Detection of chlorine-labelled chitosan in Scots pine by energy-dispersive X-ray spectroscopy

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Received: 24 June 2008 / Published online: 27 February 2010
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Abstract The aim of this study was to use energy-dispersive X-ray spectroscopy (EDX) to localize chitosan in the cell wall of chitosan-impregnated Scots pine. It was of interest to investigate the concentration of chitosan in wood to gain further knowledge and understanding of the distribution of chitosan in the wooden matrix. After deacetylation, chitosan was re-acetylated with chloroacetic anhydride to achieve a covalent bonding of chloride to the chitosan polymer. Chloride-labelled chitosan was measured by EDX using a scanning electron microscope and described as chloride intensity. Analysis of free chloride anions was performed by dialysis and inductively coupled plasma atomic emission spectroscopy. There was a significant correlation between the molecular weight of chitosan and the intensity of covalently bonded chloride to the chitosan polymer. High molecular weight chitosan showed a better interaction with the cell wall structure than low molecular chitosan.

Introduction

Due to the enhanced focus from governments, environmental organizations, and consumers on the use of environmentally benign wood preservatives, the use of traditional wood preservatives containing chromium and arsenic is restricted. The most common wood preservatives in Europe today are based on copper, which is on the reduction list of some European countries, including Norway. Application of fungicides is by far the most widely used method to control fungal decay in wood. However, chemical control may induce biocide resistance in fungi, and there are

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also health risks to consider when using fungicides. Other wood protection systems based on chemically modified wood products have to some extent become commercially available, but there is still a growing need to develop antifungal chemicals that are not toxic to humans and the surrounding environment.

Chitin, a 1-4 linked polymer of 2-acetamido-2-deoxy- β -D-glucose, is the most abundant natural nitrogen-containing polysaccharide, and its annual bio-production is estimated to be higher than 10^9 tons (Tracey 1957). According to Rubin (2003), the global annual production capacity for chitin is 8,000 tons. Approximately, 5,000 tons are used to manufacture glucosamine, the rest is used for chitin/chitosan applications. The major source of chitin is crustacean shells, which is a by-product of the seafood refining industry. Chitin is also found in the skeletons of several insects and in the cell walls of several fungi (Allan and Hadwiger 1979). Chitosan is deacetylated chitin and is mainly produced from chitin by hydrolysis of the amide C–N bond by strong alkali (Mima et al. 1983). From earlier work (Eikenes et al. 2005), it is known that to achieve sufficient protection of the wood against brown rot fungi, a certain level (35 kg chitosan/m³ wood) and distribution of chitosan in the wood structure is needed.

The molecular size of chitosan has a great impact on the properties of chitosan-treated wood. Larnøy et al. (2006) show that a molecular size of 159 kDa gave a 30% better recovery rate than 15 kDa, which again agrees with the results found by Eikenes et al. (2005). They also found that high molecular chitosan gave a better antifungal effect than low molecular chitosan. Larnøy et al. (2005) described the limitations in penetration of high molecular weight chitosan in wood, and the trade-off between high uptake and proper fixation was discussed. As molecular weight is an important factor when impregnating wood with chitosan, it was of interest to study different molecular weights of chitosan in the cell wall of pine.

To the knowledge of the authors, there has not been any study that confirms the presence, localization, and quantification of chitosan in the wood matrix. This paper describes the detection of chitosan in pine sapwood using scanning electron microscope (SEM) in conjunction with energy-dispersive X-ray spectroscopy (EDX). Chitosan consists of carbon, hydrogen, oxygen, and nitrogen and is therefore difficult to detect by EDX, partly due to their low energy spectrum, and partly because wood has a similar elemental spectral composition, and it is thus difficult to distinguish between spectra without deconvolution. By re-acetylation of the amine of chitosan with chloroacetic anhydride, covalent-bound chloride is achieved, and the chloride-modified chitosan molecules are now detectable by EDX.

Materials and methods

Preparation of wood samples

Clear samples of Scots pine sapwood (*Pinus sylvestris* L.), $5 \times 10 \times 30$ mm³ ($r \times t \times l$), were taken from boards vacuum dried at 60°C. To remove any free natural chloride atoms in the wood matrix that could interfere with the results, the

samples were leached according to EN 84 (1997) and left to dry in ambient condition before they were impregnated with chitosan.

The wood samples were weighed and impregnated using the following processes: initially weighed and evacuated for 1 h at 0.01 N/mm^2 followed by 1 h at a pressure of 1.0 N/mm^2 . The pressure was then released, and the weight of the samples was recorded for control of uptake. The impregnated samples were then left to dry in ambient conditions and were not leached after this point.

Production and analysis of chitosan solutions

The determination of F_A (fraction of *N*-acetylated residues) and the molecular weights (M_w) of the chitosan was examined according to methods described by Larnøy et al. (2006).

Acetylation with chloroacetic anhydride

Chitosan from shrimps with F_A 0.22 and flakes structure was milled to 0.5-mm particle size for better solubility. Chitosan powder (55 g, 10% moisture content) was added to 1 l of a 40% (w/v) sodium hydroxide solution while the sodium hydroxide solution was exothermic and stirred for 2 h to deacetylate the chitosan solution. The precipitated chitosan was then filtered and cleaned repeatedly in a mixture of water and ethanol (1:3 v/v) until a neutral pH was achieved. The chitosan was once again filtered and vacuum dried at 40°C and 0.01 N/mm^2 until the chitosan was dry. The deacetylated chitosan powder (10 g) was dissolved in deionized water (200 ml) by means of acetic acid (3.2 g, pH 5.5) during vigorous stirring. To the 5% (w/v) chitosan solution, chloroacetic anhydride (2.19 g, CAS 541-88-8) was then added to re-acetylate the chitosan. The solution of chloro-modified chitosan was then divided into five equal aliquots. Four of them were depolymerised according to Larnøy et al. (2006) with varying volumes of an aqueous solution of potassium nitrite (4% w/v) to achieve five different average molecular weights. The solutions were labelled “A” to “E”, where “A” is the low depolymerized high molecular chitosan, and “E” is the most depolymerized with low molecular weight. The solution with lowest average molecular weights was diluted in a dialysis tube of $24 \text{ \AA}$ to exclude the presence of any free chloride anions.

Energy-dispersive X-ray spectroscopy analysis

Interaction of the primary beam electrons with atoms in the sample causes shell transitions that result in the emission of characteristic X-rays. The emitted X-ray has an energy characteristic of the parent element. Detection and measurement of the energy permits elemental analysis (energy-dispersive X-ray spectroscopy or EDX). Chitosan-treated samples, cryotome cut to $50 \text{ }\mu\text{m}$, were picked up onto carbon double-faced sticky tape mounted onto aluminium stubs. Although some of the samples were carbon coated using a Jeol JEE-4X Vacuum Evaporator, it was soon clear that it had no effect on the quality, and most of the samples were not coated. The samples were inspected in a Zeiss EVO-50 EP SEM (scanning electron microscope).

As the samples were not coated, analyses were performed using the variable pressure mode at an accelerating voltage of 20 keV and working distance of 8–9 mm. Energy-dispersive X-ray spot analyses and acquisition of digital images in both secondary (SEI) and backscattered (BEI) electron imaging modes were performed using a INCA Energy 350 X-ray analytical system (Oxford Instruments, Oxford, UK) attached to the scanning electron microscope. The ratio of peak intensities measured on the specimen and cobalt standard gives an “apparent concentration”. “Apparent concentration” is further described as chloride intensity and is defined here as:

$$C'_{\text{sp}} = C_{\text{std}} \cdot \left(\frac{I_{\text{sp}}}{I_{\text{std}}} \right) \quad (1)$$

where C'_{sp} is apparent concentration, C_{std} is the weight per cent of the cobalt standard, I_{sp} is the intensity of the sample, and I_{std} is the intensity of the standard. The system was calibrated by cobalt standard every second hour. For each of the five chitosan molecular weights, a minimum of five repeated EDX measurements were performed on the earlywood cell wall (Fig. 1). For the latewood, only measurements of wood impregnated with solution A and D were performed (Table 1).

Fig. 1 Example picture of EDS spots for determination of chloride intensity. White dots are analysis taken in the earlywood cell wall. SEM photographs of Scots pine wood Chitosan “D”

Table 1 Average molecular weights in Daltons, potassium nitrite added to achieve this average molecular weight, and chloride intensity detected by the EDS in the earlywood and latewood cell wall

Solution	Average MW (kDa)	Added KNO ₂ (mg/g chitosan)	Chloride concentration by EDS in earlywood	Chloride concentration by EDS in latewood	Average $R_{G,Z}$ (nanometres)	Average L_w (nanometres)
Chitosan A	111.1	4.21	2.69 (0.78)	0.52 (0.08)	44.7	317.2
Chitosan B	75.7	6.27	1.97 (0.48)		36.2	216.0
Chitosan C	35.1	13.48	0.93 (0.59)		23.7	100.3
Chitosan D	14.5	26.37	0.40 (0.09)	0.64 (0.34)	14.6	41.3
Chitosan E dialysed	4.6	86.95	0.16 (0.02)		7.8	13.2

The table also shows the theoretic average hydrodynamic z -radii of gyration ($R_{G,Z}$) and the weight-average contour length (L_w) for the chitosan solutions used in this paper. Standard deviation is displayed in brackets

Results

Chemical characterization of chitosan

The samples had an average chitosan uptake of $35 \text{ kg/m}^3 \pm 3$ throughout the impregnations. The average molecular weight in Daltons and potassium nitrite added to achieve this average molecular weight are given in Table 1. The average molecular weight varies from as little as 4.6–111 kDa. By dialysis of the low molecular chitosan, the majority of the molecules smaller than $24 \text{ \AA}$ will diffuse in the surrounding media. By performing an element analysis by inductively coupled plasma atomic emission spectroscopy (IPS-AES), no increase was found in the amount of chloride anions into the solutions acetylated with chloroacetic anhydride compared to the standard chitosan solutions. This proved that there were no free chloride anions in the solutions due to acetylation with chloroacetic anhydride.

The fraction of *N*-acetylated residues in the original chitosan used in these tests had a F_A of 0.22. After deacetylation with sodium hydroxide, a F_A of 0.14 was achieved and after further acetylation with chloroacetic anhydride, a F_A of 0.23 was obtained.

Energy-dispersive X-ray analysis

The chloride intensity is shown in Table 1. The table shows the average detection level and standard deviation in the earlywood and latewood cell wall with varying molecular weights. One-way analysis of the concentration of chloride in the earlywood cell wall shows a significant correlation between molecular weight and chloride intensity ($r^2 = 0.824$, Prob $> F < 0.0001$, F ratio = 21.1), as shown in Fig. 2.

Fig. 2 Correlation between molecular weight in kDa and chloride intensity (Eq. 1) in the earlywood cell wall. The centre line in the diamonds is the mean value, the upper and lower lines describe the standard deviations

Discussion

Chloride-labelled chitosan with different molecular weights was detected with different intensity in the cell matrix of Scots pine sapwood. The detection of chitosan in the cell wall area indicates that chitosan is present. Chloride is a low reflective element that gives a rather low resolution with large area of detection in the EDX. The cross section for elastic scattering is proportional to the atomic number of the material squared. This means that for a fixed beam energy, electrons entering a high atomic number material will be scattered away from their original directions, giving the volume width and reducing penetration into the material (Baró et al. 1995). However, in a low atomic number material (e.g., pine wood), electrons will penetrate into the sample, losing energy as they undergo inelastic scattering events, until the energy of the electrons is such that the probability of elastic scattering begins to dominate. The outer detection area in some cases covered a slightly larger area than that of the wood cell wall cross section. However, since the centre of the beam is responsible for most of the chlorine X-ray signal, it is very unlikely that the signal was coming from only chitosan on the lumen wall but rather that within the secondary cell wall.

EDX analysis of the samples impregnated with the chitosan solution “E” that had undergone an exclusion of particles smaller than 24 Å, which excludes any free chloride anions, showed clear detection rates with EDX. Thus, it may be concluded that the chloride seen in the EDX is bonded to a larger molecule; chitosan. By impregnating wood with chitosan, there are two main challenges to overcome: the wood anatomical barriers and the shape of the chitosan polymer. When wood is treated with a protective system, the goal is mainly to fully penetrate the sapwood. The flow of fluids is assumed to flow through the ray tracheids to the axial tracheids

and then from axial tracheid via the margins of opened bordered pits. The transport through the cell wall is very slow, and with an intermicellular space of 10 Å and intermicellular capillary spaces up to 200 Å, only smaller molecules penetrate the cell wall (Frey-Wyssling 1937). Chitosan is a deacetylated polymer, which gives a large hydrodynamic volume due to its poly cationic structure and has been studied by several authors. Cölfen et al. (2001) and Berth and Dautzenberg (2002) summarized and calculated the average hydrodynamic z -radii of gyration ($R_{G,Z}$) and the weight-average contour length (L_w) by static and dynamic light-scattering measurements. According to Berth and Dautzenberg (2002), the average hydrodynamic z -radii of gyration of chitosan can be calculated in nanometres according to Eq. 2 when using fraction of N -acetylated residues from 0.2 to 0.3.

$$R_{G,Z} = (7.5 \times 10^{-2}) \times (M_w^{0.55}) \quad (2)$$

Cölfen et al. (2001) show a ratio of 350.2:1 between molecular weight in Daltons and the weight-average contour length in nanometres. The theoretic average hydrodynamic z -radii of gyration and the weight-average contour length for the chitosan solutions used in this paper are calculated and shown in Table 1.

Although the major part of the chitosan polymers is not expected to go through the cell wall due to their sizes, the chitosan polymer is quite flexible in an aqueous solution. It is therefore plausible that the medium to high M_w chitosan polymers are elastic and might enter the cell wall in the pressure phase of the impregnation, while the low M_w chitosan to some extent enters the cell wall without elasticating. A hypothesis is that the larger chitosan molecules elasticate into the cell wall and enter intermicellular capillary spaces, and then expand again, thus being trapped. This concurs with earlier findings by the authors, describing that high molecular chitosan fixates better than low molecular weight. It also concurs with the findings in this paper, giving higher chloride intensity, and thus higher chitosan concentration with increasing molecular weight.

The detection of chitosan in Scots pine by EDX has proved to be a quite accurate method, although time-consuming. Mehrtens (1999) described the penetration of chitosan in Scots pine to be only in the outermost tracheids. This is probably due to the use of very high molecular weight, which is difficult to bring into the wood matrix. Mehrtens (1999) used Lugol's solution to stain chitosan in order to visualize the lateral penetration of the reactant. However, this method is not quantitative.

As shown in Fig. 2, the chloride intensity in the earlywood cell wall correlates well with molecular weight.

Conclusion

Chitosan covalent bonded to chloride is detectable in the cell wall of pine by use of EDX. The chloride detected was covalently bonded to the chitosan polymer. The molecular weights of chitosan show a significant correlation with the chloride intensity of covalently bonded chloride to the chitosan polymer. Chitosan with high molecular weight clearly shows better interaction with the cell wall than low molecular chitosan.

Acknowledgments The authors would like to thank Trygve Krekling at The University of Life Sciences in Ås, Norway, for helping out with SEM/EDX related topics and Monica Fongen for laboratory work. The Research Council of Norway (NFR) is gratefully acknowledged for providing funding for this work.

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