An Improved Method for Elimination of Holotrich Protozoa from Mixed Rumen Protozoal Suspension by Mannose-Treatment

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Abstract An improvement was made of a method to eliminate holotrich protozoa from mixed rumen protozoa in this study. Mixed rumen protozoal suspension obtained from a goat was incubated at 39°C in various levels of D-mannose solutions (up to 6% (w/v) in buffer solutions). As a result, treatment with a 4% (w/v) D-mannose solution (isotonic to about 0.250 M sucrose) for 1 hr and further successive treatment for 2 hr in the same solution, which was just supplemented at this point with D-mannose equivalent to 2% (w/v) of the solution, was shown to be enough to completely eliminate holotrich protozoa from the mixed rumen protozoa, where the density of holotrich protozoa was up to about $40 \times 10^3/\text{ml}$ (*Isotricha*: up to $25 \times 10^3/\text{ml}$; *Dasytricha*: $15 \times 10^3/\text{ml}$) in the present experiment. The present improved method saved three hours to eliminate holotrichs compared to the previous method. It was also shown that *Isotricha* tended to consume D-mannose more rapidly than *Dasytricha*.

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Among rumen ciliate protozoa, the food habit and metabolism have been shown to be partly different between holotrich protozoa and entodiniomorphid ones. For example, holotrich protozoa can ferment both soluble sugars and particulate polysaccharides like starch, while entodiniomorphid protozoa can ferment starch but not soluble sugars so rapidly and are referred to as particle feeders¹⁾. Similar observation was also reported as to protein degradation^{2,3)}. Recent studies showed that plant fibre-degrading enzyme activities of entodiniomorphid protozoa were generally higher than those of holotrich protozoa^{4,5)}. In this connection, if we can establish a convenient method to eliminate holotrichs from mixed rumen protozoa, it is extremely useful for investigations directly for entodiniomorphs and indirectly for holotrichs. We have previously succeeded in eliminating all holotrich protozoa by incubating mixed rumen protozoa in 1.2% D-mannose-containing buffer solution at 39°C for 6 hr³⁾ and this method has been applied to the examination of *in vitro* digestibility trials for only entodiniomorphid protozoa^{3,6)}. But there is one problem in this method. That is the length of incubation time (6 hr) until eliminating all holotrichs.

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In the present paper, examinations were carried out for the rapid elimination of holotrichs.

Materials and Methods

1. Isolation of rumen ciliate protozoa

Rumen ciliate protozoa were collected from the rumen contents of a ruminally fistulated goat (Japanese local breed, 45 kg) fed on a ration consisting of haycube (600 g/day) and a concentrate (200 g/day, formula feed : alpha-topbeef, Chubu-shiryo, Japan) twice daily at 09 : 00 and 17 : 00. The protozoal suspension was prepared using MB 9 buffer solution⁷⁾ in the same manner as described previously⁸⁾.

2. Incubation procedure for treatment with D-mannose and sample treatment

The ciliate suspension (4% v/v) was incubated in MB 9 buffer solution or in various levels of D-mannose-containing buffer solution (Table 1) at 39°C up to 6 hr. These solutions contained 0.1 mg each/ml of dihydrostreptomycin sulfate, penicillin G potassium and chloramphenicol sodium succinate to inhibit the growth of contaminating bacteria.

After incubation, a 0.5-ml portion of the suspension was collected with stirring, mixed with 9.5 ml of MFS solution⁹⁾, a kind of fixative solution, and kept at room temperature. A 0.005-ml portion of the fixed ciliate suspension was used for counting of protozoa on a Fuchs-Rosenthal hemacytometer under microscope.

Results and Discussion

Based on the original report by ABOU AKKADA and HOWARD¹⁰⁾ that overnight incubation in 0.6% D-mannose solution killed *Dasytricha ruminantium*, we have previously tried and succeeded in total elimination of holotrich protozoa (*Dasytricha* and *Isostricha*) from mixed rumen ciliate protozoa by incubating them at 39°C for 6 hr in a 1.2% D-mannose-containing B-9 buffer solution, whose osmolarity had been adjusted so as to be isotonic to 0.25 M sucrose by decreasing NaCl³⁾. Thus the effect of the enhanced level of D-mannose in MB 9 buffer solution (where isotonicity was not adjusted) on the mortality of holotrich protozoa was examined to prospect the possibility to shorten the incubation time to kill them. Results showed that

Table 1. Composition of buffer solutions

Composition (g/l)	MB9	D-mannose solution		
		2%	4%	6%
NaCl	2.8	2.9	0	0
CaCl ₂ 2H ₂ O	0.1	0.1	0.1	0.1
MgSO ₄ 7H ₂ O	0.1	0.1	0.1	0.1
KH ₂ PO ₄	2.0	0.5	0.5	0.5
Na ₂ HPO ₄	6.0	1.5	1.5	1.5
D-mannose	0	20.0	40.0	60.0
Isotonicity to sucrose (M)	0.250	0.248	0.261	0.369

Fig. 1. Effect of D-mannose levels on the mortality of holotrich protozoa. (a) Effect on *Dasytricha*: Control (○), 0.6% (●), 1.2% (△), 2.4% (□), 3.6% (■); (b) Effect on *Isotrichidae*: Control (○), 2% (●), 4% (□), 6% (■).

enhancement of D-mannose level surely shortened the time to eliminate holotrichs and 3.6% D-mannose killed them within 2 hr as shown in Fig. 1-a. But in this case, the higher the mannose level, the higher the mortality of all protozoa including entodiniomorphid ones. Namely 5% D-mannose in MB 9 killed all protozoa within 90 min and more than 10% D-mannose within 10 min, though these were not shown in Fig. 1. These results suggested that the osmolarity of the D-mannose solution should be adjusted to be isotonic to about 0.250 M sucrose as previously reported¹¹⁾.

Then 2, 4 and 6% D-mannose-containing buffer solutions (Table 1) were prepared so as to be isotonic to near 0.250 M sucrose except for 6% in the same manner as preparing MB 9 (pH 6.8 and anaerobic)⁸⁾. Results of the first trial for incubation of the mixed rumen ciliates in these buffer solutions with or without D-mannose showed that 4 and 6% D-mannose solution killed all of *Isotrichidae* within 3 hr (Fig. 1-b). In this experiment, the densities of *Isotricha* and *Dasytricha* were both $5.3 \times 10^3/\text{ml}$, respectively.

In the second trial, however, *Dasytricha* (original density: $14.8 \times 10^3/\text{ml}$) did not disappear even after incubation for 6 hr in 6% D-mannose solution (Fig. 2-b), though *Isotricha* (original density: $18.4 \times 10^3/\text{ml}$) completely disappeared within 2 hr in each

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D-mannose level (Fig. 2-a). Other entodiniomorphid protozoa remained almost unchanged (Fig. 2-c). From these results, we surmised that high density of *Isotricha* might so quickly consume D-mannose that the protozoa died shortly and the concentration of D-mannose in the medium quickly decreased to the level not enough to kill *Dasytricha*, where *Dasytricha* might tend to incorporate D-mannose at a comparatively slow rate and their incorporation rate might be influenced by the concentration of D-mannose.

Then in the third trial, D-mannose equivalent to 2% (w/v) was supplemented to the incubation medium after 1-hr incubation of mixed rumen protozoal suspension which contained especially high density of *Isotricha* ($24.8 \times 10^3/\text{ml}$). In this case, *Isotricha* disappeared quickly again as shown in Fig. 3-a and *Dasytricha* also

Fig. 2. Effect of D-mannose levels on the mortality of *Isotricha* (a), *Dasytricha* (b) and entodiniomorphid protozoa (c). D-Mannose level: Control (○), 2% (●), 4% (□), 6% (■).

disappeared within 4 hr for 2% and 3 hr for 4 and 6% D-mannose solution (initial levels) as was expected (Fig. 3-b). Entodiniinae and Diplodiniinae remained almost unchanged in 2 and 4% D-mannose, but in 6% they tended to decrease (Fig. 3-c and d).

In the final experiment, ciliate suspensions containing two grades of densities of *Isotricha* were incubated in only 4% D-mannose solution to confirm whether the supplementation of D-mannose after 1 hr incubation could efficiently shorten the life of *Dasytricha* especially when the density of *Isotricha* was high. Results were shown in Fig. 4-a and b. *Isotricha* disappeared indubitably within 2 hr when D-mannose equivalent to 2% (w/v) of the incubation medium was supplemented after 1 hr incubation (Fig. 4-a). It was also confirmed that the life of *Dasytricha* was efficiently shortened by the supplementation of D-mannose, dying within 3 hr even when the density of *Isotricha* was very high ($23.6 \times 10^3/\text{ml}$) (Fig. 4-b). Entodiniomorphid protozoa remained almost unchanged. From these results, *Isotricha* seemed to have a tendency to consume D-mannose more rapidly than *Dasytricha* and to disappear quickly.

These results led us to the conclusion that all holotrich protozoa (density of *Isotricha* : up to $25 \times 10^3/\text{ml}$, and that of *Dasytricha* : up to $15 \times 10^3/\text{ml}$ in the present experiments) could be eliminated by incubating in 4% (w/v) D-mannose solution (Table 1) for 1 hr and then continuing incubation for 2 hr just after D-mannose

Fig. 3. Effect of the supplementation of D-mannose (2% (w/v) of the incubation medium) after 1-hr incubation of mixed rumen protozoa in the medium containing 0% (○), 2% (●), 4% (□) and 6% (■) D-mannose on the mortalities of *Isotricha* (a), *Dasytricha* (b), Entodiniinae (c) and Diplodiniinae (d).

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Fig. 4. Effect of the supplementation (broken lines with symbols ($\blacktriangle$) and ($\blacksquare$)) and non-supplementation (solid lines with symbols ($\triangle$) and ($\square$)) of D-mannose (2% (w/v) of the incubation medium) after 1-hr incubation of mixed rumen protozoal suspensions containing two grades of density of *Isotricha* (high grade: ($\triangle$) and ($\blacktriangle$); low grade: ($\square$) and ($\blacksquare$)) in 4% D-mannose solution on the mortalities of *Isotricha* (a) and *Dasytricha* (b). Control (with high density of *Isotricha* and without D-mannose) was shown by a symbol ($\circ$).

equivalent to 2% (w/v) was added to the incubation medium. Thus we could get a holotrichs-free rumen ciliate protozoal suspension after collecting the treated protozoa by centrifugation ($1000 \times g$, for 3 min) and washed with warmed MB 9 buffer solution three times. By this study, we could save three hours for the elimination of holotrich protozoa from mixed rumen protozoa.

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マンノース処理による混合ルーメン繊毛虫類からの 全毛類除去法の改良

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本研究では、混合ルーメン繊毛虫類から全毛類を完全除去する方法の改良を行なった。山羊から得た混合ルーメン繊毛虫類懸濁液を種々の濃度の D-マンノース溶液（緩衝液中に 6% (w/v) まで）中で 39°C にてインキュベートした。その結果、4% (w/v) D-マンノース溶液（約 0.250 M ショ糖液に等張）で 1 時間処理をし、続いて、この溶液に、さらに、2% (w/v) 分の D-マンノースを追加した溶液中で 2 時間処理を続けると、混合ル

ーメン繊毛虫類から全毛類が完全に除去されることが示された。本研究では、全毛類の密度は、最高時で、約 $40 \times 10^3/\text{ml}$ (*Isotricha*: $25 \times 10^3/\text{ml}$: *Dasytricha*: $15 \times 10^3/\text{ml}$) であった。本研究により、全毛類の除去に要する時間を以前よりも 3 時間節約することができた。また、*Isotricha* は、*Dasytricha* よりも速く D-マンノースを摂取する傾向が認められた。

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