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Proteolytic Activity of Rumen Microorganisms and Effects of Proteinase Inhibitors

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Proteolytic activity of the bovine rumen microflora was studied with azocasein as the substrate. Approximately 25% of the proteolytic activity of rumen contents was recovered in the strained rumen fluid fraction, and the balance of the activity was associated with the particulate fraction. The proportion of proteinase activity associated with particulate material decreased when the quantity of particulate material in rumen contents was reduced. The specific activity of the proteinase from the bacterial fraction was 6 to 10 times higher than that from the protozoal fraction. Proteinase inhibitors of synthetic, plant, and microbial origin were tested on proteolytic activity of the separated bacteria. Synthetic proteinase inhibitors that caused significant inhibition of proteolysis included phenylmethylsulfonyl fluoride, N-tosyl-1-lysine chloromethyl ketone, N-tosylphenylalanine chloromethyl ketone, EDTA, cysteine, dithiothreitol, iodoacetate, and Merthiolate. Plant proteinase inhibitors that had an inhibitory effect included soybean trypsin inhibitors types I-S and II-S and the lima bean trypsin inhibitor. Proteinase inhibitors of microbial origin that showed an inhibitory effect included antipain, leupeptin, and chymostatin; phosphoramidon and pepstatin had little effect. We tentatively concluded that rumen bacteria possess, primarily, serine, cysteine, and metalloproteinases.

Ruminal proteolysis can result in a loss of high-quality dietary protein that would otherwise be directly digested and absorbed in the small intestine of the ruminant animal (7). The first step in protein degradation in the rumen is hydrolysis of proteins by proteinases to peptides and amino acids, which are either utilized directly by the microflora or degraded further by peptidases and deaminating enzymes to short-chain fatty acids and ammonia (12, 33, 34). Proteolysis has been suggested to be the rate-limiting step in the degradation of fraction 1 protein from alfalfa (31), although with other proteins, the utilization of amino acids was considered to be the limiting step (11, 26).

Methods successful in improving protein utilization in ruminants by decreasing the apparent degradation of protein in the rumen have included chemical treatment of feed materials (11), defaunation (6), and inclusion of feed additives such as monensin and diaryliodonium compounds (13, 32).

The most direct and perhaps the most effective means of decreasing the degradation of protein within the rumen is through the selective

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inhibition of microbial proteinases. Since knowledge of the hydrolytic properties of the microbial proteinases was limited, the objective of this investigation was to examine the distribution of proteinase activity within rumen contents and to test the effects of proteinase inhibitors on proteolysis by rumen bacteria. A preliminary report of this work has already appeared (F. M. Brock and C. W. Forsberg, Abstr. 30th Annu. Meet. Can. Soc. Microbiol. 1980, F8, p. 72).

MATERIALS AND METHODS

Rumen contents. Rumen contents were obtained from a rumen-fistulated, nonlactating Holstein cow fed twice daily on alfalfa hay plus a corn grain supplement. The rumen contents were collected via the fistula 3 to 4 h after feeding by taking samples with a Nalgene beaker from different locations in the rumen and pooling them. Strained rumen fluid (SRF) was obtained by straining the contents through four layers of cheesecloth. The particulate fraction retained by the cheesecloth was saved in some experiments and used to determine the distribution of proteinase activity between the SRF and the particulate material in rumen contents.

Bacterial and protozoal fractions. Rumen bacterial and protozoal fractions were prepared from SRF as described by Forsberg (16). The separated fractions were each washed twice at 4°C in 0.1 M potassium

TABLE 1. Mode of action and solvent of each proteinase inhibitor used in this study

Inhibitor	Action[a]	Solvent[b]	Reference
Chemical			
PMSF	Inhibits serine and some cysteine proteinases	5% (vol/vol) ethanol in buffer	4, 29
TLCK	Inhibits trypsin, plasmin, thrombin, and cysteine proteinases	5% (vol/vol) ethanol in buffer	29
TPCK	Inhibits chymotrypsin and cysteine proteinases	5% (vol/vol) ethanol in buffer	29
ε-Aminocaproic acid	Inhibits plasmin and trypsin	Buffer	29
EDTA disodium	Inhibits metalloproteinases	Buffer	4, 27
L-Cysteine	Reduces disulfide bonds and inhibits metalloproteinases	Buffer	19, 37
DL-Dithiothreitol	Reduces disulfide bonds and inhibits metalloproteinases	Buffer	4, 29
Iodoacetate	Inhibits cysteine proteinases	Buffer	4
Merthiolate	Inhibits cysteine proteinases	Buffer	4, 40
Animal			
Kallikrein inhibitor	Inhibits trypsin, chymotrypsin, and kallikrein	Buffer	22
Plant			
Soybean trypsin inhibitor I-S	Inhibits trypsin	Buffer	29
Soybean trypsin inhibitor II-S	Inhibits trypsin and chymotrypsin	Buffer	29
Lima bean trypsin inhibitor	Inhibits trypsin and chymotrypsin	Buffer	29
Microbial			
Phosphoramidon	Inhibits neutral proteinases	Buffer	38
Pepstatin A	Inhibits aspartic proteinases	Suspension in buffer	4, 38
Antipain	Inhibits papain, trypsin, and cathepsin B	Buffer	38
Chymostatin	Inhibits chymotrypsin	5% (vol/vol) DMSO in buffer	38
Leupeptin	Inhibits plasmin, trypsin, papain, and cathepsin B	5% (vol/vol) DMSO in buffer	38

[a] Thiol proteinases have been renamed cysteine proteinases and carboxyl proteinases renamed aspartic proteinases (5).

[b] Buffer, 0.1 M potassium phosphate (pH 6.8). Solvents included to solubilize the inhibitors are listed at the final concentrations present in the assay mixtures. The pH of each acidic compound was adjusted to 6.8 before mixing with the buffer to give a final concentration of 0.1 M phosphate (pH 6.8).

phosphate buffer (pH 6.8) that had been purged with oxygen-free nitrogen, and resuspended in the same buffer for proteinase assays. Protein was determined by the method of Lowry et al. (24) for insoluble proteins.

Microbial cells were disrupted by using either a precooled (4°C) French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) with three passes at a pressure of 2,111 kg cm^{-2} or a Biosonik III ultrasonic disintegrator (Brownwill Scientific Inc., Rochester, N.Y.) fitted with a 0.95-cm-diameter probe, operating at a power output of 188 W cm^{-2}. Cells disrupted ultrasonically were treated in 5-ml samples under a flow of oxygen-free nitrogen gas by two 30-s treatments for protozoa or four to six 30-s treatments for bacteria. Ultrasonic treatments were separated by 1.5-min cooling periods, during which the sample was held on ice.

Proteinase assays. Proteinase activity was assayed by using azocasein (Sigma Chemical Co., St. Louis,

Mo.) as the substrate, at a final substrate concentration of 1% (wt/vol) in 0.1 M potassium phosphate buffer (pH 6.8) unless indicated otherwise. For assays designed to compare the proteinase activities of rumen contents, SRF, and the particulate fraction, the final assay volume was 5 ml, whereas for assay of activities in separated bacterial and protozoal fractions and in SRF, the assay volume was 1 ml. The assays were performed in triplicate at 39°C under nitrogen with a 2-h incubation period unless indicated otherwise. Reactions were stopped by the addition of an equal volume of 10% (wt/vol) trichloroacetic acid. The tubes were allowed to stand at room temperature for 30 min and were then centrifuged at 27,000 × g for 10 min at 4°C. A 1-ml amount of the supernatant fluid was removed from each tube and mixed with 1 ml of 1 N NaOH, and the absorbance at 450 nm was determined. Control samples were assayed in the same manner as the test samples, except that the substrate was added at the end of the incubation period and after the enzyme had
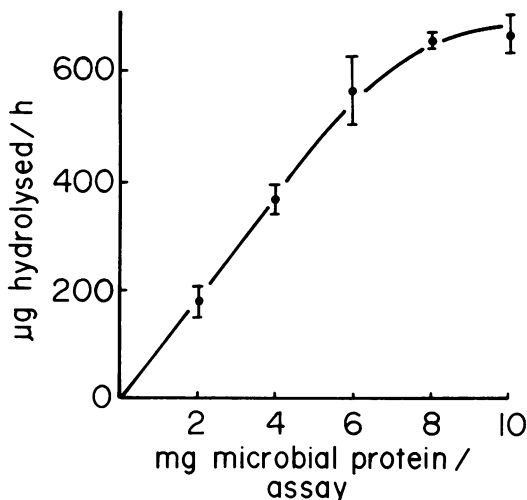
FIG. 1. Effect of rumen bacterial extract concentration on the hydrolysis of a 1% solution of azocasein (2 h of incubation). The cells were disrupted with a French pressure cell. The bars in the graph represent the mean and standard deviation of triplicate samples.

been inactivated by addition of trichloroacetic acid. An extinction curve for azocasein was prepared with azocasein diluted as described for the treated samples. The specific activity was expressed as micrograms of azocasein hydrolyzed per hour per milligram of microbial protein. Mahadevan et al. (25) have found that during assays of rumen microbial proteolytic activity with azocasein as the substrate, the color of solubilized azopeptides is destroyed when incubation periods of 19 to 20 h are used. Recovery of the azo color in the present study was essentially 100% for assays with incubation periods of up to 6 h.

Protease assays with synthetic substrates. Amino acid arylamidase activity was assayed by using L-leucine-*p*-nitroanilide hydrochloride (3) as the substrate at 39°C. The *p*-nitroaniline released during hydrolysis of the substrate was determined by diazotization and coupling to *N*-1-naphthylethylene dihydrochloride (3).

Trypsin- and chymotrypsin-like activities were measured as described by Fritz et al. (17) with *N*-benzoyl-arginine-*p*-nitroanilide and *N*-3-(carboxypropionyl)-L-phenylalanine-*p*-nitroanilide, respectively, as the substrates at 39°C under aerobic conditions. The *p*-nitroaniline formed was measured as the diazotized product to improve the sensitivity of the methods (3). The specific activity was expressed as nanomoles of *p*-nitroaniline produced per minute per milligram of protein.

Proteinase inhibitors. The proteinase inhibitors were dissolved in 0.1 M potassium phosphate buffer (pH 6.8) or in 25% (vol/vol) mixtures of either ethanol or dimethyl sulfoxide (DMSO) in the same buffer and purged with nitrogen gas. To each assay tube, 0.2 ml of the inhibitor solution was added in the place of potassium phosphate buffer, so that the final concentration of solvent in the assay was that indicated in Table 1. A total of 18 inhibitors (Table 1) were tested, each at three concentrations, with incubation periods of 0, 2, 4, and 6 h for each concentration, unless stated

otherwise. Control treatments, containing either buffer or the appropriate buffer-solvent mixture without inhibitor, were included in each experiment. The inhibitors were added to the assay tubes just before the addition of the enzyme. When the effects of trypsin and chymotrypsin inhibitors on the proteinase activity of rumen samples were tested, the effectiveness of the inhibitors was determined in separate assays by the inclusion of 2.5 µg of either trypsin or chymotrypsin per assay tube in the place of rumen microorganisms. All batches of inhibitors used effectively blocked the appropriate proteolytic enzyme. The present inhibition of proteinase activity was calculated from the difference between the reading of the untreated sample (lacking inhibitor but containing buffer or buffer-solvent mixture) minus the reading of the treated sample, divided by the untreated sample reading, multiplied by 100.

Chemicals. EDTA disodium, iodoacetate, and Merthiolate were from Fisher Scientific Co., Pittsburgh, Pa. PMSF, TLCK, ε-aminocaproic acid, L-cysteine hydrochloride, DL-dithiothreitol, kallikrein inhibitor, soybean trypsin inhibitors types I-S and II-S, and lima bean trypsin inhibitor were from Sigma Chemical Co., St. Louis, Mo. TPCK was from Calbiochem, La Jolla, Calif. Phosphoramidon, pepstatin A, antipain, chymostatin, and leupeptin were from Peptide Institute, Inc., Osaka, Japan.

RESULTS

Characteristics of the proteinase assay. Azocasein (15) and diazotized soybean protein (25) have been used as substrates for the measurement of the protease activity of suspensions of rumen microorganisms, although no systematic study of the assay parameters has been reported. The kinetic parameters of the assay were first examined in this study. It was found that the rate of hydrolysis of a 1% (wt/vol) concentration of azocasein by a mixed suspension of rumen bacteria decreased slightly over a 2-h incubation period. If more than 10% of the azocasein was hydrolyzed, the rate decreased dramatically. One percent (wt/vol) azocasein was a saturating concentration of substrate for the protease activity of both cell extracts and mixed suspensions of rumen bacteria. The extent of substrate hydrolysis was linearly related to enzyme concentration as long as no more than 12% of the azocasein was hydrolyzed. Microbial protein in cell extracts did not interfere with proteinase assays at concentrations up to 8 mg of microbial protein per 1 ml of assay volume (Fig. 1).

Proteinase assay values for mixed rumen bacteria, when determined anaerobically over a 2-hour incubation period, were greater by 42% than those for the same enzyme preparation under aerobic conditions. Cysteine and dithiothreitol at 2 mM caused 30 and 40% inhibition, respectively. Inclusion in the assay of a mixture of sodium, calcium, magnesium, iron, manganese, zinc, and cobalt salts, in the form and at

TABLE 2. Hydrolysis of azocasein by whole and disrupted preparations of rumen bacteria and protozoa in potassium phosphate buffer in the absence and presence of dithiothreitol and in a Tris buffer[a]

Sample[b]	Sp act		
	Potassium phosphate	Potassium phosphate and 1 mM dithiothreitol	Tris
Whole bacteria	152.6 ± 10.6	102.8 ± 3.6[c]	125.6 ± 6.6[c]
Disrupted bacteria	195.2 ± 0.6	133.9 ± 2.8[c]	164.2 ± 4.2[c]
Whole protozoa	14.4 ± 0.8	8.6 ± 0.8[c]	20.2 ± 1.4[c]
Disrupted protozoa	28.4 ± 1.4	22.4 ± 1.8[c]	37.2 ± 1.8[c]

[a] All buffers were 0.1 M and pH 6.8. The specific activity was expressed as micrograms of azocasein hydrolyzed per hour per milligram of microbial protein (mean ± standard deviation). Cells were disrupted by ultrasonication.

[b] Bacteria, 3.8 mg protein per assay; protozoa, 6.03 mg per assay.

[c] Significantly different ($P < 0.05$) from the values for the assays conducted in the presence of potassium phosphate buffer alone.

the culture medium concentrations used by Scott and Dehority (36), did not increase the proteinase activity of the bacteria over the corresponding activities attained in either the presence or absence of dithiothreitol (L. K. A. Lovelock and C. W. Forsberg, unpublished data).

Bacterial cell extracts had up to 45% higher proteolytic activity than whole-cell preparations when azocasein was the substrate (Table 2).

Since only those proteinases at the bacterial surface would be available for hydrolysis of dietary protein, whole-cell preparations were used for all proteinase assays unless otherwise indicated.

Distribution of proteinase activity in rumen contents. Rumen contents were separated by straining into particulate and liquid fractions, and each was assayed for proteinase activity with azocasein as the substrate (Table 3). We

TABLE 3. Distribution of proteinase activity in bovine rumen contents

Sample	Wt (g)	Proteinase activity	% of total activity
Expt 1			
Mixed rumen contents[a]	500	1,205 ± 43[b]	100
Particulate materials	255	1,110 ± 37[b]	92
SRF	247	369 ± 8[b]	31
Expt 2			
(i) SRF[c]		439 ± 14[d]	100
(ii) "Cell-free" supernatant from (i)		9 ± 6[d]	2
(iii) First wash supernatant obtained by washing the sediment from (ii) in 0.1 M potassium phosphate buffer (pH 6.8)		8 ± 5[d]	2
(iv) Second wash supernatant		7 ± 5[d]	2
(v) Resuspended microbial sediment		401 ± 5[d]	91

[a] Mixed rumen contents (500 g) were strained through four layers of cheesecloth. The solids and SRF were each made to 500 g with 0.1 M sodium phosphate buffer, and 0.75-g samples of each fraction were assayed in reaction volumes of 5.0 ml for 2 h.

[b] The mean and standard deviation for triplicate values expressed as micrograms of azocasein hydrolyzed per hour per gram of material.

[c] SRF (70 ml) was centrifuged at 16,000 × g for 10 min, and the supernatant fluid (ii) was poured off and saved. The sediment was suspended to a volume of 70 ml with 0.1 M potassium phosphate buffer (pH 6.8) and recentrifuged, and the wash supernatant was saved (iii). The sediment was washed a second time, and the supernatant was saved (iv); finally, the sediment was suspended in buffer (v). All three supernatants were centrifuged at 27,000 × g for 30 min before being assayed (0.5 ml assayed; 2 h of incubation).

[d] The mean and standard deviation for triplicate values expressed as micrograms of azocasein hydrolyzed per hour per milliliter of sample.

TABLE 4. Hydrolysis of synthetic protease substrates by rumen bacterial cell extracts[a]

Substrate	Activity	Sp act[b]
L-Leucyl-p-nitroanilide	Amino acid arylamidase	0.361 ± 0.02
N-Benzoyl-arginine-p-nitroanilide	Trypsin; some plasmin- and papain-like activity	0.84 ± 0.0001
N-3-(Carboxypropionyl)-L-phenylalanine-p-nitroanilide	Chymotrypsin-like activity	0.376 ± 0.018

[a] Cells were disrupted by ultrasonication.

[b] Nanomoles of p-nitroaniline produced per minute per milligram of protein; mean and standard deviation.

recovered at least 123% of the activity in the whole contents in the separated SRF and particulate fractions. Approximately 75% of the recovered activity was associated with the particulate fraction. In a series of five separate experiments over a period of 8 months, the proteinase activity associated with the particulate materials differed; on one occasion it was less than 50% of the recoverable activity. A reduction in the proportion of proteinase activity associated with the particulate fraction of rumen contents was always associated with a decreased proportion of particulate materials in rumen contents.

Proteinase activity corresponding to 415 μg of azocasein solubilized per h per g (dry weight) was present in the ground hay, whereas the ground cereal had no detectable proteinase activity. Based on the weight of hay fed to the cow (12 to 15 kg day^{-1}), mixed rumen contents would not be expected to contain more than 0.1 to 0.2 g (dry weight) of hay per g of wet weight. Therefore, it is likely that the proteinase activity in hay would not account for more than 5% of that in rumen contents.

When SRF was centrifuged at 16,000 × g and the microbial sediment was washed twice with 0.1 M potassium phosphate buffer (pH 6.8), 91% of the proteolytic activity was recovered in the washed microbial fraction (Table 3), and less than 4% of the activity was detected in either the "cell-free" rumen fluid or the potassium phosphate buffer washes. This indicated that the proteolytic enzymes were firmly cell bound, a property recognized by others (9, 31).

Comparative proteinase activities of separated bacteria and protozoa. When whole and disrupted bacteria and protozoa were assayed for proteinase activity, the bacteria were found to have the greatest hydrolytic activity (Table 2). Whole bacteria had a 10-fold higher specific activity than whole protozoa. The difference was less for cell extracts, but it was still appreciable. In all cases, the addition of dithiothreitol inhibited proteinase activity, whereas Tris buffer slightly decreased the activity of the bacterial preparations and stimulated the activity of protozoal preparations. In a separate experiment, the proteolytic activities of disrupted bacteria and pro-

tozoa prepared from three separate animals were determined. The proteinase of the bacterial fraction had the highest specific activity in all three cases.

Hydrolysis of chemically specific protease substrates. To provide preliminary information on the types of proteases present in rumen bacteria, cell extracts were tested for their ability to hydrolyze synthetic protease substrates which were highly specific for amino acid arylamidases, trypsin, and chymotrypsin (Table 4). All three substrates tested were hydrolyzed, indicating the probable presence of arylamidase or aminopeptidase activity and trypsin-like and chymotrypsin-like activities.

Effect of inhibitors on proteinase activity of rumen bacteria. Since proteinase from the rumen bacteria had a much higher specific activity than that from the protozoa, the objective of this series of experiments was to identify and tentatively characterize the types of proteinase inhibitors capable of inhibiting the bacterial proteinase. Figure 2 shows the effect of 1, 2, and 3 mM phenylmethylsulfonyl fluoride (PMSF) on proteolysis. PMSF, at a concentration of 1.0 mM, caused a significant degree of inhibition, which did not change during the 6-h incubation period.
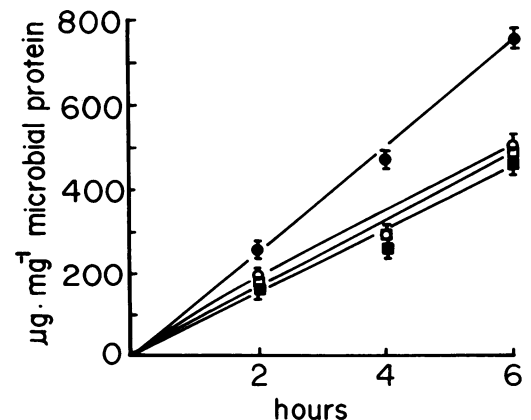

FIG. 2. Effect of PMSF on the hydrolysis of azocasein by a whole-cell preparation of rumen bacteria. PMSF concentrations: 0 (●), 1 (○), 2 (□), 3 (■) mM. The bacterial protein concentration in each assay was 0.84 mg.

TABLE 5. Effects of inhibitors of synthetic, plant, and microbial origin on the proteinase activity of whole rumen bacteria[a]

Inhibitor[b]	Concn	% Inhibition
PMSF	3 mM	36.8[c]
TLCK	1 mM	31.1[c]
TPCK	1 mM	20.8[c]
ε-Aminocaproic acid	100 μg ml^{-1}	-5.9
	500 μg ml^{-1}	7.2
EDTA	1 mM	19.4[c]
	10 mM	17.7[c]
EDTA (1 mM) plus dithiothreitol	2.0 mM	31.6[c]
Cysteine hydrochloride	2.0 mM	27.0[c]
Dithiothreitol	2.0 mM	38.0[c]
Iodoacetate	1.0 mM	24.0[c]
	5.0 mM	31.0[c]
Merthiolate	0.1 mM	14.0[c]
	1.0 mM	57.0[c]
	5.0 mM	71.0[c]
Kallikrein inhibitor	5.7 TIU[d] ml^{-1}	2.2
Soybean trypsin inhibitor type I-S	100 μg ml^{-1}	39.4[c]
	500 μg ml^{-1}	34.0[c]
Soybean trypsin inhibitor type II-S	100 μg ml^{-1}	40.6[c]
	500 μg ml^{-1}	43.2[c]
Lima bean trypsin inhibitor	25 μg ml^{-1}	29.7[c]
	125 μg ml^{-1}	31.7[c]
Phosphoramidon	0.2 mM	0.8
Pepstatin A	20 μg ml^{-1}	2.0
	50 μg ml^{-1}	6.5[c]
	200 μg ml^{-1}	5.3[c]
Antipain	50 μg ml^{-1}	26.5[c]
	250 μg ml^{-1}	32.6[c]
Chymostatin	50 μg ml^{-1}	22.3[c]
	250 μg ml^{-1}	33.6[c]
Leupeptin	50 μg ml^{-1}	28.1[c]
	250 μg ml^{-1}	42.8[c]
Antipain plus chymostatin	50 + 50 μg ml^{-1}	33.0[c]
Chymostatin plus leupeptin	50 + 50 μg ml^{-1}	33.1[c]

[a] The bacterial protein added to each assay mixture ranged from 0.84 to 1.96 mg.

[b] The solvents used to solubilize the inhibitors are indicated in Table 1 and the text.

[c] The percent inhibition differed significantly ($P <$ 0.05) from equivalent untreated controls. These data summarize five separate experiments. The percent inhibition was recorded for 6 h of incubation, except for EDTA, for which the incubation time was 4 h, and iodoacetate, Merthiolate, and pepstatin A, for which the incubation time was 2 h.

[d] One trypsin unit will hydrolyze 1 μmol of Nα-benzoyl-DL-arginine-p-nitroanilide per min at pH 7.8 and 25°C. One-half a trypsin inhibitory unit (TIU) will decrease the activity of one trypsin unit by 50%.

When the concentration of PMSF was increased to 3.0 mM, there was a small, but insignificant, increase in the degree of inhibition to 37%. Table 5 summarizes the results for the inhibitors tested. The inhibition caused by N-tosyl-1-lysine chloromethyl ketone (TLCK), N-tosylphenylal-
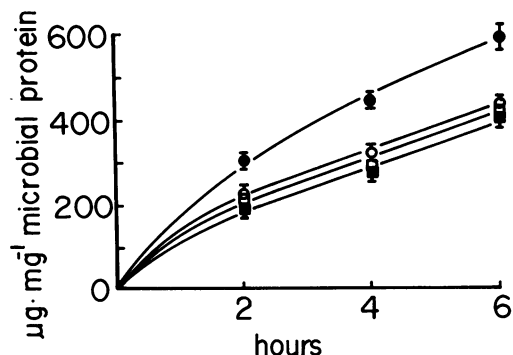

FIG. 3. Effect of antipain on the hydrolysis of azocasein by a whole-cell preparation of rumen bacteria. Antipain concentrations: 0 (●), 50 (○), 100 (□), and 250 (■) μg ml^{-1}. The bacterial protein concentration in each assay was 1.92 mg.

anine chloromethyl ketone (TPCK), ε-aminocaproic acid, cysteine hydrochloride, and dithiothreitol did not change significantly throughout the incubation period, and all the compounds tested, except ε-aminocaproic acid, caused a significant degree of inhibition. The use of ethanol to dissolve PMSF, TLCK, and TPCK caused a slight stimulation of protease activity when compared with the activity in the absence of ethanol. The results obtained with EDTA were slightly erratic. In several experiments, EDTA caused up to 30% inhibition of proteolysis.

Cysteine and dithiothreitol caused a significant degree of inhibition at concentrations of 1 and 2 mM. These concentrations were reported by Broderick (10) to have no effect in his assays of proteolysis in strained rumen fluid. Iodoacetate, an alkylating agent, at 1.0 mM caused 24% inhibition; according to Barrett (4), this is a suitable concentration for selective inhibition of cysteine proteinases. Merthiolate, because of its high solubility, was substituted for the classical thiol inhibitor 4-chloromercuribenzoate. Both of these compounds form mercaptides (40). Chloromercuribenzoate is reportedly selective for cysteine proteinases at low concentrations (0.1 mM), but selectivity decreases at higher concentrations (4); indeed, the inhibition by Merthiolate increased at the higher concentrations.

Kallikrein inhibitor, a protein widely distributed in bovine tissues, caused 15% inhibition of proteinase activity at 2 h, but the inhibition was not detectable by 6 h. The soybean and lima bean trypsin inhibitors caused 29.7 to 43.2% inhibition of proteinase activity. There was no significant difference in the degree of inhibition between the lowest and highest concentration of each inhibitor tested.

With the inhibitors of microbial origin, neither

phosphoramidon nor pepstatin had an appreciable effect on proteinase activity. Antipain caused up to 33% inhibition (Table 5 and Fig. 3). Chymostatin and leupeptin were sparingly soluble in 0.1 M potassium phosphate buffer (pH 6.8); therefore, they were dissolved in 25% (vol/vol) DMSO and added to the assay mixture to give a final DMSO concentration of 5%. At this concentration, DMSO alone caused 17% inhibition of proteinase activity, and in the reported experiments appropriate controls were included to allow for correction for this inhibitor effect. Chymostatin and leupeptin at 250 μg ml^{-1} caused 34 and 43% inhibition, respectively. As shown in Fig. 3 for antipain, chymostatin and leupeptin also interacted rapidly with the inhibitor sites on the proteolytic enzymes. Beyond 2 h of incubation, there was no change in the inhibitory effect, and the lowest concentration of inhibitor tested, 50 μg ml^{-1}, was nearly a saturating concentration.

The combination of antipain and chymostatin caused an increase in inhibition greater than that caused by either alone, although the effects were less than additive. The combination of chymostatin and leupeptin did not produce a degree of inhibition greater than that of leupeptin alone, which suggests that both inhibitors were inhibiting the same enzymes.

DISCUSSION

The use of diazotized proteins as substrates for the measurement of rumen microbial proteinase activity has been reported to be a convenient, reliable method that does not have the disadvantage of the loss of hydrolysis products, such as amino acids, due to microbial utilization (25). Results from our study support this conclusion, provided the substrate concentration is 1%, the microbial protein concentration is kept low, and the reaction is stopped before 10% of the substrate has been hydrolyzed.

Almost all studies of rumen proteolysis have employed SRF as the source of enzyme. As revealed by this study, as little as 25% of the proteolytic activity of rumen contents of animals fed hay and concentrate was present in the SRF fraction, the remainder being associated with the particulate feed fraction. Craig et al. (W. M. Craig, G. A. Broderick, and G. T. Schelling, Proc. Am. Soc. Anim. Sci., abstr. no. 645, 1981) have expanded this comparison by determining the distribution of proteinase activity in rumen contents of cattle fed either concentrate or roughage rations. As might be predicted, they observed that a greater proportion of the proteolytic activity was associated with the particulate fraction in a hay-fed animal. Thus, proteolytic microorganisms, primarily bacteria, appear to be retained by the particulate materials, which

perhaps reflects the adherent nature of a wide range of rumen bacteria (30), many of which are proteolytic (18). Whether the proteolytic activities of microbes in SRF reflect those of the flora in mixed rumen contents can be questioned. We do have preliminary data suggesting that proteolysis by the bacterial fraction is similar to that in SRF (unpublished data).

The specific activity of the bacterial proteinase was 7 to 10 times higher than that of separated protozoa when either whole cells or cell extracts were assayed. The protozoal biomass is approximately 50% of the total ruminal biomass when a ruminant receives a roughage diet, and it increases when a high grain diet is fed (20). Thus, it appears that, on roughage diets at least, bacteria are primarily responsible for hydrolysis of feed protein. Nugent and Mangan (31) have reported that protozoal cells showed neither uptake nor proteolysis of fraction 1 leaf protein from alfalfa and that the protozoal population had less than 10% of the proteolytic activity of whole rumen fluid. The contribution of protozoa is therefore probably limited primarily to bacterial engulfment and degradation, a role amply documented by Coleman (14). Nevertheless, protozoa do possess proteinases (2) and have a high endogenous proteolytic activity which can be inhibited by a number of the inhibitors used in this study (L. K. A. Lovelock and C. W. Forsberg, manuscript in preparation).

The inhibitory effects of PMSF, TLCK, and TPCK on proteolysis by rumen bacteria indicate the presence of serine or cysteine proteinases or both. The inhibitory effect of soybean trypsin inhibitor type I-S confirms the presence of a serine proteinase with trypsin-like activity. The decreased inhibitory effect of TPCK compared with that of TLCK may indicate the absence of chymotrypsin-like activity. The inhibitory effects of iodoacetate and Merthiolate confirm the presence of cysteine proteinases. The low inhibitory effect of pepstatin suggests that aspartic proteinases represent a minor component of the proteolytic activity, whereas the inhibitory effects of EDTA, cysteine, and dithiothreitol indicate the presence of metalloproteinases. The inhibitory effects of antipain, leupeptin, and chymostatin are not in disagreement with the identity of the types of proteinases. Chymostatin, for example, is specific for chymotrypsin at a low concentration (0.15 μg ml^{-1}) but is less specific at higher concentrations (38). It must also be borne in mind that proteinases are usually characterized with less complex sources of enzyme. Some of the inhibitory effects observed could be due to the indirect effects of the inhibitors, and perhaps some inhibitors would have been significantly more inhibitory had purified proteinases been tested. Undoubtedly, it will be

beneficial to conduct additional tests on combinations of inhibitors.

By using the criteria reviewed by Barrett (4, 5), it has been possible to tentatively classify the rumen bacterial proteinases as serine, cysteine, and metalloproteinases. The proteinases of only two rumen bacteria have been characterized. Blackburn (8) has reported that the proteinase of *Bacteroides amylophilus* H18 has a trypsin-like specificity. Hazlewood and Edwards (21) have found that *Bacteroides ruminicola* R8/4 possesses proteolytic activity characteristic of serine, cysteine, and aspartic proteinases. Recently, Russell et al. (35) demonstrated that *Streptococcus bovis* has a role in rumen proteolysis. Confirmation of the role of *S. bovis* in rumen proteolysis could be accomplished by determining the nature of its protease(s) activity. In view of the ubiquity of proteolytic activity among rumen bacteria (18), it appears likely that the predominant proteolytic bacteria will differ depending on diet. One possible approach for identifying the predominant proteolytic bacteria is through mapping their protease specificities by using a combination of protease inhibitors and synthetic substrates. The experiment on the hydrolysis of synthetic substrates for determination of aminopeptidase, trypsin-like, and chymotrypsin-like activities exemplifies their value, although their application may be considered to be compromised by the use of cell extracts rather than whole cells as the source of enzyme. Subsequent experiments have revealed that similar activities were obtained when either whole cells or cell extracts were assayed (Lovelock and Forsberg, unpublished data).

Because of the intense interest in methods for inhibiting proteolysis in the rumen (13), the proteinase inhibitors of plant and microbial origin require further consideration. It is worth noting that damage to growing alfalfa plants leads to increased synthesis of a trypsin inhibitor (39). The effect this might have on rumen proteolysis has not been determined. From the inhibitory effect of soybean trypsin inhibitor on proteolysis by rumen bacteria, one might postulate that raw, instead of heat-treated, soybeans fed to cattle would cause a decrease in rumen ammonia due to decreased proteolysis. Instead, it has been observed that the amount of ammonia is reduced in the rumens of cattle receiving heated soybeans (28). Unfortunately, the concentration of trypsin inhibitor in the raw soybeans was not determined, and consequently, in the context of this discussion, the data were inconclusive. They do lead, however, to the question of the stability and effectiveness of protease inhibitors in the dynamic rumen ecosystem.

The development or discovery of proteinase inhibitors for decreasing rumen proteolysis may be considered unfeasible, particularly if they inhibit proteolysis in the lower gastrointestinal tract. It is significant that ruminants, unlike some nonruminants, do not suffer ill effects from the consumption of feeds high in trypsin inhibitor (23). In rats it has been found that the small intestine responds to proteinase inhibitors by increasing the synthesis of proteinases (1). It would be worthwhile to determine the digestive response of ruminants to high concentrations of trypsin and chymotrypsin inhibitors. The reason for the lack of toxicity of trypsin inhibitor to ruminants has not been resolved.

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