A Method for Rapid Protease Substrate Evaluation and Optimization

Igor A. Kozlov, Peter C. Melnyk, Chanfeng Zhao, John P. Hachmann, Veronika Shevchenko, Anu Srinivasan, David L. Barker and Michal Lebl*

Illumina, Inc., 9885 Towne Centre Drive, San Diego, CA 92121-1975, USA

Abstract: We have developed a high throughput assay for the measurement of protease activity in solution. This technology will accelerate research in functional proteomics and enable biologists to streamline protease substrate evaluation and optimization. The peptide sequences that serve as protease substrates in this assay are labeled on the carboxy terminus with a biotin moiety and a fluorescent tag is attached to the amino terminus. Protease cleavage causes the biotin containing fragment to be detached from the labeled peptide fragment. Following the protease treatment, all biotin containing species (uncleaved substrates and the cleaved carboxy terminal fragment of the substrate) are removed by incubation with streptavidin beads. The cleaved fluorescently labeled amino terminal part of the substrate remains in solution. The measured fluorescence intensity of the solution is directly proportional to the activity of the protease. This assay was validated using trypsin, chymotrypsin, caspase-3, subtilisin-A, enterokinase and tobacco etch virus protease.

Keywords: Biotin depletion, fluorescence based assay, proteomics, protease assay.

INTRODUCTION

Proteases are important targets for the development of novel drugs due to their pivotal role in regulating biological processes – metabolism, hormonal and immune response, homeostasis, neurochemistry, viral replication, apoptosis, to name a few. Finding efficient inhibitors for proteases has been a goal of multiple academic and industrial medicinal chemistry teams. The first step in this effort is finding optimal and specific substrates for particular proteases and establishing an effective protease assay. There are multitudes of assays formats employable for this task, assays based on the chromatographic or electrophoretic evaluation of a reaction mixture, assays based on mass spectrometric analysis, or radioactivity readouts (see e.g. [1-3]). The most efficient and most widely used are the assays based on fluorescent and/or fluorogenic substrates, especially substrates utilizing the fluorescent resonance energy transfer (FRET) phenomenon (for review see [4]). FRET based assays, however, require proper size of the substrate allowing efficient quenching and their sensitivity can suffer from significant background signal.

Once the assay to study the activity of a protein is established, various methods exist for finding new substrates. The oldest method is based on the use of widely available and well characterized polypeptides – for example oxidized insulin B chain – and evaluation of products resulting from incubation with this compound. More efficient methods use "context dependent substrate libraries", i.e. multitude of modifications of a previously known substrate. Much higher throughput is achieved by the use of peptide libraries (see review [2]), either in the form of peptide mixtures created by split and mix synthesis [5-7], where each bead contains a different peptide (for the recent application of this technique in identification of serine racemase inhibitors see [8]), or in the form of positional scanning libraries [9], where mixtures of peptides are created with defined and randomized positions. Another alternative is immobilization of synthetic peptides on the solid substrate [10], or the use of microspotted substrate microarrays [11], or phage display techniques [12]. Each of the mentioned technologies has some advantages and disadvantages. The general preference is given to methods in which individually synthesized peptides are placed in microtiter plates and their activity is evaluated on an individual basis by incubation with the particular enzyme or cellular extract. A recent example of this technique is its use in the development of cathepsin S inhibitors [13]. The only limitations to this technique are the necessity to synthesize large numbers of individual peptide sequences economically and the availability of sufficient quantities of the protease or cell extract. Since we have at our disposal very fast and economical peptide synthesizer, we decided to evaluate the potential of novel one peptide per well-based assay format.

MATERIALS AND METHODS

Fmoc amino acids, BOP reagent and biotin resin (0.29 mmol/g) were purchased from Novabiochem (EMD Biosciences, Inc. San Diego, CA, USA). Solvents were from VWR International, Inc. (West Chester, PA). 4-Methylpiperidine was from Sigma-Aldrich (Milwaukee, WI, USA).

Biotin resin (300 mg) was added into a mixture of DMF and DCM (10 ml total) to form a nonsedimenting suspension which was distributed into the wells of a flat bottom polypropylene microtiterplate (Evergreen Scientific, Los Angeles, CA). The plate was placed into a centrifugal synthesizer [14, 15]. An additional 100 μL of DMF was added into the plate wells (beads sedimented) and the plate was centrifuged with a tilt of 6 degrees. A standard protocol was used for the synthesis. To remove the Fmoc protecting group, 4-Methylpiperidine was used instead of piperidine [16] to avoid working with regulated substance. Individual Fmoc protected amino acids (0.3 M solution in 0.3 M HOBt in DMF) were pipetted to the wells, and a solution of BOP (0.6 M in DMF) and 1.2 M DIEA in DMF was delivered to each

*Address correspondence to this author at the Illumina, Inc., 9885 Towne Centre Drive, San Diego, CA 92121-1975, USA;
E-mail: mlebl@illumina.com

1386-2073/06 $50.00+.00

well. Each plate was oscillated five times and rested for 50 sec. This procedure was repeated 30 times. Each plate was centrifuged, and the addition of the amino acids and reagents was repeated. After another 30 cycles of oscillation and resting, the reagents were removed by centrifugation and washing and deprotection was repeated to prepare the plate for the next cycle of synthesis.

Upon completion of the synthesis, the plate was dried in vacuo and 150 μL of mixture K [17] (TFA/thioanisol/water/phenol/EDT: 82.5:5:5:5:2.5 v/v) was added. The plate was capped and shaken on the plate shaker for 3 h. The suspension was transferred using a multichannel pipettor into a filter plate (Orochem Technologies, Lombard, IL). The eluate in the deep well plate (VWR) was precipitated with ether (600 μL). Following 2 h of refrigeration, the solution was centrifuged, the supernatant removed with a surface suction device [18], and the pellet was resuspended in ether (600 μL) and centrifuged again. The process of supernatant removal and resuspension was repeated three times. The final peptide product was dried in a Speedvac (ThermoSavant, Waltham, MA), dissolved in 200 μL of water, or 50% dimethylsulfoxide (DMSO)-50% H_2O and samples of 20 μL was taken into 180 μL of water. To assess the quality, 20 μL of the solution containing peptides were injected onto an HPLC column (Waters, Milford MA, μBondapak, C18, 10 μ particle, 125 Å pore, 3.9 x 150 mm, gradient 0.05% TFA in H_2O to 70% acetonitrile, 0.05% TFA in 15 min, flow rate 1.5 ml/min, detection by UV at 217 nm). Mass spectrometry was performed at HT-Labs (San Diego, CA, USA).

The protease assay was performed in 96-well PCR plates. Fluorescence measurements were done in black flat bottom 96-well plates on a Tecan plate reader. Two control plates were used for each experiment. The first plate was a positive control. It was scanned to obtain maximum fluorescence values for the peptides. The second plate was a negative control. The peptides in this plate were incubated with streptavidin coated magnetic beads (Seradyn). The majority of peptides were pulled out with the streptavidin magnetic beads. The solution was transferred into another plate and the plate was scanned to obtain negative background values for the peptides. The assay plates contained peptides mixed with proteases, one plate per protease. The peptides in this plate were treated with the particular protease followed by the incubation with streptavidin coated magnetic beads. The solution was transferred into another plate and the plate was scanned to obtain protease cleavage values. Peptides used in the assay were dissolved at 1 μM concentration in 100 μl of the buffer supplied by the manufacturer in the case of tobacco etch virus protease and enterokinase, in PBS buffer, pH 7.2 in the case of trypsin, chymotrypsin, subtilisin A, and papain proteases, and in 0.7 M sodium citrate buffer, pH 7.2 containing 100 mM NaCl, 1 mM EDTA, 10% sucrose, and 0.1% CHAPS in the case of caspase-3. We used 0.5 ng of trypsin (Sigma); 5 ng (0.08 U) of chymotrypsin (Calbiochem); 10 pg of subtilisin A (Aldrich); 4.6 U of papain protease (Aldrich); 0.01 U of enterokinase (Invitrogen); and 0.2 U of tobacco etch virus protease (Invitrogen) per reaction. Caspase-3 (gift from Prof. Salvesen, the Burnham Institute) was used at 0.25 nM concentration. The reactions were incubated at 37°C for 90 min. After the incubation, 30 μl of 0.6% solution of streptavidin coated magnetic beads (4.5 nmols/mg) solution were added. The binding was allowed to proceed for 15 min. 100 μl of the solution was transferred into another plate and the plate was scanned. The values for the negative controls were subtracted both from the positive control values and the assay values. The protease cleavage data are presented as a ratio between assay values and positive control values and then presented as a percent of substrate cleaved.

RESULTS AND DISCUSSION

During the development of our high throughput protease profiling assay, we have utilized our high throughput peptide synthesizer capable of simultaneous synthesis of 768 peptides in one batch in flat bottom polypropylene microtiter plates [14,15]. Synthesis of peptide substrates was performed on biotin modified resin (Fig. **1**). Peptides were assembled using Fmoc chemistry and BOP as a coupling reagent. Finally, 6(5)-carboxyfluorescein was coupled to the amino terminus using our standard amino acid coupling protocol followed by the treatment with 25% 4-methyl piperidine (removing side products resulting from coupling of carboxyfluorescein on its own hydroxyl groups [19]), and peptides were cleaved from the resin by mixture K [17].

Streptavidin interaction with biotin-labeled molecules is the basis of many assay systems (as an example of recent sophisticated use of biotin labeling see [20]). Our assay is based on the depletion of biotin containing substrates from the solution by incubation with streptavidin immobilized on magnetic beads [21,22] (see Scheme **1**). The peptide sequences were labeled on the carboxy terminus with a biotin moiety and upon protease cleavage, the biotin containing part of the molecule was detached from the rest of peptide substrate containing the fluorescent label. Following the reaction, all biotin containing species (uncleaved substrates and the cleaved carboxy terminal part of the substrate) were

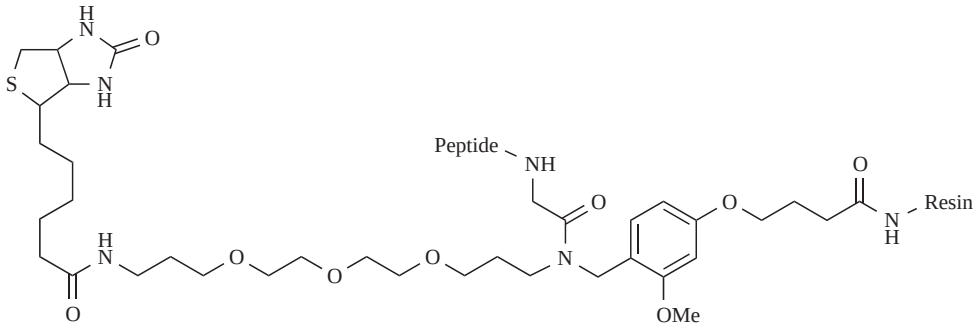
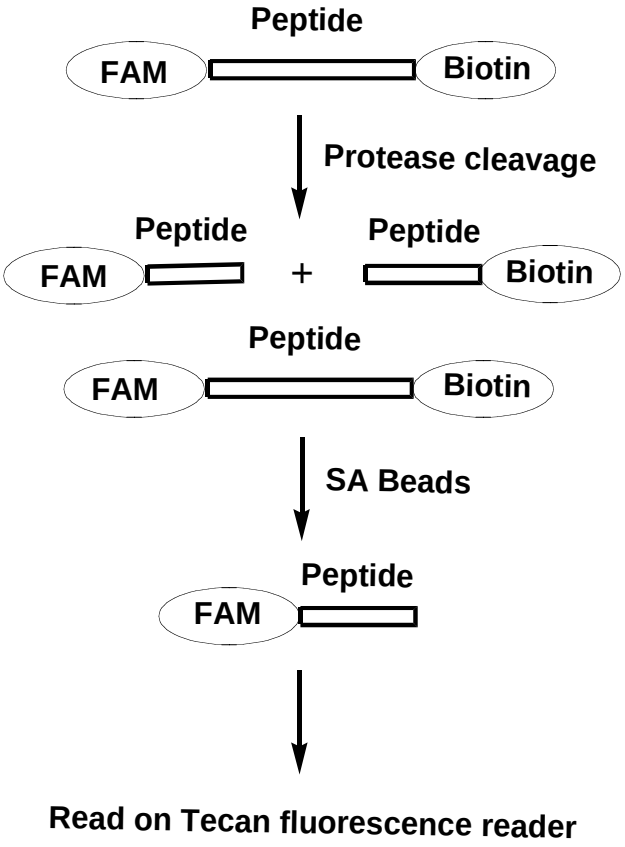


Fig. (1). Structure of biotin linker used for the substrate syntheses.

captured and removed by incubation with magnetic streptavidin beads. The cleaved amino terminal part of the substrate containing the fluorescein label remains in solution and the fluorescence intensity was read by fluorescence detector.



Scheme 1. Schematic representation of the assay.

Success of this type of an assay is dependent on (i) the complete removal of the biotin labeled molecules from the solution, and (ii) on the quality of synthesized peptides. If the synthetic product were to contain substrates with the fluorescent label but lacking the biotin tag, the background of the assay would be unacceptably high. We investigated the stability of the carboxy terminal attachment of the peptide to the biotin tag. We found that cleavage of the synthetic product from the resin using the cocktail of scavengers (mixture K), while very effective in protecting the peptide from undesired modification by linker and side chain protecting groups debris, might be cleaving the linkage to biotin as well. However, the extent of this reaction was found to be trivial, i.e. 0.2% of the peptide material was found to be missing biotin. The loss of biotin was not observed when TFA with 5% water was used for the deprotection. This condition, however, is not compatible with arginine and tryptophan containing sequences, because tryptophan is significantly damaged by cations arising from the arginine protecting group (see e.g. [23]). In our situation of high throughput synthesis, we cannot apply individually optimized conditions for different sequences, and therefore, we decided to accept small losses of biotin tag yet ensuring higher quality of the synthetic product.

Every experiment included 2 controls. The first control was a "negative control" where the substrate was not exposed to protease, but subjected to streptavidin treatment. This condition measured the completeness of the streptavidin pulldown of the biotinylated substrates and also established the assay background. The second control, "positive control", was a condition where the substrate was subjected neither to the protease nor the streptavidin. This measured the maximum possible signal from any given substrate.

To show the utility of this profiling assay we have synthesized 96 sequences (in a single plate) representing substrates for several protease groups (see Table **1**). Four sequences were synthesized in duplicates to show "in synthesis" reproducibility of the synthetic and biological results. All synthetic products were analyzed by HPLC and structure was spot checked by mass spectrometry. Four sequences

Table 1. List of Peptides Prepared for this Study (in Alphabetical Order)

ADAADGAG	ADNKDGAG	ENLYFQGA
ADAEDGAG	ADNNDGAG	ENLYFQGA
ADAFDGAG	ADNTDGAG	FSARGHRP
ADAKDGAG	ADNVDGAG	FVNQHLEG
ADANDGAG	ADTADGAG	GFFYTPKA
ADATDGAG	ADTEDGAG	GGVRGPRV
ADAVDGAG	ADTFDGAG	GPLGLKAR
ADDDKAGA	ADTKDGAG	GPLGLWAR
ADEADGAG	ADTNDGAG	GPQGIQGG
ADEEDGAG	ADTTDGAG	GPQGIWGQ
ADEFDGAG	ADTVDGAG	IEPRSFSQ
ADEKDGAG	ADVADGAG	KKKRKVGG
ADENDGAG	ADVEDGAG	LEGSHLVE
ADETDGAG	ADVFDGAG	LGIRSFRN
ADEVDGAG	ADVKDGAG	LGPVTPEI
ADFADGAG	ADVNDGAG	LRPRFKII
ADFEDGAG	ADVTDGAG	LSPRTFHP
ADFFDGAG	ADVVDGAG	LYLVEGER
ADFKDGAG	APSWLLTA	MTPRSEGS
ADFNDGAG	AQDFVQWL	NISDLTAG
ADFTDGAG	AVRWLLTA	NQHLEGSH
ADFVDGAG	DADDKAGA	PETAMSTV
ADKADGAG	DDADKAGA	PFDLLDFN
ADKEDGAG	DDDAKAGA	PVQPIGPQ
ADKFDGAG	DDDDKAGA	PVQPIGPQ
ADKKDGAG	DDDDKAGA	QGTFTSDY
ADKNDGAG	DDDDKASA	RSPFIWNG
ADKTDGAG	DVANYNFF	RYTNANTP
ADKVDGAG	ENAYFQGA	VDPRLIDG
ADNADGAG	ENLAFQGA	VIPRSGGS
ADNEDGAG	ENLYAQGA	VIPRSGGS
ADNFDGAG	ENLYFAGA	YGPKKKRK

Table 2. Results of Incubation with Particular Proteases

C3	TEV	EK	TP	CT	SA	PA	Peptide	Target Protease
100	2	0	3	3	2	2	ADVVDGAG	C14.003-caspase 3
58	1	0	1	1	1	0	ADTVDGAG	C14.003-caspase 3
57	2	0	0	2	0	0	ADAVDGAG	C14.003-caspase 3
53	2	4	1	1	2	0	ADFVDGAG	C14.003-caspase 3
52	3	0	1	0	1	0	ADEVDGAG	C14.003-caspase 3
38	1	1	0	1	0	0	ADNVDGAG	C14.003-caspase 3
38	2	0	2	2	2	1	ADVTDGAG	C14.003-caspase 3
38	1	5	1	1	1	0	ADEADGAG	C14.003-caspase 3
36	3	1	0	0	2	0	ADKVDGAG	C14.003-caspase 3
34	3	0	1	1	1	0	ADTTDGAG	C14.003-caspase 3

C3	TEV	EK	TP	CT	SA	PA	Peptide	Target Protease
0	**100**	0	0	9	9	0	ENLYFQGA	C04.004-TEV protease
0	**71**	0	0	7	23	0	ENLYAQGA	C04.004-TEV protease
0	**6**	0	0	7	7	0	ENAYFQGA	C04.004-TEV protease
0	**2**	0	0	2	8	1	ENLAFQGA	C04.004-TEV protease
0	**1**	4	3	12	12	1	ENLYFAGA	C04.004-TEV protease

C3	TEV	EK	TP	CT	SA	PA	Peptide	Target Protease
0	1	**99**	1	0	3	0	DDDDKAGA	S01.156-enterokinase
0	3	**99**	1	0	1	0	ADDDKAGA	S01.156-enterokinase
0	2	**94**	0	0	1	2	DDADKAGA	S01.156-enterokinase
0	1	**89**	0	0	1	0	DDDDKASA	S01.156-enterokinase
0	0	**83**	50	100	100	100	LGIRSFRN	S01.217-thrombin
0	2	**80**	0	0	0	0	DADDKAGA	S01.156-enterokinase
0	2	**69**	0	0	1	1	ADNKDGAG	C14.003-caspase 3
0	2	**56**	1	1	16	1	LYLVEGER	S01.131-neutrophil elastase
0	0	**54**	0	0	2	0	ADFKDGAG	C14.003-caspase 3
0	1	**18**	0	0	0	0	DDDAKAGA	S01.156-enterokinase

C3	TEV	EK	TP	CT	SA	PA	Peptide	Target Protease
0	2	14	**100**	30	30	79	LRPRFKII	S01.217-thrombin
0	3	7	**97**	27	63	86	LSPRTFHP	S01.217-thrombin
0	10	0	**64**	33	23	21	GPLGLWAR	M10.002-collagenase 2
0	1	11	**61**	3	2	24	VIPRSGGS	S01.217-thrombin
0	6	10	**58**	3	0	17	VIPRSGGS	S01.217-thrombin
0	0	7	**52**	68	66	27	FSARGHRP	S01.217-thrombin
0	0	83	**50**	100	100	100	LGIRSFRN	S01.217-thrombin
0	0	1	**37**	2	1	3	KKKRKVGG	S01.146-granzyme K
0	3	8	**34**	7	9	67	GPLGLKAR	M10.002-collagenase 2
0	0	41	**32**	40	35	15	IEPRSFSQ	S01.217-thrombin

(Table 2) contd…..

C3	TEV	EK	TP	CT	SA	PA	Peptide	Target Protease
0	0	83	50	**100**	100	100	LGIRSFRN	S01.217-thrombin
0	0	24	0	**92**	0	1	RSPFIWNG	S01.001-chymotrypsin A
0	4	1	0	**76**	8	4	AQDFVQWL	S01.001-chymotrypsin A
0	0	7	52	**68**	66	27	FSARGHRP	S01.217-thrombin
0	0	41	32	**40**	35	15	IEPRSFSQ	S01.217-thrombin
0	1	4	7	**36**	12	1	ENLYFAGA	C04.004-TEV protease
0	10	0	64	**33**	23	21	GPLGLWAR	M10.002-collagenase 2
0	2	14	100	**30**	30	79	LRPRFKII	S01.217-thrombin
0	0	0	14	**29**	42	13	APSWLLTA	M10.003-gelatinase A
0	3	7	97	**27**	63	86	LSPRTFHP	S01.217-thrombin

C3	TEV	EK	TP	CT	SA	PA	Peptide	Target Protease
0	0	83	50	100	**100**	100	LGIRSFRN	S01.217-thrombin
0	0	7	52	68	**66**	27	FSARGHRP	S01.217-thrombin
0	3	7	97	27	**63**	86	LSPRTFHP	S01.217-thrombin
24	2	0	0	3	**59**	0	PFDLLDFN	S01.133-cathepsin G
0	0	0	14	29	**42**	13	APSWLLTA	M10.003-gelatinase A
0	0	41	32	40	**35**	15	IEPRSFSQ	S01.217-thrombin
0	2	14	100	30	**30**	79	LRPRFKII	S01.217-thrombin
0	9	0	0	0	**25**	0	NISDLTAG	M10.003-gelatinase A
0	71	0	0	7	**23**	0	ENLYAQGA	C04.004-TEV protease
0	10	0	64	33	**23**	21	GPLGLWAR	M10.002-collagenase 2

C3	TEV	EK	TP	CT	SA	PA	Peptide	Target Protease
0	0	83	50	100	100	**100**	LGIRSFRN	S01.217-thrombin
0	3	7	97	27	63	**86**	LSPRTFHP	S01.217-thrombin
0	2	14	100	30	30	**79**	LRPRFKII	S01.217-thrombin
0	3	8	34	7	9	**67**	GPLGLKAR	M10.002-collagenase 2
0	0	4	6	12	6	**31**	GFFYTPKA	S01.001-chymotrypsin A
0	0	7	52	68	66	**27**	FSARGHRP	S01.217-thrombin
0	1	11	61	3	2	**24**	VIPRSGGS	S01.217-thrombin
0	10	0	64	33	23	**21**	GPLGLWAR	M10.002-collagenase 2
0	6	10	58	3	0	**17**	VIPRSGGS	S01.217-thrombin
0	0	41	32	40	35	**15**	IEPRSFSQ	S01.217-thrombin

Table is sorted in particular segments by activity for particular protease (bolded column). C3 – Caspase-3; TEV – Tobacco etch virus protease; EK – Enterokinase; TP – Trypsin; CT – Chymotrypsin; SA – Subtilisin; PA – Papain. Activity is expressed in percent of substrate cleaved.

were found to be of unacceptable quality and were excluded from the biological evaluation. The largest number of substrates synthesized targeted Caspase-3. In these forty-nine substrates, the canonical caspase cleavage site DEVDG was extended by addition of A on the amino terminus (where the fluorescein moiety is attached) and an AG sequence was added to the carboxy terminus attached to the biotin. E and V residues were exhaustively substituted by combination of seven "representative" amino acids (V,A,F,N,T,K, and E) to form a complete library of 49 ADXXDGAG sequences. The rest of the sequences were substrates for thrombin, collagenase, chymotrypsin, subtilisin, enterokinase, gelatinase, cathepsin G, elastase, granzyme K, and tobacco etch virus protease. Selection of substrates was based on the MEROPS database (http://merops.sanger.ac.uk/), where the latest references for all relevant protease specificities are found. Substrates were treated with trypsin, chymotrypsin, caspase-3, subtilisin, enterokinase and tobacco etch virus protease. Results are shown in Table **2**.

Use of the "classical" enzymes trypsin and chymotrypsin resulted in a predictable cleavage of substrates containing basic or aromatic residues. However, we were able to distinguish between highly sensitive substrates and define sequences which are less susceptible to the cleavage.

Two thrombin substrate sequences were found to be also optimal substrates for trypsin (Table **2**). On the other hand, substrates with acidic amino acids Asp and Glu in close proximity to Lys and Arg diminished the effectivity of trypsin cleavage at basic residues. All peptides with an Asp-Lys or Lys-Asp motif (caspase-3 substrates) were trypsin resistant. Resistance to cleavage of substrates with amino terminal arginine (RSPFIWNG and RYTNANTP) can be explained by the fact that the bulky fluorescein residue was attached directly to the arginine residue. However, we did not find an explanation for low levels of trypsin-substrate-like qualities of peptides APSWLLTA and ENLYFAGA, other than a possible contamination of trypsin by chymotrypsin.

Chymotrypsin substrates defined in the MEROPS database performed best in the chymotrypsin test cleavage, together with thrombin substrates (Table **2**). Again, as in the case of trypsin, we were able to define poor chymotrypsin-mediated cleavage of peptides in which an aromatic residue was flanked by an acidic residue.

Subtilisin has very broad specificity, but it shows a preference for large uncharged residues in P1. A peptide sequence defined as the Subtilisin Carlsberg substrate did not score as a good substrate for subtilisin A. Several thrombin substrates were found to be more susceptible to subtilisin cleavage. Papain, which also has very broad specificity, preferred thrombin substrates as well (Table **2**).

Enterokinase is a specific protease that cleaves after lysine at its cleavage site Asp-Asp-Asp-Asp-Lys. It also cleaves at other basic residues, depending on the conformation of the protein substrate. In our assay, enterokinase cleaved only its own substrates specifically (Table **2**). Additionally, it also cleaved elastase and caspase substrates showing a certain level of similarity (combination of acidic and basic residues). The only substrate cleaved by enterokinase that did not contain any acidic residues was the thrombin substrate LGIRSFRN. We mutated the enterokinase substrates by substitution of aspartic acid (D) residues by alanine (A). As can be seen, the significant loss of specificity could be achieved only when the aspartic acid adjacent to lysine was replaced.

In contrast to the broad specificity enzymes discussed earlier, tobacco etch virus (TEV) protease displays a unique specificity. This is often used for removal of affinity tags from recombinant proteins [24]. We did not expect to find substrates of TEV protease among the substrates tested. Therefore we included several mutations of the known TEV substrate ENLYFQGA (Table **2**). As can be seen, replacement of phenylalanine by alanine retains significant activity, but replacement of leucine, tyrosine, or glutamine eliminates substrate cleavage. This behavior concurs with previously published data [25].

Caspases are a family of proteases that are one of the main effectors of apoptosis. Caspase-3 specificity was studied with the use of a combinatorial library and DEVD was found as an optimal substrate (see Table **3**). Our limited library of 49 compounds, however, identified a superior sequence DVVD and at least three equivalent sequences (DTVD, DAVD, and DFVD). Charged (K, D) or hydrogen bonding capable (N) residues in position P2 are clearly detrimental to substrate activity of the DXXD sequence. This is in very good agreement with the published findings [26, 27].

Fig. **2** shows the activity of tested enzymes on a limited set of 96 substrates using the "star plots". In this representation the individual spokes of the wheel represent individual synthetic substrates. The length of the spokes show the degree of their proteolysis by the particular protease. All substrates are mapped to the space of 96 substrates. As can be clearly seen, even a small selection of substrates can distinguish between all tested proteases (each protease has a unique pattern of cleaved substrates), and identification of specific enzymes by their "fingerprint" [21, 28-31] is feasible.

Table 3. Results of Proteolysis of 49 Member Library of ADXXDGA by Caspase 3

Substrate	%	Substrate	%	Substrate	%	Substrate	%
ADVVDGAG	100	ADATDGAG	32	ADENDGAG	6	ADKKDGAG	0
ADTVDGAG	58	ADFTDGAG	32	ADFNDGAG	6	ADAKDGAG	0
ADAVDGAG	57	ADVADGAG	24	ADKFDGAG	3	ADVEDGAG	0
ADFVDGAG	53	ADEFDGAG	21	ADEKDGAG	2	ADNEDGAG	0
ADEVDGAG	52	ADVFDGAG	19	ADKADGAG	1	ADKEDGAG	0
ADNVDGAG	38	ADTFDGAG	16	ADVNDGAG	1	ADTNDGAG	0
ADVTDGAG	38	ADFFDGAG	16	ADKNDGAG	0	ADNFDGAG	0
ADEADGAG	38	ADAADGAG	15	ADEEDGAG	0	ADAEDGAG	0
ADKVDGAG	36	ADNTDGAG	15	ADVKDGAG	0	ADFEDGAG	0
ADTTDGAG	34	ADTADGAG	11	ADTKDGAG	0	ADTEDGAG	0
ADFADGAG	33	ADKTDGAG	9	ADANDGAG	0	ADNNDGAG	0
ADETDGAG	32	ADAFDGAG	7	ADNKDGAG	0	ADFKDGAG	0

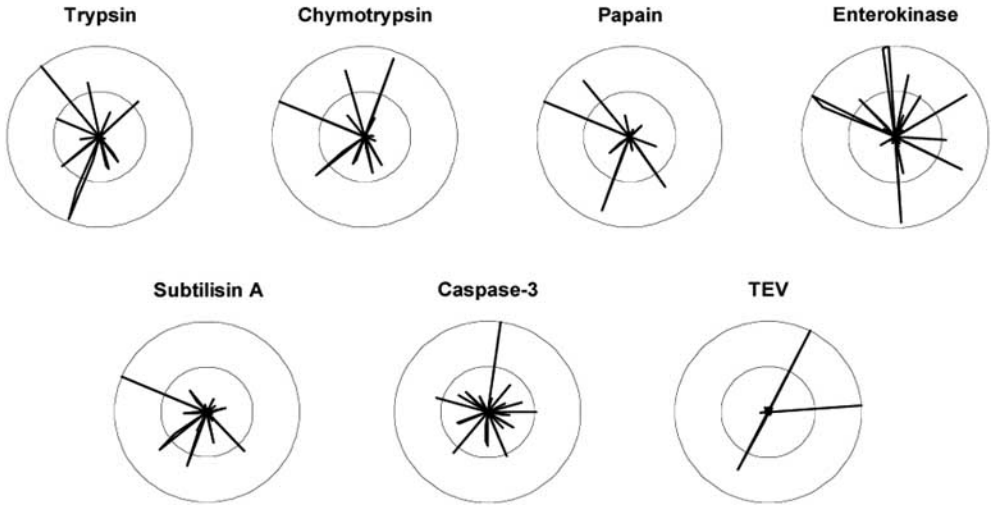


Fig. (2). "Star plot" representation of activities of synthetic peptides with a panel of proteases. The concentric circles represent signal intensities 50 and 100%. Each plot represents all 96 peptides in the same order, number 1 being at spoke pointing straight up and ascending numbers going in the clockwise direction (peptide 24 pointing to the right, peptide 48 pointing down, etc.). Activities of substrates synthesized in duplicates are included (enterokinase, TEV).

The assay system described here for interrogation of protease activities using synthetic substrates is very simple and provides a rapid and accurate assessment of substrate qualities of multiplicity of peptides. We have shown its effectiveness with both broad and narrow specificity proteases. The advantage over well established FRET assay is its universality (unlike FRET it does not depend on the length of the substrate, it can be used for very short and very long substrates) and its very low background signal allowing even small degree of cleavage to be detected. This assay has numerous applications in "fingerprinting" of biological samples and purified proteases. It can be used in targeted evaluation of specificity of novel proteases or in various drug discovery efforts.

ACKNOWLEDGEMENTS

The project was partially supported by NIH SBIR grant R44 AI056869-02. We thank Prof. Salvesen (the Burnham Institute, San Diego) for his kind gift of caspase-3.

REFERENCES

[1] Goddard, J.P.; Reymond, J.L. *Curr. Opin. Biotechnol.*, **2004,** *15*, 314.
[2] Maly, D.J.; Huang, L.; Ellman, J.A. *Chembiochem.*, **2002,** *3*, 16.
[3] Richardson, P.L. *Curr. Pharm. Des.*, **2002,** *8*, 2559.
[4] Knight, C.G. *Methods Enzymol.*, **1995,** *248*, 18.
[5] Furka, A.; Sebestyen, F.; Asgedom, M.; Dibo, G. *Int. J. Peptide Prot. Res.*, **1991,** *37*, 487.
[6] Houghten, R.A.; Pinilla, C.; Blondelle, S.E.; Appel, J.R.; Dooley, C.T.; Cuervo, J.H. *Nature*, **1991,** *354*, 84.
[7] Lam, K.S.; Salmon, S.E.; Hersh, E.M.; Hruby, V.J.; Kazmierski, W.M.; Knapp, R.J. *Nature*, **1991,** *354*, 82.
[8] Dixon, S.M.; Li, P.; Liu, R.; Wolosker, H.; Lam, K.S.; Kurth, M.J.; Toney, M.D. *J. Med. Chem.*, **2006,** *49*, 2388.
[9] Houghten, R.A.; Appel, J.R.; Blondelle, S.E.; Cuervo, J.H.; Dooley, C.T.; Pinilla, C. *BioTechniques*, **1992,** *13*, 412.
[10] Salisbury, C.M.;.Maly, D.J.; Ellman, J.A. *J. Am. Chem. Soc.*, **2002, 124,** 14868.
[11] Gosalia, D.N.; Salisbury, C.M.; Ellman, J.A.; Diamond, S.L. *Mol. Cell Proteom.*, **2005,** *45*, 626.
[12] Smith, G.P.; Petrenko, V.A. *Chem. Rev.*, **1997,** *97*, 391.
[13] Wood, W.J.L.; Patterson, A.W., Tsuruoka, H., Jain, R.K., Ellman, J.A. *J. Am. Chem. Soc.*, **2005,** *127*, 15521.
[14] Lebl, M. *Bioorg. Med. Chem. Lett.*, **1999,** *9*, 1305.
[15] Lebl, M. *J. Assoc. Lab. Autom.*, **2003,** *8*, 30.
[16] Hachmann, J.; Lebl, M. *J. Comb. Chem.*, **2006,** *8*, 149.
[17] King, D.S.; Fields, C.G.; Fields, G.B. *Int. J. Peptide Prot. Res.*, **1990,** *36*, 255.
[18] Lebl, M.; Krchnak, V.; Ibrahim, G.; Pires, J.; Burger, C.;.Chen, Y.; Ni, Y ; Podue, D.; Mudra, P.; Pokorny, V.; Poncar, P.; Zenisek, K. *Synthesis*, **1999,** 1971.
[19] Fischer, R.; Mader, O.; Jung, G.; Brock, R. *Bioconjug. Chem.*, **2003,** *14*, 653.
[20] Speers, A.E.; Cravatt, B.F. *J. Am. Chem. Soc.*, **2005,** *127*, 10018.
[21] Craig, D.B.; Wong, J.C.; Polakowski, R.; Dovichi, N.J. *Anal. Chem.*, **1998,** *70*, 3824.
[22] Turk, B.E.; Cantley, L.C. *Methods*, **2004,** *32*, 398.
[23] Stierandova, A.; Sepetov, N.F.; Nikiforovich, G.V.; Lebl, M. *Int. J. Peptide Prot. Res.*, **1994,** *43*, 31.
[24] Stevens, R.C. *Structure Fold. Des.*, **2000,** *8*, R177.
[25] Phan, J.; Zdanov, A.; Evdokimov, A.G.; Tropea, J.E.; Peters, III, H.K.; Kapust, R.B.;.Wlodawer, M.Li, A.; Waugh, D.S. *J. Biol. Chem.*, **2002,** *277*, 50564.
[26] Thornberry, N.A.; Rano, T.A.; Peterson, E.P.; Rasper, D.M.; Timkey, T.; Garcia-Calvo, M.; Houtzager, V.M.; Nordstrom, P.A.; Roy, S.; Vaillancourt, J.P.; Chapman, K.T.; Nicholson, D.W. *J. Biol. Chem.*, **1997,** *272*, 17907.
[27] Stennicke, H. R.; Renatus, M.; Meldal, M.; Salvesen, G.S. *Biochem. J.*, **2000,** *350*, 563.
[28] Grognux, J.; Reymond, J.L. *Chembiochem.*, **2004,** *5*, 826.
[29] Reymond, J.L.; Wahler, D. *Chembiochem.*, **2002,** *3*, 701.
[30] Wahler, D.; Badalassi, F.; Crotti, P.; Reymond, J.L. *Angew. Chem. Int. Ed. Engl.*, **2001,** *40*, 4457.
[31] Wahler, D.; Badalassi, F.; Crotti, P.; Reymond, J.L. *Chemistry*, **2002,** *8*, 3211.

Received: March 14, 2006 **Revised: May 1, 2006** **Accepted: May 15, 2006**