Characterization of Certain Proteinase Isoenzymes Produced by Benign and Virulent Strains of *Bacteroides nodosus*

By RICHARD S. GREEN

Wallaceville Animal Research Centre, Research Division, Ministry of Agriculture and Fisheries,
Private Bag, Upper Hutt, New Zealand

(Received 29 April 1985)

Three proteinase isoenzymes from one benign strain of *Bacteroides nodosus* and five proteinase isoenzymes from each of two virulent strains of *B. nodosus* were purified by horizontal slab polyacrylamide gel electrophoresis. The purified isoenzymes hydrolysed casein, collagen I, collagen III, elastin, α -elastin, fibrinogen, gelatin, haemoglobin and α -keratin. The pH optima of all the isoenzymes lay between 7.25 and 9.5, the range of 8.75–9.25 being common to all. The isoenzymes were inhibited by phenylmethylsulphonyl fluoride, diphenylcarbamyl chloride, L-(1-tosylamide-2-phenyl)ethyl chloromethyl ketone, EGTA and EDTA, indicating that they were chymotrypsin-like serine proteinases that require a metal ion for stability or activity. EDTA inhibition was not reversed by addition of Ca^{2+} or Mg^{2+} . Some isoenzymes were activated by Mg^{2+} , Ca^{2+} , Cr^{3+} and Se^{4+} and all were inhibited by Fe^{2+} , Co^{2+} , Cu^{2+} , Zn^{2+} , Cd^{2+} and Hg^{2+} . Isoenzymes from benign strains had a lower temperature stability, losing all activity at 55 °C, whereas those from virulent strains lost all activity at 60 °C.

INTRODUCTION

Bacteroides nodosus, the principal agent of footrot in sheep, produces extracellular proteinases *in vitro* (Beveridge, 1941; Thomas, 1963), and it has been postulated that these enzymes have a role in the pathogenesis of the disease (Thomas, 1964). The possible association between the proteolytic activity of *B. nodosus* isolates and their virulence, although not verified directly, has aroused special interest with regard to the epidemiology of benign and virulent forms of *B. nodosus* infections in sheep (Egerton & Parsonson, 1969) and other ruminants (Egerton & Laing, 1978/1979).

Crude or partially purified preparations of *B. nodosus* proteinases are serine-type enzymes requiring a divalent metal ion for activity (Kortt *et al.*, 1982). A limited range of substrates is attacked, including casein, elastin, fibrin and denatured haemoglobin (Broad & Skerman, 1976). Although Thomas (1964) considered that the digestion of hoof particles incorporated in *B. nodosus* cultures indicated keratinolytic activity, enzyme preparations do not attack native keratin substrates *in vitro* (Broad & Skerman, 1976; Kortt *et al.*, 1982).

Extracellular proteinases of isolates from benign and virulent *B. nodosus* infections have quantitatively different activities on casein and elastin (Thomas, 1962; Egerton & Parsonson, 1969; Stewart, 1979) as well as differing temperature stabilities at 37 °C (Depiazzi & Richards, 1979) and at 55 °C (Kortt *et al.*, 1982). These distinctive features of the isolates have served to differentiate the two principal clinical conditions with which they are associated (Richards *et al.*, 1980), as has a qualitative method described by Every (1982) based on the characteristic isoenzyme patterns and molecular weights of the bacterial proteinases after polyacrylamide gel

---

Abbreviations: DPCC, diphenylcarbamyl chloride; PMSF, phenylmethylsulphonyl fluoride; TPCK, L-(1-tosylamide-2-phenyl)ethyl chloromethyl ketone.

electrophoresis. As several of these isoenzymes appear to be specific to benign or virulent strains of *B. nodosus*, a method for their purification was developed (Every & Green, 1982) and their principal properties are described here.

METHODS

Enzyme preparation. *Bacteroides nodosus* strains 65 and 91, isolated from clinical cases of ovine footrot in New Zealand, were used as the source of the virulent bacterial proteinase isoenzymes. Isoenzymes were also purified from strain 134 isolated from a case of benign footrot in Australia and obtained from the McMaster Laboratory, Division of Animal Health, CSIRO, Sydney, Australia.

The organisms were grown anaerobically at 37 °C in liquid Trypticase/arginine/serine medium to maximum cell density ($1-3 \times 10^8$ cells ml⁻¹) as described by Skerman (1975). Bacteria were removed from the culture by centrifugation at 12000 g and the supernatant was concentrated 30-fold in an Amicon ultrafiltration unit with a PM 10 membrane. This concentrate containing the isoenzymes was stored at 4 °C and used within one week.

Individual proteinase isoenzymes were purified by horizontal slab polyacrylamide gel electrophoresis in a 9–17% gradient. The isoenzyme bands were detected by reaction with fluorescein-casein substrate as described by Every & Green (1982). The isoenzymes from the benign strain (benign isoenzymes) were designated Ob1, 5 and 6 and the isoenzymes from the virulent strains (virulent isoenzymes) were designated Ov1, 2, 4, 6 and 7 as described by Every (1982). These were the only isoenzymes produced in significant quantities by the strains used. Isoenzyme Ov2 was obtained from virulent strain 91. The purified isoenzymes were frozen rapidly with dry ice/ethanol, a sample being kept separate to determine the purity of the isolated proteinase isoenzymes by the quantitative proteolytic zymogram method of Every (1981). Individual isoenzymes from separate purifications were thawed, combined, divided into aliquots and refrozen with dry ice/ethanol.

Substrate preparation and substrate specificity. Collagen I was prepared from the Achilles tendon of Merino sheep by the method of Piez *et al.* (1963). Collagen III (reticulin) was prepared from the renal cortex of Merino sheep by the method of Pras & Glynn (1973). Elastin and soluble α -elastin were prepared from the ligamentum nuchae of Merino sheep by the method of Partridge *et al.* (1955). α -Keratin was prepared from the skin of the inguinal pouch of Merino sheep by the method of Steinert *et al.* (1979).

The substrate specificity of the purified isoenzyme preparations was determined by the radial diffusion-in-gel method of Schumacher & Schill (1972), with the modifications of Every & Green (1982), except that the buffer used was 100 mM-piperazine/NaCl/HCl pH 9.0. The substrate concentrations were 0.75% (w/v) casein, 0.13% (w/v) collagen I, 0.15% (w/v) collagen III, 0.15% (w/v) elastin, 0.5% (w/v) α -elastin, 0.15% (w/v) fibrinogen, 0.5% (w/v) gelatin, 0.15% (w/v) haemoglobin and 0.15% (w/v) α -keratin. All results are the mean of two to four assays.

Pronase was used to prepare the standard curves in which one pronase unit was equivalent to one *B. nodosus* proteinase unit (one pronase unit will liberate amino acids from casein equivalent in Folin colour to 1.0 mequiv. tyrosine in 1 min at pH 7.4 and 40 °C).

Hide powder azure assay. The method of Rinderknecht *et al.* (1968) was modified by using 100 mM-piperazine/NaCl/HCl buffer pH 9.0, and reducing the assay volume to 1.2 ml and the amount of hide powder azure to 6 mg per assay. The reaction was terminated by filtration through a 0.22 μ m membrane filter (Sartorius) before reading on the spectrophotometer at 595 nm. All results are the mean of two to four assays.

Protein determination. Protein was measured by the fluorometric assay of Böhlen *et al.* (1973) with samples prepared as described by Every & Green (1982).

pH optima and pH stability. pH optima were determined by the hide powder azure assay with the buffer systems piperazine, piperazine/NaCl and glycine/NaCl (all at 100 mM) and titrated with HCl or NaOH to the final pH of 5–12.

For the pH stability studies, an additional buffer system was used with potassium hydrogen phthalate/NaOH at 100 mM to extend the range to pH 4.0. Each proteinase preparation was incubated in the various buffers in the pH range 4–12 for 120 min at 37 °C. The isoenzymes were then titrated to pH 9.0 with 0.05 M-HCl or 0.05 M-NaOH and their proteinase activities determined by the hide powder azure assay.

Thermal stability. Each isoenzyme was heated for 20 min at specified temperatures within the range 35–65 °C and proteinase activity was determined at 37 °C by the hide powder azure assay.

Effect of inhibitors. The effect of inhibitors was determined using ϵ -aminocaproic acid, iodoacetamide, phenanthroline, EGTA, EDTA, PMSF (phenylmethylsulphonyl fluoride), soyabean trypsin inhibitor, ovomucoid trypsin inhibitor, TPCK [L-(1-tosylamide-2-phenyl)ethyl chloromethyl ketone] and DPCC (diphenylcarbamyl chloride). The isoenzyme preparations were pre-incubated with the inhibitors at concentrations given in Results for 30 min at 37 °C and the proteinase activity determined by the hide powder azure assay.

Effect of metal ions. Various metal ions at 1 mM final concentration were added to the hide powder azure assay mixtures immediately before incubation.

Source of chemicals. Fibrinogen, pronase and hide powder azure were obtained from Calbiochem. Haemoglobin, piperazine, ϵ -aminocaproic acid, phenanthroline, EGTA, PMSF, TPCK, soyabean trypsin inhibitor and ovomucoid trypsin inhibitor were obtained from Sigma. Casein (Hammarsten), EDTA, iodoacetamide, sodium selenite and all the metals as either their chloride, nitrate or sulphate were obtained from BDH. Gelatin was obtained from Difco and DPCC was obtained from Hopkin & Williams.

Statistics. For comparing data for the various isoenzymes on substrate specificity, effects of inhibitors and effects of metal ions, results were subjected to the Friedmann two way analysis by ranks test (Conover, 1980). Results for pH optima, pH stability and thermal stability were analysed using the Genstat analysis of variance and the minimum significant differences were determined. The Genstat is a general statistical program developed at Rothamsted Experimental Station, Harpenden, UK, and it was run on a Prime 750 computer.

RESULTS AND DISCUSSION

pH optima. These are indicated in Fig. 1. In the piperazine/piperazine-NaCl/glycine-NaCl buffer system, the benign isoenzymes Ob1, Ob5 and Ob6 had pH optima ranges of 8.5–9.25, 7.75–9.25 and 8.5–9.5, respectively. pH optima ranges for the virulent isoenzymes Ov1, Ov2, Ov4, Ov6 and Ov7 were 8.75–9.5, 7.25–9.5, 7.75–9.25, 7.5–9.25 and 8.25–9.25, respectively. All isoenzymes had an optimal range of pH 8.75 to 9.25 in common.

Substrate specificity. The activities of the various isoenzymes on soluble and insoluble natural substrates are shown in Table 1. In addition to these substrates found to be susceptible to *B. nodosus* proteinases by previous authors (see Introduction) the purified isoenzymes also hydrolysed collagen I, collagen III and α -keratin. In previous studies (Broad & Skerman, 1976; Kortt *et al.*, 1982) a hard keratin (wool) was used as a substrate, whereas the present observations were made on a soft keratin isolated from the stratum corneum, an epidermal component more allied to the site of *B. nodosus* infection. The soluble substrates fibrinogen, α -elastin, casein and gelatin were most readily hydrolysed, followed by collagen III, α -keratin and haemoglobin. Due to low enzyme concentrations, elastin and collagen I were not completely hydrolysed and therefore an accurate estimate of the rate of hydrolysis was not possible, although collagen I was apparently hydrolysed more rapidly than elastin.

When all the substrates tested were taken into account, the virulent isoenzyme Ov2, unique to strain 91, was generally the most active followed by Ov4 > Ov1 $\approx$ Ov7 $\approx$ Ob1 $\approx$ Ob6 > Ob5 > Ov6; the virulent isoenzymes generally were more actively proteolytic than the benign isoenzymes.

pH stability. Some differences in the patterns of pH stability of isoenzymes from benign and virulent strains were evident (Fig. 2). The benign isoenzymes Ob1 and Ob5 were stable throughout pH ranges 8.0–10.0 and 8.0–10.5, respectively. The benign isoenzyme Ob6 was unstable under the assay conditions. The virulent isoenzymes Ov1, Ov2, Ov4, Ov6 and Ov7 were stable at pH ranges of 7.0–10.0, 6.5–9.5, 6.5–10.0, 6.5–10.0 and 9.0–10.5, respectively.

Thermal stability. The benign and virulent isoenzymes were stable for 20 min at 40 °C, but lost about 20% of their activities at 45 °C. At 50 °C, Ov1, Ov2 and Ov4 lost approximately 30% of their activity and Ov6, Ov7, Ob1, Ob5 and Ob6 lost approximately 60%. At 55 °C, Ov6 and Ov7 had only half the remaining activity of Ov1, Ov2 and Ov4 with 13.5% and 27% remaining respectively. The benign isoenzymes had no activity after 20 min at 55 °C and the virulent isoenzymes had no remaining activity after 20 min at 60 °C. The differences between percentage readings for the assays of each isoenzyme at the stated temperatures were significant ($P < 0.01$). This distinction between benign and virulent isolates applied to both Australian and New Zealand strains as reported by Green (1984) for unpurified proteinases. However, it is in contrast to the findings for enzyme preparations from strain 92 studied by Thomas (1964) and the strains studied by Kortt *et al.* (1982), all of which were reported to be stable up to 70 °C.

Effects of inhibitors. PMSF (1 mM) caused an average inhibition of 90% (70–100%) for the benign and virulent isoenzymes, while at 0.1 mM inhibition averaged 30% (0–81%); Ob5 and Ob6 were not inhibited at this concentration. TPCK (1 mM) caused 50% inhibition (30–60%) for all isoenzymes with 0.1 mM causing 22% inhibition (0–37%) except for Ob6. DPCC caused

Fig. 1. pH optima of proteinase isoenzymes from (a) benign and (b) virulent *B. nodosus*. The pH of the isoenzymes was adjusted to that required (between 5.0 and 12.0) with the buffers and then proteinase activity was determined by the hide powder azure assay. □, Ob1; ○, Ob5; △, Ob6; ▲, Ov1; ●, Ov2; ◆, Ov4; ▼, Ov6; ■, Ov7. The 100% value for each isoenzyme activity was a change in the absorbance of 0.2, 0.25, 0.16, 0.2, 0.16, 0.16, 0.33 and 0.18 per hour respectively. Differences between percentage readings for assays of each isoenzyme at stated pH values were significant ($P < 0.05$).

Table 1. *Substrate specificity of proteinase isoenzymes from virulent and benign B. nodosus*

The substrate specificity was determined by the radial diffusion-in-gel method. The rate of substrate hydrolysis was compared with pronase.

Substrate	Substrate concn (%, w/v)	Activity of isoenzymes*							
		Ov1	Ov2	Ov4	Ov6	Ov7	Ob1	Ob5	Ob6
Casein	0.75	474	4800	791	37	289	166	58	194
Collagen I	0.13	<20	<20	<20	<20	<20	<20	<20	<20
Collagen III	0.15	27	246	224	6	40	22	21	35
Elastin	0.15	tr	tr	tr	tr	tr	tr	tr	tr
α-Elastin	0.5	154	2339	699	24	216	264	111	252
Fibrinogen	0.15	466	4246	5446	161	943	231	278	604
Gelatin	0.5	174	2154	1025	24	162	252	84	230
Haemoglobin	0.15	5	36	20	0.7	4	10	11	13
α-Keratin	0.15	11	86	93	3	30	7	7	14

tr, Trace.

* All values are expressed as pronase units per µg isoenzyme protein. Results for different isoenzymes acting on the same substrate, and for the same isoenzyme acting on different substrates were all significantly different at the 1% level.

100% inhibition at 1 mM, 94% (75–100%) inhibition at 0.1 mM and 33% (0–63%) inhibition at 0.01 mM for all isoenzymes except Ov7. EDTA (1 mM) caused 100% inhibition, 0.1 mM-EDTA caused 77% inhibition (37–100%) and 9% inhibition (0–19%) was caused by 0.01 mM-EDTA; Ob1 and Ob6 were not inhibited at this concentration. After EDTA inhibition (1 mM), isoenzyme activity was not restored by the addition of either 5 mM- Ca^{2+} or 5 mM- Mg^{2+} . EGTA (1 mM) caused 100% inhibition, 0.1 mM-EGTA caused 78% inhibition (52–100%) and 27% (0–100%) inhibition was caused by 0.01 mM-EGTA; Ov7 and Ob5 were totally inhibited and Ov1, Ov4, Ob1 and Ob6 were not inhibited. There was no inhibition by 1 mg ml⁻¹ of soyabean or ovomucoid trypsin inhibitors, or by 1 mM concentrations of the thiol inhibitor iodoacetamide, the fibrinolytic inhibitor ε-aminocaproic acid or the heavy metal chelator phenanthroline. Inhibition of the different isoenzymes by the same inhibitor was not significantly different but the effect of different inhibitors on the same isoenzyme was significant at the 1% level.

Fig. 2. pH stabilities of proteinase isoenzymes from (a) benign and (b) virulent *B. nodosus*. Each isoenzyme preparation was incubated in the pH range 4.0–12.0 for 120 min at 37 °C. The isoenzymes were titrated to pH 9.0 with 0.05 M-HCl or 0.05 M-NaOH and their proteinase activities were determined by the hide powder azure assay. □, Ob1; ○, Ob5; △, Ob6; ▲, Ov1; ●, Ov2; ◆, Ov4; ▼, Ov6; ■, Ov7. The 100% value for each isoenzyme activity was a change in the absorbance of 0.2, 0.15, 0, 0.2, 0.16, 0.16, 0.33 and 0.21 per hour respectively. Differences between percentage readings for assays of each isoenzyme at stated pH values were significant ($P < 0.05$).

The inhibition of all the isoenzymes by PMSF confirms that they are serine proteinases, the inhibition by TPCK and DPCC further indicating that they are chymotrypsin-like enzymes. The inhibition by TPCK appears similar to that reported by Kortt *et al.* (1982), but the PMSF results differ. Direct comparison with the results of Kortt *et al.* (1982) is difficult because their 'purified' enzyme would have been a mixture of isoenzymes and neither the inhibition assay method nor the concentration of PMSF was described.

Effect of metal ions. For all the isoenzymes except Ov4, Mg^{2+} (mean for all isoenzymes 112%; range 86–163%) and Ca^{2+} (121%; 79–177%) were not inhibitory and Sr^{2+} (87%; 40–104%) inhibited only Ov4 and Ov7. Cr^{3+} (90%; 45–122%) inhibited Ov4, Ov6, Ov7 and Ob6, and Mn^{2+} (87%; 58–110%) inhibited Ov4, Ov6 and Ov7. Se^{4+} (100%; 52–134%) inhibited Ov2, Ov4 and Ob6, and Pb^{2+} (77%; 34–106%) inhibited all except Ov1 and Ov2. All isoenzymes were inhibited by Ni^{2+} (53%; 21–84%), Cu^{2+} (28%; 7–49%), Cd^{2+} (14%; 0–26%), Hg^{2+} (27%; 0–38%) and Fe^{2+} (17%; 0–39%) with Cd^{2+} totally inhibiting Ov1 and Ov4, Hg^{2+} totally inhibiting Ov6 and Fe^{2+} totally inhibiting Ov1, Ov4, Ov6 and Ov7. Co^{2+} and Zn^{2+} totally inhibited all isoenzymes. Isoenzymes Ov1, Ov2, Ov7 and Ob5 were activated (>10% increase above initial activity) by the addition of Mg^{2+} ; Ov1, Ov2 and Ob5 were activated by the addition of Ca^{2+} ; Ov1 and Ob5 were activated by the addition of Cr^{3+} and Ov6, Ov7 and Ob5 were activated by the addition of Se^{4+} . Ob5 was the only benign isoenzyme activated. Results for different isoenzymes reacting with the same metal ions and for the same isoenzyme reacting with different metal ions were all significantly different at the 1% level.

There was no apparent pattern to the effect of different metal ions on the activity of the isoenzymes. No metal activated all the isoenzymes from either the virulent or benign isoenzyme groups and their effects on the activity of isoenzymes in both groups were similar.

Characteristics shared by the proteinase isoenzymes of both benign and virulent strains include their pH optima, reaction with inhibitors and their molecular weight range. The isoenzymes differed in their thermal stability, pH stability, reaction to metal ions and their rates of hydrolysis of various proteinaceous substrates; these properties could be worthy of further study as virulence determinants of *B. nodosus*.

I thank T. M. Skerman for providing the cultures.

REFERENCES

- BEVERIDGE, W. I. B. (1941). Footrot in sheep: a transmissible disease due to infection with *Fusiformis nodosus* (n. sp.). *Bulletin, Council for Scientific and Industrial Research, Australia* No. 140, pp. 1-64.
- BÖHLEN, P., STEIN, S., DAIRMAN, W. & UDENFRIEND, S. (1973). Fluorometric assay of proteins in the nanogram range. *Archives of Biochemistry and Biophysics* 155, 213-220.
- BROAD, T. E. & SKERMAN, T. M. (1976). Partial purification and properties of extracellular proteolytic activity of *Bacteroides nodosus*. *New Zealand Journal of Agricultural Research* 19, 317-322.
- CONOVER, W. J. (1980). *Practical Nonparametric Statistics*, 2nd edn, pp. 299-305. New York: John Wiley & Sons.
- DEPIAZZI, L. J. & RICHARDS, R. B. (1979). A degrading proteinase test to distinguish benign and virulent ovine isolates of *Bacteroides nodosus*. *Australian Veterinary Journal* 55, 25-28.
- EGERTON, J. R. & LAING, E. A. (1978/1979). Characteristics of *Bacteroides nodosus* isolated from cattle. *Veterinary Microbiology* 3, 269-279.
- EGERTON, J. R. & PARSONSON, I. M. (1969). Benign foot-rot - a specific interdigital dermatitis of sheep associated with infection by less proteolytic strains of *Fusiformis nodosus*. *Australian Veterinary Journal* 45, 345-349.
- EVERY, D. (1981). Quantitative measurement of protease activities in slab polyacrylamide gel electrophoretograms. *Analytical Biochemistry* 116, 519-523.
- EVERY, D. (1982). Proteinase isoenzyme patterns of *Bacteroides nodosus*: distinction between ovine virulent isolates, ovine benign isolates and bovine isolates. *Journal of General Microbiology* 128, 809-812.
- EVERY, D. & GREEN, R. S. (1982). Purification of individual proteinase isozymes from *Bacteroides nodosus* by use of polyacrylamide gel electrophoresis, a fluorogenic substrate detection system and a simple electroelution apparatus. *Analytical Biochemistry* 119, 82-85.
- GREEN, R. S. (1984). A method to differentiate between virulent and benign isolates of *Bacteroides nodosus* based on the thermal stability of their extracellular proteinases. *New Zealand Veterinary Journal* 33, 11-13.
- KORTT, A. A., O'DONNELL, I. J., STEWART, D. J. & CLARK, B. L. (1982). Activities and partial purification of extracellular proteases of *Bacteroides nodosus* from virulent and benign footrot. *Australian Journal of Biological Sciences* 35, 481-489.
- PARTRIDGE, S. M., DAVIS, H. F. & ADAIR, G. S. (1955). The chemistry of connective tissues 2. Soluble proteins derived from partial hydrolysis of elastin. *Biochemical Journal* 61, 11-21.
- PIEZ, K. A., EIGNER, E. A. & LEWIS, M. S. (1963). The chromatographic separation and amino acid composition of the subunits of several collagens. *Biochemistry* 2, 58-66.
- PRAS, M. & GLYNN, L. E. (1973). Isolation of a non-collagenous reticulin component and its primary characterization. *British Journal of Experimental Pathology* 54, 449-456.
- RICHARDS, R. B., DEPIAZZI, L. J., EDWARDS, J. R. & WILKINSON, F. C. (1980). Isolation and characterisation of *Bacteroides nodosus* from foot lesions of cattle in Western Australia. *Australian Veterinary Journal* 56, 517-521.
- RINDERKNECHT, H., GEOKAS, M. C., SILVERMAN, P. & HAVERBACK, B. J. (1968). A new ultrasensitive method for the determination of proteolytic activity. *Clinica chimica acta* 21, 197-203.
- SCHUMACHER, G. F. B. & SCHILL, W.-B. (1972). Radial diffusion in gel for microdetermination of enzymes. II. Plasminogen activator, elastase, and non-specific proteases. *Analytical Biochemistry* 48, 9-26.
- SKERMAN, T. M. (1975). Determination of some *in vitro* growth requirements of *Bacteroides nodosus*. *Journal of General Microbiology* 87, 107-119.
- STEINERT, P. M., IDLER, W. W., POIRIER, M. C., KATO, Y., STONER, G. D. & YUSPA, S. H. (1979). Subunit structure of the mouse epidermal keratin filament. *Biochimica et biophysica acta* 577, 11-21.
- STEWART, D. J. (1979). The role of elastase in the differentiation of *Bacteroides nodosus* infections in sheep and cattle. *Research in Veterinary Science* 27, 99-105.
- THOMAS, J. H. (1962). The bacteriology and histopathology of footrot in sheep. *Australian Journal of Agricultural Research* 13, 725-733.
- THOMAS, J. H. (1963). A liquid medium for the growth of *Fusiformis nodosus*. *Australian Veterinary Journal* 39, 434-437.
- THOMAS, J. H. (1964). The pathogenesis of footrot in sheep with reference to proteases of *Fusiformis nodosus*. *Australian Journal of Agricultural Research* 15, 1001-1016.