium taken was 10% rice bran extract in water. The flasks were incubated at 30°C on rotary shaker (230 rpm) for 5 days and the medium was filtered. Biomass and enzyme activity were determined.

The results are presented in Table 18. It could be seen that there was a steady increase in biomass formation depending on the volume of the medium. However, the enzyme yield increased only upto 75 ml medium in 500 ml flask. Further increase in the volume tended to decrease the enzyme titre. The optimum level was found to be 50-75 ml medium per 500 ml flask.

Effect of media pH on the enzyme production:- The effect of initial pH was studied in the case of both rice bran extract (10%) medium and glucose-peptone-mineral medium (Medium No.2). The pH from 3.0 to 11.0 was adjusted either with 1 N HCl or 1 N NaOH. After fermentation for 5 days in a rotary shaker (230 rpm) at 30°C, the final pH of the broth, the biomass and the enzyme in CF were determined.

Table - 18

Effect of surface-volume ratio on enzyme yield

Sl. No.	Volume of medium per 500 ml flask	Ratio of surface: volume	Biomass (mg)	RNase activity (units/ml)
1	25	1:20	272	91.2
2	50	1:10	508	112.8
3	75	1:66	714	114.8
4	100	1:5	1023	80.4
5	150	1:3.3	1461	70.4
6	200	1:2.5	1860	57.2

Different volumes of 10% rice bran extract were taken in 500 ml capacity Erlenmeyer flasks. Sterilised, cooled and inoculated with spore suspension the amount of which was correspondingly varied so as to supply 1 ml of inoculum per 50 ml medium. Other details were as in Table 14.

The results of experiments on the rice bran extract medium are recorded in Table 19. It could be seen that the organism was capable of growing well within this wide range of pH values and there was no significant variation in the amounts of biomass formed. However, the maximum growth was observed between pH 5.5 and 7.5. Maximum enzyme activity was observed when the initial culture pH was between 4.0 and 5.0. Then it showed a drop in enzyme titre upto pH 6.0. Again from pH 6.5 the activity increased and remained almost constant upto pH 8.5. Then it showed a steady fall upto pH 11.0. The enzyme formed at pH 3.0 and 11.0 was negligible, even though the growth was fairly good.

The data on the effect of pH on growth and enzyme formation when A. candidus M16a was grown on a semi-synthetic medium containing glucose, peptone and minerals are presented in Fig. 4 and Table 20. The organism was found to be capable of growing over a wide range of pH from 3.0 to 11. However, the growth was very good from pH 5.0 to 10.0. Within this range of pH values the biomass formed per millilitre medium was 14-16 mg. The pattern of enzyme formation at different pH values was rather interesting to observe. There seems to be two optimal pH values for the formation of enzyme, around 6.3 and 9.3, thereafter there was a steep fall and at pH 11 there was little enzyme formed. It is also interesting to observe the pattern of final pH of the broth (Table 20). It could be seen that the final pH of

Table - 19

Effect of initial media pH on the RNase production in rice bran extract medium

Initial pH of the medium	Final pH of the medium	Biomass (mg/50 ml)	Enzyme activity (units/ml)
3.0	5.0	364.0	6.35
4.0	6.0	376.5	110.00
5.0	6.4	381.5	136.00
5.5	6.7	398.0	90.00
6.0	7.2	392.5	66.00
6.5	7.3	393.5	84.00
7.0	7.4	407.0	88.00
7.5	7.5	401.0	86.00
8.0	8.3	382.0	85.00
8.5	8.5	380.0	78.00
9.0	8.5	346.0	64.00
9.5	8.7	353.0	62.00
10.0	8.8	378.5	40.00
11.0	9.0	370.5	6.05

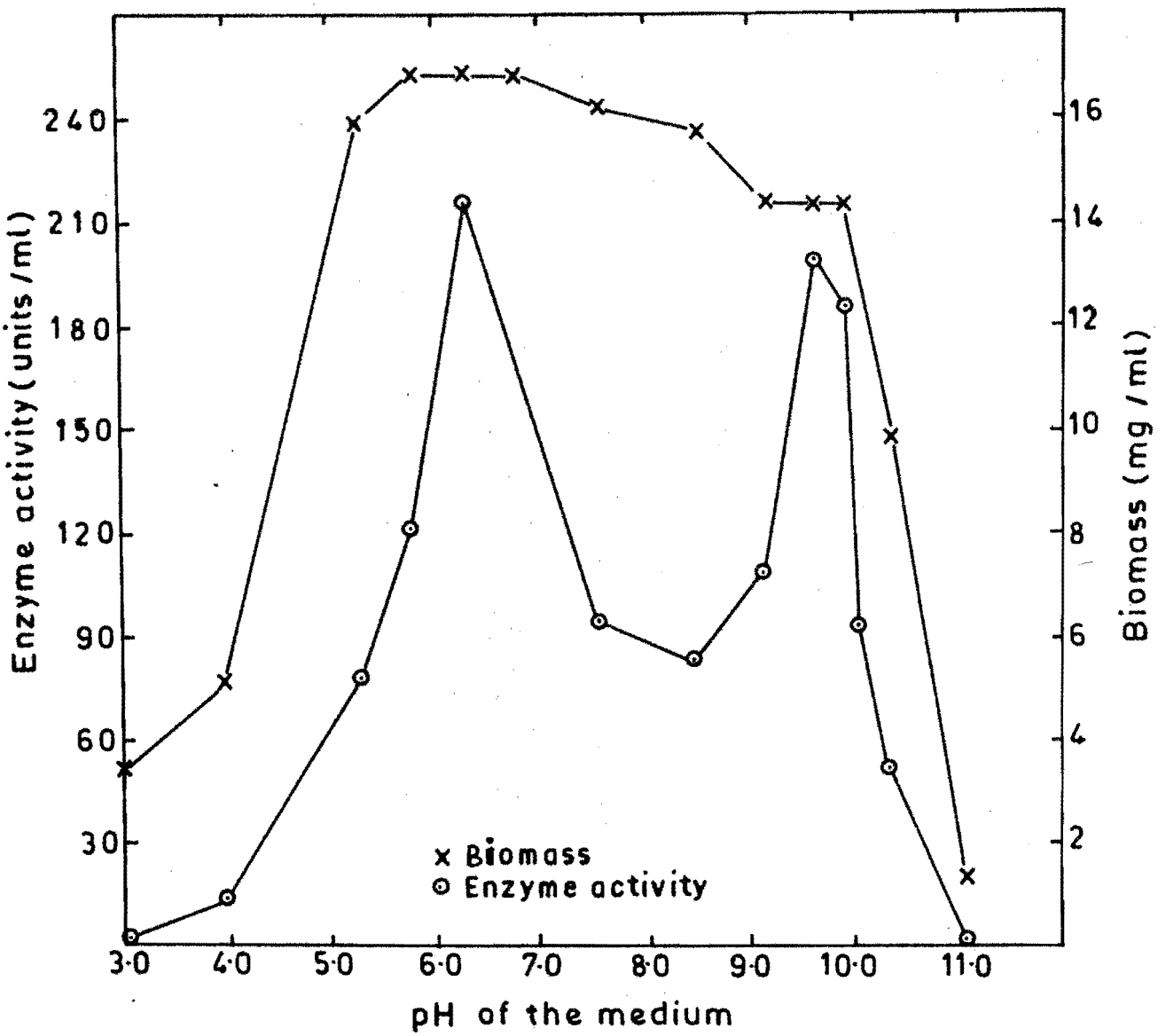

Fig. 4. Effect of the initial pH of the cultivation medium (Medium 2) on RNase and biomass production by A. candidus M16a.

Table - 20

Effect of initial media pH on the RNase formation in Medium 2 (Glucose, peptone, minerals)

Initial media pH	Final pH of the broth	Biomass (mg/50 ml)	Enzyme activity (units/ml)
3.0	3.2	173.5	0.75
4.0	3.7	257.5	13.75
5.3	4.6	663.0	87.50
5.8	4.9	636.0	122.00
6.3	5.3	657.0	216.00
7.6	6.3	685.5	95.00
8.5	6.5	781.5	83.50
9.1	6.7	715.5	110.00
9.6	5.3	718.5	195.00
9.9	5.6	719.5	186.00
10.1	5.7	744.0	94.50
10.3	5.8	486.5	52.50
11.1	9.6	93.5	2.00
11.6	9.8	86.0	0.00

the broth was within stable pH range, i.e. 5.0-6.0 (as will be given later) in the case of those which gave highest enzyme activity, i.e. pH 6.3 as well as 9.6. The fall in activity after the peak value at pH 6.3 could be very well explained by the finding that the final pH of the broth in the case of those which were grown at pH values 7.6-9.0 were between 6.0 and 6.5 at which enzyme is not very stable. The RNases which were produced at pH 6.3 as well as at pH 9.6 seems to be same by the criteria of their temperature and pH optima. But it cannot be assumed to be the same enzyme unless a detailed study is carried out. Nevertheless, it could be said that the culture A. candidus M16a is capable of producing potent RNase optimally at two different pH values.

B. SURFACE CULTURE OR SEMI-SOLID FERMENTATION

The substrates tested were wheat bran, rice bran and ragi husk. They were used alone or in combination as given in Table 21.

To 10 gms of the substrate in 250 ml Erlenmeyer flasks 6 ml of trace mineral solution prepared in 0.2 N HCl was added and thoroughly mixed. The flasks were autoclaved at 15 psi for 30 min, cooled and inoculated with 1 ml of spore suspension. They were then incubated at room temperature for 5 days in a humid chamber. The enzyme (moldy bran extract, MBE) was extracted in 50 ml of 0.05 M acetate buffer (pH 5.5), keeping for 2 hrs with intermittent stirring, and pressing through a cheese cloth. The MBE was further clarified by centrifugation (to remove spores and other suspended particles).

The effect of different moistening agents such as distilled water, tap water, 0.2 N HCl, trace mineral solution in distilled water or trace minerals in 0.2 N HCl (trace mineral composition as given in Table 21) was tested. 6 ml of these were added to 10 gm rice bran and mixed thoroughly. Other details were as above.

For studying the effect of different moisture levels 2,4,6,8 and 10 ml of mineral solution in 0.2 N HCl was added to 10 gm rice bran.

Production of RNase in trays by surface cultivation:

For large scale production of the enzyme 1.2 kg of rice bran was taken and 960 ml of tap water was added and mixed thoroughly (Medium 9). This was loosely spread on a perforated aluminium tray (1.5" x 2' x 4') and autoclaved for 45 min at 15 psi. Each tray was inoculated with seed culture (moldy bran) prepared by growing the organism for 6 days on 125 gms of rice bran as described in the previous section. The seed culture was thoroughly mixed asceptically by hand mixing and the bran was spreaded loosely on the tray again and incubated in a humidifier at room temperature and at 90% relative humidity. Temperature changes were recorded during the growth. Samples were drawn daily for determination of moisture and enzyme activity.

Study of different substrates for RNase production:

The details of cultivation in flasks and extraction of crude enzyme were as above. The substrates used were wheat bran, rice bran and ragi husk. They were used individually or in combination.

The results are presented in Table 21. All the three substrates were found to be good for enzyme production. The yield of enzyme when grown on different substrates were in the order: rice bran > wheat bran > ragi husk. None of the combinations showed any additive effect. The specific

Table - 21

Production of RNase by solid-state fermentation using various semi-solid media

Sl. No.	Media composition*	Enzyme activity units/ml of MBE	Protein in MBE mg/ml	Specific activity units/mg protein
1	Wheat bran	674	8.04	83.83
2	Rice bran	739	5.70	129.65
3	Ragi husk	642	3.24	198.15
4	W.B. + R.B.	708	6.90	102.61
5	W.B. + R.H.	606	4.95	122.42
6	R.B. + R.H.	700	5.04	138.89
7	W.B. + R.B. + R.H.	666	5.82	114.43

*Wheat bran, rice bran or ragi husk alone (10 g), a combination of two substrates with 5 g each or a combination of three substrates with 3.3 g each placed in 250 ml conical flasks, 6 ml trace mineral solution added, mixed, autoclaved at 15 psi for 30 min, cooled, inoculated with 1 ml spore suspension, mixed and incubated for 5 days at 25°C (The composition of the trace mineral solution was: in mg $CuSO_4.5H_2O$-0.7, $ZnSO_4.7H_2O$-0.7 and $FeSO_4.7H_2O$-0.07 in 1000 ml of 0.2 N HCl). Enzyme was extracted from moldy bran by addition of 50 ml of 50 mM acetate buffer (pH 5.5) for 2 hrs, filtering through cheese cloth, centrifuged and volume made up to 50 ml.

activity was the highest (198 units) in the case of ragi husk when compared with rice bran (130 units) and wheat bran (84 units). However, the total enzyme yield was highest in the case of rice bran. Hence, rice bran was selected as a substrate for further studies.

Effect of different moistening agents on RNase production:

Rice bran was moistened by wetting agents (6 ml) and the rest of the procedure was as detailed in Table 22.

The results obtained are shown in Table 22. The quantity of enzyme found ranged from 960 units/ml in the case of 0.2 N HCl and distilled water containing trace elements to 1002 units/ml in the case of 0.2 N HCl containing trace elements. However the specific activity was low in the latter case (198 units) in comparison with the one with tap water (243 units), which was the highest observed. The specific activity in other cases was almost same (194-206 units). Hence in further experiments tap water was used for moistening the rice bran.

Effect of temperature on enzyme production by surface cultivation:

The semi-solid rice bran medium was prepared as in the previous cases. The inoculated flasks were incubated at different temperatures viz. 20°, ambient temperature (24-27°C), 30°, 35° and 40°C in humid chambers for 5 days and enzyme assayed as before. The results are tabulated

Table - 22

Effect of different moistening agents on RNase production by solid state fermentation

Sl. No.	Moistening agent	Enzyme activity (units/ml of MRBE)	Protein in MRBE (mg/ml)	Specific activity (Units/mg protein)
1	Distilled water	976	4.875	200.21
2	Tap water	984	4.050	242.96
3	0.2 N Hydrochloric acid	960	4.650	206.45
4	Distilled water containing trace elements*	960	4.950	193.94
5	0.2 N HCl containing trace elements*	1002	5.050	198.42

* Trace elements composition and other details are as in the previous table.

6 ml of the different moistening agents was added to 10 g of rice bran.

in Table 23. Maximum enzyme yield was obtained at ambient temperature. At temperatures higher than this there was a steady decrease in enzyme yield. The maximum specific activity was observed in the case of 35°C. However, the total enzyme yield was low compared to ambient temperature.

Effect of moisture levels of rice bran semi-solid medium on the enzyme production:

To 10 gm rice bran different amounts of tap water (2 ml through 10 ml) were added and mixed thoroughly. Rest of the procedure was as in the previous experiments.

The results are given in Table 24. A steady increase in enzyme yield (from 970 units to 1227 units) could be observed when the moisture levels were increased from 2 ml to 8 ml/10 gm rice bran. When 10 ml water was added to 10 gm bran there was a slight fall in enzyme yield. A steady increase in specific activity also was observed in the same manner. Total enzyme yield as well as specific activity were maximum when 8 ml water was added to 10 gm bran with 13% moisture. This level worked out to give a moisture of the medium of approximately 50% after autoclaving.

Time course of production of RNase (surface fermentation):

Enzyme formation in rice bran semi-solid medium by A. candidus M16a was followed upto 6-7 days. The experiment was conducted in flasks (10 gm rice bran + 8 ml tap water in 250 ml conical flasks) as well as in large trays. The

Table - 23

Effect of temperature on enzyme production by surface fermentation

Sl. No.	Temperature °C	RNase activity (units/ml MRBE)	Protein (mg/ml MRBE)	Specific activity
1	20	540	3.42	157.9
2	Ambient temp. (24-27)	980	3.24	302.5
3	30	873	3.02	256.0
4	35	684	2.03	336.9
5	40	308	2.00	154.0

Table - 24

Effect of moisture of the rice bran semi-solid medium on enzyme production

Sl. No.	Tap water added to rice bran (ml/10 g)	RNase activity (units/ml of CRBE)	Protein in the CRBE (mg/ml)	Specific activity (units/mg protein)
1	2	970.0	3.65	265.6
2	4	1015.0	3.45	294.2
3	6	1037.6	3.40	305.0
4	8	1226.6	3.40	367.6
5	10	1140.0	3.40	335.3

experimental details were as above.

The results are given in Tables 25 and 26. In flask experiments highest specific activity was obtained at 96 hr fermentation, although maximum total yield of the enzyme increased with increased time of cultivation (Table 25). In the case of tray fermentation samples were drawn at different intervals viz. 0, 10, 24, 34, 48, 58, 72, 82, 96, 120 and 144 hrs of fermentation and analysed for moisture, enzyme activity, protein content in the MRBE etc. The relevant data are given in Table 26. The activity observed at 0 hr of fermentation (14 units/ml) was the activity present in the inoculum added. Maximum total yield of the enzyme and highest specific activity were observed at 82nd hour of fermentation. With further increase in duration there was a fall in both enzyme yield and specific activity.

It could also be observed that the yield and specific activity in the case of large scale operations were less than in the case of flask experiments.

Table - 25

Time Course of enzyme formation by surface fermentation in flasks

Sl. No.	Period of incubation (hr)	RNase (Units/ml MRBE)	Protein (mg/ml MRBE)	Specific activity (units/mg protein)
1	24	8.0	1.251	6.39
2	38	465.0	2.935	158.71
3	48	638.0	1.915	327.98
4	61	1133.5	1.900	596.57
5	72	1330.0	2.090	636.79
6	96	1353.0	1.710	792.39
7	120	1245.0	2.248	553.82
8	144	1362.0	2.780	489.92
9	168	1371.0	2.920	469.52

Rice bran was used as the substrate.

Table - 26

Time course of RNase formation in tray fermentation on rice bran semi-solid medium

Period of growth (hr)	Moisture of MRBE (%)	Enzyme activity (units/ml)	Enzyme yield units/g moldy bran (dry wt)	Specific activity (units/ mg protein)
0	49.0	14	137	12
10	49.8	35	349	24
24	46.5	262	2449	158
34	44.5	525	4730	370
48	-	551	-	314
58	43.8	763	6790	400
72	43.8	810	7473	412
82	41.8	920	7897	451
96	42.6	600	6525	350
120	41.4	675	5763	330
144	39.2	540	4441	265

DISCUSSION

Screening of a number of microorganisms is an important prerequisite when one aims at selecting a potent culture for the production of any microbial product. It has been observed that the ability to produce a particular enzyme or a secondary metabolite is restricted to a particular group of microorganisms. For example, it has been shown that the enzyme glucose isomerase is produced mostly by actinomycetes (Kowser, 1982). However, the capacity to produce this enzyme is not confined to any particular genus or species. Much variation in the enzyme productivity has been shown among strains of the same species. But in a majority of cases, particularly in the case of other enzymes, a wide variety of microorganisms belonging to a number of different species and genera has been shown to be capable of elaborating the same enzyme. Apparently it is the strain rather than the species or genera that is important in selecting an organism for industrial productions and all microorganisms have a basic capacity to produce enzymes (Davies, 1963).

In the case of RNases also it has been found that a wide variety of microorganisms are capable of producing this enzyme extracellularly (Barnard, 1969; Egami and Nakamura, 1969; Ogata et al., 1976). Marked variations have

also been observed among strains of the same species in their ability to produce this enzyme (Ogata *et al.*, 1976; Egami and Nakamura, 1969).

Hence, in the present study, 60 different fungal cultures isolated or procured from various sources were screened for their ability to produce an extracellular RNase. Majority of the cultures were found capable of excreting RNase to varying amounts (Table 5). Among the good producers of RNase, *Aspergilli* were found to be numerous than others perhaps because more *Aspergillus* strains were screened, 27 out of 60. The best RNase producer, isolate M16a was later identified to belong to *A. candidus*. However, many other fungal strains belonging to different genera are capable of producing RNase, as reported in literature.

Although much information on individual RNases produced by different organisms is available, reports on screening programme are rather scanty in literature. Ogata *et al.* (1963) examined a number of fungal and bacterial cultures for their ability to degrade RNA into 5'-nucleotides. It was shown by them that 22 fungal strains, 9 *Streptomyces*, 2 *Bacilli* and a *Pseudomonad* were capable of excreting 5'-P-forming RNases. Of the 22 fungal cultures, 8 were *Aspergillii*. They were *A. elegans*, *A. fischeri*, *A. flavipes*, *A. melleus*, *A. nidulans*, *A. quercinus*,

A. sulphureus and A. ustus. Hasegawa et al. (1964) screened 300 strains of moulds for their RNase activity and strains belonging to Pellicularia, Rhizoctonia and Phaeoisariopsis were found to degrade RNA into 5'-nucleotides. Tone and Ozaki (1968) screened 960 strains of plant pathogenic imperfect fungi and selected 48 strains capable of degrading RNA into 5'-nucleotides out of which Phoma cucurbitaciarum was the most potent one.

Of a number of microorganisms screened by Nakao and Ogata (1963), 34 fungal strains were found to be elaborating RNases that formed 3'-mononucleotides by RNA degradation. This included 2 species of Aspergillus also. They were A. fonsecaeus and A. flavus. They also showed that 18 microorganisms belonging to different groups were able to degrade RNA partially to form 3'-oligonucleotides. 32 Penicillium strains were tested for their ability to produce P_1 type nuclease (nuclease produced by P. citrinum), which hydrolyses RNA and heat denatured DNA completely into 5'-mononucleotides and also shows 3'-nucleotidase activity (Fujimoto et al., 1977). P. expansum, P. notatum, P. steckii and P. meleagrinum etc. showed this activity. In addition, many strains of Penicillium produced base-non-specific RNase forming 3'-mononucleotides via 2',3'-cyclic nucleotides.

Besides these few reports on screening, there is not much information available in literature on this aspect. Though many Aspergillii are reported to elaborate RNase, there is no report on A. candidus producing a potent extracellular RNase. The present work seems to be the first detailed study on the RNase produced by A. candidus.

Information on the nutritional requirements and cultural conditions for the production of RNases by different cultures is rather patchy. Most of the well-studied microbial RNases were isolated from other products such as digestive enzyme preparations etc. For example, RNases T_1 and T_2 were purified from a commercial digestive aid, 'Takadiastase' a product of A. oryzae, RNase Rh from 'Gluczyme' of Rhizopus niveus and RNases M and Ms from a digestive enzyme preparation, 'Molsin' of A. saitoi. All these enzyme preparations were produced by growing the organisms on a wheat bran medium. Information available regarding the media composition and cultural conditions for the formation of RNases by some of the cultures are briefly discussed below for comparison with the present work. In the present work, a fairly detailed study was carried out on the nutritional requirements and environmental factors in order to standardize conditions for the maximal production of RNase by using A. candidus M16a. The reasons for taking up such a study was (a) lack of sufficient information in the literature on this aspect, and (b) the requirement of nutritional and cultural condition usually vary from strain to strain.

Surface cultivation on wheat bran has been shown to lead to a better production of nucleolytic enzymes and has been considered to be more economical. Solid culture of P. citrinum on wheat bran has been shown to yield nuclease P_1 more than seven times of that formed in shake flasks (Kuninaka et al., 1961). Tone and Ozaki (1968) also have grown the culture Phoma cucurbitacearum in a wheat bran solid medium for the production of a 5'-nucleotide forming RNase. In the present case, however, surface culture on rice bran was found to be the best method for the maximum production of RNase, although wheat bran also showed fairly good yield. Moreover, surface cultivation on rice bran gave much higher yields than that of a liquid medium. Wide variations in the enzyme yield were observed even when the culture was grown on the same medium at similar conditions.

Different carbon substrates have been reported to be very good carbon sources for the production of RNase. Among a number of carbon sources tested xylose, glucose or molasses (at 3-10% level) was shown to be effective carbon sources for the production of nuclease P_1 by P. citrinum (Kuninaka et al., 1961). Sucrose (15%) was the best carbon source for optimal yields of an RNase from A. niger NRL.A-1-233 (Horitsu et al., 1974). RNase U_1, a guanylo-ribonuclease was produced extracellularly by growing U. sphaerogena on a medium containing RNA as the sole carbon

source (Glitz and Dekker, 1964). The medium used by Arima et al. (1968) for the production of RNases U_1, U_2, U_3 and U_4 contained glucose as the carbon source. However, in the present case starch (1.5%) was found to be the best carbon source.

Similarly, there is wide variations among the nitrogen sources for the optimal yields of different nucleolytic enzymes. NH_4NO_3 (0.2%) was the best nitrogen source among NH_4NO_3, NH_4Cl and peptone for the production of RNase by A. niger NRL.A-1-233 (Horitsu et al., 1974). However, there are instances where complex nitrogen sources such as peptone and casein were found much better sources than NH_4NO_3, NH_4Cl or $(NH_4)_2SO_4$ as N source for Nuclease P_1 production by P. citrinum (Kuninaka et al., 1961 ; the present study). In our study the yield of RNase was 10 times more in a medium containing complex N source than that in medium containing NH_4NO_3 as N source. The medium used by Arima et al. (1968) for the production of RNase U_1, U_2, U_3 and U_4 contained glycine as the N source. Glitz and Dekker's medium for the production of RNase U_1 by U. sphaerogena contained 0.05% NH_4Cl as a N source besides 0.5% RNA and 0.01% yeast extract (Glitz and Dekker, 1964). For the production of RNase U_2 by the same culture, Minato and Hirai (1979) used a medium containing $(NH_4)_2SO_4$ (0.8%) and urea (0.1%) as N sources. In the present case, NH_4NO_3 and casein or peptone were found to be the best inorganic and organic N sources, respectively.

Temperature is a critical factor which controls the growth and production of enzymes by microorganisms and the optimum temperature for production of enzyme varies with the microorganisms (Davies, 1963). P. cucurbitacearum was shown to give maximum yields of RNase on wheat bran medium when grown at 25°C (Tone and Ozaki, 1968). For the production of Nuclease P_1 in a liquid medium (glucose-peptone-minerals), P. citrinum was grown at 30°C (Kuninaka et al., 1961) whereas the same culture was grown on wheat bran at 30°C for the initial 24 hr and then at 20°C for 96 hr (Fujimoto et al., 1974a). U. sphaerogena was grown at 30°C for the production of RNase U_1 (Glitz and Dekker, 1964) and at 29°C in a different medium for the production of RNase U_2 (Minato and Hirai, 1979). In the present case, ambient temperature (24°-27°C) was found to be optimal for the RNase production when A. candidus M16a was grown on rice bran solid medium.

Hydrogen ion concentration of the medium is another important factor which controls the growth and production of any metabolite by microorganisms. Kuninaka et al. (1961) observed that the production of Nuclease P_1 was drastically affected when the pH of the medium was changed from the optimum value of 5.2; at pH 4.8 and 8.6 the enzyme yield was very low. In other organisms, the initial pH requirement for optimum RNase production varied widely e.g. 2.2 for A. niger NRL.A-1-233 (Horitsu et al., 1974) and

7.0 in the case of S. erythreus (Tanaka, 1961). In the present case the organism, A. candidus M16a was found capable of growing and producing the enzyme at a wide range of pH values (Tables 25 and 26 and Fig. 4). It produced a fairly good amount of enzyme in RBE medium within initial pH of the medium from 4.0 to 10.0, although maximum production was at pH 5.0. However, pH-enzyme formation profile in the case of glucose-peptone-mineral medium grown cultures was slightly different. In this medium maximum enzyme yield was obtained at two different initial pHs viz., 6.3 and 9.6 (Fig. 4). This is a rather interesting observation. It could be seen that the final pH of the broth in both these cases where maximum activity was observed was 5.3. When the final pH was below 5.0 or above 6.0 there was a drop in the activity. From this finding it could be speculated that the final pH of the culture broth may be responsible for the stability of the enzyme. Wherever higher enzyme activity was observed the final pH of the medium was within the stable pH range (5-6), irrespective of the initial pH of the medium.

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CHAPTER - 2

STUDIES ON THE CRUDE ENZYME PREPARATIONS OF *ASPERGILLUS CANDIDUS* M16a

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RNases from different sources are known to differ widely in their properties (Josefsson and Lagerstedt, 1962; Barnard, 1969; Egami and Nakamura, 1969; Kuninaka, 1976). As the enzyme studied in the present case is from a new source some preliminary studies on the crude enzyme were conducted. These studies led to some interesting results, particularly the role played by a contaminating protease. The results of these studies and of attempts to eliminate the protease contaminant are presented here.

Effect of pH on the RNase activity: In this study crude enzymes obtained by submerged fermentation on Medium 2 (CF) as well as by surface cultivation on rice bran (MRBE) were used. The pH of the reaction mixture was varied from 3.0 to 8.0 by using 0.5 M buffers (sodium acetate, pH 3.0-5.5; and potassium phosphate, pH 5.5-8.0). Activity of the enzyme was estimated.

It was found that the optimum pH for RNase in both the crude preparations was 4.5 (Fig. 5).

Effect of Temperature: The effect of temperature on the reaction velocity of the RNase in the crude enzymes was studied in the temperature range of 30 to 70°C. It could be seen from Fig. 6 that the temperature optimum for RNase was 55°C. This was the optimum temperature in both the cases, i.e. CF and MRBE.

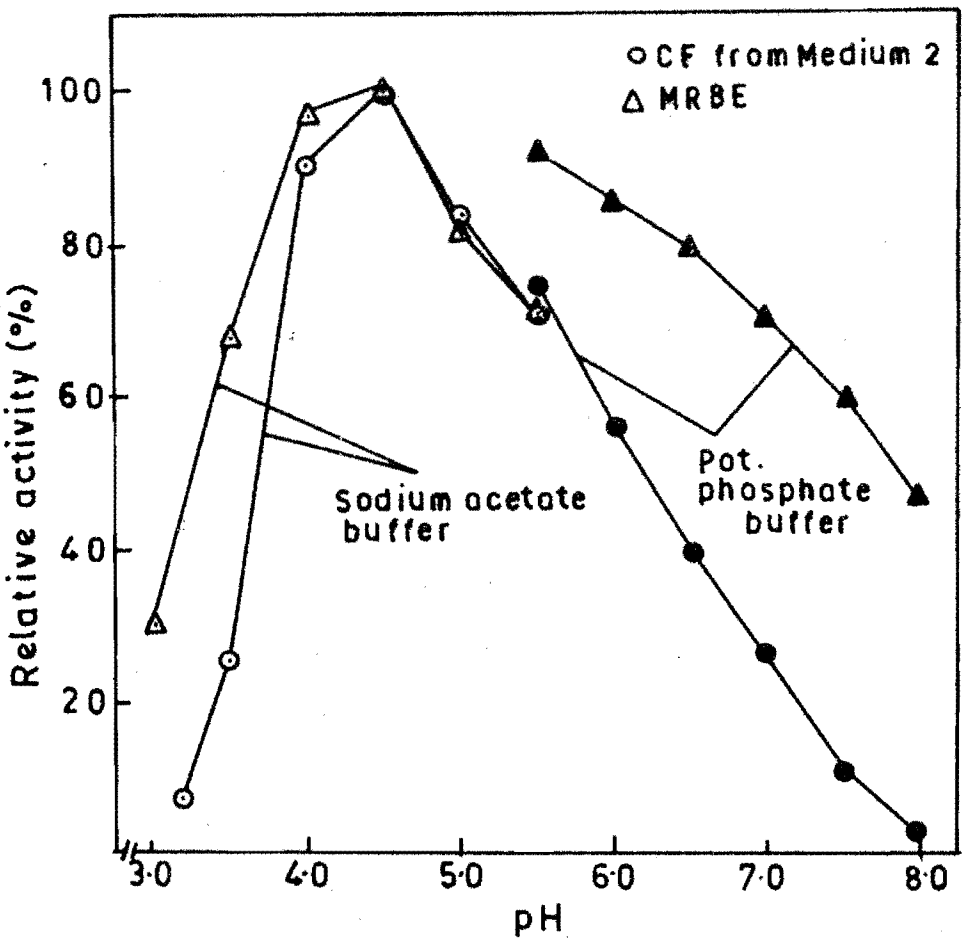

Fig. 5. Effect of pH on the RNase activity of the crude enzyme preparations.

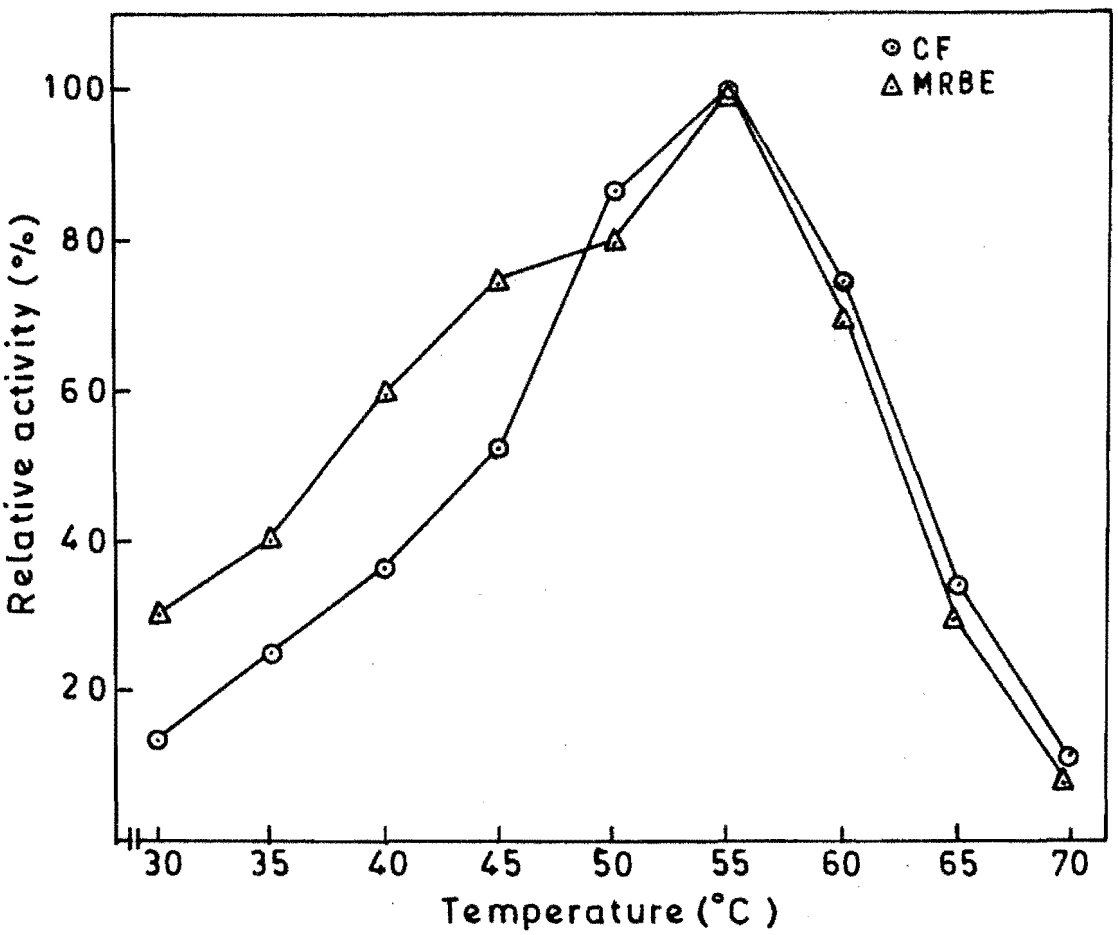

Fig. 6. Effect of temperature on the RNase activity of the crude enzyme preparations.

Substrate concentration and time course of the reaction: The effect of RNA concentration on the reaction velocity of the enzyme was studied by using three different concentrations (0.125, 0.25 and 0.5% final concentration). The reaction was allowed to continue upto 60 min. Samples were drawn at different intervals, the reaction stopped by addition of uranyl reagent and the absorbance of the supernatant determined.

The results are plotted in Fig. 7. It could be observed that the reaction was linear upto 30 min. incubation at 0.25% RNA as the substrate.

Enzyme concentration: The quantities of the crude enzyme protein added was varied from 1 µg to 20 µg in the reaction mixture (2 ml) which contained 0.25% RNA, 0.1 M sodium acetate buffer. The reaction mixture was incubated at 55°C for 30 min, and the enzyme activity determined.

It could be seen from Fig. 8 that there was a linear increase in reaction velocity with an increase in enzyme concentration upto 10 µg. It is evident that using enzyme levels resulting in any further increase beyond 0.5 OD did not show a linear response. Hence, for a reliable assay with the present system the enzyme has to be diluted to that extent so as to get the OD_{260} values below 0.5.

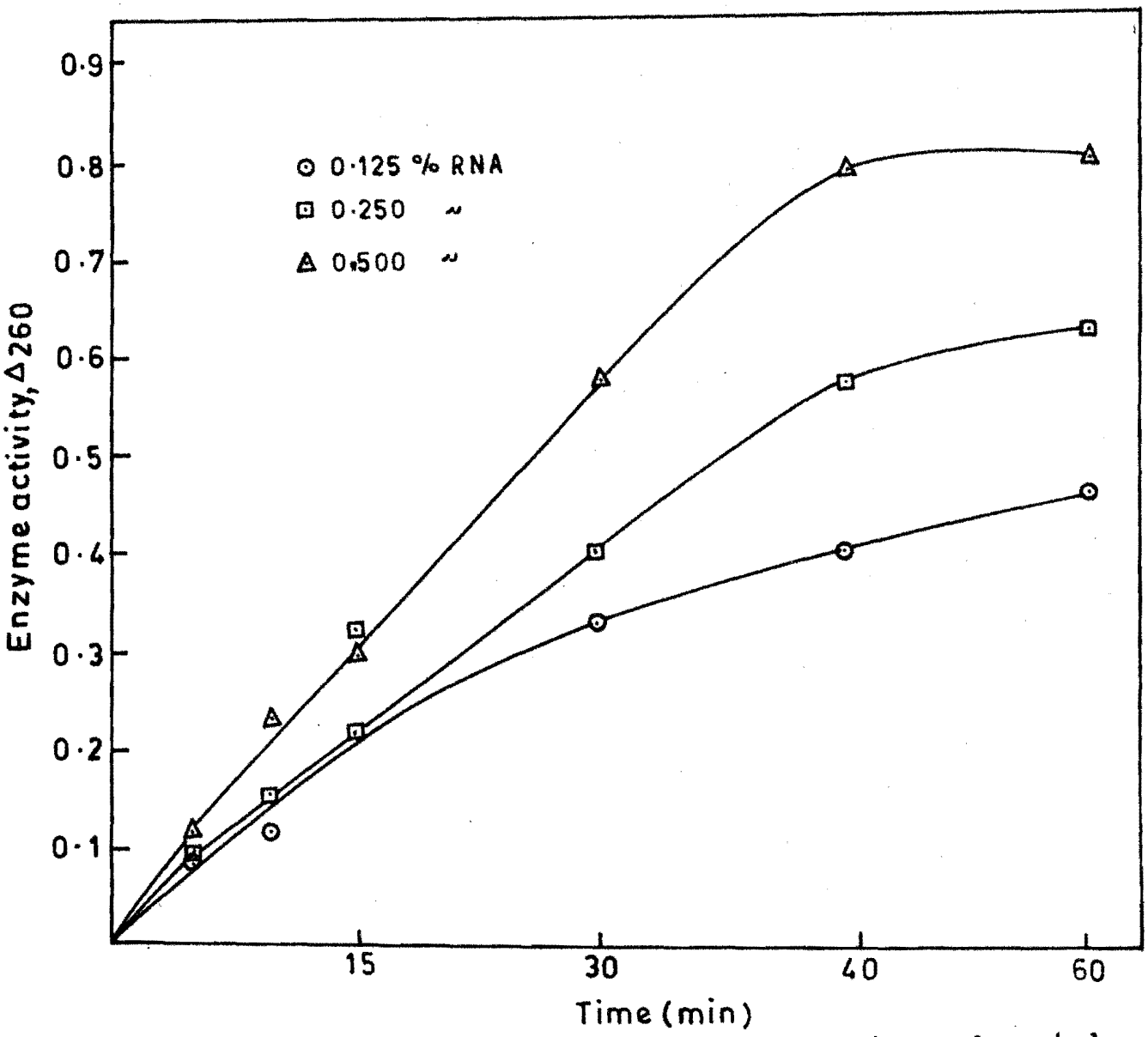

Fig. 7. Effect of substrate (RNA) concentration and period of incubation on the RNase activity of culture filtrate from Medium 2.

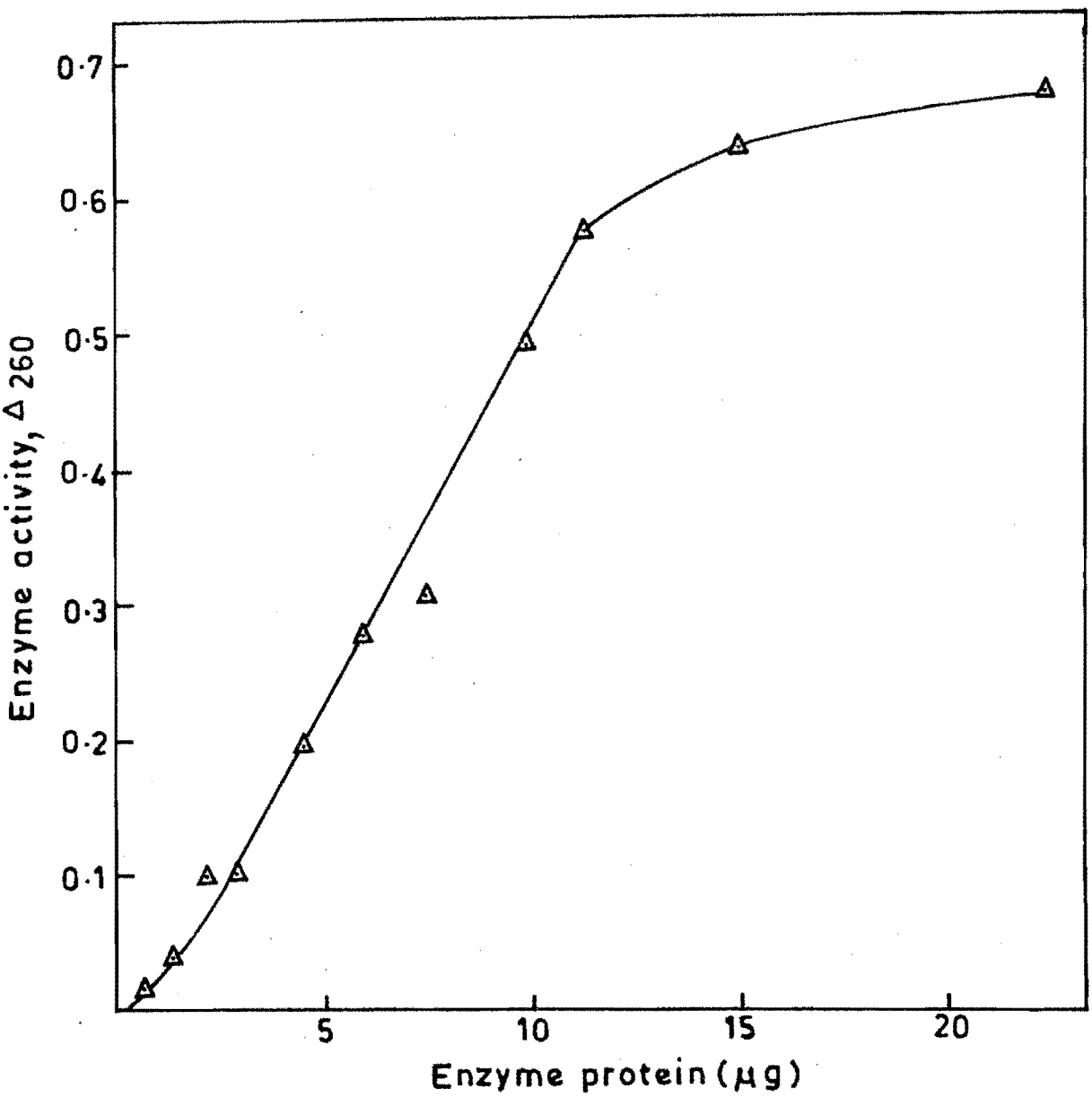

Fig. 8. Enzyme (culture filtrate from Medium 2) concentration Vs. activity. RNA concentration used was 0.25%.

Standardized procedure for RNase assay: From the above findings an assay system was formulated as follows: 2 ml reaction mixture containing 0.5 ml appropriately diluted enzyme, 0.5 ml of 1% RNA and 1 ml of 0.2 M sodium acetate buffer (pH 4.5) was incubated for 30 min. at 55°C and the reaction was stopped by the addition of 2 ml of uranyl reagent (0.25% uranyl acetate in 2.5% perchloric acid). This was incubated at 0°C for 30 min. and the precipitated residual substrate was centrifuged off at 5,000 g for 5 min. The supernatant was diluted 40 times (0.1 ml to 4.0 ml) and the absorbance at 260 nm was measured. This procedure was followed for RNase assay in all further studies.

One unit of enzyme is defined as an increase in absorbance (260 nm) by 0.1 when the assay is carried out under the above conditions. Specific activity is the number of unit enzyme activity per mg protein.

pH stability: The crude enzyme solutions (MRBE as well as CF from Medium 2) were incubated at 60°C for 15 min at different pH values (buffered with 0.1 M sodium acetate, potassium phosphate or citrate-phosphate (McIlvaine, 1921)). The residual RNase activity was then determined by the standard procedure.

The results are presented in Fig. 9. Both the preparations showed similar pH stability. It could be seen

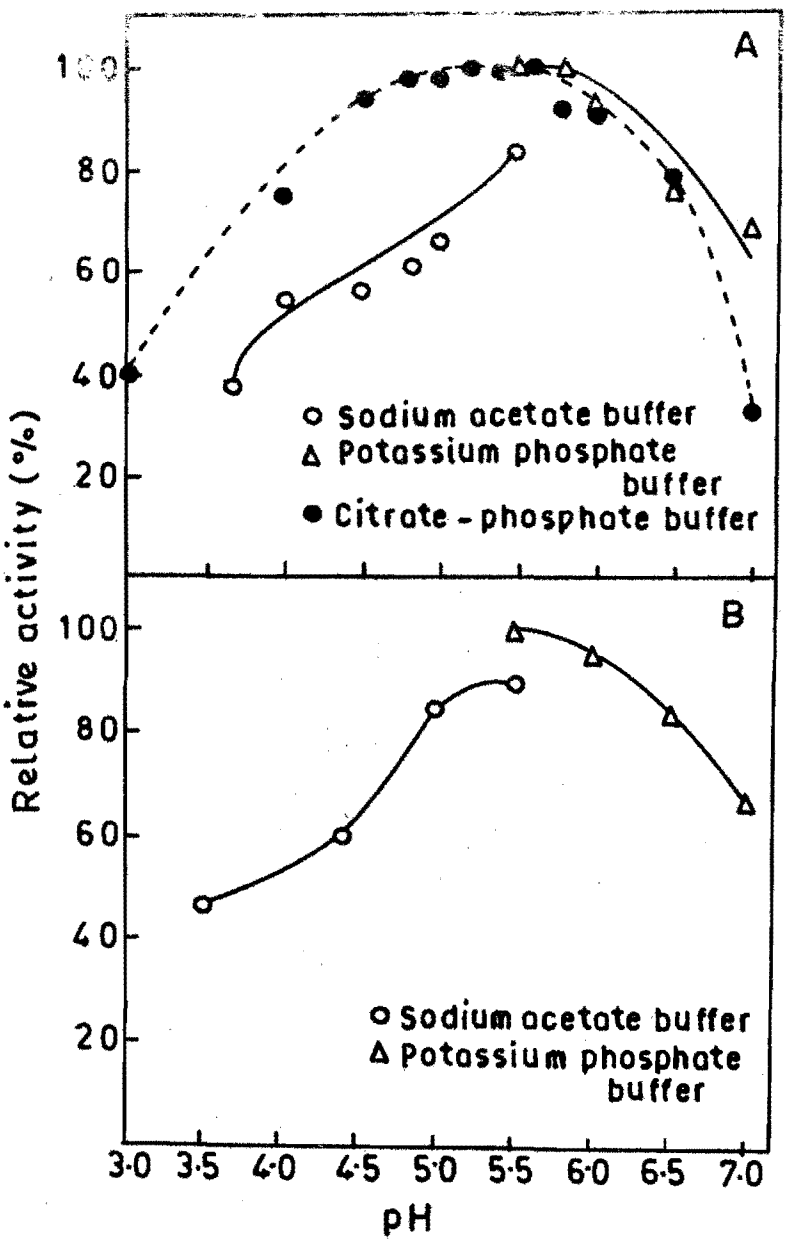

Fig. 9. Effect of pH on the stability of RNase in crude enzyme preparations; MRBE(A) and CF from Medium 2(B).

from the figure that the enzyme was less stable (85-90%) in acetate buffer than in McIlvaine's buffer or potassium phosphate buffer. The enzyme was found to be most stable in phosphate buffer and citrate-phosphate buffer within a narrow pH range of 4.8 to 5.8.

Heat stability of the RNase in crude preparations: The crude enzyme preparations at pH 6.7 were exposed for 10 min. to different temperatures varying from 30° to 97°C, cooled immediately thereafter, and then RNase activity assayed. Results are given in Fig. 10. Practically no loss of activity was observed in samples (CF from Medium 2) pre-incubated at 30 and 40°C and at 50 and 60°C there was a 50% loss, but, unexpectedly, at higher temperatures viz. 70-90°C the loss in activity was much less (only 25%) (Fig. 10A). This unusual pattern was rather puzzling. The higher loss of activity at 50-60°C was presumed to be due to the proteolytic activity. Therefore, the proteolytic activity of the crude enzyme preparations was tested at various temperatures from 30° to 90°C (Fig. 10A) and Fig. 12. In accordance with what was presumed, proteolytic activity was found to be high at 40-60°C. This explained the higher losses of RNase activity when the crude enzyme was pre-incubated at these temperatures. It was also observed that no inactivation of protease occurred when the crude enzyme from Medium 2 was incubated at temperatures upto 55°C (Table 27).

Table - 27

Heat stability of protease in culture filtrate from glucose-peptone-mineral medium.

Pre-incubation temperature (°C)	Protease Residual activity (units/ml)
30	62
55	57
75	6
90	0

The crude enzyme was pre-incubated for 30 min. at different temperatures as indicated and protease activity was assayed according to the procedure described in "Materials and Methods".

The thermal resistance of RNase from moldy rice bran extract (MRBE) was much lower than that from glucose-peptone-mineral medium (Medium 2). On heating for 5 min. at 80°C and pH 6.7 very little activity (3 to 6%) remained in the MRBE whereas in the case of CF from Medium 2 it was about 75%. Various possibilities for this abnormal behaviour were tested. It was initially assumed that the higher stability in the latter case might be due to the different residual media components present in it. Hence, those components viz. $MgSO_4$, $CaCl_2$, glucose and peptone were incorporated into MRBE individually and in combination, heat treated and RNase assay was carried out. Also the involvement of heavy metal ions in the acceleration of heat denaturation of RNase in MRBE was tested by the addition of EDTA. No significant effect of media components was observed. Interestingly, the heat stability was very much improved in the case of EDTA added samples (Table 28). The optimal concentration of EDTA to be added to the crude enzyme (MRBE) was determined and was found it to be 0.4 to 0.5% (Table 29). The results of heat treatments for different periods (1-5 min) of MRBE in the presence of 0.4% EDTA are recorded in Table 30.

From the above findings it could be inferred that the acceleration of heat-inactivation of RNase in MRBE may be due to divalent metal ions present in rice bran. In the presence of EDTA the heat-inactivation pattern of MRBE was

Table - 28

Effect of various media constituents and also of EDTA on the heat stability of the crude RNase enzyme.

Sl. No.	Compound added	Concentration (W/V)	Residual RNase (%)
1	None	-	5.8
2	$MgSO_4.7H_2O$	0.02	7.7
3	$CaCl_2.2H_2O$	0.02	5.3
4	$MgSO_4.7H_2O$ + $CaCl_2.2H_2O$	0.02+0.02	3.7
5	Glucose	0.5	6.7
6	Peptone	0.1	8.6
7	Glucose + Peptone	0.5+0.1	8.1
8	EDTA	0.2	60.9

In all cases 2 ml of MRBE containing different components (pH 5.6) were heat treated at 80°C for 5 min. and immediately cooled in an ice bath. Then the residual RNase activity was assayed.

Table - 29

Effect of different concentration of EDTA on the heat stability of RNase in crude enzyme preparation

EDTA concentration (%)	Residual RNase (%)	Specific activity of the residual RNase (units/mg)
0	5.25	-
0.1	27.43	73.0
0.2	45.71	125.0
0.3	66.29	183.4
0.4	75.43	225.6
0.5	74.51	213.8

2 ml of diluted MRBE containing different amounts of EDTA as indicated above were heated at 80°C for 5 min. and immediately cooled in ice (pH was 5.6). Then the residual RNase activity was assayed.

Table - 30

Effect of the period of heating on RNA stability in the presence of 0.4% EDTA

Period of heating (min)	Residual activity (%)	
	RNase	Protease
1	81.6	27.8
2	80.9	27.8
3	76.5	28.8
4	76.5	28.1
5	73.5	25.0

The enzyme extract (MRBE) (pH 5.6) was heated at 80°C for different periods, cocled immediately and then residual RNase as well as protease activities were assayed.

very much similar to that of CF from Medium 2 (Fig. 10). Although the pattern was similar the retention of activity at higher temperatures was lower in the former than in the latter case. It could also be seen from Fig. 10 that the heat-stability was much lower at pH 6.7 when compared to that at pH 5.6.

Effect of bovine serum albumin (BSA) on the heat-stability of RNase: Addition of an extraneous protein such as BSA to the crude enzyme should reduce the extent of action of the protease on RNase. Hence, to one set of samples BSA was added to 1% level and another set without BSA served as a control; both contained 0.5% EDTA. pH of all samples was 6.7. These were heat-treated at different temperatures and the residual RNase activity was assayed. The results obtained are presented in Fig. 11. It could be seen that the inactivation of RNase was less in the BSA added sample.

Some studies on the protease in the crude enzyme preparations: As it was found that the protease inactivated the RNase in the crude enzyme preparations, it was necessary to have a better understanding about the nature of this contaminating enzyme. Hence, some studies were carried out on this enzyme. MRBE was used for these studies.

pH optimum:- The protease assay was carried out at different pH values from 4.0 to 8.0 which was varied by

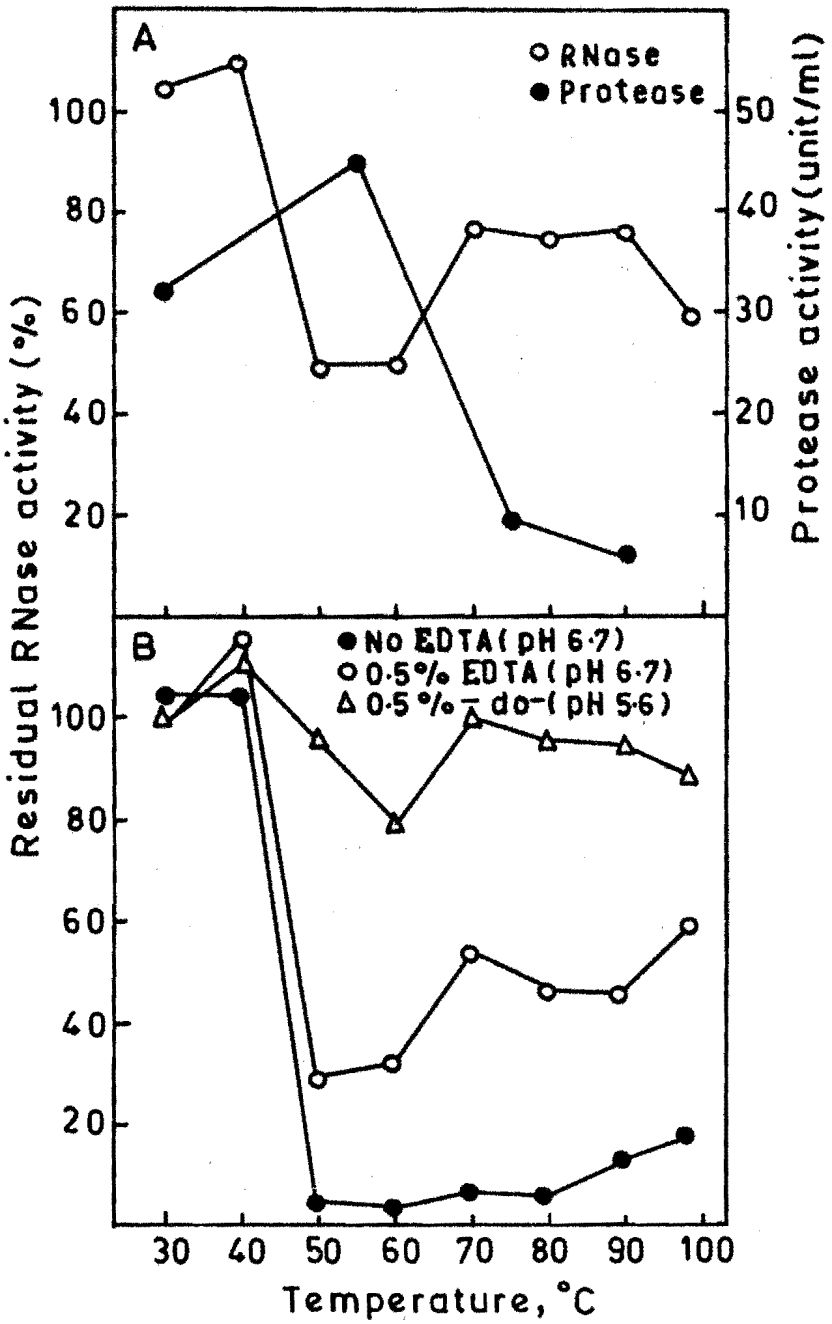

Fig. 10. Heat-stability of the RNase in crude enzyme preparations; CF from Medium 2(A) and MRBE(B).

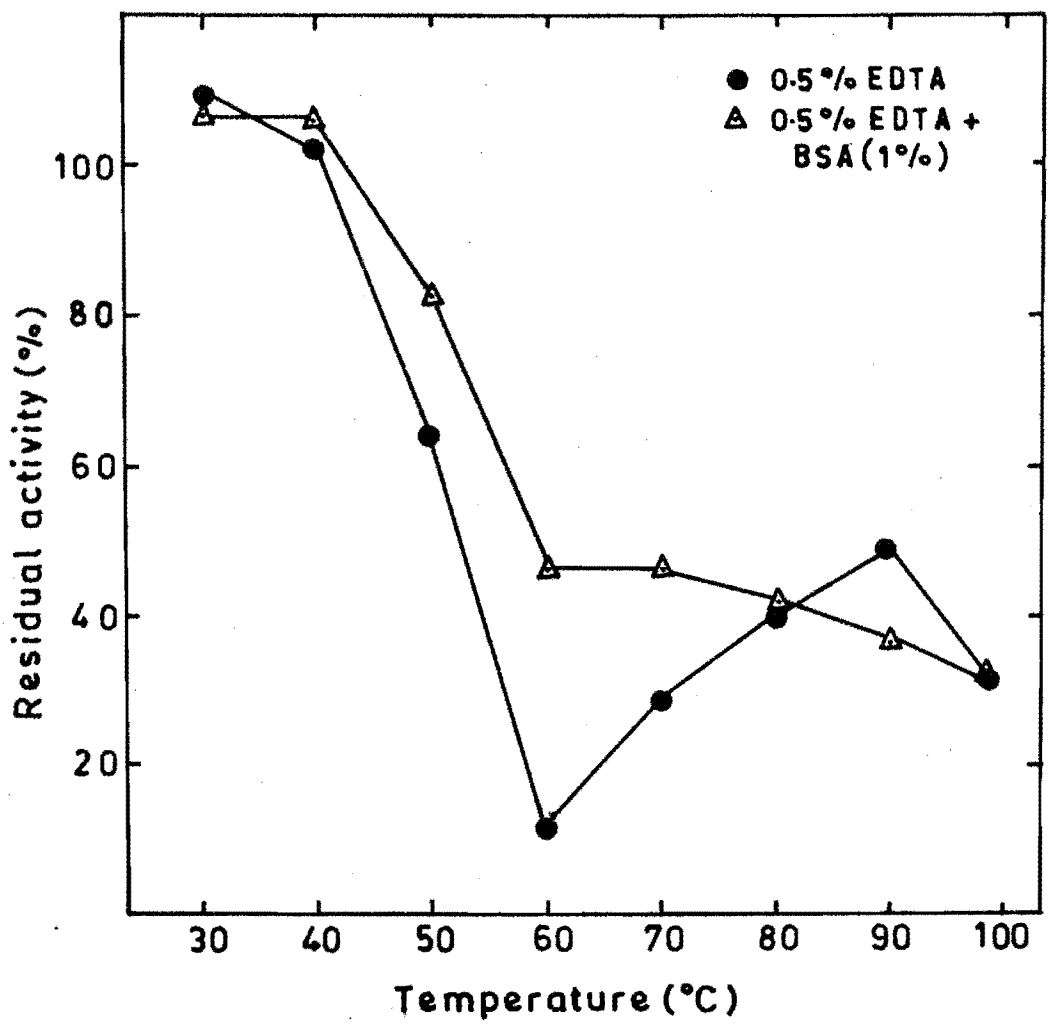

Fig. 11. Heat inactivation pattern of RNase in the crude enzyme (MRBE, pH 6.7) in the presence of 1% bovine serum albumin (BSA).

using 0.5 M acetate or phosphate buffers. The optimum pH for protease activity was found to be 6.0 to 7.0 (Fig. 12).

Temperature optimum:- Protease assay was carried out at different temperatures. The protease was found to be optimally active at temperatures 40-55°C, the maximum activity being at 50°C (Fig. 13).

Heat-stability of protease:- This is an important factor to be considered when specific inactivation of this enzyme is important in view of the use of the crude enzyme in reduction of nucleic acids in SCP. At temperatures above 60°C it was found to be heat-labile. Addition of EDTA enhanced the heat sensitivity. The loss of activity was about 80% at 50°C in the presence of 0.5% EDTA when compared to a loss of only 10% in the absence of it (Fig. 14).

Effect of Di-isopropyl fluoro phosphate (DFP) on protease and RNase activities: To one set of crude enzyme samples DFP was added to a level of 20 mM and kept at room temperature for 30 min. Another set of samples without DFP served as control. All samples contained 0.5% EDTA. All the samples were heat-treated at different temperatures and then residual RNase was assayed. The results are presented in Fig. 15. It could be very clearly seen that the heat-stability of RNase improved very much after DFP treatment when the contaminating protease was completely inactivated. This also indicates that the contaminating protease is a serine-protease.

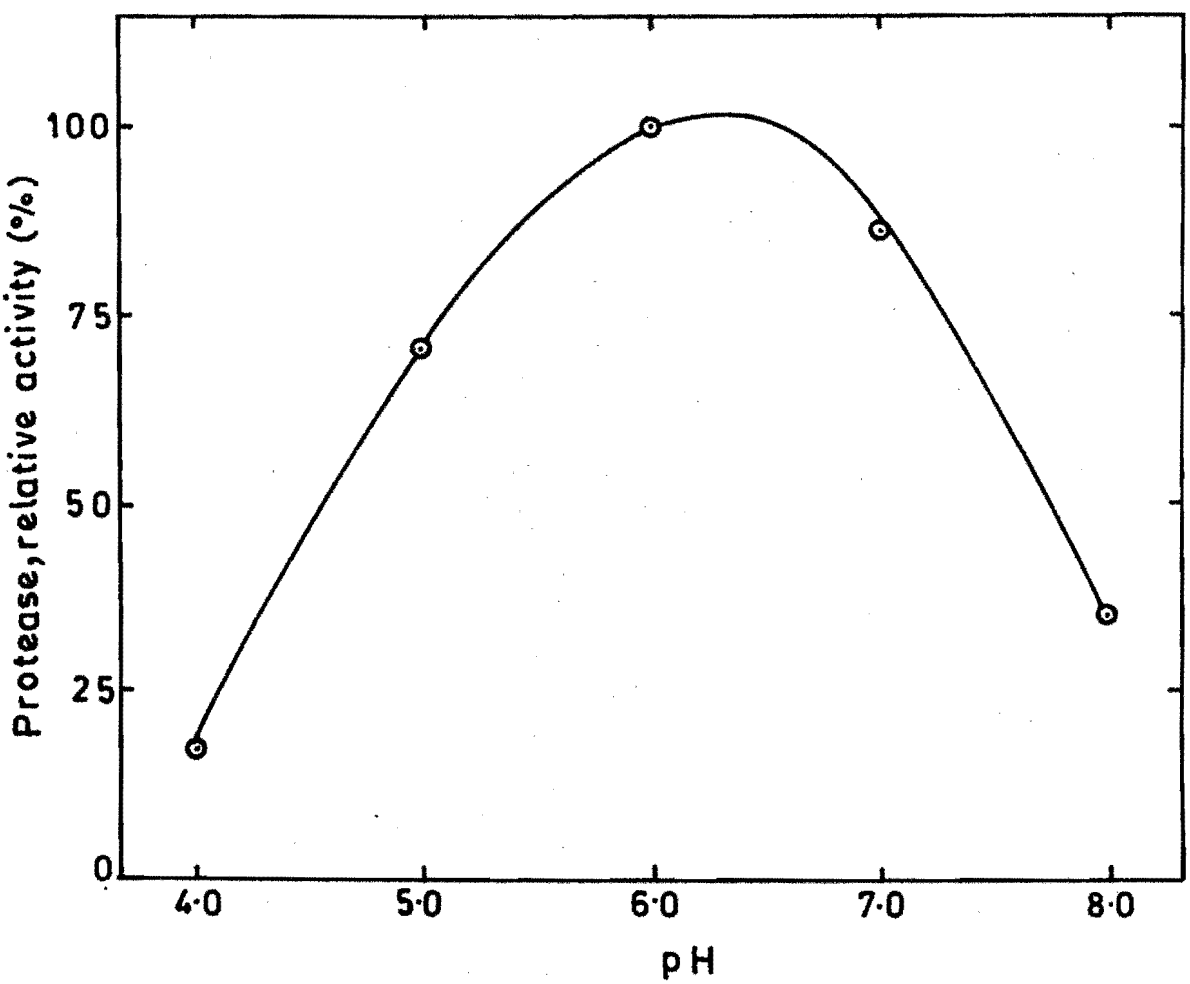

Fig. 12. Effect of pH on protease activity in crude enzyme (MRBE).

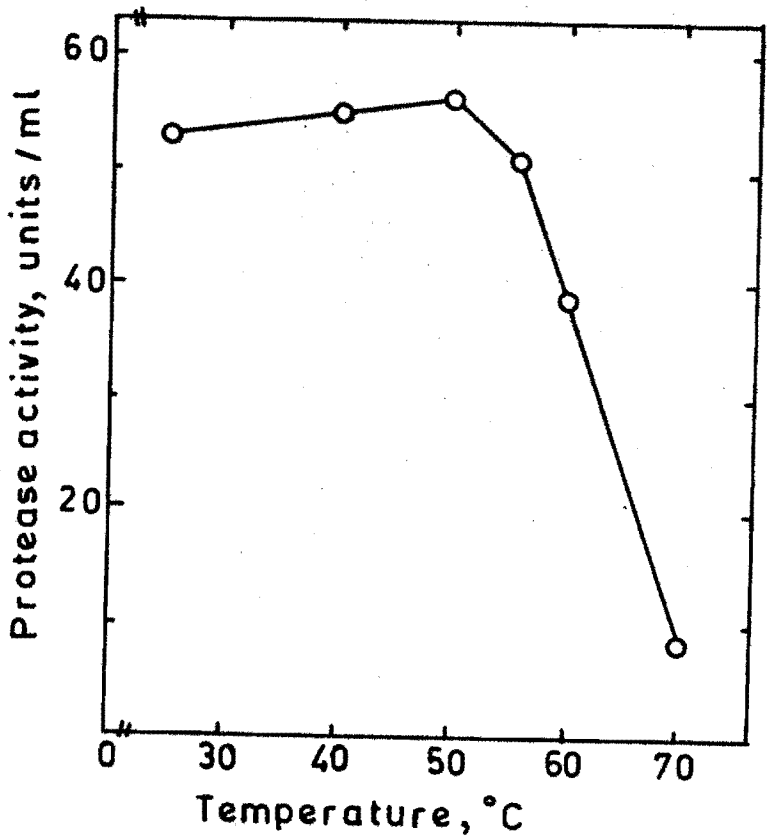

Fig. 13. Effect of temperature on the protease activity in the crude enzyme (MRBE).

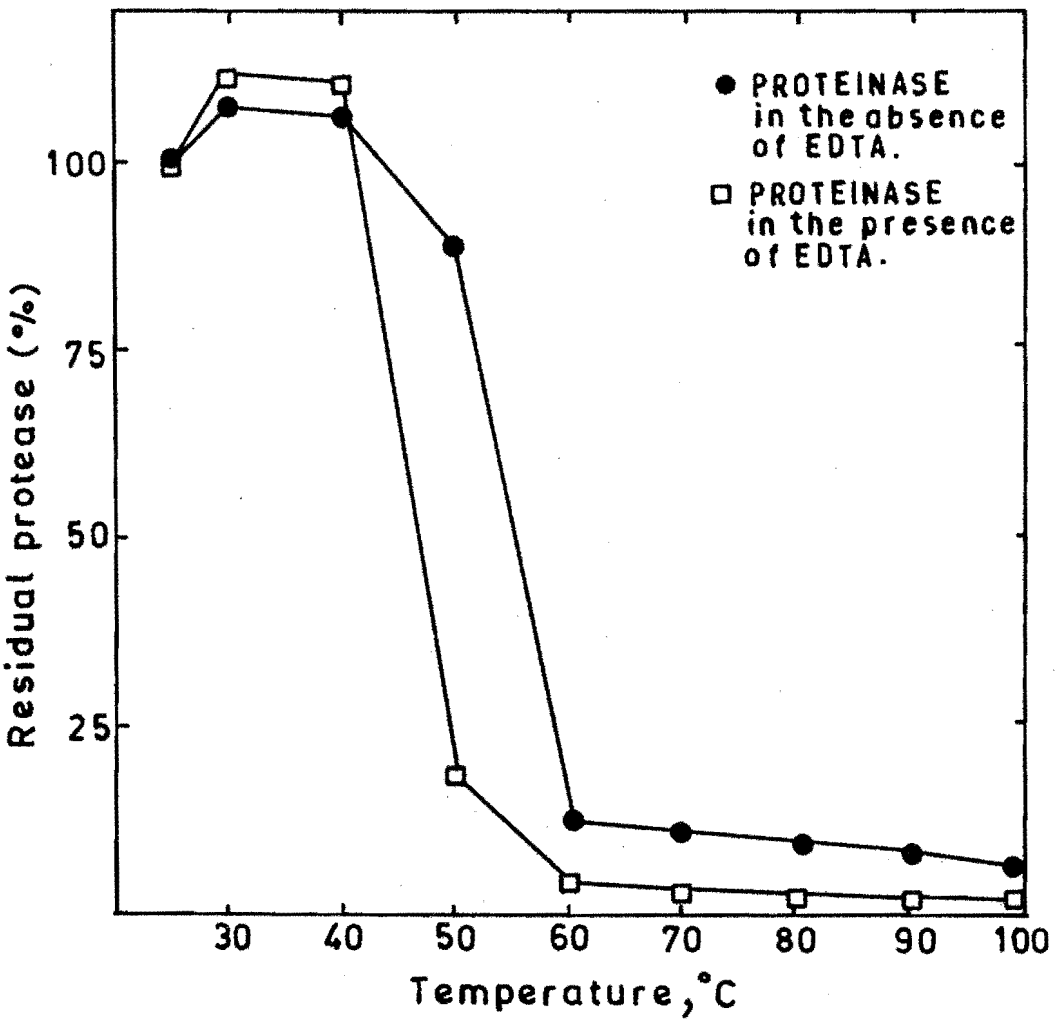

Fig. 14. Heat-stability of proteinase in the crude enzyme (MRBE) in the presence and absence of EDTA.

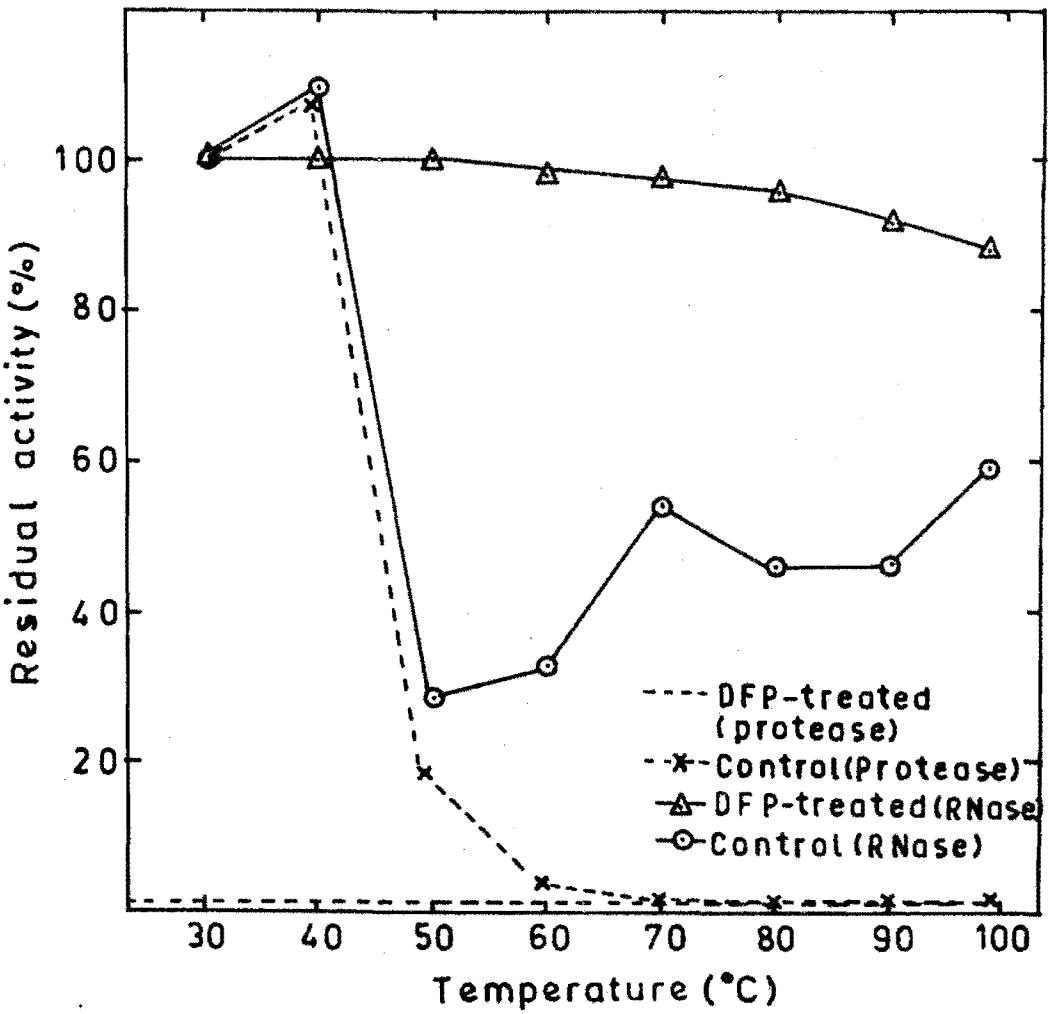

Fig. 15. Heat inactivation pattern of RNase and protease in the DFP-treated and non-treated MRBE.

Other nucleolytic enzymes in the crude preparation:

Crude enzyme preparations from a submerged culture on glucose-peptone-mineral medium and moldy rice bran extract (MRBE) of rice bran surface culture were tested for the presence of DNase (using both native and heat-denatured DNA as substrates), non-specific phosphodiesterase activity with bis-p-nitrophenyl phosphate and 3'- and 5'-nucleotidase (phosphomonoesterase) activities with 3'-AMP and 5'-AMP respectively.

The results obtained are presented in Table 31 along with RNase activities of the same preparations. Both the preparations showed fairly good activity against both native DNA and heat-denatured DNA. Heat-denatured DNA was found to be more susceptible to enzyme attack than the native DNA. Both the enzyme preparations showed almost similar activity towards the DNA substrates.

The crude enzyme extracts possessed non-specific phosphodiesterase activity also. It was slightly greater in the case of MRBE than in the culture filtrate from Medium 2 (2.07 units and 3.90 units/ml respectively).

Both preparations showed nucleotidase activity against both 3'- and 5'-AMP. However, it was significantly higher in the case of MRBE than in the culture filtrate from Medium 2, i.e. about 3-fold more activity in MRBE than in the latter.

Table - 31

Nucleic acid-degrading enzymes in crude enzyme preparations

Enzyme		Enzyme activity	
		CF[1]	MRBE[2]
RNase		590 units/ml	630 units/ml
DNase	Against native DNA	760 units/ml	765 units/ml
	Against heat denatured DNA	850 units/ml	880 units/ml
Non-specific phospho-diesterase		2.07[a]	3.90[a]
3'-Nucleotidase		6.3[b]	18.00[b]
5'-Nucleotidase		7.0[c]	23.00[c]

[a] Absorbance values at 410 nm.

[b] µg Pi liberated from 3'-AMP

[c] µg Pi liberated from 5'-AMP

[1] Concentrated culture filtrate from glucose-peptone-mineral grown submerged culture.

[2] Moldy rice bran extract from rice bran surface culture.

DISCUSSION

The characteristics of crude RNase preparations of A. candidus M16a were studied primarily in order to standardize conditions for a reliable assay method and also because of its anticipated use in reducing the nucleic content of SCP. Incidentally it was found that the crude enzyme preparations contained an active protease which would adversely affecc the products. Hence, some preliminary studies were conducted primarily to ascertain methods of inactivating it without affecting RNase activity.

A single pH optimum (4.5) indicates that the crude preparations contain, perhaps, only one major RNase activity. The pH optimum of the purified RNase also was, later found to be 4.5 (Please see next Chapter, Fig. 19). Barnard (1969) and Josefsson and Lagerstedt (1962) discussed this point about the pH optimum in crude enzymes in detail. They were of the opinion that certain contaminating substances in the crude preparation might alter the optimum pH. For example, heparin-like inhibitors in the tissue homogenates of liver, metallic contamination by Cu^{2+} and Fe^{2+} of pea leaf RNase altered the optimum pH. The pH optimum was found to vary considerably with the substrate and with ionic strength (Barnard, 1969). With the Mg^{2+} or Ca^{2+}-dependent enzymes, modest changes in the cation concentration could also shift the pH dependence (Cuatrecasas et al., 1967). 'Inactive' RNase might readily be 'activated' by

agents such as EDTA, citrate, NH_4Cl and urea within a wide pH range (Dickman and Trupin, 1958). However, in a majority of cases, pH optimum of a particular enzyme in a crude preparation would be same as that of the purified enzyme; as in the present case.

The presence of a protease in the crude enzyme preparation used in the present study is a serious drawback as for the stability of the RNase is concerned, and more so, when the enzyme is intended to be used for the removal of nucleic acids in SCP.

The protease is completely inhibited by DFP which does not affect the RNase activity. Therefore, it seems to be a serine-protease like trypsin. However it may not be practical to use DFP as it is both expensive and highly toxic. Hence, some other non-toxic specific inhibitors have to be sought. It is well-known that various legumes contain different types of serine-protease inhibitors. Perhaps these may prove useful for this purpose. This aspect, however, was not pursued further.

The heat stability of RNase in the present instance seems to be conditioned by two distinct causes. The RNase activity in CF (from Medium 2) is more stable to heat than the MRBE enzyme. It is possible that the CF enzyme may be stabilized by some component of the medium. In the case of rice bran solid medium no minerals were added exogenously.

Perhaps, some component (metallic ions?) present in the bran causes heat-lability and EDTA stabilizes the enzyme by complexing with the component.

When grown on wheat bran semi-solid medium P. citrinum produces nuclease P_1 in a complex form containing a malonogalactan complex, whereas in liquid medium the enzyme was produced in a free form, i.e. without the complex (Fujimoto et al., 1977a, 1977b). This phenomenon was reported to occur also in the case of other enzymes such as proteases and β-galactosidases (Fujimoto et al., 1977b). Probably, in the present case also such a complex formation may be taking place when grown on rice bran solid medium. This may be another possibility but unlikely since the enzyme becomes heat-stable in the presence of EDTA which is unlikely to complex with a carbohydrate moiety such as the malonogalactan. The possibility that EDTA might be inactivating the protease and thus stabilize the RNase is quite unlikely since EDTA has no significant effect on protease activity.

Heat stability of the RNases from various sources at different states of purification was found to be different. For example, on heating spleen RNase homogenate for 10 min. at 60°C and pH 3.5 abolished 65% of activity, but following a 300-fold purification the same heating at pH 3.3 reduced activity by only 20% (Josefsson and Lagerstedt, 1962). A similar phenomenon was observed in the present case also.

More than 90% of the RNase activity was destroyed when the crude enzyme (MRBE) was heated at 80°C at pH 5.6 for 5 min., whereas heating the purified enzyme even at 90°C at the same pH for the same duration caused only 50% inactivation. Heat lability of RNase preparations was also conditioned by the protease present in the culture filtrates. This has been amply demonstrated by inactivating the protease by DFP treatment and testing the heat-stability of the RNase.

The higher heat inactivation of RNase at pH 6.7 in the presence of EDTA was due to the action of protease but not mainly due to metals and the least inactivation at pH 5.6 and/in the presence of EDTA was due to the removal of metals and also due to lesser activity of protease on RNase at that pH.

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CHAPTER - 3

PURIFICATION AND PROPERTIES OF RNase FROM *ASPERGILLUS CANDIDUS* M16a

The enzyme RNase from moldy rice bran extract was purified to homogeneity and its properties studied. These studies are described in this Chapter.

A. PURIFICATION

(a) Preparation of the crude enzyme:- The culture A. candidus M16a was grown on rice bran semi-solid medium as described in Chapter 1. This was either done in conical flasks in small quantities (50 gm rice bran + 40 ml tap water in 750 ml wide-mouthed conical flasks) or on perforated aluminium trays for large scale production (1.2 kg rice bran moistened with 960 ml water per tray). After incubation for 85 hrs at ambient temperature (25-28°C) in a humidifier with 90% relative humidity (RH) the moldy bran was air-dried at room temperature (28°C) to 10% moisture content. One kg of dry moldy bran was slurried with 5 litres of distilled water and pH adjusted to 5.6. The slurry was filtered and pressed through a cheese cloth and then centrifuged at 6000xg for 15 min. The supernatant was concentrated in a forced circulation evaporator in vacuo (15-60 mm Hg pr. and at 38-40°C). Generally 5-6 fold concentration of the extract was achieved.

A typical batch of flask grown culture yielded an MRBE having 6.5 mg protein/ml and a specific activity of about 375 units/mg protein. Substantial loss of activity

was observed during the concentration process. The concentrated MRBE contained 28-30 mg protein/ml. with a specific activity of RNase of about 170 units/mg.

(b) Ethanol precipitation:- One liter of the concentrated MRBE (pH 5.6) was cooled to below 5°C. Chilled ethanol (-5° to 5°C) was slowly added dropwise with stirring to the concentrate to a final concentration of 15%, stored at 5°C for 10 min. and then centrifuged at 15,000xg for 10 min. at 10°C (The temperature during the addition of ethanol was maintained around 5°C). The precipitate was washed with 50 ml of 15% ethanol and was dried with acetone and weighed. To the supernatant ethanol was further added to get 30% concentration and the procedure was repeated as before. Similarly the precipitates were collected at 45, 60, 75 and 90% ethanol concentrations.

In another experiment, ethanol concentration was directly raised to 30% and the precipitate was removed and then the concentration was increased to 50% and this precipitate was collected.

RNase activity and protein content were determined in both precipitate and supernatant at each step. The results are presented in Table 32. It could be seen that the maximum recovery of RNase was obtained in fraction precipitating between 45 and 60% ethanol, although the highest specific activity was in the 30-45% ethanol precipitate.

Table - 32

Ethanol Fractionation

Et.OH Conc. (%)	Total protein recovered (gm)	% protein recovered	RNase specific activity	Total RNase activity recovered (units)	% activity recovered
0-15	0.190	0.66	243.0	46,118	0.94
16-30	0.587	2.05	212.9	124,955	2.55
31-45	0.203	0.71	1861.1	376,875	7.69
46-60	2.677	9.36	691.8	1,852,141	37.77
61-75	2.144	7.50	102.0	118,753	4.46
76-90	2.363	8.26	333.3	787,492	16.06
31-50	1.590	5.68	1121.0	1,781,942	35.64

One litre concentrated MRBE was used. The total initial protein content and total activity were 28.6 gms and 4,902,898 units respectively.

Hence, for further studies a fraction obtained at an ethanol concentration of 30-50% was selected. In this fraction fairly good recovery of RNase with high specific activity was obtained (Table 32). Although the yield in this particular experiment was only about 36% in later trials a yield of as much as 54% was obtained, especially when non-concentrated MRBE was used.

In all further experiments the MRBE was used without concentration as it was found that substantial loss of RNase activity occurred during concentration.

Effect of pH on ethanol precipitation of RNase:- In order to find out the optimum pH of the extract for maximum precipitation of the enzyme the MRBE was divided into lots of 100 ml and pH was adjusted to 3-8. Precipitates from 30-50% ethanol concentration were collected as in the previous case and activity and protein content were determined. The results are given in Table 33. It could be seen that the maximum protein yield with highest RNase activity was obtained at pH 4.5.

(c) Sephadex G-100 fractionation:- Sephadex G-100 of medium size was prepared according to standard procedure and 280 ml gel was packed in a glass column (2 x 85 cm) and was equilibrated with 50 mM potassium phosphate buffer (pH 5.6). The void volume was determined with Blue Dextran-2000 and was 95 ml.

Table - 33

Effect of pH on the ethanol precipitation

pH	Total protein recovered (mg)	% protein recovered	RNase specific activity	Total activity recovered (units)	% activity recovered
3.0	12.6	1.94	402.8	5,075.3	1.64
4.0	67.1	10.32	629.2	42,187.9	13.61
4.5	124.0	19.08	722.6	89,602.4	28.90
5.0	93.3	14.36	668.5	62,397.8	20.13
5.5	103.2	15.88	682.2	70,403.0	22.71
6.0	64.6	9.94	875.0	56,560.0	18.25
6.5	5.6	0.85	432.4	2,399.8	0.77
7.0	5.1	0.79	188.2	963.6	0.31
8.0	2.5	0.38	122.0	300.0	0.10

Initial protein content of MRBE was 6.5 mg/ml, i.e. 650 mg/100 ml. Initial enzyme activity was 3,100 units/ml, i.e. 310,000 units/100 ml. Specific activity was 376.9.

12 ml of the enzyme solution (30-50% alcohol precipitate in 50 mM phosphate buffer, pH 5.6) containing 300 mg protein was loaded on to the column (The RNase specific activity of the loaded sample was 1666 units/mg) and eluted with the same buffer. The flow rate was adjusted to 30-35 ml/hr and 5 ml fractions were collected.

The results are presented in Fig. 16. The protein resolved mainly into 3 fractions, first a smaller peak followed by a major and then a smaller peaks. The fractions obtained in the 3rd peak were slightly coloured (brownish). Hence, protein in all fractions was estimated by Lowry's method and also by O.D. at 280 nm. The RNase activity was present in the second peak. The fractions corresponding to elution volume from 150 ml to 185 ml were pooled. The recovery of protein in the active fraction was 70% and RNase activity recovered was 92.5%. The pooled active fraction showed a specific activity of 2,184 units/mg.

(d) DEAE-Cellulose chromatography - Batchwise elution:- DEAE-cellulose was prepared according to standard procedure (Peterson and Sobers, 1962) by treating with 0.5 N HCl and 0.5 N NaOH alternatively with thorough washing in between with distilled water. The treated DEAE-cellulose was finally washed free of alkali, equilibrated in 5 mM potassium phosphate buffer (pH 5.6) and kept overnight. This was then centrifuged and the wet cake was used for the

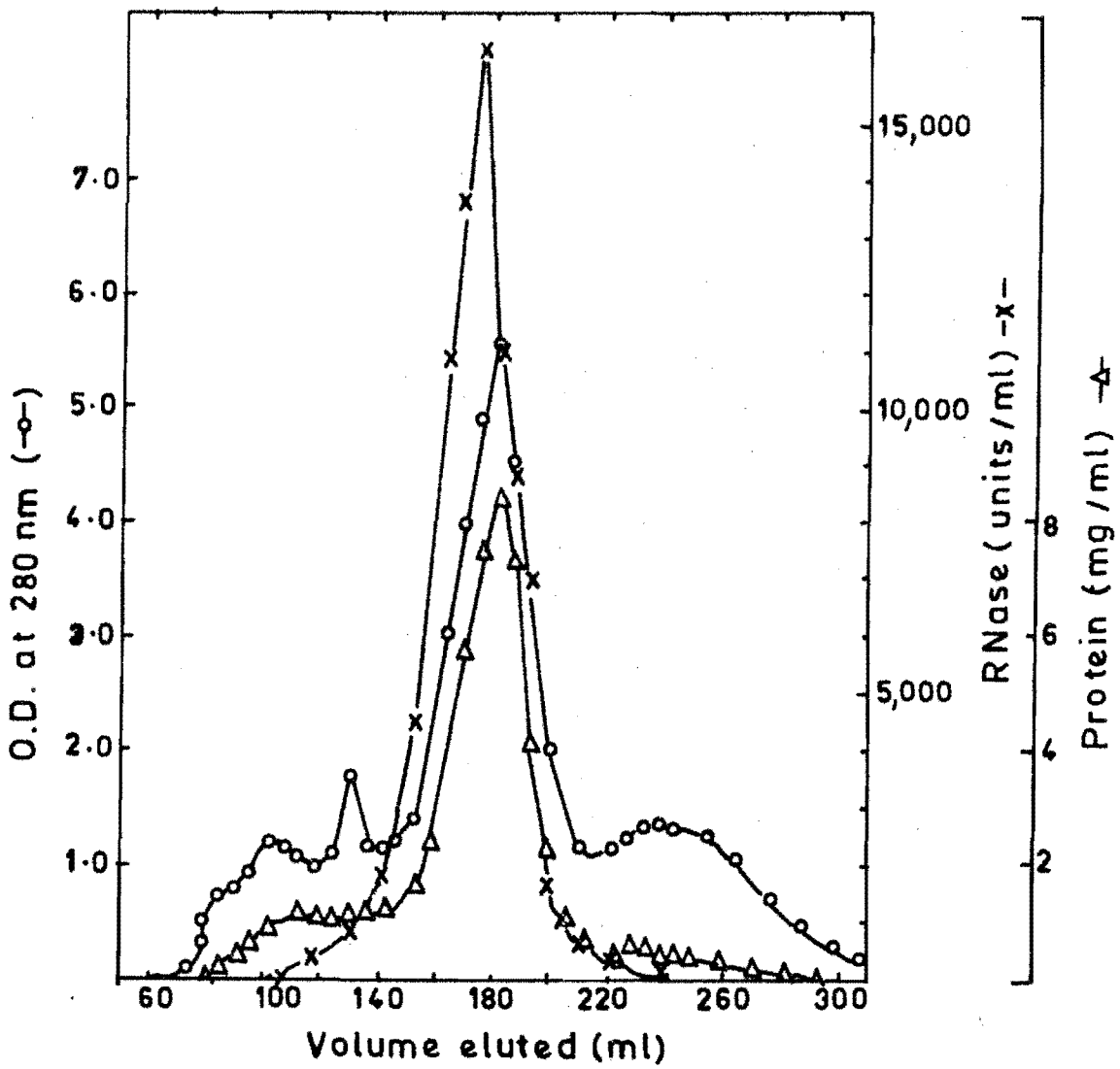

Fig. 16. Elution pattern of RNase and proteins (of 30-50% ethanol fraction) from Sephadex G-100 column. Experimental details are given in the text.

fractionation of the enzyme by batchwise elution which was carried out in two steps as follows:

Stage I:

The active fraction obtained by Sephadex G-100 gel-permeation chromatography, as described above, was dialysed against 5 mM potassium phosphate buffer (pH 5.6). To 30 ml of this solution (60 mg protein and 2,184 specific activity) DEAE-cellulose wet cake [equivalent to about 1.7 gm (dry wt)] was added and stirred thoroughly. This was kept at 5°C for 30 min., centrifuged at 10,000xg for 10 min. and washed with buffer (5 mM phosphate, pH 5.6). The supernatant and the washings were pooled which constituted fraction A. The sediment was then mixed with 20 ml of 0.16 M NaCl in 5 mM phosphate buffer, pH 5.6, stirred and allowed to stand at 5°C for 30 min. and then centrifuged. This was again eluted with another 10 ml of the same eluant. Both the supernatants were pooled which formed Fraction B. Similarly elutions were carried out with 0.26 M NaCl (Fraction C) and finally with 0.4 M NaCl (Fraction D).

The elution pattern of RNase in the different fractions are presented in Table 34. It could be seen that about 40% of the RNase activity loaded was recovered in Fraction C i.e. the fraction eluted with 0.26 M NaCl. Much activity was still retained in the ion-exchanger and about 29% was eluted with 0.4 M NaCl (Fraction D).

Table - 34

DEAE-cellulose chromatography - Stage I

Fraction	Volume eluted (ml)	RNase activity (units)	Specific activity	% activity recovered
A. Supernatant+ Washings	45.0	441	-	0.02
B. 0.16 M NaCl	31.5	32,445	1,561	14.23
C. 0.26 M NaCl	16.5	91,080	10,222	39.95
D. 0.4 M NaCl	18.8	65,363	14,834	28.67

To 30 ml of enzyme fraction from gel filtration containing 104.4 mg protein with specific activity of 2,184, 1.7 gm DEAE-cellulose cake was added, stirred and kept at 5°C for 30 min. Then centrifuged and washed with buffer (5 mM phosphate buffer pH 5.6) and then eluted stepwise with 0.16 M, 0.26 M, and 0.4 M NaCl in the same buffer.

Stage II:

As most of the RNase activity was recovered in Fraction C and D of Stage I, these were pooled. The pooled fraction was concentrated to about ¼th its volume by per evaporation and dialysed against 5 mM phosphate buffer (pH 5.6) to remove the salt.

28 ml of this dialysate containing 230 µg protein/ml were mixed with wet DEAE-cellulose cake (1 gm dry wt.) and allowed to stand at 5°C for 30 min. The rest of the procedure was the same as above except that the elutions were carried out only with two NaCl concentrations, viz. 0.16 M and 0.4 M.

The results obtained are presented in Table 35. In Fraction 3, i.e. with 0.4 M NaCl about 62% of the loaded activity was recovered. The specific activity of this fraction was 34,290 units as against 17,700 units of the loaded sample , i.e. the enzyme was purified 2-fold in the second stage of DEAE-cellulose-chromatography. RNase activity was purified from MRBE by totally about 134-fold by the above techniques, viz. ethanol fractionation (30-50%), Sephadex G-100 gel filtration and DEAE-cellulose chromatography (2 stages).

Protease activity in the active fractions:- The purified RNase activity even at this stage was found to be contaminated with protease. The pooled and concentrated

Table - 35

DEAE-cellulose chromatography - Stage II

Fraction	Volume eluted (ml)	RNase Total Units	Specific activity	% activity recovered
1. Supernatant + Washings	50.0	235	57.1	0.2
2. 0.16 M NaCl	38.0	34,124	10,564.7	29.9
3. 0.4 M NaCl	20.5	70,295	34,290.0	61.7

28 ml of pooled fractions C and D containing 230 µg protein/ml was mixed with about 1 gm DEAE-cellulose and kept at 5°C for 30 min and centrifuged and washed with the same buffer as before, then eluted with 0.16 M, and 0.4 M NaCl in the same buffer batchwise.

fractions C and D from DEAE-cellulose Stage I showed a specific activity of protease of 304 units. Again, Fraction 3 of DEAE-cellulose chromatography Stage II showed a specific activity of protease of 250 units.

Protease activity was determined at various stages of purification and the results are incorporated in Table 36. It could be seen that at almost all of the purification steps the protease also was getting eluted along with RNase. However, in the final step, i.e. DEAE-Sephadex A-50 chromatography the protease was completely eliminated (Table 36).

The active fractions obtained at two stages of DEAE-cellulose chromatography showed 2 protein bands in PAG disc gel electrophoresis, as will be explained later. The one which moved faster towards the anode at pH 8.1 was confirmed to be the RNase and the other slow moving one was found to be the protease.

(e) DEAE-Sephadex A-50 column chromatography:- The active fraction obtained from DEAE-cellulose chromatography (Stage II) was dialysed against 5 mM phosphate buffer (pH 5.6) and further purified using DEAE-Sephadex A-50.

The ion-exchange gel was equilibrated against 5 mM phosphate buffer (pH 5.6) and packed into a column (0.9 x 13 cm). The enzyme solution (16 ml) containing 1.8 mg

protein was loaded onto the column and eluted with same buffer using a sodium chloride gradient of 0 to 0.5 M. 2.4 ml fractions were collected.

The elution pattern is shown in Fig. 17. The RNase activity was eluted in fractions 23-34 at a sodium chloride concentration of 0.22 to 0.35 M. These fractions were pooled. The recovery of protein and RNase activity were 76.7 and 77.6% respectively.

The scheme of purification of RNase from moldy bran extract of A. candidus M16a is given in Table 36. The yield of pure enzyme after fractionation which involved 5 steps was about 16%. 6 mg protein was recovered as pure enzyme from 4.1 gm of total protein in the crude enzyme. The active fraction obtained after DEAE-cellulose chromatography showed a specific activity of 34,290 which after further purification by DEAE-Sephadex A-50 column chromatography came down to 28,348. 134-fold purification was observed after DEAE-cellulose chromatography which after fractionation on DEAE-Sephadex A-50 showed a calculated value of only about 111-fold purification. This may be due to inactivation of the RNase protein by the contaminating protease which was observed even after DEAE-cellulose chromatography.

Polyacrylamide gel electrophoresis (PAGE):- Disc-gel electrophoresis was carried out at two pH values, i.e. at 4.3 (β-alanine-acetic acid buffer) and 8.1 (Tris-glycine

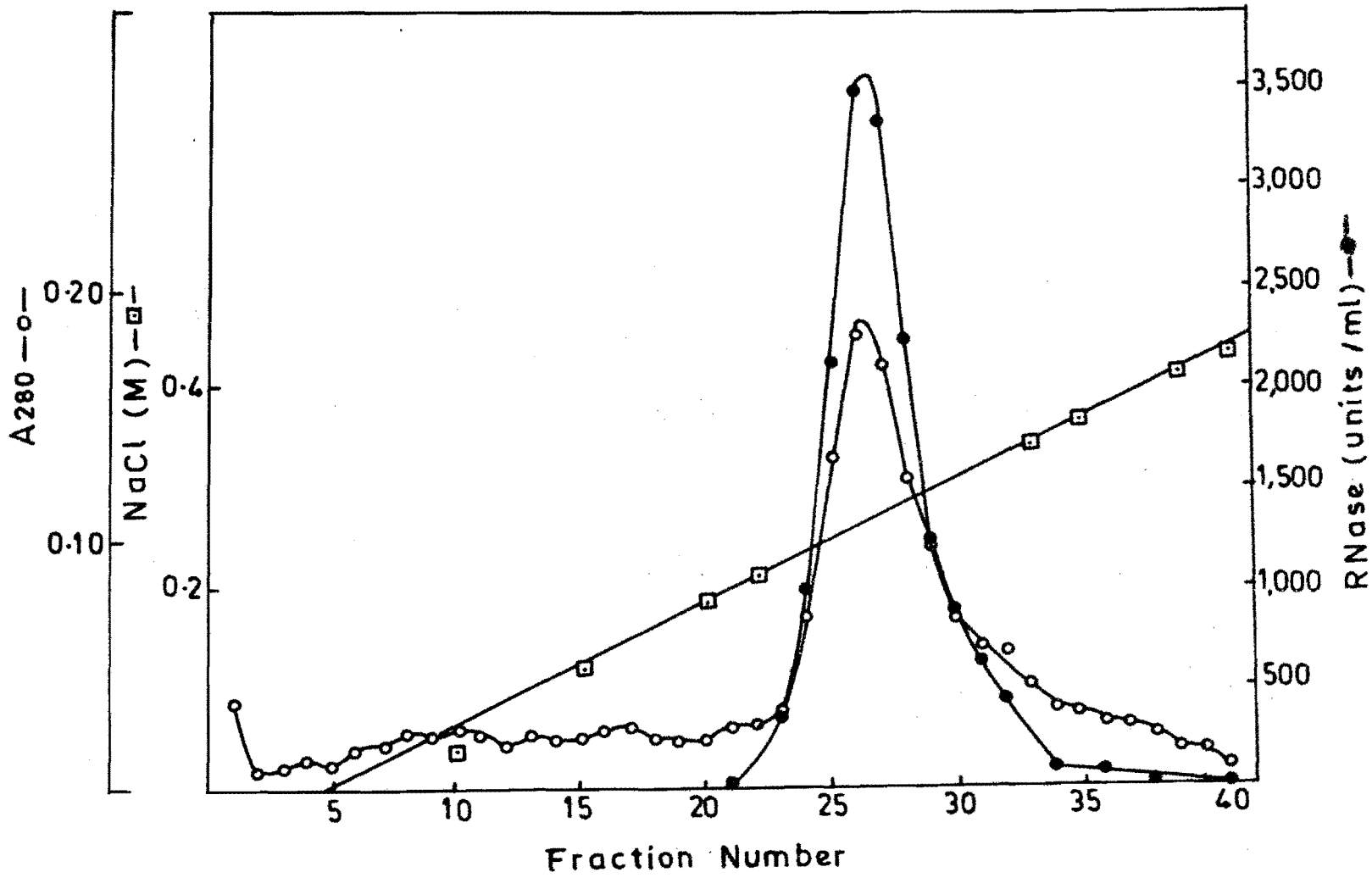

Fig. 17. Elution pattern of RNase and protein (of DEAE-cellulose II fraction) from DEAE-Sephadex A-50 column by a sodium chloride gradient. Details of the procedure are given in the text.

Table - 36

Purification of RNase from A. candidus M16a

Stages	Total protein (mg)	Total RNase activity (units)	Specific activity (units/mg)*	Fold of purification	Yield (%)
Crude enzyme (MRBE)	4,101.60	1,050,000	256(105.2)	1	100
Ethanol fraction(30-50%)	339.90	566,250	1,666(56.9)	6.5	54
Sephadex G-100 fraction	238.20	520,336	2,184(--)	8.5	50
DEAE-cellulose I fraction	20.20	357,055	17,700(304.4)	69.1	34
DEAE-cellulose II fraction	6.40	220,196	34,290(250.0)	133.9	21
DEAE-Sephadex A-50 fraction	6.03	170,038	28,348(0.0)	110.7	16

* Values given in parentheses are the specific activities of protease in different fractions.

buffer) in a 7.5% polyacrylamide gel. Samples from different stages of purification were tested. The results are given in Fig. 18.

The original crude enzyme preparation (MRBE) separated into only 5 bands at pH 4.3 whereas at pH 8.1 it resolved into 9 bands.

The two bands separated in electrophoretic gel of the sample from DEAE-cellulose Stage II (Fraction 3) were eluted and tested individually for proteolytic and RNase activities. The lower one which moved faster at pH 8.1 was found to be the RNase fraction and the upper slow moving one was detected to be the protease. The protease band was thicker than the RNase protein even at this stage of purification.

The active fraction obtained after DEAE-Sephadex A-50 chromatography showed only one band at both pH values even when about 100 µg protein was loaded, indicating the homogeneity of the sample. This did not contain any protease activity.

This pure RNase fraction was used for various kinetic and other enzyme characterization studies.

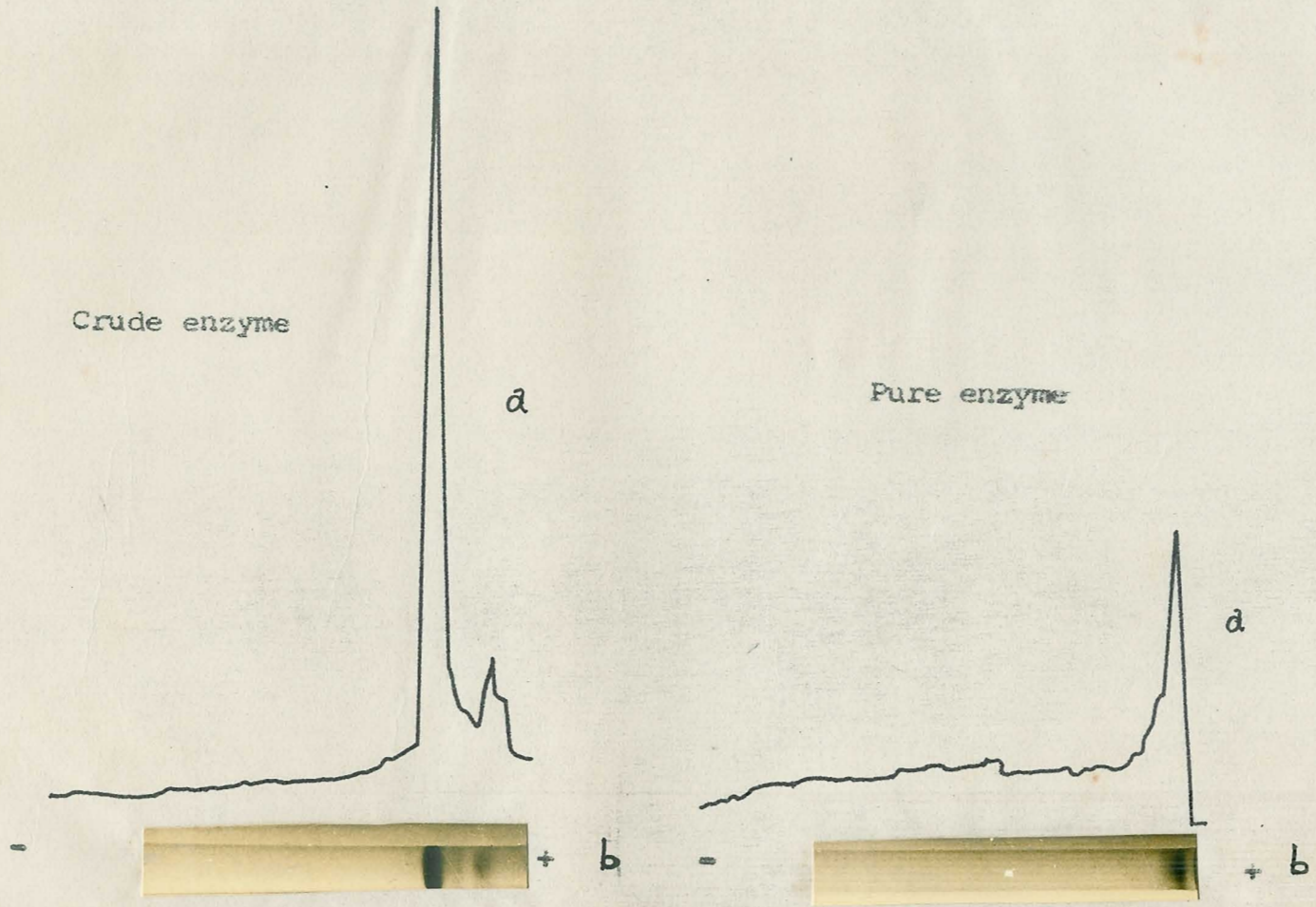

Fig. 18. PAG-electrophoresis of crude and purified RNase of A. candidus M16a
(A) at pH 4.3 - (a) chromoscan of the gel,
(b) photograph of the gel.

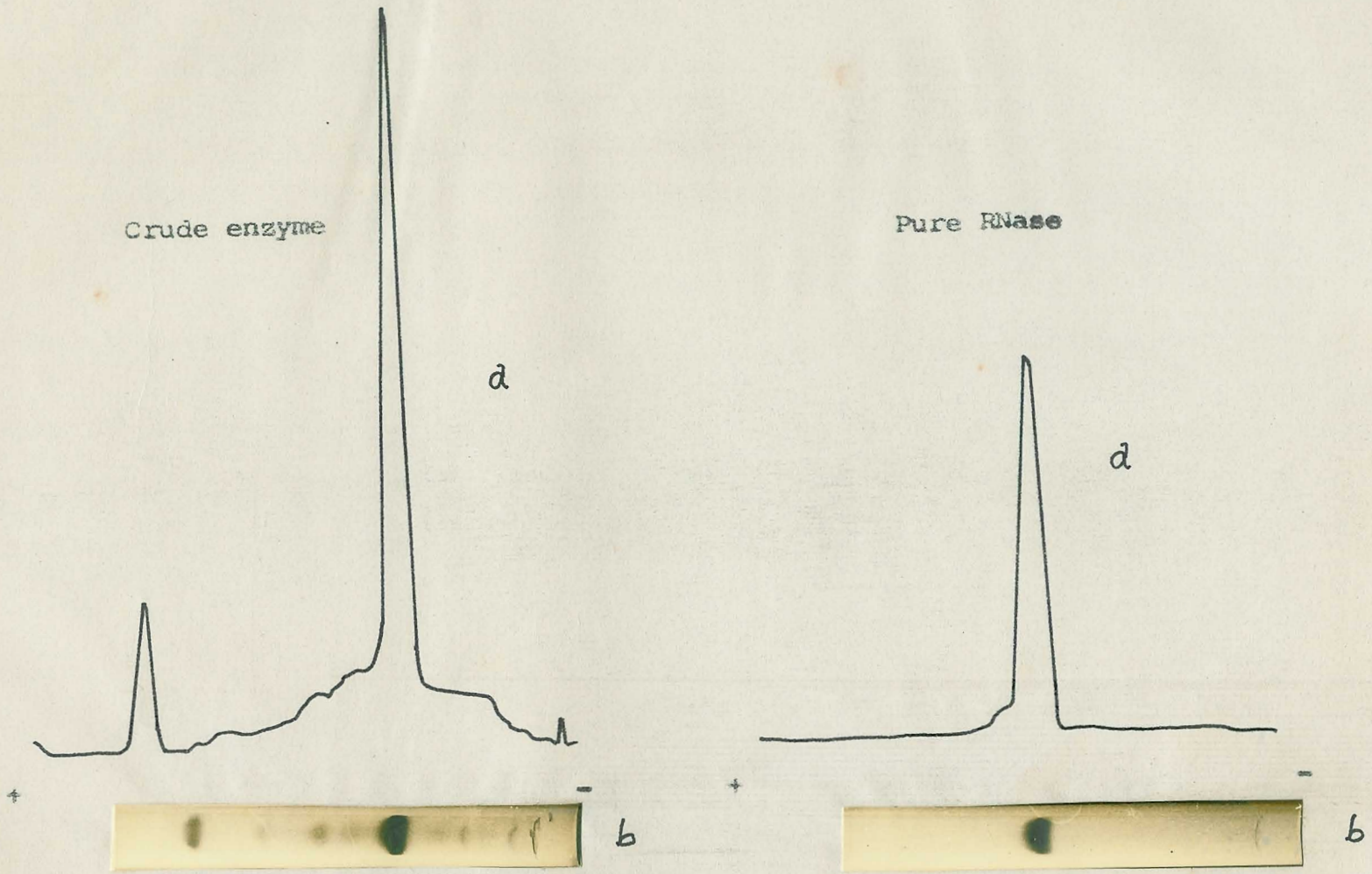

Fig. 18.(contd.).
(B) at pH 8.1. Other descriptions are as in the previous page.

B. PROPERTIES OF RNase OF A. candidus M16a

Various kinetic and other properties such as the effect of various physical and chemical parameters, specificity and mode of action of the purified RNase were studied.

1. Effect of pH:- Reaction was carried out at different pH values using 0.5 M buffers (3.0-5.5, sodium acetate buffer; 5.5-8.0 potassium phosphate buffer). Other details were as given in "Materials and Methods".

The results obtained are presented in Fig. 19. It could be seen that the enzyme was most active between pH 4.0 and 5.0, and maximally active at pH 4.5.

2. Effect of temperature:- 0.5 ml RNA solution (10 mg/ml) and 1 ml of 0.2 M acetate buffer (pH 4.5) was equilibrated at different temperatures varying from 30 to 70°C and 0.5 ml of appropriated diluted enzyme solution was added and the reaction was carried out at respective temperatures for 30 min. Other details were as in the "Materials and Methods".

The results are presented in Fig. 20. It could be seen that the enzyme activity was maximum at temperatures 55-60°C.

3. pH stability:- The enzyme samples at pH 3.0 to 8.0 in 0.1 M sodium acetate (3.0-5.5) or potassium phosphate (5.5-8.0) buffers were incubated at 60°C for 15 min. and

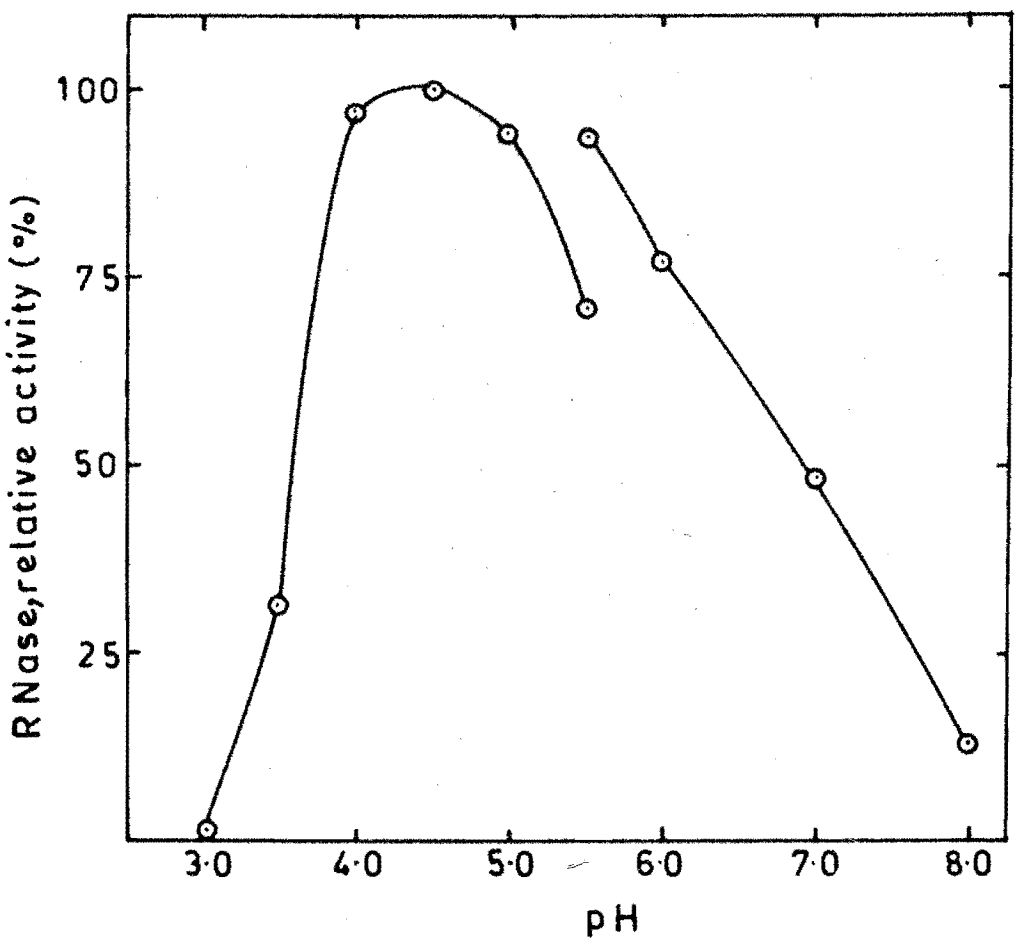

Fig. 19. Effect of pH on RNase activity.

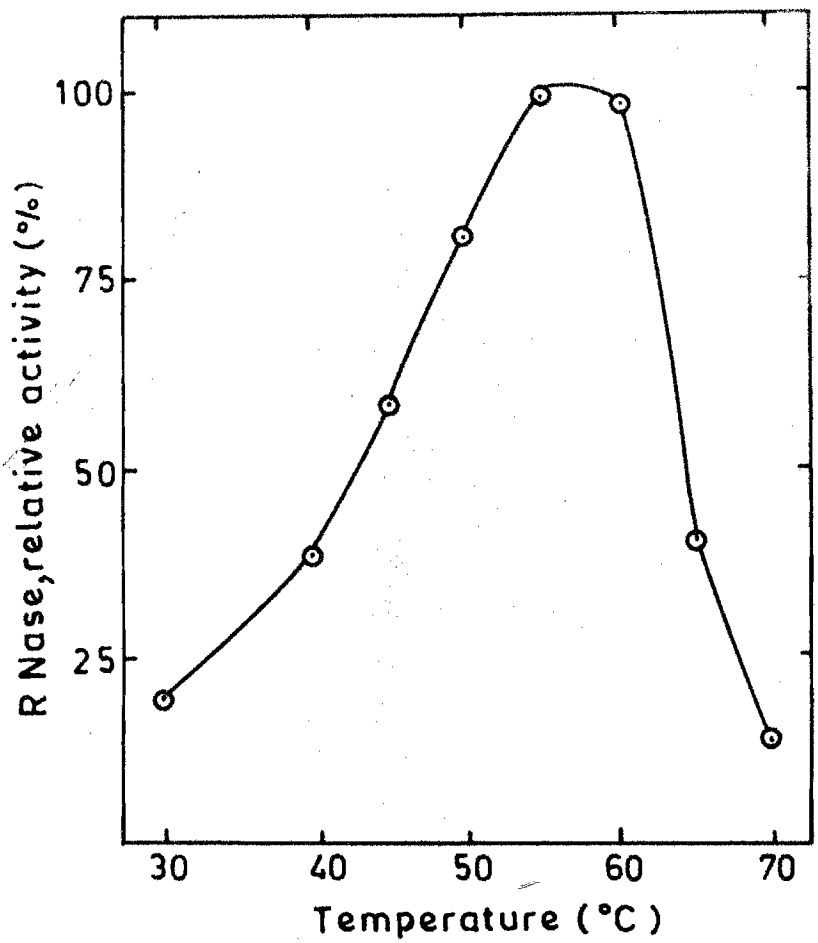

Fig. 20. Effect of temperature on RNase activity.

then immediately cooled. Residual RNase activity was determined using 0.5 ml of appropriately diluted enzyme solution.

The results are presented in Fig. 21. It could be seen that the enzyme was most stable at pH 5.5. The stability was better in phosphate buffer as compared to acetate buffer. It could also be noticed that at higher pH values (above 5.5) the stability of the purified enzyme was better than those of the crude enzymes at the same pH values, e.g. the retention of activity of the pure enzyme was about 83% at pH 7.0 as compared to about 65% in the case of crude enzymes at the same pH.

4. Heat stability:- Enzyme samples were diluted (0.3 µg protein/ml) with 0.05 M phosphate buffer (pH 5.6) and were pre-incubated at different temperatures, viz. 40°, 55°, 60°, 70° and 95°C. Samples were drawn at different intervals, viz. 5, 10, 20, 30, 45 and 60 min. and cooled immediately in ice bath. The residual RNase activity was then determined.

The results are presented in Fig. 22. About 50% and 30% of the activity were retained after heating the enzyme at 95°C for 5 min. and 10 min. respectively, while about 40% was retained after 30 min. at 70°. Only about 35% and 15% activity were lost after 30 min. heating at 60°C and 55°C respectively. Interestingly, the activity

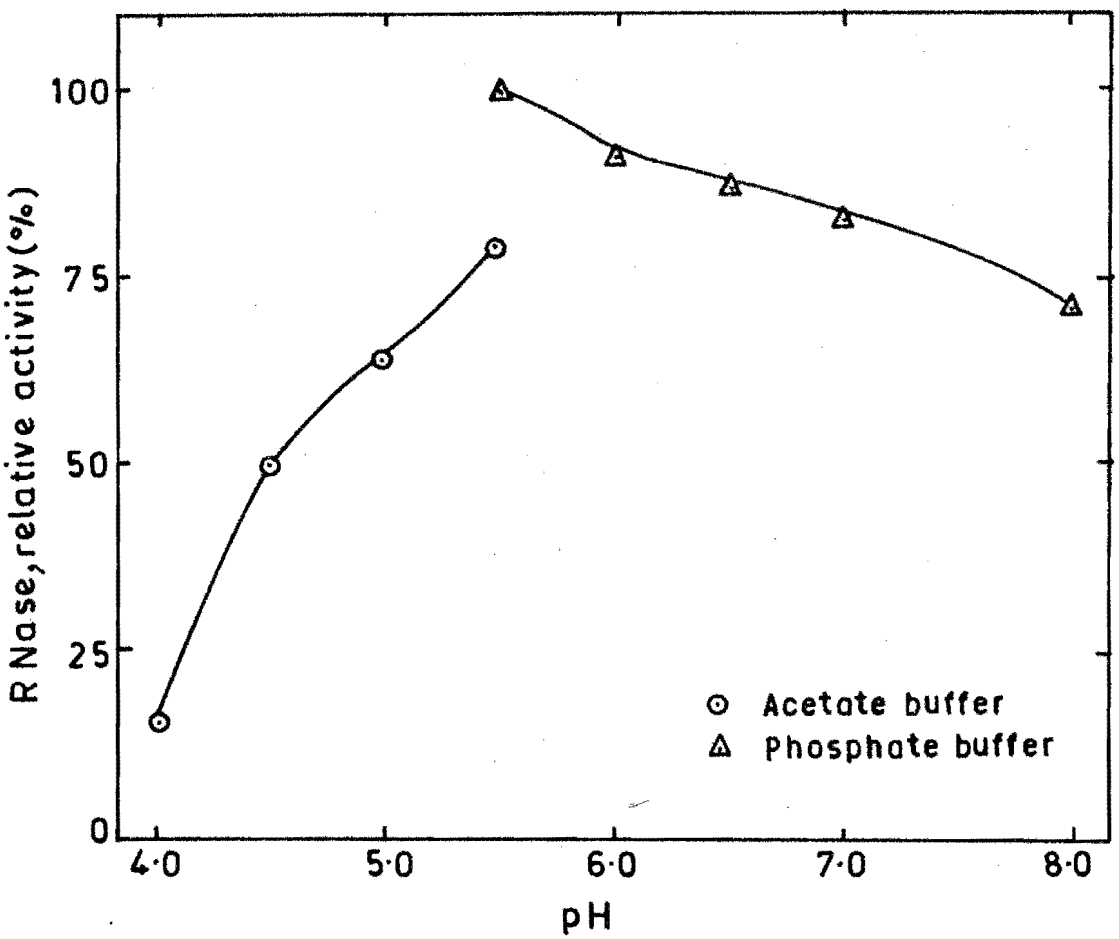

Fig. 21. Effect of pH on the stability of the purified RNase.

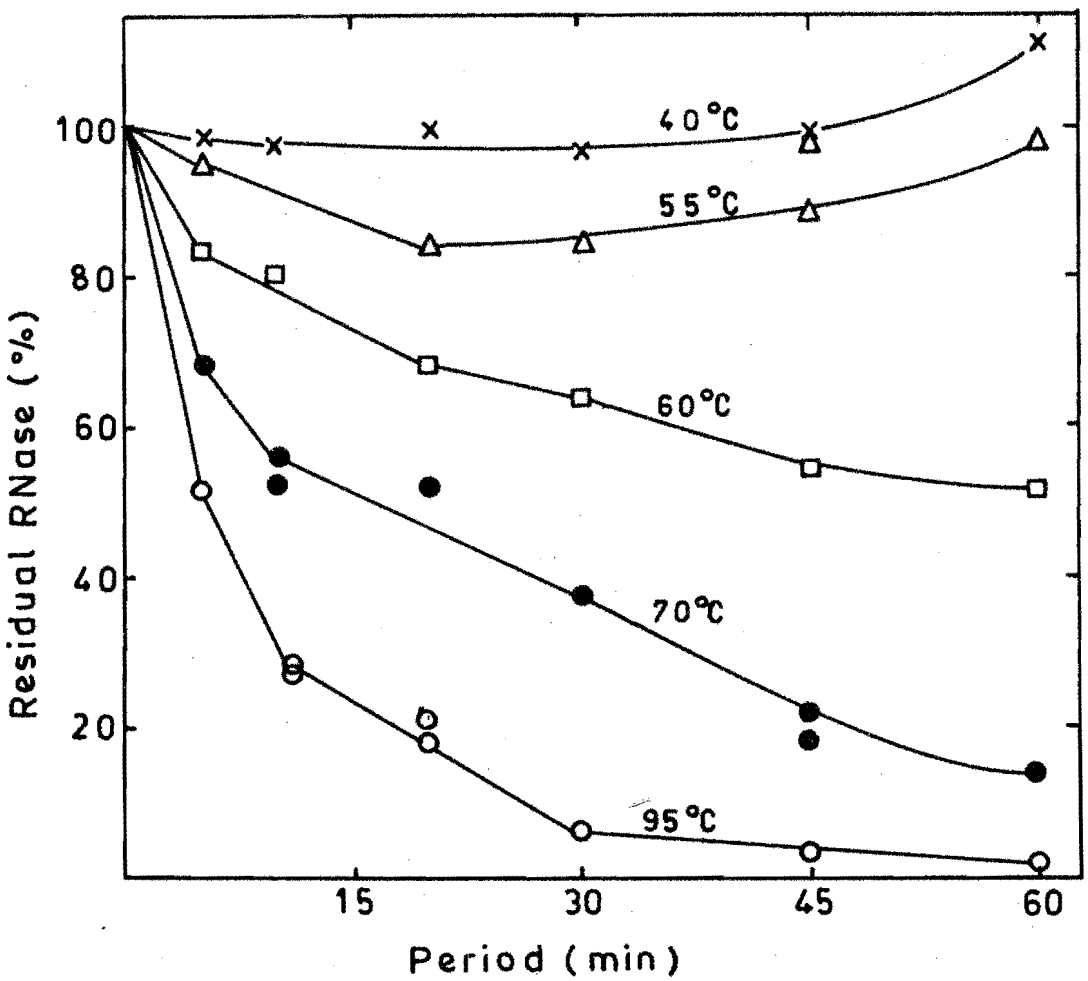

Fig. 22. Heat stability of the purified RNase at different temperatures and periods of incubation.

retention was improved after heat treatment at 55°C. For example, after 45 min. the retention was about 90% and after 60 min. it showed about 98% retention. Similarly, in the case of treatment at 40°C not only there was any significant loss of activity, but also the residual activity showed an improvement over the control, the activity being 110% after 60 min. incubation at 40°C.

5. Effect of metal ions:- The enzyme solution was dialysed against distilled water and the effect of various metal ions on the RNase activity was tested by incorporating the salts of various metals at 2 mM in the reaction mixture. EDTA concentration used was 50 mM. The results are recorded in Table 37. Most of the heavy metals tested were highly inhibitory to the enzyme in the following order: Cu^{2+} Pb^{2+} Fe^{3+} Hg^{2+} Zn^{2+}. Most others had no significant effect. EDTA also did not show any significant effect.

6. Effect of group specific reagents and of photooxidation:- The effect of certain specific chemicals such as sulphydryl reagents, amino acid specific reagents, etc. on the enzyme activity was tested. The different reagents tested, the concentrations used and their effects are given in Table 38. It could be observed that out of 6 sulphydryl reagents tested, only p-CMB and N-bromosuccinimide inactivated the enzyme. N-bromosuccinimide at 1 mM concentration completely inactivated the enzyme, whether this is due to the oxidation

Table - 37

Effect of metal ions on the activity of RNase from *A. candidus* M16a

Metal salt	Relative activity (%)	Metal salt	Relative activity (%)
Control	100.0	$CoCl_2$	97.1
$CuCl_2.2H_2O$	3.1	$MgCl_2$	98.1
$Pb(NO_3)_2$	10.0	$MnCl_2$	100.1
$FeCl_3$	12.6	KCl	111.9
$HgCl_2$	14.7	$CaCl_2.2H_2O$	112.5
$ZnCl_2$	15.7	$BaCl_2.2H_2O$	113.3
LiCl	89.8	EDTA	106.0
NaCl	93.6		

The enzyme was dialysed against distilled water and metal salts were added to a final concentration of 2 mM. EDTA was added at 50 mM concentration. RNase assay was carried out according to standard procedure.

Table - 38

Effect of group specific reagents on the RNase activity

Reagent	Concentration	Residual activity (% of the control)
para-chloromercury benzoate (p-CMB)	1 mM	35.1
	5 mM	25.9
Iodoacetamide	1 mM	107.5
	10 mM	126.2
Malathion	1 mM	98.0
	10 mM	100.0
Sodium azide	1 mM	100.0
	20 mM	100.0
N-bromosuccinimide (NBS)	1 mM	0.00
Glutathione	1 mM	99.7
	10 mM	98.5
Di-isopropylfluorophosphate (DFP)	1 mM	100.0
	20 mM	100.0

of tryptophanes or -SH is not clear. However the inhibition was 65% with 1 mM p-CMP. Therefore N-bromosuccinimide may act by oxidizing -SH also. DFP (1 mM) and glutathione (10 mM) had no effect.

Photooxidation of the enzyme was performed in the presence of dyes such as Rose Bengal and methylene blue. Enzyme concentration in the mixture was 0.285 µg/ml. Two different dye concentrations (viz. 0.3 µg/ml and 3 µg/ml) were tried. The mixtures were kept 20 cm away from a tube light, for different durations and then assayed for the RNase activity.

Photooxidation did not affect the enzyme activity even after 30 min. of exposure.

7. Effect of substrate concentration:- The activity of the enzyme (0.067 µg/ml) was determined at different concentrations of RNA (0.25 to 2.5 mg/ml) at pH 4.5.

It could be seen from the Michaelis-Menton plot made from the values obtained that the rate of enzyme reaction was quite linear upto a substrate (RNA) concentration of 1.25 mg/ml (Fig. 23).

The Lineweaver-Burk plot (Fig. 23) indicated a Km value of 3.64 mg RNA per ml for the RNase from A. candidus M16a.

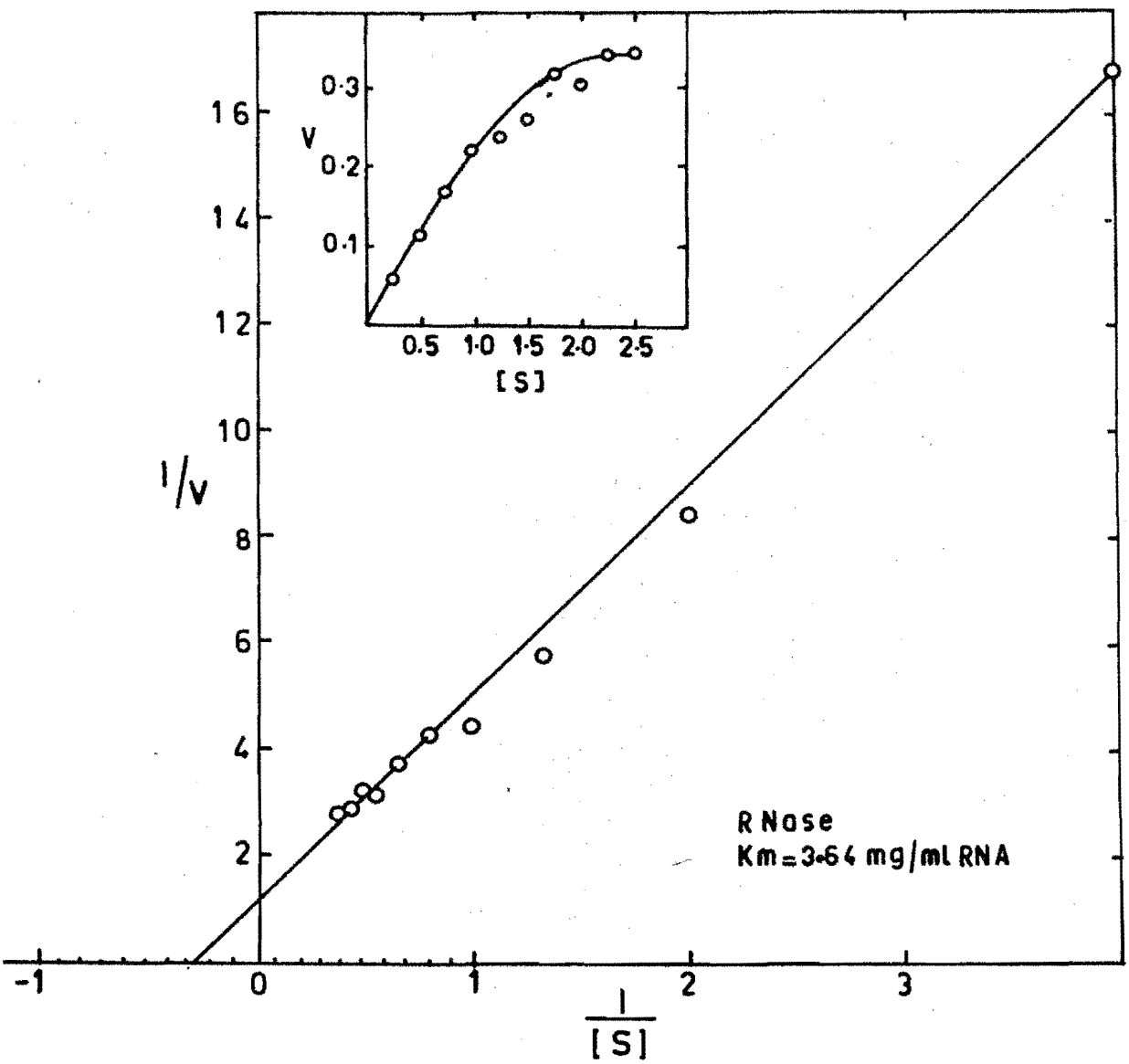

Fig. 23. Lineweaver-Burk plot for RNase (inset Michaelis-Menton plot).

8. Molecular weight:- Molecular weight was determined by gel-filtration method (Andrews, 1964, 1965). A column (1.35 x 92 cm) packed with Sephadex G-100 (130 ml) was equilibrated with 0.05 M phosphate buffer (pH 5.6). Enzyme or standard proteins were loaded individually and eluted with the same buffer with a flow rate of 20-25 ml/hr. 3 ml fractions were collected and their A_{280} was determined. Ovalbumin (M.W. 45,000), pepsin (35,000), α-chymotrypsin (22,500), myoglobin (17,000) and cytochrome C (13,000) were the standard proteins used.

The results obtained are plotted in Fig. 24. From the curve the molecular weight of RNase was extrapolated to be 26,600.

9. Action on polynucleotides:

(a) Rate of hydrolysis of different polynucleotides:- The rate of hydrolysis of different polynucleotides such as yeast RNA, calf-thymus DNA (both native and heat-denatured forms), poly A, poly G, poly U and poly C was determined. 0.4 ml reaction mixture containing 0.5 mg of substrates, 0.1 M acetate buffer (pH 4.5) and 150 units of enzyme was incubated at 55°C for 10-60 min. Reaction was stopped by the addition of 0.4 ml of uranyl reagent. Other details are as in the standard RNase assay procedure.

The results are presented in Fig. 25. It could be seen from the graph that poly U followed by yeast RNA were

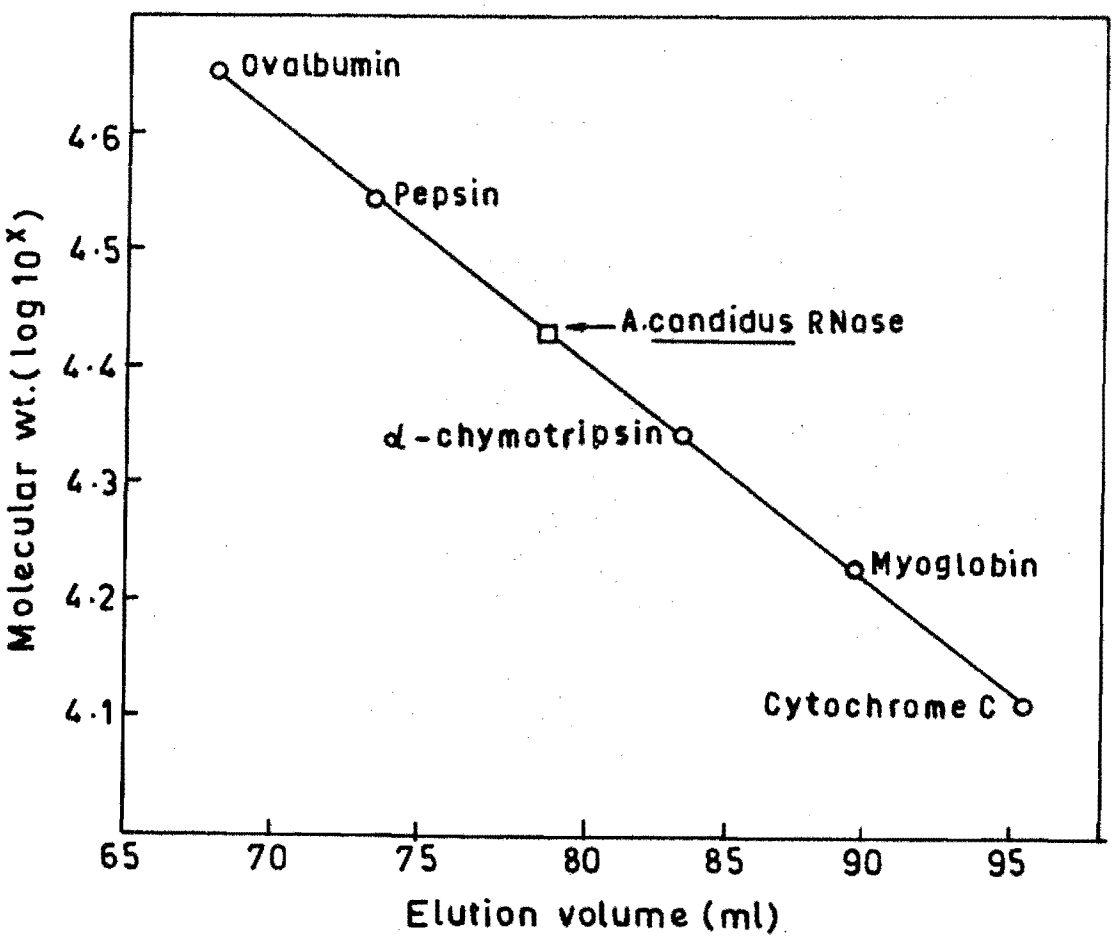

Fig. 24. Molecular weight determination of the RNase by Gel-filtration on Sephadex G-100.

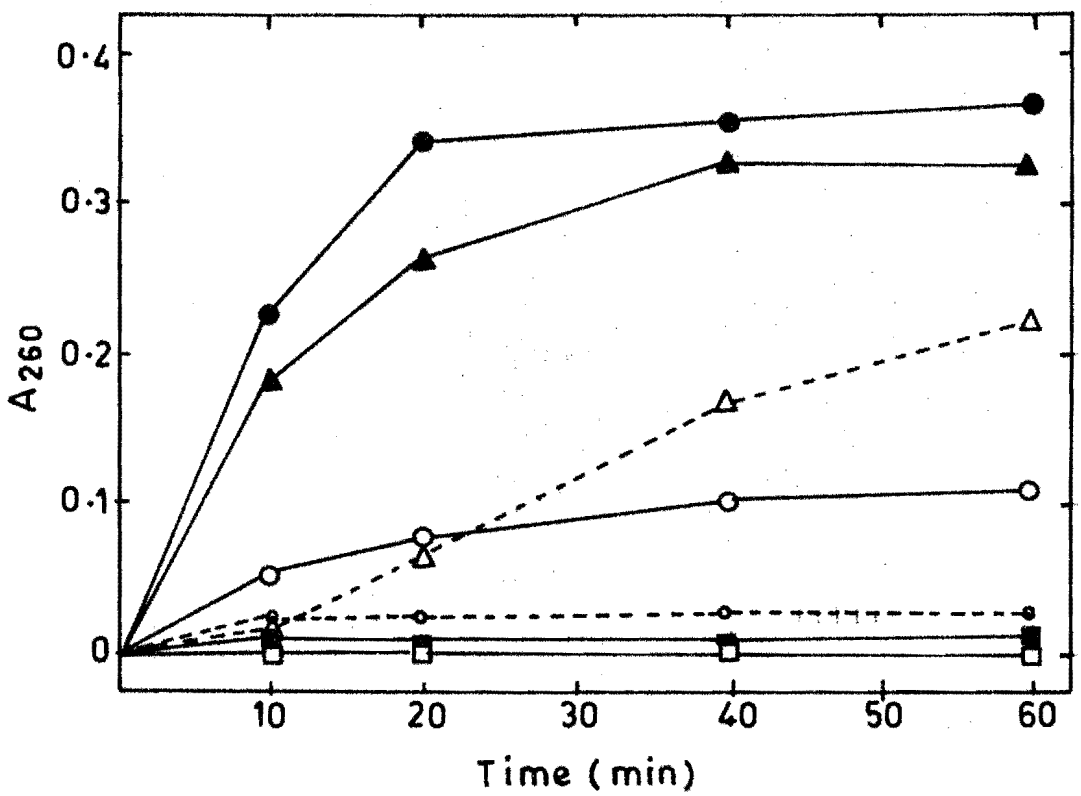

Fig. 25. Time course of degradation of various polynucleotide substrates by RNase. poly U (-●-), yeast RNA (-▲-), poly C (-△-), poly A (-○-), heat denatured DNA (—○—), native DNA (-■-) and poly G (-□-).

the most susceptible substrates for this enzyme. Hydrolysis of poly C and poly A were comparatively slow and that of both native DNA and heat-denatured DNA was negligible. Poly G was not hydrolysed even after 60 min. of incubation. The rate of hydrolysis of these different substrates were in the order: poly U> RNA >> poly C> poly A.

(b) Rate of release of mononucleotides from yeast RNA:- In order to find out the base specificity/preference of the enzyme the rate of release of the mononucleotides from yeast RNA was followed using different concentrations of the enzyme. One ml reaction mixture containing 2 mg purified RNA, 0.05 M acetate buffer (pH 4.5), and different amounts of the enzyme, viz. 0.05, 0.1, 0.2, 0.5 and 2.5 µg were incubated at 55°C for 60 min. After 60 min. incubation 50 µl of sample from each tube was spotted on a Whatman No.3 MM chromatographic paper and developed by solvent system I for paper chromatography as described in "Materials and Methods".

The spots on the paper were located by observing under U.V. light (254 nm) and identified comparing with spots of standard mononucleotides. The spots corresponding to 3' AMP, 3' UMP and 3' CMP were cut out and eluted with 0.1 N HCl overnight. The absorbance of the eluant at 260 nm was determined and plotted as percentage of the maximum value of the same nucleotide obtained in the case

of maximum concentration of enzyme. As already mentioned in the previous section, the hydrolysis of poly G was almost nil. As could be seen in the succeeding section, prolonged hydrolysis of RNA yielded a mixture of mono- and oligonucleotides of guanine.

From Fig. 26 it could be seen that the release of AMP was the fastest. This was followed by UMP and then CMP. Thus, the rate of release of mono-nucleotides from yeast RNA by RNase of A. candidus M16a was in the order: AMP > UMP >> CMP >>>> GMP. This is different from what was observed in the case of hydrolysis of homo-polymers of the four nucleotides, where the order of rate of hydrolysis was poly U > poly C >> poly A >>>>> poly G. The reason for this behaviour will be discussed later.

(c) Products of hydrolysis of RNA by RNase:- Purified yeast RNA and homopolyribonucleotides was hydrolysed with different amounts of RNase for different durations and the products formed were analysed by various techniques, viz. paper chromatography, thin layer chromatography and spectrophotometry, as described in "Materials and Methods".

Of 3 different solvent systems used Solvent System I (Isobutyric acid : glacial acetic acid : 1 N ammonium hydroxide - 10:1:5) was found to be the best for separation of 3' nucleotides from 5' nucleotides. By this system the products of hydrolysis of RNA upto 60 min. were resolved

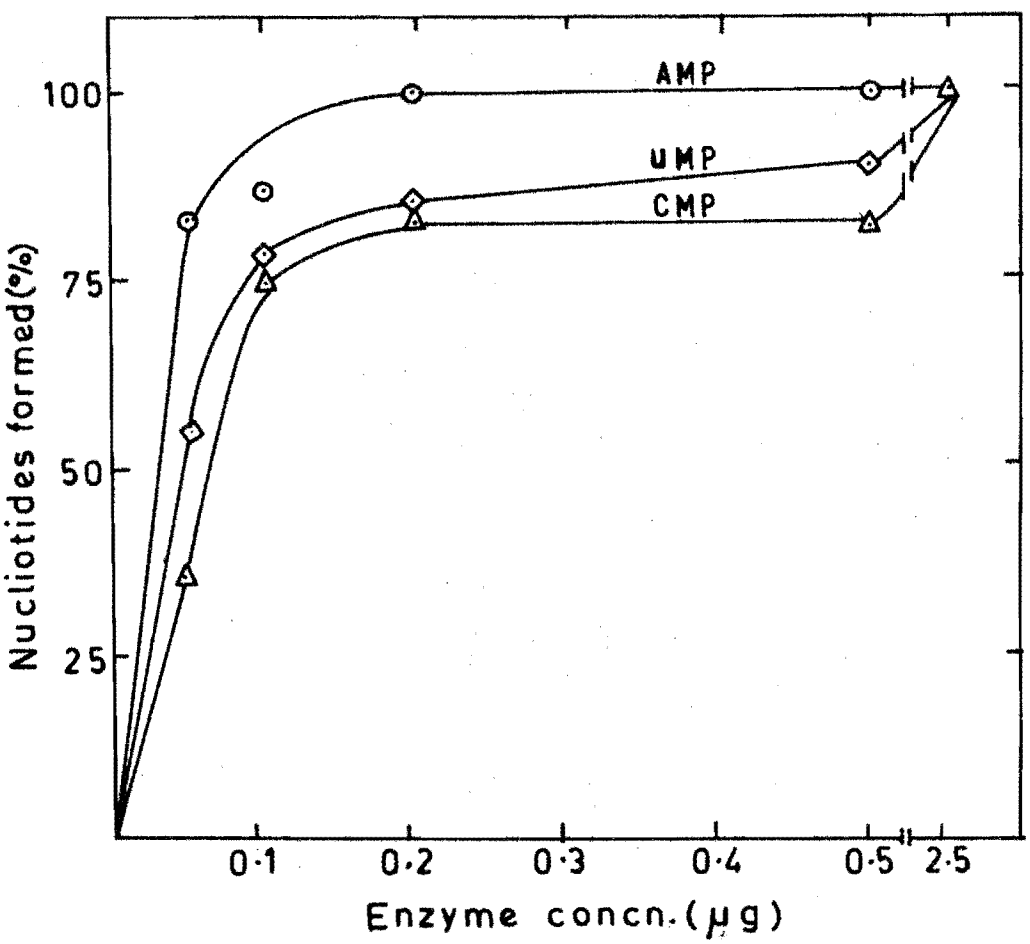

Fig. 26. Rate of release of mononucleotides from RNA with different concentrations of RNase.

into 4 major spots, the first 3 with higher Rf values being 3'-AMP, 3'-CMP and 3'-UMP respectively. The other one did not correspond with 3'-GMP. It had a lower Rf value than that of 3'-GMP. It might be an oligonucleotide of guanine. However, after 8 hrs incubation with excess enzyme a spot corresponding to 3'-GMP appeared, besides another spot with lower Rf. Similar results were obtained in TLC studies also.

Thus, the final products of hydrolysis of yeast RNA by RNase of A. candidus M16a were identified to be 3'-AMP, 3'-UMP, 3'-CMP and a mixture of 3'-GMP and oligonucleotides of guanine.

The separation pattern of these compounds in the RNA hydrolyzate is diagramatically presented in Fig. 27, as resolved in a paper chromatogram using solvent System I.

(d) Intermediate products of hydrolysis:- Attempts were made to determine the intermediate products of hydrolysis of yeast RNA, poly A, poly U, poly C and poly G. One ml reaction mixture contained 2 mg substrate, 0.1 M acetate buffer (pH 4.5) and 0.2 µg enzyme. The reaction was stopped at 5 min, 10 min, 60 min. and 8 hrs intervals and 50 µl aliquotes were used for paper chromatography using the Solvent System 4 (saturated $(NH_4)_2SO_4$, isopropanol, 1 M sodium acetate - 80:2:20) which was done according to Markham and Smith (1952).

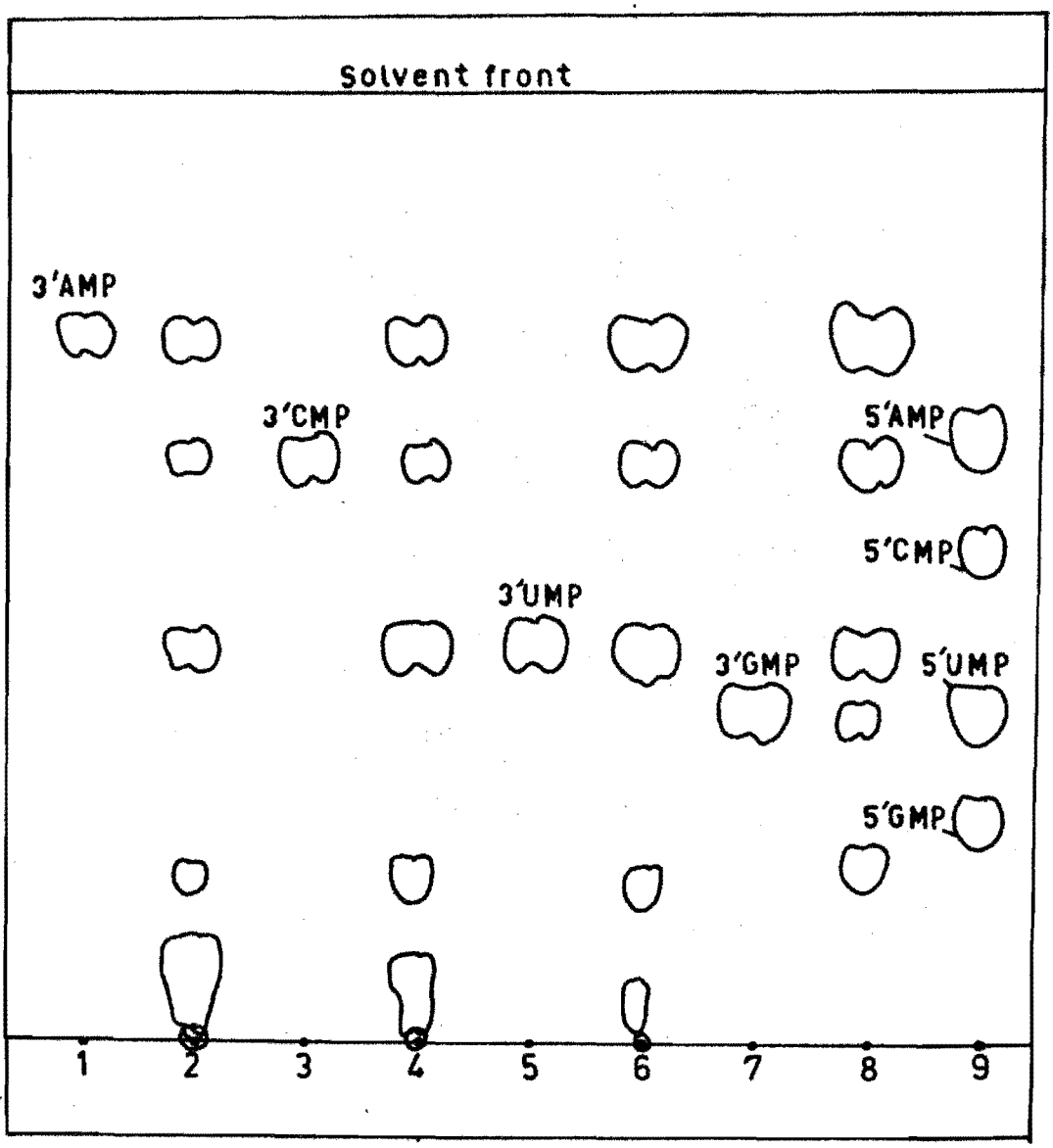

Fig. 27. Diagram of paper chromatogram of RNA hydrolysates after different periods of hydrolysis and of standard nucleotides(Developed by solvent system I). 1) 3'-AMP, 2) 10 min. hydrolysate, 3) 3'-CMP, 4) 30 min. hydrolysate, 5) 3'-UMP, 6) 60 min. hydrolysate, 7) 3'-GMP, 8) 8 hrs hydrolysate and 9) 5'-ribonucleotides.

The results obtained are diagramatically presented in Figs. 28 and 29. Spots corresponding to 2',3'-cyclic nucleotides, 3'-nucleotides and oligonucleotides appeared during short periods of incubation. Spots corresponding to 2',3'-cyclic nucleotides and oligonucleotides disappeared on prolonged incubation yielding 3'-nucleotides.

A long trailing spot was observed at the bottom which slowly faded with increase in time of incubation which finally disappeared after 8 hrs. This indicated the complete hydrolysis of RNA by the RNase. This also is suggestive of the endonucleolytic mode of action of the enzyme.

10. Mode of action:- To determine whether the enzyme acts endonucleolytically or exonucleolytically the following experiment was carried out according to the procedure of Birnboim (1966) and Omori et al. (1978).

The reaction mixture of total 2 ml containing 2 mg purified RNA and 0.5 µg enzyme and 0.02 M acetate buffer (pH 4.5) was incubated for different intervals such as 0, 2, 5, 15, 30 and 60 min. The reaction was stopped by the addition of 0.5 ml of Tris-HCl buffer (pH 7.5) and heating in a boiling water bath for 10 min. 0.3 ml aliquots of these were loaded separately on a Sephadex G-100 column (1 cm x 25 cm) and were eluted with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl (Birnboim, 1966).

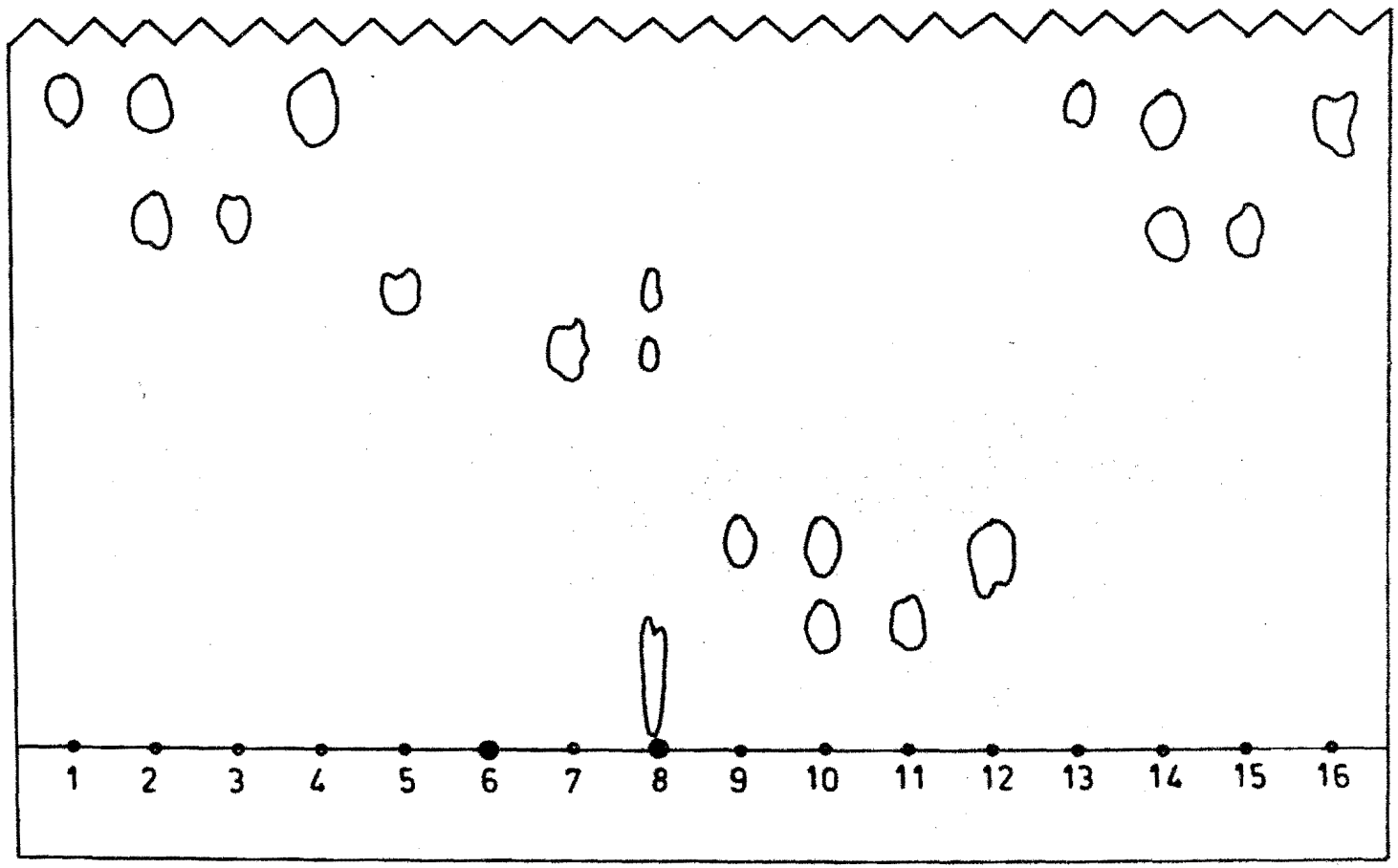

Fig. 28. Diagram of paper chromatogram of hydrolysates of various homopolyribonucleorides after different periods of hydrolysis and of standard mononucleotides (Developed by solvent system 4). 1) 3'-UMP, 2) hydrolysate of poly U (60 min.), 3) 2',3'-cyclic UMP, 4) hydrolysate of poly U (8 hrs), 5) 3'-GMP, 6) poly G (60 min), 7) 2',3'-cyclic GMP, 8) hydrolysate of poly G (8 hrs), 9) 3'-AMP, 10) hydrolysate of poly A (60 min), 11) 2',3'-cyclic AMP, 12) hydrolysate of poly A (8 hrs), 13) 3'-CMP, 14) hydrolysate of poly C (60 min), 15) 2',3'-cyclic CMP, 16) hydrolysate of poly C (8 hrs).

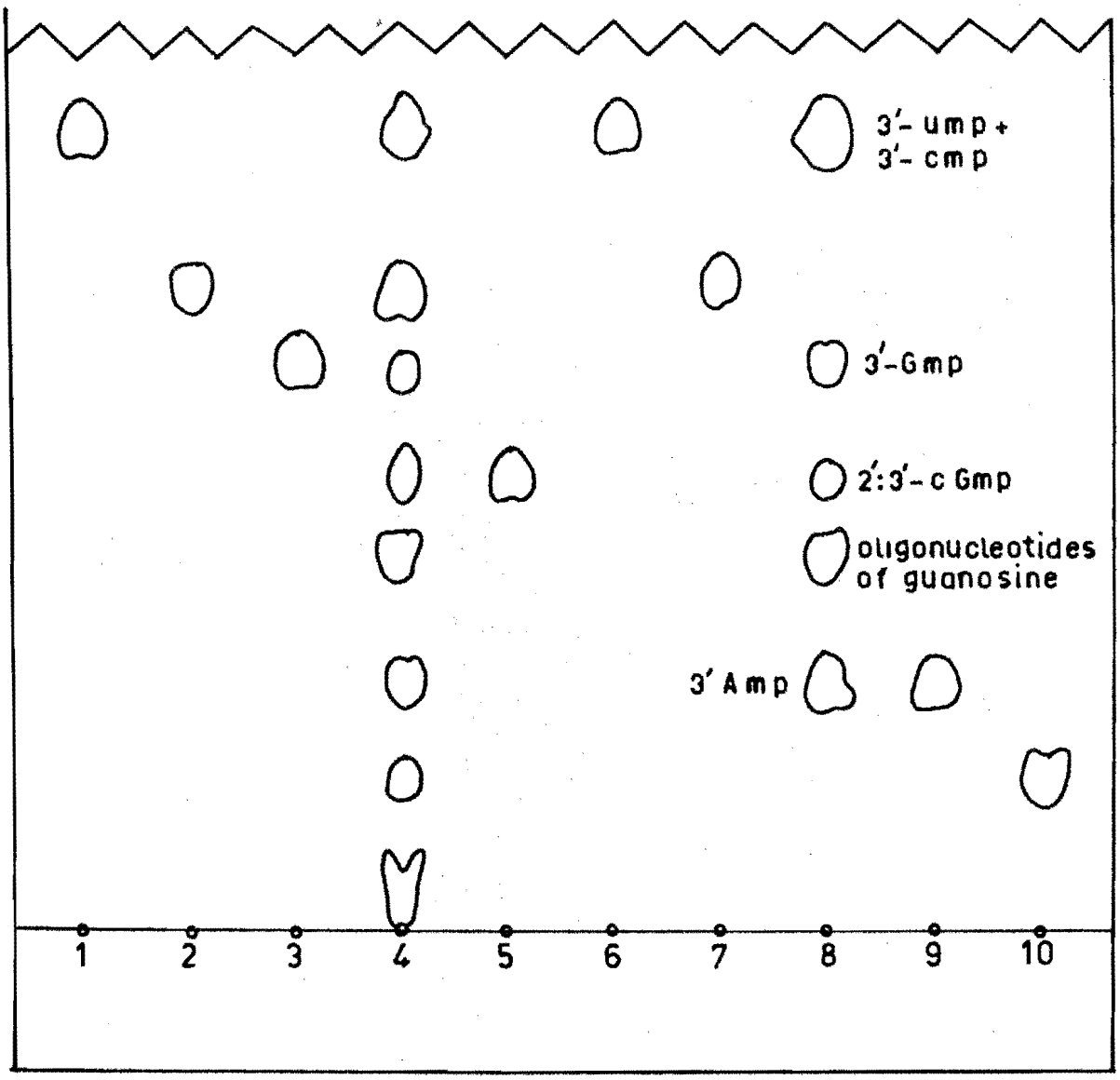

Fig. 29. Diagram of chromatogram of RNA hydrolysates and of standard nucleotides. (Developed by solvent system 4). 1) 3'-UMP, 2) 2',3'-cyclic UMP, 3) 3'-GMP, 4) RNA hydrolysate (1 hr), 5) 2',3'-cyclic GMP, 6) 3'-CMP, 7) 2',3'-cyclic CMP, 8) RNA hydrolysate (8 hrs), 9) 3'-AMP and 10) 2',3'-cyclic AMP.

The flow rate was maintained at a rate of 35 ml/hr and 2.5 ml fractions were collected. The absorbance of the fraction at 260 nm was detected spectrophotometrically.

The results are given in Fig. 30. Samples of reaction mixtures incubated for different periods resolved only into a single peak as against the expectations. However, the peak height increased with increase in reaction time, suggestive of the hyperchromicity due to breakage of the polynucleotide chain. As expected, it should have resolved into two distinct peaks, one corresponding to the mononucleotides and the other corresponding to the residual RNA chain, if the attack happened to be exonucleolytic. On the other hand, it should have been a broader peak, which would have resolved in between the two sharp peaks, if the action happened to be endonucleolytic. But, the results obtained did not correspond to either of these patterns. This indicates that this method cannot be applied to RNA as the substrate as reported in the case of DNA.

11. Other enzyme activities:- The purified RNase was tested for different activities, such as, DNase, non-specific phosphodiesterase and nucleotidase activities. The assays were carried out according to the procedures described under "Materials and Methods"

The purified enzyme did not have any of these activities whereas the crude enzyme preparations did show these activities.

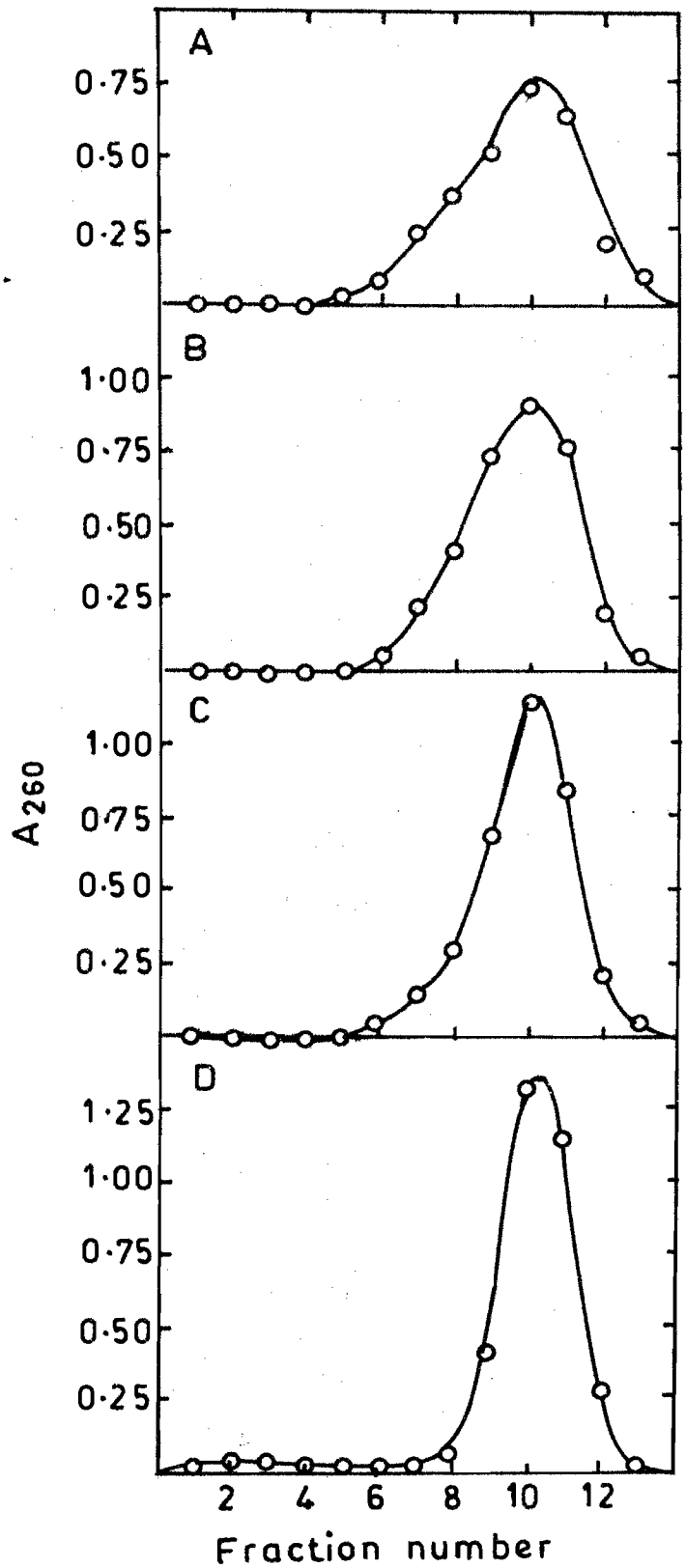

Fig. 30. Elution pattern of RNA hydrolysates after 0 min. (A), 2 min. (B), 5 min. (C) and 15 min. (D) hydrolysis from Sephadex G-100 column.

DISCUSSION

A. PURIFICATION

Purification of a number of other RNases from various sources has been described in literature. Different methods have been followed for the purification of the same RNase from the same source by different workers. Yields of the pure enzyme have been improved by using improved techniques from time to time. For example, RNase T_1 from Takadiastase powder has been purified by different workers (Takahashi, 1962; Rushizky and Sober, 1962; Uchida, 1965; Ishiwata and Yoshida, 1978). In a recent method RNase T_1 and other guanyloribonucleases such as RNase N_1, RNase F_1, and RNase St were purified by affinity chromatography on guanylyl (-2',5'-) guanosine coupled to aminohexyl Sepharose 4B (Ishiwata and Yoshida, 1978).

Most of the other procedures used for purification of RNases involved the common methods of protein purification such as $(NH_4)_2SO_4$ or solvent precipitation, gel filtration, and ion-exchange chromatography. As could be seen in literature ion-exchange chromatography on DEAE-cellulose or CM-cellulose by either gradient elution or batchwise elution is one of the important steps of purification of RNases from different sources. For example,

RNases U_1, U_2, U_3, and U_4 from U. sphaerogena (Arima et al. 1968), RNase T_1 from Takadiastase (Uchida, 1965), RNase T_2 from the same source (Uchida, 1966), RNase M from A. saitoi (Irie, 1967), RNase from A. niger NRC-A-1-233 (Horitsu et al. 1974), RNase from S. erythreus (Tanaka, 1961), and three RNase fractions from rice bran (Yokoyama, 1982) were purified by using DEAE-cellulose chromatography as one of the steps. 2,100-fold purification with 26% yield of B. subtilis K RNase involved DEAE-Sephadex A-50, Amberlite IRC-50 and Sephadex G-75 column chromatographies (Yamasaki et al., 1970). Acetone precipitation, column chromatographies on Duolite A-2 and DEAE-cellulose, repeated chromatography on DEAE-Sephadex A-50 column and affinity chromatography on 5'-AMP-Sepharose-4B column and crystallization by dialysis against $(NH_4)_2SO_4$ solution at 60% saturation were the steps involved in the purification of RNase L from Aspergillus sp. (Tomoyeda et al., 1977). When compared to these methods the procedure followed in the present case for the purification of A. candidus M16a RNase (Table 37) seems to be simpler. It involved 6 steps: fractionation with ethanol (30-50%), gel-filtration on Sephadex G-100, DEAE-cellulose adsorption and batchwise elution with a buffer containing NaCl and a final gradient elution from DEAE-Sephadex A-50 column.

It was observed that the major contaminating protein almost till the end of the purification steps was a protease

enzyme. However, this wa. finally separated by gradient elution from DEAE-Sephadex A-50 column.

A reduction of specific activity (from 34,000 units to 28,000 units/mg) observed on the second DEAE-cellulose chromatography may be, probably, due to the denaturation of RNase by protease.

B. PROPERTIES

pH optimum: Wide variation has been observed in pH optimum for RNases from different sources or different RNases from the same source (Barnard, 1969; Egami and Nakamura, 1969; Kuninaka, 1976). The pH optimum is generally neutral or alkaline for animal RNases, and either acidic, neutral or alkaline for plant and microbial RNases. RNase L from A. niger was most active at a highly acidic pH, 3.5 (Tomoyeda et al., 1977) and RNase N_1 from N. crassa at pH 7.0 (Takai et al., 1966). Many fungal RNases have been reported to be optimally active at pH 4.5. Examples are the RNase from Monascus pilosus (Rushizky et al. 1964), RNases U_2 and U_3 of U. sphaerogena (Egami and Nakamura, 1969), RNase from Endomyces (Hattori and Nakamura, 1966), RNase Ms from A. saitoi (Irie, 1967) and the well-known RNase T_2 of A. oryzae (Egami and Nakamura, 1969). Though the present enzyme is similar to these RNases in its pH optimum it has been found to be different from

these RNases in many other properties.

Josefsson and Lagerstedt (1962) and Barnard (1969) discussed the importance of pH optimum in the classification of RNases. They also cautioned about the importance of the methodology of pH optimum determination, as it was a variable feature of the enzyme (at least in some cases). In the case of pancreatic RNase it was shown that the pH optimum varied considerably with the substrate and with the ionic strength (Kalnitsky et al. 1959). With the Mg^{2+} or Ca^{2+} dependent enzymes, modest changes in the cation concentration could also shift the pH dependence (Cautrecasas et al. 1967). It is evident, now, that in the presence of different ions or inhibitors/activators (Josefsson and Lagerstedt, 1962), the pH optimum of a crude enzyme preparation might be different from that of a pure enzyme. However, in the present case, the pH optimum of the crude enzyme (Fig. 5) is same as that of the pure enzyme (Fig. 19).

Temperature optimum: is an important criterion as far as the industrial application of an enzyme is concerned. In the present case the RNase is found to be optimally active at 55°-60°C as for the RNases have been reported to be active at temperatures around 50°-60°C. For example, RNase M and RNase Ms from A. saitoi are optimally active at 50°C (Irie, 1967 and 1969).

Similarly, RNase Rh from R. niveus also is active around 50°C (Tomoyeda et al. 1969). A number of nucleases, Nuclease P_1 (from P. citrinum) and nucleases from other species of Penicillium (Fujimoto et al. 1977a), nuclease from Phoma cucurbitacearum (Tone and Ozaki, 1968), etc. however are most active at 70°C.

In the present case the crude enzyme showed a sharp peak at 55°C, the activity at 60°C being only about 80% of that at 55°C (Fig. 5), whereas the pure RNase had a broader temperature optimum from 55° to 60°C (Fig. 19). The higher thermolability of the crude enzyme is apparently due to the protease present.

pH and thermostability: The present enzyme was most stable at a narrow pH range of 5-6. It was more stable in potassium phosphate buffer than in sodium acetate buffer. When compared to the crude preparations, the pure enzyme was found to be more stable at higher pH values, i.e. above pH 5.5 (**Fig.** 21). It is interesting to note that the enzymes having their pH optima for activity at acidic side are more stable at neutral or alkaline pH and those with pH optima at neutral or alkaline side are more stable at acid pH values. For example, RNase N_1 having pH optimum 7.0 (Takai et al. 1966) and RNase T_1 with pH optimum at 7.3-7.5 (Egami and Nakamura, 1969) are highly stable at acidic pH, whereas RNases U_2 and U_3 and RNase T_2 having pH

optima at 4.5 are more stable at neutral pH (Arima et al., 1968; Egami and Nakamura, 1969). In the present case also it is more stable around pH 6.0.

The present RNase was found to be fairly heat-stable when maintained at pH values between 5 and 6. Many other microbial RNases are also heat-stable. RNases T_1, T_2, N_1 (Egami and Nakamura, 1969), U_2, U_3 (Arima et al. 1968) and a RNase from S. erythreus (Tanaka et al. 1966) are all heat-stable to varying degrees and at different pH values.

Effect of metal ions and group specific reagents: Many RNases including the one under study here are inhibited by heavy metal ions such as Cu^{2+}, Hg^{2+}, Pb^{2+} and Zn^{2+}. Mg^{2+}, Ca^{2+} and Mn^{2+} had no effect on the enzyme of A. candidus M16a.

The inhibition of various other RNases by heavy metals were as follows: RNase T_1 by Zn^{2+}, Mg^{2+}, Ca^{2+} and Mn^{2+} (Rushizky and Sober, 1962), RNase T_2 by Cu^{2+}, Zn^{2+}, and Hg^{2+} (Egami and Nakamura, 1969), RNase Rh by Zn^{2+}, Cu^{2+} and Hg^{2+} (Tomoyeda et al. 1969), RNase M by Cu^{2+}, Zn^{2+}, Hg^{2+} and Cd^{2+} (Irie, 1967), RNases Tf-3 and Tf-4 from tea leaves by Cu^{2+} and Hg^{2+} (Tsushido and Takeo, 1976), RNase RB-3 from rice bran by Cu^{2+} (Yokoyama et al. 1982), RNase Ms by Cu^{2+} and Hg^{2+} (Ohgi and Irie, 1975) and RNase from A. niger NRC-A-1-233 by Pb^{2+} and Cu^{2+}. However, no inhibition was caused by Hg^{2+}, Fe^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+},

Mg^{2+} and Mn^{2+} in the last mentioned case (Horitsu et al. 1974). It could be seen that Cu^{2+} is the most highly inhibitory metal ion in most of the cases. So was in the present case too. However, the present enzyme does not seem to be similar to any other RNase as far as its response to various metal ions is concerned, though it looks similar to RNase T_2 in its sensitivity to Cu^{2+}. But its sensitivity is greater towards Zn^{2+}, Pb^{2+} and Hg^{2+} whereas RNase T_2 is less sensitive to these metals. In conclusion it could be said that the present RNase is different from other fungal RNases in its response to metal ions.

Effect of EDTA and of temperature have been used as one of the criteria for the classification of non-specific nucleases by Fujimoto et al. (1977a). They showed that at least among strains of Penicillium, 5'-P-forming, P_1 type of nucleases are EDTA-sensitive and heat tolerant whereas 3'-P-forming nucleases are EDTA-insensitive and thermolabile. However, this method of classification does not hold good for RNases. Most of the thermostable RNases were EDTA-insensitive 3'-P-forming enzymes, for example, RNases T_1, T_2, U_1, U_2 and U_3 (Egami and Nakamura, 1969). Moreover, these enzymes were activated by EDTA. The present enzyme behaves similarly. Some enzymes such as

RNase N_1 (Egami and Nakamura, 1969) an intracellular RNase from A. oryzae (Uozumi et al. 1968) and a RNase from B. subtilis (Yamasaki and Arima, 1970) were shown to be inhibited by EDTA. But dialysis restored the enzyme activity. The present enzyme is dissimilar to these enzymes in this respect.

Modification of amino acids: Evidence that tryptophane residues may be involved in the activity of RNase is available in the case of the R. niveus enzyme (Sanda and Irie, 1980). Similarly in the present instance tryptophane seems to be part of the active site or necessary for the active conformation of the enzyme, since NBS abolished enzyme activity; however more definitive data regarding content of tryptophane, -SS- and histidine before and after NBS treatment are necessary before any definitive conclusion can be drawn.

The involvement of -SH group, serine and histidine were ruled out by its insensitivity to the various other reagents and photo-oxidation. However, in many instances the histidine moiety seems to be involved in the activity of RNases from different sources: bovine pancrease, RNase A (Weil and Seibles, 1955), A. oryzae, RNase T_1 (Takahashi, 1973), seminal vesicles (Irie, 1974), R. niveus, RNase Rh (Sanda and Irie, 1979), A. saitoi, RNase Ms (Ohgi and Irie, 1980). In this respect the present enzyme seems to be

different from many other reported RNases.

Molecular weight: RNases are usually small molecules. The molecular weight of non-specific RNases seem to range from 30,000 to 40,000 while the base-specific ones have one of 10,000 to 13,000 (Egami and Nakamura, 1969). Base-specific RNases such as RNases U_1, U_2 and U_3 from U. sphaerogena (Egami and Nakamura, 1969), RNase N_1 from N. crassa (Takai et al. 1966) have a molecular weight of 10,000 and RNase T_1 11,000 (Uozumi et al. 1968). Non-base-specific RNases such as RNase N_2 from N. crassa (Egami and Nakamura, 1969), RNase M from A. saitoi (Irie, 1967), RNase T_2 (Uozumi et al. 1968), RNase RB-2 from rice bran (Yokoyama et al. 1982) have a molecular weight around 36,000. In this respect the present base-non-specific enzyme has an intermediate molecular weight of 26,600 and again is dissimilar to these two groups of RNases. There are, however, other instances of base-non-specific RNases which have similar molecular weights; RNase Rh having molecular weight of 24,000 (Komiyama and Irie, 1971), RNase from A. niger NRC-A-1-233, molecular weight 28,500 (Horitsu et al. 1974) and a RNase from B. subtilis, molecular weight 25,000 (Yamasaki and Arima, 1970).

Base-specificity: As already mentioned many RNases are base-specific in their action on RNA substrates. They specifically cleave a phosphodiester bond next to a

particular base. Thus a number of RNases from a variety of sources has been reported to be purine specific and particularly for guanine (Table 2). Many others were found to be base-non-specific even though some of them showed preference for a particular base, i.e. they can cleave the bonds between any base, though at varying rates. It is interesting to observe that base-specificity is observed only among RNases forming 3'-nucleotides, whereas all the 5'-P-forming nucleases are base-non-specific.

The products of hydrolysis of RNA may be either 5'-mono, -di, -tri or oligonucleotides or they may be 3'-isomers of these. However, in a few cases 2',3'-cyclic nucleotides have been reported to be the final products as with RNase Rh (Imazawa et al. 1968). In most of the cases 3'-nucleotides are formed through the intermediate product formation of 2',3'-cyclic nucleotides. Non-cyclizing, 3'-P-forming RNases are very rare among microorganisms; so far the only such instance is from baker's yeast (Ohtaka et al. 1963). The RNase studied here is base-non-specific, cyclizing, 3'-P-forming RNase. Apparently many RNases release from yeast RNA 3'-adenylate more rapidly than other nucleotides as in these investigations: A, U > G > C RNase T_2 (Egami and Nakamura, 1969) and RNase M (Imazawa et al. 1968). However, the rate of release of nucleotides from RNA differs markedly: RNase Ms (Ohgi and Irie, 1975) and RNase Rh (Imazawa et al. 1968) release

nucleotides in the order: G > A > C > U and A > G > C > U respectively while RNase L (Tomoyeda et al. 1977) formed 3'-nucleotides in the order: U > C > A > G. Again it could be seen that the enzyme under study here is somewhat different from the other well studied 3'-P-forming, cyclizing RNases.

Many workers have followed the rate of hydrolysis of various homopolynucleotides as a criterion for the determination of the base-specificity of RNases (Barnard, 1969). In the present case the hydrolysis rate of different polynucleotides under similar conditions were in the order: poly U > yeast RNA >> poly C > poly A. There was apparently no hydrolysis of poly G. Only a very negligible breakdown of DNA was observed. At this juncture, it would be interesting to note that rate of hydrolysis of homopolymers of particular bases were different from the rate of release of the same base containing nucleotides from yeast RNA. As already mentioned the rate of release of nucleotides from RNA was in the order: AMP > UMP > CMP >>> GMP. The rate of hydrolysis of the homopolymer of uridine was much faster than that of poly A. But, the release of adenylic acid was faster than the other bases from yeast RNA. A similar situation was observed in the case of RNase T_2 also. It was shown that poly A was fairly resistant to RNase T_2 inspite of the preferential cleavage of adenylic acid linkages in RNA, whereas poly U was the most sensitive

(Uchida and Egami, 1967). It was explained that it may be due to the fact that poly A forms a rigid, double-stranded helical structure in acidic medium and poly U does not have any secondary structure under these conditions and is easily digested. The same explanation could be given in the present case also, as the reaction was carried out at acidic pH (4.5) in this case too. An RNase from B. subtilis K hydrolysed homopolymers in the order: poly U > poly C > poly A; poly G and DNA were not attacked (Yamasaki et al. 1970). The enzyme studied here also behaved similarly. However, B. subtilis K enzyme formed 2',3'-cyclic mononucleotides whereas in the present case the end products were 3'-mononucleotides. Other nucleases showed different patterns: nuclease O from A. oryzae, poly A > poly U > poly C and not poly G (Ouzumi et al. 1968) and rice bran enzyme RB_3, yeast RNA > poly A > poly C > poly U > poly I (Yokoyama et al. 1982).

The present enzyme seems to be endonucleolytic in its mode of action on polynucleotides. However, a method tried for testing this property, i.e. by separation of the products formed from residual RNA at various stages of hydrolysis failed to yield any definitive results as already explained in Results Section. This method has been shown to be an effective tool in distinguishing between endo and exo-nucleolytic types of degradation of DNA by different

DNases and has been suggested to be useful in the studies of RNases also (Birnboim, 1966; Omori et al. 1978). In the present case a separation of the residual RNA from the products was not possible under the conditions tried (Fig. 30). However, from paper chromatographic evidences it could be presumed to be an endonucleolytic enzyme, as long trailing spots were observed in the chromatograms of partially digested RNA by the enzyme. As already mentioned, it is a cyclizing enzyme forming 2',3'-cyclic nucleotides as intermediate products. Partially digested RNA as well as, homopolymers showed a mixture of 3'- and 2',3'-cyclic mononucleotides as products, which after longer period of hydrolysis showed only 3'-nucleotides (Figs. 28 and 29).

CHAPTER - 4

REDUCING THE NUCLEIC ACID CONTENT OF SINGLE CELL PROTEIN AND RECOVERY OF THE PRODUCTS OF HYDROLYSIS

SECTION A

APPLICATION OF RNase FROM A. CANDIDUS M16a FOR REMOVAL OF NUCLEIC ACID CONTENT IN YEAST CELLS

Single cell proteins are a promising means of enhancing protein supplies both for feed and food purposes. A variety of microorganisms and substrates are used to produce SCP (Mateles and Tannenbaum, 1968; Tannenbaum and Wang, 1975; Davis, 1974). Nutritionally the quality of SCP has been shown to be good and supplementation of cereals with SCP, especially yeasts makes them as good as animal proteins (Kihlberg, 1972; Lovland et al., 1976; Chen and Peppler, 1978). They have, in general, been found to be safe for both humans and animals from the view points of toxicity, pathogenicity and carcinogenicity (Garattini et al., 1979). However, one possible drawback or disadvantage is their high nucleic acid content. This has to be reduced to low levels if they have to be used for food purposes.

Several methods have been developed for reducing the nucleic content in SCP and they can be classified broadly into three groups: 1) Control of the growth conditions, 2) Removal of nucleic acid from whole cells, and 3) Removal of protein from cell homogenates (eliminating the nucleic acids).

Among the various methods of removal of nucleic acids from the whole cells are: i) the induction of intracellular RNase(s) by chemical or/and physical treatments (Maul et al., 1970; Cooney and Levine, 1972; Canepa et al., 1972; Chao, 1974; Sinskey and Tannenbaum, 1975) and ii) exogenous application of RNases (Schlenk and Dainko, 1965; Castro et al., 1971). Castro et al., used bovine pancreatic RNase A (RNase I) on Candida utilis and C. intermedia cells. Optimum conditions were an initial heat shock at 80°C for 30 sec. followed by an incubation at 55-60°C and pH 6.7-8.0 for upto 90 min. using an enzyme to cell ratio of 1:10,000. By this method they were able to show a reduction of nucleic acid content from the initial 7.5-9.0% to 1-2%, without any significant concomitant loss of protein. However, the prohibitive cost of this process and also the limited availability of the enzyme would make this technique impractical. Hence, an economic source of an enzyme that is capable of doing the same job had to be sought. As could be seen from the preceding sections, here is a good source of RNase with a potentiality for application. Hence, the possibility of using this enzyme for the reduction of the content of nucleic acids in yeast biomass was tested.

Experimental

Studies were carried out to optimise various parameters for the maximal removal of nucleic acids and also

to reduce the concomitant loss of protein from the cells. Five strains of yeast belonging to two genera were grown on Medium 11 for the production of biomass. The pertinent details are given in the "Materials and Methods" Section.

In all the studies, the yeast cell suspension containing 5 mg dry weight of cells per ml in 0.05 M sodium acetate (pH 4.5) was used unless otherwise mentioned.

Pre-treatment of the cells:

The cells were pre-treated to make them permeable to the enzyme.

(a) Heat treatment:- The effect of heat treatment on the improvement of permeability of cells of all five strains of yeast to RNase was tested. Cell suspensions were heated at 98°C for 5 minutes, cooled, purified RNase added (1 µg/ml) and incubated at 55°C on a shaker water bath (reciprocal shaking with an amplitude of 2 cm) for 90 min. After incubation the supernatants and the cell pellets were collected after centrifugation (6000 x g, 10 min.). Nucleic acid derivatives and protein were estimated in the supernatants and cell pellets.

The data are presented in Table 39. Heat treatment is not effective in making the cells permeable to RNase in the case of S. cerevisiae and C. lipolytica. In the case of C. tropicalis the effect was moderate, whereas in

Table - 39

Effect of A. candidus RNase on heat-treated cells of different yeast strains.

Yeast strain	Total nucleic acid in 5 mg cells (A_{260})	Nucleic acid leached (% of the total)		Total protein in 5 mg cells (µg)	Protein leached (Lowry)			
		Control (No enzyme)	Enzyme treated		Control (No enzyme)		Enzyme treated	
					(µg)	(%)	(µg)	(%)
S.cerevisiae	18.20	21.0	22.9	2500	250	10.1	290	11.6
C.lipolytica NCIM 3229	18.57	27.8	30.6	2500	540	21.6	600	24.0
C.tropicalis NCIM 3119	19.00	48.7	58.0	2703	400	14.8	450	16.7
C.utilis NCIM 3055	15.00	25.2	58.3	2400	240	10.0	330	13.8
C.utilis NCIM 3336	12.40	46.2	76.8	2600	260	10.0	400	15.4

Details of the experiment are given in the text. Protein and nucleic acid contents were determined as described in "Materials and Methods".

C. utilis strains the effect was fairly good. In C. utilis NCIM 3336 about 77% nucleic acid was removed from the cells when treated with the enzyme as compared to 46% in the control.

Protein loss from the cells was maximum in enzyme treated cells of C. lipolytica. Minimum protein loss was in the case of S. cerevisiae as was the nucleic acid leakage.

It could be concluded that the heat-treatment is not useful in improving the permeability of the cells to the enzyme in the case of S. cerevisiae, C. lipolytica and C. tropicalis, whereas this method of pre-treatment could be useful in the case of C. utilis strains for reducing the nucleic acid content.

(b) Pre-treatment with toluene and chloroform:- As heat treatment was not effective in the case of S. cerevisiae cells, toluene and chloroform were used for pre-treating the cells as they are used for accelerating the autolysis of yeast cells. S. cerevisiae cells were used in the preliminary experiment. The procedure followed was as follows:

i) Solvent treatment:- Wet cell pellet containing about 50 mg cells (dry wt.) was shaken and suspended in 5 ml of toluene or chloroform and allowed to stand for 6 hrs at room temperature (26°C). After 6 hrs the residual solvent was removed.

ii) Solvent treatment followed by heat treatment:- Treated as in (i) for 6 hrs and the residual toluene or chloroform was evaporated by heating at 62-65°C.

In all cases after the pre-treatments as described above, the cells were resuspended in 0.05 M acetate buffer of pH 4.6 (5 mg/ml) and, after addition of RNase (1 µg/ml), were incubated on a shaker water bath at 50°C for 60 min. The results obtained are presented in Table 40. It is clear that both toluene and chloroform were effective in increasing the permeability of S. cerevisiae cells to RNase, chloroform being more efficient. Exposure of cells to toluene followed by heat-treatment was less effective than toluene treatment alone, whereas in the case of chloroform a subsequent heat treatment slightly improved the permeability.

Since chloroform plus heat treatment was found to be effective on S. cerevisiae, it was tested on other four yeast strains also. The treated cells were incubated with RNase (1 µg/ml) at 55°C for 90 min. in a shaker water bath. The results are presented in Table 41. The method of pre-treatment was found to be effective on all the 5 strains, the best effect being on C. lipolytica and C. utilis NCIM 3336. However, the net effect of the enzyme on RNA removal, i.e. after subtracting the values obtained for control without enzyme from the values for the enzyme treated samples, was

Table - 40

Effect of different pre-treatments (heat and solvent treatments) of S. cerevisiae cells on the enzymatic leakage of nucleic acids.

Pre-treatment	Nucleic acid removed after enzyme treatment	
	(ug/mg cells)*	% of the total
1. No pre-treatment (control)	9.5	7.29
2. Heat[a]	28.7	22.10
3. Toluene	91.4	70.33
4. Toluene + Heat	63.1	48.50
5. Chloroform	101.4	78.00
6. Chloroform + heat	104.0	80.00

In all cases the total of 5 ml suspension contained 25 mg yeast cell (dry weight equivalent) and 10 µg RNase and pH was 4.5 (0.05 M acetate buffer).

[a] The cell suspension in buffer was heated at 98°C for 5 min.

* Total nucleic acid content of the cells was 130 µg/mg cells (dry weight).

Table - 41

Effect of RNase on chloroform and heat treated yeast

Yeast strain	Total nucleic acid in 5 mg cells (A_{260})	Nucleic acid leached (% of the total)		Total protein in 5 mg cells (µg)	Protein leached (Lowry)			
		Control (No enzyme)	Enzyme treated		Control (No enzyme) (µg)	(%)	Enzyme treated (µg)	(%)
S.cerevisiae	18.20	30.8	73.7	2500	290	11.6	320	12.8
C.lipolytica NCIM 3229	18.57	73.1	87.0	2548	581	22.8	600	23.5
C.tropicalis NCIM 3119	19.00	40.0	70.9	2710	840	31.0	820	30.2
C.utilis NCIM 3055	15.00	62.3	67.8	2353	200	8.5	210	8.9
C.utilis NCIM 3336	12.40	78.4	83.9	2396	230	9.6	250	10.4

Details of the experiment are given in the text.

marginal. On the other hand, the net effect of the enzyme treatment was significant in the case of S. cerevisiae.

Effect of the period of chloroform treatment with and without subsequent heat treatment:

In order to determine the optimum period of chloroform treatment and also of chloroform treatment with subsequent heat treatment this was carried out for different durations viz., 0.5, 1.0, 3.0, 6.0 and 9 hrs. The experimental details were as explained above.

The data obtained are presented in Table 42. It could be observed that 6 hrs treatment was optimum for the maximum removal of the nucleic acid from the cells. Further increase in duration did not show any significant effect. It could also be seen that chloroform treatment alone is sufficient to improve the permeability of the cells as far as nucleic acid leaching was concerned. But there was a concomitant leaching of protein (70%) which could be considerably reduced by a heat treatment following chloroform treatment. This may be due to the effect of heat leading to coagulation and fixing the protein in the cells, permitting only the hydrolysed products of RNA to be leached out of the cells. It could also be seen that the net effect of the RNase on the leaching of protein was just marginal in all cases (as compared to the controls incubated without enzyme).

Table - 42

Effect of time of chloroform treatment (with and without subsequent heat treatment) on the nucleic acid and protein leaching from S. cerevisiae cells.

Time of $CHCl_3$ treatment (hr)	Nucleic acid leached (% of the total)				Protein leached (% of the total)			
	Chloroform treated		Chloroform* + heat treated		Chloroform treated		Chloroform* + heat treated	
	Control[a]	Enzyme treated[b]	Control[a]	Enzyme treated[b]	Control[a]	Enzyme treated[b]	Control[a]	Enzyme treated[b]
0.5	80.5	86.7	24.9	30.5	44.8	48.0	6.8	10.8
1.0	43.0	45.1	25.1	31.1	45.6	48.4	6.8	10.8
3.0	74.6	80.1	34.2	49.3	66.8	65.6	10.0	11.2
6.0	70.1	81.6	37.8	80.5	69.6	69.6	12.0	13.2
9.0	77.9	88.7	44.5	77.5	64.0	70.4	20.0	22.4

* Chloroform treatment for different periods followed by heat treatment at 65°C for 3 min.

a Incubated for 90 min. without enzyme.

b Incubated for 90 min. with 1 µg pure enzyme/ml.

The total initial protein and nucleic acid contents in 1 mg cells were 500 µg and 130 µg respectively.

Effect of pH on the release of nucleic acids:

To study the effect of pH on the leakage of nucleic acids from the cells in the presence and absence of RNase enzyme cell suspension of chloroform pre-treated S. cerevisiae as well as C. utilis NCIM 3336 cells (2 mg/ml) were prepared in 0.25 M buffer of different pH values (pH 3.0-5.0, acetate buffer, pH 5.5-7.0, phosphate and pH 8.0 Tris glycine buffer). One set with RNase (0.5 µg/ml) and the other set without enzyme were incubated for 120 min. at 50°C in a shaker water bath.

The results are presented in Fig. 31. It could be seen that with increase in pH there was increased leakage of nucleic acids from the cells of S. cerevisiae even in the absence of RNase. However, in the presence of the enzyme it showed an enhanced rel ase of nucleic acids. Plot C indicates the net effect of the enzyme on the leakage of nucleic acids, i.e. the effect of enzyme besides the effect of pH. From this plot, it also could be observed that the enzyme was most effective between pH 4.5 and 5.0 where it is optimally active.

Fig. 31B gives the pattern for cells of C. utilis. The plots are similarly designated as in Fig. 31A. It could be seen that the net effect of enzyme on the leakage of UV-absorbing compounds was rather low, because the increase in pH itself was sufficient to release the nucleic

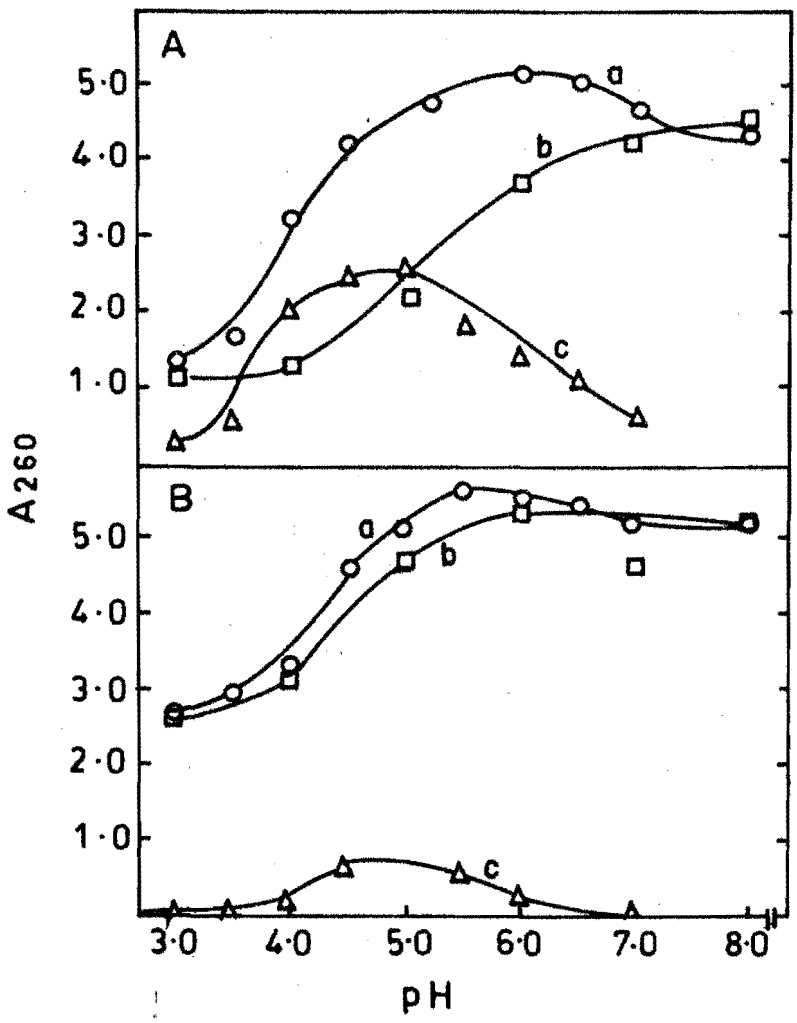

Fig. 31. Effect of pH on the leaching of nucleic acids from yeast cells by RNase treatment.
A. S. cerevisiae, B. C. utilis.
a. with enzyme, b. without enzyme (control),
c. net effect of enzyme (i.e. a-b).

acids from the cells. This may be, in turn, due to the increased cell permeability caused by the chloroform pre-treatment.

Effect of temperature on the enzymatic treatment:

The effect of temperature on the release of UV-absorbing components of S. cerevisiae cells was studied. The experimental details are similar to that of above except that the temperature of treatment was varied instead of pH. Fig. 32 shows the extent of nucleic acid derivatives which leaked out from the cells at different temperatures with increase in temperature. Upto 60°C there was an increased leakage of UV-absorbing compounds, even in the absence of RNase, but much more so when the enzyme was present. A temperature of 55°C was most effective. The net effect of enzyme was pronounced at temperatures in the range 45° to 55°C (Fig. 32C).

Effect of yeast cell concentration:

In order to determine the optimum cell to enzyme ratio for the effective removal of nucleic acids from the cells, the cell concentration in the suspension was varied (0.5-10 mg dry wt. equivalent/ml) keeping the enzyme level constant.

The results are presented in Fig. 33. There was a linear increase in the release of nucleic acid products with

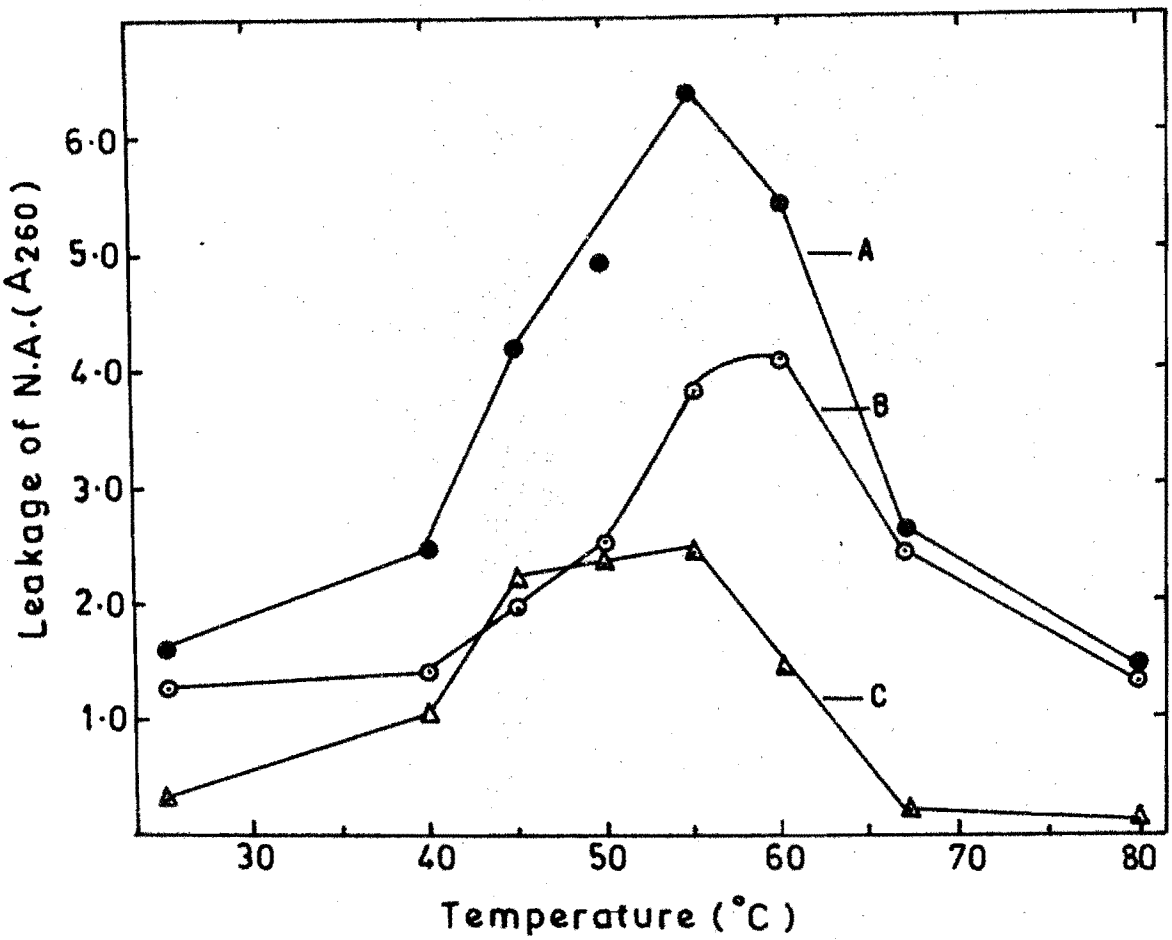

Fig. 32. Effect of temperature on the leaching of nucleic acids from the yeast cells in the presence and absence of RNase.
A - with enzyme, B - without enzyme,
C - net effect of enzyme (i.e. A-B).

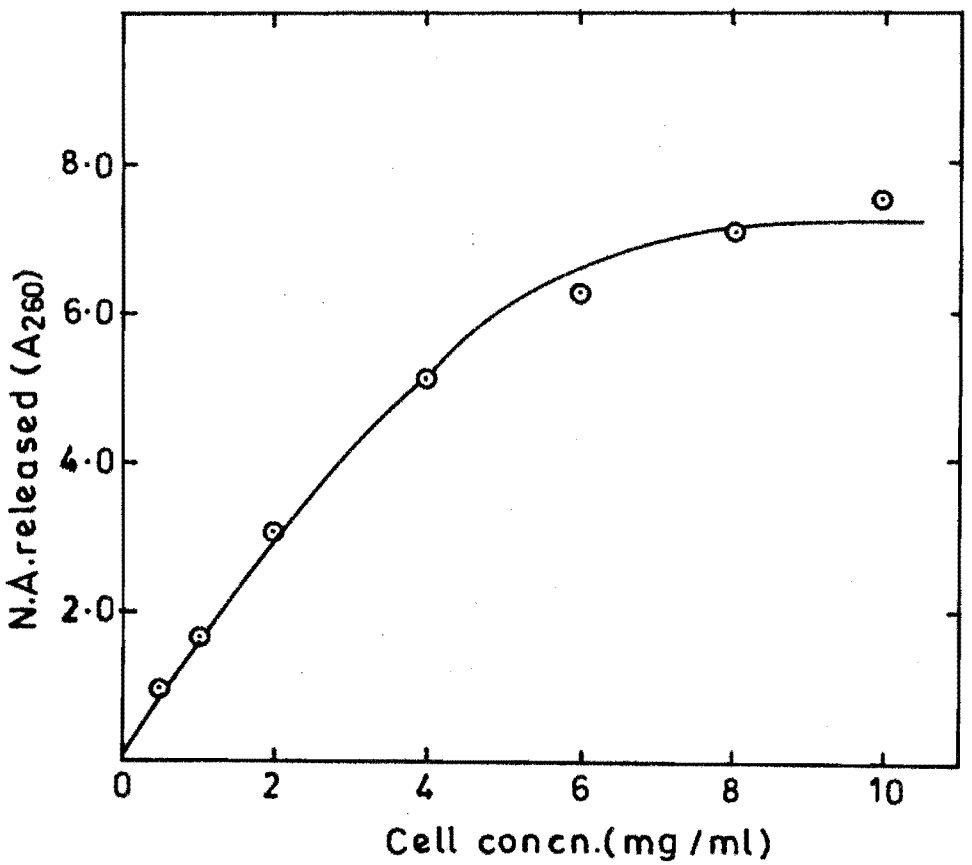

Fig. 33. Effect of yeast cell concentration on enzymatic leaching of nucleic acids. Cell suspension of different concentrations (0.5-10 mg/ml) were prepared in 0.05 M acetate buffer (pH 4.6) and incubated with RNase (1 µg/ml) at 55°C for 60 min. on a shaker water bath.

increase in cell concentration upto about 6 mg cells/ml. Further increase in cell concentration did not show any linear increase in the nucleic acid leakage. An enzyme to cell ratio of 1:6000 (W/W) seems optimum and is in agreement with the results obtained by Castro et al. (1971). They found that an enzyme to cell ratio of 1:10,000 as optimum when they used bovine pancreatic RNase A which had a molecular weight of about 13,000. The enzyme under study here has a molecular weight of 26,000, making the requirement of the enzyme almost double that of RNase A, for treating the same amount of cells.

Effect of different RNase preparations:

For the development of an economically feasible process for reducing of nucleic acids in yeast biomass by exogenous application of the enzyme one must be able to economise on the enzyme use and cost. In other words use of a purified enzyme preparation is likely to be impracticable. Hence, the efficacy and the adverse effects of the crude enzyme preparations on the products were studied.

In the present case, as already explained in the section on "Crude Enzyme", it was shown that the crude preparations contained a contaminating protease. Hence, studies were carried out with the use of crude enzyme (MRBE), heat treated MRBE (80°C for 2 min. to partially denature the

the protease), diisopropylfluorophosphate-treated MRBE (for complete inactivation of protease) as well as with pure RNase preparation for comparison.

The results are presented in Table 43. It could be seen that removal of 71% of total nucleic acids was achieved with pure enzyme, whereas 75-85% of the nucleic acids could be eliminated from the cells by the crude enzymes, even though the RNase activity of all the preparations were same.

The loss of protein in the case of pure RNase treated samples was marginal (10%) whereas with crude enzyme (MRBE), which has protease activity also, it was much higher (about 42%). With heat-treated MRBE, in which the protease was partially inactivated, there was 25% loss of protein. Nevertheless, the concomitant protein loss in the case of DFP-treated MRBE, where no protease activity was observed, was only 9.7%, as with control or with pure RNase. It clearly shows that the contaminating protease has to be eliminated from the crude enzyme preparations when the enzyme is intended to be used for reduction of nucleic acids in SCP.

Table - 43

Effect of different RNase preparations on the nucleic acid and protein loss from S. cerevisiae cells*

Enzyme preparation	Initial Nucleic acid content (µg/5 mg cells)	Nucleic acid lost		Initial protein content (µg/5 mg cells)	Protein lost	
		Total (µg)	(%)[c]		Total (µg)	(%)[c]
Control (without enzyme)	710.0	187.2	26.4	2556	230	9.0
Purified RNase	703.1	499.2	71.0	2530	253	10.0
Heat treated MRBE[a]	716.5	583.6	81.4	2520	635	25.2
DFP-treated MRBE[b]	717.2	609.6	85.0	2536	246	9.7
MRBE (untreated)	696.5	522.4	75.0	2515	1046	41.6

* Chloroform + heat-treated cells were used.

a Moldy rice bran extract (MRBE) treated at 80°C for 2 min. to partially destroy protease activity.

b MRBE treated with 20 mM diisopropylfluorophosphate to inactivate the protease completely.

c Percentage of the total of these components in 5 mg (dry weight) yeast cells.

The suspension (5 mg dry weight equivalent/ml) of chloroform + heat-treated cells in 0.05 M acetate buffer pH 4.5 incubated with RNase preparations (28 units) in all cases for 90 min. at 55°C on a shaker water bath.

SECTION B

RECOVERY OF THE HYDROLYTIC PRODUCTS OF YEAST CELL NUCLEIC ACIDS BY ION EXCHANGE COLUMN CHROMATOGRAPHY

Utilization of the products of RNA hydrolysis after treatment of cells with RNase may be important from the view point of economics of the process as the by-products recovery and their utilization may bring down the cost of the process. The first step in this is separation of the hydrolysis products. In the present case the products of the RNA hydrolysis are a mixture of 3'-mononucleotides which if separated into individual nucleotides may be useful in biochemical studies as well as in industry.

Hence, an ion-exchange chromatographic technique was tried to separate the individual nucleotides. This was carried out according to the procedure described by Cohn (1950) with slight modifications using Dowex-1 x 8 (Cl^- form, 200-400 mesh resin) (as described below).

The resin Dowex 1 x 8 (Cl^- form, 200-400 mesh) was treated with 1 N HCl twice for 30 mins. each time, washed thoroughly with distilled water, packed into a column (a bed volume of 16 cm x 1.8 cm) and again washed with distilled water. The sample (8 ml, pH 8.1) was loaded on the column. The sample used was the one which was incubated

overnight at room temperature (28°C) to ensure complete conversion of RNA into 3'-nucleotides. After adsorption the column was again washed with 500 ml of distilled water; no 260 nm absorbing material was eluted under these conditions. Then elution was started with 2 mM HCl (8 ml fraction and 40 ml/hr), followed by 3 mM, ... 4 mM, 10 mM and 20 mM HCl was used. The absorbance of the fraction was determined at 260 nm in a Spectronic 21-UV-spectrophotometer.

The elution pattern of different nucleotides are given in Fig. 34. 5 peak fractions were obtained and on chromatography were found to correspond to 3' CMP, 3' AMP, 3' UMP, 3' GMP and one unknown compound. Thus, it could be seen that the different mononucleotides formed by the action of RNase on the nucleic acid of Saccharomyces cells could be resolved into individual nucleotides by this procedure.

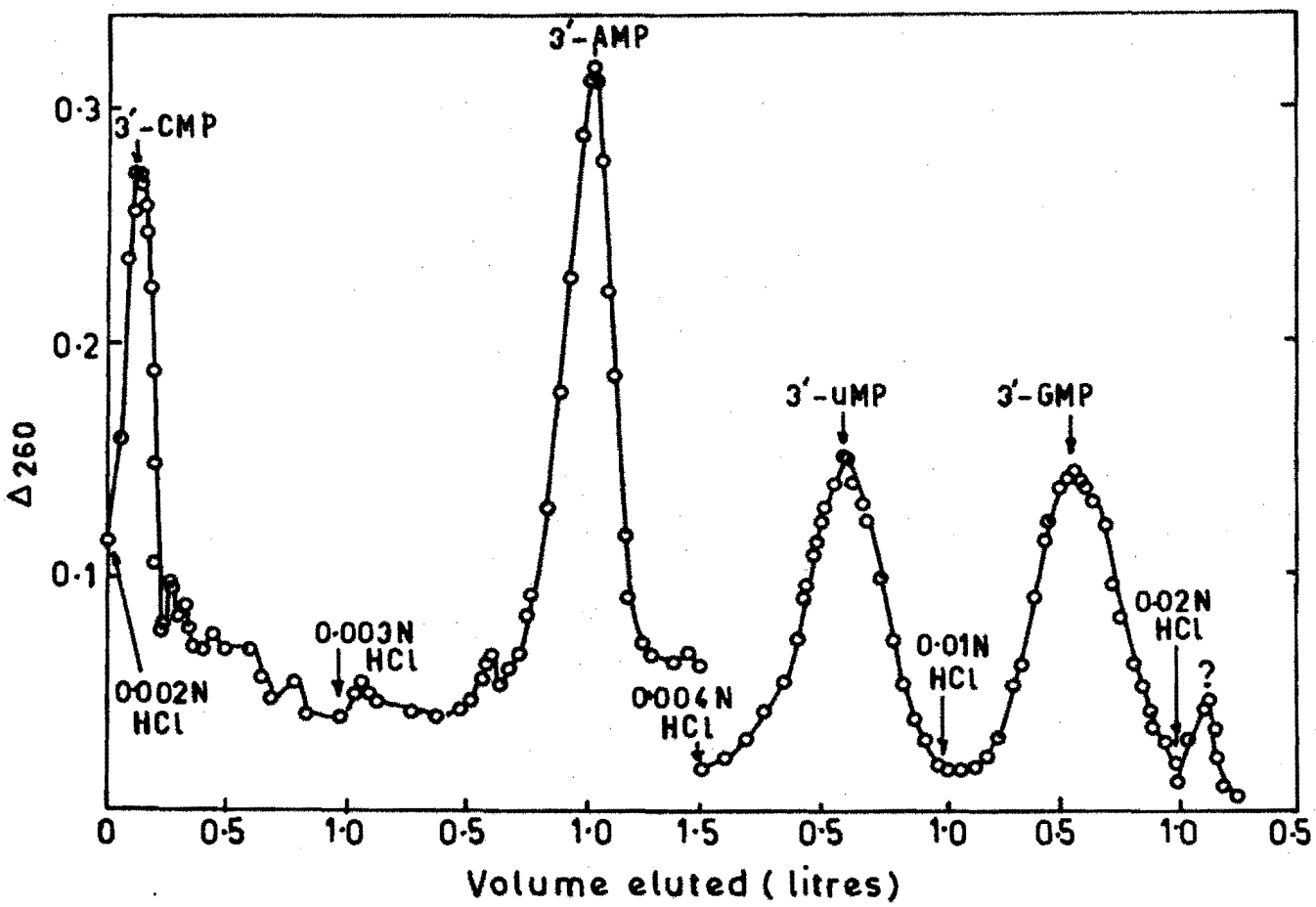

Fig. 34. Elution pattern of nucleotides (of RNase hydrolysed yeast cells) from Dowex-1x8 (Cl^- form).

DISCUSSION

Eversince the risk for humans of gout by consumption of high quantities of SCP was pointed out in the MIT Conference on Single Cell Protein in 1967, much research efforts have been oriented towards reducing the nucleic acid content in SCP. A number of methods has been tried by different groups of workers which included physical, chemical, physicochemical and physiological processing of the SCP cells (Tannenbaum, 1975; Chen and Peppler, 1978). Many processes have already been patented and some of them have been mentioned in the introduction to this Chapter.

However, most of these processes involving chemical and physical treatments were shown to affect the quality of the product. For example, the chemical processes with alkali cause chemical alteration, denaturation and destruction of desirable physico-chemical properties of the extracted proteins (DeGroot et al., 1977; Vananuvat and and Kinsella, 1978). The enzymatic processes, i.e. the activation of endogenous enzymes, result in concurrent proteolysis of the cellular protein during treatment resulting in significant reduction in yields of protein (Sinskey and Tannenbaum, 1975; Lindblom, 1977). Similarly, various other methods also have their own drawbacks. Castro et al. (1971) showed by the exogenous application of bovine pancreatic RNase A that about 80% of the nucleic

acid content of Candida cells could be removed without significant loss of protein. Sinskey and Tannenbaum (1975) have reported a cell yield of 67% and protein yield of 84% after a similar treatment of C. utilis cells. However, the limited availability and the high cost of this enzyme restrict its use in a commercial scale. Hence, our search for a similar but a cheaper enzyme capable of degrading the yeast cell nucleic acids resulted in the isolation of a fungal culture producing a very potent RNase. This enzyme was found to be suitable for the reduction of nucleic acid in yeast cells such as Candida spp. and Saccharomyces cerevisiae. The present enzyme seems to be more suitable and efficient than bovine pancreatic RNase A. The various aspects of these will be discussed in comparison and contrast with the procedure of Castro et al. (1971). The latter have tried this method only on Candida cells, whereas in the present study more emphasis was laid on Saccharomyces, although many trials were made on different species of Candida also.

Pre-treatment:- Castro et al. (1971) standardized an effective heat-shock pre-treatment procedure which involved passing of the yeast cell suspension through a heating coil at 80°C with a residence time of 30 sec. which was effective on C. utilis and C. intermedia. In the present case heat treatment of the cell suspension was done by heating at 95°C for 5 min. This treatment however made

only Candida utilis strains more permeable but S. cerevisiae, C. tropicalis and C. lypolytica cells were rather resistant (Table 39).

Among other methods tested treatment with chloroform followed by mild heating to 65°C was more effective (Table 42). This treatment overcame the undesirable effect of only $CHCl_3$ treatment, i.e. enhanced leaching of protein along with nucleic acid products and also helped in the evaporation of the solvent and its recovery for further use.

Maximum removal of nucleic acid was observed between pH 6.5 and 8.0 in Castro's method because pancreatic RNase A has its optimum pH around 7.5 which is a disadvantage since protein tend to be more soluble at neutral pH whereas in the present case optimum pH was found to be 4.5 to 5.0. This seems to be more desirable as most of the proteins are much less soluble at these pH values. In both cases 90 min. of incubation was found to be sufficient when a slurry containing 5 mg cells per ml (dry wt.) was used.

In the method of Castro et al. (1971), the enzyme to cell ratio of 1:10,000 (W/W) was shown to be optimal, whereas in the present case the optimal ratio was 1:6000. This seems to be due to the difference in the molecular weights of RNase A (13,000) and the present RNase (26,000).

Under optimal conditions about 80% nucleic acid could be eliminated by Castro's method with a concomitant loss of only 10-16% protein (Sinskey and Tannenbaum, 1975) when a pure RNase A was used. However, in the present case the pure RNase was found to be slightly less efficient releasing 70-75% of nucleic acid as compared to the crude enzyme (MRBE) by which 75-85% nucleic acid could be removed. This is a very desirable property. But the crude enzyme which contains protease as a contaminant is not suitable for this purpose as it caused high loss of proteins from the product (Table 43). Heat treatment was not effective in inactivating the protease in MRBE as it showed a fairly high loss of protein from the cells (25% as compared to 10% in the case of pure RNase). However, a DFP-treated crude enzyme in which the protease was completely destroyed was found to give excellent results leading to the removal of about 85% nucleic acid with only insignificant concomitant loss of protein (9.7%). But being a toxic chemical, DFP cannot be used in this process. Hence, it is necessary to search for a natural, non-toxic and specific inhibitor for this contaminating serine-protease. A number of protease inhibitors has been reported in legumes. It would be worthwhile to screen different pulses for a specific inhibitor for the present protease. However, this work was not carried out.

The products of hydrolysis of RNA of the yeast cells were separated by stepwise elution using dilute HCl in the present case [according to the procedure of Cohn, (1950)] rather than formic acid-ammonium formate (Hulbert et al., 1954) or acetate buffer (Anderson and Ladd, 1962) since HCl is cheaper and probably more suited for large scale operation. Gradient elution with chloride ions has been used for separation of acid-soluble nucleotides by other workers also (Strominger, 1955; Suzuki, 1963). The products were resolved into 4 fractions viz. 3'-CMP, 3'-AMP, 3'-UMP and 3'-GMP in that order and with 0.002, 0.003, 0.004 and 0.01 N HCl respectively in the present case.

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PART IV

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

1. A total of sixty strains of fungi belonging to the genera Aspergillus, Penicillium, Fusarium, Trichoderma, Trichothecium, Botryodiploidea, Gibberella, Sporotrichum, Rhizopus, Glomeralla and Rhizoctonia were isolated or obtained from various culture collections and their ability to produce RNase was tested. Among these, 5 cultures viz. A. terreus, A. ochraceous, A. carbonarius, A. oryzae and a local isolate designated as M16a showed good RNase activity of which the maximum was shown by isolate M16a. Taxonomical studies on the local isolate revealed that it belonged to Aspergillus candidus.

2. Various nutritional and cultural parameters were optimised for the maximal production of RNase by A. candidus M16a. (i) Starch was found to be the best carbon source followed by maltose and glucose. (ii) Ammonium nitrate gave the highest yield while six other inorganic nitrogenous salts did not show any significant activity. (iii) Casein was the best organic nitrogen source followed by peptone. The yield of enzyme with casein as nitrogen source was about 10 times more than that with the best inorganic nitrogen source, NH_4NO_3. (iv) The optimum ratio of ammonium nitrate to starch was 1:7.5; it was found that 1.125% starch and 0.15% ammonium nitrate were optimal. (v) Trace minerals and vitamins tested did not show any significant effect.

(vi) Maximum enzyme yields in the medium containing glucose, peptone and minerals were obtained at pH 6.3 and 9.6 in two distinct peaks, while the biomass produced was not significantly different at the pH range of 5.0 to 10.0. The enzyme produced at both the pH values were similar on the criteria of pH and temperature optima. (vii) A volume to surface ratio of 75 ml medium in 500 ml Erlenmeyer flask, shaken on a rotary shaker at 230 rpm was found to be optimal. (viii) Among the cheaper raw materials tested, 10% rice bran extract without any fortification gave good enzyme yield. Ragi flour (5%), wheat bran extract (10%), wheat bran extract fortified with groundnut meal were also beneficial of enzyme production. Supplementation with trace minerals had no effect. (ix) Rice bran moistened with tap water to about 50% moisture level was found to be the best among different semi solid media tested. (x) Incubation at 24-27°C for 80-85 hrs was most suitable for solid state fermentation.

3. Since this was the first observation of RNase produced by A. candidus it was necessary to standardize conditions for assay of this RNase. It was noted that pH 4.5, temperature of 55°C, 0.25% RNA concentration and incubation period of 30 minutes were optimum. Based on these an assay procedure was devised.

4. The properties of the crude enzyme were studied first.

(i) It was stable at pH range 5.0 to 6.0.

(ii) Heat denaturation pattern of the crude enzyme was very interesting. At 30 to 40°C or above 70°C there was no loss of activity, but at 50 and 60°C there was a 50% or more loss of activity. A proteolytic enzyme(s) active at 45-55° was responsible for this anomalous behaviour. This was further evidenced by the normal heat inactivation pattern obtained after inactivating the protease by diisopropyl fluorophosphate (DFP).

(iii) The heat inactivation pattern of moldy rice bran extract (MRBE) was different from that of culture filtrate from glucose-peptone-mineral medium. About 95% loss of activity after 5 min incubation at 80°C was observed as against 25% loss with enzyme from submerged culture. Addition of media constituents such as glucose, peptone, $CaCl_2$ and $MgSO_4$ had no effect. However, addition of EDTA at 0.4-0.5% level was found to be effective in protecting the enzyme against heat to a considerable extent indicating a possible role of metallic contaminants present in MRBE in the enhanced inactivation of the enzyme by heat.

(iv) Submerged culture filtrate and MRBE were tested for the presence of other enzymes. Both showed DNase activity against both native and heat denatured DNA. Non-specific phosphodiesterase activity and nucleotidase activity

against 3'- and 5'- AMP were also present; the latter was about 3 times more in MRBE than that in submerged culture filtrate.

5. The protease in the crude preparations showed an optimum activity at 50°C and in the pH range 6.0-7.0. It retained 25% activity when heated at 80°C and pH 5.6 for 5 min, but at higher pH the loss of activity was almost to the extent of 90%. It was inhibited completely by DFP indicating the involvement of serine in the active site. It was found to be the major contaminating protein at various steps of RNase purification.

6. RNase from MRBE was purified about 110 fold.

(i) Fractionation by ethanol (30 to 50% precipitate), gel filtration on Sephadex G-100, DEAE-cellulose chromatography (batch-wise elution with sodium chloride), and finally DEAE-Sephadex A-50 chromatography (gradient elution with sodium chloride). The yield of the pure enzyme was about 16%.

(ii) The purified enzyme was found to be homogeneous by polyacrylamide disc-gel electrophoresis at pH 4.3 and 8.1.

(iii) The purified RNase was found to be active on yeast RNA optimally at pH 4.5 and between 55° and 60°C. It showed improved stability upto pH 8.0 in contrast to that of crude enzyme.

(iv) The enzyme was fairly heat stable. At 55° and 60°C for 60 minutes it retained about 98% and 50% activity respectively. At 70° and 95°C the retention of activity after 30 minutes incubation was 38% and 6% respectively.

(v) Metallic salts such as $CuCl_2$, $Pb(NO_3)_2$, $FeCl_3$, $HgCl_2$ and $ZnCl_2$ were inhibitory at 2 mM to the enzyme.

(vi) Among group specific reagents N-bromosuccinimide (1 mM) was highly inhibitory (to the extent of 100%), followed by p-hydroxymercuribenzoate (65%). Di-isopropyl-flurophosphate, malathion, sodium azide as well as photooxidation with Rose Bengal and methylene blue did not have any effect on activity indicating that serine, -S-S- and histidine are not involved in the active site.

(vii) The molecular weight of the RNase as determined by gel filtration on Sephadex G-100 was 26,640.

(viii) Km value for RNA was 3.64 mg/ml as calculated from the Lineweaver-Burk Plot.

(ix) The rate of hydrolysis of different polynucleotides was in the order: Poly-U > Yeast-RNA > Poly-C > Poly-A. The enzyme did not degrade Poly-G and showed only very insignificant activity towards DNA. There was no non-specific phosphodiesterase activity. No strict base specificity was exhibited by the enzyme, although the rate of release of mononucleotides from yeast RNA was in the order AMP > UMP $\gg$ CMP.

The appearance of GMP was very slow. The enzyme causes the formation of 2',3'-cyclic nucleotides as intermediates during hydrolysis of RNA, the final products being 3'-mono-nucleotides of adenine, uracil and cytosine. A mixture of mono- and oligo-nucleotides of guanine is also seen as the other products. From paper chromatographic evidence, the enzyme seems to be an endonuclease.

7. Reduction of nucleic acid content in SCP:

The enzyme was employed in reducing the nucleic acid content of cells of Saccharomyces cerevisiae, Candida lipolytica, C. tropicalis and two strains of C. utilis. Parameters such as pretreatment of the yeast cells, pH, temperature and enzyme-cell ratio were studied.

(i) Among the pretreatments for improving the cell permeability, heat-treatment was effective only in the case of C. utilis and C. tropicalis strains, whereas chloroform-treatment was effective in all the strains. The concomitant loss (50-70%) of protein from the cell pellet was high in the case of chloroform treated cells. However, when heating (65°C) followed chloroform treatment of cells the protein loss (10-13%) was marginal.

(ii) Increase in the pH of cell suspensions of S. cerevisiae and C. utilis showed an increased leakage of nucleic acids. However, it was indicated that the enzyme action was optimum at pH 4.5-5.0.

(iii) Optimum temperature for the enzymatic attack of nucleic acids of yeast cells was 55°C.

(iv) The optimum enzyme to cell ratio was 1:6,000 (W/W).

(v) DFP treated MRBE having no protease activity led to a 85% decrease of nucleic acids while with purified RNase it was about 75-80%. Only 10% loss of protein was observed in both these cases. Heat treated MRBE with partially inactivated protease and non-treated MRBE were also effective in the reduction of nucleic acids. However, the protein loss was as high as 25% and 42% respectively.

(vi) The nucleotides produced by the hydrolysis of RNA from yeast cells by RNase were fractionated by ion-exchange column chromatography on Dowex-1 (Cl^- form), and were identified as 3'-CMP, 3'-AMP, 3'-UMP and 3'-GMP.

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B I B L I O G R A P H Y

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