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Extracellular Nucleases of *Rhizopus stolonifer*

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Abstract: *Rhizopus stolonifer* produces two extracellular nucleases viz., nuclease Rsn and RNAse Rs in a ratio of approximately 1:60, when grown on YPG medium. The purified nuclease Rsn is a high Mr (67 kDa), metal requiring multifunctional endonuclease with a substrate specificity in the order of ssDNA>dsDNA >>RNA. It cleaves DNA non-specifically but with RNA, shows high preference for adenylic acid linkages. RNAse Rs, on the other hand, is a 28.2 kDa atypical member of RNAse T2 family of cyclizing RNAses which produces 2', 3' cyclic nucleotides as the major end product of RNA hydrolysis. The purification, characteristics and potential application of these enzymes are reviewed.

Key words: *Rhizopus stolonifer*, extracellular nucleases, purification, characteristics

INTRODUCTION

Nucleases are versatile analytical tools and have played a major role in the determination of nucleic acid structure. Thus, sequence specific enzymes like restriction endonucleases, conformation specific enzymes like S1 nuclease, along with ribonucleases and exonucleases have played an important role in molecular biology research (Reddy and Shankar, 1993). Intracellularly, they have been implicated in cellular events viz., replication, recombination, restriction and repair whereas, extracellular enzymes have a role in nutrition (Rangarajan and Shankar, 2001a). Moreover, T2 family RNAse are reported to have a role in nutrition, phosphate remobilization, senescence, self-incompatibility and defense against pathogens (Deshpande and Shankar, 2002). *Rhizopus stolonifer* produces two extracellular nucleases viz., nuclease Rsn (Rangarajan and Shankar, 1999) and RNAse Rs (Chacko and Shankar, 1998). This compilation gives a detailed account of their physico-chemical characteristics and potential applications.

Enzyme Production

R. stolonifer (NCIM 880) was routinely maintained on potato dextrose agar (PDA) slants. Optimization studies showed that medium components viz., the type of peptone used, metal ions and inorganic phosphate influences enzyme production. Optimum nuclease Rsn (~ 60 U mL^{-1}) and RNAse Rs (~ 3000 U mL^{-1}) levels were obtained, in 5 days, when the culture was grown on YPG (yeast extract, peptone, glucose) medium containing 12 mM Mg^{2+}, 2 ppm each of Mn^{2+} and Fe^{2+} at 30°C (Rangarajan, 2001; Chacko *et al*., 1996).

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Enzyme Purification

The steps involved in the purification of nuclease Rsn and RNAse Rs to homogeneity are depicted in Fig. 1. Presence of metal ions was essential during Blue-Sepharose chromatography as the enzyme failed to bind to the matrix in their absence (Rangarajan, 2001).

Characteristics of Nuclease Rsn

Physical Properties

The Mr of the enzyme was 67 kDa and it is a tetramer and each protomer consists of two unidentical subunits of 21 and 13 kDa, respectively (Rangarajan and Shankar, 1999). Enzymes with comparable Mr have been reported from *Neurospora crassa* mitochondria (Chow and Fraser, 1983) and *Ustilago maydis* (Rushe *et al*., 1980). Unlike *N. crassa* mitochondria (Chow and Fraser, 1983) and mouse mitochondria (Tomkinson and Linn, 1986) nucleases, which are dimers with identical subunits, nuclease Rsn consists of two protomers with each protomer consisting of two non-identical monomers. Amino acid composition showed the absence of cysteine (Rangarajan, 2001) suggesting that the subunits are probably held together by non-covalent interactions. It is an acidic protein with a pI of 4.2 but not a glycoprotein (Rangarajan and Shankar, 1999). Moreover, the ssDNAse, dsDNAse and RNAse activities of nuclease Rsn showed the same pI suggesting that they are associated with the same protein (Rangarajan, 2001).

Catalytic Properties

Enzyme Assays

The DNAse and RNAse activities of nuclease Rsn were determined, at pH 7.0 and 37°C, by measuring the amount of acid soluble nucleotides, at 260 nm, following the hydrolysis of DNA and RNA. A unit is defined on the basis of μmol of acid soluble nucleotides liberated (Rangarajan and Shankar, 1999, 2001b).

Metal Ion Requirement, Optimum pH, Temperature and Stability

The ssDNAse, dsDNAse and RNAse activities of nuclease Rsn showed an obligate requirement of Mg^{2+}, Mn^{2+} and Co^{2+} for its activity but is not a metalloprotein (Rangarajan and Shankar, 1999, 2001b). Similar observations have been reported with nucleases from *N. crassa* (Chow and Fraser, 1983), *Saccharomyces cerevisiae* (Dake *et al*., 1988), *Serratia marcescens* (Nestle and Roberts, 1969) and pancreatic DNAse (Moore, 1981). In contrast to *S. marcescens* nuclease and pancreatic DNAse, no synergism was observed in presence of Mg^{2+}, Mn^{2+} and Co^{2+} with nuclease Rsn. However, the enzyme exhibited higher preference for ssDNA in presence of Co^{2+} whereas, the dsDNAse and RNAse activities were not influenced by the type of metal ion used (Rangarajan and Shankar, 1999, 2001b).

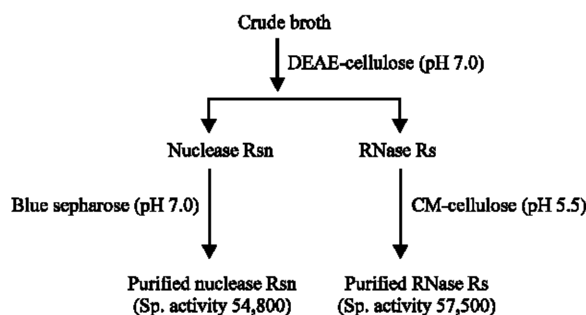

Fig. 1: Purification of nuclease Rsn and RNAse Rs

Nuclease Rsn showed an optimum pH of 7.0 for the hydrolysis of ssDNA, dsDNA and RNA and it was not influenced by the metal ion used (Rangarajan and Shankar, 1999, 2001b) as observed with *U. maydis* nuclease γ (Yarnall *et al*., 1984). In contrast, BAL 31 nuclease (Gray *et al*., 1981) and *N. crassa* mitochondria nuclease (Chow and Fraser, 1983) exhibited different pH optima for the hydrolysis of ss- and dsDNA. Although metal ions did not influence the optimum temperature of nuclease Rsn for ssDNA hydrolysis, it did in case of dsDNA hydrolysis. The optimum temperature for ss- and dsDNA hydrolysis was 40°C in presence of Mg^{2+} but it showed higher optimum temperature (45°C) for dsDNA hydrolysis in presence of Mn^{2+} and Co^{2+}. However, the associated RNAse activity of nuclease Rsn exhibited an optimum temperature of 35°C and it was not affected by the type of metal ion used. Divalent cations like, Cu^{2+}, Zn^{2+} and Hg^{2+}, EDTA, inorganic phosphate, pyrophosphate and protein denaturants were inhibitory whereas, mononucleotides had no effect (Rangarajan and Shankar, 1999, 2001b). Organic solvents stimulated endonucleases from *Streptomyces antibioticus* (De los Reys Gavlian *et al*., 1988) and *Streptomyces glaucescens* (Aparicio *et al*., 1988). However, organic solvents inhibited nuclease Rsn to a varying extent with maximum inhibition occurring with formamide. Interestingly, the inhibitory effect of ethylene glycol, dimethylsulfoxide and dimethlyformamide was pronounced on the dsDNAse activity whereas, this differential effect was not observed with formamide. Moreover, high concentration of dimethylsulfoxide completely abolished the dsDNAse activity of nuclease Rsn, with significant retention (~ 60%) of its ssDNAse activity. This observation suggests that the enzyme can be used as a strict single-strand-specific nuclease for studying DNA structure in presence of dimethylsulfoxide. Both the DNAse and RNAse activities of nuclease Rsn showed inferior stability with a half life of 60 and 15 min, respectively, at pH 7.0 and 37°C (Rangarajan and Shankar, 1999, 2001b).

Specificity and Mode of Action of the DNAse Activity

Viscometric studies showed that nuclease Rsn cleaved dsDNA by a single hit mechanism (Rangarajan and Shankar, 1999) as observed with DNAse I, acid DNAse II and *Escherichia coli* endonuclease (Bernardi and Cordonnier, 1965). However, unlike DNAse I, the type of metal ion used did not alter the mechanism of action of nuclease Rsn (Rangarajan and Shankar, 1999).

Nuclease Rsn could convert supercoiled pUC 18 DNA (form I) to linear duplex DNA (form III) via nicked circular DNA (form II) suggesting an endo mode of action (Rangarajan and Shankar, 1999). Unlike *U. maydis* nuclease γ (Yarnall *et al*., 1984), the type of metal ion used did not influence the cleavage pattern. However, the conversion of form I DNA to form III DNA and its further degradation, by nuclease Rsn, was slow in presence of Co^{2+}, pointing towards the relatively low preference of the enzyme for ds DNA in presence of Co^{2+} (Rangarajan and Shankar, 1999).

The major end products of DNA hydrolysis, by nuclease Rsn, were oligonucleotides ending in 3'hydroxyl and 5'phosphoryl termini and small amounts of 5'mononucleotides (Rangarajan and Shankar, 1999). Time course analysis of the 3'termini, of the hydrolytic products of ssDNA, revealed the presence of nucleosides in the order of dT > dG> dC, with very little, if any, of dA. The high amount of deoxythymidine, from the initial stages of hydrolysis, indicated the preference of the enzyme for thymidylic acid linkages (Fig. 2a). However, time course analysis of the 5' termini revealed the presence nucleosides in the order of dT ≅ dA > dC > dG (Fig. 2b). The predominance of deoxythymidine and deoxyadenosine at the 5'termini coupled with the presence of high amounts of deoxythymidine at the 3'termini indicated the high preference of nuclease Rsn for dTpdT and dTpdA linkages. The 3' terminal base analysis of the hydrolytic products of dsDNA showed a similar pattern i.e., dT > dG > dC with very small amounts of dA (Fig. 3a). The 5' terminal analysis, however, revealed the presence of nucleosides in the order of dT > dC > dA ≅ dG (Fig. 3b). Despite the difference in the order of appearance of the nucleosides at the 5'termini, the preference of nuclease Rsn for dTpdT linkages remained the same. The 3' and 5' terminal analysis of the hydrolytic products of

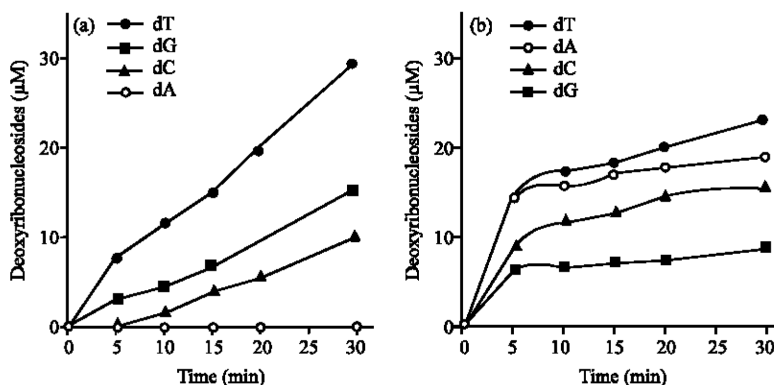

Fig. 2: Time course analysis of the 3' and 5' termini of the hydrolytic products of ssDNA. (A) 3' termini: (B) 5' termini: (Reprinted from Rangarajan and Shankar (2004), Copyright 2004, with permission from Elsevier Inc.)

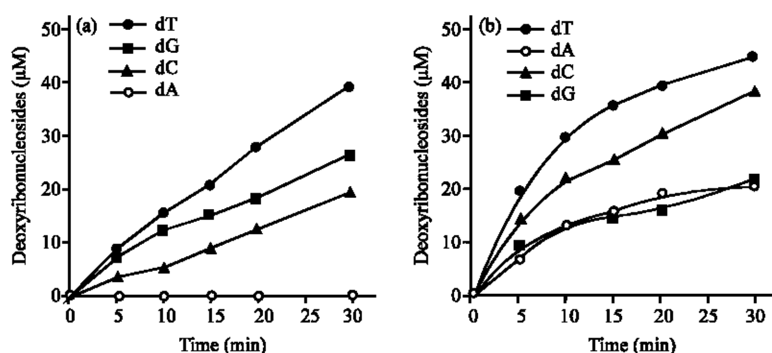

Fig. 3: Time course analysis of the 3' and 5' termini of the hydrolytic products of dsDNA. (A) 3' termini: (B) 5' termini: (Reprinted from Rangarajan and Shankar (2004), Copyright, 2004, with permission from Elsevier Inc.)

ss- and dsDNA revealed that nuclease Rsn does not exhibit any strict base preference and cleaves DNA in a non-specific manner. Moreover, small amounts of dA at the 3' termini of the hydrolytic products of both ss- and dsDNA, indicated the resistance of dApdX bonds to hydrolysis (Rangarajan and Shankar, 2004). Like nuclease Rsn, endonucleases from *S. marcescens* (Nestle and Roberts, 1969) and yeast mitochondria (Morosoli and Lusena, 1980) hydrolyzed DNA in a non-specific manner.

Separation of the hydrolytic products following the exhaustive digestion of DNA by nuclease Rsn, in presence of Mg^{2+}, $Mn^{2+}$and Co^{2+}, yielded four fractions corresponding to mono, di, tri and tetranucleotides. The relative percentages of the individual peaks revealed the predominance of tetranucleotides (35-45%) and trinucleotides (28-33%) followed dinucleotides (20-23%) and mononucleotides (7-12%) (Rangarajan and Shankar, 2004). Analysis of the hydrolytic products of DNA, by *S. marcescens* (Nestle and Roberts, 1969), yeast mitochondria (Morosoli and Lusena, 1980) and *Aspergillus nidulans* (Koa *et al*., 1990) nucleases showed comparable results.

Specificity and Mode of Action of the Associated RNAse Activity

Nuclease Rsn hydrolyzed polyA rapidly while RNA was cleaved approximately 20% the rate of polyA. PolyU, polyC and polyG were highly resistant to hydrolysis. The high susceptibility of

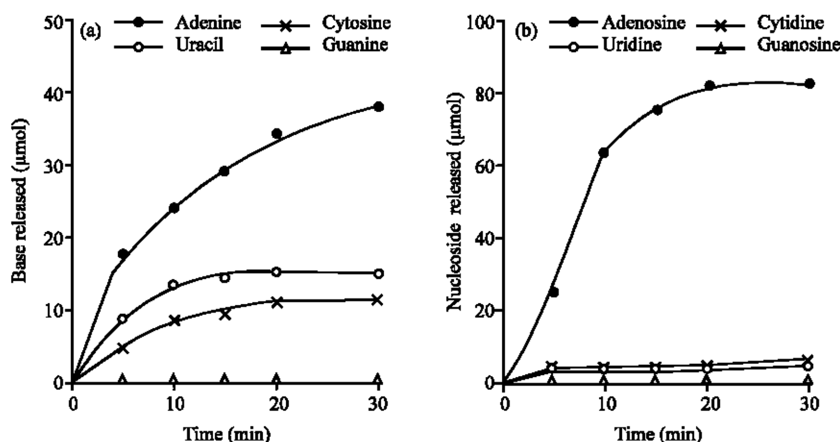

Fig. 4: Time course analysis of the 3' and 5' termini of the hydrolytic products of RNA. (A) 3' termini: (B) 5' termini: (Reprinted from Rangarajan and Shankar (2001b), Copyright 2001, with permission from Taylor and Francis.http://www.tandf.co.uk)

polyA suggested the preference of the enzyme for adenylic acid linkages. Analysis of the hydrolytic products, obtained following the exhaustive digestion of RNA, by nuclease Rsn, in presence of either Mg^{2+}, Mn^{2+} or Co^{2+} yielded oligonucleotides with 3'hydroxyl and 5'phosphoryl termini. This observation coupled with the absence of 5'AMP, during the initial stages of hydrolysis of polyA suggested an endo mode of action (Rangarajan and Shankar, 2001b). On the contrary, BAL31 nuclease F species (Benson *et al*., 1984) as well as wheat seedling nuclease (Kroekar *et al*., 1975) showed endonuclease activity towards ssDNA but exonuclease activity towards RNA. However, nuclease Rsn hydrolyzed both RNA and DNA endonucleolytically.

Time course of the 3'terminal analysis of the hydrolytic products of rRNA, by nuclease Rsn, revealed the presence of bases in the order of A >>U >C with the absence of G. Additionally, 5'terminal analysis of the hydrolytic products revealed the presence of nucleosides in the order of adenosine >> uridine ≅ cytidine with the absence of guanosine (Fig. 4a, b). The predominance of adenine at the 3' and 5' termini indicated the high preference of nuclease Rsn for ApA linkages. High levels of adenosine at the 5' termini also suggested that the enzyme recognizes adenosine residues and cleaves 5' to the phosphoribose generating 5'pA termini. Significant amount of uracil and cytosine at the 3' termini along with the very low amounts of uridine and cytidine at the 5'termini indicated that the susceptibility of cytidylic and uridylic acid linkages are probably influenced by the proximity of adenine residues. However, the absence of G either at the 3' or the 5' termini suggested the resistance of GpX or XpG to cleavage where X is any base (Rangarajan and Shankar, 2001b).

Active-Site

Nuclease Rsn catalyses the hydrolysis of both DNA and RNA. Chemical modification studies showed the involvement of two histidine, a single tryptophan and two carboxylate residues in the catalytic activity of the enzyme. The substrates of nuclease Rsn namely, DNA and RNA failed to protect the enzyme against diethylpyrocarbonate (DEPC) and carbodiimide (EDAC) mediated inactivation whereas, substrate protection was observed with N-bromosuccinimide (NBS) mediated inactivation of the enzyme. Moreover, kinetic analysis of the partially inactivated enzyme samples showed that while histidine and carboxylate are involved in catalysis, tryptophan is involved in substrate binding. In addition, fluorescence quenching studies on native and modified nuclease Rsn,

using metal ions, revealed the involvement of carboxylate in metal binding (Rangarajan and Shankar, 2001c). Nucleases exhibiting obligate requirement of metal ions for their activity, show metal ion mediated mechanism for the hydrolysis of nucleic acids. In such enzymes, carboxylate groups have been shown to be involved in metal coordination (Horton *et al*., 1998; Miller *et al*., 1999). In addition, in case of *S. marcescens* nuclease(a dimmer), the interaction of Asn 119 and Glu 127 with Mg-water cluster was shown to be responsible for the catalytic activity of the enzyme. The properties of nuclease Rsn viz., obligate requirement of metal ions, dimeric nature of the protein, substrate specificity and mode of action, resemble that of *S. marcescens* nuclease. Furthermore, the residues involved in the catalytic activity of nuclease Rsn is similar to those involved in the catalytic activity of *S. marcescens* nuclease. Hence nuclease Rsn may also follow metal-water cluster mediated mechanism for the hydrolysis of DNA and RNA (Rangarajan and Shankar, 2001c). However, studies on the crystal structure of the enzyme in presence of metal ions will help to substantiate this view.

Conclusion

In general, the properties of nuclease Rsn viz., dimeric nature, obligate requirement of metal ions, substrate specificity, mode of action and active site nature are comparable to *S. marcescens* family of non-specific endonucleases. Hence, nuclease Rsn can be assigned to the class EC. 3.1.30.2. Moreover, nuclease Rsn is similar to pancreatic DNAse with respect to metal ion requirement and mode of action and hence it can be used in conjunction with Mg^{2+} requiring enzymes like DNA polymerase I in nick translation reaction or techniques where limited digestion of DNA is required. As the associated RNAse activity of nuclease Rsn is adenine specific, it can be used in combination with other base specific RNAses for the structural determination of RNAs. The multifunctional nature of nuclease Rsn can also be exploited for eliminating nucleic acid contamination from purified recombinant proteins and single cell protein preparations.

Characteristics of RNAse Rs

Physical Properties

RNAse Rs is a single polypeptide chain of 28.2 kDa and contains 10.5% neutral sugar. It is an acidic protein with a pI of 5.0.The high Mr of the enzyme suggested that it belongs to T2 family RNAses (Chacko and Shankar, 1998). Moreover, the partial N-terminal sequence (first 15 amino acids) of RNAse Rs showed considerable similarity to T2 family enzymes viz., RNAse Rh from *Rhizopus niveus*, RNAse Trv from *Trichoderma viride*, RNAse Irp from *Irpex lacteus*, RNAse M from *Aspergillus saitoi*, RNAse Le2 from *Lentinus edodes* and RNAse T2 from *Aspergillus oryzae* (Deshpande, 2001). Earlier, we had reported a blocked N-terminus for RNAse Rs as Edman degradation of a reduced and pyridylethylated enzyme did not yield any residue till four cycles (Chacko and Shankar, 1998). Since the untreated enzyme did not show blocked N-terminus, the earlier observation can be correlated to the pretreatment of the protein (Deshpande, 2001). The enzyme contains 6 cysteine residues and all of them seem to be involved in disulfide linkages as modification of the native enzyme did not reveal the presence of free cysteine. Additionally, disulfide bridges are essential for the activity as RNAse Rs lost its activity completely in presence of DTT (Chacko and Shankar, 1998).

Catalytic Properties

Enzyme Assay

RNAse Rs was assayed, at pH 5.5 and 37°C, by measuring the amount of acid soluble nucleotides, at 260 nm, following the hydrolysis of RNA. A unit is defined on the basis of μmole of acid soluble ribonucleotides liberated (Chacko *et al*., 1996).

Optimum pH, Temperature and Stability

The optimum pH and temperature of RNAse Rs was 5.5 and 45°C, respectively. It showed high pH stability but poor temperature stability. It is neither a metal ion requiring enzyme nor a metalloenzyme. Low concentrations of divalent cations like, Zn^{2+}, Hg^{2+} and Cu^{2+}were inhibitory. In contrast, mononucleotides and in particular 2'mononucleotides did not affect the enzyme activity (Chacko and Shankar, 1998).

Effect of Denaturants

RNAse Rs showed approximately 2-fold increase in its activity in presence of 2M urea at 37°C, which was comparable to the activity at its optimum temperature i.e., 45°C. Kinetic analysis of the native enzyme at 37 and 45°C and in presence of urea at 37°C showed a decrease in the activation energy in presence of urea. Moreover, in presence of urea, there was no change in the secondary structure of the protein but minor changes in the tertiary structure were observed. Therefore, the increase in the enzyme activity in presence of urea at 37°C was correlated to lowering of the activation energy as a result of changes in the microenvironment of the active site (Deshpande *et al*., 2001). Furthermore, conformational stability of RNAse Rs, determined with chemical and thermal denaturants over the pH range 6.0-10.0, revealed that it follows a two-state F↔U unfolding mechanism during urea, guanidine hydrochloride and thermal denaturation. The pH dependence of ΔG^{H_2O} suggested that the electrostatic interactions among charged groups contribute significantly to the conformational stability of the enzyme (Deshpande *et al*., 2003).

Specificity and Mode of Action

RNAse Rs hydrolyzed RNA liberating 2', 3' cyclic nucleotides and small amounts of 3'mononucleotides suggesting it to be a cyclizing RNAse. Moreover, time course of RNA hydrolysis showed the presence of cyclic nucleotides in the order of 2', 3'cGMP > 2', 3'cAMP > 2', 3' cUMP > 2', 3' cCMP (Fig. 5). The presence of high amount of 2', 3' cGMP, from the initial stages of hydrolysis, indicated the preference of the enzyme for guanylic acid linkages. Inspite of its preference for guanylic acid linkages, the enzyme could not readily hydrolyse polyG at pH 5.5 (Chacko and Shankar, 1998). Similar observations were made in case of RNAse T2 from *A. oryzae*, which, despite

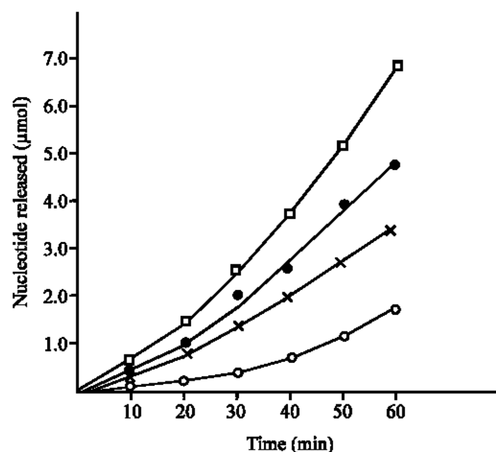

Fig. 5: Release of cyclic nucleotides from RNA by RNAse Rs. 2',3' cGMP (□), 2',3' cAMP (●), 2',3' cUMP (x) and 2',3' cCMP (○). [Reprinted from Chacko and Shankar (1998), Copyright 1998, with permission from Elsevier Science B. V.]

its preference for adenylic acid linkages, could not readily cleave polyA (Uchida and Egami, 1971). The total 3'nucleotides content, after complete solubilization of RNA by RNAse Rs, was approximately 10% of the total cyclic nucleotides. Additionally, exhaustive digestion of poly U with 5-fold excess enzyme gave only 6.5% 3'UMP, suggesting the high resistance of cyclic nucleotides to cleavage (Chacko and Shankar, 1998).

Active-Site

Active site characterization of RNAse Rs showed the involvement of a single histidine, lysine and carboxylate in the catalytic activity of the enzyme. Chemical modification in presence and absence of substrate and kinetic parameters viz., K_m and K_{cat}, of partially inactivated enzyme samples, suggested the involvement of histidine and lysine in catalysis and carboxylate in substrate binding (Rangarajan *et al*., 1999). Site directed mutagenesis studies on RNAse Rh from *Rhizopus niveus* revealed that out of three histidine residues implicated in the catalytic activity, His 109 is involved in the degradation of RNA whereas, His 46 is involved in the hydrolysis of 2', 3' cyclic nucleotides to mononucleotides (Irie *et al*., 1997). However, chemical modification studies on RNAse Rs showed the involvement of only one histidine in the catalytic activity of the enzyme. Since RNAse Rs could effectively depolymerise RNA, its inability to hydrolyse 2', 3' cyclic nucleotides to 3'mononucleotides can be due to the absence of a catalytically active second histidine residue (Rangarajan *et al*., 1999).

Immobilization

RNAse Rs bound covalently via its carbohydrate moiety to aminoethyl Bio-Gel P-2 showed high retention of activity (35-40%) and stability. The bound enzyme could convert high concentrations of RNA to 2', 3' cyclic nucleotides (Deshpande and Shankar, 1998).

Conclusions

RNAse Rs is an atypical member of T2 family RNAses since it is guanylic acid preferential and produces 2', 3' cyclic nucleotides as the major end products of RNA hydrolysis. However, cloning and comparison of the amino acid sequence of the enzyme with other T2 family RNAses followed by site-directed mutagenesis studies will help in ascertaining the cause for the inability of RNAse Rs to cleave cyclic nucleotides to mononucleotides. The high stability and conversion efficiency of immoblilized RNAse Rs suggest that it has the potential for the production of 2', 3' cyclic nucleotides. The immobilized preparation can also be used to reduce RNA content in single cell protein preparations. The properties of RNAse Rs namely, high Mr, acid pH optimum, glycoprotein nature and partial N-terminal sequence are comparable to cyclizing RNAses of T2 family. Hence RNAse Rs can be assigned to the class EC 3.1.27.1.

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