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Confirmation of non N-glycan Linked Mannose Glycosylation in Chitinase 42 kDa Secreted by *Trichoderma harzianum* BIO10671

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Abstract: Chitinase 42 kDa produced by *Trichoderma harzianum* has been proven as a prime compound to be excreted onto the hyphae of the pathogen causing localised cell wall lysis at the point of interaction. This finally initiate the process of the host cell becomes empty of cytoplasm, disintegrates and shows a rapid collapse. This study investigates the existence of N-glycan linked mannose in chitinase 42 kDa produced by the Malaysian *T. harzianum* strain BIO10671. The chitinase 42 kDa from *T. harzianum* BIO10671 was initially purified using anion exchange chromatography prior to a series of experiments such as immunoblotting against the chitinase 42 kDa antibody, lectin staining for detecting any terminal linked mannose and galactofuranose detection to determine the presence of galactofuranose components in glycoproteins. The enzyme purification harvested about 12-fold of chitinase 42 kDa from *T. harzianum* BIO10671 with strong indication of the presence chitinase 42 kDa presence on SDS-Page. This was confirmed by immunoblotting with a strong response around 42 kDa after overnight incubation in chitinase 42 kDa antibody suggesting that the gene for chitinase 42 kDa was greatly expressed in this strain. There are no intervention of galactofuranose on any of the terminal mannose in chitinase 42 kDa as shown by negative results on samples treated with or without endoglycosidase-H and lectin staining. Therefore, it can be concluded that glycosylation occurred in the chitinase 42 kDa from *T. harzianum* 42 kDa was not in the form of N-glycan linked mannose as expected.

Key words: *Trichoderma harzianum*, chitinase, glycosylation, N-glycan linked, mannose

INTRODUCTION

The chitinolytic system of *T. harzianum* is complex with several types of chitinase working together simultaneously to fulfil all the niche of *Trichoderma* sp. One of the components in the chitinolytic system is the chitinase 42 kDa which has a molecular weight of around 42 kDa. Filamentous fungi such as *T. harzianum* are able to secrete high levels of protein into the culture medium and this advantage has been used widely in biotechnology industry to produce heterologous proteins (Archer and Peberdy, 1997). Chitinase 42 kDa by itself is capable of degrading fungal cell walls and its antifungal activity can be enhanced by adding chitinase 33 kDa or combined with gliotoxin (Carsolio *et al.*, 1994). Chitinase 42 kDa from different strains display different physical characteristics such as pI values and substrate specificity (Gakul *et al.*, 2000).

Due to its high potential as biocontrol and biotechnology compounds, we are interested to study one of the interesting characteristics of chitinase 42 kDa such as glycosylation, post-translational

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modification of the protein to strengthen the protein conformation, stability and biological activity in order to produce a high-end protein. One of the problems in producing recombinant glycoprotein is the correct intracellular environment at the moment of glycosylation. Combination of correct intracellular environment, genetic combination, mannosyltransferase activity and the substrate specificity have an effect on the formation on N-linked or O-linked glycans as shown in of the *Saccharomyces cerevisiae* (Moo *et al.*, 2006) and both types of glycosylation can effect the function of the final recombinant protein produced (Yan *et al.*, 1999). However, the glycosylation process is required before glycoprotein production can truly benefit the industry.

Glycosylation is the process of saccharides addition to proteins and lipids using two types of linkage which are N-linked and O-linked glycosylations. For N-linked oligosaccharides, a 14-sugar precursor contains three glucose, nine mannose and two N-acetylglucosamine molecules is first added to the asparagine in the polypeptide chain of the target protein. Then a complex set of reactions attaches this branched chain to a carrier molecule called dolichol before being translocated into the ER lumen. While O-linked glycosylation occurs at a later stage during protein processing, probably in the Golgi apparatus which involve the addition of N-acetyl-galactosamine to serine or threonine residues by the enzyme UDP-N-acetyl-D-galactosamine: Polypeptide N-acetylgalactosaminyltransferase, followed by other carbohydrates such as galactose and sialic acid (Shoda, 2001). The aim and objectives of the present study were to obtain the evidence and to confirm the non-N-linked mannose glycosylation in chitinase 42 kDa produced by a Malaysian *T. harzianum* strain BIO10671.

MATERIALS AND METHODS

Strains

Trichoderma harzianum strain BIO10671 obtained from personal collection, Department of Biology, Faculty of Science was used in the present study. The culture was maintained on Potato Dextrose Agar (PDA) at 28°C and subcultured every fortnight.

Extracellular Chitinase Purification

The spore suspensions of *T. harzianum* BIO10671 in approximately 1×10^7 spores mL⁻¹ were added to 25 mL of Trichoderma Complete Medium (pH 5.5; 0.5% w/v glucose) to produce seed cultures. Seed cultures were shaken at 180 rpm at 28°C for 24 h before being filtered through a sterile Whatman No. 1 paper. Then were washed three times with sterile distilled water and transferred into 25 mL of Trichoderma Minimal Medium (pH 5.5; 1.0% w/v *Pleurotus sajor-caju* mycelium). Culture filtrates were harvested, filtered through a Whatman No. 1 filter paper, centrifuged at 6000 x g for 10 min before being dialysed against the distilled water for at least 24 h at 4°C.

The purification was carried out using anion-exchange method by Lima *et al.* (1997). Crude culture containing extracellular chitinase was precipitated with ice-cold 80% acetone and incubated at -20°C for 30 min. The precipitate was recovered by centrifugation at 28000 x g for 10 min at 4°C, re-dissolved in distilled water and dialysed against distilled water for another 24 h at 4°C.

Ninety microliter of buffer A (50 mM Tris-HCL pH 7.5) and 20 µL 1 M Tris pH 7.5 were added to the dialysed crude culture sample and the pH was adjusted to pH 7.0. The sample was centrifuged at 12000 x g for 10 min. Meanwhile, an anion exchange Neobar AQ column was washed with 10 column volumes of Buffer B (50 mM Tris-HCL pH 7.5; 1 M NaCl) followed by 10 column volumes of Buffer A. The resulting supernatant was loaded onto the column and eluted at a flow rate of 1 mL min⁻¹ and the bound protein was eluted with a 0-1 mM NaCl gradient. The fractions with high β-1,3-glucanase activity were pooled before the dialysis against distilled water for 24 h at 4°C.

Dialysed fraction was collected and assayed for chitinase activity using the chitinase assay method (Reissig *et al.*, 1955) which involved the estimation of N-acetylglucosamine (GlcNAc) released from chito-oligosaccharides by the β -N-acetylhexosaminidase. Discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on collected fraction in 10% acrylamide gels and stained with Coomassie R-250 brilliant blue (Sigma) according to the method of Laemmli (1970). Low molecular mass standard proteins were used for molecular mass determination.

Determination of N-Linked Glycosylation

Lyophilised sample of purified chitinase 42 kDa was resuspended in 90 μ L of nanopure water followed by adding 40 μ L 4xbuffer (100 mM sodium acetate phosphorus, pH 5.5). The sample was heated for 10 min at 100°C to denature the sample and any contaminating proteases. The sample was then divided into two portions; (i) a control sample i.e. without endoglycosidase-H enzyme (Oxford GlycoSystems) and (ii) test sample with 2 μ unit of endoglycosidase-H enzyme added. Finally, 5 μ L of toluene was added to prevent any fungal or bacteria growth during incubation at 37°C overnight. Four types of assay, namely immunoblotting, lactin staining, galactofuranose detection and general glycan detection were carried out using both of the prepared samples.

Immunoblotting

Treated purified chitinases was tested against chitinase 42 kDa antibody (CHIT42), kindly supplied by Benitez, University of Seville, Spain. Fifty microliter lyophilised CHIT42 antibody was diluted 1:2000 in 0.1% (v/v) TBS buffer. This antibody was raised in rabbits against the CHIT42 from *T. longibrachiatum*. Immunoblotting was carried out according to Wallis *et al.* (1999).

Lectin Staining: Detection of Terminal Linked Mannose

The detection of terminal linked mannose in purified chitinase 42 kDa from *T. harzianum* BIO10671 was done by staining the membrane blots of the chitinase pre and post-endoglycosidase-H enzyme treatments with *Galanthus nivalis* agglutinin (GNA) lectin (Boehringer Mannheim) according to the manufacturer's recommendation.

Galactofuranose Detection

An investigation for the presence of galactofuranose components of glycoproteins was also carried out by blotting against EBA2 antibody. The EBA2 is the antibody raised against the immunogenic polysaccharide from *Aspergillus fumigatus*, which recognises glycoproteins containing β -linked galactofuranose residues. The membrane was incubated overnight in EBA2 antibody (2.5 μ L in 5 mL 0.1% (v/v) TBS-Tween) at 4°C (Wallis *et al.*, 1999).

RESULTS AND DISCUSSION

Although chitinase-producing microorganisms are considered an effective biological control, the role of chitinase in the antagonistic process, literatures concerning the purification, molecular properties and molecular structure of extracellular chitinase from mycoparasitic fungi are still limited (Gakul *et al.*, 2000; Nampoothiri *et al.*, 2004). The crude enzyme from *T. harzianum* BIO10671 contained 26.22 μ moles μ L⁻¹ of chitinase activity was used at the beginning of the anion exchange. The presumed mixture of chitinase was purified from *T. harzianum* using acetone precipitation. The elution pattern of anion exchange chromatography of this crude enzyme fraction is shown in Fig. 1 with two peaks for chitinase activities in *T. harzianum* BIO10671 arbitrarily BIO (C1) and BIO (C2) for fraction 4-6 and 24-39, respectively. Only 75.6% (21.33 μ moles μ L⁻¹) of chitinase activity was recovered after the anion exchange, which consist of 0.18 μ moles μ L⁻¹ in BIO (C1) and 21.15 μ moles μ L⁻¹ in BIO

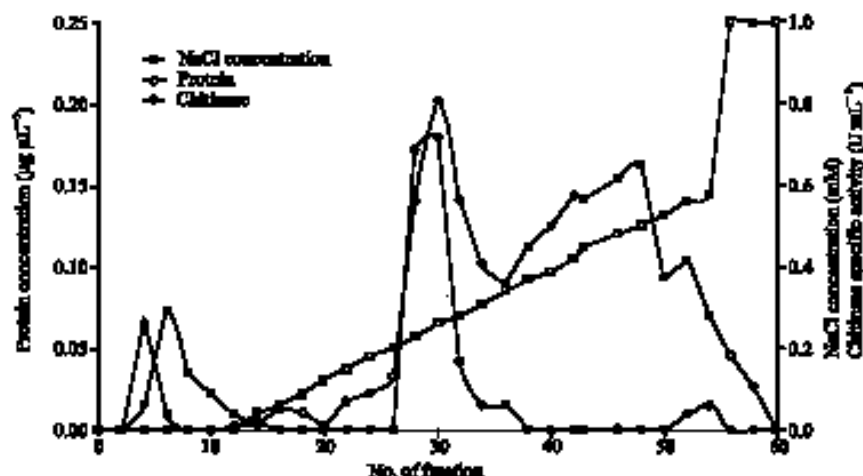


Fig. 1: Purification of chitinase by anion exchange chromatography. Bound protein was eluted with a 0 to 0.5M NaCl gradient. Elution profile of *T. haznicorum* BIO10671 for protein on Neobar AQ exchanger column with peaks at fraction 4-6 (BIO C1) and fraction 24-39 (BIO C2)

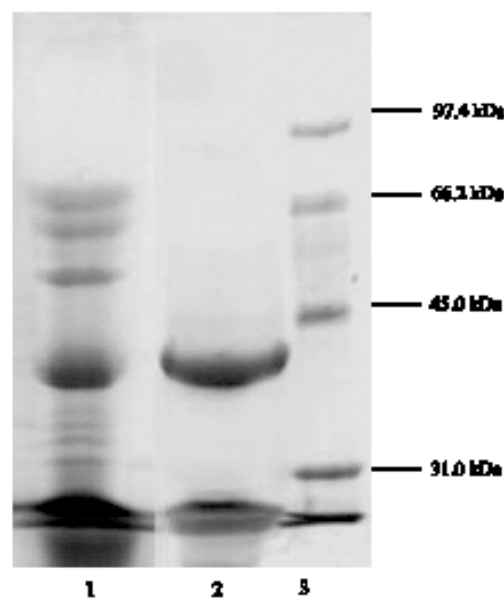


Fig. 2: Purification of chitinase by anion exchange chromatography. Bound protein was eluted with a 0 to 0.5 M NaCl gradient. SDS-PAGE (10%) of protein from pooled peaks and stained with Coomassie blue. Lane 1: BIO C1 (fraction 4-6); Lane 2: BIO C2 (fraction 24-39); Lane 3: Low range standard molecular weight marker

(C2). Since the BIO (C1) contained only a small percentage of chitinase activity, it was not used for further analysis. The analysis of both pooled fractions by SDS-PAGE (Fig. 2) showed several major proteins bands in BIO (C1) while only one band was observed in BIO (C2) at approximately 42 kDa.

Three chitinases purified by De La Cruz *et al.* (1992) showed molecular masses of 42, 37 and 33 kDa. Recently, several other chitinases have been purified and tested for their antifungal

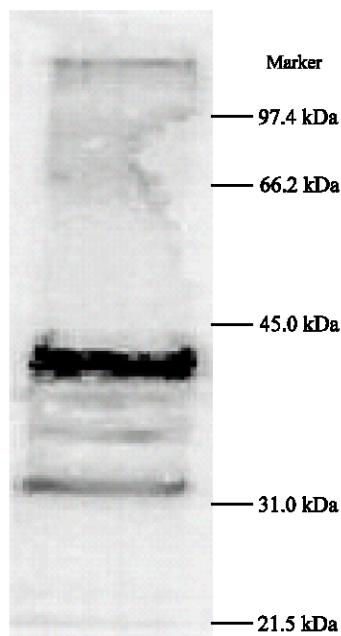


Fig. 3: Immunoblot of BIO C2 (fraction 2-39) incubated in chitinase 42 kDa antibody (CHIT 42) and detected using secondary anti-rabbit antibody for 1 h (1:1000 in 10 mL TBS-Tween)

activity (Viterbo *et al.*, 2001; De Las Mercedes *et al.*, 2001). The antibodies raised against the two higher molecular mass enzymes reacted specifically and did not cross react suggesting that each protein is encoded by a different gene. The protein size revealed by SDS-PAGE for *T. harzianum* BIO10671 was 42 kDa which was larger than the purified chitinase collected by Deane *et al.* (1998) from *T. harzianum* T198, but similar to the range of endochitinases reported previously by Haran *et al.* (1995) and Matsumiya *et al.* (2001). The molecular weight of purified chitinase may differ between species and also within species (Pitson *et al.*, 1993) and it is not known whether they are differently processed gene products from the same gene or from separate genes. Sometimes the type of growth substrate used can also influence the number of bands and molecular weight on SDS-PAGE (Vazquez-Garciduenas *et al.*, 1998). Matsuzawa *et al.* (1996) concluded that although the same type of chitinase was purified, characterisation of the purified form will be different from each other and it depends on the species, the type of reaction (exo-or endo-) and the method of purification.

Since *Trichoderma* chitinases are extracellular enzymes, they are expected to be glycosylated. In order to determine whether or not the purified chitinase from *T. harzianum* BIO10671 was N-linked glycosylated, a series of tests were carried out including immunoblotting with chitinase 42 kDa antibody, lactin staining, galactofuranose detection and general glycan detection. Immunoblotting process by incubating the membrane with CHIT42 antibody with the pooled fraction of BIO (C2) revealed four bands within the range of 31 to 45 kDa molecular weight (Fig. 2). A strong response around 42 kDa after overnight incubation in CHIT42 antibody suggested that there was a high level of chitinase 42 kDa activity in this strain. In other words, this result indicated that the gene expression for chitinase 42 kDa was greater than the other types of chitinase and similar finding was also reported by Pérez-Martínez *et al.* (2007) and Carsolio *et al.* (1994). The studies by Ike *et al.* (2006) proved the ability of chitinase 42 kDa to be expressed its activity greater than other enzyme in *Escherichia coli* but still influence by substrate specificity.

Another positive reaction was also seen on the membrane from 21 to 97 kDa although only a single chitinase band was detected in SDS-PAGE after the anion purification (Fig. 3). This would

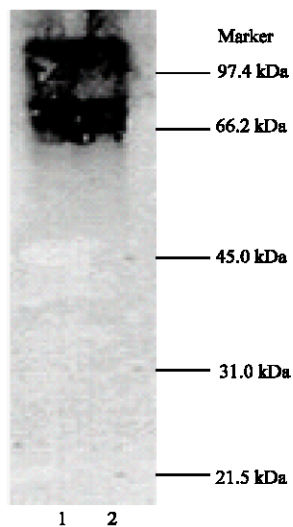


Fig. 4: Lectin staining membrane for mannose detection using *Galanthus nivalis* agglutinin (GNA). Lane 1: BIO C2 (fraction 24-39) incubated with endoglycosidase-H; Lane 2: BIO C2 (fraction 24-39) incubated without endoglycosidase-H

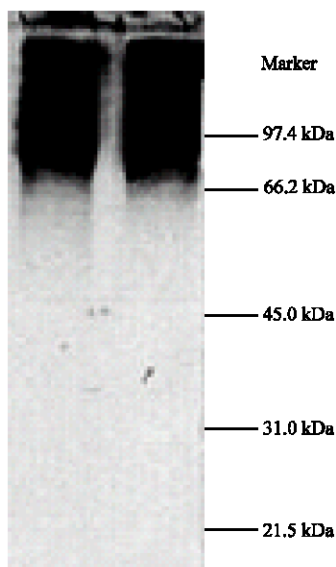


Fig. 5: Galactofuranose detection of BIO C2 (fraction 24-39) against 2.5 µL EBA2 antibody in 5 mL 1% (v/v) TBS-Tween at 4°C

indicate the existence of chitinase 42 kDa isoform in this strain. However, this result is in contrast to the findings described by Lima *et al.* (1997) who found only one band in immunoblotting of chitinase 42 kDa from *T. harzianum* T6. The differences in the number of chitinase 42 kDa form in this study and previous report might due to the difference in type of strain, carbon source and culture used in the experiment. This was explained by Steyaert *et al.* (2004) which described that regulation and expression of mycoparasitism's genes in/among *Trichoderma* species is highly variable and might be

regulated by a metabolic pathway since mycoparasitism is an alternative means of carbon assimilation, characterized by catabolite repression and induction during carbon starvation.

Lectin staining using GNA is an effective method to detect any mannose in the glycan component since most glycosylated enzymes in fungi contain mannose (Neethling and Nevalainen, 1995). Prior to this step, purified chitinases from *T. harzianum* BIO10671 was incubated in 37°C in endoglycosidase-H overnight in order to remove any N-linked oligosaccharides from the polypeptide so that mannose branches can be easily detected by GNA. There was no positive response for sample treated with or without endoglycosidase-H as shown in Fig. 4, This can be seen as an evidence of the non existence of N-glycan linked in chitinase 42 kDa as expected. The test also revealed that no lectin stained was aroused at chitinase 42 kDa's position. This showed no mannose branches formation in this glycoprotein structure.

The negative of response to endoglycosidase-H might imply a possibility that the terminal mannose was capped by galactofuranose as these residues were not detected by GNA. To investigate this possibility, a galactofuranose binding antibody was employed. However no galactofuranose could be detected in the chitinase 42 kDa area (Fig. 5), thus indicating that galactofuranose was not present in this glycoprotein structure to conceal mannose branches.

In conclusion, all the results obtained in this experiment through three experiments suggested that chitinase 42 kDa did not exist in the form of N-glycan linked mannose glycosylation.

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