Characterization of Glycoside and Polysaccharide Hydrolases Secreted by the Rumen Anaerobic Fungi *Neocallimastix frontalis*, *Sphaeromonas communis* and *Piromonas communis*

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The rumen anaerobic fungi *Neocallimastix frontalis*, *Sphaeromonas communis* and *Piromonas communis* were grown in the presence of cellulosic substrate (sisal fibres) and the properties of the glycosidases and polysaccharide-degrading enzymes produced by the organisms were studied. β -D-Glucosidase (EC 3.2.1.21), β -D-fucosidase (EC 3.2.1.38), β -D-galactosidase (EC 3.2.1.23), β -1,3-glucanase (EC 3.2.1.6), β -1,4-glucanase (EC 3.2.1.4) and β -xylanase (EC 3.2.1.8) had pH optima of 6.0 and temperature optima under the conditions of assay of 50–55 °C, whereas for β -xylosidase (EC 3.2.1.37) the optima were 6.5 and 39 °C respectively. The apparent K_m values of individual enzymes secreted by the three fungi were similar. The results show that *S. communis*, *P. communis* and *N. frontalis* produce the same range of enzymes, with similar properties, able to degrade cellulose and hemicellulose.

INTRODUCTION

Anaerobic aquatic phycomycetes occur in the rumen of cattle, sheep and goats (Bauchop, 1981; Orpin, 1984; Orpin & Munn, 1986). The species examined in the sheep rumen are *Neocallimastix frontalis*, *Neocallimastix patriciarum*, *Piromonas communis* and *Sphaeromonas communis*. Speciation has been based primarily on zoospore morphology and size, number and length of flagella. These phycomycetes are seen consistently in rumen liquid, and measurement of chitin, a polysaccharide constituent of the cell wall of these fungi, indicates that the fungal biomass may represent as much as 8% of the total microbial biomass (Orpin, 1981), suggesting a significant role in rumen function.

Zoospores of the fungi colonize and invade fibrous plant materials, and are involved in cell wall polysaccharide degradation and fibre digestion. Anaerobic fungi have been cultured on a wide range of polysaccharides, including cellulose, starch, xylan and other hemicelluloses, indicating that they produce the range of enzymes necessary to degrade these polymers to simple sugars (Orpin & Letcher, 1979; Lowe *et al.*, 1987*a*; Williams & Orpin, 1987*a*). Different isolates of *Neocallimastix* produce extracellular cellulase, carboxymethylcellulase and xylanase (Mountfort & Asher, 1985; Pearce & Bauchop, 1985; Lowe *et al.*, 1987*b*; Wood *et al.*, 1986). However, investigations have been mainly focused on *Neocallimastix frontalis*. The properties of other fungi have not been studied apart from recent reports (Williams & Orpin, 1987*a, b*) showing that *N. patriciarum*, *P. communis* and an unidentified isolate (F) produce a wide range of glycosidases. In this paper, we have characterized the glycosidases and polysaccharidases produced by three anaerobic fungi, *N. frontalis*, *S. communis* and *P. communis*, in order to determine the contribution of these fungi to rumen function.

METHODS

Strains and culture conditions. *N. frontalis*, *S. communis* and *P. communis* were isolated from different sheep and identified at the Laboratoire de Microbiologie, CRZV-INRA, Theix, France. Anaerobic techniques for the preparation of culture media were based on those described by Hungate (1969) and Fonty *et al.* (1987). The fungi

were isolated by dilution of sheep rumen fluid in anaerobic medium containing 15% (v/v) clarified rumen fluid in roll tubes (Joblin, 1981). Colonies were transferred to liquid medium containing 30 mg each of chloramphenicol, ampicillin and streptomycin ml^{-1} . Then they were reinoculated into roll tubes and reisolated to obtain pure cultures. Stock cultures were maintained on sisal medium and 0.5 ml of the culture was transferred to fresh medium every 3 or 4 d. The fungi were grown axenically at 39 °C in culture tubes containing 5 ml medium containing rumen fluid (Orpin, 1975). Sisal fibres and 0.25% glucose for *S. communis* and *P. communis* or 0.25% (w/v) cellobiose for *N. frontalis* were included in the media. The gas phase was 100% CO_2 . Experimental media were inoculated with 0.1 ml zoospore suspension from a 3 d old culture. Cultures were kept in a vertical position without shaking for 6 d.

Enzyme preparation. For each fungus, several culture tubes were pooled and centrifuged at 3000 *g* for 5 min to pellet mycelia. Pellets were rinsed by centrifugation with 10 ml sodium citrate/phosphate buffer (100 mM, pH 5.2). Supernatants were used as the extracellular fraction. Mycelia resuspended in 3 ml buffer were homogenized in a Virtis homogenizer for three periods of 30 s at high speed and 4 °C, then centrifuged at 1000 *g* for 10 min to sediment the cell wall fraction. Supernatants were centrifuged at 48000 *g* for 30 min to obtain the intracellular soluble fraction (supernatant) and the membrane fraction (pellet). Cell wall and membrane fractions were resuspended in 3 ml buffer (Fèvre, 1977).

Enzyme assays. β -1,3-Glucanase (1,3- β -D-glucan glucanohydrolase, EC 3.2.1.6), β -1,4-glucanase (1,4- β -D-glucan 4-glucanohydrolase 4-glucanohydrolase, EC 3.2.1.4) and xylanase (1,4- β -D-xylan xylanohydrolase, EC 3.2.1.8) activities were measured in a final volume of 1 ml containing respectively laminarin (1 mg), carboxymethylcellulose (3 mg) and xylan (1.5 mg) dissolved in citrate/phosphate buffer (100 mM, pH 6.0) and 10–50 μl of enzyme preparation. After 20 min incubation at 50 °C, reactions were stopped by the addition of 3 ml dinitrosalicylic acid (DNS) reagent (Miller, 1959) to measure reducing sugars liberated. Tubes were placed in a boiling water-bath for 8 min. Absorbances were read at 528 nm with glucose or xylose as standards. Enzyme and substrate controls were included in all assays. Enzyme activities are expressed as μmol glucose or xylose equivalent released min^{-1} (ml enzyme solution) $^{-1}$.

β -D-Glucosidase (β -D-glucoside glucohydrolase, EC 3.2.1.21), β -D-fucosidase (β -D-fucoside fucohydrolase, EC 3.2.1.38), β -D-xylosidase (β -D-xyloside xylohydrolase, EC 3.2.1.37) and β -D-galactosidase (β -D-galactoside galactohydrolase, EC 3.2.1.23) were determined by measuring the *p*-nitrophenol released from *p*-nitrophenyl β -D-glucoside, *p*-nitrophenyl β -D-fucoside, *p*-nitrophenyl β -D-xyloside and *p*-nitrophenyl β -D-galactoside respectively. The reaction mixtures contained 10–50 μl of enzyme solution in 1 ml substrate solution (1 mg in citrate/phosphate buffer, 100 mM, pH 6.0). After 20 min at 50 °C or 39 °C, reactions were terminated by the addition of 2 ml 1 M- Na_2CO_3 . The *p*-nitrophenol liberated was measured spectrophotometrically at 400 nm. One unit of enzyme activity is defined as that required to produce 1 nmol *p*-nitrophenol min^{-1} (ml of enzyme solution) $^{-1}$. All enzyme reactions were linear over the period of assay.

Other glycosidase activities were screened using LRA ZYM OSIDASES (API, France). Assays were conducted with 65 μl culture medium at 40 °C for 4 h. Glycosidase activity was detected by the addition of a drop of 0.1 M-NaOH.

Determination of enzyme properties. The effect of substrate concentration on each enzyme activity was tested in the range of 0.25–4 mg ml^{-1} . K_m values were determined using the Lineweaver–Burk plot. The optimum pH and temperature under the conditions of assay for each enzyme activity was determined by measuring enzyme activity in citrate/phosphate buffer (100 mM) at pH values ranging from 3.0 to 8.0 and temperatures from 20 to 70 °C. The effect of pH on enzyme stability was determined by incubating crude enzyme adjusted to pH 3.0 to 8.0 for 1 h at 20 °C. Thermal stability was determined by incubating enzyme solutions without their substrates in citrate/phosphate buffer, 100 mM, pH 6.0, for 4 h at different temperatures. Residual activities were measured using standard assay conditions.

RESULTS

Enzyme production by rumen anaerobic fungi grown on sisal fibres

When they were grown in the presence of sisal fibres, *N. frontalis*, *S. communis* and *P. communis* secreted into the medium several endo-enzymes (β -1,3-glucanase, β -1,4-glucanase, xylanase) and exo-enzymes (β -glucosidase, β -fucosidase, β -xylosidase, β -galactosidase). As maximal activities and maximal growth of the cultures occurred at the same time, analyses were done on 6 d old cultures (Table 1). *N. frontalis* had the highest β -1,4-glucanase activity while the two other fungi showed greater exo-enzyme activities. Among the exo-enzymes, β -galactosidase had a very low activity, 16 to 30 times lower than that of β -fucosidase.

Attempts to detect other glycosidase activities like mannanase, α -glucosidase, α -galactosidase, *N*-acetyl- α - or β -D-glucosaminidase, α -xylosidase, α - or β -maltosidase, α - or β -mannosidase,

Fig. 1

Fig. 1. Effect of pH on the activity of extracellular enzymes of *N. frontalis*. (a) ■, Xylanase; □, β -xylosidase. (b) ●, β -1,4-Glucanase; ○, β -glucosidase; ▼, β -galactosidase. (c) ▲, β -Fucosidase; △, β -1,3-glucanase.

Fig. 2

Fig. 2. Effect of temperature on the activity of extracellular enzymes of *N. frontalis*. (a) ■, Xylanase; □, β -xylosidase. (b) ●, β -1,4-Glucanase; ○, β -glucosidase; ▼, β -galactosidase. (c) ▲, β -Fucosidase; △, β -1,3-glucanase.

Table 1. Glycosidase and polysaccharidase activities in the culture media of rumen anaerobic fungi

Extracellular enzymic activities were measured in 6 d old cultures and are expressed as nmol *p*-nitrophenol released min⁻¹ (ml culture)⁻¹ (a) or as μ mol glucose (b) or xylose (c) equivalents produced min⁻¹ (ml culture)⁻¹. The results are the means $\pm$ SD of five experiments for *N. frontalis* and of three experiments for *S. communis* and *P. communis*.

Activity	<i>N. frontalis</i>	<i>S. communis</i>	<i>P. communis</i>
β -Glucosidase ^a	15 $\pm$ 10	46 $\pm$ 12	20 $\pm$ 8
β -Fucosidase ^a	48 $\pm$ 12	66 $\pm$ 10	55 $\pm$ 7
β -Xylosidase ^a	16 $\pm$ 6	27 $\pm$ 5	28 $\pm$ 3
β -Galactosidase ^a	3 $\pm$ 0.8	3.7 $\pm$ 0.7	2 $\pm$ 0.5
β -1,3-Glucanase ^b	0.31 $\pm$ 0.03	0.24 $\pm$ 0.03	0.26 $\pm$ 0.04
β -1,4-Glucanase ^b	0.44 $\pm$ 0.08	0.31 $\pm$ 0.04	0.27 $\pm$ 0.05
Xylanase ^c	0.36 $\pm$ 0.05	0.43 $\pm$ 0.06	0.37 $\pm$ 0.04

Fig. 3

Fig. 4

Fig. 3. pH stability of extracellular enzymes of *N. frontalis*. (a) □, β -Xylosidase; ▲, β -fucosidase; ○, β -glucosidase. (b) ■, Xylanase; △, β -1,3-glucanase; ●, β -1,4-glucanase. Crude enzymes adjusted to pH 3.0–8.0 were incubated for 1 h at 20 °C before assaying residual enzyme activity.

Fig. 4. Temperature stability of extracellular enzymes of *N. frontalis*. (a) □, β -Xylosidase; ▲, β -fucosidase; ○, β -glucosidase. (b) ■, Xylanase; △, β -1,3-glucanase; ●, β -1,4-glucanase. Enzyme preparations were incubated for 4 h at various temperatures in citrate/phosphate buffer (100 mM, pH 6.0) without substrates, before estimating residual activities.

α - or β -L-fucosidase were not convincing. The amounts of *p*-nitrophenol released from *p*-nitrophenyl substrates were so low that we could not exclude the action of non-specific hydrolases.

β -1,4-Glucanase and xylanase were found mainly in the extracellular fraction, which contained 95 and 99%, respectively, of the total activities. Most (75 and 90%) of the total β -xylosidase and β -glucosidase was also recovered in the medium. Their intracellular activities were also determined following differential centrifugation of cell-free extracts obtained with a razor blade homogenizer which allowed an efficient breakage of mycelia (Fèvre, 1977). These enzymes were associated with the cell wall fraction (10% of the total activity). β -Xylosidase was also found in the internal membranes and in the intracellular soluble fraction corresponding to about 10 and 5%, respectively, of the total activity. Only traces of β -glucosidase were detected in these fractions.

As the culture media contained most of the enzyme activities they were used as the enzyme sources for the following experiments.

Properties of the enzymes secreted by N. frontalis

Physicochemical characteristics of the enzymes produced by *N. frontalis* were determined. β -Xylosidase and β -fucosidase were active in a very narrow range of pH values and temperatures: maximal activities were obtained respectively at pH 6.5 and 39 °C and at pH 6.0 and 55 °C (Figs 1 and 2). Slight deviations from these optimal conditions resulted in a sharp decrease of the enzyme activities. β -Glucosidase, β -1,4-glucanase, β -xylosidase and β -1,3-glucanase exhibited

Table 2. Comparison of the properties of the hydrolases secreted by *N. frontalis* (*N. f.*), *S. communis* (*S. c.*) and *P. communis* (*P. c.*)

Activity	Optimum temperature* (°C)			Optimum pH*			K_m^* (mg substrate ml ⁻¹)		
	<i>N. f.</i>	<i>S. c.</i>	<i>P. c.</i>	<i>N. f.</i>	<i>S. c.</i>	<i>P. c.</i>	<i>N. f.</i>	<i>S. c.</i>	<i>P. c.</i>
β -Glucosidase	50	50	50	6.0	6.0	6.0	0.55	0.51	0.52
β -Fucosidase	55	55	55	6.0-6.5†	6.0-6.5†	6.0-6.5†	0.39	0.37	0.30
β -Xylosidase	35	39	39	6.5	6.5	6.0-6.5†	0.34	0.36	0.40
β -Galactosidase	50	50	50	6.0	6.0	6.0	1.89	1.20	1.24
β -1,3-Glucanase	55	55	55	6.0	6.0	6.0	0.97	0.85	0.77
β -1,4-Glucanase	45	50	50	5.5	6.0	6.0	2.79	2.89	2.43
Xylanase	50	50	50	6.0	6.0	5.5	1.13	0.98	1.00

* As measured under the assay conditions described in Methods.

† Plateau of pH optima.

optimal activity between pH 5.5 and 6.5 and at 50 °C. Their activities were only gradually reduced by lowering the temperature or the pH.

The effects of pH (Fig. 3) or temperature (Fig. 4) on enzyme stability showed that most of the enzymes exhibited the same properties: they were stable after 4 h at 40 °C and for 1 h at pH values between 4 and 8. However, β -xylosidase was less stable, being inactivated during incubation at pH 4 or at 50 °C.

Comparison of the properties of the enzymes secreted by different fungi

The enzymes produced by *P. communis* and *S. communis* were mainly extracellular as more than 80% of the total activity of each glycosidase was recovered in the culture medium. The properties of the enzymes secreted by these two fungi were determined (data not shown in detail) and compared to those of *N. frontalis* (Table 2). The general properties of each type of enzyme produced by the three fungi were nearly identical. The optimal pH was 6.0 for each enzyme activity except β -xylosidase (pH 6.5). Optimum temperatures under the conditions of assay were at 50 or 55 °C except for β -xylosidase which was most active at 39 °C. The apparent K_m for each type of enzyme was similar in different species.

DISCUSSION

A large population of anaerobic fungi is associated with plant material in the rumen of animals receiving fibrous diets. The different strains used in this study were isolated from the sheep rumen and were identified as *N. frontalis*, *P. communis* and *S. communis* according to the size of their zoospores, the number and size of flagella, and the size and shape of their sporangium following the descriptions of Orpin (1975, 1976, 1977). In order to evaluate the role of these fungi in fibre digestion, the nature and the properties of the glycosidases synthesized by these three species were determined. When cultured in the presence of sisal, *N. frontalis* produced β -1,3-glucanase and β -fucosidase in addition to β -1,4-glucanase, xylanase, β -xylosidase, β -glucosidase and β -galactosidase as previously reported (Pearce & Bauchop, 1985; Mountfort & Asher, 1985; Lowe *et al.*, 1987*b*; Williams & Orpin, 1987*a, b*). Most of the total activity of these enzymes was secreted and recovered in culture medium. Internal membranes, cell walls and cytosol exhibited little activity in 6 d old cultures. These results are in agreement with those of Pearce & Bauchop (1985) using 6-7 d old cultures of another strain of *N. frontalis*. As most studies have focused on the physiology of *Neocallimastix* species, it was interesting to determine and to compare the hydrolytic activities produced by two other species, *P. communis* and *S. communis*. They exhibited the same range and nature of exo- and endo-acting enzymes produced by *N. frontalis*. They were mainly secreted and their activities were of the same magnitude when the fungi were grown in the presence of sisal. The enzymic properties were similar from one enzyme to another and from one species to another. Thus, these properties

cannot be used for the identification of new isolates. The enzymes exhibited the same optimum pH (pH 6.0) and optimum temperature of 50 °C except for β -xylosidases. These values are similar to those reported for xylanase and β -1,4-glucanase from different isolates of *Neocallimastix* (Mountfort & Asher, 1985; Lowe *et al.*, 1987b) but differed from those of enzymes degrading Avicel (Pearce & Bauchop, 1985) and of a wide range of enzymes produced by *Neocallimastix patriciarum* and *P. communis* which were assayed at 39 °C (Williams & Orpin, 1987a, b). The properties of the enzymes were different to those described for enzymes of aerobic fungi, which are commonly active at more acidic pH values from pH 4.0 to 5.0, and at higher temperatures, from 55 to 70 °C (Hoebler & Brillouet, 1984; Kamagata *et al.*, 1986; Macris, 1984; Shoemaker & Brown, 1978; Sadana *et al.*, 1983). β -Xylosidases, which were more active at 39 °C and at pH 6.5, were also different to those of aerobic fungi which have optima at pH 4.0–5.0 and 50–75 °C (Lachke *et al.*, 1985; Deshpande *et al.*, 1986; Kitpreechavanich *et al.*, 1986). The enzymes, particularly the β -xylosidases, were unstable in the absence of substrate after a relatively short incubation (4 h) at 50 °C. Similar results have been reported for β -1,4-glucanase and xylanase from different *Neocallimastix* isolates (Mountfort & Asher, 1985; Lowe *et al.*, 1987b).

The apparent K_m values for each type of enzyme produced by the fungi were similar. Williams & Orpin (1987a, b) also observed that the substrate affinities of enzyme preparations from *N. patriciarum*, *P. communis* and isolate F were similar. Moreover, the K_m values, 1 mg ml⁻¹ for xylanase, 0.4 mg ml⁻¹ for β -fucosidase and 0.35–0.4 mg ml⁻¹ for β -xylosidase, were similar to those of the enzymes of three different isolates studied by Williams & Orpin (1987a, b).

The role of *S. communis* in the rumen was not clear as this species has a very limited rhizoidal system compared to *N. frontalis* and *P. communis*. Our results show that *S. communis* secretes the same wide range of glycoside hydrolases produced by *P. communis* and *N. frontalis* and is able to degrade cellulose, hemicelluloses and non-cellulosic mixed glucans of plant cell walls. Thus, although *S. communis* does not penetrate plant tissues, it probably acts as an active degrader by secreting large amounts of extracellular hydrolases. In order to elucidate the efficiency and the interactions of different polysaccharide depolymerases (β -1,3- and β -1,4-glucanases, xylanase) and glycoside hydrolases (β -glucosidase, β -fucosidase, β -xylosidase, β -galactosidase) in the degradation of plant structural polysaccharides, studies involving enzyme induction and the purification of individual enzymes will be necessary.

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