Two mannanases from *Sclerotium rolfsii* in total chlorine free bleaching of softwood kraft pulp

G.M. Gübitz*, W. Schnitzhofer, H. Balakrishnan, W. Steiner

*Institute of Biotechnology, SFB Biokatalyse, Graz University of Technology, Petersgasse 12/1, A-8010 Graz, Austria*

Received 2 May 1996; revised 17 June 1996; accepted 18 June 1996

Abstract

The plant pathogenic basidiomycete *Sclerotium rolfsii* produces a wide range of extracellular hemicellulolytic enzymes. To study the effect of β-mannanases in total chlorine free bleaching of softwood pulp, two purified β-mannanases from *S. rolfsii*, with molecular masses of 42 and 61 kDa, a xylanase preparation from *S. rolfsii* and combinations of these were tested in a O(QX)P bleaching sequence (O = oxygen delignification, X = treatment with enzymes, Q = chelation of metals, P = treatment with hydrogen peroxide in alkaline solution). A brightness increase of 1.6 and 1.9% ISO was obtained with the 42 and 61 kDa mannanase and a combination of each of these enzymes with xylanases gave a brightness increase of 2.5 and 2.8% ISO, respectively. The effect of mannanases and xylanases was nearly additive. Both mannanases alone caused a lower decrease of the kappa number as compared to xylanases. The mannanases differed in their ability to release oligosaccharides from different mannans. The 61 kDa mannanase liberated larger fragments and caused rapid depolymerisation of mannans, which seems to promote the bleaching of pulp.

**Keywords:** *Sclerotium rolfsii*; Mannanase; Bleaching; Pulp

1. Introduction

In recent years the application of enzymes in the pulp and paper industry has gained a lot of interest. Pectinases have been used for enzymatic debarking and lipases for resin degradation, solving the pitch problem. Deinking, dewatering and recycling could be enhanced mainly by the action of cellulases, while hemicellulases have shown considerable potential in fiber modification and bleaching boosting of pulps. Also, hemicellulase oxidative enzymes—such as laccase and manganese peroxide—have been successfully applied in bleaching and reduction of color in effluents pulps (Paice et al., 1995; Call and Strittmatter, 1992). Considering the pollution of the environ-
ment and annual world pulp output of 270 million tons the application of enzymes in this field will certainly gain in importance (Kirk and Jeffries, 1996).

Endo-1,4-\(\beta\)-mannanases (\(\beta\)-1,4-D-mannan mannanohydrolase, EC 3.2.1.78) are applied in combination with endo-1,4-\(\beta\)-xylanases (\(\beta\)-1,4-D-xylan xylanohydrolase, EC 3.2.1.8) in the pulp and paper industry to partially breakdown hemicelluloses in softwood pulps. This leads to a significant reduction in the amount of chemicals required for pulp bleaching and/or to an increase in brightness (Viikari et al., 1994). Furthermore, mannanases are used in the fruit juice industry and for coffee extraction (McCleary, 1990; Nicolas et al., 1995; Christgau et al., 1994). Only a few purified mannanases without xylanase activity such as those from Trichoderma reesei are reported to increase significantly the bleachability of softwood pulps (Cuevas et al., 1996). The effect of mannanases on the bleachability of pulps strongly depends on the cooking method and the bleaching sequences used (Suurnakki et al., 1996). Mannanases and xylanases seem to act differently on pulps, a synergism of these enzymes was not yet detected (Buchert et al., 1993).

The plant pathogenic basidiomycete Sclerotium rolfsii produces a wide spectrum of extracellular hemicellulolytic enzymes. Apart from polymer degrading enzymes such as endo-1,4-\(\beta\)-mannanase and endo-1,4-\(\beta\)-xylanase, the formation of several glycosidases from this fungus have been reported in the literature, including \(\beta\)-D-xylosidase, \(\beta\)-D-glucosidase, \(\alpha\)-arabinosidase and \(\alpha\)-D-galactosidase (Haltrich et al., 1994).

Two purified mannanases from S. rolfsii were used separately and in combination with xylanases from S. rolfsii to study the effect of mannanases in total chlorine free (TCF) bleaching of softwood kraft pulp.

### 2. Materials and methods

#### 2.1. Pulp

A softwood kraft pulp produced from 70% spruce, 25% pine and 5% larch was obtained from a paper mill (Zellstoff AG Pöls, Austria). The pulp sample was oxygen prebleached resulting in a kappa number of 19.2 and contained 77.3% glucose, 11.6% xylose, 0.8% arabinose, 5.0% mannoose and 0.7% galactose.

#### 2.2. Enzymes

S. rolfsii was cultivated as described previously (Gübitz and Steiner, 1995). Two mannanases were purified from the culture filtrate from S. rolfsii using ultrafiltration, anion exchange-chromatography and gel filtration as described by Gübitz et al. (1996). The mannanases had molecular masses of 41.9 and 61.2 kDa and showed similar pI values, in the region of 3.2 and 3.5, respectively, and pH-optima around pH 3. The activities of both enzymes reached a maximum at about 70°C. Both enzymes were quite stable at pH 4.5 and 50°C with half-lifes of about 700 min (Table 1). Anion exchange-chromatography was used to obtain a xylanase preparation containing at least three different xylanases.

<table>
<thead>
<tr>
<th>Properties of the purified 42 kDa mannanase and the 61 kDa mannanase from S. rolfsii Gübitz et al., 1996</th>
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<tbody>
<tr>
<td>Properties</td>
</tr>
<tr>
<td>Molecular mass (kDa)</td>
</tr>
<tr>
<td>Isoelectric point pI</td>
</tr>
<tr>
<td>Specific activity</td>
</tr>
<tr>
<td>pH-optimum (5 min, 50°C)</td>
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<tr>
<td>T-optimum (5 min, pH 4.5)</td>
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<tr>
<th>Stabilities (half life in min)</th>
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<tr>
<td>pH 4.5, 80°C</td>
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<tr>
<td>pH 4.5, 60°C</td>
</tr>
<tr>
<td>pH 4.5, 50°C</td>
</tr>
<tr>
<td>pH 3.0, 50°C</td>
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<tr>
<td>pH 6.0, 50°C</td>
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</table>

*Measured with galactomannan from Ceratonia siliqua as the substrate.
2.3. Hydrolysis experiments

Hydrolysis products were analyzed using TLC silica plates (Merck, Darmstadt, Germany) with H₂O, 1-propanol and nitromethane (2:7:1) as the eluent. Monomers and mannoooligosaccharides up to mannohexaose were quantified by HPLC using an Aminex HPX 87P and HPX 87K column from Bio-Rad, operated at 85°C with H₂O and 10 mM K₂HPO₄ as the mobile phase, respectively, and a refraction index detector. Hydrolysis of Amorphophallus konjac glucomannan (Arkopharma, Carros, France), Ceratonia siliqua galactomannan (Sigma, St. Louis, USA), Tsuga canadensis galactoglucomannan (kindly donated from J. Puls, Hamburg, Germany), ivory nut mannan and mannoooligosaccharides (Megazyme, Sydney, Australia) was carried out at pH 4.5 (10 mM KH₂PO₄) and 30°C using 1 g l⁻¹ of the substrate, 2 nkat ml⁻¹ of the enzyme and 10 mg l⁻¹ of thimerosal to prevent microbial growth. To determine the carbohydrate composition acid hydrolysis of the pulp was performed at 30°C for 60 min (72 H₂SO₄) and after dilution to 4% H₂SO₄ at 121°C (autoclave) for another min followed by neutralization with BaCO₃. The amount of solubilized saccharides after enzyme treatment of the pulp was determined using the DNS method and HPLC after acid hydrolysis of the oligosaccharides.

2.4. Enzyme assay

The standard activity of xylanases and mannanases was determined using a 1% solution of birch wood xylan (Roth, Karlsruhe, Germany) and 0.5% solution Ceratonia siliqua galactomannan, respectively, and incubating the diluted enzyme solutions for 5 min at 50°C and at pH 4.5. Released reducing sugars were assayed by adding 2-hydroxy-3,5-dinitrobenzoic acid reagent, boiling for 5 min, cooling and measuring the absorbance at 540 nm (Bailey et al., 1992). Xylanase activity was determined similarly, using a 1% (w/v) solution of xylan. For the application of the enzymes in bleaching of softwood pulp the activity assay was modified. The enzymes were dosed according to their activity under the conditions of the Q-step replacing the pulp by xylan or galactomannan and adding 0.5% EDTA, 0.84 mg l⁻¹ FeSO₄ and 2.2 mg l⁻¹ MnSO₄ for the activity assay.

2.5. Viscosity measurement

A 1% w/v solution of Ceratonia siliqua galactomannan or Amorphophallus konjac glucomannan in 50 mM sodium citrate buffer (pH 4.5) was incubated with 2 nkat ml⁻¹ of the purified enzymes at 30°C. The decrease in the viscosity was monitored using a Rheoscan 115 system with a MS 0-115 cell (Concaves, Zürich, Switzerland) at a constant shear rate of 594 s⁻¹.

2.6. Bleaching procedure

An O(QX)P bleaching sequence was performed (O = oxygen delignification, X = treatment with hemicellulolytic enzymes, Q = chelation of metals, P = treatment with hydrogen peroxide in alkaline solution). Chelation of metals combined with enzyme treatment (300 nkat xylanase g⁻¹ pulp) was carried out at pH 4.5 and 60°C min (5% consistency, 0.5% EDTA). Purified mannanases were dosed to give a final activity of 100 nkat g⁻¹ pulp. After washing, the pulp was further bleached with 3% H₂O₂ in alkaline solution (10% consistency, 2% NaOH, 0.2% MgSO₄) at 80°C for 100 min.

3. Results

3.1. Properties of the purified mannanases

Table 1 presents the molecular properties of the two purified mannanases which behaved differently in enzyme-supported TCF bleaching. Compared to standard assay conditions (50°C, 5 min), higher activities of both mannanases (about 150%) and of the xylanase preparation (about 180%) were measured at 60°C for 60 min. The addition of EDTA, FeSO₄ and MnSO₄ inhibited the two mannanases and the xylanases, resulting in activities of 70 and 80% of those measured without additives, respectively (Fig. 1).
3.2. Hydrolysis of mannans and oligosaccharides

The mannanases (61 and 42 kDa) differed in their ability to release oligosaccharides from different mannans. Both mannanases cleaved ivory nut mannan, *Ceratonia siliqua* galactomannan, *Amorphophallus konjak* glucomannan and galactoglucomannan from *Tsuga canadensis* yielding mannobiose, mannotriose and mixed oligosaccharides while xylan from birch wood, filter paper, carboxymethyl cellulose and p-nitrophenyl-β-D-mannopyranoside were not attacked by these β-mannanases. TLC of the hydrolysis products indicated that in the early stages of hydrolysis the 61 kDa mannanase liberated larger oligosaccharides from these mannans as compared to the 42 kDa mannanase. These findings were quantified for ivory nut mannan by determination of the amount of mannoooligosaccharides up to mannohexaose and the amount of larger fragments liberated by the two mannanases (Fig. 2). After 20 min the 42 kDa mannanase had released twice the amount of oligosaccharides from DP 2 to DP 6 than the 61 kDa mannanase.

Both mannanases caused a rapid decrease of the viscosity of mannan solution when incubated with galactomannan or glucomannan. A plot of the specific fluidity \( \eta^{-1} \) versus the released reducing sugars shows that the slopes of the 61 kDa mannanase for both mannans compared to the slopes of the 42 kDa mannanase are steeper, indicating a more pronounced random type breakdown of the mannans (Fig. 3).

The smallest oligosaccharides hydrolyzed by the 61 kDa mannanase were mannotetraose and α-D-galactosyl-(1-6)-β-D-mannotriose while the 42 kDa mannanase also hydrolyzed mannotriose.

3.3. Bleaching experiments

A brightness increase of 1.6 and 1.9% ISO was achieved with a 42 and 61 kDa mannanase, respectively, when these enzymes were applied in a O(XQ)P bleaching sequence of a softwood kraft pulp (Fig. 4). Without enzyme treatment (blank) a brightness of 55.6% ISO and a kappa number of 12.9 were reached. The brightness obtained with the purified mannanases was significantly higher as compared to the increase of 1.1% ISO effected by the xylanase preparation. Higher dosages than 300 nkat g\(^{-1}\) xylanase and 100 nkat g\(^{-1}\) mannanase did not further increase the brightness of the pulp. Combinations of the 42 and 61 kDa mannanase with the xylanase preparation resulted...
in a nearly additive brightness increase of 2.5 and 2.8% ISO, respectively. When applied alone or with xylanases the 61 kDa mannanase gave a higher brightness increase as compared to the 42 kDa mannanase.

Both mannanases decreased the kappa number only for 44% of the value measured for the xylanase preparation. The 42 kDa mannanase solubilized slightly higher amounts of reducing sugars than the 61 kDa mannanase, whereas the xylanase preparation liberated about twice the amount of reducing sugars than the mannanases. No mannoside was liberated by the xylanases whilst the mannanases did not solubilize xylose (Table 2).

4. Discussion

The mechanism of prebleaching of pulps is still not completely understood although extensive research has been carried out in this field in the last few years. Possible effects of hemicellulases in prebleaching include uncovering of lignin from precipitated xylan, release of chromophore and increase of the porosity. The latter effect promotes the passage of lignin or lignin-carbohydrate molecules with higher molecular masses in subsequent extraction steps (Kantelinen et al., 1993). It has been suggested that xylanases are responsible for the release of carbohydrate lignin complexes with covalent linkages during peroxide pulping (Wong et al., 1996; De Jong et al., 1996). The effect of mannanases in prebleaching strongly depends on the individual enzymes and on the cooking method and bleaching sequence used ( Cuevas et al., 1996; Suurnäkki et al., 1996). Mannanases from Trichoderma reesei increased the bleachability of softwood kraft pulps while these enzymes showed no effect on the brightness of sulfate pulps (Buchert et al., 1995b).

The performance of a great number of xylanases in bleaching of pulps has been described extensively in the literature, whilst only a few reports on the application of purified mannanases exist. Treatment of softwood kraft pulp with two purified mannanases from S. rolfssii improved the bleachability of softwood pulp in peroxide delignification (Fig. 4). A nearly additive increase of the brightness was achieved with a combination of the 42 and 61 kDa mannanases and a xylanase preparation from S. rolfssii. A synergism of xylanases and mannanases was not detected which is in accordance with results reported by Buchert et al. (1993), Lahtinen et al. (1995) and Cuevas et al. (1996).

Both mannanases (42 and 61 kDa) decreased the kappa number less than the xylanase preparation. Xylanases probably release chromophors such as 4-deoxyhex-4-enuronic acid originated from the 4-O-methylglucuronic acid groups from xylan resulting in a decrease of the kappa number (Buchert et al., 1995a). In contrast to observations reported by Saake et al. (1995) for a mannanase from A. niger, none of the mannanases (42 and 61

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**Fig. 2.** Different oligosaccharides liberated from Ivory nut mannan by the 61 (I) and 42 kDa (II) mannanase from S. rolfsii at pH 4.5 and 30°C.
kDa) solubilized xylan of softwood pulp, whereas in agreement with the results for the mannanase from *A. niger* both mannanases from *S. rohsii* solubilized less carbohydrates than the xylanases.

Treatment with 61 kDa mannanase leads to a higher brightness as compared to the 42 kDa mannanase which solubilized slightly higher amounts of carbohydrates. The 61 kDa mannanase also performed better in prebleaching when used in combination with xylanases. Out of the two xylanases from *Streptomyces sp.*, the enzyme with higher molecular mass and lower pi caused a higher brightness increase of kraft softwood and hardwood pulps (Elegir et al., 1995).

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**Fig. 3.** Relationship between the specific fluidity of galacto- and glucomannan and the released reducing sugars during hydrolysis by the 42 and 61 kDa mannanase from *S. rohsii*.

**Fig. 4.** Prebleaching of a softwood kraft pulp with the 42 and 61 kDa mannanase, a xylanase preparation from *S. rohsii* and combinations used in a O[X]P bleaching sequence.
Table 2
Carbohydrates solubilized during treatment of softwood pulp with mannanases and xylanases from *S. rolfsii*<sup>a</sup>

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Red. sugars (g l&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Carbohydrates solubilized&lt;sup&gt;b&lt;/sup&gt; (g l&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mannose</td>
</tr>
<tr>
<td>Xyl</td>
<td>0.26</td>
<td>0</td>
</tr>
<tr>
<td>Man 61 kDa</td>
<td>0.10</td>
<td>0.24</td>
</tr>
<tr>
<td>Man 42 kDa</td>
<td>0.14</td>
<td>0.29</td>
</tr>
<tr>
<td>Xyl+ Man 61 kDa</td>
<td>0.35</td>
<td>0.24</td>
</tr>
<tr>
<td>Xyl+ Man 42 kDa</td>
<td>0.41</td>
<td>0.30</td>
</tr>
</tbody>
</table>

<sup>a</sup>Experimental conditions: 5% consistency, pH 4.5, 60°C, 60 min.<sup>b</sup>Oligosaccharides were hydrolysed to monomers.

Thus, higher molecular masses do not seem to restrict the enzyme action on pulps. The 61 kDa mannanase depolymerized galacto- and gluco-mannans, liberating larger oligosaccharides than the 41 kDa mannanase. Furthermore, this enzyme rapidly decreased the viscosity of mannan solution indicating a more pronounced random type breakdown of the mannans (Esterbauer et al., 1985). These results are consistent with the hypothesis that the effect of mannanases and xylanases in prebleaching results primarily from depolymerization of hemicelluloses but not necessarily from solubilisation of oligosaccharides (Paice et al., 1992).

From the results obtained with the purified mannanases from *S. rolfsii* it can be concluded—in agreement with other reports in the literature—that there is no correlation between the ability of mannanases to release saccharides from purified mannans and their action on pulps (Saake et al., 1995; Buchert et al., 1993). More likely the mode of depolymerisation of mannan by these enzymes seems to be connected with their bleaching effect as it has been shown for the two mannanases from *S. rolfsii*.

References


McCleary, B.V. (1990) Comparison of endolytic hydrolytic hydrolases that depolymerize 1,4-β-D-Mann, 1,5-α-L-arabinan and 1,4-β-D-galactan. ACS Symp. Series, 460, 437–449.


