Characteristics and mechanism of a novel chitinase mutant from *Trichoderma harzianum* with enhanced activity

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Abstract

Background: Chitinase from *Trichoderma harzianum* could inhibit various chitin-containing pathogens. In this study, a mutant of Chit42 (Chit42m) with high enzyme activity was established by error-prone PCR method. **Results:** The Chit42m with three amino acids substitutions (D100G/I166V/A382P) was obtained from 1230 colonies. The activity of the Chit42m was enhanced by 1.26 times and affinity increased by 3.2 folds. The Chit42m showed the optimum temperature was 50 °C and pH 7.5. **Conclusion:** Structural model and molecular docking analysis suggested that A382P may affect the catalytic activity of enzymes by affecting the conformation of key residues D169 and E171 related to catalysis, while I166V probably affects the affinity of chitin and enzymes by influencing the conformation of important substrate binding residues R52 and Y293. D100G had little effect on the changes of enzyme activity. This would put insight into the study of the site-specific mutations and provide promising gene material for the directed evolution chitinase.

Background

T. harzianum (saprophytic fungus) is an effective biological control agent (BCA) against a wide range of soil-borne plant diseases including chickpeas and cucurbits wilt disease induced by *Fusarium oxysporum* [1], of rice blight, potato black scurf, tomato grey mould, of cucumber belly rot induced by *Rhizoctonia solani*, tomato damping-off caused by *Pythium spp* etc [2, 3]. The biocontrol mechanism of *T. harzianum* includes antibiosis, competition, inducing systemic resistance mycoparasitism [4]. It controls the severity of various diseases, meanwhile enhances plant growth [5, 6]. Promoting mechanisms, by which *T. harzianum* influences plant development include production of growth hormones, improving plant nutrient utilization rate, microecological regulation alteration in the rhizosphere soil [7, 8], etc. Hence, multiple mechanisms are involved in biocontrol systems, among which mycoparasitism plays an important role in biocontrol [9]. During this process, chitinase produced by *T. harzianum* [2, 10] is responsible for degrading the host cell wall, such as fungal cell wall and insect exoskeleton, which are mainly comprised up by chitin [11, 12].

Chitinase (EC 3.2.1.14) can hydrolyze chitin into chitin oligosaccharides and monosaccharides by breaking the chitosan-1,4 glycoside bonds connected by N-acetyl β -D glucosamine, Glutamate is the key residue in this hydrolytic process [13]. These hydrolytic products can greatly enhance systemic resistance as a synergistic agent of biological pesticides [14, 15]. Furthermore, with the introduction of biological control into agricultural production, chitinase is free from contamination and maintains the circulation of substance and energy since chitin acts as the second most abundant polysaccharide in nature [16]. Therefore, it is necessary to obtain a chitinase-engineered strain with enhanced activity and stability to provide an alternative to traditional biological systems for better biocontrol effects.

To expand the function of chitinase from *T. harzianum*, error-prone PCR and directed evolution are being increasingly employed to create enzymes with superior performance [17]. The molecular weight of chitinase ranges from 19 to 110 kDa, secreted 42-kDa endochitinase (Ech42) is encoded by *ech42* gene

showing the strongest and broadest antifungal activity. Moreover, constructing recombinant hybrid Chit42 by adding the chitin-binding domain (ChBD) can improve antifungal activity towards *Fusarium graminearum* and it also can hydrolyze *Botrytis cinerea* cell wall in vitro [18, 19]. In addition, overexpression of *ech42* has significantly higher ability to control *R. solani*, and it can be strongly induced during fungus-fungus interactions. Disruption of *ech42* in beans leads to the antifungal activity against *B. cinerea* reducing about 33%-40%. Therefore, this gene was selected as a template to construct an error-prone PCR system with the purpose of boosting random mutant library. The aim of this study was to screen one chitinase mutant from *T. harzianum* with enhanced enzyme activity. The characterization of the mutant was determined to provide application guidance in industrialized production. Further mechanism analysis with respect to the substrate affinity and catalytic efficiency was also done by homology model and superimposition. This novel protein was expected to apply a promising resource in enzyme engineering.

Results

Screening of random mutant libraries

In order to obtain chitinase with higher enzyme activity and stability, error-prone PCR was employed to generate random mutations. Approximately, 1230 colonies of random mutagenesis libraries were submitted to initial screening. After the clones were incubated at 30 °C for 96 hours, the clear hydrolysis zones of different sizes formed on SC-U-beta plate was shown in Fig. 1. The standard for initial screening was the diameter ratio of transparent halo and colony. It was observed that a positive clone displayed the largest hydrolytic zone. The sequence variant was identified with four base substitutions (A299G, T300G, A496G and G1144C), compared with that of Chit42wt, resulting in the substitutions of three amino acids (D100G, I166V, A382P).

Comparison of the enzymatic properties of Chit42wt and Chit42m

The enzyme activity and Kinetic properties

The activity of Chit42m was 0.48 U/g, 1.26 folds higher than that of Chit42wt with 0.38 U/g when the incubation time was 48 min, as was shown in Fig. 2. The kinetic analysis results revealed that K_m of Chit42m was 8.47 mg/ml, Chit42wt was 27.4 mg/ml, indicating that affinity of the Chit42m was 3.2 folds higher than that of the Chit42wt.

The physicochemical properties

Temperature and pH were of significance to the activity and stability of chitinase. Fig. 3(a) revealed that there was no significant difference in temperature on chitinase activity. Interestingly, they demonstrated similar activity profiles at temperatures ranging from 40-50 °C and 60-70 °C, with optimal activity at 50 °C. For further characterization, the thermostability profile was also measured (Fig. S1). The activity of Chit42m was always higher than that of the Chit42wt in the initial 50 min, but then decreased sharply, 80

% of the activity remained even after 70 min incubating at 50 °C, The relative activities of the Chit42wt and the Chit42m in the range of pH 3-8.5 were measured, exhibiting maximum activity at pH 7 and 7.5, respectively, as observed in Fig. 3(b). They both showed the uptrend of activities in acid conditions and downtrend in alkaline conditions, indicating that the enzyme was active within the neutral solution. The Chit42m demonstrated higher activity than the Chit42wt when the pH was 3-6.5 and 7-8.5, indicating that the stability of chit42m was enhanced significantly. The optimum pH of the Chit42m was 7.5.

Effect of metal ions on enzyme activity

In this study, Mg^{2+} , K^{+} slightly enhanced the activity of chitinase. Ca^{2+} almost had no effect on the enzyme, Ba^{2+} was the most effective to activate the enzyme, with the Chit42m increased by 76.3 %, higher than that of Chit42wt by 45.7 %.

Prediction of the mechanisms of the enhanced activity and affinity

Chit42wt (EC 3.2.1.14) belongs to the glycoside hydrolase (GH) families 18, which contains a $(\beta/\alpha)_8$ TIM barrel and an extra α/β domain. Homology models and docking results showed that Chit42wt contains a long substrate-binding groove similar as in other family 18 chitinases. Fig. 4 (a) showed the Chit42wt and substrate (chitohexaose) complex structure of molecular docking. Chitohexaose was bound into six sugar-binding subsites in Chit42wt, numbered from -4 (non-reducing end) to +2 (reducing end) with hydrolysis taking place on the glycosidic bond between the -1 and +1 subsites. The catalytic residue Glu171 located in the -1 subsite. Trp131 and Trp378 stacked with the sugar rings in the -1 and +1 subsite, respectively. The mutated residues (D100, I166 and A382) of Chit42 did not directly interact with sugars. Molecular docking studies showed that the binding free energy of Chit42wt and Chit42m were -12.88 and -14.74 kcal/mol, respectively, which suggested that the variant showed higher affinity for the substrate. The superimposition of the structures of Chit42wt and the Chit42m after 1ns MD simulations was shown in Fig. 4 (b). The overall structure of the Chit42m was similar to that of the Chit42wt enzyme. Though residues at position 100, 166 and 382 did not directly contact with the substrate, the mutation was predicted to change the conformations of several residues surrounding the substrate involved in substrates binding and recognition (Fig .5). In the binding site, the side chains of W131, W133, F245, Y293 and W315 in the Chit42m mutant showed a significant shift relative to the Chit42wt—thus affecting the binding of the substrate. In mutant, E171 was more closer to the bound GlcNAC unit at subsites -1 and +1, and two hydrogen bonds were formed between carboxyl oxygen of E171 and sugar units at -1 site and +1 site, respectively. While in Chit42wt, E171 only formed one hydrogen bond with +1 site sugar. The dynamic changes caused by structural changes were consistent with the experimental results.

Discussion

As for the mutation site of chitinase, the substitution N35D of chitinase Pachi improved nematicidal activities by random mutagenesis [20]. In contrast, the three mutation sites produced by *T. harzianum* with higher activity and stability in this work had never been reported before. The number of point

mutations observed was identical with the ideal mutagenesis of 1.5 to 5 base substitutions per 1000 bp [21], which could be expected from the PCR condition. Colloidal chitin with the highest activity to chitinase compared with other substrates was used as the substrate of chitinase, although chitosan had higher solubility [22].

The optimal temperature of Chit42m was 50 °C, which was the same with the chitinase from *Bacillus licheniformis* [23] and chitinase from *Streptomyces spp.* [24]. Moreover, 80 % of the activity remained even after 70 min incubating at 50 °C indicating that the thermostability was much better than the chitinase from *Bacillus licheniformis* with the activity decreasing by 50 % at 50 °C [25]. The optimum pH of Chit42m was 7.5, which was consistent with the chitinase from *Bacillus sp.* [26], similar with the chitinase from *Stenotrophomonas maltophilia* with the optimally active at pH 7 [27].

Metals ions performed different functions on enzyme in catalysis of redox reactions based on if they bind to the cofactors to form stable units [28]. Fig. S2 showed the effect of metal ions on enzyme activity. The chitinase could be stimulated by K^+ , Mn^{2+} , Mg^{2+} and Ca^{2+} [29]. The effect of Ca^{2+} is similar with that it had no influence on the chitinase of *Bacillus spp* [30]. Ba^{2+} also played active function to the chitinase from *Streptomyces roseolus* [6]. Presumably, the enhancement of Chi42wt and Chit42m activity arised from the conformation change of the enzyme and affected the binding of the enzyme and substrate, the mechanism of which would be studied henceforth. Co^{2+} often conducted an inhibitory performance on the enzyme [24].

The mechanism of its activity enhancement was revealed by molecular docking. Comparisons of these three mutation sites and surrounding residues showed in Fig. 6. D100 of Chit42wt sits in loop $\beta 6\alpha 4$ (residues 96-100), far away from binding groove of Chit42wt. D100 could form stable hydrogen bonds with S97, N99, N104 and Y106 in Chit42wt Fig. 6 (a). When Asp was mutated to Gly, it broke the original hydrogen bonds, causing this loop region more instable than Chit42wt, but these residues had no concern with substrates binding, which suggested that D100G was not the cause of the change in enzyme activity. In contrast, Chitinase from *Enterobacteriaceae* could not do the enzyme-substrate model, the analysis of its activity and catalytic property could only depend on its binding domain from its 3D structure as lacking of its crystal structure. [31]. Like other family 18 chitinase, Chit42wt has a sequence motif DXXDXDXE (residues 164-171 of Chit42) at strand $\beta 5$. I166 lies in this motif, and formed hydrophobic interactions with residues L126, I155 and I168 in Chit42wt. In mutant, though Val was also hydrophobic residue, its side-chain forcing side-chains of L126, I155 and I168 deflection Fig. 6 (b). Furthermore, I166V mutation also affected side-chains of D167, D169 and E171 in the conserved motif. E171 probably acted as the proton donor in the reaction and D169 was putative stabilizer of the positively charged oxazolonium ion intermediate [32, 33]. Thus, I166V indirectly affected catalysis of Chit42. Residue A382 in Chit42wt was surrounded by residues R52, I292, Y293, D321, Y322, K323, E379, A380, S381, D383 and K384 etc., of which R52 and Y293 were important for interacting with the substrate Fig. 6 (c). When Ala was mutated to Pro, it was possible to make the region P382 sited more rigid, and affected conformation of R52 and Y293, therefore affected the binding of substrate. These results together provided important insights into structure-function relationship of Chit42m. Amino acid substitution at

position 382 was determined to be critical for enzyme activity, while the residue at positions 199 and 116 had only marginal effect. Taken as a whole, the enhanced enzymatic activity of Chit42m was ascribed to the conformation changes of three key residues, which has reference significance for the further modification.

Conclusion

In summary, in order to obtain high enzymatic activity of chitinase, error-prone PCR was used to create Chit42 random mutations. The results identified that Chit42m mutant with three residues mutated showed high enzyme activities. Structural prediction showed that mutations could affect the conformation of the key residues that Chit42m had higher substrate affinity than the wild type. In conclusion, the Chit42m mutant has high enzymatic activity which may as a novel resource for related research and practical applications.

Methods

Materials

T. harzianum WY-1 with the CGMCC No. of 12165, *Escherichia coli* DH5 α , JM109 were preserved by the Tomato Research Institute of Northeast Agricultural University. *Saccharomyces cerevisiae* strain H158 (his-trp-leu-ura) and plasmid pYES2 (*Saccharomyces cerevisiae*) was constructed in our laboratory. *S. cerevisiae* was cultured in YPDA (2 % tryptone, 1 % yeast extract, 2 % glucose, 2 % agar). The induction expression medium of chitinase was SC-U basic culture medium added with 2 % β -D galactose. TaqDNA polymerase, dNTPs, MgCl₂, PCR Buffer were purchased from Beijing TransGen Biotech (Beijing, China). The vector pMD-18T was purchased from TAKARA (Japan). Gel Extraction Kit, DNA Purification Kit were offered by Shanghai Sangon (China).

Construction and Screening of mutant library

The full-length CDS of Chit42 (accession number; DQ132792.1) was amplified with primer pairs 5'-GCAAG CTTACCTCTACCAACATC ACAAGCAA-3' and 5'-GCGGCCGCCAGCCTAGTTCAGACCATTC' and the first cDNA strand from chitinase of *T. harzianum* as the template, and then was inserted into the binary vector pMD18-T. And the above primer pairs were also used in Error-prone PCR with *Hind* III and *Not* I restriction sites (bold and underlined). Error-prone PCR was performed using the recombinant plasmid pMD18-*Chit42* as a template, respectively. Fifty microlitres reaction mixture composed of 20 pmol template, 30 pmol the primers, 0.35 mM MgCl₂, 0.06 mM dNTP, 0.035 mM MnCl₂, 10 $\times$ Taq PCR buffer and 2.5 U Taq DNA polymerase was subjected to PCR under conditions of one cycle: 94 °C, 5mins; 35 cycles: 94 °C, 1 min, 54 °C, 1 min, 72 °C, 1 min; one cycle: 72 °C, 10 mins. The PCR products were purified, digested with *Hind* III and *Not* I, cloned into pYES2, and transformed into *S. cerevisiae* H158 strain to create the random mutant library. Mutants of the library were cultivated on LB media supplemented with ampicillin (50 μ g/mL) overnight at 37 °C and then transferred into another 96-deep well plate on the

media of SC-U- β containing 2 % β -D-galactose and 1 % (w/v) colloidal chitin. After reaction at 30 °C for 96 h, the mutants with higher activity than the wildtype chitinase Chit42 (Chit42wt) were selected for further studies. The standard for initial screening was the diameter ratio of transparent halo and colony. The selected clones were inoculated and cultivated in a SC-U medium for 20 h at 30 °C. Then, the 2 % β -D-galactose was added with a final concentration to induce the expression of recombinant enzymes. After incubation at 30 °C for 18 h, the cultured samples were removed by centrifugation (8000 r/min at 4 °C for 10 min). The supernatants were inoculated into replica 96-well plates, which contains phosphate buffer solution (0.2 mol/L, pH 6.5) and 1% (w/v) colloidal chitin. The obtained samples were used for further assays.

Determination on the enzyme activities and the Kinetic properties

The 1 mL chitinase and 1 mL colloidal chitin were mixed and reacted 20 min at 37 °C. The 1 mL DNS was added in the reaction system and then boiled at 100 °C for 15 min. The value of the supernatant was measured at OD₅₄₀. The kinetic parameters of Chit42wt and the Chit42m using different concentrations of colloidal chitin ranging from 2 to 10 mg mL⁻¹ were measured using the HPLC [34] under standard assay conditions (pH 7.0 and 37 °C) according to Lineweaver-Burk plot. The K_m and V_{max} values were calculated according to the Michaelis-Menten equation.

Characterization of Chit42wt and the mutant Chit42m

The optimal temperature of Chit42wt and the Chit42m was tested after incubating for 10 min within the 30-80 °C range. The effect of pH on the activities was determined in different buffers at 0.1 mol/L citric acid phosphate buffer and 0.1 mol/L sodium phosphate buffer (pH 3.0-8.0). The thermo-stability of chitinase was determined by four different heat treatment (45 °C, 55 °C, 65 °C or 75 °C) for 70 min under the pH of 6.5, and the residual activity was measured as described above. To determine the pH stability, the remaining activity was measured after incubation in buffers (pH 3.0-8.0) at 50 °C for 30 min. The effects of different metal ions on the activity of the enzymes were tested under standard assay conditions. They were added into 2 mM ZnCl₂, CoCl₂, BaCl₂, MgCl₂, KCl, CaCl₂ and MnCl₂ solutions. The activity of chitinase without any treatment was defined as 100 %.

Computational prediction

The homology model of Chit42wt from *T. harzianum* and Chit42m (D100G/I166V/A382P) were generated using Discovery studio 2.55 (Accelrys, USA) based on X-crystal structure of chitinase CrChi1 (PDB ID: 3G6M, resolutions 1.65 Å, <http://www.rcsb.org/>), which showed an 83 % sequence positive of the target protein. The structure refinement was accomplished by 5000 steps steepest descent energy minimization on Discovery studio 2.55 (Accelrys, USA). Chitohexaose (extracted from human cartilage glycoprotein-chitin complex, PDB ID 1HJW) was docked into the Chit42wt and its mutant Chit42m by MOE 2016.08 software (Chemical computing group Inc. Canada) for the binding analysis. After receptor preparation, the binding modes between the chitooligosaccharides and the active pocket of the protein were predicted with triangle matcher algorithm for pose generation and London dG and GBVI/WSA dG functions for

docking scoring. The enzyme-ligand complex with the lowest free binding energy was considered as the most stable binding mode based on no-bond interactions and root mean-square deviation (RMSD) values by using MOE tool. Then, 1.11 ns molecular dynamics (MD) simulations was executed on the docked protein-chitohexaose complexes with CHARMM force field, a 12 °Å nonbonded spherical cut-off and isothermal-isochoric ensemble (NVT) by Discovery studio 2.55 (Accelrys, USA). After 10000 steps steepest descent and 20000 ps adopted basis newton-raphson energy minimization, the trajectories were divided in 10 ps of heating from 50 to 300 K, 100 ps of equilibration at 300 K and 1 ns of production at 300 K. Only 1 ns production trajectories was considered for analysis with coordinates saved every 10 ps. Structures were visualized using Discovery Studio 2017 R2 Client (Biovia Co., USA).

Abbreviations

cDNA: complementary DNA

CDS: coding sequences

dNTPs: Deoxynucleotide Triphosphates

GlcNAC: N-Acetylglucosamine

PCR: polymerase chain reaction

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

X Chai analyzed and interpreted data. J Li, X Chai and J Liu wrote the article. X Chen and M Cheng carried out the experimental work and data analysis. Y Hong and W Zheng took part in partial experimental work. A Wang guided this work and revised the article. All authors read and approved the final manuscript.

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Not Applicable.

Additional files

Additional file 1: Supplementary Material (DOCX 534 kb)

Fig. S1 Thermostability of Chit42wt and Chit42m at 55 °C. The incubation time was in 70 min. The blue block represents the Chit42wt and the red circle represents the Chit42m. All assays were performed in triplicate and average values (Mean $\pm$ S.D.) were calculated.

Fig. S2 Effect of metal ions on enzyme activities of the Chit42wt and Chit42m. The activity of the enzyme was determined with the addition of 2 mM ZnCl₂, CoCl₂, BaCl₂, MgCl₂, KCl, CaCl₂ and MnCl₂ solutions. The activity without any treatment was defined as 100 %. The blue block represents the Chit42wt and the red block represents the Chit42m. All assays were performed in triplicate and average values (Mean $\pm$ S.D.) were calculated.

References

1. Zhang F, Chen C, Zhang F, Gao L, Liu J, Chen L, Fan X, Liu C, Zhang K, He YJJoPP: *Trichoderma harzianum* containing 1-aminocyclopropane-1-carboxylate deaminase and chitinase improved growth and diminished adverse effect caused by *Fusarium oxysporum* in soybean. 2017, 210:84-94.
2. Jayaraj J, Radhakrishnan NVJP, Soil: Enhanced activity of introduced biocontrol agents in solarized soils and its implications on the integrated control of tomato damping-off caused by *Pythium* spp. 2008, 304(1/2):189-197.
3. Chen J, Dou K, Gao YD, Ya Qian LIJM: Mechanism and application of *Trichoderma* spp. in biological control of corn diseases. 2014.
4. Delgado-Jarana J, Moreno-Mateos MA, Benítez TJEC: Glucose uptake in *Trichoderma harzianum*: role of gtt1. 2003, 2(4):708.

5. Zimand GJP: Effect of *Trichoderma harzianum* on *Botrytis cinerea* Pathogenicity. 1996, 86(11):1255-1260.
6. Jiang X, Chen D, Hong S, Wang W, Zou SJCP: Identification, characterization and functional analysis of a GH-18 chitinase from *Streptomyces roseolus*. 2012, 87(4):2409-2415.
7. VINALE, SIVASITHAMPARAM, GHISALBERTI, E. L, MARRA, BARBETTI, M. JJP, Pathology MP: A novel role for *Trichoderma* secondary metabolites in the interactions with plants. 2008, 72(1):80-86.
8. Hexon Angel CC, Lourdes MR, Carlos CP, José LBJPP: *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. 2009, 149(3):1579-1592.
9. Elad Y, Katan J, Aharonson N, al. e: Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Protection* 2000, 19(8):709-714.
10. Elad Y, Chet I, Henis Y: Degradation of plant pathogenic fungi by *Trichoderma harzianum*. *Canadian Journal of Microbiology* 1982, 28(7):719-725.
11. López-Mondéjar R, Ros M, Pascual JAJBC: Mycoparasitism-related genes expression of *Trichoderma harzianum* isolates to evaluate their efficacy as biological control agent. 2011, 56(1):59-66.
12. Hartl L, Zach S, Seidl-Seiboth VJAM, Biotechnology: Fungal chitinases: diversity, mechanistic properties and biotechnological potential. 2012, 93(2):533-543.
13. Lin SB, Lin YC, Chen HHJFC: Low molecular weight chitosan prepared with the aid of cellulase, lysozyme and chitinase: Characterisation and antibacterial activity. 2009, 116(1):47-53.
14. Schmoll M, Esquivelnaranjo EU, Herreraestrella AJFG, Fg B: *Trichoderma* in the light of day - physiology and development. 2010, 47(11):909-916.
15. Patil NS, Jadhav JPJC: Significance of *Penicillium ochrochloron* chitinase as a biocontrol agent against pest *Helicoverpa armigera*. 2015, 128:231-235.
16. Merino ST, Cherry JJABEB: Progress and challenges in enzyme development for biomass utilization. 2007, 108:95-120.
17. Lutz S, Patrick WMJCOB: Novel methods for directed evolution of enzymes: quality, not quantity. 2004, 15(4):291-297.
18. Sharon E, ., Bar-Eyal M, ., Chet I, ., Herrera-Estrella A, ., Kleifeld O, ., Spiegel Y, . %J Phytopathology: Biological Control of the Root-Knot Nematode *Meloidogyne javanica* by *Trichoderma harzianum*. 2001, 40(8):2016-2020.

19. Terahara T, Ikeda S, Noritake C, Minamisawa K, Ando K, Tsuneda S, Harayama SJSB, Biochemistry: Molecular diversity of bacterial chitinases in arable soils and the effects of environmental factors on the chitinolytic bacterial community. 2009, 41(3):473-480.
20. Chen J, An Y, Kumar A, Liu ZJIJoBM: Improvement of chitinase Pachi with nematicidal activities by random mutagenesis. 2017, 96:171-176.
21. Shim JH, Kim YW, Kim TJ, Chae HY, Park JH, Cha H, Kim JW, Kim YR, Schaefer T, Spendler TJPED *et al*: Improvement of cyclodextrin glucanotransferase as an antistaling enzyme by error-prone PCR. 2004, 17(3):205-211.
22. Choi YJ, Kim EJ, Zhe P, Yun YC, Yong CSJA, Microbiology E: Purification and Characterization of Chitosanase from Bacillus sp. Strain KCTC 0377BP and Its Application for the Production of Chitosan Oligosaccharides. 2004, 70(8):4522-4531.
23. Songsiriritthigul C, Lapboonrueng S, Pechsrichuang P, Pesatcha P, Yamabhai MJBT: Expression and characterization of Bacillus licheniformis chitinase (ChiA), suitable for bioconversion of chitin waste. 2010, 101(11):4096-4103.
24. Karthik N, Binod P, Pandey AJBT: Purification and characterisation of an acidic and antifungal chitinase produced by a Streptomyces sp. 2015, 188(6):195-201.
25. Neerajaa C, Moerschbacherb B, Podilea ARJBT: Fusion of cellulose binding domain to the catalytic domain improves the activity and conformational stability of chitinase in Bacilluslicheniformis DSM13. 2010, 101(10):3635-3641.
26. Lee YS, Park IH, Yoo JS, Chung SY, Lee YC, Cho YS, Ahn SC, Kim CM, Choi YLJBT: Cloning, purification, and characterization of chitinase from Bacillus sp. DAU101. 2007, 98(14):2734-2741.
27. Suma K, Podile ARJBT: Chitinase A from Stenotrophomonas maltophilia shows transglycosylation and antifungal activities. 2013, 133(133C):213-220.
28. Andreini C, Bertini I, Cavallaro G, Holliday GL, Thornton JMJJJoBIC: Metal ions in biological catalysis: from enzyme databases to general principles. 2008, 13(8):1205-1218.
29. YULI, Purwani E, SUHARTONO, Thenawidjaja M, RUKAYADI, Yaya, Hwang JK, RYANG PYUN YUJE, Technology M: Characteristics of thermostable chitinase enzymes from the indonesian Bacillus sp.13.26. 2004, 35(2):147-153.
30. Natsir H, Chandra D, Rukayadi Y, Suhartono MT, Hwang JK, Pyun YRJJob, molecular biology,, Asian bJtojobFo, Biochemists O, Biologists M: Biochemical characteristics of chitinase enzyme from Bacillus sp. of Kamojang Crater, Indonesia. 2002, 6(4):279.

31. Purushotham P, Sarma PVSRN, Podile ARJBT: Multiple chitinases of an endophytic *Serratia proteamaculans* 568 generate chitin oligomers. 2012, 112(5):261-269.
32. Boer H, Simolin H, Cottaz S, H, Koivula AJPE, Purification: Heterologous expression and site-directed mutagenesis studies of two *Trichoderma harzianum* chitinases, Chit33 and Chit42, in *Escherichia coli*. 2007, 51(2):216-226.
33. Michael L, Harry B, Arja P, Sylvain C, Anu KJG: Toward understanding of carbohydrate binding and substrate specificity of a glycosyl hydrolase 18 family (GH-18) chitinase from *Trichoderma harzianum*. 2009, 19(10):1116-1126.
34. Kruger RG, Dostal P, Mccafferty DGJAB: Development of a high-performance liquid chromatography assay and revision of kinetic parameters for the *Staphylococcus aureus* sortase transpeptidase SrtA. 2004, 326(1):42-48.

Figures

Figure 1

Screening of the randomly mutated clones with enhanced chitinase activity. Approximately, 1230 colonies of random mutagenesis libraries were subjected to initial screening on SC-U- β medium plates after incubating for 96 h at 30 °C. A represents the Chit42wt and B represents the Chit42m among the different sizes of clear hydrolysis zones of the clones on SC-U- β plates. All assays were performed in triplicate and average values (Mean $\pm$ S.D.) were calculated.

Figure 2

The enzyme activity of Chit42_{wt} and Chit42_m were determined. Enzyme activity increases the most when the incubation time was 48 min with the incubation time range of 12-84 mins. The blue block represents the Chit42_{wt} and the red circle represents the Chit42_m. All assays were performed in triplicate and average values (Mean $\pm$ S.D.) were calculated.

Figure 3

Effect of temperature and pH on the activity of Chitinases. The activity of the Chit42wt and Chit42m were compared at the temperature from 30–80 °C and pH from 3 to 8.5. The blue block represents the Chit42wt and the red circle represents the Chit42m. The blue block represents the Chit42wt and the red circle represents the Chit42m. All assays were performed in triplicate and average values (Mean $\pm$ S.D.) were calculated.

Figure 4

Enzyme-substrate complex structure model. (a) Chit42wt-substrate complex structure model. The active site groove is composed of six binding subsite (-4→+2) for GlcNAc units of chitohexaose (in green). Residues mutated in the variant are shown in cyan. Residues E171 and W378 and W131 are shown in violet and orange, respectively. (b) Superposition of equilibrium structure of Chit42wt (ribbon in grey) and Chit42m (ribbon in violet) after 1 ns MD simulation at 300K. The mutated residues were showed as stick in cyan (Chit42wt) and violet (Chit42m), respectively.

Figure 5

Comparisons of the active site of the Chit42wt (a) and Chit42m (b). Several key residues of the Chit42wt and Chit42m are shown in cyan bonds and violet bonds, respectively. Hydrogen bonds are shown as green dashed lines.

Figure 6

Comparisons of mutation sites and surrounding residues. The structures of Chit42wt and Chit42m were showed as ribbon and selected residues were showed as stick. The corresponding hydrogen bonds were labeled as dashed lines in green.

Supplementary Files

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- [SupplementaryMaterial.docx](#)