Let’s not forget: Peptidases in Alzheimer’s disease
Stargardt, A.

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KINETIC STUDIES OF CYTOPLASMIC ANTIGEN PROCESSING AND PRODUCTION OF MHC CLASS I LIGANDS

Stargardt A, Reits EA

Department of Cell Biology and Histology, Academic Medical Center, Amsterdam, The Netherlands
ABSTRACT

MHC class I molecules present peptides that are derived from intracellular proteins degraded by proteasomes. These peptides often require additional trimming by peptidases to fit into the peptide-binding groove of MHC class I. However, most peptides are rapidly recycled by the large heterogeneous pool of peptidases. Here, we describe a technique to quantify peptide degradation both in living cells and in cell lysates, using quenched peptides that contain a quencher and fluorophore. As degradation results in separation of the quencher and fluorophore, fluorescence will increase. This technique enables the examination of changes in peptide length and amino acid sequence on its half-life, and hence its chances to become presented by MHC class I.

INTRODUCTION

Inside the cell, protein synthesis and degradation is continuously ongoing. Most proteins fulfill their biological function as a mature protein, but sooner or later they become subjective to proteolysis by the proteasome which leads to the generation of short peptides ranging in size from 3 to 22 amino acids (Kisselev et al., 1999). Most peptides are immediately targeted by downstream peptidases that ultimately recycle them into single amino acids. However, some peptides escape complete degradation and are presented as MHC class I antigens to the immune system (Yewdell et al., 2003; Fruci et al., 2003; Reits et al., 2003). As most MHC class I binding peptides have a length of 8–11 amino acids, additional trimming of the proteasomal products by peptidases is then required (Rammensee et al., 1993). Various peptidases have been identified that can trim these peptides before they are translocated by the Transporter associated with Antigen Processing (TAP) into the lumen of the Endoplasmic Reticulum. Here they can be loaded onto MHC class I molecules and transported to the plasma membrane for presentation to the immune system. The various peptidases show different preferences for specific amino acid sequences and peptide lengths in vitro, which can be examined using purified peptidases together with synthesized peptides. Upon cleavage, the peptide degradation products can be analyzed by mass spectrometry to determine the substrate specificities of each peptidase. This information can subsequently be used to predict its role in the trimming of peptides into antigens (Beninga et al., 1998; Geier et al., 1999; Mo et al., 1999; Stoltze et al., 2000).

Trimming of peptides by isolated peptidases in vitro is obviously very different when compared to the processing by the heterogeneous pool of cytoplasmic peptidases in vivo. To examine peptide processing in the living cell, we previously described the use of a peptide degradation
assay to study the effect of peptide sequence or length on antigen processing (Reits et al., 2003; Reits et al., 2004). Not only does this allow to investigate the effect of alterations in peptide sequence on its half-life, but the effect of peptidase inhibitors also can be examined to identify peptidases involved in generation or degradation of the potential epitope. In addition, the effect of N-or C-terminal modifications or posttranslational modifications such as phosphorylation can be investigated (manuscript in preparation). Moreover, the incorporation of nondegradable D-amino acids at the termini can be used to examine the involvement of endo-peptidase activities, as exopeptidases are unable to degrade these peptides (Reits et al., 2004; manuscript in preparation). In addition to studying antigen processing, this assay might help to understand and improve the degradation of aggregation prone peptides as observed in neurodegenerative disorders like Alzheimer’s disease or Huntington’s disease. The technique is based on the principle that the peptide of interest is modified with a quencher and a small fluorophore like fluorescein (see Fig. 1). The quencher is an organic molecule coupled to a lysine that is able to absorb the energy emitted by the fluorophore because its absorption spectrum overlaps with the emission spectrum of the fluorophore. For efficient quenching, they should be into close proximity, e.g., separated by 4–8 amino acids. When a peptidase is cleaving the peptide, quencher and fluorophore are separated and the fluorophore is able to emit its energy as fluorescent light. This allows the determination of the half-life of the peptide of interest under different conditions including the inhibition of particular peptidases. This method can be applied to living cells as well as in vitro using cell lysates or tissue homogenates. It can also be used to study the effect of specific peptidase inhibitors, and examine whether these inhibitors are reversible, irreversible, competitive, or noncompetitive. In this protocol we describe how to prepare the quenched peptides, how to microinject cells or isolate cytoplasmic fractions, and show the possibilities of adding different peptidase inhibitors to investigate the role of peptidases in degrading the peptide of interest.

**MATERIALS**

For preparing the buffers, use ultrapure water, 18 MΩ-cm at 25°C.

**Generation of quenched peptides**

1. To detect peptide degradation using quenched peptides that become fluorescent upon degradation, peptides are synthesized with a fluorescent group (e.g., fluorescein or alexa 488, see Note 1) attached to one amino acid (usually a cysteine) and a quenching group attached
to another amino acid (usually a Dabcyl group coupled to a lysine residue). With about five amino acids between the quencher and fluorophore, the Dabcyl group quenches emission of the nearby fluorophore group, and fluorescence will only be detected when the amino acids are separated due to peptide degradation (Fig. 1).

2. The synthesis of quenched peptides can be performed by solid phase strategies using an automated multiple peptide synthesizer (Syro II, MultiSyntech) using Fmoc chemistry. Fluorescein can be covalently coupled to the cysteine residue using fluorescein-5-iodoacetamide (Molecular Probes). Various automated peptide synthesizers exist, and synthesized peptides are HPLC purified and validated by mass spectrometry.

![Figure 1 - Schematic representation of a quenched peptide, with a dabcyl group functioning as the quencher (Q) and a fluorescein moiety as a fluorophore (F). As these two groups are fused to two different amino acids, cleavage by peptidases results in the separation of the quencher and fluorophore and subsequent fluorescent light.](image)

**Degradation of quenched peptides upon microinjection in living cells**

1. Adherent cells should be used that are not too easily disturbed by micro-injection, such as HeLa cells (adherent cells such as HEK 293 cells often de-attach upon injection and remain sticking to the needle tip). Ideally, cells are seeded 1–2 days before injection on 35 mm glass coverslips in a 6-well plate and kept in regular medium with FCS in a CO₂ incubator.

2. 2× Microinjection buffer: 20 mM Tris–HCl, pH 7.4 and 0.2 mM EDTA, and subsequently autoclaved and filter (0.2 μm) sterilized. Store at −20°C.

3. Microinjection mixture: 1 μL of quenched peptide is added to 4 μL microinjection buffer, 2 μL H₂O and 1 μL 2 mM FuraRed (Molecular Probes). Prepare freshly and spin down for 10 min at minimal 20 700 × g before use.

4. HBS buffer (HEPES-buffered Saline): 21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 5.5 mM glucose. Sterilize by filtration (0.2 μm). The buffer can be CO₂ saturated, e.g., by placing the buffer prior to use in a petridish in the CO₂ incubator used for tissue culture. Store at 4°C.
5. Microinjector like the Eppendorf Femtojet coupled to an Injectman NI2 can be used.

6. Sterile microinjection needles like the Eppendorf Femtotips (5242.952.008) can be used, using thin microloader tips (5242.956.003) to load the needles with the microinjection buffer.

7. An inverted fluorescence microscope can be used equipped with a dry 40× or 63× objective and with an additional viewing port connected to a fluorescence detector. Emission of the simultaneously microinjected quenched peptide and Fura Red is measured after splitting the emitted light. Different setups can be used to detect fluorescence; we use a PTI model 612 analog photomultiplier system equipped with a 580 nm dichroic mirror with a 480–530 nm filter for fluorescein emission and a long pass 590 nm filter for Fura Red emission. The two fluorescent signals are simultaneously detected by two PTI model 612 analog photomultipliers (Fig. 2).

8. For data acquisition, software like FELIX (PTI Inc., USA) can be used.

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**Figure 2 - Microinjection setup to detect quenched peptide degradation in living cells.** Cells are grown on thin coverslips and mounted in a heated cell chamber. A high magnification objective (e.g., 40x or 63x) is used in combination with a small field diaphragm in order to create a small detection window showing only the selected cell that is to be microinjected. When exciting at 488 nm, a dichroic mirror splitting the emission light at 580 nm can be used to allow simultaneous detection of the peptide fluorophore and the injection marker.

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**Degradation of quenched peptides in cytosolic lysates**

1. 200 μM Digitonin lysis buffer: weigh 1 mg Digitonin (Sigma-Aldrich), add 55 mL H₂O. Heat for 15 min at 98°C in a heating block and cool down at room temperature. Store at 4°C.

2. Bovine Serum Albumin (BSA) solution: dissolve 10 mg BSA in 10 mL water, so that the final concentration is 10 μg/μL. Store at 4°C.

3. Bradfort reagent (Sigma-Aldrich).
4. **KMH buffer**: 110 mM KAc, 2 mM MgAc, 20 mM Hepes-KOH, pH 7.4. In an Erlenmeyer or glass beaker, weigh 260 mg 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), add water to a total volume of 50 mL and let dissolve at room temperature with a magnetic stir bar. Adjust pH to 7.4 by adding Potassium hydroxide (KOH). Then add 550 mg potassium acetate (KAc) and 14.2 mg magnesium acetate (MgAc). The buffer is filter sterilized and can be stored at room temperature.

5. **Inhibitors for specific proteases or peptidases**. Examples are butabindide for TPPII (Tocris Bioscience, Bristol, UK), bacitracin for IDE (Sigma Chemical Co., St. Louis, MO, USA), amastatin for nardilysin (Enzo Life Sciences, Lörrach, Germany), and MG132 for proteasomes (Sigma-Aldrich, Steinheim, Germany). Bath sonicator, e.g., a Bransonic-221 (Branson Ultrasonics, Danbury, LT, USA).

6. **96-Well Polystyrene Cell Culture Microplates**, black. (Greiner Bio-One) (see Note 2).

7. A microplate reader able to detect “time-resolved Fluorescence” like a Polarstar Galaxy microplate reader (BMG Labtech), using filter settings suitable to detect, e.g., fluorescein.

**METHODS**

**Measuring quenched peptide degradation in living cells**

1. Plate cells on glass coverslips and let them grow overnight until a confluence of 50–80% before mounting the coverslip in a heated stage (see Note 3). Replace the medium for CO₂-buffered HBS.

2. Prepare the microinjection mixture containing the quenched peptide and Fura Red. Fura Red is added as a control for microinjection and leakage; as it can be excited at the same wavelength as fluorescein but emits light at a much higher wavelength than fluorescein, the emitted light can be split using a dichroic mirror and separately detected. Since Fura Red is immediately fluorescent, it functions as a control for successful injection, while a decrease in Fura red signal in time indicates leakage from the cell (which will also affect peptide degradation, see Note 4).

3. About 4–6 μL of the microinjection mixture is loaded in the injection needle, and carefully ticking the needle (kept vertically with the injection tip downwards) will remove any remaining air bubbles that may obstruct the thin end of the needle upon injection (see Note 5).

4. Since all emitted light from the degraded peptides should ideally go to the detection system (e.g., the PMTs) the microinjection has to be performed “blindly” as no light is going to the oculars. Therefore, a neighboring cell has to be used to set the Z-level limit for the microinjection needle (see Note 6). When the Z-limit is set, the to-be measured cell is centered in the middle
of the field. As very little background light should pass through, the field diaphragm of the microscope can be closed until only the single cell is visible by transmitted light. Place the injection needle above the cell. Only now the emitted light can be switched from the oculars to the port connected to the PMTs (and be sure to switch off the transmission light beforehand to prevent over-exposure of the PMTs).

5. With the detection system switched on and the software running, the experiment can start. When the centered cell is microinjected, the Fura Red signal should immediately increase and remain on a plateau level (Fig. 3), which indicates a successful injection without leakage. In time, the green fluorescent signal should also increase, indicating degradation of the quenched peptides and no longer quenching of the fluorophore. Also the signal derived from the dequenched peptide should reach a plateau level when all peptides are degraded, and from this curve the half-life (\(t_{1/2}\)) of the peptide can be calculated, which is the time needed after micro-injection until 50% of the peptides are degraded (Fig. 3, see Note 7).

6. Upon completion of the experiment, a neighboring cell can be injected after being centered in the middle or a new area in the coverslip can be taken.

![Figure 3 - Model graph of a degraded quenched peptide.](image)

**Figure 3 - Model graph of a degraded quenched peptide.** Upon microinjection or addition of quenched peptides to cell lysates, the degradation of the peptides will result in fluorescence until all peptides are degraded (leading to a plateau phase). From this graph, the half-life of the peptide can be determined by calculating the time required to reach to 50% of the final plateau phase.

### Measuring quenched peptide degradation in cytosolic lysates

1. Collect cells by centrifuging at 180 × g for 3 min, 4°C and wash with PBS. Spin again to obtain the cell pellet and add ice cold Digitonin lysis buffer, 50 μL to 400,000 cells (see Note 8). Vortex and leave on ice for 30 min.
2. Centrifuge lysed cells 16 000 × g for 15 min at 4°C which will spin down membranes but leaves the cytosolic proteins soluble in the supernatant.
3. Move the supernatant to a fresh tube.
4. The protein concentration in the sample lysate can be determined by performing a Bradford assay.
   a. Pipet the standard; 0, 0.5, 1, 2, 4, and 8 μL of BSA solution (corresponding to 0, 0.5, 1, 2, 4, and 8 μg BSA) in 6 wells of a clear 96-well plate.
   b. Pipet 1 μL of sample lysate in another well.
   c. Add 200 μL of Bradford reagent to all conditions and measure the absorbance at 595 nm on the Polarstar Galaxy.
   d. Compare value of sample lysate with Standard to determine the protein concentration (see Note 9).

**Preparation of peptide and inhibitors**

1. Dissolve quenched peptide in water (see Note 10).
2. Dilute 1 μL quenched peptide in 30 μL KMH buffer (see Note 11).
3. Dilute the inhibitor in KMH buffer to the working dilution. Keep in mind that the 10 μL of the inhibitor working dilution ends up in a total volume of 200 μL. The inhibitor working dilution is thus 20 times more diluted in the final experiment and should be made 20 times more concentrated than the optimal inhibitor concentration (see Note 12).
4. Immediately prior to use, sonificate peptide solution in the ultrasonic water bath for 10 min to prevent aggregation and oligomerization and assure the peptides are soluble monomers (see Note 13).

**Measuring degradation on the polarstar galaxy microplate reader**

1. In each well of a 96-well Polystyrene Cell Culture Microplate add 20 μg lysate. For the negative control do not add lysate or use lysate pre-incubated with broad spectrum peptidase inhibitors.
2. Add 10 μL inhibitor working dilution and KMH buffer to a total of 190 μL per well. Incubate for 30 min at 4°C to assure the inhibitor is bound to the active site of the proteases of interest when adding the peptide. For the “no inhibitor” control, do not add inhibitor.
3. Add 10 μL of sonificated peptide solution to each well and place the plate in the 37°C pre-warmed Polarstar Galaxy microplate reader.
4. On the Polarstar select the configuration “Time-resolved Fluorescence”. Select the emission and excitation wavelength that correspond to the fluorophore incorporated in the quenched
peptide of interest. Set which wells to measure, for how many cycles and over what time span (see Note 14).

NOTES

1. Various fluorophores can be used to detect degradation of peptides, but ideally a small fluorophore like fluorescein should be used to prevent steric hindrance of peptide degradation. Alternatively, fluorophores like Alexa 488 can be used (which remains fluorescent at low pH, e.g., upon lysosomal degradation). When multiple cysteines are present in the peptide sequence, no specific labeling of one cysteine with a fluorophore is possible. In this case, pre-coupled fluorophores attached to an amino acid can be used, such as Edans (which is coupled to a glutamic acid).

2. Using black plates will eliminate cross talk between wells and reduce back-scattered light, making the measurements more accurate. The Polarstar Galaxy can do both top and bottom readings, but keep in mind to use plates with transparent bottoms in the last case.

3. Different setups can be used to mount living cells prior to micro-injection. We use round 24 mm glass coverslips (Menzel-Glaeser from Thermo scientific) that are mounted in an Atto fluor cell chamber (Cat. No. A-7816, Molecular Probes) which is being heated to 37°C.

4. As also shown in Fig. 3, a well-executed microinjection should not result in a perforated cell that leaks all its cytoplasm content in the medium, including the to-be-degraded peptides. Therefore, simultaneous detection of the Fura Red shows whether cells become leaky or not, and ideally the Fura Red signal should remain similar over time after microinjection.

5. When keeping the needle vertically with the injection tip down, a small air bubble can often be seen below the added peptide solution. Careful ticking the needle with your index finger will make the bubble go upwards. During the actual microinjection, the needle tip may become obstructed by dead cells that adhere to the needle. Briefly pulling the needle out of the medium and putting it back again will often result in the removal of these dead cells.

6. When microinjecting adherent cells, a threshold can be set for the injection level (Z-limit) in order to microinject numerous neighboring cells without having to set the Z-limit for each individual injection.

7. While the height of the plateau phase obviously changes when more or less amount of peptides are introduced, the half-life is much less affected by the amounts of peptides, indicating that the peptidases are not saturated. Therefore it seems not critical to inject the identical amounts of peptides during each microinjection. Still, ideally a similar concentration of (different) peptides
should be used to compare the effect of alterations of peptide sequence or upon the addition of peptidase inhibitors.

8. The amount of lysis buffer to add to the cell pellet can be adjusted to obtain the desired protein concentrations in the final cell lysate. Although this may differ for each cell type and experiment, it is important to make sure that enough lysis buffer is added to the pellet that the cells can nicely dissolve and no cell clumps are present.

9. The amount of protein in the cell lysate can be determined by comparing its absorbance value with the values of the protein standard generated with the BSA solution. This can be easily done in a program like Microsoft Office Excel by adding a linear trend line to the graph containing the values obtained for the Standard. The equation corresponding to this trend line can also be added and this is used to calculate the protein concentration in the cell lysate by inserting the measured absorbance value as x-value. The resulting y-value is the amount of protein in the lysate.

10. Peptides are usually supplied as a dry powder. This substance can be dissolved in several solvents. What to use depends on the characteristics of the peptide; non-hydrophobic peptides will most of the time easily dissolve in water, however, peptides that have hydrophobic characteristics may be difficult to dissolve in water and should be dissolved in organic solvent like DMSO. Take into account that DMSO can affect cell culture based assays; therefore make concentrated stock solutions that will be diluted in the experiment so that no more that 1% of the original solvent is present in the final experimental solution.

11. It is important to make a single diluted peptide solution that will be used for all conditions. Calculate beforehand how much diluted peptide solution is needed for the whole experiment. This will prevent little variations between conditions caused by pipeting differences or differences in preparation of the peptide solutions like sonification or pre-incubation times.

12. The optimal inhibitor concentration is the concentration at which the inhibitor has its maximum effect on peptide degradation. This concentration can be determined by making a dilution series and test what concentration maximally inhibits the degradation of a peptide that is known to be degraded by the protease that is inhibited (a positive control).

13. Peptide degradation can be influenced by peptide aggregation or oligomerization so it is essential to know in what state the peptide is when used in the experiment. Sonification of the peptides can be used to enhance solubility and (re-)dissolve possible aggregated peptides.

14. The amount and length of the measurement cycles depends on the degradation speed of the peptide of interest. When the peptide is quickly degraded (e.g., the degradation curve reaches a plateau within 30 min) then a high amount of short cycles is best (although bleaching due to
extensive measurements should be prevented). However, when the peptide is slowly degraded (e.g., the degradation curve reaches a plateau after 10 h) then cycles of a few minutes distributed over 10 hours will be best.

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REFERENCES


