

# EFFECT OF PLANT PHENOLIC ACIDS ON CELLULOLYTIC ACTIVITY OF MIXED RUMEN POPULATIONS

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## Summary

Influences of plant phenolic acids and their possible metabolites (non-phenolic aromatic acids involved) in the rumen on the cellulolytic activity of mixed rumen populations were examined by a simple *in vitro* culture technique. Initial concentrations of aromatic acids were 1, 5, 10 and 20 mM/l. All the tested aromatic acids reduced microbial cellulose digestion especially at the higher initial concentration. *p*-Coumaric acid, ferulic acid and cinnamic acid, those having unhydrogenated propenoic side chain were more inhibitory than were 3-phenylpropionic acid and phloretic acid, those having hydrogenated propanoic side chain. Lag-time for cellulose digestion was prolonged by former three acids by 16 h. Apparent reduction in *p*-coumaric acid concentration was observed at 24 h when cellulose digestion began. Volatile fatty acid productions from cellulose fermentation were shifted by former three aromatic acids to produce more acetate and less propionate. This suggests that the selection of cellulolytic organisms was induced by these aromatic acids.

(Key Words: Phenolic Acid, Rumen Cellulose Digestion)

## Introduction

Phenolic acids are involved in plant cell wall. They are known to be associated with hemicellulose (Hartley, 1972; Morrison, 1974). Ester-linkage with ferulic acid (4-hydroxy-3-methoxy-cinnamic acid) and *p*-coumaric acid (4-hydroxy-cinnamic acid) reduces the degradability of cellulose (Sawai et al., 1983). This inhibition is considered to be due to the blockage of the enzymes to access their site for action (Sawai et al., 1983). Free phenolic acids are produced from ruminant diets during rumen digestion (Martin, 1982) and they act with microorganisms. Toxicities against certain strains of bacteria (Chesson et al., 1982), entodiniomorphid ciliate (Akin, 1982), and fungi (Akin and Rigsby, 1985) were to date reported. In the present experiment, influences of phenolic acids and their possible intermediate metabolites (non-phenolic aromatic acid involved) in the rumen on the cellulolytic activity of mixed population were determined.

## Materials and Methods

### Animals and diets

Three mature cross-bred wethers, each equipped with simple rumen cannula were used as donors of rumen fluid. The sheep received 600 g alfalfa hay and 0.5 g vitamin-mineral premix (Vitamin AD<sub>3</sub> Esan, Kokin Kagaku, Osaka, Japan) twice a day at 9:00 AM and 6:00 PM.

Water and mineral block were always available to the sheep.

### *In vitro* culture for the determination of cellulolytic activity

Cellulolytic activity was estimated using a simple *in vitro* incubation technique with cellulose powder as a substrate (No. 077-10, Nakarai Chemicals Ltd, Kyoto, Japan). Rumen fluid was withdrawn via a cannula from each sheep before morning feeding and combined in a thermos. After being transported to the laboratory, the fluid was squeezed through four-layers of surgical gauze. Substrates (500 mg) were placed in 100-ml conical flasks to which was added 10 ml of strained rumen fluid and 40 ml of CO<sub>2</sub>-saturated and prewarmed McDougall buffer (pH 6.9, 39°C). The systems were then closed with bunsen valves. All the manipulations were performed

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under the stream of CO<sub>2</sub>. The flasks were incubated at 39°C for 48h with continual shaking. To define the effect of phenolic acids and their possible metabolites on cellulolytic activity of mixed rumen microorganisms, *p*-coumaric acid (PCA), ferulic acid(FA), *trans*-cinnamic acid(CA), 3-phenylpropionic acid(3PPA) and 3-(4-hydroxyphenyl)-propionic acid(phloretic acid, PHLA) were added to flasks. PCA and FA were obtained from Aldrich Chemical Co. (Milwaukee, USA), CA and 3PPA were from Nakarai Chemical Ltd., and PHLA was from Sigma Chemical Co.(St. Luis, USA). The initial concentrations of these aromatic acids(mM/l) were 1,5,10 and 20. Duplicate flasks were allotted for each concentration of acid. Two additional cultures without aromatic acid were done as untreated control for each series of experiment. Three experiments were done on every four days. A total of 126 determinations was therefore made.

Another series of *in vitro* culture was done in order to know the kinetics of cellulolysis during 48h-incubation under the presence of aromatic acids. Initial concentration of PCA, FA, CA was 10 mM/l. Triplicate flasks were allotted for each incubation time of each acid in addition to no aromatic acid-added control culture.

#### Chemical analysis

At the end of incubation, flasks were immersed in iced-water to stop fermentation. Culture fluids were then centrifuged at 1,500 x g for 30 min. Pellets were treated with neutral detergent solution to estimate residual cellulose (Ushida et al., 1987). Supernatants were analyzed for volatile fatty acid(VFA) concentration by gas chromatography as described in elsewhere (Ushida et al., 1982). Supernatants of PCA cultures in the kinetics study were analyzed for aromatic acids concentration by high performance liquid chromatography(HPLC). Aromatic acids were extracted as described by Chesson et al.(1982) and analyzed by a similar reverse-phase HPLC method to that described by Harwood and Gibson(1988) using two 510 pumping units, a 680 gradient controller, a 481 spectrophotometer and a  $\mu$ -Bondapak C-18 column. Peak areas were calculated by a 740 Data module. All the equipments were obtained from Waters Associates Inc. (Milford, USA). Portions (5  $\mu$ l) were injected into chromatograph and compounds were eluted with 1ml/min of acetonitrile/

perchloric acid(0.01 N) gradient in which acetonitrile concentration was linearly increased from 0.6% to 60% in 30 min. The detection was performed at 260 nm. In addition to five aromatic acids tested in *in vitro* cultures, retention times of 3-methoxy-phloretic acid (3-MeO-PHLA), and phenylacetic acid(PA) were determined. 3-MeO-PHLA was a gift from Dr. H. Ishibashi, Kyoto Pharmaceutical University(Misasagi, Kyoto 607, Japan) and PA was obtained from Wako Pure Chemical(Osaka, Japan). Detected aromatic acids were assigned only by comparing their absolute retention times with those of authentic compounds. Although the separation of aromatic acids by this method was not complete(e.g. CA and 3PPA, FA and 3-MeO-PHLA) and identification of metabolites was not complete (no mass or IR spectrum was determined), HPLC chromatograms were yet useful at least to know the presence of metabolism of PCA. The peak of PCA was always the highest during the first 24-h incubation and therefore easily identified. Since metabolites of FA and CA could not be separated from their parents acids, only PCA cultures were subjected to HPLC analysis.

The data were analyzed by Duncan's multiple range test.

#### Results and Discussion

PCA, FA and CA have unsaturated propenoic side chains, while 3PPA and PHLA have hydrogenated propanoic side chains. These two types of aromatic acids were apparently different at higher initial concentration in the potential of inhibitory effect on the cellulolysis(figure 1). Former three acids showed clear inhibitory effects at 10 and 20 mM, while latter two acids were much less toxic at the same concentrations. Each former acid showed similar extents of inhibition irrespective of aromatic ring substituents, such as hydroxyl or methoxyl. The latter two acids showed similar extents of inhibition irrespective of the presence or absence of hydroxyl group at para position. These results indicate that the toxicity of phenolic acids mainly results from their unsaturated propenoic side chains. Chesson et al.(1982) have shown that cellulolytic activities of three major rumen cellulolytic bacteria, *Bacteroides succinogenes*, *Ruminococcus flavefaciens*, and *R. albus* in pure culture are suppressed by PCA and FA,

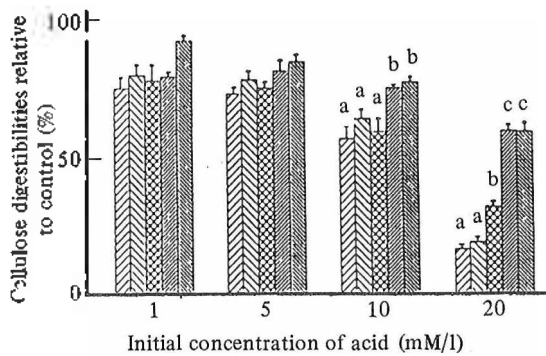


Figure 1. Influence of aromatic acids on *in vitro* cellulolysis.<sup>1</sup>

<sup>1</sup> Values are expressed as the percentages to those measured in control incubation (control=100). Values are means with standard errors of six determinations. All the values except for phloretic acid at 1 mM/l differ significantly from control ( $p < 0.05$ ). Bars with unlike superscripts within a same aromatic acid concentration differ significantly ( $p < 0.05$ ).

but to a much lesser extent by PHLA. Present results from *in vitro* culture of mixed organisms are in line with their early results.

There are two incompatible results concerning the relationship between presence of ring substituents of cinnamic acid and depressing effects of the acids on *in vitro* digestion. Akin(1982), and Jung and Fahey(1983) reported that PCA is more inhibitory to cellulose digestion than is FA, while Jung(1985) found no clear relation between ring substitutions and inhibitory effects. Present results are similar to the latter case. The reason for this incompatibility between the experiments is still unclear, but possibly related to the variety of the composition of microbial population. *B. succinogenes* is less tolerant than two Ruminococci(Chesson et al., 1982), while *Butyrivibrio fibrisolvens* is more tolerant than two Ruminococci (Borneman et al., 1986). If predominant cellulolytic organisms which developed in those experiments were different, the responses of mixed microflora to the phenolic acids should, therefore, be different. Bacteriological surveys remain to be required under present experimental condition in order to know the reason for such

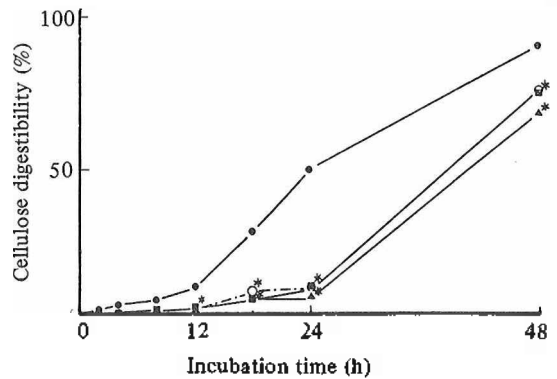


Figure 2. Kinetics of cellulolysis by mixed rumen population with or without aromatic acid.

● control (no aromatic acid)  
 ○ *p*-coumaric acid    ▲ ferulic acid  
 ■ cinnamic acid

Values are means of three determinations. Values with asterisks differ significantly from control ( $p < 0.05$ ).

inconsistency between experiments. Kinetics of cellulolysis(figure 2) indicate that PCA, FA, and CA induced the increase in lag-time. Active cellulose digestion began by 8 h in control cultures, while it began by 24 h in aromatic acid-added cultures. Such prolonged lag-times seem to be due to suppression of growth of cellulolytic organisms (Chesson et al., 1982), and inhibition of attachment of bacteria(Varel and Jung, 1986). Chesson et al.(1982) found the delay of bacterial growth induced by phenolic acids, and they considered that these delays are due to reduced specific growth rate. They also found hydrogenation of propeonic side chain in cinnamic acid derivatives by cellulolytic strains. As demonstrated in several published experiments as well as the present one, hydrogenated cinnamic acids are less toxic than are parents acids. Prolonged lag-time was likely required for the adaptation to the acids, that is in turn, for the metabolism of toxic unhydrogenated compounds to less toxic forms.

Information from present HPLC analysis was extremely limited and it is difficult to discuss the quantitative problem due to the incomplete separation of acids and the absence of internal standard, the presence of metabolism of PCA was yet apparent (figure 3): PCA reduced to approximately half of initial value in 24 h incubation and to less than 10% in 48 h. Apparent increases in

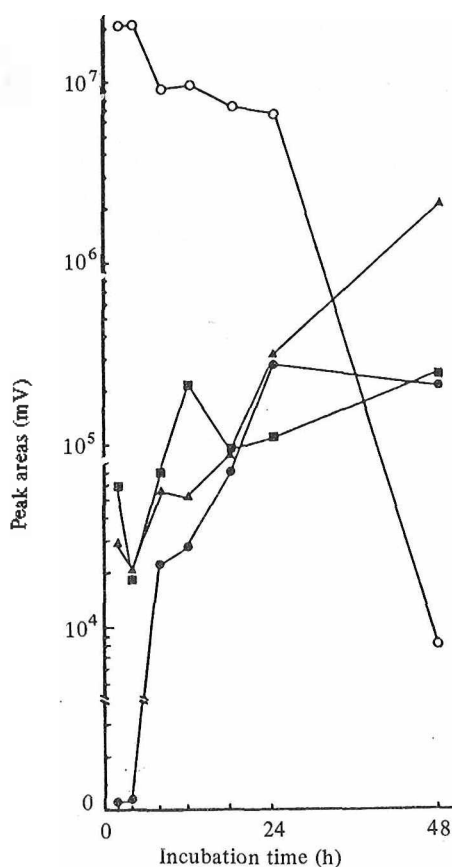


Figure 3. Changes in aromatic acid concentration<sup>1</sup> during 48-h incubation.

○ *p*-coumaric acid    ▲ phloretic acid  
 ■ phenylacetic acid  
 ● composite peak of cinnamic acid and 3-phenylpropionic acid

<sup>1</sup> Values are peak areas (mV) of each fraction.

other compounds, probably PHLA and 3PPA (CA included), was observed at the same time. A peak probably corresponding to PA was detected and this peak also gradually increased during incubation. The enzyme equipment for metabolism of phenolic acid is whether inducible or constitutive is not known, relatively long lag-time for PCA metabolism seems to suggest rather inducible characteristics.

VFA productions from cellulose decreased as a result of suppression of cellulolysis (table 1). Proportions of each VFA were affected by aromatic acids, molar proportion of acetic acid increased at the expense of propionic acid and butyric acid. The shift in VFA proportion indicates that phenolic acids affect the composition of microorganisms. *R. albus* SY3 has shown higher tolerance against PCA and FA than did *B. succinogenes* BL2 and *R. flavofaciens* 007 (Chesson et al., 1982). *R. albus* produces acetate as a major product and does not produce propionate or its precursors. While latter two organisms produce succinate which is further catabolized to propionate by other bacteria such as *Selenomonas ruminantium*. If this was in the case in the present experiment, the shifts can be explained.

Plant phenolic acids are toxic to rumen microorganisms, bacteria, protozoa, and fungi. However the results from both published and present experiments suggest that the effects are not lethal, but rather bacteriostatic. Toxic compound (PCA) was almost entirely metabolized to less toxic forms by a 48-h incubation. It is apparent

TABLE 1. TOTAL VFA CONCENTRATION INCREASED AND PROPORTION OF ACIDS (MOLAR %) DURING 48-H INCUBATION WITH AROMATIC ACID (mM/l)<sup>1</sup>

|  | Control            | PCA                | FA                 | CA                 | 3PPA                | PHLA               | SEM  |
|--|--------------------|--------------------|--------------------|--------------------|---------------------|--------------------|------|
| Acetate  | 58.4 <sup>a2</sup> | 72.2 <sup>b</sup>  | 71.2 <sup>b</sup>  | 67.4 <sup>b</sup>  | 55.9 <sup>a</sup>   | 59.2 <sup>a</sup>  | 2.1  |
| Propionate                                     | 34.9 <sup>a</sup>  | 23.8 <sup>b</sup>  | 23.4 <sup>b</sup>  | 27.7 <sup>b</sup>  | 38.7 <sup>a</sup>   | 33.0 <sup>a</sup>  | 1.9  |
| Butyrate                                       | 3.4 <sup>a</sup>   | 2.1 <sup>b</sup>   | 2.3 <sup>b</sup>   | 3.6 <sup>a</sup>   | 3.3 <sup>a</sup>    | 3.0 <sup>ab</sup>  | 0.3  |
| Other minors <sup>3</sup>                      | 3.3 <sup>a</sup>   | 1.9 <sup>a</sup>   | 3.0 <sup>a</sup>   | 1.3 <sup>b</sup>   | 2.2 <sup>a</sup>    | 4.8 <sup>a</sup>   | 0.5  |
| Total VFA concentration increased <sup>4</sup> | 393.1 <sup>a</sup> | 227.5 <sup>b</sup> | 206.9 <sup>b</sup> | 254.9 <sup>b</sup> | 306.7 <sup>ab</sup> | 240.0 <sup>b</sup> | 18.3 |

PCA, *p*-coumaric acid; FA, ferulic acid; CA, cinnamic acid; 3PPA, 3-phenylpropionic acid; PHLA, phloretic acid.

<sup>1</sup> Values are means of six determinations and initial concentration of aromatic acids was 10 mM/l.

<sup>2</sup> Values with different superscripts within a row differ significantly ( $p < 0.05$ ).

<sup>3</sup> iso-butyrate + iso-valerate + valerate

<sup>4</sup> VFA concentrations were corrected for their zero-time values.

that propenoic side chain of cinnamic acid derivatives is toxic and hydrogenation of side chain reduces the toxicity. The reasons for this apparent intoxicity of hydrogenated cinnamic acids derivatives are, however, unknown. Further studies are still required to obtain more precise information on the toxicity of phenolic acids.

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