Species of Rumen Ciliate Protozoa Which Produce Extra-Cellular Protease and Influence of Bacteria on Its Activity

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Abstract *In vitro* experiments were done in order to identify the species of rumen ciliate protozoa which produce proteolytic enzymes outside the cell and the effect of contaminated bacteria on the extra-cellular proteolytic activity. Bacteria and ciliate protozoa were isolated from the rumen liquid obtained from a Holstein cow through the fistula, and protozoa were separated into two groups of entodiniomorphs and holotrichs. The extra-cellular crude enzyme solutions were prepared by centrifuging the starved culture medium of each protozoa group and of isolated bacteria, and the proteolytic activity was assayed through the decomposition of casein incubated with crude enzyme preparations. The results obtained are summarized as follows: 1) Extra-cellular proteolytic activity was detected exclusively in the culture medium of entodiniomorphs, and not in the medium of holotrich protozoa. 2) Isolated bacteria decomposed casein, but the proteolytic activity was hardly detected in the cell-free medium prepared after the culture of bacterial suspension. 3) Extra-cellular proteolytic activity detected for entodiniomorphid protozoa was not modified by washing these organisms several times with surface-active substance (sugar-fatty acid ester) to eliminate bacteria, which may attach to the surface of ciliate protozoa. 4) the results of this work suggest that the extra-cellular proteolytic activity would be principally attributed to entodiniomorphid protozoa, and that the influence of bacteria could be neglected.

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Key words : rumen, ciliate protozoa, bacteria, extra-cellular, protease

UEDA *et al.*¹⁾ suggested that the rumen ciliate protozoa may secrete peptidase into the medium during *in vitro* incubation. SHINCHI and KANDATSU showed that the rumen ciliate protozoa excrete the extra-cellular proteolytic enzymes into the culture medium during incubation with salt buffer solution²⁾, and examined on some properties of purified enzymes³⁾. Furthermore, they demonstrated a proteolytic activity in the cell-free rumen liquid⁴⁾.

Rumen ciliate protozoa are roughly grouped into holotrichs and entodiniomorphs and it has been reported that the former decomposes both soluble and insoluble casein, but that the latter decomposes only insoluble casein⁵⁾. These experiments were done in order to investigate species of rumen ciliate protozoa which produce extra-cellular proteolytic enzymes and the effect of possible contamination of bacteria on the activity of these enzymes.

Materials and Methods

Doner of microorganisms : Rumen liquid was obtained from a rumen fistulated Holstein cow (4 year-old and weighing about 600 kg), which was fed twice daily with rations consisting of 1.5 kg rice straw and 2 kg concentrate (commercial formula feed, CP 13.5%) and given water *ad libitum*.

Fractionation of ciliate protozoa and isolation of bacteria : About 4l of rumen liquid were taken through the rumen fistula at 1 hr after feeding in the morning and were strained through two layers of gauze. Fractions of entodiniomorphid protozoa, holotrich protozoa and isolated bacteria were prepared by the method shown in Fig. 1.

Preparation of microbial suspensions : Two ciliate protozoa fractions were suspended in 60 ml of B-9 buffer solution⁶⁾ containing 50 μ g each of penicillin G potassium, streptomycin sulfate, kanamycin sulfate and chloromycetin powder per ml, and isolated bacteria fraction was suspended in an original volume of the buffer solution with or without the same amounts of antibiotics.

Separation of bacteria attached to the surface of ciliate protozoa : One part of entodiniomorphid fraction was washed twice with 0.05% sugar-fatty acid ester solution by centrifugation ($160 \times g$ for 3 min), and then washed 5 times with B-9 buffer solution. Another part of the fraction was washed 7 times in succession with B-9 buffer solution by centrifugation ($160 \times g$ for 3 min), and thereafter each fraction was suspended in 60 ml of the buffer solution containing 50 μ g each of four antibiotics per ml.

Preparation of extra-cellular crude enzyme solution : Thirty millilitres of protozoa and bacteria suspensions were cultured at 39°C without any substrate in a 100 ml ERLLENMEYER's flask sealed with rubber stopper after the air phase in the flask was

Fig. 1. Scheme for the fractionation of rumen ciliate protozoa and isolated bacteria.

replaced by mixed gasses ($N_2 : CO_2 = 95 : 5$). After 3 hr, the culture medium was centrifuged at $600 \times g$ for 5 min to remove ciliate protozoa, and the supernatant fluid was further centrifuged at $27,000 \times g$ for 40 min at $4^\circ C$ to obtain the extra-cellular crude enzyme solution. As for bacteria, extra-cellular crude enzyme solutions were prepared after 30 ml of bacteria suspensions with or without antibiotics were cultured for 3, 8 and 20 hr in a similar manner as above and centrifuged at $27,000 \times g$ for 40 min at $4^\circ C$.

Assay for extra-cellular protease : Two millilitres of crude enzyme solution and 2 ml of B-9 buffer solution containing 0.08% NaN_3 and 4 mM cysteine-HCl were added to 4 ml of casein (Merk, for biochemistry) solution (2 mg N/ml) immediately before the incubation at $39^\circ C$. The reaction was stopped by adding 8 ml of 20% trichloroacetic acid solution (final concentration : 10% W/V), and the mixture was filtrated by Whatman no. 50 paper. Non-protein nitrogen (NPN) was determined by the LOWRY method as modified by HARTREE⁷.

Assay for proteolytic activity of isolated bacteria : One hundred millilitres of bacteria suspensions, with or without antibiotics, were cultured at $39^\circ C$ with 709 mg casein (1 mg N/ml) in a 200 ml ERLLENMEYER's flask replaced its air phase by mixed gasses and sealed with rubber stopper. Ten millilitres each of the culture medium were taken periodically, and the reaction was stopped by adding 10 ml of 20% trichloroacetic acid solution. After the centrifugation at $27,000 \times g$ for 30 min at $4^\circ C$, NPN in the supernatant was determined by the above method.

Scanning electron microscopy : A small portion of entodiniomorphid fraction was dropped on the 6×6 mm slide glass on which 1% poly-L-lysine solution was painted. Within 10 min, ciliate protozoa stuck on the surface of the slide glass. The further treatments were the same as reported by IMAI and OGIMOTO⁸. The fixed and dehydrated specimen was stuck on a sample stage with stage with double-sticking tape, and the surface was coated with a thin layer of platinum-vanadium in a rotating vacuume evaporator. These specimens were observed by a scanning electron microscope (model JSM-T 20 Hitachi Electronics Co.).

Determination of microbial nitrogen : Total nitrogen of microbial suspension was determined by KJELDAL method.

Counting of protozoa : Ciliate protozoa were counted with Tatai Eosinophil Counter after the microbial suspension was diluted five-fold with FSH⁹.

Results and Discussion

SHINCHI *et al.*⁴⁾ showed that the cell-free rumen liquid has a proteolytic activity, and at least a part of the activity owes to the extra-cellular protease excreted by rumen ciliate protozoa. Proteolytic activity in cell-free rumen liquid was higher after feeding than before feeding, and the tendency was consistent with that of the variation of holotrich numbers in the rumen⁴⁾. As shown in Table 1, however, extra-cellular proteolytic activity was found in the culture medium of entodiniomorphid fraction, but not in the medium of holotrich fraction. Although protozoa number in the culture

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Table 1. Proteolytic activity of extra-cellular crude enzyme solution of entodiniomorphid and holotrich protozoa

Protozoa fraction ^{1, 2)}	Incubation time (min)	NPN in incubation medium ($\mu\text{g}/\text{ml}$)		Decomposed casein ($\mu\text{g N}/\text{ml}$)	
		Test 1	Test 2	Test 1	Test 2
Entodiniomorphs	0	16	18	—	—
	30	32	56	16	38
	60	45	96	29	78
	90	58	142	42	124
	120	76	191	60	173
	180	107	255	91	237
Holotrichs	0	9	5	—	—
	30	15	11	6	6
	60	16	10	7	5
	90	16	12	7	7
	120	17	12	8	7
	180	19	14	10	9

- 1) Protozoa nitrogen : In entodiniomorphid protozoa fraction,
256 $\mu\text{g}/\text{ml}$ in Test 1, and 305 $\mu\text{g}/\text{ml}$ in Test 2.
In holotrich protozoa fraction,
368 $\mu\text{g}/\text{ml}$ in Test 1, and 541 $\mu\text{g}/\text{ml}$ in Test 2.
- 2) Composition of protozoa ($\times 10^3/\text{ml}$) :

Entodiniomorphid protozoa fraction					Holotrich protozoa fraction			
Entodiniomorphs			Holotrichs		Entodiniomorphs		Holotrichs	
large	small		large	small	large	small	large	small
Test 1	0	600	0	4	0	13	80	74
Test 2	0	720	0	6	3	10	83	61

medium of entodiniomorphs was higher than in the medium of holotrichs, protozoa nitrogen in the former culture medium was almost equal to that in the latter. Therefore, the results shown in Table 1 suggest that entodiniomorphid protozoa excrete extra-cellular protease into the culture medium during the incubation, while that it is scarcely excreted by holotrich protozoa.

ONODERA and KANDATSU⁵⁾ reported that soluble casein was decomposed by holotrich protozoa but not by entodiniomorphid protozoa, and that insoluble casein particle was decomposed by the latter. Furthermore, ONODERA *et al.*¹⁰⁾ observed that entodiniomorphid protozoa didn't decompose soluble casein even when it was ingested with carbon powder. Some contradictions may exist between these and the results of this study, but the cause is not clear at this time.

ABOU AKKADA and HOWARD¹¹⁾ and ONODERA *et al.*¹⁰⁾ obtained a entodiniomorphid protozoa fraction after destroying holotrich protozoa byperincubation with salt buffer solution containing D-mannose. When we tried this method, however, holotrich protozoa could not be eliminated completely. In this study, protozoa were fractionated into holotrichs and entodiniomorphs by settling and the successive centrifugation of rumen liquid obtained after feeding when holotrich protozoa were remarkably higher in numbers than before feeding^{12,13)}. As shown in the footnote of Table 1, the fractiona-

tion was satisfactorily achieved by this method.

The rumen bacteria attached to the surface of rumen ciliate protozoa⁸⁾, and also free bacteria inevitably included in culture medium, may affect the proteolytic activity of extra-cellular crude enzyme solution, because rumen bacteria are known to excrete proteolytic enzymes outside the cell¹⁴⁻¹⁶⁾. It has been reported that 20% of the

Table 2. Decomposition of casein by isolated bacterial suspension¹⁾

Antibiotics ²⁾	Culture time(hr)	NPN in culture medium ($\mu\text{g}/\text{ml}$)		Decomposed casein ($\mu\text{g N}/\text{ml}$)
		Without casein	With casein	
Not added	0	4	*3)	—
	1	4	22	18
	2	4	35	31
	3	4	54	50
	5	4	96	92
	8	3	148	45
	20	2	373	371
Added	0	4	*3)	—
	1	4	20	16
	2	5	37	32
	3	5	51	46
	5	6	80	74
	8	7	117	110
	20	7	324	317

1) Bacterial nitrogen : 60 $\mu\text{g}/\text{ml}$.

2) Fifty μg each of penicilin G potassium, streptomycin sulfate, kanamycin sulfate and chloromycetin powder were added per ml medium.

3) Not determined.

Table 3. Decomposition of casein by the extra-cellular crude enzyme solution prepared after the culture of isolated bacterial suspension¹⁾ for 3, 8 and 20 hr

Antibiotics ²⁾	Incubation time (min)	NPN in incubation medium ($\mu\text{g}/\text{ml}$)			Decomposed casein ($\mu\text{g N}/\text{ml}$)		
		Culture time(hr) of bacteria			Culture time(hr) of bacteria		
		3	8	20	3	8	20
Not added	0	15	15	15	—	—	—
	30	15	16	18	0	1	3
	60	15	16	18	0	1	3
	90	18	17	18	3	2	3
	180	21	23	22	6	8	7
Added	0	15	19	21	—	—	—
	30	17	19	22	2	0	1
	60	18	21	24	3	2	3
	90	19	22	25	4	3	4
	180	20	24	27	5	5	6

1) Bacterial nitrogen : 60 $\mu\text{g}/\text{ml}$.

2) Fifty μg each of penicilin G potassium, streptomycin sulfate, kanamycin sulfate and chloromycetin powder were added per ml medium.

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protease produced by *Bacteroides amylophilus* are liberated into the growth medium, and that its production is proportional to the concentration of ammonium sulfate and maltose added to the medium¹⁴⁾. HAZLEWOOD *et al.*¹⁵⁾ showed that 90% of the proteolytic activity in the culture medium of *Bacteroides ruminicola* were derived from cell-associated protease at 16 hr of incubation, but the percentage decreased according to the time of incubation.

In order to know the influence of bacteria on the production of extra-cellular

Fig. 2. Scanning electron micrographs of entodiniomorphid protozoa washed twice with sugar-fatty acid ester solution and then washed 5 times with B-9 buffer solution.

protease, two experiments were executed. In the first, the influence of bacteria possibly contaminated in the medium was examined.

As shown in table 2, casein was degraded when it was added to bacterial suspension and cultured up to 20 hr. The addition of antibiotics to the suspension inhibited proteolysis though incompletely. While, as shown in Table 3, casein was hardly decomposed irrespectively of antibiotics when it was incubated for 3 hr with extra-cellular crude enzyme solutions, which were prepared by centrifuging bacterial suspension cultured for 3, 8 and 20 hr. These results seem to suggest that bacterial protease would be largely associated with cells, and precipitated by centrifugation without being liberated from cells. Therefore, it seems unreasonable to attribute the extra-cellular proteolytic activity in entodiniomorphid fraction to the contamination of bacteria into this fraction.

Secondly, entodiniomorphid protozoa were washed with sugar-fatty acid ester to eliminate bacteria attached to the surface of these organisms. Fig. 2 shows the effect of washing ciliate protozoa with the solution of sugar-fatty acid ester. After the washing, few bacteria were observed on the surface of entodiniomorphid protozoa by scanning electron microscopy. As shown in Table 4, the proteolytic activity of extra-cellular crude enzymes was slightly higher in the culture medium of protozoa unwashed with the sugar-fatty acid ester than in that of washed protozoa. However, because the number of protozoa in the former culture medium was more higher than in the latter, the protozoa nitrogen was 228 $\mu\text{g/ml}$ in the former culture medium and 196 $\mu\text{g/ml}$ in the latter, resulting in that the proportion of decomposed casein per protozoa nitrogen in the culture medium was similar for both unwashed and washed protozoa. These results suggest that any possible attachment of bacteria to the surface of entodiniomorphid protozoa had little influence on the proteolytic activity of extra-

Table 4. Effect of washing ciliate protozoa¹⁾ with SFAE²⁾ on the proteolytic activity of extra-cellular enzyme solution

Treatment	Incubation time(min)	NPN in incubation medium($\mu\text{g/ml}$)	Decomposed casein ($\mu\text{g N/ml}$)	Decomposed casein per protozoa nitrogen ³⁾
Unwashed	0	14	—	—
	30	65	51	0.22
	60	125	111	0.49
	90	188	174	0.76
Washed with SFAE	0	13	—	—
	30	60	47	0.24
	60	114	101	0.52
	90	172	159	0.81

1) Number of protozoa ($\times 10^3/\text{ml}$) : Entodiniomorphids		Holotrichs		
	large	small	large	small
	0	522	0	2

2) SFAE : Sugar-fatty acid ester.

3) Protozoa nitrogen : 228 $\mu\text{g/ml}$ in the culture of unwashed protozoa, and 196 $\mu\text{g/ml}$ in that of washed protozoa.

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cellular enzyme solution.

In conclusion, it is considered that the extra-cellular proteolytic activity would be principally attributed to entodiniomorphid protozoa, and that the influence of bacteria on this activity would be small enough to be neglected.

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細胞外蛋白質分解酵素を産生する反芻胃内繊毛虫の 種類とその活性に及ぼす細菌の影響

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細胞外へ蛋白質分解酵素を分泌する繊毛虫の種類と、その活性に及ぼす繊毛虫の体表付着細菌および遊離細菌の影響について検討した。第一胃フィステル装着牛から採取した反芻胃液から繊毛虫と細菌を分離し、前者は貧毛虫と全毛虫とに分画した。これらの微生物区分を飢餓培養後に遠沈して細胞外粗酵素液を調製し、そのカゼイン分解能から細胞外蛋白質分解活性を求めた。その結果は以下に示す通りであった。

1) 細胞外蛋白質分解活性は貧毛虫の培養後に調製した粗酵素液にのみ認められ、全毛虫のそれには認められなかった。

2) 繊毛虫の体表付着細菌を除去するため、ショ糖脂脂肪酸エステルで洗滌した繊毛虫の細胞外蛋白質分解活性は洗滌しない繊毛虫のそれと差がなかった。

3) 細菌はカゼインを分解したが、細菌培養液を遠沈して得た上清液中には蛋白質分解活性がほとんど認められなかった。

4) 以上の結果は、細胞外蛋白質分解活性が主として貧毛虫の産生する酵素によるものであり、遊離細菌または付着細菌の影響はほとんどないことを示唆するものと考えられた。 日畜会報, 58 (1) : 72-79, 1987