PURIFICATION AND PROPERTIES OF EXTRACELLULAR NUCLEASE(S) FROM RUMEN CONTENTS OF *BUBALUS BUBALIS*

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Summary

Extracellular nuclease(s) in buffalo rumen fluid were purified from strained rumen fluid by a procedure involving Seitz filtration, acetone fractionation and gel filtration on Sephadex G-100. The enzyme resolved into two peaks exhibiting both DNase and RNase activities. The molecular weight of enzyme corresponding to peaks I and II were approximately 30,000 and 12,000 respectively. The properties of enzymes from the two peaks, however, were same. Optimum temperature for both DNase and RNase activities was at 50°C. Whereas DNase activity was stable upto 60°C, RNase activity was stable only upto 50°C. DNase activity recorded two pH optima, one at pH 5.5 and the other at pH 7.0. RNase activity recorded a broad pH optimum between pH 6.0-8.0. pH stability of the enzyme coincided with pH optima for both the activities. DNase activity was stimulated by Mg^{2+} and Mn^{2+} and inhibited by Fe^{2+}, Zn^{2+}, Hg^{2+} and Ag^{+}. RNase activity was also stimulated by Mg^{2+} and Mn^{2+} and inhibited by Cu^{2+}, Fe^{2+}, Zn^{2+}, Hg^{2+} and Ag^{+}. Reducing agents stimulated both the activities.
(**Key Words:** Ribonuclease, Deoxyribonuclease, Rumen, Buffalo)

Introduction

Nucleic acids form an appreciable proportion of nitrogen ingested by ruminants (Smith and McAllan, 1970; Coelho Da Silva et al., 1972). These nucleic acids of feed origin are rapidly degraded in the rumen by mixed microbial population of rumen comprising of bacteria and protozoa and the enzymes released into the rumen (Sinha and Dutta, 1989). McAllan and Smith (1973) observed that at least part of the initial degradation of nucleic acids in the rumen of cow and sheep occur through the action of extracellular nucleases. Sinha (1982) also reported degradation of nucleic acids to oligo- and mono- nucleotides only by the extracellular enzymes released into the rumen fluid in buffaloes. The present paper deals with purification and properties of the extracellular nuclease(s) from the rumen fluid of buffaloes.

Materials and Methods

Animals

Two Murrah buffalo (*Bubalus bubalis*) bulls weighing about 530 kg and fitted with rumen fistulae were used.

The animals were fed berseem (*Trifolium alexandrinum) ad libitum* for 3 weeks before starting the experiments and were maintained subsequently on the same feed. The animals were fed twice daily at 8:00 a.m. and 4:00 p.m.

Collection and processing of rumen fluid

Collection and processing of rumen fluid was done according to earlier reports (Sinha and Dutta, 1986b).

Purification of nuclease(s)

Acetone precipitation. 50 ml of precooled acetone was added to 90 ml of cell-free, rumen fluid at 4°C with constant stirring and kept at 0-4°C for 1 hr. The precipitate formed was separated by centrifugation at 5,000 x g for 20 min at 0°C and discarded. To supernatant thus obtained was added with constant sirring an incremental volume of cold acetone tc get the enzyme solvent ratio of 1:1.5. It was kept at 0°C for a further period of 2 hr and the precipitate

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formed was collected by centrifugation at 5000 x g for 20 min at 0°C. The precipitate was dissolved in 14 ml of phosphate buffer (0.05 M, pH 7.0) containing 0.05% cystein-HCl and was dialysed against the same buffer for 6 hr at 4°C.

Gel filtration. 7 ml of acetone fraction was applied to the Sephadex G-100 column (1.5 x 95 cm) which was eluted with phosphate buffer (0.05 M, pH 7.0) at a flow rate of 25 ml/hr. 5 ml fractions were collected and used for estimation of DNase and RNase activities. Fraction numbers 6 to 18 from peak I and numbers 24 to 32 from peak II were pooled together and analysed for protein content and DNase and RNase activities.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Purified nuclease(s) from peaks I and II were electrophoresed on 10% polyacrylamide disc gels according to the method of Weber and Osborn (1969). Proteins were detected by staining with 0.25% Commassie brilliant blue G-250 in a mixture of 227 ml of methanol, 50 ml of glacial acetic acid and 217 ml of distilled water.

Protein determination. Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Enzyme assays

DNase assay. DNase activity was measured by the method of Kato and Ikeda (1968) using highly polymerized calf thymus DNA.

Unit of DNase activity. A unit of DNase activity was defined as the amount of enzyme required to cause an increase in absorbance of 1.0 at 260 nm under the conditions specified above.

Ribonuclease activity. RNase activity was determined by the procedure of Anfinsen et al. (1954).

Unit of RNase activity. A unit of RNase activity was defined as the amount of enzyme required to cause an increase in absorbance of 1.0 at 260 nm under the conditions specified above.

Molecular weight estimation. The approximate molecular weight was determined by gel filtration on Sephadex G-75 column (1.5 x 90 cm). The column was equilibrated, charged with the sample protein and was eluted with phosphate buffer (0.05 M, pH 7.0). Cytochrome C (12,400 daltons), chymotrysinogen A (25,000 daltons), ova albumin (45,000 daltons) and ribonuclease A (13,500 daltons) were used as the standards. The protein concentrations were determined by recording absorbance at 280 nm.

Results

Purification of nuclease(s). The results on purification are summarized in table 1. All procedures were carried out at 0-4°C unless otherwise stated.

Strained rumen fluid made free of microorganisms by centrifugation, followed by Seitz filtration was subjected to acetone fractionation

TABLE 1. SUMMARY OF PURIFICATION OF EXTRACELLULAR NUCLEASE(S) OF BUFFALO RUMEN

Step	Procedure		Volume (ml)	Enzyme activity (units/ml)		Protein (mg/ml)	Specific activity (units/mg)		Total activity		Purification fold		Yield (%)	
				DNase	RNase		DNase	RNase	DNase	RNase	DNase	RNase	DNase	RNase
1.	Strained rumen liquor		100	3.5	4.5	3.64	0.96	1.24	350	450	1	1	100	100
2.	Cell free rumen liquor		90	2.4	2.8	2.56	0.94	1.09	216	252	–	–	62	56
3.	Acetone fractionation		14	10.67	12.60	2.66	4.01	4.74	149	176	4	4	42.5	39
4.	Sephadex G-100	A	35	0.55	0.90	0.06	9.17	15.00	19	31	10	14	10.9[a]	13.8[a]
		B	25	0.60	1.60	0.11	5.45	14.50	15	40	6	13	8.6[a]	17.8[a]

[a]would have been the yield if all the samples had been applied

and gel filtration (Sephadex G-100) for purification of the extracellular nuclease(s) released into rumen fluid. The enzyme resolved into two peaks I and II on Sephadex G-100 column (figure 1) exhibiting both DNase and RNase activities.

Molecular weight. The molecular weights of peaks I and II were approximately 30,000 and 12,000 respectively as estimated by gel filtration. SDS-polyacrylamide gel electrophoresis was performed on 10% disc gels. Nuclease(s) of peaks I and II which were purified by acetone fractionation and gel filtration showed single Commassie blue-stained protein band for each peak.

Properties of purified enzyme. The properties of the enzyme in pooled fractions from peak I only are reported since the properties of the enzymes in pooled fractions of peak I and peak II were same except as regards molecular weights.

Temperature optimum. The rate of hydrolysis of DNA or RNA were examined at various temperatures ranging from 30 to 80°C (figure 2). The maximal activity was observed at 50°C.

Heat stability. With DNA as the substrate, the purified enzyme was observed to be stable upto a temperature of 60°C, whereafter a sharp decline in activity was recorded. With RNA as

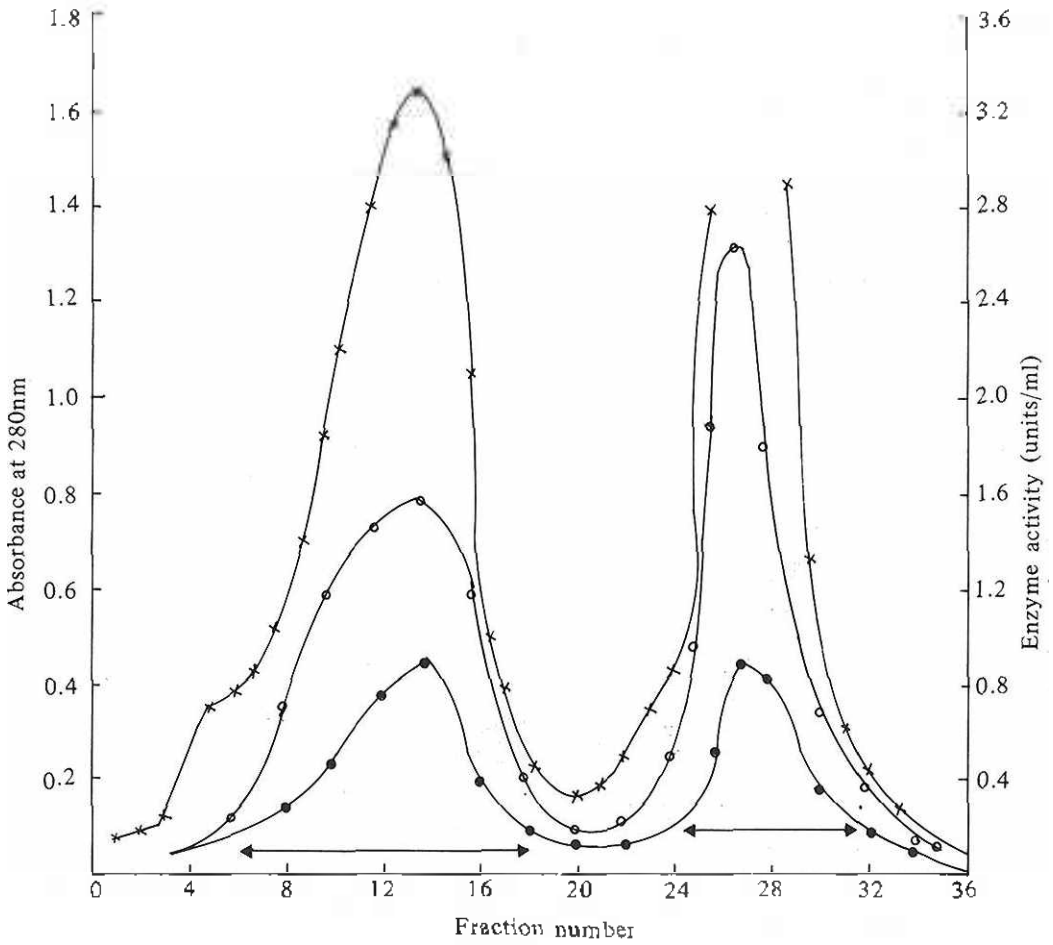

Figure 1. Separation of extracellular nuclease(s) by gel filtration on Sephadex G-100. The procedure and assay methods are described in the text.

x——x Absorbance at 280 nm
○——○ RNase activity
●——● DNase activity
◄——► Active fractions

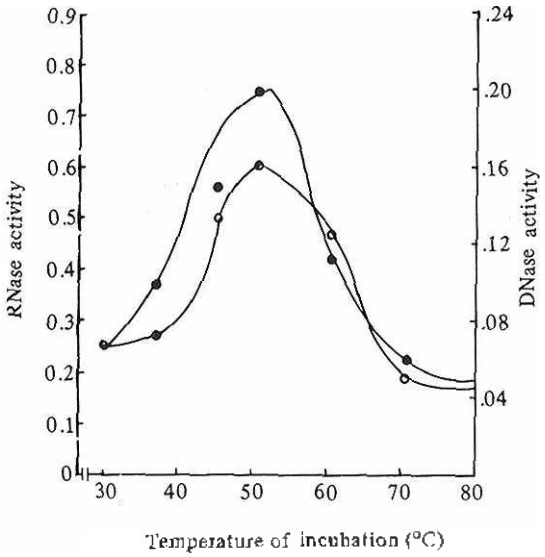

Figure 2. Effect of temperature on the activity of extracellular nuclease(s). Activities assayed as described in the text except that assay mixture was incubated at different temperatures as indicated.
○———○ RNase activity
●— —● DNase activity

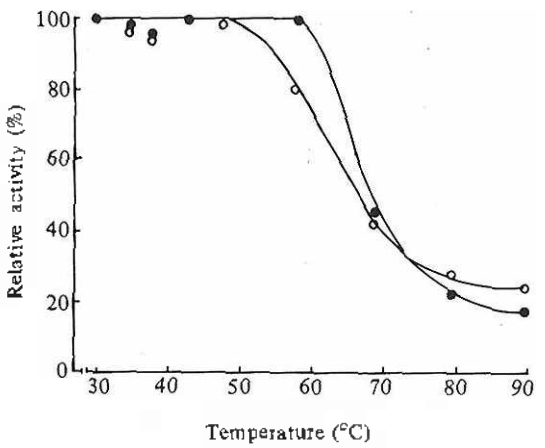

Figure 3. Heat stability of extracellular nuclease(s). Assays were conducted as described in the text except that enzyme was preincubated for 10 minutes at different temperatures as indicated.
○———○ RNase activity
●———● DNase activity

the substrate, the enzyme was found to be stable upto 50°C (figure 3).

Effect of pH. As shown in figure 4, the enzyme recorded two pH optima for hydrolysis of DNA, one at pH 5.5 and the other at pH 7.0. In case of RNase activity, a broad pH optimum between 6.0 to 8.0 was recorded.

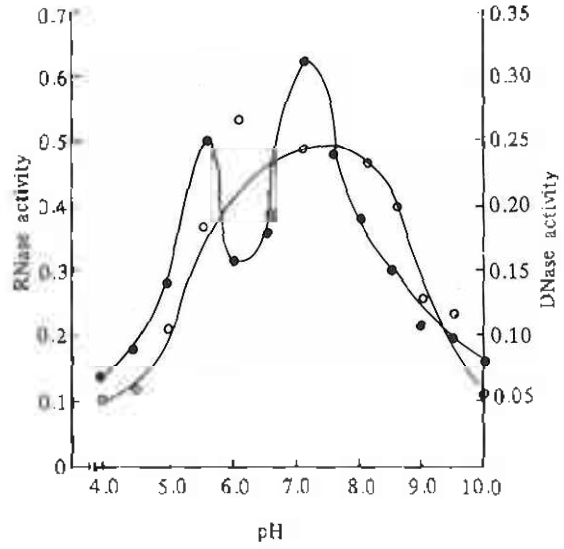

Figure 4. Effect of pH on the activity of extracellular nuclease(s). Assays were conducted as described in the text except that the pH varied as indicated.
○———○ RNase activity
●———● DNase activity

pH stability. The enzyme was observed to be stable at both the optimum pH values (figure 5)

Effect of cations. Various metal ions were examined to study their effects on hydrolysis of DNA and the results are presented in table 2. Mg^{2+} and Mn^{2+} were observed to stimulate DNase activity. The activity was, however, strongly inhibited by Fe^{2+}, Zn^{2+}, Hg^{2+} and Ag^{+}. RNase activity was also stimulated by Mg^{2+} and Mn^{2+} and strongly inhibited by Cu^{2+}, Fe^{2+}, Zn^{2+}, Hg^{2+} and Ag^{+}.

Effect of activators and inhibitors. Various group specific agents were examined to study their effect on hydrolysis of DNA and RNA (table 3). Reduced glutathione, 2-mercaptoethanol and cystein-HCl were observed to be stimulatory for hydrolysis of both DNA and RNA.

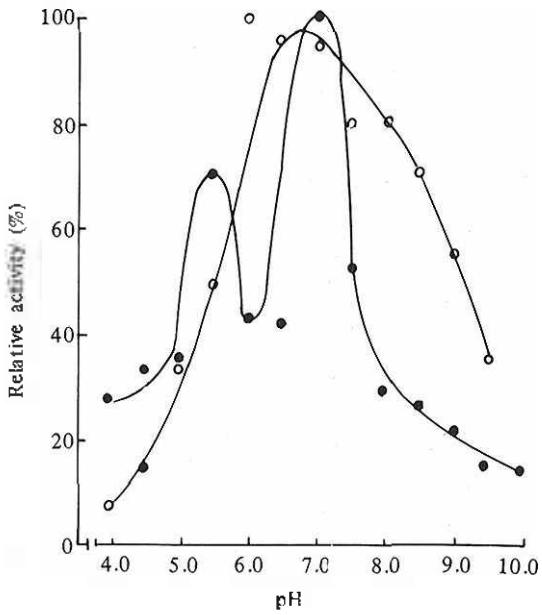

Figure 5. pH stability of extracellular nuclease(s). Assays were conducted as described in the text except that enzyme was preincubated for 1 hr at different pH as indicated.
○——○ RNase activity
●——● DNase activity

TABLE 2. EFFECT OF CATIONS ON DNASE AND RNASE ACTIVITIES[a]

Cation	Relative activity (%)	
	DNase	RNase
Control	100	100
Mg^{2+}	130	135
Mn^{2+}	123	130
Ba^{2+}	70	68
Pb^{2+}	77	20
Fe^{2+}	0	0
Ni^{2+}	63	25
CO^{2+}	40	83
Zn^{2+}	0	0
Cu^{2+}	46	0
Ca^{2+}	77	120
Hg^{2+}	13	0
K^{+}	123	120
Ag^{+}	3	0

[a]Enzyme was preincubated with various metal ions at a concentration of 10^{-3}M and assayed for DNase and RNase activities

TABLE 3. EFFECT OF ACTIVATORS AND INHIBITORS ON DNASE AND RNASE ACTIVITIES[a]

Reagent	Relative activity (%)	
	DNase	RNase
Control	100	100
Cystein-HCl	169	200
Glutathione (reduced)	184	258
2-mercaptoethanol	115	145
N-ethylmaleimide	80	77
P chloromercuribenzoate	60	78
8-hydroxy quinoline	18	0
Iodoacetamide	60	41
Iodoacetic acid	58	58
1, 10-phenanthroline	35	23
α, α - dipyridyl	65	78
Dithiothreitol	38	0
EDTA	49	50

[a]Enzyme was preincubated with various metal ions at a concentration of 10^{-3}M and assayed for DNase and RNase activities

Discussion

A probable major pathway for degradation of nucleic acids in rumen of sheep, cow and calf was proposed by McAllan and Smith in 1973. They observed that at least part of the initial degradation of RNA and DNA in rumen fluid occurs through the action of nucleases which although presumably of microbial origin are extracellular. Their observation was based on complete absence of purine and pyrimidine bases and nucleosides amongst the products formed by the action of extracellular nucleases present in the cell-free rumen fluid on yeast RNA or calf thymus DNA. Sinha and Dutta (1979) and Sinha (1982) in their studies on degradation of nucleic acids in buffalo rumen also observed that extracellular enzymes released into the rumen fluid degraded DNA or RNA into oligo- and mononucleotides only.

Identification of two peaks with molecular weights of 30,000 and 12,000 and exhibiting properties very similar to each other suggest that either the enzyme forms aggregates or might be composed of subunits. A similar observation was made by Takahashi and Uchida (1978) in their studies on extracellular exonuclease from *Thermus thermophilus* HB8. Two pH optima for degrada-

tion of DNA observed in the present study have also been observed for degradation of DNA by nucleases of rumen bacterial and protozoal origin (Sinha and Dutta, 1981, 1984, 1986a,b). Stimulation of nuclease activity by Mg^{2+} and Mn^{2+} ions in the present study have been reported for nucleases from non-rumen microorganisms also (Scher and Dubnau, 1976; Rushizky and Whitlock, 1977; Matsumoto et al., 1979; Van Tigerstrom, 1980; Rokosu and Edeko, 1981). Similarly strong inhibition by Fe^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+} and Ag^{+} observed in the present study have been reported for the enzymes from other microbial sources also (Arella and Sylvestre, 1979; Saruno et al., 1979; Shibano and Komano, 1979). Stimulation of enzyme activity by reduced glutathione, 2-mercaptoethanol, and cystein-HCl is interesting, since it would indicate the necessity of reducing atmosphere for optimal activity of the enzyme. It is important to note here that degradation of nucleic acids in rumen takes place essentially under a reducing atmosphere.

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