Definition of the Extended Substrate Specificity Determinants for \( \beta \)-Tryptases I and II*  

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Tryptases \( \beta \) and \( \beta \text{II} \) were heterologously expressed and purified in yeast to functionally characterize the substrate specificity of each enzyme. Three positional scanning combinatorial tetrapeptide substrate libraries were used to determine the primary and extended substrate specificity of the proteases. Both enzymes have a strict primary preference for cleavage after the basic amino acids, lysine and arginine, with only a slight preference for lysine over arginine. \( \beta \) and \( \beta \text{II} \) tryptase share similar extended substrate specificity, with preference for proline at P4, preference for arginine or lysine at P3, and P2 showing a slight preference for asparagine. Measurement of kinetic constants with multiple substrates designed for \( \beta \)-tryptases reveal that selectivity is highly dependent on ground state substrate binding. Coupled with the functional determinants, structural determinants of tryptase substrate specificity were identified. Molecular docking of the preferred substrate sequence to the three-dimensional tetrameric tryptase structure reveals a novel extended substrate binding mode that involves interactions from two adjacent protomers, including P4 Thr-96, P3 Asp-60B and Glu-217, and P1 Asp-189. Based on the determined substrate information, a mechanism-based tetrapeptide-chloromethylketone inhibitor was designed and shown to be a potent tryptase inhibitor. Finally, the cleavage sites of several physiologically relevant substrates of \( \beta \)-tryptases show consistency with the specificity data presented here.  

Mast cells, mediators of inflammatory and allergic response, are found throughout the body concentrated near blood vessels in connective tissue and the mucous membranes of the respiratory and gastrointestinal tract. They play an important role in innate and acquired immune responses through the release of dense granules upon activation. Mast cell activation has also been implicated as a mediator of asthma and other inflammatory diseases. The major components of mast cell secretory granules are the tryptase serine proteinases (1). Tryptases are secreted as catalytically active tetramers that are resistant to inactivation by plasma inhibitors. The 3-Å crystal structure has been solved and reveals a ringlike structure with the four active sites facing a central cavity (2). Several in vitro studies have identified multiple substrates for tryptase, including neuropetides, fibrinogen, stromelysin, prourokinase, prothrombin, and protease-activated receptor-2 (3–6). Human chromosome 16 encodes several homologous tryptase genes, designated tryptase \( \alpha \), \( \beta \), and \( \gamma \) (7, 8). The \( \beta \)-tryptases share greater than 99% sequence identity, with tryptase \( \beta \) and \( \beta \text{II} \) differing by a single \( \text{N} \)-glycosylation site. It is unclear why so many highly similar tryptases are expressed by mast cells. One possibility is that they each perform different proteolytic functions that may be reflected in their substrate specificity preferences. Indeed, it has recently been shown that a single amino acid substitution between tryptase \( \alpha \) and tryptase \( \beta \text{II} \) accounts for discrimination in substrate preference for the two enzymes (9).  

The substrate specificity of heterologously expressed human tryptase \( \beta \) and \( \beta \text{II} \) was defined using multiple positional scanning synthetic combinatorial tetrapeptide libraries. We show that \( \beta \text{II} \) and \( \beta \text{II} \) tryptase have a defined primary (P1) and extended substrate specificity (P4–P2). 1 The library profiles indicate that the substrate specificity is similar for the two enzymes. Furthermore, single substrates were designed and assayed to test the extended substrate specificity requirements, resulting in a sensitive and selective substrate for \( \beta \)-tryptases. Similarly, an irreversible inhibitor was designed from the preferred substrate sequence and shown to be a potent \( \beta \)-tryptase inhibitor. Structural determinants of specificity were examined through the modeling of the optimized substrate into the active site of the tryptase structure. Finally, it is noted that the specificity determined in this study correlates with the cleavage sites found in many of the characterized physiological substrates and may lead to the identification of additional substrates involved in both the immunological and pathological consequences of \( \beta \) and \( \beta \text{II} \) tryptase release.  

EXPERIMENTAL PROCEDURES  

Materials—DNA-modifying enzymes were obtained from Promega (Madison, WI). The Pichia pastoris expression system was purchased from Invitrogen (San Diego, CA). Native human lung tryptase was purchased from ICN (Aurora, OH). Factor Xa was purchased from New England Biolabs (Beverly, MA). tPA and uPA were purchased from England Biolabs (Beverly, MA). The abbreviations used are: tPA, tissue plasminogen activator; uPA, urokinase-type plasminogen activator; ACC, 7-amino-4-carbamoylmethylcoumarin; cmk, chloromethylketone; Nes, norleucine; Ac, acetyl; Nme, N-methyl; MES, 4-morpholineethanesulfonic acid.  

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1 Nomenclature for the substrate amino acid preference is as follows: Pn, Pn−1, . . . , P2, P1, P1′, P2′, . . . , Pm−1, Pm with amide bond hydrolysis occurring between P1 and P1′. Sn, Sn−1, . . . , S2, S1, S1′, S2′, . . . , Sm−1, Sm denotes the corresponding enzyme binding sites.  
2 The abbreviations used are: tPA, tissue plasminogen activator; uPA, urokinase-type plasminogen activator; ACC, 7-amino-4-carbamoylmethylcoumarin; cmk, chloromethylketone; Nes, norleucine; Ac, acetyl; Nme, N-methyl; MES, 4-morpholineethanesulfonic acid.
American Diagnostica (Greenwich, CT). Plasmin and thrombin were purchased from Hematologic Technologies Inc. (Essex Junction, VT). Heparin and other biochemicals were purchased from Sigma. Substrates in the positional scanning synthetic combinatorial libraries as well as Ac-PRNK-ACC, Ac-PRNR-ACC, Ac-GTAR-ACC, Ac-QFAR-ACC, and Ac-QWK-ACC were prepared as previously described (10). Ac-PRNK-cmk was synthesized by Enzyme Systems Products (Livermore, CA).

βII Tryptase Gene Construction—The pPIC9-Hu Try (human βI tryptase plasmid) (11) was subjected to site-directed mutagenesis using the GeneEditor™ in vitro site-directed mutagenesis system (Promega, Madison, WI). The mutant oligonucleotide GTCTCCAGCCAC-3′/GAGAGGCCACGTGTCCTCAGCCAC-3′ was used to introduce a substitution mutation in the DNA coding for amino acid residue 113 (N113K). Full-length nucleic acid sequencing of both strands confirmed the sequence conversion to the βII tryptase isoform.

Preparation and screening of the positional scanning synthetic combinatorial library—The concentration of each of the 361 substrates per well in the P1 lysine and tryptase II tryptase isoform.

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The proportion of catalytically active βII and βI tryptase was quantitated by active-site titration with 4-methylumbelliferyl-p-guanidinobenzoate (12). Briefly, fluorescence was monitored, with excitation at 360 nm and emission at 450 nm, upon the addition of enzyme to 4-methylumbelliferyl-p-guanidinobenzoate. The concentration of enzyme was determined from the increase in fluorescence based on a standard concentration curve.

The recombinant human βI and βII tryptases (1 μg) and native human lung tryptase were subjected to reducing SDS-polyacrylamide gel electrophoresis on a 4–20% TG gel (Novex). Following electrophoresis, the gel was stained by GelCode™ (Pierce) (Fig. 1) to verify size and purity.

Positional Scanning Synthetic Combinatorial Library Screening—Preparation and screening of the positional scanning synthetic combinatorial library were carried out as previously described (10, 13). The concentration of each of the 361 substrates per well in the P1 lysine and P1 arginine libraries was 0.25 μM. The concentration of the 6859 compounds/well in the P1-diverse library was 0.013 μM. Enzyme activity of βI and βII tryptase in the positional scanning synthetic combinatorial tetrapeptide library was assayed in 100 mM HEPES, pH 7.5, 10% glycerol, and 0 or 0.1 mg/ml heparin at excitation and emission wavelengths of 380 and 450 nm, respectively.

Single Substrate Kinetic Analysis—Tryptase activity was monitored as follows. Ac-PRNK-ACC was added to a final concentration of 5 μM to the samples containing tryptase. kcat was determined with an interatomic cut-off of 25 Å and ε = 4t. The peptide side chains (PRNK) were then added, and the concentrations of the P1–P3 side chains and the P4 proline were modeled with DOCK4.0 as previously described (19). Finally, 10 independent minimizations were carried out, and the lowest energy configuration was retained.

RESULTS

Expression of Active βI and βII Tryptase in P. pastoris—Recombinant tryptase βI and βII were produced and secreted in P. pastoris as mature enzymes. The ability to produce active mature enzyme rather than the zymogen is important for substrate specificity studies, because it obviates the need to remove the propeptide through the addition of an activating protease, whose activity may complicate subsequent specificity studies. There is a single amino acid difference between tryptase βI and tryptase βII at position 113, an asparagine and threonine, respectively. Replacement of asparagine for threonine removes an N-linked glycosylation site in tryptase βI, making it singly glycosylated. The relative degree of glycosylation can be seen in the recombinant expression of both enzymes (Fig. 1), with tryptase βI migrating as multiple glycosylated bands and tryptase βII migrating as a single glycosylated band. The only difference seen in expression and purification of the two enzymes is the final yield of active enzyme with tryptase βI expressing 10-fold more than tryptase βII. The phenomenon of reduced expression upon the removal of a glycosylation site has been observed with other proteases and has been postulated to involve decreased stability or solubility of the enzyme lacking post-translational glycosylation (20).

βI and βII Tryptase Have Equivalent Primary and Extended Substrate Specificity—To explore whether this single difference in glycosylation affects the substrate specificity of tryptase βI and βII, three combinatorial peptide libraries with fluorogenic leaving groups were used. The P1 specificity was first defined using a method in which both the propeptide and the tetrapeptide were held constant while the other three positions contained an equimolar mixture of 19 amino acids (cysteine was omitted, and norleucine replaced methionine). Both tryptase βI and βII prefer cleaving after lysine over arginine with no other amino acids being accepted at this position (Fig. 2).

To define the extended substrate specificities of the β-tryptase

Storage and transport of radioactivity are critical to the success of any experiment. The use of effective processing techniques, which include washing, drying, and storage, is essential to ensure the integrity of the samples. It is important to use appropriate excitation and emission wavelengths to ensure accurate data collection. The use of high-performance liquid chromatography (HPLC) can help in the quantification of the compounds, which is crucial for understanding the results.

In conclusion, the positional scanning synthetic combinatorial library was used to explore the substrate specificity of tryptase βI and βII. The results indicated that these enzymes have equivalent primary and extended substrate specificities, which can be used in the development of new drugs to target these proteases.
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![Fig. 1. Coomassie-stained gel of βI and βII tryptase expression products. A, recombinant βI tryptase. Non-, single-, double-, and hyperglycosylation forms are observed. B, recombinant βII tryptase. Non- and single-glycosylation forms are observed. C, native β-tryptase. D, molecular mass standards.](image)

![Fig. 2. P1 substrate specificity of β-tryptases. Results from the P1-diverse positional scanning library are shown, where the y axis represents the rate of substrate cleavage in relative fluorescence units per second (RFU/sec) and the x axis represents the P1 amino acid. The P2, P3, and P4 positions contain an equimolar mixture of 18 amino acids (Cys and Met excluded, Nle (n) included) for a total of 6,859 substrates/well.](image)

To demonstrate that information obtained from the substrate screen could be translated into a potent tryptase inhibitor, the irreversible inhibitor Ac-PRNK-cmk was tested for inhibition of tryptase. The measured association rate constant, $k_{on}$, of $5000 \pm 200 \text{ M}^{-1} \text{s}^{-1}$ for both βI and βII tryptase indicates that Ac-PRNK-cmk is a potent inhibitor of tryptase. Selectivity of the designed tryptase inhibitor, Ac-PRNK-cmk, was demonstrated through the measurement of inhibition of several tryptic plasma proteases, factor Xa, tPA, uPA, thrombin, and plasmin. At an inhibitor concentration of 10 μM, where tryptase is 95% inhibited, none of the proteases tested showed inhibition (Table II). At a 10-fold higher inhibitor concentration of inhibitor (100 μM), where tryptase is completely inhibited, only uPA and plasmin showed inhibition, 34% and 63% inhibition, respectively (Table II).

β-Tryptase Binds Its Preferred Substrate with Potential Interactions from Two Protomers—The source of the preference for basic residues at the P1 position is well known for this class of proteolytic enzyme; Asp-189 is present in all trypsin-like serine proteases and resides at the bottom of the S1 pocket. The source of extended specificity is less apparent. The structure of tryptase is unique among serine proteases in that it is a ring-like tetramer with the four active sites in close proximity within the interior pore (2). Using the program DOCK with energy scoring (22), the capped tripeptide Ac-PRNK-Nme was docked into the active site of BII tryptase. The docked molecule had a score of $-86.34$ DOCK units, consisting of an electrostatic contribution of $-56.88$ and a van der Waals contribution of $-29.46$. The unusually large electrostatic component is a result of the large negative charge concentrated within the pore of the tetramer.

The model of substrate binding suggests a paired binding site, with contributions from two tryptase protomers. Specifically, docking of the optimal peptide into the active site of tryptase predicts that the P4 and P3 side chains interact with the adjacent protomer. The P4 Pro side chain interacts with the γ-carbon of Thr-96' of the adjacent protomer (Fig. 4). A recognition site for the P3 Arg is formed by acidic residues from both protomers, Glu-217 from the cognate protomer and Asp-60B' from the adjacent protomer (Fig. 4). Formation of the P4 and P3 side chain interactions requires a somewhat noncanonical backbone configuration, resulting in the loss of a backbone hydrogen bond. By contrast, the P2 and P1 sites make the canonical interactions seen with other members of this protease class. For example, the deep S1 pocket contains Asp-189
Fig. 3. P2-P4 substrate specificity of β-tryptases. Results from the P1 Lys (A) and the P1 Arg (B) libraries are shown, where the y axis represents the rate of substrate cleavage in relative fluorescence units/second (RFU/sec) and the x axis represents the positioned P2, P3, or P4 amino acid. The two positions in the substrate that are not held constant contain an equimolar mixture of 19 amino acids (Cys and Met excluded, Nle (n) included) for a total of 361 substrates/well.
Another consequence of the structure is that each active site has an adjacent active site in close proximity, leading to potential substrate-substrate interactions (Fig. 4).

from the cognate protomer that interacts with P1 Lys (Fig. 4).

The tryptase family of serine proteases has been implicated in a variety of allergic and inflammatory diseases involving mast cells because of elevated tryptase levels found in biological fluids from patients with these disorders. However, the exact role of tryptase in the pathophysiology of disease remains to be delineated. The scope of biological functions and corresponding physiological consequences of tryptase are substan-
typically defined by their substrate specificity. In this study, high levels of mature recombinant human β and βII tryptases were expressed in *P. pastoris* (Fig. 1) for studies of primary and extended substrate specificity.

Human mast cells express at least four distinct tryptases, designated α, β, βII, and βIII. These enzymes are not controlled by blood plasma protease inhibitors and only cleave a few physiological substrates in vitro. It is currently unknown whether human tryptases perform redundant or unique functions in vivo. One recent study, which included a protein engineering approach, demonstrated that a single amino acid difference in one of the surface loops that forms the substrate-binding cleft led to a functional distinction between human tryptase α and β II (9). However, no physiological differences have been reported between β and βII tryptases, which differ only at a single amino acid residue at position 113, leading to the loss of an N-linked glycosylation site. Based on the data presented herein, β and βII tryptases have similar P4 to P1 substrate preferences (Figs. 2 and 3, Table 1). The functional similarity observed for the two enzymes is in agreement with the reported crystal structure of βII tryptase (2), in that the structure shows the glycosylation site peripheral to the active site and should therefore have minimal effect on the substrate specificity. The shared preference for peptide substrates may extend to a shared preference for physiological substrates. Indeed, the optimal sequence for β-tryptase cleavage, P4 Pro, P3 Arg/Lys, P2 X, and P1 Lys/Arg, is found in many of the macromolecular substrates previously shown by others to be cleaved by tryptase in vitro.

Tryptase is a potent activator of pro-uPA, the zymogen form of a protease associated with tumor metastasis and invasion. Activation of the plasminogen cascade, resulting in the destruction of extracellular matrix for cellular extravasation and migration, may be a function of tryptase activation of prourokinase plasminogen activator at the P4–P1 sequence of Pro-Arg-Phe-Lys (4). Vasoactive intestinal peptide, a neuropeptide that is implicated in the regulation of vascular permeability, is also cleaved by tryptase, primarily at the Thr-Arg-Leu-Arg sequence (5). The G-protein-coupled receptor PAR-2 can be cleaved by tryptase, primarily at the Thr-Arg-Leu-Arg sequence (6, 23–25).

A search of the protein data bases (Swiss-Prot) has revealed other candidate physiological substrates containing the predicted sequences for β-tryptase cleavage. These macromolecules have yet to be empirically characterized as tryptase substrates, but many, such as latent transforming growth factor-β-binding protein (cytokine modulator), annexin I and II (calcium-binding proteins that participate in the regulation of early inflammatory responses), and HGF (a growth factor implicated in tumor development and progression and in angiogenesis), are particularly intriguing because they further support the concept of a prominent role for tryptase in tissue remodeling during disease pathogenesis. In this study, the kinetic constants for βI and βII tryptase were determined using four synthetic peptide substrates derived from the positional scanning combinatorial peptide library screening results (Table 1). These substrates served to quantify the tryptase dependence on extended substrate specificity, indicating that the ground state binding and recognition are important factors in tryptase catalysis. The preferred tetrapeptide PRNK substrate and irreversible inhibitor, revealed from the combinatorial library screens, also formed the basis for a rapid, sensitive, and selective enzymatic assay for human β-tryptases in a variety of complex biological media, including serum and plasma.3

To explore the structural determinants of substrate binding, the Pro-Arg-Asn-Lys peptide was modeled into the active site of tryptase. The modeling results revealed several canonical substrate interactions in addition to interactions not seen with other serine proteases of the chymotrypsin fold. The unique tetrameric structure of tryptase allows for a substrate to interact with two promoters simultaneously. In addition, the close proximity of paired tryptase active sites raises the possibility of interactions between multiple substrates.

Tryptase has been recognized as a viable drug target, and therapeutically useful inhibitors have been under development by several pharmaceutical companies, some even taking advantage of the bifunctional active site (15, 26). Insights gained from the modeling of the optimal sequence into the active site will support further development of novel selective substrates of β-tryptases that will enhance our understanding of the pathophysiology of these enzymes as well as lead to the development of new and effective inhibitors.

In summary, recombinant human β and βII tryptase was expressed and used to determine an optimal sequence for β-tryptase cleavage: P4 Pro, P3 Arg/Lys, P2 X, and P1 Lys/Arg. This sequence has already proved useful for the development of pharmacological tools for the further study of tryptase. Moreover, this study of β and βII tryptase highlights the utility of generalized positional scanning combinatorial peptide libraries to functionally characterize similarities and differences between homologous enzymes, generate sensitive and selective substrates and inhibitors, and define a subset of potential physiological substrates.

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