A NEW INTRACELLULAR PROTEOLYTIC ACTIVITY IN MIXED RUMEN CILIATE PROTOZOA

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Introduction

It is known that rumen microoraganisms degrade proteins in feedstuffs and they utilize the degraded products for their own nutrients. Therefore, it seems to be important to determine the proteolytic activity in rumen ciliate protozoa. There is a little information on proteinases in rumen protozoa, particurally on their characteristics. Shinchi and Kandatsu (1981) have shown that rumen protozoa have mainly extracellular proteinases. However, since protozoa can engulf ruman bacteria (Onodera et al., 1974), they may digest bacteria in the cell. In this paper, we examined some properties of intracellular proteolytic activities in the mixed rumen protozoa from goats.

Materials and Methods

Mixed rumen ciliate protozoa were isolated from goats and washed ten times with MB9 buffer by the methods previously described (Onodera et al., 1988) in order to remove bacteria from the protozoal fraction as completely as possible. Then they were sonicated for 10 min and homogenate (crude enzyme) was used for the following enzyme assay. Enzyme assay was performed with neutral, alkaline and acidic pH using casein (Hammarsten, Merck) and hemoglobin(Wako Pure Chemicals, Japan) as substrate, respectively. They were dissolved so as to be 1% (W/V) in a buffer solution (0.1 M citrate, 0.1 M phoshate or 50 mM borate buffer). One ml portion of crude enzyme suspension (8 mg protein/ml) was added to a two ml portion of substrate solution and allowed to react at 39°C for 1 hr. Then the mixture was mixed with 40% (w/v) trichloroacetic acid and centrifuged at 1,500 xg for 20 min. The released peptides in the supernatant fluid were measured using Folin's reagent or absorbance at 280 nm. Proteolytic activity was expressed by increment in optical density during incubation.

The molecular weight of proteinase was estimated from elution volume on Sephadex G-100 using 10 mM sodium phosphate buffer (pH 7.0). The column was calibrated for estimating molecular weight by bovine serum albumin, ovalbumin and chymotrypsinogen. A sample for gel filtration was used after centrifugation of crude enzyme suspension at 10,000 xg for 20 min.

Results

The rumen protozoa comprised more than 99% of Entodiniiane and 1% of Dasytricha and Isotricha.

The apparent optimum pH of crude enzyme was found at pH 8.0 for casein and pH 2.5 for hemoglobin. The specific activity at pH 2.5 was four times higher than that at pH 8.0 which was a broad pH-dependent peak. The effect of temperature on the proteolytic activity was examined at 39°C and 70°C. The casein-hydrolyzing activity at 70°C was seven times higher than that at 39°C. On the other hand, the hemoglobin-hydrolyzing activity at 70°C was only 11% of that at 39°C.

The crude enzyme suspension was allowed to react in substrate solution including 1 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM cysteine or 20 mM 2-mercaptoethanol. The proteolytic activities were not affected by these metal ions. On the contrary, both enzyme activities were remarkably stimulated by cysteine, whereas 2-mercaptoethanol activated the hemoglobin-hydrilyzing ability but not the casein-hydrolyzing ability (table 1).

To know more specific characteristics of the proteolytic activities of rumen protozoa, the effects of inhibitors on the activity were examined. Serine proteinase inhibitor, phenylmethanesulfonylfluoride (1 mM, PMSF, Nacalai Tesque, Japan) and aspartic proteinase inhibitor, pepstatin (0.1 mM, Sigma Chemical, U.S.A.) had no effects on both casein- and hemoglobin proteolytic activities. Cysteine proteinase inhibitor, p-chloromercuribenzoic acid (1 mM, PCMB, Nacalai Tesque), and sodium iodoacetic acid (1 mM, IAA,

TABLE 1. EFFECTS OF ACTIVATORS AND INHIBITORS ON INTRACELLULAR PROTEOLYTIC ACTIVITY OF MIXED RUMEN CILIATE PROTOZOA

Effector	Relative activity	
	pH 2.5 (Hemoglobin) (%)	pH 8.0 (Casein) (%)
Cysteine (20 mM)	154	177
2-Mercaptoethanol (20 mM)	126	105
$CaCl_2$ (1 mM)	83	89
$MgSO_4$ (1 mM)	83	100
PMSF (1 mM)	111	109
Pepstatin (0.1 mM)	102	101
PCMB (1 mM)	101	91
IAA (1 mM)	59	63
None	100	100

Nacalai Tesque) inhibited caseinhydrolyzing activity by 9% and 37%, respectively. Hemoglobin-hydrolyzing activity was inhibited by IAA (by 37%) but not by PCMB (table 1).

Figure 1 shows the elution profile from Sehadex G-100 column. Casein-hydrolyzing activities detected a void, volume and 88 ml corresponding to more than 100,000 Da and 48,000 Da. Hemo - globin-hydrolyzing activity was eluted at 80 ml (60,000 Da)

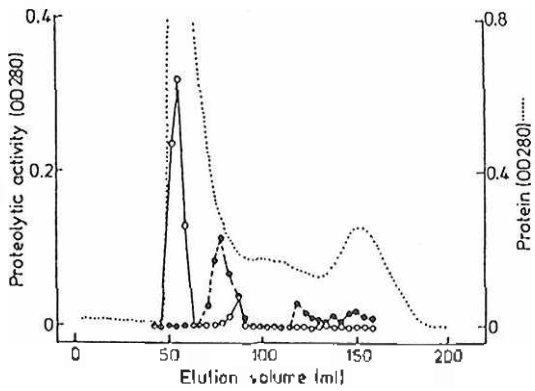

Figure 1. Sephadex G-100 chromatogram of the intracellular crude enzyme in protozoa ○, Casein hydrolyzing activity at pH 8.0 ●, Hemoglobin hydrolyzing activity at pH 2.5.

Discussion

In the present study, we found at least three intracellular proteinases in mixed rumen ciliate protozoa from the results of gel filtration. Two of them degraded casein at pH 8.0 and one degraded hemoglobin at acidic pH. It is interesting to note that the specific activity of the hemoglobin-hydrolyzing enzyme was strong compared to that of the casein-hydrolyzing enzyme which was stable under heat. From the proteolytic activity against the effectors, both proteinases appeared to be cysteine proteinases.

Shinchi and Kandatsu (1981) have demonstrated that when using casein as a substrate the proteolytic activity of rumen ciliate protozoa was mainly involved in extracellular activity. However, they did not examine hemoglobin-hydrolyzing activity at acidic pH. In the present study, we also tested extracellular proteolytic activity, but it was not much higher than the intracellular activity, though data is not shown. The characteristics of casein-hydrolyzing proteinase in the present experiment are similar to those in the report of Shinchi and Kandatsu (1981) which has shown that optimum temperature was 70°C. Furthermore, Forsberg et al. (1984) reported that the protozoa obtained from cows had endogeneous proteolytic activity for hydrolyzing casein but not for hemoglobin. This activity was inhibited by cysteine proteinase inhibitors, but it is difficult to compare their results to the present findings, because the enzyme fraction was crude and of a different origin.

There are no reports about the hemoglobin-hydrolyzing activity in rumen protozoa. Since protozoa can engulf rumen bacteria as well as proteins (Onodera et al., 1974), hemoglobin-hydrolyzing proteinases may be necessary for digestion of protein sources and for their body protein turnover. Therefore, further studies about purification and characterization of the proteinases are necessary.

(Key Words: Rumen Protozoa, Proteolysis, Hemoglobin, Casein)

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