THE ROLE OF SERPINS IN THE INHIBITION OF RAT MAST CELL PROTEINASES

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"That but this blow might be the be all and the end all, here but here upon this bank and shoal of time-We'd jump the life to come..."

Declaration.

I declare that this thesis was composed by myself and that the experimental studies reported, except where acknowledged otherwise, are my own. None of the work included in this thesis has been submitted for any other degree or professional qualification.

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ABSTRACT OF THESIS (Regulation 3.5.10)

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Abstract.

Rat Mast Cell Proteinase II (RMCPII) from mucosal mast cell was titrated into rat serum and the resulting serpin:enzyme complex (SEC) was purified by affinity chromatography on anti-RMCPII Sepharose 4B and by MonoQ anion exchange. The purified complex was used to raise polyclonal antibodies which, after cross-absorption against RMCPII Sepharose 4B, were specific for serpin and were used to affinity purify two rat serpin molecules (RSI and RSII) which inhibit RMCPII in rat serum. The kinetic and thermodynamic constants characterising the interaction between RMCPII and RSI and RSII are: k_{ass} 2.2×10^{5} $M^{-1}s^{-1}$ and $1.65\times10^{5}M^{-1}s^{-1}$ respectively; K_i, 3.6×10^{-10}M and 1.0×10^{-9}M; k_{diss}, $7.9\times10^{-5}s^{-1}$ and $1.65\times10^{-4}s^{-1}$. Amino-terminal sequence analysis indicated that RSI and II were distinct, differing at the N-terminal residues and were products of the rat SPI-1 locus. Rat Mast Cell Proteinase I (RMCPI) from connective tissue mast cells cleaved both RSI and RSII and was not inhibited.

Further antibodies were generated against RSI and II, and partially successful attempts were made to raise a monoclonal antibody against rat serpin in complex with Mouse Mast Cell Proteinase Ie. Two polyclonal antibody preparations, raised in rabbit and sheep, were used to develop an ELISA that was specific for rat serpins, although no assay developed in this work would differentiate RSI from RSII. The change in serpin concentrations in serum and perfused tissues during helminth infection was monitored with the ELISA. Serpins concentrations in control plasma were determined to be 3mg/mL. Helminth infection caused a significant ($p<0.001$) increase in this serpin concentration by day 7 of infection. Significant changes in serpin concentration were also monitored in pulmonary, and intestinal homogenates. Differences in timing, duration and intensity of acute phase response in these tissues suggested the existence of tissue specific serpins. Western blotting showed subtle differences in M_r between serpins from different sites. Purification and preliminary characterisation of pulmonary serpins showed that a pulmonary specific serpin does exist.

The implications of these findings, with regard to control of serpin synthesis during acute phase response, are discussed. Theoretical considerations indicate that RSI and RSII will modulate RMCPII activity during helminth infection.

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Chapter 1: Introduction.

1.Introduction

1.1. Proteinases

Proteolytic cleavage of peptide bonds is one of the most frequent and important enzymatic modifications of proteins. Historically, the earliest experimental studies were on the enzymatic degradation of proteins associated with digestion, and for this reason the proteinases of the pancreatic and gastric secretions are among the best characterized enzymes.

Proteinases can be categorized into 4 classes, based on structures and mechanisms of action. These are serine, cysteine, aspartic and metallo-proteinase. There are six families recognised within these 4 classes: serine proteinases (mammalian), serine proteinases (bacterial), metallo-proteinases (mammalian), metallo-proteinaes (bacterial), cysteine proteinases, and aspartic proteinases. Table 1.1 displays an archetype for each family and the characteristic active site residues. The active site is a configuration of functional residues specific to each family. Members of each family are believed to have descended from a common ancestor by divergent evolution.

The catalytic site of each class of proteinases resides in a cleft or in the case of the serine proteinases, with the notable exception of trypsin and chymotrypsin (Fersht, 1984), on the surface of the enzyme molecule. The substrate polypeptide lies along the cleft or surface, and on either side of the catalytic site are specificity subsites adapted to the binding of amino acid side chains, or the polypeptide backbone. The subsites S1, S2 and S3 bind the side chains of residues P1, P2, P3 etc numbered from the scissile bond toward the N-terminus of the substrate, whilst the

Table 1.1

Family	Archetype	Active site residues
Serine Proteinase (mammalian)	**Chymotrypsin** **Trypsin** **Elastase** **Kallikrein**	Asp-102, Ser-195, His-57
Serine Proteinase (Bacterial)	**Subtilisin**	Asp-32, Ser-221, His-64
Cysteine Proteinase	**Papain** **Actinidin** **Rat liver cathepsins B and H**	Cys-25, His-159
Aspartic Proteinase	**Penicillopepsin** **renin**	Asp-33, Asp-213
Metallo-proteinase (mammalian)	**Bovine carboxypeptidase A**	Zn, Glu-270, Tyr-248
Metallo-proteinase (bacterial)	**Thermolysin**	Zn, Glu-143, His-231

From Neurath, 1989

The numbering applies to the proteinase listed in bold in column 2.

sites S1', S2', S3' etc accept side chains of substrate residues P1', P2', P3' etc, numbered from the scissile bond towards the C-terminus of the substrate.

1.1.1. Serine Proteinases

Serine proteinases (mammalian) are the best characterized and physiologically most diversified proteinases in mammals. Members of this family include: trypsin, chymotrypsin and elastase. They are characterised by their active site which is the catalytic triad of Asp-102, His-57 and Ser-195 (chymotrypsin numbering). The overall tertiary conformation is two tightly-packed domains, symmetrically disposed around a two-fold axis of symmetry (Neurath, 1989).

Members of the chymotrypsin archetype family are found in both the animal and plant kingdoms, from prokaryotes to eukaryotes. Most of the serine proteinases in higher mammals are translated as proenzymes. Cleavage is required in the NH_2 region of the proenzyme (normally 220-260 residues from the COOH terminus) to generate active proteinase.

The serine proteinases (bacterial) are exemplified by subtilisin with the catalytic triad Asp-32, His-64 and Ser-221.

1.1.2. Mechanism of Action

The most important residues in the actual proteolysis reaction are the serine and histidine residues. The nucleophilic attack by the serine hydroxyl group on the carbonyl carbon atom of the substrate is catalysed by the histidine residue as a general base. This leads to the formation of a tetrahedral intermediate, and an imidazolium ion (addition reaction). The intermediate breaks down by general acid catalysis to acyl-enzyme, imidazole base and amine product (elimination reaction). As a result of this acylation step, the proton of the serine hydroxyl is transferred

by the imidazole group to the amine leaving group. The acyl-enzyme is hydrolysed through the reverse reaction pathway of acylation, but in this addition elimination reaction a water molecule instead of the serine residue is the attacking nucleophile (reviewed in Polgar, 1990). The members of the subtilisin family act in a similar fashion, the result of convergent evolution (Barrett, 1986).

The specificities of serine proteinases are exceedingly diverse. The P residues on the substrate protein which fit in to the S sites on the active site cleft, rather than the P' residues are important in determining specificity (Barrett, 1986). Trypsin has an overriding requirement that residue P1 must be an arginine or lysine. Other proteinases have evolved a high specificity for subsites S2 and S3, allowing S1 a more general requirement for basic residues (Barret,1986).

1.1.3. Mast Cell Proteinases.

The presence of high concentrations of serine proteinases in secretion granules is a characteristic feature of mast cells (Miller *et al,* 1990). Recent work has shown that individual mast cells may express genes for several proteinases: in man the presence of a multi-gene family encoding tryptases (proteinases with trypsin-like substrate profile) was reported (Vanderslice *et al.*, 199O) and in mouse at least six distinct serine proteinases that can be expressed in different combinations in different mast cell populations, have been identified (Reynolds *et al.*, 1990).

1.1.4. Rat Mast Cell Proteinases

Chymotrypsin-like activity was first detected histochemically in rat mast cells (Benditt and Arase, 1959), and the chymase, rat mast cell proteinase I (RMCP I) was subsequently isolated from peritoneal mast cells (Lagunoff and Pritzl, 1976). Woodbury *et al.* (1978) found that a second similar, yet distinct, proteinase (RMCP

II) first isolated by Katunuma *et al.* (1975) was present in mucosal mast cells (MMC). Recent work by Benfey *et al.*, (1987) has indicated that a third serine proteinase may be expressed in the rat.

RMCP I and II share a high degree of sequence homology (> 70%) but differ in their net charge (LeTrong *et al.*, 1987a) which may reflect different properties and physiological functions. RMCP I is a relatively insoluble protein, requiring a high concentration of salt for extraction and purification (Lagunoff and Pritzl, 1976), whilst RMCP II is readily soluble in physiological conditions (Woodbury and Neurath, 1978). The two rat mast cell proteinases, when isolated, show similarity in their substrate profiles (Yoshida *et al.*, 1980), and exhibit catalytic activity similar to that of cathepsin G. The substrate profile of RMCP I bound to secretory granules is limited to smaller molecules (Le Trong *et al.*, 1987b).

RMCPII is homologous to bovine chymotrypsin A with 33% identity (Woodbury and Neurath, 1978). A Ser(197)-Tyr(198)-Gly(199) sequence corresponds to the Ser(214)-Trp(215)-Gly(216) sequence of chymotrypsin, indicating conservation of the extended substrate binding site in RMCPII. There are, however, a number of changes in the substrate binding site of RMCPII. Asn(86) is substituted for the Ile(99) found in chymotrypsin. In addition, a disulphide bond (Cys(191) to Cys(220)) found in chymotrypsin and all other known serine proteinases (Yoshida *et al*, 1980) is replaced by Phe(178) and by a five residue deletion immediately following Ser(197)-Tyr(198)-Gly(199) in RMCPI. This indicates the possibility of a significant change in the extended substrate binding site of RMCPI, particularly in the site of S3, and may explain the unusual ability of the proteinase to accept a P3 prolyl residue in substrates (Yoshida *et al*, 1980).

The two best artificial substrates used in a study performed by Yoshida *et al* (1978) had a Phe residue in the P3 position. This indicates the presence of an hydrophobic S3 subsite. Although the natural substrate of RMCPII is unknown at present, data would suggest that it has an aromatic residue at P3 and an acidic residue at P4.

RMCPI is more likely to hydrolyze peptide bonds that have hydrophobic or non-polar residues at the P2 and P'1 positions, in addition to an hydrophobic residue at P1 (LeTrong *et al*, 1987b).

1.2. Mast Cells

1.2.1. History

The mast cell, first recognised over 100 years ago (Ehrlich, 1878; reviewed in Galli, 1990), participates in a variety of pathological processes including allergic reactions, and probably plays a fundamental role in the host defence against parasite infection.

The first clear description of the mast cell was made by Paul Ehrlich while he was a medical student. The mast cell was noted to be a deeply staining metachromatic cell which Ehrlich considered to be overfed. Therefore the use of the word mast derived from the German "Mästung", which means feeding or fatness.

The search for a physiologic function for the mast cell was stimulated by the observation, made by Webb in 1931 (reviewed in Metcalfe *et al*, 1981) that the intraperitoneal injection of various irritants, including india ink and egg white, induced mast cell degranulation. At the same time Jorpes *et al* (1937; reviewed in Metcalfe *et al*, 1981) discovered that heparin was the constituent which imparted the metachromatic staining property to the mast cell.

1.2.2. Morphology and Distribution.

In tissues the mammalian mast cell is generally ovoid and is found in the vicinity of small blood vessels and lymphatic channels. Isolated rat peritoneal mast cells in suspension average 12.6μM in diameter (Benditt and Lagunoff, 1964; reviewed in Metcalfe *et al*, 1981). The characteristic feature of these cells is the presence of dense cytoplasmic granules which occupy the cytoplasm to such a degree as to obscure the nucleus. In most mammals the granules are 0.2-0.4μM in diameter. In warm blooded vertebrates the cells predominate in the loose connective tissue surrounding blood vessels, nerves, glandular ducts and under epithelial, serous and synovial membranes (Compton, 1952; reviewed in Metcalfe *et al*, 1981). In human tissues the mast cell is relatively abundant in skin, thymus, lymphoid tissue, uterus, urinary bladder, tongue, synovia, mesentery around large and small blood vessels and in subserosal and submucosal layers of the digestive tract. In the lung, mast cells are found in both the bronchial airway connective tissues and in peripheral intraalveolar spaces.

1.2.3. Heterogeneity of Mast Cells

The extent to which a given cell type can exhibit different characteristics or functions before being considered to be heterogeneous is perhaps debatable, since some diversity may be regarded as normal. However, mast cells show considerable variation in their histochemical and biochemical properties, as well as in their functional response to a variety of stimuli. Hardy and Wesbrook are usually credited with the first observation that certain mast cells in the rat intestinal mucosa were "atypical" in their histochemical staining properties in (see Galli, 1990). Beginning in the 1960's, Enerback (1981) greatly extended the early observations of

mast cell heterogeneity and defined in detail the conditions of fixation and histochemical staining which discriminated between the "atypical" or mucosal mast cell observed in the intestinal lamina propria and the connective tissue type mast cell phenotype of the skin, peritoneal cavity and other sites in the rat. The heterogeneity of mast cell populations has been further studied by the biochemical analysis of their granule constituents. The granule proteoglycans consist of a protein core with covalently linked glycosaminoglycan side chains which are highly negatively charged due to the large numbers of sulphate and carboxylic groups (Yurt and Austen, 1977). That there is proteoglycan heterogeneity amongst sub-populations of rat mast cells was confirmed when ^{35}S labelled proteoglycan was purified from isolated rat intestinal mast cells (Stevens *et al*, 1986). The glycosaminoglycan side-chains were typical of chondroitin sulphate di-B and chondroitin sulphate A, which are not present in the glycosaminoglycans of heparin in serosal mast cells; the latter are considered to be analogous to connective tissue mast cells (Stevens *et al*, 1986). However the distinction between rat mucosal and serosal mast cells as determined by proteoglycan content is not absolute, since trace amounts of chondroitin sulphate E are present in isolated rat mucosal mast cells (Stevens *et al*, 1986).

Perhaps the most convincing evidence for the presence of different mast cell populations has come from studies on their granule proteinases. In the rat, two chymotrypsin-like mast cell proteinases have been isolated and termed rat mast cell proteinase I (RMCPI) and RMCPII (Woodbury and Neurath, 1980; Katunuma *et al*, 1975, Woodbury *et al*, 1978). The two serine proteinases can be distinguished on the basis of physical, chemical, structural and immunological properties (Woodbury

and Neurath, 1978a;Woodbury *et al*, 1978). More recently, the gene encoding RMCPII has been cloned and sequenced (Benfey *et al*, 1987) and sequence analysis together with studies on the cellular distribution of RMCPII mRNA has indicated that RMCPI and RMCPII are likely to be coded by separate, highly homologous genes in separate cell populations. Further immuno- histochemical analysis indicated that mast cells from connective tissue and from mucosa represented two major non-overlapping populations of mast cells, which contained RMCPI and RMCPII respectively (Gibson *et al*, 1987). However, tissue site may not be a reliable indicator of mast cell sub-types, since significant quantities of RMCPII and RMCPII-containing cells have been demonstrated in several non-mucosal tissues including liver and thymus (Huntley *et al* 1990a). Moreover, a minor population of mast cells expressing both RMCPI and RMCPII have been demonstrated in lung and liver, indicating further heterogeneity in mast cell phenotypes (Huntley *et al*, 1990a).

Mast cell proteinases have also been characterised in other species including man (Schechter *et al* 1986), sheep (Huntley *et al*, 1986) and mouse (Newlands *et al*, 1987). In addition to chymases, mast cell trypsin-like enzymes have been isolated from rat serosal mast cells (Kido *et al*, 1984), dog skin and mastocytoma tissues (Schechter *et al*, 1988; Caughey *et al*, 1988) and from human lung (Schwartz *et al*, 1981). Heterogeneity amongst human mast cell sub-populations has been defined according to the distribution of chymase and tryptase (Schwartz, 1989), where the majority of the mast cells in the submucosa of the small intestine and skin contain both tryptase and chymase, and the majority of the mast cells in the mucosa of the

small intestine, bronchial epithelium and lung alveoli contain tryptase alone (Schwartz, 1989).

1.2.4. Origin.

Mammalian mast cells are derived from precursors which originate in the bone marrow. This point was established by Kitamura *et al* (1978) in a series of papers describing experiments exploiting mutant mice genetically deficient of mast cells, and their congenic normal (+/+) litter mates (reviewed in Galli, 1990). A double dose of mutant genes at either the W or Sl locus of the mouse produces the pleiotropic effects of macrocytic anaemia, sterility and lack of hair pigmentation. Evidence (see Galli, 1990) indicated that $WBB6F_1$- W/W^v and $WCB6F_1$-Sl/Sl^d mice also expressed a deficiency of mast cells. However, $WBB6F_1$- W/W^v mice can develop mast cell populations if they receive bone marrow cells derived from their normal littermates or from "beige" mice. The mast cells of "beige" mice are easily distinguishable, because of the presence of giant cytoplasmic granules allowing identification of the cells as of donor origin. The deficiency of the $WCB6F_1$-Sl/Sl^d is to due to an abnormality of tissue factors regulating mast cell development, as an injection of $WCB6F_1$-Sl/Sl^d bone marrow cells cures the deficiency of $WBB6F_1$-W/W^v mice. Recent evidence has shown that Sl/Sl^d mice are deficient in stem cell factor, whereas W/W^v mice lack a functional c-kit receptor for the ligand, stem cell factor (Geissler *et al*, 1988).

Mouse bone marrow cells, cultured for 2-6 weeks in a source of conditioned medium rich in interleukin-3 (IL-3), a 25,000 Mr glycoprotein of helper T-cell origin, develop into an apparently homogeneous population of non-transformed mast cells (Razin *et al*, 1984). Recombinant IL-3, given to mice *in vivo* induces

proliferation of multiple haematopoietic lineages, including certain populations of cells similar to mucosal mast cells (Metcalf *et al*, 1986). Administration of IL-3 to nude mice confers on these animals the ability to exhibit mucosal mast cell proliferation in the absence of parasite infection (Abe *et al*, 1988).

The generation of mucosal mast cells is stimulated *in vitro* by factors derived from T-cells of helminth-infected rats (Haig *et al*, 1982). Conditioned media capable of causing the proliferation of mast cells were derived from cultures of antigen-stimulated mesenteric lymph nodes of rats infected with the nematode *Nippostrongylus brasiliensis* (Haig *et al*, 1982).

While the effects of T-cell products on mast cell recruitment and proliferation so far have been in terms of mucosal mast cells, there are also recorded effects on connective tissue mast cells, albeit of a more modest nature. IL-4 favours the *in vitro* growth and maturation of mouse mast cells with properties of connective tissue mast cells and augments the effects of IL-3 with regard to mucosal mast cells (see Galli, 1990).

The mRNA levels of a number of cytokines have been reported to be increased in mouse mast cells after IgE receptor-antigen cross linking events . These include IL-1. IL-3, IL-4, IL-5, IL-6, and TNF-α (see Gurish *et al*, 1991). TNF-α is constitutively produced by mouse mast cells but increased expression is obtained after activation (Gordon and Galli, 1990). The levels of TNF-α mRNA and IL-6 mRNA increase $>$20-fold 1 hour after activation (Gurish *et al*, 1991). Stem cell factor also appears to have a role to play in the recruitment and proliferation of mast cells, given the crucial role of the receptor for this ligand in some strains of

mice (*vide supra*). Other cytokines implicated in recruitment and growth of mast cells include IL-9, IL-10 and nerve growth factor (Woodbury et al, 1984).

1.2.5. Mast Cells and Mast cell Proteinases during parasite infection.

Among the events associated with the expulsion of nematode parasites from the gastrointestinal tract, is the extensive accumulation of mast cells in the infected tissues (Askenase, 1980; Jarrett and Miller, 1982). This phenomenon was first examined in detail by Taliaferro and Sarles in 1939 (see Miller *et al*, 1986). They noted the massive accumulation in the intestinal lamina propria of what they called connective tissue basophils which arose by differentiation and division from a stromal cell population. They also described the appearance of a cell type containing eosinophilic granules, the globule leucocyte. The populations of these cells increased even more rapidly after a second infection, with cell division occurring in both cell types.

Further studies of the histochemistry and ultrastructure of parasitized intestine led to the conclusion that the globule leucocytes were in fact mast cells; they contained proteoglycan, monoamines and basic protein (see Miller *et al*, 1986).

In the 1960's these observations were confirmed and extended. Miller and Jarrett (1971) revealed that the population of enteric mast cells expanded exponentially during primary infection with *N. brasiliensis* (see Miller *et al*, 1986), and at the same time their granules became depleted of proteoglycan (Miller and Walshaw, 1972).

Nawa and Miller (1977) described an accelerated intestinal mast cell response in infected rats adoptively immunized with putative T-cells separated from immune thoracic duct lymphocytes. The role of recirculating T-lymphocyte in mast cell

hyperplasia in parasitized gut was further confirmed in adult thymectomized, irradiated rats to which bone marrow had been restored (Mayrhoffer, 1979).

1.2.6. Function of Mast Cells

Mast cells reside in most of the major organs and in view of this ubiquity and their heterogeneity it is likely that they play important but diverse roles in the biological processes of the host.

In man perhaps the most widely known and striking activity involving these cells is their participation in inflammatory reactions,where release of potent mediators may give rise to immediate hypersensitivity reactions (Huntley, 1990). The evidence for a role in hypersensitivity reactions exists (Galli *et al*, 1990), the precise nature of the role is obscure.

1.2.7. The mast cell and IgE

The fundamental immunologic component of allergy (immediate hypersensitivity) is the production of an immunoglobulin of the ε class. IgE is a typical monomeric immunoglobulin consisting of two heavy and two light chains, the light chains are of either the κ or λ type. The heavy chains of IgE contain specific antigenic determinants which clearly distinguish IgE from other immunoglobulins. In addition IgE has a higher M_r than other monomeric immunoglobulins.

The IgE molecule, like all antibodies, has two regions; Fc and Fab. The Fc region of IgE molecules binds with unusually high affinity ($K_a = 10^{10}$ litres/mole) to a class of Fc receptors located on the surface of mast cells. The receptors are large molecules, composed of three types of domains or sub-units; α (M_r 37,000), β (M_r 33,000) and γ (M_r 7,000) (see Metzger and Kinet, 1988). The sub-units are in a 1

(α):1 (β):2 (γ) ratio. The α sub-unit is solely responsible for the binding of the IgE Fc region. Analysis of the cDNA for the α sub-unit shows a 227 residue molecule, with a 27 residue transmembrane segment and an anchoring cytoplasmic tail (Kinet *et al*, 1987). There are 7 potential glycosylation sites, and the α sub-unit is known to be 30% carbohydrate. The carbohydrate is not thought to play a part in the binding.

The reaction of binding is a simple bimolecular one

$$IgE(Fc) + Receptor \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} IgE(Fc):Receptor$$

with $k_1 = 1\text{-}2\text{x}10^5\ M^{-1}\ s^{-1}$ and $k_{-1} \leq 1\text{x}10^{-5}\ s^{-1}$. No co-factors are required for this interaction (Metzger *et al*, 1986).

The bound IgE molecules serve in their turn as receptors for antigens. The binding of the IgE alone is not a sufficient requirement for degranulation, a multivalent antigen must bind to more than one IgE bound to receptors, causing cross-linking and aggregation of receptors (see Metzger and Kinet, 1988). Dimer aggregates are sufficient to stimulate degranulation, although greater aggregations will provide a better stimulus for degranulation. Thus the IgE molecule determines which antigen can stimulate the mast cell, but plays no mechanistic role in degranulation. Degranulation releases a variety of biologically active amines such as serotonin and histamine. These amines are vaso-active and are implicated in the clinical manifestations of allergic reactions such as asthma and hay fever. It is believed that these vaso-active molecules assist blood components gaining access to sites of infection.

1.2.8. Anaphylactic release of Mucosal Mast Cell Proteinase and its relationship to Gut permeability in rat primed with *N. brasiliensis*.

A major consequence of helminth infection is the development of high titres of parasite specific, and non-specific IgE (Jarrett and Miller, 1982), although little parasite specific IgE is detectable in the early stages of infection with N. brasiliensis. The precise role of IgE in the general phenomenon of mast cell recruitment and proteinase release during helminth infection is as yet unclear. However, rats sensitized to egg albumin develop an IgE mediated reaction to subsequent intraluminal challenge, which results in abnormalities of water and electrolyte absorbtion (Perdue *et al*, 1984).

Experiments have indicated that the proliferation of mucosal mast cells was co-incident with the systemic release of RMCPII (Woodbury *et al*, 1984). Immune rats injected i.v. with soluble worm antigen had a 5-fold increase in serum RMCPII levels between 5 and 60 minutes after challenge (King and Miller, 1984). The secretory response in primed rats was both time and dose dependent, whereas no RMCPII was detected in naive rats. RMCPII was found in large amounts in the gut lumen in these experiments and the release of RMCPII was associated with very substantial changes in gut permeability. Immune rats that are challenged with soluble worm antigen show an accumulation of Evans blue in the gut lumen (King and Miller, 1984), this dye is bound to albumin and thus provides a measure of protein leakage into the gut from the epithelial and vascular systems (Rawson, 1949; see King and Miller, 1984).

Studies by Patrick *et al* (1988) have demonstrated that RMCPII is associated with the changes in the ultrastructure of the intestinal mucosa. These changes were

most noticeable in the basement membrane and underlying collagenous matrix of the intestinal mucosa.

Analysis of the distribution of RMCPII in rat infected with *N. brasiliensis* has shown that the vast bulk of this proteinase is of intestinal origin (upto 22mg/g in the proximal jejunum, Miller *et al*, 1986). Other notable sources of RMCPII are the gastric, ileal, and colonic mucosae and the immune lung.

It has also been demonstrated that the proliferation of mucosal mast cells and the release of RMCPII is co-incident with the expulsion of nematode parasites such as *N. brasiliensis* and *T. spiralis*, confirming that mucosal mast cells are functionally active during the elimination of primary nematode infections (Woodbury *et al*, 1984). The high concentrations of RMCPII found in intestinal perfusates of rats primed with *N. brasiliensis* after challenge intravenously with worm antigen (King *et al*, 1985), again indicates the potential activity of the mucosal mast cell in helminth infection.

Systemic release of mast cell proteinases also occurs in sheep following nematode infection (Huntley *et al*, 1987). Increased concentrations of sheep mast cell proteinase (SMCP) were detected in the gut mucosa of animals infected with the parasite *Haemonchus contortus*. Detection of SMCP in serum was frustrated, possibly due to the presence of proteinase inhibitors which interfere with the ELISA developed to assay sheep tissues and serum for the enzyme. Huntley *et al* (1990) have also demonstrated the systemic release of mouse mast cell proteinase I (MMCP-I) in mice infected with *T. spiralis*.

1.2.9. Biological Functions of Rat Mast Cell Proteinases.

There appear to be two principal roles for mast cell proteinases: regulation of mast cell secretion and proteolysis within the granule matrix and in the surrounding milieu after degranulation.

Non-serpin serine proteinase inhibitors, and substrates of chymases inhibit phospholipid methylation and inhibit Ca^{2+} influx into mast cells and histamine release by mast cells (see Katunuma and Kido, 1988). These findings suggest that serine proteinases in mast cells are activated by IgE- receptor bridging and play some role in triggering the process of degranulation. Mast cell granule proteinase may be involved in the process of IgE-mediated degranulation before a step of calcium entry (see Katunuma and Kido, 1988). The release of histamine from mast cells can also be prevented by inhibitors of metallo-proteinases, such as 1 10-o-phenanthroline; these results suggest that another proteinase, a metallo-proteinase, may be implicated in the degranulation process but after a calcium entry step (see Katunuma and Kido, 1988). Thus, histamine release induced by a calcium ionophore (A23187) is blocked by inhibitors of metallo-proteinase but not by F(ab')2 of antichymase. Histamine release can be inhibited *ex vivo* by inhibitors of mast cell proteinase (Dietze *et al*, 1990). The conclusion is that serine proteinases and metallo-proteinases play some common role in exocytosis.

Exogenous addition of purified RMCPI results in the degranulation of connective tissue mast cells (CTMC) (Schick, 1990); experiments involving radio-labelling of mast cell membrane components showed that a molecule (Mr 90,000) disappeared as RMCPI was added to mast cells (Schick, 1990). Loss of this band due to trypsin pre-treatment resulted in de-sensitization to subsequent

activation by mast cell proteinase, suggesting that this membrane component is involved in degranulation of CTMC by mast cell proteinases.

Little is known about the function of the mast cell proteinases released from rat mast cells *in vivo*. It has been demonstrated that they degrade type IV collagen and fibronectin, but not types I, II, or III collagen *in vitro* (Sage *et al*, 1979; Katunuma and Kido, 1988). The products of digestion of IgG by rat mast cell proteinase have potent chemotactic activity on neutrophil leucocytes *in vitro* and *in vivo*, whereas IgG itself has little chemotactic activity (Katunuma *et al*, 1986) suggesting that released mast cell proteinase may cause limited hydrolysis of IgG and produce the chemotactic factors.

RMCPII may be involved in the events leading to changes in gut permeability. By degrading the collagen in basement membrane, the mast cell proteinase may have a direct role to play in mediating permeability changes (Patrick *et al*, 1988; King and Miller, 1984). RMCPI may have a role in activating matrix metallo-proteinases (MMP) such as collagenase and stromelysin (David Woolley, personal communication). These metallo-proteinases are present in the form of zymogens and need to be activated by proteolysis for the full extent of their activity to become manifest.

1.3. Serpins

1.3.1. General

The plasma proteinase inhibitors constitute by weight the third largest group of proteins in plasma, after albumin and the immunoglobulins (Travis and Salvesen, 1983). Representing nearly 10% of total protein in plasma, they control a variety of critical events associated with connective tissue turnover, fibrinolysis, complement activation, and inflammatory reactions.

The existence of proteinase inhibitor activity in human plasma was apparently first noted by Fermi and Pernossi in 1897. Since that time a host of investigations has been made to determine the various inhibitory activities in plasma, primarily by adding proteinases of varying specificities and catalytic mechanisms to plasma and plasma fractions.

Apart from α-2-macroglobulin, which seems to function as a back-up proteinase inhibitor (Travis and Salvesen, 1983) and inhibits proteinases from several families, the most rigorously studied of the proteinase inhibitors is α-1-Proteinase Inhibitor (α-1-PI, also known as α-1-antitrypsin in man) which is the archetypal SERine Proteinase INhibitor (serpin). This is a name given to one class of proteins which inhibit serine proteinases specifically. This class of proteinase inhibitors includes antithrombin III, α-1-antichymotrypsin, α-2-antiplasmin and C1-inhibitor. These molecules all share a similarity in structure which determines their similarity in function as will be outlined below. Some other proteins are also known to have structural analogies to the serpins, although they have no known inhibitory function. Proteins such as ovalbumin (Stein *et al*, 1990), hormone binding proteins

(Pemberton *et al*, 1988) and beta-glucuronidase (Li *et al*, 1990) belong to the serpin superfamily (Hunt and Dayhoff, 1980).

The serpins all have the same mechanism of inhibition: they form 1:1 molar complexes with their target proteinase involving a reaction between the **reactive site loop** of the serpin and the active site cleft of the serine proteinase. A peptide bond in the reactive site loop is cleaved and typically the bond is X-Ser. This bond is found between the methionine at position 358 and the serine at position 359 in α-1-PI and these residues are termed the P1 and P1' residues respectively. The exact nature of the association between the serpin and proteinase is unclear (Travis and Salvesen, 1983).

There are other proteinase inhibitors which are class-specific, such as metallo-proteinase inhibitors, and cysteine proteinase inhibitors. No class specific plasma inhibitor of the aspartic proteinases is known.

1.3.2. Structure and Function

1.3.2.1. Primary Sequence and conformation.

Serpins contain approximately 400 amino acids residues (Travis and Salvesen, 1983). Attempts to crystallize native serpins in order to elucidate structure/function relationships have been unsuccessful. However, cleaved serpin has been crystallized (Loebermann *et al*, 1984). The overall dimensions of cleaved human α-1-PI delineate an asymmetrical molecule, 67Å x 32Å

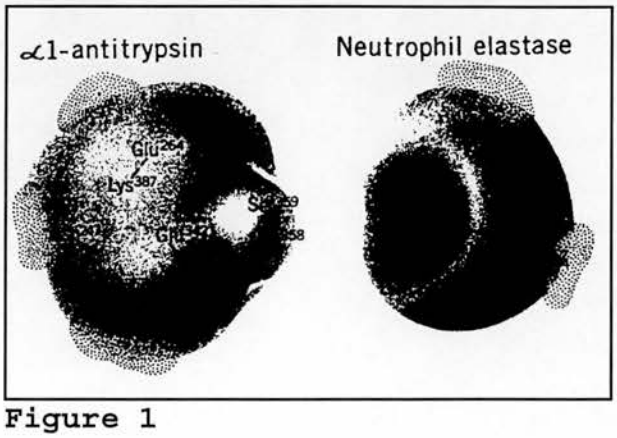


Figure 1

x 32Å, and the cleaved molecule is composed of three β-pleated sheets and 8 α-helices; the helices are formed by the first 150 residues (Loebermann *et al*, 1984). Analysis of the crystal structure of cleaved α-1-PI reveals that the two generated termini (Met 358 and Ser 359) are 70Å apart (Loebermann *et al*, 1984) indicating a major structural rearrangement of the molecule on cleavage. This observation has led to the hypothesis that native serpin is in a "stressed " form, whilst cleaved serpin is in a "relaxed" form (Carrell *et al*, 1987). To reconstruct the native inhibitor by re-forming the Met-Ser bond, it is necessary to withdraw a central strand from the major pleated sheet of the molecule to form a stretched loop of some 16 residues. This exposed and stretched loop places the reactive centre in an accessible position with probable distortion of the Met-Ser bond. It is likely that in this way the target proteinase of a serpin is presented with an ideal substrate that fits precisely into its active site cleft to give stable complex formation between enzyme and inhibitor (Carrell *et al*, 1987; see Fig 1). The structural rearrangement may well have implications for further functional activity of the complex.

Ovalbumin, although devoid of inhibitory properties, has structural similarities to the serpins which suggest that it may be part of the superfamily of serpins. Stein *et al* (1990), using crystallised ovalbumin as a model, have suggested that the protruding loop takes the form of an α-helix. This structure has 3 turns, and the putative reactive centre (Ala 358-Ser 359) is exposed on the final turn. The loop protrudes from the main body of the protein on two stalks, each 4 residues in length. The loop appears to have a measure of mobility, which may be important if it is to present itself as a substrate to a proteinase. The mobility is due to the sequence of the stalk leading from the β-sheet to the α-helix of the loop. The

primary sequence of the residues leading up to P1 and away from P1' seems to be important for the correct functioning of the serpin, and changes to these residues can have pathological implications (section 1.3.5). Evidence to support this hypothesis comes from the work of Lawrence *et al* (1990) who replaced the wild type loop Plasminogen Activator Inhibitor-1 (PAI-1) with a 19 residue region from PAI-2, AT-III, or an artificial serpin consensus loop. The chimeric proteins inhibited urokinase type plasminogen activator with k_{ass}'s similar to that of PAI-1, even though native PAI-2 has a specific activity 200-fold higher for this proteinase, indicating that structures outside the stressed loop are responsible for the major differences in specificity between PAI-1 and PAI-2.

Although there is not much doubt that the reactive site loop plays a vital role in the inhibition of serine proteinases, there may be a much more fundamental level of interaction between the serpin and proteinase. Tissue plasminogen activator (t-PA) contains an insertion of seven amino-acids (residues 296-302) that are predicted to form a loop adjacent to the active site of the enzyme. The basic and charged nature of the amino-acids in this loop (Lys-His-Arg-Arg-Ser-Pro-Gly) suggests that electrostatic interactions could contribute to the interaction between t-PA and PAI-1. Deletion of the entire loop or replacement of Arg 304, which is predicted to be located at the edge of the active site of t-PA, yield variants that are resistant to inhibition by PAI-1 and whose ability to catalyse the activation of plasminogen is essentially undiminished (Madison *et al*, 1990). These mutants are also resistant to inhibition by the complex mixture of serpins present in human plasma (Madison *et al*, 1989).

1.3.2.2. Interaction of serpins with factors other than proteinases.

PAI-1 binds to the extracellular matrix of cultured bovine aortic endothelial cells (Mimuro and Loskutoff, 1989). Bovine plasma and lung extract contain protein(s) that bind to PAI-1 and prevent this interaction. One of these proteins was purified and was found to be vitronectin, suggesting that native PAI-1 circulates in complex with vitronectin, a suggestion supported by the observation that all detectable active PAI-1 in plasma migrates in complex with another molecule, when analysed by gel filtration under non-dissociating conditions (Erickson *et al*, 1986). It has been suggested that this interaction stabilizes the serpin (Mimuro and Loskutoff, 1989), or localises and concentrates the PAI-1 in tissues where it is required.

Interaction betwen PAI-1 and vitronectin can change the specificity of the serpin (Ehrlich *et al*, 1990) and increases the efficiency of inhibition of thrombin by PAI-1. Fibrinogen and heparin also enhance the rate of inhibition of t-PA by PAI-1 (Edelberg *et al*, 1991) Kinetic studies indicate that both fibrinogen and heparin increase the second order inhibition rate constant by a maximum of four fold, whereas vitronectin increases the constant by six fold. The data suggest that heparin and fibrinogen may enhance the rate of inhibition by interacting with the proteinase and that vitronectin may enhance the inhibition by an interaction with the serpin. However there is evidence that a group of serpins are heparin dependant; this group includes antithrombin III, heparin cofactor II and protease nexin I (Ehrlich *et al*, 1991).

1.3.2.3. Glycosylation.

Members of the serpin superfamily have molecular masses ranging from 45,000 to 70,000. Much of the variation in mass can be explained by post-translational events such as glycosylation. It must also be remembered that SDS-PAGE over-estimates the M_r of glycoproteins. Several investigators examined the polymorphism in glycosylation of human α-1-PI responsible for 12% of the mass of the inhibitor. It was first reported that the protein contained two distinct sets of oligosaccharide chains attached as two sets to four positions in the protein (Chan, S. K., *et al*, 1976). However others disputed this and suggested that the inhibitor contained four attachment sites for three or four types of oligosaccharide chain (Roll *et al*, 1978). Later two types of carbohydrate chain were discovered in human α-1-PI (Hodges *et al*, 1979). The original hypothesis of four oligosaccharide chains was re-examined (Mega *et al*, 1980a; Mega *et al*,1980b) and three carbohydrate side chains in α-1-PI in positions 46, 83 and 247, numbering from the amino-terminus of the polypeptide were found. However the heterogeneity associated with individual forms of α-1-PI was later found to be the result of variations in the quantity of each of the two forms of chain (Mega *et al*, 1980b). Vaughn *et al* (1982) showed three classes of α-1-PI: class 1 had 3 biantennary oligosaccharide chains, class II had 2 biantennary and one triantennary oligosaccharide chains and class II had one bi- and two triantennary chains. The carbohydrate side chains of the major plasma serpins of the horse have been characterised by lectin analyses of protein blots from two-dimensional gels using the major plasma serpin, α-1-PI as a control (Patterson and Bell, 1990). Equine proteins seem to consist of partially sialylated

biantennary side chains, except for the most acidic proteins which have triantennary side chains.

The role of the carbohydrate in inhibitor function has not been unequivocally established, although all plasma serpins are exported from their source tissue, and glycosylation is very important in regulating secretion of proteins (West, 1986). It may also have a role to play in stability of the serpins in plasma; the half life of recombinant α-1-PI (unglycosylated) is much lower than the native glycosylated form (Mast *et al*, 1990).

1.3.3. Genetics

In the human genome there are single genes for both α-1-PI and α-1-antichymotrypsin. There is a putative pseudogene down stream from the α-1-PI gene, no transcripts from the pseudogene have been found. The α-1-PI and α-1-antichymotrypsin genes reside on chromosome 14 and are linked to the immunoglobulin heavy chain region (Inglis and Hill, 1990). These two serpin gene loci are within 120kb of each other. The α-1-PI gene has 5 exons dispersed over 12.2kb (Crystal, 1989), exons II-V code for the protein. Exon I has 3 components, which are responsible for the tissue-specific expression of the protein (see figure 2). Most hepatocyte mRNA transcripts begin in the middle of exon Ic, while macrophage transcripts begin at exon Ia (Long *et al*, 1984; Perlino *et al*, 1987). Two of the three glycosylation sites are encoded in exon II, the other is on exon III, and the reactive site loop is encoded on exon V. Exon II contains a

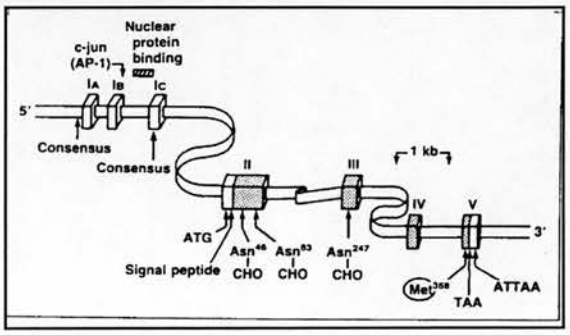


Figure 2

signal peptide of 24 residues (Brantly *et al*, 1988) to allow transport across the endoplasmic reticulum, followed by subsequent processing in the Golgi prior to secretion into the bloodstream. The gene structure for α-1-antichymotrypsin is similar to that for α-1-PI. The gene spans 10kb and contains four exons, exon II is extremely similar to exon II of α-1-PI, both contain the AUG initiation codon. However exons III and IV of the α-1-PI gene are represented by exon III of the α-1-antichymotrypsin gene. The intron structure of the two genes is radically dissimilar (Kidd and Woo, 1986).

In the mouse genome the primordial α-1-PI and α-1-antichymotrypsin genes have undergone an extensive series of duplication events (Hill *et al*, 1985). Approximately 12 closely related genes whose proteins have α-1-antichymotrypsin as their closest human relative have been identified. By the same token the α-1-PI gene has multiplied in the mouse and there appear to be 6-8 genes (Inglis and Hill, 1990). These genes are found in two clusters,the SPI-1 and -II loci. SPI-1 contains genes whose products are structurally homologous to human α-1-PI, and SPI-II contains genes which code for proteins which are the structural homologues of α-1-antichymotrypsin. The presence of antichymotrypsin activity in the mouse has yet to be shown, and gene products from the SPI-II locus (known as contrapsins) are known to have antitryptic activity (Inglis and Hill, 1990). The α-1-PI genes in the mouse are 10kb long (Krauter *et al*, 1986) and contain 5 exons.

The rat genome also contains multiple copies of genes on both the SPI-1 and -II loci (Inglis and Hill, 1990). There are 4-5 genes on each cluster in the rat, the overall picture being less clear than in the mouse genome.

1.3.4. Evolution

The serpins provide an intriguing model of the way in which evolution can provide a range of functions within one superfamily. The serpins exhibit a high degree of homology (>95%, Borriello and Krauter, 1991) within gene clusters, and to a lesser extent between clusters. Related members such as α-1-antichymotrypsin and antithrombin III have >40% homology at the nucleotide level (Kidd and Woo, 1986). Dot matrix analysis of the two sequences has shown that there is greater homology between the amino-terminal than between the carboxyl terminal portions of the proteins (Kidd and Woo, 1986). Murine α-1-PI and contrapsin share 59% and 44% homology at the genomic and amino acid level respectively (Hill *et al*, 1984), suggesting that the two proteins diverged from a common ancestral gene 200-300 million years ago.

The similarity between serpins extends across the species barrier. Hill *et al* (1984) showed that, over the region of the gene analyzed, primate and murine α-1-PI shared 70% homology at the genomic level and 60% at the amino acid level. Thus serpins are highly related to each other. It should be noted however that when serpins do diverge, it is most noticeable in the reactive site loop of the molecule. This, as discussed, is the region of the protein which confers specificity for target proteinase. However a common feature to all serpins is the stressed conformation of the native molecule, necessitated by the requirement for the loop to be extruded from the main body of the serpin. Thus the loop structure is preserved even though the P1 and P1' residues have diverged considerably even between genes on the same cluster (Borriello and Krauter, 1991).

Further examples of polymorphism were demonstrated by Potempa *et al*, (1991) in the horse. Three structurally related but functionally different serpins from horse plasma were isolated and characterised. In spite of similarities in N-terminal sequences, the reactive site loops of these proteins showed extensive variation. Only what they have termed Inhibitor I, with a P1 methionine residue, resembles human α-1-PI with regard to specificity and reactive site loop sequence. Inhibitors II and III are reported to have an arginine and alanine residue respectively at the P1 site, markedly altering specificity, susceptibility to oxidation and k_{ass}.

When comparing reactive site loop sequences of serpins within and across species (Hill and Hastie, 1987; Carrell *et al*, 1987), and also noting the chromosomal arrangement of the relevant genes (Inglis and Hill, 1990) it is compelling to hypothesise a series of duplication and divergence events. Such an hypothesis has been postulated (Inglis and Hill, 1990). Duplication events, arising from unequal crossing-over, would relieve the selection pressure on a single copy of a gene, allowing the duplicate to diverge, randomly, to an alternative form of the protein, which may or may not have a role to play in the physiology of the animal. The presence of pseudogenes at the serpin loci in man, and the abundant polymorphism exhibited by lower mammals (Potempa *et al*, 1991; Pirie-Shepherd *et al*, 1991; Inglis and Hill, 1990; Borriello and Krauter, 1990) bears witness to the validity of this hypothesis. Hill and Hastie (1987) have suggested that the selection pressure behind such evolution is the presence of extrinsic proteinases, namely those used by parasites to facilitate their spread through the host. However this hypothesis, although attractive in many ways, does not explain the lack of variety in man. As a species man is just as susceptible to a wide range of parasites as all lower

mammals. However, parasites may have a tangential role in the rapid evolution of serpins. It is known that parasites elicit a response in the host which includes the increased concentration of free mast cell serine proteinases in a variety of tissues and plasma, while many of the proteinases ar normally at extremely low concentrations normally (Miller *et al*, 1983; Huntley *et al*, 1987; Miller *et al*, 1986; Gustowska *et al*, 1983). The need to modulate the increased concentrations of mast cell serine proteinases found in plasma and tissues (Miller *et al*, 1986) which could potentially damage the host may be a sufficient selection pressure to drive evolution in the direction of diversity. The resulting variation would be partly dependent on the nature of the host response, as each species adapts to its own intrinsic response. However at the present time there is not enough evidence to support either hypothesis.

1.3.5. Pathology

Although there are only single known actively-transcribed genes for α-1-PI and α-1-antichymotrpsin in man there are a number of allelic variations present within the population which can give rise to a plethora of pathological conditions.

1.3.5.1. α-1-PI deficiency.

The most obvious causes of disease are deficiency states of the anti-elastase function of α-1-PI. The types of deficiency fall into 3 subgroups; "at risk", "null" and "change of function". Deficiency of α-1-PI is one of the most common lethal hereditary disorders of Caucasians of European descent (Crystal, 1989). In normal individuals more than 2g of α-1-PI are produced daily, the protein has an half-life of 4-5 days in blood and serum levels are 25-30uM (Cox, 1989). Concentrations in

the lung are approximately 10-20uM and in the alveolar epithelial lining are 2-5uM (Wewers, 1987; Gadek *et al*, 1981).

The normal lung is constantly exposed to foreign particles which may stimulate neutrophils to release elastase. When concentrations of α-1-PI are normal, the amount of α-1-PI in the lower respiratory tract is in excess of this neutrophil elastase burden, so protecting the lung. In contrast, in α-1-PI deficiency, the reduced concentration in plasma, and hence the lung, do not provide an adequate shield against neutrophil elastase, exposing the alveolar walls to chronic proteolytic attack (Crystal, 1989). If the individual with α-1-PI deficiency smokes, the defences of the alveoli are further compromised because the Met-358 residue at the P1 site is vulnerable to oxidation by free radicals in cigarette smoke and also by those released by inflammatory cells recruited to the lung in response to the smoke (Janoff, 1985). When the Met-358 is oxidised, the inhibitory capacity of α-1-PI is decreased markedly (Travis and Salvesen, 1983), so smoking exaggerates the deficiency of α-1-PI by reducing the number of functional molecules available to protect the lung.

The association between a deficiency of α-1-PI and human disease was first observed by Laurell and Eriksson in 1963. Emphysema, the most common manifestation of α-1-PI deficiency becomes evident by the third or fourth decade. Smoking cigarettes accelerates the disease, hastening death from respiratory failure by 10-15 years.

The α-1-PI gene is pleiomorphic, with approximately 75 alleles identified (Cox, 1989). The allelic nomenclature is based on letter assignments corresponding to the position of migration of the protein on iso-electric focusing. Approximately 90-95% of all α-1-PI alleles evaluated in a given population are "normal" in that

functionality and serum concentration are unaffected. Of the 45 known normal alleles, 10 have been sequenced (Crystal, 1989). The alleles designated M1(Ala-213), M1(Val-213), M2 and M3 represent 95% of normal alleles in US caucasians. The coding exons of the four common normal alleles differ by sequential single base changes, suggesting a pattern of evolution with M1(Ala-213) probably being the oldest human α-1-PI allele.

There is a subgroup of 12 "at risk" alleles, 9 of which have been sequenced (Cox, 1989). Most mutations causing α-1-PI deficiency appear to modify the intracellular processing of α-1-PI in its path from the rough endoplasmic reticulum (RER) to the Golgi, resulting in reduced secretion and hence reduced serum levels. The most common mutations are Z and S. The Z mutation is a single base substitution in exon V of the normal M1(Ala-213) allele causing a Glu to Lys change at position 342 in the protein (Brantly *et al*, 1988). The allele frequency is 1-2% in affected populations. Typically only 10-15% of normal levels of α-1-PI are found in serum (Brantly *et al*, 1988) although what is present is fully active. The defective molecule aggregates in the RER of the affected individual (Crystal, 1989). The S allele results from a mis-sense mutation causing replacement of Glu-264 by Val in exon III of the normal M1 (Val-213) allele; of the two mutations S is the more common. SS individuals are not at increased risk for emphysema, but SZ and ZZ individuals are (Wewers *et al*, 1987). The problem with the S form of the protein seems to be related to intracellular stability, catabolism being rapid in the lysosome (Curiel *et al*, 1989). All other mutations belonging to the "at risk" group cause deficiency because of intracellular aggregation or degradation of α-1-PI. Very recent evidence had shown that the aggregation of α-1-PI in RER is caused by

polymerisation of the molecules (Lomas et al, 1992). The polymerisation is caused by the reactive loop of one molecule inserting into a gap in the A sheet of an adjacent molecule resulting in concatenation of α-1-PI (Mast *et al*, 1992).

There are six known mutations causing the null state. All are rare except for $null_{procida}$, which is associated with the deletion of all four coding exons of the α-1-PI gene (Crystal, 1989). The other null alleles include a variety of substitution, deletion and insertion mutations which introduce stop codons into exons.

Some mutations result in altered function for the serpin, eg α-1-$PI_{pittsburgh}$ is caused by Met-358 changing to Arg-358 resulting in the decrease of anti-elastase activity and an increase in anti-thrombin activity. This mutation can lead to bleeding disorders, a direct consequence of the heparin independent anti-thrombin activity of the Pittsburgh variant (Owen *et al*, 1983).

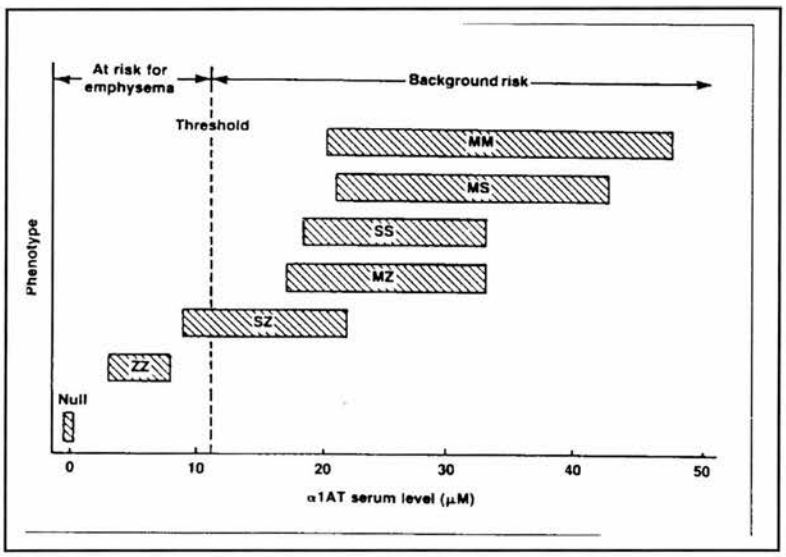


Figure 3

A diagrammatic summary of the risk of emphysema deriving from possession of various alleles of α-1-PI (Crystal,1989).

1.3.5.2. α-1-antichymotrypsin.

The above mutations of α-1-PI manifest themselves as liver, lung (Poller *et al*, 1990) or blood disorders (Crystal, 1989). Recently it has been shown that the

proteins forming the amyloid plaques in the brains of Alzheimer's disease patients are the serpins proteinase nexin II and α-1-antichymotrypsin (Abraham *et al*, 1988). The serpins are produced in the brain, particularly in areas that develop Alzheimer's lesions. Whether the protein accumulation is a cause or result of the disease is not at present understood.

1.3.5.3. Antithrombin III

Antithrombin is a major plasma protein inhibitor of the proteinases generated during blood coagulation; it plays an important role in the regulation of thrombin in the blood. Inherited deficiency of antithrombin is associated with familial thromboembolism (Lane and Caso, 1989).

1.3.5.4. C1-inhibitor

C1 is the only plasma inhibitor of activated C1r and C1s, the serine proteinase subcomponents of the first component of complement (Davis, 1989). Absence or impaired function of the protein results in angioneurotic oedema. The condition can be genetic in nature although the symptoms can be acquired in individuals with B-cell lymphoproliferative disorders.

1.3.6. Clearance of the complex

The physiological fate of the complex formed between serpins and their cognate proteinases is of importance, as the fate of the complex is essentially the fate of the proteinase.

Studies directed towards an understanding of the fate of the complex revealed that it had a shorter half-life in vivo than the corresponding serpin, eg I-125 labelled α-1-antichymotrypsin had a plasma half-life of 85 minutes, whilst complex formed between this serpin and either chymotrypsin or cathepsin G had a half-life of 12

minutes (Pizzo *et al*, 1988). It was found that clearance of this complex could be blocked by a large molar excess of complexes formed from proteinases and α-1-PI, or α-1-antichymotrypsin. Studies have demonstrated that the pathway responsible for this clearance is also responsible for the catabolism of complexes formed from heparin cofactor II and antithrombin III (Pizzo *et al*, 1988; Pratt *et al*, 1988). Thus there appears to be a common receptor -mediated pathway for the plasma clearance of serpin:proteinase complexes, but this pathway does not seem to be involved in the catabolism of serpins or proteinases. This receptor has been dubbed serpin receptor I (Pizzo, 1989).

Several recent studies suggest that the complexes formed between serpins and proteinases have intrinsic functional activities such as stimulating neutrophil chemotaxis (Banda *et al*, 1988), and mediating increases in expression of the α-1-PI gene in macrophages (Perlmutter *et al*, 1988). It is known that during complex formation there is a structural rearrangement of the inhibitor, involving hydrolysis of the serpin at the carboxyl-terminal portion of the protein. It is not known whether this hydrolysis goes to completion, but the current hypothesis is that a new domain on the serpin is exposed. This domain is recognised by the serpin receptor, allowing clearance (Perlmutter *et al*, 1990). Recently a pentapeptide domain (residues 370-374 of α-1-PI, FVFLM) has been shown to be sufficient for binding to the receptor, this sequence is exposed during complex formation and is conserved across a variety of serpins (Joslin *et al*, 1991).

The receptor seems to be responsible for the internalisation of the complex and it also would appear to play a role in subsequent catabolism of the complex intracellularly. Binding and internalisation is saturable and time dependent. SDS-

PAGE analysis of intracellular radioactivity demonstrated that intact complexes (Mr 75,000 and 66,000) were internalised. Kinetic analysis showed that a single cohort of ^{125}I-α-1-PI complexes were accumulated within 5 -15 minutes of binding to the cell surface. Evidence indicates that the subsequent degradation is lysosomal. Thus the receptor mediates internalisation and delivery of the complex to the lysosomes (Perlmutter *et al*, 1990).

It has been long known that macrophages produce α-1-PI (Remold-O'Donnel and Lewandrowski, 1983) and another serpin termed MPI (macrophage proteinase inhibitor). Using RNA blot and dot hybridisation techniques, α-1-PI mRNA has been detected in human peripheral blood monocytes, bronchalveolar and breast milk macrophages but not in lymphocytes (Perlmutter *et al*, 1985).

Expression of α-1-PI in macrophages may not be regulated by complex binding alone (Perlmutter *et al*, 1985). There is evidence to suggest that elastase and endotoxin have regulatory effects on the expression of this serpin (Perlmutter *et al*, 1988;. Barbey-Morel *et al*, 1987) in peripheral blood monocytes and bronchoalveolar macrophages. These effects have been shown to be additive, the regulatory effect of each molecule can be inhibited independently (Perlmutter and Punsal, 1988), Diisopropyl fluorophosphate inhibits the effect of elastase and a monoclonal antibody against the lipid A moiety of lipopolysaccharide inhibits the effect of endotoxin. The effect of elastase results from a specific increase in steady state levels of α-1-PI mRNA whereas endotoxin mediates a 5-9 fold increase in synthesis of α-1-PI with minimal, or no change, in α-1-PI mRNA levels.

1.4. Aims of the Project.

The concentrations of RMCP II in peripheral blood are greatly increased during systemic and local intestinal anaphylaxis (King and Miller, 1984; Patrick *et al.*, 1988) and secretion of RMCP II into blood is associated with expulsion of nematode worms in rats (Miller *et al.*, 1983). The phenomenon of increased concentrations of circulating soluble mast cell proteinase associated with worm expulsion has also been observed in mouse (Huntley *et al.*, 1990) and sheep (Huntley *et al.*, 1987). A physiological role for mast cell proteinases has not yet been unequivocally demonstrated. However, several lines of evidence suggest that human mast cell proteinases modulate the biological effect of neuropeptides in the airway, resulting in airway hypersensitivity in man (Tam and Caughey, 1990; Caughey, 1990) and dog (Nadel, 1989). Exogenous RMCP I has also been implicated in mast cell degranulation (Schick, 1990; Kido *et al.*, 1988), and in the processing of matrix metalloproteinases (Sopata *et al,* 19??).

Many serine proteinases which function in plasma or tissue during inflammation appear to be regulated by a superfamily of serine proteinase inhibitors (serpins) (Travis and Salvesen, 1983). This suggests that serine proteinases derived from mast cells also will be regulated by the serpin superfamily. For example human skin mast cell chymase is inhibited by at least two serpins (Schechter *et al.*, 1989). Work in this laboratory has shown that mouse serum has the ability to interact with mouse intestinal mast cell proteinase, resulting in the formation of higher molecular mass (c. 75kDa) complexes (Irvine *et al.*, 1990) a characteristic of the interaction of serpins with susceptible proteinases.

At least 5 plasma proteins in the rat belong to the serpin superfamily and function as serpins (Kuehn *et al.*, 1984). The purpose of this present study was to determine which, if any, of the rat serpins can regulate the activities of RMCP I and II, in an attempt to elucidate the physiological fate of these mast cell proteinases in an homologous system.

Antibodies specific for the rat serpins will be raised and used to investigate the acute phase response involving serpins during nematode infection. Attempts to correlate these data to data pertaining to rat mast cell proteinase concentrations will be made.

Chapter 2: Materials and Methods.

2. Materials and Methods.

2.1.Animals

The rat strain was randomly bred male Wistars. Average body weight was 300-400g. All rats were killed under halothane anaesthesia by cervical dislocation. The rabbit was an half-lop. This animal was killed under halothane anaesthesia and exsanguinated. The sheep was a Blackface ewe that had grazed pastures at Moredun Research Institute. All animals were obtained from the colonies maintained at the Moredun Research Institute in Edinburgh. Laboratory animals were maintained in a dark/light cycle of 12 hours and were allowed access to food and water *ad libitum*.

2.2. Parasitological Techniques

The strain of *Nippostrongylus brasiliensis* in these experiments was originally established and maintained in the Department of Experimental Parasitology, University of Glasgow and is now maintained in the Moredun institute by passage in Wistar rats. The culture and maintenance of *N. brasiliensis* was essentially as described by Nawa and Miller (1978). Briefly, seven days after infection faeces were collected into water, washed and mixed to form a thick paste. Previously washed granular charcoal (10-18 mesh, BDH Chemical Co.) was added to the faeces, and the mixture incubated at 28°C in a humidified plastic box. Seven to 14 days after incubation the infective stage larvae (L_3) were collected into water at 37°C and then transferred to a steel mesh covered with rice paper through which they migrated. After washing the larvae in sterile saline, suspensions of larvae were counted under a dissecting microscope. the concentration of the larval suspension was normally adjusted to an infective dose of 3000 L_3 in 0.5mL which was inoculated subcutaneously into the flank of anaesthetized rats.

2.3. Perfusion of animals

Animals were killed under halothane anaesthesia by cervical dislocation. The thoracic cavity of the animal was exposed and the animal was perfused with 300mL of PBS via the left ventricle of the heart. The exit point of the perfusate was the right atrium. The animal was then perfused via the right ventricle with 100mL PBS to ensure a good perfusion of pulmonary tissues. A 16 gauge needle was used. Perfusion was performed under gravity.

2.4. Preparation of serum and tissues.

2.4.1 Serum

Serum was obtained by collecting blood from the right atrium prior to any perfusion, or from the right carotid artery. Blood was allowed to clot at room temperature and serum was aspirated using a pastette. The serum was centrifuged at 800g for 15 minutes in order to pellet erythrocytes. Serum was removed from the pellet and stored at -20°C in 1mL aliquots until used.

2.4.2 Tissues

Tissues were removed from a perfused animal, trimmed to remove excess fat and stored in 1g quantities at -20°C.

Small intestine was taken 12-15cm distal to the pylorus. The 5cm of large intestine immediately distal to the caecum was removed. All other tissues were removed whole and trimmed prior to storage.

Tissues were homogenised (10%w/v) in phosphate buffered saline (PBS), using a Silverson sealed unit heavy duty laboratory Emulsifier. Gastrointestinal tissues were homogenised in the presence of 1mM phenylmethyl sulphonylfluoride to prevent proteolysis by endogenous serine proteinases. The homogenates were

centrifuged at 10,000g for 15 minutes and the supernatant was further centrifuged at 50,000g for 30 minutes. All analysis was performed on the supernatant fraction from this procedure. Tissue preparations were stored in 1mL aliquots at -20°C until analysed.

2.5. Assay Procedures

2.5.1 Protein

Protein concentration was determined using the bicinchoninic protein assay system (Pierce and Warner, Chester, UK) modified for use with microtitre plates. The standard curve was prepared using bovine serum albumin (BSA, from Sigma chemical Co., Poole, Dorset, UK) in the range 10-50μg. Absorbance at 540nm was measured. A typical standard curve can be seen in Figure 2.1.

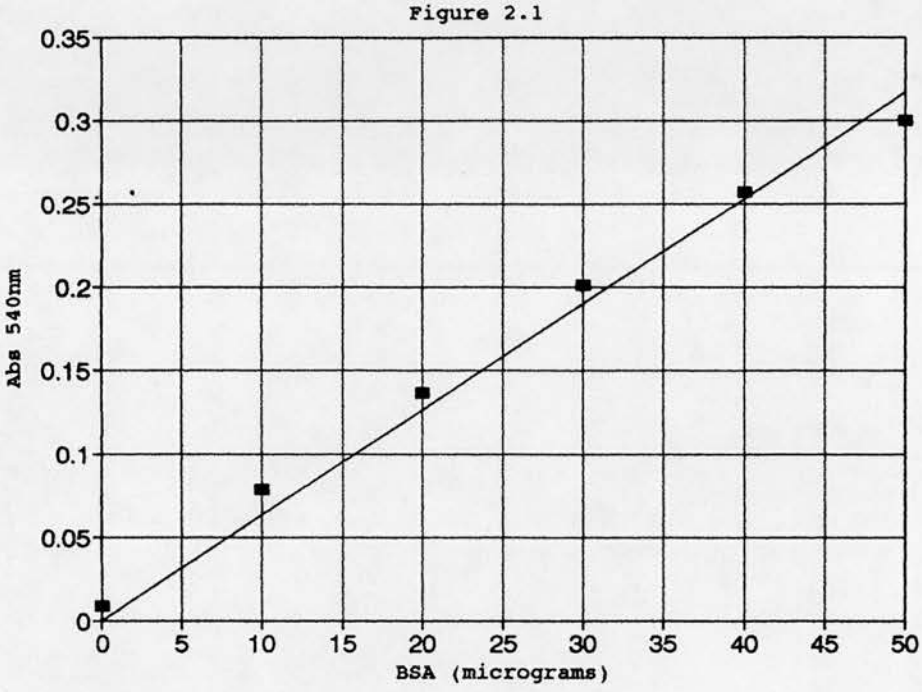
Protein concentration was also determined by measuring the absorbence of a solution at 260nm and 280nm. Protein concentration was determined using the following equation

$$[P] = (1.55x \text{ Abs } 280) - (0.77x\text{Abs } 260)$$

2.5.2 Proteinase

The standard assay for monitoring enzyme activity during the purification using the esterase substrate, benzyloxy-carbonyl-L-tyrosine-4-nitrophenyl ester (CBZ-L-Tyr-4NPE, from Sigma chemical Co. Poole, Dorset, UK) was as follows: 10μl of enzyme preparation was added to 100μl of 20mM Tris-HCl, pH 7.5. 10 μl of substrate (10mM in DMSO) was added to initiate the reaction and after 3 minutes at room temperature the formation of product was monitored at 405nm.

Figure 2.1



Standard curve for Pierce protein assay.

The assay was performed according to the manufacturers instructions. The assay was read in a microtitre plate on a titretek plate reader, with the filter wheel set at 540nm.

2.6. Preparation of antibodies

2.6.1. Polyclonal antibodies

The procedure for rabbit and sheep polyclonal antisera preparation was as follows. Equal volumes (200μL) of antigen solution (100 μg total protein) and Freund's complete adjuvant (Sigma Chemical Co., Polle, Dorset) were emulsified by drawing the mixture up a 19G needle 10-20 times. A solution of PBS/Tween-80 (2%v/v) equivalent to twice the volume of the original antigen solution was added to the mixture. This final solution was also emulsified prior to injection intramuscularly into the animal.

Subsequent immunisations were performed as above. except that the antigen was emulsified in Freund's incomplete adjuvant. Secondary immunizations were generally performed 4 weeks after the primary immunisation.

2.6.2. Monoclonal antibodies.

Mice were immunised as described in 2.6.1 and the animals were bled from the tail after 4 weeks. The serum derived from tail bleeds was assayed by enzyme linked immunoabsorbant assay ELISA, with Rat Serpin II-Mouse Mast Cell Proteinase 1e as the coating antigen. Animals which tested positive were given a secondary immunisation as described is 2.6.1. The spleen from an immune mouse was removed and washed in medium (RPMI/10% FCS/ PenStrep [50μg/mL streptomycin, 100U/mL penicillin]). The cells were liberated from the spleen using 26G needles and aspirated into a 50mL tube. This procedure was repeated with a control spleen to provide feeder cells. The fusion partner (NS0 derived from a mouse myeloma cell line) was suspended in medium and all three sets of cells were centrifuged for 5 minutes at 400g. The pellet of immune spleen cells was

resuspended in 10mL of RPMI/PenStrep (serum free) and washed once in this medium. The control spleen cells were resuspended in 5mL of RPMI/PenStrep/HAT (0.10mM hypoxanthine/0.4nM aminopterin/0.01mM Thymidine) and washed once in this medium. The fusion partner (NS0) cell pellet was resuspended in 10mL RPMI/PenStrep. Immunised spleen cells were added to fusion partner cells in a ratio of 10:1, and the suspension centrifuged for 5 minutes at 400g. The supernatent was removed, 1mL of fresh medium (serum free) was added to the pellet and the cell pellet resuspended carefully by tapping the tube. 1mL of polyethylene glycol (PEG) 1500 (Boehringer, Mannheim) was added over the course of 1 minute, the tube was in constant gentle motion. 10mL RPMI/PenStrep was slowly added and the suspension centrifuged at 300g for 5 minutes. The pellet was resuspended in 1 mL of serum free medium and RPMI/PenStrep/HAT. The cells were counted in a haemocytometer and the concentration of fused cells adjusted to 1 x 10^6/mL. Feeder cells (control spleen cells, 2 x 10^5/mL) were added to the fused cells (1 x 10^6/mL). The mixture was plated out onto 96 well polystyrene plates and cultured in RMPI/10% FCS/Penstrep/HAT/Glutamine (0.03%) at 37°C in 5% CO_2.

2.6.3. Selection of viable clones

Wells containing healthy colonies of growing hybridoma cells were assayed by ELISA, using RSII:MIMCP Ie bound to the plates as antigen. Colonies which tested positive were plated out in a limiting dilution, in order to generate one clone per well. Clones were re-assayed by ELISA. Positive clones were subcloned (see chapter 5 for specific details) in RPMI/10% FCS/PenStrep/HT (no aminopterin)/Glutamine.

2.6.4. Conjugation of antibodies

Antibodies were conjugated essentially as described in Wilson and Nakane (1978), with minor modifications. Horse radish peroxidase (HRPO) (4mg, Boehringer Mannheim) was dissolved in 1mL dH_2O. Freshly prepared 0.1M $NaIO_4$ (0.2mL)was added to the HRPO and the solution was stirred for 20 minutes at room temperature. The HRPO-aldehyde solution was dialysed against 1mM sodium acetate pH 4.4 overnight at 4°C. The pH of the HRPO-aldehyde solution was raised to 9-9.5 by the addition of 20μL of 0.2M sodium carbonate buffer, pH 9.5 and immediately 8mg IgG in 1mL 10mM sodium carbonate buffer, pH 9.5 was added. The reaction mixture was stirred for 2 hours at room temperature. Freshly prepared sodium borohydride (0.1M) was added to the mixture (4mg/mL) and the solution left for two hours at 4°C. The conjugate mixture was chromatographed on Sephadex G-100 (15 x 950mm) equilibrated in PBS. The conjugated antibody was detected by reading absorbance at 280nm and 403nm. Bovine serum albumin (fraction V) was added to a concentration of 10mg/mL and aliquots were stored in 50% glycerol at -20°C.

2.7. Preparation of affinity columns

CNBr-Sepharose 4b (Pharmacia) was washed and swollen in 1mM HCl (200mL/g). The protein to be coupled was dissolved in 0.1M sodium carbonate buffer/0.5M NaCl, pH 8.3. The protein solution was added to the swollen Sepharose 4B and the mixture was placed on a carousel for 2 hours at room temperature. The gel was then transferred to a solution of 0.2M glycine, pH 8.0 and allowed to mix for 2 hours at room temperature in order to block unreacted coupling sites. The gel was then packed under gravity into a C-16 (16 x 100mm)

type column (Pharmacia). 0.5mL of Sephadex G-25 was poured onto the top of the settled matrix and the gel was subjected to a high-low-high pH regime to remove excess blocking glycine. The column was stored at 4°C.

2.8. Column Chromatography

All column chromatography was performed using the FPLC system (Pharmacia). Proteins were routinely detected by continuously monitoring absorbance at 280nm. Columns used were all of the C-16 type (Pharmacia), or were prepacked mono-columns (HR5/5, Pharmacia).

2.9. Purification of proteins

2.9.1 Antibodies

Antibodies were purified from antiserum by affinity chromatography with, as ligand, the antigen used to raise the antiserum. Antiserum (200μL) was applied serially to a column (3mL) of ligand-Sepharose4B (0.25-0.5mg/g matrix) equilibrated with PBS. Bound material was eluted with 2mL of 0.1M citric acid/0.5M NaCl, pH 2.2. Antibody solutions (2-3mL) were immediately titrated back to neutral pH by addition of 200μL of 1M Tris, pH 10. Antibody solutions were concentrated by applying the protein solution (in 75mM Tris pH 10.00) to a MonoQ column previously equilibrated in this buffer. 95% of the antibodies in solution bound to the column under these conditions. The antibodies were eluted from the column using 2mL of 75mM Tris/1M NaCl, pH 10.00. The final volume obtained was typically 1-2mL with a protein concentration of 2-4mg/mL.

2.9.2 RMCPII

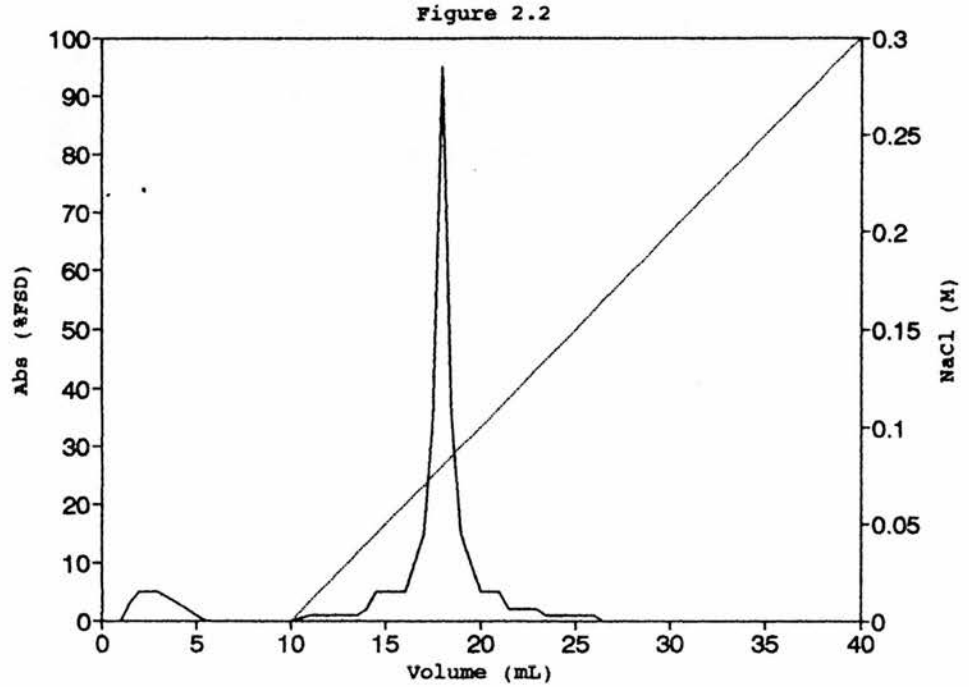
RMCPII was purified from small intestines of rats previously infected with *N.brasiliensis*. The tissue (10g) was homogenised (20% w/v) in 20mM Tris-HCl, pH

7.5, and centrifuged at 10,000g for 15 minutes. the supernatant was further centrifuged at 50,000g for 30 minutes and the supernatant from this procedure was applied to a CM-Sepharose column (16mm x 200mm) previously equilibrated in 20mM Tris-HCl, pH 7.5. The column was washed in 100mL of 20mM Tris-HCl, pH 7.5, and eluted protein was discarded. Protein bound to the column was eluted with 10mL of 20mM Tris-HCl/1M NaCl, pH 7.5, and immediately applied to a Sephadex G-25 column previously equilibrated in 20mM Tris-HCl, pH 7.5. The protein eluting from this column was applied to a MonoS (Pharmacia, Milton Keynes, Bucks, UK) column equilibrated in the same buffer. The MonoS column was washed with 20mL of 20mM Tris-HCL and a continuous salt gradient (NaCl 0-0.3M) was applied at a rate of 10mM/mL. RMCPII was eluted at 80mM NaCl. To ensure maximum purity, RMCPII was reapplied to the MonoS column three times. This was necessary as an active fragment of the proteinase was sometimes generated during the purification. The identity of the fragment was determined by amino-terminal sequence analysis. A chromatogram from the monoS column can be seen in Figure 2.2.

2.9.3 Mouse Intestinal Mast Cell Proteinase Ie

Purified Mouse Intestinal Mast Cell Proteinase Ie was a kind gift from George Newlands.

Figure 2.2



RMCPII purification: monoS column profile

The buffer system is 20mM Tris-HCl, pH 7.5. The peak is RMCPII which elutes at a salt concentration of 80mM NaCl. The UV detector was set at 100% FSD.

2.10. Electrophoresis

2.10.1 Laemmli (1970) gel system.

Buffers-

A/B- 30%acrylamide/0.8% bis-acrylamide

LT- 1.5M Tris-HCl

0.4% SDS -titrated to pH 8.8

UT- 0.5M Tris-HCl

0.4% SDS -titrated to pH 6.8

Reservoir buffer- 0.3% Tris

1.44% Glycine

0.1% SDS

AP- 2% ammonium persulphate

The solutions were mixed according to the table below to provide gels of varying properties.

Running gel

solution	7.5%	10%	12.5%	15%
H_2O (mL)	14.5	12.1	9.5	7
A/B (mL)	7.5	10	12.5	15
LT (mL)	7.5	7.5	7.5	7.5
AP (mL)	0.45	0.45	0.45	0.45
TEMED (μL)	11.3	7.5	7.5	10

Stacking gel

H2O (mL)	12.76
A/B (mL)	2.0
UT (mL)	5.0
Ammonium Persulphate (mL)	0.3
TEMED (μL)	20

All gels were cast using the Bio-Rad Protean II system or the Bio-Rad Mini-Protean II system. Sample preparation was as outlined below.

Pre-treatment buffer (PTB)

200μL 0.8% bromophenol blue in UT

200μL 40% sucrose

200μL ß-mercaptoethanol

400μL 20% SDS

Samples were diluted to the appropriate concentration in PBS, if required, to 66% of the final volume required. The sample volume was then made up using PTB. Samples were heated to 100°C for 3 minutes before loading onto the gel. Gels were run at 25mA initially. When protein had entered the resolving gel the current was increased to 35mA. Electrophoresis was stopped when the dye front was within 2cm of the bottom of the gel.

2.10.2 Schägger and von Jagow (1987) gel system.

Resolving gel

Buffer	Volume
Acrylamide (50%)/ bis-acrylamide (1.5%)	2mL
3M Tris/0.3% SDS, pH 8.45	3.33mL
H_2O	4.56mL
Ammonium persulphate (10%)	100μL
TEMED	10μL

Stacking gel

Buffer	Volume
Acrylamide (50%)/ bis-acrylamide (1.5%)	0.33mL
3M Tris/0.3% SDS pH, 8.45	1.0mL
H_2O	2.8mL
Ammonium persulphate (10%)	100μL
TEMED	10μL

Tank buffers

Upper tank buffer- 0.1MTris/0.1M Tricine/0.1% SDS

Lower tank buffer-0.2M Tris-HCl, pH 8.9

All gels were cast using the Bio-Rad Protean II system or the Bio-Rad Mini-Protean II system. Sample preparation was as outlined below.

Pre-treatment buffer (PTB)

200μL 0.8% bromophenol blue in 3M Tris/0.3% SDS

200μL 40% sucrose

200μL ß-mercaptoethanol

400μL 20% SDS

Samples were diluted to the appropriate concentration in PBS, if required, to 66% of the final volume required. The sample volume was then made up using PTB. Samples were heated to 100°C for 3 minutes before loading onto the gel. The gel was run at 110V until the dye front was within 1cm of the bottom of the gel.

2.10.3 Non-reducing native gel system.

Buffers

A/B- 30% acrylamide/0.8% bis-acrylamide

Gel buffer- 25mL 1.5M Tris

75μL ß-mercaptoethanol

0.5mL TEMED

-add water to about 70mL

-titrate to pH 7.8 with H_2SO_4

-make upto 100mL with water.

AP- 0.2% in H_2O

Tank buffer 65mM Tris-borate, pH 9.0

7.87g Tris

1g Boric Acid

-make upto 100mL with H_2O

For an 11.2% gel these components were mixed as follows.

Solution	Stack	Resolving Gel
A/B (mL)	0.5	4.48
Gel Buffer (mL)	1	3
AP (mL)	1	3
H_2O (mL)	1.5	1.52

These gels were cast using the Mini-Protean II gel system and run at 250V for 50 minutes or until the dye front had reached the bottom 1cm of the gel.

2.10.4 Blotting procedures

Proteins were transferred to inert supports using the semi-dry method of blotting (Khyse-Anderson, 1984). The transfer buffer was 10mM 3-[Cyclohexylamino]-propane-sulphonic acid (CAPS, Sigma Chemical Co., Poole, Dorset) according to the method of Matsudaira (1987). Transfer was achieved in 1 hour with a current of $2mA/cm^2$ of gel.

Protein was transferred either to polyvinylenedifluoride (Immobilon-P, Millipore, Watford, Herts, UK) or nitrocellulose (Schleicher & Schuell, Anderman & Co., Kingston upon Thames, Surrey, UK) for subsequent analysis.

2.10.5 Staining procedures.

Coomassie Brilliant Blue.

Gels were fixed and stained in 0.5% Coomassie brilliant blue (R-250, Sigma Chemical Co. Poole, Dorset, UK) dissolved in 25% methanol/10% acetic acid for

15 minutes. Gels were destained in 25% methanol/10% acetic acid for 1-2 hours, with 2-3 changes of destain solution.

Silver Stain (Morrisey, 1981)

Gels were fixed and stained in 50% methanol/10% acetic acid for 15 minutes. They were washed twice in 5% methanol/7% acetic acid for 5 minutes. After incubation in 5μg/mL dithiothreitol in H_2O for 15 minutes gels were washed 3 times in distilled H_2O. They were incubated in 0.1% silver nitrate for 20 minutes, rinsed in water for 30 seconds and developed in 100mL of 3% Na_2CO_3 with 50μL of 40% formaldehyde. Development was stopped with the addition of 10% citric acid.

2.11. Protein sequencing.

Proteins were sequenced either by the Wellmet facility in the Department of Biochemistry, University of Edinburgh, Edinburgh or by the microchemistry department at the AFRC Institute of Animal Physiology and Genetics Research, Cambridge Research Station, Babraham, Cambridgeshire. Protein was presented for sequence either in solution (25mM bis-tris, pH 6.7) or bound to Immobilon (see section 2.10.4) after electrophoresis. Protein bound to Immobilon was visualised with Coomassie Brilliant Blue (see section 2.10.5), and excised from the support with a clean scalpel.

2.12. Immunological Procedures

2.12.1 Ouchterlony

A solution of 1% (w/v) of fine agarose was made up in PBS and heated to 100°C. The solution was then poured (20mL) onto a 1850-102 Gelbond film (LKB) and allowed to set at room temperature. Wells with a diameter of 5mm were then

cut in the gel. The wells followed a standard pattern of 1 central well surrounded by 6 peripheral wells in a hexagonal arrangement. Antigen solution was pipetted (5μL) into the central well and a series of descending serial dilutions of anti-serum was pipetted into the peripheral wells. The gel was incubated overnight at 4°C in a moist atmosphere, allowing sufficient time for diffusion of the anti-serum and antigens leading to precipitate formation.

Excess fluid was removed from the gel by placing lint free tissue and paper towels on the gel and compressing this "sandwich" with a lead weight (2Kg). The gel was washed in PBS 2-3 times to remove excess protein from the anti-serum, in between each wash the gel was compressed. Finally the gel was dried in a rapid air flow at 37°C. Precipitates were visualised by staining the gel in 1% Coomassie Blue (R-250) (w/v in 20% methanol/15% acetic acid) and destaining in 20% methanol/15% acetic acid.

2.12.2 ELISA

Polystyrene plates (96 well) were coated with ligand or capture antibody dissolved in 0.1M sodium carbonate buffer, pH 9.6. The standard concentration of capture antibody or ligand was 1μg/mL (50μL per well). After 1 hour at room temperature, the plates were washed with PBS/0.05% Tween-20 (Sigma Chemical Co, Poole Dorset) and incubated with a solution of 4% BSA/PBS/Tween-20, for 15 minutes at room temperature (50μL per well). Serially descending dilutions of the antigen or antibody to be detected were then added to the plates and left for an hour (50 μL per well). The plates were then washed 2-3 times in PBS/Tween-20 solution and an antibody, conjugated to horseradish peroxidase and specific for the antigen/antibody of interest was added to the wells (50μL per well). After one hour

the conjugate was removed and the plates washed in PBS/Tween-20. The chromogenic substrate was then added to the wells, the recipe is below.

Chromogenic Substrate:

25mL 0.1M citric acid/25mL 0.2M Na di-hydrogen phosphate/50mL dH2O,pH = 5.00.

Add 40mg OPD, 5μL H_2O_2 (33% w/v) and use immediately (50μL per well).

The colour-generating reaction was stopped by the addition of 25μL of H_2SO_4 (2.5M). Plates were read using a Titretek plate reader with the filter wheel set at 405nm.

2.12.3 Western blotting

After transferring proteins (see section 2.10.4), the blot was washed in PBS/0.5% Tween-80 for 20 minutes in order to "block" any remaining reactive sites. After incubation for 1 hour at room temperature in antibody solution (made up in PBS/Tween-80). the blot was washed 4-5 times in PBS/Tween-80. If the primary antibody was conjugated directly to horse radish peroxidase, the blot was incubated in a solution of diaminobenzidine (20mL)/H_2O_2 (50μL). If the primary antibody was unconjugated the blot was incubated for another hour in a solution of conjugated anti-primary antibody before the addition of the DAB (0.4mg/mL)/H_2O_2 (50μL/20mL) solution. Blots were finally rinsed in distilled water and blotted dry using 3mm filter paper. They were stored at room temperature in the dark.

2.13. Deglycosylation of serpins.

Serpin molecules were deglycosylated as follows. An aliquot of serpin (20μL containing 1-2μg of protein) in solution was pipetted into an Eppendorf tube

(1.5mL). Deglycoslyation buffer (100mM potassium phosphate/100mM EDTA/5% β-mercaptoethanol/0.1% SDS, pH 7.5) was added to the protein solution. One unit of glycopeptidase F (PNGaseF, Boehringer Mannheim) was added to each sample. The mixture was incubated at room temperature for 18 hours. Analysis of M_r change was by SDS-10%PAGE (Schagger and von Jagow, 1987).

2.14. Statistical Analysis

All analysis of variance was performed by the Minitab program. Linear regression was performed by Excell version 4. All non-linear regression analysis was performed by the DNRP53 program written by R.G. Duggelby, University of Queensland.

Chapter 3: Purification and Characterisation of rat serpins

3. Purification and Characterisation of rat serpins.

3.1.Introduction

3.1.1. Serpins in Rodents.

At least five plasma proteins in the rat belong to the serpin superfamily and function as serpins (Kuehn *et al*, 1984). Studies on the genetics of rodents have shown that there are at least two loci in the rat and mouse that contain genes coding for serpins (Inglis and Hill, 1990). The primordial genes for α-1-PI and α-1-antichymotrypsin have undergone an extensive series of duplication events, followed by genetic divergence. In the mouse, where more work has been done, the two loci are called SPI-1 and SPI-2 (Serine Proteinase Inhibitors-1 and -2). Each locus contains 8-12 genes. Sequence analysis of SPI-1 genes shows that their overall structure is very similar to that of human α-1-PI (Inglis and Hill,1990). The gene products of this locus are known to function as inhibitors of serine proteinases. The SPI-2 locus contains genes which resemble that of human α-1 antichymotrypsin, although no true rodent antichymotrypsin activity has been discovered to date.

More recently it has been shown that there are 5 different species of mRNA from mouse that encode members of the α-1-PI family (Borriello and Krauter, 1990). These 5 species share between them 3 distinct reactive site residues at the P1 position: methionine, tyrosine and leucine. The changes govern the proteinase specificity of these serpins.

The rat also has two loci with homologous genes (Inglis and Hill, 1990), again broadly resembling human α-1-PI and human α-1 antichymotrypsin, although there are only 4-5 genes at each locus.

Attempts to purify serpin molecules from both mice (Takahara and Sinohara, 1982) and rats (Kuehn et al, 1984) have yielded interesting results. Concentrations of α-1-PI are 4-5 mg/ml in mouse serum, and it is a monomeric glycoprotein (M_r 53,000) with inhibitory activity against trypsin, chymotrypsin and elastase. Contrapsin (M_r 55,000), a product of the SPI-2 locus, is a murine serpin which inhibits trypsin but has no activity against elastase or chymotrypsin. It is present at slightly lower concentrations (2.8mg/ml) in serum, and is immunologically distinct from α-1-PI.

In the rat, purification of serpins has yielded 3 polypeptides . These were designated α-1-PI, Rat proteinase Inhibitor I and Rat Proteinase Inhibitor II (RPI-1 and RPI-2). The M_r of α-1-PI was 55,000, and of RPI-1 and -2, 66,000 and 65,000, respectively. The inhibitory spectrum of the three molecules was different: α-1-PI inhibited trypsin, chymotrypsin and elastase, whereas RPI-1 inhibited trypsin and chymotrypsin and RPI-2 inhibited trypsin only (Kuehn et al, 1984) .

Mouse serum will also inhibit mouse mast cell proteinases (Irvine et al, 1990). Gel analysis in conjunction with Western blot analysis indicated the formation of complexes in mouse serum (M_r 73,000) in the presence of mouse intestinal mast cell proteinase. Serum also blocked the binding of ^{3}H-DFP to mouse mast cell proteinases, further indicating that the higher molecular weight components detected by western blotting were formed by the interaction of proteinase and serpin.

Such evidence indicated that the best way to obtain a preparation of rat serpin which would inhibit RMCPI or II would be to use the proteinase as a "bait", allow

a complex to form, and by purifying the proteinase, co-purify the serpin involved in the formation of the complex.

Serpins account for 10%, by weight, of human serum protein (Travis and Salvesen, 1983). Their solubility at physiological salt concentrations, relative abundance and acidic pI, would suggest that they are relatively easy to purify. However, there could be as many as 10-12 proteins expressed within the SPI loci (*vida supra*). Furthermore rat serum albumin (50% of soluble serum protein) could pose a serious problem for any purification schedule as it has a similar M_r and pI, and could constitute a major contaminant in any preparation of purified serpins.

3.2. Results

3.2.1. Purification of Serpins

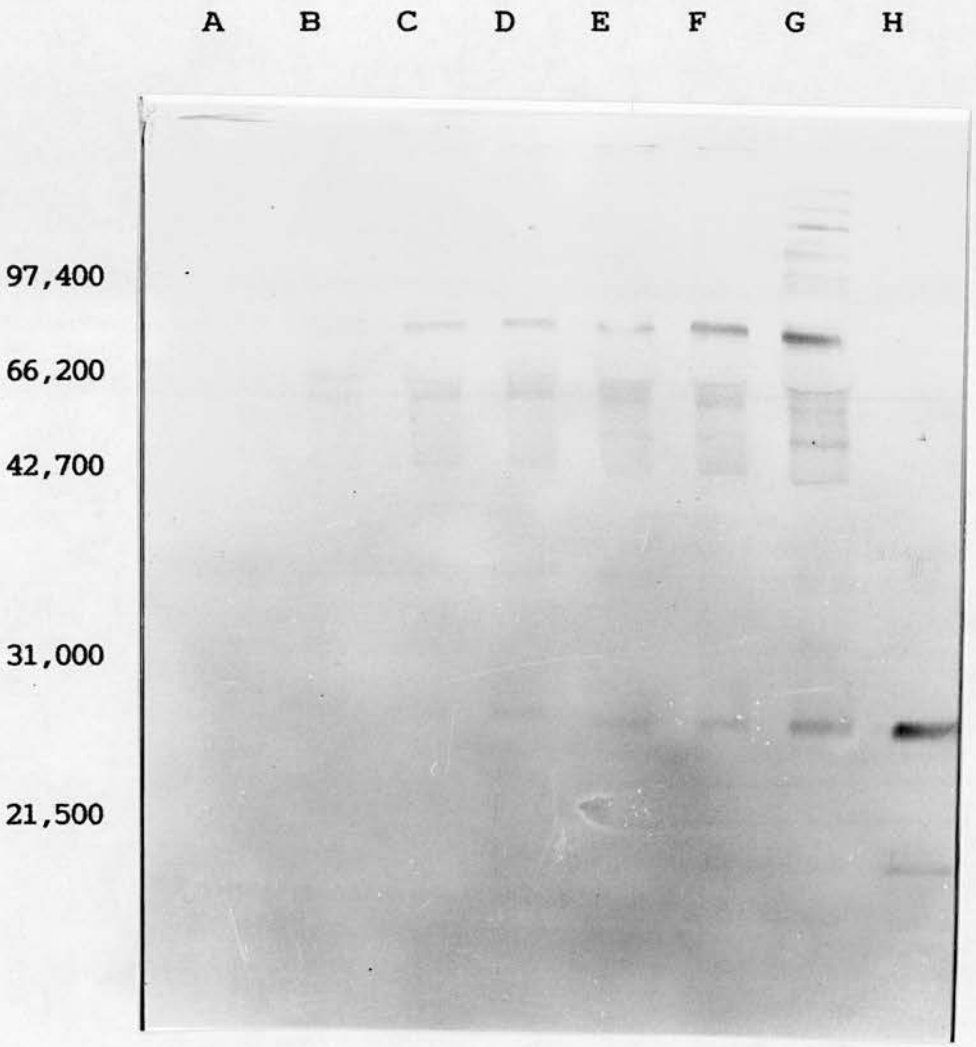
3.2.1.1. Detection of serpins and of RMCPII-Serpin complex in Rat Serum

One of the characteristics of serpins is the ability to form essentially irreversible complexes with a target proteinase (Travis and Salvesen, 1983). Thus it should be possible to detect a complex by discerning the proteinase component of the complex. By an obvious extension of this thinking, the presence of complexes containing proteinases indicates the presence of serpins with the ability to form detectable complexes with the proteinases.

Because the proteinase of interest in this work was RMCPII, and it was by no means certain that there were serpins capable of inhibiting this enzyme, it was decided to titrate RMCPII into rat serum, and try to detect any complex formed by immuno- staining of RMCPII.

Purified RMCPII (see Materials and Methods, Chapter 2) was titrated into rat serum over a range of 0-1 mg/ml. After incubation for 60 min at 37°C an aliquot (equivalent to 30μg protein) of each sample was removed and subjected to SDS-7.5% PAGE. The protein was transferred to nitrocellulose after electrophoresis, and the blot was probed with anti-RMCPII antibody. In the presence of serum, RMCPII formed multiple complexes which were visualised by Western blotting (Figure 3.1); the most abundant serpin-enzyme complex (RMCPII-serpin complex) had an approximate M_r of 79,000 suggesting that a protein(s) with M_r 50,000 is involved with the RMCPII (M_r 28,000) in this reaction.

Figure 3.1



Titration of RMCPII into rat serum and detection of RMCPII-serpin complex.
Lane A, rat serum. Lanes B-G, RMCPII titrated into rat serum at 100, 200, 300, 400, 500, 1000µg proteinase/ mL serum. Lane H, 250ng RMCPII. Samples were electrophoresed on 10%SDS-PAGE (Laemmli, 1970), transferred to nitrocellulose sheets as described (see Materials and Methods, Chapter 2) and probed with anti-RMCPII antibody conjugated to horse radish peroxidase. The band visualised at Mr 79,000 is RMCPII-serpin complex. RMCPII migrates at 28,000.

3.2.1.2. Purification of complex

The strategy adopted was to purify the complex, raise an antibody to it and use this antibody to affinity purify free serpin from serum.

RMCPII (500 μg) was added to 1 ml rat serum and incubated for 1 hr at 37°C. The serum was then applied to a Sepharose 4B anti-RMCPII affinity column (see Materials and Methods). The bound fraction, eluted with 0.1 M citric acid, and the buffer was exchanged on a PD-10 column (Pharmacia) equilibrated with 20 mM Tris-HCl, pH 7.5 (buffer A). The protein fraction was applied to a monoQ column equilibrated in buffer A and a major peak was eluted at 50 mM NaCl using a continuous gradient of 0-0.3 M NaCl (figure 3.2).

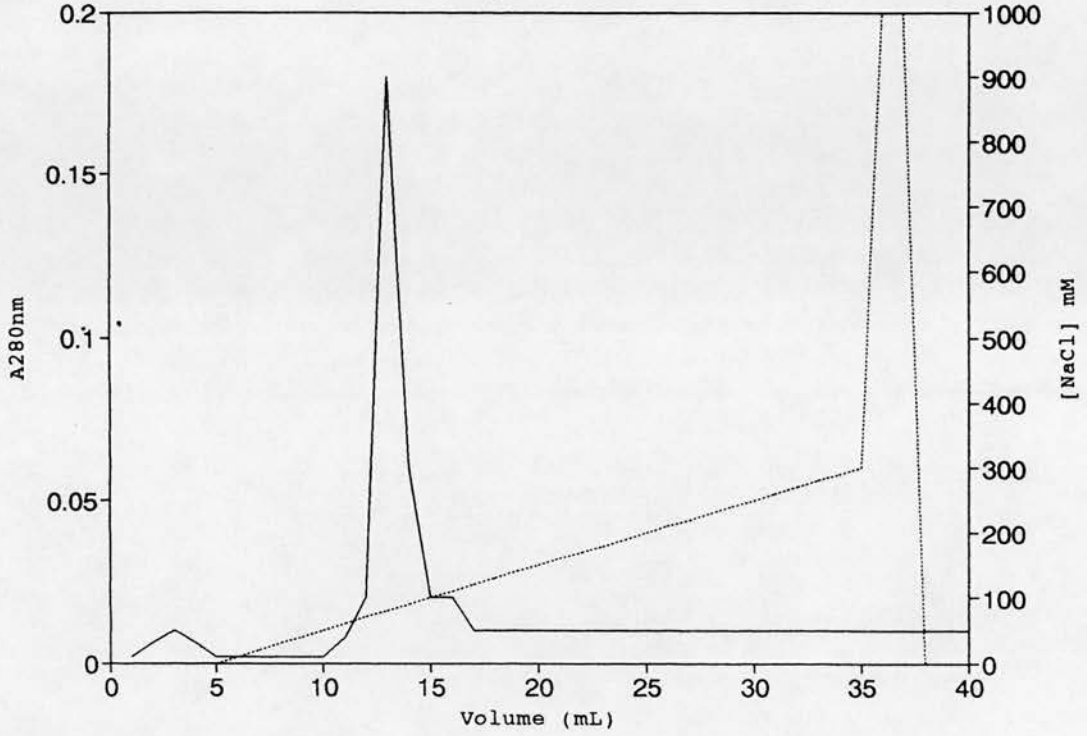
This pH (7.5) was chosen because, in theory, the complex would have a pI between that of the serpin (pI=4-5), and the proteinase (pI>9.6, demonstrated by chromatofocussing, data not shown). At pH 7.5 the complex should bind to monoQ, but not too tightly.

This peak contained 95% pure RMCPII:serpin complex (as estimated by SDS-PAGE, Figure 3.3a) and could be visualised on Western blots using anti-RMCPII antibody (figure 3.3b). RMCPII:serpin complex was used to raise polyclonal antibodies in rabbits.

3.2.1.3. Raising a polyclonal antibody to serpin

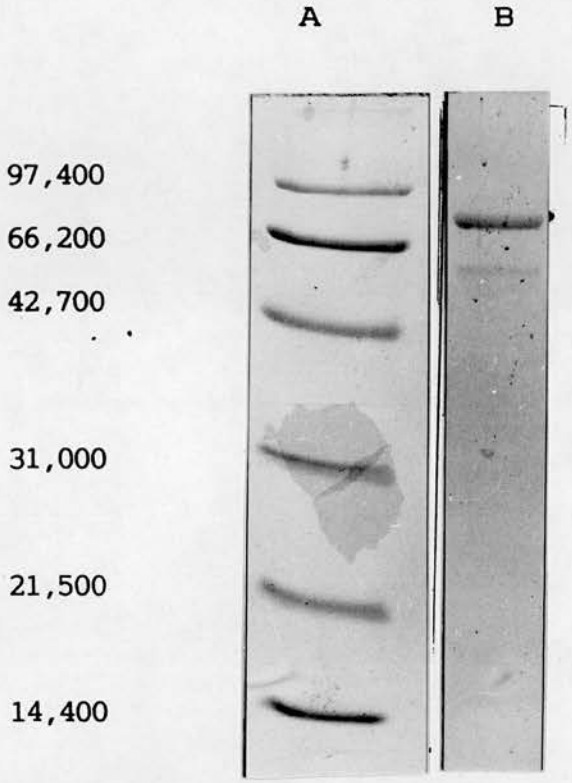
Polyclonal antibodies against complexes were prepared as described (see Materials and Methods, Chapter 2). The titre of the antibody was assessed by Ouchterlony double diffusion (Figure 3.4). Antiserum was stored at -20°C until used. The characterisation of this antibody is in chapter 5.

Figure 3.2



Purification of RMCPII-serpin complex by ion-exchange chromatography.
The affinity purified complex was further purified on a monoQ column. The equilibrating buffer was 20mM Tris-HCl, pH 7.5. A continuous NaCl gradient was generated and complex eluted at 100mM NaCl.

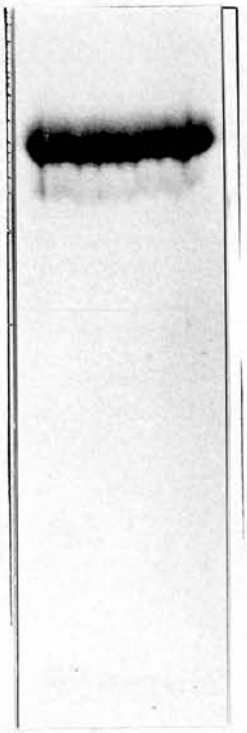
Figure 3.3a



Electrophoresis of purified complex.
Lane A, molecular weight standards (Bio-rad). Lane B, RMCPII-serpin complex, purified as outlined in the text. Electrophoresis on 10% SDS-PAGE was performed according to the method of Schagger and von Jagow (1987). Proteins were visualised using Coomassie Blue.

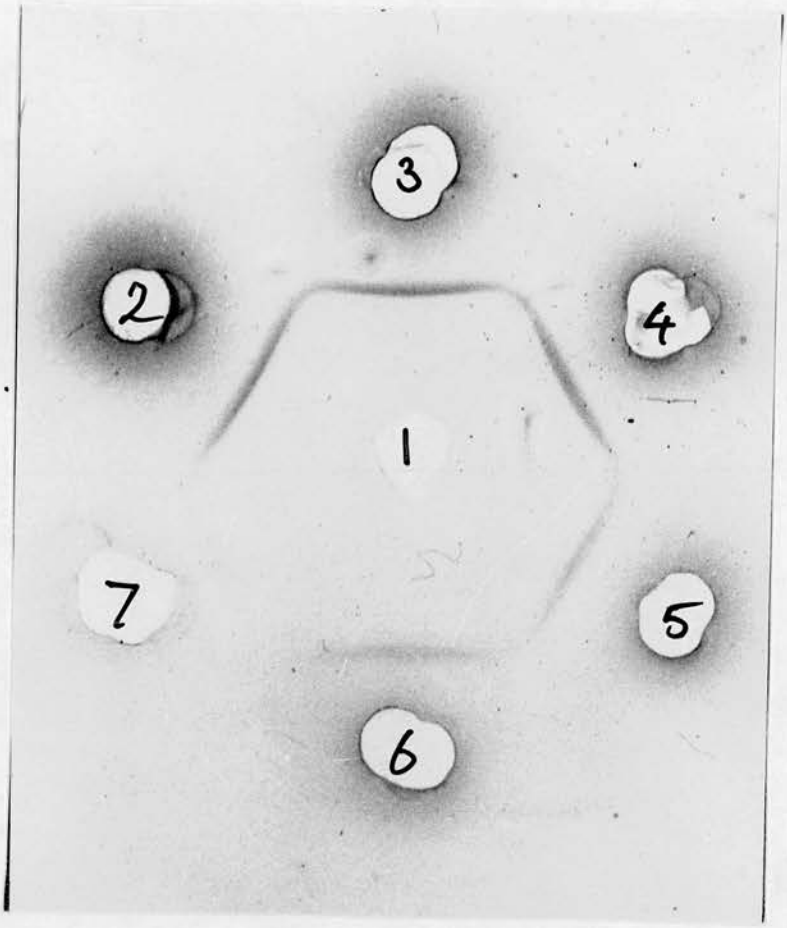
Figure 3.3b

A



Western blot of purified complex.
Lane A, RMCPII-serpin complex, purified and electrophoresed as described (see Materials and Methods, Chapter 2), was transferred to Immobilon using the method of Matsudaira (1987). The blot was probed with anti-RMCPII antibody conjugated to horse radish peroxidase. The complex migrates at M_r 79,000.

Figure 3.4



Double diffusion assay of rabbit antiserum.
The central well (1) contains 5 µL RMCPII-serpin complex (100µg/mL), and wells 2-7 contain 5µL of rabbit anti-complex antiserum diluted 1/2-1/32. Precipitates were visualised with Coomassie Blue.

3.2.1.4. Affinity purification of anti-(serpin-RMCPII)-complex antibodies

The polyclonal antiserum was applied to a RMCPII affinity column (12 mg ligand/g matrix) to remove antibody specific to this enzyme. The unbound fraction was eluted using PBS and applied to a column (5 ml, 2 mg ligand/g matrix) of RMCPII-serpin complex-sepharose. The column was then washed with 2 column volumes of PBS and bound antibodies were eluted with 1 ml of 0.1 M citrate buffer (pH 2.2) were immediately neutralised by the addition of 1M Tris base (200 μl/ml). The eluted antibody (Rab1 α-RSI/II) was used to make an "anti-serpin"-sepharose column (1.4 mg ligand/g matrix) as outlined in Materials and Methods.

3.2.1.5. Purification of serpins

From preliminary investigations it was apparent that the protein most likely to bind to any affinity column was albumin. Subsequent experiments (data not shown) demonstrated a pI of 4.5- 4.9 for rat serum albumin. Rat serum albumin has a M_r of 55,000- 60,000. These two chemical characteristics were very similar to the expected values for rat serpins and initial chromatographic steps would have to remove a large proportion of the albumin.

A comprehensive study by Gianazza and Arnaud (1982) had shown that the dye Cibacron blue could be used as an affinity ligand to bind serum albumin, moreover, a variety of serum proteins were fractionated on a column composed of Cibacron blue covalently attached to a weak anion exchange matrix. Such a material was commercially available as Affi-Gel blue (Bio-rad).

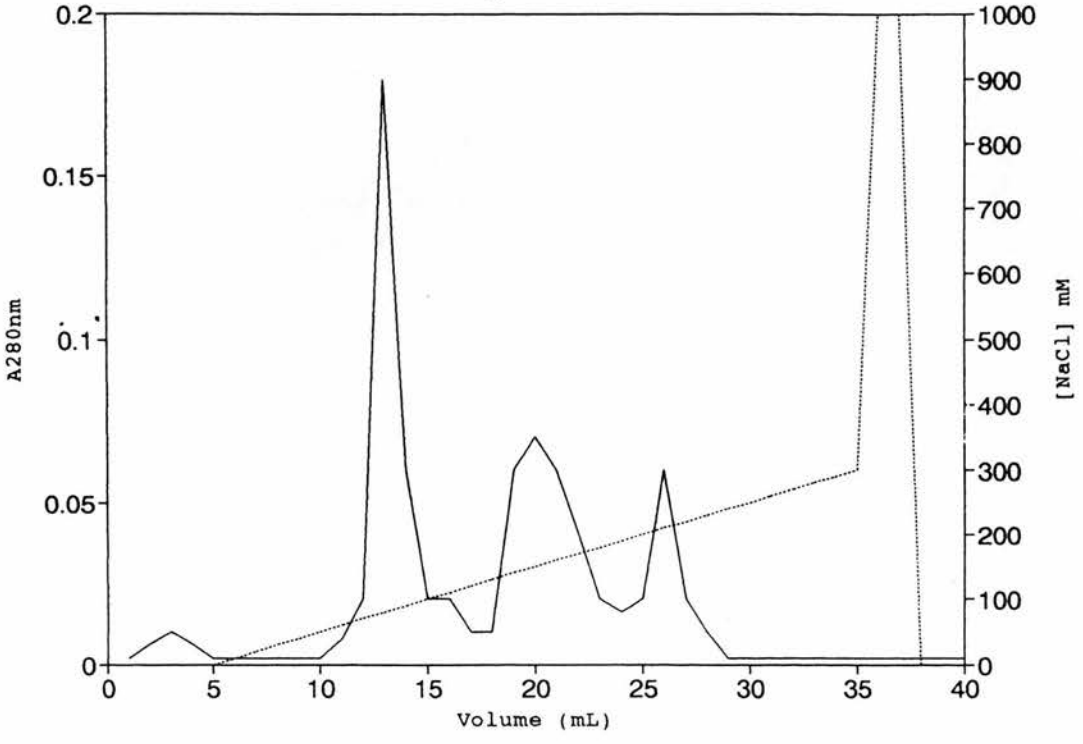
Rat serum (0.5 ml) was applied to an Affi-Gel blue column (16 mm x 50 mm) equilibrated in 25 mM bis-Tris-HCl, pH 6.7 (buffer B) and the column was

washed with this buffer in order to elute α-1-PI (Gianazza and Arnaud, 1982). The protein eluted in the wash was then applied to a sepharose 4B affinity column (16 mm x 50 mm) with, as ligand, the cross-absorbed, affinity-purified rabbit anti-rat serpin polyclonal (Rab1 α-RSI/II). The latter was obtained from a rabbit immunized with serpin-RMCPII complex. The bound serpin was eluted with 1 ml 0.1 M citric acid pH 2.2 and applied immediately to a G-25 sephadex column previously equilibrated with buffer B. The eluted protein was applied to a monoQ column equilibrated in buffer B and a continuous gradient of 0-0.3 M NaCl (10 mM/ml) was established (Figure 3.5), the eluted peaks 1 and 2 contained proteins with M_r of 52,000 and 54,500, respectively (determined on Tricine SDS-10% PAGE, Schägger and von Jagow, 1987) (Figure 3.6), which were respectively labelled rat serpin I (RSI) and rat serpin II (RSII) (Figure 3.7).

3.2.2. Complex formation between rat serpins and rat mast cell proteinases.

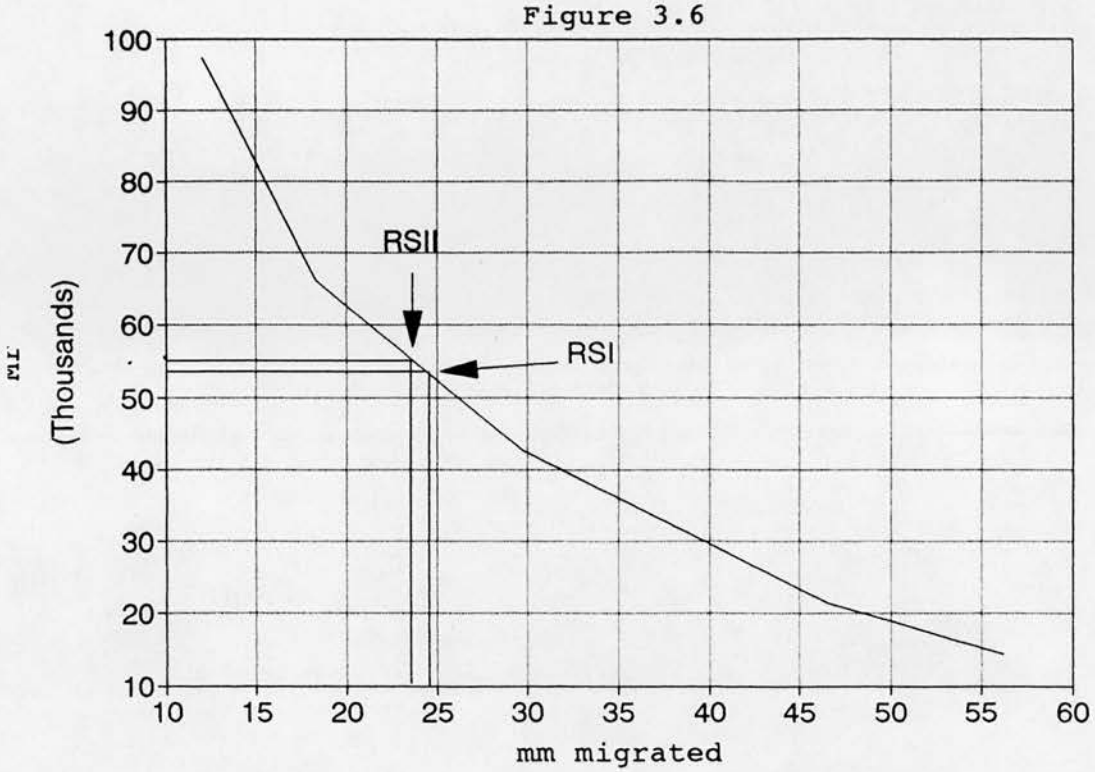
Equimolar concentrations of serpin (20μl x 150μg/ml) and rat mast cell proteinase (4μl x 400μl/ml) (based on protein concentrations, see Materials and Methods, Chapter 2) were incubated together for 5 min in an Eppendorf tube at room temperature. The samples were then analysed by SDS-10%PAGE as described (see Materials and Methods, Chapter 2) and the gel stained with Coomassie blue. The experiment in which RMCPII was added to RSI or II showed the expected staining of a novel band at M_r 75,000 indicating formation of complex which remained stable in SDS (Figures 3.8). However, the effect of adding RSI or II to RMCPI was unexpected, in that no higher molecular weight complex was formed but instead two fragments could be detected (approx M_r 25,000 and 37,000, Figures 3.9).

Figure 3 5



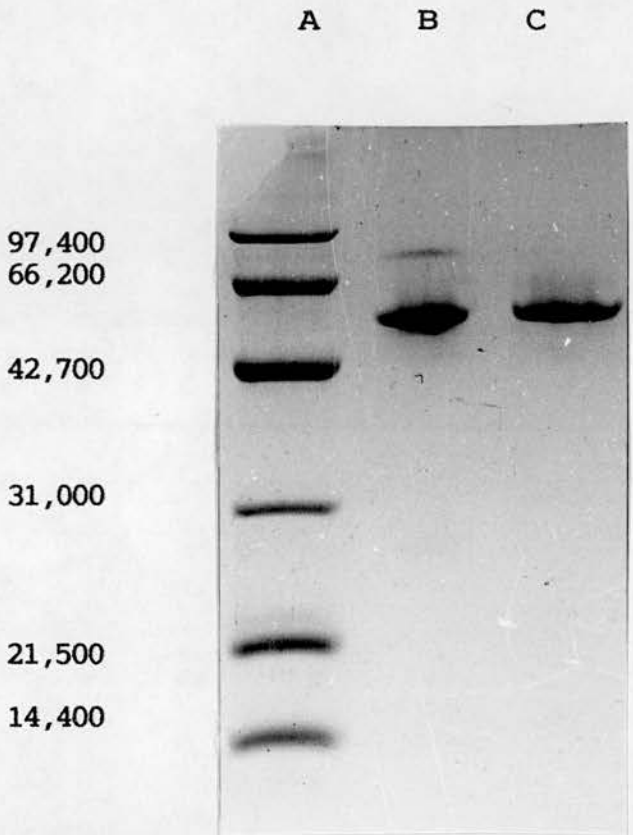
Purification of RSI and RSII by ion-exchange chromatography. Affinity purified rat serpin was applied to a monoQ column previously equilibrated in 25mM bis-Tris-HCl, pH 6.7. A continuous NaCl gradient was generated. The peak eluting at 80mM NaCl is RSI; the peak eluting at 150mM NaCl is RSII.

Figure 3.6



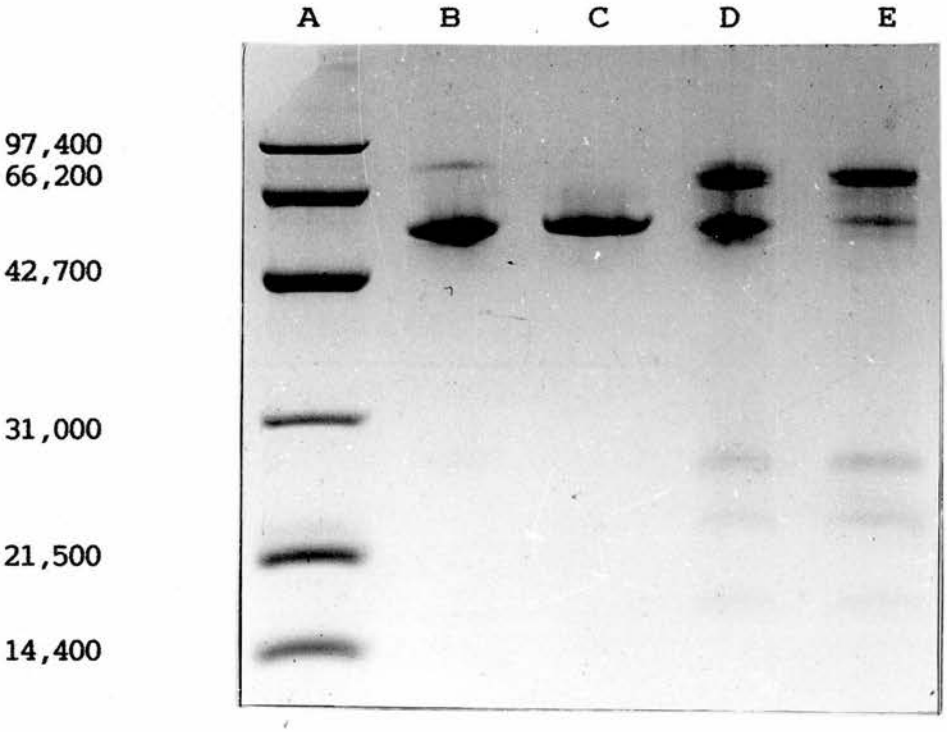
Estimation of relatative molecular mass of RSI and RSII.
The M_rs of known standards were plotted against the distance migrated from the top of the resolving gel (10%, Schägger and von Jagow, 1987). The distances migrated by RSI and RSII were measured and the M_r estimated from the y-axis.

Figure 3.7



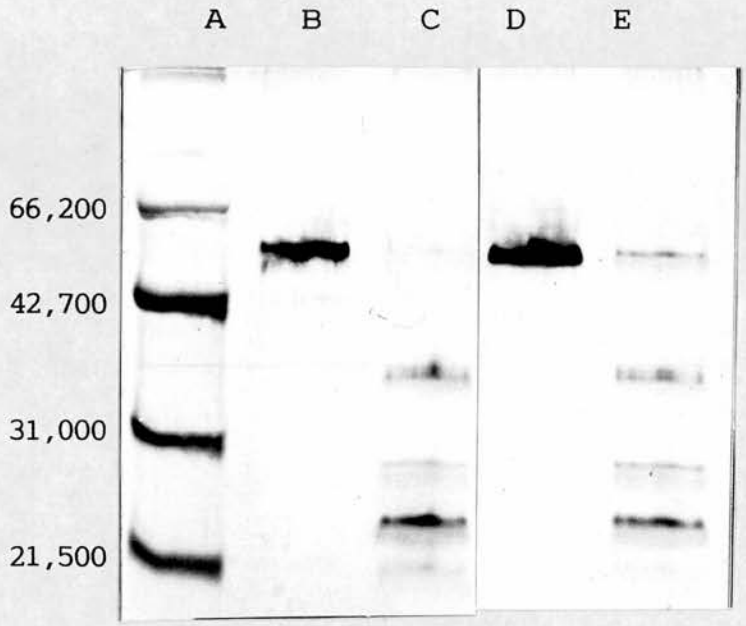
Electrophoresis of purified rat serpins.
Lane A, molecular weight standards. Lane B, RSI (2µg). Lane C, RSII (2µg). Proteins were visualised using Coomassie Blue. Electrophoresis was performed according to the method of Schagger and von Jagow (1987).

Figure 3.8



Electrophoresis of rat serpins and RMCPII-serpin complex.
Lane A, molecular weight standards (Bio-rad). Lane B, purified RSI (5µg). Lane C, purified RSII (5µg). Lane D purified RSI (5µg) + purified RMCPII (2.5µg). Lane E, purified RSII (5µg) + purified RMCPII (2.5µg). Note that Lanes D and E contain complex formed between rat serpin and RMCPII. proteins were visualised using Coomassie Blue. Electrophoresis was performed according to the method of chagger and von Jagow (1987).

Figure 3.9



Electrophoresis of purified serpins and serpins cleaved by RMCPI. Lane A, molecular weight standards (Bio-rad). Lane B, purified RSI (2µg). Lane C, purified RSI (2µg) + RMCPI (200ng). Lane D, purified RSII (2µg). Lane E, purified RSII (2µg) + purified RMCPI (200ng). Note that the rat serpins are cleaved to two major fragments with approximate M_r 25,000 and 37,000. RMCPI can be seen in lanes C and E migrating at M_r 29,000. Protein was visualised using silver stain.

In order to extend these findings, RMCPI was titrated into rat serum and the samples were electrophoresed on SDS-10%PAGE. The proteins were then transferred onto Immobilon and the blot probed with anti-RMCPI antibody. As can be seen (Figure 3.10) there are no higher molecular weight proteins detectable as there are when serum is titrated with RMCPII (Figure 3.3). This demonstrates that not only is RMCPI not inhibited by RSI or II, there does not seem to be a serpin in serum capable of inhibiting RMCPI.

3.2.3. Sequencing of serpins

Sequencing of the two serpin molecules was an essential step in determining the identity of both molecules. Were they the same molecule, the differences in M_r being due to post translational modification, or were the two molecules distinct products of separate genes?

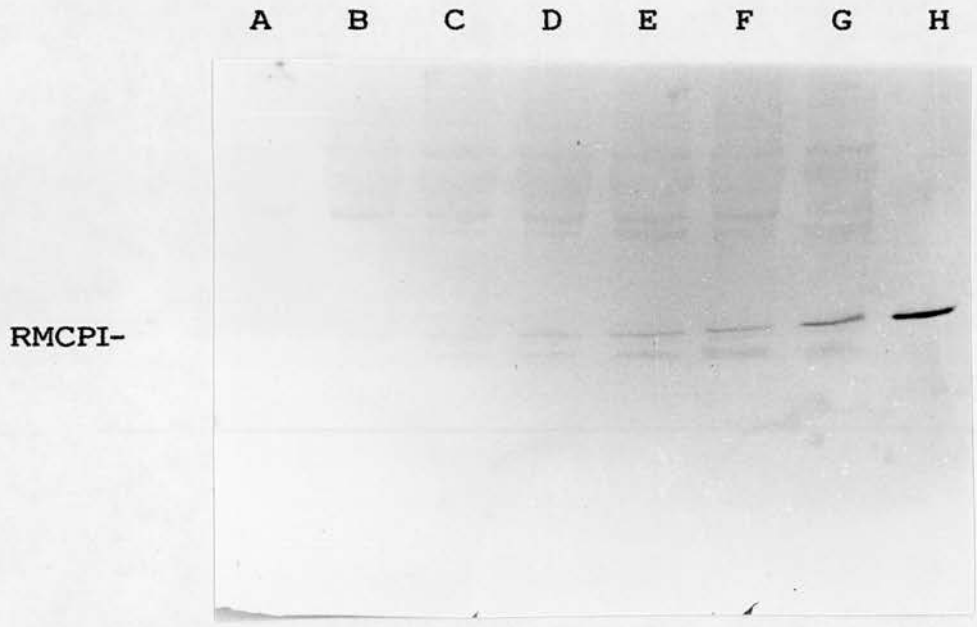
Sequencing was performed on an Applied Biosystems pulsed liquid phase machine (ABI477a). Samples were prepared for sequence analysis by chromatography of 50μg of protein on C-18 HPLC column, the largest peak was subsequently sequenced. The Amino-terminal sequence data for RSI and II is presented in Figure 3.11 where it is compared to the predicted amino-terminal sequence data for rat α-1-PI derived from nucleotide sequencing (Chao et al, 1990).

RSI is identical to rat α-1-PI over the first 17 residues, whilst RSII differs from RSI at the first residue only, where glutamic acid is replaced by threonine.

3.2.4. Glycosylation of serpins

Samples (2μg) of purified RSI and RSII were incubated for 18 hours at room temperature with 1 unit of PNGaseF (Boehringer Mannheim and see Materials and Methods, Chapter 2). The samples were electrophoresed on SDS-10%PAGE (see

Figure 3.10



Western blot of RMCPI titrated into rat serum.
Lanes A contains rat serum. Lanes B-G RMCPI titrated into rat serum at 100µg, 200µg, 300µg, 400µg, 500µg, and 1mg proteinase/mL rat serum. Lane H contains 250ng RMCPI. Proteins were electrophoresed as described (Schagger and von Jagow, 1987) and transfered to Immobilon. The blot was probed with ant-RMCPI antibody conjugated to horse radsih peroxidase. The migration of RMCPI is indicated.

Figure 3.11

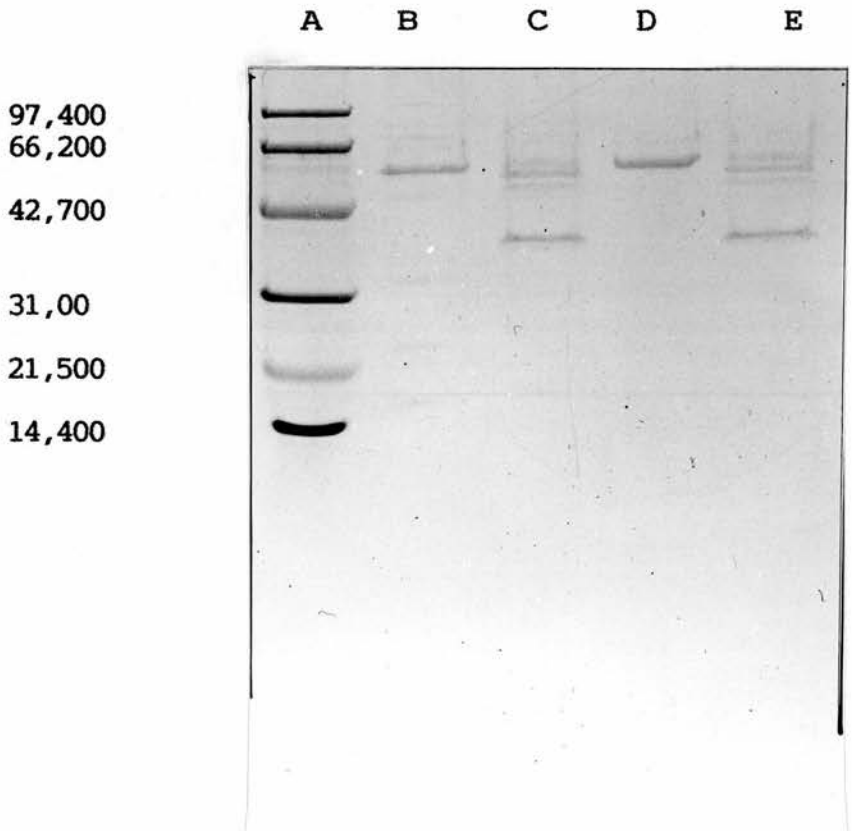
Position	1	2	3	4	5	6	7	8	9	10
α-1-PI	E	D	A	Q	E	T	D	T	S	Q
RSI	E	D	A	Q	E	T	D	T	S	Q
RSII	T	D	A	Q	E	T	D	T	S	Q

Position	11	12	13	14	15	16	17	18	19	20
α-1-PI	Q	D	Q	S	P	T	Y	R	K	I
RSI	Q	D	Q	S	P	T	Y			
RSII	Q	D	Q	S	P	T	Y	R	K	I

Amino-terminal sequence of RSI, RSII and rat α-1-PI (Chao et al, 1990)

Materials and Methods, Chapter 2) and the gel fixed and stained as described (see Materials and Methods, Chapter 2). From the result (Figure 3.12) it is obvious that both serpins are heavily glycosylated as both experience an apparent loss in M_r of 33%.

Figure 3.12



Deglycosylation of RSI and RSII
Lane A, Molecular weight standards (Bio-Rad). Lane B, RSI (500ng). Lane C, RSI (500ng) after incubation with PNGaseF (see Materials and Methods, Chapter 2). Lane D, RSII (500ng). Lane E, RSII (500ng) after incunation with PNGaseF (see Materials and Methods, Chapter 2). Samples were electrophoresed (Tricine buffer system) and proteins were visualised using Coomassie Blue.

3.3. Discussion

The work outlined above shows the presence of at least two members of the rat serpin superfamily which will inhibit RMCPII by forming essentially irreversible complexes.

The observation that no stable complex is formed between RMCPI and RSI or II, but that RMCPI degrades both the serpins is surprising given the level of homology between RMCPI and II. The inference from this observation is that the activity of RMCPI must be modulated in a different way from that of RMCPII.

From the amino-acid sequence analysis it can be seen that the two serpin molecules isolated are both likely to be transcripts of the SPI-1 locus in rats. This finding is verified by the genetic evidence (Inglis and Hill, 1990; Borriello and Krauter, 1990). RSI is most likely to be α-1-PI, whilst RSII is a variant of α-1-PI (α-1-PIb). These divergent forms of the same protein are most likely to have arisen as a result of a duplication/divergence event at the chromosomal level leading to a family of genes, each member encoding a serpin with a different target proteinase due to minor alterations in the region of the gene encoding the reactive site loop portion of the protein and at the N-terminus.

Neither of the molecules purified seem, from the amino-acid sequence analysis, to be a rat contrapsin, based on a comparison with the structure of mouse contrapsin (Inglis and Hill, 1990; Takahara and Sinohara, 1982). Nor do they appear to be RPI-1 or -2 (Kuehn et al, 1984). RPI-1 and -2 have M_r's of 66,000 and 65,000, much larger than RSI and II, a discrepancy that is too large to be explained by differences in SDS-PAGE. Also, RPI-2 is reported as inhibiting only trypsin out of the proteinases tested (Kuehn et al, 1984); the inability of RPI-2 to inhibit

chymotrypsin further discounts the possibility of it being RS-I or -II. It seems unlikely that RPI-1 and -2 are products from the same gene family as RSI and II as no protein in the correct range of M_r (65,000-66,000) was found to bind to the affinity column used to purify RSI and II. No evidence of any proteinase inhibitory activity except RSI and II was found to bind to the affinity column. The possibility that low level mRNA transcripts encoding other members of the genes from the rat SPI-1 locus may be translated cannot be ignored, but no significant concentrations of other molecules were detected in this study.

There is a difference in the M_r of RSI and II of about 2,500. If this difference is due to primary sequence then RSII is c.25 amino acids longer than RSI, but this cannot be verified until complete amino-acid sequence is available. The difference in M_r could be due to differences in glycosylation: serpins are glycosylated in general and RSI and II are no exception (see Figure 3.11).

The presence of two forms of α-1-PI in rat serum gives rise to speculation about the translation of other gene products from the SPI-1 locus. Are there low levels of 4-5 α-1-PI's in serum each with slightly different specificities? Or is there a developmental role for serpins? In the mouse it is known that foetal levels of serpins are 15% of adult levels, and it requires 25-30 days before adult levels are reached in the neonate (Lamontagne et al, 1981). Is one of the products from the SPI-1 locus a juvenile (in developmental terms) form of the inhibitor? Are some of the genes translated only as acute phase proteins? It is hoped to provide answers to these questions in the following chapters.

Chapter 4: **General Thermodynamic and Kinetic Considerations**

4. General Thermodynamic and Kinetic Considerations

4.1. Introduction

The kinetics which govern the interaction of a serpin and its target proteinase determine whether the serpin is likely to be a good inhibitor *in vivo* (Bieth, 1974; Boudier and Bieth, 1989, Smith and Johnson, 1985). Thus a serpin might inhibit a proteinase *in vitro* but the rate of reaction may be deplorably slow, rendering the serpin all but useless *in vivo*. Alternatively the dissociation rate constant may be high resulting in an unstable complex with rapid inactivation of available serpin as the proteinase complexes with the serpin, cleaves the P1-P1,' bond and dissociates leaving a non-functioning serpin.

The minimal scheme describing the interaction of serpins and proteinase is:

$$E + I \underset{k_{diss}}{\overset{k_{ass}}{\rightleftharpoons}} E{:}I$$

Where E and I stand for proteinase and serpin respectively and k_{ass} and k_{diss} are the association and dissocation rate constants respectively. The rate of association of E with I is given by equation 1:

$$\frac{-d[E]}{dt} = k_{ass}[E]\,[I] - k_{diss}[EI] \qquad (1)$$

If no significant dissociation of EI occurs during the association process, ie if the association reaction is measured over a time period considerably shorter than the

dissociation half life of the EI complex being studied, the second term of equation 1 can be neglected:

$$\frac{-d[E]}{dt} = k_{ass}[E]\,[I] \qquad (2)$$

Integration of equation 2 for $[E]_o=[I]_o$ yields

$$[E] = \left(k_{ass}.\,t+\frac{1}{[E]_o}\right)^{-1} \qquad (3)$$

The concentration of E,[E], at any time can be found from the initial rates of substrate hydrolysis. The association rate constant, k_{ass}, can be calculated by non-linear regression analysis (using the DNRP53 program of R.G. Duggleby, University of Queensland, Australia which fits experimental data to equation 3).

The K_i of the reaction between E and I is defined as k_{diss}/k_{ass}, which is equivalent to:

$$K_i = \frac{[E]\,[I]}{[EI]} \qquad (4)$$

Where the concentrations are those at equilibrium. By substituting for [EI] and [I]:

$$[EI] = ([E]_o - [E]) \qquad (5)$$

$$[I] = [I]_o - ([E]_o - [E]) \qquad (6)$$

(where $[E]_o$ is initial enzyme concentration and $[I]_o$ is initial inhibitor concentration) and re-arranging we get equation 7 (Bieth, 1974):

$$[E]^2 + (K_i + [I]_o - [E]_o)\ [E] - K_i = 0 \qquad (7)$$

which can be solved:

$$\frac{[E]}{[E]_o} = \frac{([E]_o - K_i - [I]_o) + ((([E]_o - K_i - [I]_o)^2 + 4K_i . [E]_o)^{1/2}}{2[E]_o} \qquad (8)$$

The constant K_i can thus be determined experimentally by reacting E with increasing amounts of I for 30 mins, followed by addition of substrate and the measurement of residual proteinase activity. The rate of steady state substrate hydrolysis is used to determined [E] for a given $[I]_o$. The fractional activity, $([E]/[E]_o)$, can then be used to determine the substrate-dependent K_i by fitting the data to equation 8 using non-linear regression analysis.

The rate constant which applies to the dissociation of the SEC, k_{diss}, can be determined from equation (9).

$$k_{diss} = K_i . k_{ass} \qquad (9)$$

4.2. Results:

4.2.1. Titration of proteinases and serpins

RMCP II, RMCP I and chymotrypsin were active-site titrated as described (Kezdy and Kaiser, 1970) using Cbz-L-Tyr-ONp as titrant, and trypsin was titrated with p-nitrophenyl-p'-guanidinobenzoate HCl as titrant (Chase and Shaw, 1970) . All serpins were back-titrated against RMCP II as follows; RMCP II (36 nM) was incubated with various amounts of purified serpin. After 5 min the residual activity of the proteinase was measured using Cbz-L-Tyr-ONp as substrate, absorbances at 410 nm were measured. Fractional proteinase activity ($[E]/[E]_o$) was plotted against I (mL) and the concentration of the serpin determined from the x-axis intercept of the extrapolated line through the experimental data.

4.2.2. k_{ass} of RSI and RSII

Equimolar amounts of proteinase and serpin (both active-site-titrated) were incubated in a test-tube. After a period of time (0-3 min), 50 μl aliquots were removed and added to a cuvette containing 950 μl of substrate in PBS (12.5 μM Cbz-L-Tyr-ONp for chymases, 125 μM BAPNA for trypsin) to measure the residual activity of the proteinase. This experiment was performed with two molarities of RMCP II: 9nM and 24nM. Molarity of chymotrypsin was 9.5 nM and molarity of trypsin was 6.25 nM. No inhibition of RMCPI was observed, therefore k_{ass} and K_i could not be derived.

To derive an estimate of the k_{ass}, the values of [E] obtained were plotted as 1/[E] against time. This plot allows a line with the equation

$$\frac{1}{[E]} = k_{ass} \cdot t + \frac{1}{[E]_o}$$

to be drawn. The estimate of k_{ass} derived from this manipulation was used in the DNRP53 program to derive a value of k_{ass} from non-linear regression analysis. Plots of typical experiments are shown (Figure 4.1), the theoretical curve (equation 3) fitted to experimental data can be seen in figure 4.2.

4.2.3. K_i of RSI and RSII with RMCPII

A standard concentration of RMCP II (36 nM) was incubated with increasing concentrations of serpin for 30 min at room temperature. The incubation mixture was then analysed for residual proteolytic activity using Cbz-L-Tyr-ONp (final molarity 100 μM). All measurements were made in a centrifugal analyser with a reaction volume of 250 μl PBS. Absorbances at 405 nm were measured.

A plot of

$$\frac{[I]}{(1-a)} \quad v \quad \frac{1}{a}$$

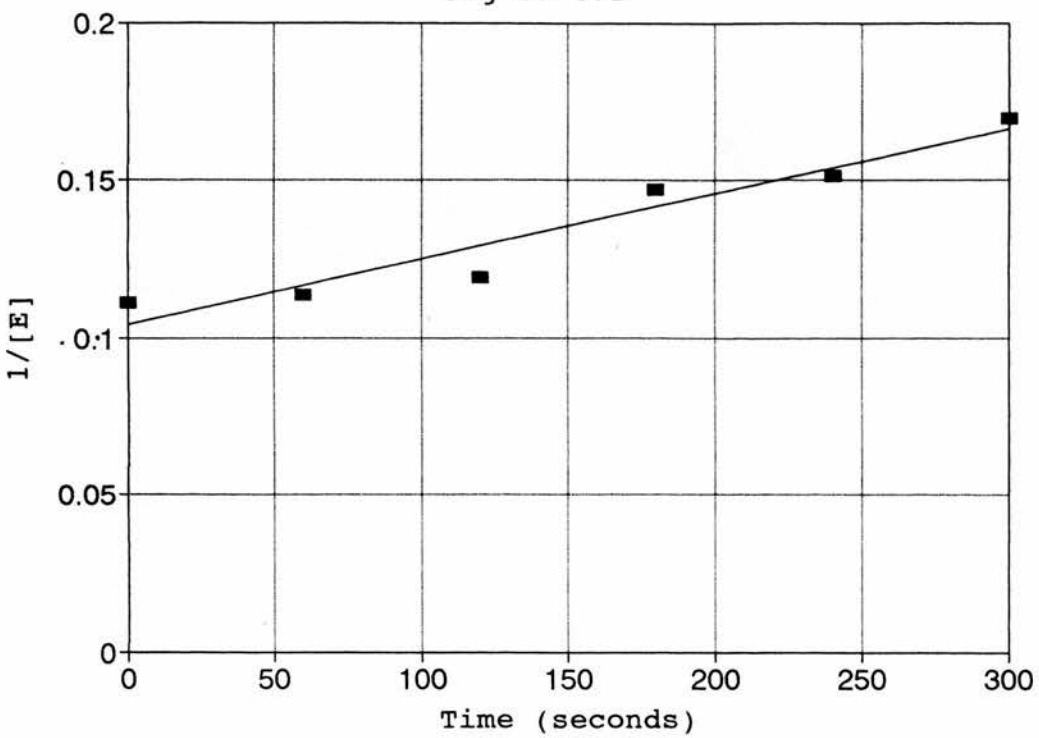
allowed a straight line to be drawn, the equation of the line is given by

$$\frac{[I]}{1-a} = \frac{K_i}{a} + [E]_o$$

The gradient of this line can be used as an estimate for the K_i, which can then be used to derive a more accurate value of K_i from the DNRP53 program. An example of this linear plot is shown in figure 4.3.

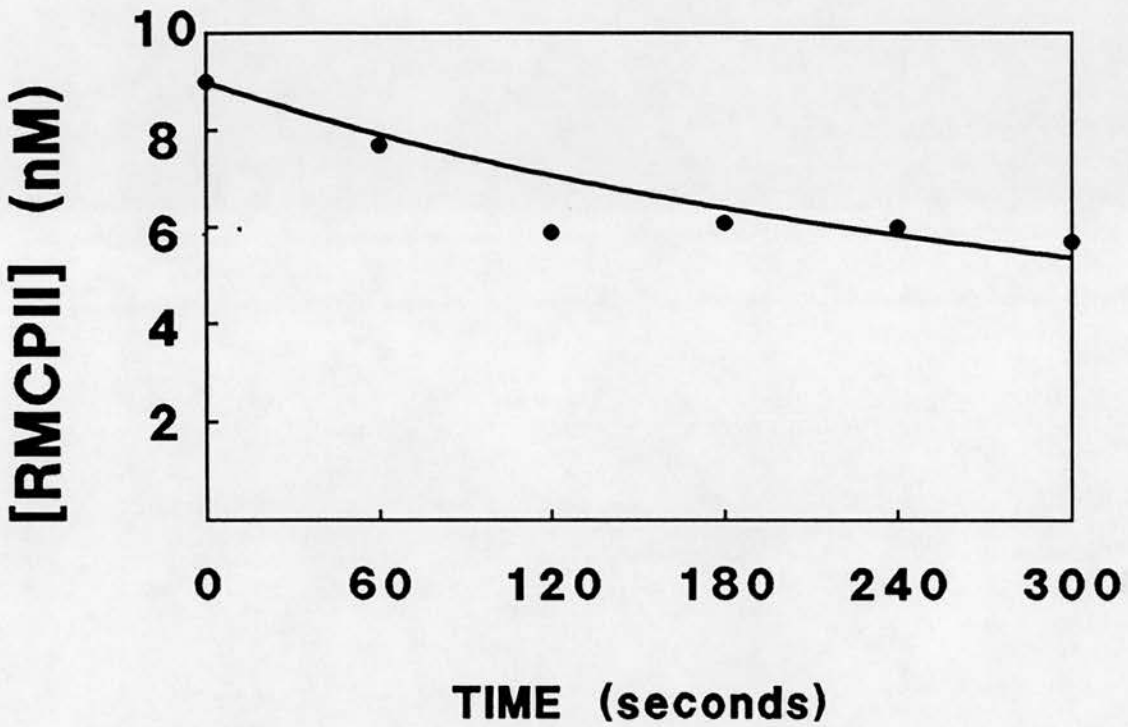
Figure 4.4 shows an example of an experiment in which the substrate-dependent K_i was determined in this way and a theoretical curve (equation 8) drawn through experimental data for the reaction between RMCP II and RSI

Figure 4.1



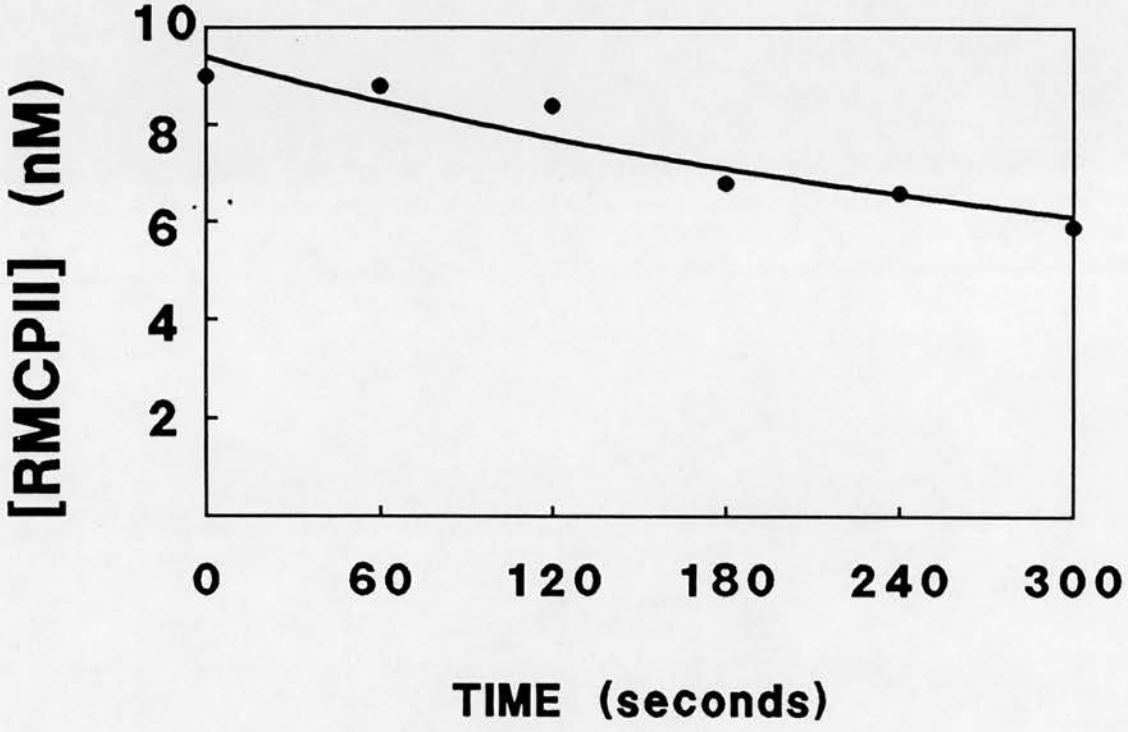
Plot of 1/[E] (enzyme activity) as a function of time.
The proteinase (RMCPII) is incubated with a serpin, in this case RSII, and aliquots are assayed at the times specified on the x-axis. The slope of this line, derived by linear regression, provides an estimate of k_{ass}. This estimate was used in the DNRP53 program of Duggleby (see text) which derives more reliable estimates of kinetic constants by an iterative, non-linear process (see text and Figure 4.2).

Figure 4.2a



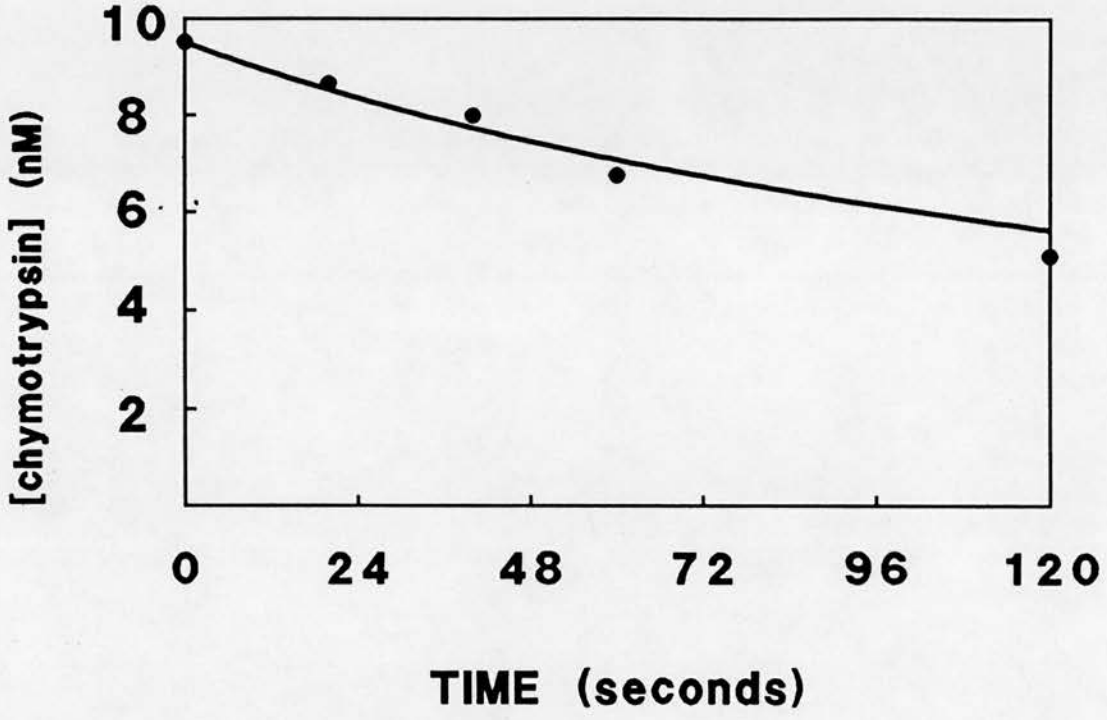
Determination of k_{ass} between RSI and RMCPII.
RMCPII was incubated with equimolar concentrations of RSI and residual enzyme activity (•) was assayed at times specified on the x-axis. The theoretical line (-) was fitted by non-linear regression using the DNRP53 program. Derived values are tabulated (Table 4.1a).

Figure 4.2b



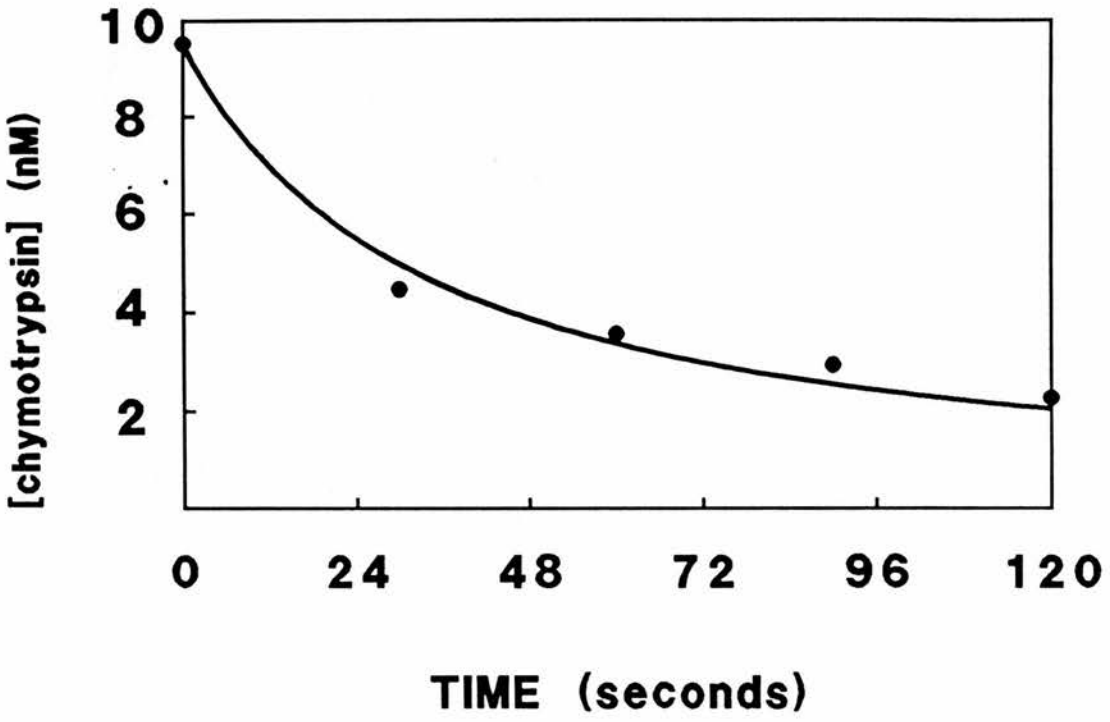
Determination of k_{ass} between RSII and RMCPII.
RMCPII was incubated with equimolar concentrations of RSII and residual enzyme activity (•) was assayed at times specified on the x-axis. The theoretical line (-) was fitted by non-linear regression using the DNRP53 program. Derived values are tabulated (Table 4.1b).

Figure 4.2c



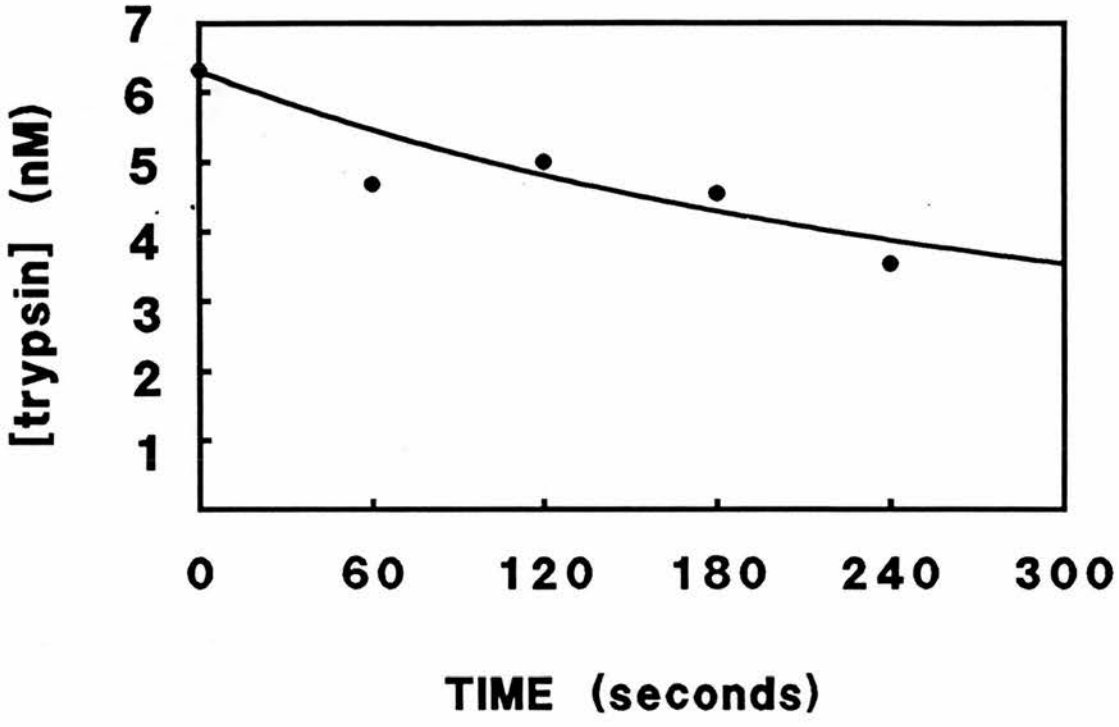
Determination of k_{ass} between RSI and chymotrypsin.
Chymotrypsin was incubated with equimolar concentrations of RSI and residual enzyme activity (•) was assayed at times specified on the x-axis. The theoretical line (-) was fitted by non-linear regression using the DNRP53 program. Derived values are tabulated (Table 4.1a).

Figure 4.2d



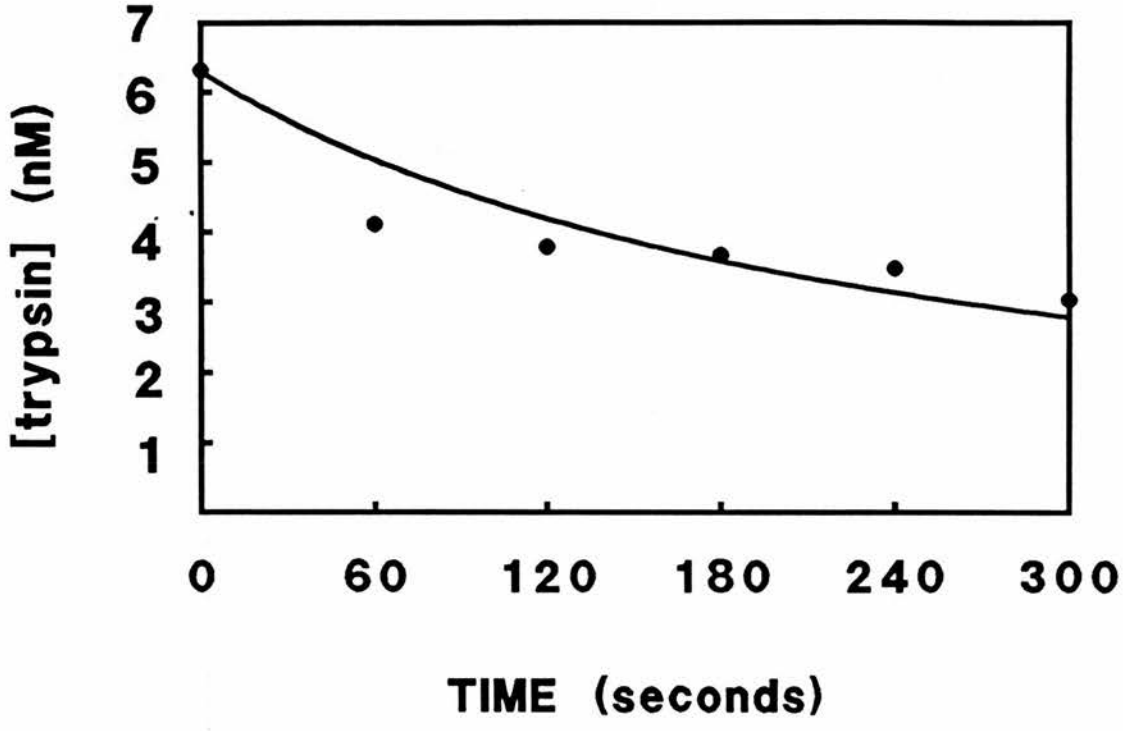
Determination of k_{ass} between RSII and chymotrypsin.
Chymotrypsin was incubated with equimolar concentrations of RSII and residual enzyme activity (•) was assayed at times specified on the x-axis. The theoretical line (-) was fitted by non-linear regression using the DNRP53 program. Derived values are tabulated (Table 4.1b).

Figure 4.2e



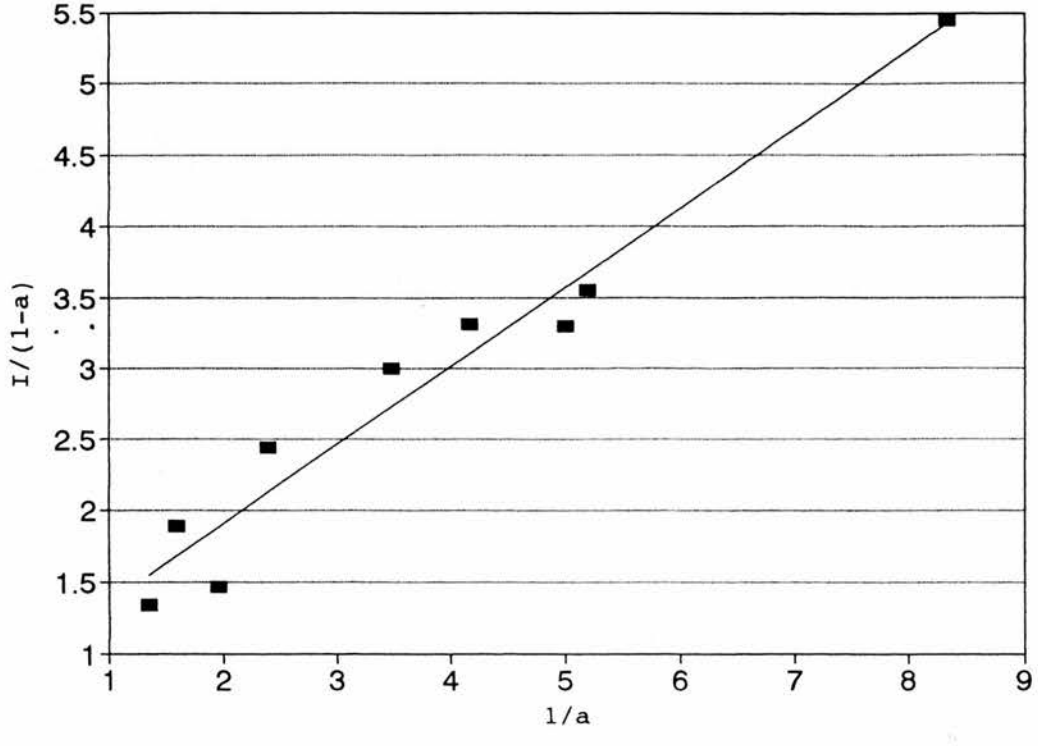
Determination of k_{ass} between RSI and trypsin.
Trypsin was incubated with equimolar concentrations of RSI and residual enzyme activity (•) was assayed at times specified on the x-axis. The theoretical line (-) was fitted by non-linear regression using the DNRP53 program. Derived values are tabulated (Table 4.1a).

Figure 4.2f



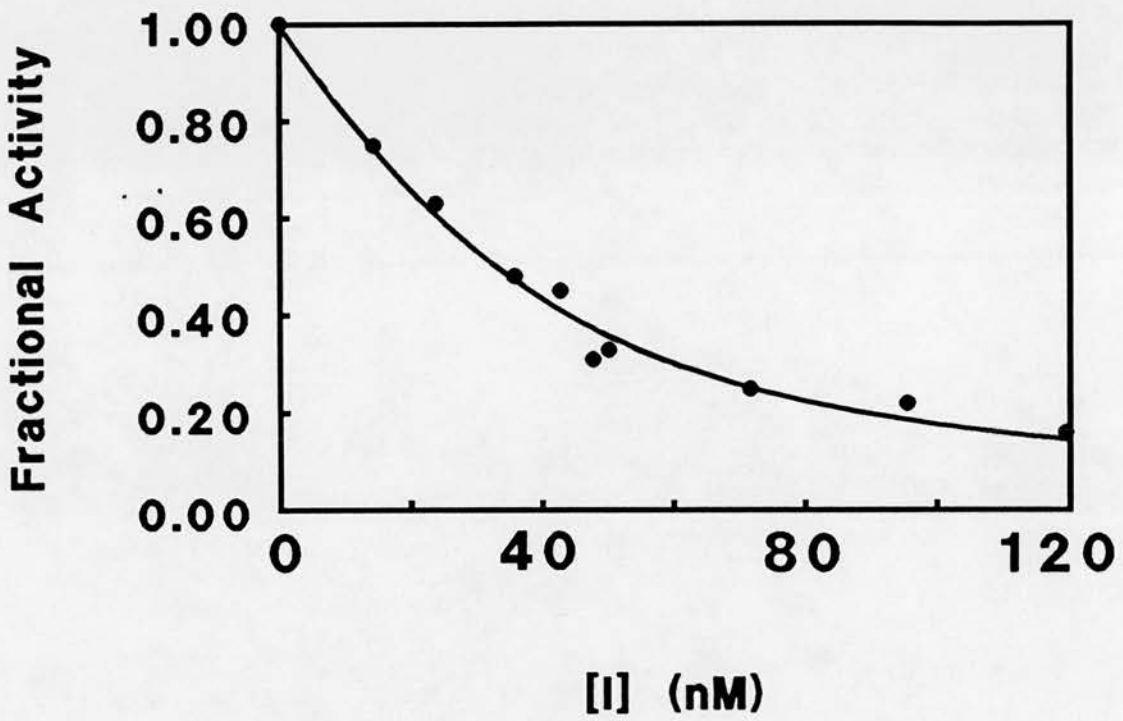
Determination of k_{ass} between RSII and trypsin.
Trypsin was incubated with equimolar concentrations of RSII and residual enzyme activity (•) was assayed at times specified on the x-axis. The theoretical line (-) was fitted by non-linear regression using the DNRP53 program. Derived values are tabulated (Table 4.1b).

Figure 4.3



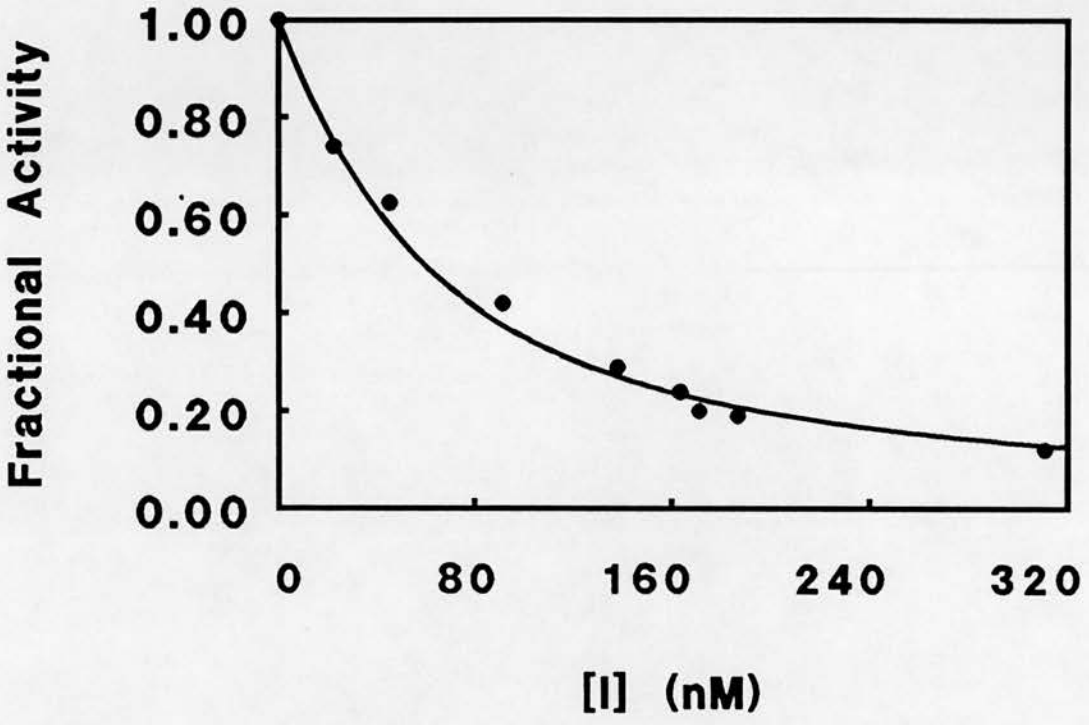
Plot of I/(1-a) as a function of 1/a.
The proteinase (RMCPII) was incubated with increasing concentrations of serpin (I) and fractional enzyme activity (a) measured (see text). The slope of this line, derived by linear regression, provides an estimate of K_i. This estimate was used in the DNRP53 program of Duggleby (see text) which derives more reliable estimates of thermodynamic constants by an iterative, non-linear process (see text and Figure 4.4).

Figure 4.4a



Determination of K_i^{app} between RSI and RMCPII.
RMCPII was incubated with increasing concentrations of RSI [I] and residual enzyme activity (•) was plotted as a function of [I]. The theoretical line (-) was fitted by non-linear regression using the DNRP53 program (see text). Derived values are tabulated (Table 4.1a).

Figure 4.4b



Determination of K_i^{app} between RSII and RMCPII.
RMCPII was incubated with increasing concentrations of RSII [I] and residual enzyme activity (•) was plotted as a function of [I]. The theoretical line (-) was fitted by non-linear regression using the DNRP53 program (see text). Derived values are tabulated (Table 4.1b).

and RSII. The substrate-dependent K_i (K_i^{app}) can be converted to the true K_i by using equation 10:

$$K_i = \frac{K_i^{app}}{(1 + \frac{[S]_o}{K_m})} \qquad (10)$$

The K_m for the RMCP II/Cbz-L-Tyr-ONp system was measured independently by classical means (Hanes plot) and found to be 2.46 μM in the buffer conditions used for all experiments described. All values of K_i are tabulated (Table 4.1).

4.2.4. k_{diss} RSI and II in complex with RMCPII

The k_{diss} was derived theoretically as described, all values are tabulated (Table 4.1).

4.3. Discussion

As can be seen from table 4.1, RSI and II are efficient inhibitors of RMCPII, trypsin and chymotrypsin. The values of k_{ass} derived are $\geq 10^5$ $M^{-1}s^{-1}$; this is typical of serpin-proteinase interactions (see Table 4.2). It should be noted that these association constants are 100-fold less than the figures expected of a serpin and its target proteinase such as α-1-PI and elastase (6.5 x $10^7 M^{-1}s^{-1}$), or α-1-antichymotrypsin and cathepsin G ($5x10^7 M^{-1}s^{-1}$,Beatty et al, 1980) . Therefore it is unlikely that either of the serpins isolated here have RMCPII as a specific target proteinase.

However, it can be seen that the association constant between RSII and bovine chymotrypsin is of the order of 10^6 $M^{-1}s^{-1}$. This is a particularly intriguing finding given that a specific rat antichymotrypsin has yet to be described, although it should be noted that this figure is similar to the constant derived for the interacton of bovine chymotrypsin and human α-1-PI (5.9 x 10^6, Beatty et al, 1980).Nonetheless the k_{ass} between RSII and chymotrypsin is 5 fold greater than the constant between RSI and chymotrypsin, suggesting the possibility of different P1 residues. Studies (Rubin et al, 1990) have shown the marked differences in association constants that arise as a result of changes to the P1 residue. Changing the leucine at residue 358 on α-1-antichymotrypsin to arginine results in a 1000-fold increase in the association constant for trypsin and a 20-fold decrease in the association constant for bovine chymotrypsin. The structural homologue of human antichymotrypsin in rodents is contrapsin which exhibits inhibitory capacity towards trypsin alone (Takahara and Sinohara, 1982) . Rodent serpins from the α-1-PI locus (SPI-1) have at least three reactive site P1 residues between them (Krauter

and Boriello, 1990) ; methionine, the classic antitrypsin P1 residue; leucine, the classic antichymotrypsin P1 residue; and tyrosine. This suggests that gene products from the SPI-1 locus have diverged thus allowing a range of proteinases to be targeted by proteins translated from the same gene cluster. However, given the range of serine proteinases that individual serpins can inhibit it would be foolish to speculate on the nature of the P1 residues in the rat serpins isolated based on kinetic data alone.

The nanomolar K_i of RSI and RSII for RMCPII indicates their efficiency as inhibitors *in vitro* (Smith and Johnson, 1985). Obviously the efficiency of RSI and II as inhibitors *in vivo* depends on the concentrations of these molecules in body fluids and tissues. Complexes formed between RSI and RMCPII have a half life of 2.4 hours, whilst the figure for RSII and RMCPII is 1 hour. Evidence (Pizzo et al, 1988) exists to suggest that the clearance time of complexes from the body is of the order of 1-2 hours. From the kinetic data presented here it would seem that RSI, when compared to RSII is the better inhibitor of RMCPII . There might be an inhibitor which has a better inhibition constant for RMCPII (RSI-RMCPII=3.6 x 10^{-10}M) but it is obviously not present in concentrations great enough to have an effect on the level of RMCPII in serum. Certainly it is not present in quantities which allow its purification. This is not to deny the importance of low level potent inhibitors of serine proteinases. From the data presented here, RSI would seem to have the most important role in the inhibition of RMCPII. High levels of RSI in serum during anaphylactic shock may be enough to compensate for the average association constant discussed (*vida supra*). Levels of RSI and II in rat tissues will be

discussed below, as will the effect of parasitic infection on the levels of these acute-phase proteins.

Table 4.1a Kinetic data for RSI

Enzyme	k_{ass} $M^{-1}s^{-1}$	k_{diss} s^{-1}	K_i M	$t_{1/2}d$ hr
RMPCI	NI	NI	NI	NI
RMCPII	2.2×10^5	7.92×10^{-5}	3.6×10^{-10}	2.4
chymotrypsin	6×10^5	ND	ND	ND
trypsin	4.13×10^5	ND	ND	ND

Table 4.1b Kinetic data for RSII

Enzyme	k_{ass} $M^{-1}s^{-1}$	k_{diss} s^{-1}	K_i M	$t_{1/2}d$ hr
RMPCI	NI	NI	NI	NI
RMCPII	1.65×10^5	1.65×10^{-4}	1.0×10^{-9}	1.2
chymotrypsin	3.17×10^6	ND	ND	ND
trypsin	6.68×10^5	ND	ND	ND

ND- not determined, NI- not inhibited

Table 4.2

Values for k_{ass} between various serine proteinases and serpins

Enzyme	Serpin	k_{ass} ($M^{-1}s^{-1}$)
bovine chymotrypsin	recombinant anti-chymotrypsin[1]	6-8 x 10^5
trypsin	recombinant anti-chymotrypsin[1]	$<5 \times 10^2$
PPE	recombinant anti-chymotrypsin[1]	8 x 10^3
HNE	recombinant anti-chymotrypsin[1]	$<10^3$
bovine chymotrypsin	human antichymotrypsin[2]	6 x 10^4
human chymotrypsin	human antichymotrypsin[2]	1 x 10^4
human cathepsin G	human antichymotrypsin[2]	5 x 10^7

[1]Rubin, ~~[1]Rubin,~~ H., Zhi, M. W.,Nickbarg, E., McLarney, S., Naidoo, N., Scheonberger, O. L., Johnson, J. L., and Copperman, B. S. (1990) *Journal of Biological Chemistry* **265** 1199-1207.

[2]Beatty, ~~[2]Beatty,~~ K., Bieth, J., and Travis, J. (1980) *Journal of Biological Chemistry* **255** 3931-3934

Chapter 5: Generation and Characterisation of Antibodies.

5. Generation and Characterisation of Antibodies.

5.1 Introduction

When studying the biochemical and physiological properties of a protein, it is of paramount importance to have an assay method which will allow detection of the protein in a variety of circumstances. Many proteins, especially enzymes, can be detected by monitoring a chemical reaction and other, such as serpins by monitoring the decreased activity of a target proteinase. However many enzyme families can display overlapping substrate specificities and most serpins will inhibit more than their "target" proteinase. More specific techniques are, therefore, required to identify and quantify serpins in complex biological samples such as plasma or tissue homogenates.

Antibodies can surmount many of the aforementioned problems. An antibody can detect a protein in a variety of tissue sources. Polyclonal antibodies allow detection of antigenically similar proteins. The specificity can be improved by affinity purifying antibody preparations using a specific antigen as ligand. Monoclonal antibodies, although highly specific, must be used in conjunction with another antibody if quantification of antigen by sandwich ELISA is the aim. A single monoclonal antibody can be used in a competition ELISA, but one needs a ready supply of antigen. The multivalent nature of polyclonal antibodies can lead to the formation of lattices of antigen and antibody, leading to precipitation of the antibody-antigen complex. This property of polyclonal antibody preparations allows for the development of Radial ImmunoDiffusion assays (RID). Directly labeled antibody preparations can be used to probe Western blots, or it can be used to localise antigens in immunocytological studies.

Antibodies can also be used to affinity purify antigen rapidly and efficiently. Antibodies can also prove useful in the screening of expression libraries, leading to the elucidation of gene sequence data.

Many antibody preparations raised against one member of a family of related proteins will cross react with all members of the family, or even structurally similar but functionally dissimilar proteins (Lafferty *et al*, 1991). This very cross reactivity can, however, be extremely useful in the elucidation of phylogenetic relationships. Selection of a unique peptide as antigen can increase to specificity of the antibody preparation.

Antibodies have been employed extensively in the study of serpins. The localisation of protease nexin-I, a potent inhibitor of urinary plasminogen activator (u-PA) and thrombin, to the amyloid plaques found in Alzheimers patients was achieved with antibodies specific for protease nexin-I. Western blotting studies subsequently localised this serpin in the cerebro-spinal fluid of normal individuals (Festoff *et al*, 1989). Further immunocytochemical studies showed that an antigenically similar molecule was found in mouse skeletal muscle. The molecule co-localised with acetylcholine receptor clusters at synaptic neuro-muscular junctions, implicating protease nexin-I in the remodeling events that occur in synapse formation and elimination (Festoff *et al*, 1991).

Antibodies have also been employed in the quantitation of serpins in a variety of species. Electro-immunoassay was utilised to determine the concentrations of α-1-PI and contrapsin in mice (Takahara and Sinohara, 1981), the antibody prepartion was whole antiserum raised against mouse α-1-PI. LaMontagne *et al*, (1981) implemented rocket immuno- -electrophoresis and ELISA to determine

concentrations of α1-PI in mouse serum and a variety of other biological fluids. The ELISA, based on a sheep polyclonal antibody raised against purified mouse α-1-PI was used to assay bronchoalveolar lavage, and a concentration of 65μg α-1-PI/lung was reported. Detectable concentrations of α-1-PI were reported in amniotic fluid, bile, breast milk and gastrointestinal washings. The ontogeny of α-1-PI in mice was also monitored.

A monoclonal antibody against a novel epitope of human α-1-PI has been shown to detect certain disease conditions (Silvestrini *et al*, 1990). The concentration of human α-1-PI was estimated to be 0.4mg/mL using this antibody in ELISA. Further evidence from the same group suggested that the epitope on α-1-PI recognised by this monoclonal antibody is only present on 19% of α-1-PI isoforms; the true concentration of α-1-PI in serum is nearer to 2-3mg/mL. The effects of diet on α-1-PI concentrations was investigated in rats using a radial immunodiffusion assay (RID) with a rabbit anti-rat α-1-PI antiserum to quantify this serpin in control and experimental animals (Lewis *et al*, 1985). This study reported concentrations of 1.9mg α-1-PI/mL in normal rats.

Antibodies have also been essential in the elucidation of tissue distribution of mast cell proteinases (and by extension the distribution of mast cell populations) in the rat, mouse and sheep (Woodbury and Neurath, 1978; Woodbury and Miller, 1981; King *et al*, 1986; Huntley *et al*, 1990b; Newlands *et al*, 1987; Huntley *et al*, 1987). Antibodies were instrumental in determining the effects of parasite infection on concentrations of mast cell proteinases. In these latter studies the technique utilised was ELISA analysis.

In this present study the aim was to detect and quantify serpin concentrations in a variety of rat tissues and in serum. A polyclonal antibody (Rab1 α-RSI/II) had been raised against RMCPII-serpin complex (see chapter 3). This antibody preparation had been used as an affinity ligand in the subsequent purification of RSI and II (see chapter 3). It was reasoned that the best approach to developing a sandwich ELISA, to quantify serpin concentrations, would be to raise monoclonal antibodies. In order to raise a panel of antibodies directed againstnative and cleaved serpins mice were immunised with mouse mast cell proteinase Ie in complex with RSII. Since mouse mast cell proteinase Ie would not be antigenic antibodies would most probably be raised against epitopes unique to cleaved serpins and against native serpin. Antibodies detecting cleaved serpin (ie serpin in complex with proteinases), should not react with uncleaved serpin and vice versa. In this way, it was hoped to quantify complexes and serpins in traumatised rats. Uncleaved serpin was also used as antigen in the attempt to generate monoclonal antibodies.

For reasons which will be dealt with below it was also necessary to generate further polyclonal antibody preparations. The following studies describe the generation and characterisation of the polyclonal and monoclonal antibody preparations utilised in Chapters 6 and 7 to detect and quantify serpin concentrations in rat serum and tissues.

5.2 Results

5.2.1. Rabbit Polyclonal anti—rat serpin:RMCPII complex antibody

A rabbit was immunized with RMCPII—serpin complex as described in Materials and methods (see Chapter 2). By double diffusion, immuno-precipitation occurred at dilutions of 1/32 (Figure 3.4) and following a further immunization the rabbit was bled out fourteen days later, the anti—serum was collected in 5mL aliquots and stored at —20°C. Antibody specific to RSI/II (Rab1 α-RSI/II) was purified as described in chapter 3.

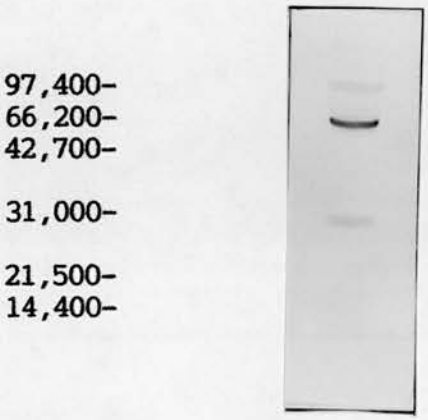
5.2.2. Specificity of the rabbit polyclonal antibody.

Normal rat serum and serum from *N. brasiliensis* infected animals was electrophoresed on polyacrylamide gels under reducing conditions (see Materials and Methods, Chapter 2) and the resolved proteins transferred to Immobilon as described (see Materials and Methods, Chapter 2). The blot was probed with Rab1 α-RSI/II conjugated to horse radish peroxidase (Po) (see Materials and Methods, Chapter 2). Rab1 α-RSI/II-Po detects a protein(s) that co-migrates with uncleaved rat serpins (Figure 5.1) in control rat serum.

5.2.3. Mouse Monoclonal anti—(cleaved rat serpin) antibody

To generate further probes, attempts were made to raise monoclonal antibodies specific for RSI. Mice were immunized with RSI (100μg) and after 4 weeks and a further immunization spleen cells were obtained and hybridomas were generated (see Materials and Methods, Chapter 2). However, hybridomas did not survive for more than 72 hours, and no successful fusion was detected. The fusion partner, NS-0 cell line, may have been responsible for the failure of this experiment (D. Deane and G. Entrican, personal communication).

Figure 5.1

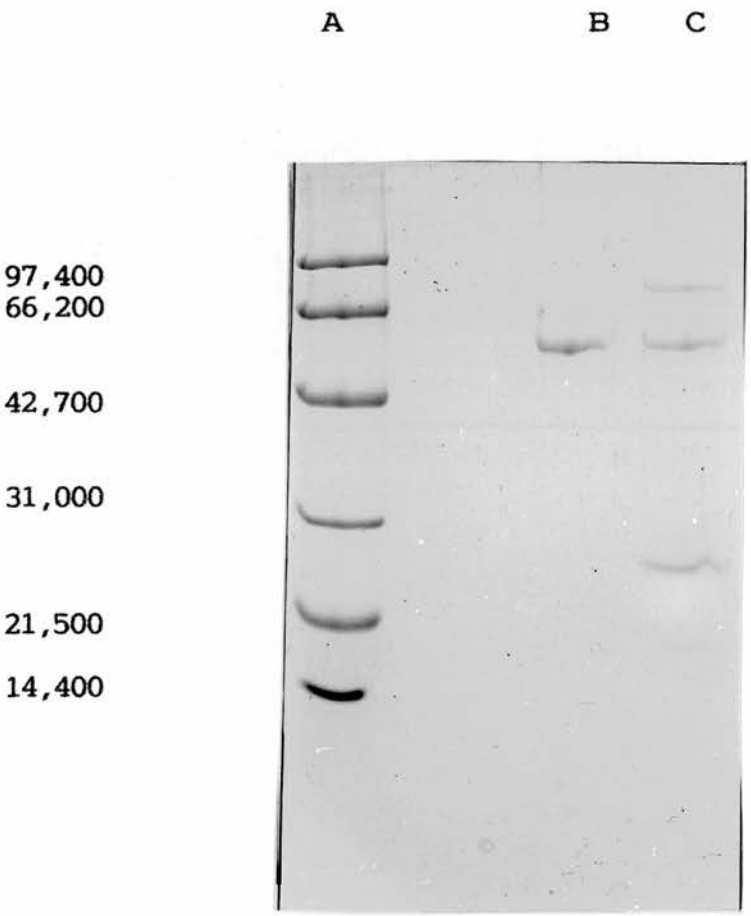


Specificity of Rab1 α-RSI/II
Normal rat serum was electrophoresed under reducing conditions (tricine buffer system) and protein was transferred to Immobilon as described (see Materials and Methods, Chapter 2). The blot was probed with Rab1 α-RSI/II-po. The darkly staining band is rat serpin. Note the staining of bands at apparent M_r 72,000 and 25,000.

A fresh source of NS-0 cells was obtained and a second attempt to raise monoclonal antibodies was made. The antigen was complex formed between RSII and mouse mast cell proteinase-1E (MMCP-IE), an isoform of the soluble chymotrypsin-like proteinase from murine intestinal mast cells (Newlands *et al*, 1993). Complex formation between RSII and MMCP-1E was shown by SDS-PAGE after incubation of varying concentrations of RSII and MMCP-1E at room temperature for 5 minutes (Figure 5.2). Since a structural rearrangement of the serpin is believed to occur at complex formation, it was reasoned that this would provide novel epitopes, and that MMCP-1E would not be immunogenic. Some epitopes generated by complex formation would therefore be specific for complex, and certain antibodies that recognised novel epitopes generated by complex formation would bind to cleaved RSII but not to native serpin. This approach would possibly allow development of a complex-specific ELISA.

Three Mice were immunised *intra peritoneally* with 100μg of RSII:MMCP-1E. The animals were further immunised with 100μg of antigen at 3 and 5 week intervals and spleen cells obtained as described (see Materials and Methods, Chapter 2). The hybridoma fusions were plated out in 96 well plates and assayed every 2—3 days by ELISA using RSII-MMCP 1E as coating antigen (see Materials and Methods, Chapter 2). Four hybridoma cultures tested positively for RSII:MMCP-1E reactivity, but only one clone retained reactivity with the antigen after 5 days. This culture was sub—cloned 3 times and used to generate ascitic fluid in mice as described (see Materials and Methods, Chapter 2).

Figure 5.2



Formation of RSII-mouse mast cell proteinase Ie complex
Lane A, molecular mass standards (Bio-Rad). Lane B, purified RSII (1µg). Lane C, RSII (1µg) + MMCP Ie (0.5µg). Proteins were electrophoresed according to the method of Schägger and von Jagow, 1987. Note the presence of RSII-MMCP Ie complex in lane C. MMCP Ie migrates at M_r 26,000. Proteins were visualised using Coomassie Brilliant Blue.

5.2.4. Specificity of the mouse monoclonal antibody

5.2.4.1. ELISA

Supernatant from the third sub—clone was tested for antigenic reactivity by ELISA. The results of this assay (Figure 5.3) suggest that the monoclonal antibody (MAb1) binds RSI and II only when they are complexed to a serine proteinase, in this instance RMCPII. Uncleaved RSI and II do not bind to the antibody. The antibody binds to RSII in complex with MMCP-1E, as the hybridoma cultures were selected on this criterion.

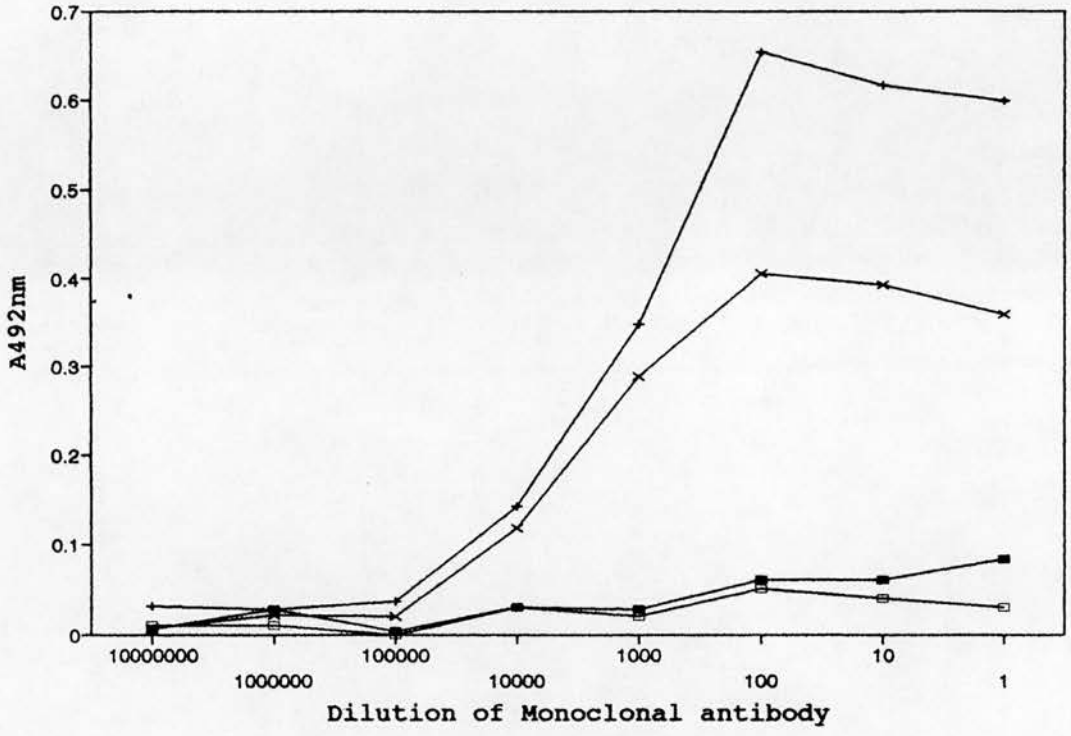
5.2.4.2. . Western Blotting.

The specificity of the MAb1 was further analyzed by Western blotting. Normal rat serum was electrophoresed on a polyacrylamide gel under reducing conditions (see Materials and Methods, Chapter 2). The proteins were transferred to Immobilon as described (see Materials and Methods, Chapter 2) and the blot was probed with MAb1-Po (Figure 5.4a). Under these conditions MAb1 recognises uncleaved serpin. The specificity of MAb1 was also determined on Western blots of non-reducing gels. Control serum, serum taken 14 days after infection with *N. brasiliensis* and purified RMCPII:serpin complex were electrophoresed under non reducing conditions, and the subsequent blots probed with MAb1-po (Figure 5.4b). This result demonstrates that MAb1-po can be used to detect, if not quantify, complex under certain conditions.

5.2.5. Sheep polyclonal anti—rat serpin antibody.

Attempts to use a combination of Rab1 α-RSI/II and the mouse monoclonal anti—complex antibodies in the development of a capture ELISA were unsuccessful.

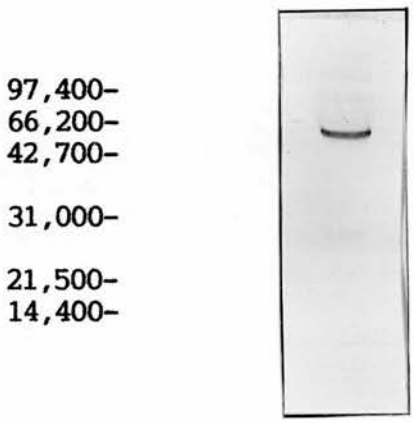
Figure 5.3



Cross reactivity of MAb1

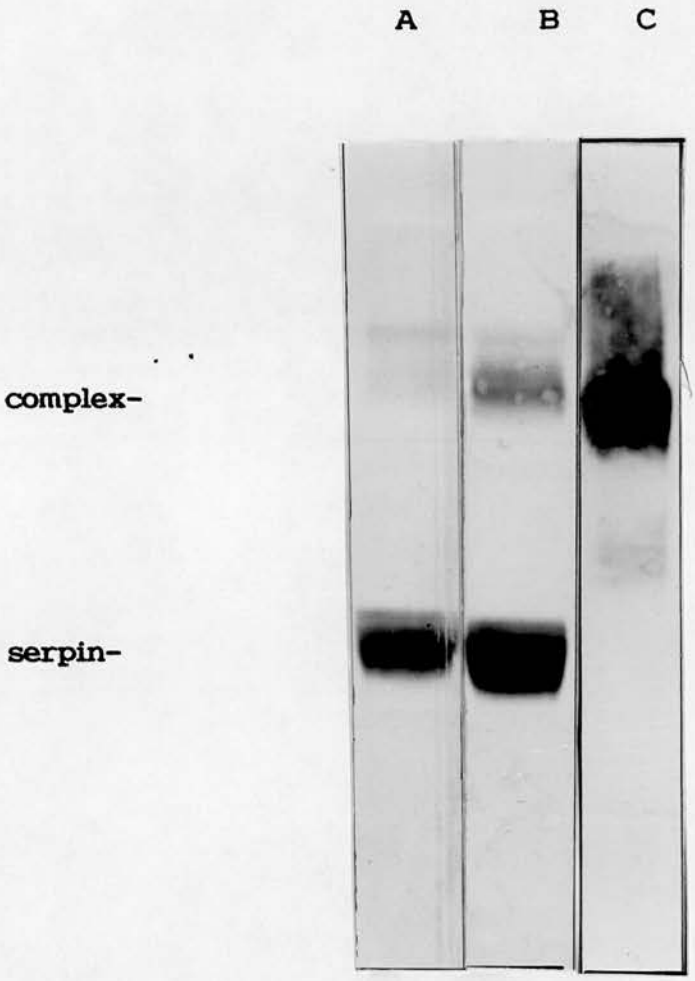
Antigen was bound to polystyrene plates as described (see Materials and methods, Chapter 2). The supernatant from the 3rd subclone was of the monoclonal antibody was then added to the wells in a series of 10-fold dilutions as indicated on the x-axis. the colour reaction was generated by adding a sheep anti-mouse antibody conjugated to horse radish peroxidase, and the OPD substrate. MAb1 is cross reactive with RSI-RMCPII complexes (+-+) and RSII-RMCPII complexes (x-x) under these conditions. It does not react with uncomplexed RSI (■-■) or RSII (□-□). Reasons for the higher cross-reactivity with RSI-RMCPII complexes are discussed in the text.

Figure 5.4a



Specificity of MAb1 antibody
Normal rat serum was electrophoresed under reducing conditions (tricine buffer system) and protein was transferred to Immobilon as described (see Materials and Methods, Chapter 2). The blot was probed with MAb1-po. The darkly staining band is rat serpin.

Figure 5.4b



Specificity of MAb1 antibody.
Lane A, Normal rat serum. Lane B, Serum from a rat taken 14 days after primary infection. Lane C, purified RS-RMCPII complex. Samples were electrophoresed under nonreducing conditions and the proteins transferred to Immobilon as described (see Materials and Methods, Chapter 2). The blot was probed with MAb1-po. Note the staining of complex in lane B.

A second polyclonal antibody was raised. A sheep was immunised with RSI. After 4 weeks antiserum was tested by Ouchterlonie double diffusion, against a solution of RSI. A precipitin arc was detected at an antiserum dilution of 1/2 (Figure 5.5a). The animal was given a second immunisation and bled again 4 weeks later. The antiserum was again assayed by double diffusion. A precipitin arc was visible at an anti—serum dilution of 1/32 (Figure 5.5b). Antiserum (200mL) was drawn from the animal and frozen in 10mL aliquots.

Sheep antibody was affinity purified against RSI/II-RMCPII complex as described (see Materials and Methods, Chapter 2). Purified antibody (Sh1 α-RSI/II, 2mg/mL) was stored at —20°C in 100μl aliquots.

5.2.6. Specificity of sheep polyclonal antibody by Western Blotting.

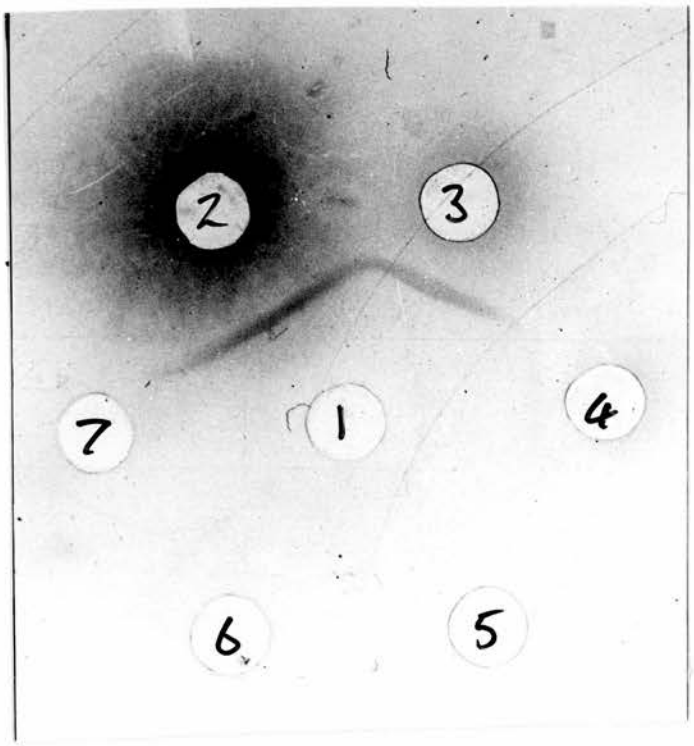
The specificity of Sh1 α-RSI/II was determined by Western blot analysis. Rat serum was transferred from a reducing gel to Immobilon and the blot was probed with Sh1 α-RSI/II-po. The result (Figure 5.6) shows a protein(s) migrating at Mr approximately 54,000.

5.2.7. Development of Capture ELISAs

The availability of 2 polyclonal antibodies and one monoclonal antibody permitted a choice of 8 different capture ELISAs. As has already been mentioned (*vide supra*) the assay wherein antigen was captured by Rab1 α-RSI/II and detected by MAb1 was not successful. The likely reason is that Rab1 α-RSI/II and MAb1 recognise a common epitope.

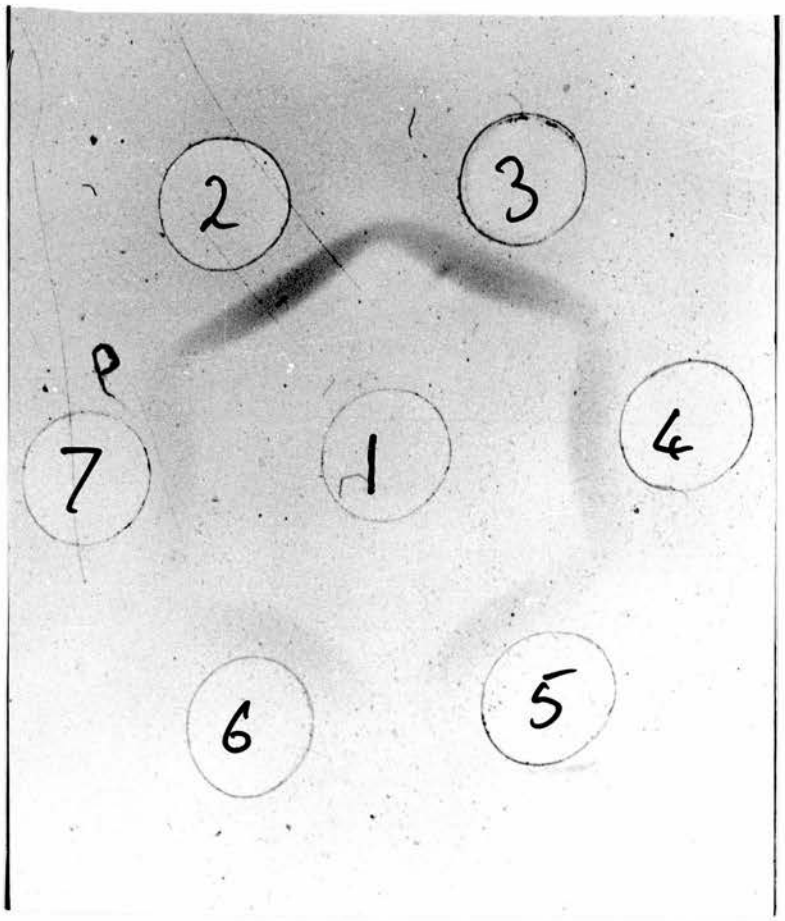
Attempts to capture with MAb1 were not successful, possibly because the antibody was not affinity purified from the ascitic fluid. Thus, attempts to coat an

Figure 5.5a



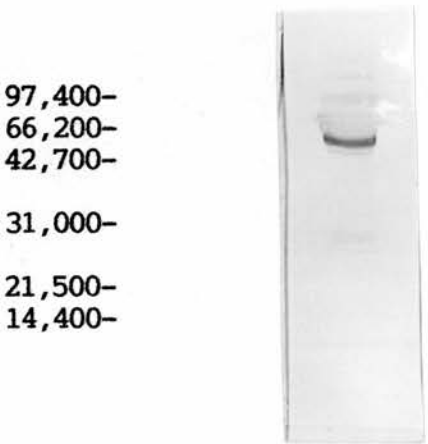
Double diffusion assay of sheep anti-RSII antiserum.
The central well (1) contains 5µL of RSII (100µg/mL). Wells 2-7 contain 5µL of sheep anti-RSII antiserum diluted 1/2-1/32.

Figure 5.5b



Double diffusion assay of sheep anti-RSII antiserum
The central well contains 5µL RSII (100µg/mL), and wells 2-7 contain 5 µL sheep antiRSII antserum diluted 1/2-1/32.

Figure 5.6



Specificity of Sh1 α-RSI/II antibody
Normal rat serum was electrophoresed under reducing conditions (tricine buffer system) and protein was transferred to Immobilon as described (see Materials and Methods, Chapter 2). The blot was probed with Sh1 α-RSI/II. The darkly staining band is rat serpin.

ELISA plate with MAb1 would not have resulted in a high enough concentration of antibody to be effective as a capture antibody.

This left 5 antibody combinations, of which it was decided to investigate further ELISA (1) with Sh1 α-RSI/II as the capture antibody and MAb1 as detection antibody, ELISA (2) with Sh1 α-RSI/II capture antibody and Rab1 α-RSI/II for detection, and ELISA (3) with Rab1 α-RSI/II as the capture antibody and Sh1 α-RSI/II for detection.

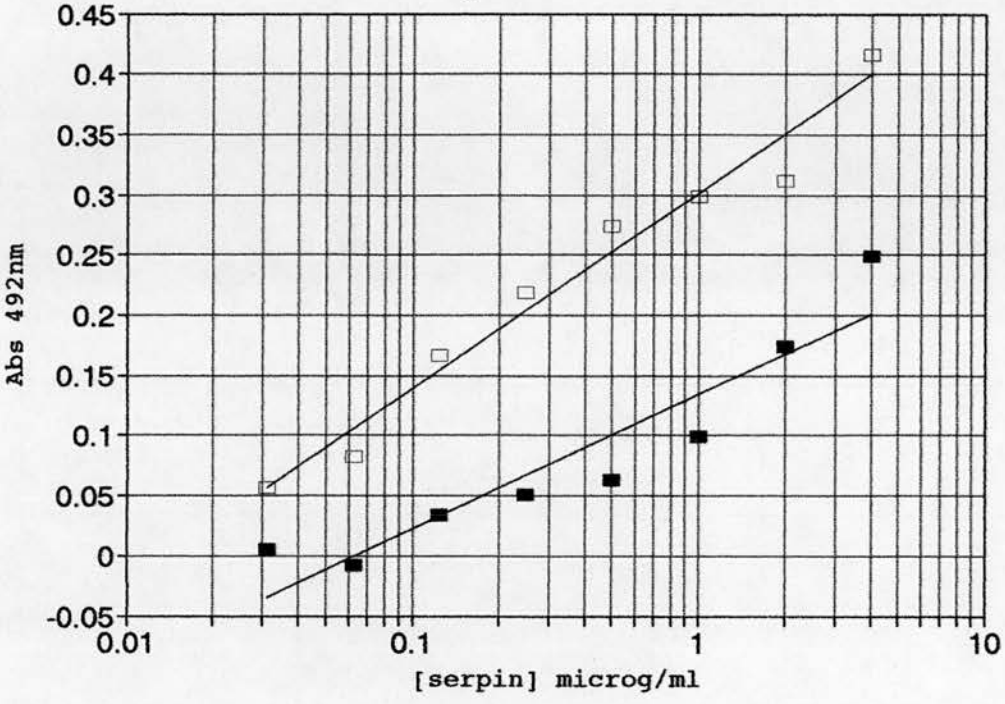
5.2.7.1. ELISA (1) employing Sh1 α-RSI/II as the antigen capture antibody and MAb1 as the antigen detection antibody.

Microtitre plates were coated with Sh1 α-RSI/II at a concentration of 1μg/mL. A broad range of concentrations of RSI and II was used to generate a standard curve. Captured antigen was detected with MAb1-Po. The results (Figure 5.7) suggest RSII is detected with a greater sensitivity than RSI. The apparent bias is greater than 5-fold with respect to RSII detection.

5.2.7.2. ELISA (2) employing Sh1 α-RSI/II as the antigen capture antibody and Rab1 α-RSI/II as the antigen detection antibody.

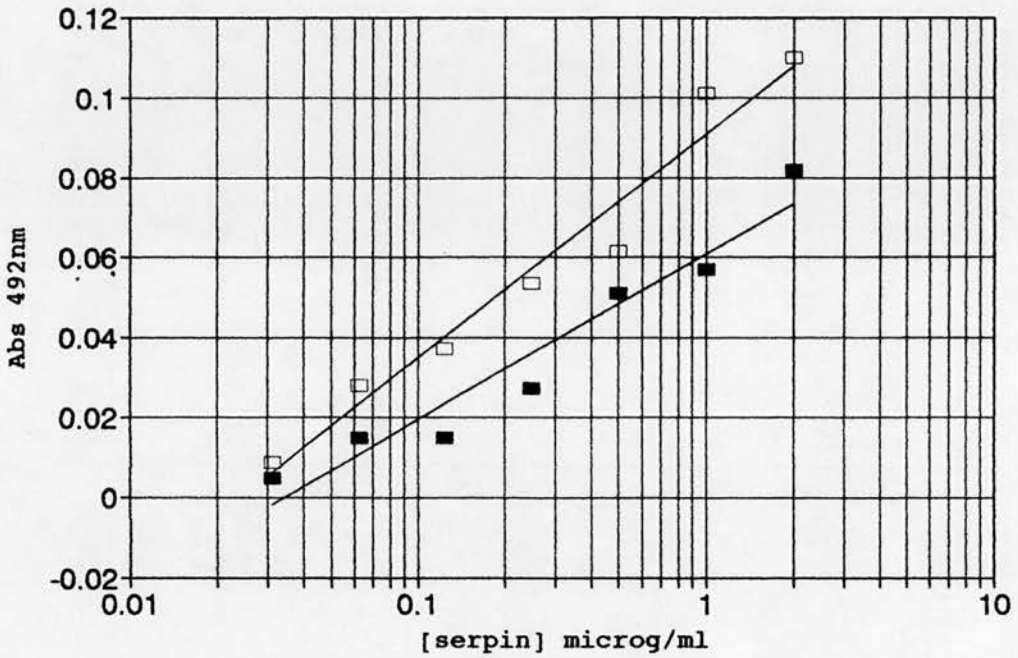
Microtitre plates were coated with Sh1 α-RSI/II (1 μg/mL). A broad range of concentrations of RSI and II was used to generate a standard curve. Captured antigen was detected with Rab1 α-RSI/II-Po. This ELISA (Figure 5.8) is also biased towards detection of RSII, although the sensitivity of detection is only 2.5-fold greater for RSII. This is less than the > 5-fold sensitivity that ELISA (1) displays (see Figure 5.7). The colour generation in this ELISA was lower than for ELISA (1) for the same incubation period.

Figure 5.7



Detection of purified serpins by ELISA 1
Polystyrene plates were coated with Sh1 α-RSI/II polyclonal antibody (1µg/mL) then washed and blocked as described (see Materials and Methods, Chapter 2). Serial dilutions of RSI (■) and RSII (□) were incubated in the wells for an hour. The plates were washed and captured antigen was detected by MAb1 conjugated to horse radish peroxidase and reacted with OPD (a peroxidase substrate). Colour generation is plotted as a function of serpin concentration. This assay is biased towards detection of RSII.

Figure 5.8



Detection of purified serpins by ELISA 2
Polystyrene plates were coated with Sh1 α-RSI/II polyclonal antibody (1μg/mL) then washed and blocked as described (see Materials and Methods, Chapter 2). Serial dilutions of RSI (■) and RSII (□) were incubated in the wells for an hour. The plates were washed and captured antigen was detected by Rab1 α-RSI/II-Po and reacted with OPD (a peroxidase substrate). Colour generation is plotted as a function of serpin concentration. This assay is biased towards detection of RSII.

5.2.7.3. ELISA (3) employing Rab1 α-RSI/II as the antigen capture antibody and Sh1 α-RSI/II as the antigen detection antibody.

Microtitre plates were coated with Rab1 α-RSI/II (1μg/mL). The same concentration range of RSI and II was used as in the previous experiments. Captured antigen was detected with Sh1 α-RSI/II-Po. This ELISA was again biased towards detection of RSII, the bias being approximately 10-fold in favour of RSII (Figure 5.9).

5.2.7.4. Further purification of rabbit and sheep polyclonal antibodies.

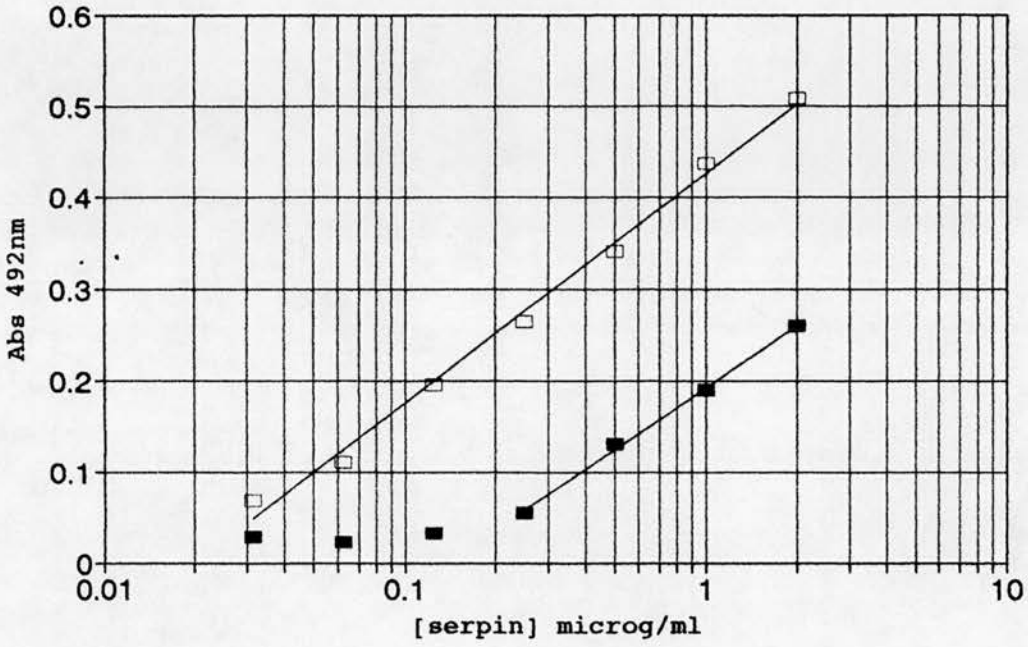
All of the ELISAs investigated display a bias towards the detection of RSII. From the purification studies of Chapter 3 it is known that RSI and II are present in serum, but the ratios are unknown. Any assay system that shows greater sensitivity towards one of the serpins in rats will give grossly incorrect results when used to quantify serpin concentrations, particularly if RSI and II are not present in approximately equal ratios.

An attempt was made to minimise the bias towards RSII seen in the ELISAs. Antibodies from rabbit and sheep antisera were affinity purified using RSI-sepharose 4B or RSII-sepharose 4B. All sheep antibodies that bound to RSI-sepharose 4B subsequently bound to RSII-sepharose 4B, suggesting that RSI and II have a similar epitope that is recognised by the sheep antiserum. It was possible to obtain antibody preparations from rabbit antiserum that bound to RSI-sepharose 4B but not to RSII-sepharose 4B (Rab2a α-RSI) or vice-versa (Rab2b α-RSII).

5.2.7.5. ELISA 4

When Rab2a α-RSI and Rab2b α-RSII were used as antigen capture antibodies in ELISA it became apparent these antibodies (Rab2a α-RSI and Rab2b

Figure 5.9

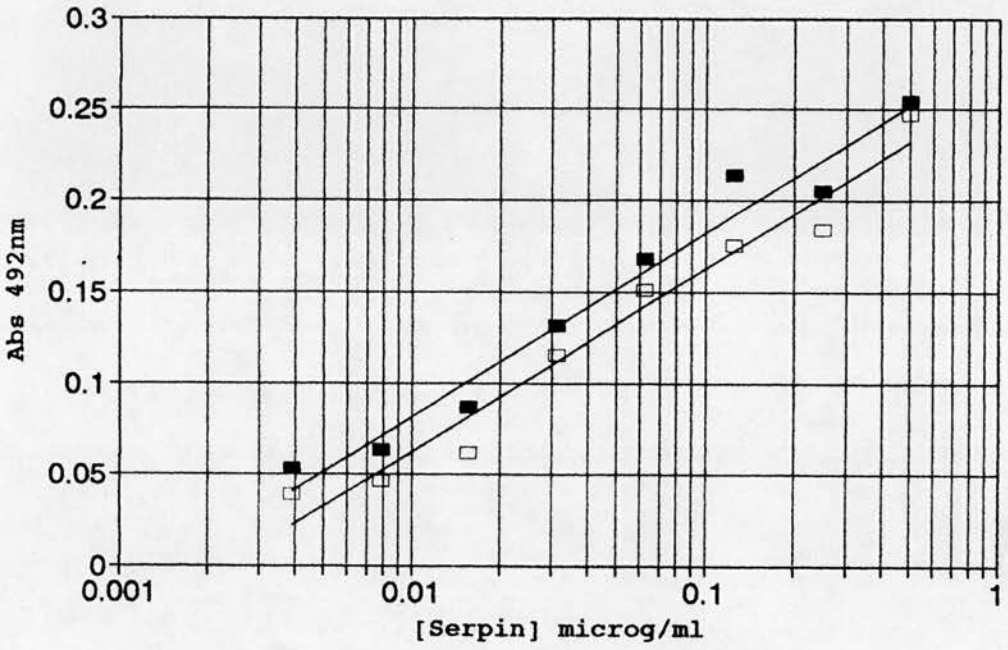


Detection of purified serpins by ELISA 3
Polystyrene plates were coated with Rab1 α-RSI/II polyclonal antibody (1µg/mL) then washed and blocked as described (see Materials and Methods, Chapter 2). Serial dilutions of RSI (■) and RSII (□) were incubated in the wells for an hour. The plates were washed and captured antigen was detected by Sh1 α-RSI/II-Po and reacted with OPD (a peroxidase substrate). Colour generation is plotted as a function of serpin concentration. This assay is biased towards detection of RSII.

α-RSII) were cross reactive with both RSI and RSII (data not shown). However ELISA (4) employing Rab2a α-RSI as the antigen capture antibody with Sh1 α-RSI/II-Po for detection proved to recognise RSI and RSII with only a 2-fold bias towards RSI (Figure 5.10). This was the lowest partiality observed to date. Rab2a α-RSII was not a good capture antibody; colour generation was very reduced with Sh1 α-RSI/II-Po for detection.

ELISA (4) was used to quantify serpin concentrations in rat serum and tissue samples in control rats and helminth infected rats (see Chapter 6) because of this low bias. Mab1 was used to probe Western blots of samples electrophoresed on nonreducing gels as it appeared to demonstrate the highest specificity.

Figure 5.10



Detection of purified serpins by ELISA 4
Polystyrene plates were coated with Rab2 α-RSI/II polyclonal antibody (1µg/mL) then washed and blocked as described (see Materials and Methods, Chapter 2). Serial dilutions of RSI (■) and RSII (□) were incubated in the wells for an hour. The plates were washed and captured antigen was detected by Sh1 α-RSI/II-Po and reacted with OPD (a peroxidase substrate). Colour generation is plotted as a function of serpin concentration. This assay is biased towards detection of RSI, however the degree of bias is less than that evident in ELISA's 1,2, and 3.

5.3 Discussion

Apart from Rab1 α-RSI/II, which was raised to facilitate the purification of rat serpins, the main purpose in generating the antibodies characterised in this study was to expedite the detection and quantification of serpins and serpin complexes in plasma and tissues. To this end it was imperative that the antibodies should be highly specific for their target antigens. Only one antibody (Sh1 α-RSI/II) was raised specifically against uncleaved serpin. This antibody was shown to be specific for serpins by Western blotting (Figure 5.6). Rab1 α-RSI/II was raised against RMCPII:serpin complexes and, by removing cross reactivity against RMCPII, it was possible to obtain a preparation that was specific for serpins by Western blotting. More importantly this antibody preparation was used to affinity purify RSI and RSII. Thus two serpin-specific antibody preparations were available to develop ELISA's.

The quantification and detection of serpins is complicated by the presence of uncleaved serpin and serpin complexed to proteinases in biological samples. Many workers are aware of the limitations of immunological techniques. In order to quantify ATIII:factor IX complexes in human blood (Takahashi *et al*, 1991), it was necessary to remove free ATII by absorbtion onto barium citrate. Complex was then detected with a combination of ATIII antibodies and factor IX antibodies. Further complications can arise if the epitope on the serpin or proteinase is obscured when the molecule is in a complex. Kurokawa *et al* (1991) have described a monoclonal antibody directed against tissue type plasminogen activator (tPA) which will not bind to tPA complexed to plasminogen activator inhibitor 1 (PAI-1).

An antibody with specific binding to a free proteinase can be used to quantify free proteinase.

MAb1 was generated against RSII:MMCPIe with the goal of developing an ELISA specific for complex, this approach was partially successful. Uncleaved serpin bound to a plate is not detected by MAb1, complexed serpin is detected (Figure 5.3). However, MAb1 will detect uncleaved serpin when used to probe Western blots of gels run under reducing conditions (Figure 5.4a), the antibody will also detect uncleaved serpin and complexed serpin when used to probe Western blots made from nonreducing gels (Figure 5.4b).

Uncleaved serpin captured in an ELISA (1) by Sh1 α-RSI/II was detectable with MAb1. This was unexpected as MAb1 was presumed to be complex specific, detection of uncleaved serpin by Western blotting was conjectured to be a result of the appropriate epitope being exposed by electrophoresis and blotting. The fact that complex was undetected under reducing conditions was thought to be due to steric hindrance by the proteinase component of the complex, hindrance that was not so critical under nonreducing conditions. It may be that the conformation of the serpin:proteinase complex is subtly altered when bound by Sh1 α-RSI/II, and this change is enough to allow MAb1 access to its epitope.

An additional problem with ELISA (1), was the bias towards detection of RSII. This bias was also noted when Rab1 α-RSI/II was used to detect antigen captured by Sh1 α-RSI/II (ELISA (2)). This suggests that the sheep antibody may have a higher affinity for, and an inherent bias towards RSII. Using Rab1 α-RSI/II as capture antibody and Sh1 α-RSI/II as detection antibody further exacerbated this bias. Further evidence of this inherent bias was found in radial immunodiffusion

assays using sheep antiserum (data not shown). It was decided to try and isolate RSI and RSII specific antibodies from the affinity purified rabbit (Rab1 α-RSI/II) and sheep (Sh1 α-RSI/II) antibody preparations. This resulted in the production of rabbit antibodies which bound to RSI-Sepharose 4B (Rab2 α-RSI) or RSII-Sepharose 4B (Rab2 α-RSII). Rab2 α-RSI antibodies were used as capture antibodies in ELISA (4), with Sh1 α-RSI/II used as the detection antibody. Sheep antibodies that bound to RSI-Sepharose 4B also bound to RSII-Sepharose 4B, indicating that RSI and RSII had similar epitopes that would bind to Sh1 α-RSI/II. ELISA (4) was deemed to be the most suitable as there was good colour development, and minimal bias towards RSI. The lack of bias was crucial to the choice of ELISA. Approximately equal masses of RSI and II were obtained from each purification procedure and this is the limit of the information regarding the ratios of RSI and II at present. ELISA (4) would be used to quantify serpin concentrations in helminth infected rats. If the serpins should be differentially expressed as a result of such an infection, a biased assay would only distort the true picture.

ELISA (4) would quantify serpin and serpin:proteinase complexes in plasma and tissue homogenates. Although detection of complex, distinct from serpin, was possible by probing Western blots of nonreducing gels, it was not possible to quantify serpin:proteinase complex unequivocally. It may be possible to develop a specific ELISA for complex using anti-RMCPII antibody as a capture antibody, and serpin antibody as a detection reagent, or vice versa. Such ELISA's have been developed for the detection of neutrophil elastase and elastase:α-1-PI complexes (Hamaguchi *et al*, 1991).

The difference between concentrations determined by ELISA (4), and a complex-specific ELISA would obviously provide a more accurate value for uncleaved serpins. Such an ELISA would also be very useful in determining that fraction of RMCPII which is inactive and bound to serpin, when used in conjunction with a RMCPII specific ELISA.

In conclusion, three antibody preparations were successfully raised against serpins. All of these were shown to be serpin specific by Western blotting analysis of serum. MAb1 was capable of detecting complex under certain conditions. ELISA (4) was able to detect RSI and II with a slight bias towards detection of RSI, but this was lower than the bias towards RSII apparent in ELISAs (1), (2), and (3). No assay capable of quantifying complex alone was developed, although such an assay should be possible by utilising antibodies specific for cognate proteinases in conjunction with serpin specific antibodies. Equipped with such techniques it was decided to investigate the distribution of serpins in a variety of tissues and in plasma (see chapter 6).

Chapter 6: Serpins as Acute Phase proteins in Helminth Infection

6. Serpins as acute phase proteins in Helminth infection

6.1. Introduction.

6.1.1 The acute phase response.

Inflammation comprises a series of cellular changes, which can facilitate phagocytosis and elimination of microorganisms. This is followed by proliferation of connective tissue cells and repair of the intracellular matrix. Both events require the complex control of various cell types at a local level. The constellation of systemic responses that accompany trauma and inflammation, regardless of cause, is termed the acute phase response.

There are approximately 30 plasma proteins in the acute phase response. They are all produced in increased amounts in the liver in inflammation. When classified according to function it is clear that they all have roles to play in inflammation or the healing process that follows (Kushner and Mackiewicz, 1987). The serpins α-1-PI and α-1-antichymotrypsin are part of this response, with 2-4 fold increases in production during the response (Kushner and Mackiewicz, 1987). There are also negative acute phase proteins, which include albumin and prealbumin. There is little evidence that these negative acute phase proteins play an active part in inflammation and the reduced concentrations may simply reflect redistribution in the extravascular space (Ballantyne *et al,* 1973).

The serpins involved in the acute phase response are primarily concerned with modulating the activity of the cellular proteinases released during infection. In man, α-1-PI inhibits leucocyte elastase and α-antichymotrypsin inhibits granule derived cathepsin G.

6.1.2. The Acute Phase Protein Response in Helminth Infection in Rodents.

In a study to determine the effects of infection with *Trichinella spiralis* on the hepatic responses it was discovered that this parasite does not elicit the acute phase response (Stadnyk et al, 1990). Similarly, intestinal infection with *N. brasiliensis*, did not elicit a response although infection via the cutaneous route, which involves a stage in the lung, did elicit the acute phase response. This and the results with *T. spiralis* suggest that intestinal helminth infections do not invoke systemic hepatic responses.

6.1.3. Mast Cell Proteinases in the Gut during parasite infection.

Rat Serpins I and II inhibit RMCPII and mouse intestinal mast cell proteinase, but not RMCPI (see chapters 3, 4 and 5). If these serpins have a physiological role to play in modulating the activity of mast cell proteinases, then it might be a beneficial evolutionary trend to have both serpins and proteinases expressed in the same tissues.

There are detectable concentrations of serpins in liver, lung and serum (Travis and Salvesen, 1983). The concentrations of RMCPII in liver (8.27μg/g tissue) and lung (11μg/g tissue) have been quantified (Huntley *et al*, 1990b) but most RMCPII is located in the gastrointestinal tissues (Table 6.1).

Enteric RMCPII is within mast cells of the mucosal and submucosal layers of the tissue (Gibson *et al*, 1987). In fact $>$98% of enteric mast cells stain exclusively for RMCPII. As a result RMCPII is almost the exclusive mast cell proteinase in the gut from the duodenum to the colon (Gibson *et al*, 1987). There are also substantial concentrations of RMCPII in the gastric and rectal mucosae (Gibson *et al*, 1987).

During intestinal nematodiasis there is recruitment of mast cells in the gastrointestinal mucosa, as discussed (see chapter 1). This recruitment is coincident with the presence of increased concentrations of RMCPII in serum in the rat and the expulsion of the parasite from the host (see chapter 1). Similar events also occur in mice after infection with the parasite *T. spiralis* (Huntley *et al.* 1990a). The distribution of MMCP-I, like RMCPII, is predominantly in the gastrointestinal tract of normal mouse (Huntley *et al*, 1990a). After infection with the parasite *T. spiralis*, the concentrations of MMCP-1 (at day 10) increase 30-fold in the stomach, 4,000-fold in the duodenum, 2,000-fold in the jejunum, 2,000-fold in the ileum and 1,000-fold in the colon (Huntley *et al*, 1990a) when compared with values in uninfected controls.

6.1.4. The effect of *N. brasiliensis* on the expression of rat serpins.

Although the modulation of concentrations of RMCPII by nematode infection is well documented (see section 6.1.3) the specific effect of infection on the serpin inhibitors of RMCPII is not. In this chapter the acute phase response was investigated by measuring the concentration of rat serpins.

The strategy was to examine the concentrations of serpins in serum and then to quantify serpins in tissues which had been extensively perfused to remove plasma proteins. This approach would possibly detect tissue specific serpins, if they existed. The techniques employed, Western blot analysis and ELISA with the antibody preparations discussed in Chapter 5, would allow not only the quantification of serpin concentrations in serum and perfused tissue, but would also render the identification of distinct, yet antigenically similar proteins possible. Non denaturing gel electrophoresis was used to analyse the serum and perfused tissue samples. This

technique was chosen as MAb1 would not detect complexed serpin under reducing conditions (data not shown) but would detect complex in serum from rats infected with *N. brasiliensis* (Figure 5.5).

6.2 Results

6.2.1. Concentrations of Serpins in normal rat tissues and serum.

The first stage of this study involved determining the concentrations of serpins in normal rats. The ELISA (4) employing Rab2a α-RSI as the capture antibody and Sh1 α-RSI/II as the detection antibody made no distinction between RSI & II, (see chapter 5).

Serum samples were taken from control animals prior to whole body vascular perfusion as outlined (see Materials and Methods, Chapter 2). Perfused tissues were treated as described (see Materials and Methods, Chapter 2). All samples were assayed by ELISA (4) using RSI as the standard protein. The results are tabulated (table 6.1).

Table 6.1 Concentration of serpins in serum and perfused rat tissues.

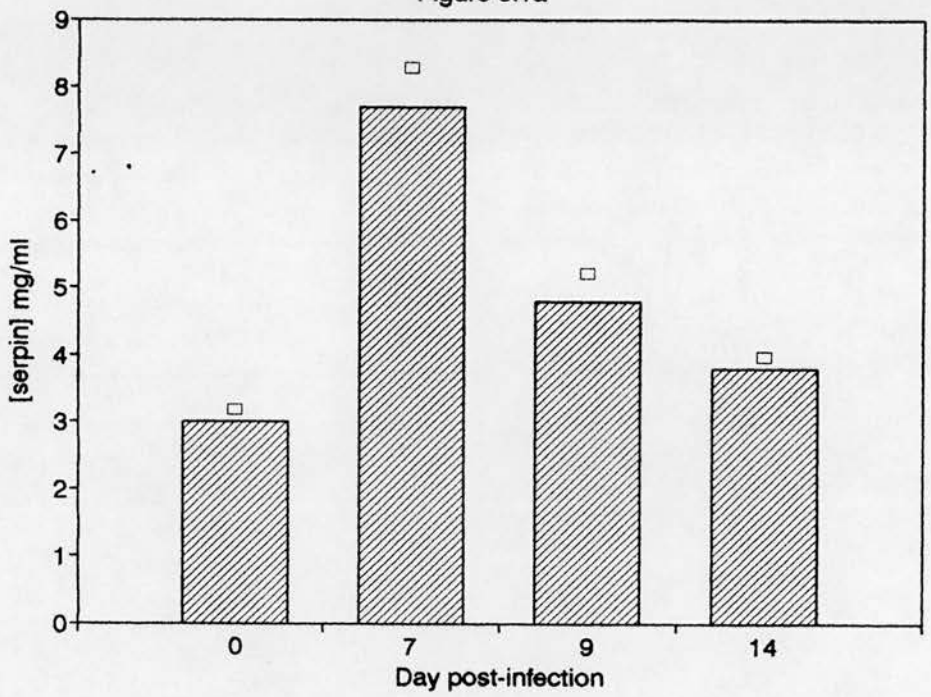
Sample	Mean (n=5)	Standard error
Serum	3 mg/mL	0.17mg/mL
Liver	76.9 μg/g	12.5μg/g
Lung	54 μg/g	7.5μg/g
Stomach	69.5μg/g	9.4μg/g
Small intestine	48μg/g	7.07μg/g
Large intestine	14.1μg/g	3.02μg/g

The highest concentration of serpin molecules is located in plasma. The liver contains the highest concentration of serpins compared with the other perfused tissues assayed.

6.2.2. Effect of N. brasiliensis infection on the concentrations of serpins in tissues and serum.

Animals were infected with *N. brasiliensis* as described (see Materials and Methods, Chapter 2) and were killed, and their vasculature perfused. Tissues were removed and homogenised for analysis at 7,9 and 14 days after primary infection. The tissues were assayed for serpins by ELISA (4) using RSI as the standard protein. The data is tabulated (Table 6.2). The effect of *N. brasiliensis* on each tissue is displayed as a histogram in Figure 6.1.

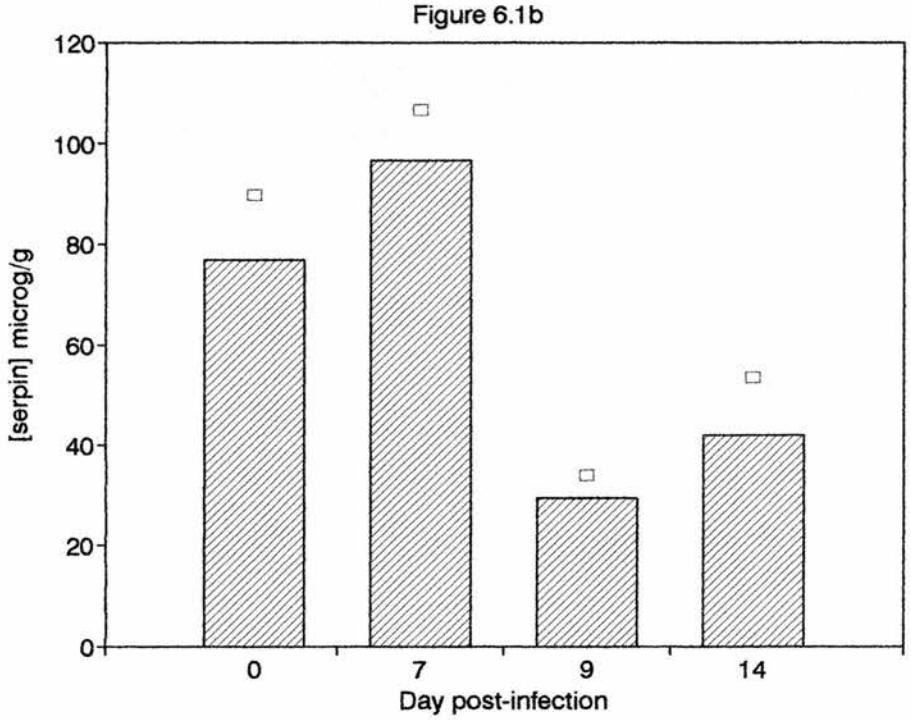
Figure 6.1a



Concentration of serpins in rat serum at different times after infection with <u>N. brasiliensis.</u>

Serum samples were assayed using ELISA 4. Boxes show the SEM.

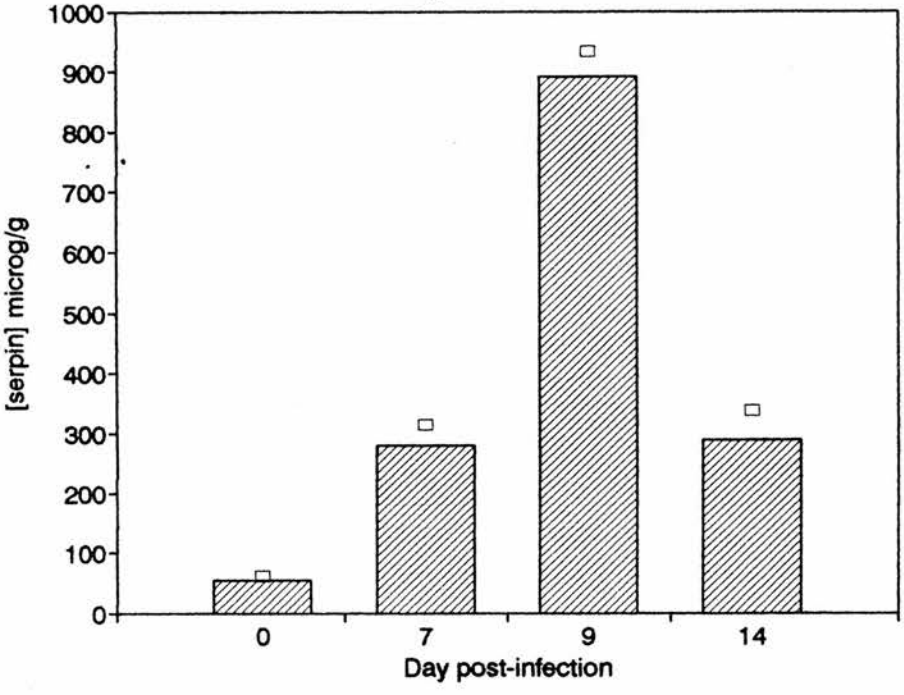
Figure 6.1b



Concentration of serpins in rat liver at different times after infection with N. brasiliensis.

Hepatic homogenates were assayed using ELISA 4. Boxes show the SEM.

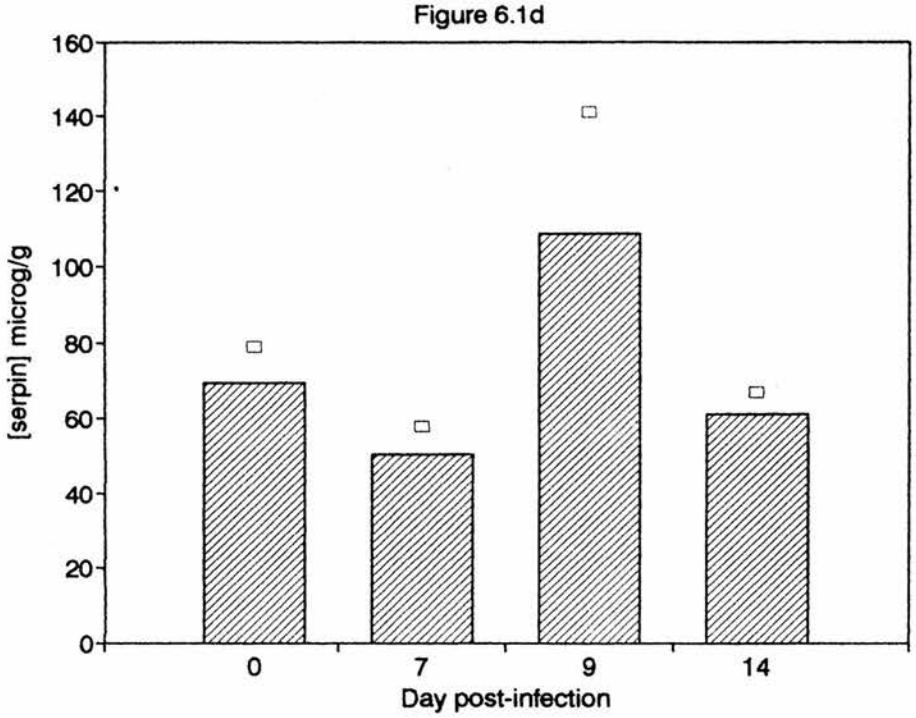
Figure 6.1c



Concentration of serpins in rat lung at different times after infection with N. brasiliensis.

Pulmonary homogenates were assayed using ELISA 4. Boxes show the SEM.

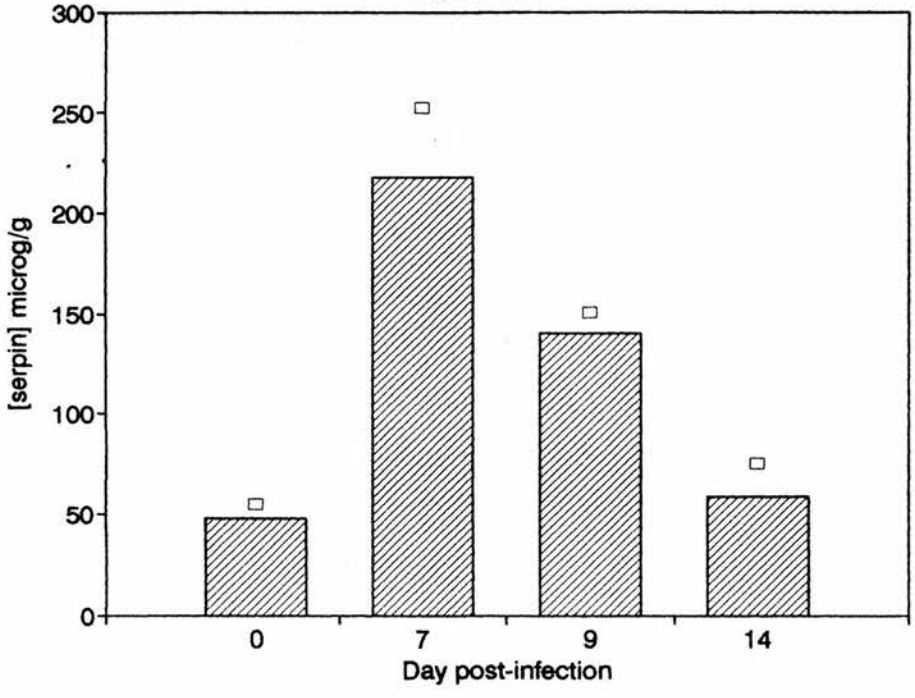
Figure 6.1d



Concentration of serpins in rat stomach at different times after infection with N. brasiliensis.

Gastric homogenates were assayed using ELISA 4. Boxes show the SEM.

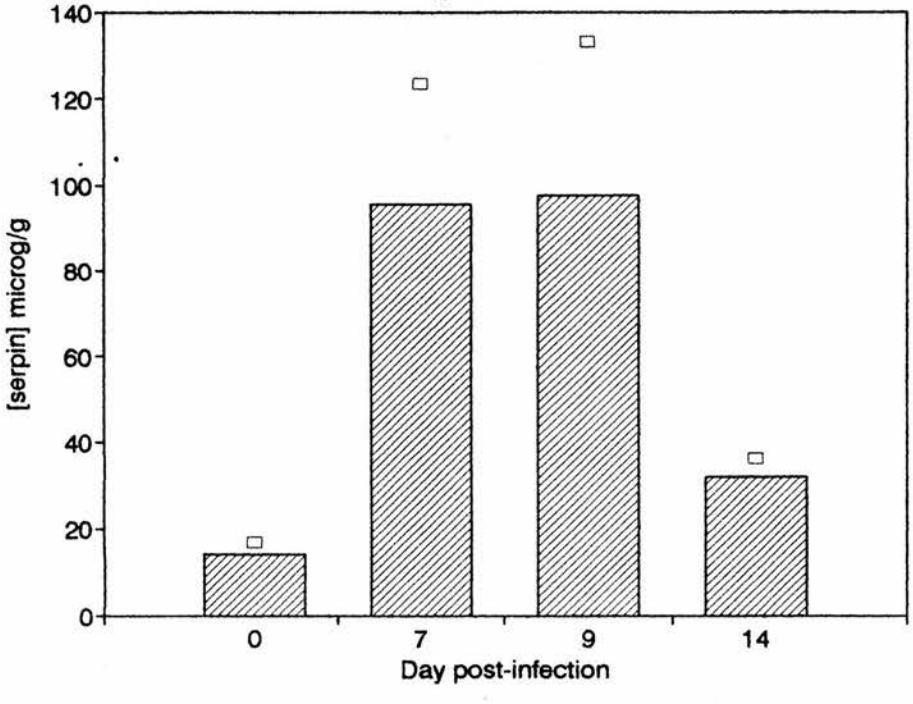
Figure 6.1e



Concentration of serpins in rat small intestine at different times after infection with N. brasiliensis.

Intestinal homogenates were assayed using ELISA 4. Boxes show the SEM.

Figure 6.1f



Concentration of serpins in rat large intestine at different times after infection with N. brasiliensis.

Intestinal homogenates were assayed using ELISA 4. Boxes show the SEM.

Table 6.2

Concentrations (mean ± SE, n=5) of serpins in Rat serum (mg/mL) and tissues (μg/g wet weight) following infection with *N. brasiliensis*.

Sample	Day 0	Day 7	Day 9	Day 14
Serum[1]	3±0.17	7.7±0.58***	4.8±0.42**	3.8±0.17
Liver[2]	76.9±12.5	96.5±20.2	29.5±4.4*	42±11.44*
Lung[2]	54±7.5	280±35***	892.5±40.5***	290±48***
Stomach[2]	69.5±9.4	50.25±7.5	108±32.4	61±5.5
Small Intestine[2]	48±7.05	217.5±34.5***	140±11.5**	58.7±16.5
Large Intestine[2]	14.1±3.02	95.5±27.5***	97.5±35.4***	32±4.2**

1. mg/mL serum, 2.μg/g wet weight tissue, * significant difference $p<0.05$, ** significant difference $p<0.01$, *** significant difference $p<0.001$. Significance is measured in relation to control sample (day 0).

There are significant increases in concentrations of serpins in serum 7 ($p<0.0001$) and 9 ($p<0.001$) days after infection (Table 6.2, Figure 6.1a) with a return to normal values by day 14. The analyses of liver (Table 6.2, Figure 6.1b) show a slight, but insignificant, rise in concentration by day 7 of infection, with a significant decrease below control values on day 9 and 14. The response in lung (Table 6.2, Figure 6.1c) involved a highly significant ($p<0.001$) increase in serpin concentration by day 7 and a further increase by day 9 of infection. At day 14 concentrations are returning to normal, although they are still significantly higher than in controls. There is no significant change in the stomach (Table 6.2, Figure 6.1d), but increases occurred in small (Table 6.2, Figure 6.1e) and large intestine (Table 6.2, Figure 6.1f). In both organs, the concentrations increase by day 7 to values significantly ($p<0.001$) greater than those in control rats (450% and 700% respectively), are maintained until day 9, and then return to control values by day 14 of infection. The % increase in concentrations in pulmonary and gastrointestinal tissue is greater than the increase in plasma concentrations.

6.2.3. Analysis of samples by non-denaturing electrophoresis and Western blotting.

In order to determine whether the serpins in these samples were all of the same molecular weight, and whether complexed serpin was detectable, samples were subjected to non denaturing electrophoresis (see Materials and Methods, Chapter 2) and the separated proteins were transferred to Immobilon for analysis with MAb1-Po.

Serum

The blot of serum proteins (Figure 6.2a) probed with MAb1 shows distinctive changes in the profile of serpins. The sample taken from rats at day 7 of infection shows a slight downward shift in M_r, although the intensity of the band appears unaltered. It is possible to detect an immunoeactive polypeptide in the higher M_r range (approximate M_r 75,000) at days 7, 9 and 14. This is likely to be complexed serpin. There are high levels of RMCPII (a target proteinase for rat serpins) in serum at this point in the infection (Miller *et al*, 1983) although concentrations of RMCPII are measured in μg/mL rather than mg/mL (Huntley et al, 1992).

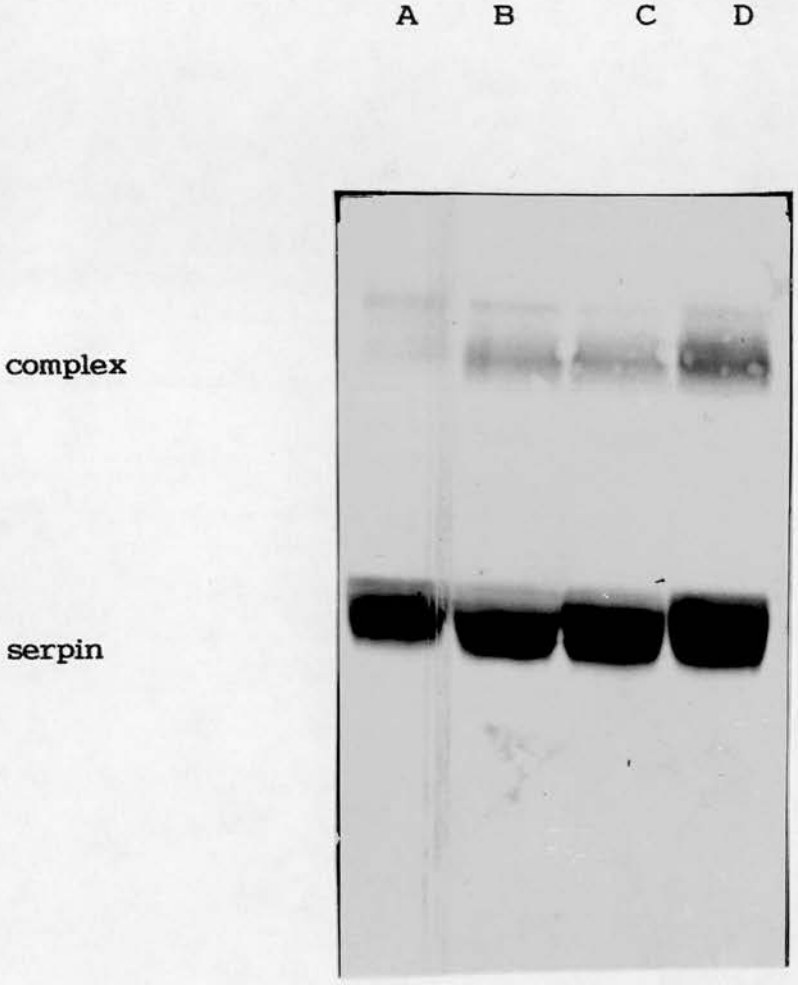
Liver

The analysis of hepatic homogenates (Figure 6.2b) shows the presence of immunoreactive protein(s) that migrate at the same M_r as complex. There is not sufficient free serpin to be detected by this method. The apparent concentrations of complex appear to increase over the course of the infection. An additional band, approximately 5000-10,000 Da. heavier than complex is immunoreactive in these samples. The identity of this band is unknown, although it may be a serpin (translated because of the infection) in complex with proteinase. The other possibility is that the serpins in the liver undergo a modification in glycosylation, accounting for the dissimilarity in M_r. In the samples from days 9 and 14 of infection there are also immunoreactive bands at a $M_r > 100,000$.

Lung

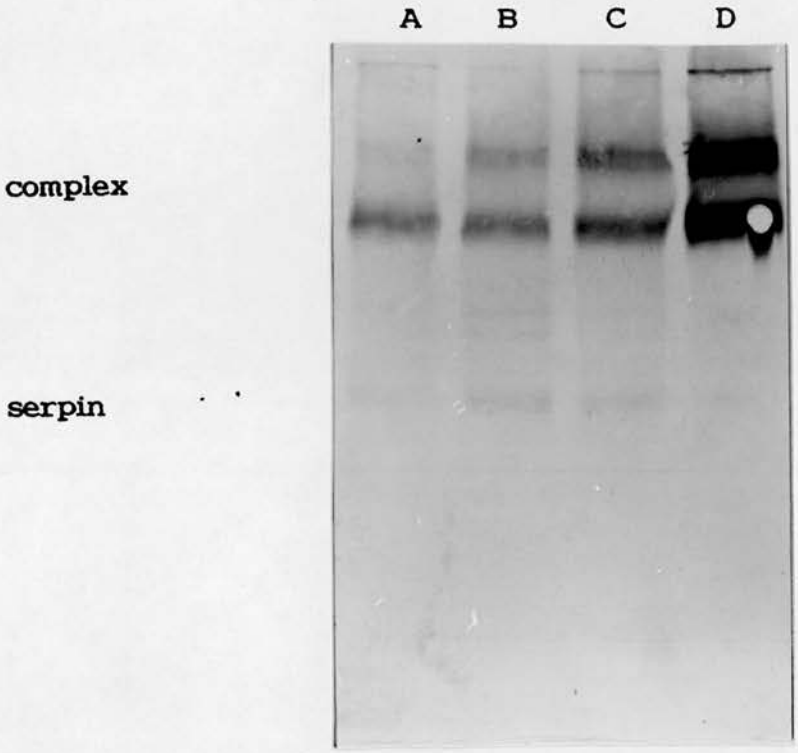
Homogenates of lung (Figure 6.2c) also display increasing concentrations of the c.M_r 75,000 protein(s) and free serpin can also be detected. Pulmonary

Figure 6.2a



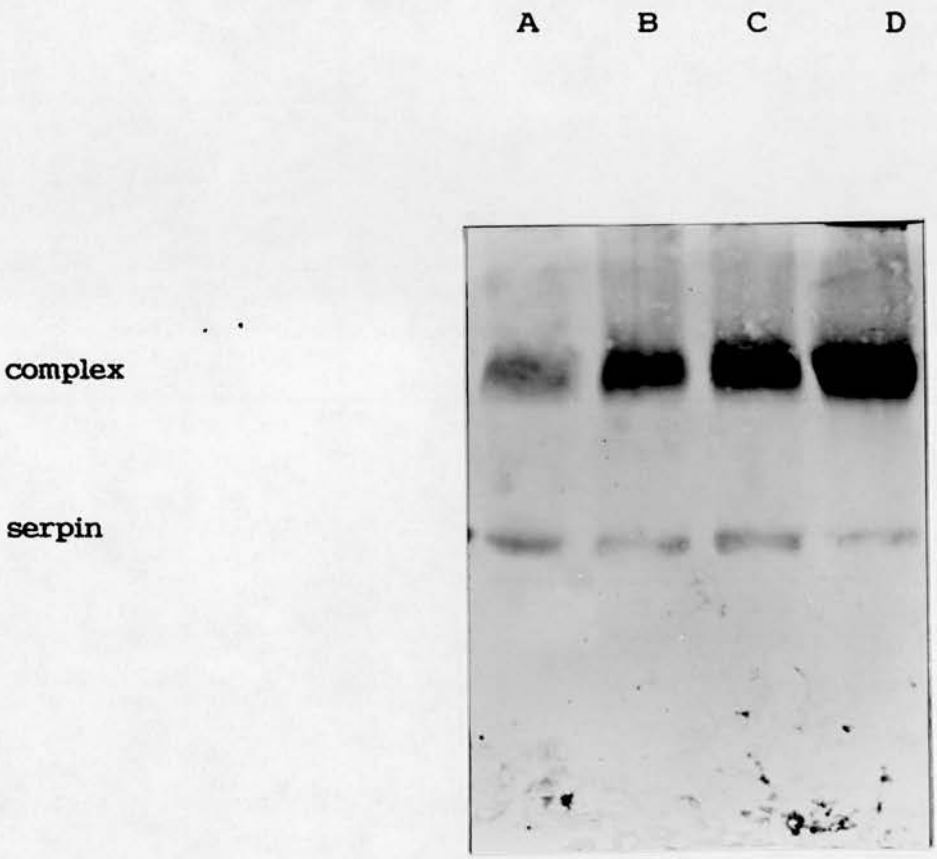
Detection of serpin and serpin proeinase complex in rat serum after infection with N. brasiliensis
Lane A, normal serum. Lanes B-D, serum samples from days 7,9,and 14 after infection with N. brasiliensis. Proteins were elctrophoresed under nonreducing conditions and transferred to to Immobilon as described (see Materials and Methods, Chapter 2). The blots were probed with MAb1-po antibody. Note the change in intensity and M_r of the serpin as the infection proceeds. Note also the appearance of protein migrating with a similar M_r to complexed serpin as the infection progresses.

Figure 6.2b



Detection by Western blotting of serpin and serpin proeinase complex in hepatic homgenates after infection with N. brasiliensis.
Lane A, homogenate of control liver. Lanes B-D, hepatic homogenates from days 7,9,and 14 after infection with N. brasiliensis. Proteins were elctrophoresed under nonreducing conditions and transferred to to Immobilon as described (see Materials and Methods, Chapter 2). The blots were probed with MAb1-po antibody. Note the apparent absence of serpin as the infection proceeds. Note also the appearance of two immunoreactive polypeptides migrating with a similar M_r to complexed serpin as the infection progresses.

Figure 6.2c



Detection by Western blotting of serpin and serpin proteinase complex in pulmonary homgenates after infection with N. brasiliensis.
Lane A, control pulmonary homogenate. Lanes B-D, pulmonary homogenates from days 7,9,and 14 after infection with N. brasiliensis. Proteins were electrophoresed under nonreducing conditions and transferred to to Immobilon as described (see Materials and Methods, Chapter 2). The blots were probed with MAb1-po antibody. Note the presence of serpin-proteinase complex in all samples analysed. An immunoreactive polypeptide co-migrating with rat serpin is visible in all the samples. This protein increases in intensity as the infection progresses.

homogenates also contain the immunoreactive protein migrating with an apparent M_r of > 100,000.

Stomach, small and large intestine.

Analysis of gastric and of small and large intestinal homogenates shows the presence of protein(s) with the same mobility under non reducing conditions as complex purified from rat serum titrated with RMCPII (Figure 6.2d). It is also possible to see what may be a free serpin, particularly in homogenates from the small intestine. The free serpin would appear to have a slightly higher M_r than RSI. Concentrations of free serpin in stomach and large intestine, quantified by ELISA, are such that they may be undetectable by blotting. There does not appear to be any protein migrating with a M_r > 100,000 in these samples.

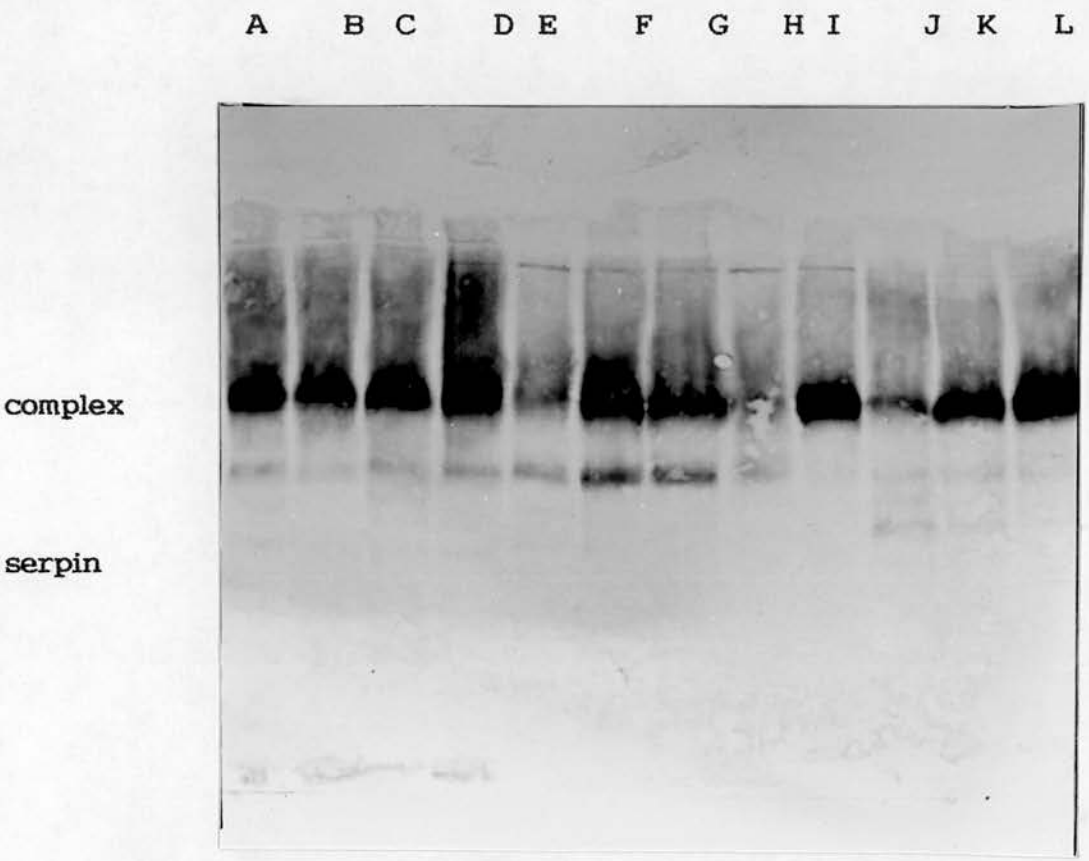
So as to further characterise the immunoreactive proteins with similar mobility to RMCPII-serpin complex, serum and perfused thoracic tissue homogenates were subjected to non reducing electrophoresis and the gels were blotted. These blots were probed with anti-RMCPII antibody.

Pulmonary homogenates displayed an immunoreactive protein, with a similar mobility to RMCPII-serpin complex (Figure 6.2e). Thus, some component of the complex in lung at day 14 of infection is RMCPII-RSI/II complex. No immunoreactive proteins were detectable in serum or liver homogenates (data not shown), although this could be because the concentrations of these proteins are below the limit of detection by this technique.

6.2.4. Albumin levels in homogenates.

In order to be sure that the serpins detected in various tissues were derived from those tissue and were not residual plasma proteins following incomplete

Figure 6.2d



Detection by Western blotting of serpin and serpin proteinase complex in gastrointestinal homgenates after infection with N. brasiliensis.
Lane A, control gastric homogenate. Lanes B-D, gastric homogenates from days 7,9,and 14 after infection with N. brasiliensis. Lane E, control small intestinal homogenate. Lanes F-H, small intestinal homogenates from 7,9,and 14 days after infection with N. brasiliensis. Lane I, control large intestinal homogenate. Lanes J-L, large intestinal homogenates 7, 9, and 14 days after infection with N. brasiliensis. Proteins were electrophoresed under nonreducing conditions and transferred to to Immobilon as described (see Materials and Methods, Chapter 2). The blots were probed with MAb1-po antibody. Note the presence of serpin-proteinase complex in all samples analysed. In the small intestinal samples (Lanes E-H) the intensity of these serpin bands appears to rise and decrease during the course of the infection. An immunoreactive polypeptide migrating slightly above rat serpin is visible in all the samples.

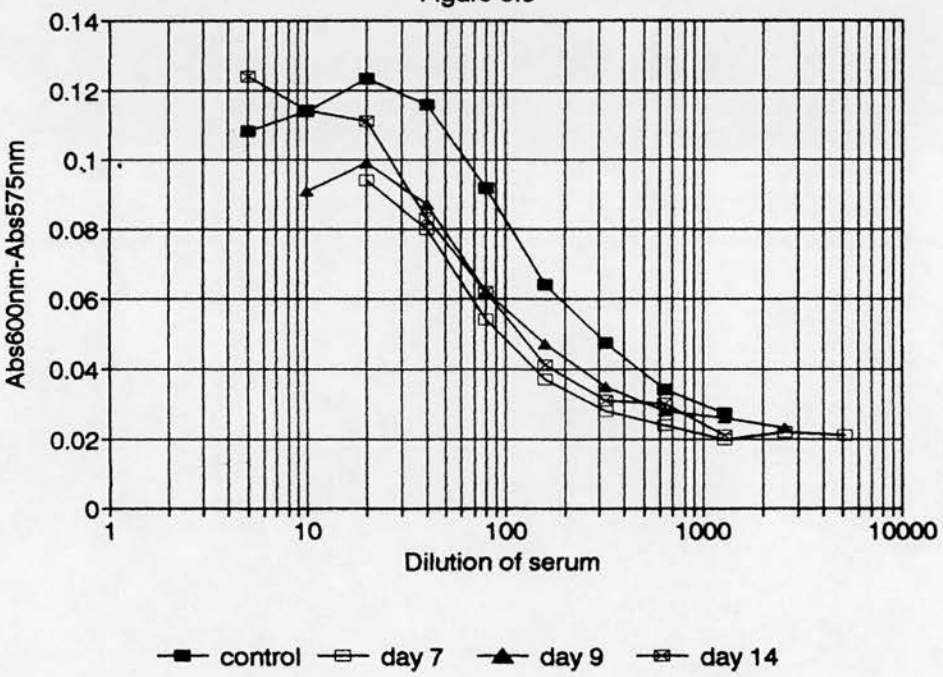
Figure 6.2e

Detection by Western blotting of RMCPII-serpin complex in pulmonary homgenates after infection with N. brasiliensis.
Lane A, purified RMCPII-serpin complex. Lane B, pulmonary homogenate 14 days after infection wityh N. brasiliensis. The proteins were electrophoresed under nonreducing conditions and protein was transferred to Immobilon as described (see Materials and Methods, Chapter 2). The blot was probed with anti-RMCPII antibody, conjugated to horse radish peroxidase.

perfusion, albumin concentrations were compared in perfused tissue and in plasma from control and infected rats. Bromophenol blue was added to all of the samples and the difference in absorbance between 600nm and 575nm was measured. The data are presented as percentage of serum albumin concentrations at the time of infection. The serum dilution curves are shown in Figure 6.3, and the data are presented in Figure 6.4.

The only organ that displays a notable change in albumin levels is the liver. The peak of albumin synthesis implied from this analysis is day 7 post-infection.

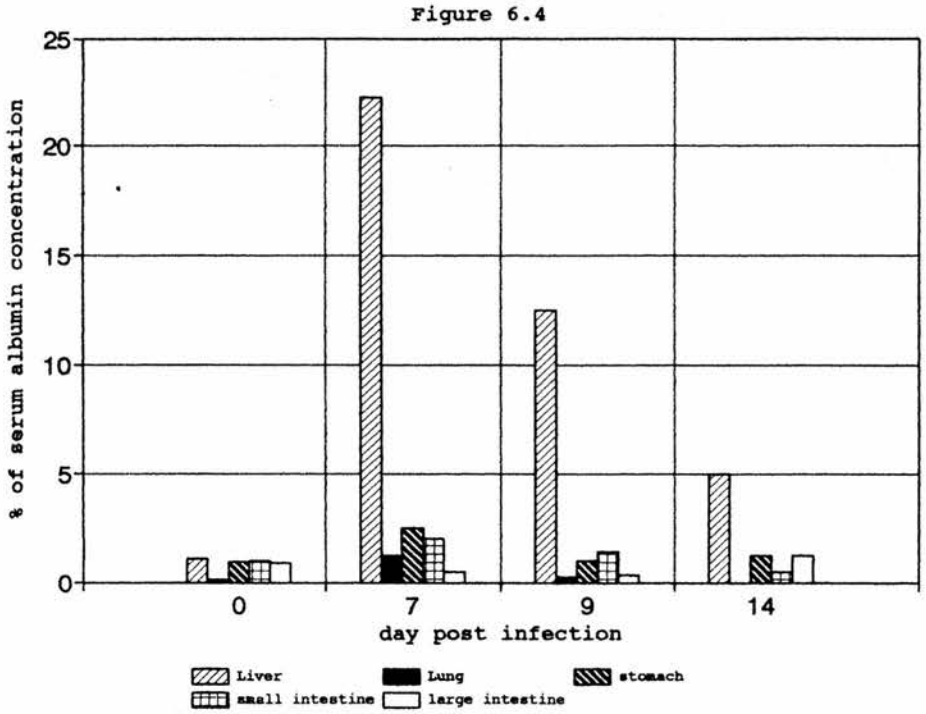
Figure 6.3



Binding of Bromophenol blue by rat serum albumin at different times after infection with N. brasiliensis

Serum was diluted as indicated on the x-axis. Samples were taken from control animals (■), 7 days after primary infection (□), 9 days after primary infection (▲) and 14 days after primary infection (▣). Note the decrease in binding of the dye in all infected samples, indicating a decrewase in albumin concentration. This may be due to the negative acute phase response of albumin or substantial plasma leakage in the gut.

Figure 6.4



Albumin concentrations in tissues after whole body vascular perfusion displayed as a percentage of serum albumin concentration.
The albumin concentration was determined as described in the text. Each value is indexed to the serum albumin concentration at the appropriate stage of infection.

6.3. Discussion

The results of this study indicate that the concentration of plasma serpins in control rats is approximately 3mg/mL. This is in excellent agreement with the values reported by other workers (Takahara and Sinohara, 1981; LaMontagne *et al*, 1981; Lewis *et al*, 1985). This value is also very similar to the serpin concentration reported in human plasma (Silvestrini *et al*, 1991).

Previous studies (LaMontagne *et al*, 1981) have indicated the presence of serpins in various fluids in the body, notably bile, breast milk, bronchiolavage fluid and gastrointestinal washings. Although the serpins in these fluids were shown to be electrophoretically heterogeneous, it was hypothesised that this was due to post-translational modification. The source tissue of all the serpins quantified was postulated to be the liver. The serpins in rodent gut were believed to have been transported to that location in bile, secreted from the liver.

The present investigation of serpins aimed to eliminate plasma-, and, by extension, liver-derived serpins by perfusing the vasculature. Although perfused liver contained the greatest concentrations of serpins (as quantified by ELISA) of all the perfused tissues assayed, there were appreciable serpin concentrations in all of the other organs. The gastric and gastrointestinal homogenates contained over 100μg serpin/g wet weight tissue (Table 6.1).

Infection of rats with the helminth *N.brasiliensis* significantly alters the serpin concentration in plasma ($p < 0.001$), lung ($p < 0.001$) and small and large intestine ($p < 0.001$). Previous reports (Stadnyk *et al*, 1990) have shown that *N.brasiliensis* will elicit an acute phase response in rats if allowed to follow its normal course of infection. The sites that the parasite resides in all show significant increases in serpin

concentration as a result of infection. The data presented here are in broad agreement with the data of Stadnyk *et al* (1990), who reported a 200-500% rise in a variety of serum proteins of hepatic origin by day 8 of *N. brasiliensis* infection, with a return to normal protein concentrations by day 10-14 of infection.

Concentrations of serpins increase significantly in the sites where mast cell recruitment and increases in concentrations of RMCPII occur following infection with *N. brasiliensis* (see section 1.2.8 for references). Mast cell recruitment in response to intestinal nematodiasis occurs principally in the intestinal mucosa, and lung (Arizono *et al*, 1987; Mayrhofer *et al*, 1976; Taliaferro and Sarles, 1939).

Analysis of the Western blots of serum show the increase in staining intensity of an immunoreactive protein that co-migrates with complex (Figure 6.2a) as the infection proceeds. The appearance of this protein may correlate with increased RMCPII concentrations, resulting in complex formation in plasma. The blot also shows slight M_r shifts in the densely staining polypeptide that co-migrates with serpin. This may be the result of altered glycosylation events (LaMontagne *et al*, 1981).

Further analysis of the serpins in perfused organs, by Western blotting, indicated that only pulmonary homogenates contained immunoreactive polypeptides that migrated in SDS-PAGE in an identical fashion to plasma serpins (Figure 6.2c, Lane b). It is known that alveolar macrophages will synthesise serpins (Downing *et al*, 1985), and this might explain the relatively high concentration of serpin found in lung. Both hepatic and pulmonary homogenates contained immunoreactive bands that migrated more slowly than plasma serpins (Figure 6.2b, lane b and 6.2c, lane b). These "heavier" bands had a similar mobility to proteinase-complexed serpin.

Further analysis of the putative complex in pulmonary homogenates, by probing blots with anti-RMCPII antibody, reveals that this protein does indeed contain RMCPII. On both blots (Figure 6.2c, lane E and Figure 6.2e), this complex migrates with an apparent M_r that is less than complex derived from plasma serpins, implying that the serpin component of this complex is of a different M_r from plasma serpins. This putative complex also appears to increase in density as the infection proceeds. Concentrations of RMCPII in pulmonary tissue increase by almost 2000% to $>100\mu g/g$ on day 9 of infection (Huntley *et al*, 1992). The concentrations of serpins in lung at this time would be sufficient to modulate locally-released RMCPII and evidence presented here does suggest a RMCPII component to at least some complexed serpin detected in lung by western blotting (Figure 6.2e). Thus, rat serpins may have a role to play in the lung in the modulation of mast cell proteinase activity.

Western blots of pulmonary and hepatic homogenates also showed the presence of immunoreactive proteins migrating with apparent M_r in excess of 100,000. These proteins were absent in serum samples analysed in the same way. The identity of these bands is unknown, but their absence from serum is intriguing.

The densest staining bands in gastric and intestinal homogenates exhibited a reduced mobility on non-reducing PAGE. In figure 6.2d (lanes B, F and J) it is possible to discern an immunoreactive protein that migrates more slowly than plasma serpin as well as the more obvious band that migrates fractionally faster than complex. It is tempting to classify these slow immunoreactive proteins as complexed serpin of some form, and the faster bands as serpins, as they display antigenic similarity to plasma serpins. There have been reports of altered electrophoretic

mobility of serpins in rodents (Lewis *et al*, 1985). This phenomenon was shown to be due to microheterogeneity in carbohydrate content, caused by protein deficiency. The altered α-1-PI contained more sialic acid and consequently was more acidic.

The variation in M_r could also be due to local synthesis of serpins in the gut, proteins translated from different genes on the serpin locus would not all have identical masses. There is evidence that the α-1-PI gene is expressed in a human intestinal epithelial cell line (Perlmutter *et al*, 1989). The tissues and organs investigated were all perfused prior to homogenisation. The levels of albumin present in the perfusates were less than 5% of serum albumin concentrations as adjudged by the albumin assay (Figure 6.4), lending further credence to the hypothesis that novel, locally synthesised, serpins are visualised in these experiments.

Gastric and intestinal homogenates displayed a slight rise in albumin concentrations by day 7 of infection. This may be a result of increased permeability at these sites because of the increased concentration of RMCPII (see King and Miller, 1984).

Western blot analysis of gastric and gastrointestinal homogenates also indicates that the immunoreactive protein with the greater M_r is the predominant immunoreactive band in these homogenates. This protein has a similar M_r to RMCPII-serpin complex. It is possible to detect immunoreactive bands in small intestinal homogenates that display a slightly slower mobility when compared to serpin. The density of the immunoreactive proteins appears to vary with the infection. This variation indicates a rise at day 7 and 9 with less densely staining proteins at day 14 and in control animals. This pattern correlates well with the

ELISA data presented in this work. However there is some doubt as to whether the ELISA (which employs two polyclonal antibody preparations, affinity purified as discusses in Chapter 5) detects these slower migrating immunoreactive proteins. The patterns of intensity of staining in the Western blots do not correlate well with the ELISA data, except the small intestinal homogenates. The apparent continual rise in density of the RMCPII-serpin complex in pulmonary homogenates is not reflected in the ELISA data. A possible explanation is that the apparent increase in intensity of the detected proteins is due more to post translational modifications, rather than increased synthesis of serpin alone. The Western blots are of nonreducing gels, glycoproteins can migrate as a broad band under these conditions. Increased glycosylation of a protein would appear as a broader band on a gel or blot. The band width in each lane of each blot does appear to broaden as infection time increases, possibly as a result of increased glycosylation.

In earlier studies of rodent infection with *N. brasiliensis* it has been shown that the message to trigger the acute phase response arose from the site of inflammation, in particular from alveolar macrophages (Lamontagne *et al*, 1984). Alveolar macrophages from infected rats secrete increased amounts of IL-6 and IL-1 (Gauldie *et al*, 1987; Lamontagne *et al*, 1985). Whether the serpin acute phase response is part of the general response as mediated by cytokines or is mediated by the mast cell proteinase release or indeed by another factor derived from mast cells is unknown at present. Stadnyk *et al* (1990) favours a dual response; one in skin and lung mediated by cytokines from macrophages, and a separate intestinal response. This theoretical intestinal response must either be induced as a result of the skin and pulmonary response or be induced locally in the sites where the parasite resides. It

is known that elevated concentrations of elastase will induce macrophages to synthesise and secrete α-1-PI (Perlmutter *et al*, 1988). This induction of serpin synthesis by a target proteinase is hypothesised to be mediated by the receptor believed to bind serpin-proteinase complex. It is possible that RMCPII can also mediate such a response in macrophages or other cell types, although no evidence exists of such a phenomenon. The evidence of Stadnyk *et al*, (1990) for a dual response is that intestinal only infection with *N. brasiliensis* does not elicit the serum and lung response seen in this present study, although such an infection will still immunize the recipient against a challenge infection (Ogilvie, 1965). Thus, stimulation of intestinal macrophages alone is not enough to trigger the systemic acute phase response.

The evidence presented in this work suggested that serpins located in the lungs and small intestine had different M_rs compared to plasma serpins. It was unknown whether these molecules were locally synthesised or whether the differences in electrophoretic mobility were due to post-translational modification. It was decided to purify these molecules in an attempt to resolve these issues. The next chapter of this thesis deals with the preliminary results of these investigations.

Chapter 7: Preliminary studies of tissue specific serpins

7. Preliminary studies of tissue specific serpins in rat.

7.1 Introduction

The serpin molecules detected by Western blot analysis of hepatic and pulmonary homogenates (Figure 6.2b and c) display a different electrophoretic mobility to the serpin molecules detected in plasma (Figure 6.2a). A difference in electrophoretic mobility is also apparent in gastrointestinal homogenates (Figure 6.2d). Although there are reports of electrophoretic differences due to variation in glycosylation (Lewis *et al*, 1985) in traumatised rats, there are also differences in the nature of the serpin response reported in this work (see Chapter 6). The increase in concentration of serpins is not found in all the sites assayed, eg the intestinal response is markedly different from that of the stomach. The timing of the increase also varies from tissue to tissue. The increase in serpins in pulmonary homogenates peaks 7 days after infection with *N. brasiliensis*, and then begins to return to control values (Table 6.2 and Figure 6.1c). The increase in serpin concentrations appears to be sustained in small and large intestine 7 to 9 days after primary infection with the parasite (Table 6.2 and Figures 6.1e and f). As all assays were performed on perfused tissue homogenates this evidence points to the existence of locally synthesised serpins.

Although serpins are found predominantly in plasma (Travis and Salvesen, 1983; and this work, Chapter 6) and are synthesised in the liver, there is also evidence that cells other than hepatocytes can and do synthesise serpins. Using RNA blot and dot hybridisation, α-1-PI mRNA was detected in human peripheral blood monocytes and in bronchoalveolar and breast milk macrophages, but not in T or B lymphocytes (Perlmutter *et al*, 1985). The α-1-PI synthesised by these cells

is secreted in a functionally active form, as evinced by complex formation with serine proteinases (Perlmutter *et al*, 1985).

Further evidence of a pulmonary source of α-1-PI was provided by Greening *et al* (1985) suggesting that macrophages may modulate the proteinase/proteinase inhibitor balance in the macrophage microenvironment.

Local synthesis of serpins may occur in other sites in man. For example a single 1.6 kilobase α-1-PI-specific mRNA was found (Perlmutter *et al*, 1989) in human jejunum and in a human colonic epithelial tumour cell line, Caco2. The mRNA was identical in size to that found in human HepG2 cells, but smaller than the message in macrophages in which an alternative upstream transcriptional start site is used (Perlino *et al*, 1987). The synthesis and secretion of the peptide in Caco2 cells reveals a protein with M_r 52,000. Results of these experiments provide further evidence for the hypothesis that expression of the α-1-PI gene in different cell types is controlled by distinct regulatory factors.

As there is only one known gene encoding α-1-PI in man, it seems likely that Kb differences in message and M_r differences in secreted peptide would be due to alternative initiation of translation and splicing, and alternative post-translational modification. In other species however, novel inhibitors have been discovered in peritoneal macrophages. Remold-O'Donnell and Lewandrowski (1983) report the presence of two inhibitors of serine proteinases in peritoneal macrophages of the guinea pig. One of these proteins formed complexes with M_r 78,000 and was subsequently shown to be α-1-PI by immunological criteria. The other protein was termed MPI (Macrophage Proteinase Inhibitor). This second inhibitor formed complexes with M_r 66,000, suggesting a smaller inhibitor than α-1-PI. As well as

inhibiting elastase, this protein was secreted constitutively in cultured macrophages, whereas α-1-PI was not.

It is known (Inglis and Hill, 1990) that there is more than one gene encoding α-1-PI in the rat Spi-1 locus. The possibility exists that the serpins synthesised in lung and intestine in the rat are transcribed/translated from genes other than those expressed in hepatocytes.

To test this hypothesis it was decided to further characterise serpins from lung and intestine. The first stage in this characterisation involved the attempted purification of serpins from rat lung and small intestine. The purification protocol was based on the affinity column, with Rab1 α-RSI/II as the ligand, utilised in Chapter 3. Purification of serpins would allow preliminary sequence data to be obtained and hopefully a more positive indication of the inhibitory capacities of any molecules isolated.

7.2 Methods and Results

7.2.1 Perfusion of animals

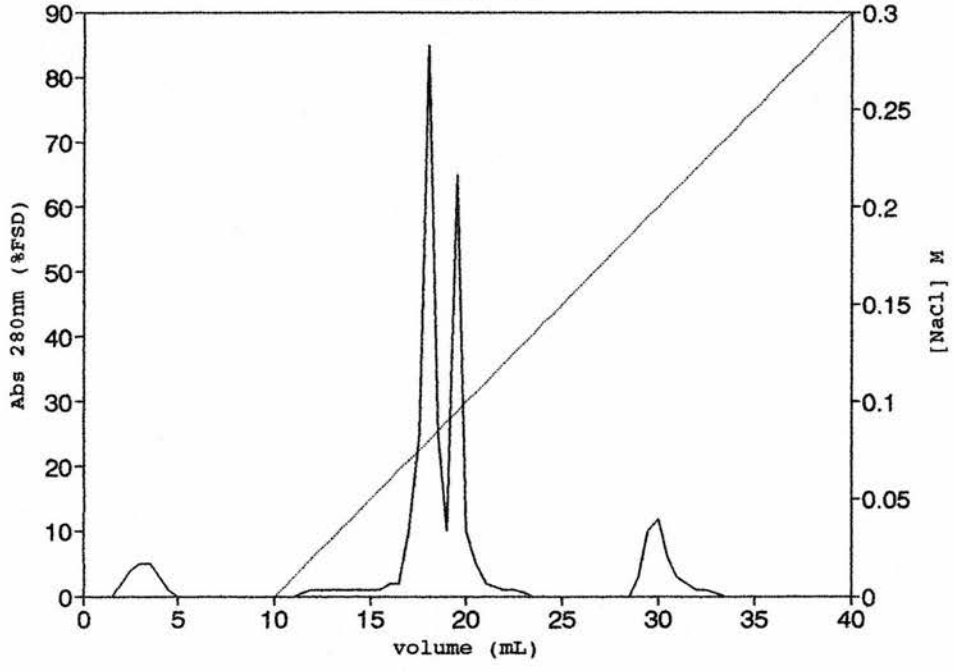
Male wistar rats (6 months old, 250-300g body weight) were infected with *N.brasiliensis* as described (see Materials and methods, Chapter 2) and killed 10 days after the primary infection. The animals were perfused and the organs removed and washed in PBS. Tissue was trimmed and stored at -20°C until processed.

7.2.2 Purification of serpins from rat pulmonary tissue

Putative serpins were purified using a combination of anion exchange chromatography and affinity chromatography (see Materials and Methods, Chapter 2). Briefly, soluble proteins from pulmonary homogenates were fractionated on DEAE-Sepharose. Proteins that bound to this column, previously equilibrated in 20mM Tris-HCl, pH 7.5, were eluted using 1M NaCl and applied to a Rab1 α-RSI/II-sepharose 4B column. Cross reactive protein eluting from this column was applied to a monoQ column previously equilibrated in buffer B. Unbound protein was washed off the column using 20mM Tris-HCl buffer, pH 7.5 and a continuous salt gradient (0-0.3M NaCl) was generated. Two peaks were eluted from this column, the first at 80mM NaCl and the second at 100mM NaCl (Figure 7.1). The eluted protein was concentrated by vacuum centrifugation and stored at-20°C prior to analysis.

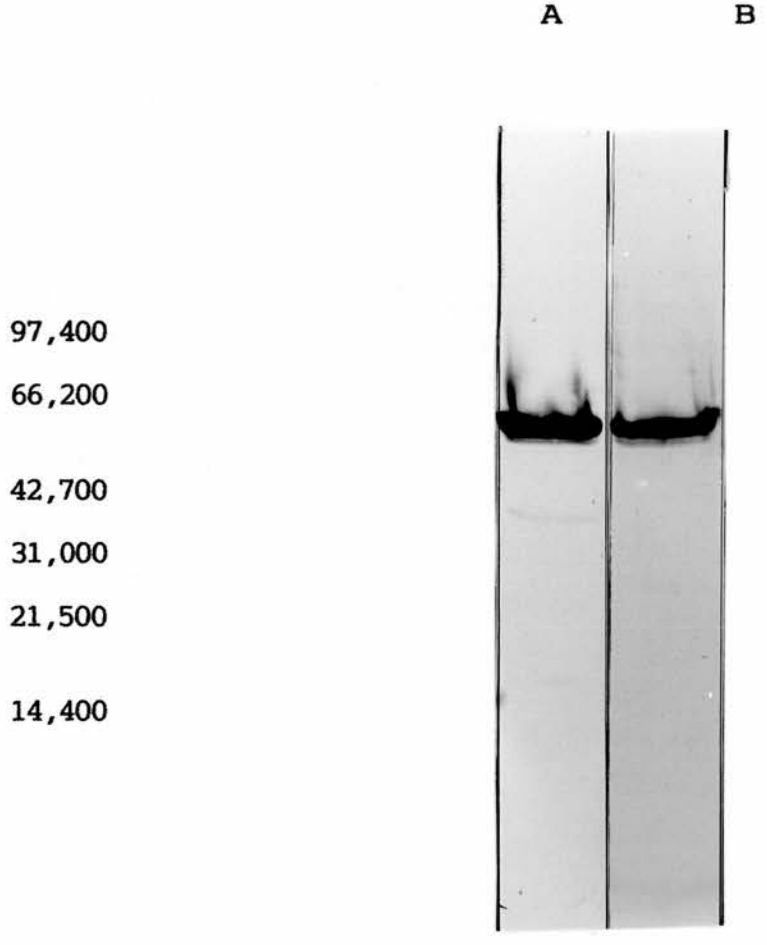
The two proteins isolated from pulmonary tissue were subjected to reducing SDS-10%PAGE (tricine buffer system) and the proteins transferred to Immobilon for Western blotting analysis. Both proteins are cross reactive with the MAb1 (Figure 7.2), the protein eluting from the monoQ column at 80mM NaCl migrated in a similar fashion to RSI, whilst the protein eluting from the column at 100mM

Figure 7.1



Purification of pulmonary serpins by anion exchange chromatography. Pulmonary protein affinity purified with Rab1 α-RSI/RSII antibody was applied to a monoQ column previously equilibrated in 25mM bis-tris-HCl, pH 6.7. A continuous salt gradient was generated (0-0.3M NaCl). Two main peaks eluted at 80 and 100mM NaCl.

Figure 7.2



Western blot of purified pulmonary serpins.
Pulmonary serpins were purified as described (see text) and electrophoresed under reducing conditions (Tricine gel system). The proteins were transferred to Immobilon sheets as described (see Materials and Methods, Chapter 2) and the blots were probed with MAb1-po. Lane A, pulmonary serpin eluting at 80mM NaCl. Lane B, pulmonary serpin eluting at 100mM NaCl. M_r standards are as indicated.

NaCl migrated to a lower M_r. Both purified proteins had a similar M_r to the uncleaved serpin detected in pulmonary homogenates (Figure 6.2c), indicating that this protein had been successfully purified. Sequence analysis showed that the protein eluting at 80mM NaCl had complete homology to RSI over the first 10 residues (Figure 7.3). The protein eluting at 100mM NaCl has complete homology to RSI over the first 10 residues, but then diverges with at least 4 changes of amino acid in the next 10 residues (Figure 7.3). The protein eluting at 100mM NaCl was not detected in serum, the preliminary sequence presented here suggests that both proteins purifed from pulmonary homogenates may be gene products of the Spi-1 locus. The protein eluting at 100mM NaCl was tentatively named RSIa. An attempt to confirm this sequence data was unsuccessful, due to technical difficulties.

To determine whether this new serpin (RSIa) has inhibitory properties, an equimolar concentration (500nM) of RMCPII was added to RS1a and the results analysed by SDS-10% PAGE. The result (Figure 7.4) shows the presence of a higher molecular weight protein in samples containing RS1a and RMCPII, characteristic of complex formation.

7.2.3 Purification of serpins from rat stomach.

The protocol was essentially that of section 7.2.2. The purified protein was concentrated and analysed after the affinity chromatography stage. As protein concentrations were very low, it was feared that a further fractionation step would result in an unacceptable loss of protein.

Electrophoresis of this protein fraction followed by Western blotting showed the presence of two major bands when the blot was probed with MAb1-po, at approximate M_r's of 60,000, and 50,000 (Figure 7.5). It is difficult to directly

Figure 7.3

Sequence of Lung Serpin I and Lung Serpin II

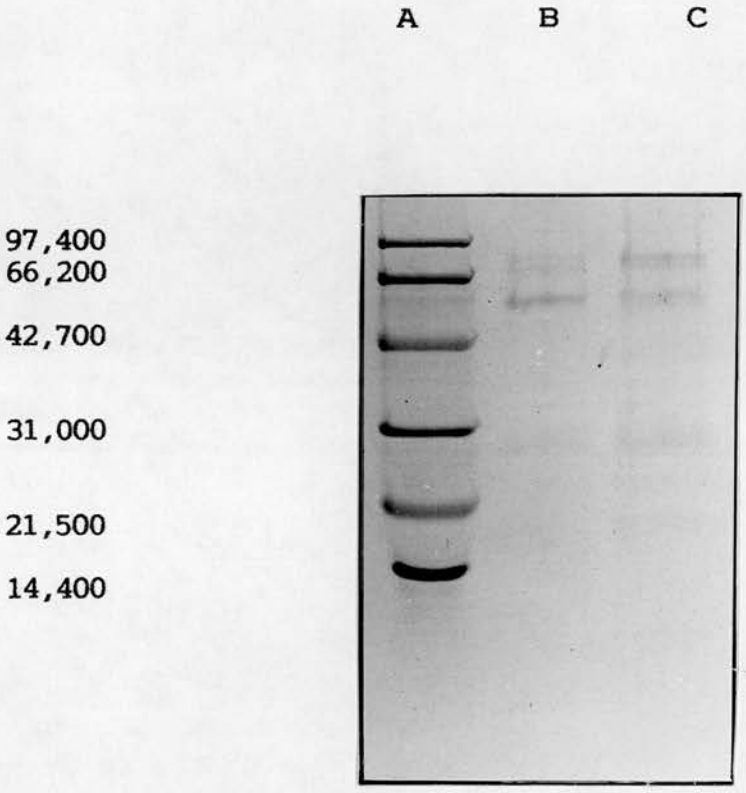
Compared to sequence data derived from RSI and RSII

purified from serum.

Position	1	2	3	4	5	6	7	8	9	10
LSI	E	D	A	Q	E	T	D	T	S	Q
LSII	E	D	A	Q	E	T	D	T	S	Q
RSI	E	D	A	Q	E	T	D	T	S	Q
RSII	T	D	A	Q	E	T	D	T	S	Q
Position	11	12	13	14	15	16	17	18	19	20
LSI										
LSII	Q	D	R	X	G	T	S	G	K	I
RSI	Q	D	Q	S	P	T	Y	R	K	I
RSII	Q	D	Q	S	P	T	Y	R	K	I

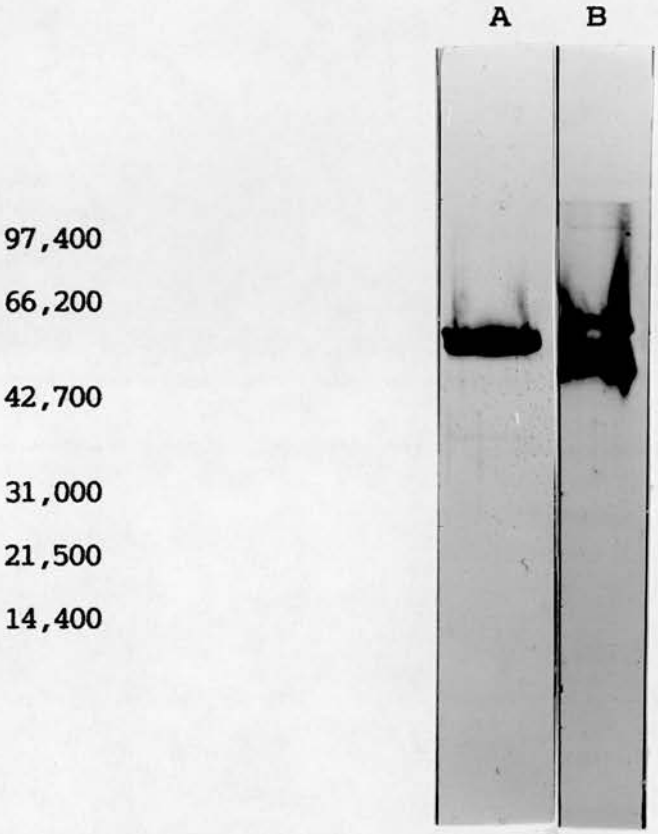
All data derived from this work.

Figure 7.4



Complex formation between purified pulmonary serpin (RSIa) and RMCPII. Lane A, M_r standards. Lane B, Pulmonary serpin (500ng). Lane C, Pulmonary serpin (500ng) + RMCPII (250ng). Samples were electrophoresed acording to the method of Schägger and von Jagow (1987). Protein was visualised with Coomassie blue.

Figure 7.5



Detection of purified gastric serpins
Lane A, RSI (1µg). Lane B, affinity purified gastric serpin (1µg). Protein was electrophoresed under reducing conditions (Tricine buffer system) and the protein was transferred to Immobilon. the blot was probed with MAb1-po. Note the presence of two immunoreactive polypeptides in the gastric sample.

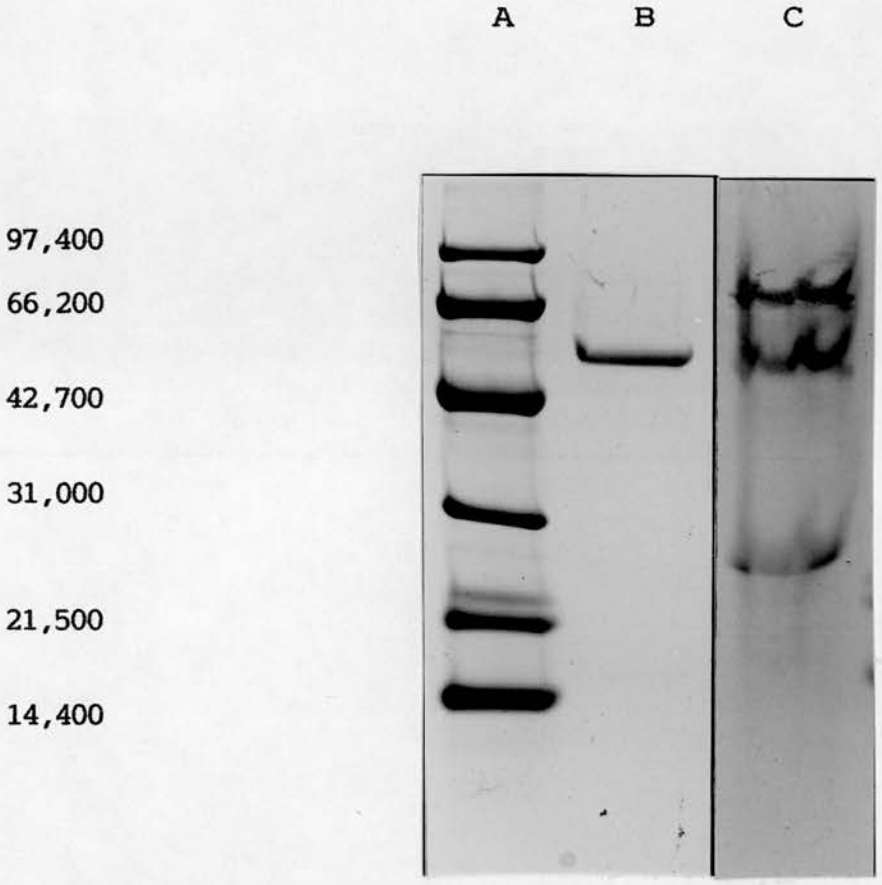
compare these purified proteins with the immunoreactive proteins detected in gastric homogenates, as the gastric homogenates were analysed under non reducing conditions (Figure 6.2d).

7.2.4. Partial purification of serpins from rat intestinal tissue

Intestinal serpins were purified as described in section 7.2.2. The protein fraction was then concentrated by vacuum centrifugation and stored at -20°C prior to analysis.

Reducing SDS-PAGE (tricine buffer system) analysis of this protein showed 3 main bands (Figure 7.6). The upper two proteins showed cross reactivity with MAb1-po, under non-reducing conditions (Figure 7.7). These immunoreactive proteins show similar electrophoretic mobility to the two immunoreactive bands present in intestinal homogenates (Figure 6.2d).

Figure 7.6



Detection of purified intestinal serpins
Lane A, M_r standards. Lane B, RSI (1µg),. Lane C, affinity purified intestinal serpin (1µg). Protein was electrophoresed under reducing conditions (Tricine buffer system). Note the presence of three polypeptides in the gastric sample. The identity of the lowest polypeptide in unknown.

Figure 7.7

Detection of purified intestinal serpins
Lane A, purified intestinal serpins. Proteins were electrophoresed under nonreducing conditions and transferred to Immobilon as described (see Materials and Methods, Chapter 2). The blot was probed with MAb1-po. The left edge of the blot shows the approximate migration of purified RSI and RSI-RMCPII complex. Note that the upper band in the intestinal sample co-migrates with complex, whilst the lower band appears to have a slightly greater mass than RSI.

7.3. Discussion.

The aim of these preliminary investigations was to partially characterise serpin tissue derived serpins. Previous studies of perfused tissues (see Chapter 6, Figure 6.2c and d) had indicated that there were electrophoretic differences between tissue and plasma derived imunoreactive proteins detected by MAb1-po, under non reducing conditions. Differences in the timing and duration of significant ($p < 0.001$) changes in concentrations of serpins (Table 6.2) were also noted in the lung and intestine.

Two molecules isolated from pulmonary homogenates were both cross reactive with MAb1-po under reducing conditions. The M_rs of these molecules were similar to those of RSI and RSII, and both proteins had a similar elution profile to plasma serpins on anion exchange chromatography, implying similar pI's. The proteins can both be classified as serpins, based on amino-terminal sequence data (Figure 7.3). It is equivocal whether the pulmonary serpin with complete identity to RSI over the first 10 residues is RSI. The pulmonary serpin which displays incomplete homology to RSI and RSII also displays a slightly different pI, as adjudged by its anion exchange elution characteristics. The pI would appear to be approximately midway between the pI's of RSI and RSII (see Chapter 3). This provides further indication, along with the preliminary sequence data, that a novel pulmonary specific serpin exists in rats. The serpin band detected in pulmonary homogenates (Figure 6.2c) is likely to be a tight doublet composed of the two serpins purified and partially sequenced. Further confirmation of the inhibitory capacity of the pulmonary serpin eluting at 100mM NaCl is provided by the ability of this protein to form an SDS stable complex (Figure 7.4). An immunoreactive

polypeptide with similar electrophoretic mobility to complexed serpin and detected with anti-RMCPII antibody was present in pulmonary homogenates (Figure 6.2e). The synthesis of this novel serpin, which would appear to be translated from the Spi-1 locus (Inglis and Hill, 1991) based on preliminary sequence data, may be responsible for the significant increased concentrations of serpin detected in pulmonary homogenates during the infection with *N. brasiliensis* (see Chapter 6). However, no attempt was made to purify serpins from the lungs of control animals, due to the limitations of time.

It was not possible to characterise putative gastrointestinal serpins with the same degree of confidence. Immunoreactive proteins from stomach and small intestine were partially purified and the two proteins isolated from gastric homogenate both cross react with MAb1-po under reducing conditions. This indicates that neither of these proteins is complexed with proteinase, as MAb1 will not detect complex under reducing conditions. This result, in conjunction with the apparent molecular weight of the molecules (approximate M_rs 50,000 and 60,000) may indicate that both molecules are serpins.

The partially purified preparation of intestinal protein exhibited 3 bands under reducing SDS-PAGE (Figure 7.6), two of which were cross reactive with MAb1 under non reducing conditions (Figure 7.7). The lack of resolution in Figure 7.7 is probably because the protein was electrophoresed directly after affinity chromatography, and was in a solution of 0.1M citric acid/0.5M NaCl, pH 2.2.

These preliminary investigations indicate that there are tissue specific serpins in rat. Any hypothesis as to the regulation of synthesis of these molecules at the moment would be speculative in the extreme. If time had allowed it was hoped to

further purify the immunoreactive molecules from pulmonary and gastrointestinal homogenates, and properly characterise them. Sequence analysis would provide more proof of the identity of these proteins, and kinetic studies of the inhibitory properties of these molecules would provide a basis for determining their role in the modulation of RMCPII during helminth infection.

Chapter 8: Concluding Remarks

8. Concluding Remarks.

The purpose of this work was to determine which, if any, serpins in rat would inhibit mast cell proteinases. By titrating RMCPII into rat serum it was possible to purify complex formed between RMCPII and serpin(s) molecules. Antibodies raised against this complex were used to affinity purify two plasma serpins. Amino acid sequence analysis of these two molecules showed that they were similar, but distinct, proteins. One of the two plasma serpins isolated (RSI) had 100% amino terminal sequence homology with the predicted amino acid sequence of an α-1-PI clone (Chao *et al*, 1990), the other molecule (RSII) exhibited incomplete homology to this clone. This evidence suggests that both RSI and II are transcribed/translated from the SPI-1 locus in rat, which encodes 4-5 genes for α-1-PI (Inglis and Hill, 1990).

Before this work, only one α-1-PI molecule had been purified and partially characterised from the rat (Kuehn et al, 1985). This protein had displayed inhibitory capacities towards a variety of serine proteinases, but inhibition of mast cell proteinases had not been investigated. Both RSI and RSII inhibit RMCPII, the K_i's are sufficiently low ($\leq 10^{-9}$M) to indicate efficient inhibition (Beatty *et al*, 1980). The observation that RMCPI is not inhibited by RSI and II, and will in fact cleave the serpins into two smaller fragments is unexpected. RMCPI is chemically and enzymatically similar to RMCPII. The inference from this data is that RMCPI activity will be modulated in some other way, possibly by the mast cell granule (LeTrong et al, 1987b).

A variety of antibodies were raised against the purified rat serpins, and complexes prepared from these serpins and mouse mast cell proteinases. All of the

antibodies developed in the course of this work were shown to be specific for rat serpins. Attempts to generate monoclonal antibodies against a cleaved form of rat serpin were only partially succesful, however further attempts may prove to have more success. These antibodies were used to develop ELISA's, which aided in the quantification of serpin concentrations in plasma and in perfused tissues. The data obtained suggest that tissue specific serpins exist in lung and gastrointestinal tissues. The synthesis of serpins locally in these tissues has been described in man (Greening *et al*, 1985; Perlmutter *et al*, 1989) and, in rats infected with *N brasiliensis*, the changes in concentrations of serpins were indicative of an acute phase response. The differenct tissue responses, in terms of timing and intensity, provided further credence to the hypothesis that there were tissue specific serpins in the rat.

Electrophoresis followed by Western blotting of perfused tissue homogenates indicated subtle differences in molecular mass between plasma serpins and serpins from lung, stomach and intestinal tract. An attempt to purify serpins from pulmonary tissue suggested the presence of at least one serpin that was not found in plasma. This serpin had inhibitory capacity against RMCPII and had a similar pI to RSI and RSII. Preliminary sequence analysis also indicated that at least one of the molecules found in lung was tissue specific. The maximal concentrations of serpins were on day 7 of infection in serum and day 9 in the lung. In both cases the serpin concentration is greater than the RMCPII concentration at this point in *N. brasiliensis* infection (Miller *et al*, 1986). In serum the molar ratio of serpins to RMCPII is about 2500 and in lung about 10. RSI and II, since they are involved in the acute phase response, will doubtless inhibit other target proteinases such as neutrophil elastase. It may be reasonable to hypothesise that RSI, RSIa and II have

a role to play in the modulation and clearance of RMCPII although this will not be their sole function. A Western blot of pulmonary homogenates probed with α-RMCPII antibody suggests that at least some fraction of the serpin-proteinase complex present has an RMCPII component.

There is an apparent abundance of serpin in gastrointestinal tissue, and this increases during infection with kinetics which are distinct from those in plasma and lung. There is a rise in concentration of protein recognised by antibodies specific for serpins, but this rise is sustained from day 7 until day 9. Liver or bloodare unlikely to be the source of these proteins since they were isolated from perfused tissues, and were never co-purified in preparations of RSI and II derived from serum. The elution characteristics of these proteins from the monoQ column allows a tenative assignment of pI's in the 5-6 range. However as discussed in chapter 7, a precise assignment of Mr was not feasible.

It is believed that the trigger for the acute phase response during *N. brasiliensis* infection is the stimulation of alveolar macrophages to produce cytokines (Lamontagne *et al*, 1984). If this is the case then the lung is stimulated to produce pulmonary-specific serpins in addition to plasma-specific serpins, based on the evidence presented here.

In a similar study to this present work, Stadnyk *et al*, (1989) reported two peaks of plasma glycoprotein during the first 9 days of infection. However this present study did not sample plasma until day 7 of infection so any early increase resulting from an acute phase response was not measured.

Stadnyk *et al* (1984) noted that an *N. brasiliensis* infection when confined to the intestine did not elicit the acute phase response, leading to speculation that

intestinal macrophages lack the required mediators to trigger the systemic acute phase response. This present study has demonstrated a response in gut by day 7 of infection.

No attempt was made to show the presence of serpins in gut macrophages nor in enterocytes, although such a study should be very interesting. It is known that elastase can induce the expression of α-1-PI in mononuclear phagocytes (Perlmutter and Punsal, 1988), and the secretion of RMCPII by MMC in the gut may, similarly, promote local synthesis of serpins. Studies on the clearance of complexed elastase by macrophages have indicated that a biological feedback mechanism may be in operation (Perlmutter *et al*, 1990) such that the endocytosis of serpin-enzyme complex can stimulate the expression of serpins in macrophages. Thus we can see how an excess of serine proteinase, forming complex in the microenvironment of the macrophage with low levels of constitutively produced serpin, could stimulate the expression of further serpins from macrophages.

The three α-1-PI-like rat serpins will all form complexes with RMCPII. Further, it is known that RSI and RSII will inhibit trypsin and chymotrypsin (see chapter 4). The two plasma serpins have association constants of the order 10^5 $M^{-1}s^{-1}$ and inhibition constants of the order 10^{-9} M and below, indicating that they are efficient inhibitors.

If we try to determine whether or not the serpins are physiologically relevant inhibitors of RMCPII we have to make certain assumptions based on the data presented in this thesis. The total concentration of serpin is 3mg/mL in plasma, and assuming that RSI and II are present in equimolar concentrations (implied from the amounts of each serpin obtained from the purification procedure) and with M_rs

of 50,000, the plasma concentration of each serpin is approximately 30μM. We are now in a position to calculate certain kinetic parameters as outlined by Bieth (1980).

The time needed for complete inhibition of a proteinase is given by the equation

$$delay\ time = \frac{5}{k_{ass} \times [I]_o}$$

This equation is based upon the time for the concentration of active enzyme to fall to 1% of original values. This assumes that the [inhibitor] remains essentially unchanged, ie the inhibitor is present in large excess. The molar ratio of serpin to RMCPII is about 2500 in plasma. By using the assumed concentrations for RSI and II (vide supra) we can calculate that complete inhibition of RMCPII occurs within 0.75 seconds (RSI) and 1 second (RSII) in control rats. The half-lives if dissociation for complexes formed between RSI and RMCPII, or RSII and RMCPII are at least 1 hour (see table 4.1), the half-life for complex clearance has been shown to be 12-15 minutes (Pizzo *et al*, 1988). Thus the kinetic and thermodynamic constants calculated in this present study, coupled with other evidence (from other laboratories) pertaining to the clearance of complexed serine proteinases, suggests that RSI and II may have a physiological role in the clearance of RMCPII.

The data presented indicate that RMCPII, a chymase, is inhibited by members of the family of proteins that are α-1-PI-like (classically the anti-elastase/trypsin serpins). The proteins translated from the Spi-2 locus (Inglis and Hill, 1990) are homologous to the human α-1-antichymotrypsin proteins, but no evidence has been found here of involvement of these proteins in inhibition of

RMCPII. Interestingly, RSII displays a high k_{ass} for chymotrypsin, indicating that the divergence of the genes on the Spi-1 locus (Inglis and Hill, 1990; Borriello and Krauter, 1991) has resulted in an anti-elastase activity evolving into an anti-chymotrypsin activity (possibly a mutation from methionine as p1' residue to leucine). However, RSI appears to be the physiologically more relevant inhibitor as far as RMCPII is concerned based on thermodynamic evidence.

Evidence presented here and elsewhere (Inglis and Hill, 1990) has indicated the presence of multiple serpin genes as opposed to the single functional gene and a pseudo gene coding for the homologous protein in man (Inglis and Hill, 1990). Hill and Hastie (1987) have proposed that the diversity of serpins in rodents is a result of parasitic infection. Recent evidence has shown that there are many more genes for mast cell proteinases than had been thought (Reynolds *et al*, 1990; Vanderslice *et al*, 1990). As mentioned in the Introduction to this work, it may be that the diversity of serpins has evolved to inhibit the multitude of proteinases in mast cells and other cells. There is evidence for a diversity of mast cell proteinases in human mast cells (Vanderslice *et al*, 1990), but so far only one gene for human α-1-PI is known to be expressed, this does not exclude the possibility that other genes are responsible for the production of human serpins which will modulate mast cell proteinase expression. It is already known that α-1-antichymotrypsin, as well as α-1-PI, will inhibit mast cell proteinases from human skin mast cells (Schechter *et al*, 1989).

As shown in this thesis there are a multiplicity of Spi-1 serpins expressed in the rat and their expression is affected dramatically by helminth infection. There are several questioned raised by this work. Firstly, the known multiplicity of rodent

serpins (Inglis and Hill, 1991) coupled with the present findings raises questions regarding the tissue distribution of serpins. It is feasible that individual genes on the SPI-1 cluster may be transcribed /translated in different tissues and, furthermore, transcription of each gene may be controlled by a unique set of regulators. The data in Chapter 6 and 7 which suggests differences in timing and intensity of response may indicate that different physiological traumas are responsible for regulating the synthesis of tissue specific serpins. The questions that need to be answered concern the stimulus for increasing concentrations of serpins, especially in the gut. It is not known if intestinal inoculation of adult *N. brasiliensis* induces local synthesis of serpins, although it has been suggested that such an infection will not stimulate the acute phase response (Stadnyk *et al*, 1990). If an increase does occur then further investigation of the regulation of such an event would be worthwhile. For example does circulating serpin-RMCPII complex provoke further synthesis in the gut. This line of investigation is inextricably bound to the question of complex clearance in helminth infection. Which cell types are responsible for complex clearance, and will they be stimulated to synthesise serpin? The answers to such questions will require the use of recombinant DNA technology. This will be necessary because of the need for *in situ* hybridisation studies to determine the precise source of transcription/translation of serpins in a variety of tissues. However the similarity in primary sequence between serpins will require the utilisation of very specific antibody preparations in addition to the above mentioned techniques. Development of capture ELISAs, specific for RMCPII:RSI/II complexes, may be possible with the judicious use of α-RMCPII antibodies in conjunction with the α-serpins antibodies generated in this work. Such an assay could provide information on the fate of

RMCPII in helminth infection. Such an ELISA would also be very useful in characterising the clearance of complex from rat plasma.

One very intriguing flight of fancy is that mast cells themselves may synthesise serpins, and may themselves be stimulated to synthesise serpins by helminth infection. In the course of this study, it was not possible to demonstrate a serpin which would inhibit RMCPI. Tissue concentrations of RMCPI are known to be affected by helminth infection (Huntley *et al*, 1992), and although it is believed that the mast cell granule is responsible for the modulation of the activity of this enzyme (LeTrong *et al*, 1987b) the physiological significance of this has yet to be demonstrated.

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Differential Inhibition of Rat Mast Cell Proteinase I and II by Members of the α-1-Proteinase Inhibitor Family of Serine Proteinase Inhibitors*

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Rat mast cell proteinase II (RMCP II) from mucosal mast cells was titrated into rat serum, and the resulting serine proteinase inhibitor (serpin)-enzyme complex was purified by affinity chromatography on anti-RMCP II-Sepharose 4B and by Mono-Q anion-exchange. The purified complex was used to raise polyclonal antibodies which, after cross-absorption against RMCP II-Sepharose 4B, were specific for serpin and were used to affinity purify two rat serpin molecules (RSI and RSII) that inhibit RMCP II in rat serum. The kinetic constants characterizing the interaction between RMCP II and RSI and RSII are k_a, 2.2×10^5 and 1.65×10^5 M^{-1} s^{-1}, respectively; K_i, 3.6×10^{-10} and 1.0×10^{-9} M; and k_d, 7.9×10^{-5} and 1.65×10^{-4} s^{-1}. Amino-terminal sequence analysis indicated that RSI and RSII are distinct, differing at the amino-terminal residues, and are products of the rat Spi-1 locus. Rat mast cell proteinase I (RMCP I) from connective tissue mast cells cleaved both RSI and RSII and was not inhibited.

The presence of high concentrations of serine proteinases in secretion granules is a characteristic feature of mast cells.

Recent work has shown that individual mast cells may express genes for several proteinases; in man the presence of a multigene family encoding tryptases was reported (Vanderslice *et al.*, 1990), and in mouse at least six distinct serine proteinases that can be expressed in different combinations in different mast cell populations have been identified (Reynolds *et al.*, 1990).

Chymotrypsin-like activity was first detected histochemically in rat mast cells (Benditt and Arase, 1959) and the chymase, rat mast cell proteinase I (RMCP I),[1] was subsequently isolated from peritoneal mast cells (Lagunoff and Pritzl, 1976). Woodbury *et al.* (1978) found that a second similar, yet distinct, proteinase (RMCP II), first isolated by Katunuma *et al.* (1975), was present in mucosal mast cells. Recent work by Benfey *et al.* (1987) has indicated that a third serine proteinase may be expressed in the rat.

RMCP I and II share a high degree of sequence homology (>70%) but differ in their net charge (LeTrong *et al.*, 1987a), which may reflect different properties and physiological functions. RMCP I is a relatively insoluble protein, requiring a high concentration of salt for extraction and purification (Lagunoff and Pritzl, 1976), whereas RMCP II is readily soluble in physiological conditions (Woodbury and Neurath, 1978). The two rat mast cell proteinases, when isolated, show similarity in their substrate profiles (Yoshida *et al.*, 1980) and exhibit catalytic activity similar to that of cathepsin G. The substrate profile of RMCP I bound to secretory granules is limited to smaller molecules (LeTrong *et al.*, 1987b).

The concentrations of RMCP II in peripheral blood are greatly increased during systemic and local intestinal anaphylaxis (King and Miller, 1984; Patrick *et al.*, 1988), and secretion of RMCP II into blood is associated with expulsion of nematode worms in rats (Miller *et al.*, 1983). The phenomenon of increased concentrations of circulating soluble mast cell proteinase associated with worm expulsion has also been observed in mouse (Huntley *et al.*, 1990) and sheep (Huntley *et al.*, 1987). A physiological role for mast cell proteinases has not yet been unequivocally demonstrated. However, several lines of evidence suggest that human mast cell proteinases modulate the biological effect of neuropeptides in the airway, resulting in airway hypersensitivity in man (Tam and Caughey, 1990; Caughey, 1989) and dog (Nadel, 1989). Exogenous RMCP I has also been implicated in mast cell degranulation (Schick, 1990; Kido *et al.*, 1988) and in the processing of matrix metalloproteinases (Sopata *et al.*, 1988).

Many serine proteinases that function in plasma or tissue during inflammation appear to be regulated by a superfamily of serine proteinase inhibitors (serpins) (Travis and Salvesen, 1983).

This suggests that serine proteinases derived from mast cells also will be regulated by the serpin superfamily. For example, human skin mast cell chymase is inhibited by at least two serpins (Schechter *et al.*, 1989). Work in this laboratory has shown that mouse serum has the ability to interact with mouse intestinal mast cell proteinase, resulting in the formation of higher molecular mass (~75-kDa) complexes (Irvine *et al.*, 1990), a characteristic of the interaction of serpins with susceptible proteinases.

At least five plasma proteins in the rat belong to the serpin superfamily and function as serpins (Kuehn *et al.*, 1984). The purpose of the present study was to determine which, if any, of the rat serpins can regulate the activities of RMCP I and II, in an attempt to elucidate the physiological fate of these mast cell proteinases in an homologous system.

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[1] The abbreviations used are: RMCP, rat mast cell proteinase; RS, rat serpin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; serpin, serine proteinase inhibitor; bis-Tris, 2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

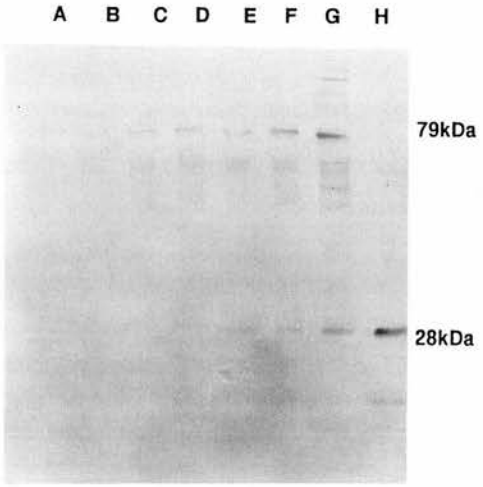

FIG. 1. **Titration of RMCP II into rat serum and detection of RMCP II-serpin complex.** *Lane A*, 0.25 μl of rat serum; *lane B*, 0.25 μl of rat serum + 100 μg of RMCP II/ml of serum; *lane C*, 0.25 μl of rat serum + 200 μg of RMCP II/ml of rat serum; *lane D*, 0.25 μl of rat serum + 300 μg RMCP II/ml of rat serum; *lane E*, 0.25 μl of rat serum + 400 μg of RMCP II/ml of rat serum; *lane F*, 0.25 μl of rat serum + 500 μg of RMCP II/ml of rat serum; *lane G*, 0.25 μl of rat serum + 1 mg of RMCP II/ml of rat serum; *lane H*, 250 ng of RMCP II. Samples were electrophoresed on 10% SDS-PAGE (Laemmli, 1970), transferred to nitrocellulose sheets as described in the Miniprint, and probed with anti-RMCP II antibody conjugated to horseradish peroxidase. The band visualized at 79 kDa is RMCP II-serpin complex; RMCP II migrates at 28 kDa.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Titration of RMCP I and II into Rat Serum—Purified RMCP I or II was titrated into rat serum over a range of 0–1 mg/ml. After incubation for 60 min at 37 °C, an aliquot of each sample was removed and subjected to SDS-PAGE (7.5%). The protein was transferred to nitrocellulose after electrophoresis, and the blot was probed with anti-RMCP I or II antibody. In the presence of serum, RMCP II formed multiple complexes that were visualized by Western blotting (Fig. 1); the most abundant serpin-enzyme complex had an approximate molecular mass of 79 kDa, suggesting that a 50-kDa protein(s) is involved with the 28-kDa RMCP II in this reaction. At a titration of >500 μg of RMCP II/ml of serum, free protease could be observed (Fig. 1). RMCP I formed no complexes that could be detected with anti-RMCP I antibody (Fig. 2).

Purification of the Serpin-Enzyme Complex—RMCP II (500 μg) was added to 1 ml of rat serum and incubated for 1 h at 37 °C. The serum was then applied to a Sepharose 4B anti-RMCP II affinity column. The bound fraction, eluted with 0.1 M citrate, was immediately desalted on a PD-10 column (Pharmacia LKB Biotechology Inc.) equilibrated with 20 mM Tris-HCl, pH 7.5 (buffer A). The protein fraction was applied to a Mono-Q column equilibrated in buffer A, and a major peak was eluted at 50 mM NaCl using a continuous gradient of 0–0.3 M NaCl. This peak contained 95% pure serpin-proteinase complex (by SDS-PAGE) and could be visualized on Western blots using anti-RMCP II antibody. Serpin-proteinase complex was used to raise polyclonal antibodies in rabbits.

Purification of Serpins—Rabbit antibodies raised against serpin-proteinase complex were affinity-purified as outlined under "Materials" and "Methods."

Rat serum (0.5-ml aliquot) was applied to an Affi-Gel Blue column (16 × 50 mm) equilibrated in 25 mM bis-Tris-HCl, pH 6.7 (buffer B), and the column was washed with this buffer. This step was incorporated to remove most of the

[2] Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 5–8) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

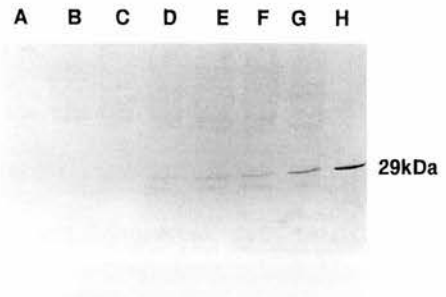

FIG. 2. **Titration of RMCP I into rat serum and the absence of complex formation.** *Lane A*, 0.25 μl of rat serum; *lane B*, 0.25 μl of rat serum + 100 μg of RMCP I/ml of serum; *lane C*, 0.25 μl of rat serum + 200 μg of RMCP I/ml of rat serum; *lane D*, 0.25 μl of rat serum + 300 μg RMCP I/ml of rat serum; *lane E*, 0.25 μl of rat serum + 400 μg of RMCP I/ml of rat serum; *lane F*, 0.25 μl of rat serum + 500 μg of RMCP I/ml of rat serum; *lane G*, 0.25 μl of rat serum + 1 mg of RMCP I/ml of rat serum; *lane H*, 250 ng of RMCP I. Techniques were as described for Fig. 1, except that anti-RMCP I antibody was used to probe the blot. RMCP I migrates at 29 kDa. No RMCP I-serpin complex was visualized.

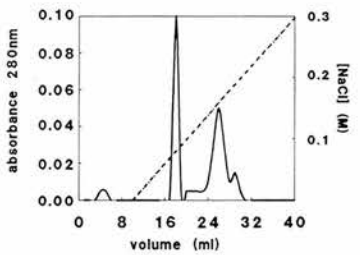

FIG. 3. **Purification of RSI and RSII by ion-exchange chromatography showing the Mono-Q column profile, 25 mM Bis-tris-HCl, pH 6.7.** The peak eluting at 80 mM NaCl is RSI; the peak eluting at 150 mM NaCl is RS II.

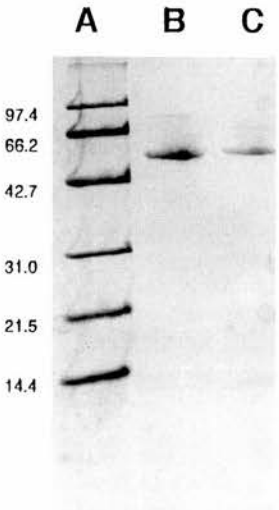

FIG. 4. **Electrophoresis of purified rat serpins.** *Lane A*, molecular weight standards (Bio-Rad); *lane B*, RSI (2 μg); *lane C*, RSII (2 μg). Proteins were visualized using Coomassie Blue. Electrophoresis was performed according to the method of Schagger and von Jagow (1987).

TABLE I

Kinetic Data for RS I and RS II

All data were determined as described in the Miniprint.

Enzyme	k_a	k_i	k_d	$t_{1/2\,d}$
	$M^{-1}\,s^{-1}$	M	s^{-1}	h
	RSI			
RMCP I	NI[a]	NI	NI	NI
RMCP II	2.2×10^5	3.6×10^{-10}	7.92×10^{-5}	2.4
Chymotrypsin	6.0×10^5	ND	ND	ND
Trypsin	4.13×10^5	ND	ND	ND
	RSII			
RMCP I	NI	NI	NI	NI
RMCP II	1.65×10^5	1.0×10^{-9}	1.65×10^{-4}	1.0
Chymotrypsin	3.17×10^6	ND	ND	ND
Trypsin	6.68×10^5	ND	ND	ND

[a] NI, not inhibited; ND, not done.

position

	1	2	3	4	5	6	7	8	9	10	11
alpha-1-PI	E	D	A	Q	E	T	D	T	S	Q	Q
RSI	E	D	A	Q	E	T	D	T	S	Q	Q
RSII	T	D	A	Q	E	T	D	T	S	Q	Q

	12	13	14	15	16	17	18	19	20	21
alpha-1-PI	D	Q	S	P	T	Y	R	K	I	S
RSI	D	Q	S	P	T	Y				
RSII	D	Q	S	P	T	Y	R	K	I	S

FIG. 9. **Sequence of RSI and RSII compared with rat α-1-proteinase inhibitor (*alpha-1-PI*).** The data is from Chao *et al.* (1990) and this work.

albumin in the sample (Gianazzi and Arnaud, 1982). The protein eluted in the wash was then applied to an anti-rat serpin-RMCP II-complex Sepharose 4B column (16 × 50 mm) with, as ligand, the cross-absorbed, affinity-purified polyclonal antibody. The bound serpin was eluted with 1 ml of 0.1 M citrate, pH 2.2, and applied immediately to a Sephadex G-25 column previously equilibrated with buffer B. The eluted protein was applied to a Mono-Q column equilibrated in buffer B and a continuous gradient of 0–0.3 M NaCl (10 mM/ml) was established. The chromatogram of this column can be seen in Fig. 3; the eluted peaks 1 and 2 contained proteins with molecular masses of 52 and 54.5 kDa, respectively (determined on Tricine SDS-PAGE (10%), Schagger and von Jagow (1987)), which were, respectively, labeled RSI and RSII (Fig. 4).

Inhibition Kinetics of RSI and II with RMCP I and II—The k_a values of the reactions between RSI and II and RMCP II, chymotrypsin, and trypsin were determined as outlined in the Miniprint, and the inhibition constant, K_i, of the interaction between RSI and II and RMCP II was also determined experimentally as outlined. All results are tabulated (Table 1).

Sequencing of RSI and RSII—RSI and II were sequenced on an Applied Biosystems 477A pulsed-liquid phase Protein Sequencer. The proteins were prepared for sequence analysis by high pressure liquid chromatography on a C18 column and eluted with an acetonitrile gradient. The amino-terminal sequences are shown in Fig. 9, where they are compared with the predicted sequence of the gene product of the rat α-1-proteinase inhibitor gene (Chao *et al.*, 1990). The partial sequences of RSI and II are identical apart from the amino-terminal residue.

DISCUSSION

Serpins act on their target proteinases by forming 1:1 molar complexes with them. A short peptide loop on the surface of the inhibitor is bound by the cognate proteinase and is cleaved between the p1 and p1′ residues (Travis and Salvesen, 1983). This results in the structural rearrangement of the serpin such that the proteinase is covalently bound to the serpin, resulting in inactivation of the proteinase. The p1 residue is crucial in determining the specificity of the serpin.

The data presented provide evidence that there are at least two serpins in rat plasma that will inhibit RMCP II by forming essentially irreversible complexes. The kinetic data (Table I) show that the inhibition is of a similar order to those of other proteinase-serpin interactions (Beatty *et al.*, 1980). However, RMCP II is unlikely to be the natural target proteinase for RSI and II, as the k_a values determined are approximately 10-fold less than values obtained for α-1-proteinase inhibitor and its target proteinase elastase or α-1-antichymotrypsin and its target proteinase cathepsin G (Beatty *et al.*, 1980).

The observation that there is no complex formed between equimolar concentrations of RMCP I and RSI or II is unexpected. RMCP I, as has been discussed (see above), is chemically and enzymatically similar to RMCP II. The inference from the data is that proteolysis by RMCP I must be modulated in a different way from RMCP II. The fact that no serpin-RMCP I-complex is seen on Western blots of rat serum titrated with RMCP I (Fig. 2), in conjunction with the observation that RMCP I will cleave RSI and II under physiological conditions (Fig. 6) supports the hypothesis that the mast cell granule may be responsible for modulating RMCP I activity (LeTrong *et al.*, 1987b). Currently, investigations are underway to determine the role of the rat α-2-macroglobulin-like molecule, α-1-inhibitor3 (Enghild *et al.*, 1989), in the modulation of RMCP I activity.

Sequence analysis of the amino terminus of RSI and II indicated that both molecules are probably gene products of the Spi-1 locus of the rat (Inglis and Hill, 1990). RSI is most likely to be rat α-1-proteinase inhibitor, the serpin believed to control elastase activity *in vivo*. RSII differs from RSI in that it has threonine at the amino-terminal position, as opposed to glutamate (Fig. 9). This is the first time that a member of the rat Spi-1 protein family, other than α-1-proteinase inhibitor (Kuehn *et al.*, 1964), has been purified and identified.

Recent evidence (Borriello and Krauter, 1990) has shown that at least five members of the Spi-1 multigene family are expressed in the mouse. Analysis of the reactive site region of the genes (generated by polymerase chain reaction) has shown that there are three variant p1 residues, methionine, tyrosine, and leucine. The p1 site residue determines effectiveness against the various target proteinases, and a methionine residue would be expected at the p1 site of α-1-proteinase inhibitor. Leucine is the residue found at the p1 site of human α-1-antichymotrypsin (Morii and Travis, 1983). Interestingly, the molecule designated RSII is more potent as an inhibitor of chymotrypsin than of trypsin or chymase (see Table I).

In the mouse, this family of genes has less than 4% overall predicted amino acid sequence divergence (Hill *et al.*, 1984). The presence of residues other than methionine at the p1 site has led to speculation (Borriello and Krauter, 1990) that proteins encoded by the Spi-1 locus in mouse may not all

have anti-elastase activity. The evidence (Table I) that RSII inhibits chymotrypsin more efficiently than other proteinases used in this work supports this hypothesis. Further investigations should reveal the extent of the polymorphism in the rat Spi-1 locus.

Future work in this laboratory will determine whether any proteins encoded by the Spi-2 (Inglis and Hill, 1990) locus can form complexes with rat mast cell proteinases. The Spi-2 locus encodes proteins that are the structural homologues of human α-1-antichymotrypsin, although no evidence previous to this has reported a rodent α-1-antichymotrypsin activity.

It will also be of interest to determine the biochemistry of RMCP II-serpin complexes in the rat, and to this end we have initiated a series of experiments designed to investigate the clearance of the proteinase-serpin complex from body tissues and fluids, enabling us to understand more fully the physiological fate of rat mast cell proteinases after the events stimulating degranulation.

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Supplementary Material to
Differential inhibition of rat mast cell proteinase I and II by members of the alpha 1-proteinase inhibitor family of serpins
Steven R. Pirie-Shepherd, Hugh R.P. Miller and Andrew Ryle

EXPERIMENTAL

1. Materials

Animals

Male Wistar rats were used in all experiments; the animals were kept in a 12 hr dark 12 hr light cycle and allowed access to food and water ad libitum. Animals were killed at 6 months by exsanguination under deep anaesthesia. Blood was allowed to clot at room temperature and serum, obtained by centrifugation, was stored in 5 ml aliquots at -20°C until used.

Chemicals

The substrates, benzyloxylcarbonyl-L-tyrosine-paranitrophenol ester (Cbz-L-Tyr-ONp) and benzoyl-L-arginine-p-nitroanilide (BAPNA) were purchased from Sigma (Poole, Dorset). The proteinases RMCP I and RMCP II were purified in this laboratory as described (Gibson and Miller, 1986); bovine pancreatic trypsin was purchased from Sigma and bovine pancreatic chymotrypsin from Boehringer Mannheim (Lewes, East Sussex, UK). Sephadex G-25 and affi-gel blue were purchased from Bio-Rad, monoQ HR5/5 columns, CNBr sepharose 4B and all columns (C16 type) were purchased from Pharmacia. All columns were run on Pharmacia/LKB FPLC equipment. Polyclonal and monoclonal antibodies against RMCP I and II were a kind gift from G. Newlands and J. Huntley (MRI, Edinburgh). Affinity-ligands were coupled to CNBr-sepharose as per manufacturer's instructions. Tricine was purchased from Sigma. Proteins were blotted onto nitrocellulose or Immobilon for visualisation with antibodies. All other chemicals were of the highest purity available.

2. Methods

Electrophoresis

Electrophoresis was with the Bio-Rad mini Protean II system using the buffer systems of Laemmli (1970) and Schagger and von Jagow (1987). Western blotting was performed using the semi-day method (Khyse-Anderson, 1984) using the buffer system of Matsudaira (1987).

Production of antibody against serpin-RMCP II-complex (SEC)

Polyclonal antibodies against complexes (see Results: purification of serpin-RMCP II-complex (SEC)) were prepared as follows; 100 ug of antigen in an emulsion of Freund's complete adjuvant was injected intramuscularly into a rabbit. The rabbit was bled from the ear after two weeks and the titre of the antibody assessed by Ouchterlony double diffusion. When the titre began to decrease the animal was given a further intramuscular injection of 100 ug antigen in an emulsion containing Freund's incomplete adjuvant. After a further test bleed to ensure that the titre of the antibody was maximal the animal was killed and bled. The blood was allowed to clot at room temperature, and antiserum was obtained by centrifugation. Antiserum was stored at -20°C until used.

Affinity purification of anti-(serpin-RMCP II-complex) antibodies

17 mg ligand/g matrix) to remove antibody specificity to this enzyme. The unbound fraction was eluted using PBS and applied to a column (5 ml, 2 mg ligand/g matrix) of SEC-sepharose. The column was then washed with 2 column volumes of PBS and bound antibodies, eluted with 1 ml of 0.1 M citrate buffer (pH 2.7), were immediately neutralised by the addition of 1M Tris base (200 uL/ml). The eluted antibody was used to make an "anti-serpin"-sepharose column (1.4 mg ligand/g matrix).

Titration of proteinases and serpins

RMCP II, RMCP I and chymotrypsin were active-site titrated as described by Kezdy and Kaiser (1970) using Cbz-L-Tyr-ONp as titrant, trypsin was titrated as described by Chase and Shaw (1970) using p-nitrophenyl-p'-guanidinobenzoate HCl as titrant. All serpins were back-titrated against RMCP II as follows; RMCP II (36 nM) was incubated with various amounts of purified serpin. After 5 min the residual activity of the proteinase was measured using Cbz-L-Tyr-ONp as substrate, absorbances at 410 nm were measured.

6.75 nM.

Determination of K_i.

A standard concentration of RMCP II (36 nM) was incubated with increasing concentrations of serpin for 30 min at room temperature. The incubation mixture was then analysed for residual proteolytic activity using Cbz-L-Tyr-ONp (final molarity 100 uM). All measurements were made in a centrifugal analyser with a reaction volume of 750 ul PBS. Absorbances at 405 nm were measured.

RESULTS

Kinetics of serpin-rat mast cell proteinase-complex formation

Aliquots of RMCP II and RMCP I were added to RSI and RSII in an equimolar ratio and incubated for 10 second before being analysed by SDS-10% PAGE. Stable serpin-proteinase complexes were formed between RMCP II and RSI and II (Figure 5) whereas RMCP I cleaved both serpins to give two major cleavage products of approx. 37 and 25 kDa (Figure 6). In order to determine the capacities of RSI and RSII to inhibit RMCP I and RMCP II the kass for each reaction was determined experimentally.

Determination of k_{ass}:

The minimal scheme describing the interaction of serpins and proteinase is:

$$E + I \xrightarrow{k_{ass}} E{:}I$$

$$\downarrow k_{diss}$$

Where E and I stand for proteinase and serpin respectively and k_{ass} and k_{diss} are the association and dissocation rate constants respectively. The rate of association of E with I is given by equation 1:

$$-\frac{d[E]}{dt} = k_{ass}[E][I] - k_{diss}[EI] \qquad (1)$$

No significant dissociation of EI occurred during the association process as the association reaction was measured over a time period (300 seconds) considerably shorter than the half life of the SEC (> 1 hr), see below). Hence the second term of equation 1 could be neglected:

$$-\frac{d[E]}{dt} = k_{ass}[E][I] \qquad (2)$$

Integration of equation 2 for [E]≠[I] yields

$$[E] = (k_{ass}\, t + 1/[E]_0)^{-1} \qquad (3)$$

The concentration of E,[E], at any time was derived from the initial rates of substrate hydrolysis. The association rate constant, k_{ass}, was calculated by non-linear regression analysis (using the DNRP53 program of R.G. Duggleby, University of Queensland, Australia which fitted the experimental data to equation 3).

Figure 7 shows an example of the time-dependent decrease of RMCP II, bovine chymotrypsin and bovine trypsin activity due to inhibition by RSI and RSII: there is a good fit between the experimental data and the theoretical curve generated using equation 3. All parameters. All data are shown in Table 1.

Determination of K_i

The K_i of the reaction between E and I is defined as kdiss/kass, which is equivalent to:

$$K_i = \frac{[E].[I]}{[EI]} \qquad (4)$$

By rearrangement with respect to [E] and substituting the term:

$$[EI] = ([E]_0 - [E]) \qquad (5)$$

and

$$[I] = [I]_0 - ([E]_0 - [E]) \qquad (6)$$

(where $[E]_0$ is initial enzyme concentration and $[I]_0$ is initial inhhibitor concentration) we get equation 7 (Bieth, 1974):

$$\frac{[E]}{[E]_0} = \frac{([E]_0 - K_i - [I]_0) + ((([E]_0 - K_i - [I]_0)^2 + 4K_i.[E]_0)^{1/2}}{2[E]_0} \qquad (7)$$

The constant K_i can be determined experimentally by reacting E with increasing amounts of I for 30 mins, followed by addition of substrate and the measurement of residual proteinase activity. The addition of substrate will initially upset the equilibrium of the reaction mixture but after 2 minutes a steady state is reached (Boudier and Bieth, 1989). The rate of steady state substrate hydrolysis was used to determined [E] for a given $[I]_0$. The fractional activity, $([E]/[E]_0)$, can then be used to determine the substrate-dependent K_i by fitting the data to equation 7 using non-linear regression analysis. Figure 8 shows an example of an experiment in which the substrate-dependent K_i was determined for the reaction between RMCP II and RSI and RSII. The substrate-dependent K_i (K_i^{app}) can be converted to the true K_i by using equation 8:

$$K_i = K_i^{app}/(1 + [S]_0/K_m) \qquad (8)$$

The K_m for the RMCP II/Cbz-L-Tyr-ONp system was measured independently by classical means and found to be 2.46 uM in the buffer conditions used for all experiments described. All values of K_i are tabulated (Table 1).

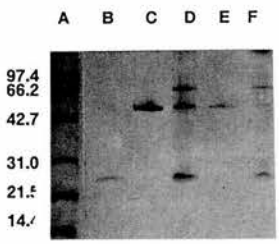
Determination of k_{diss}

The rate constant which applies to the dissociation of the SEC, k_{diss}, can be determined

$$k_{diss} = K_i.k_{ass} \qquad (9)$$

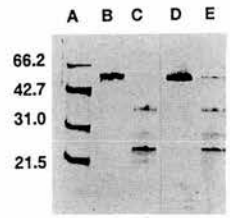
Values of k_{diss} and the half-life of dissocation are shown in Table 1

Figure 5



Lane A; molecular weight standards, lane B; 1 µg RMCP II, lane C; 2 µg RSI, lane D; 1 µg RMCP II/2 µg RS1, lane E; 2 µg RSII, lane F; 1 µg RMCP II/2 µg RSII. Lanes D and F contain serpin:enzyme complex (band staining at 79kDA). Electrophoresis performed as outlined in experimental section using the buffer system of Schagger and von Jagow.

Figure 6



Lane A; molecular weight standards, lane B 2 µg RSI, lane C 2 µg RSI & 200 ng RMCP I lane D, 2 µg RSII, lane E, 2 µg RSII & 200 ng RMCP I. Electrophoresis performed as outlined in experimental section using the buffer system of Schagger and von Jagow.

Figure 7a

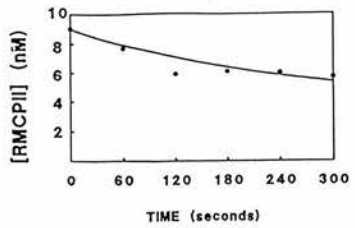
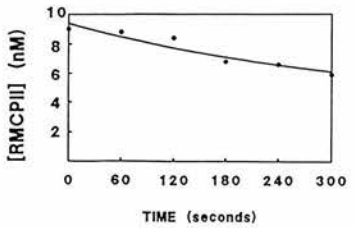


Figure 7b



Decrease in the activity of RMCPII as a result of incubation with equimolar amounts of RSI (Figure 7a) and RSII (Figure 7b) for increasing lengths of time. Molarity of RMCPII was 9nM. Details of procedure are in experimental section. The theoretical line was fitted using non-linear regression as described.

Figure 7c

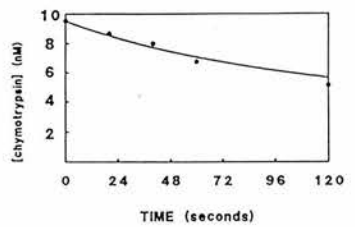
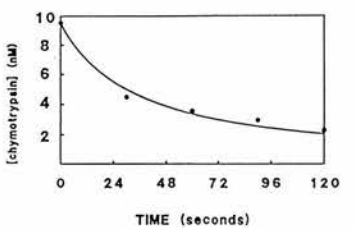


Figure 7d



Decrease in the activity of chymotrypsin as a result of incubation with equimolar amounts of RSI (Figure 7c) and RSII (Figure 7d) for increasing lengths of time. Molarity of chymotrypsin was 9.5nM. Details of procedure are in experimental section. The theoretical line was fitted using non-linear regression as described.

Figure 7e

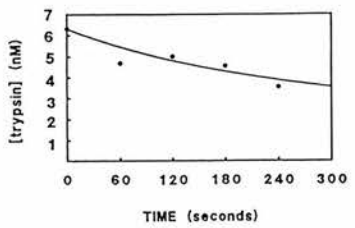
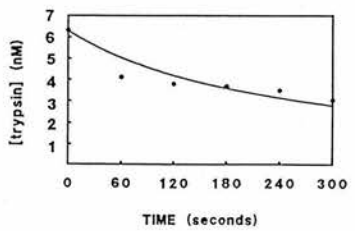


Figure 7f



Decrease in the activity of trypsin as a result of incubation with equimolar amounts of RSI (Figure 7e) and RSII (Figure 7f) for increasing lengths of time. Molarity of trypsin was 6.25nM. Details of procedure are in experimental section. The theoretical line was fitted using non-linear regression as described.

Figure 8a

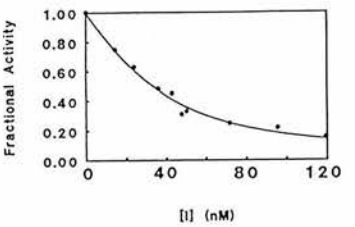
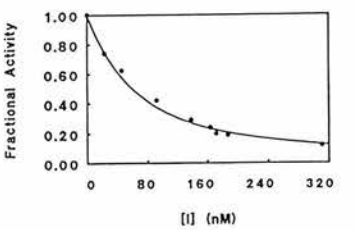


Figure 8b



Inhibition of RMCPII activity plotted against increasing concentrations of RSI (Figure 8a) and RSII (Figure 8b). Conditions were as described in the experimental section. The theoretical curve was fitted to experimental data using non-linear regression analysis as outlined. The K_i^{app} between RMCPII and RSI was 15nM, the K_i^{app} between RMCPII and RSII was 45nM.