

Analysis of Proteasome Generated Antigenic Peptides by Mass Spectrometry

Kathrin Textoris-Taube, Christin Keller, Ulrike Kuckelkorn,
and Peter-M. Kloetzel

Abstract

Mass spectrometry (MS) is today one of the most important analytical techniques in biosciences. The development of electro spray ionization (ESI) as a gentle ionization method, in which molecules are not destroyed, has revolutionized the analytic of peptides. MS is an ideal technique for detection and analysis of peptides generated by in vitro experiments using purified 20S proteasomes. It also provides a convenient and sensitive way to monitor the processing activity of enzymes. The combination of high performance liquid chromatography (HPLC) with ESI-MS allows the analysis of complex samples with separation in their specific constituents by LC and their subsequent detection by MS.

Key words: Proteasome, Mass spectrometry, HPLC, Antigen processing, Antigenic peptides, Polypeptides, Substrate degradation, Fragment generation, Epitope

1. Introduction

The protease responsible for the degradation of polyubiquitylated proteins and generation of antigenic peptides is the 26S proteasome, which is composed of the 20S proteasome, representing the catalytic core, and two 19S regulator complexes that confer the binding and unfolding of ubiquitylated substrates (1, 2). The hydrolyzing activities of the 20S core are conferred by three of the seven β subunits located in the two inner heptameric β -rings, whereas the 19S regulator complexes (composed of six ATPase subunits as well as 9–10 non-ATPase subunits) attach to the outer heptameric α rings of the 20S core (3, 4). Major histocompatibility complex (MHC) class I presented peptides are predominantly generated by the proteasome system. Thereby, IFN- γ strongly influences the processing efficiency by inducing immunoproteasome formation and proteasome activator PA28 synthesis. Depending on the protein substrate,

the presence of immunoproteasomes and PA28 (5–10) influence epitope liberation either positively or negatively.

To study proteasomal antigen processing simple in vitro systems have been established that allow mimicking the in vivo situation with surprisingly high fidelity. These assays generally involve the processing of a synthetic polypeptide of 20–30 amino acid residues in length that harbor a known or a potential MHC class I epitope by purified 20S proteasomes, HPLC-separation of the generated peptides followed by MS analysis of the generated peptide products (11) (see Note 1).

2. Materials

2.1. Proteasome Isolation

All solutions are prepared using ultra pure water (MilliQ) or *aqua demineralisata* (A. dem.)

1. Dounce homogenizer.
2. JA20 rotor and 50 ml tubes (Avanti J-E; Beckman & Coulter) and SW40Ti rotor and 14 ml tube (Optima LE-80K; Beckman & Coulter).
3. Beaker.
4. Ponceau red: 1% Ponceau in 5% trifluoroacetic acid (TFA).
5. Ammonium sulfate crystals.
6. 10×TEA buffer: Dissolve 200 mM 2-amino-2-(hydroxymethyl)propan-1,3-diol (12.1 g TRIS), 1 mM ethylenediaminetetraacetate (1.86 g Na-EDTA), and 1 mM sodium acid (0.3 g NaN_3) in 450 ml water. Mix and adjust the pH with 25% HCl to 7.2 and add the volume to 500 ml.
7. Stock 0.2 M DTT: Dissolve 308 mg dithiothreitol ($\text{DTT} = \text{C}_4\text{H}_{10}\text{O}_2\text{S}_2$) fresh in 10 ml water (see Note 2).
8. Stock solution 2 M NaCl: Dissolve 58.44 g NaCl in 500 ml water.
9. TEAD buffers: Prepare the different TEAD buffers from the 10× TEA stock solution (see Subheading 2.1, item 6), supplement the adequate volume of NaCl from a 2 M stock and 5 ml 0.2 M DTT/l (see below).
 - (a) *TEAD-50*: 10 ml 10× TEAD, 2.5 ml 2 M NaCl, 0.5 ml 0.2 M DTT add to 100 ml.
 - (b) *TEAD-100*: 10 ml 10× TEAD, 25 ml 2 M NaCl, 2.5 ml 0.2 M DTT add to 500 ml.
 - (c) *TEAD-350*: 10 ml 10× TEAD, 17.5 ml 2 M NaCl, 0.5 ml 0.2 M DTT add to 100 ml.
 - (d) *TEAD-1000*: 50 ml 10× TEAD, 250 ml 2 M NaCl, 2.5 ml 0.2 M DTT add to 500 ml.

10. Stock solution 10% NP40: add 9 ml water to 1 g nonyl phenoxypolyethoxylethanol (NP40 = tergitol-type NP-40).
11. 40% Sucrose: add 30 ml 1× TEAD to 20 g sucrose.
12. 10% Sucrose: add 45 ml 1× TEAD to 5 g sucrose.
13. Proteasome assay buffer: 50 mM Tris-HCl (pH 7.5), 1 mM DTT in deionized water: dissolve 0.146 g TRIS in 40 ml water, adjust the pH to 7.2 with 25% HCl, pipette 0.25 ml 0.2 M DTT and add the volume to 50 ml (see Note 2).
14. 20 mM Proteasome substrate stock: dissolve 7.64 mg of Suc-LLVY-AMC (Succinyl-leucine-leucine-valine-tyrosine-7-amino-4-methylcoumarin) in 500 µl DMSO (dimethyl sulfoxide).
15. Lysis buffer: supplement 10 ml TEAD-50 with 0.1 ml 0.1% (w/v) NP-40.
16. Diethylaminoethyl-(DEAE) Sephacel®: pre-swollen Suspension in 20% ethanol is washed by water to remove the ethanol. The DEAE-Sephacel has been equilibrated in TEAD-50 over night.
17. Open 50 ml column (BioRad).
18. Anion exchange column: MonoQ 5/50 GL, 1 ml column volume (CV) (GE Healthcare).
19. Fast protein liquid chromatography (FPLC) buffers are filtrated (Millipore filter 0.2 µ) to remove particles and degassed.
 - (a) Buffer A: TEAD-100.
 - (b) Buffer B: TEAD-1000.
20. Black 96-well microtiter plate and plate fluorescence reader (i.e., BioTek Synergy HT).

2.2. Proteasome Digestion Assay Mix

1. Stock solution 10× TEA buffer: see Subheading 2.1, item 2.
2. Stock solution 2 mM DTT: dissolve 3.08 mg DTT in 10 ml water (see Note 2).
3. Digestion buffer: 1 ml solution 10× TEA and 1 ml solution 2 mM DTT in 10 ml water.
4. Substrate solution: 1 mg peptide in 1 ml 0.5% trifluoroacetic acid (TFA) in water (see Note 3).
5. Reagent to stop digestion: 3% TFA solution in water (final: 0.3% TFA).

2.3. HPLC Analyses

All reagents in particular the mobile phases used for HPLC and MS analysis should fulfill the following criteria: HPLC or better MS grade.

1. Analytical reversed phase-(RP) chromatography.

Stationary phase:

C18 RP material column, 0.3 cm long with a core of 4.6 mm and a pore size of 1.5 µm.

Mobile phases:

- (a) Solvent A: 0.5% TFA in water, add 5 ml 100 %TFA to 995 ml water.
- (b) Solvent B: 0.45 %TFA in acetonitrile (ACN).

2.4. HPLC-MS Analyses

1. HPLC/ESI-MS:

Stationary phases:

- (a) Analytical column: RP18 ID 1 mm, length 10 cm, particle size 3 μ m, pore size 150 Å.
- (b) Precolumn: RP18, ID 1 mm, length 1 cm, 3 μ m particle size.

Mobile phases:

- (a) Solvent A: 0.05% TFA in water.
- (b) Solvent B: 0.045 %TFA in 70% ACN and 30% water (see Note 4).

Stock solution of the 9GPS standard:

- 1 mg YPHFMPTNLGPS (9GPS, MG1359.6) is dissolved in 1 ml 50% methanol (MeOH)–50% water (H₂O), 1% acetic acid (AcOH).

9GPS standard for analysis:

- Dissolve 10 μ l stock solution of the 9GPS standard in 990 μ l 50% MeOH–50% water, 1% (AcOH). Freeze five aliquots a 200 μ l (see Note 5).

2. Analysis software.

3. Methods

3.1. Proteasome Preparation (12)

20S proteasomes are purified from cultured cells (see Note 6).

1. The cells are lysed mechanically in 20 ml lysis buffer using a dounce homogenizer (25–30 strokes). Store the cells for 10 min on ice to complete lysis. The cell debris is removed by centrifugation at 17,000 rpm (40,000 $\times g$) for 30 min at 4°C (Avanti J-E centrifuge, JA20 rotor). The cleared supernatant is used for further purification.
2. DEAE-anion-exchange-chromatography
 - (a) First the DEAE Sephacel is equilibrated in TEA buffer (see Note 7) over night (about 20 ml).
 - (b) To bind the protein-fractions the supernatant of the first purification step is incubated with the DEAE sephacel on a bottle roller at 4°C for 1 h.

- (c) The DEAE gel is filled into an open 50 ml column and washed six times with about 10 ml TEAD-50 controlling the protein amount in the flow through (absorption at 280 nm or spotting drops from the eluates onto nitrocellulose and staining with Ponceau red (see Note 8)). The amount of proteins in the flow through should be markedly reduced (see Note 8).
 - (d) In the next step, DEAE bound proteins are eluted with TEAD-350 and collected in about 20 fractions à 2 ml.
 - (e) The proteolytic activity is determined (see Subheading 3.1, step 6).
3. The proteolytically active fractions are combined and subjected to fractional ammonium sulfate precipitation (see Note 9).
 - (a) First the volume of the pooled fractions is estimated, and then filled in a beaker, which is placed in an ice bath.
 - (b) Under continuous stirring ammonium sulfate crystals are added very slowly to achieve a 35% saturation (1.94 g ammonium sulfate ((NH₄)₂SO₄) per 10 ml solution).
 - (c) The mixture is centrifuged at 12,000 rpm (29,000 × *g*; JA20 rotor) for 10 min at 4°C.
 - (d) The supernatant is filled in a fresh beaker and 2.91 g of ammonium sulfate crystals per 10 ml are added to 80% saturation.
 - (e) The precipitate is collected by centrifugation at 19,000 rpm (46,000 × *g*; JA20 rotor) at 4°C for 10 min. The pellet is slowly dissolved in 1 ml TEAD-50 on ice.
4. The completely resolved ammonium sulfate precipitate is overlaid onto a 10–40% sucrose gradient in a 14 ml SW40 tube (see Note 10).
 - (a) Centrifugation has to be performed for 16 h at 40,000 rpm (285,000 × *g*) in a SW40Ti rotor at 4°C (corresponding to $1 \times 10^{12} \omega^2 t$).
 - (b) The gradients are fractionated in 0.6 ml aliquots and the proteolytic activity is determined (see Subheading 3.1, step 6).
 - (c) The proteolytically active fractions are pooled and dialyzed with TEAD-100 over night at 4°C.
5. The dialyzed samples are diluted with 9 ml buffer A. Non-dissolved proteins are removed by a disposable filter holder (0.2 μm).
 - (a) The anion exchange column is equilibrated with 5 CV buffer A.
 - (b) The filtrate is applied with a flow of 1 ml/min.
 - (c) After washing the column with buffer A (2CV), proteins are eluted with a linear NaCl-gradient. Within 5 min the

concentration of buffer is raised from 0 to 20%, thereafter the gradient increases from 20 to 40% in 20 min and in 2 min from 40 to 100%. At a concentration of 1 M NaCl (100 %B), the column is washed for 5 min and returned to buffer A within 2 min.

- (d) Fractions are collected in a volume of 1 ml during the elution with 20–40% buffer B (see Note 11).
 - (e) The proteolytic activity is determined (see Subheading 3.1, step 6).
6. To prepare the proteasome activity assay:
- (a) The substrate (Suc-LLVY-AMC; 20 mM stock solution) measuring the chymotrypsin-like activity is added freshly to the proteasome assay buffer (10 μ l to 10 ml buffer to a final concentration 20 μ M).
 - (b) 10 μ l sample or 10 μ l water (reference value) are placed into the wells of a black 96-well microtiter plate. 100 μ l substrate assay buffer is added. This reaction mixture is incubated for 60 min at 37°C.
 - (c) The released fluorogenic AMC is measured at an emission of 460 nm (excitation at 360 nm) in a plate fluorescence reader.
 - (d) Highly proteolytically active fractions are combined and aliquots are frozen or stored on ice.

3.2. Digestion

1. For time-dependent processing experiments (signal intensity versus time of digestion) 1 mg of the synthetic polypeptide substrate is incubated with 0.1 mg enzyme in an eppendorf tube at 37°C (2).
2. For a five time points kinetic experiment (0, 1, 4, 8, and 24 h to qualify) a master mix of approximately 150 μ l is used. The mixture is partitioned in a volume of 29 μ l to five labeled tubes and incubated for the indicated time.
3. The digestions are stopped with 3 μ l 3% TFA (see Notes 13 and 14) or freezing at –20°C.

3.3. HPLC (See Note 15)

Proteasomal processing products are separated on an analytical reversed phase-(RP) column and generated peptides are monitored by UV at 220 nm.

1. 20 μ l of the digestion solution (see Subheading 3.2) is injected into a sample loop of 50 μ l (see Note 16) and measured by RP chromatography. The flow through of the mobile phase is 1 ml. Peptide bonds are specifically detected at a wavelength of 220 nm.
2. A short binary gradient (6% in 15 min from 5 to 95% mobile phase B) (see Note 17) is performed to monitor the substrate degradation (Fig. 1).

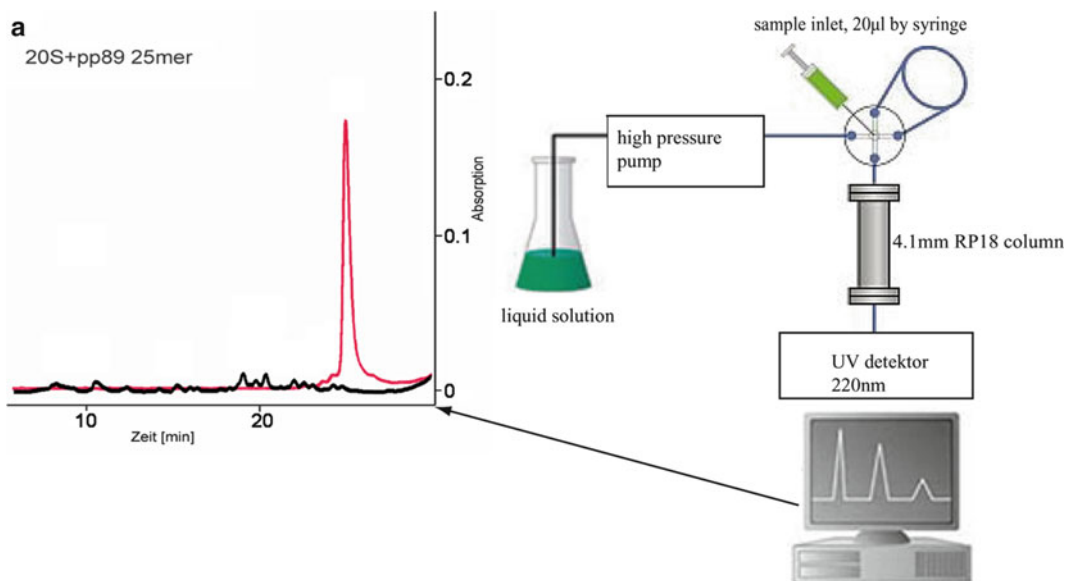


Fig. 1. Schematic configuration of a HPLC instrument with UV detection. The display at the head (a) illustrates two digestion experiments of a peptide with the 20S proteasome. Two chromatograms are displayed; the 0-h incubation period corresponds to the *red marked graph*, the 20-h digestion to the *black one*. Non-cleaved pp89 25mer peptide substrate (RLMYDMYPHFMPPTNLGPSEKRVWMS, (18)) runs under the peak at 22.5 min and it is no longer detectable after digestion by 20S, but on the other hand, many individual peaks of the digestion products can be observed (A. Voigt, unpublished data). Using this experimental set up various options of the digestion approach can be optimized conditions (concentration of substrate and proteasomes, time points of digestion) (see Note 18).

3.4. HPLC/ESI-MS

1. 10 µl of digestion and 1 µl of 9GPS standard for analysis are mixed in a vial. A cooled autosampler (4°C) picks up 10 µl of the sample and loads it onto a 40°C heated (see Note 19) RP18-column with a flow rate of 30 µl/min. The sample is analyzed by a binary gradient starting with 3% eluent B and increasing up to 67% with a slope of 2% for each RP-analysis (see Note 20).
2. The resulting peaks are detected by ESI-MS in a data dependent experiment (see Note 21). Data are used for the identification and quantification of the proteasomal generated polypeptides. The identified fragments are described in a table called cleavage map (Fig. 2).

3.5. Analyze Data

Analysis by mass spectrometry allows identification and quantification of in vitro generated digestion products.

1. Identification (Fig. 4): The processed peptides can be identified in the ion trap by the mass to charge ratio determined by full (Fig. 4b) and zoomscan (Fig. 4c) and subsequent fragmentation (MS/MS, Fig. 4d). An evaluation software (mascot, bioworks (see Note 22)) compares theoretically calculated patterns of

murine cytomegalovirus (MCMV) IE pp89 protein (14)
RLMYDMYPHFMPPTNLGPSEKRVWMS-CONH2
mono MG: 3085.4
Proteasome: e.g. T2, T2.27 20S proteasomes
method
gradient: for example 3 - 67 % B (in 30 min), flow 30 µl/min
standard: 9GPS (YPHFMPPTNLGPS)

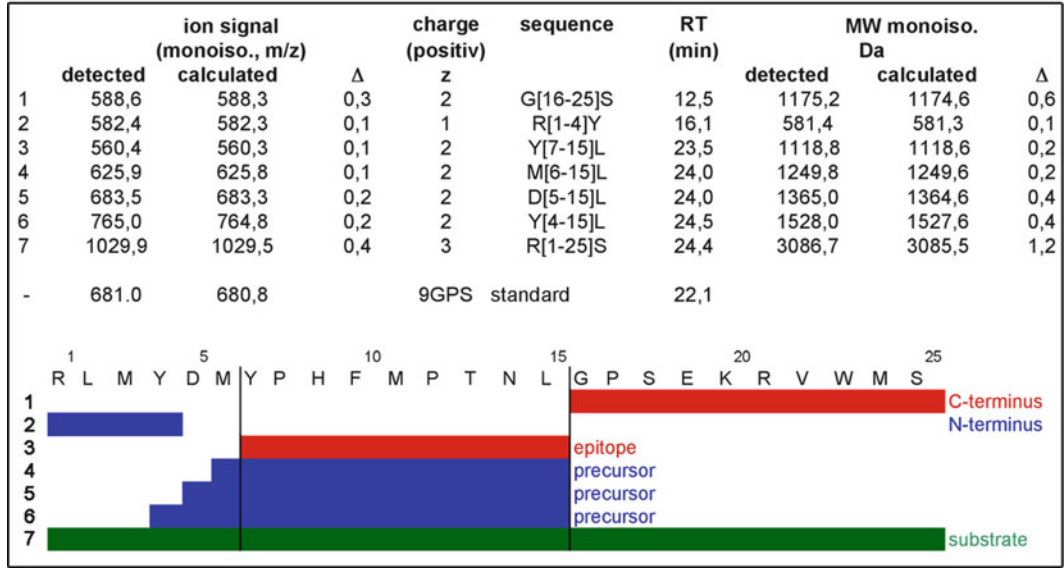


Fig. 2. This figure shows a so-called cleavage map, an Excel file containing all the important data for identification. The example shows some of the important pp89 (18) antigenic fragments (substrate, epitope, N-terminal elongated precursors of different length, C- and N-terminus).

- peptides with the experimentally measured MSMS. Database search is performed using databases of the peptides (in fasta-format: x.fasta) and the following parameters: no enzyme, mass tolerance for fragment ions 1 amu. To get more reliable results the peptide product pattern and retention time should be compared to that of synthetic peptides (13).
2. Quantification (Fig. 3): The number of detected ions is proportional to the produced signal. The integration of the signal measured by the profile mode describes the relative rate of product formation (see Notes 22 and 23).

4. Notes

1. The mass range of ESI MS (200–2,000 Da) and the column (particle and pore size), which is used for chromatography limit the length of the peptide sequence that can be analyzed. Peptides with a length of up to 35 amino acid residues can be

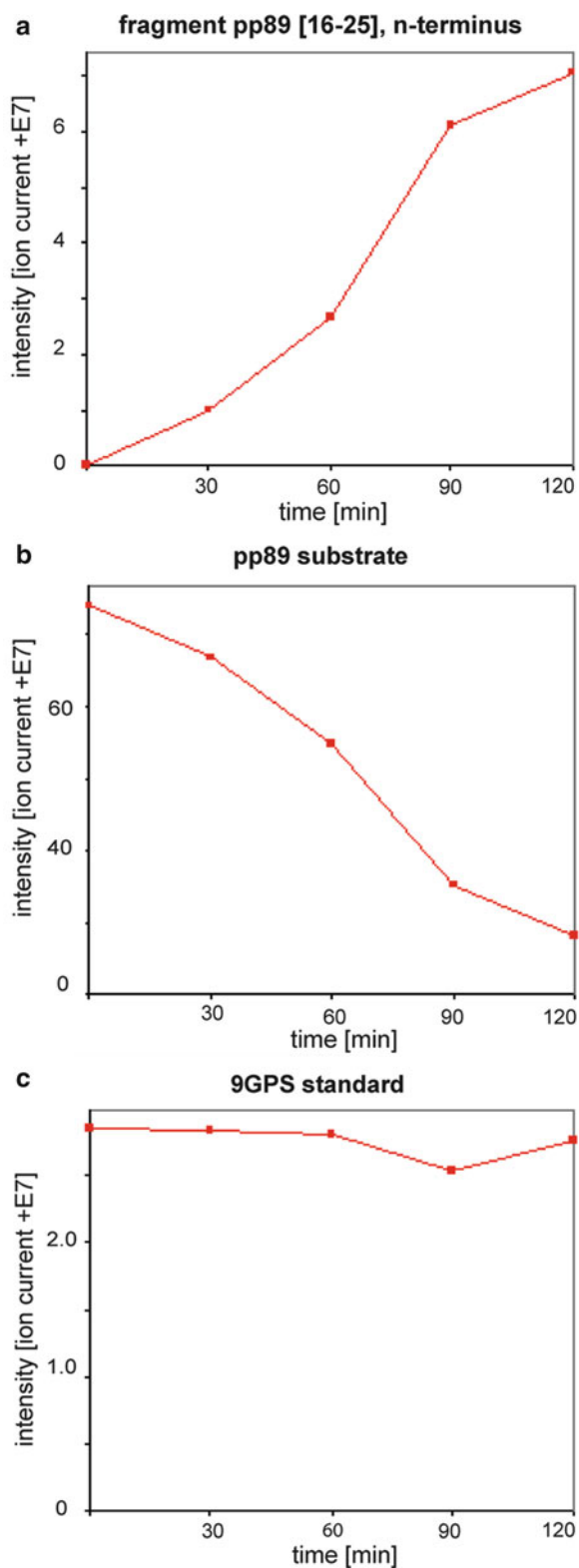


Fig. 3. The graphical plot of the measured intensity versus the incubation time forms so-called digestion kinetics of pp89 (14). The graphs illustrate (a) fragment generation, (b) substrate degradation, and (c) the 9GPS standard as a control for measurement.

measured and analyzed using this ionization method. The peptide sequences derived from proteins of choice (tumor antigen, virus etc.) harboring epitope sequences have a length between 8 and 12 aa residues.

For efficient analysis the amino- (N-) and carboxy- (C-) terminal flanking regions of the epitope sequence should encompass at least 6 residues. Shorter flanking sequences may negatively affect the processing efficiency and analysis by mass spectrometry.

Some of the amino acids tend to be modified: cysteine (C) = dimerization, oxidation, methionine (M) = oxidation.

The analysis of leucine and isoleucine cluster (L, I) is difficult using LC, because of their hydrophobicity. To avoid these methodical problems the number of these amino acid residues may be limited in the flanking regions. In some exceptional cases it is also possible to change an amino acid (for example C to serine (S)).

2. It is useful to prepare 10 ml 0.2 M DTT fresh in deionized water. Store it at -20°C .
3. Some peptides are poorly soluble, on this account we carefully tried to dissolve the solid substance in 100% dimethylformamide (DMF) and dilute it to 20% with mobile phase A (Subheading 2.4). The precipitation of a gelatinous, insoluble pellet should be avoided.

In case a gel-like, insoluble pellet is formed this should be discarded. For physiological reasons the solvent concentration should not be higher than 20% (v/v). To prevent oxidation it is useful to use DMF.

4. We normally prepare a 10% TFA solution. To mix the mobile phase A (Subheading 2.3) 5 ml TFA (10%) is dissolved in 995 ml water.
5. Due to the complexity of the samples it is thus possible to monitor the conditions of measurement via LC and MS. It is not intended to quantify fragments.

9GPS standard derived from pp89 is a peptide, which is not generated in the proteasomal digestion of pp89. This fragment is added prior the MS analysis and serves as a standard. During the whole analysis the peak area of 9GPS in comparable samples should be approximately the same. In addition, the amount of standard must be high enough to generate significant MSMS.

6. For 150–200 μg proteasome 10^9 – 10^{10} cells should be used.
7. It is important to eliminate the ethanol.
8. A280 control: The absorbance at 280 nm is applicable to calculate the amount of protein.

9. The 20S proteasome is precipitated between 38 and 80% saturation of $(\text{NH}_4)_2\text{SO}_4$.
10. For SW40 tube, prepare the gradient with a gradient mixer: 6.5 ml 10% sucrose in the front chamber and 6.5 ml 40% sucrose in the retral chamber (the higher density solution is underlayed to the lower density solution). The proteins are separated in a SW40Ti rotor (BECKMAN ultra centrifuge Optima LE-80 K at $\omega^2 t = 1.0 \text{E}12$, that means 40,000 rpm or $285,000 \times g$ for 16 h).
11. Proteasome is eluted in 28–30% B.
12. In a substrate/enzyme reaction the ratio of mass, not of volume, is critical. In general, an approximate 1,000-fold excess of substrate should be added.
13. The digest is stopped by adding of 0.1 Vol 3% TFA or by freezing. Adding TFA can increase the salt concentration. This may negatively affect the mass spectrometric analysis.
14. The following points should be considered when performing the digest:

Partitioning of the master mix provides reproducible results. The incubation in the whole master mix raises several problems:

- (a) To achieve a constant temperature during digestion in the whole approach is difficult.
- (b) The formation of condensation products in the lid of the sample tube can falsify results.

The biological matrix affects the MS signal. The 0-h-value has to include buffer and enzyme. To stop the reaction as quickly as possible, TFA should be placed in the tube for the zero hour time point. Be careful with the TFA concentration, because a high concentration of salt is able to quench the MS signal.

15. The separation of individual substances is based on the differential distribution of samples in two phases. One phase (liquid, mobile) moves to another phase (liquid, stationary) and takes over the constituents with different speeds.
16. For an optimal analysis the sample loop should be half filled.
17. The solvent B increases in 1 min with a slope of 6%, in this example (Subheading 3.3) the eluent B moves from 5 to 95% in 15 min. Eluate A decreases in 1 min 6%, this means it moves from 95 to 5% in the same time.
18. It is important that not more than 50% of the substrate is degraded at the time points that are used for the analyses (better 30–40% degradation time points). This prevents “reentry” of processing products in the 20S proteasome core particle and

reprocessing of the degradation products. To achieve this, we normally increase or decrease substrate or enzyme concentrations accordingly to optimize the substrate turnover. It is also possible to choose different incubation times.

Reliable kinetics should contain at least 5 measured time points (for example, 0, 1, 2, 4, and 8 h). To generate convincing fragmentation pattern (MSMS) the 24 h value is often essential. After 24 h a sufficiently large concentration of fragments has been generated to identify the processed peptides by MS.

19. Hydrophobic or high molecular weight peptides elute in a non-sharp, quenched flat peak. To optimize the resolution, the column temperature can be increased. Higher temperature will lead to a shorter column lifetime and some columns may not be able to tolerate 60°C. But to our experience peaks will be sharper and elute earlier with higher temperature.
20. Usually in the beginning the hydrophilic solvent A rinses (at least 10 min) the polar salts from the column. Salts are able to quench the MS signal.

The hydrophobic eluent B is able to wash the major (non polar) proteins (for example the proteasomes or other enzymes) from the column, because high molecular proteins may block the column. After performing the gradient we generally wash about 10 min with 100% B (if possible longer). Finally, the column is equilibrated for 10 min.

To improve peak separation, the concentration of buffer B should be determined at which the substrate elutes in the chromatogram. At this point in most of the cases the entire elution of the sample is achieved (see Note 17). Ending with this concentration of buffer B makes the gradient shallower and possibly leads to a better separation. The substrate should not elute in the equilibration phase, but also be well separated from processed fragments during the whole gradient.

Chromatography methods often start with a higher non-polar hydrophobic ACN concentration but this can lead to a loss of small polar fragments. To avoid this, these analytical methods (13) often begin with an initially steep gradient, flattening in the course of the chromatogram.

This should be considered depending on substrate and complexity of the sample.

TFA has been reported to suppress MS ionization and often in mass spectrometry a lower percentage of TFA (to 0.02%) is used without significant loss in chromatographic efficiency.

21. To facilitate the identification of many different peptides fragments with little sample consumption a data dependent scan experiment with dynamic exclusion is often used. Data dependent experiments automatically switch between different

scan modes. Without input from the operator the MS System processes the information generated in the experiment to decide about the next step. The mass with the highest intensity is automatically zoomed and fragmented by MS. After several scans (normally 2 or 3) the parent ion (m/z) is set briefly on an exclusion list and the next highest precursor (m/z) can be analyzed. This enables the user to identify as many peptides in a HPLC run as technical possible.

A so-called triple run (Fig. 4) includes a chromatogram (Fig. 4a) and three forms of mass spectra:

- (a) Fullscan (Fig. 4b): all masses in a given range, most of the cases 200–2,000 Da are collected in this kind of scan.
 - (b) Zoomscan (Fig. 4c): the growth of the highest mass and its environment to determine the charge state of the parent ion and calculate the molecular weight.
 - (c) MSMS-scan (Fig. 4d): the identification of the peptide by fragmentation of the parent ion into product ions.
22. We normally work with Bioworks Rev. 3.3, LCquan 2.5 SUR1, and Xcalibur 2.0 SR2 from Thermo Electron Corporation 1998–2006.
23. To characterize proteasome subtypes and their different cleavage strength and preferences, it is essential to determine the 50% substrate degradation. Whether a proteasome type degrades a substrate only faster or generates more efficiently the fragments in total (epitopes, N-terminal elongated precursors of different length etc. (Fig. 2)) can be seen at this time point. On the other hand, longer incubation periods will result in “reentering” of large cleavage fragments (producing artifacts). The 50% substrate degradation time point is determined from graph analysis displaying 50% substrate concentration compared to time point zero. The example (Fig. 3) shows a 50% substrate degradation in about 70 min.

The generated amount of one fragment cannot be compared with another processed peptide, as intensities in mass spectrometry are directly dependent on the peptide sequence and its individual amino acids. Therefore, a direct quantification is not possible.

To determine the epitope–antitope ratio (14) represents an attempt to quantify antigenic peptides. Another possibility is the usage of a so called “Aqua-peptide” (heavy peptide). This is a peptide with heavy amino acids in the sequence to be analyzed used as an internal standard for quantifying the “natural” peptide (15, 16). To quantify fragments the titration method and QME (17) show success simulating the biological matrix (inactive proteasomes have to be added to the sample).

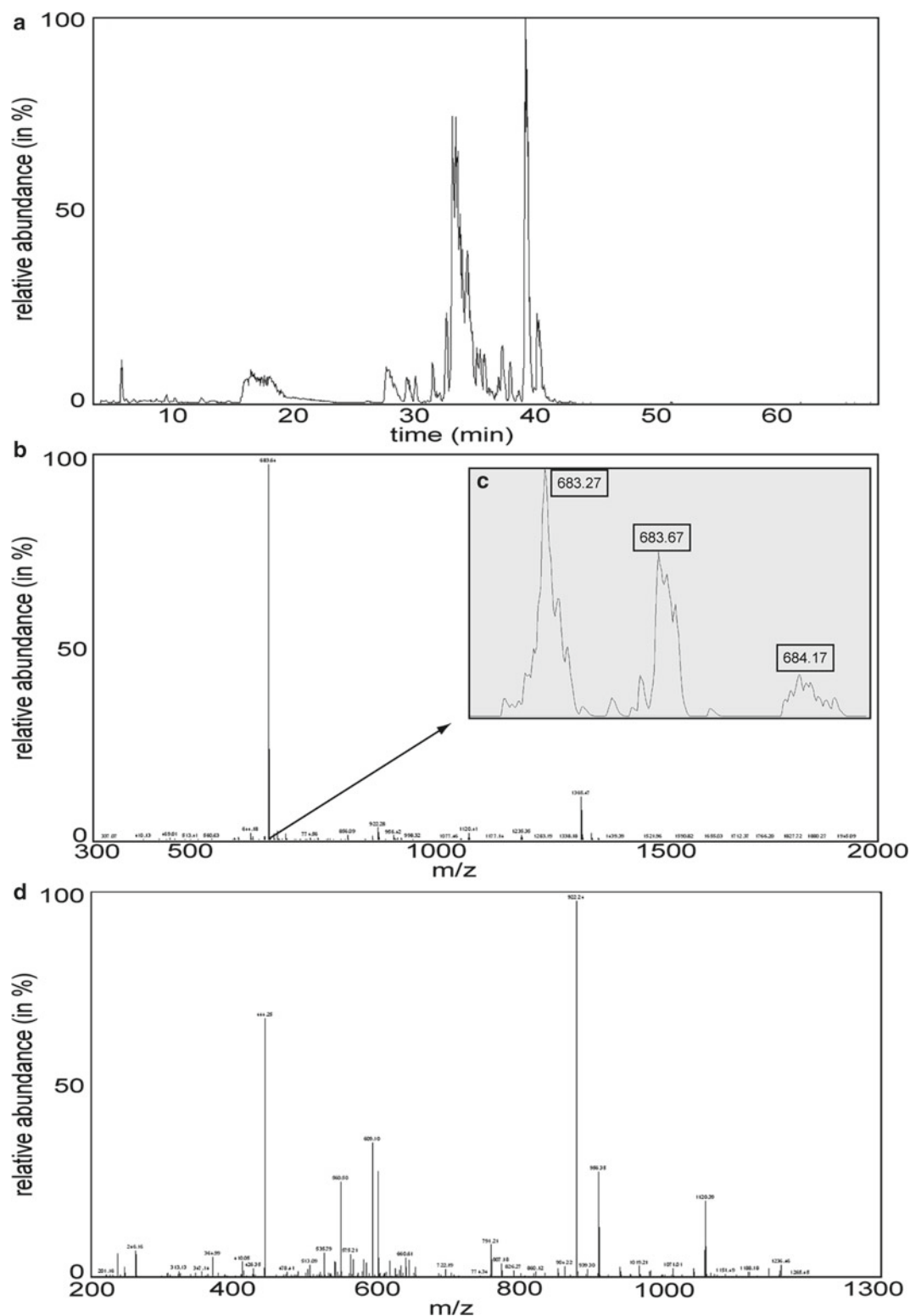


Fig. 4. This figure shows a chromatogram with all generated spectra in a data-dependent experiment. (a) Chromatogram intensity versus time, (b) mass spectra 300–2,000 Da, (c) zoomscan in the spectra (b) concentrated on one (m/z 683.1) mass, charge +2, and (d) MSMS-spectra 150–1,400 Da on this mass (parent ion m/z 683.56).

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