Chapter 2

Positional Scanning Substrate Combinatorial Library (PS-SCL) Approach to Define Caspase Substrate Specificity

Marcin Poręba, Aleksandra Szalek, Paulina Kasperkiewicz, and Marcin Drąg

Abstract

Positional scanning substrate combinatorial library (PS-SCL) is a powerful tool for studying substrate specificity of proteolytic enzymes. Here, we describe the protocol for analyzing S4-S2 pockets preferences of caspases using PS-SCL. Additionally, we describe procedures for the identification of optimal substrates sequence after PS-SCL, solid phase synthesis, and purification of selected fluorogenic substrates, as well as their kinetic analysis.

Key words Substrate specificity, Caspase, Fluorogenic substrate, Combinatorial library, Cysteine protease

1 Introduction

The accurate knowledge of substrate specificity of an enzyme provides important information about its functions and aids in the understanding of molecular pathways [1]. Moreover, this knowledge is invaluable in the development of potent and specific inhibitors and activity-based probes. One of the best understood family of proteases are caspases, which are involved in apoptosis regulation and inflammatory response [2–4]. Most caspases cleave substrates after aspartic acid residue in the P1 position, and amino acids after aspartate (P2, P3, P4…) what collectively determines their substrate specificity [5].

At present, there are several strategies to identify the substrate specificity of proteolytic enzymes [6]. Positional scanning substrate combinatorial library (PS-SCL) is one of the most reliable and powerful tools in determining substrate-enzyme interaction in binding pockets around protease active site. In this method, libraries of substrate mixtures with conjugated fluorescent molecule are synthesized. Fluorescent molecules or fluorophores are fixed in

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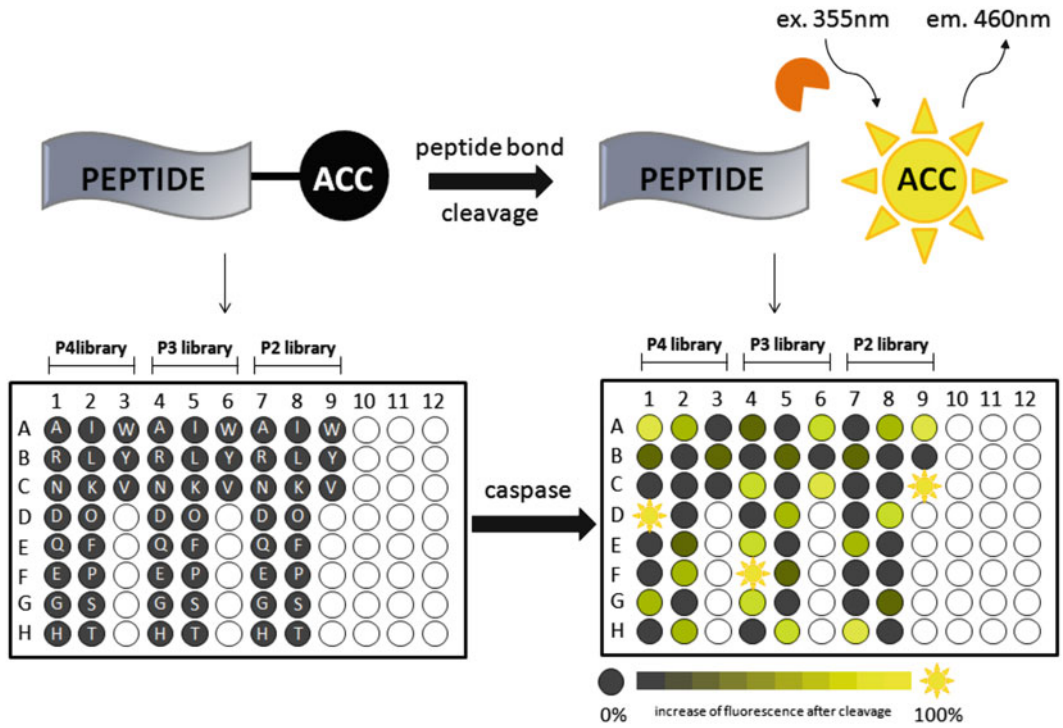


Fig. 1 General outline for caspase substrate specificity analysis using SCL screening with ACC as fluorescent reporter group

P1′ position. In intact substrates, fluorescence emission is either absent or very weak. Following protease hydrolysis, fluorophore is released and emits fluorescence when excited by an appropriate wavelength. Fluorescence can be quantified thereby providing the data on reaction kinetics. This method enables fast and reliable analysis of protease specificity. Noteworthy, apart from fluorophores, chromophores and luminophores can be also used as reporter groups. However, the sensitivity of each group differs, with luminophores being the most sensitive and chromophores possessing the lowest sensitivity. Fluorophores are the best choice of reporter group, since they are quite easy to synthesize and yield strong fluorescence in biological tests [6] (Fig. 1).

PS-SCL are composed of mixtures of peptidic substrates, which are divided into sublibraries. In each sublibrary, one or more positions are fixed with a defined amino acid, whereas the remaining positions contain equimolar concentrations of amino acids. It allows to establish the effect of the fixed amino acids independently. The same degree of substitution for each amino acid is necessary to obtain reliable results [6]. A procedure for the synthesis of equimolar mixture of natural amino acids on solid support was developed in 1994 by Ostresh et al. [7]. First, the level of substitution of the particular amino acids was established, which

was followed by the identification of the exact composition of isokinetic mixture determining equimolar levels of substitution. The following Fmoc-amino acid composition was established (the numbers in parentheses are percentages of molar fraction): Fmoc-Ala-OH (3.4), Fmoc-Arg(Pbf)-OH (6.5), Fmoc-Asn(Trt)-OH (5.3), Fmoc-Asp(O-*t*-Bu)-OH (3.5), Fmoc-Glu(O-*t*-Bu)-OH (3.6), Fmoc-Gln(Trt)-OH (5.3), Fmoc-Gly-OH (2.9), Fmoc-His(Boc)-OH (3.5), Fmoc-Ile-OH (17.4), Fmoc-Leu-OH (4.9), Fmoc-Lys(Boc)-OH (6.2), Fmoc-Nle-OH (3.8), Fmoc-Phe-OH (2.5), Fmoc-Pro-OH (4.3), Fmoc-Ser(O-*t*-Bu)-OH (2.8), Fmoc-Thr(O-*t*-Bu)-OH (4.8), Fmoc-Trp(Boc)-OH (3.8), Fmoc-Tyr(O-*t*-Bu)-OH (4.1), and Fmoc-Val-OH (11.3). Nowadays, Ostresh procedure is commonly used during synthesis of SCL. Utilizing combinatorial chemistry techniques, the libraries can be synthesized quickly and efficiently.

The first broad study of caspase substrate specificity using PS-SCL was carried out in 1997 by Rano et al. [8] for interleukin-1β converting enzyme (ICE, caspase-1). In this study, three separate combinatorial sublibraries of tetrapeptides conjugated with 7-amino-4-methyl-coumarin (AMC) were synthesized. Each sublibrary contained 8,000 compounds. The P1 position was occupied by aspartic acid residue, what was in line with previous findings of a strong requirement for Asp in that position [9–11]. In each sublibrary, one position was fixed with a defined amino acid and the other contained equimolar concentrations of natural amino acids (cysteine was omitted and methionine was replaced by norleucine in order to avoid their oxidation), according to Ostresh et al. procedure [7]. This study has revealed that the WEHD sequence is the most favorable tetrapeptide recognition motif for caspase-1 [8]. The result was surprising as it was inconsistent with formerly found optimal sequence YVAD [12] and differed from caspase 1 cleavage site YVHD present in its natural substrate pro-IL-1β. Further investigations confirmed that WEHD is an optimal caspase 1 substrate and proved that PS-SCL is a trustworthy method [8].

In the same year, Thornberry group used an identical library to examine the substrate specificity of almost all members of caspase family [13]. It was a seminal study, which has resulted in a division of caspases into three main groups based on their substrate specificity. The P4 position played an important role in this classification. The first group constituted the inflammatory caspases (caspases 1, 4, and 5), which prefer the (W/L)EHD tetrapeptide sequences. Group II consisted of caspases 2, 3, and 7, all favoring DEXD (X indicates that several amino acids are tolerated in this position), and group III was composed of caspases 6, 8, and 9 showing specificity for (L/V)EXD. Soon after, Garcia-Calvo et al. [14] examined the substrate specificity of caspase 10 using the same method, and the enzyme was classified to the third group based on the optimal recognition sequence LEXD. Further studies

conducted by Wachmann et al. [15] with a use of PS-SCL confirmed this finding and established LEHD as the best substrate for caspase-10. Mikolajczyk et al. [16] studied optimal substrates for caspase 14 employing the same type of library as Garcia-Calvo, but with AFC as a reporter group. It was found that the substrate specificity of caspase 14 matches that of group I.

All caspases show strong preference for aspartic acid and glutamic acid residues in the P1 and P3 positions, respectively. The library constructed by Thornberry et al. [13] can be used only for proteases with strong preferences for aspartic acid in the P1 position of their substrates. Later, some modifications to this method were published [17, 18]; however, it is still not easy to examine proteases with unknown P1 specificity using such a library.

In 2000, new strategy to synthesize fluorogenic libraries, which employed bifunctional fluorogenic group 7-amino-4-carbamoylmethylcoumarin (ACC) was reported [19]. In this method, fluorophore ACC is bound to the solid support and the amino acids are being directly attached one by one [20], significantly simplifying the synthesis of fluorogenic libraries. The method enables incorporating any amino acid in any position, and consequently, complete diversification of peptide libraries is possible. Moreover, the assay sensitivity is enhanced as ACC has higher fluorescent yield than AMC [19]. When caspase 3 substrate specificity was investigated using this approach [21], the results were identical to those obtained by Thornberry et al. [13] demonstrating that the type of fluorophore does not affect substrate specificity. Various proteases were examined with ACC-based libraries, including papain, bromelain, human cathepsins [22], kallikreins [23], human paracaspase MALT1 [24], and DUBs (deubiquitinating enzymes) [25].

In this chapter, we describe how to profile substrate specificity of caspases using PS-SCL. In addition, we present step by step the synthesis of individual tetrapeptide substrates with ACC fluorescent tag. Finally, we describe kinetic analysis of caspase fluorogenic substrates.

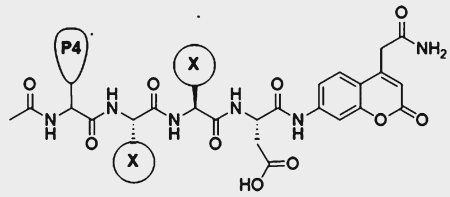
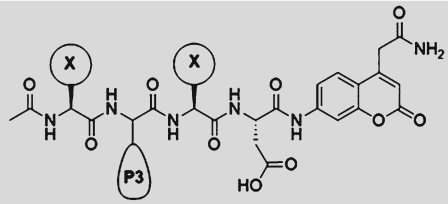
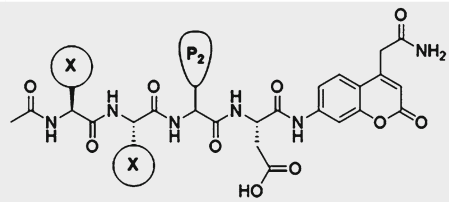
2 Materials

2.1 Caspase Profiling by SCL

1. Combinatorial library of tetrapeptides conjugated with fluorescent tag (AMC or ACC) and composed of the sublibraries (*see* Table 1):
   - P4 sublibrary: Ac-Aaa-Mix-Mix-Asp-ACC (or AMC).
   - P3 sublibrary: Ac-Mix-Aaa-Mix-Asp-ACC (or AMC).
   - P2 sublibrary: Ac-Mix-Mix-Aaa-Asp-ACC (or AMC).

   The sublibraries are dissolved in peptide grade DMSO to the final concentration of 5 mM (*see* **Notes 1, 2**).

Table 1
Complete tetrapeptide-ACC libraries for studying P4-P2 substrate specificity of caspases

Library	Structure	Number of sublibraries	Compounds
P4		19	361
P3		19	361
P2		19	361

(X) Equimolar mixture of natural amino acids (cysteine was omitted and methionine was replaced by norleucine)

(Px) Fixed natural amino acid residue

2. Human caspases expressed according to standard procedures described in literature [8, 13, 26, 27] or purchased from commercial sources (*see* **Notes 3, 4**).
3. Standard caspase reaction buffer: 20 mM pipes, 100 mM NaCl, 10 % (w/v) sucrose, 10 mM DTT, 1 mM EDTA, 0.1 % (w/v) CHAPS, pH 7.2. Sometimes, caspase buffer can be supplemented with 0.7–0.75 M sodium citrate (kosmotropic salt) to increase caspase activity [28] (*see* **Notes 5, 6**).
4. Plate reader to monitor fluorescence upon substrate hydrolysis (e.g., Molecular Devices SpectraMax Gemini).
5. Round-bottom 96-well plates suitable for plate reader (e.g., Corning® 96-well plates, opaque bottom).
6. A set of single channel pipettes with different capacity and one multichannel pipette for delivering 8 × 100 µl.
7. 15 ml Falcon tubes for caspase incubation.
8. Reagent reservoir (50 ml size) for 8 channel pipettes.
9. 37°C incubator.
10. Vortex mixer.

2.2 Synthesis of ACC-Conjugated Tetrapeptide Substrates

1. Rink amide RA resin, particle size 200–300 mesh, loading 0.48 mmol/g (*see* **Note 7**).
2. 7-Fmoc-aminocoumarin-4-acetic acid, purity >98 %, commercial or synthesized according to the method described by Maly et al. [20].
3. Fmoc-protected amino acids: Fmoc-Asp(O-*t*-Bu)-OH, Fmoc-Glu(O-*t*-Bu)-OH, Fmoc-Val-OH, purity >99 %.
4. *N*-Hydroxybenzotriazole, HOBt, purity >98 %, commercial or synthesized according to the method described by Fu et al. [29].
5. *N,N′*-Diisopropylcarbodiimide, DICI, peptide grade.
6. *N,N′*-Diisopropylethylamine, DIPEA, peptide grade.
7. *O*-Benzotriazole-*N*, *N*, *N′*, *N′*-tetramethyluronium-hexafluorophosphate, HBTU, peptide grade.
8. 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uranium hexafluorophosphate methanaminium, HATU, peptide grade.
9. 2,4,6-Trimethylpyridine, collidine, peptide grade.
10. *N,N′*-Dimethylformamide, DMF, peptide grade.
11. Dichloromethane, DCM, pure for analysis.
12. Methanol, MeOH, pure for analysis.
13. Acetonitrile, ACN, HPLC gradient grade.
14. Diethyl ether, Et_2O, pure for analysis.
15. Piperidine, PIP, purity >99 %.
16. Cleavage mixture: 1.9 ml trifluoroacetic acid (TFA, purity 99 %), 50 µl triisopropylsilane (TIPS, purity 99 %), and 50 µl H_2O.
17. Distilled H_2O.
18. Acetic acid, AcOH, purity >98 %.
19. Phosphorus pentoxide, P_2O_5, purity 98 %.
20. Vacuum line with trap.
21. Ten milliliter solid phase peptide synthesis vessel (e.g., Chemglass).
22. Microcentrifuge.
23. Shaker.
24. High-performance liquid chromatography system (HPLC).
25. Lyophilizer.

2.3 Kinetic Analysis (K_M, k_{cat}/K_M, k_{cat}) of Caspase Fluorogenic Substrates

1. Individual tetrapeptide caspase substrate (e.g., Ac-DEVD-ACC for caspase 3 assay) dissolved in peptide grade DMSO to the final concentration of 10, 25, or 50 mM (*see* **Notes 1, 2, 8**).
2. Other materials as described in section 2.1 under points 2–10.

3 Methods

3.1 Caspase Profiling by SCL

1. To ensure that the caspase of interest will display high enough activity during kinetic assay, each of caspase activity (P4-P2) should be tested in the initial screening. To perform the screening, prepare several samples in caspase buffer with different enzyme concentrations ranging from 1 to 500 nM (e.g., 1, 5, 25, 100, and 500 nM). Next, select several substrates from one sublibrary (start from P4) that are known or expected to be good caspase substrates.
2. Perform the initial caspase activity screening (*see* **Notes 1**, **9–11**). Caspase should be preactivated in the assay buffer for 10 min at 37°C [28]. The combinatorial fluorogenic substrates tailored for caspases should be tested at the final concentration of 50 μM. If library substrates are dissolved in DMSO to the final concentration of 5 mM, vortex and spot 1 μl of selected substrates in each well of a 96-well plate and to each well add 99 μl of caspase-containing buffer starting from the lowest caspase concentration (e.g., from 1 to 500 nM) using multichannel pipette. For this caspase, samples with different concentrations should be placed in five separate reagent reservoirs. The general outline of the initial screening is presented in Fig. 2.
3. Monitor proteolytic reaction on a plate reader. Read fluorescence every 15 or 30 s for 15–60 min with excitation 355 nm and emission 460 nm (the overall time of the assay depends on the caspase activity).
4. Appropriate caspase concentration is when the best substrate gives 50–100 relative fluorescence units (RFU) per sec (RFU/s). Under these conditions even very poor caspase substrate (around 1 % of the best one) can be detected during

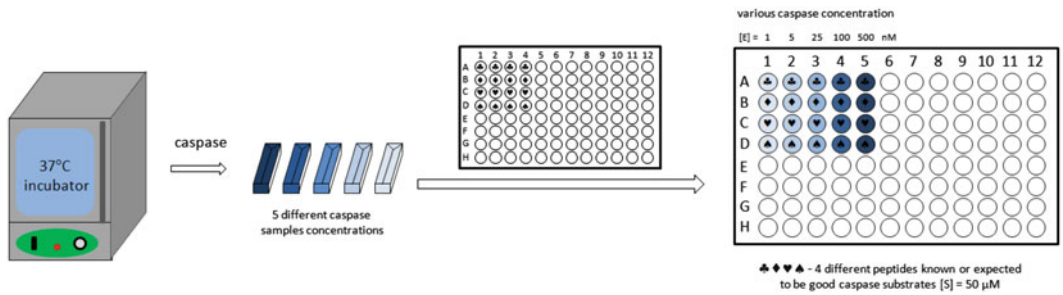


Fig. 2 The initial screening for determining optimal caspase concentration for a particular sublibrary screening. Several substrates from the combinatorial library have been selected, and their concentration was held constant (50 μM) during the whole assay. Caspase has been tested in five different concentrations (from 1 to 500 nM). Such a wide range of enzyme concentrations will enable the identification of the optimal enzyme concentration for each library assay. Characters *clubs*, *diamonds*, *hearts*, and *spades* represent four different substrates selected from the library

the assay (its RFU/s will be in the range 0.5–1.0). Substrates displaying RFU/s lower than 1 % of that of the best caspase substrate(s) cannot be considered to be caspase substrates. Note that RFU/s value depends on several factors, such as fluorescent tag, excitation and emission wavelengths, type of spectrofluorimeter, and software. The RFU/s values presented above were obtained with ACC tag, excitation 355 nm, emission 460 nm, Molecular Devices SpectraMax Gemini XPS, and Soft Max Pro 5 software.

5. Repeat the initial caspase activity screening for the P3 and P2 sublibraries separately (**steps 2–4**). In most cases all three sublibraries (P4, P3, and P2) can be screened with the same caspase concentration; however, there are some exceptions (e.g., caspase 9 screening).
6. After selecting the optimal caspase concentration for each sublibrary, perform the kinetic analysis of the whole CSL. Start with P4 sublibrary. One 96-well plate can accommodate four independent 19-membered sublibraries screenings (first set—columns 1–3, second set—columns 4–6, third set—columns 7–9, fourth set—columns 10–12). However, it is beneficial to perform only one experiment (one sublibrary) at a time. This is because pipetting takes a while and if we run too many columns in parallel, some very active substrates from the first columns might be already significantly hydrolyzed before we start an assay. To profile the caspase substrate specificity in P4 position, prepare the enzyme sample with the concentration established in **steps 2–4**: 3 columns × 8 wells × 99 µl = 2,378 µl (*see* **Note 12**). Incubate the enzyme sample in a 15 ml Falcon tube at 37 °C for 10 min (*see* **Note 11**).
7. In parallel, prepare the P4 sublibrary substrates. If the substrates are dissolved in DMSO to the final concentration of 5 mM, vortex and spot 1 µl of substrate into wells of the 96-well plate (columns 1–3). The pattern of spotting substrates from the P4 sublibrary on the 96-well plate is presented in Fig. 3.

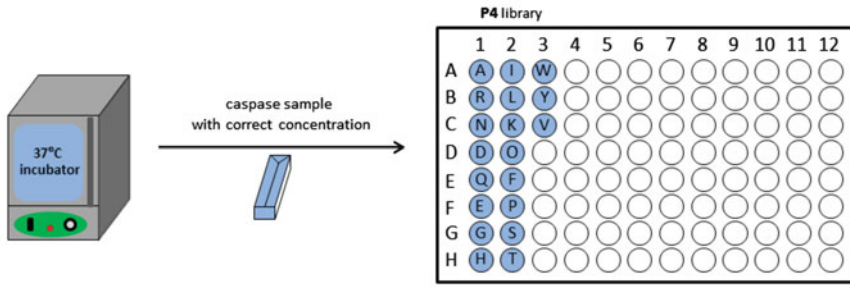


Fig. 3 The pattern of the P4 sublibrary substrates spotting on the 96-well plate. *Single letters* denote different amino acids

8. After 10 min of incubation, transfer the enzyme sample from the 15 ml Falcon tube to the reagent reservoir and add 99 μl in each well with substrates using 8-channel pipette. Mix well and run the experiment.
9. Monitor proteolytic reaction on a plate reader. Read fluorescence every 15 or 30 s for up to 60 min with excitation 355 nm and emission 460 nm. Stop reading when the very poor substrates will produce signal strong enough to be detected by the plate reader. For each substrate, extract only the linear part of the plot (*see* **Note 13**). In many cases it is possible to select the same time interval from hydrolysis curves for all substrates. However, sometimes each substrate hydrolysis should be considered separately, because the time range for linear plot differs among substrates and it is shorter for good substrates and longer for poor substrates. Figure 4 shows how to select the correct time range for various substrates.
10. To obtain the substrate specificity of a particular caspase in P4 position, analyze the data from kinetic assays. Find the best substrate with the highest RFU/s value and set this value as 100 %. Normalize cleavage rates of other substrates (in %) to that of the best substrate.
11. Repeat P4 sublibrary screening two more times (**steps 6–10**) and calculate the means and standard deviations for different substrates. If the standard deviation for any of the substrates is higher than 10 % of the mean, the assay must be repeated.
12. An alternative strategy to determine caspase substrate specificity is a simultaneous screening of P4, P3, and P2 sublibraries on a single 96-well plate. Note that for most caspases, the same enzyme concentration can be used for P4, P3, and P2 sublibraries profiling; however, there are some exceptions (e.g., caspase 9). The general idea of the approach with different enzyme concentrations is presented in Fig. 5. In any case, each experiment should be repeated at least two times.
13. To obtain substrate specificity profiles of particular caspase in P3 and P2 positions, repeat **steps 8–11**.
14. Select most active or specific amino acid in P4, P3, and P2 position (P1 is fixed with Asp) and proceed with synthesis of optimal tetrapeptide substrate (*see* **Note 14**) (Fig. 6).

3.2 Synthesis of ACC-Conjugated Tetrapeptide Substrates (See Fig. 7)

3.2.1 ACC-Resin Synthesis

1. Swell 0.048 mmol (100 mg) of Rink amide AM resin in a 5 ml DCM in a solid phase peptide synthesis vessel for 1 h with gentle stirring, once per 10 min to make functional group accessible [20] (*see* **Note 7**).
2. Remove DCM by vacuum filtration.
3. Wash the resin thoroughly with DMF, three times with 5 ml aliquots.

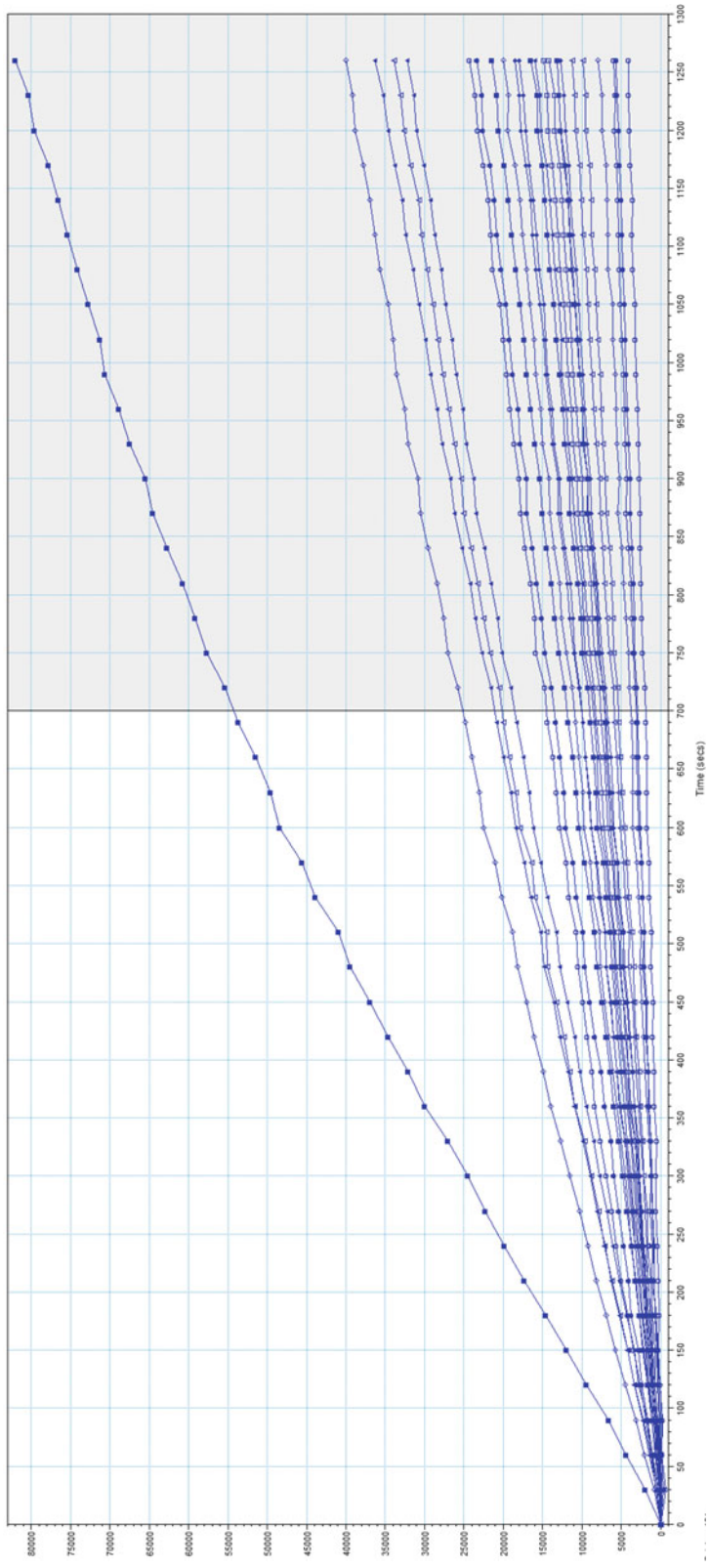


Fig. 4 The general scheme for how the linear part of the plot from library screening should be selected

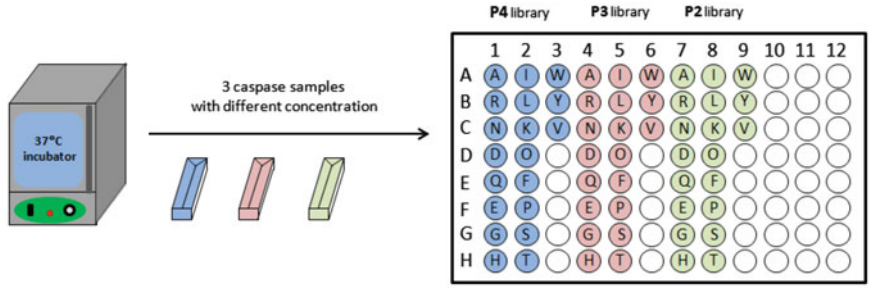


Fig. 5 The pattern of P4-P2 SCL spotting on the 96-well plate. This pattern is an alternative to the one presented in Fig. 3

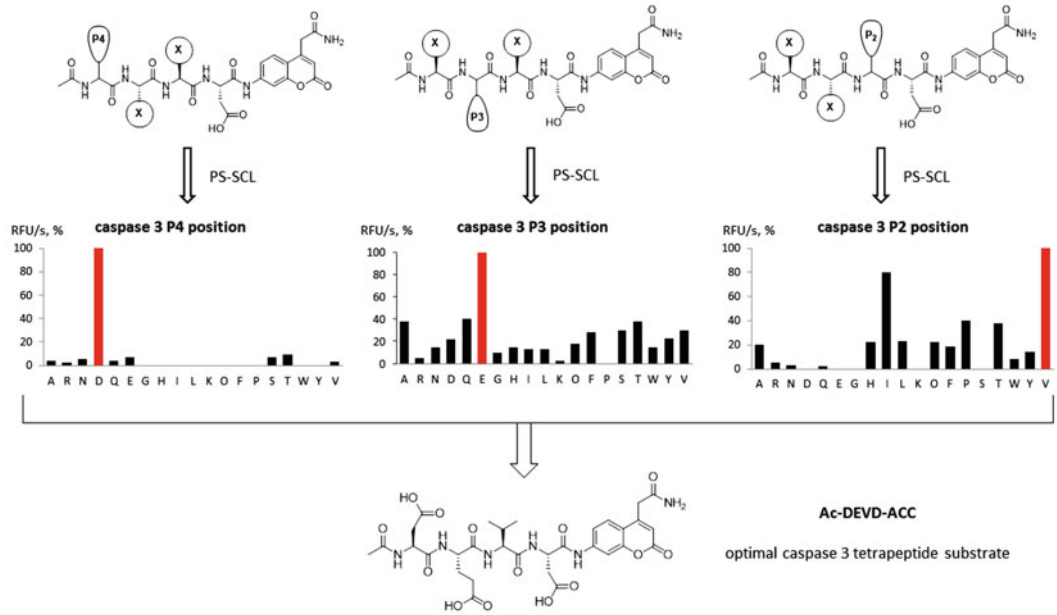


Fig. 6 Selection of optimal amino acids in P4-P2 positions from PS-SCL screening

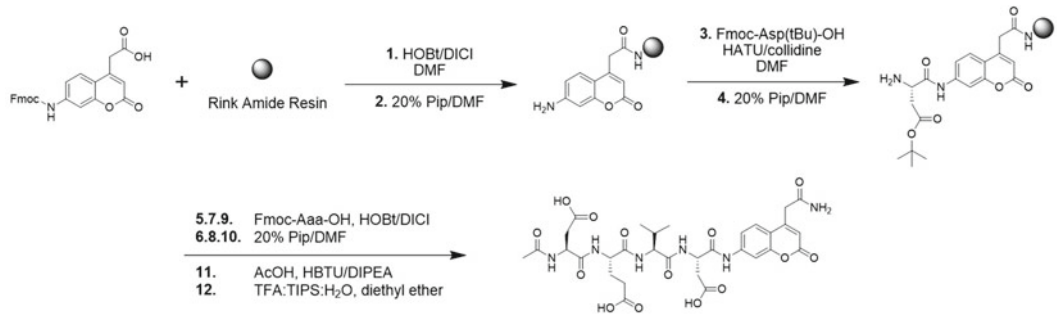


Fig. 7 Scheme for synthesis of Ac-DEVD-ACC. The numbers from *1* to *12* indicate successive steps of the synthesis. (*1*) Fmoc-ACC-OH coupling to the resin; (*2*, *4*, *6*, *8*, and *10*) Fmoc group deprotection; (*3*) Coupling the first amino acid to the NH_2-ACC-resin; (*5*, *7*, and *9*) peptide chain elongation; (*11*) acetylation of NH_2-tetrapeptide-ACC; (*12*) substrate cleavage from the resin (removing protecting groups from the substrate) and substrate precipitation

4. Remove *N*-Fmoc groups by incubating the resin in 5 ml of 20 % piperidine in DMF for 5, 5, and 30 min. Each time, agitate gently and remove solution by vacuum filtration [30] (*see* **Note 15**).
5. Wash the resin three times with 5 ml aliquots of DMF, then three times with 5 ml aliquots of DCM, and again three times with DMF. Agitate gently and remove solution each time.
6. Transfer a few resin beads to a glass test tube and perform a ninhydrin test to ensure that amine groups of resin are free (beads color should change from yellow to dark blue). If test is positive (beads are dark blue), proceed with synthesis, if negative (beads are yellow), repeat **steps 4–6** until the test is positive [31] (*see* **Note 16**).
7. Add Fmoc-ACC-OH (0.144 mmol, 63.5 mg), HOBt (0.144 mmol, 21.6 mg), and DICI (0.144 mmol, 22.55 μl) to 1 ml of DMF in an Eppendorf tube. After 5 min activation of functional groups, transfer solution to synthesis vessel with resin and incubate for 24 h with slow agitation [20] (*see* **Note 17**).
8. Filter the resin and wash three times with DMF. Then repeat **step 7** with a half amount of reagents to increase coupling efficiency.
9. Remove reaction mixture by vacuum filtration and wash resin three times with DMF. Test for unreacted amine by ninhydrin test. If test is positive (beads are dark blue), repeat **step 8**, if negative (beads are yellow), proceed with **step 10** [31] (*see* **Note 16**).
10. To cap any unreacted resin, add 3 molar equivalents of acetic acid (0.144 mmol, 8.23 μl), DIPEA (0.144 mmol, 25.08 μl), and HBTU (0.144 mmol, 54 mg) in 1 ml of DMF in an Eppendorf tube. After 1 min of preactivation, transfer capping mixture to the resin and shake gently for 1 h [20].
11. Filter resin and wash three times in 5 ml aliquots of DMF.
12. To remove the *N*-Fmoc group from ACC-resin, repeat **steps 4–6** (positive ninhydrin test for free amine groups of ACC should yield dark red color; *see* **Note 16**).

3.2.2 NH_2-Asp-ACC-Resin Synthesis

1. Add 3 molar equivalents of each of Fmoc-*L*-Asp(O-*t*-Bu)-OH (0.144 mmol, 59.26 mg), HATU (0.144 mmol, 54.72 mg), and collidine (0.144 mmol, 18.74 μl) in 1 ml of DMF to an Eppendorf tube. After 1 min of preactivation, transfer mixture to the reaction vessel containing NH_2-ACC-resin and incubate for 24 h with gentle shaking (*see* **Note 17**).
2. Filter the resin and wash three times with 5 ml aliquots of DMF.
3. Repeat **step 1** using half amount of reagents to increase coupling efficiency and then repeat **step 2**.

4. Add 3 ml of 20 % piperidine in DMF to synthesis vessel and gently agitate for 5 min. Remove piperidine solution and then repeat deprotection treatment for 5 min and 30 min while agitating [32] (*see* **Note 15**).
5. Remove piperidine solution and wash three times with 5 ml aliquots of DMF, three times with 5 ml aliquots of DCM, and again three times with 5 ml aliquots of DMF.
6. Check for the presence of free amine group by ninhydrin test. If the test is negative (beads are yellow), repeat **steps 4–5**, if positive (beads are blue), continue with synthesis [31].

3.2.3 Peptide Chain Elongation: P2-P4 Positions Coupling [33, 34]

1. Add 3 molar equivalents of each Fmoc-*L*-Val-OH (0.144 mmol, 48.87 mg), HOBt (0.144 mmol, 22 mg), and DICI (0.144 mmol, 22 µl) in 1 ml to an Eppendorf tube. After 3 min of preactivation, transfer mixture to the reaction vessel containing NH_2-Asp-ACC-resin and incubate for 3 h with gentle shaking (*see* **Note 17**).
2. Filter the resin and wash three times with 5 ml aliquots of DMF.
3. Transfer a few resin beads to a test tube and perform ninhydrin test. If test is positive (beads are blue), repeat **steps 1–3**. If test is negative, proceed with **step 4** [31].
4. Remove Fmoc-protecting groups by incubating with three aliquots of 20 % piperidine in DMF for 5, 5, and 30 min removing piperidine solution each time [32] (*see* **Note 15**).
5. Wash resin three times with 5 ml aliquots of DMF, three times with 5 ml aliquots of DCM, and again three times with 5 ml aliquots of DMF.
6. Perform ninhydrin test. If test is positive (beads are blue), proceed with **step 7**. If test is negative (beads are yellow), repeat **steps 4–6** [31].
7. To substitute P3 position, repeat **steps 1–6**, but replace Fmoc-*L*-Val-OH by 3 equivalents of Fmoc-*L*-Glu(O-*t*-Bu)-OH (0.144 mmol, 64 mg).
8. To substitute P4 position, repeat **steps 1–6**, but replace Fmoc-*L*-Val-OH by 3 equivalents of Fmoc-*L*-Asp(O-*t*-Bu)-OH (0.144 mmol, 59 mg).
9. Protect free N-terminal amino group of resulting NH_2-DEVD-ACC-resin with acetyl group. Add 3 equivalents of acetic acid (0.144 mmol, 8.23 µl), DIPEA (0.144 mmol, 25.08 µl), and HBTU (0.144 mmol, 54 mg) in 1 ml of DMF to Eppendorf tube and activate for 1 min. Then transfer capping mixture to the vessel with resin and incubate with gentle agitation for 1 h.
10. Remove capping mixture and wash resin 3 times with 5 ml aliquots of DMF.

11. Transfer a few resin beads to a test tube and perform ninhydrin test. If test is positive (beads are blue), repeat **steps 9–11**. If test is negative (beads are yellow), proceed with **step 12** [31].
12. Wash resin three times with 5 ml aliquots of DCM and three times with 5 ml aliquots of methanol.
13. Dry resin over P_2O_5 under vacuum for 5–12 h.
14. Cleave peptide from resin by incubating in cleavage mixture with gentle mixing (*see* **Note 18**).
15. Collect the cleaved substrate in plastic tube and precipitate in 12 ml of cold diethyl ether for 30 min. The general scheme of Ac-DEVD-ACC synthesis is presented in Fig. 7.
16. Centrifuge precipitate at 3,000 × *g* for 5 min at 4 °C and remove supernatant. Wash pellet with 5 ml of diethyl ether, shake and centrifuge again at the same conditions. Remove supernatant. Dry crude peptide substrate on air.
17. Dissolve substrate in 1 ml of DMSO, purify on reverse phase HPLC (acetonitrile/water), and lyophilize.
18. Dissolve peptide in DMSO to final concentration of 50, 20, or 10 mM (*see* **Note 8**).
19. Store at −80 °C until use (*see* **Note 1**).

3.3 Kinetic Analysis (K_M, k_{cat}/K_M, k_{cat}) of Caspase Fluorogenic Substrates

1. The small molecule substrates of caspase are usually tetrapeptides conjugated with fluorophore [13, 14, 35]. To determine the kinetic parameters of a substrate, it is necessary to establish how much fluorescence signal (RFU) is produced by a certain amount of fluorophore. For this, vortex and spot 1 μl of 1 mM good caspase substrate in the well of the 96-well plate and add 99 μl of buffer containing active caspase (the final concentration of substrate in the well is 10 μM). Monitor proteolytic reaction on a plate reader. Read fluorescence every 10–15 s with excitation 355 nm and emission 460 nm. The time of measurement depends on the enzyme concentration, and the time after which all substrate is hydrolyzed can range from 30 min to 2 h. After all substrate is hydrolyzed, take the maximum RFU (not RFU/s) value and divide it by 10 to obtain the amount of fluorescence produced by 1 μM fluorophore.
2. To measure K_M for a selected substrate, prepare its serial dilution. Take the substrate stock in DMSO, vortex it, and dilute in assay buffer to known concentration (e.g., 500 μM for Ac-DEVD-ACC). Prepare at least 60 μl of this substrate. Next, vortex the sample, take 60 μl of this substrate, and transfer it into A1 well of 96-well plate. To the other wells (B1 to H1), add 20 μl of assay buffer. Next, transfer 40 μl of substrate from well A1 to B1 and mix it several times using single channel pipette. Then, transfer 40 μl of substrate from well B1 to C1

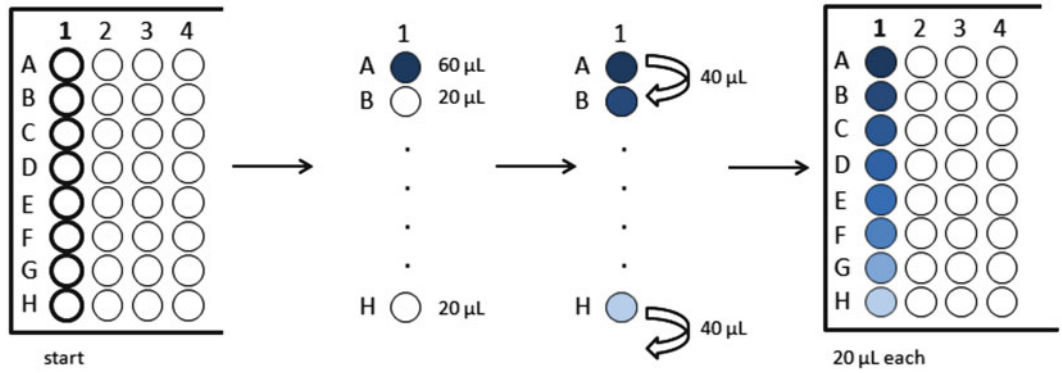


Fig. 8 The general outline of how serial dilution for K_m measurement should be prepared

and mix it several times. Repeat this procedure until the last well is filled with diluted substrate. Mix solution in H1 well and discard 40 µl. Now all wells in the first column contains 20 µl serial dilutions of Ac-DEVD-ACC in various concentration (from 500 µM to 29.3 µM; concentration of substrate in a given well is 2/3 of the concentration in the previous well) (*see* **Note 19**) (Fig. 8).

3. Prepare enzyme sample. For one column of wells, 640 µl of enzyme in buffer is required, but for comfortable pipetting, prepare around 700 µl (*see* **Note 12**). The enzyme concentration depends on the substrate (e.g., for assay with Ac-DEVD-ACC, the 0.5–1.0 nM solution of caspase 3 is usually used).
4. Transfer enzyme to a reagent reservoir and add 80 µl to each well using 8-channel pipette.
5. Monitor proteolytic reaction on a plate reader. Record fluorescence every 10–15 s with excitation 355 nm and emission 460 nm for 15–60 min. Calculate RFU/s for each well (from A1 to H1) using linear part of the plots.
6. Draw Michaelis-Menten plot: *y* axis is RFU/s or µM of ACC/s and *x* axis is substrate concentration. Calculate from the plot three kinetic parameters: K_M, k_{cat}/K_M, and k_{cat}. Substrate concentration range is often mismatched. When there are too many points on the plateau, decrease substrate concentration in the next experiment. On the other hand, if none of points achieve the V_{max}, the substrate concentration range should be shifted to higher values.
7. When the substrate concentration range is properly selected, repeat this experiment at least two times and present the results as average values with standard deviation. It is beneficial to carry out several experiments on one 96-well plate, but always calculate the total volume of caspase-containing buffer required for comfortable pipetting.

8. If K_M experiment has been carried out at least three times, it is possible to calculate three main substrate kinetic parameters K_M, k_{cat}/K_M, and k_{cat}. K_M can be determined directly by transferring obtained data into GraphPad Prism software (XY analyses → nonlinear regression (curve fit) → enzymes kinetic → Michaelis-Menten). In addition to the K_M value, one can also obtain a V_{max} value expressed in RFU/s. To calculate k_{cat}, divide V_{max} by the amount of fluorescence produced by 1 μM fluorophore (*see* **step 1**) and enzyme concentration used in the assay. Finally, calculate k_{cat}/K_M.

4 Notes

1. All substrates containing a fluorescent tag (e.g., ACC, AMC of AFC) are light sensitive so try to use dark-colored Eppendorfs. If you use transparent Eppendorfs, make sure that substrates are not exposed to UV light for a long time (use aluminum foil or hide tubes in a dark place when you are not using them at the moment).
2. P4, P3, and P2 sublibraries, as well as individual substrates (powders or DMSO solutions), should be stored at −80°C. Under such conditions they can be stored for up to several years.
3. Recombinant caspases can be stored at −80°C for several years.
4. Caspases are not very stable enzymes at higher temperatures, so after thawing they need to be stored at 4°C and used for kinetic assay within several hours. Therefore, it is a good practice to prepare many small (10–50 μl) aliquots of recombinant enzymes after expression.
5. Caspase reaction buffer contains DTT, so it needs to be prepared just before kinetic assays. We recommend not to store assay buffer at 4°C overnight. Freshly prepared buffer ensures good repeatability of results.
6. Different caspases have different optimal conditions for catalysis. The buffer presented here can be used for all caspases, but it is not the optimal one. Optimal buffers for individual caspases can be found elsewhere [14, 28, 36].
7. If the resin appears to be clammy, dry it overnight in the vacuum over P_2O_5.
8. Use only peptide grade DMSO to dissolve caspase substrates. Avoid long-stored open-bottle DMSO, because it is hygroscopic and this can lead to sample dilution.

9. Combinatorial libraries and individual substrates should be brought from −80°C to room temperature very slowly (over at least 3 h), without additional heating (i.e., in 37°C incubator).
10. All caspase substrates contain Asp residue in the P1 position and are well soluble in DMSO or water buffers at room temperature, so there is no need to warm them up.
11. For incubation, recombinant caspase should be added to the buffer heated up to 37°C, otherwise 10-min incubation of caspase in a large volume of buffers (10–20 ml) will not be sufficient to achieve 37°C.
12. Always prepare larger volume of enzyme in assay buffer than necessary to ensure comfortable pipetting.
13. It is very important to identify the linear part of the plot. However, there is no common range of time, which should be chosen. All sublibraries are scanned at the final concentration of 50 μM to ensure that velocity data are proportional to k_{cat}/K_m. Good substrates are hydrolyzed with a high rate (RFU/s = 50–100 units), so in this case only the first 5 min (or even shorter) period of reaction is enough for the kinetic analysis. Poor substrates are hydrolyzed much slower (RFU/s = 0.5–1.0) and require more time (>30 min) to produce a linear part of the plot.
14. SCL with Asp at the P1 position (Ac-X-X-X-Asp-ACC) can be used to determine substrate specificity profiles of almost all caspases from different organisms following above described protocol. However, some enzymes assigned to caspases family do not recognize aspartic acid in the P1 position of their substrates. A good example is paracaspase MALT1, which displays strict arginine specificity [24]. The P4-P2 specificity of MAL1 can be profiled using above described protocol; however, a different SCL is required (Ac-X-X-X-Arg-ACC).
15. Piperidine deprotection can lead to side reactions, such as diketopiperazine and aspartimide formation. Removing all piperidine solution is therefore critical. On the other hand, poor deprotection results in slow or incomplete coupling, thus repeated piperidine treatment helps to overcome the problem.
16. Primary amines condensed with ninhydrin can be detected by ninhydrin test (formed Schiff base, dark blue color, NH_2-amino acids). However, NH_2-ACC is an aromatic amine, which is detected by ninhydrin test by an appearance of orange to red color.
17. It is important to gently shake resin in vessel, because beads are susceptible to rubbing. Improper handling of resin during reaction can significantly decrease yield.

18. Silane derivative (TIPS) shows good efficacy in quenching carbocations. Cleavage should be completed in 2–3 h at room temperature to minimize side reactions.
19. Note that during K_M measurement experiments substrate concentration in particular well after serial dilution is not the final concentration during the assay, because substrate (20 μl) will be diluted 5 times with the enzyme-containing buffer (80 μl).

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