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Interactions between proteolytic and cellulolytic rumen bacteria during hydrolysis of plant cell wall protein

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Summary — During the degradation of the plant cell wall protein of dried alfalfa, interactions may occur between hydrolytic activities of cellulolytic *(Ruminococcus albus* or *Fibrobacter succinogenes)* and proteolytic *(Prevotella ruminicola* or *Butyrivibrio fibrisolvens)* bacteria. *In vitro* the hydrolysis of these protein compounds begins after the depolymerization of the cell wall polysaccharides has started. Maximal degradation of cell wall protein of dried alfalfa (37.2%) was obtained with cocultures of *Prevotella ruminicola* and *Ruminococcus albus*.

proteolysis / cellulolysis / rumen / NDF / NDIN / alfalfa

Résumé — Interactions entre les bactéries cellulolytiques et protéolytiques du rumen durant l'hydrolyse des protéines des parois cellulaires végétales. *Lors de la dégradation des protéines associées aux parois végétales de la luzerne déshydratée, il existe une interaction entre les activités hydrolytiques des bactéries cellulolytiques* (Ruminococcus albus *ou* Fibrobacter succinogenes) *et protéolytiques* (Prevotella ruminicola *ou* Butyrivibrio fibrosolvens). *L'hydrolyse* in vitro *de ces composés protéiques est précédée par une dépolymérisation des polysaccharides pariétaux. Une dégradation maximale des protéines des parois de la luzerne (37,2%) est obtenue lors de la coculture de* Prevotella ruminicola *et* Ruminicoccus albus.

protéolyse / cellulolyse / rumen / NDF / NDIN / luzerne

INTRODUCTION

In roughages, polysaccharides unsolubilized part of the protein compounds that are covalently bound to the plant cell wall. The unsolubilized proteins are only slightly hydrolyzed by rumen microorganisms (Leng and Nolan, 1984) and never by the gastrointestinal enzymes of the animals. Some *in sacco* research has shown interactions between the degradation of plant cell-wall polysaccharides and the hydrolysis of their associated proteins (Lindberg, 1981). The degradation of nitrogen enclosed in the plant cell wall is higher when the polysaccharides are more degraded (Lindberg, 1981). Different studies using varied feed have shown that fermentation of nitrogenous compounds was increased as lignocellulose (ADF) constitutes a small proportion of the cell wall (NDF) (Blanchart, 1988; Blanchart and Vignon, 1990). Moreover, Wallace and Kopecny (1983) assumed that stabilization of extensin conformation by its polysaccharide bonds could constitute an obstruction to the digestion of the other fibrous compounds of the plant cell wall. The cellulolytic bacteria *Fibrobacter succinogenes* and *Ruminococcus albus* and the proteolytic bacteria *Prevotella ruminicola* and *Butyrivibrio fibrisolvens* have regularly been shown to be associated with alfalfa residues (Miron and Yokoyama, 1990). These results lead to the hypothesis that both groups of bacteria play a major role in the degradation of the plant cell wall. Due to their hydrolytic activity these bacteria could participate in the hydrolysis of cell-wall polysaccharides and their associated proteins.

The aim of this study was to determine the interactions between cellulolytic and proteolytic bacteria during the degradation of the nitrogenous fraction bound to the alfalfa cell wall.

MATERIALS AND METHODS

Organisms

The pure cellulolytic strains used, *Fibrobacter succinogenes* S85 and *Ruminococcus albus* 7, were kindly provided by D Fonty (INRA, Theix). The proteolytic strains *Butyrivibrio fibrisolvens* S23 and *Prevotella ruminicola* S17/3 were isolated in our laboratory by the rumen anaerobic roll-tube technique of Hungate (1950), as modified by Bryant (1972). The proteolytic strains were maintained in glucose–cellobiose–soluble starch–rumen fluid (MG) (Hobson, 1969) and the cellulolytic strains in the medium described by Hungate (1957).

Growth condition

The anaerobic culture techniques were similar to those described by Hungate (1950). The basal growth medium contained 40% (v/v) clarified rumen fluid (25 000 *g* for 10 min) 15% (v/v) of both mineral solutions I and II (Bryant and Burkey, 1953), 0.0001% resazurin, 0.4% sodium carbonate and 0.05% cysteine (Dehority and Scott, 1967). The medium was sterilized for 20 min at 120°C. Dried alfalfa was used as a substrate (NDF, 43.9%; ADF, 31.9%; ADL, 9.0%). It was sterilized by γ-irradiation (10 kGy) to avoid any denaturing of the proteins. The sterilization was carried out by the Station d'Amélioration des Plantes (INRA, Dijon). The addition of substrate to the liquid medium (2% w/v) and inoculation were performed in an anaerobic glove box. The inoculum was prepared by diluting a fresh bacterial culture (< 24 h) grown on MG, to with 0.3% tryptose was added until the OD equalled 1. For the monocultures, 2 ml inoculum (OD = 1) were mixed with 2 ml distilled water. For the cocultures, 2 ml inoculum of each strain (OD = 1) were mixed. The final fermentation volume was 200 ml. Fermentation was maintained at 39°C for 6 d. Three replicates of each culture were performed. For each fermentation, 10 ml supernatant were stored at –20°C for subsequent analysis. The presence of the bacteria in monocultures and cocultures was confirmed performing a Gram reaction and a morphological observation on each culture.

Preparation of plant residues

The treatments of fermented alfalfa were carried out to eliminate bacteria associated with the plant cell wall. After incubation and filtration on nylon cloth, the fermentation residues were transferred to NaCl 0.85% (w/v) and stored for 12 h at 4°C.

Samples were then strongly shaken for 5 min using a magnetic stirrer. The supernatant was discarded and replaced with fresh NaCl. Samples were then stored at 4°C. This procedure was applied twice at 6-h intervals. A Stomacher treatment was finally carried out for 5 min.

The fermented alfalfa was lyophilized before analysis. This method avoided the Maillard reactions that could have bound soluble proteins to the plant cell wall (Giger and Pochet, 1987).

Chemical analysis

Procedures for analysis of dietary fiber

The NDF (plant cell wall), ADF (lignocellulose) and ADL (lignin) were extracted according to the method of Goering and Van Soest (1970). The nitrogen cell-wall content (NDIN) was measured using the Kjeldahl method on the NDF residue.

Volatile fatty acid determination

Samples were centrifuged at 5 000 *g* for 5 min; 0.950 ml supernatant was mixed with 0.05 ml 4-methyl valeric acid (0.1%) (internal standard) and 0.1 ml orthophosphoric acid (5% v/v). A standard solution containing a known mixture of the different pure volatile fatty acids (VFA) was used to determine the molar response of the individual VFA. Gas chromatographic determination was performed using a Delsi DN 250 gas chromatograph with a flame-ionization detector. Both the injector and detector temperatures were 250°C. The oven temperature was 120°C. Helium was used as carrier gas (4 ml/min). The capillary column was a Spirawax FS 2602 (25 m x 0.32 mm) (Spiral, Dijon, France).

Estimation of forage digestion and statistical analysis

The alfalfa was extensively solubilized in the medium. To compute the dry weight loss of different compounds of plant cell wall, the lignin was used as internal marker. This compound was not altered by the microorganisms used in this study and its concentration was constant. Three non-inoculated controls were used to calculate the lignin concentration and the disappearance of NDF, ADF and NDIN. Data were analyzed by ANOVA analysis. The Fisher PLSD test was used to compare the means when the effect was significant ($P < 0.05$).

RESULTS

After 6 d incubation at 39°C, 20.7 and 30.6% of the plant cell wall and 25.8 and 34.2% of the lignocellulose were degraded by *Fibrobacter succinogenes* and *Ruminococcus albus* respectively (table I). For the same incubation time, these components were not degraded by the proteolytic strains alone. The disappearance of the cell-wall fractions increased when the proteolytic strains were associated with the cellulolytic bacteria. In most cases these cocultures did not induce significantly higher NDF or ADF degradation than the monocultures of cellulolytic bacteria alone. Only *Fibrobacter succinogenes* activity was enhanced when associated with *Butyrivibrio fibrisolvens.*

The monocultures of either cellulolytic or proteolytic bacteria have a rather low effect on the disappearance of the NDIN fraction (tables I, II). The breakdown of NDIN was only 1% with *Fibrobacter succinogenes* and 3.9% with *Ruminococcus albus.* It also reached only 3% during alfalfa fermentation by *Prevotella ruminocola* and 2.6% with *Butyrivibrio fibrisolvens.* All

Table I. Disappearance (%) of plant cell wall (NDF), lignocellulose (ADF), wall-bound protein (NDIN) of alfalfa and VFA (mmol/l) production *in vitro*. Incubation at 39°C for 6 d.

	Prevotella ruminicola	Fibrobacter succinogenes	Ruminococcus albus	Fibrobacter succinogenes + Prevotella ruminicola	Ruminococcus albus + Prevotella ruminicola	P
Disappearance (%)						
NDF	0^{c}	$20.7^{a,b}$	30.6^{d}	17.1^{b}	$24.2^{a,d}$	0.001
ADF	0^{c}	25.8^{a}	34.2^{b}	$21.5^{a,d}$	$28.6^{a,b}$	0.004
NDIN	$3.0^{a,c}$	1.0^{a}	$3.9^{a,c}$	11.7^{c}	37.6^{b}	0.02
VFA (mmol/l)						
Acetic acid	5.2^{c}	9.6^{d}	11.5^{a}	$9.1^{c,d}$	15.9^{b}	0.03

Propionic, isobutyric, isovaleric and valeric concentrations remained unchanged; $^{a, b, c, d}$ within rows, means followed by the same letter do not differ statistically ($P < 0.05$).

Table II. Disappearance (%) of plant cell wall (NDF), lignocellulose (ADF), wall-bound protein (NDIN) of alfalfa and VFA (mmol/l) production *in vitro*. Incubation at 39°C for 6 d.

	Butyrivibrio fibrisolvens	Fibrobacter succinogenes	Ruminococcus albus	Fibrobacter succinogenes + Butyrivibrio fibrisolvens	Ruminococcus albus + Butyrivibrio fibrisolvens	P
Disappearance (%)						
NDF	0.2^{a}	20.7^{b}	30.6^{c}	26.3^{b}	22.0^{b}	0.001
ADF	0.3^{a}	25.8^{d}	34.2^{c}	34.8^{c}	30.9^{b}	0.002
NDIN	2.6	1.0	3.9	16.9	14.9	0.06
VFA (mmol/l)						
Acetic acid	-1.1^{c}	9.6^{a}	11.5^{a}	4.8^{b}	5.3^{b}	0.04
Butyric acid	6.2^{a}	0^{b}	0^{b}	9.7^{c}	12.0^{d}	0.001

Propionic, isobutyric, isovaleric and valeric concentrations remained unchanged; $^{a, b, c, d}$ within rows, means followed by the same letter do not differ statistically ($P < 0.05$).

the cocultures of proteolytic and cellulolytic bacteria improved the disappearance of NDIN. The highest degradation rate (37.6%) was obtained with coculture of *Prevotella ruminicola* and *Ruminococcus albus*.

Acetate was a fermentation end-product of *Prevotella ruminicola, Fibrobacter succinogenes* and *Ruminococcus albus*. The coculture of *Ruminococcus albus* and *Prevotella ruminicola* increased the production of this acid. In the presence of *Butyrivibrio*

fibrisolvens, butyrate was produced and acetate was consumed. This bacteria can be an acetate-utilizer (Hungate, 1966). In the cocultures with cellulolytic bacteria, the production of butyrate was increased. The concentrations of branched chain VFA were not modified after incubation.

DISCUSSION

In this study the monocultures of *Prevotella ruminicola* and *Butyrivibrio fibrisolvens* did not solubilize part of the alfalfa cell wall. However, both strains had previously been characterized as xylan-hydrolyzing and *Butyrivibrio fibrisolvens* S23 was also able to slighty solubilize filter paper.

The alfalfa cell-wall polysaccharides are surely more difficult to hydrolyze than pure polymers. Thus Miron and Yokoyama (1990) showed that *Prevotella ruminicola* and *Butyrivibrio fibrisolvens* were hemicellulose utilizers, especially after solubilization.

The slight disappearance of plant cell wall nitrogen by monocultures of *Ruminococcus albus* or *Fibrobacter succinogenes* cannot be due to the hydrolysis of plant cell-wall protein because these strains do not synthesize any extracellular proteolytic enzyme (Hungate, 1966). This loss of nitrogen could be induced by the cellulase and the hemicellulase system or by the action of intracellular proteases liberated during the cell lysis that commonly occurs in batch cultures (Russell and Hespell, 1981).

Under our study conditions, it was not possible to measure the growth of each bacterial strain in cocultures. An analysis of VFA is useful in assessing microbial activity. A more complete screening of the fermentation end-products (other acids, alcohols and gas production) would have been necessary to determine the possible metabolic interactions between strains. The increase in the concentrations of VFA in the culture media of the proteolytic bacteria (acetate with *Prevotella ruminicola* and butyrate with *Butyrivibrio fibrisolvens)* demonstrated that these strains were able to grow with alfalfa as sole energy source. They probably used the water-soluble carbohydrates that represent ≈ 10% of the alfalfa dry matter (Chesson and Forsberg, 1988). The final concentrations of VFA in the cocultures and in the monoculture media were different, except with the association of *Fibrobacter succinogenes* and *Prevotella ruminicola.* This result leads to the assumption that both strains have in fact grown in cocultures. It was not possible to determine whether these strains were maintained in a constant ratio. VFA production depended on the strains used and the extent to which solubilized components and the non-NDF fraction were utilized. This utilization can differ between monocultures and cocultures (Miron and Ben-Ghedalia, 1992).

The association of cellulolytic and proteolytic bacteria increased NDIN degradation. Polysaccharide hydrolysis is therefore useful for the hydrolysis of cell-wall proteins. These results show that plant cell-wall polysaccharides reduce proteolytic enzyme access to the protein fraction. It can also be assumed that an increase in proteolytic activity due to higher monosaccharide levels was responsible for part of the hydrolysis of the cell-wall protein.

The cellulolytic bacteria are firmly bound to fibers, unlike the proteolytic bacteria which are located in free suspension or loosely associated with fibers (Czerkawski and Cheng, 1988). This difference in compartmentation could explain the low hydrolysis efficiency of plant cell-wall proteins. Only the rumen anaerobic fungi possess both proteolytic and cellulolytic activities (Orpin and Joblin, 1988) and are firmly bound to the cell wall by their mycelium. However, it would be of interest to deter-

mine the factors that could improve the joint expression of proteolytic and cellulolytic activities in these organisms.

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