limited proteolysis protocol

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Materials

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| **protease** | **Sigma-Aldrich catalog number** | **notes** |
| trypsin | T1426 | TPCK treated |
| chymotrypsin | C3142 | TLCK treated |
| thermolysin | P1512 | may be substantially contaminated with other enzymes |
| V8 (endoproteinase Glu-C) | P2922 |  |
| endoproteinase Asp-N | P3303 |  |
| endoproteinase Arg-C | P6056 |  |
| endoproteinase Lys-C | P3428 |  |

Lyophilized proteases should be stored desiccated at 4 °C or -20 °C as recommended by the manufacturer.

Preparation of proteases

1. Prior to preparing solutions, allow lyophilized proteases to warm to room temperature to prevent condensation on the surface of the powder.
2. Follow the manufacturer’s protocol for resuspension of the protease (if available) and proceed to step 5. If no instructions for resuspension are provided, follow steps 3-5.
3. Weigh out protease into a tared micro-centrifuge tube and transfer to ice.
4. Add the appropriate amount of ice-cold protease-specific storage buffer and resuspend the protease thoroughly but gently- avoid foaming.
5. Divide the protease solution into working aliquots, snap freeze in liquid nitrogen, and store at -80 °C.
   1. For the most reproducible results, do not freeze and thaw the protease multiple times.

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| **protease** | **recommended storage buffer** | **recommended concentration (mg/mL)** |
| trypsin | 1 mM HCl, 20 mM CaCl2 | 5 |
| chymotrypsin | 1 mM HCl, 2 mM CaCl2 | 5 |
| thermolysin | 20 mM CaCl2 | 5 |
| V8 (endoproteinase Glu-C) | ddH2O | 1 |
| endoproteinase Asp-N | 10 mM Tris (8.0) | 0.04 |
| endoproteinase Arg-C | 1 mM HCl | 0.1 |
| endoproteinase Lys-C | 50 mM Tricine, 10 mM EDTA (8.0) | 0.1 |

Limited proteolysis of your favorite protein

1. Quickly thaw the stock solution of protease and transfer to ice.
2. Make the appropriate serial dilutions of the protease using the same buffer the protease was frozen in (**please see *Important Notes*, below**). Prepare your dilutions such that the same volume of protease will be added to each reaction.
3. Quickly thaw your protein and transfer to ice.
4. Dilute your protein as necessary- ideally with the same buffer it was frozen in.
5. Combine all reaction components except the protein and protease and keep on ice. A final volume of 25 L is recommended, but this can be scaled as necessary.
   1. Reasonable controls include (**i**) your protein without any protease and (**ii**) protease without any of your protein- use the highest amount of protease that you used to digest your protein.
6. Add your protein to each reaction tube.
7. Working quickly and still on ice, add the appropriate amount of protease to each tube and mix gently by pipette.
8. Transfer all reactions to a water bath at the appropriate temperature, or keep on ice.
9. After the desired incubation time, stop the reaction by adding SDS-PAGE sample buffer, and boil the samples for 5 minutes.
   1. Note that many proteases retain some activity in the presence of reducing agents and SDS. They also have varying degrees of resistance to thermal denaturation. Therefore, it is advisable to load the samples onto an SDS-PAGE gel as soon as possible. If necessary, the boiled samples can be stored at -20 °C overnight. Storage of samples for longer periods is not recommended.

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| **protease** | **recommended reaction buffer** | **notes** |
| trypsin | 100 mM Tris (8.5) |  |
| chymotrypsin | 100 mM Tris (8.5), 10 mM CaCl2 |  |
| thermolysin | 200 mM ammonium acetate (8.5), 5 mM CaCl2 |  |
| V8 (endoproteinase Glu-C) | 100 mM ammonium bicarbonate (8.0) | to extend cleavage to the C-terminal side of Asp, use 100 mM sodium phosphate (7.8) |
| endoproteinase Asp-N | 100 mM ammonium bicarbonate (8.5) |  |
| endoproteinase Arg-C | 100 mM ammonium bicarbonate (8.5) |  |
| endoproteinase Lys-C | 100 mM ammonium bicarbonate (8.5) |  |

***Important Notes***

The optimal conditions for generating proteolytic fragments are quite protein-specific. Therefore, most parameters of the experiment must be determined empirically for each protein-protease pair, including: (**i**) the amount of protein in the reaction, (**ii**) the amount of protease, (**iii**) the length of digestion, and (**iv**) the temperature. Included below are some ***suggestions*** to guide initial experiments. These parameters will likely need to be refined for your particular protein, but should serve as a reasonable starting point.

1. **protein amount**: you need enough starting material to ensure that each of your proteolytic fragments is stained reasonably well with Coomassie Brilliant blue. As a general guideline, each reaction should contain 10X the amount of intact protein you need to see a well-stained band after SDS-PAGE. For example, 0.5 g of PLC-1 is readily detectable on a gel, so I use 5 g of protein per reaction.
2. **protease amount**: a reasonable starting range of protease:protein ratios is 1:50 – 1:10,000 (w/w).

**(iii) and (iv)**: **time and temperature**

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| **protease** | **recommended temperature** | **recommended incubation time** |
| trypsin | on ice | 1 hour |
| chymotrypsin | on ice | 1 hour |
| thermolysin | 25 °C | 1 hour |
| V8 (endoproteinase Glu-C) | 30 °C | 2 hours |
| endoproteinase Asp-N | 30 °C | 2 hours |
| endoproteinase Arg-C | 30 °C | 2 hours |
| endoproteinase Lys-C | 30 °C | 2 hours |

References

1. Carey, J. (2000) A systematic and general proteolytic method for defining structural and functional domains of proteins. *Methods in Enzymology* 328, 499-514.
2. Kleifeld, O. *et al*. (2010) Isotopic labeling of terminal amines in complex samples identifies protein N-termini and protease cleavage products. *Nature Biotechnology* 28, 281-288.
3. Ambler R. P. and Meadway R. J. (1968) The use of thermolysin in amino acid sequence determination. *Biochemical Journal* 108, 893-895.