Function-based isolation of novel enzymes from a large library

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Here we describe a high-throughput, quantitative method for the isolation of enzymes with novel substrate specificities from large libraries of protein variants. Protein variants are displayed on the surface of microorganisms and incubated with a synthetic substrate consisting of (1) a fluorescent dye (2) a positively charged moiety (3) the target scissile bond, and (4) a fluorescence resonance energy transfer (FRET) quenching partner. Enzymatic cleavage of the scissile bond results in release of the FRET quenching partner while the fluorescent product is retained on the cell surface, allowing isolation of catalytically active clones by fluorescence-activated cell sorting (FACS). Using a synthetic substrate with these characteristics, we enriched *Escherichia coli* expressing the serine protease OmpT from cells expressing an inactive OmpT variant by over 5,000-fold in a single round. Screening a library of 6×10^5 random OmpT variants by FACS using a FRET peptide substrate with a nonpreferred Arg-Val cleavage sequence resulted in the isolation of variant proteases with catalytic activities enhanced by as much as 60-fold. This approach represents a potentially widely applicable method for high-throughput screening of large libraries on the basis of catalytic turnover.

Keywords: Flow cytometry, directed enzyme evolution, fluorescence

Directed evolution of a desired catalytic activity from libraries of random mutants represents a powerful route to enhancing the stability and substrate specificity of enzymes[1,2]. Mutant library construction using targeted random mutagenesis and/or DNA shuffling followed by colony-based plate assays has led to the isolation of remarkable enzyme variants from relatively small libraries, typically containing 10^3–10^4 members[3–6]. Rapid screening of very large libraries, however, has been possible for only a few enzymatic reactions in which the desired function can be linked to a selectable phenotype[7–11]. Phage display offers a possible alternative to colony-based plate screening approaches because it is amenable to screening large libraries; however, enrichment is generally based on ligand binding rather than catalytic turnover[12–15].

Herein we present a new strategy for the catalytic turnover-based isolation of desired enzyme variants from large libraries. As shown in Figure 1, enzyme variants are displayed on the cell surface of microorganisms[16], giving them free access to substrates. We synthesized a fluorescence resonance energy transfer (FRET) substrate consisting of (1) a fluorophore (Fl), (2) a moiety with a +3 charge, (3) the scissile bond, and (4) a quenching fluorophore that acts as an intramolecular FRET partner (Q). Because the surface of *Escherichia coli* is negatively charged (z potential –25 to –30 mV)[17], the positively charged FRET substrate associates with the cell surface. The F1 and Q moieties are then separated by enzymatic cleavage of the scissile bond, resulting in disruption of intramolecular FRET quenching.

Using such cell surface–retained substrates and reaction products allowed us to link an enzyme-encoding gene to catalytic turnover. We then analyzed cells for fluorescence in real time and with high throughput using multiparameter flow cytometry[18–21]. Clones exhibiting high catalytic activities were enriched by fluorescence-activated cell sorting (FACS), which resulted in the isolation of a small number of catalytically active clones that are easily analyzed in detail using robotic 96-well plate assays.

The *E. coli* OmpT (EC 3.4.21.87), a surface-displayed protease, is the prototypical enzyme of the omptin family that includes several serine proteases implicated in microbial pathogenicity[22,23]. Examination of the cleavage specificity of OmpT using substrate phage revealed a strong preference for basic residues (Arg, Lys) in both the P1 and P1' subsites, a specificity similar to the prohormone convertases (J.J. McCarter, D.L. Stephens, G. Georgiou and J.F. Kirsch, unpublished data). Consistent with earlier reports[24,25], OmpT was found to be an efficient enzyme, catalyzing the hydrolysis of optimal peptide substrates such as AcWGG**RR**IKGWGTI-CONH$_2$ with an efficiency of $k_{cat}/K_M = 3.3 \times 10^6$ s^{-1} M^{-1} (ref. 22). Because of its well-characterized cleavage specificity, ease of purification, and natural localization on the cell surface, we used OmpT for the present studies.

Results and discussion

Substrate design. The FRET substrate **1** (Fig. 2) contains the BODIPY (Fl) and tetramethylrhodamine (Q) fluorophores on either side of an Arg-Arg sequence optimal for OmpT cleavage. Substrate **1** has an overall +3 charge that promotes electrostatic binding to the *E. coli* surface in low-ionic-strength solutions. The low-ionic-strength buffers used contain sucrose to avoid plasmolysis. Hydrolysis of substrate **1** by OmpT at the Arg-Arg peptide bond gives rise to a C-terminal product containing the BODIPY (Fl) fluorophore on a short peptide with a +3 overall charge (two arginine guanidinium groups as well as the unmasked N-terminal amine). In contrast, the N-terminal product containing the tetramethylrhodamine (Q) fluorophore has no net charge, and presumably diffuses away from the cell.

Using substrate **1**, FACS analysis can discriminate between three different cell populations: (1) cells with no OmpT on the surface, (2) cells with active OmpT catalysts on the surface, and (3) cells expressing an impaired OmpT variant. In particular, incubation of the *ompT*-
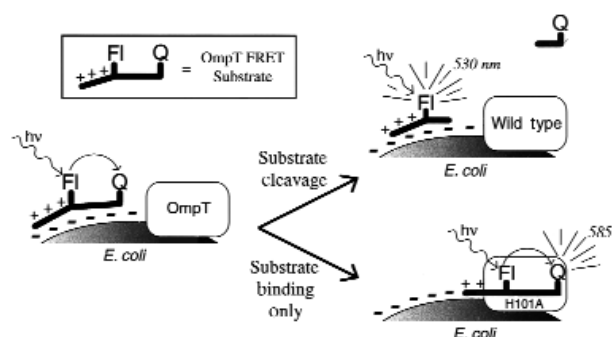
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Figure 1. FRET substrate binding and fluorescence emission upon catalytic turnover or binding to protein displayed on the cell surface. FI, BODIPY; Q, tetramethylrhodamine.

strain UT5600 with substrate **1** results in baseline emission at 530 ± 30 nm (FL1; from BODIPY) and 585 ± 42 nm (FL2; from tetramethylrhodamine) wavelengths (population 1 in Fig. 3A). However, UT5600 cells transformed with the plasmid pML19, a pUC derivative expressing OmpT from its native promoter (D. Stephens & G. Georgiou, unpublished data), exhibit over 50-fold greater FL1 fluorescence, consistent with cleavage and cell surface product capture (population 3 in Fig. 3A). Cells transformed with a pML19 derivative encoding an OmpT (H101A) variant with approximately 4% of the specific activity of the wild-type enzyme (D. Stephens & G. Georgiou, unpublished data) exhibit a strikingly different fluorescence profile, characterized by moderate FL1 fluorescence but 20-fold greater FL2 fluorescence (population 2 in Fig. 3A). This increased FL2 emission could be due to the tetramethylrhodamine fluorophore being sequestered inside a protein-binding pocket, away from the solvent water molecules that would ordinarily limit emission resulting from collisional quenching.

Apparently substrate **1** partitions onto the cell surface, because a concentration of only 50 nM was sufficient to give the population discrimination shown in Figure 3A. The fluorescence profiles of the three cell populations remained virtually unchanged for at least 30 min of incubation with substrate **1**, indicating that catalytic turnover and product capture rapidly reach steady state.

Catalytic enrichment. A 5,000-fold enrichment of clones expressing active catalysts was obtained in a single round using the FRET/FACS system. For these studies, we used cells in which the expression of OmpT was reduced by using the lower copy number vector pML319 (pBR322 origin of replication), which displays about four-fold lower FL1 fluorescence relative to expression from the higher copy number vector pML19 (pUC replication origin) when incubated with substrate **1** under identical conditions. In the enrichment experiment, UT5600/pML319 was mixed with a 5,000-fold excess of UT5600/pDS327, a pML319 derivative encoding a H212A mutation that results in a reduction in catalytic activity by about 10^3 (D. Stephens & G. Georgiou, unpublished data). The mixed cells were incubated with **1**, and clones exhibiting high fluorescence were isolated by FACS, then grown in liquid media. Consistent with the expected frequency of active clones in the mixture, 32 highly fluorescent cells were detected in the positive window, out of approximately 150,000 bacteria analyzed. These 32 highly fluorescent cells were grown overnight; the culture (post-sort) was then labeled with substrate **1** and analyzed by flow cytometry. The fluorescence profile of the post-sort population was essentially indistinguishable from that of UT5600/pML319 cells expressing wild-type OmpT. Nine of nine clones chosen at random from the selected population were shown to contain pML319 as expected.

Evolution of substrate specificity. An enhancement of the rate of cleavage of substrates containing the Arg-
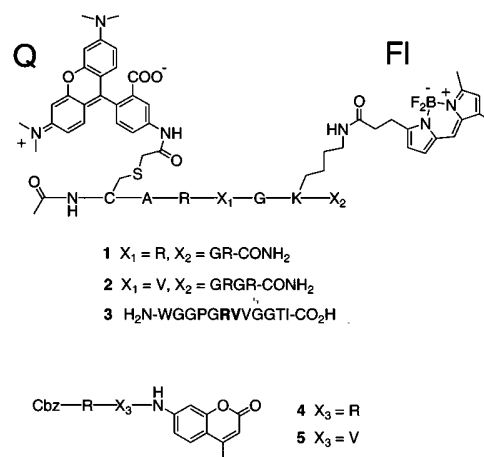

Figure 2. Substrates for the detection of catalytic activity.

Val sequence specificity was chosen as the target of initial directed-evolution experiments, because wild-type OmpT possesses a modest ability to cleave this sequence. In fact, the weak Arg-Val cleavage activity of OmpT is responsible for its role as a plasminogen activator, an function possibly related to bacterial pathogenicity[22]. Thus, peptide **3**, a sequence derived from human plasminogen, is cleaved by OmpT at Arg-Val with a catalytic efficiency $k_{cat}/K_M = 24$ s^{-1} M^{-1}.

The FRET substrate **2**, containing a putative Arg-Val cleavage site in the place of the Arg-Arg site in substrate **1**, was synthesized and used to screen libraries of OmpT random variants. The additional Gly-Arg sequence in **2** compared to **1** was necessary to confer a +3 charge on the BODIPY-containing product. Because the optimal rate of random mutagenesis that must be used in order to obtain protein variants with altered function is not a priori obvious[26], the *ompT* gene was subjected to random mutagenesis by error-prone polymerase chain reaction (PCR) at different Mn^{2+} concentrations. The mutagenized DNA was amplified and then inserted within a high copy vector without ligation using the CloneAmp cloning strategy[27]. A total of 6×10^5 transformants were obtained. The library was incubated with substrate **2** at 50 nM, and clones falling within an FL1-FL2 window expected for active clones were isolated by FACS. A total of 1.9×10^6 cells were evaluated in 24 min, and 352 individual clones were recovered, corresponding to an enrichment of 5,400-fold in one round (Fig. 3C).

The FACS-isolated clones were further ranked on the basis of hydrolysis of the simpler 7-amino-4-methylcoumarin (AMC) substrates **4** and **5** containing putative Arg-Arg and Arg-Val cleavage sites, respectively. These assays were carried out using a robotically implemented, coupled assay with aminopeptidase M. Cleavage of **4** and **5** by OmpT gives rise to H$_2$N-Arg-AMC and H$_2$N-Val-AMC,

Table 1. Catalytic efficiencies as well as cleavage products observed during cleavage of substrate 3 by OmpT and the three variants isolated in this study[a]

Enzyme	k_{cat}/K_M (s^{-1}M^{-1})[b]	Cleavage site (%)			
		Arg-Val	Val-Val	Val-Gly	Gly-Thr
OmpT	24	100	ND	ND	ND
C5	1440	89	8	1	2
B4	310	32	35	12	21
G11	200	10	41	23	26

Substrate 3: H-WGGPGR•V•V•GG•TI-CONH$_2$

[a]Catalytic activities were monitored by HPLC. Identities of the individual cleavage products were determined by mass spectral analysis, and relative amounts were determined by HPLC. ND, None detected. For reference, the sequence of peptide substrate 3 is shown beneath the table, and the observed cleavage sites are indicated (•).
[b]These apparent values were calculated by measuring rates of cumulative appearance of products during the cleavage reaction.
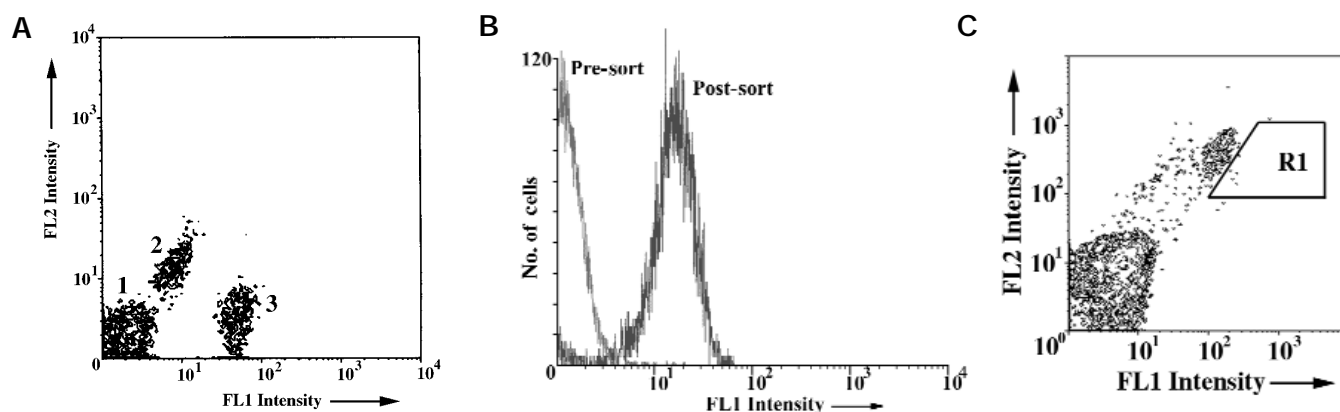
Figure 3. Flow cytometric discrimination of *E. coli* on the basis of OmpT activity. (A) UT5600 (population 1); pDS26 encoding OmpT H101A (population 2); pML19 encoding the wild-type OmpT (population 3). **(B)** Single-step enrichment of cells with wild-type OmpT (UT5600/pDS319) from a 5,000-fold excess of cells expressing the OmpT H212A variant (UT5600/pDS327). **(C)** Library data obtained when sorting OmpT library for increased RV cleavage activity using FRET substrate **2**. The sort gate used to isolate 352 clones is shown as region R1 in the figure.

respectively, which are not fluorescent. These products, however, unlike substrates **4** and **5** in which the N terminus is blocked by a carbonylbenzyloxy (Cbz) group, are substrates for aminopeptidase M. Aminopeptidase M removes the amino acid, releasing the highly fluoresecent AMC moiety. Three clones out of the selected 352 exhibited a high ratio of cleavage activity of substrate **5** relative to wild type. For comparison, only one out of 1,200 randomly selected clones from the pre-sort library population gave fluorescent product that was slightly above background when analyzed for hydrolysis of substrate **5**.

Kinetic analysis. Purified OmpT[28] from all three clones was found to hydrolyze substrate **5** with rates at least threefold higher than purified, wild-type OmpT. Surprisingly, the major hydrolysis product generated by all three variants with substrate **5** was AMC, whereas the expected product H_2N-Val-AMC was produced in lesser amounts. The fraction of **5** converted to AMC ranged from 78% (clone B4) to >96% (clone G11). In contrast, hydrolysis of **5** by the wild-type enzyme gave only the expected H_2N-Val-AMC cleavage product, with no detectable AMC. Subsequent experiments confirmed that the three variants did not possess detectable amino peptidase activity with H_2N-Val-AMC as substrate. Thus, cleavage of the Val-AMC bond of the intact substrate **5**, as opposed to sequential Arg-Val then H_2N-Val-AMC cleavage, was responsible for the formation of the AMC cleavage product.

In order to assess the cleavage activities of the three variants in the context of a peptide substrate, cleavage activity with substrate **3** was analyzed (Table 1). Peptide **3** corresponds to the cleavage sequence of human plasminogen, a known substrate of OmpT, which is cleaved predominantly at the Arg-Val sequence. The variants C5, B4, and G11 hydrolyzed peptide **3** with apparent k_{cat}/K_M values of 1,440 s^{-1} M^{-1}, 310 s^{-1} M^{-1}, and 200 s^{-1} M^{-1}, respectively, corresponding to catalytic efficiencies that are 60-fold, 13-fold, and 9-fold better than the wild-type OmpT enzyme.

Mass spectrometric analysis was used to determine cleavage products of peptide **3** (Table 1). Variant C5 cleaved peptide **3** at predominantly the expected Arg-Val cleavage site, as did wild-type OmpT. Thus, the most active variant, C5, displayed a significantly higher level of catalytic activity, yet overall similar specificity, with this peptide compared to the wild-type OmpT.

DNA sequencing of the three variants revealed that C5, B4, and G11 had 11, 7, and 9 single-base changes, respectively, corresponding to 8, 4, and 6 amino acid substitutions (Table 2). There is little in the way of homology among the amino acid changes. In fact, none of the sub-stitutions with B4 coincide with changes in either C5 or G11, even though the catalytic activity of B4 appears to be a hybrid of C5 and G11 activities. In general terms, results for these OmpT variants having relatively high numbers of mutations are consistent with previous investigations of antibody plasticity in which a surprising number of gain-of-function variants have been found in highly mutated libraries.[26]

The fact that variant C5 displays 60-fold enhanced Arg-Val catalytic efficiency compared to the wild-type enzyme emphasizes the potential benefits of screening large libraries for novel catalytic activities. The catalytic activity of the isolated OmpT variants leading to the release of AMC from cleavage of substrate **5** is a fully consistent, albeit unexpected, solution to the selection criteria employed in the 96-well plate assay. This latter result indicates that alteration of certain protease substrate cleavage activities may not be limited to incremental changes, but may, in fact, be amenable to a more striking degree of modulation. Experiments are currently being planned to explore how far OmpT substrate specificity can be altered by directed evolution.

Although OmpT is a native surface enzyme, the strategy shown in Figure 1 can be employed with a wide variety of heterologous enzymes using well-established methods for protein surface display in *E. coli*, *Saccharomyces cerevisae*, or *Streptococcus* spp[16]. Current FACS technology allows the screening of 10^9 cells per hour, and the ability to screen quantitatively libraries of such size for catalytic activity may open new avenues to the directed evolution of enzyme substrate preferences, physical properties, and catalytic chemistry.

Experimental protocol

Plasmid construction. Unique site elimination was used to construct mutations in *omp*T (ref. 29) at codons 101 (pDS26) and 212 (pDS27), in each case changing from histidine to alanine. The *Hin*dIII-*Mlu*I site change primer 5′-CAGGCATGC**ACGCGT**GGCGTAATC-3′ was used to construct all point mutants. Targeted mutations were made using primers 5′-GTG-TATCAGG**AGC**TCTACTTTCATC-3′ (H101A) and 5′-CGGGT-CATAA**GC**TTCATCGTTATCAG-3′ (H212A)[30]. Changed bases are in bold-face type. All mutations were confirmed by dideoxynucleotide sequencing.

To transfer the native *omp*T and H212A *omp*T to a lower copy number background, the 2.0 kB *Pst*I-*Eco*RI fragment from pML19 and pDS27,

Table 2. Mutational analysis of unique isolated clones selected for altered substrate specificity

							Position									
	33	**87**	**111**	**131**	**137**	**141**	**149**	**152**	**186**	**200**	**240**	**276**	**278**	**284**	**286**	**288**
WT	Glu	Met	Glu	Met	Ser	Phe	Ile	Ser	Ser	Tyr	Tyr	Ser	Asn	Asn	Asn	Ile
G11		Leu		Lys		Ile	Val						Ile		Ser	Val
C5	Lys	Leu	Val		Asn				Cys	Phe				Ile		Phe
B4						Ala					Phe	Arg		Ile		

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respectively, was cloned into pBR322. The resultant plasmids were called pDS319 (native *omp*T) and pDS327 (H212A *omp*T).

Substrate synthesis. FRET substrates **1** and **2** were synthesized by reacting the appropriate deprotected peptides (University of Texas Peptide Synthesis Facility) with the following reagents: (1) Tetramethylrhodamine iodoacetamide (Molecular Probes, Eugene, OR), dimethyl formamide (DMF); (2) BODIPY-FL-SE (Molecular Probes, Eugene OR), DMF, according to the manufacturer's protocols. Substrates **1** and **2** were purified by medium-pressure preparative reverse-phase liquid chromatography (FPLC; Pharmacia, Piscataway, NJ), eluted with a 120 min linear gradient of acetonitrile (ACN) in H_2O with .07% trifluoroacetic acid (TFA). Compound **4** was purchased (Sigma Co, St. Louis, MO), and compound **5** was synthesized from valinyl-7-amido-4-methylcoumarin (9 mg, 32 μmol) (Bachem, Bubendorf, Switzerland), Cbz-Arg-OH (10 mg, 32 μmol) (Novabiochem, La Jolla, CA), PyBOP (33 mg, 64 μmol) (Novabiochem, La Jolla, CA), *N*-methylmorpholine (14 μl, 128 μmol) (Aldrich, St. Louis, MO) in 1 ml anhydrous DMF, stirred at room temperature for 4 h. Purification was carried out using C18 Sep-Pak cartridges (Waters Corp., Milford, MA) eluted with ACN–H_2O mixtures. Yield was 14 mg (25 mmol, 78%) of compound **5** following lyophilization. Peptide **3** was purchased (Chiron, Clayton, Australia).

Flow cytometric analysis. UT5600 was transformed with either pDS26 or pML19 encoding the wild-type OmpT. Overnight cultures of UT5600, UT5600/pDS26, and UT5600/pML19 were washed with 1% sucrose, diluted to 0.01 OD_{600}, labeled for 10 min with 50 nM substrate **1** in 1% sucrose, diluted into 1 ml 1% sucrose, and analyzed using a Becton-Dickinson FACSort.

Single-step enrichment of UT5600/pML319 from a 5,000 fold excess of cells expressing the OmpT H212A variant (UT5600/pDS327). Overnight cultures of UT5600/pML319 and UT5600/pDS327 were mixed at a ratio of 1:5,000 (pML319:pDS327), labeled as above, and analyzed by FACS. A total of 159,715 cells of the labeled mixture were examined in 9.5 min. Thirty-two cells falling inside of R1 were collected in 45 ml of sheath fluid, and were regrown overnight at 37°C in a total of 100 ml Luria–Bertani (LB) medium. Cells were harvested, washed with 1% sucrose, labeled with substrate **1** as above, and analyzed by FACS.

Library construction. A library of random mutants was constructed by error-prone PCR using 0.01, 0.15, 0.25, or 0.5 mM Mn^{2+} (ref. 31). The PCR product, pAMP1 vector DNA (Life Technologies, Rockville, MD), annealing buffer, and 2 units uracil DNA glycosylase in a total volume of 20 μl were incubated for 40 min at 37°C, followed by 1 h at 4°C. Each reaction mixture was then electroporated into electrocompetent XL-1 Blue Stratagene cells, and serial dilutions were plated on selective plates to determine the number of independent transformants. The libraries were pooled, and the cells grown at 37°C overnight in LB medium with antibiotic selection. Plasmid DNA was isolated and subsequently transformed by electroporation into UT5600 for library screening.

Library screening. Transformants were grown at 37°C in LB medium, harvested at 16 h, washed with 1% sucrose, and reactions were prepared by incubating with 50 nM substrate **2** for 40 min. A 5 μl aliquot of the reaction was then diluted into 1 ml 1% sucrose. FACS gates were set based upon FSC/SSC and FL1/FL2. A total of 1,929,783 cells were examined in 24 min, and 352 viable cells were collected. The collected solution was filtered, and the filters were placed on agar plates containing 100 μg/ml carbenicillin. At 12 h, the 352 colonies were inoculated into minimal M63 medium containing amino acids and glycerol as the carbon source in quadruplicate 96-well plates. After 20 h of growth at 37°C, 20 μM of substrate **4** or **5** and 5 U/ml aminopeptidase M (Sigma) were added to all wells, and plates were incubated at 37°C for 16 h. Fluorescence intensities were measured on a Bio-Tek FL600 (Bio-Tek Instrument, Winooski, VT) fluorescence plate reader, with excitation at 360/40 and emission at 460/40. Optical densities were also measured at 600 nm to correct for cell growth effects. All liquid handling steps were performed with a Beckman Biomek 2000 robotic station (Beckman Instruments, Fullerton, CA).

Enzyme purification and kinetic analysis. OmpT was isolated as previously described[28], and 1.5 μM of purified enzyme (>90% by SDS–PAGE) was incubated with 20 μM substrate **4** or **5**, and the products were analyzed by high-performance liquid chromatography (HPLC) on a Vydac C_{18} column using: 5% ACN/95% H_2O for 1 min, gradually increasing to 95% ACN/5% H_2O by 31 min, 95% ACN/5% H_2O for an additional 5 min, returning to 5% ACN/95% H_2O over 5 min, followed by 5 min at 5% ACN/95% H_2O. Cleavage kinetics for compound **3** with wild-type OmpT and the variants were performed using the same HPLC conditions as above, and substrate concentrations varied from 20 μM to 300 μM. Apparent cleavage rates were determined by monitoring the amount of cumulative products produced as a function of substrate peptide concentration, and using a hyperbolic regression analysis to determine apparent k_{cat} and K_M values. Cleavage products were determined by

liquid chromatography–mass spectrometry (LC-MS) (Echellette Spectrograph and Imager, ESI) as well as matrix-assisted laser desorption ionization mass spectral (MALDI-MS) analysis. The product ratios were determined by HPLC, using the integrated peak areas monitored at 280 nm.

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