Pertanika J. Trop. Agric. Sci. 28(1): 23 -31 (2005) ISSN: 1511-3701


# Purification and Characterisation of β-1,3-glucanase from *Trichoderma harzianum* BIO 10671

*MUSKHAZLI MUSTAFA, SALFARINA RAMLI, NALISHA ITHNIN & NOR FARIZAN TOHFAH
*Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

**Keywords:** ***Tricoderma harzianum*, β-1,3-glucanase, purification, characterisation, pH, metal ion**

## ABSTRAK

*Enzim β-1,3-glukanase dari cecair kultur* Trichoderma harzianum *BIO 10671 telah berjaya ditulenkan melalui pemendakan dengan 80% aseton, diikuti kromatografi penukaran ion menggunakan Neobar AQ dan pemfokusan kromatografi menggunakan kolum Mono P HR 5/20. Dua β-1,3-glucanase bersaiz 32kDa dan 66kDa telah ditulenkan dan ditunjukkan pada SDS-PAGE. pH optimum bagi aktiviti enzim ini ialah pada pH 4.5 dan aktiviti maksimum dicerap pada 45°C. Manakala aktiviti enzim direncat 20-45% oleh 20mM $Zn^{2+}$, $Ca^{2+}$, $Co^{2+}$, $Mg^{2+}$, $Cu^{2+}$, $Mn^{2+}$ dan $Fe^{2+}$. Aktiviti hidrolisis β-1,3-glucanase tertinggi didapati pada laminarin disebabkan persamaan ikatan β-glikosidik dan diikuti masing-masing pada pustulan, glukan dan selulosa.*

## ABSTRACT

*β-1,3-glucanase enzyme from culture filtrate of* Trichoderma harzianum *BIO 10671 was successively purified by precipitation with 80% acetone followed by anion-exchange chromatography on Neobar AQ and chromatofocusing on a Mono P HR 5/20 column. Two β-1,3-glucanases of 32kDa and 66kDa were purified to homogeneity as judged by SDS-PAGE. The pH optimum for the enzymes activity was pH 4-5 and maximum activity was obtained at 45°C. Enzyme activity was slightly inhibited by 20-45% in its activity by 20mM of $Zn^{2+}$, $Ca^{2+}$, $Co^{2+}$, $Mg^{2+}$, $Cu^{2+}$, $Mn^{2+}$ and $Fe^{2+}$. The highest β-1,3-glucanase hydrolysis activity was obtained on laminarin due to the similarity on β-glucosidic bonds and followed on pustulan, glucan and cellulose, respectively.*

## INTRODUCTION

*Trichoderma* spp. has long been recognised as an effective biocontrol agent of plant pathogens. The antagonistic mechanisms involve are chemotropism (Chet *et al.* 1981), lectin-mediated recognition (Inbar and Chet 1994) and the formation of trapping and penetrating structures (Elad *et al.* 1983a,b). These processes are enhanced by the secretion of extracellular enzymes such as chitinases, β-glucanases and proteases (De la Cruz *et al.* 1992; Lorito *et al.* 1994; Flores *et al.* 1997).

*Trichoderma harzianum* produces β-glucanases which target the β–glucan chain in fungal cell walls. These enzymes are common in fungi and are classified according to their cleave type of ß-glucosidic linkage and the mechanism of substrate attack. β-1,6-glucan and α-1,3-glucan represent relatively minor components of fungal cell walls (Lora *et al.* 1995). Both β-1,3 and β-1,6-glucanase activities are secreted simultaneously in *T. harzianum* (De la Cruz *et al.* 1993).

Enzymes with ß-1,3-glucanase activity have been reported in fungi, bacteria, actinomycetes and higher plants (Bielecki and Galas 1991). β-1,3-glucanases hydrolyse the *O*-glycosidic linkages of β-1,3-glucan chains by two mechanisms. Exo-β-glucanase hydrolyses the β-glucan chain by sequentially cleaving the glucose residues sequentially from the non-reducing ends, while endo-β-glucanase cleaves β-linkages at random sites along the polysaccharide chain, thus

* Correspondence author:
*muskhazli@science.upm.edu.my*

releasing smaller oligosaccharides (Pitson *et al.* 1993). In most cases, multiple b-glucanases have been found rather than a single enzyme (Vazquez *et al.* 1998).

At least three endo-$\beta$-1,3-glucanases with different molecular weights which are 17kDa, 29kDa and 78kDA have been purified from *T. harzianum* (Mrsa *et al.* 1993; De la Cruz *et al.* 1995; Thrane *et al.* 1997). Purified endo-ß-1,3-glucanase isolated from *T. harzianum* inhibited the germination of encysted zoospores and the elongation of germ tubes of *Pythium ultimum in vitro* (Thane *et al.* 1997).

The role of fungal $\beta$-glucanases depends on their types and the most recognised functions of $\beta$-1,3-glucanases in *Trichoderma* is mycoparasitism (Pitson *et al.* 1993) since it can destroy the polymer fabric of the pathogen cell wall more effectively (Mauch *et al.* 1988). ß-1,3-glucanase also has other several functions such as (i) physiological role in cell wall formation and morphogenetic processes during the growth and differentiation of fungal hyphae (Wong and Maclachlan 1980); (ii) as autolytic enzymes to mobilise ß-glucan under conditions of carbon and energy source exhaustion (Papavizas 1985) and (iii) a nutritional role in saprophytes and mycoparasites (De la Cruz *et al.*, 1995). In this study, we try to purify and characterise the $\beta$-1,3-glucanase of local *T. harzianum* strain.

## MATERIALS AND METHODS

*Strains*

*T. harzianum* BIO 10671 was obtained from the laboratory collection, Department of Biology and grown on Potato Dextrose Agar (PDA) for 4 days at 28°C.

*Extracellular $\beta$-1,3-glucanase Purification*

The spore suspensions of *Trichoderma harzianum* BIO 10671 in approximately $1x10^7$ spores/ml 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 180rpm at 28°C for 24 hours before filtering through sterile Whatman No-1, paper then 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 Whatman no.1 filter paper, centrifuged at 6000xg for 10 minutes then dialysed against distilled water for at least 24 hours at 4°C.

The purification was carried out using anion-exchange method as described by Lima *et al.* (1997). Crude extracellular $\beta$-1,3-glucanase was precipitated with ice-cold 80% acetone and incubated at –20°C for 30 minutes. The precipitate was recovered by centrifugation at 28000xg for 10 minutes at 4°C, re-dissolved in distilled water and dialysed against distilled water for another 24 hours at 4°C.

Ninety microliter (90$\mu$l) of Buffer A (50mM Tris-HCL, pH 7.5) and 20$\mu$l of 1M 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 12000xg for 10 min. Meanwhile, an anion exchange Neobar AQ column was washed with 10 times column volumes of buffer B (50mM Tris-HCL pH 7.5; 1M NaCl) and followed by 10 times column volumes of buffer A. The resulting supernatant was loaded onto the column and eluted at a flow rate of 1ml/min and bound protein was eluted with a 0-1mM NaCl gradient. The fractions with high b-1,3-glucanase activity were pooled before being dialysed against distilled water for 24 hours at 4°C.

Chromatofocusing was performed on a Mono P HR 5/20 column equilibrated with 25 mM Tris-$CH_3COOH$, pH 8. Freeze dried anion exchange samples were dissolved in 1ml of distilled water and adjusted to pH 8.0 by the addition of 25 mM Tris pH 7.5. Following this, centrifugation was done at 1200xg for 10 minutes and then the supernatant was loaded onto the column. Protein were eluted $1ml.min^{-1}$ with a pH gradient from pH 8.0 to pH 5.5 formed by Polybuffer PB 74/96. The fractions were collected and assayed for $\beta$-1,3-glucanase activity. The active fractions were pooled and dialysed against distilled water for 24 hours at 4°C.

The dialysed fraction was collected and assayed for $\beta$-1,3-glucanase activity using the b-glucanase assay method (Somogyi 1952) which involves the estimation of reducing sugars amount released from laminarin. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on collected fraction according to Laemmli (1970) using 10% acrylamide gels and stained with Coomasie R-250 brilliant blue (Sigma). Low molecular mass standard proteins were used for molecular mass determination.

*The Characterisation of Purified β-1,3-glucanase*

In this experiment, β-1,3-glucanase activity was assessed under standard conditions as recommended by Lorito *et al.* (1994) with three replicates.

1. Optimal temperature and temperature stability of β-1,3-glucanase activity

The optimal temperature for β-1,3-glucanase activity was determined by measuring the reducing sugars released after 30 minutes incubation at temperatures between 25°C-75°C at increments of 5°C. Meanwhile, the temperature stability for β-1,3-glucanase activity was examined by maintaining the purified β-1,3-glucanase for 1h at temperatures between 25°C-75°C at increments of 5°C before determining the β-1,3-glucanase activity.

2. Optimal pH and pH stability of β-1,3-glucanase activity

The effect of pH on enzyme activity was determined by varying the pH of the reaction mixture between pH 4-8 at increments of 1 pH unit. The pH of the mixture was adjusted to intended pH with 50mM sodium citrate buffer. pH stability was determined by incubating purified β-1,3-glucanase enzyme at pH 4-8 for 1 hours at 37°C before the pH was changed to pH 5 prior to β-1,3-glucanase activity determination.

3. Effect of metal ions on β-1,3-glucanase activity

The effect of several metal ions on the activity of b-1,3-glucanase was investigated. The metal ions used for this study were $Zn^{2+}$, $Co^{2+}$, $Ca^{2+}$, $Mn^{2+}$, $Mg^{2+}$, $Fe^{2+}$ and $Cu^{2+}$. Twenty millimolar (20mM) of metal ion solution in sodium acetate buffer pH 5.5 was prepared and added into purified β-1,3-glucanase enzyme prior to activity determination.

4. β-1,3-glucanase activity on different substrates

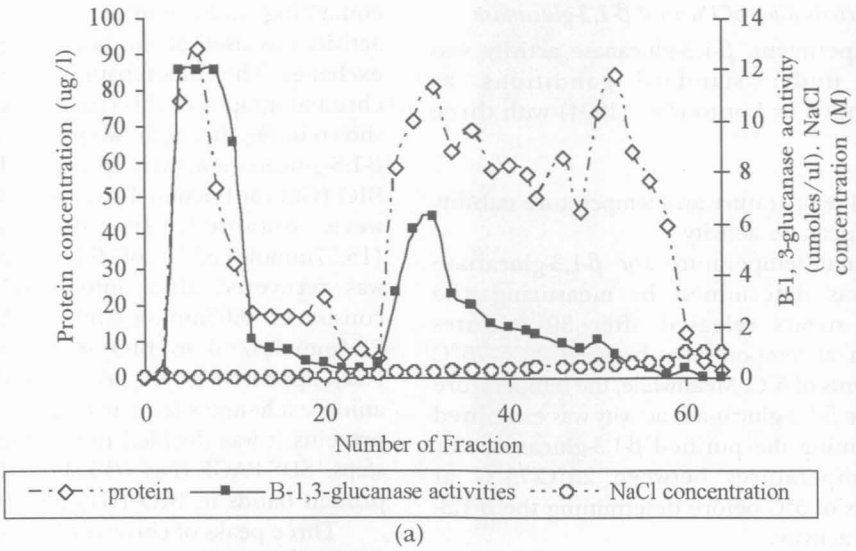
β-1,3-glucanase activity on several substrates was determined. These included pustulan (1.0% w/v), laminarin (1.0% w/v), cellulose (1.0% w/v) and glucan (1.0% w/v). Each substrate was prepared in 0.05M sodium acetate buffer pH 5.5.

## RESULTS AND DISCUSSIONS

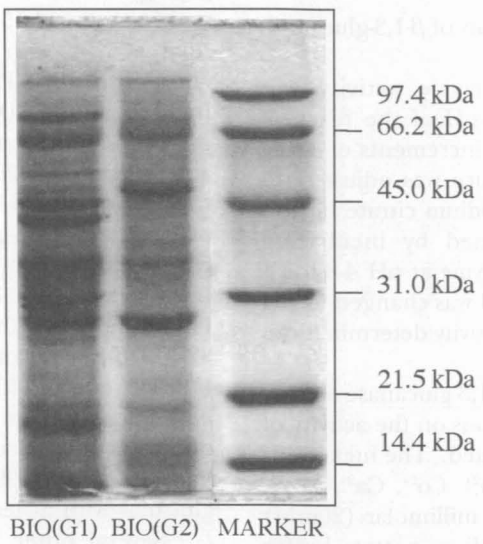
The mixture of β-1,3-glucanase was purified from *T. harzianum* using acetone precipitation. The crude enzyme from *T. harzianum* BIO 10671 containing 23.87mmoles/ml of β-1,3-glucanase activity was used at the beginning of the anion exchange. The elution pattern of anion exchange chromatography of this crude enzyme fraction is shown in *Fig. 1(a)* with two peaks for protein and β-1,3-glucanase activity arbitrarily BIO (G1) and BIO (G2) for fraction 4-10 and 28-36, respectively were obtained. In total, only 69.4% (16.57mmoles.$ml^{-1}$ ) of β-1,3-glucanase activity was recovered after anion exchange, which consist of 9.03mmoles/ml in BIO (G1) and 7.54mmoles/ml in BIO (G2). Since the BIO (G2) appeared to be bound at the middle of anion exchange that may contain contaminated proteins it was decided not to use it in further steps. SDS-PAGE (*Fig. 1(b)*) showed several major protein bands in BIO (G1) and (G2).

Three peaks of chromatofocusing containing β-1,3-glucanase activity were detected (*Fig. 2(a)*) and fractions 11-15 which comprised 60% of the total activity with 30.21μmoles/ml had been chosen for SDS-PAGE. Analysis of this pool fraction using SDS-PAGE revealed two clear protein bands at molecular mass around 66kDa and 32kDa (*Fig. 2b*) suggesting the presence of two isoforms of β-1,3-glucanase. The existence of purified β-1,3-glucanase around 33kDa to 66kDa with isoform has been reported previously. Lorito *et al.* (1994) and Noronha and Ulhoa (1996) described the characterisation of purified 32kDa β-1,3-glucanase, while De la Cruz *et al.* (1995) reported that β-1,3-glucanase from *T. harzianum* with a molecular weight of 66kDa was due to at least 4 sub-unit proteins. Meanwhile Vazquez *et al.* (1998) obtained a 39kDa b-1,3-glucanas with at least six sub unit proteins.

Several other isoform or subunit of β-1,3-glucanase with different catalytic activities, molecular weights and substrate specificities have been found in supernatants from *T. harzianum* culture and the differences in number of isoform or protein subunit for purified β-1,3-glucanase is not new because the molecular mass of β-1,3-glucanase appears to vary between species and also within species (Pitson *et al.* 1993). It is not known whether the existence of isoform for same β-1,3-glucanase molecular weight is the product of the same or separate β-1,3-glucanase gene. According to Mrsa *et al.* (1993), one of the reasons for the differences is the anomalous migration of protein in the gels rather than to the post-translation processing of the polypeptide chain; sometimes the type of growth substrate




(a)

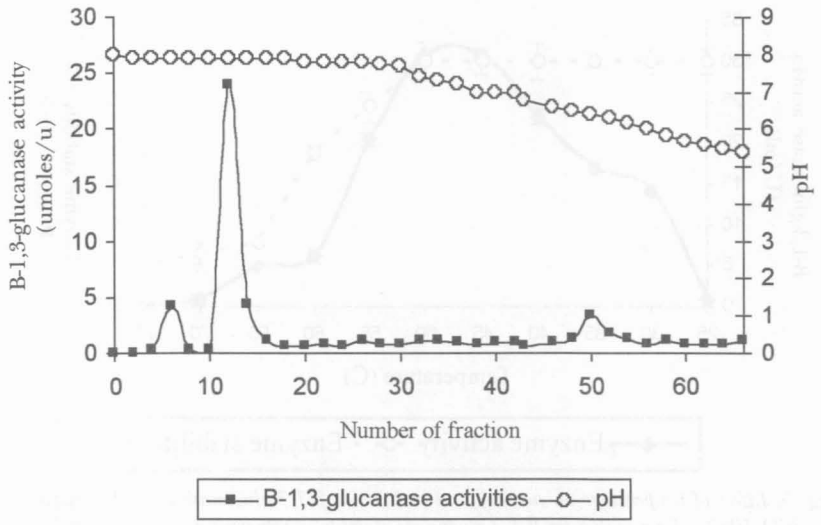



(b)

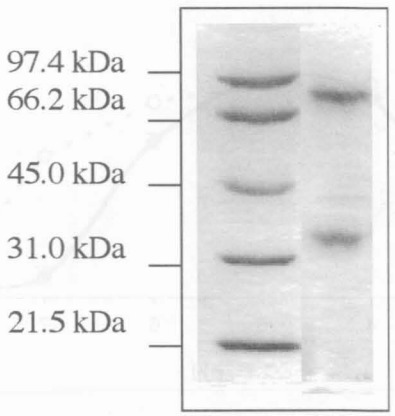
*Fig. 1: Purification of b-glucanases by anion exchange chromatography. Bound protein was eluted with a 0 to 0.5M NaCl gradient (a) Elution profile of BIO 10671 anion exchange for protein on Neobar AQ exchanger column with fraction 4-10 (BIO G1) and 28-36 (BIO G2). (b) SDS-PAGE (10%) of protein from pooled peaks and stained with Coomassie blue*

used can also influence the number of bands on SDS-PAGE (Vazquez *et al.* 1998). Species, type of reaction (exo- or endo-) and method of purification may have an effect on characterisation even for the same type of purified β-glucanase (Matsuzawa *et al.* 1996).

Optimal activity for short term incubation is often seen at temperatures in the range of 30°C to 50°C and many fungal b-glucanases appear stable at temperatures up to 50°C to 60°C (Pitson *et al.* 1993). The effect of temperature on β-1,3-glucanase is shown in *Fig. 3* an optimum operation temperature at 45°C. β-1,3-glucanase activities are not affected by the temperature of incubation up to 50-55°C. In general the temperature optimum and stability for β-1,3-glucanase in this work were lower than those reported previously by Tangarone *et al.* (1989) and Matsuzawa *et al.* (1996) which had optimal temperatures of 55°C and 60°C, respectively.




(a)




(b)

*Fig. 2: Chromatofocusing of BIO 10671 (G1) on mono P HR 5/20 with a 8.5 to 5.5 pH gradient. (a) Elution profile b-1,3-glucanase. (b) SDS-Polyacrylamide (10%) electrophoresis of for b-1,3-glucanase protein from pooled peaks (fraction 11-15) and stained with Coomassie blue*

Very low activity was observed after the temperature exceeded 60°C and this can be concluded that even though *T. harzianum* was categorised as warm climate fungi, some of the enzymes was unable to sustain their function at the maximum level in hot condition (Danielson and Davey 1973). Many of the enzymes have optimum temperature higher than their thermal stability which indicates that they may be stabilised by their substrate (Bodenmann *et al.* 1985).

Optimal pH was determined by varying the pH of the reaction mixture at 37°C. The optimal activity of fungal β-1,3 glucanase usually appears in acidic conditions, often between pH 4.0 to 6.0. Most fungal β-glucanases have a broad pH optima, retaining their activity over 2 to 3 pH units while some have a wider range (Pitson *et al.* 1993). As shown in *Fig. 4*, there is not much difference in activities related to pH with the enzymes being stable over a pH range of approximately 4-6 and most being active at a pH of between 4-5. Theodore and Panda (1995) found that the optimum pH for β-1,3-glucanase production in *T. harzianum* in both surface and submerged culture processes was at an initial pH

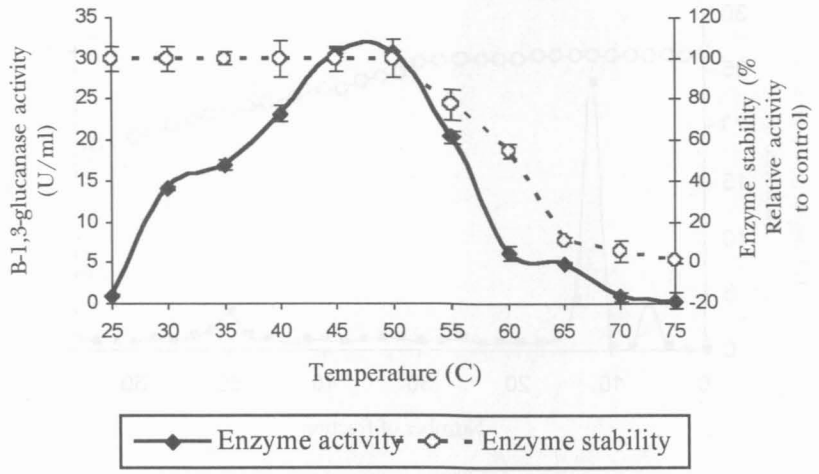



*Fig. 3: Effect of temperature on activity and stability of β-1,3-glucanase in* T. harzianum *BIO 10671. Each value for β-1,3-glucanase stability is represented as a percentage compared to control which is taken as 100%*

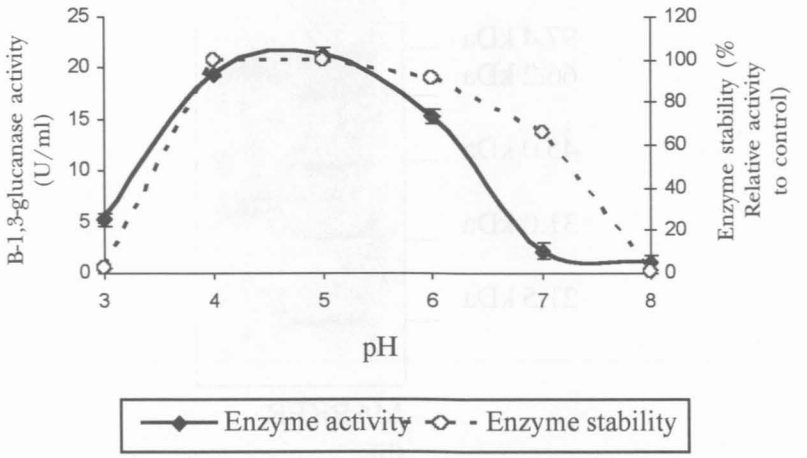



*Fig. 4: Effect of pH on activity and stability of β-1,3-glucanase in* T. harzianum *BIO 10671. Each value for β-1,3-glucanase stability is represented as a percentage compared to control which is taken as 100%*

of 4.7. However, it is considered low optimum compared to that has been observed for *T. harzianum* BIO 10671 in this work. The maximum activity for β-1,3-glucanase observed in the acidic conditions for this work was correlated with the observation on *Trichoderma* growth, where *Trichoderma* spp. appeared to be more prevalent in acidic soils (Gochenaur 1970). The concentration of $H^+$ may have a strong impact on the nutrient transport mechanism and also on the purified enzyme activity (Vazquez *et al.* 1998).

Enzyme activity was slightly inhibited (20-35%) by 1mM of $Co^{2+}$, $Hg^{2+}$, $Cu^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ (Hiura *et al.* 1987) but significantly inhibited in the presence of 1mM $HgCl_2$, $MnCl_2$, $KmnO_4$ (Tangarone *et al.* 1989); 1% (w/v) SDS and 1% (w/v) β-mercaptoethanol (Thrane *et al.* 1997). The effect of several known metal ion inhibitors on the activity of the purified enzymes is shown in Table 1. β-1,3-glucanase activity was inhibited by these metal ions with $Cu^{2+}$ ion in the assay mixture resulting in the highest relative inhibition for β-1,3-glucanase (47.5%). A similar inhibition by $Mg^{2+}$ and $Cu^{2+}$ ions on b-1,3-glucanase activity was also obtained in this work. Metal ions affected the level of β-1,3-glucanase activity by binding to the recognition site in

TABLE 1
Effect of metal ions (20mM) and 1% (w/v) of different substrate on β-1,3-glucanase activity in *T. harzianum* BIO 10671

| | | B-1,3-glucanase activity ($U.ml^{-1}$) ± s.d* | Relative activity (%)** |
|---|---|---|---|
| Metal ions (20mM) | None | 30.70 ± 0.016 | 100 |
| | $Zn^{2+}$ | 25.26 ± 0.610 | 82.3 |
| | $Ca^{2+}$ | 25.45 ± 0.03 | 82.9 |
| | $Co^{2+}$ | 21.61 ± 0.280 | 70.4 |
| | $Mg^{2+}$ | 21.02 ± 0.950 | 68.5 |
| | $Cu^{2+}$ | 16.67 ± 0.010 | 54.3 |
| | $Mn^{2+}$ | 21.76 ± 0.630 | 70.9 |
| | $Fe^{2+}$ | 23.66 ± 0.97 | 77.1 |
| Substrate (1% w/v) | Pustulan | 26.52 ± 0.722 | 86.4 |
| | Laminarin | 30.70 ± 0.610 | 100 |
| | Cellulose | 0.33 ± 0.015 | 2.5 |
| | Glucan | 9.67 ± 0.775 | 31.5 |

*The results are mean values of triplicate tests ± standard error.
**Relative activity (%) is expressed as a percentage compared to control which is taken as 100%.

enzyme for substrate hydrolysis and prevented the activity from reaching the maximum level (Watanabe *et al.* 1988). Therefore, it disturbed the environmental condition, which caused the slowing down of the activity (Widden and Scattolin 1988).

Table 1 also showed the activity detected when different substrates with different linkage bonds were used. Purified β-1,3-glucanase had an ability to hydrolyse all the substrates especially on laminarin because most of the linkages in laminarin are β-1,3-linked glucan and produced a large amounts of glucose (De la Cruz *et al.* 1995). β-1,3-glucanase still can split the linkage for non specific substrates such as pustulan (β-1,6-glucan bonds) and cellulose (β-1,4-glucan bonds) but at a very low level of activity. *T. harzianum* EP-1 appeared to be a typical exo-β-1,3-glucanase but was still able to hydrolyse b-glucans containing β-1,3- and β-1,4-lingkages such as in barley glucan, or β-1,3- and β-1,6-linkage such as yeast glucan (Matsuzawa *et al.* 1996). The highest activity was obtained when the enzyme hydrolysed substrate containing the same β-glucosidic bonds specific for the enzymes and the type of linkage in the substrate influenced the determination of activity. This may due to the fact that the Cys-rich domain of *bgn13.1* (β-1,3-glucanase) may function by interacting with other components of fungal cell walls that are normally not a substrate for β-1,3-glucanase (De la Cruz *et al.* 1995). However, not all purified b-glucanase have this ability as Vazquez *et al.* (1998) found that the purified β-1,3-glucanase can only hydrolyse laminarin.

## ACKNOWLEDGEMENT

The authors are indebted to Universiti Putra Malaysia (UPM) for their technical assistance and Ministry of Science, Technology and Innovation for the research funding under Fundamental Research Program (05-11-02-0060F).

## REFERENCES

BIELECKI, S. and E. GALAS. 1991. Microbial beta-glucanases different from cellulases. *Critical Reviews in Biotechnology* **10:** 275- 304.

BODENMANN, J. 1985. Extracellular enzymes of *Phytophthora infestans* – endocellulase, beta-glucosidase and 1,3-beta-glucanase. *Canadian Journal of Microbiology* **31:** 75-82

CHET, I., G.E. HARMAN and R. BAKER. 1981. *Trichoderma hamatum* - its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. *Microbial Ecology* **7:** 29-38.

DANIELSON, R.M. and C.B. DAVEY. 1973. The abundance of *Trichoderma* propagules and distribution of species in forest soil. *Soil Biology and Biochemistry* **5:** 485-494.

DE LA CRUZ, J., A. HIDALGO-GALLEGO, J.M. LORA, T. BENITEZ, J.A. PINTOR-TARO and A. LLOBELL.

1992. Isolation and characterization of three chitinase in *Trichoderma harzianum. European Journal of Biochemistry* **206:** 859-867.

DE LA CRUZ, J., M. REY, J.M. LORA, A. HIDALGO-GALLEGO, F. DOMINGUEZ, J.A. PINTOR-TORO, A. LLOBELL and T. BENITEZ. 1993. Carbon source control on b-glucanases, chitobiase and chitinase from *Trichoderma harzianum. Archives of Microbiology* **159:** 316-322.

DE LA CRUZ, J., J.A. PINTOR-TARO, T. BENITEZ, A. LLOBELL and L.C. ROMERO. 1995. A novel endo- ß-1,3-glucanase, BGN 13.1, involved in mycoparasitism of *Trichoderma harzianum. Journal of Bacteriology* **177:** 6937-6945.

ELAD, Y., I. CHET, P. BOYLE and Y. HENIS. 1983a. Parasitism of *Trichoderma* spp. *Rhizoctonia solani* and *Sclerotium rolfsii*- Scanning electron microscopy and fluorescence microscopy. *Phytopathology* **73:** 85-88.

ELAD, Y., R. BARAK and I. CHET. 1983b. Possible role of lectins in mycoparasitism. *Journal of Bacteriology* **154:** 1431-1435.

FLORES, A., I. CHET and A. HERRERA-ESTRELLA. 1997. Improved biocontrol activity of *Trichoderma harzianum* strains by overexpression of the proteinase encoding gene *prb*1. *Current Genetic* **31:** 30-37.

GOCHENAUR, S.E. 1970. Soil microflora of Peru. *Mycopathology and Mycologica Application* **14:** 1-4.

HIURA, N., T. NAKAJIMA and K. MATSUDA. 1987. Purification and some properties of an endo-b-1,6-glucanase from *neurospora crassa. Agriculture Biology and Chemistry* **51:** 3315-3321.

INBAR, J. and I. CHET. 1994. A newly isolated lectin from the plant pathogenic fungus *Sclerotium rolfsii*: purification, characterization and role in mycoparasitism. *Microbiology* **140:** 651-657.

LAEMMLI, U.K. 1970. Clavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.

LIMA, H.C., C.J. ULHOA, A.P. FERNANDES and C.R. FELIX. 1997. Purification of a chitinase from *Trichoderma* sp. and its action on *Sclerotium rofsii* and *Rhzioctonia solani* cell walls. *Journal of Genetic Applied Microbiology* **43:** 31-37.

LORA, M., J. DE LA CRUZ, T. BENITEZ, A. LLOBELL and J.A. PINTOR-TORO. 1995. Molecular characterization and heterologous expression of an endo-1,6-b-glucanase gene from the mycoparasitic fungus *Trichoderma harzianum. Molecular and General Genetics* **247:** 639-645.

LORITO, M., C.K. HAYES, A. DI PIETRO, S.L. WOO and G.E. HARMAN. 1994. Purification, characterization, and synergistic activity of a glucan 1,3-b-glucosidase and an N-acetyl-b-glucosaminidase from *Trichoderma harzianum. Phytopathology* **84:** 398-405.

MATSUZAWA, T., Y. AMANO, M. KUBO and T. KANDAT. 1996. Mode of action of exo-b-1,3-glucanase from *Trichoderma pseudokoningii* TM37 on various b-D-glucans. *Journal of the Society for Fermentation and Bioengineering* **74:** 261-267.

MAUCH, F.B., MAUCH-MANI and T. BOLLER. 1988. Antifungal hydrolases in pea tissue: II. Purification and characterization of two chitinase and two $\beta$-1,3-glucanase differentially regulated during development and in response to fungal infection. *Plant Physiology* **87:** 325-333.

MRSA, V., F. KLEBL and W. TANNER. 1993. Purification and characterization of the *Saccharomyces cerevisiae* BGL2 gene product, a cell wall endo-1,3-b-glucanase. *Journal of Bacteriology* **175:** 2102-2106.

NORONHA, C.F. and C.J. ULHOA. 1996. Purification and characterization of an endo-$\beta$-1, 3-glucanase from *Trichoderma harzianum. Canada Journal of Microbiology* **42:** 1039-1044.

PAPAVIZAS, G.C. 1985. *Trichoderma* and *Gliocladium*: Biology, ecology and the potential for biocontrol. *Annual Review of Phytopathology* **23:** 23-54.

PITSON, S.M., R.J. SEVIOR and B.M. MC DOUGAL. 1993. Non-celluloytic fungal $\beta$-glucanase: their physiology and regulation. *Enzyme Microbiology Technology* **15:** 178-192.

SOMOGYI, M. 1952. Notes on sugar determination. *Journal of Biology and Chemistry* **195:** 19-23.

TANGARONE, B., J.C. ROYER and J.P. NAKAS. 1989. Purification and characterization of an endo-(1,3)-b-D-Glucanase from *Trichoderma longibrachiatum. Applied and Environmental Microbiology* **55:** 177-184.

THEODORE, K. and T. PANDA. 1995. Application of response surface methodology to evaluate the influence of temperature and initial pH on the production of β-1,3-glucanase and carboxymethylcellulase from *Trichoderma harzianum*. *Enzyme and Microbial Technology* **17:** 1043-1049.

THRANE, C., A. TRONSMO and D.F. JENSEN. 1997 Endo-1,3-β-glucanase and cellulase from *Trichoderma harzianum*: purification and partial characterization, induction of and biological activity against plant pathogenic *Pythium* spp. *European Journal of Plant Pathology* **103:** 331-344.

VAZQUEZ-GARCIDUENAS, A., C.A. LEAL-MORALES and A. HERRERA-ESTRELLA. 1998. Analysis of the β-1,3-glucanolytic system of the biocontrol agent *Trichoderma harzianum*. *Applied and Environmental Microbiology* **64:** 1442-1446.

WATANABE, R., N. OGASAWARA, H. TANAKA and T. UCHIYAMA. 1988. Effect of fungal lytic enzymes and non-ionic detergents on the actions of some fungicides against *Pyricularia oryzae*. *Agriculture Biological Chemistry* **52:** 895-901.

WIDDEN, P. and V. SCATTOLIN. 1988. Competitive interaction and ecoligical strategies of *Trichoderma* species colonizing spruce litter. *Mycologia* **80:** 795-803.

WONG, Y. and G.A. MACLACHLAN. 1980. β-1,3-glucanase from *Pisum sativum* seedling. *Plant Physiology* **65:** 222-228.

*(Received: 25 February 2005)*
*(Accepted: 21 September 2005)*