ENZYMES FORMING KININ-LIKE PEPTIDES IN CARDIOVASCULAR TISSUES.

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ABSTRACT

That kinins are released during myocardial ischaemia has been known for sometime now but it is only recently they have been shown to improve metabolic and haemodynamic parameters following an ischaemic insult. This work was an attempt to investigate the possibility that the heart contains kinin-forming enzymes which become active during ischaemia. Dog ventricles and thoracic aorta and rat ventricles were used for this study.

Aqueous extracts of dog ventricles released rat uterus contracting substance (UCS) optimally at pH 5.2 - 5.4. It showed no obvious optimum at neutral pH. Release of UCS was completely inhibited by pepstatin. It was inhibited 32.8 % by aprotinin and 13.5 % by lima bean trypsin inhibitor but not by SBTI. Gel filtration (Sephacryl S-300) showed that this enzymic activity was related to a fraction, molecular weight 42.6 $\pm$ 0.9 kDa which at pH 5.2, cleaved D-Val-Leu-Arg-pNA and released bradykinin-like immunoreactivity (BKIR) from a plasma kinin-forming substrate.

Aqueous extracts of dog aorta released UCS optimally at both acid (pH 5.4) and neutral (pH 8.0) pH and cleaved D-Val-Leu-Arg-pNA optimally at pH 5.5 and pH 7.5. Release of UCS at acid pH was completely inhibited by pepstatin; 38.9 % by aprotinin and 33.3 % by LBTI. Gel filtration produced two acid optimum peaks, molecular weights 42.0 $\pm$ 4.6 kDa and 252.0 $\pm$ 39.0 kDa. Both cleaved D-Val-Leu-Arg-pNA and released BKIR from plasma kininogen. It also showed one neutral enzyme peak, molecular weight 48.5 $\pm$ 4.0 kDa, which released BKIR (pH 8.0) from plasma kininogen. The release of BKIR by the neutral enzyme peak was inhibited 94 % by aprotinin.

Aqueous extracts of rat ventricles showed one pH-related peak of UCS formation, at pH 4.7 - 5.3. There was no release of UCS at neutral pH. Release of UCS was completely inhibited by pepstatin. It was inhibited 20 % by aprotinin, 33.1 % by SBTI but not by LBTI. The extract released BKIR (pH 5.0) from a plasma kinin-forming substrate and from L-kininogen but not from H-kininogen. Gel filtration revealed one enzyme peak, molecular weight 42.8 $\pm$ 4.9 kDa, which cleaved D-Val-Leu-Arg-pNA optimally at pH 4.5 and released BKIR (pH 5.0) from L-kininogen and not from H-kininogen. Ligation of the left main coronary artery for 10 and 30 minutes reduced BKIR-releasing activity in extracts of rat left ventricles by 30 and 48.1 %, respectively. It also increased levels of free kinin in mixed venous blood from the right atrium by up to 7 fold after ligation for 10 minutes.

We have shown that dog and rat ventricles contain one cathepsin D-like aspartic proteinase which releases immunoreactive kinin from human L-kininogen. This enzyme in the rat is released from the ventricles during ischaemia and concomitantly cleaves L-kininogen which results in elevation of kinins in the coronary effluent. Activation and or release of this enzyme and the release of kinins may be an adaptive mechanism for both haemodynamic and metabolic readjustment by the ischaemic myocardium.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 ISCHAEMIC HEART DISEASE AND SUDDEN CARDIAC DEATH

Sudden cardiac arrest is a major cause of death in the industrialized world. In the United States, for example, about 1200 people succumb to it daily (Lown, 1979; Goldstein, 1974; Lucchesi, 1984). In the majority of cases sudden death is caused by ventricular arrhythmias and in particular ventricular fibrillation (Gradham et al, 1977; Nikolic et al, 1982; and Roelandt et al, 1984). The underlying pathology which, in most cases, causes these arrhythmias is atherosclerotic coronary artery disease, which narrows or occludes the artery and in turn causes cardiac ischaemia and infarction (Blumgart et al, 1940; Davies, 1981; Kannel et al 1975). Additional lethal mechanisms are thought to include acute ventricular failure, severe reflex hypotension, and maliginant bradycardia, each occuring rapidly during acute ischaemic episodes (Goldstein, 1974).

Acute regional myocardial ischaemia, whatever its aetiology, invariably triggers off profound metabolic, biochemical, humoral and neurohumoral events which impinge deleteriously on the ionic and electrical stability of the heart, and ultimately provoke or provide the setting for the onset of the potentially lethal ventricular dysrhythmias (Hearse and Dennis, 1982). A typical feature of an ischaemic episode consists of a shift from aerobic to anaerobic metabolism in the ischaemic myocardium resulting in a derangement in substrate uptake and utilization patterns. This triggers a wide range of cellular events including lowering of myocardial pH and loss of ionic homeostasis leading to extracellular accumulation of K^+ and intracellular accumulation of Ca^{2+}. Other changes include ultrastructural damage, loss of intracellular components, depletion of high energy bond

sources (ATP and creatine phosphate), increase in cyclic AMP, increase in lysophosphoglycerides and long-chain acyl carnitines, increased levels of catecholamines, increase in α-adrenoceptors, release of prostaglandins, and increase in myocardial lactate (Van Gilst, 1989; Golikov et al, 1977; Wilde et al, 1988).

In concert with these changes is the triggering of a cascade of proteolytic activities with a wide range of consequences. The release of proteolytic enzymes is a result of increased fragility of myocardial lysosomes which then release lysosomal enzymes into the cell cytosol. This is thought to promote cell damage (Wildenthal, 1978). Evidence of this is seen in the increased release of lysosomal-derived cathepsin D (Decker et al, 1977). Some of the released enzymes cleave and release components of the complement cascade (Hill and Ward, 1971). Activated complement components are potent chemotactic and stimulatory agents for neutrophils and may initiate leukocyte infiltration into the extravascular myocardial tissue (Lucchesi and Mullane, 1986). The neutrophils which are attracted to the ischaemic myocardium also release proteolytic enzymes, including cathepsin G, elastase and collagenase. Human polymorphonuclear leucocytes were also shown to contain tissue kallikrein (Figueroa et al, 1989). The neutrophil-derived enzymes are capable of proteolytic attack on ischaemic myocytes. The neutrophil-derived lysosomal enzymes have been shown to contribute to the degenerative tissue damage associated with rheumatoid arthritis (Weissmann, 1982). However, the extent of myocardial cell injury caused by proteolytic enzymes is not clear. It is likely that the lysosomal enzyme activity and the extent of myocardial damage are not linked. This conclusion was reached following the observation that inhibition of lysosomal protease

activity with leupeptin, antipain, pepstatin and chymostatin totally suppressed proteolysis but did not influence infarct size (Lucchesi and Mullane, 1986). On the other hand the experience with aprotinin give a second thought about the role of proteolytic enzymes. Aprotinin was reported to have a protective and lysosomal stabilizing effect during acute myocardial ischaemia (Lefer et al, 1974). It is possible that aprotinin inhibits serine proteases from neutrophils and the myocardium and in so doing limits proteolytic cell damage. Another place where we find activation of proteolytic enzymes is during tissue damage and consequently inflammation (Beloff and Peters, 1945; Unger, 1947). Haberland (1975), likened the role of proteolytic enzymes in inflammation to that in myocardial infarction.

Apart from the degenerative effects the released enzymes may serve the role of processing a variety of biologically active peptides such as atrial natriuretic peptide and kinins. This would constitute release and activation of enzymes which would otherwise remain inactive under physiological conditions. Kininogens are mainly plasma based proteins and under normal conditions they can not get access to the intracellularly stored enzymes which are either stored inactive or are not accessible to the kininogens. The on site processing of peptides like the kinins in the myocardium may be of physiological importance because most of the kinins released in the peripheral blood are destroyed both in plasma and in the lungs before they reach the various organs and tissues (Ryan, 1985; Erdos et al, 1963).

Ischaemic cell injury leading to myocardial infarction, like the inflammatory response to tissue damage, is characterized by pain, vasodilatation, increased permeability and accummulation of leukocytes, all of which are

known actions of kinins (Armstrong et al, 1953; Armstrong et al, 1954; Armstrong et al, 1957; Elliot et al, 1960; Konzett and Sturmer, 1960; Lewis, 1962). Indeed kinins and other vasoactive substances have been detected at the site of local injury (Rocha e Silva and Antonio, 1960; Rocha e Silva and Rosenthal, 1961; Melmon et al, 1967). Similarly kinin levels in the coronary effluent are elevated following experimental occlusion of the left anterior descending coronary artery in dogs (Kimura et al, 1973; Matsuki et al, 1987). The accumulation of kinins may be a result of activation of the kallikrein-kinin system due to lowered pH at the site of injury (Werle, 1934; Frey et al, 1950; Pitt et al, 1970). On the other hand it is an established fact that acid pH or, specifically, pH's below 7 are known to inactivate the kinin-destroying enzymes (Edery and Lewis, 1962). Inactivation of these enzymes could be a possible explanation for the accumulation of kinins, although it is doubted if this could sufficiently account for the large amounts of kinins found in the coronary sinus blood during myocardial ischaemia (Matsuki et al, 1987). The presence of kinin-forming enzymes in the heart activatable during myocardial ischaemia to release kinins may account for the amounts of kinins detected in the coronary circulation.

1.2. CANDIDATE KININOGENASES IN MYOCARDIAL AND VASCULAR TISSUES.

The generation of kinins locally in the coronary circulation may be from three sources; from the ventricular myocardium, from the coronary vasculature, and from plasma. There are several enzymes which are capable of releasing kinins from kininogens. Among the kinin-forming enzymes there are those like kallikrein which are quite efficient kininogenases. At the same time we

find other enzymes which are not so efficient as kininogenases, but they process other peptides with cleavage sites similar to those required for the processing of kinins from kininogens. Much as they are able to release kinins, possibly in vivo, they are not so efficient as to have a biological significance or are stored at such remote sites that they are not readily deployed for the release of kinins. For example, cathepsin D can release bradykinin from low molecular weight kininogen (Ogloblina et al, 1981) but it is a lysosomal enzyme and unless there is tissue damage it is not available for the release of kinins.

Kinin releasing enzymes can be grouped into two categories; acid and neutral proteases.

1.2.1 ACID PROTEASES.

Among the acid proteases there are two categories, cysteine or thiol proteases and the aspartic proteases. So far no member of the cysteine proteases has been reported to release kinins. The cysteine proteases include cathepsins B, H, L, N, and S (Barrett and MacDonald, 1980).

The aspartic proteases include the pepsins, cathepsin D, renin, pseudorenin, chymosin, gastric cathepsin, plasma aspartic proteinase, mouse submandibular renin, thyroid aspartic proteinase, lymphocyte aspartic proteinase, human ascites aspartic proteinase and cathepsin E. Among the group, cathepsin D (EC 3.4.23.5) is known to release kinins from kininogens (Ogloblina et al, 1981; Kobayashi and Ohata, 1981; Okamoto and Greenbaum, 1983 (a); Kariya and Hirota, 1984, Kariya et al, 1984; Shisheva et al, 1985; Sakamoto et al, 1988). The substrate for cathepsin D

was reported to be low molecular weight kininogen (Ogloblina et al, 1980) but it has also been shown to release T-kinin from T-kininogen (Okamoto and Greenbaum, 1983 (b); Sakamoto et al, 1988). Cathepsin D is a 42 kdalton glycoprotein that has endopeptidase activity. Apart from the release of kinins from kininogens it also releases g-endorphin from b-endorphin (Graf et al, 1979). Other substrates include somatostatin (Benuck et al, 1977), angiotensinogen (Hackenthal et al, 1978), and b-lipotropin (Barat et al, 1979). Recently it has also been shown to be the processing enzyme for endothelin in aortic endothelial cells (Ohnaka et al, 1990; Samawura et al, 1990).

Pepsin has been reported to release methionyl-lysyl-bradykinin from both low and high molecular weight kininogen (Guimaraes et al, 1976). This is, however, an unlikely candidate because of its location in the gastric juice.

1.2.2. RELEASE OF KININS BY NEUTRAL PROTEASES.

The enzymes in this group include serine proteinases like plasmin, trypsin, plasma kallikrein and tissue kallikrein and non-serine proteinases like calpain.

1.2.2.1 CALPAIN

Calpain (EC 3.4.22.17) is a Ca^{2+}-dependent cysteine proteinase which is widely distributed in mammalian and avian cells. Two distinct types of the enzyme are known to coexist in most cells; A low (micromolar order) Ca^{2+}-requiring form, calpain I and a high (millimolar order) Ca^{2+}-requiring form,

calpain II. They are present in rat kidney (Yoshimura et al, 1983), human erythrocytes (Sasaki et al, 1984) and in porcine kidney and erythrocytes (Sasaki et al, 1984). They are involved in diverse cell functions such as degradation of cytoskeletal and muscle proteins, activation or modification of enzymes and alteration of membrane receptors. In many instances, a limited proteolysis by calpains is coupled to a transient influx of calcium across the cell membranes (Murachi et al, 1981; Imahori, 1982).

Analysis of DNA sequence indicate that the catalytic subunits of both calpains I and II contain a papain-like thiol protease domain and a calmodulin-like calcium binding domain (Aoki et al, 1986; Emori et al, 1986; Ohno et al, 1984). Due to the presence of the papain-like thiol protease domain the calpains behave like the cysteine proteases and they are inhibited by the cysteine protease inhibitors. However, they are rather unusual because, unlike the other cysteine proteases, they act at neutral pH's.

Both the calpains release lysyl-bradykinin from low and high molecular weight kininogen. However, they have a limited kininogenase activity because in addition to releasing kinins they are also inhibited by the kininogens (cysteine protease inhibitors). This is thought to be a useful aspect of kininogens for while they allow for the release of lysyl-bradykinin they also check further inflammatory consequences of calpains as they are released from damaged tissues (Higashiyama et al, 1986). Other substrates include α-neoendorphin, angiotensin I, luteinizing hormone-releasing hormone, neurotensin, dynorphin, glucagon, and oxidized insulin B-chain (Sasaki et al, 1984). During substrate cleavage they show a general

preference for a Tyr, Met, or Arg residue in the P_1 position preceded by a Leu or Val residue in the P_2 position (Sasaki et al, 1984).

1.2.2.2 PLASMIN

Plasmin (EC 3.4.21.7) is a serine protease present in plasma, especially of rabbit and man (Barrett and McDonald, 1980). It degrades fibrin, casein and other proteins. It preferentially cleaves -Lys-X- and -Arg-X- bonds, but it is more selective than trypsin (Barrett and McDonald, 1980). It releases kinin from both H-kininogen and L-kininogen at about the same rate (Habal et al, 1976).

1.2.2.3 PLASMA KALLIKREIN (EC 3.4.21.8)

It liberates bradykinin from high molecular weight kininogen by cleavage of -Lys-Arg- and -Arg-Ser- bonds. It also activates coagulation factors XII and VII and plasminogen (Barrett and McDonald, 1980). It may, therefore, be considered as a clotting factor. The ability of plasma kallikrein to cleave peptides flanked between pairs of basic amino acids make it a candidate enzyme for the processing of other biologically active peptides. It cleaves bonds COOH-terminal to or between pairs of basic amino acids such as Lys-Arg, Lys-Lys and Arg-Arg (Metters et al, 1988) It is postulated to be involved in the extracellular maturation of precursors either during or after their release into plasma (Metters et al, 1988).

1.2.2.4 TISSUE KALLIKREIN

Tissue or glandular kallikrein is a simple serine protease related in many regards to trypsin. It differs from trypsin in that it has a higher specificity for polypeptide substrate cleavage sites (McDonald et al, 1988). Historically, tissue kallikrein was first named glandular kallikrein, because it was found in high concentrations in exocrine glands such as the salivary glands and the pancreas. It is now known that in some species kallikrein or its closely related family members have a much wider distribution outside these glands.

Tissue kallikrein is synthesized on the rough endoplasmic reticulum and glycosylated and processed in the golgi apparatus. It contains carbohydrates which contribute to the microheterogeneity of human kallikrein in electrophoresis (Morichi et al, 1984; Takada et al, 1985). Pig pancreatic kallikrein contains fucose, mannose, galactose, glucosamine, and sialic acid (Fiedler et al, 1977; Moriya et al, 1983). It is obtained from autolysed pancreas mainly as β-kallikrein, a two chain form apparently arising from cleavage of a single-chain form during isolation. It occurs as β-kallikrein A (mol. wt. 27,100; 5.6% carbohydrate) and B (mol. wt. 28,900; 11.5% carbohydrate) of similar amino acid composition (Barrett and McDonald, 1980). The submandibular and urinary kallikreins are both single-chain α-kallikreins. They show immunological identity with β-kallikrein, and have the same N-terminus sequence, but contain more carbohydrate (Barrett and Mc Donald, 1980) Human urinary kallikrein has a relatively higher total carbohydrate content (Moriya et al, 1983). The size of the human urinary kallikrein is estimated to be between 25,000 and 48,000 (Barrett and McDonald, 1980).

In each species tissue kallikrein is probably one enzyme, but there are other proteases which are closely related to tissue kallikrein. This constitute the kallikrein-like enzyme group which is a group of enzymes encoded by a family of very similar genes, the kallikrein gene family. In the mouse, for example, there are 24 genes all arranged in tandem on chromosome 7 (Evans et al, 1987). The kallikrein gene family is named after the protein product of its most prominent and first sequenced member, tissue kallikrein (Tschesche et al, 1979). The kallikrein-like enzymes are similar in enzymic and physical properties. They have similar amino acid sequences and often cross-react with polyclonal and occasionally monoclonal antibodies to tissue kallikrein (Woodley et al, 1985). The major difference among them is substrate specificity. The other proteases do not appear to function physiologically as alternative kallikreins. For example gamma nerve growth factor (γ-NGF) and epidermal growth factor binding protein (EGF-BP) do have some kininogenase activity but their kininogenase activity is one to two orders of magnitude less than that of renal kallikrein (Bothwell et al, 1979). When γ-NGF is added to proEGF it does not release any EGF even when molar ratios of up to 500:1 are used (Frey et al, 1979). Likewise, only one member of the mouse kallikrein family, γ-renin, cleaves synthetic renin substrate (Poe et al, 1983).

The number of genes encoding for the kallikrein-like enzymes vary greatly among mammals. The mouse has 24 member genes (Evans et al, 1987) while the rat has 10 (Ashley and MacDonald, 1985) to 17 (Gerald et al, 1986). The hamster has 3 (Howles et al, 1984). The size of the human gene family is also smaller than that of the rat and mouse (Fukushima et al,

1985; Baker and Shine, 1985). It contains at least 4 distinct genes, kallikrein and unidentified partial gene sequence (Fukushima et al, 1985; Baker and shine 1985), prostate specific antigen (Watt et al, 1986; Lundwall and Lilja, 1987), and the characterized but unidentified gene termed hGK-1 (Schedlich et al, 1987). In each species not all the genes are functional; for example, in the rat only 8 of the 10 or 17 gene are functional genes.

Tissue kallikrein releases kallidin from kininogen by cleavage of the amino-terminal -Met-Lys- and the C-terminal -Arg-Ser- bonds of kininogen. The resulting lysyl-bradykinin is converted to bradykinin by an amino peptidase (Erdos, 1977). Rat urinary (Alhenc-Gelas et al, 1981), kidney (Yamada and Erdos, 1982) kallikrein and kallikrein of human colonic mucosa (Seki et al, 1972) release bradykinin directly from kininogen. There are other serine proteases which also release kinins (Schachter, 1980) but their release is inefficient.

Apart from the processing of kinins from kininogens, tissue kallikrein is thought to participate in the processing of other biologically active peptides. Evidence accumulated from in vitro experiments show that tissue kallikrein hydrolyses apolipoprotein B-100 (Cardin et al, 1984), atrial natriuretic peptide (Currie et al, 1984), prorenin (Sealey et al, 1980), proinsulin (Ole-Moiyoi et al, 1979), vasoactive intestinal polypeptide (VIP; Mutt and Said, 1974) and procollagenase (Eeckhout and Vaes, 1977).

1.3. THE PROCESSING OF BIOLOGICALLY ACTIVE PEPTIDES

Most biologically active peptides are synthesized as large precursor molecules, called pre-propeptides (Steiner et al, 1980). The synthesis of large precursor molecules capable of being cleaved to release biologically active peptides is a common phenomenon for most hormones and growth factors in single-celled eukaryotes and multicellular organisms. In some cases the precursor molecule may be a source of more than one product. For example, pro-opiomelanocortin and the products of processing differ in each of two cell types expressing the gene (Eipper and Mains, 1980). Other precursors, for example kininogens, have multifunctional domains which appear to act at different sites in the one physiological process (Muller-Esterl, 1987).

The biologically active peptides are released from the parent peptide by a limited post-translational proteolysis of the precursor molecules (Docherty and Steiner, 1982). The biologically active peptides are usually flanked in the precursor molecule, by pairs of basic amino acids, usually lysine and/or arginine (Rholam et al, 1986). For example lysyl-bradykinin is released by the cleavage of -Met-Lys- and -Arg-Ser- bonds. In some cases the release of the active peptide is a sequential action of a trypsin-like enzyme followed by a carboxypeptidase B-like enzyme (Docherty and Steiner, 1982).

1.3.1 THE PROCESSING OF EGF

Mouse epidermal growth factor (EGF) is a 53 amino acid protein (6 kDa) with potent mitogenic activity. Its precursor molecule is a 1217 amino acid protein

(Drinkwater et al, 1988). This is processed by a series of steps, through at least two intermediates (125kDa and 110 kDa) to a 9 kDa EGF precursor. Finally the carboxy-terminal fragment is removed by EGF-binding protein (EGF-BP), a member of the tissue kallikrein family, to produce the mature growth factor (Drinkwater et al, 1988).

1.3.2 THE PROCESSING OF ENDOTHELIN

Another example is that of endothelin. Endothelin is a 21-amino acid peptide found in at least 3 isoforms, ET-1, ET-2, and ET-3. Endothelin is synthesized by endothelial cells as pre-proendothelin containing approximately 200 amino acids. This is proteolytically cleaved to form a 38- or 39-amino acid "big endothelin" (Yanagisawa et al, 1988; Inoue et al, 1989). The big endothelin is cleaved at Trp^{21}-Val^{22} by, probably, cathepsin D (Ohnaka et al, 1990; Samawura et al, 1990) to release the mature endothelin.

1.3.3 THE PROCESSING OF ANF

Atrial natriuretic factor (peptide) is a family of peptide hormones secreted by the mammalian heart. The major source of atrial natriuretic factor is the atria where it was first located (de Bold, 1982). Small amounts of this peptide have been found in the ventricles (Gardner et al, 1986; Day et al, 1987; Lattion et al, 1986; Bloch et al, 1986). It has also been located in the aortic arch (Gardner et al, 1987) and in brain (Saper et al, 1985).

Atrial natriuretic factor is synthesized as a large precursor molecule, prepro-

ANP, containing between 149 - 153 amino acids depending on the species (Maki et al, 1984; Seidman et al, 1984; Yamanaka et al, 1984; Zivin et al, 1984; Nakayma et al, 1984; Oikawa et al, 1984; Oikawa et al, 1985). Each prepro-ANP molecule contains a signal peptide sequence at its amino terminus thought to provide the signal for co-translational transport of the nascent polypeptide chain across the membrane of the endoplasmic reticulum. This signal peptide is removed from the prepro-ANP molecule as the transport process takes place, resulting in the generation of pro-ANP, a protein of 126 amino acids (Baxter et al, 1988). Pro-ANP is cleaved to generate an amino-terminal fragment of about 13 kDa and a shorter biologically active carboxy-terminal ANP molecule. In addition, two arginine (Arg) residues present at the carboxy-termini of rat, mouse, and rabbit are absent from smaller bioactive peptides suggesting that they are removed as an early processing event (Baxter et al, 1988). The Arg-Arg dipeptide is not present in human pro-ANP (Baxter et al, 1988). Amino terminal fragments appear to be secreted along with ANP and circulate in plasma (Nakao et al, 1987). Experiments using the perfused rat heart (Thibault et al, 1986), and direct detection in plasma using HPLC and radioimmunoassay have demonstrated that the heart releases the small 28-amino acid ANP (Sugawura et al, 1985; Schwartz et al, 1985; and Cantin and Genest, 1985). This means that the processing of ANP takes place within the cardiac myocytes.

Some enzymes have been located in cardiac tissues which could be candidates for the processing of ANP. Cathepsin B, a cysteine proteinase with endopeptidase activity, has been localized in the same granules as ANP (Watanabe et al, 1989). Tissue kallikrein (Xiong et al, 1990) and

arginine esterase A (Simson et al, 1989) have been shown to appear in granules containing pro-ANP. Tissue kallikrein has been demonstrated invitro to be capable of releasing ANP from pro-ANP (Currie et al, 1984). A serine protease capable of releasing ANP from pro-ANP was also isolated from membranes of atrial tissue (Imada et al, 1987; Imada et al, 1988).

Atrial natriuretic peptide has potent natriuretic, diuretic, sympatholytic, and vasodilator activity. It suppresses the release of aldosterone, renin and vasopressin. It is involved in the physiological regulation of water and electrolyte balance and the regulation of blood pressure (Cantin and Genest, 1985; Needleman and Greenwald, 1986; Cole and Needleman, 1985; Kuchel et al, 1987; Debinski et al, 1986; Kakamaru et al, 1986; Obana et al, 1985). ANP seems to be important in different cardiovascular conditions including, congestive heart failure (Tikkanen et al, 1985; Hartter et al, 1985; Nakaoka et al, 1985; Pettersson et al, 1986; Arendt et al, 1986; Schriffrin et al, 1986), Valvular heart disease (Lang et al, 1986; Hirata et al, 1987), acute myocardial infarction (Matsubara et al, 1987), supraventricular tachycardia (Tsai et al, 1988; Nilsson et al, 1987) and hypertension (Sagnella et al, 1986).

1.3.4 THE PROCESSING OF KININS

Kinins, like other biologically active peptides, are released from larger precursor peptides, the kininogens. Kininogens are single chain acidic glycoproteins synthesized by the liver from which they are secreted into plasma via the normal secretory pathways (Habermann, 1963; Suzuki, 1965). Habermann(1963) and Suzuki(1965) were the first to recognize that

plasma of mammalian species contains at least two distinct kinin precursors which became later known as high molecular weight and low molecular weight kininogens (Jacobson, 1966). A third type of kininogen, T-kininogen, was isolated from rat plasma (Okamoto and Greenbaum, 1983 (b)). T-kininogen is a good substrate for trypsin. T-kininogen is a poor substrate for tissue kallikrein. It generates 28 % of kinin with rat urinary kallikrein and 14 % with rat submaxillary kallikrein. Two types of T-kininogen, TI- and TII-kininogen have been isolated from rat plasma (Enjyoji et al, 1988 b). Both T1 and T2 kininogens are present in the plasma of adjuvant treated rats (Enjyoji et al, 1988 b), but only T1 is present in the non-inflamed rat plasma (Enjyoji et al, 1988 a). The two kininogens are similar in amino acid composition and antigenicity but differ in sialic acid content (Enjyoji et al, 1988 b).

The three main types of kininogens share their gross structure. They are composed of a heavy chain located at the amino terminal end followed by the kinin segment, and a light chain of variable length at the carboxyl terminal end (Muller Esterl, 1987). A common feature among the three kininogens is that they are all susceptible to cleavage by proteases, resulting in the release of kinins. Until recently four types of kinins, bradykinin, lysyl-bradykinin, Met-lysyl-bradykinin, and Ile-ser-bradykinin (named T-kinin by Okamoto and Greenbaum) were known to exist in mammalian species. Most recently two additional kinins have been discovered; [Hyp^3]-lysl-bradykinin, which is released from human heat inactivated plasma by urinary kallikrein and from Cohn's fraction IV-4 by hog pancreatic kallikrein (Sasaguri et al, 1988; Maier et al, Sasaguri et al, and Matsumura et al, 1987) and [Hyp^3]-bradykinin isolated from human and monkey kininogens (Enjyoji and Kato, 1988) and from urine (Kato et al, 1988).

Bradykinin H_2N-R-P-P-G-F-S-P-F-R-COOH

[Hyp^3]-bradykinin H_2N-R-P-T-G-F-S-P-F-R-COOH

Kallidin H_2N-K-R-P-P-G-F-S-P-F-R-COOH

[Hyp^4]-kallidin H_2N-K-R-P-T-G-F-S-P-F-R-COOH

Met-lysyl-bradykinin H_2N-M-K-R-P-P-G-F-S-P-F-R-COOH

T-kinin H_2N-I-S-R-P-P-G-F-S-P-F-R-COOH

FIGURE 1.1 The kinin moieties released from different kininogens by kininogenases. They all share a common C-terminal sequence while variations on the amino-terminal end makes the distinction among them and sometimes this may be used to identify the kininogenase responsible for the release of a particular kinin. The respective amino acids are: R = Arginine, P = Proline, G = Glycine, F = Phenylalanine, S = Serine, K = Lysine, T = Hydroxyproline, M = Methionine, I = Isoleucine.

1.4. THE EFFECT OF KININS ON THE CARDIOVASCULAR SYSTEM.

Kinins are potent endogeneous vasodilators and hypotensive peptides. They cause smooth muscle relaxation and vasodilatation, and when given systemically they cause a fall in vascular resistance and increase in local and systemic blood flow in both animals and man (Harrison et al, 1968). The effect of bradykinin on blood pressure is biphasic. Initially it causes a fall in blood pressure and then this response is followed by a second phase in which the blood pressure rises, often overshooting the control values (Haddy et al, 1970). The pressure response is thought to be mediated via both central and peripheral sympathetic reflexes (Haddy et al, 1970). It is difficult to distinguish the direct effects of kinins from reflex sympatho-adrenergic

activity, as a consequence of hypotension. Many studies in the past attribute the circulatory action of bradykinin to its action on the adrenal medulla in releasing catecholamines (Feldberg and Lewis, 1963; Staszewska and Vane, 1967), to a direct stimulating action on sympathetic ganglia (Parratt, 1964), and indirectly to the fall in arterial pressure which might then produce a sympathetic response via baroreceptor-sensed reflexes (Parratt, 1964; Mason and Melmon, 1965). In addition it has been suggested that bradykinin directly activates parasympathetic centres (Erdos, 1963). Harrison et al (1968) studied the systemic effects of bradykinin in the presence of a blockade of the autonomic nervous system. He came up with a conclusion that the circulatory action of bradykinin is primarily peripheral vasodilatation and this response is not mediated by way of the autonomic nervous system. Kinins are also known to stimulate renin secretion from kidneys (Carretero and Beierwaltes, 1984) and vasopressin from the central nervous system (Baertschi et al, 1981).

The catecholamines released by kinins from the adrenal medulla seem to decrease plasma kininogen (Erdos, 1961; Castania and Rothschild, 1974). This effect is thought to be mediated through a_2-adrenoceptors (Zebrowska-Lupina et al, 1987). When adrenaline is injected kinin levels are not increased, instead there is an increase in plasma kininase activity (Sicuteri, 1970).

The recent development of kinin antagonists by Vavrek and Stewart has provided a means for evaluating the effects of kinins. Studies indicate that kinins contribute to the regulation of blood pressure. This effect on blood pressure is probably achieved by attenuation of the pressor effect of

angiotensin II, vasopressin, and stimulation of a-adrenoceptors (Carbonell et al, 1988). In support of this, circulating levels of bradykinin have been found in humans to be directly related to the rate of renin secretion, the highest kinin levels occurring together with the highest renin levels (Wong et al, 1975; Vinci et al, 1979). Using the bradykinin antagonist D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg-TFA (B4162), it was shown that circulating bradykinin may be involved in the blood pressure control of spontaneously hypertensive rats when the renin-angiotensin system is stimulated by salt depletion (Waeber et al, 1987).

In the heart, kinins cause dilatation of coronary arteries both invivo and in isolated heart preparations (Maxwell et al, 1962; Antonio et al, 1962; Montague et al, 1963; Parratt, 1964; Lochner and Parratt, 1966; and Brecher and Brobmann, 1970). They increase cardiac output, myocardial oxygen extraction and myocardial metabolic heat production (Lochner and Parratt, 1966). Initially kinins were also thought to exert a positive inotropic effect on the myocardium (Montague et al, 1963; Reichgott and Melmon, 1970) but subsequent experiments have failed to prove this effect (Lochner and Parratt, 1966; Hashimoto et al, 1977). The positive chronotropic effect of kinins is, however, a well established effect (Parratt, 1964; Lochner and Parratt, 1966; Maxwell et al, 1962). This together with coronary vasodilatation contribute to increased cardiac output.

Bradykinin causes dilatation of arteries and arterioles both in peripheral and cerebral circulation. The vasodilator effect of bradykinin is mediated by the release of either EDRF or prostaglandins or both (Crutchley et al, 1983; De Nucci et al, 1988; Terragno et al, 1975). Which mediator is involved

depends on the species involved and the particular type of artery. Indomethacin, a cyclo-oxygenase inhibitor, blocks the vasodilator effect of bradykinin in the isolated perfused rabbit heart (Needleman et al, 1975) confirming the involvement of prostaglandins in the vasodilator effect of bradykinin. The vasodilatation is mediated by prostaglandins in rabbit coeliac (Aiken, 1974; Forstermann et al, 1986), coronary (Needleman et al, 1975), and mesenteric arteries (Blumberg et al, 1977; Cherry et al, 1981; 1982; Forstermann et al, 1986), cat coeliac and mesenteric arteries (Cherry et al, 1981; 1982) and guinea pig coronary vasculature (Messina et al, 1975). EDRF is thought to mediate vasodilatation in dog coeliac, coronary, femoral, gastric, intrapulmonary, renal and splenic arteries (Altura and Chand, 1981; Chand and Altura, 1981; Cherry et al, 1981; 1982). Porcine coronary arteries stimulated by bradykinin liberate PGI_2 and EDRF, both of which produce relaxation of bovine coronary arteries denuded of endothelium(Gryglewski et al, 1986). In some species the vasodilator effect of bradykinin is endothelium-dependent while in others it is endothelium-independent. Bradykinin causes endothelium-dependent relaxation of canine arteries excluding those from brain (Altura and Chand, 1981; Chand and Altura, 1981; Cherry et al, 1981; 1982) and in bovine (Cocks et al, 1985; de Nucci et al, 1988) and porcine (Gryglewski et al, 1986) aorta. In rabbits and cats bradykinin causes endothelium-independent relaxation of arteries (Cherry et al, 1982).

The effects of kinins are mediated by basically two types of receptors, B_1 and B_2-bradykinin receptors. Bradykinin and its analogues induce endothelium-dependent relaxation of canine arterial rings via B_2 kinin receptors (Regoli et al, 1986) and of rabbit mesenteric artery strips via B_1

receptors (Churchill and Ward, 1986; Deblois and Marceau, 1987). Kinins act on B_1 and B_2- receptors to release conjointly EDRF and PGI_2 in bovine aortic endothelial cells (D'Orleans-Juste et al, 1989; de Nucci et al, 1988). In bovine pulmonary arteries endothelial cells the release of PGI_2 by bradykinin is 100 times more than with des-Arg^9-bradykinin (Crutchley et al, 1983), suggesting involvement of B_2-receptors. The existence of a third bradykinin receptor, B_3- receptor, was recently reported in the guinea pig trachea (Farmer et al, 1989).

1.5 KININS IN MYOCARDIAL ISCHAEMIA AND INFARCTION.

Cardiogenic shock is still a major cause of death in patients with acute myocardial infarction (Goldstein, 1974; Anderson, 1968). The ability of bradykinin to cause vasodilatation and pain makes it a possible participant in the pathophysiology of acute myocardial infarction. Several studies show that kinins are involved in both experimental and clinical myocardial ischaemia and infarction. Sicuteri et al (1967) determined plasma kininogen in 14 patients who were in the early phase of myocardial infarction. He found that plasma kininogen levels were decreased, suggesting activation of plasma kinin system. Wiegershausen and co-workers (1970) measured kininogen levels in 24 patients with myocardial infarction. They showed that kininogen levels in these patients were decreased, being lowest 48 hours after the onset, and the levels increased thereafter. They found no difference in kininogen levels between patients with and without shock. They also found no correlation between serum GOT activity and the minimum kininogen levels. These results indicate that the severity of infarction was not related to plasma kininogen and hence kinin levels. In another study

involving 70 patients with acute myocardial infarction opposite results were reported (Dzizinskii and Kuimov, 1972). They noted a decrease in plasma kininogen. This decrease was lower in patients who had no accompanying shock. Trypsin inhibitory activity was increased in all patients with coronary insufficiency and it was highest in the acute phase of myocardial infarction. They observed a big variation in plasma kallikrein-like activity (spontaneous BAEe-esterase activity). Pitt and co-workers (1970) studied eleven patients with ischaemia as characterized by both angina and/or ST-segment changes in ECG pacing tests. Seven of these patients showed activation of the plasma kallikrein system; as characterized by increased spontaneous TAMe-esterase activity.

Sicuteri et al (1972) studied 16 infarcted patients. They found no change in spontaneous plasma TAMe-esterase activity (kallikrein). However, prekallikrein levels fell and a minimum was registered on the eighth day after the onset of symptoms.

Kedra and co-workers (1973) found that plasma kininogen levels were decreased on the first and second day of infarction. The decrease was greater in patients with marked haemodynamic disturbances. However, there was no correlation between plasma kininogen levels and the extent of infarction. The study was repeated, on 54 patients, by Kolber-Postepska (1975). He found that kininogen and prekallikrein levels fell to the lowest simultaneously and they were both normalized after 2 weeks. He found no difference in prekallikrein levels between patients with haemodynamic disturbances and the non-complicated ones. There was no correlation between the extensiveness of infarction and the measured parameters. He

concluded that reduced kininase activity in patients with myocardial infarction may prolong the presence of kinins in plasma and thus increase biological activity.

Gomaskov (1974) reported on 20 patients with myocardial infarction. He found that spontantaneous TAMe-esterase activity (kallikrein) was elevated and prekallikrein, kallikrein inhibitory activity and plasma kininogen levels were decreased. The levels of prekallikrein and kallikrein inhibitory activity were associated with the severity of infarction. Similar results were reported in a study on 50 patients with acute myocardial infarction all of whom did not survive (Golikov et al, 1977). They also found that the levels of catecholamines increased within 6 hours after the onset of the disease.

Hashimoto et al (1978) studied 23 patients with myocardial infarction. Sixteen survived and 7 died. They measured both plasma kininogen and kinin concentrations. Plasma kininogen levels decreased reaching a minimum level on the second day. There was a simultaneous increase in levels of kinin and both parameters significantly correlated with the lowering of blood pressure. There was a significant correlation between the decrease in plasma kininogen and increase in kinin levels, decrease in total peripheral resistance, and the increase in circulation time. However, there was no correlation with the decrease in cardiac output. Also interestingly, they found that the levels of kinin in the non-survivors remained within normal limits. They concluded that the release of kinins at the local myocardial site may perhaps be a beneficial compensatory mechanism contributing towards survival. This study and that by Kolber-Postepska (1975), suggest that possibly kininase activity is decreased in the survivors. This observation was

confirmed in a later study (Hashimoto et al, 1976). They found that kininase levels were higher in non-survivors.

Elevated kinin levels have also been observed in aortic and coronary sinus blood in the dog following the experimental occlusion of the left anterior descending coronary artery (Kimura et al, 1973; Hashimoto et al, 1977; Matsuki et al, 1986; Matsuki et al, 1987). As in the human studies, the results are conflicting and there is no clear cut evidence of whether or not kinins subserve a purely pathological role and hence offer an explanation for the cause of cardiogenic shock and anginal pain. Likewise there is no clear cut evidence for a beneficial role in terms of improving coronary haemodynamics and metabolic parameters during acute myocardial infarction.

1.6. ANGIOTENSIN CONVERTING ENZYME INHIBITORS - ROLE PLAYED IN THE TREATMENT OF ACUTE MYOCARDIAL INFARCTION.

The available evidence shows that the kallikrein-kinin system is involved in ischaemic heart disease and acute myocardial infarction. A lot of the evidence shows that kinins accumulate in the ischaemic myocardium, as indicated by measurements taken in plasma from the coronary sinus. However, until recently, the exact role of kinins in ischaemic heart disease remained unknown. A new insight has now been provided by the introduction of angiotensin-converting enzyme inhibitors.

The inhibition of angiotensin-converting enzyme (ACE) results in

accumulation of bradykinin and decrease in the formation of angiotensin II. Inhibition of ACE is now one of the new approaches in the treatment of hypertension and congestive heart failure. Inhibition of ACE has been associated with the reduction of ventricular arrhythmias in patients with congestive heart failure after myocardial infarction (Cleland et al, 1984; Cleland et al, 1985; Webster et al, 1985; and Packer et al, 1987). Angiotensin-converting enzyme inhibitors prevented reperfusion-induced ventricular fibrillation in the isolated rat heart (Van Gilst et al, 1986; Linz et al, 1986). Both bradykinin, at doses not affecting haemodynamic parameters, and converting enzyme inhibitors, improved metabolic parameters and showed a significant cardioprotective effect against ischaemic-reperfusion injury (Martorana et al, 1990; Linz et al, 1989, De Langen et al, 1989). Using a bradykinin antagonist all these beneficial effects were abolished showing that they are due to bradykinin (Scholkens et al, 1988; Scholkens and Linz, 1988). Both converting enzyme inhibitors and bradykinin inhibit noradrenaline efflux in ischaemic myocardium in rat heart (Carlsson and Abrahamson, 1989). This further contributes to amelioration of the ischaemic consequences owing to the reduction of ventricular arrhythmias caused by noradrenaline.

1.7. KININ METABOLIZING ENZYMES AND MYOCARDIAL ISCHAEMIA.

Kinins are metabolized by a host of enzymes collectively refered to as kininases. Kininases are widely distributed in the body. They are present in plasma, vascular tissues, the heart and other organs. Kininases are classified into two categories, kininase I-type and kininase II-type.

Kininase I is a carboxypeptidase that releases the C-terminal Arg^9 of bradykinin (Erdos and Sloane, 1962; Erdos et al, 1964; Erdos et al, 1967). The two members which appear in this group are carboxypeptidase N and carboxypeptidase M. Carboxypeptidase N is present in plasma and the liver. Carboxypeptidase M is a membrane bound enzyme but it is also present in urine.

The kininase II group includes those enzymes which remove a C-terminal dipeptide Phe^8-Arg^9 from bradykinin. Angiotensin-converting enzyme and the neutral endopeptidase 24.11 fall under this group. Angiotensin-I-converting enzyme is a widely distributed enzyme. It is present in endothelial, epithelial and neuroepithelial cells (Erdos, 1990). Neutral endopeptidase 24.11 is present in epithelial and neuroepithelial cells (Erdos, 1990). It is present on membranes in kidneys (Kenny and Stephenson, 1987).

The activity of these enzymes is impaired under acidic conditions and hence their kininase activity (Edery and Lewis, 1962). At the time they did this study no actual measurement of kinins in ischaemic myocardium had been made, but they speculated that the lowering of pH in ischaemic myocardium may lead to accumulation of kinins due to reduced kininase activity. A similar observation was made by Kolber-Postepska (1975). He postulated that reduced kininase activity in patients with myocardial infarction may prolong the presence of kinins in plasma and thus increase biological activity.

The recent reports on the beneficial effects of angiotensin-converting enzyme

inhibitors in ischaemic heart conditions is a clear support of the theory of decreased kininase activity resulting in accumulation of kinins although, to this end, for beneficial and not for deleterious effects. However, it is unlikely that the inhibition of kininases by itself is enough to account for the levels of kinins in the coronary effluent following an ischaemic episode. Sometimes during ischaemia the kinin levels in the coronary sinus are more than double the normal levels as was observed in an experimental model of myocardial ischaemia (Matsuki et al, 1987).

The presence of kininogenases in the myocardium, which may be activated during an ischaemic episode to release kinins, seems a more plausible explanation. Indeed this would be very important since most of the kinins released in the periphery can hardly reach the heart because most of them would be metabolized as they pass through the lungs. Most of kinins are metabolized by kininases on the pulmonary vascular endothelium (Ryan, 1985).

1.8 THE PRESENCE OF KININ-FORMING ENZYMES IN CARDIOVASCULAR TISSUES.

Kinins are involved in the regulation of blood flow and blood pressure (Mills, 1979), as well as in the pathogenesis of experimental and human hypertension (Carretero and Scicli, 1978). In myocardial infarction it is also becoming increasingly obvious that kinins participate in improving coronary haemodynamics and metabolic parameters (Scholkens et al, 1988), also they reduce the incidence and severity of ventricular arrhythmias by reducing the release of catecholamines (Carlsson and Abrahamsson, 1989).

For kinins to exert their effects they have to be available in sufficient amounts at the site of action. This points to the importance of local kinin formation because if the only source of kinins is through generation in plasma then it will not be possible to get a high enough concentration at the site of action due to destruction both in plasma and on the pulmonary vascular endothelium (Ryan, 1985). All Vascular endothelium also contains kinin-destroying enzymes, thus making it increasingly difficult for kinins generated in plasma to reach a remote site of action in the tissues.

A lot of information is now accumulating to show that kinin-forming enzymes are present in both vascular and cardiac tissues. Kinin-forming enzymes were first reported in human, bovine, and rat cardiac tissues.
The cardiac tissues of the three species were reported to contain an acid optimum enzyme, although the cardiac tissue used was not specified (Nolly et al, 1981a). This enzyme was localized in the lysosomal fraction of rat "cardiac tissue" (Britos and Nolly, 1981).

In the same year two kinin-forming enzymes, one with a pH-optimum at pH 4-5 and another at pH 7-9, were reported to be present in rat mesenteric arteries (Nolly et al, 1981b). The acid optimum enzyme was inhibited by pepstatin between 10^{-3} and 10^{-5} M, showing that it had properties similar to those of aspartic proteases. The neutral enzyme was resistant to inhibition by pepstatin but it was inhibited by aprotinin. Both were resistant to inhibition by soybean trypsin inhibitor (Nolly et al, 1981b). The neutral enzyme in rat mesenteric arteries was at first suspected to be due to contamination with pancreatic trypsin. However, in a subsequent study it was proved to be a

kallikrein-like enzyme (Nolly and Lama, 1982).

Nolly et al (1985) isolated a neutral enzyme from rat tail arteries and veins. This enzyme had properties similar to urinary kallikrein. Both purified rat vascular kininogenase and urinary kallikrein had similar molecular weights (37 kDa), isoelectric points, electrophoretic mobilities, and inhibition profiles (Nolly et al, 1985).

The aortae of both normotensive and hypertensive rats were reported to contain two kininogenases, both resistant to inhibition by soybean trypsin inhibitor (Nolly et al, 1987). One had a molecular weight of 37 kDa, and it was bound by kallikrein antibodies. The levels of this kininogenase were lower in the aortae of hypertensive rats than in the normotensive rats (Nolly et al, 1987). The second soybean trypsin inhibitor-resistant kininogenase had a molecular weight of 33 kDa. This enzyme was not further characterized. Recently, using culture cells the rat aorta has been confirmed to contain glandular kallikrein (Oza et al, 1990). In this work it was shown that the rat aorta vascular smooth muscle synthesize both glandular kallikrein and kininogen. Oza and co-workers (1990) also showed that the same cells synthesize kinin-destroying enzymes. This was the first report of its kind to show that an independent kinin-generating system is present on the vasculature and that while it is possible to generate kinins for immediate local action the same system is capable of inactivating the released kinins. It will, probably, be interesting to determine the relative rates of local release and inactivation of kinins. It might be the case in ischaemic heart disease that, the kinin-forming enzymes are activated in circumstances which render the kinin-destroying enzymes inactive (Edery

and Lewis, 1962).

The dog ventricular myocardium and coronary arteries were reported to contain both acid and neutral kinin-forming enzymes (Zeitlin et al, 1989). The acid optimum enzyme, like that in rat mesenteric arteries, was inhibited by pepstatin. It had properties similar to an aspartic proteinase. The neutral enzyme had properties similar to plasma kallikrein. This was suspected to be plasma kallikrein resulting from contamination with blood (Zeitlin et al, 1989).

The neutral enzymes, tissue kallikrein (Xiong et al, 1990), and arginine esterase A (Simson et al, 1989) have been reported to be present in the rat atrial tissue. Arginine esterase A is a kallikrein-like enzyme and is located in the same granules as atrial natriuretic peptide (Simson et al, 1989). Tissue kallikrein was also found to be present in the atrial granules (Xiong et al, 1990). It seems, therefore, that in all cardiovascular tissues there are present both acid and neutral kinin-forming enzymes, mainly in the inactive form (Nolly et al, 1981a; Xiong et al, 1990) and whenever required they can be activated for the release of kinins and other biologically active peptides.

1.10 AIM OF THE STUDY

Levels of kinins in the coronary sinus and aortic blood are elevated following the experimental occlusion of the LAD coronary artery in the dog (Kimura et al, 1973; Matsuki et al, 1987). Also activated kallikrein-like activity was found in 7 out 11 patients with myocardial infarction (Pitt et al, 1970). It has been speculated that there are present in both cardiac and vascular tissues

kinin-forming enzymes which are activated during myocardial ischaemia. Initial studies cited above report the presence of such enzymes in mammalian cardiac and vascular tissues.

The aims of this study were:

1. To screen rat and dog cardiac and vascular tissues for kinin-forming enzymes using ventricular myocardium and aorta.
2. To characterize the kinin-forming enzymes in these tissues
3. To determine the effect of acute myocardial ischaemia on the levels of these enzymes and how they may be related to plasma kinin levels.

CHAPTER 2

METHODS VALIDATION EXPERIMENTS

Three main types of assays were used in this study, amidase assays, using the chromogenic substrate S 2266 (D-Val-Leu-Arg-pNA); bioassay using the isolated oestrous rat uterus and radioimmunoassay. These assays were assessed to evaluate their suitability.

STATISTICS.

The results are presented as mean ± S.D, except where indicated otherwise. Comparison of group results was done using the non-parametric Mann-Whitney U test. Where more than two groups were involved one way analysis of variance was used followed by the Neuman-Keuls range test. The results were considered significantly different when $P \leq 0.05$.

2.1 AMIDASE ASSAYS.

INTRODUCTION

Kallikreins are endogenous enzymes which rapidly and specifically liberate kinins from either or both low and high molecular weight kininogens. The release of kinins from kininogens occurs upon the cleavage of bonds between or to the right of basic amino acids such as arginine or lysine. Tissue kallikrein for example releases lysyl-bradykinin by the cleavage of an NH_2-terminal Met-Lys- and a COOH-terminal Arg-Ser bond. The realization of the bonds required for cleavage has made it possible to make synthetic substrates specific for different enzymes. The tripeptide substrate D-Val-Leu-Arg-pNA (S 2266), the substrate for tissue kallikrein, is one such example. Tissue kallikrein cleaves the paranitroaniline moiety to the right of arginine (basic amino acid) of this tripeptide substrate releasing

paranitroaniline which can be monitored spectrophotometrically at 405 nm. The tripeptide substrate for tissue kallikrein was chosen for use in this work following the previous work done in our laboratory (Zeitlin et al, 1989). In this work it was shown that both acid and neutral kinin-forming enzymes in dog ventricles and coronary arteries were capable of cleaving this substrate.

2.1.1 ASSESSMENT OF LINEARITY OF CLEAVING PNA FROM THE TRIPEPTIDE SUBSTRATE D-VAL-LEU-ARG-pNA WITH ENZYME CONCENTRATION.

The progression of an enzymatic reaction is only linear to a certain point, that is up to about 60 - 70 % substrate utilization, and beyond this the reaction slows down and the reaction curve flattens. For the comparison of the rates of different reactions only the linear part is useful when there is still an excess of the substrate. In all the assays done in this work 0.1 ml of either 1.5mM or 0.75 mM solutions of the substrate were used. The 1.5 mM solution was chosen for this experiment. Trypsin, a serine proteinase from the pancreas, was used. It is similar in many ways to tissue kallikrein and hydrolyses the tripeptide D-Val-Leu-Arg-pNA.

Method

Pure crystalline trypsin (10 mg) was weighed and dissolved in Tris buffer (pH 8.5) containing 0.001M $CaCl_2$ to give a concentration of 100 µg/ml. Further dilutions were made with the same buffer to make 0.02, 0.05, 0.1, 0.2, 0.3, 0.4, 0.8, 1.0, 2.0, and 4.0 µg/ml. Tris buffer (0.5 ml, pH 8.5) containing 0.001 M $CaCl_2$ was pre-incubated at 37 ^{0}C for 10 minutes. Each of the trypsin concentrations were added (0.4 ml) and pre-incubation was continued

for another 5 minutes. The reaction was started by the addition of 0.1 ml (1.5 mM) of substrate and continued for 30 minutes. The reaction was stopped by the addition of 0.1 ml (50 % v/v) acetic acid. For each dilution a blank was included in which the enzyme was inactivated with 50 % acetic acid before the substrate was added. Released pNA was measured as a change in absorbance and then converted to concentration in nmoles/ml using a pNA concentration/absorbance calibration curve.

RESULTS.

Figure 2.1 shows the release of pNA by different trypsin concentrations. The release of pNA was linear with increasing trypsin concentrations up to 0.4 µg/ml ($r = 0.9955$; $P < 0.001$). At this concentration there is 70 % utilization above which the curve flattens into a plateau. Incubation times and enzyme concentrations were subsequently adjusted so as to work below this limit of substrate utilization.

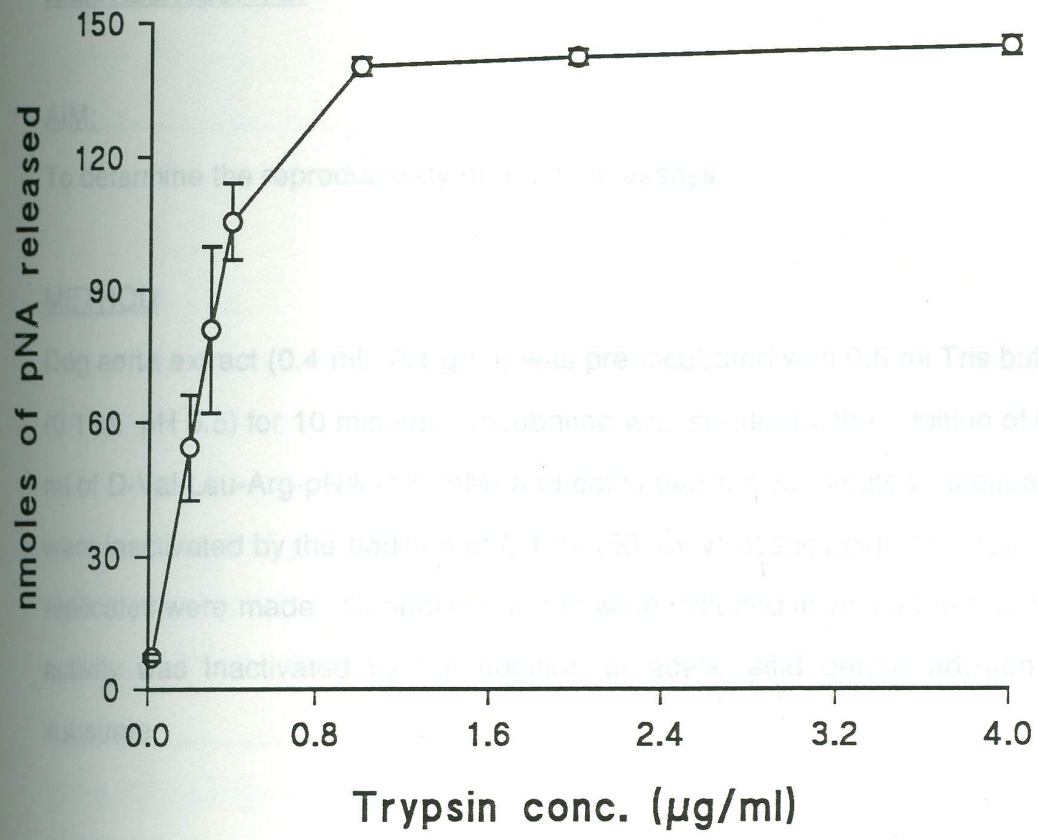


Figure 2.1 The curve for the hydrolysis of D-Val-Leu-Arg-pNA by trypsin. Trypsin was used to assess the linearity of cleavage of pNA from this substrate with enzyme concentration. Each point represents mean ± S.D (n = 3).

2.1.2 DETERMINATION OF THE COEFFICIENT OF VARIATION FOR AMIDASE ASSAYS.

AIM:

To determine the reproducibility of amidase assays.

METHOD

Dog aorta extract (0.4 ml, 0.1 g/ml) was pre-incubated with 0.5 ml Tris buffer (0.1 M, pH 8.5) for 10 minutes. Incubation was started by the addition of 0.1 ml of D-Val-Leu-Arg-pNA (1.5 mM) and continued for 90 minutes. Incubates were inactivated by the addition of 0.1 ml (50 %v/v) acetic acid. Six incubate replicates were made. Control incubates were included in which the enzymic activity was inactivated by the addition of acetic acid before addition of substrate.

RESULTS.

Table 2.1 shows the release of pNA by 40 mg (wet tissue equivalent) of dog aorta from the tripeptide substrate S2266 at pH 8.5. Six incubate replicates were made. The released pNA was calculated by changing absorbance units into nmoles of pNA. The coefficient of variation which is defined as S.D/mean X 100 was 6.8 % .

Experiment no.	nmoles of pNA/ml
1	1.83
2	2.06
3	1.83
4	2.17
5	1.94
6	1.94

Mean $\pm$ S.D = 1.96 $\pm$ 0.13 nmoles pNA/ml

Coefficient of variation (C.V) = (S.D / Mean) x 100 =

0.13 x 100 / 1.96 = 6.8 %

Table 2.1 The determination of the coefficient of variation for amidase assays. Incubates of dog aorta were made with the tripeptide substrate D-Val-Leu-Arg-pNA. The released pNA was determined, the mean and standard deviation calculated, and from these the coefficient of variation was determined.

2.2 RADIOIMMUNOASSAY.

Radioimmunoassay of released and plasma kinins was done according to a protocol supplied by Sera-Lab with some modifications introduced in our laboratory.

Assay protocol.

Assay samples (0.1 ml) were incubated with 0.1 ml of polyclonal rabbit anti-bradykinin antiserum (diluted in potassium-phosphate buffer, pH 7.4) and 0.1 ml of potassium phosphate buffer (containing 1 g/litre BSA, 0.1 g/l sodium azide and 9g/l NaCl) for 18 - 24 hours. Bradykinin-tyrosine-8-[I^{125}] (0.1 ml, 15000 cpm) was then added and incubation continued for another 48 hours. The antibody-antigen complex was precipitated by the addition of 20 µl of normal rabbit serum and 1 ml of isopropyl alcohol. The bound radioactivity was then calculated.

Standard curve.

The standard curve was made using synthetic bradykinin. The working range for the standard curve was fixed between 8 - 8000 pg/tube. Beyond these two points it is not certain if the accuracy is dependable. Unknown sample concentrations were interpolated in the standard curve using a computer programme built into the gamma counter (Packard Multi-Prias).

2.2.1 DETERMINATION OF THE COEFFICIENT OF VARIATION FOR RADIOIMMUNOASSAYS

AIM.

To determine the amount of variation inherent in the radioimmunoassay.

METHOD.

Aqueous extract of rat ventricles (0.2 ml, 10 mg/ml) was pre-incubated with 0.25 ml citrate-phosphate buffer (0.1 M, pH 5.0) for 10 minutes. Incubation was started by the addition of 50 µl (50 µg/ml) L-kininogen and continued for 10 minutes. Incubation was terminated by boiling the tubes on a water bath for 10 minutes.

Extraction of released kinins.

The incubates (0.2 ml) were pipetted into new tubes and 20 µl (0.1 g/ml) bovine serum albumin added. Absolute alcohol (1 ml) was added, the mixture heated at 70 °C on a water bath for 10 minutes and then centrifuged (2000 g, 4 °C, 10 minutes). The supernatant was evaporated over a stream of oxygen free nitrogen on a water bath at 70 °C.

Radioimmunoassay.

Radioimmunoassay was done as indicated above after reconstituting the tube contents in 0.2 ml of the assay buffer. No further buffer was added.

RESULTS.

Six replicates were used for the incubates which were extracted and assayed separately. The values obtained vary from 1262.7 pg/0.2 ml to 1706.4

representing a mean ± S.D of 1527.3 ± 186.7 pg/0.2 ml of incubate (Table 2.2). The coefficient of variation is 12.2 %. This is a relatively big variation, especially when compared with the amidase assays in which the coefficient of variation was only about 7 %. However, a lot more procedures are involved in a radioimmunoassay and this will increase errors in the values finally obtained.

Experiment no.	pg/100 µl
1	1706.4
2	1699.0
3	1576.4
4.	1576.4
5	1242.9
6.	1362.7

Mean ± S.D 1527.3 ± 186.7 .

C.V = (S.D / Mean) x 100 = 12.2 %

Table 2.2 The determination of the coefficient of variation for radioimmunoassay. Aqueous extract of rat ventricles was incubated with L-kininogen (pH 5.0) and the incubates assayed by radioimmunoassay. Six incubate replicates were made and the mean and standard deviation determined and these were used to calculate coefficient of variation.

2.3 ASSESSMENT OF LINEARITY FOR THE RELEASE OF BRADYKININ-LIKE IMMUNOREACTIVITY BY DIFFERENT CONCENTRATIONS OF AQUEOUS EXTRACT OF RAT VENTRICLES.

AIM

To determine the concentration dependent hydrolysis of L-kininogen by aqueous extracts of rat ventricular myocardium.

METHOD

Male Sprague Dawley rats 250 - 300 g were used. They were killed by a blow on the head followed by cervical dislocation. The abdomen and chest were opened alone the midline and the aorta cannulated. The hearts were perfused retrogradely with a Watson-Marlow slow rate flow pump at the rate of 4 ml min^{-1} until the hearts were cleared of blood. The ventricles were isolated, chopped into small pieces with a pair of scissors and then homogenized in distilled water (0.1 g ml^{-1}) containing 0.1 % Triton X-100. The homogenate was centrifuged (2000 g, 4 ^{o} C, 30 min), dialysed under tap water for 18 hours. The dialysed extract was diluted in distilled water to give concentrations of 5, 10, 20, 30, and 50 mg/ml. The extracts (0.2 ml) were each pre-incubated with 0.25 ml citrate-phosphate buffer (0.1 M, pH 5.0) for 10 minutes. Incubations were started by the addition of 50 µl (50 µg/ml) of L-kininogen and continued for 10 minutes. The incubates were inactivated by boiling in a water bath for 10 minutes. Both enzyme and substrate blanks were included. The incubates were extracted with 80 % ethanol in the presence of 1 % BSA (Section 2.6) and the released bradykinin-like immunoreactivity was assayed by radioimmunoassay.

Figure 2.3 shows the release of bradykinin-like immunoreactivity (BKIR) from L-kininogen by different concentrations of an aqueous extract of rat ventricles. The release of BKIR increased linearly with increasing concentration of the ventricular extract up to 30 mg/ml ($r = 0.9714$; $P < 0.001$). Above this concentration the rate of release decreased and at 50 mg/ml, although there was still an increase in the released BKIR, the linearity had stopped.

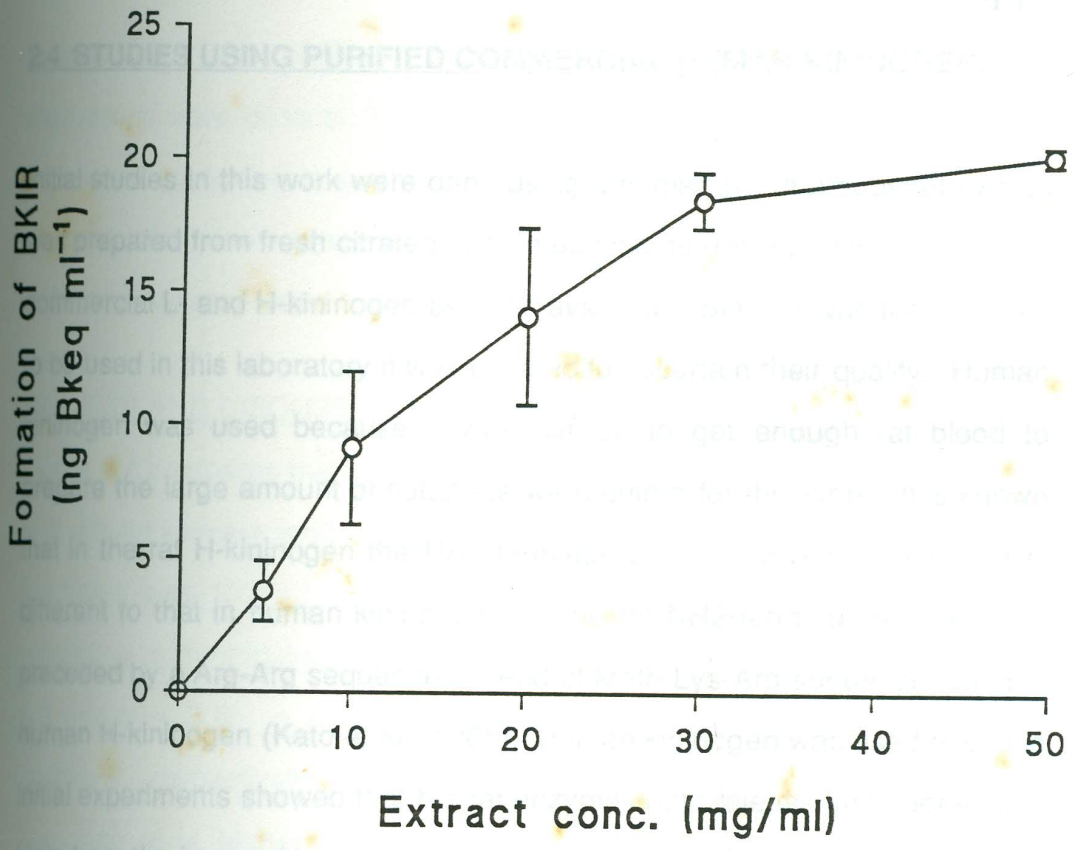


Figure 2.3 Concentration related release of bradykinin-like immunoreactivity (BKIR) from L-kininogen by aqueous extracts of rat ventricles. The aqueous extract (5 - 50 mg/ml) was incubated with 50 µl (50 µg/ml) of L-kininogen for 10 minutes (see methods). The released BKIR was separated by selective precipitation of intact kininogen with 80 % ethanol in the presence of 1 % BSA and assayed by radioimmunoassay. The results are presented as mean ± S.D (n = 6).

2.4 STUDIES USING PURIFIED COMMERCIAL HUMAN KININOGEN.

Initial studies in this work were done using a human plasma substrate which was prepared from fresh citrated human plasma as described later. Later on commercial L- and H-kininogen became available. Since it was the first time to be used in this laboratory it was decided to ascertain their quality. Human kininogen was used because it was difficult to get enough rat blood to prepare the large amount of substrate we required for this work. It is known that in the rat H-kininogen the NH2-terminus of the bradykinin sequence is different to that in human kininogen. At the rat NH2-terminus bradykinin is preceded by a Arg-Arg sequence instead of Meth-Lys-Arg sequence found in human H-kininogen (Kato et al, 1985). Human kininogen was used because initial experiments showed that the rat enzyme could release immunoreactive kinin from the human kininogen.

2.4.1 ASSAY FOR KININ DESTROYING ACTIVITY

AIM

To determine whether commercial kininogens used in assays contained any kinin destroying activity.

Method:

To assay for the presence of kininase activity in the substrates incubates of L-kininogen and H-kininogen were made with and without kininase inhibitors. Incubates without kininase inhibitors contained 1.6 ml Tris buffer (0.1 M, pH 8.0), 0.3 ml of L-kininogen or H-kininogen (20 µg/ml) and 0.1 ml of synthetic bradykinin (100 ng/ml). When the inhibitors were used 0.2 ml of buffer were

replaced by 0.1 ml each of EDTA(0.3 M) and o-phenanthroline (0.01 M). Incubations were done at 37 °C for 30 minutes. The incubates were then inactivated by boiling in a water bath for 10 minutes. The incubates were assayed for the presence of bradykinin-like immunoreactivity by radioimmunoassay using the polyclonal rabbit anti-bradykinin antiserum.

RESULTS AND DISCUSSION

Figure 2.4 compares the amount of BKIR measured in incubates made with and without kininase inhibitors. The total amount of bradykinin added into the incubates was 10 ng, giving a concentration of 5 ng/ml. The amount remaining in the incubates was 5.93 ± 1.7 (mean ± S.D; n = 3) and 5.52 ± 0.8 (mean ± S.D; n = 3) ng/ml in the presence and in the absence of inhibitors, respectively. The amount of bradykinin in the incubates of L-kininogen were higher than the amount of bradykinin added. It could be assumed there was no destruction. However, for H-kininogen the mean values obtained were slightly lower than the amount of bradykinin which was originally added. The amount of BKIR in the presence and in the absence of inhibitors was 3.75 ± 0.80 (mean ± S.D; n = 3) and 3.50 ± 0.60 (mean ± S.D; n = 3) ng/ml, respectively. There is no apparent difference between incubates with kininase inhibitors and those without. The difference could be attributed to variations in the assay since the actual value is within 2 standard deviations of the mean values obtained. For both kininogens there was no significant difference between incubates with and without kininase inhibitors ($P > 0.05$).

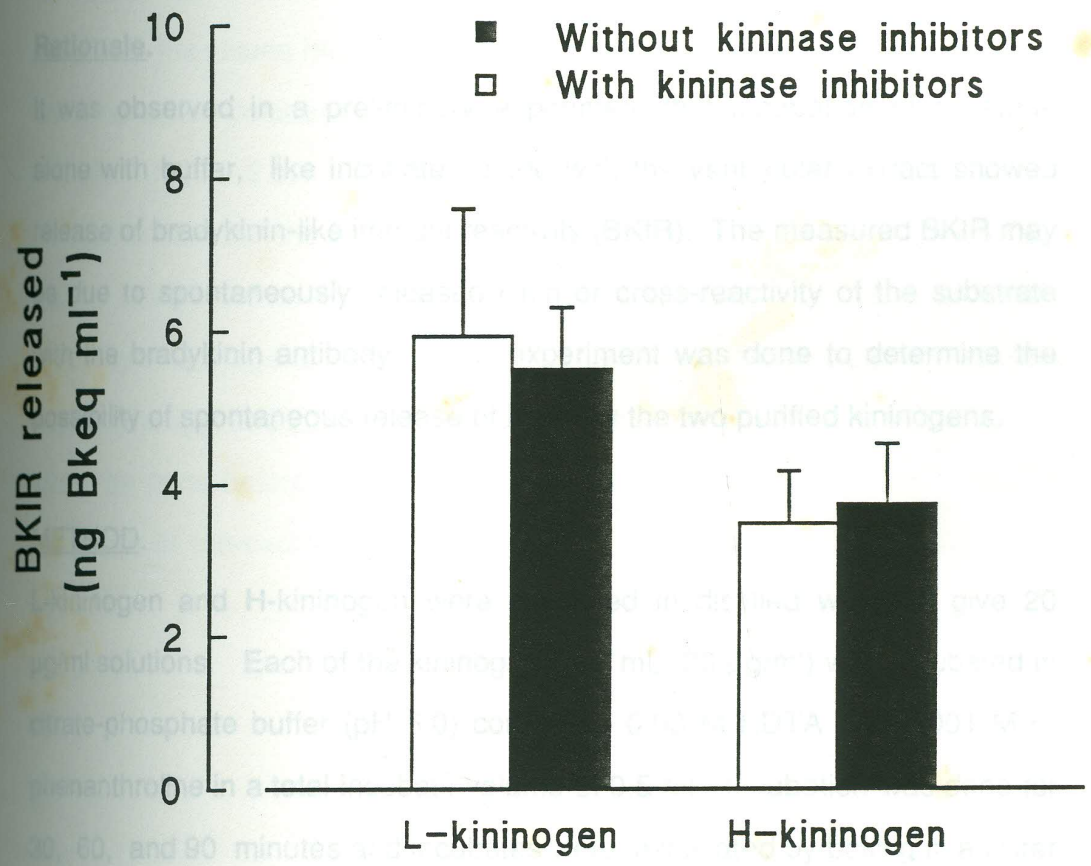


Figure 2.4. The assay of H-kininogen and L-kininogen for kininase activity. 5 ng/ml of synthetic bradykinin were incubated in buffer with either L- or H-kininogen for 30 minutes. Two sets for each were made; one with kininase inhibitors (0.03M EDTA and 0.001M o-phenanthroline) and another without kininase inhibitors. The results are presented as mean $\pm$ S.D (n = 3).

2.5 ASSAY FOR SPONTANEOUS RELEASE OF KININS BY L-KININOGEN AND H-KININOGEN

Rationale.

It was observed in a preliminary experiment that incubation of substrate alone with buffer, like incubates made with the ventricular extract showed release of bradykinin-like immunoreactivity (BKIR). The measured BKIR may be due to spontaneously released kinin or cross-reactivity of the substrate with the bradykinin antibody. This experiment was done to determine the possibility of spontaneous release of BKIR by the two purified kininogens.

METHOD.

L-kininogen and H-kininogen were dissolved in distilled water to give 20 µg/ml solutions. Each of the kininogens(0.3 ml, 20 µg/ml) was incubated in citrate-phosphate buffer (pH 5.0) containing 0.03 M EDTA and 0.001 M o-phenanthroline in a total incubate volume of 0.5 ml. Incubation was done for 30, 60, and 90 minutes and incubates were inactivated by boiling in a water bath for 10 min. For each kininogen two other sets of incubates were included in which after addition of kininogen to buffer and mixing assay for BKIR was done immediately, with one set boiled and another without boiling (incubation time 0 minutes). Released BKIR was assayed by radioimmunoassay.

RESULTS AND DISCUSSION.

Figures 2.5.1 and 2.5.2 show the release of BKIR by incubates of L-kininogen and H-kininogen in buffer at 0 - 90 minutes of incubation. Higher values of BKIR were recorded in incubates which were assayed without

boiling. This is true for both high and low molecular weight kininogen. Boiling caused a mean decrease in the amount of the measured BKIR of 30 % and 59 % for low and high molecular weight kininogen respectively. However, the values measured for the two kininogens at different incubation times remained constant up to 90 minutes, regardless of incubation time ($P > 0.05$). Figure 2.5.1 shows that the measured BKIR was decreasing with incubation time but the difference was not statistically significant ($P > 0.05$). This is interpreted to mean that the observed BKIR is a result of cross-reactivity of kininogens with the bradykinin antibody, a phenomenon reported before (Spragg et al, 1976). This requires, therefore, that extraction of kinins be done before radioimmunoassay is done. Two methods, ethanol extraction or ion-exchange chromatography could be used (Spragg et al, 1976)

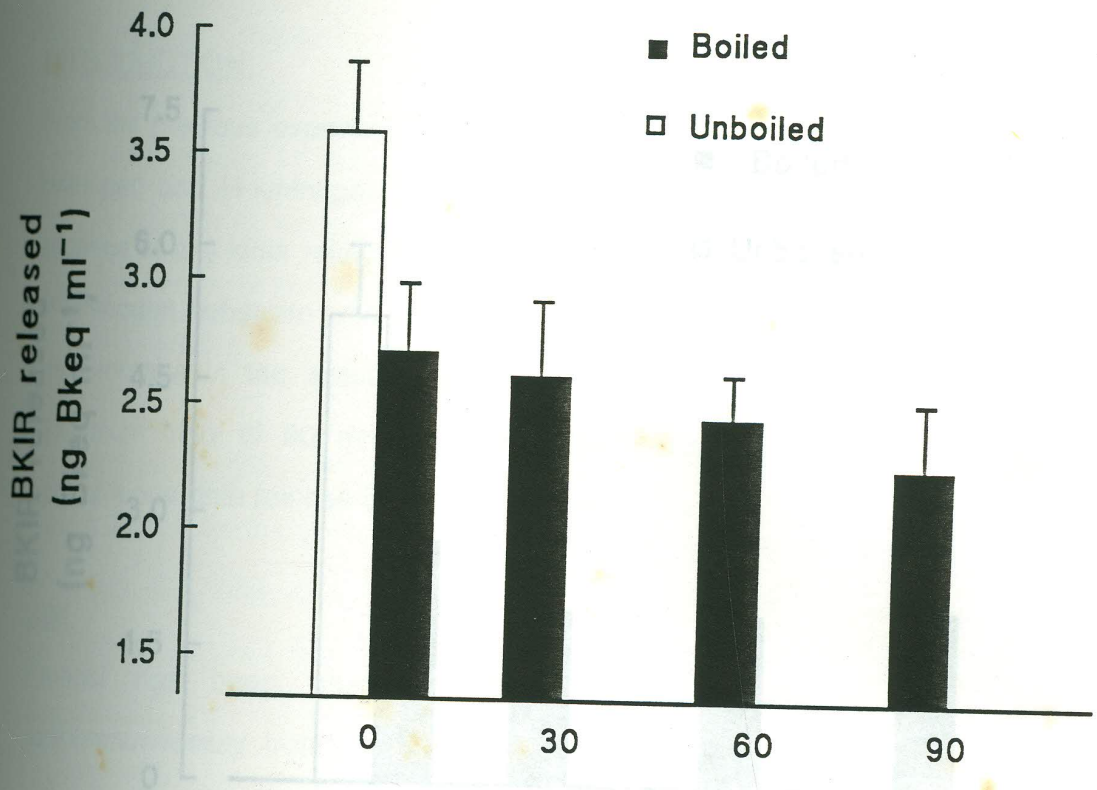


Figure 2.5.1 Time-dependent release of BKIR from L-kininogen. L-Kininogen was incubated in citrate-phosphate buffer (0.1 M, pH 5.0) for 0 to 90 minutes. The incubates at each point were inactivated by boiling. One set of incubates at time 0 was not boiled. The incubates were assayed by radioimmunoassay. The results are presented as mean ± S.D (n = 5).

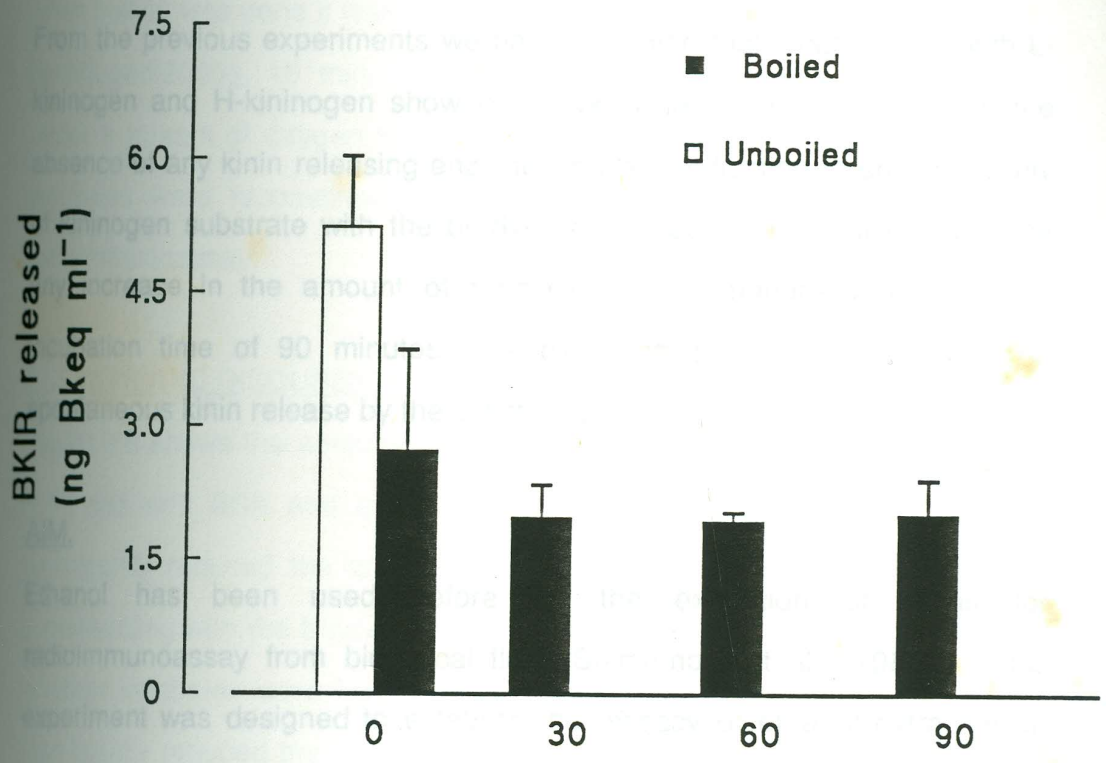


Figure 2.5.2 Time-dependent release of BKIR from H-kininogen. H-Kininogen was incubated in citrate-phosphate buffer (0.1 M, pH 5.0) for 0 to 90 minutes. The incubates at each point were inactivated by boiling. One set of incubates at time 0 was not boiled. The incubates were assayed by radioimmunoassay. The results are presented as mean $\pm$ S.D (n = 5).

2.6. THE USE OF ETHANOL TO EXTRACT BKIR FROM INCUBATES.

INTRODUCTION:

From the previous experiments we have observed that incubates of both L-kininogen and H-kininogen showed bradykinin-like immunoreactivity in the absence of any kinin releasing enzyme, a clear indication of crossreactivity of kininogen substrate with the bradykinin antibody. The failure to observe any increase in the amount of bradykinin-like immunoreactivity over an incubation time of 90 minutes is a clear indication that there was no spontaneous kinin release by the substrates.

AIM.

Ethanol has been used before for the extraction of kinins for radioimmunoassay from biological fluids(Shimamoto et al, 1988). This experiment was designed to establish the efficacy of ethanol extraction in removing uncleaved kininogen from incubates.

METHOD.

The concentrations of kininogen used for assays are in the microgramme range. It was anticipated that in the presence of such low concentrations it might be difficult to precipitate out the kininogen with alcohol. It was decided to use BSA at a concentration of 10 mg/ml(1%) to act as a nucleus for precipitation of the kininogen.

Three sets of incubates were made :

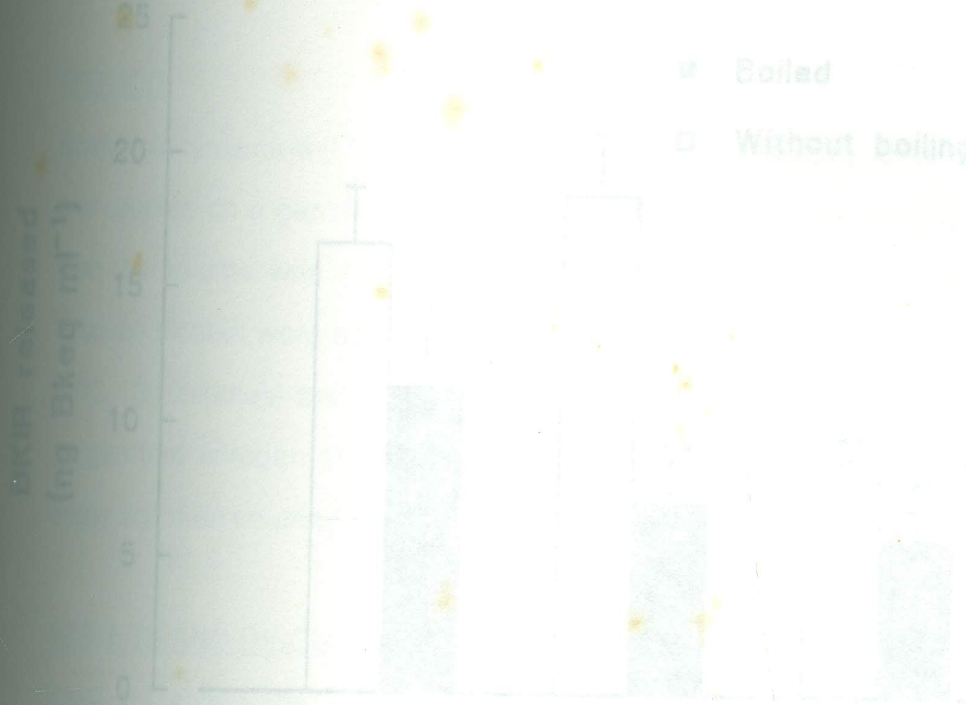
Set 1 consisted of 0.44ml citrate-phosphate buffer (0.1 M, pH 5.0) and 0.1ml L-kininogen (25 µg/ml). Set 2 contained 0.4 ml buffer, 0.1 ml substrate and 0.04 ml BSA (135 mg/ml). Set 3 contained 0.4 ml buffer, 0.1 ml substrate

and 0.04 ml of BSA (135 mg/ml). The three sets were made in duplicate so that one set was boiled at 70 ^{o} C, while the other one was assayed directly without boiling. Incubates in set three were extracted with 80 % ethanol and when boiling was done it was done in the presence of ethanol. All sets were centrifuged(2000g, 15 min, 4 ^{o}C) supernatant separated and evaporated under a stream of oxygen free nitrogen on a water bath at 70 ^{o} C. The incubates were reconstituted in 0.54 ml 0f buffer and then assayed by radioimmunoassay.

RESULTS AND DISCUSSION.

Figure 2.6 shows the amounts of kininogen in incubates without BSA, with BSA and with BSA and extracted with ethanol. Extraction with ethanol significantly reduced the amount of the substrate kininogens in incubates crossreacting with the bradykinin antibody. This was achieved regardless of whether incubates were boiled or not. In the previous experiment boiling significantly reduced the amount of measured BKIR in incubates ($P < 0.05$). This is also true in this experiment; smaller amounts of crossreactive kininogen remained behind after incubates were boiled. The percentage decrease in BKIR is the same for both boiled and unboiled incubates but overall the values obtained after boiling are much lower. The use of BSA was adopted from the Sera-Lab protocol for bradykinin RIA. The protocol uses BSA to aid the precipitation of antigen-antibody complex. The results show that addition of BSA does not have any significant effect on the amount of measured BKIR ($P > 0.05$). The values of BKIR obtained without and with BSA were 1668.2 $\pm$ 210.6 pg/0.1 ml and 1855.0 $\pm$ 224.73 pg/0.1 ml (mean $\pm$ S D) respectively. After boiling incubates containing BSA showed a

significantly lower amount of BKIR; 1142.4 ± 205.1 and 721.5 ± 148.1 without and with BSA respectively ($P < 0.05$).



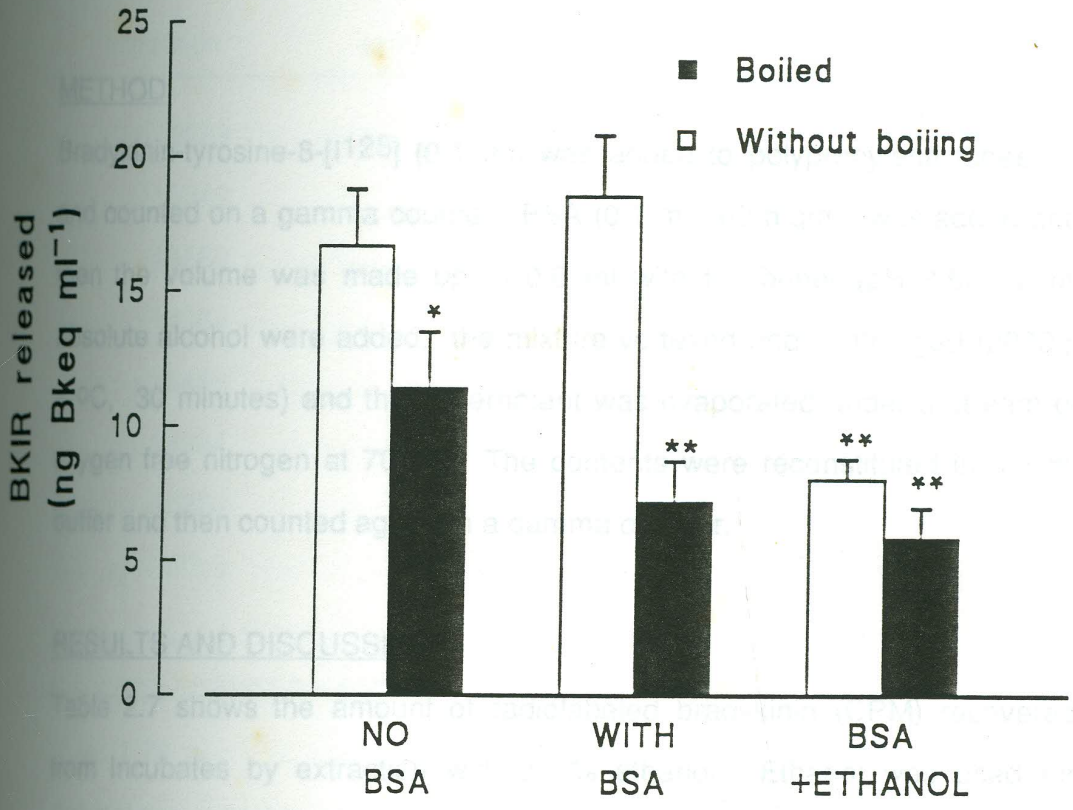


Figure 2.6. The extraction of L-kininogen by ethanol in the presence of 1 % bovine serum albumin. The figure shows measured values of BKIR from crossreacting kininogen with and without BSA and with BSA followed by ethanol extraction. The results are presented as mean ± S.D (n = 6).

2.7 DETERMINATION OF RECOVERY FOR ETHANOL EXTRACTION OF BRADYKININ IN THE PRESENCE OF 1% BSA.

METHOD.

Bradykinin-tyrosine-8-[I^{125}] (0.1 ml) was added to polypropylene tubes (6) and counted on a gamma counter. BSA (0.1 ml, 60 mg/ml) was added and then the volume was made up to 0.6 ml with tris buffer (pH 8.6). 2 ml absolute alcohol were added, the mixture vortexed and centrifuged (2000g, 4 ^{o}C, 30 minutes) and the supernatant was evaporated under a stream of oxygen free nitrogen at 70 ^{o}C. The contents were reconstituted in 0.2 ml buffer and then counted again on a gamma counter.

RESULTS AND DISCUSSION .

Table 2.7 shows the amount of radiolabeled bradykinin (CPM) recovered from incubates by extraction with 80 % ethanol. Ethanol was used for selective precipitation of kininogen and the extraction of bradykinin. The concentrations of kininogens used in these assays are, however, very small and it was thought that the addition of BSA might help to serve as a nucleus for precipitating out the kininogen. In the previous experiment it was shown that addition of BSA increased the efficiency of extraction. Proteins precipitate at acidic pH's and they tend to solubilize at alkaline pH's. It was thought that since the method would probably be used for extraction of both acid and alkaline incubates it was a good idea to do the extraction at an alkaline pH. The results show a high recovery rate (94.2 %) even though this time an alkaline pH was used. This shows that the precipitation of BSA and probably kininogens takes place effectively regardless of the pH of incubates. The variation within and between the extraction values were also quite low.

	cpm before extraction	cpm after extraction
	15533	14715
	15349	14300
	15560	14539
	14964	14264
	15458	14696
	14964	14007
Mean	15304	14420
S.D	273.8	278.1

C.V = 273.8/15304.7 x 100 = 1.79 %

C.V = 278.1 x 14420.2 x 100 = 1.93 %

Percentage recovery = 94.2 %

Table 2.7 The extraction of radiolabeled bradykinin with ethanol in the presence of 1 % BSA. Radiolabeled bradykinin was placed in tubes containing 1 % BSA (final concentration). The bradykinin was recovered by selective precipitation of BSA. The supernatant was separated (see methods) and the recovered radioactivity counted and percent recovery calculated.

2.8 BIOASSAY USING THE CONTRACTIONS OF THE OESTROUS RAT UTERUS.

2.8.1 PREPARATION AND STANDARDIZATION OF HUMAN PLASMA KININ-FORMING SUBSTRATE.

Preparation of substrate.

Fresh citrated human blood (1 part 3.8 % sodium citrate to 9 parts blood) was obtained from Glasgow Royal Infirmary Haematology Clinic. The blood was donated at the outpatient clinic by a disease and drug free donor. A kinin-forming plasma substrate was prepared according to the method of Zeitlin and co-workers (Zeitlin et al, 1976). Plasma was obtained by centrifuging the fresh blood (1300g, 4 ° C, 45 min) and kept in plastic containers under ice. The plasma was shaken 3 times at room temperature with glass beads; first with 0.1 g/ml of plasma for 5 minutes and then twice with 0.05 g/ ml of plasma for 10 minutes and 60 minutes, respectively. Each time the supernatant was separated by centrifugation (2000g, 4°C, 10 minutes). The supernatant was heated at 60 °C for 60 minutes, allowed to cool, and pH brought to pH 2 with 2N HCl. After incubation for 10 minutes at 37 °C the pH was raised to pH 7.4 with 2N NaoH, the mixture centrifuged and supernatant was stored at -20 °C ready for use as substrate.

Assay for total kinin releasing capacity.

The substrate was assayed for spontaneous release of kinins, the presence of kinin destroying activity, and the maximum kinin-forming capacity. Incubations were carried out as indicated in appendix 2.

Bioassay.

Female Sprague Dawley rats 130 - 200 g were used. The rats were injected with 200 µg/kg body weight of 30 % ethanolic diethylstilboestrol 18 - 24 hours before the assay. The rats were killed by a blow on the head followed by cervical dislocation. The abdomen was opened and the two uterus horns removed. One horn was used for the assay while the other was stored in De Jalon's solution at 4 °C. The horn was opened up and a piece 3 cm long was mounted in a 10 ml bath containing De Jalon's solution at 31 ± 0.5 °C constantly bubbled with 95 % O_2 and 5 % CO_2. Synthetic bradykinin was used as a standard in a bracketing assay to estimate the amount of total kinins released. The determination was done by bracketing the unknown doses between two close concentrations of the standard.

Calculation of test sample concentration by the bracketing assay.

The "three point" or "2+1" assay method was used. Let d_1 be the smaller dose of standard, d_2 the larger dose, t the dose of the "test" and suppose the mean effects they produce are S_1, S_2 and T, respectively. To increase the size of the response from S_1 to S_2 it is necessary to increase the log(dose) by $\log(d_2/d_1)$. So to increase the response from S_1 to T it should be necessary to increase the log(dose) by $(T-S_1)/(S_2-S_1) \times \log(d_2/d_1)$. The amount of standard which should produce the same response as the dose of the test is $d_1 \times \text{antilog } (T-S_1)/(S_1-S_2)\log(d_2/d_1)$.

The strength of the test solution will be this amount divided by t.

RESULTS.

High concentration of trypsin (200 µg/ml) was used for the maximum release of total kinins. To determine how the substrate keeps by storage at - 20 °C the assay was repeated one year later. The amount of total kinins released was 6.84 ± 1.5 µg of bradykinin equivalent/ ml. The same values were obtained after storage for one year. The substrate was free from kinin destroying activity. The total of 1.5 µg of bradykinin was added into 1.5 ml of incubate. The amount recovered was 1.44 ± 0.18 µg (n = 6). There was no difference between incubates with and those without o-phenanthroline. Incubates containing the substrate in buffer did not show any uterus contracting activity. The minimum detectable amount was 0.3 ng/ml 0f synthetic bradykinin.

DISCUSSION.

Excess trypsin was used to assess the maximum kinin releasing capacity of the plasma substrate. Assays done immediately after preparing the substrate and one year later gave the same values of released total kinins; 5.12 - 7.72 µg of bradykinin equivalent/ml (6.84 ± 1.5) and no detectable spontaneous kinin release within the sensitivity of the assay. It is observed from these results that storage at -20 °C keeps the substrate intact for at least one year even with freezing and thawing that may occur during use. The substrate was not, however, reused after it was thawed for any experiment.

NB: For all assays in this study the incubates were first centrifuged before assayed.

CHAPTER 3.

STUDIES ON KININ-FORMING ENZYMES IN DOG VENTRICULAR MYOCARDIUM.

INTRODUCTION

Levels of kinin in the coronary sinus and aortic blood are elevated following coronary artery occlusion in dogs (Kimura et al, 1973; Hashimoto et al, 1977; Matsuki et al, 1987), while increased plasma kinin levels have been reported in patients with myocardial ischaemia (Hashimoto et al, 1978). The mechanism by which myocardial ischaemia or acute myocardial infarction is related to plasma kinin release is unclear. The presence of enzymes either in plasma or in cardiac tissue which can be activated to release kinins during myocardial ischaemia has been suggested.

Nolly and co-workers reported the presence of an acid optimum kinin-forming enzyme in unspecified rat, human and bovine cardiac tissues (Nolly et al, 1981a). Recently tissue kallikrein was reported to be present in rat atrial tissue (Xiong et al, 1990). Canine ventricular myocardium and coronary arteries were reported to contain acid optimum proteinases which release a uterus contracting vasodilator peptide (Zeitlin et al, 1989). Also reported in this work was the presence, in the ventricular myocardium and coronary arteries of a neutral enzyme with properties similar to plasma kallikrein. This was the first report showing the presence of a plasma kallikrein-like enzyme in cardiovascular tissues and it differs from the results reported by Nolly and co-workers who have so far reported the presence of a tissue kallikrein-like enzyme in rat vascular tissues (Nolly et al, 1981b; Nolly et al, 1987; Nolly et al, 1985). This work was done to resolve this difference and also to determine if there was any other kinin-forming enzyme in dog ventricular myocardium.

3.1. pH-DEPENDENT RELEASE OF A UTERUS CONTRACTING SUBSTANCE (UCS) BY AQUEOUS EXTRACTS OF DOG VENTRICULAR MYOCARDIUM.

INTRODUCTION

The dog ventricular myocardium and coronary arteries were reported to have acid and neutral UCS-forming enzyme activities (Zeitlin et al, 1989). It was not very clear if the neutral enzyme was a new enzyme or plasma kallikrein resulting from contamination of the extracts with blood. This experiment was done to detect the presence of acid and neutral kinin-forming enzymes in aqueous extracts of dog ventricles.

METHOD

Preparation of ventricular extract.

Hearts were obtained from greyhounds at the end of experiments which involved exposure of the dogs to experimental myocardial ischaemia. Greyhounds (21-30 kg body weight) were anaesthetized (chloralose, 80 mg kg^{-1} i.v) and subjected to occlusion of the left anterior descending coronary artery (Coker et al, 1982) followed by reperfusion. The animals died within 2-3 minutes of reperfusion. All the hearts used were from dogs used in control experiments and they received no drug treatment apart from the anaesthetics. Hearts from dogs subjected to this procedure contained at least 80 % normally perfused tissue. Immediately after death the hearts were perfused through the left anterior descending coronary artery with heparinized saline (10 IU/ml) before being excised. The excised hearts were perfused through the same vessel with saline to clear out blood. The hearts were cleared of most of the visible blood vessels, fat and connective tissue.

The ventricles were removed, cut into small pieces with a pair of scissors, and homogenized in distilled water (0.1 g ml^{-1}) containing 0.1% v/v Triton X-100 . The homogenate was incubated under toluene at 18-20°C for 20 hours to allow for autolytic activation. The homogenate was centrifuged (2000g, 4°C, 30 min) and the supernatant obtained was dialysed under tap water for 20-24 hrs. The extract was stored at -20 °C until used.

Release of Uterus contracting substance (UCS).

Ventricular extract was lyophilized and concentrated. The extract (0.2 ml, 0.2 g ml^{-1}) was pre-incubated with 0.55 ml, 0.05 M acetate-barbitone buffer and 0.1 ml of a mixture of o-phenanthroline (0.01 M) and EDTA (0.3 M) for 10 minutes. Incubation was started by the addition of 0.25 ml of a human plasma kinin-forming substrate (prepared as indicated in chapter 2.8) and continued for one hour. Incubation was terminated by boiling the incubates on a water bath for 10 minutes. To determine the pH optima for the release of UCS, incubations were done at pHs from 4.0 to 9.0. Incubates were centrifuged and assayed using the contractions of the oestrous rat uterus. The released UCS was quantified by a" bracketing assay" using synthetic bradykinin as a standard (Zeitlin et al, 1976) and measured as ng Bk equivalent $hr^{-1}g^{-1}$ wet tissue equivalent.

RESULTS AND DISCUSSION.

Aqueous extracts of dog ventricles released a uterus contracting substance from the plasma kinin-forming substrate with a maximum at pH 5.2 to 5.4 (Figure 3.1.1). Figure 3.1.2 shows the effect on the oestrous rat uterus of incubates (made at pH 5.2) containing the extract alone, substrate alone and both extract and substrate in buffer. Both incubates containing substrate

alone and the extract alone in buffer were incapable of releasing a uterus contracting substance. This shows that the observed release of UCS originates from the action of an enzyme/s in the extract and not any activity originally present in either the substrate or the extract alone (no spontaneous release of UCS by either the extract or the substrate alone). Figure 3.1.1 shows that there was some release of UCS up to pH 7.5 but there was no obvious peak of UCS formation. These results suggest that if there is any neutral activity at all in the ventricular myocardium then it is very small relative to the acid optimum enzyme activity. This is in contrast to the results obtained before (Zeitlin et al, 1989) in which the dog ventricles were reported to have both acid and neutral UCS releasing enzymes. Recently, using immunocytochemistry and electron microscopy, tissue kallikrein was shown to be present in the sarcoplasmic reticulum and granules of rat atrial myocytes (Xiong et al, 1990). It was not clearly stated in this study if the ventricular myocardium contained tissue kallikrein. In another study a kallikrein-like serine proteinase, arginine esterase A, was localized by immunohistochemical staining in the myocytes in both the atria and ventricles in the rat (Simson et al, 1989). This enzyme, also identified in urine (urinary esterase A), has neutral activity (Chao, 1983). The content in the ventricles relative to the atria and whether or not the ventricular extract could release detectable amounts of kinins remains to be determined.

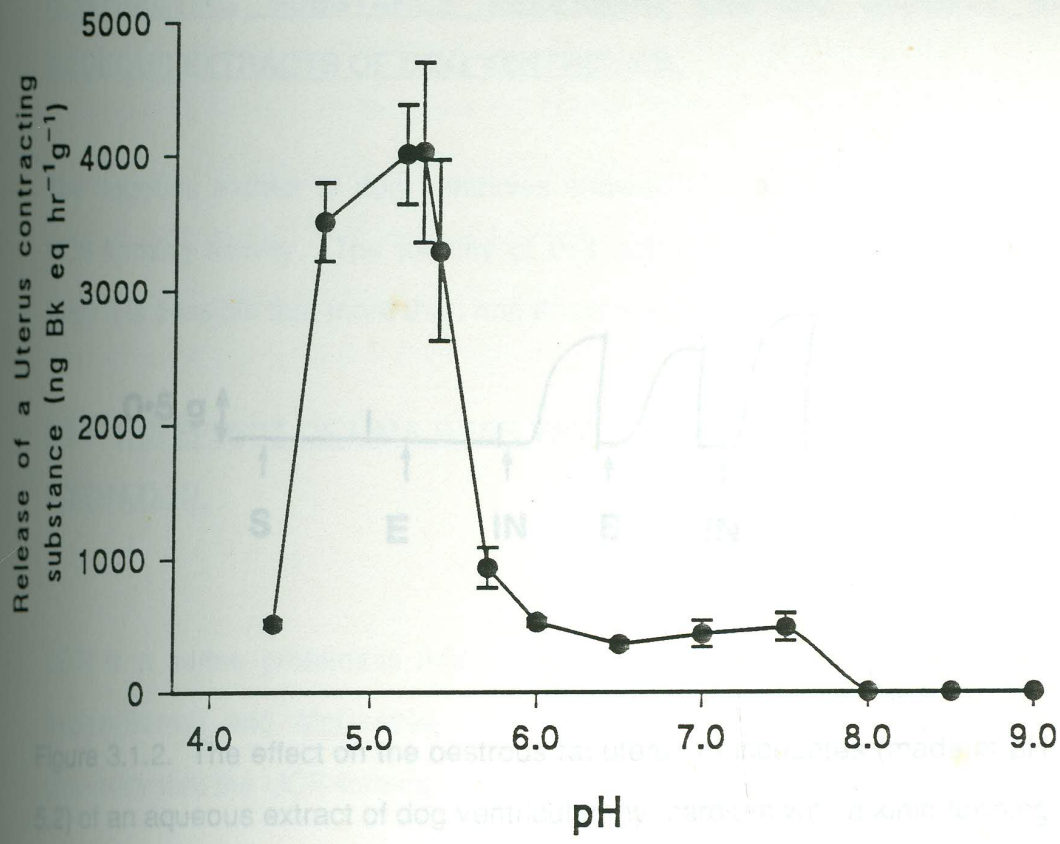


Figure 3.1.1. The effect of pH on the release of a uterus contracting substance(UCS) from a plasma kinin-forming substrate by aqueous extracts of dog ventricular myocardium. The aqueous extract was incubated with the kinin-forming substrate at pH 4 - pH 9 for 1 hour. Incubates were inactivated by boiling and assayed using contractions of the isolated oestrous rat uterus using synthetic bradykinin as a standard and the released UCS was expressed as ng Bk eq hr^{-1} g^{-1} wet tissue equivalent. Each point represents mean $\pm$ S.D (n = 5).

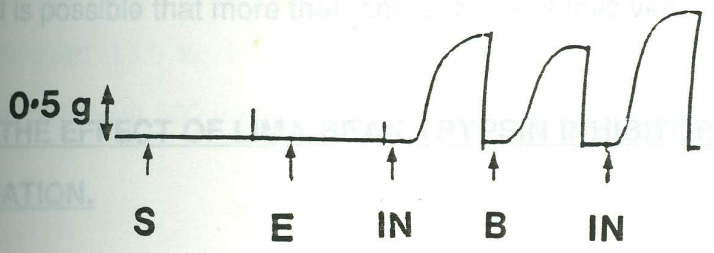


Figure 3.1.2. The effect on the oestrous rat uterus of incubates (made at pH 5.2) of an aqueous extract of dog ventricular myocardium with a kinin-forming substrate. The figure shows a tracing of isometric contractions of the oestrous rat uterus in response to: 0.2 ml of an incubate containing substrate and buffer only (S). 0.2 ml of an incubate containing the ventricular extract and buffer only (E). 0.2 ml of an incubate containing both the extract and substrate in buffer (IN). 2ng/ml, final bath concentration of synthetic bradykinin (B)

3.2 INHIBITOR STUDIES ON THE ACID OPTIMUM UTERUS CONTRACTING SUBSTANCE RELEASING ENZYMIC ACTIVITY IN AQUEOUS EXTRACTS OF DOG VENTRICLES.

The aqueous extract of dog ventricles showed one peak of acid optimum UCS-forming activity. The identity of this activity needs to be determined since it is possible that more than one enzyme is involved.

3.2.1 THE EFFECT OF LIMA BEAN TRYPSIN INHIBITOR (LBTI) ON UCS FORMATION.

LBTI is a serine proteinase inhibitor. It inhibits the Hageman factor and trypsin(Barrett and McDonald, 1980). It was used to investigate the possibility that the UCS-forming activity had an LBTI sensitive component.

METHOD.

An aqueous extract of dog ventricles (0.2 ml, 0.2 g/ml) was pre-incubated with 0.55ml, 0.05M acetate-barbitone buffer (pH 5.2) and 0.1 ml of o-phenanthroline (0.01 M) and EDTA (0.3 M) for 10 minutes. Lima bean trypsin inhibitor (100 - 500 µg/ml) was included in the pre-incubate Incubation was started by the addition of 0.25 ml of the plasma kinin-forming substrate and continued for one hour. Incubation was terminated by boiling on a water bath for 10 minutes. Incubates were centrifuged and supernatant stored at -20 ^{o}C until assayed. Assays were done using contractions of the oestrous rat uterus.

RESULTS.

Figure 3.2.1 shows the release of UCS by an aqueous extract of dog ventricles, at pH 5.2, in the presence of lima bean trypsin inhibitor (LBTI). LBTI at 100 and 200 µg/ml did not have a significant effect on the release of UCS as compared to the control incubates ($P > 0.05$). When the concentration was increased to 400 µg/ml a slight but significant inhibition was achieved (13.5 %; $P < 0.05$ vs control). The same amount of inhibition was obtained with 500 µg/ml.

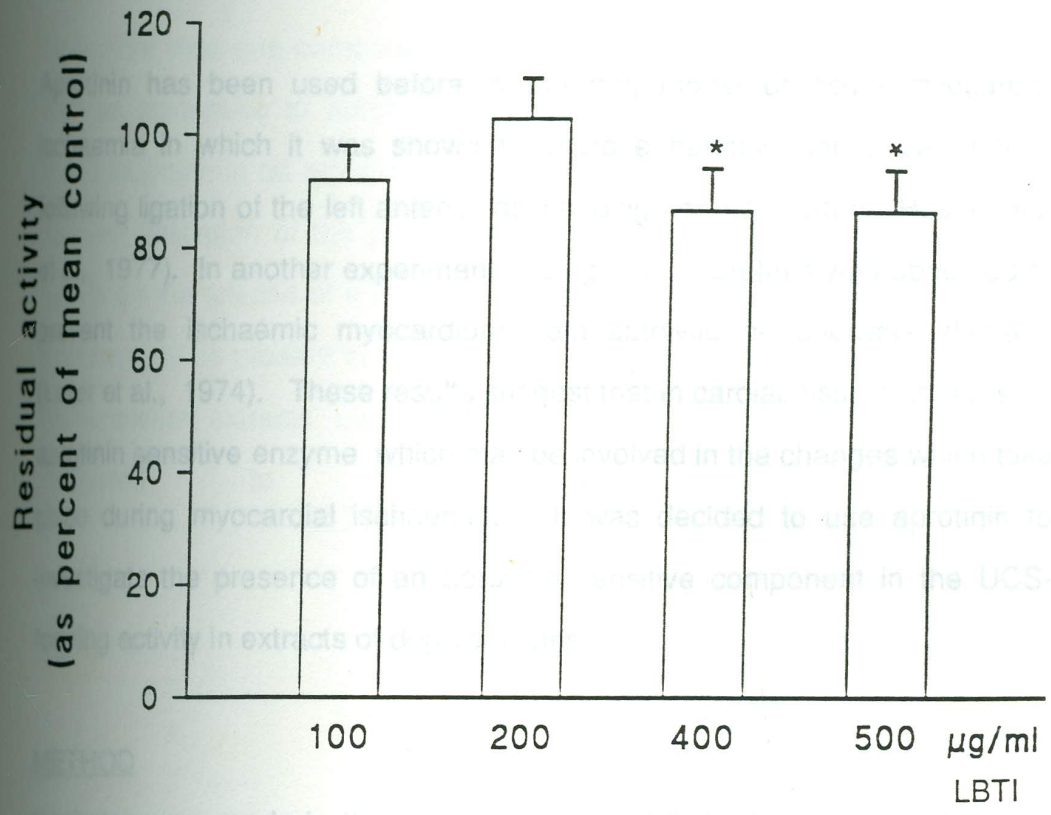


Figure 3.2.1. The effect of lima bean trypsin inhibitor (LBTI) on the release of UCS from a plasma kinin-forming substrate (at pH 5.2) by extracts of dog ventricles . LBTI was pre-incubated with the ventricular extract for 10 minutes before the addition of the kinin-forming substrate (see methods). Incubates were inactivated by boiling (10 min) and the released UCS was assayed using contractions of the isolated oestrous rat uterus. The released UCS in the presence of LBTI is expressed as a percent of the mean value of UCS in the control incubates. The figure represent mean ± S.D (n = 5).

3.2.2 THE EFFECT OF APROTININ ON THE RELEASE OF UCS BY EXTRACTS OF DOG VENTRICLES.

Aprotinin has been used before in the dog model of acute myocardial ischaemia in which it was shown to improve haemodynamic parameters following ligation of the left anterior descending coronary artery (Hashimoto, et al, 1977). In another experiment, using cats, aprotinin was observed to prevent the ischaemic myocardium from autolytic degenerative damage (Lefer et al., 1974). These results suggest that in cardiac tissues there is an aprotinin sensitive enzyme which may be involved in the changes which take place during myocardial ischaemia. It was decided to use aprotinin to investigate the presence of an aprotinin sensitive component in the UCS-forming activity in extracts of dog ventricles.

METHOD

Incubates were made in the same way as for LBTI. Aprotinin was used at concentrations of 540 to 2700 KIU/ml pre-incubated with the extract. The incubates were also assayed using the contractions of the oestrous rat uterus.

RESULTS AND DISCUSSION.

Aprotinin at concentrations of 540, 1080, 2160, and 2700 KIU/ml caused a concentration dependent inhibition of the release of UCS ($r = -0.992$; $P < 0.001$) by ventricular extracts (Figure 3.2.2). At 540 KIU/ml there was no significant inhibition ($P > 0.05$) as compared to control. Increasing the concentration to 1080 and 2160 KIU caused a significant reduction in the release of UCS ($P < 0.05$). No further inhibition was achieved at 2700 KIU/ml

($P > 0.05$). A maximum inhibition of 32.8% ($P < 0.05$) was obtained. The failure to obtain total inhibition suggests that the UCS-forming activity may have more than one component, part of which is sensitive to aprotinin and the rest insensitive to aprotinin. Recent reports suggest the presence of tissue kallikrein in rat atrial myocardium (Xiong et al, 1990), but there was no clear indication of the presence of this enzyme in the ventricles. The results on the release of a uterus contracting substance show that a neutral enzyme may be present in the ventricles since there was release of UCS by the ventricular extracts up to pH 7.5. The activity was, however, small as compared to the acid optimum activity.

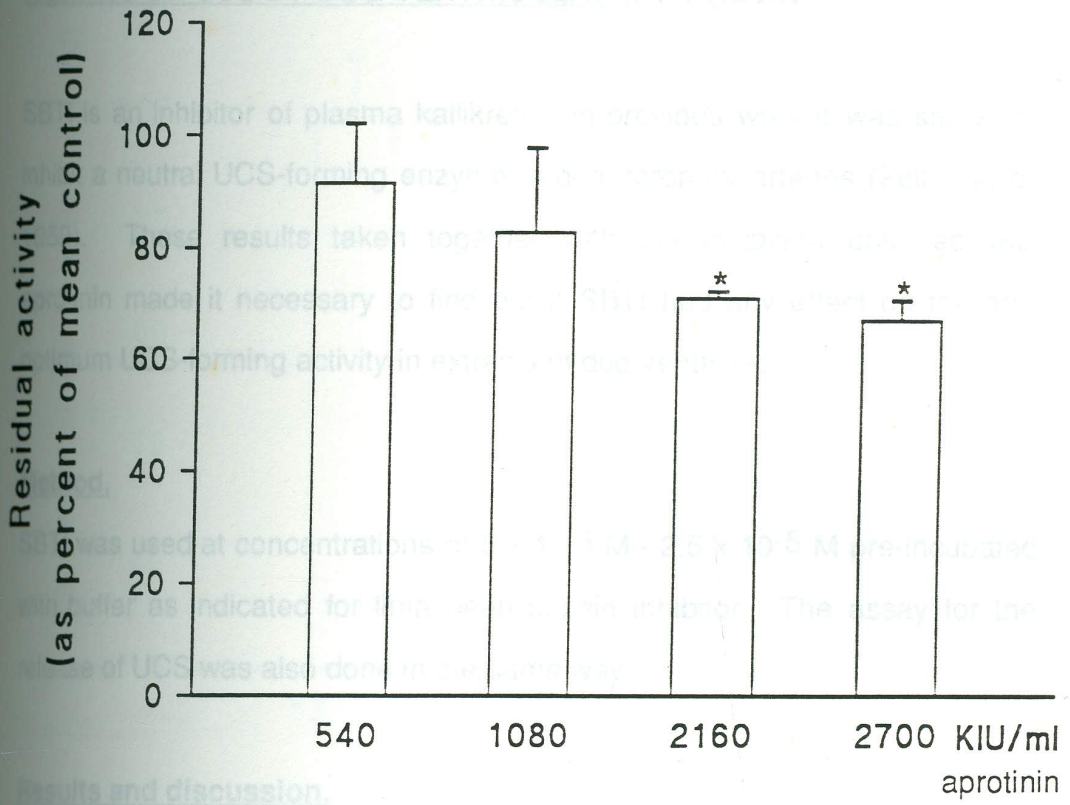


Figure 3.2.2. The effect of aprotinin on the release of UCS from a plasma kinin-forming substrate (at pH 5.2) by extracts of dog ventricles. Aprotinin at concentrations of 540 - 2700 KIU/ml was pre-incubated with the ventricular extract for 10 minutes before adding the substrate. Incubates were inactivated by boiling in a water bath and released UCS was assayed on the isolated oestrous rat uterus. The released UCS in the presence of aprotinin is expressed as a percent of the mean value of UCS in the control incubates. The figure represent mean $\pm$ S.D (n= 5).

3.2.3 THE EFFECT OF SOYBEAN TRYPSIN INHIBITOR (SBTI) ON THE RELEASE OF UCS BY DOG VENTRICULAR EXTRACTS.

SBTI is an inhibitor of plasma kallikrein. In previous work it was shown to inhibit a neutral UCS-forming enzyme in dog coronary arteries (Zeitlin et al, 1989). These results taken together with the inhibition obtained with aprotinin made it necessary to find out if SBTI had any effect on the acid optimum UCS-forming activity in extracts of dog ventricles.

Method.

SBTI was used at concentrations of 5×10^{-6} M - 2.5×10^{-5} M pre-incubated with buffer as indicated for lima bean trypsin inhibitor. The assay for the release of UCS was also done in the same way.

Results and discussion.

Soybean trypsin inhibitor, up to 2.5×10^{-5} M (500 µg/ml) showed no significant effect on the release of UCS by extracts of dog ventricles (Figure 3.2.3). It is clear from these results that the aprotinin sensitive component is not SBTI sensitive, an indication that it is unrelated to plasma kallikrein, which might be expected if the extracts were contaminated with blood.

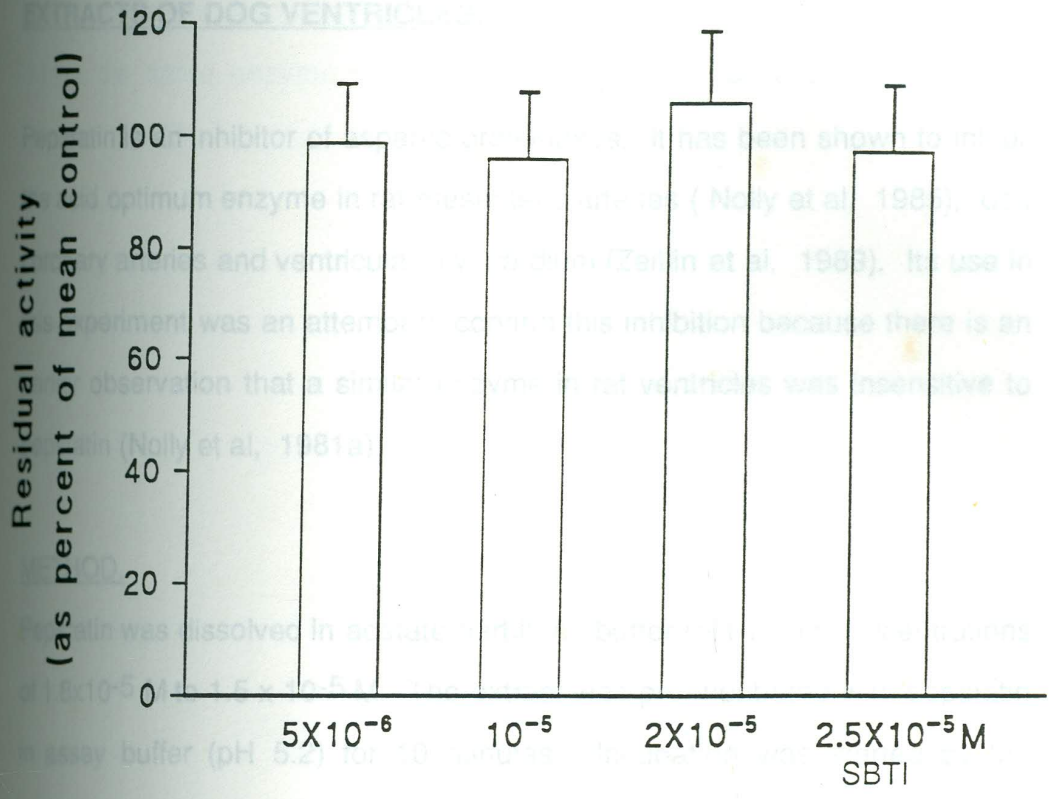


Figure 3.2.3. The effect of soybean trypsin inhibitor (SBTI) on the release of UCS from a plasma kinin-forming substrate (pH 5.2) by aqueous extracts of dog ventricles. SBTI was pre-incubated with the ventricular extract for 10 minutes before adding the substrate. Incubates were inactivated by boiling in a water bath and then assayed on the isolated oestrous rat uterus. The released UCS in the presence of SBTI is expressed as a percent of the mean value of UCS in control incubates. The figure represent mean ± S.D (n =5).

3.2.4 THE EFFECT OF PEPSTATIN ON THE RELEASE OF UCS BY EXTRACTS OF DOG VENTRICLES.

Pepstatin is an inhibitor of aspartic proteinases. It has been shown to inhibit the acid optimum enzyme in rat mesenteric arteries (Nolly et al, 1985), dog coronary arteries and ventricular myocardium (Zeitlin et al, 1989). Its use in this experiment was an attempt to confirm this inhibition because there is an earlier observation that a similar enzyme in rat ventricles was insensitive to pepstatin (Nolly et al, 1981a).

METHOD.

Pepstatin was dissolved in acetate-barbitone buffer (pH 10) to concentrations of 1.8×10^{-5} M to 1.5×10^{-5} M. The extract was pre-incubated with pepstatin in assay buffer (pH 5.2) for 10 minutes. Incubation was started by the addition of 0.25 ml of the kinin-forming substrate and continued for 1 hour. Inactivated incubates were assayed using contractions of the oestrous rat uterus.

RESULTS AND DISCUSSION.

Pepstatin inhibited the release of UCS to undetectable levels at 1.8×10^{-5} M (Figure 3.2.4). Undetectable levels were below 265 ngBkeq hr^{-1} g^{-1}, equivalent to 0.3 ng/ml using a 4 ml organ bath. Therefore at 1.8×10^{-5} M the released UCS was less than 265 ng BK equivalent hr^{-1} g^{-1} wet tissue equivalent. This means less than 6.6% of the mean UCS releasable in control incubates (4007 ± 485.6). The results obtained with pepstatin are similar to those obtained previously using both normal and ischaemic ventricles (Zeitlin et al, 1989) and rat mesenteric arteries (Nolly et al,

1981b). While pepstatin completely inhibited the release of UCS by this acid optimum enzyme aprotinin showed only 32.8 % inhibition. The inhibition may be on the same enzyme or on different enzymes all responsible for the release of UCS.

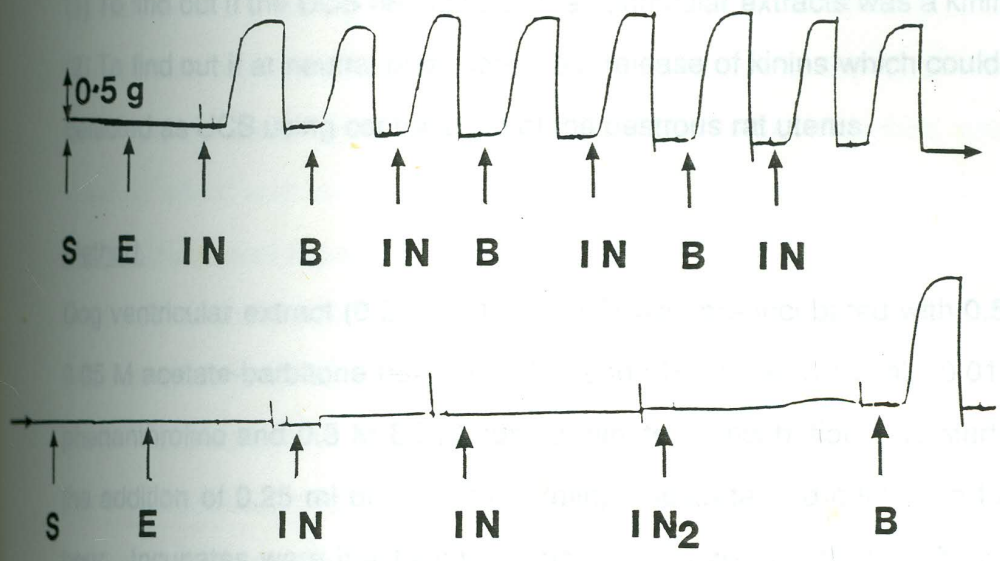


Figure 3.2.4. The effect of pepstatin on the release of a uterus contracting substance (UCS) by incubates of dog ventricular extract with a kinin-forming substrate (pH 5.2). Incubates were assayed using the contractions of the oestrous rat uterus and synthetic bradykinin as a standard. The upper tracing represents the effect of incubates without pepstatin and the lower tracing represents the effect of incubates containing 1.8×10^{-5} M pepstatin, pre-incubated with the extract for 10 minutes before addition of substrate. On both tracings 0.2 ml of either substrate control (S) or extract control (E) did not release UCS. On the upper tracing 0.2 ml of incubates containing both the ventricular extract and substrate (IN) released UCS. 0.2 ml of similar incubates containing pepstatin showed no release of UCS. Even when 0.3 ml of incubate was used (IN_2) there was still no effect on the uterus. B = bradykinin standards.
No control containing bradykinin and pepstatin was used.

3.3 RELEASE OF BRADYKININ-LIKE IMMUNOREACTIVITY (BKIR) BY AQUEOUS EXTRACTS OF DOG VENTRICLES.

This experiment was done for two purposes:

(1) To find out if the UCS released by the ventricular extracts was a kinin.

(2) To find out if at neutral pHs there was release of kinins which couldn't be detected as UCS using contractions of the oestrous rat uterus.

Method.

Dog ventricular extract (0.2 ml, 0.2 g ml^{-1}) was pre-incubated with 0.55 ml, 0.05 M acetate-barbitone buffer (pH 5.2 and pH 8.0) and 0.1 ml, 0.01 M o-phenanthroline and 0.3 M EDTA for 10 minutes. Incubation was started by the addition of 0.25 ml of the kinin-forming substrate and continued for one hour. Incubates were inactivated by boiling in a water bath for 10 minutes, centrifuged and stored at -20 ^{o}C until assayed by radioimmunoassay using a polyclonal rabbit anti-bradykinin antiserum (chapter 2.2). Assays were done without further extraction of kinins.

RESULTS AND DISCUSSION.

Dilutions (1 in 10) of incubates were assayed. Figure 3.3 shows bradykinin-like immunoreactivity released by incubates of the aqueous ventricular extract with a kinin-forming substrate at pH 5.2 and pH 8.0. When the aqueous ventricular extract was incubated with a plasma kinin-forming substrate at pH 5.2 the formation of BKIR increased by 856.5 % in the incubates containing both extract and substrate as compared to incubates containing substrate and buffer only. The net release of BKIR at pH 5.2 was

197.67 ng Bkeq, equivalent to 4941.8 ngBkeq hr^{-1} g^{-1} wet tissue equivalent (n=2). The amount of UCS released at the same pH was 4007 $\pm$ 485.6 ngBkeq hr^{-1} g^{-1} (mean $\pm$ S.D, n=20); a value which is very similar to that of BKIR. It could be said here that a large percentage of the measured UCS represents BKIR released from the human plasma kinin- forming substrate. Nolly and co-workers showed that the uterus contracting activity of the UCS was inhibited 85% by a bradykinin antibody, while its blood pressure lowering effect was inhibited by 90% (Nolly et al, 1981a). These results suggest that the released BKIR is a kinin. At pH 8 both substrate blanks and incubates containing both the ventricular extract and substrate released the same amount of BKIR. There was no net BKIR released into incubates. These results support the results obtained in the determination of pH-optima that in the aqueous dog ventricular extract there is no kinin release measured as UCS-forming activity above pH 7.5.

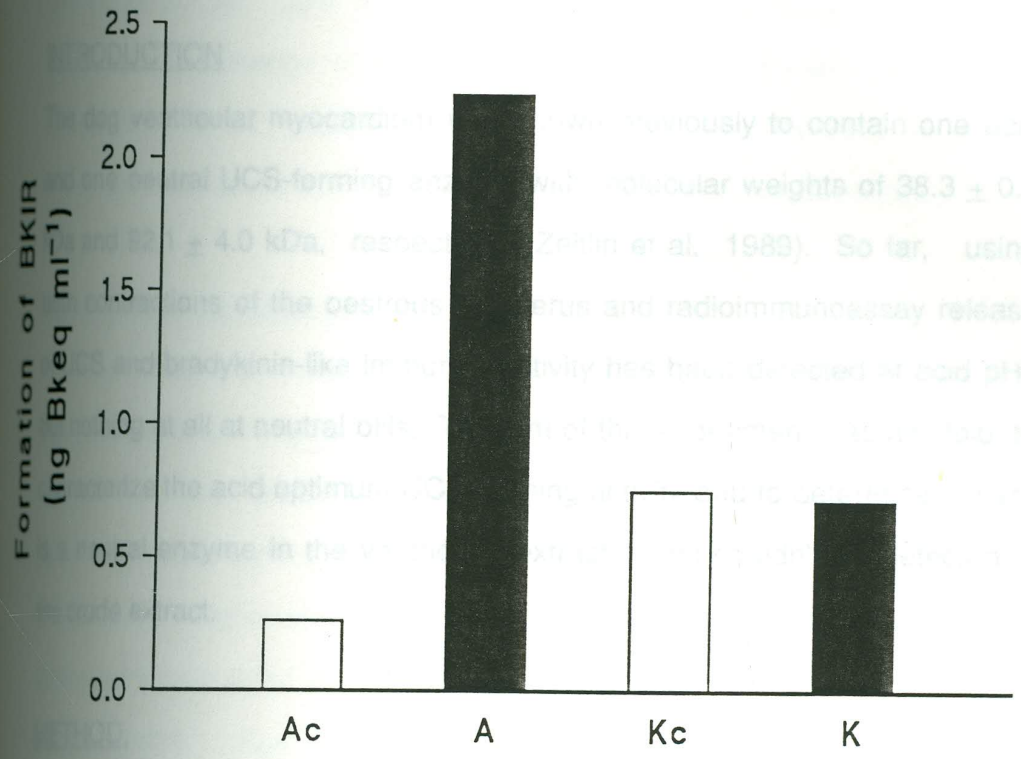


Figure 3.3. The release of bradykinin-like immunoreactivity (BKIR) by aqueous extracts of dog ventricles at acid pH 5.2 and alkaline pH 8.0. The figure shows the released BKIR by an incubate of : the kinin-forming substrate alone in buffer, at pH 5.2 (Ac); the ventricular extract and substrate at pH 5.2 (A); the substrate alone in buffer, at pH 8.0 (Kc); and the ventricular extract and kinin-forming substrate at pH 8.0 (K). The figure represents mean values(n =2).

3.4 GEL FILTRATION OF ENZYMES CLEAVING D-VAL-LEU-ARG-pNA IN AQUEOUS EXTRACTS OF DOG VENTRICLES.

INTRODUCTION

The dog ventricular myocardium was shown previously to contain one acid and one neutral UCS-forming enzyme with molecular weights of 38.3 ± 0.8 kDa and 92.1 ± 4.0 kDa, respectively (Zeitlin et al, 1989). So far, using both contractions of the oestrous rat uterus and radioimmunoassay release of UCS and bradykinin-like immunoreactivity has been detected at acid pHs but nothing at all at neutral pHs. The aim of this experiment was two fold; to characterize the acid optimum UCS-forming activity and to determine if there is a neutral enzyme in the ventricular extract which couldn't be detected in the crude extract.

METHOD.

Aqueous extract of dog ventricles (0.1 g/ml) was lyophilized and reconstituted in 0.1 M Tris buffer, pH 8.5 to a concentration of 0.4 g ml^{-1}. The extract was centrifuged (2000g, 4°C, 15 minutes) and filtered through a Whatman number 1 filter paper. The filtered extract (3 ml) was fractionated on a Sephacryl S-300 column (2.6 cm x 86 cm) equilibrated with 0.1 M Tris buffer, pH 8.5. The column was eluted with the same buffer at the rate of 5.7 ml hr^{-1} cm^{-2} and 3 ml fractions were collected. Elution of proteins was monitored by measuring absorbance at 280 nm (A_{280}). Enzyme peaks were determined by measuring amidase activity against the tripeptide D-Val-Leu-Arg-pNA, a substrate for tissue kallikrein and trypsin (Zeitlin et al, 1989). Aliquots (0.4 ml) of the fractions were pre-incubated with 0.5 ml of either 0.1 M citrate phosphate buffer (pH 5.2) or 0.1 M, Tris buffer (pH 8.0) for 10

minutes. Incubations were started by the addition of 0.1 ml, 1.5 mM D-Val-Leu-Arg-pNA and continued for 3 hours. Incubations were terminated by the addition of 0.1 ml, 50% v/v glacial acetic acid. The released pNA was measured as a change in absorbance at 405 nm and then converted to nmoles/ml. In all incubates enzyme blanks were included in which inactivation with acetic acid was done before addition of the substrate.

Determination of molecular weights.

For determination of molecular weights the column was calibrated using the standard proteins myoglobin (17.2 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), and apoferritin (443 kDa). K_{av} values for the standard proteins were plotted against the logarithm of molecular weights and the molecular weights of the unknown enzyme peaks were calculated by interpolating their respective K_{av} values in the standard curve. $K_{av} = (V_e - V_o)/ (V_t - V_o)$; where V_e = peak elution volume, V_o = elution volume for dextran blue (the void volume), and V_t = the total packed column volume (Andrews, 1965).

RESULTS AND DISCUSSION.

Gel filtration of aqueous extracts of dog ventricles produced only one peak of arginine amidase activity, obtained by assaying aliquots at pH 5.2 (Figure 3.4). This peak had a molecular weight of 42.6 $\pm$ 9.0 kDa . When the eluates were assayed at pH 8.0 no peak of arginine amidase activity could be detected. This is in contrast to the results obtained in a previous study (Zeitlin et al, 1989) in which a neutral enzyme with a molecular weight of 92.1 kDa was detected. These results confirm the previous suspicion (Zeitlin et al, 1989) that this neutral enzyme was probably plasma kallikrein

arising from contamination with blood. The acid optimum enzyme has a molecular size similar to that obtained before of 38.3 $\pm$ 0.8 kDa.

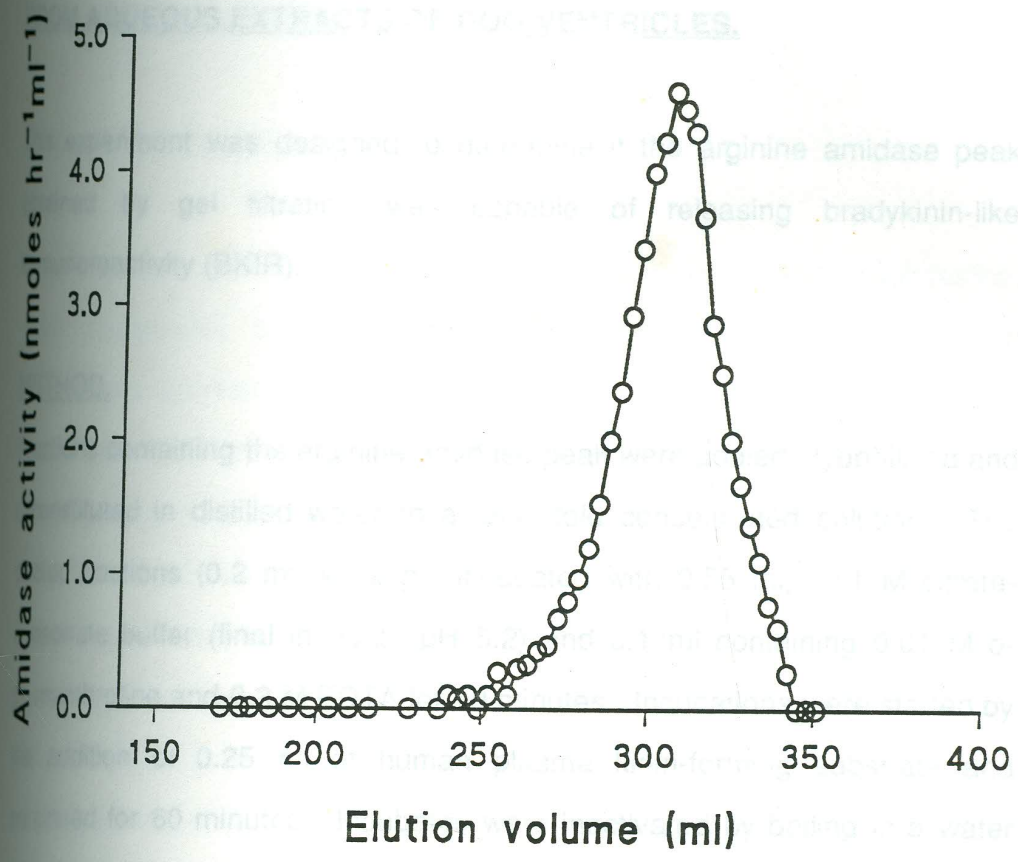


Figure 3.4. Gel filtration pattern of acid optimum enzymes cleaving D-Val-Leu-Arg-pNA at pH 5.2 in aqueous extracts of dog ventricular myocardium. The figure represents mean values of 5 column runs. Only one peak of amidase activity with a molecular weight of 42.6 ± 0.9 kDa was obtained.

3.5. RELEASE OF BRADYKININ-LIKE IMMUNOREACTIVITY BY POOLED FRACTIONS OF ARGININE AMIDASE PEAKS OBTAINED FROM AQUEOUS EXTRACTS OF DOG VENTRICLES.

This experiment was designed to determine if the arginine amidase peak obtained by gel filtration was capable of releasing bradykinin-like immunoreactivity (BKIR).

METHOD.

Fractions containing the arginine amidase peak were pooled, lyophilized and reconstituted in distilled water to a four- fold concentrated solution. The pooled fractions (0.2 ml) were pre-incubated with 0.55 ml, 0.1 M citrate-phosphate buffer (final incubate pH 5.2) and 0.1 ml containing 0.01 M o-phenanthroline and 0.3 M EDTA for 10 minutes. Incubations were started by the addition of 0.25 ml of human plasma kinin-forming substrate and continued for 60 minutes. Incubates were inactivated by boiling in a water bath for 10 minutes. Incubates were centrifuged and the released BKIR was measured by radioimmunoassay using a polyclonal rabbit anti-bradykinin antiserum. Substrate blanks were also included. Protein concentrations were determined by using a Pierce micro BCA protein assay kit.

RESULTS AND DISCUSSION.

Pooled fractions of arginine amidase peaks from aqueous ventricular extracts released bradykinin-like immunoreactivity with a mean specific activity of 24.5 $\pm$ 12.9 ngBkeq $hr^{-1}mg^{-1}$ protein (mean $\pm$ S.D, n=5). This value is equivalent to 24500 ngBkeq hr^{-1} g^{-1} protein. Compared with the value 4941.8 obtained with whole extract this represents a 5 fold increase in specific activity. These

results support the previous work that arginine amidase activity against D-val-leu-arg-pNA can be used to detect the acid optimum UCS and BKIR releasing enzyme in aqueous extracts of dog ventricles.

GENERAL DISCUSSION AND SUMMARY.

Three methods, pH-dependent release of a uterus contracting substance, radioimmunoassay, and gel-filtration were used to detect and characterize kinin-forming enzymes in aqueous extracts of dog ventricular myocardium. Results from the three methods show that aqueous extracts of dog ventricles contain one acid optimum UCS and BKIR releasing enzymic moiety with a molecular weight of 42.6 $\pm$ 0.9 kDa (mean $\pm$ S D). Comparable amounts of UCS and BKIR were measured when the crude extract was incubated with a plasma kinin-forming substrate. The UCS and BKIR released were 4007 $\pm$ 485 (mean $\pm$ S.D, n = 20), and 4941.8 (n = 2) ngBKeq $hr^{-1}g^{-1}$ wet tissue equivalent, respectively. Both values were measured against standards of synthetic bradykinin. Gel filtration increased the BKIR-releasing activity 5 fold.

Four inhibitors were used based on previous experience, both in other laboratories and from our own laboratory, to try to characterize the UCS and BKIR forming enzymic activity. The acid optimum UCS-forming activity was not affected by SBTI. Aprotinin, on the other hand, showed a significant inhibition (32.8 %; $P < 0.05$) of UCS formation. It showed a concentration-dependent inhibition ($r = -0.992$; $P < 0.001$) of UCS-formation between 540 and 2160 KIU/ml at which maximum inhibition was achieved. When the concentration was increased to 2700 KIU/ml there was no further increase in

inhibition ($P > 0.05$). The question at this point is what enzyme did aprotinin act on and why is the inhibition only 32.8 %. At pH 5.2, the pH used for the inhibitor studies, some kallikrein-like activity can be detected (Chakder, 1988). Based on the results on the formation of UCS and BKIR there is no evidence of the presence of such activity in the dog ventricular extract. Recently tissue kallikrein was reported in combined aqueous rat atrial and ventricular extracts at a concentration of 0.35 ± 0.05 ng/mg protein (Xiong et al, 1990). This enzyme was localized to the sarcoplasmic reticulum and granules of atrial myocytes (Xiong et al, 1990). Simson et al (1989) reported the presence of arginine esterase A, a kallikrein-like serine protease in both rat atrial and ventricular myocytes. Both reports support the possibility of the presence of tissue kallikrein-like enzymes in the ventricles. This may explain the inhibition obtained with aprotinin, although being a non-specific serine proteinase inhibitor, the inhibition obtained could be inhibition of an entirely different enzyme. Aprotinin has been reported to have a protective and lysosomal stabilizing effect during acute myocardial ischaemia (Lefer et al, 1974). Whether or not this is related to the partial inhibition of UCS formation and prevention of the increase in plasma kinin levels following occlusion of the left main coronary artery in the dog remains to be further looked into. LBTI, the inhibitor of the Hageman factor, also partially inhibited the release of UCS at acid pH by the ventricular extract. It caused 13.5 % ($P < 0.05$) inhibition of the release of UCS. UCS formation was completely inhibited by pepstatin at 1.8×10^{-5} M; an aspartic proteinase inhibitor. Included in this group are cathepsins D, renin, pseudorenin, the pepsins, uropepsin, chymosin, gastric cathepsin, cathepsin E, lymphocyte aspartic proteinase, and thyroid aspartic proteinase (Barrett and McDonald, 1980). The UCS-forming enzyme is similar to cathepsin D and renin. However, the pH

optimum at pH 5.2 is a bit disturbing because cathepsin D is known to have an optimum at pH 3.5. This enzyme could as well be one of the renin-like enzymes (Rosenthal et al, 1990). Cathepsin D has been reported to be the major acid proteinase in the heart (Decker et al, 1977) and recent work show that it is also present in vascular tissues (Samawura et al, 1990). Cathepsin D is capable of releasing kinins from L-kininogen (Ogloblina et al, 1981).

CONCLUSION.

Aqueous extracts of dog ventricles contain one acid optimum UCS and BKIR forming enzymic activity. This enzymic activity was partially inhibited by aprotinin. It was completely inhibited by pepstatin, an aspartic proteinase inhibitor. Gel filtration using Sephacryl S-300 revealed an arginine amidase and BKIR forming activity with a molecular weight of 42.6 $\pm$ 0.9 k.dalton. The UCS and BKIR forming enzymic activity is similar to cathepsin D and the renin-like enzymes in molecular size and in its sensitivity to pepstatin. The partial inhibition by aprotinin is not very characteristic of cathepsin D and the explanation for this remains to be found.

CHAPTER 4.

STUDIES ON KININ-FORMING ENZYMES IN DOG AORTA.

4.1 INTRODUCTION

Levels of kinins in the coronary sinus and aortic blood are elevated following the occlusion of the left anterior descending coronary artery in the dog (Kimura et al, 1973; Hashimoto et al, 1977; Matsuki et al, 1987). These reports are suggestive of possible local generation of kinins within the heart and in the aorta. Considering that most of the kinins formed in the peripheral blood are destroyed in the lungs before reaching most organs, local generation of kinins would be important to produce enough concentrations for local vasodilatation. Vascular tissues, including rat mesenteric arteries (Nolly et al, 1981b; Nolly and Lama, 1982) and rat aorta (Nolly et al, 1987; Oza et al, 1990) have been reported to contain neutral kinin-forming enzymes with properties similar to tissue kallikrein. In dog coronary arteries the neutral enzyme was reported to be similar to plasma kallikrein (Zeitlin et al, 1989). An acid optimum kinin-forming enzyme with properties similar to cathepsin D was reported in dog coronary arteries (Zeitlin et al, 1989) and rat mesenteric arteries (Nolly et al, 1981b). To date there is no report of an acid optimum kinin-forming enzyme in the aorta. The recent work by Oza and co-workers (1990), showed that the rat aorta contains tissue kallikrein, kininogen and a kininase. The levels of tissue kallikrein are postulated to be decreased in hypertension (Nolly et al, 1987). Nolly et al (1987), reported the presence of another kinin-forming enzyme in rat aorta which is resistant to inhibition by soybean trypsin inhibitor. However, there was no further characterization of the enzyme. The present work was done in an attempt to characterize kinin-forming enzymes in the dog aorta and to compare the

results with those obtained using dog coronary arteries and the ventricular myocardium.

4.2. DETERMINATION OF THE LINEARITY WITH TIME FOR THE RELEASE OF PARANITROANILINE (pNA) BY AN AQUEOUS EXTRACT OF DOG AORTA.

The chromogenic substrate S-2266 is a selective substrate for tissue kallikrein. It is also hydrolysed by plasma kallikrein. Since it is hydrolysed by both enzymes it can only be used to measure tissue kallikrein-like activity in blood free tissues . However, for comparative studies, the assays must be restricted to that part of reaction in which the hydrolysis of the substrate is linear with time. This experiment was done with the aim of establishing a suitable incubation time for further chromogenic assays.

METHOD.

Collection of tissues and preparation of extract.

The thoracic aorta was obtained from the same greyhounds used for the ventricular studies. Both tissues were washed free of blood as indicated for the ventricular tissue. The aorta was cleared of fat, connective tissue and any traces of blood. It was chopped into small pieces with a pair of scissors and homogenized in distilled water (4^{o}C, 0.1 g ml^{-1}) with an Ultra-Turrax Janke & Kunkel homogenizer. The homogenate was subjected to autolytic degradation under toluene (18 - 20 ^{o}C) for 20 - 24 hours, centrifuged (2000g, 4^{o}C, 30 minutes) and the supernatant obtained was dialysed under tap water for 24 hours.

Determination of the time course for the hydrolysis of S-2266.

To determine the time course, 0.5 ml Tris buffer (0.1 M, pH 8.5), was pre-incubated for 10 minutes. The aqueous aorta extract (0.4 ml, 0.1 g ml^{-1}) was added and pre-incubation continued for another 5 minutes. Incubation was started by the addition of 0.1 ml (1.5 mM) of S-2266. To determine the time course incubations were done for 30, 60, 90, 120, 150, and 180 minutes. The incubations were terminated by the addition of 0.1 ml (50 % v/v) acetic acid. The tubes were centrifuged (2000g, 4°C, 15 min); 0.9 ml of supernatant mixed with 0.1 ml SLS (20 % w/v) and absorbancies read at 405 nm. The amount of pNA released was calculated using the pNA calibration curve.

RESULTS

Figure 4.2 shows the formation of pNA by an aqueous extract of dog aorta at 30 to 180 minutes. The release of pNA was linear with time ($r = 0.997$, $P < 0.001$) up to 180 minutes of incubation. The maximum releasable pNA from 0.1 ml (1.5 mM) of the substrate is about 140 nmoles ml^{-1} (Chapter 2.1.1). During the 180 minute incubation period only about 20 nmoles ml^{-1} were consumed. It can be said that using 0.1 g ml^{-1} of extract only a small proportion of substrate was consumed and even with higher activity in the tissue there would be enough substrate to allow for working within the linear range.

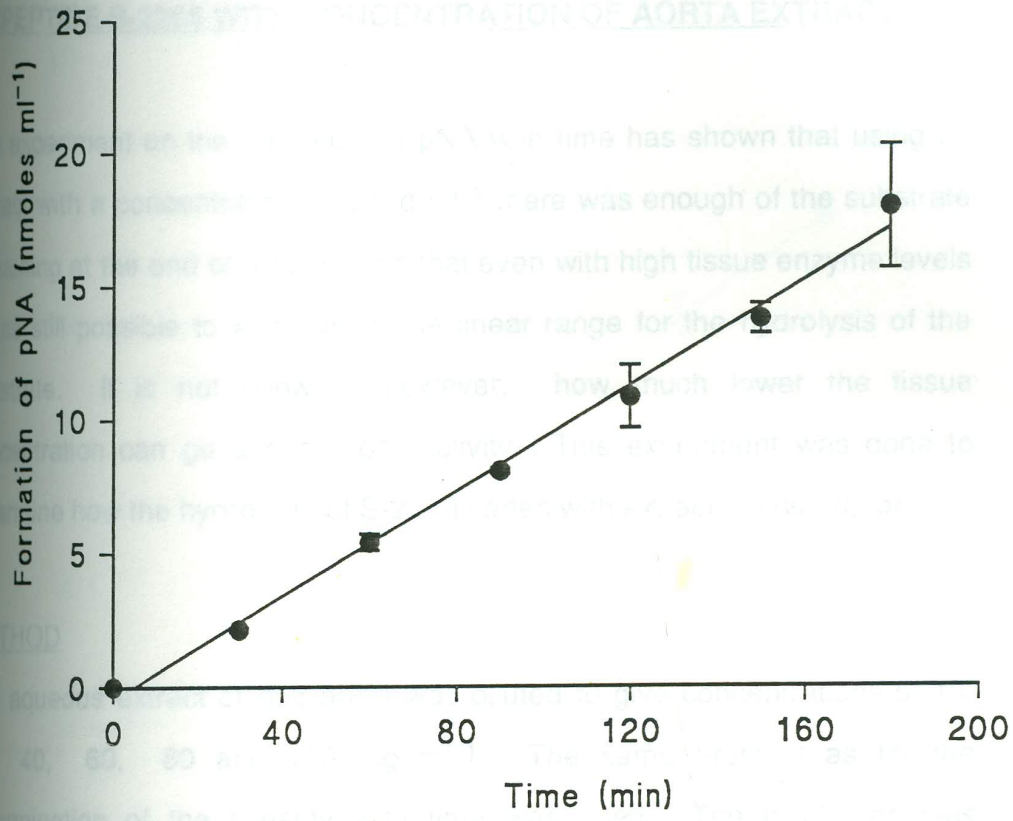


Figure 4.2. Time-dependent hydrolysis of the tripeptide S-2266 by an aqueous extract of dog aorta. The aqueous extract (0.1 g ml^{-1}) was incubated with the substrate for 30 - 180 minutes and the released pNA measured. Each point represents mean ± S.D (n = 3).

4.3. DETERMINATION OF LINEARITY FOR THE HYDROLYSIS OF THE TRIPEPTIDE S-2266 WITH CONCENTRATION OF AORTA EXTRACT.

The experiment on the formation of pNA with time has shown that using an extract with a concentration of 0.1 g ml^{-1} there was enough of the substrate remaining at the end of 3 hours and that even with high tissue enzyme levels it was still possible to work within the linear range for the hydrolysis of the substrate. It is not known, however, how much lower the tissue concentration can go and still get activity. This experiment was done to determine how the hydrolysis of S-2266 varies with extract concentration.

METHOD

The aqueous extract of dog aorta was diluted to give concentrations of 10, 20, 40, 60, 80 and 100 mg ml^{-1}. The same protocol as for the determination of the linearity with time was used. The incubation was continued for 90 minutes.

RESULTS.

Figure 4.3 shows the release of pNA by different concentrations of the aorta extract. The figure shows that the hydrolysis of the substrate was linear with increasing extract concentration ($r = 0.998$, $P < 0.001$). All the concentrations of extract used down to 10 mg ml^{-1} released detectable amounts of pNA.

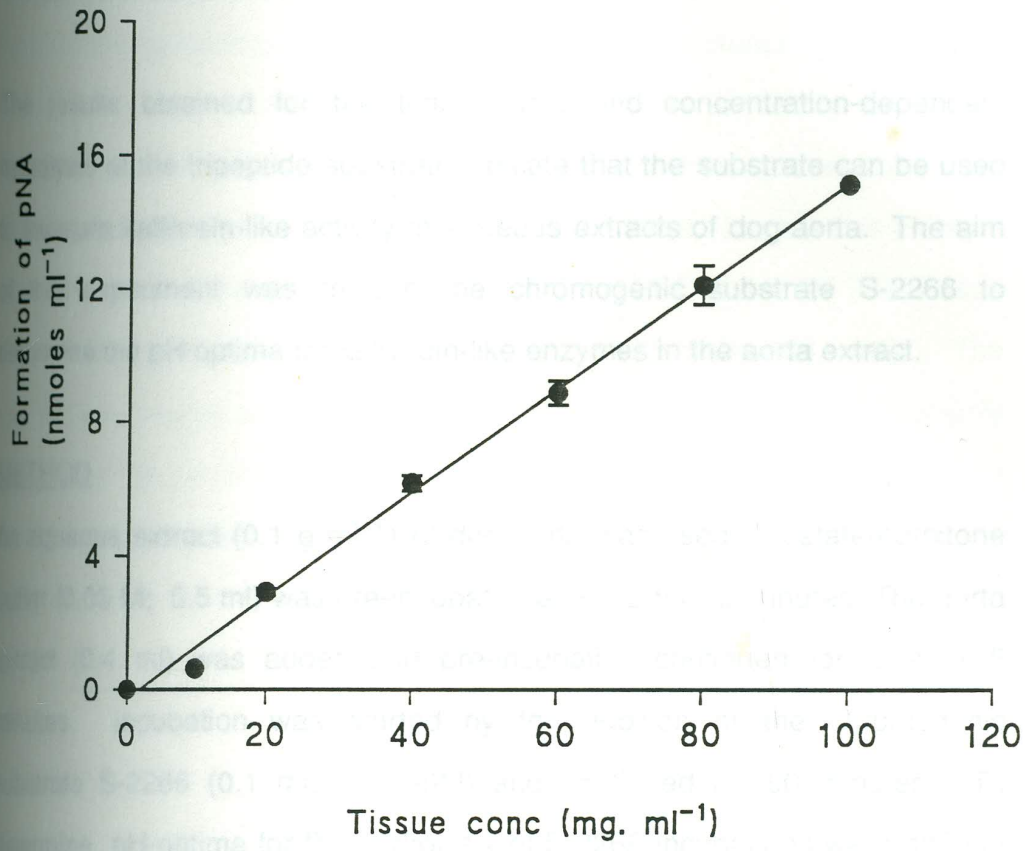


Figure 4.3. The hydrolysis of the tripeptide S-2266 by different concentrations of an aqueous extract of dog aorta. Dilutions of the extract from 10 - 100 mg ml^{-1} were incubated with the substrate and the released pNA in 90 minutes was measured. Each point represents mean $\pm$ S.D (n = 3) and where no S.D is shown the value is too small to indicate on the graph.

4.4. pH-DEPENDENT HYDROLYSIS OF THE CHROMOGENIC SUBSTRATE S-2266 BY AQUEOUS EXTRACTS OF DOG AORTA.

The results obtained for the time course and concentration-dependent hydrolysis of the tripeptide substrate indicate that the substrate can be used to measure kallikrein-like activity in aqueous extracts of dog aorta. The aim of this experiment was to use the chromogenic substrate S-2266 to determine the pH optima for kallikrein-like enzymes in the aorta extract.

METHOD

An aqueous extract (0.1 g ml^{-1}) of dog aorta was used. Acetate-barbitone buffer (0.05 M; 0.5 ml) was pre-incubated at 37°C for 10 minutes. The aorta extract (0.4 ml) was added and pre-incubation continued for another 5 minutes. Incubation was started by the addition of the chromogenic substrate S-2266 (0.1 ml; 1.5 mM) and continued for 90 minutes. To determine pH-optima for the hydrolysis of S-2266 incubations were done in the pH range from pH 4.0 to pH 9.0. The incubations were terminated by the addition of 0.1 ml (50 % v/v) acetic acid. The tubes were centrifuged (2000g, 4°C, 15 min); 0.9 ml of supernatant mixed with 0.1 ml SLS (20 % w/v) and absorbancies read at 405 nm.

RESULTS AND DISCUSSION.

Figure 4.4 shows the hydrolysis of the tripeptide S-2266 between pH 4.0 and pH 9.0. Negative changes in absorbance were obtained at pH 4, 4.5, 5.0 and pH 6.0. It was observed that at acid pH's there was precipitation of some material in the aorta extract. This effect was independent of the type of buffer used. When the aorta extract was fractionated by gel filtration it was

shown that the extract contained a high molecular weight substance (molecular weight > 400 kDa) which precipitates in acid pH's. Incubates of the fractions containing this high molecular weight substance in acid buffers always gave negative changes in absorbance relative to the blanks. It was observed that once the high molecular weight material was separated out there was no further problem with the amidase assays. Despite this problem two peaks of amidase activity were obtained at pH 5.5 and at pH 7.5. Tissue kallikrein has a pH-optimum at pH 7 - 8 (Barrett and McDonald, 1980). The neutral peak may be due to a tissue kallikrein-like enzyme. There is possibly a true peak at pH 5.5 but due to the precipitation problem it is difficult to confirm the presence of this peak.

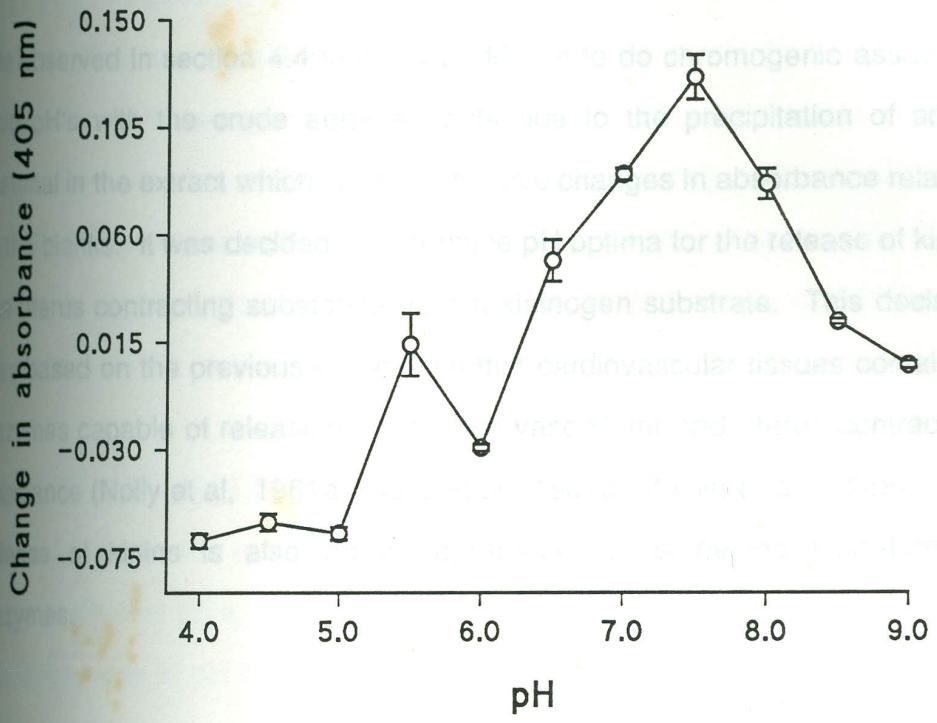


Figure 4.4. pH-dependent hydrolysis of S-2266 by an aqueous extract of dog aorta. The aqueous extract (0.4 ml, 0.1 g ml^{-1}) was incubated with 0.1 ml (1.5 mM) of the substrate at pH 4 - pH 9.0 for 90 minutes. The released pNA was measured as a change in absorbance (405 nm) as compared to controls. Each point represents mean $\pm$ S.D (n = 3).

4.5. pH-DEPENDENT RELEASE OF A UTERUS CONTRACTING SUBSTANCE (UCS) BY AN AQUEOUS EXTRACT OF DOG AORTA.

We observed in section 4.4 that it was difficult to do chromogenic assays at acid pH's with the crude aorta extracts due to the precipitation of some material in the extract which caused negative changes in absorbance relative to the blanks. It was decided to determine pH optima for the release of kinin-like uterus contracting substance from a kininogen substrate. This decision was based on the previous experience that cardiovascular tissues contained enzymes capable of releasing a kinin-like vasodilator and uterus contracting substance (Nolly et al, 1981a; Nolly et al, 1981b; Zeitlin et al, 1989). The release of kinins is also more appropriate for screening kinin-forming enzymes.

METHODS.

Collection of tissue and preparation of extract.

The collection of tissue was done as indicated for the chromogenic assays. The aorta was chopped into small pieces with a pair of scissors and homogenized in distilled water (4^{o}C, 0.1 g ml^{-1}) containing 0.1 % Triton X-100. The homogenate was subjected to autolytic activation under toluene (18 - 20 ^{o}C; 20 - 24 hrs), centrifuged (2000g, 4 ^{o}C, 30 minutes) and the supernatant obtained was dialysed under tap water for 24 hours. The dialysed supernatant was lyophilized and stored at -20 ^{o}C until used.

Release of a uterus contracting substance (UCS).

Reconstituted extract (0.2 ml, 0.4 g ml^{-1}) was pre-incubated with 0.55 ml, acetate-barbitone buffer (0.05 M) and 0.1 ml containing o-phenanthroline

(0.01 M) and EDTA (0.3 M) for 10 minutes. Incubations were started by the addition of 0.25 ml of a kinin-forming substrate (Chapter 2.8) and continued for 1 hour. Incubates were inactivated by boiling in a water bath for 10 minutes. The incubates were stored at -20 ^{o}C until assayed. On the assay day the incubates were thawed, centrifuged and the supernatants assayed for the presence of a uterus contracting substance using contractions of the isolated oestrous rat uterus and synthetic bradykinin as a standard. To determine pH-dependent release of UCS incubations were done in the pH range from pH 4.0 - pH 9.0.

RESULTS AND DISCUSSION.

Figure 4.5 shows the release of a uterus contracting substance by incubates made at different pH's. The figure shows the presence of two peaks of UCS formation, one at pH 5.4 and another at pH 8.0. The acid optimum peak is characteristically narrow as compared to the neutral peak which is quite broad. Surprisingly, despite the precipitation observed at acid pH's when chromogenic assays were used the results obtained using the two methods are comparable. Two pH optima were obtained, at pH 5.5 and pH 7.5, when the chromogenic assays were used. As for the release of UCS the acid peak was narrow and the neutral peak was broad, both extending over the same range of pH's. These results support the observation made earlier (Zeitlin et al, 1989) that the tripeptide substrate S-2266 can be used to monitor both acid and neutral kinin (UCS)-forming enzymes in cardiovascular tissues. Similar observations were made when using the dog ventricular myocardium but the substrate could not be used on the unfractionated ventricular extract due to the difficulty of monitoring release of pNA in the background of a pink coloration of myoglobin which is present in the extract.

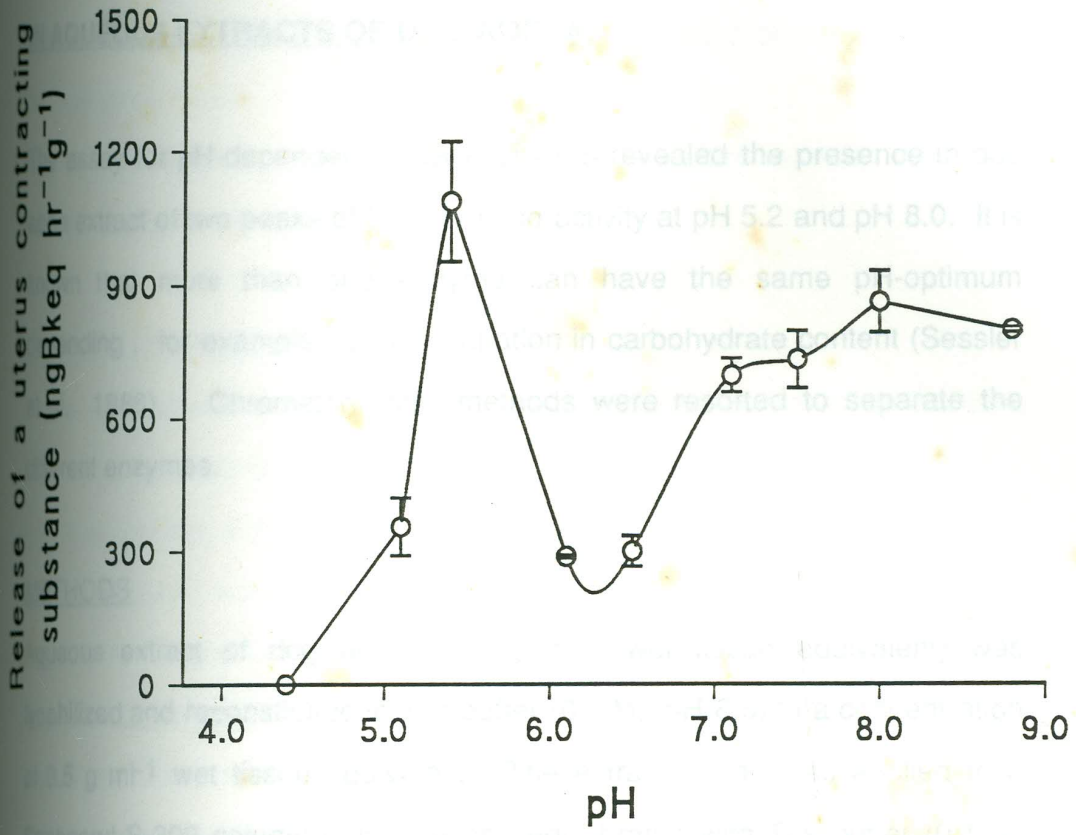


Figure 4.5. pH-dependent release of a uterus contracting substance by aqueous extracts of dog aorta. An aqueous extract of aorta was incubated with a kinin-forming substrate at pH 4 - pH 9 and incubates assayed on the isolated oestrous rat uterus. Two pH-related peaks of UCS-formation were obtained at pH 5.4 and pH 8.0. Each point represents mean ± S.D (n = 5).

4.6. GEL FILTRATION OF ENZYMES CLEAVING D-VAL-LEU-ARG-pNA IN AQUEOUS EXTRACTS OF DOG AORTA.

The assay for pH-dependent release of UCS revealed the presence in dog aorta extract of two peaks of UCS forming activity at pH 5.2 and pH 8.0. It is known that more than one enzyme can have the same pH-optimum depending , for example, on the variation in carbohydrate content (Sessler et al, 1986). Chromatographic methods were resorted to separate the different enzymes.

METHODS

Aqueous extract of dog aorta (0.1 g ml^{-1} wet tissue equivalent) was lyophilized and reconstituted in Tris buffer (0.1 M, pH 8.5) to a concentration of 0.5 g ml^{-1} wet tissue equivalent. The extract (3 ml) was applied to a Sephacryl S-300 column (2.6 x 86 cm) equilibrated with Tris buffer (0.1 M, pH 8.5). The column was eluted with the same buffer at the rate of 5.7 ml hr^{-1} cm^{-2} and 3 ml fractions were collected. Enzyme peaks were monitored by measuring the absorbance of the eluted fractions at 280 nm (A_{280}) and by assaying the fractions for arginine amidase activity. The fractions were assayed at pH 5.2 (0.1 M, citrate-phosphate buffer) or at pH 8.0 (0.1 M, Tris buffer). The tripeptide substrate D-Val-Leu-Arg-pNA was used. The chromogenic assays were possible because the high molecular weight substance in the extract fractionates near the void volume leaving the other fractions free of the precipitating material. The column was calibrated for the determination of molecular weights using the standard proteins myoglobin (17.2 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and apoferritin (443 kDa). K_{av} values were

determined and then plotted against the logarithm of molecular weights. The unknown molecular weights were calculated by interpolation of K_{av} values in the calibration curve.

$K_{av} = (V_e - V_o)/ (V_t - V_o)$; where V_e = sample elution volume; V_o = void volume and V_t = total packed column volume.

RESULTS AND DISCUSSION

Figure 4.6.1 shows a typical elution pattern obtained by assaying fractions from an aqueous aorta extract at pH 8.0. The figure shows 4 peaks A, B, C, and D of amidase activity. Four column runs were made and the calculated mean molecular weights were $\geq$ 400 kDa for the high molecular weight fractions A, B and C and 48.5 $\pm$ 4.0 kDa for the low molecular weight peak D. There is probably a second low molecular weight peak, molecular weight < 48.5 kDa which was not very obvious in all the four runs which were used for calculating the mean molecular weights. On figure 4.6.2 the second low molecular weight peak, peak E, is more distinct but smaller in size. Since the two low molecular weight peaks were not always completely separated in all subsequent studies they were pooled together and treated as one peak. Figure 4.6.3 shows a typical elution pattern obtained by assaying fractions from aqueous aorta extract at pH 5.2. The figure shows two peaks of amidase activity. Four column runs were made and the mean molecular weights for the two peaks were 252.0 $\pm$ 39.0 kDa and 42.0 $\pm$ 4.6 kDa for the low and high molecular weight peaks, respectively.

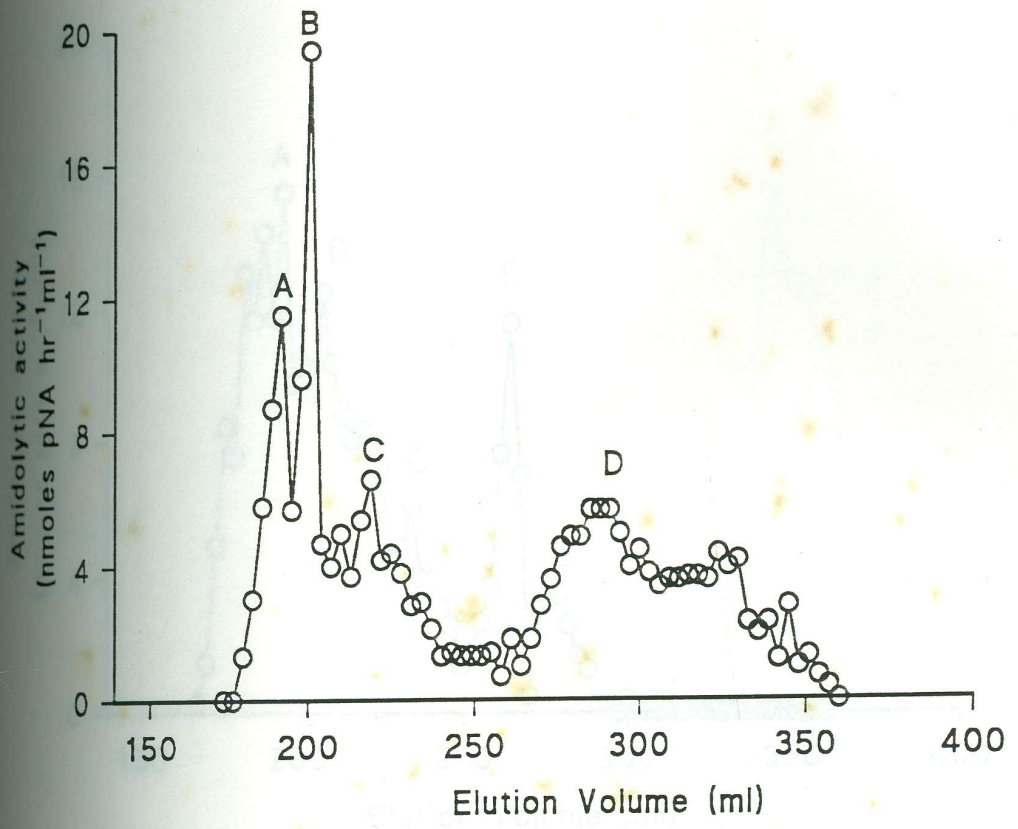


Figure 4.6.1. A typical gel filtration pattern of neutral enzymes cleaving D-Val-Leu-Arg-pNA at pH 8.0 in aqueous extracts of dog aorta. The figure represents only one column run. Three peaks of molecular weights $\geq$ 400 kDa and one peak molecular weight 48.5 $\pm$ 4.0 kDa were obtained.

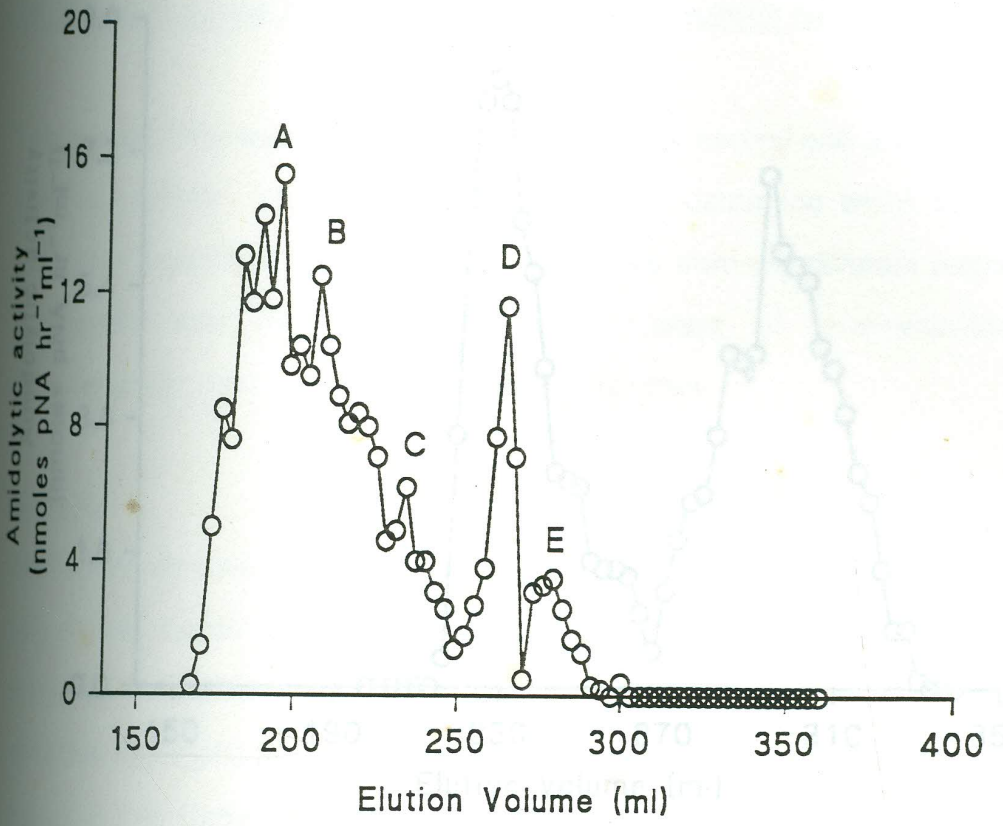


Figure 4.6.2. A typical gel filtration pattern of neutral enzymes cleaving D-Val-Leu-Arg-pNA at pH 8.0 in aqueous aorta extract. Compared to figure 4.6.1 this run shows a separate small peak, E, molecular weight < 48.5 kDa. In subsequent studies the two peaks were pooled and treated as one peak.

4.7. DETERMINATION OF FRACTIONS FROM THE AQUEOUS EXTRACT OF DOG AORTA WHICH ARE CAPABLE OF RELEASING BKIR.

Fractionation of the aqueous aorta extract gave both neutral and acid peaks of amidase activity. This experiment was done to determine which of the peaks were capable of releasing kinins. Fractions from the different peaks were pooled and then assayed for the release of bradykinin-like immunoreactivity from a plasma kinin-forming substrate.

METHOD

The fractions containing each of the amidase peaks at pH 5.2 and pH 8.0 were pooled, lyophilized and reconstituted in distilled water to double the original concentration. The reconstituted extract (0.2 ml) was pre-incubated with either citrate-phosphate buffer (0.1 M, pH 5.2) or Tris buffer (0.1 M, pH 8.0) and 0.1 ml containing o-phenanthroline (0.01 M) and EDTA (0.3 M) for 10 minutes. Incubation was started by the addition of 0.25 ml of the kinin-forming substrate and continued for 1 hour. Incubates were inactivated by boiling in a water bath for 10 minutes. Protein concentrations were measured using a microBCA protein assay reagent(Pierce Ltd).

RESULTS.

Table 4.7 shows that both the high and low molecular weight acid optimum peaks were capable of releasing BKIR with a specific activity of 163.8 $\pm$ 46.3 and 23.3 $\pm$ 9.2 ngBkeq hr^{-1} mg^{-1} protein , respectively. When the peaks at pH 8.0 were assayed for the release of BKIR only the low molecular weight peak released BKIR with a specific activity of 61.8 $\pm$ 20.0 ng Bkeq hr^{-1} mg^{-1}

protein. The three high molecular weight peaks pooled together were incapable of releasing BKIR.

pH 5.2		pH 8.0	
Mwt (kDa)	Specific activity (ngBkeq hr^{-1} mg^{-1}	Mwt (kDa)	Specific activity (ngBkeq hr^{-1} mg^{-1})
252.0 ± 39.0	163.8 ± 46.3	≥ 400	Nil
42.0 ± 4.6	23.3 ± 9.2	48.5 ± 4.0	61.8 ± 20.0

Table 4.7. The release of bradykinin-like immunoreactivity (BKIR) associated with peaks of amidase activity fractionated from an aqueous extract of dog aorta. The table shows molecular weights (mean ± S.D; n = 4) and the mean specific activities for the release of BKIR (mean ± S.D; n = 5).

4.8. INHIBITOR STUDIES ON THE ACID OPTIMUM UTERUS CONTRACTING SUBSTANCE RELEASING ENZYMIC ACTIVITY IN AQUEOUS EXTRACTS OF DOG AORTA.

Four inhibitors were used, aprotinin, soybean trypsin inhibitor, lima bean trypsin inhibitor and pepstatin. The inhibitors were chosen based on previous reports. Previous experience shows that these inhibitors might have one effect or the other on the consequences of acute myocardial infarction (Hashimoto et al, 1977; Lefer et al, 1974) or directly on the kinin-forming enzymes in cardiovascular tissues (Nolly et al, 1981b; Zeitlin et al, 1989).

4.8.1. THE EFFECT OF APROTININ ON THE RELEASE OF UCS BY THE ACID OPTIMUM ENZYME PEAK IN AQUEOUS EXTRACTS OF DOG AORTA.

Aprotinin caused a concentration-dependent inhibition of the acid optimum enzyme in aqueous extracts of dog ventricular myocardium. This experiment was done to determine its effect on the acid optimum UCS-forming activity in the aqueous extract of dog aorta.

METHOD

The aorta extract (0.2 ml, 0.4 g ml^{-1}) was pre-incubated, at 37 ^{o}C, with 0.55 ml acetate-barbitone buffer (0.05 M, pH 5.2) and 0.1 ml containing o-phenanthroline (0.01 M) and EDTA (0.3 M) for 10 minutes. Three concentrations of aprotinin, 540, 1080, and 2700 KIU/ml were used. These were included in the pre-incubates. Incubations were started by the addition of 0.25 ml of the kinin -forming substrate and continued for one hour.

Incubates were inactivated by boiling in a water bath for 10 minutes. The incubates were centrifuged and the supernatants were assayed on the isolated oestrous rat uterus.

RESULTS.

Figure 4.8.1 shows the effect of 540 - 2700 KIU of aprotinin on the release of UCS by aqueous extracts of dog aorta. A significant inhibition in the release of UCS was achieved (31.5 %; $P < 0.05$) at the concentration of 540 KIU/ml. When the concentration was increased to 1080 and 2700 KIU no further inhibition was achieved as compared to 540 KIU ($P > 0.05$). The maximum inhibition obtained was 38.9 % .

4.8.2. THE EFFECT OF SOYBEAN TRYPSIN INHIBITOR ON THE RELEASE OF UCS BY THE ACID OPTIMUM PEAK IN AQUEOUS EXTRACTS OF DOG AORTA.

Soybean trypsin inhibitor , the inhibitor of plasma kallikrein, did not have any effect on the acid optimum UCS-forming enzyme in the aqueous extract of dog ventricles. It was used in this experiment to compare its effect on the aqueous extract of aorta with that on the ventricular extract.

METHOD

The aorta extract (0.2 ml, 0.4 g ml^{-1}) was pre-incubated, at 37 ^{o}C, with 0.55 ml acetate-barbitone buffer (0.05 M, pH 5.2) and 0.1 ml containing o-phenanthroline (0.01 M) and EDTA (0.3 M) for 10 minutes. Soybean trypsin inhibitor was used at concentrations of 5×10^{-6} M - 2.5×10^{-5} M. These were included in the pre-incubates. Incubations were started by the addition of 0.25 ml of the kinin -forming substrate and continued for one hour.

Incubates were inactivated by boiling in a water bath for 10 minutes. The incubates were centrifuged and the supernatants were assayed on the isolated oestrous rat uterus.

RESULTS.

Figure 4.8.2 shows that SBTI at 5 x 10^{-6} M - 2.5 X 10^{-5} M did not have any effect on the release of a uterus contracting substance by the acid optimum enzymic activity in the aqueous extracts of dog aorta.

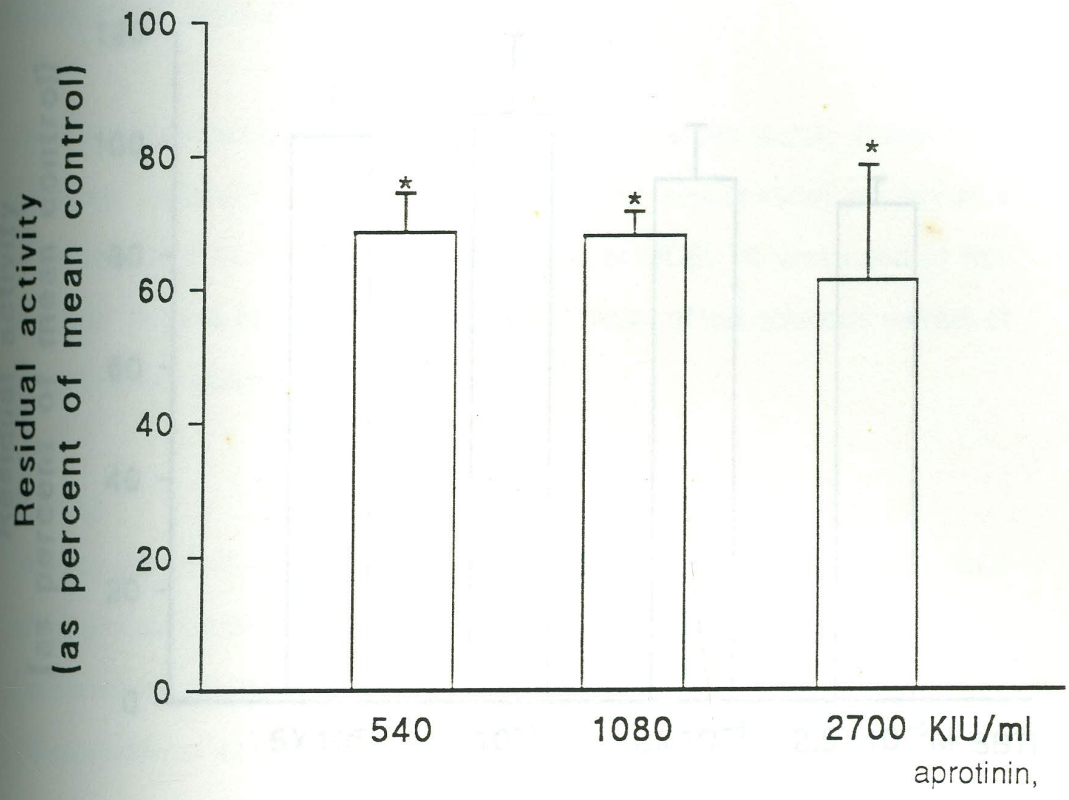
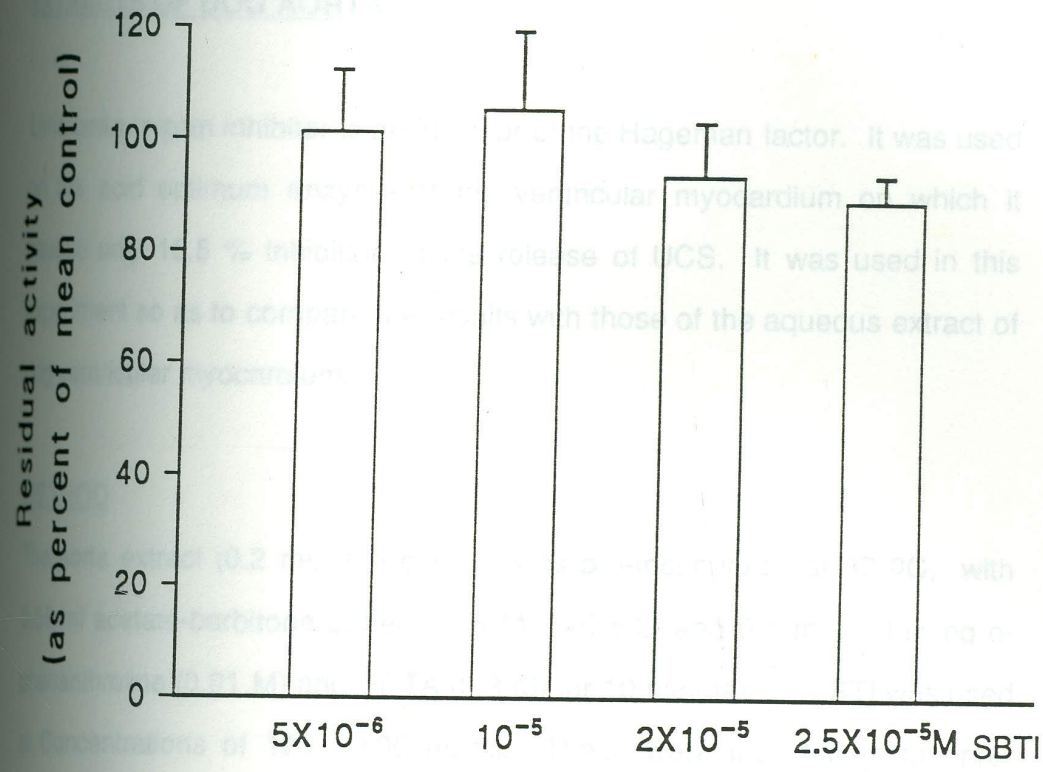


Figure 4.8.1. The effect of aprotinin on the release of a uterus contracting substance (UCS), at pH 5.2, by aqueous extracts of dog aorta. Aprotinin was pre-incubated with the extract for 10 minutes before adding the substrate. The results are presented as mean ± S.D (n =5). Aprotinin up to 2700 KIU/ml caused a maximum inhibition of 38.9 % ($P < 0.05$).



...e 4.8.2. The effect of soybean trypsin inhibitor on the release of a uterus ...acting substance (UCS), at pH 5.2, by aqueous extracts of dog aorta. The ...e extract was pre-incubated with SBTI for 10 minutes before adding the kinin-...ng substrate. The results are presented as mean ± S.D (n = 5).

4.8.3 THE EFFECT OF LIMA BEAN TRYPSIN INHIBITOR ON THE RELEASE OF UCS BY THE ACID OPTIMUM PEAK IN AQUEOUS EXTRACTS OF DOG AORTA.

Lima bean trypsin inhibitor is an inhibitor of the Hageman factor. It was used on the acid optimum enzyme of the ventricular myocardium on which it caused only 13.5 % inhibition of the release of UCS. It was used in this experiment so as to compare the results with those of the aqueous extract of dog ventricular myocardium.

METHOD

The aorta extract (0.2 ml, 0.4 g ml^{-1}) was pre-incubated, at 37 ^{o}C, with 0.55 ml acetate-barbitone buffer (0.05 M, pH 5.2) and 0.1 ml containing o-phenanthroline (0.01 M) and EDTA (0.3 M) for 10 minutes. LBTI was used at Concentrations of 125 - 500 µg/ml. These were included in the pre-incubates. Incubations were started by the addition of 0.25 ml of the kinin - forming substrate and continued for one hour. Incubates were inactivated by boiling in a water bath for 10 minutes. The incubates were centrifuged and the supernatants were assayed on the isolated oestrous rat uterus.

RESULTS.

Figure 4.8.3 shows the effect of LBTI on the release of UCS, at pH 5.2, by aqueous extracts of dog aorta. LBTI at 125 µg/ml had no significant effect on the release of UCS by the acid optimum enzyme in aqueous extracts of dog aorta. Increasing the concentration to 500 µg/ml caused 33.3 % ($P < 0.05$) inhibition. Using the aqueous extract of dog ventricles LBTI caused 13.5 % inhibition of the release of UCS at pH 5.2. The results show that the release

of UCS by extracts of both tissues at acid pH is slightly inhibited by LBTI. The release of UCS by the aorta extract was more affected.

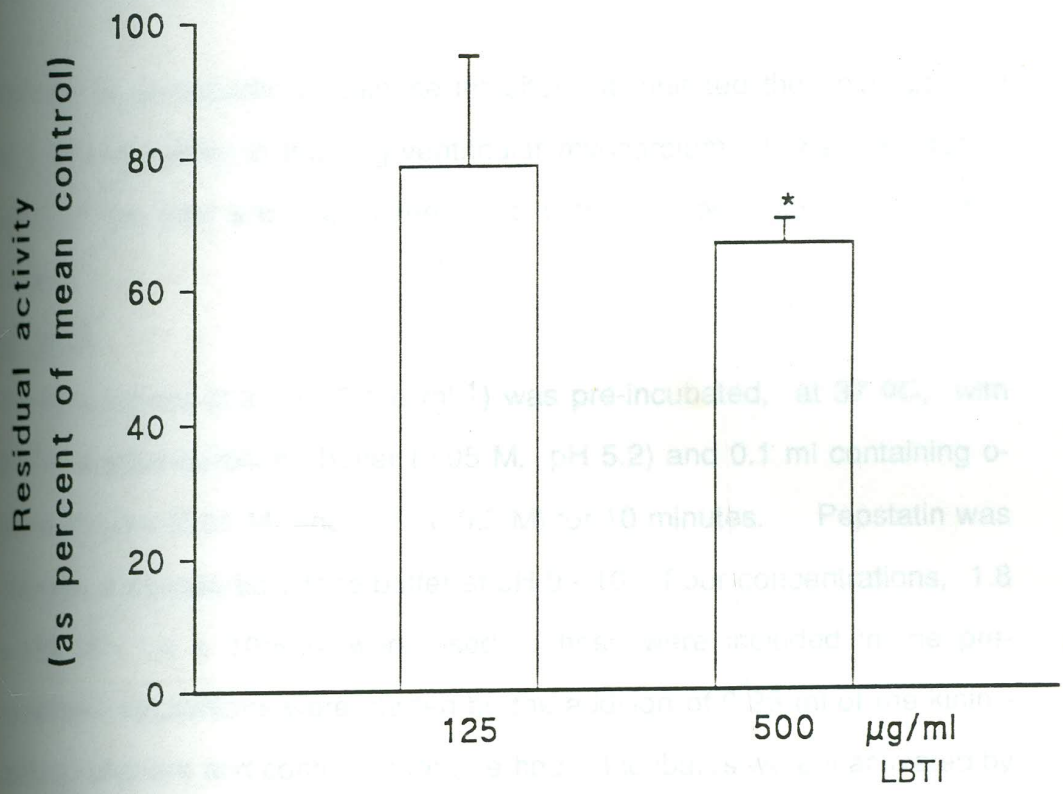


Figure 4.8.3. The effect of lima bean trypsin inhibitor (LBTI) on the release of a uterus contracting substance (UCS), at pH 5.2, by aqueous extracts of dog aorta. The aqueous extract was pre-incubated with LBTI for 10 minutes before adding the kinin-forming substrate. The results are presented as mean $\pm$ S.D (n = 5).

4.8.4. THE EFFECT OF PEPSTATIN ON THE RELEASE OF UCS BY THE ACID OPTIMUM PEAK IN AQUEOUS EXTRACTS OF DOG AORTA.

Pepstatin is an aspartic proteinase inhibitor. It inhibited the acid optimum UCS-forming activity in the dog ventricular myocardium. It was decided to use it on the acid enzyme in the aorta extract to determine if it is also inhibited.

METHOD

The aorta extract (0.2 ml, 0.4 g ml^{-1}) was pre-incubated, at 37 ^{o}C, with 0.55 ml acetate-barbitone buffer (0.05 M, pH 5.2) and 0.1 ml containing o-phenanthroline (0.01 M) and EDTA (0.3 M) for 10 minutes. Pepstatin was dissolved in acetate-barbitone buffer at pH 9 - 10 . Four concentrations, 1.8 x 10^{-5} M - 1.4 x 10^{-4} M were used. These were included in the pre-incubates. Incubations were started by the addition of 0.25 ml of the kinin - forming substrate and continued for one hour. Incubates were inactivated by boiling in a water bath for 10 minutes. The incubates were centrifuged and the supernatants were assayed on the isolated oestrous rat uterus.

RESULTS.

Figure 4.8.4 shows the release of UCS by incubates with and without pepstatin. Substrate and extract blanks were free of uterus contracting activity. The incubates without pepstatin released a uterus contracting substance. Pepstatin at 1.8 x 10^{-5} M completely inhibited the release of UCS by incubates of aqueous extracts of aorta with the kinin-forming substrate. The higher concentrations of pepstatin showed similar inhibition.

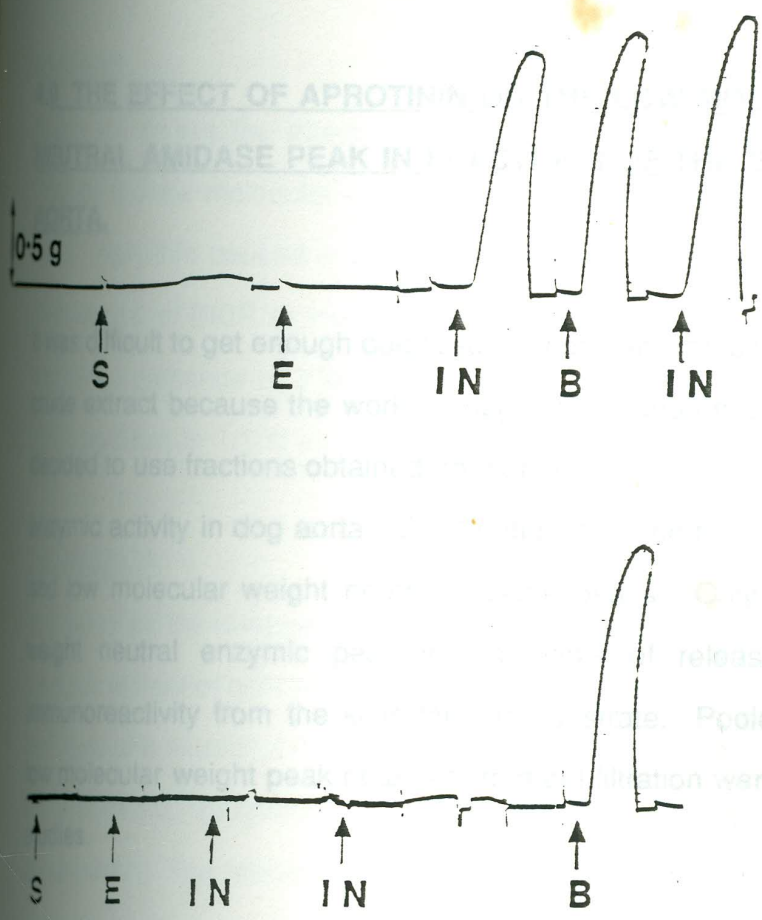


Figure 4.8.4. The effect of pepstatin on the release of a uterus contracting substance (UCS), (pH 5.2), by aqueous extracts of dog aorta. Incubates were assayed using the contractions of the oestrous rat uterus and synthetic bradykinin as a standard. The upper tracing shows the effect of incubates without pepstatin and the lower tracing shows the effect of incubates containing 1.8×10^{-5} M pepstatin, pre-incubated with the extract for 10 minutes before addition of substrate. On both tracings both substrate (S) and extract (E) controls did not release UCS. On the upper tracing 0.1 ml of incubate (IN) released UCS. B = 2ng/ml bradykinin. On the lower tracing up to 0.2 ml of incubate (IN) were used but there was no release of UCS. No control containing bradykinin and pepstatin was used.

4.9 THE EFFECT OF APROTININ ON THE LOW MOLECULAR WEIGHT NEUTRAL AMIDASE PEAK IN FRACTIONS OF THE EXTRACT OF DOG AORTA.

It was difficult to get enough dog tissue to complete inhibitor studies using the crude extract because the work on dogs in the department stopped. It was decided to use fractions obtained from gel filtration to characterize the neutral enzymic activity in dog aorta. Gel filtration gave both high molecular weight and low molecular weight neutral enzyme peaks. Only the low molecular weight neutral enzymic peak was capable of releasing bradykinin-like immunoreactivity from the kinin-forming substrate. Pooled fractions of this low molecular weight peak obtained from gel filtration were used for inhibitor studies.

METHOD

Lyophilized and reconstituted pooled fractions of the low molecular weight neutral enzyme were used. Two sets of incubates were made, one with and another without aprotinin. The fractions (0.2 ml) were pre-incubated with 0.6 ml Tris buffer (pH 8.0, 0.1 M) and 0.1 ml containing o-phenanthroline (0.01 M) and EDTA (0.3 M) for 10 minutes. When aprotinin was used it was included in the pre-incubates (2700KIU/ml). Incubation was started by the addition of 0.25 ml of the kinin-forming substrate and continued for 1 hour. Incubates were inactivated by boiling in a water bath for 10 minutes. The incubates were centrifuged and the supernants assayed by radioimmunoassay (Chapter 2.2).

RESULTS.

Figure 4.9 shows the effect of 2700 KIU/ml of aprotinin on the release of BKIR by the low molecular weight neutral enzyme in aqueous extracts of dog aorta. Aprotinin caused a mean inhibition in the release of BKIR of 94 %. The release of BKIR in the controls was 6145.3 $\pm$ 996 pg Bkeq hr^{-1}/100 µl and in the presence of 2700 KIU aprotinin the release of BKIR was 368.7 $\pm$ 368.7 pg Bkeq hr^{-1}/100 µl.

DISCUSSION

The assay for the release of a uterus contracting substance showed that the aqueous extract of canine aorta releases UCS from a kininogen substrate at both acid and neutral pH's. The release of UCS showed two optima at pH 5.4 and pH 8.0, suggesting the presence of two enzymic activities in the aorta extract. The release of UCS at acid pH 5.2 was inhibited 33.3 % by LBTI. This is a higher inhibition as compared to that obtained using the dog ventricles (13.5 %). Aprotinin, the non-selective serine proteinase inhibitor, inhibited this acid optimum activity by 38.9 %. It produced almost the same inhibition on the release of UCS by the acid optimum enzyme in the dog ventricles (32.8 %). Pre-treatment with aprotinin prevented the elevation of levels of kinin in the coronary sinus blood in the dog following ligation of the left anterior descending coronary artery (Hashimoto et al, 1977). It is possible this effect is related to the inhibition of a kinin-forming enzyme in vasculature and or in the cardiac tissues. Like with the ventricular extract, the release of UCS by the aorta extract was unaffected by SBTI. This is probably an indication that the release of UCS by the aorta extract is not due to the presence of plasma kallikrein. SBTI also inhibits tissue kallikrein, but to a lesser extent (Barrett and McDonald, 1980). This suggests that the

inhibition obtained with aprotinin may not be related to an effect on tissue kallikrein. Pepstatin, the aspartic proteinase inhibitor, completely inhibited the release of UCS by aqueous extracts of dog aorta at pH 5.2. This shows that the release of UCS at acid pH's may be due to the presence, in the extract, of an aspartic proteinase. Gel filtration and assay of the fractions at pH 5.2 showed that the aqueous extract of dog aorta contains two acid optimum enzymes, molecular weights 252 $\pm$ 39 kDa and 42.0 $\pm$ 4.6 kDa. The low molecular weight enzyme has a molecular weight similar to that of cathepsin D and renin. The identity of the high molecular weight enzyme is not clear. Based on the results obtained with pepstatin it is tempting to speculate that the high molecular weight enzyme is the low molecular weight enzyme complexed to a high molecular weight substance like an α_2-macroglobulin monomer (Enghild et al, 1989). When fractions of the aorta were assayed at pH 8.0 three high molecular weight amidase peaks (mwt > 400 kDa) and one low molecular weight peak (mwt 48.5 $\pm$ 4.0 kDa) were obtained. All the high molecular weight peaks pooled together failed to release BKIR from a plasma kinin-forming substrate. The low molecular weight peak released BKIR at the rate of 61.8 $\pm$ 20.0 ng Bkeq hr^{-1} mg^{-1} protein. This low molecular weight enzyme was almost completely inhibited by aprotinin (2700 KIU/ml). It was not possible to get enough tissue to do all the four inhibitors on the neutral enzyme due to lack of dog tissues. The inhibition by aprotinin and the molecular weight of 48.5 kDa suggests that the neutral enzyme could be tissue kallikrein. However, it is difficult to make conclusions about the identity of the enzyme from these results. Purification to homogeneity and the use of specific antibodies will be required to confirm its identity.

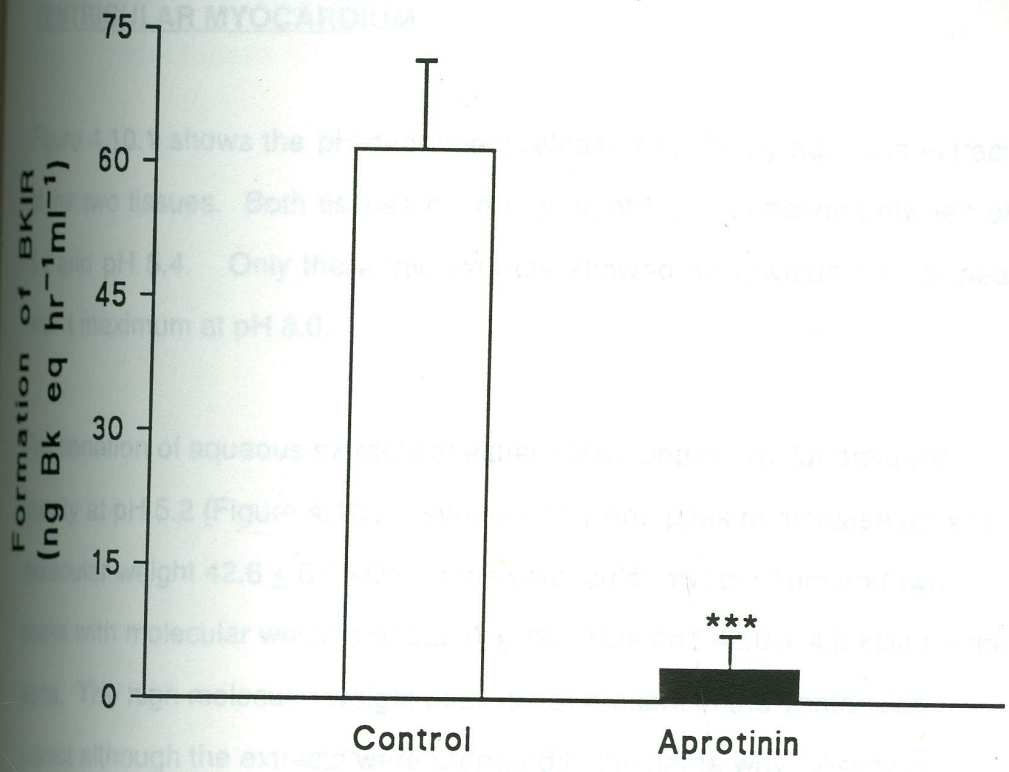


Figure 4.9. The effect of aprotinin on the release of bradykinin-like immunoreactivity (BKIR) by the low molecular weight neutral enzyme (pH 8.0) fractionated from dog aorta extract. Fractions containing the low molecular weight peak were pooled and then assayed (pH 8.0) for the release of BKIR in the presence and in the absence of aprotinin (2700 KIU/ml). The results are presented as mean ± S.D (n =5).

4.10 COMPARISON OF ENZYMES IN DOG AORTA AND DOG VENTRICULAR MYOCARDIUM.

Figure 4.10.1 shows the pH-dependent release of UCS by aqueous extracts of the two tissues. Both tissues have a peak of UCS-formation between pH 5.2 and pH 5.4. Only the aortic extracts showed an obvious neutral peak with a maximum at pH 8.0.

Fractionation of aqueous extracts of either tissue and assay for amidase activity at pH 5.2 (Figure 4.10.2) revealed only one peak of amidase activity, molecular weight 42.6 $\pm$ 0.9 kDa for the ventricular myocardium and two peaks with molecular weights of 252.0 $\pm$ 39.0 kDa and 42.0 $\pm$ 4.6 kDa for the aorta. The high molecular weight peak is not present in the ventricular extract although the extracts were prepared in the same way. Assay of fractions from the dog aorta at pH 8.0 gave three high molecular weight, non-BKIR forming peaks, and a low molecular weight peak (molecular weight 48.5 $\pm$ 4.0 kDa) capable of releasing BKIR. There was no descernible peak of neutral activity in the ventricular extract. Table 4.10.1 compares the molecular weights in the two tissues and the respective specific activities for the release of BKIR. The low molecular weight acid optimum enzyme in the aorta extract is similar in size and specific activity for the release of BKIR to that in the ventricular extract. Comparing the effect of inhibitors on the acid optimum UCS-forming activity in the two extracts (Table 4.10.2) shows that they are both completely inhibited by pepstatin and partially by aprotinin. The inhibition caused by aprotinin was almost the same for the two tissues. Lima bean trypsin inhibitor also partially inhibited the release of UCS by extracts of the two tissues. Only 13.5 % inhibition was

obtained with the enzyme in the ventricles but with the aorta a higher inhibition was obtained (33.3 %). The release of UCS by the acid optimum activity in both tissues was not affected by SBTI.

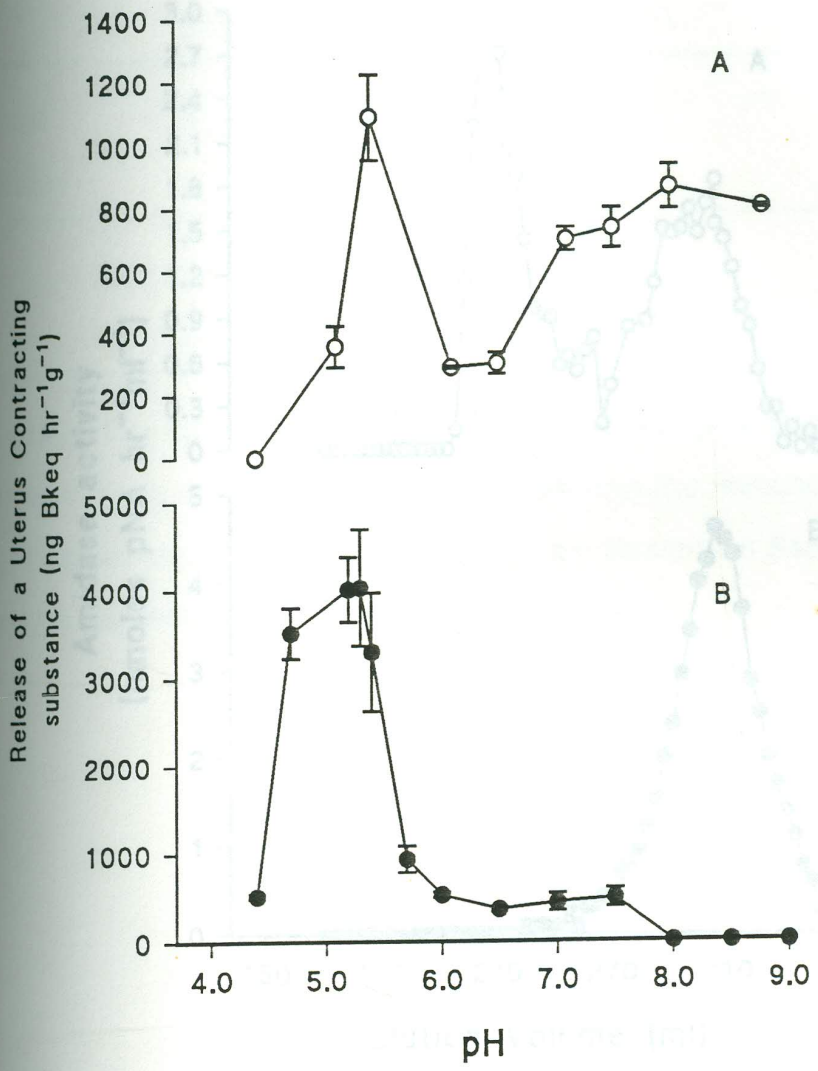


Figure 4.10.1. pH-dependent release of a uterus contracting substance by aqueous extracts of dog aorta (A) and dog ventricular myocardium (B). The aorta extract gave two peaks of UCS-formation, at pH 5.4 and pH 8.0. The ventricular extract gave only one peak at pH 5.2 - 5.4. Each point represents mean $\pm$ S.D (n = 5).

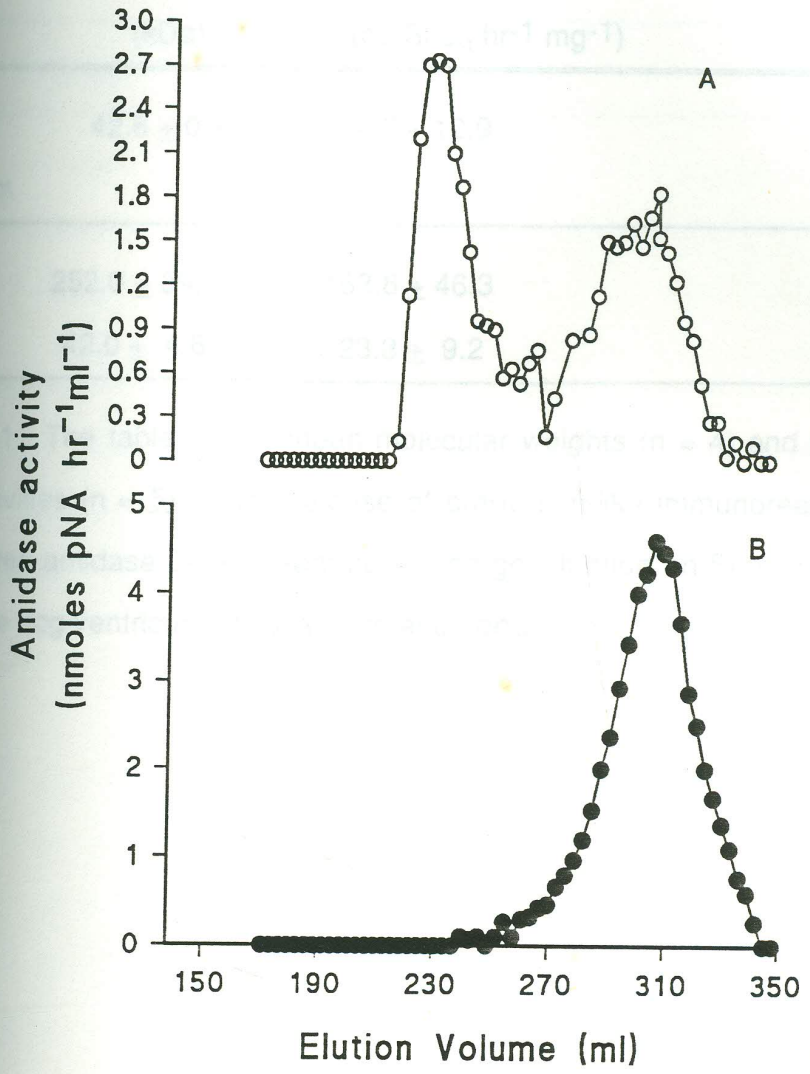


Figure 4.10.2. Gel filtration pattern of acid optimum enzymes cleaving D-Val-Leu-Arg-pNA at pH 5.2 in aqueous extracts of dog aorta (A) and dog ventricular myocardium (B). The aorta extract gave two peaks of amidase activity molecular weights 259.0 ± 39.0 kDa and 42.0 ± 4.6 kDa. The ventricular extract gave only one peak, molecular weight 42.6 ± 0.9 kDa.

Source of peak	Molecular weight (kDa)	Specific activity (ng Bkeq hr^{-1} mg^{-1})
Ventricular myocardium	42.6 ± 0.9	24.5 ± 12.9
Aorta	252.0 ± 39.0	163.8 ± 46.3
	42.0 ± 4.6	23.3 ± 9.2

Table 4.10.1. The table shows mean molecular weights (n = 4) and mean specific activities (n = 5) for the release of bradykinin-like immunoreactivity (BKIR) related amidase peaks separated using gel filtration on Sephacryl S-300 from the dog ventricular myocardium and aorta.

Inhibitor	PERCENTAGE INHIBITION	
	Aorta	Ventricular myocardium
Pepstatin	100*	100*
Aprotinin	38.9*	32.8*
LBTI	33.3*	13.5
SBTI	10.9	2.87

Table 4.10.2. Actions of proteinase inhibitors on UCS forming enzymic activity in aqueous extracts of dog aorta and ventricular myocardium. Assays were done at pH 5.2. * $P < 0.05$, $n = 5$.

GENERAL DISCUSSION.

The dog coronary arteries were reported to contain acid and neutral UCS-forming enzymes (Zeitlin et al, 1989). The acid enzyme was similar to the enzyme in rat mesenteric arteries (Nolly et al, 1981b) and that in rat cardiac tissue (Britos and Nolly, 1981). The acid enzyme in rat cardiac tissue had a molecular weight of 30 - 35 kDa (Britos and Nolly , 1981) which is very close to that of the acid enzyme in dog coronary arteries (38.3 kDa; Zeitlin et al, 1989). The neutral enzyme in the dog coronary arteries was similar to plasma kallikrein in molecular size and it was inhibited by soybean trypsin inhibitor (Zeitlin et al, 1989). Other reports indicate that the neutral enzyme in rat mesenteric arteries (Nolly et al, 1981b, Nolly and Lama, 1982), rat tail arteries (Nolly et al, 1985) and rat aorta (Nolly et al, 1987; Oza et al, 1990) is a tissue kallikrein-like enzyme.

The present work shows that aqueous extracts of dog aorta contain two enzymic activities releasing UCS from a kinin-forming substrate. It showed two peaks of UCS formation, one at pH 5.4 and another at pH 8.0. Using the amidase assay similar results were obtained, two pH-related peaks of amidase activity, one at pH 5.5 and another at pH 7.5.

Gel filtration of the canine aorta extracts and assay of the eluted arginine amidase activity at pH 5.2 gave two peaks of activity, one eluting with a molecular weight of 252.0 $\pm$ 39.0 kDa and the second with a molecular weight of 42.0 $\pm$ 4.6 kDa. Both peaks released bradykinin-like immunoreactivity (BKIR) from a plasma kinin-forming substrate, the high molecular weight peak with a specific activity of 163.8 $\pm$ 46.3 ng Bkeq hr^{-1} mg^{-1} protein, the low molecular weight peak with a specific activity of 23.3 $\pm$

9.2 ngBkeq hr^{-1} mg^{-1} protein. When assays were done at pH 8.0 four peaks of amidase activity were obtained. Three had high molecular weights $\geq$ 400 kDa and were incapable of releasing BKIR from the kinin-forming substrate. The fourth peak had a molecular weight of 48.5 $\pm$ 4.0 kDa and released BKIR with a specific activity of 61.8 $\pm$ 20.0 ngBkeq $hr^{-1}mg^{-1}$ protein. The acid optimum enzyme was completely inhibited by pepstatin in the crude extract; however, on fractionation instead of getting only one enzyme two enzymes were obtained, one with a high molecular weight and another with a low molecular weight. The low molecular weight acid optimum enzyme in dog aorta is similar, in molecular size and in its sensitivity to pepstatin, to the acid optimum enzyme in the dog ventricular myocardium and coronary arteries (Zeitlin et al, 1989). A similar enzyme in rat cardiac tissues had a molecular weight of 30 - 35 kDa (Britos and Nolly, 1981). This low molecular weight acid optimum kininogenase is similar to cathepsin D. Cathepsin D (E.C. 3.4.23.5) is an acid optimum aspartic proteinase. It has a molecular weight of about 42 kDa, it is inhibited by pepstatin (Barrett and McDonald, 1980) and it is capable of releasing kinins from low molecular weight kininogen (Ogloblina et al, 1981). The dog enzyme had an optimum at pH 5.2 - 5.5, while cathepsin D has its optimum between pH 2.8 - 5.0 depending on the source and substrate (Barrett and McDonald, 1980). Two other aspartic proteinases, renin and rat submandibular renin resemble the acid optimum enzyme in molecular size and pH-optimum (Barrett and McDonald, 1980). Rat submandibular renin has a variable pH optimum depending on the substrate and its source but with octapeptide substrates the pH optimum is at pH 5.4. Likewise rat renin has its optimum at pH 3.5 with octapeptide substrates and pH 6.0 with angiotensinogen (Barrett and McDonald, 1980). The identity of the high molecular weight acid optimum kininogenase with a molecular weight at about 250 kDa, inhibited by

pepstatin, and pH optimum at pH 5.4 - 5.5 is not very obvious. It could be a new enzyme, or an aggregated or polymeric form of an existing enzyme. It has been reported that different carbohydrate contents could give an enzyme different molecular sizes and yet retain the same pH optimum (Sessler et al, 1986). One other possibility derives from the observation that α_2-macroglobulin combines with some aspartic proteinases, including cathepsin D (Thomas et al, 1989). The α_2-macroglobulin is a tetramer consisting of monomers with a molecular weight of approximately 180 - 190 kDa (Sotrup-Jensen et al, 1983). The monomers are also capable of combining with proteinases and partially inhibiting their enzymic activity (Enghild et al, 1989). A complex with the low molecular weight monomer would fall well within one standard error of the present molecular weight determinations (234 - 269 kDa.). The reason for the presence of a high molecular weight acid optimum kinin-forming enzyme in the canine aorta and its absence from the ventricular tissue remains to be determined.

The neutral enzyme in the aorta extract was completely inhibited (94 %) by aprotinin. It may be tissue kallikrein, but since no other inhibitors were used to distinguish it from other enzymes, it is difficult to make any conclusions about its identity based only on these results. The enzyme requires further purification to homogeneity following which specific antibodies can be used for its proper characterization.

CHAPTER 5

STUDIES ON KININ-FORMING ENZYMES IN THE RAT VENTRICULAR MYOCARDIUM.

INTRODUCTION.

Nolly and his colleagues reported that rat, human, and bovine cardiac tissues contain an acid optimum kinin-forming enzyme (Nolly et al., 1981a; Britos and Nolly, 1981). However, while they went as far as identifying the subcellular localization of this enzyme it was not specified which area of the heart contained this enzyme. Since 1967 a lot of work has been done, particularly, to determine the role played by the kallikrein-kinin system in the pathophysiology of myocardial ischaemia (Sicuteri et al., 1967; Sicuteri et al., 1972; Pitt et al., 1970; Hashimoto et al., 1977; Matsuki et al, 1986; Matsuki et al, 1987; Zeitlin et al, 1989). To date the work done, so far, shows that kinins are released during myocardial ischaemia (Kimura et al, 1973; Hashimoto et al, 1977; Matsuki et al, 1987) and that kinin-forming enzymes are present in cardiac tissues (Nolly et al, 1981a; Britos and Nolly, 1981; Zeitlin et al, 1989; Xiong et al, 1990), in the coronary vasculature (Zeitlin et al, 1989) and in the aorta (Nolly et al, 1987; Oza et al, 1990). It seems logical to assume that under ischaemic conditions a kinin-forming enzyme in cardiac tissues, coronary vasculature, or in plasma is activated or released to form kinins from kininogens in plasma and possibly in interstitial fluid. The data linking the formation of kinins and activation or release of these enzymes is still missing. The present work was done as part of the effort to close the existing gap of information. Different methods were employed in the survey for candidate kininogenases which may be responsible for the release of kinins during myocardial ischaemia.

5.1. pH-DEPENDENT RELEASE OF A UTERUS CONTRACTING SUBSTANCE BY AQUEOUS EXTRACTS OF RAT VENTRICLES.

The dog ventricular myocardium was shown to contain one peak of UCS forming activity with an optimum at pH 5.2 -pH 5.4. On the alkaline range there was detectable release of UCS up to pH 7.5 but the released UCS was very small as compared to that at the acid pH's. There was no obvious peak of neutral activity. The aim of this experiment was to determine the pH of optimum activity of enzymes capable of releasing kinins from a plasma kinin-forming substrate in aqueous extracts of rat ventricles.

METHOD.

Collection of heart tissues.

Male Sprague Dawley rats 250-350 g were killed by a blow on the head followed by cervical dislocation. Both the chest and abdomen were opened up through the midline and the ribs cut along either side to allow for easy cannulation and drainage of perfusate. The thoracic aortae were cannulated with polyethylene (PE 50) tubing connected to a Watson-Marlow slow rate flow pump. Retrograde perfusion of the hearts was done (Nolly et al, 1981a), at 4 ml min^{-1}, with Kreb's Henseleit (37°C, 95 % O_2, 5% CO_2) containing heparin (10 IU/ml), followed by heparin-free Kreb's until a clear perfusate was obtained. The ventricles were excised and stored at -20°C until used.

Preparation of homogenate.

The ventricles were thawed, chopped into small pieces, and homogenized in distilled water (0.1 g ml^{-1}) containing 0.1 % v/v Triton X-100, using an

Ultra Turrax Junke & Kunkel homogenizer. Homogenization was carried out under ice for 3 minutes. The homogenate was allowed to undergo autolytic activation under toluene (18-20 ^{o}C) for 20 hours, centrifuged (2000g, 4 ^{o}C, 30 minutes) and the supernatant obtained was dialysed under tap water for 18 hours. The extract was lyophilized and then stored at -20 ^{o}C until used.

Assay for the release of UCS.

Reconstituted extract (0.2 ml, 0.2 g/ml in distilled water) was pre-incubated with 0.55 ml, acetate-barbitone buffer (0.05 M) and 0.1 ml containing EDTA (0.3 M) and o-phenanthroline (0.01 M) for 10 minutes. Incubation was started by the addition of 0.25 ml of a human kinin-forming plasma substrate and continued for 1 hour. Incubates were inactivated by boiling in a water bath for 10 minutes, centrifuged and then assayed using the contractions of the isolated oestrous rat uterus. The unknowns were determined by bracketing between standards of synthetic bradykinin. To determine the pH optimum for the release of UCS incubates were made from pH 4 to pH 9.0.

RESULTS.

Incubates of rat ventricular extract with the kinin-forming substrate between pH 4 and 9 released UCS only at acid pHs with a maximum between pH 4.7 and pH 5.3 (Figure 5.1.1). The UCS-forming activity decreased with increasing pH and at neutral pH there was less UCS released than could be detected within the sensitivity of the assay. The minimum that could be detected was 165 ngBkeq hr^{-1} g^{-1} (equivalent to 0.3 ng/ml using a 4 ml organ bath). There was no neutral UCS-forming activity detected. Incubates made with either substrate and buffer alone or rat extract and buffer alone did not show any release of the UCS (Figure 5.1.2).

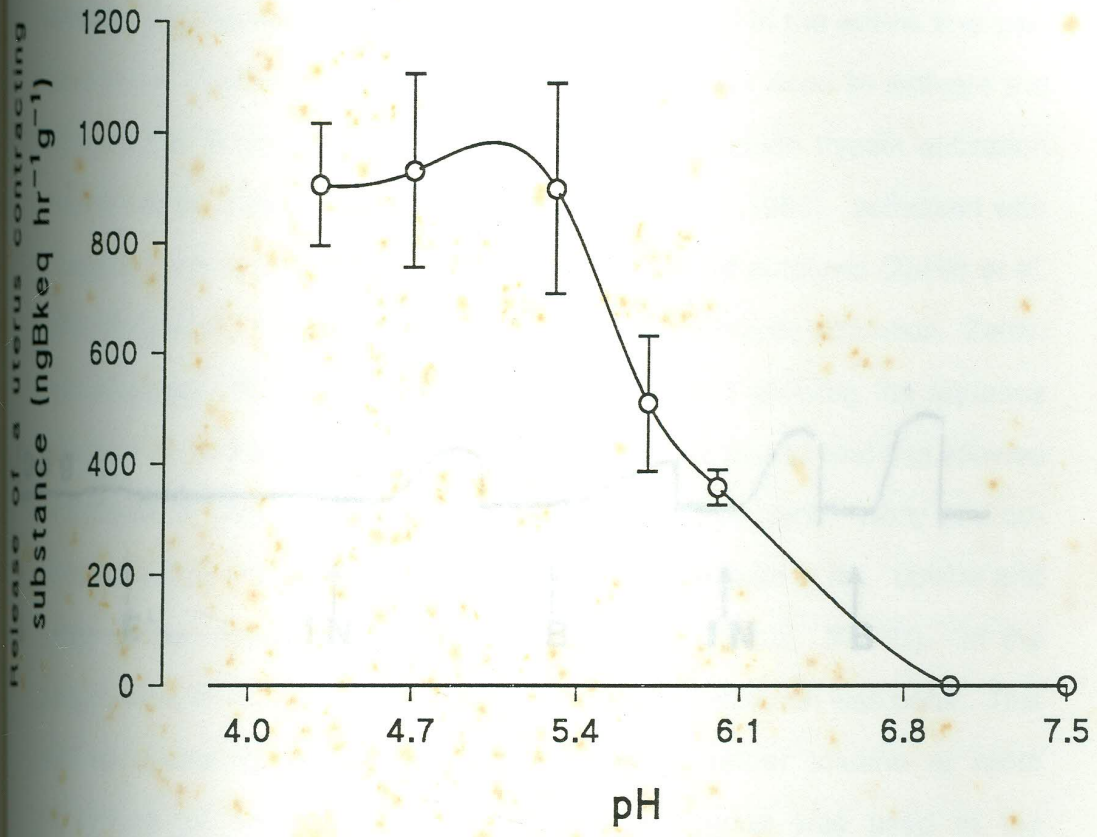


Figure 5.1.1. The effect of pH on the release of a uterus contracting substance(UCS) from a plasma kinin-forming substrate by aqueous extracts of rat ventricular myocardium. Incubates were made in 0.05 M acetate-barbitone buffer at pH 4 - pH 9. Incubates were assayed using contractions of the oestrous rat uterus using synthetic bradykinin as a standard and the released UCS was expressed as ng Bk eq hr^{-1} g^{-1} wet tissue equivalent. Each point represents mean $\pm$ S.D (n = 5).

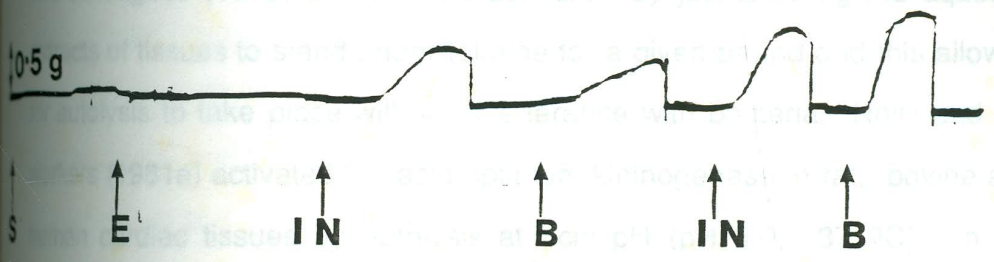


Figure 5.1.2. The effect on the oestrous rat uterus of incubates (made at pH 5.0) of an aqueous extract of rat ventricular myocardium with a kinin-forming substrate. The figure shows a tracing of isometric contractions of the oestrous rat uterus in response to: (A) 0.2 ml of an incubate of substrate and buffer only (S). (B) 0.2 ml of an incubate of ventricular extract and buffer only (E). (C) 0.2 ml of an incubate containing the ventricular extract, substrate and buffer (IN). (D) 1 ng/ml of synthetic bradykinin (B). The substrate blank (A) and extract blank (B) did not have any effect on the rat uterus.

5.2 THE EFFECT OF AUTOLYTIC ACTIVATION ON THE ACID OPTIMUM ENZYMIC ACTIVITY IN AQUEOUS EXTRACTS OF RAT VENTRICLES.

Kinin-forming enzymes in different tissues occur both in the active and pre-active forms. Different activation methods have been used to activate the kininogenases in various tissues. Such methods include trypsin activation (Woodley et al, 1985; Guder et al, 1987; Powers , 1986), activation with detergent (Berg et al, 1987; Guder et al, 1987) and autolysis (Zeitlin et al, 1976; Nolly et al, 1981a). There is a variation in autolytic activation. Zeitlin and colleagues (1976) did autolytic activation by just allowing the aqueous extracts of tissues to stand under toluene for a given period and this allowed for autolysis to take place without interferance with bacteria. Nolly and co-workers (1981a) activated the acid optimum kininogenase in rat, bovine and human cardiac tissues by autolysis at acid pH (pH 5.0, 37 ^{o}C). In the previous work on dog (Zeitlin et al, 1989) autolytic activation was done. This involved incubating the aqueous tissue extracts under toluene at room temperature for 18 - 24 hours. The same method was used in the experiments on dog ventricles and aorta in this work. This work was done to determine if the aqueous extracts of rat ventricles release bradykinin-like immunoreactivity (BKIR) from the plasma kinin-forming substrate (Chapter 2.8) and also to determine the effect of autolytic activation on the release of BKIR.

METHOD

Preparation of extracts.

Ventricles were collected from male Sprague Dawley rats as indicated in section 5.1. The ventricles were chopped into small pieces and

homogenized in distilled water (0.2 g ml^{-1}) at 4 ^{o}C for 3 minutes. The homogenate was divided into two portions; one portion was immediately centrifuged (2000g, 4 ^{o}C, 30 minutes), the supernatant dialysed under tap water for 18 hours and then stored at -20 ^{o}C until used. The second portion was subjected to autolytic activation under toluene (18 - 20 ^{o}C) for 20 hours, centrifuged (2000g, 4 ^{o}C, 30 minutes) and the supernatant obtained was dialysed under tap water for 18 hours and stored at -20 ^{o}C until used.

Release of bradykinin-like immunoreactivity (BKIR).

The ventricular extract (0.2 ml, 0.2 g ml^{-1}) was pre-incubated, at 37 ^{o}C, with acetate -barbitone buffer (0.05 M, pH 5.0) and 0.1 ml containing o-phenanthroline (0.01 M) and EDTA (0.3 M) for 10 minutes. Incubations were started by the addition of 0.25 ml of a human plasma kinin-forming substrate (Chapter 2.8) and continued for 1 hour. Incubates were inactivated by boiling in a water bath for 10 minutes. The incubates were stored at -20 ^{o}C until used. On the assay day they were centrifuged and the supernatant obtained was used for radioimmunoassay. Radioimmunoassay was done as indicated in chapter 2.2.

RESULTS AND DISCUSSION.

Figure 5.2 shows the release of bradykinin-like immunoreactivity (BKIR) from a plasma kinin-forming substrate by a normal aqueous extract of rat ventricles and that of an aqueous extract subjected to autolytic activation (Autolysed). Autolytic activation significantly reduced the amount of BKIR released by the rat ventricular extract ($P < 0.05$ vs control). Following autolytic activation the aqueous extract of rat ventricles released only 72.6 % of the BKIR released by the same extract without autolytic activation. The

results show that the ventricular extract is capable of releasing bradykinin-like immunoreactivity (BKIR) from a plasma kinin-forming substrate. Autolytic activation of the extract reduced its ability to release BKIR , at acid pH, from the plasma kinin-forming substrate. Nolly et al (1981a) showed that the acid optimum kininogenase in rat, human and bovine cardiac tissues did not occur in the active form. It needs activation at pH 5 (37 ^{o}C) to release the active enzyme. The activation process was less at pH 4.0 and at pH 6.0. The autolytic activation as adopted from Zeitlin and co-workers (Zeitlin et al, 1976) involves incubating the tissue extracts at room temperature without change of the pH of the extract. This is the method which was used in this experiment and on the dog experiments. When the pH of the aqueous extracts of rat ventricles was measured (0.1 g ml^{-1} and 0.2 g ml^{-1}) it was found to vary between pH 6.25 - pH 6.3. This is the pH at which autolysis was done. It seems that activation under these conditions offer no advantage. Although the same conditions have been used for autolytic activation of kallikrein before (Zeitlin et al, 1976) and found to be useful it is concluded that autolysis is not applicable in all cases and that each tissue and the enzyme involved should be assessed for the necessity of activation and the method to be used for activation.

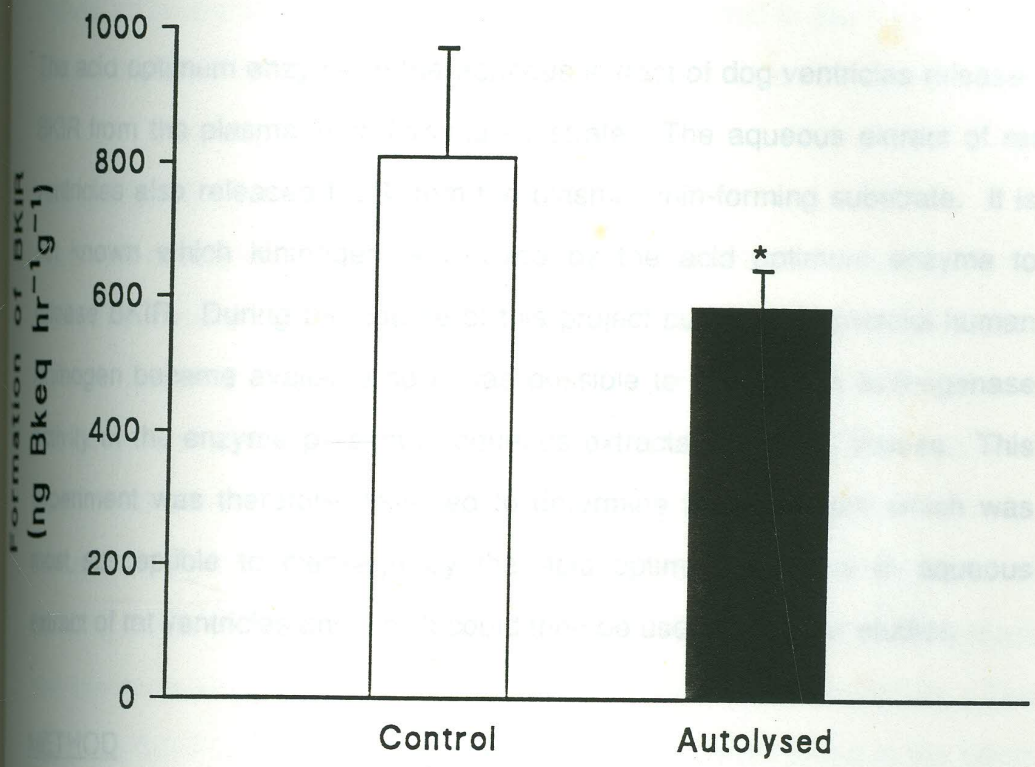


Figure 5.2. The release of bradykinin-like immunoreactivity by normal and autolysed aqueous extracts of rat ventricles. A homogenate of rat ventricles in distilled water was divided into two portions. One portion was centrifuged, dialysed and the extract obtained was incubated with plasma kinin-forming substrate. The second portion was first subjected to autolytic activation and then used. Inactivated incubates of both were assayed for the release of BKIR. The results are presented as mean ± S.D (n = 5).

5.3 DETERMINATION OF KININOGEN CLEAVED BY THE ACID OPTIMUM ENZYME IN AQUEOUS EXTRACTS OF RAT VENTRICLES.

The acid optimum enzyme in the aqueous extract of dog ventricles released BKIR from the plasma kinin-forming substrate. The aqueous extract of rat ventricles also released UCS from the plasma kinin-forming substrate. It is not known which kininogen is cleaved by the acid optimum enzyme to release BKIR. During the course of this project purified commercial human kininogen became available so it was possible to assess the kininogenase activity of the enzyme present in aqueous extracts of cardiac tissues. This experiment was therefore designed to determine the kininogen which was most susceptible to cleavage by the acid optimum enzyme in aqueous extract of rat ventricles and which could then be used for further studies.

METHOD

Aqueous extract of rat ventricles (0.2 ml, 10 mg/ml) was pre-incubated, at 37 ^{o}C, with 0.25 ml citrate-phosphate buffer (0.1M, pH 5.0) containing o-phenanthroline (0.001 M) and EDTA (0.03 M)for 10 minutes. Incubation was started by the addition of 50 µl (50 µg/ml) of either low molecular weight or high molecular weight kininogen (L- or H-kininogen) and continued for 15 minutes. Control incubates were included in which the ventricular extract used was boiled before being used for incubation with the kininogens. Incubates were inactivated by boiling in a water bath for 10 minutes. Aliquots of the incubates (0.2 ml) were put into polypropylene tubes and BSA added to a final concentration of 1 %. Four volumes of absolute alcohol were added (80 % ethanol) and the mixture heated on a water bath (70 ^{o}C, 10 minutes). The mixture was centrifuged (2000g, 4 ^{o}C, 15 minutes) and the precipitated

BSA and uncleaved kininogen was separated out and the supernatant was evaporated on a water bath (70 ^{o}C) over a stream of oxygen-free nitrogen. Potassium phosphate buffer (0.2 ml, pH 7.4) was added to each of the tubes and the released BKIR was assayed by radioimmunoassay (Chapter 2.2).

RESULTS AND DISCUSSION.

Figure 5.3 shows the release of bradykinin-like immunoreactivity (BKIR) by boiled and unboiled aqueous extracts of rat ventricles at pH 5.0. Incubation of H-kininogen with the unboiled rat ventricular extract showed no net release of BKIR as compared to the incubates which were made with the pre-boiled rat extract ($P > 0.05$). Since both boiled and unboiled extract released similar amounts of BKIR it means that the rat ventricular extract was incapable of releasing BKIR from H-kininogen. H-kininogen was shown to cross-react with the bradykinin antibody used in this experiment (Chapter 2) The measured BKIR is probably due to H-kininogen which remained in the tubes after precipitation with 80 % ethanol. When L-kininogen was incubated with the unboiled ventricular extract there was significantly higher bradykinin-like immunoreactivity released as compared to the incubates containing boiled ventricular extract and L- kininogen ($P < 0.01$). Using the unboiled ventricular extract (4 mg/ml) the released BKIR was 595 % higher as compared to incubates containing boiled extract (Figure 5.3). It is concluded here, therefore, that the acid optimum enzyme in rat ventricular extract cleaves only L-kininogen and not H-kininogen. The rate of release of BKIR calculated after subtracting the background BKIR was 36.830 µg Bkeq hr^{-1} g^{-1} wet tissue equivalent.

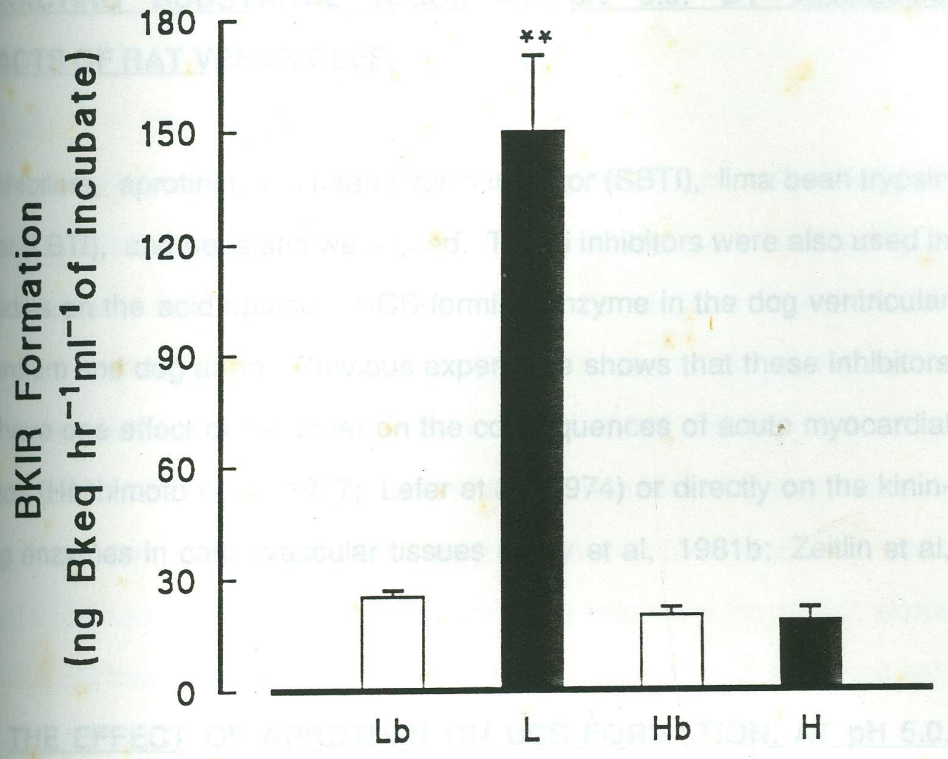


Figure 5.3. The hydrolysis of L-kininogen and H-kininogen, at pH 5.0, by aqueous extracts of rat ventricular myocardium. Two sets of incubates were made; one containing a boiled ventricular extract with either L-kininogen (Lb) or H-kininogen (Hb) and the second set contained unboiled extract with either L-kininogen (L) or H-kininogen (H). The results are presented as mean ± S.D (n = 6).

5.4 THE EFFECT OF INHIBITORS ON THE RELEASE OF A UTERUS CONTRACTING SUBSTANCE (UCS), AT pH 5.0, BY AQUEOUS EXTRACTS OF RAT VENTRICLES.

Four inhibitors, aprotinin, soybean trypsin inhibitor (SBTI), lima bean trypsin inhibitor (LBTI), and pepstatin were used. These inhibitors were also used in the studies on the acid optimum UCS-forming enzyme in the dog ventricular myocardium and dog aorta. Previous experience shows that these inhibitors might have one effect or the other on the consequences of acute myocardial infarction (Hashimoto et al, 1977; Lefer et al, 1974) or directly on the kinin-forming enzymes in cardiovascular tissues (Nolly et al, 1981b; Zeitlin et al, 1989).

5.4.1 THE EFFECT OF APROTININ ON UCS FORMATION, AT pH 5.0, BY THE AQUEOUS EXTRACTS OF RAT VENTRICLES.

Ligation of the left anterior descending coronary artery in the dog results in the elevation of levels of kinin in the coronary sinus and aortic blood (Kimura et al, 1973; Hashimoto et al, 1977; Matsuki et al, 1987). When Aprotinin was administered to dogs before ligation of the left anterior descending coronary artery it prevented the post-ligation elevation of kinin levels (Hashimoto et al, 1977). In addition to the effect on kinins aprotinin was also reported to reduce infarct size when it was administered to cats before ligation of the coronary artery (Lefer et al, 1974). This effect could be related to the inhibition of an enzyme in the myocardium or the coronary vasculature or non-specific. This experiment was done to determine the effect of aprotinin on the release of UCS by aqueous extracts of rat ventricles

at the acid pH 5.0, which is within the range of optimum activity for the release of UCS.

METHOD.

Aqueous extract of rat ventricles (0.2 ml, 0.2 g/ml) was pre-incubated with 0.55 ml, acetate-barbitone buffer (0.05 M, pH 5.0) and 0.1 ml containing o-phenanthroline (0.01 M) and EDTA (0.3 M) for 10 minutes. Aprotinin was dissolved in distilled water and when used it was added to the pre-incubation buffer to give final incubate concentrations of 675 - 2700 KIU/ml. Incubations were started by the addition of 0.25 ml of the human kinin-forming substrate and continued for 1 hour. Incubates were inactivated by boiling on a water bath for 10 minutes. Incubates were centrifuged and the supernatant stored at -20 ^{o}C until assayed. Assays were done using contractions of the isolated oestrous rat uterus and bradykinin as a standard. The amount of UCS released in the presence of aprotinin was expressed as a percent of the mean value of UCS released in the control incubates (i.e residual activity as percent of mean control).

RESULTS AND DISCUSSION.

Figure 5.4.1 shows the release of UCS in the presence of aprotinin expressed as percent of the mean control values. Three concentrations of aprotinin, 675, 1350, and 2700 KIU/ml were used. When the lowest concentration, 675 KIU/ml, was used the release of UCS was not significantly different from that released in the control incubates ($P > 0.05$). When the concentration of aprotinin was increased to 1350 KIU/ml there was about 20 % reduction in the release of UCS. Doubling this concentration to 2700 KIU/ml seems to have further decreased the release of UCS, but the

difference as compared to that at 1350 KIU/ml was not statistically significant ($P > 0.05$). Aprotinin is a nospecific serine proteinase inhibitor. It inhibits both tissue kallikrein and plasma kallikrein (Barrett and McDonald, 1980). At pH 5.0 it is possible to measure some activity due to kallikrein-like enzymes (Chakder, 1988). The inhibition obtained may be due to inhibition of tissue kallikrein or possibly plasma kallikrein present in the extract due to contamination with blood. The possibility that aprotinin inhibits the acid optimum enzyme can not be ruled out. Similar results were obtained with the acid optimum enzyme in the dog ventricular myocardium and aorta.

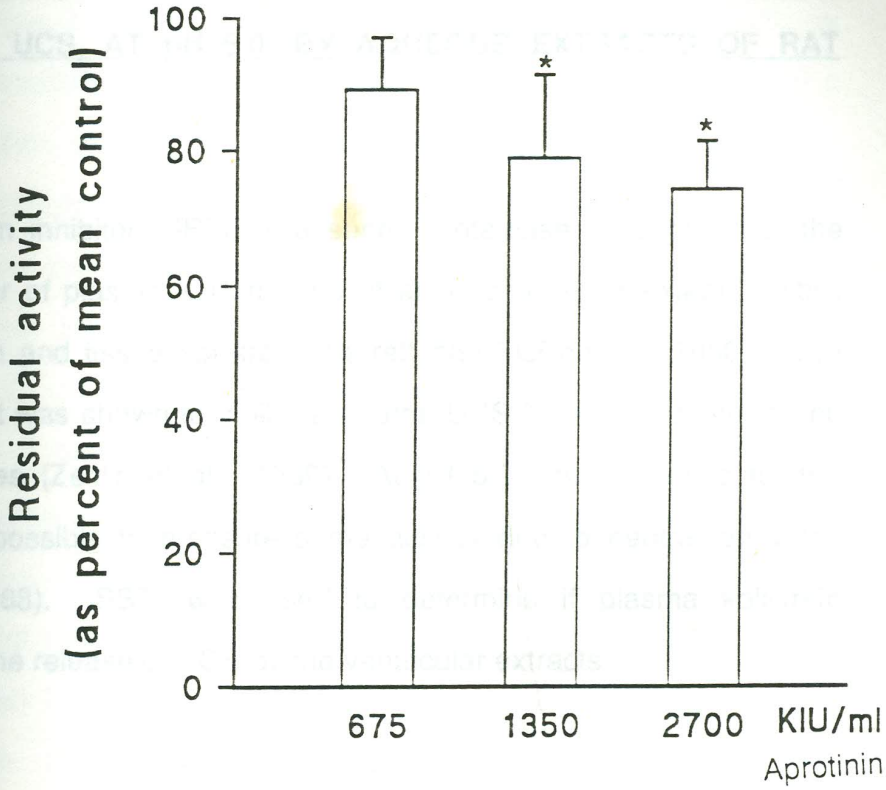


Figure 5.4.1. The effect of aprotinin (at pH 5.0) on the acid optimum UCS-forming activity in aqueous extracts of rat ventricles. The ventricular extract was pre-incubated with 675 - 2700 KIU/ml of aprotinin for 10 minutes before the addition of the kinin-forming substrate. The released UCS was assayed using contractions of the isolated oestrous rat uterus using synthetic bradykinin as a standard. The results are presented as mean $\pm$ S.D (n= 5).

5.4.2 THE EFFECT OF SOYABEAN TRYPSIN INHIBITOR (SBTI) ON THE RELEASE OF UCS, AT pH 5.0, BY AQUEOUS EXTRACTS OF RAT VENTRICLES.

Soybean trypsin inhibitor (SBTI) is a serine proteinase inhibitor. It is the specific inhibitor of plasma kallikrein but it also, to a lesser extent, inhibits trypsin, plasmin and tissue kallikrein (Barrett and McDonald, 1980). In a previous work it was shown to inhibit a neutral UCS-forming enzyme in dog coronary arteries (Zeitlin et al, 1989). At pH 5.0, the pH used for the assays, it is possible to measure some activity due to neutral enzymes (Chakder, 1988). SBTI was used to determine if plasma kallikrein contributed to the release of UCS by the ventricular extracts.

METHOD.

Aqueous extract of rat ventricles (0.2 ml, 0.2 g/ml) was pre-incubated (at 37 °C) with 0.55 ml, acetate-barbitone buffer (0.05 M, pH 5.0) and 0.1 ml containing o-phenanthroline (0.01 M) and EDTA (0.3 M) for 10 minutes. When SBTI was used it was dissolved in distilled water and then added to the pre-incubation buffer to give final incubate concentrations of 6.25×10^{-6} - 2.5×10^{-5} M. Incubations were started by the addition of 0.25 ml of a human plasma kinin-forming substrate and continued for 1 hour. Incubates were inactivated by boiling in a water bath for 10 minutes. The incubates were centrifuged and the supernatant was assayed using contractions of the isolated oestrous rat uterus. The released UCS in the presence of SBTI was expressed as a percent of the mean value of the UCS released in the control incubates.

RESULTS AND DISCUSSION.

Figure 5.4.2 shows the effect of SBTI on the release of UCS, at pH 5.0, by the aqueous extracts of rat ventricles. SBTI at 6.25 x 10^{-6} M caused a significant inhibition of the release of UCS (33.1 %; $P < 0.05$). Almost the same amount of inhibition was obtained at 1.25 x 10^{-5} M but at 2.5 x 10^{-5} M there was a much lower inhibition (22.2 %). However, this is probably due to variations and lack of accuracy inherent in bioassays because the inhibition at this concentration was not significantly different from that obtained at 1.25 x 10^{-5} M ($P > 0.05$). The acid optimum enzyme in the dog ventricles and aorta was not inhibited by SBTI. It is not clear why SBTI has produced different results on the rat acid optimum enzyme. Since it has been shown that some kallikrein-like activity can be detected at pH 5.0 (Chakder, 1988) it is possible the observed effect is due to inhibtion of plasma kallikrein, plasmin or another enzyme present in the rat extract as a result of contamination with blood.

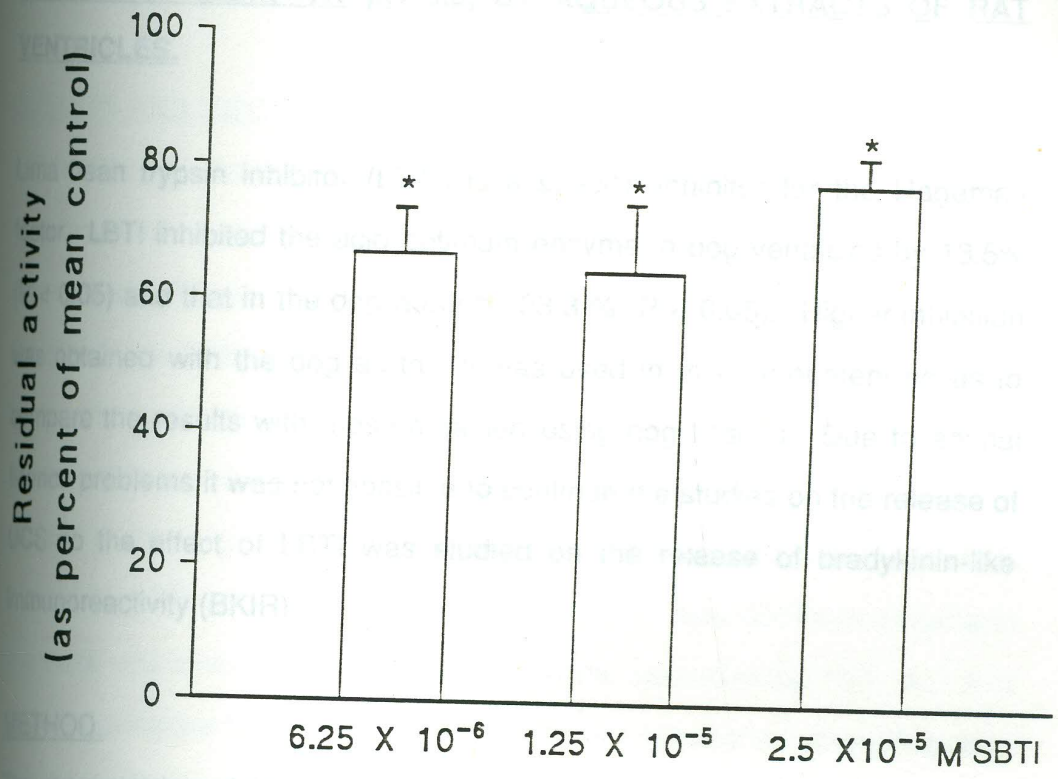


Figure 5.4.2. The effect of soybean trypsin inhibitor (SBTI), at pH 5.0, on the release of UCS by aqueous extracts of rat ventricles. The aqueous ventricular extract was pre-incubated with SBTI at 6.25 x 10-6 M - 2.5 x 10-5 M for 10 minutes before incubation with the kinin-forming substrate. The released UCS was measured using contractions of the isolated oestrous rat uterus. The results are presented as mean ± S.D (n =5).

5.4.3 THE EFFECT OF LIMA BEAN TRYPSIN INHIBITOR (LBTI) ON THE RELEASE OF BKIR, AT pH 5.0, BY AQUEOUS EXTRACTS OF RAT VENTRICLES.

Lima bean trypsin inhibitor (LBTI) is a specific inhibitor for the Hageman factor. LBTI inhibited the acid optimum enzyme in dog ventricles by 13.5% ($P < 0.05$) and that in the dog aorta by 33.3 % ($P < 0.05$). Higher inhibition was obtained with the dog aorta. It was used in this experiment so as to compare the results with those obtained using dog tissues. Due to animal licence problems it was not possible to continue the studies on the release of UCS so the effect of LBTI was studied on the release of bradykinin-like immunoreactivity (BKIR).

METHOD.

The concentration of 500 µg/ml was used based on the results from the work on dog ventricular myocardial extract. Aqueous extract (0.2 ml, 50 mg/ml) was pre-incubated with 0.25 ml of 0.1 M citrate-phosphate buffer (pH 5.0) containing 0.01 M o-phenanthroline and 0.3 M EDTA for 10 minutes. LBTI was dissolved in distilled water and then added to the pre-incubation buffer. Incubation was started by the addition of 50 µl of L-kininogen (50 µg/ml) and continued for 30 minutes. The incubates were inactivated by boiling on a water bath for 10 minutes. Both extract and substrate blanks were included. Aliquots of the centrifuged incubates (0.2 ml) were placed into polypropylene tubes containing 20 µl of BSA (0.1 g/ml). Absolute alcohol was added (80 %) and the mixture heated at 70 °C on a water bath for 10 minutes. The tubes were centrifuged (2000g, 4 °C, 15 minutes) and the supernatant transfered into new tubes. The supernatant was evaporated over a stream of oxygen-

free nitrogen, reconstituted in 0.2 ml, 0.1 M potassium-phosphate buffer (pH 7.4) and then assayed by radioimmunoassay (Chapter 2.2).

RESULTS AND DISCUSSION.

LBTI at 500 µg/ml did not show any inhibitory activity on the release of BKIR by extracts of rat ventricles from L-kininogen. The release of BKIR in the presence of LBTI was not significantly different from that released by control incubates ($P > 0.05$). In the presence of LBTI the release of BKIR was 120 % that of the controls. The effect of LBTI on the acid optimum enzyme in aqueous extracts of rat ventricles differs from that on the acid optimum enzyme in the dog ventricles and dog aorta, both of which were partially inhibited by LBTI at the same concentration. The acid optimum enzyme in dog aorta was inhibited 33.3 % and that in the ventricles by 13.5 %.. It is difficult to compare the dog results with those on rat because different assays were used. While it is possible for the differences to be real it is also likely that the differences derive from the different assays used. The inhibition observed with dog aorta may be unrelated to inhibition of the acid optimum enzyme.

5.4.4 THE EFFECT OF PEPSTATIN ON THE RELEASE OF BKIR, AT pH 5.0, BY AQUEOUS EXTRACTS OF RAT VENTRICLES.

Nolly et al (1981 b), reported that rat mesenteric arteries contained an acid optimum kinin-forming enzyme which was inhibited by pepstatin. Dog coronary arteries and ventricular myocardium were also reported to contain an acid optimum enzyme sensitive to pepstatin (Zeitlin et al., 1989). This

experiment was done to determine the effect of pepstatin on the release of BKIR by the acid optimum enzyme in rat ventricles.

METHOD.

Incubates were made and assayed as for LBTI (Section 5.4.3). Pepstatin was used at concentrations of 8.65×10^{-5} M and 1.5×10^{-4} M.

RESULTS AND DISCUSSION.

Pepstatin significantly inhibited the formation of BKIR by extracts of rat ventricular myocardium from L-kininogen. Figure 5.4.4 shows the effect of 8.6×10^{-5} M and 1.5×10^{-4} M pepstatin on the release of BKIR.

At 8.6×10^{-5} M there was 80 % inhibition and at 1.5×10^{-4} M the release of BKIR was almost completely inhibited (96.9 $\pm$ 5.8 %). The acid optimum enzyme in aqueous extracts of dog ventricles was completely inhibited by pepstatin. Similar results were obtained with the acid optimum UCS-forming activity in dog aorta, although the activity was found to be due to two enzymes; a low molecular weight enzyme, and a high molecular weight enzyme. The two enzymes were, however, suspected to be variations of the same enzyme. The fact that the rat ventricular enzyme is inhibited by pepstatin indicates that this enzyme is an aspartic proteinase.

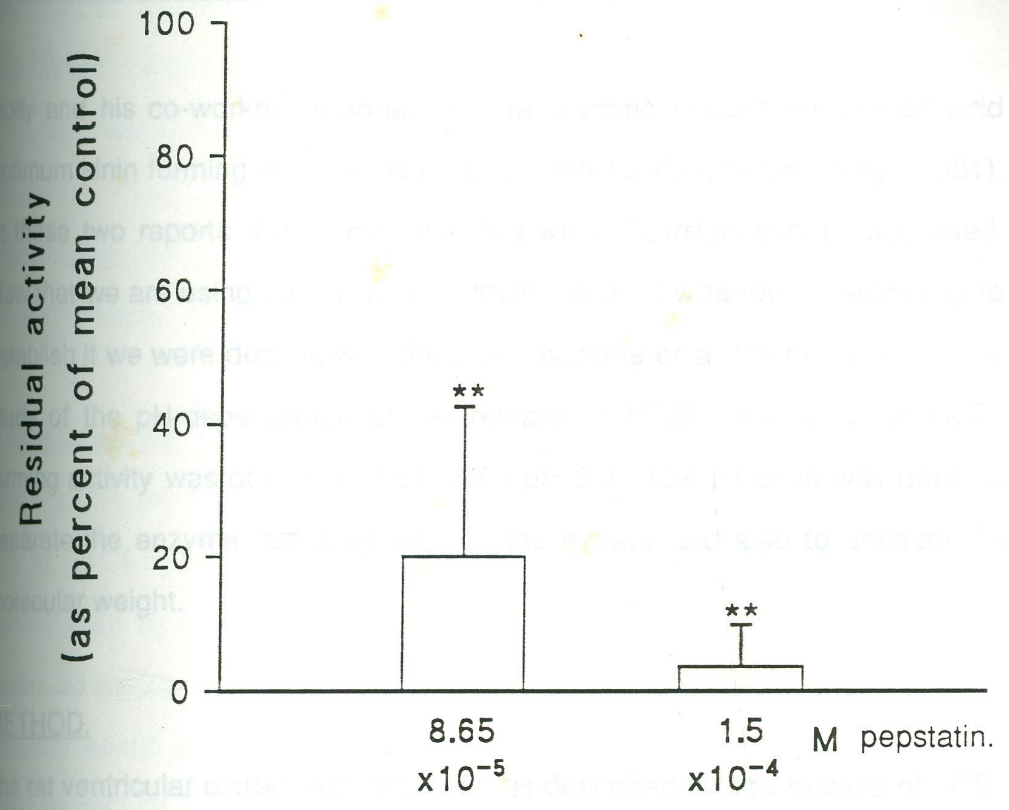


Figure 5.4.4 The effect of pepstatin on the release of bradykinin-like immunoreactivity (BKIR) by an aqueous extract of rat ventricles from L-kininogen. Aqueous extract of rat ventricles was incubated with L-kininogen (pH 5.0) in the presence of pepstatin (see methods). The released BKIR was separated by selective precipitation of intact kininogen with 80 % ethanol in the presence of 1 % BSA and then assayed by radioimmunoassay. The released BKIR in the presence of pepstatin is expressed as a percent of the mean value of BKIR released in control incubates. The results are presented as mean $\pm$ S.D (n = 6).

5.5. GEL FILTRATION OF ENZYMES CLEAVING D-VAL-LEU-ARG-pNA IN AQUEOUS EXTRACTS OF RAT VENTRICLES.

Nolly and his co-workers reported that rat cardiac tissues contain an acid optimum kinin forming enzyme (Nolly et al, 1981a; Britos and Nolly, 1981). In these two reports it was not indicated which "cardiac tissue" was used. Now that we are using the ventricular myocardium it was found necessary to establish if we were dealing with the same enzyme or a different one. In the study of the pH-dependence of the release of UCS, one peak of UCS-forming activity was obtained at pH 4.7 - pH 5.3. Gel filtration was done to separate the enzyme releasing UCS in the extract and also to estimate its molecular weight.

METHOD.

The rat ventricular extract was prepared as described for the release of UCS. The extract (0.1 g/ml) was lyophilized and reconstituted in Tris buffer (0.1 M, pH 8.5) to a concentration of 0.5 g/ml wet tissue equivalent, centrifuged (2000g, 4 ^{o}C, 15 minutes) and then filtered through Whatman number 1 filter paper. The filtered extract (3 ml) was applied to a Sephacryl S-300, superfine, column (2.6 x 86 cm) equilibrated with Tris buffer (0.1 M, pH 8.5) at 4 ^{o}C. The column was eluted at the rate of 5.7 ml hr^{-1} cm^{-2} and 3 ml fractions were collected. The fractions were assayed for arginine amidase activity (Zeitlin et al, 1989) using the tripeptide substrate D-Val-Leu-Arg-pNA. Elution of proteins was monitored by measuring absorbancies at 280 nm (A_{280}). The column was calibrated for molecular weight determination using the standard proteins, myoglobin (17.2 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and apoferritin

(443 kDa). A regression line was obtained by plotting K_{av} against the logarithm of molecular weights of the standard proteins. The molecular weights of unknown enzymes were calculated by interpolation in the regression line (Andrews, 1965). The constant K_{av} was calculated as: $K_{av} = (V_e - V_o)/(V_t - V_o)$, Where, V_e = sample elution volume; V_o = the void volume (the elution volume for dextran blue); V_t = total volume of the packed column.

Amidase Assay.

Aliquots (0.4 ml) were pre-incubated with 0.5 ml of citrate-phosphate buffer (0.1 M, pH 5.0) or Tris buffer (0.1 M, pH 8.0) for 10 minutes. Incubations were started by the addition of 0.1 ml D-Val-Leu-Arg-pNA (0.75 mM) and continued for 3 hours. Incubations were terminated by the addition of 0.1 ml, 50% v/v acetic acid. The released pNA was measured as a change in absorbance and then converted to nmoles/ml using a pNA calibration curve.

RESULTS AND DISCUSSION

Gel filtration of the aqueous extract of rat ventricular myocardium and assay of the fractions at pH 5.0 showed the presence of two arginine amidase peaks (Figure 5.5). Five column runs were made and all contained two peaks of amidase activity. The mean molecular weights as determined by interpolation of the K_{av} values are 42.8 $\pm$ 4.9 kDa for the low molecular weight peak (mean $\pm$ S.D; n = 5) and $\geq$ 800 kDa for the high molecular weight peak. The high molecular weight of the high molecular weight peak could not be determined since it lay outside the working range for the Sephacryl S-300. When the fractions were assayed at pH 8.0 there was no peak of arginine amidase activity detected. Gel filtration of dog ventricles

revealed one peak of amidase activity (at pH 5.2) with a molecular weight 42.0 ± 0.9 kDa. The high molecular weight peak was not present. Like the rat ventricular extract the dog ventricular extract showed no peak of amidase activity at pH 8.0.

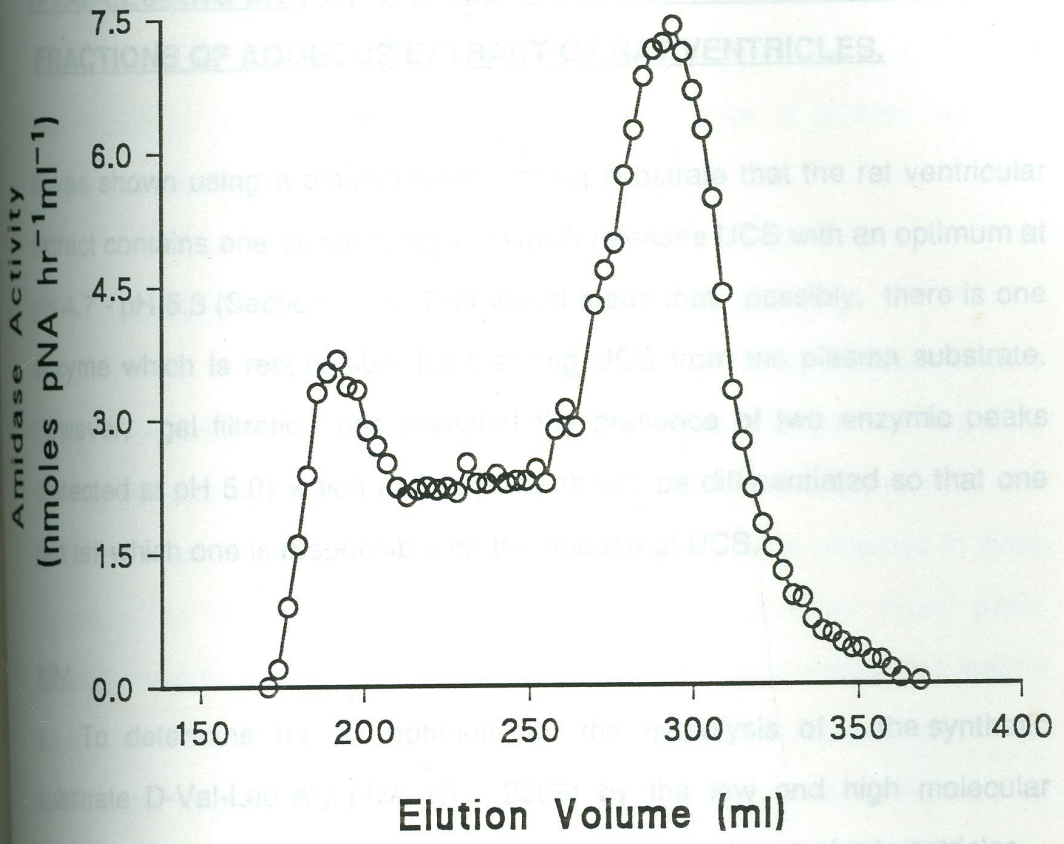


Figure 5.5. Gel filtration pattern of acid optimum enzymes cleaving D-Val-Leu-Arg-pNA , at pH 5.0, in aqueous extracts of rat ventricular myocardium. The figure represents mean values for 4 column runs. Two peaks of amidase activity with molecular weights of 42.8 $\pm$ 4.9 kDa and $\geq$ 800 kDa were obtained.

5.6 pH-DEPENDENT HYDROLYSIS OF THE TRIPEPTIDE SUBSTRATE D-VAL-LEU-ARG-pNA BY THE HIGH AND LOW MOLECULAR WEIGHT FRACTIONS OF AQUEOUS EXTRACT OF RAT VENTRICLES.

It was shown using a plasma kinin-forming substrate that the rat ventricular extract contains one enzymic activity which releases UCS with an optimum at pH 4.7 - pH 5.3 (Section 5.1). This would mean that, possibly, there is one enzyme which is responsible for cleaving UCS from the plasma substrate. However, gel filtration has revealed the presence of two enzymic peaks (detected at pH 5.0) which at this point should be differentiated so that one can tell which one is responsible for the release of UCS.

AIM.

1. To determine the pH-optimum for the hydrolysis of the synthetic substrate D-Val-Leu-Arg-pNA (S- 2266) by the low and high molecular weight enzyme peaks fractionated from aqueous extracts of rat ventricles.
2. To compare the pH-optimum obtained with that for the release of UCS from the human plasma kinin-forming substrate.

METHOD.

Column fractions were obtained during the determination of molecular weights (Section 5.5). The enzymic peaks were pooled and then stored at -20 °C until used.

Determination of pH-optimum.

Pooled column fractions (0.4 ml) were pre-incubated with 0.5 ml, 0.1 M citrate-phosphate buffer at pH 4 to 8.2 for 10 minutes. Incubation was

started by the addition of 0.1 ml D-Val-Leu-Arg-pNA (0.75 mM) and continued for 3 hours. Incubation was stopped by the addition of 0.1 ml, 50 % v/v acetic acid. The released pNA was measured as a change in absorbance at 405 nm and then converted to nmoles of pNA/ml released using a pNA calibration curve.

RESULTS AND DISCUSSION.

Both the high molecular weight and low molecular weight fractions were assayed for pH-dependent hydrolysis of the tripeptide D-Val-Leu-Arg-pNA. The low molecular weight fraction produced one peak of amidase activity with an optimum at pH 4.5 (Figure 5.6.1). Similar results were obtained in three assays. The high molecular weight fraction gave a rather broad peak, possibly multiple peaks. The major peak for the high molecular weight fraction had a maximum at pH 5.3 to 5.5. There was a minor peak at pH 4.5 and another at pH 4.1 (Figure 5.6.2). It would seem from these results that the low molecular weight fraction and the high molecular weight fraction represent different enzymes. One possibility is that the high molecular weight enzyme is the low molecular weight enzyme bound to high molecular weight substances like α_2-macroglobulins (Thomas et al, 1989; Sotrup-Jensen et al, 1983.). However figure 5.5 suggests that this is unlikely since there was only one peak of UCS formation and the separated peaks gave more than one peak of amidase activity. This is evidence that they are different enzymes because if an enzyme appears in different forms, say due to variations in carbohydrate content, the pH-optimum is supposed to be the same (Sessler et al, 1986).

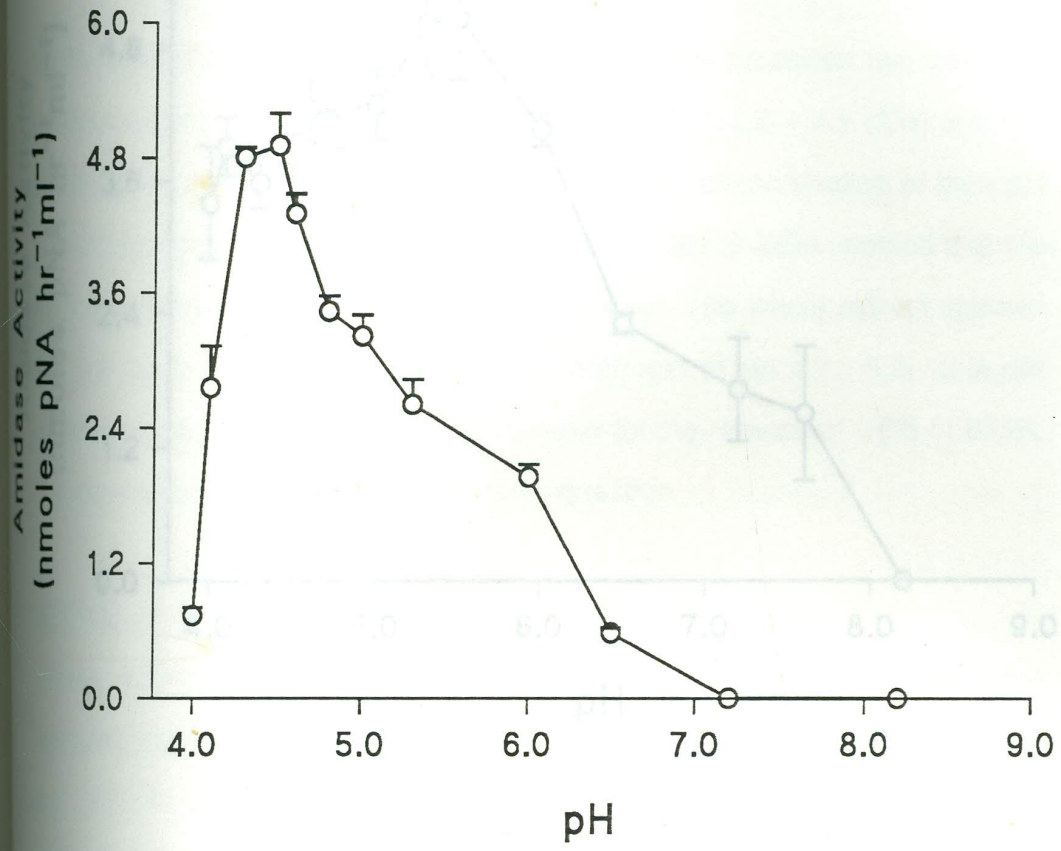


Figure 5.6.1. The effect of pH on the amidase activity of the low molecular weight enzymic peak in the pooled eluate of rat ventricular myocardium. Aliquots of the pooled eluate of the enzyme peak were incubated with the tripeptide substrate S-2266 in 0.1 M citrate-phoshate buffer at pH 4.0 to pH 8.2. The released pNA was measured as a change in absorbance (405 nm) and then converted to nmoles of pNA. Each point represents mean ± S.D (n = 5).

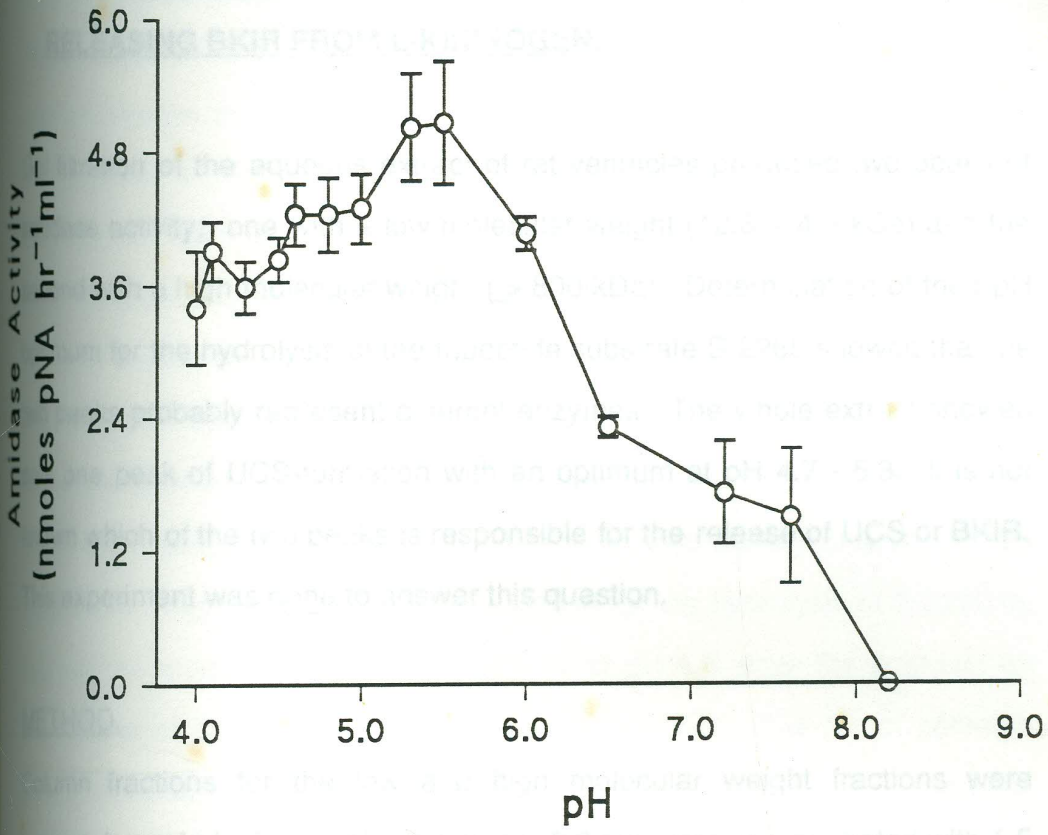


Figure 5.6.2. The effect of pH on the amidase activity of the high molecular weight enzymic peak in the pooled eluate of rat ventricular myocardium. Aliquots of the pooled eluate of the enzyme peak were incubated with the tripeptide substrate S-2266 in 0.1 M citrate-phoshate buffer at pH 4.0 to pH 8.2. The released pNA was measured as a change in absorbance (405 nm) and then converted to nmoles of pNA. Each point represents mean $\pm$ S.D (n = 5).

5.7 DETERMINATION OF THE FRACTION RESPONSIBLE FOR RELEASING BKIR FROM L-KININOGEN.

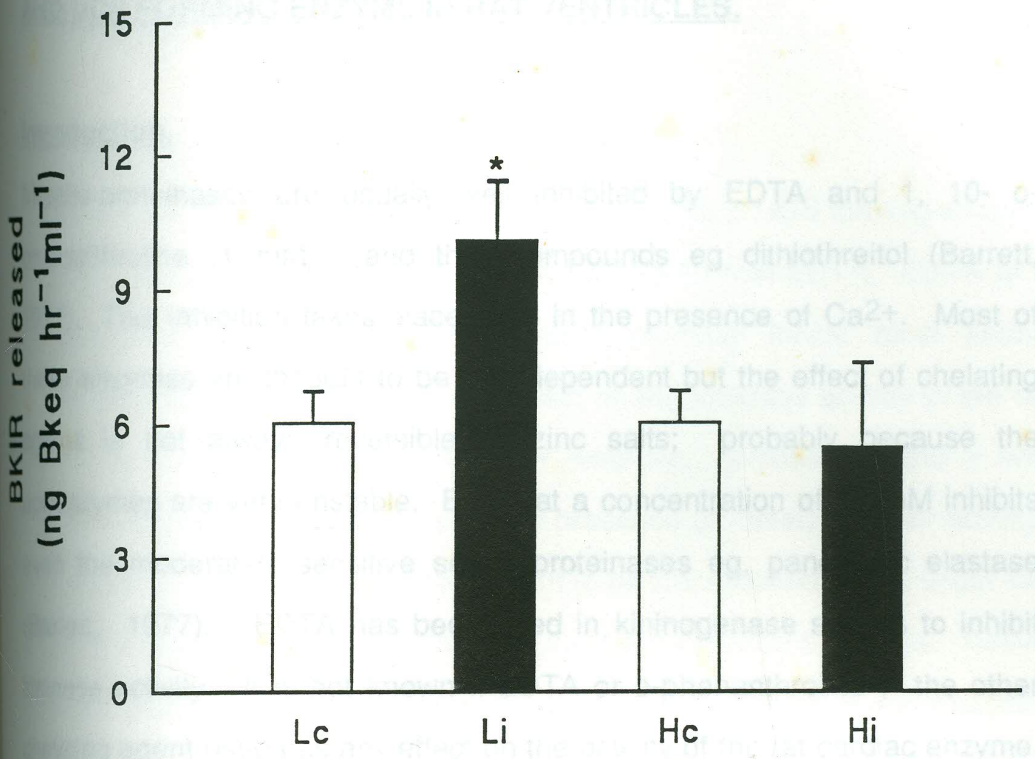
Gel filtration of the aqueous extract of rat ventricles produced two peaks of amidase activity; one with a low molecular weight (42.8 $\pm$ 4.9 kDa) and the second with a high molecular weight ($\geq$ 800 kDa). Determination of their pH optimum for the hydrolysis of the tripeptide substrate S-2266 showed that the two peaks probably represent different enzymes. The whole extract showed only one peak of UCS-formation with an optimum at pH 4.7 - 5.3. It is not known which of the two peaks is responsible for the release of UCS or BKIR. This experiment was done to answer this question.

METHOD.

Column fractions for the low and high molecular weight fractions were separately pooled. The pooled fractions (0.2 ml) were pre-incubated with 1.5 ml, 0.1 M citrate-phosphate buffer (pH 5.0) containing 0.01 M o-phenanthroline and 0.3 M EDTA for 10 minutes. Incubation was started by the addition of 0.3 ml of L-kininogen (25 µg/ml) and continued for 1 hour. Control incubates were included in which the enzyme fractions were first boiled before they were incubated with L-kininogen. Incubates were inactivated by boiling on a water bath for 10 minutes. Incubates were extracted with ethanol and then assayed by radioimmunoassay. Protein concentrations were measured using a microBCA protein assay reagent from Pierce.

RESULTS AND DISCUSSION.

When fractions containing the low molecular weight peak were incubated with L-kininogen there was 167 % increase in the release of BKIR ($P < 0.05$) as compared to incubates made using boiled fractions of this peak (Figure 5.7). On the other hand incubation of fractions of the high molecular weight peak with L-kininogen did not have any significant effect on the released BKIR as compared to the incubates made using boiled fractions ($P > 0.05$; Figure 5.7). The results show that the high molecular weight peak is incapable of releasing BKIR from L-kininogen. The low molecular weight peak was capable of releasing BKIR with a specific activity of 87.0 ± 11.24 ng Bkeq $hr^{-1}mg^{-1}$ protein. The pH-optimum for the hydrolysis of S-2266 by this low molecular weight enzymic peak is at pH 4.5 while the optimum for the release of UCS is between pH 4.7 and pH 5.3. The pH of optimum activity depends on the type and the source of the substrate. For example, rat submandibular renin has an optimum at pH 5.4 for octapeptide substrates, pH 6.5 for tetradecapeptide substrates and pH 8.3 for sheep plasma substrate (Barrett and McDonald, 1980).



5.7. The hydrolysis of L-kininogen, at pH 5.0, by the low and high molecular enzymes fractionated from aqueous extracts of rat ventricles. Two sets of es were made. In the first set L-kininogen was incubated with unboiled low ular weight fraction (Li) and high molecular weight fraction (Hi). In the second ininogen was incubated with boiled low molecular weight fraction (Lc) and high ular weight fractions (Hc). Released BKIR was assayed by munoassay. The results are presented as Mean $\pm$ S.D (n = 6).

5.8 THE EFFECT OF EDTA ON THE AMIDASE ACTIVITY OF THE BKIR AND UCS FORMING ENZYME IN RAT VENTRICLES.

Introduction.

Metallo-proteinases are usually well inhibited by EDTA and 1, 10- o-phenanthroline (1 mM), and thiol compounds eg dithiothreitol (Barrett, 1977). This inhibition takes place even in the presence of Ca^{2+}. Most of these enzymes are thought to be zinc-dependent but the effect of chelating agents is not always reversible by zinc salts; probably because the apoenzymes are very unstable. EDTA at a concentration of 10 mM inhibits even the moderately sensitive serine proteinases eg. pancreatic elastase (Barrett, 1977). EDTA has been used in kininogenase studies to inhibit kininase activity. It is not known if EDTA or o-phenanthroline , the other chelating agent used has any effect on the activity of the rat cardiac enzyme. Using gel filtration and amidase assay with the tripeptide substrate S-2266 made it possible to separate the enzyme fraction that is responsible for the kininogenase activity. It was decided to use the same assay to determine if the rat cardiac enzyme was metal dependent.

METHOD.

Pooled column fractions of the kinin-forming enzyme peak were used. Pooled fractions (0.4 ml) were pre-incubated with 0.5 ml of citrate-phosphate buffer (0.1 M, pH 4.5) for 10 minutes. Two sets were made, one containing 10 mM EDTA (final incubate concentration) and another without EDTA. Incubations were started by the addition of 0.1 ml of D-Val-Leu-arg-pNA (1.5 mM) and continued for 3 hours. Incubates were inactivated by the addition of 0.1 ml, 50 % v/v acetic acid. An enzyme blank in which the enzyme was

inactivated with acetic acid before addition of substrate was included for each of the two sets of incubates.

RESULTS AND DISCUSSION.

Figure 5.8 shows the amidase activity of the rat cardiac UCS forming enzyme in control incubates and in incubates containing 10 mM EDTA. EDTA at a concentration of 10 mM, which would normally inhibit most serine proteinases (Barrett, 1977), had no effect on the amidase activity of the rat cardiac enzyme. Some enzymes, for example, kininases I and II and neutral endopeptidase 24.11 require the presence of metals for their activity. Most of these enzymes which are thought to be zinc-dependent fall under the group of metalloproteinases. Other enzymes are inhibited by metals. Tissue kallikrein and acrosin, the enzyme present in the acrosome of spermatozoa of mammals, are both inhibited by Zn^{2+} (Barrett and McDonald, 1980). Pancreatic elastase is inhibited by Cu^{2+} but not by Zn^{2+} (Barrett and McDonald, 1980). The use of chelating agents can be expected to either stimulate enzymic activity or be inhibitory depending on the type of enzyme. EDTA at 10 mM did not have any effect on the amidase activity of the rat cardiac enzyme, so it is concluded that the enzyme is not metal dependent.

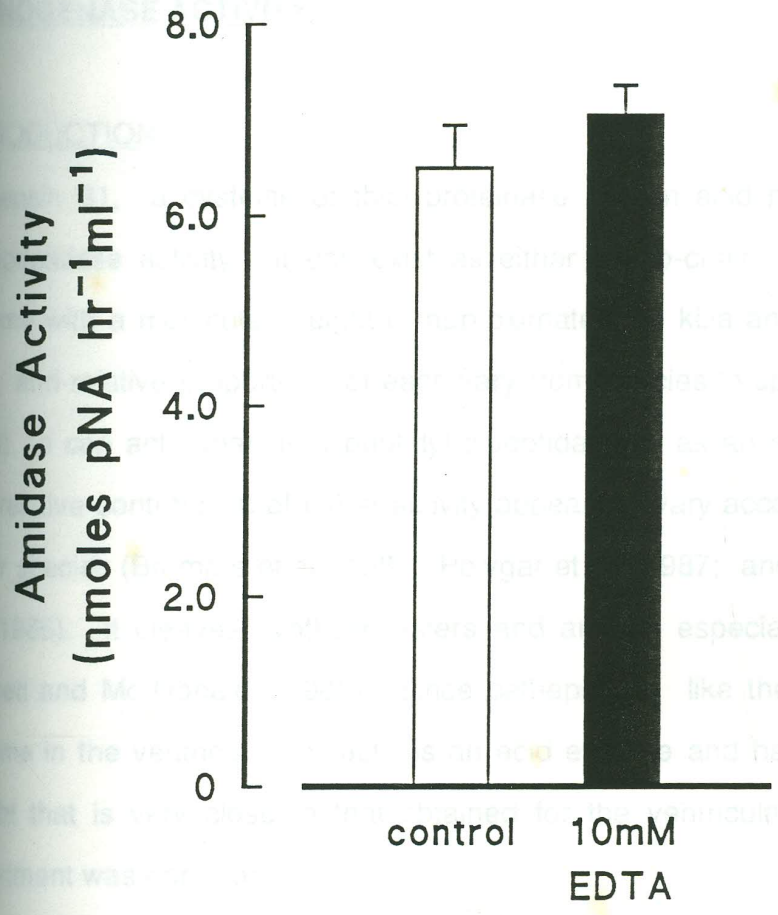


Figure 5.8. The effect of EDTA (pH 4.5) on the amidase activity of the low molecular weight acid optimum enzyme in rat ventricles. Aliquots of pooled fractions of the low molecular weight enzyme peak were assayed for the hydrolysis of the tripeptide S-2266 in the presence or in the absence of 10 mM EDTA. The results are presented as mean $\pm$ S.D (n = 5).

5.9 ASSAY OF CATHEPSIN B1 (EC 3.4.22.1) FOR AMIDASE AND KININOGENASE ACTIVITY.

INTRODUCTION.

Cathepsin B1, a cysteine or thiol proteinase, is an acid proteinase with endopeptidase activity. It can exist as either a two-chain or single-chain enzyme with a molecular weight of approximately 25 kDa and 5 kDa or 30 kDa; and relative proportions of each vary from species to species (Mason, 1989). It can act either as a peptidyl dipeptidase or as an endopeptidase. The relative contribution of either activity appears to vary according to either pH or species (Bromme et al, 1987; Polygar et al, 1987; and Takahashi et al, 1986). It cleaves synthetic esters and amides especially of arginine (Barrett and Mc Donald, 1980). Since cathepsin B, like the kinin-forming enzyme in the ventricular extract, is an acid enzyme and has a molecular weight that is very close to that obtained for the ventricular enzyme this experiment was done to:

1. find out if cathepsin B has amidase activity against the tripeptide substrate, D-Val-Leu-Arg-pNA.
2. to find out if cathepsin B is capable of releasing bradykinin-like immunoreactivity from either L-kininogen or H-kininogen.

5.9.1 ASSAY OF CATHEPSIN B1 FOR AMIDASE ACTIVITY AGAINST D-VAL-LEU-ARG-pNA.

METHOD.

Ten units of bovine spleen cathepsin B1, equivalent to 1 mg of protein, were dissolved in distilled water to give a 2 units/ml solution. The solution (0.4 ml)

was pre-incubated with 0.5 ml citrate-phosphate buffer (pH 5.0) for 10 minutes. The incubation was started by the addition of 0.1 ml (1.5 mM) of the substrate and continued for 1 hour. The incubation was terminated by the addition of 0.1 ml, 50 % v/v acetic acid. A blank was included in which inactivation with acetic acid was done before the addition of substrate.

RESULTS.

The chromogenic substrate D-Val-Leu-Arg-pNA was readily cleaved by cathepsin B1. Only 0.8 units were used but within half of an hour release of pNA was visible as the incubates turned yellow. The specific activity for the release of pNA was 71.0 $\pm$ 1.84 (mean $\pm$ S.D, n = 4) nmoles pNA hr^{-1} $unit^{-1}$

5.9.2 ASSAY OF CATHEPSIN B1 FOR KININOGENASE ACTIVITY.

Cathepsin B1, like the rat cardiac enzyme is an acid optimum enzyme and it has a similar molecular size. The results in section 5.9.1 show that it also cleaves the tripeptide substrate S-2266. This experiment was done to determine if cathepsin B1 is capable of releasing bradykinin-like immunoreactivity from either L-kininogen or H-kininogen.

METHOD

Bovine spleen cathepsin B1 (0.2 ml, 0.4 units/ml) was pre-incubated with 0.55 ml of citrate-phosphate buffer (pH 5.0) containing 0.01 M o-phenanthroline and 0.3 M EDTA for 10 minutes. Incubation was started by the addition of 0.25 ml of either L- or H-kininogen (25 µg/ml) and continued for 1 hour. For each kininogen incubates were included in which cathepsin B1 was first boiled before addition of the kininogens. Incubation was stopped by boiling the tubes in a water bath for 10 minutes. Aliquots of the incubates (0.2 ml) were put into polypropylene tubes containing 20 µl (0.1 g/ml) BSA. Absolute alcohol (1 ml) was added and the mixture heated on a water bath (70 ^{o}C) for 10 minutes. Precipitated kininogens and BSA were separated by centrifuging (2000g, 4 ^{o}C, 15 minutes) and the supernatant was evaporated (70 ^{o}C) using a stream of oxygen-free nitrogen. The incubates were assayed by radioimmunoassay using a polyclonal rabbit anti-bradykinin antiserum (Chapter 2.2).

RESULTS AND DISCUSSION.

Figure 5.9.2 shows the release of bradykinin-like immunoreactivity from L-kininogen and H-kininogen by cathepsin B1. Similar amount of BKIR were

released from L-kininogen by both boiled and unboiled cathepsin B1 ($P > 0.05$). Similarly using H-kininogen comparable amounts of BKIR were released by both boiled and unboiled cathepsin B1. The results show that cathepsin B1 was incapable of releasing BKIR from either L-kininogen or H-kininogen. In the previous experiment it was shown to have acid optimum amidase activity against the tripeptide S-2266. This enzyme can occur with a molecular weight of 30 kDa (Mason, 1989) which puts it in the same molecular size range as the cardiac kinin-forming enzyme. However, since it failed to release any BKIR from L-kininogen it is unlikely to be the enzyme present in the rat ventricular myocardium.

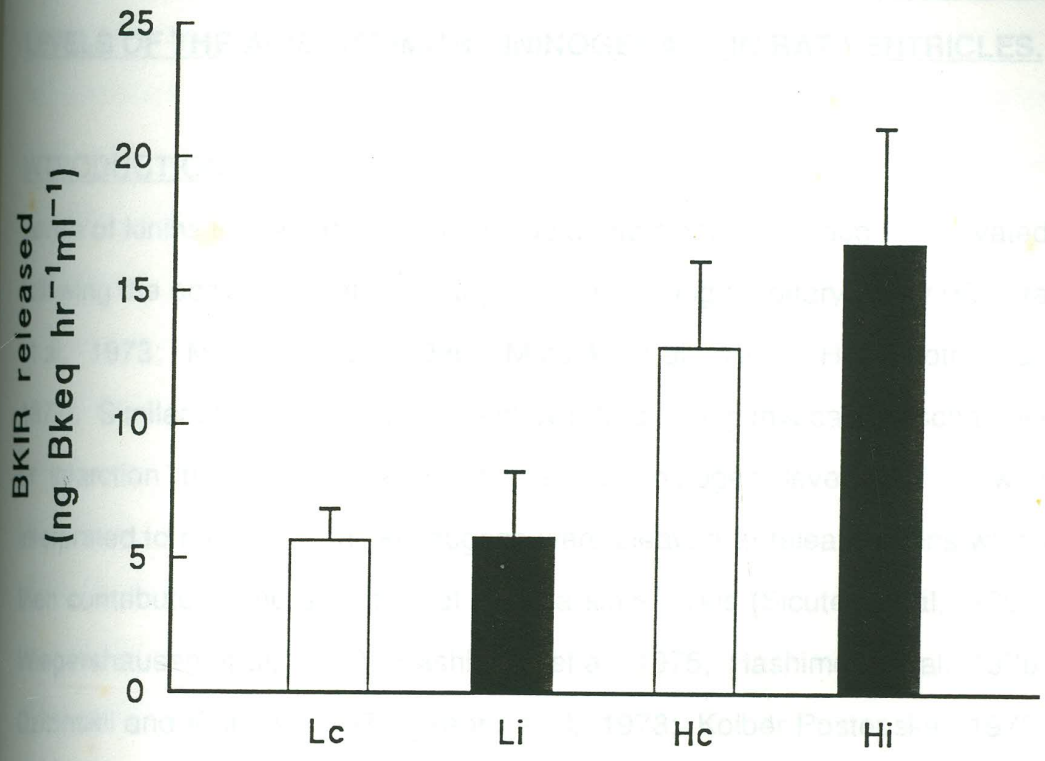


Figure 5.9.2. The assay of cathepsin B1 for kininogenase activity. Two sets of incubates were made for each kininogen. One set contained unboiled cathepsin B1 and L-kininogen (Li) or H-kininogen (Hi). The second set contained boiled cathepsin B1 and L-kininogen (Lc) or H-kininogen (Hc). Released BKIR was assayed by RIA after separation with 80 % ethanol. The figure shows mean $\pm$ S.D (n = 6).

5.10 THE EFFECT OF ACUTE EXPERIMENTAL ISCHAEMIA ON THE LEVELS OF THE ACID OPTIMUM KININOGENASE IN RAT VENTRICLES.

INTRODUCTION.

Levels of kinins in the coronary sinus and aortic blood in the dog are elevated following the occlusion of the left anterior descending coronary artery (Kimura et al, 1973; Matsuki et al, 1986; Matsuki et al, 1987; Hashimoto et al, 1977). Similar studies on man have shown that during myocardial ischaemia or infarction there is depletion of plasma kininogen levels. This was interpreted to mean that the kininogens were cleaved to release kinins which then contribute to the elevation of plasma kinin levels (Sicuteri et al, 1967; Wiegershausen et al, 1970; Hashimoto et al, 1975; Hashimoto et al, 1978; Dzizinskii and Kuimov , 1972; Kedra et al, 1973; Kolber-Postepska, 1975; Torstila, 1978). A negative correlation was found between levels of kinins and that of kininogens (Hashimoto et al, 1978) suggesting the involvement of plasma and/or intracardiac kininogenase/s. Activation of the plasma kallikrein system was observed in 7 out 11 patients with myocardial ischaemia (Pitt et al, 1970). Similar results were reported by Sicuteri and co-workers (Sicuteri et al, 1970). An acid optimum kininogenase has been reported to be present in unspecified human, bovine and rat cardiac tissues (Nolly et al, 1981a; Britos and Nolly, 1981) and in the dog ventricular myocardium and coronary arteries (Zeitlin et al, 1989). Recent reports indicate that aqueous extracts of combined atria and ventricles contain tissue kallikrein (Xiong et al, 1990). There is a possibility that these enzymes are either activated or made available for the release of kinins during myocardial ischaemia. The distribution of cathepsin D in the heart in rabbits (Decker et al, 1977) and in dogs (Araki et al, 1975) was reported to be significantly

altered during myocardial ischaemia resulting in depletion from the sedimentable fraction and its enrichment in the non-sedimentable fraction. Such changes in the distribution of enzymes may be a contributing factor which eventually render the kinin-forming enzymes available for the release of kinins. This work was done to determine the effect of acute ischaemia on the levels of the acid optimum kininogenase in the rat ventricular myocardium and also how acute ischaemia affects the levels of plasma kinins in the coronary sinus.

METHOD.

Male Sprague Dawley rats 310-450 g were used. The rats were anaesthetized with pentobarbitone sodium (60 mg/kg, i.p). Thorachotomy was performed and rats kept on artificial ventilation with a C. F. Palmer (London) respirator pump. The carotid artery was cannulated for the recording of blood pressure. Electrocardiogram (ECG) was recorded using standard limb leads. Both recordings were done on a Grass polygraph recorder (model MDL 7, USA). A catheter was passed through the jugular vein down to the right atrium for the collection of blood samples. The chest was opened at the fourth left intercostal space to expose the heart. Ischaemia was induced by the ligation of the left main coronary artery (Clark et al, 1980). Three groups of 6 rats, were used. Group one, the control group, was subjected to all the surgical procedures and a suture was passed below the left main coronary artery but no ligation was done. Groups 2 and 3, each containing 6 rats, were subjected to ligation of the left main coronary artery for 10 and 30 minutes, respectively. Rats in all groups were finally killed by a large i.v dose of pentobarbitone sodium containing 10 units/ml heparin.

Collection of blood samples for determination of kinins.

Blood samples were collected from rats which had been subjected to ligation of the left main coronary artery. Each rat acted as its own control and blood samples were collected before and after ligation of the coronary artery. After all surgical procedures were completed rats were allowed to stabilize for 10 minutes before collecting the control blood samples. Ligation was done immediately after collection of the control blood samples. Ischaemic blood was collected 10 minutes after ligation. The 10 minute point was chosen because this was the time at which most rats experienced arrhythmias. Each time 0.7-0.8 ml blood samples were collected and 0.3 ml, for each sample, were put in duplicate, into polypropylene tubes containing 1.2 ml of absolute alcohol containing 1 mg/ml o-phenanthroline and immediately shaken. The tubes were centrifuged (1500g, 4 ^{o}C, 10 min), the supernatant was collected into new polypropylene tubes and evaporated to dryness on a water bath, at 70 ^{o}C, under a stream of oxygen-free nitrogen. Contents were reconstituted in 0.2 ml of potassium phosphate buffer (pH 7.4) and assayed by radioimmunoassay using the polyclonal rabbit anti-bradykinin antiserum (Chapter 2.2).

Measurement of kininogenase levels.

After rats were killed by an excess dose of pentobarbitone the abdomen and chests were opened up, the aorta cannulated with polyethylene (PE 50) tubing and the hearts were cleared of blood by retrograde perfusion with Kreb's Henseleit solution (37 ^{o}C, 95% O_2, 5% CO_2) containing 10 units/ml heparin, followed by heparin free Krebs. The perfusion was done at 4 ml/minute using a Watson Marlow slow rate flow pump. The left ventricles

were excised, chopped into small pieces with a pair of scissors, and homogenized in distilled water (40 mg/ml) containing 0.1 % Triton X-100. The homogenate was centrifuged (2000g, 4 °C, 30 min), supernatant dialysed under tap water for 20 hours and then stored at -20 °C until used.

Release of BKIR.

Aqueous extracts of the rat ventricles (0.2 ml, 0.01 g ml^{-1}) were pre-incubated with 0.25 ml, 0.1 M citrate-phosphate buffer (pH 5.0) for 10 minutes. Incubation was started by the addition of 0.05 ml of human L-kininogen (50 µg/ml). Incubates were inactivated by boiling in a water bath for 10 minutes. BKIR was separated by selective precipitation of intact kininogen with 80% v/v ethanol in the presence of 1% BSA and then assayed by radioimmunoassay (Chapter 2.2).

RESULTS AND DISCUSSION.

Figure 5.10.1 shows that ligation of the left main coronary artery significantly decreased the release of bradykinin-like immunoreactivity (BKIR) by aqueous extracts of rat ventricles. The released BKIR after ligation for 10 minutes was 70 % of the mean control value ($P < 0.05$). After ligation for 30 minutes the value of the released BKIR dropped to 51.9 % of the mean control value ($P < 0.05$). Two rats showed very low levels of released BKIR. The released BKIR from the same amount of L-kininogen was 6 % and 5.7 % for the two rats. It would seem from these results that ligation of the left main coronary artery in the rat causes a decrease in the levels of the acid optimum BKIR-forming enzyme. Ligation of the left main coronary artery resulted in an increase in the levels of free kinins in mixed venous blood sampled from the right atrium. Table 5.10.2 shows the concentrations of kinin measured in

mixed venous blood from the right atrium before ligation and 10 minutes after the ligation of the left main coronary artery. There was a big variation in the percentage increase in plasma kinin levels ranging from 16.4 % to 702.6 % of the control pre-ligation levels (Figure 5.10.2). The work done by Kimura and co-workers (1973), also showed variation in the level of kinins in the coronary sinus after ligation of the left anterior descending coronary artery. In their study there was also a variation in the time at which kinin levels peaked and in one case kinin levels were not at all elevated. It would have been interesting to follow up the changes of kinin levels with time. This was necessary so as to be able to determine the peaking time and possibly the maximum kinin levels achievable in the right atrial venous blood . However, it was noted that after collection of two samples of blood (0.8 ml) most of the rats died due to hypotension. This problem could be avoided by using bigger animals in which it will be possible to draw several samples of blood without a significant effect on the blood pressure. The formation and destruction of kinins as the samples are collected is a continuous process. Since both processes are in operation at the same time any variation in the collection time or delays of any sort is likely to reflect in the levels of kinin measured. This may be a contributing factor to the variations of kinin levels obtained in this study. To get a better explanation for the variation in kinin levels in the mixed venous blood from the right atrium the time course would have been of some help. This is confirmed by the work by Kimura and co-workers (Kimura et al, 1973) which shows that the changes in kinin levels did not take place at the same time in all the dogs used.

Concentration of kinin in mixed venous blood from the right atrium pg/ml		
Rat no	Control	Ischaemic
1	51.0	121.0
2	42.0	49.0
3	36.0	74.0
4	87.0	153.0
5	61.0	73.0
6	96.0	771.0
7	447.0	728.0

Table 5.10.2. Kinin concentration in mixed venous blood from the rat right atrium. The table shows levels of kinin in the mixed venous blood from the right atrium of rats before ligation (control) and after ligation of the left main coronary artery for 10 minutes. Each value is a mean of two measurements.

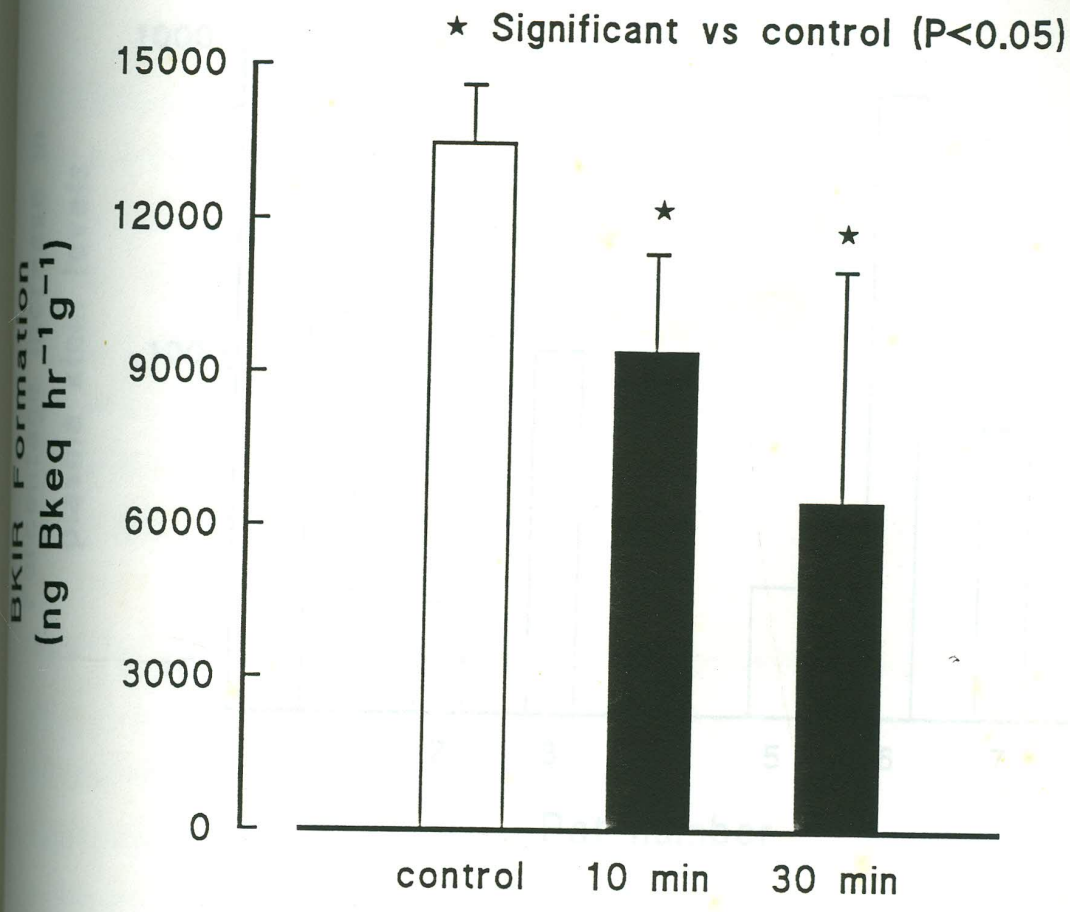


Figure 5.10.1. The effect of acute ischaemia on the release of bradykinin-like immunoreactivity from L-kininogen, at pH 5.0, by the aqueous extracts of rat left ventricles. Three groups 6 rats each were used. The control group was operated on but was not ligated. Groups 2 and 3 were subjected to ligation of the left main coronary artery for 10 and 30 minutes, respectively. Blood-free extracts of the left ventricles were incubated with L-kininogen and released BKIR was measured. The results are presented as mean ± S.D (n = 6).

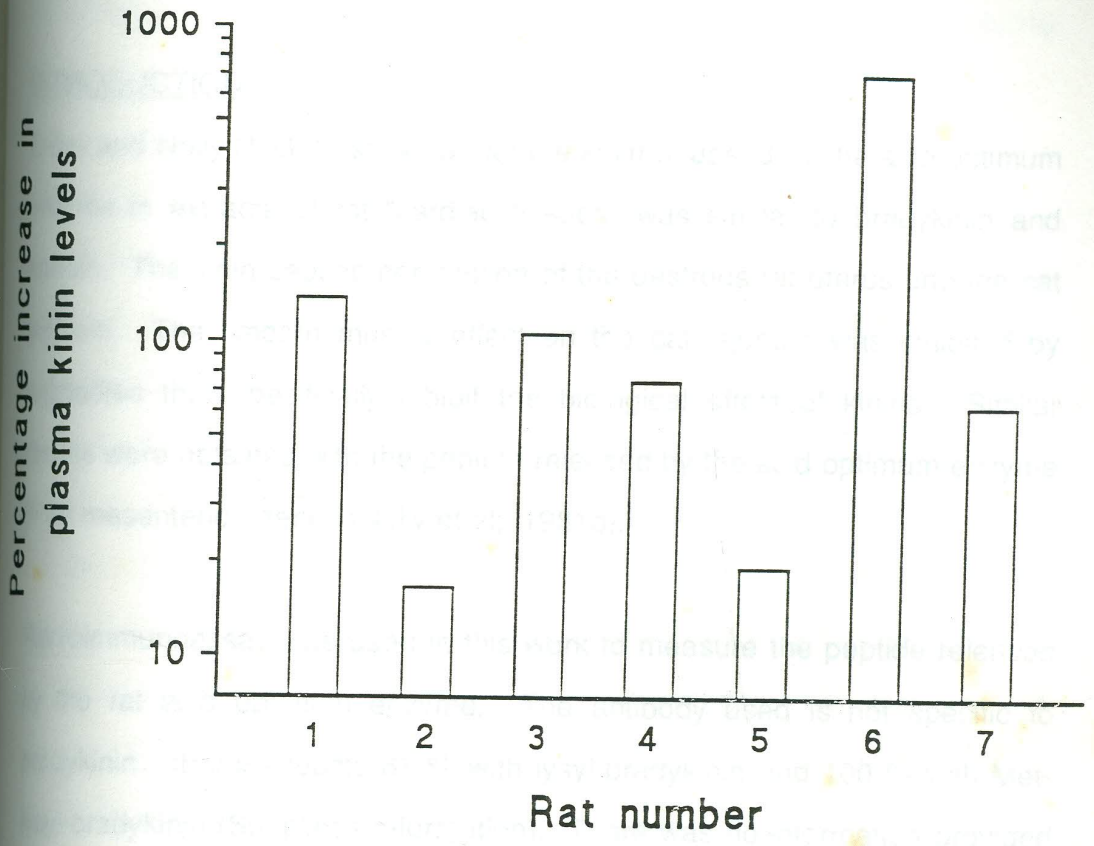


Figure 5.10.2. The effect of acute ischaemia on levels of kinins in mixed venous blood from rat right atria. After surgical procedures the rats were allowed to stabilize for 10 minutes before collection of the control blood samples. Ligation of the coronary artery was done for 10 minutes and then blood samples collected, kinins extracted and assayed by RIA. The figure represent percentage increase in kinin levels after ligation of the left main coronary artery for 10 minutes as compared to pre-ligation levels. The numbers 1 to 7 represent individual rats.

5.11. CHARACTERIZATION OF THE PEPTIDE RELEASED BY THE ACID OPTIMUM ENZYME IN RAT VENTRICULAR MYOCARDIUM.

INTRODUCTION

Britos and Nolly (1981), showed that the kinin released by the acid optimum enzyme in extracts of rat "cardiac tissues" was similar to bradykinin and kallidin. The kinin caused contraction of the oestrous rat uterus and the cat jejunum. The smooth muscle effect on the cat jejunum was inhibited by antibodies that specifically inhibit the biological effect of kinins. Similar results were obtained with the peptide released by the acid optimum enzyme in rat mesenteric arteries (Nolly et al, 1981b).

Radioimmunoassay was used in this work to measure the peptide released by the rat acid optimum enzyme. The antibody used is not specific to bradykinin. It cross-reacts 81 % with lysyl-bradykinin and 100 % with Met-lysyl-bradykinin (Supplier's information). There was no information provided on the cross-reactivity with T-kinin (Ile-Ser-bradykinin). T-kinin is present in both normal and inflamed rat plasma so any measurements of plasma kinins should take into consideration the presence of this kinin.

5.11.1. DETERMINATION OF THE CROSS-REACTIVITY OF T-KININ WITH THE BRADYKININ ANTIBODY.

METHOD.

Pure synthetic bradykinin (molecular weight 1060)and T-kinin (molecular weight 1260) were used. Bradykinin was diluted to give concentrations of 147.4 pM to 75.5 μM ; and T-kinin 124 pM to 63.5 μM (i.e 15.625 - 8000

pg/100 µl each). Binding curves for the two peptides were obtained using the protocol indicated in the methods. The binding curves were analysed for parallelism using a computer statistical programme (Tallarida and Murray, 1981).

RESULTS AND DISCUSSION.

T-kinin cross-reacted with the bradykinin antibody. Figure 5.11.1 shows that the binding curves for the two peptides are parallel at concentrations above 500 pg/100 µl (i.e 500 pM and 590 pM for T-kinin and bradykinin respectively). It seems at high concentrations the antibody shows the same recognition for the two peptides. Below this concentration the two curves deviate slightly from parallelism and they cross each other at approximately 2 µM for T-kinin and 2.4 µM for bradykinin. When the two binding curves were tested for parallelism using a computer statistical programme, they were not significantly different from one another($P > 0.05$). It is concluded that within the limits of experimental error the two binding curves are parallel. Visual inspection shows that T-kinin has about 80 % cross-reactivity with the polyclonal rabbit anti-bradykinin antiserum.

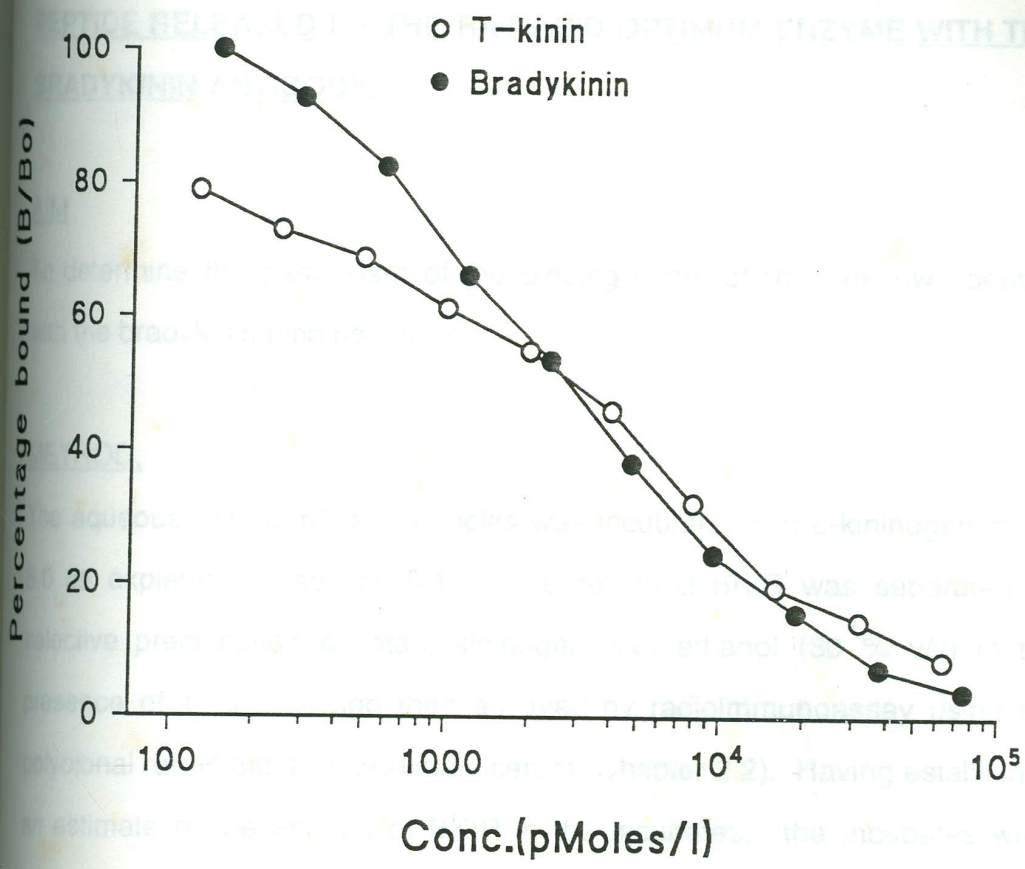


Figure 5.11.1. The cross-reactivity of the polyclonal bradykinin antibody with T-kinin. Synthetic bradykinin and T-kinin were diluted to give concentrations of 147.4 pM - 75.5 μM and 124 pM - 63.5 μM, respectively. Radioimmunoassay was done in parallel for the two peptides and the percentage binding plotted against log (concentrations). Each point is a mean of 2 values.

5.11.2. DETERMINATION OF THE CROSS-REACTIVITY OF THE PEPTIDE RELEASED BY THE RAT ACID OPTIMUM ENZYME WITH THE BRADYKININ ANTIBODY.

AIM

To determine the parallelism of the binding curve of the unknown peptide with the bradykinin binding curve.

METHOD.

The aqueous extract of rat ventricles was incubated with L-kininogen at pH 5.0 as explained in section 5.10. The released BKIR was separated by selective precipitation of intact kininogen with ethanol (80 % v/v) in the presence of 1 % BSA and then assayed by radioimmunoassay using the polyclonal rabbit anti-bradykinin antiserum (Chapter 2.2). Having established an estimate of the amount of BKIR in the incubates, the incubates were pooled and serial dilutions made. Dilutions of 150, 300, 375, 750, 1500, and 3000 pg/0.1 ml were made. Dilutions of synthetic bradykinin were made to produce concentrations of 15.625 - 8000 pg/0.1 ml. These were used to make a binding curve for bradykinin as shown in the methods. The dilutions for the unknown kinin were assayed in parallel with bradykinin standards.

RESULTS AND DISCUSSION.

Two almost superimposable binding curves were obtained for both bradykinin and the unknown peptide (Figure 5.11.2). The two curves were tested for parallelism using a statistical package by Tallarida and Murray (1981). The equations obtained for the two binding curves were $y = -0.01812*X + 60.627$ and $y = -0.010587*X + 69.645$ for the unknown kinin and bradykinin

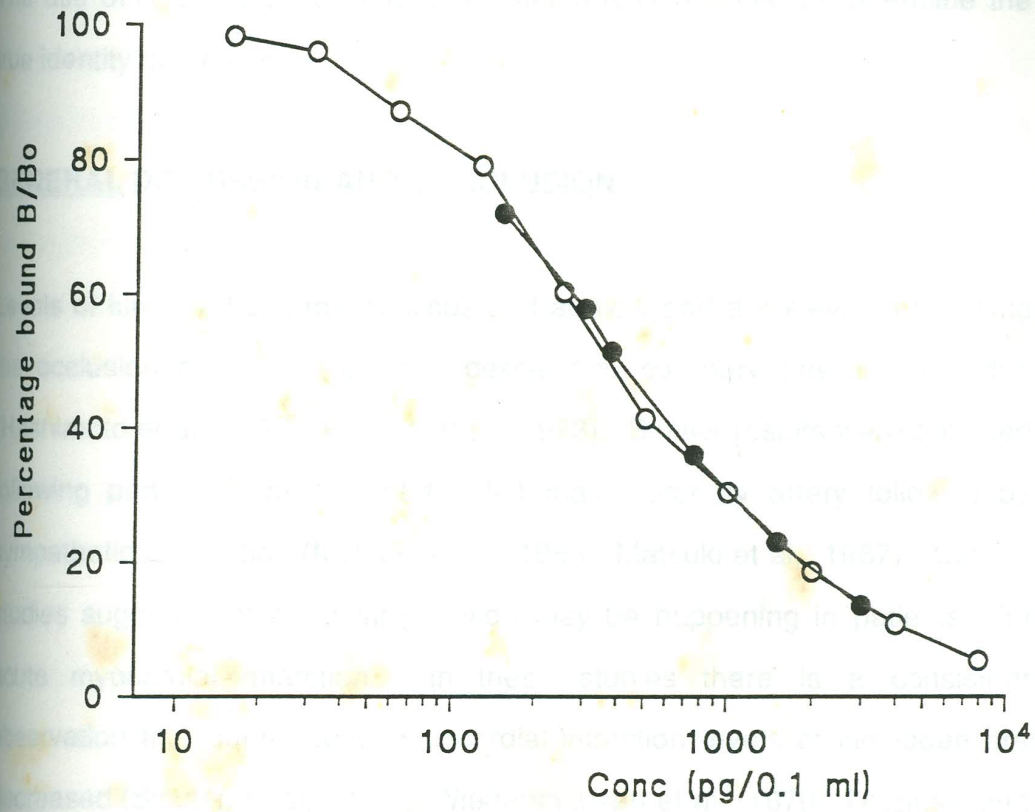


Figure 5.11.2. The cross-reactivity of the peptide released by the ACOP enzyme in rat ventricles with a bradykinin antibody. Aqueous extract of rat ventricles was incubated with L-kininogen (pH 5.0). The released BKIR was separated using 80 % ethanol and then assayed by RIA against bradykinin. The estimated concentration was used for making dilutions which were used to make a binding curve at the same time with bradykinin. Each point is a mean of 3 values.

respectively. The gradients for the two curves were not significantly different ($P > 0.05$). It is concluded that the released peptide is similar to bradykinin. The use of HPLC and possibly other methods is required to determine the true identity of the kinin.

GENERAL DISCUSSION AND CONCLUSION

Levels of kinin in the coronary sinus and aortic blood are elevated following the occlusion of the left anterior descending coronary artery in the dog (Hashimoto et al, 1977; Kimura et al, 1973). Similar results were obtained following partial constriction of the left main coronary artery followed by sympathetic stimulation (Matsuki et al, 1986; Matsuki et al, 1987). Clinical studies suggest that a similar situation may be happening in patients with acute myocardial infarction. In these studies there is a consistent observation that during acute myocardial infarction levels of kininogen are decreased (Sicuteri et al, 1967; Wiegershausen et al, 1970; Dzizinski and Kuimov, 1972; Kedra et al, 1973; Kolber-Postepska, 1975; Torstila, 1978). It was interpreted in these studies that the fall in kininogen levels directly contribute to increase in plasma kinin levels, although no actual measurement was made. In all these studies there was a great tendency to consider kinins as a pathological agent and hence offering an explanation to pain in angina pectoris and shock during acute myocardial infarction. Levels of kinin in plasma during clinical acute myocardial infarction were first measured by Hashimoto and co-workers in blood from the cubital vein (Hashimoto et al, 1978). This study was the first of its kind to identify elevation of plasma kinins to have a beneficial effect as opposed to being a causative of anginal pain and shock. Two groups of patients were identified

in this study; the group of survivors and that of non-survivors. In the survival group increased kinin and decreased kininogen levels were observed and these changes correlated with concomitant lowering of blood pressure, decreased total vascular resistance and prolongation of circulation time. In the non-survival group while there was a decrease in kininogen levels there was no increase in kinin levels; likewise there was no correlation with haemodynamic changes. Lack of change in kinin level was interpreted to be due to the presence of strong kininase activity; a view also shared by another worker (Kolber-Postepska, 1975). The elevation of kinin level in the survivors was interpreted to indicate beneficial vasodilatation effects, possibly by increasing coronary flow and reducing the afterload of the left ventricle.

The advent of angiotensin-converting enzyme inhibitors and kinin receptor antagonists has recently been useful in evaluating the role of kinins in acute myocardial ischaemia and acute myocardial infarction. Through the use of these agents it has been shown that bradykinin improves both metabolic and haemodynamic parameters during acute myocardial ischaemia (Scholkens et al, 1988) and also reduces infarct size (Martorana et al, 1990a; Martorana et al, 1990b) in the dog model of acute myocardial ischaemia. The two studies suggest that the beneficial effects of ACE-inhibitors are due to elevation of plasma kinins and possibly the reduction of plasma angiotensin II.

The major question has been, and still remains to be, whether or not the elevation of kinins in the coronary sinus and aortic blood is due to the presence of kinin-forming enzymes either in plasma, myocardium or

coronary vasculature which are activated during acute myocardial ischaemia and acute myocardial infarction.

An acid optimum kinin-forming enzyme was reported to be present in unspecified rat cardiac tissues (Nolly et al, 1981a). This enzyme had a molecular weight between 30 - 35 kDa. In the present study the ventricular myocardium was used. The aqueous ventricular extract was shown to contain one peak of UCS-formation with an optimum between pH 4.7 and 5.3. Gel filtration of the extract followed by assay of the fractions for arginine amidase activity gave two acid optimum peaks with molecular weights of 42.8 $\pm$ 4.9 kDa and $\geq$ 800 kDa. There was no alkaline peak of UCS formation and assay of the fractions at pH 8.0 did not reveal any alkaline peak of amidase activity. When the pH-optima for the hydrolysis of the tripeptide S-2266 by the two peaks were determined they were found to be different. The low molecular weight peak had an optimum at pH 4.5 while the high molecular weight peak showed two pH optima at pH 5.5 and pH 4.5. There was a minor peak at pH 4.1. It seems the high molecular weight peak was a mixture of more than one enzyme or different forms of an enzyme.

Both the crude extract and the enzyme fractions were assayed for the release of bradykinin-like immunoreactivity from L-kininogen and H-kininogen. Using the crude extract it was shown that the rat enzyme hydrolyses only L-kininogen and not H-kininogen. The release of BKIR by the crude extract was 36.83 ngBkeq hr^{-1} mg^{-1} wet tissue equivalent (mean; n = 6). When the two peaks were assayed for the release of BKIR only the low molecular weight peak was capable of releasing BKIR from L-kininogen;

also not from H-kininogen, at the rate of 87.0 ± 11.24 ngBkeq hr^{-1} mg^{-1} protein (n = 6).

Nolly et al (1981a) showed that the uterus contracting activity and the blood pressure lowering effect of the peptide released by this enzyme in the rat were inhibited 90 % and 85 %, respectively, by a bradykinin antibody. They concluded that the released peptide was a kinin and that it was similar to bradykinin and kallidin. In this study we have shown that the peptide cleaved from both the crude plasma substrate and L-kininogen can be detected using a polyclonal bradykinin antibody. When the two peptides were assayed in parallel they showed similar binding characteristics and the two binding curves were parallel to one another. The released peptide may be bradykinin or a very closely related peptide. Further studies using HPLC and may be Edman degradation are required to identify the structure of the peptide.

Inhibitor studies were done using the crude ventricular extract. The acid optimum enzyme in the ventricular extract was shown to release both a uterus contracting substance and bradykinin-like immunoreactivity. Both are probably the same substance; a peptide similar to bradykinin or kallidin. Due to animal licence problems it became necessary to use both the release of UCS and BKIR for the inhibitor studies. For pepstatin and lima bean trypsin inhibitor the effect on the release of BKIR was used. Pepstatin at 1.5 x 10^{-4} M almost completely inhibited the release of BKIR, at pH 5.0, by the aqueous extract of rat ventricles. Lima bean trypsin inhibitor at the concentration of 500 µg/ml did not have any effect on the release of BKIR, at pH 5.0, by the rat ventricular extract. The effect of aprotinin and SBTI was studied on the release of UCS. Both inhibitors partially inhibited the release

of UCS, at pH 5.0, by the rat ventricular extract. The release of UCS by the acid optimum enzyme in aqueous extracts of dog ventricles was also completely inhibited by pepstatin and only partially by aprotinin. Lima bean trypsin inhibitor inhibited the acid optimum enzyme in dog ventricles by 13.5 % ($P < 0.05$). These results differ from those obtained with the rat extract. The use of different methods may be the reason for the difference. While the release of UCS by the rat ventricular extract is affected by SBTI, the release of UCS by the dog ventricular extract was not affected by SBTI. The inhibition by SBTI is very likely to be due to contamination of the extracts with blood. Considering its molecular size and optimum at acid pH's the rat cardiac enzyme is similar to cathepsin B1, a cysteine proteinase, and cathepsin D and the renin-like aspartic proteinases. Comparison of the rat enzyme with cathepsin B1 (bovine) showed that they are different since, although cathepsin B cleaves the substrate S-2266, it could not release BKIR from either L-kininogen or H-kininogen. The remaining possibility is that of cathepsin D and the renin-like aspartic proteinases all of which are inhibited by pepstatin. Cathepsin D has a molecular weight of 42 kDa (Barrett and McDonald, 1980), it releases bradykinin from L-kininogen (Ogloblina et al, 1981) and it is inhibited by pepstatin (Barrett and McDonald, 1980). Cathepsin D is reported to be the major acid proteinase in the heart (Decker et al, 1977). Two previous studies, one on rabbits (Decker et al, 1977) and another on dogs (Araki et al, 1975) suggest that the distribution of this enzyme is altered during myocardial ischaemia.

In the present study an attempt was made to determine the consequence of acute myocardial ischaemia on the ability of the rat left ventricles to release bradykinin-like immunoreactivity from L-kininogen. Ligation of the left main

coronary artery for 10 and 30 minutes reduced the ability of the aqueous extract of the left ventricles to release bradykinin-like immunoreactivity from L-kininogen by 30 % and 48.1 % , respectively. There was a wide variation, especially at the 30 minute ligation period, in the amount of released BKIR. Two rats showed only 6% and 5.7 %, respectively, of the control values of released BKIR.

Seven rats were selected to measure kinin levels. After 10 minutes of ligation of the left main coronary artery, levels of kinin in venous blood from the right atrium increased in all the seven rats. However, there was a big variation in the percentage increase, ranging from 16.4 % to 702 %. Kimura et al (1973) showed a similar variation in increase in kinin levels and also in the time at which the plasma kinin concentrations peaked. No attempt was made to follow the change with time due to the fact that taking 0.8 ml samples would result in killing the rats due to hypotension after the second blood sample or so. Replacement with saline also would be a problem as it would lead to dilution of the blood. It was for this reason that it was difficult to do a time course for the levels of kinin in the mixed venous blood from the right atrium. This would have been important to determine when kinin levels started to increase and time at which the concentrations were maximum.

CONCLUSION.

Aqueous extracts of rat ventricular myocardium contain one acid optimum UCS and BKIR forming enzyme. The enzyme has a pH optimum at pH 4.7 - pH 5.3 for the release of UCS from a human plasma substrate and pH 4.5 against the chromogenic substrate S2266. This enzyme cleaves L-kininogen

to release a kinin very similar immunologically to bradykinin. It is completely inhibited by pepstatin and only partially by aprotinin. The release of bradykinin-like immunoreactivity from L-kininogen by aqueous extracts of rat left ventricles decreased following ligation of the left main coronary artery in the rat. At the same time levels of kinin in mixed venous blood from the right atrium were found to be elevated by 16.4 to 702 % of the control pre-ligation levels. This is interpreted to mean that during acute ischaemia the acid optimum enzyme leaks from the cardiac myocytes into the interstitial fluid and lymph where it cleaves L-kininogen present in these fluids to release kinin. The released kinin is then delivered to the venous blood as part of the coronary effluent. The acid enzyme in the rat ventricles is similar, in molecular size and in its sensitivity to inhibitors, to cathepsin D and the renin-like aspartic proteinases. Further studies are required to establish the identity of this enzyme.

CHAPTER 6

SUMMARY AND CONCLUSION

Pain and hypotension are the two most common manifestations of myocardial infarction but to date the mechanism by which they occur remains unknown. Several substances have been proposed to be the cause of anginal pain. Katz (1935), postulated that the pain was produced by the metabolites released during ischaemia which include acid metabolites like lactic acid, phosphoric acid, pyruvic acid and succinic acid and non-acid metabolites such as histamine, phosphocreatine, adenosine and potassium. Raab (1953), postulated that anginal pain was caused by catecholamines the release of which is increased in myocardial ischaemia. Kinins are other substances which have also been suspected to be responsible for anginal pain owing to the fact that they have been shown to have a strong pain-producing effect (Armstrong et al, 1953; Armstrong et al, 1954). Administration of large doses of bradykinin into the brachial artery in man caused pain (Fox et al, 1961). Sicuteri and co-workers also observed that injection of bradykinin into the common carotid artery produced headache (Sicuteri et al, 1967) and this led them to speculate that the pain of angina pectoris, myocardial infarction, intermittent claudication and migraine headache could be caused by bradykinin. Kinins are potent vasodilators and vascular smooth muscle relaxants. Kinins caused coronary vasodilatation in the intact dog (Maxwell, 1964; Lochner and Parratt, 1966). Such observations have led people in the past (Sicuteri et al, 1967; Wiegershausen et al, 1970 and Dzizinski and Kuimov, 1972) to associate kinins with the hypotension in myocardial infarction. However, all these reports have been mainly speculative and there has not been any clear-cut evidence associating kinins with these effects in myocardial infarction. On the contrary kinins have been shown to increase cardiac output, myocardial oxygen extraction and myocardial metabolic heat production in dogs (Lochner and Parratt, 1966). Recently, following the development of kinin

antagonists it has become easier to evaluate the role of kinins in myocardial ischaemia. Bradykinin increased coronary blood flow in the guinea pig heart; this effect was potentiated by a single oral dose of ramiprilat (an angiotensin converting enzyme inhibitor) and it was abolished by perfusion with the bradykinin antagonist D-Arg [Hyp^{2}, Thi^{5,8}, D-Phe^{7}]Bk (Scholkens et al, 1988). When ischaemic rat hearts were perfused with bradykinin (1 x 10^{-10} mol/l) it reduced enzymatic activities in the perfusate and improved metabolic parameters in the myocardium (Scholkens et al, 1988). These protective effects of bradykinin were abolished when the bradykinin antagonist D-Arg [Hyp^{2}, Thi^{5,8}, D-Phe^{7}]Bk was added to the perfusate. They were only partially attenuated by indomethacin. Higher concentrations of bradykinin (1 x 10^{-7} mol/l) or ramiprilat (2.58 x 10^{-5} mol/l) overcame the bradykinin antagonist suggesting a direct effect of bradykinin itself on the ischaemic myocardium (Scholkens et al, 1988). Recently also, bradykinin was shown to have a significant cardioprotective effect against ischaemic reperfusion injury and it improved metabolic parameters in dogs following occlusion of the left coronary artery (Martorana et al, 1990a, 1990 b) and in rats (Scholkens et al, 1988; Linz et al, 1990).

Until now the evidence that has been presented was obtained from experiments involving the in vitro administration of bradykinin. The question that one would like to ask is whether or not kinins are released invivo during myocardial ischaemia. If they are released what is the mechanism of release and what is the resultant effect?. Ligation of the left anterior descending coronary artery in the dog was reported to result in the elevation of levels of kinin in the coronary sinus and aortic blood (Kimura et al, 1973; Hashimoto et al, 1977). Similar results were obtained in dogs by partial coronary artery stenosis followed by sympathetic stimulation (Matsuki et al, 1987). Kinin

levels were also elevated in the blood from the cubital vein in patients with acute myocardial infarction (Hashimoto et al, 1978). There are two possible explanations for the elevation of levels of kinin. Kinins could be elevated by preventing their metabolism or through increased release of kinins. During ischaemia the myocardium becomes acidic. Recently it was shown that intracellular pH in the myocardial cells can fall to as low as pH 5.8 following a 30 minute period of zero flow ischaemia (Khandoudi et al, 1990). Edery and Lewis (1962) showed that the metabolism of bradykinin decreases at pH's below 7. It is, however, unlikely that decreased metabolism by itself can account for more than 10 fold increase in plasma kinin levels which was reported by Matsuki and co-workers (Matsuki et al, 1986). An alternative explanation is that during myocardial ischaemia there is release or activation of enzymes in plasma, in the cardiac tissues or in the coronary vasculature which then release kinins from kininogens. A small fall in pH, as is the case in myocardial ischaemia, can activate the kinin system (Wilkens et al, 1970). However, there are doubts as to the involvement of plasma kallikrein in the release of kinins during myocardial ischaemia. This was shown in the work done by Pitt and co-workers (1970), involving 17 patients undergoing a diagnostic evaluation of chest pains. These patients were subjected to a pacing stress test in which the heart rates were increased up to 170 - 180/minute. Eleven out of the 17 patients experienced myocardial ischaemia which was evident in the elevation of the ST-segment. However, only 7 patients showed evidence of activation of plasma kallikrein system (TAME - esterase activity) while 4 of them did not show any changes of the plasma kallikrein system despite clear signs of ischaemia as shown by the elevation of the ST-segment. This leaves us with only two alternatives, the cardiac tissues and the coronary vasculature. There is already evidence of the

presence of kinin-forming enzymes in these tissues (Nolly et al, 1981a; Britos and Nolly, 1981; Nolly et al, 1987; Zeitlin et al, 1989).

In the present work the dog ventricular myocardium, the dog aorta and rat ventricular myocardium were assayed for the presence of kinin-forming enzymes. Different methods were used including release of a uterus contracting substance (UCS) from a plasma kinin-forming substrate, release of bradykinin-like immunoreactivity (BKIR) from a plasma kinin-forming substrate and purified commercial L-kininogen and H-kininogen and amidase assays.

Aqueous extracts of dog ventricles were assayed for the release of UCS from a plasma kinin-forming substrate. They released UCS and optimum release occurred between pH 5.2 and pH 5.4. Release of UCS was detected up to pH 7.5 but there was no obvious alkaline peak of UCS formation. The release of UCS at pH 5.2 by the dog ventricular extract was completely inhibited by pepstatin but it was inhibited 32.8 % by aprotinin and 13.5 % by LBTI. It was not inhibited by soybean trypsin inhibitor. Aqueous extracts of dog ventricles also released bradykinin-like immunoreactivity from a plasma kinin-forming substrate. The amount of BKIR released at pH 5.2 was 4.9 µg Bk eq hr^{-1} g^{-1} while the amount of UCS released at the same pH was 4.0 $\pm$ 0.47 µg Bk eq hr^{-1} g^{-1}. The amount of BKIR which was released is very close to that of UCS indicating that most of the UCS formed was due to kinin. Gel filtration showed that the enzyme responsible for the release of UCS and BKIR at acid pH's has a molecular weight of 42.6 $\pm$ 0.9 kDa. Pooled eluate fractions containing this enzyme released BKIR from the kinin-forming substrate at the rate of 24.5 $\pm$ 12.9 ng Bk eq hr^{-1} mg^{-1} protein. Zeitlin et al (1989) showed that the dog ventricular myocardium contains one acid

optimum enzyme sensitive to pepstatin. This enzyme had properties similar to an aspartic proteinase. They also reported the presence of a neutral enzyme similar to plasma kallikrein in molecular size and it was inhibited by SBTI. The presence of the acid optimum enzyme in the dog ventricular myocardium has been confirmed in this study . The identity of this enzyme, however, is not completely known. It is very similar to cathepsin D. Cathepsin D is an aspartic proteinase with a molecular weight of 42 kDa and like the dog enzyme it is inhibited by pepstatin (Barrett and McDonald, 1980). Cathepsin D is known to release kinins from kininogen substrates (Ogloblina et al, 1981). Cathepsin D has a pH optimum between pH 2.8 - pH 5.0 depending on the source and substrate (Barrett and McDonald, 1980). The pH optimum for the ventricular enzyme was at pH 5.2 - 5.4. Another possible candidate is renin and the related enzymes like the rat submandibular renin (Barrett and McDonald, 1980) and the renin-like enzymes reported in human vascular tissues and the heart (Rosenthal et al, 1990). Renin has a pH optimum between pH 3.5 - 6.0 depending on substrate. The rat submandibular renin has pH optimum between pH 5.4 - 8.3 depending on the substrate (Barrett and McDonald, 1980). The renin-like enzymes are also aspartic proteinases with molecular weights similar to cathepsin D and they are all inhibited by pepstatin. Since none of these other enzymes apart from cathepsin D have been shown to release kinins the comparison with the dog ventricular enzyme remains a speculation. The use of specific antibodies to these enzymes may help to identify the rat enzyme. The presence of a neutral enzyme in the dog ventricles was not shown in this study. Examination of pH-dependent release of UCS showed that UCS was released up to pH 7.5 but this was probably a continuation of the activity of the acid optimum enzyme. Similarly when the ventricular extract was incubated with the plasma kinin-forming substrate at pH 8.0 and the

incubates assayed by radioimmunoassay, no BKIR was detected. Gel filtration of the aqueous extract of dog ventricles and assay of the fractions for amidase activity at pH 8.0 failed to show any peak of enzymic activity. These results all together failed to show the presence of enzymic activity at pH 8.0. It is concluded that the neutral enzyme observed in the previous study (Zeitlin et al, 1989) was plasma kallikrein resulting from contamination of the extracts used, by blood.

Aqueous extracts of dog aorta showed two optima for the release of a uterus contracting substance from a plasma kinin-forming substrate; one at pH 5.4 and the second at pH 8.0. When the extract was assayed with the tripeptide substrate D-Val-Leu-Arg-pNA two pH optima were also obtained; one at pH 5.5 and the second at pH 7.5. Despite precipitation problems when using the chromogenic substrate at acid pH's both methods produced similar results. The acid optimum UCS-forming peak was completely inhibited by pepstatin. It was inhibited 38.9 % by aprotinin and and 33.3 % by lima bean trypsin inhibitor. It was not inhibited by SBTI. Gel filtration showed that the acid optimum UCS forming activity derives from two enzyme moieties; a low molecular weight moiety, molecular weight 42.0 $\pm$ 4.6 kDa and a high molecular weight moiety, molecular weight 252.0 $\pm$ 39.0 kDa. Both released BKIR from a plasma kinin-forming substrate. The low molecular weight enzyme was similar to that in the ventricular myocardium. It had the same molecular size and released BKIR at a similar rate (see table 4.10.1). Gel filtration revealed the presence of a neutral enzyme with a molecular weight 48.5 $\pm$ 4.0 kDa which also released BKIR from the plasma kinin-forming substrate. The release of BKIR by this enzyme was almost completely inhibited by aprotinin. Since no other inhibitor was used it is difficult to say whether or not it was tissue kallikrein or a related enzyme.

Similar studies were done on the rat ventricular myocardium. Nolly and co-workers reported that rat cardiac tissues contain an acid optimum kinin-forming enzyme (Nolly et al, 1981a) The enzyme was reported to occur in an inactive form which required activation by incubation at pH 5.0 (37 ^{o}C) for 2 hours. It released a peptide which contracted the isolated rat uterus and lowered rat blood pressure. Incubation of the peptide released by the rat cardiac enzyme with a bradykinin antibody inhibited its oxytocic effect by 90 % and the blood pressure lowering effect by 85 %, showing that the peptide is similar to bradykinin and kallidin. Britos and Nolly (1981), reported that the rat cardiac enzyme had an approximate molecular weight between 30 and 35 kDa and it released kinin from a substrate present in rat plasma, at the rate similar to that by kallikrein and trypsin. The release was unaffected by incubation of the enzyme with aprotinin or SBTI. They also reported that the rat enzyme was unaffected by pepstatin in the concentrations of 10^{-5} - 10^{-3} M. In both studies it was not indicated which cardiac tissue was used. In the present study it was shown that aqueous extracts of rat ventricles released a uterus contracting substance from a plasma kinin-forming substrate only at acid pH's. Optimum release occurred between pH 4.7 and pH 5.3. There was no release of UCS at alkaline pH's. Aqueous extracts of rat ventricles also released BKIR at pH 5.0 from the plasma kinin-forming substrate. Contrary to the previous report (Nolly et al, 1981a) no activation process was found necessary for the rat acid optimum enzyme. Autolytic activation done by incubating aqueous extract of the ventricles (18 - 20 ^{o}C) for 20 hours resulted in 27.4 % reduction of enzymatic activity. Following this observation all the subsequent studies were done without autolytic activation. The rat enzyme released BKIR from L-kininogen and not H-kininogen. This was confirmed using both the crude aqueous extract and fractions of the

amidase peak obtained from gel filtration of the aqueous extract. Radioimmunoassay of the released peptide was done using a polyclonal rabbit anti-bradykinin antiserum. The binding of the peptide released from L-kininogen by the rat ventricular enzyme to the bradykinin antibody was compared to that of bradykinin and T-kinin. The binding curves were parallel. The peptide released from L-kininogen is similar to bradykinin and T-kinin. It is concluded here that the substrate for this acid optimum enzyme is L-kininogen. The molecular weight of the acid optimum enzyme in rat ventricles determined by gel filtration was 42.8 $\pm$ 4.9 kDa. This enzyme had an optimum at pH 4.5 for the hydrolysis of the tripeptide substrate D-Val-Leu-Arg-pNA. It was completely inhibited by pepstatin and only partially by aprotinin. Table 6.1 summarizes the comparison of the effect of inhibitors on the release of UCS by the aqueous extracts of the three tissues used. The table shows that the acid optimum enzymes in the three tissues were completely inhibited by pepstatin. They were partially inhibited by aprotinin. SBTI and LBTI did not have a uniform effect on all the three tissues. SBTI did not inhibit the acid optimum enzyme in dog ventricles or in the dog aorta, but it inhibited the rat enzyme by 33.1 %. It was suspected that the inhibition was due to the presence of plasma kallikrein in the rat ventricular extract. Nolly et al (1981a), showed that the rat enzyme was not inhibited by SBTI. LBTI inhibited the dog ventricular enzyme by 13.5 % and that in dog aorta by 33.3 % but it had no effect on the rat enzyme. The cause of the difference is not clear. Table 6.2 compares the molecular weights and the mean rates for the release of BKIR. The rat ventricular enzyme had a similar molecular size to the dog ventricular enzyme and the low molecular weight enzyme in the dog aorta. The low molecular weight enzyme in dog aorta and that in the dog ventricles released BKIR at comparable rates. The rat ventricular enzyme released BKIR at a higher rate. Purified L-kininogen was used to calculate

release of BKIR by the rat ventricles while for the dog tissues a crude plasma kinin-forming substrate was used. This may be the source of the difference, or otherwise species differences may be another factor. It is concluded that the acid optimum enzyme in the rat ventricles is similar to that in the dog ventricles and the low molecular weight acid optimum enzyme in the dog aorta. Considering the molecular size of about 42 kDa and inhibition by pepstatin this enzyme resembles cathepsin D. Cathepsin D is an aspartic proteinase with a molecular weight of 42 kDa and it is inhibited by pepstatin (Barrett and McDonald, 1980). It is reported to be the most abundant acid enzyme in the heart (Decker et al, 1977). It releases bradykinin from L-kininogen and not from H-kininogen (Ogloblina et al, 1981). The rat enzyme also released a bradykinin-like peptide from L-kininogen and not from H-kininogen. The effect of aprotinin on cathepsin D is not known so it is difficult to relate the effect on the rat cardiac enzyme to that on cathepsin D. Renin and rat submandibular renin which are also aspartic proteinases share similar properties with cathepsin D (Barrett and McDonald, 1980). They have a molecular weight of 42 kDa and they are inhibited by pepstatin. These are other likely candidates although at the moment there is no sufficient evidence showing that they could be similar to the rat cardiac enzyme. Renin-like enzymes with optimum activity between pH 4 and pH 6 were recently reported in human vascular tissues and the heart (Rosenthal et al, 1990). The identity of the high molecular weight acid optimum enzyme in the dog aorta is not clear. Since there was only one pH-optimum for the release of UCS at acid pH, it is likely that the high molecular weight enzyme is a variation of the low molecular weight enzyme. It is possible that the high molecular weight enzyme is a polymeric form of the low molecular weight enzyme. It is reported that different carbohydrate contents could give an enzyme different molecular sizes and yet retain the same pH-optimum

(Sessler et al, 1986). Another possibility derives from the observation that α_2-macroglobulin combines with some aspartic proteinases, including cathepsin D (Thomas et al, 1989). The α_2-macroglobulin is a tetramer consisting of monomers with a molecular weight of 180 - 190 kDa (Sotrup-Jensen et al, 1983). The monomers can also combine with proteinases and partially inhibit their enzymic activity (Enghild et al, 1989). The complex formed of the low molecular weight enzyme with an α_2-macroglobulin monomer falls within one standard error of the present molecular weight determinations (234 - 269 kDa). This high molecular weight acid enzyme was absent in dog and rat ventricles.

The effect of acute ischaemia on the levels of the acid optimum enzyme in rat ventricles was investigated. Rats were exposed to acute ischaemia by ligation of the left main coronary artery. Ligation of the left main coronary artery reduced the formation of BKIR by aqueous extracts of the rat left ventricles as compared to extracts from control rats. The formation of BKIR after 10 and 30 minutes of ligation was 70 % and 51.9 % of the mean control values, respectively. There are two possible explanations for the decreased ability to release BKIR. One possibility is that following ischaemia an inhibitor is released which inhibits the enzyme in rat ventricles and in so doing decrease the ability to cleave kininogen. Little credibility can be given to this theory at the moment because there is so far no evidence suggesting that an inhibitor might be present in the myocardium for this acid enzyme. Kallikrein or trypsin inhibitory activity, in plasma, has been reported to be elevated during myocardial infarction (Dzizinski and Kuimov, 1972; Torstila, 1978). Other groups have also reported a decrease of kallikrein inhibitory activity during myocardial infarction (Sicuteri et al, 1972). These reports so far talk about inhibitory activity against plasma kallikrein which according to Pitt et al

(1970), may not be important in the events occurring during myocardial ischaemia. Only 7 out of 11 patients with myocardial ischaemia in their study showed activation of the plasma kallikrein system. The second possibility is that during an ischaemic episode the kinin-forming enzyme in the rat ventricles leaks out from the myocardial cells into the interstitial fluid and possibly plasma. The result of this is that as it leaks out the enzyme is depleted from the myocardium and enriched in the interstitial fluid and plasma where it then cleaves kininogen to release kinins. Decker and co-workers showed, using immunofluorescent techniques, that ligation of the circumflex coronary artery in rabbits resulted in the release of cathepsin D from the lysosomes into the cytosol (Decker et al, 1977). In a similar study on dogs ligation of the left coronary artery resulted in the increase of the activity of cathepsin D in the cardiac lymph and pericardial fluid (Araki and Takenaka, 1975). There was no increased content of cathepsin D in plasma. It has been reported that serum or plasma enhance the activity of cathepsin D (Kolski et al, 1987); so any little amount released into plasma is likely to see enhanced activity and this will contribute to increasing plasma kinin concentrations. This second theory seems to agree with the increased levels of kinin in the coronary sinus blood. Ligation of the left main coronary artery for 10 minutes resulted in the elevation of kinin levels in the mixed venous blood from the right atrium by 16.4 - 702.6 % as compared to pre-ligation levels. Matsuki et al (1986) showed that stenosis of the coronary artery, followed by sympathetic stimulation in dogs, increased kinin levels in the coronary sinus blood from 12.8 $\pm$ 3.3 to 142.4 $\pm$ 45.4 pg/ml. This represents 1112.5% increase as compared to the control values. The question one would like to ask at this point is about the link between the decreased enzyme levels in the myocardium and the elevation of levels of kinin in the mixed venous blood from the right atrium. Are the two, in any

way, related?. There are two ways of approaching this problem. One is by measuring the levels of this enzyme in plasma and probably interstitial fluid before and after ligation. This is probably not a very direct method and is likely to experience problems as the activity of other enzymes in plasma might not be excluded. The other alternative is the use of enzyme inhibitors, and in this case pepstatin, to see how the levels of kinin are going to be affected. Hashimoto et al (1977) reported that administration of aprotinin to dogs before ligation of the left coronary artery prevented the post-ligation elevation of kinin levels in the coronary sinus blood. However, aprotinin only partially inhibited the rat enzyme (20 %) so it is not known how effective or useful it will be. Still the use of inhibitors appears to be necessary so as to establish the relationship between changes of enzyme levels and the elevation of levels of kinin in the coronary sinus blood (mixed venous blood from the right atrium.

Inhibitor	Dog ventricles	Rat Ventricles	Dog aorta
pepstatin	100*	100*	100*
aprotinin	32.8*	20*	38.9*
LBTI	13.5*	0	33.3*
SBTI	0	33.3*	0

Table 6.1 Actions of proteinase inhibitors on acid optimum UCS-forming enzymic activity in aqueous extracts of dog aorta, dog ventricular myocardium and rat ventricular myocardium. Assays were done at pH 5.2 or pH 5.0 (Rat ventricles). * $P < 0.05$; $n = 5$.

Source of peak	Mwt (kDa)	Specific activity (ng Bkeq hr^{-1} mg^{-1})
Source of peak		
Rat ventricles	42.8 ± 4.9	87.0 ± 11.3
Dog ventricles	42.6 ± 0.9	24.5 ± 12.9
Dog aorta	252.0 ± 39.0	163.8 ± 46.3
Dog aorta	42.0 ± 4.6	23.3 ± 9.2

Table 6.2 The table shows mean molecular weights (n= 5) and mean specific activities (n = 5) for the release of BKIR related to amidase peaks separated using gel filtration on Sephacryl S-300 from dog ventricular myocardium, dog aorta and rat ventricular myocardium.

Proposals for future work.

1. The dog and rat ventricles have been shown to contain one kinin-forming enzyme. The enzyme is similar to cathepsin D and the renin-like aspartic proteinases. Further studies are required to confirm the identity of this enzyme. The enzyme requires further purification to uniformity. This together with the use of specific antibodies is required to confirm its identity.

2. The peptide released by the acid optimum enzyme requires further characterization. The use of HPLC and possibly Edman degradation is proposed.

3. It is possible that the processing of kinins is not the primary role of the acid optimum enzyme in the heart and vascular tissues. For example cathepsin D releases endothelin from big endothelin in bovine aortic endothelial cells (Ohnaka et al, 1990; Samawura et al, 1990). The role of this enzyme in the processing of other biologically active peptides requires further studies.

4. The use of proteinase inhibitors is required to establish the link between the elevation of levels of kinin in the coronary sinus blood and the decrease of enzyme levels in the rat left ventricles during ischaemia.

5. The use of kinin receptor antagonists is required for more understanding of the role of kinins in coronary heart diseases.

6. The high molecular weight enzyme in dog aorta requires further characterization. At the same time the role of the vascular endothelium in the formation of kinins requires further investigation. Oza et al (1990) showed that the endothelium of rat aorta contains tissue kallikrein, kininases, and

kininogen. The vascular endothelium in the aorta and in the coronary vasculature may be playing an important role in the formation of kinins.

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