Enzyme Screening

Profiling Primary Protease Specificity by Peptide Synthesis on a Solid Support**

*Ron H. P. Doezé, Beatrice A. Maltman, Claire L. Egan, Rein V. Ulijn, and Sabine L. Flitsch**

An estimated 2–3% of the mammalian proteome consists of proteases: enzymes that hydrolyze proteins and peptides.[1] Many of these enzymes are involved in essential physiological functions such as immunological defense and cell differentiation. Several proteases are involved in disease states, such as HIV, Alzheimer's disease, hepatitis C, Candida infections, and pancreatitis.[2] In addition, proteases have found applications as (industrial) biocatalysts in synthetic organic chemistry as a result of their ability to selectively hydrolyze (or synthesize) peptide bonds with high specificity

[*] R. H. P. Doezé, Dr. B. A. Maltman, C. L. Egan, Prof. S. L. Flitsch
School of Chemistry
The University of Edinburgh
King's Buildings, West Mains Road, Edinburgh, EH9 3JJ (UK)
Fax: (+44) 131-650-4737
E-mail: slf01@staffmail.ed.ac.uk

Dr. R. V. Ulijn
Manchester Materials Science Centre
University of Manchester/UMIST
Grosvenor Street, Manchester, M1 7HS (UK)

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under mild conditions.[3] Given the large number of proteases and their importance in both synthetic chemistry and biomedicinal science, high-throughput screening methods for the measurement of protease specificity are required.

Key to the characterization of this class of enzymes is the definition of substrate specificity, that is, the selectivity for amino acids flanking the scissile peptide bond. The number of potential substrates for proteases can be large. There are 400 possible dipeptide sequences if only the "primary" amino acids directly adjacent to the scissile bond are considered. This number increases to 160000 when secondary sites are considered. Several methods have been developed to determine the specificity of proteases. Large peptide libraries are used where the library members are attached on solid supports such as polymer beads[4] or microarrays.[5] For example, Meldal and co-workers have developed screening methods for large combinatorial peptide (or peptide mimic) libraries by using quenched fluorogenic labels that become fluorescent upon peptide hydrolysis. Both the synthesis and the biological screening of these libraries could be carried out directly on polyacrylamide/poly(ethylene glycol) (PEGA copolymer) polymers that are compatible with enzyme activity.[4] Recently, the research groups of Ellman and Yao independently developed successful peptide chips where the members of a peptide library were directly arrayed onto glass slides. This method is so far restricted to the analysis of the specificity for amino acids positioned on the carboxylic side of the cleaved peptide bond.[5a,b]

Here we describe a simple new method for the profiling of the primary specificities of proteases. The primary specificity describes the protease preference for the amino acids on either side of the amide bond (P_1 and P_1').[6] The assay is based on our recently reported discovery that the peptide hydrolysis equilibrium can be reversed toward peptide synthesis on PEGA polymer beads.[7] Instead of studying the cleavage of peptides, the reverse reaction, that is, the protease-catalyzed coupling of amino acids is monitored (Figure 1).

Biocompatible polymer materials are required for the successful analysis of enzymatic reactions on polymer supports. $PEGA_{1900}$ has been identified as a suitable biologically inert material where all reactive sites on the polymer are accessible to small enzymes. Literature reports have shown that proteases are catalytically active within $PEGA_{1900}$ beads.[4,6]

To assess the validity of our approach, the selectivity of the protease thermolysin from *Bacillus thermoproteolyticus Rokko* in the hydrolysis and synthesis directions was compared by analytical HPLC analysis. Attachment of two amino acids (Asp and Phe) and two Fmoc-protected dipeptides (Fmoc–Asp–Phe and Fmoc–Phe–Asp) through the Wang linker to $PEGA_{1900}$ resin provided solid-supported substrates for the synthesis and hydrolysis reactions, respectively. Analysis of the products (Fmoc–Phe–Asp and Fmoc–Asp–Phe after acidic cleavage of the Wang linker or of Fmoc–Asp and Fmoc–Phe, respectively) was performed by LC-MS. Figure 2 shows that thermolysin-catalyzed synthesis and hydrolysis follow the same selectivity, with a marked preference for the Fmoc–Asp–Phe over the Fmoc–Phe–Asp sequence. The rates of the reactions appear similar because the kinetics are dominated by the rate-limiting diffusion of enzyme into the beads, as shown previously.[8]

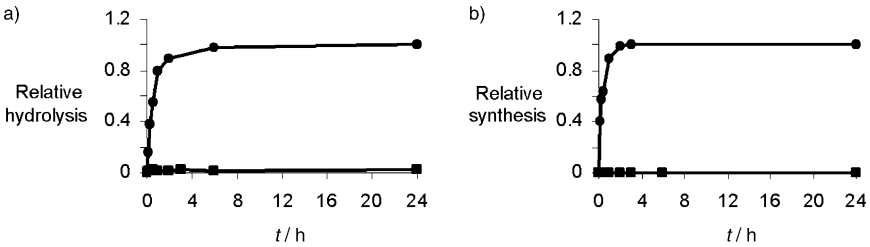

Figure 2. Thermolysin-catalyzed hydrolysis and synthesis of Fmoc–Asp–Phe–Wang–$PEGA_{1900}$ and Fmoc–Phe–Asp–Wang–$PEGA_{1900}$. a) Hydrolysis of Fmoc–Asp–Phe (circles) is favored over hydrolysis of Fmoc–Phe–Asp (squares). b) In the presence of excess Fmoc–Asp or Fmoc–Phe, synthesis of Fmoc–Asp–Phe (circles) is favored over synthesis of Fmoc–Phe–Asp (squares). Analyses were performed by LC-MS.

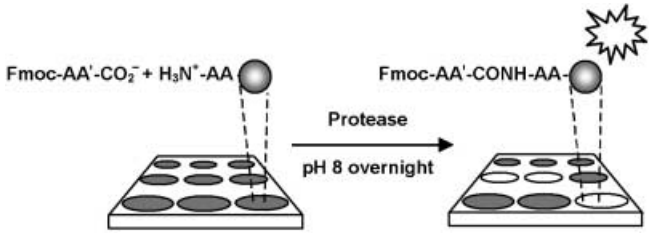

Figure 1. Protease specificity assay on a microtiter plate. Each well contains 1 of the 20 different P_1' amino acids (AA) directly linked to PEGA-NH_2 through the carboxylic acid terminus. The P_1 amino acid AA′ carries a fluorescent 9-fluorenylmethoxycarbonyl (Fmoc) protecting group and is added in a buffer solution containing the protease of interest. After incubation of the beads for 16 h at RT, they are washed and the Fmoc fluorescence is read by using a plate reader.

For comparison of two different proteases, commercially available bovine pancreatic α-chymotrypsin and thermolysin from *B. thermoproteolyticus Rokko*, which display opposite but complementary specificities for certain P_1/P_1' amino acid combinations were selected. While α-chymotrypsin is selective for aromatic amino acids in the P_1 position, it is rather unspecific for the P_1' residue. By contrast, thermolysin exhibits a preference for large hydrophobic residues in the P_1' position and is nonspecific for the P_1 residue. Hence, one would expect hydrolysis/synthesis of the amide bond in Asp–Phe to be catalyzed preferentially by thermolysin and that of Phe–Asp by α-chymotrypsin.[9]

To test whether this specificity could be measured with the present method of peptide synthesis, PEGA-immobilized Phe and Asp were exposed to Fmoc–Asp and Fmoc–Phe in the presence of either of the two enzymes. After overnight incubation, the relative fluorescence intensities were measured; these directly indicated the level of protease-catalyzed peptide synthesis. Figure 3 shows that the relative fluorescence intensities observed indeed corresponded to the reported specificities for both enzymes. These experiments

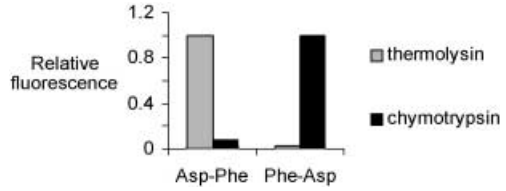

Figure 3. Comparison of substrate selectivity of two complementary proteases. Reactions were performed as outlined in Figure 1 with either Asp or Phe in positions P_1 or P_1'. "Relative fluorescence" is a measure of transfer of the respective P_1 amino acid by thermolysin (gray) or chymotrypsin (black). The experiment shows that thermolysin favors formation of Fmoc–Asp–Phe–PEGA while chymotrypsin favors formation of Fmoc–Phe–Asp–PEGA.

suggest that solid-phase screening through peptide synthesis can give the same specificity as observed in peptide hydrolysis. Indeed, protease specificity is expected to be identical in either the hydrolysis or synthesis directions because the same transition states are involved.

The assay was then applied in a 96-well microtiter plate format by using thermolysin as a model system. Thermolysin specificity against three P_1 amino acids carrying side chains with different physical properties was monitored: phenylalanine (aromatic side chain), aspartic acid (negatively charged), and glycine (smallest amino acid). The fluorescence intensities were measured, compared directly (Figure 4, top images), normalized, and converted into a gray-scale (bottom image) with white representing the highest fluorescence intensity and black representing no signal above the background level.

The reported specificity for large hydrophobic amino acids, specifically leucine (L), isoleucine (I), methionine (M), and phenylalanine (F) is indeed observed for the three P_1 amino acids studied here. Alanine (A), glycine (G), and tyrosine (Y) were recognized to a lesser extent as they are smaller or less hydrophobic than the preferred substrates. Tryptophan (W) appears to be too bulky to fit into the active site and is not accepted by thermolysin.[10]

Figure 4 also shows that the nature of P_1 significantly affects the selectivity for P_1' in thermolysin. It appears that a large hydrophobic phenyl group in the P_1 position restricts P_1' to large hydrophobic amino acids (mainly Ile, Leu, and to a lesser extent Val, Met, and Phe), while for smaller Gly residues the substrate specificity is more relaxed and a wider range of substrates are accepted. This highlights the importance of screening all combinations of P_1 and P_1', rather than both sites independently, to fully characterize substrate specificity.

In summary, we have presented a new approach to screening for protease specificity. Primary screening of protease specificity is greatly simplified by taking advantage of the reversal of peptide hydrolysis (that is, peptide synthesis) on a solid support. The choice of label for each added amino acid is flexible; indeed, different fluorescent labels could be used for different amino acids and thus might aid simultaneous detection and analysis. The methodology has been demonstrated for a microtiter plate system but should be also applicable to the array format. Specificity for sites away from the primary positions can also be monitored by

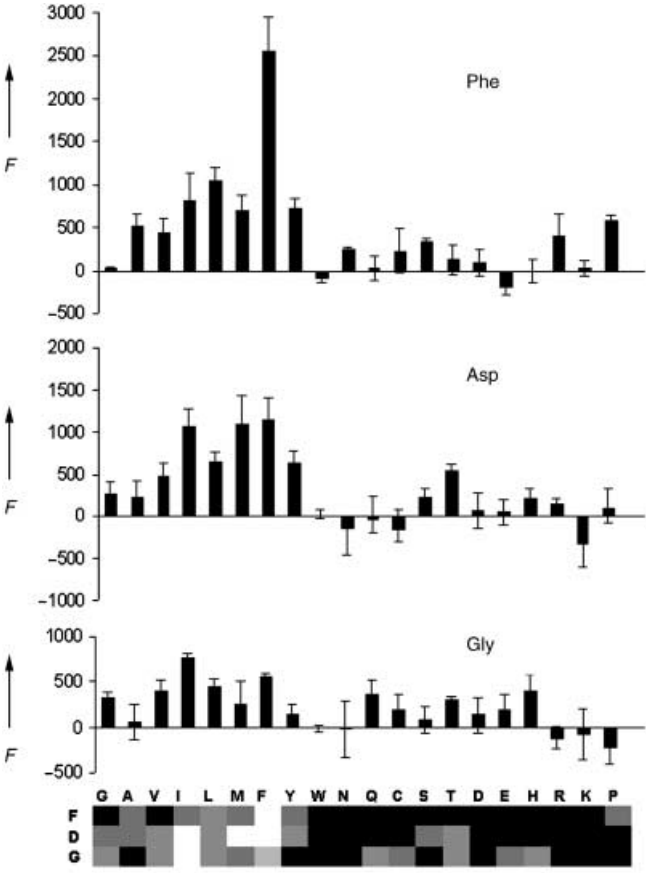

Figure 4. Primary thermolysin specificity as determined by reverse peptide hydrolysis on a solid support. Three different P_1 amino acids were supplied as Fmoc derivatives in separate experiments and all of the natural P_1' amino acids were directly linked to $PEGA_{1900}$. The histograms show the average fluorescence response obtained over three measurements. (The large error bars on the data are mainly as a result of inaccuracies resulting from dispensing bead suspensions in microtiter plates.) The bottom image shows the relative fluorescence normalized against the P_1' amino acid which gave rise to the highest intensity. Lighter shades indicate increased fluorescence observed.

incubation with di-, tri-, and polypeptides instead of Fmoc-protected amino acids. Work on such an extension of the methodology is currently in progress. We believe that the present approach paves the way for flexible rapid high-throughput identification and characterization of proteases without the need for expensively labeled peptide arrays.

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