Gel-based mass spectrometric analysis of a strongly hydrophobic $GABA_A$-receptor subunit containing four transmembrane domains

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The analysis of highly hydrophobic proteins is still an analytical challenge. Using a recombinant gamma-aminobutyric acid A ($GABA_A$)-receptor subunit as a model protein, we developed a gel-based proteomic approach for high MS/MS-peptide sequence coverage identification. Protein samples were separated by multi-dimensional gel electrophoresis and the three protein spots representing the $GABA_A$-receptor subunit α-1 from the last electrophoretic step were used for in-gel digestion with trypsin, chymotrypsin and subtilisin, followed by subsequent mass-spectrometric identification by nano-ESI-LC-MS/MS Qstar XL (quadrupole time-of-flight (qQTOF)) and linear ion trap (LIT) LTQ XL identification. This protocol allows the unambiguous identification of the $GABA_A$-receptor α-1 subunit protein with 100% sequence coverage, thus covering all four hydrophobic transmembrane domains. This protocol differs from other methods in the selection of enzymes, digestion conditions and use of the two mass spectrometry principles. The protocol takes ~10 d to complete and may represent a step forward in the complex analysis of other membrane or hydrophobic proteins.

INTRODUCTION

The analysis of highly insoluble and/or hydrophobic proteins is still an analytical problem and is a major technical challenge in proteomics technology[1,2]. Well-known difficulties encountered when working with hydrophobic proteins include solubilization, separation by gel electrophoresis and extraction of hydrophobic peptides from gels. Rabilloud *et al.*[3] recently reviewed the problems associated with working on hydrophobic proteins. It is possible to use gel-free methods to aid in the isolation of hydrophobic proteins, but the pre-fractionation and pre-separation steps required to do this are extremely laborious[4]. Moreover, such methods are not able to detect splice variants, and once the proteins are solubilized the peptides need to be pieced back together.

The complete sequence of proteins is required to investigate protein conformation and aid in modelling studies. A complete structural analysis is required to generate antibodies against individual epitopes of receptor proteins. These antibodies are mandatory for immunochemical and immunohistochemical studies in biology. Moreover, the complete protein sequence is necessary to detect sequence conflicts (discrepancies between experimental data and database information on the sequence) and mutations, as well as splice variants. In addition, the protein sequence is crucial to study post-translational modifications that cannot be predicted from nucleic-acid sequences, and to investigate protein–protein interactions.

In our laboratory, we are interested in studying the receptors and transporters that are involved in brain homeostasis and disease. The gamma-aminobutyric acid A ($GABA_A$) receptor is an important protein regulating brain physiology[5–7]. This receptor is involved in many pivotal mechanisms in brain physiology, including neural transmission in motor, sensory, behavioral and emotional functions. It is important for the modulation of anxiety, feeding, drinking behavior, circadian rhythm and cognitive functions, such as vigilance, memory and learning[8,9].

The GABA receptor is a chloride channel opened by GABA and can be modulated by several drugs, including benzodiazepines, barbiturates, steroids, anaesthetics and convulsants[10]. Biochemically, the $GABA_A$ receptor is a protein complex consisting of a vast number of subunits (α, β, γ, δ and ε and their splice variants) resulting in the formation of heterogeneous complexes[11], and at present only part of the $GABA_A$ receptor–protein sequence has been published[12,13]. Analysis of this receptor is hampered by the hydrophobicity of the protein and by the need for complex enzymatic digestion conditions as well as fragmentation by mass spectrometrical tools. The $GABA_A$ α-1 subunit of the $GABA_A$ receptor contains four transmembrane domains and was chosen for investigation in this study because of the availability of the recombinant protein expressed in insect cells[14]. This protein serves as a model protein, demonstrating that the complete sequencing of the protein can be achieved using this protocol. Therefore, the use of this protocol may allow for the analysis of other strongly hydrophobic and highly insoluble proteins.

In this protocol, based on our previous work[14,15], we describe a gel-based proteomic approach (**Fig. 1**) that combines recent advances in gel separation methods and mass-spectrometric protein identification[16–18] to elucidate the sequence of hydrophobic proteins. The flow diagram in **Figure 1** outlines the steps of the protocol. Hydrophobic and highly insoluble proteins and protein complexes are pre-separated using blue native (BN) gel electrophoresis in a manner similar to that of our previous protocol[16]. Protein complexes are then easily dissociated by taking BN-gel-derived bands and carrying out a second electrophoretic step. The optimization of separation and protein purification is enabled by a third electrophoretic step. Multienzyme digestion of the purified protein then leads to 100% sequence coverage of the peptides using MS/MS.

The combination of the gel procedures with specific enzymes and incubation conditions, together with the use of two mass-spectrometry principles, is a major advantage of our method when compared with other techniques. Recent methodologies, including the use of two-dimensional benzyldimethyl-*n*-hexadecylammonium chloride SDS-PAGE (BAC/SDS-PAGE)[4], complex gel methods or free-flow electrophoresis and the use of different individual detergents[5], have been used to study hydrophobic proteins; however, they have not significantly contributed to the complete protein sequencing of proteins, such as the $GABA_A$ receptor. The protocol outlined here is highly specific, in contrast to the immunochemical analytical approaches carried out so far to characterize the receptor-complex stoichiometry, distribution and quantification of subunits[19–22]. Although mass spectrometry is a pure protein chemical method that can unambiguously identify a protein, immunochemical methods rely on antibody specificity and avidity, and a protein can be identified as 'immunoreactive' with the given antibody only. However, the present protocol carries several disadvantages compared with other previously published methods, including lower sensitivity, high costs for instrumentation, a requirement for trained personnel and specific knowledge of data mining[23].

Experimental design

Protein samples. Proteins extracted from the brain (and most probably from other organs) or a recombinant protein can be analyzed. A mixture of hydrophobic proteins from the brain can be used with a minimum protein amount of 200 µg. If a single protein is applied, a protein amount of ~50 µg is optimal. The buffer in which the protein is dissolved has to be exchanged by dialysis against the BN-sample buffer to enable running the protein on BN-gel electrophoresis.

The main principle of the protocol (**Fig. 1**) is to separate and thus pre-purify a strongly hydrophobic protein by a sequence of three gel procedures, named BN/SDS^n-PAGE (*n* means 'multi'). Before separation, proteins are quantified using a protein determination kit. This bicinchoninic acid (BCA) protein assay is recommended, as it is more sensitive and accurate than the Bradford assay for measurement of hydrophobic protein concentrations because the kit has a higher tolerance of the presence of detergents and buffers containing high molar salt.

BN-PAGE and SDS-PAGE. BN-PAGE, which is run first, relies on the principle that hydrophobic and highly insoluble proteins migrate through the gel because of their coating by a dye, Coomassie blue. This electrophoretic step is under native conditions, i.e., without protein denaturation, and keeps protein complexes intact. In the second electrophoretic step, the proteins are denatured by a SDS equilibration buffer, thus dissociating the complex into its components. Many hydrophobic proteins—as in particular receptors—are present in complexes and therefore pre-separation of such a complex is mandatory. Following the first electrophoretic separation, individual high-molecular-weight bands are excised from the gel[24,25]. The size of the gel pieces containing bands to be cut out from the gel depends on the density and the expected apparent molecular weight of the protein or the protein complex to be analyzed. A broad band of strong density should be cut out into a 2-cm piece, whereas weaker bands should be cut to 1-cm pieces. A single band containing the $GABA_A$-receptor subunit is re-run using one-dimensional and then subsequently two-dimensional SDS-PAGE. The first SDS-PAGE gel is not stained, but is an intermediate for use in the two-dimensional SDS-PAGE. These two gel systems significantly improve the separation of protein spots in two-dimensional SDS-PAGE. The use of gradient gels is mandatory for clear separation of the protein spots for one- and two-dimensional gels. The gradient given here is appropriate for the apparent molecular weight of a GABA subunit, but may be modified according to the molecular weight and electrophoretic mobility of any other protein to be analyzed. Proteins larger than the subunits of the $GABA_A$-receptor subunits require a lower percentage of acrylamide concentration, e.g., 5%–10%. However, the optimal gradient to be used has to be determined experimentally.

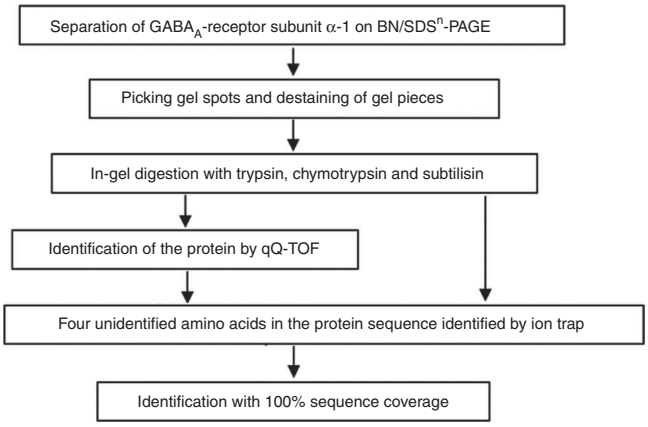

Figure 1 | Flow diagram of the analytical procedure.

Enzymatic digestion of proteins. Following the successful electrophoretic steps, the spots are then excised from the two-dimensional gel. Before enzymatic digestion of the proteins, in-gel reduction and alkylation reactions are carried out to prevent oxidation, disulfide formation and therefore aggregation of the proteins. The proteins are digested with trypsin, chymotrypsin and subtilisin under specific conditions provided in the tables below (see PROCEDURE). The three different enzymes were selected because they represent the gold standard as starting enzymes for protein digestion. This results in the cleavage of the majority of proteins into peptides of suitable length (700–3500 kDa) for identification by mass spectrometry.

The use of the three enzymatic protein digestion principles is a key factor for the successful complete digestion of the proteins into useful peptides[26]. These proteases cleave substantial parts of the protein sequences of hydrophilic and hydrophobic peptides. A description of the characteristics of the proteases that make them ideal for cutting proteins into individual specific peptides can be found at http://www.expasy.ch/tools/peptidecutter/. Briefly, trypsin cleaves N-terminal bonds of lysine and arginine, and chymotrypsin cleaves C-terminal bonds of phenylalanine, tyrosine and tryptophan, whereas subtilisin generates a non-specific cleavage pattern. In our laboratory the use of other proteases, including AspN, LysC, ArgC and protease K, did not increase the sequence coverage (data not shown).

The individual incubation conditions, such as time, temperature or buffers used, were determined experimentally and are of pivotal

importance. Variations lead to qualitative and quantitative changes in the cleavage of the protein to peptides. In **Supplementary Table 1** peptides resulting from individual enzymes at different incubation conditions are listed, thus allowing specific information about the enzyme-cleavage patterns of a hydrophobic protein.

Following enzymatic digestion the samples are transferred to lobind tubes before mass-spectrometry analysis as the use of these tubes significantly reduces the binding of hydrophobic proteins to the wall of the tubes. This is of importance as binding of hydrophobic proteins to vial walls is a major source of protein loss during analysis.

Mass spectrometry (MS). Two different mass spectrometers are used in this protocol; the quadrupole time-of-flight (qQTOF) mass spectrometer (ions selected and separated by voltage across the quadrupole and time-of-flight detection) is complementary to the linear ion-trap (LIT) mass spectrometer (ions trapped by electric and magnetic potentials with the advantage that specific ions can be selected) and only the combination of both instruments, i.e., the generation of useful ionization and fragmentation, leads to the high sequence coverage. Our previous experience has shown that LIT is the most suitable mass spectrometry approach to sequence peptides generated by subtilisin cleavage (data not shown). Thus, peptides generated by subtilisin are analyzed by LIT and qTOF, whereas peptides generated by trypsin and chymotrypsin digestion are analyzed by qTOF only.

qQTOF. The qQTOF mass spectrometer system can be described as a triple quadrupole with the last quadrupole section replaced by a time-of-flight (TOF) analyzer. This mass spectrometer generates high-quality MS/MS data from electrospray ionization (ESI) when operated in the filter mode to transmit only the parent ion of interest and when a mass window from 1 to 3 Thomson (Th) is selected. ESI–qQTOF mass spectrometry is based on the principle of producing molecular ions in the gas phase for subsequent separation and analysis by collision-induced fragmentation in neutral gases. After leaving the collision cell, ions are re-accelerated to the required energy and focused by ion optics into a parallel beam that continuously enters the ion modulator of the TOF analyzer. In the mass analyzer, molecular ions are separated on the basis of their mass and charge. All mass spectra from the TOF system are recorded with a time-to-digital converter. Ions generated by an ESI source usually carry several charges, enabling better sequencing in MS/MS modes.

LIT. The LTQ XL is a linear ion-trap mass spectrometer, which is operated with a nano-electrospray ion source and directly coupled with the UltiMate nano HPLC System. The multiple-charged peptide ions generated in the ion source are analyzed by a two-dimensional ion trap. In the first stage, a full MS spectrum is recorded by scanning out all ionic species from the analyzer according to their mass/charge ratios and detecting their signals in one of the two detectors. A list of peptide masses will be generated according to the results of the full MS measurement, and in the second stage the four most-intensive ions will be chosen for fragmentation. The ion trap is filled a second time with the peptides eluting from the HPLC, and all but the one with the highest intensity is ejected from the analyzer. The trapped ions are activated and fragmented by collision. The fragment ions will be scanned out according to their mass/charge ratios and detected. In a second fragmentation cycle the analyzer will again be filled and the ion with the second-highest intensity will be trapped and fragmented. After a third and a fourth MS/MS cycle, a new full MS will be carried out and a new peptide-mass list will be generated. The peptide masses from the first cycles will be set on an exclusion list for a certain time, which correlates with the peak width of the HPLC separation, and again, the four most-intensive ions will be chosen for fragmentation. The main advantages of the LIT mass spectrometer are its short duty cycle (MS and MS/MS cycles) and high sensitivity, which makes it ideal for the analysis of complex mixtures and low abundant peptides.

Peptide identification. Peptides are unambiguously identified using the Mascot MS/MS peptide identification system. Mascot is a powerful search engine, which uses mass spectrometry data to identify proteins from primary sequence databases. Mascot is unique in that it integrates all of the proven methods of searching, peptide mass fingerprinting, sequence queries and MS/MS ion searches. Mascot will match the peptides to proteins within its database; if there is no exact match it will match the peptides to those with the closest homology; these are often orthologues.

MATERIALS

REAGENTS

▲ CRITICAL All chemicals and plastic consumables should be of the highest quality, all solvents used for HPLC and MS sample preparation should be stored in glass bottles to avoid plasticisers, and polymers leaching from low-quality plastic cans or laboratory wares.

- $GABA_A$-receptor subunit α-1-complex (obtained from the Division of Biochemistry and Molecular Biology, Center for Brain Research, Medical University of Vienna, Vienna, Austria)
- HPLC-grade water (Millipore, cat. no. ZRQS0P0WW)
- Dithiothreitol (DTT) (Bio-Rad, cat. no. 161-0611)
- Ethylenediaminetetraacetic acid (EDTA) (Sigma, cat. no. 431788-100G)
- Slyde-A-Lyzer mini dialysis kit (Pierce, cat. no. 66372)
- Triton-X100 (Promega, cat. no. H5141-500 ml)
- BCA protein assay kit (Pierce, cat. no. 23225)
- Iodoacetamide (Bio-Rad, cat. no. 163-2109)
- Urea (Sigma, cat. no. 431788-100G)
- Tricine (Sigma, cat. no. T0377-250G)
- Glycine (Sigma, cat. no. G8790-1KG)
- Sodium dodecyl sulfate (SDS) (Sigma, cat. no. L6026-250G)
- Acrylamide/piperazine-di-acrylamide (PDA) (Bio-Rad, cat. no. 161-0108/161-0202) ! CAUTION Acrylamide is toxic and a potential genotoxin and carcinogen. When weighing powdered acrylamide, work in a chemical fume hood, wear gloves, eye protection and a mask.
- Glycerol (Sigma, cat. no. G8773-1L)
- 6-Aminocaproic acid (Sigma, cat. no. A2504-500G)
- 2-Mercaptoethanol (Bio-Rad, cat. no. 161-0710) ! CAUTION 2-Mercaptoethanol is toxic. When handling this chemical, work in a chemical fume hood, wear gloves, a mask and use a pipetting aid.
- 2,2-Bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (Bis–Tris) (Fluka, cat. no. 14880-500G)
- Precision plus protein standards (Bio-Rad, cat. no. 161-0363)
- NativeMark unstained protein standard (Invitrogen, cat. no. LC0725)
- HMW Native marker kit (GE Healthcare, cat. no. 17-0445-01)
- Colloidal Coomassie blue staining kit (Invitrogen, cat. no. LC6025)
- Trifluoroacetic acid (TFA) (Sigma, cat. no. 431788-100G)
- Formic acid (FA) (Sigma, cat. no. 27001-500mL) ! CAUTION FA is dangerously irritating to the skin, eyes and mucous membranes.

When handling this chemical, work in a chemical fume hood, wear gloves, eye protection and a mask.
- Brilliant Blue G (Sigma, cat. no. B0770-25G)
- Ammonium bicarbonate (Sigma, cat. no. 40867-50G-F)
- CHROMASOLV grade iso-propanol (Sigma, cat. no. 4959-1L)
- LC-MS CHROMASOLV acetonitrile (ACN) (Sigma, cat. no. 34967-1L) **! CAUTION** Acetonitrile may show inhalation toxicity, with the development of symptoms in the central nervous system (CNS). When handling this chemical, work in a chemical fume hood, wear gloves and use a pipetting aid.
- LC-MS CHROMASOLV methanol (Sigma, cat. no. 34966-1L) **! CAUTION** Methanol is an inhalation toxin leading to CNS depression. When handling this chemical, work in a chemical fume hood, wear gloves and use a pipetting aid.
- HPLC-grade FA (Sigma, cat. no. 27001-500ML-R) **! CAUTION** FA is dangerously irritating to the skin, eyes, and mucous membranes. When handling this chemical, work in a chemical fume hood with wearing gloves and use a pipetting aid.
- Cytochrome C digest (Dionex, cat. no. 161089)

EQUIPMENT
- Qstar XL (Applied Biosystems, cat. no. 4345820)
- Ultimate 3000 nano HPLC system (Dionex, cat. nos. 5035.9230 (solvent rack with degasser), 5035.0050 (dual micro-gradient pump), 5074.0010 (variable wavelength detector), 5720.0030 (micro-switching valve), 5820.0010 (autosampler with temperature control))
- Chromelon 6.7 software (Dionex, provided with Ultimate 3000 nano HPLC system)
- Mascot 2.2.04 software (Matrix Science Ltd; inquiries regarding licensing should be sent to the Matrix Science offices (info@matrixscience.com))
- Mascot.dll 1.6b21 (Matrix Science Ltd.)
- 250 µl HPLC vial (Dionex, cat. no. 6820.0029)
- AnalystQS 1.1 software (Applied Biosystems, provided with Qstar XL)
- Thermo LTQ-XL (Thermo, cat. no. ETD 10195)
- UltiMate nano HPLC system equipped with large volume injection kit (Dionex, cat. no. 30065) and a cooled sample tray for 0.2 ml vials
- XCalibur 2.0.7 software (Thermo, provided with Thermo LTQ-XL)
- Proxeon nano ES ion source (Proxeon, cat. no. ES012)
- Nanospray adapter (New Objective, cat. no. ADPC-PRO)
- Nanospray emitter; distal coated (New Objective, cat. no. FS360-20-10-D-20)
- Mascot Daemon 2.2.2 software (Matrix Science Ltd; inquiries regarding licensing should be sent to the Matrix Science offices (info@matrixscience.com))
- C18 PepMap100 solid-phase extraction µ-Precolumn cartridge (particle size 5 µm, pore size 100 Å, 300 µm inner diameter) (Dionex, cat. no. 160454)
- Emitter needle tip (no coating, 20 ìm inner diameter) (New Objective, cat. no. FS360-20-10-N-20-C10.5)
- Eppendorf microcentrifuge 5417C (Eppendorf, cat. no. 5417 000.315)
- Eppendorf SpeedVac concentrator (Eppendorf, cat. no. 5301 000.210)
- Nano-column PepMap C18 reversed-phase material (particle size 3 µm, pore size 100 Å, 75 µm inner diameter) (Dionex, cat. no. 160321)
- PROTEAN II xi multi-gel casting chamber (Bio-Rad, cat. no. 165-2025)
- Protein lobind tube (Eppendorf, cat. no. 0030 108.094)

REAGENT SETUP

Trypsin Dissolve the trypsin in the resuspension buffer provided (contains 50 mM acetic acid; the final trypsin concentration of this solution is 12.5 ng µl^{-1}). Once made, the solution is stable for 2–4 weeks at 4 °C. The trypsin is stable for at least three freeze–thaw cycles. The purchased powder is stable for 1 year, if stored at −20 °C.

Chymotrypsin Dissolve the chymotrypsin in 1 mM HCl (to a final concentration of 12.5 ng µl^{-1}). Once made, the solution is stable for 1 to 2 d at 4 °C. The chymotrypsin is stable for at least three freeze–thaw cycles. The purchased powder is stable for 1 year, if stored at 4 °C.

Subtilisin A Dissolve the subtilisin A in 1 mM HCl (final concentration of 12.5 ng µl^{-1}). The subtilisin solution is stable for 1 or 2 d at 4 °C.

BN-gel sample buffer 50 mM sodium phosphate, 250 mM NaCl, 0.1% Triton X-100 (wt/vol) and 1 mM EDTA, adjusted to pH 8.0. Prepare fresh on the day of use.

BN-gel electrophoresis buffer 250 mM 6-aminocaproic acid and 25 mM Bis–Tris, pH 7.0. Prepare fresh on the day of use.

Cathode buffer 50 mM Tricine and 15 mM Bis–Tris, 0.05 % (wt/vol) Coomassie G250, pH 7.0. Prepare fresh on the day of use.

Anode buffer 50 mM Bis–Tris, pH 7.0. Prepare fresh on the day of use.

G250 solution 5 % (wt/vol) Coomassie G250 in 10 mM 6-aminocaproic acid. Prepare fresh on the day of use.

SDS equilibration buffer 1% (wt/vol) SDS in 0.1 M Tris-HCl and 1% (vol/vol) mercaptoethanol, pH 6.8. Prepare fresh on the day of use.

SDS-PAGE electrophoresis buffer 25 mM Tris-HCl and 192 mM glycine and 0.1% (wt/vol) SDS, pH 8.3. Prepare fresh on the day of use.

Staining solution 0.1% (wt/vol) Colloidal Coomassie blue. Prepare fresh on the day of use.

For qQTOF
- **Solution A:** 0.1% FA (vol/vol) in water. Prepare fresh on the day of use.
- **Solution B:** 80% acetonitrile/0.08% (vol/vol) formic acid in water. Prepare fresh on the day of use.

For LIT
- **Loading buffer:** 0.1 % TFA. Prepare fresh on the day of use.
- **Solvent A:** 5% ACN/0.1% FA (vol/vol) in water. Prepare fresh on the day of use.
- **Solvent B:** 80% ACN/0.08% FA (vol/vol) in water. Prepare fresh on the day of use.

EQUIPMENT SETUP

qQTOF The qQTOF is set up as described previously[16]. Connect an Ultimate 3000 nano-LC system to the qQTOF to set up the nano-ESI-LC-MS/MS system. The flow rate of the loading pump (A) is 20 µl min^{-1}, and the flow rate of the micro pump is 300 nl min^{-1}. Load the sample onto a Pepmap100 C18 precolumn from 0 to 4 min and then separate on a PepMap100 C18 nano-column with a gradient of 96% A and 4% B to 60% from 0 min to 30 min, 90% B constant from 30 to 35 min and 4% B from 35 min to 60 min. Ion spray voltage is 3200 V and the gas is set at 10. The mass spectrum is recorded from 10 min to 50 min for a 7-s cycle with one 1-s MS following three 2-s MS/MS fragmentation of highest-intensity precursor ions.

LIT The LIT is set up as described previously[16]. The UltiMate nano HPLC System was directly interfaced to the LTQ–LIT mass spectrometer. Use 0.1% TFA on the Switchos module for the binding of the peptides with a flow rate of 20 µl min^{-1}, and a linear gradient of ACN and 0.1% (vol/vol) FA in water for the elution at a flow rate of 300 nl min^{-1}. Wash the peptides adsorbed on the precolumn for 15 min with ACN and 0.1% FA; then switch the precolumn on-line with the analytical column and elute peptides.

Use the following gradient: 0% B for 8 min, 50% B for 60 min, 95% B for 1 min, 100% B for 5 min, 0% B for 1 min, 0% B for 22 min. Set the electrospray voltage to 1,500 V. Record the peptide spectra over the mass range of m/z 450–1,600. Use helium as the collision gas. Operate the instrument in data-dependent modus; set fragmented ions onto an exclusion list for 20 s. Although doubly and triply charged peptides fragment work better in both systems, do not omit singly charged peptides if you work with hydrophobic peptides generated by chymotrypsin or unspecific proteases. Peptides originating from transmembrane regions may carry only one charge.

PROCEDURE

BN-gel electrophoresis ● TIMING 2 d

▲ CRITICAL Unless stated otherwise, all procedures are carried out at room temperature (20–22 °C).

1| Dialyze the samples (herein an affinity-purified mixture of 60 µg rat recombinant $GABA_A$-receptor subunits[14]) against 10 ml of buffer containing 750 mM 6-aminocaproic acid, 50 mM Bis–Tris, 5% (wt/vol) glycerol, 0.5 mM EDTA, 0.1% (vol/vol) Triton X-100 (vol/vol), pH 7.0 using a Slyde-A-Lyzer mini dialysis unit according to the manufacturer's instructions. The protein amount is sufficient for the following digestions and was prepared as described previously[14]. Prepare six aliquots of the protein of interest to test the multiple enzymes and conditions in Step 21.

2| Determine the protein concentration of the dialyzed samples using the BCA protein assay[27]. Transfer 5 ml of each sample to Ultrafree-4 centrifugal filter 50 ml tubes, centrifuge at 2,500*g* and 4 °C until the remaining volume is ~1 μg μl^{-1}. For example, if 5 ml of the sample contained 60 μg, concentrate the protein to a final volume of ~60 μl. Measure the amount of protein again and adjust to 1 μg μl^{-1} with BN-gel sample buffer. This takes ~2 h.
▲ CRITICAL STEP An amount of between 50 and 100 μg is required for resolution of BN-PAGE and BN/SDS/SDS-PAGE and reproducibility.

3| Prepare a BN-gel in a PROTEAN II xi cell using a 4% stacking and a 5%–13% separating gel[28].

4| Add 10 μl of G250 solution to each 60 μl of sample (protein content 1 μg μl^{-1}) and load onto the BN gel. Also load HMW Native marker kit size markers.

5| Run the gel for 2 h at 70 V, and then increase the voltage to 250 V (10 mA/gel) until the dye front reaches the bottom of the gel. Cut the gel lanes of interest into small pieces of ~1–3 cm, depending on the intensity and broadness of the protein bands at the expected apparent molecular weight for the BN/SDS/SDS-PAGE.
? TROUBLESHOOTING

BN/SDS^n-PAGE ● TIMING 3 d

6| Prepare the SDS-PAGE gels in a PROTEAN II xi cell using a 4% stacking and a 6%–13% separating gel for the first-dimension SDS-PAGE, and using a 4% stacking and a 7.5%–17% separating gel for the second-dimension SDS-PAGE.
▲ CRITICAL STEP Fix the parameters of the gel-casting system to obtain a reproducible gradient.

7| Soak the 1–3 cm gel pieces from Step 5 for 1 h in a solution of 1% (wt/vol) SDS and 1% (vol/vol) 2-mercaptoethanol. Wash the gel pieces twice with 10 ml of SDS-PAGE electrophoresis buffer for 10 min.

8| Place the gel pieces onto the first-dimension SDS-PAGE gel. Run the gel for 1 h at 70 V, 25 °C, then increase the voltage to 100 V for a further 12 h (overnight). Run the gel at 200 V until the dye front has moved 17 cm from the top of the separation gel.
▲ CRITICAL STEP In order to obtain high-quality gel patterns, ensure that no air bubbles exist between the gel piece and the first-dimension SDS-PAGE gel surface. For complete attachment between them, we recommend the use of a 'glue', an additional 4% stacking solution or 1% agarose solution (wt/vol).

9| Cut the gel into lanes and soak the gel strips from each lane in 10 ml of 1% (wt/vol) SDS and 1% (vol/vol) 2-mercaptoethanol for 20 min. Rinse the gel strips twice with SDS-PAGE electrophoresis buffer for 10 min.

10| Place the gel strips onto the second-dimension SDS-PAGE gel and use Precision plus protein standards size markers. Run the gel as described in Step 8.
▲ CRITICAL STEP In order to obtain high-quality gel patterns, ensure that no air bubbles exist between the gel piece and the second-dimension SDS-PAGE gel surface. For complete attachment between them, we recommend the use of an additional 4% stacking solution or 1% agarose solution (wt/vol).

11| To fix the gels, incubate in 50% methanol (vol/vol) and 10% acetic acid (vol/vol) for 12 h.

12| Stain the gels with 300 ml of 0.1% (wt/vol) Colloidal Coomassie blue for 8 h and wash away the excess dye with distilled water.
? TROUBLESHOOTING

Spot excision and in-gel digestion ● TIMING 2 d

13| Cut the stained gel pieces from Step 12 into 5 mm × 5 mm pieces and place in a 0.6-ml tube.

14| Add 100 μl of 50 mM ammonium bicarbonate and incubate for 10 min and then remove the ammonium bicarbonate.

15| Add 100 μl of 50% 50 mM ammonium bicarbonate (wt/vol)/50% ACN (vol/vol) and incubate for 30 min with occasional vortexing. Remove the solution.

16| Repeat Step 15.

17| Add 100 μl of 100% ACN to each tube to cover the gel piece completely and incubate for at least 5 min.

18| Dry the gel pieces completely in a SpeedVac concentrator 5301.

19| Add 10 μl of 10 mM dithiothreitol solution in 0.1 M ammonium bicarbonate, pH 8.6, and incubate for 60 min at 56 °C.

20| Add the same volume of 55 mM iodoacetamide in 0.1 M ammonium bicarbonate buffer, pH 8.6, and incubate in the dark for 45 min at 25 °C.

21| Discard the solutions, add 40 µl of 50 mM ammonium bicarbonate and incubate for 10 min at room temperature under ambient light.

22| Transfer the gel pieces to a clean tube, add 100 µl of ACN for 5 min then remove the ACN and dry the gel pieces completely using the SpeedVac concentrator.
■ **PAUSE POINT** Samples can be kept at −20 °C for several weeks, if wrapped tightly with a parafilm.

23| Prepare the following enzyme solutions:

Condition no.	Enzyme	Concentration (ng µl^{-1})	Buffer
1	Trypsin	12.5	25 mM ammonium bicarbonate pH 8.6
2	Trypsin	12.5	25 mM ammonium bicarbonate/40% acetonitrile (vol/vol), adjusted to pH 8.6 with ammonium hydroxide
3	Chymotrypsin	12.5	25 mM ammonium bicarbonate, pH 8.6
4	Chymotrypsin	12.5	25 mM ammonium bicarbonate/5% acetonitrile (vol/vol), pH 8.6
5	Chymotrypsin	12.5	25 mM ammonium bicarbonate/30% acetonitrile (vol/vol), pH 8.6
6	Subtilisin	10	Dilute in 1 mM HCl, and then dilute 1:100 with 9 parts of 6M urea and 1 part of 1M Tris, pH 8.5.

24| To each tube from Step 22 add one of the above buffers. The buffer volume must be sufficient to cover the gel pieces.

25| Incubate the samples using the conditions described below:

Enzyme	Incubation conditions
Trypsin	16 h (overnight) at 37 °C
Chymotrypsin	16 h (overnight) at 21 °C
Subtilisin	1 h at 37 °C (gently shaking)

26| Remove the buffers from the gel pieces and add 50 µl of 0.5% FA (vol/vol)/20% ACN (vol/vol) (conditions 1–5) or 5% FA (condition 6) for 15 min. Place the tubes in a sonication bath at room temperature.

27| Remove and retain the solutions from each condition. Repeat the addition of the solutions to the gel pieces and the sonication as described in Step 26.

28| Repeat Step 27. Pool the three, 50-µl solutions for each condition in a 0.6-ml tube. Use the SpeedVac to reduce the volume to ~10 µl and then add 10 µl of water.
▲ **CRITICAL STEP** Keep the sample volume as low as possible, but do not allow the samples to completely dry out. If this happens re-solubilize the samples in 0.1% FA (vol/vol)/20% ACN (vol/vol) for a minimum of 20 min in a sonication bath; this may result in a significantly lower intensity of spectra during mass spectrometry.

29| Transfer the solution into 0.2-ml tubes as stock solution for further analysis by the two mass spectrometers.
▲ **CRITICAL STEP** Samples should be injected without further treatment directly from the 0.2 ml tubes as soon as possible, to avoid sample loss due to adsorption of hydrophobic peptides onto the vial wall.

qQTOF ● TIMING 2 d

30| Transfer 50 µl of the extracted peptides to 0.5 ml protein lobind tubes.

31| Use Chromelon 6.7 software to control the Ultimate 3000 nano-LC system. Inject 6 µl of the sample and separate in a PepMap100 C18 nano-column, as described in the EXPERIMENTAL SETUP.

32| Use AnalystQS to control QSTAR XL. Record the MS spectra over the mass range of *m/z* 350–1300, and MS/MS spectra in information-dependent data acquisition over the mass range of *m/z* 50–1300. Repeatedly, one MS spectrum is generated, followed by three MS/MS spectra on the QSTAR XL instrument; the accumulation time is 1 s for MS spectra and 2 s for MS/MS spectra. The collision energy is set automatically according to the mass and charge state of the peptides chosen for fragmentation. Choose doubly or triply charged peptides for MS/MS experiments because of their good fragmentation characteristics[16].

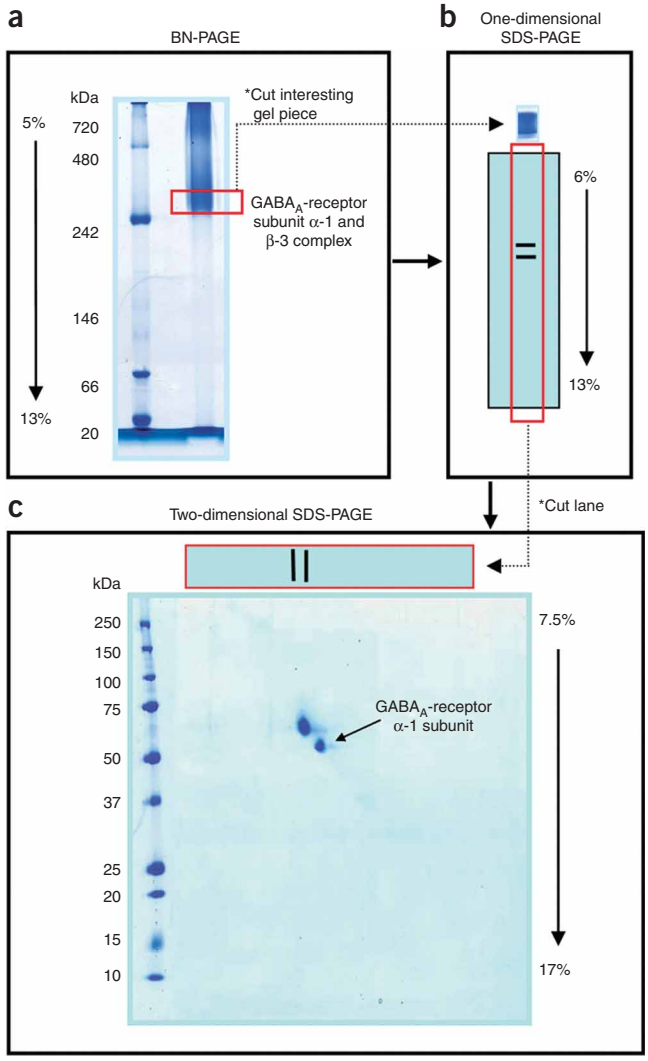

Figure 2 | Separation of gamma-amino butyric acid A ($GABA_A$) receptors using multi-dimensional gel electrophoresis—blue native (BN) and two-dimensional SDS-PAGE. (**a**) Purified recombinant $GABA_A$ receptors (60 μg) were separated by BN-PAGE using a 5%–13% separating gel. (**b**) The recombinant $GABA_A$-receptor complex band from the BN gel was dissected and separated on a 6%–13% SDS gel. (**c**) The resulting unstained SDS gel is then applied to a 7.5%–17% SDS gel. Colloidal Coomassie blue staining was used for protein visualization, and Precision plus protein standards were used as size markers. Adapted with permission from Kang *et al.*[14]. Copyright 2008 American Chemical Society.

▲ **CRITICAL STEP** The length of the exposed part of the ion-spray tip should not be more than 2 mm. Adjust the position of the ion-spray tip, ion-spray voltage and gas carefully to get high intensity before analyzing real samples. Clean the ion source approximately every 50 samples to prevent the spectrum quality from declining. Make the connection between nano-LC and QstarXL as short as possible to prevent separated peptides from getting merged before ionization. Make zero dead-volume connection for every connection.

33| Use mascot.dll 1.6b21 in AnalystQS to interpret MS/MS spectra from qQTOF, and search against the MSDB 20051115 database and UniProtKB/Swissprot 53.3 to identify proteins. The searching parameters should be set as follows: a mass tolerance of 500 ppm for peptide tolerance, 0.2 Da for MS/MS tolerance, 2 missing cleavage sites, fixed modification of carbamidomethyl (C) and variable modification of oxidation of methionine residues.

? TROUBLESHOOTING

LIT ● TIMING 2 h

34| Use the peptide extract solutions from the subtilisin digest (condition 6), from Step 28, centrifuge and transfer to a new 0.2-ml vial and inject directly into the UltiMate nano HPLC System, as described in the section Experimental design.

35| Use XCalibur 2.0.7 to control LIT. Record the peptide spectra over the mass range of *m/z* 450–1600, record MS/MS spectra in data-dependent data acquisition and set the default charge state to 3. The mass range for MS/MS measurements is calculated automatically according to the masses of the parent ions. Record one full spectrum followed by four MS/MS spectra for the most intense ions; automatic gain control should be applied. Set the collision energy to the arbitrary value of 35. Set fragmented ions onto an exclusion list for 20 s.

36| Use Mascot Daemon 2.2.2 software to merge and interpret MS/MS spectra recorded by the LIT. Search parameters should be set as follows: 2 Da mass tolerance for peptide masses, and 0.8 Da mass tolerance for fragment ion masses. Use 'no enzyme' option for subtilisin. Carbamidomethyl should be chosen for fixed modifications and oxidation (M) as variable modification. Use the newest version of UniProtKB (Swiss Institute of Bioinformatics, Basel, Switzerland) or the NR (NCBI Resources, NIH, Bethesda, MD, USA) protein database to identify proteins.

▲ **CRITICAL STEP** Searching without enzyme specificity implies that the running time can be long. Limiting the number of entries, which should be searched by choosing the right taxonomy, will shorten the search.

● TIMING

Steps 1–5, BN-gel electrophoresis: 2 d
Steps 6–12, BN/SDS/SDS-PAGE: 3 d
Steps 13–29, Spot excision and in-gel digestion: 2 d
Steps 30–33, qQTOF: 2 d
Steps 34–36, LIT: 2 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Cause	Solution
Step 5	Aggregation of hydrophobic proteins (no mobility into gel, remaining at application site)	Keeping extracted hydrophobic proteins in refrigerator	Use freshly prepared samples when hydrophobic proteins are extracted
	Several spots from purified protein	Protein amount too high	Decrease protein amount
	Poor resolution in BN-PAGE	Inappropriate detergent concentration	Dialyze samples with buffers containing concentration of detergents suitable for solubilization
	Strong blue color at starting lines of BN-PAGE	Aggregation from high amount of sample	Decrease sample amount
		Blocking entering of samples into the gel because of undissolved Coomassie blue in cathode buffer and/or in sample buffer	Stir and completely dissolve Coomassie blue in cathode and sample buffer
	Vertical streaking in BN-PAGE	Inappropriate concentration of detergents Short protein extraction time	Decrease or increase detergents concentration Increase extraction time
Step 12	Horizontal streaking in two dimensional SDS-PAGE gels	Gap or bubble between gel pieces and stacking gel	Use warmer (45–55 °C) 1% agarose to fill space (20–30 °C 1% agarose generates bubbles)
Step 33	No detection of transmembrane peptides	In case of using a strong signal spot, hydrophobic peptides can be aggregated during concentration	Depending on spot intensity, reduce the amount or make smaller gel piece
		After complete dryness of sample, re-solubilization of peptides insufficient	Use more than 40 μl of 30% of ACN with 1% FA for peptide solubilization, then reduce volume again to 10 μl and add 20 μl of HPLC-grade water
	Low sequence coverage	Low accuracy in MS/MS Aggregation of sample peptides	Calibrate mass spectrometers Re-solubilize sample peptides with 10%–20% ACN
		Complete dryness of the sample and re-solubilization	Carefully dry and use freshly prepared sample peptides
	Low intensity spectra	Contamination	Carefully prepare sample on clean bench and hood; use caps and gloves
		Gradient in HPLC too steep	Use slower and longer gradient in HPLC

ACN, acetonitrile; BN-PAGE, Blue native polyacrylamide gel electrophoresis; FA, formic acid; HPLC, high performance liquid chromatography.

ANTICIPATED RESULTS

We used an affinity-purified/enriched fraction of recombinant rat $GABA_A$ receptors[14] for separation by multi-dimensional gel electrophoresis (**Fig. 2**). This procedure was followed by in-gel digestion with three proteases, trypsin, chymotrypsin and

TABLE 2 | Comparison of sequence coverage and identified peptides from transmembrane domains (TM) resulting from the digestion of $GABA_A$-receptor α-1 subunit using six different conditions.

Condition no.	Enzyme	Incubation conditions	Sequence coverage obtained (%)	Total ion score[a]	Identified TM[b]	Mass spectrometer
1	Trypsin	12.5 ng μl^{-1} trypsin solution buffered in 25 mM ammonium bicarbonate pH 8.6	73	1,044	PTM2, pTM4	qQTOF
2		12.5 ng μl^{-1} trypsin solution buffered in 25 mM ammonium bicarbonate/40% acetonitrile pH 8.6 adjusted with ammonium hydroxide	64	1,025	TM2, TM4	
3	Chymotrypsin	12.5 ng μl^{-1} chymotrypsin solution buffered in 25 mM ammonium bicarbonate pH 8.6	66	1,188	TM1, pTM2, pTM3, pTM4	
4		12.5 ng μl^{-1} chymotrypsin solution buffered in 25 mM ammonium bicarbonate/5% acetonitrile pH 8.6	49	1,129	PTM1, pTM2, pTM4	
5		12.5 ng μl^{-1} chymotrypsin solution buffered in 25 mM ammonium bicarbonate/30% acetonitrile pH 8.6 adjusted with ammonium hydroxide	52	972	PTM1, pTM2, pTM4	
6	Subtilisin	100 ng μl^{-1} subtilisin solution in 5.4 M Urea and 100mM Tris (pH8.5)	60	589	PTM2, TM3, pTM4	qQTOF, LIT

GABA, gamma-amino butyric acid; qQTOF, quadrapole time-of-flight; LIT, linear ion trap; TM, transmembrane domain. Modified with permission from Kang *et al.*[14]. Copyright 2008 American Chemical Society.
[a]The total ion score represents the sum of scores of all identified peptide scores. [b]TM stands for transmembrane domain (TMs1-4).

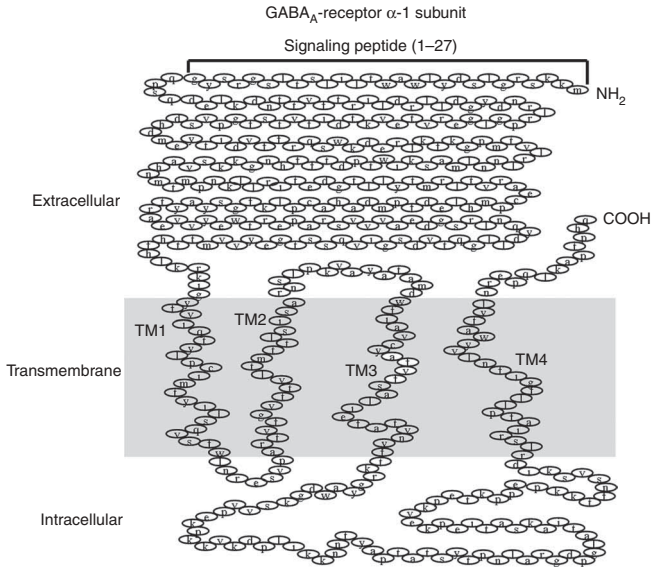

Figure 3 | Gamma-amino butyric acid A (GABA$_A$)-receptor α-1 subunit identified by multi-enzyme digestion and mass spectrometry. For mass spectrometric analysis of GABA$_A$-receptor α-1 subunit, the protein spot was excised from the two-dimensional SDS-PAGE gel and subjected to six different digestion conditions using different enzymes and buffers. The resulting peptides were then analyzed using quadrapole time-of-flight (qQTOF) and linear ion trap (LIT) (results in white). Excluding the signal peptide, the total sequence coverage for the α-1 subunit is 100%. These data allowed for the GABA$_A$-receptor α-1 subunit structure to be predicted. Adapted with permission from Kang *et al.*[14]. Copyright 2008 American Chemical Society.

subtilisin, under various conditions. Only the combination of these three enzymes under the specific incubation conditions (time, temperature, ACN or urea) described allowed determination of the complete protein sequence (**Table 2**). The simple use of the peptide-cutter software described above would not have been sufficient, as the conditions described were a limiting factor. The resulting peptides were then analyzed by qTOF for all conditions and by LIT for the subtilisin digestion. Representative spectra are given in **Supplementary Figures 1** and **2**. The peptide sequences obtained are listed in **Supplementary Table 1**. Without the use of the ion trap 99.1% sequence coverage (MS/MS) was obtained and the four individual amino acids, alanine, phenylalanine, valine and phenylalanine, in the transmembrane 3 (TM3) region of the sequence were not detectable (**Fig. 3**). **Supplementary Table 1** shows the peptides obtained from the use of individual enzyme digestions. The most successful enzyme in terms of highest sequence coverage was trypsin at condition 1.

Unambiguous high-score identification of the GABA$_A$ α-1 subunit covering the whole sequence of this receptor subunit was achieved using this protocol. The score for an MS/MS match was based on the absolute probability that the observed match between the experimental data and the database sequence was a random event. The results are in agreement with a nucleic-acid sequence of this receptor subunit (P62813, entry name: GBRA1_RAT) provided in the UniProtKB database (16 Aug 2004; http://www.expasy.org/uniprot/P62813) and represent the first complete protein sequencing of one of the GABA$_A$-receptor subunits (**Fig. 3**), including the four highly hydrophobic transmembrane domains; previously only fragments of this receptor sequence had been published[13]. This gel-based proteomic approach (**Fig. 1**) may therefore be recommended for the analysis of receptors and carriers, membrane proteins, as well as for other hydrophobic proteins. Moreover, downstream analysis including that of post-translational modifications and splice variants can be based on this gel-based technique.

Note: Supplementary information is available via the HTML version of this article.

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