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# Purification and properties of an α-(1→3)-glucanase (EC 3.2.1.84) from *Trichoderma harzianum* and its use for reduction of artificial dental plaque accumulation

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**Extracellular α-(1→3)-glucanase (mutanase, EC 3.2.1.84) produced by *Trichoderma harzianum* CCM F-340 was purified to homogeneity by ultrafiltration followed by ion exchange and hydrophobic interaction chromatography, and final chromatofocusing. The enzyme was recovered with an 18.4-fold increase in specific activity and a yield of 4.3%. Some properties of the α-(1→3)-glucanase were investigated. The molecular mass of the enzyme is 67 kDa, as estimated by SDS/PAGE, its isoelectric point 7.1, and the carbohydrate content 3%. The pH and temperature optima are 5.5 and 45°C, respectively. The enzyme is stable over a pH range of 4.5-6.0 and up to 45°C for 1 h. The $K_m$ and $V_{max}$ under standard assay conditions are 0.73 mg/ml and 11.39 x $10^{-2}$ μmol/min/mg protein, respectively. The enzyme activity is stimulated by addition of $Mg^{2+}$ and $Na^+$, and significantly inhibited by $Hg^{2+}$. The α-(1→3)-glucanase preparation preferentially catalyzed the hydrolysis of various streptococcal mutans and fungal α-(1→3)-glucans. The 20-residue N-terminal sequence of the enzyme is identical with those of other α-(1→3)-glucanases from the genus *Trichoderma*, and highly similar to those from other fungi. The purified α-(1→3)-glucanase was effective in preventing artificial dental plaque formation. The easy purification from fermentation broth and high stability, and the effective inhibition of oral biofilm accumulation make this α-(1→3)-glucanase highly useful for industrial and medical application.**

**Key words:** α-(1→3)-glucanase, mutanase, purification, streptococcal film, *Trichoderma harzianum*

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## INTRODUCTION

The dental plaque matrix contains polysaccharides, among which glucans with a large proportion of α-(1→3) linkages have been recognized as virulence factors in the pathogenesis of dental caries (Yamashita *et al.*, 1993). Such glucans have been termed mutans (α-(1→3)-, α-(1→6)-glucans). Mutans are tenacious and water insoluble; in contrast to other matrix components, they are not attacked by enzymes present in the plaque and are synthesized from dietary sucrose by tooth-colonizing streptococci such as *Streptococcus mutans* (Kopec *et al.*, 1997, Wiater *et al.*, 2012). The presence of α-glucans in dental plaque has prompted testing of various preparations of glucanohydrolases as potential anti-plaque agents. In particular, mutanase (α-1→3-glucan 3-glucohydrolase, EC 3.2.1.84) has been tested for its potential as a caries-preventive agent, due to its ability to remove biofilms created by oral bacteria *in vitro* (Wiater *et al.*, 2004) and to reduce plaque formation *in vivo* (Pleszczyńska *et al.*, 2011). Moreover, applications of these enzymes are related to their antifungal effect against phytopathogenic fungi containing α-(1→3)-glucan in their cell wall, like *Fusarium oxysporum* or *Botrytis cinerea* (Calo *et al.*, 2006). Currently, filamentous fungi, mostly *Trichoderma*, are the most extensively studied and reviewed sources of α-(1→3)-glucanases (Sanz *et al.*, 2005; Wiater *et al.*, 2011). It should be noted that some of the α-(1→3)-glucanase preparations tested in caries prophylaxis contained large amounts of protease impurities, which caused adverse local side effects. Therefore, it is important to obtain highly purified α-(1→3)-glucanase preparations that will be safe for oral applications. In this article, we report a procedure for purification of a fungal α-(1→3)-glucanase, providing also some details on its biochemical and catalytic properties. We also show that purified α-(1→3)-glucanase efficiently reduces artificial dental plaque accumulation.

## MATERIALS AND METHODS

**Organisms and culture conditions.** Stock cultures of *T. harzianum* CCM F-340 (Czech Collection of Microorganisms, Brno, Czech Republic) maintained at 4°C on potato dextrose agar slants were used for inoculations. Minimal salts medium A (pH 5.3) as described by Mandels *et al.* (1962) supplemented with 0.4% CWP (cell wall preparation from *Laetiporus sulphureus*), 0.05% proteose peptone, and 0.1% Tween 80 was used for α-(1→3)-glucanase production. The medium (2 l) was autoclaved for 30 min at 117°C in a 5-l glass fermenter (Biostat, Sartorius, Germany) and inoculated with a 10% (v/v) suspension of *T. harzianum* mycelium grown for 48 h in shaken flasks in the same medium but with 0.5% glucose in place of CWP. The fermenter culture was run at 30°C for 3 days at an aeration rate of 1 l air/l/min and with a stirrer speed of 300 rpm. The mycelium was separated by centrifugation (20 min at 17 000×*g*) and the clarified supernatant was used as the starting material for the enzyme purification. The cariogenic streptococci used

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**Abbreviations**: CWP, cell wall preparation from *Laetiporus sulphureus*; DTM, dextranase-pretreated mutan

in this study included *Streptococcus mutans* CAPM 6067 and *S. sobrinus* CAPM 6070 (The Collection of Animal Pathogenic Microorganisms, Brno, Czech Republic); *S. sobrinus* GCM 20381 (The German Collection of Microorganisms, Braunschweig, Germany); and *S. sobrinus/downei* CCUG 21020 (The Culture Collection, University of Göteborg, Göteborg, Sweden). Fruiting bodies of *Pleurotus precoce*, *P. djamor*, *P. citrinopileatus*, *P. eryngii*, *Piptoporus betulinus*, *Laetiporus sulphureus,* and *Hericium erinaceus* were a gift from Prof. M. Siwulski (Department of Vegetable Crops, Poznań University of Life Sciences, Poznań, Poland). *Aspergillus* species: *A. fumigatus* CCM F-614 (Czech Collection of Microorganisms, Brno, Czech Republic), *A. wentii* CIM 449 (Collection of Industrial Microorganisms, Warsaw, Poland), and *A. nidulans* DIM AN1 (Department of Industrial Microbiology, Maria Curie-Skłodowska University, Lublin, Poland) were used as a source of α-(1→3)-glucans.

***L. sulphureus* cell wall preparation (CWP).** The cell wall preparation (CWP) from fruiting bodies of *L. sulphureus* was performed according to the procedure described by Wiater *et al.* (2008). Lyophilized fungal material was milled and the resulting powder was treated with water at 121°C for 1.5 h (×3). The wall material was removed by centrifugation (17 000 × *g* for 30 min) and freeze-dried (cell wall preparation, CWP).

**Enzyme purification.** Crude extracellular α-(1→3)-glucanase was concentrated by ultrafiltration using a Prep/Scale® TFF Cartridge with a 10-kDa cut-off polyethersulfone (PTGC, 0.09 $m^2$) membrane (Millipore Corporation, Billerica, MA, USA) at 15 psi. The retentate was clarified by centrifugation (17 000 × *g*, 30 min at 4°C) and its pH was adjusted to 5.5. Chromatography was performed using Econo-System (Bio-Rad, Richmond, CA, USA). The enzyme solution was loaded on a DEAE-Sepharose column (2.5 × 15 cm) preequilibrated with 0.02 M Tris-HCl buffer (pH 6.8), and proteins were eluted with a 0.0–1.0 M linear gradient of NaCl at a flow rate of 1 ml/min. Fractions showing high α-(1→3)-glucanase activity were pooled and applied to a Butyl-Sepharose column (1.5 × 15 cm) equilibrated with 0.02 M Tris/HCl buffer (pH 6.8) containing 1.25 M $(NH_4)_2SO_4$. The adsorbed enzyme was eluted with 150 ml of a decreasing linear gradient of 1.25–0.0 M $(NH_4)_2SO_4$ in 0.02 M Tris-HCl buffer (pH 6.8) at a flow rate of 1 ml/min. Active α-(1→3)-glucanase fractions obtained were chromatofocused on an Econo-chromatography column (Bio-Rad, 1 × 30 cm, packed to a bed height of 20 cm) of Polybuffer Exchanger PBE-94 equilibrated with 250 ml of 0.02 M imidazole-HCl buffer (pH 7.8). A sample showing α-(1→3)-glucanase activity (5 ml) was injected onto the column and the enzyme was desorbed by elution with 200 ml of Polybuffer 74-HCl (pH 3.4) at a flow rate of 0.5 ml/min. Active fractions were pooled and used for further experiments.

**Analytical methods.** A standard α-(1→3)-glucanase assay mixture contained 0.5 ml of 0.2% (w/v) dextranase-pretreated mutan (DTM) in 0.2 M sodium acetate buffer (pH 5.5) and 0.5 ml of a suitably diluted enzyme solution. After a 1-h incubation at 45°C, the reducing sugars released were quantified by the Somogyi-Nelson method (Nelson, 1944; Somogyi, 1945). Appropriate substrate and enzyme blanks were included to correct for any reducing groups unrelated to the enzymatic hydrolysis of DTM. One unit of α-(1→3)-glucanase activity (U) was defined as the amount of enzyme hydrolyzing DTM to yield reducing sugars equivalent to 1 μmol of glucose/min and expressed as units per ml of culture (U/ml). One unit corresponds to 16.67 nkat. The protein content was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. The carbohydrate content in the purified α-(1→3)-glucanase was determined by the method of Dubois *et al.* (1956) using glucose as a standard.

**Preparation of dextranase-pretreated mutan (DTM).** Dextranase-pretreated mutan (DTM) was prepared (using 50 U of dextranase/mg of native mutan, pH 6.0, 37°C, 3 × 24 h) as a substrate for α-(1→3)-glucanase activity. Native mutan was synthesized from sucrose with the use of a mixture of crude glucosyltransferases of cariogenic *S. sobrinus/downei* CCUG 21020 as described previously (Wiater *et al.*, 2005). Dextranase from *Penicillium* sp. with an enzyme activity of 12.9 U/mg preparation was purchased from Sigma-Aldrich (St. Louis, MO, USA). The linkage structure of the native and the dextranase-pretreated mutan determined by $^1H$ NMR showed that they were mixed-linkage α-(1→3) and α-(1→6) biopolymers with a greater proportion of α-(1→3) than α-(1→6) linkages, namely, 59.1 and 40.9 mol% for native mutan and 79.8 and 20.2 mol% for DTM, respectively.

### Characterization of purified α-(1→3)-glucanase

**Molecular mass determination**. Denaturing sodium dodecyl sulphate gel electrophoresis (SDS/PAGE) was used to check protein purity and molecular mass. Discontinuous SDS/PAGE was prepared according to the method of Laemmli (1970) using a 4% (stacking) and 10% (separating) polyacrylamide gel. Proteins were stained with Coomassie Brilliant Blue. The molecular mass of the α-(1→3)-glucanase was determined by comparing its electrophoretic mobility with those of standard proteins (Thermo Scientific, Rockford, IL, USA).

**Effect of pH and temperature on the activity and stability of α-(1→3)-glucanase.** The influence of pH on α-(1→3)-glucanase activity was examined in a standard assay mixture, except that 0.2 M phosphate-citrate buffers (pH 3.0–8.0) were used instead of acetate buffer. The pH stability was determined in a similar way. Following preincubation of the enzyme solution at various pH values at 25°C for 24 h aliquots were removed and the enzyme activity was assayed by the standard method at the optimum pH of 5.5. The effect of temperature on enzyme activity was analyzed at temperatures ranging from 20 to 60°C in 0.2 M acetate buffer at the optimum pH 5.5 for 60 min. Thermal stability was measured in the same way as the temperature optimization. The enzyme solution was incubated without DTM at different temperatures (from 20 to 60°C) for 1 h under optimum pH conditions (5.5). Then it was cooled and the activity was assayed by the standard method at the optimum pH of 5.5 and 45°C. The relative activity at each pH and temperature value was expressed as the percentage of the maximum activity.

**Effects of metal ions on enzyme activity**. Purified α-(1→3)-glucanase was preincubated for 15 min at 4°C with various metal ions at 1 mM in 0.2 M acetate buffer, pH 5.5, then its activity was measured under the optimum conditions (45°C, pH 5.5, 60 min).

**Substrate specificity**. The activity of the purified α-(1→3)-glucanase toward different substrates (1%, w/v) was determined under optimum assay conditions. Streptococcal α-glucans (mutans) were synthesized from sucrose using cell-free glucosyltransferases of streptococci (*S. sobrinus/downei* CCUG 21020 — mutans DTM, $B_3$ and $D_6$; *S. sobrinus* GCM 20381 — mutan $A_{10}$; *S. sobrinus* CAPM 6070 — mutan $A_{15}$) under different condi-

tions (Wiater *et al.*, 2012). Fungal alkali-soluble α-(1→3)-glucans were prepared according to the method described by Kiho *et al.* (1994). These glucans were isolated from fruiting bodies of *Pleurotus precoce*, *P. djamor*, *P. citrinopileatus*, *P. eryngii*, *Piptoporus betulinus*, *Laetiporus sulphureus* and *Hericium erinaceus*, and from mycelia of three species of *Aspergillus*: *A. fumigatus* CCM F-614, *A. wentii* CIM 449, and *A. nidulans* DIM AN1.

**Enzyme kinetics studies.** The reaction rate was determined at seven different substrate (DTM) concentrations in the range of 0.05 to 1%. The measurements were made at apparent pH and temperature optima in the assay reaction mixtures, and under the conditions outlined above. The kinetic constants ($K_m$, $V_{max}$) for purified α-(1→3)-glucanase were determined by Lineweaver–Burk transformation of data using the SigmaPlot 12 software (SPSS Inc., Chicago, USA).

**N-terminal amino acid sequencing.** For N-terminal amino acid sequencing, the purified α-(1→3)-glucanase was subjected to SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The sequence was determined by the Edman degradation method using the Procise Protein Sequencing System (Applied Biosystems, Foster City, CA, USA).

**Inhibition of artificial dental plaque formation.** To test the influence of α-(1→3)-glucanase on streptococcal biofilm formation, a laboratory system simulating the growth of oral plaque was prepared. The formation of mutans streptococci biofilm was conducted in a CBR 90 CDC Biofilm Reactor (BioSurface Technologies Corp., Bozeman, MT, USA) which allowed obtaining reproducible biofilm samples under reproducible growth conditions. The CDC biofilm reactor consists of a glass vessel with a polyethylene top supporting eight removable polypropylene rods (Fig. 1). Each polypropylene rod holds three removable glass coupons (12.5 mm diameter) on which biofilms can form and is oriented in such a way that the coupon is perpendicular to the rotating baffle. In this reactor, the magnetic stirrer in the center of the vessel provides a continuous flow of nutrients over colonized surfaces (Coenye & Nelis, 2010). The bioreactor with 500 ml of brain-heart infusion medium (BHI) (Baltimore Biological Laboratory, Cockeysville, MD, USA) was autoclaved for 30 min at 117°C. When cooled to room temperature, the rods with the coupons were submerged for 2 h in a jar with sterile artificial saliva (Ledder *et al.*, 2009) for formation of a pellicle, and then placed in the bioreactor. The CDC bioreactor was inoculated with 5 ml of an overnight mixed culture of mutans streptococci (including *Streptococcus mutans* CAPM 6067, *S. sobrinus* CAPM 6070, *S. sobrinus* GCM 20381 and *S. sobrinus/downei* CCUG 21020) cultivated in BHI medium. Biofilms (artificial dental plaque) were grown on the coupons for 3 days at 37°C with stirring (50 rpm). The culture medium (BHI) was replaced every 24 hours, and inoculated with 5 ml of a fresh overnight mixed culture of the mutans streptococci. Three times daily, the rods with the coupons were removed from the culture medium, submerged in BHI with 3% sucrose ("a meal") for 0.5 h, and then washed gently with PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$; pH 7.4) Afterwards, the biofilm-coated coupons were submerged in α-(1→3)-glucanase solution (1 U/ml, test trials) or PBS buffer (vehicle controls) for 3 min. Then, after another washing with PBS buffer, all the rods were again placed in the bioreactor medium. At the end of the experimental period, the biofilm-coated coupons were used to measure the artificial dental plaque level after staining with erythrosine (Wiater *et al.*, 2004).

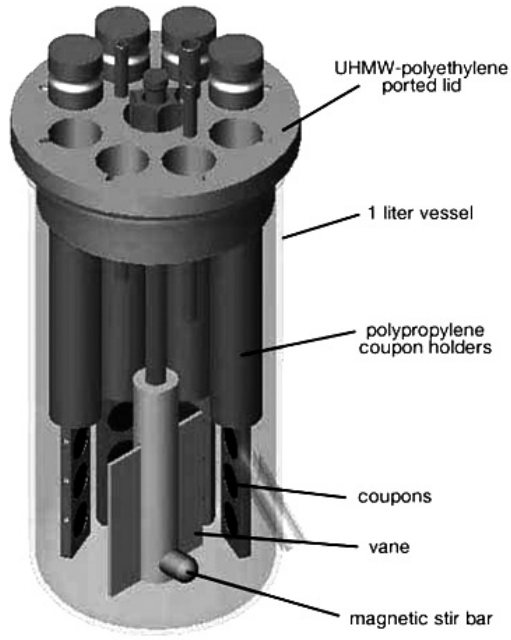


**Figure 1. CBR 90 CDC Biofilm Reactor (BioSurface Technologies Corp., Bozeman, MT, USA; http://www.biofilms.biz).**

## RESULTS AND DISCUSSION

For α-(1→3)-glucanase production on a larger scale, *T. harzianum* CCM F-340 was grown on the optimized medium containing CWP from *L. sulphureus* as the sole carbon source in a fermenter. The enzyme activity in the medium increased with culture time, reached the peak of activity (0.85 U/ml, 6.3 U/mg protein) on the 3rd day, and then declined slowly. Consequently, the α-(1→3)-glucanase purification was carried out from *T. harzianum* cultures grown for 3 days.

α-(1→3)-Glucanase purification was achieved by a combination of concentrations by ultrafiltration followed by chromatographic resolution using ion exchange, hydrophobic interaction, and chromatofocusing. The results of a representative purification are shown in Table 1. The crude enzyme was concentrated by ultrafiltration (10 kDa) and the retentate was subjected to anion exchange chromatography (DEAE-Sepharose) where a

**Table 1. Purification of α-(1→3)-glucanase of *T. harzianum* CCM F-340**

| Purification step | Total protein (mg) | Total activity (U) | Specific activity (U/mg protein) | Yield (%) | Fold purification |
|---|---|---|---|---|---|
| Crude enzyme solution | 339.03 | 2135.28 | 6.29 | 100 | 1 |
| Ultrafiltration (10 kDa) | 297.68 | 2026.60 | 6.80 | 94.90 | 1.08 |
| DEAE-Sepharose | 32.40 | 389.78 | 12.03 | 18.25 | 1.91 |
| Butyl-Sepharose | 6.99 | 199.08 | 28.48 | 9.32 | 4.52 |
| Chromatofocusing (PBE 94) | 0.79 | 91.33 | 115.60 | 4.27 | 18.37 |

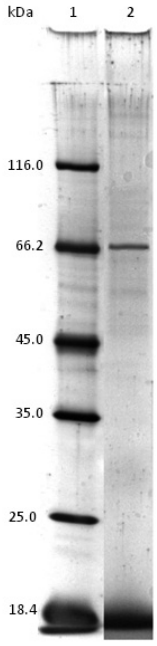


**Figure 2. SDS/PAGE of α-(1→3)-glucanase preparation obtained after chromatofocusing.**
Lane 1, standard molecular mass markers (Thermo Scientific, Rockford, IL, USA); lane 2, purified α-(1→3)-glucanase.

major peak of α-(1→3)-glucanase activity was detected. The enzyme solution obtained in this step was applied to a Butyl-Sepharose column and proteins were eluted by decreasing the salt concentration (1.25 M to 0 ammonium sulfate). During the hydrophobic interaction chromatography, all the α-(1→3)-glucanase activity was bound to the column and eluted as a single peak (0.20–0.25 M $(NH_4)_2SO_4$). The main active fractions were pooled and were further fractionated by chromatofocusing on Polybuffer exchanger PBE 94. The α-(1→3)-glucanase activity was concentrated in one narrow peak that coincided closely with the protein peak. Its isoelectric point (pI) was estimated to be 7.1. The observed pI is in the range obtained for the *T. harzianum* CCM-F470 α-(1→3)-glucanase (pI 7.1) (Wiater *et al.*, 2001) and other enzymes from *T. harzianum* QMZ779 (pI 7.1) (Guggenheim & Haller, 1972), *T. harzianum* CECT2413 (pI 7.5) (Ait-Lahsen *et al.*, 2001), and *T. harzianum* SP234 (pI 6.7–7.5) (Fuglsang *et al.*, 2000). As shown in Table 1, the purification fold of the α-(1→3)-glucanase from *T. harzianum* CCM-F340 was 18.4 times with the recovery yield of 4.3%. The purified α-(1→3)-glucanase showed a high specific activity (about 116 U/mg protein), which was comparable to the specific activities of purified α-(1→3)-glucanase from *T. harzianum* CCM F-470 (108 U/mg protein) (Wiater *et al.*, 2001) and was 2.5-times higher than that reported for the highly purified enzyme from *T. viride* (Hasegawa *et al.*, 1969).

The homogeneity of the α-(1→3)-glucanase preparation obtained from *T. harzianum* was examined and confirmed by SDS/PAGE where the final purified enzymatic preparation appeared as a single protein band of 67 kDa (Fig. 2), which was in the range reported for enzymes isolated from *T. harzianum* CCM-F470 (Wiater *et al.*, 2001), *T. harzianum* CECT2413 (Ait-Lahsen *et al.*, 2001), and *T. asperellum* CECT20539 (Sanz *et al.*, 2005). The carbohydrate content in the purified α-(1→3)-glucanase was 3%, indicating that it is a typical glycoprotein. Kinetic constants ($K_m$, $V_{max}$) for the purified α-(1→3)-glucanase (Table 2) were basically similar to those reported for enzymes isolated from *T. asperellum* CECT20539 (Sanz *et al.*, 2005), Aspergillus nidulans (Zonneveld, 1972), and *T. viride* (Hasegawa *et al.*, 1969).

The effects of pH and temperature on the activity and stability of *T. harzianum* α-(1→3)-glucanase are summarized in Table 2. The optimum pH for the enzyme ac-

**Table 2. Properties of purified α-(1→3)-glucanase**

| Properties | Values |
|---|---|
| Isoelectric point (pI) | 7.1±0.1 |
| Carbohydrate content (%) | 3±0.2 |
| Molecular mass (kDa) | 67±1.7 |
| $K_m$ (mg/ml) | 0.73±0.03 |
| $V_{max}$ (μmol/min/mg protein) | 11.39 x $10^{-2}$±0.25 x $10^{-2}$ |
| pH | |
| - optimum | 5.5 |
| - stability | 3.5–6.0 |
| Temperature (°C) | |
| - optimum | 45 |
| - stability | <45 |

The data are expressed as mean ±S.D. calculated from three independent experiments.

**Table 3. Effect of metal ions on α-(1→3)-glucanase activity**

| Metal | Relative activity (%) |
|---|---|
| None | 100.0±0.4 |
| $Mg^{2+}$ | 110.3±0.9 |
| $Na^{+}$ | 105.3±1.1 |
| $Ba^{2+}$ | 100.4±0.7 |
| $K^{+}$ | 96.0±1.8 |
| $Fe^{2+}$ | 96.0±0.4 |
| $Sn^{2+}$ | 91.7±1.1 |
| $Mn^{2+}$ | 87.3±0.5 |
| $Fe^{3+}$ | 85.2±0.7 |
| $Co^{2+}$ | 81.8±0.8 |
| $Zn^{2+}$ | 79.6±1.5 |
| $Ca^{2+}$ | 69.4±0.4 |
| $Cu^{2+}$ | 68.6±0.8 |
| $Hg^{2+}$ | 0.0±0.0 |

The relative activity is expressed as the percentage of activity in the absence of metal ions. The results are shown as mean ±S.D. of three independent experiments.

**Table 4. Substrate specificity of α-(1→3)-glucanase**

| Substrate | α-(1→3)-glucosidic linkages (mol%) | Relative activity (%) |
|---|---|---|
| Streptococcal mutan: | | |
| DTM | 79.8 | 100.0±1.2 |
| $A_{10}$ | 59.9 | 74.1±1.4 |
| $A_{15}$ | 51.2 | 91.0±0.8 |
| $B_3$ | 60.1 | 76.0±0.6 |
| $D_6$ | 73.4 | 154.8±1.2 |
| Purified α-glucan from mycelium: | | |
| *Aspergillus fumigatus* | 95.4 | 139.5±0.5 |
| *Aspergillus nidulans* | 97.8 | 147.0±0.9 |
| *Aspergillus wentii* | 94.2 | 147.4±2.1 |
| Purified α-glucan from fruiting body: | | |
| *Hericium erinaceus* | 95.5 | 155.7±1.9 |
| *Laetiporus sulphureus* | 91.2 | 107.1±0.7 |
| *Piptoporus betulinus* | 84.6 | 57.7±0.5 |
| *Pleurotus citrinopileatus* | 66.2 | 114.1±1.7 |
| *Pleurotus djamor* | 82.3 | 133.2±1.2 |
| *Pleurotus eryngii* | 60.6 | 73.5±0.8 |

The relative activity of the purified α-(1→3)-glucanase for each substrate is expressed as the percentage of that obtained with DTM. The results are shown as mean ±standard deviation of three independent experiments.

**Table 5. Alignment of N-terminal amino acid sequences of α-(1→3)-glucanase from *T. harzianum* CCM F-340 and other fungi**

| Species | Sequence |
|---|---|
| *T. harzianum* CCM F-340 | 1 ASSADRLVFCHFMIGIVGDR |
| *T. harzianum* CECT 2413 | 39 ASSADRLVFCHFMIGIVGDR |
| *T. atroviridis* ATCC 20476 | 42 ASSADRLVFCHFMIGIVGDR |
| *T. asperellum* CECT 20539 | 42 ASSADRLVFCHFMIGIVGDR |
| *T. asperellum* LST 1 | 42 ASSADRLVFCHFMIGIVGDR |
| *Metarhizium acridum* CQMa 102 | 41 AAAGDRLVFCHFMIGIVGDR |
| *M. anisopliae* ARSEF 23 | 41 AAAGDRLVFCHFMIGIVGDR |
| *Aspergillus fumigatus* Af293 | 36 VSSTDRLVFCHFMIGITSNR |
| *A. fumigatus* A1163 | 36 VSSTDRLVFCHFMIGITSNR |
| *A. nidulans* FGSC A4 | 31 QSSDDRLVFAHFMIGIVSNR |

The sequences were from the following accession numbers: *T. harzianum* CECT 2413 (CAC80493), *T. atroviridis* ATCC 20476 (EHK46766), *T. asperellum* CECT 20539 (CAH04880), *T. asperellum* LST 1 (ABG56439), *Metarhizium acridum* CQMa 102 (EFY89453), *M. anisopliae* ARSEF 23 (EFY96730), *Aspergillus fumigatus* (EAL87492), *A. fumigatus* A1163 (EDP54055), *A. nidulans* FGSC A4 (CBF84404). Numbers indicate the amino acid position in the published sequence. Red letters are used to indicate identity to the *T. harzianum* CCM F-340 α-(1→3)-glucanase sequence.

tivity was pH 5.5, and the enzyme was the most stable over a pH range of 3.5–6.0. The effect of temperature on the α-(1→3)-glucanase activity was investigated over the range of 20–60°C. The temperature optimum for the purified α-(1→3)-glucanase was found to be 45°C. Thermostability studies showed that after a 1-h incubation the enzyme was stable at temperatures up to 45°C. At 50°C, the enzyme lost above 20% of its activity, above 55°C its activity decreased rapidly, and at 60°C it was totally lost. Correspondingly, fungal α-(1→3)-glucanases from *T. harzianum* CCM-F470 (Wiater *et al.*, 2001), *T. harzianum* QMZ779 (Guggenheim & Haller, 1972), *T. harzianum* CECT2413 (Ait-Lahsen *et al.*, 2001), *T. harzianum* SP234 (Fuglsang *et al.*, 2000), *T. reesei* QM6A (Hasegawa *et al.*, 1969), and *T. asperellum* CECT20539 (Sanz *et al.*, 2005) have similar (5.5), lower (3.5–5.0) or higher (6.0) pH optima, and similar temperature optima ranging from 40 to 55°C. Although the pH in the oral cavity is around pH 6-7, the slightly acidic pH profile of the fungal α-(1→3)-glucanase may be of importance in its application for plaque removal, as low pH values have been observed locally in the plaque.

The activity of the purified α-(1→3)-glucanase in the presence of a range of metal ions was assessed (Table 3). Among the various cations, only $Mg^{2+}$ and $Na^{+}$ showed a predominant positive influence on the enzyme activity. The activity was not affected by $Ba^{2+}$, while $Hg^{2+}$ inhibited the enzyme completely. Other tested ions partially inhibited the α-(1→3)-glucanase activity. These results are broadly similar to those reported for α-(1→3)-glucanase purified from *T. viride* (Hasegawa *et al.*, 1969).

The α-(1→3)-glucanolytic activity of *T. harzianum* α-(1→3)-glucanase was tested by a reducing sugar assay on a number of streptococcal and fungal α-glucans (Table 4). α-glucans are a very diverse group of polysaccharides and exhibit a great structural heterogeneity. The α-(1→3)-glucanase effectively degraded all the substrates tested but the relative enzyme activity varied from 58 to 155% (Table 4). Although, on the whole, glucans with a higher content of α-(1→3)-linkages were more susceptible to the enzymatic attack of α-(1→3)-glucanase, it seems that the substrate specificity cannot be directly related to the number of α-(1→3)-bonds. Similar findings were observed for other α-(1→3)-glucanases (Takehara *et al.*, 1981; Pleszczyńska *et al.*, 2012). Based on these results, it can be supposed that the substrate specificity depends not only on the number of α-(1→3)-linkages in the glucan but also on other factors such as spatial arrangement of the linkages in the polymer molecule and accessibility of the α-(1→3) bonds to the enzyme.

A comparison of the N-terminal sequence of α-(1→3)-glucanase from *T. harzianum* CCM F-340 with other known sequences through BLAST search (Table 5) indicated its 100% identity with α-(1→3)-glucanases from the genus Trichoderma and high similarity to that isolated from other fungi such as *Metarhizium acridum* and *M. anisopliae* (85% identity); *Aspergillus fumigatus* Af293, *A. fumigatus* A1163, and *A. nidulans* FGSC A4 (78.9% identity).

In our previous studies, we demonstrated an excellent mutanolytic activity of α-(1→3)-glucanase from *T. harzianum* CCM F-340 towards native mutans in saccharification and solubilization experiments (Wiater *et al.*, 2004; 2008). In this study, the purified α-(1→3)-glucanase was used for reducing artificial dental plaque formation. For this purpose, in simulated oral conditions (CBR 90 CDC bioreactor), artificial dental plaque (mixed streptococcal biofilm) was formed on glass coupons. Oral cavity biofilms were „fed" three times a day by immersing in sucrose solution. Then, after each „meal", the coupons with biofilms were submerged in an α-(1→3)-glucanase solution for 3 min (which simulated everyday proper oral hygiene). The mutan-hydrolysing activity was visualized as a decrease in redness of erythrosine-dyed streptococcal biofilm. As shown in Fig. 3, the use of α-(1→3)-glucanase quite efficiently prevented the accumulation of biofilm on the coupons (nearly 30% reduction). Moreover, the enzyme degraded the biofilm in a relatively brief treatment (3 × 3 min a day). Therefore, the tested α-(1→3)-glucanase could be applied as an active ingredient in toothpaste, mouthwash, dental gel, or chewing

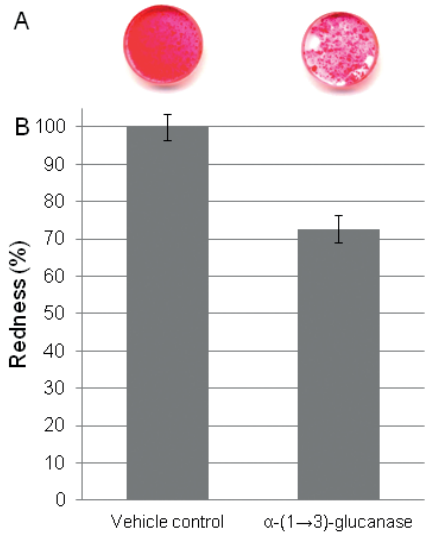


**Figure 3. Effect of purified α-(1→3)-glucanase on the formation of artificial dental plaque.**
(**A**) Biofilm-coated coupons after dyeing with erythrosin. Twelve samples were observed and typical images are presented. (**B**) The reduction of artificial dental plaque accumulation was expressed as a decrease in redness of dyed biofilm. Redness was quantified as optical density at 560 nm and given as the percentage of the maximum attained in the control test. Bars represent standard deviations of three independent experiments.

gum to prevent accumulation of dental plaque, given the fact that an enzymatically damaged biofilm remaining on teeth is easier to remove by application of conventional oral hygiene methods (e.g., brushing).

Summing up, the α-(1→3)-glucanase from *T. harzianum* CCM F-340 has the same general properties as those shown by other fungal α-(1→3)-glucanases, making it a potentially viable enzyme for biotechnological purposes. Moreover, the α-(1→3)-glucanase has the potential to aid the removal of dental plaque and reduce the incidence of dental caries, but further experiments are needed to confirm its clinical effectiveness.

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