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Active-site characterization of nuclease Stn α from *Streptomyces thermonitrificans*

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ABSTRACT

Chemical modification studies on purified nuclease Stn a revealed the involvement of Histidine, Carboxylate and Arginine in the active site of the enzyme. DNA, the substrate of the enzyme could protect the DEP (Histidine) and WRK (Carboxylate) inactivated enzyme but not Phenylglyoxal (Arginine) inactivated enzyme suggesting that histidine and carboxylate are present in substrate binding while arginine is in catalysis. Kinetics of inactivation indicated involvement of two histidines, two carboxylates in substrate binding while a single arginine in catalysis.

KEYWORDS

Streptomyces thermonitrificans;
Extracellular nuclease;
Active site;
Substrate binding;
Catalysis.

INTRODUCTION

Streptomyces thermonitrificans endonuclease (nuclease Stn α) is a multifunctional enzyme and catalyses the hydrolysis of DNA and RNA[3]. Recently few reports are available on purification and properties of DNase from Streptomyces cultures[1,21] but no report exists on active site studies of these nucleases. In the previous papers, we had reported purification and characterization of two nucleases stn a and β[3,13]. Associated RNase activity of stn a which is adenylic acid preferencial was also reported[4]. In case of sugar non specific S_1 nuclease, [6]nucleases from Serratia marcescens family[5] nuclease Rsn from Rhizopus stolonifer[15] and RNase Rs from Rhizopus stolonifer[14] histidine and carboxylate has been implicated in the catalytic activity of the enzyme. In case of enzymes acting on anionic substrates lysine and or arginine has been implicated in substrate binding[16,17] As nuclease Stn α is sugar non specific acting on both DNA and RNA, modification studies of histidine, carboxylate and arginine residues were carried out to evaluate their role in the catalytic activity of the enzyme and the results are presented in this communication.

EXPERIMENTAL

Materials

Diethylpyrocarbonate (DEP), 5,5'-dithiobis (2 -nitrobenzoic acid) (DTNB), N-acetylimidazole (NAI), Phenylglyoxal, Woodwards reagent K, BSA and calf thymus DNA (Sigma Chemical Co., USA) and hydroxylamine hydrochloride (BDH, India) were used. All other chemicals used were of analytical grade.

Methods

Determination of DNase activity: This was carried out as described earlier[3] The amount of acid soluble deoxyribonucleotides liberated following the hydrolysis of DNA, at pH 7.0 (in presence of 2mM Mn^{2+}) and 37˚C, was calculated by assuming a molar absorption coefficient of 10,000 M_^{1}cm_^{1} for the deoxyribonucleotides mixture[2]. One unit of DNase activity is defined as the amount of enzyme required to liberate 1µmol of acid soluble nucleotides min under the assay conditions.

Protein determination

Protein concentration was determined according to Stoscheck[19].

Purification of nuclease Stn α: The nuclease was purified to homogeneity as described earlier [3].

Reaction with DEP

Nuclease Stn α (10 µg), in 1 ml of 30 mM Tris- HCl buffer pH 7.0 (containing 2 mM Mn^{2+} and 2% v/v glycerol) was incubated for 10 min with various concentrations of DEP freshly diluted with absolute ethanol. Aliquots of 20 µl were removed at suitable time intervals and the reaction was arrested by the addition of 5 ml of 10 mM imidazole buffer, pH 7.5. Subsequently, the residual activity was determined under standard assay conditions. Enzyme samples incubated in the absence of DEP served as control. The DEP concentration in the diluted sample was determined by mixing an aliquot of the diluted sample with 3 ml of 10 mM imidazole buffer (pH 7.5) followed by monitoring the increase in the absorbance at 230 nm. The amount of N- carbethoxyimidazole formed was calculated by using a molar absorption coefficient of 3000 $M^{-1} cm^{-1}$[9]. The DEP concentration of the diluted sample was 2.7mM. The ethanol concentration in the reaction mixture did not exceed 1% (v/v) and had no effect on the activity and stability of the enzyme during the incubation period. DEP mediated modification of nuclease Stn α was also monitored spectrophotometrically by measuring the change in the absorbance at, 240 nm, as described by Ovadi et al [12]. Reaction with hydroxylamine: Decarbethoxylation was carried according to Miles[10] DEP modified enzyme samples were incubated with 500 mM hydroxylamine hydrodrochloride at pH 7.0 and 4˚C for 15 h and the enzyme activity was determined at under standard assay conditions.

Reaction with NAI

The enzyme (10 µg),in 1 ml of 30 mM Tris-HCl buffer pH 7.0 (containing 2mM Mn^{2+} and 2% v/v glycerol) was incubated with 2 mM NAI at 30º C for 10 min and the residual activity was determined under standard assay conditions. Enzyme samples incubated in the absence of NAI served as control. The number of tyrosine residues modified was determined, at 278 nm, using a molar absorption coefficient of 1160 M^{-1} cm -1[8].

Reaction with DTNB

Nuclease Stn α (10µg), in 1 ml of 30 mM Tris -HCl buffer pH 7.0(containing 2mM Mn2+ and 2% v/v glycerol) was incubated with 2 mM DTNB for 10 min at 37˚C and the residual activity was determined under standard assay conditions. Enzyme incubated in the absence of DTNB served as control. The number of cysteine resi-

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dues modified was determined, at 412 nm, using a molar absorption coefficient of 13600 M^{-1} cm^{-1}[8].

Reaction with phenylglyoxal

Purified nuclease Stn a 10(g) in 30 mM HEPES buffer pH 8 was incubated with varying concentrations of phenylglyoxal at fixed time interval, aliquots were removed at 5, 10, 15 and 20 mins and assayed for residual activity.

Reaction with Woodward's reagent K

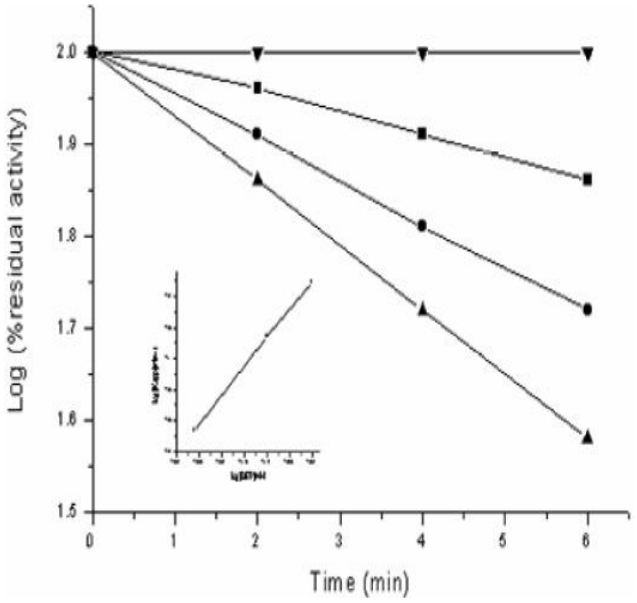
Nuclease Stn (15ig) in Tris HCl buffer pH 7.0 was treated with different concentrations of Woodward's reagent K (0.5-2mM). Aliquots of enzyme were removed and excess reagent is removed by passing it through Sephadex G-25 and assayed for residual activity. Numbers of carboxylate residues modified were determined spectrophotometrically as described by Sinha and Brewer[18]. Substrate and metal ion protection studies, The effect of substrate and metal ion protection was studied by pre incubating the enzyme with excess amounts of DNA or the metal ion (Mn^{2+}) followed by treatment with modifying agent.

RESULTS AND DISCUSSION

Modification of histidine residues

Carbethoxylation of nuclease Stn α, at pH 7.0, resulted in approximately 80-90% loss of its initial activity and the inactivation was dependent on the concentration of the reagent. However, no loss of activity was observed in the control samples. The logarithm of the residual activity plotted as a function of time at various DEP concentrations was linear up to 20% of the initial activity (Figure 1).

The DEP modified reaction followed pseudo first order kinetics at any fixed concentration of the reagent and the order of the reaction (inset Figure 1) suggested that the loss of enzyme activity occurred as a result of modification of 2 histidine residues. Additionally, carbethoxylation of the enzyme as a result of DEP treatment was accompanied by an increase in the absorbance of the modified protein at 240 nm, characteristic of histidine modification. Incubation of the DEP modified enzyme with 500mM hydroxylamine, at pH 7.0 and 4˚C for 15h, restored 80-90 % of its original activity. These results suggest that histidine may have a role in the catalytic activity of nuclease Stn α.



Concentrations of DEP were 0mM (▼), 0.027mM (■), 0.040mM (•) and 0.054mM (▲).
Insets: second-order plots of the pseudo –first order rate constants (K_{app})(min^{-1}) of inactivation at different concentrations of DEP.

Figure 1: Pseudo first order plots for inactivation of nuclease Stn a by DEP.

Though DEP is specific for histidine at neutral pH, it also reacts to a lesser extent with tyrosine, cysteine and lysine residues[10]. DEP mediated inactivation of tyrosine was excluded by the observation that there was no decrease in the absorbance of the modified enzyme at 278 nm. Moreover, modification of the tyrosine residues by a tyrosine specific reagent viz. NAI, though resulting in the modification of 6 residues did not have any significant effect on the enzyme activity,

suggesting that tyrosine may not have a role in the catalytic activity of nuclease Stn α (TABLE 1).

TABLE 1 : Effect of different modifying reagents on the activity of nuclease Stn α.

Incubation Mixture	No. of residues modified	Residual activity (%)
Control	0	100
Histidine (DEP)	2	20
Decarbethoxylation (Hydroxylamine 500mM pH7)	-	85
Tyrosine (NAI)	6	100
Cysteine (DTNB)	2	100
Lysine	-	100
Carboxylate (WRK)	2	22
Arginine (Phenylglyoxal)	1	20

Though these observations support the presence of histidine at or near the active site, they still do not rule out the possible involvement of cysteine. However, modification of the native enzyme with a cysteine specific reagent namely, DTNB, though resulted in the modification of 2 residues did not have any significant effect on the activity of the enzyme suggesting that cysteine may not have a role in the catalytic activity of nuclease Stn (TABLE 1). Furthermore, the DEP mediated inactivation of nuclease Stn α can not be correlated to lysine modification as hydroxylamine could restore the activity. If the inactivation had been due to lysine modification, hydroxylamine would not have restored the activity. DEP mediated inactivation could be prevented by incubating the enzyme with excess amounts of DNA prior to the modification reaction. On the contrary, Mn^{2+} could not protect the enzyme (TABLE 2).

Furthermore, DEP modified, partially inactivated enzyme samples showed an increase in the Km but no change in Kcat (TABLE 3) pointing towards the involvement of histidine in substrate binding.

In case of P1 nuclease, histidine has been implicated in metal binding[20]. However, florescence quenching studies on native and histidine modified enzyme samples with Mn^{2+} showed a similar level of quenching with no difference in the emission maxima (335nm) indicating that histidine residues are not involved in metal binding. (data not shown.)

TABLE 2 : Substrate and metal ion protection studies of nuclease Stn α using Histidine, Carboxylate and Arginine modification.

Incubation mixture	Residual Activity (%)
Enzyme	100
Enzyme + DEP (2.7mm)	20
Decarbethoxylation (hydroxylamine 500 mM pH 7.0)	85
Enzyme + DNA+ DEP (2.7mM)	100
Enzyme +DEP (2.7mM) + Mn	20
Enzyme	100
Enzyme +Woodwards Reagent K (2mM)	22
Enzyme + Woodwords Reagent K+DNA +Mn	100
Enzyme + Woodwards Reagent K (2mM) +Mn	50
Enzyme	100
Enzyme + Phenyl glyoxal (2mM)	20
Enzyme + DNA+ Phenylglyoxal	-

TABLE 3 : K_m and K_{cat} values for partially inactivated nuclease Stn α.

Activity	Residue modified	Residueal activity (%)	Km (mg/ml)	Kcat (min^{-1})
DNase	None	100	0.125	127
	Histidine	52	0.142	127
		29	0.285	127
	Carboxylate	20	0.225	127
		21	0.285	127
	Arginine	30	0.125	112

Modification of arginine residue

Purified nuclease Stn a when incubated with different concentrations of phenylglyoxal lost 80-90% of its activity. No loss of activity was observed in phenylglyoxal control samples. Semi-logarithmic plots of residual activity as a function of time of inactivation for various concentrations of phenylglyoxal was linear up to 20% of initial activity indicating that the inactivation follows

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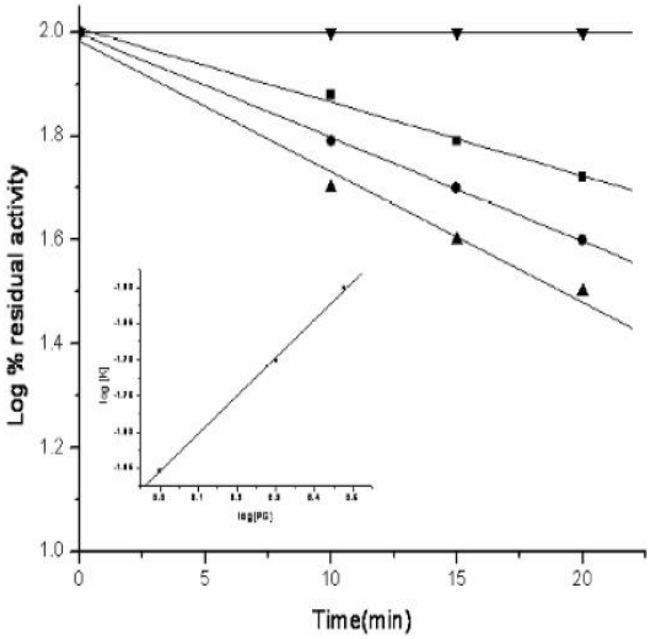
pseudo first order kinetics (Figure 2).

Analysis of the order of the reaction of phenylglyoxal (Figure 2 inset) gave a slope of 0.6 indicating that the loss of enzyme activity occurred as a result of modification of a single arginine residue per molecule of the enzyme. Furthermore, phenylglyoxal modified and partially inactivated enzyme samples showed no change in Km but decrease in Kcat (TABLE 3) values pointing towards involvement of arginine residues in catalysis.

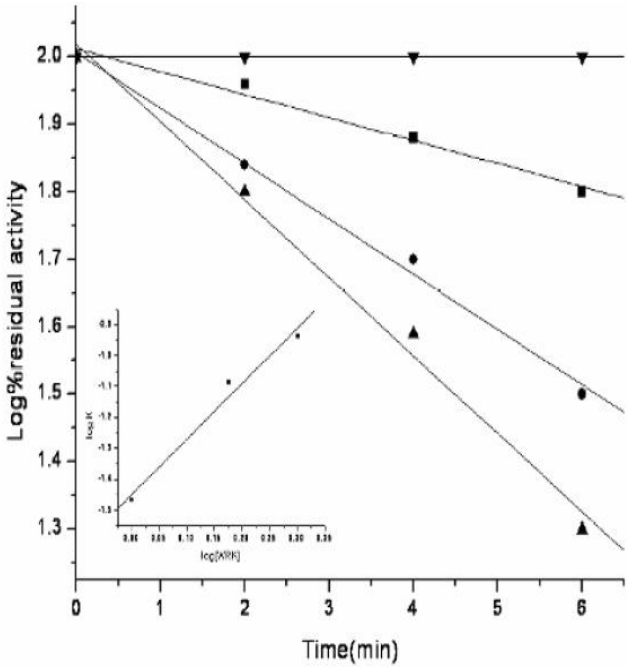


Insets: second -order plots of the pseudo –first order rate constants (K_{app})(min^{-1}) of inactivation at different concentrations of phenyl-glyoxal.
Concentration of PG were 0.0mM (▼), 1.0 mM (■), 2.0 mM (•), and 3.0 mM (▲).

Figure 2: Pseudo first order plots for inactivation of nuclease Stn α by phenyl glyoxal.

Modification of carboxylate groups

Purified nuclease when modified with Woodward's reagent K showed initially fast inactivation and lost 78% activity with modification of 2 carboxylate residues. (Figure 3) Substrate protection studies could restore 100 % activity in presence of DNA (TABLE 2) suggesting the involvement of carboxylate residues in substrate binding. Increase in Km and no change in Kcat also points towards the presence of carboxylate groups in substrate binding (TABLE 3).



Insets: second-order plots of the pseudo –first order rate constants (K_{app})(min^{-1}) of inactivation at different concentrations of WRK. Concentrations of WRK were 0mM (▼), 1.0mM (■), 1.5mM (•) and 2.0mM (▲).

Figure 3: Pseudo first order plots for inactivation of nuclease Stn a by WRK.

X-ray crystallographic studies on type II restriction endonuclease EcoRV (a dimmer), showed that the binding of metal ion occurs through two Asp and one Glu residues, which forms a common motif for this classs of enzymes [7]. In case of *S.* marcescens nuclease, the residues Asn 119 and Glu127 involved in contact with water cluster coordination (required for the integrity of magnesium binding site) are conserved throughout in the Serria endonuclease family[11,7].

The properties of nuclease Stn α viz. obligate requirement of metal ions, substrate specificity and mode of action and involvement of similar residues closely resembles that of *S.* marcescens nuclease. Hence, it is reasonable to assume that nuclease Stn α may also follow the metal-water cluster mediated mechanism for the hydrolysis of DNA.

CONCLUSION

Chemical modification studies, on nuclease Stn α revealed that the active site of the enzyme consists of a substrate binding site and a catalytic site. Histidine and carboxylate are involved in substrate binding, while arginine is involved in catalysis.

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