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STRUCTURAL MODELING, EXPRESSION AND PURIFICATION OF CHIMERIC CHITINASE 42 CONTAINING HIS-TAG IN *NICOTIANA TABACUM* HAIRY ROOT SYSTEM

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Abstract: Chimeric chitinase42 (Chit42 containing ChBD) has great potential as a candidate for digesting and recycling chitin as a beneficial nutrient, which can be produced in bioreactors. The plant is one of the most efficient bioreactors that can produce the eukaryotic proteins in active forms. With the plant hairy root system, it is possible to express a variety of recombinant proteins cost-effectively, easily, and quickly. Due to the huge amount of proteins in plants, protein purification can be facilitated by the use of the His-tag. In this research, different computer programs were used for the three-dimensional structural analysis of Chimeric chitinase42 containing His-tag. The results showed that these comparative modeling approaches had a remarkable degree of accuracy in predicting the fused protein structure. The Z-score of -9.38 and -3.64 predicted for Chit42 and ChBD by ProSA represents the good quality of the model. In addition, bioinformatic observations showed that the His-tag was exposed and can be used to purify the Chimeric chitinase42. The Chimeric chitinase42 containing a His-tag was expressed in *Nicotiana tabacum* hairy roots, and the role of the His-tag in the detection by Western blot and purification using a Ni-NTA column was investigated. The presence of the Chimeric chitinase42 was confirmed by analyzing root extracts using SDS-PAGE and Western blot. The purification step was achieved using the His-tag and the Ni-NTA column. The plant-derived Chimeric chitinase42 was confirmed to be biologically active by measuring the chitinase activity of the purified protein on a media plate containing colloidal chitin.

Key words: Chimeric chitinase 42, His-tag, tobacco hairy root, structural modeling.

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Introduction

Chitin, a polymer found in abundance in nature, is made up of β-(1,4)-linked N-Acetyl-D-glucosamine (GlcNAc) units and is the primary structural element of the cell walls of numerous fungi and arthropod exoskeletons (Akeed et al., 2020; Bhattacharya et al., 2007). During the past decades, more attention has been paid to the degradation of chitin in order to recycle it as a nutrient in nature (Deng et al., 2019). Chitinases (EC.3.2.1.14) are enzymes that catalyze the hydrolysis of chitin, a polysaccharide found in the cell walls of fungi, plants, bacteria, insects, viruses, and vertebrates. These enzymes are present in a wide variety of organisms and play an important role in the breakdown of chitin (Duo-Chuan, 2006; Bhattacharya et al., 2007; Churklam and Aunpad, 2020). Fungal chitinases have been described as more efficient enzymes compared to other chitinases. They offer a promising and sustainable solution for industrial production (Poria et al., 2021). ChBDs are important for the recognition and binding of chitin, and the linker region is thought to be important for the efficient transfer of the substrate to the active site (Arakane et al., 2003; Kowsari et al., 2014). To further improve the efficiency of chitin degradation, researchers have developed a novel approach that combines the presence of ChBD domains with the use of engineered chitinases. This approach has been shown to significantly increase the rate of chitin degradation, resulting in improved efficiency and productivity (Matroodi et al., 2013; Ataei et al., 2016). Among Trichoderma chitinases, Chit42, which lacks a ChBD, is important due to its role in biocontrol (Guthrie et al., 2005; Kowsari et al., 2016). Using protein engineering, the activity of this enzyme can be increased by the adding of a ChBD (Matroodi et al., 2013; Kowsari et al., 2014). Due to the various applications of chitinase, many attempts have been made to improve its activity and produce it in many organisms (Fan et al., 2007; Matroodi et al., 2013; Karthik et al., 2014).

Plants are one of the safest, most scalable, and economic systems for the production of recombinant proteins (Lico et al., 2008). Hairy roots (HRs), among plant tissues, are considered a bioreactor for large-scale production without the requirement for expensive hormones and light, which is cost-effective (Srivastava et al., 2018; Varasteh-Shams et al., 2020). In addition, HRs have genotypic and phenotypic stability, and they are capable of secreting expressed proteins into the culture media (Pham et al., 2012; Gutierrez-Valdes et al., 2020).

The goal of this study is to express the Chimeric chitinase42 containing ChBD and His-tag in the C-terminal end to facilitate the detection and purification of this enzyme, using the *Nicotiana tabacum* hairy root expression system.

Material and Methods

Structural analysis

The ExPASy website (https://web.expasy.org/translate/) was used to translate the nucleotide sequences of Chimeric chitinase42 and ChBD into amino acid sequences. The three-dimensional structures of Chimeric chitinase42 and ChBD were modeled by the comparative modeling approach using Modeller v9.18. The quality and reliability of the models were then evaluated through model validation using the ProSA web server (https://prosa.services.came.sbg.ac.at/prosa.php) as recommended by Nezafat et al. (2016).

Microorganisms, plasmids, and plants

The *E. coli* strain DH5α and the pJET 1.2 cloning vector from Novagen were used for cloning, while the pARM2 expression vector containing a His-tag from the National Institute of Genetic Engineering and Biotechnology in Iran was used for the plant expression experiments. The transformation of the plants was done using the *Agrobacterium rhizogenes* strain ATCC 15834 from Invitrogen. Seeds of *Nicotiana tabacum* cv. *xanthi* were kindly provided by Dr. T. Lohrasebi.

Construction of recombinant plasmids

A Chimeric chitinase42 DNA was used in this study (Ghiasi-sis, 2016). This recombinant construct contained the *chit*42 from *T. atroviride* joined with the ChBD of *Rhizopus oligosporus chi*1. The amplification of the *chit*42-ChBD was performed using PCR and the *Pfu* DNA polymerase (Fermentas, Germany), along with specific primer pairs, (*Xba*I-F*chit*42f, 5'GCTCTAGAATGTTGGGCTTCCTCGGAAAG3' and *Xho*I-R*chit*42f, 5'CCGCTCGAGCAG ACAGTGCCGGAGGG3'), which were designed with *Xba*I and *Xho*I sites at their 5′ ends, respectively. The amplified fragment, purified with the PCR Product Purification Kit (Roche, Germany), was then inserted into the pJET 1.2 cloning vector. The *chit*42-ChBD sequence was digested from the pJET 1.2 vector using *Xba*I and *Xho*I restriction enzymes and inserted into the corresponding sites of the pARM2 expression vector. The expression of the recombinant construct was driven by the CaMV 35S promoter. The recombinant construct (pARMFS) contains a polyhistidine tag at its C-terminus (Figure 1). For comparison, a control was established using tobacco hairy roots transformed with an empty pARM2 vector. The pARM2 is an empty vector containing a multiple cloning site under the CaMV35S promoter in which the gene of interest can be cloned.

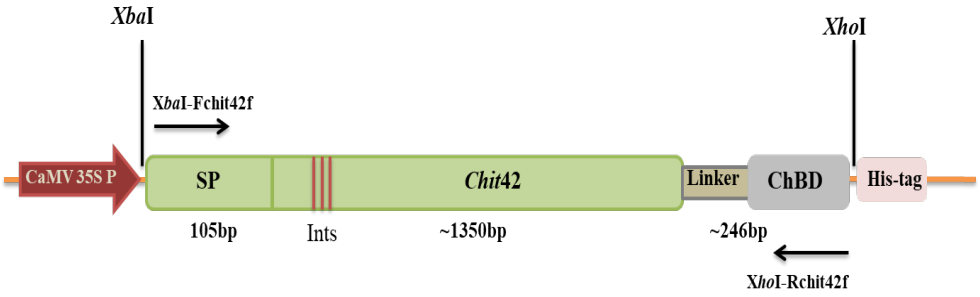

Figure 1. A representation of the pARMFS construct is shown, which includes the *chit*42 gene with three short introns, the ChBD domain, a linker region, and a His-tag sequence. The arrows show the specific primers (*Xba*I-F*chit*42f and *Xho*I-R*chit*42f) of the Chimeric chitinase42. SP: Signal peptide

Transgenic tobacco hairy root establishment by recombinant *Agrobacterium rhizogenes*

Tobacco seeds were cultured in sterile conditions, and leaf discs (2 cm^2) were prepared as explants. These explants were co-cultured with *A. rhizogenes* cultures containing pARMFS expression vectors for 7 minutes under shaking. The leaf discs were dried on the sterile filter papers and placed on the Murashige and Skoog (MS) solid medium (pH 5.6) for 2–3 days under dark conditions. After inoculation, the explants were transferred to MS medium containing antibiotics (400 mg/L cefotaxime and 50 mg/L kanamycin) and incubated for two weeks under a 16/8-hour photoperiod at 24°C. Engendered hairy roots were transferred to fresh liquid MS medium without antibiotics every two weeks at 24°C and 120 rpm for further growth. Negative control hairy roots were produced by leaf disc transformation with *A. rhizogenes* carrying an empty pARM2 vector.

Extraction of genomic DNA and confirmation of transgenic hairy roots

Genomic DNA was extracted from the transgenic tissue samples using the CTAB method (Doyle and Doyle, 1987). The presence of the *chit*42-ChBD in the hairy roots was confirmed via multiplex PCR amplification of the transgenic samples. The absence of *A. rhizogenes* infection was also detected by *Vir*G-specific primers (F-*Vir*G, 5'GGTCGCTATGCGGCATC3' and R-*Vir*G, 5'CCTGA GATTAAGTGTCCAGTCAG3').

Protein extraction and detection of recombinant protein in hairy roots

For the immunological detection of the expressed recombinant protein, the obtained hairy roots were ground in liquid nitrogen. One ml of protein extraction

buffer (1:1, w/v) (200mM Tris−HCl, pH 8.0, 10mM EDTA, 400mM sucrose, 14mM 2-mercaptoethanol, 100mM NaCl, 1mM phenylmethyl sulfonyl fluoride (PMSF), 0.05% Tween-20) was added to each sample. The total soluble protein (TSP) was measured by the Bradford method (1976) after centrifugation (15 min, 16,000×g, 4°C). Bovine serum albumin (BSA) was used as the standard protein.

The total soluble protein samples (70 µg) were loaded onto a 12% SDS-PAGE. The protein bands were then visualized by Coomassie Blue R250 staining. For the Western blot, the treated samples were separated using 12% SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane (Roche, Germany) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, the USA). The transfer was performed at 4°C for 4 hours with a transfer buffer consisting of 40 mM glycine, 50 mM Tris-base, 0.04% SDS, and 20% methanol, at a pH of 8.3, and at a voltage of 100 V. The protein was detected using anti-His-tag antibody as per the manufacturer's instructions (Qiagen, Catalogue No. 34460).

Purification of protein under native conditions

The enzyme was purified by a Ni-NTA column (Qiagen, Catalogue No. 30210). The column was prepared by loading 600 µL of lysis buffer (50 mM NaH2PO4, 10 mM imidazole, 300 mM NaCl, pH 8.0). One ml of the transgenic hairy root-extracted protein containing the 6×His-tagged Chimeric chitinase42 was then loaded onto the column, and the flow-through was collected. Then, 600 µL of wash buffer (50 mM NaH2PO4, 20 mM imidazole, 300 mM NaCl, pH 8.0) was used for washing the column two times. In the end, the protein was eluted with 200 µL of elution buffer (50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole, pH 8.0). The concentration of the purified protein was measured using the Bradford method and stored at -70°C.

Enzymatic activity test

The activity of the purified expressed protein was determined using a chitinase-detection agar (CHDA). CHDA was prepared using (g/L): 0.5 NH4Cl, 0.65 NaHPO4, 0.25 NaCl, 1.5 KH2PO4, 0.005 CaCl2, 0.12 MgSO4, and 20.0 agar-agar at a pH of 6.5, with 10% (w/v) colloidal chitin as described by Barboza-Corona et al. (1999). The method of Roberts and Selitrennikoff (1988) with some modifications was used for preparing the colloidal chitin. Five g of commercial chitin (Sigma-Aldrich, USA) was slowly added into 100 ml of cold concentrated HCl and kept overnight at 4◦C with gentle agitation on a magnetic stirrer. Then, 500 ml of ice-cold 96% ethanol was added, and the mixture was stirred rapidly for 24 hours at 4°C. The precipitate was centrifuged at 10,000 g for 20 minutes at 4°C

and then washed repeatedly with sterile distilled water until colloidal chitin reached a pH of 7.0 and was cleared. It was then stored at 4°C for future use.

Results and Discussion

Analysis of the structural modeling

The BLASTp webserver was employed to match the Chimeric chitinase42 of *T. atroviride* and the ChBD domain of *R. oligosporus chi*1 against the PDB database. This was to determine the template sequences that had the greatest sequence identity. A structural model was then predicted by selecting Chit42, which had an 82.7% sequence identity with a template (PDB code: 6EPB_A), and the ChBD domain, which had a 100% sequence identity with a template (PDB code: P29026.1) for protein model building.

The 3D structures of the Chit42 and ChBD domains were modeled by the comparative modeling approach using Modeller. Using the ProSA server, the "Z" scores of Chit42 and ChBD were -9.38 (Figure 2a) and -3.64 (Figure 2b), respectively. The results show that the models were of good quality and had a remarkable degree of accuracy in predicting the fused protein structure. The 3D model of the Chimeric chitinase42 and His-tag sequence was visualized using the Discovery studio 2020 client software (Figure 3). Bioinformatic observations demonstrated that the tertiary structure of Chimeric chitinase42 and the His-tag was exposed and can be used for the purification procedures of Chimeric chitinase42.

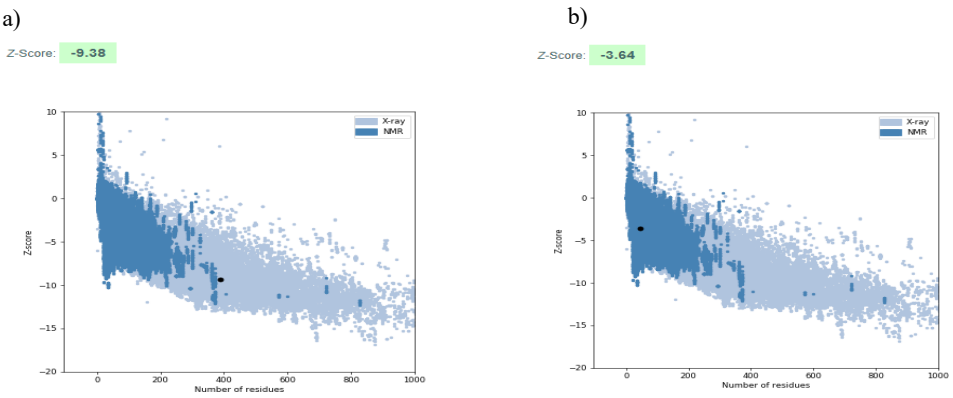

Figure 2. "Z" scores of the models in the ProSA server, (a) "Z" score plot of the Chit42 model (−9.38), (b) "Z" score plot of the ChBD model (-3.64).

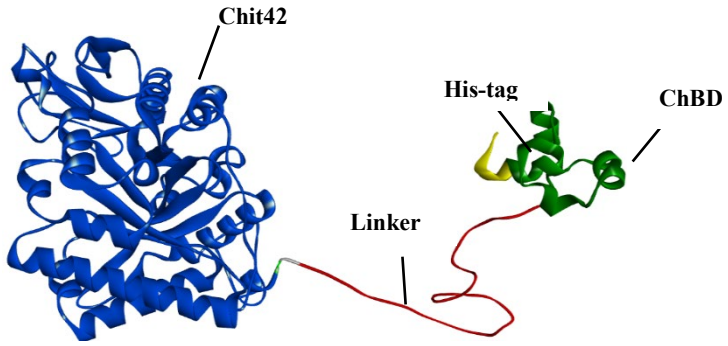

Figure 3. "Z" scores of the models in the ProSA server, (a) "Z" score plot of the Chit42 model (-9.38), (b) "Z" score plot of the ChBD model (-3.64).

Expression analysis in *N. tabacum*

The purpose of this study was to produce a new Chimeric chitinase42 enzyme, which contains a chitin-binding domain (ChBD) and a histidine-tag (His-tag), using a plant expression system. Since the T-DNA was transferred to the plant by *Agrobacterium*, the genomic DNA of *chit*42, containing three short introns, was used to express it only through the plant expression system (not via *Agrobacterium*). Chimeric chitinase 42 DNA (Ghiasi-sis, 2016) was sub-cloned in the pARM2 expression vector containing the His-tag sequence and designated as pARMFS. The chimeric sequence was confirmed through sequencing. This construct expressed a Chimeric chitinase 42 protein tagged at the C-terminal end with a 6xHis tag that can be easily detected (using Western blotting) and purified.

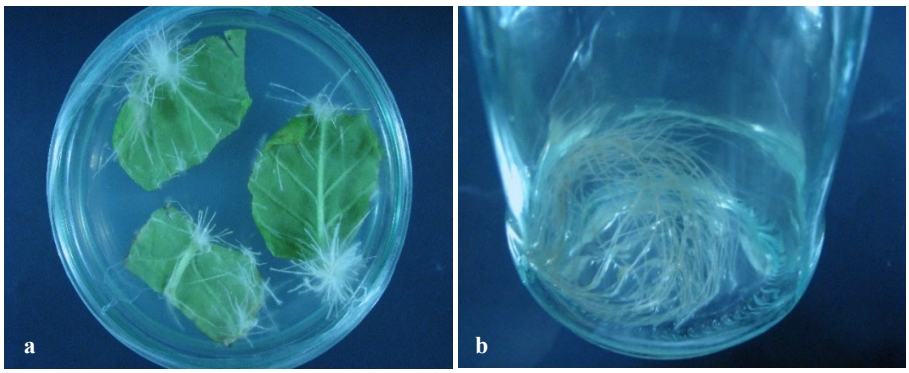

Figure 4. (a) A view of explants demonstrating the growth of hairy roots. (b) An observation of the propagation of hairy roots in MS medium at 24°C and 120 rpm.

The transformation via *A. rhizogenes* was used to transfer the construct to *N. tabacum*. The results of the experiment show that out of 25 inoculated leaf discs, 21 explants produced hairy roots with an average of three roots per explant (Figure 4), suggesting an 84% transformation efficiency. Based on the growth conditions of the hairy roots, ten explants were chosen for further molecular analysis. PCR amplification with *VirG* primers did not detect any *A. rhizogenes* contamination in the hairy roots. The cloned fragment (1699 bp) was confirmed using chimeric chitinase 42-specific primers, which showed the expected size of *chit*42-ChBD following the electrophoresis (Figure 5).

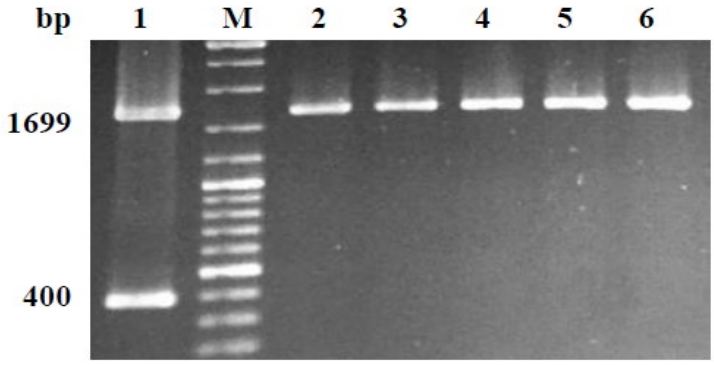

Figure 5. Screening transgenic hairy roots by multiplex PCR. The presence of a 1699-bp fragment of *chit*42-ChBD was detected by PCR amplification using specific primers and DNA extracted from tobacco hairy roots. *Vir*G specific primers also confirmed the *A. rhizogenes* infection elimination. 1) PCR on *A. rhizogenes* DNA harboring pARMFS, which shows a 1699-bp fragment of *chit*42-ChBD and a 400-bp fragment of *Vir*G. 2,3,4,5 and 6) The amplified fragment from transgenic hairy root explants. M) DNA ladder m.

Fifteen days after the growth of the transgenic hairy roots in a liquid medium, the total soluble protein was extracted and used to analyze the expression of the recombinant protein. The molecular weight of the expressed protein, estimated to be near 56 kDa, was confirmed by the analysis of SDS-PAGE (Figure 6a). Western blot analysis using an anti-His-tag antibody was performed for further validation. The results confirmed the presence of the recombinant protein with a 6×His tag and with a molecular weight of 56 kDa, which matched the expected molecular weight of the expressed protein (Figure 6b). The total cell protein of the hairy root harboring the empty pARM2 vector was used as a negative control.

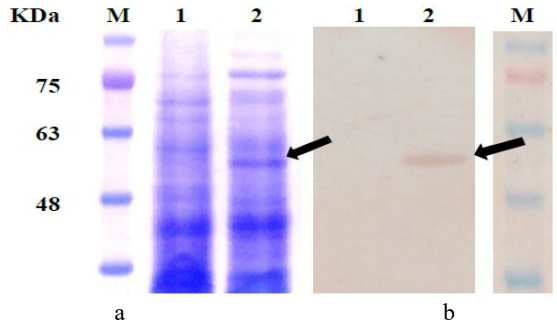

Figure 6. a) SDS-PAGE analysis of the total extracted proteins. b) Western blot analysis of the recombinant protein using the anti-His-tag antibody. 1) Extraction of proteins from hairy roots containing the empty pARM2 vector (negative control). 2) Proteins extracted from the transgenic hairy root. The arrows indicate the presence of a band of approximately 56 kDa in the samples of the transgenic hairy roots. M) Protein molecular weight marker.

Enzyme purification

After confirming the presence of the expressed protein in the hairy roots, the 6xHis/Ni-NTA purification system was carried out under native conditions to determine the role of the addition of His-tag for purifying the recombinant enzyme. The His-tagged protein was purified from the total hairy root protein extract using a Ni-NTA column. The expected 56-kDa band was detected by SDS-PAGE and confirmed through Western blot analysis (Figure 7).

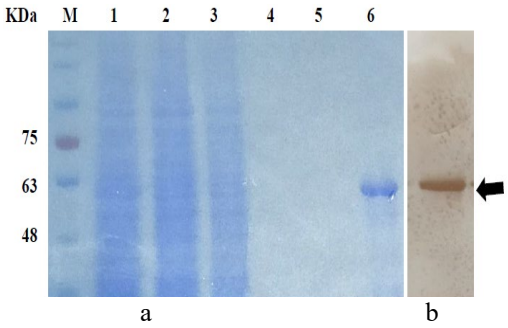

Figure 7. Purification of the Chit42-ChBD-6His protein by native chromatography on a Ni-NTA column. a) The protein was visualized on an SDS-PAGE gel. 1) Protein sample extracted from the hairy root; 2) Flow-through; 3, 4 and 5) Wash with 20 mM imidazole (pH 8); 6) Elution by 500 mM imidazole (pH 5.6); M) Protein molecular weight marker; b) Western blot analysis of the purified Chit42-ChBD-6His using anti-His-tag. The arrow indicates the 56-kDa band of the purified enzyme.

Chitinase activity assay

To verify the enzyme activity of the purified expressed enzyme, the Chitinase detection agar assay was used. Seven µg of the purified protein was loaded into each well, and after 24–48 h, the activity of the enzyme was assessed by observing the creation of a clear zone around the well on the media plate with colloidal chitin. The clear zone indicates the degradation of chitin by the enzyme, and its size can be used to quantify the activity of the enzyme (Figure 8).

a b

Figure 8. Chitinase detection of hairy root protein extract by agar assay. a) The well was loaded with the purified Chimeric chitinase 42 sample at a concentration of 7 µg. The enzyme activity was detected by a clearing zone around the well on the media containing the colloidal chitin. b) The well was loaded with buffer as a negative control. No clearing zone was observed around the well.

Chitinase [EC3.2.1.14] enzymes have the capacity to digest and recycle chitin as a beneficial nutrient. In this study, Chitinase42 from *T. atroviride* containing ChBD of *R. oligosporus chi1* (Kowsari et al., 2016) was used. In their study, adding ChBD resulted in a 1.56-fold increase in the chitinase activity of Chtinase42. Studies have demonstrated the impact of ChBD on enhancing the chitinase activity of Chimeric chitinases. Matroodi et al. (2013) showed that combining a ChBD from *Serratia marcescens Chi*B with Chit42 increased the activity of the resulting Chimeric enzyme. The same finding was also reported by Kowsari et al. (2014), who showed that the addition of a ChBD from *T. atroviride* Chitinase 18-10 to Chit42 of *T. harzianum* increased 1.7-fold of the enzyme specific activity. Furthermore, Limón et al. (2001) demonstrated that incorporating a ChBD from *N. tabacum* into Chit42 of *T. harzianum* increased the enzyme activity.

The purification of proteins by conventional affinity chromatography procedures has disadvantages, such as high cost and time consumption, thus,

affinity chromatography methods are alternative procedures for recombinant protein purification. A protein affinity tag provides an essential mechanism for protein purification. There are a number of tags such as streptavidin-binding tags, calmodulin-binding tags, polyarginine or histidine tags, which facilitate the purification of the various proteins (Amarasinghe and Jin, 2015; Oliveira and Domingues, 2018; Terpe, 2003).

In this study, prior to the experimental analysis of the protein expression using His-tag, different computer programs were used for the analysis of the structural three-dimension of Chimeric chitinase42 containing His-tag. The results showed that these comparative modeling approaches exhibited a remarkable degree of accuracy in predicting the fused protein structure. In addition, bioinformatic observations showed that the His-tag was exposed and can be used for the purification of Chimeric chitinase42. We used the polyhistidine tag (6×His-tag) which is a useful tool for easy, efficient, and high-quality protein purification to facilitate the detection (using Western blot) and purification of Chimeric chitinase. Using the enzyme activity assay, we have confirmed that the His-tag does not affect the activity of Chimeric chitinase. Some reports indicate that the His-tag does not change protein properties in most cases (Hung et al., 2002; Aslantas and Surmeli, 2019; Zhou et al., 2020). Kowolik and Hengstenberg (1998) reported that the location of the His-tag on the lactose enzyme II of *Staphylococcus aureus* (either at the C-terminal or N-terminal end) had no impact on its surface affinity. Nevertheless, it can sometimes influence the biological functions of the protein (Mohanty and Wiener, 2004; Kenig et al., 2006). It has been reported that one of the His-tagged subunits of the bacteriophage λ terminase enzyme demonstrated a change in its interaction with DNA (Hang et al., 1999). In a study by Zayakina et al. (2009), the addition of a polyhistidine tag to the coat protein of potato virus X resulted in the loss of its ability to form helical nucleoprotein virus-like particles with viral RNA.

In the present work, we utilized an expression vector containing the His-tag sequence (pARM2), in which the Chimeric chitinase 42 containing the His-tag at its C-terminal can be produced, and the plant expression system was used as a bioreactor. Protein purification from biochemically complex plant tissues containing too many different biomolecules and proteins is usually an important and challenging task in biochemistry (Sánchez-Ferrer et al., 1994; Borisjuk et al., 1999). The time required and the method of purification depend on the specific protein and the aim of using this protein.

The various recombinant protein production systems have some limitations, such as the inability of the bacterial systems to produce functional complex proteins or the high risk of contamination when producing recombinant proteins with toxic molecules in mammalian cells (Cardon et al., 2019). It has been reported that the recombinant protein production using a plant expression system as a

bioreactor has several advantages over other expression systems, including the production of a large amount of products, the sustainability of the molecules, high safety issues, and low upstream costs (Gutierrez-Valdes et al., 2020). In comparison to other plant-based expression systems, hairy root cultures have been recommended as a promising method for the production of recombinant proteins such as enzymes (Woods et al., 2008) and antibodies (Sharp and Doran, 2001; Martínez et al., 2005) due to their genetic stability and high productivity (Gutierrez-Valdes et al., 2020). In our study, we employed a hairy root of the tobacco plant as a platform for producing the recombinant Chimeric chitinase 42 protein, which was verified through SDS-PAGE and Western blot analysis.

Conclusion

In conclusion, our study has shown that a Chimeric chitinase 42 can be produced using a hairy root expression system. Additionally, it was shown that the His-tag did not affect the activity of Chimeric chitinase 42, making it a viable option for purifying the active recombinant protein.

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STRUKTURNO MODELIRANJE, IZRAŽAVANJE I PREČIŠĆAVANJE HIMERNE KITINAZE 42 SA HIS-OZNAKOM U ŽILIČASTOM KORENOVOM SISTEMU BILJKE *NICOTIANA TABACUM*

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R e z i m e

Himerna hitinaza 42 (Chit42 koji sadrži ChBD) ima veliki potencijal kao kandidat za razlaganje i recikliranje hitina kao korisne hranljive materije, koja se može proizvesti u bioreaktorima. Biljka je jedan od najefikasnijih bioreaktora koji mogu proizvesti eukariotske proteine u aktivnim oblicima. Pomoću žiličastog korenovog sistema biljke, moguće je ekonomično, lako i brzo dobiti različite rekombinantne proteine. Zbog velike količine proteina u biljkama, prečišćavanje proteina može biti olakšano korišćenjem His-oznake. U ovom istraživanju, korišćeni su različiti kompjuterski programi za trodimenzionalnu strukturnu analizu himerne hitinaze 42 koja sadrži His-oznaku. Rezultati su pokazali da su ovi pristupi uporednom modeliranju imali izuzetan stepen tačnosti u predviđanju strukture fuzionisanog proteina. Z-skor od -9,38 i -3,64 za Chit42 i ChBD koji je ProSA predvideo predstavlja dobar kvalitet modela. Pored toga, bioinformatička posmatranja su pokazala da je His-oznaka bila ekspresivna i da se može koristiti za prečišćavanje himerne hitinaze 42. Himerna hitinaza42 koja sadrži His-oznaku izražena je u žiličastim korenovima biljke *Nicotiana tabacum*, a istražena je i uloga His-oznake u detekciji putem analize Vestern blot i prečišćavanju pomoću kolone Ni-NTA. Prisustvo himerne hitinaze 42 potvrđeno je analizom ekstrakata korena korišćenjem tehnike SDS-PAGE i analize Vestern blot. Prečišćavanje je postignuto korišćenjem His-oznake i kolone Ni-NTA. Potvrđeno je da je dobijena himerna hitinaza 42 biološki aktivna merenjem hitinazne aktivnosti prečišćenog proteina na podlozi koja sadrži koloidni hitin.

Ključne reči: himerna hitinaza 42, His-oznaka, žiličast koren duvana, strukturno modeliranje.

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