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THE DIGESTIVE ASPARTIC PROTEINASES OF THE BROWN EAR TICK

Rhipicephalus appendiculatus Neuman.

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The Digestive Aspartic Proteinases of the Brown Ear Tick, Rhipicephalus appendiculatus Neuman

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Le doyen:

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CHAPTER 1. INTRODUCTION

Bloodmeal digestion is known to play a central role in the tick, all important processes such as moulting, mating and vitellogenesis being preceded by a bloodmeal (Arthur, 1965; Aeschlimann & Grandjean, 1973). Despite its central role, however, little is known about the general physiology of bloodmeal digestion in ticks (Bogin & Hadani, 1973; Akov, 1982) and even less is known about the enzymes involved in the process (Coons et al. 1986). To date, only a few brief studies have dealt with the *in vitro* activity of tick digestive proteinases in adult *Argas persicus* (Tatchell, 1964), female *Hyalomma excavatum* (Bogin & Hadani, 1973), female *Ornithodoros tholozani* (Akov et al., 1976), the larvae of *Boophilus microplus* (Reich & Zorzopulos, 1978) and in *Ixodes ricinus* (Girardin, 1987). Moreover, no parallel studies appear to have been done for the brown ear tick, *Rhipicephalus appendiculatus*.

R. appendiculatus is the vector of *Theileria parva* which causes East Coast Fever, one of the most serious diseases of livestock in Eastern Africa. (Purnell & Joyner, 1968). Consequently, it was felt that the biochemistry of tick digestion deserved attention

for the following reasons:

1/ It would improve our knowledge of tick digestion and also lead to a better understanding of the associated processes.

2/ It may facilitate selective tick control by the vaccination of host animals with purified tick digestive enzymes.

The aim of this project is therefore twofold:

1/ To identify, purify and characterize two of the digestive enzymes of *R. appendiculatus*, which though not the only digestive enzymes, are probably key enzymes in the process. This is because both enzymes are endopeptidases and may therefore be involved in the initiation of protein breakdown, an important process since protein is the most abundant nutrient in blood.

2/ to determine if the said enzymes can be employed as target antigens for tick control.

It is the hope of the author that the results of this study will add to our knowledge of the biochemistry of tick digestion and hence contribute to ongoing efforts to control this important vector of livestock disease.

1.1 *Physiology of Bloodmeal Digestion in the Ixodidae*

1.1.1 *The Site of Digestion*

As in all Acari (Wigglesworth, 1943) digestion is intracellular (Balashov, 1972; Grandjean, 1984) at acid pH (Tatchell, 1964; Reich & Zorzopulos, 1978), well below the pH 6.3 - 6.5 of the gut contents (Tatchell et al., 1972; Bogin & Hadani, 1973), and corresponds to a classical lysosome concept (de Duve & Wattiaux, 1966) in a process known as heterophagy. This is in sharp contrast to most insects in which digestion is extracellular, is much more rapid and occurs at neutral or near-neutral pH (Gooding, 1972). Though phylogenetically primitive, intracellular digestion is economical (Tatchell et al., 1972; Grandjean, 1983; Connat et al., 1986) and therefore important in the ability of the tick to survive prolonged periods of starvation.

1.1.2 *The Phases of Digestion*

Bloodmeal digestion in the Ixodidae is divisible into three phases (see review by Coons et al., 1986), continuous digestion, delayed or reduced digestion and a second phase of continuous digestion (Balashov, 1972; Araman, 1979; Raikhel, 1983). Each phase of digestion is dependent on a specific event in the life cycle of the tick.

The first continuous phase of digestion is initiated by the onset of feeding and is characterized by the concentration of the bloodmeal, red cell haemolysis and the uptake and subsequent digestion of the bloodmeal by the digestive cells (Coons et al., 1986). The bloodmeal is concentrated by the elimination of water and ions via the salivary glands (Tatchell, 1967; 1969; Kaufman and Sauer, 1982). For *I. ricinus*, Lees (1946) reported a 3-fold concentration of the bloodmeal. The actual mechanism of red cell haemolysis is unknown, but evidence points to a gradual increase in red cell fragility due to continuous damage of the membrane (Hughes, 1954; Osterhoff & Gothe, 1966). A Periodic Acid-Schiff (PAS)-positive substance which is secreted into the midgut approximately 24 hours after the onset of feeding, has been implicated in haemolysis (Hughes, 1954; Tatchell, 1964; Grandjean & Aeschlimann, 1973). Finally, there is uptake and subsequent digestion of the bloodmeal by the digestive cells (see review by Coons et al., 1986). Bogin and Hadani (1973) demonstrated a rapid increase in proteinase activity after the attachment of *H. excavatum*. Similar observations were made in the soft ticks *A. persicus* and *A. arboreus* by Tatchell et al., (1972). Histologically, large cells with haemoglobin-filled vacuoles have been observed at this stage (Belozerov & Tymopheev, 1971). Waste products of digestion accumulate in the cells as residual bodies (Holtzman & Noviboff, 1984) which once present in large amounts

cause the cells to be detached and released into the gut lumen where they are lysed. The nutrients resulting from the first continuous phase of digestion are mobilised for metabolic purposes, organ growth and development and not for vitellogenesis (Araman, 1979; Raikhel, 1983). Similar conclusions were arrived at for A. *persicus* (Tatchell, 1964).

The delayed or reduced phase of digestion in ixodids is initiated by mating (Pappas & Oliver, 1972; Bogin & Hadani, 1973) and occurs 12-24 hours prior to the detachment of the tick from the host (Balashov, 1972; Araman, 1979). The phase is characterized by the acquisition of a large bloodmeal by the mated female (Bogin & Hadani, 1973) and a marked reduction in intracellular digestion (Bogin & Hadani, 1973; Coons et al., 1986). Bogin and Hadani (1973) made observations which pointed to the presence of a proteinase inhibitor at this stage. Histologically, large endosomes and few residual bodies are present, leading to the suggestion that the decrease in digestion may be due to great reduction or cessation of lysosome production which would lead to the observed accumulation of large endosomes (Coons et al., 1986). It may well be that both enzyme inhibition and cessation of lysosome function are important. Since digestion is reduced, digestive cells are no longer sloughed into the midgut lumen and the vitellogenic cell appears in the midgut epithelium at this

stage (Coons et al., 1986). Virgin females neither undergo delayed digestion, nor do they engorge fully (Snow, 1967; Aeschlimann & Grandjean, 1973; McSwain et al., 1982), while male ixodids feed for much shorter periods of time, depending on the species, and then detach to mate. For example, male *Dermacentor variabilis* feed for maximum of three days and then detach (Sonenshine et al., 1982). Ultrastructurally, digestion in the male ixodid corresponds to the first phase of continuous digestion and characteristically, there are no vitellogenic cells (Coons et al., 1986).

The second continuous phase is the third and final stage of digestion in the mated ixodid female and is initiated by detachment from the host. As described by Coons and his colleagues (1986), the lysosomal system once again becomes active and digestive cells accumulate numerous residual bodies as oviposition proceeds. However, no digestive cells detach into the midgut lumen and there is progressive growth of the vitellogenic cells, accompanied by a shrinkage of digestive cells. It has been shown that in addition to the fat body, the midgut is also a site of vitellogenin production (Coons et al., 1982; Tarnowski & Coons, 1989). Unlike the first phase of continuous digestion, the products of this phase are utilised for egg formation (Arthur & Snow, 1966; Snow & Arthur, 1966). At the end of oviposition, both

digestive and vitellogenic cells have an organisation suggestive of cell death (Coons et al., 1986).

1.1.3 *The Digestive Enzymes*

The most abundant nutrient in blood is protein and as in other haematophagous arthropods, the work on digestive enzymes in ticks has centered on proteinases (Tatchell, 1964; Bogin & Hadani, 1973; Akov et al., 1976; Reich & Zorzopulos, 1978; Girardin, 1987). The data obtained by the various workers suggest an enzyme or enzymes very similar to mammalian cathepsin D (see section 1.2). In addition to this, Tatchell (1964) demonstrated the presence of a non-specific esterase and an aminopeptidase activity in the cell border microvilli of A. *persicus* during protein uptake.

1.2 ***The Aspartic Proteinases***

It has been mentioned that the principal digestive enzyme of the tick appears to be similar to cathepsin D and must therefore be an aspartic proteinase. A brief review of some of the properties of this class of proteinases is therefore necessary.

1.2.1 *Historical Background*

The history of aspartic proteinases can be traced back to German physiological chemists of about 1890, whose interest in autolysis

led to the partial purification from autolysed liver, of what we now know as cathepsin D (Jacoby, 1900). Parallel to the autolysis work, a growing interest in the proteinases of phagocytes led to the demonstration of a distinct group of proteinases acting under acid conditions (Opie 1905; 1906). Later work (Dernby, 1918) led to the description of these enzymes as 'pepsin-like' proteinases of leucocytes and tissues, which differed from pepsin by their higher pH optima.

1.2.2 *Distribution, Localisation and Evolution*

Today, we know that the aspartic proteinases are widely distributed throughout the plant and animal Kingdoms, including the fungi and protozoa (Bovey & Yanari, 1960; Fukumoto et al., 1967; Barrett, 1970; Sodek & Hoffman, 1970) and that they are very diverse in their localisation within the different organs (see review by Barrett, 1977). In invertebrates, aspartic proteinases have been described in various locations including the midgut of various insect (Pendola & Greenberg, 1975; Houseman & Downe, 1981; Kawamura et al., 1987) and tick species (Tatchell, 1964; Tatchell et al., 1972; Bogin & Hadani, 1973; Akov et al., 1976; Girardin, 1987). Regardless of the tissue however, most aspartic proteinases are located within lysosomes or other organelles that are functionally related to lysosomes (Cook & Pickering, 1962; Yago & Bowers, 1975).

Aspartic proteinases are thought to have evolved from a common digestive enzyme which may have acted extracellularly as in saprophytic fungi and the gastric proteinases or intracellularly as in the digestive vacuoles of protozoa and the tissues of higher animals (Barrett, 1977). This common 'ancestor' theory is supported by similarities in the catalytic mechanism, and sensitivity to inhibitors of aspartic proteinases from diverse sources (Bayliss et al., 1969; Sodek & Hofmann 1970; Takahashi et al., 1974; Tang, 1979) and some immunological recognition (cross-reactions) between cathepsin D preparations from equally diverse sources (Weston & Pool, 1973). Furthermore, the NH_2-termini of different aspartic proteinases have amino acid sequences that are highly homologous (see section 1.2.5).

1.2.3 *Definition*

The best known member of the aspartic proteinases is swine pepsin A (EC 3.4.23.3), M_r 36,000 (Bovey & Yanari, 1960). Others include chymosin (EC 3.4.23.4 ; Foltmann et al., 1977), cathepsin D (EC 3.4.23.5), M_r 42,000 (Barrett, 1971; Woesner, 1971), cathepsin E, M_r 100,000 (Lapresle & Webb, 1962) and renin, M_r 42,000 (Cook & Pickering, 1962). Also in the group are the acid proteinases of several moulds including *Rhizopus chinensis* (Fukumoto et al., 1967) and *Penicillium janthinellum* (Sodek & Hofmann, 1970). They are essentially endopeptidases whose optimal action on protein

substrates is in the pH range 2 - 5 (see review by Barrett, 1977). All aspartic proteinases have two essential aspartyl groups in their active site, correspondig to residues 32 and 215 of pepsin. This renders them susceptible to irreversible inhibition by epoxy inactivators such as 1,2-epoxy-3-(p-nitrophenoxy) propane which react irreversibly with the carboxyl group of Asp-32 (Tang, 1971; Sepulveda et al., 1975) and diazo-inhibitors such as the methyl and ethyl esters of diazoacetylnor-leucine which specifically esterify Asp-215 (Bayliss et al, 1969). But the single most useful reagent in the identification of aspartic proteinases is the pentapeptide inhibitor from actinomycetes, pepstatin (see review by Umezawa & Aoyagi,1977). Pepstatin is a powerful reversible inhibitor of all aspartic proteinases but has little or no inhibitory effect on other classes of proteinases (Knight & Barrett, 1976).

1.2.4 *Molecular and Isoenzyme Forms*

Most aspartic proteinases have molecular weights of about 35,000 (Barrett, 1977). Nevertheless, variations abound. Pepsin, the best known and most studied of the enzymes is composed of a single polypeptide chain of M_r 36,000 (Huang et al., 1979). However, cathepsin E, located in the bone marrow and spleen of rabbits, has a molecular weight of 100,000 (Lapresle & Webb, 1960; Lapresle, 1971). Turk et al. (1968) reported a cathepsin E

of M_r 305,000 from bovine spleen. This rather high value was attributed to possible association with some acidic high molecular weight proteins (Barrett, 1977). The angiotensin-forming enzyme, renin, was found to have a molecular weight of about 40,000 - 42,000 (Waldhäusl et al., 1970; Cohen et al., 1972; Murakami & Inagami, 1975). Cathepsin D occurs in several molecular forms, ranging in molecular weight from 30,000 - 60,000 (Press et al., 1960; Smith et al., 1969; Keilova, 1971; Erickson & Blobel, 1979; Huang et al., 1979; Barth & Afting, 1984). It is now widely accepted that mature cathepsin D occurs in two forms, the single chain form (M_r 40,000 - 42,000) and the two-chain form, which is derived from the single chain-enzyme and is composed of light (M_r 13,000 - 15,000) and heavy (M_r 25,000 - 30,000) chains held together by non-covalent bonds (Sapolsky & Woessner, 1972; Huang et al., 1979; Erickson et al., 1981). In some cases however, the enzyme occurs only in the single-chain form (Yamamoto et al., 1979; Kawamura et al., 1987). In sharp contrast to cathepsin D and other proteinases of this class, the rodent lymphocyte aspartic proteinase occurs as 45,000 molecular weight monomers and disulphide-linked dimeric forms of M_r 95,000 (Yago & Bowers, 1975).

The question arises as to the significance of the multiple forms of cathepsin D. Like all lysosomal proteins, cathepsin D

undergoes post-translational modifications (Erickson et al., 1983). But in addition to this, it is now accepted that the prepro- and pro- forms of cathepsin D are zymogens of the enzyme, functionally similar to the pepsinogens (see reviews by Kirschke & Barrett, 1987; Conner et al., 1987). It is noteworthy that renin, another aspartic proteinase with many similarities to cathepsin D is also thought to have large molecular weight precursors (Inagami et al., 1977).

Besides the different molecular forms, aspartic proteinases also tend to occur in multiple isoelectric forms. Cathepsin D from various sources occurs in at least three isoenzyme forms having isoelectric points (pI) in the range 5.0 - 7.0 (Barrett, 1970; 1971; Sapolsky & Woessner, 1972; Afting & Becker, 1981; Kawamura et al., 1987). Renin also exists in several isoenzyme forms (Cohen et al., 1972; Murakami & Inagami, 1975).

1.2.5 *Amino Acid and Carbohydrate Composition*

The amino acid sequences of pepsin (Tang et al., 1973), chymosin (Foltmann et al., 1977) and cathepsin D (Keilova et al., 1969; Barrett, 1971) have been determined and in all three enzymes there are twice as many acidic residues (together with their amides) as there are basic residues. Cathepsin D has 8 cystein residues per mole of 45,000 (Barrett, 1971) while pepsin has 6

such residues (Tang et al., 1973). There are no free -SH groups in either cathepsin D or pepsin, the cysteine residues forming intra-chain disulphide bridges thought to be important in the stabilisation and conformation of the respective enzyme molecule (Barrett, 1971; Tang et al., 1973). The amino acid sequences of the amino termini of several aspartic proteinases - porcine and bovine cathepsin D, porcine pepsin (Tang et al., 1973), bovine chymosin (Foltmann et al., 1977), an aspartic proteinase from *R. chinensis* (Sepulveda et al., 1975) and penicillopepsin (Hsu et al., 1977) were compared by Huang et al. (1979) and found to be highly homologous. Particularly striking were the sequences around the aspartate residue corresponding with Asp-32 of the active centre of pepsin (Knowles, 1970) which were identical in all six enzymes. Aspartic proteinases are glycoproteins containing mannose-6-phosphate and N-acetyl glucosamine (Huang et al., 1979).

1.2.6 *Active Site and Catalytic Mechanism*

The similarities in the susceptibility of aspartic proteinases to inhibitors (Sodek & Hoffmann, 1970; Tang, 1971; Takahashi et al.,1974; Umezawa & Aoyagi, 1977), substrate specificity (Fruton, 1971), pH dependence (Barrett, 1977) and the homology of their respective amino acid sequences around the essential aspartate residue of the binding site (Knowles, 1970), suggest a common

catalytic mechanism. Sampath-Kumar and Fruton (1974) examined the specificity of pepsin, cathepsin D and *Rhizopus* pepsin and concluded that the three aspartic proteinases possessed extended active sites of comparable dimensions. Indeed, it is now generally agreed that the aspartic proteinases have very similar active centres and that their respective catalytic sites contain a pair of carboxyl groups acting in the way proposed by Knowles (1970) for pepsin and Hsu et al. (1977) for penicillopepsin.

1.2.7 *Substrate Specificity*

The specificity of most aspartyl proteinases is rather broad, but they exibit similar preferences for large, hydrophobic L-amino acid residues on both sides of the sensitive bond (Fruton, 1971), which must be at least two residues removed from either end of the peptide molecule (Schwabe & Sweeney, 1972). Furthermore, the amino donor tends to be aromatic, though leucine is an acceptable alternative in some cases. Fully charged amino acid residues are never found as either donor of hydrolysed bonds (Schwabe & Sweeney, 1972).

Aspartic proteinases differ in their preferences for protein substrates. Thus, haemoglobin is the best substrate for cathepsin D, serum albumin being degraded at only 5 - 10 % of the rate of haemoglobin hydrolysis (Press et al., 1960). In contrast to this,

pepsin and cathepsin E both hydrolyse the two substrates equally well (Barrett, 1967), and while collagen and gelatin are resistant to cathepsin D, they are readily digested by pepsin (Burleigh et al., 1974). However, for protein substrates to be hydrolysed by aspartic proteinases, they must first be denatured (Barrett, 1977). In contrast to pepsin-like enzymes which hydrolyse synthetic substrates such as N-acetyl-L-phenylalanyl-3,5-diido-L-tyrosine (Kress et al., 1966, Woessner & Shamberger, 1971), cathepsin D has a low affinity for peptide substrates containing less than five amino acid residues and therefore has no activity with these substrates (Press et al., 1960).

1.2.8 *Inhibitors*

The active site-directed diazo and epoxy compounds are undoubtedly the most important synthetic inhibitors of aspartic proteinases (see section 1.2.3). But these inhibitors also readily inhibit other classes of enzymes including the cysteine proteinases (Knight & Barrett, 1976). Other synthetic inhibitors of aspartic proteinases include 3-phenylpyruvate (Barrett, 1967; Williams & Lin, 1971).

Aspartic proteinases are generally unaffected by most inhibitors of serine, cysteine and metallo-proteinases (see review by Barrett, 1977) but several exceptions have been reported

including the inhibition of rabbit cathepsin D by dithiothreitol (Barrett, 1967) and *Rhodnius prolixus* midgut proteinase by mercaptoethanol (Houseman & Downe, 1982).

Pepstatin, the pentapeptide inhibitor from actinomycetes (Aoyagi et al., 1972; Umezawa, 1972) is the most powerful and most specific inhibitor of aspartic proteinases known. Classified as a slow tight-binding reversible inhibitor (Rich & Sun, 1980), it is thought to bind aspartic proteinases at their active sites (Aoyagi et al., 1972). Because of its strong equimolar binding to pepsin and cathepsin D, pepstatin can be used to titrate these enzymes. The binding of pepstatin to aspartic proteinases is strongly pH-dependent and decreases with increasing pH (Knight & Barrett, 1976). Like other proteolytic enzymes, the aspartic proteinases are inhibited by α_2-macroglobulin (Barrett & Starky, 1973). Finally, another important inhibitor is the pepsin inhibitor from the body wall of *Ascaris lumbricoides* which inhibits pepsin and cathepsin E but has no effect on cathepsin D or chymosin (Keilova & Tomasek, 1972).

1.2.9 *The Effect of pH and Temperature*

Aspartic proteinases generally have their pH optima in the acid range. Typically, the optimum pH for the hydrolysis of haemoglobin by cathepsin D from various sources is between pH 2.8

- 4.0 (Barrett, 1977). Other aspartic proteinases have been shown to have similar pH optima, including pH 2.5 for cathepsin E (Stefanovic et al., 1962; Keilova & Lapresle, 1970), 2.6 for penicillopepsin (Takahashi & Hofmann, 1975) and a characteristically low 1.7 for porcine pepsin (Christensen, 1955). For the midgut aspartic proteinases from the various tick species, the optimum pH was found to be between pH 2.6 - 3.5 (Tatchell, 1964; Bogin and Hadani, 1973; Akov et al., 1976; Reich & Zorzopulos, 1978). Similar enzymes from various insect species also had comparable pH optima (Okasha, 1968; Smith & Birt, 1971; Pendola & Greenberg, 1975; Houseman & Downe, 1982). Most cathepsin D preparations however, show a distinct shoulder or even a second peak at about pH 4.0 - 5.0 (Barrett, 1971; Cunningham & Tang, 1976). A similar phenomenon has been reported for *A. persicus* gut proteinase by Tatchell (1964) and for the aspartic proteinase from *Lucilia cuprina* (Smith & Birt, 1971). Working with cathepsin D, Cunningham and Tang (1976) showed that this bimodal character is the property of a single enzyme species and is not due to changes in the substrate. Unlike cathepsin D, pepsin is irreversibly denatured at pH values above 6.0 (Steinhardt, 1936) while penicillopepsin loses activity rapidly at pH 2.0 or below and pH 7.0 or above (Sodek & Hofmann, 1970). Another interesting point is that the optimum pH of aspartic proteinases may vary to a surprising degree depending on the nature and source of

substrate. This has been demonstrated for several enzymes including cathepsin D (Woessner, 1967), renin (Waldhäusl et al., 1970) and penicillopepsin (Hofmann & Shaw, 1964; Takahashi & Hofmann, 1975).

The aspartic proteinases appear to have similar thermal stabilities. Cathepsin D (Cunningham & Tang, 1976; Barth & Afting, 1984), penicillopepsin (Sodek & Hofmann, 1970), renin (Reinharz & Roth, 1969) and pepsin (Herriot, 1941) all lost activity rapidly above 50 °C. But pH was important in all cases, the enzyme being more stable to thermal inactivation at pH 6.0 or above. Herriot (1941) reported the stabilisation of pepsin by a peptide at pH values above 4.0, while Puizdar & Turk (1981) reported findings that pointed to a similar mechanism for cathepsin D.

1.3 *Immunoinhibition*

1.3.1 *Tick Proteins as Target Antigens for Tick Control*

It has been known for sometime that resistance to ticks can be induced in animals by vaccination with antigens which are not normally "seen" by the host during tick feeding, and therefore play no part in naturally acquired resistance (see review by Willadsen, 1987). Known as concealed antigens, they form a wide range of potential and important targets in the parasite. The

idea of immunity directed against concealed tick antigens was first proposed by Galun in 1985, and has since gained wide acceptance.

Immunisation with concealed tick antigens was undoubtedly inspired by earlier work on other haematophagous arthropods. Alger and Cabrera (1972) demonstrated that the vaccination of rabbits with midgut homogenates of *Anopheles stephensi* led to increased death rates in the mosquitoes after feeding. Similar results were reported by Schlein & Lewis (1976) who vaccinated rabbits with tissues from *Stomoxys calcitrans*. In the latter case, vaccination with thoracic muscle, abdominal tissue and wing bud extracts led to a variety of effects including paralysis of the legs and wings. According to Willadsen (1987) such effects were suggestive of immunity directed against internal antigens. Recently, Bowles et al. (1987) immunised sheep with larval extracts of the blowfly, *L. cuprina*, inducing some protection against infection by the fly larvae.

Vaccination against ticks using concealed antigens is possible, since several groups working with different tick species have shown that host blood components including IgG can cross from the tick gut to the haemolymph (Ackerman et al., 1981; Brossard & Rais, 1984). Brossard and Rais have further shown that such

immunoglobulins retain their specificity and biological activity.

To date, host resistance to ticks has been induced by antigens prepared from a variety of tick tissues. Allen and Humphreys (1979) used extracts of the internal organs (midgut and reproductive organs) from partially engorged *D. andersoni* females to immunise guinea pigs and cattle. The result was a drastic reduction in the engorgement weights of the ticks, accompanied by a reduction or total abolition of egg laying, or the production of non-viable eggs. Similar results have been reported in several other systems after inoculation with antigens prepared from different tick tissues including whole tick extract (Ackerman et al., 1980; McGowan et al., 1980; McGowan et al., 1981; Mongi et al., 1986; Dhadialla et al., 1990); midgut extract (Ackerman et al., 1980;); salivary gland extract (Garin & Grabarev, 1972; Brossard, 1976; Wikel, 1981; Brown et al., 1984); vitellin (Chinzei & Minoura, 1988); and tick integumental protein (Rutti and Brossard, in press; Aeschlimann et al., 1990).

The most thoroughly studied system however, is that of induced immunity to *B. microplus* in cattle. Immunity to *B. microplus* was induced in cattle by vaccination with a crude extract from partially engorged adult female ticks (Johnston et al., 1986; Kemp et al., 1986; Opdebeeck et al., 1988). In all cases, a

highly significant reduction in tick burden following challenge was observed, as well as a reduction in engorgement weights and egg production, similar to observations made for *D. andersoni* by Allen and Humphreys (1979). Histological examination showed extensive damage to the gut epithelium, particularly the digestive cells. Subsequent rupture of the gut allowed host leucocytes to enter the haemocoel and attack other tissues including the muscle and Malpighian tubules. In males, the accessory glands were also damaged (Agbede & Kemp, 1986). Externally, the affected ticks were often malformed and had red colouration due to the leakage of bovine erythrocytes into the tick haemolymph (Kemp et al., 1986).

The *B. microplus* data are important because a great deal is already known about acquired resistance to this tick, thus making it possible to compare natural and induced immunity in the *B. microplus*/bovine system. Such a comparison shows that the mechanism of induced immunity is clearly different from that acquired by tick infestation and apparently stimulates a different mechanism (Agbede & Kemp, 1986; Kemp et al., 1986). Moreover, immunity to concealed antigens may have considerable advantages. As stated by Willadsen (1987), natural immunity does not exhaust all the immunological possibilities, thus overlooking important internal targets. Secondly, the tick is less likely to

have developed sophisticated forms of immune evasion against antibodies to concealed antigens, since the antigens do not normally come in contact with the host. Induced immunity also has a greater effect on the fecundity of those ticks that engorge fully (Kemp et al., 1986). Induced immunity may therefore act synergistically with acquired immunity, leading to a two-pronged and hence more efficient attack on the tick.

1.3.2 *Immunoinhibition of Proteolytic Enzymes*

Antibodies have an advantage over other enzyme inhibitors in that they are non-toxic and are highly specific. Moreover, in addition to their potential in biological control strategies, they provide potent tools for the study of intracellular digestion in various cell types (Weston, 1969; Weston et al., 1969; Poole et al., 1972; Dingle et al., 1973; Weston & Poole, 1973).

Antibodies have been raised to several proteolytic enzymes including pepsin, trypsin, chymotrypsin and papain (Arnon, 1965; review by Cinader, 1967) and crude midgut proteases of the tsetse fly, *Glossina morsitans morsitans* (Otieno et al., 1984). The immunogenic properties of none of these enzymes have, however, been studied as thoroughly as those of cathepsin D (reviewed by Barrett, 1971; Weston & Pool, 1973). Antisera have been raised to the chicken, rabbit and human enzymes (Weston, 1969; Dingle et

al., 1971; Dingle et al., 1973) which proved to be good immunogens, resulting in the production of specific and potent antisera.

The availability of specific antisera to cathepsin D has enabled workers to inhibit the autolysis of cartilage *in vitro* (Dingle et al., 1971) and the intracellular digestion of sheep IgG, haemoglobin and proteoglycan in living rabbit alveolar macrophages (Dingle et al, 1973), thus underscoring the importance of cathepsin D in these processes. In the tick, Reich and Zorzopulos (1978) used subcellular extracts of larval *B. microplus* to raise anti-phosphomonoesterases in guinea pigs. Similar studies using tick aspartic proteinases have however, not been done. Against this background, it was felt that immunising hosts with purified tick digestive enzymes may result in the *in vivo* inhibition of tick digestion and consequently, the gonadotrophic cycle.

CHAPTER 2. MATERIALS AND METHODS

2.1 *Animals*

New Zealand white rabbits were used. Ticks were Muguga Strain *R. appendiculatus* maintained at the International Centre for Insect Physiology and Ecology (ICIPE). In all experiments ticks were confined to rabbit ears by using ear bags and rabbits were fitted with collars to prevent them from grooming.

2.2 *Materials*

All centrifugation was done on a Sorval Centrifuge using rotor SS34/05 (DuPont, Newton CT 06470, USA). For spectrophotometry, a Beckmann Du-50 Spectrophotometer was used (Beckman, INC Palo Alto, CA 94304, USA). Gel permeation was performed on a Pharmacia C16/100 column (Pharmacia Fine Chemicals, Uppsala, Sweden) equipped with an LKB 2138 UVICORD S monitor, an LKB 2232 Microperpex S Peristaltic Pump, an LKB Ultrorac 7000 fraction collector and an LKB 2210 1-Channel Recorder (LKB-Producter AB, S-16125 Bromma, Sweden). Anion exchange chromatography was on a Pharmacia Fast Protein Liquid Chromatography (FPLC) system comprising a Mono Q HR 5/5 column, a GP 250 Gradient Programmer,

a pair of P500 pumps, a Single Path Monitor UV-1, a Frac-100 fraction collector and a Two-Channel Recorder REC-482. Hydrophobic interaction chromatography was performed by FPLC on a Phenyl-Superose HR 5/5 column. All chromatography media were from Pharmacia. Gels for polyacrylamide gel electrophoresis (PAGE) were cast using a Bio-Rad Model 385 Gradient Former (Bio-Rad Laboratories, Richmond, California 94804, USA) and run on a Bio-Rad slab gel equipment connected to an LKB 2103 Power Supply. Molecular weight markers were from Pharmacia. Isoelectrofocusing equipment and media were from LKB and comprised Ampholine PAG-Plates and a 2117 Multiphor connected to a 2103 Power Supply. Cooling was by tap water at 11 - 15 °C. Ultrafiltration equipment, membranes and dialysis tubing were from Amicon Coorporation, Danvers, MA, USA. All filter paper was from Whatman Ltd. U.K. Bovine serum albumin (BSA), commercial cathepsin D (calf spleen), pepstatin and ninhydrin reagent solution were from Sigma Chemical Company, U.K. BCA Protein Assay Reagent was from Pierce Chemical Company, USA. All other reagents were of analytical grade and were obtained from Sigma.

Enzyme-linked immunosorbent assays (ELISA) were performed on Microelisa plates from Dynatech Laboratories (Santa Monica, California, USA) and the reaction monitored on a Dynatech MR 580 Microelisa Auto Reader. Nitrocellulose paper for immunoblots was

obtained from Louis Schleiffer Ag, Feldbach, Switzerland and transfers were performed on an LKB 2117-250 Electrophoretic Transfer Kit connected to a 2103 Power Supply. Saponin DAB and 1,2-phenylendiamine (OPD) were from Fluka Ag, Buchs, Switzerland while peroxidase-labelled anti-rabbit immunoglobulin G (IgG) was from Cooper Biomedical Inc. Malvern, PA 19355, USA.

2.3 *Biochemical Experiments*

2.3.1 *Preparation of Midgut Homogenates*

Male and female adult ticks were placed on the ear of a rabbit in a 1:1 ratio. On day 5 after infestation (the end of the first continuous phase of digestion), partially engorged female ticks were detached from the rabbit and the midguts (~ 500 in each batch) were removed and immediately placed in chilled (4 °C) 0.15M NaCl solution. Guts were washed 10 times in 50 volumes of the same solution to remove as much of the rabbit blood as possible and then homogenised in PBS (20mM potassium phosphate; 0.2M NaCl; 0.02% NaN_3, pH 7.4) containing 0.5% Triton X-100, using a manual glass homogeniser with a teflon pestle. The homogenate was allowed to stand for 2 hours at 4 °C and then centrifuged for 30 minutes at 31,000 g. The pellet was washed 5 times with PBS and the washings combined with the original supernatant to give a final volume of 15 ml (about 700 mg protein).

2.3.2 *Acid Precipitation*

The supernatant described in section 2.3.1 was brought to pH 3.0 with 1M formic acid, allowed to stand for 30 min at 4 °C and then centrifuged for 30 min at 31,000 g. The acid was removed by ultrafiltration against PBS, in an Amicon 8050 concentrator using an Amicon 10 membrane (M_r 10,000 cut off).

2.3.3 *Gel Permeation Chromatography*

The supernatant described in section 2.3.2 was applied to a 90 x 1.6 cm Sephadex G-100 column equilibrated with PBS and calibrated with the following standards: aldolase (M_r 158,000), albumin (M_r 67,000), ovalbumin (M_r 43,000), chymotrypsinogen A (M_r 25,000), ribonuclease A (M_r 13,700). Fractions (3.8 ml) were collected and those having enzyme activity were pooled and concentrated by ultrafiltration as described in 2.3.2.

2.3.4 *Anion Exchange Chromatography*

The enzyme fraction from section 2.3.3 was equilibrated with 20mM tris-HCl buffer pH 7.4 using an Amicon concentrator, then applied to a Mono Q anion exchange column equilibrated with the same buffer and eluted with a linear NaCl gradient 0 - 0.35M. Two peaks of enzyme activity designated proteinase 1 and proteinase 2 were obtained (see section 3.1). The fractions corresponding to proteinase 2 were equilibrated with 0.02M histidine-HCl buffer pH

6.0 and rechromatographed on the same column. The enzyme was eluted with a linear NaCl gradient, 0 - 0.35M.

2.3.5 *Hydrophobic Interaction Chromatography*

The proteinase 1 fraction from section 2.3.4 was treated with 40% ammonium sulphate and centrifuged at 31,000 g for 30 min. The supernatant was applied to a Phenyl-Superose column and eluted with a decreasing linear ammonium sulphate gradient, 40 - 0%.

2.3.6 *Polyacrylamide Gel Electrophoresis*

Electrophoresis was conducted on 10 - 20% SDS-polyacrylamide slab gels in 50mM tris, 384mM glycine buffer pH 8.3 as described by Laemmli (1970). SDS-PAGE was also performed using 10 - 20% slab gels copolymerised with substrate as described for plasminogen activators by Heussen & Dowdle (1980). Electrophoresis under non-denaturing conditions was carried out on 10 - 20% slab gels copolymerised with substrate (Heussen and Dowdle, 1980). Gels were prepared in the normal way, but contained 20 $mg.ml^{-1}$ substrate (denatured Hb). Electrophoresis was at 4 °C with a constant current of 20mA for about 2 h. The protein standards used for both types of gel were thyroglobulin (M_r 669,000), ferritin (M_r 440,000), catalase (M_r 232,000), lactate dehydrogenase (M_r 140,000), phosphorylase b (M_r 94,000), BSA (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000),

soybean trypsin inhibitor (M_r 20,100), lactalbumin (M_r 14,400). After electrophoresis, SDS gels were stained for protein by the silver staining method of Wray et al. (1981). The molecular weight of enzyme fractions 1 and 2 were determined from a plot of log molecular weight versus relative migration of the standards. Native gels were transferred to enzyme assay buffer (section 2.3.10.1) and incubated for 48 h at 37 °C and then stained with Coomassie Brilliant Blue R250. SDS gels copolymerised with substrate were transferred to 2.5% Triton X-100 for 2 h (room temperature on a shaker) to wash off the SDS, and then incubated and stained as for the native gels.

2.3.7 *Isoelectrofocusing*

Analytical isoelectrofocusing was conducted at 11 - 15 °C on Ampholine PAG plates, pH 3.5 - 9.5 as described in the LKB application note 250 (1977). One half of the gel was stained for protein with silver stain. Gel slices (0.5 cm) from the unstained half of the gel were soaked overnight in water or phosphate buffer and assayed for pH or proteinase activity respectively (Barth & Afting, 1984).

2.3.8 *Protein Determination*

Protein concentrations were determined using the BCA Protein Assay Reagent with BSA as protein standard (Pierce application

note 23225, 1984).

2.3.9 *Preparation of Protein Substrates*

Acid-denatured haemoglobin was prepared from bovine blood as described by Barrett (1967; 1970). Acid-denatured bovine serum albumin was prepared from commercial BSA as described by Barrett (1977). Both substrates were dialysed for 48 h against water and then filtered with Whatman No 4 filter paper. NaCl was then added to each substrate to give a final concentration of 0.2M. Each substrate was filtered once more with Whatman No 4 filter paper.

2.3.10 *Enzyme Assays*

2.3.10.1 *Assays With Protein Substrates*

The proteinase assay was based on the method of Anson (1937; 1938) with minor modifications. Assays were performed in 0.25M sodium formate-formic acid buffer pH 3.3 containing 0.2M NaCl, 10mg.ml^{-1} acid-denatured Hb or BSA in a total reaction volume of 0.5 ml. Incubation was for 20 min at 45 °C and the activity was terminated with 5 ml 3% trichloroacetic acid (TCA). The TCA-insoluble peptides were filtered off using Whatman No 542 filter paper and the A_{278} of the filtrate determined. All assays were done in duplicate and for every assay, a blank was included in which the enzyme was added after TCA. Unless otherwise stated, 50 µl enzyme was used in this and all other assays. 20 min

incubation period was adopted after it was experimentally shown that it fell within the linear portion when activity was compared with time.

2.3.10.2 *Assays with N-acetyl-phenylalanyl-3,5-diido-L-tyrosine (Ac-Phe-DIT)*

Assays were carried out by a modification of the method used for pepsin by Jackson et al. (1965). They were performed in sodium formate-formic acid buffer pH 3.3, 0.1mM Ace-Phe-DIT in dimethyl sulphoxide (DMSO). Hydrolysis was initiated by the addition of 0.1 ml enzyme solution in a total reaction volume of 0.5ml. Reactions were run in duplicate at 37 °C and arrested after 5 min by adding 0.3 ml of 1M NaOH. The degree of hydrolysis was determined by the ninhydrin method as described by Moore (1968). The absorbance was measured at 410 nm.

2.3.10.3 *Assays with Other Synthetic Substrates*

Aspartic proteinase fractions 1 and 2 were tested for the hydrolysis of the following synthetic substrates of serine and thiol proteinases: two substrates of trypsin, α-N-benzoyl-DL-arginine-p-nitroanilide (BApNA; Σ_{410} = 8,800; Erlanger et al., 1961), p-tosyl-L-arginine methyl ester (TAME; Σ_{247} = 540; Hummel, 1959); two chymotrypsin substrates, N-acetyl-L-tyrosine ethyl ester (ATEE; Schwert & Takenaka, 1955; (Σ_{237} = 400; Patat & Hirch,

1966)), N-benzoyl-L-tyrosine-p-nitroanilide (BTpNA; Σ_{410} = 8,800; Erlanger et al., 1961); a substrate of carboxypeptidase A, hippuryl-L-phenylalanine (HP; Σ_{254} = 280; Davies et al.,1968); a substrate of carboxypeptidase B, hippuryl-L-arginine (HA; Σ_{254} = 360; Folk et al., 1960); L-leucine-p-nitroanilide (LpNA; Wachsmuth et al., 1966 (Σ_{410} = 8,800; Erlanger et al., 1961)) for aminopeptidase. Assays were conducted in duplicate at 37 °C and followed spectrophotometrically for 10 min against a blank into which no enzyme had been added.

2.3.11 *Kinetic Studies*

2.3.11.1 *Determination of K_M*

The initial velocity, v_o of the enzyme catalysed hydrolysis of acid-denatured haemoglobin, was measured at different substrate concentrations (1 - 20 mg.ml^{-1}). The Michaelis constant (K_M) and the maximum velocity (V_{max}) were calculated from plots of $1/v_o$ versus $1/s$ and s/v_o versus s.

2.3.11.2 *Inhibition by Pepstatin*

In all assays involving pepstatin, the enzyme fraction and inhibitor were preincubated in the assay system for 15 min prior to the addition of substrate. Each enzyme fraction was assayed in the presence of increasing concentrations (1 - 8nM in the assay mixture) of pepstatin and 20 mg.ml^{-1} haemoglobin at pH 3.3. The

reaction volume was 0.5ml. To determine the type of inhibition, assays were performed at different substrate concentrations (see section 2.3.11.1) in the presence of 1nM and 3nM pepstatin for enzymes 1 and 2 respectively. The apparent K_M and V_{max} were calculated from double reciprocal plots (Lineweaver & Burk).

2.3.11.3 *Determination of K_I*

The inhibition constant (K_I) for pepstatin was determined by conducting assays in the presence of increasing concentrations of inhibitor (section 2.3.11.2) at two different substrate concentrations (5 and 10 $mg.ml^{-1}$). K_I was obtained from the intersect of Dixon plots of I/v_o versus I for the two substrate concentrations.

2.3.11.4 *Assays with other inhibitors*

The following inhibitors were tested on enzyme fractions 1 and 2 in the presence of 20 $mg.ml^{-1}$ haemoglobin. As described for pepstatin (2.3.11.2), assays were preceded by incubation of each enzyme with increasing concentrations of EDTA (0.5 - 2mM), soybean trypsin inhibitor (STI: 2.5 -20$\mu g.ml^{-1}$), aprotinin (1 - 10$\mu g.ml^{-1}$) and phenylmethylsulphonyl fluoride (PMSF: 0.5 - 2mM).

2.3.11.5 *Assays with antisera*

Assays were performed by a modification of the method of Dingle et al. (1971). Each serum (3 parts) was preincubated with each enzyme fraction (1 part) for 15 min at room temperature and pH 6.5. Assays (total volume 0.5ml) were then performed in the usual way, but at pH 3.8.

2.3.11.6 *pH Studies*

Proteinase fractions 1 and 2 were assayed in 0.15M buffers in the pH range 1.8 - 6.5, prepared from a mixture of phosphoric, formic and acetic acids as described by Barrett (1967) and containing 0.2M NaCl. The substrate concentration was 20 $mg.ml^{-1}$. The effect of pH on pepstatic inhibition of haemoglobin hydrolysis was tested by assaying the proteinase fractions in the presence of pepstatin (2.3.11.2) at pH 1.8, 2.6 and 3.3 for proteinase fraction 2, and at pH 2.6, 3.3 and 3.8 for proteinase fraction 1.

2.3.11.7 *Thermal Stability Studies*

Aliquots of each enzyme fraction were incubated at temperatures between 25 °C - 65 °C for 10 min at a) pH 3.3 and b) pH 7.4. Each sample was then brought to 4 °C and assayed at 45 °C and pH 3.3.

2.3.11.8 *Stability Studies*

Aliquots of each enzyme were stored in Eppendorf tubes at 4 °C or -20 °C with or without glycerol. Samples were assayed for hydrolysis of haemoglobin on days 0, 5, 10, 15, and after the onset of the experiment. Activities were expressed as a ratio of the untreated enzyme activity on day 0.

2.4 ***Immunological Experiments***

2.4.1 *Immunisation of animals*

Each rabbit was bled from the marginal ear vein for preimmune serum and then injected subcutaneously with 60 µg of enzyme fraction 1 (group 1) or enzyme fraction 2 (group 2) or 120 µg of the G-100 fraction (group 3) in an equal volume of saponin solution (50 $\mu g.ml^{-1}$; total volume of 0.1ml) and boosted on day 8 after the initial injection. On day 24, a second booster was given in which rabbits were injected intradermally with the respective enzyme fractions emulsified with an equal volume of Freunds Complete Adjuvant (FCA). Control rabbits were similarly injected with a 1:1 solution of PBS and saponin or PBS emulsified with FCA (group 4). Untreated controls (group 5) were kept under the same conditions as the rabbits in groups 1 - 4. Animals were bled from the marginal ear vein on the 7^{th} day after each injection. There were 3 rabbits in each group.

2.4.2 *Preparation of Antiserum*

Blood was obtained from experimental and control rabbits and allowed to clot in glass tubes for 2 h at room temperature and then stored overnight at 4 °C. Serum was separated from clots by centrifugation for 20 min at 1,500 rpm and stored at -20 °C. For the *in vitro* enzyme inhibition studies, ammonium sulphate was added to each serum to give a final concentration of 50%. Sera were then centrifuged at 31,000 g for 30 min. The pellete was washed 3 times with the same ammonium sulphate solution and dialysed against water overnight to remove the ammonium sulphate. Sera were centrifuged once again at 31,000 g (Heide & Schwick, 1973).

2.4.3 *Detection of Antibodies*

Antibodies (Ab) were detected by ELISA. MicroELISA plates were coated with 100 µl of proteinase fraction 1, 2 or the G-100 fraction (protein concentration = 5 $\mu g.ml^{-1}$) in 50 mM carbonate buffer pH 9.6. Plates were incubated overnight at 4 °C then washed 3 times with 50mM PBS pH 7.4 containing 0.05% Tween 20 (PBS-Tween). Unblocked sites were saturated with 1% BSA in PBS (200 µl per well) and incubated for 30 min at room temperature. The plates were then washed as before. Test sera were diluted 200x in PBS-Tween containing 1% BSA (PBS-Tween-BSA), incubated with antigen (Ag) for 30 min at 37 °C and washed as described

above. The peroxidase-labelled secondary Ab (IgG) was diluted 5,000x in PBS-Tween-BSA, incubated with primary Ab for 30 min at 37 °C, and washed as described previously. The substrate was 3.7 mM 1,2-phenylendiamine (OPD) in citrate phosphate buffer pH 5.0 and containing 1.5 µl H_2O_2 (30%) per ml. Incubation was for 30 min at room temperature in the dark and the reaction was stopped with 50 µl of 2.5M H_2SO_4. The absorbance was read at 492 nm.

2.4.4 *Immunoblotting*

The electrophoretic transfer of antigens from polyacrylamide gels to nitrocellulose was as described by Towbin et al. (1979), using an LKB 2117- NOVABLOT electrophoretic transfer kit. The transfer was conducted for 1 h in 48 mM tris-39 mM glycine pH 8.5 containing 20% ethanol, at 0.8mA.sq.cm.$^{-1}$. After the transfer, the nitrocellulose paper was rinsed with 20mM tris-HCl buffer pH 7.5 containing 0.5M NaCl (TBS), washed twice for 15 min each in TBS containing 5% fat-free powdered milk (TBS-milk 5%) and then immersed in the same solution for 2.5 h. The blots were then washed with TBS for 5 min and placed overnight in antiserum solution diluted 100x in TBS-milk 1%, at room temperature with shaking. The blots were washed 4x for 15 min each in TBS-milk 5% and then once with TBS. After washing, the nitrocellulose blots were immersed in peroxidase-labelled anti-rabbit IgG diluted 1,000x in TBS-milk 1%, and incubated for 2 h at room temperature

with shaking. The blots were rinsed once in TBS and then in 10mM tris-HCl buffer pH 6.8 (TB) and then immersed in substrate solution (0.3% 4-chloro-1-naphthol in methanol diluted 5x with TB and containing 0.33 µl of H_2O_2 (30%) per ml of final substrate solution) until the bands became visible (10 - 20 min). Excess substrate was washed off with distilled water.

2.4.5 *Challenge Experiments*

Immunised rabbits were challenged with 10 female and 15 male ticks per ear. Starting from the 5th day after infestation, earbags were opened twice daily and engorged females weighed and kept individually in preweighed vials at 27 °C and approximately 80% relative humidity. For each tick, eggs were weighed on the 20th day after the onset of oviposition. The egg conversion factor (ECF) was calculated by dividing the egg batch weight for each female with its engorgement weight.

2.5 ***Statistical Analysis***

The statistical analysis on the immunological experiments was by analysis of variance (ANOVA). Straight line graphs for the enzyme kinetic studies were drawn by linear regression.

CHAPTER 3. RESULTS AND DISCUSSION

Prior to the onset of the enzyme assays from which the results reported below were obtained, it was acertained that the release of the TCA soluble material was linear over the 20 min. incubation period used in the assays. This is further indicated by the fact that both enzymes showed Michaelis kinetics (3.2.3).

3.1 *Purification of Proteinases 1 and 2*

Acid precipitation proved to be a useful purification step with good recovery (table 1). For the second step of purification, Sephadex G-100 was chosen because the molecular weights of the known aspartic proteinases are between $2x10^4$ - 10^5. After gel filtration on Sephadex G-100, two broad protein peaks were obtained (fig. 1). In the trough between the two peaks, a broad peak of enzyme activity, hydrolysing haemoglobin at acid pH was demonstrated. Native PAGE of the active fractions on gels co-polymerised with substrate (Hb) showed that there were infact two proteinases (fig. 1). The active fractions from this step were next chromatographed on a Mono-Q column which resolved the proteinase activity into two peaks. Native PAGE on gels co-polymerised with substrate showed that the two activity peaks

Fig. 5 **Analytical Isoelectrofocusing of Proteinase Fractions 1 and 2 at varios stages of Purification**

Fractions from different purification steps were applied onto the gel and electrofocused over a pH range of 3.5 - 9.5 at -12 °C. The approximate amount of protein applied to the gel is shown in parenthesis for each fraction.

1 - Crude homogenate (10 µg)
2 - After acid precipitation (5 µg)
3 - Sephadex G-100 fraction (0.6 µg)
4 - Sephadex G-100 fraction (conc. 1.5 µg)
5 - Proteinase fraction 1 after Mono-Q (0.6 µg)
6 - Proteinase fraction 1 after Phenyl-superose (0.4 µg)
7 - Proteinase fraction 2 after Mono-Q (0.6 µg)
8 - Proteinase fraction 2 after 2nd Mono-Q (0.3 µg)

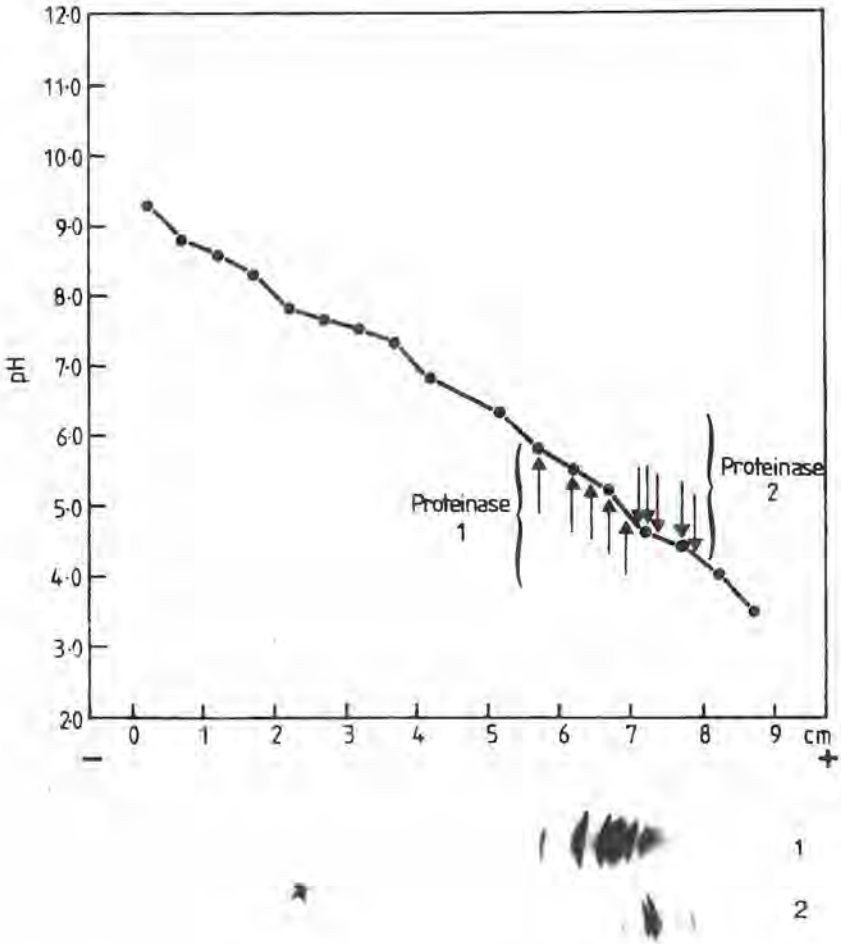

Fig. 6 **Analytical Isoelectrofocusing of Enriched proteinases 1 and 2**

Approximately 0.4 µg of proteinase fraction 1 (after the hydrophobic column) and 0.3 µg of proteinase fraction 2 (after 2nd anion exchange chromatography) were applied onto the gel. Electrofocusing was over a pH range of 3.5 - 9.5 at -12 °C. Sections of the gel (0.5 cm) were assayed for pH and proteinase activity. The latter could not be detected.

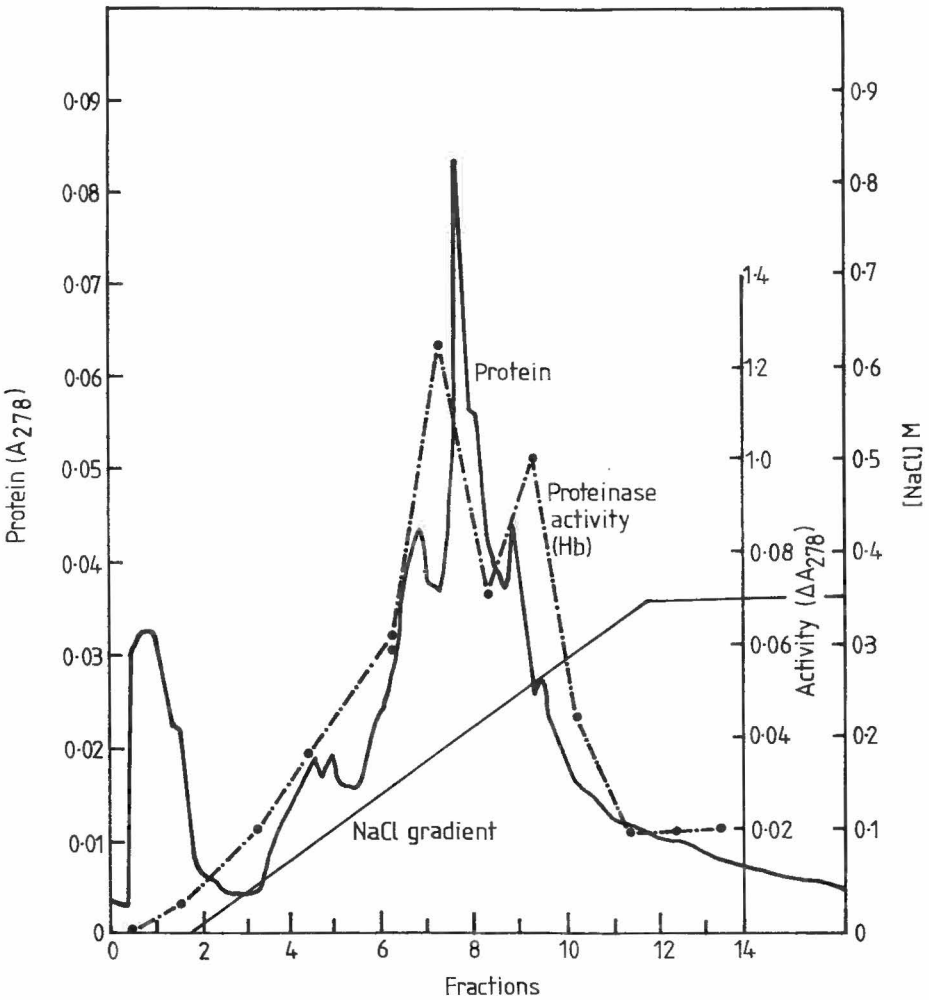

Fig. 3 **Purification of Aspartic Proteinase 2 by Anion exchange chromatogrphy**

Approximately 0.2 mg of protein showing proteinase 2 activity was loaded onto the column in 20mM histidine-HCl buffer pH 6.0, containing 0.02% NaN_3. The flow rate was 0.5ml.min^{-1} and elution was by a linear NaCl gradient, 0 - 0.35M. Fractions (2ml) showing proteinase activity (3 - 12) were combined.

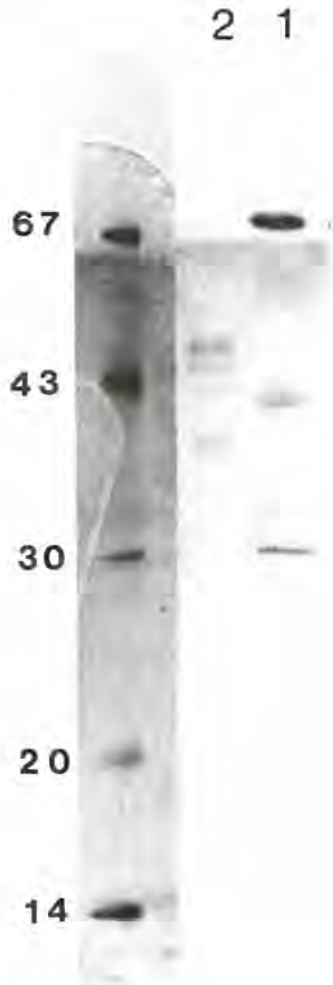

Fig. 7 **Estimation of the Molecular Weight of Proteinases 1 and 2**

Approximately 1.2 µg of proteinase fraction 1 and 0.6 µg of proteinase fraction 2 were applied to 10 - 20% SDS slab gels. The buffer was 50mM tris, 348 mM glycine, pH 8.3 containing 0.1% SDS. Electophoresis was for ~2h at 20 mA and 4 °C. The molecular weight markers shown are: BSA (67 kD); ovalbumin (43 kD); carbonic anhydrase (30 kD); STI (20.1 kD); lactalbumin (14.4 kD). The gel was stained by the silver staining method.

from the Mono-Q column corresponded with the two proteinase activities described above (fig. 2). Proteinase fraction 2 was cleaned up by further chromatography on a Mono-Q column using histidine buffer pH 6.0 (fig. 3) while proteinase fraction 1 from the Mono-Q column was subjected to hydrophobic interaction chromatography which separated it from several other proteins including BSA, a major contaminant (fig. 4).

Typical purification data for the two proteinase fractions are summarised in Table 1. Proteinase fractions 1 and 2 were purified 466-fold and 417-fold respectively, over the crude extract. The specific activities were 44.55 U.mg protein^{-1} for proteinase fraction 1 and 40.04 U.mg protein^{-1} for proteinase fraction 2, with yields of 7.5% and 8.5% respectively. Figure 5 shows an IEF gel of the different purification steps.

Acid precipitation has been used in the purification of cathepsin D by several workers including Yamamoto et al. (1978), Huang et al. (1979) and Takahashi & Tang (1981). As in the present study, the procedure proved to be a useful purification step with good recovery.

The drastic loss in activity after the anion exchange step is similar to the observations of Huang et al. (1979) during the

Table 1 **Purification of Proteinases 1 and 2**

	Total Prot. (mg)	Sp.Act. (U/mg)	Total Act. (Units)	Purif. (fold)	Yield (%)
Crude	742.5	0.096	70.1	1	100
Acid ppt.	45.9	1.243	57.0	13	80
G 100	5.58	5.439	30.4	57	43
Prot. 1(M-Q)	0.30	28.820	8.7	300	12
Prot. 2(M-Q)	0.36	24.017	8.7	250	12
Prot. 1(P-S)	0.12	44.550	5.5	466	7.5
Prot. 2 (2nd.M-Q)	0.15	40.04	6.0	417	8.5

Typical purification data for the *R.appendiculatus* aspartic proteinases 1 and 2. 1 unit of enzyme activity is that amount which causes an increase of 1, in absorbance at 278 nm per minute per mg of protein. M-Q = Mono-Q, P-S = Phenyl Superose.

purification of cathepsin D from bovine spleen. They obtained a purification factor and recovery similar to ours after a procedure which included acid precipitation, anion exchange chromatography and affinity chromatography on a pepstatin-Sepharose column. They showed that the apparent loss in activity was due to the separation of 'activator' proteins after the pepstatin-Sepharose step. Takahashi & Tang (1981) observed similar losses in activity after pepstatin-Sepharose chromatography during the purification of porcine and bovine spleen cathepsin D, and attributed it to the loss in the synergistic effect of sulfhydryl proteases with cathepsin D. As in the present study where the loss in activity was sudden (occuring after the Mono-Q step), both Huang et al. (1979) and Takahashi & Tang (1981) observed the loss in enzyme activity after their respective pepstatin-Sepharose steps. The loss in the activities of both proteinase fractions reported in the present study may therefore be due to factors similar to those reported by Huang et al. (1979) and Takahashi & Tang (1981). Alternatively, it may be due to the loss in synergistic activity between proteinases 1 and 2, as the two are separated from one another during the Mono-Q step.

3.2 ***Properties of Proteinases 1 and 2***

3.2.1 *Isoelectric Points*

Each enriched proteinase fraction focused into at least 3 major bands and several other minor forms, in the pH range 4.65 - 5.8 for proteinase fraction 1 and 4.3 - 4.6 for proteinase fraction 2. The Proteinase 1 fraction focused into five major bands of pI 4.8, 5.1, 5.3, 5.5 and 5.8. For proteinase fraction 2 there were 2 bands of pI 4.3, 4.4, 4.55, 4.6, and a less prominent band of pI 4.5. (fig. 6).

Although an attempt was made to assay each protein band for proteinase activity, the activity in the bands was too low to be detected. It was therefore not possible to determine whether the isoelectric bands observed for each enriched proteinase fraction were isoenzymes or contaminants. It can therefore be concluded that the pI of the *R. appendiculatus* aspartic proteinase 1 is between pH 4.65 - 5.8, while for proteinase 2, the pI is between pH 4.3 - 4.6. However most studies of aspartic proteinases have revealed the presence of at least three major isoenzymes and several other minor forms (Press et al., 1960; Barrett, 1971; Sapolsky & Woessner, 1972; Murakami & Inagami, 1975; Yamamoto et al., 1978). This is also true for the Cathepsin D from the blowfly, *Aldrichina grahami*, which had 2 pI forms of 5.4 and 6.2 (Kawamura et al., 1987) and appears to be a common phenomenon

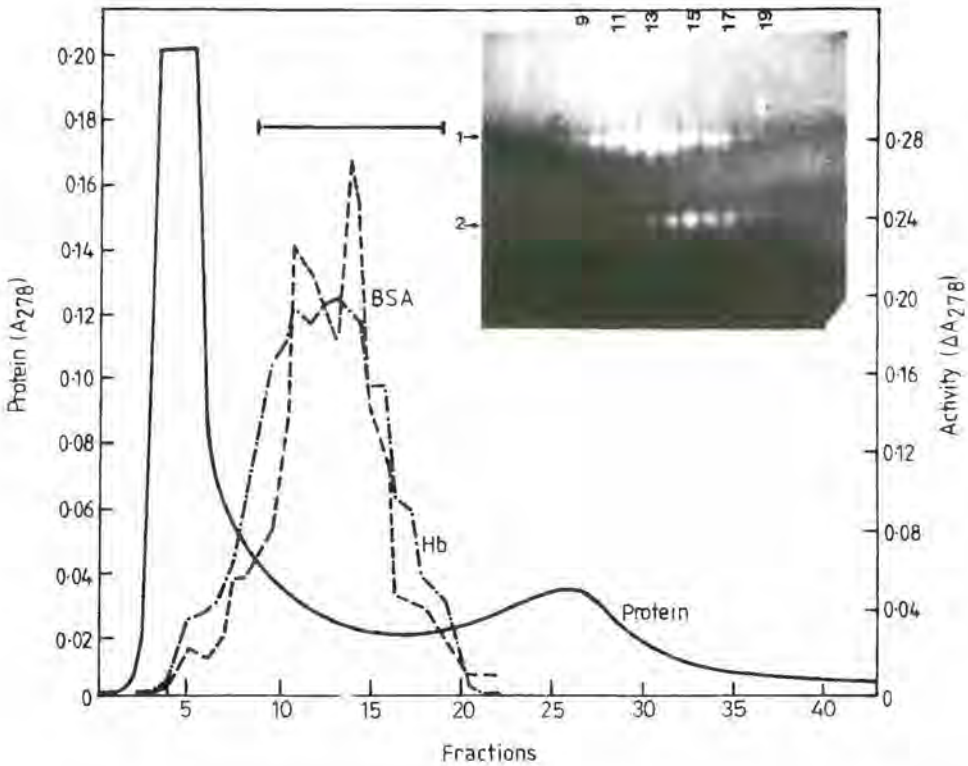

Fig. 1 **Gel Permeation of Tick Aspartic Proteinases on Sephadex G-100**

Approximately 46 mg of protein extract obtained after acid precipitation of the crude homogenate was loaded onto a 90x1.6 cm column equilibrated with 20mM potassium phosphate buffer pH 7.4, containing 0.2M NaCl and 0.02% NaN_3. The flow rate was 30ml.h^{-1}. Fractions (3.8 ml) showing enzyme activity (9 - 19; indicated by horizontal bar) were combined for further purification.

Insert shows native PAGE of chromatographed fractions on 10 - 20% slab gels co-polymerised with Hb. The buffer was 50mM tris, 384mM glycine, pH 8.3. Gels were run at 20mA and 4 °C for -2h and then stained for proteinase activity (arrows).

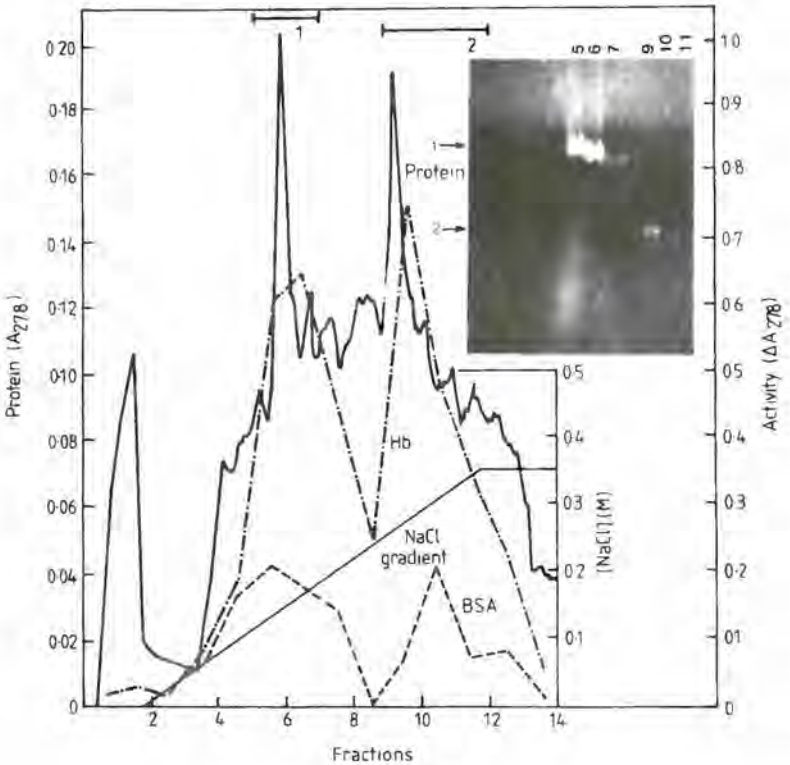

Fig. 2 **Separation of Tick Aspartic Proteinases 1 and 2 by Anion Exchange Chromatography**

Approximately 7 mg of the active fraction from the gel permeation step was loaded onto the column, equilibrated with 20mM tris-HCl, pH 7.4 containing 0.02% NaN_3. The flow rate was 0.5ml.min^{-1} and elution was by a linear NaCl gradient, 0 - 0.35M. Fractions (2ml) 5 - 7 were combined (proteinase fraction 1). Fractions 9 - 12 were similarly combined (proteinase fraction 2).

The insert shows the corresponding gel, stained for proteinase activity (arrows). Other details are as for fig. 1.

among the aspartic proteinases (see 1.2.4). The pI reported here for the enriched fractions of the *R. appendiculatus* enzymes are within the range reported for other aspartic proteinases (~ 4.0 - 7.5).

Several workers including Barrett (1971), Sapolsky and Woessner (1972) and Huang et al., (1979) did experiments to determine the origin of the multiple isoenzyme forms of aspartic proteinases. They concluded that the different forms were not generated by autodigestion (Huang et al., 1979), that there was no evidence of interconversion of isoenzymes during prolonged storage at 4 °C or under any other conditions (Barrett, 1971) and that the different forms arose *in vivo* and were not due to proteolysis or any other artificial conditions (Sapolsky & Woessner, 1972). Further work by Huang et al. (1979) showed that the isoenzymes of cathepsin D could be partly traced back to the heavy chain region of the enzyme, but that structural differences which were as yet unobserved were also involved.

3.2.2 *Molecular Weights*

A broad peak of enzyme activity was obtained after gel filtration, due to the overlap of the two enzymes (fig.1). It was therefore not possible to determine molecular weights by this method. SDS PAGE of the enriched proteinase fraction 1 revealed a

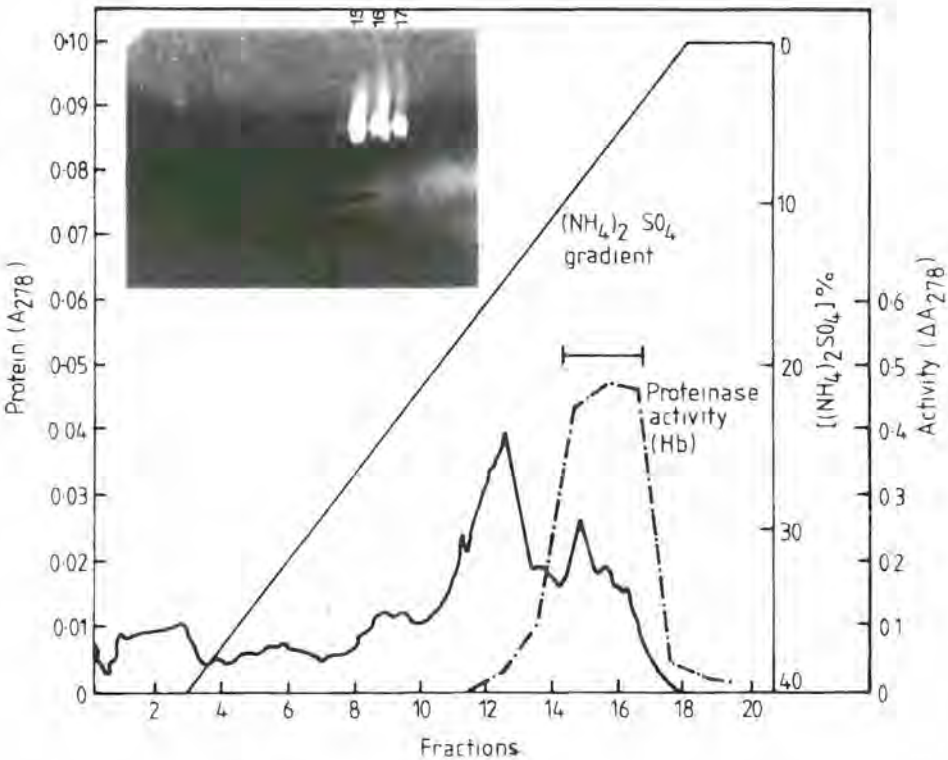

Fig. 4 **Purification of Aspartic Proteinase 1 by Hydrophobic Interaction Chromatography**

Approximately 0.3 mg of protein showing proteinase 1 activity was loaded onto the column in 0.2M potassium phosphate buffer pH 7.4, containing 40% $(NH_4)_2SO_4$ and 0.02% NaN_3. The flow rate was 0.5ml.min^{-1} and elution was by a linear $(NH_4)_2SO_4$ gradient, 40 - 0%. Fractions (2 ml) showing proteinase activity (15 - 17; horizontal bar) were combined.

Insert shows the corresponding gel, stained for proteinase activity. Details are as for fig. 1.

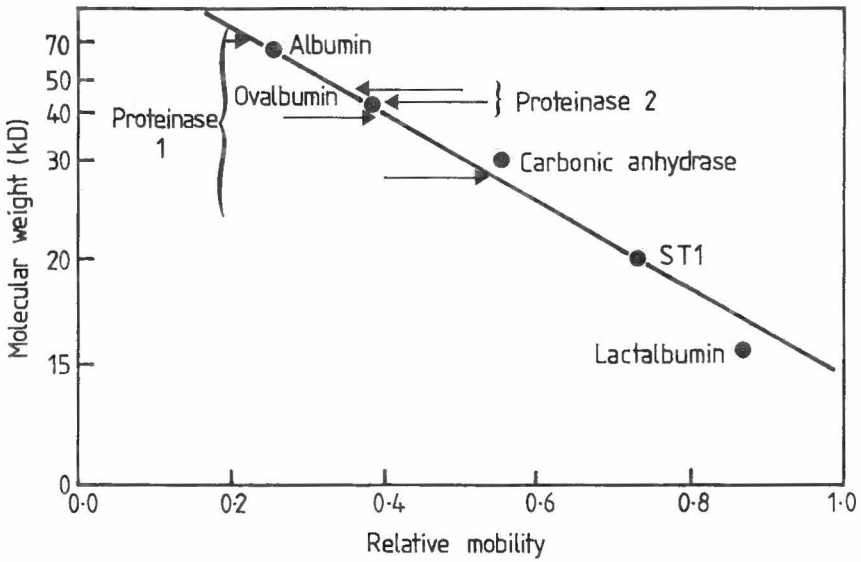

Fig. 8 **SDS-PAGE molecular weight analysis of proteinase fractions 1 and 2.**

Analysis of the data obtained from SDS-PAGE by semi-log transformation.

major band with a molecular weight of about 70,000 and two less prominant bands having molecular weights of about 40,000 and 29,000. For enriched proteinase fraction 2, there were two bands of about 46,000 and 44,000 (figs.7 & 8).

It was not possible to demonstrate enzyme activity for any of the protein bands. This was undoubtedly due to the denaturation of the enzymes. For proteinase fraction 1, however, the molecular weights of the two smaller bands, 40,000 and 29,000 add up to 69,000, about the same as the molecular weight (70,000) of the major band. It is therefore possible that the two less prominant bands are formed as a result of a "nick" of the major form. This feature is well documented for cathepsin D (Sapolsky & Woessner, 1972; Huang et al., 1979; Erickson et al., 1981). It is not possible to conclude whether both or only one of the two bands obtained after SDS PAGE of the proteinase 2 fraction belong to the enzyme. However, it can be concluded that the molecular weight of the *R. appendiculatus* proteinase 2 is approximately between 44,000 and 46,000.

Although most aspartic proteinases have molecular weights of about 35,000 (eg. swine pepsin, M_r 36,000; Huang et al., 1979), there are nevertherless many variations including 100,000 for cathepsin E (Lapresle & Webb, 1960); 30,000 - 90,000 for

cathepsin D (see review by Barrett, 1977); 40,000 - 42,000 for renin (Waldhäusl et al., 1970; Murakami & Inagami, 1975); monomers of 45,000 and disulphide-linked 90,000 dimers for the rodent lymphocyte aspartic proteinase (Yago & Bowers, 1975). The cathepsin D from blowfly pupae had a molecular weight of 41,000 (Kawamura et al., 1987). Our estimates for proteinase fractions 1 and 2 therefore fall within the expected range for aspartic proteinases. To date, none of the work on tick digestive proteinases has included molecular weight determinations.

3.2.3 *Substrate Specificity*

3.2.3.1 *Protein Substrates*

Crude and semicrude preparations of the *R. appendiculatus* gut proteinases hydrolysed Hb and BSA equally well, BSA being hydrolysed slightly better than Hb (fig. 1). With increasing purification and the separation of proteinases 1 and 2 from each other, the ability of the two enzymes to hydrolyse BSA drastically diminished, resulting in rates of hydrolysis (relative to that of Hb) of ~ 32% for proteinase 1 and ~ 28% for proteinase 2 (fig. 2). Both proteinases reached maximum activity when the haemoglobin concentration was 20 $mg.ml^{-1}$. There was no inhibition of either enzyme at higher concentrations of this substrate.

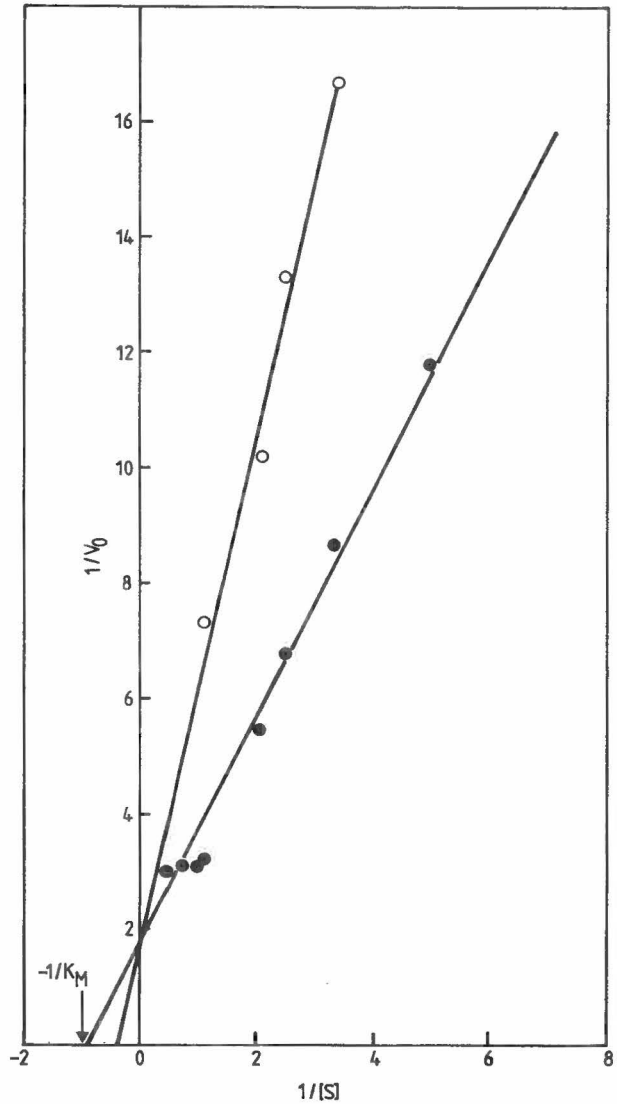

Fig. 9a **Determination of the Michaelis Constant, K_M, and Maximum Velocity, V_{max}, of Aspartic Proteinase 1**

The K_M and V_{max} of proteinase 1 for the hydrolysis of Hb at pH 3.3 and 45 °C as determined by a Lineweaver-Burk transformation. (●——●), normal reaction; (o——o), apparent competitive inhibition by 1nM pepstatin. [s] is expressed in g per 100 ml. n = 6.

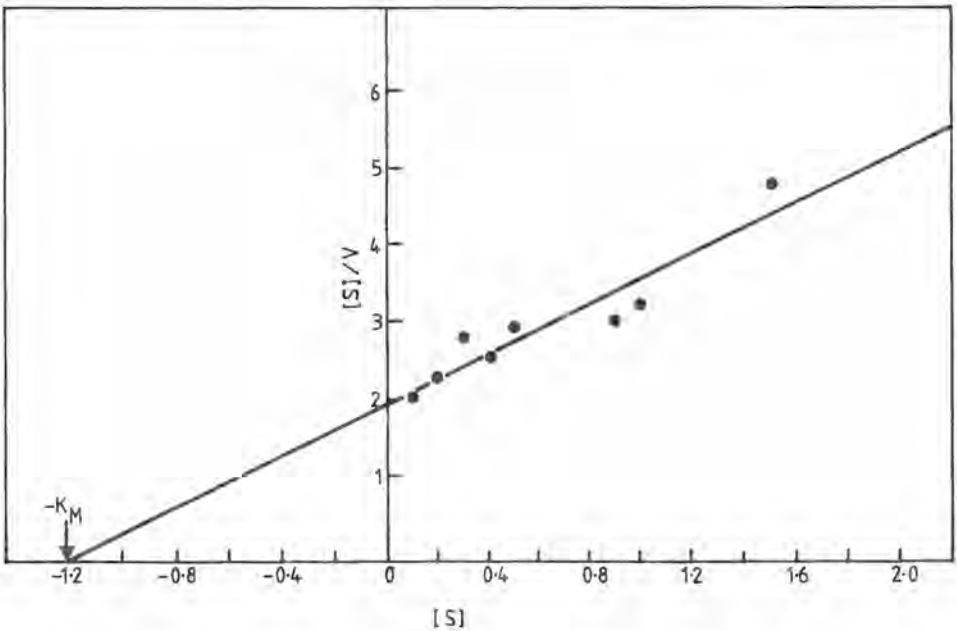

Fig. 9b **Determination of the Michaelis Constant, K_M, of Aspartic Proteinase 1**

The estimation of K_M for the hydrolysis of Hb by aspartic proteinase 1 using [s]/v versus [s] transformation. Details are as for fig. 9a. n = 6.

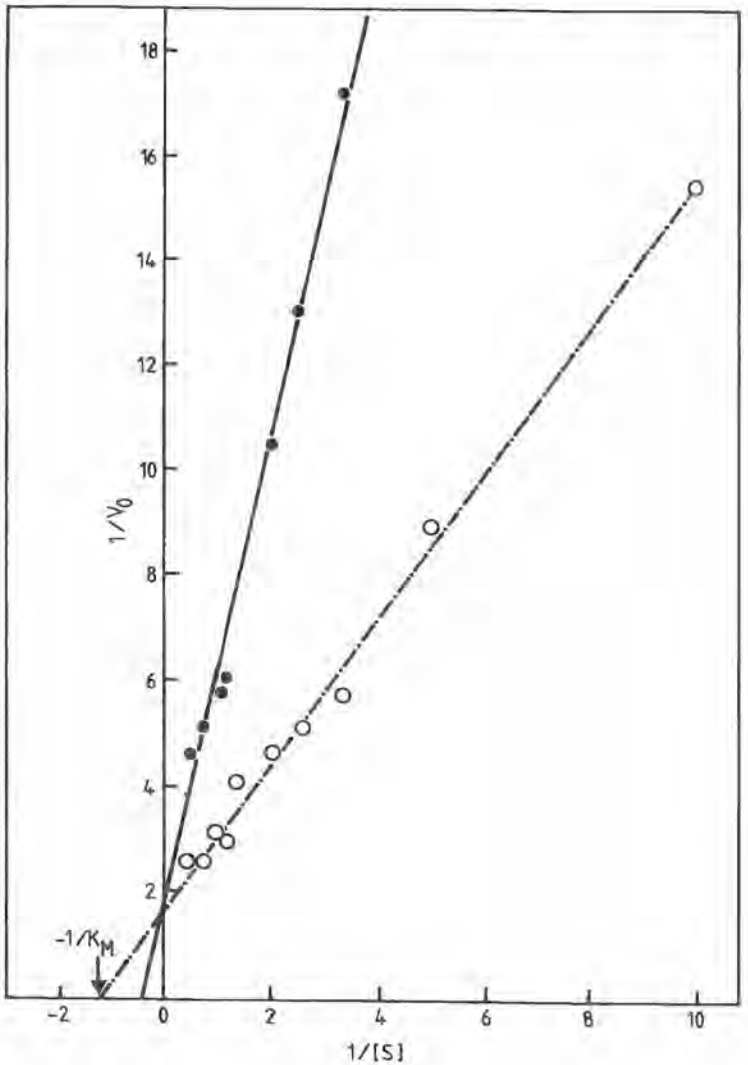

Fig. 10a **Determination of Michaelis Constant, K_M, and Maximum Velocity, Vmax, of Aspartic Proteinase 2**

The K_M and V_{max} of proteinase 2 for the hydrolysis of Hb at pH 3.3 and 45 °C as determined by a Lineweaver-Burk transformation. (o-.-o), normal reaction; (●——●), apparent competitive inhibition by 3 nM pepstatin. [s] is expressed in g per 100 ml. n = 6.

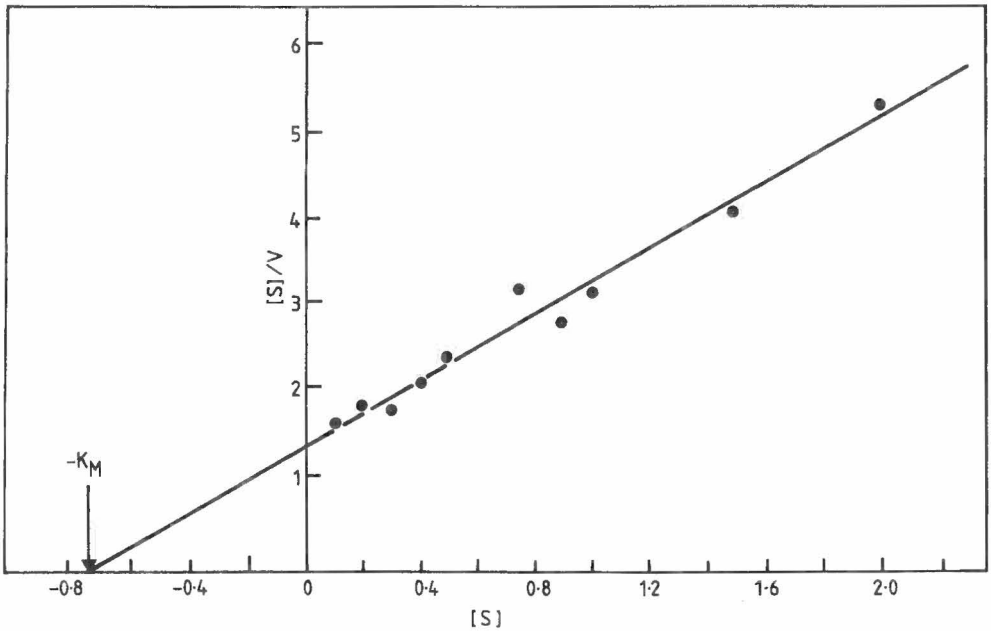

Fig. 10b **Determination of the Michaelis Constant, K_M, of Aspartic Proteinase 2**

The estimation of K_M for the hydrolysis of Hb by aspartic proteinase 2 using [s]/v versus s transformation. Details are as for fig. 9a. n = 6

The Michaelis constant (K_M) for the hydrolysis of haemoglobin by proteinase 1 was determined to be 11.1 $mg.ml^{-1}$ by a Lineweaver-Burk plot (fig. 9a) and 11.9 $mg.ml^{-1}$ by an s/v versus s plot (fig. 9b). The maximum velocity (V_{max}) was 0.556 as determined by the Lineweaver-Burk method (fig. 9a). For proteinase 2, the K_M for haemoglobin hydrolysis was 8 $mg.ml^{-1}$ (fig. 10a) and 7.1 by the s/v versus s plot (fig. 10b). The V_{max} was 0.588 as determined by the method of Lineweaver and Burk.

The results for the relative hydrolysis of serum albumin and haemoglobin differ from those obtained by other workers including Akov et al. (1976). Whereas we have consistently observed equally efficient hydrolysis of BSA and Hb by crude or semi-crude preparations of the *R. appendiculatus* gut proteinase, Akov and her co-workers, also working with crude enzyme preparations reported that for a substrate concentration similar to that used in our study (10 $mg.ml^{-1}$), the *O. tholozani* gut proteinase degraded BSA at only 27% of the rate at which Hb was hydrolysed. This compares well with 22% reported by Houseman and Downe (1982) for an acid proteinase from *R. prolixus* gut, but considerably higher than the 10% reported by Kawamura et al. (1987) for the cathepsin D from the larvae of the blowfly, *A. grahami*. Tatchell (1964) did not compare the relative rates of hydrolysis of the two substrates by *A. persicus* gut proteinase, but he used serum

albumin as the sole substrate in all his studies, an indication that the rate of hydrolysis must have been substantial.

The aspartic proteinases are known to differ in their preferences for protein substrates. It has been shown by many workers that haemoglobin is the best substrate for cathepsin D, serum albumin being degraded at only 5 - 10% of the rate of haemoglobin hydrolysis (see review by Barrett, 1977). In contrast to this, pepsin and cathepsin E degrade the two protein substrates equally well (Barrett, 1967). Renin is similar to cathepsin D in its molecular weight, pI, isoenzyme forms and the fact that both are lysosomal glycoproteins (Cohen et al., 1972). Yet the activity of renin against haemoglobin is 10,000 - fold less than that of cathepsin D (Misono et al., 1974). Instead, renin has a high degree of specificity for a particular -Leu-Leu- bond in angiotensinogen whose hydrolysis leads to the formation of angiotensin. According to Barrett (1977), renin could be regarded as being homologous with cathepsin D but having diverged during evolution to perform its special function.

A similar argument could be advanced for the *R. appendiculatus* aspartic proteinases whose primary function is bloodmeal digestion. Though haemoglobin is the major protein in blood, serum proteins, of which albumin is the most abundant, are also

major constituents. The tick digestive proteinases could therefore have evolved in such a way as to degrade serum proteins just as efficiently as haemoglobin. Moreover, the results of Boctor and Araman (1971) and Tatchell et al. (1972) suggest that in *Argas* spp. plasma protein digestion proceeds during the first two days before the haemoglobin becomes available from lysing erythrocytes. Like renin therefore, the *R. appendiculatus* proteinases could be regarded as having diverged to perform their special function.

The differences between our results and those of Akov et al. (1976) may be due to interspecific variations between *R. appendiculatus* and *O.tholozani* as well as the fact that whereas we used partially engorged mated female *R. appendiculatus*, Akov et al. (1976) used fully engorged, unmated females. This may therefore be a reflection of the species and stage of ticks used in the respective studies.

Although our observations with crude gut homogenates differed from those of previous workers, the pattern changed after anion exchange chromatography, when proteinases 1 and 2 hydrolysed BSA at only 32 and 28% of the rate of haemoglobin hydrolysis respectively, thus becoming similar to the observations of Akov et al. (1976). This reduction in the ability to hydrolyse BSA by

the two proteinases may be associated with the equally drastic loss in activity observed after this purification step (see section 3.1) and the accompanying inability to hydrolyse the synthetic substrate Ace-Phe-DIT, despite some hydrolysis by the less pure fractions (section 3.2.3.2).

Akov et al. (1976) reported maximum activity for *O. tholozani* gut proteinase when the haemoglobin concentration was 5 $mg.ml^{-1}$, which differs from the 20 $mg.ml^{-1}$ obtained for both proteinases 1 and 2 in this study. Neither *R. appendiculatus* proteinase was inhibited by high concentrations of haemoglobin as reported for cathepsin D and an acid proteinase from rat spleen by Yamamoto et al. (1978). The present results are in this respect, similar to those obtained for pig myometrium cathepsin D by Barth and Afting (1984). To the best of our knowledge, this aspect has not been considered by any of the other studies on insect and tick acid proteinases. For BSA, Reich and Zorzopulos (1978) obtained maximum activity of *B. microplus* larval acid proteinase when the substrate concentration was 8 $mg.ml^{-1}$. Higher substrate concentrations inhibited the enzyme. Similar results were obtained by us for the hydrolysis of BSA by a mixture of *R. appendiculatus* proteinases 1 and 2 (data not shown) in which maximum enzyme activity was attained when the concentration of BSA was 10 $mg.ml^{-1}$ and as in the *B. microplus* studies, higher

substrate concentrations inhibited the enzyme.

The K_M and V_{max} for the hydrolysis of haemoglobin by proteinases 1 and 2 were analysed by two methods so as to achieve a good estimate of the two parameters. A wide variety of K_M values have been reported for the hydrolysis of haemoglobin by acid proteinases from different sources. These include 1.9 $mg.ml^{-1}$ for an enzyme from rat spleen (Yamamoto et al., 1978), ~ 0.413 $mg.ml^{-1}$ for pig myometrium cathepsin D (Barth & Afting, 1984) and 3.7$mg.ml^{-1}$ for cathepsin D from muscle of the fish *Mujil auratus* (Bonete et al., 1984). These values are different from those reported in the present study though the values for the rat spleen and fish muscle enzymes are within the same order of magnitude as those for the *R. appendiculatus* proteinases. None of the previous studies on tick gut proteinases had included a determination of the K_M for haemoglobin. However Tatchell (1964), obtained a K_M of 3.2 $mg.ml^{-1}$ for the hydrolysis of BSA by *A. persicus* gut proteinase. This value compares well with 4.04 $mg.ml^{-1}$ reported by Lapresle and Webb (1960) for the hydrolysis of BSA by rabbit spleen cathepsin D, and both are closer to our values for the hydrolysis of haemoglobin by the *R. appendiculatus* proteinases. It must, however, be pointed out that the K_M values obtained for these protein substrates are not true kinetic constants since each represents an average value for the

hydrolysis of several bonds, occurring at different velocities. They are however, useful for the purposes of comparison between similar enzymes from different sources.

3.2.3.2 *Synthetic Substrates.*

Enriched *R. appendiculatus* proteinases 1 and 2 did not hydrolyse the pepsin substrate, Ace-Phe-DIT, although there was very slight hydrolysis by the crude enzyme (results not shown). Likewise, a wide variety of serine and cysteine proteinase substrates (2.3.10.3) were not hydrolysed under optimum assay conditions. These included BApNA, TAME, ATEE, BTpNA, HA and LpNA (2.3.10.3).

The inability of the *R. appendiculatus* aspartic proteinases to hydrolyse the substrates of serine and cysteine proteinases was as expected, while the lack of hydrolysis of Ace-Phe-DIT is an indication that these particular aspartic proteinases are closer to cathepsin D than they are to pepsin (Press et al., 1960).

3.2.4 *Effect of Inhibitors*

3.2.4.1 *Inhibition by Pepstatin*

The effect of pepstatin on the hydrolysis of haemoglobin by proteinase 1 is shown in figure 11. The inhibitor had the same effect on the enzyme at the 3 different conditions of pH used in

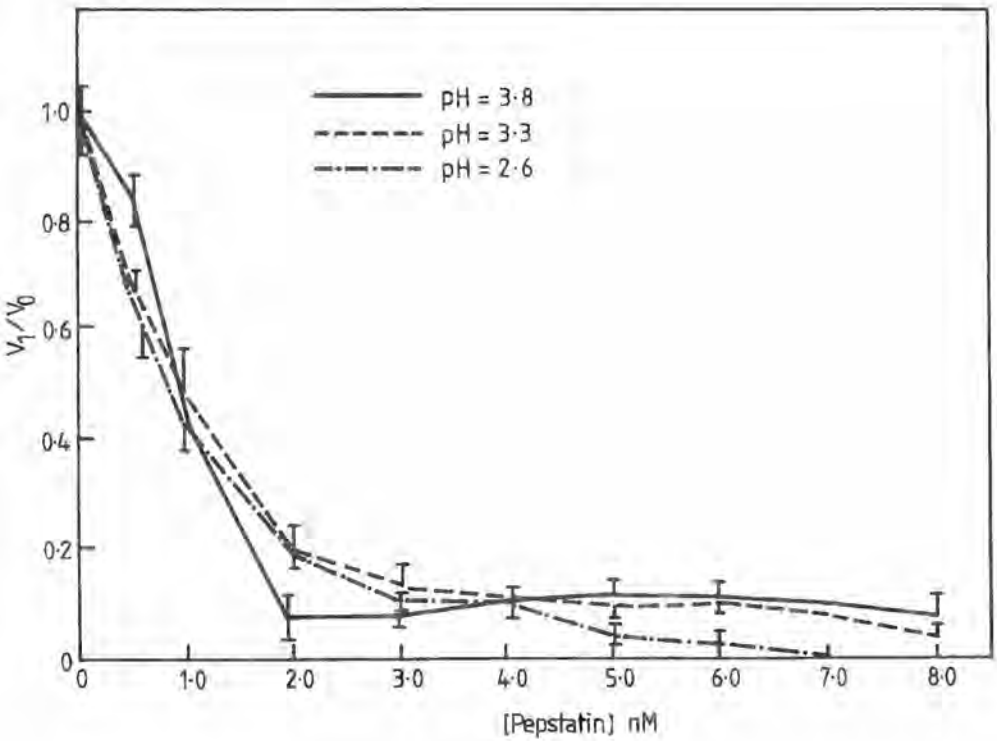

Fig. 11 **Inhibition of Aspartic Proteinase 1 by Pepstatin**

Proteinase 1 was assayed in the presence of increasing concentrations of pepstatin and 20 mg Hb per ml under different conditions of pH. V_1 = treated enzyme; V_0 = untreated enzyme. Each point respresents the mean±SEM; n = 6.

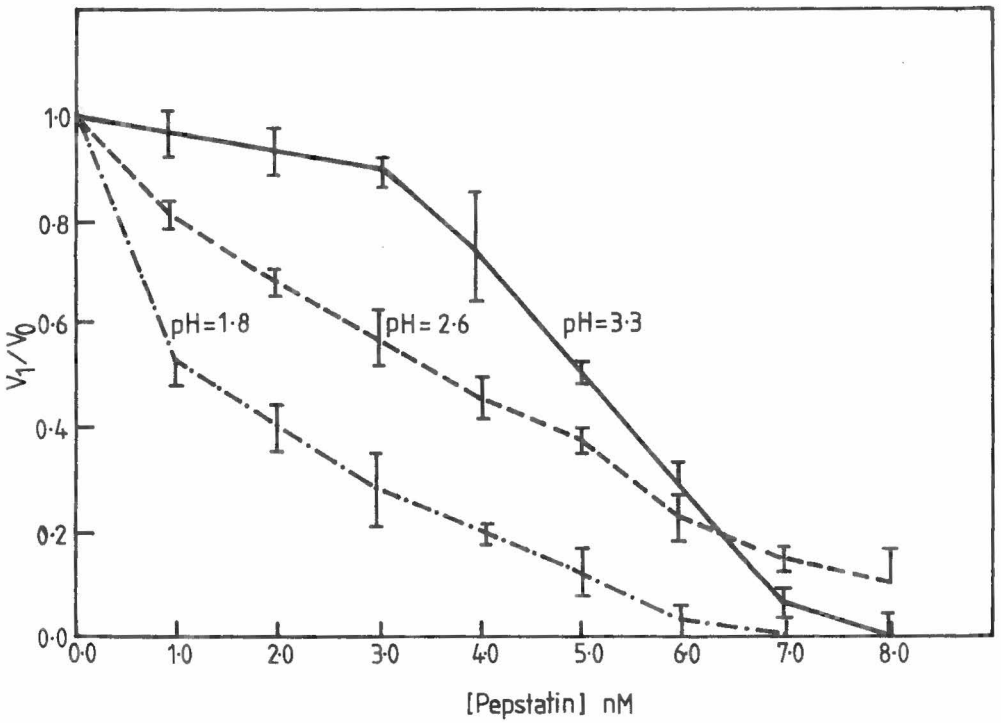

Fig. 12 **The Inhibition of Aspartic Proteinase 2 by Pepstatin**

Proteinase 2 was assayed in the presence of increasing concentrations of pepstatin and 20 mg Hb per ml under different conditions of pH. V_1 = treated enzyme; V_0 = untreated enzyme. Each point represents the mean±SEM; n = 6.

the experiment. In contrast to this, the inhibition of proteinase 2 by pepstatin was strongly pH-dependent (fig. 12). For each concentration of pepstatin, inhibition decreased with increasing pH. At pH 3.3, pepstatin concentrations below 3nM had very little effect on enzyme activity in contrast to the marked inhibition observed at pH 1.8 and 2.6. Regardless of the assay pH, proteinase 1 was much more sensitive to pepstatic inhibition than proteinase 2. The inhibition of both proteinases 1 and 2 was apparently competitive since in both cases the inhibitor raised the K_M while the V_{max} was unaltered (figs. 9a and 10a respectively). Figure 13 shows the Dixon plot of 1/V versus I from which a K_I of 1.7×10^{-9}M for proteinase 2 was calculated.

The powerful inhibition of proteinases 1 and 2 by pepstatin identifies the two *R. appendiculatus* enzymes as aspartic proteinases. Pepstatin is a specific inhibitor for this class of enzymes (see review by Umezawa & Aoyagi, 1977).

Barth and Afting (1984) reported a lack of pH dependence on the inhibition of pig myometrium cathepsin D by pepstatin, similar to the observations made for proteinase 1 in the present study. The strong pH dependence observed for the inhibition of proteinase 2 is, however, in agreement with observations made for cathepsin D (Knight & Barrett, 1976; Afting & Becker, 1981) and renin

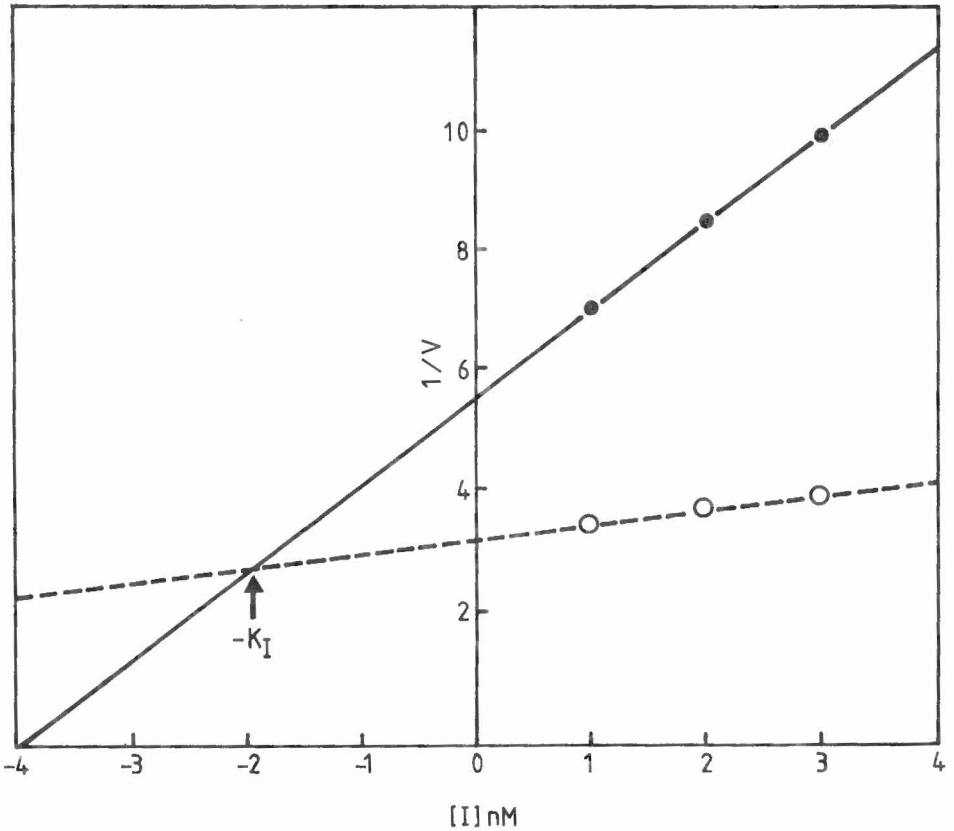

Fig. 13 **Determination of the Inhibition Constant, K_I, of Pepstatin for Aspartic Proteinase 2**

Dixon transformation of 1/v versus [I]. Assays with 5 mg Hb per ml (●—●), and 10 mg Hb per ml (0---0 were done at pH 3.3. n = 4.

(Murakami et al., 1973). The phenomenon has infact been exploited by various workers in the purification of the two enzymes (Murakami et al., 1973; Kregar et al., 1977; Afting & Becker, 1981). The difference between proteinases 1 and 2 in the effect of pH on their inhibition by pepstatin is not unusual since as mentioned above, evidence points to variations in this phenomenon among the aspartic proteinases. Bonete et al. (1984) did not investigate the effect of pH on the inhibition of *M. auratus* muscle cathepsin D, but they observed that 1.3 nM pepstatin strongly inhibited the enzyme at pH 4.0. In the present study, the same inhibitor concentration was similarly effective against proteinase 1 at pH 3.8, but had little effect on proteinase 2 at pH 3.3.

The competitive nature of the inhibition of the *R. appendiculatus* proteinases by pepstatin was as expected, since it is known to be a competitive inhibitor of the aspartic proteinases (Kunimoto et al., 1974), thought to bind at the active site of the enzyme (Aoyagi et al., 1972). The K_I values obtained for the inhibition of haemoglobin hydrolysis by the two *R. appendiculatus* enzymes are in good agreement with the value of 2.1×10^{-9}M reported for pig myometrium cathepsin D by Barth and Afting (1984).

3.2.4.2 *Other Inhibitors*

R. appendiculatus proteinases 1 and 2 were unaffected by several inhibitors of trypsin and chymotrypsin-like enzymes including soybean trypsin inhibitor (STI), aprotinin, N-p-tosyl-L-lysine chloromethyl ketone (TLCK), tosyl-phenylalanyl chloromethyl ketone (TPCK) and EDTA. Contrary to expectations however, both enzymes were inhibited by phenylmethylsulphonyl fluoride (PMSF). At pH 3.3, 1mM PMSF reduced the activity of proteinase 1 to 25% and that of proteinase 2 to 50% of the respective uninhibited reactions (fig. 14).

Aspartic proteinases are generally unaffected by most inhibitors of serine and cysteine proteinases or by chelating agents (Barrett, 1977). Nevertherless, many exceptions similar to our observations with the *R. appendiculatus* proteinases and PMSF have been reported. Bogin and Hadani (1973) reported the inhibition of *H. excavatum* gut aspartic proteinase by L-cysteine and mercaptoethanol, and slight inhibition by 1mM PMSF while Marks and Lajtha (1965) observed the inhibition of an aspartic proteinase from rat brain by TPCK. TLCK and mercaptoethanol inhibited an acid proteinase from the gut of a hemipteran, *R. prolixus* (Houseman & Downe, 1982). Rabbit cathepsin D was rapidly inactivated when preincubated with 1mM dithiothreitol at pH 8.0 (Barrett, 1967) while the cathepsin D from pig myometrium was

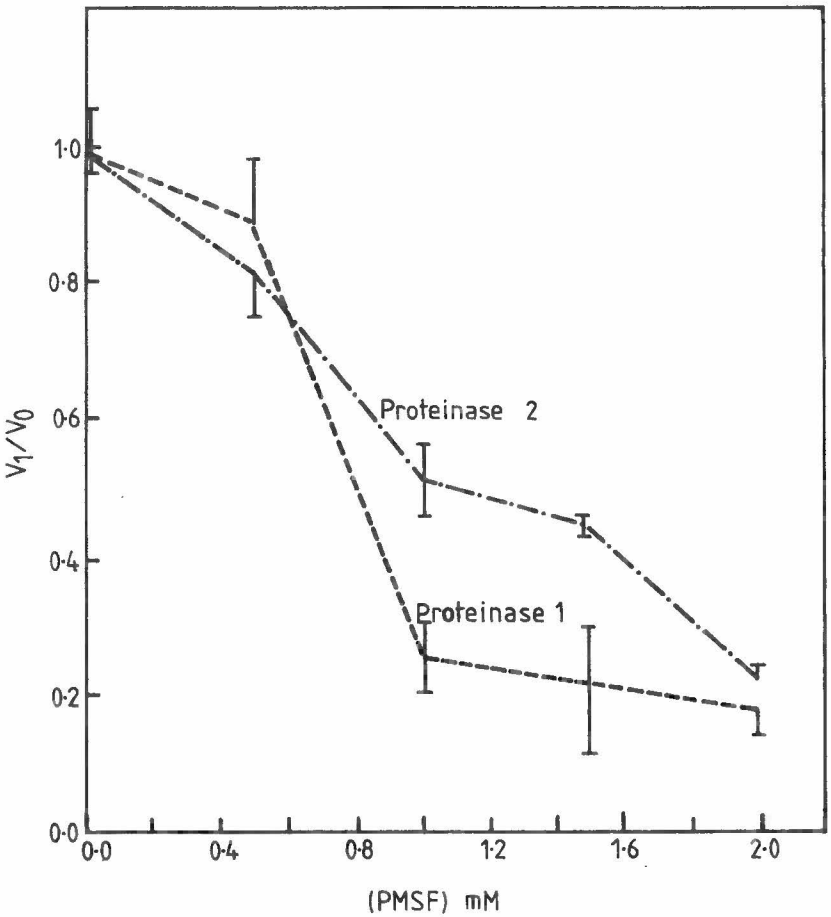

Fig. 14 **Inhibition of Aspartic Proteinases 1 and 2 by PMSF**

Proteinases were assayed with increasing concentrations of PMSF at 20 mg of Hb per ml and pH 3.3. V_1 = treated enzyme; V_0 = untreated enzyme. Each point represents the mean±SEM. n = 6.

slightly inhibited by PMSF, TPCK and STI (Barth & Afting, 1984). There is therefore no uniformity among the aspartic proteinases with regard to possible inhibition by some of the inhibitors discussed above.

As mentioned in the introduction, aspartic proteinases contain intra-chain disulphide bridges, thought to be important in the stabilisation and conformation of the enzyme molecule (Northrop, 1930; Barrett, 1971). Barrett (1977), suggested that the inhibition of aspartic proteinases by thiol compounds such as dithiothreitol may be due to the reduction of one or more strained disulphide bridges in the enzyme molecule. Perhaps such a strained disulphide bridge or a free cysteine may react with PMSF, resulting in enzyme inhibition.

The inhibition of *R. appendiculatus* proteinases by 1mM PMSF is perhaps not remarkable, considering that the inhibitor concentration is really quite high. What is important however, is that 1mM PMSF is routinely used by different workers as a protease inhibitor during the purification of aspartic proteinases. In view of our observations, one would be wise to test this inhibitor against their proteinase of interest before including it in their purification system.

3.2.5 *Effect of pH*

The effect of pH on the hydrolysis of haemoglobin by proteinases 1 and 2 is shown in figure 15a. Proteinase 1 was optimally active at pH 3.0, with a distinct, though much smaller second peak at pH 4.6. Under the same conditions, the activity of proteinase 2 was optimal at pH 2.6, with a second smaller peak at pH 5.0. At pH 1.8 the activity of proteinase 1 was only 17.6% of its activity at optimum pH, while proteinase 2 had a residual activity of 78% at the same pH, suggesting that the latter is able to withstand very low conditions of pH better. On the more basic side of the curve we observed a consistent dramatic drop in activity between pH 3.8 and 4.0 for both proteinases. Neither enzyme was active at pH 6.0 or above.

The pH optima for the hydrolysis of haemoglobin by the two *R. appendiculatus* enzymes are in agreement with those reported by other workers for this class of proteinases. Tatchell (1964) reported two peaks of activity for the *A. persicus* gut proteinase, a major peak at pH 2.6 and a subsidiary one at pH 3.8, both falling within a broad range of high activity (pH 2 - 4.5). For the hard tick, *H. excavatum*, Bogin and Hadani (1973) obtained a similar broad region of high activity between pH 2.8 and 3.2, with a peak at pH 2.9. The gut proteinase from *O. tholozani* was maximally active at pH 2.7 - 3.3 (Akov et al.,

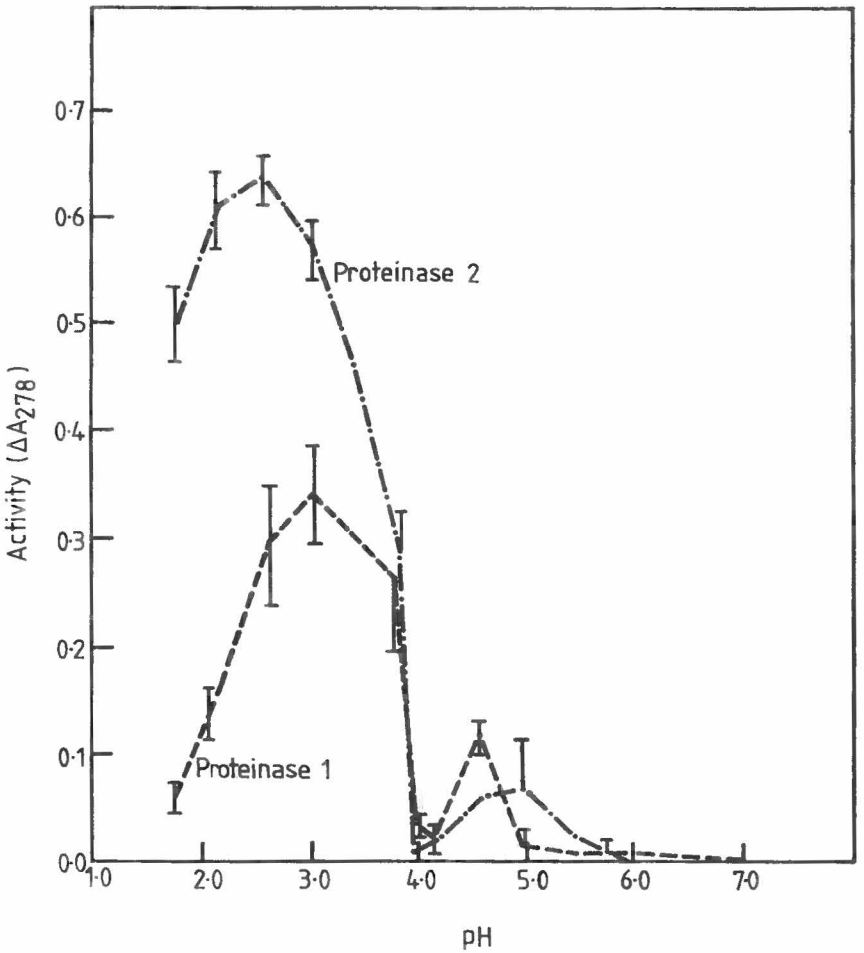

Fig. 15a **Determination of the pH Optima for Aspartic Proteinases 1 and 2**

Each proteinase was assayed at increasing pH with 20 mg Hb per ml. Each point represents the mean±SEM. n = 5.

1976) while the *B. microplus* enzyme had its optimum at pH 3.0 - 3.5 (Reich & Zorzopulos, 1978). Acid proteinases from various insect species also had comparable pH optima (Okasha, 1968; Smith & Birt, 1971; Pendola & Greenberg, 1975; Houseman, 1978). Similar pH optima have been reported for other aspartic proteinases including pH 2.8 - 4.0 for cathepsin D from various sources (see review by Barrett, 1977); pH 2.5 for cathepsin E (Keilova & Lapresle, 1970); 2.6 for penicillopepsin (Takahashi & Hofmann, 1975) and 1.7 for porcine pepsin (Christensen, 1955). The broad pH optima reported for the various tick species are most probably due to the fact that as in *R. appendiculatus*, at least two aspartic proteinases having slightly different pH optima are involved. A similar broad pH peak was obtained for *R. appendiculatus* when a mixture of proteinases 1 and 2 (G-100 eluate) was assayed (fig. 15b).

A second pH peak similar to those observed for each of the *R. appendiculatus* proteinases was reported for *A. persicus* by Tatchell (1964), and for the dipteran, *L. cuprina* by Smith and Birt (1971). This bimodal nature is well documented for cathepsin D and has been reported for the chicken and human enzymes (Barrett, 1971), the porcine spleen enzyme (Cunningham & Tang, 1976) and for bovine uterus cathepsin D (Woessner & Shamberger, 1971). It has been shown to be the property of a single enzyme

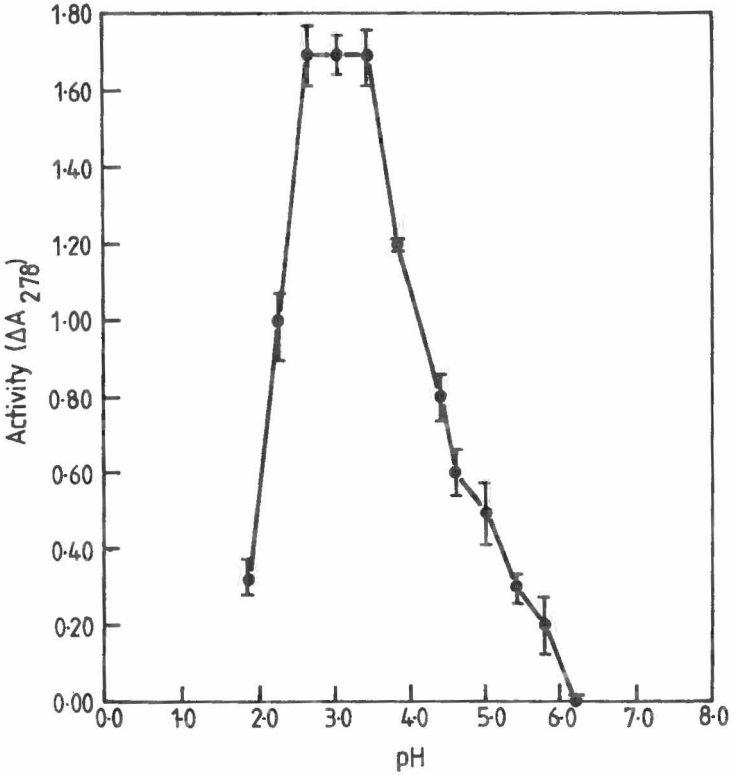

Fig 15b **Determination of the pH Optimum of a Mixture of Aspartic Proteinases 1 and 2**

The proteinase fraction after G-100 chromatography (proteinases 1 and 2 are unseparated) was assayed at increasing pH with 20 mg Hb per ml. Each point represents the mean±SEM. n = 4.

species and is not due to changes in the substrate (Cunningham & Tang, 1976).

In terms of pH optimum and its ability to withstand very low conditions of pH, the *R. appendiculatus* proteinase 2 is rather similar to pepsin and cathepsin E while proteinase 1 is more like cathepsin D. But unlike pepsin which is irreversibly denatured above pH 6.0 (Steinhardt, 1936), both *R. appendiculatus* proteinases, though inactive at pH 6.0 and above are nevertheless not denatured. Proteinase 2 was infact rendered more stable to thermal inactivation at conditions of relatively high pH (see 3.2.6).

3.2.6 *Thermal Stability and Stability to Storage*

The results obtained when proteinases 1 and 2 were incubated at different temperatures are shown in figure 16. Both enzymes lost activity rapidly at 50 °C and above. But the pH had an effect on the thermal inactivation of proteinase 2, the enzyme being more susceptible when incubated at pH 3.3. Hence, after incubation at 65 °C and pH 3.3, proteinase 2 had only 12% of the original activity compared to 62% for the same temperature at pH 7.4. pH had no effect on the thermal stability of proteinase 1. Some conditions of preincubation (35 - 45 °C) led to enzyme activation.

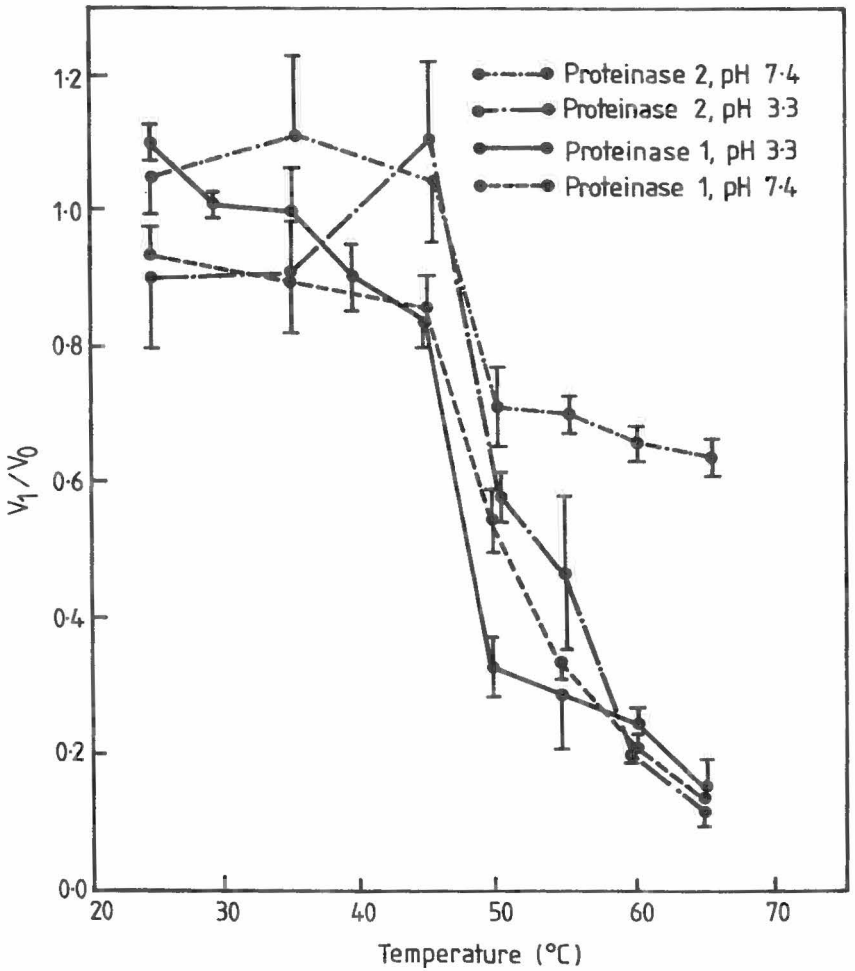

Fig. 16 **Determination of the Thermal Stability of Aspartic Proteinases 1 and 2**

Each enzyme fraction was preincubated for 10 min at the respective temperature and pH and then brought to 4 °C. Assays were at 45 °C and 20 mg Hb per ml. V_1 = treated enzyme; V_0 = untreated enzyme. Each point represents the mean±SEM. n = 5.

On storage, proteinases 1 and 2 were relatively unstable, losing more than 30% of their initial activity when stored for up to 15 days at 4 °C. The two proteinases were even more unstable when stored frozen at -20 °C, and were extremely unstable in the presence of glycerol at either temperature. The best condition for the storage of the two *R. appendiculatus* proteinases was therefore found to be 4 °C at pH 7.4 (table 2).

The progressive loss in enzyme activity above 50 °C observed in this study has been reported for other proteinases of this class including cathepsin D preparations from *M. auratus* muscle (Bonete et al., 1984) and pig myometrium (Barth & Afting, 1984) and for the mouse submaxillary gland renin (Reinharz & Roth, 1969). But as observed in our study, pH is an important factor in the thermal stability of some of these enzymes. Thus, cathepsin D from porcine myometrium (Barth & Afting, 1984) and spleen (Cunningham & Tang, 1976), penicillopepsin (Sodek & Hofmann, 1970), pepsin (Herriot, 1941) and an enzyme from rat spleen (Yamamoto et al., 1978) were all more temperature stable at intermediate or slightly alkaline pH. The mechanism involved in this phenomenon is not known but for pepsin it has been shown that the enzyme is stabilised by a peptide at pH values above 4.0 (Herriot, 1941), and evidence points to a similar mechanism for

Table 2 **The Effect of Storage on Proteinases 1 and 2**

	Proteinase Activity (V_1/V_0)			
Time (day)	0	5	10	15
Treatment				
			Proteinase 1	
4 °C	1	0.69±0.02	0.64±0.03	0.58±0.01
4 °C + Glycerol	0.55±0.03	0.33±0.01	0.26±0.03	0.24±0.03
-20 °C	-	0.25±0.05	0.21±0.02	0.19±0.02
-20 °C + Glycerol	-	0.10±0.01	0.07±0.01	0.04±0.01
			Proteinase 2	
4 °C	1	0.74±0.02	0.66±0.01	0.63±0.01
4 °C + Glycerol	0.74±0.05	0.30±0.03	0.23±0.01	0.14±0.01
-20 °C	-	0.55±0.07	0.56±0.03	0.49±0.04
-20 °C + Glycerol	-	0.36±0.03	0.22±0.03	0.19±0.01

Proteinases 1 and 2 were assayed for hydrolysis of Hb after storage as described. V_1 = treated enzyme; V_0 = untreated enzyme. Each value = mean±SEM. n = 3.

cathepsin D (Puizdar & Turk, 1981).

Storage conditions that are optimum appear to vary depending on the particular aspartic proteinase. Thus, while rabbit cathepsin D was very stable when frozen or kept in glycerol at -20 °C (Barrett, 1977), both *R. appendiculatus* proteinases rapidly lost activity when stored under similar conditions. In this respect the two tick proteinases are similar to chicken cathepsin D which is unstable to freezing at pH 7.0 and is completely inactivated when frozen at pH 8.0. Freezing at pH 8.0 also caused some inactivation of human cathepsin D (Barrett, 1977). Unlike blowfly pupal cathepsin D which was unstable when stored above pH 6.5 (Kawamura et al., 1987), the *R. appendiculatus* proteinases were quite stable at pH 7.4 when stored at 4 °C. However, like the blowfly enzyme, the tick proteinases rapidly lost activity in the presence of glycerol. Proteinases 1 and 2 therefore resemble some aspartic proteinases in their behavior under different conditions of storage.

3.3 ***Immunoinhibition***

3.3.1 *Immunogenicity of proteinase fractions 1 & 2*

The immunisation of rabbits with each of the three enzyme preparations resulted in the production of high titre antibodies, each of which cross reacted with each of the three antigen

preparations (fig. 17). The antibodies to enriched fractions 1 and 2 reacted with both fractions as demonstrated by immunoblotting (fig. 18).

That poteinase fractions 1 and 2 proved to be good immunogens was not surprising, since similar results have been reported for cathepsin D preparations from chicken, rabbit and man (Weston, 1969; Dingle et al., 1971; Dingle et al., 1973). As in these cases, potent antisera were raised by the injection of relatively small quantities of each of the *R. appendiculatus* proteinase preparations.

3.3.2 *The in vitro inhibition of proteinases 1 and 2 by antisera*
The effect of the three antisera on the activity of proteinases 1 and 2 *in vitro* is summarised on fig. 19. Anti-proteinase fraction 1 significantly inhibited both proteinases 1 and 2 ($p<0.05$) as did anti-proteinase fraction 2 ($p<0.05$) and serum raised against the G-100 fraction ($p<0.01$). The antiserum to the G-100 fraction was a better inhibitor of both proteinases than their own sera ($p<0.05$). Proteinase 2 was more susceptible to *in vitro* inhibition than proteinase 1 ($p<0.05$). In figure 20, a breakdown of the results shown on figure 19 has been made to show the effect of antisera from individual rabbits on proteinases 1 and 2. There were large individual variations between rabbits.

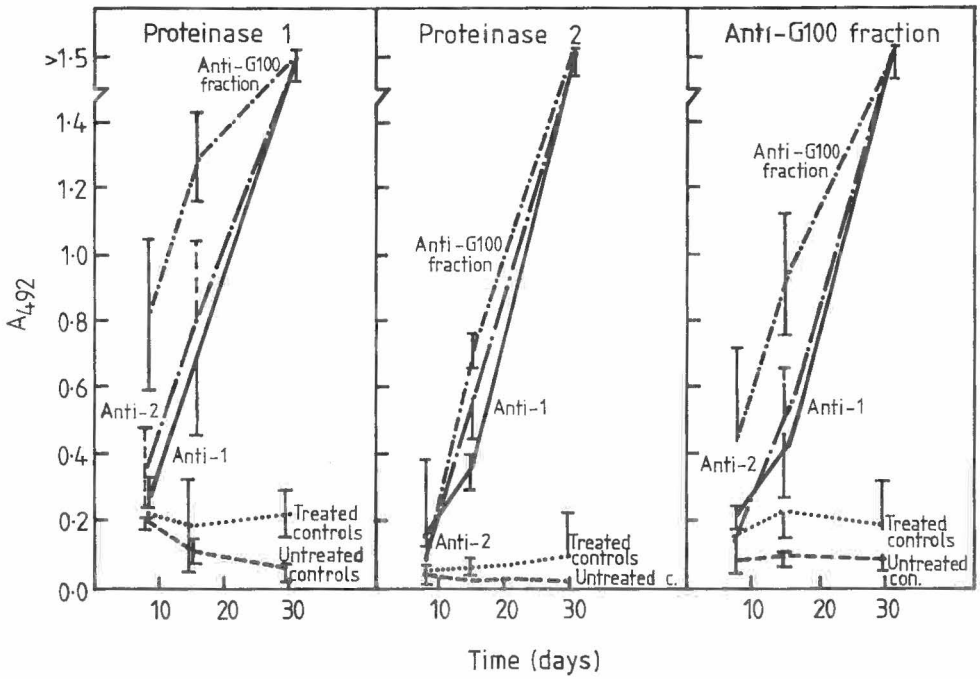

Fig. 17 **The Production of Antibody (IgG) by Rabbits Immunised With Aspartic Proteinase Fractions**

Antibody titres in rabbits immunised with enriched aspartic proteinase fraction 1, 2, or the G-100 fraction were tested against each of the three protein preparations by ELISA, as were treated and untreated controls. Each point represents the mean±SEM. n = 3.

Fig. 18 **Immunological Reactions of Enriched proteinases 1 and 2**

Approximately 0.4µg of proteinase fraction 1 and 0.3µg of proteinase fraction 2 were subjected to isoelectrofocusing at pH 3.5 - 9.5 and -12 °C. The proteins were then electrophoretically transferred to nitrocellulose paper and reacted with the corresponding antiserum.

1 - Proteinase fraction 1

2 - Immunoblot of proteinase fraction 1

3 - Proteinase fraction 2

4 - Immunoblot of proteinase fraction 2

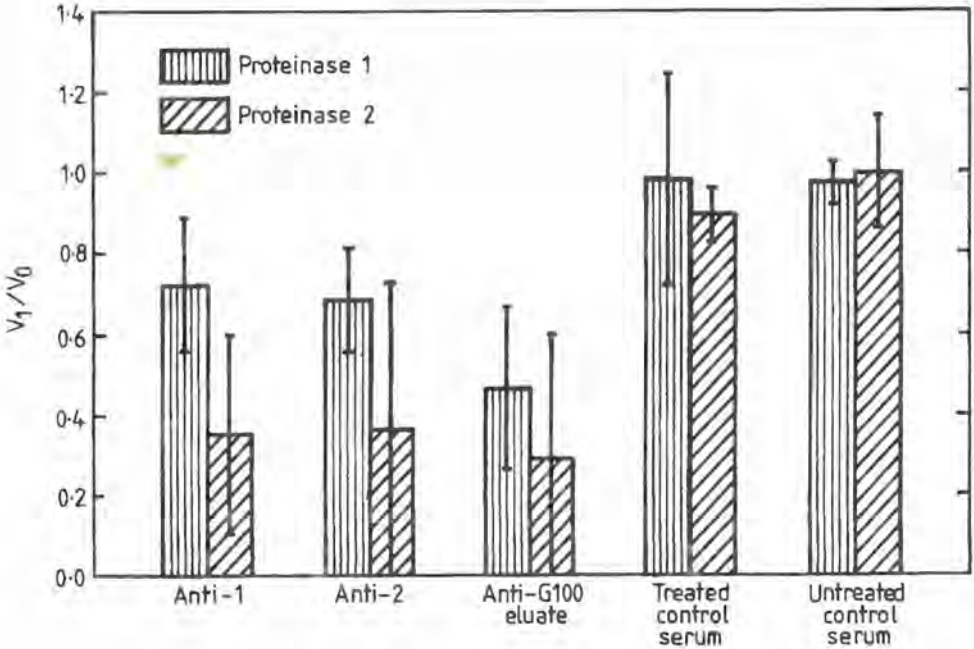

Fig. 19 **The Inhibition of Proteinases 1 and 2 by antisera *in vitro***

Each of the three enzyme preparations (enriched proteinase 1, enriched proteinase 2, the G-100 fraction) were assayed in the presence of each of the three antisera, and the two control sera, with 20 mg Hb per ml as substrate. Each histogram represents the mean±SEM. V_1 = reaction with serum; V_0 = reaction without serum. n = 3.

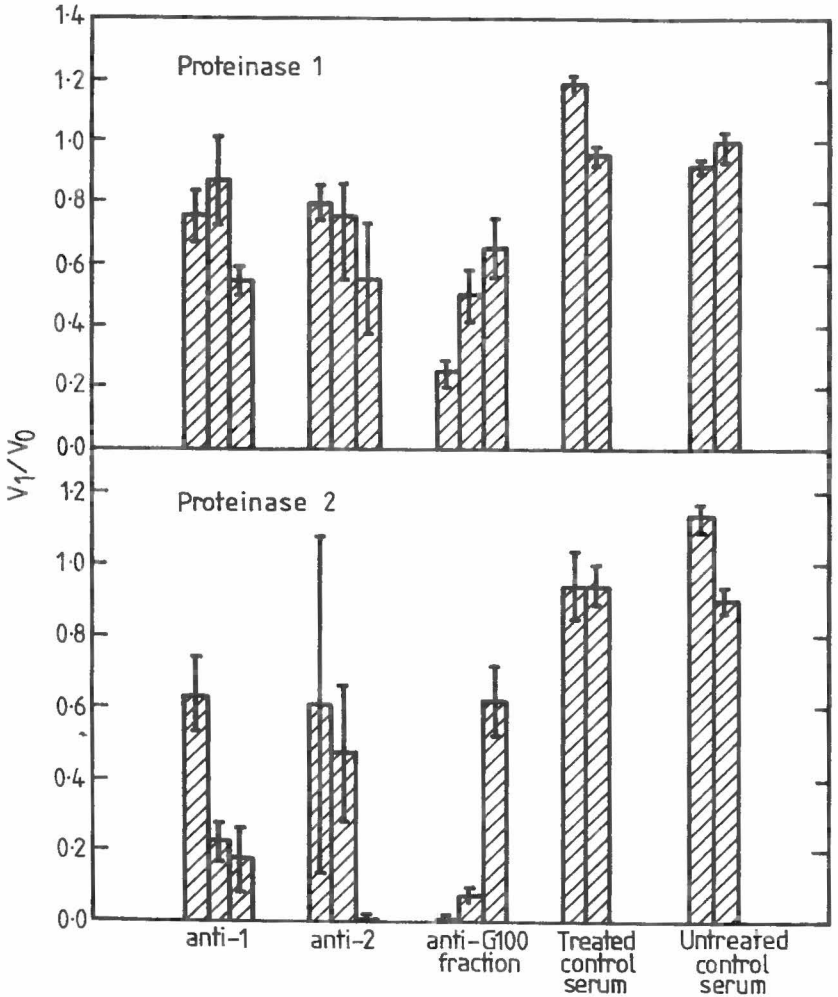

Fig. 20 **The Inhibition of Proteinases 1 and 2 by Antisera *in vitro***

A breakdown of the results shown on fig. 19. Each histogram represents the mean±SEM of a single serum tested with different enzyme preparations. V_1 = reaction with serum; V_0 = reaction without serum. n = 3.

Control sera had little or no effect on either proteinase.

The inhibition of aspartic proteinases by the corresponding antiserum in an *in vitro* system similar to that observed in the current study was reported by Dingle et al. (1971) for the autolysis of cartilage by cathepsin D. That the serum to the G100 fraction proved to be a more efficient inhibitor than the antisera to the partially purified enzymes may be an indication of important factors which are lost during purification. The greater susceptibility of proteinase 2 to inhibition by all three sera can be explained by its molecular weight. Immunoinhibition is normally the result of steric blockage of the enzyme catalytic site by surrounding antibody molecules (Cinader & Lafferty, 1964; Arnon, 1967; Dingle, 1971). Since proteinase 2 is so much smaller than proteinase 1, it would take fewer antibody molecules to surround it, thereby blocking it from its substrate.

It must be pointed out that the data presented here represent averages of three antisera in each case. There is however, a great deal of individual variation between sera (as shown by the large standard errors and also by fig. 20).

3.3.3 *The effect of immunised rabbits on tick biology*

The results obtained when immunised rabbits were challenged with ticks are summarised in table 3. The immunisation of rabbits with the three enzyme preparations had no effect on the biology of the tick.

The lack of effect of antibody in an *in vivo* system similar to the one in the current study may be attributed to some or all of the following factors:

a/ Low diffusibility of antibody

b/ Diluting effect

c/ Continuous flow of enzyme from primary to secondary lysosomes

d/ The effect of substrate

e/ The involvement of other enzymes

f/ Hydrolysis of the immunoglobulins by the lysosomal proteases

g/ Homeostasis

h/ The dissociation of antibody-antigen complexes at low pH.

One of the major drawbacks to the use of antibodies as enzyme inhibitors in *in vivo* systems is their poor diffusibility which makes it difficult to achieve the local concentrations necessary for efficient inhibition. This would however not be a limiting factor in the current system since the antibody would be taken up into gut lysosomes with the bloodmeal.

Table 3 **The effect of immunised rabbits on female ticks**

Variables	Anti-1	Anti-2	Anti-G100F	C/T	C/U
	Mean ± SEM (n)				
Drop-off (day)	7.9±0.3 (39)	7.9±0.1 (53)	8.1±0.2 (43)	8.0±0.2 (51)	8.3±0.3 (44)
Eng. wt (mg)	290±130 (39)	280±10 (53)	250±13 (43)	260±130 (51)	230±20 (44)
Egg-laying (day)	4.2±0.1 (36)	4.2±0.1 (53)	4.2±0.1 (38)	4.1±0.1 (42)	4.7±0.2 (33)
E/batch wt (mg)	160±10 (36)	160±10 (53)	130±10 (38)	160±10 (42)	120±10 (33)
ECF	0.5±0.02 (36)	0.6±0.01 (53)	0.5±0.02 (38)	0.6±0.01 (42)	0.5±0.03 (33)

Female ticks were monitored individually for each parameter after attachment to the respective rabbit. The results are pesented as the mean±SEM. The number of observations (ticks) for each treatment is shown in parenthesis. C/T and C/U = treated and untreated controls respectively. There were no significant differences between the different treatements for any of the parameters examined.

The diluting effect is however, a probable factor in the current system. A study of similar inhibition as a function of the antibody:antigen ratio showed that for cathepsin D, the binding of about 6 antibody molecules per enzyme molecule was necessary for complete inhibition, consistent with a steric mechanism of inhibition (Dingle et al., 1971). Coupled to this is the fact that the aspartic proteinases are thought to be major digestive enzymes in the tick gut (Akov, 1982). A large excess of antibody would therefore be required to neutralise all the enzyme. In addition to this, it has been shown in *in vitro* enzyme systems that there is often residual enzyme activity even when sufficient antibody is present, and evidence points to a system that is more complex than simple steric mechanism alone (Cinader, 1967).

The continuous flow of enzyme from primary to secondary lysosomes may also be a factor in the present system. Dingle et al. (1973) inhibited the intracellular digestion of sheep IgG, haemoglobin, and proteoglycan in living rabbit alveolar macrophages. Only about 42% inhibition was attained however, and the authors attributed this partly to the continuous flow of cathepsin D from primary to secondary lysosomes, thus necessitating a large excess of antibody for complete inhibition. This is a very likely system in the *R. appendiculatus* gut in which digestion occurs over several days (Coons et al., 1986).

According to Cinader (1967), antibody is less effective as enzyme inhibitor when combining with enzyme in the presence rather than in the absence of substrate. In our *in vitro* studies antibody and enzyme were preincubated before the addition of substrate. In the *in vivo* system however, the antibody combines with enzyme in the presence of a very large excess of substrate. This could have resulted in reduced efficiency of the antibody.

The involvement of proteinases other than the aspartic proteinases in the tick digestive system is yet another likely factor. These would not be inhibited by sera raised against fractions enriched for proteinases 1 and 2. When Dingle et al. (1973) obtained only 42% inhibition in their rabbit alveolar system, they attributed this partly to the involvement of proteinases other than cathepsin D.

Since immunoglobulins are proteins, it is likely that they would be hydrolysed by the lysosomal proteases. This could therefore be a factor in the failure of antisera to proteinase fractions 1 and 2 to affect tick digestion.

The dissociation of antibody-antigen complexes at low pH is well documented (see review by Barrett, 1977). This could be a factor

in the *R. appendiculatus* system.

Finally, the tick may have a homeostatic mechanism whereby the removal of enzyme from the system would trigger the stimulation of enzyme production. If such a mechanism does exist, then there would be an unlimited supply of enzyme to be neutralised by a limited amount of antibody.

It is likely that several of the factors discussed above are jointly responsible for the lack of inhibition *in vivo* of tick digestion by antisera raised against preparations of proteinases 1 and 2.

3.4 *The origin of proteinases 1 and 2*

An important question in this work concerns the origin of proteinases 1 and 2, since similar enzymes occur in rabbit blood. Preparations of tick gut were washed prior to homogenisation. This procedure removed some of the rabbit blood (section 2.3.1). In addition to this, proteinase 2 was shown to be of tick origin by its presence in unfed *R. appendiculatus* larvae (fig. 21). The same technique showed that proteinase 1 is not present in unfed larvae. However, rabbit cathepsin D has been extensively studied and a comparison of some of its properties with those of proteinase 1 indicate that this proteinase is of

tick origin (table 4).

These include:

i/ The efficient hydrolysis of serum albumin by proteinase 1 (≥100% of Hb hydrolysis for the crude enzyme; 32% for enriched proteinase 1) as compared to 5 - 10% for both crude and purified rabbit enzyme (Press et al., 1960).

ii/ Pure rabbit cathepsin D is stable for long periods when stored at 4 °C, frozen or in glycerol at -20 °C at pH 5.6 and 8.0 (Barrett, 1977). In contrast to this, both *R. appendiculatus* proteinases were relatively unstable when stored for up to 15 days at 4 °C or -20 °C. Both enzymes were very unstable in glycerol at either temperature (3.2.5). In this respect, the two tick enzymes were similar to blowfly pupa cathepsin D (Kawamura et al. 1987).

iii/ The pI of the major isoenzymes of proteinase 1 are in the pH range 4.65 - 5.8. This is in contrast to rabbit cathepsin D isoenzymes whose pI are in the range 5.2 - 6.9 (Lin & Fletcher, 1973).

iv/ The injection of proteinase 1 into rabbits resulted in the formation of potent antibodies which inhibited the activity of the tick proteinases *in vitro*. This would not have been possible if the protein in question had been of rabbit origin. Moreover, antisera raised against the proteinase 1 preparation cross-reacted with proteinase 2 and viceversa.

Fig. 21 **The Presence of Proteinase 2 in Unfed *R. appendiculatus* larvae**

Native PAGE of a homogenate of unfed larvae (1) and a fraction obtained after chromatography on Sephadex G-100 (2) on 10 - 20% slab gels co-polymerised with Hb. Other details are as for fig. 1. The gel was stained for proteinase activity.

3.5 *Conclusions and Summary*

The aim of this project was to purify and characterize some of the enzymes involved in bloodmeal digestion in the tick, *R. appendiculatus*, and to determine whether such enzymes could be used to improve the resistance of animals to tick infestation.

1/ Earlier work (Akov, 1982) indicated that the major digestive proteinase of the tick appeared to be rather similar to mammalian cathepsin D. This project not only confirms this, but also shows that there are infact two such proteinases.

2/ The two *R. appendiculatus* digestive proteinases, proteinase 1 and proteinase 2 have been identified and for the first time, partially purified.

3/ The inhibition experiments have shown that pepstatin is a powerful competitive inhibitor of both proteinases, thus proving for the first time that the two *R. appendiculatus* enzymes are indeed aspartic proteinases. Prior to the current study, there was no conclusive evidence as to the nature of the enzyme which hydrolysed protein substrates at acid pH in the tick gut.

4/ The observation that crude and semi-crude preparations of both enzymes hydrolysed haemoglobin and BSA equally well underscores the role of the two proteinases in the hydrolysis of blood proteins as opposed to other similar proteinases whose primary function is to hydrolyse proteins other than blood proteins.

5/ This project has shed light on many of the physical and chemical properties of the two aspartic proteinases. The two enzymes differed in molecular weights, kinetic and electrical properties, sensitivity to pH, inhibitors, and thermal inactivation. Overall the project has shown that the *R. appendiculatus* proteinases are generally similar to other aspartic proteinases (table 4).

6/ Immunologically, both proteinases were good immunogens and the two cross reacted with each other. The immune sera inhibited both proteinases *in vitro*, but had no observable effect on the digestion of the tick *in vivo*.

In conclusion, the project has given considerable insight into the biochemistry of tick digestion, an area of tick physiology which though important, had hitherto been neglected. However, much work remains to be done. The two enzymes described here are both endopeptidases and are therefore incapable of completing bloodmeal digestion on their own. Future efforts should therefore be focused on the exopeptidases, dipeptidyl peptidases and of course, the haemolysin. On the failure of the immune sera to inhibit tick digestion *in vivo*, future efforts could be directed at targeting the factors involved in the conversion of the zymogens to the active aspartic proteinases. Unlike proteinases 1 and 2, such factors would be present only in trace ammounts, thus

Table 4 **Properties of *R. appendiculatus* Proteinases 1 and 2, Compared to Other Aspartic proteinases**

	Proteinase 1	Proteinase 2	Other
Substrates			
Hb			
K_M	11 $mg.ml^{-1}$	8 $mg.ml^{-1}$	1.9 $mg.ml^{-1}$ (Yamamoto et al., 1978) 0.41 $mg.ml^{-1}$ Porcine (Barth & Afting, 1984) 3.7 $mg.ml^{-1}$ *M. auratus* (Bonete et al., 1984)
Substrate inhibition	None	None	Yes, Rat cathepsin D (Yamamoto et al.,1978). None, Porcine cathepsin D (Barth & Afting, 1984).
BSA			
Hydrolysis (% of Hb)	Crude 100 %	Crude 100 %	27 %, Crude *O. tholozani* (Akov et al., 1976). 10 %, Pure *A. grahami* (Kawamura et al., 1987). 5 - 10 %, Pure Cathepsin D (Barret, 1977).
	Enriched 32 %	Enriched 28 %	100 %, Pepsin & Cathepsin E (Barrett, 1967)
Ace-Phe-DIT			
Hydrolysis	None	None	Yes, Pepsin (Jackson et al., 1965).
Inhibitors			
Pepstatin	Apparent Competitive	Apparent Competitive	Competitive, Pepsin, Cathepsin D (Umezawa & Aoyagi, 1977).
K_I	-	1.7 nM	2.1 nM, Porcine Cathepsin D (Barth & Afting, 1984).

pH Effect	Independent	Dependent	Independent, Porcine Cathepsin D (Barth & Afting, 1984). Dependent, Human Cathepsin D (Knight & Barrett, 1976).
PM$F	Inhibited	Inhibited	Inhibited, *H. excavatum* Gut Aspartic Proteinase (Bogin & Hadani, 1973). Inhibited, Porcine Cathepsin D (Barth & Afting, 1984).
Antisera			
In vitro	Inhibited	Inhibited	Inhibited, Cathepsin D (Dingle et al., 1971).
In vivo	None	None	Inhibited, Sheep Cathepsin D (Dingle et al., 1973).
Molecular Wt.			
SDS-PAGE	70 kD 40 kD 29 kD	46 kD 44 kD	41 kD, *A. grahami* (Kawamura et al., 1987). 36 - 100 kD (Barrett, 1977).
pI	4.65 - 5.8	4.3 - 4.6	5.4, 6.2, *A. grahami* (Kawamura et al., 1987). 5.0 - 7.0, (Barrett, 1970).
pH Optimum	3.0 4.6 (smaller)	2.6 5.0 (smaller)	2.6, 3.8 (smaller), *A. persicus* (Tatchell, 1964). 2.7 - 3.3, *O. tholozani* (Akov et al., 1967). 2.0 - 4.0, 4.0 - 5.0 (smaller), (Barrett, 1977).

Temperature			
Inactivation	50 °C +	50 °C +	50 °C +, *M. auratus* (Bonete et al., 1984).
pH Effect	Independent	Dependent	Dependent, Penicillopepsin (Sodek & Hofmann, 1970). Dependent, Pepsin (Herriot, 1941).
Storage			
4 °C	Best	Best	Best, *A. grahami* (Kawamura et al., 1987). Stable, Rabbit Cathepsin D (Barret, 1977).
-20 °C	Very unstable	Very unstable	Very unstable, *A. grahami* (Kawamura et al., 1987). Very unstable, Chicken Cathepsin D (Barrett, 1977). Stable, Rabbit Cathepsin D (Barrett, 1977).
+ Glycerol	Very unstable	Very unstable	Very unstable, *A. grahami* (Kawamura et al., 1987). Stable, Rabbit Cathepsin D (Barrett, 1977).

making better targets. In addition to this, the haemolysin would be an exellent target, since unlike the aspartic proteinases, its point of action is the gut lumen, a location easily accessible to antibodies. Moreover, digestion of such antibodies would not be a problem since no protein digestion occurs in the lumen of the tick gut.

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