MECHANISMS FOR KAPPA REDUCTION AND COLOR REMOVAL BY XYLANASES

Thomas W. Jeffries, Mark Davis,

Brian Rosin and Larry L. Landucci
USDA, Forest Service
Forrest Products Laboratory
Madison, WI 53705

ABSTRACT

Xylanases reduce kappa and release UV- and visibly-absorptive materials from kraft pulps. The extents of these actions depend on the origin and processing of the pulp, access of enzymes to the substrate, and the natures of the enzymes. Hexeneuronic acid (HexA) is a component of kraft pulp xylans that accounts for a fraction of the kappa content. It absorbs strongly in the UV region (235 nm), and its release can be used to monitor xylanase activity. This report shows that HexA release correlates closely with release of xylooligo-saccharides, but not with the reduction in kappa.

INTRODUCTION

Viikari et al. first showed in 1986 that xylanases could enhance the bleaching of kraft pulps [1]. Numerous investigators have confirmed the observation and various companies have commercialized the technology. Xylanases comprise an economical and efficient means for the displacement of elemental chlorine in pulp bleaching. The roles of xylanases in bleaching have recently been reviewed from both fundamental [2] and applied [3] aspects.

Much research has focused on the mechanism by which xylanases enhance bleaching. Paice et al. reported that xylanase prebleaching "results primarily from depolymerization but not necessarily solubilization of xylan-derived hemicellulose" [4]. Kantelinen et al. proposed that re-precipitated xylan forms an insoluble barrier that interferes with pulp bleaching and that removal of the surface xylans thereby enhances extraction [5]. Clark et al. suggested that xylanases loosen the hemicellulose structure to facilitate extraction of lignin [6], and Saake et al. concluded that various interactions of the enzyme with the pulp surface determines the efficacy of the treatment [7]. As noted by Wong et al., the exact mechanism by which bleach boosting occurs is not clear because the "carbohydrate degrading enzymes are not expected to act directly on the residual lignin in pulp" [8].

In 1993, Pate1 et al. showed that purified xylanases from *Streptomyces roseiscleroticus* differed from one another in their abilities to reduce kappa and release visible- and W-absorbing materials from a red oak kraft pulp [9]. Some of the materials had a distinct UV absorption at 237 nm. Other released materials had weaker but broad absorbance in the visible region. Extraction with alkaline peroxide removed additional material with absorptivity around 260 nm. The amounts of materials released differed with the isozyme.

It is not a simple matter to compare treatments with different enzymes, because it is difficult to attain constant enzyme dosing. The activities of xylanases are usually assessed by their capacities to release reducing sugars as measured by the dinitrosalicylic acid (DNS) method [10], or the arsenomolybdate (ARS) method [11]. Neither of these approaches are satisfactory, however, because DNS tends to overestimate while ARS tends to underestimate the number of bonds broken during hydrolysis [12]. Direct measurement of xylooligosaccharides by ion chromatography (IC) and pulsed amperometric detection (PAD) is much-more accurate.

The kappa reduction and chemical savings resulting from enzyme treatment are a complex function of the pulp type, the way it is processed both before and after enzyme treatment, and the nature of the bleaching process. In the past, kappa reduction has been attributed to removal of lignin. More recently, hexeneutonic acid (HexA), which is an unsaturated moiety formed from the 4-O-methyl-D-glucuronic acid component of xylan during kraft pulping, has been shown to account for a significant fraction of the oxidizable components in kraft pulps [13]. We have developed a chromatographic assay that enables simultaneous measurement of neutral and HexA oligosaccharides [14]. We were interested in knowing whether the release of HexA could account for the reduction in kappa and chemical demand by xylanase treatment. The results of our present study show that there is an excellent correlation between xylanase activity and HexA release, but that kappa reduction does not correlate directly with either of these factors. These findings indicate that other materials released during enzyme treatment also contribute to kappa reduction. NMR analysis of materials released by enzyme treatment shows that they have no methoxy content, so the balance of the material released by xylanase action is probably carbohydrate degradation products.

MATERIALS AND METHODS

Pulps Mixed hardwood and softwood pulps were gifts from Thilmany Pulp and Paper Company, a division of International Paper (Kaukauna, WI). They were stored wet at 3°C until used. The consistencies were 8.8 and 16.9 g moisture/100 g pulp for the hardwood (Hw) and softwood (Sw) pulp, respectively. The initial kappa levels were 14.4 and 37.2 for the Hw and Sw, respectively.

Enzymes Pulpzyme HC™, SP342™ (Nova Nordisk) and Ecozyme™ (Swan Chemicals) were gifts from the respective companies. Their xylanase activities were measured in 10 min assays at pH 7.0, 50°C using 1% (w/v) birch xylan as the substrate. The final reaction volume was 0.5 ml. The arsenomolybdate method [1] was used to measure the release of reducing sugars. Reactions were stopped by the addition of reagent A and B. The enzyme preparations were successively diluted in two- to ten-fold stages until the reactions were not substrate limited. Enzyme activities are reported as averages of duplicate reducing sugar determinations of two or more dilutions with apparent activities within 10% of one another. One IU of xylanase is defined as that amount of enzyme liberating 1 μmole of xylose reducing sugar equivalents under the conditions employed.

Pulp preparation The Hw and Sw pulps were suspended in water to approximately 2% consistency and defibrillated in a British disintegrator for 3 min. The pulps were drained on filter paper (Fisher P8) to remove excess water. Each pulp sample was divided into two batches. One-half was suspended in distilled water, the other half was suspended in 100 mM of sodium acetate, (pH 7.8); each was at 10% consistency. The pulp samples were equilibrated at 50°C for 20 h. The pulps were then recovered by filtration as previously and adjusted to a consistency of approximately 16% by the addition of water or acetate buffer, respectively.

Enzyme treatments HW and SW acetate (A) or water (W) washed pulp samples (33.7 and 26.3 g OD, respectively) were placed in Ziploc™ bags and enzyme solutions diluted in water (25 IU enzyme/g of oven dry pulp) were added to each; four of the samples were used as controls and received only

1

water. This resulted in a total of 16 pulps and treatment conditions. Final consistency after addition of diluted enzymes or water was approximately 10%. The pulps were incubated at 50°C for 3 h with kneading every 30 min. The samples were then drained on Fisher P8 filter paper on a Büchner funnel. The free solutions containing solubilized sugars, HexA and chromophores were frozen and the drained pulps were steamed for 30 min to kill any residual enzymatic activity. Pulps were then refrigerated at 3°C until analyzed. Kappa was determined by TAPPI method T236. Reported values are the average of two determinations.

Sugar and HexA determinations Chromatographic analysis was performed by high pH anion exchange on a Dionex CarboPac PA1™ column. Initial conditions eluted neutral xylan oligosaccharides up to a degree of polymerization (DP) of 5 within 6 min. A gradient of increasing ionic strength was used to elute the more strongly retained acidic moieties. Carbohydrates were detected by their oxidation at a gold electrode with a pulsed amperometric detector (PAD). For detection of HexA, a diode array detector (DAD) plumbed in series with the PAD was used to monitor the column effluent at 230 mn. Oxidizable, UV-absorbing components were identified as a series of HexA-xylose oligosaccharides based upon criteria described previously [14]. The areas under the UV-peaks were summed and this value was used as a relative measure of the total chromophore, *i.e.,* the HexA content of the series.

RESULTS

Each of the three commercial enzyme preparations, Pulpzyme HC (P), Ecozyme (E) and SP342 (S) had similar xylanase activities (Table 1). The exact origins and compositions of these preparations are proprietary, but both P and E are derived from cloned genes while SP342 is derived from a fungal fermentation *(Humicola insolens)* and contains both cellulase and xylanase activities. It is most commonly used for deinking or denim treatment.

Table 1. Xylanase activities.

Preparation	IU/ml
Pulpzyme HC (P)	3,734
Ecozyme (E)	4,606
SP342 (S)	2,319

The enzyme levels employed here (25 IU/g OD pulp) were much higher than what one would normally use for commercial treatment. This was done in order to assure that the amount of enzyme would not be limiting and our observations would reflect the characteristics of the pulp, the nature of the pulp treatment or the properties of the enzyme.

An ion chromatogram of an SP342 reaction are shown in Figure 1. This product profile was typical except that with Pulpzyme and Ecozyme, the first two acidic products (eluting between 20 and 22 min) were not observed. Under the conditions employed for chromatography, all of the neutral oligosaccharides eluted within 7 min, and the bulk of the acidic oligosaccharides eluted between 21 and 26 min. No glucose or cellulooligosaccharides were present in only trace amounts. This is not surprising in the case of the pure xylanase preparations, but SP342 is sold primarily for its cellulase activity. No neutral or HexA products were released from the control pulps.

We examined the effect of sodium acetate treatment because Buchert et al. [15] had shown that sodium counter ions increase pulp reactivity. As can be seen from Table 2, the bulk of the neutral sugars were recovered as xylotriose (X3). Only trace amounts of neutral products were observed

larger than xylopentaose (X5). Similar amounts of xylan and HexA were liberated from Hw by S and P, but only about 1/3 of this amount was liberated by E. On Sw, S activity remained much greater than that of E, but the activity of P was reduced to an intermediate level. Each of the enzymes released more sugar and HexA from the acetate-washed pulp than from the water-washed pulp except in the case of softwood treated with Ecozyme. In general, much more HexA was released from Hw than from Sw, but Ecozyme released relatively little xylan or HexA from any of the four pulp samples (HwA, HwW, SwA, SwW). The xylan released from Hw by S or P corresponds to roughly 2% of the total mass of the pulp.
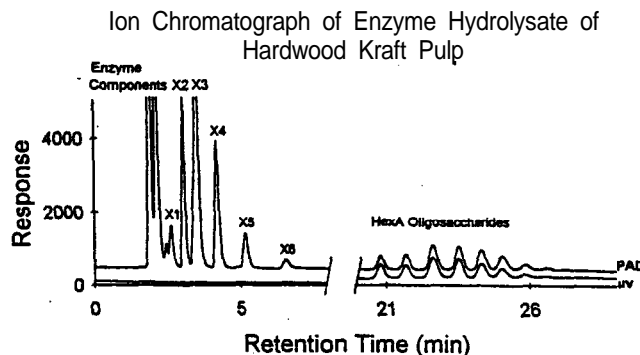

Figure 1.

Overall, there was a close correlation between the release of neutral and HexA xylooligosaccharides from any one sample by any one enzyme (Fig. 2). The observed reduction in kappa following enzyme treatment did not correlate with either total neutral or HexA products released. In the case of hardwood pulps, it was higher in all of the acetate-washed samples and lower in all of the water-washed samples, respectively.
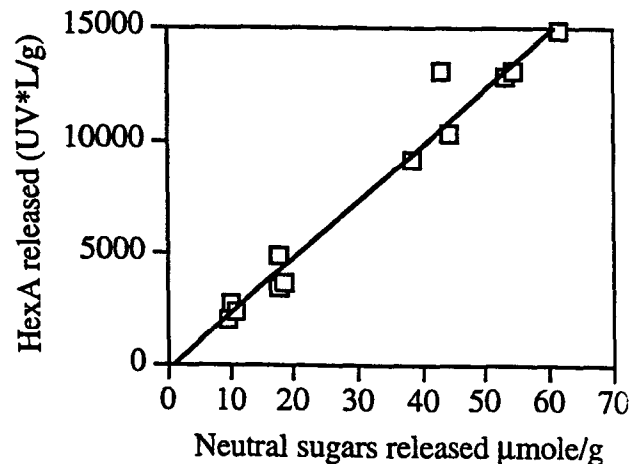

Figure 2. Release of neutral and HexA xylooligosaccharides from hardwood and softwood pulps by three different xylanase preparations. ($r^2 = 0.967$)

DISCUSSION

These results indicate that the enzymatic release of neutral and HexA oligosaccharides from pulps correlate closely and both represent xylanase activity. The apparent activity against pulps, however, does net correlate well with apparent activity against birch xylan in solution: Even though all three enzymes were titered against a single substrate under the same conditions, sugar and HexA released varied greatly with the enzyme, pulp and the washing conditions employed.

Enzyme treatment appeared to reduce kappa, but the observed reduction did not correlate well with the neutral or

2

HexA oligosaccharides released. Thus, a significant portion of the xylanase-induced reduction in kappa must be due to mechanisms distinct from HexA liberation. Because the solubilized material did not appear to contain methoxy groups, it probably is not derived from lignin. Rather, it is probably derived from degraded carbohydrate components.

REFERENCES

1. VIIKARI, L., RANUA, M., KANTELINEN, A., SUNDQUIST, J., AND LINKO, M. "Bleaching with Enzymes. Proc. 3rd Int. Conf. Biotechnology in the Pulp and Paper Industry, STFI, Stockholm, Sweden, 67-69 (1986).

2. SUURNAKKI, A., TENKANEN, M., BUCHERT, J. AND VIIKARI, L. "Hemicellulases in the Bleaching of Chemical Pulps", *Adv. Biochem. Eng. Biotechnol.* 57:261-287 (1997).

3. TOLAN, J.S. AND GUENETTE, M. "Using Enzymes in Pulp Bleaching: Mill Applications. *Adv. Biochem. Eng. Biotechnol.* 57:289-310 (1997).

4. PAICE, M.G., GURNAGUL, N., PAGE, D.H AND JURASEK, L. "Mechanism of Hemicellulose-Directed Prebleaching of Kraft Pulps", *Enzyme. Microb. Technol.* 14(4):272-276 (1992).

5. KANTELINEN, A., HORTLING, B., SUNDQUIST, J., LINKO, M. AND VIIKARI, L. "Proposed Mechanism of the Enzymatic Bleaching of Kraft Pulp with Xylanases", *Holzforschung* 47(4):318-324 (1993).

6. CLARK, T.A., STEWARD, D., BRUCE, M. McDONALD, A., SINGH, A. AND SENIOR, D. "Improved Bleachability of Radiata Pine Kraft Pulps Following Treatment with Hemicellulosic Enzymes", *Appita* 44:389-383 (1991).

7. SAAKE, B., CLARK, C., AND PULS, J. "Investigations on the Reaction mechanism of Xylanases and Mannanases on Sprucewood Chemical Pulps", *Holzforschung* 49:60-68 (1995).

8. WONG, K.K.Y., DeJONG, E. SADDLER, J.N. AND ALLISON, R.W. "Mechanisms of Xylanase Aided Bleaching of Kraft Pulp", *Appita* 50(5):415-422 (1997).

9. PATEL, R.N., GRABSKI, A.C. AND JEFFRIES, T.W. "Chromophore Release from Kraft Pulp by Purified *Streptomyces roseiscleroticus* xylanases". *Appl. Environ. Microbiol.* 34:405-412 (1993).

10. MILLER, G.L. "Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar", *Anal. Chem.* 31:426-428 (1959).

11. SOMOGYI, M. "Notes on Sugar Determination", *J. Biol. Chem.* 195:19-23 (1952).

12. JEFFRIES, T.W., YANG, V. and DAVIS, M. "Comparative Study of Xylanase Kinetics Using Dinitrosalicylic, Arsenomolybdate and Ion Chromatographic Assays", *Appl. Biochem Biotechnol.* - in press)

13. GELLERSTEDT, G., "An HPLC Method for the Quantitative Determination of Hexeneuronic Acid Groups in Chemical Pulps", *Carbohyd. Res.* 294(1): 41-51 (1996).

14. DAVIS, M., ROSIN, B., LANDUCCI, L.L. AND JEFFRIES, T.W. "Characterization of UV Absorbing Products Released from Kraft Pulps by Xylanases", Tappi Biol. Sci. Symp. 435-442 (1997).

15. BUCHERT, J., TENKANEN, M., PITKÄNEN, M. and VIIKARI, L. "Role of surface charge and Swelling on the Action of Xylanases on Birch Kraft Pulp", Tappi J. 76(11):131-135 (1993).

Table 2. Release of Neutral and HexA Acidic Oligosaccharides from Hardwood and Softwood Pulps.

Sample†	μmoles/g pulp					μmole/g X1-X5	UV*L/g HexA	Kappa reduction*
	X1	X2	X3	X4	X5			
HwAE	0.12	2.38	10.70	3.98	0.33	17.5	4847	3.2
HwAP	1.37	18.70	26.96	7.38	0.27	54.7	13032	3.3
HwAS	4.82	18.82	28.20	8.41	1.59	61.8	14791	4.0
HwWE	0.18	1.87	6.05	2.01	0.00	10.1	2777	0.7
HwWP	1.63	18.83	25.73	6.85	0.37	53.4	12830	1.6
HwWS	2.72	11.90	18.91	7.36	2.04	43.0	13001	1.7
SwAE	0.12	1.33	5.58	2.30	0.31	9.6	2030	nd
SwAP	0.86	7.10	8.02	2.25	0.11	18.3	3724	nd
SwAS	5.83	12.76	20.82	4.25	0.87	44.5	10359	nd
SwWE	0.20	2.18	6.28	2.31	0.15	11.1	2418	nd
SwWP	0.77	7.72	7.19	1.82	0.07	17.6	3381	nd
SwWs	5.15	11.92	17.21	3.42	0.79	38.5	9074	nd

†Hw = hardwood; Sw = softwood   *Compared to water or acetate controls
A = acetate-washed; W = water-washed;
E = Ecozyme; P = Pulpzyme; S = SP342

3

Hyatt Regency Hotel
Vancouver, BC Canada
June 16 – 19, 1998

7th International Conference on

Biotechnology

in the

Pulp and Paper Industry

Poster

Presentations

Vol. C

Jeffries, Thomas W.; Davis, Mark; Rosin, Brian; Landucci, Larry L. 1998. Mechanisms for kappa reduction and color removal by xylanases. In: Seventh international conference on biotechnology in the pulp and paper industry: Poster presentations vol. C; 1998 JUNE 16-19; Vancouver, BC, Canada. Montreal, Quebec, Canada: Canadian Pulp and Paper Association: C41-C43.