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Analysis of Electroblotted Proteins by Mass Spectrometry

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Summary

Identification of proteins by mass spectrometry is crucial for better understanding of many biological, biochemical, and biomedical processes. Here we describe two methods for the identification of electroblotted proteins by on-membrane digestion prior to analysis by mass spectrometry. These on-membrane methods take approximately half the time of in-gel digestion and provide better digestion efficiency, due to the better accessibility of the protease to the proteins adsorbed onto the nitrocellulose, and better protein sequence coverage, especially for membrane proteins where large and hydrophobic peptides are commonly present.

Keywords

On-membrane digestion; mass spectrometry; nitrocellulose membranes; tryptic digestion; protein identification; protein coverage; membrane proteins

1. Introduction

Identification of gel-separated proteins is commonly followed by in-gel enzymatic digestion of the proteins immobilized in the gel followed by analysis of the digested peptides by mass spectrometry ***(1)***. However, the effectiveness of in-gel digestion can be compromised by (a) the limited accessibility of the enzyme or the chemical cleavage to the proteins embedded into the gel and (b) the low recovery of large and/or hydrophobic digested peptides from the gel ***(2, 3)***.

Different alternatives based on the electroblotting of the proteins in a gel onto nitrocellulose or poly(vinyldifluoride) (PVDF) membranes followed by on-membrane digestion have been developed over the years ***(4, 5)***. One of the advantages of electroblotting the proteins is that after the transfer, the proteins are adsorbed onto the membrane and therefore, they are more available to the proteases, thus allowing more efficient digestions ***(2)***and solving one of the limitations associated to in-gel digestion.

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The peptides generated by on-membrane digestion can be analyzed by MS using several different approaches: (a) They can be directly analyzed on the membrane by MALDI-MS ***(6-8)***. In this case the matrix solution used to dissolve the nitrocellulose-bound peptides is crucial in order for the peptides to be incorporated properly into the matrix crystals. (b) The peptides can be extracted from the membrane ***(9, 10)***. This approach, as for in-gel digestion, has an extraction step that may result in low recovery of large and/or hydrophobic peptides due to the strong protein-membrane binding ***(11)***. (c) They can be dissolved together with the membrane. In this approach, nitrocellulose membranes are used exclusively. It is based on the dissolution of the nitrocellulose in an organic solvent and thus, of the peptides adsorbed onto it. This last approach overcomes the limitations associated with in-gel digestion commented on before because it bypasses the low-yield step of peptide extraction. This approach was first described by Liang et al. ***(12)*** using MALDI matrix solution prepared in 100% acetone to dissolve the nitrocellulose membrane followed by MS analysis of the digested peptides. However, the 100% acetone used to dissolve the nitrocellulose can lead to partial peptide precipitation resulting in loss of peptides and thus, decreased sensitivity.

Here we describe two methods ***(2)*** in which most of the steps involved in the procedure are identical (those steps have been described just once in the text); the methods only vary in the way in which the peptides generated by on-membrane digestion are recovered for MS analysis. The "direct dissolution method" is an improvement of the method described by Liang et al ***(12)*** and it uses a mixture of acetonitrile:methanol instead of 100% acetone to prepare the MALDI matrix solution, therefore minimizing protein loss most likely due to precipitation. On the other hand, in the "nitrocellulose-free method", the nitrocellulose is removed from the sample before MS analysis allowing the possibility of using (LC)-ESI-based mass spectrometers and increasing the sensitivity.

The methods described here are suitable for either soluble or membrane proteins although they are especially recommended for the study of the latter, which can be very problematic due to the presence of hydrophobic domains. It is also noteworthy that the on-membrane methods take approximately half the time of conventional in-gel digestion, and can provide more efficient tryptic digestions, thus producing fewer missed cleavage peptides.

2. Materials

2.1. SDS-PAGE and Electroblotting

1. Separating buffer (10X): 1.5 *M* Tris-HCl, pH 8.8, 1% SDS. Store at room temperature. (*See* **Note 1**).
2. Stacking buffer (10X): 0.5 *M* Tris-HCL, pH 6.8, 1% SDS. Store at room temperature.
3. Thirty percent acrylamide/bis solution (29:1 acrylamide monomer:cross-linker ratio) (Bio-Rad, Hercules, CA) (this is a neurotoxin when unpolymerized so care should be taken to prevent skin contact) Store at 2-8°C.

[1]All solutions and buffers should be prepared in MilliQ water or HPLC-grade water (referred as "water" in this text).

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4. N.N.N.N′-Tetramethyl-ethylenediamine (TEMED, Bio-Rad, Hercules, CA). Store at room temperature in a desiccator.
5. Ammonium persulfate: prepare 10% solution in water and immediately freeze in single use aliquots at −20°C.
6. Isopropanol. Store at room temperature.
7. Laemmli sample buffer (Bio-Rad, Hercules, CA). Store at room temperature.
8. Running buffer (5X): 125 m*M* Tris, 960 m*M* glycine, 0.5% SDS, pH 8.3. Store at room temperature.
9. Kaleidoscope prestained molecular weight markers (Bio-Rad, Hercules, CA). Store at −20°C.
10. 100% Triton-free nitrocellulose membranes (pore size, 0.2 μm) (Bio-Rad, Hercules, CA). (*See* **Note 2**)
11. 3 mm Whatman chromatography paper (Whatman International, U.K.).
12. Transfer buffer: 25 m*M* Tris, 190 m*M* glycine, 20% methanol, 0.05% SDS, pH 8.3. Store at room temperature.

2.2. Membrane staining and destaining

2.2.1. Ponceau-S staining

1. Ponceau-S solution: 5% acetic acid, 0.2% Ponceau-S (w/v) (Sigma, St. Louis, MO). Store at room temperature.

2.2.2. MemCode™ staining

1. MemCode™ reversible protein stain kit for nitrocellulose membranes (Pierce, Rockford, IL). Store at room temperature.

2.3. Blocking of the membrane before digestion

1. PVP-40 solution: 100 m*M* acetic acid, 0.5% poly(vinylpyrrolidone) (PVP-40) (w/v) (Sigma, St. Louis, MO).

2.4. On-membrane digestion

1. Digestion buffer: 50 m*M* ammonium bicarbonate, pH 7.8. Use freshly prepared buffer.
2. Trypsin Gold, Mass Spectrometry Grade (Promega, Madison, WI). Store the lyophilized powder at −20°C. Reconstitute in 50 m*M* ammonium bicarbonate (pH 7.8) and store in single use aliquots at −20°C (*see* **Note 3**).

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[2]Make sure the nitrocellulose membrane used is 100% Triton-free to provide the best results for mass spectrometry analysis.
[3]Reconstituted trypsin can be stored at −20°C for up to one month. For long-term storage, freeze reconstituted trypsin at −70°C. Before use, thaw the reconstituted trypsin at room temperature, placing on ice immediately after thawing. To maintain sufficient enzymatic activity, limit the number of free-thaw cycles to 5.

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2.5. Peptide recovery

1. HPLC-grade acetone, acetonitrile and methanol (Fisher Scientific, Morris Plains, NJ).

2.6. Mass Spectrometry analysis

2.6.1. MALDI-TOF

1. α-cyano-4-hydroxycinnamic acid (α-CHCA) (Sigma, St. Louis, MO). Store at −20°C. (*See* **Note 4**).

2.6.2. (LC)-ESI-MS/MS

1. Sample solution: 2% acetonitrile, 0.1% formic acid

3. Methods

As with any sample preparation method for MS analysis, special attention must be paid to avoid loss or contamination of the samples during processing. Please, be very careful not to use bare hands, loose hair, dirty glass- and plastic-ware and always wear gloves (powder free and rinsed with water and ethanol before use) to eliminate contamination by keratins, or these proteins will overwhelm low level protein samples and preclude successful identification of the proteins of interest.

3.1. SDS-PAGE and Electroblotting

1. Prepare a 1 mm thick 10% gel (8 × 7.3 cm) by mixing 1 mL of separating buffer with 1 mL acrylamide/bis solution, 1.94 mL water, 15 μL ammonium persulfate solution, and 5 μL TEMED. Pour the gel, leaving space for the stacking gel, and overlay with isopropanol. The gel should be polymerized in about 30 min.
2. Pour off the isopropanol and rinse the top of the gel with water.
3. Prepare the stacking gel by mixing 0.5 mL of the stacking buffer with 0.2 mL acrylamide/bis solution, 1.272 mL water, 7.5 μL ammonium persulfate, and 2.5 μL TEMED. Use about 0.5 mL of this to quickly rinse the top of the gel and then pour the stacking solution and insert the comb. The gel should polymerize overnight to eliminate cross-linking of unpolymerized acrylamide to proteins during electrophoresis (*see* **Note 5**).
4. Prepare the running buffer by diluting 200 mL of the 5X running buffer with 800 mL of water.
5. Mix the sample with the Laemmli sample buffer 1:1, vortex, centrifuge and incubate at 95°C for 5 min to denature the proteins.

[4]If the dry matrix has a mustard-yellow color instead of bright yellow color, it may contain impurities. To recrystallize (purify), dissolve the CHCA matrix in warm ethanol (exact temperature is not important). Filter and add two volumes of deionized water. Let stand overnight in an explosion proof-refrigerator. Filter and wash the precipitate with cold water.

[5]Unpolymerized acrylamide cross-linked to proteins may interfere with tryptic digestion. Thus, gels should be prepared the day before the experiment is carried out. Gels can be stored at 4°C for several days wrapped in a sturdy lab tissue paper (suck as Kimwipes) wetted with running buffer and sealed with plastic wrap to prevent drying.

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6. Add the running buffer to the chambers of the gel unit and carefully remove the comb. Load the sample in a well. Include one well for pre-stained molecular weight markers.
7. Complete the assembly of the gel unit and connect to a power supply. Run the gel at 50 V until all the sample enters the gel (around 20 min) and then increase voltage to 80-100 V (maintain below 20 mA) until the dye front reaches the bottom of the gel.
8. Prepare a tray of water large enough to lay out a transfer cassette with its two pieces of foam. Cut a sheet of nitrocellulose slightly larger than the size of the separating gel.
9. Wet a sheet of Whatman chromatography paper of approximately the same size as the nitrocellulose membrane and place it on top of one of the wet foam sheets. Then submerge the nitrocellulose sheet on top of the Whatman paper.
10. Disassemble the gel unit. Remove the stacking gel and discard. After briefly rinsing the separating gel with water, place it on top of the nitrocellulose membrane.
11. Wet an additional sheet of Whatman paper and carefully place it on top of the gel, ensuring no bubbles are trapped in the resulting sandwich. Place the second wet foam sheet on top and close the transfer cassette.
12. Place the cassette into the transfer tank such that the nitrocellulose membrane is between the gel and the anode. It is very important to ensure this orientation to avoid the loss of the proteins into the transfer buffer instead of being electrotransferred to the nitrocellulose membrane.
13. Place the transfer tank into an ice bath, put on the lid and activate the power supply. Carry out the transfer at 100 V for 1 h.
14. Once the transfer is complete, disassemble the cassette, discard the gel and the Whatman sheets. Transfer the nitrocellulose membrane to a clean tray and rinse briefly with water. The colored molecular weight markers should be clearly visible on the membrane.

3.2. Membrane staining and destaining

Either of two different protein stains, both of them reversible and MS-compatible, can be used. While Ponceau-S is less expensive, it has a limit of detection of ~250 ng of protein on the membrane. MemCode™ is more sensitive, with a limit of detection of ~25 ng of protein on the membrane. Thus, the staining procedure should be selected according to the concentration of the protein/s of interest.

3.2.1. Ponceau-S staining

1. Add 10 mL of Ponceau-S solution to the membrane. Agitate at room temperature for 1-2 min on a rotary platform shaker or until the stained proteins appear as red bands. (*See* **Note 6**).

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2. Remove the Ponceau-S solution and wash the membrane with water several times until the background staining is completely removed.

3. Excise the band/s of interest with a new clean scalpel or razor blade and transfer to clean 1.5 mL Eppendorf tubes.

4. Wash several times with 1 mL of water for 15 min with agitation (1000 rpm) on a thermomixer at room temperature until the stain is completely removed. (If not digested immediately, membrane samples should be stored at −20°C in water).

3.2.2. MemCode™ staining

1. Rinse the nitrocellulose membrane with water and quickly decant. (*S*ee **Note 6**).

2. Add ~25 mL of MemCode™ reversible protein stain to the membrane. Agitate for 30 seconds on a platform shaker at room temperature. Stained proteins appear as turquoise-blue bands.

3. To remove the background, add ~25 mL MemCode™ destaining reagent to the nitrocellulose membrane and quickly decant. Repeat this step two additional times.

4. Add ~25 mL of the MemCode™ destain reagent to the membrane and gently agitate for 5 min on a rotary platform shaker at room temperature.

5. Decant the destaining solution and rinse the membrane four times by adding water to the tray and quickly decanting.

6. Wash the membrane with water for 5 min with agitation.

7. Excise the band/s of interest with a new clean scalpel or razor blade and transfer to 1.5 mL eppendorf tubes. Add 1 mL of MemCode™ stain eraser to the membrane and agitate for 2 min on a thermomixer at 1000 rpm and room temperature (2 min of agitation with stain eraser is optimal for most proteins but it may be extended to 5 min).

8. Rinse the membrane four times by adding 1 mL of water and quickly decanting.

9. Wash the membrane with water for 5 min with agitation using a thermomixer at room temperature and 1000 rpm. (If not digested immediately, samples should be stored at −20°C in water).

3.3. Blocking of the membrane before digestion

1. Remove the water and add 1 mL of PVP-40 solution to block nonspecific protein (trypsin) binding sites on the nitrocellulose. Incubate at 37°C for 30 min with gentle agitation using a thermomixer at 300 rpm.

2. Wash the membrane 6-10 times with 1 mL of water to remove excess PVP-40.

3. Transfer the nitrocellulose membranes to a 200 μL eppendorf tube (PCR style).

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[6]For all steps, use sufficient volumes to completely immerse the membrane, and agitate at a moderate speed on a rotary platform shaker. Do not allow the membrane to become dry during the procedure.

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3.4. On-membrane digestion

1. Add 20 μL of trypsin at 12.5 ng/ μL in 50 m*M* ammonium bicarbonate buffer and add enough digestion buffer to completely cover the gel slices (~20 μL).
2. Incubate at 37°C for 5 h or overnight with gentle agitation using a thermomixer at 300 rpm. Cap the tubes tightly and cover with parafilm to avoid evaporation.
3. After completing the digestion, dry the samples under vacuum.

3.5. Recovery of peptides

There are two different methods for recovering the peptides for MS analysis. The first one (direct dissolution method) is based on the direct dissolution of the nitrocellulose membrane, and thus of the peptides adsorbed onto it, in the MALDI matrix solution. This method can be used only for MALDI-based mass spectrometers. The second method (nitrocellulose-free method) is based on the removal of the nitrocellulose before MS analysis. This second method takes longer but it can be used for either MALDI- or (LC)-ESI-based mass spectrometers.

3.5.1. Direct dissolution method

1. Prepare the MALDI matrix solution consisting of 10 mg of α-CHCA, 300 μL of methanol, 700 μL of acetonitrile and 10 μL of TFA.
2. Add approximately 10 μL of MALDI matrix solution per mm^2 of nitrocellulose (40 μL for a typical 4 mm^2 protein band).
3. Vortex and sonicate for 10 min.
4. Spot 1 μL of the final solution onto the MALDI plate for MS analysis.

3.5.2. Nitrocellulose-free method

1. Add 90 μL of acetone per 4 mm^2 of nitrocellulose. Vortex and incubate for 30 min at room temperature to allow complete dissolution of the nitrocellulose and precipitation of the tryptic peptides adsorbed onto it. The nitrocellulose should dissolve completely. Add additional acetone if necessary.
2. Centrifuge 10 min at 14000 × g. and carefully remove the acetone containing the dissolved nitrocellulose. Air-dry the precipitated peptides.
3. Resuspend the peptides. For MALDI-MS analysis, add 20 μL of MALDI matrix solution (10 mg/mL of α-CHCA prepared in 50:50 acetonitrile:water and 1% TFA). For LC-MS/MS analysis, add 20 μL of sample solution (2% acetonitrile, 0.1% formic acid).
4. Sonicate 10 min.

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3.6. Mass Spectrometry analysis

3.6.1. MALDI-TOF

1. Calibrate the instrument in reflectron mode using molecular weight standard peptides such as angiotensin I (average mass, 1296.5 Da) and corticotropin-like intermediate lobe peptide (ACTH clip 18-39, average mass, 2465.7).
2. Analyze the samples of interest. Typically, 100-200 laser shots are summed into each mass spectrum. Mass spectra should be acquired in an interactive (manual) mode that allows modifying the laser energy and other key acquisition parameters as well as the visual selection of the sample area targeted to obtain a good signal-to-noise ratio (*see* **Note 7**). Examples of results obtained using this method as compared to in-gel digestion are shown in **Fig. 1** and **Fig. 2**.

3.6.2. (LC)-ESI-MS/MS

1. The sample is transferred to a sample vial compatible with the autosampler of the liquid chromatograph, or injected manually into the HPLC. Some remaining nitrocellulose may precipitate in the presence of water in the final solution. Transfer the sample carefully to the sample vial avoiding the transfer of the nitrocellulose which otherwise could cause clogging of the LC system. (*See* **Note 8**).
2. The following instructions assume the use of a CapLC (Waters) HPLC system coupled directly to a Q-TOF micro (Micromass) mass spectrometer, or similar instruments. Load the peptides onto a 0.3 × 1-mm C18 nano-precolumn and wash 5 min with 2% acetonitrile in 0.1% formic acid at a flow rate of 20 μL/min.
3. After washing, reverse the flow through the precolumn and elute the peptides with a gradient of 2-90% acetonitrile in 0.1% formic acid. The gradient is delivered over 120 min at a flow rate of 200 nL/min, using a 15:1 precolumn flow split, through a 75 μm × 15 cm fused silica capillary C18 HPLC column to a fused silica distal end-coated tip nano electrospray needle. The electrospray needle can vary depending on the ion source of the mass spectrometer.
4. Use a database search engine such as Mascot (Matrix Science) for database searching and protein identification.

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[7]Usually, the laarge amount of dissolved nitrocellulose in the sample creates a heterogeneous crystal. Best signal is usually obtained in those areas with the least nitrocellulose. Those with large amounts of nitrocellulose require greater laser energy to provide a good signal. Also, after selecting an area, the first shots may provide a low signal-to-noise ratio that will increase with the number of shots.

[8]To ensure that remaining traces of nitrocellulose do not reach the analytical column, we strongly recommend the use of a precolumn between the injection valve and the analytical column in the LC system.

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References

1. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 1996; 68:850–858. [PubMed: 8779443]
2. Luque-Garcia JL, Zhou G, Sun TT, Neubert TA. Use of nitrocellulose membranes for protein characterization by matrix-assisted laser desorption/ionization mass spectrometry. Anal. Chem. 2006; 78:5102–5108. [PubMed: 16841935]
3. Jonsson AP, Aissouni Y, Palmberg C, Percipalle P, Nordling E, Daneholt B, Jornvall H, Bergman T. Recovery of gel-separated proteins for in-solution digestion and mass spectrometry. Anal. Chem. 2001; 73:5370–5377. [PubMed: 11816562]
4. Bai J, Qian MG, Liu Y, Liang X, Lubman DM. Peptide Mapping by CNBr Degradation on a Nitrocellulose Membrane with Analysis by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. Anal. Chem. 1995; 67:1705–1710.
5. Dukan S, Turlin E, Biville F, Bolbach G, Touati D, Tabet JC, Blais JC. Coupling 2D SDS-PAGE with CNBr cleavage and MALDI-TOFMS: a strategy applied to the identification of proteins induced by a hypochlorous acid stress in Escherichia coli. Anal. Chem. 1998; 70:4433–4440. [PubMed: 9796426]
6. Schleuder D, Hillenkamp F, Strupat K. IR-MALDI-mass analysis of electroblotted proteins directly from the membrane: comparison of different membranes, application to on-membrane digestion, and protein identification by database searching. Anal. Chem. 1999; 71:3238–3247. [PubMed: 10450165]
7. Strupat K, Karas M, Hillenkamp F, Eckerson C, Lottspelch F. Matrix-assisted laser desorption ionization mass spectrometry of proteins electroblotted after polyacrylamide gel electrophoresis. Anal. Chem. 1994; 66:464–470.
8. Vestling MM, Fenselau C. Poly(vinylidene difluoride) membranes as the interface between laser desorption mass spectrometry, gel electrophoresis, and in situ proteolysis. Anal. Chem. 1994; 66:471–477.
9. van Oostveen I, Ducret A, Aebersold R. Colloidal silver staining of electroblotted proteins for high sensitivity peptide mapping by liquid chromatography-electrospray ionization tandem mass spectrometry. Anal. Biochem. 1997; 247:310–318. [PubMed: 9177693]
10. Bunai K, Nozaki M, Hamano M, Ogane S, Inoue T, Nemoto T, Nakanishi H, Yamane K. Proteomic analysis of acrylamide gel separated proteins immobilized on polyvinylidene difluoride membranes following proteolytic digestion in the presence of 80% acetonitrile. Proteomics. 2003; 3:1738–1749. [PubMed: 12973734]
11. Jorgensen CS, Jagd M, Sorensen BK, McGuire J, Barkholt V, Hojrup P, Houen G. Efficacy and compatibility with mass spectrometry of methods for elution of proteins from sodium dodecyl sulfate-polyacrylamide gels and polyvinyldifluoride membranes. Anal. Biochem. 2004; 330:87–97. [PubMed: 15183766]
12. Liang X, Bai J, Liu YH, Lubman DM. Characterization of SDS--PAGE-separated proteins by matrix-assisted laser desorption/ionization mass spectrometry. Anal. Chem. 1996; 68:1012–1018. [PubMed: 8651486]

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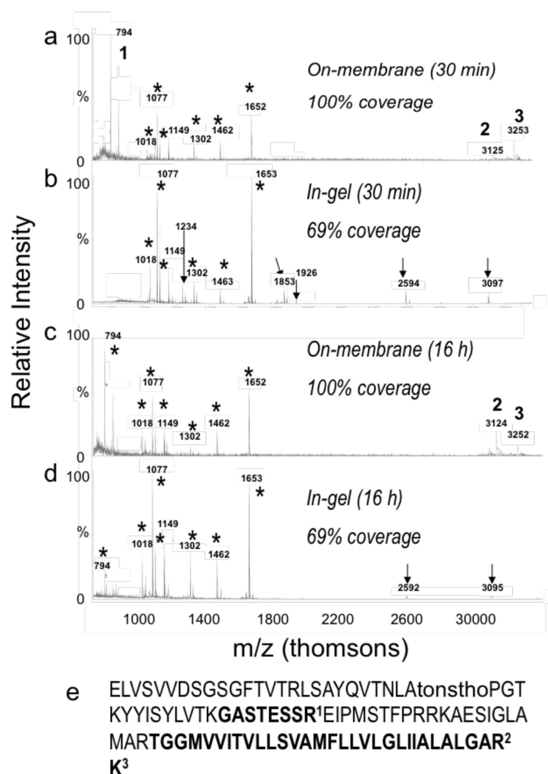

Figure 1.

Comparison of MALDI MS spectra obtained from 10 pmol of uroplakin II (UPII) after (a) 30 min on-membrane digestion, (b) 30 min in-gel digestion, (c) 16 h on-membrane digestion, (d) 16 h in-gel digestion. (e) Amino acid sequence of bovine mature UPII. The underlined amino acids correspond to the transmembrane domain of the protein. In the spectra, the stars indicate UPII peptides detected after both in-gel and on-membrane digestion, arrows indicate missed cleavage peptides that appear only after in-gel digestion, and the numbers indicate peptides from the UPII sequence shown in (e) detected only after on-membrane digestion. (Reproduced from **ref. *2*** with permission from the American Chemical Society).

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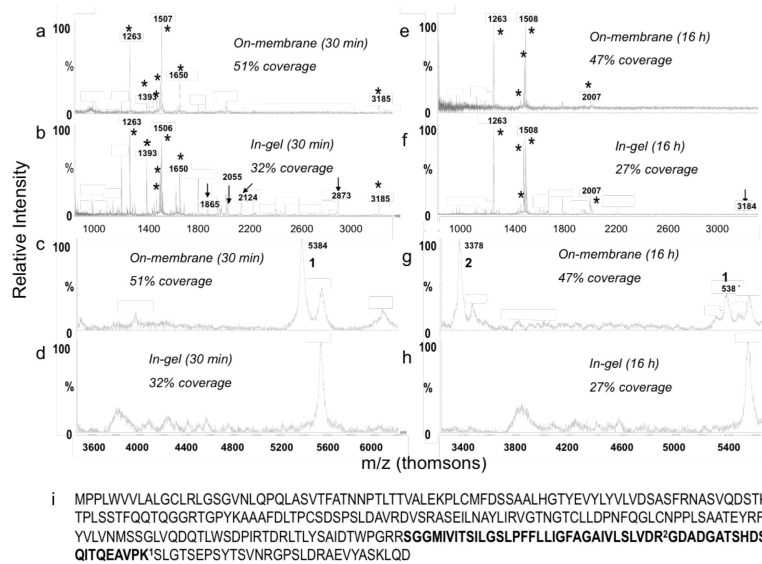

Figure 2.

Comparison of MALDI MS spectra obtained from 3 pmol of uroplakin III (UPIII) after (a, c) 30 min on-membrane digestion, (b, d) 30 min in-gel digestion, (e, g) 16 h on-membrane digestion, (f, h) 16 h in-gel digestion. (i) Amino acid sequence of bovine UPIII. The underlined amino acids correspond to the transmembrane domain of the protein. In the spectra, the stars indicate UPII peptides detected after both in-gel and on-membrane digestion, arrows indicate missed cleavage peptides that appear only after in-gel digestion, and the numbers indicate peptides from the UPII sequence shown in (i) detected only after on-membrane digestion. Spectra shown in a, b, e and f were collected in reflectron mode and spectra shown in c, d, g, and h in linear mode. (Reproduced from **ref. *2*** with permission from the American Chemical Society).