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Identification of sulphur-rich proteins which resist rumen degradation and are hydrolysed rapidly by intestinal proteases

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Several proteins with high proportions of S-containing essential amino acids were incubated in sheep rumen fluid *in vitro* and their rate of digestion was examined by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. The S-rich proteins rice prolamin (10 kDa), maize zein (10 kDa) and the 3·2 kDa pumpkin (*Cucurbita maxima* L.) trypsin inhibitor-1 (CMTI-1) were highly resistant to rumen fluid degradation, relative to control proteins of known degradation rate (casein, bovine serum albumin (BSA) and pea (*Pisum sativum*) albumin-1 (PA1)). Comparison of PA1 and a recombinant N-terminal epitope-tagged PA1 indicated that addition of the epitope caused a slight increase in resistance to rumen degradation. The proteins were also incubated with a mixture of trypsin (*EC* 3.4.21.4) and chymotrypsin (*EC* 3.4.21.1). PA1, BSA and casein were hydrolysed less rapidly than rice prolamin, maize zein and CMTI-1. Digestion by these intestinal proteases appeared to be complete. Thus, the prolamin, zein and CMTI-1 proteins are suitable candidates for expression as foreign proteins in pasture plants to increase throughput and uptake of essential amino acids in sheep.

Sulphur-rich proteins: Rumen proteolysis: Ruminant nutrition

Considerable losses of protein occur (up to 40%) from the rumen of animals grazing on temperate legumes (Ulyatt *et al.* 1988). S-containing and other essential amino acids (such as lysine, histidine and arginine) that are released by proteolytic digestion in the rumen are deaminated and lost to the digestive system via urinary urea excretion or reabsorbed and converted to microbial biomass. In this process S, also, can be lost to the rumen system as H_2S (Broderick *et al.* 1991).

In general the balance of essential amino acids reaching the abomasum and intestines of the ruminant, therefore, can be adversely affected, resulting in detrimental effects on ruminant growth rates and production yields (Reis & Colebrook, 1972; Ørskov & Chen, 1989). Supplementation of dietary methionine by direct abomasal infusion gave increased wool yields in sheep under certain conditions (Reis & Colebrook, 1972), and co-supplementation of methionine with other essential amino acids has resulted in improved lamb growth rates (Barry, 1981) and increases in milk yields in dairy cows (Rulquin & Verite, 1993).

Some dietary proteins are more resistant to rumen degradation than others (Mangan, 1972; Nugent & Mangan, 1981) and this can be assessed by *in vitro* rumen digestion under anaerobic conditions followed by analysis by sodium dodecyl sulphate (SDS)–polyacrylamide-gel electrophoresis (PAGE) (Spencer *et al.* 1988). The S-rich protein pea (*Pisum sativum*) albumin-1 (PA1) is known to be resistant to *in vitro* rumen digestion for

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up to 8 h compared with casein which is digested within 1 h (Spencer *et al.* 1988). The expression of such rumen-protected essential amino acid-rich proteins in transgenic pasture plants may lead to an improved nutrition for grazing ruminant animals.

In the present study the *in vitro* rumen-fluid stability of PA1 is compared with other high-S proteins to try to identify further candidates with still higher S content that meet the criteria of resistance to rumen degradation, as well as sensitivity to intestinal protease digestion. Since immunological detection is essential to determine the levels of foreign protein accumulation in transgenic plants we also investigated whether the presence of an epitope which allows for protein detection (Field *et al.* 1988) would interfere with the structure of PA1 and, hence, its resistance to degradation once in the rumen. The results described here are the first step in a programme designed to optimize the throughput and delivery of essential amino acids for improved ruminant nutrition.

MATERIALS AND METHODS

Preparation of rumen fluid

A 4-year-old male Romney sheep that had been rumen-fistulated at 6 months of age was used for these experiments. The sheep was grazed on a white clover (*Trifolium repens*) and ryegrass (*Lolium perenne*) sward typical of that found in the lower half of the North Island of New Zealand. Rumen fluid was collected in an Ar-flushed container and later strained through muslin and diluted with 4 vol. McDougall's (1948) artificial saliva. Rumen fluid and buffer were maintained at 39° in an anaerobic cabinet (Forma Scientific model 1024; Biolab Scientific, Northcote, Auckland, New Zealand) in a CO_2–H_2 (95:5, v/v) atmosphere.

Isolation of proteins

Bovine serum albumin (BSA) and α- and β-casein were purchased from Sigma Chemical Co., St Louis, MO, USA. PA1 was isolated according to Higgins *et al.* (1986). Pumpkin (*Cucurbita maxima* L.) trypsin (*EC* 3.4.21.4) inhibitor-1 (CMTI-1) was obtained from Dr John Christeller (New Zealand Institute of Horticultural and Crop Research, Palmerston North). Pumpkin seeds were homogenized in 100 mM-Tris–HCl pH 8·0 containing 150 mM-NaCl and 10 mM-$CaCl_2$ (buffer A). The homogenate was brought to pH 2·0 by addition of 1 M-glycylglycine and heated to 80° for 10 min. The supernatant fraction was applied to an anhydrotrypsin-affinity column, prepared according to Ako *et al.* (1972), washed extensively with wash buffer (0·5 M-NaCl, 10 mM-$CaCl_2$, 10 mM-Tris–HCl, pH 7·5) and eluted with 10 mM-$CaCl_2$ in 50 mM-HCl. The resulting protein was stored at −20°. The epitope-tagged PA1 protein (EpiPA1) was overexpressed as a maltose-binding-protein fusion in *Escherichia coli* strain DH5α. An oligonucleotide encoding the nine amino acid epitope HA1 (YPYDVPDYA; Field *et al.* 1988), a peptide linker (GGP) and a Kozac's translation consensus start codon were synthesized on an ABI model 391-A DNA synthesizer (Applied Biosystems, Burwood, Victoria, Australia). The sequence of the oligonucleotide was 5′-GCGAAGCTTGAATTCACCATGTATCCATACGATTGT-TCCAGATTATGCTGGTGGTCCAGCAAGCTGCAATGGG-3′. This seventy-two-base oligonucleotide was used in conjunction with a 3′ PA1 primer (complementary to base pairs 685–700; Higgins *et al.* 1986) to produce by polymerase chain reaction amplification a double-stranded DNA fragment encoding an epitope derivative form of the PA1 protein. This was then cloned into the maltose-binding-protein fusion vector pMAL-C1 (New England Biolabs, Beverly, MA, USA). Overexpressing strains of *E. coli* were lysed by sonication and the fusion protein was isolated by amylose-resin-affinity purification. The EpiPA1 was then cleaved from the fusion protein by treatment with protease factor Xa (Riggs, 1990).

Rice prolamin was partly purified from commercially available rice flour. The flour was defatted with acetone, dried and then homogenized in a mortar and pestle with *n*-propanol (550 ml/l) at 4°. The homogenate was centrifuged and the supernatant fraction containing the extracted prolamin was collected. The propanol was removed under vacuum and the resultant white precipitate was washed with distilled water and stored at 4° (Sugimoto *et al.* 1986).

Maize zein-2 fraction (containing the 14 kDa and 10 kDa zein proteins) was isolated from coarse maize flour by homogenizing in propan-2-ol (550 ml/l) containing *β*-mercaptoethanol (6 ml/l) at 20°. The resulting supernatant fraction containing the zein was placed under vacuum to remove the propan-2-ol. The resultant yellow precipitate was washed in distilled water and stored at 4° (Sodek & Wilson, 1971).

In vitro *rumen fluid digests*

Protein samples (100–500 μg) were prepared as concentrates and added to anaerobically collected and strained rumen fluid diluted 1:4 (v/v) with artificial saliva. The mixture was incubated in an anaerobic cabinet (CO_2–H_2; 95:5, v/v) at 39° and portions removed at appropriate times and frozen at −20°. The samples were then analysed on SDS–PAGE gradient gels using the Protean II gel system (Biorad Laboratories Pty Ltd, Northshore, Auckland, New Zealand). A gradient separating gel (12·5–25%) and stacking gel was prepared essentially as described in Spencer *et al.* (1988). Before electrophoresis, all samples were boiled for 2 min in 1 vol. sample buffer (60 mM-Tris–HCl, pH 8·0, containing (l): SDS 40 ml, *β*-mercaptoethanol 10 ml, glycerol 200 ml, bromophenol blue 0·2 g).

Electrophoresis was carried out for 18 h at 15 mA. The gels were stained in staining solution (containing (l): propan-2-ol 250 ml, acetic acid 100 ml, Coomassie Blue R 2 g) for 2 h, and then destained overnight in ethanol–acetic acid washes (20:7, v/v) to allow resolution of the protein bands.

Trypsin–chymotrypsin digests

Protein samples (20–50 μg) were rapidly mixed with a 1:1 (w/w) mixture of trypsin (type III, 10400 *n*-benzoyl-L-tyrosine ethyl ester (BTEE) units/mg; Sigma) and chymotrypsin (*EC* 3.4.21.1; type VII, 50 *α*-benzoyl-L-arginine ethyl ester (BAEE) units/mg; Sigma) at 50 μg/ml. (One unit of substrate (BTEE or BAEE) used to define trypsin (BTEE)- or chymotrypsin (BAEE)-like activity is an A_{253} of 0·001/min with the substrate at pH 7·6 and 25°.) Serial dilutions of this mixture were made in 50 μl 100 mM-Tris–HCl, pH 8·0, and incubated at 37° for 1 h before 1 vol. sample buffer was added and the samples frozen. Samples were boiled for 2 min, followed by electrophoresis on 12·5–25% SDS–PAGE gradient gels and treated essentially as described previously.

RESULTS

The method of Spencer *et al.* (1988) was used to assess stability in rumen fluid of seven proteins including three controls (BSA, casein and PA1), some seed proteins rich in S amino acids (10 kDa rice prolamin, 10 kDa maize zein, and 3·2 kDa CMTI-1 and EpiPA1 (epitope-tagged PA1 protein). Fig. 1 shows the protein fractions used in our study. Casein and BSA were obtained commercially, whilst PA1 was a methanol-soluble fraction of maturing pea seed and contained predominantly the 6 kDa PA1a fraction (Higgins *et al.* 1986). EpiPA1 was overexpressed in *E. coli* as a maltose-binding-protein fusion. Rice prolamin fraction was extracted with *n*-propanol and consisted of the mainly methionine-rich polypeptides, the 16 kDa and 10 kDa prolamins. CMTI-1 was isolated by acid extraction of pumpkin seeds followed by anhydrotrypsin-affinity chromatography and

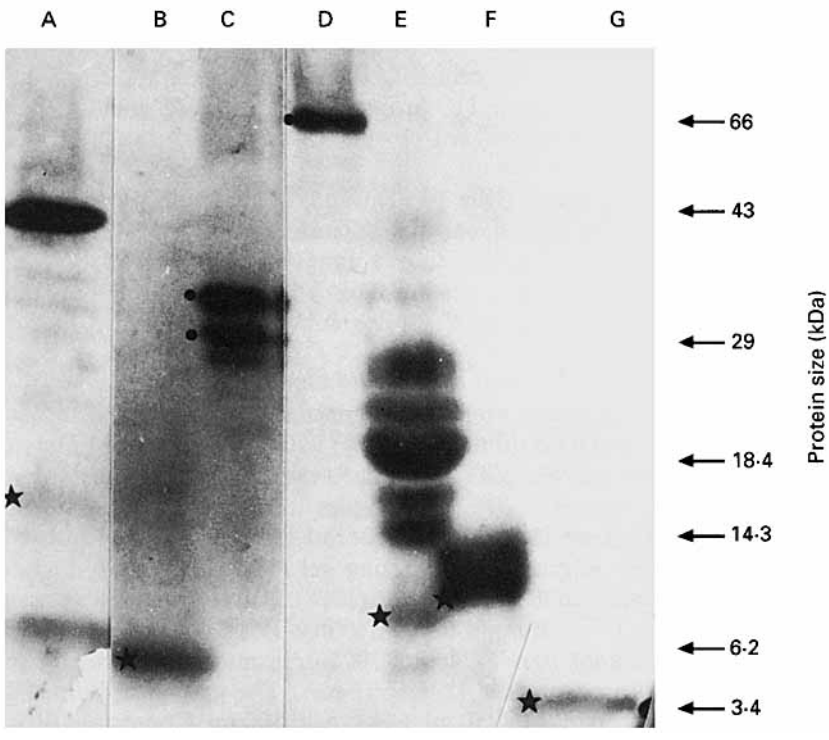

Fig. 1. Protein fractions used for *in vitro* digestion assays. The protein fractions are: Protease XA cleaved pea (*Pisum sativum*) albumin-1 (PA1) fusion protein (lane A (✱), 15 kDa EPIPA). Methanol-soluble developing pea seed extract (mainly 6 kDa PA1a (✱) lane B); α- and β-casein (lane C (●); Sigma); bovine serum albumin (lane D (●); Sigma); maize-flour zein-2 fraction (lane E, 10 kDa zein-2 (✱)); rice-flour prolamin fraction (lane F, 10 kDa rice prolamin (✱)); pumpkin (*cucurbita maxima* L.) trypsin (*EC* 3.4.21.4) inhibitor-1 (lane G (✱)). For details of procedures, see pp. 856–857.

consisted almost entirely of a single polypeptide of approximately 3·2 kDa. The maize zein-2 fraction was isolated from commercially available maize seed and comprised the 10, 15, 16, 22 and 24 kDa polypeptides characteristic of the zein-2 fraction.

Fig. 2 shows the time-course of degradation of each protein in rumen fluid *in vitro*. Casein was degraded very rapidly (within 1 h), BSA was relatively resistant to degradation (disappeared after 12–16 h) and PA1 was characteristically resistant for up to 4–8 h.

EpiPA1 was degraded more slowly than PA1, and was resistant to rumen digestion for up to 8–12 h. The remaining three proteins were all very slowly degraded in the rumen fluid. Rice prolamin and the 3·2 kDa CMTI-1 appeared to be totally resistant to degradation for up to 24 h, whereas the 10 kDa maize zein started to degrade slowly after 16–24 h, but was still highly resistant.

The extent to which each protein was sensitive to intestinal proteolytic digestion was determined using serial dilutions of a 1:1 (w/w) mixture of trypsin and chymotrypsin (Fig. 3). Casein, BSA and PA1 were relatively insensitive to complete digestion by these serine proteases at relatively high concentrations. PA1 was resistant to all but the highest concentrations (50 μg/ml for 1 h). In contrast, the other proteins tested (EpiPA1, 10 kDa rice prolamin, 3·2 kDa CMTI-1 and 10 kDa maize zein) were very sensitive, with CMTI-1 being completely digested by relatively low concentrations of these enzymes (0·046 μg/ml) in 1 h.

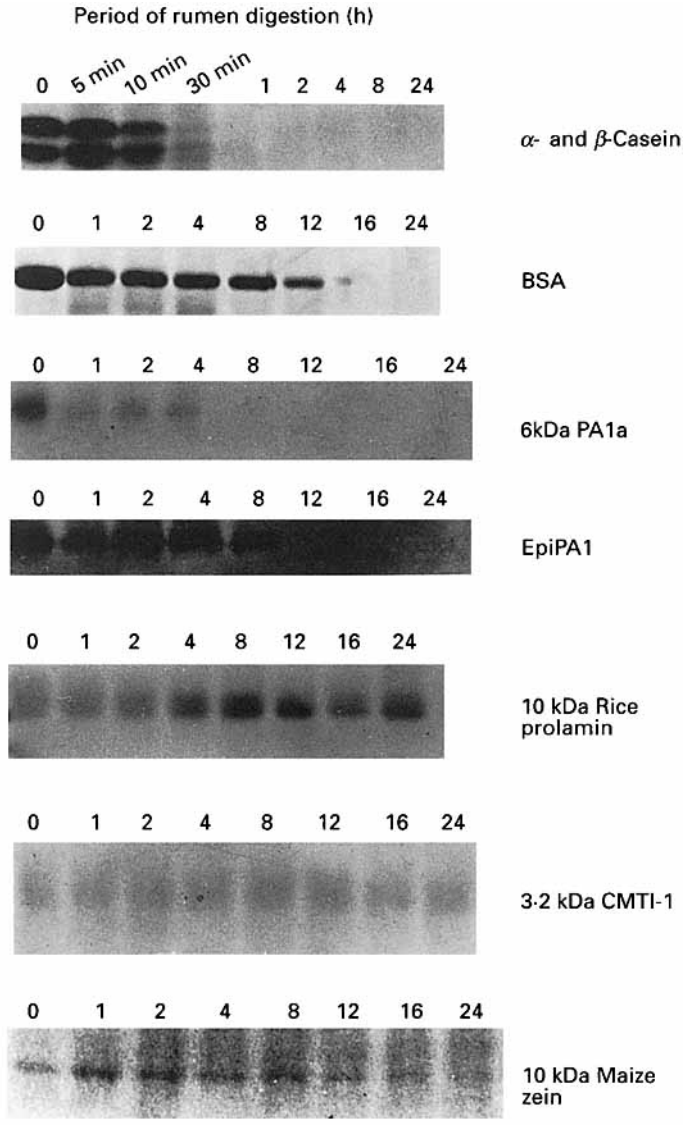

Fig. 2. Digestion of sulphur-rich proteins in rumen fluid *in vitro*. Protein fractions (100–500 μg) were quickly mixed with freshly collected sheep rumen fluid in an anaerobic cabinet at 39°. Samples were taken at the times indicated. Samples were separated electrophoretically on 12·5–25% sodium dodecyl sulphate–polyacrylamide gradient gels. For details of procedures, see pp. 856–857. BSA, bovine serum albumin; PA1a, pea (*Pisum sativum*) albumin-1 fraction; EpiPA1, epitope-tagged PA1; CMTI-1, pumpkin (*Cucurbita maxima* L.) trypsin (*EC* 3.4.21.4) inhibitor-1.

DISCUSSION

Increasing throughput and uptake of essential amino acids from the diet of grazing ruminants may lead to an improvement in animal nutrition. By the overexpression of rumen-protected essential amino acid-rich proteins in transgenic pasture plants it may be possible to achieve this nutritional improvement in ruminants. In the present study we have identified S amino acid-rich proteins that meet the required criteria of rumen stability and intestinal protease sensitivity.

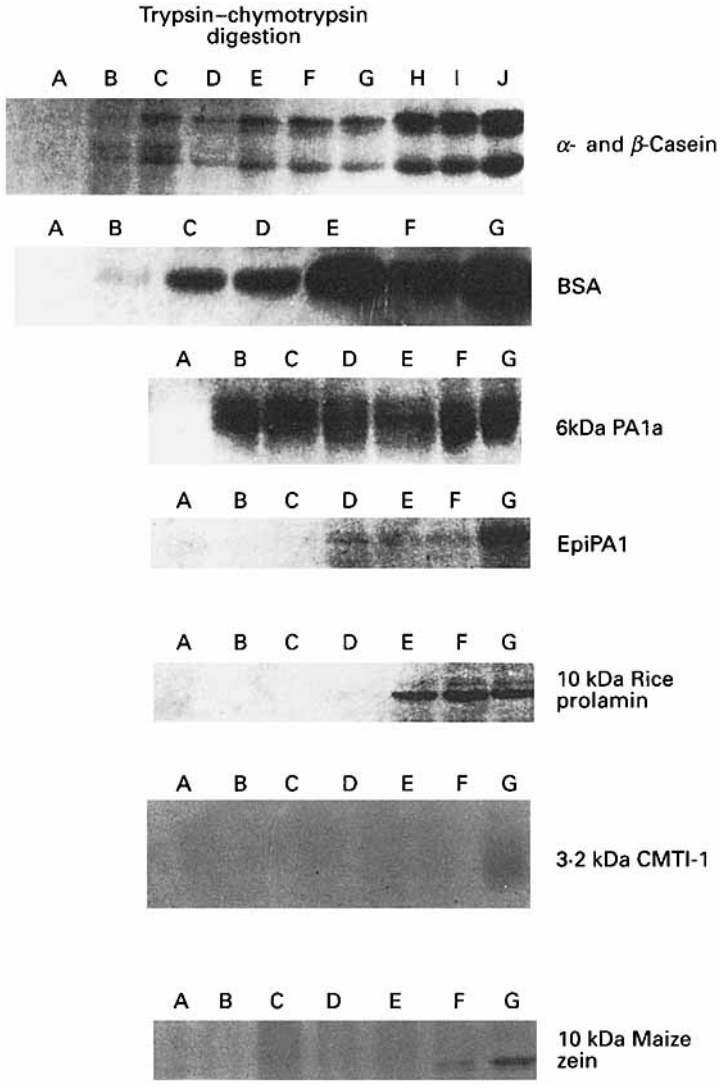

Fig. 3. Concentration dependence of protein degradation in trypsin (*EC* 3.4.21.4)–chymotrypsin (*EC* 3.4.21.1) mixtures. Protein samples (20–50 μg) were incubated in 50 μg trypsin–chymotrypsin mixture in 100 mM-Tris–HCl, pH 8·0, for 1 h at 37° then were added to 50 μl sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (SDS–PAGE)-loading buffer and electrophoresis on 12·5–25% SDS–PAGE gradient gels. For α- and β-casein, enzyme concentrations (μg/ml) were: A 50, B 25, C 12·5 etc. in serial 2-fold dilution to J 0·09 76; for all other proteins (μg/ml): A 50, B 12·5, C 3·125, etc. in serial 4-fold dilution to G 0·012. For details of procedures, see pp. 856–857. BSA, bovine serum albumin; PA1a, pea (*Pisum sativum*) albumin-1 fraction; EpiPA1, epitope-tagged PA1; CMTI-1, pumpkin (*Cucurbita maxima* L.) trypsin-inhibitor-1.

The three new proteins we have identified (10 kDa maize zein, 10 kDa rice prolamin and the 3·2 kDa CMTI-1) are all superior to PA1 for the first three criteria outlined (see Table 1). All three are higher in S amino acids (20–30%), and more resistant to degradation in the rumen than PA1. Also, the values in Fig. 3 suggest that they will break down even more rapidly and completely in the intestines of ruminants than PA1 which is relatively more resistant to chymotrypsin and trypsin digestion *in vitro*. This insensitivity of PA1 to chymotrypsin and trypsin digestion may slow its uptake in the sheep intestine. In two cases

Table 1. *Comparison of some features of sulphur-rich protein candidates for genetic engineering of forage plants for nutritional improvement*

Protein	Size (kDa)	S content (mg/g)	Rumen digestion period (h)	Sensitivity to intestinal protease (μg/ml per h)
PA1a*	6	7·5	8	6
EpiPA1	15	10	12	3
Rice prolamin	10	30	> 24	0·75
CMTI-1	3·2	24	> 24	0·046
Maize zein-10	10	27	24	0·19

PA1, pea (*Pisum sativum*) albumin-1 proprotein, EpiPA1, epitope-tagged PA1; CMTI-1, pumpkin (*Cucurbita maxima* L.) trypsin (*EC* 3.4.21.4)-inhibitor-1.
* PA1 is cleaved to form PA1a (6 kDa) and PA1b (4 kDa).

(10 kDa zein and 10 kDa prolamin) the protein is known to be antigenic (Ludevid *et al.* 1985; Masumura *et al.* 1989). However, epitope tagging allows facile detection of foreign proteins in transgenic plants and from the evidence using EpiPA1 (Fig. 2) this particular epitope does not appear to interfere with the property of resistance to rumen degradation.

Although the very complex nature of rumen microflora would suggest many influences on the degradation of proteins within the rumen, the early assumptions that the solubility of proteins in rumen fluid or buffer was an index of its degradability no longer holds true (McDonald & Hall, 1957; Nugent & Mangan, 1981). Mangan (1972) showed that whereas soluble casein was extremely sensitive to degradation in the rumen, other soluble proteins such as serum albumin had considerable resistance to degradation. More recently, by demonstrating that soluble and insoluble proteins of soya-bean meal were hydrolysed at almost identical rates, Mahadevan *et al.* (1980) showed that solubility or insolubility of a protein is not by itself an indication of the protein's resistance or susceptibility to hydrolysis by rumen bacterial proteases.

Our results confirm these findings, showing that solubility in the case of the three proteins we investigated is not correlated with susceptibility to proteolysis via rumen proteases. CMTI-1 is extremely soluble in water, yet it is more resistant to degradation than BSA. The 10 kDa zein and the 10 kDa prolamin are also highly resistant to degradation yet are totally insoluble in aqueous buffer. This difference in degradation rates is more likely to be due to the secondary and tertiary structural characteristics of these proteins, together with the presence of a number of disulphide crosslinkages.

Rates of degradation of four proteins (casein, α-lactoalbumin, BSA and γ-globulin) were shown to be in proportion to the disulphide content of each protein; the greater the number of disulphide bonds, the higher the resistance to degradation (Broderick *et al.* 1991). Treatment with performic acid resulting in disulphide cleavage increased degradation rates up to thirty times the former rate.

Casein which has essentially a linear secondary and tertiary structure and possesses a single cysteine amino acid residue (and hence no disulphide bonds) is very sensitive to degradation (Mangan, 1972). However, BSA has a complex tertiary structure with 6% cysteine, disulphide bonds and possesses a greater resistance to degradation. Degradation appears to accelerate with time, reflecting increasing proteolysis as the disruption in structure proceeds (Broderick & Craig, 1989).

Mahadevan *et al.* (1980) showed that treating the resistant proteins (regardless of solubility characteristics) with denaturing β-mercaptoethanol in 8 M-urea or oxidation with performic acid resulted in these proteins becoming susceptible to hydrolysis. Since these

substances directly effect crosslinkage one can assume removal of cysteine-S bonds resulted in the sensitivity to proteases. In contrast, the addition of artificial crosslinks via chemical treatment resulted in slower degradation of albumins compared with the untreated protein (Mahadevan *et al.* 1980). The treatment of casein with formaldehyde, resulting in chemical bonds, also caused insolubility and resistance to rumen proteases, with its passage through the sheep rumen enhanced, resulting in an increase in wool growth (Broderick *et al.* 1991).

Our findings tend to support the hypothesis that the degree of cross-linking by cysteine disulphide bonds has a direct correlation with the degree of resistance of a protein to proteolysis within the rumen environment. All three new proteins examined have a high percentage of cysteine residues and disulphide bonds with a corresponding high resistance to degradation by rumen proteases.

The genes encoding CMTI-1, the 10 kDa zein and 10 kDa prolamin are presently being utilized for incorporation into pasture plants for analysis of expression and accumulation in leaf tissue. It remains to be seen whether the three genes encoding these proteins will be expressed in pasture plant leaf tissue and to what level they accumulate, or can be engineered to do so, by the manipulation of subcellular target such as the incorporation of the endoplasmic reticulum retention signal amino acid sequence KDEL (Wandelt *et al.* 1992).

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