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G. M. Gübitz · D. Haltrich · B. Latal · W. Steiner

Mode of depolymerisation of hemicellulose by various mannanases and xylanases in relation to their ability to bleach softwood pulp

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Abstract Endo-mannanases and endo-xylanases cleave different heteromannans and xylans yielding mainly dimers and trimers of the corresponding sugars as end-products. However, in the early stages of hydrolysis, four purified mannanases and four xylanases from fungal and bacterial origin, examined in this study, showed a different pattern of released oligomers (determined up to the pentamers). Furthermore, some of these enzymes showed a preference for cleaving the polysaccharides in the middle of the chain while others acted more at the end. When the increase in the specific fluidity of mannan and xylan solutions per reducing sugar released (K_v) was measured against the bleaching effect of the enzymes on softwood kraft pulp, a correlation was found. A xylanase from *Penicillium simplicissimum* ($K_v = 0.15\ l\ mPa^{-1}s^{-1}g^{-1}$) and a mannanase from *Sclerotium rolfsii* ($K_v = 0.12\ l\ mPa^{-1}s^{-1}g^{-1}$) applied in a O(QX)P bleaching sequence (O = oxygen delignification, X = treatment with hemicellulolytic enzymes, Q = chelation of metals, P = treatment with hydrogen peroxide in alkaline solution) gave a high brightness increase of 3.0% and 1.9% ISO respectively. A less significant brightness increase was obtained with enzymes showing lower K_v values, such as a xylanase from *Schizophyllum commune* ($K_v = 0.051\ l\ mPa^{-1}s^{-1}g^{-1}$, 0.2% ISO) and a bacterial mannanase ($K_v = 0.061\ l\ mPa^{-1}s^{-1}g^{-1}$, 0.5% ISO).

G. M. Gübitz (✉) · B. Latal · W. Steiner
Institute of Biotechnology, SFB Biokatalyse,
Graz University of Technology
Petersgasse 12, A-8010 Graz, Austria
Tel.: (+43) 316/873/8408
Fax: (+43) 316/873/8434
e-mail: guebitz@biote.tu-graz.ac.at

D. Haltrich
Institute of Food Technology, University of Agriculture, Vienna
Muthgasse 18, A-1190 Wien, Austria

Introduction

Many investigations have been carried out in the last few years to evaluate the potential of enzymes in the pulp and paper industry. Lipases have shown considerable potential in resin degradation, solving the pitch problem, while pectinases have been used for enzymatic debarking. Treatment of mechanical and kraft pulps with cellulases or preferably with endoglucanases has improved pulp properties such as handsheet density and smoothness, and has resulted in increased pulp freeness (Mansfield et al. 1996).

Hemicellulases, the enzymes responsible for the degradation of wood xylans and mannans in nature (Eriksson et al. 1990), have been used for the modification of pulp fibres and for their bleach-boosting effects on pulps. Biobleaching using endo-1,4-β-mannanases (β-1,4-D-mannan mannanohydrolase, EC 3.2.1.78) in combination with endo-1,4-β-xylanases (β-1,4-D-xylan xylanohydrolase, EC 3.2.1.8) led to a significant reduction in the amount of chemicals required for pulp bleaching and/or to an increase in brightness (Viikari et al. 1994). In addition to hemicellulases, oxidative enzymes, such as laccase and manganese peroxidase, have been successfully applied in pulp bleaching and for the reduction of colour in effluents (Paice et al. 1995; Call and Strittmatter 1992).

The performance of a great number of hemicellulases used in the bleaching of pulps has been described extensively in the literature. Pronounced differences have been reported between the effects of xylanases and mannanases from different organisms on pulp. No correlation was found between carbohydrates released from pulp and the increase of brightness achieved with the enzymes (Cuevas et al. 1996). Neither differences in p*I* values nor those in molecular masses of the enzymes could explain their different performances on pulp (Viikari et al. 1993; Elegir et al. 1995).

In this study the relation between the mode of action of mannanases and xylanases from different microbial

origin on purified xylans and mannans was investigated and also their effect in total-chlorine-free bleaching of softwood kraft pulp.

Materials and methods

Enzymes

Four mannanase and four xylanase preparations from different microorganisms were tested. A mannanase and a xylanase from *Sclerotium rolfsii* (ATCC 200224) and xylanases from *Thermomyces lanuginosus* (DSM 5826), *Schizophyllum commune* (BT 2115) and *Penicillium simplicissimum* (BT 2246) were isolated as described by Gübitz et al. (1996, 1995), Schlacher et al. (1996) and Lischnig (1996). The two latter strains were obtained from the Austrian TU-Graz culture collection. A purified mannanase from *Trichoderma reesei* (ATCC 56765) as well as semipurified mannanase preparations from *Aspergillus sp*. (MF) and *Bacillus amyloliquifaciens* (MB, ATCC 23842) were kindly provided by Genencor International (San Francisco, USA). The molecular parameters of these enzymes are listed in Table 1.

Pulp

Softwood kraft pulp was obtained from the Zellstoff AG (Pöls, Austria) paper mill. The pulp was cooked from 70% spruce, 25% pine and 5% larch, and oxygen prebleached, resulting in a κ number of 19.2. The carbohydrate content of the samples consisted of 77.3% glucose, 11.6% xylose, 0.8% arabinose, 5.0% mannose and 0.7% galactose.

Enzyme assay

Mannanase and xylanase activities were measured according to Bailey et al. (1992) using a 0.5% solution of locust bean galactomannan (Sigma, St. Louis, USA) and a 1% solution of birch wood xylan (Roth, Karlsruhe, Germany) respectively, incubated with the diluted enzyme, solutions for 5 min at 50 °C and pH 4.5. Released reducing sugars were assayed by adding 2-hydroxy-3,5-dinitrobenzoic acid reagent, boiling for 5 min and cooling, and the absorbance was measured at 540 nm. One nanokatal was defined as the amount (nmoles) of xylose and mannose released from xylan and mannan, respectively, in one second. For their application in pulp bleaching, the enzymes were dosed according to their activity under conditions similar to the chelation step, replacing the pulp by xylan or galactomannan. EDTA (0.5%), 0.84 mg l^{-1} $FeSO_4$ and 2.2 mg l^{-1} $MnSO_4$ were added and reducing sugars liberated were measured as described above. Similarly, for the hydrolysis experiments the enzymes were dosed according to their activity at 30 °C.

Hydrolysis experiments

Hydrolysis of locust bean galactomannan (Sigma, St. Louis, USA), ivory nut mannan, wheat arabinoxylan (Megazyme, Sydney, Australia) and birch wood xylan (Roth, Karlsruhe, Germany) was carried out for 90 min at pH 4.5 (50 mM sodium citrate) and 30 °C using 10 g l^{-1} substrate, 2 nkat ml^{-1} enzymes and 10 mg l^{-1} ethylmercurithiosalicylic acid to prevent microbial growth. Monomers and oligosaccharides up to the pentamers (standards from Megazyme) were quantified by HPLC using an Aminex HPX 87P and HPX 87K column (Bio-Rad, Richmond, USA), operated at 85 °C with H_2O and 10 mM K_2HPO_4 as the mobile phase and with a refractive index detector. Acid hydrolysis of the pulp was performed at 30 °C for 60 min (72% H_2SO_4) and after dilution to 4% H_2SO_4 at 121 °C (autoclave) for another 60 min followed by neutralization with $BaCO_3$.

Viscosity measurement

Solutions of 1% w/v locust been galactomannan and wheat arabinoxylan in 50 mM sodium citrate buffer (pH 4.5) were incubated with 2 nkat ml^{-1} purified enzymes at 30 °C. The decrease in the viscosity was monitored using a Rheoscan 115 system with a MS 0–115 cell (Contraves, Zürich, Switzerland) at a constant shear rate of 594 s^{-1}. Depolymerisation (K_v) of hemicellulose by the different enzymes was defined as the increase of the specific fluidity per released reducing sugars (1 $mPa^{-1}s^{-1}g^{-1}$).

Bleaching procedure

Softwood kraft pulp was bleached using an O(QX)P bleaching sequence (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 mannanase or xylanase g^{-1} pulp) was carried out at pH 4.5 and 60 °C for 60 min (5% consistency, 0.5% EDTA). After washing, the pulp was further bleached with 3% H_2O_2 in alkaline solution (10% consistency, 2% NaOH, 0.2% $MgSO_4$) at 80 °C for 100 min. κ number and brightness of handsheets were determined according to the TAPPI standard method T236 cm-85 (1996) and ISO 2469 (1994) respectively.

Results

Hydrolysis of mannans and xylans

Mannans from ivory nut and locust bean as well as xylans from birch wood and wheat were used as model substrate to study the action of mannanases and

Table 1 Molecular properties of the enzymes employed in this study

Enzyme	Molecular mass (kDa)	Isoelectric point	pH optimum	Temperature optimum (°C)
Mannanases				
S. rolfsii	61	3.5	2.9	74
T. reesei[a]	52	4.6	3.5–4	60
MF[a]	42	3.0	4	60–70
MB[a]	35	5.4	5	ND
Xylanases				
P. simplicissimum	35	7.7	5.6	67
T. lanuginosus	26	4.1	6.5	82
S. rolfsii	53	3.8	3.2	72
S. commune	41	5.5	5.9	65

[a] Data from Cuevas et al. (1996)

xylanases respectively. The mannanases from *S. rolfsii*, *T. reesei*, MF and MB all hydrolysed both mannans, yielding mainly oligosaccharides and no mannose, and they did not attack the xylans. However, the enzymes differed in their ability to release oligosaccharides from ivory mannan.

While, in the case of the mannanases from *T. reesei* and MF, the amount of oligomers liberated decreased with their chain length, the mannanases from *S. rolfsii* and MB released similar amounts of the different oligomers (Fig. 1). The mannanase from *T. reesei* released more than twice as much mannobiose from this mannan than did any of the other mannanases tested.

Galactomannan from locust bean was incubated with each of the mannanases and the decrease in viscosity of the mannan solution was followed during hydrolysis. Although equal enzyme dosages were used, the viscosity decreased in a different manner. The mannanase from *S. rolfsii* caused a rapid decrease in viscosity, indicating an endo-type breakdown of the polymer, whereas MF seems to act mainly at the ends of the chains. A steeper slope in the plot of the specific fluidity

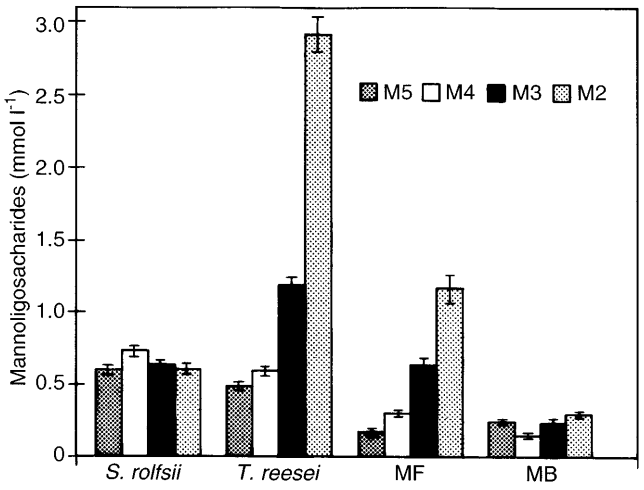

Fig. 1 Mannobiose (*M2*), -triose (*M3*), -tetraose (*M4*) and -pentaose (*M5*) released from ivory nut mannan after 90 min incubation with the mannanases from *S. rolfsii* and *T. reesei*, and semipurified fungal (MF) and bacterial (MB) mannanase preparations

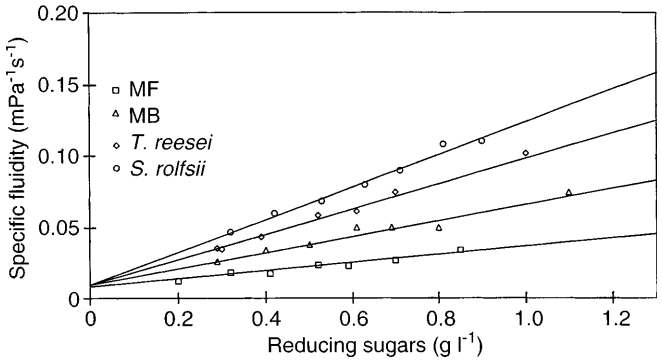

Fig. 2 Depolymerisation of locust bean galactomannan by mannanases from *S. rolfsii* , *T. reesei*, MF and MB

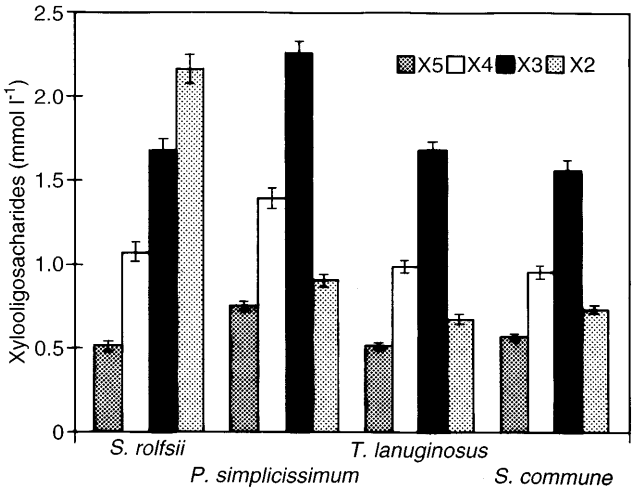

Fig. 3 Xylobiose (*X2*), -triose (*X3*), -tetraose (*X4*) and -pentaose (*X5*) released from birch wood xylan after 90 min incubation with the xylanases from *T. lanuginosus*, *S. rolfsii*, *P. simplicissimum* and *S. commune*

η^{-1} versus the released reducing sugars (Fig. 2) thus attested a more pronounced random type of breakdown of the mannan (Esterbauer et al. 1985). However, these findings did not correlate with the amounts of dimers, trimers, tetramers and pentamers liberated by these enzymes.

The xylanases from *T. lanuginosus*, *P. simplicissimum* and *S. commune* hydrolysed xylan from birchwood, yielding xylotriose as the main product, and liberated only relatively small amounts of xylobiose, showing a very similar pattern of oligomers liberated. However, the xylanase from *S. rolfsii* liberated twice as much xylobiose as did any of the other enzymes. There was no significant amount of xylose released by these enzymes (Fig. 3).

A xylan from wheat, showing a higher viscosity than solutions of birch wood xylan, was chosen to study the action of the xylanases on this polymer. The xylanase of *P. simplicissimum* showed the steepest ascent in a plot of the specific fluidity η^{-1} versus the released reducing sugars, indicating a breakdown starting in the middle of the chain (Fig. 4). Similar to the experiments carried out with mannans and mannanases, no correlation between

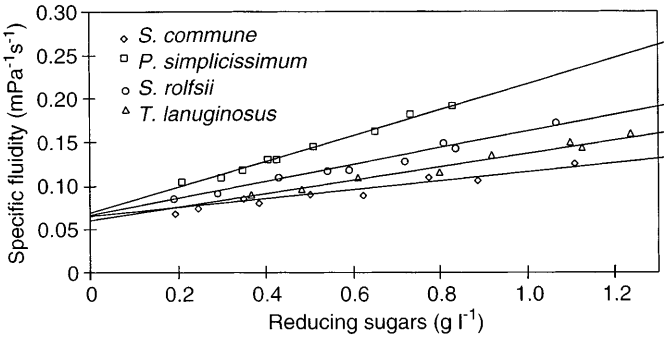

Fig. 4 Depolymerisation of wheat xylan by xylanases from *T. lanuginosus*, *S. rolfsii*, *P. simplicissimum* and *S. commune*

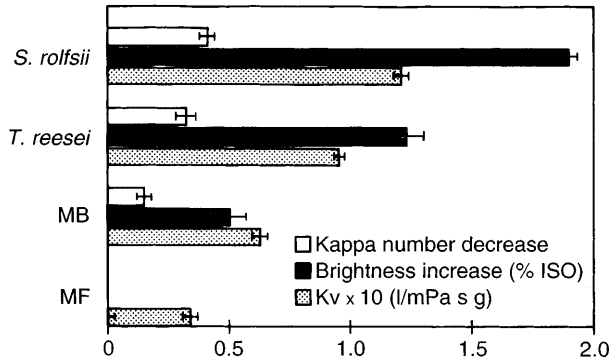

Fig. 5 Relation between brightness increase, κ number decrease of softwood pulp and depolymerisation (K_v) of locust bean galactomannan for mannanases from *S. rolfsii*, *T. reesei*, MF and MB

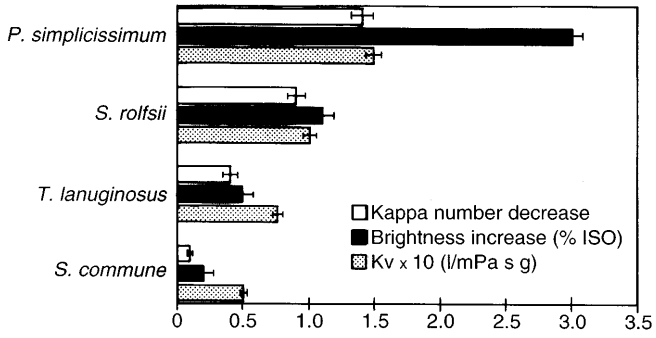

Fig. 6 Relation between brightness increase, κ number decrease of softwood pulp and depolymerisation (K_v) of wheat xylan for xylanases from *T. lanuginosus*, *S. rolfsii*, *P. simplicissimum* and *S. commune*

this effect and the amounts of xylobiose, -triose, -tetraose, and -pentaose released was found.

Bleaching experiments

The highest brightness increases of 1.9% and 3.0% ISO were achieved with the mannanase from *S. rolfsii* and the xylanase from *P. simplicissimum* respectively, when these enzymes were applied in a O(XQ)P bleaching sequence of a softwood kraft pulp (Figs. 5, 6). Without enzyme treatment (blank) a brightness of 55.6% ISO and a κ number of 12.9 was reached. The incubation of the pulp with MF showed a negative effect on the resulting brightness. In general, the decrease of κ number obtained with the mannanases was lower when compared to the decrease seen when the xylanase preparations of *S. rolfsii*, *T. lanuginosus*, and *S. commune* were used.

Discussion

Several explanations for the pulp prebleaching effect of hemicellulases have been discussed in the literature during the least few years. Effects of xylanases and mannanases in prebleaching comprise uncovering lignin from reprecipitated xylan and mannan, release of chromophors and increase of the porosity (Kantelinen et al. 1993; Wong et al. 1996; De Jong et al. 1996). Mannanases and xylanases from a great number of bacteria and fungi were tested in these studies, which showed pronounced different effects on the pulps, although the same activities according to the standard assay were dosed.

Treatment of softwood kraft pulp with purified xylanases from *S. rolfsii*, *S. commune*, *P. simplicissimum*, and *T. lanuginosus* and with mannanases from *S. rolfsii*, *T. reesei* and the bacterial mannanase MB improved the bleachability of softwood pulp in peroxide delignification. This was indicated optically by a pulp brightness increase and by the decrease of the κ number as a measure for the lignin content. Only the fungal mannanase MF decrease the brightness compared to a blank. The highest brightness increases achieved with a xylanase (3% ISO, *P. simplicissimum*) and a mannanase (1.9% ISO, *S. rolfsii*) are in agreement with results from Buchert et al. (1993) and Cuevas et al. (1996), though pulps and/or bleaching conditions were not the same in those studies.

Purified xylans and mannans were used as model substrates to find any characteristics of the individual hemicellulases that could be of importance with regard to their effect in prebleaching of softwood pulp. All tested mannanases and xylanases liberated oligosaccharides from ivory nut mannan and from birch wood xylan respectively. Despite the fact that individual enzymes showed a different pattern of oligosaccharides released, there was no relation between these characteristics and the prebleaching effect. Neither of the patterns of oligomers liberated from softwood pulp by the mannanases from *T. reesei*, MF and MB correlated with the brightness obtained in bleaching experiments (Cuevas et al. 1996). In agreement with these results, Clark et al. (1991) found, for both mannanases and xylanases, that there was no relation between the extent of hemicellulose removal from radiata pine kraft pulp and improved bleachability.

The mannanases and xylanases investigated hydrolysed locust bean galactomannan and wheat xylan, respectively, in a different manner, acting, either more in an endo or in an exo fashion. Depolymerisation was quantified as the increase of the specific fluidity per released reducing sugars (K_v). A correlation between K_v and the effect of the enzymes in the bleaching of softwood kraft pulp was found (Figs. 5, 6). A xylanase from *P. simplicissimum* (K_v = 0.15 l $mPa^{-1}s^{-1}g^{-1}$) and a mannanase from *S. rolfsii* (K_v = 0.12 l $mPa^{-1}s^{-1}g^{-1}$) effected a high brightness increase of 3.0% and 1.9% ISO respectively. A low brightness increase was obtained only when enzymes showing lower K_v values, such as a xylanase from *S. commune* (K_v = 0.05 $mPa^{-1}s^{-1}g^{-1}$, 0.2% ISO) and a bacterial mannanase (K_v = 0.06 l $mPa^{-1}s^{-1}g^{-1}$, 0.5% ISO), were studied. These results are consistent with the hypothesis that the effect of mannanases and xylanases in prebleaching results primarily

from depolymerisation of hemicellulose but not necessarily from solubilisation of oligosaccharides (Paice et al. 1992).

Besides the specific mode of action of the individual enzymes on the pulp, their molecular mass might also be an important factor, considering the limited accessibility of the hemicellulose in the fibre matrix. In this study, almost a contrary effect was found for mannanases. Out of the enzymes tested, those mannanases with higher molecular masses from *S. rolfsii* (61 kDa) and *T. reesei* (52 kDa) showed a more pronounced effect on the increase of pulp brightness. Between xylanases with molecular masses in the range from 26 kDa (*T. lanuginosus*) to 53 kDa (*S. rolfsii*), no tendency was found with regard to their effect on the pulp. In agreement with these results, no correlation between hemicellulases with molecular masses ranging from 20 kDa to 50 kDa and the achieved degree of hydrolysis and bleachability was found by Viikari et al. (1993). Out of two xylanases from *Streptomyces* sp., the enzyme with higher molecular mass and lower p*I* caused a higher brightness increase of kraft softwood and hardwood pulps (Elegir et al. 1995). Mannanases from *T. reesei* and *B. subtilis* had different effects when applied in a peroxide bleaching sequence of softwood pulp, which could not be explained by differences in molecular mass or isoelectric points (Buchert et al. 1993).

Concluding the results of this study, neither the molecular mass of mannanases and xylanases nor the amount of oligosaccharides they released from hemicellulose seemed to be related to their prebleaching effect. On the other hand, the mode of depolymerisation of hemicellulose caused by the different enzymes, simply measured as the increase of fluidity, correlated well with the resulting brightness increase.

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