Anaerobe 29 (2014) 68–72

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Molecular biology, genetics and biotechnology

Can fungal zoospores be the source of energy for the rumen protozoa *Eudiplodinium maggii*?

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ARTICLE INFO

Article history:
Received 19 April 2013
Received in revised form
1 August 2013
Accepted 19 August 2013
Available online 4 September 2013

Keywords:
Fungal zoospores
Protozoa
Chitin content
Fermentation
Rumen

ABSTRACT

Results of our earlier studies showed the ability of ciliates *Eudiplodinium maggii* to digest and metabolize commercial chitin. The natural source of this polysaccharide in the rumen are fungi. The objectives of present research were to determine the effect of fungal zoospores on the survival and population density of *E. maggii* to quantify the concentration of chitin in the cells of protozoa and to examine the ability of *E. maggii*, to ferment chitin of fungal zoospores. The cultivation experiment showed that the survival of protozoa was shorter than 4 days when the culture medium was composed of buffer solution and lyophilized fungal spores. An enrichment of this medium with wheat gluten prolonged the survival of ciliates up to 8 days. The supplementation of the last medium with meadow hay enabled the protozoa to survive for 28 days but a positive effect was observed only during the last 8 days of experiment. The chitin content was 0.27 ng and 0.21–0.35 ng per single zoospore and ciliate, respectively. An increase in the concentration of volatile fatty acids (VFA) was found when protozoa were incubated with zoospores. The production rate of VFA was 46.3 pM/protozoan per h whereas the endogenous production did not exceed 31 pM/protozoan per h. The molar proportion of acetic acid was 77.7% and these of butyric and propionic acids–12.2 and 11.0%, respectively. The obtained results make it evident that carbohydrates present in fungal zoospores were utilized by protozoa in energy yielding processes.

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1. Introduction

Rumen is the largest chamber of the complex stomach of ruminants. It is colonized by numerous microorganisms representing three different taxonomic groups i.e. bacteria, fungi and protozoa. Protozoa are having an important impact on the metabolism of nutrients in the rumen [29]. The ability of these microorganisms to digest and metabolize dietary structural and storage polysaccharides was the subject of numerous studies [25,28,19,2]. Their contribution to the conversion of the complex carbohydrates synthesized in the rumen has been very poorly recognized to date.

The structural carbohydrates synthesized in the rumen are chitin and β-glucans of murein [9,3]. They are the components of the fungal and bacterial cell wall, respectively. According to the commonly accepted opinion, bacteria are the major source of protein for protozoa [30]. On the other hand, the role of fungi in the covering of nutritional requirements of rumen ciliates still remains unknown in spite of its potential role as a source of protein and carbohydrates. The literature data show that rumen ophryoscolecid protozoa engulf the fungal zoospores, which are rich in chitin [30,16]. Results of our earlier studies demonstrated that ciliates *Eudiplodinium maggii* posses the chitinolytic enzymes [22–24]. Thus, they may contribute to digestion and metabolism of chitin present in the cell wall of ruminal fungi.

The objectives of present research were a) to determine the effect of the supplementation of culture medium with rumen fungi zoospores on the survival and population density of *E. maggii*; b) to quantify the concentration of chitin in the cell of protozoa isolated from the rumen; c) to examine the ability of *E. maggii* to digest and ferment carbohydrates present in fungal zoospores.

2. Materials and methods

2.1. Protozoa

Ciliates *E. maggii* were obtained by isolation and cultivation of individuals showing typical features of this species [10]. Twenty to thirty cells were picked and introduced to the Erlenmeyer flask containing culture medium (40 ml) with a well developed population of rumen bacteria. Ciliates were cultured by the routine method [20] in "*caudatum* type" salt solution (Table 1) and fed

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http://dx.doi.org/10.1016/j.anaerobe.2013.08.005

Table 1
The chemical composition of the salt solution used to prepare the culture medium (g/l).

Ingredient	"*Caudatum*" type solution	"Hungate type" solution
K_2HPO_4	6.3	0.0
KH_2PO_4	5.0	1.0
$NaHCO_3$	0.0	5.0
NaCl	0.65	6.0
$CaCl_2 \times 6H_2O$	0.09	0.2
$MgSO_4 \times 7H_2O$	0.09	0.2
CH_3COONa	0.75	0.0
pH	6.84	7.76

The particular buffer solution was prepared according to Coleman et al. [6] and Hungate [12].

Table 2
The production rate of VFA (pM/protozoa per h) and molar proportion of particular acids (% of total acids). Mean values ± SD ($n = 3$).

	Control cultures (starved)	Experimental cultures (supplemented with zoospores)
Production rate of VFA	$28.6^a \pm 14.50$	$46.3^b \pm 30.13$
Acetic acid	$81.7^a \pm 0.73$	$77.7^b \pm 1.22$
Propionic acid	$10.8^a \pm 0.40$	$11.0^a \pm 0.74$
Butyric acid	$11.5^a \pm 0.42$	$12.2^a \pm 0.51$

Values in the row with different superscript differ significantly at $P < 0.05$.

powdered meadow hay and wheat gluten. Some maintained cultures were used to initiate the growth experiment when the population density increased to about 10^3 cells/ml. The remaining cultures were transferred into the continuous culture system [18] and were cultured there for over two months. The latter protozoa were used as an inoculum to develop the population of *E. maggii* in the rumen of three ciliate free permanently rumen-fistulated adult rams according to Bełżecki et al. [3]. After development period lasting 3 weeks [14] the protozoa were isolated from the rumen and used to determine the concentration of chitin in their cells and to perform the fermentation studies. The animals weighting 65 kg ± 3 kg were fed hay (750 g) and ground barley (130 g) every 12 h. Drinking water was available *ad libitum*.

2.1.1. Cultivation experiment

Ciliates were cultured in a "*caudatum* type" salt solution (Table 1.). The culture medium was composed of a "*caudatum*" buffer solution and lyophilized fungal zoospores alone (medium A) or the same solution plus zoospores and wheat gluten (medium B) or all listed components and powdered meadow hay (medium C). The control medium (D) was composed of the same salt solution supplemented with powdered meadow hay and wheat gluten. The particular components were supplemented in the proportion of 0.25 (fungal zoospores), 0.08 (wheat gluten), 0.3 (meadow hay) mg/ml salt solution per d. They were treated as food components.

The ciliates were fed every day and were diluted with fresh "*caudatum*" medium (1:1; v:v) every fourth day. The protozoa were counted on the dilution days according to Michałowski [17]. The cultivation period lasted 28 days.

2.1.1.1. Preparation of food components. Meadow hay was ground in a high speed grinder and the smallest particles which settled on the lid of the grinder were collected and used to prepare the food.

Wheat gluten was prepared according to Klein [15] and Pace [26].

The zoospores were isolated from the culture of rumen fungi maintained *in vitro* in the medium composed of (l^{-1}): 850 ml/l "Hungate" salt solution (Table 1), 150 ml/l of rumen fluid, 2.5 g/l Yeast extract, 10.0 g/l peptone, 5.0 g/l glucose, 1.0 g/l L-cysteine hydrochloride and 6.0 g/l $NaHCO_3$ [8]. The rumen fluid was strained through one layer of muslin before use and the filtrate was collected and centrifuged at 1000 ×*g* for 20 min at 4 °C. The obtained supernatant was centrifuged again at 20,000 ×*g* for 30 min at 4 °C and autoclaved. It was used to prepare the culture medium.

To initiate each culture of fungi, the 50 ml sample of fresh rumen fluid was diluted with 450 ml of warm (40 °C) culture medium followed with supplementation of chopped wheat straw (particle length was 20 mm) up to the proportion of 1% w/v. Finally chloramphenicol, streptomycin and ampicillin were added to a final concentration of 50 μg/ml per antibiotic. The cultivation of cultures was performed at 40 °C. Twice a week they were transferred into a fresh medium and a new portion of antibiotics as well as wheat straw were supplemented. Fungal growth was monitored microscopically on the dilution days. Isolation of zoospores was performed when their concentration was not lower than 500 cells/ml. For this purpose, the selected cultures were filtrated through the nylon filters of three different pore sizes i.e. 170 μm, followed with 40 μm and then with 10 μm. The filtrate was collected and centrifuged at 7000 ×*g* for 20 min at 4 °C. The sediment was washed with fresh culture medium and centrifuged again. The sediment composed of zoospores was suspended in a culture buffer to the final volume of 100 ml. The suspension was thoroughly mixed and the 5 ml sample was preserved with an equal volume of 4% aqueous solution of formaldehyde (1:1; v:v) to enumerate zoospores by the same method as described by Michałowski [17]. The remaining part of suspension was sedimented and washed 2 times with distilled water. Finally the water was eliminated by delicate sucking whereas zoospores were collected, lyophilized. The lyophilizate was weighed and stored at −80 °C. It was used to determine the dry matter (DM) and chitin content in zoospores.

2.2. Purification of ciliates

Ciliates grown in the rumen of monofaunated sheep (see above) were separated from food debris and external bacteria by repeated sedimentation according to Miltko et al. [22]. The sample of rumen content (about 1 kg) was diluted with warm "*caudatum*" salt solution in the proportion of 1:2 (w/v) and squeezed through a screen of the pore size 250 μm. The filtrate was collected, poured into a separatory funnel and allowed to stand at 40 °C for about 30–40 min. The protozoa forming a gray-white pellet at the bottom of the funnel were carefully collected, re-suspended in the same salt solution and sedimented again. The washing procedure was repeated several times and the effect of purification was controlled microscopically. Finally, well purified protozoa were collected and suspended in the warm salt solution of Hungate [12]. They were transferred into the cultivation vessels of the continuous culture system [18] and incubated aerobically overnight in the presence of chloramphenicol, streptomycin and ampicillin according to the method of Bełżecki [1]. On the next day the ciliates were washed with warm "*caudatum*" salt solution (Table 1) and were immediately used to perform the fermentation experiment or were washed with cold "*caudatum*" salt solution and allowed to sediment in an ice bath. The sediment was diluted with deionized water to the final volume of 100 ml. The suspension of protozoa was thoroughly mixed and the samples of 2 × 5 ml were preserved with an equal volume of 4% aqueous formaldehyde solution to count ciliates. The protozoa present in the remaining part of the suspension were allowed to sediment. The sediment was collected and lyophilized. The lyophilizate was weighed and stored at −80 °C. It was used to determine the DM and chitin content in protozoa cells.

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