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Tryptase from Rat Skin: Purification and Properties[†,‡]

Vincent J. Braganza[§] and William H. Simmons*
Department of Molecular and Cellular Biochemistry, Stritch School of Medicine, Loyola University of Chicago, Maywood, Illinois 60153

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ABSTRACT: Tryptase was purified 13 000-fold to apparent homogeneity from rat skin. The two-step procedure involved ammonium sulfate fractionation of the initial extract followed by combined sequential affinity chromatography on agarose–glycyl-glycyl-*p*-aminobenzamidine and concanavalin A–agarose. The purified enzyme had a specific activity toward *N*-benzoylarginine ethyl ester (BzArgOEt) of 170 μmol/min mg^{-1} and was obtained in a yield of 28% as determined by the specific substrate, H-D-Ile-Pro-Arg-*p*-nitroanilide. Rat skin tryptase was thermal labile, losing 50% of its activity when preincubated for 30 min at 30 °C. The presence of NaCl (1 M) improved thermal stability and was necessary for long-term storage. Heparin did not stabilize the enzyme against thermal denaturation, and heparin–agarose failed to bind the enzyme. Rat skin tryptase was inhibited by diisopropylphosphofluoridate, antipain, leupeptin, and aprotinin but not by α_1-antitrypsin, ovomucoid, or soybean or lima bean trypsin inhibitors. Substrate specificity studies using a series of tri- and tetrapeptidyl-*p*-nitroanilide and peptidyl-7-amino-4-methylcoumarin substrates demonstrated the existence of an extended substrate binding site. Rat skin tryptase hydrolyzed [Arg^8]vasopressin, neurotensin, and the oxidized B-chain of insulin at the -Arg^8-Gly^9-NH$_2$, -Arg^8-Arg^9-, and -Arg^{22}-Gly^{23}-bonds, respectively. No general proteinase activity was observed toward casein, hemoglobin, or azocoll. Rat skin tryptase had a M_r of 145 000 by gel filtration. The subunit M_r was either 34 000 or 30 000 depending on the electrophoretic technique used. Treatment of the enzyme with peptide N-glycosidase F (N-glycanase) decreased the subunit M_r by 4000. The enzyme exhibited multiple isoelectric forms (p*I*'s of 4.5–4.9). Rat skin tryptase was found to be related statistically to other tryptases on the basis of amino acid composition. The N-terminal amino acid sequence was Ile^1-Val^2-Gly^3-Gly^4-Gln^5-Glu^6-Ala^7-Ser^8-Gly^9-Asn^{10}-Lys^{11}-Trp^{12}-Pro^{13}-Trp^{14}-Gln^{15}-Val^{16}-Ser^{17}-Leu^{18}-Arg^{19}-Val^{20}---^{21}-Asp^{22}Thr^{23}-Tyr^{24}-Trp^{25}-, with a putative glycosylation site at residue 21. This sequence was 72–80% homologous with the N-terminus of other tryptases but only 40% homologous with that of bovine trypsin.

Tryptase is a protease that is stored within the secretory granules of mast cells and released in active form upon mast cell activation (Schwartz et al., 1981a). Tryptase is presumed to play a role in the immunoglobulin E dependent and drug-induced immediate hypersensitivity reactions mediated by mast cells and has recently been used as a specific marker of these events in humans (Schwartz et al., 1987). Although the precise in vivo function of tryptase is unknown, in vitro studies have shown that certain tryptase preparations can activate complement factor C3 to C3a anaphylatoxin (Schwartz et al., 1983), convert prothrombin to thrombin (Kido et al., 1985a), destroy both fibrinogen (Schwartz et al., 1985) and high molecular weight kininogen (Maier et al., 1983), activate latent rheumatoid synovial collagenase (Gruber et al., 1988), and hydrolyze vasoactive intestinal peptide, peptide histidine–methionine (PHM), calcitonin gene-regulated peptide (Tam & Caughey, 1990), and ACTH (1–39) (Cromlish et al., 1987).

To date, tryptase has been purified from various human tissues [pulmonary mast cells (Schwartz et al., 1981b), lung (Smith et al., 1984; Harvima et al., 1988), pituitary (Cromlish et al., 1987), and skin (Harvima et al., 1988)] as well as from dog mastocytoma cells (Caughey et al., 1987) and rat peritoneal mast cells (Kido et al., 1985b). These different tryptase preparations have many properties in common. However,

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[‡]Submitted by V.J.B. in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Loyola University of Chicago.
*Author to whom correspondence should be addressed.
[§]Present address: St. Xavier's College, Ahmedabad 380009, India.

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recent cloning experiments have demonstrated the presence within a species of multiple mast cell tryptase mRNAs (four from human and two from mouse) indicating the existence of a new multigene serine protease family (Miller et al., 1989, Vanderslice et al., 1990; Chu et al., 1990; Dr. David A. Johnson, personal communication). Whether each gene product plays a specific role in mast cell function remains to be determined. The answer to this question will depend in part on the purification and characterization of additional forms of tryptase.

In this paper, we report on the purification to homogeneity of a novel tryptase-like protease from rat skin. The procedure used to isolate this enzyme represents a modification of techniques used previously to purify carboxamidopeptidase (Simmons & Walter, 1980, 1981). Preliminary accounts of this work have appeared elsewhere (Braganza & Simmons, 1986, 1987, 1988; Simmons & Braganza, 1988).

Experimental Procedures

Materials

The peptidyl-pNA[1] substrates shown in Table III were the products of either KabiVitrum and Helena Labs, American Diagnostica, Immuno AG, Diagnostica Stago, or Boehringer Mannheim and were the generous gifts of Dr. Jaweed Fareed. 7-Amino-4-methylcoumarin substrates were from Sigma except for Z-Ala-Arg-Arg-AMC (Bachem Bioscience). N-Glycanase (peptide N-glycosidase F) and endoglycosidase H were from Genzyme and Boehringer Mannheim, respectively. Heparin from bovine lung (sodium salt, H-9133), oxidized B-chain of bovine insulin, and agarose–glycyl-glycyl-*p*-aminobenzamidine were from Sigma. Neurotensin, Ac-Arg-Arg-Pro-Tyr-Ile-Leu, and [Arg^8]vasopressin were from Bachem, Inc. Concanavalin A–agarose was from Bethesda Research Laboratories. iPr_2PF was from Aldrich, and [^{3}H]iPr_2PF (3.9 Ci/mmol) was from New England Nuclear. Aprotinin (Trasylol) was the product of Farbenfabriken Bayer AG. [Arg^8,^{14}C-Gly^9-NH_2]vasopressin was synthesized in the laboratory of the late Dr. Roderich Walter. Des(Gly^9-NH_2)-[Arg^8]vasopressin was from Peninsula.

Methods

Enzyme Assays. Unless otherwise indicated, all assays were done at 25 °C with use of 1 mM substrate in phos–EDTA–7.5 buffer.[1] The standard assay for tryptase was the esterase activity toward BzArgOEt determined by the spectrophotometric method of Schwert and Takenaka (1955). The hydrolysis of *p*-nitroanilide (pNA) substrates was determined spectrophotometrically at 405 nm (Svendsen et al., 1972). The hydrolysis of peptidyl-7-amino-4-methylcoumarin (-AMC) substrates was determined fluorometrically according to Barrett (1980).

Protein Determination. Protein concentration was determined by the micro dye-binding method of Bradford (1976) with bovine serum albumin as the standard.

Tissue Preparation and Extraction. Male Sprague-Dawley rats were sacrificed by decapitation and their skins removed. Skins not immediately used for enzyme extraction were stored frozen at −20 °C and were thawed out overnight at 4 °C before use. About 12–15 skins weighing a total of 300–450 g were used for each enzyme preparation. The fur was clipped away as close as possible to the epidermis with Oster animal grooming clippers. Facia, adipose tissue, and the thin muscle sheet that is closely appended to the dermis (*m. cutaneous-trunci*) were scraped off by use of an anatomical razor. The skin was then washed in cold 0.65% saline and minced in a Universal No. 3 meat grinder through two cycles. All subsequent purification procedures were performed at 4 °C unless otherwise indicated. The minced skin was homogenized in phos–EDTA–0.5 M NaCl–7.0 (1:9 w/v) in a Waring blender. Homogenization was carried out at high speed for six 20-s intervals with 20-s rest intervals in between. The resulting homogenate was filtered under vacuum through a Buchner funnel lined with a double-layer nylon mesh.

Ammonium Sulfate Fractionation. The filtrate from the previous step was collected and slowly brought to 25% $(NH_4)_2SO_4$ saturation. The solution was then allowed to stand undisturbed for 4–6 h and was then centrifuged at 13700*g* for 15 min. The solid material was discarded and the resulting solution rapidly brought to 80% $(NH_4)_2SO_4$ saturation. This mixture was allowed to stand unstirred for 14 h and was then centrifuged as above. The sediment was collected and resuspended in 100 mL of phos–EDTA–0.5 M NaCl–6.0 and then dialyzed against the same buffer. Insoluble material in the retentate was removed by centrifugation.

Sequential Affinity Chromatography on Agarose–Glycyl-glycyl-p-aminobenzamidine and Concanavalin A–Agarose. The centrifuged retentate was applied at 25 mL/h to an agarose–glycyl-glycyl-*p*-aminobenzamidine (A-Gly-Gly-PAB) affinity column (1.5 × 20 cm) that had been equilibrated with phos–EDTA–0.5 M NaCl–6.0. The sample reservoir was maintained on ice during the procedure while the column itself and the inlet tubing were equilibrated at room temperature. The column was then washed at room temperature with one column volume (25 mL) of phos–NaN_3–0.5 M NaCl–6.0. The column was placed for 1 h at 4 °C and then washed with one column volume of cold phos–NaN_3–0.5 M NaCl–6.0. The enzyme was eluted with phos–NaN_3–0.5 M NaCl–6.0 containing 0.1 M benzamidine hydrochloride. When one-third of a column volume (approximately 10 mL) of the elution buffer had been passed through the A-Gly-Gly-PAB column, the column outlet tubing was connected to a concanavalin A–agarose column (1 × 10 cm) equilibrated with phos–NaN_3–0.5 M NaCl–6.0. The flow rate was immediately reduced to 10 mL/h. The two affinity columns connected in series were further washed with 40 mL of the benzamidine-containing buffer. The first column was subsequently removed from the system and the concanavalin A–agarose column was then washed with one column volume (10 mL) of phos–NaN_3–0.5 M NaCl–6.0 at a flow rate of 5 mL/h. One-third of a column volume (3.5 mL) of a solution of 100 mg/mL of methyl α-D-mannoside in phos–NaN_3–0.5 M NaCl–6.0 was passed through the column and the flow was stopped. One hour later, elution was continued with use of the same buffer and fractions of 1 mL were collected. Fractions containing BzArgOEt activity were pooled and then concentrated with use of Centricon-30 microconcentrator units (Amicon). The

[1] Abbreviations: pNA, *p*-nitroanilide; AMC, 7-amino-4-methylcoumarin; BzArgOEt, *N*-benzoyl-L-arginine ethyl ester; BzArgOMe, *N*-benzoyl-L-arginine methyl ester; TosArgOMe, *N*-tosyl-L-arginine methyl ester; AcTyrOEt, *N*-acetyl-L-tyrosine ethyl ester; A-Gly-Gly-PAB, agarose–glycyl-glycyl-*p*-aminobenzamidine; SDS–PAGE; sodium dodecyl sulfate–polyacrylamide gel electrophoresis; iPr_2PF, diisopropylphosphofluoridate; EDTA, ethylenediaminetetraacetic acid; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; MU, methylumbelliferone; Tos-Lys-CH_2Cl, N^{α}-tosyl-L-lysine chloromethyl ketone; Tos-Phe-CH_2Cl, N^{α}-tosyl-L-phenylalanine chloromethyl ketone; Z-Phe-CH_2Cl, *N*-(benzyloxycarbonyl)-L-phenylalanine chloromethyl ketone; PCMB, *p*-(chloromercuri)benzoate. Buffer abbreviations refer to 0.025 M sodium phosphate containing 0.001 M EDTA (or 0.02% NaN_3) and the indicated concentration of NaCl at the indicated pH (e.g., phos–EDTA–0.5 M NaCl–7.5).

sample buffer was changed to phos–EDTA–0.5 M NaCl–6.0 in the microconcentrators by use of two dilution–concentration cycles. The final preparation contained about 75 μg of pure enzyme in 2.5 mL of buffer, which was typically stored at 4 °C.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli (1970) with use of a discontinuous vertical slab gel system with 12.5% separation gels. Protein samples were prepared for electrophoresis by heating them to 100 °C for 5 min in a solution of 5 M urea–2% sodium dodecyl sulfate either with or without 2% 2-mercaptoethanol (reducing vs nonreducing conditions, respectively). Gels were stained with Coomassie Brilliant Blue. Alternatively, SDS–PAGE was carried out under reducing conditions with use of a Pharmacia PhastSystem with homogeneous 12.5% gels and PhastGel SDS buffer strips. Gels were silver stained by use of the protocol provided with the PhastSystem development unit.

[^3H]iPr$_2$PF-Labeled Enzyme. Purified enzyme was incubated with 25 μM [1,3-^{3}H]diisopropylphosphofluoridate ([^{3}H]iPr$_2$PF) (3.9 Ci/mmol) at 25 °C for 72 h in 0.5 mL of phos–EDTA–0.5 M NaCl–6.0. Unlabeled iPr$_2$PF (2 μL of 250 mM) was then added, and the incubation continued for 1 h. Free [^{3}H]iPr$_2$PF was removed by repeated concentration and dilution of the sample with use of a Centricon-30 microconcentrator. The final concentrated, labeled enzyme was subjected to SDS–PAGE according to the vertical slab gel method described above. The gel lane containing labeled enzyme was cut into 2-mm slices that were each placed into 0.3 mL of 1 N NaOH for 12 h at 25 °C. Each NaOH extract was diluted with 0.35 mL of 1 N HCl and then added to 10 mL of scintillation fluid and counted for radioactivity.

Active Site Titration. The concentration of active enzyme was determined by active site titration with use of 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB) according to the method of Jameson et al. (1973).

Heparin–Agarose Binding. Potential binding of purified rat skin tryptase to heparin–agarose at 4 °C was determined essentially as described by Alter et al. (1987). A 1-mL column of heparin–agarose (GIBCO BRL, No. 5907SA, lot AEOA01) (containing 0.9 mg of heparin) was prepared, and the column was subsequently washed with 20 column volumes of phos–EDTA–7.5. Purified tryptase (0.5 μg in 83 μL of 0.025 M sodium phosphate containing 1 mM EDTA and 0.12 M NaCl, pH 7.3) was applied to the column. The column was then washed with phos–EDTA–7.5, and 0.5 mL fractions were collected and assayed for BzArgOEt activity.

In order to remove any bound endogenous heparin glycosaminoglycan from tryptase prior to heparin–agarose chromatography, a modification of the method of Schwartz et al. (1981b) was used. Purified tryptase (1.8 μg) was applied to a 1-mL AG-1X2 (Bio-Rad) anion-exchange column equilibrated with phos–EDTA–0.5 M NaCl–6.0 at 4 °C. An aliquot (0.5 μg) of enzyme that eluted from this column was brought to 0.025 M sodium phosphate, 1 mM EDTA, and 0.12 M NaCl, pH 7.3, and applied to a 1-mL heparin–agarose column essentially as described above.

Hydrolysis of Peptide Hormones and Fragments. Purified rat skin tryptase (2–4 pmol) was incubated with various peptides (0.1 μmol) in phos–EDTA–0.5 M NaCl–7.5 at 25 °C for varying lengths of time. The reaction mixtures were filtered through an Amicon PM-30 membrane and subjected to high-performance liquid chromatography (HPLC) with detection at 206 nm. For vasopressin, an Ultrasphere ODS reversed-phase column (3.9 mm × 30 cm) (5 μm) was used. Separation was performed isocratically at 1 mL/min with use of a mobile phase of 0.1% trifluoroacetic acid–acetonitrile (84:16) that was capable of separating vasopressin from its des(Gly-NH$_2$)-metabolite, which was used as a standard.

The products of other cleaved peptides were separated with gradient programs utilizing 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). For the oxidized B-chain of insulin, a μBondapak column was used with a linear gradient of 0–60% B in 30 min (1 mL/min). For neurotensin and its analogue (Ac-Arg-Arg-Pro-Tyr-Ile-Leu), an Ultrasphere ODS column was used with the following program: 0–25% B in 30 min followed by 25% B from 30 to 60 min (1 mL/min). In each case, product peaks were collected and subjected to amino acid sequence analysis.

Molecular Weight Determination. The subunit molecular weight was determined with the vertical slab SDS–PAGE system described above with use of the Dalton Mark VII-L mixture of molecular weight standards (Sigma). Alternatively, SDS–PAGE was carried out with the Pharmacia PhastSystem as described above with use of the Sigma Mark VI molecular weight standard kit. The molecular weight of undenatured tryptase was performed by high-performance gel permeation chromatography at 25 °C with use of a Synchropak GPC300 column (25 cm × 4.6 mm) equilibrated with phos–EDTA–0.5 M NaCl–6.0. The column was run at 0.5 mL/min, and protein peaks were monitored at 280 nm.

Enzymatic Deglycosylation. Purified tryptase (4.7 μg) was incubated with 0.6 unit (0.03 μg) of peptide N-glycosidase F (N-glycanase) from *Flavobacterium meningosepticum* (Tarentino et al., 1985) in 0.3 M sodium phosphate, pH 7.5, at 37 °C overnight. In a parallel experiment, tryptase was boiled for 5 min in the presence of 0.024% sodium dodecyl sulfate and 0.05 M 2-mercaptoethanol prior to the addition of peptide N-glycosidase F. Both samples and an untreated tryptase control were subjected to SDS–PAGE along with molecular weight standards with use of the Pharmacia PhastSystem as described above.

Amino Acid Analysis. Amino acid analysis was performed by Alan J. Smith at the Protein Structure Research Labs of the School of Medicine, University of California at Davis, using a Beckman Model 6300 amino acid analyzer. Samples of enzyme (approximately 0.2 nmol) were hydrolyzed in 6 N HCl at 100 °C for either 24, 48, or 72 h. The amino acid composition of a buffer control was subtracted from the composition at each time point. Serine and threonine were determined by extrapolation to zero-time hydrolysis. The 72-h values were used for valine, isoleucine, and leucine. Cysteine and methionine were determined in the form of cysteic acid and methionine sulfone, respectively, following performic acid oxidation. The values for all other amino acids were averages of all three time points. Tryptophan was not determined.

Amino Acid Sequence. The N-terminal amino acid sequence of rat skin tryptase was determined by the automated sequential Edman procedure (Edman, 1950) by Mr. Russ Blacher at Applied Biosystems, Inc. The amino acid sequences of the tryptase-generated fragments of the oxidized B-chain of bovine insulin, neurotensin, and the neurotensin analogue were determined at Loyola University's Macromolecular Analysis Facility by Dr. Bassam T. Wakim using an Applied Biosystems 477A pulsed liquid phase protein/peptide sequencer.

Results

Purification of Rat Skin Tryptase. The summary of a representative purification of rat skin tryptase is shown in Table I. Enzyme activity in Table I is expressed in terms of

Table I: Purification of Rat Skin Tryptase

step	substrate	total activity (units[a])	total protein (mg)	sp. activity (units/mg)	% recovery	purification
rat skin extract[b]	BzArgOEt	153	3320	0.046	100	1
	H-D-Ile-Pro-Arg-pNA	340	3320	0.102	100	1
25–80% $(NH_4)_2SO_4$	BzArgOEt	86.8	1500	0.058	57	1.3
	H-D-Ile-Pro-Arg-pNA	197	1500	0.131	58	1.3
sequential affinity chromatography[c]	BzArgOEt	12.1	0.073	170	8	3700
	H-D-Ile-Pro-Arg-pNA	95.7	0.073	1300	28	13000

[a] 1 unit = 1 μmol of substrate hydrolyzed/min at 25 °C with use of 1 mM substrate in phos-EDTA-7.5. [b] An aliquot of filtered rat skin extract was centrifuged at 13000*g*, and the resulting supernatant was assayed. [c] Sequential affinity chromatography was performed on agarose–Gly-Gly-*p*-aminobenzamidine + concanavalin A–agarose.

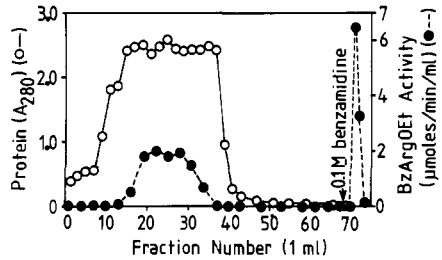

FIGURE 1: Affinity chromatography of rat skin tryptase on agarose–glycyl-glycyl-*p*-aminobenzamidine (A-Gly-Gly-PAB). Enzyme from the ammonium sulfate precipitation step was applied to the A-Gly-Gly-PAB column essentially as described in Methods except that 1-mL fractions were collected at this stage instead of using the sequential column technique. The BzArgOEt activity that bound to the column was eluted with buffer containing 0.1 M benzamidine. Key: Protein (A_{280}) (O), BzArgOEt activity (●).

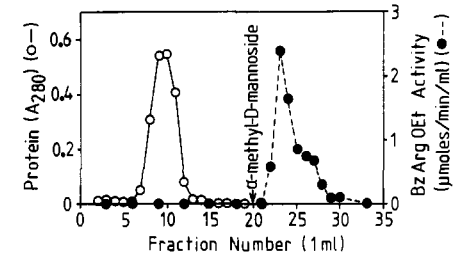

FIGURE 2: Concanavalin A–agarose affinity chromatography. BzArgOEt activity that eluted from the A-Gly-Gly-PAB column with benzamidine was applied to a concanavalin A–agarose column by use of the conditions described in Methods. Enzyme activity was eluted with buffer containing 100 mg/mL methyl α-D-mannoside. Key: Protein (A_{280}) (O), BzArgOEt activity (●).

the routine nonspecific substrate BzArgOEt and the more specific substrate D-Ile-Pro-Arg-pNA.

BzArgOEt activity was optimally extracted from rat skin with buffer containing 0.5 M NaCl. Higher concentrations of salt did not extract additional BzArgOEt activity and prevented proper sedimentation of the enzyme upon ammonium sulfate fractionation. The precipitation of enzyme activity with ammonium sulfate between 25 and 80% saturation removed about half of the contaminating protein and served to concentrate the enzyme for affinity chromatography.

Tryptase was observed to bind to an affinity column consisting of *p*-aminobenzamidine immobilized to agarose through a glycyl-glycine spacer arm (A-Gly-Gly-PAB) (Figure 1). Bound tryptase could be eluted from the column with 0.1 M benzamidine. The bulk of the contaminating protein, as well as considerable non-tryptase BzArgOEt activity (70–80% of the applied activity), did not bind to the column. The breakthrough BzArgOEt activity also did not bind when reapplied to a regenerated A-Gly-Gly-PAB column, indicating that the breakthrough activity did not result from column overloading. Furthermore, only the benzamidine-eluted enzyme (not the breakthrough activity) could hydrolyze the more specific tryptase substrate D-Ile-Pro-Arg-pNA.

Preliminary experiments had indicated that the degree of purification achieved with the A-Gly-Gly-PAB column was highly dependent on temperature and pH. When enzyme was applied to a A-Gly-Gly-PAB column equilibrated at room temperature, a nearly 10-fold higher purification was obtained compared to that achieved at 4 °C. This phenomenon was due to a decrease in binding of low-affinity contaminating protein to the column. If the column was equilibrated at room temperature at pH 6.0 rather than at pH 7.0, an additional doubling of the purification factor was obtained. The optimized A-Gly-Gly-PAB affinity chromatography procedure resulted in an approximately 3300-fold purification of the enzyme compared to that of the applied ammonium sulfate fraction.

The tryptase activity that eluted from the A-Gly-Gly-PAB column was further purified on concanavalin A–agarose (Figure 2), suggesting that the enzyme is a glycoprotein. Enzyme activity bound to this column while the remaining contaminating protein eluted in the breakthrough fractions. The enzyme was then eluted from the column with buffer containing 100 mg/mL methyl α-D-mannoside. This affinity step provided an additional 3-fold purification.

While Figures 1 and 2 show the results of the individual A-Gly-Gly-PAB and concanavalin A–agarose steps, the two columns were routinely connected in series at one point in the procedure and run as a single chromatography step. After the contaminating protein had completely eluted from the A-Gly-Gly-PAB column, the outlet tubing from this column was connected to the inlet of the concanavalin A–agarose column. Benzamidine was then used to elute the enzyme from the A-Gly-Gly-PAB column directly onto the concanavalin A–agarose column as described in Methods. The concanavalin A–agarose column was then disconnected and washed and the enzyme subsequently eluted with methyl α-D-mannoside. This procedure resulted in an approximately 10000-fold purification over the ammonium sulfate fraction in one step. The overall procedure, shown in Table I, resulted in a 13000-fold purification and a 28% recovery of activity when assayed with the specific substrate D-Ile-Pro-Arg-pNA. (The lower recovery and resulting lower magnitude of purification using BzArgOEt as a substrate in Table I are consequences of the multiple non-tryptase BzArgOEt-hydrolyzing enzymes in the crude extract, which were removed during the purification procedure.)

Purity. Figure 3 shows that when the purified enzyme was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under nonreducing conditions, a single band was observed with use of Coomassie Brilliant Blue stain. When the enzyme was labeled by the covalent incorporation of [^{3}H]diisopropylphosphofluoridate prior to SDS–PAGE electrophoresis, a single peak of radioactivity was observed in the gel that comigrated with the Coomassie-stained band (Figure 3). These results suggest that the stained band

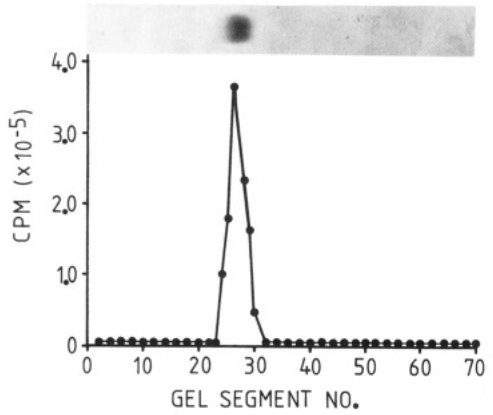

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of purified and radiolabeled rat skin tryptase. Purified tryptase was prelabeled with [^{3}H]iPr_2PF and subjected to SDS–PAGE under nonreducing conditions according to the vertical slab gel technique as described in Methods. The gel was stained with Coomassie Brilliant Blue (insert) and then cut into 2-mm sections that were extracted and counted for radioactivity as described in Methods.

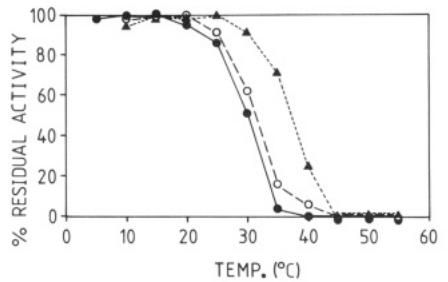

FIGURE 4: Thermal stability of purified rat skin tryptase. Purified enzyme (approximately 0.2 μg) was preincubated at various temperatures for 30 min in 0.1 mL of either phos–EDTA–7.5 (●), phos–EDTA–7.5 containing 1.8 μg/mL bovine lung heparin (○), or phos–EDTA–7.5 containing 1 M NaCl (▲). The residual activity of the entire preincubation volume was determined by use of the standard BzArgOEt assay (1 mL total volume) at 25 °C. Percent residual activity was plotted as a function of preincubation temperature.

was in fact tryptase and that the preparation was homogeneous by this electrophoretic technique. N-Terminal amino acid sequence analysis of the purified enzyme by the Edman technique revealed a single unambiquous sequence.

Stability to Storage. When stored in phos–EDTA–0.5 M NaCl–6.0, the purified enzyme was stable for at least six months at 4 °C and for at least eight days at 25 °C. The enzyme was also stable in this buffer to repeated (at least six) freeze/thaw cycles (–20 °C). However, storage of the purified enzyme at 4 °C in buffer not containing NaCl resulted in the rapid loss of at least half of the original activity. When the purified enzyme was evaporated to dryness in a Savant Speed-vac concentrator from phos–EDTA–0.5 M NaCl–6.0 containing 2% 20 M Carbowax, 80% of the activity was recovered upon reconstitution with water following eight days of storage at either 4 or 25 °C.

pH Stability. The purified enzyme was relatively stable over the pH range of 3.5–7.5 when preincubated in various 0.1 M buffers at 25 °C for 2 h. However, essentially all activity was lost upon preincubation at pH values below 3.5 or above 7.5 under the same conditions.

Thermal Stability. Enzyme was preincubated at various temperatures for 30 min in phos–EDTA–7.5. The residual BzArgOEt-hydrolyzing activity was then determined by the standard assay at 25 °C. The results are shown in Figure 4. The enzyme was stable under these preincubation conditions only up to 25 °C. At 30 °C, 50% of the activity was irreversibly lost. Essentially no activity remained at temperatures at or above 35 °C. For this reason, routine assays were performed at 25 °C.

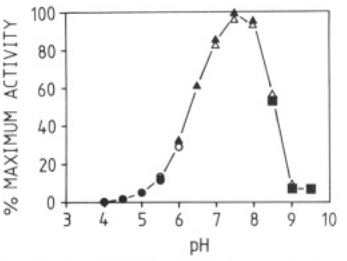

FIGURE 5: Relative BzArgOEt-hydrolyzing activity as a function of pH. The hydrolysis of 1 mM BzArgOEt by tryptase was determined spectrophotometrically as described in Methods except that phos–EDTA–7.5 was replaced with various 0.1 M buffers. Each value represents the relative rate of hydrolysis of substrate averaged over the 15 min incubation period at 25 °C. Rates were corrected for nonenzymatic hydrolysis of BzArgOEt at the higher pH values. The change in absorbance at 253 nm for complete cleavage of substrate was determined at each pH value. Buffers: acetate (●); succinate (○); phosphate (▲); Tris (△); borate (■).

NaCl and heparin were tested for their ability to stabilize the enzyme against thermal denaturation. NaCl, at 1 M, had a stabilizing effect, allowing 71% of the activity to survive a 30-min preincubation at 35 °C (Figure 4). Heparin (from bovine lung) (1.8 μg/mL), giving a heparin:enzyme weight ratio of approximately 1:1, had little effect on thermal stability (Figure 4). In another experiment, a much higher concentration of heparin (165 μg/mL) failed to stabilize the enzyme against denaturation at 30 °C.

Heparin–Agarose Binding. Purified rat skin tryptase failed to bind to a heparin–agarose column under conditions similar to those used to show tight binding of purified human pulmonary mast cell tryptase (Alter et al., 1987). Essential all (96%) of the applied activity was recovered in breakthrough fractions. To show that the failure of the enzyme to bind to heparin–agarose was not due to the presence of bound endogenous heparin, the enzyme was first passed through an AG-1X2 anion-exchange resin. This resin has been shown to remove heparin from human pulmonary mast cell tryptase (Schwartz et al., 1981b). Heparin binds tightly to this resin, eluting only at 3 M NaCl (Metcalfe et al., 1979). Purified rat skin tryptase did not bind to an AG-1X2 column that was equilibrated with phos–EDTA–0.5 M NaCl–6.0. Enzyme eluting from the AG-1X2 column was then applied to a heparin–agarose column under the same conditions used above. Again, the enzyme failed to bind to heparin–agarose (recovery of activity in the breakthrough fractions was 83%).

Effect of Temperature on Activity. Initial reaction rates were determined at various temperatures (10–50 °C) with use of a saturating concentration of substrate (1 mM BzArgOEt) in phos–EDTA–7.5. An Arrhenius plot (log of velocity versus 1/K) (Segel, 1976) yielded two slopes. The energy of activation was 10.2 kcal/mol in the range of 10–25 °C and 12.7 kcal/mol in the range of 25–50 °C.

pH Optimum. Figure 5 shows the relative BzArgOEt-hydrolyzing activity of the enzyme as a function of pH. Optimal activity was observed at pH 7.5–8.0. Little or no activity was observed below pH 6.0 or above pH 8.5.

Inhibitor Studies. A variety of agents were tested for their effect on enzyme activity with the standard BzArgOEt assay. Some of the results are shown in Table II. Tryptase was almost completely inhibited by a 10-min preincubation with the serine protease inhibitor diisopropylphosphofluoridate (iPr_2PF) (1.0 mM). The time course of inactivation of the enzyme by iPr_2PF (0.1 mM) was carried out as described by

Table II: Effect of Various Agents on Tryptase Activity

agent	concn	% inhibn[a]
iPr_2PF[b]	1.0 mM	93
Tos-Lys-CH_2Cl[b]	1.0 mM	54
NPGB (*p*-nitrophenyl-p′-guanidinobenzoate)[c]	10 μM	100
MUGB (p-methylumbelliferyl-p′-guanidinobenzoate)[c]	10 μM	99
aprotinin (basic pancreatic trypsin inhibitor)[d]	0.1 mg/mL	94
antipain[e]	50 μg/mL	80
leupeptin[e]	50 μg/mL	79
$HgCl_2$[e]	2.0 mM	98
	0.1 mM	27

[a] Each value is the mean of triplicate determinations. [b] The enzyme was preincubated with the agent at the indicated concentration in a volume of 40 μL of phos–EDTA–7.5 for 10 min at 25 °C. The entire volume was then assayed for residual activity by use of the standard BzArgOEt assay (1 mL). Activity was compared to that of the control enzyme preincubated under the same conditions but without the test agent. [c] The enzyme was preincubated with the agent in phos–EDTA–7.5 for 5 min at 25 °C in a total volume of 0.9 mL. Activity was determined with the standard assay by the addition of 0.1 mL of 10 mM BzArgOEt. The indicated concentration of agent is that present during the enzyme assay. [d] Same as footnote *b* except that enzyme was preincubated with the indicated concentration of agent in 0.1 mL for 5 min. [e] As in footnote *c* but 0.1 M Tris, pH 7.5, was used as the assay buffer.

Simmons and Walter (1980). The pseudo-first-order rate constant (k_{obsd}) for this reaction was 1.3×10^{-3} s^{-1}. The second-order rate constant ($k_{obsd}/[iPr_2PF]$) was 13 M^{-1} s^{-1}. Tos-Lys-CH_2Cl, which alkylates the active site histidine residue in trypsin-like proteases, inhibited tryptase. Little or no inhibition was seen by Tos-Phe-CH_2Cl and Z-Phe-CH_2Cl, which inhibit chymotrypsin-like proteases by an analogous mechanism.

Aprotinin (basic pancreatic trypsin inhibitor) (Trasylol) (0.1 mg/mL) (15 μM) inhibited tryptase 94% when preincubated with the enzyme for 5 min (Table II). When the enzyme was added to a mixture of substrate (1 mM BzArgOEt) and aprotinin (10 μM) (i.e., no enzyme–inhibitor preincubation), inhibition of the enzyme occurred slowly and continuously, reaching 98% only after 80 min of incubation. Thus, in the presence of the substrate, the "on-rate" for aprotinin was relatively slow. The same experiment carried out with 0.1 μM aprotinin instead of 10 μM produced only 9% inhibition during the same time period (80 min). This behavior is consistent with the formation of a relatively weak collision complex followed by conversion of the complex to a tight-binding form (Broze et al., 1990). Other protein proteinase inhibitors such as lima bean trypsin inhibitor, ovomucoid, soybean trypsin inhibitor, and α_1-antitrypsin had no effect on activity at 1 mg/mL. (All commercial samples of these inhibitors were found in control experiments to inhibit trypsin.) Although the rat skin tryptase is a glycoprotein and can bind to concanavalin A–agarose, concanavalin A (1 mg/mL) did not inhibit the enzyme. The thrombin inhibitor hirudin (1 unit/mL) had no effect. Various proteinase inhibitors of microbial origin were tested. Antipain and leupeptin, which both have a terminal argininal residue, were good inhibitors of tryptase (Table II). However, peptides having either a terminal phenylalaninal (chymostatin) (100 μg/mL) or alaninal (elastatinal) (200 μg/mL) did not inhibit.

Of several metal ions tested, only Hg^{2+} was inhibitory (Table II). $MgCl_2$, $MnCl_2$, $CoCl_2$, and $CaCl_2$ (all at 10 mM) had no effect. NaCl in concentrations up to 1 M had no significant effect on activity under the conditions of the standard assay that utilized highly saturating concentrations of BzArgOEt. However, when peptidyl-7-amino-4-methylcoumarin (-AMC) substrates were assayed at low substrate concentrations (10 μM) (see below), an effect of NaCl on activity was seen. For example, activity toward t-Boc-Val-Pro-Arg-AMC and Z-Gly-Gly-Arg-AMC was inhibited 61% and 64%, respectively, by 0.9 M NaCl. (Cleavage of these same substrates by bovine trypsin was unaffected by salt.) Addition of increasing concentrations of KCl caused increases in the K_m value for BzArgOEt (to about 7-fold at 1 M) without significantly affecting V_{max}.

The effect of different concentrations of dimethyl sulfoxide on the initial rate of cleavage of 0.2 mM BzArgOEt was also tested. Dimethyl sulfoxide, present in the assay mixture at 5, 10, 15, and 20% (v/v), inhibited activity 21, 43, 76, and 95%, respectively.

Active Site Titration. Table II shows that *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) and 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB) completely inhibited tryptase at 10 μM. These agents are known to be active site titrants of trypsin-like enzymes. MUGB was therefore tested as an active site titrant of tryptase. Tryptase hydrolyzed MUGB, releasing the fluoroscent product, methylumbelliferone (MU), with "burst"-type kinetics. A rapid increase in fluorescence (essentially instantaneous by the method used) was followed by a plateau phase during which no substrate turnover was observed. This phenomenon suggested that a stable *p*-guanidinobenzoyl–enzyme intermediate was formed (Chase & Shaw, 1970). The size of the "burst" was directly proportional to enzyme concentration and, for a given amount of enzyme, was independent of the amount of MUGB present (0.25–2.0 μM). The number of picomoles of MU released during the "burst" was assumed to be equivalent to the number of picomoles of enzyme subunit present.

Substrate Specificity. (1) Single amino acid substrates: Tryptase hydrolyzed BzArgOEt with a V_{max} of 170 μmols/min mg^{-1} and a K_m of 0.013 mM. On the basis of active site titration, the k_{cat} was 100 s^{-1} while k_{cat}/K_m was 7.7×10^6 M^{-1} s^{-1}. The corresponding methyl ester, BzArgOMe, was hydrolyzed with the following kinetic parameters: K_m = 0.020 mM; k_{cat} = 150 s^{-1}; $k_{cat}/K_m = 7.5 \times 10^6$ M^{-1} s^{-1}. TosArgOMe, and D,L-BzArg-pNA (both at 1 mM) were hydrolyzed at rates of 75 μmol/min mg^{-1} and 9.4 μmols/min mg^{-1}, respectively. The K_m for L-BzArg-pNA was 0.061 mM.

(2) Tri- and tetrapeptidyl-*p*-nitroanilides: Table III shows the relative rates of hydrolysis of several *p*-nitroanilide substrates, all at 1 mM. Tryptase hydrolyzed peptides with either an arginine or lysine residue in the P_1 position [nomenclature of Schechter and Berger (1967)]. The enzyme was unable to cleave CH_3-Suc-Arg-Pro-Try-pNA, ⟨Glu-Pro-Val-pNA, H-D-Pro-HHT-Arg-pNA, and H-D-Val-CHA-Arg-pNA (CHA = cyclohexylalanyl). Although definitive statements about substrate specificity cannot be made without knowledge of relative specificity constants (k_{cat}/K_m), it appears that tryptase favored substrates with a glycine, proline, or proline analogue (pipecolic acid) in the P_2 position. Peptides with a large hydrophobic residue at P_2 (e.g., leucine, phenylalanine, valine, hexahydrotyrosine) were cleaved relatively slowly. D-amino acids were readily accommodated in the P_3 position.

Several of the peptidyl-*p*-nitroanilide substrates were assayed for their ability to specifically detect tryptase in the crude 13700*g* supernatant of the initial rat skin extract. The specificity of the substrates was determined by measuring the relative percent recoveries of activity in the final purified enzyme preparation compared to that in the 13700*g* supernatant. A low percent recovery of activity for a given substrate

Table III: Relative Rates of Hydrolysis of Peptidyl-*p*-nitroanilide Substrates

substrate[a]	relative rate[b] (% maximum)
P_3 P_2 P_1	
H-D-Ile-Pro-Arg-pNA	100
H-D-Pro-Pro-Arg-pNA	89
H-D-Phe-Pip-Arg-pNA	67
Z-Val-Gly-Arg-pNA	44
Z-Gly-Pro-Lys-pNA	38
Bz-Ile-Glu-(OR[c])Gly-Arg-pNA	34
Tos-Gly-Pro-Arg-pNA	29
Bz-Ile-Glu-(OR[d])Gly-Arg-pNA	24
CH_3CO-Lys(Z)-Gly-Arg-pNA	19
H-D-Val-Leu-Arg-pNA	14
H-D-CHG-But-Arg-pNA	11
Bz-Phe-Val-Arg-pNA	7.4
CH_3-SO_2-D-Leu-Gly-Arg-pNA	5.6
H-D-Val-Leu-Lys-pNA	3.9
Bz-Pro-Phe-Arg-pNA	3.3
H-D-Pro-Phe-Arg-pNA	2.9
H-D-Nle-HHT-Lys-pNA	2.5

[a] Abbreviations: pNA, *p*-nitroanilide; Pip, pipecolic acid; CHG, cyclohexylglycyl; But, L-α-aminobutyryl; HHT, hexahydrotyrosine. [b] Values are initial rates [relative to that for H-D-Ile-Pro-Arg-pNA (=100%)] determined for the hydrolysis of 1 mM substrate in phos-EDTA-7.5 at 30 °C with use of a Multistat III F/LS centrifugal analyzer. [c] R = CH_3 (50%) and H (50%). [d] R = piperidyl.

Table IV: Relative Rates of Hydrolysis of Peptidyl-7-amino-4-methylcoumarin Substrates

substrate[a] (10 μM)	relative rate[b] (%)
Z-Ala-Arg-Arg-AMC	290
t-BOC-Phe-Ser-Arg-AMC	100
t-BOC-Val-Pro-Arg-AMC	52
Z-Gly-Gly-Arg-AMC	14
Pro-Phe-Arg-AMC	2.8
Bz-Arg-AMC	2.4
t-BOC-Val-Leu-Lys-AMC	2.0
t-BOC-Ile-Glu-Gly-Arg-AMC	0.3
Glut-Gly-Arg-AMC	0.2
Suc-Leu-Leu-Val-Tyr-AMC	0

[a] AMC = 7-amino-4-methylcoumarin. [b] The enzyme was incubated with substrate (10 μM) at 25 °C in phos-EDTA-7.5. The release of AMC was monitored with a recording fluorescence spectrophotometer as indicated in Methods. Values are relative initial rates and are compared to t-BOC-Phe-Ser-Arg-AMC (=100%) to facilitate comparison with Kido et al. (1985).

indicates the presence of competing enzymes in the crude sample acting on that substrate which were removed during purification. H-D-Ile-Pro-Arg-pNA (used in Table I), Bz-Ile-Glu(OR)-Gly-Arg-pNA, and two substrates not shown in Table III, Tos-Gly-Pro-Lys-pNA and ⟨Glu-Gly-Arg-pNA, exhibited essentially equivalent percent recoveries and were therefore equally specific for tryptase. H-D-Lys(Z)-Pro-Arg-pNA, H-D-Val-Leu-Lys-pNA, and H-D-Val-Leu-Arg-pNA yielded recoveries that were only about one-third of those for the other substrates and hence were much less specific for tryptase in the crude supernatant. H-D-Phe-Pip-Arg-pNA gave two-thirds, and Z-Val-Gly-Arg-pNA three-fourths, of the recovery obtained for the first group of substrates.

(3) Peptidyl-7-amino-4-methylcoumarin (-AMC) substrates: Table IV shows the relative rates of release of AMC from several peptidyl-AMC substrates (all at 10 μM) determined with a fluorescent assay. The best substrate of the series was Z-Ala-Arg-Arg-AMC, indicating that a basic residue is easily tolerated in the P_2 position.

(4) Peptide hormones and hormone fragments: Table V shows the results of incubating rat skin tryptase with various peptides of biological significance. [Arg[8]]vasopressin was hydrolyzed by tryptase as determined by HPLC analysis of the reaction mixture. The major UV-absorbing product had a retention time identical with the standard, des(Gly[9]-NH_2)-[Arg[8]]vasopressin (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-OH) (17.8 min) (vasopressin eluted at 16.3 min). This suggested that the enzyme hydrolyzed the -Arg[8]–Gly[9]–NH_2 bond. The release of Gly-NH_2 was confirmed by use of [Arg[8],[14]C-Gly[9]-NH_2]vasopressin as the substrate. In this case, a radioactive peak was detected by HPLC with use of a radioactive flow-through scintillation detector (Radiomatic Flo-one HS) that had the same retention time as an authentic Gly-NH_2 standard (but different from Arg-Gly-NH_2 and higher homologues). The pH optimum for the hydrolysis of [Arg[8]]vasopressin with various 0.1 M buffers was 7.5. No activity was seen at or below pH 5.5 and at or above pH 8.5.

Neurotensin was hydrolyzed by tryptase to give two major products that were observed by their UV absorbance following HPLC. An active neurotensin analogue, Ac-Arg-Arg-Pro-Tyr-Ile-Leu, was also hydrolyzed and yielded two products, one of which had the same retention time (29 min) as one of

Table V: Sites of Cleavage of Peptide Hormones and Hormone Fragments

the neurotensin products. The apparently identical product peaks for neurotensin and its analogue were collected and subjected to amino acid sequence analysis by use of the automated Edman technique. Each peak gave the same sequence, namely, Arg-Pro-Tyr-Ile-Leu. This data indicated that both neurotensin and the active analogue were hydrolyzed at the Arg–Arg bond. Neurotensin was hydrolyzed 4 times faster than the active analogue (both at 0.5 mM), indicating that additional amino acid residues in the N-terminal direction from the scissile bond contribute to rate enhancement. The Lys–Pro and Arg–Pro bonds of neurotensin were not cleaved, suggesting that tryptase (like trypsin) may have difficulty with imino-containing peptide bonds. Interestingly, cleavage of both neurotensin and [Arg^8]vasopressin occurred at a site in which a proline residue occupied the P_2 position. As discussed above, *p*-nitroanilide substrates with proline in the P_2 position were among the most rapidly hydrolyzed substrates.

When the enzyme was incubated with the oxidized B-chain of bovine insulin, two product peaks were observed by HPLC that were then subjected to amino acid sequence analysis. Peak 1 was found to be Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala, while peak 2 was Phe-Val-Asn-Gln-His-Leu---Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val---Gly-Glu-Arg. Thus, tryptase hydrolyzed the oxidized B-chain of bovine insulin at the Arg^{22}–Gly^{23} bond (Table V). It was not quantitatively possible, using sequence analysis, to determine whether some fraction of the molecules in peak 1 lacked the C-terminal alanine, which would indicate slow cleavage at the C-terminal Lys^{29}–Ala^{30} bond.

Neither bradykinin nor oxytocin were hydrolyzed under conditions in which [Arg^8]vasopressin was hydrolyzed to the extent of 75%.

(5) Protein substrates: Tryptase was assayed for its ability to hydrolyze casein at pH 7.2 by use of an agar gel technique (Bio-Rad protease detection kit). Rat skin tryptase and bovine trypsin (equivalent amounts of BzArgOEt activity) were placed in wells in the casein gel plate. After approximately 20 h of incubation at 25 °C, bovine trypsin produced broad zones of lysis, whereas rat skin tryptase showed no evidence of casein hydrolysis. Also in contrast to bovine trypsin, rat skin tryptase (with twice the amount of BzArgOEt units of activity compared to trypsin) was unable to produce trichloroacetic acid soluble peptides from hemoglobin or azocoll following a 30-min incubation at pH 7.5 and 25 °C, (Walter, 1984). The results suggest that rat skin tryptase does not possess general proteinase activity.

Isoelectric Point. Isoelectric focusing was carried out with use of an LKB Model 2117 multiphor system with precast 0.5-mm polyacrylamide gels having an ampholyte range of 3.5–9.5. Purified rat skin tryptase yielded five closely spaced bands in the pH range of 4.5–4.9, with the most prominent band at pH 4.8. The observed microheterogeneity is presumably due to the glycoprotein nature of the enzyme.

Molecular Weight. SDS–PAGE in vertical gels using the system of Laemmli (1970) gave a subunit molecular weight for purified rat skin tryptase of 34 000 ± 2000 under both reducing and nonreducing conditions. SDS–PAGE using the Pharmacia PhastSystem gave a somewhat lower molecular weight of 30 000 ± 1000 (Figure 6, lane 1).

Since tryptase appeared to be a glycoprotein on the basis of its ability to bind to a concanavalin A–agarose column, the purified enzyme was treated with peptide N-glycosidase F (N-glycanase) to remove any N-linked carbohydrate moieties (Tarentino et al., 1985). Figure 6 shows that peptide N-glycosidase F was able to generate a second protein band of

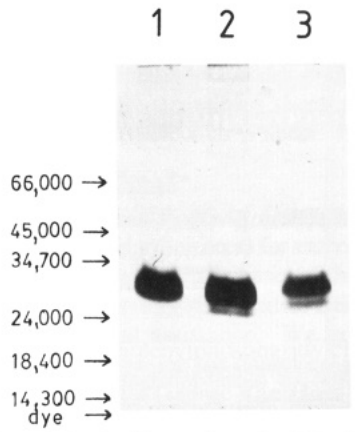

FIGURE 6: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of peptide N-glycosidase F treated tryptase. SDS–PAGE of peptide N-glycosidase F treated and untreated tryptase was carried out under reducing conditions using a Pharmacia PhastSystem with a 12.5% homogeneous gel and standards at the indicated molecular weights as described in Methods. The gel was developed with silver stain. The individual lanes represent (1) untreated tryptase (0.7 μg), (2) tryptase (0.7 μg) treated with peptide N-glycosidase F, and (3) tryptase (0.7 μg) that was denatured prior to treatment with peptide N-glycosidase F.

molecular weight 26 000. Similar results were obtained following treatment of the enzyme with endoglycosidase H (Trimble & Maley, 1984) (data not shown).

High-performance gel permeation chromatography of tryptase gave a single sharp protein peak (detection at 214 or 280 nm), which was coincident with activity. The molecular weight of purified rat skin tryptase determined by this technique was previously reported to be 160 000 (Braganza & Simmons, 1986). A repeat of that experiment using a more extensive set of molecular weight standards gave a molecular weight estimate of 145 000. The molecular weight did not change when gel permeation chromatography was carried out at different ionic strengths (0.05, 0.5, or 1 M NaCl).

Amino Acid Analysis. The amino acid composition of purified rat skin tryptase was determined. The results are given as mol % as well as the nearest integer number of residues (given in parentheses, assuming 236 total residues excluding Trp): Asx, 12.6% (30); Thr, 6.0% (14); Ser, 5.6% (13); Glx, 8.1% (19); Pro, 7.7% (18); Gly, 8.6% (20); Ala, 4.7% (11); Val, 10.2% (24); Ile, 5.1% (12); Leu, 10.2% (24); Tyr, 4.4% (10); Phe, 1.8% (4); Lys, 3.7 (9); His, 3.9% (9); Arg, 3.4% (8); Cys, 2.9% (7); Met, 1.0% (2). This composition was compared to the amino acid compositions of dog mastocytoma tryptase (Vanderslice et al., 1989) and human pulmonary mast cell tryptase (Miller et al., 1989) calculated from the respective cDNA sequences. The three tryptases exhibited very similar amino acid compositions. Tests developed by Cornish-Bowden (1980) for statistically determining the relatedness of protein sequences on the basis of amino acid composition were applied to the mol% data. Rat skin tryptase and human pulmonary mast cell tryptase were predicted with near certainty to have related amino acid sequences by this method (DI = 7.15), as was human mast cell tryptase compared to dog mastocytoma tryptase (DI = 8.85). The rat skin enzyme and dog mastocytoma tryptase passed the "weak" test for relatedness of sequence (DI = 12.7). In contrast, none of the three tryptases were found to pass even the "weak" test for relatedness when compared to bovine trypsin (DI = 20.9–25.6).

N-Terminal Sequence. Table VI shows the sequence of the first 25 amino acid residues of rat skin tryptase determined by the automated Edman procedure. Cycle 21 did not yield a prominent PTH-amino acid. The presence of a threonine

Table VI: N-Terminal Sequence of Rat Skin Tryptase

	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	% Homology
Tryptase (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	
Tryptase (human lung mast cell)[a]	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		80
Tryptase (clone I) (human skin mast cell)[b]	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		76
Tryptase (dog mastocytoma)[c]	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		72
Tryptase (human pituitary)[d]	Ile	Val	Gly	Gly	Gln	Glu	Ala	Pro	-																		
Kallikrein (human plasma)[e]	Ile	Val	Gly	Gly	Thr	Asn	Ser	Ser	Trp	Gly	Glu	Trp	Pro	Trp	Gln	Val	Ser	Leu	Gln	Val	Lys	Leu	Thr	Ala	Gln		56
Elastase (porcine)[f]	Val	Val	Gly	Gly	Thr	Glu	Ala	Gln	Arg	Asn	Ser	Trp	Pro	Ser	Gln	Ile	Ser	Leu	Gln	Tyr	Arg	Ser	Gly	Ser	Ser		48
Trypsin (bovine)[g]	Ile	Val	Gly	Gly	Tyr	Thr	Cys	Gly	Ala	Asn	Thr	Val	Pro	Tyr	Gln	Val	Ser	Leu	Asn	Ser	Gly	Tyr	His	Phe	Cys		40
Chymotrypsin A (bovine)[i]	Ile	Val	Asn	Gly	Glu	Glu	Ala	Val	Pro	Gly	Ser	Trp	Pro	Trp	Gln	Val	Ser	Leu	Gln	Asp	Lys	Thr	Gly	Phe	His		40
Chymase II (rat)[h]	Ile	Ile	Gly	Gly	Val	Glu	Ser	Ile	Pro	His	Ser	Arg	Pro	Tyr	Met	Ala	His	Leu	Asp	Ile	Val	Thr	Glu	Lys	Gly		24

[a] Miller et al. (1989). [b] Vanderslice et al. (1990). [c] Vanderslice et al. (1989). [d] Cromlish et al. (1987). [e] Chung et al. (1986). [f] Shotton and Hartley (1970). [g] Mikes et al. (1966). [h] Woodbury et al. (1978).

at position 23 suggests that position 21 may be a glycosylation site containing an asparagine with an N-linked carbohydrate moiety.

Discussion

The protease described in this paper was purified to apparent homogeneity from rat skin initially on the basis of its ability to hydrolyze BzArgOEt, a nonspecific substrate for trypsin and related enzymes. However, structural and enzymatic properties of this protease suggested that it closely resembles various forms of tryptase described in the literature. Among the properties that the rat skin protease has in common with other tryptases are (1) the presence of an active site serine residue, (2) the binding specificity for basic amino acid residues in the P_1 site of the substrate, (3) a similar molecular weight and the ability to exist as a noncovalently linked tetramer, (4) the presence of carbohydrate moieties, (5) an instability in the absence of a high concentration of salt, (6) an inhibition of activity by salt, (7) the lack of nonspecific proteinase activity, and (8) an inability to be inhibited by most natural serine protease inhibitors. Furthermore, the rat skin protease described in this report has both an amino acid composition and an N-terminal amino acid sequence closely related to those of other tryptases. Affinity-purified antisera to human lung tryptase (which cross-reacts to the extent of 0.1% with bovine trypsin) showed a 10% cross-reactivity with rat skin tryptase in an immunoblot assay (Dr. David A. Johnson, personal communication).

Some significant differences exist, however, between rat skin tryptase and certain other tryptases. A prominent characteristic of tryptases purified from human sources [pulmonary mast cells (Schwartz et al., 1981b), lung (Smith et al., 1984; Harvima et al., 1988), pituitary (Cromlish et al., 1987), and skin (Harvima et al., 1988)], as well as from dog mastocytoma (Caughey et al., 1987), is their ability to bind to, and be stabilized by, heparin. Heparin, which coexists with tryptase in mast cell granules, has been postulated to regulate both the activity and the extent of diffusion of secreted tryptase (Schwartz & Bradford, 1986; Alter et al., 1987). However, heparin was found to have little or no ability to protect purified rat skin tryptase from thermal inactivation (Figure 4). Furthermore, the purified enzyme failed to bind to heparin–agarose under conditions similar to those utilized by Alter et al. (1987). The same results were obtained with heparin–agarose when the purified enzyme was first passed through an anion-exchange resin that can remove endogenous heparin (Schwartz et al., 1981b). These observations are consistent with a recent report that tryptase from rat peritoneal mast cells also fails to interact with heparin (Katumuma & Kido, 1988). Unless other stabilizing factors are present in vivo, rat tryptase might be expected to be very labile under physiological conditions.

It has been proposed that human pulmonary mast cell and dog mastocytoma tryptases, which are both anionic at physiological pH, have specific regions of positive charge responsible for heparin binding (Miller et al., 1989; Vanderslice et al., 1989). Comparison of the amino acid compositions indicates that rat skin tryptase has fewer positively charged residues than human pulmonary mast cell tryptase and possibly also dog mastocytoma tryptase. It is possible that rat skin tryptase may lack positive charges at key positions responsible for heparin binding. Eventual comparison of the amino acid sequence of rat tryptase with those of the human and dog tryptases may shed some light on the nature of the interaction of these latter forms of the enzyme with heparin.

The amino acid sequence of the N-terminal 25 residues of rat skin tryptase (Table VI) shows a high degree of homology with the same region in human pulmonary mast cell tryptase (80%), human skin mast cell tryptase (clone I) (76%), and dog mastocytoma tryptase (72%). The most notable differences in the sequences occur at residues 8–10 and 21–23. Rat skin tryptase gave a blank cycle for residue 21 in two independent runs. It is proposed that position 21 is an Asn and that this residue is N-glycosylated because of the existence of an Asn-X-Thr glycosylation signal. Recent cloning experiments using a mouse mast cell line (Chu et al., 1990) have revealed the existence of two mouse tryptase messages, one of which also has a putative glycosylation site at residue 21 (Dr. David A. Johnson, personal communication). Thus, glycosylation at this position may be characteristic of a specific type of tryptase, at least in rodents, that may serve a specific physiological function.

Additional Edman protein sequencing of purified rat skin tryptase (Chen et al., 1990) has revealed a second glycosylation site. This site is identical with the single putative glycosylation site in the dog enzyme (Asn^{102}) (Vanderslice et al., 1989) and one of the two putative sites in the human pulmonary mast cell enzyme (Asn^{101}) (Miller et al., 1989). The presence of at least two glycosylation sites with the potential for charge heterogeneity in the carbohydrate moieties presumably explains the multiple bands seen upon PAGE isoelectric focusing (five closely spaced bands, p_I 4.5–4.9). Different molecular weight subunit forms were not seen in SDS–PAGE experiments, although the dye-stained band tended to be rather broad. All human tryptases and dog tryptase, but not rat peritoneal mast cell tryptase (Katunuma & Kido, 1988), have exhibited sub-

units of different sizes, a phenomenon usually attributed to differences in posttranslational modification.

Rat skin tryptase was not inhibited by *p*-(chloromercuri)-benzoate (PCMB). In contrast, PCMB inhibited human pituitary tryptase (Cromlish et al., 1987) but not human lung tryptase (Smith et al., 1984). Dog mastocytoma tryptase has an odd number of cysteine residues (Vanderslice et al., 1989) but was not inhibited by PCMB (Caughey et al., 1987).

Tryptases have been found to be relatively resistant to inhibition by natural protein proteinase inhibitors. Aprotinin (basic pancreatic trypsin inhibitor, Kunitz) (Trasylol) was the only inhibitor of this type that was found to block the activity of rat skin tryptase (Table II). Aprotinin has also been shown to inhibit dog tryptase (Caughey et al., 1987) and rat peritoneal mast cell tryptase (Kido et al., 1985b) but none of the human tryptase preparations (Smith et al., 1984; Cromlish et al., 1987; Harvima et al., 1988). While rat peritoneal mast cell tryptase has been reported to be inhibited by soybean trypsin inhibitor and α_1-antitrypsin, rat skin tryptase was resistant to these inhibitors.

Kido et al. (1985b) found that tryptase from rat peritoneal mast cells copurified with an endogenous Kunitz-type protease inhibitor referred to as trypstatin (Kido et al., 1988). To date, we have seen no direct evidence for the presence of trypstatin in the purified rat skin tryptase preparation. The observed inhibition of rat skin tryptase by aprotinin is inconsistent with the presence of an endogenous inhibitor–enzyme complex (Kido et al., 1985b). SDS–PAGE, amino acid analysis, and N-terminal sequence analysis of the native enzyme (performed by Dr. Bassam Wakim), failed to detect a second protein. The enzyme preparation, either untreated or heat denatured, failed to inhibit Factor X_a activity as might be expected if trypstatin were present (Kido et al., 1988) (data not shown). Gel filtration of the heat-denatured enzyme showed the conversion from the tetrameric to the monomeric form but no clear evidence for the release of a Kunitz-type inhibitor of M_r 6600 (data not shown). Interestingly, the observed pH optimum and specificity toward peptidyl-AMC substrates for purified rat skin tryptase (Figure 5 and Table IV, respectively) resemble more closely the data reported for the rat peritoneal mast cell tryptase complexed with trypstatin than for the enzyme free of inhibitor (Kido et al., 1985b).

The substrate specificity of rat skin tryptase was found to be similar to that of other tryptases. Activity of purified rat skin tryptase toward TosArgOMe was 75 μmol/min mg^{-1} compared with 125, 101, and 97 μmols/min mg^{-1} for purified dog mastocytoma (Caughey et al., 1987), human lung (Smith et al., 1984), and human pulmonary mast cell (Schwartz et al., 1981b) tryptases, respectively, assayed under somewhat different conditions. The relative rates of cleavage of the tri- and tetrapeptidyl-pNA (Table III) substrates and peptidyl-AMC substrates (Table IV) demonstrate that rat skin tryptase specifically cleaves on the carboxyl side of arginine and lysine residues. The ability of amino acids in substrate positions P_2 and P_3 to influence the catalytic rate indicates that rat skin tryptase has an extended substrate binding site as has been observed for other tryptases (Tanaka et al., 1983; Caughey et al., 1987; Kido et al., 1985b; Cromlish et al., 1987; Harvima et al., 1988). Larger peptides such as vasopressin, neurotensin, and the oxidized B-chain of insulin were hydrolyzed by purified rat skin tryptase on the carboxyl side of arginine residues (Table V). The inability to cleave the Arg–Pro and Lys–Pro bonds of neurotensin is consistent with the inability of human pituitary tryptase (Chromlish et al., 1987) and a human lung tryptase preparation (Tam & Caughey, 1990) to hydrolyze imino-containing peptide bonds in ACTH (1–39) and substance P, respectively. Nonspecific proteinase activity was not observed for rat skin tryptase toward casein, hemoglobin, and azocoll. Human lung tryptase, on the other hand, has been reported to hydrolyze azocasein (Smith et al., 1984).

The high degree of purification required to obtain a homogeneous preparation of tryptase from rat skin (13000-fold) suggests that the enzyme is not present in great abundance in this tissue. Tryptases from whole human skin (Harvima et al., 1988) and human lung (Smith et al., 1984), by comparison, required only a 448- and 232-fold purification, respectively. The tryptase described in this report represented only 20–30% of the tryptic-like BzArgOEt activity present in the rat skin extract. The agarose–glycyl-glycyl-*p*-aminobenzamidine (A-Gly-Gly-PAB) column resulted in the selective adsorption of this tryptase activity, while the remaining BzArgOEt activity passed straight through the column. The specificity of this column may reside in the glycyl-glycine spacer arm since a *p*-aminobenzamidine column with a caproic acid spacer arm absorbed more of the contaminating BzArgOEt activity.

The use of a one-step chromatographic procedure consisting of a A-Gly-Gly-PAB column in sequence with a concanavalin A–agarose column provided a rapid and highly reproducible method for purifying tryptase to homogeneity from rat skin. The method has now been performed successfully many dozens of times by different laboratory personnel using different lots of affinity matrices. This method, based on affinity chromatography, may be useful for purifying tryptases from other tissues, particularly if an enzyme is present in low amounts or cannot be purified by previously described methods based on affinity of the enzyme for heparin.

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