Biochemical Pharmacology, Vol. 52, pp. 331–340, 1996.

ELSEVIER

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00211-0

Guinea Pig Lung Tryptase

LOCALISATION TO MAST CELLS AND CHARACTERISATION OF THE PARTIALLY PURIFIED ENZYME

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ABSTRACT. Tryptase (EC 3.4.21.59), the major secretory product of human mast cells, has become widely used as a biochemical marker for mast cells and mast cell activation, and is attracting attention as a mediator of allergic disease. However, there is little information available on the properties, or even the presence, of this protease in commonly used species of laboratory animals. We, here, report the demonstration and characterisation of this enzyme in the guinea pig lung. Tryptic activity resistant to α1-proteinase inhibitor and soybean trypsin inhibitor was detected in sections of guinea pig lung tissue with the histochemical substrate Z-Gly-Pro-Arg-MNA. It was localised to mast cells and appeared to be present in all mast cells staining with Alcian Blue. A tryptic protease was purified 2400-fold from whole lung tissue by high salt extraction, cetylpyridinium chloride precipitation, heparin agarose chromatography, and gel filtration. This enzyme was found to be multimeric with a subunit of 38 kDa and a native molecular mass of 860 ± 100 kDa. Inhibitor studies identified it as a serine protease. Like human tryptase, it was inhibited by leupeptin, benzamidine, and APC 366 (*N*-(1-hydroxy-2-naphthoyl)-L-arginyl-L-prolinamide hydrochloride), but not by α1-proteinase inhibitor, soybean trypsin inhibitor, or antithrombin III. Its response to changes in pH and ionic strength was similar to that of human tryptase. Differences between the guinea pig and human enzymes were seen in activity toward a panel of 10 tryptic *p*-nitroanilide peptide substrates. Kinetic constants were determined for two of these: with L-Pyr-Pro-Arg-pNA the guinea pig tryptase had a similar K_m but a 5-fold lower k_{cat} than human tryptase, and with L-Pyr-Gly-Arg-pNA the guinea pig enzyme had a 10-fold lower K_m and a 30% greater k_{cat} than its human counterpart. Heparin stabilised guinea pig tryptase, but did not alter its kinetic parameters as it did with human tryptase, decreasing the K_m towards both substrates. The presence of a protease with similarities to human tryptase in the mast cells of guinea pigs suggests that this species may be an appropriate model to investigate the actions of tryptase *in vivo*, provided cognizance is taken of the differences that do exist. BIOCHEM PHARMACOL 52;2:331–340, 1996.

KEY WORDS. tryptase; mast cell; guinea pig; APC 366; heparin; protease

Tryptase (EC 3.4.21.59), a serine protease with trypsin-like substrate specificity, is the major secretory product of human mast cells [1]. It is present in both major subsets of mast cells, the MC_T and MC_{TC} cells, and is released in response to mast cell activators along with histamine, heparin, and other mast cell proteases. Through the availability of monoclonal antibodies, it has become widely used as an immunohistochemical marker for mast cells, and as a selective marker for mast cell activation [2].

The actions of tryptase include the cleavage of regulatory neuropeptides [3, 4], the generation of kinins [5], and the inactivation of fibrinogen as a clottable substrate for thrombin [6]. A role for tryptase has been implicated in tissue remodelling because it can degrade the extracellular matrix both directly [7, 8] and indirectly through the activation of other proteases [9], and can promote the proliferation of fibroblasts [10] and epithelial cells [11]. It can induce leukocyte accumulation at sites of injection in experimental animals [12], stimulate epithelial cells to produce interleukin-8 (a chemokine that can provide a potent stimulus for granulocyte recruitment) [11], and act directly as a chemoattractant for neutrophils and eosinophils [13, 14]. These actions of tryptase parallel events seen in the development of allergic diseases such as asthma, and raise the possibility that this enzyme could be an important target for therapeutic intervention.

A more critical appraisal of the pathophysiological role of tryptase requires the availability of specific inhibitors for *in vivo* studies. The synthetic tryptase inhibitor, APC 366,† has recently been reported to have efficacy in a sheep model of allergic airways disease [15]. Administration of

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† *Abbreviations:* ACES, *N*-[2-acetamido]-2-aminoethanesulphonic acid; AMP, 2-amino-2-methylpropan-1-ol; APC 366, *N*-(1-hydroxy-2-naphthoyl)-L-arginyl-L-prolinamide hydrochloride; DFP, diisopropylfluorophosphate; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; MES,2-[*N*-morpholino]ethanesulphonic acid; -MNA,-(4-methoxy-)2-naphthylamide; Nle, norleucine; Pip, pipecolic acid; PMSF, phenylmethylsulphonylfluoride; -pNA, *p*-nitroanilide; Pyr, pyroglutamate; Z-, *N*-benzyloxycarbonyl-

Received 24 October 1995; accepted 8 March 1996.

this inhibitor reduced the early and late increases in specific lung resistance induced by allergen, as well as protecting against airway hyperresponsiveness. Moreover, treatment with APC 366 decreased vascular leakage and eosinophil infiltration into lung tissues. *In vitro* studies with purified human tryptase demonstrate that inhibition by APC 366 is reversible, but has complex kinetics with a time-dependent K_i (app) [16]. Because evaluation of the therapeutic potential of inhibitors of tryptase must, of necessity, involve experimental animals, it is important that the tryptases from these species are well characterised.

Of the various species that have been used in the development of models of asthma [17], tryptase has been characterised from only two, the dog [18, 19] and the rat [20, 21]. Guinea pigs are, arguably, the most extensively studied species employed in models of asthma and other allergic conditions [22]. Moreover, the first investigations of the potential actions of tryptase *in vivo* involved injection of human tryptase into the skin of guinea pigs [12]. Glenner and Cohen [23], in their seminal paper reporting the presence of tryptase in human mast cells, failed to detect any trypsin-like activity in the mast cells of rat, rabbit, guinea pig, or mouse. The subsequent isolation of tryptase from the tissues of rat and demonstration of mRNA for two tryptases in the mouse [24, 25] suggest that the original histochemical substrate (*N*-benzoyl-DL-arginine-2-naphthylamide) may not have been sufficiently sensitive, and that a radical reappraisal of the early studies of mast cell proteases in nonhuman species is needed. In the present study, we have applied the more sensitive and specific substrate Z-Gly-Pro-Arg-MNA and have demonstrated by histochemical means the presence of tryptase in guinea pig mast cells. We have also partially purified the major tryptic enzyme in guinea pig lung tissues and report on its characterisation. A portion of this work was previously published in an abstract [26].

MATERIALS AND METHODS

Materials

Guinea pigs (ex-breeders) were purchased from Harlan (Bicester, Oxon., U.K.), and frozen isolated guinea pig lungs from Interfauna (Huntingdon, Cambs., U.K.). Heparin agarose was obtained from Muratech (Aylesbury, Bucks., U.K.) and Sephacryl S-200 and S-300 from Pharmacia (Milton Keynes, U.K.). The chromogenic substrates methoxycarbonyl-D-Nle-Gly-Arg-pNA, tosyl-Gly-Pro-Arg-pNA and tosyl-Gly-Pro-Lys-pNA were purchased from Boehringer (Lewes, East Surrey, U.K.); L-Pyr-Gly-Arg-pNA, L-Pyr-Pro-Arg-pNA, Z-D-Arg-Gly-Arg-pNA, D-Phe-Pip-Arg-pNA, D-Val-Leu-Arg-pNA, D-Pro-Phe-Arg-pNA, and MeSuccinyl-Arg-Pro-Tyr-pNA from Quadratech (Epsom, Surrey, U.K.); and Z-Gly-Pro-Arg-MNA from Bachem (Bubendorf, Switzerland). [1,3^{3}H]-diisopropylfluorophosphate ([^{3}H]-DFP) and En^{3}Hance scintillant for autoradiography were purchased from DuPont New England Nuclear (Stevenage, Herts., U.K.). Heparin (porcine intestinal mucosa, molecular mass range of 13–15 kDa) was purchased from Calbiochem (Nottingham, U.K.), Coomassie protein assay reagent from Pierce & Warriner (Chester, U.K.), and biotinylated antibodies to mouse IgG from Dako (High Wycombe, Bucks., U.K.). Electrophoresis reagents were obtained from Bio-Rad (Hemel Hempstead, U.K.) or BDH (Poole, U.K.), and all other reagents from Sigma (Poole, U.K.) or BDH. AA1 and AA5 (monoclonal antibodies specific for human mast cell tryptase) were prepared as described previously [27], and APC 366 was provided by Arris Pharmaceutical Corp. (South San Francisco, CA, U.S.A.).

Histology

Histochemical staining for tryptase activity was performed by a modification of the method of Harvima *et al.* [28]. Briefly, cryosections of guinea pig lung tissue, fixed in 0.6% formaldehyde and 0.5% acetic acid, were incubated in 0.1 M Tris buffer (pH 7.5) containing 0.5 mg mL^{-1} Z-Gly-Pro-Arg-MNA, 0.5 mg mL^{-1} Fast Garnet GBC and 0.5 mg mL^{-1} α1-proteinase inhibitor. After rinsing with tap water, slides were examined and photographed. Selected slides were then treated with 0.1% trypsin at 37°C for 15 min followed by staining with 1% Alcian blue (pH 0.5). Slides were then aligned under the microscope according to the recorded scales and the same fields photographed.

Immunocytochemical staining of sections of guinea pig lung tissue with the monoclonal antibodies AA1 and AA5 was performed as previously described [29]. Sections of human lung tissue were employed as positive controls.

Purification of Guinea Pig Tryptase

A protocol for the isolation of human tryptase [*vide infra*] was employed, with some modifications. Lungs were either removed from freshly killed guinea pigs and frozen until use or obtained frozen from the supplier. Lungs were washed and roughly chopped prior to extraction. Lung tissue (100–200 g) was homogenised in a Waring blender 4 times with 1 L low-salt buffer (0.15 M NaCl, 1 mM EDTA, 20 mM MES, adjusted with NaOH to pH 6.1), then 3 times with 250 mL high-salt buffer (2.0 M NaCl, 1 mM EDTA, 10 mM MES, pH 6.1). The pooled high-salt extracts were mixed with 3% cetylpyridinium chloride to give a final concentration of 1.0%, and left with stirring overnight at 4°C to precipitate proteoglycans [30]. After centrifugation at 33,000 × *g* for 40 min, the supernatant was dialysed, first against distilled water, then against 0.3 M NaCl, 20 mM MES, pH 6.8. After further centrifugation (38,000 × *g* for 40 min), the supernatant was successively filtered through Whatman GF/F and 0.45 μm pore Millipore membranes, and then applied to a 6.3 × 1.5 cm column of heparin-agarose equilibrated with 0.3 M NaCl, 20 mM MES, pH 6.8 at a flow rate of 0.4 mL min^{-1} cm^{-2}. The column was washed with 10 bed-volumes of equilibration buffer followed by a 100-mL gradient from 0.30 to 1.15 M NaCl in 20 mM MES, pH 6.8. Fractions containing tryptic activity were pooled and concentrated in C-30 Centricon centrifu-

gal concentrators (Amicon) prior to being loaded on an 88 × 2.6 cm column of Sephacryl S-200 equilibrated with 2.0 M NaCl, 100 mM MES, pH 6.8. This procedure was repeated with a 66.5 × 2.6 cm column of Sephacryl S-300, and the tryptase-containing fractions were pooled and concentrated as above and dispensed into 100-μl samples before storage at −70°C.

Purification of Human Tryptases

Tryptase from human skin was isolated as a byproduct from the protocol used for the purification of chymase [31]. Briefly, after high-salt extraction of finely minced skin and cetylpyridinium chloride precipitation, the tryptase was partially resolved from chymase by gradient elution from a column of heparin agarose. Final purification was achieved by gel filtration on Sephacryl S-200. Tryptase from human lung was purified by a similar protocol, except that all buffers were at pH 6.1 and a shallower gradient was used for heparin chromatography to resolve tryptase from a major contaminant in lung extracts that is apparently absent from skin extracts.

Enzyme Assays

Tryptic activity during purification was monitored by the hydrolysis of 0.5 mM L-Pyr-Pro-Arg-pNA in 1 M glycerol, 0.1 M Tris-HCl, pH 8.0 containing 1% BSA. Assays were performed in microtitre plates (total reaction volume = 100 μL) at ambient temperature (20°C), as previously described [31]. With purified enzymes, other substrates were investigated using the same buffer, although the NaCl concentration of the enzyme samples was adjusted so that the final reaction mix contained 0.1 M NaCl. However, during the course of this investigation, it was found that some commercial preparations of BSA suppressed enzyme activity. Omission of BSA from the assay medium did not adversely affect the stability of the enzyme over the time-course of the assay (10 min), so it was omitted from all subsequent characterisation studies. K_m and k_{cat} determinations and all inhibitor studies were performed in a buffer containing 0.1 M NaCl, 1 M glycerol, 0.1 M Tris-HCl, pH 8.0 (I = 0.15). K_m(app) and k_{cat}(app) values were calculated by linear regression with the Hanes plot. Confidence limits were calculated using SPSS statistical software package. K_i(app) values were calculated from a secondary plot of K_m(app) against inhibitor concentration. Buffers used for pH profile studies were formulated to maintain a constant ionic strength [32], and contained either 50 mM acetic acid, 50 mM ACES, 100 mM Tris, or 100 mM ACES, 52 mM Tris, 52 mM AMP, adjusted to the appropriate pH with either HCl or NaOH.

Other Assays

The enzyme concentration was determined by titration of the active site with 4-methylumbelliferyl-*p*-guanidinobenzoate [33] in a Millipore CytoFluor 2350 fluorescence platereader (excitation λ = 360 nm, emission λ = 460 nm), and expressed as moles of active site. Protein was determined with Brilliant Blue G [34] with bovine serum albumin as standard.

Electrophoresis and Western Blotting

SDS-PAGE was performed on 10% gels or on precast gradient (4–20%) gels (Bio-Rad). The gels were stained with the Bio-Rad silver stain according to manufacturer's specifications. Western blotting was carried out in a wet transfer system and, after blocking with 3% gelatine, blots were probed with the appropriate antibody and developed by treatment with the corresponding secondary antibody followed, if appropriate, by a streptavidin-alkaline phosphatase conjugate. Colour was developed with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue tetrazolium.

Radiolabelling with [^{3}H]-DFP

Human and guinea pig tryptases were incubated with 40 μM [^{3}H]-DFP (6.0 Ci mmol^{-1}) for 2 hr at room temperature in 0.13 M NaCl, 20 mM Tris-HCl, pH 8.0 containing 100 μg mL^{-1} heparin. After addition of BSA as carrier protein, radiolabelled tryptase was precipitated with 25% (final concentration) trichloroacetic acid, and washed with trichloroacetic acid solutions of decreasing concentration before being redissolved in SDS sample buffer [35]. After SDS-PAGE, gels were stained with Brilliant Blue R, then incubated in En^{3}Hance following the manufacturer's protocol prior to exposure to X-ray film at −70°C.

RESULTS

Histology

When cryostat sections of guinea pig lung tissue were incubated with the chromogenic substrate Z-Gly-Pro-Arg-MNA and co-reagent Fast Garnet GBC, a red azo dye product was deposited in a population of cells (Fig. 1A). This tryptic activity was inhibited by leupeptin but not by α1-proteinase inhibitor or soybean trypsin inhibitor. The staining was granular and the cells had the morphology and distribution typical of mast cells identified in separate sections by Alcian Blue staining. Subsequent staining of the same sections with Alcian Blue leached away the red azo dye product to give a similar pattern of light blue staining (Fig. 1B). Examination of 200 fields of view under the 40× objective (0.19 mm^2/field) revealed 130 Alcian Blue stained cells, all of which stained positively for tryprase. In the same area, a total of 255 cells stained positively for tryptase. Monoclonal antibodies specific for human tryptase did not bind to mast cells or to any other cell type in these guinea pig tissues, but did bind to human mast cells in control sections.

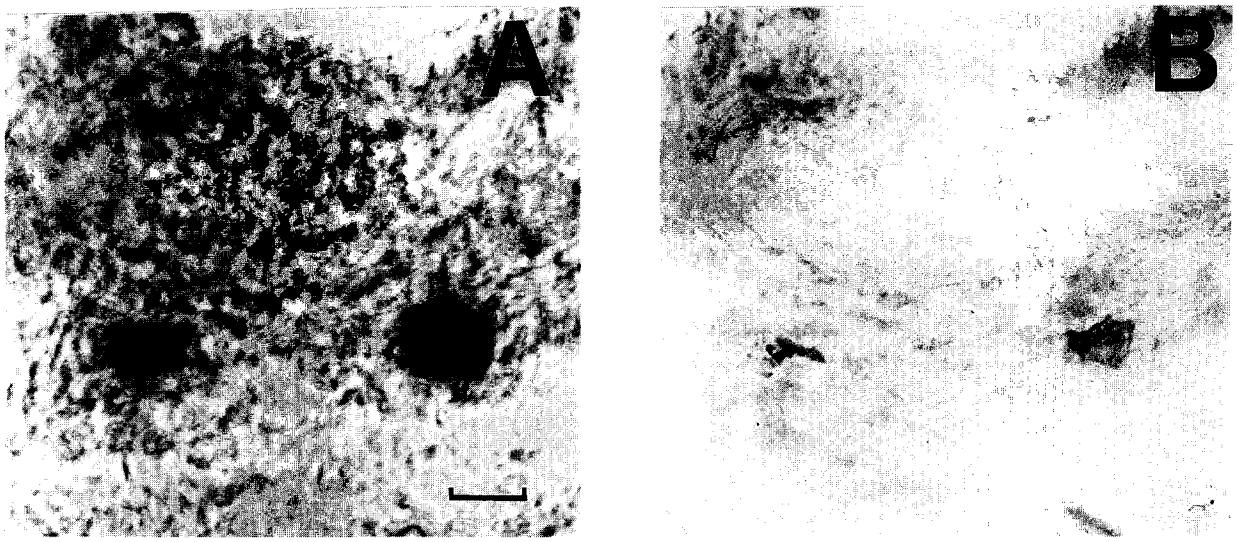

FIG. 1. Histochemical detection of tryptase in guinea pig lung mast cells. (A) Staining of a cryostat section of guinea pig lung tissue with Z-Gly-Pro-Arg-MNA and Fast Garnet GBC in the presence of 0.5 mg mL^{-1} α1-proteinase inhibitor. (B) Same section subsequently stained with Alcian Blue (pH 0.5). (The azo dye complex was bleached by this latter process). The bar in (A) represents 20 μm.

Purification

Tryptic activity was detected in high-salt extracts of guinea pig lung tissue with the chromogenic substrate L-Pyr-Pro-Arg-pNA. As is the case with human tryptase, the guinea pig enzyme bound to heparin agarose after removal of endogenous proteoglycans from the extract by precipitation with cetylpyridinium chloride. However, the tryptic activity was eluted at a lower salt concentration: approximately 0.7 M NaCl compared to 0.9 M for human tryptase, with the same matrix and a similar gradient. A greater fraction of the proteins in the guinea pig extract were found to bind to heparin agarose than in human extracts; therefore, to avoid overloading the column, the extract was chromatographed in 4 separate portions. Fractions containing tryptic activity were then pooled (= Heparin 1 in Table 1), and applied to the heparin column a second time, which served as both a concentrating step and a further purification step (= Heparin 2 in Table 1). After application to a column of Sephacryl S-200, the guinea pig enzyme eluted very close to the void volume, so gel permeation chromatography was repeated using a Sephacryl S-300 column (Table 1).

The tryptic activity eluted from Sephacryl S-300 before the largest marker protein available (thyroglobulin, 660 kDa) (Fig. 2). By extrapolation, this is equivalent to a molecular mass of 860 ± 100 kDa. To see whether or not this high molecular weight was a consequence of the high salt concentration incorporated into the buffer to stabilise the enzyme [36], a sample was rechromatographed on the same column equilibrated with phosphate buffered saline (I = 0.15 M). Most of the activity (>80%) eluted at the same V_e, with a minor peak eluting at the void volume.

SDS-PAGE, followed by silver staining, indicated that the final preparation was not homogeneous despite having been purified 2400-fold. However, reaction with radiolabelled DFP followed by SDS-PAGE and fluorography yielded a single band of 38 kDa (Fig. 3). This band did not react with the monoclonal antibody AA5 against human tryptase in Western blots. As a positive control, human skin tryptase treated with [^{3}H]-DFP under the same conditions yielded a doublet of 33 and 36 kDa on SDS-PAGE.

Action of Inhibitors

The tryptic protease isolated from guinea pig lung was inactivated by DFP and PMSF, which are specific inhibitors

TABLE 1. Summary of the purification of tryptase from guinea pig lung

Step	Vol (mL)	Tryptase activity (mU/mL)	Tryptase activity (mU Total)	Recovery (%)	Protein (mg/mL)	Sp. activity (mU/mg)	Purification
High-salt extract	745	6.9	5100	100	12.4	0.56	1
CPC pptn	980	3.4	3300	65	4.1	0.83	1.5
Heparin 1	1004	3.3	3300	65	0.48	6.9	12
Heparin 2	65	34.7	2300	45	1.85	18.8	33
S-200	22	67.8	1500	29	0.15	452	807
S-300	17	49.5	840	17	0.036	1360	2440

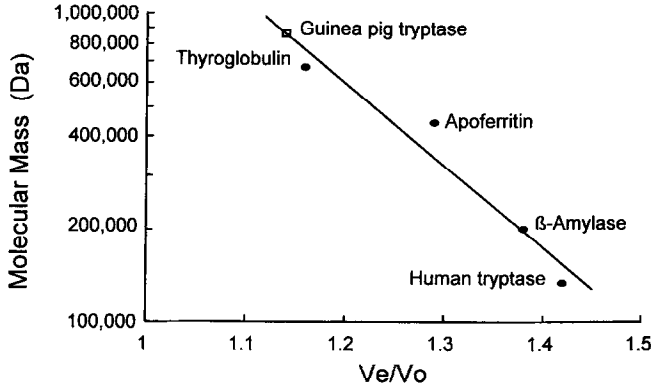

FIG. 2. Molecular mass determination of guinea pig lung tryptase by chromatography on Sephacryl S-300.

of serine proteases, but not by E-64 (an inhibitor of cysteine proteases), pepstatin A (an inhibitor of aspartic proteases), or 1,10-phenanthroline (an inhibitor of metalloproteases) (Table 2). The inactivation of trypsin by 1,10-phenanthroline can be ascribed to its dependence on calcium for stability and activity; neither the guinea pig nor the human tryptases showed such a dependence. The rate of reaction with PMSF was relatively slow—little or no inactivation of the human tryptases was seen when incubations were carried out at 0°C (Table 2), hence, the need to repeat the incubations with the protease class inhibitors at 20°C. The guinea pig enzyme was considerably more reactive with PMSF than the human tryptases and even more reactive than trypsin.

Incubation with a range of protease inhibitors showed

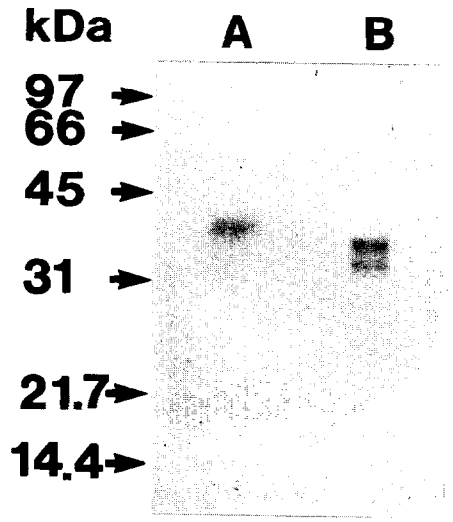

FIG. 3. Determination of subunit size by SDS-PAGE of [^{3}H]-DFP-labelled proteases followed by fluorography. Purified guinea pig lung and human lung tryptases were reacted with [^{3}H]-DFP and analysed by SDS-PAGE on a 10% gel. After staining with Brilliant Blue R to reveal MW marker proteins, the gel was treated with En^{3}Hance scintillant and exposed to X-ray film. Lane (A) guinea pig lung tryptase. Lane (B) human lung tryptase.

broad similarities between the guinea pig enzyme and human tryptases. None of the proteinaceous inhibitors (α1-proteinase inhibitor, soybean trypsin inhibitor, antithrombin III) had any inhibitory effect on these proteases, although they were quite potent against trypsin. However, a number of low-molecular-mass tryptic inhibitors (benzamidine, leupeptin, antipain, and APC 366) were effective against guinea pig and human tryptases. As the values reported in Table 2 are relative to samples incubated simultaneously under identical conditions but without any inhibitor, and all 4 proteases lost some activity under such conditions, the apparent stimulation of enzyme activity observed in some cases could be due to stabilisation during incubation, rather than activation of the enzyme.

Inhibition by leupeptin, benzamidine, and APC 366 was examined further. When incubation times were varied between 2 and 30 min, the inhibitory effects of leupeptin and benzamidine were relatively constant, but the potency of APC 366 increased dramatically with time, in agreement with the studies of Moore *et al.* [16]. K_i(app) values were determined under conditions involving no prior incubation (<2 min) of enzyme with inhibitor (Table 3). Within the time-course of the assay (10 min), all behaved as competitive inhibitors. The guinea pig lung tryptic enzyme was more resistant to inhibition by leupeptin [5-fold greater K_i(app)] than were the human tryptases, but had comparable sensitivity to APC 366 and benzamidine [differences in K_i(app) values were 2-fold or less].

Effects of Ionic Strength and pH

As has been reported for human tryptase, the guinea pig enzyme was found to lose activity at physiological ionic strength (*vide infra*), but was stable for several hours at 4°C in the presence of 1 or 2 M NaCl. However, the inclusion of 1 M NaCl in the assay medium inhibited the activity of the guinea pig enzyme by 34 ± 2%, of human lung tryptase by 55 ± 1%, and of human skin tryptase by 54 ± 2% (mean ± SEM of triplicate assays).

The pH profile was determined under conditions of constant ionic strength (Fig. 4). At pH 5.5, which is the pH calculated for the mast cell granule [37], the activity was very low. Activity was 15-fold higher at pH 7.5, which approximates the pH of the extracellular space, and optimal at pH 9.0. Prior incubation in the same buffers for 1 hr at 25°C in the presence of heparin indicated that the enzyme is stable at acidic and neutral pH, but becomes progressively less stable above pH 8.0.

Substrate Specificity

The action of the guinea pig lung tryptic enzyme was tested against a range of commercially available substrates and compared to the results obtained with human lung tryptase (Table 4). Enzyme concentration was determined with 4-methylumbelliferyl-*p*-guanidinobenzoate, and results expressed as katals per mole of active site. Both proteases preferred Arg to Lys in the P1 position, and substrates with

TABLE 2. Per cent inhibition of guinea pig tryptase and related proteases by selected compounds*

Inhibitor	Inhibition of enzyme activity (%)			
	Guinea pig lung tryptase	Human lung tryptase	Human skin tryptase	Bovine pancreatic trypsin
Incubation at 20°C				
DFP (1 mM)	98	99	99	99
PMSF (1 mM)	94	54	53	84
E-64 (5 μM)	3	4	10	-89
Pepstatin A (1 μM)	1	9	9	-44
1,10-Phenanthroline (1 mM)	2	4	5	94
Incubation at 0°C				
PMSF (1 mM)	44	2	14	29
SBTI (50 μg mL^{-1})	0	-14	-59	100
α1-PI (50 μg mL^{-1})	0	-35	-52	95
ATIII (4 U mL^{-1}) + heparin	0	-15	0	99
Chymostatin (500 μM)	23	16	16	-96
Antipain (500 μM)	90	94	93	96
Leupeptin (500 μM)	97	97	98	98
Benzamidine (500 μM)	27	50	51	-17
APC 366 (100 μM)	64	58	70	90

* Samples were incubated with the compound and concentration indicated for 1 hr in 0.1 M NaCl, 0.1 M Tris-HCl, pH 8.0 (I = 0.15 M), containing 1 μg mL^{-1} heparin (20°C) or 1.0 M glycerol (0°C). Samples (20 μL in a final volume of 100 μL) were assayed with 0.5 mM L-Pyr-Pro-Arg-pNA in 1.0 M glycerol, 0.1 M NaCl, 0.1 M Tris-HCl, pH 8.0 (I = 0.15 M). Values are means of determinations performed in triplicate and are expressed relative to those of samples incubated simultaneously under identical conditions, but without any additive (except for antithrombin III + heparin, which was expressed relative to a control sample containing heparin). A negative value indicates that the activity was greater than the control value.

either Pro or Gly at P2 were preferred over those with Leu or Phe. However, whereas human tryptase showed a strong preference for Pro over Gly in the P2 position, the guinea pig lung tryptic enzyme showed no clear preference. This is best illustrated with the two substrates L-Pyr-Pro-Arg-pNA and L-Pyr-Gly-Arg-pNA, which differ solely at the P2 position: the guinea pig protease favoured L-Pyr-Gly-Arg-pNA by a factor of 1.3, whereas human tryptase favoured L-Pyr-Pro-Arg-pNA by a factor of 9. Another major difference was the apparent inability of the guinea pig enzyme to cleave benzoyl-DL-Arg-pNA, a substrate that has been frequently employed in studies of human tryptase.

The kinetics of hydrolysis of L-Pyr-Pro-Arg-pNA and L-Pyr-Gly-Arg-pNA were investigated further (Table 5). With L-Pyr-Pro-Arg-pNA, and in the absence of heparin, the guinea pig enzyme had a lower K_m than either human tryptase, but also a lower k_{cat}, so that the ratio k_{cat}/K_m was approximately 4-fold lower for the guinea pig enzyme. Replacement of Pro with Gly resulted in a moderate increase (3.4-fold) in the K_m value for the guinea pig protease, but a massive increase (approximately 25-fold) for the two human enzymes. This was accompanied by an increase in the k_{cat} of the guinea pig enzyme and a decrease in the k_{cat} of both human enzymes, so that the k_{cat}/K_m ratio for L-Pyr-

TABLE 3. Inhibition constants [K_i(app) values] for guinea pig and human tryptases*

Inhibitor	Guinea pig lung tryptase (μM)	Human lung tryptase (μM)	Human skin tryptase (μM)
Leupeptin	2.7 ± 0.3	0.52 ± 0.02	0.54 ± 0.08
Benzamidine	82 ± 10	37 ± 1	41 ± 21
APC 366	116 ± 14	175 ± 43	273 ± 76

* Enzyme activity was determined by cleavage of L-Pyr-Pro-Arg-pNA in the presence of inhibitor without prior incubation (<2 min) in 1.0 M glycerol, 0.1 M NaCl, 0.1 M Tris-HCl, pH 8.0 (I = 0.15 M). Values are expressed ± SE.

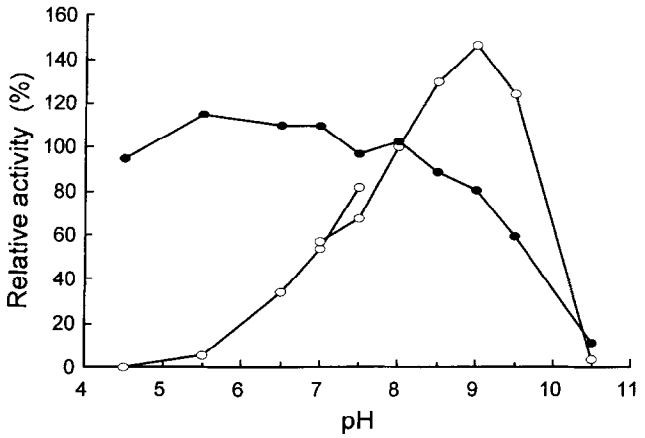

FIG. 4. pH activity and stability profiles of guinea pig lung tryptase. (○) Activity assayed with 0.5 mM L-Pyr-Pro-Arg-pNA in the presence of 10 μg mL^{-1} heparin, 25 mM NaCl, in buffers formulated to maintain constant ionic strength, either 50 mM acetic acid, 50 mM ACES, 100 mM Tris (pH 4.5–7.5), or 100 mM ACES, 52 mM Tris, 52 mM AMP (pH 7.0–10.5). (●) Enzyme incubated for 1 hr at 25°C in the presence of 100 μg mL^{-1} heparin, 250 mM NaCl, in either acetic acid-ACES-Tris (pH 4.5–7.0), or ACES-Tris-AMP (pH 7.5–10.5) and, subsequently, assayed with 0.5 mM L-Pyr-Pro-Arg-pNA in 0.2 M Tris-HCl, pH 8.0. All activities are expressed relative to that obtained in ACES-Tris-AMP, pH 8.0, without prior incubation.

TABLE 4. Substrate profile of guinea pig and human lung tryptases*

Substrate	Molar catalytic activity (katal/mol active site)			
	Guinea pig lung tryptase		Human lung tryptase	
L-Pyr-Gly-Arg-pNA	28.4	(±2.3)	5.23	(±0.06)
L-Pyr-Pro-Arg-pNA	22.0	(±0.7)	43.8	(±1.3)
Z-D-Arg-Gly-Arg-pNA	19.4	(±0.7)	3.40	(±0.17)
Methoxycarbonyl-D-Nle-Gly-Arg-pNA	18.7	(±0.4)	9.85	(±0.12)
Tosyl-Gly-Pro-Arg-pNA	17.7	(±0.4)	32.8	(±0.7)
D-Phe-Pip-Arg-pNA	17.5	(±0.4)	15.9	(±0.6)
Tosyl-Gly-Pro-Lys-pNA	8.97	(±0.08)	20.3	(±0.2)
D-Val-Leu-Arg-pNA	3.33	(±0.16)	2.90	(±0.03)
D-Pro-Phe-Arg-pNA	1.90	(±0.02)	1.30	(±0.05)
Benzoyl-DL-Arg-pNA	<0.01	–	1.35	(±0.07)
Succinyl-Ala-Ala-Pro-Phe-pNA	<0.01	–	<0.002	–
MeSuccinyl-Arg-Pro-Tyr-pNA	<0.01	–	<0.002	–
Succinyl-Ala-Ala-Ala-pNA	<0.01	–	<0.002	–

* Assays were performed at substrate concentrations of 0.5 mM in 1 mg mL^{-1} BSA, 1.0 M glycerol, 0.1 M NaCl, 0.1 M Tris-HCl, pH 8.0 (I = 0.15 M). The concentration of active sites was determined by titration with 4-methylumbelliferyl-p-guanidinobenzoate. Values are means (±SEM) of determinations performed in triplicate.

Gly-Arg-pNA was 14-fold greater for the guinea pig lung tryptic enzyme. The addition of heparin did not significantly affect the k_{cat} of any of the proteases with either substrate (i.e. the calculated value of k_{cat} in the presence of heparin was within the 95% confidence limits of the mean for the value of k_{cat} in its absence), but did decrease the K_m values. This decrease was not significant for the guinea pig enzyme with either substrate, but was for both human enzymes with L-Pyr-Pro-Arg-pNA and for human lung tryptase with L-Pyr-Gly-Arg-pNA (i.e. there was no overlap between the respective 95% confidence intervals). Human skin tryptase had similar K_m values to human lung tryptase with L-Pyr-Gly-Arg-pNA as substrate, in the presence and absence of heparin but, on this occasion, failed to reach significance.

Heparin did, however, stabilise the guinea pig lung tryptic enzyme. Under the conditions of the assay, the progress curve of product formation was linear for both guinea pig and human tryptases over the period of the assay (10 min). If the reaction was allowed to proceed for 16 min, deviation from linearity became apparent in the reaction wells without heparin. However, the progress curves for both human and guinea pig enzymes remained linear in wells containing heparin, until substrate depletion became significant. Moreover, when the guinea pig enzyme was incubated for 1 hr in ice under conditions of low ionic strength (0.1 M NaCl, 0.1 M Tris-HCl, pH 8.0, containing 1.0 M glycerol), it lost 25% of its activity relative to a control sample incubated simultaneously with 100 μg mL^{-1} heparin.

DISCUSSION

Using the histochemical substrate Z-Gly-Pro-Arg-MNA, we have demonstrated, for the first time, that guinea pig mast cells contain tryptase. This enzyme was inhibited by leupeptin but not by soybean trypsin inhibitor or α1-proteinase inhibitor, properties it shares with tryptases from human [present study, 38], rat [20, 21], canine [18], and bovine [39] sources. The failure of previous histochemical studies with benzoyl-DL-Arg-naphthylamide [23] or D-Val-Leu-Arg-MNA [40] to detect tryptase in guinea pig tissue could be explained by the substrate preference of this enzyme. With the p-nitroanilide analogs of these histochemical substrates, the tryptic enzyme isolated from guinea pig lungs showed more than 5 times as much reactivity towards

TABLE 5. Kinetic constants for guinea pig and human tryptases*

Source of tryptase	Heparin (μg mL^{-1})	L-Pyr-Pro-Arg-pNA			L-Pyr-Gly-Arg-pNA		
		K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} M^{-1})	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} M^{-1})
Guinea pig lung	none	0.26 ± 0.06	20.9 ± 1.6	80,000 ± 9000	0.79 ± 0.15	103 ± 9	130,000 ± 13,000
	100	0.24 ± 0.06	22.3 ± 1.9	93,000 ± 11,000	0.77 ± 0.11	106 ± 7	138,000 ± 11,000
Human lung	none	0.43 ± 0.10	135 ± 15	313,000 ± 38,000	9.3 ± 2.0	83 ± 11	8900 ± 1000
	100	0.29 ± 0.03	130 ± 6	447,000 ± 27,000	6.3 ± 0.9	71 ± 6	11,300 ± 800
Human skin	none	0.38 ± 0.08	104 ± 11	277,000 ± 33,000	8.4 ± 3.4	72 ± 18	8600 ± 1600
	100	0.24 ± 0.03	95 ± 5	402,000 ± 29,000	6.7 ± 4.1	65 ± 24	9700 ± 2100

* Assays were performed in 1.0 M glycerol, 0.1 M NaCl, 0.1 M Tris-HCl, pH 8.0 (I = 0.15 M). The concentration of active sites was determined by titration with 4-methylumbelliferyl-p-guanidinobenzoate. Calculated values for kinetic constants are expressed ±95% confidence limits of the mean.

tosyl-Gly-Pro-Arg-pNA than towards D-Val-Leu-Arg-pNA, and no reactivity towards benzoyl-DL-Arg-pNA.

All guinea pig lung mast cells identified by staining with Alcian Blue contained tryptase. This is similar to what is seen in human tissues, where virtually all mast cells contain tryptase [1] and contrasts with the situation in rat [41] and mouse [24, 25] where tryptases are confined to a specific subset of mast cells. Whether or not this pattern is true for other guinea pig tissues remains to be seen. However, not all tryptase-containing cells were subsequently stained with Alcian Blue, suggesting either that tryptase may be present in other cell types, or that Alcian Blue is not a sufficiently sensitive method for detecting all mast cells. Immunohistochemical methods utilising antibodies to tryptase [29] or to histamine [42] have been reported to be more sensitive than conventional staining with basic dyes, and Harvima *et al.* [28] found comparable sensitivity between immunohistochemistry and enzyme histochemistry with the substrate used in this study (Z-Gly-Pro-Arg-MNA) (other substrates tested being noticeably less sensitive). It, therefore, seems likely that the discrepancy between the 2 staining methods is one of sensitivity, rather than being due to the presence of tryptase in other cell types.

A tryptic protease was purified 2400-fold from guinea pig lung tissue, but not to homogeneity. Because the final preparation was still heterogeneous, the extent to which the behaviour of guinea pig tryptase was modified by other proteins present cannot be immediately assessed. However, similar inhibitor profiles (leupeptin, aprotinin, SBTI) were observed *in situ* and at different stages of purification (results not shown). Also, nearly identical substrate profiles were obtained from 2 separate preparations of guinea pig lung tryptase of different degrees of purity. If a modifier protein were present, it would have to be closely associated with the protease to have stayed with it through a number of purification steps and under differing conditions of ionic strength. It is, therefore, likely that the properties of this preparation are representative of the native enzyme. These results are also consistent with the fact that no endogenous inhibitor of tryptase has been reported [1]. Although other proteins were still present in the final preparation, only a single [^{3}H]-DFP-reactive band was detected by fluorography. Thus, only one serine protease appears to be present in the preparation. This contrasts with human tryptase, which appears as a characteristic doublet [1, 29]. Little and Johnson [30] have resolved this doublet by chromatography on cellulose phosphate, although it is not clear whether or not these represent separate gene products.

The tryptic protease isolated from guinea pig lung had similar inhibition characteristics to the mast cell tryptase detected by histochemistry. Furthermore, it was isolated by a procedure which, when used with human lung, selects for mast cell tryptase. It is, therefore, likely that the purified protease and the tryptase demonstrated histochemically are the same enzyme. The isolated protease also has a number of properties in common with well-characterised tryptases from other sources. It is a serine protease with trypsin-like substrate specificity. It is resistant to α1-proteinase inhibitor and other proteinaceous inhibitors. Its pattern of inhibition by a range of compounds is broadly similar to that of human tryptases isolated from both lung and skin, as are its apparent K_i values for benzamidine and APC 366 (although it does appear to be significantly more resistant to leupeptin). It also displays the reported time-dependency for inhibition by APC 366 [16]. It pH activity and stability profiles are very similar to those reported for human lung tryptase [43], but extends to slightly higher pH values than does rat tryptase [21]. Like human [36], canine [18], and bovine [39] tryptase, the guinea pig enzyme was both stabilised and inhibited by high concentrations of NaCl, but steadily lost activity at physiological ionic strength unless heparin was present. Heparin binding (and, related to it, extraction from source tissue by high but not low salt) is a property it shares with tryptase from human [44], canine [18], and bovine [39] sources, but not with rat tryptase (which does not bind heparin and can be extracted with low ionic strength buffers) [20, 21].

All tryptases isolated, to date, are oligomeric in structure: human [43], canine [18, 19], and rat [21] tryptase are tetrameric (MW = 130–145 kDa, subunit MW = 30–35 kDa), and bovine tryptase is a higher order structure (MW = 360 kDa, subunit MW = 40 kDa) [39]. The guinea pig tryptic enzyme appears to be of a still higher order (MW = 860 kDa, subunit MW = 38 kDa). This highly oligomeric structure cannot be ascribed to the increase in the strength of hydrophobic interactions caused by the high-salt concentration used, as rechromatography on Sephacryl S-300 equilibrated with phosphate buffered saline (I = 0.15) gave the same high apparent molecular mass. These similarities with authentic tryptases strengthen the conclusion that the purified protease is guinea pig mast cell tryptase. However, final proof must await the availability of specific antibodies to the purified protease for immunohistochemical confirmation.

The high molecular mass of guinea pig tryptase is unusual, but not without precedent because intracellular proteosomes are approximately 700 kDa and the ATP-dependent protease of the ubiquitin pathway is even larger [45]. Because the final preparation was still heterogeneous, it is uncertain as to whether this complex consists of a single type of subunit (possibly a homoeicosamer or a homotetraeicosamer) or different subunits. If there is still some heparin or other proteoglycan complexed with the tryptase, it must be very tenaciously bound to remain associated at such high ionic strength (2 M NaCl). This high molecular mass with attendant low diffusion rates may not produce a very great alteration in its physiological function relative to human tryptase, because the human enzyme is secreted in proteoglycan complexes with apparent molecular masses of 200–250 and 400–560 kDa [46].

Although guinea pig tryptase bound to heparin and was stabilised by it, there were qualitative as well as quantitative

differences in the effect of heparin on guinea pig and human tryptases. The binding of guinea pig tryptase to heparin would appear to be weaker on the basis of the lower concentration of NaCl required to elute the enzyme from a heparin agarose column. Also, when included in the assay medium without prior incubation with the enzyme, heparin had no significant effect on the activity of guinea pig tryptase, but did enhance the activity of both lung and skin human tryptase by causing a moderate decrease in the apparent K_m without altering k_{cat}. This is the first report of this effect on human tryptase. Whereas there have been a number of studies on the effect of heparin stabilising tryptase [36, 44, 47], they have all included heparin in the assay medium when determining K_m and k_{cat} values. We have found this precaution not to be necessary for assays conducted at 20°C rather than 37°C, for reaction times of 10 min or less.

The substrate specificities seen in this study for human and guinea pig tryptase are broadly similar to those reported for other species. Arg is preferred over Lys at position P1 for both canine [18] and rat [21] tryptases, and Pro or Gly is preferred over Phe or Leu at P2 with rat tryptase [21]. (No substrate with Phe or Leu at P2 was tested on dog tryptase [18]). Differences were seen, however, in the relative importance of Gly, Pro, or the proline analog Pip at P2. For example, comparison of the three substrates D-Phe-Pip-Arg-pNA, L-Pyr-Gly-Arg-pNA, and tosyl-Gly-Pro-Arg-pNA implied a P2 preference of dog tryptase of Pip ≈ Gly ≈ Pro, of rat tryptase of Pip > Pro (L-Pyr-Gly-Arg-pNA not tested), of guinea pig tryptase of Gly ≥ Pip ≈ Pro, and of human tryptase of Pro > Pip > Gly. Comparison of the molar catalytic activities of guinea pig and human tryptases shows a decrease in the molar catalytic activity of the guinea pig enzyme towards substrates with Pro at P2 relative to human tryptase, with a simultaneous increase towards substrates with Gly at P2, to give more or less the same activity towards all. Kinetic studies showed that this change in activity involves changes in both K_m and k_{cat}. The role of the residue at P3 remains unclear. Guinea pig tryptase showed a slight preference for pyroglutamate at P3, but tolerated such disparate residues as Arg, Nle, Gly, and Phe equally well. However, the absence of a residue at P3, as is the case with benzoyl-DL-Arg-pNA, resulted in a complete loss of activity, which suggests the presence of an extended binding site with the accompanying need for an extended substrate.

The demonstration that tryptase is present in the mast cells of guinea pigs, and that it shares a number of properties with human tryptase, suggests that this species may be an appropriate model to investigate the actions of tryptase *in vivo*. However, the differences in reactivity towards peptide substrates suggest that guinea pig tryptase may act on different targets, or at different relative rates on the same targets compared to human tryptase. Alternatively, there may be small changes in target molecules to complement the changes in substrate specificity. Therefore, some caution must be exercised when interpreting studies of tryptase in guinea pig models of disease.

This work was funded by Arris Pharmaceutical Corp. and the Allergy, Asthma and Inflammation Research Trust, Southampton.

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