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Moderate Ferulate and Diferulate Levels Do Not Impede Maize Cell Wall Degradation by Human Intestinal Microbiota

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The degradation of plant fiber by human gut microbiota could be restricted by xylan substitution and cross-linking by ferulate and diferulates, for example, by hindering the association of enzymes such as xylanases with their substrates. To test the influence of feruloylation on cell wall degradability by human intestinal microbiota, nonlignified primary cell walls from maize cell suspensions, containing various degrees of ferulate substitution and diferulate cross-linking, were incubated in nylon bags in vitro with human fecal microbiota. Degradation rates were determined gravimetrically, and the cell walls were analyzed for carbohydrates, ferulate monomers, dehydrodiferulates, dehydrotriferulates, and other minor phenolic constituents. Shifting cell wall concentrations of total ferulates from 1.5 to 15.8 mg/g and those of diferulates from 0.8 to 2.6 mg/g did not alter the release of carbohydrates or the overall degradation of cell walls. After 24 h of fermentation, the degradation of xylans and pectins exceeded 90%, whereas cellulose remained undegraded. The results indicate that low to moderate levels of ferulates and diferulates do not interfere with hydrolysis of nonlignified cell walls by human gut microbiota.

KEYWORDS: Ferulic acid; diferulic acids; triferulic acids; cross-links; arabinoxylans; maize cell walls; fermentation; human fecal microbiota; degradability

INTRODUCTION

According to a widely accepted definition, dietary fiber is the edible part of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine (*1*). Consequently, polysaccharides, oligosaccharides, lignin, and associated plant substances are included as dietary fiber constituents. Major constituents of cereal dietary fibers are arabinoxylans, mixed-linked β-glucans, and cellulose. The physiological effects of arabinoxylans are dependent on their physicochemical properties, which are related to their degree of polymerization, the arabinose/xylose ratio, the distribution of side chains, and the degree of cross-linking, especially via hydroxycinnamates. Ferulate is the predominant hydroxycinnamate in cell walls of cereal grains, acylating arabinose side chains at the *O*-5-position (*2*). Radical coupling of ferulates yields ferulate dehydrodimers (*3*) with lesser amounts of ferulate dehydrotrimers (*4*−*8*) and ferulate dehydrotetramers (*8*), which mediate intra-/intermolecular coupling of arabinoxylans (*9*, *10*). Arabinoxylans may also be coupled to lignin (*11*, *12*) and proteins (*13*) via a radical mechanism involving ferulates, further bolstering cell wall cross-linking. Although lignification is a major determinant to fiber degradability (*14*), ferulates and diferulate cross-links are also thought to inhibit polysaccharide degradation. Whereas early studies suggested a direct toxicity of phenolic acids on ruminal microbes, more recent studies proposed that ferulate substitution and especially diferulate cross-linking impede binding of endoxylanases, thus limiting xylan degradation (*15*−*17*). For example, studies with nonlignified cell walls from maize cell suspensions revealed that diferulate cross-links restrict the rate and, to a lesser degree, the extent of cell wall and xylan degradation by fungal enzymes (*18*). Other work with whole and fractionated wheat bran indicates that xylan degradation by endoxylanase was related to the extent of arabinose substitution and diferulate cross-linking (*19*, *20*). These restrictions may be partially overcome by esterases able to cleave diferulate esters. However, feruloyl esterases, including the esterase produced by the intestinal bacterium *Lactobacillus acidophilus*, are much more efficient in releasing ferulate and its dimers from soluble substrates than

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from untreated insoluble cell walls (*21*−*24*). The aim of this study was to establish whether ferulate substitution and diferulate cross-linking influence the degradation of nonlignified cell walls and cell wall arabinoxylans by human intestinal microbiota. In this study, we used a cell wall model system in which ferulate substitution and diferulate cross-linking were specifically manipulated without altering other cell wall properties.

MATERIALS AND METHODS

General. Nylon bags (4.5 × 9.0 cm) used in the fermentation experiments were made from polyamide monofilament fabrics (NITEX, polyamide 6.6, mesh opening = 10 μm) (Sefar, Freibach, Switzerland). Prior to use, nylon bags were heated for 30 min at 100 °C and stored at 55−60 °C to minimize bacterial contamination. Brain-heart infusion (BHI) broth was from Roth (Karlsruhe, Germany). Sephadex LH-20 was from Pharmacia Biotech (Freiburg, Germany). Chemicals used in chromatographic procedures were of HPLC grade.

Synthesis of Primary Maize Cell Walls. Cell walls with different concentrations of ferulates and diferulates were prepared according to the method of Grabber et al. (*25*, *26*). Cell suspensions (350 mL) of maize (*Zea mays* L. cv. Black Mexican) were grown in 1 L flasks. On the third and ninth days after culture initiation, 2-aminoindan-2-phosphonic acid (AIP) was added at 0 or 5.4 mg/L to produce cells with normal or reduced levels of cell wall feruloylation. After 14 days of culture, cells were collected at the early stationary growth phase on a nylon mesh (20 μm pore size) and washed with cold 1,4-piperazinediethanesulfonic acid (PIPES) buffer (10 mM, pH 7.0). Cells were suspended in PIPES buffer and ruptured by two passages (15 min) through a Parr nitrogen bomb maintained at 1500 psi. After cell rupture, cell wall fragments were collected on a nylon mesh (20 μm pore size) and washed sequentially with PIPES buffer, aqueous $CaCl_2$ (50 mM), and water to remove cytoplasmic contaminants. Cell walls from normal and AIP-treated cultures were resuspended in water, and then an aqueous solution of H_2O_2 (0 or 0.3 mmol per gram of dry weight of cell walls) was added with stirring over a 60 min period to promote diferulate formation via wall-bound peroxidase. After an additional 30 min of stirring, cell walls were collected on glass microfiber filters (3.1 μm retention), washed thoroughly with water and acetone, and then oven-dried at 55 °C.

Characterization of Cell Walls. Analysis of neutral carbohydrate components was performed as described previously (*27*, *28*). Briefly, cell walls were pretreated with 12 M H_2SO_4 at 35 °C for 2 h. Following dilution, hydrolysis was performed with 2 M H_2SO_4 for 1 h at 100 °C. Cellulose crystallinity is a main barrier to its chemical degradation. Therefore, prehydrolysis using 12 M H_2SO_4 is needed to alter the fine structure of cellulose. In contrast, acid hydrolysis of hemicelluloses using 2 M H_2SO_4 is possible without pretreatment using 12 M H_2SO_4. Uronic acids in acid hydrolysates were determined colorimetrically according to the method of Blumenkrantz and Asboe-Hansen (*29*). Following reduction of neutral monosaccharides with $NaBH_4$, the resulting alditols were acetylated with acetic anhydride by using 1-methylimidazole as a catalyst. Erythritol was used as internal standard compound. Alditolacetates were determined by GC-FID (GC Focus Series, Thermo Electron Corp., Milan, Italy) using a 0.32 mm × 30 m DB-5 capillary column (0.25 μm film thickness) (J&W Scientific Inc., Folsom, CA). GC conditions were as follows: initial column temperature, 150 °C, held for 3 min, ramped at 4 °C/min to 220 °C, held for 2 min, ramped at 10 °C/min to 300 °C, and held for 10 min; injector temperature, 250 °C; split ratio, 1:6; flame ionization detection (detector temperature, 290 °C). He (1.6 mL/min) was used as carrier gas.

Phenolic monomers and di-/triferulates were analyzed by HPLC with photodiode array detection (DAD) following saponification and a preseparation using small-scale Sephadex LH-20 chromatography, as will be described in detail elsewhere. In brief, cell walls were saponified for 18 h in the dark using 2 M NaOH (degassed with N_2). Monomethylated 5-5-dehydrodiferulic acid and *o*-coumaric acid were added as internal standards just prior to saponification. The alkaline hydrolysates were acidified (pH <2), and phenolic compounds were extracted into diethyl ether (three times). Combined ether extracts were evaporated under a stream of N_2, redissolved in methanol (MeOH)/H_2O (50:50, v/v), and applied to a SPE column filled with 1 g of Sephadex LH-20 swollen in H_2O. Phenolic monomers were eluted with 3 mM aqueous HCl and recovered by extraction into ethyl acetate. Di-/triferulates were then eluted from the column with ethanol. After evaporation, phenolic monomers [redissolved in MeOH/H_2O (20:80, v/v)] and di-/triferulates [in MeOH/H_2O (70:30, v/v)] were analyzed by HPLC-DAD (L-6200 Intelligent Pump, Merck Hitachi, Tokyo, Japan; HPLC 540 diode array detector, BioTek Kontron Instruments, Milan, Italy; Spectra Series Autosampler AS100, Thermo Separation Products, Dreieich, Germany; 2155 HPLC column oven, LKB, Bromma, Sweden) on a 250 × 4.6 mm i.d., 5 μm Luna Phenyl-Hexyl 100 Å column (Phenomenex, Aschaffenburg, Germany). The injection volume was 20 μL, the column temperature was 45 °C, and the flow rate was maintained at 1 mL/min. Separation of phenolic monomers was performed using a ternary elution system consisting of 1 mM aqueous trifluoracetic acid (A), acetonitrile (B), and MeOH (C). Elution was carried out as follows: initially A 87%, B 13%, and C 0%, held isocratically for 10 min, linear over 10 min to A 77%, B 20%, and C 3%, linear over 5 min to A 70%, B 25%, and C 5%, linear over 5 min to A 25%, B 50%, and C 25%, followed by an equilibration step. Elution of di-/triferulates was performed as follows: initially A 85%, B 15%, and C 0%, linear over 15 min to A 82%, B 18%, and C 0%, linear over 5 min to A 80%, B 20%, and C 0%, linear over 5 min to A 72%, B 25%, and C 3%, linear over 5 min to A 70%, B 25%, and C 5%, held isocratically for 5 min, linear over 5 min to A 65%, B 30%, and C 5%, held isocratically for 5 min, linear over 5 min to A 55%, B 40%, and C 5%, linear over 10 min to A 10%, B 50%, and C 40%, followed by an equilibration step. Detection wavelength was 280 nm.

Cell Wall Fermentation. To provide a broad spectrum of microbiota, aliquots (∼10 g) of freshly voided human feces were combined from five female and three male donors (ranging from 26 to 61 years of age, mean age of 39 years) who had not undergone antibiotic therapy within 6 months prior to the study. The pooled fecal sample (81.2 g) was mixed with 320 mL of BHI broth (pH 7.4) in a stomacher bag and homogenized (Stomacher model 3500, Seward Ltd., London, U.K.). The resulting suspension was filtered through a sieve with a 1 mm mesh opening to remove large particles, and 10 mL of the resulting suspension was added to 500 mL bottles containing 190 mL of BHI broth. To each bottle was added a nylon bag containing 500 mg of cell walls, and the bottles were sealed with natural rubber stoppers and screw caps. Except for feces homogenization, the preparation of the fecal suspension and fermentation bottles was done under anoxic conditions using a glove box (MACS Anaerobic Workstation, Don Whitley Scientific, Shipley, U.K.) containing a gas atmosphere of $N_2/CO_2/H_2$ (80:10:10, v/v/v). Fermentation bottles were incubated in duplicate on a rotary shaker (200 rpm) at 37 °C for 4, 8, and 24 h. Following fermentation, the bags were removed from the bottles and thoroughly washed with water, followed by acetone. Fermentation residues were carefully dried under N_2 and further dried for 2 days at 55 °C before weighing for gravimetric determination of degradation rates. To estimate cell wall losses during the incubation procedure and further sample handling, additional cell walls were incubated in a similar manner but without feces addition, washed, and dried as described above. The fermentation residues were chemically characterized as described above for unfermented cell walls.

Statistical Analysis. Data were statistically analyzed by two-factorial analysis of variances at the 0.05 level of significance using SPSS software for Windows, version 11.5.2.1.

RESULTS AND DISCUSSION

Cell Wall Characteristics. Maize cell suspension cultures were grown normally or with AIP, a specific inhibitor of phenylalanine ammonia-lyase (*30*), to produce cell walls with normal or reduced levels of xylan feruloylation. Cell walls isolated from the suspension cultures were in turn incubated in water or in water with H_2O_2 to yield normal or elevated levels of di-/triferulate cross-links formed via wall-bound peroxidase. Dry cell walls produced without AIP addition (AIP −,

H_2O_2 −) contained 15.8 mg/g of total ferulates, 86% of which were ferulate monomers. Treatment with H_2O_2 of these cell walls (AIP −, H_2O_2 +) increased peroxidase-mediated diferulate formation from 2.1 to 2.6 mg/g (total ferulate concentration = 14.8 mg/g) without changing the diferulate pattern. Adding AIP to cell suspensions (AIP +, H_2O_2 −) reduced total ferulate concentrations by 82%. Addition of H_2O_2 to these cell walls (AIP +, H_2O_2 +) had marginal impact on diferulate cross-linking, suggesting that H_2O_2 production during cell growth was sufficient for maximal formation of diferulates. In all treatments, ferulate dimers were dominated by 8-5-coupled diferulic acids (49%) with the cyclic, the open, and the decarboxylated forms (*3*) being detected in alkaline hydrolysates. The cyclic 8-5-coupled diferulate is probably the natural form in cell walls, whereas the other structures were presumably formed during the saponification procedure. The 5-5-coupled and 8-8-coupled diferulic acids contributed 21 and 19% of diferulates, respectively. The 8-8-coupled dimers included the open, the cyclic, and the newly discovered tetrahydrofuran forms (*31*). The 8-*O*-4-coupled structure was the least abundant, accounting for 11% of the diferulates. Three triferulates were detected [5-5,8-*O*-4-coupled triferulic acid, 8-8(cyclic),8-*O*-4-coupled triferulic acid, and 8-*O*-4,8-*O*-4-coupled triferulic acid] in alkaline hydrolysates, but the low abundance of each trimer (<0.05 mg/g) made quantification difficult. Because each triferulic acid molecule is potentially attached to three xylan chains, they can play a major role in cell wall cross-linking. Cell walls also contained about 0.3 mg/g of *p*-coumaric acid, whereas other phenolic monomers, such as vanillin, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, caffeic acid, and vanillic acid, were detected in lower amounts (<0.15 mg/g). The concentrations of ferulate and diferulates we observed were comparable to that found in insoluble dietary fiber isolated from wheat and spelt grains but lower than that reported for maize bran (*26*, *32*).

The polysaccharide composition of the prepared maize cell walls was determined and shown to be typical for primary cereal walls (*25*). AIP and H_2O_2 treatments did not affect the polysaccharide composition. Dry cell walls contained 204−232 mg/g of glucose, 151−165 mg/g of arabinose, 135−142 mg/g of xylose, 115−120 mg/g of uronic acids, 80−86 mg/g of galactose, 8−11 mg/g of mannose, and ≤5 mg/g of rhamnose. Our acid hydrolysis procedures indicated about two-thirds of the glucose was derived from cellulose with the balance being derived from hemicelluloses such as β-glucans. Arabinose and xylose originated predominantly from arabinoxylans. The high arabinose/xylose ratio may be due to additional arabinose contribution from pectins, particularly arabinan side chains. However, small amounts of xyloglucans were also supposed to be present (*33*). Galactose, mannose, and rhamnose presumably originated from hemicelluloses and pectins. The detected uronic acids also indicated the presence of pectins but may partially also stem from glucuronoarabinoxylans.

Cell Wall Fermentation. Fermentation studies were performed under conditions mimicking the human colon. Cell walls were fermented using nylon bags with a pore size of 10 μm to permit ingress of fecal microorganisms while minimizing the loss of unfermented cell walls. Diffusion of unfermented cell wall components through the nylon pores is regarded as highly unlikely because the cell walls were obtained by filtration over a nylon mesh with a pore size of 20 μm. Indeed, in tests of the incubation (8 and 24 h) and washing procedures without fecal microbiota, only ∼6% of the cell walls were lost. These losses were considered in calculations of cell wall degradation. The overall degradation of all investigated cell walls averaged 5% (4−6%) after 4 h, 24% (23−30%) after 8 h, and 65% (64−69%) after 24 h of fermentation (**Figure 1**).

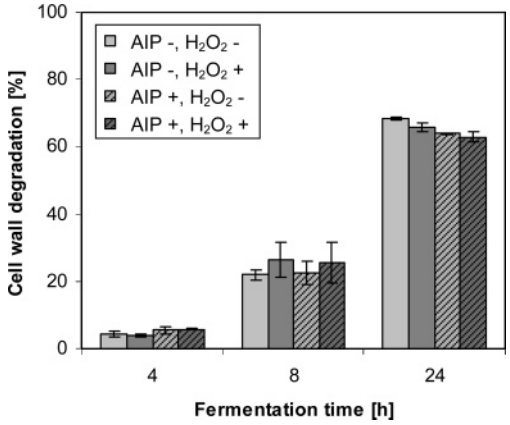

Figure 1. Time-dependent degradation of maize cell walls during fermentation with human fecal microbiota. Cell walls differed in treatment with (+) or without (–) 2-aminoindan-2-phosphonic acid (AIP) and H_2O_2. Bars represent standard deviations of repeat determinations.

The degradation of cell walls after 4, 8, and 24 h of fermentation was not affected ($P > 0.05$) by AIP or H_2O_2 treatments (**Figure 1**), indicating that variations in xylan feruloylation and diferulate cross-linking, as generated within this study, did not influence whole cell wall fermentation by human fecal microbiota.

To investigate the influence of AIP and/or H_2O_2 treatments on the degradation of individual cell wall polysaccharides, especially arabinoxylans, the monomer carbohydrate composition of the residual cell walls was analyzed following acidic hydrolysis. These studies revealed that cell wall polysaccharides, with the exception of cellulose, were degraded extensively during fermentation (**Figure 2a**). Arabinoxylan degradation was not affected by either ferulate concentrations or ferulate cross-linking. Arabinoxylans were only slightly degraded after 4 h as indicated by minor reductions in xylose concentrations (**Figure 2a**). From **Figure 3** it might be assumed that reduced ferulate concentrations lead to an enhanced degradation of arabinoxylans after 4 h of fermentation. However, differences in xylose removal from residual cell walls were not significant ($P > 0.05$). Similar findings were observed for the release of arabinose after 4 h, suggesting that the total concentration of ferulates may influence arabinoxylan degradation within the first hours of fermentation. However, this needs to be investigated in further studies using shorter time periods. Removal of arabinose and xylose from residual cell wall components after 8 and 24 h was not significantly affected by AIP and/or H_2O_2 treatments. After 24 h of fermentation, the extensive loss of arabinose (95−99%) and xylose (89−94%) further indicated that modest levels of ferulate substitution and diferulate cross-linking did not impede xylan degradation (**Figure 3**).

Galactose and uronic acid concentrations were also efficiently reduced during fermentation. Cell walls contained <1% of these polysaccharide constituents after 24 h of fermentation (**Figure 2a**), indicating that pectins were almost completely degraded. By contrast, glucose-containing polysaccharides were slowly degraded with only about 27% of glucans lost after 24 h of fermentation. As a consequence, the residual cell wall material was highly enriched in glucose-containing polysaccharides (**Figure 2b**). Performing carbohydrate analysis with and without prehydrolysis in 12 M H_2SO_4 revealed that all glucose lost during fermentation was derived from hemicelluloses such as β-glucans, whereas cellulose remained essentially undegraded.

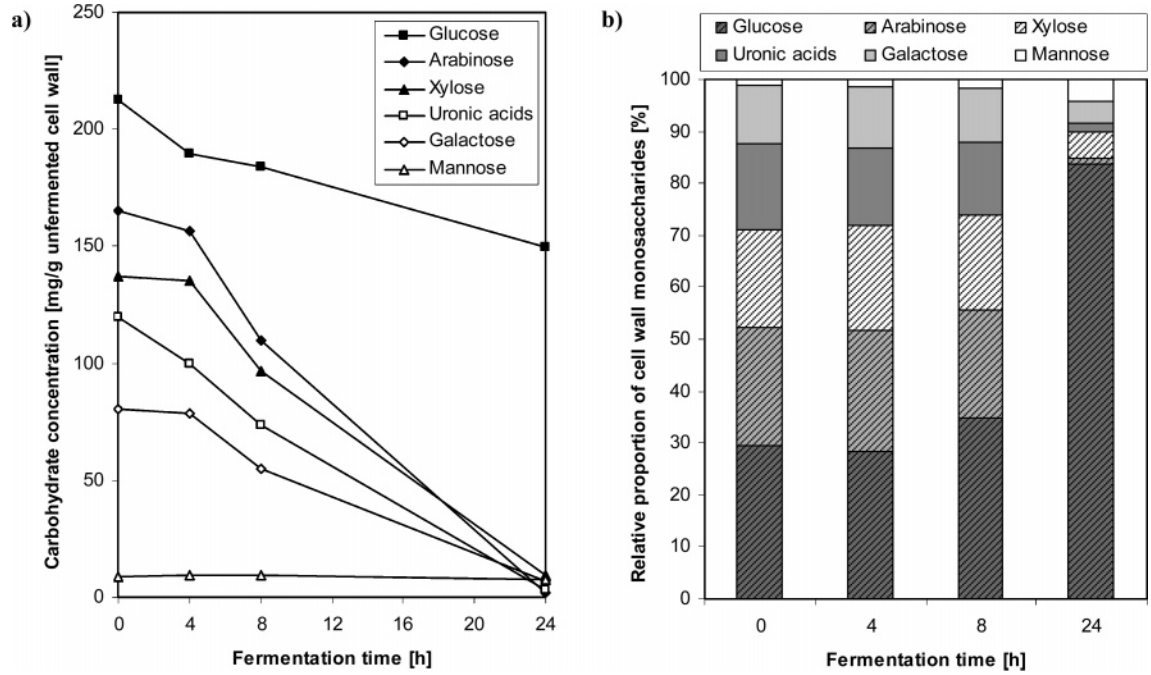

Figure 2. Degradation of carbohydrate constituents in primary maize cell walls during fermentation with human fecal microbiota: (**a**) carbohydrate monomer concentrations of residual maize cell walls fermented with human fecal microbiota for varying time periods (carbohydrate concentrations are referred to the original cell wall before fermentation); (**b**) proportions of monosaccharides in cell wall residues (% of total carbohydrate). Because degradation patterns did not vary between cell wall types, carbohydrate degradation is exemplified by cell walls with nonmanipulated feruloylation (AIP –, H_2O_2 –).

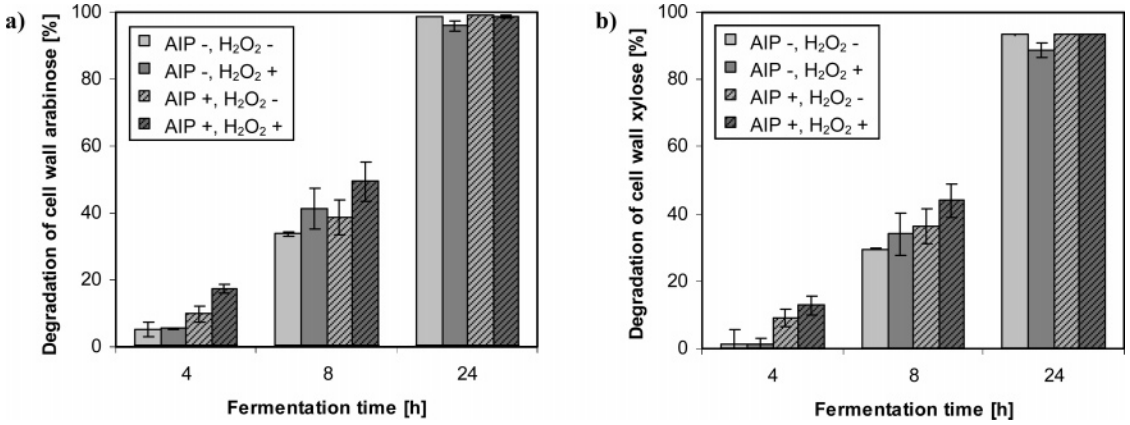

Figure 3. Time-dependent degradation of (**a**) arabinose and (**b**) xylose from maize cell wall polysaccharides during fermentation with human fecal microbiota. Cell walls differed in treatment with (+) or without (–) 2-aminoindan-2-phosphonic acid (AIP) and H_2O_2. Bars represent standard deviations of repeat determinations.

Mannose, a minor carbohydrate in unfermented cell walls, was not influenced by fermentation.

The analysis of monomeric and oligomeric ferulates in cell wall residues revealed that monomeric ferulate was slowly released during the first hours of fermentation (<10%). After 8 and 24 h of fermentation, ferulate concentrations were reduced by about 25 and 85−95%, respectively. Diferulate levels changed only slightly after 4 h of fermentation as illustrated in **Figure 4a**. Diferulate release was more distinct after 8 (15−30%) and 24 h (93−96%) of fermentation. The ratio of diferulate regioisomers in the cell wall remained fairly constant during the fermentation (**Figure 4b**). Although ineffective for inhibiting arabinoxylan degradation by human fecal microbiota, diferulates accumulated on undegraded xylans during fermentation of H_2O_2-treated (AIP −, H_2O_2 +) cell walls (**Figure 5**). Triferulates were removed effectively from all cell wall preparations. However due to low abundance, reliable estimates of trimer concentrations could not be determined.

In light of previous studies that indicate inhibitory effects of ferulates, and particularly diferulates, on degradation (*15*, *17*−*19*), the results of our studies were somewhat surprising. However, enzyme isolates or isolated hemicelluloses were often used in previous studies, and these may not fully reflect the degradation of cell walls by fecal microbiota. Although endoxylanases play a predominant role in degrading xylans in cell walls, feruloyl esterases produced by human gut microbiota may help to overcome the restrictions caused by ferulate cross-linking (*21*, *34*, *35*). Recently, a bacterial feruloyl esterase was purified and characterized from a human gut-relevant species, *Lactobacillus acidophilus* (*24*). Whereas the feruloyl esterase from *L. acidophilus* was not able to release ferulates from highly polymerized xylans, Kroon et al. (*23*) described a feruloyl

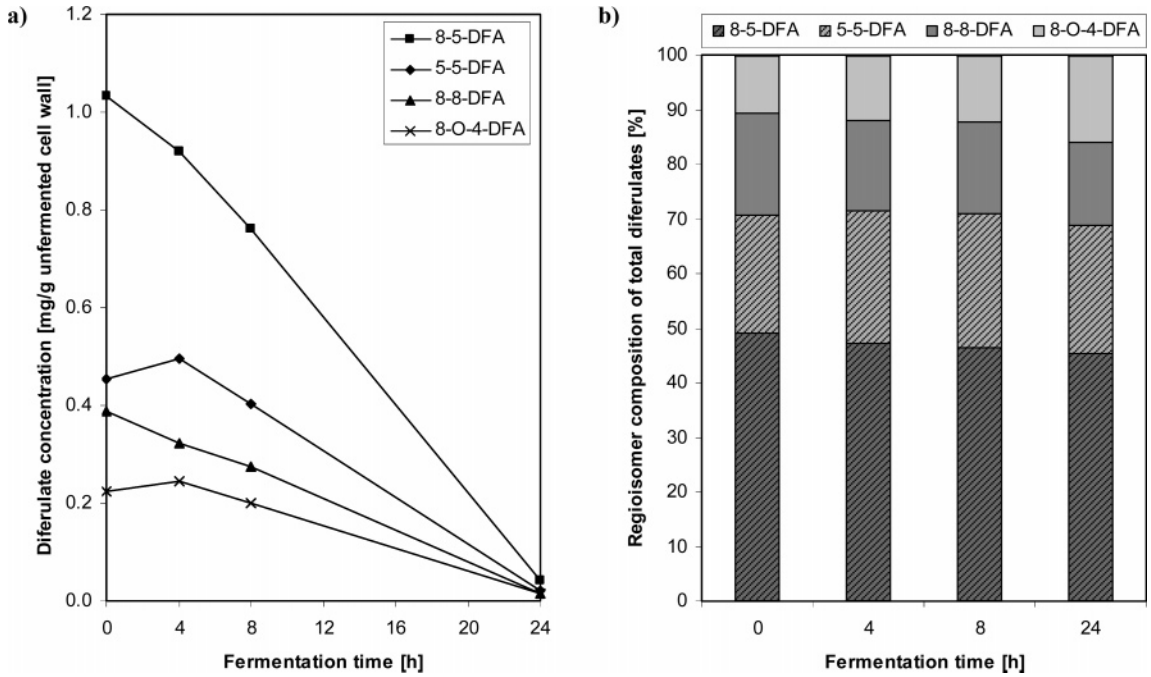

Figure 4. Release of diferulates from maize cell walls during fermentation with human fecal microbiota exemplified by cell walls with nonmanipulated feruloylation (AIP –, H_2O_2 –): (**a**) diferulate (DFA) concentrations of cell walls fermented for various time periods; (**b**) regioisomer composition of total diferulates of cell wall residues during fermentation.

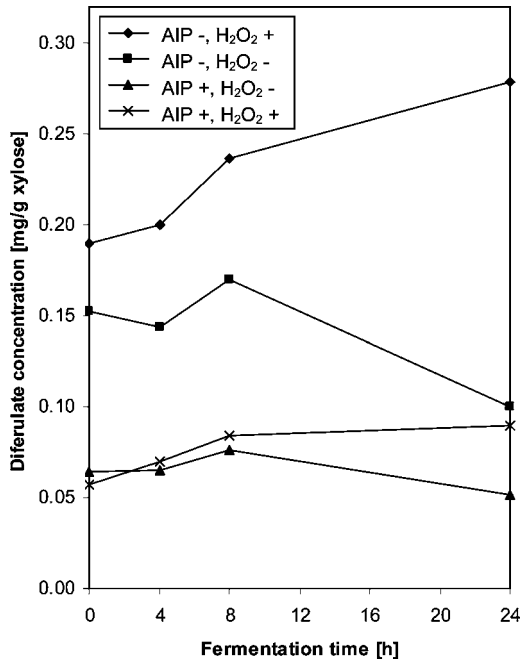

Figure 5. Ratio of residual total diferulates and xylose from cell walls during fermentation with human fecal microbiota. Cell walls differed in treatment with (+) or without (–) 2-aminoindan-2-phosphonic acid (AIP) and H_2O_2.

esterase from the gut-irrelevant fungus *Penicillium funiculosum* that was able to release ferulic acid from wheat bran and sugar beet pulp. Although the activity was enhanced by a xylanase, the presence of this enzyme was not mandatory. However, the release of diferulates has not been observed from complex matrices to date. From our study we cannot conclude whether ferulates, di-, and triferulates are liberated as free acids or ester-linked to arabinoxylan fragments. Preliminary work also suggests that cell wall cross-links might partly be broken by cleavage of ether linkages in diferulates by human gut microbiota (Braune et al., unpublished results). Although the fairly constant diferulate pattern during fermentation suggests that this mechanism is probably minor in our studies, such a mechanism should additionally be considered and investigated in more detail. Apart from this less investigated mechanism and a potential effect of feruloyl esterases, it should be considered that the degree of cross-linking in the investigated cell walls was simply not effectual to inhibit xylanases from degrading arabinoxylans. In some types of fiber, poor xylan degradation is associated with extremely high ferulate and diferulate concentrations. Maize bran insoluble dietary fiber, for example, contains 25 mg/g of ferulates and 13 mg/g of diferulates (*32*, *36*). Unfortunately, our attempts to elicit higher degrees of ferulate substitution in maize cell suspensions and more extensive diferulate cross-linking proved to be unsuccessful.

Conclusions. Cell walls prepared from maize cell suspensions enabled us to investigate the specific role of ferulate monomers and di-/triferulate cross-linking as constraints to cell wall fermentation by human fecal microbiota. Fermentation studies showed that low to moderate levels of ferulate substitution or diferulate cross-linking do not impede the degradation of nonlignified cell walls. A slight enrichment of diferulates on xylans was observed in fermentation residues from cell walls with the highest degree of cross-linking, but the efficient overall degradation of xylans was not affected. We suppose that human fecal microbiota exhibit sufficient xylanase and/or feruloyl esterase and cross-link cleaving activities to overcome any inhibitory effects of ferulates and diferulates found in many types of insoluble cereal dietary fibers.

ABBREVIATIONS USED

AIP, 2-aminoindan-2-phosphonic acid; BHI, brain-heart infusion; PIPES, 1,4-piperazinediethanesulfonic acid.

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