Journal of Applied Microbiology 2002, **92**, 140–146

Assessment of the fructanolytic activities in the rumen bacterium *Treponema saccharophilum* strain S

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829/7/01: received 18 April 2001, revised 17 July 2001 and accepted 23 July 2001

A. KASPEROWICZ AND T. MÍCHALOWSKI. 2002.

Aims: To characterize the fructose polymer degrading enzymes of rumen bacterium *Treponema saccharophilum* strain S.

Methods and Results: Conventional methods were used to examine bacterial growth and enzyme activities. Electrophoretic zymogram under native conditions, and thin layer chromatography, were applied to identify and characterize the enzymes. *Treponema saccharophilum* utilized Timothy grass fructan, inulin and sucrose but not free fructose. Timothy grass fructan was degraded at a significantly higher rate than sucrose and inulin. Two fructanolytic enzymes were found in the soluble, and one in the membrane fraction of bacterial cell extract. The first degraded each mentioned carbohydrate to monosaccharides. The second released oligosaccharides only from Timothy grass fructan.

Conclusions: The bacterium *T. saccharophilum* strain S is capable of synthesizing non-specific β-fructofuranosidases and 2,6-β-D-fructan fructanohydrolase. The enzymes are of constitutive character.

Significance and Impact of the Study: It has been stated for the first time that the 2,6-β-D-fructan fructanohydrolase is synthesized by the rumen bacterium *T. saccharophilum*. This organism appears to be responsible for grass fructan degradation in the rumen.

INTRODUCTION

Fructose polymers (fructans) are important storage carbohydrates in some plants that are ingested by herbivore mammals as feed. Fructofuranosyl units in fructans are linked by either β-2-1 or β-2-6 glycosidic bonds and termed accordingly inulins or levans; the first are present in roots and tubers of the *Compositae* family while the second are in leaves and stems of temperate grasses (Chesson and Forsberg 1988).

It is generally accepted that fructan digestion in mammals depends on the activity of the microbes harboured in the digestive tract of the host. The microbial enzymes with an exo and endo mode of action, such as β-fructofuranosidase (EC 3.2.1.80 or EC 3.2.1.26), inulinase (2-1-β-D-fructan fructanohydrolase, EC 3.2.1.7) and levanase (2-6-β-D-fructan fructanohydrolase, EC 3.2.1.65), are involved in degradation of the fructose polymers (Uchiyama 1993).

Inspection of the available literature shows that information on both the degradation and fermentation of grass fructan in the rumen, and the fructanolytic enzymes synthesized by rumen bacteria, is limited (Thomas 1960; Czerkawski and Lumsden 1971; Biggs and Hancock 1998; Ziolecki *et al*. 1992). However, current opinion contends that inulin and levan type fructose polymers are readily and completely digested and metabolized in the rumen ecosystem (Chesson and Forsberg 1997).

Previously published results of an investigation on the fructanolytic activity of some strains of rumen bacteria, which was carried out in this laboratory, suggested that β-fructofuranosidase could be responsible for Timothy grass fructan degradation (Ziolecki *et al*. 1992). This study was performed to obtain more detailed information concerning the fructanolytic enzymes of *Treponema saccharophilum* strain S.

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MATERIALS AND METHODS

Bacteria and culture medium

The rumen bacterium *Treponema saccharophilum* strain S originated from the collection maintained in this laboratory. The method of isolation and characteristics of this strain of bacterium have been described previously (Ziolecki *et al*.

1992). The culture medium was prepared according to the Anaerobe Laboratory Manual (Anon. 1973) and Hungate (1969), and consisted of (g l^{-1}): 0·45 $(NH_4)_2SO_4$, 0·45 KH_2PO_4, 0·9 NaCl, 0·09 $MgSO_4.7H_2O$, 0·09 $CaCl_2$, 0·45 K_2HPO_4, 7·5 $NaHCO_3$, 1 peptone, 1 yeast extract, 0·25 cysteine hydrochloride, 0·001 resazurine and 0·3 ml l^{-1} thioglycolic acid. The medium was supplemented with clarified and pasteurized rumen fluid at 300 ml l^{-1}. Fructose, glucose, sucrose, inulin and Timothy grass fructan were separately pasteurized twice at 24 h intervals and added to the sterilized medium at a final concentration of 5 g l^{-1}.

Growth experiments

Experimental cultures were initiated by inoculation of bacteria in the early stationary phase of growth into tubes containing 7 ml of the medium supplemented with the appropriate carbohydrate. They were cultured overnight (16 h) at 38°C, followed by measurements of both the optical density (O.D.) and carbohydrate content in the culture medium. This latter was performed in order to determine saccharide utilization.

Cell extract preparation

Bacteria were centrifuged at 17 000 *g* for 25 min at 4°C, washed once with cold 0·9% (w/v) NaCl and sedimented again under the same centrifugation conditions. The pellet was resuspended in 7 ml cold (4°C) water, and then disrupted in a sonicator (Measuring and Scientific Equipment Ltd, London, UK). The sonicated material was centrifuged as described above to remove the unbroken cells and other particulate matter. The supernatant fluid was collected and used as the bacterial cell extract whereas the sediment was discarded.

Cell extract fractionation

Cell extract was prepared from large-scale sucrose or glucose bacterial cultures (1·5 l) by the method described above, except that washed bacterial cells were resuspended in 20 ml cold water and then disrupted. The cell extract obtained was then centrifuged at 100 000 *g* and 4°C for 1 h. The supernatant fluid was collected as the soluble fraction. The pellet was washed once with cold water and centrifuged again at 100 000 *g*. The supernatant fluid was discarded whereas the sediment was suspended in 20 ml water (4°C) and used as the sedimentable, i.e. the membrane fraction.

Protein separation

Protein was precipitated with ammonium sulphate at either 80% (cell extract and soluble fraction) or 45% (w/v) saturation (membrane fraction). The precipitate was dissolved in a small volume of cold (4°C) distilled water, dialysed against the same water, then lyophilized and stored at –20°C. To release the protein from the membrane fragments, both the cell extract and its sedimentable fraction (see above) were treated with a Triton X-100 solution at a final concentration of 0·4% (w/v) for 24 h at 4°C, prior to protein precipitation (Mäntsala and Zalkin 1979). The preparation was then centrifuged at 100 000 *g* as described above and protein was precipitated from the collected supernatant fluid.

Enzyme assay

The optimum pH was determined by incubation of Timothy grass fructan, inulin and sucrose with bacterial cell extract, and measurement of the released reducing sugars. The incubated mixture contained 0·2 ml bacterial cell extract (approximately 40 μg protein), 0·25 ml 1% (w/v) aqueous solution of the appropriate carbohydrate and 0·8 ml 100 mmol l^{-1} McIlvaine's buffer (citric acid/sodium phosphate, pH 4·0 and 4·6) or 100 mmol l^{-1} potassium phosphate buffer (pH 5–8). The mixtures were incubated at optimum temperature (45°C) for 30–60 min in the case of Timothy fructan, or 3–5 h in the case of sucrose and inulin.

The degradation rate of Timothy grass fructan, inulin and sucrose was determined in samples composed of 0·25 ml bacterial cell extract, 0·25 ml aqueous substrate solution at a concentration of 1% (w/v) and 0·75 ml 0·02 mol l^{-1} potassium phosphate buffer (pH 6·0). Samples at a final volume of 1·25 ml were incubated for 4 h at 45°C.

The reducing sugars released from substrates were determined immediately after incubation had finished.

Fructanolytic enzyme identification

For this purpose, the fructanolytic enzyme zymogram was prepared following native polyacrylamide gel electrophoresis (NPAGE) of bacterial protein. NPAGE was performed using a 5% gel. Timothy grass fructan at a final concentration of 0·2% (w/v) was co-polymerized with polyacrylamide during gel preparation. Buffers containing 5 and 50 mmol l^{-1} Tris/glycine (pH 8·3) were used as electrode and gel buffer, respectively. The pH 8·3 of the gel buffer was used to prevent the co-polymerized fructan from being digested by fructanolytic enzymes during the electrophoretic separation of proteins. Samples (10 μl) were applied to each lane. Electrophoresis was carried out at a constant current of 94 V in a mini dual gel unit for slab electrophoresis (Sigma). The electrophoretic separation of proteins was followed by incubation of the gel for 15 min at 45°C in 100 mmol l^{-1} citric acid/sodium phosphate McIlvaine's buffer (pH 6·0), and fructanolytic activities on the gel were visualized by

staining with 2,3,5-triphenyltetrazolium chloride according to Gabriel and Wang (1969). The protein was routinely stained by Coomassie brilliant blue.

Fructanolytic enzyme isolation

Enzymes of the soluble fraction were isolated from the polyacrylamide gel following the electrophoretic separation of protein (see above). Gel slices containing active enzymes were excised, immersed in 100 mmol l^{-1} McIlvaine's buffer (pH 6·0), disintegrated in a glass homogenizer at 4°C and the homogenate used as an enzyme source.

End products identification

Products of Timothy grass fructan, inulin and sucrose hydrolysis were identified by thin layer chromatography. The reaction mixture consisted of 4·5 mg of appropriate carbohydrate and 0·9 mg of the lyophilized soluble or membrane fraction protein preparation, or 0·5 ml of homogenate obtained by disintegration of gel slices with separated enzyme (see above). Protein content in the lyophilizates was 48 and 31%, respectively. Samples were adjusted to a final volume of 1·65 ml with 100 mmol l^{-1} McIlvaine's buffer (pH 6·0) and incubated for 48 h at 40°C. Suspensions of excised enzyme and substrates alone were incubated simultaneously as controls. To prevent any bacterial growth in incubated material, one drop of toluene was added to each tube. Enzyme digests (10 μl) were spotted on TLC silufol plates (Sklo Union, Votice, Czech Republic). The plates were developed three times with *n*-butanol–isopropanol–acetic acid–water (35:25:20:10) [v/v] according to Lüscher *et al.* (1993) and visualized with ketose-specific urea/phosphoric acid reagent (Wise *et al.* 1955).

Quantitative measurements

These were performed using a Beckman DU-64 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA). Optical density of the bacterial cultures was determined at 660 nm. Reducing sugars released from Timothy grass fructan, inulin and sucrose by bacterial cell extract were measured using dinitrosalicilic acid reagent according to Groleau and Forsberg (1981), with fructose as standard. Enzyme activity was expressed as micromoles of fructose released from substrate per milligram of protein per hour. Total sugars in the culture medium before and after cultivation of bacteria were quantified by the anthrone method (Southgate 1991) with Timothy grass fructan, inulin, sucrose and glucose as standard, respectively. Protein was quantified by the bicinchoninic acid reagent using bovine serum albumine (BSA) as a standard (Smith *et al.* 1985).

Chemicals

All chemicals for electrophoresis and protein determination were supplied by Sigma Chemical Co. Glucose, sucrose and inorganic reagents were from POCH Gliwice (Poland). The chicory inulin was supplied by Aldrich Chemical Company, Inc., and fructose by Loba-Chemie, Wien-Fischamend. Yeast extract and bacto-casitone were obtained from Difco Laboratories. All chemicals were of analytical grade. The Timothy grass fructan was isolated and purified in the laboratory according to Ziolecki *et al.* (1992).

Statistical analysis

Mean values and their standard deviations (S.D.) were calculated from the quantitative results obtained. Significance of differences between mean values were examined using Student's *t*-test. All statistical calculations were made according to Ruszczyc (1970).

RESULTS

Bacterial growth

Bacteria were able to grow on Timothy grass fructan, inulin, sucrose and glucose as the sole carbon source. No growth was observed when the culture medium was supplemented with fructose. The optical densities of the Timothy grass fructan- and sucrose-grown population (Table 1) did not differ significantly from each other, while that of the glucose-grown population was lower ($P < 0·05$). The optical density of the inulin-grown culture was about twofold lower than those of the other cultures ($P < 0·001$). Carbohydrate utilization varied from about 33% to over 77% of the initial doses and was closely related to optical density of the cultures ($r = 0·96$; $P < 0·01$).

Carbohydrate digestion

Bacterial cell extract degraded Timothy grass fructan, inulin and sucrose; the activity was highest at pH 6·2, 6·0 and 6·2, respectively (Fig. 1). The degradation rate of the carbohydrates varied from about 0·7 to 34·7 μmol reducing sugars released from substrate mg $protein^{-1}$ h^{-1} depending on the carbon source in the culture medium (Table 2). Sucrose was hydrolysed at a significantly lower rate than Timothy grass fructan ($P < 0·01$), whereas inulin was degraded at the lowest rate of the three carbohydrates tested ($P < 0·05$). No fructanolytic activity was found in the post-culture medium.

Enzyme detection

Two fructanolytic activities were found following electrophoretic separation of protein precipitated from the bacterial

Table 1 Population density of *Treponema saccharophilum* strain S and substrate utilization from the culture medium by bacteria during 16 h growth on the media initially containing 5g l^{-1} different carbohydrates as sole carbon source

Item	Carbon source in medium			
	T.g. fructan	Inulin	Sucrose	Glucose
Population density (O.D. 660 nm cm^{-1})	1·36* ± 0·02	0·68 ± 0·02	1·35 ± 0·07	1·22 ± 0·08
Substrate utilization (% of initial concentration)	77·1 ± 1·60	33·7 ± 0·8	73·4 ± 1·50	57·4 ± 3·78

*Mean value ± S.D.; $n = 4$.
T.g. = Timothy grass.

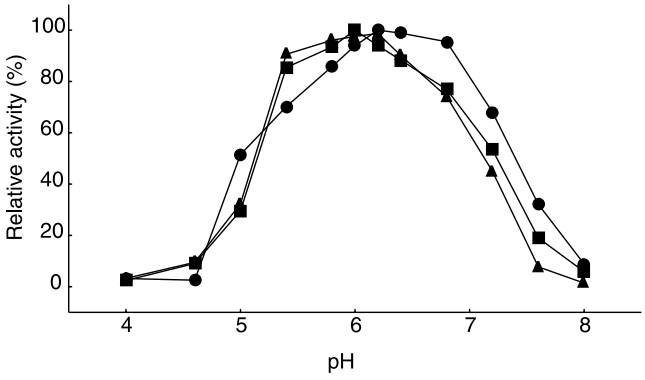

Fig. 1 Effect of pH on the degradation of Timothy grass fructan (●), inulin (■) and sucrose (▲) by cell extract of *Treponema saccharophilum* strain S. Results are the mean of the three independent experiments performed at optimum temperature of 45°C as described in Material and Methods. The highest value in each experiment was taken as 100% to calculate the relative activity at a particular pH

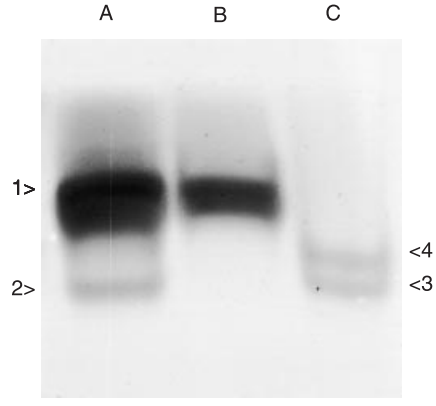

Fig. 2 Fructanolytic activity detected in different fractions of protein of *Treponema saccharophilum* strain S by the zymogram technique. Lane A: bacterial cell extract; lane B: membrane fraction of bacterial cell extract; lane C: soluble fraction of the bacterial cell extract. About 29, 20 and 27 μg protein were analysed in lanes A, B and C, respectively

Table 2 The degradation rate of Timothy grass fructan, inulin and sucrose by cell extract of bacteria growing in media initially containing 5g l^{-1} different carbohydrates as sole carbon source

Carbon source in the medium	Degradation rate (μmol fructose released mg $protein^{-1}$ h^{-1}) of		
	T.g. fructan	Inulin	Sucrose
Timothy grass fructan	25·9* ± 5·04	0·8 ± 0·29	2·1 ± 0·47
Inulin	34·7 ± 5·19	0·9 ± 0·26	1·2 ± 0·55
Sucrose	16·4 ± 3·85	0·7 ± 0·17	1·6 ± 0·47
Glucose	28·4 ± 7·35	0·8 ± 0·63	1·8 ± 0·55

*Mean value ± S.D.; $n = 9$.

cell extract (Fig. 2, lane A), but treatment of this preparation with Triton X-100 solution, prior to protein precipitation, was necessary to separate enzyme no. 1 from membrane fragments. Omission of this step in the protein separation procedure resulted in location of the enzyme at the top of the line (data not shown). Centrifugation of the bacterial cell extract at 100 000 *g* showed that fructanolytic activities were present in both the membrane (sediment) and soluble (supernatant) fractions. Protein precipitated from the membrane fraction was shown to contain a single fructanolytic activity, which corresponded to enzyme no. 1 of the bacterial cell extract as detected by the zymogram technique (Fig. 2, lane B). Two activities were found when a similar procedure was applied to the examination of protein precipitated from the soluble fraction (Fig. 2, lane C).

Further investigations showed that ammonium sulphate at 45% w/v saturation precipitated the fructanolytic activity from the membrane fraction, giving partially purified enzyme (Fig. 3).

End products of carbohydrate hydrolysis

Enzymes present in the protein precipitated from the soluble fraction of the cell extract degraded Timothy grass fructan to fructose. Fructose was also found to be the end product of sucrose and inulin degradation (Fig. 4). A similar activity pattern was shown by both soluble enzymes isolated from the gel by excision (Fig. 5). The membrane-bound

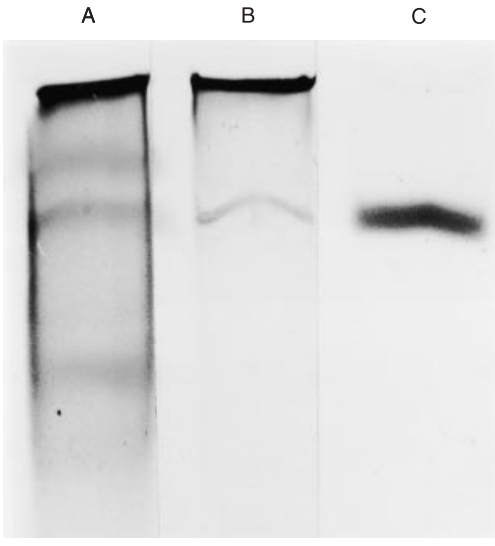

Fig. 3 Coomassie brilliant blue stained protein from the cell extract of *Treponema saccharophilum* strain S precipitated with ammonium sulphate at 80% saturation (lane A), and from its sedimentable (membrane) fraction at 45% saturation (lane B); lane C: fructanolytic activity detected in protein from the membrane fraction precipitated with ammonium sulphate at 45% saturation. About 18 (lane A) and 17 μg protein (lanes B and C) were analysed

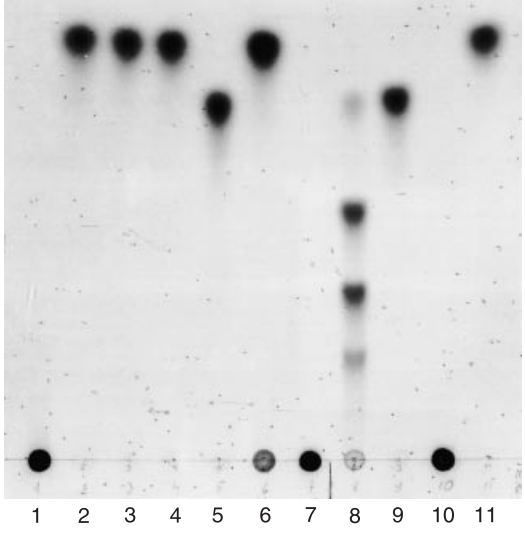

Fig. 4 TLC of the products released from different fructose polymers by the enzymes from *Treponema saccharophilum* strain S: Timothy grass fructan (1), fructose (3 and 11), sucrose (5), inulin (7) standard. Products released from Timothy grass fructan (2), sucrose (4) and inulin (6) by the enzymes from the soluble fraction of the bacterial cell extract. Products released from Timothy grass fructan (8), sucrose (9) and inulin (10) by the enzyme from the membrane fraction of the cell extract

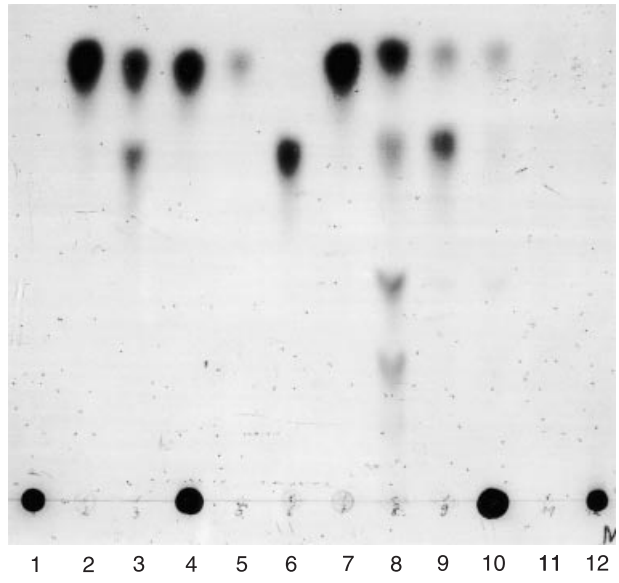

Fig. 5 TLC of the products released from different fructose polymers by the enzymes extracted from excised gel pieces following native PAGE of soluble fraction of cell extract protein (see Fig. 2, lane C in detail). Timothy grass fructan (1), sucrose (6), fructose (7) and inulin (12) standard. Products released from Timothy grass fructan (2), sucrose (3) and inulin (4) by the enzyme 3. Products released from Timothy grass fructan (8), sucrose (9) and inulin (10) by enzyme 4. Enzymes 3 and 4 incubated without substrate (5 and 11, respectively)

enzyme hydrolysed Timothy grass fructan to oligosaccharides. Neither sucrose nor inulin was degraded.

DISCUSSION

It was found that *T. sacharophilum* strain S was able to grow *in vitro* in the culture medium supplemented with Timothy grass fructan, inulin, sucrose and glucose, although the density of the bacterial population in the medium with the inulin was significantly lower. Restricted growth on inulin could be the result of a limited energy supply to the cells, as the ability of *T. sacharophilum* strain S to utilize this carbohydrate was significantly lower than the others used in the experiment. Paster and Canale-Parola (1985), who described this species of rumen bacterium, also found that growth yield was better on glucose and sucrose than on inulin.

Treponema saccharophilum strain S grew well on sucrose whereas no growth was observed when fructose was used as carbon source in the culture medium. A similar phenomenon was observed in the case of the cellulolytic bacterium, *Ruminococcus flavefaciens*, which utilized cellobiose but not glucose because of its inability to transport this hexose across the cell membrane (Helaszek and White 1991). It is possible that a fructose transport mechanism was lacking in the cell

membrane of *T. saccharophilum* strain S and that due to this, they were unable to take up the extracellular fructose.

High fructanolytic activity was detected in the glucose-grown cultures. This could suggest a constitutive character of the fructose polymer degrading enzyme(s), consistent with the findings of Walker *et al*. (1983) and Jacques *et al*. (1985) who postulated the constitutive character of fructanase produced by some strains of *Streptococcus mutans* growing in medium supplemented with D-glucose.

Timothy grass fructan was degraded at the lowest rate by the cell extract obtained from sucrose-grown bacteria. This could result from a restricting effect of sucrose present in the culture medium upon the production of enzymes involved in degradation of this polysaccharide. This is in good agreement with the findings of McGavin *et al*. (1990) who did not observe synthesis of one of the three β-endoglucanases by the cellobiose-grown cellulolytic bacterium *Fibrobacter succinogenes* S85.

Maximal enzyme activities were obtained at pH 6·0–6·2. There are no data concerning the optimum conditions for degradation of the levan type fructan by rumen micro-organisms. Sucrose, however, was degraded at the highest rate by extract from the rumen holotrich ciliates *Isotricha prostoma* at pH 5·5–6 (Dauvrin and Thinès-Sempoux 1989). A similar pH optimum was also found for inulinase originated from *Bacillus subtilis* (Allais *et al*. 1987), and for fructanase from oral bacteria (Walker *et al*. 1983).

The present results showed that the rumen bacterium *T. saccharophilum* strain S synthesized two types of fructanolytic enzymes. The enzyme associated with the membrane fraction seems to be an endo-hydrolase which specifically cleaves the 2,6-β-D glycosidic linkages inside the fructose polymer chain, giving different oligosaccharides as end products. An enzyme with a very similar mode of action is 2,6-β-D-fructan fructanohydrolase (EC 3.2.1.65), also termed endo-levanase (Uchiyama 1993). As far as is known, no such enzymes have been identified in the rumen bacteria yet (Chesson and Forsberg 1997). It was, however, produced by *Bacillus* sp. isolated from soil (Murakami *et al*. 1992).

In contrast to the membrane-bound enzyme, both enzymes from the soluble fraction of the cell extract degraded all three carbohydrates tested to monosaccharide as the main or the only product. The presence of oligosaccharides in the end products of Timothy grass fructan hydrolysis catalysed by one of these enzymes could suggest that the reaction was not completed, presumably due to a low concentration of protein in the reaction mixture and/or low activity of this enzyme. In fact, the protein content in the enzyme sample was too low to be estimated by the method used here. The ability to hydrolyse the 2,6-β-D linkages present in Timothy grass fructan, as well as 2,1-β-D-glycosidic linkages in chicory inulin or sucrose, is in good accord with the mode of action of fructan-β-fructosidase (EC 3.2.1.80) and/or β-D-fructofuranosidase (EC 3.2.1.26), which are mentioned as non-specific β-fructofuranosidases (Uchiyama 1993). Non-specific β-fructofuranosidase has been found in mixed bacteria isolated from the rumen (Thomas 1960; Czerkawski and Lumsden 1971) and rumen holotrich protozoa (Howard 1959; Thomas 1960; Dauvrin and Thinès-Sempoux 1989). The presence of this type of enzyme in different strains of rumen bacteria has been postulated previously (Ziolecki *et al*. 1992). It has also been synthesized by *Lactobacillus paracasei* subsp. *paracasei* P 4134 (Müller and Seyfarth 1997).

The data obtained indicate that the enzymes identified were associated with two different fractions of bacterial cells. It is proposed that the endo-levanase is associated with the outer membrane of the bacterial cells and that this makes possible the utilization of energy from fructose polymers in which the fructofuranosyl residues are bound by the 2,6-β-D-linkages through the following mechanism: the membrane-bound enzyme degrades the fructose polymers to oligosaccharides; oligosaccharides are transported into the bacteria across the cell membrane and are digested by the intracellular β-fructofuranosidase to monosaccharides. This hypothesis needs experimental confirmation.

The mechanism described above does not explain utilization of inulin by *T. saccharophilum*, as the membrane-bound enzyme is unable to degrade this polysaccharide. It cannot be precluded that inulin is transported into the bacterial cell and degraded there by a non-specific β-fructofuranosidase type of enzyme to fructose. A similar model has been proposed by Anderson (1995) for the utilization of starch by *Ruminobacter amylophilus*.

ACKNOWLEDGEMENT

Some of these results were briefly reported at the 8th Seminar on Inulin, 1–2 July 1999, Lille, France.

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