A Study on Cellulolytic and Hemicellulolytic Enzymes of Anaerobic Rumen Bacterium *Ruminococcus flavefaciens* Strain 17

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Abstract: Polysaccharide-degrading enzymes of the anaerobic rumen bacterium *Ruminococcus flavefaciens* strain 17 were exmined. This work demonstrated that *R. flavefaciens* strain 17 produces a complex array of enzymes active against cellulose, hemicellulose (xylan) and lignin. These enzymes are xylanase, avicellase, acid-swollen cellulase, carboxymethyl cellulase (CMCase), pNP-cellobiosidase (pNPC), pNP-glucopyranosidase (pNPG), pNP-xylopyranosidase (pNPX) and pNP-arabinofuranosidase (pNPA). Although *R. flavefaciens* is an anaerobic bacterium, its enzymes worked optimally under aerobic conditions and with dithiothreitol (DTT), except in the case of pNP-xylopyranosidase. pNP-xylopyranosidase exhibited a higher level of activity under anaerobic conditions with DTT. It was demonstrated that the cross-links between arabinose side chains, which esterified via ferulic acid and p-coumaric acid, were cleaved by *R. flavefaciens* esterase and α-arabinofuranosidase.

Key Words: *Ruminococcus flavefaciens*, polysaccharidase, esterase, enzymes

Anaerobik Rumen Bakterisi *Ruminococcus flavefaciens* 17 suşunun Selülolitik ve Hemiselülolitik Enzimleri Üzerine Bir Çalışma

Özet: Anaerobik rumen bakterisi *Ruminococcus flavefaciens* 17 suşuna ait, polisakkaritleri parçalayan enzimler çalışıldı. Bu çalışma *R. flavefaciens* 17 suşunun selüloz, hemiselüloz (xylan) ve lignine karşı aktif olan kompleks bir takım enzimler ürettiğini gösterdi. Bu enzimler ksilanaz, avisellaz, asid artırılmış selülaz, karboksimetil selülaz, pNP-sellobiozidaz, pNP-glukopiranozidaz, pNP-ksilopiranozidaz ve pNP-arabinofuranozidaz. *R. flavefaciens* 17 suşu anaerobik bir bakteri olmasına rağmen enzimlerinin (pNP-ksilopiranozidaz hariç) optimum çalışma koşullarının aerobik şartlar altında DTT ilavesi ile olduğu bulundu. pNP-ksilopiranozidaz anaerobik şartlarda DTT ilavesi ile daha fazla enzim aktivitesi gösterdi. Ferulik asit ve p-koumarik asit ile esterlenmiş arabinoz yan zincirleri arasındaki çapraz bağların *R. flavefaciens* suşunun esteraz ve α-arabinofuranozidaz enzimleri ile parçalandığı gösterildi.

Anahtar Sözcükler: *Ruminococcus flavefaciens*, polisakkaridaz, esteraz, enzim

Introduction

Plant-cell-wall polysaccharides only become available as a source of energy to the host animal through the activities of the rumen microflora (1-3). Cellulases and xylanases encompass a collection of enzymes whose primary function is to hydrolyse $\beta(1-4)$ glycosidic linkages in the major plant structural polysaccharides, cellulose and xylan. In converting cellulose and xylan to their constituent sugars, these enzymes play an essential role in the digestive processes of herbivores and in the recycling of photosynthetically fixed carbon.

The xylan chains are substituted to varying degrees with arabinose, methyl glucuronic acid and acetyl groups and are also cross-linked via esterified ferulic acid between arabinose side chains. The in-vitro esterification of phenolic acid to plant cell walls significantly reduces the biodegradability of plant cell walls. These cross-linked bonds are cleaved by ferulic acid esterase and α-arabinofuranosidase (2).

The major cellulose-degrading rumen bacteria are *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *R. albus* (4-7). *R. flavefaciens* is a Gram-positive, non-motile organism, which occurs singly or in pairs and chains (8). A yellow pigment is produced, particularly during growth on cellulose. This bacterium is recognised as being among the most numerous, strictly anaerobic bacteria active in the rumen that are capable of degrading both the cellulose and hemicellulose components of plant

cell walls (9). Its cellulases and xylanases have been partly characterised (5,6). Polysaccharidase activity has been shown to be strongly influenced by growth substrate (10-12). Cellodextrins are hydrolysed extracellularly prior to transport into the cell (13). Many strains of *R. flavefaciens* are able to grow with crystalline cellulose or xylan as the sole energy source in vitro. Hydrolysis products derived from polysaccharide breakdown are presumably utilised mainly as disaccharides or oligosaccharides, since neither glucose nor xylose appear to be utilised for growth. Cellobiose is utilized for growth; it has been proposed that this involves a cellobiose phosphorylase (bacteria provide energy from the cellobiose while limited energy sources are available) (14).

In continuous cultures, cells of *R. flavefaciens* wash out at pH 6.1 or below (15). Addition of readily fermentable carbohydrates to cellulose broths reduces cellulolysis, but not enzyme synthesis (12,16), as a consequence of the fall in pH during the fermentation (17,18). Because of this capacity, the plant-cell-wall degrading system of *R. flavefaciens* has received considerable attention. A number of cellulosic and noncellulosic polysaccharidase genes have been well documented (19-23), and these genes have been expressed in *E. coli*, in *S. bovis* (24-26), and in tobacco plants (27). *R. flavefaciens* is a truly cellulolytic species, being able to degrade crystalline cellulose, and is known to possess multiple genes encoding cellulases and xylanases (19,20). The precise role and interaction of the different gene products in the process of plant cell-wall breakdown are not yet understood, but significant functional differences are apparent.

This study concerns *R. flavefaciens* 17, a strain that expresses activities against crystalline cellulose and noncellulosic plant-cell-wall polysaccharides, xylan and b-(1-3,1-4) glucan. Regulation of its enzymes by growth substrate and the expression level of these enzymes under different growth conditions were examined.

Materials and Methods

Anaerobic media and growth conditions

Anaerobic M2 and HS (28) media were prepared by boiling of the ingredients in a conical flask 3 times for a few minutes in order to remove O_2. Between waiting times, the medium bubbled with O_2-free absolute CO_2. Then all the ingredients were poured into CO_2-filled bottles and bubbled with O_2-free absolute CO_2 in order to remove the remaining O_2 for a few minutes according to Bryant (29), and then 0.1% cysteine (Sigma) was added. The required volumes were dispensed under absolute CO_2 into Hungate tubes (Belco Glass, Inc., Vineland, N.J.), or into 100ml crimp-top bottles (Alltech Ltd., Carnforth, Lancashire, U.K.) before autoclaving. Agar was included at 1.5% (w/v) for plates, 0.75% (w/v) for stab cultures, and 2% (w/v) for roll tubes. Roll tubes containing 5-ml medium were kept molten at 45ºC, and, subsequently, all the tubes were solidified by rotating in a cold-water bath after inoculation. Otherwise, sterile molten media were equilibrated to 50-55ºC before any required sterile additions (e.g., antibiotics) were made. Additions to the media in 100ml crimp-top bottles were made by syringe, the pressure being equilibrated to atmospheric (or above) by the addition of 100% CO_2 by syringe.

The M2 medium of Hobson (30), containing 0.2% cellobiose (Sigma), and medium HS, containing either 0.5% freezer-milled barley straw or oat spelt xylan (Sigma) as the sole carbon and energy source, was used to grow *R. flavefaciens* 17 according to the methods of Bryant (29).

Preparation of cell extracts for enzyme assay

Anaerobic cells were grown on appropriate medium (500 ml) for 72 and 168 hours (3 and 7 days), then whole cultures were centrifuged in GSA rotor in a Sorvall RC5B centrifuge (7,500 rpm, 4ºC, 10 minutes) in CO_2-filled 300ml bottles with tight-fitting caps (black caps with O rings).

The supernatants were removed and frozen at -70ºC under CO_2 for enzyme assays. The pellet was resuspended in 1/10 volume Na phosphate buffer (pH 6.7, 0.05M) and subjected to sonication at 0ºC, using an MSE Soniprep (4 min in total - 1 min at a time with 1 min cooling intervals on ice; sonication time can be extended depending on the type of bacterial cell). This sonicate was frozen to -70ºC for subsequent analysis. Another anaerobically prepared cell suspension from 3- and 7-day-old cultures was treated with 1% toluene. These toluenised cells were stored at –70ºC for subsequent analysis.

Enzyme assay

Lever assay

All the substrates were prepared either aerobically or anaerobically in 0.05% sodium phosphate buffer (pH 6.5) with or without 2mM DTT, containing 1% of the appropriate polysaccharide. The CMCase (carboxymethyl cellulase), avicellase, acid-swollen cellulase and xylanase activity in the samples was determined by measuring reducing-sugar release (nmol/min mg cell protein) as determined by the method of Lever (31) and as described by Flint et al. (32). Incubations were carried out at 37ºC for 15-120 min in 0.05 M sodium phosphate buffer (pH 6.5) containing 1% polysaccharide substrate. The absorbance was measured at 410 nm. Protein was determined by the modified method of Lowry et al., (33) using 4 mg/ml bovine serum albumin (BSA) as the standard.

Para-nitrophenol (pNP) Assays

All the substrates were prepared either aerobically or anaerobically in 0.05% sodium phosphate buffer (pH 6.5), with or without 2mM DTT, containing 3mM pNP substrates (32). The release of para-nitrophenol (pNP) (yellow colour) was measured (nmol/min mg cell protein) (32). The assay with para-nitrophenol (pNP) derivatives (3 mM final concentration) as the substrates was incubated in 0.05 M sodium phosphate buffer (pH 6.5) for 30-60 min at 37ºC. Absorbance was measured after the addition of 2M Na_2CO_3 at 420 nm. This assay was performed for pNP-α-L-arabinofuranosidase (pNPA), pNP-β-D-cellobiosidase (pNPC), pNP-β-glucopyranosidase (pNPG) and pNP-β-D-xylopyranosidase (pNPX). All the substrates, pNP-α-L-arabinofuranoside, pNP-β-D-cellobioside, pNP-β-glucopyranoside, pNP-β-D-xylopyranoside, oat spelt xylan, and CMC (Carboxymethyl cellulose) were obtained from Sigma (Chemical Company Ltd.). All the other chemicals and media, unless otherwise stated, were supplied by Difco laboratories, Michigan, U.S.A.

Methyl coumarate or methyl ferulate dispersed (1.5 mg/ml) in 50 mM, pH 6.5, and potassium phosphate buffer as the substrate (the method was improved at the Rowett Research Institute, U.K. It has yet to be published).

Results

The total cell and supernatant enzyme activities (nanomol of sugar or pNP released min/ml culture) and the corresponding specific activities (nanomol/min/mg cell protein) for the studied enzymes were calculated.

The total cell and supernatant activities shown in Table 1 were determined on toluene treated cell extracts from a 3-day-old culture. Although there were no significant differences detected between enzyme activities with dithiothreitol (DTT) and those without DTT under both anaerobic and aerobic conditions, the optimal enzyme activities were detected under aerobic conditions with DTT, except in the case of pNP-xylopyranosidase (Table 1). The level of this enzyme activity was found to be approximately two times higher under anaerobic conditions with DTT than it was under aerobic conditions (+DTT, -DTT). Supernatant activities were also assayed under the same conditions (Table 1), and the optimal supernatant activities were determined under aerobic conditions with DTT, except in the case of pNP-xylopyranosidase. This supernatant activity was found to be approximately 76% higher under anaerobic conditions with DTT than it was under aerobic conditions (+DTT, -DTT).

The total cell and supernatant activities shown in Table 2 were determined on toluene treated cell extracts from newly grown 3- and 7-day-old culture and from sonicated cell extract from 3-day-old culture. The percentage distribution of total activity between the cells and supernatant was also calculated (Table 3). However, it should be stressed that the 'cell' fraction could include any substrate-bound enzyme, since the straw centrifuged down with the cells. The specific xylanase activities were found to be higher than the other enzyme activities under all conditions. The avicellase, acid-swollen cellulase and CMCase activities were found to be similar in the toluenised cells and these enzyme activities were also found to be similar in the sonicated cells (Table 2). However, sonication resulted in a q-to-20-fold increase in these enzyme activities for each enzyme when they were compared with toluenised-cell activities (Table 2). The highest total supernatant activities of avicellase, acid-swollen cellulase and CMCase were also found in the culture grown in oat spelt xylan (Table 2). The

Table 1. Total cell and supernatant activities [nmol min^{-1} (mg cell protein)$^{-1}$] of *R. flavefaciens* 17 cultures grown in defined medium (28). Inocula were from a culture grown in medium containing 0.2% cellobiose. The 3-day growth period and toluene treated values are based on samples taken from 500 ml cultures (see methods), and represent ±SD of duplicate (each has 3 repetitions, total 6 assays) assays.

Enzyme	Cell			Supernatant		
	Aerobic		Anaerobic	Aerobic		Anaerobic
	+DTT	-DTT	+DTT	+DTT	-DTT	+DTT
Xylanase	187±36.2	159±28.4	159±26.7	92±21.8	51±8.613.4	57±7.56
Avicellase	1.1±0.16	0.6±0.08	0.9±0.12	8.5±2.4	7.0±2.2	7.0±1.9
Acid swollen cellulase	1.3±0.18	0.9±0.17	0.9±0.16	6.2±2.05	6.0±1.76	6.0±1.85
Carboxymethyl cellulase	1.9±0.25	1.8±0.22	1.7±0.18	8.7±1.86	9.0±1.96	8.6±1.57
pNP-cellobiosidase	1.1±0.13	0.8±0.07	1.0±0.13	0.7±0.08	0.4±0.01	0.4±0.01
pNP-glucopyranosidase	12.4±3.2	11.5±2.8	10.6±2.6	2.7±0.98	1.8±0.82	1.9±0.94
pNP-xylopyranosidase	0.7±0.06	0.5±0.09	1.1±0.18	0.13±0.01	ND	0.23±0.01
pNP-arabinofuranosidase	3.3±0.98	2.3±0.84	2.0±0.52	4.3±1.15	4.3±1.23	4.0±1.08

ND= Not determined

Table 2. Total cell and supernatant enzyme activities [nmol min^{-1} (ml culture)$^{-1}$] of *R. flavefaciens* 17 from (A) 3- and (B) 7-day-old toluenised culture grown in barley straw and from (C) 3-day-old sonicated (C) culture grown in oat spelt xylan.

Enzymes	Aerobic +DTT					
	Cell			Supernatant		
	A	B	C	A	B	C **
Xylanase	187±36.2	155±32.4	160±35.8	92±21.8	132±28.3	304±48.9
Avicellase	1.1±0.16	1.1±0.18	20±4.7	8.5±2.4	14.3±3.4	28±5.9
Acid-swollen cellulase	1.3±0.18	0.8±0.09	19±4.2	6.2±2.05	10±3.4	23±4.6
Carboxymethyl cellulase	1.9±0.25	2.0±0.96	19±4.8	8.7±1.86	14±3.8	24±4.2
pNP-cellobiosidase	1.1±0.13	0.4±0.07	1.5±0.22	0.7±0.08	0.7±0.08	0.5±0.04
pNP-glucopyranosidase	12.4±3.2	5.0±1.87	4.3±1.84	2.7±0.98	1.2±0.12	0.9±0.08
pNP-xylopyranosidase	0.7±0.06	0.3±0.08	0.9±0.12	0.13±0.01	0.9±0.1	0.12±0.02
pNP-xylopyranosidase*	1.1±0.16	0.7±0.1	1.5±0.24	0.23±0.01	0.4±0.07	0.2±0.06
pNP-arabinofuranosidase	3.3±0.98	2.0±0.86	3.4±1.07	4.3±1.15	4.2±1.49	1.8±0.32

* Under anaerobic conditions with dithiothreitol.

** Concentrated supernatant with AMİCON PM10 filter

supernatant activities of avicellase, acid swollen cellulase and CMCase were found to be between 55% and 92% higher than the cell activities (Table 3). The supernatant- and cell-associated activities of pNP-cellobiosidase and pNP-xylopyranosidase (anaerobic) were found to be similar for both toluenised and sonicated cells (Table 2). The cell-associated activities of the pNP-glucopyranosidase were found to be the highest of all the pNP activities (Table 2). The specific pNP-arabinofuranosidase activity was similar for both toluenised and sonicated cells. However, pNP-arabinofuranosidase activity was found to be higher than both the pNP-cellobiosidase and pNP-xylopyranosidase activities, and lower than the pNP-glucopyranosidase activity (Table 2). The total supernatant activity of pNP-arabinofuranosidase was found to be similar for both 3- and 7-day-old cultures, and these supernatant activities were found to be higher than for the culture grown on oat spelt xylan (Table 2).

Enzymes	Aerobic +DTT					
	Cell			Supernatant		
	A	B	C	A	B	C **
Xylanase	67	54	35	33	46	65
Avicellase	12	8	42	88	92	58
Acid swollen cellulase	17	8	45	83	92	55
Carboxymethyl cellulase	18	13	44	82	87	56
pNP-cellobiosidase	63	36	75	37	64	25
pNP-glucopyranosidase	82	80	83	18	20	17
pNP-xylopyranosidase	84	30	88	16	70	12
pNP-xylopyranosidase*	83	62	88	17	38	12
pNP-arabinofuranosidase	44	32	65	56	68	35

Table 3. Total cell and supernatant enzyme activities % [$nmol\ min^{-1}$ (ml culture)$^{-1}$] of *R. flavefaciens* 17 from (A) 3- and (B) 7-day-old toluenised culture grown in barley straw and from (C) 3-day-old sonicated culture grown in oat spelt xylan.

* Under the anaerobic condition with dithiothreitol.
** Concentrated supernatant with AMİCON PM10 filter

This work also demonstrated that *R. flavefaciens* has significant detectable methyl ferulate and methyl coumarate esterase activities (Table 4) when methyl ferulate and methyl coumarate (1.5 mg/ml) are used as the substrate, even hough previous studies have reported that *R. flavefaciens* has little feruloyl and exhibits no p-coumaroyl esterase activity. Methyl ferulate and methyl coumarate esterase activities were found to be similar for both toluene- treated cells from 3- and 7-day-old cultures and sonicated cells activities were found to be lower than those of both toluenised cultures. No supernatant activities for either enzyme were detected in the cultures.

Table 4. Methyl esterase activities [$\mu mol\ min^{-1}$ (ml enzyme)$^{-1}$] of *R. flavefaciens* 17 cell extracts from (A) 3- and (B) 7-day-old toluenised culture and from (C) 3-day-old sonicated culture.

Substrate	A	B	C
Methyl ferulate	0.38±0.05	0.35±0.06	0.21±0.03
Methyl coumarate	0.46±0.07	0.35±0.07	0.30±0.04

Discussion

This work demonstrates that *R. flavefaciens* strain 17 produces a complex array of enzymes active against cellulose, hemicellulose (xylan) and lignin under aerobic conditions with DTT, except in the case of pNP-xylopyranosidase. This enzyme has higher activity under anaerobic conditions with DTT. DTT is required for enhancing the hydrolysis of crystalline cellulose (34), and for debranching activities, but is not needed for CMCase activity (35), which supports the finding from our experiments. Although sonicated xylanase and debranching activities were found to be similar for both toluenised cultures, the sonicated avicellase, acid-swollen cellulase and CMCase activities were found to be 10-to-20 times higher than both toluenised-culture cellulase activities. Toluene makes holes in the cell wall of the bacteria, and allows short chains of cellulose such as cellobiose and glucose to go into the cell. Longer chains of cellulose such as cellodextrine cannot go into the cell to digest, and cellulose is degraded only by the cell surface enzymes (36). Xylanase, pNPA and pNPC enzyme activities were found to be equal in terms of intracellular and extracellular action on the substrate. The PNPX and pNPG enzyme activities were found to be approximately 80% intracellular, while the avicellase, acid swollen cellulase and CMCase activities were found to have an extracellular action on the substrate. Xylan substituted to various degrees with acetyl, arabinosyl and glucuronosyl residues and is cross-linked via esterified ferulic acid and p-coumaric acid between arabinose side chains. These cross-linked bonds were cleaved by *R. flavefaciens* esterase and α-arabinofuranosidase was also detected.

Whereas, according to Borneman and Akin (37), enzymatic studies using *O*-[5-*O*-(E)-*p*-coumaroyl]- α-L-arabinofuranosyl]-(1-3)-*O*-β-D-xylopyranosyl-(1-4)-D-xylopyranose (PAXX), and *O*-[5-*O*-(E)-feruloyl)- α-L-

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arabinofuranosyl]-(1-3)-*O*-β-D-xylopyranosyl-(1-4)-D-xylopyranose (FAXX) as substrates showed that three major fibre-degrading rumen bacteria (*F. succinogenes, Butyrovibrio fibrosolvens* and *R. flavefaciens*) produced

little feruloyl and no p-coumaroyl esterase activity this work showed that *R. flavefaciens* 17 has both methyl ferulate and methyl coumarate esterase activities, using methyl ferulate and methyl coumarate as substrates.

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