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Peroxidase-Mediated Oxidative Cross-Linking and Its Potential To Modify Mechanical Properties in Water-Soluble Polysaccharide Extracts and Cereal Grain Residues

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Analysis of wheat bran and spent grain shows that concentrations of ferulate and diferulates offer considerable scope to modify the cross-linking of feruloylated polysaccharides and hence the mechanical properties of these residues. In solution ferulic acid (FA) can be readily polymerized by horseradish peroxidase, but when esterified to a polysaccharide, the profile of diferulates becomes restricted. This situation also exists *in muro* and suggests structural constraints may limit the availability of FA for cross-linking. At relatively low polysaccharide concentration, (~3%), steric restrictions were apparent in gels prepared using isolated polysaccharides. Mechanical properties such as swelling also appear to be fixed at these relatively low polysaccharide concentrations. This limits the potential to modify mechanical properties *in muro* through oxidoreductase activity. To modify mechanical properties such treatments will need to focus on increasing the "flexibility" of the cell wall matrix and the accessibility of enzymes to the cell wall matrix.

KEYWORDS: Peroxidase; ferulate; cross-linking; swelling; wheat bran; spent grain

INTRODUCTION

Cross-linking of water-extractable polysaccharides through peroxidase treatment is a recognized method to develop products with designed functional properties (*1–5*). Chemical structure and polymer conformation and the propensity for molecular interaction are important considerations for designing functional properties (*6*). As well as isolated polysaccharides, such as pectins and arabinoxylans (AX), oxidative cross-linking is also applicable to proteins and protein−polysaccharide mixtures (*7*). This greatly adds to the diversity of potential gelling agents that might become available for biomedical use (*8*) and to control texture and stability properties in foods (*6, 9, 10*).

Feruloylated polysaccharides have a recognized ability to be oxidatively cross-linked by oxidoreductases, such as peroxidases and laccases (*1–4, 11*). Although there has been much activity centered on the use of such polysaccharides when extracted from the cell wall, little information is available on the potential to modify cross-linking *in muro* and hence to manipulate the properties of cell wall-rich food processing residues, such as wheat bran (WB) and brewers' spent grain (BSG).

These cereal-derived residues have a large proportion of ferulate (FA) as well as diferulates (diFA) in the cell wall (*12*). There may be a structural or functional requirement to maintain FA and diFA in the cell wall, but equally the persistence of FA may indicate these moieties are unavailable for cross-linking. Unavailability may be related to an inaccessibility of enzyme to the cell wall matrix or the spatial distribution of FA and diferulates being sterically unfavorable for cross-linking. The chemical profiling within a semiflexible polysaccharide matrix of ferulates gives little information on their spatial location either within the cell wall or in different tissue types. It is known that different tissues within the cereal grain can have distinct profiles of phenolic acids (*13, 14*) and that a manganese-dependent peroxidase can induce modification to mechanical properties, but also this can be tissue specific (*10*).

The ratio of FA to sugars is important for gelation, with an ara/xyl/FA (600:2000:1) ratio considered to be limiting for gelation in AX (*6*). It is presumed that the important ratio is the degree of substitution of arabinose, although other substitutions and esterification to a polysaccharide may also influence the steric accessibility of FA for cross-linking. For example, in ester substitution of pectic polysaccharides *O*-2 linked arabinose predominates (~60% *O*-2-linked arabinose and ~40% *O*-6-linked galactose), whereas in AX it is *O*-5-linked arabinose (*15, 16*).

The overall ratio of FA to AX sugars in WB and BSG (*12*) was apparently in excess of the ratio reported to be required for oxidative cross-linked gelation (*14*). However, when ratios are considered, the total spectrum of AX present in each residue and relative distribution of FA and diFA has to be taken into

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account. Thus, ratios will vary for specific AX found in different tissues and will also be influenced by the extraction methods used.

Using model systems, based on free FA and FA ester-linked to either a sugar beet pectin (SBP) or AX extracts, the effects of horseradish peroxidase (HRP) on oxidative cross-linking have been characterized. The solubility and swelling capacity of gelled extracts have been related to conditions considered to be prevalent within the cell wall matrix to identify factors that may limit the potential to modify cereal residues *in muro*.

MATERIALS AND METHODS

Materials. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) was obtained from Sigma (Sigma, Poole, Dorset, U.K.). A sugar beet derived pectin fraction (SBP) with demonstrated gel-forming properties (kindly provided by Herbstreith & Fox KG, Neuenburg, Germany) was used a standard reference material. Water-soluble arabinoxylans were obtained from Megazyme (Megazyme, Bray, Ireland) as a wheat arabinoxylan (WAX), medium viscosity (code P-WAXYM -20cSt), and a rye arabinoxylan (RAX) (code P-RAXY). WB was a destarched sample (ARD, Pomacle, France), and BSG was obtained from Scottish Courage (Scottish Courage Ltd., Edinburgh, U.K.). WB was used as supplied, and BSG was freeze-dried prior to experimental use. When indicated, samples were milled to pass a specific sieve size, as indicated in the text or figure. HRP (Sigma, P-8125) had a stated activity of 113 purpurogallin units mg^{-1} and was used as supplied. Hydrogen peroxide (H_2O_2) was obtained as a 50% solution ($\sim$17 mol L^{-1}) in water (Sigma-Aldrich: 51,681-3) and diluted to 5%/$\sim$1.7 mol L^{-1} prior to use. All other chemicals used were of at least Analar quality.

Sugars Analysis. Sugars were released from samples following dispersion in 72% H_2SO_4 for 3 h followed by dilution to 1 M H_2SO_4 and hydrolysis for 1 h at 105 °C (*12*). Sugars present in hydrolysates were derivatized as their alditol acetates and analyzed by GLC using a flame ionization detector (*17*). Total uronic acid content was determined spectrophotometrically using glucuronic acid as a standard (*18*).

Peroxidase Treatments. *Treatment of FA with HRP in Solution.* FA (0.8 mg mL^{-1}; 0.4 mM) in either deionized water or HCl (pH $\sim$3.6) was treated with HRP (1 mg mL^{-1}) and H_2O_2 (5%) in the volume ratio 1:0.015:0.010 for 16 h at room temperature in the dark. Thereafter, the reaction mixture was treated with the same protocol used for alkali-extracted phenolic acids (see below) except that NaCl (230 mg mL^{-1}) was added to the final reaction mixture to provide a salt concentration ($\sim$4 M) equivalent to that present to facilitate extracting phenolic acids from alkali-treated samples following acidification.

Polysaccharide Gelling. SBP was dispersed in hot (90−95 °C) deionized water, with UltraTurraxing to assist dispersion. Nondispersed material was removed by centrifugation, and a sample of the stock solution supernatant was freeze-dried to determine dispersed solids concentration. Stock solution was diluted to provide the required concentrations for gelling experiments. Arabinoxylan powders were dispersed in hot (90−95 °C) deionized water at 25 mg mL^{-1}, with vortexing to assist dispersion as recommended by the supplier. Unlike the SBP, AX samples were readily dispersed and did not require centrifugation prior to use.

For gel formation sample solutions of polysaccharide were gelled in duplicate. Polysaccharide solutions of known concentration, HRP (1 mg mL^{-1}), and H_2O_2 (5%) were mixed in the volume ratio 1:0.015:0.010, respectively, using gentle vortexing. Gelling generally commenced within 2 min. Samples were left overnight (16 h) at room temperature to equilibrate.

Cereal Residues. Samples of WB, in duplicate, were hydrated (3 h) in deionized water (25 mg mL^{-1}) with constant stirring, prior to the addition of HRP and H_2O_2 to achieve the same final volume ratio used for polysaccharide gelling (above). After 16 h of incubation at room temperature, the insoluble material was recovered by filtration and freeze-dried.

Alkali Extraction and Quantification of Phenolic Acids. The total alkali-extractable phenolic content was determined in duplicate from each sample treatment following saponification of samples with 4 M NaOH ($\sim$5 mg mL^{-1}), under N_2, for 18 h in the dark at room temperature and with gentle stirring. Samples were gently centrifuged (1000 rpm bench centrifuge), and an aliquot of supernatant (0.8 mL) was acidified (pH $\sim$2) using concentrated HCl, prior to extraction with ethyl acetate (3 × 3 mL). Extracts were reduced to dryness and resuspended in methanol/H_2O (50:50, v/v). Alkali-soluble phenolic acids (as documented in Figures 1–3), were separated using HPLC (Luna C18 reverse phase HPLC column (Phenonomex, Macclesfield, U.K.) and quantified according to the method of Waldron et al. (*19*), using *trans*-cinnamic acid as internal standard.

Milling and Sieving. WB was used as supplied, and a sample was milled in a laboratory grinder (Janke & Kunkel, Labortechnik) to a mean particle size estimated as $\sim$1 mm. BSG samples were freeze-dried prior to milling and sieving. BSG was milled under the same conditions used to mill the WB. Samples of BSG were also milled to pass 1 and 0.5 mm screens using a Ditton Laboratory Mill. A sample of BSG milled to pass the 1 mm screen was further dry sieved to obtain samples retained on Endecotts sieves at 500, 212, and 106 μm. Negligible amounts of material passed through sieves below 106 μm.

Swelling. *Polysaccharide Gels.* Samples, in duplicate, of SBP and AX gelled using HRP in deionized water, estimated to contain $\sim$50 mg dry weight, were disrupted and transferred to a 10 mL graduated measuring cylinder. The supernatant volume was adjusted to 10 mL with either deionized water or salt solution. The contents were left overnight (16 h) before noting the settled gel volume (*20*). The effect of cations on swelling was measured in the presence of either 100 mM calcium chloride or 100 mM sodium chloride. Final volume was calculated on the basis of original sample weight.

Cereal Residues. Samples, in duplicate, $\sim$250 mg, in a 10 mL graduated measuring cylinder, were wetted with 0.5 mL of 40% ethanol and then dispersed in 10 mL of deionized water. The settled volume was noted after 16 h, with swollen volume calculated on the basis of the original sample weight. The same treatment regimen was used to test for effects of particle size on swelling.

Solubility. Samples of pectin gel ($\sim$50 mg), in duplicate, were disrupted in deionized water (10 mL) and gently stirred overnight at room temperature in sealed soviral tubes. Tubes were centrifuged (5000*g*; 10 min) to recover the insoluble gel material, which was freeze-dried. Sample solubility was determined as

$$\text{solubility (\%)} = [(\text{original dry weight} - \text{final dry weight})/\text{original dry weight}] \times 100$$

RESULTS AND DISCUSSION

Phenolic Acid Profiling. The total saponifable phenolic acid contents of cereal grain residues and SBP were similar, 8–9 mg g^{-1} dry weight, (**Table 1**), whereas in the AX extracts, phenolic acid concentration was 1–1.5 mg g^{-1}. In the SBP and AX extracts, FA was the exclusive phenolic acid released during saponification of the original untreated samples. However, the cereal residues (**Figure 1**) had a variable contribution from FA, diferulates and coumaric acid were detected, especially in the BSG. The initial concentration of phenolic acids in cereal residues and their profile (**Figure 1**) were also similar to those reported in WB and BSG (*12*).

Sugar Composition. Uronic acid (420 mg g^{-1} of sample) and arabinose (192 mg g^{-1} of sample) were the only sugars detected in significant amount in the SBP fraction used, consistent with the presence of feruloylated arabinans in sugar beet pectin (*21*) and indicating feruloylated galactans had been removed during preparation of the SBP fraction. In the WAX and RAX extracts arabinose and xylose accounted for >95% of the sugars present, and for each extract the ara/xyl ratio was $\sim$40:60. This ratio is consistent with the ratio for endosperm-derived AX found in water-soluble extracts of wheat and rye (*4*). The ratio ara/xyl also suggests an apparently similar degree of substitution in the WAX and RAX, but a more detailed

Table 1. Total Phenolics Extractable Using 4 M KOH[a]

sample	ferulic acid (mg g^{-1})	diferulate (mg g^{-1})	total phenolics (mg g^{-1} ± SD)
sugar beet pectin			
soluble	7.70	nd	7.70 ± 0.03
2% gel	0.31	3.61	3.90 ± 0.34
4% gel	0.45	3.67	4.13 ± 0.26
6% gel	0.58	3.53	4.16 ± 0.08
8% gel	0.54	4.04	4.58 ± 0.30
10% gel	0.78	3.17	3.96 ± 0.71
arabinoxylan extracts			
WAX			
soluble	0.98	nd	0.98 ± 0.07
2.5% gel	0.27	0.26	0.53 ± 0.09
RAX			
soluble	1.55	nd	1.55 ± 0.12
2.5% gel	0.18	0.34	0.52 ± 0.08
cereal grain residues			
wheat bran			
untreated	4.68	2.56	7.61 ± 0.27
peroxidase, pH 6.5	4.37	2.60	7.38 ± 0.05
peroxidase, pH 3.6	4.70	2.71	7.96 ± 0.01
brewers' grain			
untreated	3.91	2.52	9.17 ± 0.40
peroxidase, pH 6.5	3.58	2.86	9.07 ± 0.13

[a] WAX, wheat arabinoxylan; RAX, rye arabinoxylan; nd, not detected. Results are shown as mean + SD for samples analyzed as duplicates. Sample preparation and peroxidase treatment were as described under Materials and Methods.

analysis will be required to confirm the profile of attachment in each AX. The cereal residue samples used corresponded to those analyzed previously (*12*). Arabinose and xylose were major sugars present in each residue, and with glucose these accounted for around 90% of the total sugars present in each residue. In WB arabinose + xylose accounted for 513 mg g^{-1}, from a total sugars content of 774 mg g^{-1} alcohol-insoluble residue (AIR), and the ratio ara/xyl was 1:1.33. In BSG, arabinose + xylose accounted for 373 mg g^{-1} from a total sugars content of 714 mg g^{-1} AIR, and the ratio ara/xyl was 1:2.45.

Oxidative Cross-Linking Treatment. The conditions used for oxidative cross-linking were based on the observed ability to gel SBP. The conditions used also resulted in gel formation from the AX extracts and reaction products from FA in solution. Thus, it was expected that any potential for further cross-linking in the cereal residues should be realized under the experimental conditions used. Conditions used also considered the effect of pH on oxidative cross-linking because an optimum of ~4.5 has been reported for ferulic acid oxidation (*22*). Acid pH (~3.5) has been preferred to polymerize FA using a lignin peroxidise (*23*), and acid pH is favored for the oxidation of methoxybenzenes by lignin peroxidase, laccase, and HRP (*11*). However, it should also be noted that for overall peroxidise-based cross-linking there may be no apparent pH optimum (*24*).

As shown in **Figure 2**, treating FA in solution with HRP at either neutral pH or acid pH generated a broad spectrum of FA dimers. Other compounds, as yet unidentified, would also be expected to be formed through decarboxylated products generated under the conditions used, but as shown previously for lignin peroxidase (*23*) dimers and trimers would be expected as significant reaction products. The formation of phenolic derivatives was more pronounced at acid pH, consistent with an acidic pH optimum for enzyme activity. Unidentified derivatives generally chromatographed later than the recognized phenolic monomers and dimers and were presumed to be trimers. Although recent work to characterize novel dimers and

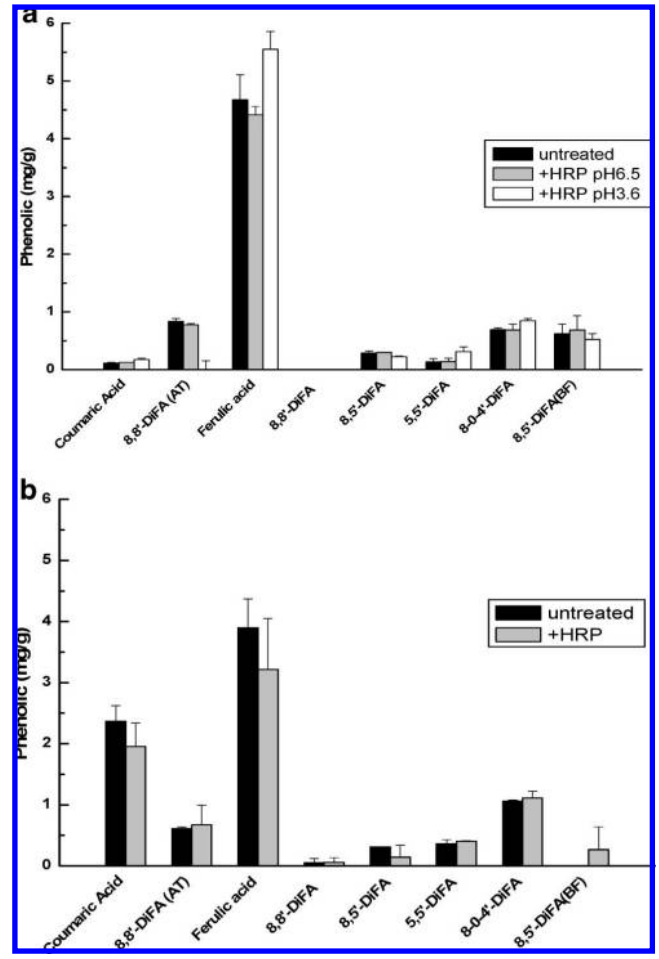

Figure 1. Profile of phenolic acids following alkali extraction from (**a**) WB and (**b**) BSG. Results are shown as mean ± SD for samples analyzed as duplicates. For experimental details see Materials and Methods.

trimers from maize bran (*25*) has shown that saponification can result in an apparent modification of trimers, given the noted effect of acidic treatment compared to neutral treatment on phenolic derivatization in the current work, then formation of trimers and tetramers through saponification should be excluded. Also, under both acid and neutral treatment conditions there was an apparent loss of phenolic acids and an accompanying precipitate formation, presumed to result from other peroxidase-associated reactions resulting in polymerization. This implies the HRP was promoting cross-linking rather than breakdown of ferulic acid.

Of the diferulates identified, the 8,5′ form predominated, with a trace of 5,5′ diFA and 8-*O*-4′ diFA, but there was no detectable 8,8′ diFA. Similar treatment of FA, using a lignin peroxidase (*23*), showed 8,5′ diFA was the preferred product, but significant amounts of 8,8′ diFA were also produced. The 8,8′ diFA has also been reported to be the favored radical coupling from FA with different peroxidases and laccases (*26*). One possibility why 8,8′ diFA was not detected is that 8,8′ diFA is more transient and involved in further polymerization to form more complex dehydropolymers (DHPs), whereas the less reactive diferulates accumulate.

In gelled SBP, the loss of FA was accompanied by the appearance of 8,8′ diFA and 8,5′ diFA and 8-*O*-4′ diFA at all gel concentrations tested, but 5,5′ diFA was not detected (**Figure 3a**). Because the apparent loss of FA and the concentration of diferulates show little change at increasing gel concentration (**Table 1**), this implies that steric restrictions on cross-linking

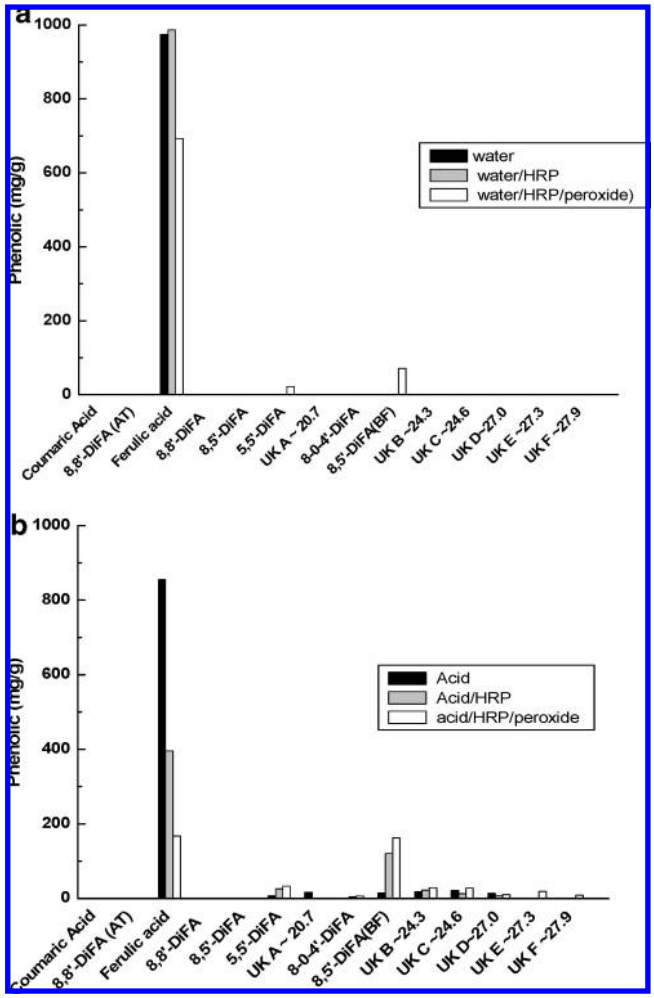

Figure 2. Effect of pH on the profile of phenolic acids obtained following HRP-mediated oxidative cross-linking of ferulic acid in solution: (**a**) in deionized water (pH 6.7); (**b**) in acid (pH 3.6). Results are shown as mean ± SD for samples analyzed as duplicates. For experimental details see Materials and Methods.

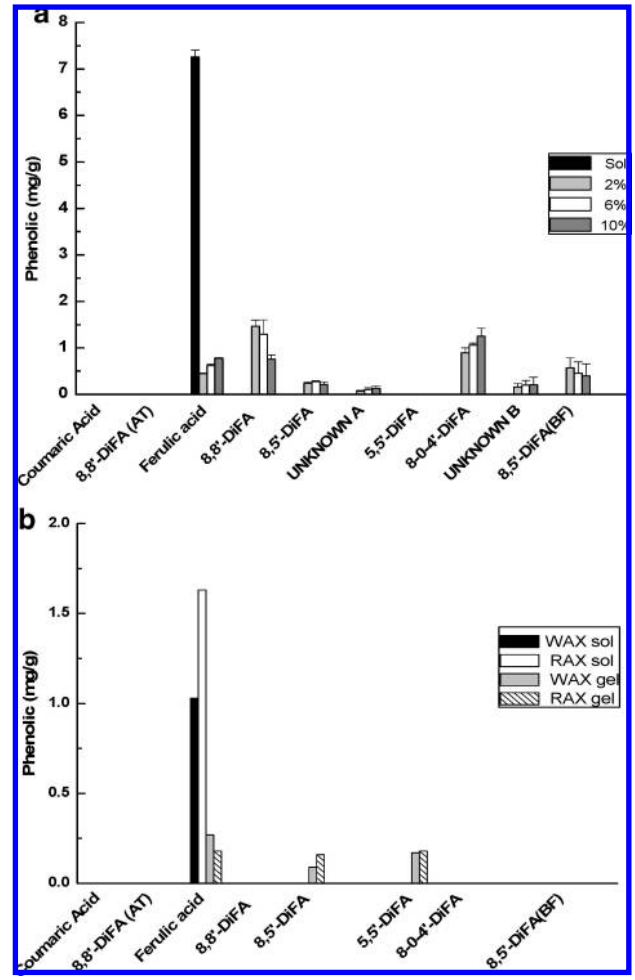

Figure 3. Effect of HRP-mediated oxidative cross-linking on the profile of phenolic acids from water-soluble polysaccharide extracts: (**a**) SBP in solution and at increasing gel concentration; (**b**) WAX and RAX in solution and at increasing gel concentration. Results are shown as mean ± SD for samples analyzed as duplicates. For experimental details see Materials and Methods.

are imposed at relatively low concentration of SBP. For the gelled WAX and RAX, neither 8,8′ diFA nor 8-*O*-4′ diFA was detected, whereas 8,5′ diFA and 5,5′ diFA were present. The formation of the 8,5′ diFA in the AX extracts indicates that while its formation may be intracellular *in vivo* (*27*), it can also be formed extracellularly and, structurally, may be a preferred steric product.

Water-extractable cereal endosperm AX has also been reported to be deficient in 8,8′ diFA (*4*), but 8-*O*-4′ diFA was detected in the current study. The existence of differences in diFA profile between sample sources in different investigations emphasizes how the availability of FA for cross-linking may be affected by esterification to a polysaccharide and the location of the ester link to the sugar. Differences in ester substitution, for example, to arabinopyranose in pectic polysaccharides in SBP or arabinofuranose in AX in cereals (*15, 16*), structural differences in the arabinose chain, and distribution of FA substituents can be proposed to affect the mobility and availability of FA for cross-linking. Also, the ratio of FA to sugars could be important for gel formation (**Table 2**), considering reported work with an extracted AX (*6*), which has shown that an ara/xyl/FA ratio of 600:2000:1 was limiting for gelation. For the WAX and RAX extracts in the current work the proposed limiting ratio was being approached, although gelation still occurred. The SBP was relatively highly substituted with FA

Table 2. Ferulic Acid Concentration and Molar Ratio in Substrates prior to HRP Treatment[a]

sample	ferulic acid (mg g^{-1})	molar ratios Ara/Xyl/FA
SB pectin	7.7	25:0:1
WAX	1.0	590:840:1
RAX	1.6	340:530:1
wheat bran	4.7	30:40:1
BSG	3.9	27:67:1

[a] Substrates are as described in **Table 1** and under Materials and Methods. Ferulic acid was extracted in 4 M KOH and quantified using HPLC, and sugars were measured as their alditol acetates from 1 M acid hydrolysates.

(**Table 2**) with an ara/FA ratio ~25:1. This was similar to the ara/FA ratio in the WB and BSG, but in the cereal residues this ratio encompasses the total spectrum of AX present within the cell wall and will mask tissue-specific differences known to exist (*13, 14*).

Extraction of phenolic acids from WB and BSG following treatment with peroxidase (**Figure 1**) showed no significant effect on either the total extractable phenolics or the spectrum of phenolic acids. Also, unlike the treatment of free FA at low pH, treatment of WB at low pH had no apparent effect on the profile of cross-linking. The residues showed a similar spectrum

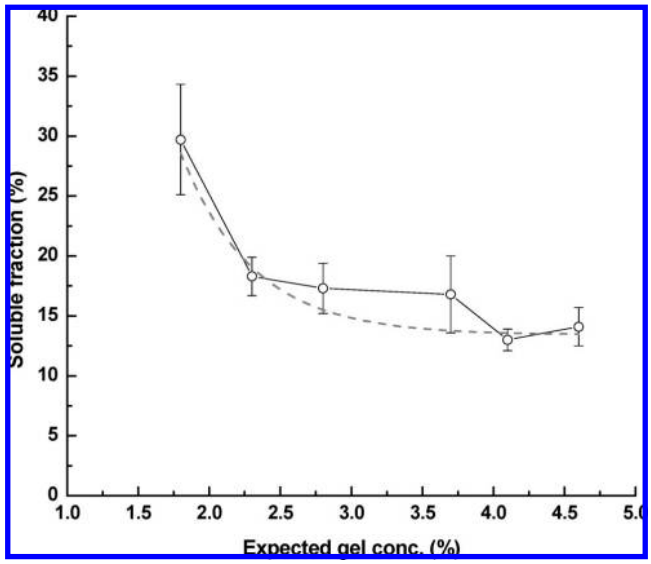

Figure 4. Apparent solubility of SBP in gels prepared at increasing concentration of SBP: (solid line) observed; (dashed line) fitted to first-order exponential decay ($y = y_0 + 533e^{-x/t_1}$) $y_0 = 13.4$, $A_1 = 533$, $t_1 = 0.21$. Results are shown as mean ± SD for samples analyzed as duplicates. For experimental details see Materials and Methods.

of diferulates pre- and post-treatments, including the 8,8′ diFA, 8,5′ diFA, 5,5′ diFA and 8-*O*-4′ diFA, and were not restricted to the 8,5′ diFA and 5,5′ diFA noted for the AX extracts tested. Treatments also showed no effect on the content of FA. This implies that the constituent phenolic acids within the cell wall matrix are unavailable for further cross-linking, through either their steric restrictions within the cell wall matrix or an inability of HRP to access and cross-link phenolic acids *in muro*.

Solubility. At above ~2% concentration, SBP and the AX extracts gelled when treated with HRP/peroxide. Samples did not gel without the presence of enzyme. Above a 3% solution the AX extracts became very viscous; hence, gels were prepared at only 2.5% concentration. The SBP showed little apparent change in viscosity below ~5%, consistent with what has been reported for other sugar beet pectin extracts (*2*).

At lower gel concentrations a significant proportion of the SBP gels prepared could be solubilized (**Figure 4**), which may be related to a bias for intramolecular cross-linking at lower concentrations of SBP and with higher concentrations required to promote intermolecular cross-linking and the formation of a more rigid gel network. Formation of a more rigid network is implicit with a reduced flexibility and hence implies a restriction on the potential to form novel cross-links.

The change in solubility with increasing concentration of SBP (**Figure 3**) showed that in gels prepared above ~4% concentration the proportion of material which could be solubilized could be extrapolated to ~13% of the constituent SBP. It remains unknown whether the nongelled material arises from a population of nonferuloylated pectins in the sample or whether above a critical density of cross-linker a proportion of the SBP was being sterically excluded from participating in gel formation. Extrapolation to conditions *in muro* then suggests that where an oxidatively cross-linked gel-like network exists, generation of novel intermolecular cross-links will be difficult. Enzymatic modification of polysaccharides may allow some scope for *in muro* modification of cross-linking, such as that reported using an α-L-arabinofuranosidase to modify a sugar beet pectin to increase accessibility to feruloyl groups carried by arabinose (*28*).

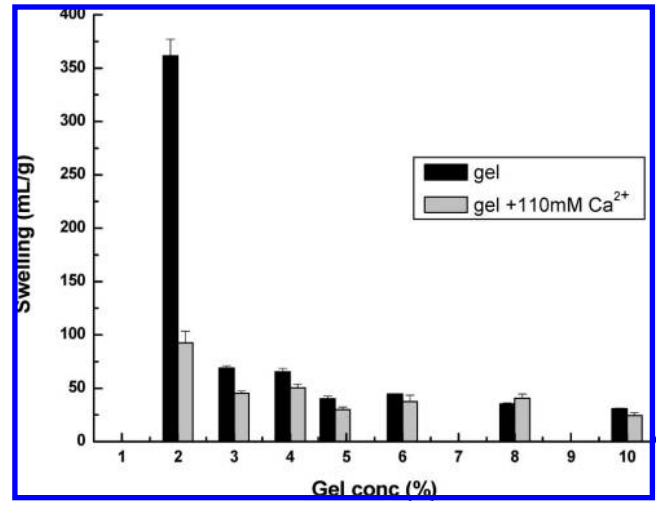

Figure 5. Swelling capacity of SBP gels in the presence and absence of Ca^{2+} following HRP-mediated oxidative cross-linking. Results are shown as mean ± SD for samples analyzed as duplicates. For experimental details see Materials and Methods.

Swelling. *Gelled Extracts.* Although the apparent density of cross-links per gram of SBP dry weight remained relatively constant at all gel concentrations, there was a notable effect of gel concentration on swelling (**Figure 5**). Whereas a 2% gel had a relatively high swelling capacity (~350 mL g^{-1}), at 3% and above gel concentration the SBP gel had a much reduced swelling capacity (<70 mL g^{-1}). Thereafter, only a modest decrease in swelling was noted with increased gel concentration. A similar profile for swelling has been reported for a sugar beet pectin treated with a laccase (*9*). The swelling capacity of the AX gels (~100 mL g^{-1}) was lower than the swelling capacity of SBP gels at similar gel concentration, although the FA content and hence apparent cross-linking density should be lower in the AX extracts. The concentration of FA in the WAX and RAX extracts (~1.6 mg g^{-1}) approached the lower limit reported for gelation to occur, as modeled using a water-extractable AX (WEAX) (*6*). In the WEAX, gelation was dependent on the degree of feruloylation. As reported (*6*), a 1% WEAX gel, composed of ~1.6 mg of FA g^{-1}, had a swelling capacity similar to that noted for the SBP at ~2% concentration; at higher degrees of feruloyation the swelling capacity of the WEAX decreased. This would also be consistent with swelling capacity being controlled by the density of cross-links. Because not all of the FA present is used for cross-link formation, some steric restrictions must exist to limit the potential to form cross-links and hence the density of cross-links which can be realized. It should not be assumed that cross-link formation is directly proportional to the FA concentration, and models should account for steric restrictions imposed by cross-link formation. Differences in swelling between SBP and AX at equivalent concentration also indicate that factors other than oxidative cross-linking may contribute to mechanical properties.

Salt Treatment of Gelled Extracts. Incubation in excess calcium (**Figure 5**), especially at lower gel concentration, reduced the swelling capacity of SBP gels, such that the swelling capacities of SBP and AX gels were similar at equivalent concentrations. Therefore, at low gel concentration, oxidative cross-linking in SBP must leave some flexibility within the gel network. However, a relatively modest increase in gel concentration (~3%) can result in the apparent loss of any flexibility in SBP. No similar effect of calcium was noted for the AX gels, and given that uronic acid is present in the SBP but not detected in the AX extracts, then the effects of calcium on the SBP gel

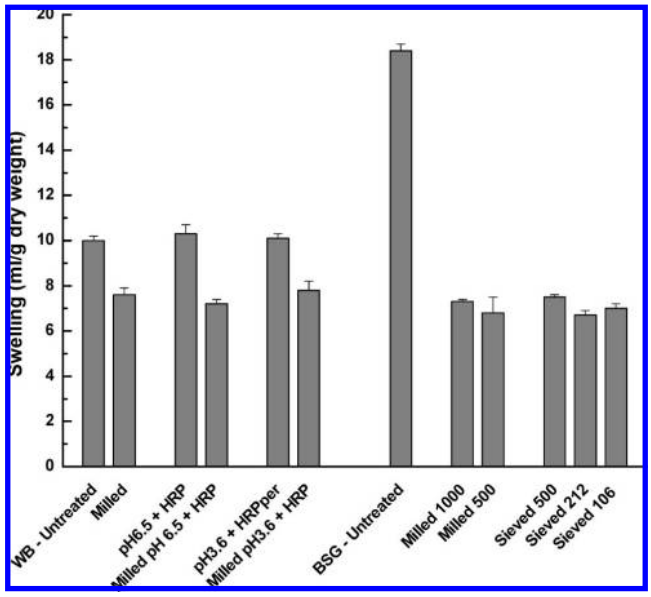

Figure 6. Effect of particle size and HRP treatment on the swelling capacity of WB and BSG. Results are shown as mean ± SD for samples analyzed as duplicates. For experimental details see Materials and Methods.

can be accounted for by the presence of a pectin gel formed through calcium cross-links (*29*). The result is a covalently cross-linked gel interacting with an ionically cross-linked gel. Such gels are considered to have potential medical and pharmaceutical applications (*8*). Gels incubated in NaCl, at equivalent molar concentration to $CaCl_2$, to distinguish whether salts were having an osmotic effect on swelling (results not shown), showed a decrease in swelling of the SBP gel in the presence of Na, similar to that reported previously (*9*). However, the effect of both NaCl and $CaCl_2$ was negligible for AX gels. At 2%, SBP gel concentration swelling decreased by ~10% in the presence of sodium, compared to 80% in the presence of calcium. At higher gel concentrations the effect of sodium and calcium was negligible. At increased gel concentration the expected increase in the density of oxidative cross-links is presumed to restrict formation of calcium cross-links.

The trends in swelling suggest that *in muro*, where the apparent concentration of pectic polysaccharide (and AX) may exceed 30% (*30*), the swelling potential should be similar to that measured in the 10% SBP gel.

Cereal Residues. In the cereal residues (**Figure 6**), HRP treatment had no apparent effect on swelling. Given the observed existence of free FA in the WB and BSG and its persistence following exposure to HRP, this suggests there are major steric constraints on the availability of FA for cross-linking in the cereal residues.

The swelling capacity of the cereal residues is controlled more by particle size than by HRP treatment. Even at pH 3.6, at which there was extensive oxidative cross-linking of free FA in solution, there was no change detectable in the swelling of WB. Although there may be the potential to induce changes in oxidative cross-links and modify mechanical properties in specific tissues, as shown using isolated strips of WB (*10*), to realize this potential in general will apparently require selective modification to the tissues to release steric constraints and make constituent phenolics available for further cross-linking. Even with considerable disruption to the cellular network in the BSG and sieving to isolate different particle sizes (**Figure 5**), no apparent change in swelling capacity for particle size below 500 μm was observed. Bulk measurements such as swelling may be too imprecise to be able to detect changes in mechanical properties originating *in muro*, where constraints on swelling will be imposed by the presence of a rigid lignocellulosic network. This does not preclude changes occurring, as noted using the isolated strips of WB (*10*), but rather indicates that more specific methods are required to monitor changes in mechanical properties (*30*).

General Discussion. Oxidoreductases, such as HRP, can be used to modify oxidative cross-links between phenolic acids and give the potential to modify the mechanical properties of cereal residues, such as WB and BSG. When free in solution, FA is readily polymerized by HRP under the experimental conditions used, and from the spectrum of diferulates isolated, there is little apparent steric limitation on interphenolic linkages. However, when esterified to a polysaccharide, the linkage profile of diferulates becomes more restricted. The persistence of free FA also indicates that steric limitations must exist on the availability of FA for cross-linking when esterified to a polysaccharide. This situation also exists *in muro* in the cereal residues, where there is a relatively high concentration of FA that cannot be induced to form cross-links.

Gels formed from SBP through treatment with HRP show that even at relatively low polysaccharide concentration swelling capacity becomes restricted, implying a rigid network is formed. Structural constraints imposed by such networks will restrict the ability to form further cross-links, and at gel concentrations >3% in SBP the limited swelling capacity showed that most of the flexibility in the system was lost. The estimated polysaccharide concentration *in muro*, equivalent to ~30% gels, allows little flexibility to form novel cross-links without an associated treatment to either remove or modify existing cross-links or to modify the lignocellulosic network.

It would appear that to exploit cereal residues through the controlled modification of their mechanical properties, oxidative cross-linking treatment will have to be considered in conjunction with other physical and chemical treatments. Such treatments need to focus on increasing the "flexibility" of the cell wall matrix and the accessibility of enzymes such as HRP to the matrix. Also, more precise and controlled methods are required to monitor effects of treatment on the mechanical properties.

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