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Inhibition of Structural Carbohydrate Fermentation by Forage Phenolics

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The benzoic and cinnamic acids found in forages were tested for their inhibitory effects on digestion of cellulose and hemicellulose by mixed cultures of rumen microorganisms *in vitro*. Rate and extent of cellulose digestion with the addition of cinnamic acids were determined. The benzoic acids and aldehydes were also compared for their inhibitory potential. Of the benzoic acid derivatives, only the parent compound (benzoic acid) inhibited in vitro dry matter disappearance (IVDMD) of cellulose after 48 h, whereas most of the cinnamic acids were inhibitory. None of these compounds depressed IVDMD of hemicellulose. Extent of cellulose digestion was unaffected by cinnamic acids, but rate of digestion was significantly reduced. The ranking of compounds for inhibitory potential was essentially reversed between 48 and 168 h of fermentation as compared with the ranking for 0 to 48 h of fermentation. There were also shifts in the molar proportions of volatile fatty acids produced between these times. The rapid reduction in concentration of cinnamic acids by microbial fermentation may explain these shifts. Benzoic aldehydes were more inhibitory of structural carbohydrate fermentation than were acids. No clear relationship existed between addition of hydroxyl or methoxyl groups to benzoic or cinnamic acids and their depressing effects on IVDMD. Cinnamic acids, which are more abundant in forages, were more inhibitory than the benzoic acids; but for all compounds, it appeared that hemicellulolytic bacteria were generally more tolerant of these compounds than the cellulolytic bacteria.

Keywords: Benzoic acid; benzoic aldehydes; cinnamic acid; forage phenolics; cellulose; fermentation.

1. Introduction

Plants are known to contain a wide variety of simple phenolic compounds,[1] of which the benzoic and hydroxycinnamic acids are among the most common.[2] These phenolic acids are present in both the cell soluble and cell wall fractions of plants.[3,4] The cinnamic acids are relatively abundant in forages, whereas benzoic acids and aldehydes occur in trace amounts only.[4] Certain cinnamic acids have been suggested to function as cross-linking moieties between lignin and hemicellulose in the cell wall.[5] Microbial degradation of forages has been demonstrated to result in release of phenolic acids from cell wall material, both *in vitro* and *in vivo*.[4,6,7] The ratio of ferulic acid to *p*-coumaric acid has been shown to be correlated with cell wall digestibility.[6] These data suggest that phenolic acids may influence forage digestion by ruminants.

Recently, it has been shown that certain phenolic acids inhibit growth of rumen microorganisms[8] and depress cellulose digestion *in vitro*.[9,10] The results indicate that phenolic acids may differ in the degree to which they alter fibre digestion. The present investigation attempted to determine the relationship between chemical structure of phenolic acids and inhibitory effects on microbial degradation of structural carbohydrates.

2. Experimental

In all experiments a simple in vitro fermentation procedure was used to test the effects of phenolics on digestion. The substrates consisted of 90% carbohydrate and 10% silica, with phenolic compounds being included in the substrate at the expense of the silica. The carbohydrates used were isolated cellulose (solka floc; Brown Co., Des Plaines, IL, USA) or hemicellulose (Larchwood xylan; United States Biochemical Co., Cleveland, Ohio, USA). The hemicellulose was composed of xylose (30%), glucose (30%) and other sugar residues (primarily arabinose and mannose). Substrates (500 mg) were placed in 50 ml culture tubes to which was added 6 ml of strained rumen fluid from a steer and 24 ml of anaerobic McDougall's buffer.[11] Substrate and buffer were not autoclaved prior to inoculation. The donor animal was maintained on a lucerne–cocksfoot (*Medicago sativa–Dactylis glomerata*) hay, and rumen fluid was collected after an overnight fast. Samples were allowed to ferment at 39°C with periodic shaking. Fermentations were halted by freezing the samples at −10°C. In vitro dry matter disappearance (IVDMD) was determined gravimetrically after drying the residual pellet at 100°C following centrifugation. The IVDMD values were adjusted for the solubility of the phenolic compounds in the buffer solution. Solubilities ranged from 0.7 to 1.5 mg ml^{-1}, which resulted in concentrations of 2.2 to 12.3 mM of phenolic compound in solution.

In experiment 1, the major benzoic and cinnamic acid derivatives of forages were tested. These compounds were added at concentrations of 30, 60 and 90 g kg^{-1} of the substrate. The lowest concentration represents the ratio of phenolic acids to structural carbohydrate usually found in grasses. The highest concentration is several times the highest values reported. Each treatment was run in triplicate and the experiment was repeated four times. Samples were allowed to ferment for 48 h for IVDMD determination. The inhibition of both cellulose and hemicellulose degradation by phenolic acids was tested.

Based on the results of experiment 1, in the second experiment the effects of cinnamic acids on rate and extent of cellulose digestion were examined. The phenolics were added at a concentration of 60 g kg^{-1} and fermented for various lengths of time (0 to 168 h). The equation of Graham and Aman[12] was used to estimate the rate and extent of digestion:

$$R=Ae^{-kt}+B$$

where R equals the residue (%) remaining after fermentation, A is the potentially digestible fraction (%), k is the rate of digestion (h^{-1}), t denotes the time (h) of fermentation, and B is the indigestible fraction (%). The value for A was set equal to $100-B$ and $B\geq 0$. The equation was solved by the non-linear regression program of SAS.[13] The IVDMD curve for each phenolic acid was determined in triplicate. Production of volatile fatty acids was determined after 48 and 168 h.[14] Concentrations of cinnamic acids were monitored by measuring the absorbance at 300 nm of the supernatants.

In experiment 3, the major pairs of benzoic acids and aldehydes were compared for their inhibitory effects on cellulose and hemicellulose IVDMD. All compounds were tested at a concentration of 60 g kg^{-1} and fermented for 48 h. Treatments were run in triplicate and the experiment was repeated three times.

3. Results

The benzoic and cinnamic acid derivatives were quite different in their effects on IVDMD of cellulose (Table 1). Of the benzoic acids, only the unsubstituted parent compound (benzoic acid) depressed ($P<0.05$) IVDMD of cellulose relative to the control. Substitution of hydroxyl (−OH) or methoxyl ($-OCH_3$) groups at the 3, 4 and/or 5 position on the aromatic ring eliminated the inhibitory properties of benzoic acid. In contrast, cinnamic acid and two derivatives (caffeic and ferulic acids) all resulted in cellulose IVDMD less ($P<0.05$) than the control. If the results for *p*-coumaric acid are ignored, the IVDMD of the cinnamic acid derivatives suggest that, in general, substitution with either −OH or $-OCH_3$ groups reduced the inhibitory activity of

Table 1. In vitro dry matter disappearance (IVDMD) of cellulose and hemicellulose fermented in the presence of benzoic and cinnamic acid derivatives. Values are the mean of 30, 60 and 90 g kg^{-1} phenolic acids in the substrate

	IVDMD (%)	
Treatment	Cellulose	Hemicellulose
Control	30.9	36.4
Benzoic acid	25.5^{a*}	35.6^{a}
p-Hydroxybenzoic acid	29.5^{b}	37.0^{bc}
Protocatechuic acid	29.9^{b}	35.8^{ab}
Gallic acid	29.3^{b}	37.6^{c}
Vanillic acid	30.6^{b}	37.0^{bc}
Syringic acid	30.4^{b}	37.4^{c}
Cinnamic acid	26.1^{d*}	34.8^{d}
p-Coumaric acid	29.3^{f}	38.5^{e*}
Caffeic acid	27.5^{de*}	37.5^{ef}
Ferulic acid	28.2^{ef*}	36.3^{fg}
Sinapic acid	28.6^{ef}	36.0^{g}
SEM	0.6	0.5

^{abc} Means in the same column, for benzoic acid derivatives, not sharing a common superscript differ ($P<0.05$).

^{defg} Means in the same column, for cinnamic acid derivatives, not sharing a common superscript differ ($P<0.05$).

* Different from the control ($P<0.05$).

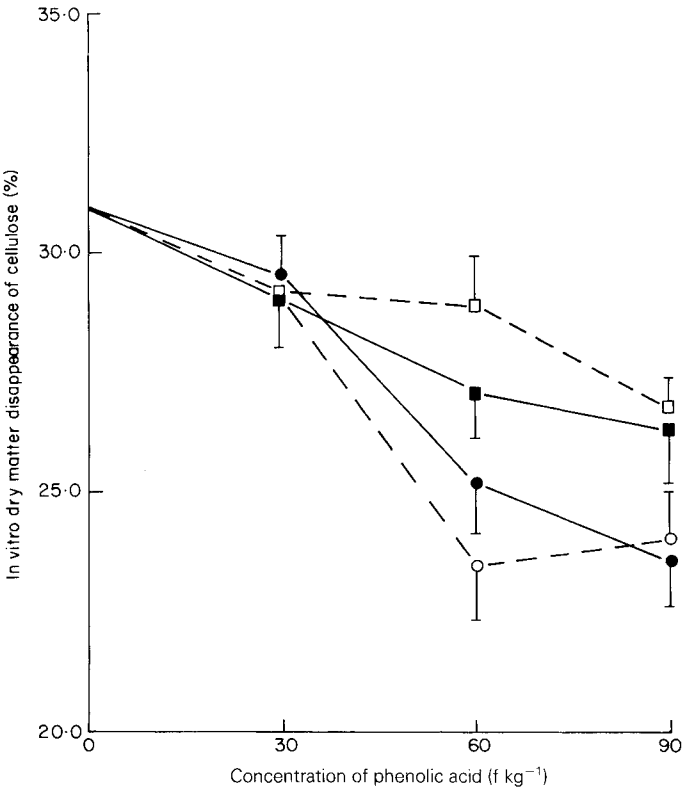

Figure 1. Depression of in vitro dry matter disappearance of cellulose with increasing concentrations of benzoic acid (○), cinnamic acid (●), caffeic acid (■) and ferulic acid (□).

cinnamic acid and that this reduction was increased as the number of substitutions increased. Also, the addition of a $-OCH_3$ group apparently reduced the inhibitory activity of cinnamic acid to a greater extent than addition of a $-OH$ group. The results for *p*-coumaric acid do not follow this trend, however. Figure 1 illustrates that for those compounds that had an overall inhibitory effect on cellulose IVDMD, the effect of increasing phenolic acid concentration on IVDMD was basically linear. The IVDMD value for benzoic acid at 90 g kg^{-1} may relate to the fact that the solubility of benzoic acid in McDougall's buffer was exceeded at this concentration.

The effects of phenolic acids on hemicellulose disappearance were very different (Table 1) than those for cellulose. None of the compounds depressed IVDMD significantly and *p*-coumaric acid actually stimulated ($P<0.05$) hemicellulose IVDMD. Within the phenolic acid groups there were significant differences among compounds, but no obvious pattern emerged.

In experiment 2, there was a lag period in the fermentation of cellulose *in vitro* (Table 2). Although not statistically different, the 12 h IVDMD values suggest that the cinnamic acids extended the lag period relative to the control. Control IVDMD was greater than values for all cinnamic acid treatments after 48 h and subsequent lengths of fermentation. As time of fermentation increased, the cinnamic acids segregated into two groups. Caffeic, ferulic and sinapic acids were the most inhibitory compounds, while cinnamic and *p*-coumaric acids inhibited cellulose IVDMD to a lesser extent. This is different than the pattern seen in experiment 1 (Table 1) after 48 h fermentation.

When the equation for rate and extent of digestion was fitted to the data, differences ($P<0.05$) were found between the control and the cinnamic acids for rate of digestion only (Table 2). The control treatment had the fastest rate of digestion, followed by cinnamic acid and then

Table 2. In vitro dry matter disappearance (IVDMD) of cellulose after fermentation by rumen microorganisms for various time periods with cinnamic acid derivatives. Rate of digestion (k) and indigestible fraction (B) of the cellulose are compared among the phenolic acids. The cinnamic acids were included as 60 g kg^{-1} of the substrate

	IVDMD (%)										
	Time (h)								k	B	
Compound	12	24	48	72	96	120	144	168	(h^{-1})	(%)	r^2
Control	2.1	11.4^a	29.3^a	49.3^a	65.5^a	76.7^a	81.5^a	83.3^a	0.0100^a	0	0.95
Cinnamic acid	0	5.1^b	21.4^{bc}	45.6^b	60.5^b	69.1^b	72.3^b	75.7^b	0.0087^b	1.7	0.92
p-Coumaric acid	0.2	9.5^{ac}	20.2^{bc}	45.6^b	57.2^c	69.1^b	70.6^b	73.7^b	0.0080^c	0	0.94
Caffeic acid	0	7.8^{bc}	19.2^c	42.4^c	53.9^d	61.4^c	66.4^c	68.8^c	0.0073^d	1.3	0.94
Ferulic acid	0	7.8^{bc}	19.4^{bc}	41.4^c	53.0^d	61.1^c	66.2^c	67.6^c	0.0070^d	0.3	0.94
Sinapic acid	0	9.6^a	22.2^b	42.1^c	52.2^d	60.1^c	62.7^d	66.6^c	0.0070^d	0.4	0.95
SEM	0.2	0.5	0.9	0.7	0.8	1.5	1.5	1.4	0.0002	0.7	

^{abcd} Means in the same column not sharing a common superscript differ ($P<0.05$).

Table 3. Volatile fatty acid (VFA) production and molar proportions after fermentation of cellulose with cinnamic acid derivatives. The cinnamic acids were included as 60 g kg^{-1} of the substrate

			Molar proportions					
	VFA production(μmol)		Acetate		Propionate		Butyrate	
Compound	48 h	168 h	48 h	168 h	48 h	168 h	48 h	168 h
Control	1161^a	3879	0.602^a	0.545^a	0.345^a	0.381^a	0.052^a	0.073^a
Cinnamic acid	829^b	3671	0.655^b	0.541^{ab}	0.299^b	0.392^{ab}	0.046	0.067^b
p-Coumaric acid	1149^a	3791	0.671^b	0.523^b	0.276^c	0.409^b	0.052^a	0.068^{ab}
Caffeic acid	595^b	3811	0.646^b	0.551^a	0.302^b	0.381^a	0.051^{ab}	0.068^{ab}
Ferulic acid	765^b	3778	0.642^b	0.537^{ab}	0.310^b	0.398^{ab}	0.048^{ab}	0.065^b
Sinapic acid	1105^a	3797	0.645^b	0.539^{ab}	0.305^b	0.386^a	0.050^{ab}	0.075^a
SEM	68	29	0.006	0.003	0.006	0.003	0.001	0.001

^{abc} Means in the same column not sharing a common superscript differ ($P<0.05$).

p-coumaric acid. The other three phenolic acids were not different from one another, but did result in a slower ($P<0.05$) rate of digestion than did all other treatments. The ranking of treatments for rate of digestion was very similar to IVDMD at 168 h. The extent of digestion, as estimated by the indigestible fraction, was not different among treatments and did not differ from 100% digestion. Complete digestibility of isolated cellulose was not unexpected. For all the compounds, the fitted equation accounted for greater than 90% of the variation in the data.

The VFA production data for in vitro fermentation of cellulose with cinnamic acids is shown in Table 3. After 48 h, the total VFA production was different ($P<0.05$) among the treatments and the ranking of compounds agreed closely with IVDMD at this time point (Table 2). However, after 168 h there were no detectable differences in VFA production among the treatments, even though IVDMD were substantially different. Further evidence for a shift in VFA production between 48 and 168 h was seen in the molar proportion of the VFA at this time (Table 3). The proportion of acetate in the total VFA production declined ($P<0.05$) between 48 and 168 h for all treatments, whereas the proportions of both propionate and butyrate increased ($P<0.05$) between these time intervals for all treatments. The control treatment had a lower content of acetate and a greater proportion of propionate after 48 h than did the phenolic acid treatments. After 168 h, these differences were substantially eliminated (Table 3).

The concentration of the cinnamic acids was monitored after 12, 24, 48 and 168 h of fermentation (Table 4). Caffeic and sinapic acid concentrations were reduced markedly after only 12 h of fermentation by rumen microorganisms. By 48 h, all the compounds had declined in

Table 4. Cinnamic acid derivatives remaining after fermentation by rumen microorganisms *in vitro*. The cinnamic acids were included as 60 g kg^{-1} of the substrate

	Percentage remaining			
Compound	12 h	24 h	48 h	168 h
Cinnamic acid	95.7^{a}	59.5^{ab}	5.2	1.7
p-Coumaric acid	112.1^{a}	75.2^{b}	14.3	0.1
Caffeic acid	57.5^{b}	59.4^{ab}	11.6	0.6
Ferulic acid	87.6^{a}	41.7^{ac}	5.9	0.7
Sinapic acid	53.3^{b}	18.1^{c}	20.6	2.6
SEM	7.4	7.0	2.2	0.3

^{abc} Means in the same column not sharing a common superscript differ ($P<0.05$).

Table 5. In vitro dry matter disappearance (IVDMD) of cellulose and hemicellulose fermented in the presence of benzoic acids and aldehydes. Phenolic compounds were included as 60 g kg^{-1} of the substrate

	IVDMD (%)	
Treatment	Cellulose	Hemicellulose
Control	31.4	31.6
p-Hydroxybenzoic acid	30.5^{a}	31.1^{a}
p-Hydroxybenzaldehyde	17.1^{b*}	27.7^{b*}
Vanillic acid	33.6^{a}	33.8^{a}
Vanillin	18.1^{b*}	25.2^{b*}
Syringic acid	31.2^{a}	32.1^{a}
Syringaldehyde	17.7^{b*}	25.3^{b*}
SEM	1.1	1.1

^{ab} Means in the same column not sharing a common superscript differ ($P<0.05$).

* Different from control ($P<0.05$).

concentration to low levels and after 168 h there were virtually no cinnamic acids remaining in solution. The rate of reduction in concentration of individual phenolic acids did not appear to be related to differences in IVDMD or rate of digestion.

Results from experiment 3 with benzoic acids and aldehydes are given in Table 5. As expected, the benzoic acids tested did not depress IVDMD of either cellulose or hemicellulose relative to the control. Both substrates, however, had reduced ($P<0.05$) IVDMD when the benzoic aldehydes were added to the media. The benzoic aldehydes did not differ in the degree to which they inhibited IVDMD and all the aldehydes depressed fermentation of cellulose to a much greater extent than hemicellulose IVDMD.

4. Discussion

Previous work with mixed cultures of rumen microbes has shown that *p*-coumaric acid is more inhibitory to cellulose degradation than ferulic acid.[9,10] This contrasts with results reported here where the ranking of these compounds is reversed. Working with pure cultures of rumen cellulolytic bacteria, Chesson *et al.* [8] found that *Bacteroides succinogenes* and *Ruminococcus flavefaciens* exhibited a somewhat greater depression in cellulolytic activity when ferulic acid was added to the media than seen with *p*-coumaric acid. The predominant cellulolytic organism isolated from the 48 h in vitro fermentations in the current studies was found to be *B. succinogenes* (Varel, V. H. and Jung, H. G., unpublished). Enrichment for this particular organism may explain the differences between *p*-coumaric and ferulic acids in their relative inhibitory properties among the studies.

There does not appear to be a straightforward relationship between depression of IVDMD of cellulose or hemicellulose with changes in substitutions to the aromatic ring of phenolics. For cellulose degradation, addition of any group to benzoic acid eliminated the inhibitory property of the parent compound. However, the cinnamic acid derivatives do not appear to follow this rule, especially as length of microbial fermentation time increased. There does not appear to be a correlation between hemicellulose IVDMD and chemical structure of phenolic acids. It does not seem possible to assign any biological consequences to specific chemical changes in ring substitutions of phenolic acids. It does seem clear that aldehydes are significantly more inhibitory of structural carbohydrate digestion than their related acids. The lack of any difference among the aldehydes in their inhibition of IVDMD provides support for the previous statement that ring substitutions are relatively unpredictable in their effects.

The major differences between the effects of phenolic compounds on cellulose and hemicellulose IVDMD suggest that these substrates are selective for microbial populations which differ in their susceptibility to phenolic inhibition. It is also possible that the phenolics affect the cellulose and hemicellulose degrading enzymes differently, although the extra-cellular enzyme of *Bacteroides succinogenes* which hydrolyses carboxymethycellulose is unaffected by cinnamic acid or vanillin (Varel, V. H. and Jung, H. G., unpublished). Chesson *et al.*[8] found that pure cultures of rumen bacteria were different in their tolerance of phenolic acids. It is of interest that the hemicellulolytic populations in the present work were more tolerant of phenolics than the cellulolytic bacteria because phenolic acids are felt to be linked to the hemicellulose fraction of forage cell walls.[6] This suggests that those bacterial species which are exposed to the greatest concentrations of phenolic compounds under natural conditions have evolved a higher tolerance to these compounds. It is possible that hemicellulose of different composition than that used in this study might result in a different conclusion by selecting for a different microbial population and/or enzyme system.

Chesson *et al.*[8] and Jung and Fahey[10] reported very low recoveries of added phenolic acids after fermentation by mixed cultures of rumen microorganisms. Anaerobic microbes are known to metabolise benzoic and cinnamic acids extensively.[5,16] The rate of digestion study reported here demonstrated how rapidly the concentration of cinnamic acids can be reduced by microbial fermentation. The shift in molar proportions of VFA with time of fermentation indicate that the population of microbes was probably changing with time. After long fermentations, the VFA

proportions were similar among the treatments which suggests that the treatments had all shifted to a similar microbial population. This can be explained by the elimination of the cinnamic acids from the media via microbial metabolism.

A note of caution must be expressed relative to the interpretation of this work. The concentrations of phenolic compounds used to test for inhibitory properties were substantially higher than those found in forages.[4,17] Also, in this work and that of others,[8–10] the phenolic compounds used were free acids and aldehydes. The available evidence indicates that phenolics in forages do not exist in the free form to an appreciable extent.[3] Ester-linked phenolics are the natural form which must be tested for inhibitory effects. Johanning *et al.*[18] have reported that a water-insoluble fraction from lucerne, which probably contained ester-linked cinnamic acids, inhibited growth of a cellulolytic bacteria isolated from the guinea pig. Preliminary data for studies of forage fractions containing ester-linked phenolics indicate inhibition of IVDMD by mixed cultures of rumen microbes at near physiological phenolic concentrations (Jung, H. G., unpublished). Sawai *et al.*[19] have recently reported that when *p*-coumaric and ferulic acids were linked in the laboratory to cocksfoot cell walls or isolated cellulose via ester bonds, hydrolysis by a fungal cellulase was depressed. Degradation of the cell wall preparation was inhibited to a greater extent by ferulic acid than by *p*-coumaric acid.[19] These data suggest that results from studies with purified, free phenolics may be reasonable indicators of the effects of the bound forms of phenolics found in forages.

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