Extended abstract

Fibre degrading enzymes, their origin and diversity

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In the rumen, plant cell wall polysaccharides, which represent the main energy source in forages, are degraded by a community of interacting microorganisms belonging to the three microbial populations : bacteria, fungi and protozoa. Each population contains several different species able to digest part or all of the plant structural carbohydrates. In the case of bacteria, which are considered as the main cellulose degraders in the rumen, the cellulolytic species have been shown to be composed of different subspecies that could be relatively distant phylogenetically from each other. Biochemical studies as well as gene cloning have shown that, at the cellular level, each microorganism produces many different glycosylhydrolases. Thus, the rumen is characterized by a great diversity of enzymes, species and populations able to hydrolyse polysaccharides and this diversity contributes to its extreme efficiency in plant degradation.

The diversity of enzymes produced by the rumen microorganisms is a consequence of the diversity of the polysaccharides of the plant cell walls, composed of many different monosaccharides that are linked by many different glycosidic bonds. Indeed, the plant cell walls consist of cellulose fibrils embedded in a matrix of other polymers, primarily hemicelluloses, pectin and proteins, with varying degrees of lignification. The cellulolytic rumen microorganisms possess an array of enzymes allowing them to cleave nearly all of the bonds found in the cell wall, except those of lignins.

Fibre-degrading microorganisms

Based on the ability to degrade purified and intact forage cellulose, and on enumeration, the main cellulolytic rumen bacterial species are *Fibrobacter succinogenes*, *Ruminococcus albus* and *R. flavefaciens* [1]. Some strains of *Butyrivibrio fibrisolvens* are also cellulolytic, but the major role of this species is considered to be xylan degradation. Other secondary cellulolytic species (found sporadically or in low numbers) include clostridial species [1]. Some species unable to grow on cellulose show hemicellulolytic activity or have the capability in degrading and utilizing cellodextrins, such as *Prevotella ru*

minicola.

The rumen fungi can solubilize a high proportion of plant fragments with high efficiency, and they seem to have the ability to penetrate plant cell walls with their rhizoids, thus gaining access to polysaccharides not available to cellulolytic bacteria [2]. Five genera of fungi have been described, although they seem to be very close phylogenetically [3]. They include *Neocallimastix*, *Caecomyces*, *Piromyces*, *Orpinomyces* and *Anaeromyces*.

The exact contribution of rumen protozoa to cellulose digestion in the rumen is uncertain. More than 10 species of rumen protozoa have been shown to digest cellulose or hemicelluloses [4], but the posibility that this activity is due to intracellular bacteria or bacterial enzymes has not yet been ruled out.

Description of the plant cell wall hydrolyzing systems

Biochemical analysis and gene cloning have led to a description and characterization of the enzymes of the cellulolytic systems of rumen microorganisms. However, the molecular organization of the enzymes into an efficient hydrolyzing system is not fully understood. Biochemical analysis suggested the existence of multienzyme complexes at the surface or in the extracellular culture medium of the main cellulolytic bacterial species [5] Similarly, high molecular weight complexes exhibiting cellulase and hemicellulase activities were found in the rumen fungi *N. frontalis* and *Piromyces* [6].

Cloning of the genes encoding these enzymes into *E. coli* and their sequencing has allowed some of them to be studied at a molecular level. It appears that many of the individual enzymes are modular proteins, comprising one or more catalytic domain(s) linked to ancillary domains. Twenty-one glycosyl-hydrolases genes have been cloned from four strains of *F. succinogenes*, most of them being isolated from the S85 strain.Twelve of the genes code for cellulases, and nine of them have been sequenced and characterized; five belong to family 5 of glycosyl-hydrolases [7], and four belong to family 9. One gene codes for a lichenase (family 16). From the six xylanase genes isolated, two have been sequenced; the first one carries two family 11 catalytic domains and the second a family 10 catalytic domain [8-11]. Basic terminal domains (BTD) were found in some of the enzymes encoded by these genes : endoglucanases EGC (that is identical to EGE), EGD, EGG, lichenase LichA and xylanase XynC were shown to possess a BTD at their C-terminal end [9-11].The role of these BTD is not known. Endoglucanase EG2, that was purified from *F. succinogenes* S85 cultures, was shown to possess a cellulose binding domain distinct from the catalytic domain, but the corresponding gene has still to be cloned. A gene coding for a protein characterized as a cellulose-binding-protein was also isolated from S85, and it was shown to carry a CBD [12].

Some of the cellulase genes were found in many different strains of *F. succinogenes*, but others seemed to be restricted to some isolates [9]. Thus, the cellulolytic equipment may be different in strains belonging to different subspecies, although the overall cellulolytic and xylanolytic activity is similar in several isolates tested (unpublished results). In the case of the strain S85, as many as 25 enzymes involved in plant cell wall degradation were identified.

Eight cellulase genes and one xylanase gene were isolated from five strains of *R. albus*, and eight cellulase genes and 3 xylanase genes from three strains of *R. flavefaciens* [8, 13]. The majority of the cellulases belong to family 5, but some of them are in family 3, 9 and 44. One enzyme from *R. albus* F40 was recently reported to carry a CBD, as well as reiterated sequences at its C-terminal end [15]. The xylanase from *R. albus* and one from *R. flavefaciens* were in family 11, and the two other xylanases from *R. flavefaciens* were multidomain enzymes, one carrying a family 10 and a family 11 catalytic domain, and the other a xylanase family 11 catalytic domain and a lichenase (family 16) domain. Genes coding for a family 3 β-glucosidase, family 5 and 9 cellulases as well as genes coding for family 10 and 43 xylanases have been cloned from four strains of *B. fibrisolvens* [5, 8, 13]. A sequence homologous to a CBD was found at the C-terminus of endoglucanase END1 from this bacterium, and a CBD was also present in the only gene sequenced from *Clostridium longisporum*, coding for a family 5 cellulase. Endoglucanase and xylanase genes were also isolated from several strains of *P. ruminicola* : the enzymes belong to family 3, 5, 10 and 43 [13].

Cellulase and hemicellulase genes were also cloned from the rumen fungi; two cellulase genes, encoding CELA in family 6 and CELB in family 5 were isolated from *Neocallimastix patriciarum*, together with two xylanase genes encoding XYLB in family 10 and XYLA which carries two family 11 domains [6, 8]. One cellulase (family 5) and two xylanases from *N. frontalis* (one in family 11 and another similar to XYLA from *N. patriciarum)* were also characterized by gene sequencing [8, 13]. Genes have also been isolated from *Piromyces*; they code for three mannanases (family 26) and one xylanase (containing two family 11 domains) [6, 14].

Organization of the cellulase-hemicellulase systems

In most of the enzymes encoded by the genes isolated from the fungi, a 40-residue reiterated sequence is found at the C-terminus (in XYLA and CELB from *N. patriciarum* and CELA from *N. frontalis*, as well as the mannanases from *Piromyces*), or separating the two catalytic domains (in the case of XYLA from *Piromyces)*. These reiterated noncatalytic sequences were shown to interact with polypeptides of 116 and 97 kDa that are present in the multienzyme cellulase-hemicellulase complexes of *Neocallimastix* and *Piromyces* respectively [6]. It was thus proposed that these duplicated domains function as protein docking sequences in a manner similar to the 23-residue reiterated sequence, present in many cellulases and hemicellulases from *Clostridium thermocellum* and *C. cellulolyticum*, that has been shown to be involved in the docking of enzymes to a non-catalytic scaffolding protein to form the cellulosome multienzyme complex [16]. Such reiterated sequence was also recently reported in one enzyme from *R. albus* [15]. However, as it was found in only one of the 16 depolymerase genes characterized from this species, it is not known whether a cellulosome-like structure is also present in the ruminococci. Concerning *Fibrobacter*, it is possible that the BTD found in several enzymes plays a role in protein association, but there is no experimental evidence at present.

In conclusion, numerous cellulase and

hemicellulase genes from rumen microorganisms were characterized in the last few years, and new ones are still being characterized. Each of the microorganisms studied produces multiple endoglucanases and multiple xylanases. Taking into account the uniform chemical structure of cellulose, it is not easy to explain the multiplicity of endoglucanases produced by one microorganism, particularly when the enzymes belong to the same glycosylhydrolase family and show quite similar specificity and physiochemical properties, as in the case of family 9 endoglucanases from *F. succinogenes*. Different bacterial species and the rumen fungi produce similar enzymes from the same family, although some specificity may be found in the nature of the ancillary domains. Finally, the diversity of the plant cell wall hydrolyzing enzymes of rumen microorganisms is comparable to that found in microorganisms from other anaerobic ecosystems [13]. At the molecular level, the cellulolytic and hemicellulolytic enzymes appear to have arisen by extensive domain shuffling with a significant degree of horizontal transfer, as evidenced for many other bacterial and eukaryotic proteins [17].

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