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Use of tannic acid to protect barley meal against ruminal degradation

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Abstract: The inefficiency of starch utilisation by ruminants fed readily fermentable cereal grains, such as barley, decreases the metabolic potential of such feeds and can cause serious dysfunctions related to acidosis or pre-acidosis status in animals with higher energy requirements. The rate and extent of ruminal disappearance of grain dry matter (DM) are determined largely by the morphological features of the seed endosperm, but the presence of polyphenols may also constitute a limiting factor in ruminal hydrolysis. To assess the impact of tannins on the rate and extent of ruminal fermentation of barley grain, commercial grade tannic acid (TA) was included at 0, 1.0, 2.5 or 5.0% (wt/wt, DM basis) with ground barley grain in incubation *in situ*, and disappearance of DM and crude protein were monitored over 48 h. A dose-dependent effect of TA on *in situ* degradation of barley meal was observed; significance was attained ($p < 0.05$) at the 5% treatment level. Scanning electron microscopy revealed limited microbial hydrolysis of endosperm cell walls in TA-treated samples, although TA did not prevent microbial attachment to or hydrolysis of starch granules. Tannins may be effective for slowing ruminal disappearance of barley to improve starch utilisation by ruminants.

Keywords: by-pass starch; concentrate feeds; scanning electron microscopy; tannins

INTRODUCTION

Tannins comprise a complex group of plant polyphenols characterized by their propensity for binding to macromolecules. They bind mainly to proteins, but also to starch and structural polysaccharides,[1,2] which results in reduced bioavailability of nutrients in the digestive tract. Nevertheless, recent investigations have considered the possibility of exploiting tannins as agents for reducing the degradation of plant proteins in the rumen and thus increase the intestinal bioavailability of amino acids to ruminant livestock.[3,4] Tannin–protein interactions are reversible and pH-dependent,[5] which favours their dissociation in the abomasum, thereby increasing the provision of plant protein to the small intestine.[2] Several studies with forages,[6] legume concentrates[7] and isolated proteins[8] have been carried out to investigate this hypothesis. Despite this interest in tannins and protein, knowledge about the mechanisms involved in the interactions between tannins and dietary starch is currently limited.

With some exceptions (eg corn, sorghum), hydrolysis of starch from grains in the ruminant digestive tract occurs primarily in the rumen.[9] However, rapid ruminal fermentation of starch to produce volatile fatty acids is a less efficient metabolic utilisation of these carbohydrates than is intestinal digestion to glucose.[10,11] In addition, feeding excessive amounts of cereal grains to meet the energy requirements of high-producing ruminants in response to this digestive inefficiency is known to give rise to acidosis and digestive disturbances.[12,13] Manipulation of starch fermentation in the rumen, therefore, has become an important research topic in animal nutrition with some studies focussing on modifying the susceptibility of starch sources to microbial degradation. The effects on barley of chemical treatments such as alkali[14] and aldehydes[15,16] have been assessed, but their practical application has been limited because of concerns for human health and safety.

Tannins as agents for protection of barley from ruminal degradation have scarcely been explored, and studies to date have focussed on feeding grain varieties containing low levels of naturally occurring polyphenols.[17] Experimental addition of incrementally larger amounts of tannins to barley grain would allow assessment of their contribution to retarding ruminal degradation of the barley, an approach similar to that taken in earlier studies focussed on legume

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seeds.[18] Scanning electron microscopy (SEM) has been used effectively to augment knowledge of the effects of physical and chemical manipulations of grains,[15,19] and could also be used to reveal the effects of tannins on digestion of cereal endosperm. The aim of the present paper was to evaluate the potential of pre-treatment with tannic acid as a means of slowing the ruminal degradation of barley grain, using *in situ* incubation to determine effects on ruminal disappearance kinetics and, with SEM, to examine its effects on the ultrastructure of barley grain subjected to ruminal incubation.

MATERIALS AND METHODS

Animals

The study was conducted using four ruminally cannulated, non-lactating Segureña ewes (local breed) The sheep were fed twice daily a diet containing oat-vetch hay and milled barley grain [4:1 ratio, dry matter (DM) basis] with vitamin and mineral block supplement, and had free access to water. The ewes were cared for in accordance with EU requirements related to laboratory animal welfare.

Sample preparation

Barley grain milled to pass through a 2-mm mesh sieve was combined with 10% (wt/vol) aqueous tannic acid (TA; Sigma Chemical Co, St Louis, MO, USA) in quantities to yield final TA concentrations of 0 (control), 10, 25 and 50 g kg^{-1} barley DM. Distilled water was added as required to reach complete homogeneity of suspension. After 20 min of stirring, the slurries were dried in a forced-air oven at 45 °C. The dried residues were gently crushed using a mortar in order to break lumps and were passed again through a 2-mm mesh sieve. For SEM studies, the same procedure was followed, but a 3-mm mesh sieve was used so that the particles generated were handled more easily.

Ruminal disappearance of dry matter and crude protein

Barley meals were incubated *in situ* according to the procedure described by Mehrez and Ørskov (1977).[20] The meals (approximately 3 g per bag) were loaded into 9 cm × 5 cm polyester bags with 50-μm pore size (Ankom Technology Co, Fairport, NY, USA). Duplicate bags were prepared for each of six sampling times (2, 4, 8, 12, 24 and 48 h) as well for an incubation of 0 h in buffer. The 12 *in situ* incubation bags were introduced into the rumen simultaneously (one treatment per ewe). Upon retrieval from the rumen, the bags were washed thoroughly under cold tap water until it ran clear, then placed immediately in a forced-air oven at 50 °C for 48 h. After drying, the contents of duplicate bags were combined for determination of crude protein (CP) content.[21] The incubation was conducted three times, with each treatment incubated in three of the four ewes, yielding triplicate measurements. Disappearances of DM and CP at each incubation time (%) were calculated from the proportion of material remaining in the *in situ* bags. Data were fitted to the equation of Ørskov and McDonald:[22]

$$D = a + b(1 - e^{-ct})$$

where D = the proportion (%) of loss from the bag at time t (h), a = the immediately soluble fraction (including fine particulate matter lost passively from bags), b = the slowly degradable fraction and c = the rate of degradation of fraction b (h^{-1}). The immediately soluble fraction was estimated from the 0-h bags, which were shaken in 0.2 M phosphate buffer (pH 6.8) at 39 °C for 60 min.

The non-linear parameters a, b, and c were estimated by an iterative least-squares procedure, and best-fit values were chosen using the smallest sum of squares after convergence, using a specific application (Table Curve 2D, Jandel Scientific, AISN Software, 1994). Effective degradabilities of DM and CP, denoted ED_{DM} and ED_{CP}, respectively, were calculated[22] as:

$$ED_x = a + [(bc)/(c + k)]$$

where k is the estimated rate of outflow from the rumen, which was set at 0.02, 0.05 or 0.08 h^{-1}, to represent slow, medium or rapid turnover rates, respectively.[23]

Scanning electron microscopy

Samples from the control (0 g kg^{-1}) and 50 g TA kg^{-1} DM treatments were selected for examination by SEM. Residues from the *in situ* incubation were fixed in 5% (vol/vol) glutaraldehyde for 3 h, then washed five times with 1.0 M cacodylate buffer (pH 7.2). Washed samples were stored in the cacodylate buffer at 4 °C prior to dehydration in a graded ethanol series. The dehydrated specimens were critical-point dried under CO_2, affixed to aluminum stubs with carbon paste and sputter-coated with gold (Polaron E5000, Quorum Technologies Ltd, Newhaven, East Sussex, UK). The specimens were viewed using a Zeiss DSM 950 scanning electron microscope.

Statistical analyses

Mean effective degradabilities and disappearances of DM and CP, expressed as percentages, were normalised by arc sine transformation[24] prior to statistical analysis. Data were analysed by one-way ANOVA followed by a comparison of means by Fisher's LSD procedure to determine the significance of differences within TA treatments and within outflow rates. The contribution of these two factors to effective degradability was assessed by performing two-way ANOVA analysis. In this case, the contribution of each factor was measured having removed the effect of the other factor. All analyses were conducted

using Statgraphics Plus 4.0 (Statistical Graphics Corp, Rockville, MD, USA).

RESULTS

Disappearances of dry matter and crude protein

At 2, 4, 8 and 12 h, disappearances of DM and CP from barley were lower ($p < 0.05$) in all TA-treated samples than in the controls, and the response became more pronounced as TA treatment level increased (Fig 1). At 24 h, the difference was significant only at the 50 g kg^{-1} treatment level, and by 48 h no differences among treatments were evident ($p > 0.05$). Effective degradabilities of DM and CP decreased ($p < 0.05$) as TA treatment level increased (Table 1), and they were also lower when outflow rates were set at 0.05 h^{-1} and (or) 0.08 h^{-1} when compared with 0.02 h^{-1}.

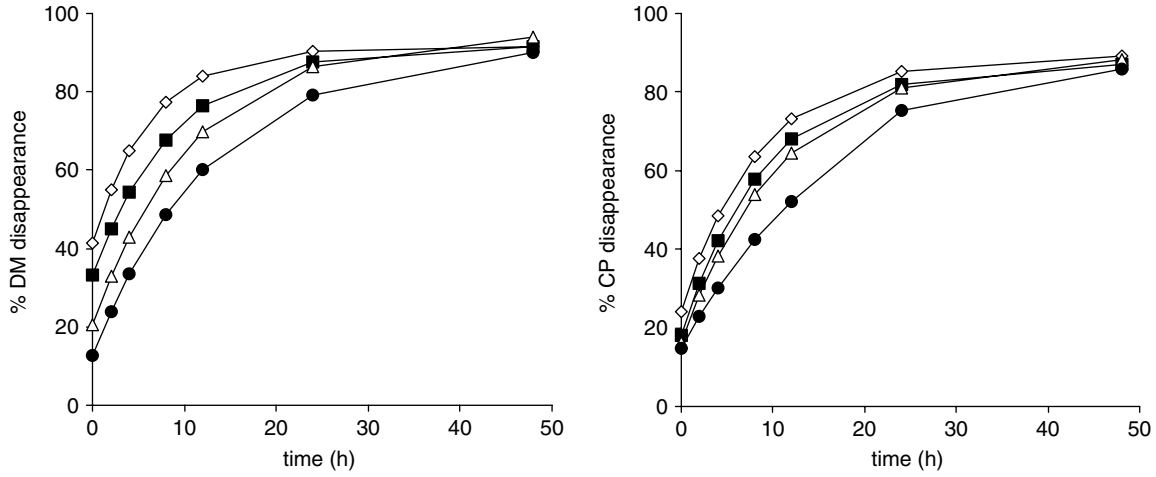

Figure 1. Disappearance of (A) dry matter and (B) crude protein during 48 h of *in situ* ruminal incubation of barley meal treated with tannic acid at 0 (control, —◇—), 10 (—■—), 25 (—△—) or 50 (—●—) g kg barley DM^{-1}.

Table 1. *In situ* digestion kinetics[a] of DM and crude protein in ground barley grain untreated or treated with tannic acid

	Tannic acid treatment level (g kg^{-1} barley DM)[d]			
	0 (control)	10	25	50
Dry matter (DM)				
a	41.36a	33.38b	20.56c	12.73d
b	50.18a	58.50b	74.34c	79.44c
c	0.159a	0.110b	0.090c	0.080d
Adjustment (r^2)[b]	0.983	0.958	0.969	0.975
RSD[c]	2.33	4.24	4.50	4.21
Effective degradability				
$k = 0.02$ h^{-1}	85.92a,A	82.89ab,A	81.38ab,A	75.51b,A
$k = 0.05$ h^{-1}	79.51a,B	73.62ab,B	68.34bc,B	60.50c,B
$k = 0.08$ h^{-1}	74.71a,B	67.27ab,B	59.91 b,B	51.27c,B
Crude protein (CP)				
a	24.11a	17.97b	16.27bc	14.88c
b	65.21a	69.47ab	72.96bc	76.11c
c	0.116a	0.110a	0.090b	0.060c
Adjustment (r^2)	0.989	0.978	0.988	0.995
RSD	2.33	3.58	2.74	1.73
Effective degradability				
$k = 0.02$ h^{-1}	79.75a,A	76.46ab,A	76.00ab,A	71.04b,A
$k = 0.05$ h^{-1}	69.71a,B	65.24ab,B	63.23b,B	55.19c,B
$k = 0.08$ h^{-1}	62.74a,C	57.64ab,B	54.96 b,C	46.32c,C

[a] Determined by fitting data to the equation: $D = a + b(1 - e^{-ct})$ where D = % disappearance of material (DM or CP) at time t; a = immediately soluble fraction (%); b = slowly degradable fraction (%); c = rate at which b is degraded (% h^{-1}); and t = duration of incubation *in situ* (h). Effective degradabilities (ED, %) were calculated from the equation: $ED = a + [(bc)/(c + k)]$ where k = ruminal outflow rate (h^{-1}), set as shown at 0.02, 0.05 or 0.08 h^{-1}.

[b] The adjustment r^2 represents the better adjust to the non-linear model that can be achieved from the experimental data.

[c] RSD = residual standard deviation (after fitting data to equation).

[d] a–c: Treatment effect. Within a row, values followed by different letters differ ($p < 0.05$). A–C: Effect of outflow rate. Within a column and material (DM or CP), values followed by different letters differ ($p < 0.05$) ($n = 6$).

All levels of TA treatment decreased ($p < 0.05$) the soluble fraction (*a*), the potentially degradable fraction (*b*) and the degradation rate (*c*) of barley DM (Table 1). The impact of TA on kinetics of *in situ* digestion of CP was similar to the effect on DM digestibility, but decreases in slowly degradable fraction (*b*) and digestibility rate (*c*) were significant ($p < 0.05$) only at TA treatment levels 25 and 50 g kg barley DM^{-1}. The effects of TA on ED_{DM} and ED_{CP} varied with the passage rate selected for calculation. At an outflow of $0.02\,h^{-1}$, the TA-mediated decreases in ED were significant only at the highest treatment level, whereas at outflow rates of 0.05 or $0.08\,h^{-1}$, a treatment effect ($p < 0.05$) was observed also with TA at $25\,g\,kg^{-1}$ barley DM. Two-way ANOVA confirmed that the main factor contributing to determination of ED_{DM} was ruminal outflow rate ($F = 48.98$), followed by treatment level ($F = 24.63$). Similarly, these were the main factors influencing ED_{CP} ($F = 142.31$ and $F = 30.60$ for outflow rate and TA treatment, respectively).

Scanning electron microscopy

Scanning electron micrographs of untreated and TA-treated barley meal are presented in Figs 2, 3 and 4. Effects of TA on ruminal degradation of the barley were evident in the samples collected after 4 h of incubation (Figs 2-1 and 2-2). Hydrolysis of cell walls enclosing the starch granules in barley endosperm was evident in untreated meals (Fig 2-1). However, in the control barley, the starch granules themselves showed no sign of hydrolysis (Fig 2-1), whereas colonisation and hydrolysis of TA-treated starch granules was shown by this point in the incubation (Fig 2-2). Degradation of starch granules in untreated barley lagged behind that observed in the TA-treated meal at

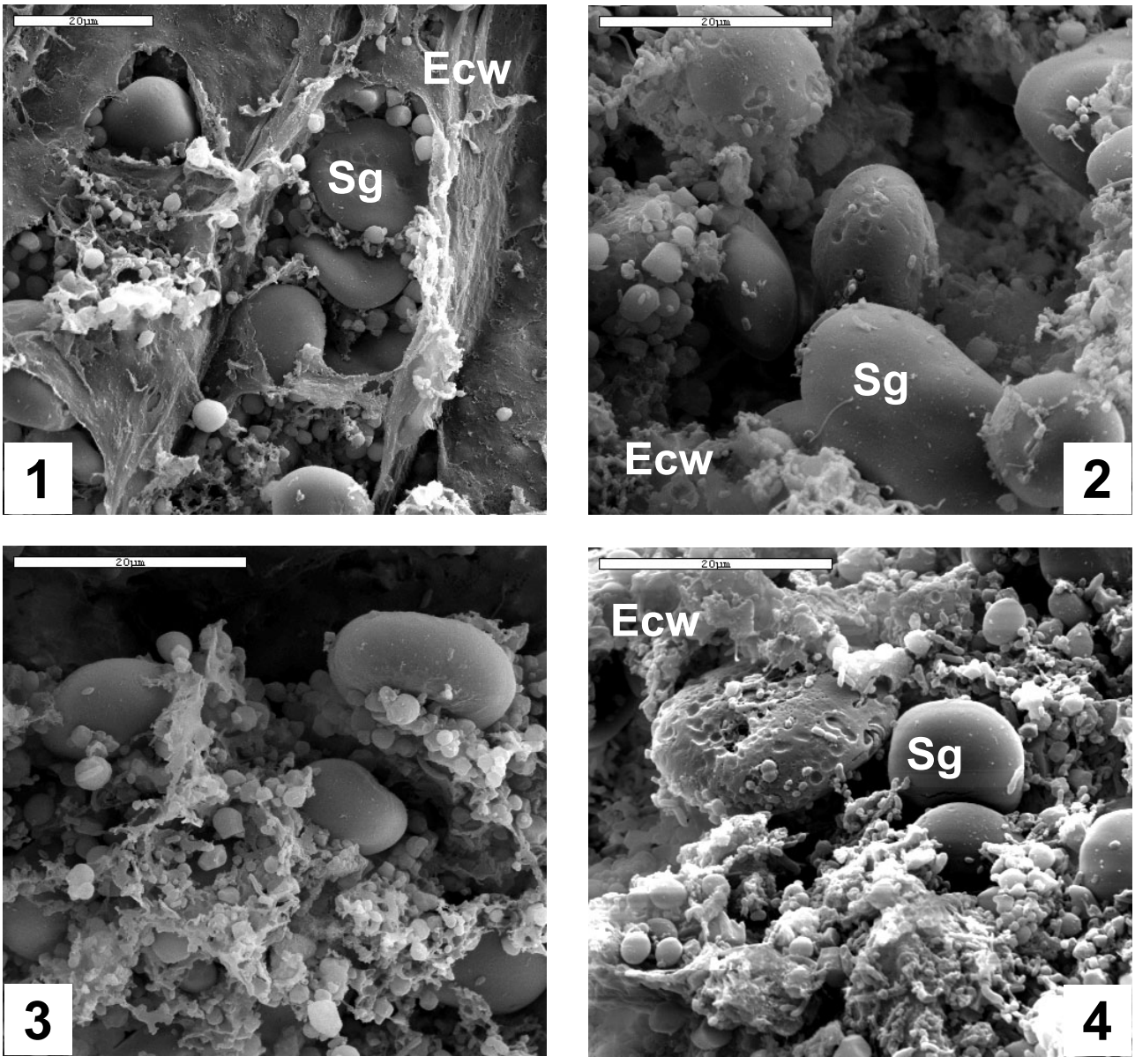

Figure 2. Scanning electron micrographs of the endosperm of barley meal treated with (1, 3) no tannic acid or (2, 4) with tannic acid applied at 50 g kg barley DM^{-1} following (1, 2) 4 h or (3, 4) 8 h of *in situ* incubation. **Ecw** = endosperm cell wall; **Sg** = starch granule.

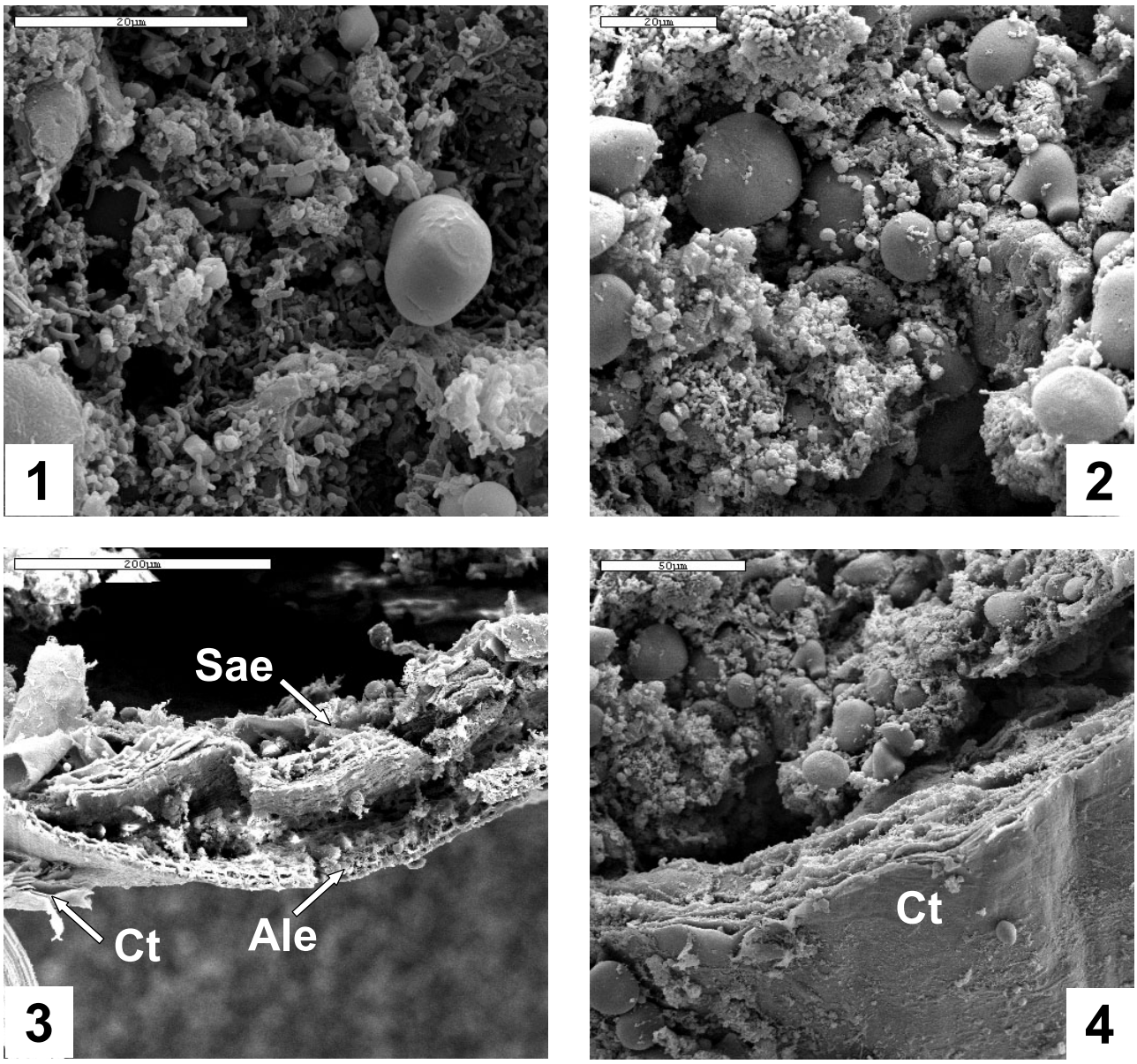

Figure 3. Scanning electron micrographs of the endosperm of barley meal treated with (1, 3) no tannic acid or (2, 4) with tannic acid applied at 50 g kg barley DM^{-1} following (1, 2) 12 h or (3, 4) 24 h of *in situ* incubation. **Ct** = cuticle; **Ecw** = endosperm cell wall; **Sae** = sub-aleuronic endosperm; **Sg** = starch granule.

8 and 12 h (Fig 2-3, 2-4; Fig 3-1, 3-2). After 24 h of *in situ* incubation, only the cuticle and sub-aleuronic endosperm tissue remained in untreated samples (Fig 3-3), but considerable amount of the endosperm persisted in TA-treated barley (Fig 3–4, Fig 4).

DISCUSSION

Ruminal microorganisms utilise the starch contained in readily fermentable cereal grains extensively as an energy source. In barley, for example, over 90% of starch is hydrolysed in the rumen,[9] but substantial variability in the rates and extents of ruminal fermentation of starch has been observed among different cereal grains. It has been proposed that the internal structure of the starch granules themselves, such as the amylose:amylopectin ratio, could affect the rapidity with which they are hydrolysed in the rumen.[25,26] This factor alone, however, cannot account for all the differences observed, and recent findings suggest that other characteristics of the cereal grains, principally the endosperm protein and cell wall architecture, are the primary factors regulating their digestibility.[27]

Digestion of barley and wheat is more rapid than that of corn or sorghum because of the natural abundance of proteins of low digestibility (eg prolamins) in the vitreous (horny) endosperm of the latter.[28] In addition, the presence of polyphenols in some cereals, including sorghum, is believed to contribute to the higher resistance of those grains to ruminal hydrolysis.[29]

Tannic acid was first investigated as an agent for protecting soybean meal from ruminal degradation in the 1970s,[30] and beneficial effects on growth and nitrogen retention in association with TA treatment

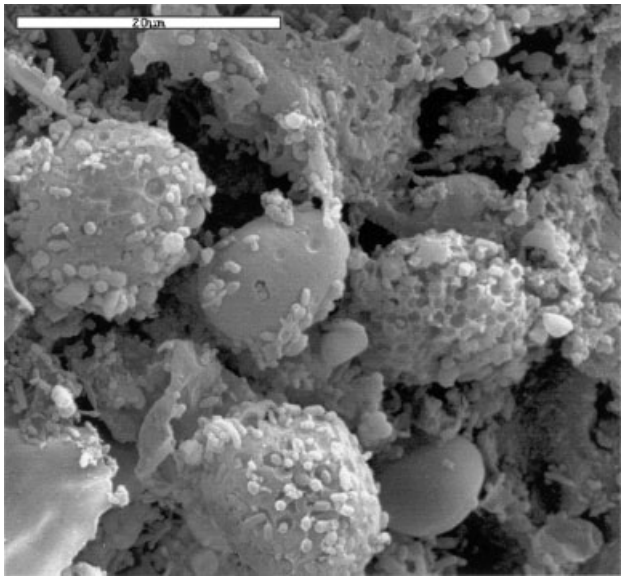

Figure 4. Scanning electron micrographs of starch granules of barley meal treated with tannic acid at 50 g kg barley DM^{-1} following 24 h of *in situ* incubation.

were reported. Since then, the majority of studies in this area have focussed on protein utilisation, but similar studies with cereal grains have not been reported. In 1973, Waldo[31] described an effect of tannins on the ruminal digestibility of starch, and the inhibition by tannins of *in vitro* hydrolysis of starch has also been reported.[32] It was not determined, however, whether the starch–tannin interactions were dependent more on properties of the starch or on tannin chemistry.[33]

In the present study, TA decreased the rate and extent of ruminal disappearance of barley in an apparently dose-dependent manner, although a significant response in effective degradability was recorded only at 25 or 50 g TA kg barley DM^{-1} for DM, and at the highest treatment level only for CP. *In situ* incubation alone does not indicate which components of barley grains are susceptible to the TA effects that inhibited the ruminal degradation. Some studies have described limitations of the technique for use with starch-rich cereals that can lead to over-estimation of their ruminal disappearance.[34] This arises in part from the fact that the fate of non-hydrolysed starch granules passing out of the *in situ* sample bags is unknown.

Scanning electron microscopy revealed that starch granules in the barley used in this incubation were smaller than the pore size of the fabric from which the bags were constructed. Consequently, the granules could pass from the bags and were likely to be extensively hydrolysed in the rumen, by both bacteria and protozoa. In contrast, granules entrapped in the endosperm of TA-treated barley persisted longer in bags, leading to lower values for DM and CP disappearances, and also allowing visualisation of more extensive microbial hydrolysis of the granules.

Although not quantitative, the results of the SEM study indicate that starch granules themselves are not directly involved in the protective effect of TA against *in situ* disappearance of barley meal. Instead, the SEM images suggest an interaction of TA with proteins and structural polysaccharides constituting the endosperm cell matrix in which the granules are embedded. The apparent lack of interaction between starch granules and TA is in contrast to some earlier reports,[29] but the involvement of the matrix is consistent with the mechanism of protection proposed for formaldehyde treatment of barley,[15] ie a selective interaction of the aldehyde with the proteinaceous matrix of the endosperm rather than a direct effect on the starch granules. Streeter *et al*[35] suggested that polyphenols could also contribute to denaturation of protein in the matrix of sorghum seeds. Even without clear definition of the exact mechanism, the overall effect of TA was to decrease the disappearance of DM and CP from the bags, suggesting the possibility of increased ruminal outflow of starch to the small intestine.

The post-ruminal availability of nutrients in TA-treated barley is not clearly defined. The interactions of tannins with other molecules are based primarily on non-covalent hydrogen bonds, and thus are reversible at pH values found in the abomasum and small intestine.[5] Other studies have shown that polyphenols in legume forages improve protein utilisation, mainly through increased flow of undegraded protein from the rumen and, after breakage of tannin–protein bonds, increased bioavailability of the protein in the small intestine.[36] However, data are lacking on the possible influence of tannins on post-ruminal utilisation of starch. Although further study is necessary, a reversible mechanism similar to that described for tannin–protein interactions in legumes may exist for cereals, given two assumptions: (i) a lack of involvement of the starch granules themselves in the interaction between TA and barley endosperm, and (ii) a non-covalent interaction between TA and barley ultrastructures.[37]

In the present study, effects of TA on *in situ* DM and CP disappearance were not evident at sampling times beyond 24 h, and at 24 h were significant only with TA included at 50 g kg barley DM^{-1}. Given the rapid hydrolysis of cereal meal in the rumen, however, strategies which effectively slow its ruminal disappearance over the initial 12 h would be of interest from a practical point of view. Microbial metabolism of tannins has been proposed as a possible explanation for the lack of persistence of the effects of polyphenols,[38] and in fact several species of ruminal are able to hydrolyse tannins.[39–41] Most of these studies, however, were characterised by weak tannase activity under culture conditions and incubation intervals quite distinct from the *in vivo* ruminal environment. Outflow of cereal meals from the rumen is relatively quick,[42] so that it is questionable whether or not bacterial activity could decisively affect hydrolysis of TA. Moreover, a lack of persistence of effect in the final stages of *in situ* incubation has

also been described for aldehyde-treated barley,[16] which suggests a mechanism other than the microbial hydrolysis proposed for tannins. One explanation for the short duration of effects of both TA and formaldehyde on barley could be a non-homogeneous distribution of exogenous chemical during treatment, leaving areas of grain surfaces insufficiently treated. Another possible reason could be the soaking of the grain fragments during the incubations, which produce a swelling of its volume, thus breaking the external protective layer provided by the treatments. In both cases, subsequent access to underlying tissue and effective circumvention of the chemical barrier are enabled.

The effect of TA on disappearances of DM and CP was much lower than has been observed with formaldehyde applied at similar concentrations to barley[16] and corn.[43,44] The greater effect exerted by formaldehyde, however, is achieved through permanent denaturation of proteins, which limits their subsequent bioavailability as amino acids in the intestine.[45]

The present study suggests that tannins could be valuable as agents to depress ruminal degradability and increase the by-pass value of barley for ruminants. In contrast to many other chemicals proposed for manipulating ruminal fermentation, tannins are naturally occurring compounds present in many plant species, and may therefore be judged more acceptable by livestock producers and consumers. From a practical standpoint, the main interest in tannins in this application is not based on their use as exogenous agents (possible depression in feed intake, as well as risk of direct toxicity[46]), but rather on the fact that their expression in plants is genetically regulated.[47] Selection of tannin-containing cereals, or genetic modification of tannin-free varieties to increase tannin content may have promise as an alternative strategy for improving starch utilisation in ruminants.

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