Proteolytic Activities of a Rumen Bacterium, *Bacteroides ruminicola* R8/4

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Proteolysis of leaf Fraction 1 protein, casein, azocasein and bovine serum albumin by the cell-associated proteinases of the rumen bacterium *Bacteroides ruminicola* R8/4 was investigated and the kinetic parameters $V_{\max}$ and K_m were evaluated for each substrate. A variety of proteinase inhibitors was used to show that the proteolytic activity comprises a mixture of at least three different classes of proteinase. With respect to substrate specificity and some inhibition characteristics, the proteolytic activity of *B. ruminicola* R8/4 was similar to that of rumen contents.

INTRODUCTION

The proteolysis of soluble proteins in the rumen is effected primarily by cell-associated enzymes of the rumen bacterial fraction (Nugent & Mangan, 1981; Brock & Forsberg, 1980). The rate of degradation of different soluble proteins by rumen contents varies considerably (Mangan, 1972; Nugent & Mangan, 1978) indicating that not only protein solubility, but also protein structure, influences rumen proteolysis.

Comparatively little is known of the nature of rumen proteinases, but a recent study showed the bacterial fraction to contain chymotrypsin- and trypsin-like enzymes, as well as other neutral proteinases and peptidases (Brock & Forsberg, 1980). By contrast, the proteolytic rumen bacterium *Bacteroides amylophilus* strain H18 produced only a trypsin-like activity which could be almost totally inhibited by the serine proteinase inhibitor, diisopropyl fluorophosphate (Blackburn, 1968; Lesk & Blackburn, 1971).

We describe here experiments to determine the nature of the proteolytic activity of *Bacteroides ruminicola* strain R8/4, a rumen bacterium which will utilize soluble proteins as a sole source of nitrogen for its growth (Hazlewood & Nugent, 1978; Hazlewood *et al.*, 1981).

METHODS

Growth of organism. *Bacteroides ruminicola* R8/4 was grown in 800 ml batch cultures as described previously using leaf Fraction 1 protein (ribulose-1,5-bisphosphate carboxylase; EC 4.1.1.39) (1 g l^{-1}) as sole nitrogen source (Hazlewood *et al.*, 1981). Bacteria in which the specific activity of proteinase was maximal were harvested during exponential growth by centrifugation ($12\,000 \text{ g}$, 4°C , 20 min) in capped tubes under an atmosphere of O_2 -free N_2 , and washed once with 75 mM-potassium phosphate buffer, pH 6.8, containing 1.3 mM-dithiothreitol. Washed cells (25 mg dry wt) in 4 ml of the same buffer were stored under N_2 at -70°C until required.

Bacterial growth. Culture turbidity (A_{650}) was measured with an EEL colorimeter and a red filter which transmits light of wavelength exceeding 650 nm. Standard curves were constructed relating turbidity of bacterial suspensions to dry weight.

Proteins. Unlabelled and [$\text{U-}^{14}\text{C}$]Fraction 1 protein were prepared as described previously (Hazlewood *et al.*, 1981). Bovine serum albumin (BSA, crystalline), azocasein and ovalbumin (Fraction V) were from Sigma and casein from Hopkin & Williams.

Inhibitors. Diisopropyl fluorophosphate, iodoacetic acid, phenylmethanesulphonyl fluoride, *p*-chloromercuribenzoate, 1,10-phenanthroline and soybean trypsin inhibitor were from Sigma. Proteinase inhibitors derived from cultures of actinomycetes (antipain, chymostatin, elastatinal, leupeptin and pepstatin A; Ūmezawa & Aoyagi, 1977) were obtained from the Peptide Institute (476 Ina, Minoh-shi, Osaka 562, Japan) and sterile stock solutions were prepared with regard to published solubility data.

Analytical procedures. Protein nitrogen was estimated by a micro-Kjeldahl method (Mangan & West, 1977), the ammonia liberated from digested samples being determined by the phenol-hypochlorite reaction (Russell, 1944).

Proteinase assay. Washed *B. ruminicola* R8/4 cells (final concentration 0.45 mg dry wt ml⁻¹) suspended in 75 mM-phosphate buffer, pH 6.8, containing 1.3 mM-dithiothreitol at 39 °C were added to the same buffer containing the substrate (0.1 mg ml⁻¹ unless otherwise stated) and any additions. Dithiothreitol could be omitted from the reaction mixture if cells were preincubated for 30 min at 0 °C in the presence of 1 mM-dithiothreitol and then resuspended in 75 mM-phosphate buffer, pH 6.8, prior to assay. All assays were at 39 °C and were terminated by rapid cooling in an ice-water bath. Depending on the substrate used, the extent of proteolysis was determined by the following procedures.

(i) [U-¹⁴C]Fraction 1 protein as substrate. Bacteria were removed by centrifugation (2500 g, 4 °C, 10 min), and soluble radioactivity was determined before and after acid deproteinization [trichloroacetic acid (TCA) final concentration 10%, w/v] as described by Hazlewood *et al.* (1981).

(ii) Unlabelled Fraction 1 protein, casein, BSA or ovalbumin as substrate. Bacteria were removed by centrifugation (2500 g, 4 °C, 10 min), and residual protein in the cell-free supernatant was sedimented by centrifugation (10000 g, 4 °C, 20 min) after the addition of TCA (final concentration 10%, w/v) and determined by the Lowry method as modified by Miller (1959). A standard curve was constructed for each protein studied. The method proved highly reproducible with variation between duplicates of less than 5%, but it was unsuitable for monitoring product formation when dithiothreitol was added to satisfy the requirement of the proteinase activity for a thiol reagent (Hazlewood *et al.*, 1981) as the latter interfered with colour development (1.3 μmol dithiothreitol gave $A_{750} = 1.46$) and no satisfactory way was found to remove or oxidize it.

(iii) Azocasein as substrate. In a modification of the method of Charney & Tomarelli (1947), cell-free supernatant (2500 g, 4 °C, 10 min) was deproteinized with TCA and centrifuged as described above, and a sample of the TCA-soluble fraction was mixed with an equal volume of 1 M-NaOH. A_{440} was measured and compared with a standard curve for azocasein in 0.5 M-NaOH. Where required, residual protein in the TCA-pellet was redissolved in 0.5 M-NaOH and determined similarly.

All assays, and substrate and enzyme controls, were carried out in duplicate.

RESULTS

Substrate specificity

The proteolytic activity of washed, exponential phase *B. ruminicola* R8/4 cells degraded casein more rapidly than either Fraction 1 protein or BSA (Fig. 1). Both Fraction 1 protein and casein were more completely hydrolysed than BSA or ovalbumin which, under the conditions of the experiments, was degraded very slowly; after 2 h incubation, only about 10% of the ovalbumin had been converted to TCA-soluble products.

The rate of proteolysis of each of a number of different protein substrates at a fixed concentration of R8/4 cells (0.45 mg dry wt ml⁻¹) depended on the initial substrate concentration. For the proteolysis of BSA, casein and Fraction 1 protein, the respective values of K_m and V_{max} were 3.18, 0.43 and 0.15 mg protein ml⁻¹ and 0.45, 3.01 and 0.30 mg protein hydrolysed (mg dry wt cells)⁻¹ h⁻¹. With casein and Fraction 1 protein, substrate inhibition was apparent at concentrations above 0.4 and 0.1 mg ml⁻¹, respectively. Diazotized casein was rapidly degraded by R8/4 proteinase with unchanged K_m (0.45 mg ml⁻¹) but the value of V_{max} obtained with azocasein as substrate [1.53 mg protein hydrolysed (mg dry wt cells)⁻¹ h⁻¹] was considerably less than that obtained with casein. Substrate inhibition occurred when the initial azocasein concentration exceeded 0.5 mg ml⁻¹.

Inhibition characteristics of proteolytic activity

Proteolysis of casein and Fraction 1 protein was inhibited 9% and 12%, respectively, by 1 mM-iodoacetic acid. *p*-Chloromercuribenzoate (at 0.1 mM) inhibited the activity towards

Fig. 1. Proteolysis of Fraction 1 protein (■), casein (●) and BSA (▲) by cell-associated proteinases of *B. ruminicola* R8/4. Washed exponential phase cells ($0.45 \text{ mg dry wt ml}^{-1}$) suspended in 75 mM -phosphate buffer, pH 6.8, containing 1.3 mM -dithiothreitol, were incubated at 39°C with each protein substrate (0.1 mg ml^{-1}). The extent of protein degradation was determined as described in Methods.

Table 1. *Effect of inhibitors on the cell-associated proteolytic activity of B. ruminicola* R8/4

Washed exponential phase cells ($0.45 \text{ mg dry wt ml}^{-1}$) suspended in 75 mM -phosphate buffer, pH 6.8, containing 1.3 mM -dithiothreitol, were incubated at 39°C with substrate in the presence or absence of proteinase inhibitors. Proteolysis was determined as described in Methods.

Inhibitor	Concn (μM)	Percentage inhibition of proteolytic activity		
		Casein as substrate: 0.1 mg ml^{-1}	Fraction 1 protein as substrate:	
			0.1 mg ml^{-1}	0.05 mg ml^{-1}
Antipain	8.3	40	13–14	57
Chymostatin	11.7	38	48–60	63
Elastatinal	8.5	18	31–36	53
Leupeptin	11.7	42	20–24	58
Pepstatin A	7.3	32	28–35	33
Soybean trypsin inhibitor	2.5	18	ND	18
Leupeptin + EDTA (10 mM)	11.7	ND	ND	79
EDTA (10 mM)	—	57	46	28
EDTA (20 mM)	—	93	24	ND

ND, Not determined.

casein by 56% but caused precipitation of Fraction 1 protein and could not be tested with this substrate.

At 5 mM, EDTA inhibited the rate of proteolysis of Fraction 1 protein by 75%, but inhibition decreased with increasing EDTA concentration to 24% with 20 mM-EDTA. Breakdown of azocasein and casein was similarly strongly inhibited (60 to 70%) by 10 mM-EDTA but this inhibition increased to 90% with 20 mM-EDTA. Another metal chelator, 1 mM-1,10-phenanthroline, inhibited by 77% the proteolysis of azocasein by cell-associated proteinases of R8/4. In the absence of inhibitors, proteolytic activity towards both Fraction 1 protein and casein was stimulated up to 30% by the addition of 10 to 20 mM- Mg^{2+} ions.

The serine proteinase inhibitors diisopropyl fluorophosphate and phenylmethanesulphonyl fluoride (each at 1 mM) inhibited the proteolysis of 0.5 mg azocasein ml^{-1} by 26% and 21%, respectively.

The effect of naturally occurring proteinase inhibitors and EDTA on the proteolytic activity of R8/4 towards two substrates is shown in Table 1. With the exception of pepstatin A, the degree of inhibition produced by the microbial proteinase inhibitors depended on the nature of the protein substrate and, for a given protein, also on the initial substrate concentration. The inhibitory effect of leupeptin on hydrolysis of azocasein and Fraction 1 protein was maximal at a leupeptin concentration of $1.2\ \mu\text{M}$ and remained unchanged when the concentration of inhibitor was raised to $11.7\ \mu\text{M}$.

DISCUSSION

In hydrolysing casein more rapidly than Fraction 1 protein or BSA the proteolytic activity of *B. ruminicola* R8/4 is similar to that of whole rumen contents (Nugent & Mangan, 1978). Mangan (1972) suggested that the recalcitrance in the rumen of soluble proteins such as ovalbumin compared with casein or Fraction 1 protein may be a consequence of their tightly-folded globular structure. While the same would appear to be true for hydrolysis of BSA and ovalbumin by R8/4 proteinases, the proteolysis of BSA can be sufficiently rapid and complete to permit growth of the organism in a medium which contains BSA as sole nitrogen source (Hazlewood & Nugent, 1978; Hazlewood *et al.*, 1981).

Although casein was most rapidly degraded, K_m values indicate that the proteinases of *B. ruminicola* R8/4 have a higher affinity for Fraction 1 protein than for any of the other soluble proteins examined. This is probably because Fraction 1 protein is the major soluble protein of the leaves of green plants (Lyttleton & T'so, 1958) and as such it is likely to be the main substrate for the proteinases of this rumen organism *in vivo*.

The proteolytic activity of *B. ruminicola* R8/4 was stimulated by dithiothreitol and cysteine proteinase could account for at least part (probably up to about 35%) of the total activity (Hazlewood *et al.*, 1981). The present work has shown that R8/4 proteinases, like those of the rumen bacterial fraction (Brock & Forsberg, 1980), constitute a complex mixture of different activities. The use of specific inhibitors of serine proteinases (diisopropyl fluorophosphate and phenylmethanesulphonyl fluoride) and soybean trypsin inhibitor indicate that around 25% of the R8/4 proteinase activity may be serine proteinase with a possible 20% being trypsin-like. If the maximal inhibition of proteinase activity which was obtained using leupeptin, antipain, elastatinal and chymostatin with $0.05\ \text{mg}$ Fraction 1 protein ml^{-1} as substrate (Table 1) represented inhibition of both serine and cysteine proteinases, then these two types of activity could account for up to 60% of the total activity. However, inhibition of azocasein hydrolysis by iodoacetic acid indicated that only about 10% was cysteine proteinase. The proteolysis of casein and Fraction 1 protein was inhibited to the same extent (about 30%) by pepstatin A, a powerful and specific inhibitor of aspartic acid proteinases (Barrett, 1977), indicating that roughly one-third of the bacterial activity could belong to this class.

The greater part of the proteolytic activity of *B. ruminicola* R8/4 could be inhibited by the metal chelators EDTA (5 to 20 mM) or 1,10-phenanthroline (1 mM), suggesting that the bulk of the activity is composed of enzymes dependent on metal ions for their function; stimulation of activity by Mg^{2+} ions reinforces this conclusion. However, inhibition by chelating agents is not definitive proof of the existence of a metalloenzyme since loss of activity may result from denaturation (see Knight, 1977). Further experiments would therefore be required to confirm the presence of a metalloproteinase.

On the basis of the scheme reviewed by Barrett (1977) it can tentatively be concluded that the cell-associated proteolytic activity of *B. ruminicola* R8/4 comprises a mixture of serine, cysteine and aspartic acid proteinases with the possibility that some of the activity is dependent on the presence of metal ions.

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