Turkish Journal of Biology

Volume 25 | Number 2 Article 2

1-1-2001

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Turk J Biol
25 (2001) 113-122

Physiological Studies on the Plant Cell Wall Degrading Enzymes of the Rumen Bacterium *Ruminococcus flavefaciens*

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Received: 03.09.1999

Abstract: Two forms of *Ruminococcus flavefaciens*, the wild type and a trained culture of strain 17 were compared for their cellulose and xylan degradative ability when then were grown in ryegrass, cellobiose and xylan for different incubation times. No cell-associated corboxymethylcellulase (CMCase) activity was observed after 1-day incubation in cellobiose, as no supernatant activity was detected after 1 and 3 days incubation in xylan and 2 days incubation in cellobiose grown culture. Both CMCase and xylanase activities of trained culture were higher than those of wild type culture when they were grown in ryegrass. Wild type cultures, however, showed higher levels of both activities when grown in medium containing xylan.

Key Words: *Ruminococcus flavefaciens*, cellulase, xylanase.

Bitki Hücre Duvarını Parçalayan Rumen Bakterisi *Ruminococcus flavefaciens*'e Ait Enzimler Üzerine Fizyolojik Bir Çalışma

Özet: *R. flavefaciens*'in iki formu; yabani form ile ksilene karşı eğitilmiş form, farklı inkübasyon zamanlarında ve enerji kaynağı olarak cavdar samanı, sellobioz, ve ksilen içeren besi yerlerinde yetiştirilerek selulozu ve ksileni parçalama kabiliyetleri karşılaştırılmıştır. Sellobioz üzerinde 1 gün inkübasyondan sonra hücresel; ksilen üzerinde 1 ve 3 gün inkübasyondan sonra ve sellobioz üzerinde 2 gün inkübasyondan sonra supernatant karboksimetilsellulaz (CMCaz) aktivitesi gözlenmemiştir. Eğitilmiş formun çavdar samanı üzerindeki CMCaz ve ksilenaz etkinlikleri yabani formdan daha yüksektir. Bunun yanında yabani formun ksilen içeren ortamdaki her iki etkinliği daha iyidir.

Anahtar Sözcükler: *Ruminococcus flavefaciens*, ksilenaz, selülaz.

Introduction

The forage plant cell wall is a complex and fascinating biological structure. It is made up of polymers of cellulose and other noncellulosic polysaccharides, such as xylans and pectins in association with lignin, proteins, ions, and water (1-6).

Cellulose, the major component of the plant cell wall, is composed of β1, 4-linked glucose residues, which form chains up to 14,000 residues in length (7), tightly packed parallel molecules linked together by hydrogen bonds and the resulting fibrils are embedded in a ligno-hemicellulose complex. However, lignin does not appear to be directly bound to cellulose itself (8,9). Cellulose is hydrolysed by the synergistic action of a number of different enzymes including, β–1,4-endoglucanase (EC 3.2.1.4), β–1,4 exoglucanase (cellobiohydrolase (EC 3.2.1.91), β–1,4-glucosidase (EC 3.2.1.21), (10) and cellodextrinase. These enzymes act in synergy to degrade cellulose (11).

Xylan, the most abundant hemicellulose, constitutes up to 35% of the total dry weight of higher plants (12). Because of the heterogeneity of xylans, several types of endo-and exo-acting xylanases are required to effect hydrolysis. These include (Figure.1.2.2.1.1) β–1,4-endoxylanase (Xylanases; EC 3.2.1.8), β–1,4-exoxylanase; α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1), O-acetyl xylan esterase (EC 3.1.1.6), β–1,4-xylosidase (EC 3.2.1.37), (13) and ferulic and p-coumaric acid esterases. The β–1,3-1,4 glucans are hydrolysed by β–1,3-glucanases and mixed linkage β–1,3-1,4-glucanases, respectively. (14-17).

The ability of ruminant animals to make productive use of low-quality plant materials depends on the ability of the microorganisms that live in the rumen to digest the plant structural polysaccharides, primarily cellulose and hemicelluloses. While rumen protozoa have been shown to play a role in plant fibre degradation (18), and rumen fungi display a somewhat greater potential for the degradation of more heavily lignified plant tissues (19), the bacterial population is generally the most effective in digesting plant polysaccharides (20). On the basis of the relative numbers in the rumen and ability to hydrolyse purified and intact forage cellulose, the principal cellulolytic species of rumen bacteria appear to be *Fibrobacter succinogenes* and *R. flavefaciens* (17).

R. flavefaciens is a cellulolytic bacterium, which is a normal inhabitant of the rumen in ruminant animals. Their cells are Gram-positive, non-motile, and occur singly and in pairs and chains (21). A yellow pigment is produced, particularly during growth on cellulose. *R. flavefaciens is* recognised among the most numerous, strictly anaerobic active bacteria in the rumen that are capable of degrading both the cellulose and hemicellulose components of plant cell walls (22). 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 utilised for growth; it has been proposed that this involves a cellobiose phosphorylase (bacteria provide its energy from the cellobiose while limit energy source present) (23). *R. flavefaciens* cellulases and xylanases have been partly characterised (17). The polysaccharidase activities have been shown to be strongly influenced by the growth substrate (24-26).

The aim of this study was to investigate regulation of the polysaccharidase activities of wild and trained strains of *R. flavefaciens* by using different growth substrates.

Materials and Methods

Bacterial strains, media and growth conditions

Wild type strain of *R. flavefaciens* 17 was isolated and characterised by Flint et al., (27) and the trained (28) form of that strain was used in this experiment.

M2 medium (29) was supplemented with 3.1% xylan and 2.5% cellobiose as the sole energy sources. Ryegrass and xylan were directly added to M2 medium and autoclaved before usage while filter sterilised cellobiose was added to the medium after autoclaving. Anaerobic growth conditions and media preparation was performed according to Hungate (30) and Bryant (31).

Enzyme assay

The supernatants were taken off and frozen to -70 °C 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 0 °C, using an MSE Soniprep (4 min in total 1 min at a time with 1 min cooling on ice in between, sonication time can be extended depending on the type of the bacterial cell). Sonicated cell extracts were frozen to -70 °C for subsequent analysis. Six samples for each enzyme was assayed and t-test used for statistical analysis.

Lever assay

All substrates were prepared either aerobically or anaerobically in 0.05M sodium phosphate buffer (pH 6.5) containing either 2mM DTT or not and 1% of appropriate polysaccharide. CMCase and xylanase activities in samples were determined by measuring reducing-sugar release as determined by the method of Lever (32) and as described by Flint et al., (33). Incubations were at 37 °C for 15 min to 2 h in 0.05 M sodium phosphate buffer (pH 6.5) containing 1% polysaccharide substrate and absorbance measured at A410 nm. Protein was determined by the modified method of Lowry et al., (34) using the 4 mg/ml bovine serum albumin (BSA) as standard. All substrates, Cellobiose, Oat spelt xylan; CMC (Carboxymethylcellulose) and ryegrass were obtained from Sigma (Chemical Company Ltd.).

Results and Discussion

CMCase activities

Regulation of cellulase production in rumen environment is crucial for rumen microorganisms to survive. Enzyme quantity and activity produced by rumen microorganisms can be affected either positively or negatively by different energy sources (35). Cellulolytic and hemicellulolytic enzyme activities of *Bacteroides ruminicola, Butyrivibrio fibrosolvens,*

Fibrobacter succinogenes, Ruminococcus albus and *R. flavefaciens* are affected by the supplied energy sources and their end products (24,25,33). It has been put forward that cellulase enzyme is synthesised under the control of two basic mechanisms: a) cellulase enzymes are repressed in the presence of readily available low molecular weight carbon sources; b) cellulase production is encouraged in the presence of xylan or cellulose (8). However, it has been shown that *R. flavefaciens* can lose its ability to adhere to cotton after continued sub-culturing on the soluble energy sources (36). Similarly, Saluzzi (28) has shown that in a different growth medium different enzyme activities can be obtained and sub-culturing on the ryegrass stimulated xylanase activity. In this study, cellulolytic and hemicellulolytic enzymes of wild and trained types of *R. flavefaciens* 17 and the effects of different growth medium and incubation time on the enzyme activities were investigated. Like in previous studies (24,25,28) we observed that either CMCase or xylanase activities of *R. flavefaciens* 17 were stimulated or repressed by different nutrient sources. Effects of three different growth environments and four different incubation times on CMCase activities of the both wild and trained cultures are summarised in Table 1. Assayable cell-associated, from 1-day-old cultures, and supernatant, from 2-day-old cultures, CMCase activities were detected from cellobiose grown cultures. However, no CMCase activities in culture supernatant were detected from 1 and 3-day-old xylan grown cultures.

It has been observed that 1-day-old trained ryegrass grown cultures gave higher total supernatant activity than the wild cultures ($P<0.01$). However, 1-day-old wild xylan grown cultures gave higher both cell-associated specific activity and total supernatant activity than the trained cultures ($P<0.1$ and $P<0.05$ respectively) as supported by Saluzzi's (28) results. While no significant differences in total cell-associated activities between 3-day-old ryegrass grown trained and wild cultures were observed, specific cell-associated activity of trained culture found to be higher ($P<0.1$). However, in terms of specific and total supernatant activities wild types were found to be more successful ($P<0.05$ and $P<0.1$ respectively). While in cultures grown in xylan no distinct specific activity between them was observed, wild types gave higher activity when grown in cellobiose ($P<0.01$). Statistically no significant differences were observed between cultures after 7 days incubation in xylan and ryegrass.

CMCase activities were found to be mainly cell-associated as reported earlier by Pettipher and Latham (17). Along with lengthening of incubation time in media containing ryegrass and cellobiose, both specific and cell-associated activities of bacterial cultures also increased. This elevation might be due to increase in incubation time along with cell density. However, why the same thing was not observed in the xylan grown cultures is still hard to interpret.

Xylanase activities

So far limited research has been devoted to the control mechanism of the xylanase genes that are produced by microorganisms. These enzymes are synthesised under a different regulation mechanism from that of the cellulase enzymes in fungi (37). However, it is known that there are relationships between different enzymes according to regulation mechanism in

Table 1. CMCase activity of two different forms of *R. flavafaciens* 17 grown in different growth substrates at the different time intervals.

Carboxymethylcellulase (CMCase) activities						
			Total activity (nm/ml culture/min)		Specific activity (nm/mg protein/min)	
Incubation time	Energy source	Culture	Cell	Supernatant	Cell	Supernatant
1 day	Ryegrass	WT	0.49±0.23	0.70±0.05***	5.69±1.98	119±14.0
		TT	0.55±0.20	1.17±0.13	5.31±1.97	111±26.5
1 day	Cellobiose	WD	ND	0.42±0.10	ND	83.1±39.4
		TT	ND	0.43±0.02	ND	35.5±8.82
1 day	Xylan	WT	3.93±0.7*	ND	47.7±9.9*	ND
		TT	2.70±0.8	ND	26.7±9.4	ND
3 days	Ryegrass	WT	0.99±0.31	0.82±0.33**	10.23±3.0	101.8±51.6*
		TT	1.43±0.41	0.28±0.21	16.54±5.3	26.5±21.2
2 days	Cellobiose	WT	2.35±0.16***	ND	26.5±3.0***	ND
		TT	1.25±0.25	ND	15.6±3.8	ND
3 days	Xylan	WT	1.44±0.48	ND	14.13±3.9	ND
		TT	1.37±0.30	ND	13.03±2.5	ND
7 days	Ryegrass	WD	5.0±2.01	0.45±0.28	50.96±17.5	69.31±24.58
		TT	3.42±0.38	0.26±0.16	39.88±7.78	52.01±13.03
7 days	Xylan	WT	1.21±0.33	0.553±0.15	12.47±3.74	31.08±7.20
		TT	1.27±0.11	0.525±0.13	12.03±1.26	30.98±6.25

WT: wild type of *R. flavefaciens* 17 strain
TT: trained type of *R. flavefaciens* 17 strain
*, **. *** Statistically significant value, $P<0.1$, $P<0.05$, and $P<0.01$ respectively
ND: enzyme activity not determined

bacteria and energy sources in growth medium regulate xylanase activities (27,33,38). In this study both wild and trained *R. flavefaciens* strains grown in xylan gave higher xylanase activity than the cultures grown in medium containing cellobiose. This finding is also supported by previous studies that state that xylanase is induced by xylan (28).

Effects of three different growth environments and four different incubation times on xylanase activities of both wild and trained cultures are summarised in Table 2. Total cell, supernatant and specific cell xylanase activities of the trained strain were found to be higher than the wild strain when grown in ryegrass for 1 day ($P<0.05$, $P<0.01$ and $P<0.05$ respectively). However, when grown in medium containing cellobiose only, differences between specific supernatant activities were found to be significant ($P<0.01$). When xylan was used as the energy and carbon source, wild type gave higher total and specific cell activity ($P<0.1$ and $P<0.01$ respectively). However, higher supernatant activity was obtained from the trained type ($P<0.05$).

Total cell, supernatant and specific cell activities of the trained strain were found to be higher than that those of the wild strain when grown in ryegrass for 3 days ($P<0.01$, $P<0.1$ and $P<0.01$ respectively). When cellobiose was used as the energy source wild types gave higher total and specific supernatant activity ($P<0.01$). No statistically significant cell-associated activity was found between the two types. When strains were grown in medium containing xylan, gave higher performance in terms of total and specific cell activity. These data show that trained types gave higher xylanase activity when only grown in medium containing ryegrass. This was probably due to sub-culturing bacteria for a long time in ryegrass resulting in adaptation of the strain to degrade ryegrass or a mutation might have occurred in the enzyme control mechanism (28).

Cell-associated xylanase activity of the trained strain was found to be higher than that of the wild strain when grown in ryegrass for 7 days ($P<0.05$). When xylan was used as the energy source supernatant activity of wild types was found to be higher ($P<0.01$). However, along with increasing incubation time, cell-associated xylanese activity of both ryegrass grown strains increased while no significant effect was observed in cellobiose grown cultures. When xylan was used as the energy source, as observed for CMCase activity, a negative relation between incubation time and xylanase activity was observed.

Finally, it has been shown that cellulase and xylanase activities of *R. flavefaciens* were affected by the different energy sources probably due to regulation of genes that are encoding these activities by the growth busbtrates (17,36). According to SDS-PAGE zymogram analysis, different cellulase and xylanase active protein bands were observed from different cultures grown in different sources (data not shown). This finding also supports the regulation of cellulase and xylanase enzymes, and each growth substrate (Table 1, 2) may switch on or switch off the different genes that encode cellulase and xylanase activities (27,33,39).

Table 2. Xylanase activity of two different forms of *R. flavafaciens* 17 grown in different growth substrates at the different time intervals.

Xylanase activities						
			Total activities (nm/ml culture/min)		Specific activity (nm/mg protein/min)	
Incubation time	Energy source	Culture	Cell	Supernatant	Cell	Supernatant
1 day	Ryegrass	WT	0.99±0.46**	0.70±0.05***	10.83±4.12**	87.00±13
		TT	2.74±0.97	1.20±0.13	27.00±9.97	91±21
1 day	Cellobiose	WT	4.64±0.77	1.01±0.20	52.52±7.4	201.4±40.9***
		TT	3.95±0.35	0.93±0.06	44.16±4.7	76.17±24.2
1 day	Xylan	WT	11.26±2.8*	1.33±0.09**	132.8±24.6***	209.4±65.5
		TT	7.46±1.7	1.64±0.18	74.1±19.4	225.5±42.5
3 days	Ryegrass	WT	2.49±0.5***	1.63±0.29*	25.3±4.3***	172.3±11.5
		TT	4.63±0.7	1.96±0.11	53.5±8.3	183.7±34.5
2 days	Cellobiose	WT	3.44±0.2	1.57±0.03***	38.7±3.1	167.4±11.5***
		TT	3.22±0.05	0.96±0.18	40.7±2.2	58.3±16.7
3 days	Xylan	WT	5.81±1.1**	1.70±0.38*	59.8±22.5*	257.3±80.9**
		TT	3.08±1.1	1.00±0.50	29.6±10.8	110.7±47.8
7 days	Ryegrass	WT	5.22±0.3**	1.33±0.18	53.9±5.9**	218.1±57.3
		TT	7.44±1.6	1.31±0.11	85.7±20.1	261.6±22.1
7 days	Xylan	WT	5.15±1.5	2.56±0.23***	52.9±15.6	476.5±125.9***
		TT	5.05±1.9	1.82±0.22	48.2±18.7	108.2±17.59

WT: wild type of *R. flavefaciens* 17 strain
TT: trained type of *R. flavefaciens* 17 strain
*, **. *** Statistically significant value, P<0.1, P<0.05, and P<0.01 respectively

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