

## Invited commentary

# The contribution of plant-derived proteinases to the breakdown of fresh pasture protein in the rumen

Pastoral agriculture in New Zealand has a long history of improving the efficiency with which fresh pasture is converted into meat, wool and dairy products. New Zealanders live in a temperate climate where rainfall and pasture growth are the main driving forces in low-input pastoral systems. The process whereby protein in fresh pasture is broken down in the rumen and reassimilated into microbial protein for use by the animal is consequently of fundamental importance to ruminant nutrition in New Zealand, and indeed elsewhere. The article appearing in a previous issue of the *British Journal of Nutrition* by Kingston-Smith *et al.* (2005) provides further evidence for a role of plant proteinases in protein breakdown in the rumen and is therefore of interest.

A scan of the literature in the area of ruminal protein degradation reveals numerous reports from the points of view of animal nutrition and rumen microbiology but very few on the contributions of plants to their own demise once ingested into the rumen. Up until 1996, the generally accepted version of events in the initial stages of degradation of soluble plant protein in the rumen involved the release of proteins from masticated plant tissue, the adsorption of protein to the surface of rumen bacteria and their subsequent hydrolysis by mainly cysteine proteinases (Wallace *et al.* 1997). Much of the information on this process was derived from the study of animals receiving dried or ensiled forage or concentrate diets (Brock *et al.* 1982; Kopecny & Wallace, 1982; Prins *et al.* 1983; Falconer & Wallace, 1998), and protein solubility, degree of folding and cross-linking were found to be important determinants of degradability. Under these circumstances, protein breakdown was mediated by rumen microbes, principally the bacteria, while protozoa were also important in degrading insoluble protein and recycling N within the rumen by predation of bacteria (Nugent & Mangan, 1981; Brock *et al.* 1982). Detailed studies on rumen proteinases followed and defined the main enzymatic types found in rumen contents and correlated these with their occurrence in rumen microbial species (summarised in Wallace *et al.* 1997).

Since 1996, a series of publications from the Institute of Grassland and Environmental Research in Aberystwyth, Wales, have promoted the concept that plant proteinases may play an important role in the breakdown of protein in fresh forage diets. Theodorou *et al.* (1996) first raised this possibility, arguing that pasture-grazed ruminants ingest plant material that is largely composed of intact cells that are still metabolically active. Plant cell vacuoles contain proteinases that are known to be involved in proteolysis during ensilage and it was proposed that conditions found in the rumen (elevated temperature, pH and lack of O<sub>2</sub>) favoured plant cell death and increased plant proteinase activity. They also pointed out that those rumen microbes most closely associated with plant material in the rumen (cellulolytic bacteria and

fungi) were either not proteolytic or had very low proteolytic activity. Subsequently, Zhu *et al.* (1999) examined ryegrass, red clover, white clover and bird's-foot trefoil *in vitro* in the presence and absence of rumen micro-organisms and measured volatile fatty acid (VFA) production and the disappearance of the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). They found similar patterns of protein degradation both in the presence and absence of rumen micro-organisms and concluded that plant proteinases contributed significantly to plant protein breakdown in the rumen. However, significant amounts of VFA (mainly acetate) were produced during these incubations, suggesting at least some growth of microbes associated with the plant material had occurred, thus raising the possibility of protein degradation by the epiphytic microbial population.

In response to these observations, Wallace *et al.* (2001) tested the plant proteinase hypothesis by autoclaving fresh ryegrass to rule out the action of plant-associated microbes and to inactivate plant proteinases. They found that autoclaving markedly decreased the rate of NH<sub>3</sub> release *in vitro* from both chopped and blended grass compared with non-autoclaved material. However, the rate of protein breakdown in blended grass as measured by <sup>14</sup>C-labelled casein hydrolysis was approximately 10% of that usually observed in rumen fluid. They concluded that although plant proteinases had low activity, their co-location with their protein substrate within plant cells gave them a disproportional contribution to the initial stages of protein breakdown in the rumen.

Further *in vitro* experiments by Beha *et al.* (2002) examined a number of plant protein and senescence parameters in ryegrass under temperature and anoxic stresses. Leaf blades incubated under rumen-like conditions in the absence of microbes released protein and amino acids, which subsequently appeared in the incubation buffer. The amount of amino acids appearing in the buffer exceeded that originally observed in the plant material, suggesting breakdown of protein in the plant tissue and release of amino acids. Proteinase zymograms indicated that the main activity in mature leaf samples was a non-cysteine proteinase.

Measurement of *in situ* protein degradation using Rubisco antisera indicated that some of the large and small subunit proteins were lost from the chloroplast after 6 h incubation. The authors concluded that ingested plant cells in the rumen entered a metabolic state similar to, but not exactly the same as, senescence, where protein was lost at a greater rate than either cell nuclei or chlorophyll. More recently, Kingston-Smith *et al.* (2003a) made similar observations in white and red clover leaves. The protein content of leaves decreased rapidly over the first 6 h of incubation and was accompanied by increased proteinase activity and more proteinase isoforms. These clovers also

showed progressive DNA damage and degradation over the incubation period, which was associated with increased ionic leakage.

Kingston-Smith *et al.* (2003b) also investigated the effect of rumen conditions on the availability of nutrients from a variety of fresh forages *in vitro*. All of the forage crops exhibited significant leakage within 2 h, suggesting damage to cell membranes. Using betanin release from cubes of red beet tubers as an indicator of plant vacuole integrity, it was found that the addition of rumen fluid gave best release. This suggests that microbial activity plays a significant role in release of vacuolar contents, although incubations in the absence of rumen fluid still accounted for approximately 20% of betanin release. The efflux of water-soluble carbohydrates from timothy grass under the same conditions saw 68% released over a 6 h period. The different rates of release of ions *v.* betanin and carbohydrates was suggested as evidence that small molecules were released in a constant linear fashion from intercellular pools while the cell wall acted as a barrier to release of higher molecular weight compounds. The authors proposed that upon ingestion, plant protein and proteases would be released from membrane-bound compartments such as the vacuole and chloroplast and result in proteolysis within the boundary of the cell wall. Shorter peptides and amino acids would be generated and would be small enough to be released from the cell and therefore be available for use by rumen microbes.

The current paper by Kingston-Smith *et al.* (2005) adds more evidence to the case for a significant role of plant proteinases in protein degradation in the rumen. Bags of varying permeability were used to assess the contribution of plant and rumen microbial proteinases to protein breakdown in freshly cut ryegrass incubated *in situ* in the rumens of dairy cows. The pore size of the bags determined different levels of microbial access to plant material; woven polyester bags (50  $\mu\text{m}$  pore size) allowed free access by all rumen microbes, dialysis tubing (10 kDa molecular weight cut off) excluded rumen microbes and enzymes and metabolites > 10 kDa, while polythene bags were essentially impermeable, so prevented access by rumen microbes, enzymes and most metabolites, while retaining components released from the plant material during incubation. Significant protein loss from leaf material was seen in all of the bag types over the 16 h incubation period, irrespective of permeability. The disappearance of 95–97% of leaf protein from dialysis tubing and polythene bags where rumen microbes were excluded is strong evidence for a significant role of plant proteinases. Some growth of epiphytic microbes within these incubations probably occurred, as shown by the appearance of low levels of VFA (mainly acetate) in the polythene bags; however, this is unlikely to account for the protein losses observed. The ultimate fate of the leaf protein in these experiments was not measured, but analysis of the liquid phase in the dialysis tubing suggests that approximately 5% was present as protein or peptides > 10 kDa while the remainder was presumably peptides and amino acids < 10 kDa. Analysis of the protein loss via SDS–PAGE showed that protein degradation was very similar in all three bag incubations. A large proportion of the large subunits and small subunits of Rubisco disappeared regardless of the presence of rumen microbes, again supporting the involvement of plant proteinases. A protein band corresponding to the light-harvesting complex protein of photosystem 2 (LHCPII) appeared to remain undegraded in all bags. It was suggested that its resistance to degradation was due to association with chlorophyll in the thylakoid membrane of

the chloroplast. This is consistent with previous observations that chlorophyll is lost only slowly from plant cells under rumen-like conditions (Beha *et al.* 2002).

These publications provide clear evidence that plant-derived proteinases contribute significantly to the first stages of protein degradation in fresh forages. Under rumen conditions, intact plant cells appear to undergo a type of autolysis, which bears some similarities to senescence in which proteinases break down protein for remobilisation within the plant. However, the protein breakdown induced by ingestion into the rumen is rapid compared with senescence processes. Measurements of ion leakage and release rates of protein and carbohydrates from leaf material suggest there are differential release rates of plant components and their breakdown products based on size. As in the case of LHCPII and chlorophyll, cellular location also appears to influence the rate of breakdown and release. It should be recognised that plant-derived proteinases account for approximately 10% of the activity recovered from rumen fluid and that significant amounts of plant cells are ruptured during the browsing and rumination processes, making protein available for rumen microbes. However, co-location of plant proteinases within cells gives them first access to protein and therefore a chance to contribute disproportionately to protein breakdown. The overall significance of plant proteinases to protein breakdown in the rumen remains to be established. However, the fact that plant proteinases do have a role in the initial stages of protein breakdown offers an opportunity for plant breeders interested in improving N availability in forages for ruminants. It may be possible to select forage plants that have slower rates of autolysis in the rumen, and preferably this would be coordinated with release of carbohydrates to improve N capture. The benefits from such plants would be enhanced efficiency of N utilisation in the rumen and therefore improved animal performance, decreased demands on the animal for  $\text{NH}_3$  detoxification and lower impact on the environment through reduced excretion of nitrogenous wastes.

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