Activities and Partial Purification of Extracellular Proteases of *Bacteroides nodosus* from Virulent and Benign Footrot

A. A. Kortt,^A I. J. O'Donnell,^A D. J. Stewart^B and B. L. Clark^B

^A Division of Protein Chemistry, CSIRO, Parkville, Vic. 3052.

^B Division of Animal Health, CSIRO, Parkville, Vic. 3052.

Abstract

In an attempt to differentiate virulent and benign strains of *B. nodosus*, the extracellular proteolytic activity of these cultures was assayed with elastin, casein and hide powder azure, and the stability to heating at 55°C was determined. Broth cultures of both strains hydrolysed ¹²⁵I-labelled elastin, indicating that this activity is not a unique marker of virulence. When cultures were grown in Trypticase-arginine-serine broth medium modified by omitting Na₂CO₃ and thioglycollic acid, the total proteolytic activity and its stability at 55°C could be used to differentiate isolates causing virulent or benign footrot lesions. However, when other broth cultures were used, these parameters could no longer be used to make such a distinction. The proteases of a virulent and benign strain of *B. nodosus* were partially purified and characterized. Four to five closely related proteases were detected by polyacrylamide gel electrophoresis at pH 8·8 in both types of isolates. The proteases are serine-type enzymes requiring a divalent metal ion such as calcium for activity. The proteases of the benign strain were somewhat less stable to heat than the enzymes of the virulent strain. Differences in the relative mobilities of the proteases of virulent and benign strains of *B. nodosus*, on electrophoresis at pH 8·8, suggest that this property may be used to distinguish virulent and benign strains.

Introduction

The activities of the extracellular proteases produced by Bacteroides nodosus have been used in laboratory tests to distinguish virulent and benign strains of B. nodosus, the causative organism of ovine footrot. Depiazzi and Richards (1979), using hide powder azure as the assay substrate, showed that the relative stability of B. nodosus proteases at 37°C was greater for virulent than for benign strains over a 12-day Furthermore, Stewart (1979) demonstrated that virulent strains slowly period. digested elastin particles incorporated in an agar medium on which they were growing after a minimum period of 6-7 days whereas benign strains did not, even though the incubation period was extended to 21-28 days. Stewart (1979) also reported a more marked difference between virulent and benign strains in the stability of proteolytic activity than that recorded by Depiazzi and Richards (1979). Several colony types of B. nodosus have been identified by Thorley (1976) and Skerman et al. (1981). Skerman et al. (1981) found that colonies from virulent footrot in sheep hydrolysed insoluble elastin whereas colonies from benign strains from sheep and cattle did not. Colony variants from virulent primary isolates showed reduced virulence and hydrolysed elastin to a lesser extent. A major drawback of the tests for distinguishing the different strains of *B. nodosus* is the time taken for an identification to be made.

The present work was undertaken to characterize the proteolytic activity of *B. nodosus* strains and to determine whether it was possible to develop a more rapid differential diagnostic test than those previously described. The properties of the purified proteases from virulent and benign strains of *B. nodosus* were also examined.

Materials and Methods

Bacteria

Bacteroides nodosus strains 134, 198, 217, 243, 265, 269, 291 and 305 from the McMaster Laboratory culture collection were used. Strains NZ-10 and NZ-80 were obtained from Dr T. M. Skerman, Wallaceville Animal Research Centre, Ministry of Agriculture and Fisheries, Upper Hutt, New Zealand.

Culture Methods

Cultures of *B. nodosus* were grown anaerobically in an atmosphere of 90% H₂ and 10% CO₂ at 37°C in a variety of broth media. Medium A was Trypticase-arginine-serine (TAS) broth, pH 7.8 (Skerman 1975), modified by omitting Na₂CO₃ and thioglycollic acid. Medium B was TAS broth containing the two above ingredients and was modified to contain 2.5 g/l of L-arginine-HCl (BDH Chemicals Ltd). Medium C was medium B plus 15% (w/v) gelatin (Oxoid). Medium D was Eugon broth (BBL, Division of Bioquest, Becton, Dickinson and Co., Maryland, U.S.A.) plus 0.2% (w/v) yeast extract (Difco), 15% (w/v) gelatin and 0.2% (w/v) Na₂CO₃. Eugon broth alone and Eugon broth with either 0.2% (w/v) yeast extract or 15% (w/v) gelatin were also used.

Assays of Proteolytic Activity

Elastase activity was measured using elastin (Sigma, E-1625) iodinated with ¹²⁵I as described by Robert and Robert (1969). The iodinated elastin was stored in phosphate-buffered saline containing 5% (v/v) tertiary butanol, which promoted even suspension and sampling of the elastin for assay. Aliquots, containing 0.25 mg of labelled elastin, were pipetted into a 3.5-ml clear polystyrene tube for assay, and were counted. The counts per minute were of the order of $500\,000 \pm$ 10000. A suitable aliquot of enzyme and 2.5 ml of 0.05 M Tris-HCl-0.01 M CaCl₂-0.2% (v/v) Triton X-100, pH 8.6, was added and incubated on a shaker at room temperature for 1 h. The tubes were then centrifuged and 200-µl aliquots counted.

Proteolytic activity was measured with hide powder azure (Calbiochem-Behring Corp., B grade) as described by Depiazzi and Richards (1979) except that the buffer used was 0.05 M Tris-HCl--0.01 M CaCl₂, pH 8. For the assay with casein (Merck), the method used was a modification of that of Kakade *et al.* (1970). To 1 ml of casein (1% w/v) in 0.05 M Tris-HCl--0.1 M NaCl, pH 8), a suitable aliquot (0.05-0.1 ml) of enzyme and 0.05 M Tris-HCl--0.01 M CaCl₂ buffer, pH 8, was added to a final volume of 2 ml and incubated at 40° C for 2 h. The reaction was stopped by adding 3 ml of buffered trichloroacetic acid (Kakade *et al.* 1970) and the absorbance of the filtrate was measured at 280 nm. For all assay systems, suitable controls were performed.

Effect of Heating on the Stability of Proteolytic Activity

Broth cultures (media A, B, C, D) of *B. nodosus*, in sealed tubes, were incubated in a water bath at $55\pm0.2^{\circ}$ C for various times (15, 30, 45 and 60 min), cooled and assayed for proteolytic activity using hide powder azure, casein and elastin as substrates. For the temperature-stability study, partially purified proteases (Sephadex G100 peak concentrate diluted to approximately the activity of the broth culture) were incubated in sealed tubes at various temperatures for 30 min, cooled in ice and 50-µl aliquots assayed. The assays of pH stability were carried out using the same enzyme preparations. The protease concentrate was diluted (to approximately the activity of the culture filtrate) in an appropriate buffer (pH 2–10) and assayed after 1 h and 24 h incubation at room temperature. The pH of the incubation mixture was checked at the end of the experiment.

Purification of the Proteases

Three- or four-day broth cultures were grown in medium B for the purification and electrophoretic analyses. The cultures were centrifuged to remove insoluble material and concentrated approximately 30-fold in an Amicon ultrafiltration cell using a Diaflo YM-10 membrane. The concentrate was chromatographed on a column of Sephadex G100. The proteolytic activity eluted as a single peak and the active fractions were pooled, concentrated (to approximately 100-fold of the original volume) and dialysed against 0.02 M Tris-HCl-5 mM CaCl₂, pH 8. This material (for strains 198S and 305) was then fractionated on a column of DEAE-Sephadex A25. A gradient of 0-0.2 M NaCl in 800 ml of starting buffer was applied to elute the bound proteolytic activity. A second gradient, 0.2-1.0 M NaCl in 800 ml, was then applied to elute the remainder of the bound protein. The peaks with protease activity were pooled and concentrated and their purity assessed by polyacrylamide gel electrophoresis at pH 8.8. Chromatography and sample concentration were carried out at room temperature.

Electrophoretic Experiments

Discontinuous polyacrylamide gel electrophoresis in 3-mm slabs was carried out using the buffer system of Davis (1964) with 5 and 7.5% (v/v) acrylamide. The protease peaks from the Sephadex G100 column and the purified proteases were analysed. Disc gel electrophoresis at pH 8.8 was carried out to identify the protein bands with protease activity. Slices (5 mm) of the disc gel were incubated with the substrate (hide powder azure) in a normal assay mixture to locate the activity and a duplicate gel was stained for protein. The gels were stained with 0.25% (w/v) Coomassie brilliant blue G250 and destained in 20% (v/v) methanol-10% (v/v) acetic acid.

Results and Discussion

Proteolytic Activity

The proteolytic activities of various cultures grown in TAS broth (medium A) are shown in Table 1. The relative proteolytic activity measured with hide powder azure, an insoluble substrate, was found to be similar to that measured with casein, a soluble substrate. As the hide-powder-azure method is the simplest, it was used

Table 1. Proteolytic activities and stabilities of broth cultures of virulent and benign strains of B. nodosus grown in medium A

The strains were grown in TAS broth (medium A) containing Bacto beef-extract paste (Difco). Cultures were maintained at 37°C under anaerobic conditions and proteolytic activities were assayed at day 4, before heating and after heating for 30 min at 55°C, and at day 13. Activity against ¹²⁵I-labelled elastin powder is expressed as No. of counts per min, that against casein as ΔA_{280} per 0.1 ml of culture per 2 h, and that against hide powder azure as ΔA_{595} per 0.05 ml of culture per 30 min

Lesion ^A	Strain	Activity of 4-day culture						Activity of 13-day culture		
		Agains Before heating	f elastin After heating	Agains Before heating	After heating	Against hi Before heating	After After heating	elastin	casein	hide powder
VF	243	9900	2300	0.42	0.15	0.65	0.40	2400	0.18	0.56
VF	265	11300	3000	0.48	0.20	0.79	0.55	2200	0.18	0.59
BF	269	6400	1000	0.23	0.01	0.38	0.01	0	0.01	0.03
BF	291	2400	670	0.18	0.46	0.03	0.02	500	0.00	0.04
BF	305	1700	1000	0.22	0.02	0.39	0.03	300	0.00	0.04
BF	134	3700	1000	0.36	0.02	0.56	0.02	200	0.00	0.02

^A VF, virulent footrot; BF, benign footrot.

for routine assays. In contrast to the findings using elastin-agar plates (Stewart 1979), the benign cultures hydrolysed elastin. Thus, the ability to hydrolyse elastin is not restricted to virulent strains of *B. nodosus* (Stewart 1979; Skerman *et al.* 1981). The virulent strains showed much higher levels of elastolytic activity than the benign strains. However, the difference in the level of activities was not as marked with the casein and hide-powder-azure substrates. Benign strain 269 showed a higher

level of elastolytic activity than the other benign strains. This difference was not found with the other substrates. Essentially the same results were obtained in a duplicate experiment in which the same strains in Table 1 were grown in medium A containing Lab Lemco powder (Oxoid) rather than Bacto beef-extract paste (Difco).

In medium A, the stability of the proteolytic activity to heating at 55° C for 30 min was significantly greater for virulent broth cultures than for benign broth cultures (Table 1). The experiments at 55° C gave the same result as 13-day cultures maintained at 37° C. This agrees with the earlier work of Depiazzi and Richards (1979) who found that the proteolytic activity of broth cultures of virulent isolates was relatively stable when maintained at 37° C over a period of 12 days whereas that of benign isolates was significantly less stable. By performing the test at 55° C, the time for the test is markedly decreased.

Table 2. Proteolytic activities and stabilities of broth cultures of virulent and benign strains of B. nodosus

Activities were assayed with hide powder azure and casein as substrates. The strains were grown in three different broth culture media and were assayed after 3 days of growth, before heating and after heating for 30 min at 55°C. Activity against hide powder azure is expressed as ΔA_{595} per 0.05 ml of culture per 30 min, and that against casein as ΔA_{280} per 0.1 ml of culture per 2 h

Lesion ^A	Strain	Growth	Proteolytic activity				
		medium [®]	Against hi Before heating	After heating	Agains Before heating	t casein After heating	
VF	198-L	В	0.35	0.31	0.42	0.19	
VF	198-S	В	0.35	0.17	0.26	0.14	
VF	NZ-80	В	0.35	0.23	0.31	0.10	
BF	291	В	0.40	0.12	0.28	0.11	
VF	198 - S	С	n.d.	n.d.	0.56	0.38	
VF	NZ-80	С	0.46	0.45	0.78	0.54	
BF	291	С	0.51	0.36	0.51	0.33	
VF	198 -S	D	0.45	0.33	0.47	0.32	
VF	NZ-80	D	0.41	0.28	0.57	0.36	
VF	291	D	0.25	0·19	0.18	0.09	

^A VF, virulent footrot; BF, benign footrot.

^B B, medium B; C, medium B+15% added gelatin; D, Eugon broth +0.2% yeast +15% gelatin +0.2% Na₂CO₃.

In an attempt to stimulate the production of proteases by *B. nodosus* in broth culture, different media were tested. The different media influenced the total amount of proteolytic activity produced. The addition of gelatin (15% w/v) to medium B and Eugon broth increased the amount of proteolytic activity by up to twofold in 3-day cultures. However, when virulent and benign strains of *B. nodosus* were grown in culture media other than medium A, they were not always able to be differentiated by the stability of their proteases to heating at 55°C, irrespective of the substrate used for assay (Table 2). Hence, we conclude that media components may enhance (or decrease) the stability of the proteolytic activity to heat. These results show that only TAS broth (medium A) is useful for the growth of *B. nodosus* cultures for testing the heat stability of the proteases to distinguish virulent and benign organisms.

Purification of the Proteolytic Activity

A substantial purification of the proteolytic activity was achieved by gel filtration of concentrated culture supernatant on Sephadex G100 (Fig. 1). All *B. nodosus* strains examined yielded a single peak of proteolytic activity (when assayed with hide powder azure, casein or ¹²⁵I-labelled elastin), which eluted between a major protein peak at the column void volume and a broad peak of lower molecular weight, which contained much of the pigmented material present in the culture supernatant.



Fig. 1. Gel filtration of concentrated culture supernatant of *B. nodosus* strain 305 on Sephadex G100 (150 by 2.5 cm) equilibrated with 0.05 M Tris-HCl-5 mM CaCl₂, pH 8. A flow rate of 36.8 ml/h was maintained by a peristaltic pump. Fraction volume was 9.2 ml; sample volume was 26 ml. Aliquots (50 µl) were assayed using hide powder azure as substrate and the active fractions were pooled as indicated by the bar. —— Absorbance at 280 nm. \circ — \circ Proteolytic activity.

Fig. 2. DEAE-Sephadex A25 chromatography of the protease peak from the Sephadex G100 column. The column (20 by 1 cm) was equilibrated with 0.02 M Tris-HCl-5 mM CaCl₂, pH 8. A flow rate of 21 ml/h was maintained by a peristaltic pump. Fraction volume was 5.3 ml. Aliquots (100 μ l) were assayed as for Fig. 1 and elution conditions are described in Materials and Methods. (a) Virulent strain 198-S. (b) Benign strain 305. •—•• Proteolytic activity. ——Salt gradient. Active fractions were pooled as indicated by the bars.

The recovery of proteolytic activity was ~95%. Chromatography on DEAE-Sephadex A25 (Fig. 2) of the protease peaks isolated on Sephadex G100, from virulent and benign strains of *B. nodosus*, separated several broad peaks of proteolytic activity. Several of the protease fractions contained a single protein band (Fig. 3). Electrophoretic analysis (Fig. 3) of all the protease peaks showed the presence of four to five closely related proteases in both strains. The protein bands observed in the gels that had protease activity are referred to as virulent 1 (vl), etc. and benign 1 (bl), etc. The assignment of these protein bands as active proteases was confirmed in separate experiments.

The purification procedure described yielded highly purified proteases of B. nodosus and the loss of proteolytic activity associated with the use of calcium phosphate gel

adsorption described by previous workers (Thomas 1964; Broad and Skerman 1976) was avoided. Broad and Skerman (1976) reported that their purified preparation contained four protein bands.

Comparison of the Protease Components of Various Strains

The protease peak from the Sephadex G100 gel filtration (Fig. 1) was found to give a clear and unique protein-banding pattern on polyacrylamide gel electrophoresis at pH $8 \cdot 8$. The protein bands with protease activity, as indicated in Figs 3 and 4, were identified by direct assay of gel slices and these agreed with the assay of gel slices obtained with the purified proteases following DEAE-Sephadex A25 chromato-graphy (see Fig. 3).



Fig. 3. Polyacrylamide gel electrophoresis at pH 8 · 8 of the protease fractions separated by DEAE-Sephadex A25. (a) 5% (w/v) polyacrylamide gel slab of virulent strain 198-S: (i) protease peak from Sephadex G100; (ii) fraction 1; (iii) fraction 2; and (iv) fraction 3 from Fig. 2A. (b) $7 \cdot 5\%$ (w/v) Polyacrylamide gel slab of benign strain 305: (i) fraction 1; (iii) fraction 2; (iii) peak tubes 52–56; (iv) fraction 3 from Fig. 2b; and (v) protease peak from Sephadex G100.

The protein patterns of the protease peak from a number of different strains of *B. nodosus* were compared. Virulent strains contained essentially the same protein protease bands although quantitative differences in the bands were apparent. Benign strains had a similar protease band pattern, which was clearly different from the virulent pattern. The protease peak of a virulent strain and the peaks of two benign strains are compared in Fig. 4. These results suggest that the differences observed in these protein protease patterns may be useful in distinguishing virulent and benign strains of *B. nodosus*. This method, however, is not rapid as a partial purification and concentration of the proteases are required to obtain the protein patterns described. Furthermore, the non-protease protein bands present in the protease peak

isolated on Sephadex G100 can interfere with the identification of the protease bands. Detection directly of the protease bands with activity in the polyacrylamide gels would overcome this problem. Recently, Every (1981) reported that virulent strains have a characteristic protease isozyme pattern in electrophoretograms, which was distinct from the pattern for benign strains. He suggested that such protease isozyme patterns could be useful for distinguishing *B. nodosus* from virulent and benign footrot. Our findings appear to differ from Every's in that he reported up to nine protease bands spread over the entire gel whereas we found four to five protease bands in a discreet region of the gel.



Fig. 4. Comparison of the protein patterns of the protease peaks (from Sephadex G100) of *B. nodosus* strains by polyacrylamide gel electrophoresis at pH 8.8 using a 7.5% (w/v) polyacrylamide slab gel. (i) Benign strain 291; (ii) benign strain 305; and (iii) virulent strain 198-S.

Properties of B. nodosus Proteases

Stability

The purified proteases of virulent and benign *B. nodosus* were found to be remarkably stable. Little activity was lost on storage at pH 8 in the presence of 5 mM CaCl₂ at 4°C for several months. The electrophoretic pattern of the protease peak isolated on Sephadex G100 and of the purified proteases was unchanged after 6 months storage at 4°C, indicating that they do not undergo significant autolysis. Stability experiments showed that the proteases of virulent and benign *B. nodosus* were stable at pH > 6 whereas their activity was rapidly lost at pH < 6. These results are in agreement with the earlier work of Thomas (1964).

Temperature-stability experiments with partially purified proteases showed that the proteases of virulent *B. nodosus* were stable to heating to 50° C and that above 60° C their activity rapidly decreased (Fig. 5). The proteases of benign *B. nodosus* showed a similar temperature-stability profile except that the loss of activity above 60° C was more marked (Fig. 5). These findings demonstrate that *in vitro* the proteases of benign *B. nodosus* are less stable than those of the virulent *B. nodosus* and indicate that the basis of the degrading proteinase test (Depiazzi and Richards 1979) is indeed due to their different stabilities and not solely an artefact arising from the use of unfiltered broth cultures.





Benign strain 305.

Effects of various inhibitors and metal ions

The effects of various inhibitors on the proteases of virulent and benign *B. nodosus* Both protease activities were completely inhibited by phenyl were examined. methylsulfonyl fluoride. N-Tosyl-L-phenylalanyl chloromethylketone (TPCK), a specific inhibitor of α -chymotrypsin, at a final concentration of 10^{-4} M, inhibited $\sim 30\%$ of the activity of both *B. nodosus* proteases. Iodoacetate (final concentration, 10^{-2} M) inhibited ~20 and ~30% of the activity of proteases of virulent and benign B. nodosus, respectively. Reducing agents did not significantly affect their activity. The protease activity was inhibited by the metal ion chelator EDTA but not by 1,10-phenanthroline, indicating that divalent ions such as Ca^{2+} , Mg^{2+} and Mn^{2+} are essential for activity but not the heavy metal ions such as Zn^{2+} and Fe^{2+} . These results indicate that these proteases are serine proteases and require a divalent metal ion (such as Ca²⁺) for activity. The proteases from a New Zealand strain, B. nodosus 10 (Broad and Skerman 1976), showed a similar requirement for divalent ions but were reported not to be inhibited by TPCK.

Substrate specificity

The proteases digested casein, hide powder azure and ¹²⁵I-labelled elastin, and denatured haemoglobin. There was no digestion of native haemoglobin, native bovine serum albumin, or keratin (as finely ground wool). They did not hydrolyse the synthetic substrates α -N-benzoyl-L-arginine ethyl ester or N-benzoyl-L-tyrosine ethyl ester. The substrate specificity is similar to that reported by Broad and Skerman (1976) with the exception that NZ strain 10 in our hands hydrolysed ¹²⁵I-labelled elastin (unpublished data). The specific activity per microgram of purified proteases of *B. nodosus* with casein as substrate is about the same as that observed with bovine trypsin.

Acknowledgments

The authors wish to thank Charles Ng for expert technical assistance in performance of the electrophoretic experiments. We also wish to thank Dr T. M. Skerman, Wallaceville Animal Research Centre, Ministry of Agriculture and Fisheries, Upper Hutt, New Zealand, for kindly supplying the two *B. nodosus* strains 10 and 80. The technical assistance of Jill Vaughan and Robin Hatswell in preparation of broth cultures and the helpful advice of Dr P. Willadsen and Dr T. Rothwell are greatly appreciated.

References

- Broad, T. F., and Skerman, T. M. (1976). Partial purification and properties of extracellular proteolytic activity of *Bacteroides nodosus*. N.Z. J. Agric. Res. 19, 317-22.
- Davis, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121, 404-27.
- Depiazzi, L. J., and Richards, R. B. (1979). A degrading proteinase test to distinguish benign and virulent ovine isolates of *Bacteroides nodosus*. Aust. Vet. J. 55, 25-8.
- Every, D. (1981). Surface structures and proteases of *Bacteroides nodosus* in relation to virulence and immunoprotective properties in sheep. Ovine Footrot, Report of Workshop, University of Sydney, May 3-6, pp. 8-15.
- Kakade, M. L., Swenson, D. H., and Liener, I. E. (1970). Note on the determination of chymotrypsin and chymotrypsin inhibitor activity using casein. *Anal. Biochem.* 33, 255–8.
- Robert, B., and Robert, L. (1969). Determination of elastolytic activity with ¹²⁵I and ¹³¹I labelled elastin. *Eur. J. Biochem.* 11, 62–7.
- Skerman, T. M. (1975). Determination of some *in vitro* growth requirements of *Bacteroides nodosus*. J. Gen. Microbiol. 87, 197–19.
- Skerman, T. M., Erasmuson, S. K., and Every, D. (1981). Differentiation of *Bacteroides nodosus* biotypes and colony variants in relation to their virulence and immunoprotective properties in sheep. *Infect. Immun.* 32, 788–95.
- Stewart, D. J. (1979). The role of elastase in the differentiation of *Bacteroides nodosus* in sheep and cattle. *Res. Vet. Sci.* 27, 99-105.
- Thomas, J. H. (1964). Proteolytic enzymes produced in liquid media by *Fusiformis nodosus*. Aust. J. Agric. Res. 15, 417-26.
- Thorley, C. M. (1976). A simplified method for the isolation of *Bacteroides nodosus* from ovine footrot and studies on its colony morphology and serology. J. Appl. Bacteriol. 49, 301-9.

Manuscript received 5 January 1982, accepted 30 June 1982

