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AMINO ACIDS PROTOCOLS

The use of native gels for the concomitant determination of protein sequences and modifications by mass spectrometry with subsequent conformational and functional analysis of native proteins following electro-elution

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Abstract The protocol consists of running a native gel with in-gel digestion by proteases, subsequent mass spectrometrical determination of protein sequence and modifications, followed by electro-elution and conformational analysis using melting point and circular dichroism. Finally, the eluted protein is tested for preserved function. Herein, C1 esterase inhibitor is applied on a native gel; in-gel digestion by proteases is carried out and peptides are identified by nano-LC-ESI-CID/ETD-MS/MS using an ion trap for generation of peptide sequences and protein modifications. Protein from replicate bands from the same gel is electro-eluted and used for determination of the melting point and used for circular dichroism analysis. Additional bands from the native gel are either in-gel digested with asparaginase to generate deamidation or PNGase F for deglycosylation, followed by mass spectrometry, conformational and functional studies. Preserved conformation and function of the C1 esterase inhibitor was shown. This protocol can be completed in 1 week.

Keywords Conformation · CD · Protein structure · Native gel

Introduction

The technique under discussion is using native gels for separation and purification of proteins. Concomitantly with separation of the protein, in-gel digestion with several proteases to generate a sufficient amount of peptides resulting into high-sequence coverage when run on mass spectrometry for sequencing and the determination of protein modifications (including post-translational modifications) is carried out. On the identical gel also digestions with other protein-modifying enzymes, including deamidases or glycosidases, can be performed and verified by subsequent proteolytic enzyme in-gel digestions followed by mass spectrometry as shown herein for deamidation and partial *N*-deglycosylation. Electro-elution under strict cooling conditions is performed and conformational studies as differential scanning fluorimetry (DSF) for the evaluation of the melting point and circular dichroism (CD) for additional structural information are made possible. Moreover, the corresponding preserved functions of proteins, herein the inhibitory function of the C1 esterase inhibitor, can be tested.

Human C1 esterase inhibitor (C1INH) is a multifunctional plasma glycoprotein belonging to the superfamily of serine protease inhibitors (serpins). C1INH is a major inhibitor of the enzymatic activity of C1 esterase, activated forms of factor XII and kallikrein and several other enzymes; thus, C1INH is a key regulator of coagulation, complement and fibrinolytic systems (Cugno et al. 2009). Deficiency or low levels of functional C1INH in the circulation are associated with hereditary angioedema that can be lethal (Banerji and Sheffer 2009; Carugati et al. 2001).

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Human C1INH is comprised of a single polypeptide chain (478 amino acid residues) that is heavily glycosylated (26 %) at 13 glycosylation sites, including 6 N- and 7 O-attached glycans (Bock et al. 1986; Perkins 1993; Perkins et al. 1990). Its crystal structure has not been resolved yet. Due to its size, complex glycosylation and the peptide fragment repeats, C1INH is a highly heterogeneous protein and represents an analytical challenge.

As to the development of the protocol, the individual methods have been reported for the preparation of native gels, for mass spectrometrical analysis, for DSF and CD as well as for the function assay used herein (Albertini et al. 2007; Dunahay and Staehelin 1985; Evans and Anderson 1987; Ford 1987; Ford et al. 1987; Heinz and Siefermann-Harms 1981; Knoetzel et al. 1988; Krause 2006; Lancaster et al. 1999; Madhavarao et al. 2003; Poetsch et al. 2000; Schafer et al. 2006, 2007; Schagger 2002; Schagger et al. 2004; Seelert et al. 2003; Seelert and Krause 2008; Siefermann-Harms et al. 1982; Singh and Wasserman 1970; Tsiotis et al. 1993).

The combination of these methods using the principle of native gels including the adaptations necessary are reported herein and are based on previous work showing that native gels can be used for purification and subsequent electro-elution at low temperature of growth hormone that described preserved conformation as expressed by synchrotron radiation circular dichroism spectroscopy, synchrotron small-angle X ray scattering and DSF; a receptor binding assay was applied to show preserved function, i.e. receptor binding (Chen et al. 2010).

The applications of this system are numerous: apart from separation and purification, native proteins can be analysed in the native state by mass spectrometrical techniques, studying sequence and modification analyses that may not be valid when the protein is analysed under denaturing conditions.

Effects of protein modifying enzymes or chemicals can be studied as, e.g. the effect of deamidating enzymes as asparaginase on structure and function or the effect of partial N-deglycosylation by peptide *N*-glycosidase F (PNGase F) on conformation and function. And indeed, the effects on the protein per se can be easily evaluated by mass spectrometry revealing deamidation of amino acids or deglycosylation. Although not shown herein, the protocol can be easily extended to study protein–protein interactions or the effect of binding of other chemicals and their effects on structure and function. In addition, the effect of binding of a large series of compounds can be tested using appropriate methodology including ultramorphological/ultrastructural techniques.

The advantages are that proteins can be studied concomitantly using different individual techniques. For example, lane one may contain the molecular weight standard, lane 2 the native protein, the protein in lane 3 can be digested by trypsin, that from lanes 4–6 by chymotrypsin, subtilisin, pepsin and the band from lane 7 can be used for incubation with specific enzymes or chemical compounds to induce specific protein modifications that in turn can be tested in terms of conformation or function. Thus information from the identical proteins can be obtained. Another advantage is the simple use for sample preparation in conformational studies, even for complex analytical systems as synchrotron radiation circular dichroism spectroscopy, synchrotron small-angle X-ray scattering or, alternatively, for the determination of the melting point or traditional CD studies, that are available in classical protein laboratories.

Another advantage is that proteins in general do not lose conformation when electro-eluted and subsequently lyophilised: the eluted protein samples can be shipped abroad for special studies in this form or can be collected for own advanced studies in the laboratory.

In comparison with the potentially important outcome the time spent for the experiments is rather low. Moreover, the experiments or at least a part of the experiments can be carried out in most protein laboratories.

The disadvantages are the cost-intensive running of mass spectrometrical analyses that needs well-educated and well-trained personnel. The need for a cold room—all parts of the experiment have to be carried out there—is mandatory. Finally, the recovery of the electro-elution is not satisfactory yet due to the many steps involved and one has to find out, e.g. the appropriate plastic ware to warrant lowest binding of the protein to vessels used.

A work flow diagram of the protocol is shown in Fig. 1.

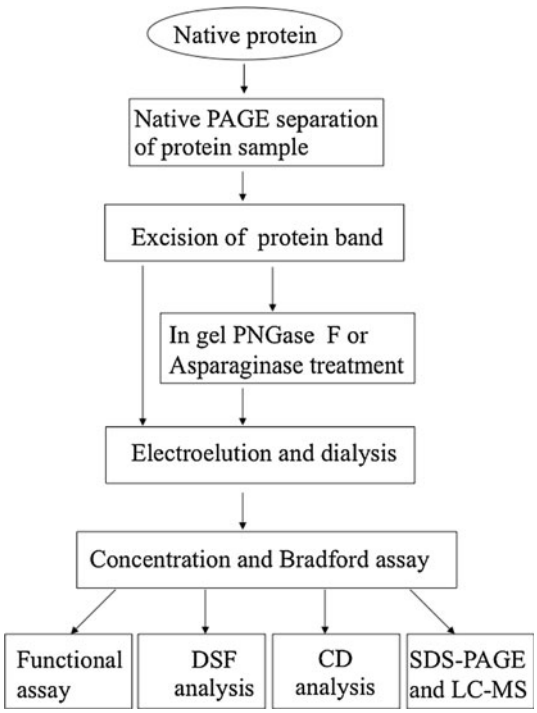

Fig. 1 Work flow diagram on the procedures described in this protocol

Materials

Reagents

- Acrylamide (Bio-Rad; http://www.bio-rad.com; cat. no. 161-0108)

! CAUTION Acrylamide is toxic. When handling these chemicals, wear gloves and use a pipetting aid.

- Piperazine-di-acrylamide (PDA) (Bio-Rad; http://www.bio-rad.com; cat. no. 161-0202)

! CAUTION PDA is toxic. When handling these chemicals, wear gloves and use a pipetting aid.

- C1 esterase inhibitor (Calbiochem; http://www.merck-chemicals.com; cat. no. 204883-1MG)
- Native protein markers (Invitrogen; http://www.invitrogen.com; cat. no. LC0725)
- E-Zinc reversible staining kit® (Pierce; http://www.piercenet.com; cat. no. 24582)
- PNGase F (NewEngland Biolabs; http://www.neb.com; cat. no. P0704L)
- Asparaginase (Sigma; http://www.sigmaaldrich.com; cat. no. A3809)
- SYPRO Orange (Invitrogen; http://www.invitrogen.com; cat. no. S6651)
- Colloidal Coomassie blue staining kit (Invitrogen; http://www.invitrogen.com; cat. no. LC6025)
- DTT (Bio-Rad; http://www.bio-rad.com; cat. no. 161-0611)
- Iodoacetamide (IDA) (Bio-Rad; http://www.bio-rad.com; cat. no. 163-2109)
- Ammonium bicarbonate (Sigma; http://www.sigmaaldrich.com; cat. no. 40867-50G-F)
- Octyl β-D-glucopyranoside (OGP) (Sigma; http://www.sigmaaldrich.com; cat. no. 75083-5G)
- Trypsin (sequencing grade; Promega; http://www.promega.com; cat. no. V5111)
- ProteinaseK (Sigma; http://www.sigmaaldrich.com; cat. no. P2308-25MG)
- Subtilisin (Sigma; http://www.sigmaaldrich.com; cat. no. P5459-5G)
- Pepsin (Sigma; http://www.sigmaaldrich.com; cat. no. P6887-1G)
- Chymotrypsin (sequencing grade; Roche Diagnostic; http://www.roche-diagnostics.com; cat. no. 1141846 7001)
- LC–MS CHROMASOLV acetonitrile (ACN) (Sigma; http://www.sigmaaldrich.com; cat. no. 34967-1L)

! CAUTION ACN is toxic. When handling, wear gloves and use a pipetting aid.

- HPLC-grade formic acid (Sigma; http://www.sigmaaldrich.com; cat. no. 27001-500ML-R)
- LC–MS CHROMASOLV water (Sigma; http://www.sigmaaldrich.com; cat. no. 39253-1L-R)
- Phosphate buffered saline (PBS) pH 7.4 (Quality Biological, Inc.; http://www.qualitybiological.com; cat. no. 119-068-131)
- Ethyl alcohol anhydrous, USP grade (Warner-Graham Co.; http://www.warnergraham.com; cat. no. 64-17-5)
- (1S)-(+)-Camphor-10-sulfonic acid (CSA) (Sigma; http://www.sigmaaldrich.com; cat. no. C2107)
- Fisherbrand Chromerge cleaning solution (Fisher Scientific; http://www.fishersci.com; cat. no. C577-12)

! CAUTION Be cautious using Fisherbrand Chromerge cleaning solution (potassium chromate in concentrated sulfuric acid), wear gloves, glasses, lab coat).

Equipment

- Mini-PROTEAN 3 electrophoresis module (Bio-Rad; http://www.bio-rad.com; cat. no. 165-3302)
- D-Tube midi (3.5 kDa cutoff, Novagen; http://www.merck-chemicals.com; cat. no. 71506-3)
- Sub-Cell R GT cell horizontal electrophoresis system (Bio-Rad; http://www.bio-rad.com; cat. no. 170-4484)
- 0.5 mL Amicon Ultracel tubes with 10 K membrane (Millipore; http://www.Millipore.com; cat. no. UFC501096)
- TECHNOCHROMTM C1-INH kit (Technoclone Gmbh; http://www.technoclone.com; cat. no. 5345003)
- iQ5 multicolour real-time PCR detection system (Bio-Rad; http://www.bio-rad.com)
- Bio-Rad iQ5 software (Bio-Rad; http://www.bio-rad.com)
- CONTIN software (http://lamar.colostate.edu/~sreeram/CDPro/)
- SELCON 3 software (http://lamar.colostate.edu/~sreeram/CDPro/)
- CDSSTR software (http://lamar.colostate.edu/~sreeram/CDPro/)
- Speedvac Concentrator 5301 (Eppendorf; http://www.eppendorf.com; cat. no. 5305 000.215)
- Ultimate 3000 nano HPLC system (Dionex Corporation; http://www.dionex.com)
- HCT ultra ETD II (Bruker Daltonics; http://www.bruker.com)
- EsquireControl 6.2 software (Bruker Daltonics; http://www.bruker.com)
- Hystar 3.2 software (Bruker Daltonics; http://www.bruker.com)
- DataAnalysis 4.0 (Bruker Daltonics; http://www.bruker.com)
- Biotools 3.1 software (Bruker Daltonics; http://www.bruker.com)
- Mascot server 2.3.0 software (Matrix Science Ltd.; http://www.matrixscience.com)

- J-815 Spectropolarimeter (JASCO Co.; http://www.jasco.co.jp).

Reagent setup

30 % acrylamide/PDA 29.25 g acrylamide, 0.75 g PDA in 100 ml. Can be kept at 4 °C for 4 weeks.

Running buffer 25 mM Tris, 192 mM Glycine, pH 8.3. Can be kept at 4 °C for 4 weeks.

Loading buffer 125 mM Tris–HCl pH 6.8, 20 % (v/v) glycerol, 0.2 % (w/v) bromophenol blue. Can be kept at 4 °C for 4 weeks.

Solution A 0.1 % (v/v) formic acid in LC–MS water. Freshly prepared and can be used for 2 weeks.

Solution B 0.08 % (v/v) formic acid in LC–MS acetonitrile. Freshly prepared and can be used for 2 weeks.

Equipment setup

Differential scanning fluorimetry (DSF)

DSF analysis is used to check protein structure before and after electro-elution assays. The temperature of protein unfolding is monitored via the rise in the dye SYPRO Orange fluorescence signal while binding to hydrophobic patches of the protein that become accessible during the heating process (20 to 95 °C in 1 h). The experiments were carried out in a Real-Time PCR instrument with filters calibrated to absorb signals from the SYPRO Orange dye: iQ5 multicolor Real-Time PCR Detection System.

Circular dichroism (CD)

CD measurements were performed using a spectropolarimeter at 25 ± 0.2 °C. The far-UV CD spectra were recorded within the 180–260 nm range in a quartz cuvette of 0.5 mm pathlength using the following parameters: bandwidth 1.0 nm, resolution 0.2 nm and scan speed 20 nm/min. All spectra were accumulated in triplicate; baseline (PBS) was subtracted.

▲ **CRITICAL** CD instrument should be calibrated regularly to assure the correct measurements of ellipticity and wavelength (Kelly et al. 2005). CSA serves as calibration standard.

▲ **CRITICAL** CD cuvette must be clean and dry after each measurement because protein can stick to the cuvette surface. Careful washing with Chromerge solution followed by continuous rinsing with water, rinsing with ethanol and drying by a stream of air or nitrogen is the most effective way for cleaning of thin cuvettes.

Nano-LC–ESI-CID/ETD-MS/MS system

The HPLC used was an Ultimate 3000 nano HPLC system equipped with a PepMap100C-18 trap column (300 µm × 5 mm) and PepMap100C-18 analytic column (75 µm × 150 mm). The gradient was 8–25 % solution B from 0 to 100 min, 25–80 % solution B from 100 to 105 min, 80 % solution B from 105 to 110 min, 8 % solution B from 110 to 130 min. A HCT ultra ETD II was used to record peptide spectra over the mass range of m/z 350–1,500 and MS/MS spectra in information-dependent data acquisition over the mass range of m/z 100–2,800. Repeatedly, MS spectra were recorded followed by four data-dependent CID MS/MS spectra and four ETD MS/MS spectra generated from four highest intensity precursor ions. An active exclusion of 0.4 min after two spectra was used to detect low abundant peptides. The voltage between ion spray tip and spray shield was set to 1,500 V. Drying nitrogen gas was heated to 150 °C and the flow rate was 10 l/min. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics (Kang et al. 2009).

Procedure

Native PAGE ● **TIMING** *4 h*

1. Prepare 8 % separation polyacrylamide gel by mixing 1.2 ml 30 % acrylamide/PDA, 1.125 ml 1.5 M Tris–HCl pH 8.8, 2.175 ml deionized water, 2.7 µl TEMED and 27 µl of 10 % (w/v) ammonium persulfate and cast into mini-PROTEAN 3 plates. Add 1 ml of 50 % 2-propanol (v/v) to the gel surface and keep at 25 °C for 10 min to polymerize.

! CAUTION Acrylamide and PDA are toxic. When handling these chemicals, wear gloves and use a pipetting aid.

2. Clean the gel surface with deionized water and insert the sample loading comb.

3. Prepare 4.5 % stacking gel by mixing 0.13 ml 30 % acrylamide/PDA, 0.25 ml 0.5 M Tris–HCl pH 6.8, 0.62 ml deionized water, 1.4 µl TEMED, 10 µl 10 % (w/v) ammonium persulfate; cast into mini-PROTEAN 3 plates and keep at 25 °C for 10 min to polymerize.

! CAUTION Acrylamide and PDA are toxic. When handling these chemicals, wear gloves and use a pipetting aid.

4. Set up the mini-PROTEAN 3 electrophoresis cell and add 900 ml running buffer to the electrophoresis chamber. Pre-run at 100 V for 1 h to remove unpolymerised acrylamide and other constituents in cold room at 4 °C.

5. Mix 36 µl C1INH with 4 µl loading buffer. Load sample and native protein markers. Electrophorese at a

constant voltage of 50 V in the cold room at 4 °C for 30 min and then 100 V for 1 h 30 min.

▲ **CRITICAL STEP** Perform electrophoresis in cold room at 4 °C to keep the protein activity.

6. Cut out two longitudinal strips, i.e. one lane containing markers and one lane containing C1INH and stain with E-Zinc staining kit while keeping the rest of the gel on a glass plate on ice.

7. Line up the stained strips along the edges of the unstained gel and use as a guide to cut out bands of interest from the unstained gel.

N-deglycosylation and asparaginase treatment ● **TIMING** *16 h*

8. Put cut gel bands into 2 ml Eppendorf tubes. Add 500 µl 10 mM phosphate buffer pH 7.0, keep at 4 °C for 16 h.

9. Put cut gel band into 2 ml Eppendorf tube. Add 10 µl PNGase F, 50 µl 50 mM pH 7.5 phosphate buffer and 440 µl deionized water; keep at 37 °C for 16 h to remove N-glycan.

10. Put cut gel band into 2 ml Eppendorf tube. Add 20 µl/6 units asparaginase, 25 µl 1.5 M Tris–HCl pH 8.8 and 455 µl deionized water; keep at 37 °C for 2 h 30 min.

Electro-elution and dialysis ● **TIMING** *2d*

11. Transfer the gel containing the protein band of interest into D-Tube, transfer the corresponding buffer also into D-Tube, add 200 µl running buffer and fix the D-Tube on a horizontal electrophoresis chamber, Sub-Cell R GT cell.

12. Fill the chamber with running buffer and perform electro-elution at 100 V for 5 h in the cold room at 4 °C. Reversely electrophorese at 100 V for 2 min to recover proteins attached to the membrane of D-Tube.

▲ **CRITICAL STEP** Use only one piece of gel band per D-tube to increase recovery. Electro-elute at 4 °C to keep the protein activity.

13. Fix D-Tubes in a floater and keep in 2.5 l of 10 mM phosphate buffer pH 7.0 overnight with gentle stirring in cold room at 4 °C for 16 h and repeat this procedure twice.

14. Aspirate and dispense 10 times to improve the protein recovery from D-Tube.

? TROUBLE SHOOTING

Protein concentration and Bradford assay ● **TIMING** *1 h*

15. Transfer the electro-eluted and dialyzed sample to 0.5 ml Amicon Ultracel tubes with 10 K membrane and centrifuge at 14,000×*g* at 4 °C for about 40 min to reduce the volume to about 100 µl. Reversely spin down the concentrated sample into another tube at 1,000×*g* at 4 °C for 2 min.

▲ **CRITICAL STEP** Spin down the concentrated sample immediately after centrifugation to increase the sample recovery.

? TROUBLE SHOOTING

16. Use 1 µl of the sample to measure protein concentration by the Bradford assay (Bradford 1976).

■ **PAUSE POINT** The sample can be kept at 4 °C for 1 week or lyophilized for 6 months at −20 °C.

Activity assay ● **TIMING** *1 h*

17. Measure C1INH activity using TECHNOCHROM™ C1INH kit. Dilute C1INH samples to 1 µg/10 µl. Prepare reference tubes, 0mUnit, 2.4 mUnits, 6mUnits, 12mUnits.

18. Add 100 µl buffer A to each tube, add 100 µl C1 esterase to each tube and keep tubes at 37 °C for 5 min. Add 500 µl substrate to each tube. Keep tubes at 37 °C for 3 min. Add 200 µl 50 % (v/v) acetic acid to each tube to stop the reaction. Measure the extinction at 405 nm against water.

19. Plot a standard curve and calculate the activity of C1INH samples.

Differential scanning fluorimetry ● **TIMING** *2 h*

20. Dilute C1INH samples to 0.12 mg/ml. Add 4.5 µl to 96-well plate and add 0.5 µl 10 × SYPRO Orange dye. Seal the 96-well plate with film. Spin down the samples to the bottom.

21. Put the sample plater into a Real-Time PCR instrument to measure melting curve. Calculate the melting point using Bio-Rad iQ5 software.

? TROUBLE SHOOTING

CD spectroscopy ● **TIMING** *5 h*

22. Reconstitute the lyophilized protein samples (C1-1, C1-2, C1-3 and C1-4) in deionized water at 21 ± 1 °C to achieve a concentration of 0.4–0.6 mg/ml immediately before the measurement. Apply gentle shaking, minimize foaming of the samples and avoid vortexing.

23. Measure far-UV CD spectra using a spectropolarimeter at 25 ± 0.2 °C in a 0.5-mm path length quartz cuvette over the wavelength range of 180–260 nm. Measure buffer as a blank as the first step.

▲ **CRITICAL STEP** When loading the protein solution, make sure there are no air bubbles left in the cuvette. Between measurements of protein samples, wash and dry cuvette as described above. Make sure that CD spectra of the cuvette itself (air filled) or with water do not show any traces of remaining protein impurities.

24. Accumulate 5–6 consecutive scans, smooth the resulting data and overlay spectra for comparison with other results.

25. In case of observed conformational changes, follow the protocol (Greenfield 2006) to estimate the protein secondary structure using the deconvolution programs CONTIN, SELCON 3 and CDSSTR.

? TROUBLE SHOOTING

Nano-LC-ESI-CID/ETD-MS/MS analysis ● ***TIMING*** *2d*

26. Cut C1INH band from native gel and put into a 1.5-ml tube. Wash with 10 mM ammonium bicarbonate and 50 % ACN (v/v) in 10 mM ammonium bicarbonate repeatedly.

! CAUTION ACN is toxic. When handling, wear gloves and use a pipetting aid.

27. Keep gel band in 30 mg/ml DTT in 100 mM ammonium bicarbonate at 37 °C for 30 min and in 185 mg/ml IDA in 100 mM ammonium bicarbonate at 25 °C for 30 min in the dark. Wash gel plug in 10 mM ammonium bicarbonate and repeat twice.

28. Add ACN to dehydrate and dry gel plugs in a Speedvac.

! CAUTION ACN is toxic. When handling, wear gloves and use a pipetting aid.

29. Re-swell the dried gel pieces, in-gel digest with 40 ng/µl trypsin in 5 mM OGP, 10 mM ammonium bicarbonate and incubate for 2 h at 37 °C.

30. Perform chymotrypsin digestion using 25 mM ammonium bicarbonate containing 25 ng/µl chymotrypsin and incubate for 1 h 30 min at 30 °C.

31. Carry out proteinase K digestion using 25 ng/µl in 50 mM ammonium bicarbonate and keep at 37 °C for 1 h.

32. Perform subtilisin digestion using 25 ng/µl in 6 M urea, 1 M Tris–HCl pH 8.8 and keep at 37 °C for 1 h.

33. Perform pepsin digestion using 25 ng/µl in 0.1 M HCl and keep at 37 °C for 4 h.

34. Perform peptide extraction with 15 µl of 1 % formic acid (FA) (v/v) in 5 mM OGP for 30 min, 15 µl 0.1 % FA (v/v) for 30 min and 15 µl 0.1 % FA (v/v) in 20 % ACN (v/v) for 30 min. Pool the extracted peptides.

! CAUTION ACN is toxic. When handling, wear gloves and use a pipetting aid.

35. Load 40 µl sample on nano-LC-ESI-CID/ETD-MS/MS and generate MS/MS spectra as given in equipment setup and published previously (Kang et al. 2009).

36. Interpret MS/MS spectra and generate peak lists using Data Analysis 4.0.

37. Use Mascot 2.3.0 to search against latest Uniprot KB database for protein identification. Set searching parameters as follows: select enzyme as trypsin or corresponding enzyme with three maximum missing cleavage sites, limit species to human, use a mass tolerance of 0.2 Da for peptide tolerance, use 0.2 Da for MS/MS tolerance, set fixed modification as carbamidomethyl (C) and variable modification as methionine oxidation, asparagine deamidation (caused by PNGase F or asparaginase treatment) and phosphorylation (Tyr, Thr and Ser). Identify protein based on a significant MOWSE score (Kang et al. 2009).

● **TIMING**

Step 1–7 **Native PAGE** 4 h
Step 8–10 **N-deglycosylation and asparaginase treatment** 16 h
Step 11–14 **Electro-elution and dialysis** 2d
Step 15–16 **Protein concentration and Bradford assay** 1 h
Step 17–19 **Activity assay** 1 h
Step 20–21 **Differential scanning fluorimetry** 2 h
Step 22–25 **CD spectroscopy** 5 h
Step 26–37 **Nano-LC-ESI-CID/ETD-MS/MS analysis** 2d

? TROUBLE SHOOTING

Step	Problem	Possible reason	Solution
At any step	Low protein recovery	Insufficient cooling	Eventually use pack of ice in addition to work in cold room
Step 11–14 (Supplementary Figure 2a)		Proteins sticking to membrane	Repeated pipetting to flush
Step 11–14		Wrong selection of membrane	Take membranes with appropriate cutoff (molecular selectivity should be below 1/6th of molecular weight of the protein)
Step 15 (Supplementary Figure 2b)		Sticking to tube or membrane	Change tubes with other material or flush and spin down inversely. Spin down concentrated samples immediately after centrifugation
Step 20–21	Poor results	Inappropriate fluorescent dye/protein concentration	Test dye/protein concentration in separate experiment
Step 22–25		Salts, buffers or contaminants	Use phosphate buffer, dialyse or reconsider solvent

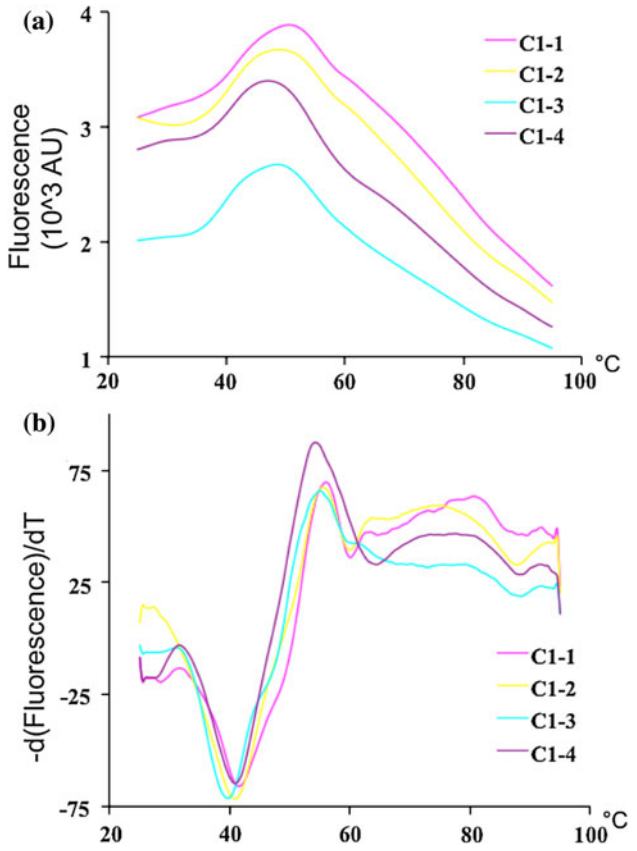

Fig. 2 The melting curves (**a**) and the melting peaks (**b**) of C1-1, C1-2, C1-3 and C1-4. C1-1 is C1INH in 10 mM phosphate buffer. C1-2 is C1INH after electro-elution. C1-3 is C1INH after PNGase F treatment. C1-4 is C1INH after asparaginase treatment. The melting points of C1-1, C1-2, C1-3 and C1-4 are 41.17 ± 0.29 °C, 40.67 ± 0.29 °C, 39.33 ± 0.29 °C and 40.83 ± 0.29 °C, respectively. Comparable melting points reflected comparable protein conformation

Anticipated results

C1INH in phosphate buffer without any electrophoretic step (C1-1) was used as a control for the native protein. Differential scanning fluorimetry spectra (DSF; Fig. 2: melting point 41.17 ± 0.29 °C, (all results in triplicate), circular dichroism spectra (Fig. 3) and inhibitory activity provided the basis for comparison with samples following electro-elution, enzyme treatments and the functional assay. Conformation studies revealed spectra showing helical elements as expected from known C1INH structural information. Using the standard kit for the determination of C1 esterase inhibitory activity, inhibition of 3.81 ± 0.69 Units/mg C1INH (triplicates) was observed.

When C1INH was run and electro-eluted from the native gel (C1-2) DSF (melting point 40.67 ± 0.29 °C; Fig. 2) and CD spectra (Fig. 3) were comparable to the control (C1-1) and so was the functional result showing inhibition

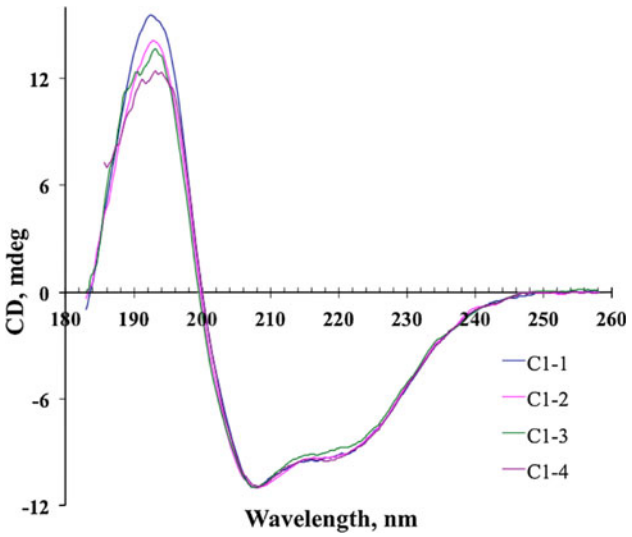

Fig. 3 The circular dichroism spectrum of C1-1, C1-2, C1-3 and C1-4. C1-1 is C1INH in 10 mM phosphate buffer. C1-2 is C1INH after electro-elution. C1-3 is C1INH after PNGase F treatment. C1-4 is C1INH after asparaginase treatment. Spectra were comparable between treated and un-treated groups

of 3.25 ± 0.69 Units/mg C1INH. The results clearly indicate that basic conformation and function were preserved using this native gel and electro-elution.

Mass spectrometrical identification and characterisation of C1INH obtained from this native gel- based proteomic method (Supplemental Figure 1b) showed high sequence coverage (70.5 %, Supplemental Table 1) due to multi-enzyme digestion of the C1INH. No protein modifications other than technical artifacts from the analytical procedure were observed.

In-gel treatment with peptide N-glycosidase F (PNGase F; C1-3) to show the effect of partial deglycosylation on structure and function of C1INH resulted in partial N-deglycosylation as reflected by a shift in SDS-PAGE and different expressional pattern on native PAGE (Supplemental Figure 1). Moreover, sequence coverage as shown by nano-LC-ESI-CID/ETD-MS/MS following in-gel digestion with the proteases was remarkably increased to 79.9 % probably due to increased accessibility of proteases to partially N-deglycosylated C1INH. Following N-deglycosylation, mass spectrometrical analysis showed glycosylation-induced deamidation of the glycosylated peptide at asparagine residue (N238, N253, N352; Supplemental Table 1). DSF revealed a melting point of 39.33 ± 0.29 °C (Fig. 2) and was, therefore, comparable to the control sample. CD studies as given in Fig. 3 also indicated preserved conformation despite partial N-deglycosylation and inhibitory activity of C1INH was comparable (3.87 ± 0.61 Units/mg C1INH) to controls as well.

Results of the effect of deamidation on C1INH structure and function by asparaginase treatment (C1-4) showed that

deamidation on N238, N252, N253, N291 and N353 indeed occurred as shown by mass spectrometry (supplemental table 1). No effect by asparagine deamidation on DSF (melting point 40.83 ± 0.29 °C, Fig. 2) and CD spectra was observed indicating preserved conformation by this protein modification (Fig. 3). Again, function was preserved as indicated by comparable inhibitory activity (3.36 ± 0.61 Units/mg C1INH). Deamidation did not lead to a shift in electrophoretic mobility (Supplemental Figure 1).

As to the recovery rates from all procedures from electro elution to dialysis and concentration, these were 51.7 % for C1-2, 47.0 % for C1-3 and 63.5 % for C1-4. These low recovery rates have to be taken into account and are the result from the many procedures/analytical steps. It would vary from protein to protein and cannot be predicted because of the individual protein properties and modifications as in this case work were carried out with a heavily glycosylated protein.

Previous work (Chen et al. 2010) from our laboratory used another protein, human growth hormone (hGH) using the same analytical principles: hGH was separated and purified on native gels, identified and characterised by mass spectrometry (no post-translational modifications were analysed because the recombinant protein was used). The protein was electro-eluted with an approximate recovery rate of 32.5 % and preserved; conformation in terms of melting point determination by DSF, synchrotron radiation circular dichroism spectroscopy and synchrotron small-angle X-ray scattering was shown. Function expressed as binding of eluted hGH to the hGH receptor was preserved.

Taken together, this analytical system has a broad application spectrum allowing purification, gel-based proteomic studies using multi-enzyme digestion followed by mass spectrometrical analysis with information on sequence and modifications. From the same gels, more native protein can be eluted for conformational analyses and the corresponding functional tests.

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