Carbohydrate Polymers 17 (1992) 19–28

Crosslinked xylan as an affinity adsorbent for endo-xylanases

H. Rozie[a, c]*, W. Somers[b], A. Bonte[a, c], F.M. Rombouts[a] & J. Visser[c]

[a]*Department of Food Chemistry,* [b]*Department of Food and Bioprocess Engineering, Agricultural University Wageningen, 6703 HD Wageningen, The Netherlands*

[c]*Department of Genetics, Agricultural University Wageningen, 6703 HA Wageningen, The Netherlands*

(Received 17 August 1990; revised version received 5 October 1990; accepted 19 October 1990)

In order to facilitate the purification of xylanases from *Aspergillus niger*, an affinity adsorbent has been developed from oat spelts xylan. A suitable adsorbent was only obtained by crosslinking oat spelts xylan with epichlorohydrin in water but not in ethanol or ethanol–water mixtures. After some initial degradation of the adsorbent (approximately 4%), no significant biodegradation was measured with a reused adsorbent. Up to 60% of the xylanase activity from an *Aspergillus niger* enzyme mixture (50 mU/ml) was adsorbed at pH 4 (50 mM sodium acetate buffer). The degree of adsorption to crosslinked xylan of four fractions of this preparation, previously separated by DEAE-Biogel A chromatography, varied between 40 and 90%.

Adsorption was strongly dependent on pH and ionic strength and desorption was easily accomplished by an increase in ionic strength. In addition to xylanases, polygalacturonases were also adsorbed to the matrix. No significant adsorption of β-D-xylosidase, α-L-arabinofuranosidase, β-D-galactosidase, β-(1,4)-galactanase, β-(1-3/6)-D-galactanase or cellulase activities was found.

INTRODUCTION

Xylanases play an important role in the decomposition of plant waste material by microorganisms. Significant improvement in the economics of bioconversion of biomass to fuels and chemicals can be achieved if the hemicellulosic component of biomass, which includes xylan, is utilized (Woodward, 1984). This application, and also the liquefaction of fruits and vegetables, requires the joint activity of xylanase and cellulolytic and pectinolytic enzyme systems (Biely, 1985). Cellulolytic enzymes must be absent if xylanases are used to remove xylan from wood pulp in the paper manufacturing process. In this way the xylan of the pulp can be converted to a mixture of sugars with economic value, whereas the alkaline extraction of pulp results in xylan-containing effluent streams (Paice & Jurasek, 1984).

Due to its complex structure, complete breakdown of naturally occurring branched acetylated xylan requires the action of several hydrolases. Important are endo-β-(1,4)-xylanase (EC 3.2.1.8) that degrades the xylan backbone and β-xylosidase (EC 3.2.1.37) that converts xylo-oligosaccharides to D-xylose. The xylan backbone, however, is not completely accessible to xylanases. The enzymes α-D-glucuronidase and α-L-arabinofuranosidase are required to remove sugar residues from side chains of the xylan backbone. An acetylesterase should be present to remove acetyl groups from the xylose residues in the polymer backbone.

We are interested in developing methods whereby these enzymes can be easily recovered from complex extracellular enzyme mixtures. Ideally, affinity chromatography could provide a single-step procedure to isolate a particular enzyme. Xylanase from *Irpex lacteus* was purified by pseudo-affinity chromatography (hydrophobic interaction, dye ligand (Hoebler & Brillouet, 1984)). However, an affinity method would be more specific if xylan could be used as an adsorbent, since it can be recognized by the desired enzymes. Modification of original substrates by crosslinking with epichlorohydrin resulted in suitable adsorbents with pectate (endo-polygalacturonase (Rexová-Benková & Tibensky, 1972), endopectate lyase (Visser *et al.*, 1979)), starch (α-amylase (Weber *et al.*, 1976 and Rozie *et al.*, 1990))

*Present address: DSM Research, CP-Chemicals, Postbox 18, 6160 MD Geleen, The Netherlands.

Carbohydrate Polymers 263-8223/92/$03.50

and mannan (1,2-α-mannosidase (Tanimoto *et al.*, 1986)). In this paper the development of a suitable adsorbent for xylanase, by crosslinking of xylan with epichlorohydrin, is described.

MATERIALS AND METHODS

The xylans used to prepare crosslinked adsorbents were from oat spelts (batch no. X-0376 and 107-F-0802 from Sigma, St Louis, Missouri, USA) and from larch (batch no. 90581 from Koch & Light, Haverhill, UK). Oat spelts xylan (batch no. X-0376) was used to determine xylanase activity.

Sodium pectate obtained from Sigma (batch no. P-1879) was used to determine polygalacturonase activity. The content of polygalacturonic acid was 85–90%.

CMC-cellulose (Akucell type AF 0305, Akzo, Arnhem, The Netherlands) was used to determine cellulase activity.

Coffee arabino-β-(1,3/6)-D-galactan, isolated from green coffee beans (*Coffea arabica*) according to the method of Wolfrom & Patin (1965), was used to determine β-(1,3/6)-D-galactanase. Potato arabino-β-(1,4)-D-galactan, isolated from destarched potato fibre according to the method of Labavitch *et al.* (1976) was used to determine β-(1,4)-D-galactanase activity.

p-Nitrophenyl derivatives of α-L-arabinofuranose (Sigma), β-D-galactopyranose and β-D-xylopyranose (Koch & Light) were used to determine glycosidase activities.

The pectinolytic enzyme mixture KPB 00I 3XL from *Aspergillus niger*, obtained from Novo Ferment AG (Basel, Switzerland), was used in adsorption and degradation experiments. From preliminary experiments it was known that this enzyme mixture contained a considerable amount of xylanase activity (166 U/ml).

Determination of sugar composition

The uronide content was estimated using the method of Ahmed & Labavitch (1977) using glucuronic acid as a standard. The neutral sugar composition was determined by gas chromatography after hydrolysis according to Seaman *et al.* (1963) and derivatization to alditol acetates (Jones & Albersheim, 1972).

Elemental analysis

Elemental analysis of the original oat spelts xylan and a crosslinked oat spelts xylan was conducted with a Carlo Erba CHN Elemental Analyzer (type 1106, Milan, Italy).

Determination of xylanase and other polysaccharide splitting activities

Xylanase activity was determined with a modified ferricyanide test (Rozie *et al.*, 1988). The reaction mixture contained 100 μl of 0·5% (w/v) oat spelts xylan in 50 mM sodium acetate buffer (pH 5·0), 350 μl of this buffer without substrate and 50 μl of an enzyme solution (0–50 mU/ml). The mixture was incubated for 60 min at 30°C. The reaction was stopped by the addition of 500 μl of 2% sodium carbonate after which the mixture was cooled in an ice bath. Next, 2 ml of a freshly prepared mixture (1:1) of a cyanide solution (0·25% KCN, 1% Na_2CO_3) and a ferricyanide solution (0·08% $K_3Fe(CN)_6$, 1% Na_2CO_3) was added. After standing for 20 min at room temperature, the reaction mixture (3 ml) was immersed in a boiling-water bath for 10 min and then immediately cooled in an ice bath. Discoloration was measured spectrophotometrically at 420 nm after 1 h. The absorbance changes were interpreted in terms of an increase in the concentration of reducing sugars by means of a calibration curve for D-xylose. One unit (U) was defined as the amount of enzyme which released 1 μmol of reducing groups per min.

Determinations of other endo-enzyme activities such as polygalacturonase, cellulase, β-(1,3/6)-D-galactanase and β-(1,4)-D-galactanase were conducted in a similar way. Instead of a 0·5% (w/v) xylan solution, 0·5% (w/v) solutions of the desired substrates, described above, were used. For all these enzymes the calibration curve for D-xylose was used. Calibration curves for various sugars differ by 5–15% from that obtained for xylose.

Determination of β-D-xylosidase, β-D-galactosidase and α-L-arabinofuranosidase activities

For the determination of β-D-xylosidase activity 50 μl enzyme sample (0–20 mU/ml) was mixed with 350 μl 50 mM sodium acetate buffer (pH 5). Next, 100 μl 0·1% *p*-nitrophenyl-β-D-xylopyranoside was added. The reaction mixture was incubated for 1 h at 30°C. The reaction was stopped by adding 500 μl 500 mM glycine buffer (pH 9, 2 mM EDTA). The absorbance changes were measured spectrophotometrically at 400 nm. One unit (U) was defined as the amount of enzyme which released 1 μmol of *p*-nitrophenol per min.

Determinations of β-D-galactosidase and α-L-arabinofuranosidase activities were conducted in a similar way. Solutions of 0·1% *p*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-α-L-arabinofuranoside were used, respectively.

Protein determination

Protein determinations were performed with the Lowry method and measured spectrophotometrically at 690 nm

(Lowry *et al.*, 1951). To spare sample, small amounts of protein (150 µl, 0–50 µg/ml) were determined with this test in a micro titerplate scanner EAR 400 (SLT GmbH, Salzburg, Switzerland). Lowry reagent (50 µl) was prepared by mixing an alkaline solution (4% sodium carbonate in 1·0 M sodium hydroxide) with a copper solution (2% copper (II) sulfate · 5 H_2O) and a tartrate solution (4% potassium sodium tartrate) in the ratio of 46:2:2.

Determination of the matrix biodegradation

The biodegradability of the adsorbent was determined by measuring the total amount of saccharides in the supernatant after incubation of the adsorbent with an enzyme mixture. According to the method of Dubois *et al.* (1956) 900 µl phenol (2·5% w/v) was added to a 100 µl sample. The solution was mixed and then 2·5 ml sulphuric acid (96%) was added. The reaction mixture was mixed again thoroughly. The solution was cooled for 1 h at ambient temperature. The colour was measured spectrophotometrically at 490 nm. The absorbance changes were interpreted by means of a standard graph for the original xylan (0–1 mg/ml) and corrected for the enzyme blank. The percentages of degradation were corrected for the increased weight of the crosslinked xylan with respect to the original polysaccharide.

Synthesis of crosslinked xylans in ethanolic solvents

In 10 ml test tubes amounts of ethanol (96%) or ethanol/water (1:1) were added to portions of 200 mg oat spelts xylan (Sigma, X-0376) in such a way that the final total reaction volume was always 2 ml. Amounts of epichlorohydrin (12–60 µl) and 5 M NaOH in water (30–152 µl) were added to the reaction mixtures. A molar NaOH epichlorohydrin ratio of 1·0 was maintained. The test tubes were shaken for 24 h at 30°C. The suspensions were neutralized with 5 ml 7% acetic acid and centrifuged, after which the supernatant was decanted. The residue was washed successively with 5 ml water (twice), ethanol (twice) and acetone (twice).

Synthesis of crosslinked xylan in water

Oat spelts xylan and larch sawdust xylan were crosslinked in water in different reaction volumes, keeping the amounts of reagents constant. Water (0·706–3·484 ml) and 5 M NaOH (1·52 ml) were added to portions of 500 mg xylan in 20 ml test tubes. The tubes were agitated with a Vortex mixer to homogenize the brownish sludges. Epichlorohydrin (0·59 ml) was added while the reaction mixtures were agitated. The reaction was allowed to proceed for 22 h at 40°C. To mix the epichlorohydrin thoroughly with the other reagents the tubes were agitated for 15 s in time spans of 5 min at the start of the reaction (1 h). The gels obtained were cut with a spatula, neutralized with 10 ml 7% acetic acid and centrifuged, after which the supernatants were decanted. The gel particles were washed five times with 10 ml water. Dehydration occurred by freeze-drying.

Synthesis of crosslinked xylan suitable for adsorption experiments

8·88 g oat spelts xylan was suspended in 26·54 ml 5 N NaOH and 12·52 ml water in a 100 ml flask. The suspension was stirred with a magnetic stirrer. The magnetic follower had a diameter of 10 mm and a length of 45 mm. In some cases the viscous suspension had to be homogenized with a spatula. The mixture acquired a dark brown colour on addition of NaOH. At 40°C 10·55 ml epichlorohydrin was added. Magnetic stirring became easier because of the increase of the volume of the reaction mixture. After 15–30 min the mixture gelatinized and stirring was no longer possible. The reaction continued for 24 h at 40°C. Next the temperature was increased up to 70°C (for about 16 h). The gel was crushed with a spatula and homogenized in a Waring Blendor 801E (model 32BL80, Dynamics Corp., New Hartford, USA). The fine particles were transferred to a 1 litre flask. About 1 litre of 7% acetic acid was added. The mixture was stirred thoroughly after which the particles settled down. After decantation of the supernatant and the finest particles, the residue was washed 3 times with 1 litre of water. After that a brown-yellow powder was isolated by lyophilization (yield 12·4 g). This product was used in adsorption studies.

Enzymatic degradation of adsorbents

10 mg of crosslinked xylan was incubated for 20 h at 25°C with 1 ml KPB xylanase (50 mU/ml) in 50 mM sodium acetate (pH 4) in an Eppendorf centrifuge tube. The adsorbent was centrifuged and the degree of degradation was measured from the xylan dissolved in the supernatant. The degree of adsorption was calculated from the xylanase activity in the supernatant. After washing of the adsorbent (1 ml 500 mM NaCl (twice), 1 ml water four times) and subsequent lyophilization, the remaining adsorbent was exposed again to the enzyme in a similar incubation.

Composition of enzymatically degraded (crosslinked) xylan

Oat spelts xylan and its crosslinked derivatives were suspended (10 mg/ml) in 0·05% KPB xylanase in 50 mM sodium acetate buffer pH 4·0. The enzyme mixture had

been previously dialyzed to remove interfering saccharides. The xylans were incubated for 48 h at 30°C. The breakdown products were determined by HPLC (SP 8000, Spectraphysics, San José, USA) equipped with a CH-Pb column (300 × 7·8 mm, Merck, Darmstadt, FRG) and a guard column (50 × 4·6 mm) packed with a mixture of equivalent amounts of dried AG50W-X4 (H^+, 400 mesh) and AG3-X4A (OH^-, 200–400 mesh, Bio-Rad, Richmond, USA). The analytical column was operated at 85°C, the guard column at ambient temperature. Elution was done with water at a flow rate of 0·4 ml/min. Sugars were detected with a Shodex refractive index detector.

Effect of degraded crosslinked xylan on xylanase activity

An aliquot of 100 μl KPB was added to a suspension of 0·5 g crosslinked xylan (Table 3, 150 mg/ml) in 10 ml 50 mM sodium acetate buffer (pH 5·5). The test-tube was rotated at room temperature for 24 h, after which the material was centrifuged. The supernatant with the degraded polysaccharides was heated for 5 min at 100°C. The effect of this material on xylanase activity was measured in the normal xylanase activity assay.

Influence of pH and ionic strength on adsorption

Portions of 10 mg crosslinked xylan were incubated with 1 ml of a KPB solution (50 mU/ml) in 50 mM sodium acetate buffer of different pH values (pH 4–6) in Eppendorf centrifuge tubes. Experiments to study the influence of the ionic strength were performed at pH 4. The NaCl concentration in the buffer was varied in the range 0–200 mM. The solutions were mixed for 20 h at 25°C in a test-tube rotator. Next, the adsorbent was centrifuged, and the degree of adsorption of the xylanase activity was determined taking the difference between the total enzyme activity and the activity in the supernatant.

Separation of different xylanase activities by DEAE chromatography

50 ml KPB commercial enzyme solution was desalted with a Biogel-P10 column (800 × 45 mm) in 20 mM sodium acetate buffer (pH 5). 10 ml of the eluate (in total 110 ml) was separated on a DEAE-Biogel-A column (150 × 40 mm) in 50 mM sodium acetate buffer (pH 5) using a stepwise increase in NaCl concentration.

Influence of pH on xylanase activity of KPB and DEAE purified fractions

Xylanase activity of the crude KPB xylanase and of the fractions purified by DEAE column chromatography was measured as described above. The buffers used in the assay were 100 mM sodium acetate (pH 3·0, pH 4·0, pH 5·0, pH 6·0), 50 mM sodium phosphate (pH 7·0, pH 8·0, pH 9·0) and 50 mM sodium carbonate (pH 10·0). The pH of the assay solutions was measured again after addition of the enzyme sample (50 mM sodium acetate, pH 5·0). Percentages of activity were plotted, taking the activity at pH 5·0 as 100%.

Adsorption of KPB and of DEAE-Biogel-A separated fractions on crosslinked xylan

Portions of 10 mg crosslinked xylan were incubated with 1 ml of an adsorbate (50 mU/ml xylanase activity) in 50 mM sodium acetate buffer (pH 4 and pH 5) in an Eppendorf centrifuge tube. The solutions were mixed for 20 h at 25°C in a test tube rotator. Next, the adsorbent was centrifuged and the degree of adsorption of enzyme was calculated from the enzyme activity in the supernatant.

RESULTS

Composition of xylan

In our attempts to synthesize a suitable adsorbent for xylanases from the natural substrate of the enzyme, we used two xylans. One originated from larch sawdust, the other from oat spelts. The saccharide composition of these complex carbohydrates was determined after hydrolysis and derivatization to alditol acetates (Table 1). Relative to larch xylan, oat spelts xylan contained less xylose and mannose and more glucose and arabinose. Determination of the amount of reducing groups (Rozie *et al.*, 1988) revealed for both xylans an average size of 150 residues per molecule. However, the linear xylose backbone is known to carry a large number of side groups. According to Reilly (1981) larchwood xylan is a linear chain of xylopyranose residues, in which every fifth or sixth residue is substituted at C-2 with a 4-*O*-methyl-D-glucuronic acid unit and in which a small number of xylopyranose residues is substituted at C-3 with arabinofuranose. In

Table 1. Sugar composition of the xylans used in adsorbent synthesis

Sugar	Larch sawdust xylan (mol%)	Oat spelts xylan (mol%)
Arabinose	7·8	10·4
Xylose	76·7	72·1
Mannose	3·1	0·5
Galactose	1·3	1·5
Glucose	5·2	9·3
Uronic Acid[a]	5·9	6·2

[a]Uronic acid is expressed as glucuronic acid.

view of the lower xylose content it may be presumed that oat spelts xylan is more branched than larch wood xylan.

Crosslinking in an ethanolic solvent

In preliminary experiments oat spelts xylan was crosslinked in an ethanolic solvent. The epichlorohydrin concentration and the ethanol/water ratio were varied. The solvent composition itself had no effect on the properties of the product with respect to its degradability by xylanase. However, an increase of the ephichlorohydrin concentration in the crosslinking reaction resulted in products with a higher degree of crosslinking, as is reflected by the reduced biodegradability when the crosslinked xylans were incubated with a commercial xylanase preparation (Table 2). The product prepared with the lowest epichlorohydrin concentration (76 μmol/ml) dissolved completely with a xylanase enzyme mixture. Although the crosslinked xylans prepared in ethanolic solvents cover a broad range of more or less degradable matrices, in none of the cases was enzyme adsorption measured. Thus it seems that this lack of adsorption cannot be attributed to an unfavourable degree of crosslinking. It may be due to the low accessibility for xylanases of the relatively hard and compact adsorbents prepared in ethanolic solvents.

Crosslinking of xylan in water

Next, crosslinking in a larger effective reaction volume was carried out to achieve high accessibility of the adsorbent. In an ethanolic solvent the reaction is heterogeneous. Intermolecular and intramolecular crosslinking of the polysaccharide chains with glyceryl bridges is very likely to occur as the chains are reasonably close to each other. Crosslinking of polysaccharides solubilized in water, e.g. dextran (Flodin, 1962), starch (Kuniak & Marchessault, 1972) and arabic gum (Fujita *et al.*, 1975) resulted in gelation of the total reaction mixture. In those cases the polysaccharide is homogeneously distributed over the total reaction volume. This volume is an important parameter in determining the degree of crosslinking and finally the accessibility of the modified polysaccharide to enzymes.

Table 2. Degree of enzymatic degradation of crosslinked xylans prepared in an ethanolic solvent relative to unmodified xylan

Epichlorohydrin concentration (μmol/ml)	Solvent	
	Ethanol degradation (%)	Ethanol/water (1:1) degradation (%)
0	100	100
76	93	91
152	40	39
228	8·0	8·1
304	5·6	6·8
380	2·0	4·1

Crosslinking in water was carried out both with oat spelts and larch sawdust xylan. More epichlorohydrin (2 moles per mole xylose monomer) was used since preliminary experiments showed that with regard to the crosslinking in ethanolic solvent, higher amounts of epichlorohydrin were necessary to prepare a product which was less than 10% degradable by xylanase activity. The total reaction volume was varied (2·8–5·6 ml), keeping the amount of epichlorohydrin constant. The resulting gels were weaker with increasing reaction volume. The gels were crushed, neutralized and washed. Subsequent dehydration with ethanol resulted in hard stone-like particles. A powder was obtained if dehydration of the water-washed gel particles was carried out by freeze-drying.

Portions of 10 mg of the adsorbents prepared in this way were incubated with 1 ml of a KPB solution (50 mU/ml) in 50 mM sodium acetate buffer (pH 5) in an Eppendorf centrifuge tube and mixed for 20 h at 25°C in a test-tube rotator. The modified xylans were centrifuged and the degree of degradation of the adsorbents was calculated from the xylan material in the supernatant. In some cases limited degradation and appreciable adsorption of xylanase activity was found (Table 3).

Larch sawdust xylan cannot be crosslinked as effectively as oat spelts xylan (Table 3), probably because of the difference in composition and in average chain length. The low yield at the largest reaction volume is due to the high solubility of the product in the washing fluids. All crosslinked larch xylans were degraded by xylanase and no adsorption of enzyme was found. The yields with regard to the original polysaccharide of the crosslinked xylans that did not dissolve in water were relatively high.

The concentrations of xylan in Table 3 could not be increased further because of mixing problems with the extremely viscous solutions. Also the amount of epichlorohydrin added to the reaction mixtures could hardly be increased. Larger amounts of epichlorohydrin did not mix properly with the other reagents, thus forming a two phase system. The values in Table 3 show that the degradation of an adsorbent by xylanase was minimized if oat spelts xylan was crosslinked in water in a reaction volume as small as possible. In that case adsorption of xylanase to the matrix could be detected. The synthesis on a larger scale of such a crosslinked xylan suitable for adsorption studies was carried out under vigorous stirring in a 100 ml flask. The disruption of the gel and the washing procedures were

Table 3. Properties of crosslinked xylans from oat spelts and larch sawdust prepared in water solvent

Xylan concentration[a] (mg/ml)	Yield[b] (%)	Elemental composition[c] C (%)	H (%)	Degree of degradation (%)	Degree of adsorption (%)
Oat spelts xylan					
100	173			43	
125	165	44·7	6·6	41	
150	173			34	
175	146	45·0	6·8	11	12 (31)[d]
200	136			3	28 (55)
225	138	46·5	7·0	4	29 (59)
Larch sawdust xylan					
100	22				
125	132			98	
150	121			85	
175	144			56	
200	137			48	
225	111	42·9	6·5	39	

[a]Mg/ml xylan in the crosslinking reaction mixture before the addition of epichlorohydrin.
[b]Yield after freeze-drying as percentage of the original xylan.
[c]Elemental analysis was carried out for some modified xylan. In all cases 0% nitrogen was detected. The carbon and hydrogen content of the original xylans were: C 41·5%, H 6·4% for oat spelts xylan; C 40·2%, H 5·9% for larch wood xylan. The margin of error in these measurements is 0·3%.
[d]Values are given for adsorption at pH 5 and at pH 4 (in parenthesis), both in 50 mM sodium acetate.

the same as those used to prepare Sephadex (Flodin, 1962).

The elemental composition of this product was shown to be equal to that of the product prepared on a smaller scale (xylan concentration 225 mg/ml, Table 3). The carbon content was increased compared to the original oat spelts xylan. From the yield of the crosslinking reaction (140%) one can calculate that about one glyceryl moiety is present per sugar monomer. In that case the high carbon content of the modified xylan is in agreement with a fully crosslinked xylan, in which every hydroxyl group is connected to another hydroxyl group by a glyceryl diether bridge. However, these calculations are based on a hypothetical xylan that consists of D-xylose residues, with two available reactive hydroxyl groups, only. One can expect an increase in carbon content from 40·0% to 46·6% if this hypothetical xylan is fully crosslinked by epichlorohydrin. In fact, the increase in carbon content, due to the modification of oat spelts xylan, was not that high (from 41·5% to 46·5%). This means that a part of the glyceryl moieties is present as glyceryl monoether residues.

With increasing reaction volume there is an obvious trend of increasing yield and decreasing carbon content. This is caused by a shift from glyceryl diether bridges to glyceryl monoether side chains in crosslinked xylan. From the yield (173%) of a modified oat spelts xylan, crosslinked at a lower xylan concentration (100 mg/ml), it can be calculated that *c.* 1·5 glycerol moieties are present per sugar monomer. In that case only a small part of the glyceryl moieties is present as a connecting bridge between polysaccharide chains. Such an adsorbent is apparently easily degraded by enzymatic action, although most of the hydroxyl groups in the crosslinked xylan are substituted.

Polysaccharides, present in the supernatant of the degraded crosslinked xylan (150 mg/ml), had no inhibitory effect on the xylanase activity of KPB-enzyme. This shows that the dissolved polysaccharide fragments are hardly recognized by the xylanase enzymes, which may be primarily due to the high substitution grade of those polysaccharides.

Composition of enzymatically degraded crosslinked xylan

Oat spelts xylan and its crosslinked derivatives were degraded as described in Materials and Methods. The supernatants with the solubilized polysaccharides were injected on HPLC. Xylose was the main component (80%) in the supernatant of the degraded unmodified oat spelts xylan. The remainder consisted of 7% arabinose and two oligosaccharides (3% and 8%). The supernatants of the degraded crosslinked oat spelts xylans showed on HPLC a large amount of oligosaccharides, which overshadows the relatively small xylose peak (3–10%). Arabinose peaks were detected

but could not be quantified in this material. From these results it is noticeable that, although it was measured only in the degraded material, there is an amount of unsubstituted xylan present in the crosslinked oat spelts xylan.

Repeated degradation

About 4% of the adsorbent, prepared on a larger scale as described above, was degradable by xylanase activity. The degree of adsorption of KPB xylanase was 60%. The adsorbent was reused in a similar degradation experiment after a washing procedure and subsequent lyophilization. No significant degradation was measured with the reused adsorbent. The degree of adsorption did not change significantly (63%).

The influence of pH and ionic strength on adsorption of *A. niger* xylanase

The influence of pH was studied with sodium acetate buffers at a low ionic strength (Fig. 1(A)). Adsorption was maximal at pH 4, the lowest pH studied, although the optimal pH for enzyme activity of crude KPB xylanase was 5·0. Adsorption was also studied in 50 mM sodium citrate buffers of pH 3·0, 3·5 and 4·0. However, with 50 mM sodium citrate buffers no significant adsorption was found. Figure 1(B) shows that this is due to the higher ionic strength of the citrate buffer solutions (54 mM at pH 4) compared to the sodium acetate buffers (7·4 mM at pH 4). The addition of 50 mM NaCl to the acetate buffer reduces the adsorption to approximately 10%.

Separation of different xylanase activities by anion-exchange chromatography

Since there are different xylanases present in fungal extracellular enzyme preparations (Dekker & Richards, 1976), these activities were separated in a conventional way to study the adsorption properties of the individual xylanases to the crosslinked xylan affinity adsorbent. Therefore xylanases present in KPB were treated by anion-exchange chromatography using DEAE-Biogel-A (Fig. 2). Four fractions with xylanase activities were pooled. The fraction which was not bound by the column material contained about 80% of the xylanase activity and some other endopolysaccharide splitting activities (e.g. polygalacturonase). This xylanase had no activity at pH 3·4 contrary to the xylanases that were eluted from the column with the stepwise salt gradient (Fig. 3). Enzyme activities found in the different fractions are shown in Table 4. The xylanolytic activities of fractions I to IV did not add up to that of the crude KPB preparation: the yield was only 66%. This effect can be explained partly by synergistic phenomena which are known to exist when these enzymes are allowed to work simultaneously (Dekker & Richards, 1976).

Adsorption of crude KPB and DEAE-separated enzyme fractions to crosslinked xylan

The crude commerical xylanase preparation and the xylanase fractions separated by DEAE chgromatography were incubated with crosslinked xylan as described in Materials and Methods. The xylanase fraction I that was not retained by the DEAE-Biogel-A column, did not adsorb to crosslinked xylan at pH 5 but it did bind to the adsorbent at pH 4. Xylanase activity from the fractions II and III was adsorbed at pH 5 but binding did improve at pH 4. With fraction IV there was a slight decrease in the degree of adsorption at a lower pH.

In these adsorption experiments it was found that β-D-xylosidase, α-L-arabinofuranosidase and β-D-galactosidase activity did not adsorb significantly

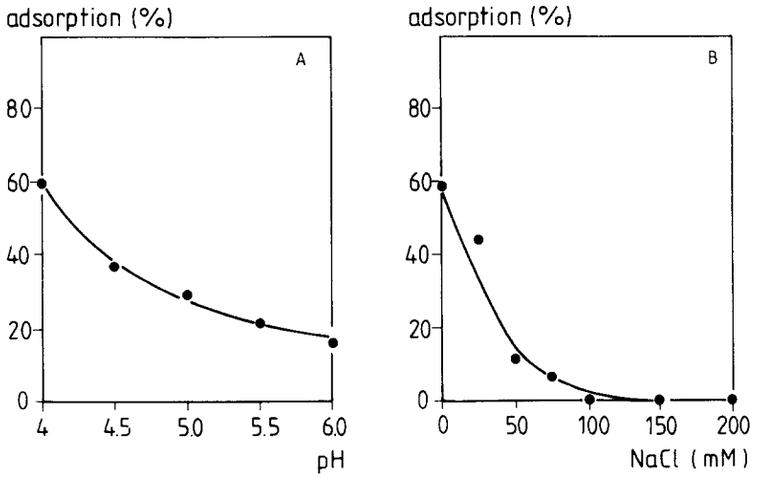

Fig. 1. Adsorption of xylanase with (A) pH and (B) ionic strength (pH 4) in 50 mM sodium acetate buffers. The original activity was 50 mU/ml. The amount of adsorbent was 10 mg/ml.

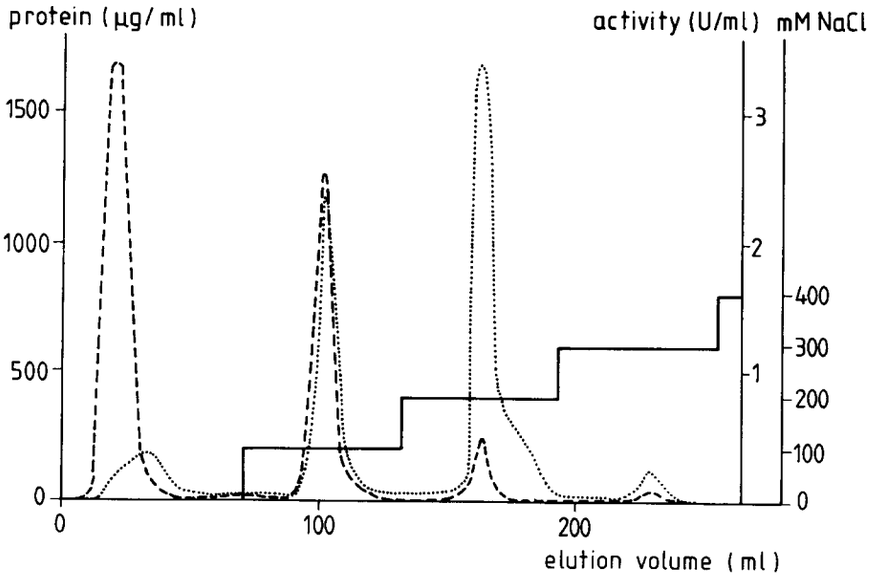

Fig. 2. Separation of different xylanase activities by DEAE chromatography. ····· protein (μg/ml); — — — — xylanase activity (mU/ml); ——— NaCl concentration (mM).

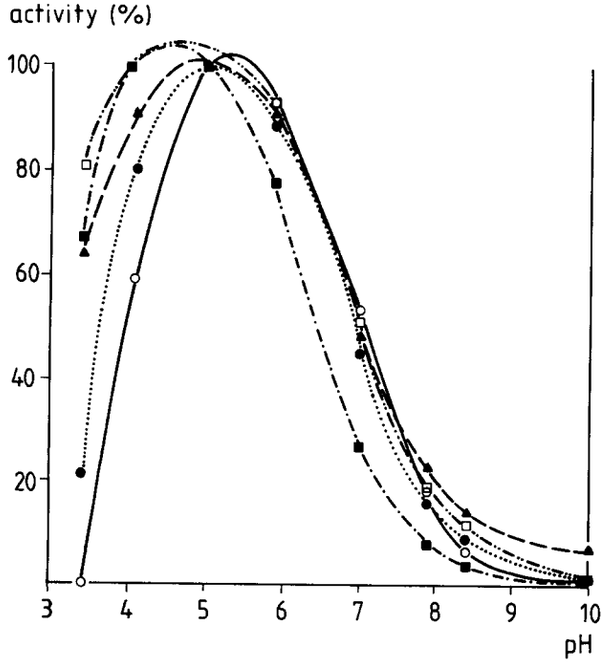

Fig. 3. pH dependency of xylanase related to their activity at pH 5. ● crude KPB; ○ fraction I; ■ fraction II; □ fraction III; ▲ fraction IV.

to the crosslinked xylan adsorbent. There was also no adsorption for endo activities such as cellulose, β-(1,3/6)-D-galactanase and β-(1,4)-D-galactanase. Apart from xylanase, only some polygalacturonase enzyme activity did adsorb to the modified xylan. Polygalacturonase behaved similar to xylanase as it was also bound to a larger extent at pH 4 than at pH 5. The fractions I to IV showed decreasing degrees of polygalacturonase adsorption (Table 5).

DISCUSSION

Modification of polysaccharides, such as pectate (Rexová-Benková & Tibensky, 1972) and starch (Weber *et al.*, 1976), by crosslinking these polymers with epichlorohydrin in water as solvent resulted in products which could still adsorb enzymes involved in the breakdown of such polysaccharides. These adsorbents, however, were still partly degraded by these enzymes. Better reproducibility and a higher effective degree of crosslinking was obtained for pectate (Visser *et al.*, 1979) and starch (Rozie *et al.*, 1991) when the crosslinking procedure was conducted in an ethanolic solvent. When the polysaccharide was more effectively crosslinked, there was a decrease of adsorbent biodegradation together with a decrease in the degree of adsorption of the substrate splitting enzymes. For crosslinked starch some biodegradation was inevitable if suitable adsorption properties were to be achieved (Rozie *et al.*, 1991).

A similar strategy was thought to be necessary in order to prepare crosslinked xylan for the adsorption of xylanases. However, it appeared that oat spelts xylan cannot be crosslinked in an ethanolic solvent to an adsorbent that binds xylanases. This is probably due to a limited accessibility of the crosslinked polymer chains. Accessibility cannot be improved by grinding the crosslinked polymers to a powder (0·5 mm). However, by dissolving xylan in water and by crosslinking it in a homogeneous reaction, useful adsorbents were obtained. High concentrations of xylan were required in the reaction mixture to get a sufficiently high degree of crosslinking, in which biodegradation was limited (⩽4%). The product yield of the crosslinking reaction and elemental analysis of xylan and

Table 4. Protein content and enzyme activities in fractions pooled after DEAE-Biogel-A chromatography of KPB enzyme

	Fraction			
	I	II	III	IV
Protein (mg)	11·0	39·0	73·5	24·9
Xylanase (U)	420	71	11	1·8
Polygalacturonase (U)	3200	1900	730	173
Cellulase (U)	2·7	3·7	33	11
β-(1,3/6)-D-galactanase (U)	6·4	31	280	69
β-(1,4)-D-galactanase (U)	0·97	57	28	63
β-D-xylosidase (U)	n.d.[a]	7·1	8·5	0·27
α-L-arabinofuranosidase (U)	n.d.	0·96	490	160
β-D-galactosidase (U)	n.d.	190	39	2

[a] n.d. = not detected.

Table 5. Degrees of adsorption of crude and DEAE-purified xylanases on crosslinked xylan

	Degree of adsorption		
	Xylanase pH 4 (%)	Xylanase pH 5 (%)	Polygalacturonase pH 4 (%)
KPB	60	29	84
Fraction I	91	0	90
Fraction II	58	15	72
Fraction III	72	40	54
Fraction IV	41	49	45

modified xylan showed that a large number of the available hydroxyl groups in xylan were crosslinked. HPLC analysis of enzymatically degraded crosslinked xylan showed, however, that there are still xylose residues present that are not substituted. Adsorption of xylanase (60%) was measured with a crosslinked xylan. The same degree of adsorption but no further biodegradation was found if the adsorbent was used in a second adsorption experiment.

No suitable adsorbent could be prepared with larch sawdust xylan, although this material had a higher xylose content than the suitable oat spelts xylan. The latter material is expected to have high degrees of substitution, as was concluded from the large product yields. With decreasing reaction volume, larch sawdust xylan shows a similar decrease in product yield as oat spelts xylan, probably due to a shift from mono- to di-substituted glyceryl. However, the large product yields, and with that the apparently necessary degree of substitution, are not reached with larch sawdust xylan. The low glyceryl content compared to oat spelts xylan is confirmed by elemental analysis.

Since there are different xylanases present in the commercial enzyme preparation, these activities were separated by anion exchange chromatography to study the adsorption of the individual enzymes to the matrix separately. From the four xylanase fractions that were obtained, one xylanase (I) adsorbed for more than 90% to the adsorbent prepared at pH 4. With this isolated xylanase no adsorption on crosslinked xylan was found at pH 5. It differs from the others as it has no enzyme activity on the original substrate at pH 3·4. None of the other xylanases obtained had these two features, although the adsorption of the xylanases in fraction II and III was improved at pH 4 compared to that at pH 5. The xylanase in fraction IV adsorbed better at pH 5.

About 80% of the xylanase activity, eluted from the DEAE-Biogel-A column, is present in fraction I. A large amount (90%) of this xylanase and reasonable amounts (41-72%) of the xylanases in the other fractions adsorb to the matrix. Of the original enzyme preparation only 60% of the activity adsorbs to the adsorbent. This difference can be due to synergism between the enzymes that remain in the adsorbate or to inhibitory components in the crude enzyme preparation.

Crosslinking of heteropolymers as xylan could result in an adsorbent with affinity for many enzymes of the complex enzyme mixture that has activity on the original xylan. It appeared from the enzymes involved in xylan degradation that only endo-xylanases were bound to the synthesized adsorbent.

Adsorption experiments were also conducted for cellulase and polygalacturonase, since these activities mostly occur in commercial fungal enzyme mixtures in which xylanase activity is found. Polygalacturonase activity was adsorbed by the matrix, cellulase was not. The adsorption of polygalacturonase, an enzyme that does not have activity on the natural xylan, is probably due to the uronic acid content of xylan, which acts thereby as an ion exchange matrix.

Polygalacturonase and xylanase adsorption decreased when the ionic strength is decreased. Also, xylanase adsorption was less at 4°C compared to the values shown at 25°C (results not shown). These results show that xylanase adsorption by crosslinked xylan is due

primarily to electrostatic interactions between the enzyme and the adsorbent. The question may be raised whether this association is due to ion-exchange properties of the adsorbent or to biomolecular recognition of the xylan backbone. Investigations in that matter will be discussed in another paper, as well as capacity and possible applications of the adsorbent.

ACKNOWLEDGEMENTS

These investigations were supported in part by the Netherlands Technology Foundation (S.T.W.), Grant WLM 47.0700. We are grateful to H. van de Vis (Department of Food Chemistry) for providing the galactan substrates, to F. Kormelink for carrying out HPLC analyses and to H. Jongejan (Department of Organic Chemistry) for carrying out the elemental analyses.

REFERENCES

Ahmed, A.E.R. & Labavitch, J.M. (1977). *J. Food Biochem.*, **1**, 361–5.

Biely, P. (1985). *Trends in Biotechnol.*, **3**, 286–90.

Dekker, R.F.H. & Richards, G.N. (1976). In *Advances in Carbohydrate Chemistry and Biochemistry*, ed. R. Stuart Tyrson & D. Horton. Academic Press, New York, pp. 278–352.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, F.A. & Smith, F. (1956). *Anal. Chem.*, **28**, 350–6.

Flodin, P. (1962). Dextran gels and their applications in gel filtration. Ph.D. thesis, Uppsala, p. 15.

Fujita, Y., Oishi, K., Suzuki, K. & Imahori, K. (1975). *Biochem.*, **14**, 4465–70.

Hoebler, C. & Brillouet, J.M. (1984). *Carbohydr. Res.*, **128**, 141–55.

Jones, T.M. & Albersheim, P. (1972). *Plant Physiol.*, **49**, 926–36.

Kuniak, L. & Marchessault, R.H. (1972). *Stärke*, **24**(4), 110–16.

Labavitch, J.M., Freeman, L.E. & Albersheim, P. (1976). *J. Biol. Chem.*, **251**, 5904–10.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). *J. Biol. Chem.*, **193**, 265–75.

Paice, M.G. & Jurasek, L. (1984). *J. Wood Chem. Technol.*, **4**, 187–98.

Reilly, P.J. (1981). In *Trends in the Biology of Fermentations for Fuels and Chemicals*, ed. A. Hollaender. Plenum Press, New York, pp. 111–29.

Rexová-Benková, L. & Tibensky, V. (1972). *Biochim. Biophys. Acta*, **268**, 187–93.

Rozie, H., Somers, W., Bonte, A., Visser, J. van 't Riet, K. & Rombouts, F.M. (1988). *Biotechnol. Appl. Biochem.*, **10**, 346–58.

Rozie, H., Somers, W., van 't Riet, K., Rombouts, F.M. & Visser, J. (1991). *Carbohydr. Polym.*, **15**, 349–65.

Seaman, J.F., Moore, W.E. & Millet, M.A. (1963). *Methods Carbohydr. Chem.*, **3**, 54–69.

Tanimoto, K., Nishimoto, T., Saitoh, F. & Yamaguchi, H. (1986). *J. Biochem.*, **99**, 601–4.

Visser, J., Maeyer, R., Topp, R. & Rombouts, F.M. (1979). *Les Colloques de l'INSERM*, **86**, 51–62.

Weber, M., Foglietti, M. & Percheron, F. (1976). *Biochimie*, **58**, 1299–302.

Wolfrom, M.L. & Patin, D.L. (1965). *J. Org. Chem.*, **30**, 4060–3.

Woodward, J. (1984). *Top. Enzyme Ferment. Biotechnol.*, **8**, 9–30.