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Kinetic and Thermo-Inactivation Thermodynamic Parameters of a Novel Isolated *Serratia Marcescens* B4A Chitinase

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ABSTRACT

Chitinase is one of the essential enzymes that have broad applications in industrial, agriculture, medical through biodegradation of chitin and hence are in demand. Chitinase-producing Serratia marcescens B4A was cultured in the medium containing chitin and then was purified through ammonium sulfate precipitation and DEAE ion-exchange chromatography. The purity of chitinase was determined by one and two-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Its optimum activity was at pH 7.0 and temperature 50 °C and was stable at 90 °C for 60 min and over a pH range from 5.0-8.0. The K_m, V_{max}, k_{cat} and k_{cat}/K_m values were 3.72 mg ml^{-1}, 0.19 U ml^{-1}, 134.75 min^{-1} and 36.17 mg min^{-1} ml^{-1}, respectively that showed a high affinity of chitinase to the substrate and exhibited excellent catalytic efficiency. Investigation of irreversible thermo-inactivation of chitinase at a range of 60-90 °C revealed a high value of $\Delta G^{\#}$ with the low value of $\Delta H^{\#}$ and a negative value of $\Delta S^{\#}$. These characteristics of Serratia marcescens B4A chitinase showed high tolerance to thermal denaturation. Therefore, it may have a positive impact on industrial, antifungal applications and biodegrade chitin waste. The present study is the first report on the thermodynamic and kinetic characterization of chitinase from Serratia marcescens B4A.

Keywords: Chitinase, Serratia marcescens B4A, Purification, Kinetic characterization, Thermodynamic parameters

INTRODUCTION

Chitin is an insoluble homopolymer of β-1,4-N-acetyl-D-glucosamine units that is one of the most abundant polysaccharides in nature [1]. It is found mainly in many agronomically important pests, including fungi, nematodes and the exoskeleton of many crustaceans, insects and other arthropods (*e.g*., crabs, shrimp) and therefore, it is an excellent target for controlling pests and diseases [2-4].

On the other hand, N-acetyl-D-glucosamine and its oligosaccharides derived from hydrolysis of chitin have applications in industrial, biotechnology, agriculture, medical, food, and nutrition sciences [5,6], so degradation of chitin is a crucial issue in the world.

Unlike chemical degradation, enzymatic hydrolysis of chitin by chitinases has several benefits, including wholly digested chitooligosaccharides, low costs products, and no environmental pollution [7]. Chitinases (EC 3.2.1.14) glycosyl hydrolases 18, 19 families are a group of enzymes that received increasing attention because of their applications in the bioconversions of chitin waste and various industrial, pharmacological, and clinical applications. Besides, these enzymes are involved in the biocontrol of plant-pathogenic fungi by destroying major components of fungal cell walls [4,8]. Also, these enzymes have several other essential roles, such as valid biomarker in cancer [9], treatment of cystic fibrosis-associated fungal disease [10], diagnosis of multiple sclerosis [11,12].

Chitinases are produced by various organisms, such as fungi, plants, insects, some vertebrates and bacteria [13,14]. The useful catalytic properties of chitinolytic microorganisms made it important to screen and

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characterized these microorganisms. Some chitinolytic bacteria have been isolated and was also investigated their chitinase activity [15-19].

Serratia marcescens is one of the critical bacterium in chitin degradation [20]. In the *Serratia* genus, *S. polymuthica* was reported as a biocontrol agent [21], *S. marcescens* GG5 and *S. marcescens* B4A introduced as antifungal agents because of theirs high-chitinolytic activity [22,23].

Industrial hydrolysis of chitin by chitinase is associated with the chemical and physical nature of chitin [24]. Moreover, the enzymes' catalytic properties, such as temperature, pH, enzymatic deactivation and thermodynamic stability, are involved in chitin hydrolysis [24]. The determination of the enzyme's kinetic and thermodynamic parameters is essential for improving and optimizing the biological processes. In the present study, a native Iranian chitinase from *Serratia marcescens* B4A was purified and characterized its biochemical, kinetic, and thermodynamic properties to use for many applications.

METHODS AND MATERIALS

Materials

N-acetyl D-glucosamine, 3,5-dinitrosalicylic acid (DNS), Bovine serum albumin (BSA), and flake crab shell chitin were purchased from Sigma (St. Louis, MO, USA). Colloidal chitin was prepared from commercial chitin according to the optimized method of Roberts and Selitrennikoff [25]. All other reagent grade chemicals were purchased from Merck (Darmstadt, Germany).

Chitinase Production

Serratia marcescens B4A strain was isolated from the shrimp farming waste-water in the south of Iran [26], and the 16S rRNA gene sequence deposited in the GenBank nucleotide sequence database under the accession number HM535665 [26]. For chitinase production, *Serratia marcescens* B4A was cultured, according to zarei *et al.* [26].

Enzyme Assay

For the measurement of chitinase activity, colloidal chitin (1% w/v chitin in 20 mM phosphate buffer, pH 7.5) was used as a substrate by the modified method of Roberts and Selitrennikoff [25]. The reaction mixture containing 0.2 ml of the substrate and 0.2 ml enzyme solution was incubated at 50 ºC for one hour. The hydrolysis of chitin was assessed using the DNS method, according to Miller [27]. The amount of enzymatic hydrolysis was measured at 530 nm using a UV spectrophotometer. The Enzyme unit was determined by preparing serial dilutions of N-acetyl-glucosamine (from 0 to 50 mM). One unit (U) of the chitinase activity was defined as the amount of enzyme required to release 1 mmol of N-acetyl D-glucosamine (as a standard) from chitin/min under the conditions of assay.

Purification of Chitinase

All purification steps of chitinase were done at 4 °C. After cultivation, undegraded chitin and microbial cells were removed by centrifugation at 3,000 × g for 20 min. The supernatant containing extracellular chitinase was precipitated by ammonium sulfate at 80% saturation. After 4 h, the precipitate was then recovered by centrifugation at 10,000 g for 15 min, resuspended in 20 mM Tris buffer, pH 7.5, and dialyzed overnight against 20 mM Tris-HCl buffer adjusted to pH 7.5. The dialyzed sample was subjected to a DEAE-ion change column chromatography, which equilibrated with Tris-HCl buffer. The enzyme was eluted with a linear NaCl gradient (0.0-1.0 M in Tris-HCl buffer) at a 1 ml min^{-1} flow rate. The absorbance of the collected fraction's protein content was measured at 280 nm and assayed chitinase activity. The fractions with chitinase activity were pooled and stored at 4 °C.

Protein Quantification and Electrophoresis

According to the Bradford method, total protein contents were determined using BSA as a standard [28]. Collected protein samples in the purification steps of ammonium sulfate and DEAE chromatography were analyzed by sodium dodecyl sulfate (SDS-PAGE) according to the Laemmli method under reducing conditions [29]. The two-dimensional gel was also performed at pH 3.0-10.0 to check the enzyme preparations' homogeneity and determine enzymes with similar molecular weight and charge different [29]. After electrophoresis, the gel was stained with 0.1% (w/v) Coomassie brilliant blue R-250.

Characterization and Kinetics Studies of Purified Chitinase

To assay optimum pH and temperature, chitinase activity was assessed in a pH range of 2-12 using 50 mM mixed buffer (glycine-acetate-phosphate) and various temperatures of 10 to 90 °C in 20 mM phosphate buffer, respectively, under the assay conditions.

The enzyme was assayed after preparing the chitinase in the buffers with different pH values in a range of 3-12 for 90 minutes to determine the pH stability. The standard assay measured the residual activity.

The colloidal chitin concentration did the measurement of Michaelis-Menten constant (K_m), maximal reaction velocity (V_{max}), turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m), ranging from 0-20 mg ml^{-1}, calculation of this kinetic constants were performed from Michaelis-Menten curves using GraphPad Prism software and the standard deviations were <5% of the experimental values [30].

Irreversible Thermo-inactivation and Calculation of Thermodynamic Parameters

Irreversible thermal inactivation of enzyme samples was investigated at different temperatures ranging from 60-90 °C for various times from 0 to 60 min. For determination of the enzyme half-life ($t_{1/2}$), first, the constant of enzyme inactivation rate (k_{in}) was calculated from the slope of the thermostability graph [by regression plot of log relative activity (%) *versus* time (min)]. It was then determined by Eq. (1).

$$t_{1/2} = \ln 2/k_{in} \qquad (1)$$

The activation energy for irreversible deactivation (Ea(d)) of chitinase was obtained from the slope of the Arrhenius plot [regression of log inactivation rate constant versus reciprocal of absolute temperature in Kelvin] according to Eq. (2) [31].

$$Slop = -E_a/R \qquad (2)$$

The thermodynamics of the chitinase's irreversible inactivation was determined using Eyring's absolute reaction rate theory and the same equation in the other studies [32-34].

Calculation of the activation enthalpy ($\Delta H^{\#}$), Gibb's free energy of activation ($\Delta G^{\#}$), and activation entropy ($\Delta S^{\#}$) were estimated from Eqs. ((3), (4) and (5).

$$\Delta H^{\#} = E_a - RT \qquad (3)$$

$$\Delta G^{\#} = -RT\ln(k_{in}h/KBT) \qquad (4)$$

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

Where R is gas constant, T = absolute temperature, h = Planck's constant, and KB = Boltzman's constant.

Statistical Analysis

All data in the present study were analyzed using SPSS software, and data represented as means ± standard deviation of means.

RESULTS

Chitinase Purification and Electrophoresis

Purification of chitinase was done first by ammonium sulfate precipitation with 3.91-fold purification and 79% recovery, then dialyzed and was subjected to DEAE ion-exchange chromatography, which gave one peak with chitinase activity. The enzyme was purified by DEAE column to 10.65-fold with a 62% recovery and specific activity 2.45 u mg^{-1} (Table 1). A chromatogram of chitinase purification with DEAE ion-exchange chromatography was presented in Fig. 1. One- and two-dimensional electrophoresis of the purified enzyme in the purification steps of ammonium sulfate and DEAE chromatography showed that there was one enzyme with the molecular weight of 55 kDa and the pI almost 4.3 (Fig. 2).

Biochemical Characterization and Kinetics Studies of Purified Chitinase

Investigation of kinetics properties of chitinase showed that the enzyme was active at various pH of 3-12 with optimal activity at pH 7.0 and was stable over a pH range from 5.0-8.0 (Fig. 3A). Besides, the enzyme activity was in a range of 10- 90 °C with optimal activity at 50 °C and exhibited over 50% activity up to about 70 °C (Fig. 3B).

Table 1. Purification Steps of Extracellular Chitinase from *Serratia Marcescens* B4A

Purification step	Volume (ml)	Total activity (unit)	protein (mg ml^{-1})	Total protein (mg)	Specific activity (unit mg^{-1})	Fold purification	Yield (%)
Crude	25	1.73	0.9	22.5	0.23	1	100
Ammonium sulfate	17	1.38	0.09	1.53	0.90	3.91	79
DEAE	11	1.08	0.04	0.44	2.45	10.65	62

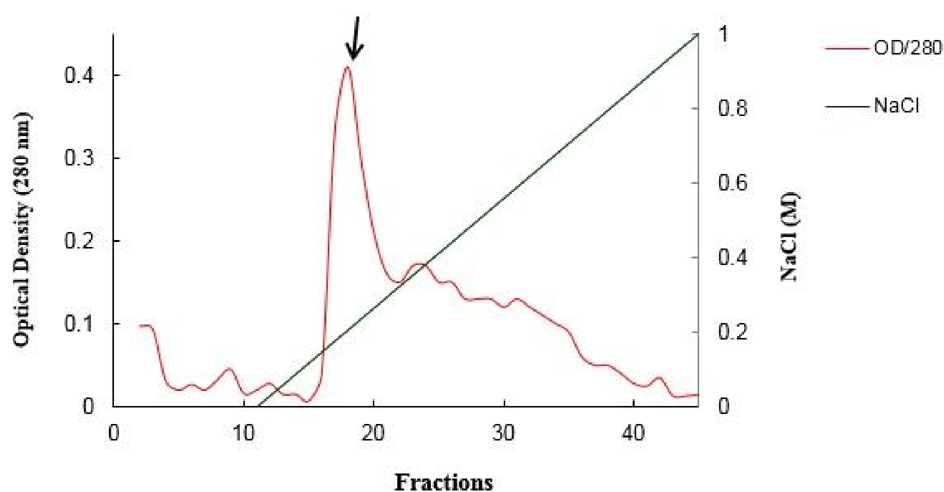

Fig. 1. Elution profile of *Serratia marcescens* B4A chitinase on the DEAE-ion change column. The fraction 18 that was showed by a filled arrow was a fraction with high activity of chitinase.

The values of K_m, V_{max}, k_{cat} and k_{cat}/K_m obtained from Michaelis-Menten curve was 3.72 mg ml^{-1}, 0.19 U ml^{-1}, 134.75 min^{-1} and 36.17 mg min^{-1} ml^{-1}, respectively (Fig. 3C).

Irreversible Thermo-inactivation Thermodynamic Parameters of the Chitinase

A thermal stability study of chitinase was carried out to understand the behavior of molecules at different temperatures (Fig. 3D). Enzyme k_{in} at various temperatures was calculated from thermal inactivation kinetic curve (Fig. 4A), and $Ea_{(d)}$ of purified chitinase, which obtained from the slope of the Arrhenius plot was 23.354 kJ mol^{-1} (Fig. 4B). The $t_{1/2}$ and thermodynamic parameters $\Delta G^{\#}$, $\Delta H^{\#}$ and $\Delta S^{\#}$ of chitinase for irreversible thermal inactivation at different temperatures are given in Table 2, which showed as the temperature rises, the values of $t_{1/2}$, $\Delta H^{\#}$ and $\Delta S^{\#}$ decreased, and $\Delta G^{\#}$ increased.

DISCUSSION

With the enormous consumption of most natural resources, enzymatic technology has dramatically helped many industries. Several studies have been performed on

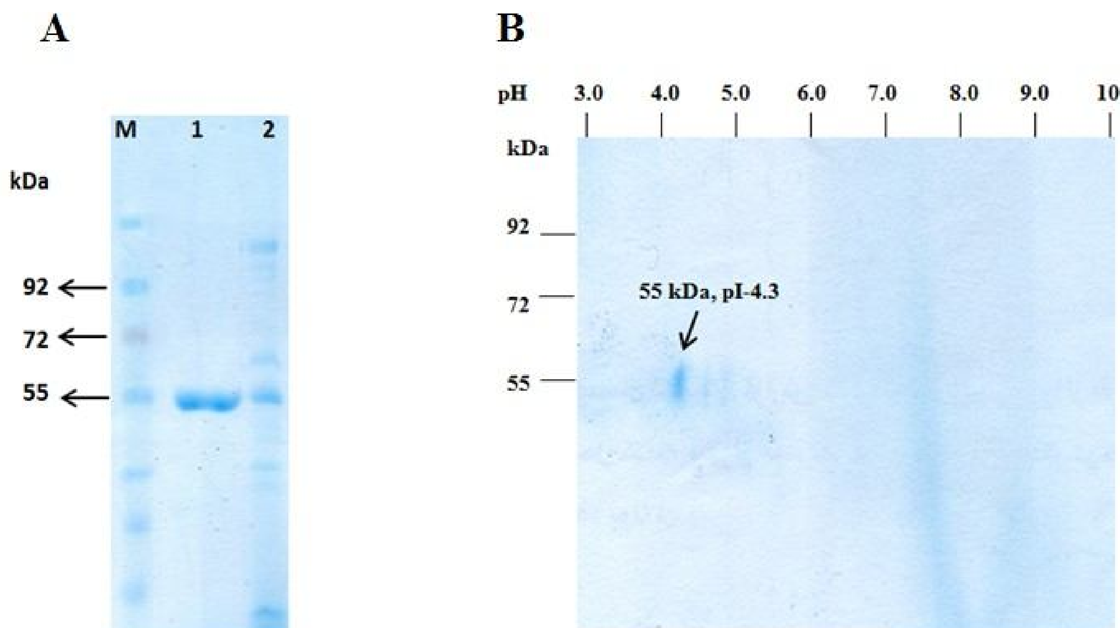

Fig. 2. SDS-PAGE electrophoretic pattern of chitinase enzyme. A) Lane M: marker protein, lane 1: purified chitinase from DEAE, lane 2: ammonium sulfate fractionated. B) Two-dimensional gel electrophoresis of purified chitinase from DEAE. The filled arrow showed one enzyme with a molecular weight of 55 kDa, and the pI almost 4.3.

the screening, purification, and characterization of chitinase due to its significant applications in various fields such as medicine, biotechnology, and industry [4,8]. The different biochemical and kinetic properties of microbial chitinases have made it essential to identify and characterize native bacterial chitinases. In between, one of the most effective bacteria for hydrolysis of chitin was *Serratia marcescens* [35].

In this study, chitinase was purified from *S. marcescens* B4A isolated from the shrimp farming soils in different areas of southern Iran [26]. Unlike previous reports, this chitinase's purification fold was 2.3 fold higher than chitinase from *Paenibacillus* thermo-aerophilous [36] and 4.9 fold lower than chitinase from *Paenibacillus sp.* D1, [33]. The purified chitinase's optimum temperature was 5-15 °C higher than reported *S. marcescens* chitinase [37,38] and was similar to chitinase from the other organisms such as *Aeromonas sp.* PTCC 1691 [16], and *S. marcescens* [35] had an optimum temperature of 50- 60 °C. The absolute advantage of the present enzyme purified from *S. marcescens* B4A is the over 50% activity up to 70 °C as compared to purified enzymes from mentioned microorganisms useful for industrial applications.

This enzyme showed the optimal activity at pH 7.0 higher than chitinases extracted from *S. marcescens* and *Aeromonas sp.* PTCC and was stable over a pH range similar to these reports [16,35]. The K_m value of chitinase was meager compared with other chitinases from different organisms *Bacillus licheniformis* [39] and *S. marcescens* [15] that reflect the high affinity of the enzyme for substrate. The catalytic efficiency obtained by the k_{cat}/K_m is an excellent factor for a more efficient selection of catalysts suitable for industrial applications. This enzyme has a higher advantage based on catalytic efficiency analysis than other extracted enzymes [33,40].

Also, the calculation of the thermodynamic parameters for enzyme deactivation is essential to investigate the enzyme behavior during thermal unfolding [41]. Investigation of enzyme thermostability through irreversible thermal inactivation at different temperatures and various times showed that this enzyme was stable at 70 °C for 60 min with about 20% activity. Also, $t_{1/2}$ of chitinase at

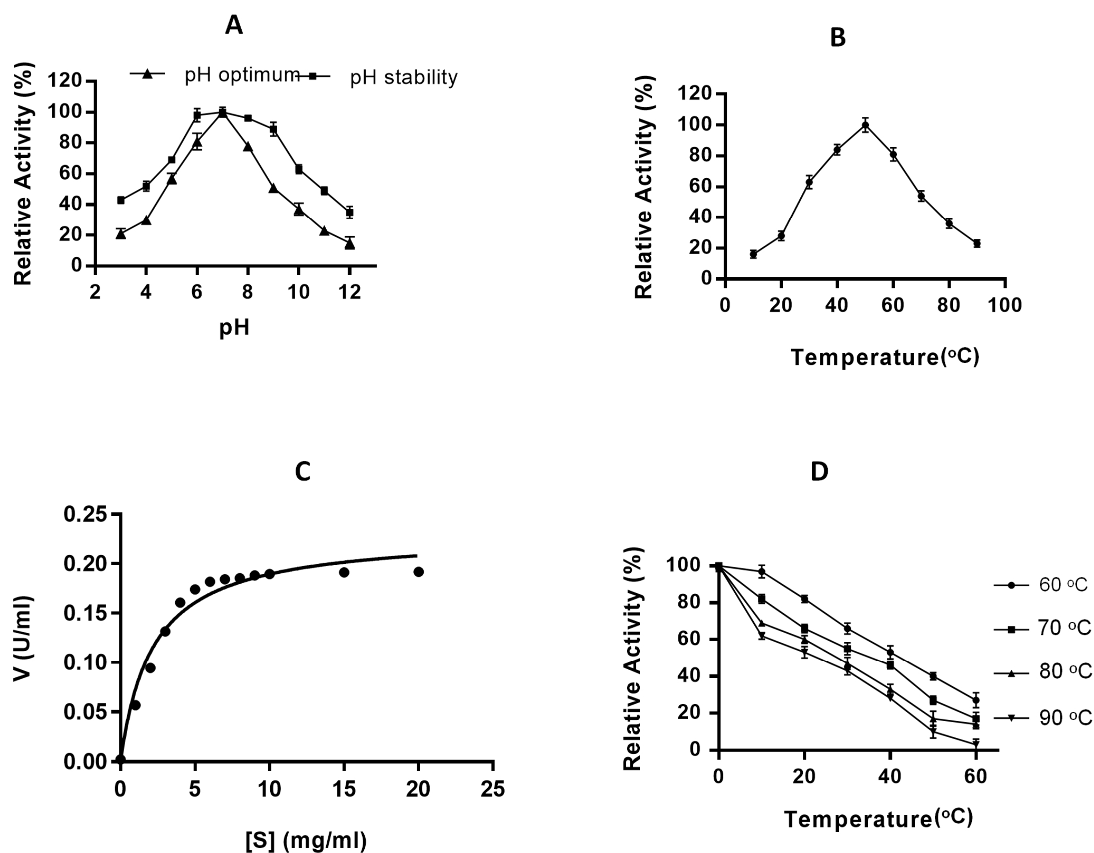

Fig. 3. Kinetic properties of chitinase from *Serratia marcescens* B4A. A) Effect of pH on activity and stability of chitinase. B) Effect of temperature on the activity of chitinase. C) The K_m and V_{max} values of purified chitinase were found to be 3.72 mg ml^{-1} and 0.19 U ml^{-1}, respectively. These Kinetics properties were calculated from the Michaelis-Menten curve. D) Thermal stability of chitinase at different temperatures, 60, 70, 80 and 90 °C. The maximum activity was set to 100%.

Table 2. Thermodynamic Parameters for Irreversible Thermal Inactivation of Chitinase

Temperature (°C)	$t_{1/2}$ (min)	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})
60	31.6	20.585	92.439	- 215
70	24.57	20.502	94.578	- 215
80	20.87	20.419	96.941	- 216
90	13.1	20.336	98.365	- 214

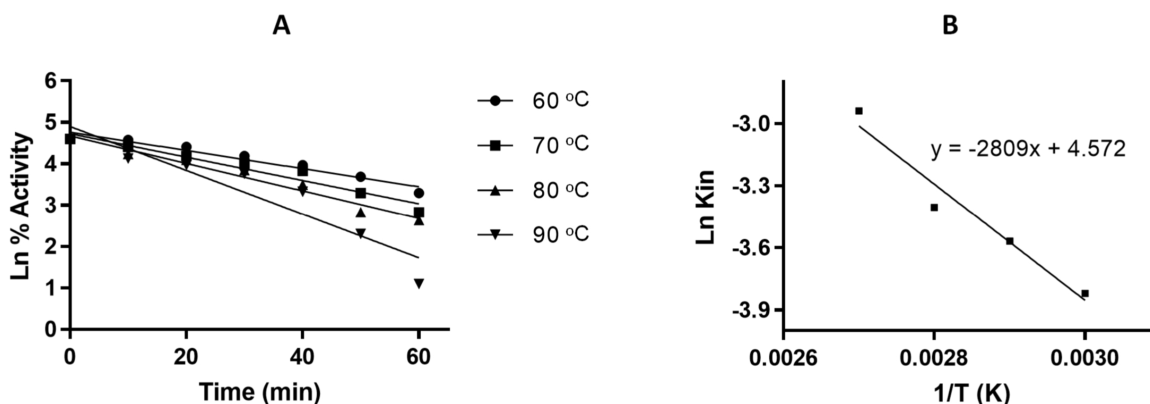

Fig. 4. Thermal activation and irreversible inactivation of chitinase. A) Thermal inactivation kinetic curve of chitinase at 60, 70, 80 and 90 °C. B) Determination of the activation energy for irreversible inactivation based on Arrhenius plots.

Table 3. Irreversible Thermo-inactivation Thermodynamic Parameters of the Chitinases

Source of chitinase	$t_{1/2}$ (min)	$Ea_{(d)}$ (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})	Ref.
Serratia marcescens B4A	31.6	23.35	20.58	92.43	- 215	Current study
Paenibacillus sp. D1	128.6	55.08	52.36	96.36	-129.97	[33]
Trichoderma harzianum	4.47	72.83	70.01	98.37	-85.12	[42]
Chitinase I from *Pantoea dispersa*	71.5	50.96	29.24	93.81	-53.38	[45]
Chitinase II from *Pantoea dispersa*	60	47.82	24.31	92.19	-55.57	[45]
Chitinase III from *Pantoea dispersa*	55	48.62	22.32	92.11	-56.31	[45]

60 °C was 31.6 min higher than chitinase from *Trichoderma harzianum* and lower than *Paenibacillus sp.* D1 chitinase, which was 4.47 and 128.6 min, respectively [33,42]. With the elevation of temperature, decreased $t_{1/2}$, and increased the k_{in}. Elevation of inactivation constant with the temperature indicated that high temperatures increase the rate of inactivation.

Another significant parameter that could describe the enzyme thermostability is the inactivation energy ($Ea_{(d)}$) that is the amount of energy required for the enzyme to begin the denaturation process. Our findings are in line with previous studies that reported that Ea's value exhibited that the

chitinase is relatively tolerant of the denaturation and requirements a more considerable amount of energy before protein unfolding takes place and initiates the process of thermal inactivation [43,44]. At a moderate temperature, the rate-determining step for the enzymatically irreversible thermal reaction is forming the unfold enzyme. The calculated $\Delta G^{\#}$ value at 60 °C was 92.43 kJ mol^{-1} higher than chitinase II and III from *Pantoea dispersa* that was 92.19 and 92.11 kJ mol^{-1}, respectively [45] and was lower than chitinase from *Paenibacillus sp.* D1, which was 96.36 kJ mol^{-1} [33]. This value increased with elevation of temperature that was the expected value for most protein denaturation. Positive $\Delta G^{\#}$ values of chitinase indicated that the process of thermo-inactivation was not thermodynamically spontaneous [46]. The high $\Delta G^{\#}$ was related to the low $\Delta S^{\#}$ for heat-stable enzyme and to resist against thermal denaturation observed in the present study and was similar to previous reports [33]. The $\Delta G^{\#}$ is a critical element for determining the $\Delta H^{\#}$ and the $\Delta S^{\#}$. High $\Delta G^{\#}$ relates to the low $\Delta H^{\#}$ and $\Delta S^{\#}$ for the enzyme's thermal inactivation [34].

The $\Delta H^{\#}$ value is a characteristic of protein denaturation, and this value in thermal unfolding showed the number of broken non-covalent bonds. In the current study, $\Delta H^{\#}$ value at 60 °C was 20.58 kJ mol^{-1} lower than chitinase from *Paenibacillus sp.* D1 and *Trichoderma harzianum* were 52.36 and 70.01 kJ mol^{-1}, respectively [33,42]. The low $\Delta H^{\#}$ value means the enzyme was heat stable.

Besides, the $\Delta S^{\#}$ value of thermal unfolding represented the amount of structure disorder at the chitinase in the transition state during the thermal inactivation [47,48]. Therefore, an enzyme's unfolding is a rate-limiting step for irreversible thermal inactivation [33]. In this study, $\Delta S^{\#}$ value of chitinase, similar to chitinase from *Paenibacillus sp.* D1, *Trichoderma harzianum* and other reports, was negative [33,34,42]. The negative value of $\Delta S^{\#}$ indicates which unfolded enzymatic molecules were aggregated and be in the order form. The comparison of irreversible thermo-inactivation thermodynamic parameters of the *Serratia marcescens* B4A chitinase with other presented in Table 3.

These properties were unique characteristics of biological processes that reflect conformational stability and resistance to denaturation [41,49]. The present study is the first report on the thermodynamic and kinetic characterization of *Serratia marcescens* B4A chitinase.

CONCLUSIONS

Purified chitinase from Iranian-native bacteria, *Serratia marcescens B4A*, could hydrolyze chitin to chitooligosaccharides. The enzyme's characterization showed high activity levels and stability in a relatively wide range of temperature and pH of the bacterial chitinase. Also, this enzyme had an excellent specific activity and a high affinity to a substrate. Besides, the high value of $\Delta G^{\#}$ with the low value of $\Delta H^{\#}$ and the negative value of $\Delta S^{\#}$ showed high thermostability and resistance to thermal denaturation of chitinase. These characteristics presented that this enzyme has a high tolerance to thermal denaturation and could be an applicable candidate for biodegradation of chitin and various industrial, pharmacological, antifungal, and clinical applications.

Abbreviations

DNS: 3,5-dinitrosalicylic acid; BSA: Bovine serum albumin; SDS PAGE: Sodium Dodecyl Sulfate.

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Availability of Data and Materials

All data generated or analyzed during this study are included in this published article or available from the corresponding author on reasonable request.

Authors' Contributions

ZE and DGh collected, analysis and interpretation of the

experimental materials and wrote the manuscript. SA designed, analysis and interpretation of the manuscript for submission. MK collected experimental materials. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable

Consent for Publication

Not applicable

Competing Interests

The authors declare that they have no competing interests.

REFERENCES

[1] D. Elieh-Ali-Komi, M.R. Hamblin, Int. J. Adv. Res. 4 (2016) 411.
[2] N.P. Price, T.A. Naumann, Anal. Biochem. 411 (2011) 94.
[3] S. Babashpour, S. Aminzadeh, N. Farrokhi, A. Karkhane, K. Haghbeen, Biochem. Genet. 50 (2012) 722.
[4] A. Oyeleye, Y.M. Normi, Biosci. Rep. 38 (2018).
[5] S. Lordan, R.P. Ross, C. Stanton, Mar Drugs. 9 (2011) 1056.
[6] S. Mine, T. Ikegami, K. Kawasaki, T. Nakamura, K. Uegaki, Protein Expr. Purif. 84 (2012) 265.
[7] N. Annamalai, M.V. Rajeswari, S. Vijayalakshmi, T. Balasubramanian, Ann. Microbiol. 61 (2011) 801.
[8] T.I. Kim, D.H. Lim, K.S. Baek, S.S Jang, B.Y. Park, V. Mayakrishnan, Int. J. Biol. Macromol. 112 (2018) 1115.
[9] X.W. Wang, C.L. Cai, J.M. Xu, H. Jin, Z.Y. Xu, Tumor Biol. 36 (2015) 901.
[10] A. Hector, S.H. Chotirmall, G.M. Lavelle, B. Mirković, D. Horan, L. Eichler, M. Mezger, A. Singh, A. Ralhan, S. Berenbrinker, J. Allergy Clin. Immunol. 138 (2016) 1183.
[11] M.A. Elmonem, P.V. Lambertus, E.N. Levtchenko, Enzyme Res. 2016.
[12] M. Møllgaard, M. Degn, F. Sellebjerg, J. Frederiksen, S. Modvig, Eur. J. Neurol. 23 (2016) 898.
[13] A. Makhdoumi, Z. Dehghani-Joybari, M. Mashreghi, K. Jamialahmadi, A. Asoodeh, Int. J. Environ. Sci. Technol. 12 (2015) 3895.
[14] F. Jabeen, A. Hussain, M. Manzoor, T. Younis, A. Rasul, J.I. Qazi, Egypt. J. Biol. Pest. Cont. 28 (2018) 86.
[15] M. Zarei, S. Aminzadeh, H Zolgharnein, A. Safahieh, M. Daliri, K.A. Noghabi, A. Ghoroghi, A. Motallebi, Braz. J. Microbiol. 42 (2011) 1017.
[16] R. Jahangiri, K. Jamialahmadi, J. Behravan, M.F. Najafi, J. Mater. Environ. Sci. 10 (2019) 590.
[17] M. Yahiaoui, H. Laribi-Habchi, K. Bouacem, K.L Asmani, S. Mechri, M. Harir, H. Bendif, E.l. Aïssani, R. Fertas, B. Jaouadi, Carbohydr. Res. 483 (2019) 107747.
[18] S.M. Korany, A.N. Mansour, H.H. El-Hendawy, A.N.A. Kobisi, H.H. Aly, Egypt. J. Biol. Pest. Cont. 29 (2019) 16.
[19] S. Shaikh, S. Wani, R. Sayyed, R. Thakur, A. Gulati, Nopr Niscair Res. in. 2018.
[20] A. Bjørk, B. Dalhus, D. Mantzilas, V.G. Eijsink, R. Sirevåg, J. Mol. Biol. 334 (203) 811.
[21] J. Frankowski, M. Lorito, F. Scala, R. Schmid, G. Berg, H. Bahl, Arch. Microbiol. 176 (2001) 421.
[22] G. Singh, A. Bhalla, G.S. Hoondal, IUP J Life Sci. 4 (2010).
[23] S. Babashpour, S. Aminzadeh, N. Farrokhi, A. Karkhane, K. Haghbeen, Biochem. Genet. 50 (2012) 722.
[24] A. Sanromán, M.A. Murado, J.M. Lema, Appl. Biochem. Biotech. 59 (1996) 329.
[25] Y. Takiguchi, Physical Properties of Chitinous Materials. Adv. Chitin. Sci. 111 (1991) 1.
[26] M. Zarei, S. Aminzadeh, H. Zolgharnein, A. Safahieh, A. Ghoroghi, A. Motallebi, M Daliri, A. Sahebghadam Lotfi, Iran. J. Biotechnol. 8 (2010) 252.
[27] G.L. Miller, Anal. Chem. 31 (1959) 426.
[28] M. Bradford, Anal. Biochem. 7 (1976) 248.
[29] U.K. Laemmli, Nature 227 (1970) 680.
[30] H. Lineweaver, D. Burk, J. Am. Chem. Soc. 56

(1934) 658.
[31] M. Dixon, E. Webb, Enzymes, 3rd ed Academic Press Inc, New York. 3 (1979) 7.
[32] H. Eyring, A.E. Stearn, Chem. Rev. 24 (1934) 253.
[33] A.K. Singh, H.S. Chhatpar, Appl. Biochem. Biotechnol. 164 (2011) 77.
[34] S.H. Mansour, A.M. Abdel-Fattah, M.A. Esawy, E.F. Ahmed, A.H. Haroun, A.H. Hussein, M.M. Hassanien, H.M. Khater, Immobilization, Egypt. J. Aqua. Biol. Fish. 23 (2019) 527.
[35] J. Li, J. Zheng, Y. Liang, R. Yan, X. Xu, J. Lin, Pro. Exp. Purif. 171 (2020) 105613.
[36] J. Ueda, N. Kurosawa, World J. Microbiol. Biotechnol. 31 (2015) 135.
[37] S. Okay, B. Tefon, M. Özkan, G. Özcengiz, J. Appl. Microbiol. 104 (2008) 161.
[38] N. Nawani, B. Kapadnis, J. Appl. Microbiol. 90 (2001) 803.
[39] S. Kudan, R. Pichyangkura, Appl. Biochem. Biotechnol. 157 (2009) 23.
[40] G.J. Joo, Biotechnol. Lett. 27 (2005) 1483.
[41] V. Maisuria, A. Nerurkar, Biochem. Eng. J. 63 (2012) 22.
[42] A. Kapat, T. Panda, Bioproc. Eng. 16 (1997) 269.
[43] D.T. Tran, J.S. Chang, Bioproc. Biosystems. Eng. 37 (2014) 481.
[44] F.A. Mostafa, W.A.A. Wahab, H.A. Salah, G.A. Nawwar, M.A. Esawy, Int. J. Biol. Macromol. 119 (2018) 232.
[45] V. Gohel, D. Naseby, Biochem. Eng. J. 35 (2007) 150.
[46] A.P. Tavares, C.G. Silva, G. Dražić, A.M. Silva, J.M. Loureiro, J.L. Faria, J. Colloid Interface Sci. 454 (2015) 52.
[47] K.R.M. Ramos, K.N.G. Valdehuesa, G.M. Nisola, W.K. Lee, W.J. Chung, New Biotechnol. 40 (2018) 261.
[48] V. Sant'Anna, F. Cladera-Olivera, A. Brandelli, Food Chem. 130 (2012) 84.
[49] S. Nagar, A. Mittal, D. Kumar, L. Kumar, V.K. Gupta, Process Biochem. 47 (2012) 1402.