

10. ABSTRACT

Green plants and specially some grasses are the important components of the natural diet of ruminants. The major constituents of green plants are the structural polysaccharides like cellulose and hemicellulose. Numerous plants synthesize also sucrose and complex carbohydrates which consist of fructose and deposited them as storage carbohydrates. Ruminants do not synthesize enzymes involved in the digestion of carbohydrates listed above and depend entirely on the activity of microorganisms inhabiting their rumen. There is the abundant literature on digestion and metabolism of structural carbohydrates in this chamber. In contrast to that the bibliography concerning utilization of sucrose and fructans in the rumen is scanty. This opinion concerns both identification of microbial species involved in metabolism of sucrose and fructans as well as their ability to digest mentioned carbohydrates and to use the obtained products to cover the requirements for energy.

The aims of the studies were: 1) to verify the species affiliation of bacteria selected to perform the study, 2) to determine the ability of the selected strain of bacteria to grow on culture medium supplemented with sucrose or fructose polymers as a sole carbon source, 3) to examine the ability of bacteria to digest sucrose and fructans and to identify enzymes involved in this process, 4) to examine the ability of the tested bacteria to ferment sucrose, polymers and oligomers of fructose and to identify the major end products of fermentation.

The subject of the study was strain k3 of bacteria which were isolated in 1999 from the ovine rumen with an use of culture medium supplemented with Timothy grass fructan as a sole carbon source. On the basis of morphological and physiological features, the isolated bacteria were identified to be the representatives *Butyrvibrio fibrisolvens*. Verification of this affiliation was done with an use of genetic methods. It revealed that the examined strain of bacteria represents the species *Pseudobutyrvibrio ruminis*.

The results of cultivation of bacteria with an use of media supplemented separately with sucrose, as well as with the polymers and oligomers of fructose and either fructose or glucose showed very intense growth of the optical density of bacterial cultures in the course of the first 8-12 h of incubation period. It was stated there that the rate of increase in population density depended on the kind of carbohydrate

supplemented to the culture medium. The highest growth was found when culture medium was supplemented with glucose and Timothy grass fructan whereas the lowest followed the supplementation of growth medium with inulin. It was observed that an continuous decrease in optical density of bacterial cultures up to 50 – 64% followed the achievement of maximum values of this parameter. It was also stated that changes in optical density were accompanied by the changes in concentration of carbohydrate in culture medium. The concentration of the Timothy grass fructan, inulo-oligosaccharides, sucrose, glucose and fructose diminished by 94 – 96% of initial value during the first 8 – 12 hours of incubation period. The inulin concentration decreased, however, by 34% only. No statistically significant changes in carbohydrate concentration were observed during the last 12 – 16 hours of experiment. So, the obtained results show that increase in optical density of cultures was accompanied by the continuous decrease in concentration of carbohydrates added to the culture medium. This can suggests that the loss in carbohydrate concentration resulted from their intake by cultivated bacteria. This suggestion has been confirmed by results of the identification of carbohydrates present in post-culture medium. They showed the presence of polysaccharide in the medium supplemented with inulin. Thus due to this perhaps the growth of bacteria was restricted when culture medium was supplemented with this polysaccharide.

Enzymatic studies were performed using crude enzyme preparation as well as its sedimentable and soluble fractions and selected subfractions of the latter. It was stated that digestion activity of bacteria depended on the carbohydrate supplemented to the culture medium. It was stated that the crude enzyme preparation of the Timothy grass fructan grown bacteria degraded the same carbohydrate with the highest velocity. On the other hand, the crude enzyme preparation of inulin and sucrose grown bacteria exhibited the highest activity in digestion of sucrose. It was also stated there that crude enzyme preparations originating from bacteria maintained on the both simple sugars exhibited distinctly lower activity in comparison to the other examined enzyme preparations. So, these finding suggested that carbohydrates supplemented to the culture medium were the factors inducing selectively synthesis of fructanolytic and saccharolytic enzymes in the cells of examined bacteria.

The crude enzyme preparation, it's sedimentable as well as soluble fractions and the most active subfractions of the latter were used to identify and characterize enzymes. Four enzymes have been identified in general. Two of them were able to

catalyze digestion of only the Timothy grass fructan i.e. the polymers composed of fructose residues linked to each other with β -2,6-glycosidic bonds. It was also stated there that fructo-oligosaccharides consisting of 2 to 6 fructose residues were end products of substrate degradation. On the basis of the obtained results the both enzymes were identified as 2,6- β -D-fructan fructanohydrolases (EC 3.2.1.65), called commonly "endolevanases". It was also found that the both enzymes exhibited the maximum activity at 6,0 pH and at 45°C. The molecular masses of identified endolevanase enzymes were 60,000 and 53,000, respectively. In order to get more detailed characterization of enzymes, the Michaelis constant (K_m) and the maximal velocity of reaction (V_{max}) were determined. The K_m of particular enzymes were 0,75 and 1,75%, respectively. These results show that the first endolevanase exhibited higher affinity to substrate than the second. The maximal velocity of reaction was 2,03 and 2,6 μ mol product/mg protein per minute, respectively. The specific activity of partially purified endolevanases were 0,13 and 0,23 μ mol of released product/mg protein per minute.

The third identified enzyme was unspecific β -fructofuranosidase i.e. β -D-fructan fructohydrolase (EC 3.2.1.80) and/or β -D- fructofuranoside fructohydrolase (EC 3.2.1.26). The enzyme catalyzes degradation of sucrose as well as releases the terminal fructose from the chain of fructose residues linked by β -2,6- and β -2,1-glycosidic bonds. The molecular mass of enzyme was 121,500. It was stated, that the highest rate of inulin digestion took place at pH 6,5 and these of Timothy grass fructan and sucrose - at pH 6,0. The optimal temperature of degradation was 40°C. No saturation of enzyme with sucrose as well as straight line relationship between the degradation rate of sucrose and its concentration were found. These findings did not allow any calculation as K_m as well as V_{max} of relevant reactions.

Specific sucrose phosphorylase was the fourth identified enzyme. The identified enzyme is chemically the sucrose glucosyltransferase (EC 2.4.1.7) The performed analysis showed that fructose, glucose-1-phosphate and small quantities of free glucose were the end products of sucrose digestion. It was also stated that the identified enzyme possessed also the synthase features. The activity of it depended on presence of inorganic phosphate in reaction mixture and it's optimal concentration was 56 mmol. The identified enzyme exhibited maximum activity at pH 6,0 and temperature 45°C. It was stated that K_m of glucose-1-phosphate formation and fructose release were 4,4mmol and 6,39 mmol of sucrose, respectively. On the other hand, the maximal velocities of mentioned reactions were 1,19 and 1,56 mmol of products/mg of protein per minute,

respectively. The obtained results suggested also some ability of enzyme to catalyze the hydrolytic breakdown of sucrose. Thus it seems to be probable that phosphorolytic cleavage of this disaccharide could be supported by its hydrolytic breakdown, especially at high substrate concentration in the reaction mixture. The performed study showed that sucrose phosphorylase was present in soluble fractions of crude enzyme preparation originating from Timothy grass fructan, inulin and sucrose grown bacteria. The specific activity of partially purified enzyme was 0,29 μmol of released products/mg protein per minute and its molecular mass – 48,000.

In fermentation experiments was found that during incubation of bacteria with particular carbohydrates, the L-lactic and butyric acids were released by these microorganisms to the culture medium. The concentration of listed acids increased from 0,04-0,05 mmol/100ml to 1,1-3,5 mmol/100ml and from 0,018-0,019 mmol/100ml to 0,3-0,44 mmol/100ml, respectively. It depended on fermented carbohydrate. The smallest concentration of lactate was found when inulin was fermented and the highest – when fructose was incubated with bacteria. Likewise, the smallest increase in butyrate concentration was observed during fermentation of inulin. The highest was stated, however when sucrose and Timothy grass fructan were fermented. It was shown that the lactate production rate ranged from 0,30 to 7,32 μmol /mg protein per hour and this of butyrate from 0,1 to 0,35 μmol /mg protein per hour in relation to the fermented carbohydrate and the time of incubation. The obtained results indicated that lactate fermentation was the main pathway of metabolism of examined carbohydrates in the cells of bacteria *Pseudobutyrvibrio ruminis* strain k3.

Summary: The ability of rumen bacteria *Pseudobutyrvibrio ruminis* stain k3 to utilize sucrose as well as the Timothy grass fructan and inulo-oligosaccharides from the one side and inability of them to utilize large molecule of inulin from the other could suggest an adaptation of mentioned microorganisms to use carbohydrates present mainly in grasses, which belong to the permanent components of the natural diet of ruminants.