Appl Biochem Biotechnol (2011) 164:77–88
DOI 10.1007/s12010-010-9116-8

Purification and Characterization of Chitinase from *Paenibacillus* sp. D1

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Received: 26 June 2010 / Accepted: 18 October 2010 /
Published online: 29 October 2010

Abstract A 56.56-kDa extracellular chitinase from *Paenibacillus* sp. D1 was purified to 52.3-fold by ion exchange chromatography using SP Sepharose. Maximum enzyme activity was recorded at pH 5.0 and 50 °C. MALDI-LC-MS/MS analysis identified the purified enzyme as chitinase with 60% similarity to chitinase Chi55 of *Paenibacillus ehimensis*. The activation energy (E_a) for chitin hydrolysis and temperature quotient (Q_{10}) at optimum temperature was found to be 19.14 kJ/mol and 1.25, respectively. Determination of kinetic constants k_m, V_{max}, k_{cat}, and k_{cat}/k_m and thermodynamic parameters ΔH^*, ΔS^*, ΔG^*, ΔG^*_{E-S}, and ΔG^*_{E-T} revealed high affinity of the enzyme for chitin. The enzyme exhibited higher stability in presence of commonly used protectant fungicides Captan, Carbendazim, and Mancozeb compared to control as reflected from the $t_{1/2}$ values suggesting its applicability in integrated pest management for control of soil-borne fungal phytopathogens. The order of stability of chitinase in presence of fungicides at 80 °C as revealed from $t_{1/2}$ values and thermodynamic parameters $E_{a(d)}$ (activation energy for irreversible deactivation), ΔH^*, ΔG^*, and ΔS^* was: Captan > Carbendazim > Mancozeb > control. The present study is the first report on thermodynamic and kinetic characterization of chitinase from *Paenibacillus* sp. D1.

Keywords Chitinase · Fungicides · IPM · Kinetics · *Paenibacillus* · Thermodynamics

Introduction

Chitinases (EC 3.2.1.14) are glycoside hydrolases that catalyzes the degradation of chitin, a linear β-1,4-linked polymer of N-acetylglucosamine. Chitinases occur in a wide range of organisms including bacteria, fungi, plants, insects, and animals. The role of chitinase in these organisms is diverse [1]. In recent years, chitinases have received increased attention due to their wide range of biotechnological applications, especially in production of chito-oligosaccharides and N-acetyl D-glucosamine (which function as antibacterial agents, elicitors of lysozyme inducers, and immunoenhancers) [2, 3], preparation of sphaeroplasts and protoplasts from yeast and fungal species [4], and bioconversion of chitin waste to

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single cell protein [5]. In agriculture, chitinolytic microorganisms have been widely used for biocontrol of fungal phytopathogens as cell wall of most of the fungal pathogens contains chitin to varying extent [6].

Paenibacillus species are widely distributed in nature and have been reported for their biocontrol potential against bacteria and/or fungi [7] owing to production of antimicrobial substances (antibiotics, bacteriocins, and/or small active peptides) and cell wall degrading enzymes (β-1,3-glucanases, cellulases, chitinases, and proteases) [8].

Our earlier work with chitinase from *Paenibacillus* sp. D1 has revealed its potential in controlling Fusarium wilt of *Cajanus cajan* both at laboratory and field levels (unpublished). Moreover, the chitinase from *Paenibacillus* sp. D1 exhibited strong tolerance towards range of pesticides commonly used in fields [9]. Therefore, chitinase from *Paenibacillus* sp. D1 can have potential to be used as nontoxic additive to commercially used formulations of fungicides and can contribute in reducing the dose of toxic chemical fungicides in fields.

Such agricultural application of chitinases requires an understanding of its enzymatic and thermodynamic properties. The most important environmental factor effecting the catalysis and stability of enzymes in such applications is temperature. Agricultural fields are natural habitats where temperature fluctuations are frequent. Therefore, success of fungicide formulations containing chitinases will depend upon enzyme's stability under such conditions. Studies on the thermodynamic stability of enzymes have provided some fundamental insights into factors that determine enzyme stability [10]. However, no such studies have been reported with chitinases in presence of fungicides.

The present investigation was therefore carried out to purify and characterize the catalytic and thermodynamic properties of chitinase from *Paenibacillus* sp. D1.

Materials and Methods

Organism and Culture Conditions

The medium used for growth and chitinase production by *Paenibacillus* sp. D1 was as mentioned earlier [11]. The culture was centrifuged at 10,000×*g* for 10 min (4 °C), and the culture supernatant was used as source of chitinase.

Enzyme Purification

The culture supernatant was precipitated using ammonium sulphate (saturation up to 70%). The precipitate was dissolved in 50 mM sodium acetate buffer, pH 4.0 and dialysed overnight against the same buffer. Dialysed enzyme was subjected to ion exchange chromatography using SP Sepharose column (4×2 cm). The column was equilibrated with 50 mM acetate buffer (pH 4.0). Two millilitres of the dialysed enzyme was loaded and eluted using a linear gradient of NaCl (0–0.5 M) at a flow rate of 0.5 ml per minute. Fractions collected were assayed for chitinase activity, and protein was estimated by measuring absorbance at 280 nm.

Chitinase Assay

Chitinolytic activity was estimated as described by Vyas and Deshpande [12]. One unit of chitinase activity was defined as the amount of enzyme required to liberate 1 μmole of *N*-acetyl-D-glucosamine equivalent at 50 °C per hour.

Polyacrylamide Gel Electrophoresis and Zymogram Analysis

The samples having chitinase activity were examined by electrophoresis on 10% polyacrylamide gel containing 0.1% sodium dodecyl sulphate (SDS PAGE) according to the method of Laemmli [13]. Protein bands on gel were detected by silver staining method of Sambrook and Russell [14].

For zymogram analysis, 1% ethylene glycol chitin and 0.001% fluorescent brightener (Calcofluor white M2R, Sigma) was incorporated in the polyacrylamide gel and the samples loaded by mixing with 5× Laemmli sample buffer (containing SDS but not the reducing agent) without boiling. The samples were electrophoresed at 4 °C after which the gel was immersed in 50 mM sodium acetate buffer (pH 5.0) containing 2.5% triton X-100, for 15 min to remove SDS. The gel was washed twice with the same buffer (without triton X-100) and incubated for 1 h at 50 °C. Finally, the gel was visualised under UV light. Clear zone on a fluorescent background indicated chitinase activity.

Effect of pH and Temperature on Chitinase Activity

Chitinase activity was measured at different pH values by the standard assay method using acid swollen chitin as the substrate. The pH of reaction mixtures was varied using the 50 mM buffers (pH 3.0, sodium citrate and citrate phosphate; pH 4.0, sodium citrate and sodium acetate; pH 5.0, sodium acetate and sodium succinate; pH 6.0, sodium phosphate and maleate; pH 7.0, sodium phosphate, Tris–maleate, and Tris–Cl; pH 8.0, sodium phosphate and Tris–Cl; pH 9.0, Tris–Cl and glycine NaOH; pH 10.0, glycine NaOH and carbonate–biocarbonate). The optimum temperature for enzyme activity was determined by incubating the reaction mixture at different temperatures like 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, and 60 °C and assaying the enzyme activity.

Activation energy (E_a) for enzyme activity was determined by assaying the chitinase activity at different temperatures ranging from 30 °C to 80 °C. E_a was calculated using Arrhenius plot [15] of ln [chitinase activity] versus 1/T, where $E_a = -\text{slope} \times R$, $R(\text{gas constant}) = 8.314\text{J/K/mol}$.

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 [16].

$$Q_{10} = \text{antilog}_{\varepsilon}\left(E \times 10/RT^2\right) \tag{1}$$

$E=E_a$ Activation energy

Catalytic Constants for Chitin Hydrolysis

Kinetic constants (V_{max}, K_m, k_{cat}, and k_{cat}/K_m) were determined using Lineweaver–Burk plot of $1/v$ versus 1/[S] [15] by assaying chitinase activity at a fixed enzyme concentration with varying concentrations of substrate (acid swollen chitin), 0.1–2.0 mg/ml for 10 min at 50 °C. For determination of k_{cat} and k_{cat}/K_m, the value of V_{max} was expressed in terms of micromoles per minute per millilitre.

Estimation of Thermodynamic Parameters for Chitin Hydrolysis

The thermodynamic parameters for substrate hydrolysis were calculated using Eyring's absolute rate equation derived from the transition state theory [16].

$$k_{\mathrm{cat}} = (k_{\mathrm{b}}T/h) \times e^{(-\Delta H^*/RT)} \times e^{(\Delta S^*/R)} \text{where,} \tag{2}$$

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

$$\Delta H^* = E_{\mathrm{a}} - RT \tag{3}$$

$$\Delta G^* \text{ (Gibb's free energy of activation)} = -RT\ln(k_{\mathrm{cat}}h/k_{\mathrm{b}} \times T) \tag{4}$$

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \tag{5}$$

The free energy of substrate binding and transition state formation was calculated using the following derivations:

$$\Delta G^*_{\mathrm{E-S}}(\text{free energy of substrate binding}) = -RT\ln K_{\mathrm{a}} \tag{6}$$

where $K_{\mathrm{a}}=1/K_{\mathrm{m}}$

$$\Delta G^*_{\mathrm{E-T}}(\text{free energy for transition state formation}) = -RT\ln(k_{\mathrm{cat}}/K_{\mathrm{m}}) \tag{7}$$

Thermal Stability

The purified enzyme was incubated with 50 mM sodium acetate buffer pH 5.0 at different temperatures 30–80 °C for 120 min in presence of 100 μg/ml of fungicides (Mancozeb, Captan, and Carbendazim). The chitinolytic activity was determined at 50 °C with intervals of 15 min. The residual activity was expressed as percentage of the initial activity.

Estimation of Deactivation Rate Constant

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

$$\mathrm{d}E/dt = -K_{\mathrm{d}}E \tag{8}$$

So that

$$\ln[E_t/E_0] = -K_{\mathrm{d}}t \tag{9}$$

The K_d (deactivation rate constant or first order rate constant) values were calculated from slopes obtained by a plot of $\ln[E_t/E_0]$ or ln[residual activity] versus t (time) at a particular temperature and apparent half-lives were estimated using Eq. 10.

$$t_{1/2} = \ln 2/K_d \tag{10}$$

The half-life is known as the time where the residual activity reaches 50%.

Estimation of Thermodynamic Parameters for Chitinase Deactivation

Energy of deactivation was calculated from the slope of a linear plot of $\ln[K_d]$ versus $1/T$ using the Arrhenius Eq. 12 [17]:

$$K_d = Ae^{(-E/RT)} \tag{11}$$

So that

$$\ln[K_d] = -E/RT + \ln A \tag{12}$$

Thermodynamics of irreversible inactivation of the chitinase was determined by rearranging the Eyring's absolute rate Eq. 13 derived from the transition state theory.

$$K_d = (k_b T/h) \times e^{(-\Delta H^*/RT)} \times e^{(\Delta S^*/R)} \tag{13}$$

Eyring's equation was rearranged to give

$$\ln[K_d/T] = -(\Delta H^*/R)(1/T) + (\ln(k_b/h) + \Delta S^*/R) \tag{14}$$

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

So that,

$$\Delta H^* = -(\text{slope})R \tag{15}$$

$$\Delta S^* = R[\text{intercept} - (k_b/h)] \tag{16}$$

Free energy change (ΔG^*) for inactivation of chitinase were calculated by applying the following equations:

$$\Delta G^* \text{ (Gibb's free energy change)} = -RT\ln(K_d h/k_b \times T) \tag{17}$$

Results

Purification and Characterization of Chitinase

The active chitinase form *Paenibacillus* sp. D1 was purified to 52.3-fold by ion exchange chromatography using SP Sepharose with a final yield of 10.17% and specific activity 492.4 units/mg protein (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 56.56 kDa was obtained (Fig. 2a). The identity of the band as chitinase was confirmed by zymogram analysis (Fig. 2b).

Table 1 Purification of chitinase form *Paenibacillus* sp. D1

Purification steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Culture medium supernatant	32,730	3475	9.42	1	100
Ammonium sulphate precipitation	26,555	2794	9.5	1.01	81.13
Ion exchange (SP Sepharose)	3,329	6.76	492.4	52.3	10.17

The purified chitinase was also identified by MALDI-LC-MS/MS analysis. The peptide sequences obtained after tryptic digestion, R.QWDDVAK.A and R.TAFANSALQYIR.A, showed ion score of 60 in Mascot search revealing identity or extensive homology (p value <0.05) of *Paenibacillus* sp. D1 chitinase with chitinase Chi55 of *Paenibacillus ehimensis*.

The enzyme exhibited highest activity in the pH range 4.0–8.0 with maximum activity at pH 5.0 (100±0.57%) and 85.63±1.52% and 90.61±1.2% activity at pH 4 and 8, respectively (Fig. 3). These results indicated that chitinase from *Paenibacillus* sp. D1 was active over a broad pH range.

Optimum temperature for enzyme activity was recorded as 50 °C (100±0.67%) with relative activities of 76.60±2.32% and 86.07±2.04% at 40 °C and 60 °C, respectively (Fig. 4). These results were suggestive of a broad temperature optima for activity of the purified enzyme. Activation energy (E_a) for substrate hydrolysis by purified chitinase was 19.14 KJ/mol. The effect of temperature on rate of reaction was measured in terms of temperature quotient (Q_{10}). The Q_{10} value (temperature quotient) of *Paenibacillus* sp. D1 chitinase for chitin hydrolysis was 1.29–1.23 between temperatures 25–60 °C.

Catalytic Constants and Thermodynamic Parameters for Chitin Hydrolysis

Purified chitinase showed typical Michaelis–Mentan profile. The K_m and V_{max} values as determined by Lineweaver–Burk plot for chitin hydrolysis at 50 °C and pH 5.0 were 4.97 mg and 55.25 units/ml, respectively. The turn over number (k_{cat}) of enzyme was 83.46/s and the apparent second-order rate constant (k_{cat}/K_m) was 16.79/mg/s.

The thermodynamic parameters enthalpy (ΔH^*), Gibbs free energy (ΔG^*), and entropy (ΔS^*) of activation for chitin hydrolysis and free energy for activation of substrate binding

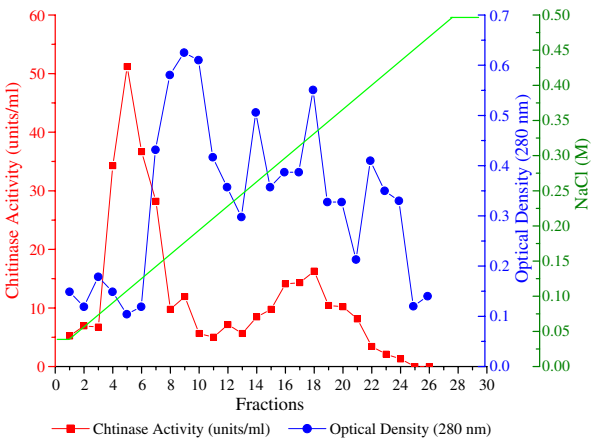

Fig. 1 Elution profile of chitinase form *Paenibacillus* sp. D1 on SP Sepharose column

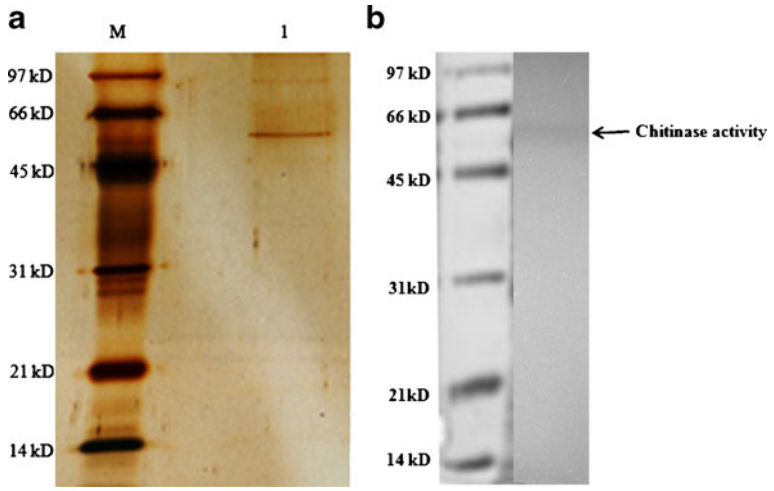

Fig. 2 **a** SDS PAGE of the purified chitinase from *Paenibacillus* sp. D1. *Lane M* shows protein molecular weight marker 97–14 kDa, and *lane 1* shows the purified chitinase from SP Sepharose fraction having highest specific activity. **b** Zymogram analysis of purified chitinase from *Paenibacillus* sp. D1. *Clear zone* indicates chitinase activity

(ΔG^*_{E-S}) and formation of activated (transition) complex (ΔG^*_{E-T}) were investigated (Table 2).

Thermal Stability and Deactivation Rate Constants for Chitinase

Kinetics of thermal inactivation of chitinase from *Paenibacillus* sp. D1 was measured (Fig. 5). The enzyme was found to be highly stable at maximum temperatures prevailing under fields conditions during summers (40–50 °C) in Gujarat region of India with half-life ($t_{1/2}$) values 370.68 and 255.15 min, respectively. Further increase in temperature increased the inactivation rate. However, the enzyme was quite stable at 80 °C with $t_{1/2}$ of 22.61 min (Table 3).

Thermal Deactivation of Chitinase in Presence of Fungicides

The deactivation rate for chitinase was studied at different temperatures 30–80 °C in presence of fungicides Mancozeb, Captan, and Carbendazim (Fig. 5). All the test fungicides

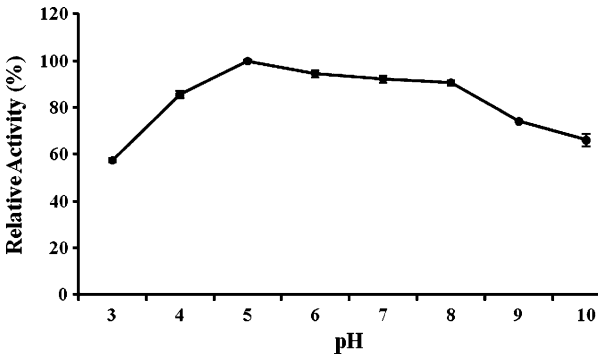

Fig. 3 Effect of pH on chitinase activity

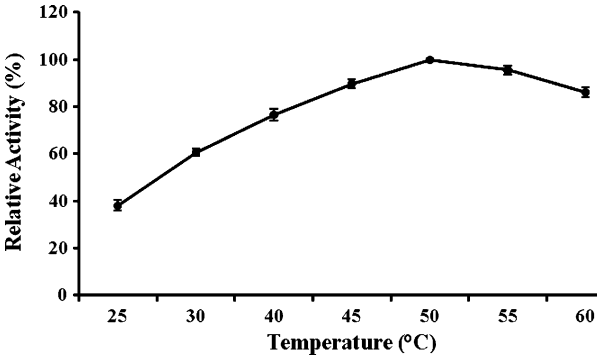

Fig. 4 Effect of temperature on chitinase activity

increased the thermal stability of chitinase. Maximum stability of chitinase was observed in presence of Captan with $t_{1/2}$ values of 542.95 and 469.41 min at maximum field temperatures (40–50 °C) and 119.17 min at 80 °C. The stability of the three fungicides was in the order: Captan > Carbendazim > Mancozeb > control (Table 3).

Thermodynamic Parameters for Chitinase Deactivation

Investigation of thermodynamic parameters like change in enthalpy (ΔH^*), change in entropy (ΔS^*), change in free energy (ΔG^*), and activation energy ($E_{a(d)}$) of deactivation of enzyme was carried out to understand the behaviour of molecules at different temperatures in presence of fungicides. Values for all the thermodynamic parameters are given in Tables 4 and 5. The $E_{a(d)}$ for chitinase deactivation calculated in presence of Captan, Carbendazim, Mancozeb, and control was 28.95, 38.59, 42.91, and 55.08 KJ/mol, respectively.

Discussion

Health hazards and environmental risks associated with excessive use of toxic pesticides in agriculture has prompted greater interest in alternative pest control strategies. Biocontrol

Table 2 Kinetic and thermodynamic parameters for chitin hydrolysis by chitinase of *Paenibacillus* sp. D1

Parameters	
Kinetic	
K_m (mg)	4.97
V_{max} (units/ml)	55.25
k_{cat} (/s)	83.46
k_{cat}/K_m (/mg/s)	16.79
Thermodynamic (KJ/mol)	
ΔH^*	16.45
ΔS^* (J/mol)	−157.89
ΔG^*	67.47
ΔG^*_{E-S}	4.31
ΔG^*_{E-T}	−7.58

K_m Michaelis constant, k_{cat} turnover number, k_{cat}/K_m second-order rate constant, ΔH^* change in enthalpy, ΔS^* change in entropy, ΔG^* free energy of activation, ΔG^*_{E-S} free energy for substrate binding, ΔG^*_{E-T} free energy for transition state formation

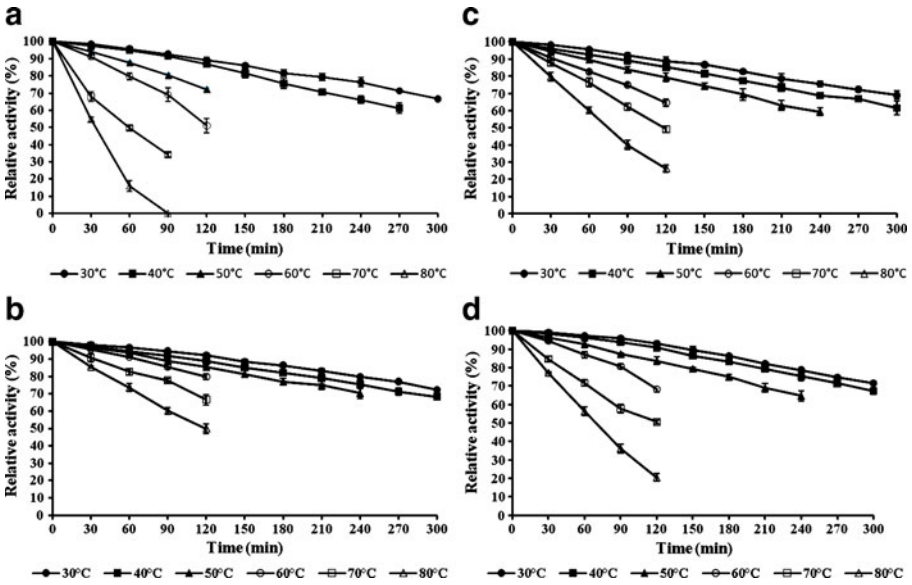

Fig. 5 Thermal stability of chitinase at different temperatures. **a** Control. **b** In presence of Captan. **c** In presence of Carbendazim. **d** In presence of Mancozeb

and integrated pest management (IPM) methods that completely or significantly reduce the use of pesticides offers best alternative to chemical control. Besides, being ecofriendly, these methods are more economic and target specific [18]. Although biocontrol has been used and proved effective under controlled conditions, results have been highly variable when applied in fields, mainly due to environmental fluctuations and residual pesticides in fields. One of the ways to overcome the ineffectiveness of most of the biocontrol agents is to apply them in mixture with chemical pesticides to which they are tolerant [19].

In our earlier work, we had shown strong tolerance of *Paenibacillus* sp. D1 and its chitinase towards number of commonly used pesticides in for control of fungal and insect pests [9]. Since chitinases are major mycolytic enzymes against fungal phytopathogens [20], it was of interest to investigate the catalytic properties and thermal stability of chitinase from *Paenibacillus* sp. D1 chitinase in presence of fungicides for their possible

Table 3 Deactivation rate constants (K_d) and half-life ($t_{1/2}$) of chitinase at different temperatures in presence of Mancozeb, Captan, and Carbendazim

Temperature (°C)	Control		Mancozeb		Captan		Carbendazim	
	K_d ($\times 10^{-3}$)	$t_{1/2}$ (min)	K_d ($\times 10^{-3}$)	$t_{1/2}$ (min)	K_d ($\times 10^{-3}$)	$t_{1/2}$ (min)	K_d ($\times 10^{-3}$)	$t_{1/2}$ (min)
30	1.33	519.88	1.15	601.01	1.06	656.0	1.27	547.24
40	1.87	370.68	1.34	517.29	1.28	542.95	1.59	435.95
50	2.72	255.15	1.8	384.38	1.48	469.41	2.18	317.48
60	5.39	128.6	3.06	226.53	1.87	370.68	3.55	195.08
70	11.74	59.04	5.81	119.24	3.22	215.27	5.86	118.29
80	30.65	22.61	13.09	52.95	5.82	119.17	11.21	61.82

ND not determined

Table 4 Thermodynamic parameter for chitinase deactivation in presence of Mancozeb, Captan, and Carbendazim

Treatment	ΔH^* (KJ/mole)	ΔS^* (J/mole)	$E_{a(d)}$ (KJ/mole)
Control	52.36	−129.97	55.08
Mancozeb	40.19	−171.35	42.91
Captan	26.23	−177.99	28.95
Carbendazim	35.87	−183.90	38.59

ΔH^* change in enthalpy, ΔS^* change in entropy, $E_{a(d)}$ activation energy for deactivation

use in integrated pest management strategies. In IPM, mixture of Bacillus-based biocontrol agents and fungicides are mainly used for seed dressing. For example, today nearly all cotton plantations are treated with Kodiak (*Bacillus subtilis* GB03) and fungicides [21]. Since, *Paenibacillus* sp. D1 and its chitinase exhibited strong tolerance towards protectant fungicides (Mancozeb, Captan, and Carbendazim), used for seed dressing to protect plants against soil-borne fungal pathogens, they were selected for the present study.

The molecular mass of purified chitinase (56.56 kDa) was found to be close to Chi55 (55.35 kDa) of *P. ehimensis*. The mascot search following LC-MS analysis of the purified chitinase also revealed its close homology to Chi55 of *P. ehimensis*. The pH and temperature are most important factors affecting the activity and stability of enzymes to be used under harsh industrial conditions and fluctuating environmental conditions in fields for agricultural applications. The soil pH and temperature in Gujarat (India) ranges from neutral to alkaline and 40–45 °C, respectively (www.agri.gujarat.gov.in). High activity (90.61± 1.2%) of chitinase from *Paenibacillus* sp. D1 at pH 8.0 and maximum activity at 40–60 °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 for catalysis. The effect of temperature on rate of reaction was measured in terms of temperature quotient (Q_{10}). Q_{10} values are used to infer whether or not the metabolic reactions being examined are controlled by temperature or by some other factor. Generally, enzymatic reactions show Q_{10} values between 1 and 2 and any deviation from this value is indicative of involvement of some factor other than temperature in controlling the rate of reaction. Q_{10} value of 2 suggests doubling of rate of reaction with every 10 °C rise in temperature [15]. The Q_{10} value for chitin hydrolysis by chitinase of *Paenibacillus* sp. D1 was found to be 1.29–1.23

Table 5 Values of ΔG^* (Gibb's free energy) for deactivation of chitinase at different temperatures

Temperature (°C)	ΔG^* (KJ/mole) for deactivation of chitinase			
	Control	Mancozeb	Captan	Carbendazim
30	90.97	91.33	91.55	91.10
40	93.17	94.04	94.17	93.59
50	95.23	96.33	96.87	95.82
60	96.36	97.93	99.29	97.52
70	97.12	99.12	100.81	99.10
80	97.22	99.71	102.10	100.17

between temperatures 25–60 °C reflecting that every 10 °C raise in temperature increased the rate of reaction by 29–23%.

The purified chitinase exhibited high affinity for chitin and hydrolysed it efficiently as revealed from low k_m and high V_{max} values. Thermodynamic parameters for substrate hydrolysis were also calculated. Low values of enthalpy and negative values of entropy suggested formation of a more efficient and ordered transition state complex between enzyme and substrate. ΔG^* is the measure of spontaneity of any reaction. Low ΔG^* values suggest that the conversion of transition state complex (ES*) into product was more spontaneous. The feasibility and extent of an enzyme catalysed reaction is best determined by measuring change in ΔG^* for substrate hydrolysis, i.e., the conversion of E–S complex into product [20]. Low values of free energy for substrate binding (ΔG^*_{E-S}) and formation of activated complex (ΔG^*_{E-T}) confirmed that the chitinase had high affinity for chitin.

Stability of enzymes is an important parameter that determines the economic feasibility of applying them for agricultural process. Thermal stability represents the capability of enzyme molecule to resist thermal unfolding in absence of substrate, while thermophilicity is the ability of an enzyme to work at elevated temperatures in presence of substrate. 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 [10]. Effect of pesticide on stability of chitinase is important if they are to be used in combination for integrated pest management. Maximum stability of chitinase was observed in presence of Captan followed by Carbendazim and Mancozeb as revealed by $t_{1/2}$ values at 80 °C. The high stability of purified chitinase at temperatures prevailing under field conditions (40–50 °C) in presence of pesticides suggested its applicability in integrated pest management for control of fungal plant pathogens. The stability was high in all the treatments compared to control. Although there are few reports on integrated use of chitinases with pesticides for control of fungal phytopathogens [22, 23], but studies on stability and thermodynamics of chitinases in presence of pesticides at temperatures prevailing in fields is lacking. 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. These parameters were effective in the order: Captan > Carbendazim > Mancozeb > control. 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 conformational stability and resistance to denaturation [24]. The study of these thermodynamic parameters for chitinase deactivation in presence of pesticides at different temperatures 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.

Thus, an improved knowledge of enzyme deactivation kinetics at different temperatures in presence of different fungicides can explore feasibility of such fungicide formulations for agricultural applications. Moreover, enhanced stability of chitinase in presence of frequently used protectant fungicides against soil-borne fungal pathogens suggested its potential in integrated pest management.

Acknowledgment The work was supported by UGC/DRS Phase-II, New Delhi, India.

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