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Limited Proteolysis of Clu and Clu-DMPC Complexes

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We use this protocol and it's working

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Abstract

This protocol details how to perform limited proteolysis with Chymotrypsin of free Clusterin (Clu) and Clu-1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) complexes followed by SDS-PAGE or Native-PAGE analysis.

Guidelines

Limited proteolysis is a structural biology technique used to probe proteins for flexible, protease-accessible regions, i.e., to identify ordered, protected fragments, often domains. Typically, unspecific proteases such as Proteinase-K or subtilisin are used. Amino acid specific proteases such as trypsin (cutting after Lys/Arg) or chymotrypsin (preferentially cutting after Phe/Tyr) may give results easier to interpret. To demonstrate differential protease sensitivity, either a series of protease concentrations or an incubation time series is used. The protease concentrations and incubation time/temperature have to be adjusted to visualize transiently protected protein fragments. The experiments are subsequently analyzed by SDS-PAGE analysis. In case of Clusterin, native PAGE analysis was useful to reveal removal of a small fragment. The identity of the protected fragments can be analyzed by mass spectrometry proteomics.

Materials

Buffers, reagents and consumables:

- 10x TBS-C buffer (10x TBS buffer ([M] 100 millimolar (mM) Tris-HCl pH 7.5 , [M] 1500 millimolar (mM) NaCl) containing [M] 10 millimolar (mM) CaCl_2)
- Phenylmethylsulfonyl fluoride (PMSF) stock ([M] 100 millimolar (mM) PMSF in ethanol)

Note

PMSF is toxic: Wear protective goggles and mask. PMSF is poorly soluble ($<$ [M] 1 millimolar (mM)) and prone to rapid hydrolysis in water. Store PMSF stock in a closed vessel at 4°C .

-  α -Chymotrypsin from bovine pancreas **Merck Catalog #C7762**
- Clusterin wildtype and TL4 mutant (see protocol "Clusterin purification from HEK293E cells", [dx.doi.org/10.17504/protocols.io.bvvkn64w](https://doi.org/10.17504/protocols.io.bvvkn64w)).
- Clusterin-DMPC lipoprotein particles
- NuPAGE 4–12% Bis-Tris SDS gels (Thermo Fisher Scientific)
- NuPAGE MES SDS running buffer (Thermo Fisher Scientific)
- 2x SDS-PAGE sample buffer ([M] 100 millimolar (mM) Tris-HCl pH 6.8 , [M] 0.2 Molarity (M) dithiothreitol (DTT), 4% sodium dodecyl sulfate (SDS),  2 g I-1 bromophenol blue and 20% glycerol)

Note

Prepare by mixing  1.54 g DTT, 0.10 g bromophenol blue,  10 mL glycerol,  5 mL [M] 1 Molarity (M) Tris-HCl pH 6.8 stock and  20 mL 10% (w/v) SDS stock. Top up with Milli-Q de-ionized water to  50 mL total volume. Make  1 mL aliquots and store at -20°C .

- Prestained molecular weight SDS-PAGE marker (PageRuler Prestained Protein Ladder, 10 to 180 kDa, Thermo Fisher Scientific)
-  NativePAGE 3%–12% Bis-Tris SDS gel **Thermo Fisher Scientific Catalog #BN1001BOX**
-  NativePAGE[®] Running Buffer (20X) **Thermo Fisher Catalog #BN2001**
-  NativePAGE[®] Sample Buffer (4X) **Thermo Fisher Catalog #BN2003**
-  NativeMark[®] Unstained Protein Standard **Thermo Fisher Catalog #LC0725**
- Coomassie blue staining solution



- Coomassie blue destaining solution

Instruments

- Microcentrifuge for 1.5 ml reaction tubes (Eppendorf)
- Vortex mixer (Vortex-Genie 2, Scientific Industries)
- Thermo mixer (Eppendorf Thermomixer Comfort)

Preparatory Work

- 1 Chymotrypsin stock: Dissolve approximately [M] 2 mg/mL bovine chymotrypsin in [I] 1 mL [M] 1 millimolar (mM) hydrochloric acid (HCl). Determine actual concentration by absorbance at 280 nm using an absorbance coefficient of 2.04 ml/mg/cm. The molecular weight of bovine chymotrypsin is 25,000 g/mol. Aliquot stock in 1.5 ml reaction tubes, snap-freeze aliquots in liquid nitrogen and store at [I] -70 °C . 
- 2 Prepare a concentration series of [M] 0.2 mg/mL , [M] 0.02 mg/mL , [M] 0.002 mg/mL and [M] 0.0002 mg/mL chymotrypsin (labels Ct/1E1, Ct/1E2, Ct/1E3, Ct/1E4, respectively) by serially diluting with ice-chilled 1x TBS-C. Store dilutions [I] On ice . 
- 3 Prepare each [I] 75 µL of [M] 20 micromolar (µM) Clusterin stocks of free Clusterin and Clusterin-DMPC lipoprotein complex by diluting with 1x TBS-C buffer. For clearer gel electrophoresis results, use Clusterin with oligo-mannose N-glycans, i.e., produced in presence of the α-mannosidase-I inhibitor kifunensine. Store [I] On ice . 
- 4 Set the thermostat in the thermomixer to [I] 25 °C . 

Limited Proteolysis

30m

- 5 Label 1.5 ml reaction tubes with C0-C6 (Clu) and CD0-CD6 (Clu-DMPC) (14 in total). Chill the tubes in an ice-cooled metal block.
- 6 Use the following pipetting scheme (in µl): Pre-mix 10x TBS-C and water before adding Clu or Clu-DMPC. Pipet the chymotrypsin dilutions into the tube caps. 

	A	B	C	D	E	F	G	H
	Name	Ct/1E 2	Ct/1E3	Ct/1E 4	Clu	Clu- DMPC	10xTBS- C	Water
	C6	1.25			10.00		2.00	6.75
	C5		5.00		10.00		2.00	3.00

	A	B	C	D	E	F	G	H
	C4		2.50		10.00		2.00	5.50
	C3		1.25		10.00		2.00	6.75
	C2			5.00	10.00		2.00	3.00
	C1			2.50	10.00		2.00	5.50
	C0				10.00		2.00	8.00
	CD6	1.25				10.00	2.00	6.75
	CD5		5.00			10.00	2.00	3.00
	CD4		2.50			10.00	2.00	5.50
	CD3		1.25			10.00	2.00	6.75
	CD2			5.00		10.00	2.00	3.00
	CD1			2.50		10.00	2.00	5.50
	CD0					10.00	2.00	8.00

7 Mix each sample by simultaneously spinning down the chymotrypsin solutions in a microcentrifuge.



8 Incubate the samples in the thermomixer at **25 °C** for **00:30:00**.

30m



9 Transfer the samples to an ice-chilled metal block.

10 Pipet **2 µL** PMSF stock into the tube cap of each sample.



11 Stop the limited proteolysis reactions by simultaneously spinning down PMSF in a microcentrifuge.



12 Vortex samples and keep **On ice**.



SDS-PAGE

51m



- 13 Label 1.5 ml reaction tubes with C0-C6 and CD0-CD6 (14 in total).
- 14 Prepare SDS-PAGE samples by mixing  5 μL sample with 2x SDS-PAGE sample buffer at 1:1 (v/v) ratio. Then heat SDS-PAGE samples to  95 °C for  00:05:00 in metal heating block, followed by cooling in a cold metal block and centrifugation for  14000 rpm, 00:01:00 in microcentrifuge.    
- Note**
- The DTT in the 2x SDS-PAGE sample buffer quickly perishes by air oxidation. Keep chilled  On ice ! To avoid Clusterin gel artifacts by incomplete reduction of its five disulfide bonds, do not re-use 2x SDS-PAGE sample buffer, once it was thawed.
- 15 Assemble electrophoresis apparatus with NuPAGE 4–12% Bis-Tris SDS gels. Fill inner and outer chamber with NuPAGE MES SDS running buffer.
- 16 Load NuPAGE 4–12% Bis-Tris SDS gels with SDS-PAGE samples ( 10 μL). Use pre-stained SDS-PAGE molecular weight marker as a standard. 
- 17 Run electrophoresis for  00:45:00 at a constant voltage of  200 V . 
- 18 Carefully pry open the plastic casing of the NuPAGE 4–12% Bis-Tris SDS gel by using a so-called gel knife (Invitrogen) or similar.
- 19 Place the gel into a staining dish and stain the protein bands with Coomassie-blue.

Native PAGE

1h 30m

20

Note

In case of Clusterin, this is a useful addition to SDS-PAGE.

Label 1.5 ml reaction tubes with C0-C6 and CD0-CD6 (14 in total).

- 21 Prepare native PAGE samples by mixing  15 μL sample with  5 μL native PAGE sample buffer (4x). Vortex and spin down in microcentrifuge.



- 22 Assemble electrophoresis apparatus with native PAGE 3–12% Bis-Tris gels. Fill inner and outer chamber with ice-chilled Native PAGE running buffer (1X).

Note

Native PAGE samples are faintly colored pink. Put a white paper towel underneath the apparatus to improve visibility during sample application.

- 23 Load native PAGE 3–12% Bis-Tris gels with native PAGE samples (each  15 μL). Use NativeMark unstained native protein standard ( 5 μL) as a marker.



- 24 Run electrophoresis for  01:30:00 at a constant voltage of  140 V .

1h 30m

- 25 Carefully pry open the plastic casing of the native PAGE 3–12% Bis-Tris gels by using a so-called gel knife (Invitrogen) or similar. Remove the loading pockets with a scalpel while the gel is still attached to the plastic.

Note

The native PAGE gradient gels are highly fragile and easily fold back onto itself. Getting rid of the loading pockets at the beginning simplifies handling.

- 26 Place the gel into a staining dish and stain the protein bands with Coomassie-blue.

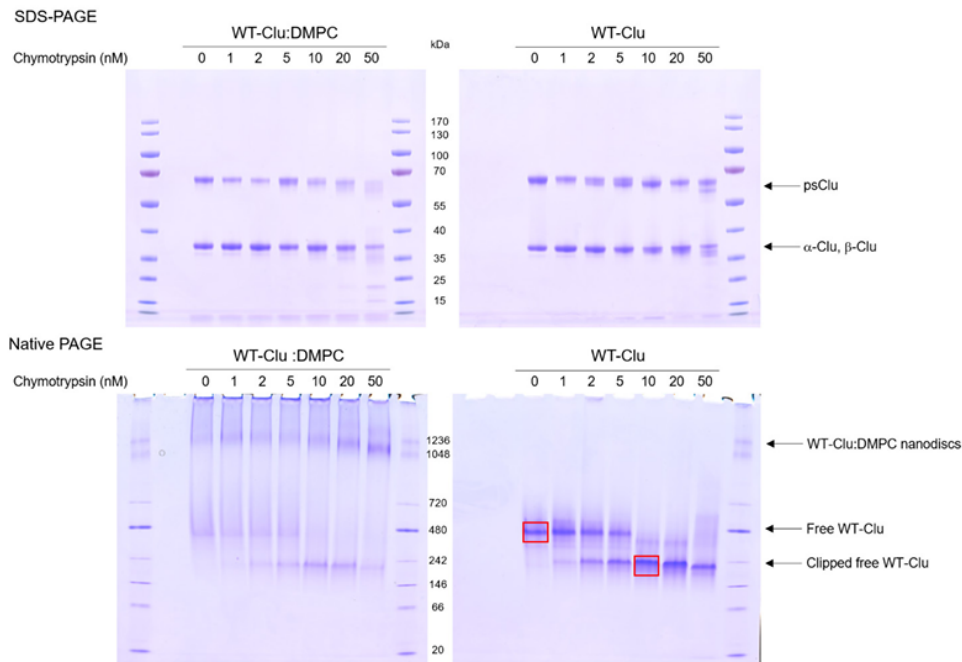


Fig. 1 SDS-PAGE (top) and native PAGE (bottom) analysis of limited Chymotrypsin proteolysis experiments of Clusterin lipoprotein complexes (WT-Clu:DMPC, left) and free Clusterin (Clu, right). The chymotrypsin concentration (nM) in the respective lane is shown above the gels; Clusterin concentration was 10 μ M. The bands for “pre-secretory Clusterin” (psClu), covalently linked Clusterin α and β chains, Clusterin α and β chains (α -Clu, β -Clu, these bands are overlapping), Clusterin lipoprotein complexes (WT-Clu:DMPC nanodiscs) and free WT-Clu are indicated. The additional free Clusterin in the lipoprotein complex preparation was released from the complex. The psClu band is likely an SDS-PAGE gel artifact caused by incomplete disulfide bond cleavage during sample preparation. After limited proteolysis of free Clusterin with 10 nM chymotrypsin, a faster moving proteolysis product is clearly visible in the native PAGE gel, whereas no electrophoretic mobility shift is apparent in SDS-PAGE. Mass spectrometry 7 proteomics of the boxed bands showed that the peptide Clu(210-227) in the C-terminal flexible tail of the Clusterin β chain was removed. This peptide is highly positively charged bearing one Lys and four Arg residues and no Asp/Glu, and the charge difference between unprocessed and processed Clusterin heterodimer seems to cause the large change in electrophoretic mobility. The proteolysis patterns in SDS-PAGE for Clusterin lipoprotein complex and free protein were similar, suggesting that the flexible tail of the Clusterin β chains is exposed to solvent in both species. Of note, this result became clearer with the single-chain Clusterin mutant TL4.