Isolation and characterization of tryase, a serine proteinase from rat liver

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1. A new serine proteinase, tryase, was isolated from the membrane fraction of a post-nuclear supernatant of rat liver homogenate. The enzyme was solubilized with 1M-MgCl₂ and purified to homogeneity by DEAE-cellulose chromatography and affinity chromatography with soya-bean trypsin inhibitor linked to Sepharose 4B. 2. The enzyme was identified on sodium dodecyl sulphate/polyacrylamide gels by reaction with radiolabelled di-isopropyl phosphorofluoridate. Unreduced its molecular weight was 32 500, reduced it was 28 000. 3. The enzyme readily hydrolysed azocasein and tripeptide nitroanilide substrates with an arginine or lysine residue adjacent to the leaving group. D-Pro-Phe-Arg-NPhNO₂ was used routinely ($K_m = 0.25$ mM). Tryase showed little activity on blocked arginine esters or amides. 4. It was inhibited by di-isopropyl phosphorofluoridate, benzamidine, aprotinin, soya-bean and lima-bean trypsin inhibitors, Ile-Leu-Arg-CH₂Cl and Phe-Ala-Arg-CH₂Cl. It was not inhibited by Tos-Lys-CH₂Cl. 5. Subcellular-fractionation studies showed that tryase was associated with particles similar in their sedimentation properties to lysosomes, but, since it was not present in tritosomes, it was not in the classical lysosome. 6. Rat liver contained other neutral proteinases; one of these was a serine proteinase with an apparent molecular weight of 90 000 on gel chromatography.

In the present paper we describe the isolation and characterization of a new serine proteinase from rat liver that was found during an investigation of the neutral proteinases of this tissue. The enzyme has been named 'tryase', because catalytically it belongs to the trypsin family.

Tissue proteinases have been implicated in intracellular protein catabolism (Barrett, 1977) and in the post-translational modification of proteins that occurs intracellularly when precursors of secreted proteins are processed to their secreted forms by removal of pre- and pro-pieces (Hales, 1978) and extracellularly when zymogens are split to give active products (Neurath, 1975). Much of the intracellular degradation of protein occurs in the lysosomal system, and several lysosomal protein-

ases have been isolated and characterized (Barrett, 1977; Kirschke *et al.*, 1977).

Although there have been many reports of proteolytic activity associated with nuclei, mitochondria and microsomal fraction of tissues, including rat liver (see the Discussion section), few of the cellular proteinases that have been purified and well characterized have been shown to come from subcellular compartments other than the lysosome. The only proteinase that has been purified from rat liver is chymase (Jusic *et al.*, 1976; Haas *et al.*, 1979), which originated from mast cells dispersed through the hepatic connective tissue. We have therefore re-investigated the neutral proteinases of rat liver and have found a new trypsin-like proteinase. We have characterized the enzyme and studied its subcellular distribution by using a synthetic substrate.

Materials

Substrates

Bz-Arg-OEt, Bz-Arg-NPhNO₂, Bz-DL-Phe-ONap, Bz-Tyr-OEt and Tos-Arg-OMe were from Sigma Chemical Co., Poole, Dorset, U.K.; Bz-Arg-NNap and Z-Ala-ONap were prepared by Dr. C. G. Knight of the Strangeways Research Labora-

Abbreviations used: the abbreviations used are the standard ones for amino acids and *N*- and *C*-terminal blocking groups [Biochem. J. (1972) 126, 773–780]; additional *C*-terminal groups are: -NNap, 2-naphthylamide; -NPhNO₂, 4-nitroanilide; -ONap, 2-naphthyl ester; -CH₂Cl, -chloromethane; also, Dip-F, diisopropyl phosphorofluoridate; STI, soya-bean trypsin inhibitor (Kunitz); SDS, sodium dodecyl sulphate.

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tory, Cambridge, U.K. D-Pro-Phe-Arg-NPhNO₂, D-Val-Leu-Arg-NPhNO₂ and D-Val-Leu-Lys-NPhNO₂ were from Kabi Diagnostica, Stockholm, Sweden; Bz-Pro-Phe-Arg-NPhNO₂ was from Pentapharm, Basle, Switzerland; Suc-Ala-Ala-Ala-NPhNO₂ was from Bachem Feinchemikalien A.G., Bubendorf, Switzerland. Azocasein was prepared by treating casein with diazotized sodium sulphanilate, the method being otherwise as described by Charney & Tomarelli (1947). Acid-denatured bovine haemoglobin was prepared as described by Barrett (1970).

Inhibitors

Benzamidine, Dip-F, STI, Tos-Lys-CH₂Cl and Tos-Phe-CH₂Cl were from Sigma. Ile-Leu-Arg-CH₂Cl and Phe-Ala-Arg-CH₂Cl were given by Dr. Elliot Shaw, Brookhaven National Laboratory, Upton, NY, U.S.A. Chymostatin and leupeptin were from the Peptide Research Foundation, Tokyo, Japan. Aprotinin (Trasylol) was a gift from Dr. E. Phillip, Bayer A.G., Wuppertal 1, West Germany. Chicken ovomithin was purified as described by Barrett (1974). [³²P]Dip-F was from The Radiochemical Centre, Amersham, Bucks., U.K.

Miscellaneous

Brij 35 was from Fisons, Loughborough, Leics., U.K. Triton WR-1339 was from Ruger Chemical Co., Irvington, NJ, U.S.A. Microgranular pre-swollen DEAE-cellulose (DE-52) was from Whatman Biochemicals, Maidstone, Kent, U.K. Sepharose 4B was from Pharmacia, Hounslow, Middx., U.K. Ultrogel AcA-44 and AcA-34 were from LKB Instruments, Croydon, Surrey, U.K. Carbowax 20M [poly(ethylene glycol)] was from Raymond A. Lamb, London NW10 6JL, U.K. Fluorescamine was from F. Hoffman-La Roche and Co., Basel, Switzerland. Coomassie Brilliant Blue G, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride, sodium urate and protein standards for SDS/polyacrylamide-gel electrophoresis were from Sigma. All other chemicals were analytical-reagent grade.

Methods

Enzyme assays

Activity against azocasein. Reaction mixtures (1.0 ml) contained 0.1 ml of 1 M-Tris/HCl buffer, pH 7.5, 0.25 ml of 6% (w/v) azocasein and 0.65 ml of enzyme solution diluted with 0.1% Brij 35. After incubation at 50°C for 3 h, the reaction was stopped by adding 3 ml of 5% (w/v) trichloroacetic acid and the mixture was filtered. The A_{366} of the filtrate was measured and the amount of azocasein hydrolysed was calculated. One unit of activity corresponded to the hydrolysis of 1 mg of azocasein/h under the assay conditions specified.

Activity against tripeptide nitroanilide substrates. Incubation mixtures (0.5 ml) contained 0.05 ml of 1 M-Tris/HCl buffer, pH 7.5, 0.1 ml of 4 mM substrate solution and 0.35 ml of enzyme solution diluted with 0.1% Brij 35. After incubation for 15 min at 37°C the reaction was stopped by adding 0.05 ml of 50% (v/v) acetic acid. The A_{410} was measured and activity was expressed as μmol of substrate hydrolysed/min. For measurement of K_m the rate of hydrolysis was measured at substrate concentrations over the range 1–0.1 mM, at enzyme concentrations of 50–100 mg/ml. K_m was calculated from values of s and V_0 by non-linear regression as described by Wilkinson (1961).

Assays for activity against other proteinase substrates. Spectrophotometric assays were used for the measurement of hydrolysis of Tos-Arg-OMe (Walsh, 1970), Bz-Arg-OEt (Schwert & Takenaka, 1955), Bz-Tyr-OEt (Walsh & Wilcox, 1970) and Bz-Arg-NPhNO₂ (Erlanger *et al.*, 1961). Assays were done in Tris/HCl buffers (100 mM final concentration) containing 10 mM-CaCl₂, at pH 7.1 and pH 7.8. Hydrolysis of Z-Ala-ONap and Bz-DL-Phe-ONap was measured as described by Starkey & Barrett (1976). Cathepsin D was assayed on haemoglobin substrate (Barrett, 1970) and cathepsin B on Bz-Arg-NNap (Barrett, 1976).

Inhibition assays. The effect of potential inhibitors of proteinase activity was investigated with both azocasein and D-Pro-Phe-Arg-NPhNO₂ as substrate. The inhibitor under test was allowed to react with the enzyme for 30 min at 20°C before the substrate was added. The assay was then performed as described.

Succinate dehydrogenase. This was measured as succinate-tetrazolium reductase with the substrate 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium as described by Pennington (1961).

Urate oxidase. The procedure of Leighton *et al.* (1968) was followed, with sodium urate as substrate.

Other methods

Protein determination. Protein concentration was measured by the method of Lowry *et al.* (1951), but the concentration of the purified enzyme was also determined with fluorescamine (Weigle *et al.*, 1972). Both assays were calibrated with bovine serum albumin.

SDS/polyacrylamide-gel electrophoresis and radioautography. Electrophoresis was done in slabs of 10% polyacrylamide gel with a modification of the glycine/2-amino-2-methylpropane-1,3-diol (Ammediol)/HCl buffer system described by Wyc-koff *et al.* (1977) (Barrett *et al.*, 1979).

Gels were stained for protein with Coomassie Brilliant Blue G250. After being destained they were washed in water and dried on Whatman 3MM

chromatography paper. Some samples for electrophoresis were treated overnight at 4°C with 1 mM- $[^{32}\text{P}]$ Dip-F before treatment with SDS. Dried gels containing such samples were radioautographed for periods ranging from 12 to 48 h with Kodak X-omat film against an image-intensifying screen.

Perfusion and homogenization of rat livers. Young adult Lister Hooded rats (Strangeways colony) were killed by cervical dislocation and their livers were immediately perfused with 0.9% NaCl (150 ml/animal). The livers were removed, weighed and put in 0.25 M-sucrose solution containing 1 mM-EDTA and adjusted to pH 7.0. A 9 ml volume of sucrose solution was used per gram of tissue and the mixture was maintained at 4°C. The livers were then homogenized in a Silverson mixer-emulsifier (Silverson Machines, Chesham, Bucks., U.K.) for 40 s (two 20 s bursts at full speed).

Subcellular fractionation. All centrifugation was at 4°C. The homogenate was successively centrifuged at 600 g for 10 min, 5000 g for 10 min, 20 000 g for 10 min and 100 000 g for 60 min. The pellets from these centrifugations are referred to as the nuclear, mitochondrial, lysosomal and microsomal fractions respectively. The final supernatant was the cytosol. The pellets were resuspended in 0.1 M-Tris/HCl buffer, pH 7.5, to one-tenth of the original volume of the homogenate.

Preparation of tritosomes. Tritosomes were made as described by Trouet (1974). Two rats were each injected intraperitoneally with 1% (w/v) Triton WR-1339 in 0.9% NaCl at a dose of 1 ml/100 g body wt. The rats were killed 76 h later, and their livers were perfused with saline and homogenized as described above. The homogenate was centrifuged at 600 g for 10 min, and the resulting supernatant was centrifuged at 34 000 g for 10 min. Fluffy material above the pellet was discarded, and the pellet was suspended in the solution used for homogenization. It was re-centrifuged and resuspended in 20 ml of 45% (w/v) sucrose. The mixture was placed in centrifuge tubes (10 ml/tube). Then 6 ml of 45% (w/v) sucrose solution was layered over the sample, followed by 12 ml of 34.5% (w/v) sucrose and 6 ml of 14% (w/v) sucrose. Centrifugation was done at 25 000 rev./min for 2 h in a swinging-bucket rotor (MSE 6 × 38 ml) in an MSE 65 Super-Speed ultracentrifuge. After centrifugation the tritosomes (fraction G1) were retrieved from the interface of the lighter sucrose layers; the intermediate layer (fraction G2) and the pellet (fraction G3) were also kept.

Purification of tryase

Preparation of particle-bound neutral proteinase and its solubilization. A homogenate made from ten rat livers was centrifuged at 600 g for 10 min. The supernatant was kept. The pellet was resuspended

in one-half the original volume of 0.25 M-sucrose solution, homogenized again and re-centrifuged at 600 g for 10 min. This second supernatant was combined with the first and centrifuged at 20 000 g for 4 h. The pellet was suspended in 200 ml of 0.1 M-Tris/HCl buffer, pH 7.5, frozen and thawed four times (to solubilize lysosomal enzymes) and re-centrifuged at 100 000 g for 1 h. It was then washed three times by resuspending it in the same volume of Tris buffer and re-centrifuging it. After the final wash it was suspended in 100 ml of Tris buffer containing 1 M-MgCl₂ and centrifuged at 100 000 g for 1 h. The MgCl₂ treatment was repeated on the pellet, and the two supernatants were combined.

Chromatography on DEAE-cellulose. The MgCl₂ extract was made 0.1% with respect to Brij 35 and dialysed twice against 10 vol. of 10 mM-Tris/HCl buffer, pH 8.5, containing 0.1% Brij 35. Next 30 g of DEAE-cellulose was equilibrated with the same buffer and was mixed with the dialysed extract. The cellulose was allowed to settle and the supernatant was poured off. The cellulose was washed once with 3 vol. of the Tris buffer and packed into a chromatography column (3 cm × 25 cm). The bed was washed with 3 vol. of the buffer, and eluted with 0.2 M-NaCl in the buffer. The A_{280} of the eluate was monitored and the fractions containing protein were combined.

Affinity chromatography. STI was coupled to Sepharose 4B by the method of Porath *et al.* (1973). The coupling mixture contained 4 mg of STI/g (wet wt.) of CNBr-activated Sepharose 4B. A column (1.5 cm × 15 cm) containing 3 ml of STI-Sepharose was equilibrated with 5 mM-Tris/HCl buffer, pH 7.5, containing 0.1% Brij 35 and 1 M-NaCl. The sample eluted from the DEAE-cellulose column was adjusted to 1 M-NaCl and pumped through the STI-Sepharose column at a flow rate of 0.3 ml/cm² per min; 4 ml fractions were collected. The column was washed with 50 ml of Tris buffer and then eluted with 50 mM-glycine/HCl buffer, pH 3.0, containing 1 M-NaCl and 0.1% Brij 35. The fractions were immediately adjusted to pH 7.0, and those containing proteinase activity were combined and concentrated 5-fold in dialysis tubing surrounded by Carbowax. The concentrated preparation was dialysed against 3 × 1 litre of 10 mM-sodium citrate buffer, pH 6.5, containing 0.1% Brij 35 and 0.15 M-NaCl. This purified proteinase was stored at -20°C.

Results

Purification of tryase

The results of a typical purification of tryase are summarized in Table 1.

Solubilization of neutral proteinase. Preliminary experiments showed that 85–90% of the neutral proteolytic activity of post-nuclear supernatants of

Table 1. *Purification of trypsin from rat liver*

The fractions are those described in the Methods section. Proteinase activity is expressed as units of azocasein hydrolysis.

Fraction	Total protein (mg)	Total proteinase (units)	Specific activity (units/mg)	Purification factor	Recovery of proteinase (%)
Liver cytoplasmic extract	30600	367	0.012	1.0	100
High-speed pellet	8300	241	0.029	2.2	65
Washed high-speed pellet	3200	185	0.058	4.8	51
MgCl ₂ extract	1180	87	0.074	6.1	24
DEAE-cellulose	507	77*	0.152	11.5	21
STI-Sepharose (pH 3.0)	0.045	13	290	24200	3.5

* Only 57 units of enzyme activity were applied to the STI-Sepharose column, the remaining 20 units being used for gel chromatography.

Fig. 1. *Subcellular fractionation of rat liver*

Rat liver homogenate was centrifuged as described in the Methods section to yield nuclear (N), mitochondrial (M), lysosomal (L), microsomal (E) and cytosol (C) fractions. These were assayed for succinate dehydrogenase (a), cathepsin D (b), activity against D-Pro-Phe-Arg-NPhNO₂ at pH 7.5 (c) and activity against azocasein at pH 7.5 (d). Activity is expressed as relative specific activity [(% activity)/(% protein)] and is plotted against the percentage of the protein in each fraction.

the rat liver homogenates was associated with sedimentable material. The distribution of the activity on azocasein at pH 7.5 among subcellular

fractions is shown in Fig. 1(d): 47% was in the nuclear fraction, 22% in the mitochondrial fraction, 13% in the lysosomal fraction, 12% in the micro-

somal fraction and 6% in the cytosol. The high percentage of activity in the nuclear fraction may have been due in part to the presence of unbroken cells. Because of the uniform distribution of activity between the mitochondrial, lysosomal and mitochondrial fractions, we prepared from the post-nuclear supernatant a washed high-speed pellet that contained all the sedimentable particles. Table 1 shows that 24% of the enzymic activity was lost from this pellet during the washing. The proteinases in the pellet were solubilized by 1M-MgCl₂. This resulted in 75% solubilization with an overall recovery (pellet + supernatant) of 61%. Other solubilizing agents were tried: 1M-KCl solubilized only 20% and Triton X-100 at 0.1% solubilized only 25%, though at 1% it solubilized the whole pellet along with the enzyme. 1M-NaSCN also solubilized activity, but with an unacceptably low recovery (25–30%). MgCl₂ was chosen, since it gave selective solubilization with reasonable recovery.

Column chromatography. The material solubilized by MgCl₂ was chromatographed on DEAE-cellulose (Table 1). All of the proteolytic activity was adsorbed by the cellulose, and 88% was recovered by elution with 0.2M-NaCl in the buffer.

The major purification step was affinity chromatography on STI–Sephacel. A sample containing 57 units of azocaseinase activity was applied to the column. The greater part (31 units) failed to be adsorbed on the column, but 13 units of activity were desorbed at pH 3.0, with a specific activity of 290 units/mg (Table 1); this represents a 24 200-fold increase in specific activity over the liver cytoplasmic extract, and the overall recovery was 3.5% of the proteolytic activity. The activity that was not

adsorbed by the STI–Sephacel column was probably due to other enzymes (see below).

Electrophoresis. On SDS/polyacrylamide-gel electrophoresis the purified proteinase ran as a single protein band of 32 500 mol.wt. (Fig. 2a). A faint trace of another protein was visible in the position of the major 70 000-mol.wt. protein seen in the DEAE-cellulose fraction. Since the enzyme was inhibited by Dip-F (see below), it was treated with [³²P]Dip-F before electrophoresis. A radioautograph of the gel (Fig. 2b) showed a radioactive band coinciding with the protein band and identifying it as the proteinase. When the enzyme was reduced before electrophoresis, it ran with an apparent molecular weight of 28 000 (Fig. 2c), which suggested that it might consist of a 28 000-mol.wt. polypeptide chain, with a 4500-mol.wt. peptide attached by disulphide bonds, although no low-molecular-weight fragments were visible on the gel.

Action of the purified proteinase on synthetic substrates

The enzyme was tested on a number of ester and amide substrates (Table 2). The tripeptide substrates containing an arginine or lysine residue adjacent to the leaving group were hydrolysed rapidly. Suc-Ala-Ala-NPhNO₂ was not hydrolysed. The *K_m* was 0.25 mM for D-Pro-Phe-Arg-NPhNO₂ and 0.53 mM for D-Val-Leu-Arg-NPhNO₂ under the conditions shown in Table 2. The trypsin substrates Tos-Arg-OMe, Bz-Arg-OEt and Bz-Arg-NPhNO₂ were only slowly hydrolysed. The enzyme was 40-fold less active than trypsin against Tos-Arg-OMe and 5-fold less active against Bz-Arg-OEt. Low rates of hydrolysis were also found for

Fig. 2. SDS/polyacrylamide-gel electrophoresis and radioautography of rat liver proteinase purification (a) A dried SDS/10% polyacrylamide-gel that was run under non-reducing conditions. Samples were: S, standard proteins; 1, MgCl₂ extract of high-speed pellet (50 µg of protein); 2, material eluted from DEAE-cellulose by 0.2M-NaCl in the column buffer (50 µg of protein); 3, purified proteinase eluted from STI–Sephacel at pH 3.0 (0.2 µg of protein). Samples were incubated overnight with [³²P]Dip-F before treatment with SDS as described in the Methods section. Standard proteins were those used by Barrett *et al.* (1979). (b) Radioautograph of (a) after 24 h exposure. The sample tracks are numbered as for (a), the positions of the standard proteins are shown by the arrows and the numbers are 10⁻³ × mol.wt. (c) Radioautograph of a gel run as for (a) except that the samples were run under reducing conditions.

Table 2. *Hydrolysis of synthetic substrates by purified tryase*

The assays were done as described in the Methods section with 0.05 M-Tris/HCl buffers and 0.1% Brij 35.

Substrate	Concn. (mM)	pH	Temperature (°C)	Rate of hydrolysis ($\mu\text{mol/min per mg}$)
D-Pro-Phe-Arg-NPhNO ₂	0.8	7.5	37	42.0
D-Val-Leu-Arg-NPhNO ₂	0.8	7.5	37	34.0
D-Val-Leu-Lys-NPhNO ₂	0.8	7.5	37	33.0
Bz-Pro-Phe-Arg-NPhNO ₂	1.0	7.5	37	6.0
Suc-Ala-Ala-Ala-NPhNO ₂	1.0	7.5	37	0.1
Tos-Arg-OMe	1.0	7.8	25	7.4
Tos-Arg-OMe	1.0	7.1	25	5.4
Bz-Arg-OEt	1.0	7.8	25	8
Bz-Arg-OEt	1.0	7.1	25	10
Bz-Arg-NPhNO ₂	1.0	7.8	25	0.03
Bz-Tyr-OEt	0.4	7.1	25	1.2
Bz-Tyr-OEt	0.4	7.8	25	0.8
Z-Ala-ONap	1.0	7.5	50	2.2
Bz-DL-Phe-ONap	1.0	7.5	50	0.54

Bz-Tyr-OEt (50-fold less than chymotrypsin), Z-Ala-ONap (30-fold less than leucocyte elastase) and Bz-DL-Phe-ONap (20-fold less than cathepsin G). For further assays D-Pro-Phe-Arg-NPhNO₂ was chosen as the best substrate.

Effect of inhibitors on the proteinase

Table 3 shows the effects of various potential inhibitors on the proteinase. It was inhibited by Dip-F, benzamidine, aprotinin, STI and lima-bean trypsin inhibitor, but not by chicken ovinhibitor. The peptidyl-chloromethanes Ile-Leu-Arg-CH₂Cl and Phe-Ala-Arg-CH₂Cl were inhibitory at 1 μM , but Tos-Phe-CH₂Cl and Tos-Lys-CH₂Cl were not inhibitory, even when Tos-Lys-CH₂Cl was pre-incubated with the enzyme for 5 h. The enzyme was not affected by EDTA, Ca²⁺, Mg²⁺ or Mn²⁺. The effects of the inhibitors used were the same whether azocasein or the peptide substrate was used, indicating that both were hydrolysed by the same enzyme.

Since the enzyme was inhibited by Dip-F it was of the serine proteinase catalytic class, and it was trypsin-like in that it readily hydrolysed bonds containing a basic amino acid adjacent to the leaving group in the tripeptide substrates. It also readily reacted with the chloromethanes containing arginine as the reactive amino acid. For these reasons it was called tryptase.

pH-dependence of the activity of tryase

The pH-dependence of the activity of the enzyme is shown in Fig. 3. For the substrate D-Pro-Phe-Arg-NPhNO₂, greatest activity was seen in the range pH 7–8, the maximum being at pH 7.3. When azocasein was used as substrate the optimum was found to be broader, in the range pH 6–8.

Subcellular fractionation

The distribution of tryase was studied in sub-

Table 3. *Effect of potential inhibitor on tryase*

Enzymic activity is expressed as a percentage of activity in the absence of inhibitor. Results are shown for the substrates D-Pro-Phe-Arg-NPhNO₂ and azocasein. Inhibitors marked (a) were dissolved in propan-2-ol, and the final solvent concentration was 5% at 0.1 mM inhibitor concentration. Abbreviation: N.D., not determined.

Compound	Final concn. (mM)	Activity remaining (%)	
		D-Pro-Phe-Arg-NPhNO ₂	Azo- casein
Dip-F	1.0	4	0
Benzamidine	2.0	8	N.D.
Tos-Lys-CH ₂ Cl	1.0	84	85
Tos-Phe-CH ₂ Cl (a)	0.1	83	100
Ile-Leu-Arg-CH ₂ Cl (a)	0.1	1	N.D.
Ile-Leu-Arg-CH ₂ Cl (a)	0.01	31	N.D.
Ile-Leu-Arg-CH ₂ Cl (a)	0.001	57	N.D.
Phe-Ala-Arg-CH ₂ Cl (a)	0.1	2	N.D.
Phe-Ala-Arg-CH ₂ Cl (a)	0.01	41	N.D.
Phe-Ala-Arg-CH ₂ Cl (a)	0.001	60	N.D.
6-Aminohexanoate	1.0	90	N.D.
STI	100 $\mu\text{g/ml}$	18	14
Lima-bean trypsin inhibitor	100 $\mu\text{g/ml}$	22	20
Aprotinin	100 $\mu\text{g/ml}$	2	0
Chicken ovinhibitor	100 $\mu\text{g/ml}$	N.D.	96
CaCl ₂	1.0	105	N.D.
MgCl ₂	1.0	101	N.D.
MnCl ₂	1.0	105	N.D.
EDTA	1.0	106	N.D.
Chymostatin	100 $\mu\text{g/ml}$	N.D.	100
Leupeptin	100 $\mu\text{g/ml}$	N.D.	34

cellular fractions of a homogenate of two perfused rat livers. The fractions were assayed for activity against D-Pro-Phe-Arg-NPhNO₂ and azocasein at

Fig. 3. *pH-dependence of the enzymic activity of tryase* Activity against 0.8 mM-D-Pro-Phe-Arg-NPhNO₂ (●) was measured at 37°C in 0.05 M-Tris/HCl buffers with 50 ng of enzyme/assay. Activity against azocasein was measured at 50°C in 0.1 M-Tris/HCl (▲) and 0.1 M-citrate (△) buffers. Activities are expressed as a percentage of the maximum.

pH 7.5. Cathepsin D was assayed as a lysosomal marker and succinate dehydrogenase as a mitochondrial marker. The results are shown in Fig. 1. The specific activities of cathepsin D and succinate dehydrogenase were highest in the lysosomal and mitochondrial fractions respectively. About 50% of the activity against D-Pro-Phe-Arg-NPhNO₂ was in the lysosomal fraction, and showed nearly 6-fold enrichment; 40% was in the mitochondrial fraction, and the remainder was in the nuclear and microsomal fractions. The activity in the lysosomal and mitochondrial fractions was inhibited by Dip-F, aprotinin and Ile-Leu-Arg-CH₂Cl (Table 4). Since these substances inhibited the purified tryase, the activity localized in the lysosomal fraction could be attributed to it. The sharp localization of the enzyme was in marked contrast with the uniform distribution of neutral proteolytic activity against azocasein throughout the fractions (Fig. 1), which showed that other proteinases must have been present.

Tritosome experiments

Although the tryase was localized in the 'lysosomal' fraction, it was not necessarily in lysosomes; it could have originated from another organelle of similar sedimentation properties. To test this possibility lysosomes were more thoroughly purified in the form of 'tritosomes'. The detergent Triton WR-1339 is taken up into the lysosomal system, producing modified lysosomes of diminished buoyant density that are more easily isolated than are natural lysosomes. The tritosomes were separated from other granules on a discontinuous sucrose gradient as described in the Methods section. The

Table 4. *Inhibition of tryase activity in subcellular fractions*

The subcellular fractions shown in Fig. 1 were assayed on the substrate D-Pro-Phe-Arg-NPhNO₂ at pH 7.5 in the presence of inhibitors. Enzymic activity is expressed as a percentage of activity in the absence of inhibitor.

Inhibitor	Final concn.	Activity remaining (%)	
		Lysosomal fraction	Mitochondrial fraction
Dip-F	5 mM	0	11
Ile-Leu-Arg-CH ₂ Cl	0.1 mM	16	18
Aprotinin	100 µg/ml	19	23

tritosome layer (fraction G1) contained only 1.3% of the recovered protein, the intermediate layer (fraction G2) had 6.0%, and the pellet (fraction G3) 92.7%. Fraction G1 was 18- and 25-fold enriched in cathepsins B and D respectively; these are both lysosomal proteinases. All the succinate dehydrogenase and urate oxidase (a peroxisomal marker) were found in fraction G3. Only 1% of the activity on D-Pro-Phe-Arg-NPhNO₂ was in fraction G1, and 81% was in fraction G3. The detergent was not inhibitory to the purified tryase, and the same amount of activity on D-Pro-Phe-Arg-NPhNO₂ was found in livers from normal and injected rats. The distribution of azocaseinase activity was similar to that found with the peptide substrate except that there was a small amount (6%) in fraction G1. Since the tryase showed a distribution different to that of the lysosomal proteinases, it must be localized in a different organelle.

Further investigation of neutral proteinases by gel chromatography

Some of the sample from DEAE-cellulose chromatography (see Table 1) was subjected to gel chromatography on Ultrogel AcA-44 (Fig. 4). Two peaks of enzymic activity against azocasein were detected, with elution volumes corresponding to (A) 90 000 mol.wt. and (B) 35 000 mol.wt. The enzyme in peak A was inhibited 90% by 1 mM-Dip-F, which indicated that it was a serine proteinase, although it failed to be adsorbed on STI-Sepharose. On rechromatography on Ultrogel AcA-34 it was eluted at a position corresponding to 100 000 mol.wt. It hydrolysed D-Pro-Phe-Arg-NPhNO₂ at one-twelfth of the rate of tryase (relative to the rates of action on azocasein).

When material from peak B was passed through a 2 ml STI-Sepharose column 40% of the activity against azocasein was adsorbed and could be eluted at pH 3.0. Enzyme purified in this way was electrophoretically identical with the tryase shown in Fig. 2, and it also hydrolysed D-Pro-Phe-Arg-NPhNO₂ at

Fig. 4. Gel chromatography of rat liver material from DEAE-cellulose

Chromatography was done on a column (1.5 cm × 90 cm) of Ultrogel AcA-44 with 0.1 M-Tris/HCl buffer containing 0.1% Brij and 1 M-NaCl at pH 7.5. The sample was that from DEAE-cellulose (see Table 1) and contained 20 units of activity against azocasein. A_{280} (—) and hydrolysis of azocasein at pH 7.5 (----) of the fractions are shown.

the same rate. About 30% of the activity failed to be adsorbed on STI-Sepharose, and 30% was not recovered. On rechromatography of the unadsorbed material most of its activity failed to be adsorbed, but 15% was recovered by elution at pH 3.0. It was not possible to prove whether peak B contained proteinases other than trypase that could be isolated by affinity chromatography.

Discussion

The livers were perfused before homogenization in order to remove blood cells, which contain proteinases, and plasma inhibitors, which could have masked proteolytic activity.

The purified proteinase was unequivocally identified on electrophoresis by its labelling with [32 P]-Dip-F, and the molecular weights determined by electrophoresis (32 500) and gel chromatography (35 000) were in close agreement.

Although the enzyme showed a trypsin-like specificity for bonds involving basic amino acid residues in the tripeptide substrates, there was little hydrolysis of blocked arginine ester and amides. The enzyme apparently required more than a single amino acid residue in the substrate for hydrolysis to occur. This may account for the failure of Tos-Lys-CH₂Cl to cause inhibition although the tripeptidyl-chloromethanes were inhibitory at 1 μ M. The enzyme belongs to the trypsin family, and rapidly hydrolysed D-Pro-Phe-Arg-NPhNO₂ (a substrate for plasma kallikrein), D-Val-Leu-Arg-NPhNO₂ (a substrate for glandular kallikrein) and D-Val-Leu-Lys-NPhNO₂ (a substrate for plasmin). It differed from the kallikreins in being a non-specific

proteinase; its specific activity on azocasein was about the same as that of elastase or cathepsin G. Its substrate-specificity was quite different from that of the 'mitochondrial' proteinase (or chymase) of rat liver described by Jusic *et al.* (1976) and Haas *et al.* (1979), which hydrolysed the chymotrypsin substrate Ac-Tyr-OEt, was inhibited by Tos-Phe-CH₂Cl and was of lower molecular weight (23 500).

Neutral proteinases have been found associated with chromatin and nuclei of rat liver (Carter & Chae, 1976; Ramponi *et al.*, 1978) and of other tissues (Furlan *et al.*, 1968; Kurecki *et al.*, 1975; Katz *et al.*, 1976, 1977). We found neutral proteolytic activity in the nuclei, but it could not be attributed to trypase, since there was little activity against D-Pro-Phe-Arg-NPhNO₂. Neutral proteinase activity has also been found in microsomal fractions of rat and monkey livers (Sogawa & Takahashi, 1978, 1979). The rat liver microsomal fraction was shown to contain a serine proteinase of 90 000 mol.wt. This could be the same enzyme that was eluted in peak A from the Ultrogel AcA-44 column in the present study.

A trypsin-like enzyme was found in extracts of rat intestinal muscle by Beynon & Kay (1978). It was of similar size to trypase (33 000 mol.wt. on gel filtration), but, because it was inhibited by Tos-Lys-CH₂Cl at low concentration (37 μ M), it was probably a different enzyme. A trypsin-like proteinase has also been found in the granules of mast cells of humans, dogs, cats and mice, but not of rats (Lagunoff, 1968; Vensel *et al.*, 1971). This enzyme was called tryptase by Lagunoff & Benditt (1963); it has not been purified or well characterized, but it hydrolyses Tos-Arg-OMe and is inhibited by Tos-Lys-CH₂Cl (Vensel *et al.*, 1971).

Subcellular fractionation of rat liver showed that the trypase was associated with particles similar to lysosomes in their sedimentation properties, but its complete absence from tritosomes was strong evidence against it being in the lysosomes themselves. Its solubilization characteristics also were not typical of a lysosomal enzyme. It was possible that it was in peroxisomes, which sediment with lysosomes during rate-sedimentation, and there has been a report of neutral proteolytic activity associated with peroxisome-rich fractions of rat liver (Gray *et al.*, 1970), although that activity was not characterized. However, the intermediate layer (fraction G2) from the density gradient contained 18% of the proteinase, but no urate oxidase. Indeed, fraction G2 contained no succinate dehydrogenase either, suggesting that some of the proteinase was associated with organelles of a lower buoyant density than mitochondria (peroxisomes are of higher buoyant density).

The function of trypase is unknown, but its specificity for basic amino acid residues means that

it could be capable of processing pro-polypeptides to their natural secreted forms. Proalbumin is converted into albumin in the hepatocyte by cleavage at a pair of basic (-Arg-Arg-Glu-) amino acid residues that removes the pro-peptide (Russell & Geller, 1975). Conversion of pro-polypeptides by cleavage at dibasic sites has been found for a number of secreted proteins, including proinsulin (Kemmler *et al.*, 1973), proparathyrin (Hamilton *et al.*, 1974) and the corticotropin- β -lipotropin precursor (Mains *et al.*, 1977; Nakanishi *et al.*, 1979). The site of these conversions and the enzymes involved are unknown. Proalbumin may be converted in the secretory vesicles (Ikehara *et al.*, 1976), and lysosomal proteinases have been proposed as processing enzymes (Hales, 1978; Quinn & Judah, 1978). Clearly tissue proteinases that show a specificity for basic amino acid residues must be considered as possible protein-processing enzymes.

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