Characterization of a basic serine proteinase (pI ~ 9.5) secreted by virulent strains of *Dichelobacter nodosus* and identification of a distinct, but closely related, proteinase secreted by benign strains

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An extracellular serine proteinase with a pI ~ 9.5 (referred to as 'basic proteinase') was purified to homogeneity, from strains of *Dichelobacter nodosus* that cause virulent foot-rot, by gel filtration of concentrated culture supernatant on Sephadex G-100 and chromatography on sulphopropyl-Sephadex C-25 at pH 8.6. *D. nodosus* strains that cause benign foot-rot do not secrete a corresponding basic proteinase with a pI of ~ 9.5. Benign strains secrete a closely related, but distinct, proteinase which has the same molecular mass and N-terminal sequences as the 'virulent' basic proteinase, but a lower pI of ~ 8.6. The basic proteinases from both strains appear to interact with other proteins present in the culture medium, which results in anomalous behaviour on

gel filtration. Pure *D. nodosus* 'virulent' basic proteinase has a molecular mass of 36 kDa and showed a low solubility at $I < 0.05$ precipitating quantitatively from solution as microcrystals. The proteinase shows optimal activity at pH 8.0 and is stable to heating to 55 °C for 30 min, but at higher temperatures activity is rapidly lost. Bivalent-metal ions (e.g. Ca^{2+}) are required to maintain the structural integrity and stability of the proteinase; in the presence of EDTA or conditions that cause protein unfolding, the proteinase undergoes rapid and complete autolysis. Cleavage of oxidized insulin A- and B-chain showed that the basic proteinase has a broad specificity, including cleavage at lysine and arginine bonds.

INTRODUCTION

Dichelobacter (Bacteroides) nodosus [1], an anaerobic Gram-negative bacterium, is the essential causative organism of ovine foot-rot. Virulent foot-rot is a contagious disease of the sheep's foot which is characterized by separation of the hoof from the living epidermal tissue, due to a spreading infection beneath the horn from an interdigital dermatitis. Benign foot-rot is caused by less invasive strains (lower virulence) of *D. nodosus*, and the resulting interdigital dermatitis is usually the only lesion observed [2–4]. The tissue damage which accompanies the infection indicates the involvement of extracellular proteinases in the degradation of host tissue [5]. *D. nodosus* secretes a number of extracellular proteinases which readily degrade the proteins present in skin and hoof of sheep, including elastin [6–8], keratin, fibrinogen and collagen [8]. Furthermore, virulence of *D. nodosus* correlates with differences in the properties of the proteinases, and these have been used in diagnostic tests to distinguish virulent and benign strains of the organism [7,9–11]. Electrophoretic analyses of *D. nodosus* culture supernatants of virulent and benign strains showed the presence of four or five proteinases with pI values ranging from 4.9 to 6.0 [12] and apparent molecular masses of 40–42 kDa [6]. The partial purification of these 'acidic' proteinases has been described [6,13].

In the present paper we describe the properties of a serine proteinase, secreted by virulent strains of *D. nodosus*, which has a pI of ~ 9.5 and a molecular mass of ~ 36 kDa. The amino acid and DNA sequences of this proteinase were recently determined [14], and it was shown that this proteinase is a member of the subtilisin family of proteinases. Benign strains of *D. nodosus* do not secrete this basic proteinase, but a closely related proteinase with a lower pI (~ 8.6) has been identified in the culture supernatants of these strains.

MATERIALS AND METHODS

D. nodosus strains from the CSIRO Division of Animal Health Collection were grown as described in [14]. The basic proteinase from virulent strains of *D. nodosus* was purified from concentrated culture supernatants by gel filtration on Sephadex G-100 and chromatography on sulphopropyl-Sephadex C-25 at pH 8.6 as described [14]. This procedure also yielded a partly purified preparation of 'benign' basic proteinase from benign strains of *D. nodosus*.

Assay of proteinase activity

Proteolytic activity was measured using of hide powder azure (20 mg) as substrate in 5.0 ml of 0.02 M Tris/HCl/5 mM CaCl_2 /0.2 M NaCl, pH 8.0, as described in [6]. Esterase activity was measured spectrophotometrically using α -N-benzoyl-L-tyrosine ethyl ester (BTEE). To 3.0 ml of 0.02 M Tris/HCl/5 mM CaCl_2 /0.2 M NaCl, pH 8.0 (TBS), in a quartz cuvette, 100 μ l of BTEE in acetonitrile (final concn. 2.91×10^{-4} M) was added, followed by 50 μ l of enzyme solution to start the reaction. Hydrolysis of BTEE was measured at 256 nm at 20 °C [15].

PAGE

SDS/PAGE was performed by the method of Laemmli [16], using 12% (w/v)-polyacrylamide gels as described in [14]. For Western blots, proteins were transferred to nitrocellulose (Schleicher and Schuell) with 25 mM Tris/glycine, pH 8.5, containing 20% (v/v) methanol as the transfer buffer and probed with sheep antiserum raised against the purified basic proteinase. The detection reagent was donkey anti-sheep IgG-horseradish peroxidase conjugate as the second antibody (Silenius Laboratories) and 4-chloro-1-naphthol the colour reagent. PAGE

under non-denaturing conditions was also carried out in 1.5 mm slabs [5% (w/v) polyacrylamide] at pH 4.3 [17] and pH 8.8 [18,19]. Isoelectric focusing was carried out using Ampholine (LKB) polyacrylamide-gel plates (pH range 3.5–9.5) according to the manufacturer's instructions.

Immunoblots of gels run under non-denaturing conditions were performed as follows. After electrophoresis the proteins were transferred to nitrocellulose coated with a monoclonal antibody specific for either *D. nodosus* basic proteinase or acidic proteinase B2. The nitrocellulose was coated with either Mab (at a dilution of 1 in 500) in TBS for 2 h at room temperature and then washed with TBS for 1 h. The transfer was achieved by overlaying the Mab coated nitrocellulose on to the gel for 1.5 h using a light weight to ensure good contact. The nitrocellulose was then removed and blocked with 0.4% powdered milk in TBS for 1 h at room temperature and probed with either a sheep polyclonal anti-(basic proteinase) antibody or a sheep polyclonal anti-(acidic proteinase) antibody which recognizes 'benign' acidic proteinases B1–B4, as well as the virulent acidic proteinases V1–V3, but not acidic proteinases V5 and B5. The detection system was the same as that used for the Western blots.

pH optima

The pH activity profile of 'virulent' basic proteinase was determined with BTEE as substrate in 0.2 M NaCl/5 mM CaCl₂ in the following buffers: 20 mM acetate, pH 5.0; 20 mM Mes, pH 5.0–7.0; 20 mM Tris/HCl, pH 7.0–9.0; 20 mM cyclohexylaminoethanesulphonic acid, pH 8.5–10.0.

Sedimentation equilibrium

The molecular mass of virulent basic proteinase was determined in 0.05 M Tris/HCl/5 mM CaCl₂/0.2 M NaCl, pH 8.0, by sedimentation equilibrium in a Beckman model XLA centrifuge. A partial specific volume of 0.71 was calculated from the composition. The samples for ultracentrifugal analysis were prepared by size-exclusion h.p.l.c. on Superose 12.

Temperature stability

Aliquots of 'virulent' basic proteinase (at a concentration of 1 mg/ml) in 20 mM Tris/HCl/5 mM CaCl₂/0.2 M NaCl, pH 8.5, were heated in sealed Eppendorf tubes in a water bath for 30 min at various temperatures (40–70 °C), cooled on ice and residual activity assayed with BTEE.

Proteinase inhibitors

The effect of the inhibitors listed in Table 2 (below) on the activity of 'virulent' basic proteinase, *D. nodosus* acidic proteinase V5 and *B. subtilis* subtilisin I 168 was determined. Stock solutions of tosyl-lysylchloromethane ('TLCK'), soybean trypsin inhibitor, wheat subtilisin inhibitor [20] and winged-bean chymotrypsin inhibitor [21] were made up in 0.02 M Tris/HCl/5 mM CaCl₂, pH 8.0, phenylmethanesulphonyl fluoride (PMSF) was dissolved in n-propanol and the other inhibitors were dissolved in dimethyl sulphoxide. The final concentration of each inhibitor and the inhibition conditions are given in Table 2 (below).

Substrate specificity

The cleavage specificity of basic proteinase was determined using oxidized insulin A- and B-chains (Sigma). The oxidized insulin

chains (200 µg) were digested at 37 °C for 4 h at a peptide/enzyme ratio of 100:1 in 0.1 M NH₄HCO₃, pH 8, or 0.02 M Tris/HCl/5 mM CaCl₂/0.2 M NaCl, pH 8. The digests were stopped with PMSF (1 mM final concn.) and dried at 50 °C in a Savant drier, redissolved in 0.1% trifluoroacetic acid and the peptides separated by reverse-phase h.p.l.c. on a Vydac 218 TP54 column (4.6 mm × 250 mm) at 45 °C [22]. Cleavage sites were identified by amino-acid-sequence analysis and the amino acid compositions of the isolated peptides. Amino-acid and amino-acid-sequence analyses were performed as described in [22]. For N-terminal sequence analysis of purified acidic proteinase, the protein band obtained on SDS/PAGE was blotted on to a Selex-20 (Schleicher and Schuell) membrane, excised and sequenced using an Applied Biosystems Model 470A gas-phase sequencer.

RESULTS AND DISCUSSION

Basic proteinase from virulent strains

Gel filtration of concentrated culture supernatants of *D. nodosus* virulent strains on Sephadex G-100 resolved two peaks of proteolytic activity. Chromatography of proteolytic activity peak 1, which was eluted just after the column void volume, on sulphopropyl-Sephadex C-25 at pH 8.6 yielded a single protein peak with proteolytic activity at ~0.2 M NaCl, and this proteinase is referred to as 'virulent' basic proteinase. The yield of purified protease was ~0.5 mg/litre.

On SDS/PAGE the proteinase migrated as a single protein band of about 37 kDa, considerably less than the 42 kDa band found for *D. nodosus* acidic proteinase V5 (Figure 1a). On a non-denaturing gel at pH 4.3 the basic proteinase migrated as a single band (not shown), and on isoelectric focusing it migrated as a single component with a pI of ~9.5 (see Figure 4 below).

On gel filtration on Superose 12 in 0.01 M Tris/HCl/5 mM CaCl₂/0.2 M NaCl, pH 8.6, purified basic proteinase and acidic proteinase V5, which does not show anomalous behaviour on Sephadex G-100, were eluted in the same volume. The finding that the basic proteinase was eluted at a higher-than-expected molecular mass on Sephadex G-100 suggests that it interacts with other proteins in the culture supernatant. Furthermore, the *D. nodosus* proteinases subtilisin and proteinase K were retarded on Superose 12, being eluted after soybean trypsin inhibitor (20 kDa). Retardation on Sephadex G-100 and Bio-Gel has been reported for a number of purified bacterial and fungal proteinases [25]. As gel filtration gave anomalous results, sedimentation-equilibrium experiments were performed to determine the molecular mass of the basic proteinase, and a value of 35.8 kDa was found with no evidence of aggregation.

Figure 1 SDS/PAGE of purified *D. nodosus* basic proteinase

(a) Comparison of the basic proteinase (lane 2) with *D. nodosus* acidic proteinase V5 (lane 1). (b) Basic proteinase isolated from different strains of *D. nodosus*. Lanes 1–7 are the following strains: 1, 198; 2, 198; 3, 332; 4, 265; 5, 312; 6, 450; and 7, 334.

Table 1 Amino acid compositions of hydrolysates of basic proteinase from four virulent strains of *D. nodosus*

Samples were hydrolysed in 6.0 M HCl for 24 h at 110 °C. Values for Ser and Thr are uncorrected, and those for Val and Ile were obtained after 72 h hydrolysis. The values in parentheses are the compositions based on the determined sequence [14].

Amino acid	<i>D. nodosus</i> strain ...	Amino acid composition (mol/mol)				Sequence
		198	312	334	450	
Lys		10.7	11.2	11.7	11.2	11
His		6.7	6.2	6.5	7.1	7
Arg		17.4	17.3	18.2	17.4	16
Asp		50.3	49.2	51.8	50.1	26
Asn						27
Thr		16.1	16.4	16.9	16.6	18
Ser		22.5	22.9	24.0	22.7	26
Glu		16.7	16.5	17.9	16.7	4
Gln						11
Pro		17.0	17.0	17.0	17.0	17
Gly		42.7	42.7	42.2	42.4	47
Ala		33.1	33.1	32.2	32.1	35
$\frac{1}{2}$ -Cys		3.2	3.3	2.5	3.2	4
Val		29.9	30.4	29.5	29.9	32
Met		5.4	5.8	5.9	5.9	6
Ile		14.4	14.7	14.0	14.1	15
Leu		16.0	17.1	16.8	16.4	18
Tyr		9.6	9.8	10.2	9.7	10
Phe		6.8	7.3	7.3	7.1	7
Trp		—	—	—	—	7
Total						344

The basic proteinase isolated from a number of virulent strains of *D. nodosus*, including strains 198 (serogroup A), 265 (serogroup H) and 334 (serogroup B) and intermediate strains 312 (serogroup B) and 450 (serogroup B), were identical as indicated by SDS/PAGE analysis (Figure 1b), N-terminal sequence, identical peptide profiles obtained on complete autolysis of the basic proteinase (results not shown) and amino acid composition (Table 1). The amino acid compositions were in good agreement with the composition obtained from the sequence [14], which showed that the protein contains 30 acidic and 27 basic residues. The binding of Ca²⁺ by a number of acidic residues results in the protein having an excess of basic residues and a basic pI.

Identification of 'benign' basic proteinase

The concentrated culture supernatants of *D. nodosus* benign strains on Sephadex G-100 also resolved into two peaks, but the yield of proteolytic activity in peak 1 was considerably lower than that found for virulent strains. Chromatography of the Sephadex G-100 peak 1 from benign strains 305 and 309, on sulphopropyl-Sephadex C-25 at pH 8.6, yielded a single protein peak with proteolytic activity which was eluted at ~0.1 M NaCl and not at 0.2 M NaCl as found for the virulent basic proteinases (results not shown). SDS/PAGE analysis of this peak (lane 2, Figure 2a) showed the presence of two protein bands: ~37 kDa and ~40 kDa. The faster-migrating band (band 2; 37 kDa) showed the same mobility as the 'virulent' basic proteinase (lane 1, Figure 2a), and Western-blot analysis showed that the 37 kDa protein band (lane 2, Figure 2b) cross-reacted strongly with a polyclonal anti-(basic proteinase) antibody, while the 40 kDa band (lane 2, Figure 2b) reacted only weakly with the anti-(basic proteinase) antibody. The benign acidic proteinases

Figure 2 (a) SDS/PAGE and (b) Western-blot analysis of the proteinase peak obtained on chromatography of the Sephadex G-100 peak 1 from benign strains 305 and 309 on sulphopropyl-Sephadex C-25

(1) Basic proteinase from virulent strain 198; (2) sulphopropyl-Sephadex C-25 proteinase peak (eluted at 0.1 M NaCl) from benign strain 305; (3) same proteinase peak from strain 309; (4) Sephadex G-100 peak 2 of benign strain 309 containing acidic proteinase B1–B5. The Western blot was probed with a sheep polyclonal antibody raised against the basic proteinase from virulent strain 198. This antibody also cross-reacts weakly with acidic isoenzymes B1–B4, but not with B5, as indicated in lane 4.

from peak 2 of the Sephadex G-100 column also reacted weakly with this antibody (lane 4, Figure 2). This result indicates that the 37 kDa protein is related to the 'virulent' basic proteinase.

Native PAGE at pH 8.8 showed that the 'benign' proteinase peak that was eluted at 0.1 M NaCl (lanes 1 and 2, Figure 3a) contained three protein bands with same mobility as acidic proteinases B2, B3, B4 which are present in peak 2 of the Sephadex G-100 column (lane 3, Figure 3a). An immunoblot of this gel showed that these bands reacted specifically with a monoclonal anti-(proteinase B2) antibody, which only recognizes acidic proteinases B1, B2, B3 and B4 (Figure 3b); an immunoblot of the same gel, using a monoclonal anti-(basic proteinase) antibody that only recognizes 'virulent' basic proteinase, did not detect any cross-reacting proteinase bands. This result confirmed that the 37 kDa protein band that cross-reacted with the polyclonal anti-(basic proteinase) antibody on SDS/PAGE (band 2, lane 2, Figure 2a) has a basic pI and that the 40 kDa band (lane 2, Figure 2b) corresponds to proteinases B2, B3 and B4.

Isoelectric focusing of the 'benign'-proteinase peak revealed the presence of a protein band with a pI of ~8.6 and protein bands with pI values of ~5.6 corresponding to acidic proteinases B2 to B4 (Figure 4). It is noteworthy that proteins with such different pI values should be co-eluted as a single peak from the sulphopropyl-Sephadex column at pH 8.6. One explanation is that the benign basic proteinase interacts with acidic proteinases B1–B4 to form a complex which binds to the cation-exchanger at pH 8.6. Interactions of the basic proteinases with other proteins may also account for their anomalous elution behaviour on Sephadex G-100.

The identity of the 37 and 40 kDa bands (Figure 2) was established by N-terminal-sequence analysis. The 37 kDa band (lane 2, Figure 2a) gave the same N-terminal sequence (A A P N-D P S Y R Q Q) as found for the 'virulent' basic proteinase, while the 40 kDa band (lane 2, Figure 2a) gave the same N-terminal sequence as found for acidic proteinase B2 (A A P N D Q H Y-R E Q). These results show that benign strains secrete a basic proteinase which has the same molecular mass, N-terminal sequence and cross-reactivity with anti-(basic proteinase) antibodies as the virulent basic proteinase, but a significantly lower pI. Recently, a gene encoding a basic proteinase from benign strain 305 was cloned and sequenced (G. G. Lilley, M. C. Riffkin, A. A. Kortt and D. J. Stewart, unpublished work). The deduced amino acid sequence of the 'benign' basic-proteinase gene

Figure 3 Native PAGE at pH 8.8 of sulphopropyl-Sephadex C-25 peak 2 (at 0.1 M NaCl) from benign strains 305 and 309

(a) Gel stained with Coomassie Brilliant Blue G-250; (b) immunoblot with proteins bound to nitrocellulose on transfer probed with a sheep polyclonal antibody which recognizes acidic isoenzymes V1 (B1)–V3 (B4), but not V5 (B5). Lanes are: (1) strain 305 sulphopropyl-Sephadex C-25 peak 2; (2) same peak from strain 309; (3) acidic proteinases of Sephadex G-100 peak 2 from benign strain 309; (4) acidic proteinases of Sephadex G-100 peak 2 from virulent strain 312.

Figure 4 Isoelectric focusing on Ampholine polyacrylamide-gel plates (pH 3.5–9.5 gradient) of (1) *D. nodosus* basic proteinase (BP) from virulent strain 198, (2), strain 305 sulphopropyl-Sephadex C-25 peak 2, (3) acidic proteinases of Sephadex G-100 peak 2 from benign strain 309

product showed 97% identity with the 'virulent' basic-proteinase sequence. Comparison of the two sequences has revealed that the mature region of the 'benign' basic proteinase contained only ten amino acid differences which resulted in two additional negative charges (two Asp residues) in the 'benign' basic proteinase, consistent with the lower pI observed.

Properties of 'virulent' basic proteinase

D. nodosus 'virulent' basic proteinase requires an *I* of about 0.2 for solubility at pH 8.6 and to exhibit maximum activity during assay. Dialysis against 0.01 M Tris/HCl/5 mM CaCl₂, pH 8.6, to remove salt after the sulphopropyl-Sephadex C-25 chromatography resulted in the quantitative precipitation of the protein as microcrystals which are subsequently difficult to redissolve. In contrast, the 'benign' basic proteinase and acidic proteinases V2, B2/B3 and V5/B5 are soluble at low *I* values. Subtilisins from *B. subtilis* strain *amylosacchariticus* and from *B. mesentericus*, which are also basic proteins (pI ~ 9.2), show very low solubility in water [23,24] and yield crystals on dialysis [24], whereas subtilisin novo/BPN (from *B. amyloliquefaciens*) with a lower pI (~ 7.8) is very soluble in water [23].

Basic proteinase was stable at room temperature for at least 24 h and storage at –20 °C for 12 months resulted in no loss of

activity or generation of autolysis products. The basic proteinase was stable to heating up to 55 °C for at least 30 min, but at higher temperatures activity was rapidly lost. SDS/PAGE analysis of heat-inactivated proteinase showed that the protein had been totally degraded, indicating that the loss of activity was due to autolysis induced by the loss of Ca²⁺ ions on thermal unfolding of the protein.

The effect of pH on the rate of hydrolysis of BTEE by basic proteinase gave a bell-shaped pH-activity profile with an optimum around pH 8.0. The activity is apparently dependent on the ionization of two groups with pK_as of about 6.6 and 9.5 consistent with pK_as found for other serine proteinases.

Effect of proteinase inhibitors

The effects of the various proteinase inhibitors on the activity of *D. nodosus* basic proteinase, acidic proteinase V5 and subtilisin I 168, as a representative of the subtilisin family of proteinases, are shown in Table 2. Inhibition by PMSF and di-isopropyl fluorophosphate confirmed that the two *D. nodosus* proteinases are serine proteinases. The two *D. nodosus* proteinases showed an interesting difference in their inhibition by the chymotrypsin inhibitors Tos-Phe-CH₂Cl (tosylphenylalanylchloromethane; 'TPCK') and chymostatin. Tos-Phe-CH₂Cl did not inhibit the basic proteinase, and only weakly inhibited proteinase V5, whereas chymostatin weakly inhibited the basic proteinase, but strongly inhibited proteinase V5 and subtilisin I 168.

The peptidyl-chloromethanes with phenylalanine at the P1 position and Ala or Gly at P2 (according to the nomenclature of Schechter and Berger [26]) effectively inhibited the proteinases, indicating that the large tosyl group of Tos-Phe-CH₂Cl is not favoured at the P2 position. A further difference was noted with the Ala-Ala-Phe-CH₂Cl, in that the inactivation was slower and that complete inhibition was not obtained even after 24 h for the

Table 2 Effect of various proteinase inhibitors on the activity of *D. nodosus* basic proteinases acidic proteinase V5 and subtilisin I168

Inhibitions were set up as follows: for inhibitors dissolved in dimethyl sulphoxide 10 µl aliquot of stock inhibitor was incubated with 90 µl of proteinase (1 mg/ml) in 0.02 M Tris/HCl/5 mM CaCl₂/0.2 M NaCl, pH 8.0; for those dissolved in buffer an equal aliquot of proteinase plus inhibitor was incubated at room temperature. Residual activity was assayed with BTEE after 2 and 24 h incubation. The values in parentheses were the activities remaining after 24 h incubation of the reaction mixture at room temperature. Abbreviation: Tos-Lys-CH₂Cl, tosyl-lysylchloromethane ('TLCK'); Z, benzylloxycarbonyl; MeoSuc, methoxysuccinyl.

Inhibitor	Concn.	Residual activity (%)		
		Basic proteinase	Proteinase V5	Subtilisin I168
PMSF	1 mM	0	0	0
Di-isopropyl fluorophosphate	1 mM	0	0	0
Tos-Phe-CH ₂ Cl	1 mM	100	70 (68)	94
Tos-Lys-CH ₂ Cl	1 mM	100	100	86
Ala-Ala-Phe-CH ₂ Cl	1 mM	80 (55)	33 (0)	49
Z-Ala-Ala-Phe-CH ₂ Cl	1 mM	0	0	0
Z-Gly-Gly-Phe-CH ₂ Cl	1 mM	0	0	0
N-MeoSuc-Ala-Ala-Pro-ValCO-CH ₂ Cl	1 mM	2 (0)	39 (0)	0
Z-Gly-Pro-Phe-Pro-Leu	2 mM	88	78	89
Chymostatin	0.1 mg/ml	77	0	2
Elastinal	0.1 mg/ml	100	100	–
Leupeptin	0.1 mg/ml	95	100	–
Soybean trypsin inhibitor	0.1 mg/ml	100	100	100
Wheat subtilisin inhibitor	0.1 mg/ml	100	100	12
Winged-bean chymotrypsin inhibitor	0.1 mg/ml	100	100	100
EDTA	10 mM	0	0	–

Figure 5 Cleavage of oxidized bovine (a) insulin A- and (b) B-chains by *D. nodosus* virulent basic proteinase

The double-headed arrows indicate the major cleavage sites and the open arrows the secondary sites. The peptides isolated are shown below the sequences.

basic proteinase (Table 2), indicating that the free amino group was not favoured at the P4 position. With a Val at P1 as in the case of *N*-methoxysuccinyl-Ala-Ala-Pro-Val-CH₂Cl the inactivation was significantly slower with proteinase V5 than with the basic proteinase, indicating that proteinase V5 may show a different residue preference to the basic proteinase at the P1 position, although the influence of the Pro at P2 on the inactivation rate cannot be ruled out at present. The other inhibitors tested did not inhibit the two *D. nodosus* proteinases, but the wheat subtilisin/ α -amylase inhibitor inhibited subtilisin I 168.

Treatment with the chelating agent EDTA produced an irreversible loss of activity, whereas the heavy-metal-ion chelator 1,10-phenanthroline had no inhibitory effect. SDS/PAGE analysis of proteinase samples treated with EDTA showed that the 37 kDa basic proteinase band had been totally degraded. Reverse-phase h.p.l.c. analysis of EDTA-treated proteinases showed that they had completely autolysed to small peptide fragments (results not shown). Thus bivalent-metal ions are essential for maintaining the integrity of the proteinase structure and stabilizing the *D. nodosus* proteinase against autolysis or hydrolysis by other proteinases.

Substrate specificity

Basic proteinase showed esterase and amidase activity and extensively hydrolysed native proteins such as BSA, ovalbumin, carbonic anhydrase and lysozyme. Denatured proteins (reduced and alkylated) such as *S*-carboxymethylated haemoglobin and lysozyme, and peptides such as glucagon and oxidized insulin A- and B-chains were extensively hydrolysed.

The cleavage specificity of *D. nodosus* basic proteinase was determined at pH 8.0 using the A- and B-chains of oxidized bovine insulin. The sites of cleavage in the A- and B-chains of

oxidized insulin are summarized in Figure 5. The A-chain was cleaved initially at Gln-Cys(5–6) and Leu-Glu(16–17) followed by cleavages at Cys-Ser(11–12), Leu-Tyr(13–14), Tyr-Gln(14–15) and Glu-Asn(17–18) to yield a series of overlapping peptides (Figure 5). In the B-chain basic proteinase initially hydrolysed the Leu-Cys(6–7) and Leu-Tyr(15–16) bonds, and then the peptides generated were further cleaved to yield a series of overlapping peptides, as shown in Figure 5. Basic-proteinase cleavage of other peptides [27] and *S*-carboxymethylated lysozyme produced cleavages at bonds with Ala, Asn, Leu, Tyr, Trp, Phe, Met, Ser, Thr, Gln, Arg and Lys residues at the P1 position. The specificity of *D. nodosus* basic proteinase is therefore very broad, with cleavage at hydrophobic, aromatic and hydrophilic residues as well as the basic residues lysine and arginine. No evidence of residue preferences at subunits P2, P3 and P4 was apparent.

We thank Mr. J. E. Burns for technical assistance, Mr. N. Bartone for performing the amino acid analyses, Mr. P. M. Strike for gas-phase-sequencer analyses and Mr. K. J. Burns for concentrating the *D. nodosus* bulk cultures. We thank Dr. G. J. Howlett, Biochemistry Department, University of Melbourne, for performing the sedimentation-equilibrium experiments, Dr. L.-F. Wang, Australian Animal Health Laboratories, CSIRO, for providing *B. subtilis* culture supernatant for purification of subtilisin I168, and Dr. K. Maeda, Nisshin Flour Mills Co. Ltd., Saitama, Japan, for providing the wheat amylase/subtilisin inhibitor. This work was supported in part by the Wool Research and Development Fund of the Australian Wool Corporation.

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