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The Roles of Beta and Alpha Tryptases in Asthma: Genetic and Immunopharmacological Studies

by

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I, Ahmed Asem Mahrous Abdelmotelb

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Abstract

Faculty of Medicine

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Doctor of Philosophy

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Tryptases, the dominant secretory granular proteins from human mast cells, are emerging as important mediators in asthma and allergy. The β- and α- tryptases have highly similar nucleotide sequences and located on the same locus. While the entire population expresses β-tryptase, the α-tryptase gene exhibits copy number variation (CNV). We have studied the association of expression of these allelic variants with asthma or allergic diseases. We have investigated also the potential actions of β- and α-tryptases *in vitro* and *in vivo*. We have found that the one alpha tryptase copy allele was significantly associated with lower total serum IgE levels (Z= -2.39, p=0.01) and a tri-allelic architecture with alleles carrying no, one or two copies of the α-tryptase gene was postulated. The addition of β-tryptase to epithelial cells induced upregulation of mRNA for IL-8, IL-6 and TNF-α, while α-tryptase on the other hand was without effect in this model. Injection of β-tryptase into the mouse peritoneum induced great accumulation of neutrophils but accumulation of other cell types was less marked. Under the same conditions, injection of α- tryptase induced less neutrophilia but eosinophils, macrophages and mast cells numbers were significantly increased. The actions of β-tryptase seemed be independent of PAR-2 receptors but not the case for α-tryptase, where PAR-2 pathway might take the leads. In conclusion, recombinant α-tryptase may be a stimulus for the recruitment of inflammatory cells and altered cytokine gene expression with effects distinct from those of β-tryptase.

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Abbreviations

AUC	Area under the curve
BAL	Bronchoalveolar lavage
BAPNA	N-α-benzoyl-DL-argenine-*p*-nitroaniline
BHR	Bronchial hyperresponsiveness
CNV	Copy number variation
CGRP	Calcitonin gene related-peptide
COPD	Chronic obstructive pulmonary disease
CysLT	Cysteinyl leukotriene receptor
DPP1	Dipeptidyl peptidase I
ECl	Extracellular loop
ECM	Extracellular matrix
FLAP	5-Lipoxygenase activating protein
FCS	Foetal calf serum
FEV_1	Forced expiratory volume in one second
GM-CSF	Granulocyte macrophage-colony stimulating factor
GST	Glutathione S-transferase
GPCR	G-protein coupled receptor
HMWK	High molecular-weight kininogen
HPR	Horse-radish peroxidise
HRM	High resolution melting
HBE	Human bronchial epithelial
ICL	Intracellular loop
IgE	Immunoglobulin E
IL	Interleukin
Kb	Kilobase
KDa	Kilodalton
LDH	Lactate dehydrogenase
LT	Leukotriene
MC_T	Mast cell containing tryptase, not chymase
MC_{TC}	Mast cell containing tryptase and chymase
MCP	Monocyte chemoattractant protein
MEM	Modified Eagle's medium

MES	2-[*n*-morpholino]ethane-sulphonic acid
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
Nt	Nucleotide
PAGE	Polyacrylamide gel electrophoresis
PAR	Protease activated receptor
PAF	platelet activation factor
PG	Prostaglandin
SCF	Stem cell factor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TXA	Thromboxane

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Chapter 1
General Introduction

1. General Introduction

Mast cells have been implicated in many inflammatory and allergic conditions, and particularly in asthma. The major secretory product released upon degranulation is the trypsin-like serine protease tryptase, and its levels are elevated in the airways of asthmatic people. Tryptase has been reported to have a range of pro-inflammatory actions and is able to alter the behaviour of a number of cell types.

Our knowledge of tryptase has drawn predominantly from studies with the β-form of the tryptase, and investigation of α-tryptase, the first form to be cloned, has been neglected. Genes for these closely related forms of tryptase are located on the same region of chromosome 16, but it is not clear how they are arranged. A substantial proportion of the population lack the α-tryptase, but the potential for the gene for this tryptase variant to be associated with asthma or other allergic conditions has not been explored. General substrates for β-tryptase have been proposed, including protease activated receptor-2 (PAR-2); however the key substrate remains to be defined, and the actions of α-tryptase remain to be clearly demonstrated.

1.1 The mast cell in asthma

Asthma is defined as a chronic inflammatory disorder of the airways in which many cells and cell product elements can play a role. Present among these is the mast cell. Patients with asthma suffer from recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or early morning. These episodes are usually associated with widespread but variable airflow obstruction, which is often reversible even without treatment, and also increased bronchial hyperresponsiveness (BHR) to a variety of stimuli, which is one of the main asthma characteristics [1].

The prevalence rates of asthma depend on the definitions that have been used to assess the disease as a doctor's diagnosis, patient's symptoms and the way BHR is defined. For example, a difference between the doctor's diagnosis of asthma and the presence of BHR has been reported [2], where only 7.2% of children suffered from BHR, were prior diagnosed by doctor as asthmatic. Different environmental factors have been investigated to explain the rise in prevalence of asthma.

Factors like exposure to allergen, smoking, air pollution, and some diets, have been implicated as provoking factors for asthma [3].

Two key alterations of the airways typically associated with asthma are variable airway obstruction and BHR. Several factors have been found to contribute to the airway narrowing, such as smooth muscle contraction, airway wall thickening, oedema and increased secretion of mucus. Airway obstruction can be measured by forced expiratory tests or measurements of airway resistance and conductance. Stimuli that evoke BHR can act directly on receptors of airway smooth muscle or may act through cellular and neurogenic pathways. In general, the concentrations of an agent that can cause a drop of 20% in forced expiratory volume in one second (FEV_1) is lower than that producing the same effect in healthy subjects [4].

Asthma affects people of all races and ethnic groups. The prevalence of asthma has increased dramatically over the past few decades in Western countries [5] and more recently similar trends are being reported in less-developed nations [6]. Asthma is a major health problem that significantly affects the lives of its many sufferers especially children. It has a substantial economic impact on public health spending [7], and there is a need for better understanding of the condition.

The pathology of asthma has been attributed to the dysregulation of a number of physiological processes. These include inflammatory cell infiltration of the airways in which eosinophilia is often prominent. Accompanying structural changes include subepithelial collagen deposition and hyperplasia of mucus glands and airway smooth muscle cells [8]. Increased numbers of mast cells are present within the bronchial smooth muscle bundles [9], as well as in the epithelial lining, mucosa, sub-mucosa and even in the airways lumen [10] and in the alveolar walls in asthma [11], pointing to a pivotal role for mast cells in asthma.

Mast cell activation is a characteristic feature of the acute asthmatic attack, which occurs 10-20 minutes after allergen challenge and is followed by gradual recovery in the following 2 hours [12]. Mast cells can be activated when allergen cross-links the high affinity immunoglobulin E (IgE) receptors (FcεRI) on their cell surface. When adjacent receptors are aggregated by multivalent antigens, a signaling cascade culminates in secretion of an array of mediators [13], including

histamine, proteases, prostaglandin D_2 (PGD_2) and leukotriene C_4 (LTC_4). Their release leads to bronchoconstriction, mucosal oedema and increased mucus secretion, which are responsible for the acute airway narrowing in response to allergen [14]. The levels of mast cell tryptase and histamine in broncho-alveolar lavage (BAL) fluid may be dramatically increased within minutes of bronchial allergen challenge [15], supporting the idea that mast cell activation is an early event in asthma pathogenesis.

Asthma is a chronic condition with evidence of continuous mast cell degranulation, as assessed by electron microscopy, in both the epithelium and submucosa [16]. Increased numbers of mast cells can also be recovered in BAL fluid from asthmatics compared to healthy controls, as well as increased concentrations of histamine and tryptase, providing further evidence for on-going mast cell degranulation [17].

Airflow obstruction, airway hyperresponsiveness and inflammation and remodelling in the tissue will be mediated by a complex interplay of many cell types. However, among these the mast cell is likely to make a particularly important contribution in these processes. The next section will review the key characteristics of this cell type and the potential involvement of cellular constituents

1.2 Mast Cells

Paul Ehrlich (1878) was the first to identify mast cells in human connective tissues on the basis of the metachromatic (change from reddish to purple color) staining properties of their prominent cytoplasmic granules with aniline dyes. He called them “mästzellen” a term that may have referred to feeding or could be interpreted as “well-fed” based on account of the presence of numerous granules [18].

Mast cells are derived from $CD34^+$ progenitor cells in the bone marrow [19]. These cells express the receptor (c-kit) for stem cell factor (SCF) a key growth factor for mast cells [20]. SCF has multiple biological effects on mast cells, including stimulating cell differentiation and homing, prolonging cell viability, inducing cell hyperplasia, and enhancing mediator production [21]. Mast cell

precursors leave the circulation and migrate into tissues where they acquire their mature phenotype characterized by the presence of the serine protease tryptase.

Human mast cells have been divided into two major sub-populations on the basis of their protease content. Mast cells expressing the protease tryptase but not chymase (MC_T) have been localized predominantly to respiratory and gastrointestinal mucosal tissues and their granules appear as scroll-like on electron microscopy [22]. A major subtype of mast cells contains both tryptase and chymase (MC_{TC}) and may also express carboxypeptidase, and cathepsin G. MC_{TC} cells predominate in the skin, the submucosa of respiratory and gastrointestinal tracts, in blood vessel walls and in the heart. At the ultrastructural level, the granules have a lattice or grating appearance and the scroll-like pattern is less prominent [22]. Rarely a subpopulation of mast cells containing only chymase (MCc) but not tryptase has been reported [23].

Human mast cell heterogeneity based on expression of proteases may be linked with heterogeneity in expression of certain cytokines. Interleukin-4 (IL-4) has been found to be mainly expressed by MC_{TC} and MC_T in bronchial biopsy tissue from asthmatic subjects, while IL-5 and IL-6 are only expressed by MC_T in such tissues [24]. In addition to that, heterogeneity in proteoglycan contents has also been reported, where the density and/or molecular size of heparin have varied between different sites [25]. This heterogeneity may be reflecting the range of actions that could be promoted by the mast cells in different tissues.

Activation of mast cells can be induced by immunological or nonimmunological stimuli. The first is mediated through aggregation of FcεRI, which leads to mast cell activation, granule exocytosis, and mediator release [26]. Mast cells may also be activated by non-immunologic stimuli including certain neuropeptides, basic compounds [27], complement components [28], and some drugs (such as opiates). Morphologically, both mechanisms appear similar though the biochemical processes that lead to mediator release may differ.

The remarkable variety of mediators derived from mast cells has pointed to the possibility that these cells may actively participate in a range of inflammatory and physiological processes [29]. Changes in mast cell number in various anatomical sites and evidence of degranulation have been observed in a wide spectrum of

innate and adaptive immune responses [30], anaphylaxis [31], conditions often with asthma, these can be features of fibrosis [32], wound healing [33], and autoimmune disease [34]. The potential roles of mast cell mediators will be considered in more detail in the following section.

1.3 Mast cell mediators

The more abundant mediators of inflammation released from human mast cells are illustrated in (Figure 1-1).

1.3.1 Histamine

Histamine (β-imidazolylethylamine) is a biogenic amine and the only one of its kind found in human mast cells. Histamine is formed within the cells, and is stored in the secretory granules in close association with the carboxyl groups of proteoglycans or proteins with which they form ionic linkages. Upon degranulation, histamine is externalized where it rapidly dissociates from the proteoglycan-protein complex and is rapidly metabolized, within minutes of release. Thus it is likely to act primarily at the site of its release. Histamine has been implicated in the pathogenesis of asthma, and levels in BAL fluid collected from asthmatic subjects are greater than those in healthy subjects [35].

1.3.2 Proteoglycans

Proteoglycans are highly sulphated acidic macromolecules located in the secretory granules of mast cells. They are composed of glycosaminoglycan side chains covalently linked to a single-chain protein core. Two proteoglycans, heparin and chondroitin sulphate E, have been identified in the granules of purified human mast cells [36]. These molecules serve to bind to histamine, neutral proteases and acid hydrolases. Their binding to histamine seems to maintain the osmolarity, thereby preventing swelling of the granules. Proteoglycans appear to be essential for the storage of histamine and tryptase in mast cell granules [37;38].

1.3.3 Neutral proteases

The term neutral protease is applied where the optimal enzyme activity is at neutral pH (such as that in the respiratory tract). Three distinct proteases have been isolated from human mast cells so far, tryptase, chymase and carboxypeptidase [39]. Proteases are enzymes that hydrolyse peptide bonds and they can be sub-classified into those enzymes that cleave peptide bonds distant

from the ends of polypeptide chains (referred to as endopeptidases) and those that cleave peptide bonds close to the ends of the chains (referred to as exopeptidases). Endopeptidases have been further classified into four groups based on the nature of their catalytic site; serine, cysteine, aspartic and metalloproteases [40].

The serine proteases comprise the largest group of these enzymes and include tryptase, chymase and cathepsin G. Chymase and cathepsin G, cleave peptides at sites next to amino acids with aromatic residues, whereas tryptase cuts the peptide bonds next to amino acids with basic residues (lysine and arginine). As the main focus of the present study is tryptase, this protease will be considered separately.

Human chymase is a monomeric enzyme of 30kDa and is the principal enzyme accounting for the chymotrypsin-like activity in human cutaneous mast cells. The enzyme is an endopeptidase, stored in a fully active form in association with proteoglycans in the mast cell granules, bound to heparin and/or chondroitin sulphated E. However chymase resides on separate proteoglycan molecules from those for tryptase, and unlike tryptase, its stability is not affected by heparin [41].

Cathepsin G is a 30kDa neutral protease, also found in neutrophils and monocytes. The presence of a cathepsin G-like enzyme in MC_{TC} cells was demonstrated using immunohistochemical, western blotting and enzymatic techniques [42]. When mast cells are activated, cathepsin G together with chymase and carboxypeptidase are released in a 400-500 kDa complex associated with proteoglycan, and are likely to act in concert with other enzymes to promote proteolysis [41].

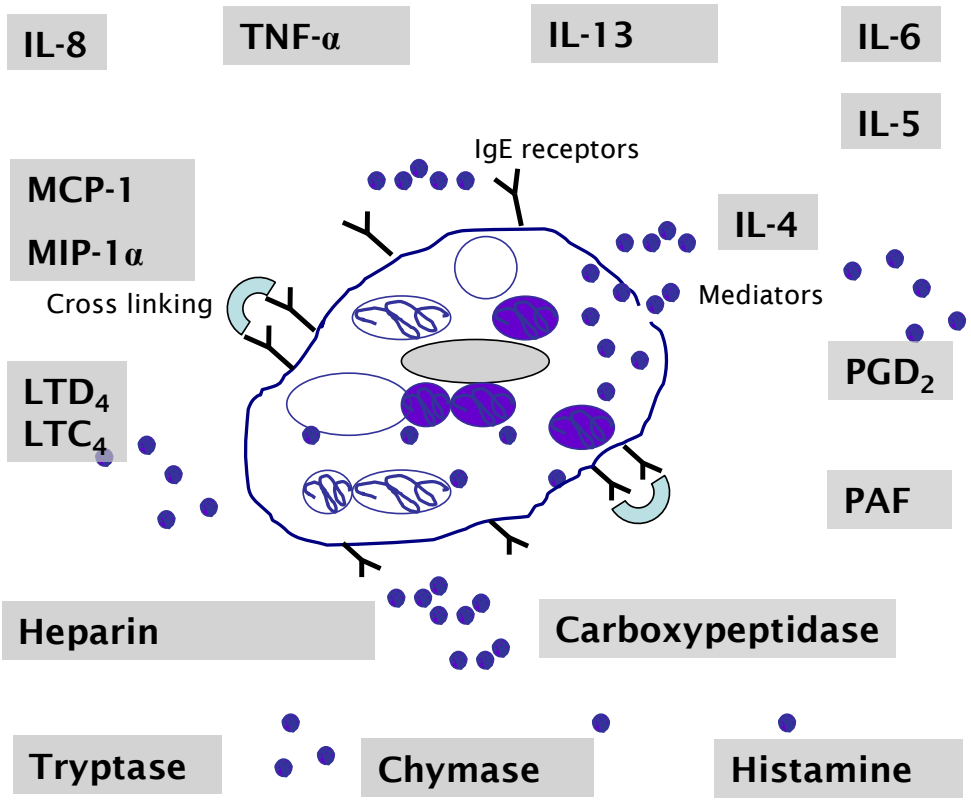


Figure 1-1. Products of human mast cell activation.

Mast cell carboxypeptidase A, is an enzyme with a molecular mass of 34.7 kDa. It appears to be selectively present in MC_{TC} cells only [43]. It is released with chymase and cathepsin G and has been shown to be bound to proteoglycans in the same macromolecular complex as chymase [41]. Its biological role is not clear.

1.3.4 Lipid mediators

The activation of mast cells initiates the de novo synthesis of lipid-derived substances which occurs in parallel with the release of the preformed granule-associated mediators. The newly generated mediators are generated by the cyclooxygenase and lipoxygenase pathways. The cyclooxygenase products include prostaglandins and thromboxanes, whereas those generated by lipoxygenase are leukotrienes (LT), hydroperoxy eicosatetraenoic acids (HPETEs), and the reduced products of the HPETEs, hydroxyl eicosatetraenoic acids (HETEs).

Prostaglandin D_2 (PGD_2) has been detected in BAL fluid from asthmatic patients and it constricts human bronchial smooth muscle in vitro. It has also been found to elicit eosinophil infiltration in the airways of dogs [44;45]. PGD_2 acts through two G-protein coupled receptors termed DP 1 and DP 2. DP1 deficient mice are protected from induction of allergic inflammation in a model of allergic asthma [46], and DP1 antagonists have been reported to block airway inflammation in animal models [47].

Prostaglandin E_2 (PGE_2) has four receptors designated EP1 to EP4 which are coupled to a multitude of intracellular signal transduction pathways. Both pro-and anti-inflammatory roles have been proposed in allergic disease. Studies with EP3 gene deficient mice and with EP3 agonists have suggested anti inflammatory actions for this receptor [48], while EP-2 and EP-4 receptor agonist have been proposed to mediate protective actions in ovalbumin induced bronchoconstriction in animal models[49]. On the other hand, PGE_2 has been demonstrated to augment IgE production and histamine release through EP1 and EP3 receptors [50].

Prostaglandin I_2 (PGI_2) is produced mainly by vascular endothelial cells but its release from lung tissue during allergic reactions has been reported, associated with airways smooth muscle relaxation and suppression of leukotriene production

[51]. A role in suppression of chronic airway inflammation and remodeling has been proposed on the basis of studies with IP receptor gene deficient mice [52]. In addition, PGI_2 has been found to reduce the responsiveness of Th2 cells to inhaled allergen in mice [53].

Thromboxane A_2 (TXA_2) is a potent constrictor of bronchial smooth muscle via TP and DP receptors and an inducer of airway smooth muscle cell proliferation [54]. Thromboxane A_2 receptor agonists have been found to stimulate bronchial smooth muscle cell hyperplasia , consistent with the changes in airway remodelling which occur in the asthmatic airways [55].

The 5-lipoxygenase pathway forms 5-HPETE, which is subsequently converted into an unstable intermediate, LTA_4, by the enzyme 5-lipoxygenase which works in concert with the 5-lipoxygenase activating protein (FLAP). Neutrophils preferentially hydrolyze LTA_4 by the enzymatic addition of water to the dihydroxy leukotriene, LTB_4, while in mast cells, the preference is to form LTC_4 by the enzymatic addition of glutathione. LTC_4 is released into extracellular fluid and converted to LTD_4 and LTE_4 through the release of glutamic acid and glycine, respectively.

Elevated concentrations of LTC_4 have been detected in BAL fluid collected from atopic human subjects after allergen challenge [56], and high levels of LTE_4 have been found in urine samples from patients following asthma exacerbations [57]. The use of 5-lipoxygenase inhibitors has been shown to improve lung function in mild to moderate asthmatic patients on other asthma treatment [58], but the rationale for single use of such drugs is still unclear.

Cysteinyl leukotriene receptors (CysLT1 and CysLT2) are G-protein coupled receptors with different pharmacologic profiles and incompletely overlapping tissue distribution. The CysLT1 has higher affinity for LTD_4 than for LTC_4 or LTE_4, whilst CysLT2 has equal affinity for them suggesting different functions for these receptors. The use of CysLT1 receptor antagonists has been found effective in the treatment of asthma, particularly in asthma phrophylaxis.

1.3.5 Cytokines

This ability of mast cells to produce a range of cytokines confers on them the potential for interaction with various cell types to modulate homeostatic, allergic, inflammatory and host defence functions [59]. Human mast cells are able to synthesize and secrete a number of cytokines including: IL-4, IL-5, IL-6, IL-8, IL-10, IL-15, IL-13, IL-16, granulocyte macrophage-colony stimulating factor (GM-CSF), transforming growth factor (TGF)-β, tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-1α (MIP-1α),and monocyte chemoattractant protein-1 (MCP-1) [60].

IL-4, IL-5, IL-6 and TNF-α have been implicated in asthma and have been identified within mast cells by immunohistochemical staining in the bronchial mucosa [61]. IL-4 and IL-13 can regulate IgE synthesis, and a IL-4/IL-13 receptor antagonist has proven effective in preventing AHR in a model of asthma in the monkey [62]. This has yet to be applied in human asthma. A possible role for TGF-β in the remodeling process of chronic asthma has been postulated on account of its ability to induce epithelial mesenchymal transition, a principal step in the remodeling process [63].

IL-15 has been found to be able to regulate expression of the chymotryptic enzyme mast cell protease-2 in a mouse model [64], through controlling transcription synthesis of relevant mast cell proteases, but such effect with human mast cell protease has not been explored. IL-10 can exert either anti-inflammatory or pro-inflammatory effects on a variety of cell types. Thus IL-10 is a potent inhibitor of monocyte/macrophage function and can suppress the release of TNF-α, IL-6, MIP-1α and IL-8 from blood mononuclear cells [65], but on the other hand, stimulate the release of MCP-1 [66]. IL-10 released from mast cell granules and fibroblasts, have been found to decrease IgE receptor expression and function in human skin mast cells [67], and can block IgE-mediated cytokine production.

High levels of IL-6 have been documented in BAL fluid [68], and serum [69] in asthma, but its precise role is not clear. Transgenic animal models with over expression of IL-6, have shown alveolar air space enlargement and subepithelial fibrosis, a hallmark of the airway remodelling process [70]. However, as it is difficult to separate the effects of IL-6 from the other cytokines in these models

and because IL-6 is a multifunctional cytokine, it may exert distinct effects, depending on the phase of airway inflammation. Recently, a role for exogenous IgE in mast cell survival has been proposed, and this may be dependent on an autocrine function of IL-6 [71].

A possible role for tumor necrosis factor (TNF-α) in asthma has been indicated in studies with mice deficient in this cytokine, as they have reduced airway hyperresponsiveness, inflammation and goblet cell metaplasia following allergen challenge [72]. Thus, mast cell cytokines are likely to contribute to allergic airway disease through a complex network with other mediators.

1.4 Mast cell tryptase

Human tryptase enzyme (E.C.3.4.21.59) was first isolated from human lung mast cells in 1981 [73] and later from whole tissues including pituitary, lung and skin. There are six to seven human tryptases identified so far (Table 1-1). The best studied is β-tryptase (with 90% sequence identity to the less studied α-tryptase). Other tryptases are δ- tryptase [74], γ-tryptases [75] and ε-tryptase [76]. A novel protein, HtrA1 has been described as a member of the tryptase family [77]; this has been found to be secreted constitutively but its function remains unclear. In the next section, the β- and α- tryptases will be the main focus of the review as they are directly relevant to the present studies.

1.4.1 Alpha and β-tryptases

To date, three similar β-tryptases have been identified and termed: β-I, β-II and β-III tryptases. The amino acid sequences of β-I and β-II tryptases are almost identical, but β-III has differences in positions 60-63, which encode RDR instead of the HGP in β-I and β-II tryptases [78]. Beta-tryptases are stored in the active form, bind to heparin and are released as a complex with proteoglycans on mast cell activation. The removal of the 12-residue propeptide of β-tryptase is needed for processing and activation, and if inhibited, it leads to constitutive secretion of pro-β-tryptase [79].

Two very similar α-tryptases have been identified and termed: α-I and α-II tryptases. Sequance for αI-tryptase was the first to be described and later the sequence for αII tryptase was identified. Unlike β-tryptase, α-tryptase has not been isolated from mast cells, and our understanding is based on studies

Table 1-1. Tryptase genes and proteins identified and their unique characteristics.

Tryptase gene	protein	GenBank identifier	Location	Function
TPSAB1	Tryptase, αI Tryptase, αII	M30038, AF098328	Constitutively secreted as a proform	Not known
TPSB2	Tryptase, βI Tryptase, βII Tryptase, βIII	M33494, AF099145, AF099143	Stored in MC and secreted in pro and active forms	Proinflammatory functions (animal and human studies)
TPSG1	Tryptase, γI Tryptase, γII	NM_012467, AAF76458	Membrane bound tryptase, stored in MC	BHR and increase IL-13 expression (mouse study)
TPSD1	Tryptase, δ	NM_012217	Secreted as a proform	Inactive in human but active in monkey
TPSE1	Tryptase, ε	NM_022119	Expressed in epithelium	Play a role in fibrinolysis in lung (mouse study)
HtrA1	PRSS11	NM_002775	MC_T only and is constitutively secreted	Linked to macular degeneration

with recombinant forms. Recombinant α-tryptase has been proposed to be incapable of being processed into its mature form due to a mutation in the propeptide form [80]. The consequences may include a failure to adopt an active conformation and to accumulate in the secretory granules. Instead, inactive pro-enzyme may divert into a constitutive secretory pathway [81]. While α-tryptase can be assembled into a tetramer, the activity detected is negligible compared with that of recombinant β-tryptase. The crystal structure of recombinant α-tryptase has revealed that the overall folding and tetramer assembly is essentially similar to β-II tryptase although packing of the tetramers in the crystal is different [82].

1.4.2 Biochemical characteristics

Mast cell tryptase is a tetrameric protease with a molecular weight of about 134 KDa. It is made up of four non-covalently bound subunits. Each subunit has a molecular weight of about 31 to 38 KDa, as a consequence of variable glycosylation that was revealed by gel electrophoresis and gel filtration under both denaturing and nondenaturing conditions [73]. Determination of the crystal structure has revealed that the four active sites of the tetramer are directed towards an oval central pore (Figure 1-2), which restricts the access of macromolecular substrates and natural enzyme inhibitors [83].

Although the site for activation of tryptases from their precursors is unknown, it is most likely that it occurs in the Golgi apparatus before it is packaged into in the mast cell secretory granules. *In vitro* experiments with recombinant human α- and β- pro-tryptases indicate that two steps may be required for β-tryptase activation. The first step appears to be autocatalytic and the process is completed in a second step in which the pro-dipeptide is removed by dipeptidyl peptidase I (DPP1), also known as cathepsin C. The activation of pro α-tryptase does not proceed possibly on the account of a mutation in the propeptide, which blocks the autocatalytic process [80;84]. However, all studies have been *in vitro*, and other factors may contribute towards the activation of tryptases.

Optimal catalytic activity is expressed for β-tryptase when it is in a tetrameric form. However, when incubated at neutral pH (generally present in the tissues), or at physiological ionic strength, or in the absence of a stabilizing polyanion such

as heparin, the activity is rapidly lost as the active enzyme dissociates into inactive monomers [85;86].

1.4.3 Tryptase substrates and inhibitors

Beta tryptase preferentially cleaves peptide and ester bonds on the carboxyl side of basic amino acids [87]. Several neuropeptides are substrates and peptidergic nerves are present in the immediate vicinity of mast cells [88]. Tryptase has been also shown to cleave vasoactive intestinal peptide (VIP) [89], peptide histidine methionine (PHM) and calcitonin-gene related peptide (CGRP) [90], and tryptase may play a role in the normal physiological control of these neuropeptides. Proteins that are cleaved by tryptase include, fibrinogen [91] and high and low - molecular-weight kininogen (HMWK) [92;93]. Tryptase can also activate pro-matrix metalloproteinase 3 (MMP3) [94], and pro-urokinase [95]. Although a wide range of substrates have been identified for β- tryptase, such studies are lacking for α-tryptase.

Biological control over tryptase activity has been demonstrated at several levels. Firstly the acidic pH within the mast cell granules may suppress enzymatic activity within the mast cell [96]. Also, histamine is a reversible inhibitor and could limit tryptase activity inside granules or in the vicinity of degranulated mast cells [97]. The tetrameric conformation of β-tryptase is likely to restrict access of big protease inhibitors to the catalytic sites and this is likely to explain its resistance to known biological inhibitors, including α-1 antitrypsin, antithrombin III, α-2-macroglobulin, bovine aprotinin and soybean trypsin inhibitor [98]. Over the last few years, several synthetic β-tryptase inhibitors have been defined. Leupeptin has been widely employed as an investigative tool in studies with tryptase. It is produced by Actinomycetes, and it inhibits various serine and cysteine proteases reversibly and in a competitive way [99;100].

A peptide-based inhibitor, APC-366 was the first low molecular mass inhibitor for β-tryptase to be employed in clinical trials. The drug was able to reduce allergen-induced air-way inflammation but not BHR in mild asthmatics [101], but such effects need to be tested in a larger number and in other disease stages. The uses of dibasic inhibitors, which bind to two catalytic sites of the tryptase tetramer, have proven to be highly potent and selective. AMG-126737 is an

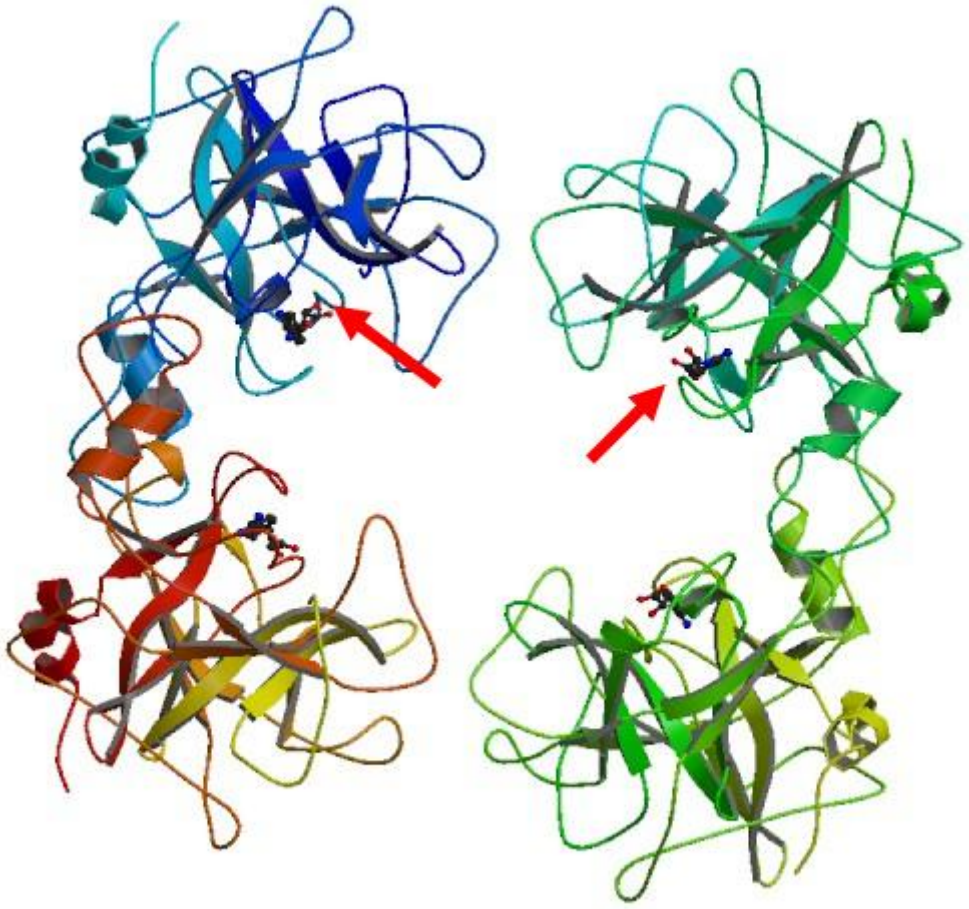


Figure 1-2. Tetrameric β-tryptase crystal structure, each of the four monomeric subunits has an active sites (red arrow) facing towards a central pore in the tetramer.

example, which has been proved successful in preventing both early and late phase bronchoconstriction and BHR in sheep and pigs [102], but clinical studies are lacking on this drug.

A leech-derived tryptase inhibitor, secretory leukoprotease inhibitor, a serpin-type proteinaceous inhibitor (SERPINB6), and a tick derived tryptase inhibitor may prove useful in developing new drugs to control tryptase activity [103-105]. Protamine sulphate that binds to heparin has also been reported to be able to displace tryptase from heparin and hence destabilize the active tryptase tetramer [106].

1.4.4 Role of tryptase in asthmatic disease

Tryptase levels have been found to be higher in BAL fluid and sputum from asthmatic subjects than in non-asthmatic control subjects [107] and an increase in BAL fluid levels following endotracheal challenge with house dust mite allergen has also been found in mild atopic asthmatic subjects, in the early asthmatic reaction but not in the late asthmatic reaction [108]. Tryptase can also induce bronchoconstriction and increase bronchial hyperresponsiveness in vitro, possibly through stimulating histamine release from mast cells [109], degradation of bronchodilator neuropeptides and potentiation of inflammatory responses [60].

Tryptase may cause bronchoconstriction by cleaving VIP and PHM, both of which are potent relaxants of smooth muscle, and tryptase can also attenuate the vasodilator effects of CGRP [90]. Tryptase may contribute to neurogenic inflammation through stimulating release of substance P and CGRP [110], and certain components of the extracellular matrix could be cleaved by tryptase that include type IV collagen and fibronectin [111;112]. This involvement in the degradation of the ECM implicates tryptase in tissue destruction, and this along with tissue remodelling is an important process in chronic asthma. Tryptase has been shown to stimulate the proliferation of various human cell types, including lung fibroblasts [113], airway smooth muscle cells [114], epithelial cells [115] and microvascular endothelial cells [116]. Other actions on fibroblasts includes stimulating the synthesis and release of type 1 collagen [32], and fibroblast chemotaxis [117].

Most studies of tryptase function have been with the enzyme purified from human lung tissue. The potential roles of β-tryptase and α-tryptase have not been studied, and the study of α-tryptase function has been neglected. In the next section, the possible contribution of protease activated receptor-2 (PAR-2) to altered cell function will be considered.

1.5 Protease activated receptor-2 (PAR-2)

PAR-2 is a member of a large family of seven trans-membrane domain G-protein coupled receptors (GPCR). These receptors have a unique mechanism of irreversible activation by proteases, and the ligand is physically part of the receptor itself. PAR-2 is cleaved by trypsin and mast cell tryptase at specific sites within the extracellular amino terminus of the receptor. Cleavage leads to exposure of a new amino terminus that serves as a tethered ligand domain, that binds to conserved regions in the second extracellular loop of the receptor (Figure 1-3), resulting in the initiation of the signal transduction [118]. Four members of the PAR family have been identified and cloned, PAR-1, PAR-2, PAR-3 and PAR-4. Table 1-1 details protease agonists found for these receptors, the sequence of the tethered legend exposed on activation, and the cellular distribution.

1.5.1 Characteristics of PAR-2

A DNA sequence encoding a protein of 395 residues with the typical characteristics of a GPCR and which has about 30% amino acid identity to thrombin receptor (PAR-1) was first cloned from a mouse genomic library [119]. Following that discovery, PAR-2 was cloned in humans [120]. It is 397 amino acids in length and has about 83% homology with the corresponding mouse sequence. It is located on chromosome region 5q13 adjacent to PAR-1.

1.5.2 Mechanisms of PAR-2 activation

The activation of most known GPCRs involves reversible activation by soluble ligands but the protease agonists of PAR-2 have a unique mechanism of activating the receptor, with the ligand physically part of the receptor sequence (Figure 1-3).

Activation by trypsin [121] and mast cell tryptase [122] has been demonstrated. PAR-2 contains a putative enzymatic cleavage site $SKGR^{34} \downarrow S^{35}$ LIGRL [119] and is cleaved at the Arg^{34}-Ser^{35} bond, thus leading to exposure of a new tethered ligand (NH_2-SILGKV). Synthetic five to six amino acid peptides corresponding to this

tethered ligand (SLIGRL in mouse, SLIGKV in human) can activate PAR-2 without the need for receptor cleavage [123]. A selective PAR-2 agonist, trans-cinnamoyl-LIGRLO-NH_2 (tc-LIGRLO-NH_2) has been found to be more potent than the peptide whose sequence corresponds to that of the tethered ligand of human PAR-2, SLIGKV-NH_2 [124]. Generally, the rat/mouse peptide SLIGRL is slightly more potent than the human agonist SLIGKV [125]. Comparing different synthetic peptides efficiency using alanine scanning indicates that Leu^2 and Arg^5 are essential for activity and it also showed that replacing Ser^1 or Arg^5 with Ala, leads to reduced activity, whereas substitution of Gly^4 or Leu^6 has only a slight effect on the PAR-2 activation [126].

1.5.3 PAR-2 in the airways

PAR-2 is abundantly expressed on airway epithelium and smooth muscle [127]. There is evidence that PAR-2 activating peptides can act on epithelial PAR-2 and initiate the release of PGE_2 which can prevent contraction of airway smooth muscle cells [128]. Also, intravenous injection of PAR-2 activating peptides has been reported to significantly inhibit the histamine-induced increase in lung resistance in guinea-pigs, by a mechanism independent of prostanoids or nitric oxide release [129]. This supports the idea of a protective role for PAR-2 in the airways in lung disease but such observations have not been made in human studies. On the other hand, there is strong evidence for a proinflammatory role for PAR-2 receptors. Activation of PAR-2 by intratracheal instillation or intravenous injection of PAR-2 activating peptide in the guinea-pig airways causes bronchoconstriction and other motor effects, partly mediated by neural mechanisms [130].

Importantly, PAR-2 is expressed on mast cells (both the surface and on the membrane of secretory granules), and therefore activation of PAR-2 receptors on mast cells by tryptase secreted from their granules may provide a positive feedback mechanism to stimulate further degranulation [131]. Matrix metalloproteinase-9 (MMP-9), a protease which is important in tissue remodelling and repair, may be released by human airway epithelial cells in response to stimulation by PAR-2 activating peptides [132], suggesting that PAR-2 may be involved in tissue repair and remodelling. The extent to which PAR-2 represents a key substrate for tryptase is not known, and the potential for the various forms of tryptase to cleave this receptor has not been studied.

Table 1-2. PAR activating proteases, disabling proteases, activating peptides, localization.

	PAR-1	PAR-2	PAR-3	PAR-4
Activating proteases	Thrombin, Trypsin, FXa	Trypsin, Tryptase, Proteinase-3, FVIIa	Thrombin	Thrombin, Trypsin, Cathepsin G
Inactivating proteases	Cathepsin G, Proteinase-3, Elastase	Elastase, Cathepsin G	Cathepsin G	
Cleavage site AP	LDPR41↓S42FLLRN	SKGR34↓S35 LIGKV	LPIK38↓T39FRGAP	PAPR47↓G48YPGQV
Tethered ligand	SFLLRN	SLIGKV(human), SLIGRL(mouse)	TFRGAP	GYPGQV
Localization	Platelets(human), endothelium, epithelium, neurons	Epithelium, endothelium, fibroblast, myocytes, neurons	Platelets (mouse), endothelium, myocytes	Platelets (human), endothelium, myocytes

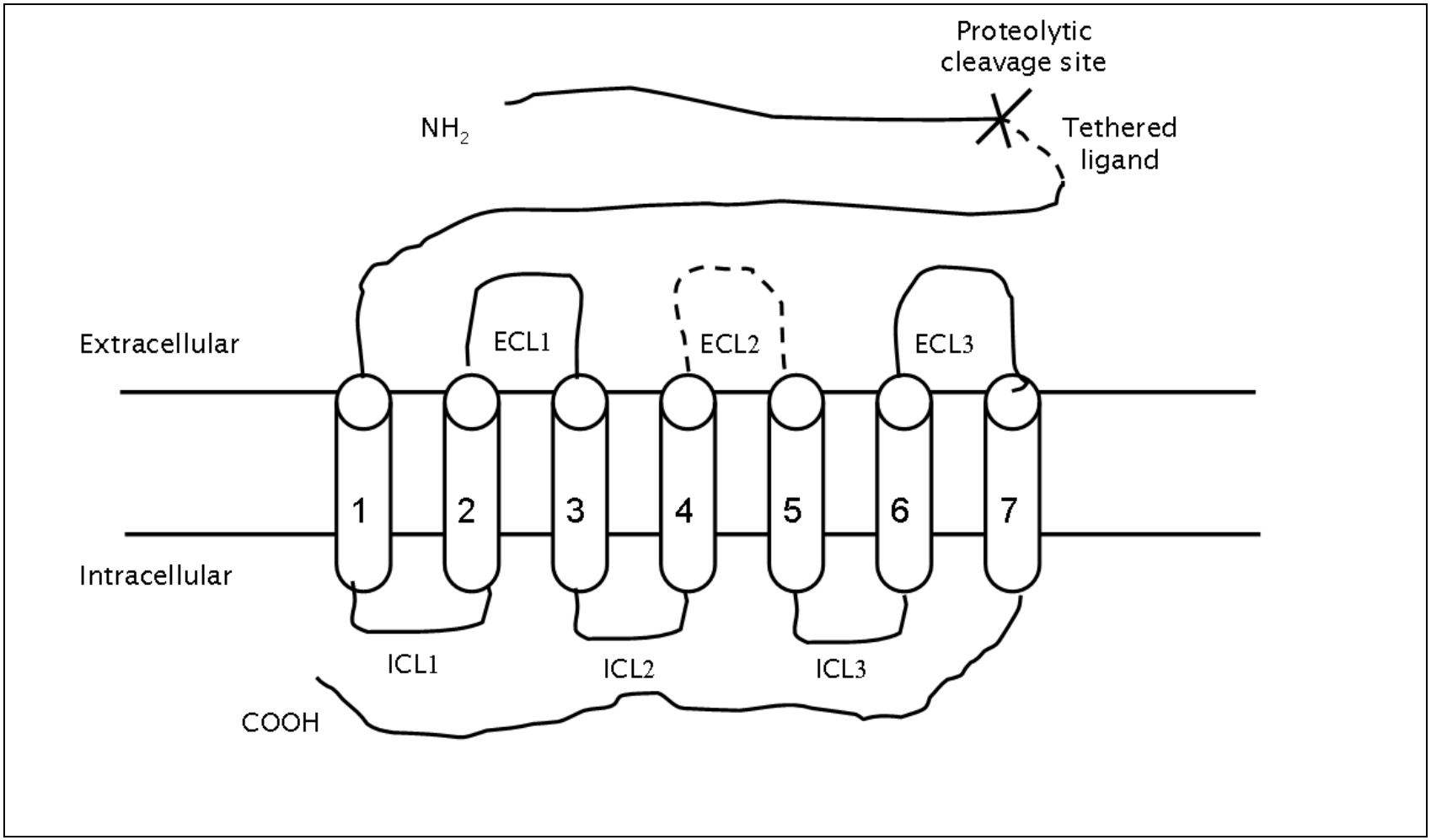


Figure 1-3. Diagrammatic representation of PAR-2 showing the 7 transmembrane domains, and the intracellular (ICL) and extracellular (ECL) loops. The cleaved ligand (X) or the PAR-2 AP bind to ECL2 which is shown as dashed line.

1.6 Asthma genetics

1.6.1 Defining asthma in genetic studies

Asthma has been long recognized to have a familial basis. In 1976, Wagatsuma *et al*, have studied four families with at least three members with asthma and they concluded that inheritance is a definite factor [133]. However the exact mechanisms that underlie this familial basis were unclear. In the last two decades, new tools in molecular biology and genetic epidemiology have become available that are facilitating understanding of the familial basis of asthma [134].

Asthma is a complex disease that is influenced both by genetic and environmental factors. There appears to be no simple relationship between genotypes and phenotypes of asthma. This may be due to different genes causing the same phenotype (genetic heterogeneity) or the same genotype resulting in different phenotypes.

Different phenotypes of the disease are illustrated by broad variations in the age at onset, the severity of asthma and the progression of the disease; while the genetic heterogeneity involves multiple genes that interact with each other and with the environment. Evidence for this is provided by the results of family studies, where the inheritance of asthma has not been explained by a single gene model but rather by oligogenic or polygenic models. With the more recent advances in molecular technology, our understanding of the genetic basis is being refined by the discovery of gene copy number variation (CNV), a feature of many genes implicated in the etiology of asthma [134].

1.6.2 Gene copy variation and disease susceptibility

An early sign of gene CNV and association with a phenotype was described in the last century. Duplication of the Bar gene in *Drosphila melanogaster* was shown to be able to cause the bar eye phenotype [135]. CNVs influence gene expression and phenotypic variation and adaptation by disrupting genes and/or altering gene dosage, and can cause disease, as in microdeletion or microduplication disorders, or may confer risk to complex disease traits [136;137].

Over 118 genes have been associated with asthma in at least one clinical study, and findings with at least 54 of these have been replicated in one or more follow-up studies by different investigators [138]. For example, the copy number of the glutathione S-transferase (GST) gene superfamily, has been found to be associated with increased risk of asthma in children in Tunisia [139], although such results have not been replicated in a Caucasian population [140].

Diseases such as asthma are typically caused by multiple genes with their interaction with each other as well as environmental factors. The search for susceptibility loci using existing statistical methods has been found slow and the results often inconsistent and unrepeatable from one study population to another [141], but still the mapping of such complex traits is one of the most important and central areas of human genetics.

Although tryptase genes have not been demonstrated to be asthma candidate genes, the established role of tryptase in allergic and inflammatory conditions, would argue the case for studying the tryptase genes and tryptase gene CNV in asthma. Current understanding of tryptase gene structure and location is discussed in the next section.

1.6.3 Tryptase loci

All tryptase genes (1.8-2.3Kb) have a six exon/five intron organization, with highly conserved intron phase and size (about 80-200 nt), and intron/exon boundary position where the codons for the catalytic triad residues are located in separate exons, namely exons 3, 4 and 6 [142;143].

Genes corresponding to known mast cell tryptases band together at a site about 1.2Mb from the tip of the short arm of chromosome 16 [144] (Figure 1-4). The genomic neighbourhood of this particular region of 16p13.3 is rich in repetitive sequences and therefore is subject to exceptionally high rates of recombination and breakage, and it has been one of the most difficult regions in various genomic investigations to be assembled and sequenced [145].

There is some disagreement in the order and number of tryptase genes present in this region based on genomic sequence data [146], compared with the restriction map data previously reported [144], and the presence of two or three or even more loci remains unclear.

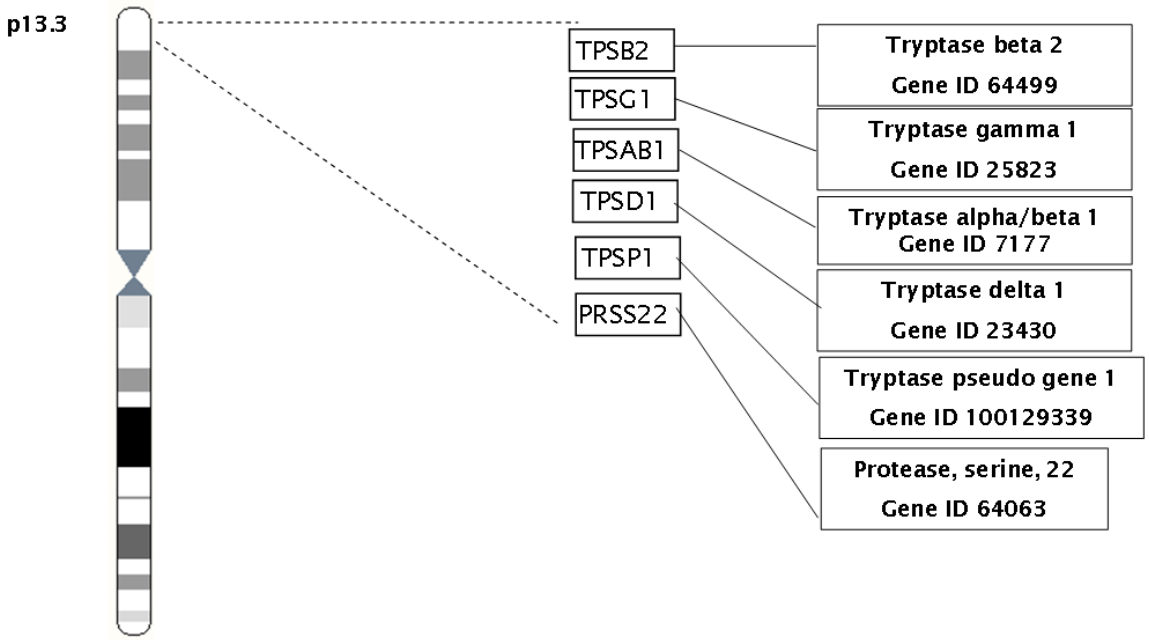


Figure 1-4. Schematic arrangement of chromosome region 13.3 for the family members of the tryptase, with their identification number. The exact arrangement of these genes remains unclear.

1.6.4 Tryptase heterogeneity

Tryptases exhibit considerable heterogeneity in size, charge and kinetic behavior, and this may reflect the sequence differences as well as the difference in their glycosylation patterns. Two groups of tryptase molecules have been cloned from human lung mast cell library, the α- and β–tryptases [142;147], with about 90% amino acid identity between both groups. In addition, three clones cloned independently from a human skin mast cell library are named tryptase I, II and III. Consequently, tryptase I, II and III have been considered together as the β-tryptases but distinguished as β-I, β-II and β-III [143].

Studies of polymorphisms have provided evidence for at least two genetic loci for α- and β- tryptases. Soto *et al*. have proposed that α- and β-I tryptase may be allelic at one locus, and β-II and β-III tryptase at the other [148], while Guida *et al*. have suggested that α, βI and βII tryptases may each occupy separate loci [146]. In addition, Vanderslice *et al*. found multiple cDNAs in genomic clones from skin tryptase, which suggested the presence of at least two and probably three or even more tryptase genes in the human genome [143].

The finding that about 29% of the general population may lack the α-tryptase gene [148], raises the question of possible effects of CNVs on the outcome of known tryptase actions, and highlight the importance of α-tryptase as a counter neighbour to β-tryptase.

The emerging concept of tryptase copy number seems likely to be important; but its association with asthma susceptibility has not been studied.

1.7 Aim of the work

The purpose of these studies was to investigate the hypothesis that mast cell tryptases may play key roles in asthma and allergic conditions, and that α- and β-tryptases may make distinctive contributions to allergic inflammation.

The approach has been to:

1- Determine the structure of the tryptase gene locus, examine copy number variation and investigate the association with asthma phenotypes.

2- Study the potential of α- and β-tryptase to upregulate expression of mRNA for cytokines in human bronchial epithelial cells.

3- Investigate the actions of α- and β-tryptases in vivo in a mouse model, exploring dependency on catalytic activity and activation of PAR-2 receptors.

Chapter 2
Materials and General Methods

2. Materials and General Methods

2.1 Materials

Chemicals

Stock solutions of chemicals were stored at room temperature unless otherwise stated. Most solutions were made in de-ionized water obtained from a Ri Os Reserve Osmosis system/Milli Q Ultrapure Water Systems apparatus (Millipore, Billerica, USA). All chemicals required for general laboratory use were of analytical or molecular grade. Reagents required for a specific purpose and their abbreviations are listed below:

Bromophenol blue, ammonium chloride, hydrochloric acid, ethanol (absolute), ethidium bromide, glycerol (Sigma ultra), dimethyl sulphoxide (DMSO), leupeptin, isopropanol, chloroform, sodium hydroxide, Tri® reagent, N-α-benzoyl-D,L-arginine-p-nitroanilide (BApNA), Trizma®base, sodium chloride, bovine serum albumin (BSA, fraction V-approx 90%), 4-morpholino ethanesulphonic acid (MES) hydrate (minimum 99.5% titration), Tween® 20 and heparin agarose type 1,β-mercaptoethanol (β-ME) were from Sigma Chemical Company (Poole, Dorset, UK). Sodium dodecyl sulphate (SDS), tetramethylethylenediamine (TEMED), ammonium persulphate, protein size standard and 30% acrylamide/bis, 29:1 (3.3% C) were from Bio-Rad Laboratories (Hemel Hempstead, Herts, UK). Butyl Sepharose™ 4 fast flow was from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Agarose was from Eurogentec (Fawley, Southampton, UK). Deoxyribonucleotide triphosphates (dNTPs) were from Promega (Chilworth Science Park, Southampton, UK). BioSep 3000 size exclusion column was from Phenomenex (Macclesfield, Cheshire, UK). TBE buffer 10X liquid was from Amresco (Cochran, Ohio, USA). Polyclonal rabbit antimouse immunoglobulin horseradish peroxidase was from Dakocytomation (Ely, Cambridgeshire, UK). Magic Marker, molecular weight markers ™XP were from Invitrogen (Inchinnan Business Park, Paisley, UK). Amicon filters and ultrafiltration devices, with nominal 30KDa cut-off were from Millipore Bioscience (Hatters Lane, Watford, UK). Methanol and acetic acid were from Fisher Scientific (Loughborough, Leicestershire, UK). Pichia pastoris supernatant was kindly provided for these studies by Roche Pharmaceuticals (Hertfordshire, UK).

Culture media, DNA and enzymes

Enzymes and DNA were stored at -20ºC unless otherwise stated. Separation buffer, DNA size standard kit-600, fragment analysis test sample, separation gel and sample loading solution (SLS) were from Beckman Coulter (High Wycombe, Buckinghamshire, UK). Pro-oligo primer, foetal calf serum (FCS), (100X) penicillin/streptomycin were from Sigma. Primers/oligonucleotides, Taq DNA polymerase and mastermix RT-QP2X-03 were from Eurogentec. RNase inhibitor was from Ambion (Huntingdon, Warrington, UK). Precision mastermix was from Primer Design (Millbrook, Southampton, UK). Minimal essential medium (MEM) media, trypsin (bovine and porcine), EDTA (100X) were from Invitrogen. EcoRV-R0195S, Phusion™, High-Fidelity DNA polymerase and buffers were from New England Bio-labs (Hitchin, UK).

Molecular biology kits

The RNA extraction kits (RNeasy mini kit) were from Qiagen (Crawley, UK). Reverse transcription kit and geNorm house-keeping gene selection kit were from Primer Design. Silver staining kits were from Bio-Rad. Supersignal® West Pico chemiluminescent substrate was from Piercenet (Cramlington, Northumberland, UK). Rapid Romanowsky stain pack-HS705 was from Raymond A Lamb (Eastbourne, East Sussex, UK). Bicinchoninic acids protein assay kit (BCA1 and B9643) was from Sigma. QCL-1000 chromogenic LAL (50-647U) was from Lonza (Slough, Berkshire, UK). Zero Blunt® PCR cloning kit was from Invitrogen.

2.2 Cell Culture

Cells

The SV40 transformed bronchial epithelial cell line, 16 HBE 14o- [149] was a kind gift from Dr D.C. Gruenert. The 16HBE growth medium employed was MEM supplemented with 10% FCS, 100U/ml penicillin and 100ug/ml streptomycin.

Foetal calf serum (FCS) was stored at -20ºC prior to use. It was thawed at room temperature and heat inactivated by incubation at 56ºC for 30 min. Aliquots were stored at -20ºC and a 10% FCS working concentration was used. The penicillin/streptomycin was used as a 1% working stock.

Maintenance of cell line

16-HBE cells are adherent cells and grow predominantly in monolayers. Cells were maintained at 50-75 % confluence by routinely passaging every 3-4 days using trypsin/EDTA followed by centrifugation and were then re-suspended and diluted in fresh medium. Cells were grown at a temperature of 37ºC and in a humidified atmosphere of 95% air and 5 % CO_2. Cells of each passage number were stored in complete medium with 10% DMSO in liquid nitrogen.

16HBE cell harvesting

Medium was removed by aspiration and the cells were washed at least twice with HBSS or PBS. The HBSS was removed and an appropriate volume of trypsin/EDTA was added (25ml flask 1ml, 75ml flask 5ml, 175 ml flask 10 ml). The cells were then incubated at 37ºC for 5-10 minutes to allow trypsin to act. Gentle agitation was used to ensure the detachment of cells, and this was checked using the microscope. An equal volume of the complete medium was then added to stop the action of trypsin; the cells were centrifuged and resuspended in an appropriate volume of medium for counting.

Cell counting

An aliquot of the cell suspension (10 µl) was removed and 90 µl of trypan blue was added and mixed by aspirating with a pipette. The improved Neubauer haemocytometer was cleaned and the cover-slip moistened with water and both sides of the chamber were filled with 5-10 µl of the suspension. Viable (bright cells) and non-viable cells (stained blue) were counted in four areas of the counting chamber, then multiplied by the dilution factor (10) and the correction factor (10^4) to provide the number of cells per ml.

Using the 16HBE-14o bronchial epithelial cell line, the actions of tryptase on the pattern of gene expression were studied. After reaching 75% confluence, cells were detached by trypsin-EDTA, centrifuged, counted and dispensed into each well of a 6-well plate, about $1x10^6$ cells per well and grown for 24 hr and maintained to a confluent layer for another 24 hr.

Cells were treated in 2% medium in the presence or absence of tryptase and other protease inhibitors. The activity of the tryptase was determined at the time it was added. RNA was extracted for the generation of cDNA. Supernatants from treated cells were collected and kept for further analysis.

2.3 RNA extraction

Total cellular RNA was isolated from human bronchial epithelial cells using TRI reagent followed by Qiagen miniprep kit according to the manufacturer's instructions. The purity of the RNA was checked using the Agilent 2100 Bioanalyzer chips and quantified using a NanoDrop ND-1000 spectrophotometer, (V 3.0.1, Thermo) following the manufacturer's protocols. The RNA was stored at -80ºC until use.

2.3.1 Tri RNA extraction

Cells were grown in 6-well plates, at a maximum of $1x10^7$ cells/well. The plate was centrifuged at 120 g for 5 minutes and then the supernatant was collected and kept at -20ºC for further assay. Wells were washed twice with 1 ml of ice cold PBS and to each well, Tri reagent (1ml/well) was added. Cells were homogenized by passing through a 1ml pipette tip 10 times, then transferred to 1.5 ml tubes.

For a sample in 1ml TRI solution, 200 µl of chloroform was added and mixed by flicking by hand, following centrifugation at 10,000g for 10 min at 4ºC. The top layer was transferred into a new tube (about 7-800 µl) and a further 200 µl chloroform was added and mixed by hand flicking. A further centrifugation step at 10,000 g at 4ºC for 15 min was carried out. The top layer was transferred into a new 1.5 ml tube. An aliquot of isopropanol (1 ml) was added, mixed gently by shaking and incubated at room temperature for 10 min. Samples were then centrifuged at 10,000 g at 4ºC for 10 min, the supernatant poured off, the last drop being removed by pipette. An aliquot of ice cold 75% ethanol (1 ml) was added, the tube vortexed briefly and incubated for 10-15 min at room temperature before centrifugation at 8,000 g at 4ºC for 5 min. The supernatant was removed and after a further centrifugation step, the last drop of ethanol was removed by pipette. The lid of the tubes was left open at room temperature for 10 to 20 min, to air-dry the RNA pellet. RNase-free water (100 µl) was added to each tube and incubated at 50-60ºC in a water bath or heat block, for 10 min before placing back on ice. RNA samples at this stage were stored at -80ºC or used right away in the Qiagen assay.

2.3.2 Qiagen RNeasy

To the sample in 100 µl RNase-free water, 350 µl of RLT buffer (with β-ME 10 µl/ml RLT) was added and gently mixed by pipette. Next 250 µl of 100% ethanol was added, then mixed gently by pipetting and transferred into a RNeasy column. After centrifugation at 10,000 g for 15 s, the flow-through was discarded.

The column was washed by the addition of 500 µl of RPE buffer and centrifuged at 10,000 g for 15 s. The flow-through was discarded and washed again with another 500 µl of the same buffer. The column was transferred into a new 2 ml tube and centrifuged at 10,000 g for 2 min to dry the column and then placed into a new 1.5 ml tube. RNase-Free water (30 µl) was added to the centre of the column and incubated at room temperature for 10min before centrifugation at 10,000g for 2 min. The RNA sample was stored at -70ºC until further analysis.

2.3.3 RNA Quantitation

The concentration and purity of the extracted RNA was assessed using a Nanodrop 1000 spectrophotometer, using 1 µl for calibration. Absorbance readings were taken for each sample at wavelengths of 260 nm and 280 nm. A reading at 260 nm was only for calculation of the concentration of nucleic acid in the sample. The ratio of the readings at 260 nm and 280 nm (A260/A280) was taken in to estimate the purity of the nucleic acid. The ratio of A230/A260 was used to assess the presence of protein contaminant in the sample.

To further confirm the integrity and quality of the extracted RNA, 1 µl of the sample was run on a Bioanalyzer 2100 (Stockport, Cheshire, Agilent Technologies, UK) electrophoresis and RNA integrity number (RIN) was generated by the software of the machine to calculate the ribosomal ratio of the total RNA sample.

2.4 DNA extraction

2.4.1 Trizol extraction

After collection of the aqueous phase with RNA, the tubes containing the interphase/organic phase were centrifuged at 12,000 g for 5 min at 4ºC. Any remaining aqueous phase, which could contaminate DNA sample with RNA, was carefully removed. With the interphase now containing DNA, extraction

was carried out through three stages: DNA was precipitated by adding 300 µl 100% ethanol for each 1 ml Trizol, mixed gently by inversion. The sample was kept for 2-3 min and then DNA sedimented by centrifugation at 2,000 g for 5min at 4ºC.

DNA was washed, by tilting the tube to allow the white layer to go down and to remove supernatant (containing protein). The DNA pellet was washed twice for 30 min each time with periodic mixing, in 0.1 M Tri-sodium citrate solution with 10% ethanol, then centrifuged at 2,000 g for 5 minutes at 4ºC. The DNA was re-suspended in 1 ml 75% ethanol and incubated at room temperature for 10-20 minutes (with periodic mixing), and then centrifuged at 2,000 g for 5 minutes. The pellet was dried for 2-5 minutes under vacuum or left uncovered on the bench.

Finally the DNA in the pellet was solubilised, dissolving in 100 µl of sterile water with a pipette. Samples were centrifuged at 2,000 g for 10 minutes to remove insoluble material, and supernatant containing DNA was transferred to a new, autoclaved, labeled flip-top 1.5 ml tube.

2.4.2 DNA quantitation

DNA concentration was determined by measurement of absorbance of the sample at 260 nm using a nanodrop 1000 spectrophotometer.

2.5 Reverse-transcribed polymerase chain reaction (RT-PCR)

A 100 ng quantity of RNA was used as a template for reverse transcribing into cDNA using a Primer Design reverse transcriptase kit in a 20 µl reaction volume according to manufacture instructions using oligo-(dT) primers. The cDNA was stored at -20ºC until use.

2.6 Primer design

The primers and probes were designed with the assistance of Primer Express® software V3.0 (Applied Biosystems) and obtained from Eurogentec. It also calculates the hybridization temperature and secondary structure of an olignucleotide based on nearest-neighbour thermal stability values, which provides a more accurate measure than other methods [150]. PCR efficiency

was optimized so that the annealing temperatures could be accurately matched. Genomic DNA and mRNA sequences were obtained from Gene Bank. BLAST® (Basic Local Alignment Search Tool) was used to test the primers or whole amplicon specificity. Primer pairs were designed to have melt temperatures as close to 60ºC as possible. The following primers and probes were designed using this method (Table 2-1).

Table 2-1. Primers and probes sequence designed in-house and used in the study

DNA primers & probes

Primer Name	**Primer Sequence(5'-3')**	**Amplicon size**
Tryptase F	GAGTGGGATCCTCCGCTGC	
Tryptase R2	CGGCACACAGCATGTCGT	544bp
Forwarddel	GGCCAGGGTCTTAGCCACA	138bp α
Reversedel	TATGGGGACCTTCACCTGCTT	&150bp β
Reverseextended	GGTTTTCCATTATGGGGACCTT	
α-tryptase Probe	CAGCCCCTGGGCTCCAGGTG	
β-tryptase Probe	CCTGGGCTCCCTCTGGGCTCC	
TrypMeltFwr	ATCATCGTGCACCCACAGTTCT	
TrypMeltRev	GCTCCTCCAGCTCCAGCAG	73bp

RNA primers

Name	Sequence	Amplicon size
IL6F	TGCAGAAAAAGGCAAAGAATCTAGA	57bp
IL6R	CATTTGTGGTTGGGTCAGGG	
IL8F	GCTCTGTGTGAAGGTGCAGTTT	141bp
IL8R	TGTGTTGGCGCAGTGTGG	
GM-CSFFwr	CCTGGGAGCATGTGAATGC	81bp
GM-CSFRev	TTTCATTCATCTCAGCAGCAGTGT	
GM-CSF//Fwr	CCTCCAACCCCGGAAACT	87bp
GM-CSF//Rev	CTGAAGGACTTTCTGCTTGTCATC	

Pro oligo primer

Name	Sequence:(5'-3')	size	MW g/mole	Epsilon1/(mMcm)
Tryptase_R2	CGGCACACAGCATGTCGT	18	6024	169.5

Mice genotyping assay primers

Name	Sequence	Amplicon size
PAR2-F (P1)	GAGAGGAGGTCGGCCAAGGCC	380bp
PAR2-R (P2)	GAGAGGAGGTCGGCCAAGGCC	
NeoFwr (P3)	GAGGAAGCGGTCAGCCCATT	281bp
NeoRev (P4)	TCTTCCTATTGACTAAACGG	

NB. Other primers used in this study were purchased from Primer Design.

2.7 Polymerase chain reaction (PCR)

Steps were taken to minimize the risk of PCR reaction contamination, particularly with the carry over of PCR products into subsequent PCR reactions. Two separate areas were used for setting and analyzing PCR products. The first area was used exclusively for preparation of master mixes, the addition of primers and templates to master mix reagents in aliquots. The PCR machine and gel electrophoresis equipment were located in a second area and PCR products were never brought into the first area. Nitrile gloves were changed when moving between the two areas.

Plasticware

All PCR reactions were carried out in thin-walled 0.2 ml tubes unless otherwise stated. Primers and master mix reagents were stored in 0.6 ml or 1.5 ml micro-centrifuge tubes and all the plastic-ware was autoclaved before hand.

Reagents

All PCR reagents were purchased from Eurogentec, and were stored at -20ºC. The dNTPs, buffers and primers were aliquoted to minimize freeze/thaw damage and contamination. Water for PCR reactions was from a RiOs Reverse Osmosis System/Milli Q Ultrapure Water system apparatus. The water was autoclaved and aliquoted into sterile 1.5 ml tubes and stored at -20ºC.

PCR was carried out using a standard Taq DNA polymerase protocol, with 10-20 µl volumes. The final concentrations of PCR reaction components were those recommended by the manufacturer.

The $MgCl_2$ concentration of 2mM was in the middle of the recommended range, and worked well for most primer pairs. Primers were used at 100 nM in the standard PCR reaction, and in the DNA template between 10-20 ng per reaction. In order to detect any possible contamination, water controls were employed in all reactions.

Reaction set-up

For maximum efficiency and high polymerase fidelity, all tubes were kept in ice until the heat block had reached 95ºC. After centrifugation of all tubes, they were put in place and the thermal protocol was allowed to proceed. In this way non-specific priming of polymerase reactions at sub-optimal temperatures was minimized.

Thermal protocols

All PCR reactions were carried out according to the protocols optimized for each primer pair. Generally 35 cycles of amplification were performed with an initial denaturation step of 95ºC for 1 min, then of 60ºC for 20 s /cycle, 30 s extension at 72ºC /cycle, and a final extension (last cycle) at 72ºC for 10 min. PCR reactions were finished by cooling down to 4ºC. PCR products were analyzed immediately or stored at 4ºC for further analysis.

2.8 Electrophoresis

Horizontal electrophoresis was routinely performed using a Horizon 58 electrophoresis tank (Thistle Scientific Ltd, Uddingston, Glasgow, UK) for smaller gels or a Kodak MP-1015 unit for larger gels (Sigma-Poole-UK) with a Bio-Rad (model 500/200) power supply. TBE buffer (1x), was used as running buffer. Gels were stained with ethidium bromide (500ng/ml) at a 7% concentration for 20 min and photographed under fluorescent light using the Molecular Dynamics Fluroimager 595 or Typhoone imager (GE Health Care, UK). Two types of gel techniques were used:

2.8.1 **Polyacrylamide gel electrophoresis** (Microplate Array Diagonal Gel Electrophoresis (MADGE)

The following reagents were mixed in the order listed below in a 20ml universal tube (Sterilin): TBE x1(10 ml), acrylamide/bisacrylamide (3ml), 20% ammonium persulphate (150 μl), TEMED (22.5 μl) with water (7 ml).

The gel mix was poured between glass plates (18x16 cm) (Hoefer Scientific Instruments from Holliston, MA, USA) and a comb inserted to form the wells of the gel. This was allowed to set for 30 minutes. The gel was placed in a horizontal slab gel electrophoresis tank. A 10 µl aliquot of PCR product was mixed with 2 µl of loading dye and loaded onto the gel. PCR products were run in parallel with molecular weight markers, and electrophoresed for one hour at 240 V. Gels were stained as previously described.

2.8.2 Agarose gel electrophoresis

A quantity of 10 g agarose powder was dissolved in 500 ml TBE concentrate by heating in a microwave oven. When the agarose was completely dissolved, it was left to cool to approximately 50ºC, at which point it was carefully poured in one movement onto a horizontal tray and combs inserted to form the wells of the gel. The gel was allowed to set for 40 minutes, after which the combs were removed and the gel placed in a horizontal slab gel. Volumes of 10 µl the PCR products were mixed with 2 µl of loading buffer and loaded onto the gel and run for 25 minutes at 100V. DNA was routinely electrophoresed on 1.5-2% agarose. Molecular weight markers (Invitrogen) ranging from 50bp up to 1000bp were used.

2.9 Restriction digest

Restriction fragment length polymorphism (RFLP) analysis was carried out using *Eco*RV enzyme. The following mix was incubated at 37ºC for 3 hours: 1µl of 10 x Reaction buffer, 0.1 µl of 100 x BSA (10 µg/µl), 0.5 µl of *Eco*RV (20 µg/µl), and 5.0 µl of PCR template and up to 10 µl of dH_2O. Reactions were carried out in either 10 or 20 µl volume reactions

2.10 Fragment sizing

The CEQ 8800 Fragment analysis module (Beckman Coulter) was used. This estimates DNA fragment sizes and amounts and identifies alleles represented

by specific DNA fragments. This used a WellRED reverse primer (Sigma), which is a DNA, D4-PA, dye labeled oligo designed to be used with the CEQ Genetic analysis system.

Reaction protocol

The PCR reactions were prepared as described before, but in 96 well plates using the same cycling conditions. The PCR products were digested with the *Eco*RV enzyme for 3 h and to each well, SLS (25 µl), size standard 600 (0.5 µl) and digested PCR fragments (5 µl) were added and mixed. Using the pre-set method Frag 1, with the following parameters: capillary temperature was set to 37ºC and the denaturing temperature to 90ºC both for 120 s, the injection voltage was 20KV (15 s), and the separation force was 2.4 KV (60 m). The dye calibration used was PA Ver1.

2.11 House keeping genes (HKGs)

Using housekeeping genes as a reference is an essential step in all quantitative studies as such genes are involved in basic functions needed for the sustenance of the cell and so are constitutively expressed. The appropriate HKGs were selected using a kit with 6 identified genes (Primer Design) to test their expression in our cell model and the least variable taken as reference genes. The kit was chosen to include primers for human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ubiquitin (*UBC*), 18srRNA, actin-beta (*ACTB*), beta-2-microglobulin (*B2M*) and splicing factor 3a, subunit 1(*SF3A1*) mRNA. Applying RNA samples from cells treated for different time and dose points, information was obtained using the software provided as to which were the most stable genes and the smallest number required in each run for quantitation.

2.12 Real time PCR

Principle

A quantitative PCR method was employed for the determination of the copy number of PCR templates such as DNA or the expression level for the cDNA in a PCR reaction. There are two types of real-time PCR: probe-based and intercalator-based. Both methods require a special thermo cycler equipped with a sensitive camera that monitors the fluorescence in each well of the 96-384 well plate at frequent intervals during the PCR reaction.

Probe-based real-time PCR, also known as TaqMan PCR, requires a pair of PCR primers (as does regular PCR), and also a fluorogenic probe which is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. Two PCR primers with a preferred product size of 50-150 bp, a probe with a fluorescent reporter or fluorophore such as 6-carboxyfluorescein (FAM) and tetrachlorofluorescin (TET) and quencher such as tetramethylrhodamine (TAMRA) covalently attached to its 5' and 3' ends, respectively.

The intercalator-based method, also known as the SYBR Green method, requires a double-stranded DNA dye in the PCR reaction which binds to newly synthesized double-stranded DNA and gives fluorescence. The TaqMan method is more accurate and reliable than the SYBR green method, but also more expensive.

Optimization protocol

All reagents used in the qPCR reactions were optimized first to test quality and reproducibility of the results. So, primers were optimized to find the best concentrations with steep amplification and reproducibility. This was tested among a range of concentrations; the same was done for the probes. After amplification, melting of the amplicons was carried out to test specificity (one melting peak for each amplicon amplified) and to test the presence of contamination. Samples volumes were calculated at 10% more to avoid pipetting variation. Every plate included two wells of non template control and two wells of duplication of two control samples.

Program

The PCR protocol consisted of three programs: denaturation, amplification, and melting curve analysis. The denaturation-extension and amplification conditions were 95ºC for 15 min followed by 40 cycles of PCR. Each cycle included denaturation at 95ºC (15 s) and annealing at 60ºC (30 s). The temperature transition rate was 10ºC/s.

Analysis modules

Using the LightCycler® 480 software, different analysis modules were used according to the study applied. Four modules were employed: relative quantitative analysis for cDNA work, Tm calling, gene scanning, and melting curve analysis. The lightCycler 480 System provides a unique format in which

the entire experiment, including real-time and post-PCR analysis, can be carried out on one instrument in a 96- or 384-well format. Protocols were carried out according to the manufacturer's instructions (Roche).

High resolution melting (HRM)

HRM was introduced as a homogeneous closed-tube system that allows mutation scanning and genotyping without the need for costly labeled oligonucleotides. The method relies on the usage of a new generation of double stranded DNA (dsDNA) binding dyes. Heteroduplex products from PCR are identified by the presence of a second low-temperature melting transition. The use of SYTO09, which could be used at high saturation concentration without inhibiting or adversely affecting the PCR reaction, could be applied to detect heteroduplexes, perhaps due to better dye redistribution during melting step.

Gene scanning module

Analysis was performed using the Tm calling module, where the Tm were determined by the manual method for two Tm of interest for amplicons which allows a good estimate of the area under the peaks. Using the gene scanning module of the software, and after choosing the negative samples, analysis was commenced with normalisation of the melting curves to both 0 and 100 signal values of all samples, then temperature shifted analyses were carried out before grouping of the samples by LightCycler 480 gene scanning software.

2.13 DNA templates

ECACC Human Random Control (HRC1) DNA samples (www.ecacc.org.uk): were used to optimise reaction conditions, to determine α-tryptase genotyping frequency and to validate subsequent experiments as positive controls.

Southampton asthmatic families' DNA:

Caucasian families (n=341) from the Southampton area had been recruited prior to the commencement of this project with at least two biological siblings with a current physician diagnosis of asthma and taking medication on a regular basis [151]. Serum total IgE levels and specific IgE levels for grass, house dust mite, cat, dog, *Alternaria* and tree allergens were determined by RAST. Skin prick testing was also completed for the same allergens. Baseline lung function tests (forced expiratory volume in 1s (FEV_1) best of three values within 5%) were performed using a Vitalograph® dry wedge bellows spirometer

(Vitalograph, Maids Moreton, UK). Bronchial hyper-responsiveness (BHR) was measured with the provocation concentration of inhaled methacholine required to reduce FEV_1 by 20 % (PC20 FEV_1). Methacholine dilutions (0-16 mg/ml) were given at 5 min intervals by a DeVilbiss nebuliser with KoKo dosimeter (PDS Instruments Inc. Louisville, CA, USA). This population and the generation of the phenotypic scores has been described previously [152]. Phenotypes examined in the association analysis included; asthma, FEV_1 (percent predicted), BHR (methacholine), total IgE, atopy severity and asthma severity. Ethical approval was obtained from the Southampton & Southwest Hampshire joint Ethics Committee.

2.14 The Southampton Phenotype characteristics

Atopy measurement

Total and specific IgE levels (for house dust mite, cat, dog, grass) had been measured in the blood samples using the ImmunoCAP™ system (Pharmacia Diagnostics). Asthmatic individuals were dichotomized using an age-specific cut-off for elevated total IgE levels (age 5–9 yr, >52 kilounits (kU/L; age 10–14, >63 kU/L; age 15–18, >75 kU/L; age ≥ 19, >81 kU/L). An individual was assigned a positive specific IgE value if his or her level was positive (to grass or tree) or elevated for at least one source of allergens (≥0.35 kU/L for cat, dog, *Dermatophagoides pteronyssinus, D. farinae, Alternaria*, ragweed).

Bronchial hyperresponsiveness

Bronchial hyperresponsiveness was measured using a methacholine challenge. Bronchial responsiveness was only measured in participants with a baseline forced expired volume in one second (FEV_1) of ≥70% predicted. Doubling methacholine concentrations (0.06 – 16.0 mg/ml) were administered at 5 minute intervals until a 20% reduction in FEV_1 or the progressively highest concentration of the methacholine was reached. FEV_1 values were recorded 30 seconds after each dose and continued at 1 minute intervals thereafter. A linear model was then fitted to the FEV_1 values recorded and the cumulative dose measured. The least squares slope value was then derived and used to provide a measure of each participant's bronchial responsiveness according to the equation:

BHR variable =

$$\left[\frac{1}{(\text{least squares slope}+30)}\right]\times 1000$$

The reciprocal transformation was used to improve the uniformity of variance, while the constant value of 30 was added to avoid negative values. The multiplication factor of 1000 avoided the need for three decimal places in the figure derived. The value of BHR variable is inversely correlated with BHR.

Lung function measurements

A baseline FEV_1 measurement was established by monitoring the participant's performance in pulmonary function tests at 60 s intervals. Values of percentage predicted FEV_1 were calculated using the formula and the population-based sample used to calculate the predicted FEV_1 values described [153].

Atopy Severity Score

Each patient's atopy score was derived via an equation involving the following components:

1. The 1st principal component analysis (PCA) which transforms a number of possibly correlated variables into a smaller number of uncorrelated variables has been performed for mean wheal diameter from the skin prick test. This represents the magnitude of the response.
2. The number of positive (diameter ≥ 3 mm) skin prick responses to the panel of allergen tested.
3. The level of specific IgE response as measured by the 1st PC of specific IgE's. This represents the magnitude of the response.
4. The number of positive responses to specific IgE. This represented the range of the response.

An appropriate constant value was added to the severity score to ensure all values were positive.

Asthma Severity score

Each patient's asthma score was calculated via an equation involving three components:

1-BHR variable =

$$\left[\frac{1}{(\text{least squares slope}+30)}\right]\times 1000$$

2-A treatment score, representing a value assigned to each patient according to the British Thoracic Society's guidelines [154]. This was dependent on the type of medication they were receiving. A high treatment score indicates the patient is being treated for severe asthma.

3-A symptom score, derived from ten questions relating to asthma on the health survey questionnaire. The score was calculated by assigning a weight indicating the degree of importance to each question. The weighting was based on the opinion of general practioners, chest physicians, paediatricians with a respiratory interest and respiratory nurse specialists.

2.15 Purification and characterization of recombinant mast cell β- tryptase

The method applied for purification of recombinant human β tryptase from supernatants of *Pichia* was modified from the protocol described by Niles and colleagues [155]. Purification was carried out with about 110 ml of cell-free medium containing the recombinant protein, which was applied sequentially on butyl Sepharose then heparin agarose columns, using low pressure Econo system chromatography equipment (Bio-Rad) and then through a size exclusion column using an HPLC from Dionex ICS-3000 (Camberley, Surrey, UK). Aliquots from each purification stage were saved for determination of BApNA activity and protein content.

2.15.1 Purification steps

Butyl Sepharose chromatography

A column with a bed volume of 30 ml of butyl Sepharose 4 fast flow matrix was used at room temperature. The column (1.6 mm/ 30ml) was filled with matrix and packed at 1 ml/min using the pump. Equilibration was with 10 mM MES, 1 M $(NH_4)_2SO_4$, 0.5 M NaCl, 10 % (V/V glycerol), and the pH 6.1. The Pichia

supernatant was loaded at the same rate and the column washed with 230 ml of the same buffer. The washout fractions were kept for further analysis.

The bound protein was eluted from the column with 10 mM Mes, 0.2 M NaCl, 10 % (V/V glycerol), and pH 5.5. Fractions were collected every 5 minutes (5 ml) and assayed for activity. High activity fractions were pooled, and then loaded to the heparin column.

Heparin agarose chromatography

A column with similar bed volume of heparin agarose was employed at 4ºC and equilibration was carried out with fractions eluted buffer (300 ml), before loading the active fractions from butyl Sepharose column. The elution step was carried out with a gradient buffer (0.2-2.0 M NaCl).

Elution was carried out, with a linear salt gradient of 0% to 100% of buffer containing 10 mM Mes, 2 M NaCl, 10 % (V/V glycerol) and pH 6.1, followed by 60 ml of 100%. The fraction volume was adjusted, so that the entire gradient, including the final wash with buffer C, was collected in the 80 tubes collector rack. Tryptase activities of each fraction, including the "run through" were determined.

Size exclusion chromatography

The high activity fractions were pooled together and injected (0.5 ml) into the HPLC pump with BioSep-Sec-S-3000 columnfrom Phenomenex (Macclesfield, Cheshire, UK) and 0.5 ml fractions were collected. Fractions with peaked chromatogram were selected and confirmed with activity for BApNA.

2.15.2 Characterization of the purified protein

The activity of the enzyme in each fraction was assayed with BApNA and a sample (10ul) was loaded into SDS-polyacrylamide gel for each active fraction. Further analysis of the protein purity was carried out by silver staining and finally the specificity of the purified protein was tested by Western blotting with tryptase specific monoclonal antibody AA5 [156].

Activity assays

An aliquot of 10 µl sample/well was added to a microtitre plate. Each sample was run in duplicate and both a positive and a negative control were included in each plate. With a multi-channel pipette, 90 µL of the assay mixture,

including BApNA substrate (88.9 mM BAPNA in 1 ml DMSO) and assay buffer (1.0 M glycerol, 0.1 M Tris base, pH 8.0) was added to each well.

The changes in absorbance (mOD/min) were taken at wavelength 410 nm, at 25ºC, applying the kinetic L1 module mixing once before readings. The run time was 10 minutes and 14 readings were taken every 43 second.

One BAPNA Unit of activity was defined as the amount of tryptase that can cleave BAPNA and release 1 umol p-nitroanalide (the extinct coefficient (ε) = 8800 M cm^{-2}) per min at 25ºC, pH 8.0. Activity was expressed as BApNA units per ml sample, and specific activity was as BApNA units per mg protein.

Bicichoninic acid (BCA) protein assay

The BCA reagent was prepared by mixing 1 ml of 4% $CuSO_4$ with 50ml BCA solution (Sigma). Ten microliters of sample were added to 200 µl of BCA reagent in a 96 well plate, and the plate was incubated at 37ºC for 30 min. The absorbance was read at 550 nm using a microtitre plate reader. The values were compared to a standard curve prepared using bovine serum albumin (BSA), diluted between 25 µg/ml and 2000 µg/ml using water or buffer relevant for the sample.

SDS-PAGE

The purified protein was analysed by electrophoretic separation on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE). Tryptase-containing samples were dissolved in Laemmli loading buffer 3X (1M Tris-base (pH6.8) 2.4 ml, 20 % SDS 3 ml, β-mercaptoethanol 1.6 ml, 100 % glycerol (3 ml) and 0.006 g bromophenol blue). Two volumes of sample and one volume of the buffer were mixed and heated to 95ºC for 5 minutes. A 20-40 µl sample was layered on to a 4% polyacrylamide SDS stacking gel (18 g Tris-base and 5.8 g SDS to 100ml H_2O, pH 8.8) and electrophoresis was performed in a 10% acrylamide separating gel (6.05g Tris-base and 0.4 g SDS to 100ml H_2O, pH 6.8) at 80 mA for 2 hours in the running buffer (Tris base 30.3g, glycine 144 g and SDS 10.0 g up to 1L H_2O). Standard markers employed in these studies were Precision Plus Protein™ standards (Bio-Rad).

Coomassie blue staining

After electrophoresis of the gel, it was transferred to a round staining tray, and 200 ml Coomassie blue stain (1.2 g Coomassie blue, 300 ml methanol, 60 ml acetic acid and 240 ml H_2O) was added, heated for about 45 seconds, and then incubated at room temperature with gentle shaking for 10-15 min. About 200 ml of protein gel destain (50 ml methanol, 50 ml acetic acid and 400 ml H_2O) was added and left overnight.

Silver staining

The silver staining protocol employed was based on the instructions provided by the manufacturer's protocol. Gels were immersed in a fixative solution for at least 30 minutes followed by washing briefly for about 30 s before being immersed in 10% oxidizer for 5 min. This was followed by washing for 15 min in deionised water.

The gel then was immersed in silver reagent 10% for 20 minutes, followed by washing with water for 30 seconds. The gel was then incubated in 3.2% developing solution W/V until clear and dense bands appeared. The reaction was stopped by addition of 5% acetic acid.

Western Blotting

The unstained gel was incubated for 30 minutes in the transfer buffer (3.03g Tris-base, 14.4 g glycine in 800 ml H_2O and 200 ml methanol) and was sandwiched with nitrocellulose membrane between Scotch-Brite pads in a hinged cassette and inserted into a trans-blot apparatus.

Electrophoretic blotting was carried out for 12 hours at 22V. Nitrocellulose membranes were blocked with dried milk powder (Bio-Rad) 2.5% in PBST for 1 h at room temperature and then washed three times with PBST for 15 min each wash. The membrane was incubated with the primary antibody (AA5) at a 1:2,000 dilution for 30 min, before washing again with PBST in the same way as before and then the membrane was incubated with the secondary antibody (anti-mouse peroxidase conjugate IgG; Dakocytomation) at a dilution of 1:10,000 for 30 minutes .

The membrane was washed again three times for 15 min, and the immunoperoxidase staining on transferred protein bands was developed with

chemiluminescent substrate for detection of horse radish peroxidase (HRP) by (SuperSignal® West Pico Chemiluminescent Substrate, and the image recorded using a SynGene Gene Snap version 6.03 Gamma camera or Versa-doc machine (Bio-Rad).

Endotoxin assay

Endotoxin levels in the biological material used in this study was assessed by Limulus Amebocyte Lysate (LAL) QCL-1000 (Lonza) following the manufacturer's instructions applying the microplate method. Standards were employed in the range of 0.1- 1.0 EU/ml, applying the LAL water supplied. Glacial acetic acid (25%) was added as the stop reagent.

Recombinant α-tryptase

Alpha-tryptase that had been expressed and purified using the same expression system as β-tryptase [157], was a kind gift from Dr. Christian Sommerhoff (University of Munich, Germany). The enzyme was kept in 1 M NaCl buffer at a concentration of 1.48mg/ml.

2.16 Mouse model

2.16.1 Animals

Experiments were performed on adult wild-type C57BL/6J mice (body weight ranging from 25-35 g), which were housed in standard cages, with food and water available *ad libitum*, and maintained in a thermo neutral environment. Colonies of PAR-$2^{+/+}$ (wild type) and PAR-2-deficient (PAR-$2^{-/-}$) mice, which had been genetically modified as described previously [158], were a gift from Kowa Company Limited, Japan.

2.16.2 Mouse tail biopsy for genotyping

Biopsy samples of mouse tail tissue were collected for DNA genotyping and were taken in accordance with the approved Animal Study Proposal (ASP). Three samples from each genotype were kept in -80C before processing. Samples were transferred to 1.5 ml epid Hybrid Ribolyser in 1.5 ml Lysing Matrix D tubes (MP Biomedicals, Solon, OH, USA) with added Trizol.

The genotype was confirmed using PCR applying the primers P1, P2 for the wild type, P3 and P4 for the knockout type. The conditions applied with P1

and P2 were as follows: generally 35 cycles of amplification were performed with an initial denaturation at 95ºC for 5 min, annealing at 51ºC then for 2 min/cycle, 3 min extension at 72ºC/cycle, and a final extension (last cycle) at 72ºC for 10 min. PCR reactions were concluded by cooling to 4ºC.

The conditions used for P3 and P4 were as follows: 33 cycles of amplification with initial denaturation of 95ºC for 10 min, followed by 68ºC for 1 min and 30 second extension at 72ºC with a final one for 10 min. PCR products were analyzed immediately or stored at 4ºC for further work.

2.16.3 Study design

Animals were injected intra-peritoneally (ip) with saline alone as vehicle or with recombinant β-tryptase, α-tryptase, protease inhibitors leupeptin or a new tryptase inhibitor (inhibitor x). Each drug was injected in a concentration range from 1.0 - 0.01 µg/ml and each mouse received one injection of 500 µl. Animals were allowed to recover and were killed at 6, 12 and 24h following the injection and peritoneal lavage was performed.

Care was taken in the preparation of recombinant beta tryptase to minimize spontaneous inactivation, so tryptase was maintained on ice and diluted from a concentrated stock solution with 0.9% saline just before injection into mice. For some mice, tryptase was pre-incubated for 1h on ice with 10ug/ml leupeptin or with a selective small molecule tryptase inhibitor 50 µg/ml. This is from the drug development programme of Sanofi-Aventis company, and while its use is subject to a confidentiality agreement, it is being termed inhibitor X. The degree of inhibition of catalytic activity was confirmed with chromogenic substrate BAPNA. Saline diluent alone was employed as a negative control.

2.16.4 Peritoneal lavage

Animals were killed by cervical dislocation. The ventral side was swabbed with 70% ethanol, and part of the anterior abdominal wall skin was removed. Five ml saline was injected by syringe ip and the abdomen gently massaged for 2 minutes. The peritoneal cavity then opened and peritoneal fluid recovered by pasteur pipetting. A total of 4-5 ml peritoneal lavage fluid was consistently recovered by this technique and kept on ice until the next stage.

The lavage fluid was centrifuged at 500 g for 10 minutes, the pelleted cells were resuspended in Eagle's Minimal Essential Medium (MEM) and the supernatant was stored in aliquots at -20ºC until analysis.

2.16.5 Peritoneal cell count

After centrifugation (500 x g for 10 min at 4ºC), cells were resuspended in 2.5 ml MEM, stained with 0.4% trypan blue, and enumerated using an improved Neubauer haemocytometer. The cell number in the suspension was then adjusted to $1x10^6$ cells/ml. Cells were subjected to cyto-centrifugation (Cytospin; Shandon Southern, Runcorn, UK) with 10^6 cells in 100 µl/slide and slides left to dry for 12 hrs. One slide from each sample was stained with rapid Romanowsky stain (according to the manufacturer's instructions). Differential cell counts were performed, counting a minimum of 500 cells using *Nikon* plan microscope (100 X/1.30 Oil), Nikion (Kingston, Surry, UK). Data from animals which received the same treatment on different occasions were pooled and analyzed together.

2.16.6 Albumin assay

Quantitative colorimetric albumin bromocresol green (BCG) assay kits (QuantiChrom, BioAssay Systems, Hayward, USA) with detection range 0.01 g/dl to 5 g/dl were employed to determine albumin concentrations in peritoneal lavage fluid. Standard preparations of bovine serum albumin (BSA) were dissolved in distilled water at concentrations from zero to 50 mg/mL. Five microlitres of diluted standards and samples were transferred to wells of a clear bottom 96 well plate. Working reagent (200µl) was added to each well and the plate was tapped lightly to mix. The plates were incubated for 5 minutes at room temperature and the optical density (OD) was read between 570-670 nm (peak absorbance at 620 nm) using a Thermo Max plate-reader (Molecular Devices).

Blank OD was subtracted from the standard OD values and a standard curve was drawn by plotting the OD values against standard concentrations. Albumin concentrations in samples were determined using the standard curve using conversions of 0.1 g/dL albumin equals 15 µM, 0.1% or 1000 ppm.

2.16.7 Gelatin Zymography

Supernatants of peritoneal lavage samples were mixed 3:1 with a nonreducing sample buffer (0.5 M Tris [pH 6.8], 10% glycerol, 10% sodium dodecylsulphate [SDS], and 0.5% bromophenol blue) and separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 8% polyacrylamide gels which had been copolymerized with gelatin (20 mg/ml) VWR (Lutterworth, Leicestershire, UK). The gels were washed for 30 min in 2.5% Triton X-100 to refold proteins and then incubated overnight at 37°C in 50 mM Tris buffer (pH 7.8) containing 50 mM $CaCl_2$, and 0.5 M NaCl. In some experiments proteolysis buffer was prepared without $CaCl_2$ and 10 mM ethylenediaminetetraacetic acid (EDTA) was added to the incubation buffer to inhibit metalloproteinases by chelating Zn^{2+} ions required for proteolytic activity.

After incubation, the gels were stained with Coomassie Blue (Brilliant Blue R250 [Sigma] in 40% methanol/10% acetic acid) and destained with 10 % methanol, 10 % acetic acid in 80 % of dH_2O. This destaining procedure produced reproducible background staining between individual gels. Areas of gelatinolytic activity appeared as clear bands against a dark blue background. Images of the gels were scanned with a densitometer (BioRad G800) and the image obtained was analyzed using Quantity One 4.5.2 software (BioRad). The density of individual bands of gelatinolytic activity was estimated and expressed in arbitrary units.

2.17 Statistics

Values are presented as mean± SEM or medians (interquartile ranges) unless otherwise specified. Nonparametric 2-group comparison was made with the Mann-Whitney U test, and multiple group comparisons were made with Kruskal-Wallis 1-way ANOVA. A 2-tailed *P* value of less than 0.05 was taken as statistically significant.

2.17.1 Family Based Association Tests

This section details the Family Based Association Test (FBAT) method that was used to analysis α-copy number variation haplotypes and the asthma phenotypes described in chapter 2. The FBAT association anaylsis described was performed with the assistance of Ms. Sheila Barton.

2.17.2 Priniciple of Transmission Disequilibrium test

A transmission disequilibrium test (TDT) uses genotype data from families with at least one affected child and evaluates the transmission of the associated marker allele from an informative parent to an affected offspring. TDT tests can detect linkage between the marker locus and the disease locus onlu if association (due to linkage disequilibrium) is present.

As the test compares the number of times a heterozygous parent transmits the associated marker to an affected offspring to the number of times they transmit the alternate marker allele within the family pedigree, this test is not affected by the presence of population stratification.

2.17.3 The FBAT software

FBAT version 1.4 is a computer program was built on the TDT method and integrates tests of different genetic models, sample designs, disease phenotypes, missing parents and tests of different null hpotheses.

2.17.4 Haplotype Based Association Tests using the FBAT software

An extension of the FBAT analysis program is the ability to test for linkage and association between a haplotype and a disease susceptibility locus. The association analysis was performed under a null hpothesis of no linkage and no association. Association analysis for the diagnosis of asthma was performed on asthmatic and non-asthamtic individuals (siblings); while the remaining asthma phenotypes were tested for association in asthmatic siblings only.

FBAT test hypotheses were as follow:
H1= associationbetween the presence or absence of the gene and asthma phenotype
H0= no association and no linkage between the presence or absence of the gene and asthma phenotype.
As there is more than one affected sibling in some families the null hpothesis was set to “no association and no linkage” so that the family pedigree can be broken down into nuclear families that can be treated as independent.

Chapter 3
Copy Number Variation for Tryptase Loci

3. Quantification of DNA Copy Number Variation for Tryptase Loci.

3.1 Introduction

Study of the genetic basis of common, complex phenotypes can help to explain the inherited basis of human variation, providing more insight into human physiology and disease. Copy number variants (CNVs) for several years identified in human and animal models have been found to be associated with various diseases and phenotypes. CNVs are likely to have important roles in contributing to phenotype variation, but it is not clear what proportion of complex diseases may be explained by CNVs.

CNVs could offer clues as to the genetic background of complex disorders, which could harbour genes that cause or predispose to phenotype variation [159]. The potential effect of CNVs spanning functional genetic elements such as genes, is exemplified by studies of the glutathione-S-transferase Mu1 (GSTM1) locus. A complete copy of the GSTM1 gene (two copies) has been found in only half of the Caucasian population, with the other half carrying a homozygous deletion that has been associated with increased susceptibility to some cancers, possibly by reduction of the metabolism of some carcinogenic compounds in the environment [160]. Other individuals carry three copies of that gene due to the presence of gene duplication alleles in the population and in these individuals 'ultra' GSTM1 activity [161] may confer protection by increasing the breakdown of harmful environmental toxins.

Not all gene CNVs are associated with corresponding changes in the expression level. Thus for example in individuals carrying CNVs of the α-defensin gene, no relationship has been found between total mRNA levels and gene copy numbers [137]. It is important to note that gene dosage effects are not the only consequences of CNVs that could affect gene expression. The variation in copy number or disruption of gene coding or regulatory sequences could also contribute to such changes [136].

Additional copies of a gene may provide redundancy allowing some copies to evolve new or modified functions or different expression patterns while the other copies keep the original function [162]. The influence of copy number is

likely to extend far beyond what has been established so far as different copies of the gene could have different functions.

As much as 12% of the human genome is variable in copy number, and this diversity is likely to be responsible for a significant proportion of normal phenotypic variation. The study of such variation requires the development of methods able to detect CNVs at higher resolution. Methods such as qPCR, multiplex amplified probe hybridization, multiplex ligation-dependent probe amplification and dynamic allele-specific hybridization, are able to detect CNVs, but with restricted throughput and scale [163].

Having a genetic predisposition is undoubtedly a substantial risk factor for developing asthma, as has been demonstrated by several family twin studies [164] but the genes underlying susceptibility to asthma are still largely unknown. Inflammation is a prominent feature of asthma and a range of proinflammatory actions have been described for the mast cell tryptase.

A high degree of genetic variation in tryptase genes has been indicated by the report that α-tryptase is lacking in approximately 29% of a healthy population [148]. However, it is not known to what extent such variation may have an impact on the severity of inflammatory processes. The aim of the studies reported in this chapter has been to develop a robust, reliable, and validated assay for α-tryptase copy number determination that will allow high throughput analysis of the α-tryptase genotype, and to investigate the copy number association with asthmatic phenotypes.

3.2 Sequence studies

3.2.1 Human tryptase locus sequences

All the available sequences for the tryptase locus genes were identified utilising the Entrez nucleotide database (http://www.ncbi.nlm.nih.gov/entrez) Table 3-1, and aligned together, using the software Bio Edit version 5.0.9.1. This allowed identification of sequence differences between them, and with α- and β-tryptase gene sequences that could be used to either allow gene specific amplification or distinguish between the co-amplified transcripts. Sequences for the related and highly homologous γ- and δ- tryptase genes were also compared to assess the specificity of the primers designed to amplify α- and/or β- tryptase specific amplicons.

3.2.2 Differences between α-and β-tryptase gene sequences

Close examination of the available sequences for β- and α-tryptases revealed two regions in intron 4 could be used to develop a PCR based assay enabling differentiation between both genes (Figure 3-1). These differences included the presence of a restriction site polymorphism and a 10-11bp region that is absent from the α-tryptase gene sequences in the gene bank, while present in all the β-tryptase gene sequences. Several approaches are possible in developing an α-tryptase copy number assay. The first involves amplification of an α-tryptase specific amplicon followed by quantification using real-time PCR. This would require developing a primer set that would selectively amplify the α-tryptase gene and not the highly homologous β-tryptase gene. A second option could be to use the single nucleotide difference between the α- and β-tryptase in intron-4, which forms part of the *EcoRV* restriction enzyme recognition site, and to develop a restriction fragment length polymorphism (RFLP) assay to distinguish α- from β-tryptase fragments. This would avoid having to generate primers that amplified α-tryptase exclusively, and would help to distinguish individuals that carry copies of the α-tryptase gene from those who do not have a copy. However, this provides no information on α-tryptase copy number.

A disadvantage in using the intron 4 sequences to distinguish α- from β-tryptase, is the availability of the sequence for the αII-tryptase variant (AF0983828) but not for αI-tryptase for that intron. Thus sequence differences

between α- and β-tryptase in intron-4 may be unique to αII-tryptase and not to other α-tryptase isoforms.

3.2.3 Allele specific primers

One approach to genotyping the α-tryptase copy number is the generation of an α-tryptase specific PCR amplicon that could be utilised in quantitative real-time PCR to accurately assess differences in α-tryptase copy numbers between individuals. This approach has been used successfully in the past to genotype other copy number variations such as those exhibited by the GSTM1 and glutathione-S-transferase theta 1 (GSTT1) loci [140].

A pair of primers was designed with the reverse primer overlying a single-nucleotide sequence difference between α- and β-tryptase at the 3' end (analogous to an amplification refractory mutation system (ARMS) primer for SNP genotyping), which may have allowed only amplification of α-tryptase amplicons. Despite repeated attempts to optimize the amplification conditions using a range of $MgCl_2$ concentrations and different annealing temperatures, positive DNA control samples from the HMC-1 cell line which carry no α-tryptase copy revealed that allele specific amplification was not occurring (data not shown). Thus, it appeared that the best possible approach for α-tryptase genotyping would be using a non-specific amplification strategy. Therefore an alternative reverse primer was designed for co-amplification of α- and β-tryptase.

3.2.4 Allele non-specific primers

As gene specific amplification did not appear to be possible, given the high similarity between α- and β-tryptase genes, non-specific primers were designed to allow co-amplification of a segment of all α- & and β-tryptase genes. This segment contained two specific α-tryptase sequence features. First, the α-tryptase amplicons were 10-11bp shorter (542bp) than the β-tryptase amplicons (552bp). Second, the α-amplicons contained a recognition site for the *Eco*RV restriction enzyme. Applying the *Eco*RV enzyme to α-tryptase amplicons would result in two segments of 151 and 391bp in length, while the β-tryptase amplicons would remain intact.

To optimise the primers and to confirm the co-amplification of α- and β-tryptase, DNA from cell lines with known tryptase genotype was used as positive controls; HMC-1 cells which express only β-tryptases and KU812 cells which express both α- and β-tryptases (with undefined α/β ratio) [165]. Following amplification and digestion with the restriction enzyme *Eco*RV, the expected pattern of digestion was seen with 2 bands representing the digested α-tryptase amplicon and one (undigested) band for β-tryptase (Figure 3-2). Individuals having three bands were therefore taken as having the αβ-tryptase genotype and those with just one band the β-tryptase only genotype.

The population frequency of subjects carrying at least one α-tryptase allele in the UK Caucasian population was established using 96 UK Caucasian samples from ECACC HRC-1 plate samples (section 2.13). It was found that 29.6 % of the samples were α-tryptase deficient. This is similar to the published frequencies observed in other Caucasian population samples [148]. Moreover, no individual was found to have an α-tryptase only genotype. However, while confirming the presence or absence of an α-tryptase allele in an individual, this digest method does not allow the quantification of α-tryptase copy number, i.e. it is not possible to distinguish between individuals carrying 1 or 2 (or more) α-tryptase alleles.

Table 3-1. Accession numbers of all human tryptase sequences accessed in our study

Accession No.	Description
NM_012467	Homo sapiens tryptase gamma 1 (TPSG1).
NM_012217	Homo sapiens tryptase delta 1 (TPSD1).
M33494	Homo sapiens tryptase-1 gene, complete cds.
M30038	Homo sapiens α-1 tryptase mRNA, complete cds.
M37488	Homo sapiens β-tryptase mRNA, complete cds.
M33491	Tryptase I cDNA.
M33492	Tryptase II cDNA.
M33493	Tryptase III cDNA.
AF191031	Homo sapiens gamma I-tryptase gene, complete cds.
AF195508	Homo sapiens gamma II-tryptase gene, complete cds.
AF098328	Homo sapiens tryptase alpha II gene, complete cds.
AF099143	Homo sapiens mast cell tryptase beta III gene, complete cds.
AF099144	Homo sapiens mast cell tryptase beta I gene, complete cds.
AF099145	Homo sapiens mast cell tryptase beta II gene, partial cds.
AF099146	Homo sapiens mast cell tryptase beta II gene, partial cds.

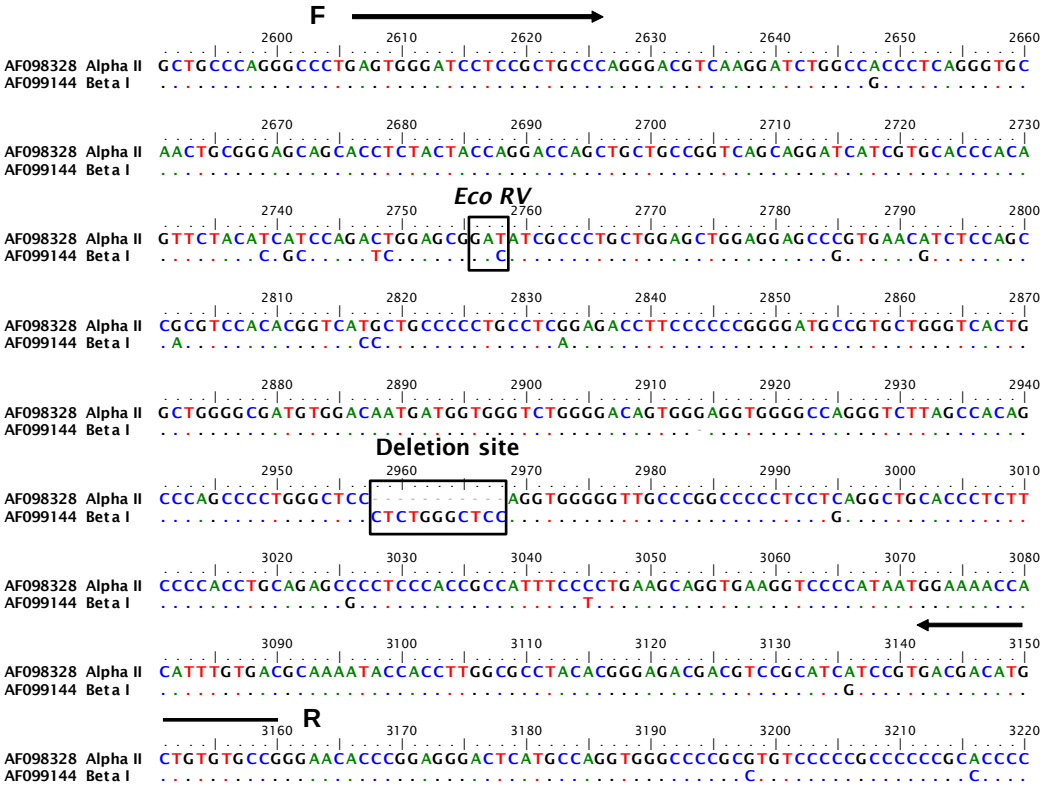


Figure 3-1. Sequence differences used to design the PCR assays for α- and β-tryptase genes. The only possible difference was the *Eco*RV site present in α-tryptase but not in β-tryptase. F, forward primer, R, reverse primer.

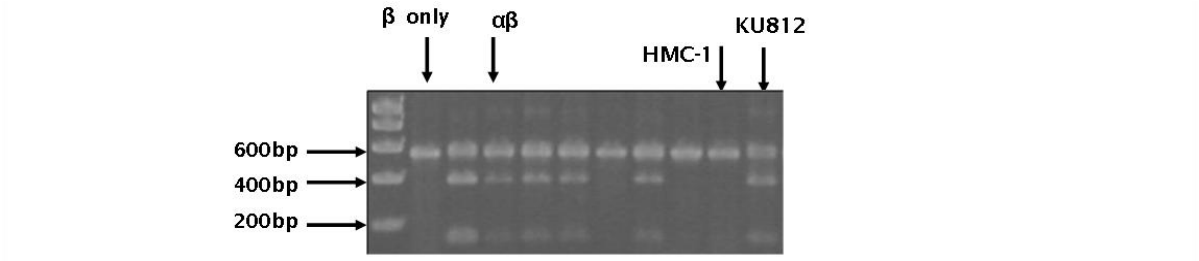


Figure 3-2. Restriction digests of a co-amplified amplicon. Restriction digest with *Eco*RV leads to digestion of the α-tryptase amplicon, leaving the β-tryptase amplicon uncut. The control genotyped cell line, HMC-1 is known to have only β-tryptase (one band) and the KU812 had α- and β-tryptase (three bands). The presence of three bands denote αβ-tryptase genotype, while one band denote β-tryptase only genotype.

3.3 Assessment of α-tryptase copy number using capillary electrophoresis

In order to establish a method that would allow accurate assessment of α-tryptase copy number, it was decided to analyse the α- and β-tryptase PCR amplicons using capillary electrophoresis on a Beckman Coulter CEQ8800 automated DNA sequencer. This relied on the high resolution of the capillary electrophoresis to be able to confirm that all α-tryptase alleles carried the 10-11bp deletion compared to β-tryptase. As the area under the peak observed is proportional to the amount of PCR product loaded this provides a potential way of measuring the copy number.

The ECACC HRC-1 DNA samples were amplified using the same primer pair used for the RFLP assay (section 2.9), but with the reverse primer being labelled with dye (D4-PA) to allow the detection of amplicons by the automated DNA sequencer. Capillary electrophoresis of the undigested amplicons indicated fragments of approximately the accepted size, with one peak in β-tryptase only individuals and two peaks in αβ-tryptase individuals and one peak being 10-11bp shorter. The estimated size of the amplicons was 555bp in length for β-tryptase and 544bp for α-tryptase, due to the 10-11bp deletion present in α-tryptase amplicons compared to those from the β-tryptase amplicon.

It was important to confirm that α-tryptase amplicons only (and not β-tryptase variants) would be carrying the 10-11bp deletion; so the amplicons were digested with *Eco*RV and re-analysed. As the *Eco*RV site was only present in α-tryptase, the presence of 544bp amplicons would suggest that the restriction site was not present in all α-tryptase alleles, and therefore would not be suitable for use as a feature distinguishing between α- and β-tryptases. However, after digestion with *Eco*RV, all the 67 samples that had the short 544bp α-tryptase fragments were digested into two parts, with only the longer fragments of 391 bp being seen (Figure 3-3) as that was the part labelled with the dye. This suggested that the frequency of α-tryptase alleles not containing the 10-11bp deletion must be below 0.5% in the UK population. So, capillary electrophoresis was able to confirm the finding obtained using RFLP analysis of the ECAAC HRC-1 plate.

In order to estimate the copy number variation for α- and β-tryptase genes by measuring the area under the amplicon peaks, it was necessary to ensure that the amplification, under the conditions applied, was still in the linear phase of the PCR reaction. In order to establish this, samples with both α- and β-tryptase were amplified for 20, 25, 30, and 35 cycles and the resulting products were resolved using a 3% agarose gel. This was stained with 0.1% Vista Green dye and the image of the gel captured on the Typhoon fluorimager.

The density of the bands was quantified to allow the assessment of the amount of amplicon generated after the different cycle numbers. We found that the reaction was in the linear phase between 20 and the 25 cycles, and then reached a plateau by 30-35 cycles. For this reason, the 20 cycle amplification conditions were chosen to assess the allele copy number (Figure 3-4). All 96 ECACC HRC-1 DNA samples were amplified (for 20 cycles) and resolved on the capillary DNA sequencer. The areas under the α- and β-tryptase fragment peaks were quantified using Beckmann Coulter software data exported to Excel. The ratio of the α-tryptase to the β-tryptase peak area was calculated.

Two models of the tryptase gene locus have been proposed [144;146]. We expected to see the ratio of α/β correlated with one of these models, but our results did not fit with either model. Quantification of α-and β-tryptase copy numbers by comparing the area under the peaks of the undigested fragments yielded promising findings. However, no definite conclusions could be made as good controls were not available and our data did not fit any of the published models. In addition, while being able to identify accurately those individuals carrying the α-tryptase allele, the method was not suitable for high throughput genotyping of a large population cohort and it was not therefore possible to assess potential associations between the presence or absence of α-tryptase and the risk of allergic disease. It was therefore decided to develop a high-resolution melt (HRM) curve assay to allow rapid genotyping for individuals.

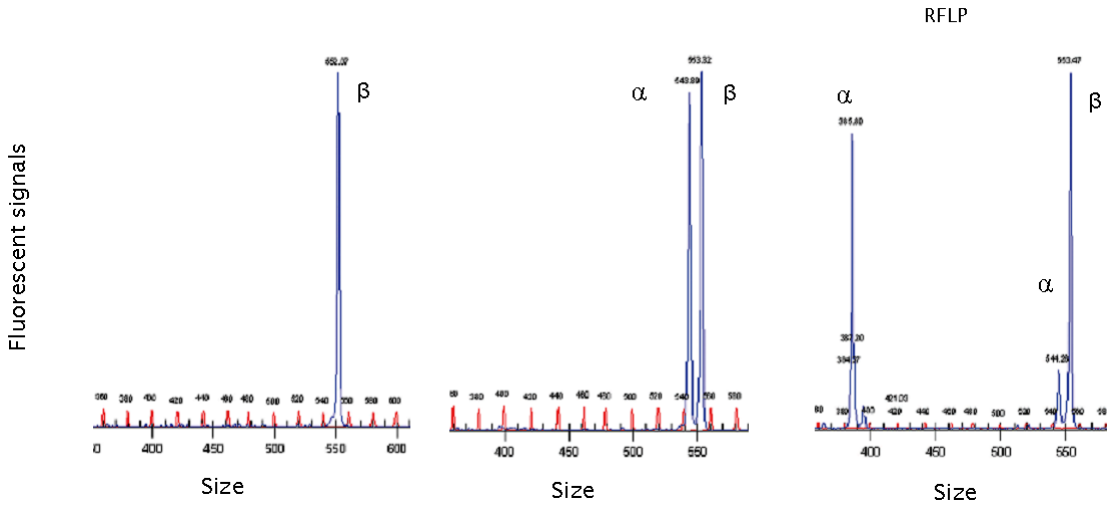


Figure 3-3. Fragment sizing using selected samples, showing one peak for β-tryptase only genotype, two peaks for αβ-tryptase genotyped individuals and three peaks after applying RFLP which cuts α- tryptase but leave β-tryptase uncut, confirming αβ-tryptase genotype.

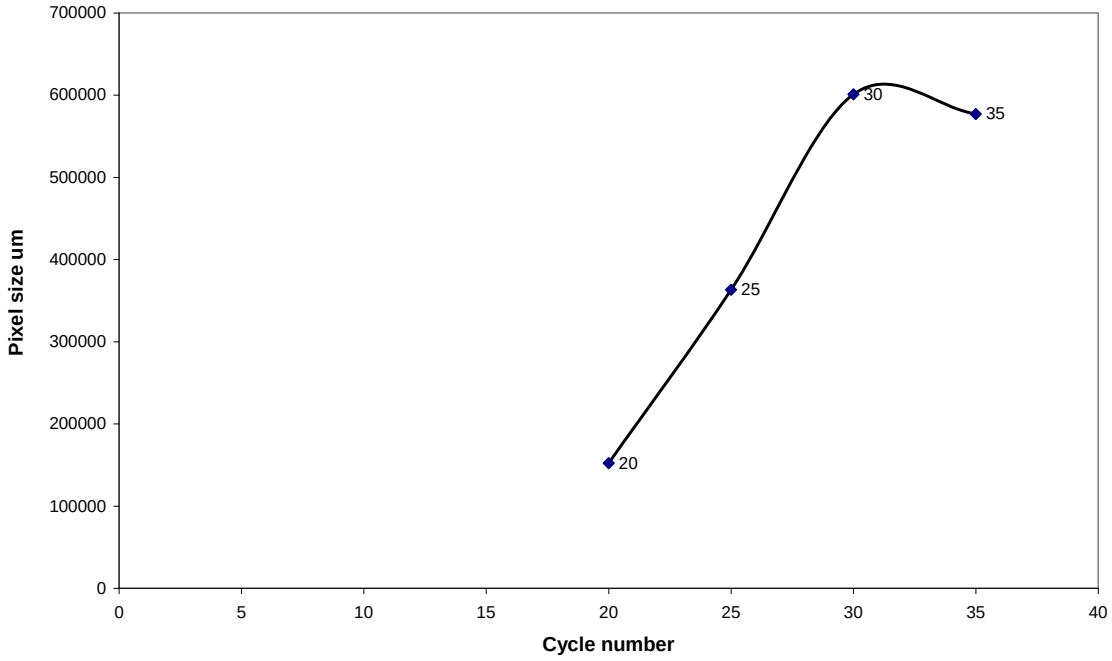


Figure 3-4. Quantitation of PCR products and reaction linearity using the Typhoon fluorimager. The slope was linear within the first 20 and 25 cycles but reached a plateau after 30 cycles.

3.4 Development of a high-throughput, high resolution melt assay for α-tryptase genotyping

3.4.1 Non-specific dsDNA dyes

Using Primer Express™ V3.0 (Applied Biosystems) software, a pair of primers (TrypMeltFwr and TrypMeltRev) were designed to amplify a 73bp amplicon of both α-and β-tryptase genes. The predicted length chosen for the α- and β-tryptase amplicons was identical so as to achieve similar amplification efficiencies between the two amplicons and thus no preferential amplification of one allele over the other). However, the amplicons differed at six nucleotide positions, including the *Eco*RV restriction site mentioned earlier. This would allow differentiation of amplicons on the basis of both altered melting profile and restriction digest (Figure 3-5).

The primers were optimised, using standard HRM reaction conditions (see 2.12) and the optimal primer concentrations were found to be 600/600 nM F/R. Control DNA samples from the cell lines were amplified using the Roche LC480 qPCR instrument using Cyto-09 double stranded DNA (dsDNA) binding dye to assess the melting profile of the amplicons. Using the T*m* calling module of the LightCycler software, it was possible to see two definite peaks, and by using the control DNA from the HMC-1, KU812 and LAD2 cell lines as a reference, we were able to distinguish between α- and β-tryptase peaks (Figure 3-6). HRM analysis was found to provide a fast, robust means for identifying the presence or absence of the α-tryptase allele.

The observed difference in the melting peaks was due to the six nucleotide differences in the amplicon sequences between the α- and β-tryptase genes. *In silico* analysis of the expected melting temperatures (T*m*) of different amplified amplicons predicted T*m* values as follows: for homozygous α-tryptase (94.96ºC), β-tryptase (98.46ºC) and for the α/β-tryptase heteroduplex it was 96.71ºC. The percentage of each type in the sample is likely to affect the pattern of the melting peaks. It was straightforward to predict the patterns in homozygous individuals but not in those who were heterozygous or in heteroduplexes.

To enable assessment of CNV of the α- and β-tryptase loci, two analytical approaches were utilised. First, the gene scanning module of the LC480 instrument was used, following a protocol supplied by Roche. This software clustered samples together based on their melting profile and identified several different genotype groups from the data. Alternatively, the area under the curve (AUC) of the two peaks from the T*m* calling modules was calculated and a ratio between the two peaks calculated using the known genotyped cell line as a standard.

According to previously reported models of human tryptase gene architecture [144], three different genotypes may be postulated: ββββ, ββαα and βββα, with expected ratios between α- and β- tryptase of 100, 1 and 0.3 respectively. This was tested by using 96 DNA UK Caucasian control samples which were genotyped by measuring the AUC.

The AUC for the α- and β-tryptase peaks did not fit the expected ratios, raising the possibility that the architecture of the α- & β-tryptase locus may be different from that proposed. The population lacking α-tryptase was found to be 27.3%, a figure slightly different from the 29.4% calculated when genotyping was performed by RFLP. The difference may reflect the increased accuracy of the new technique.

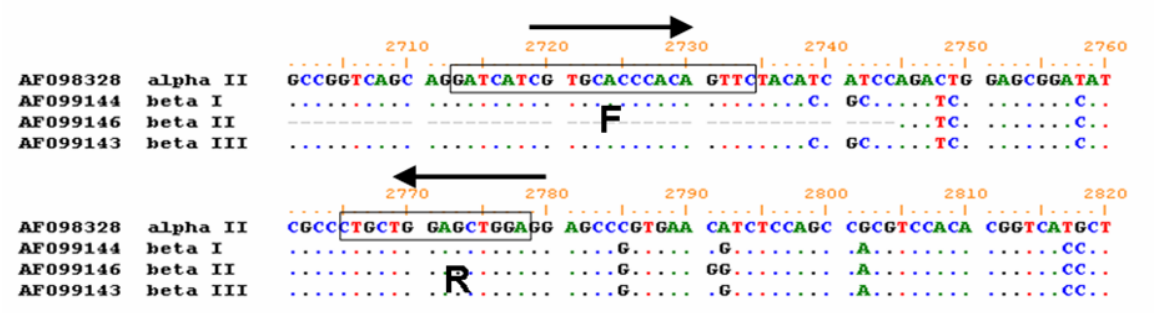


Figure 3-5. Sequences for human α- & β-tryptase employed in the tryptase melting assay where amplicons for the published sequences were aligned. The presence of six single nucleotide differences in the predicted amplicons for α- and β-tryptases amplified during the PCR, resulted in differences in melting peaks. F, forward and R, reverse primer positions.

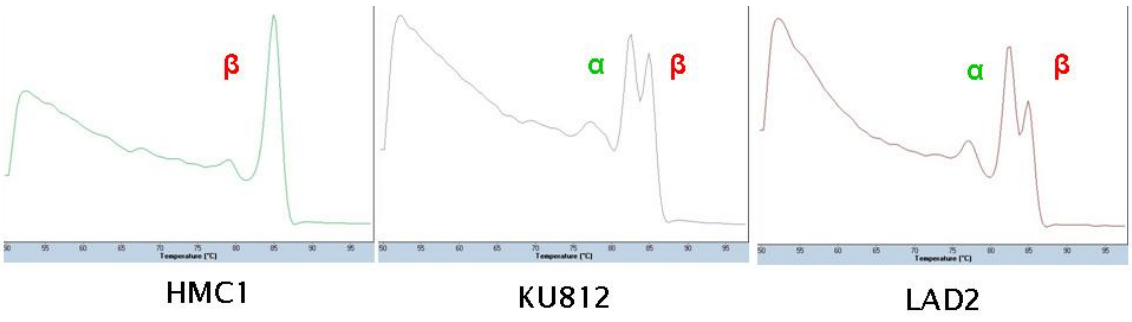


Figure 3-6. Melting curve profiles for cell lines with known tryptase genotype, with only one peak for β-tryptase genotype (HMC1) and two peaks for the αβ-tryptase genotype. The variable ratios between α- and β-tryptase peaks confirm the ability of the assay to detect the nucleotide differences between the two sequences.

3.5 Cloning of αβ-tryptase mixed controls

To improve the accuracy when determining tryptase copy number, it was decided to generate artificial α- and β-tryptase standards which could be mixed in different ratios. Small amplicons (75bp) were cloned for α- and β-tryptase. After purification these were then mixed in different proportions. These acted as standards in investigating possible genotypes.

3.5.1 Separation of α- and β-tryptase amplicons

Making use of the size difference (10-11bp) between the two tryptase genes, we ran the product of a PCR reaction (using a proofreading polymerase enzyme) on a 3% agarose gel for better separation of products. The templates amplified were the DNA samples genotyped earlier using the RFLP assay and the identity was confirmed by capillary electrophoresis. Bands on the gel were at the expected molecular weights of 542-552bp (Figure 3-7). These were better resolved by running the products for a longer time (3 h). Other non-specific bands were seen on the gel, which were outside of the expected size range. The specificity of the two main bands was confirmed by RFLP assay.

After separation of the bands, they were purified using the QIAquick PCR purification kit. The amount purified was 26.9 ng/μl for the β-tryptase only sample, 29.4 ng/μl for upper band and 74.8 ng/μl for lower band (αβ-tryptase genotype). The ratio of absorbance at the 260 nm wave length to that of the 280 nm wave-length (260/280) was 1.76-1.97, indicating high purity of the DNA samples.

3.5.2 PCR cloning

PCR cloning was carried out using a Zero Blunt® PCR cloning kit (Invitrogen). Starting with 158 ng/μl of plasmid with a 260/280 absorbance ratio of 1.8, three different ligation reactions were performed using DNA purified from the gel, in a 20 μl reaction volume. Following the manufacture protocol, plasmids were transformed into *E. coli* TOP 10 cells and after processing, 100 μl of each reaction was plated on to agar plates. The plates were checked for growing colonies and then stored at 4ºC.

3.5.3 Screening of E coli colonies and selection

PCR screening was used to check colonies of E coli for the presence of tryptase amplicons. Five separate colonies were chosen from each plate to run the PCR, and control DNA samples from both HMC1 and KU812 cell lines were included. Colonies of the appropriate size were selected and an *Eco*RV restriction digest assay performed (Figure 3-8).

Colonies selected for α- and β-tryptase were regrown and the plasmid with the correct insert was purified using the Qiagen plasmid miniprep purification kit. The plasmid was quantified using a Nanodrop-1000 and was sent to be sequenced using M13F and M13R (by Geneservice Ltd, Oxford). Sequence results were aligned with all known tryptase sequences for that region. One sample was confirmed to be identical to α-tryptase only and another one to β-tryptase only.

3.5.4 Copy number calculations

After obtaining the correct insert inside the plasmid, the difference was calculated between the plasmid and human genomic DNA copy number, so as to be able to construct precisely the relative amount of mixed controls from the plasmid DNA. Using the assumption that the average weight of a base pair is 650 Da, the molecular weight of any double stranded DNA template can be estimated by calculating the length of the product.

The formula employed was that created by Andrew Staroscik (www.uri.edu/research/gsc/resources/cndna.html). Quantitation of the selected plasmid DNA using this formula indicated that there were 5.21×10^{10} copies/μl for the α-tryptase sample and 5.19×10^{10} copies/μl for the β-tryptase sample. Relative to human DNA, the copy number for genomic DNA was found to be 2.81×10^{12}/μl. The plasmid DNA was diluted to have the same number of copies in each PCR reaction for purpose of quantitation.

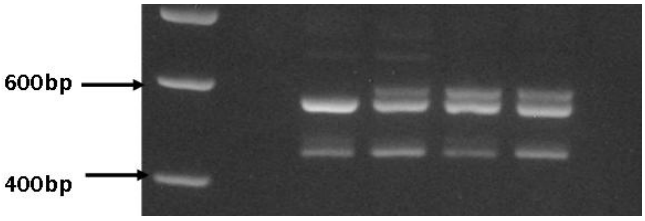


Figure 3-7. PCR of the co-amplified amplicons of α- and β-tryptase. Four DNA samples, with β-tryptase only to the left and the rest genotyped as αβ-tryptase are shown. The size difference in the upper bands on the gel confirmed the genotype of the sample selected. Under UV-light screen, these bands were cut and collected separately as upper bands and lower bands and then purified for further analysis.

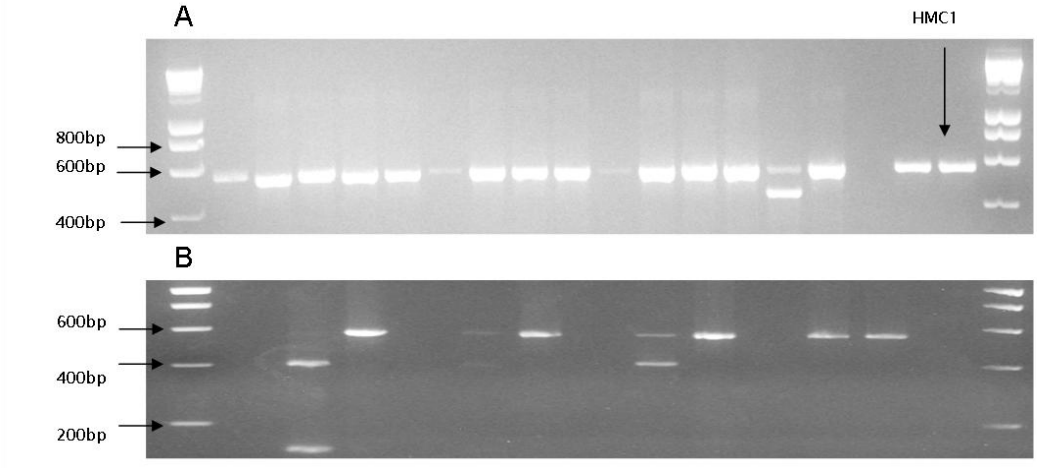


Figure 3-8. PCR screening and selection. (A) Screening colonies for amplicons of the expected size carried out by simple PCR including DNA from the cell line HMC-1 as a control. (B) Amplicons with the expected size digested with *Eco*RV One amplicon was cleaved into two fragments of about the expected size (391&151bp) (arrowed).

3.5.5 Mixing clones

Plasmids containing α- and β-tryptase specific fragments were mixed in defined proportions to create control templates for various possible α-and β-tryptase copy numbers. Using the two and three loci models that have been suggested, all the possibilities are listed in Table 3-2. Using a template of the mixed positive control DNA plasmids, the HRM qPCR assay was undertaken using previously established conditions. The analysis showed that the changes in the melting curve profiles were due to changes in gene copy number (Figure 3-9). Examination of the series of samples with a fixed β-tryptase copy number mixed with different numbers of α-tryptase copies revealed that the changes in the High Resolution Melting (HRM)-profile were actually due to changes in the α-tryptase template copies only (Figure 3-10). Some of the melting curves representing different combinations; (eg. the homogenous α- and β-tryptases) gave similar melting profiles although different in number of copies (Table 3-2). Other mixtures having similar melting profiles were αβββ & ααββββ and ααββ & αααββββ, possibly because they had the same α-tryptase/β-tryptase ratio.

3.6 Genotyping of cohort of families with asthma

DNA samples were available from asthmatic families with at least two siblings diagnosed with asthma. The Genome Therapeutics Corporation (GTC) family cohort consisted of 341 families with asthma recruited from the Wessex region of the UK. Applying the artificial standards created to analysis the melting curve profiles for these DNA samples, the samples were grouped into four genotypes. Some of the samples did not fit in the pattern observed with the standards, possibly on account of heterozygous combinations or other mutations present in these samples. A 96 DNA samples were regenotyped to estimate the error rate and it was found to be 1.08% in the genotype (Table 3-12), which represent the same haplotype. The four different groups identified in the analysis were, ββββ, βββα, ββαα and βααα. There was no individual genotyped as having α-tryptase only copies (Figure 3-11). The α-tryptase gene was absent in 28% of the population, a figure similar to that for Caucasian population frequency reported before [148]. The frequency of the four genotype groups identified was 23.1, 53.3, 21.5 and 2 % for ββββ, βββα, βαβα and βααα respectively in the unrelated parental samples. These genotype frequencies met the Hardy-Weinberg equilibrium.

Table 3-2. Possible genotypes for α- and β-tryptase using two loci and three loci models

Two loci model	Three loci model
αββ	αβββββ
ααββ	ααββββ
αααβ	αααββ
αααα	αααααβ
ββββ	αααααα
	ββββββ

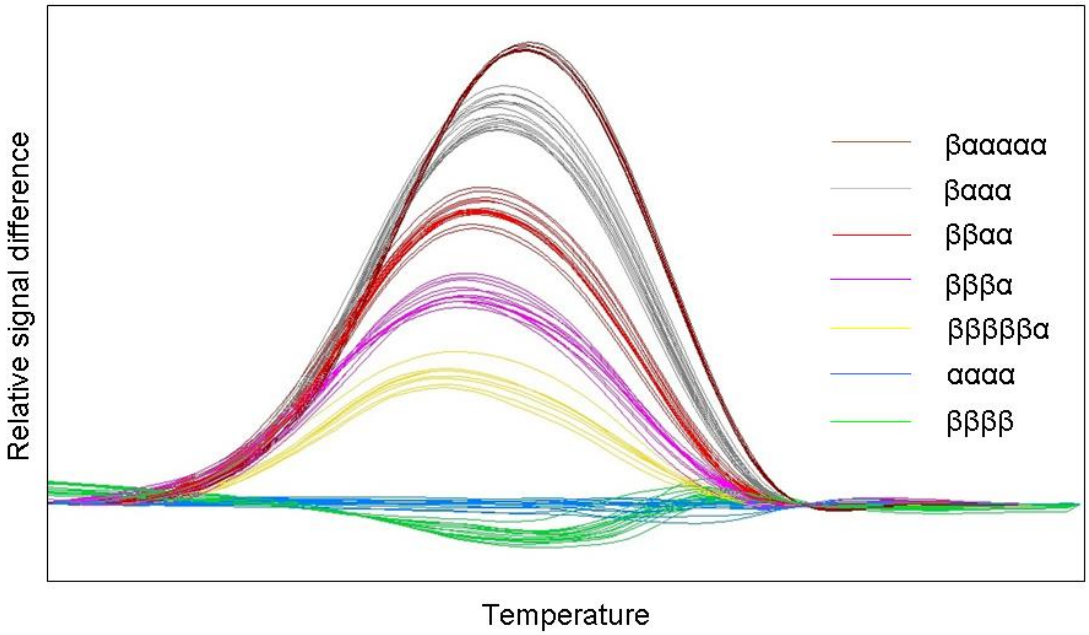


Figure 3-9. Normalized and temperature shifted difference blots for different gene copy numbers for α- and β-tryptases. The melting curve patterns were altered by the changing the α/β template ratio.

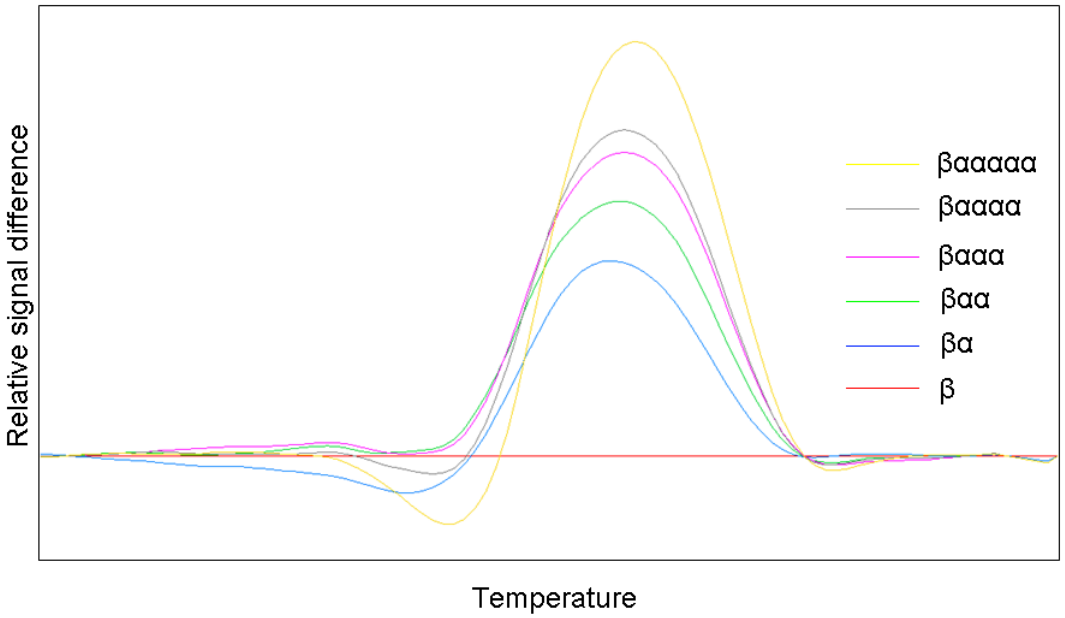


Figure 3-10. Normalized and temperature shifted difference blot for different α-tryptase template copies mixed with fixed numbers of β-tryptase template copies. The changes in the melting curve profile were due to changes in α-tryptase copy number only.

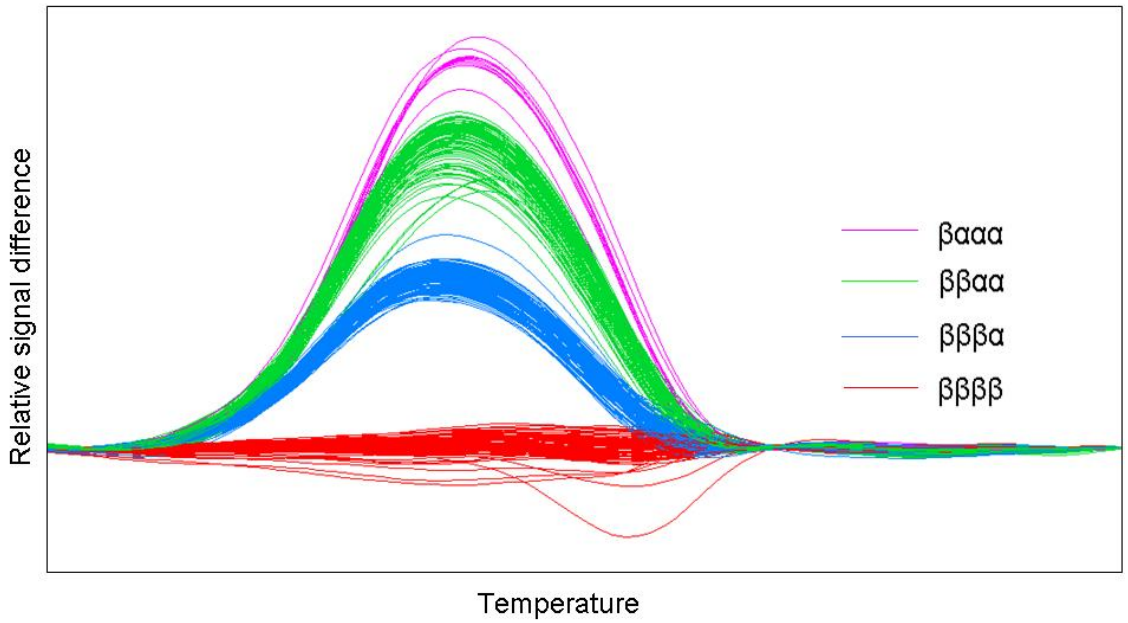
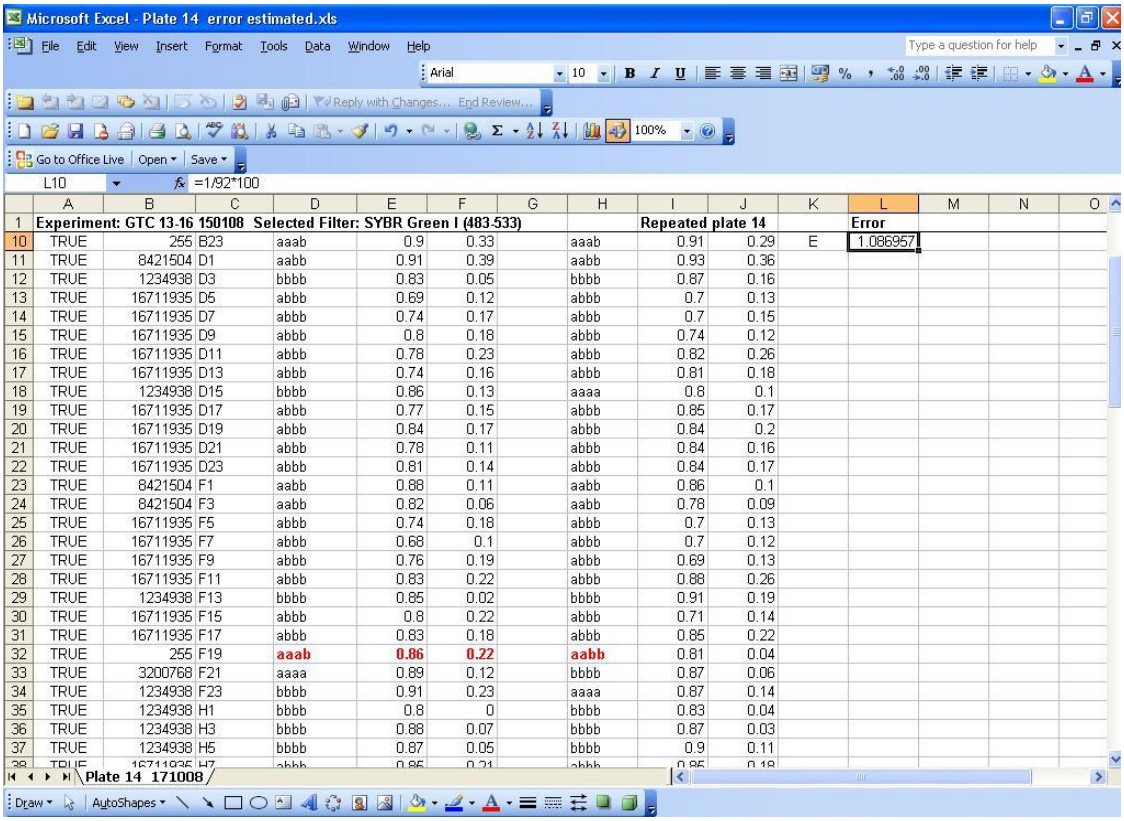


Figure 3-11. Normalized and temperature shifted difference blot for DNA samples from asthmatic individuals. Using the standard controls for the genotypes indicated the samples fell into four distinct genotypes.



Microsoft Excel - Plate 14 error estimated.xls

L10 =1/92*100

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	Experiment: GTC 13-16 150108 Selected Filter: SYBR Green I (483-533)								Repeated plate 14			Error			
10	TRUE	255	B23	aaab	0.9	0.33		aaab	0.91	0.29	E	1.086957			
11	TRUE	8421504	D1	aabb	0.91	0.39		aabb	0.93	0.36					
12	TRUE	1234938	D3	bbbb	0.83	0.05		bbbb	0.87	0.16					
13	TRUE	16711935	D5	abbb	0.69	0.12		abbb	0.7	0.13					
14	TRUE	16711935	D7	abbb	0.74	0.17		abbb	0.7	0.15					
15	TRUE	16711935	D9	abbb	0.8	0.18		abbb	0.74	0.12					
16	TRUE	16711935	D11	abbb	0.78	0.23		abbb	0.82	0.26					
17	TRUE	16711935	D13	abbb	0.74	0.16		abbb	0.81	0.18					
18	TRUE	1234938	D15	bbbb	0.86	0.13		aaaa	0.8	0.1					
19	TRUE	16711935	D17	abbb	0.77	0.15		abbb	0.85	0.17					
20	TRUE	16711935	D19	abbb	0.84	0.17		abbb	0.84	0.2					
21	TRUE	16711935	D21	abbb	0.78	0.11		abbb	0.84	0.16					
22	TRUE	16711935	D23	abbb	0.81	0.14		abbb	0.84	0.17					
23	TRUE	8421504	F1	aabb	0.88	0.11		aabb	0.86	0.1					
24	TRUE	8421504	F3	aabb	0.82	0.06		aabb	0.78	0.09					
25	TRUE	16711935	F5	abbb	0.74	0.18		abbb	0.7	0.13					
26	TRUE	16711935	F7	abbb	0.68	0.1		abbb	0.7	0.12					
27	TRUE	16711935	F9	abbb	0.76	0.19		abbb	0.69	0.13					
28	TRUE	16711935	F11	abbb	0.83	0.22		abbb	0.88	0.26					
29	TRUE	1234938	F13	bbbb	0.85	0.02		bbbb	0.91	0.19					
30	TRUE	16711935	F15	abbb	0.8	0.22		abbb	0.71	0.14					
31	TRUE	16711935	F17	abbb	0.83	0.18		abbb	0.85	0.22					
32	TRUE	255	F19	**aaab**	**0.86**	**0.22**		**aabb**	0.81	0.04					
33	TRUE	3200768	F21	aaaa	0.89	0.12		bbbb	0.87	0.06					
34	TRUE	1234938	F23	bbbb	0.91	0.23		aaaa	0.87	0.14					
35	TRUE	1234938	H1	bbbb	0.8	0		bbbb	0.83	0.04					
36	TRUE	1234938	H3	bbbb	0.88	0.07		bbbb	0.87	0.03					
37	TRUE	1234938	H5	bbbb	0.87	0.05		bbbb	0.9	0.11					

Figure 3-12: Assesment of error rate in genotyping using the HRM. The genotyping data of sample data was repeated and the error rate for this sample was 1.08% in 96 samples and it is reflecting the same gene haplotype applied in the analysis.

3.7 Analysis of the influence of α-tryptase copy number on asthma susceptibility in a family based cohort

Given the key roles that tryptases are postulated to play in allergic inflammatory processes, it was hypothesised that variation in the copy number of α-tryptase might influence susceptibility to asthma and atopy. To test this hypothesis, the HRM α-tryptase assay described (Section 3.4) was used to genotype DNA samples of the asthmatic family cohort (Section 2.14).

3.7.1 Clinical phenotypes of the GTC families' cohort

The clinical characteristics of the cohort are shown in Table 3-3. All of the measurements were performed at recruitment as previously described [152;166]. Numerous measurements were made but only six phenotypes were used in the GTC CNV FBAT analysis: asthma diagnosis, log age corrected total IgE levels, bronchial hyperresponsiveness (BHR), % predicted FEV_1, positive atopy severity score and positive asthma severity score (Section 2.14).

3.7.2 The Family Based Association Test (FBAT)

The association analysis was undertaken using Family Based Association Test (FBAT) software, version 1.3 [167]. FBAT association statistics were calculated for each nuclear family separately, after which the grand total was taken. Analysis of 336 families, comprising 1455 persons, was undertaken for three alleles null, α and αα, (i.e. having no, one or two copies of the α-tryptase gene).

Association analysis of these alleles was studied for the diagnosis of asthma (Table 3-4), and related phenotypes including total serum IgE (Table 3-5), FEV_1 (Table 3-6), bronchial hyperresponsiveness (BHR) to methacholine (Table 3-7), atopy and asthma severity scores for these alleles were studied (Table 3-8 and Table 3-9). Where S is the test statistic, E (S) and Var (S) are the expected value and variance of the test statistic under null hypothesis (H0); Z and P are the Z statistic (S normalized using E (S) and Var (S)) and its corresponding p-value.

There was an apparent trend towards an association between possessing one copy of the α-tryptase alleles and reduced susceptibility of asthma (p=0.07)

(Table 3-4). Such an association was not observed with the other two alleles. The association tests indicated that, one copy allele was also associated significantly with lower total IgE levels (p=0.01) (Table 3-5). On the other hand, the two copy allele was associated with higher IgE level (p=0.05). The measures of FEV_1 and BHR did not show an association with the pattern of α-tryptase copy numbers (Table 3-6 and Table 3-7). There was an apparent trend towards there being an association between reduced atopy severity score and possessing the one copy allele of α–tryptase while with the two copy allele there was a significant association with increased atopy severity score (Table 3-8). There was no association identified between the different α-tryptase copy alleles and the asthma severity score (Table 3-9).

3.7.3 Association of serum IgE levels in parental samples with the α-tryptase genotype

To explore further the association of α-tryptase expression with unrelated samples, analysis of parental samples was carried out. Median IgE levels in subjects carrying one copy of the α-tryptase allele (i.e. those with a genotype of null/α) was not significantly different from those in subjects with no copies of the α-tryptase (null / null genotype), or those carrying two or three copies of the α-tryptase gene (Figure 3-13).

There were two opposing trends, one for the affected (i.e. with asthma) and the other for the unaffected parents (i.e. non-asthmatics). The unaffected parents had lower IgE levels (high median Log IgE, 0.41) in the null (ββββ) group and a similar level (0.40) was seen in the (βααα) group. While the other two genotypes (βββα and βαβα) had higher levels (0.11 and 0.30 respectively). On the other hand, in affected parents the log IgE levels appeared to be related to some extent to the α-tryptase copy number (with log IgE values of 0.67, 0.91, 0.88 and 1.5 for ββββ, βββα, βαβα and βααα respectively). The association between the parents' genotypes and the IgE levels could not be studied on account of insufficient numbers in some groups, but those did not appear to be a relationship between the genotype for α-tryptase and the IgE levels.

Table 3-3. Clinical characteristics of the Southampton asthma families' cohort

	Pedigrees (n=1508)	Parents (n=681)	Non- asthmatic parents (n=492)	Asthmatic parents (n=189)	Sibling 1 (n=341)	Sibling 2 (n=328)
Age (y), mean (SD)	24.6	40.5	40.7	40.2	13.0	9.9
Gender (% male)	51.8	49.9	51.0	47.1	56.9	53.6
Asthma (doctor diagnosis)	60.1	27.8	0	100	100	100
Eczema (% questionnaire)	45.6	32.7	25.8	50.8	57.8	62.4
Hayfever (% questionnaire)	48.9	46.8	38.0	69.8	64.2	47.0
FEV_1 (% predicted),mean	98.05	100.8	103.4	94.12	94.7	95.6
BHR (methacholine)* (1/Lslope+30) x1000	19.03	24.3	26.8	17.22	14.5	12.0
IgE Iu/ml median (range) (Age corrected)	105 (29.5-381.5)	56.3 (26.4-151.3)	42.2 (168-115)	107 (47.3-248)	252 (70.3-779)	

* The PC_{20} of the population studied were as follow: 736 (>16mg/ml), 210 (4-16), 262 (1-4) and 227 (<0.1) according to the ATS classification of asthma-enriched population.

Table 3-4. FBAT results from association analysis involving α-tryptase CN genotypes and the diagnosis of asthma.

Marker	Allele	Allelic Frequency	Fam	S	E (S)	Var (S)	Z	P
α-tryptase alleles	0	0.577	271	628	614.5	215.25	0.92	0.36
	1	0.312	255	319	342.5	169.25	-1.8	0.07
	2	0.11	120	149	139	69.00	1.2	0.23

Fam, number of informative nuclear families, i.e., families with minimum one informative phenotype; S, the score statistics; E (S), and Var (S) are the expected value and variance of the test statistics under H0; Z and P are the Z statistic (S normalized using E (S) and Var (S) and its corresponding p-value.

Table 3-5. FBAT results from association analysis involving α-tryptase CN genotypes and log age corrected total serum IgE level.

Marker	No. of Copies	Allelic Frequency	Fam	S	E (S)	Var (S)	Z	P
α-tryptase alleles	0	0.577	271	1248	1213.2	1136.77	1.03	0.28
	1	0.312	255	602	673.9	894.45	-2.4	0.01
	2	0.11	120	315	279.3	361.24	1.92	0.05

Table 3-6. FBAT results from association analysis involving α-tryptase CN genotypes and FEV1 % Predicted.

Marker	No. of Copies	Allelic Frequency	Fam	S	E (S)	Var (S)	Z	P
α-tryptase alleles	0	0.577	272	6858	67021	2329357	1.02	0.30
	1	0.312	257	34288	36842	1783595	-1.9	0.30
	2	0.11	123	17515	16519	800087	1.11	0.26

Table 3-7. FBAT results from association analysis involving α-tryptase CN genotypes and the measure of BHR on asthmatic siblings.

Marker	No. of Copies	Allelic Frequency	Fam	S	E(S)	Var(S)	Z	P
α-tryptase alleles	0	0.577	264	9.89	9.55	0.08	1.19	0.23
	1	0.312	247	4.73	5.15	0.06	-1.7	0.08
	2	0.11	117	2.44	2.35	0.03	0.51	0.60

Table 3-8. FBAT results from association analysis involving α-tryptase CNV genotypes and Atopy Severity Score.

Marker	No. of Copies	Allelic Frequency	Fam	S	E(S)	Var(S)	Z	P
α-tryptase alleles	0	0.577	245	760.2	748.9	575	0.47	0.63
	1	0.312	233	384.9	424.1	440.3	-1.9	0.06
	2	0.11	111	209.8	182	195.9	1.98	0.04

Table 3-9. FBAT results from association analysis involving α-tryptase CNV genotypes and Asthma Severity Score.

Marker	No. of Copies	Allelic Frequency	Fam	S	E(S)	Var(S)	Z	P
α-tryptase alleles	0	0.577	270	2605	2549	3852.6	0.9	0.36
	1	0.312	255	1312	1398	2949.7	-1.6	0.11
	2	0.11	122	652.5	622	1298.6	0.84	0.3

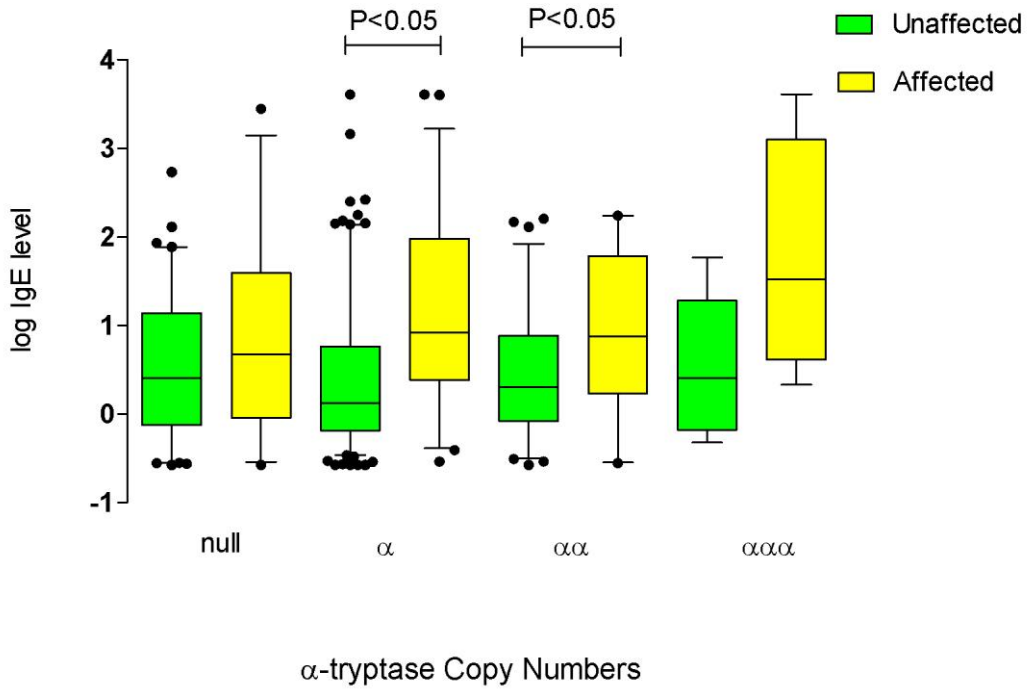


Figure 3-13. Association of total serum IgE level of parents (affected & unaffected) and the α-tryptase copy numbers. Data represented as box plot with median. The log IgE level differed (P<0.05) between the affected (asthmatic) and the unaffected (healthy) parents with α or αα–tryptase copy numbers, using Mann-Whitney U test.

3.8 Discussion

The present study provides information for the first time on α-tryptase copy number and its association with asthma susceptibility and related asthma phenotypes. The genotyping assay developed used a range of approaches and the findings were consistent. The creation of a standard from cloned α-tryptase products allowed quantitative melt curve analysis to be applied to determine the copy numbers of α-tryptase.

The development of genotyping techniques has been rapid over the last 10 years, with high accuracy in application of the melting curve analysis for genotyping [168-170]. The assay developed in this study was based on a 6 bp mismatch between all α- and β-tryptase sequences and the use of the HRM technique has proven highly accurate in the analysis of SNP even in heterozygous or heteroduplex individuals [171;172]. Using BLAST searching against the NCBI database, the assay was applied to all α- and β-tryptase sequences, and assay specificity was confirmed using melt curve analysis. The use of HRM analysis for copy number variation resulted in a robust, high throughput assay, with an error rate of less than 1% based on repeated genotyping of a proportion of subjects and analysis of inheritance errors in the family cohort.

Previous models of the architecture of the tryptase locus on chromosome 16p13.3 have used data derived from sequencing and restriction mapping of two bacterial artificial chromosomes (BACs) [144]. The presence of two tryptase loci was described as one exclusively for β-tryptase and the other allelic between α- and β-tryptase. These loci have also been shown to be highly polymorphic with approximately one SNP every 90 bp, and differences between ethnic groups [146]. Our findings have led to a new model for the architecture of the α- and β-tryptase locus. We hypothesise that there is a single α-tryptase locus that exhibits copy number variation due to segmental duplications and/or deletions spanning the α-tryptase gene. This results in alleles carrying 0, 1, 2 or 3 copies of the α-tryptase gene, with individuals having potential genotypes of 0, 1, 2, 3, 4, 5 and 6 α-tryptase copies. A consequence is that there are likely to be more than two loci for tryptase. Future work will be required to determine the copy number of β-tryptase to provide definitive information on the structure of that region.

Copy number variation has been shown to be associated with several common inflammatory and autoimmune disorders, including rheumatoid arthritis [173], Crohn's disease [174] and psoriasis [175]. Previous observations of α-tryptase copy number variation have led to studies examining the relationship between tryptase genotypes and some common diseases. Akin *et al* [176] found a strong correlation between the category or severity of mastocytosis and plasma levels of tryptase but not with tryptase genotypes, while Min *et al* [177] in 106 healthy subjects reported a strong association between plasma tryptase levels and tryptase haplotypes. The apparent conflict between these two findings could be due to differences in serum tryptase level between diseased and healthy individuals. Moreover, current assays for serum tryptase level detect the mature tryptase form, which may not include α-tryptase as it is not processed to a mature form [81].

In the present study, using a well characterized cohort of 341 asthmatic families (1419 subjects), the α-tryptase copy number was found to be associated with total serum IgE levels. Carrying an allele of α-tryptase was significantly associated with lower total serum IgE levels and there was also a trend for an association with lower risk of asthma diagnosis. In contrast, there was a trend for one allele of α-tryptase to be associated with increased BHR.

This could be explained by the recent discovery of tryptase splice variants for all known tryptase genes [178] and for a non-functional form of β-III tryptase [179], which could account for the presence of variable or non-functional tryptase variant other than α-tryptase. Alternatively, it could be that α-tryptase genes are in linkage disequilibrium with nearby genes which would produce such effect in asthma. There was no apparent dosage effect for α-tryptase copy numbers with all phenotypes studied.

The observation that a one copy allele of α- tryptase is associated with a lower serum IgE level raise the possibility that there is a direct beneficial effect of having more α-tryptase genes or of linked genes nearby. In support of this idea, β-tryptase has been found able to cleave IgE level *in vitro*, in a way that could provide a natural feedback control of allergic inflammation [180]. Where there is reduced expression of functioning β-tryptase alleles, this mechanism would be unexpected to be the case. The importance of serum IgE level as a marker of the severity of allergic disease is unclear. Gerdes *et al* [181] found

no relationship between serum IgE levels and the severity of atopic disease while Gergen *et al* [182] reported an association between IgE serum levels and the prevalence of asthma. These apparent discrepancies could be due to differences in the study populations or to differences in disease phenotypes.

In conclusion, we have identified a strong association between expression of one copy α-tryptase allele and total serum IgE levels. The relationship between the α-tryptase CNV and serum IgE levels does not appear to be additive with increasing copy number, suggesting that the effect may be a consequence of linkage disequilibrium between the α-tryptase CNV and other variation at the α-tryptase locus or at an adjacent locus (e.g. β-tryptase). To confirm these findings and to determine the precise site of the causal variation, this observation will need to be replicated in additional study populations. The development of an assay for β-tryptase copy number may enable a distinction to be made between effects of α-and β-tryptase variation on total serum IgE levels.

Chapter 4
Expression profiles of β- and α-tryptases

4. Expression profile of β- and α- tryptases

4.1 Introduction

The respiratory epithelium was once thought of as a relatively inert barrier to the outside environment. However, it is now recognised that it can regulate fluid and ion transport across the airway lumen, modulate airway smooth muscle tone, and contribute to the control of inflammatory cell accumulation and mediator secretion [183]. In addition, it plays a key role in protecting the airway from the external environment. Disruption of these functions may contribute substantially to airway inflammation, oedema, mucus production and airway constriction in respiratory disease. Airway epithelial cells are the first line of defence, and activation of these cells by mediators derived from inflammatory cells or inhaled allergens can release a plethora of pro-inflammatory mediators [184].

Endogenous and exogenous proteases are emerging as important mediators of inflammatory processes and may play critical roles in the pathogenesis of asthma and rhinitis [185]. A compelling case can be made for mast cell tryptase as a key mediator in asthma. The levels of this protease are higher in BAL fluid and sputum collected from asthmatic subjects than that from non-asthmatic subjects [107]. Several studies have shown that β-tryptase can not only contribute to airway bronchoconstriction and hyperresponsiveness, but also have a key role in fibrosis and extracellular matrix turnover which are hallmarks of the remodelling process [186-188]. Beta-tryptase has been shown to induce proliferation of airway smooth muscle resulting in release of TGF-β1 and it can stimulate an increase in mast cell numbers in many tissues [189]. Tryptase can also play a critical role in epithelial repair [115], and by controlling airway smooth muscle cell migration [190], it may play a key role in airway remodelling in asthma.

The effects of mast cell tryptase in allergic disease have been studied in terms of β-tryptase. However, little is known about the contribution of α-tryptase to cellular responses. Evidence for α-tryptase having biological activity is conflicting. The recombinant form of α-tryptase was shown to be able to induce cell proliferation in one study [122], but not in another [191]. In the latter study, α-tryptase had been engineered to have catalytic activity and it is

not clear if a catalytically active form of α-tryptase may occur naturally. The observation in the present studies that α-tryptase copy number is associated with some features of asthma and atopy (chapter 3), raised further questions over whether α-tryptase may have a function in allergic disease.

We have aimed to investigate and compare the actions of recombinant α- and β-tryptase on epithelial cells and have examined in particular the cytokine expression profile. We have also examined the extent to which actions may be dependent on an intact catalytic site.

4.2 Expression Profiles of β-Tryptase

4.2.1 Purification of recombinant β-tryptase

Recombinant β-tryptase was expressed in a *Pichia pastoris* expression system [155], and was successfully purified from culture supernatant. Elution profiles for tryptase are illustrated with the culture supernatant applied to butyl Sepharose, a hydrophobic interaction column and then to a heparin agarose affinity column, eluting with salt gradient molarities (0.2-2.0 M) (Figure 4-1). A trace for BAPNA cleaving activity is shown also for a BioSep-Sec-S-3000, size exclusion column, employed in HPLC for the final polishing stage of the purification (Figure 4-2). The elution position was consistent with tryptase having a molecular weight of 132 KDa for the active tetrameric form.

The purity of the protein was confirmed by SDS-PAGE analysis, in which it appeared as a single band (Figure 4-3A). The molecular mass of 30-35 kDa calculated on the Coomassie blue and silver stained gels is consistent with that of the monomeric form of tryptase. Its identity as tryptase was confirmed by western blotting with the tryptase-specific monoclonal antibody AA5 (Figure 4-3B). The specific activity of the β-tryptase preparations employed ranged from 1.6-2.1 U/mg where 1 unit was taken as the amount of tryptase that can cleave BApNA and release 1 umol p-nitoanalide per min at 25ºC. The endotoxin levels as assayed by the LAL assay were less than 0.08 EU/ 1U tryptase in all preparations used in the study.

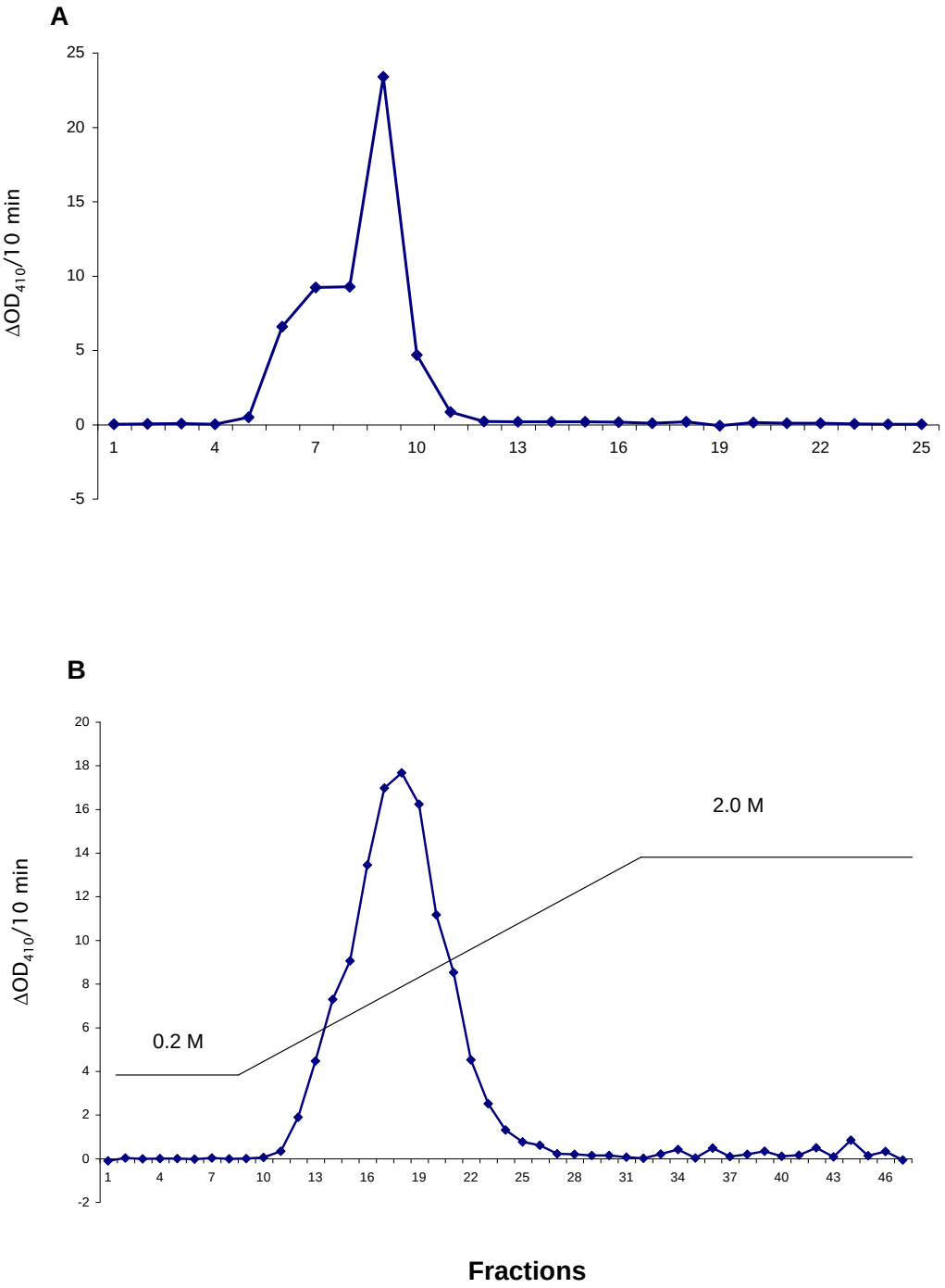


Figure 4-1. BApNA substrate activity for fractions eluted from A: butyl Sepharose column. B: on 0.2-2.0 M NaCl gradient from a heparin agarose column. The activity was expressed as the change in the optical density of the BAPNA substrate at 410 nm for each fraction.

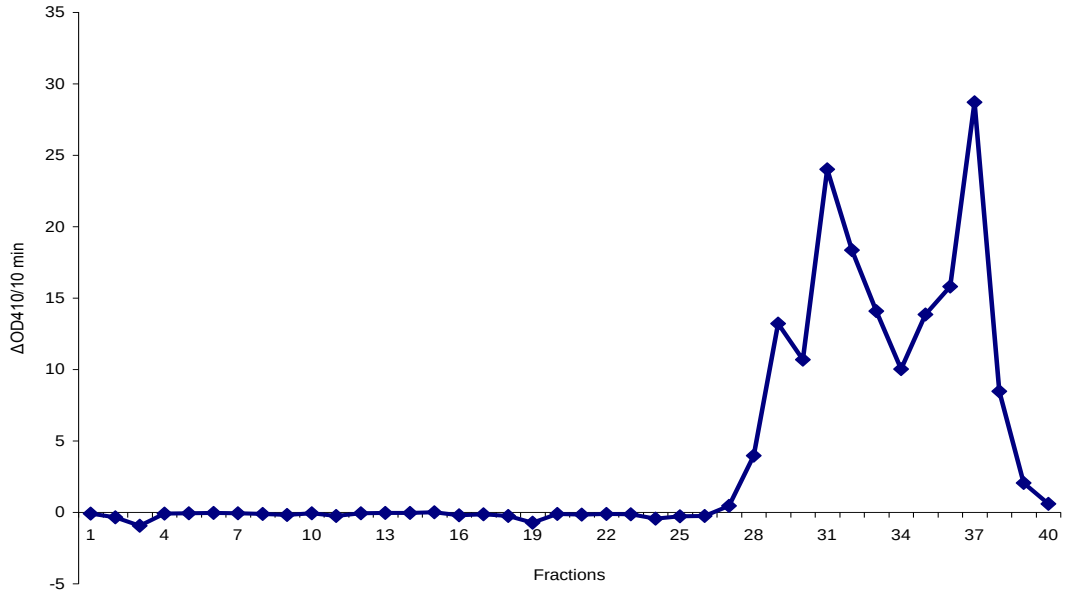


Figure 4-2. Final stage in tryptase purification using a BioSep-Sec-S-3000 size exclusion column in HPLC. The BAPNA cleaving activity expressed as change in substrate colour at 410 nm.

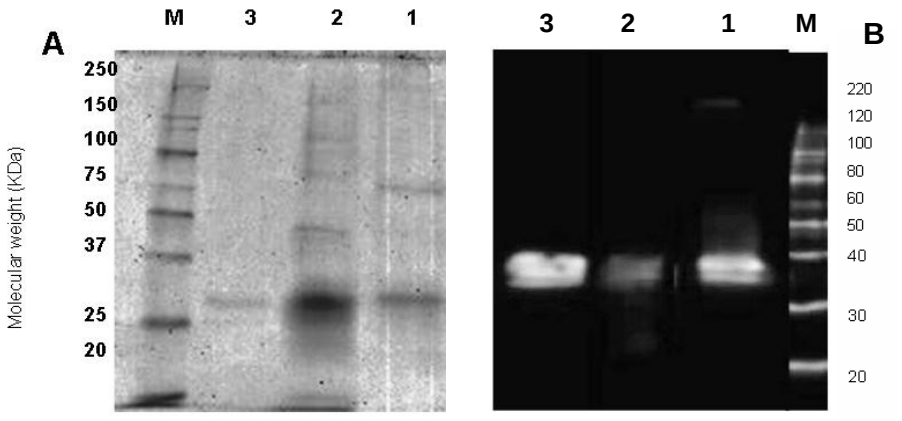


Figure 4-3. (A) Coomassie blue stained SDS PAGE gel (10% W/V) under reducing conditions, showing bands for (1) supernatant from yeast expression system, (2) the tryptase-rich fraction from a butyl agarose column, (3) purified β-tryptase eluted from heparin agarose column and molecular weight markers (M). (B) Western blot of the same preparations with tryptase-specific monoclonal antibody AA5.

4.10 Optimisation of RT-PCR for quantification of gene expression

Accurate normalisation of gene expression levels is essential for obtaining reliable results, especially when the biological importance of subtle gene expression differences is studied [192]. In this section, we have reported the normalization procedures performed when investigating the effects on gene expression of human bronchial epithelial cells following addition of β-tryptase.

4.10.1 RNA quality control

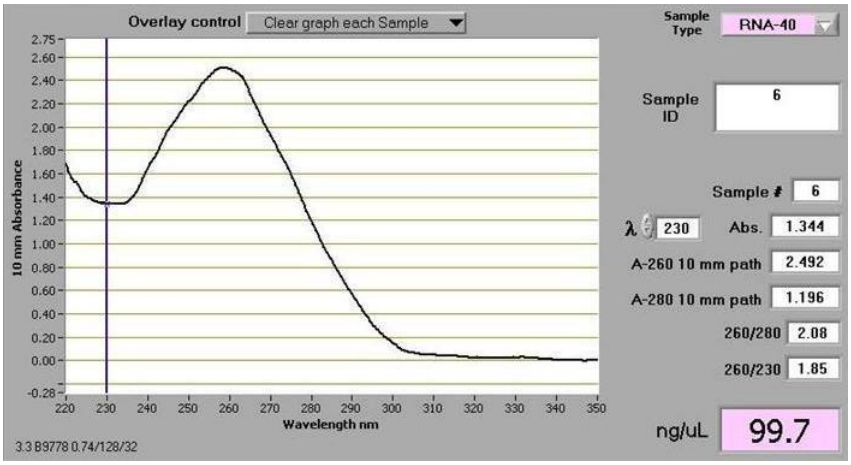
The quality and quantity of RNA extracted was assayed using a Nanodrop ND1000 spectrophotometer (average yield/well = 50-150 ng/µl). RNA sample quality was further assessed using the Agilent Bioanalyzer 2100 (Figure 4-4), both peaks were clearly visible with no assay noise and a ratio of over 1.8 for all samples. RNA samples were further separated electrophoretically on a micro-fabricated chip and detected by laser induced fluorescence detection. The 18S and 28S ribosomal bands showed no evidence of degradation products.

4.10.2 Expression profiling of house keeping genes for 16HBE 14o- cells

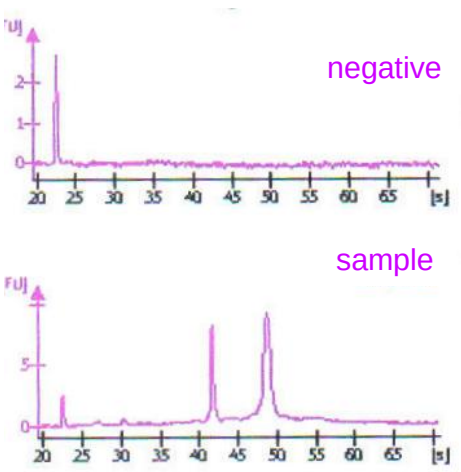
The ideal reference genes should be expressed at similar levels to those of the target to be analysed and should show little to no variation in expression levels under different experimental conditions. The expression of six potential reference genes (UBC, GAPDH, ACTB, 18SrRNA, SF3A1 and B2M) was assessed for 16 HBE 14o- cells treated with tryptase. Special attention was paid to selecting genes that belong to different functional classes, so as to reduce the chance that genes might be co-regulated [193]. Visual basic application (VBA applet) geNorm software ranked the housekeeping genes in order of stability of expression and output graphs identified the least variable housekeeping genes for the experiment.

The geNorm software provided information on the systematic variation. This was expressed in terms of pairwise variation, where the levels of variation in average reference gene stability was calculated following sequential addition of each reference gene to the equation (for calculation of the normalization factor). This allowed estimation of how many genes were needed to reduce the experimental variation.

A



B



C

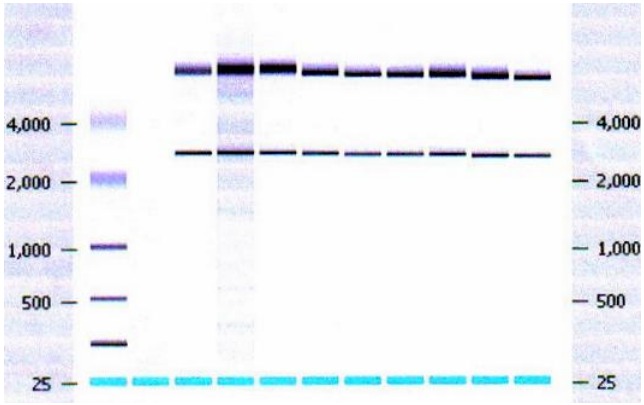


Figure 4-4. Quantitative and qualitative analysis of extracted RNA. (A) Nanodrop spectrophotometer and fluorometer traces of an RNA sample. (B) Segment of an electropherogram: the 18S-region and 28S-region covering the 18S peak and 28S peak respectively. (C) RNA samples on a micro-fabricated chip and detected by laser induced fluorescence detection.

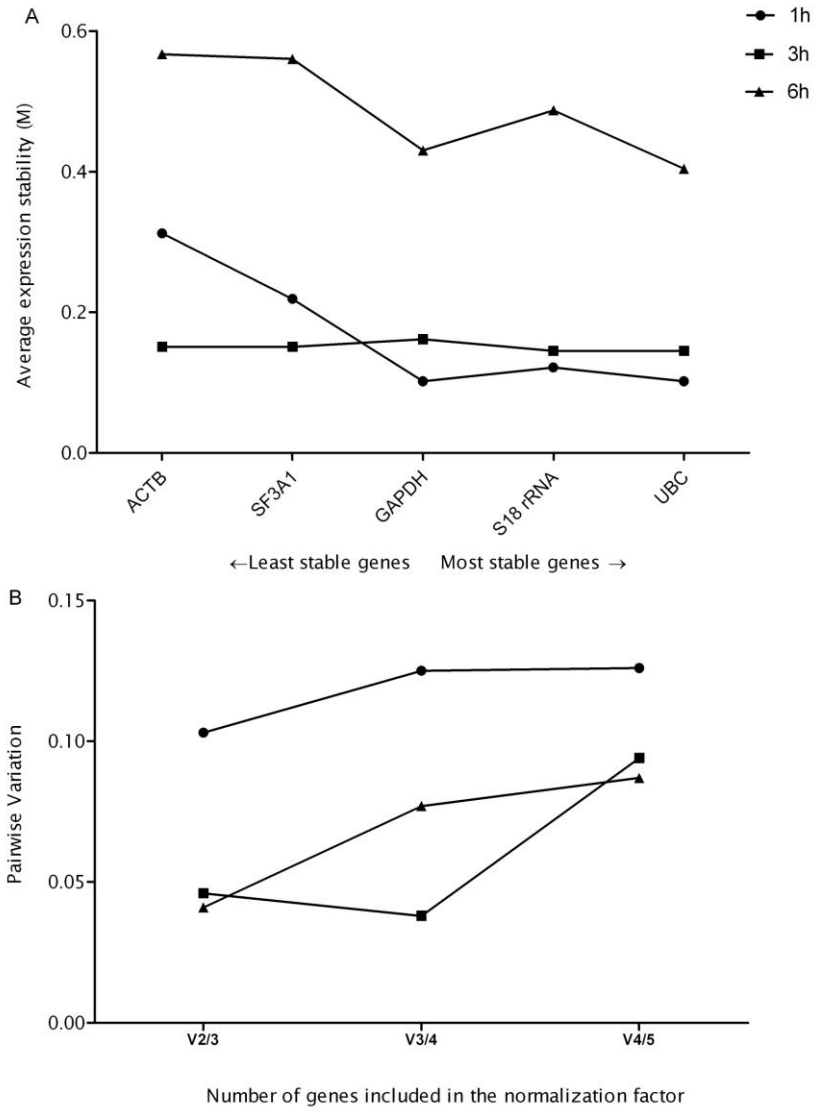


Figure 4-5. Selection of appropriate normalizing reference genes for quantitative PCR. (A) Average variation of the expression stability value (M) for the six candidate housekeeping genes during stepwise exclusion of the least stable control gene (from the left to the right on the X-axis). (B) Pairwise variation analysis.

In all subsequent experiments the geometric mean of these three gene transcripts was used as the reference value to assess expression of genes of interest (section 2.11)

4.10.3 Amplification efficiency

In a real time PCR assay a positive reaction is detected by the accumulation of fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (ie exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample).

In order to be able to analyse genes expression data statistically using the ΔΔCT method, it was necessary to confirm that amplification efficiency throughout the experiments was the same. That was validated using variable cDNA concentrations for each gene primer used. Standard curves were generated with various dilutions of the stock cDNA and the Ct values for each gene. The range of template concentrations applied was designed to cover any variation in the template amounts throughout the whole experiment.

4.10.4 Medium conditions

In previous investigations of gene expression in 16-HBE-14o- cells that have been reported previously, 24-hour cultures in serum-free medium have been tested, so that cells are synchronised to the same phase of the cell cycle [194]. Initially the same methodology was adopted in the present studies, but it was found that the use of serum-free medium rendered the cells poorly responsive to treatment with the stimuli employed. Therefore other conditions were explored that might improve cell responsiveness. Cellular responses to tryptase treatment were compared between serum-free medium, medium with 2% FCS or 10% FCS 'full medium'. It was found that the use of 2% FCS medium was associated with stronger effects on cytokine mRNA responses to tryptase. Expression of IL-6 and IL-8 mRNA was greater in cells incubated with β-tryptase in 2% medium than in cells incubated with serum-free medium (0% FCS) (Figure 4-6). The medium with 2% FCS was used in all subsequent experiments.

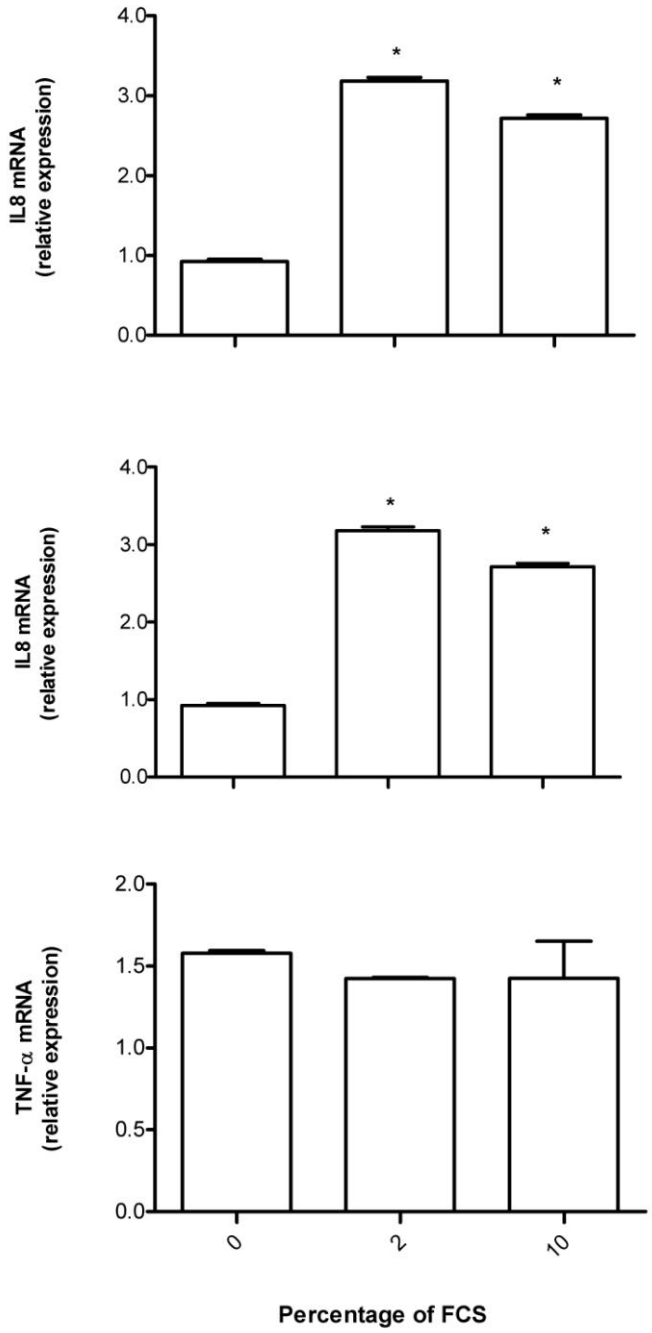


Figure 4-6. The effect of cell culture medium on cytokine gene expression profiles following treatment of epithelial cells with β-tryptase (40 mU/ml for 3 hours). Data for each culture condition are expressed relative to its own control, and represent the mean ± SEM for n = 4. *P< 0.01 compared to response with 0% FCS medium using the unpaired t-test with Welch's correction

4.10.5 Concentration response and time course studies.

When cells were treated with 10, 20, 40, or 80 mU/ml of β-tryptase for 6 hours, increased expression of mRNA for IL-6 was observed with a concentration of 40 and 80 mU/ml and for IL-8 with concentrations of 40 mU/ml (Figure 4-7A). When cells were treated with 40 mU/ml β-tryptase for periods ranging from 1 to 24h, increased expression of IL-6 was observed at 6, 9, and 24 hours, and for IL-8 at 6 and 24 h (Figure 4-7 B).

On the basis of these experiments, studies with β-tryptase were conducted at 40 mU/ml and this was added for 6 h (Figure 4-8). It was found that β-tryptase was able to stimulate an increase in expression of mRNA for IL-8 and TNF-α by some seven to eight fold. The increase in IL-6 mRNA expression was minimal, with only a two-fold increase, relative to expression of untreated cells.

4.11 Catalytic dependency of β-tryptase actions

The ability of β-tryptase to cleave BAPNA was abolished by heating for 95 min at 60°C or at 95°C for 35 minutes. Incubation of β-tryptase with leupeptin and the selective small molecule inhibitor X, for 60 minutes at 4°C, reduced the activity of β-tryptase by 90-95% (Figure 4-9). The β-tryptase induced increases in mRNA for IL-6, IL-8, and TNF-α was in all cases significantly reduced by heat inactivation of tryptase (Figure 4-10).

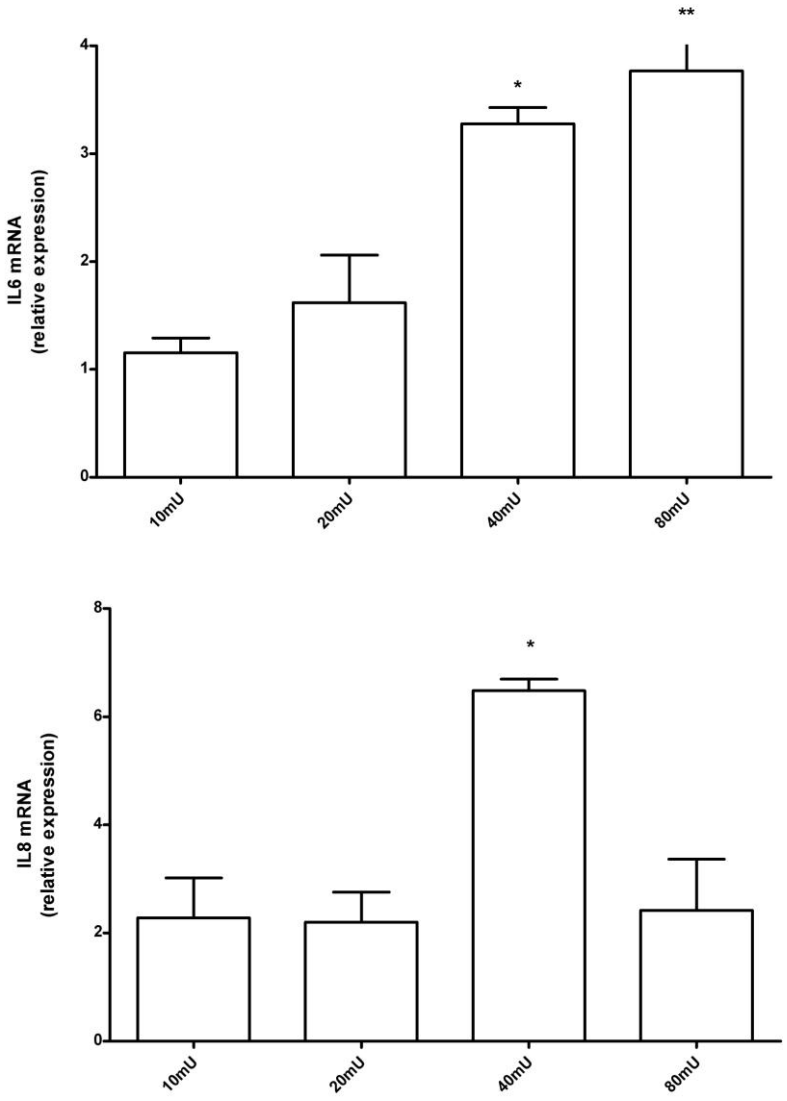


Figure 4-7 A. Relative expression of mRNA for IL-6 and IL-8 in epithelial cells following treatment with recombinant β-tryptase for the 6h time point, at concentrations of 10 to 80 mU/ml (n=3 to 6). Data were normalized to the house keeping genes and expressed as mean ± SEM for 2 to 3 separate experiments performed in duplicate. *P< 0.05 **p<0.01 compared with response in cells incubated with medium alone.

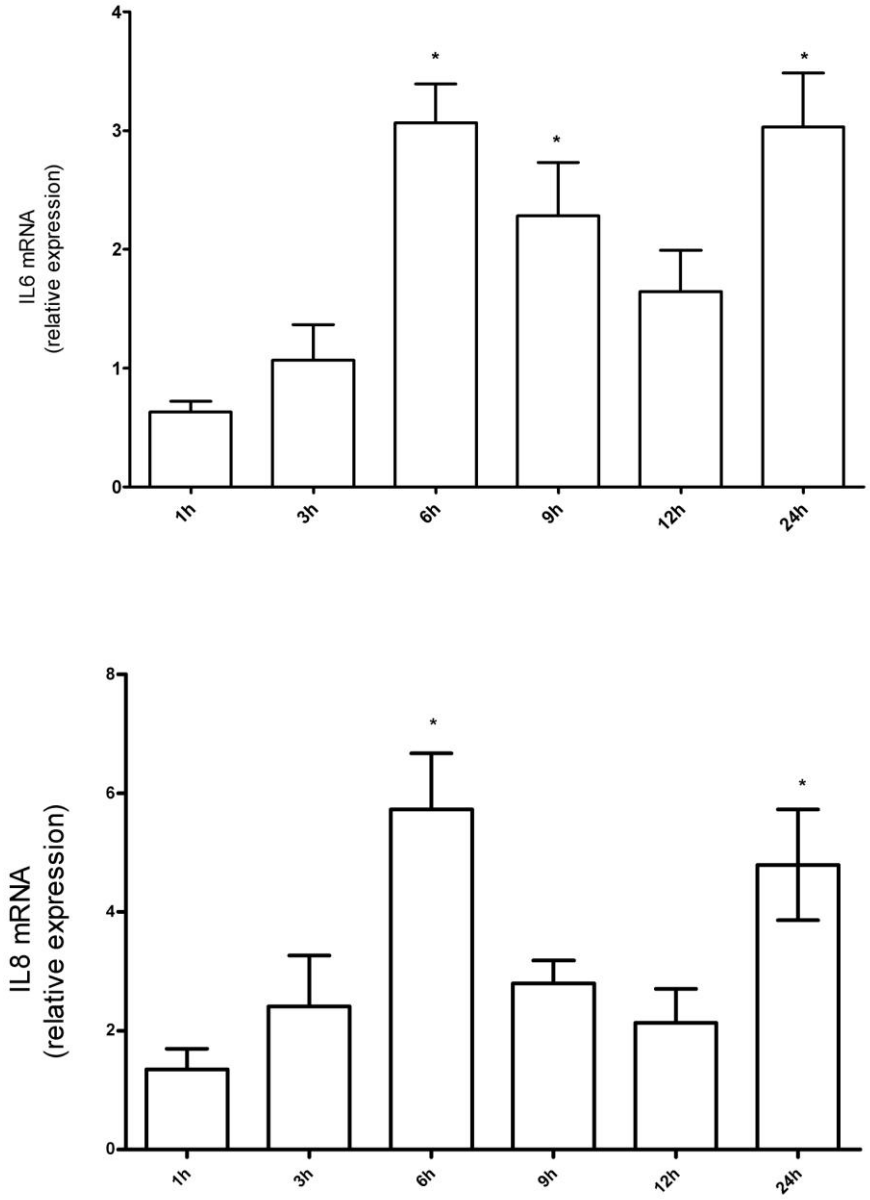


Figure 4-7 B: Treatment points form 1 to 24h with 40 mU/ml tryptase (n=3 to 6). Data were normalized to the house keeping genes and expressed as mean ± SEM for 2 to 3 separate experiments performed in duplicate. *P< 0.05 compared with response in cells incubated with medium alone.

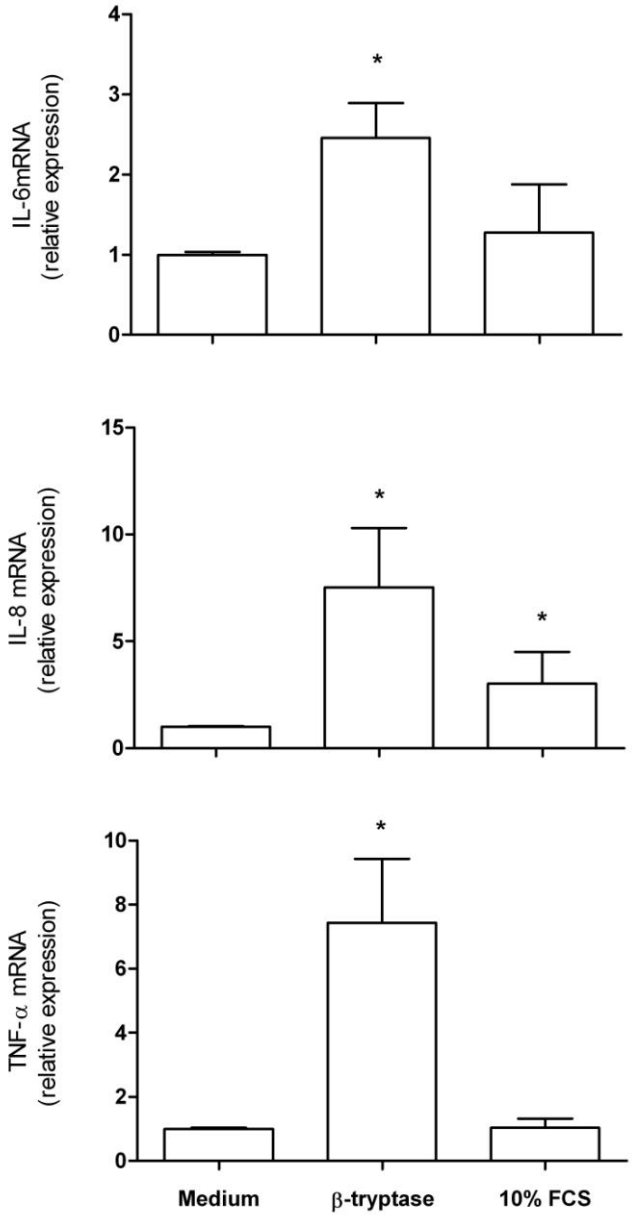


Figure 4-8. Expression of mRNA for IL-6, IL-8 and TNF-α in epithelial cells 6 h following treatment with 40mU/ml β-tryptase or with 10% FCS. The data were normalized to the house-keeping genes and expressed as mean ± SEM for 2 to 3 separate experiments performed in duplicate. *P< 0.05 compared with response in cells incubated with medium alone.

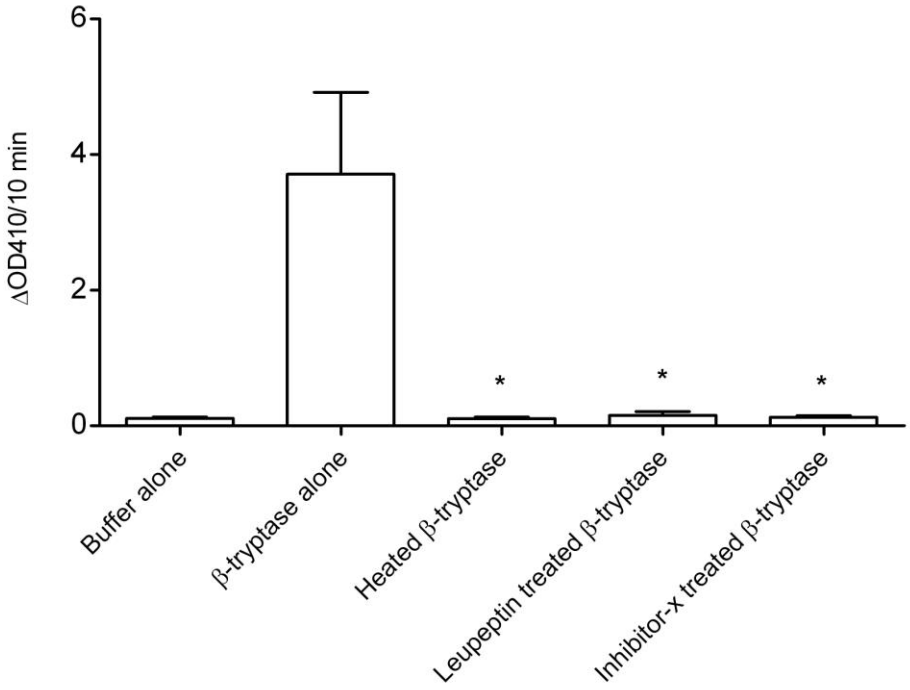


Figure 4-9. Effect of heat treatment and addition of protease inhibitors on the catalytic activity of β-tryptase. The rate of cleavage of BAPNA is shown for 40mU/ml β-tryptase and for preparations of the original concentration that had been heat inactivated (95ºC for 30 min) or incubated for 1 h with 40 ug/ml leupeptin or with 10^{-3} M inhibitor X at 4˚C. Data represent the the change in absorbance for 2-3 wells per treatment. *P<0.05 relative to activity of untreated β-tryptase.

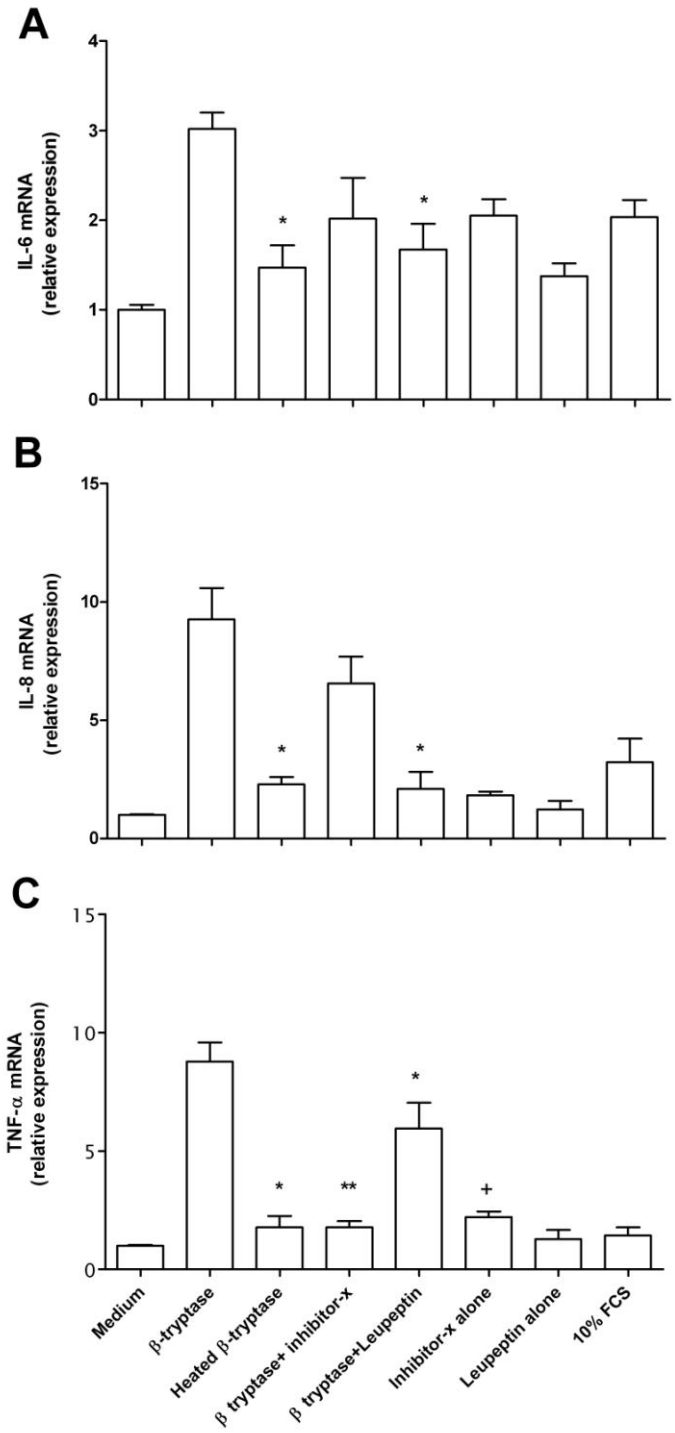


Figure 4-10. The expression of mRNA (A) IL-6, (B) IL-8, and (C) TNF-α in epithelial cells treated for 6 h with active β-tryptase (40 mU/ml) or with β-tryptase heat inactivated or inhibited with inhibitor-x (10^{-3} M) for 1 h at 4˚C or Leupeptin (0.2 ug/ml for 1 h at 4˚C). Data indicate mRNA expression levels for 3 to 4 independent experiments, * P<0.05 **P< 0.01 compared with response in cells treated with active β-tryptase, +P< 0.05 with response of untreated cells.

4.12 Effect of α-tryptase treatment on epithelial cell gene expression

To examine the possible contribution of α-tryptase in inflammatory processes, the epithelial cells were incubated with α-tryptase under conditions similar to those for the studies with β-tryptase.

4.12.1 Catalytic activity of α-tryptase

No activity for α-tryptase towards BAPNA (10 µl of 1 µg/ml) was detected and for purposes of comparison, the quantity of α-tryptase used in this study was adjusted to the same amount of β-tryptase on a weight basis.

4.12.2 Expression profile of α-tryptase

The concentration of α-tryptase added to cells for 6 h was in the range 12-24 µg/ml (10^{-6} M). On a weight basis, this was similar to that of β-tryptase at an activity of 40 mU/ml. The use of the same concentrations and time points employed for β-tryptase was important in evaluating the actions of α-tryptase in comparison with those of β-tryptase. Under these conditions, α-tryptase was unable to induce expression of any of the genes examined for β-tryptase (Figure 4-11).

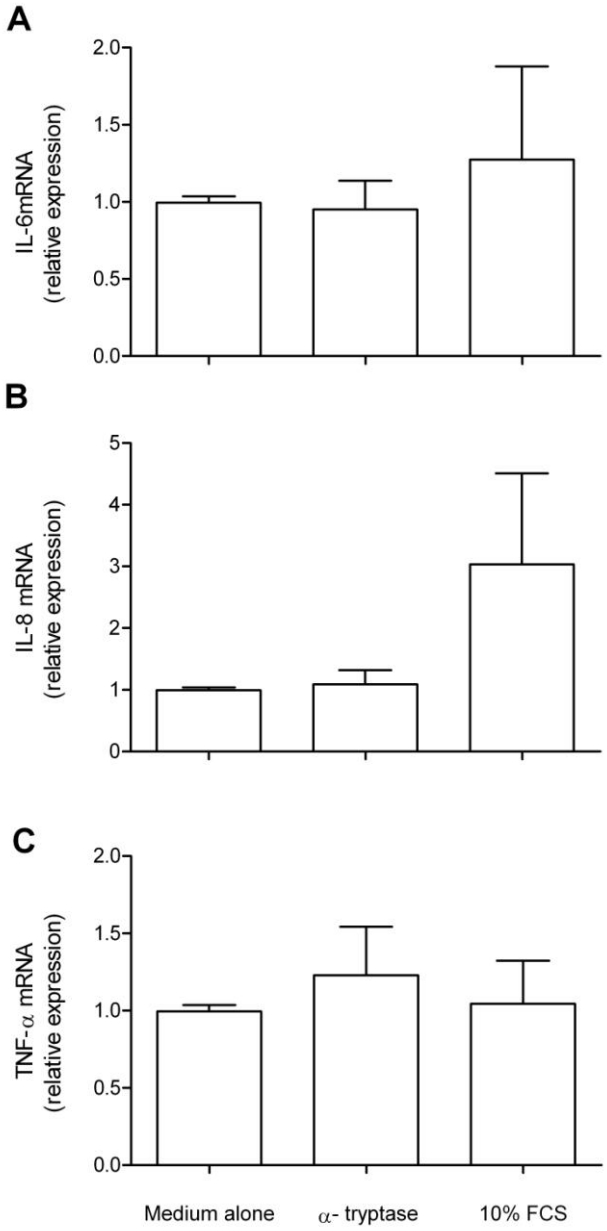


Figure 4-11. Expression of IL-6, IL-8 and TNF-α in epithelial cells following the addition of α-tryptase for 6h. The data represents the mean ± SEM of three independent experiments performed in duplicate.

4.13 Discussion

Beta tryptase was found to be a potent stimulus for the upregulation of mRNA for key inflammatory cytokines in epithelial cells. Our findings suggest that the effect was dependent on an intact catalytic site. A role for α-tryptase in upregulating expression of these cytokines in epithelial cells was not found.

Care was taken to ensure that the β-tryptase was of high purity. There was a single band for the tryptase isolated on SDS PAGE, and preparations had low levels of endotoxin contamination. Appropriate house-keeping genes were carefully selected and extraction methods validated ensuring that RNA was of good quantity and quality. Dual methods of RNA extraction and assessment were employed using NanoDrop and Bioanalyzer 2100 equipment, and the RNA integrity number was generally over 8. A high quality and integrity for RNA is recognised as being critical in ensuring good qPCR performance [195].

The selected house-keeping genes spanned different essential cell functions: GAPDH in glycolysis and gluconeogenesis, 18S for protein synthesis and UBC for protein degradation. This approach should have ensured accurate normalisation even with variation in conditions associated with the treatment, medium or RNA extraction efficiency [192]. It has been reported previously that GAPDH can be variably expressed in BAL fluid cells from asthmatic airways [193;196] and in response to serum treatment [197], but in the present studies with the defined cell line and conditions, there was no variation in gene expression for GAPDH. The geNorm application did not allow identification of a single HKG to use, so a combination of three genes represented by their geometric mean was employed. Such methods have been reported to provide an accurate estimate of mRNA transcript fraction [192].

Our finding that β-tryptase was able to upregulate expression of certain cytokines is in keeping with some previous studies with tryptase purified from human lung tissue which have indicated the potential for this protease to stimulate the generation of inflammatory cytokines. This lung tryptase has been reported to induce the generation and release of IL-8 from H292 epithelial cell line [115], IL-8 from primary culture of human umbilical vein

endothelial cells [116;198], and TGF-β1 and stem cell factor from airway smooth muscle cells [189].

Ti is not possible to establish the precise composition of human lung tryptase in terms of the isoforms of tryptase present, but it seems likely that it will be composed predominantly of β-tryptase, as this is the form stored in mast cell granules. The generation of IL-8 by β-tryptase released on mast cell activation could be an important stimulus for granulocyte recruitment. IL-8 is a potent chemoattractant for neutrophils and eosinophils [199]. IL-6 acts as a pro-inflammatory cytokine in chronic inflammation with diverse actions [200]. TNF-α is a key proinflammatory cytokine, which has been implicated in many aspects of airway pathology in asthma, and application of drugs that target this cytokine have highlighted its importance in refractory asthma [201]. This is the first report that β-tryptase may act as a stimulus for upregulation of mRNA for IL-6 and TNF-α. A more comprehensive, genome-wide assessment of changes in epithelial gene expression in response to tryptase treatment will be needed to establish in detail the biological pathways that mediate the responses to tryptase.

Heat treatment of β-tryptase was effective at inactivating the enzyme, and also in inhibiting tryptase-induced upregulation of mRNA for cytokines. Consistent with this, pre-incubation of tryptase with leupeptin or inhibitor X (both of which abolished the catalytic activity of β-tryptase towards a chromogenic substrate) also appeared to reduce upregulation of mRNA expression.

Addition of recombinant α-tryptase to epithelial cells failed to replicate the actions of β-tryptase on epithelial cells in these studies. We found that α-tryptase is without catalytic activity on a chromogenic substrate readily cleaved by β-tryptase. The absence of actions of α-tryptase on the epithelial cells is likely to reflect this lack of catalytic activity. The actions of α-tryptase on cell function have been little investigated previously. However, using a concentration of α-tryptase similar to that employed in the present study, Mirza *et al*, reported that this variant of tryptase was able to induce the proliferation of a lymphoid cell line at 24 h. In contrast Huang *et al*, even though they used a processed form of α-tryptase which had been engineered to have enzymatic activity [191], they did not show any proliferation response to treatment with α-tryptase.

In seeking to determine the potential functions of α-tryptase, it was felt better to employ a broader approach. In the next chapter are described studies involving the injection of human α- and β-tryptases into a mouse model.

Chapter 5
Actions of β- and α-Tryptases *in vivo*

5. Actions of Beta- and Alpha-Tryptases *in vivo*.

5.1 Introduction

In seminal early studies, the pro-inflammatory potential of human tryptase was established by injecting purified material into the mouse peritoneum [202]. Purified tryptase was found to be effective at inducing the accumulation of neutrophils and eosinophils even at very low doses. It may be assumed that tryptase purified from human lung will be composed predominantly of β-tryptase. Somewhat surprisingly, no effect was observed following injection of recombinant β1-tryptase in a similar mouse model by another group [191]. In the same study, there was no effect either seen when recombinant α-tryptase was injected with heparin, though there were limited data and these were not shown. In contrast, it was reported that instillation of β-tryptase into the trachea was associated with neutrophilia, in BAL fluid and lung parenchyma, an effect not observed with α-tryptase [191]. There is clearly a need to examine in more detail the actions of tryptases *in vivo*.

The actions of β-tryptase on cells and tissues have been assumed to be through activation of PAR-2 [203]. A role for α-tryptase in PAR-2 activation has also been proposed, albeit with a form of α-tryptase that has been engineered to have enzymatic activity. Both similarities and differences have been reported in the actions of tryptase and PAR-2 agonists on cells in vitro, but the lack of an antagonist has prevented direct investigation of a role of PAR-2 in mediating tryptase-induced alterations in cell function. The advent of PAR-2 knockout mice provides a way of examining this issue.

In this chapter, we have injected recombinant α- and β-tryptases into the peritoneum of mice and investigated cell accumulation, microvascular leakage, and the activation of matrix metalloprotineases. Also we have studied the requirement for an intact catalytic site and have investigated the contribution of PAR-2 in a PAR-2 knock-out mouse model.

5.2 Actions of β-and α-tryptase.

The study design is indicated diagrammatically in Figure 5-1. Essentially mice both wild-type and PAR-2 deficient C57BL6 mice were injected intraperitoneally with tryptases, killed at 6, 12 or 24 h thereafter, and peritoneal lavage fluid collected. Differential cell counts were performed and levels of albumin, total protein and MMP activity determined.

5.2.1 Leukocyte accumulation

Injection of 0.5 μg β-tryptase failed to induce a significant increase in leukocyte numbers in the peritoneum over those in the saline-injected mice (Figure 5-2). Injection of 0.5 μg α-tryptase stimulated an apparent increase in the total number of leukocytes recovered from the peritoneum at 6 h and 24 h. Injection of α-tryptase in a quantity 100 fold less provoked no significant change in the total cell number at the 24 h time point.

5.2.2 Differential cell responses

Identification of the inflammatory cells recruited following β- and α-tryptase injection indicated distinct patterns of cell accumulation for these tryptases. Injection of β-tryptase was associated with neutrophilia and not with alterations in other cell types under the conditions examined while α-tryptase-injected mice exhibited increased numbers not only of neutrophils but also of eosinophils and mast cells.

The injection of 0.5 μg β-tryptase was able to induce an increase in neutrophil counts as early as 6 h following injection, while a 21-fold increase in median numbers was observed at 24 h (Figure 5-3 A). There were no apparent alterations in numbers of other cell types in response to β-tryptase injection, apart from a slight decline in mast cell numbers at 12 h.

At 12h following injection of β-tryptase, neutrophil numbers tended to be lower than at the other time points studied. This was reflected in the numbers of some of the other cell types enumerated and may possibly be a consequence of diurnal variation rather than of a biphasic response, but this issue was not examined further.

Injection α-tryptase, as with β-tryptase, at a similar concentration, was associated with an increase in neutrophil numbers at 24 h five-fold increase in the median value at compared with those of the saline-injected mice.

Though such an effect was not observed in mice injected with β-tryptase, eosinophilia was evident in the α-tryptase-injected mice (Figure 3-5 B). Median numbers of eosinophils in lavage fluid from α-tryptase-injected mice were some two- or four-fold greater than in mice injected with saline alone at 6h or 24 h respectively. Increased numbers of macrophages (Figure 5-3 C) and mast cells (Figure 5-3 E) were also seen following injection of α-tryptase but significant differences in lymphocyte numbers, were not seen (Figure 5-3 D).

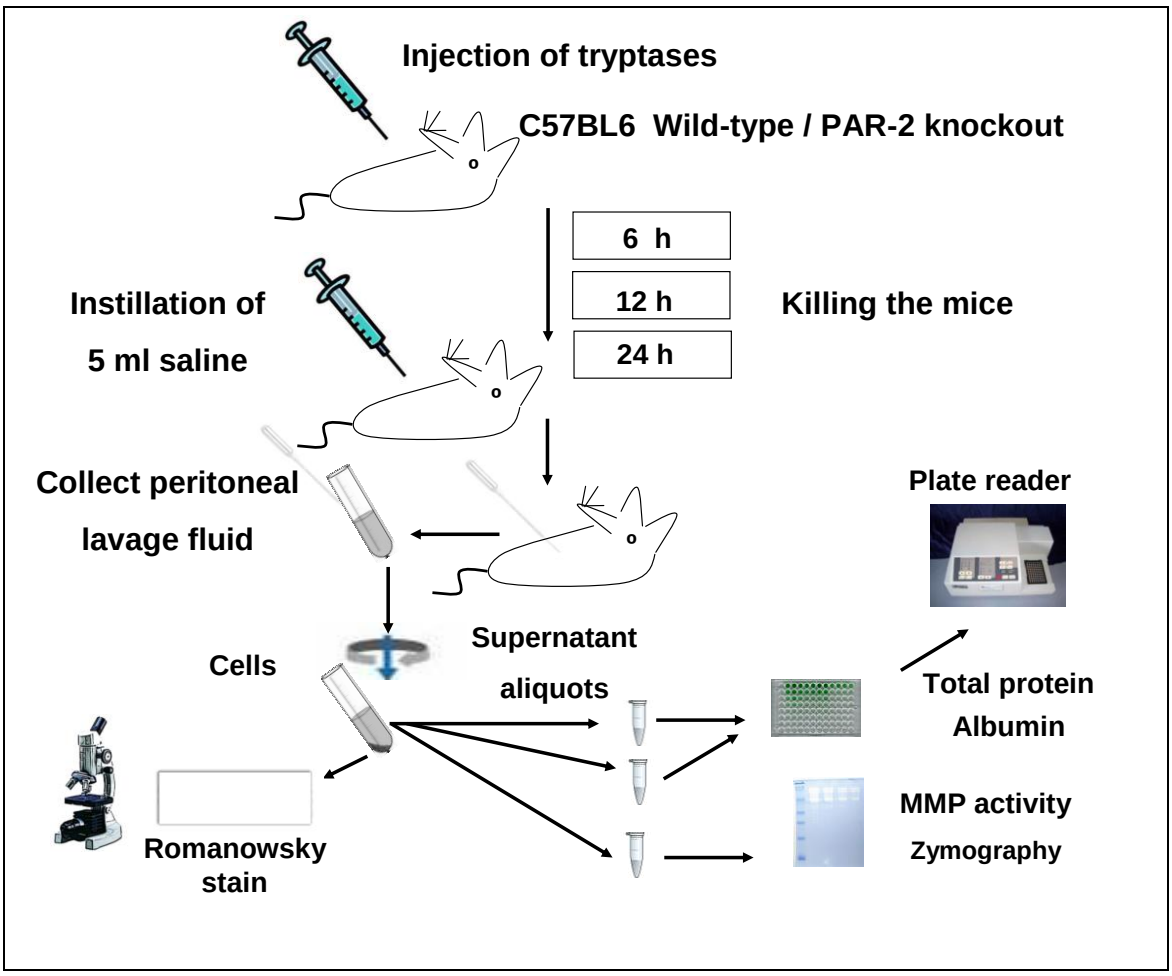


Figure 5-1. Schematic diagram of the study involving injection of tryptases into the mouse peritoneum, and analysis of peritoneal lavage fluid.

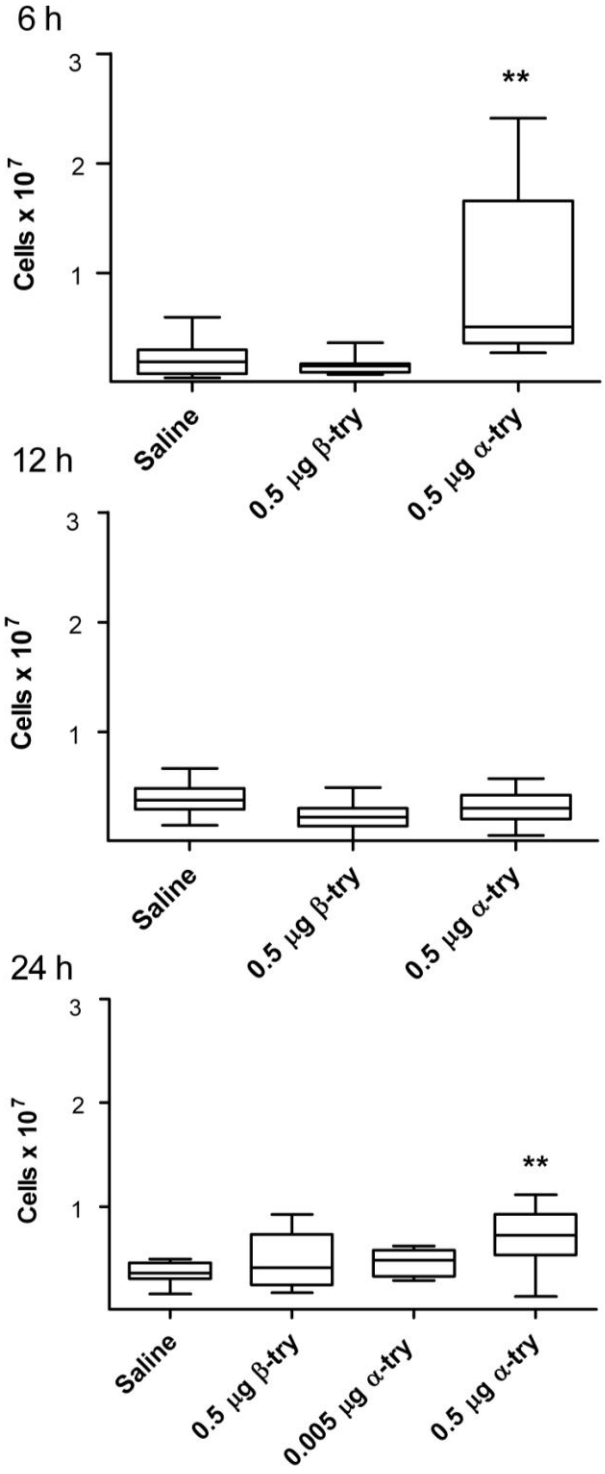


Figure 5-2. Total numbers of nucleated cells recovered in the peritoneal lavage fluid of wild-type mice 6 h, 12 h or 24 h following injection of α-tryptase (0.005 or 0.5 μg), β-tryptase (0.5 μg) or saline alone. Data are displayed as box blots with the median and interquartile range and the largest and smallest values that are not outliers (whiskers) for six to twelve mice in each group. *$p<0.05$ and **$p<0.01$ compared with response with the saline diluent alone (Mann-Whitney U test).

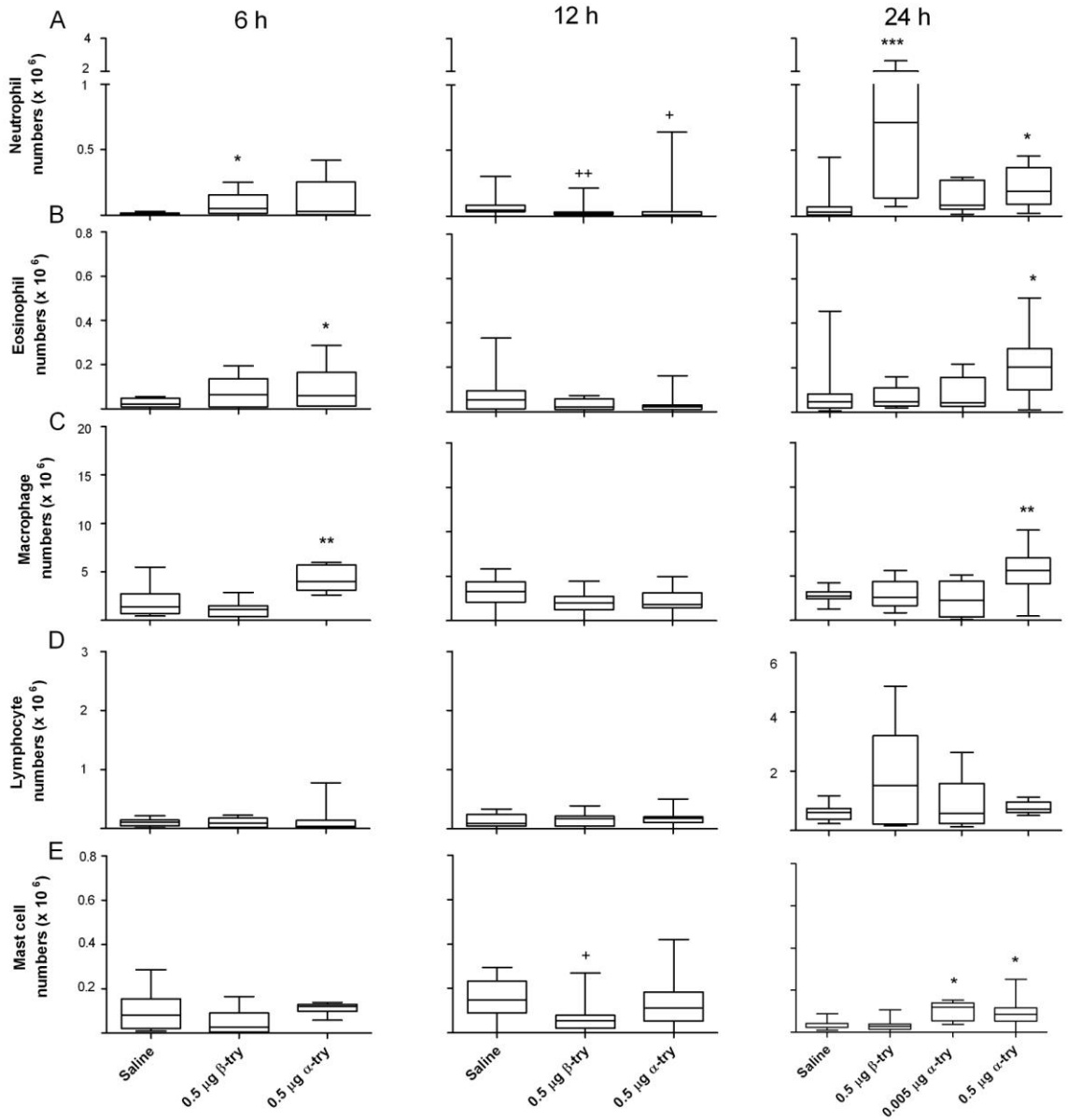


Figure 5-3. Numbers of (A) neutrophils, (B) eosinophils, (C) macrophages, (D) lymphocyte and (E) mast cells in mouse peritoneal lavage fluid following injection of β-and α-tryptases. Data are represented as outlined in the legend to Figure 5-2. *p<0.05, **p<0.01, ***p<0.001 for an increase and $^{+}$ p<0.05, $^{++}$ p<0.01 for a decrease in the response compared with that of the saline.

5.2.3 Dependency on catalytic site

The neutrophilia induced by the injection of β-tryptase was abolished by heating the enzyme (Figure 5-4). This finding would support the contention that the neutrophilia provoked was not a consequence of contamination of the tryptase with bacterial endotoxin, a potent stimulus of granulocyte accumulation which is heat resistant [204]. Moreover, pre-incubation of β-tryptase with the inhibitors leupeptin (final concentration 5μg/mouse) or inhibitor X (25 μg/mouse) for 1 h prior to injection into mice also significantly reduced neutrophilia without having any effect when injected alone. The enzymatic activity for β-tryptase towards BAPNA was abolished by these inhibitors under the conditions employed (data not shown).

The purpose of pre-incubating β-tryptase with protease inhibitors was to study their effects on tryptase-induced neutrophilia. Numbers of the other cell types were not altered by injecting β-tryptase alone in the model, but pre-incubation of β-tryptase with the inhibitors, actually appeared to stimulate an increase in numbers of some of the other cell types (Table 5-1).

Thus injection of inhibitor X either alone or in combination with tryptase provoked an increase in numbers of macrophages recovered (also reflected in total leukocytes recovered) and a decrease in numbers of lymphocytes. Injection of leupeptin with tryptase was also associated with increased numbers of leukocytes with an increase in macrophage and eosinophil numbers. Each of the inhibitors appeared to stimulate a small increase in numbers of mast cells, albeit from a low base.

As reported in Chapter 4, α-tryptase was without enzymatic activity, but so as to investigate the possibility that α-tryptase may be activated to acquire enzymatic activity after being injected into the mouse, α-tryptase (0.5 μg/ml) was incubated at 37ºC with peritoneal lavage fluid and the hydrolysis of BAPNA was monitored after 6, 12, 24, 36 h. No detectable substrate cleavage was detected by α-tryptase with or without incubation with peritoneal lavage fluid (data not shown).

Applying the same heat treatment protocol to α-tryptase that was applied to β-tryptase, it was found that heating α-tryptase abolished the increase in total

cell number observed with unheated α-tryptase, but not the neutrophilia or mast cells (Table 5-2).

5.2.4 Role of PAR-2

PAR-2 knock-out mice were injected with 0.5 μg α- or β-tryptase according to the same protocol as employed with the wild type mice. Neither α- or β-tryptase injection was associated with an alteration in the total numbers of leukocytes in the knock-out mice (Table 5-3). As in the wild-type mice, injection of β-tryptase provoked significant neutrophilia in the knock-out mice (Figure 5-5). As in the wild-type mice also, there was little alteration in numbers of other cell types in response to injection of β-tryptase (apart from small decline in mast cell numbers).

Eosinophilia, a feature of α-tryptase injection in the wild-type mice, was also observed in the PAR-2 knock-out animals, at least at one of the two concentrations examined. On the other hand, the α-tryptase-induced increase in neutrophil and macrophage numbers in the wild type mice was not replicated in the PAR-2 knock-out mice.

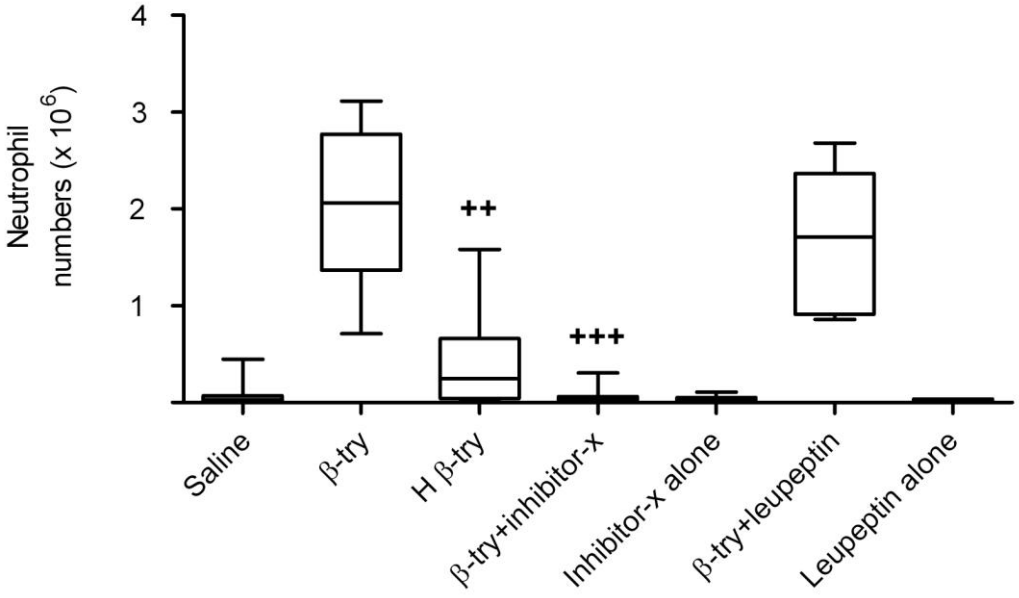


Figure 5-4. Neutrophil numbers in mouse peritoneal lavage fluid at 24 h following the injection with β-tryptase alone, heated (H) β-tryptase or β-tryptase pre-incubated with inhibitor X or with the inhibitors alone. Data are presented in the same form as in Figure 5-3. $^{++}P<0.01$, $^{+++}P<0.001$ compared with the response elicited by injection of β-tryptase alone.

Table 5-1. Total and differential cell counts in mouse peritoneal lavage fluid at 24 h following the injection of β-tryptase alone, pre incubated with X-inhibitor, leupeptin or the compounds itself as controls. Data shown are median numbers with interquartile range for eight to twelve animals. $^{+}$P<0.05, $^{++}$P0.01, and $^{+++}$P<0.001< compared to untreated β-tryptase and *P<0.05, **p<0.01 and ***p<0.001 compared with saline-injected animals.

Compound injected	Total cells (X 10^{6})	Eosinophils (X 10^{6})	Lymphocytes (X 10^{6})	Macrophages (X 10^{6})	Mast cells (X 10^{6})
Saline	3.6 (3.06-4.59)	0.04 (0.02-0.08)	0.61 (0.36-0.74)	2.74 (2.44-3.22)	0.04 (0.02-0.04)
β-tryptase	4.1 (2.54-6.58)	0.04 (0.02-0.11)	0.72 (0.59-0.96)	2.59 (1.67-4.37)	0.02 (0.01-0.03)
β-try+ inhibitor X	10.2^{+} (4.14-12.9)	0.11 (0.06-0.28)	0.24^{+++} (0.17-0.4)	9.44^{++} (3.85-12.3)	0.08 (0.0-0.14)
Inhibitor X alone	7.15* (3.75-1.09)	0.14 (0.05-0.22)	0.11*** (0.03-0.16)	4.55* (3.23-10.25)	0.17** (0.063-0.28)
β-try + leupeptin	6.88 (5.82-9.65)	0.35** (0.27-0.45)	0.55 (0.42-0.76)	4.35^{+} (3.86-6.2)	0.06^{+} (0.03-0.08)
Leupeptin alone	4.18 (3.44-5.25)	0.09 (0.03-0.12)	0.53 (0.28-0.88)	3.4 (2.75-4.43)	0.07* (0.069-0.11)

Table 5-2. Total and differential cell counts in the peritoneal lavage fluid of mice 24h following injection of α-tryptase with and without a heat treatment. Data shown are median numbers with interquartile range for eight to twelve animals. *P<0.05, **p<0.01 and ***p<0.001 compared with saline-injected animals and $^{+}$P<0.05 compared to the unheated α-tryptase.

Compound injected (μg)	Total cells (X 10^6)	Neutrophils (X 10^6)	Eosinophils (X 10^6)	Macrophages (X 10^6)	Mast cells (X 10^6)
Saline	3.6 (3.06-4.59)	0.03 (0.01-0.07)	0.04 (0.02-0.08)	2.74 (2.44-3.22)	0.04 (0.02-0.04)
0.005 μg α-tryptase	6.43 (3.27-5.74)	0.08 (0.05-0.27)	0.04 (0.02-0.15)	2.25 (0.38-4.41)	0.11* (0.05-0.13)
0.005 μg α-tryptase heated	3.63 (2.76-7.19)	0.08 (0.01-0.19)	0.06 (0.01-0.22)	2.78 (1.83-3.98)	0.09* (0.05-0.11)
0.5 μg α-tryptase	7.27** (5.33-9.24)	0.18* (0.09-0.37)	0.2* (0.1-0.28)	5.61* (4.12-7.04)	0.08* (0.05-0.11)
0.5 μg α-tryptase heated	5.37 (3.17-6.97)	0.27* (0.16-0.67)	0.21 (0.03-0.29)	2.48^{+} (0.26-5.81)	0.06 (0.3-0.11)

Table 5-3. Total leukocyte counts in the peritoneal lavage fluid of mice 6, 12 or 24 h following injection of α- or β-tryptase in PAR-2 knock-out mice.

Compound injected (μg)	6 h	12 h	24 h
Saline	4 (1.99-4.41)	5.22 (3.93-7)	4.43 (3.03-5.65)
0.005 μg α-tryptase	ND	ND	4.23 (3.04-5.16)
0.5 μg α-tryptase	ND	5.22 (3.57-6.61)	4.79 (4.09-6.25)
0.5 μg β-tryptase	3.9 (2.35-7.11)	4.55 (3.15-8.61)	3.95 (3.21-5.52)

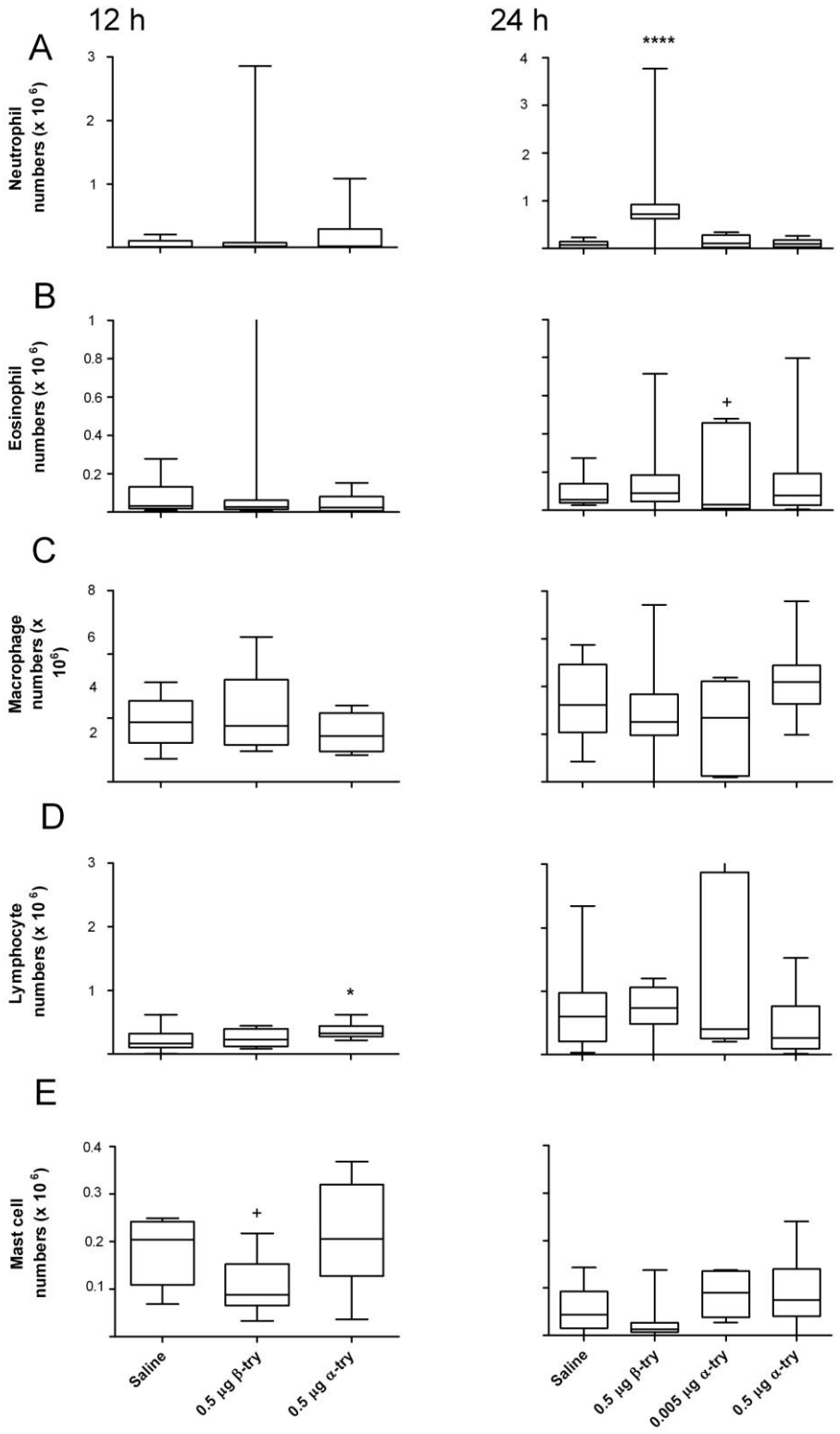


Figure 5-5. Numbers of (A) neutrophils, (B) eosinophils, (C) macrophages, (D) lymphocyte and (E) mast cells in peritoneal lavage fluid at 12 h or 24 h following injection of β-tryptase (0.5 µg), α-tryptase (0.005 and 0.5 µg), or the saline vehicle in PAR-2 knock-out mice. Data are presented in the same way as in Figure 5-2.

5.2.5 Microvascular permeability

In mice injected with β-tryptase, the levels of albumin in peritoneal fluid were greater than in saline-injected mice (Figure 5-6). Heated β-tryptase did not provoke an increase in albumin concentration. When mice were injected with inhibitor X alone or with β-tryptase and this inhibitor together, both provoked an increase comparable to that obtained with the untreated β-tryptase. Injection of α-tryptase (0.5 μg) failed to decrease the albumin levels below those in the saline-injected mice. Albumin levels in the PAR-2 knock-out mice injected with β- or α-tryptase were similar to those in the wild-type.

Protein levels in peritoneal lavage fluid from tryptase injected mice were highly variable (Figure 5-7), though Kruskal-Wallis analysis indicated that there were differences between groups of mice (P= 0.03). There was actually a decrease in protein concentration observed in peritoneal lavage fluid from mice injected with β-tryptase compared with that in the saline-injected mice, but otherwise no clear trends were seen.

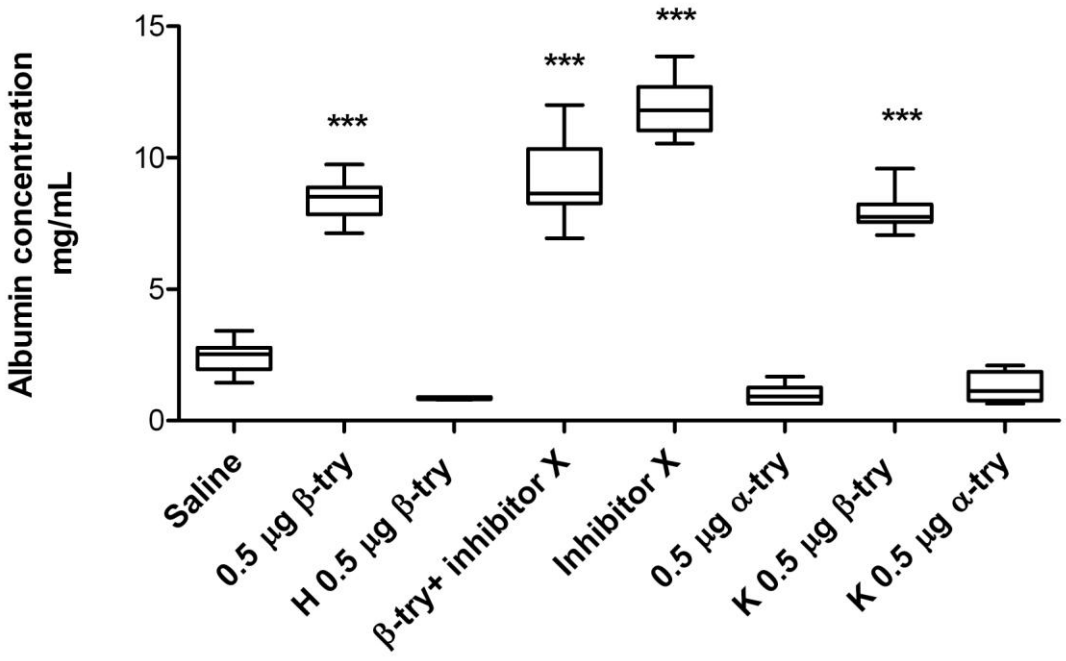


Figure 5-6. Albumin levels in the peritoneal lavage fluid of mice 24 h following injection of β- or α-tryptase. Data is presented as outlined in the legend for Figure 5-2. K knok-out mice

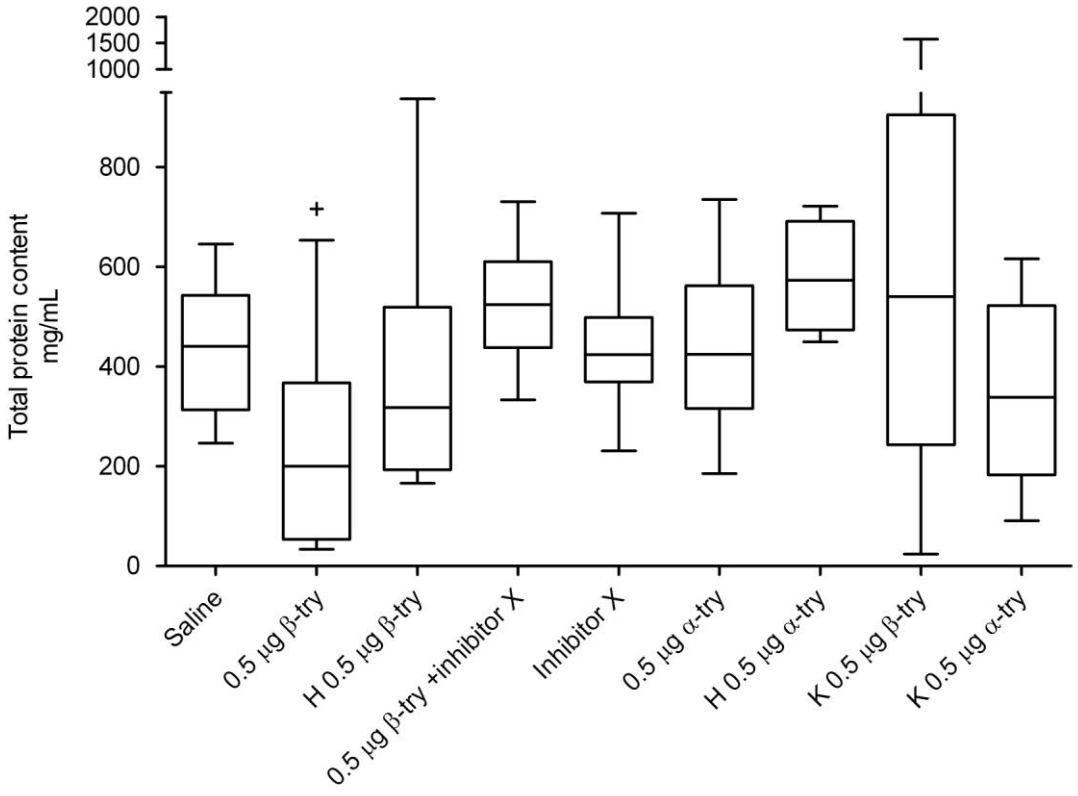


Figure 5-7. Protein concentrations in peritoneal lavage fluid injected with β-tryptase (0.5 μg), α-tryptase (0.5 μg), heated (H) forms or β-tryptase with or without inhibitor X, or the saline vehicle alone. $^{+}P<0.05$ compared to the levels in saline-injected mice. Data are presented in the same form as outlined in the legend for Figure 5-2.

5.2.6 Activities of matrix metalloproteinase (MMP)

Two bands of gelatinolytic activity were observed, one at approximately 92 KDa and the other at 72 KDa, when mouse peritoneal lavage fluid was subject to gelatine Zymography (Figure 5-8). These bands were at positions in the gel corresponding to those for pro-MMP 9 and pro-MMP 2, respectively. The presence of the pro-MMP 2 band was an invariable feature of all samples of peritoneal lavage fluid analysed, whereas proMMP-9 was largely restricted to mice injected with β-tryptase (Figure 5-9 A). A heat treatment abolished β-tryptase induced release of pro-MMP 9, as did pre-incubating β-tryptase with inhibitor X. Injection of α-tryptase failed to induce the appearance of pro-MMP 9 in peritoneal lavage fluid.

Injection of β-tryptase was associated with higher levels of pro-MMP 2 in the peritoneal lavage fluid, than those in saline-injected mice (Figure 5-9 B). Heat treatment or pre-incubation of b-tryptase with inhibitor X, in both cases reduced levels to those in the mice that had received saline alone. In PAR-2 knockout mice, injection of tryptases elicit a pattern of pro-MMP 9 and pro-MMP 2 levels (Figure 5-10) that was very similar to those in the wild-type mice.

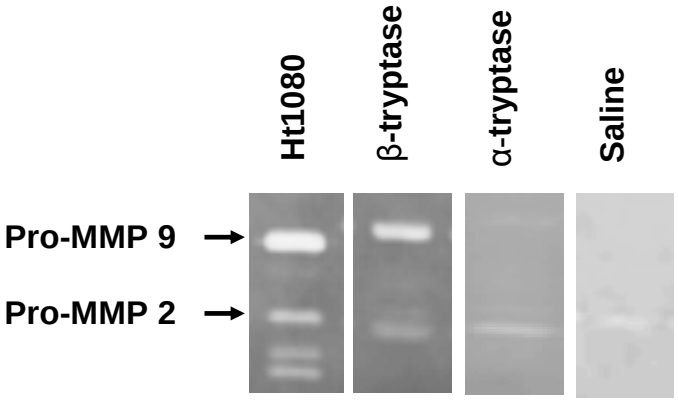


Figure 5-8. Representative gelatine zymography images for peritoneal lavage fluid from mice injected with β-tryptase, α-tryptase or with saline vehicle alone. The location of pro-MMP 9 (92 KD) and pro-MMP 2 (72 KD) are indicated. Culture supernatant of the Ht1080 fibrosarcoma cell line was included as a control.

Wild-type

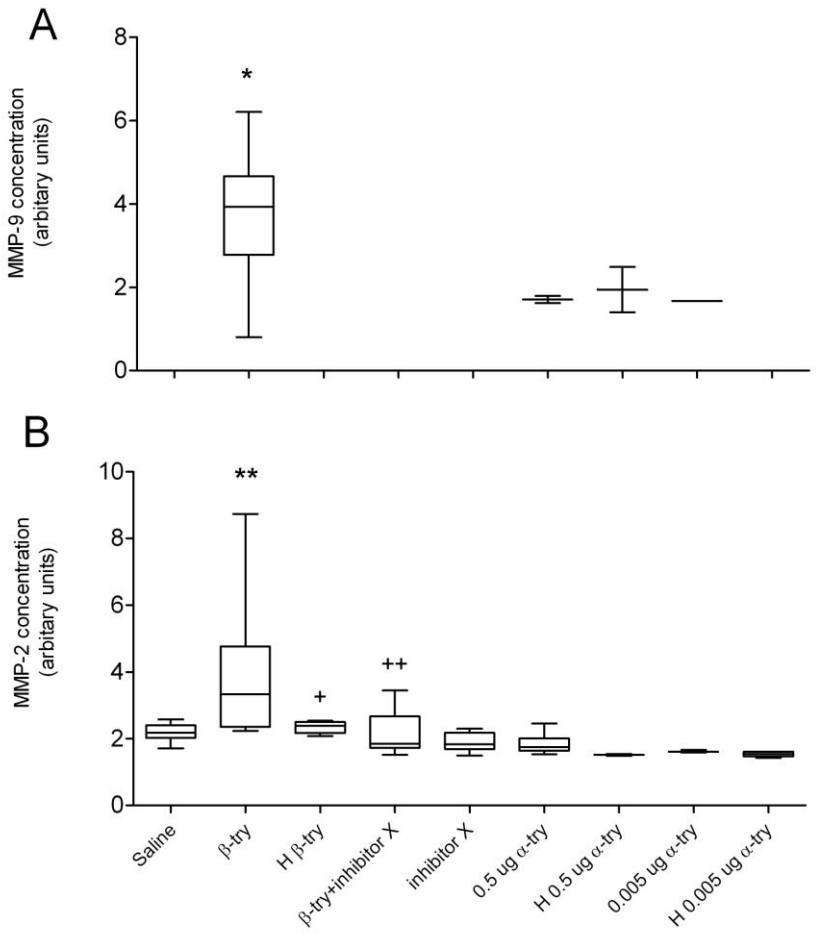


Figure 5-9. (A) pro-MMP 9 and (B) pro-MMP 2 concentrations as assessed by densitometric analysis of gelatine zymography in peritoneal lavage fluid from wild-type mice 24 h following injection with β-tryptase (0.5 ug), α-tryptase (0.5 and 0.005 ug), their heated forms, β-tryptase pretreated with inhibitor X, inhibitor X alone or the saline vehicle alone. **P< 0.01 compared to saline group and $^{+}$P< 0.05, $^{++}$P< 0.01 compared to untreated β-tryptase. Data are presented in the same way as outlined in the legend for Figure 5-2.

Knock-out type

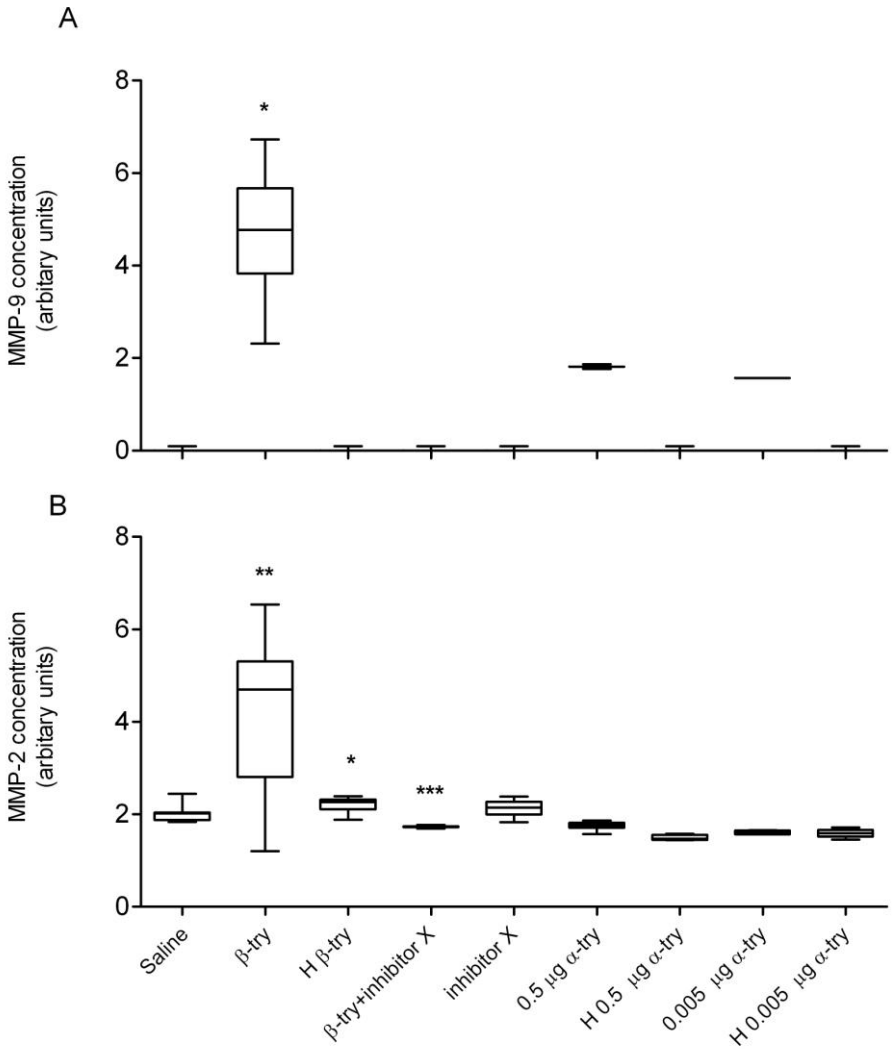


Figure 5-10. (A) pro-MMP 9 and (B) pro-MMP 2 concentrations as assessed by densitometric analysis of gelatine zymography in peritoneal lavage fluid from knock-out mice 24 h following injection with β-tryptase (0.5 ug), α-tryptase (0.5 and 0.005 ug), their heated forms, β-tryptase pretreated with inhibitor X, inhibitor X alone or the saline vehicle alone. **P< 0.01 compared to saline group and $^{+}$P< 0.05, $^{++}$P< 0.01 compared to untreated β-tryptase. Data are presented in the same way as outlined in the legend for Figure 5-2.

5.3 Discussion

Our studies indicate that β-tryptase can provide a potent stimulus for neutrophil accumulation, microvascular leakage and for the release of MMPs. Injection of this protease into the peritoneum of mice elicited neutrophilia and increase in levels of albumin and pro-MMP 2 and pro-MMP 9 in the lavage fluid. Also were able to show for the first time to our knowledge in the same model, α-tryptase can provide a potent stimulus for recruitment of neutrophils, eosinophils and mast cells, through alterations in peritoneal lavage fluid concentrations of albumin and proMMP 2 and pro-MMP 9 were not observed.

Contamination of the tryptase preparations by bacterial endotoxins, a potent stimulus of granulocyte accumulation [205], was unlikely to have been a factor in the present work. Endotoxin levels were exceedingly low for α- and β-tryptase (0.82 and 2.62 EU/ml respectively), and were less these limits indicated in guidelines for pre-clinical research for injecting mice (7.2 EU/ml for injection of 30 g mice for 24 h time point) [204].

The finding that injection of recombinant β-tryptase can stimulate a significant increase in the neutrophil counts is in accord with the findings of He et al, [202], who observed this effect following injection of β-tryptase purified from human lung into mouse peritoneum and into guinea pig skin. However, in contrast to that study neutrophilia was not accompanied by eosinophilia. A key difference between the studies that may account for this different pattern of cell accumulation could be the strains of the mice employed.

The Balb C mice employed in the previous study have been reported previously to be more prone to eosinophilia than the C57BL 6 strain employed in the present study [206]. The quantities of tryptase injected were similar in both studies, but differences in the composition of preparations of lung tryptase and recombinant β-tryptase can't be excluded at this stage. Differences between tryptases may account also for the failure of Huang et al [191] to detect a change in the cell numbers recovered from peritoneal cavities of mice following injection of recombinant β-tryptase. In that study, the tryptase used has been expressed in an insect cell expression system [207] rather than Pichia pastoris expression system as in the present study and the glycosylation patterns of the tryptases are likely to differ.

Neutrophilia induced by injection of β-tryptase was abolished by heat treatment, again arguing against endotoxin contamination as a factor in our findings. The selective tryptase inhibitor (inhibitor X) was also able to block tryptase-induced neutrophil accumulation, though leupeptin, broad spectrum proteinase inhibitor, failed to prevent neutrophilia. While the possibility can not be excluded that a region of the tryptase molecule other than the active site could have contributed in part to the neutrophilia, a more likely explanation is that the inhibitory effects of leupeptin, a reversible inhibitor, may have been partially lost through dilution following injection.

Though dependant on an intact catalytic site, the actions of tryptase can not readily be ascribed to activation of PAR-2. Neutrophilia was induced by β-tryptase in PAR-2 knock-out mice just as effectively as in the wild type mice. There is a need for further investigations of the mechanisms involved, but PAR-2 activation appears initially as a key underlying process.

Injection of α-tryptase in quantities similar to those employed in studies of b-tryptase elicited leukocyte influx in the peritoneum of mice. This was less marked than that for β-tryptase, but there was a concomitant increase in eosinophils, macrophages and mast cells. A pro-inflammatory role for α-tryptase has not been descriped previously.

Huang et al [191] alluded to a preliminary study involving injection of recombinant α-tryptase into the mouse peritoneum but few details were given and neither the dose nor number of mice used was stated. It appears that the α-tryptase employed had been engineered to be processed by enterokinase into a catalytically active form, but this failed to show any effect.

We found that heating α-tryptase was able to abolish α-tryptase its ability to induce cell accumulation, which could provide some insight into the mechanisms of action for α-tryptase, indicating that the region of the α-tryptase responsible was heat sensitive. Mirza et al [122], have reported that α-tryptase was able to induce the proliferation of BaF3 cell transfected with PAR-2, an effect that was not shown for the mock-transfected cells. In that study, the α-tryptase used also appears to been engineered to have proteolytic activity.

In out study, the α-tryptase employed was the enzymatically inactive proform, and we were unable to detect activity even when incubated for several hours with peritoneal lavage fluid. It is thus paradocially that in our studies, injection of α-tryptase into PAR-2 deficient mice failed to reproduce leukocyte accumulation to the degree seen in wild type mice. While activation of α-tryptase in vivo can not be excluded, a direct role in activating PAR-2 in the mouse model seems likely and downstream effects may have been more important in this case.

Following β-tryptase injection, there was an apparent increase in albumin levels detected in peritoneal lavage fluid. This was not reflected in levels of total protein in peritoneal lavage, but this is likely to provide a less reliable measure of microvascular permeability and be affected by the local generation of protein mediators. On the other hand, Albumin is produced predominantly by the live and it s presence in peritoneal lavage fluid will almost certainly be a consequence of microvascular leakage.

Heat treatment of β-tryptase abolished its ability to provoke increased albumin levels, but possibly on account of inhibitor related effects, only pretreatment with the protease inhibitors did not block the microvascular leakage.

Injection of α-tryptase did not lead to significant alteration in albumin levels detected in the peritoneal lavage fluid, whether heat treated or not, no evidence was found for a role for PAR-2 in microvascular leakage, and there was likely difference in findings between albumin levels in PAR-2 knock-out mice or wild-type, whether injected with β - or α-tryptase.

Our finding that b-tryptase can stimulate increased levels of pro-MMP 9 and pro-MMP 2 in peritoneal lavage fluid highlighted the potential role of tryptase in process of tissue remodeling. Previously it has been reported that tryptase purified from human tonsils can activate proMMP-9 [208] and that recombinant β-tryptase on corneal fibroblast proMMP-2 [209]. Our observation of increased levels of pro-MMP 9 and proMMP 2 suggest that β-tryptase is also able to stimulate the generation of these MMPs. Both of MMP 9 and MMP 2 are key enzymes involved in the degradation of extracellular matrix. The β-tryptase-induced release of pro-MMP 9 and pro-MMP 2 was abolished by heat treatment of β -tryptase or its pre-incubation with inhibitor X.

Consistent with other parts of the study, generation of pro-MMP 9 and pro-MMP 2 appeared to be PAR-2 independent, with similar levels PAR-2 knock-out mice as in the wild type animals following injection of β-tryptase. Injection of α-tryptase, had no effect on pro-MMP 9 and pro-MMP 2 levels.

Chapter 6
General Discussion

6. General Discussion

Our findings support the hypothesis that mast cell tryptases may play key roles in the pathogenesis of asthma and in allergic inflammation. Association were found between CNV and features of atopy and asthma, and tryptases were found to elicit a range of potent actions *in vitro* and *in vivo*. These include the ability to stimulate the upregulation of inflammatory cytokines in bronchial epithelial cells, and the recruitment of neutrophils and eosinophils, induction of microvascular leakage, and the generation of pro-MMP 9 and pro-MMP 2 in a mouse model. The α- and β- variants of tryptase were found to make distinct contributions in these processes and involve proteolytic and possibly also non-proteolytic mechanisms. By establishing a robust and well validated genotyping assay for tryptase gene copy number, we were able to analyse population variability in tryptase gene copies.

In so doing, we have been able to shed further light on the complexity of gene architecture for tryptases. The tryptase gene cluster located on chromosome 16, comprises several related genes occupying distinct loci. Alpha and β-tryptases share the same locus and have 93-95% sequence homology [143;144] and are ancestrally related, having developed from a gene duplication event with differences acquired during primate evolution [210]. Further unequal recombination events have subsequently led to the presence of α-tryptase null allele present at a frequency of about 45% in the Caucasian population [148]. Further diversity in tryptase forms is elicited through the generation of different tryptase splice variants which may lead to non-functional proteins [178;179]. However, to date, it has been unclear to what extent the variation in α-tryptase gene copies in the population alters disease susceptibility.

We have proposed a new structure for the α/β-tryptase locus, with four different genotypes detected for α-tryptase in a study of α-tryptase copy number in 1455 individuals. Pallaoro *et al.* [144], using two BAC clones, and Miller *et al.*, using a lung specimen from a single donor [142], proposed a model in which α-tryptase is allelic to β-tryptase at one tryptase locus, with an adjacent β-tryptase only locus. This model implies the presence of only three possible α-tryptase genotypes. However, that study involved few specimens and the structural and genetic heterogeneity of mast cell tryptases may have

been missed for this reason. Furthermore, due to the linkage disequilibrium between allelic variants at both loci, the data observed by Pallaoro *et al.* and Miller *et al.* are not consistent with the data presented in the present study. Instead of α- and β-tryptases being allelic at one locus, β-tryptase may also exhibit copy number variation that in most individuals is linked to equal variation at the α-tryptase locus.

The association of α-tryptase genotypes with allergy and asthma phenotypes in this study, did not show a clear gene dosage effect, with only the one copy allele (βαβα or βββα) found to be associated with lower total serum IgE level and a trend toward less asthmatic disease. This could lend support to a model of parallel variation at the α- and β-tryptase loci, as one possible explanation is that variation at the α-tryptase locus is acting as a proxy marker for variation at an adjacent locus (e.g. β-tryptase) that is the true causal variant.

Alternative explanations for the lack of a clearly observed α-tryptase gene dosage effect on IgE levels, could include lack of power due to the low frequency of the α-tryptase duplication allele, and analytical problems with study of subjects with higher copy numbers where imprecision may be more likely to affect the analysis [211].

Akin *et al* [176], found no relationship between the tryptase genotype and either total or mature tryptase serum levels in patients with mastocytosis. Differences between this study and that of Akin *et al.*, such as the larger number of subjects studied, differences in patient population (mastocytosis vs. asthma enriched) and genotyping methods (α-tryptase present/absent versus α-tryptase gene dosage) may account for the differences in these observations.

Given these results, the view of α-tryptase copy number effect is still unclear, especially with no specific assay available so far, to measure the serum α-tryptase. The development of such assay would clarify the consequences of gene copy number variations on an serum α-tryptase levels. There is a need also for the development of similar assay strategies to allow a clear assessment of β-tryptase copy number variation, which may be a determinant of α-tryptase variation.

Using purified recombinant α- and β-tryptases, new information was obtained on the potential functions of these major tryptase variants. Considerable care was taken in conducting the studies, a search made for appropriate house-keeping genes for normalisation between different samples. The ideal stable gene for all experiments is a myth, and even supposed 'stably expressed house keeping genes' exhibit variation in expression with different cell types and experimental conditions [212]. In the present studies, three out of six genes tested were selected for our experimental conditions as these were the most stably expressed at different time points and with different concentrations of stimulus.

The finding that β-tryptase can upregulate expression of mRNA for IL-6, IL-8, and TNF-α could be important in the airways in asthma. These studies should be extended to primary epithelial cells, and studies of global gene expression should provide valuable information. Our findings are however in accord with the finding that lung tryptase can stimulate the generation of IL-8 from another epithelial cell line (H292) [115]. In addition to stimulating epithelial cell proliferation, tryptase is also able to induce profound alterations in the behaviour of a number of other cell types. Thus, tryptase can stimulate fibroblast proliferation and chemotaxis [213], and increase the synthesis and secretion of type 1 collagen, and the amount of collagenolytic activity found in this cells [32]. It can also stimulate the proliferation of bronchial smooth muscle [186] and endothelial cells [214], and stimulate cytokine release from these cells [215;216].

It remain to be determined the precise cellular processes that may underlie the pro-inflammatory actions of β-tryptase *in vivo* but it seems likely that the triggering of cytokine release from various cell types will be involved. In the present studies, injection of β-tryptase in the mouse peritoneum model was associated not only with a marked neutrophil accumulation but with increased microvascular leakage and the generation of pro-MMP 9 and pro-MMP 2.

The actions of β-tryptase wether in the studies with epithelial cells in vitro or in vivo following injection of mice were invariably dependant on an intact catalytic site. The various actions were blocked by heat inactivating β-tryptase or pre-incubating it with the protease inhibitors. The potential involvement of a non-catalytic mechanism whereby β-tryptase can mediate altered function in airway smooth muscle cells has been proposed on the basis that actions have not

been inhibited completely by protease inhibitors [217]. However, compelling direct evidence is not so far available in the present studies or elsewhere.

The demonstration that tryptase can activate PAR-2 has led to the assumption that many of its actions on cellular targets are mediated through this receptor. Support for this idea has not come from our studies with PAR-2 knock-out mice. In mice deficient in PAR-2, the consequences of injecting b-tryptase were in almost all cases similar to those in the wild-type mice. The key cellular substrate for β-tryptase remains unclear.

For β-tryptase there is a going body of evidence for this protease having an important mediator role. Studies with α-tryptase have been limited and largely restricted to form engineered to have catalytic activity. While the only study indicated the potential of a-tryptase to proliferate a lymphoid cell line [122], another, no such response was seen on a fibroblast cell line [191]. Addition of α-tryptase to the epithelial cell line in the present studies was also without effect on expression of mRNA for IL-6, IL-8 or TNF-α, using concentration similar to those employed with β-tryptase. On the other hand, injection of mice with α-tryptase did induce the accumulation of neutrophils and eosinophils, though not microvascular leakage or the generation of pro-MMP 9 or pro-MMP 2. No evidence was found for α-tryptase catalytic activity following injection and the only clue to the mechanism of action as a stimulus of leukocyte accumulation was that it was found to be susceptible to heat treatment.

Alpha and beta tryptases are likely to fulfil quite different roles following release from mast cells. Our findings suggest that α-tryptase can have potent pro-inflammatory actions though further work will be required to define the mechanisms of actions in more details, and in a wider range of experimental models. Investigation of copy number variation for both α- and β-tryptases in new populations should also aid understanding of the roles of these proteses in health and disease.

The search for key cellular substrates of β-tryptase must continue, but evidence continues to accumulate of the ability to participate in numerous processes that seem fundamental to the pathogenesis of asthma and allergic disease.

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