Engineering improved forage degradation characteristics

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Summary

Cell walls of forage plants are of major importance in the nutrition of ruminants, providing the major source of energy-yielding carbohydrate and controlling the release of nitrogen-rich cell contents. The spatial (3–D) distribution of materials in cell walls, the distribution of lignin, the structural integrity of the cells, the amount of cell wall surface intrinsic to a specific cell type and the area of surface exposed and actually available for attack are key elements affecting rate of dry matter degradation in the rumen. Total surface area can be measured and the surface area potentially available to microbial enzymes estimated. Information on these and other chemical and anatomical factors can be incorporated into 'rules' describing the behaviour of a 3-D cellular automaton model describing the degradation process. Used in combination with 3-D confocal microscopy, to provide 'real' images of forage particles, discrete mathematical modelling allows the consequences of introducing specific changes to forage anatomy or chemistry to be explored. With this approach, desirable features of a forage can be engineered by design rather than selected by chance.

Introduction

In systems of ruminant production in which pasture is plentiful (>2 tonnes dry matter ha^{-1}) and sources of additional rumen–degradable nitrogen relatively cheap, there may be little incentive to more closely match the nitrogen and energy needs of the animal to its supply. However this system of production is biologically inefficient and environmentally unfriendly. The producer encounters problems when stocking density is high and where nitrogen and phosphorus excretion is recognised as an environmental hazard likely to attract a punitive response. This is increasingly the case in large areas of Europe and northern America and, for this reason, Tamminga (1992) has recommended that herbivore diets should not contain greater than 30g N kg^{-1} dry matter intake.

Nitrogen losses from the rumen can be reduced by more accurately balancing rurnen-degradable protein and rumen-degradable carbohydrate (Tamminga et *al.* 1990; Sinclair et *al.* 1993). While this can be achieved with good feeding management in the feedlots of northern America, the same balance is far more difficult to maintain in animals at pasture. The high nitrogen content of young, leafy forages presents particular difficulties because the asynchrony of nitrogen and fermentable carbohydrate release results in a sizeable nitrogen loss as NH, absorbed from the rumen (Van Vuuren et al. 1990). Options available to the producer include reducing nitrogen intake by diluting young forage with a conserved forage of lower nitrogen content and/or to incorporating a supplement which is a high soluble carbohydrate to increase the energy available for microbial fermentation and protein synthesis. In intensive systems of production, feeding all or part of the diet totally mixed is seen as appropriate only for dairy cattle where animals can be individually fed. For pasture-based sheep and beef production, concentrate supplied separately, usually in the form of cereal grains or maize silage, is the preferred solution. While exact synchronisation is probably not necessary (or even achievable) because of the various fluxes and delays in supply imposed by the host animal (Sauvant and Milligan, 1995), once–a–day feeding of concentrate cannot hope to provide a continuous balance with a day-long intake of forage. More frequent provision of concentrates to animals at pasture might be beneficial, but the higher labour requirement makes it economically unattractive. An idealised solution for a grazing animal would be a single or mixed species sward able, at all stages of growth, to provide rumen degradable nitrogen and carbohydrate in a ratio which allows good production but minimises nitrogen loss.

The soluble nitrogen components released from plant cells on mastication/rumination consist primarily of the numerous proteins involved in cell activities including photosynthesis. Manipulation of such proteins to alter their amount or solubility is theoretically possible but inevitably would have unpredictable consequences for cellular function. A more practical target for improving forage quality is the cell wall fraction, since it is the integrity of the cell wall that controls the release of cell contents and the cell wall itself, which contributes the bulk of the carbohydrate, supply to the rumen microflora.

Any attempt to balance the supply of degradable protein and carbohydrate to the rumen ecosystem assumes an ability to predict release rates from data provided by feed evaluation. Predicting the rate at which cells are disrupted and the cell wall fraction is degraded in the rumen presents particular problems. Analytical methods have helped in predicting both intake and degradation characteristics but none have proved wholly satisfactory. One reason is that these methods were generally developed pragmatically and any underlying biological interpretation sought subsequently. Of the various analytical methods, neutral detergent fibre (NDF) has proved one of the more robust, probably because it is the one most readily related to a biological entity (the plant cell wall fraction), at least in members of the Poaceae (Gramineae). Thus there is a broad negative relationship between NDF as a fraction of dry matter and the degradation rate of potentially digestible NDF (Sauvant et *al.* 1995) and between NDF content and intake (Mertens, 1992). However, the amount of cell wall present is a poor index of other factors important to the digestive process of herbivores such as salivation, pH control in the rumen or particle comminution. Researchers studying these aspects of ruminal metabolism have recognised that forages have physical properties which may be used to refine compositional data or even replace it in importance. In an attempt to integrate a physical concept into feed evaluation, a measure of 'diet fibrosity' was proposed (Sudweeks ***et al.*** 198 1) and shown to relate to VFA production (Beauchemin, 199 1), nutrient utilisation (Grant ***et al.*** 1990) and the rate of dry matter intake (Sauvant *et al.* 1995). Unfortunately fibrosity, measured as time spent eating and ruminating per kg^{-1} dry matter intake, is not a readily measured parameter. The energy input required to grind a feed to a known particle size proposed earlier (Paul and Mika, 198 1) is perhaps an easier measure, although used in isolation it is as unidimensional in value as a single chemical analysis. The 'roughage value unit' proposed by Mertens (1992) and defined as the dry matter NDF content in particles <1.2 mm diameter also acknowledges the physical attributes of forages and is more readily determined than the fibrosity index.

Although there is implicit acknowledgement in such measures as fibrosity of the importance of the three-dimensional nature of feed particles, the spatial distribution of the cell wall and other nutrient fractions has been largely ignored in feed evaluation. This review seeks to establish the importance of spatial distribution in any conceptual model of the degradation of cell walls and to introduce some of the methodology which could be used to provide the quantitative data required to underpin any programme designed to manipulate forage quality.

Spatial distribution of cell wall material

Plant material is a heterogeneous mixture of different cell types, each with different characteristics. The chemical composition, and anatomical features of these cell types are the result of many physiological processes occurring within the growing plant which control the partitioning of dry matter between different tissues and organs and the differentiation of cells within tissues. The amount of dry matter present in different tissues and the chemical nature of the cell walls are determined by a wide variety of genetic and environmental factors in addition to the endogenous responses of the plant to pest or pathogen attack. Environmental factors such as light intensity may be important in determining forage degradability by their direct effects on anatomy and histology (Deinum et ***al.*** 1996).

The pioneering work of, in particular, Akin in the USA (Akin, 1979; 1989) and Wilson in Australia (Wilson, 1993; Minson and Wilson, 1994; Wilson and Kennedy, 1996), has led to the recognition of the importance of anatomy in determining the nutritive value of forages to the animal. However, there has been a gap between a the recognition of the importance of cell anatomy and the ability to apply knowledge of anatomy to the breeding of new forage varieties. This is largely because of the lack of tools for quantitative assessment of anatomy, which could be applied to the large number of samples typically, involved in any breeding programme. Analysis of images from microscope sections has revolutionised approaches to the measurement of anatomical features, although the time involved in the quantification of anatomy still remains a problem. Further development of the automated recognition of cell types and tissues will substantially reduce operator time and enable image analysis methods to be used as routine selection criteria (Travis ***et al.*** 1996a).

The value of being able to describe differences in anatomy was amply demonstrated when chemical and anatomical factors contributing to variation in cereal straw degradation were compared using step-wise multiple regression. Chemical composition alone was not the best predictor of degradability and was little better than anatomy alone. The greatest part of the variance was explained when both chemical and anatomical data was include in a five-variable model which included three anatomical terms (thickness of sclerenchyma and epidermis cell walls and density of epidermis) (Travis ***et al.*** 1996b). There is considerable overlap in the information provided by anatomical and chemical measurements. Schlerenchyma, because of the thickness of its walls, contributes most to the cell wall content of forage. It is also one of the most lignified tissues in forages and so it is not surprising that variation in the amount of sclerenchyma present has an impact on degradability or that anatomical features relating to sclerenchyrna give a better predition of degradability than lignin content alone.

Variations in the distribution of cell wall material can influence the degradative process at a number of levels. A shift in distribution towards large, thin-walled cells provides a higher surface area to weight ratio, while an increase in the proportion of thick-walled cells with a small lumen, such as sclerenchyma, has the opposite effect (Figure 1) Resistance to fragmentation can also vary, as is shown by the quite considerable differences in the energy needed to grind different forages to a constant particle size (Paul and Mika, 1981). This has consequences for mastication and the initial release of cell contents and subsequently for particle size reduction, which is a product of both chewing by the animal and the action of its rumen microflora thinning individual cell walls.

Many anatomical features can be extracted from two-dimensional images. Other components of the digestive process, however, are a product of the three–dimensional nature of ingested plant material. The actual fate of a cell wall is often determined not by its intrinsic properties, but by its location within a forage particle and its accessibility to rumen microorganisms. Serial sectioning of plant fragments can provide three-dimensional information but is laborious and time–consuming. An alternative approach is to measure cell integrity and cell wall degradation in situ on intact tissue using confocal microscopy. The integrity and degradability characteristics of specific cell types, or of individual cells, can be measured directly in the presence of neighbouring cells using this approach (Travis *et al.* 1997). Only cells in which the wall is physically disrupted have their luminal surface exposed to microbial colonisation and attack in the rumen.

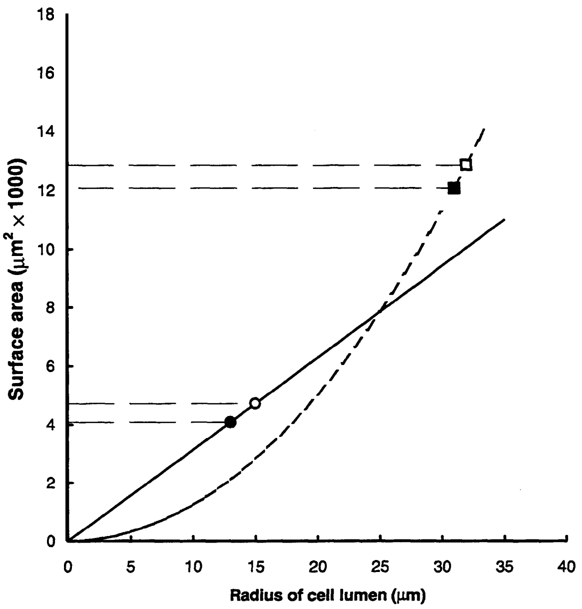


Figure 1 Theoretical calculations of lumen surface area for sclerenchyma and parenchyma cells (adapted from Stewart, 1985). The surface area of sclerenchyma lumens was calculated using a cylindrical model: Area = $2\pi rl$, where r = lumen radius and l = lumen length (50 μm). The surface area of parenchyma lumens was calculated using a spherical model: Area = $4\pi r^2$, where r = lumen radius. Values indicated for sclerenchyma (●) and parenchyma (■) are based on observations of cell dimensions in wheat and barley internodes (data from Travis *et al.* 1996b) before (solid symbols) and after (open symbols) degradation.

Available surface as a factor in degradation

Measurement of the area associated with individual plant cell walls is essentially fractal in nature and defined by the nature of the probe used (Yano, 1996). The surface available to a low molecular weight compound such as sodium hydroxide is substantially greater than that available to a much larger enzyme or to an even larger microorganism. A variety of physical and chemical methods are available for the study of porous materials and their surface, but only gas adsorption can be satisfactorily applied to plant material (Chesson *et al.* 1997). Even gas adsorption, using nitrogen gas as the probe molecule, is required to operate near the limits of detection simply because plant cell walls have very limited porosity and the amount of gas absorbed per unit of cell wall is low compared to that for the materials for which the method was developed. Information about surface features is derived from the amount of gas that physically absorbs as a monolayer onto the surface of the cell wall sample measured at various partial pressures. From this volume measurement, values for total surface area, pore sizes and pore size distribution can be obtained.

Surface area and porosity of forage cell walls

Values for total surface vary depending on the forage or forage tissue examined and the method of sample preparation. The primary, thin-walled mesophyll cells isolated from ryegrass (*Lolium perenne*) leaf had a relatively high total surface area of 56.6 $m^2 g^{-1}$ compared to that of the cell walls of timothy grass (*Phleum pratense*) gave a value of 5.0 $m^2 g^{-1}$ — more typical of the values obtained from preparations of whole forage plants. The large difference reflects both the thinness of the mesophyll cell walls, and hence a high surface area to weight ratio, and their loose packing in the leaf which leaves both luminal and outer surfaces available. Most other cells found in forges have much thicker walls and are found in tissues in a form which approximates to hexagonal close packing in which cells

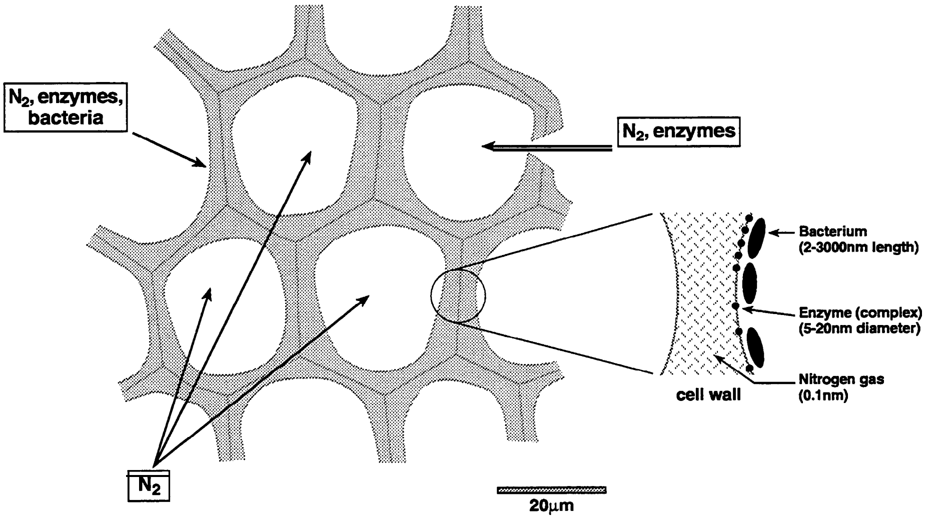


Figure 2 Diagram showing the differential access of nitrogen gas, enzymes and microorganisms to the lumen of entire and broken cells and (expanded insert) to an individual cell wall.

walls abut, leaving only a luminal surface available to a probe (Figure 2).

Most surfaces available to very small probes, such as nitrogen, are associated with pores defined by the spacing within and between the polymers-which make up the cell wall. The pore structure of cell walls from the vegetative parts of vascular plants appears very similar, reflecting the essential similarity in the architecture of their cell walls. Cell walls of forage plants have a regime of pores with discrete sizes ranging between 0.5 to 5 nm radius with the distribution skewed toward the lower pore sizes (Figure 3). These values are in good agreement with the mean pore size values (~ 2.5 nm radius) obtained in hydrated systems by the traditional method based on solute exclusion (Neuman and Walker, 1992; Floumoy et ***al.*** 1993). Cell walls in which there is little polymer mobility tend to have well-defined pore regimes, whilst walls in which there is a greater degree of disorder and in which polymer spacing is more variable, produce a more diffuse pattern. This is particularly true of primary vegetative cells such as leaf mesophyll (Figure 3) or the walls of storage cells which only have a minor structural function.

In all vegetative tissues, pores with a radius of 1.5 – 3 nm predominate. These are below the size which would allow free penetration of the wall by degrading enzymes with molecular weights of >20 kDa and a Stokes radius of 2.5 nm. The cell wall degrading enzymes produced by rumen microorganisms have molecular weights well in excess of 20 kDa and this has a number of important implications for the metabolism of the fibre fraction by herbivores. In the absence of any diffusion of enzyme into the wall there can be no selective degradation of polymers; attack by microorganisms must be limited to available surfaces and must involve the erosion of the wall as a whole. As a result neither the pore structure nor chemistry of the wall changes greatly during degradation (Chesson, 1993). This can be clearly seen in Figure 4 where the pore regimes of cell walls of the timothy of grass are compared before and after 72 h degradation. Despite a 70% loss of dry matter after 72 h the two pore regimes are essentially the same.

Total, potentially available, and actually available surface areas.

As those surfaces with pores whose radius is below the Stokes radius of the attacking enzyme are not accessible, the total surface area potentially available

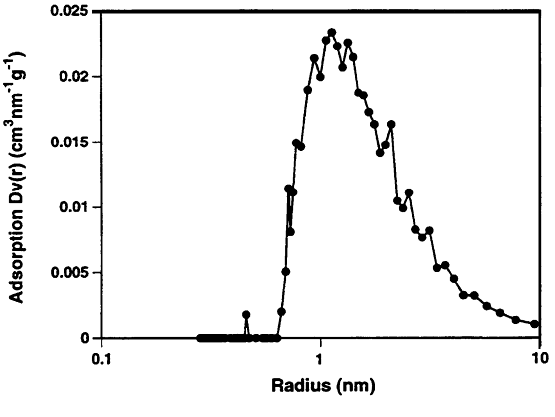


Figure 3 Pore size distribution in mesophyll cell walls isolated from ryegrass leaves. Pore radii are shown as a function of Dv(r) the volume-based pore size distribution, which provides a better estimate of relative pore number.

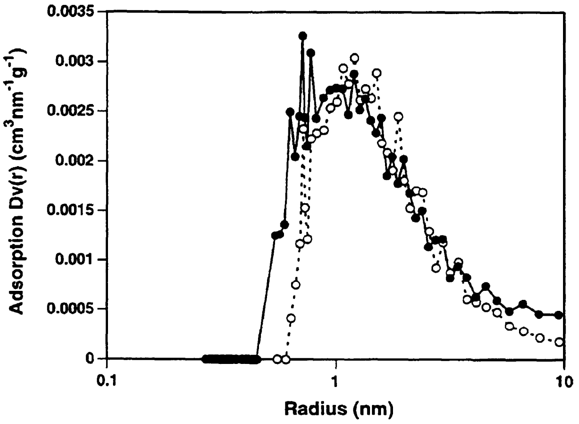


Figure 4 Pore size distribution in timothy cell walls shown as a function of Dv(r) before (●) and after (O) 72 degration.

to a protein is substantially less than that available to nitrogen gas (Figure 2). An estimate of surface area theoretically available to a protein can be made by ignoring the area associated with pores of less than a selected radius. Discounting the surface area associated with pores of <3 nm radius, for example, generally reduces the potentially available surface by 5040%. In the case of the timothy preparation described above, the surface area falls to 2.5 m^2 g^{-1}; half that of the total area. Where cellular integrity has been lost, the potentially available surface area calculated from gas adsorption data will approximate to the surface actually accessible to a protein. Where, however, cells remain entire, access to luminal surfaces is denied to an enzyme and its microbial delivery system but not to a gaseous probe, and the two values will differ. Although total surface area is readily measured and potentially available surface estimated, no routine method exists to determine the actual available surface although this value is the single most important determinant of degradation rate.

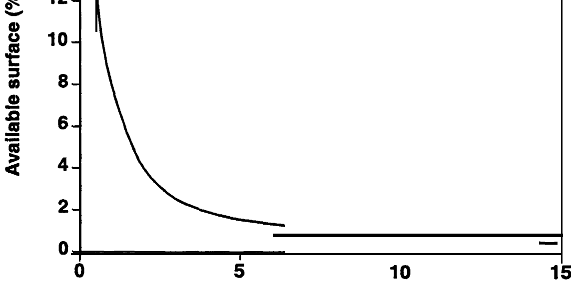


Figure 5 Effect of particle size on the percentage of total area available for microbial attack.

The potentially available surface area is an intrinsic property of the plant cell wall which defines the maximum surface area available to microbial attack and therefore the maximum possible initial rate of attack. If the cell wall sample is prepared in such a way that all cells are disrupted, the actual and potential values for surface area are the same. Under these conditions it can be shown that this initial rate is a constant independent of cell type and age (degree of lignification) (Lopez et *al.* 1993).

Rumination and the creation of surface

Rumination is commonly thought of in terms of particle size reduction and as encouraging the release of cell contents such as soluble protein. Although particle size reduction and increased particle density are important factors in determining the clearance rate of poorly degraded material from the rumen (Lechner–Doll et *al.* 1995), it can be argued that the single most important role for rumination is the creation of available surface. This cannot be achieved by microbial attack alone. Microbial attack results in the gradual erosion of the inner (luminal) surface of cell walls and the thinning of individual walls but the shape and size of the ingested feed particle is otherwise retained. Fine grinding of any feedstuff would, in theory, allow the maximum initial rate of degradation to be achieved. However a simple calculation shows that the actual surface approaches the potential maximum value only when particle sizes fall well below 1 mm diameter, at which size outflow from the rumen would severely compromise the extent of ruminal degradation (Figure 5). Simple chopping of a feed, as can be seen from Figure 5, has little impact on available surface. Only the shear forces generated by some processing technologies and by the animal itself during rumination are able to break open cells, releasing cell contents and exposing new surfaces, without a simultaneous large reduction in overall particle size.

Effect of lignification

Denial of surface

Lignin can be considered to be an essentially inert polymer under the anaerobic conditions found in the rumen and, where it occurs in cell walls, it serves to deny surface area to attacking organisms and their enzymes. This can operate at the level of cell wall degrading bacteria, most of which become closely associated with the cut edges of forage particles and primary walled material, tend to be found in reduced numbers adhering to the more heavily lignified cell types such as xylem vessels (Latham et *al.* 1978). Colonisation of lignified tissues can be stimulated by various treatments known to solubilise a significant part of the lignin present (Kerley et *al.* 1985). However, it is still is not known whether the limited colonisation of the more lignified tissues is the product of a reduced ability to adhere or simply a lack of accessible nutrients.

Although the presence of lignin effectively reduces the surface area of cell walls available to attac, this is not a static position. Rumen microorganisms have evolved a strategy for the removal of lignin which does not dependent on lignin degradation. Dissolution of the polysaccharide to which lignin is covalently bonded will release lignin into the rumen liquor as soluble lignin–carbohydrate complexes (Conchie *et al.* 1988). This undermining and release is dependent on lignin existing within the wall in small discrete units (Chesson, 1993). This condition is met only in plants with a relatively low lignin content such as the grasses and legumes. As the degree of lignification increases, lignin molecules tend to coalesce and cannot so readily be removed by the hydrolysis of underlying polysaccharide. In practice, the practical limit for any single feed material appears to be in the region of 10–12% lignin. Above this value insufficient carbohydrate is accessible to meet the animal's requirement for maintenance.

If the process of lignin removal was wholly effective then lignin would not present any barriers to degradation, at least not in most forages. The rate of degradation would be linear or increase slightly with time as each cell lumen becomes larger and the thickness of the cell wall diminishes, increasing the surface to mass ratio. In practice, forage degradation curves are sigmoidal (Groot *et al.* 1996) and degradation rates, after an initial increase associated with full colonisation of available surfaces, decrease with time. This is due primarily to the accumulation of lignin at the surface steadily converting a carbohydrate-rich surface to one that is more phenolic based. Removal of lignin by chemical or biological means will reduce the rate of conversion from carbohydrate to phenolic surface and will increase both the rate of the later part of the degradation curve and the extent of degradation.

Removal of lignin from plant residues

Many hydrothermal, alkaline and oxidative methods exist for the disruption of lignin in plant cell walls, producing treated residues with improved degradability characteristics (Chesson, 1981, 1993; Kerley *et al.* 1985). Few, however, are used on a commercial scale and then only when local shortages of feed distort the normal feed market or when some form of subsidy is available. Experience to date would suggest that it is unlikely that any chemical treatment with the potential to be developed as a widespread on-farm process would be sufficiently cheap to apply (given the limited scope for added value), sufficiently safe to use and free from environmental problems. The alternative biological treatments have also proved to be of limited value (Reid, 1989; Zadrazil *et al.* 1995). Degradation of lignin is thermodynamically unfavourable and, although some bacteria able to grow at the expense of lignin methoxyl groups are known, no fungus has been isolated that is able to use lignin as a sole carbon source (Zadrazil *et al.* 1995). Instead, cell-wall carbohydrate is used to provide the energy for lignin dissolution and available carbohydrate is always used in preference to carbohydrate associated with lignin. As a result, plant residues composted with lignolytic fungi generally show no or very limited improvement in degradability and then only at the cost of a substantial loss of dry matter. Strains of white and soft-rot fungi have been isolated which use less carbohydrate per unit of lignin oxidised than the average, which may point to a potential for reducing dry matter losses associated with composting systems. As more is learnt about the biochemistry and genetics of lignolytic systems, recombinant technology may be able to further refine this capability (Gold and Alic, 1993). However, delignification will always be energetically demanding and organisms modified to make less use of carbohydrate will find it difficult to compete with naturally occurring, faster growing strains. Sterilisation/pasteurisation of the substrate can overcome this problem, but at some cost. Coupling biological delignification with mushroom production is also possible but, because of the relative value of the end products, the process has to be optimised for fruiting body production and not for improved degradability of the spent compost.

Genetic manipulation of lignin structure and content

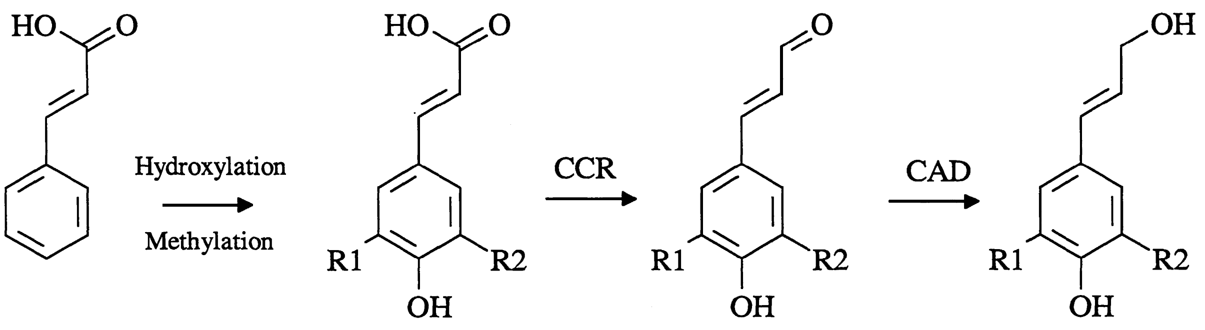
While chemical or biological treatments may produce short-term gains, genetic manipulation of lignin structure or concentration is permanent and has become a topic of some interest to seed producers, particularly to those selling hybrid crops such as maize. The section of the phenylpropanoid pathway leading to the production of the lignin precursors has been mapped and the relevant enzymes identified, enabling targets for manipulation to be chosen. Attempts have been made to modify virtually all stages in the formation of lignin with varying degrees of success. In general, down regulation of enzymes with low specificity which occur in multiple forms such as cytochrome P450 or peroxidase (Sherf *et al.* 1993) or those enzymes occurring very early in biosynthetic pathways such as phenylalanine ammonia lyase (PAL) has proved unsuccessful (Elkind *et al.* 1990). Either the down-regulated activity is replaced by a similar gene product or the early changes induced have multiple and usually deleterious consequences for the plant. As a result interest has focused on those activities specifically involved in the formation of the lignin precursors.

Entry to the phenylpropanoid pathway is via cinnamic acid which is hydroxylated at the 4 position to give 4–hydroxycinnamic acid, more commonly referred to a *p*–coumaric acid (Figure 6). Further hydoxylation reactions at positions 3 and 5, followed by rapid methylation under the control of O-methyl transferases (OMT) produce the mono- and dimethoxylated derivatives, 4–hydroxy–3–methoxycinnamic acid and 3,5–dimethoxy–4–hydroxycinnamic acids (ferulic and sinapic acids respectively). Each of these 4–hydroxycinnamic acids can be reduced through the aldehyde to the primary alcohol. It is these alcohols which, in the majority of plants, are exported from the cell into the cell wall where, in the presence of peroxidase, they polymerise to form the lignins.

In the Poaceae (Gramineae) and related families, the 4–hydroxcinnamic acids, *p*–coumarate and ferulate, are additionally incorporated into lignin structure. The two acids serve different purposes and little interchange between functions is observed. Ferulic acid appears to be ester linked to polysaccharide (arabinoxylan), presumably by the action of a feruloyl transferase, before the polysaccharide is exported to the wall (Myton and Fry, 1994). Once in the wall, ferulate units may couple to cross-link polysaccharide chains (Ralph *et al.* 1994a: Grabber *et al.* 1995). This is a characteristic of the primary wall. Alternatively, they may serve as a nucleation site for lignin formation (Lam *et al.* 1992: 1994: Ralph *et al.* 1995). In contrast, *p*–coumaric acid is laid down in parallel with lignin occurring as terminal units ester linked to, possibly, syringyl units (Chabbert *et al.* 1994: Ralph *et al.* 1994b)

The nature of the lignin formed is primarily determined by the capacity of the precursor molecule to reduce activated peroxidase by the donation of an electron, resulting in the formation of a phenoxyl radical, by its subsequent capacity to delocalise the remaining unpaired electron. The degree of ring methoxylation profoundly influences this process by determining the nature of the inter-unit bonding that occurs between radical species (Russell *et al.* 1996).

Inhibiting the introduction of methoxyl groups or the reduction of the acid group on C9 would be expected to influence the nature but not necessarily the amount of lignin in a plant. There is evidence from chemically–induced and naturally-occurring brown midrib (*bm*) mutants of maize, sorghum and pearl millet that this expectation is true and that such changes may benefit degradability (Cherney *et al.* 1991). The activities of two of the enzymes active in lignin precursor biogenesis are known to be modified in the *bm* mutants of maize and sorghum. The most widely studied has been the OMT–deficient bm_3 mutant of maize, in which the second methylation step leading to sinapic acid is disrupted (Lapierre *et al.* 1981). Less studied are mutants deficient in cinnamyl alcohol dehydrogenase (CAD) such as the bm_1 maize (Provan *et al.* 1997) or the bmr, sorghum (Buchholz, 1980) which leads to the increased incorporation of aldehydes into lignin (Provan *et al.* 1997). Although it is possible to model the consequences of these mutations (reduced syringyl units, increased aldehyde), the implications for the degradation process are not fully understood. Although the reduced syringyl content of the OMT mutants appears to have more impact on degradability than the incorporation of aldehyde, any improvement in degradability of these mutants appears to be more the consequence of an incidental reduction in overall lignin content than of the chemical changes. The effect of mutagenesis is highly variable and unpredictable and was, for example, seen in only one of the maize mutants studied by Barière *et al.* (1994). Nonetheless OMT, CAD and cinnamyl CoA reductase (CCR) are the present targets for genetic manipulation and antisense constructs for all three have been made and expressed in various host plants including forages. The degradability of such genetically altered forages has yet to be reported. However, expression of the antisense gene to CAD in tobacco led to reduced enzyme activity, incorporation of aldehyde (Figure 6) into lignin and development of the colour characteristic of the ***bm***



R1 = R2 = H	*p*-coumaric acid	*p*-coumarly aldehyde	*p*-coumaryl alcohol
R1 = H, R2 = OMe	ferulic acid	coniferyl aldehyde	coniferyl alcohol
R1 = R2 = OMe	sinapic acid	sinapyl aldehyde	sinapyl alcohol

Figure 6 The conversion of cinnamic acid to the monolignol precursors of lignin. CCR, cinnamy CoA reductase; CAD, cinnamyl alcohol dehydrogenase.

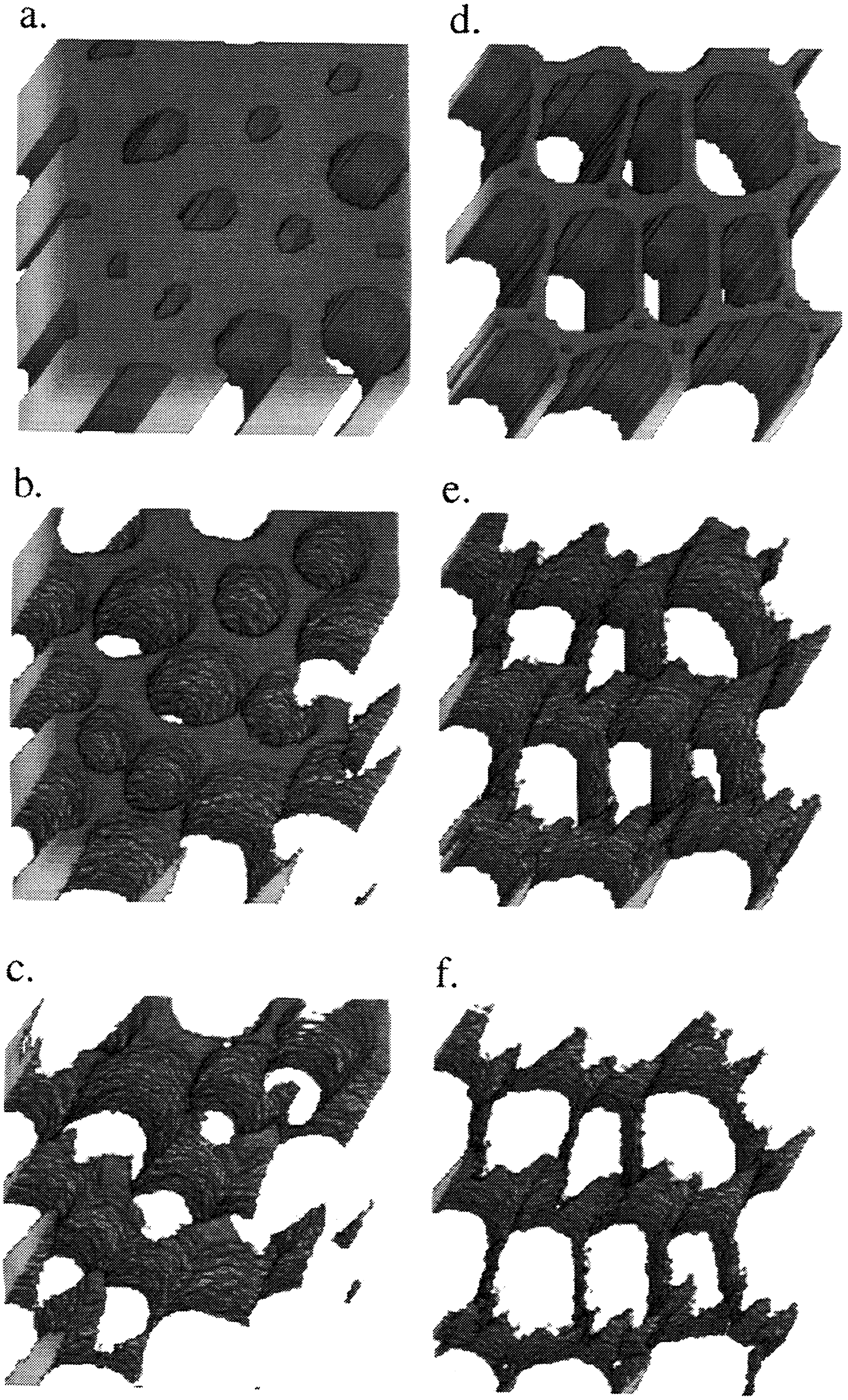


Figure 7 Mathematical simulation of cell wall degradation using a cellular automaton model in which automata change state at each iteration of the model according to rules describing the diffusion of cell-free enzymes into the lumen from the cut surface of a thick (50 μm) section of a recently expanded maize internode (*Zea mays*, cell line W401). Images of sections taken at the top (oldest) part of the internode are shown as 3-D reconstructions of the partly degraded cell walls after 0, 50 and 80 iterations of the model (a, b, c), and from the bottom (youngest) part of the internode after 0, 50 and 80 iterations of the degradation model (d, e, f). The area of cell walls shown in the reconstruction were sampled from within a larger area of tissue and, in this model, diffusion of enzyme from the edges of the tissue was not permitted. Only the cut surface of the section was exposed to attack by cell wall degrading enzymes.

phenotype, but no improvement in degradability (Halpin et *al.* 1994). An increase in in vitro degradability was noted with sense and antisense OMT constructs of tobacco with no apparent reduction in the amount of lignin present (Atanossova et *al.* 1995; Bernard–Vailé et *al.* 1996). However, the extent of improvement was very small (~ 5%) and it is questionable whether this would have been detectable in vivo.

The brown midrib mutants probably do not provide the most appropriate guide to future constructs. Reducing the supply of precursors, inhibiting their coupling by targeting the apoplastic and wall bound glycosidases and oxidases, and reducing the degree of cross-linking to carbohydrate may all prove to be better targets for genetic manipulations intended to improve degradability. Molecular engineering is time--consuming and expensive in terms of staff involvement and the present pragmatic approach to modifying lignin in forage plants is unlikely to be the most cost-effective. Predictive modelling of the degradative process would introduce some refinement to the selection of desirable traits.

Modelling the degradation process

The kinetics of plant cell wall degradation by rumen microorganism in vivo are complex and difficult to model mathematically in a biologically meaningful way. Theories about the degradation process occurring in the rumen have been proposed for many years, these have been based on first-order reaction kinetics and the use of differential equations to construct predictive models of forage degradability (France et *al.* 1990). More recent models seek to describe the rumen degradation process in its entirety (Dijkstra et *al.* 1992). Despite the use of increasingly sophisticated mathematical models, non-linear approaches have been necessary to explain deviations from exponential behaviour (Dhanoa *et al.* 1995). While these models are of considerable help in understanding the overall degradation process, the simplifying assumptions needed to permit the use of continuous functions may obscure critical areas of forage quality that cannot be modelled in a biologically meaningful way using this approach.

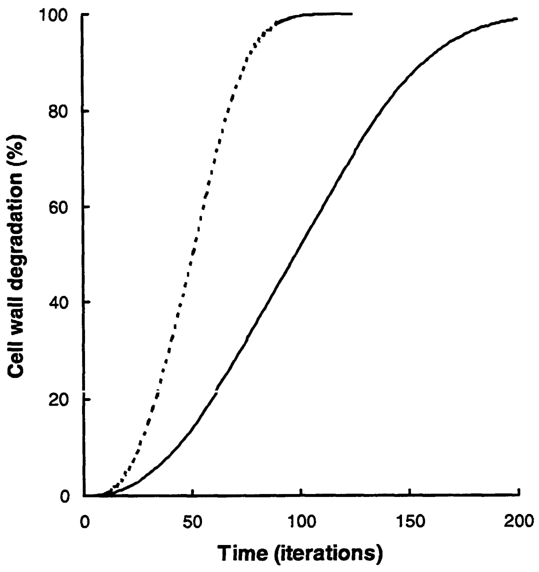


Figure 8 Cell wall degradation curves for the tissue sections shown in Figure 7. The dashed line represents degradation the section from the youngest part of the internode, and the solid line represents degradation of the section from the oldest part of the internode. Degradation is expressed as the proportion of cell volume lost over time and, in this model, the cell walls were assumed to be fully degradable.

An alternative method is to simulate the degradation process using a discrete computational model in which the distribution of cell wall material over time and the changing environment in the cell lumen are taken into account. The spatial distribution of dry matter in cell walls, the distribution of lignin, the structural integrity of the cells, the amount of cell wall surface intrinsic to a specific cell type and the area of exposed surface actually available for attack are key elements in the degradative process. Information on these factors can be incorporated into ‘rules’ describing the behaviour of a 3-D cellular automaton in which each processing unit corresponds to a small cube of cell wall material or part of the cell lumen. This type of model is illustrated in Figure 7 where digitised images of microscope sections from the top (oldest) and bottom (youngest) regions of a newly expanded maize internode (see Scobbie *et al.* 1993) have been ‘degraded’ mathematically according to a set of rules that define the transition of automata from one state to another. The automata change state at each generation (iteration) of the model simulating the loss of cell wall material over time as degradation proceeds. The degradation curves for sclerenchyma cells from the top and bottom of the internode is shown in Figure 8.

An important feature of the cellular automaton model is that detailed information about the predicted changes in surface area during degradation, and the progressive exposure of new cell wall surfaces to attack can readily be obtained from digitised images of sections taken from plant material with known degradability characteristics. In this way, the relative importance of different anatomical features in limiting the rate of degradation can be assessed. The initial automaton model is intended to simulate the degradation of tissue sections by cell-free enzymes in vitro and to provide some understanding of the consequences of selection for different anatomical features.

Anatomical features alone are unlikely to be sufficient to explain the degradation patterns observed in different forages The distribution of lignin also plays a critical role in the development of impermeable barriers to enzyme diffusion as degradation proceeds. A similar automaton could be used to simulate the degradation process at the more detailed level of the surface of an individual cell wall using the discrete model of lignin distribution proposed by Chesson (1993). In this type of model, the consequences of lignin distribution within the cell wall can be investigated in relation to changes in the nature of the surface under attack. Ultimately, a better understanding of the factors limiting the degradation of forage requires a better understanding of the degradation process itself. That, in turn, requires the necessary tools to investigate the three–dimensional process that occurs as forage particles are degraded. A combination of 3-D confocal microscopy, and discrete mathematical modelling allows the exploration of the consequences of introducing specific changes to forage anatomy or chemistry. With this approach, desirable features of a forage can be engineered by design rather than selected by chance.

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