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Synthesis and Physical Characterization of a P_1 Arginine Combinatorial Library, and Its Application to the Determination of the Substrate Specificity of Serine Peptidases

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Abstract—Serine peptidases are a large, well-studied, and medically important class of peptidases. Despite the attention these enzymes have received, details concerning the substrate specificity of even some of the best known enzymes in this class are lacking. One approach to rapidly characterizing substrate specificity for peptidases is the use of positional scanning combinatorial substrate libraries. We recently synthesized such a library for enzymes with a preference for arginine at P_1 and demonstrated the use of this library with thrombin (Edwards et al. *Bioorg. Med. Chem. Lett.* **2000**, 10, 2291). In the present work, we extend these studies by demonstrating good agreement between the theroretical and measured content of portions of this library and by showing that the library permits rapid characterization of the substrate specificity of additional SA clan serine peptidases including factor Xa, tryptase, and trypsin. These results were consistent both with cleavage sites in natural substrates and cleavage of commercially available synthetic substrates. We also demonstrate that pH or salt concentration have a quantitative effect on the rate of cleavage of the pooled library substrates but that correct prediction of optimal substrates for the enzymes studied appeared to be independent of these parameters. These studies provide new substrate specificity data on an important class of peptidases and are the first to provide physical characterization of a peptidase substrate library.

Introduction

A key to understanding the activity of any peptidase is knowledge of the substrate specificity of the enzyme. Such understanding provides a route to the design of enzyme assays, and, potentially, a starting place for structure-based design of peptidic and/or non-peptidic inhibitors. Until recently, determining substrate specificity for peptidases required considerable effort, typically involving studies with native or synthetic substrates, sequence and structural data, and naturally occurring or synthetic inhibitors. Traditionally, most such investigations of substrate specificity have been conducted with a single or small number of enzymes, and this work can be complex and time consuming. Recently, however, combinatorial substrate libraries have been devised for revealing peptidase substrate specificity and these libraries provide the opportunity for rapid study of substrate specificity for many enzymes in parallel.[1] In principle, substrate library approaches can result in detailed knowledge of enzyme specificity for a peptidase without prior knowledge of the native substrate. However, surprising new results can be obtained even when characterizing well-studied enzymes.

Recently, we synthesized an aminocoumarin positional scanning library for determining substrate specificity for serine peptidases.[2] This library is suitable for use with serine peptidases that prefer Arg at the P_1 position and is similar to other recent work with positional scanning libraries for peptidases with a preference for Asp at P_1.[3] Since many serine peptidases are known to have a preference for Arg at P_1, such a library is potentially useful in the comprehensive characterization of a large subset of all the known enzymes in this class or for revealing substrate specificity of novel serine peptidases. In the present study, we have tested 12 serine peptidases with this combinatorial library. These studies have revealed

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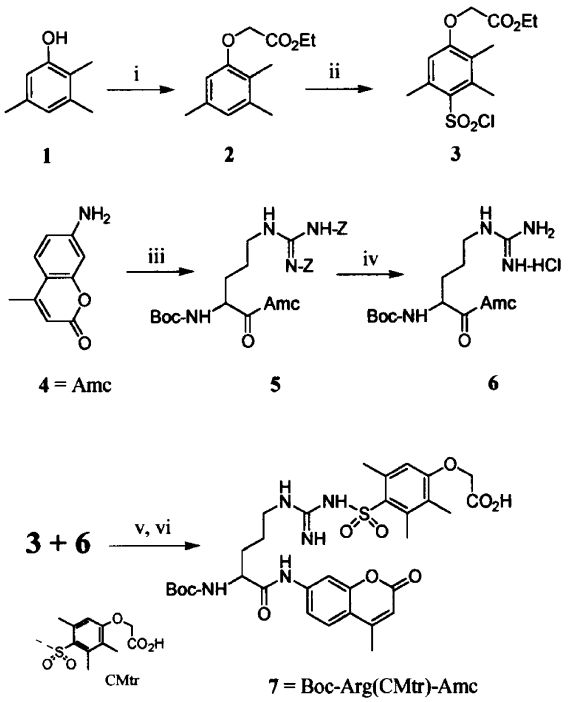
detailed information on the substrate specificity of four of these enzymes. In addition, we describe here the complete synthetic details for construction of the library, and a method for physical characterization of the library to ensure synthetic fidelity.

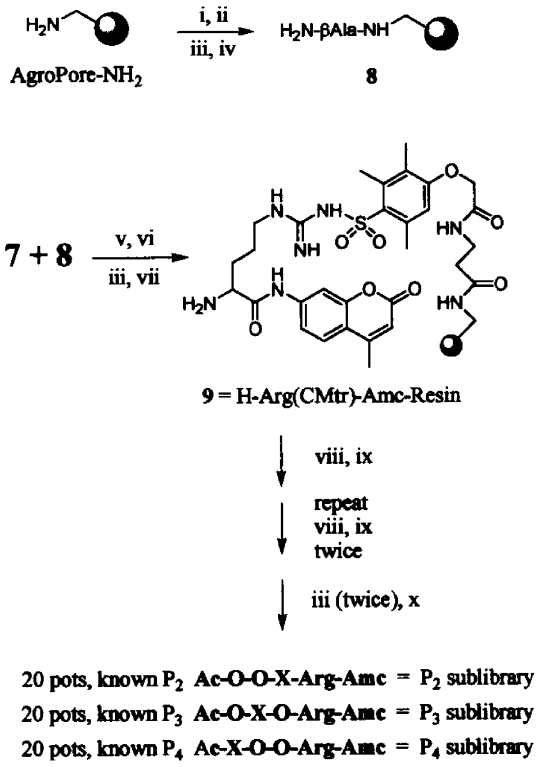
Chemistry

The methodology we developed to determine the substrate specificity of SA clan serine peptidases[2] centered around the construction of a positional scanning combinatorial substrate library (PS-CSL) via solid phase techniques. This library was used to evaluate the specificity of the P_4–P_2 positions of tetrapeptides containing a P_1 arginine aminocoumarin (AMC). This is accomplished by constructing separate P_2, P_3 and P_4 sublibraries. In order to evaluate 20 different amino acids at each position, 20 reaction vessels or 'pots' are prepared in each sub-library, with a known residue. Using the P_2 library for illustration, the resin bound P_1-Arg-AMC is placed in 20 pots and coupled with a single, different amino acid residue in each pot. Each pot is then coupled with the same isokinetic mixture of all 20 amino acids[4] to generate an equimolar mixture of all possible 20 tripeptides with a known P_2 residue (e.g., *O*-V-Arg-AMC). Next, each pot is again coupled with the same isokinetic mixture to generate all 400 tetrapeptides (e.g., Ac-*O*-*O*-V-Arg-AMC). Following cleavage from the resin, each pot is assayed for its relative rate of hydrolysis by the enzyme under evaluation. Since each pot contains all possible tetrapeptides with a known, but different P_2 residue, the pots displaying the greatest level of hydrolysis possess the preferred P_2 residues. Similar sublibraries are prepared for the P_3 and P_4 positions. Following assay of these libraries, the combined data is used to determine the preferred substrate specificity at each position. While there have been several reports of the solid-phase synthesis of amidines and guanidines,[5] little has been published on the solid-phase synthesis of peptides containing arginine linked to the resin through the side chain guanidine group. As we began considering a strategy for attaching an arginine to a solid-phase resin via its side chain guanidine group, two reports appeared in the literature describing such a procedure.[6,7] Zhong et al. employed an aromatic sulfonyl linker, which is conceptually similar to the procedure we ultimately employed. However, attachment of the arginine guanidine to the resin-bound sulfonyl group required 4 N KOH at 75 °C for 2 days, conditions incompatible with the presence of an aminocoumarin amide required in our synthesis. Most relevant to our needs was a poster presentation by Urban and Dattilo describing the solid phase preparation of a P_1 arginine tripeptide *p*-nitroanilide substrate using a novel CMtr linker. The CMtr is a modified Mtr containing a carboxyl group for attachment to the resin.[7] We used a modified version of this procedure for preparing the intermediate Boc-Arg(CMtr)-Amc **7** (Scheme 1). The chemistry is straightforward with a few notable exceptions. The sulfonyl chloride **3** is relatively unstable, and the reaction time for its formation should be limited to 1 h. Furthermore, **3** should be used in the subsequent



Scheme 1. (i) Ethyl bromoacetate, K_2CO_3, DMF, rt, 18 h, 75%; (ii) HSO_3Cl, CH_2Cl_2, 4 °C, 1 h, 85%; (iii) Boc-Arg(Z)$_2$-OH, Boc_2O, Py, THF, rt, 18 h, 76%; (iv) $Pd(OH)_2$, EtOH, 1 N HCl, H_2/48 psi, 4 h, 93%; (v) 1 N NaOH (2 equiv), acetone, rt, 0.5 h, 32%; (vi) 1 N NaOH (1 equiv), THF/EtOH (1:1), rt, 3 h, 82%.

sulfonamide forming reaction immediately upon solvent removal. We have also found that the best overall yields are obtained when sulfonamide formation and ester hydrolysis are carried out in two independent, sequential reactions. A significant amount of effort was required to identify appropriate conditions for attaching **7** to a solid support (Scheme 2). We initially investigated two different PEG polystyrene resins. Since the amino group attachment point in these resins is distal to the cross-linked resin backbone, we felt that these resins would afford a more efficient amide coupling with the relatively bulky acid **7**. However, the physical properties of the coupled products were such that severe mechanical losses were encountered. These losses continued to plague us throughout the solid-phase synthetic sequence. To overcome this problem and still maintain an extended attachment point to the resin, we attached a β-alanine linker to an AgroPore^{TM}-NH_2 resin. When coupled with **7**, the resin bound intermediate **9** had excellent handling properties and no significant mechanical losses were observed. The couplings of both the β-alanine to the resin and of **7** to **8** were monitored by following the growth of the amide carbonyl absorption peak of a bead using solid-phase IR since a negative Kaiser test could not be obtained. Maximum loading was considered the point where there was no further increase in the carbonyl absorption, and was achieved using a two-step sequence involving basic coupling conditions (HATU and DIPEA) followed by a more acidic system (DIPCI and HOBt). Unreacted amino groups on the resin were then capped with acetic anhydride. Recently, a procedure has been reported for the solid-phase synthesis of peptide substrates with a P_1 argininne-aminocoumarin using a safety-catch linker approach.[8]



Scheme 2. (i) Boc-β Ala-OH, HATU, DIPEA, DMF, 18 h, repeat for 3 h; (ii) HOBT, DIPCI, DMAP (cat), DMF, 18 h, repeat for 3 h; (iii) Ac_2O, DIPEA, DMF, 2 h; (iv) 40% TFA/CH_2Cl_2, 0.5 h; (v) HATU, DIPEA, DMAP (cat), DMF, 18 h; (vi) HOBt, DIPCI, DMAP (cat), DMF, 18 h; (vii) 40% TFA/CH_2Cl_2, 1 h; (viii) isokinetic mixture or known amino acid, HATU, DIPEA, DMF, 2 h, repeat procedure once; (ix) 20% piperidine, DMF, 15 min, repeat procedure once; (x) thioanisole/TMSBr/ triisopropyl silane/phenol/TFA.

Results

Physical characterization of the Arg P_1 positional scanning library

Although there have been several reports on the preparation and use of positional scanning combinatorial substrate libraries, these libraries have only been characterized biochemically (functionally). The assumption is made that since isokinetic mixtures of amino acids are used in the construction of the libraries, all possible peptides will be present in equimolar mixtures. However, since the efficiency of solid-phase amide couplings is dependent upon the exact sequence of the resin-bound substrate, the experimentally derived isokinetic mixtures may not afford equimolar products for a particular sublibrary. In order to validate the synthetic fidelity of our libraries, we have developed a procedure for physical analysis of the libraries. Using the P_3 sub-libraries Ac-*O*-Val-*O*-Arg-Amc and Ac-*O*-Asp-*O*-Arg-Amc as examples, the pots were analyzed by means of MALDI-TOF mass spectrometry (Fig. 1). The mass spectral data was assessed for the presence of three features: (1) that the smallest (G-V-G-; G-D-G-) and largest (W-V-W-; W-D-W) molecular weight peptides were present; (2) that the center of the distribution for each of the two pots was shifted from each other by 16, the difference in molecular weight between valine and aspartic acid; and (3) that the molecular weight distribution was centered

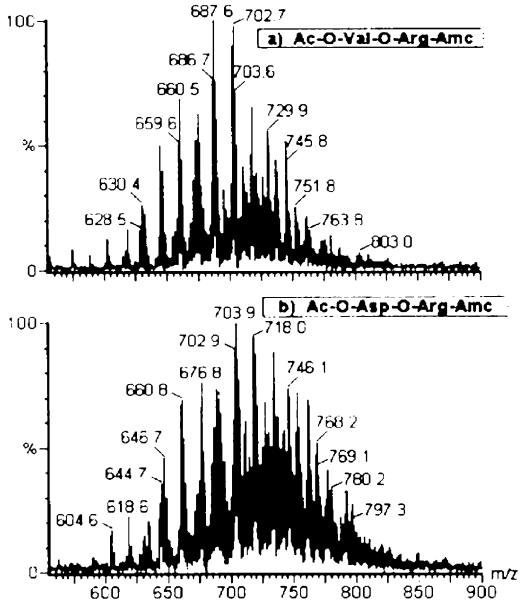


Figure 1. MALDI-TOF mass spectra of P_3 sublibrary pots: (a) Ac-*O*-Val-*O*-Arg-Amc; (b) Ac-*O*-Asp-*O*-Arg-Amc.

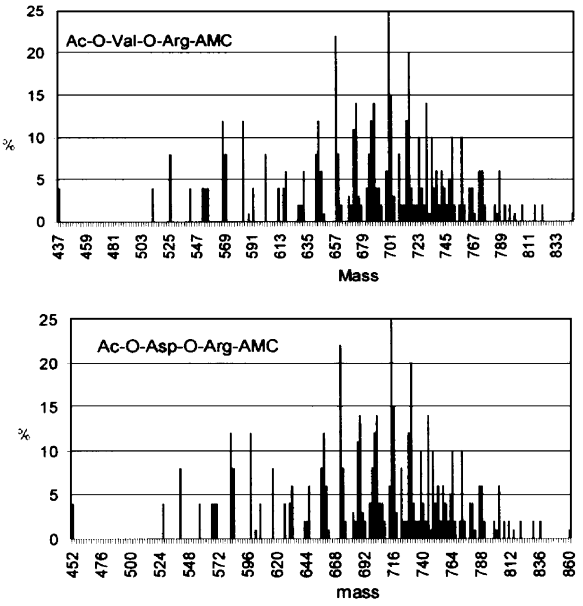


Figure 2. Simulated MALDI-TOF mass spectra of P_3 sublibrary pots: (a) Ac-*O*-Val-*O*-Arg-AMC; (b) Ac-*O*-Asp-*O*-Arg-AMC.

around the average molecular weight. Figure 1 is a comparison of the mass spectra for the-Val- and-Asp-pots. As anticipated, the high and low molecular weight compounds were present and the center of distribution between each library was shifted by 16. The average molecular weights for the Ac-*O*-Val-*O*-Arg and Ac-*O*-Asp-*O*-Arg pots are 705 and 721, respectively. The centers of distribution in Figure 1 are in good agreement with these calculated values. In addition, we have developed a program for computationally simulating the predicted MALDI-TOF spectrum of an individual sublibrary (MALDIsym, see Experimental). The simulated mass spectra for the Ac-*O*-Val-*O*-Arg-Amc and Ac-*O*-Asp-*O*-Arg-Amc are depicted in Figure 2, and agree favorably with the experimental mass spectra in

Figure 1. The mass spectral analysis we have presented here provides an easy and valuable method for evaluating the fidelity of a combinatorial substrate library. *Since interpretation of the results from the biological evaluation of a substrate library is dependent upon the synthetic fidelity of the library, we believe that an analysis of the library such as the MALDI-TOF analysis presented here is a prerequisite for utilization of combinatorial substrate libraries for screening against target enzymes.*

Screening of the Arg P_1 positional scanning library

In our previous report using the present library with thrombin,[2] we demonstrated cleavage of library substrates consistent with the known properties of this enzyme.[9] Specifically, thrombin has a strong P_2 preference for Pro and is less specific at P_3 and P_4. In the present study, we expand on this initial result by comparing the results of thrombin with this library to results using other serine peptidases: Factor Xa, trypsin, and tryptase. As depicted in Figure 3, in addition to substrates with proline at P_2, thrombin also cleaved substrates with aliphatic amino acids at this position such as alanine, isoleucine, valine, norleucine, valine or leucine. However, this cleavage occurred at markedly lower rates than for substrates with proline at P_2. Generally, substrates with charged amino acids at P_2 were not cleaved at a significant rate by thrombin. By contrast, tryptase generally

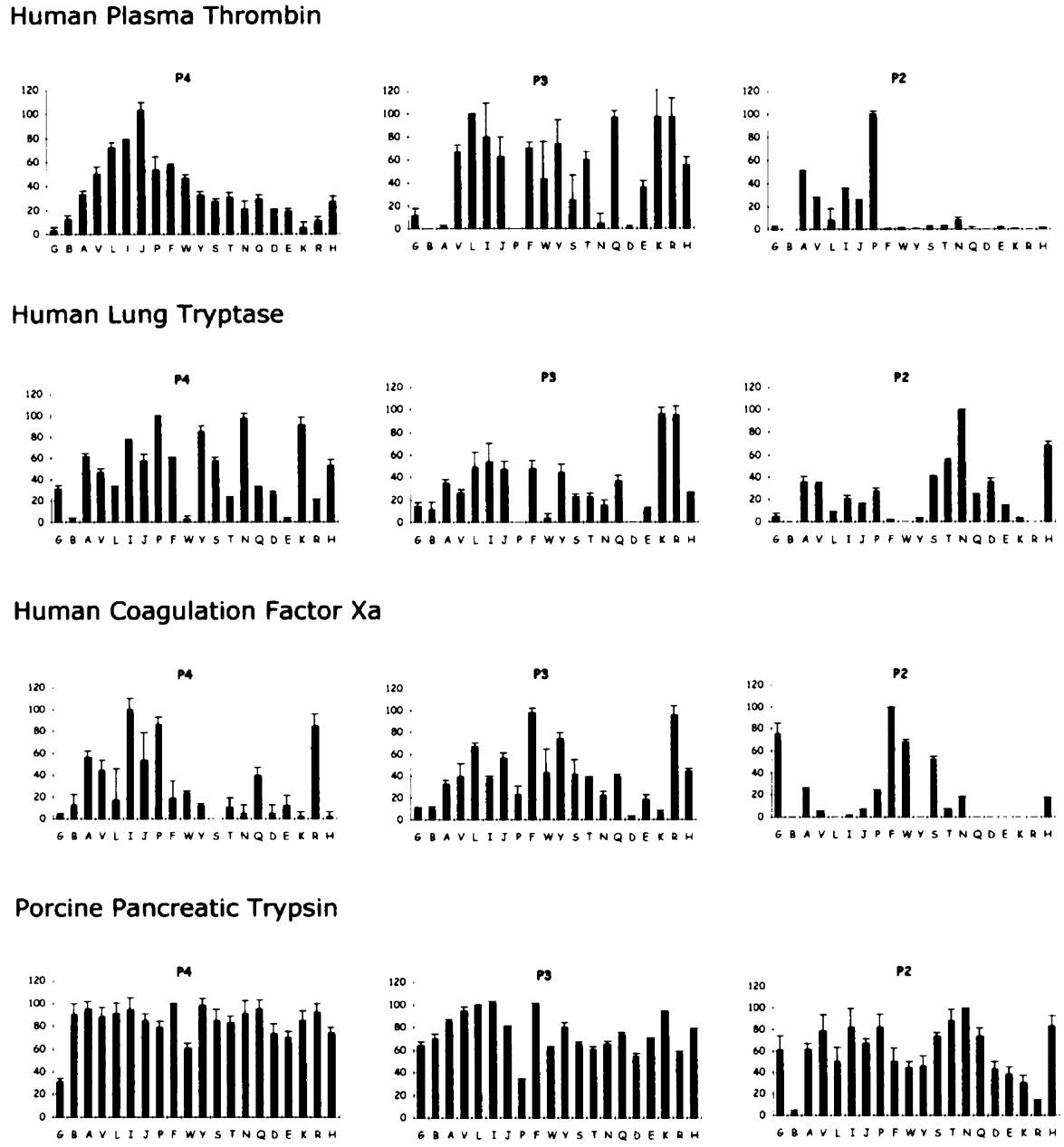


Figure 3. Substrate specificities for serine peptidases. Assays were conducted for four serine peptidases with a known preference for arginine at P_1. The *y* axis shows the rate of AMC production expressed as a percentage of the maximum rate observed in each experiment. The *x* axis represents the amino acids (B = D-alanine; *J* = norleucine). The data indicate more selectivity at the P_2- and P_3-positions for thrombin, factor Xa, and tryptase. Porcine pancreatic trypsin shows less specificity than the other enzymes at all positions. Each bar represents mean ± standard deviation for an *n* of three assays carried out on different days.

preferred neutral-polar amino acids at P_2 and also tolerated hydrophobic aliphatic, but not aromatic amino acids. Factor Xa preferred the aromatic residues phenylalanine and tryptophan at P_2 but, interestingly, did not cleave substrates with tyrosine in that position. Factor Xa also cleaved substrates with glycine or serine at P_2 at a significant rate but, like thrombin, generally did not cleave substrates with charged P_2 residues. As might be expected for a digestive enzyme, trypsin showed little preference at P_2. Like the other three enzymes illustrated, though, trypsin did not cleave substrates with the non-physiological D-alanine at P_2. For all four enzymes a wide variety of amino acids were tolerated at P_3. With the exception of trypsin, which showed little preference at P_3, and tryptase, which demonstrated a clear preference for the basic amino acids arginine and lysine at this position, general patterns were less clear at P_3 than at P_2. At P_4, again trypsin showed little preference and all enzymes tolerated a wide variety of amino acids. Both thrombin and Factor Xa generally seemed to prefer hydrophobic-aliphatic residues at P_4 and both cleaved substrates containing charged amino acids at a lower rate (with the exception of glutamine or arginine containing substrates by Xa). Tryptase cleaved substrates containing glutamate, tryptophan and D-alanine at P_4 poorly, and those containing lysine, asparagine, tyrosine and proline at P_4 best.

Cleavage of homogeneous synthetic substrates by tryptase

Using a library such as that which we describe here, a comparison of cleavage for substrates containing a specific amino acid at positions P_2–P_4 can be obtained. These results are obtained by using mixtures of synthetic substrates in each 'pot', however, so it would be of interest to compare library results to those obtained using homogeneous substrates. Since a variety of homogeneous aminocoumarin peptide substrates are commercially available we chose to compare tryptase cleavage of selected homogeneous substrates with the results obtained using the combinatorial library. Tryptase was chosen as the representative enzyme with which to conduct these studies because it was shown to have clear specificity at positions P_2–P_4 and two tripeptide aminocoumarin substrates, H-Val-Pro-Arg-AMC and H-Phe-Ser-Arg-AMC are known to be cleaved by this enzyme. However, based on our initial substrate library results with tryptase, other substrates were also expected to be cleaved. This prediction proved correct. As shown in Figure 4, H-Val-Pro-Arg-AMC and H-Phe-Ser-Arg-AMC were efficiently cleaved by tryptase but, consistent with the library results, three other substrates, H-Gly-Pro-Arg-AMC, H-Gln-Ala-Arg-AMC and H-Asp-Pro-Arg-AMC, were superior. Also consistent with the library observations for tryptase, H-Arg-Arg-Arg-AMC and H-Phe-Arg-Arg-AMC are not good substrates. Somewhat surprisingly, several of the substrates that contained K, G or R at P_2 also showed a reasonable rate of cleavage albeit at a lower rate than predicted better substrates. It should be noted that the homogeneous substrates used in the present studies were not terminated with an acetyl group and this may have had some effect on the cleavage rate, especially since our studies have revealed that tryptase prefers basic residues at P_3.

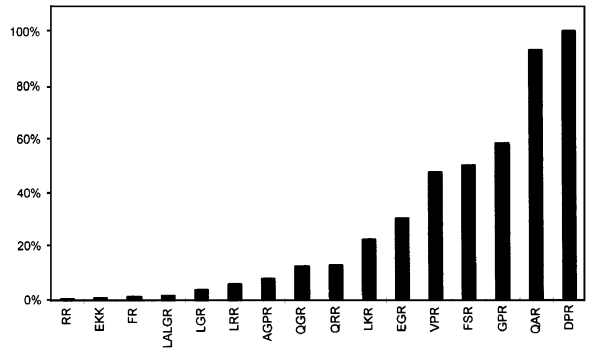


Figure 4. Cleavage of a panel of commercially available aminocoumarin-labeled synthetic substrates by tryptase. Assays were carried out under identical pH and buffer conditions to those described for assays with the combinatorial library. All substrates assayed were AMC labeled peptides (shown by single-letter amino acid designations), most with R at P_1. Results are expressed as rate of cleavage by comparison to that for the most efficiently cleaved substrate (DPR-AMC).

Effect of salt and pH on substrate cleavage

It is possible that assay conditions could affect the *relative* cleavage rate of different substrates and thus produce confusing or misleading results. This possibility could be particularly important when evaluating a new or poorly characterized enzyme. Two specific assay conditions that might influence the relative rate of cleavage of substrates (either within or between pots) using the positional scanning approach are salt concentration and pH. To our knowledge, these affects have not as yet been investigated. As a comparison, the effect of salt concentration on cleavage of one tripeptide tryptase substrate, H-Gly-Pro-Arg-AMC, was compared to results obtained using substrate pots. The best cleavage of H-Gly-Pro-Arg-AMC by tryptase was observed in the absence of salt and a dose dependent inhibition by sodium chloride was observed. Similar results were observed using selected pots from the library. The pots selected for this experiment were those from the P_2 libraries in which tryptase had been shown to produce the most efficient cleavage. As shown in Figure 5, results from the selected pots showed a dose dependent inhibition by salt comparable to that observed with the tripeptide substrate. Significantly, however, the rank order of substrate preference was unaffected. As a further comparison, two P_2 pots were chosen to compare salt effect on thombin cleavage. Again these pots were chosen as representative pots that contain substrates efficiently cleaved by the enzyme. There was no evidence, however, that the salt concentration at which the assays were conducted would markedly alter the rank order substrate preferences of the enzyme. Thrombin was further chosen to test the effect of pH on library screening assays and in particular to determine if the marked preference for Pro at P_2 could be altered by changing this parameter. Figure 6 shows results from assays carried out at pH 9.0. For the P_2 library relative cleavage at pH 9.0 was essentially identical to that

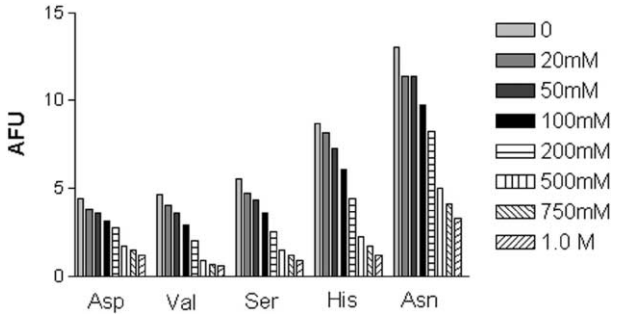


Figure 5. Effect of NaCl concentration on human lung tryptase cleavage of synthetic substrates. Each substrate has arginine at P_1. The *y* axis shows the rate of AMC production in arbitrary fluorescence units/min. The *x* axis represents the amino acids at P_2. The data shows a decrease in the potency of the substrate with increasing concentrations of salt. Although there is a decrease in the rate of cleavage with increasing salt concentration, for all the amino acids shown there is variation in the amount of the decrease that is dependent on the specific amino acid. Each bar represents the mean ± standard deviation for three assays.

shown at lower pH (Fig. 3). By contrast, thrombin cleavage in the P_3 pot showed a marked pH dependence. For example at pH 9.0, the P_3 'N' pot contained the best substrates, while at lower pH (Fig. 3), this represented one of the least efficiently cleaved substrates, and the 'R', 'L', and 'Q' pots were preferred. This is not unexpected in that charge–charge electrostatic interactions between enzyme and substrate would be expected to be affected by pH. In general, it appeared that there was less specificity at pH 9.0 than at pH 7.5. Generally, the results showed that there was no pH dependence at the P_4 position.

Discussion and Summary

In the present study, we have used our Arg P_1 positional scanning library to characterize the substrate specificity of a panel of serine peptidases, most of which were already known to exhibit a preference for Arg at P_1. This work demonstrates that, with this library, these serine peptidases show substrate specificity unique to each enzyme which is highly reproducible and consistent with previously published data. Furthermore, although there were kinetic differences in the cleavage of these mixed substrate pools depending on salt concentration and pH, the specific assay conditions had only minor impact on the ability to predict the substrate specificity of a particular enzyme.

The substrate specificity for each of the enzymes examined in the present study are summarized in Table 1. In this work we have shown that thrombin has a preference but not an absolute requirement for proline at P_2; other hydrophobic residues such as alanine and isoleucine were also tolerated at P_2. By contrast, although less specificity was demonstrated by thrombin at P_3 than P_2, generally, there were some amino acids that were not tolerated such as A, G, P, N and D. At P_4, the strongest preference was shown for nor-leucine or other hydrophobic amino acids. Overall, these results for thrombin agree with previous studies both in terms of known cleavage sites in naturally occurring substrates and from the results of other studies using positional scanning libraries. For example, thrombin cleavage sites for substrates in the clotting cascade, including multiple sites in Factors V (709, 1018, 1545, 372) and VIII (373, 740, 1689), are consistent with the results shown here. Similarly, known thrombin cleavage sites in other substrates, including PAR1, Protein C, uPA, Factors XI, and Factor XIII, could also have been predicted. Interestingly, while other studies yielded similar results to those shown here, using a different approach to the synthesis of the library, the results with our library yielded results more consistent with the thrombin substrate specificity for known substrates, overall. Specifically, while the earlier studies suggested a nearly absolute requirement for P at P_2,[10] we have shown here that thrombin has a preference for P at P_2 but will cleave substrates with other hydrophobic residues at P_2 nearly as well. Factor Xa also yielded results with our library consistent with but not identical to other studies.[10] At P_2 we found that Xa prefers F > G, W > S > > A, P, N, H; had a preference for basic and aromatic hydrophobic residues at P_3; and preferred hydrophobic residues and R at P_4.

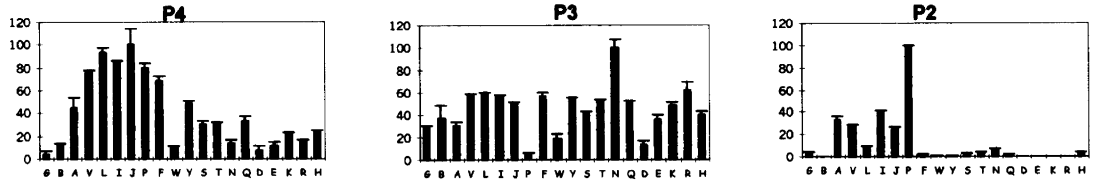


Figure 6. Cleavage of library substrates by human plasma thrombin at pH 9.0. Assays were conducted identically to those described in Figure 1 except that the pH was 9.0. Each bar represents mean ± standard deviation for three separate assays.

Table 1. Summary of substrate preferences for enzymes tested (enzyme assays were carried out as described and reported in Fig. 3)

Enzyme	Preference at P_2	Preference at P_3	Preference at P_4
Factor Xa (human plasma)	F, G, W, S	Basic, aromatic hydrophobic	Hydrophobic and R
Thrombin (human plasma)	P then hydrophobic	Everything except A, G, P, N, D	Prefers J and other hydrophobic
Trypsin (human pancreatic)	Everything but D-Ala and R	Everything but P	Everything but G
Tryptase (human lung)	Prefers N and other polar neutral, and H	Prefers K, R, then hydrophobic	Fairly broad, with significant activity for all classes except acidic

Table 2. Substrate preferences for enzymes tested that produced no detectable cleavage using the substrate library[a]

Enzyme	P_1 Substrate preference	Enzyme	P_1 Substrate preference
C1s	Arg	CVFBb/Factor B	Arg
C1r	Arg	Factor D	Arg
m-Calpain	Lys/Try, Arg/Lys	Plasmin	Lys/Arg
Chymotrypsin	Tyr, Phe, Trp, Leu	Semenogelase	Tyr/Phe

[a]All enzymes shown were tested at concentrations equal to or higher than those reported in other studies. Other than enzyme concentration, assay conditions were identical to those used for enzymes that showed activity with this library.

Harris et al.,[10] however, reported that Xa has a strong preference for G at P_2 and very little preference at P_3 and P_4. In general, despite some discrepancies between the studies reported here and those previous, there is an overall remarkable degree of agreement.

Two other serine peptidases tested included porcine pancreatic trypsin and human lung tryptase. As might be expected for a digestive enzyme, beyond the requirement for R at P_1, trypsin showed very little preference for a particular substrate. Trypsin did not efficiently cleave substrates with D-Ala and R at P_2 but the presence of any other amino acids at P_2 made little difference. Similarly, trypsin cleaved substrates with P at P_3 and G at P_4 less efficiently, but otherwise little specificity was observed. Tryptase, however, prefers N, other polar neutral amino acids, and H at P_2, a preference not previously described. Tryptase prefers K, R, and Q at P_3 but also tolerates other hydrophobic residues. Little specificity was exhibited at P_4 with the exception of W, E and D-Ala, which were not cleaved. Although a complete analysis of tryptase specificity has not been accomplished prior to the present study, these results were generally consistent both with synthetic substrates known to be cleaved by this enzyme as well as a panel of commercially available tripeptide synthetic substrates tested for the first time here (Fig. 2). For example, natural substrates include vasoactive intestinal peptide, fibrinogen, complement C3 and H-kininogen.[11]

Other enzymes were tested with this library that may have been expected (based on existing information) to show activity with the Arg P_1 library but did not (Table 2). In this category were enzymes of the complement cascade including C1s, C1r, CVFBb, Factor B and Factor D. For each of these enzymes, no cleavage was observed even at high concentrations of enzyme. However, these enzymes generally appear to be atypical serine peptidases for which inability to cleave small synthetic substrates (or inefficient cleavage) has been demonstrated.[12] Thus, it is not surprising that cleavage of library substrates could not be demonstrated here. Another enzyme tested that might have been expected to yield some cleavage but did not was plasmin. This enzyme has been reported to have a preference for Lys or Arg at P_1 with an overall specificity similar to trypsin. However, this plasmin is also known to be much less efficient than trypsin and cleaves only some of these bonds in proteins.[13] Three other enzymes that were tested with the library for which no cleavage was observed included calpain (preference for Lys/Tyr at P_1), chymotrypsin (preference for Tyr, Phe, Trp, or Leu at P_1), and PSA (preference for Phe at P_1). The fact that no cleavage was observed with these latter enzymes with the P_1 Arg library is consistent with expectations and provides further proof for the specificity of this library since none of these enzymes prefer Arg at this position. A possible explanation for why a particular enzyme did not work with this library is that enzyme concentration was too low. This explanation seems unlikely, however, since all of the enzymes in this category were tested at concentrations at least ten-fold higher than those reported in other studies.

In summary, in the present work we have synthesized and characterized a P_1 Arginine substrate library. Physical analysis of this library yielded results in good agreement with theoretical expectations. Biochemical studies with four serine proteases demonstrated that, as expected, experimental conditions, including salt and pH, affect cleavage rates of library substrates. However, determinations of substrate preferences for the enzymes tested were largely independent of experimental conditions confirming the usefulness of this and similar libraries for predicting substrate preferences. Further studies with additional enzymes may be required to confirm these observations more broadly.

Experimental

Analytical samples were homogeneous by TLC and afforded spectroscopic results consistent with the assigned structures. Proton NMR spectra were obtained using a Bruker AM-300 spectrometer. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane as internal standard. Mass spectra (MS) were recorded on a Micromass VG electrospray instrument (ES) or a Finnigan MAT-60 operating in the chemical ionization (APCI) mode (only peaks $\geq$10% of the base peak are reported). Analytical thin-layer chromatography (TLC) was conducted on pre-layered silica gel GHLF plates (Analtech, Newark, DE, USA). Visualization of the plates was accomplished using UV light, phosphomolybdic acid-ethanol and/or iodoplatinate charring. Flash chromatography was conducted on Kieselgel 60, 230–400 mesh (E. Merck, Darmstadt, West Germany). Analytical HPLC was conducted on an HP series 1050 instrument with a Vydac RP-C18 column. Solvents were either reagent or HPLC grade. Reactions were run at ambient temperature and under a nitrogen atmosphere unless otherwise noted. Solvent mixtures are expressed as volume/volume ratios. Solutions were evaporated under reduced pressure on a rotary evaporator.

All starting materials were commercially available unless otherwise indicated. Enzymes were purchased from Sigma, Calbiochem or Enzyme Systems Products. The buffers and salts were purchased from Fisher Scientific and the plates used were Costar #3912 opaque white. The following synthetic substrates were purchased from Sigma: Cbz-Arg-Arg-AMC, Boc-Glu-Lys-Lys-AMC, Cbz-Phe-Arg-AMC hydrochloride, Boc-Nle-Gln-Leu-Gly-Arg-AMC, Boc-Leu-Gly-Arg-AMC acetate, Boc-Leu-Arg-Arg-AMC dihydrochloride, Boc-Ala-Gly-Pro-Arg-AMC hydrochloride, Boc-Gln-Gly-Arg-AMC hydrochloride, Boc-Gln-Arg-Arg-AMC acetate, Boc-Leu-Lys-Arg-AMC hydrochloride, Boc-g-benzyl-Glu-Gly-Arg, Boc-Val-Pro-Arg-AMC, Boc-Phe-Ser-Arg, N-p-tosyl-Gly-Pro-Arg-AMC hydrochloride, Boc-Gln-Ala-Arg-AMC hydrochloride, and Boc-b-benzyl-Asp-Pro-Arg-AMC hydrochloride.

2,3,5-Trimethylphenoxyethylacetate (2). A solution of 2,3,5-trimethylphenol (12 g, 88 mmol) in DMF (50 mL) was treated with K_2CO_3 (13.4 g, 97 mmol). After stirring at room temperature for 15 min, ethylbromoacetate (11.73 mL, 106 mmol) was added dropwise. The solution was stirred for 16 h at room temperature and the solvent removed under reduced pressure. The residue was dissolved in EtOAc (500 mL), washed with 1 N HCl (2 × 250 mL), 1 N NaOH (2 × 250 mL), and then brine (1 × 250 mL). The organic phase was dried (Na_2SO_4), filtered, and the solvent removed under reduced pressure. The crude product was purified by flash chromatography on silica gel eluting with ethyl acetate/hexane (5:95) to afford **2** (13.4 g, 68%). HPLC $R_t = 17.3$ min (RP C-18 at 210 nm, acetonitrile/water/trifluoroacetic acid; 10:90:0.1); MS (APCI) $m/z = 223$ (M + 1, base); ^{1}H NMR (300 MHz, DMSO-d_6/TFA) δ 6.77 (s, 1H), 6.51 (s, 1H), 4.79 (s, 2H), 4.21 (q, 2H, $J = 7.2$ Hz), 2.21 (s, 3H), 2.18 (s, 3H), 2.08 (s, 3H), 1.25 (t, 3H, $J = 7.2$ Hz).

2,3,5-Trimethylphenoxyethylacetate-4-sulfonyl chloride (3). A solution of **2** (500 mg, 2.25 mmol) in dichloromethane (10 mL) was cooled to 4 °C followed by the addition of chlorosulphonic acid (0.37 mL, 5.63 mmol). The cloudy pink solution was stirred for 1 h at 4 °C. Cold water (50 mL) was added and the mixture extracted with dichloromethane (3 × 50 mL). The combined organic extracts were washed with water (2 × 50 mL) and brine (1 × 50 mL). The organic phase was dried ($MgSO_4$), filtered, and the solvent removed under reduced pressure affording **3** (648 mg, 90%). HPLC $R_t = 5.5$ min (RP C-18 at 210 nm, acetonitrile/water/trifluoroacetic acid; 10:90:0.1). ^{1}H NMR (300 MHz, DMSO-d_6/TFA) δ 6.77 (s, 1H), 4.79 (s, 2H), 4.15 (q, 2H, $J = 7.2$ Hz), 2.55 (s, 6H), 2.12 (s, 3H), 1.22 (t, 3H, $J = 7.2$ Hz).

BOC-Arg(di-CBZ)-Aminomethylcoumarin (5). A solution of BOC-Arg(di-CBZ)-OH (25 g, 46.07 mmol) in THF (150 mL) was treated dropwise with anhydrous pyridine (3.72 mL, 46.07 mmol) followed by the addition of di-*tert*-butyl dicarbonate (10.05 g, 46.07 mmol). The solution was stirred for 1 h at room temperature to form the activated ester, followed by the addition of 7-amino-4-methylcoumarin **4** (2.70 g, 15.36 mmol) in THF (150 mL). The solution was stirred at room temperature for 16 h, diluted with EtOAc (300 mL), and washed with saturated $NaHCO_3$ (2 × 200 mL), 1 N HCl (2 × 200 mL), and brine (1 × 200 mL). The organic phase was dried ($Na_2SO)_4$, filtered, and the solvent removed under reduced pressure. The crude product was purified by flash chromatography on silica gel eluting with acetone/hexane (30:70) to afford **5** (8.21 g, 76%). HPLC $R_t = 18.02$ min. (RP C-18 at 210 nm, acetonitrile/water/trifluoroacetic acid, (10:90:0.1); MS (ES) $m/z = 700$ (M + 1, base); ^{1}H NMR (300 MHz, DMSO-d_6/TFA) δ 7.84 (s, 1H), 7.30 (d, 1H, $J = 8.7$ Hz), 7.52 (d, 1H, $J = 8.5$ Hz), 7.33–7.44 (m, 10H), 6.27 (s, 1H), 5.28 (s, 2H), 5.13 (s, 2H), 3.90 (br s, 1H), 3.80 (br s, 2H), 2.42 (s, 3H), 1.70 (br s, 4H), 1.41 (s, 9H).

BOC-Arg-Aminomethylcoumarin (6). A solution of **5** (8.21 g, 11.73 mmol) in absolute ethanol (400 mL) was treated with $Pd(OH)_2$ on carbon (1.23 g) followed by the dropwise addition of 1 N HCl (12.9 mL, 12.9 mmol). The reaction vessel was charged with hydrogen (50 psi) and shook for 4 h on a Parr Shaker. The catalyst was filtered and the filter cake was washed with absolute ethanol (4 × 50 mL). The solvent was removed under reduced pressure to afford **6** (5.10 g, 93%), which was used without further purification. HPLC $R_t = 8.44$ min. (RP C-18 at 210 nm, acetonitrile/water/trifluoroacetic, 10:90:0.1); MS (ES) $m/z = 432$ (M + 1, base); ^{1}H NMR (300 MHz, DMSO-d_6/TFA) δ 7.92 (s, 1H), 7.86 (d, 1H, $J = 8.7$ Hz), 7.55 (d, 1H, $J = 8.5$ Hz), 6.27 (s, 1H), 4.16 (br s, 1H), 3.18 (br t, 2H), 2.42 (s, 3H), 1.20–1.80 (br m, 4H), 1.41 (s, 9H).

BOC-Arg(CMtr)-Aminomethylcoumarin (7). A suspension of **6** (8.35 g, 17.84 mmol) in anhydrous acetone (200 mL) was treated with 1 N NaOH (35.7 mL, 35.68 mmol). DMF (25 mL) was added to increase solubility. Sulfonyl chloride **3** (6.01 g) in anhydrous acetone (160 mL) was added and the cloudy pale yellow solution was stirred at room temperature for 30 min. The reaction mixture was adjusted to pH 4 with 1 N HCl and the product was extracted with EtOAc (500 mL). The organic extract was washed with 1 N HCl (2 × 300 mL) and brine (1 × 100 mL). The organic phase was dried ($MgSO_4$), filtered, and the solvent removed under reduced pressure. The crude material was purified by flash chromatography on silica gel eluting with EtOAc/CH_2Cl_2 (1:1) to afford the intermediate ester (1.67 g, 20%). HPLC $R_t = 14.98$ min. (RP C-18 at 210 nm, acetonitrile/water/trifluoroacetic acid, 10:90:0.1); MS (ES) $m/z = 716$ (M + 1), 660 (M-55, base), 616; ^{1}H NMR (300 MHz, DMSO-d_6/TFA) δ 7.97 (s, 1H), 7.80 (d, 1H, $J = 8.7$ Hz), 7.52 (d, 1H, $J = 8.5$ Hz), 6.60 (br s, 1H), 6.27 (s, 1H), 4.80 (s, 2H), 4.13 (q, 2H, $J = 6.5$ Hz), 4.07 (br t, 1H), 3.12 (br t, 2H), 2.59 (s, 3H), 2.55 (s, 3H), 2.42 (s, 3H), 2.13 (s, 3H) 1.20–1.80 (br m, 4H), 1.41 (s, 9H), 1.26 (t, 3H, $J = 6.3$ Hz). The intermediate ester (2.35 g, 3.28 mmol) in absolute ethanol/THF (66 mL, 1:1) was treated with 1 N NaOH (3.45 mL, 3.45 mmol). The solution was stirred at room temperature for 3 h and the solvent was removed under reduced pressure. The gummy residue was redissolved in water (10 mL) with a minimum amount of MeOH (0.5 mL) to

insure homogeneity. The solution was acidified to pH 4 with 1 N HCl. The resulting white powder was filtered and dried in a heated (50 °C) vacuum oven for 16 h to yield pure **7** (1.86 g, 82%). HPLC $R_t = 11.43$ min. (RP C-18 at 210 nm, acetonitrile/water/trifluoroacetic acid, 10:90:0.1); MS (ES) $m/z = 688$ (M + 1), 632 (M-55, base), 588; ^{1}H NMR (300 MHz, DMSO-d_6/TFA) δ 7.83 (s, 1H), 7.75 (d, 1H, $J = 8.7$ Hz), 7.52 (d, 1H, $J = 8.5$ Hz), 6.60 (br s, 1H), 6.32 (s, 1H), 4.80 (s, 2H), 4.16 (br s, 1H), 3.12 (br t, 2H), 2.59 (s, 3H), 2.55 (s, 3H), 2.42 (s, 3H), 2.13 (s, 3H) 1.20–1.80 (br m, 4H), 1.41 (s, 9H).

β-Ala-Aminomethyl-AgroPore™ resin (8). Aminomethyl AgroPore™ resin (3.85 g, 2.77 mmol, 0.72 mmol/g resin loading) was swelled in 50 mL of DMF for 15 min and then the solvent was drained. BOC-β-Alanine (2.67 g, 14.1 mmol), HATU (5.27 g, 13.8 mmol), and diisopropylethylamine (4.83 mL, 27.7 mmol) were dissolved in 20 mL of DMF and 10 mL of this reaction cocktail was added to the resin and agitated with positive nitrogen gas pressure for 2 h. The reaction mixture was drained and the resin was washed with DMF (2 × 20 mL). Another 10 mL of the reaction cocktail was added to the resin and the resin agitated for 16 h with positive nitrogen gas pressure to complete the double coupling. The reaction mixture was drained and the resin washed with DMF (8 × 20 mL). The resin was treated with acetic anhydride (15.7 mL, 166 mmol) and diisopropylethylamine (28.9 mL, 166 mmol) in DMF (30 mL) and agitated with nitrogen gas for 2 h to endcap any residual free NH_2 sites on the resin. The reaction mixture was drained and the resin washed with DMF (8 × 10 mL) and CH_2Cl_2 (5 × 10 mL) affording BOC-β-Ala-aminomethyl-AgroPore™ resin. The Kaiser test was performed on a small aliquot of resin beads and gave a pale orange color indicating no free amine was present. This resin was then treated with trifluoroacetic acid/CH_2Cl_2 (20 mL, 2:3) and the resin agitated for 30 min with positive nitrogen gas. The reaction mixture was drained and the resin washed with CH_2Cl_2 (8 × 20 mL), ether (3 × 20 mL) and dried with nitrogen gas to afford β-Ala-aminomethyl-AgroPore™ resin **8**. The Kaiser test was performed on a small aliquot of resin beads giving a dark blue color indicating free amine was present.

H-Arg(CMtr)-AMC Resin (9). β-Ala-Aminomethyl-AgroPore™ resin **8** (3.85, 2.77 mmol) was swelled in DMF (50 mL) for 15 min and the solvent drained. A solution of acid **7** (2.00 g, 2.91 mmol), HATU (1.07 g, 2.83 mmol), and diisopropylethylamine (1.00 mL, 5.65 mmol) in DMF (5 mL) was added to the resin and agitated with positive nitrogen gas pressure for 16 h. The reaction mixture was drained and the resin washed with DMF (5 × 20 mL). A second coupling was performed by adding to the resin a solution of acid **7** (481 mg, 0.700 mmol), HOBT (86 mg, 0.64 mmol), diisopropylcarbodiimide (160 mg, 1.27 mmol), and DMAP (17 mg, 0.14 mmol) in DMF (5 mL) and then agitating the resin with positive nitrogen gas pressure for 16 h. The reaction mixture was drained and the resin washed with DMF (5 × 20 mL). The Kaiser test was performed on a small aliquot of resin beads to give a very pale blue color indicating some free amine is still present on the resin. To cap these free amino groups, a 5 mL aliquot of a reaction cocktail consisting of acetic anhydride (5.30 mL, 55.4 mmol) and diisopropylethylamine (9.7 mL, 55.4 mmol) in DMF (10 mL) was added to the resin and agitated with positive nitrogen gas pressure for 5 h. The reaction mixture was drained and the resin washed with DMF (3 × 20 mL). Another 5 mL of the acetic anhydride reaction cocktail was added to the resin followed by agitation for 16 h with positive nitrogen gas pressure. The resin was drained and washed with DMF (8 × 10 mL), CH_2Cl_2 (5 × 10 mL), ether (3 × 20 mL), and dried with positive nitrogen gas to afford Boc-Arg(CMtr)-AMC resin **9**. The Kaiser test was performed on a small aliquot of resin beads and gave a pale orange color indicating no free amine was present. The Boc-Arg(CMtr)-AMC resin was swelled in 20 mL CH_2Cl_2 for 15 min and the solvent drained. Trifluoroacetic acid/CH_2Cl_2 (20 mL, 2:3) was added to the resin followed by agitation for 1 h with positive nitrogen gas. The reaction mixture was drained and resin washed with CH_2Cl_2 (8 × 20 mL), ether (5 × 20 mL) and dried with nitrogen gas to afford H-Arg(CMtr)-AMC resin **9**. The Kaiser test was performed on a small aliquot of resin beads giving a dark blue color indicating free amine was present.

Library synthesis example: Ac-*O*-X-*O*-Arg-AMC. The resin **9** (6.00 g, 4.32 mmol) was divided equally into 20 reaction vessels, swelled with 10 mL DMF for 15 min and the solvent was drained. A reaction cocktail consisting of the isokinetic mixture of 20 amino acids (various wt, 35.3 mmol of each amino acid), HATU (13.7 g, 33.5 mmol), and diisopropylethylamine (12.3 mL, 70.5 mmol) in DMF (88 mL) was prepared. An aliquot of the reaction cocktail (2.5 mL) was added to each of the 20 reaction pots containing resin **9** and the resin agitated with positive nitrogen gas pressure for 4 h. The reaction mixture was drained and the resin washed with DMF (2 × 5 mL). Another 2.5 mL of the isokinetic amino acid reaction cocktail was added to the resin and the resin agitated for 16 h with positive nitrogen gas pressure to complete the double coupling. The reaction mixture was drained and the resin washed with DMF (5 × 5 mL). The Kaiser test was performed on a small aliquot of resin beads giving a dark orange color. A solution of piperidine/DMF (3 mL, 1:4) was added to each reaction vessel and the resin agitated with positive nitrogen pressure for 10 min. The reaction mixture was drained and the resin washed with DMF (3 × 5 mL). A solution of piperidine/DMF (3 mL, 1:4) was then added to resin and the resin agitated for 10 min with nitrogen gas. The reaction mixture was drained and the resin washed with DMF (8 × 5 mL) affording **H-*O*-Arg-AMC** resin. The Kaiser test was performed on a small aliquot of resin beads giving a dark blue color indicating free amine was present and Fmoc cleavage was complete.

A reaction cocktail was prepared by placing the 20 distinct amino acids, **X**, (various wts, 1.73 mmol) in 20 distinct vessels, along with HATU (624 mg, 1.64 mmol), diisopropylethylamine (0.60 mL, 3.46 mmol) and DMF (6 mL). 3 mL of the 20 distinct reaction cocktails was

then added to each of the 20 reaction pots containing the resin from the step above and the resin agitated with positive nitrogen gas pressure for 4 h. The reaction mixture was drained and the resin washed with DMF (4 × 5 mL). Another 3 mL of the distinct amino acid reaction cocktail was added to the resin and the resin agitated for 16 h with positive nitrogen gas pressure to complete the double coupling. The reaction mixture was drained and the resin washed with DMF (8 × 5 mL). The Kaiser test was performed on a small aliquot of resin beads, giving a dark orange color. A solution of piperidine/DMF (3 mL 1:4) was added to each reaction vessel and the resin agitated with positive nitrogen pressure for 10 min, the reaction mixture was drained and the resin washed with DMF (3 × 5 mL). Another solution of piperidine/DMF (3 mL, 1:4) was added to each reaction vessel and the resin agitated with positive nitrogen pressure for 10 min, the reaction mixture drained and the resin washed with DMF (3 × 5 mL) to give **H-X-*O*-Arg-AMC resin**. The Kaiser test was performed on a small aliquot of resin beads giving a dark blue color indicating free amine was present and the Fmoc cleavage was complete. The isokinetic amino acid coupling and the FMOC cleavage procedure described in the first step of the library synthesis was repeated to afford **H-*O*-X-*O*-Arg-AMC** resin. A reaction cocktail of acetic anhydride (16.4 mL, 172.8 mmol) and diisopropylethylamine (30 mL, 172.8 mmol) in DMF (80 mL) was prepared. 3.1 mL of this reaction cocktail was added to each of the 20 reaction vessels and the resin agitated with positive nitrogen gas pressure for 1.5 h. The reaction mixture was drained and the resin washed with DMF (3 × 5 mL). Another 3.1 mL of the reaction cocktail was added to the reaction vessels and the resin agitated for 1 h with positive nitrogen gas pressure to complete the resin-endcapping procedure. The resin was drained and washed with DMF (8 × 5 mL), CH_2Cl_2 (5 × 5 mL), ether (3 × 5 mL), and dried with positive nitrogen gas. The Kaiser test was performed on a small aliquot of resin beads giving a pale orange color indicating no free amine is present and coupling is complete to give **Ac-*O*-X-*O*-Arg-AMC** resin.

A cleavage cocktail consisting of thioanisole (17.6 mL), phenol (1.07 g), and triisopropylsilane (5 mL) in trifluoroacetic acid (100 mL) was prepared and 4 mL of the cleavage cocktail was added to each of the 20 reaction pots and the pots cooled to 0 °C. Bromotrimethylsilane[14] (0.66 mL) was added to the 20 reaction pots at 0 °C and shaken gently for 0.5 h, allowed to warm to room temperature and shaken for an additional 3 h. The solvents were removed under reduced pressure and the residue triturated with ether (5 mL) and filtered affording the **Ac-*O*-X-*O*-AMC** library as yellow powders (~85 mg from each pot). The **Ac-*O*-*O*-X-AMC** and **Ac-X-*O*-*O*-AMC** libraries were also synthesized using the above procedures.

Mass spectral analysis of libraries. Analyses were performed on a Micromass (VG) TofSpec SE operating with a flat plate target, pulsed ion extraction and a reflectron analyzer. Samples were dissolved in 2:2:1 acetonitrile/ethanol/water to approximately 10 pmol/mL, mixed 1:1 with matrix and 1 mL deposited on the target. The matrix solution was 10 mg/mL alpha-cyano-3-hydroxycinnamic acid in 2:2:1 acetonitrile/ethanol/water. The mass range was calibrated to 7000 Da. A nominal number of scans were acquired with each recorded spectrum consisting of an average of 10 laser shots. Data were combined, smoothed and background subtracted to produce the final spectrum.

For the simulated mass spectrum, the molecular mass of the peptides in a library resulting from all possible combinations of 20 amino acids is calculated using the averaged atomic mass. According to the isokinetic assumption, each peptide in the library is given equal weight, and a MALDI spectrum for a library is simply the distribution of molecular mass of the peptides in the library. The computation is carried out with a computer program MALDIsym, which is available upon request.

Enzyme assays. All fluorescent measurements were conducted on a Perkin–Elmer HTS 7000 with the following parameters: Excitation filter 360 nm; Emission 465 nm; Gain 40–80; Number of flashes 20; Integration time 1000 μs; intervals between measurements were typically 1 min. The substrates were prepared at a concentration of 1.0 mM in DMSO. The enzymes were prepared in buffer (100 mM HEPES, 20 mM NaCl, 10 mM $MgCl_2$ pH 7.5). Enzyme concentrations were as follows: trypsin, 0.11 mg/well; thrombin, 10.1 U/well; chymotrypsin, 2.5 mg/well; human urine kallikrein, 2.0 mU/well; human plasma kallikrein, 5.0 mU/well; porcine pancreatic kallikrein, 0.1 ng/well; PSA, 0.05–0.5 mg/well; calpain, 0.013–0.05 U/well; human lung tryptase, 40 ng/well; coagulation factor Xa, 0.2 U/well. The standard buffer defined above was used for most experiments. However, for some experiments the effect of different pH or salt conditions was also tested. 80 μL of buffer and 10 μL of substrate were added to appropriate wells in the plate and mixed. The reaction was initiated by the addition of 10 μL of enzyme and the change in fluorescent intensity at ex/em 360/465 nm was measured for 30 min at 25 °C. The slope was determined by linear regression and the results expressed as percent maximum observed rate for each enzyme and substrate library.

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