Research Paper

Purification, characterization and thermodynamics of antifungal protease from *Streptomyces* sp. A6

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A 20 kDa antifungal serine protease from *Streptomyces* sp. A6 was purified to 34.56 folds by gel permeation chromatography. The enzyme exhibited highest activity at neutral to near alkaline pH 7–9 and 55 °C. Neutral surfactant triton X-100 enhanced the activity by 4.12 fold. The protease activity also increased (109.9–119%) with increasing concentration of urea (2–8 mole/l). The enzyme was identified as serine protease with 67% similarity to SFase 2 of *Streptomyces fradiae* by MALDI-LC-MS/MS analysis. Determination of kinetic constants k_m, $V_{\max}$, k_{cat} and k_{cat}/k_m suggested higher affinity of enzyme for N-Suc-Ala-Ala-Val-Ala-*p*NA (synthetic substrate for chymotrypsin activity). The enzyme was highly stable at temperature prevailing under field conditions (40 °C) as apparent from K_d and $t_{1/2}$ values, 0.0065 and 106.75 min, respectively and high ΔG^* and negative ΔS^* values, 87.17 KJ/mole and –126.95 J/mole, respectively. Thermal stability and increased activity of protease in presence of commonly used chemical fertilizer, urea, suggested its feasibility for agricultural applications. The present study is the first report on thermodynamic and kinetic properties of an antifungal protease from *Streptomyces* sp. A6. The study reflects potential of this enzyme for biocontrol of fungal plant pathogens.

Keywords: Antifungal protease / Kinetics / Purification / *Streptomyces* / Thermodynamics

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Introduction

Streptomyces are quantitatively the most abundant and qualitatively the most important group among soil bacterial communities [1]. The ability to efficiently colonize the rhizhosphere and form desiccation resistant spores makes them excellent candidates for biocontrol studies. Furthermore, several members of the group are known to produce mycoltyic enzymes, siderophores and antimicrobials as principal antifungal agents [2]. Although chitinases and glucanases are considered as major mycolytic enzymes, few recent reports have also identified role of proteases in fungal biocontrol. Role of proteolytic enzymes produced by the *Trichoderma harzianum* and *Pseudomonas aeruginosa* M-1001 in biological control of fungal pathogens has been demonstrated [3, 4]. Studies have shown that overproduction of extracellular protease by *Stenotrophomonas maltophilia* strain W81 enhanced its biocontrol efficacy against the fungal pathogen [5]. Antifungal action of protease suggests their potential in agriculture for control of fungal phytopathogens. Such novel agricultural application will lead to increased demand of proteases and thus search for newer sources. Although proteases have been reported from several *Streptomyces* species [6, 7], there are no reports on their antifungal potential. We have recently investigated the antagonistic properties of *Streptomyces* sp. A6 against number of fungal plant pathogens. Indirect evidence suggested that *Streptomyces* sp. A6 antagonized first by antibiosis leading to cell death followed by degradation of cell wall by mycolytic enzymes. *Streptomyces* sp. A6 when grown in liquid

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medium with fungal biomass as sole carbon and nitrogen source exhibited considerable proteolytic activity suggesting role of protease in antagonistic process and its potential in biocontrol of fungal plant pathogens. However, for such applications the enzyme must be active and stable at various temperatures especially those prevailing in fields. Therefore the present investigation was carried out to purify, characterize and study the catalytic and thermodynamic parameters of antifungal protease from *Streptomyces* sp. A6.

Materials and methods

Organism and culture conditions

Protease producing bacterium was isolated from the intertidal zone 2 km way from the sea coast of Diu (Gujarat, India) and was selected based on antifungal activity against *Fusarium udum* on casein agar plate. The organism was identified as *Streptomyces* sp. A6 by morphological, biochemical and 16S rDNA sequence (1.2 kb) analysis (96% similarity to *Streptomyces fradiae*). The GeneBank Accession No. for the nucleotide sequence is DQ908927.1 [8].

The organism was grown in medium containing (g/l): Shrimp waste, 14; $FeCl_3$, 0.035; $ZnSO_4$, 0.065 and pH, 8.0 for 72 h at 30 °C under shaking conditions (180 rpm). The culture was centrifuged at 10,000 × *g* for 10 min (4 °C) and the culture supernatant was used as a source of protease [8].

Enzyme purification

The culture supernatant was precipitated using ammonium sulphate (saturation up to 80%). The precipitate was dissolved in 50 mM Tris-Cl, pH 7.2 and dialysed overnight against the same buffer. Dialysed enzyme was subjected to ultrafiltration using 100 kDa cut off membrane filter to remove high molecular weight impurities. The filtrate obtained was concentrated using 10 kDa cut off membrane filter. The concentrated enzyme (~0.6 ml i.e. 2% of the column volume) was applied to sephacryl S-100-HR (Sigma) gel permeation column (1.0 × 40 cm) having column volume of 31.4 ml equilibrated with 50 mM Tris-Cl buffer pH 7.2. The fractions (1.5 ml) were collected at a flow rate of 0.4 ml per min and analysed for protein content and caseinolytic activity. All the purification steps were carried out at 4 °C. The protein content of fractions was determined spectrophotometrically at 280 nm using crystalline bovine serum albumin (Sigma) as the standard.

Enzyme assay

Caseinolytic activity was determined as described by Oceguera-Cervantes *et al.* [9]. One unit of caseinolytic activity was defined as the amount of enzyme that released 1 μg of tyrosine per hour under standard assay conditions.

Polyacrylamide gel electrophoresis and zymogram analysis

The samples were examined by electrophoresis on 10% polyacrylamide gel containing 0.1% sodium dodecyl sulphate (SDS PAGE) according to the method of Laemmli [10]. Protein bands on gel were detected by silver staining method of Sambrook and Russell [11].

For zymogram analysis, the samples were mixed with 5 × Laemmli sample buffer containing SDS without reducing agent and were kept at 45 °C for one hour for binding of SDS to protein and then loaded on to the gel. The samples were electrophoresed at 4 °C after which the gel was immersed in 2.5% triton X-100, 100 mM Tris-Cl (pH 7.2) for 15 min. The gel was overlaid with the same buffer containing 2% gelatine without triton X-100 and incubated for 1 h at 55 °C. Finally the gel was stained using Coomassie Brilliant Blue R-250. Clear zone on a blue background indicated protease activity.

Determination of antifungal activity of purified protease

Antifungal activity of the purified protease was studied by fungal spore germination inhibition assay. 0.1 ml of fungal spore suspension (10^6 conidia/ml) of *Fusarium udum* was incubated at 25 °C for 24 h with varying amounts (20–100 units/ml) of purified protease. Samples from each mixture were analysed for spore germination using a Neubauer's chamber. EC_{50} dose of protease for inhibition of fungal spore germination was determined by plotting percentage inhibition *vs* protease activity.

Antifungal activity of the purified protease was also studied by well diffusion technique. A standard cork borer of 8 mm was used to cut a well in the center on surface of potato dextrose agar plate. Three agar blocks (8 mm diameter) containing confluent growth of the test fungus was placed at equal distance from the central well. 600 units of purified enzyme (5.56 mg) was introduced in central well and was allowed to diffuse for 30 min at 4 °C and was then incubated at 25 °C for 7 d. Zone of inhibition was considered as a measure of antifungal activity. Chemical fungicide mancozeb (50 μg/ml of active ingredient), Dow agrosciences, India,

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Pvt. Ltd. and heat inactivated protease were used as positive and negative controls, respectively.

Effect of pH on protease activity

The activity of purified enzyme was determined at various pH from 5 to 11 using substrates (1% casein) prepared in different buffers, 100 mM: Sodium citrate buffer pH 5.0; Phosphate buffer pH 6.0; Tris-Cl buffer pH 7.0, 8.0 and 9.0; Glycine-NaOH buffer pH 10.0 and 11.0. The caseinolytic activity was determined using these substrates in the assay system at 55 °C.

Effect of temperature on protease activity

The optimum temperature and activation energy (E_a) for enzyme activity was determined by assaying the caseinolytic activity at different temperatures ranging from 30–80 °C. E_a was calculated using Arrhenius plot [12] of ln [protease activity] versus $1/T$, where $E_a = -$ slope $\times R$, R (gas constant) = 8.314 J/K/mol.

The effect of temperature on the rate of reaction was expressed in terms of temperature quotient (Q_{10}), which is the factor by which the rate increases due to a raise in the temperature by 10 °C. Q_{10} was calculated using the Eq. (1) as given by Dixon and Webb [12].

$$Q_{10} = \text{antilog}_e (E \times 10/RT^2) \quad (1)$$

$E = E_a$ = Activation energy.

Effect of inhibitors, metal ions and detergents on enzyme activity

The enzyme was incubated with different inhibitors (1 mM): phenyl methyl sulfonyl fluoride (PMSF), dithiotritol (DTT) and ethylene diamine tetraacetic acid (EDTA); metal ions (5 mM): $ZnSO_4$, $MnSO_4$, $MgSO_4$, $CaCl_2$, $FeSO_4$, $CuSO_4$; detergents (0.5%): sodium dodecyl sufate (SDS), cetyl trimethyl ammonium bromide (CTAB), triton X-100, tween 80, span 80, dicotylsulfosuccinate; and urea at concentrations 2, 4 and 8 M at 20 °C for 30 min. The caseinolytic activity was determined at 55 °C using Tris-Cl pH 7.2. The relative activity was calculated with respect to the control without treatment.

Substrate specificity

The activity of purified enzyme was estimated using the synthetic peptide substrates N-Succinyl-Ala-Ala-Pro-Phe-*p*NA, N-Succinyl-L-Phe-*p*NA, N-Succinyl-Ala-Ala-Val-Ala-*p*NA and N-Succinyl-Ala-Ala-Ala-*p*NA (para nitroanilide) as described by Izotova *et al.* [13]. 1.25 ml of 50 mM Tris-HCl buffer pH 7.2 was added to 12.5 µl of substrate (from the stock of 10 mg/ml in dimethylformamide, DMF) and preincubated at 55 °C for 5 min. The reaction was initiated with 100 µl of enzyme. After incubation for 15 min the reaction was stopped with 0.25 ml of 2 M sodium citrate buffer pH 5.0 and the released *p*-nitroaniline was estimated spectrophometrically at 410 nm. For blank tubes, the enzyme was added after sodium citrate buffer. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmole of *p*-nitroaniline per min under these standard conditions.

Determination of catalytic constants

Kinetic constants ($V_{\max}$, k_m, k_{cat} and k_{cat}/k_m) were determined using Lineweaver–Burk double reciprocal plot [12] by assaying protease activity at a fixed enzyme concentration with varying concentrations of substrates: 0.88–6.16 µM casein and 0.036–0.36 µM N-Suc-Ala-Ala-Val-Ala-*p*NA (chymotrypsin specific substrate) at 55 °C.

Thermal stability

The purified enzyme was incubated with 100 mM Tris-HCl buffer pH 7.2 at different temperatures 30, 40, 50, 60 and 70 °C for 120 min. The caseinolytic activity was determined at 55 °C with intervals of 15 min. The residual activity was expressed as percentage of the initial activity.

Estimation of deactivation rate constant and thermodynamic parameters for protease deactivation

Thermal inactivation kinetics of the purified protease was determined by first order expression:

$$dE/dt = -K_d E \, . \quad (2)$$

So that

$$\ln [E_t/E_0] = -K_d t \, . \quad (3)$$

The K_d (deactivation rate constant or first order rate constant) values were calculated from slopes obtained by a plot of ln $[E_t/E_0]$ versus t (time) at a particular temperature and apparent half-lives were estimated using Eq. (4).

$$t_{1/2} = \ln 2/K_d \, . \quad (4)$$

Energy of deactivation was calculated from the slope of a linear plot of ln $[K_d]$ versus $1/T$ using the Arrhenius Eq. (6):

$$K_d = A \, e^{(-E/RT)} \, . \quad (5)$$

So that

$$\ln [K_d] = -E/RT + \ln A \, . \quad (6)$$

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Table 1. Purification of extracellular protease form *Streptomyces* sp. A6.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude	2236.35	433.71	5.16	1	100
Ammonium sulphate precipitation	1813.25	231.31	7.84	1.52	81.08
Dialysed sample	1659.79	137.72	12.05	2.34	74.22
100 kDa filtrate	921.99	28.17	32.73	6.35	41.23
10 kDa concentrate	969.77	20.25	47.88	9.28	43.36
Gel permeation sephacryl S-200	111.04	0.62	178.23	34.56	4.96

Thermodynamics of irreversible inactivation of the protease was determined by rearranging the Eyring's absolute rate Eq. (7) derived from the transition state theory [14].

$$K_d = (k_b T/h) \times e^{(-\Delta H^*/RT)} \times e^{(\Delta S^*/R)} \quad (7)$$

where,

k_b Boltzmann's constant (R/N) = 1.38×10^{-23} J/K
T Absolute temperature (K)
h Planck's constant = 6.626×10^{-34} Js
N Avogadro's number = 6.02×10^{23}/mol
R Gas constant = 8.314 J/K/mol
ΔH^* Change in enthalpy
ΔS^* Change in entropy

Eyring's equation was rearranged to give

$$\ln [K_d/T] = -(\Delta H^*/R)(1/T) + (\ln (k_b/h) + \Delta S^*/R) \quad (8)$$

ΔH^* and ΔS^* values were calculated from the slope and intercept of a ln [Kd/T] versus $1/T$ plot, respectively.

So that,

$$\Delta H^* = -\text{(slope)}\ R \quad (9)$$

$$\Delta S^* = R\ [\text{intercept} - (k_b/h)]\ . \quad (10)$$

Free energy change (ΔG^*) for inactivation of protease were calculated by applying Eq. (11):

$$\Delta G^* \text{ (Gibb's free energy change)} = -RT \ln (K_d h/k_b \times T)\ . \quad (11)$$

MALDI–LC–MS/MS analysis

The enzyme was identified by MALDI–LC–MS/MS peptide sequence analysis at The Centre for Genomic Applications, New Delhi (TCGA). In-gel tryptic digestion was performed and the digested mixture of peptides was subjected to MALDI–TOF and LC–MS/MS for peptide mass fingerprinting and peptide sequencing. The data was analyzed using MASCOT search engine tool.

Results

Enzyme purification and identification

The active protease form *Streptomyces* sp. A6 was purified to 34.56 folds using gel permeation chromatography with a final yield of 4.96% (Table 1). The elution pattern of gel permeation fractions is depicted in Fig. 1.

The purity of enzyme was confirmed by SDS PAGE analysis. A single band of molecular mass 20 kDa was obtained (Fig. 2a). The identity of the band as protease was confirmed by zymogram analysis (Fig. 2b).

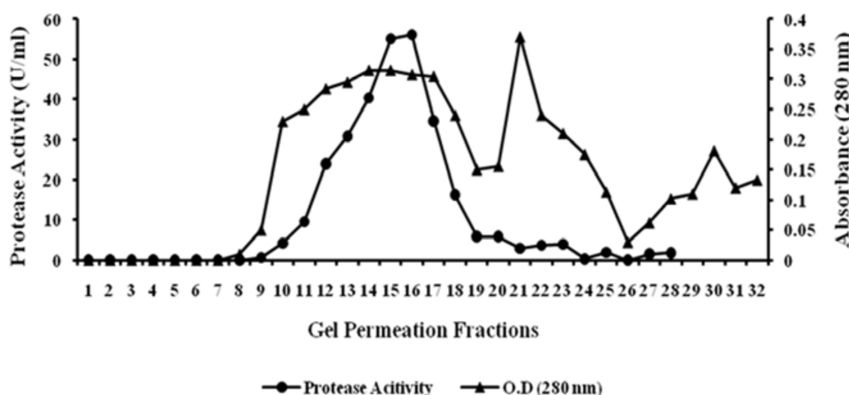

Figure 1. Elution profile of protease form *Streptomyces* sp. A6 on sephacryl S-100.

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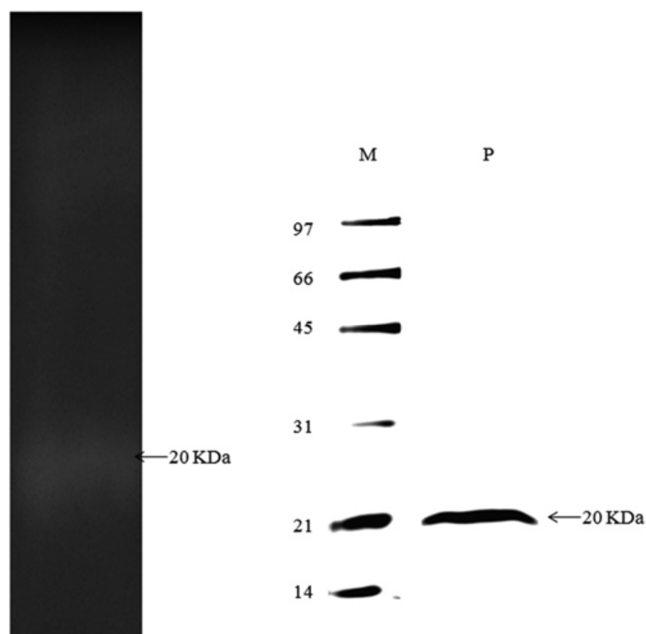

Figure 2. (a) Zymogram analysis of purified protease from *Streptomyces* sp. A6. Clear zone indicates protease activity. (b) SDS PAGE of the purified protease from *Streptomyces* sp. A6. Lane M shows protein molecular weight marker 97–14 kDa and lane P shows the purified protease from gel permeation fraction containing highest specific activity.

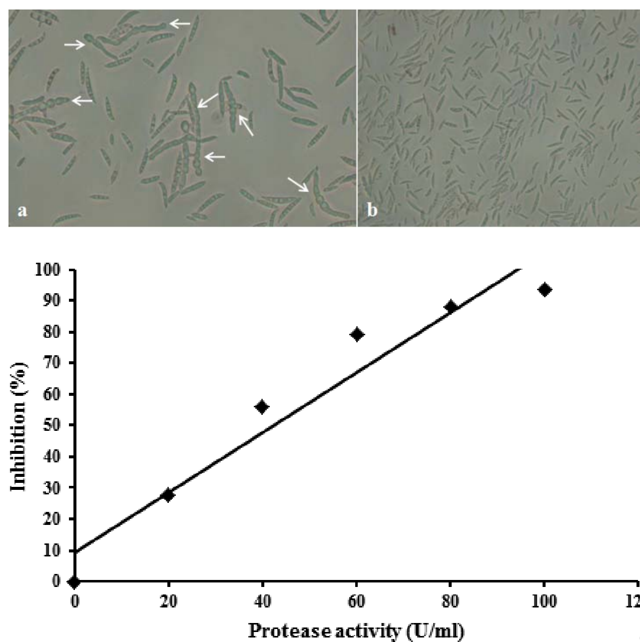

Figure 3. Phase contrast microscopy of *Fusarium udum* spore germination assay (magnification 100X). (a) Germination of spores after treatment with heat inactivated purified protease from *Streptomyces* sp. A6 (arrow shows germinated spores); (b) Inhibition of spore germination after treatment with active purified protease from *Streptomyces* sp. A6. (c) Regression plot of *Fusarium udum* inhibition by antifungal protease from *Streptomyces* sp. A6. $R^2 = 0.94$.

Antifungal activity of purified protease

Inhibition of *Fusarium udum* spore germination and hyphal extension by purified protease from *Streptomyces* sp. A6 was studied using phase contrast microscopy and well diffusion method, respectively (Figs. 3a, b and 4). The purified protease inhibited germination of *Fusarium udum* spores in dose dependent manner. EC_{50} dose of protease was found to be 37.0 units/ml as calculated from the plot of % inhibition *vs* protease activity (Fig. 3c). Inhibition of the advancing edge of growth of *Fusarium udum* indicated the antifungal property of protease from *Streptomyces* sp. A6. Antifungal activity of protease was also observed for other fungal plant pathogens like *Rhizoctonia* sp. and *Alternaria* sp. (data not shown).

Effect of pH and temperature on enzyme activity

The enzyme exhibited highest activity in the pH range 7.0–9.0 with maximum activity at pH 7.0 (100 ± 6.61%) and 94.05 ± 1.3 and 94.32 ± 6.96% activity at pH 8 and 9, respectively (Fig. 5a). These results indicated that the extracellular protease from *Streptomyces* sp. A6 is a neutral to near alkaline protease.

Optimum temperature for enzyme activity was recorded as 55 °C (100 ± 1.4%) with relative activities of 83.33 ± 0.315 and 70.55 ± 0.49% at 40 and 70 °C, respectively (Fig. 5b). These results were suggestive of a broad range of temperature optima for activity of the purified enzyme. The activation energy (E_a) for substrate hydrolysis by purified protease was 7.96 KJ/mol. The Q_{10} value (temperature quotient) was 1.1 between temperatures 20–80 °C.

Effect of various chemical on enzyme activity

Table 2 represents the effect of various chemicals on activity of purified protease. The enzyme was strongly inhibited by 1 mM PMSF, a serine protease inhibitor, which indicated it to be a serine protease. EDTA inhibited the enzyme activity by 58% whereas DTT had no effect. Amongst the metal ions tested, maximum activity was retained in presence of $CaCl_2$ (104.20 ± 2.42%) and $ZnSO_4$ (99.53 ± 6.38%) after incubation for 30 min while maximum inhibition was observed with $CuSO_4$ retaining only 16.76 ± 2.05% activity. Most of the detergents tested enhanced the activity of protease. Highest enzyme activity was observed in presence of 1% triton-X 100 which was 4.12 fold higher the untreated control. Urea exhibited positive effect on enzyme activity. Increase in enzyme activity was observed with increasing concentration of urea from 2 to 8 M (109.9–119% activity).

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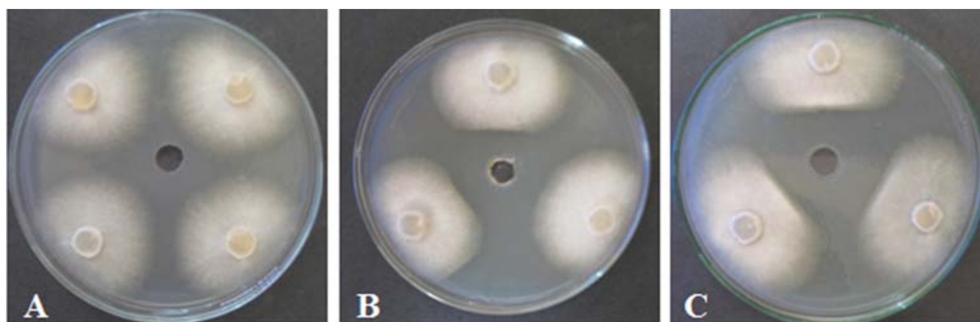

Figure 4. Antifungal activity of (A) heat inactivated purified protease from *Streptomyces* sp. A6 (negative control); (B) purified protease from *Streptomyces* sp. A6; (C) chemical fungicide mancozeb, 50 μg/ml of active ingredient (positive control), by well diffusion method.

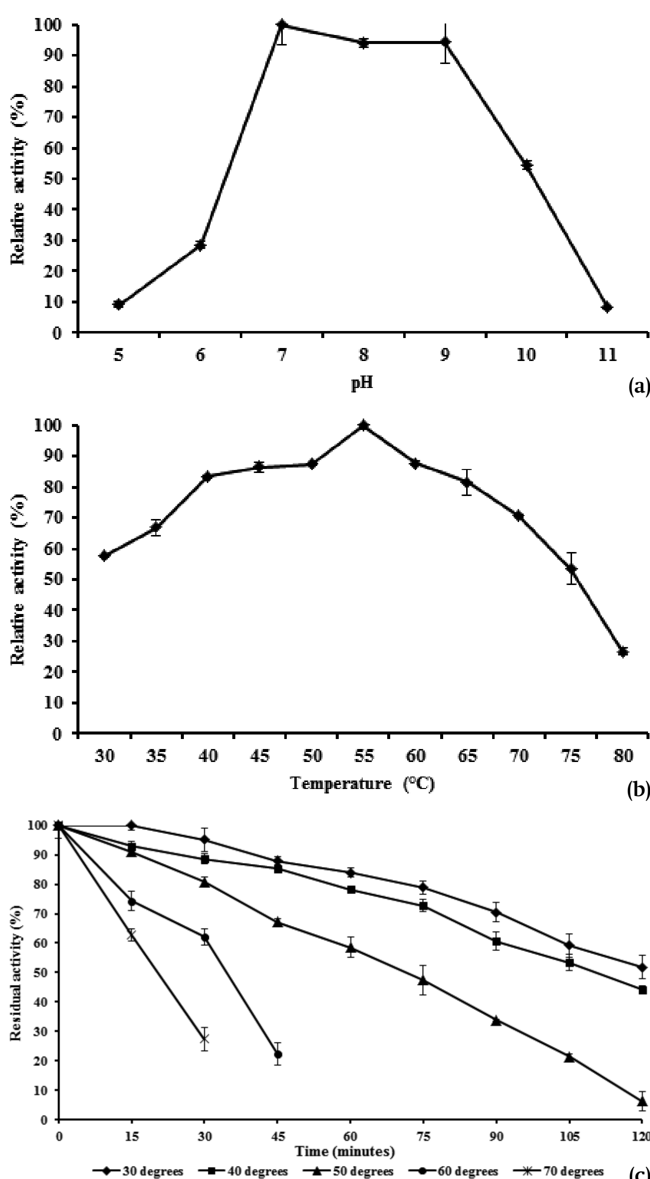

Figure 5. (a) Effect of pH on protease activity; (b) Effect of temperature on protease activity; (c) Thermal stability of protease at different temperatures.

Table 2. Effect of various chemical agents on protease activity.

Chemical agents	Residual activity (%)
Control	100 ± 0.17
Inhibitors (1 mM)	
PMSF	2.45 ± 0.71
EDTA	42.15 ± 0.16
DTT	78.33 ± 0.34
Surfactants (0.5%)	
SDS	25.21 ± 1.89
CTAB	31.78 ± 0.72
Triton X-100	412.33 ± 0.00
Tween 80	127.18 ± 4.71
Span 80	109.30 ± 3.50
Dicotylsulfosuccinate	50.43 ± 3.62
Metal ions (5 mM)	
$ZnSO_4$	99.53 ± 6.38
$MnSO_4$	62.85 ± 0.20
$MgSO_4$	91.705 ± 6.39
$CaCl_2$	104.20 ± 2.42
$FeSO_4$	83.06 ± 6.37
$CuSO_4$	16.76 ± 2.05
Urea (M)	
2	109.90 ± 2.76
4	116.27 ± 3.26
8	119.00 ± 2.75

Substrate specificity and catalytic constants for protease

Among the various synthetic substrates tested, highest enzyme activity (304.29 ± 13.23 U/ml) was observed with N-Suc-Ala-Ala-Val-Ala-pNA. No activity was observed with N-Suc-L-Phe-pNA (Table 3).

The enzyme had a higher affinity for chymotrypsin as revealed by low K_m, 0.201, compared to that for casein ($K_m = 4.26$). The V_{max} for chymotrypsin hydrolysis (344.83 U/ml) by the enzyme was also found to be higher compared to casein hydrolysis. The results of kinetic parameters, k_m, V_{max}, k_{cat} (turnover number) and k_{cat}/k_m (catalytic efficiency) are summarized in Table 4.

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Table 3. Substrate specificity of protease with synthetic peptides.

Substrate	Activity (U/ml)
N-Suc-Ala-Ala-Val-Ala-*p*NA	304.29 ± 13.23
N-Suc-Ala-Ala-Pro-Phe-*p*NA	33.1 ± 5.29
N-Suc-Ala-Ala-Ala-*p*NA	29.33 ± 0.5
N-Suc-L-Phenylalanine-pNA	0

Table 4. Kinetic parameters for protease.

Treatments	Kinetic constants			
	k_m (μM)	V_{max} (U/ml)	k_{cat} (s^{-1})	k_{cat}/k_m (s^{-1}/μM)
Casein	4.26	285.71	1.92	0.451
N-Suc-Ala-Ala-Val-Ala-*p*NA	0.207	344.83	2.31	11.19

Thermal stability and deactivation rate constants for protease

Fig. 5c shows the temperature stability of protease from *Streptomyces* sp. A6. The deactivation rate constant (K_d) increased with increasing temperature whilst the half-life ($t_{1/2}$) decreased with increasing temperature (Table 5). The enzyme was highly stable at 40 °C as apparent from the K_d and $t_{1/2}$ values 0.0065 and 106.75 min, respectively. The enzyme was also stable at higher temperatures up to 70 °C with K_d and $t_{1/2}$ value of 0.043 and 16.07 min.

Thermodynamic parameters for protease deactivation

The activation energy for protease deactivation was calculated as 50.014 KJ/mole. Change in enthalpy (ΔH^*), entropy (ΔS^*) and free energy (ΔG^*) for deactivation of protease at 40 °C were 47.41 KJ/mole, –126.95 J/mole and 87.17 KJ/mole, respectively (Table 5).

MALDI–LC–MS/MS analysis

The purified protease was identified by MALDI-LC-MS/MS analysis. The peptide sequences obtained after tryptic digestion were –.IAGGEAIYAAGGGR.C, R.CSLGFNVR.S, R.AGTSFPGNDYGLIR.H, R.HSNASAA-DGR.V, R.VYLYNGSYR.D, R.DITGAGNAYVGQTVQR.S. The MALDI-LC-MS/MS generatedpeptides sequences, when compared to NCBI protein database by BLAST search analysis revealed 67% similarity of *Streptomyces* sp. A6 protease with SFase 2 of *Streptomyces fradiae*. The calculated molecular mass of enzyme was 19.219 kDa with pI of 6.99.

Discussion

Streptomyces are known to produce several antifungal agents, mycolytic enzymes being one of them. Although chitinases and glucanases form the major components of mycolytic enzymes, some recent reports have identified proteases as another antifungal principal [3–5]. Chitinases and glucanases from *Streptomyces* species have been well studied for their antifungal activities; however, there are no reports on antifungal property of proteases from these organisms. We have previously isolated a high protease producing strain (A6) of *Streptomyces* sp. and optimized its protease production using statistical approach [8]. The present investigation was carried out to purify and explore the antifungal potential of *Streptomyces* sp. A6 protease. The molecular mass of purified protease (20 kDa) as revealed by SDS PAGE analysis was close to that reported for protease of *Streptomyces fradiae*, SFase-2, (19 kDa). However, the substrate specificity of *Streptomyces* sp. A6 protease was different from SFase-2. The purified protease exhibited antifungal activity against *Fusarium udum* and other fungal plant pathogens by inhibiting spore germination and hyphal extension. Although the biochemical mechanism of antifungal activity of proteases is not yet clear, inhibition of fungal spore germination and hyphal extension by protease has also been reported from *P. aeruginosa* M-1001 [4], while extracellular serine protease produced by *Stenotrophomonas malto-*

Table 5. Deactivation rate constants (K_d) and half life ($t_{1/2}$) and thermodynamic parameters $\Delta E_{a(d)}$[a], ΔH^*, ΔS^* and ΔG^* for deactivation of protease at different temperatures.

Temperature (°C)	K_d (× 10^{-3})	$t_{1/2}$ (min)	ΔH^* (KJ/mol)	ΔS^* (J/mol)	ΔG^* (KJ/mol)
30	5.453	127.105	47.49	–130.52	87.06
40	6.493	106.747	47.41	–126.95	87.17
50	19.4	35.729	47.33	–127.61	88.56
60	33.5	20.691	47.24	–130.40	90.69
70	43.127	16.072	47.16	–138.82	93.41

[a] Value of $\Delta E_{a(d)}$ was calculated as 50.014 KJ/mol. ΔH^* is change in enthalpy; ΔS^* is change in entropy and ΔG^* is Gibb's free energy.

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philia strain W81 exhibited biological control by dissolving the mycellium of *Pythium ultimum* [5].

Cajanus cajan is the principal pulse crop of Gujarat region in India. Major loss (around 30%) in yield of this crop is caused by *Fusarium udum*. Laboratory scale studies and field trials with *Streptomyces* sp. A6 have revealed its potential in controlling fusarium wilt of *Cajanus cajan* (unpublished). Moreover, protease from *Streptomyces* sp. A6 was tolerant to a range of pesticides commonly used in fields (unpublished) and can thus prove as a nontoxic additive to commercially used formulation of fungicides.

Optimum pH, temperature and tolerance towards various chemicals are important considerations when a protease is used for industrial or agricultural purposes. The soil pH and temperature in Gujarat (India) ranges from neutral to alkaline and 40–45 °C, respectively (www.agri.gujarat.gov.in). The maximum activity of protease from *Streptomyces* sp. A6 in neutral to near alkaline range of pH and more than 80% activity at 40–45 °C revealed its feasibility for application in fields of Gujarat. Activation energy (E_a) for substrate hydrolysis was calculated. The low value of E_a explains the correct conformation of active site for favorable ES* complex formation, hence requring less energy. The effect of temperature on rate of reaction was measured in terms of temperature quotient (Q_{10}). The usefulness of calculating a Q_{10} value is that it suggests whether or not the metabolic reactions being examined are controlled by temperature or by some other factor. For enzymatic reactions the values of Q_{10} is between 1–2 and any deviation from this value indicates that some factor other than temperature is controlling the rate of reaction. Q_{10} value of 2 suggests doubling of rate of reaction with every 10 °C rise in temperature [12]. The Q_{10} value for casein hydrolysis by protease of *Streptomyces* sp. A6 was found to be 1.1 reflecting that every 10 °C raise in temperature increased the rate of reaction by 10%. Complete inhibition of enzyme activity by PMSF was suggestive of it being a serine protease. LC-MS analysis of the purified enzyme also revealed its identity as serine protease with similarity to 19 kDa serine protease of *Streptomyces fradiae*. Enhanced enzyme activity in presence of a neutral detergent triton X-100 signified the stabilizing effect of such detergents. Ezgimen *et al.* has reported 2 fold increase in activity of West Nile virus protease by triton X-100 [15]. Urea is a most commonly used fertilizer in agricultural fields. Increase in protease activity of *Streptomyces* sp. A6 with increasing concentration of urea upto 8 M suggested that the enzyme is resistant to the denaturing effect of urea and therefore, can have potential for agricultural applications. Dodia *et al.* have reported salt dependent resistance of alkaline protease to chemical denaturants from a newly isolated haloalkaliphilic *Bacillus* sp. [16].

The purified protease had higher affinity for chymotrypsin specific substrate and hydrolysed it more efficiently compared to casein as evident from the low k_m and high $V_{\max}$, k_{cat} and k_{cat}/k_m values.

Stability of enzymes is important parameter which determines the economic feasibility of applying them for agricultural process. Enzyme thermostability encompasses thermodynamic and kinetic stabilities. Thermodynamic stability is defined by the enzyme's free energy of stabilization whereas; the enzyme's kinetic stability is often expressed as its half-life ($t_{1/2}$) at defined temperatures. The high stability of purified protease at 40 °C as revealed by high $t_{1/2}$ value suggested its applicability in agricultural fields for biocontrol of fungal plant pathogens. Investigation of other thermodynamic parameters like activation energy of deactivation (E) and change in enthalpy (ΔH^*), entropy (ΔS^*) and free energy (ΔG^*) of enzyme is necessary to understand the behaviour of molecules in different conditions. The low ΔG^* value for the heat labile enzyme corresponds to the large ΔH^* and ΔS^* contributions and conversely the high ΔG^* corresponds to the low ΔH^* and ΔS^* for heat-stable enzyme. The value of $\Delta S^* > 0$ is suggestive of increased randomness of the activated transition state reflecting an increased disorder (of the active site or of the structure), which is the main driving force of heat denaturation. Conversely, low ΔS^* value reflects conformation stability and resistance to denaturation [17]. The study of these thermodynamic parameters at 40 °C suggested that the thermal stability of enzyme was due to higher value of ΔG^* and negative value of ΔS^* which enabled the enzyme to resist against thermal denaturation. Similar results have been reported for the endoglucanase (CMCase) of *Aspergillus oryzae* cmc-1 and chitinase of *Pantoea dispersa* [18, 19].

Thus, the present study revealed the biocontrol potential of antifungal protease from *Streptomyces* sp. A6. Moreover, thermal stability of the purified protease at temperatures prevailing under field conditions suggested its feasible for agricultural applications.

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Conflict of interest

Authors have no conflict of interest.

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