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Effects of Sainfoin (*Onobrychis viciifolia* Scop.) Condensed Tannins on Growth and Proteolysis by Four Strains of Ruminal Bacteria†

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Sainfoin leaf condensed tannins inhibited growth and protease activity in *Butyrivibrio fibrisolvens* A38 and *Streptococcus bovis* 45S1 but had little effect on *Prevotella ruminicola* B_14 or *Ruminobacter amylophilus* WP225. Tannins bound to cell coat polymers in all strains. Morphological changes in *B. fibrisolvens* and *S. bovis* implicated the cell wall as a target of tannin toxicity.

Condensed tannins (CTs) are generally regarded as antinutritional for ruminants because of depression of feed intake and dry matter digestibility (13, 24), but their capacity to precipitate proteins reversibly at rumen pH may be nutritionally beneficial (14, 15, 20). When CT-containing herbage is masticated, insoluble CT-protein complexes are formed; these are stable over the pH range 3.5 to 7.0 but dissociate in the abomasum and anterior duodenum (11, 16). This protects the protein from microbial hydrolysis and deamination in the rumen and increases the proportion of plant amino acids available for postruminal absorption. Furthermore, the "bloat-safe" property of pasture legumes such as sainfoin (*Onobrychis viciifolia* Scop.) and birdsfoot trefoil (*Lotus corniculatus* L.) is correlated with CT content (14, 15). Most of the proteolytic activity of rumen contents resides with the bacterial fraction (3, 8, 17). Soluble plant proteins typically adsorb to the surface of proteolytic bacteria (21) and are hydrolyzed by constitutively expressed cell coat proteases (12, 22). Our objective was to determine some effects of CTs on growth and proteolysis by four strains of ruminal bacteria representing functionally important proteolytic species (23). The CTs used were purified (2) from leaves of sainfoin (cv. Melrose) and had a high protein-precipitating capacity compared with those of CTs from eight other forage legume sources (9). A single batch of sainfoin leaf CTs was used for all experiments.

Effects of CTs on bacterial growth. *Butyrivibrio fibrisolvens* A38, *Prevotella ruminicola* B_14, *Ruminobacter amylophilus* WP225, and *Streptococcus bovis* 45S1 were grown anaerobically (4) in a basal medium, which was the medium of Scott and Dehority (19) modified by omission of casein hydrolysate and cellobiose and addition of 0.0001% (wt/vol) hemin. The basal medium is referred to as modified Scott-Dehority medium. Omission of casein hydrolysate was necessary to avoid precipitate formation on the addition of CT. *B. fibrisolvens* grew poorly in the absence of casein hydrolysate, however, and 10% (vol/vol) clarified rumen fluid (CRF2 [5]) was added to the medium for this strain. To prepare CRF2, rumen fluid was obtained from a fistulated steer fed alfalfa hay cubes. Similarly, precipitate formation in the presence of CT precluded use of starch, the preferred energy source for growth of *R. amylophilus*. Maltose (12.5 mM) was therefore used as the substrate for this strain, and glucose (25 mM) was used for all other strains. Supplemented modified Scott-Dehority medium was sterilized by autoclaving under CO_2, and CTs were added subsequently as a prereduced, filter-sterilized (0.22-μm-pore-size filter) solution in modified Scott-Dehority medium lacking volatile fatty acids, vitamins, and hemin. The complete medium (final pH, 6.5 to 6.6) was inoculated in duplicate with a standardized cell suspension of each strain (10), and cultures were incubated under CO_2 at 39°C. Growth was measured as the increase in optical density at 660 nm (OD_{660}); maximum OD_{660} values were corrected for zero time readings and then converted to adjusted OD to correct for deviations from Beer's law (10). Results are expressed as mean adjusted OD ± standard error.

B. fibrisolvens grew in the presence of 100 μg of CTs ml^{-1} but did not grow in the presence of 200 μg ml^{-1}, whereas the other strains grew with 600 μg of CTs ml^{-1} (Table 1). CTs had little effect on the growth of *P. ruminicola* or *R. amylophilus* but did cause flocculation of *S. bovis* cultures; growth in these cultures was therefore estimated visually, and markedly extended lag times were observed as the CT concentration was increased.

Cultures grown with and without CTs were examined by scanning and transmission electron microscopy (2). Cells of *P. ruminicola* grown with CTs were interconnected by condensed extracellular material which was absent from cells grown without CTs (Fig. 1A and B). This material probably resulted from precipitation of cell coat polymers by CTs. *B. fibrisolvens* cells grown with CTs were elongated compared with cells from control cultures (Fig. 1C and D). CTs caused extensive chain formation and flocculation in cultures of *S. bovis*, and deposits of electron-dense material, presumably precipitated tannin, occurred on the surface of cells (Fig. 2A and B). The chains were composed of irregularly shaped cells, resulting from the initiation of multiple division planes and failure of the daughter cells to separate (Fig. 2C and D). These results suggest that the cell wall is involved in the action of CTs on *B. fibrisolvens* and *S. bovis*.

Effects of CTs on proteolysis. Two sets of experiments were carried out. In the first set, the proteolytic activity of cultures grown with CTs was determined and cell-associated CTs were assayed. Cultures were prepared as described above, grown to mid-logarithmic phase in the presence or absence of CTs, and then separated into cell and supernatant fractions by centrifugation under CO_2 at 8,000 × *g* for 15 min at 5°C. The supernatant fraction was retained. The cell pellet was resuspended under N_2 in cold, N_2-saturated 0.1 M phosphate buffer (pH 6.8), containing 0.1% (wt/vol) cysteine hydrochloride

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TABLE 1. Effect of sainfoin leaf CTs on growth of four strains of proteolytic ruminal bacteria[a]

Strain	Growth (increase in adjusted OD ± SE) with the following amt of CTs (μg ml^{-1}):				
	0	100	200	400	600
B. fibrisolvens	0.422 ± 0.004 (19)	0.319 ± 0.010 (32)	0	0	0
P. ruminicola	1.255 ± 0.004 (10)	1.395 ± 0.003 (10)	1.295 ± 0.002 (11)	1.128 ± 0.004 (12)	1.072 ± 0.049 (20)
R. amylophilus	0.277 ± 0 (9)	0.238 ± 0.006 (9)	0.224 ± 0.003 (9)	0.197 ± 0.003 (10)	0.197 ± 0.003 (10)
S. bovis[b]	0.719 ± 0.005 (5)				

[a] Modified Scott-Dehority medium contained 10% CRF2 (5) for *B. fibrisolvens*; the energy sources were 12.5 mM maltose for *R. amylophilus* and 25 mM glucose for all other strains. Numbers in parentheses are the numbers of hours of incubation required to reach maximum adjusted OD.

[b] Flocculation was seen for *S. bovis* at CT concentrations of 100 to 600 μg ml^{-1} and with ~15 to ~140 h of incubation required to reach maximum adjusted OD.

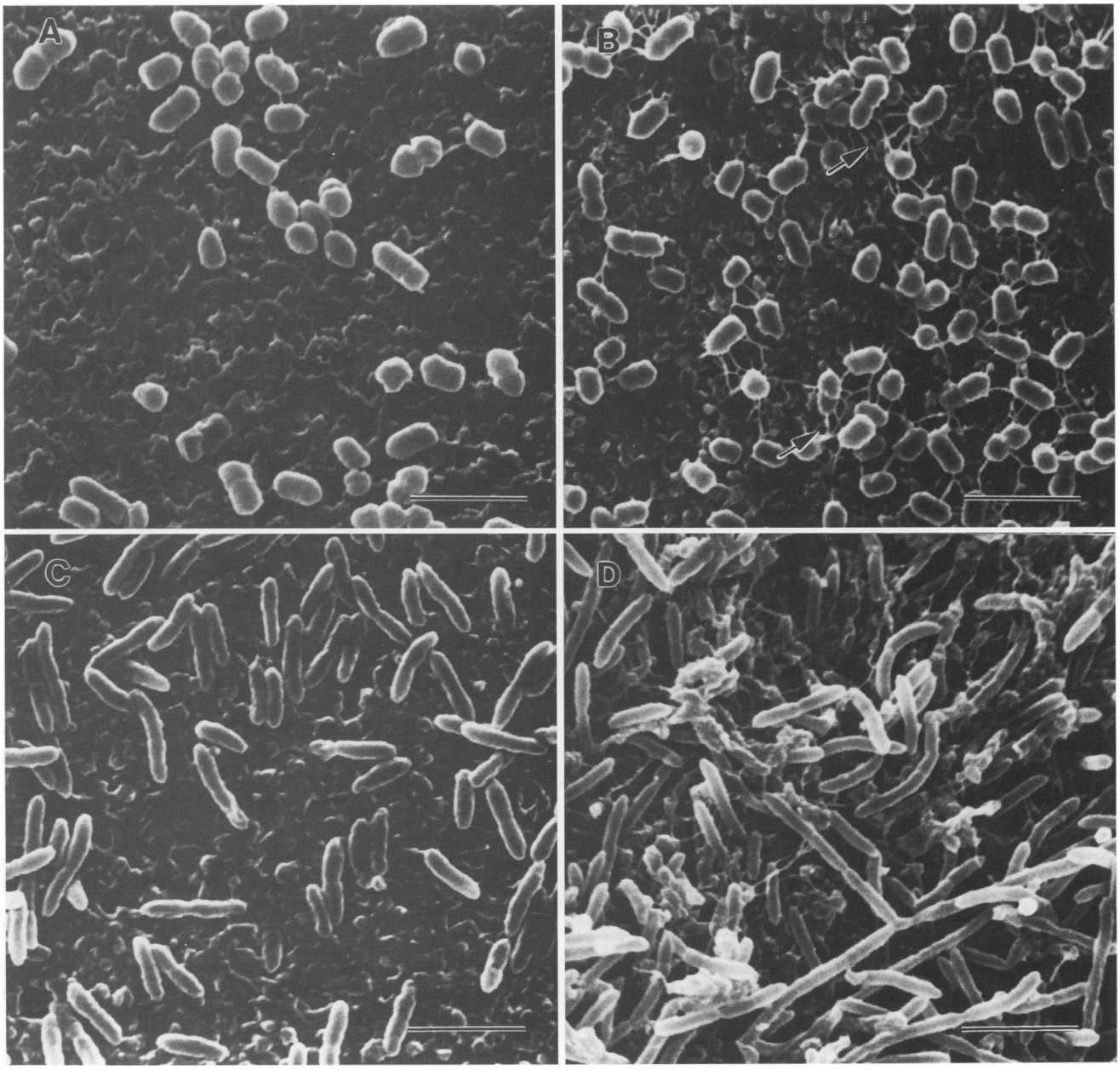

FIG. 1. Scanning electron micrographs of *P. ruminicola* grown without (A) and with (B) 500 μg of CTs ml^{-1} and *B. fibrisolvens* grown without (C) and with (D) 100 μg of CTs ml^{-1}. Arrows in panel B indicate interconnecting network of condensed extracellular material. Bars, 2 μm.

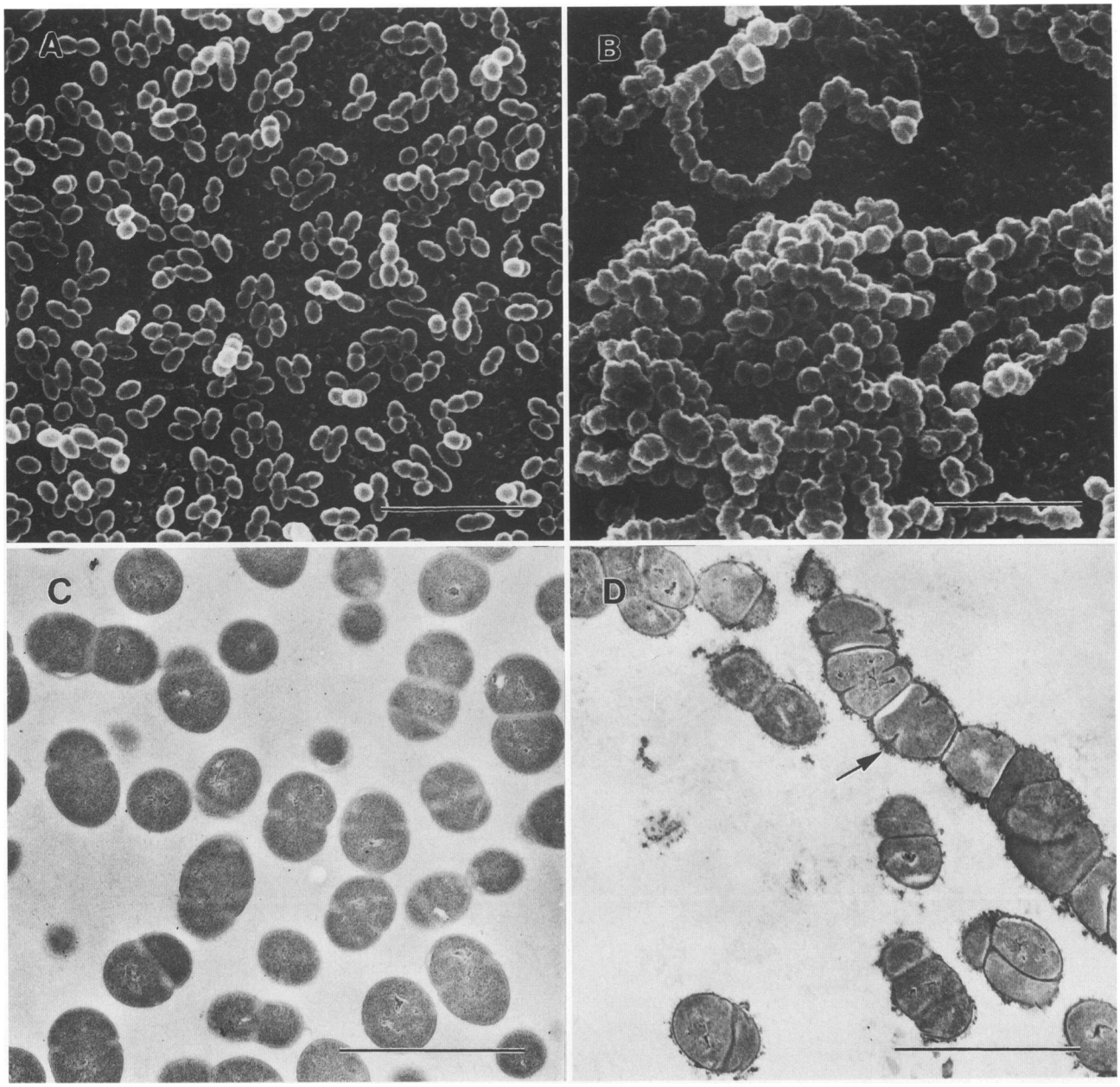

FIG. 2. Electron micrographs of *S. bovis* obtained with scanning electron microscopy (A and B) and transmission electron microscopy (C and D). The organism was grown without (A and C) and with (B and D) 500 μg of CTs ml^{-1}. Arrow in panel D indicates electron-dense deposits on cell surface. Bars, 5 μm in panels A and B and 2 μm in panels C and D.

(anaerobic buffer), with gentle agitation to minimize removal of cell coat proteases (12). Centrifugation and resuspension were repeated, thus providing three fractions of each culture—supernatant, washed cells, and wash. Protease activities of the fractions were assayed and summed to obtain total activity. The washed cell fractions were assayed for cell-associated CTs.

In the second set of experiments, CTs were added to suspensions of washed cells grown to mid-logarithmic phase in the absence of tannins. After addition of CTs, the suspensions (1 mg of cells [dry weight] ml^{-1} in a total volume of 3 ml) were incubated in a water bath at 39°C for 15 min and immediately cooled in ice. They were then centrifuged at 8,000 × *g* for 15 min at 5°C, and the cell pellets were washed twice to remove free tannins. After the final centrifugation, the pellets were resuspended in 3 ml of anaerobic buffer and were assayed for protease activity (3) and cell-associated CTs (1). Cell dry weight was estimated from the OD_{660} of cultures at the time of harvest by reference to previously constructed standard curves. To measure cell dry weight, suspensions of washed cells grown in the absence of CTs were dried at 105°C for 2 h and then held overnight in a desiccator before being reweighed. An OD_{660} of 0.500 was equivalent to cell dry weights of 244.6, 205.5, 238.9, and 154.6 μg ml^{-1} in the case of *B. fibrisolvens*, *P. ruminicola*, *R. amylophilus*, and *S. bovis*, respectively. In both sets of

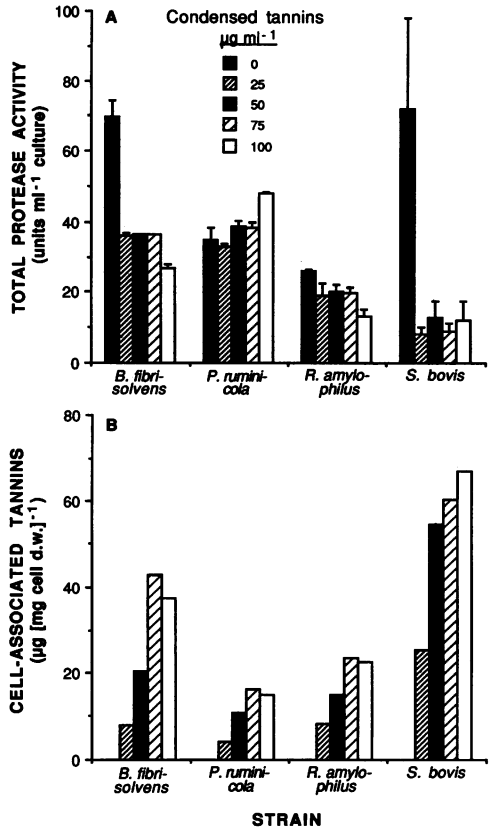

FIG. 3. Effects of CTs on total protease activity (A) and cell-associated tannin concentrations (B) in cultures of four strains of ruminal bacteria. Error bars in panel A represent 1 standard error. One unit of protease activity hydrolyzed 1 μg of azocasein in 1 h. d.w., dry weight.

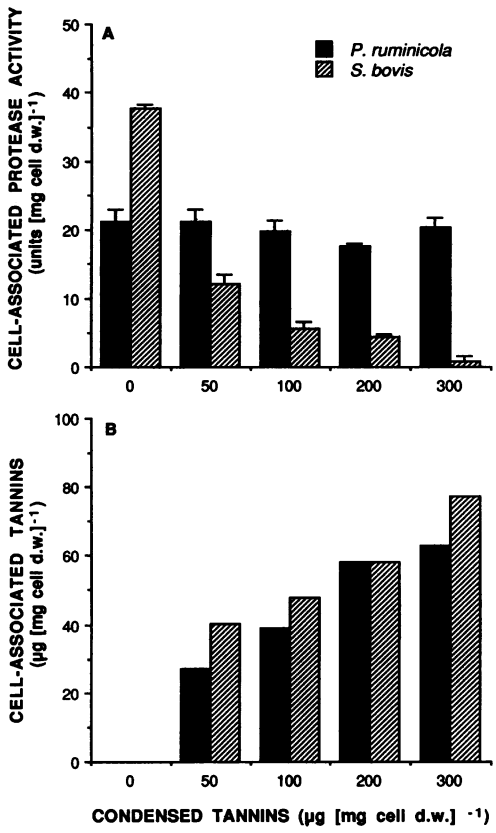

FIG. 4. Effects of CTs on cell-associated protease activity (A) and cell-associated tannin concentrations (B) in washed cell suspensions of *P. ruminicola* and *S. bovis*. Error bars in panel A represent 1 standard error. One unit of protease activity hydrolyzed 1 μg of azocasein in 1 h. d.w., dry weight.

experiments, results are expressed as the mean of replicate procedures ± standard error; where appropriate, treatment means were compared with the means of controls lacking CTs by Dunnett's procedure (6).

In cultures grown without CTs, the proportion of total protease activity in the wash fractions of all strains was less than 5%, indicating that the fractionation procedure did not cause significant removal of cell-associated proteases. Cell-associated activity represented 40 to 45% of the total activity in cultures of *P. ruminicola* and *R. amylophilus* and 20 to 30% of the total activity in cultures of *B. fibrisolvens* and *S. bovis*. Total activity in cultures of *B. fibrisolvens* and *S. bovis* was reduced by 48 and 92%, respectively ($P < 0.01$), in the presence of 25 μg of CTs ml^{-1} (Fig. 3A). In both strains, the relative proportions of cell-associated and soluble activity in the presence of 25 μg of CTs ml^{-1} were similar to those in the control cultures (data not shown). The total activity in cultures of *P. ruminicola* was 36% higher in the presence of 100 μg of CTs ml^{-1} ($P < 0.05$) than in the control (Fig. 3A). CTs did not inhibit the activity of *R. amylophilus* cultures at concentrations below 100 μg ml^{-1} ($P > 0.05$). Concentrations of cell-associated CTs in the cultures were generally in proportion to the reductions in protease activity observed (Fig. 3B).

In *S. bovis*, cell-associated protease activity was similar to that of the control ($P > 0.05$) when washed cells were treated with 10 or 25 μg of CTs mg of cells (dry weight)$^{-1}$ (data not shown), but at 50 μg mg of cells (dry weight)$^{-1}$, the activity was 32% of that shown by untreated cells ($P < 0.01$), and at 300 μg mg of cells (dry weight)$^{-1}$, it was 2% ($P < 0.01$) (Fig. 4A). In contrast, there was no reduction ($P > 0.05$) in the cell-associated protease activity of *P. ruminicola* at any CT concentration tested up to 300 μg mg of cells (dry weight)$^{-1}$. At 50 μg of CTs mg of cells (dry weight)$^{-1}$, 53% of the tannins became associated with *P. ruminicola* cells, and 80% became associated with *S. bovis* cells (Fig. 4B); at 300 μg mg of cells (dry weight)$^{-1}$, the respective proportions were 20 and 25%.

Several mechanisms have been proposed to account for the antimicrobial properties of tannins, including inhibition of extracellular enzymes (18). In the present work, CTs became bound to cell coat polymers of both growing and washed cells in all of the strains tested. The inhibition of cell-associated proteolytic activity in *B. fibrisolvens* and *S. bovis* implicates cell coat proteases in complex formation. The effects of CTs on the morphology of these strains also implicate the cell wall as a target of tannin toxicity. The wall composition in *S. bovis* and probably also in *B. fibrisolvens* A38 (7) is characteristic of gram-positive bacteria. We conclude that, despite complex formation with cell coat polymers in these strains, CTs penetrated to the cell wall in sufficient concentration to react with one or more ultrastructural components and to selectively inhibit cell wall synthesis. Decreased proteolysis in these strains may also reflect reduced export of proteases from the cell in the presence of CTs.

Little is known of the affinity of microbial cell surfaces for CTs relative to that of other components of the rumen ecosystem in vivo, but they clearly represent potential binding sites. In this respect, the resistance of *P. ruminicola* and its proteases to high concentrations of CTs is of particular interest because of the functional importance of this species in rumen proteolysis (23).

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