PROTOCOL

"De Novo" Sequencing of Peptides Recovered from In-Gel Digested Proteins by Nanoelectrospray Tandem Mass Spectrometry

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Abstract

Proteins separated by one-dimensional or two-dimensional gel electrophoresis can be digested in-gel with trypsin and the recovered peptides can be sequenced *de novo* using triple quadrupole or hybrid quadrupole time-of-flight instruments equipped with a nanoelectrospray ion source. The peptide sequences determined provide useful information for identification of proteins by homology searching for cloning of the cognate genes by PCR based approaches.

Index Entries: Proteomics; nanoelectrospray mass spectrometry; tandem mass spectrometry; protein sequencing.

1. Introduction

Recent developments in technology and instrumentation have made mass spectrometry the method of choice for the identification of gel separated proteins in rapidly growing sequence databases *(1–3)*. Proteins with a full-length sequence present in a database can be identified with high certainty and high throughput using the accurate peptide masses determined by matrix-assisted laser desorption/ionization (MALDI) peptide mapping *(4,5)*. Only 1–3% of a total digest is typically consumed for MALDI analysis even if the protein of interest was present on a gel in subpicomole amount. If no conclusive identification has been achieved, the remaining digest can be further analyzed by nanoelectrospray tandem mass spectrometry (nanoES MS/MS) *(6)*. NanoES MS/MS allows identification of proteins in complex mixtures *(7)* and can pinpoint relevant clones in an EST database *(8)* if the full-length sequence of the protein is not available. Importantly, it is not necessary to determine the complete sequence of peptides in order to search a database. A sequence stretch consisting of three to four amino acid residues provides enough search specificity when combined with the intact mass of the peptide and the masses of corresponding fragment ions in a peptide sequence tag *(9)* (**Subheading 3.4.**). Furthermore, proteins not present in a database that are, however, homologous to a known protein can be identified by modified BLAST homology searching protocol *(10)*.

The emerging MALDI Quadrupole Time-of-Flight mass spectrometry enables to combine rapid peptide mass fingerprinting of a protein digest with extensive characterization of individual peptide precursors by low-energy collision-induced dissociation *(11)*. Proteins can be identified in all types of sequence databases either by peptide mass mapping or by tandem mass spectra acquired from multiple peptide precursors in the course of a single experiment *(12)*.

However, despite the success of ongoing genomic sequencing the demand for *"de novo"* peptide sequencing has not been eliminated. Long and accurate peptide sequences are required for

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the cloning of new genes. Degenerate oligonucleotide probes are designed on the basis of peptide sequences obtained in this way, and subsequently used in polymerase chain reaction-based cloning strategies.

Continuous series of fragment ions containing the C-terminus (Y" ions) *(13)* which are usually observed in tandem mass spectra of tryptic peptides have been successfully used for *de novo* sequencing *(14)*. The peptide sequence can be deduced by considering precise mass differences between the adjacent Y"- ions. However, it is necessary to obtain additional evidence that the particular fragment ion indeed belongs to the Y"-series. To this end, a separate portion of the digest is esterified using 2 *M* HCl in anhydrous methanol (**Fig. 1A**) (**Subheading 3.2.**). Upon esterification, a methyl group is attached to the C-terminal carboxyl group of each peptide, as well as to the carboxyl group in the side chain of aspartic and glutamic acid residues. Therefore the m/z of each peptide ion is shifted by 14(n + 1)/ z, where n is the number of aspartic and glutamic acid residues in the peptide, and z is the charge of the peptide ion. The derivatized digest is then also analyzed by nanoES MS/MS, and for each peptide, fragment ion spectra acquired from underivatized and derivatized forms are matched. An accurate peptide sequence is called by considering mass differences between the adjacent Y"-ions as well as characteristic mass shifts induced by esterification (**Subheading 3.4.1.**) (**Fig. 2**). Since esterification with methanol significantly shifts the m/z of Y"-ions (by 14, 28, 42, ... Da), it is possible to use low resolution settings when sequencing is performed on a triple quadrupole mass spectrometer, thus attaining high sensitivity on the instrument. The sequencing approach employing esterification is laborious and time consuming, and requires much expertise in the interpretation of tandem mass spectra. However, it allows the determination of accurate peptide sequences even from protein spots that can only be visualized by staining with silver *(15,16)*.

An alternative approach to de novo sequencing has become feasible after a novel type of mass spectrometric instrumentation—a hybrid quadrupole time-of-flight mass spectrometer ("Q-TOF") *(17)* or "QqTOF" *(18)* was introduced. Q(q)TOF instruments allow to acquire tandem mass spectra with very high mass resolution (> 10,000 full-width at half maximum [FWHM]) without compromising sensitivity. These features make it possible and practical to apply selective isotopic labeling of the peptide C-terminal carboxyl group in order to distinguish Y"- ions from other fragment ions in tandem mass spectra (**Subheading 3.4.2.**). Proteins are digested with trypsin in a buffer containing 50% of $H_2{}^{16}O$ and 50% $H_2{}^{18}O$ (v/v) (**Subheading 3.1.**) so that half of the resulting tryptic peptide molecules incorporate ^{18}O atoms in their C-terminal carboxyl group, whereas the other half incorporate ^{16}O atoms (**Fig. 1B**). During subsequent sequencing by MS/MS the entire isotopic cluster of each peptide ion, in turn, is selected by the quadrupole mass filter, and fragmented in the collision cell. Since only the fragments containing the C-terminal carboxyl group of the peptide appear to be partially (50%) isotopically labeled, Y"- ions are distinguished by a characteristic isotopic pattern—a doublet of peaks split by 2 mass units (**Subheading 3.4.2.**) (**Fig. 3**). Other fragment ions have normal isotopic distribution. Thus, only a single analysis is required, peptide sequence readout is much faster and the approach lends itself to automation *(19)*.

2. Materials (*see Note 1*)

2.1. In-gel Digestion (*see Note 2*)

1. 100 m*M* ammonium bicarbonate in water (high-performance liquid chromatography [HPLC] grade, [LabScan, Dublin, Ireland]).
2. Acetonitrile (HPLC grade [LabScan]).
3. 10 m*M* dithiotreitol in 100 m*M* ammonium bicarbonate.
4. 55 m*M* iodoacetamide in 100 m*M* ammonium bicarbonate.
5. 100 m*M* CaCl2 in water.
6. 15 µL aliquots of trypsin unmodified, sequencing grade (Roche Diagnostics GmbH, Mannheim, Germany) in 1 m*M* HCl (*see* **Note 3**).

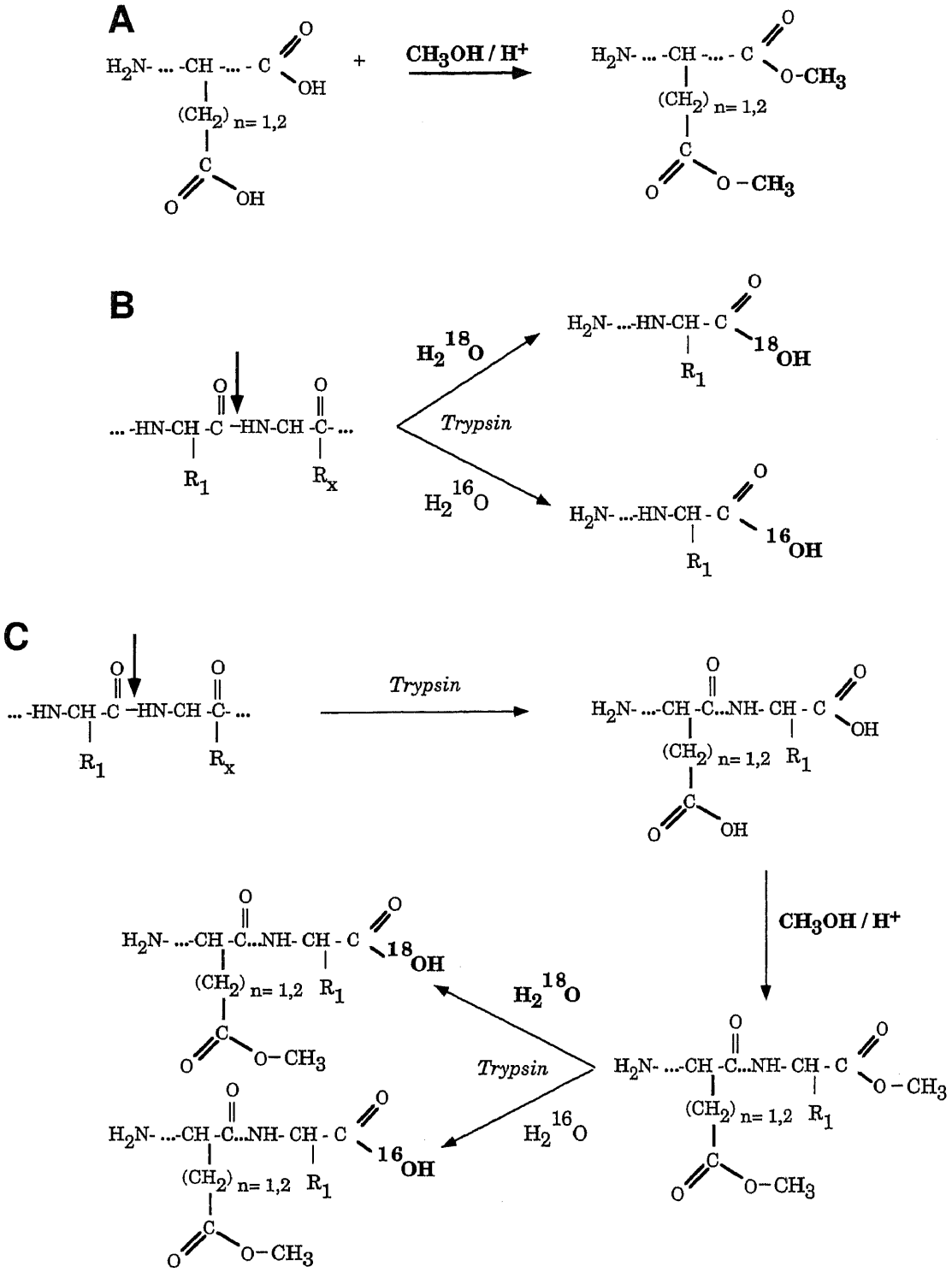

Fig. 1. Chemical derivatization for mass spectrometric *"de novo"* sequencing of peptides recovered from digests of gel separated proteins. **Panel A:** a protein is digested *in-gel* (*see* **Subheading 3.1.**) with trypsin and a portion of the unseparated digest is esterified by 2 *M* HCl in anhydrous methanol (*see* **Subheading 3.2.**). **Panel B:** Protein is digested in-gel with trypsin in the buffer containing 50% (v/v) of $H_2{}^{18}O$ and 50 % (v/v) $H_2{}^{16}O$ (*see* **Subheading 3.1**). **Panel C:** Protein is digested *in-gel* with trypsin, the digest is esterified and subsequently treated with trypsin in the buffer containing 50% (v/v) of $H_2{}^{18}O$ and 50% (v/v) $H_2{}^{16}O$ (*see* **Note 22**). Here R_1 stands for the side chain of arginine or lysine amino acid residues (representing trypsin cleavage sites), R_x stands for the side chain of any other amino acid residue except proline.

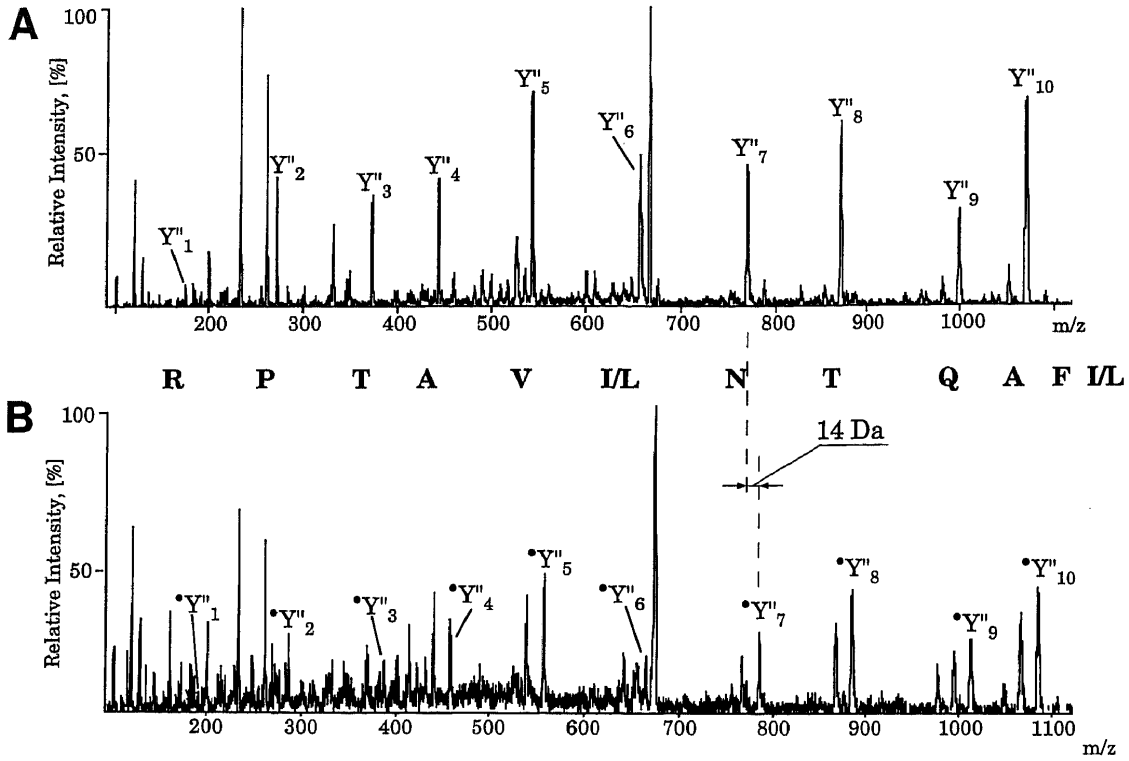

Fig.2. Peptide *"de novo"* sequencing by comparison of tandem mass spectra acquired from intact and esterified peptide. A 120 kDa protein from *E. aediculatis* was purified by one dimensional gel electrophoresis *(31)*, digested *in-gel* with trypsin and a part of the digest was analyzed by nanoES MS/MS on a API III triple quadrupole mass spectrometer (MDS Sciex, Ontario, Canada). A separate part of the digest was esterified and then also analyzed by nanoES MS/MS. **Panel A:** tandem mass spectrum acquired from the doubly charged ion with m/z 666.0 observed in the Q1 spectrum of intact digest. **Panel B:** matching tandem spectrum acquired from the ion with m/z 673.0 observed in the Q1 spectrum of esterified digest. The peptide sequence was determined by software assisted comparison of spectra in **panel A** and **panel B**. The only methyl group was attached to the C-terminal carboxyl of peptide molecule (designated as filled circle) and therefore the masses of Y"-ions in the spectrum in **panel B** were shifted by 14 Da compared with corresponding Y"-ions in the spectrum in **panel A**.

7. 5% formic acid in water (v/v).
8. Heating blocks at 56°C and at 37°C.
9. Ice bucket.
10. Laminar flow hood (optional) (*see* **Note 2**).

2.2. Esterification with Methanol

1. Methanol (HPLC grade), distilled shortly before the derivatization.
2. Acetyl chloride (reagent grade), distilled shortly before the derivatization (*see* **Note 4**).

2.3. Isotopic Labeling Using $H_2{}^{18}O$

1. Reagents as in **Subheading 2.1.**
2. $H_2{}^{18}O$ (Cambridge Isotopic Laboratories, Cambridge MA), distilled (*see* **Note 5**).

2.4. Desalting and Concentrating of In-gel Tryptic Digests Prior to the Analysis by nano ES MS/MS

1. 5% formic acid in water (v/v).
2. 60% methanol in 5% aqueous formic acid (v/v).
3. Perfusion sorbent POROS 50 R2 (PerSeptive Biosystems, Framingham, MA) (*see* **Note 6**).
4. Borosilicate glass capillaries GC120F-10 1.2 mm OD × 0.69 mm ID; (Harvard Apparatus Ltd, Edenbridge, UK) (*see* **Note 7**).
5. Purification needle holder, made as described in *(20)* or purchased from Protana (Odense, Denmark).
6. Benchtop minicentrifuge (as "PicoFuge," Stratagene, Palo Alto CA).

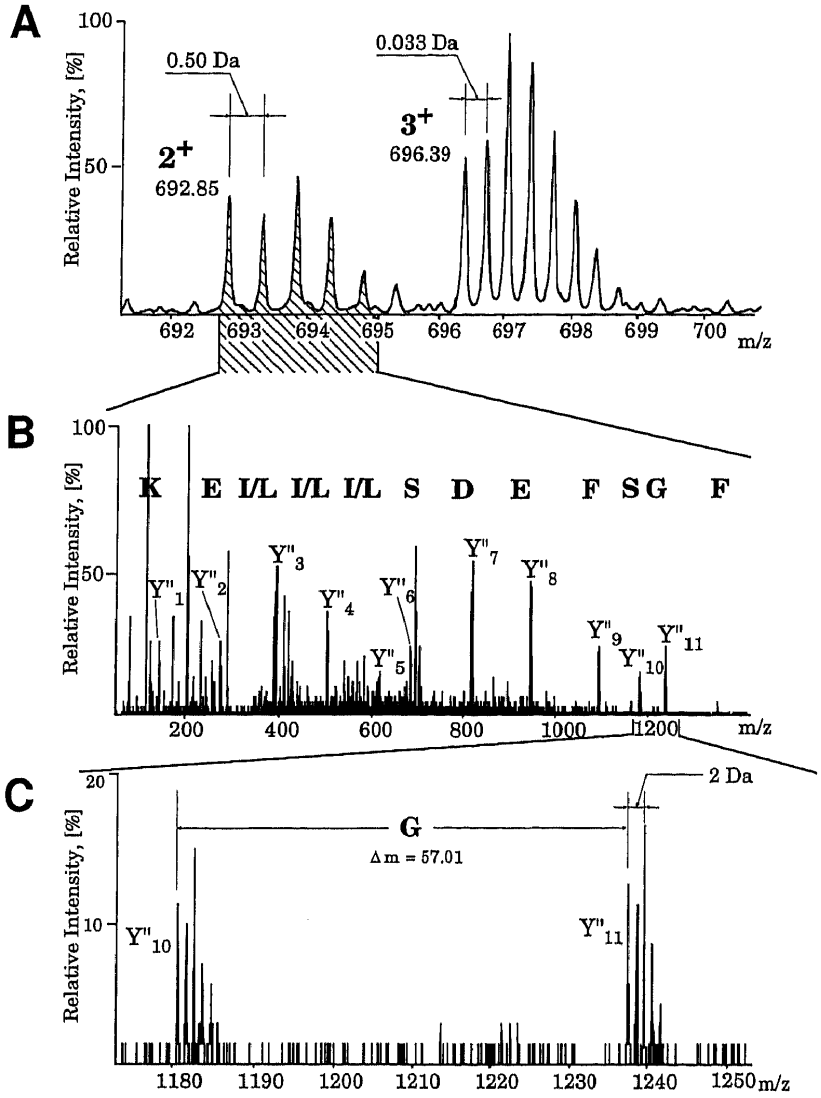

Fig. 3. Sequencing of ^{18}O C-terminally labeled tryptic peptides by Nano ES MS/MS. A 35 kDa protein from *Drosophila* was purified by gel electrophoresis, digested *in-gel* in a buffer containing 50% (v/v) $H_2{}^{18}O$ and analyzed using a QqTOF mass spectrometer (MDS Sciex, Ontario, Canada). A part of the spectrum of the unseparated digest is shown in **panel A**. Although the isotopic pattern of labeled peptides is relatively complex the high resolution of QqTOF instrument allowed to determine the charge of the ions. The entire isotopic cluster of doubly charged ion with m/z 692.85 was isolated by a quadrupole mass analyzer, transmitted to the collision cell and its fragment ion spectrum was acquired (**panel B**). **Panel C:** a zoom of the region of the fragment ion spectrum (**panel B**) close to m/z 1200. Isotopically labeled Y" ions are observed as doublets split by 2 Da. The peptide sequence was determined by considering the mass difference between adjacent ^{18}O-labeled Y"- ions.

3. Methods

3.1. In-gel Digestion (see Note 8)

3.1.1. Excision of Protein Bands (spots) from Gels

1. Rinse the entire gel with water. Excise bands of interest with clean scalpel cutting as close to the edge of the band as possible. Chop the excised bands into cubes (1 × 1 mm). Transfer gel particles into a microcentrifuge tube (0.5 mL or 1.5 mL Eppendorf test tube).

3.1.2. In-gel Reduction and Alkylation (see **Note 10**)

1. Wash gel pieces with 100–150 µL of water (5 min). Spin down and remove all liquid. Add

acetonitrile (the volume of acetonitrile should be at least two times larger than the volume of gel pieces) and wait for 10–15 min until gel pieces have shrunk (they become white and stick together). Spin gel pieces down, remove all liquid. Dry gel pieces down in a vacuum centrifuge.
2. Swell gel pieces in 10 m*M* dithiotreitol in 100 m*M* NH_4HCO_3 (add enough reducing buffer to cover gel pieces completely) and incubate for 30 min at 56°C to reduce the protein. Spin gel pieces down and remove excess liquid. Shrink gel pieces with acetonitrile. Replace acetonitrile with 55 m*M* iodoacetamide in 100 m*M* NH_4HCO_3. Incubate for 20 min at room temperature in the dark.
3. Remove iodoacetamide solution and wash the gel pieces as described in **Subheading 3.1.3.** Spin gel pieces down and remove all liquid. Shrink gel pieces with acetonitrile and remove all liquid. Dry gel pieces down in a vacuum centrifuge.

3.1.3. Additional Washing of Gel Pieces (see ***Note 11***)

1. Rehydrate gel pieces in 100–150 µL of 100 m*M* NH_4HCO_3 and after 10–15 min. add equal volume of acetonitrile (thus reaching the ratio 1:1 v/v of 100 m*M* NH_4HCO_3 /acetonitrile).
2. Vortex the tube for 20 min., spin gel pieces down and remove all liquid. Shrink gel pieces with acetonitrile and remove all liquid.
3. Dry gel pieces down in a vacuum centrifuge.

3.1.4. Application of Trypsin (see ***Note 12***)

1. Rehydrate gel pieces in the digestion buffer containing 50 m*M* NH_4HCO_3, 5 m*M* $CaCl_2$ and 12.5 ng/µL of trypsin at 4°C (use ice bucket) for 30–45 min. After 15–20 min check the samples and add more buffer if all liquid is absorbed by gel pieces.
2. Remove remaining buffer. Add 10–20 µL of the same buffer but prepared without trypsin to cover gel pieces and keep them wet during enzymatic cleavage.
3. Leave samples in a heating block at 37°C overnight.
4. To perform isotopic labeling of C-terminal carboxyl groups of tryptic peptides with ^{18}O atoms, prepare the buffers (both the buffer containing trypsin and the buffer without trypsin) in 50% of $H_2{}^{16}O$ and 50% $H_2{}^{18}O$ (v/v) (*see* **Note 12**).

3.1.5. Extraction of Peptides

1. Add 10–15 µL of water to the digest, spin gel pieces down and incubate at 37°C for 15 min in a thermomixer. Spin gel pieces down and add acetonitrile (add the volume two times larger than the volume of gel pieces). Incubate at 37°C for 15 min with shaking. Spin gel pieces down and collect the supernatant into the separate Eppendorf test tube.
2. Add 40–50 µL of 5% formic acid to gel pieces. Vortex and incubate for 15 min at 37°C with shaking. Spin gel pieces down and add equal volume of acetonitrile. Incubate at 37°C for 15 min with shaking. Spin gel pieces down, collect the supernatant and pool the extracts. Dry down pooled extracts in a vacuum centrifuge.

3.2. Esterification of In-gel Digests with Methanol

1. Put 1 mL of methanol (for the preparation of reagents *see* **Subheading** 2.2.) into a 1.5 mL Eppendorf test tube. Place the tube in a freezer with the temperature –20°C or lower for 15 min.
2. Take the tube from the freezer and immediately add 150 µL of acetyl chloride (Caution! Put on safety goggles and gloves! The mixture may boil up instantly!) . Leave the tube to warm up to room temperature and use the reagent 10 min later.
3. Add 10–15 µL of the reagent (*see* **Note 13**) prepared as in **step 2**, to a dried portion of the peptide pool recovered after in-gel digestion of the protein (*see* **Subheading 3.1.5.**).
4. Incubate for 45 min at room temperature.
5. Dry down the reaction mixture in a vacuum centrifuge.

3.3. Desalting and Concentration of In-gel Digest Prior to NanoES MS/MS Sequencing (see *Note 14*)

1. Pipet 5 µL of POROS R2 slurry prepared in methanol into the pulled glass capillary (here and further down referred as a "column"). Spin the beads down and then open the pulled end of the column by gentle touching against a bench

top. Wash the beads with 5 µL of 5% formic acid and make sure the liquid can easily be spinned out of the column by gentle centrifuging. Open the column end wider if necessary. Mount the column into the micropurification holder (*see* **Subheading 2.4.**).

2. Dissolve the dried digest (*see* **Subheading 3.1.5.**) or the esterified portion of the digest (*see* **Subheading 3.2.**) in 10 µL of 5% formic acid and load onto the column. Pass the sample through the beads layer by centrifuging.
3. Wash adsorbed peptides with another 5 µL of 5% formic acid.
4. Align the column and the nanoelectrospray needle in the micropurification holder and elute peptides directly into the needle with 1 µL of 60% of methanol in 5% formic acid by gentle centrifuging.
5. Mount the spraying needle with the sample into the nanoelectrospray ion source and acquire mass spectra (*see* **Note 15**).

3.4. Acquisition of Mass Spectra and Data Interpretation

1. Before the analysis, the tandem mass spectrometers—triple quadrupole or a quadrupole time-of-flight mass spectrometer should be tuned as discussed in **Note 16** and **Note 17**, respectively.
2. *In-gel* digestion using unmodified trypsin is accompanied by trypsin autolysis. Therefore it is necessary to acquire the spectrum of a control sample (blank gel pieces processed as described in **Subheading 3.1.**) in advance **(Note 18)**. Spectra should be acquired both in conventional single MS mode and in precursor ion scanning mode (as in **Subheading 3.4.1., step 1**)

3.4.1. Sequencing on a Triple Quadrupole Mass Spectrometer

1. After desalting and concentration (*see* **Subheading 3.3.**), initiate spraying and acquire Q1 spectrum of the peptide mixture. Turn on collision gas and acquire the spectrum in the precursor scan mode (scanning for precursor ions producing fragment ions with m/z 86 upon their collisional fragmentation) *(21)* (*see* **Note 19**).
2. Stop spraying by dropping spraying voltage to zero. Drop air pressure applied to the spraying capillary. Move the spraying capillary away from the inlet of the mass spectrometer.
3. Examine the acquired spectra and compare them with the spectra acquired from the control sample. Select precursor ions for subsequent tandem mass spectrometric sequencing.
4. Add 0.3–0.5 µL of 60% of methanol in 5% formic acid directly to the spraying capillary if the remaining sample volume is less than 0.5 µL. Reestablish spraying and acquire tandem mass spectra from selected precursor ions.
5. Interpret acquired spectra. A m/z region above the multiply charged precursor ion is usually free from chemical noise and is dominated by Y"-ions in tandem mass spectra of tryptic peptides. Therefore in this region it is relatively easy to retrieve short sequence stretches considering the masses of fragment ions. Assemble peptide sequence tags and perform database search using PeptideSearch software installed on a Macintosh computer or via Internet. Some servers also accessible over the web allow database searching using MS/MS spectra of peptides without preliminary interpretation (*see* **Note 20**).
6. If the protein turns out to be unknown (i.e., not present in a sequence database) take the remaining portion of the digest, esterify with methanol (**Subheading 3.2.**), redissolve in 10 µL of 5% formic acid, perform desalting and concentration (**Subheading 3.3.**) and sequence peptide peaks by nanoelectrospray as described above.
7. Correlate peptide molecular ions detected in the spectra of native and esterified digests (*see* **Note 21**). Deduce peptide sequences by comparison of tandem mass spectra of modified and unmodified peptides **(Fig. 2)**.

3.4.2. Sequencing of ^{18}O Labeled Peptides on a Quadrupole Time-of-Flight Mass Spectrometer

1. Perform nanoelectrospray analysis of in-gel digests as described for a triple quadrupole instrument (**Subheading 3.4.1. steps 1–4**; *see also* **Note 22**).
2. Interpret fragment spectra and deduce peptide sequences (*see* **Note 23**) **(Fig. 3)**.
3. Note that in principle only one set of acquired data is required to deduce the peptide sequence. However, if necessary, the remaining portion

of a digest could be esterified (**Subheading 3.2.**) and analyzed separately, thus allowing independent verification of the peptide sequence determined as described in **step 2** (*see* **Note 24**).

4. Notes

1. All chemicals should be of the highest degree of purity available. Solutions of dithiothreitol and iodoacetamide should be freshly prepared. It is recommended to use 50–100 mL stocks of water, ammonium bicarbonate buffer and acetonitrile and discard old solvents before starting the preparation of new series of samples. In our experience stock solutions rapidly accumulate dust, pieces of hair, threads, and so on from laboratory environment. Plastic ware (pipet tips, gloves, dishes, and so on) may acquire a static charge and also attract dust. Accumulation of even a minute amount of dust in solutions and reagents results in massive contamination of samples with human and sheep keratins and makes sequencing exceedingly difficult if not impossible. Any polymeric detergents (Twin, Triton, and so on) should not be used for cleaning the laboratory dishes and tools.
2. All possible precautions should be taken to avoid the contamination of samples with keratins and polymeric detergents (*see* **Note 1**). Gloves should be worn at all times during operations with gels (staining, documenting, excision of the bands or spot of interest) and sample preparation. It is necessary to rinse new gloves with water to wash away the talcum powder. It is also recommended to rinse gloves with water occasionally in a course of sample preparation since gloves with a static charge attract dust. In our experience it is advisable to perform all operations in a laminar flow hood, which helps to preserve a dust-free environment.
3. Add 250 µL of 1 m*M* HCl to the commercially available vial containing 25 µg of trypsin. Vortex the vial and aliquot trypsin stock solution in 0.5 mL Eppendorf test tubes (15 µL per each tube). Freeze the aliquots and store at –20°C before use. Unfreeze the aliquot shortly before the preparation of the digestion buffer. Discard the rest of the aliquot if its volume is not totally consumed. Surplus digestion buffer containing trypsin (**Subheading 3.1.4.** and *see also* **Note 12**) should be also discarded.
4. A glass tubing filled with calcium chloride or molecular sieves should be used to protect acetyl chloride during distillation.
5. Commercially available $H_2{}^{18}O$ has chemical purity of *ca.* 95% and is unsuitable for protein sequencing by mass spectrometry. Therefore 0.5 mL portion of $H_2{}^{18}O$ is purified by microdistillation in a sealed glass apparatus and stored at –20°C in 15 µL aliquots until used. Each aliquot is used only once.
6. Methanol (1 mL) is added to 30 µL of POROS R2 resin to prepare a slurry. A submicrometer fraction of the resin beads, whose presence increases the resistance to liquid flow, is removed by repetitive sedimentation. Vortex the test tube containing the slurry and then let it stay in a rack until the major part of the resin reaches the bottom of the tube. Aspirate the supernatant with pipet and discard it. Repeat the procedure 3–5 times if necessary.
7. Capillaries for micropurification are manufactured in the same way as capillaries for nanoelectropsray *(22)* but are not coated with a metal film.
8. The *in-gel* digestion procedure described in **Subheading 3.1.** *(23)* is applied with no modifications to spots (bands) excised from one-dimensional (two-dimensional) polyacrylamide gels stained with Coomassie Brilliant Blue R 250 or G 250, as well as to silver stained (*see* **Note 9**) or negatively stained gels *(24)*. Bands (spots) excised from negatively stained gels should be fixed and destained by incubating in 45:2:45 (v/v/v) methanol: acetic acid: water for 15 min.
9. Any convenient protocol for silver staining can be employed to visualize proteins present on a gel in subpicomole amount. However, the reagents used to improve the sensitivity and the contrast of staining must not modify proteins covalently. Thus treatment of gels with the crosslinking reagents as glutaraldehyde or strong oxidizers such as chromates and permanganates should be avoided. Note that the intensity of silver staining strongly depends on the time for which the gel was exposed to developing solution *(25)*. It is therefore recommended to allo-

cate two lanes on a one-dimensional gel for a protein standard (for example, 100 fmols and 500 fmols of bovine serum albumin). Semi-quantitative estimation of the amount of protein available for sequencing helps to choose optimal sequencing strategy.

10. *In-gel* reduction and subsequent alkylation of free SH-groups of cysteine residues is recommended even if proteins have been reduced prior to electrophoresis. Note that sometime alkylation of free cysteine residues by acrylamide occurs during separation by electrophoresis. Treatment with dithiotreitol does not cleave acrylamide off the modified cysteines. Other common protein modifications are mono-oxidation of methionine residues and double oxidation of tryptophane residues *(26)*.
11. This step of the protocol is applied only when Coomassie stained gel pieces still look blue after reduction and alkylation of the protein are completed. It usually occurs when intense bands (spots) containing picomoles of protein material are being analyzed. If a single washing cycle does not remove the residual staining, the procedure is repeated.
12. To prepare the digestion buffer, add 50 µL of 100 m*M* NH_4HCO_3, 50 µL of water and 5 µL of 100 m*M* $CaCl_2$ to 15 µL aliquot of trypsin stock solution (*see* **Note 3**). Keep the test tube with digestion buffer on ice before usage. To prepare the buffer for ^{18}O labeling use $H_2{}^{18}O$ instead of $H_2{}^{16}O$ and the same stock solution of 100 m*M* NH_4HCO_3.
13. The added volume of the reagent should just cover the solid residue at the bottom of the tube. Avoid an excessive volume since it increases chemical background observed in the mass spectra.
14. Rapid clean-up of the tryptic digest can also be performed using commercially available micropipet tips packed with a reversed phase material (ZipTip C18, [Millipore Corporation, Bedford, MA]). However the bead volume of ZipTip's is fairly large and careful precautions should be taken in order to elute the purified peptide mixture in less than 1 µL vol. Since intensity of detected signals depends on a concentration of the analyte unnecessary dilution of the sample severely compromises the sensitivity.
15. For detailed instructions on the manufacturing of the nanoelectropsray needles and on the operation of nanoelectrospray ion source *see (22)*.
16. The calibration of a triple quadrupole mass spectrometer is performed in accordance with the instructions from manufacturer. However for femtomole sequencing of tryptic peptides, several settings should be specially tuned. Make sure that the settings controlling resolution of the first quadrupole (Q1) allow good transmission of precursor ions. On the other hand, unnecessary low resolution of Q1 results in the transmission of too many background ions, which may densely populate the low m/z region of the MS/MS spectra. The third quadrupole (Q3) should likewise be operated at a low resolution settings in order to improve its transmission and to achieve acceptable ion statistics in the MS/MS spectra. In our experience, resolution of Q3 as low as 250 (FWHM) still allows accurate readout of peptide sequences. The Q1 and Q3 resolution settings can be tuned in tandem mass spectrometric experiment using synthetic peptides.
17. Calibration of a QqTOF instrument is performed by acquiring a spectrum of a mixture of synthetic peptides. External calibration with two peptide masses allows better than 10 ppm mass accuracy for both conventional and tandem mass spectra, if calibration and sequencing experiments are performed within 2 h. If the instrument was calibrated using a conventional mass spectrum, the calibration does not change when the instrument is switched to MS/MS mode. Resolution of the first quadrupole (Q1) should be tuned in a similar way as described for a triple quadrupole mass spectrometer (*see* **Note 16**).
18. Useful list of masses of trypsin autolysis products and minor background peptides originating from human and sheep keratins *(27)* is maintained at Protana's web site http://www.protana.com.
19. Scanning for precursor ions producing the characteristic fragment ions with m/z 86 (immonium ion of leucine or isoleucine) helps to distinguish genuine peptide ions from chemi-

cal noise and is therefore indispensable for sequencing at low levels. However, it is also recommended to acquire such spectrum even if a somewhat larger (picomole) amount of protein is present on a gel. For example, precursor ion scanning helps to recognize and reject ions of polyethylenglycol-like contaminants, which are often detected in the low m/z part of Q1 spectra as series of intense peaks spaced by 44 or 22 mass unit intervals.

20. PeptideSearch v 3.0 software can be downloaded from EMBL Peptide & Protein Group WWW-page (http://www.mann.embl-heidelberg.de/). The available software for searching sequence databases using mass spectrometric data is reviewed in *(28)*.
21. The number of residues of aspartic and glutamic acids present in any particular peptide is not known. To identify the matching peptide ion in the spectrum of the esterified digest, it is necessary to consider all ions shifted from the m/z of the unmodified peptide by $14(n + 1) / z$ (where $n = 0, 1, 2, 3...$) (**Subheading 1**) and to fragment all of them.
22. Because of the low duty cycle of QqTOF mass spectrometers, precursor ion scanning mode was previously regarded as being far less sensitive in these instruments compared to triple quadrupoles. In this mode of operation, the 2nd mass analyzer (Q3 or TOF) is used in a nonscanning mode (recording ions with m/z = 86 only), and the TOF analyzer does not benefit from recording all fragment ions without scanning. However, the recently introduced "ion trapping and bunching" technology enabled to overcome those limitations. Importantly, QqTOF mass spectrometers allow simultaneous acquisition of precursor ion spectra for several selected fragment ions *(29)* It is also relatively easy to distinguish precursor ions from chemical background by taking advantage of the high resolution of the QqTOF instrument. Genuine peptide ions are in most cases detected as characteristic isotopic patterns with sharp peaks superimposed on broad, irregularly shaped, background *(30)* Isotopic peaks of multiply charged ions are very well resolved, and the charge of the precursor ion can be instantly calculated from the mass difference between the isotopic peaks. If a conventional mass spectrum of the digest is noisy, it is not always straightforward to recognize the peak of the first isotope in complex isotopic pattern of multiply charged ^{18}O-labeled peptide ion. In this case the isotopic pattern of singly charged fragment ions yielded upon collisional fragmentation has to be examined. If the isotopic pattern of fragment ions is disturbed (for example, there is only one isotopic peak for unlabeled ions, or isotopic peaks of the ^{18}O-labeled fragments are missing), then the selection of the precursor ion has to be corrected.
23. Y" ions are distinguished from other fragment ions by their characteristic isotopic profile (**Subheading 1.**). It is easier to start the interpretation in the m/z region above the precursor ion where fragment spectra usually contain less background ions, and isotopic profiles of labeled ions are clearly visible. The series of Y"-ions is followed downwards and should terminate at the labeled Y" ion of arginine or lysine. Upwards, the Y"-series can be extended to the mass of the singly protonated ion of an intact peptide. The high resolution of a QqTOF instrument greatly assists in spectra interpretation and allows one to obtain additional pieces of information which are not available in low resolution tandem mass spectra acquired on a triple quadrupole instruments. Fragmentation of doubly charged precursor ions mainly results in a series of singly charged fragments. A series of doubly charged fragments usually has a much lower intensity. However, the high resolution of the QqTOF instrument enables them to be identified and used as independent verification of the sequence determined from the series of singly charged fragment ions. Since only the C-terminal carboxyl group of peptides is labeled during tryptic digestion, the N-terminal series of fragment ions (b-series) appear to be unlabeled. Although these ions often have low intensity, they can be recognized in the fragment ion spectrum and are useful for data interpretation. High mass accuracy of QqTOF

instruments also makes it possible to distinguish phenylalanine from methionine-sulfoxide (their masses are different by 0.033 Da), as well as glutamine from lysine (mass difference 0.037 Da).

24. If the protein was *in-gel* digested with trypsin in a buffer that did not contain $H_2{}^{18}O$, selective C-terminal isotopic labeling can still be performed. The digest should be esterified with methanol (**Subheading 3.2.**), dissolved in a buffer containing 50% (v/v) of $H_2{}^{18}O$, treated with trypsin for 30 min and dried down in a vacuum centrifuge. Treatment with trypsin efficiently removes the ester group from the C-terminal carboxyl group of tryptic peptides. At the same time the C-terminal carboxyl group of peptides incorporates ^{18}O or ^{16}O atoms from the buffer (**Fig. 1C**). Carboxyl groups in side chains of aspartic and glutamic acid residues remain esterified. However, the procedure results in a much higher chemical noise and in an increased level of keratin peptides. Therefore it is only recommended for sequencing of peptides from chromatographically isolated fractions, containing only a small number of peptides.

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