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Separation, purification and *N*-terminal sequence analysis of a novel leupeptin-sensitive serine endopeptidase present in chemically induced rat mammary tumour

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Leupeptin is a small peptide microbially derived inhibitor of certain proteolytic enzymes. Using *N*-α-benzoyl-DL-arginine 4-nitroanilide as substrate, we found a novel leupeptin-sensitive proteolytic enzyme in *N*-methyl-*N*-nitrosourea(MNU)-induced rat mammary adenocarcinoma. This enzyme was apparently different from urokinase-type plasminogen activator or cathepsin B and was present in mammary tumour at levels at least 20 times higher than those in normal mammary tissue. This enzyme was separated and purified from crude extracts of MNU-induced mammary adenocarcinoma approx. 1900-fold with 34% yield. It was a trypsin-like serine endopeptidase and had a pH optimum at 7.0. The native enzyme had an apparent M_r of 180000 and exhibited four isoelectric points ranging from 4.3 to 5.0. Electrophoresis of denatured enzyme, however, yielded, with reduction, a major band with an apparent M_r of 37500 and a minor band with an apparent M_r of 35500. The *N*-terminal 23 residues of the major band were Ile[1]-Val[2]-Gly[3]-Gly[4]-Gln[5]-Glu[6]-Ala[7]-Ser[8]-Gly[9]-Asn[10]-Lys[11]-Xaa[12]-Pro[13]-Val[14]-Gln[15]-Val[16]-Xaa[17]-Leu[18]-Xaa[19]-Val[20]-Trp[21]-Leu[22]-Pro[23]. These and other properties of this enzyme suggested that it most closely resembles rat skin tryptase, followed by rat peritoneal mast-cell tryptase and then by tryptases from other species. The rat, like human and mouse, may carry multiple tryptase genes, and this mammary-tumour enzyme may be an additional form of rat tryptase within a new serine-proteinase family.

INTRODUCTION

Leupeptin (*N*-acetyl-(or *N*-propyl)L-leucyl-L-leucyl-L-arginal), isolated from *Actinomycetes* (Aoyagi *et al.*, 1969*a*,*b*), is an inhibitor of certain proteolytic enzymes which preferentially hydrolyse endopeptidase substrates at the carboxy side of a basic amino acid residue, either arginine or lysine (Aoyagi & Umezawa, 1975; Umezawa, 1976). The argininal residue at the *C*-terminus of leupeptin is essential for its inhibitory activity (Kawamura *et al.*, 1969; Kondo *et al.*, 1969). In general, the proteolytic enzymes that are inhibited by leupeptin are grouped into two classes: trypsin-like serine endopeptidases and cathepsin B-like cysteine endopeptidases (Aoyagi & Umezawa, 1975; Umezawa, 1976).

An increased production of leupeptin-sensitive proteolytic enzymes has been observed in neoplastic mammary tissues. Cultured explants of mouse mammary adenocarcinoma and malignant human breast tumours have been shown to release excessive amounts of cathepsin B when compared with normal counterparts (Poole *et al.*, 1978; Mort *et al.*, 1980; Recklies *et al.*, 1980, 1982*a*,*b*; Mort & Recklies, 1986). Urokinase-type plasminogen activator, a trypsin-like serine endopeptidase, has been found to be secreted in increased quantities from human breast cancer (Peterson *et al.*, 1973; Nagy *et al.*, 1977; Sherman *et al.*, 1980; Sutherland, 1980; Evers *et al.*, 1982; Thorsen, 1982; Needhan *et al.*, 1988; Duffy *et al.*, 1988; Pacheco *et al.*, 1988), rat mammary adenocarcinoma (O'Grady *et al.*, 1981; Mira-y-Lopez *et al.*, 1983) and mouse mammary tumours (Mira-y-Lopez *et al.*, 1983; Pereyra-Alfonso *et al.*, 1988; Pereyra-Alfonso & Bal de Kier Joffe, 1989).

There is both direct and indirect evidence to suggest that proteolytic enzymes are involved in the process of tumour invasion and metastasis. Both cathepsin B and urokinase-type plasminogen activator have been implicated in playing a central role in the destruction and invasion of connective tissues by tumour cells (Mullins & Rohrlich, 1983; Dano *et al.*, 1985; Goldfarb & Liotta, 1986; Mignatti *et al.*, 1986; Hart & Rehemtulla, 1988; Clavel & Birembaut, 1988; Pollanen *et al.*, 1988; Laiho & Keski-Oja, 1989). Also, leupeptin has been shown to inhibit blood-borne lung metastases in rats injected intravenously with Yoshida ascites hepatoma cells (Saito *et al.*, 1980). Other studies have shown that leupeptin blocks tumour promotion in mouse skin (Hozumi *et al.*, 1972) and suppresses carcinogenesis in many experimentally induced animal cancer models, including the 7,12-dimethylbenz[*a*]anthracene(DMBA)-induced rat mammary cancer model (Matsushima *et al.*, 1976).

Using *N*-α-benzoyl-DL-arginine 4-nitroanilide (Bz-Arg-4NA) as substrate, we found a novel leupeptin-sensitive serine endopeptidase in *N*-methyl-*N*-nitrosourea(MNU)-induced rat mammary adenocarcinoma (Eto & Bandy, 1990). This enzyme appeared to be different from other leupeptin-sensitive serine endopeptidases, including urokinase-type plasminogen activator, and was detected at levels at least 20 times higher than those in normal mammary tissue. Here we describe the separation, purification and *N*-terminal sequence determination of this enzyme.

EXPERIMENTAL

Materials

All chemicals, except for those listed below, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephacryl S-300 and S-200 (Superfine grade), Polybuffer Exchanger 94 and Polybuffer 74 were from Pharmacia–LKB, Uppsala, Sweden. The protein assay kit, freeze-dried BSA, Protean II vertical-electrophoresis cells, Mini-Protean II dual-slab electrophoresis cells, Mini Trans-Blot electrophoretic-transfer cells, SDS/PAGE

Abbreviations used: -4NA, 4-nitroanilide. MNU, *N*-methyl-*N*-nitrosourea; DMBA, dimethylbenz[*a*]anthracene; Bz, *N*-α-benzoyl; Cbz, *N*-α-benzyloxycarbonyl; Tos, *N*-α-*p*-tosyl (toluenesulphonyl); -CH_2Cl, chloromethane; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

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standards, and other electrophoresis reagents were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Immobilon-P transfer membranes were from Millipore Corp., Bedford, MA, U.S.A. Stirred ultrafiltration cells and YM10 disc membranes were purchased from Amicon Division, W. R. Grace and Co., Danvers, MA, U.S.A.

Animals, tumours and tissues

Female Sprague–Dawley rats (virus-free colony 202) were purchased from Harlan Sprague–Dawley, Indianapolis, IN, U.S.A., at 35–40 days of age. Mammary tumours were induced in these animals with MNU or DMBA according to the procedures of Grubbs *et al.* (1983, 1985) and Moon *et al.* (1976). All tumours used in the present study were classified histologically as either mammary adenocarcinomas or fibroadenomas. The tumours larger than approx. 3 cm in average diameter were often necrotic at the centre. Therefore, only the tumours smaller than 3 cm were used in the present study. In addition, all tumours used in the present study were sliced into two or three pieces with a pair of scissors and those that were found to be necrotic at the centre were discarded. Normal mammary tissues were obtained from the same stock of female rats (at about 230 days of age), except that they did not receive MNU or DMBA.

Enzyme assays

Hydrolysis of 4-nitroanilide substrates, including Bz-Arg-4NA, was assayed by continuously monitoring the absorbance of the product, 4-nitroaniline, at 410 nm using Varian DMS 200 u.v.-visible spectrophotometer. The preincubation mixture contained 0.1 M-potassium phosphate buffer, pH 6.9, and the enzyme preparation in a final volume of 1.0 ml. After incubation for 5 min at 37 °C, the reaction was initiated by the addition of 10 μl of 0.1 M-4-nitroanilide substrate in dimethyl sulphoxide to the mixture. Enzyme blank without 4-nitroanilide substrate served as reference mixture. A substrate blank, which consisted of 1.0 ml of 0.1 M-potassium phosphate buffer, pH 6.9, and 10 μl of 0.1 M-4-nitroanilide substrate in dimethyl sulphoxide, was used to correct the absorbance reading of the sample mixture. The molar absorption coefficient (ϵ) of the product, 4-nitroaniline, at 410 nm was 1.016×10^4 $M^{-1} \cdot cm^{-1}$ in 0.1 M-potassium phosphate buffer, pH 6.9. Initial velocities were calculated from the progress curve by the method of Waley (1981).

Protein estimation

The protein concentration was determined by the dye-binding procedure of Bradford (1976) (Bio-Rad Protein Assay Kit), with BSA (Bio-Rad Protein Standard II) as the standard.

Preparation of crude extract

Tissues were homogenized (Polytron; Brinkman Instruments, Westbury, NY, U.S.A.) at 4 °C with 3 vol. of 0.1 M-potassium phosphate buffer, pH 6.5, containing pepstatin A (10 μg/ml) and bestatin hydrochloride (10 μg/ml). Crude extracts were obtained by centrifugation of the homogenate for 20 min at 4 °C and 23400 *g*.

Preparation of partially purified enzyme

A crude extract (approx. 60 ml) of MNU-induced mammary adenocarcinoma tissue was mixed with 2 vol. of 0.1 M-potassium phosphate buffer, pH 6.5, containing 0.6 M-NaCl and the mixture was divided into two equal parts and applied to two *p*-aminobenzamidine–agarose columns (1.5 cm × 12.5 cm). The columns were washed with 0.1 M-potassium phosphate buffer, pH 6.5, containing 0.4 M-NaCl (100 ml each) and the enzyme was eluted from the column with 0.1 M-sodium acetate buffer, pH 4.0, containing 0.4 M-NaCl (200 ml each). The pH of this enzyme solution was raised to 6.2 with 1 M-NaOH and the solution was concentrated to a small volume (2–4 ml) by stirred-cell ultrafiltration using a YM10 disc membrane (Amicon). The concentrated enzyme solution was mixed with glycerol (to a final concentration of 50%, w/v) and stored at −20 °C.

Five batches of the concentrated enzyme solution, which represented approx. 100 g of MNU-induced mammary-adenocarcinoma tissue, were pooled, concentrated to approx. 2 ml, and applied to a Sephacryl S-200 (Superfine grade) column (2.5 cm × 48 cm) that had been previously equilibrated with 0.1 M-potassium phosphate buffer, pH 6.5. The enzyme was eluted with the same buffer and fractions of 30 drops (approx. 1.5 ml) were collected. Active fractions were pooled, concentrated to approx. 2 ml, mixed with glycerol (to 50%, w/v), and stored at −20 °C.

Estimation of apparent M_r by gel-filtration chromatography

Gel-filtration chromatography was carried out with a Sephacryl S-300 (Superfine grade) column (1.0 cm × 50 cm) equilibrated and eluted at 4 °C with 0.1 M-potassium phosphate buffer, pH 6.5. The protein standards used were bovine thyroglobulin (M_r 669000), horse spleen apoferritin (443000), sweet-potato (*Ipomoea batatas*) β-amylase (200000), yeast alcohol dehydrogenase (150000), BSA (66000) and bovine erythrocyte carbonic anhydrase (29000). Blue Dextran (M_r 2000000) was used to estimate the void volume (V_0) of the column.

Polybuffer Exchanger-94 chromatofocusing

The partially purified enzyme in 25 mM-Bistris/HCl buffer, pH 6.5, was applied to a Polybuffer Exchanger-94 column (1.0 cm × 50 cm), which had been equilibrated at 4 °C with the same buffer. The elution buffer was 560 ml of Polybuffer 74, which had been previously diluted 1:8 with degassed water and adjusted to pH 4.0 with HCl. Fractions consisting of 70 drops (approx. 3.4 ml) were collected.

PAGE

The apparent M_r of the denatured enzyme was estimated by gradient (5–15%, w/v) PAGE in 0.1% SDS using Protean II vertical-electrophoresis cells (Bio-Rad). Enzyme samples in glycerol sample buffer were boiled for 2 min in the presence or absence of 5% (v/v) 2-mercaptoethanol and 2% (w/v) SDS. Gels were stained for protein with Coomassie Blue. The marker proteins used were rabbit muscle phosphorylase *b* (M_r 97400), BSA (66200), hen's-egg-white ovalbumin (42699), bovine carbonic anhydrase (31000), soybean trypsin inhibitor (21500) and hen's-egg-white lysozyme (14400). Bromophenol Blue was used as the tracking dye.

Mini-Protean II dual slab-electrophoresis cells (Bio-Rad) were used for discontinuous SDS/12%-PAGE (Laemmli, 1970). Enzyme samples in sucrose sample buffer containing 5% (v/v) 2-mercaptoethanol and 2% (w/v) SDS were boiled for 2 min, loaded on to 1.0 mm-thick slab mini-gels (5% stacking gel; 12% running gel) and run at 300 mA and 200 V for approx. 45 min. Twice-recrystallized SDS was used for the sample buffer, stacking gel, and separating gel. Phenol Red was used as tracking dye.

N-Terminal amino-acid-sequence analysis

Approx. 30 μg of enzyme protein was applied to a discontinuous (Laemmli) SDS/polyacrylamide mini-gel (5% stacking gel; 12% running gel) in a Mini-Protean II dual slab-electrophoresis cell (Bio-Rad). The proteins were transferred to

Table 1. Partial purification of Bz-Arg-4NA-hydrolysing activity from MNU-induced mammary adenocarcinoma of rat

The enzyme was partially purified from 105 g of mammary adenocarcinoma. Crude extracts were obtained by homogenization of the tissues in 0.1 M-potassium phosphate buffer, pH 6.5, containing pepstatin A (10 μg/ml) and bestatin hydrochloride (10 μg/ml) and centrifugation of the homogenate for 20 min at 23400 *g*. Approx. 60 ml of the supernatant was divided into two portions and loaded on to two *p*-aminobenzamidine–agarose affinity columns (1.5 cm × 12.5 cm) at pH 6.5 and the enzyme was eluted at pH 4.0 as shown in Fig. 1. The active fractions were pooled and concentrated. Five batches of the concentrated enzyme solutions were then applied to a Sephacryl S-200 (Superfine) column (2.5 cm × 48 cm), as shown in Fig. 2. Values are typical for 13 separate purifications. For other details, see the Experimental section.

Procedure	Protein (μg)	Total activity (μmol/min)	Specific activity (μmol/min per mg of protein)	Yield (%)	Purification (-fold)
Crude extract	3.51×10^6	17.9	5.10×10^{-3}	100	1
p-Aminobenzamidine–agarose	965	8.03	8.33	44.9	1630
Sephacryl S-200	627	6.08	9.70	34.0	1900

the Immobilon-P membrane (Millipore) using a Mini Trans-Blot electrophoretic-transfer cell [100 V (300 mA) for 60 min; Bio-Rad] and stained with Coomassie Blue [0.1% in 10% (v/v) methanol] for 15 min. The membrane was destained with 50% (v/v) methanol (no acetic acid). The protein band on the membrane was cut out, destained thoroughly, washed with distilled water and sealed in an Eppendorf tube. The *N*-terminal amino acid sequence of the protein band on the membrane was analysed by using an Applied Biosystems 470A automated gas-phase protein sequencer with an online 120A phenylthiohydantoin-derivative analyser.

RESULTS

Bz-Arg-4NA-hydrolysing activity in the crude extracts of mammary tumours and normal mammary tissue

The Bz-Arg-4NA-hydrolysing activities in the crude extracts of mammary tumours and normal mammary tissue were assayed at pH 6.9. 2-Mercaptoethanol and EDTA were not included in the reaction mixture because these reagents did not increase or decrease specific Bz-Arg-4NA-hydrolysing activities. In addition, NaCl and Triton X-100 were omitted from the extraction buffer (0.1 M-potassium phosphate, pH 6.5) because inclusion of these reagents significantly decreased the specific enzyme activities by increasing the amount of proteins extracted without increasing the yield of enzyme activities. With these improvements incorporated in the extraction and enzyme-assay procedures, MNU-induced mammary adenocarcinoma contained the highest Bz-Arg-4NA-hydrolysing activity (4.94 ± 0.33 nmol of 4-nitroaniline released/min per mg of protein at 37 °C; $n = 7$). DMBA-induced mammary adenocarcinoma and fibroadenoma were also found to contain considerable Bz-Arg-4NA-hydrolysing activities: 2.95 ± 0.31 ($n = 5$) and 1.13 ± 0.32 ($n = 2$) nmol/min per mg of protein respectively. Normal mammary tissue exhibited the lowest activity (0.19 ± 0.02 nmol/min per mg of protein; $n = 5$). All activities were completely inhibited by the addition of *N*-acetyl-leupeptin hemisulphate to the preincubation mixture at a final concentration of 50 μg/ml.

Separation and partial purification of Bz-Arg-4NA-hydrolysing activity from a crude extract of MNU-induced mammary adenocarcinoma

Since a crude extract of MNU-induced mammary adenocarcinoma contained the highest levels of Bz-Arg-4NA-hydrolysing activity, this tissue was used to separate and partially purify the Bz-Arg-4NA-hydrolysing activity. Pepstatin A and bestatin hydrochloride were added to the extraction buffer to suppress aspartate endopeptidase and leucine aminopeptidase activities present in the crude extract of MNU-induced mammary adenocarcinoma. As shown in Table 1, *p*-aminobenzamidine–agarose affinity chromatography was the most effective step in the separation and partial-purification procedure. Fig. 1 shows that most of the Bz-Arg-4NA-hydrolysing activity was absorbed to *p*-aminobenzamidine–agarose beads at pH 6.5 and eluted from the beads at pH 4.0. The bulk of the proteins in the extract were not absorbed at pH 6.5. Since the activity of the enzyme was decreased substantially when it was exposed to elution buffer, the pH of the enzyme solution was quickly raised to 6.2 after elution to avoid inactivation. Addition of 0.1 M-benzamidine to the elution buffer did not increase the yield of enzyme from the column. The activity of the enzyme obtained after *p*-aminobenzamidine–agarose chromatography did not decrease for at

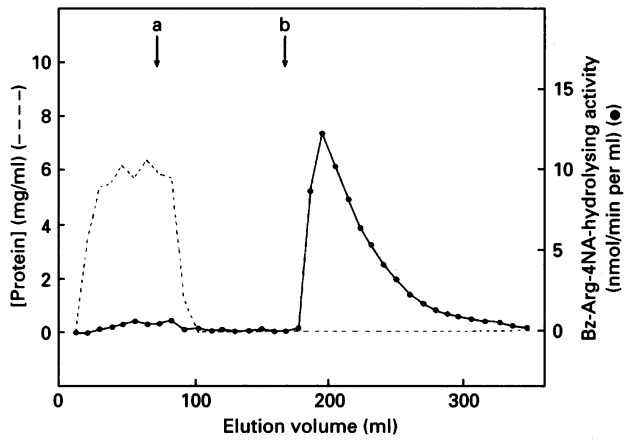

Fig. 1. *p*-Aminobenzamidine–agarose affinity chromatography of the Bz-Arg-4NA-hydrolysing activity present in the crude extracts of MNU-induced mammary adenocarcinoma

A crude extract (approx. 30 ml), which was prepared in 0.1 M-potassium phosphate buffer, pH 6.5, was mixed with 2 vol. of the same buffer containing 0.6 M-NaCl and applied to *p*-aminobenzamidine–agarose column (1.5 cm × 12.5 cm). Elution buffers were (*a*) 0.1 M-potassium phosphate buffer, pH 6.5 (100 ml) and (*b*) 0.1 M-sodium acetate buffer, pH 4.0 (200 ml). Both buffers contained 0.4 M-NaCl. The Bz-Arg-4NA-hydrolysing activity was assayed in 0.1 M-potassium phosphate buffer, pH 6.9, without 2-mercaptoethanol and EDTA. The pH of the eluted enzyme solution was raised to 6.2 and the solution was concentrated to a small volume by ultrafiltration.

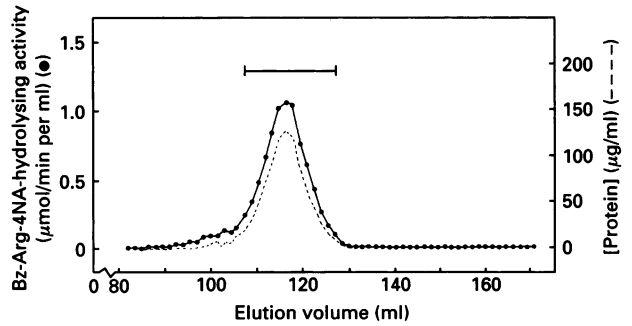

Fig. 2. Sephacryl S-200 chromatography of the Bz-Arg-4NA-hydrolysing activity obtained after *p*-aminobenzamidine–agarose chromatography

Five batches of the concentrated solution which represented 105 g of MNU-induced mammary-adenocarcinoma tissue were applied to a Sephacryl S-200 (Superfine) column (2.5 cm × 48 cm). The 0.1 M-potassium phosphate buffer, pH 6.5, was used to equilibrate the column and elute the enzyme. Fractions (30 drops; approx. 1.5 ml) were collected. Active fractions, which are indicated in the Figure as a horizontal bar, were pooled, concentrated to approx. 2 ml, mixed with glycerol (to 50%, w/v) and stored at −20 °C.

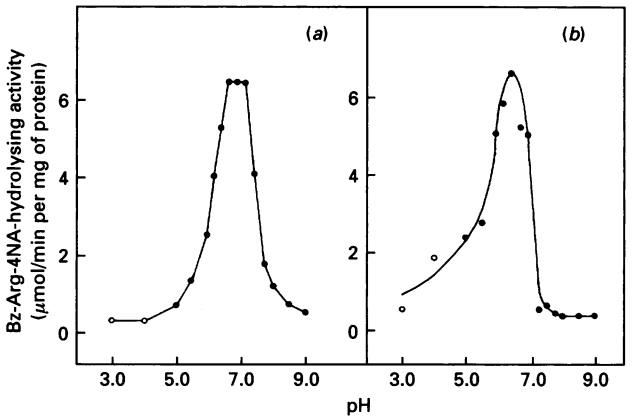

Fig. 3. pH optimum and stability of the partially purified Bz-Arg-4NA-hydrolysing activity

The enzyme was separated and partially purified as described in the Experimental section. (*a*) pH optimum profile. Preincubation mixtures contained portions (5 μl) of the enzyme solution in 0.1 M buffers (1.0 ml; ●, phosphate; ○, acetate). A known amount of 4-nitroaniline dissolved in dimethyl sulphoxide was added to each buffer and the absorption at 410 nm was measured in order to calculate accurately the amount of 4-nitroaniline released from Bz-Arg-4NA in different buffers. (*b*) pH stability profile. Portions (5 μl) of the enzyme solution were diluted with 0.1 M buffers to a final volume of 0.2 ml. After incubation for 30 min at 37 °C without Bz-Arg-4NA, 0.5 ml of 0.2 M-potassium phosphate buffer, pH 6.9, was added to the mixtures and the pH values were adjusted to 6.9 using either NaOH or HCl. The volumes were then adjusted to 1.0 ml before the addition of Bz-Arg-4NA.

least 6 months at −20 °C in the presence of 50% (w/v) glycerol. Sephacryl S-200 chromatography of the active fraction (Fig. 2) showed that Bz-Arg-4NA-hydrolysing activity was eluted as a single peak between β-amylase (M_r 200000) and alcohol dehydrogenase (M_r 150000). Starting with 105 g of mammary adenocarcinoma tissue, 627 μg of partially-purified enzyme was obtained with 34% yield and 1900-fold purification.

Attempts to resolve this partially purified enzyme into two or more peaks were unsuccessful when the enzyme was chromatographed on heparin–agarose, CM–Sepharose Fast Flow, DEAE-Sepharose Fast Flow and aprotinin–agarose columns under various conditions.

Table 2. Hydrolysis of 4NA substrates by partially purified Bz-Arg-4NA-hydrolysing enzyme from MNU-induced mammary adenocarcinoma

Partially purified enzyme was prepared as described in the Experimental section and Table 1. The enzyme solution was diluted 1:100 with 0.1 M-potassium phosphate buffer, pH 6.5, and portions (5 μl) of this diluted solution (0.505 nmol of 4-nitroaniline released/min per 0.05 μg of protein in 5 μl with 1 mM-Bz-Arg-4NA as substrate) were preincubated in 0.1 M-potassium phosphate buffer, pH 6.9, for 5 min at 37 °C before the addition of 10 μl of 0.1 M-4NA substrates dissolved in dimethyl sulphoxide. Hydrolysis of the 4NA substrates at 37 °C was assayed by monitoring the increase in the absorption of the product 4NA at 410 nm. Each value is the mean for duplicate determinations. For other details of the enzyme assay, see the Experimental section. The arginine residue in Bz-Arg-4NA is a mixture of D- and L-configuration; all other optically active amino acids are of the L-configuration. Further abbreviation: Suc, *N*-α-succinyl.

Expt.	Substrate	4NA-hydrolysing activity (relative activity, %)
1	Cbz-Gly-Pro-Arg-4NA	100.0
	Bz-Arg-4NA	1.5
	Gly-Pro-4NA	0
	Ala-4NA	0
	Arg-4NA	0
	Cbz-Ala-Ala-Pro-Leu-4NA	0
	Cbz-Gly-Gly-Leu-4NA	0
	Methoxy-Suc-Ala-Ala-Pro-Val-4NA	0
	Suc-Ala-Ala-Pro-Phe-4NA	0
	Acetyl-Phe-4NA	0
2	Cbz-Gly-Pro-Arg-4NA	100.0
	Cbz-Val-Gly-Arg-4NA	82.1
	Tos-Gly-Pro-Lys-4NA	81.6
	Tos-Gly-Pro-Arg-4NA	77.2
	Bz-Phe-Val-Arg-4NA	43.9
	Bz-Val-Gly-Arg-4NA	7.9
	D-Val-Leu-Lys-4NA	4.9
	Bz-Pro-Phe-Arg-4NA	3.5
	Bz-Ile-Glu-Gly-Arg-4NA	2.6
	DL-Val-Leu-Arg-4NA	2.6
	Bz-Arg-4NA	1.6

pH optimum and stability

The partially purified enzyme was most active at pH values between 6.75 and 7.25 and most stable at pH 6.5 (Fig. 3). The enzyme was unstable above pH 7.0 and below pH 5.5 (Fig. 3). The apparent M_r of the enzyme as determined by gel-filtration chromatography on the Sepharose S-300 column did not change when the enzyme was exposed to pH 7.5 or 8.0 for 30 min at 37 °C.

Substrate specificity and activation–inactivation studies

As shown in Table 2 (Expt. 1), the partially purified enzyme did not hydrolyse seven substrates which had Pro, Ala, Leu, Val or Phe adjacent to the 4NA group. The two substrates which had Arg adjacent to the 4NA group (Cbz-Gly-Pro-Arg-4NA and Bz-Arg-4NA) were hydrolysed, but the aminopeptidase substrate (Arg-4NA) was not hydrolysed.

The specificity of hydrolysis of 11 Arg- or Lys-4NA endopeptidase substrates was then examined (Expt. 2 in Table 2). The

Table 3. Effects of inhibitors and activators on Bz-Arg-4NA-hydrolysing enzyme partially purified from MNU-induced mammary adenocarcinoma of rat

The partially purified enzyme solution was prepared from MNU-induced mammary adenocarcinoma as described in the Experimental section and Table 1. The solution was diluted 1:10 with 0.1 M-potassium phosphate buffer, pH 6.5, and portions (5 μl) of this diluted solution (which represented 4.82 nmol of 4-NA released/0.5 μg of protein per 0.5 μl) were preincubated in 0.1 M-potassium phosphate buffer, pH 6.9, with or without potential inhibitors or activators for 5 min at 37 °C before the addition of Bz-Arg-4NA. Many of these inhibitors or activators were dissolved in dimethyl sulphoxide, which had no effect on the assay at a final concentration of 5% (v/v). Each value is the mean of duplicate determinations. For other details of the enzyme assay, see the Experimental section.

Expt.	Addition	Concn. [mM (Expt. 1) or μg/ml (Expts. 2 and 3)]	Bz-Arg-4NA-hydrolysing activity (% of control)
1	None	–	100
	2-Mercaptoethanol	10	101
	EDTA	1.5	99.1
	2-Mercaptoethanol + EDTA	10 + 1.5	98.5
	4-(Hydroxymercuri)benzoic acid	0.15	90.7
	E-64	0.15	96.2
2	Leupeptin	50	3.8
	Antipain	50	4.1
	Benzamidine	50	22.2
	Tos-Lys-CH_2Cl	100	29.3
	Phenylmethanesulphonyl fluoride	100	34.3
	Elastatinal	200	97.8
	Pepstatin	5	98.1
	Amastatin	100	99.0
	Tos-Phe-CH_2Cl	100	99.7
	Chymostatin	15	101
	Epiamastatin	100	101
	Bestatin	100	102
	Phosphoramidon	50	103
3	Bovine pancreatic trypsin inhibitor	100	49.8
	Aprotinin, bovine plasma	100	50.0
	Turkey egg-white trypsin inhibitor	100	99.8
	Trypsin ovoinhibitor (chicken egg white)	100	99.8
	α_2-Antiplasmin (human plasma)	100	101
	Bowman–Birk trypsin–chymotrypsin inhibitor (soybean)	100	101
	Ovomucoid trypsin inhibitor (chicken egg white)	100	102
	Antithrombin III, rat plasma	100	102
	Lima-bean trypsin inhibitor	100	103
	Soybean trypsin inhibitor	100	103

partially purified enzyme hydrolysed Arg- and Lys-4NA substrates equally well (Tos-Gly-Pro-Arg-4NA versus Tos-Gly-Pro-Lys-4NA). The *N*-terminal group of the substrates appeared to influence the rate of hydrolysis. For example, Cbz was the preferred *N*-terminal group over Tos (Cbz-Gly-Pro-Arg-4NA versus Tos-Gly-Pro-Arg-4NA) or Bz (Cbz-Val-Gly-Arg-4NA versus Bz-Val-Gly-Arg-4NA).

The activation–inhibition profiles of the partially purified Bz-Arg-4NA-hydrolysing enzyme are presented in Table 3. As shown in Expt. 1 (Table 3), 2-mercaptoethanol and/or EDTA did not activate the enzyme and 4-(hydroxymercuri)benzoic acid and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) did not inhibit enzyme activity. The results of Expt. 2 (Table 3) shows that the enzyme was inhibited by leupeptin, antipain, benzamidine, tosyl-lysylchloromethane (Tos-Lys-CH_2Cl) and phenylmethanesulphonyl fluoride, but not by elastase inhibitor (elastatinal), aspartate endopeptidase inhibitor (pepstatin), aminopeptidase inhibitors (amastatin, epiamastatin and bestatin), chymotrypsin inhibitors [chymostatin and tosylphenylalanylchloromethane (Tos-Phe-CH_2Cl)] or metallo-endopeptidase inhibitor (phosphoramidon). Of ten polypeptide inhibitors tested (Expt. 3 in Table 3), only two, namely bovine pancreatic trypsin inhibitor and bovine plasma aprotinin, were found to inhibit enzyme activity.

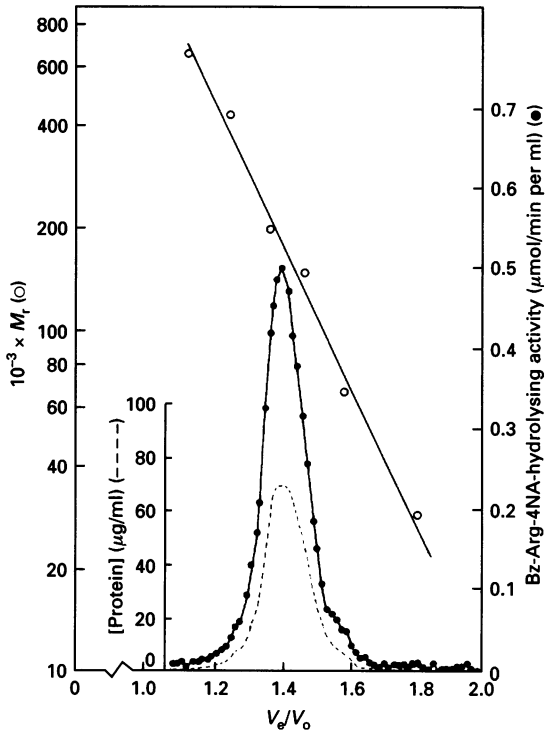

Fig. 4. Estimation of the apparent M_r of partially purified enzyme by gel filtration

Sephacryl S-300 (Superfine) column (1.0 cm × 50.0 cm) was calibrated with the protein standards thyroglobulin (M_r 669000), apoferritin (443000), β-amylase (200000), alcohol dehydrogenase (150000), albumin (66000) and carbonic anhydrase (29000). The 0.1 M-potassium phosphate buffer, pH 6.5, was used for equilibration and elution. The enzyme was partially purified as described in the Experimental section and Table 1. Glycerol was removed from the enzyme solution by ultrafiltration (YM10) and replaced with 0.1 M-potassium phosphate buffer, pH 6.5. A 500 μl portion of this solution was applied to the column and fractions (15 drops; approx. 0.63 ml) were collected. The Bz-Arg-4NA-hydrolysing activity of each fraction was assayed in 0.1 M-potassium phosphate buffer, pH 6.9, as described in the Experimental section.

Analytical gel filtration and gel electrophoresis

The apparent M_r of the partially purified Bz-Arg-4NA-hydrolysing enzyme was estimated by gel-filtration chromatography. A single peak of Bz-Arg-4NA-hydrolysing activity was eluted from a calibrated Sephacryl S-300 gel at a position slightly behind β-amylase (M_r 200000) and ahead of alcohol dehydrogenase (M_r 150000) (Fig. 4). On the basis of this

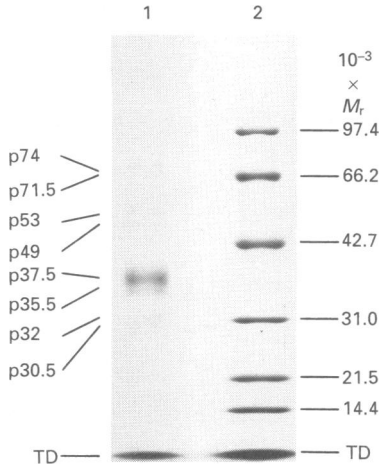

Fig. 5. SDS/5–15%-gradient PAGE of partially purified enzyme

The Bz-Arg-4NA-hydrolysing activity was partially purified as described in the Experimental section and Table 1. A 10 μg portion of this partially purified enzyme was boiled for 2 min in the presence of 5% (v/v) 2-mercaptoethanol and 2% (w/v) SDS and subjected to SDS/gradient (5–15%) PAGE (lane 1). Marker proteins (lane 2) were rabbit muscle phosphorylase *b* (M_r 97400), BSA (66200), hen's-egg-white ovalbumin (42699), bovine carbonic anhydrase (31000), soybean trypsin inhibitor (21500) and hen's-egg-white lysozyme (14400). TD indicates the position of tracking dye (Bromophenol Blue). Gels were stained for protein with Coomassie Blue.

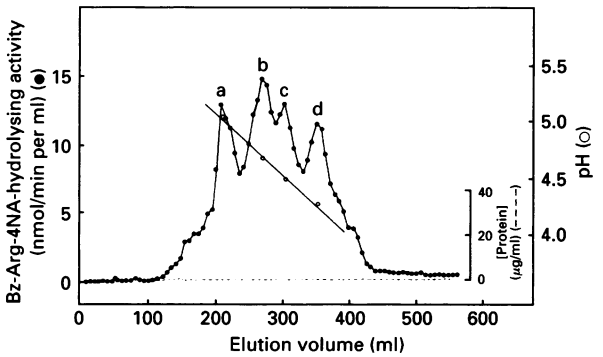

Fig. 6. Estimation of pI values by Polybuffer Exchanger-94 chromatofocusing

The buffer (0.1 M-potassium phosphate buffer, pH 6.5) of the partially purified enzyme was replaced with 25 mM-Bistris/HCl buffer, pH 6.5, by ultrafiltration (Amicon's YM10 membrane) and 2 ml of this solution (containing approx. 300 μg of protein) was applied to Polybuffer Exchanger-94 column (1.0 cm × 50.0 cm) which had been equilibrated with 25 mM-Bistris/HCl buffer, pH 6.5. Elution buffer was 560 ml of Polybuffer 74, which had previously been diluted 1:8 with water and adjusted to pH 4.0 with HCl. Fraction (70 drops; approx. 3.4 ml) were collected. The Bz-Arg-4NA-hydrolysing activity was assayed in 0.1 M potassium phosphate buffer, pH 6.9, as described in the Experimental section.

observation its apparent M_r was estimated to be approx. 180000. The apparent M_r remained unchanged for at least 6 months at 4 °C in 0.1 M-potassium phosphate buffer, pH 6.5.

The SDS/5–15%-gradient-PAGE of denatured and reduced enzyme (Fig. 5) did not yield a band with an apparent M_r of 180000. Instead it yielded a major band (p37.5) with an apparent

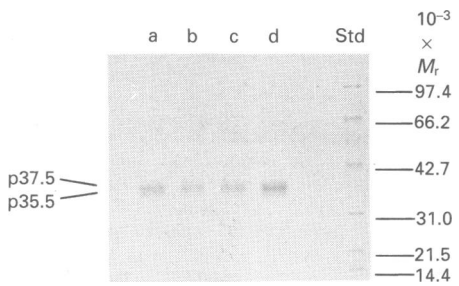

Fig. 7. Gradient SDS/PAGE analysis of the partially purified enzyme resolved into four fractions (corresponding to peaks a, b, c, and d in Fig. 6) by Polybuffer Exchanger-94 chromatofocusing

The fractions containing each of the four peaks, except for those that corresponded to the trough of two peaks, were pooled and concentrated to about 50–100 μl by ultrafiltration using an Amicon YM10 membrane. The concentrated solution was boiled for 2 min in the presence of 2-mercaptoethanol and SDS and subjected to SDS/gradient-5–15%-PAGE as described in the legend to Fig. 5. Approx. 1–3 μg of enzyme protein from each peak was applied to the gel. Lanes a, b, c, and d correspond to peaks a (pI 5.0), b (pI 4.7), c (pI 4.5) and d (pI 4.3) on the Polybuffer Exchanger-94 column respectively.

M_r of 37500 and a minor band (p35.5) with apparent M_r of 35500. There were six additional bands (p74, p71.5, p53, p49, p32, and p30.5) which stained faintly after prolonged Coomassie Blue staining. The position and composition of the bands did not change when the enzyme sample was boiled with SDS in the absence of 2-mercaptoethanol (results not shown).

Estimation of pI values by chromatofocusing

pI values for partially purified enzyme were estimated by chromatofocusing on Polybuffer Exchanger 94. As shown in Fig. 6, the enzyme exhibited four isoelectric points, i.e. 5.0 (peak a), 4.7 (peak b), 4.5 (peak c), and 4.3 (peak d). SDS/5–15%-gradient-PAGE analysis of these four peaks indicated that only two bands (p37.5 and p35.5) with apparent M_r values of 37500 and 35500 were present in all four peaks (Fig. 7).

N-Terminal amino acid sequence analysis

Approx. 30 μg of the partially purified enzyme was reduced, denatured and subjected to a discontinuous (Laemmli) SDS/12%-PAGE. Twice-recrystallized SDS was used for the sample buffer, stacking gel and separating gel. The protein bands were electroblotted on to a poly(vinylidene difluoride) membrane (Immobilon-P) as described in the Experimental section. The major protein band (p37.5) with an apparent M_r of 37500 was cut out, thoroughly destained and sequenced from the *N*-terminus by using an automated gas-phase sequencer with an online phenylthiohydantoin-derivative analyser.

The *N*-terminal amino acid sequence of the band p37.5 was Ile[1]-Val[2]-Gly[3]-Gly[4]-Gln[5]-Glu[6]-Ala[7]-Ser[8]-Gly[9]-Asn[10]-Lys[11]-Xaa[12]-Pro[13]-Val[14]-Gln[15]-Val[16]-Xaa[17]-Leu[18]-Xaa[19]-Val[20]-Trp[21]-Leu[22]-Pro[23].

The *N*-terminal sequence of the minor band (p35.5) with apparent M_r of 35500 could not be determined.

DISCUSSION

Various properties of this enzyme suggest that it is a serine endopeptidase. This assignment is based on its (*a*) affinity adsorption on to *p*-aminobenzamidine–agarose (Fig. 1), (*b*) sensitivity toward benzamidine, phenylmethanesulphonyl fluoride, bovine pancreatic trypsin inhibitor and bovine plasma aprotinin

Table 4. *N*-Terminal sequence of the novel trypsin-like enzyme present in rat mammary tumour

Numbers indicate the amino acid position relative to the *N*-terminal residue of tryptases, including rat skin tryptase (Braganza & Simmons, 1991). Residues identical with those in rat mammary-tumour enzyme are boxed.

Enzyme	Sequence																									Identity (%)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
Rat mammary tumour Tryptase	Ile-	Val-	Gly-	Gly-	Gln-	Glu-	Ala-	Ser-	Gly-	Asn-	Lys-		-Pro-	Val-	Gln-	Val-		-Leu-			-Val-	Trp-	Leu-	Pro-		
Rat skin	Ile-	Val-	Gly-	Gly-	Gln-	Glu-	Ala-	Ser-	Gly-	Asn-	Lys-	Trp-	Pro-	Trp	Gln-	Val-	Ser-	Leu-	Arg-	Val		-Asp-	Thr-	Tyr-	Trp-	80
Clone I, human skin mast cell	Ile-	Val-	Gly-	Gly-	Gln-	Glu-	Ala	Pro-	Arg-	Ser-	Lys-	Trp-	Pro-	Trp	Gln-	Val-	Ser-	Leu-	Arg-	Val	His-	Gly-	Pro	Tyr-	Trp-	70
Human lung mast cell	Ile-	Val-	Gly-	Gly-	Gln-	Glu-	Ala	Pro-	Arg-	Ser-	Lys-	Trp-	Pro-	Trp	Gln-	Val-	Ser-	Leu-	Arg-	Val	Arg-	Asp-	Arg-	Tyr-	Trp-	65
Dog mastocytoma	Ile-	Val-	Gly-	Gly	Arg	Glu-	Ala	Pro	Gly-	Ser-	Lys-	Trp-	Pro-	Trp	Gln-	Val-	Ser-	Leu-	Arg	Leu-	Lys-	Gly-	Gln-	Tyr-	Trp-	60
Human pituitary	Ile-	Val-	Gly-	Gly-	Gln-	Glu-	Ala	Pro-																		

(Table 3) and (*c*) the Ile[1]-Val[2]-Gly[3]-Gly[4] sequence and Pro[13] residue, which are identical with those found in pancreatic trypsin and many other serine endopeptidases, where the *N*-terminal isoleucine residue has been shown to form an internal salt bridge on zymogen activation (Ruhlman *et al.*, 1973; Young *et al.*, 1978). In addition, the results of the activation–inhibition studies (Table 3) preclude its assignment to the cysteine family [E64, 2-mercaptoethanol, 4-(hydroxymercuri)benzoic acid and EDTA], aspartic family (pepstatin), metalloproteinase family (phosphoramidon), and aminopeptidase family (amastatin, epiamastatin and bestatin).

This enzyme is a trypsin-like endopeptidase. It preferentially hydrolyses various synthetic endopeptidase substrates at the carboxy side of an arginine or lysine residue (Table 2). It is also inhibited by leupeptin, antipain and Tos-Lys-CH_2Cl (Table 3). Furthermore, the results of the study of substrate specificity (Table 2) and sensitivity to various inhibitors (Table 3) indicate that it does not belong to the chymotrypsin subfamily (Phe-4-NA substrates, Tos-Phe-CH_2Cl and chymostatin) or elastase subfamily (Leu- or Val-4NA substrates and elastatinal).

Although this is a trypsin-like enzyme, it is apparently different from other trypsin-like enzymes. No perfect match was found when the first 13 residues of the *N*-terminal sequence of this enzyme were compared with those in either the National Biomedical Research Foundation (NBRF) Protein Database or the University of Geneva Protein Sequence Data Bank (Swiss-Prot). This result suggested that either (*a*) this enzyme belongs to a known family of trypsin-like enzymes, but the *N*-terminal sequence of the rat enzyme has not yet been reported, or (*b*) it belongs to a new family distinct from any known trypsin-like enzymes.

To explore the first possibility, the 20 residues of this sequence were manually compared with those of other known trypsin-like enzymes and we found that they had the highest sequence similarity to those of mast-cell tryptases. The number of matched residues were 14 (70 %), 13 (65 %), and 12 (60 %) for human skin mast-cell tryptase, human lung mast-cell tryptase and dog mastocytoma tryptase respectively (Table 4). Recently, the *N*-terminal amino acid sequence of rat skin tryptase has also become available in the literature (Braganza & Simmons, 1991) and comparison of the two sequences has revealed an even higher degree of similarity; the number of matched residues is 16 (80 % identity) (Table 4). Although this is not a perfect match, it suggests that this enzyme is similar to rat skin tryptase.

The structural and enzymic properties of this enzyme also suggest that it is similar to rat skin tryptase. (*a*) The apparent M_r of native enzyme is 180000 for this enzyme, 145000 for rat skin tryptase (Braganza & Simmons, 1991) and 110000–144000 for rat peritoneal (Kido *et al.*, 1985; Muramatu *et al.*, 1988), human (Smith *et al.*, 1984; Schwartz, 1985, 1989; Cromlish *et al.*, 1987; Rochefort *et al.*, 1987) and dog (Caughey *et al.*, 1987) tryptases. (*b*) The apparent M_r of the denatured enzyme is 37500 (major) and 35500 (minor) for this enzyme and 30000–37500 for tryptases with two predominant bands approx. 1000–2000-M_r equivalents apart from one another. (*c*) pI values range from 4.3 to 5.0 for this enzyme and 4.5 to 4.9 for rat skin tryptase. (*d*) This enzyme is also relatively resistant to inactivation by many natural serine-proteinase inhibitors (Smith *et al.*, 1984; Caughey *et al.*, 1987; Cromlish *et al.*, 1987); however, aptrotinin (or basic pancreatic trypsin inhibitor) inhibits this enzyme, rat skin tryptase, rat peritoneal mast-cell tryptase and dog mastocytoma tryptase, but it does not inhibit human tryptase. Also, soybean trypsin inhibitor and lima-bean trypsin inhibitor inhibit rat peritoneal-mast-cell tryptase (Kido *et al.*, 1985), but they do not inhibit this enzyme or any other tryptases, including rat skin tryptase.

This enzyme appears to be more similar to rat tryptase than to other tryptases. Although this enzyme and all tryptases are rapidly inactivated at weakly alkaline pH values, heparin glycosaminoglycan does not protect this enzyme (results not shown), rat skin tryptase or rat peritoneal-mast-cell tryptase, whereas it protects other tryptases (Alter *et al.*, 1987). Also the inactivation of this mammary-tumour enzyme does not seem to be attributable to the dissociation of the active multimer, because the apparent M_r of the native enzyme, as determined by gel filtration, does not change under these conditions (results not shown).

In summary, all available evidence indicates that this enzyme most closely resembles rat skin tryptase, followed by rat peritoneal-mast-cell tryptase and then by tryptases from other species. The rat, like human and mouse, may carry multiple tryptase genes, and this mammary-tumour enzyme may be an additional form of rat tryptase within a new serine-proteinase family (Miller *et al.*, 1990; Vanderslice *et al.*, 1990; Braganza & Simmons, 1991).

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