

Mechanism of action of the *endo*-(1 → 3)- α -glucanase MutAp from the mycoparasitic fungus *Trichoderma harzianum*

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Abstract (1 → 3)- α -Glucanases catalyze the hydrolysis of fungal cell wall (1 → 3)- α -glucan, and function during cell division of yeasts containing this cell wall component or act in mycoparasitic processes. Here, we characterize the mechanism of action of the (1 → 3)- α -glucanase MutAp from the mycoparasitic fungus *Trichoderma harzianum*. We observed that MutAp releases predominantly β -glucose upon hydrolysis of crystalline (1 → 3)- α -glucan, indicating inversion of the anomeric configuration. After having identified (1 → 3)- α -glucan tetrasaccharide as the minimal substrate for MutAp, we showed that reduced (1 → 3)- α -glucan pentasaccharide is cleaved into a trisaccharide and a reduced disaccharide, demonstrating that MutAp displays *endo*-hydrolytic activity. We propose a model for the catalytic mechanism of MutAp, whereby the enzyme breaks an intrachain glycosidic linkage of (1 → 3)- α -glucan, and then continues its hydrolysis towards the non-reducing end by releasing β -glucose residues in a processive manner.

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1. Introduction

(1 → 3)- α -Glucanases are glycoside hydrolases that catalyze the degradation of (1 → 3)- α -glucan, a water-insoluble polysaccharide in the cell wall of many fungi. Both bacteria [1–5] and fungi [6–16] are known to produce (1 → 3)- α -glucanases, but their respective glucanases are quite distinct in amino acid sequence. Based on amino acid sequence similarities, the fungal (1 → 3)- α -glucanases have been assigned a separate family of glycoside hydrolases, GH-71. Its founding member is the (1 → 3)- α -glucanase MutAp from the fungus *T. harzianum*. This mycoparasite has gained special interest because of its use in the biocontrol of fungal plant pathogens [17,18] and its secretion of a wide range of carbohydrate-active hydrolases, such as (1 → 3)- β -glucanases, (1 → 6)- β -glucanases, chitinases, and cellulases, as well as MutAp [19].

MutAp was first described by Guggenheim and Haller as an enzyme able to hydrolyze mutan, a mainly (1 → 3)- α -glucan-

containing extracellular polysaccharide produced by tooth-colonizing streptococci [14]. Later, Fugsang and colleagues indicated that the carboxyl-terminal domain of MutAp forms a polysaccharide-binding domain, whereas the amino-terminal domain represents the actual catalytic domain [8]. Ait-Lahsen and colleagues characterized the hydrolytic activity of MutAp (denoted AGN13.1 in their report) and showed that glucose is its major hydrolysis product after incubation with crystalline (1 → 3)- α -glucan [20]. Furthermore, they reported that MutAp was unable to hydrolyze periodate-oxidized (1 → 3)- α -glucan, leading them to propose that this enzyme possesses an *exo*-hydrolytic activity [20]. Recently, we characterized another member of the GH-71 family, the (1 → 3)- α -glucanase Agn1p from the fission yeast *Schizosaccharomyces pombe* [21]. Agn1p shares strong amino acid sequence similarities with the catalytic domain of MutAp but lacks a separate polysaccharide-binding domain. Furthermore, it hydrolyzes (1 → 3)- α -glucan predominantly into pentasaccharides, rather than glucose residues [21]. Based on these data, we concluded that Agn1p has *endo*-hydrolytic, rather than *exo*-hydrolytic, activity.

Here, we perform an in-depth analysis into the mechanism of action of MutAp and address the apparently-contradictory results regarding the hydrolytic activities of MutAp and Agn1p. By using crystalline (1 → 3)- α -glucan as well as defined (1 → 3)- α -glucan oligosaccharides as substrates, we show that MutAp exhibits an *endo*-hydrolytic activity. Furthermore, we demonstrate that, after the initial intrachain attack, hydrolysis proceeds in a repetitive manner towards the non-reducing end, releasing β -glucose residues as the major digestion product. To our knowledge, this is the first time a processive mechanism has been ascribed to a GH-71 enzyme.

2. Materials and methods

2.1. MutAp isolation and α -glucan preparations

MutAp was purified as described previously [21] and its identity was reconfirmed by mass spectrometric analysis [21], which detected a total of 11 tryptic peptides that matched the MutAp amino acid sequence [8], providing a coverage of 18%. His-tagged Agn1p and α -glucan were isolated as described [21]. To obtain water-soluble substrate, crystalline α -glucan was partly *O*-(carboxy)methylated as described by Kiho et al. [22], ensuring that the α -glucan was solubilized without affecting enzyme activity. Carboxymethyl groups were hardly observed by nuclear magnetic resonance (NMR) spectroscopy. To lower background signals in the colorimetric enzyme assays (see below), the reducing ends of carboxymethylated α -glucan were reduced with excess sodium borohydride.

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To ensure a complete removal of (1 → 4)-linked residues and a complete oxidation of the terminal residues, crystalline α -glucan preparations were incubated with sodium periodate at 4 °C in the dark over a period of two weeks as described by Grün et al. [23]. Linkage analysis of the reaction product using gas chromatography electron-impact mass spectrometry showed complete removal of 4-substituted glucose residues, indicating complete oxidation (data not shown). Sodium borohydride reduction and mild acid hydrolysis (Smith degradation) were performed as described [23].

2.2. Enzyme assays

Activity of Agn1p-his (at a final concentration of 0.9 μ g/ml) or MutAp (0.4 μ g/ml) on crystalline (1 → 3)- α -glucan (4 mg/ml) was measured in 50 mM sodium acetate, pH 5.6, at 37 °C for 30 min. Subsequently, remaining insoluble (1 → 3)- α -glucan was removed by three subsequent centrifugation steps, each for 3 min at 10000 \times g, and hydrolysis products were analyzed using colorimetric assays with *p*-hydroxybenzoic acid hydrazide (PAHBAH) [24] or glucose oxidase (GOD) [25]. The same assays were used to monitor the ability of MutAp (0.4 μ g/ml) to hydrolyze crystalline (1 → 3)- α -glucan that was oxidized, oxidized and then reduced, or Smith-degraded (4 mg/ml each).

Enzyme assays for high-performance anion-exchange chromatography (HPAEC) analyses were performed in 5 mM sodium azide, 50 mM sodium acetate, pH 5.5, at 37 °C. Samples were desalted using Carbo-graph SPE solid phase extraction columns (Alltech Associates, Deerfield, IL) according to Packer et al. [26].

To test the effects of inhibitors, 5 mM 1-deoxynojirimycin (Fluka 31128), *N*-methyl-1-deoxynojirimycin (Fluka 66570), castanospermine (Sigma C-3784), freshly prepared D-glucono-1,5-lactone (Sigma G-9766), conduritol- β -epoxide (Biomol Research Laboratories, Philadelphia, PA), *N*-(5 adamantane-1-yl-methoxy)pentyl-deoxynojirimycin [27], or buffer was preincubated on ice for 15 min with MutAp (0.2 μ g/ml), glucoamylase (0.5 μ g/ml) (Roche 1202332), or glucocerebrosidase (0.2 μ g/ml) (Genzyme, Cambridge, MA). Remaining MutAp activity was measured by incubation with carboxymethylated, then reduced, (1 → 3)- α -glucan (1 mg/ml) in 50 mM sodium acetate, pH 5.6, at 37 °C for 15 min (final inhibitor concentration of 250 μ M). Remaining glucoamylase activity was measured in 50 mM sodium acetate, pH 5.6, containing 5 mg/ml of starch treated according to Zulkowsky (Fluka 85642), at 37 °C for 15 min (final inhibitor concentration of 250 μ M). Hydrolysis products were analyzed using the PAHBAH colorimetric assay [24]. Remaining glucocerebrosidase activity was measured with 3.7 mM 4-methylumbelliferyl- β -glucopyranoside in 0.25% (w/v) sodium taurocholate, 0.1 M citrate, 0.2 M phosphate buffer, pH 5.2, at 37 °C for 10 min (final inhibitor concentration of 500 μ M). The reaction was stopped by the addition of 0.3 M glycine-NaOH, pH 10.6, and the amount of liberated 4-methylumbelliferone was determined with a LS2 fluorometer (Perkin-Elmer Corp.) at 445 nm wavelength. All enzyme reactions occurred in a linear manner with the amounts of enzyme used and during the indicated time of incubation (data not shown).

2.3. ¹H NMR spectroscopy

Carboxymethylated α -glucan (0.4 mg/ml) was taken up in 10% deuterium oxide, 5 mM sodium azide, 50 mM sodium acetate, pH 5.5, and incubated at 37 °C for 10 min with MutAp (0.4 μ g/ml), as indicated. Mutarotation was accelerated by adding 2.4 M ammonium hydroxide. Spectra were recorded at 500.13 MHz on a DRX500 instrument (Bruker Biospin, Karlsruhe, Germany) at 10 °C. Under these conditions, the proton signal corresponding to water is situated exactly between the two anomeric signals of glucose, and, therefore, suppression of the water signal will not disturb signals corresponding to glucose. Chemical shifts were referred to internal acetone (¹H: 2.225 ppm).

2.4. Preparation of defined (1 → 3)- α -glucan oligosaccharide substrates

One hundred mg of (1 → 3)- α -glucan from fruiting bodies of *Laetiporus sulphureus* were resuspended in 98% (vol/vol) formic acid and heated at 100 °C for 10 min. After evaporation of the acid under a stream of nitrogen, hydrolysis was continued by addition of 0.5 M trifluoroacetic acid and heating at 100 °C for 12 min, after which the clear solution was lyophilized.

Products obtained after acid hydrolysis were separated on a Bio-Gel P4 column (1.6 \times 96 cm, BioRad, Hercules, CA). The column was thermostated at 58 °C. Oligosaccharides were eluted with 5 mM

ammonium bicarbonate at a flow-rate of approximately 5 ml/h and the eluent was monitored using a Bischoff Differential Refractometer (Bischoff Chromatography, Leonberg, Germany). Oligosaccharides were further purified to homogeneity by HPAEC. Fractions were desalted using Carbo-graph SPE solid phase extraction columns (Alltech Associates, Deerfield, IL) according to Packer et al. [26].

2.5. Labeling of reducing ends

Oligosaccharide samples were dissolved in 0.5 M ammonium hydroxide and reduced by adding excess sodium borohydride at 20 °C for 4 h. The excess of borohydride was removed by adding 10% (vol/vol) acetic acid. Boric acid was removed by co-evaporation from methanol under reduced pressure.

2.6. High-performance anion-exchange chromatography

The HPAEC system consisted of a Dionex DX 500 apparatus equipped with a GP 40 gradient pump and an ED 40 electrochemical detector (Dionex Corporation, Sunnyvale, CA). A CarboPac PA1 column (9 \times 250 mm, Dionex Corporation) at a flow rate of 4.0 ml/min was used for semi-preparative purification of (1 → 3)- α -glucan oligosaccharides obtained after partial acid hydrolysis of (1 → 3)- α -glucan and BioGel P4 separation. For analysis of enzymatic hydrolysis products, 30-min linear gradients were applied from 0 to 500 mM sodium acetate in 100 mM sodium hydroxide using an analytical CarboPac PA1 column with dimensions 4.6 \times 250 mm at a flow rate of 1.0 ml/min. Unseparated (1 → 3)- α -glucan oligosaccharides in their native or reduced forms were used as a ladder to calibrate the analytical column.

3. Results and discussion

3.1. MutAp hydrolyzes crystalline (1 → 3)- α -glucan with glucose as the main product

To compare the hydrolysis products of MutAp with those of His-tagged Agn1p, we incubated crystalline (1 → 3)- α -glucan with purified MutAp or Agn1p-his and analyzed the reaction products by using two different colorimetric assays. The assay with *p*-hydroxybenzoic acid hydrazide (PAHBAH) was used to quantify the amount of soluble reducing ends, whereas that with glucose oxidase (GOD) was used to quantify the amount of free glucose. For MutAp, both the PAHBAH and GOD assays gave similar values, indicating that most reducing ends (i.e., more than 80%) are produced in the form of glucose. By contrast, hardly any glucose was released after incubation with Agn1p-his (Fig. 1). These data are in complete agreement with previous data on the actions of MutAp and Agn1p-his [20,21] and demonstrate that, although both enzymes are members of family GH-71, their hydrolysis products are remarkably distinct.

3.2. MutAp hydrolyzes (1 → 3)- α -glycosidic linkages with inversion of anomeric configuration

Enzymatic hydrolysis of carbohydrates involves a nucleophilic substitution reaction at the anomeric carbon and leads to either inversion or retention of the anomeric configuration of the hydrolysis product [28]. To determine the anomeric configuration of the hydrolysis product of MutAp (i.e., glucose), we incubated carboxymethylated (1 → 3)- α -glucan with MutAp and analyzed the product by ¹H NMR spectroscopy. As a reference, the ¹H NMR spectrum of an equilibrium mixture of α -glucopyranose (α -Glc_p) and β -Glc_p was included to assign the signals specific for the anomeric configuration of free glucose (Fig. 2A). The ¹H NMR spectrum of untreated carboxymethylated (1 → 3)- α -glucan showed only signals corresponding to (1 → 3)-linked α -Glc_p residues and signals

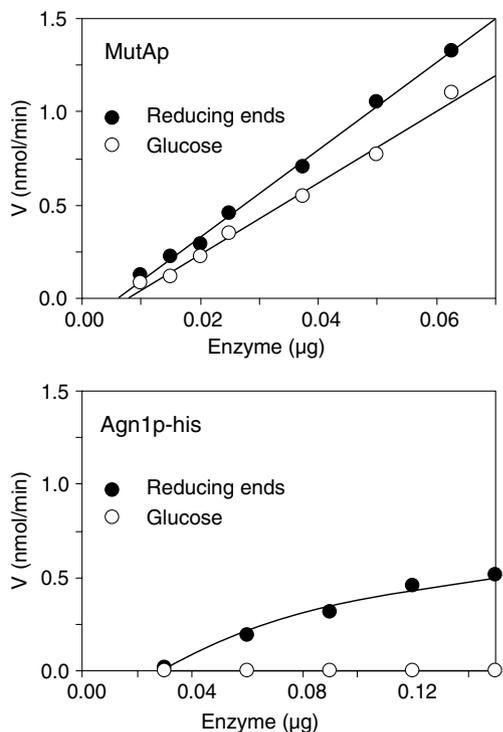


Fig. 1. MutAp hydrolyzes crystalline (1 \rightarrow 3)- α -glucan into glucose residues. Crystalline (1 \rightarrow 3)- α -glucan was incubated with purified MutAp (Upper) or Agn1p-his (Lower), and release of reducing ends was measured by using the PAHBAH assay (closed circles) whereas free glucose residues were measured by using the GOD assay (open circles).

corresponding to free glucose were absent (Fig. 2B). This spectrum was essentially identical to that taken immediately after addition of MutAp (data not shown). However, after a 10-min digestion with MutAp at 37 $^{\circ}\text{C}$, signals corresponding to β -Glc_p (denoted H-1 β) became apparent, whereas α -Glc_p signals remained absent (Fig. 2C). As an internal control, we created an equilibrium of α -Glc_p and β -Glc_p by accelerating mutarotation through addition of ammonium hydroxide and observed the appearance of signals corresponding to α -Glc_p (H-1 α) and a decrease in the β -Glc_p signal (Fig. 2D). These results show that MutAp liberates β -Glc_p during hydrolysis of (1 \rightarrow 3)- α -glucan, demonstrating that MutAp acts by inversion of the anomeric configuration.

3.3. A tetrasaccharide is the minimal substrate for MutAp

To identify its minimal substrate, MutAp was incubated with crystalline (1 \rightarrow 3)- α -glucan. At selected time intervals, samples were taken and analyzed by high-performance anion-exchange chromatography (HPAEC). At $t = 0$, virtually no background peaks were visible, indicating that by our method only hydrolysis products are analyzed (data not shown). The HPAEC profile of the sample taken after a 1-h incubation showed two peaks, corresponding to G1¹ and G3 in the molar ratio of 7.5:1 (Fig. 3A, upper panel). Increasing the incubation period to 16 h led to an increase of these

¹G1, G2, G3, etc. correspond to the degree of polymerization of (1 \rightarrow 3)- α -glucan oligosaccharides; i.e., glucose, nigerose, nigerotriose, etc. Similarly, the suffix -ol indicates the alditol form of these oligosaccharides.

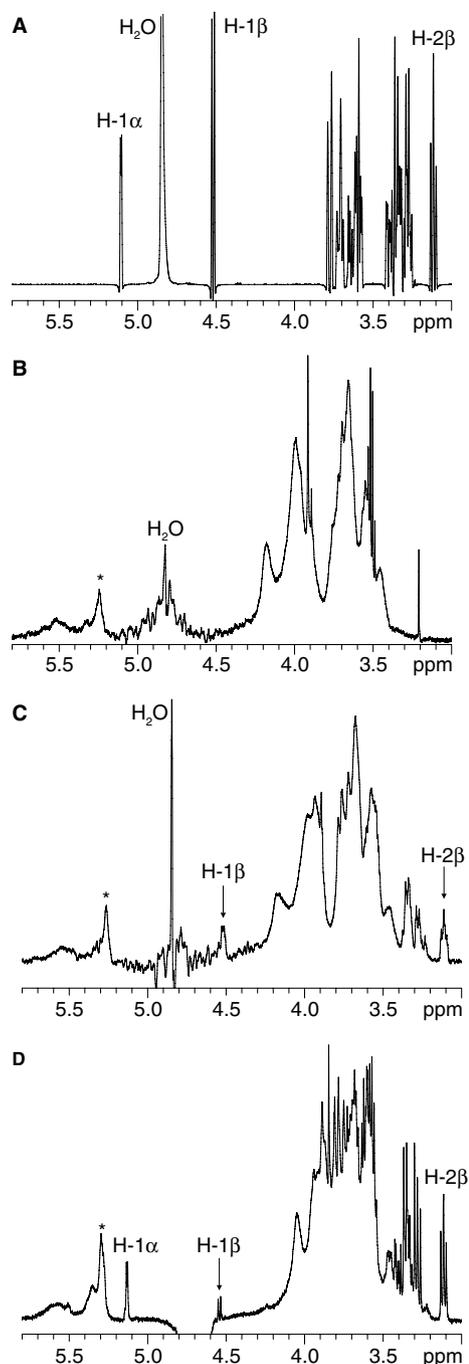


Fig. 2. MutAp cleaves glycosidic linkages with inversion of anomeric configuration. (A) The ¹H NMR spectrum of glucose shows α -anomeric and β -anomeric signals in the ratio of 2:3. (B) Untreated carboxymethylated (1 \rightarrow 3)- α -glucan from *Laetiporus sulphureus*. (C) Reaction mixture after a 10-min incubation of carboxymethylated (1 \rightarrow 3)- α -glucan with MutAp. (D) Same as in C after addition of ammonium hydroxide to accelerate mutarotation. * marks the anomeric signal of the (1 \rightarrow 3)- α -glucan and H₂O marks the water signal.

products but not in a major change in their molar ratio (Fig. 3A, lower panel). In addition, we now observed a small peak corresponding to G2, whereas peaks corresponding to G4 or higher oligosaccharides remained almost absent. Together, these data indicate that G3 is a poor substrate for MutAp, whereas G4 is its minimal substrate.

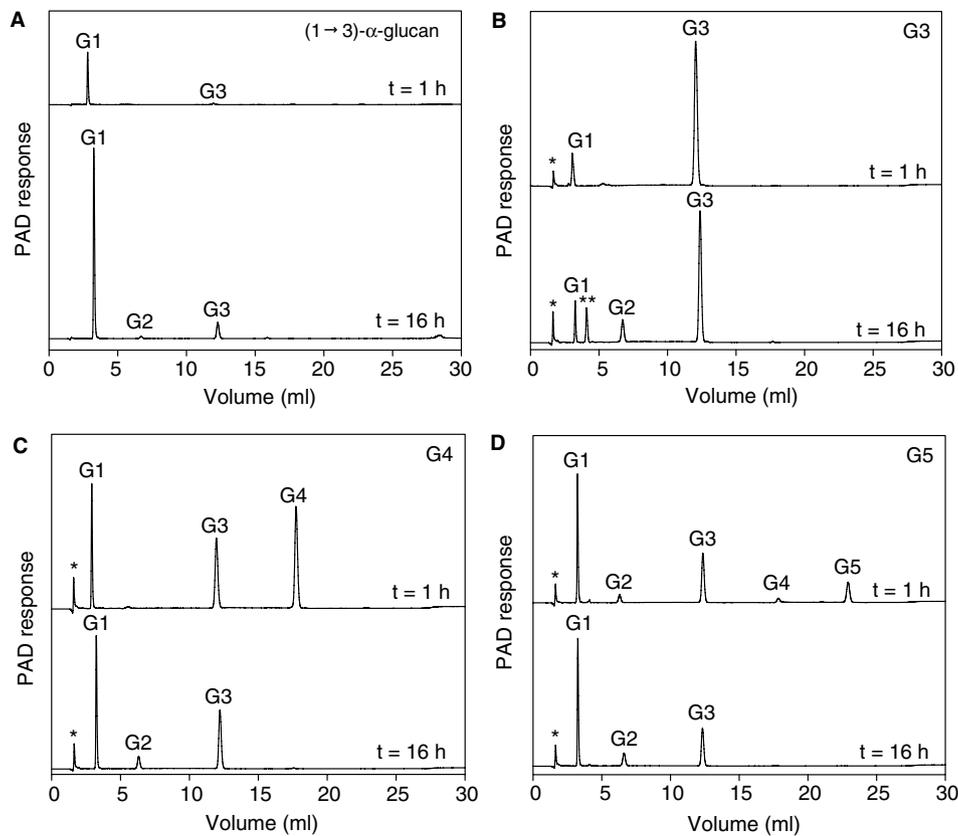


Fig. 3. (1 → 3)- α -Glucan tetrasaccharide is the minimal substrate for MutAp. (A) Incubation of crystalline (1 → 3)- α -glucan with MutAp releases glucose as the main hydrolysis product. (B) G3 is hydrolyzed only slowly by MutAp, whereas (C) G4 and (D) G5 are hydrolyzed readily into G3 and G1. HPAEC analysis of hydrolysis products taken after the specified time intervals. * indicates the injection peak and ** indicates an unknown contaminant.

To confirm these findings more directly, we tested the ability of MutAp to use purified (1 → 3)- α -glucan oligosaccharides as a substrate. Purified disaccharide G2 was not hydrolyzed at all by MutAp (data not shown), while purified G3 was hydrolyzed only poorly to G2 and G1 (Fig. 3B). By contrast, purified G4 was hydrolyzed partially to G3 and G1 after a 1-h incubation, while after 16 h G4 was cleaved completely into the major products G3 and G1, together with only a small amount of G2 (Fig. 3C). Finally, purified G5 was hydrolyzed efficiently after a 1-h incubation to the main hydrolysis products G3 and G1, while a small amount of the intermediate product G4 was also formed, as well as some G2 (Fig. 3D, upper panel). After extending the incubation period to 16 h, G5 and G4 were completely digested (Fig. 3D, lower panel), resulting in a hydrolysis profile that is similar to that of purified G4 (Fig. 3C and D, compare lower panels). From these data, we conclude that MutAp uses G4 efficiently as a minimal substrate.

3.4. MutAp shows *endo*-hydrolytic activity

To determine whether MutAp starts hydrolysis of its substrate (1 → 3)- α -glucan from the non-reducing end (*exo*-hydrolytic activity) or at an intrachain glycosidic linkage (*endo*-hydrolytic activity), we explored three different approaches.

First, we tested MutAp activity in the presence of the *exo*-glycosidase inhibitors deoxynojirimycin, *N*-methyl deoxynojirimycin, castanospermine, D-glucono-1,5-lactone, con-

duritol- β -epoxide, or *N*-(5-adamantane-1-yl-methoxy)pentyl-deoxynojirimycin [27]. None of these inhibitors affected the activity of MutAp (Fig. 4A), whereas the activities of the *exo*-type control glycosidases glucoamylase (Fig. 4B) and glucocerebrosidase (Fig. 4C) strongly decreased under similar reaction conditions. These results are consistent with our earlier finding that these types of inhibitors do not affect the activity of the *endo*-(1 → 3)- α -glucanase Agn1p-his [21], suggesting that MutAp also lacks *exo*-hydrolytic activity.

Second, we studied the ability of MutAp to hydrolyze (1 → 3)- α -glucan, whose non-reducing and reducing ends had been modified to contain aldehyde or hydroxyl groups, by using periodate oxidation or periodate oxidation followed by borohydride reduction, respectively. These treatments should render the (1 → 3)- α -glucan inaccessible to *exo*-type glycosidases, whereas *endo*-type glycosidases should remain active because their internal site of initial attack is located away from the modified ends [12,15]. Periodate oxidation alone reduced MutAp activity to ~40% of control values (Fig. 5A, compare lanes 1 and 4). Rather than the partial inhibition observed here, a previous report described a complete inhibition of MutAp activity following periodate oxidation of (1 → 3)- α -glucan, leading the authors to propose an *exo*-hydrolytic activity [20]. An alternative explanation of the observed decrease in MutAp activity is a potential inactivation and immobilization of MutAp caused by a reaction of the introduced aldehydes with MutAp through primary amines in its amino terminus or 19 lysine residues, forming Schiff bases [29]. Formation of undesirable Schiff bases

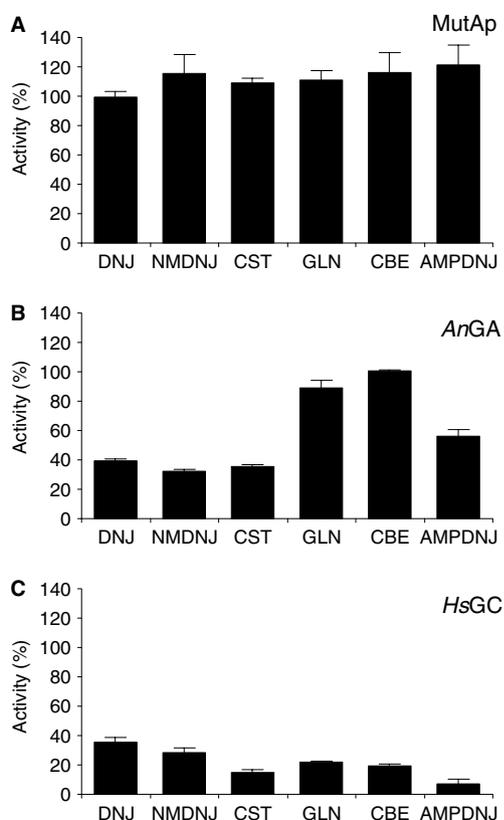


Fig. 4. MutAp activity is not inhibited by *exo*-glycosidase inhibitors. Effects of the *exo*-glycosidase inhibitors 1-deoxynojirimycin (DNJ), *N*-methyl-1-deoxynojirimycin (NMDNJ), castanospermine (CST), *D*-glucono-1,5-lactone (GLN), conduritol- β -epoxide (CBE), or *N*-(5 adamantane-1-yl-methoxy)pentyl-deoxynojirimycin (AMPDNJ) on the activities of (A) MutAp, or the *exo*-glycosidases (B) glucoamylase from *Aspergillus nidulans* (AnGA) or (C) recombinant human glucocerebrosidase (HsGC).

can be prevented by carrying out a borohydride reduction on periodate-treated (1 \rightarrow 3)- α -glucan, converting the aldehyde groups to hydroxyl groups but leaving the ends modified and therefore inaccessible to *exo*-type glycanases. This sequential treatment of (1 \rightarrow 3)- α -glucan with periodate and borohydride did not inhibit enzyme activity at all (Fig. 5A, lane 2), suggesting that MutAp has *endo*-hydrolytic activity. As a positive control, we treated oxidized, then reduced, (1 \rightarrow 3)- α -glucan with acid (Smith degradation) to completely remove the modified ends, and observed control levels of enzyme activity (Fig. 5A, compare lanes 3 and 4).

Third, to analyze the hydrolysis activity of MutAp directly, we labeled purified (1 \rightarrow 3)- α -glucan pentasaccharide at its reducing end by means of reduction, incubated the labeled substrate with MutAp, and analyzed the hydrolysis products. HPAEC analysis showed that G5-ol (in effect a tetrasaccharide due to the ring opening of the reducing residue) eluted as a single peak (data not shown). After a 1-h incubation, hydrolysis products corresponding to G2-ol and G3 were formed (Fig. 5B, upper panel), whereas after 16 h G5-ol was digested completely into G3 and G2-ol, together with some G1 and G2 (Fig. 5B, lower panel). As a control, G4-ol also was purified and incubated with MutAp, and, as expected from our above findings (Fig. 3C), it hydrolyzed slowly and proved a poor substrate for MutAp (data not shown). Together, our

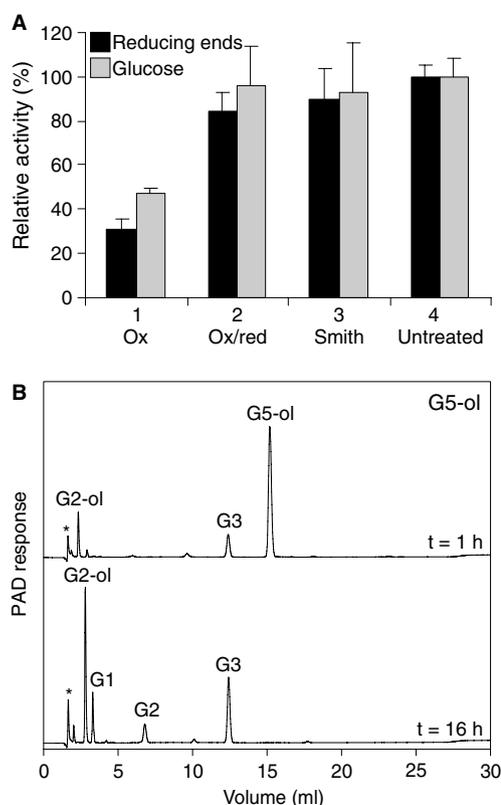


Fig. 5. MutAp possesses *endo*-hydrolytic activity. (A) MutAp enzyme activity is not affected by modification of its ends. Activity levels are the means \pm S.D. of percentages relative to the hydrolysis of untreated (1 \rightarrow 3)- α -glucan in three independent experiments. Substrates are crystalline (1 \rightarrow 3)- α -glucans that were: (1) oxidized; (2) oxidized and then reduced; (3) Smith-degraded; or (4) untreated. The assays are the PAHBAH (black bars) or GOD (grey bars) assays. (B) G5-ol is hydrolyzed readily into G2-ol and G3. Note that no G4-ol is formed. * indicates the injection peak.

results show that G5-ol is hydrolyzed primarily into G3 and G2-ol, demonstrating that MutAp hydrolyzes intrachain glycosidic linkages. Fully consistent with this finding is the absence of G4-ol from the G5-ol hydrolysis products, clearly demonstrating a lack of *exo*-hydrolytic activity. In summary, we conclude that MutAp can be characterized as an *endo*-hydrolytic enzyme.

3.5. Model for the catalytic mechanism of MutAp

Despite its *endo*-hydrolytic mechanism of action, MutAp produces mainly glucose moieties rather than a series of oligosaccharides of different length. This finding suggests that MutAp possesses processivity, the process by which an enzyme remains attached to its substrate after the initial attack, transferring it along its active site (sliding) for multiple rounds of hydrolysis [30,31]. Processivity is common among β -amylases, *exo*-glycosidases which bind (1 \rightarrow 4)- α -glucan and liberate multiple β -maltose units [32]. Also a number of *endo*-glycosidases, such as α -amylases [33–36] and some *endo*-polygalacturonases from *Aspergillus niger* [37–40], display processivity following the initial random intrachain attack. Regarding the processive mechanism of MutAp, we propose a model in which the enzyme first binds to its substrate (Fig. 6, step 1) and hydrolyzes an intrachain glycosidic linkage. Subsequently, the piece with the original reducing end dissociates and is

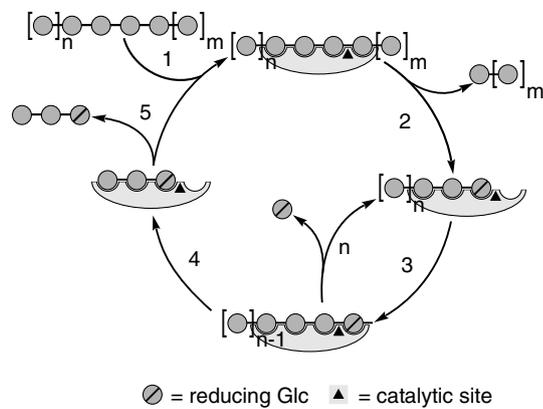


Fig. 6. Proposed catalytic mechanism of the *endo*-(1 → 3)- α -glucanase MutAp from *T. harzianum*. MutAp (1) binds to the substrate, hydrolyzes a random internal glycosidic linkage, and (2) releases a piece of the substrate. The other piece slides across the catalytic center of the enzyme, (3) releasing a β -glucose moiety in every round of hydrolysis. This process continues until (4) only a trisaccharide remains, which (5) is released from the enzyme.

released into the medium, whereas the other piece remains bound to the substrate binding site (Fig. 6, step 2). The retained piece then slides through the catalytic site for repetitive attack (Fig. 6, step 3), releasing a single glucose moiety in each round of hydrolysis. In our model, hydrolysis continues until the substrate is shortened to a trisaccharide (Fig. 6, step 4), which will be released (Fig. 6, step 5). This model is in complete agreement with our results on the digestion pattern of defined (1 → 3)- α -glucan oligosaccharide substrates (Fig. 3). These results showed that in G4 the glycosidic linkage next to the reducing residue is hydrolyzed by MutAp. The same holds for G5 as a substrate, since the amount of G2 formed after a short period of incubation is very small compared to glucose (G1) formation. Also G4 is not released into the medium in large quantities, indicating that the tetrasaccharide is still bound to the enzyme and is hydrolyzed into G1 and G3.

Although MutAp and Agn1p are both *endo*-(1 → 3)- α -glucanases, their physiological roles are quite distinct. MutAp is secreted by a mycoparasitic fungus and its processive mechanism may find its physiological significance in an effective digestion of fungal cell-wall (1 → 3)- α -glucan material, releasing glucose moieties, which may be used as a carbon and energy source. By contrast, Agn1p is secreted during a specific stage in the cell division cycle of a unicellular fungus (a yeast) to hydrolyze a specific region of the mother cell wall overlaying the division septum, called the septum edging, and to facilitate the physical separation of daughter cells [21]. Agn1p lacks processivity and produces oligosaccharides with a pentasaccharide as the main hydrolysis product. This mechanism is consistent with its physiological role of localized cleavage of cell-wall (1 → 3)- α -glucan rather than complete hydrolysis of this structural polysaccharide, which might compromise the structural integrity of the dividing cell. Interestingly, Agn1p lacks a polysaccharide-binding domain, whereas MutAp contains such a domain at its carboxyl terminus. We hypothesize that the polysaccharide-binding domain in MutAp may be involved in processivity, either by partially disrupting the crystalline structure of (1 → 3)- α -glucan and thereby making it more accessible to hydrolysis, or by assisting in retention of (1 → 3)- α -glucan after each round of hydrolysis.

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