Phosphorolytic Cleavage of Sucrose by Sucrose-Grown Ruminal Bacterium *Pseudobutyribrio ruminis* Strain k3

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**ABSTRACT.** Rumen bacterium *Pseudobutyribrio ruminis* strain k3 utilized over 90 % sucrose added to the growth medium as a sole carbon source. Zymographic studies of the bacterial cell extract revealed the presence of a single enzyme involved in sucrose digestion. Thin layer chromatography showed fructose and glucose-1-phosphate (Glc1P) as end products of the digestion of sucrose by identified enzyme. The activity of the enzyme depended on the presence of inorganic phosphate and was the highest at the concentration of phosphate 56 mmol/L. The enzyme was identified as the sucrose phosphorylase (EC 2.4.1.7) of molar mass \( \approx 54 \text{ kDa} \) and maximum activity at pH 6.0 and 45 °C. The calculated Michaelis constant (\( K_m \)) for Glc1P formation and release of fructose by partially purified enzyme were 4.4 and 8.56 mmol/L while the maximum velocities of the reaction (\( v_{\text{lim}} \)) were 1.19 and 0.64 µmol/L per mg protein per min, respectively.

Sucrose (storage disaccharide in some plants) consists of \( \alpha \)-D-glucose and \( \beta \)-D-fructose linked by 1,2-glycosidic bond. Ruminants do not synthesize saccharolytic enzymes and depend on the activity of rumen microbiota, mainly bacteria. Unfortunately, neither saccharolytic species of bacteria inhabiting the rumen nor enzymes involved in the digestion of sucrose have as yet been well recognized. The studies performed to date suggest that *Streptococcus bovis*, *Selenomonas ruminantium* and some treponemes represent the saccharolytic microorganisms of the rumen (Martin and Russell 1987; Ziołek et al. 1992). The ability of the bacterium *Pseudobutyribrio ruminis* strain A to utilize sucrose has been recently stated by Kasperowicz et al. (2009). The objective of this study was to characterize the saccharolytic property of strain k3 belonging to the same species and isolated from the ovine rumen (Guczyńska 1999).

**MATERIALS AND METHODS**

Microorganisms and culture media. The examined strain of bacteria originated from our own collection of pure cultures. It was isolated from the ovine rumen by Guczyńska (1999) and identified as *Butyrivibrio fibrisolvens* strain k3. The comparison of its 16S rRNA gene with the data from GenBank performed last year showed \( \geq 99 \% \) identity with *Pseudobutyribrio ruminis* (EU714408). The culture medium consisted of salt solution (Hungate 1969), filtered and pasteurized rumen fluid was supplemented with casitone, yeast extract, cysteine hydrochloride, thioglycolic acid and resazurine according to Guczyńska (1999). The medium was supplemented with sucrose to a final concentration of 0.5 % as a sole source of carbon.

Growth experiments. Bacterial cultures were initiated by an inoculation of a 2.5-mL sample of the stock culture from our collection to Erlenmeyer flasks filled with 25 mL of complete culture medium, and were grown anaerobically for 24 h at 40 °C. The absorbance of bacterial cultures as well as saccharide concentration in culture medium were measured just after beginning of incubation and 2, 5, 8, 10, 12 and 24 h thereafter.

Cell extract preparation, fractionation and molar mass estimation. Cultures of bacteria (750 mL) used to prepare the bacterial cell extract were grown overnight on sucrose as a sole carbon source. The bacteria were centrifuged (20000 \( g \), 25 min, 4 °C) and washed with 0.9 % NaCl (\( W/V \)). The pellet was resolved in 20 mL of cold (4 °C) distilled water and disrupted in an ultrasonic disintegrator (*MSE Ltd.*, UK) and centrifuged (20000 \( g \), 15 min). The supernatant was then collected and used as bacterial cell extract. The supernatant was centrifuged again (100000 \( g \), 1 h, 4 °C) to separate the membrane fragments (Kasperowicz and Michałowski 2002). The liquid (soluble) fraction was collected and used to purify saccharolytic enzyme(s)
and to estimate the molar mass. This was done by molecular filtration according to the protocol MW-GF 200 (Sigma) using a glass column (400 × 16 mm) packed with Sephadex G-150 (Pharmacia).

**Enzyme assays.** The saccharolytic activity was determined by quantification of the products released from sucrose by examined enzyme preparation. The reaction mixture consisted of 0.75 mL of 0.1 mol/L sodium–potassium phosphate buffer (pH 6.0), 0.25 mL sucrose solution and 0.25 mL of bacterial cell extract or its appropriate fraction. The mixture was incubated for 15–60 min at 40 °C. The reaction was stopped by immersion of the tubes in boiling water and the products released from sucrose were measured spectrophotometrically. McIlvaine buffer (0.1 mol/L) of pH ranging from 4 to 8 and temperature in the range of 20–70 °C was applied to determine the optimum conditions for the activity of examined enzyme(s). The influence of inorganic phosphorus on the activity of saccharolytic enzyme(s) was examined using either 20 mmol/L Tris-HCl buffer or sodium–potassium phosphate buffer of different phosphate concentration.

The saccharolytic enzymes were identified by zymogram technique (Gabriel and Wang 1969), which followed the native polyacrylamide gel electrophoresis (PAGE) according to Kasperowicz and Michałowski (2002). The digestion products of sucrose were identified by TLC according to Kasperowicz and Michałowski (2002). Analyses and calculations. The total reducing sugars released from sucrose were quantified using dinitrosalicylic acid reagent (Miller et al. 1959) and the total saccharides by anthrone method according to Southgate (1991). Glucose, glucose-1-phosphate and fructose were measured with the help of glucose/fructose assay kit (Megazyme) and phosphoglucomutase (Sigma) according to Kasperowicz et al. (2009). The protein was quantified using the bicinchoninic acid reagent (Smith et al. 1985) with bovine serum albumin as standard. The absorbance of bacterial cultures was determined spectrophotometrically at 660 nm on Beckman DU-64 Spectrophotometer. The Michaelis constant ($K_m$) and the maximum velocity of the reaction ($v_{lim}$) were determined according to Lineweaver–Burk equation.

**RESULTS AND DISCUSSION**

**Bacterial growth and saccharide utilization.** The absorbance of cultures of the sucrose-grown *P. ruminis* strain k3 increased logarithmically from about 0.04 ± 0.009 to >1.63 ± 0.086 AU (absorbance units) during the first 8 h of incubation; this increase was accompanied by continuous decrease in the concentration of sucrose added to the culture medium up to 98 % of the initial value. No further changes in the sucrose content were observed during the remaining 16 h of incubation period whereas the cell number in the population continuously decreased. This suggests that cultured bacteria utilized sucrose to cover the requirement for energy needed to support the synthesis of the cellular matter. This also shows that the examined microorganisms were equipped with enzyme(s) capable of catalyzing the first step of sucrose utilization, i.e. its cleavage to simple components.

**Enzyme detection and identification.** The zymographic studies of purified enzyme revealed the presence of a single band, which was active against sucrose (*data not shown*). Moreover, fructose and glucose-1-phosphate were the only products of sucrose degradation by the enzyme purified by molecular filtration (Fig. 1). It was also stated that the enzyme required inorganic phosphate in the reaction mixture; the optimum concentration of phosphate was found to be 56 mmol/L (Fig. 2). We supposed that the identified enzyme is a sucrose phosphorylase (Purich and Allison 2002). An enzyme of similar properties was found earlier in *Streptococcus bovis* (Martin and Russel 1987) and *P. ruminis* strain A (Kasperowicz et al. 2009) as well as in some non-ruminal bacteria (Doudoroff 1943; Russell et al. 1988; Kim et al. 2003).

Further studies revealed that the molar mass of the identified sucrose phosphorylase was ≈54 kDa. The enzyme exhibited the maximum activity at pH 6.0 and temperature 45 °C. In comparison, sucrose phosphorylase of *Pseudomonas saccharophila* was 80–100 kDa (Silverstein et al. 1967). The calculated $K_m$ for glucose-1-phosphate formation and for release of fructose were 4.4 and 8.56 mmol/L while the maximum velocities of the reaction ($v_{lim}$) were 1.19 and 0.64 µmol/L per mg protein per min, respectively. In the case of *P. ruminis* strain A the relevant figures were 3.88 and 5.56 mmol/L for $K_m$ and 0.579 and 0.9 µmol/L per mg protein per min for the maximum velocity, respectively (Kasperowicz et al. 2009).

Our data also showed that independently of initial concentration of sucrose in the reaction mixture fructose, glucose-1-phosphate and small quantities of free glucose were the products of substrate degradation by cell extract (Fig. 3). These findings suggest that phosphorolysis could be the main pathway of sucrose digestion by examined strain of rumen bacteria. On the other hand, the increasing quantities of free glucose suggest that the hydrolytic cleavage could support the phosphorolysis of sucrose, in particular when its concentration exceeds 5.85 mmol/L. Similar relationship was found in the case of cellobiose and cellopentaose digestion by *Clostridium thermocellum* (Zhang and Lynd 2004).