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Regulation of human mast cell tryptase

Effects of enzyme concentration, ionic strength and the structure and negative charge density of polysaccharides

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Tryptase was previously shown to undergo rapid inactivation under physiological conditions unless stabilized by the presence of heparin. The current study shows that increasing the concentration of free tryptase enhances the preservation of enzymic activity, consistent with dissociation of the tetramer, rather than autodegradation, as the mechanism of inactivation. Heparin glycosaminoglycan fragments of M_r greater than 5700 are necessary for complete stabilization of tryptase activity. This stabilizing effect depends upon negative charge density rather than carbohydrate composition. Thus, keratan sulphate or hyaluronic acid were no better than physiological buffer alone; chondroitin monosulphates and heparan sulphate each prolonged the $t_{\frac{1}{2}}$ about 20-fold over buffer alone; chondroitin sulphate E prolonged the $t_{\frac{1}{2}}$ 69-fold; and dextran sulphate and heparin provided complete stabilization of tryptase activity for 120 min. Poly-D-glutamic acid prolonged the $t_{\frac{1}{2}}$ 55-fold. In each case the loss of tryptase activity followed apparent first-order kinetics. Increasing the NaCl concentration from 0.01 M to 1.0 M increased the stability of free tryptase. In contrast, increasing the NaCl concentration in the presence of stabilizing polysaccharides decreased the stability of tryptase until dissociation of tryptase from each polysaccharide presumably occurred; thereafter tryptase stability increased as did that of free tryptase. The effect of salt concentration on heparin-stabilized tryptase activity (as opposed to stability) was also evaluated. The mast cell proteoglycans heparin and chondroitin sulphate E, by virtue of containing the naturally occurring glycosaminoglycans of highest negative charge density, may play a major role in the regulation of mast cell tryptase activity *in vivo*.

INTRODUCTION

Tryptase is the most abundant neutral protease and protein component released from activated human mast cells [1–3]. It is a tetramer of M_r 134000 [2,4–6]; each subunit shares common antigenic determinants [3,7] and has one active site [2]. Like other mast cell proteases, tryptase is stored in secretory granules in its active form, presumably bound to the highly sulphated proteoglycans that also reside in these granules [8–10]. Expression of tryptase activity in human mast cell secretory granules should be suppressed by the acidic environment predicted to be present in these organelles by analogy with the situation in rodent mast cells [11]; in the extracellular environment, with near-neutral pH, tryptase activity would be optimal.

The regulation of tryptase activity after its release *in vivo* may depend on the presence of proteoglycans. For example, tryptase binds to heparin in physiological buffer or in human plasma and is stabilized in an active tetrameric configuration [5]; tryptase is eluted from heparin–agarose at 0.8 M-NaCl (4 °C). Free tryptase, however, is unstable and rapidly becomes inactive under physiological conditions [5,12]. The mechanism by which the activity of purified tryptase is lost appears to involve dissociation of the tetramer to monomeric subunits in the absence of any autodegradation [5]. The presence of protease inhibitors endogenous to plasma and tissues, including α_1-proteinase inhibitor and α_2-macroglobulin, seems not to alter tryptase activity [2,4–6]. Thus, the ultimate biological expression of tryptase activity may be governed by the tryptase–proteoglycan interaction.

Tryptase is present in each of the two types of human mast cells thus far defined [3,13,14] at 10 pg/T mast cell (tryptase positive, chymase negative) and 35 pg/TC mast cell (tryptase positive, chymase positive). Heparin has been found in preparations of human mast cells from mastocytosis skin [9] where TC mast cells normally predominate, and from lung [8] where the T type accounts for about 90% of the mast cells. Preparations of human colon tissue synthesize and release chondroitin sulphate E [10], with the presumed source being mast cells (most likely a combination of T and TC subtypes). Thus, classes of proteoglycans that have different charge densities and carbohydrate compositions may be present in different types of human mast cells. This is somewhat analogous to the situation in rodents where heparin, chondroitin sulphate E and chondroitin sulphate di-B predominate in different types of mast cells [15–21]. In contrast, human basophils contain predominantly chon-

Abbreviations used: Tos-Gly-Pro-Lys-pNA, tosyl-glycyl-L-prolyl-L-lysine *p*-nitroanilide; Tos-Arg-OMe, tosyl-L-arginine methyl ester.

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droitin sulphate A [22,23]. The ability of these proteoglycans to stabilize tryptase after its release *in vivo* may be an important factor in the chronicity of immediate hypersensitivity events.

This report describes the effects of tryptase concentration and the size and structure of various polysaccharides (including heparin and chondroitin sulphate E proteoglycans) on tryptase stability. The complex effects of salt concentration on the stability and activity of tryptase as well as on the binding of tryptase to various sulphated polysaccharides are also examined.

EXPERIMENTAL

Materials

Tos-Gly-Pro-Lys-*p*-NA (Boehringer-Mannheim); Tos-Arg-OMe, bovine serum albumin, whale cartilage chondroitin sulphate A (M_r 25000–50000), bovine kidney heparan sulphate (M_r 8000–10000), human umbilical cord hyaluronic acid (M_r 4000–8000000), poly-D-glutamic acid (M_r 50000–100000) (Sigma); RD Heparin 3000 (M_r 3500), RD Heparin 5000 (M_r 5700), Hepes (Calbiochem); Tris (Fisher); porcine skin chondroitin sulphate B (M_r 11000–25000), shark cartilage chondroitin sulphate C (M_r 40000–80000), bovine cornea keratan sulphate (M_r 4000–19000) (ICN Immunobiologicals, Napervile, IL, U.S.A.); heparin–agarose (Bethesda Research Labs); and dextran sulphate (M_r 500000) (Pharmacia) were obtained as indicated. Porcine heparin glycosaminoglycan (M_r 12000–20000) (Sigma) was purified by chromatography on Dowex 1-X2 [24]. Heparin proteoglycan (M_r approx. 750000) was prepared from rat serosal mast cells by chromatography on Dowex 1-X2 and phenyl–agarose [24]. Both preparations were dialysed (8000 Da cut-off) against 0.15 M-NaCl, adjusted to 1.1 mg/ml, and stored at −75 °C. Chondroitin sulphate E proteoglycan was prepared from the PT-18 cloned mouse mast cell line by guanidine extraction and Dowex 1-X2 chromatography as described previously [19]. Digestion by chondroitinase ABC followed by Partisil-10-Pack chromatography [20] has shown that the PT-18 derived chondroitin sulphate E proteoglycan consists of approx. 80% chondroitin 4-sulphate and 20% chondroitin 4,6-disulphate disaccharides; no heparin synthesis by this cell line was detected. Tryptase was purified to homogeneity from human lung mast cells by $(NH_4)_2SO_4$ fractionation and sequential chromatography on decyl–agarose, DEAE-Sephadex (A-25), and heparin–agarose as described [5,7]. Total recovery routinely exceeded 50% and the enzyme appeared pure by electrophoresis in polyacrylamide gels under denaturing conditions.

Methods

Rates of hydrolysis of Tos-Gly-Pro-Lys-pNA were determined by addition of 5–20 μl of tryptase preparations to 0.6 ml of 0.05 M-Tris/HCl, pH 7.6, containing 0.12 M-NaCl and 0.1 mM-substrate at 25 °C unless indicated otherwise. Tos-Gly-Pro-Lys-pNA concentration was determined by the change in absorbance at 405 nm after complete substrate cleavage (molar absorption coefficient 8800 $M^{-1} \cdot cm^{-1}$) and by its absorbance at 316 nm (molar absorption coefficient 12700 $M^{-1} \cdot cm^{-1}$). The bovine serum albumin preparation (50 μg) produced no detectable cleavage of the substrate. Tos-Arg-OMe esterase activity was determined by addition of 2–20 μl of tryptase to 1 ml of 0.04 M-Tris/HCl, pH 8.1, containing 0.15 M-NaCl, 10 mM-$CaCl_2$ and 1 mM-Tos-Arg-OMe at 25 °C, unless stated otherwise. The molar absorption coefficient for the change in absorbance at 247 nm with cleavage of Tos-Arg-OMe is 540 $M^{-1} \cdot cm^{-1}$. The change in absorbance at 405 nm (for Tos-Gly-Pro-Lys-pNA) or 247 nm (for Tos-Arg-OMe) was followed on a Cary 2200 spectrophotometer (Varian Associates). Kinetic constants were determined by Lineweaver–Burk analysis from initial velocities of substrate cleavage at five substrate concentrations during a time period when no more than 10% of the substrate had been cleaved. An M_r value for the holoenzyme of 134000 was used for the calculation of $K_{cat.}$ values. Activity is expressed in units, where one unit of enzyme cleaves 1 μmol of substrate/min. Protein concentration was determined by the method of Lowry *et al.* [25].

Experiments examining the time course of loss of tryptase activity were initiated by addition of tryptase to various solutions in which the NaCl concentration and polysaccharide type were varied. Where indicated, bovine serum albumin (1 mg/ml) was included to prevent adherence of dilute tryptase to plastic tubes. Samples of tryptase were removed at various times and combined with excess heparin glycosaminoglycan, which serves to stabilize the remaining tryptase activity without regenerating lost activity [5], and immediately assayed with Tos-Gly-Pro-Lys-pNA as described above.

To assess the binding of tryptase to solid-phase macromolecules, dextran sulphate, chondroitin sulphate E and chondroitin sulphate A were coupled to CNBr-activated Sepharose 4B (Sigma) as described by Iverius [26]. A control gel with only ethanolamine coupled to Sepharose 4B was also prepared. After coupling in 0.1 M-Na_2CO_3 (24 h, 4 °C) and blocking unbound sites with ethanolamine (24 h, 4 °C) the gels were washed extensively with 1 M-NaCl, water, and equilibrating buffer (0.05 M-NaCl/0.01 M-Hepes, pH 7.6) and stored at 4 °C in equilibrating buffer containing 0.02% NaN_3.

RESULTS

Effect of initial tryptase concentration on enzymic stability

The effect of tryptase concentration on its enzymic stability was determined as shown in Fig. 1. At four concentrations of tryptase over a 10-fold range (2.3–23 μg/ml) the enzyme was incubated at 25 °C in 0.12 M-NaCl/0.05 M-Tris/HCl, pH 7.6, containing 1.0 mg of bovine serum albumin/ml. The loss in enzymic activity followed apparent first-order kinetics in each case, as demonstrated by the linearity ($r = 0.99$) of the semilogarithmic plots of percentage residual enzymic activity versus time (Fig. 1*a*). Increasing concentrations of tryptase increased the time for 50% loss of enzymic activity ($t_{\frac{1}{2}}$). The relationship of $t_{\frac{1}{2}}$ versus initial tryptase concentration (Fig. 1*b*) was linear ($r = 0.99$) with a slope of 0.16 min/μg per ml and a projected y-axis intercept of 2.9 min, the apparent $t_{\frac{1}{2}}$ for an infinite dilution of tryptase at 25 °C.

Stabilization of tryptase by negatively charged polymers

The stability of tryptase was determined in the presence of a 1.2:1 weight ratio (polysaccharide:tryptase) of dextran sulphate, heparin proteoglycan and glycosaminoglycan, chondroitin sulphates A, B, C and E,

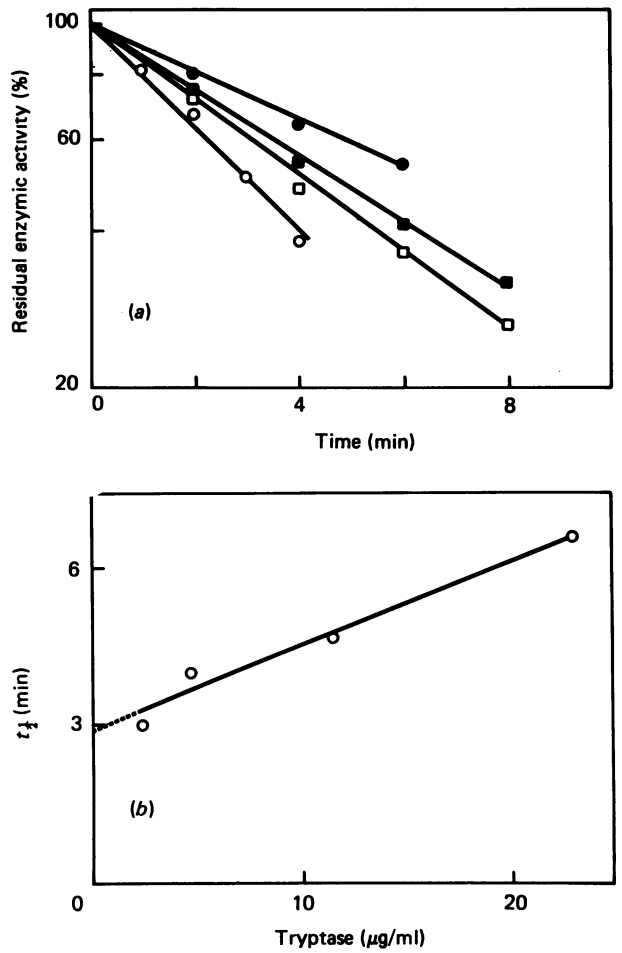

Fig. 1. Effect of tryptase concentration on the stability of tryptase activity

(*a*) Tryptase at 2.3 μg/ml (○), 4.6 μg/ml (□), 11.5 μg/ml (■) and 23 μg/ml (●) was incubated at 25 °C in 0.12 M-NaCl/0.05 M-Tris/HCl, pH 7.6, containing 1.0 mg of bovine serum albumin/ml. Samples containing 0.012–0.12 μg of tryptase were removed, combined with 0.5 μg of heparin glycosaminoglycan, and assayed for residual tryptase activity with the substrate Tos-Gly-Pro-Lys-pNA. 100% residual activity represents 27 units/mg at $t = 0$, which was constant throughout the range of tryptase concentrations used. Values represent the average of duplicate determinations. (*b*) Times of 50% inactivation ($t_{\frac{1}{2}}$) calculated from the individual slopes in (*a*) are also plotted versus tryptase concentration.

heparan sulphate, keratan sulphate and hyaluronic acid, and compared with that of tryptase in buffer alone. A weight ratio of 1:1 was previously shown to provide near-optimal stabilization of tryptase by heparin glycosaminoglycan [5]. An apparent first-order time course for loss of tryptase activity was observed in each case, except for heparin and dextran sulphate where no loss in activity occurred within 2 h at 37 °C. The stabilizing effect increased with negative charge density (Table 1) and was greatest for dextran sulphate and heparin. Chondroitin disulphate type E provided about 3-fold greater stability than the chondroitin monosulphates A, B or C, or heparan sulphate, whereas the stability of tryptase with keratan sulphate and hyaluronic acid were not appreciably different from that of tryptase in buffer alone. Increasing the concentration of polysaccharide by 10-fold increased the $t_{\frac{1}{2}}$ of tryptase inactivation from 24 to 44 min for chondroitin sulphate A, and from 69 to 207 min for chondroitin sulphate E. In order to determine whether sulphated carbohydrate residues are required for stabilization of tryptase, poly-D-glutamic acid (two carboxyl groups per dipeptide) was also tested. Under the same conditions used in Table 1, a 1.2:1 and 12:1 weight ratio of poly-D-glutamic acid to tryptase produced apparent first-order kinetics, with $t_{\frac{1}{2}}$ values for tryptase inactivation equal to 55 min and 75 min, respectively.

Relationship between heparin size and tryptase stabilization

Tryptase was incubated for 2 h at 37 °C at a 1.2:1 weight ratio (polysaccharide:tryptase) with heparin proteoglycan (M_r approx. 750000 with glycosaminoglycan chains of M_r approx. 60000), heparin glycosaminoglycan (M_r 12000–20000) and heparin glycosaminoglycan fragments of M_r 5500 and M_r 3700. Residual enzymic activity was determined at various times and plotted as log (% residual activity) versus time (not shown); the plot was linear in each case. Heparin proteoglycan and intact glycosaminoglycan provided complete protection of tryptase activity throughout the 2 h incubation. The heparin fragments had appreciable but diminished (when compared with the larger heparin forms) capacity to stabilize tryptase, resulting in calculated $t_{\frac{1}{2}}$ values of 348 min and 122 min for the larger and smaller fragments respectively. Thus heparin with M_r greater than 5500 appears necessary for optimal stabilization of tryptase.

Binding of tryptase to glycosaminoglycans and dextran sulphate

The relative strength of binding of tryptase to each type of polysaccharide was determined by the experiment in Fig. 2. Tryptase was applied, at 4 °C, to columns of dextran sulphate and chondroitin sulphates A and E conjugated to CNBr-activated Sepharose 4B, and to commercially available heparin–agarose. At low ionic strength almost all tryptase was bound to each of these columns (Fig. 2), but not to ethanolamine-conjugated Sepharose 4B (results not shown). Bound tryptase was eluted in a single peak of activity by application of a linear salt gradient (0.05–1.0 M-NaCl). Peak elution of activity occurred at 0.2 M-NaCl for chondroitin sulphate A, 0.4 M-NaCl for chondroitin sulphate E, 0.8 M-NaCl for heparin and 0.9 M-NaCl for dextran sulphate. Total recoveries of tryptase activity were 60%, 60%, 89% and 59%, respectively. In a separate experiment performed with heparin–agarose at 37 °C, the peak of tryptase was eluted at 0.6 M-NaCl, Thus, the relative affinity of these macromolecules for tryptase increases with their increasing charge densities in the same fashion as their stabilizing potencies.

Effect of NaCl on stabilization of tryptase

The effect of NaCl concentration on tryptase stability in the presence of chondroitin sulphates A and E, heparin, or dextran sulphate (1.2:1 weight ratio of polysaccharide to tryptase) or in buffer alone is shown in Fig. 3. Results of a similar experiment using heparin proteoglycan plus tryptase (not shown) closely followed the plot for tryptase plus heparin glycosaminoglycan shown in Fig. 3. All samples were assayed at 0.12 M-NaCl

Table 1. Stabilization of tryptase activity by glycosaminoglycans and dextran sulphate at 37 °C

Tryptase (0.27 μg) and polysaccharide (0.33 μg or 3.3 μg) were incubated together at 37 °C in 0.05 M-Tris/HCl/0.12 M-NaCl, pH 7.6, containing 1.0 mg of bovine serum albumin/ml in a total volume of 50 μl. Duplicate 5 μl samples were removed at various times (up to 120 min), mixed with 0.5 μg of heparin glycosaminoglycan, and immediately assayed for residual tryptase activity against the substrate Tos-Gly-Pro-Lys-pNA. The $t_{\frac{1}{2}}$ values for tryptase inactivation were calculated from a plot of log(% residual activity) versus time and represent the average of duplicate determinations. Tryptase in buffer alone exhibits a $t_{\frac{1}{2}}$ of 1 min under these conditions.

Polysaccharide	Sulphate residues per disaccharide	Carboxyl residues per disaccharide	$t_{\frac{1}{2}}$ (min) at polysaccharide:tryptase weight ratio of: 1.2:1	12:1
Dextran sulphate	4–5	0	> 120*	
Heparin proteoglycan	2–3	1	> 120*	
Heparin glycosaminoglycan	2–3	1	> 120*	
Chondroitin sulphate E	1–2	1	69	207
Chondroitin sulphate A	1	1	24	44
Chondroitin sulphate B	1	1	22	46
Chondroitin sulphate C	1	1	19	35
Heparan sulphate	1	1	22	
Keratan sulphate	1	0	1	1
Hyaluronic acid	0	1	1	1

* No loss of activity during 120 min incubation.

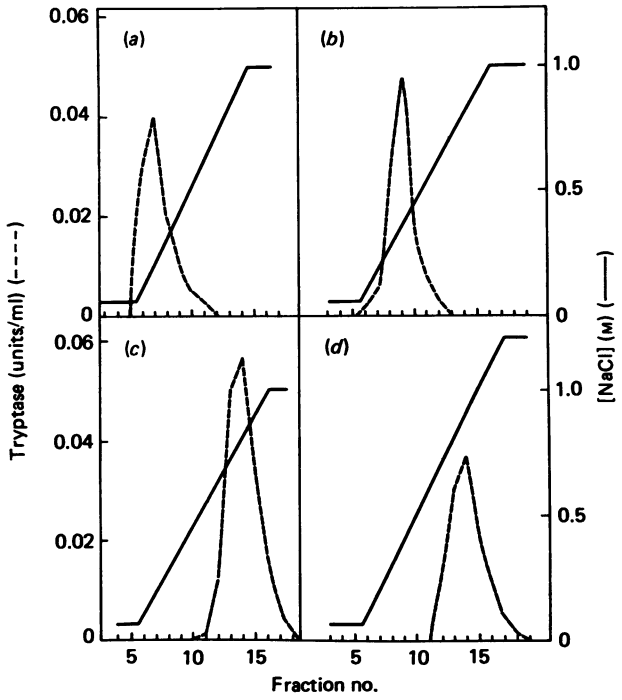

Fig. 2. Elution of tryptase from immobilized polysaccharides

Tryptase (0.9 unit, assayed with Tos-Gly-Pro-Lys-pNA) at approximately 0.15 M-NaCl was loaded at 4 °C onto 1.0 ml columns containing immobilized (*a*) chondroitin sulphate A, (*b*) chondroitin sulphate E, (*c*) heparin or (*d*) dextran sulphate equilibrated with 0.05 M-NaCl/0.01 M-Hepes, pH 7.6. After washing with 5 ml of equilibrating buffer, retained tryptase was eluted at 4 °C with a 10 ml linear gradient of 0.05–1.0 M-NaCl (0.05–1.2 M-NaCl for dextran sulphate). Fractions (1.0 ml) were collected in tubes containing 100 μg of dextran sulphate to preserve eluted activity. Each fraction was assayed for tryptase activity (----) and NaCl concentration (——).

after a 10 min incubation at 37 °C. Stabilization of tryptase in buffer alone increases dramatically with increasing concentrations of NaCl and approaches a plateau near 1 M-NaCl. This curve acts as a lower limit for stabilization of tryptase in the presence of the sulphated macromolecules. Stabilizing effects of these substances were maximal at low ionic strength and decreased with increasing ionic strength until the salt concentration was reached where the tryptase stability was the same in the presence or the absence of each compound. The salt concentration at the intercept is highest for dextran sulphate, and decreases with heparin, chondroitin sulphate E and chondroitin sulphate A in that order. This sequence corresponds to the above-mentioned capacities of each agent to stabilize tryptase (Table 1), the apparent binding strength of tryptase to each agent (Fig. 2), and the negative charge density of each agent. The NaCl concentration at the intercept for tryptase plus heparin (0.6 M-NaCl) is the same NaCl concentration at which tryptase is eluted from heparin–agarose at 37 °C. At salt concentrations higher than the intercept the stability of tryptase increased and was no different in the presence of a stabilizing macromolecule compared to buffer alone, presumably because tryptase is free in solution at the higher ionic strengths.

Effect of NaCl on heparin-stabilized tryptase activity

The effects of NaCl concentration on the $k_{cat.}$, K_m and $k_{cat.}/K_m$ ratio for tryptase stabilized with heparin glycosaminoglycan were determined with Tos-Gly-Pro-Lys-pNA and Tos-Arg-OMe as shown in Table 2. The K_m rises with increasing [NaCl], but much more for Tos-Gly-Pro-Lys-pNA than for Tos-Arg-OMe. Changing [NaCl] from 0.015 M to 0.15 M raises the $k_{cat.}$ with Tos-Gly-Pro-Lys-pNA, but causes little change in the $k_{cat.}$ with Tos-Arg-OMe, in each case resulting in a lower $k_{cat.}/K_m$ ratio. Because both K_m and $k_{cat.}$ increase with increasing [NaCl] for Tos-Gly-Pro-Lys-pNA, the sub-

strate concentration will determine whether the rate of hydrolysis increases, decreases or remains unchanged at these two different salt concentrations. For example, with the data in Table 2 for Tos-Gly-Pro-Lys-pNA one can solve the two simultaneous Michaelis–Menten equations for the substrate concentration at which the velocities at 0.015 M- and 0.15 M-NaCl will be equal. That concentration is 0.56 mM. With substrate concentrations of less than 0.56 mM the rate will be faster in 0.015 M- than in 0.15 M-NaCl, while at higher substrate concentrations the rate in 0.015 M-NaCl will be slower. A similar calculation for the data with Tos-Arg-OMe indicates that rates of hydrolysis of 0.015 M-NaCl will be higher than at 0.15 M-NaCl at all substrate concentrations, whereas at 0.15 M and 0.5 M-NaCl equivalent rates of hydrolysis should occur at 1.54 mM-Tos-Arg-OMe.

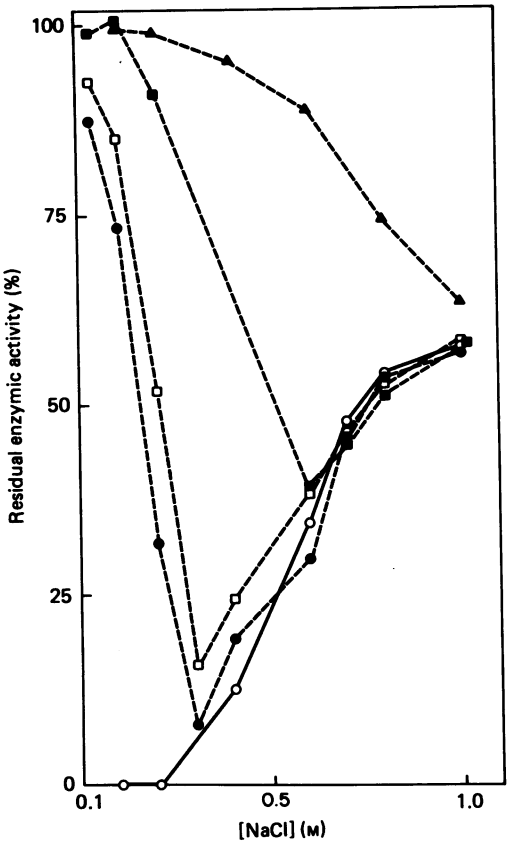

Fig. 3. Effect of NaCl concentration on the stability of tryptase activity

Tryptase (0.13 μg) was incubated alone (○) or with 0.16 μg of chondroitin sulphate A (●), chondroitin sulphate E (□), heparin (■) or dextran sulphate (▲) at 37 °C in buffer (0.01 M-Hepes, pH 7.6) with increasing NaCl concentrations. At 0 and 10 min samples were removed, combined with 0.5 μg of heparin glycosaminoglycan, and assayed immediately with Tos-Gly-Pro-Lys-pNA under standard conditions. The percentage of the initial activity remaining after 10 min of incubation was calculated; the average of duplicate determinations is shown.

To test this prediction, initial velocities for tryptase-catalysed cleavage of 0.1 mM- and 1 mM-Tos-Gly-Pro-Lys-pNA at both 0.015 M- and 0.15 M-NaCl in the presence of heparin glycosaminoglycan were measured in triplicate at 37 °C and averaged. At 0.1 mM-Tos-Gly-Pro-Lys-pNA the initial rates of hydrolysis with 38 ng of enzyme were 36 units/mg at 0.015 M-NaCl and 15 units/mg at 0.15 M-NaCl, while at 1.0 mM-Tos-Gly-Pro-Lys-pNA the respective rates were 79 and 94 units/mg. Predicted ratios of rates of hydrolysis for low over high salt concentrations calculated from the K_m for $k_{cat.}$ data in Table 2 are 0.4 at 0.1 mM- and 1.4 at 1.0 mM-Tos-Gly-Pro-Lys-pNA, which are not significantly different than the respective experimentally determined values of 0.4 and 1.2.

Initial rates of hydrolysis of Tos-Arg-OMe (1.0 mM) and Tos-Gly-Pro-Lys-pNA (0.1 mM) are plotted against NaCl concentration in Fig. 4. With Tos-Gly-Pro-Lys-pNA, as predicted from the preceding analysis, reaction rate falls with increasing salt concentration from a maximal value at 0.015 M-NaCl to a plateau at about 0.5 M-NaCl of 14% of maximal. With Tos-Arg-OMe the reaction rate was maximal and relatively constant between 0.015 M- and 0.2 M-NaCl, and decreased to 60% of maximal at 2 M-NaCl.

DISCUSSION

Factors affecting the stabilization and activity of tryptase were examined in the current study. In physiological buffer at 25 °C loss of tryptase activity follows apparent first-order kinetics, suggesting that inactivation of each active site occurs independent of other sites. The $t_{\frac{1}{2}}$ values increased linearly with increasing tryptase concentrations from about 3 min at 2.3 μg of tryptase/ml to 6.6 min at 23 μg of tryptase/ml. This behaviour is consistent with a mechanism of inactivation of active

Table 2. Effect of NaCl on $k_{cat.}$, K_m and $k_{cat.}/K_m$ values for heparin-stabilized tryptase with Tos-Gly-Pro-Lys-pNA and Tos-Arg-OMe

With Tos-Gly-Pro-Lys-pNA, 30 ng of tryptase and 0.1 μg of heparin glycosaminoglycan in 5 μl were used to start each assay; with Tos-Arg-OMe, 360 ng of tryptase and 1 μg of heparin glycosaminoglycan were used. Kinetic constants were calculated from Lineweaver–Burk analyses. $k_{cat.}$ and K_m values at 0.5 M-NaCl with Tos-Gly-Pro-Lys-pNA were not obtained because the Lineweaver–Burk plot passed through the origin. Assays were performed at 37 °C.

[NaCl] (M)	Tos-Gly-Pro-Lys-pNA			Tos-Arg-OMe		
	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ $[(s \cdot M)^{-1}]$	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ $[(s \cdot M)^{-1}]$
0.015	509	0.15	3.4×10^6	461	0.17	2.7×10^6
0.15	2225	2.5	0.9×10^6	401	0.24	1.7×10^6
0.5	–	–	0.3×10^6	494	0.65	0.8×10^6

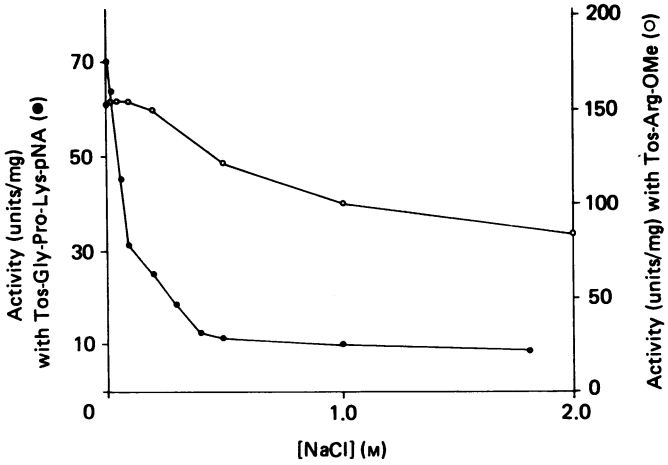

Fig. 4. Effect of NaCl concentration on the enzyme activity of heparin-stabilized tryptase against 0.1 mM-Tos-Gly-Pro-Lys-pNA (●) and 1 mM-Tos-Arg-OMe (○)

With Tos-Gly-Pro-Lys-pNA 0.04 μg of tryptase and 0.25 μg of heparin glycosaminoglycan were used in each assay, and with Tos-Arg-OMe, 0.36 μg of tryptase and 0.83 μg of heparin glycosaminoglycan were used. Data represent the average of duplicate determinations.

tetrameric tryptase that involves dissociation into monomeric subunits rather than autodegradation [5], because higher enzyme concentration would be expected to accelerate autodegradation. Compelling experimental support for this mechanism was provided previously: active and inactive tryptase produced identical patterns after electrophoresis in polyacrylamide gels under denaturing conditions with SDS, and diisopropylfluorophosphate-inactivated tryptase became monomeric after incubation under physiological conditions. Higher concentrations of tryptase therefore should favour preservation of the tetramer and catalytic activity. The calculated $t_{\frac{1}{2}}$ value of 2.9 min for tryptase at infinite dilution indicates in theory the $t_{\frac{1}{2}}$ of an individual subunit to become inactive. The possibility that two equilibria are involved, one being the dissociation of tetramer to monomer and the other the shift from active to inactive enzyme, still needs to be considered. Monomeric tryptase with enzymic activity has not been found after gel filtration; whether inactivation occurs prior to, with or immediately after subunit dissociation is not known.

The ability of naturally occurring polysaccharides, dextran sulphate and poly-D-glutamic acid to stabilize tryptase at 37 °C was evaluated at a 1.2:1 weight ratio of each polyanion to tryptase. An apparent first-order time course for loss of tryptase activity was observed in each case where loss of activity was detected, suggesting that this amount of polyanion was sufficient to bind all of the tryptase. The relative effectiveness of the various polymers depends primarily on the negative charge density rather than the chemical structure or conformation of the polyanions (Table 1). The primary importance of charge-density-related interactions between enzymes and proteoglycans is also seen in the anti-complement effects of various glycosaminoglycans [27–29], the ability of both heparin and chondroitin sulphate E to activate Hageman factor [30], and the interaction of bovine testicular hyaluronidase with its substrate [31]. In contrast, certain enzyme cofactors have been shown to be influenced by structural components of polysaccharides; antithrombin III has been shown to bind to a defined tetrasaccharide structure of heparin [32], and the binding of platelet factor 4 to different chondroitin sulphates is influenced by the type of uronic acid moiety encountered [33]. In addition, the effect of polysaccharide size on stabilization of tryptase was evaluated for heparin, indicating a minimum glycosaminoglycan size of M_r greater than 5700 for optimal stabilization.

The retention of tryptase by columns containing immobilized chondroitin sulphates A and E, heparin and dextran sulphate (Fig. 2) demonstrates the binding of tryptase to each of these polysaccharides, which was demonstrated previously only for heparin [5]. The relative abilities of these compounds to retain tryptase under conditions of increasing NaCl concentrations correlates with their abilities to stabilize tryptase activity in high ionic strength buffer (Fig. 3). In solution, stability of the free enzyme increases with increasing salt concentrations from 0 to 1 M-NaCl. Polysaccharide-bound enzyme is most stable at low ionic strength (where binding of tryptase is stronger) and decreases with increasing ionic strength until the stability of tryptase in the presence of polysaccharide approaches that of tryptase alone. The salt concentration at which this occurs increases with increasing polysaccharide charge density and presumably reflects the ionic strength which dissociates the tryptase–polysaccharide complex. Stability at higher ionic strengths increases, following the same curve as for free tryptase.

The concentration of NaCl exerts a considerable effect on the activity of heparin-stabilized tryptase, quite separate from the effects on stability discussed above. For the peptide substrate Tos-Gly-Pro-Lys-pNA, the apparent $k_{cat.}/K_m$ ratio decreases as the NaCl concentration is increased (Table 2). However, because $k_{cat.}$ and K_m both increase with NaCl, the relationship between initial rates of hydrolysis at two NaCl concentrations depends upon the initial substrate concentration. High substrate concentrations will result in higher rates of hydrolysis at the higher salt level, while low substrate concentrations (Fig. 4) result in higher rates of hydrolysis at the lower salt level. An analogous substrate-concentration-dependent variation is seen in the rate of hydrolysis of the ester substrate, Tos-Arg-OMe. Thus the relationship of enzyme activity to NaCl concentration depends upon the K_m and $k_{cat.}$ at each salt concentration together with the substrate concentration. High salt concentrations were noted previously to decrease the catalytic activity of tryptase from human lung [4,12], skin [12] and pituitary [6]. A similar inhibitory effect has also been observed with a tryptase-like protease from human cervix [34]. In contrast with tryptase, which recognizes amino acids with basic residues, chymase from human skin mast cells, which recognizes aromatic amino acid residues, exhibits increasing activity with increasing NaCl concentrations [35].

It is likely that heparin and/or chondroitin sulphate E occur together with tryptase in the human mast cell granule by design. The ability of both heparin and chondroitin sulphate E to stabilize mast cell tryptase *in vitro* suggests that their coexistence in mast cells occurs, in part, to stabilize the stored enzyme *in situ* and to extend greatly the duration of expression of tryptase activity after release into the extracellular environment.

Stabilization of tryptase activity by the chondroitin sulphate and heparan sulphate proteoglycans (but not by keratan sulphate or hyaluronic acid) found in the extracellular environment may also occur to a lesser extent. Such proteoglycan–tryptase complexes most likely play a major role in the expression of the biological function of mast cells.

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