CHARACTERIZATION OF UV ABSORBING PRODUCTS RELEASED FROM KRAFT PULPS BY XYLANASES

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ABSTRACT

Xylanase treatment of kraft pulps concomitantly decreases the amount of chemicals required for bleaching and releases chromophores. Enzymatically-released products absorb strongly in the visible and ultraviolet regions. Release of materials absorbing at 235 nm correlates positively with the efficacy of enzymatic prebleaching. Proton and carbon NMR revealed no evidence of aromatic moieties in the enzymatically-liberated material. Xylanase products were fractionated by solid-phase extraction and analyzed by ion exchange chromatography. Polar materials passing through the column did not show appreciable color but did include most of the 235 nm absorbing material. Absorbance at 235 nm was associated with a group of acidic oligosaccharides tentatively identified as Hex-A-xylooligosaccharides based upon their UV spectra and chromatographic properties. Sequential UV-absorbance and electrochemical oxidation of moieties separated by high pH anion exchange elucidated striking differences in the action patterns of three commercial enzyme preparations with respect to HexA-xylan release. This analytical approach provides a means for the rapid, routine assessment of molecular events associated with enzymatic prebleaching.

INTRODUCTION

Viikari et al. (1) first showed that treating kraft pulps with fungal xylanases decreases the amount of bleach chemical required to attain a given brightness. Subsequent numerous advancements have moved the technology toward commercialization (see (2) and references therein). More recently, it has been shown that enzyme treatment reduces the pulp kappa number (3). The molecular basis of these effects are not well understood. The mechanism behind xylanase-enhanced bleaching is still being elucidated. One hypothesis is removal of xylan allows lignin extraction (4). However, xylanase activity, as measured by reducing sugar release, does not correlate well with pulp brightness or the decreased chemical demand brought about by a given enzyme treatment (2).

Previous work by our group has demonstrated the enzymatically-catalyzed liberation of materials from pulps with UV absorption at *ca.* 237 and 260 nm (3) and liberation of material absorbing at 237 correlates well with enhanced brightness following bleaching (5). Different purified xylanase isozyme fractions release UV-absorbing materials to different extents, and in some instances, activities of the isozymes are synergistic (6).

Recent work in Finland (7,8) and Sweden (9,10) has verified the presence of a moiety in kraft pulps that could be responsible for these observations: 4-deoxy-β-L-*threo*-hex-4-enopyramosyluronic acid (hexenuronic acid, HexA). This species is created during kraft pulping by β-elimination of the 4-O-methyl-D-glucuronic acid (MGlcA) component of xylan (11). The resulting HexA has an unsaturated C4-C5 bond, giving it a W-absorbance in the 230-235 nm region (8,10).

A positive interference by HexA on kappa determinations of hardwood pulps on the order of 30-40% has been demonstrated (9). However, the role of this species in the increase in brightness and decrease in chemical consumption during bleaching has not been elucidated. This report describes the putative identification of HexA-xylose oligosaccharides liberated by xylanase treatment of pulps, and discusses the relevance of this finding to mechanistic studies of the enzymatic prebleaching of pulps.

METHODOLOGY

Xylan, purchased from Sigma Chemical Co., was prepared by alkaline extraction of birch wood. Aspen kraft pulp was obtained as a gift from Potlatch Corporation. Pulp (200 g oven-dry) was suspended in water at 3% consistency and dispersed with a British disintegrator. Fibers were washed three times by suspending in water at 10% consistency and holding at 50°C for 18 h. Commercial xylanase preparations were obtained from three suppliers: Enzyme 1 was S P 342 and Enzyme 3 was Pulpzyme HC; both were from Novo Nordisk. Enzyme 2 was Ecozyme from Zeneca Bio Products. Xylanase treatments of pulp and xylan hydrolyses have been described elsewhere (4-6). Pulp treatments were carried out at 50°C at pH 8.5 In one experiment, pulps were treated with 3 IU/g of Enzyme 1 or Enzyme 2 for 2 h. In a second experiment, pulp was treated with 23 IU/g for 24 h. Supernatant solutions were recovered by filtration and absorption profiles of enzyme hydrolysates were obtained using a scanning *W* spectrometer following dilution. Solid phase extraction employed a SPE-C18 Sep-Pak (Waters)

cartridge. The resin-was wetted with methanol prior to sample application in water. The column was washed 3 times with water then bound materials were eluted with methanol, acetone and hexane.

Chromatographic analysis was performed by high pH anion exchange on a Dionex CarboPac PA1 column. Initial conditions were employed which eluted neutral xylan oligosaccharides up to a degree of polymerization (DP) of 5 within 6 min. A gradient of increasing ionic strength was then 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). A diode array detector (DAD) plumbed in series with the PAD was used to monitor the column effluent at 230 nm. Spectral scans were obtained by the DAD for chromatographic peaks as identified by absorbance at 230 nm.

The proton (250 MHz) and carbon (62.9 MHz) NMR spectra were obtained with a Bruker Avarice DMX-250 spectrometer. The NMR sample was prepared by concentrating the hydrolysate on a rotary evaporator (40°C/15 mm) and dissolving the residue in 38% acetone d_6/D_2O. A 5 mm 4-nucleus probe was used and standard proton and proton-decoupled carbon spectra were acquired. The proton chemical shifts were referenced to tetramethylsilane (δ = 0.0 ppm) and the carbon chemical shifts to the acetone-d_6 multiplet (δ = 29.83).

RESULTS

Identification of HexA oligosaccharides. The UV absorbance profile of material released from a hardwood pulp by enzymatic treatment is shown in Fig. 1. Substantial increase in chromophores in the xylanase hydrolysate as compared to a control treatment without enzyme is evident. Absorbance decreased rapidly with increasing wavelength from its maximum of 190 nm, with a shoulder apparent at *ca.* 230-235 nm. This shoulder was more defined in the polar fraction following solid phase extraction on C18 silica cartridges (Fig. 2). The fact that this material is not retained by the C18 solid phase indicates that it is hydrophilic, and is consistent with a carbohydrate structure. The bulk of the material absorbing in the visible region was retained on the column and only partially released by methanol washing.

Hydrolysates were assessed for the presence of lignin-derived materials by NMR and chromatographically with detection by UV-absorbance at 275 nm (bandwidth 45 nm). Proton and carbon NMR revealed no evidence of aromatic moieties in the crude enzymatically-liberated material. However the NMR spectra were consistent with the presence of xylooligosaccharides. In addition, no phenolic materials were retained by solid phase extraction, as determined by elution of cartridges with methanol or acetonitrile and five-fold concentration prior to chromatographic analysis.

Earlier work (3) indicated that alkaline extraction of xylanase-treated pulps liberates substantial amounts of material absorbing at 260 nm, although whether this absorption is due to lignin components or to carbohydrate degradation products is not presently known.

High pH anion exchange, the method of choice for chromatographic analysis of carbohydrates, was used to analyze the hydrolysates. Chromatograms of solid phase extracted hydrolysates did not differ from those of non-extracted hydrolysates, and all subsequently described analyses involved the latter. The elution order of carbohydrates with this chromatographic system follows the pattern of neutral prior to acidic, and smaller prior to larger.

Preliminary chromatographic analysis with PAD detection indicated the presence of three discrete series of peaks in xylanase hydrolysates of pulp and xylan. The first series, eluting within 6 min after injection, consisted of high concentrations of neutral xylose and its oligomers up to the pentomer (data not shown). The second series, presumably acidic due to their longer retention times, eluted between 16 and 21 min (Fig. 3), A third series, also presumably acidic, eluted between 21 and 26 min (Fig. 4).

Given that MGlcA is the only acidic component expected in non-modified xylan, it is probable that one of these acidic series is HexA derived from MGlcA. The predominance of the 21 to 26 min series of peaks in pulp hydrolysates (Figs. 4 to 6) led us to hypothesize that this series represents HexA-containing oligosaccharides. The later elution of HexA-containing oligomers is consistent with structural considerations as well: The unsaturated bond at C4-C5 would be expected to render the carboxylic moiety of HexA more acidic than that of the parent MGlcA, and consequently more strongly retained by anion exchange resins. This hypothesis was tested by linking DAD to PAD in a dual detection scheme for the simultaneous determination of UV-absorbance and electrochemical properties of the chromatographically separated components. As predicted, the only substantial absorbance at 230 nm occurred in the third series of peaks (Figs. 4 to 6).

The UV-scans of peaks C to E of Fig. 6 are shown as overlays in Fig. 7. We conclude from their identical UV-spectra that the same chromophore is present in each chromatographically distinct species. An absorbance maximum of 235 nm was observed, in good agreement with literature references to UV-absorbance properties of HexA (8,10). In contrast, the absorbance profile of MGlcA falls rather steadily from a maximum of >210 nm with increasing wavelengths to low absorbance values at 235 nm (data not shown). Taken together, these observations strongly support the conclusion that the series of peaks described above represent xylose oligomers containing HexA as a chromophore. The novel non-chromophoric peak in the Enzyme 2 hydrolysate (Fig. 5) underscores the importance of coupling UV-absorbance to electrochemical detection: This peak is

thereby identified as an *oligomer* lacking HexA, in spite of its retention time within the HexA series.

Analysis of xylanase action. As shown in Fig. 7, the same chromophore, presumably HexA, is associated with the acidic W-absorbing peaks of pulp xylanase hydrolysates. In general, elution time in this chromatographic system is positively correlated with DP. Thus, it is reasonable to interpret this series of peaks as representing oligomers of HexA with consecutive additions of xylose. The difficulty lies in assigning the correct number of xylose units to the first member of the series: First, production of HexA *per se* by xylanase treatment is unlikely because its bond to xylan is probably stable. Second, the HexA-xylose linkage is an unnatural consequence of an industrial process, and is therefore an improbable candidate for enzyme action. Third the presence of the HexA residue may inhibit enzyme action at nearby xylose-xylose bonds, such that the smallest oligomer produced by a given enzyme might be *e.g.* HexA-xylotriose.

If the HexA series obtained with Enzyme 1 treatment is interpreted as being headed by HexA-xylose (Peak A, Fig. 4), this is precisely the interpretation which must be given the first major component of the series obtained with the other enzyme treatments (Peak C, Figs. 5 and 6). However, it must be stressed that the general tendency of carbohydrates to elute in order with their DP may not be strictly observed for this strongly acidic class of oligomers. Exceptions to this tendency have been reported (12,13), although under different conditions than those used in the present work. Resolution of this issue awaits the crucial task of isolation and analysis by GC/MS of HexA oligomers.

The correlation between pulp brightness and xylanase-catalyzed liberation from pulp of material absorbing at 237 nm (5), presumably HexA, has been mentioned. The analytical approach described here provides a means to assess the molecular basis of this correlation. In one approach, enzyme action patterns can be determined by direct analysis of pulp hydrolysates. The pattern of reaction products shown in Figs. 4 to 6 illustrate the power of this approach in the elucidation of mechanisms and limitations of enzyme action. Comparison of Enzyme 1 and Enzyme 2 treatments under identical incubation conditions indicate that given the retention time-DP relationship discussed above, the former (Fig. 4) is able to liberate smaller HexA oligomers than is the latter (Fig. 5). Further, even though treatment with Enzyme 3 was 12 times longer and with almost 8 times more activity, it was also unable to generate substantial quantities of the smaller HexA oligomers characteristic of the action of Enzyme 1 (Fig. 6).

An alternative approach to analysis of enzyme action patterns is the compositional analysis of materials liberated by a given treatment. Compositional analysis would be simplified by a second, exhaustive enzymatic digestion of liberated materials to generate the simplest possible product profiles. The potential failure to achieve a single HexA component for quantitation is obviated by the expectation that the molar absorption coefficient of the chromophore will be identical regardless of the size of the oligomer with which it is associated. Thus, given standards for calibration, the total absorbance at 230 nm exhibited by all the HexA peaks can be used to quantitate total liberated HexA. This measure of HexA will be vastly superior to that obtained by simple spectrometric means, due to the contribution of undefined interferences in the latter case (see Figs. 1 and 2). Both approaches provide us with powerful tools with which to assess the liberation of HexA from pulp and its role in enzymatic prebleaching.

Finally, it should be mentioned that the use of two distinct detectors provides the opportunity to use the ratio of the two signals as a measure of peak purity. Spectral assessments may be inadequate for this task for the same reason that the three distinct HexA oligomers in Fig. 6 exhibited identical spectra (Fig. 7): Likely contaminants are carbohydrates with poor W-absorbing properties. However, to the extent that non-chromophoric carbohydrate components are included in the peak, the PAD/UV ratio will be increased The consistency of this ratio in pulp hydrolysates obtained by treatment with three different enzymes indicates that the peaks C and D are highly pure (Table 1). (The alternative hypothesis, that the three distinct treatments give precisely the same ratio of HexA oligomer and contaminant is highly unlikely.) The less consistent result for peak E may indicate impurity, or may be a simple consequence of the error inherent in the measurement of this more minor component. In contrast, experiments with xylan preparations have given higher PAD/UV ratios (data not shown), indicating a lack of purity in those cases.

Table 1. Detector ratio as a purity indicator.

Treatment	Detector Ratio (PAD/UV)		
	Peak C	Peak D	Peak E
Enzyme 1	5.26	6.44	6.73
Enzyme 2	5.19	6.35	8.02
Enzyme 3	5.29	6.14	7.58
Mean	5.25	6.31	7.44
Std Dev	0.05	0.16	0.66

CONCLUSION

An acidic series of oligosaccharides liberated from pulp by xylanase treatment was tentatively identified as a series of HexA-xylooligosaccharides. A dual detection system capitalizing upon the electrochemical and W-absorption properties of these moieties was developed. The system is suitable for the rapid, routine analysis of enzyme action patterns, and

potentially for quantitation of liberated HexA as well. Work continues to refine and calibrate the system, and to correlate molecular observations with the efficacy of enzymatic prebleaching treatments.

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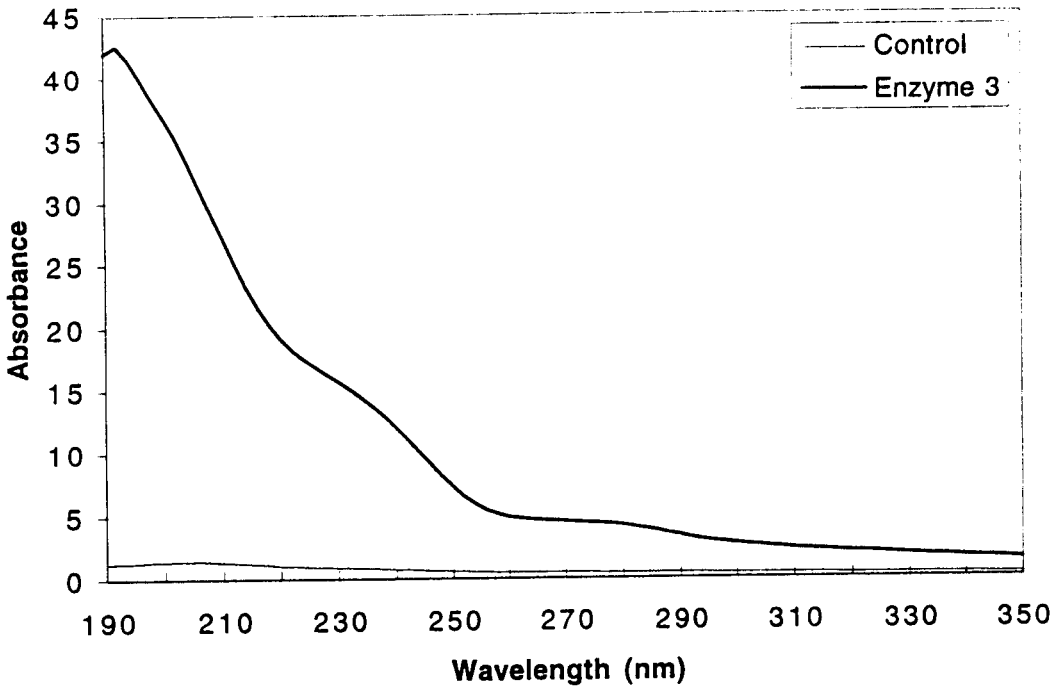

Figure 1. Comparative absorptivity of sample and control supernatant solutions. Aspen kraft pulp was washed three times then treated with 25 IU/g of Enzyme 3 for 3 h at 50°C and the resulting supernatant solution was recovered by filtration. A washed control pulp was treated in the same manner but without enzyme. Enzyme-treated samples were diluted 50:1 prior to scanning and the dilution factor was used to calculate absorbance.

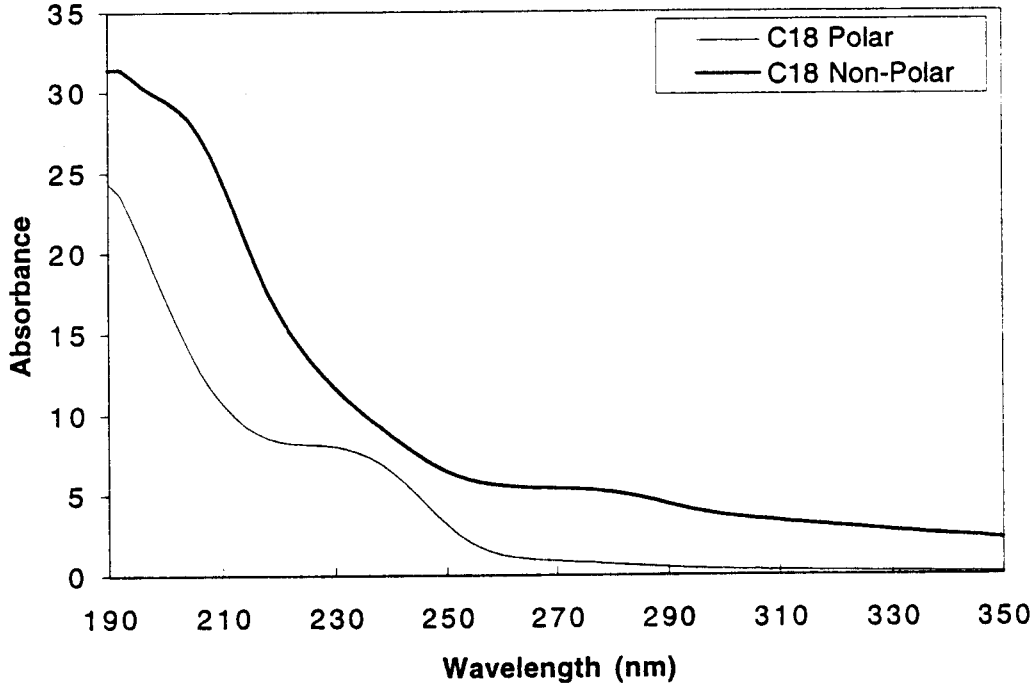

Figure 2. Comparative absorptivity of polar and non-polar fractions from enzyme treatment.

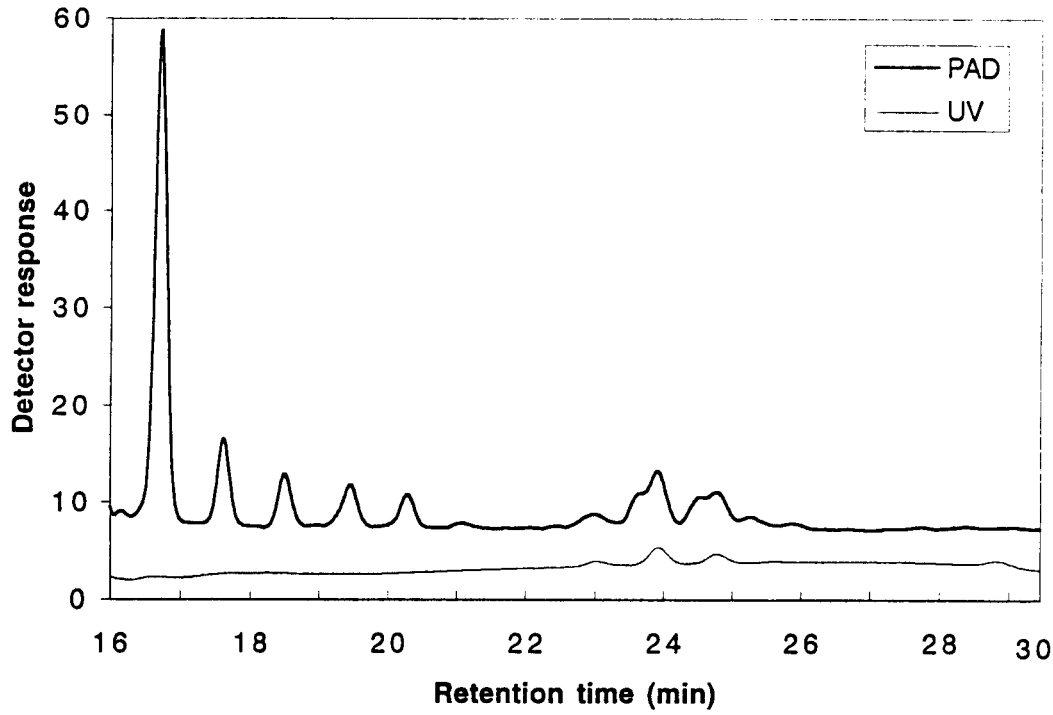

Figure 3. High pH anion exchange chromatogram with coupled pulsed amperometric detection and UV detection of hydrolysate resulting from xylanase treatment of birch xylan for 2 hr with Enzyme 2 in 10 mM phosphate buffer. PAD and UV detector output (μvolts) has been reduced by factors of 2500 and 1000, respectively, for graphical presentation.

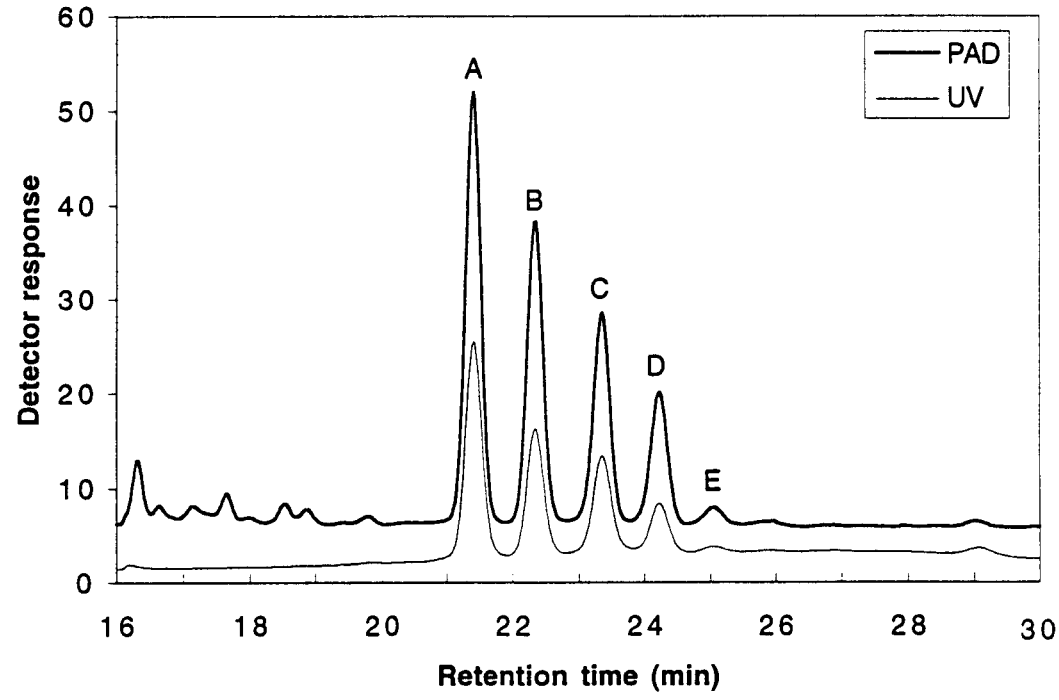

Figure 4. Chromatogram of hydrolysate resulting from xylanase treatment of hardwood kraft pulp for 2 hr with Enzyme 1. Detection and scaling as in Fig. 3. Pulp consistency was 12.9%; enzyme loading rate was 3 IU/g dry wt pulp.

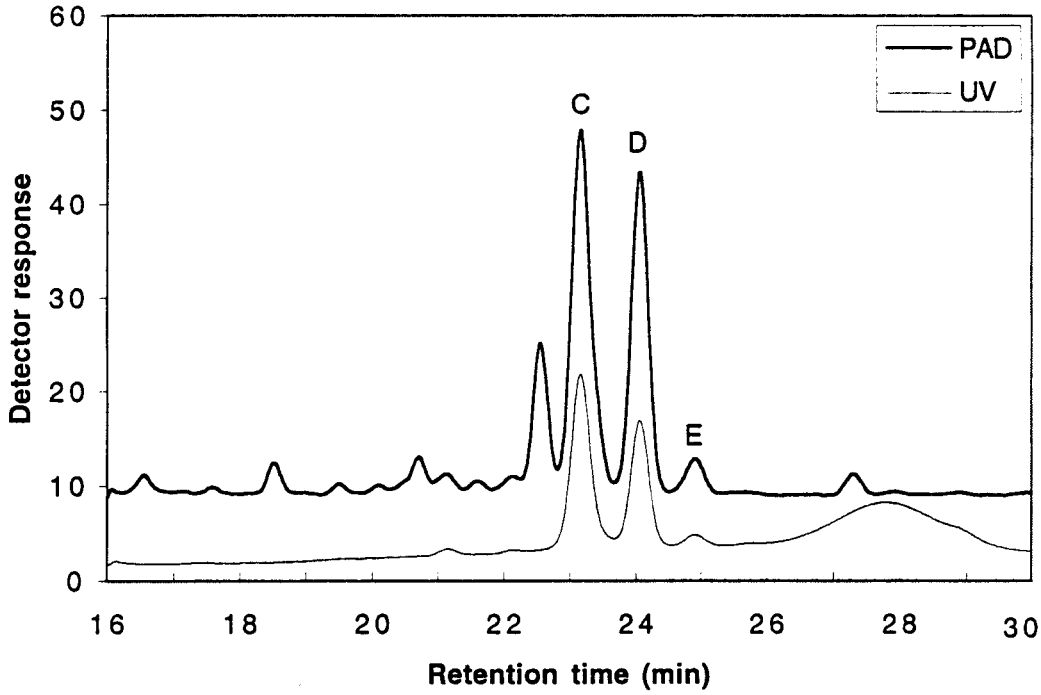

Figure 5. Chromatogram of hydrolysate resulting from xylanase treatment of hardwood kraft pulp for 2 hr with Enzyme 2. Detection and scaling as in Fig. 3. Pulp consistency was 12.9% enzyme loading rate was 3 IU/g dry wt pulp.

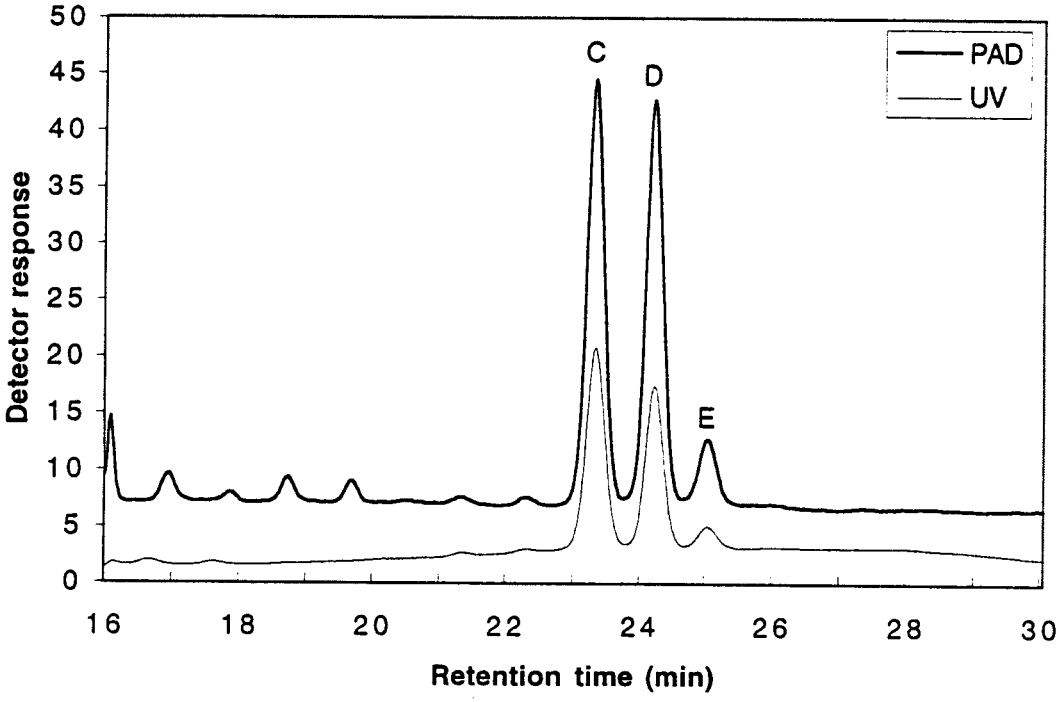

Figure 6. Chromatogram of hydrolysate resulting from xylanase treatment of hardwood kraft pulp for 24 hr with Enzyme 3. Detection and scaling as in Fig. 3. Pulp consistency was 10.9%. Enzyme loading rate was 22.9 IU/g dry wt pulp.

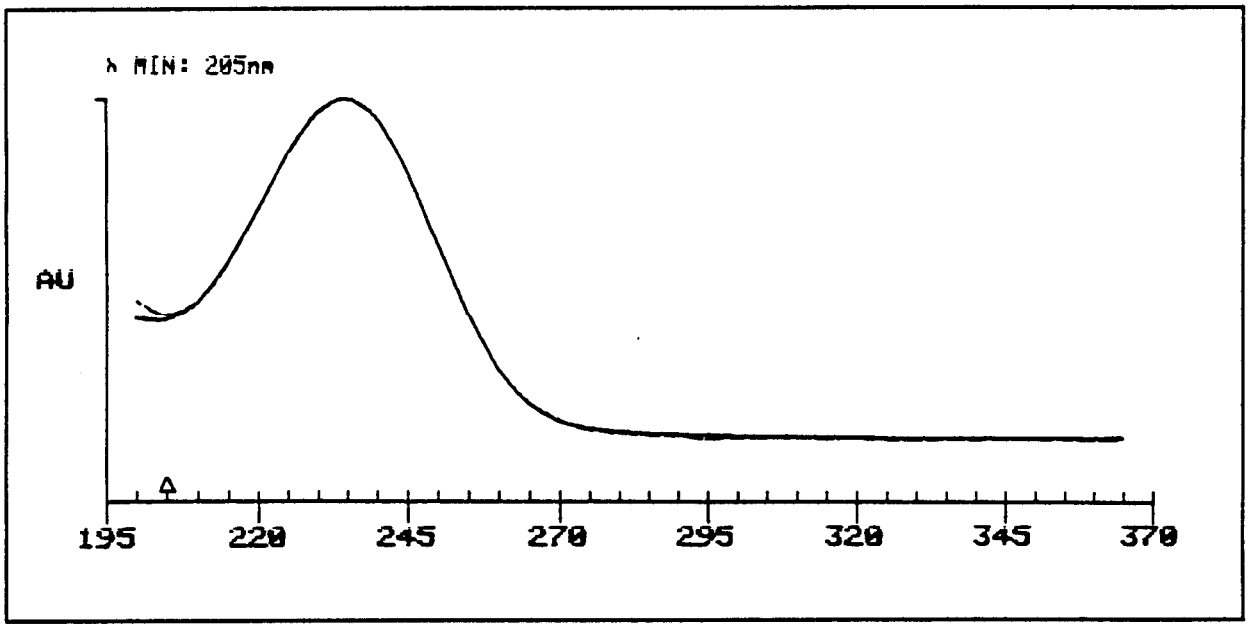

Figure 7. Spectral overlays of peaks C, D and E from Fig. 6, as obtained by diode array detection of the chromatogram.

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