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Purification and characterization of *Aspergillus* β-D-galactanases acting on β-1,4- and β-1,3/6-linked arabinogalactans

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Abstract

Arabinogalactan and arabinan fractions were isolated from kraft pulping black liquor. Both type I and type II arabinogalactans consisting of 1,4- and 1,3-linked β-D-galactose backbones, respectively, were found. Samples contained more arabino-1,3/6-galactan than arabino-1,4-galactan. Arabinan was mainly 1,5-linked slightly branched polysaccharide. Two enzymes acting on galactans, an *endo*-β-1,4-D-galactanase and a β-1,6-D-galactanase, were isolated from commercial pectinase preparations produced by *Aspergillus aculeatus* and *A. niger*, respectively. The purified enzymes showed molecular masses of 38 and 58 kDa, respectively. Based on its N-terminal amino acid sequence the *endo*-β-1,4-D-galactanase was the same as the previously studied GAL1 from *A. aculeatus*. It acted on β-1,4-linked galactan, producing a range of galacto-oligosaccharides. It was also able to liberate galactose from a lignin–carbohydrate complex isolated from softwood kraft pulp. No activity was detected towards β-1,3-liked galactan. The β-1,6-D-galactanase was active on arabino-1,3/6-galactan, liberating galactose and 1,6-β-D-galactobiose. It was found to be active only on β-1,6-linkages and no detectable hydrolysis of β-1,3-galactose linkages occurred. It also showed no activity on 1,4-β-D-galactan. However, β-1,6-D-galactanase was able to liberate arabinose from arabinan. Although chemical pulps contain only a minute quantity of galactans, both galactanases have recently been shown to enhance the bleachability of spruce kraft pulp.

Keywords: Galactan; Kraft pulp; Black liquor; *endo*-β-1,4-galactanase; β-1,6-galactanase; *Aspergillus*

1. Introduction

Galactans and arabinogalactans are polysaccharides which are widely distributed in higher plants. Arabinogalactans can be grouped into three main structural types. Type I arabinogalactans are composed of a linear β-1,4-linked D-galactopyranose backbone, which is substituted mainly at C-6 with α-L-arabinofuranoside side groups and 1,5-linked side-chains of varying degrees of polymerization, and in some cases also with β-D-galactopyranose. Polymers of this type are present in pectic complexes e.g. in seeds, bulbs and leaves. Arabino-1,3/6-β-galactans (type II) exist in various plant tissues, including e.g. coniferous woods (especially larch), gums, saps and exudates of angiosperms, seeds, roots etc. They are highly branched polysaccharides composed of a β-1,3-linked galactopyranose backbone, which is frequently substituted at C-6. The third, somewhat distinct group is formed by the cell wall glycoproteins containing arabinose and galactose (Aspinall, 1980; Clarke, Anderson, & Stone, 1979; Timell, 1967).

The structures of arabinogalactans isolated from various larch species, in which arabinogalactans are the major hemicelluloses, have extensively been studied by several groups (Bouveng & Lindberg, 1956; Jiang & Timell, 1972; Karácsonyi, Kovácik, Alfäldi, & Kubackova, 1984; Manley-Harris, 1997; Ponder & Richards, 1997). According to these studies, larchwood arabinogalactan contains a highly branched β-1,3-galactan backbone. The branches consist of single 1,6-linked

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β-D-galactopyranosyl or α-L-arabinofuranosyl residues or of two to three 1,6-linked β-D-galactopyranosyl residues. In addition, 1,3-linked β-D-arabinopyranosyl residues have been found in the terminal position of the side-chains. After partial acid hydrolysis of tamarack (*Larix laricina*) compression wood, a series of β-1,4-linked galacto-oligosaccharides, slightly branched at C-6, was also observed (Jiang & Timell, 1972).

In other wood species galactans comprise only up to few pecent of the total wood. Galactans exist mainly in connection with pectin (polygalacturonic acid). Thus young fibres, which are composed of middle lamella and a primary wall only, contain a large proportion of galactan (Meier, 1959). Galactans are also found in larger amounts in reaction wood, where the primary cell wall is thicker than in normal wood. The structure of galactans is heterogenous, varying within different species and tissues. Most studies report 1,3/6-linked type II arabinogalactans isolated from various pine species (Aspinall & Wood, 1963; Timell, 1967). Arabinogalactan-proteins, in which the carbohydrate moiety comprises type II arabinogalactan, have been isolated from *Picea abies* L. Karst (Karácsonyi, Pätoprstý, & Kubacková, 1998). Recently Willför, Sjöholm, and Holmbom (1999) and Willför, Sjöholm, Laine, and Holmbom (2002) reported structures of water soluble type II arabino-1,3/6-galactans from Norway spruce and Scots pine heartwood. These arabinogalactans also contained some glucuronic acid side groups, their amount being higher in spruce arabinogalactan than in pine. Galactan associated with highly lignified parts of the cell wall has been reported to have a linear 1,4-linked structure (Minor, 1991). β-1,4-Linked galactose residues have been found to exist in Norway spruce compression wood (*Picea abies* Karst.) (Bouveng & Meier, 1959) and in white birch (*Betula papyrifera*) (Gillham & Timell, 1958). Both β-1,4- and β-1,3/6-linked galactans have been found in pine kraft cooking liquors (Vilkkula, Hortling, Tamminen, Teleman, Tenkanen, & Vuorinen, 1997) and in oxygen delignification filtrates (Laine & Tamminen, 2002).

In chemical pulp fibres galactans are enriched on the outer cell wall layers and they have been suggested to be chemically linked to lignin (Engström, Vilkkula, Teleman, & Vuorinen, 1995; Hortling, Tamminen, & Pekkala, 2001; Hortling, Tamminen, & Ranua, 1998; Hortling, Tamminen, & Turunen, 1997; Laine, Haakana, Hortling, & Tamminen, 1999; Tamminen, Vuorinen, Tenkanen, Hausalo, & Hortling, 1995; Vilkkula et al., 1997). These lignin–carbohydrate complexes (LCC) are assumed to be difficult to remove during delignification of pulp, and galactans have indeed been found to be enriched in residual lignins after chemical cooking of wood fibres (Hortling et al., 2001; Hortling et al., 1998; Laine et al., 1999; Minor, 1991). The galactans in the residual LCC (RLCC) are of both types, a mostly linear β-1,4-linked galactan and a β-1,3-linked linear and branched (C-6) galactan. In the RLCC from the fibre surface, β-1,4-linked galactan is the major galactan (Laine et al. 1999; Minor, 1991).

Enzymes randomly degrading the backbone of galactans have been classified into two groups: *endo*-β-1,4-D-galactanases (EC 3.2.1.89) are specific for β-1,4-D-galactopyranosyl linkages, whereas *endo*-β-1,3-D-galactanases degrade 1,3-linked β-D-galactans (Dekker & Richards, 1976). Galactanases are known to be produced by bacteria, fungi and plants. *endo*-β-1,4-galactanases have been isolated and purified from species of *Aspergillus* (Christgau, Sandal, Kofod, & Dalboge, 1995; Kimura, Yoshioka, & Tajima, 1998; Lahaye, Vigouroux, & Thibault, 1991; van de Vis, Searle-van Leeuwen, Siliha, Kormelink, & Voragen, 1991; Yamaguchi, Inoue, & Hatanaka, 1995), *Penicillium* (Nakano, Takenishi, & Watanabe, 1985) and *Bacillus* (Emi & Yamamoto, 1972; Labawitch, Freeman, & Albersheim, 1976; Tsumura, Hashimoto, Akiba, & Horikoshi, 1991). *endo*-β-1,3-galactanase has hitherto been isolated only from *Rhizopus niveus* (Hashimoto, Tsujisaka, & Fukumoto, 1969) and *endo*-β-1,6-galactanase from *Aspergillus niger* (Brillouet, Williams, & Moutounet, 1991). In addition to the *endo*-acting enzymes, some *exo*-galactanases acting on β-1,3- (Pellerin & Brillouet, 1994; Tsumuraya, Mochizuki, Hashimoto, & Kovác, 1990) and β-1,4-linked (Bonnin, Lahaye, Vigouroux, & Thibault, 1995; Carey et al., 1995; Nakano, Takenishi, Kitahata, Kinugasa, & Watanabe, 1990) galactose moieties have been reported.

The objective of this study was to isolate, purify and characterize *endo*galactanases able to attack the type I and II galactans, with the main emphasis on galactans present in wood and chemical wood pulp. Furthermore, the structures of galactan and arabinan isolated from kraft pulp black liquor were analysed.

2. Materials and methods

2.1. Isolation of galactans

Linear (α-arabinofuranoside-treated) lupin β-1,4-galactan and branched larchwood arabino-β-1,3/6-galactan were obtained from Megazyme (P-GALLU) and Sigma (A-2012), respectively. Larchwood arabinogalactan is highly substituted and it contains about 16% of arabinose of the total monosaccharides as determined by acid hydrolysis. In order to make the substrate more accessible for galactanases, some of these arabinose side-groups were removed by incubating the polymer (10 mg ml^{-1}) in 1 M HCl at 40 °C for 24 h. After incubation the pH of the solution was adjusted to pH 4.8 with NaOH. The polymer was precipitated from the solution with two volumes of ethanol overnight at 4 °C. The precipitate was separated by centrifugation and washed twice with water–ethanol mixture (volume ratio 1:2). The washed precipitate was dissolved in water and lyophilized. The polymer was used as a substrate in the β-1,6-galactanase activity assay and in the hydrolysis experiments (acid-treated larchwood arabinogalactan).

Softwood galactans and arabinan were isolated from a polysaccharide sample, which was obtained from the kraft cooking black liquor by dioxane–acetic acid precipitation according to Engström et al. (1995). The sample contained about 14 g of carbohydrates and 4 g of lignin. It was kindly provided by Tapani Vuorinen (Helsinki University of Technology, Finland). Xylans, mannans and glucans were removed by enzymatic hydrolysis (20,000 nkat g^{-1} xylanase pI 9, 200 nkat g^{-1} β-xylosidase, 1000 nkat g^{-1} mannanase, 2000 nkat g^{-1} *endo*glucanase II, all from *Trichoderma reesei*, purified at VTT, and 100 nkat g^{-1} β-glucosidase from *A. niger*; Megazyme; incubation: pH 5.0, 40 °C, 48 h). The reaction was terminated by heat treatment at 100 °C for 10 min. The residual polymeric material was separated by two ethanol precipitations (1st precipitation → galactan I, solution: 96% EtOH 1:2 (vol/vol), 4 °C, 60 h; 2nd precipitation → galactan II, solution: 96% EtOH 1:3, 4 °C, overnight). The supernatant (1390 ml) was concentrated by evaporation to 260 ml and lyophilized. The dry material (10.5 g) was dissolved in 77 ml of distilled water and gel filtered (BioGel P2, Amersham Biosciences) with 20 mM ammonium acetate, pH 5.0 as eluent. Fractions were analysed by thin-layer chromatography (TLC) and those containing polymeric material (arabinan) were pooled.

Lignin–carbohydrate complex isolated from residual lignin of softwood kraft pulp (RLCC) was obtained from Bo Hortling (KCL, Finland). The sample contained about 9.9 mg carbohydrates per 100 mg of RLCC, the main carbohydrate being galactose (Table 1). A water-soluble arabinogalactan, isolated from Norway spruce, was kindly provided by Jeff Thornton (Åbo Akademi, Finland/SCA, Sweden).

2.2. Structural analysis of the isolated polysaccharides

The monosaccharide composition was determined by HPAEC after acid hydrolysis with 1.5% sulphuric acid at 120 °C for 40 min (Table 1) (Puls, Poutanen, & Körner, 1985). Methylation was performed using a modification of the method of Ciucane and Kerek (Ciucane & Kerek, 1984; Laine, Tamminen, Vilkkula, & Vuorinen, 2002). After the methylation, acid methanolysis was performed as described by Sundberg, Sundberg, Lillandt, and Holmbom (1996). The samples were then silylated and run on a GC/MS (Laine et al., 2002).

2.3. Activity assays

Endo-β-1,4-galactanase (*endo*-1,4-gal) activity was assayed by modifying the method used for *endo*-glucanase assay (IUPAC, 1987). Lupin β-1,4-galactan (0.5% (w/v) in 50 mM sodium citrate buffer, pH 5.0) was used as a substrate. Diluted sample (0.1 ml) in 50 mM sodium citrate, pH 5.0 was added to a tube containing 0.9 ml of preheated (50 °C) substrate solution and incubated for 10 min at 50 °C. The reaction was stopped by adding 1.5 ml of DNS (3,5-dinitrosalisylic acid) reagent. The colour was developed by heating the samples in a boiling water bath for 5 min. After cooling the absorbances were measured at 540 nm against a reaction blank containing 50 mM sodium citrate, pH 5.0, instead of the sample. Galactose (Merck 4058) solutions were used as standards. The assay was modified for microtiter plates for the detection of activity during the purification. In this case 20 μl of sample and 180 μl of preheated substrate solution were incubated in Eppendorf tubes for 50 min at 50 °C. The reaction was stopped by adding 150 μl of DNS-reagent. The colour was developed (100 °C, 5 min), the samples were applied to microtiter plates and the absorbances measured at 540 nm as described above.

β-1,6-Galactanase (1,6-Gal) activity was assayed analogously to *endo*-1,4-gal activity by using acid-treated larchwood arabinogalactan as a substrate. As the enzyme reaction was not linear, a quantitative activity assay could not be developed. The products were analysed qualitatively by TLC. Sample (20 μl) was incubated with 20 μl of 1% (w/v) substrate solution in 50 mM sodium acetate, pH 4.5, at 50 °C for 1 h. Immediately after the incubation, 2.5 μl of the sample was applied to a TLC plate and run as described below. The enzyme activity was evaluated on the basis of the release of galactose and oligosaccharides.

Table 1
Monosaccharide compositions of the polymeric substrates used as determined by HPAEC after acid hydrolysis

Sample	Gal (%)	Ara (%)	Glc (%)	Xyl (%)	Man (%)	Gal: Ara
Linear lupin galactan[a]	98	2	–	Trace	–	49
Acid-treated larchwood arabinogalactan	85.6	14.4	Trace	–	–	5.9
Arabinogalactan from Norway spruce[b]	87.8	10.2	0.9	0.7	0.4	8.6
Black liquor polysaccharide	24.4	24.9	9.4	39.6	1.7	1.0
Galactan I[c]	71.4	6.9	14.5	1.9	5.3	10.4
Galactan II	41.3	27.8	8.9	16.8	5.2	1.5
Arabinan	5.4	90.6	3.1	1.3	–	0.06
RLCC[d]	69.0	2.5	13.3	6.7	8.5	27.6

[a] Values from Megazyme. Also contained some rhamnose and glucuronic acid.
[b] Values from Jeff Thornton. Also contained some glucuronic acid.
[c] Contains some rhamnose and glucuronic acid.
[d] Residual lignin carbohydrate complex from softwood kraft pulp.

Endomannanase, *endoxylanase* and *endoglucanase* activities were assayed according to the methods of Stålbrand, Siika-aho, Tenkanen, and Viikari (1993), Bailey, Biely, and Poutanen (1992) and IUPAC (1987). α-Arabinofuranosidase, α-galactoside and β-galactosidase activities were evaluated on the basis of their ability to hydrolyse α-1,5-linked arabinobiose, raffinose and lactose, respecyively. Oligosaccharides (2 mg ml^{-1}) were incubated with purified enzymes (0.5 mg ml^{-1}) at pH 5 and 40 °C overnight. The released arabinose and galactose were analysed by TLC.

2.4. Enzyme sources

To select the enzyme preparations for the purification of galactanases, some fungal culture filtrates already available in our laboratory and several commercial enzyme preparations were tested for their activities against lupin β-1,4-galactan and acid-treated larchwood arabinogalactan. Samples were analysed by TLC for the released sugars and the preparations for the enzyme purification were chosen on the basis of galactose and oligosaccharide formation (results not shown).

Endo-β-1,4-galactanase was purified from a commercial pectinase product Pectinex Ultra SP-L (Novo Ferment Enzymes) produced by *Aspergillus aculeatus*. 800 ml of Pectinex Ultra SP-L was diluted 1:4 in 10 mM sodium acetate buffer, pH 5.5, and buffered in a column of Sephadex G-25 coarse (25.2 cm × 40 cm, Amersham Biosciences, Sweden) pre-equilibrated with the same buffer. After elution, the conductivity and pH were measured and adjusted to correspond to those of 10 mM sodium acetate, pH 5.5. The buffered sample (6680 ml) was applied to a column of DEAE Sepharose FF (25.2 cm × 20 cm, Amersham Biosciences) and the unabsorbed proteins were eluted with the equilibration buffer (10 mM Na acetate, pH 5.5). The elution was continued with a step of 250 mM NaCl, a linear gradient of 250–600 mM NaCl and a step of 600 mM NaCl. The elution was completed with 1 M NaCl. All NaCl-solutions were made in 10 mM sodium acetate buffer, pH 5.5. The fractions containing *endo*-β-1,4-galactanase activity were pooled (12.7 l) and concentrated by ultrafiltration (Millipore, Prep/Scale™ -TFF 1 ft^2 Cartridge, cut-off 10,000) to 1900 ml.

The concentrate was rebuffered to 10 mM sodium acetate buffer, pH 3.7, as described above, after which the sample (3000 ml) was applied to a column of CM Sepharose FF (11.3 cm × 20 cm, Amersham Biosciences) pre-equilibrated with the same buffer. After elution of the unabsorbed proteins a gradient from 10 mM sodium acetate, pH 3.7, to 25 mM sodium acetate, pH 4.4, was applied. The rest of the proteins were eluted with steps of 50 mM NaCl and 1 M NaCl in 25 mM sodium acetate, pH 4.4. The fractions containing *endo*-β-1,4-galactanase activity were again combined (4050 ml) and concentrated to 290 ml as described above.

The concentrate from the cation exchange chromatography run was applied to a column of Sephacryl S-100 HR (11.3 cm × 105 cm, Amersham Biosciences) pre-equilibrated with 20 mM sodium acetate buffer, pH 4.0, containing 100 mM NaCl in three equal batches (á 97 ml). The elution was carried out with the same buffer. The fractions containing *endo*-β-1,4-galactanase were combined in two pools (pool I: 3580 ml; pool II: 2120 ml) and concentrated by ultrafiltration to final volumes of 380 ml (pool I) and 280 ml (pool II), respectively, as described above.

β-*1,6-Galactanase* was purified from a commercial pectinase preparation Pectinex 3XL (Novo Nordisk) produced by *A. niger*. Pectinex 3XL (2.5 l) diluted with 1.5 l of distilled water was buffered in a column of Sephadex G-25 coarse (25.2 cm × 40 cm, Amersham Biosciences) pre-equilibrated with 10 mM sodium acetate buffer, pH 5.0. After elution, the conductivity and pH of the sample were adjusted to correspond to those of the equilibration buffer. The buffered sample was applied in two equal batches to a column of DEAE Sepharose FF (25.2 cm × 20 cm, Amersham Biosciences) and the unabsorbed proteins were eluted with the equilibration buffer (10 mM Na acetate, pH 5). The elution was continued with a linear gradient of 0–250 mM NaCl, a second linear gradient of 250–500 mM NaCl and a step of 500 mM NaCl. The elution was completed with 1 M NaCl. All NaCl-solutions were made in 10 mM sodium acetate buffer, pH 5.0. The fractions from both runs containing β-1,6-galactanase activity (15 + 13 l) were pooled. The pH of the combined solution was adjusted to 5.2 and it was concentrated by ultrafiltration to 4.2 l.

The concentrate was rebuffered to 10 mM sodium acetate buffer, pH 4.0, as described above (6.3 l), and applied to a column of CM Sepharose FF (25.2 cm × 12 cm, Amersham Biosciences) pre-equilibrated with the same buffer. After elution of the unabsorbed proteins a linear gradient from 0 to 75 mM NaCl in 10 mM sodium acetate buffer, pH 4, was applied. After a step of 75 mM NaCl a second gradient from 75 to 200 mM NaCl in the same buffer was applied. The rest of the proteins were eluted with steps of 200 mM NaCl and 1 M NaCl in 10 mM NaAc, pH 4. The fractions containing β-1,6-galactanase activity were combined in three pools (12, 12 and 19 l). Each of the pools was concentrated by ultrafiltration (Millipore, Prep/Scale™ -TFF 1 ft^2 Cartridge, cut-off 10,000) to 1–2 liters. The pools were further concentrated separately on a DEAE Sepharose FF column (5 cm × 20 cm, Amersham Biosciences) equilibrated with 10 mM Na-acetate buffer, pH 5.0, after their pH and conductivity had been adjusted to correspond to those of the equilibration buffer. Proteins were eluted with 10 mM Na-acetate buffer, pH 5.0, containing 100 mM sodium chloride, in volumes of 250–380 ml.

The concentrates were applied in several batches of 95 ml to a column of Sephacryl S-100HR (11.3 cm × 105 cm, Amersham Biosciences) pre-equilibrated with 25 mM sodium acetate buffer, pH 5.0, containing 100 mM NaCl. The proteins were eluted with the same buffer. The fractions

containing β-1,6-galactanase were also analysed for other hydrolytic enzymes. The best fractions were combined (6.5 l) and concentrated by ultrafiltration (Millipore, Prep/Scale™ -TFF 1 ft^2 Cartridge, cut-off 10,000) to a final volume of 415 ml.

2.5. Characterization of the purified enzymes

Molecular masses of the purified proteins were determined by SDS-PAGE on PhastSystems (Amersham Biosciences) according to the manufacturer's instructions. *Isoelectric point* was determined either by gel electrophoresis on PhastSystems (Amersham Biosciences) according to the manufacturer's instructions or by chromatofocusing (*endo*-β-1,4-galactanase). In chromatofocusing the sample (3 ml) was buffered on an Econo-Pac® 10DG-column (Bio-Rad) with 25 mM imidazole (Merck 4716)—HCl-buffer, pH 7.6, and applied to a PBE94-column (1 cm × 30 cm; Amersham Biosciences) pre-equilibrated with the same buffer. Proteins were eluted with Polybuffer-74-HCl-buffer, pH 4.0, and the elution was completed with 25 mM imidazole–HCl-buffer, pH 4.0, containing 1 M NaCl. *endo*-β-1,4-galactanase activity and pH were determined in each fraction.

The possible glycosylation of the purified proteins was detected after SDS-PAGE gel electrophoresis by staining the gel with Schiff's reagent according to Zacharius and Zell (1969). *N-terminal amino acid sequences* of the purified proteins were determined by automated Edman degradation using an Applied Biosystems 477A Pulsed Liquid Phase Protein Sequencer with 120A Analyzer (HPLC equipped with a C18 reverse phase column) at the University of Kuopio, Finland.

Optimal pH for *endo*-β-1,4-galactanase activity was determined using the standard activity assay method but with 0.5% (w/v) substrate in McIlvane buffer solutions at different pH values between pH 3.3 and pH 8.2. *Temperature and pH-stabilities* of the purified *endo*-β-1,4-galactanase were determined by incubating 6 μg ml^{-1} protein in 100 mM sodium citrate buffer, pH 4.5, at various temperatures between 4 and 45 °C, and in McIlvane buffer solutions at different pH values (pH 3.3–8.2) at 40 °C for 24 h, respectively. In both cases, the residual enzyme activities were determined immediately after incubation by the standard acitivity assay.

2.6. Hydrolysis experiments

The abilities of the purified galactanases to hydrolyse various wood- or pulp-derived arabinogalactans and arabinans were studied using enzyme dosages of 1000 and 10,000 nkat $g^{-1}_{substrate}$ (0.02 and 0.2 $mg_{protein}$ $g^{-1}_{substrate}$, respectively) for *endo*-β-1,4-galactanase and 5 and 10 $mg_{protein}$ $g^{-1}_{substrate}$ for β-1,6-galactanase. The final substrate concentration in 50 mM Na-citrate buffer, pH 5.0, was 5 mg ml^{-1}. Samples were incubated at 40 °C for 24 h. The incubation was terminated by heat treatment (5–10 min, 100 °C). In addition, the purified enzymes were incubated with polymeric mannans (galactomannan, locust bean gum, Sigma G-0753; polymannose, ivory nut mannan, Megazyme, Ireland), xylans (birch 4-*O*-methylglucuronoxylan, Roth, No. 7500; Kraft pulp arabinoglucuronoxylan, Jan Jansson, KCL, Finland), barley β-1,3/4-glucan (Megazyme) and amorphous cellulose (Whalseth prepared from Avicel) (final concentration 2–10 mg ml^{-1}), with galacto-oligosaccahrides (0.5 mg ml^{-1}; raffinose, α-D-galactopyranosyl-1,6-α-D-glucopyranosyl-1,2-β-D-fructofuranose, Merck 7549; lactose, β-D-galactopyranosyl-1,4-D-glucopyranose; 1,4-galactobiose, β-D-galactopyranosyl-1,4-D-galactopyranose, Megazyme; 1,6-galactobiose, β-D-galactopyranosyl-1,6-D-galactopyranose, Sigma G-5643; 1,3-galactosyl arabinose, β-D-galactopyranosyl-1,3-D-arabinose, Sigma G-3508) and α-1,5-L-arabino-oligosaccharides (Ara_2, Ara_4, Ara_5, Megazyme) at dosages of 10,000 nkat $g^{-1}_{substrate}$ for *endo*-β-1,4-galactanase and 13.5 $mg_{protein}$ $g^{-1}_{substrate}$ (polymers) and 10 $mg_{protein}$ $g^{-1}_{substrate}$ (oligosaccharides) for β-1,6-galactanase at pH 5.0 and at 40 °C for 24 h. The hydrolyzates were analysed by HPAEC.

The RLCC (40 $mg_{substrate}$ ml^{-1} = 3.2 $mg_{carbohydrate}$ ml^{-1}) was treated with *endo*-β-1,4-galactanase (2000 nkat $g^{-1}_{substrate}$ = 25,000 nkat $g^{-1}_{carbohydrate}$) in 100 mM sodium acetate, pH 5, at 40 °C for 16 h. The RLCC did not dissolve in the buffer and thus mixing was applied during the hydrolysis. The reaction was stopped by heat treatment (10 min, 100 °C) and the precipitate was separated by centrifugation. The supernatant was analysed for mono- and oligosaccharides by HPAEC directly and after secondary enzymatic hydrolysis of soluble oligosaccharides to monosaccharides.

The mode of action of β-1,6-galactanase was studied further by incubating 0.14 mg of enzyme protein with 5 mg of acid-treated larchwood arabinogalactan (final volume 1 ml) for 2 and 24 h at pH 4.5 and 40 °C. The incubation was terminated by heat treatment, after which the samples were lyophilized. The samples were analysed by HPAEC and 1D ^1H NMR.

2.7. Other analysis methods

Soluble protein content of the samples was analysed according to Lowry, Rosebrough, Farr, and Randall (1951) using bovin serum albumin (Sigma, A-8022) as standard. The protein contents of raw preparations were assayed after precipitation with 10% trichloroacetic acid. Thin layer chromatography (TLC) on silica plates (Kieselgel 60) was used for rapid screening of active fractions during enzyme purifications. The eluent system contained ethyl acetate, acetic acid and water in a volume ratio of 60:40:40. Plates were stained with a solution containing 10% water, 10% sulphuric acid, 80% ethanol and 0.2% Orsin and developed in an oven (105 °C) for 5 min.

Different hydrolysates were analysed by HPAEC on a Dionex CarboPac PA-1 column directly according to the method for oligosaccharides described by Tenkanen and Siika-aho (2000) Galactose, 1,4-galactobiose, 1,6-galactobiose, arabinose and 1,5-arabinobiose were used as standards.

NMR spectroscopy was carried out on a Varian UNITY 600 MHz spectrometer. Samples were lyophilized and redissolved in $^{2}H_2O$ (99.8 atom%, Fluka). pD was adjusted to 6.5–7.0 by additions of 0.2 M NaOD. 1D ^{1}H NMR spectra were obtained at 599.94 MHz at 70 °C. The chemical shifts are reported relative to internal sodium 3-(trimethyl)-3,3,2,2-tetra-deuteropropionate at 0 ppm.

3. Results

3.1. Isolation and structural analysis of the wood galactans

Larchwood arabinogalactan was treated with mild acid to remove arabinose side-groups and thus make the substrate more accessible for the galactanases (Table 1). The removal of arabinose with acid treatment did not succeed very well and the polymer obtained still contained arabinose side groups. The treatment was not optimized in this work. However, the acid-treated larchwood arabinogalactan could be used for screening and purification of the galactanase. Enzymatic removal of side groups with α-arabinofuranosidase would possible have been a better option, as α-arabinofuranosidases are reported to possess activity on arabinogalactans (Luonteri, Beldman, & Tenkanen, 1998; Brillouet et al., 1991). Softwood arabino-1,3/6-galactan was isolated from Norway spruce by water extraction (Willför et al., 2002).

Arabinogalactan fractions were isolated from polysaccharides obtained from kraft cooking liquor in order to test enzyme actions on galactans present in kraft pulp. In addition to galactose and arabinose, the raw polysaccharide sample contained a notable amount of xylose and minor amounts of glucose and mannose (Table 1). In order to isolate the (arabino)galactan, other polymeric carbohydrates were hydrolysed enzymatically to mono- and/or oligosaccharides, after which the residual polymeric material was separated by two ethanol precipitations and gel permeation chromatography. In the first EtOH precipitation about 28% of the original carbohydrates was precipitated. The precipitate (galactan I) contained mainly galactose, arabinose and glucose (Table 1). The second precipitate (galactan II; yield 10%) had lower galactose and higher arabinose and xylose contents than precipitate I. After ethanol precipitations the supernatant was lyophilized. The residual lyophilized material was dissolved in water and applied to a gel permeation chromatography column. The polymeric material eluted from the column was mainly composed of arabinose (arabinan; yield 3.6%) (Table 1).

The major constituent of galactan I was a 1,3-linked arabinogalactan with C-6 substitution at on average every second unit (Table 2). The substituent was most probably either directly a non-reducing galactopyranose or arabinofuranose endgroup or a side-chain of 1,6-linked galactose with one of the non-reducing endgroups (30%). A smaller proportion of a 1,4-linked galactan was present, estimated at approximately one third of the amount of 1,3-linked galactan. The ratio of the 1,3 to 1,4-linked galactan could not be determined accurately, because it was not known excatly how the non-reducing galactose endgroups were divided between them. Arabinose was mainly present as non-reducing endgroups in furanose form, and only traces of arabinan were present. Glucose and small amounts of xylose units present in the sample originated from 1,4-linked oligomers of glucan and xylan, respectively. Thus galactan I was a mixture of type I and type II arabinogalactans.

Galactan II was not as pure an arabinogalactan as galactan I. It was composed mainly of 1,3-linked galactan (approximately 30%), 1,4-linked galactan (approximately 10%) and 1,5-linked arabinan (approximately 30%) (Table 2). The galactan structure was comparable to that of galactan I, but there were more non-reducing endgroups, most probably due to shorter chains. Furthermore, the degree of substitution was lower. The ratio of 1,4-linked galactan to 1,3-linked galactan was approximately the same as in galactan I. The arabinan in galactan II was a slightly branched 1,5-linked arabinan, which was branched at C-2 or C-3 at approximately every 10th arabinose unit. In addition, 1,4-linked, slightly substituted xylan and traces of short-chain oligomers of glucose and mannose were present in the sample.

Table 2
Structural distribution of the polysaccaride samples isolated from black liquor expressed as mol% of identified units as determined by methylation analysis

Sample	Gal						Ara				
	T, p	1,4	1,3	1,6	1,3,6	Tot	T, f	1,5	1,2 or 1,3[a]	1,2,5 or 1,3,5[a]	Tot
Galactan I	15.5	15.8	20.4	4.7	17.1	73.5	6.3	+	+		6.3
Galactan II	18.8	8.0	9.9	1.8	6.2	44.7	16.4	12.3	1.3	1.0	31.0
Arabinan	2.1	+	+	–	+	2.1	22.4	46.3	12.4	2.1	83.2

T = non-reducing end (terminal), p = pyranose form, f = furanose form, no terminal arabinose was found in pyranose form. Tot = total.
[a] Linkage not confirmed by retention time comparison with a model compound.

The arabinan fraction was mainly 1,5-linked slightly branched arabinose (Table 2). Approximately 4/5 of the arabinose units were 1,5-linked and every fifth arabinose unit was 1,2- or 1,3-linked. Short-chain xylan and 1,4- and 1,3-linked glucose (DP < 4) were present in traces. The structural elements in galactan I, II and arabinan were also verified by NMR spectroscopy. Galactan I and arabinan were used later to analyse the action of purified galactanases.

3.2. Purification of galactanases

The starting materials for purification of the galactanases were chosen on the basis of the ability of various culture filtrates and commercial pectinase preparations to degrade lupin β-1,4-galactan and/or acid-treated larchwood arabinogalactan (results not shown). *endo*-β-1,4-galactanase and β-1,6-galactanase were purified from the commercial enzyme preparations Pectinex Ultra SP-L and 3XL, respectively, containing several pectin-degrading enzymes. As the starting materials were rather viscous, samples had to be diluted before the first buffering step. Both purification procedures consisted of three chromatographic steps: anion exchange, cation exchange, and gel permeation chromatography as a final step.

In the anion exchange chromatography the major part of *endo*-β-1,4-galactanase eluted with 0.5–0.6 M NaCl. In addition, two smaller peaks containing minor amounts of galactanase activity were detected already with the salt concentration of 0.25 M but they were not studied further. Xylanase, mannanase and β-glucanase eluted either during the 0.25 M NaCl step or at the beginning of the salt gradient (0.25–0.4 M NaCl). The yield of *endo*-β-1,4-galactanase was 52% after the first purification step (Table 3). In the following cation exchange chromatography, *endo*-β-1,4-galactanase was only weakly bound to the gel. It eluted at the beginning of the pH-gradient as a single sharp peak and the activity yield was 94% from the sample applied to the column. No mannanase, xylanase or β-glucanase activities were detected in the fractions containing *endo*-β-1,4-galactanase. The final purification of the protein was obtained by gel permeation chromatography. In this run a small protein peak was observed prior to *endo*-β-1,4-galactanase. The total final yield of *endo*-β-1,4-galactanase was 15% of the original activity and 0.1% of the original protein of the sample (Table 3).

β-1,6-Galactanase eluted in the anion exchange chromatography during the salt gradient with 0.35–0.45 M NaCl. In the subsequent cation exchange chromatography, the enzyme eluted in several fractions starting from the beginning of the second salt gradient (75 mM NaCl) to about 0.14 M NaCl. Final purification of the protein was achieved by gel permeation chromatography, in which two major protein peaks eluted before the β-1,6-galactanase. The purified enzyme accounted for 0.6% of the total protein in the original sample (Table 4). Due to the lack of an appropriate substrate and to the non-linearity of the enzyme reaction on the substrate used, the activity yield could not be determined.

3.3. Characterization of the purified proteins

endo-β-1,4-galactanase was a monomeric protein with a molecular mass of 38 kDa as determined by SDS-PAGE (Fig. 1, Table 5) and by gel permeation chromatography. Its isoelectric point (pI) could not be determined by isoelectric focusing (IEF) because surprisingly the sample did not move in the gel. In chromatofocusing the enzyme was eluted with 25 mM imidazole-HCl buffer, pH 4.0, containing 1 M NaCl, thus indicating that the pI of the protein was below 4.0. After staining with Schiff's reagent no colour was detected, indicating that the protein was not significantly glycosylated (results not shown).

The optimal pH for the *endo*-β-1,4-galactanase activity was at pH 4.5 but it acted efficiently over a pH range from 3.4 to 5.0 (Table 5). The enzyme retained at least 80% of its activity in the pH range from 4.4 to 7.0 when incubated at 40 °C for 24 h but the activity decreased sharply outside this range: at pH-values below pH 3.2 and over pH 7.5 less than 50% of the activity remained. In addition, it retained at least 80% of its activity at temperatures below 40 °C when incubated at pH 4.5 for 24 h. At 45 °C only 47% of the activity was retained. The specific activity of *endo*-β-1,4-galactanase was remarkably high, about 51,000 nkat mg^{-1}. The purified enzyme did not show any significant activity on the other polymeric substrates tested.

Table 3
Purification of *endo*-β-1,4-galactanase from Pectinex Ultra SP-L

Sample	Volume (ml)	Activity (mkat)	Protein (mg)	Spec. activity (nkat mg^{-1})	Yield (%)
Pectinex Ultra SP-L	800	26.7	78,500	340	100
DEAE Sepharose FF	1900	14.0	n.d.	–	52
CM Sepharose FF	290	13.0	n.d.	–	49
Sephacryl S-100 HR	380	3.5	68.4	51,000	13

n.d. = not determined.

Table 4
Purification of β-1,6-galactanase from Pectinex 3XL

Sample	Volume[a] (ml)	Protein (g)	Yield (%)
Pectinex 3XL	2500	44.3	100
DEAE Sepharose FF	4200 conc.	15.0	34
CM Sepharose FF	260 conc.	n.d.	n.d.
Sephacryl S-100 HR	420 conc.	0.29	0.6

n.d. = not determined.
[a] Conc = volume after concentration.

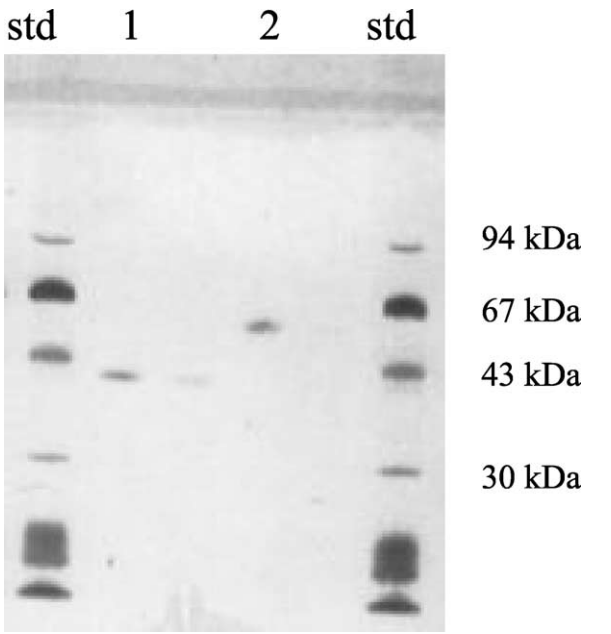

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified *endo*-1,4-galactanase (line 1) and 1,6-galactanase (line 2). The molecular mass standards of 94, 67, 43, 30, 20 and 14.4 kDa (from the top) are show on both sides.

The second enzyme preparation, β-1,6-galactanase, contained 0.7 mg ml^{-1} of protein. It gave a single band on SDS-PAGE, corresponding to a molecular mass of 58 kDa (Fig. 1, Table 5). The value determined by gel permeation chromatography was 17 kDa. The reason for the lower apparent molecular weight obtained by gel permeation chromatography is unclear but a similar phenomenon has often been reported with other proteins and may be due to some interaction of the protein with the gel matrix used. The specific activity of the enzyme could not be determined because of the non-linearity of the reaction with acid-treated larchwood arabinogalactan.

The N-terminal amino acid sequences (15 aa) of the purified proteins were determined by automated Edman degradation (Fig. 2). The N-terminal aa sequence of *endo*-β-1,4-galactanase was identical with that of *endo*-β-1,4-galactanase of *A. aculeatus* (Christgau et al., 1995), whereas no similarity was found for the 15 aa sequence of β-1,6-galactanase in a BLAST search on public databases.

Table 5
Properties of the purified *endo*-β-1,4-galactanase and β-1,6-galactanase

	Method	*endo*-β-1,4-gal	β-1,6-Gal
Molecular mass	SDS-PAGE	38 kDa	58 kDa
	Gel filtration	38 kDa	17 kDa
Isoelectric point	Isoelectric focusing	<4.0	nd
pH-optimum	Activity assay	3.4–5.0	nd[b]
pH-stability[a]	40 °C, 24 h	4.4–7.0	nd[b]
T-stability[a]	pH 4.5, 24 h	≤40 °C	nd[b]

[a] At least 80% of the activity retained.
[b] Not determined due to the lack of a linear activity assay.

3.4. Hydrolytic properties of the galactanases

endo-β-1,4-galactanase was able to hydrolyse β-1,4-galactosidic linkages in lupin β-1,4-galactan and in the galactan I isolated from black liquor (Fig. 3, Table 6). After extensive hydrolysis with a high enzyme dosage (10,000 nkat/g, 0.2 mg/g), the main products formed were galactose and galactobiose. Minor amount of other oligosaccharides were also observed after extensive hydrolysis. The enzyme did not hydrolyse acid-treated larchwood or the Norway spruce arabinogalactan, which contain β-1,3- and β-1,6-linked galactose residues, and had no action on black liquor arabinan. Lactose, raffinose, 1,6-galactobiose and 1,3-galactosylarabinose were also not hydrolysed. *endo*-β-1,4-galactanase did not only act on isolated polysaccharides containing β-1,4-linked galactose, but it was also able to liberate galactose (2.3% of the theoretical amount of galactose) and galactobiose from residual lignin–carbohydrate complex (RLCC) (results not shown).

β-1,6-Galactanase liberated galactose and 1,6-galactobiose from acid-treated larchwood and Norway spruce arabinogalactans (Fig. 4(A) and (B), Table 6). Furthermore, it liberated a minute amount of galactose and galactobiose from lupin β-1,4-galactan, when a high enzyme dosage (10 $mg_{protein}\ g^{-1}_{substrate}$) was used (Fig. 4(C)). From the black liquor galactan I the enzyme liberated mainly galactose and 1,6-galactobiose (Fig. 4(D)). In addition a small amount of free arabinose was detected. Interestigly, arabinose was the major product from arabinan isolated from black liquor (Fig. 4(E), Table 6). However, the isolated α-1,5-linked arabinofuranoside-oligosaccharides with a degree of polymerization of from 2 to 5 were not degraded, indicating that the enzyme did not posses α-1,5-arabinan or α-arabinofuranosidase activity. In addition it did not show any α-galactosidase or β-galactosidase activity as evaluated by the action on raffinose and lactose, respectively. It was able to liberate a very small amount of galactose from 1,3-galactosylarabinose (results not shown). It appears that in the presence of both galactose and arabinose side groups in the polymer the enzyme preferred to release the previous moieties. In the absence of galactose β-1,6-galactanase liberated arabinose, probably from a side-group position.

The activity of β-1,6-galactanase on acid-treated larchwood arabinogalactan was further analysed by HPAEC and ^{1}H NMR in order to understand its mode of action. The enzyme was able to release about 9% of the theoretical amount of galactose after 2 h and about 54% after 24 h of incubation. In addition, 1,6-galactobiose and some smaller unknown peaks were detected on the chromatogram. According to NMR-analysis the relative intensity of the Gal-β(1 → 3) resonance (4.7–4.75 ppm) remained the same and that of the Gal-β(1 → 6)

Endo-ß-1,4-galactanase

5 10 15

Ala - Leu - Thr - Tyr - Arg - Gly - Ala - Asp - Ile - Ser - Ser - Leu - Leu - Leu - Leu -

ß-1,6-galactanase

5 10 15

Ile - Ser - Ser - Ser - Pro - Leu - Ser - Thr - Ser - Gly - Gly - (Asn) - Ile - Val - Asp -

Fig. 2. N-terminal amino acid sequences of the purified proteins as determined by automated Edman degradation. Brackets indicate uncertainty in interpretation.

resonance (4.4–4.5 ppm) decreased during the β-1,6-galactanase treatment (Fig. 5(A)–(C)). During the hydrolysis two new resonances were observed in the ^{1}H NMR spectra. The relative intensities of the groups at 5.29 and 4.60 ppm, representing α- and β-reducing ends of galactose, respectively, increased during the enzyme treatment (Fig. 5(B) and (C)). Concomitantly the three sharp doublets between 4.4 and 4.5 ppm decreased. Thus it appears that the galactooligosaccharides produced were hydrolysed further. This was also supported by the HPAEC analysis. In conclusion, it appears that the enzyme was hydrolysing the β-1,6-linked galactose side-chains from the larchwood arabinogalactan.

4. Discussion

Hemicellulose-degrading enzymes, mainly xylanases, are currently used as bleaching aids for kraft pulps (Viikari, Kantelinen, Sundqvist, & Linko, 1994). The exact mechanism of xylanase 'bleaching' is not known but it is believed to be due at least partly to the action of enzymes on lignin-bound xylans and to enhanced leachability of lignin due to the more open fibre structure after xylanase treatment. Galactans are present in woods only in trace amounts. However, the outer cell wall layers of wood fibres are enriched with lignin and galactans, which have been suggested to be chemically linked to each other (Hortling et al., 2001; Hortling et al., 1997). The galactans in the lignin carbohydrate complexes have been found to be both type I and type II galactans, i.e. mainly linear β-1,4-linked galactan and branched β-1,3/6-linked galactan (Laine et al. 1999; Minor, 1991). In addition, galactose is one of the major sugars released from kraft pulps during oxygen delignification (Laine and Tamminen, 2002). Breaking the bonds between lignin and galactans could facilitate the removal of residual lignin from kraft pulps during bleaching operations. Thus the aim of this work was to isolate *endo*galactanases able to degrade wood galactans efficiently.

Softwood galactans were isolated for substrates from the kraft cooking liquor, as kraft wood fibres contain only minute quantities of galactans. The presence of both β-1,4-linked and β-1,3/6-linked galactans was verified. α-1,5-linked arabinan was also isolated from the cooking liquor. The major component in galactan I was branched arabino-1,3/6-galactan, which is reasonable as type II galactans are more soluble than type I galactans. About 20% of galactose in galactan I was in the form of β-1,4-linked galactan. The degree of substitution, especially the amount of arabinofuranoside side groups, was lower in black liquor arabino-1,3/6-galactan than in that solubilized already with water from Norway spruce (Willför et al., 1999; Willför et al., 2002). The structural differences were also reflected in the action of enzymes, i.e. less galactose

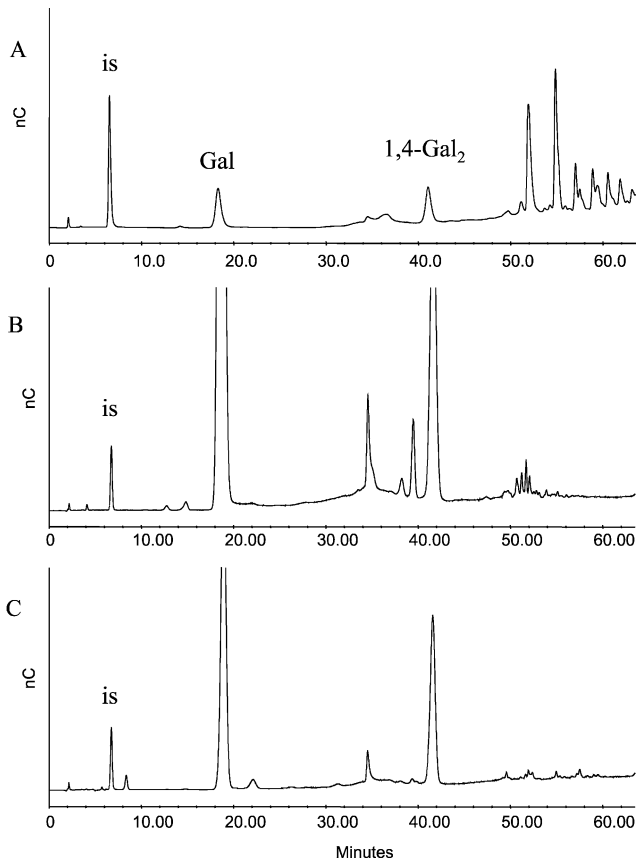

Fig. 3. Hydrolysis of lupin β-1,4-galactan (A, B) and black liquor galactan I (C) by *endo*-β-1,4-galactanase. Enzyme dosages used were 1000 nkat $g^{-1}_{substrate}$ (A) and 10,000 nkat $g^{-1}_{substrate}$ (B,C). Incubation at pH 5.0 and 40 °C for 24 h. Is = internal standard, Gal = galactose, 1,4-Gal_2 = β-1,4-D-galactobiose.

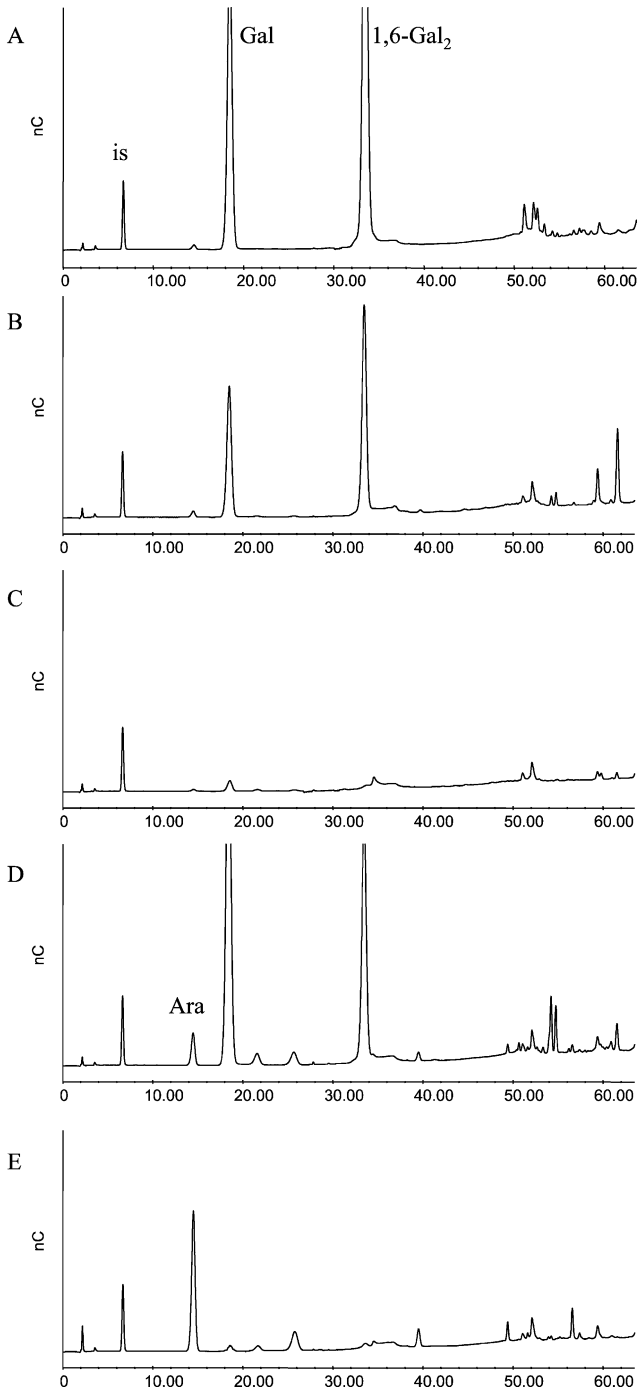

Fig. 4. Hydrolysis of acid-treated larchwood arabinogalactan (A), Norway spruce arabinogalactan (B), lupin β-1,4-galactan (C), black liquor galactan I (D) and black liquor arabinan (E) by β-1,6-galactanase (10 $mg_{protein}$ $g^{-1}_{substrate}$) at pH 5.0 and 40 °C for 24 h. Is = internal standard, Gal = galactose, 1,6-Gal_2 = β-1,6-D-galactobiose.

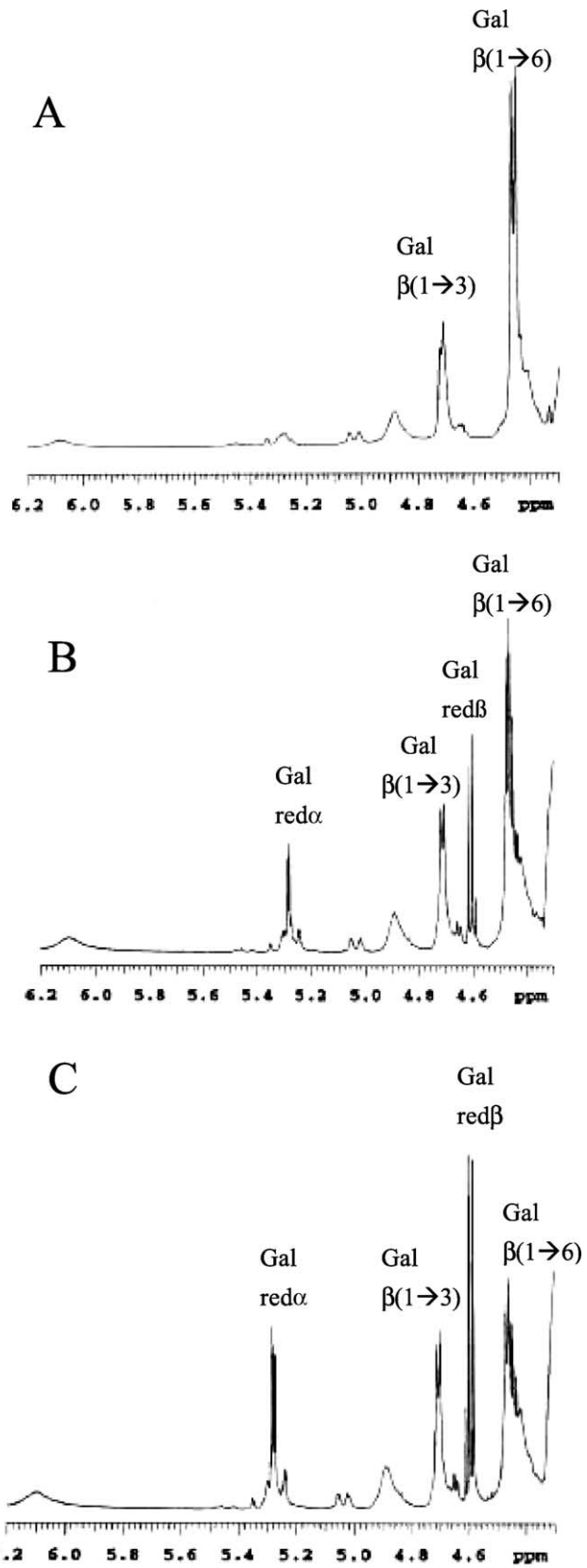

Fig. 5. NMR-spectra of acid-treated larchwood arabinogalactan before (A) and after 2 h (B) and 24 h (C) hydrolysis with β-1,6-galactanase (27 $mg_{protein}$ $g^{-1}_{substrate}$). Incubation at pH 4.5 and 40 °C.

was liberated by 1,6-galactanase from the more highly branched acid-treated larchwood arabinogalactan and Norway spruce arabinogalactan than from galactan I.

Two galactanases with different substrate specificities were purified from commercial pectinase preparations. The enzyme purified from *Aspergillus aculeatus* was able to

hydrolyse lupin type I β-1,4-galactan to galactose and a mixture of β-1,4-galacto-oligosaccharides but it was inactive towards type II arabinogalactan isolated from larchwood. Thus it was clearly an *endo*-β-1,4-galactanase (EC 3.2.1.89). In the final stage of the reaction galactose and galactobiose accumulated as end products. Moreover, the enzyme was able to release galactose and galactobiose from a residual lignin–carbohydrate complex isolated from softwood kraft pulp. On the basis of its molecular and hydrolytic properties, as well as the N-terminal amino acid sequence similarity, it was the same enzyme as has previously been purified from the culture filtrate of *A. aculeatus,* having a molecular mass of 42–43 kDa, isoelectric point below 3 and pH optimum between 4.0 and 4.5 (Christgau et al., 1995; Lahaye et al., 1991; van de Vis et al., 1991). The gene encoding this enzyme has also been isolated and characterized and based on this the *endo*-β-1,4-galactanase from *A. aculeatus* was assigned to the glycosyl hydrolase family 53 (Christgau et al., 1995).

Specific *endo*-β-1,4-galactanases have also been purified from *A. niger* (van de Vis et al., 1991; Yamaguchi et al., 1995) and *A. sojae* (Kimura et al., 1998). Their molecular masses ranged from 32 to 43 kDa. These enzymes readily hydrolysed β-1,4-linked arabinogalactans to produce galactose and short chain-length (2–4 residues) galacto-oligosaccharides without liberation of arabinose.

The other enzyme purified was from *A. niger* and it liberated galactose and 1,6-galactobiose from various arabino-1,3/6-galactans. It was also able to release arabinose residues. This might be explained by the structural similarity of β-D-galactopyranose and α-L-arabinopyranose (or α-D-galactopyranose and β-L-arabinopyranose) residues, which have the same ring structure but different groups attached to the C-5 ($-CH_2OH$ and $-H$, respectively). This relative lack of specificity has been observed e.g. in several galactosidases, which have been able to release both galactose and arabinose residues (Dey and Del Campillo, 1984). Arabinose was clearly liberated by 1,6-galactanase from galactan I and from arabinan isolated from black liquor. Only a minor amount of arabinose was liberated from acid-treated larchwood arabino-1,3/6-galactan. In larchwood arabinogalactan, arabinose is mainly present in the furanose form, but some L-arabinopyranose is also found, most commonly in the sequence: β-L-Ara*p*-(1 → 3)-L-Ara*f*-(1- (Aspinall, 1980). Arabino-1,4-galactans are reported to have mainly 1,5-linked α-L-arabinofuranoside side groups (van de Vis, 1994), and arabinans are reported to be solely composed of α-L-arabinofuranose residues (Aspinall, 1970).

According to the NMR analysis the enzyme acted on β-1,6-linkages in larchwood arabinogalactan. Based on this result the enzyme was named a β-1,6-galactanase. The β-1,6-galactanase purified in this work appeares to be very similar to the previously described *endo*-β-1,6-galactanase from *A. niger* (Brillouet et al., 1991). This enzyme liberated a mixture of oligosaccharides, predominantly β-1,6-galactobiose, and to a lesser extent galactose from native grape arabinogalactan-protein. However, the enzyme purified here was not yet named as either an *endo*- or *exo*-1,6-galactanase as the actual action mechanism is still unclear. From the arabinogalactans tested it did not produce any other galactooligosaccharides in any of the tested conditions. 1,6-galactobiose was already liberated in the first stages of the hydrolysis of acid-treated larchwood arabinogalactan, thus indicating digalactose-producing *exo*-action. However, as the action was not tested on polymeric 1,6-linked galactan we cannot rule out the *endo*-action.

The previously purified *endo*-β-1,6-galactanase (Brillouet et al., 1991) had a molecular mass of 60 kDa by SDS-PAGE, which is close to our result for β-1,6-galactanase (58 kDa). In addition both enzymes showed similar behaviour in gel filtration, giving clearly lower molecular masses (29 and 17 kDa, respectively) by this method. *endo*-β-1,6-galactanase had a rather low pH optimum of 3.5. It was most stable in near-neutral pH in temperatures below 30 °C. Unfortunately these properties could not be determined for the β-1,6-galactanase reported here. The main difference observed between these two galactanases was that the previously purified *endo*-β-1,6-galactanase liberated almost solely 1,6-galactobiose, and only a small amount of free galactose was detected in the hydrolysis of grape arabino-1,3/6-galactan-protein, whereas the enzyme purified in the present work was able to produce

Table 6
Liberation of galactose and arabinose from various galactans by *endo*-1,4-galactanase and 1,6-galactanase

Sample	Galactose			Arabinose		
	1,4-Gal	1,6-Gal		1,4-Gal	1,6-Gal	
	0.2 mg/g	5 mg/g	10 mg/g	0.2 mg/g	1,6-Gal 5 mg/g	10 mg/g
Linear 1,4-galactan from lupin	1470	–	8	7	–	–
Acid-treated larchwood arabinogalactan	–	65	200	–	–	(+)
Arabinogalactan from Norway spruce	–	49	105	–	–	5
Galactan I from black liquor	295	125	295	–	11	25
Arabinan from black liquor	–	–	(+)	–	68	115

a significant amount of galactose from wood arabino-1,3/6-galactans. *endo*-β-1,6-galactanase was also inactive on native grape arabino-1,3/6-galactan without removal of arabinose side groups.

A galactanase has also been partially purified from *Rhizopus niveus*. This enzyme showed rather similar activity to that of the β-1,6-galactanase in this work, acting efficiently on native coffee bean arabinogalactan and liberating galactobiose, galactose and arabinose (Hashimoto et al., 1969; Hashimoto, 1971). The enzyme from *R. niveus* was classified as an *endo*-β-1,3-galactanase. As no clear data was reported on its action on 1,3-linkages, it might also be a β-1,6-galactanase. Hitherto there are no other reports of *endo*-β-1,3-galactanases and the corresponding EC number (EC 3.2.1.90) was recently deleted from the Enzyme Nomenclature by the International Union of Biochemistry and Molecular Biology (www.chem.qmw.ac.uk/iubmb/) due to insufficient characterization of the enzyme. An enzyme named *exo*-galactanase has been purified from radish seeds. This enzyme cleaved both β-1,3- and β-1,6-D-galacto-oligomers to galactose, preferentially splitting β-1,6-linkages in de-arabinosylated radish galactan (Sekimata, Ogura, Tsumuraya, Hashimoto, & Yamamoto, 1989). Two previously purified galactanases from *A. niger* and *Irpex lacteus*, which have both been named *exo*-β-1,3-galactanases, show rather similar action to that of the β-1,6-galactanase purified here, producing mainly galactose and 1,6-galactobiose (Pellerin and Brillouet, 1994; Tsumuraya et al., 1990).

Unfortunately we were not able to purify *endo*-β-1,3-galactanase, even though this was our target enzyme rather than β-1,6-galactanase. No clear indication of this type of *endo*-activity was found during the work even though several commercial enzyme preparations, as well as self-produced culture filtrates, were screened. Furthermore there is currently no reliable information in the literature for the existence of *endo*-β-1,3-galactanases. One option is that arabino-1,3/6-galactans have a special sequential enzymatic degradation mechanism, e.g. arabinose- and galactose-containing side groups and chains are cleaved first and then a processive *exo*-β-1,3-galactanase hydrolyses the residual backbone to galactose.

The potential of the enzymes purified in the current work in breaking up lignin carbohydrate connections, and further enhancing the bleachability of spruce kraft pulp, was recently demonstrated by Tamminen et al. (1999). Both *endo*-1,4-galactanase and β-1,6-galactanase were found to give a positive response in chorine-free TCF bleaching. The enzyme treatments resulted in improved efficiency of following bleaching chemicals if applied to brown stock pulp prior to oxygen delignification. Only a small amount of galactose was released resulting in an insignificant yield loss due to the treatment. It was also shown that RLCCs isolated from pulps contained less galactose after galactanase treatments. Thus even though the chemical pulp fibres contain only trace amounts of galactans, the specific galactan-degrading enzymes facilitate clearly the removal of residual lignin. The effect could be improved further by a mixture of various galactanases, and one obvious candidate for more efficient action would be an enzyme hydrolysing β-1,3-linkages in arabino-1,3/6-galactans.

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References

Aspinall, G. O. (1970). Pectins, plant gums, and other plant polysaccharides. In W. Pigman, & D. Horton (Eds.), (*Vol. IIB*) (pp. 515–536). *The carbohydrates chemistry and biochemistry*, New York: Academic Press.

Aspinall, G. O. (1980). Chemistry of cell wall polysaccharides. In J. Preiss (Ed.), (*Vol. 3*) (pp. 473–500). *The biochemistry of plants*, New York: Academic Press.

Aspinall, G. O., & Wood, T. M. (1963). The structures of two water-soluble polysaccharides from Scotts pine. *Journal of Chemical Society*, 1686–1696.

Bailey, M. J., Biely, P., & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology*, *23*, 257–270.

Bonnin, E., Lahaye, M., Vigouroux, J., & Thibault, J.-F. (1995). Preliminary characterization of a new *exo*-β-(1,4)-galactanase with transferase activity. *International Journal of Biological Macromolecules*, *17*, 345–351.

Bouveng, H., & Lindberg, B. (1956). Studies on arabinogalactanas; I. Products from the mild hydrolysis of arabinogalactan from *Larix occidentalis*. *Acta Chemica Scandinvica*, *10*, 1515–1519.

Bouveng, H. O., & Meier, H. (1959). Studies on a galactan from Norwegian spruce compression wood (*Picea abies* Karst.). *Acta Chemica Scandinvica*, *13*, 1884–1889.

Brillouet, J.-M., Williams, P., & Moutounet, M. (1991). Purification and some properties of a novel *endo*-β-(1 → 6)-D-galactanase from *Aspergillus niger*. *Agricultural and Biological Chemistry*, *55*, 1565–1571.

Carey, A. T., Holt, K., Picard, S., Wilde, R., Tucker, G. A., Bird, C. R., Schuch, W., & Seymour, G. B. (1995). Tomato *exo*-(1 → 4)-β-D-galactanase. *Plant Physiology*, *108*, 1099–1107.

Christgau, S., Sandal, T., Kofod, L. V., & Dalboge, H. (1995). Expression cloning, purification and characterization of a beta-1,4-galactanase from *Aspergillus aculeatus*. *Current Genetics*, *27*, 135–141.

Ciucane, I., & Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, *131*, 209–217.

Clarke, A. E., Anderson, R. L., & Stone, B. A. (1979). Form and function of arabinogalactans and arabinogalactan-proteins. *Phytochemistry*, *18*, 521–540.

Dekker, R. F. H., & Richards, G. N. (1976). Hemicellulases: Their occurrence, purification, properties, and mode of action. *Advances in Carbohydrate Chemistry and Biochemistry*, *32*, 277–352.

Dey, P. M., & Del Campillo, E. (1984). Biochemistry of the multiple forms of glycosidases in plants. *Advances in Enzymology*, *56*, 141–249.

Emi, S., & Yamamoto, T. (1972). Purification and properties of several galactanases of *Bacillus subtilis* var. *amylosacchariticus*. *Agricultural and Biological Chemistry*, *36*, 1945–1954.

Engström, N., Vilkkula, A., Teleman, A., & Vuorinen, T. (1995). Structure of hemicelluloses in pine kraft cooking liquors. *Eighth International Symposium on Wood and Pulping Chemistry, Helsinki, Finland*, *3*, 195–200.

Gillham, J. K., & Timell, T. E. (1958). The polysaccharides of white birch (*Betula papyrifera*). *Svensk Papperstidning*, *61*, 540–544.

Hashimoto, Y. (1971). Studies of the enzyme treatment of coffee beans part V. Structure of coffee arabinogalactan. *Nippon Nogeikagaku Kaishi*, *45*, 147–150.

Hashimoto, Y., Tsujisaka, Y., & Fukumoto, J. (1969). Studies on the enzyme treatment of coffee beans, Part III. Purification and some properties of galactanase from *Rhizopus niveus*. *Nippon Nogeikagaku Kaishi*, *43*, 831–836.

Hortling, B., Tamminen, T., & Pekkala, O. (2001). Effects of delignification on residual lignin–carbohydrate complexes in normal pine wood and pine wood enriched in compression wood. 1. Kraft pulping. *Nordic Pulp Paper Research Journal*, *16*, 219–224.

Hortling, B., Tamminen, T., & Ranua, M. (1998). Oxygen and chlorine dioxide delignification of compression wood enriched and normal pine kraft pulp 1. Differences in delignification behaviour and residual lignin structure. *International Pulp Bleaching Conference, Helsinki, Finland*, 295–300. Book 2.

Hortling, B., Tamminen, T., & Turunen, E. (1997). Structures and carbohydrate composition of kraft pulp residual lignin fractions of different molar masses. *Ninth International Symposium on Wood and Pulping Chemistry, Montreal, Canada*, *1*, B6-1-4.

IUPAC (1987). Measurement of cellulase activities. *Pure and Applied Chemistry*, *59*, 257–268.

Jiang, K.-S., & Timell, T. E. (1972). Polysaccharides in compession wood of tamarack (*Larix laricina*). *Svensk Papperstidning*, *75*, 592–594.

Karácsonyi, Š., Kovácik, V., Alfäldi, J., & Kubacková, M. (1984). Chemical and ^{13}C-NMR studies of an arabinogalactan from *Larix sibirica* L. *Carbohydrate Research*, *134*, 265–274.

Karácsonyi, Š., Pätoprstý, V., & Kubacková, M. (1998). Structural study on arabinogalactan-proteins from *Picea abies* L. Karst. *Carbohydrate Research*, *307*, 271–279.

Kimura, I., Yoshioka, N., & Tajima, S. (1998). Purification and characterizytion of an *endo*-1,4-β-D-galactanase from *Aspergillus sojae*. *Journal of Fermentation and Bioengineering*, *85*, 48–52.

Labawitch, J. M., Freeman, L. E., & Albersheim, P. (1976). Structure of plant cell walls. Purification and characterization of a β-1,4-galactanase which degrades a structural component of the primary cell wall of dicots. *Journal of Biological Chemistry*, *251*, 5904–5910.

Lahaye, M., Vigouroux, J., & Thibault, J.-F. (1991). *endo*-β-1,4-D-galactanase from *Aspergillus niger* var. *aculeatus*: Purification and some properties. *Carbohydrate Polymers*, *15*, 431–444.

Laine, C., Haakana, S., Hortling, B., & Tamminen, T. (1999). Application of metylation analysis to samples derived from spruce kraft pulp. *10th International Symposium on Wood and Pulping Chemistry, Yokohama, Japan*, *1*, 72–77.

Laine, C., & Tamminen, T. (2002). Origin of carbohydrates dissolved during oxygen delignification of birch and pine kraft pulp. *Nordic Pulp Paper Research Journal*, *17*, 46–49.

Laine, C., Tamminen, T., Vilkkula, A., & Vuorinen, T. (2002). Methylation analysis as a tool for structural analysis of wood polysaccharides. *Holzforschung*, *56*, 607–614.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Luonteri, E., Beldman, G., & Tenkanen, M. (1998). Substrate specificity of *Aspergillus terreus* α-arabinofuranosidases. *Carbohydrate Polymers*, *37*, 131–141.

Manley-Harris, M. (1997). Structural studies by NMR spectroscopy of the major oligomers from alkali-degraded arabinogalactan from *Larix occidentalis*. *Carbohydrate Polymers*, *34*, 243–249.

Meier, H. (1959). Fördelning av hemicellulosakomponenterna I en tallvedsfiber. *Svensk Papperstidning*, *62*, 687–691.

Minor, J. L. (1991). Location of lignin-bonded pectic polysaccharides. *Journal of Wood Chemistry and Technology*, *11*, 159–169.

Nakano, H., Takenishi, S., Kitahata, S., Kinugasa, H., & Watanabe, Y. (1990). Purification and characterization of and *exo*-1,4-β-galactanase from a strain of *Bacillus subtilis*. *European Journal of Biochemistry*, *193*, 61–67.

Nakano, H., Takenishi, S., & Watanabe, Y. (1985). Purification and properties of two galactanases from *Penicillium citrinum*. *Agricultural and Biological Chemistry*, *49*, 3445–3454.

Pellerin, P., & Brillouet, J.-M. (1994). Purification and properties of an *exo*-(1 → 3)-β-D-galactanase from *Aspergillus niger*. *Carbohydrate Research*, *264*, 281–291.

Ponder, G. R., & Richards, G. N. (1997). Arabinogalactan from western larch. Part III: Alkaline degradation revisited, with novel conclusions on molecular structure. *Carbohydrate Polymers*, *34*, 251–261.

Puls, J., Poutanen, K., & Körner, H.-U. (1985). Biotechnical utilization of wood hemicelluloses. *Applied Microbiology and Biotechnology*, 416–423.

Sekimata, M., Ogura, K., Tsumuraya, Y., Hashimoto, Y., & Yamamoto, S. (1989). A β-galactosidase from radish (*Raphanus sativus* L.) seeds. *Plant Physiology*, *90*, 567–574.

Stålbrand, H., Siika-aho, M., Tenkanen, M., & Viikari, L. (1993). Purification and characterization of two β-mannanases from *Trichoderma reesei*. *Journal of Biotechnology*, *29*, 229–242.

Sundberg, A., Sundberg, K., Lillandt, C., & Holmbom, B. (1996). Determination of hemicelluloses and pectins in wood and pulp fibres by acid methanolysis and gas chromatography. *Nordic Pulp Paper Journal*, *11*(4), 216–220.

Tamminen, T., Hortling, B., Ranua, M., Luonteri, E., Suurnäkki, A., Tenkanen, M., & Buchert, J. (1999). Enhanced bleachability of spruce kraft pulp by mechanical and enzymatic treatments. *10th International Symposium on Wood and Pulping Chemistry, Yokohama, Japan*, *1*, 584–588.

Tamminen, T., Vuorinen, T., Tenkanen, M., Hausalo, T., & Hortling, B. (1995). Analysis of lignin and lignin–carbohydrate complexes isolated from black liquor. *Eighth International Symposium on Wood and Pulping Chemistry, Helsinki, Finland*, *2*, 297–302.

Tenkanen, M., & Siika-aho, M. (2000). An α-glucuronidase of *Schizophyllum commune* acting on polymeric xylan. *Journal of Biotechnology*, *78*, 149–161.

Timell, T. E. (1967). Recent progress in the chemistry of wood hemicelluloses. *Wood Science and Technology*, *1*, 45–70.

Tsumura, K., Hashimoto, Y., Akiba, T., & Horikoshi, K. (1991). Purifications and properties of galactanases from alkalophilic *Bacillus* sp. S-2 and S-39. *Agricultural and Biological Chemistry*, *55*, 1265–1271.

Tsumuraya, Y., Mochizuki, N., Hashimoto, Y., & Kovác, P. (1990). Purification of an *exo*-β-(1 → 3)-D-galactanase of *Irpex lacteus* (*Polyporus tulipiferae*) and its action on arabinogalactan-proteins. *Journal of Biological Chemistry*, *265*, 7207–7215.

Viikari, L., Kantelinen, A., Sundqvist, J., & Linko, M. (1994). From an idea to the industry. *FEMS Microbiological Review*, *13*, 335–350.

Vilkkula, A., Hortling, B., Tamminen, T., Teleman, A., Tenkanen, M., & Vuorinen, T. (1997). Structure of galactans in kraft cooking liquors. *Ninth International Symposium on Wood and Pulping Chemistry, Montreal, Canada*, *2*, 117-1-3.

van de Vis, J. W (1994). *Characterization and mode of action of enzymes degrading galactan structures of arabinogalactans*. PhD Thesis, Wageningen Agricultural University, The Netherlands.

van de Vis, J. W., Searle-van Leeuwen, M. J. F., Siliha, H. A., Kormelink, F. J. M., & Voragen, A. G. J. (1991). Purification and characterization of *endo*-1,4-β-D-galactanases from *Aspergillus niger* and *Aspergillus aculeatus*: Use in combination with arabinanases from *Aspergillus niger* in enzymic conversion of potato arabinogalactan. *Carbohydrate Polymers*, *16*, 167–187.

Willför, S., Sjöholm, R., & Holmbom, B. (1999). Isolation and characterization of water-soluble arabinogalactans from the heartwood of Norway spruce and Scots pine. *10th International Symposium on Wood and Pulping Chemistry, Yokohama, Japan*, *2*, 32–34.

Willför, S., Sjöholm, R., Laine, C., & Holmbom, B. (2002). Structural features of water-soluble arabinogalactans from Norway spruce and Scots pine heartwood. *Wood Science and Technology*, *36*, 101–110.

Yamaguchi, F., Inoue, S., & Hatanaka, C. (1995). Purification and properties of *endo*-β-1,4-galactanase from *Aspergillus niger*. *Bioscience Biotechnology and Biochemistry*, *59*, 1742–1744.

Zacharius, R. M., & Zell, T. E. (1969). Glycoprotein staining following electrophoresis on acrylamide gel. *Analytical Biochemistry*, *30*, 148–152.