Iranian Journal of Fisheries Sciences 18(4) 1046-1059 2019
DOI: 10.22092/ijfs.2019.119292.

Serratia marcescens B4A chitinase thermostability enhancement by S390I QuikChange site directed mutagenesis

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Received: December 2016 Accepted: May 2017

Abstract

Thermostable chitinases are useful for industrial and biotechnological applications. This paper reports the stabilization of chitinase from *Serratia marcescens* B4A through rational mutagenesis. Changing of Ser 390 to Ile in *S. marcescens*. The stabilization was enhanced through entropic stabilization by reduction of the loop length and also by increasing of the beta chain length. With this replacement, polar uncharged residue changed to non-polar one and increased the hydrophobic interactions. Furthermore Isoleucine has branched β-carbon that restricts the backbone conformation more than non-branched residues. Finally all of these factors lead to entropic stabilization and thermal stabilization. The results exhibited that the optimal temperature and pH for enzyme activity of native chitinase were not changed by mutagenesis which showed that mutation didn't affect the original characteristics of the enzyme, the K_m values of native and mutant chitinase were different very little, showing that the affinity of enzyme towards the substrate and also the natural flexibility of chitinase did not change by mutation. Besides the V_{max} value of the mutant chitinase was decreased, while its pH stability was increased briefly, but its thermal stability was increased remarkably. Mutation made chitinase to tolerate high temperatures up to 90°C. In addition its activity was increased at 50°C, 60°C for 120 min and up to 2 hours of incubation period and the mutant chitinase demonstrated a high level of activity at 60°C. These results show that entropic stabilization works well for chitinase and this approach may be generally applicable for stabilization of other proteins.

Keywords: Chitinase, Thermal stability, *Serratia marcescens* B4A, Loop, β chain, unfolding

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[DOR: 20.1001.1.15622916.2019.18.4.21.2]

Introduction

Chitin, a polysaccharide made of β-(1,4) linked N-acetyl D-glucosamine units, is the second most plentiful biopolymer and a source of renewable raw materials (Patil *et al*., 2000; Annamalai *et al*., 2011; Wang *et al*., 2015). Chitin has applications in industrial, biotechnology, agriculture, material and medical, food and nutrition sciences (Xia *et al*., 2011). Chitinases (EC 3.2.1.14) are widespread enzymes in nature with ability to degrade chitin into smaller chito-oligosaccharide segments (Bhattacharya *et al*., 2007; Tsuji *et al*., 2010). These enzymes are efficient biomarkers in cancer disease (Wang *et al*., 2015) and are used for treatment of CF-associated fungal disease (Hector *et al*., 2016). Some chitinases such as chitinase 3-like 2 and chitotriosidase are used in early MS diagnosis (Elmonem *et al*., 2016; Møllgaard *et al*., 2016).

Thermostable chitinases are in demand in various biotechnology sectors (Bjørk *et al*., 2003; Aliabadi *et al*., 2016). The need, similar to the other interesting enzymes, has been addressed through protein engineering. However, the use of a rational design for protein stability improvement seems to be protein specific and any of such attempts would require to be directed by the detailed structural information (Giver *et al*., 1998; Gåseidnes *et al*., 2003; Khurana *et al*., 2011; Matroodi *et al*., 2013) and understanding the correlations between the sequence, function and the structure (Eijsink *et al*., 2004; Khurana *et al*., 2011).

Several reasons have been ascribed to the superior stability of the thermophilic proteins including greater hydrophobicity (Mozhaev and Martinek, 1984; Haney *et al*., 1997; Kumar *et al*., 2000) better packing, shorter loops (Russell *et al*., 1997), smaller and less numerous cavities (Salminen *et al*., 1996; Kumar *et al*., 2000), less uncharged polar residues (Haney *et al*., 1999; Fields, 2001), increased occurrence of proline residues (Bogin *et al*., 1998; Kumar *et al*., 2000), and increased hydrogen bonding (Vogt and Argos, 1997).

Serratia marcescens is one of the pioneer bacterium in chitin degradation (Watanabe *et al*., 1997; Bjørk *et al*., 2003). *S. marcescens* B4A chitinase (Babashpour *et al*., 2012) plays an important role in its plant anti-pathogenic properties. 3D-Structure analysis of this enzyme suggests that substitution of Serine 390 with Isoleucine could improve its thermostability by shortening the loop size and increasing the β-sheet length. To examine the assumption, Quik Change Site Directed Mutagenesis (QCSDM) was employed to modify *S. marcescens* B4A chitinase structure and its thermostability. The outcome of the research has been presented in this paper.

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Materials and methods

Materials

The plasmid pQE60 chitinase (previous work) (Babashpour *et al*., 2012; Zarei *et al*., 2012) was used for amplification of chitinase gene. *Escherichia coli* strain DH5α (Invitrogen. Carlsbad, CA, USA) was used as the host for molecular cloning of recombinant pTZ57R/T (Fermentas. Waltham, Massachusetts, USA). pET26b (Fermentas. Waltham, Massachusetts, USA) plasmids harboring native and mutant chitinase gene, respectively. BL21 (DE3) *Escherichia coli* strain (Invitrogen. Carlsbad, CA, USA) was also used for protein expression. Flake crab shell chitin and 3, 5-dinitrosalicylic acid (DNS) were purchased from Sigma (St. Louis, MO, USA). Colloidal chitin was prepared according to the modified method of Roberts and Selitrennikoff and use of commercial chitin (Takiguchi, 1991).

In silico studies

Homology modeling and 3D-structure analysis

The amino acid sequence of chitinase retrieved from NCBI database was used as a template in BLASTP search against PDB to find appropriate templates for homology modeling. Amino acid sequence of *Serratia marcescens* B4A chitinase (accession number HM473183) was retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov).

BLASTP search (Altschul *et al*., 1997) of a query (chitinase sequence) was performed against Protein Data Bank (PDB). Homology modeling for the native and the mutant chitinase of *Serratia marcescens* B4A was performed by MODELLER v9.10 (http://www.salilab.org/modeller/) (Webb and Sali, 2014) using opened form of chitinase (1edq_A) downloaded from PDB server as the template. The MODELLER generated structure of the mutant chitinase was further analyzed by Ramachandran analysis generated by Procheck (Laskowski *et al*., 1993), Qmean server (Benkert *et al*., 2008), ProSA-web (Wiederstein and Sippl, 2007), and RMSD value calculation (Humphrey *et al*., 1996). Qmean is a parameter (between 0 and 1) for assessment of model credibility and ProSA shows quality score of the predicted model in the context of all known protein structures. Furthermore, the structural similarity between the native and mutant chitinase was checked by the RMSD value calculation using VMD software (Humphrey *et al*., 1996).

Experimental studies

Mutagenesis

The Quik Change Site Directed Mutagenesis strategy was used for mutagenesis. The plasmid pQE60 harboring the chitinase gene (previous work) (Babashpour *et al*., 2012; Zarei *et al*., 2012) was used as the template for producing the mutant chitinase. The forward (5'-CACATCTTCCTGATGATCTACGACTTCTACG-3') and reverse (5'

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CGTAGAAGTCGTAGATCATCAGG AAGATGTG-3') mutant primers were used in polymerase chain reaction. PCR product was digested with 1-2 unit of *Dpn*I (Jena. science. Germany) at 37°C for 30 min. The correctness of mutation in the chitinase gene was verified by sequencing of the chitinase gene.

Cloning and expression

For reinforcement of the mutant chitinase gene and insertion of restriction sites in two sides of gene were designed two oligonucleotide primers, a forward primer (Emr-F-chit: 5'-ATAATCATATGGGGCGCAAATTT AATAAACC-3', *Nde*I site with ATG as the start codon) and reverse primer (Emr-R-chit: 5'-ATATACTCGAGATCTTTGAACGC CGGCGC-3', *Xho*I site). Following, the PCR product (1,701 bp) was cloned into pTZ57R/T then recombinant plasmid was digested with *Nde*I and *Xho*I and the restricted segments were isolated and ligated into the pET26b expression vector which has been digested with the same enzymes. A single colony of recombinant plasmid carrying the mutant chitinase which had been transformed into BL21 (DE3) *Escherichia coli* strain was cultured in LB medium. Therefore protein expression was induced by addition of 1 mM isopropyl-cD-thiogalactoside (IPTG) and was verified by SDS-PAGE analysis. The protein content of the extracts was determined by the Bradford method (Bradford, 1976).

Enzyme activity

Chitinase activity was assayed by the modified method of Roberts and Selitrennikoff (Takiguchi, 1991) using colloidal chitin (1% w/v chitin in 20 mM phosphate buffer, pH 7.5) as the substrate. The enzymatic reaction was run for 60 min at 50°C. To stop the reaction, the mixture was boiled. Then, DNS (1% w/v) was added and the resulting mixture was maintained at 100°C for 10 min. The extent of the enzymatic hydrolysis was evaluated spectrophotometrically at 540 nm (Miller, 1959).

Stability and kinetics of the native and mutant recombinant chitinases

To determine the optimum pH, the chitinases activity was measured at various pH (s) ranging from 3–12 using 50 mM glycine–acetate–phosphate buffer. The enzyme stability at various pH(s), was determined by preincubation of the enzyme at the desired pH in the absence of the substrate for 90 min, followed by the enzyme assay at optimum pH.

To determine the optimum temperature, the enzymatic reaction was run in the presence of the substrate at different temperatures between 10–90°C in 20 mM phosphate buffer (pH 6) for 45 min, followed by the enzyme assay at pH and °C.

Thermal stability was measured by two methods: (1) measurement of the thermal stability at different temperatures. To do so, the enzyme was incubated at different temperatures

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ranging from 50 to 90°C for 90 min, then colloidal chitin was added, and the enzyme assay was done at 50°C (optimum temperature). (2) Measurement of the thermal stability at different times at two selected temperatures of 50 and 60°C. Every 20 min (up to 120 min), the reaction mixture was sampled and the samples were placed on ice for 30 min, then colloidal chitin was added to assay the enzyme at 50°C (optimum temperature).

Kinetics parameters of the chtinases were calculated by double-reciprocal analysis of the corresponding kinetics curves obtained from the corresponding enzymatic reactions in the presence of various concentrations of the substrate ranging from 0 to 50 mg mL^{-1} at pH and °C (Lineweaver and Burk, 1934).

Results

Homology modeling and structure validation

Using *Serratia marcescens* B4A chitinases (PDB: HM473183) as the reference structure in the computational studies, it was found that Ser390 could be more important to the structural stability of HM473183 as compared with the other suggestions. Therefore, substitution of Ser390 with Ile was further studied. This substitution not only decreases the number of amino acids with polar residues, but it also increases the chance of the beta sheet extension in the protein skeleton since Ile, as a branched amino acid, has conformational preference for the beta sheet structure. As a result, the suggested substitution was anticipated to shorten the loop section (Fig. XX), where ser390 was placed, in order to enhance the structural stability through the reduction of the entropy of the whole molecule. To appraise the change in protein stability after having the substitution, the thermodynamic stability (ΔΔG) using flexible and fixed backbone method was measured by Eris server (Yin *et al*., 2007). This resulted in $\Delta\Delta\mathbf{G}$ of 2.18 kcal mol^{-1} for the mutant chitinase. Moreover, the appropriateness of the mutation site was verified by an automated web site: http://bioinformatics.org/primerx (Zheng *et al*., 2004).

Homology modeling was performed to predict the 3D structure (opened form) of the mutant. Based on the sequence identity between the query and the template protein sequence, the PDB number of 1edq_A with 99% identity was recognized for prognostication of the opened form of both chitinases. Then, the 3D structures of native and mutant chitinase were optimized using MODELLER v9.10 (Fig. 1). ProSA and QMEAN scores were found to be −9.75 and 0.837 (Z-score=0.88) for the mutant chitinase and -9.56 and 0.84 (Z-score=0.91) for native chitinase, respectively.

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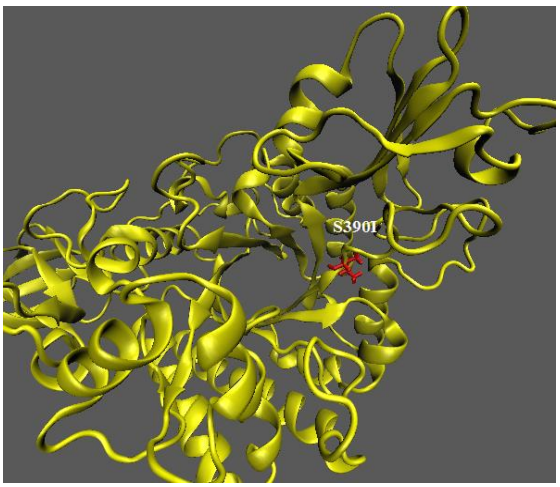


Figure 1: Optimized 3D structure of the opened form of mutant chitinase generated by MODELLER v9.10 using 1edq_A as the template. The protein structures are shown as new cartoon representation. Visualization was performed using VMD software.

Ramachandran plot demonstrated that 93.7 % of the amino acid residues of the mutant chitinase were in the most favored region and 6.3 % in the additional allowed region. Superimposition of native and the mutant resulted in RMSD value of 2.9 Å for the Cα atoms. These results indicated that the structures of the mutant and template are similar and the homology model is reliable.

Mutagenesis and expression

After mutagenesis with the QCSDM strategy, the accuracy of mutation was verified by DNA sequencing. Then, the mutant chitinase gene was sub cloned in pET26b expression vector which resulted in successful expression of the enzyme in BL21 (DE3) *Escherichia coli* strain. The protein produced a 62 kD band on SDS-PAGE (Fig. 2).

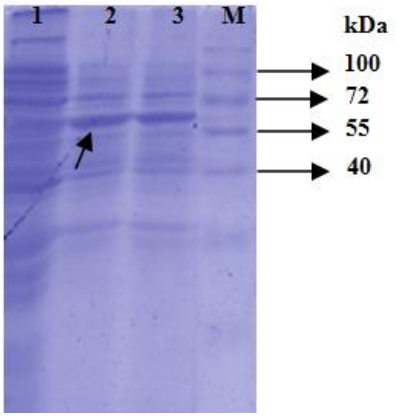


Figure 2: SDS–PAGE analysis of native and mutant chitinase. Lanes 1 total recombinant before induction, 2-3 native and mutant chitinase after induction (filled arrow is 62 kDa band refer to chitinase), M protein size marker.

Kinetics parameters

Measurement of the Michaelis constant was showed that the K_m values of native and mutant chitinases were 4/5 mg mL (Fig. 3a) and 4/7 mg mL^{-1} (Fig. 3b), respectively. Very little different of the K_m values of native and mutant

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chitinase showing that the affinity of enzyme towards the substrate has not been changed. The V_{max} value of native and mutant chitinase were observed to be 588/23 Unit (μM min^{-1}) of protein (Fig. 3a) and 153/84 Unit (μM/min) of protein (Fig. 3b), respectively.

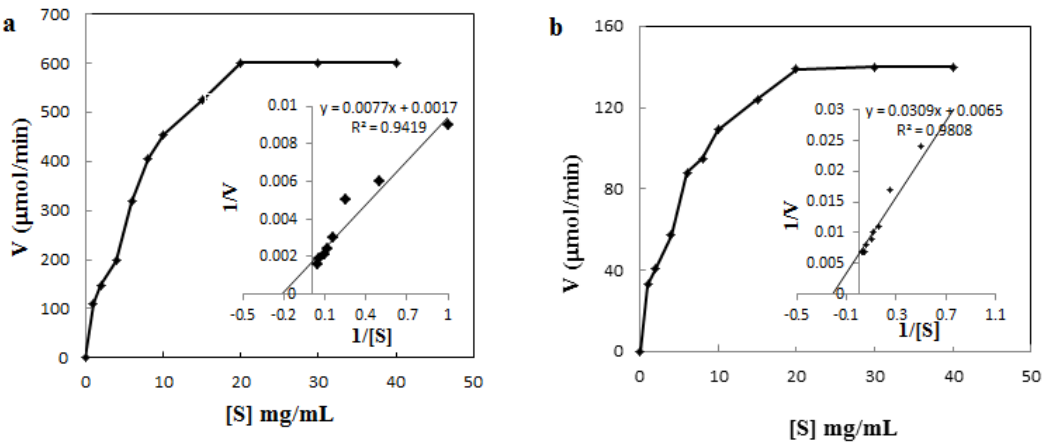


Figure 3: Michaelis–Menten curve of native and mutant chitinase activity. *Inset* Lineweaver–Burk plot. a) The Km values and the Vmax value of native chitinase were found to be 4/5 mg mL^{-1} and 588/23 Unit (μmol min^{-1}), respectively. b) The Km values and the Vmax value of mutant chitinase were found to be 4/7 mg/ml and 153/84 Unit (μmol min^{-1}) of protein, respectively.

Effect of pH and Temperature

The optimal pH(s) for native and the mutant chitinases were found to be 6 and 5, (Fig. 4a), respectively. However, the enzymes were stable between pH range of 6-10 for native and 5-10 for the mutant chitinase (Fig. 4b). The optimal temperatures of 50°C and 45°C were obtained for native and the mutant, (Fig. 4c), respectively.

Thermal stability

Thermal stability of chitinases was measured at different temperatures. Although the activities of both chitinases were reduced with the rise of temperature, native chitinase activity was reduced faster and reached zero at 70°C, while the mutant chitinase could maintain about 20 percent of its activity (Fig. 5a). Measurement of temperature stability during the time showed that the native enzyme activity at 50 and 60°C was decreased, but the activity of mutant chitinase increased over the time (Fig. 5b).

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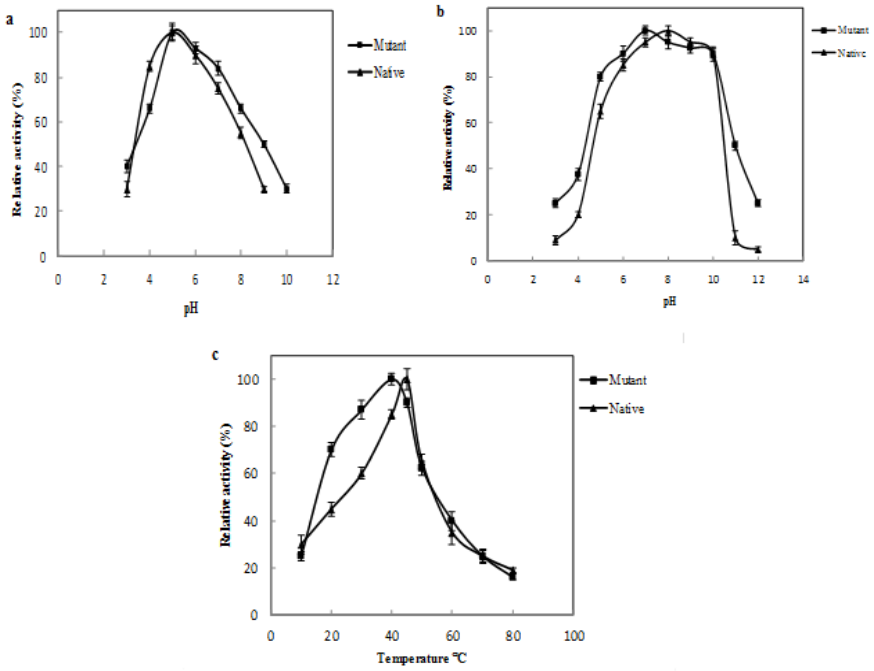


Figure 4: Effects of pH and temperature on the activity and stability of the native and mutant chitinase. a) Effect of pH on enzymes activity, enzymes assays performed over a range of pH values (3–10) using colloidal chitin as substrate under standard assay conditions. The optimal pH for the chitinase activity of native and mutant chitinases was found to be 6 and 5 respectively. b) Effect of pH on enzyme stability, enzymes preincubated at various levels of pH (3–12) without the substrate for 90 min. Following adjustment of the enzymes to pH 6, colloidal chitin was added and then enzymes assays performed under standard assay conditions. The enzymes were stable between pH range of 6-10 for native and 5-10 for mutant chitinase. c) Effect of temperature on enzymes activity, enzymes assays performed in the range of 10–90°C in pH 6 using colloidal chitin as substrate under standard assay conditions. The optimal temperature for enzyme activity of native and mutant was found to be 50°C and 45°C, respectively.

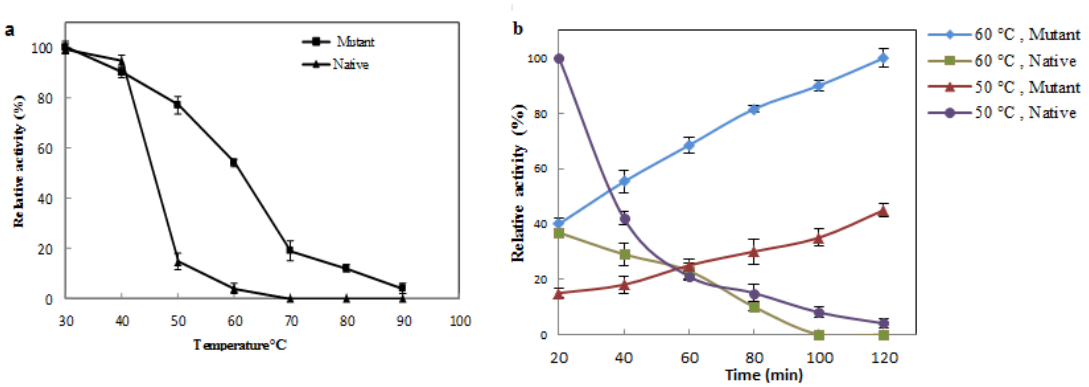


Figure 5: Thermal stability of chitinase. a) Effect of different temperatures on the activity and stability of native and mutant chitinase after incubation for 90 min. b) Effect of temperature on the stability of native and mutant chitinase after incubation at 50, 60°C in various time intervals.

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Discussion

Research on the thermostable chitinases is attractive for finding efficient enzymes with more usage in industrial process (Roodi *et al*., 2017). Recent studies clearly exhibit that the kinetic stability of proteins might be better by rational design, but that it may be difficult to exactly determine which region and amino acid of the protein should be considered. There are a commonplace strategy for increasing protein thermal stability through shortening of exposed loop regions which leads to decreasing the entropy and elevating the free energy of protein unfolding (Thompson and Eisenberg, 1999). Furthermore other researchers showed that the proportion of beta sheet amino acids is meaningfully high in thermophiles. This result might be obtained through enhancing hydrogen bonding which leads to increased thermal stability (Chakravarty and Varadarajan, 2000). In this study we wanted to stabilize the chitinase by reduction of the loop length and increasing of beta sheet length altogether. For this purpose we determined amino acids that locate in the border of the loop and beta sheet, and considered that candidate sites did not have unfavorable steric interactions.

Also researchers showed that for increasing thermal stability, declining uncharged polar amino acids is very significant. The elimination of these residues may also reduce deamination of asparagine and glutamine by serine and threonine at high temperatures (Vogt and Argos, 1997; Haney *et al*., 1999). Serine due to its short side chain mostly involve in local interactions, but large side chains amino acids generally contribute in short and long range interactions (Hornby, 1993; Kumar *et al*., 2000). Thus we selected Serine 390 resides in the end of the loop and replaced it with Isoleucine residue because of several reasons: Isoleucine residue tends to place in beta sheet and this replacement causes the reduction of the loop length and enhancement of the beta sheet length. Furthermore, increase of protein hydrophobicity stabilizez thermophile proteins (Mozhaev and Martinek, 1984; Haney *et al*., 1999). Also entropy of unfolding dependent on the kind of the side chain, for example in this case flexibility of the selected loop is decreased by the presence of long or bulky side chains (Némethy *et al*., 1966). Also studies showed that polar uncharged residue in comparison of one non-polar is less favorable in thermostable protein (Mozhaev and Martinek, 1984; Chakravarty and Varadarajan, 2000). Our studies demonstrated that mutant chitinase has been compacted and required time and temperature for its activity. Native and mutant chitinase kinetic studies showed that mutant chitinase had more activity than the native type because the native chitinase could not tolerate the assay condition, but this condition is favorable for the mutant chitinase to increase its flexibility and exposed catalytic site for substrate. Because the enzyme was partially purified, in

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chitinase activity assay two negative controls were used. The first negative control was cocktail without chitinase; the second negative control was cocktail with BL21 containing pET26b bacterial cell extract. Any enzymatic activity was observed in two negative controls. Alteration in enzyme binding site may lead to increase in K_m (Fields and Somero, 1998; Fields, 2001). In our study the brief change of mutant chitinase K_m showed that the enzyme substrate affinity was similar to the native enzyme. The V_{max} of mutant chitinase is smaller than native chitinase because the calculation of the V_{max} value was performed in routine condition while mutant chitinase was rigided and at this condition showed low activity. Reduction of mutant chitinase V_{max} without change of K_m was similar to the noncompetitive inhibition. Studies have shown that there is correlation between thermal stability and the optimum temperature (Danson *et al*., 1996; Daniel *et al*., 2001). Optimum temperature is increased in several engineered thermostabilized enzymes and doesn't change in the others (Thomas and Scopes, 1998; Bjørk *et al*., 2003). In our research the optimum temperature and pH didn't change remarkably for both the mutant and native chitinases, indicating that mutation did not affect the main characteristic of the enzyme. pH stability of the mutant and native chitinases were the same, but thermal stability was increased remarkably. Mutation caused the chitinase to tolerate high temperatures up to 90°C and it remained stable at 50°C, 60°C for up to 120 minutes. This characteristic is very important for industrial and biotechnological applications.

Acknowledgments

This work was financially supported by the Iran National Science Foundation (grant number 89002350) and the National Institute of Genetic Engineering and Biotechnology (grant number: 453). The authors also wish to thank Denazist Company of Iran-Mashhad.

References

Aliabadi, N., Aminzadeh, S., Karkhane, A.A. and Haghbeen, K., 2016. Thermostable chitinase from Cohnella sp. A01: isolation and product optimization. *Brazilian Journal of Microbiology*, 47(**4**), 931-940.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25(**17**), 3389-3402.

Annamalai, N., Rajeswari, M.V., Vijayalakshmi, S. and Balasubramanian, T., 2011. Purification and characterization of chitinase from *Alcaligenes faecalis* AU02 by utilizing marine wastes and its antioxidant activity. *Annals of Microbiology*, 61(**4**), 801-807.

[Downloaded from jifro.ir on 2026-11-12]

[DOR: 20.1001.1.15622916.2019.18.4.21.2]

Babashpour, S., Aminzadeh, S., Farrokhi, N., Karkhane, A. and Haghbeen, K., 2012. Characterization of a chitinase (Chit62) from *Serratia marcescens* B4A and its efficacy as a bioshield against plant fungal pathogens. *Biochemical Genetics*, 50(**9-10**), 722-735.

Benkert, P., Tosatto, S.C. and Schomburg, D., 2008. QMEAN: A comprehensive scoring function for model quality assessment. *Proteins: Structure, Function, and Bioinformatics*, 71(**1**), 261-277.

Bhattacharya, D., Nagpure, A. and Gupta, R.K., 2007. Bacterial chitinases: properties and potential. *Critical Reviews in Biotechnology*, 27(**1**), 21-28.

Bjørk, A., Dalhus, B., Mantzilas, D., Eijsink, V.G. and Sirevåg, R., 2003. Stabilization of a tetrameric malate dehydrogenase by introduction of a disulfide bridge at the dimer–dimer interface. *Journal of Molecular Biology*, 334(**4**), 811-821.

Bogin, O., Peretz, M., Hacham, Y., Burstein, Y., Korkhin, Y. and Frolow, F., 1998. Enhanced thermal stability of *Clostridium beijerinckii* alcohol dehydrogenase after strategic substitution of amino acid residues with prolines from the homologous thermophilic *Thermoanaerobacter brockii* alcohol dehydrogenase. *Protein Science*, 7(**5**), 1156-1163.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(**1-2**), 248-254.

Chakravarty, S. and Varadarajan, R., 2000. Elucidation of determinants of protein stability through genome sequence analysis. *Febs Letters*, 470(**1**), 65-69.

Daniel, R.M., Danson, M. J. and Eisenthal, R., 2001. The temperature optima of enzymes: a new perspective on an old phenomenon. *Trends in Biochemical Sciences*, 26(**4**), 223-225.

Danson, M.J., Hough, D.W., Russell, R.J., Taylor, G.L. and Pearl, L., 1996. Enzyme thermostability and thermoactivity. *Protein Engineering*, 9(**8**), 629-630.

Eijsink, V.G., Bjørk, A., Gåseidnes, S., Sirevåg, R., Synstad, B., van den Burg, B. and Vriend, G., 2004. Rational engineering of enzyme stability. *Journal of Biotechnology*, 113(**1**), 105-120.

Elmonem, M.A., van den Heuvel, L.P. and Levtchenko, E.N., 2016. Immunomodulatory effects of chitotriosidase enzyme. *Enzyme Research*, 2016, 19..

Fields, P.A. and Somero, G.N., 1998. Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A4 orthologs of Antarctic notothenioid fishes. *Proceedings of the National Academy of Sciences*, 95(**19**), 11476-11481.

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[DOR: 20.1001.1.15622916.2019.18.4.21.2]

Fields, P.A., 2001. Review: Protein function at thermal extremes: balancing stability and flexibility. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, 129(**2**), 417-431.

Gåseidnes, S., Synstad, B., Nielsen, J.E. and Eijsink, V.G., 2003. Rational engineering of the stability and the catalytic performance of enzymes. *Journal of Molecular Catalysis B: Enzymatic*, 21(**1**), 3-8.

Giver, L., Gershenson, A., Freskgard, P.O. and Arnold, F.H., 1998. Directed evolution of a thermostable esterase. *Proceedings of the National Academy of Sciences*, 95(**22**), 12809-12813.

Haney, P., Konisky, J., Koretke, K., Luthey-Schulten, Z. and Wolynes, P., 1997. Structural basis for thermostability and identification of potential active site residues for adenylate kinases from the archaeal genus Methanococcus. *Proteins Structure Function and Genetics*, 28(**1**), 117-130.

Haney, P.J., Badger, J.H., Buldak, G.L., Reich, C.I., Woese, C.R. and Olsen, G.J., 1999. Thermal adaptation analyzed by comparison of protein sequences from mesophilic and extremely thermophilic Methanococcus species. *Proceedings of the National Academy of Sciences*, 96(**7**), 3578-3583.

Hector, A., Chotirmall, S.H., Lavelle, G.M., Mirković, B., Horan, D., Eichler, L., Mezger, M., Singh, A., Ralhan, A. and Berenbrinker, S., 2016. Chitinase activation in patients with fungus-associated cystic fibrosis lung disease. *Journal of Allergy and Clinical Immunology*. 138(**4**), 1183–1189**.**

Hornby, D., 1993. Hydrogen bonding in biological structures. Wiley Online Library. 623-624.

Humphrey, W., Dalke, A. and Schulten, K., 1996. VMD: visual molecular dynamics. *Journal of Molecular Graphics*, 14(**1**), 33-38.

Khurana, J., Singh, R. and Kaur, J., 2011. Engineering of Bacillus lipase by directed evolution for enhanced thermal stability: effect of isoleucine to threonine mutation at protein surface. *Molecular Biology Reports*, 38(**5**), 2919-2926.

Kumar, S., Tsai, C.J. and Nussinov, R., 2000. Factors enhancing protein thermostability. *Protein Engineering*, 13(**3**), 179-191.

Laskowski, R.A., MacArthur, M.W., Moss, D.S. and Thornton, J.M., 1993. PROCHECK: a program to check the stereochemical quality of protein structures. *Journal of Applied Crystallography*, 26(**2**), 283-291.

Lineweaver, H. and Burk, D., 1934. The determination of enzyme dissociation constants. *Journal of the American Chemical Society*, 56(**3**), 658-666.

Matroodi, S., Zamani, M., Haghbeen, K., Motallebi, M. and Aminzadeh, S., 2013. Physicochemical study of a

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[DOR: 20.1001.1.15622916.2019.18.4.21.2]

novel chimeric chitinase with enhanced binding ability. *Acta Biochim Biophys Sin (Shanghai),* 45(**845-856**). 1-12.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry,* 31(**3**), 426-428.

Møllgaard, M., Degn, M., Sellebjerg, F., Frederiksen, J. and Modvig, S., 2016. Cerebrospinal fluid chitinase-3-like 2 and chitotriosidase are potential prognostic biomarkers in early multiple sclerosis. *European Journal of Neurology,* 23(**5**), 898-905.

Mozhaev, V. and Martinek, K., 1984. Structure-stability relationships in proteins: new approaches to stabilizing enzymes. *Enzyme and Microbial Technology,* 6(**2**), 50-59.

Némethy, G., Leach, S. and Scheraga, H.A., 1966. The influence of amino acid side chains on the free energy of helix-coil transitions1. *The Journal of Physical Chemistry,* 70(**4**), 998-1004.

Patil, R.S., Ghormade, V. and Deshpande, M.V., 2000. Chitinolytic enzymes: an exploration. *Enzyme and Microbial Technology,* 26(**7**), 473-483.

Roodi, F.Z., Aminzadeh, S., Farrokhi, N., Karkhane, A.A. and Haghbeen, K., 2017. *Cohnella* amylopullulanases: Biochemical characterization of two recombinant thermophilic enzymes. *PLoS ONE* 12(**4**), e0175013.

Russell, R.J., Ferguson, J.M., Hough, D.W., Danson, M.J. and Taylor, G.L., 1997. The crystal structure of citrate synthase from the hyperthermophilic archaeon Pyrococcus furiosus at 1.9 Å resolution. *Biochemistry,* 36(**33**), 9983-9994.

Salminen, T., Teplyakov, A., Kankare, J., Cooperman, B.S., Lahti, R. and Goldman, A., 1996. An unusual route to thermostability disclosed by the comparison of Thermus thermophilus and *Escherichia coli* inorganic pyrophosphatases. *Protein Science,* 5(**6**), 1014-1025.

Takiguchi, Y., 1991. Physical properties of chitinous materials. *Advances in Chitin Science,* 3(**1-7**) 30-80.

Thomas, M.T. and Scopes, K.R., 1998. The effects of temperature on the kinetics and stability of mesophilic and thermophilic 3-phosphoglycerate kinases. *Biochemical Journal,* 330(**3**), 1087-1095.

Thompson, M.J. and Eisenberg, D., 1999. Transproteomic evidence of a loop-deletion mechanism for enhancing protein thermostability. *Journal of Molecular Biology,* 290(**2**), 595-604.

Tsuji, H., Nishimura, S., Inui, T., Kado, Y., Ishikawa, K., Nakamura, T. and Uegaki, K., 2010. Kinetic and crystallographic analyses of the catalytic domain of chitinase from *Pyrococcus furiosus*–

[Downloaded from jifro.ir on 2026-11-12]

[DOR: 20.1001.1.15622916.2019.18.4.21.2]

the role of conserved residues in the active site. *FEBS Journal*, 277(**12**), 2683-2695.

Vogt, G. and Argos, P., 1997. Protein thermal stability: hydrogen bonds or internal packing? *Folding and Design*, 2, 40-46).

Wang, X.W., Cai, C.L., Xu, J.M., Jin, H. and Xu, Z.Y., 2015. Increased expression of chitinase 3-like 1 is a prognosis marker for non-small cell lung cancer correlated with tumor angiogenesis. *Tumor Biology*, 36(**2**), 901-907.

Watanabe, T., Kimura, K., Sumiya, T., Nikaidou, N., Suzuki, K., Suzuki, M., Taiyoji, M., Ferrer, S. and Regue, M., 1997. Genetic analysis of the chitinase system of *Serratia marcescens* 2170. *Journal of Bacteriology*, 179(**22**), 7111-7117.

Webb, B. and Sali, A., 2014. Comparative protein structure modeling using Modeller. *Current protocols in bioinformatics*, 5.6. 1-5.6. 32.

Wiederstein, M. and Sippl, M.J., 2007. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Research*, 35(**suppl 2**), 407-410.

Xia, J.L., Xiong, J., Zhang, R.Y., Liu, K.K., Huang, B. and Nie, Z.Y., 2011. Production of chitinase and its optimization from a novel Isolate *Serratia marcescens* XJ-01. *Indian Journal of Microbiology*, 51(**3**), 301-306.

Yin, S., Ding, F. and Dokholyan, N.V., 2007. Eris: an automated estimator of protein stability. *Nature Mmethods*, 4(**6**), 466-467.

Zarei, M., Aminzadeh, S., Ghoroghi, A., Motalebi, A., Alikhajeh, J. and Daliri, M., 2012. Chitinase isolated from water and soil bacteria in shrimp farming ponds. *Iranian Journal of Fisheries Sciences*, 11(**4**), 911-925.

Zheng, L., Baumann, U. and Reymond, J.L., 2004. An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Research*, 32(**14**), e115-e115.

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