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Efficient Digestion and Mass Spectral Analysis of Vesicular Glutamate Transporter 1: A Recombinant Membrane Protein Expressed in Yeast

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Abstract

Attempts to characterize recombinant integral membrane proteins (IMPs) by mass spectrometry are frequently hindered by several factors including the detergents required for extraction and purification that interferes with analysis, poor solubility, incomplete digestion, and limited identification of the transmembrane domain-spanning peptides. The goal of this study was to examine and develop methods for purification of an IMP that are amenable to downstream digestion of the protein and peptide analysis by mass spectrometry. In this study, we have overexpressed a candidate IMP, the vesicular glutamate transporter 1 (VGLUT1) in *Pichia pastoris* and examined conditions for the efficient affinity purification, in-solution digestion, and analysis of the protein. Analysis of the intact purified protein without detergent was performed by MALDI-TOF mass spectrometry. The purified IMP was digested with trypsin, and the resulting peptides were identified. A method that utilizes differential solubility and ionization properties of hydrophobic and hydrophilic peptides was developed. Large hydrophobic peptides were only detected in solutions containing 50% formic acid. Ionization of hydrophilic peptides was suppressed in formic acid, but they produced a strong signal in 50% acetonitrile. Eighty-seven percent sequence coverage of the protein was obtained with only one large hydrophobic peptide that remained unidentified. The results demonstrate a simple method to purify and digest a recombinant IMP for analysis by mass spectrometry.

Keywords

VGLUT1; membrane protein; MALDI-TOF; recombinant; sequence coverage; trypsin digestion; differential solubility

Introduction

Heterologous expression, purification, and digestion of recombinant membrane proteins for structural analysis by mass spectrometry are often problematic. Typically, to extract proteins from the membrane and maintain solubility during subsequent affinity purification steps, large amounts of detergent must be added. The detergent selected must allow efficient extraction from the membrane, allow for exposure of the affinity tag for column binding when applicable, and prevent nonspecific binding of proteins to the column. Most importantly, nonspecific column binding of the recombinant membrane protein can result in

low yields and nonspecific aggregation and adherence to other proteins thus resulting in poor purity. The importance of detergent choice is illustrated in the extraction of the multidrug resistance protein 1 (ABCC1), which was inefficiently extracted in the detergents 3-[(3-cholamidopropyl) dimethylammonio]-propanesulfonate (CHAPS), taurocholic acid, and *n*-dodecyl- β -D-maltoside (DDM) but was successfully extracted using lysophosphatidyl glycerol.¹ Affinity purification of the δ opioid receptor required four complementary column chromatography methods in DDM.² Alternatively, membrane proteins have been overexpressed as inclusion bodies in *E. coli* in a relatively pure form but then require strong anionic detergents or denaturants, such as sarkosyl or 7 M urea to solubilize them, leaving downstream digestion and analysis difficult.

For analysis by mass spectrometry, detergents used for purification must be removed from the protein while preventing significant absorptive loss of the protein on nonspecific surfaces. The problem of detergent removal has frequently been circumvented using in-gel digestion of the purified membrane protein after SDS-PAGE. For example, in-gel digestion of the δ opioid receptor resulted in 28% sequence coverage of the protein.² However, the in-gel digestion of ABCC1 required the sequential digestion of the protein by several two-enzyme combinations, which is undesirable because it increases the number of potential cleavage sites, missed cleavages, and enzyme autolytic peptides. Thus, in-gel digestion can make the identification of *m/z* peaks more difficult resulting in more unidentifiable peaks and peptide misassignments. Further, in-gel digestion of the purified cannabinoid receptors CB1 and CB2 failed to identify several transmembrane domain-spanning peptides.³⁻⁵ Overall, in-gel digestion methods are typically less effective than in-solution digestion and rarely improve the identification of transmembrane domain-spanning peptides.⁶ This is likely due to poor digestion of the protein aggregates produced by gel fixation and inefficient extraction and solubilization of large hydrophobic peptides from the gel piece, which probably explains the failure to obtain sufficient sequence coverage particularly of transmembrane domain-spanning peptides.

Methods developed for the in-solution digestion of integral membrane proteins have demonstrated success in the analysis of abundant endogenously occurring proteins, such as bacteriorhodopsin⁷ and lens aquaporin,⁸ but may have limited applicability to recombinant affinity-purified proteins. Digestion of bacteriorhodopsin in 60% buffered methanol allowed tryptic cleavage in transmembrane domain-spanning regions, while the native protein was still folded inside the membrane. It is likely that this strategy will be ineffective for an affinity-purified protein that has been extracted from the membrane, denatured, and aggregated. In-solution digestion of endogenous lens aquaporin in 10% buffered acetonitrile improved tryptic cleavage of the protein after precipitation in ethanol but first required isolation of the hydrophobic peptides by ultra-centrifugation.⁸ Digestion of membrane proteins using cyanogen bromide usually results in the production of very large hydrophobic peptides that are poorly soluble in aqueous solutions, are difficult to resolve in reflectron mode using MALDI-TOF mass spectrometry, and often do not acquire sufficient ionization for analysis by ESI-MS/MS. Additionally, cyanogen bromide and the strong acid coreagents used can produce multiple side reactions with the peptides.

In this study, we developed conditions for the efficient purification, in-solution digestion, and analysis of the vesicular glutamate transporter 1 (VGLUT1), overexpressed in *Pichia pastoris*. VGLUT1 is a 61 kDa membrane protein hypothesized to contain 10–12 transmembrane domains with a total GRAVY score of 0.225. Using the HMMTOP prediction algorithms,^{9,10} we developed the proposed model of VGLUT1 containing 12 transmembrane domains (Figure 1). Jung et al.¹¹ previously reported a similar 12 transmembrane domain model of VGLUT using antibodies to demonstrate that the amino-terminal and the carboxyl-terminal may be cytosolic. Analysis of the VGLUT1 topology by

mass spectrometry would greatly refine the existing computer models and validate the overall structure predictions.

VGLUT1 is responsible for transporting the neurotransmitter glutamate into synaptic vesicles and may be a potential drug target for diseases involving glutamate imbalance. The structure, transport mechanism, and ligand-binding domain of VGLUT1 are currently unknown, and as a result, numerous efforts are underway to solve VGLUT1 structure and/or define the transmembrane domains and the direction of the N- and C-terminus and cytosolic loops. A review of the VGLUT1 transporter covering the structure of the main inhibitors has been reported.¹² This is the first report to characterize VGLUT1 by mass spectrometry, and through this challenge methods have been developed that can be applied to the analysis of other recombinant membrane proteins by mass spectrometry.

Materials and Methods

Materials

The rat brain cDNA library, yeast shuttle vector pZAP α , *E. coli* strain DH5 α , *P. pastoris* strain X-33, antimyc monoclonal antibody, and Zeocin were obtained from Invitrogen (Carlsbad, CA). Protease inhibitor cocktail was obtained from Roche Applied Science (Indianapolis, IN). Hi-Trap chelating columns were obtained from Amersham Biosciences (Piscataway, NJ). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI). Bovine serum albumin (BSA) protein standard was obtained from Pierce (Rockport, IL). Formic acid (98%) was obtained from EM Scientific (Carson City, NV). α -Cyano-4-hydroxycinnamic acid (CHCA) matrix, 3, 5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) matrix, tris-(2-carboxyethyl)phosphine (TCEP), iodoacetamide, *n*-dodecyl- β -D-maltoside (DDM), 3-[(3-cholamidopropyl) dimethylammonio]-propanesulfonate (CHAPS), octyl- β -D-glucoside, methanol, nickel sulfate, and imidazole were obtained from Sigma (St. Louis, MO). The solvents, acetonitrile and isopropanol, were both 99% and used without further purification. Peptide calibration standards were obtained from Bruker Instruments (Billerica, MA) and Invitrogen (Carlsbad, CA).

Cloning of VGLUT1 and Construction of Plasmids

To facilitate the manipulation of the rat VGLUT1, gene primers for PCR were synthesized to introduce a unique *Eco*RI site upstream of the ATG initiation codon. A unique *Not* I site was inserted downstream of the coding sequence with the deletion of the termination codon to allow C-terminal *c-myc*/HIS tagged fusion proteins to be generated. Rat VGLUT1 (Genbank accession number U07609) sequence was amplified by PCR using specific primers, sense 5'-GAA TAA ACG ATG GAG TTC CGG CAG GAG GAG TTT-3' and antisense 5'-GCG GCC GCG TAG TCC CGG ACA GGG GGT G-3' in a 50 μ L reaction containing 0.2 μ M primers, 200 μ M dNTPs, 2.5 mM MgCl₂, 1 ng of rat brain quick-clone cDNA as template, and 2.5 U *Pfu* Ultra DNA polymerase to generate full length blunt ended fragments. This fragment was incorporated into pCR2.1 Blunt II-TOPO vector and transformed into competent cells. Sequence analysis confirmed homology of isolated VGLUT1 ORF to that in the NCBI database (GI: 16758725).

Yeast Transformation and Culture

Rat VGLUT1 sequence was subcloned into the *Eco*RI and *Not* I sites of the *Pichia pastoris* yeast expression vector pGAPZ B/*c-myc* HIS. The resulting plasmid pGAPZ B VGLUT1 was linearized by restriction digestion using *Avr* II and transformed into wild type *P. pastoris* strain X-33 by electroporation. Positive transformants were screened by PCR of the genomic DNA using pGAP Forward and 3'*AOXI* primers.

Yeast Membrane Preparation

All steps were performed on ice or at 4 °C unless otherwise stated. Sterile media (1 L) containing yeast extract, peptone, and dextrose (YEPD) were inoculated with a 2 mL starter culture and incubated at 30 °C with shaking at 300 rpm for 48 h. Yeast cells were pelleted by centrifugation at 500g for 10 min and washed once with cold PBS. The yeast pellet was resuspended in a buffer containing 1.0 M sorbitol, 40 mM HEPES pH 7.4, 10 mM MgCl₂, 2 mM dithiothreitol, and protease inhibitor cocktail and incubated at 30 °C for 10 min. Yeast cell walls were removed to create spheroplasts by treatment of the cells with 1 mg of zymolase per gram of yeast cell pellet and incubated at 30 °C for 30 min. Spheroplasted yeast cells were collected by centrifugation, resuspended in a lysis buffer (200 mM sorbitol, 50 mM HEPES buffer, pH 7.0, and protease inhibitor cocktail), and incubated on ice for 1 h. Cells were lysed by homogenization in a Dounce glass mortar and pestle. The cell lysis solution was centrifuged at 1000g for 5 min to pellet the unbroken cells and nuclei. The supernatant was transferred to a fresh tube and centrifuged at 13 000g for 20 min to pellet the crude large membrane fraction that was used as the source of VGLUT1 protein.

IMAC Purification of Hexahistidine-Tagged VGLUT1

Ten milligrams of yeast crude membranes in 1 mL were solubilized in 20 mL of binding buffer containing 1% SDS or alternative detergents as described, 20 mM sodium phosphate, pH 7.4, 100 mM NaCl, 10 mM imidazole, and 20% glycerol with shaking for 30 min. Insoluble material was removed by centrifugation at 15 000g for 20 min. Solubilized membranes were applied to a 1 mL HiTrap Chelating column charged with Ni²⁺ and equilibrated in binding buffer. The column was washed twice with 10 mL of binding buffer, and hexahistidine-tagged VGLUT1 protein was eluted in a 20 mL linear gradient of imidazole (10–150 mM) in binding buffer. Fractions (0.5 mL) were collected and screened for VGLUT1 protein by SDS-PAGE with colloidal Coomassie blue staining and Western blot analysis. The concentration of protein in each VGLUT1-containing fraction was quantified using the Lowry method.¹³

SDS-PAGE and Western Blot

An aliquot (20 µL) of each fraction eluted from the IMAC column was mixed with 20 µL of Laemmli sample buffer (Bio-Rad, Hercules, CA) containing 5% β-mercaptoethanol. The samples were not boiled to prevent oligomerization of the VGLUT1 protein. Samples were loaded into a 10% precast SDS-PAGE minigel and run at 100 V for 1 h. Proteins were either stained with colloidal Coomassie blue (Bio-Rad, Hercules, CA) or transferred to PVDF membranes for immunodetection. Recombinant VGLUT1 protein was detected on membranes by probing with a monoclonal antimyc antibody (Invitrogen, Calsbad, CA) at 1:5000 dilution in phosphate buffered saline buffer, pH 7.4, 0.1% Tween (PBS-T), followed by probing with a horseradish peroxidase-conjugated anti-mouse secondary antibody (Cell Signaling, Beverly, MA) at 1:4000 dilution in PBS-T. Protein bands were visualized with enhanced chemiluminescence reagents, ECL Plus (Amersham, Piscataway, NJ).

Intact Protein Analysis

The protein used for intact protein analysis was neither reduced nor alkylated. Purified VGLUT1 protein (10 µg) or BSA protein standard was precipitated in 10% trichloroacetic acid (TCA) at –20 °C for 30 min. The protein pellet was collected by centrifugation at 15 000g for 5 min and washed with a 1:1 solution of ethanol:ether at RT. The washed pellet was briefly air-dried and immediately resuspended in 10 µL of 50% acetonitrile and 0.1% TFA. The solution was analyzed immediately by MALDI-TOF mass spectrometry as described below and was not stored.

In-Gel Digest

Diced gel pieces were washed three times in a solution containing 50% acetonitrile in 25 mM ammonium bicarbonate buffer, pH 8.0 (AMBIC) for 1 h each or until all visible blue dye was removed. Gel pieces were dried in a SpeedVac, rehydrated in a solution of 100 mM AMBIC and 10 mM dithiothreitol, and incubated at 56 °C for 1 h. This solution was removed, and a fresh solution containing 100 mM AMBIC and 55 mM iodoacetamide was added and incubated with the gel pieces in the dark, at room temperature for 1 h. This solution was removed, and the gel pieces were washed twice in a solution containing 50% acetonitrile in 25 mM AMBIC and dried in a SpeedVac. Gel pieces were rehydrated in a solution containing 25 mM AMBIC and 12.5 ng/ μ L of trypsin at 4 °C for 15 min. After rehydration, the excess trypsin solution was removed and replaced with 25 mM AMBIC, and the gel pieces were incubated at 37 °C for 16 h. After digestion, peptides were extracted from the gel pieces in 60% acetonitrile and 0.1% TFA twice for 1 h each. The digestion and extraction solutions were combined in a separate tube and dried in a SpeedVac.

Trypsin Digestion In-Solution

The pH of a solution containing 10 μ g of purified VGLUT1 or BSA protein standard in binding buffer with imidazole was adjusted to >pH 8.0 with Tris-HCl buffer. Protein was reduced with 5 mM TCEP at RT for 1 h and alkylated with 100 mM iodoacetamide in the dark for 1 h. The protein was then precipitated in 10% trichloroacetic acid at -20 °C for 30 min. The protein pellet was collected by centrifugation at 15 000g for 5 min and washed with a 1:1 solution of ethanol:ether at RT. The washed pellet was briefly air-dried and resuspended in a 20 μ L solution containing 50 mM AMBIC buffer, pH 8.0, alone or 50 mM AMBIC buffer combined with 10% acetonitrile or 60% methanol. Sequencing grade trypsin was added at a 1:20 ratio, and digestion was allowed to proceed at 37 °C for 16 h. Tryptic peptides were analyzed directly from the digest solution or were dried in a SpeedVac. Dried peptides were resuspended in one of three solutions: (1) 50% acetonitrile, 0.1% TFA; (2) 50% formic acid; or (3) 50% formic acid, 25% acetonitrile, 15% isopropanol, 10% water (FAPH buffer).¹⁴

MALDI-MS

MALDI-MS analysis was performed on a Voyager DE PRO (Applied Biosystems, Foster City, CA). For peptide analysis, an aliquot of each sample was mixed with an equal volume of CHCA matrix solution in 50% acetonitrile and 0.3% TFA, and 0.5 μ L was spotted onto the target plate. For low molecular weight peptide analysis the CHCA matrix was spiked with a 1:250 dilution of Bruker peptide standards for internal calibration. For monoisotopic masses obtained in reflectron mode, m/z tolerance window was set at 50 ppm. For larger molecular weight peptides the CHCA matrix was spiked with a 1:10 dilution of Invitrogen peptide standards (InvitroCal 3 and 4) for internal calibration. For average masses obtained in linear mode, mass accuracy was set at 200 ppm. Typically, 500 laser shots were accumulated for each spectrum. Masses were searched against the SwissProt and NCBI protein databases or a VGLUT1 database using Mascot (www.matrixscience.com). Modifications for carbamidomethylated cysteines and partial methionine oxidation were included. For intact protein analysis an aliquot of sample was mixed with an equal volume of saturated sinapinic acid matrix in 50% acetonitrile and 0.3% TFA, and 0.5 μ L was spotted on the target plate. External calibration was performed using Bruker protein standards II.

Transmembrane Topology Prediction

The transmembrane model was generated by HMMTOP (www.enzim.hu/hmmtop/)^{9,10} based on the amino acid composition of the recombinant VGLUT1 protein.

Results and Discussion

Extraction and Purification of Hexahistidine-Tagged VGLUT1

The large number of transmembrane domains and hydrophobic nature of VGLUT1 make extraction of the transporter from the membrane difficult and cause it to strongly adhere to surfaces nonspecifically. Initial studies using 2% DDM detergent indicated that VGLUT1 eluted from the Hi-Trap Chelating column at low concentrations of imidazole, below 150 mM. Denaturing the protein in 8 M urea and 2% DDM detergent during purification did not permit elution at higher concentrations of imidazole and further enhanced formation of high molecular weight VGLUT1 oligomers as detected by Western blot analysis. After treating the column with a gradient of 10–150 mM imidazole, three additional column washes were performed in 250 mM imidazole, 500 mM imidazole, and finally 500 mM imidazole containing 1% SDS. Western blot analysis indicated that most of the VGLUT1 protein remained bound to the column until 1% SDS was added. Since the hexahistidine-tagged protein was not eluted with imidazole alone but required the presence of a strong denaturing detergent, it appeared that VGLUT1 was bound to the column and possibly other proteins in a nonspecific manner. SDS is not compatible with mass spectrometry analysis, and four detergent solutions were tested (2% CHAPS, 2% octyl- β -D-glucoside, 2% DDM and 1% SDS) for their ability to desorb VGLUT1 protein from the column. However, only 1% SDS prevented nonspecific adherence of VGLUT1 to the column and also prevented nonspecific binding of yeast proteins that were coeluting with VGLUT1 in the presence of other detergents. By extraction of VGLUT1 in 1% SDS followed by purification in a shallow gradient of 10–150 mM imidazole containing 1% SDS, fractions could be collected that contained a single band on a Coomassie blue stained gel corresponding to the approximate molecular weight of VGLUT1 (Figure 2B). While the abundance of VGLUT1 in yeast crude cell membranes was not high enough to make a predominant band on the PAGE gel (Figure 2B), the typical yields obtained from a standard 1 L culture (1 mg VGLUT1 protein) were good for a large, mammalian membrane protein. Also shown to coelute in the same fraction on the gel as VGLUT1 are two higher molecular weight bands representing VGLUT1 oligomers (approximately 180–220 kDa) that likely form due to the extreme hydrophobic nature of the protein. All three bands are detected by the antimyocitope antibody by Western blot (Figure 2A) indicating that they are derived from the recombinant VGLUT1 epitope-tagged protein and not a protein from yeast. Due to the high purity of VGLUT1 in the column-eluted fractions, it was diluted 1000-fold relative to the crude cell membranes for Western blot analysis shown in Figure 2A. Interestingly, the VGLUT1 band migrates on Coomassie blue stained SDS-PAGE gels and Western blots at approximately 60 kDa, even though the predicted molecular weight of epitope-tagged VGLUT1 is 64 721 Da.

As an added test of protein purity, the VGLUT1 protein band from the Coomassie blue stained gel was excised and digested with trypsin. Peptides extracted from the gel spot were analyzed by MALDI-MS and searched against the yeast database of protein sequences in SwissProt using MASCOT. While no yeast proteins were identified, 10 peptides matching VGLUT1 (sequence coverage 18%) were identified when searched against the rat NCBI database. Since VGLUT1 is a membrane protein that tends to aggregate in solution and is difficult to digest in AMBIC buffer alone, it is not surprising that the in-gel digest of VGLUT1 produced few peptides.

Effects of Residual SDS Detergent

Few efficient methods exist to remove detergent from membrane protein solutions. Use of dialysis or ultrafiltration devices frequently fails due to detergent micelle formation, and in cases where the detergent has been efficiently removed, significant loss of the membrane

protein can occur by nonspecific adherence of the protein to surfaces. In practice, we found that VGLUT1 strongly adhered to ultrafiltration membranes.

A very effective way to remove detergent from a protein solution is protein precipitation typically performed by chloroform:methanol extraction¹⁵ or in ice-cold 10% TCA. TCA precipitation to remove SDS followed by protein quantitation has demonstrated efficient recovery of the protein.^{16,17} For dilute solutions, the use of ribonucleic acid as a carrier may be needed to obtain efficient protein precipitation in the presence of SDS.¹⁶ With VGLUT1, TCA precipitation produced a more soluble protein pellet that digested more efficiently with trypsin than chloroform:methanol extraction (data not shown). The average protein concentration of VGLUT1 in 1% SDS was 0.1 mg/mL, and at this concentration the protein was easily TCA precipitated without a carrier.

SDS is also known to tightly bind to proteins making detergent removal difficult and quenching peptide ionization by ESI-MS.¹⁸ While SDS solutions containing VGLUT1 were not boiled, residual SDS could remain bound to the TCA precipitated protein that was not removed by the ethanol:ether solution and could quench peptide ionization. Residual SDS has been detected on proteins precipitated by chloroform: methanol extraction at the concentration of $2 \times 10^{-4} \%$; however, this concentration of SDS did not affect subsequent protein analysis by MALDI-MS.¹⁹ The SDS measurement method used cannot be performed on TCA precipitated proteins because TCA causes high background levels in the assay.²⁰ To determine if residual SDS bound to TCA precipitated proteins could affect ionization by MALDI, a BSA protein standard was diluted into two solutions containing 25 mM Tris buffer, one with 1% SDS and one without. The protein concentration was the same as that of VGLUT1 (0.1 mg/mL). The two BSA solutions were TCA precipitated and washed with ethanol:ether (1:1). The pellets were resuspended in 50% acetonitrile and 0.1% TFA, and the mass of the intact protein was measured in six identical spots by MALDI-MS (Figure 3A/3B). The experimental mass of BSA when TCA precipitated in 1% SDS was 66407 ± 47 Da, and the experimental mass of BSA TCA precipitated in 25 mM Tris was 66398 ± 38 Da. The intensity and signal-to-noise ratio of the BSA m/z signal was similar in both solutions. A second experiment was performed in which the TCA precipitated pellets were resuspended in 10% buffered acetonitrile and digested with trypsin. The resulting peptides were analyzed by MALDI-MS (Figure 3C/3D). After digestion with trypsin, the mass spectrum of peptides from the protein precipitated in SDS had a similar intensity and signal-to-noise ratio to the mass spectrum of peptides from the protein precipitated in Tris buffer. One m/z peak at 1724.8 Da matching the carbamidomethylated peptide from position 469–482 in the protein was significantly larger in the trypsin digest sample prepared from 1% SDS. This is likely due to the fact that the protein alkylation step was more efficient in 1% SDS than in 25 mM Tris buffer because the protein was denatured. Two peptides at m/z 1639 and 960 Da show a decreased intensity in the spectra from protein samples treated with SDS, and this may be the result of a low level of signal quenching due to residual SDS in the sample. Overall, Mascot analysis of the m/z peaks from the BSA digest with SDS identified 39 peptides with 59% sequence coverage. Similar analysis of the m/z peaks from the BSA digest with Tris identified 36 peptides with 57% sequence coverage. The results demonstrate that if SDS remains bound to the protein after TCA precipitation it does not adversely affect downstream analysis by MALDI-MS.

MALDI Analysis of Intact VGLUT1 Protein

MALDI-MS analysis of the intact protein after TCA precipitation was performed to determine if the experimental mass of recombinant VGLUT1 protein expressed in yeast matched the predicted mass of the epitope-tagged protein (64 721 Da). After TCA precipitation and washing, the pellet was resuspended in 50% acetonitrile and 0.1% TFA and immediately mixed 1:1 with sinapinic acid matrix and spotted on the target plate. [Note:

Due to the very poor solubility of VGLUT1, storage of pellet solutions in 50% acetonitrile at -20°C created an insoluble pellet that could not be reanalyzed later. Boiling or sonicating the pellet in acetonitrile did not improve solubility as determined by MALDI-MS. Solutions of 0.1% octyl glucoside did not solubilize the pellet, and while 50% formic acid did produce a MALDI signal, it was less than that obtained using 50% acetonitrile. The MALDI spectrum of the intact protein displays a small doubly charged peak at 32.5 kDa and a very small high molecular weight peak representing the dimer at 130 kDa (Figure 4). The singly charged monomer has a mass of $65\,067 \pm 19$ Da, which is 346 Da larger than the predicted mass. This mass is the average of six measurements with each measurement consisting of 500 accumulated laser shots. The additional mass on the protein may be caused by a post-translational modification that has not yet been identified. The experimental mass determined by MALDI-MS is significantly different from the experimental mass estimated with SDS-PAGE molecular weight standards, which appeared to be closer to 60 kDa (Figure 2). The altered SDS-PAGE migration may also indicate the presence of a post-translational modification. As discussed below, only the amino-terminal methionine and a single peptide covering amino acids 206–271 were not identified, and all other peptides were identified without modification. This implies that the modification may exist in one of the two unidentified regions.

Trypsin Digestion of VGLUT1

Trypsin digestion of the purified VGLUT1 protein was performed to analyze the peptides by MALDI-MS, which will be used in future studies of VGLUT1 structure and its post-translational or chemical modifications. Before digestion, VGLUT1 protein was eluted from the Ni^{2+} -chelating column in 1% SDS, reduced with TCEP, and alkylated with iodoacetamide. Next, the protein was concentrated by TCA precipitation, and the pellet was washed with ethanol:ether (1:1). As determined prior, this method was shown to effectively remove SDS to a level that does not interfere with MALDI-MS analysis as demonstrated with BSA protein (Figure 3). The washed pellet containing 10 μg of denatured VGLUT1 protein was resuspended in a small volume of buffer (20 μL), and trypsin was added at an enzyme to a protein ratio of 1:20. Although a lower protein concentration (0.1 mg/mL) was used for the digestion of lens aquaporin, a high protein concentration (0.5 mg/mL) was essential to obtain the best digestion of VGLUT1. Decreasing the protein concentration to 0.1 mg/mL resulted in the identification of fewer peptides. It is possible that the TCA precipitated VGLUT1 protein was not completely resolubilized in digestion buffer; therefore, the true concentration of VGLUT1 protein available for digestion may be lower than calculated.

Organic solvents, such as 10% acetonitrile or 60% methanol, have been shown to improve the digestion of membrane proteins in solution. To optimize pellet solubilization and digestion of VGLUT1, three organic solvent-containing solutions were compared: 50 mM AMBIC, pH 8.0, 10% acetonitrile in 50 mM AMBIC, and 60% methanol in 50 mM AMBIC. After digestion, each solution was dried by speed vacuum, and the peptides were resuspended in 50% acetonitrile, 0.1% TFA, thereby allowing the direct comparison of the three solutions for their ability to improve digestion efficiency regardless of the relative peptide solubility in the three solutions. Digestion in the 10% acetonitrile or 60% methanol solutions produced similar mass spectra of equal intensity. Digestion in AMBIC alone produced fewer identified peptides with a lower signal intensity than digestion in organic solvents (data not shown). Therefore, organic solvents improved the solubility of the denatured and aggregated protein pellet to provide accessibility to tryptic cleavage sites. Peptides less than 2500 Da were identified as monoisotopic masses in reflectron mode (Figure 5). Larger peptides were identified as average masses in linear mode. Peptides containing cysteines were identified as the carbamidomethylated species, indicating that all

sites on the protein were successfully reduced and alkylated and a low level of methionine oxidation was observed. The first 18 amino acids in the protein sequence contain four cleavage sites for trypsin producing several peptides that are smaller than 500 Da. These peptides were too small for direct identification but were found as part of peptides with multiple missed cleavages (Table 1). The N-term peptide MEFR ($m/z = 582.27$) was not identified alone or in modified form, and the presence of pyro-glutamate, formylation, and acetylation was also searched. However, the peptide from amino acids 2–18 was identified and may indicate that the methionine in position one of the protein is cleaved during protein processing. Two consensus sequences for N-linked glycosylation are found at positions 92 and 93 in the VGLUT1 sequence, but the tryptic peptide containing both sites (81–98) was identified without modification (Figure 5). It is possible that the site is partially modified or that it is modified in mammalian cells, but not in yeast. As an additional measure of protein purity, m/z peaks from the spectrum of the VGLUT1 digest were also searched against the yeast protein database in SwissProt using MASCOT, but no peptides from additional proteins were identified. Digestion in 10% acetonitrile followed by peptide resuspension in 50% acetonitrile resulted in only 56% sequence coverage. Large peaks in the mass spectrum were very hydrophilic and few hydrophobic peptides were identified. Peptide hydrophobicity was calculated using the grand average hydropathy score (GRAVY)²¹ whereby a positive number indicates a hydrophobic peptide and a negative number indicates a hydrophilic peptide.

It was previously demonstrated that hydrophobic peptides from lens aquaporin were successfully solubilized in 50% formic acid after they were collected by ultracentrifugation and separated from the water-soluble peptides.⁸ Collection of hydrophobic peptides by ultracentrifugation requires digestion volumes of at least 250 μ L to prevent collapse of the microfuge tube and is unfortunately not applicable to the small volume used for VGLUT1 digestion, as described above. Therefore, to solubilize hydrophobic peptides for MALDI-MS analysis, the digestion solution was dried in a SpeedVac and resuspended in 50% formic acid. The mass spectrum of peptides in 50% formic acid was significantly different than the spectrum of peptides in 50% acetonitrile (Figures 6 and 7). Major peptide peaks found in the mass spectrum from 50% formic acid were large, hydrophobic peptides, while signals from the hydrophilic peptides was suppressed. By comparison, hydrophobic peptides in 50% acetonitrile were smaller than 3000 Da and had very low relative signal intensity when compared to the hydrophilic peptides (Figures 6 and 7). Additionally, no signal was observed for most small peptides, less than 1900 Da in 50% formic acid.

Protein incubation in heated solutions of formic acid is known to cause cleavage at aspartate residues and can result in the formylation of lysine residues.²² While peptides solubilized in 50% formic acid were not heated for solubilization, the mass spectrum was searched for cleavage at aspartate residues and peptide formylation adducts. When peptides in formic acid were analyzed immediately after resuspension, no cleavage at aspartate residues or formylation adducts were identified. After storage of the peptides in formic acid at -80°C for two weeks or more, a low level of both aspartate cleavage and formylation adducts was observed.

These side reactions produced only minor peaks that did not significantly change the quality of mass spectra or the peptides identified.

Hydrophobic peptides from in-gel digests have been solubilized in solutions containing formic acid and isopropanol.²³ Additionally, a solution of 50% formic acid, 25% acetonitrile, 15% isopropanol, and 10% water (FAPH) has been used to extract intact proteins from gel pieces for MALDI-MS analysis.¹⁴ Therefore the FAPH solution was tested to solubilize hydrophobic VGLUT1 peptides after speed vacuum drying. As shown in

Figures 6 and 7, the FAPH solution clearly improved the signal-to-noise ratio, which allowed identification of two low intensity hydrophobic peptides at m/z 2485 and 3016 Da. Signal intensity for most peptides was improved and most pronounced for peptides m/z 2831 and 2019 Da. The FAPH solution was more effective at suppressing the signal of peptides identified in 50% acetonitrile as peptides m/z 2229 and 2508 Da were present in 50% acetonitrile solution and 50% formic acid solution but completely suppressed in FAPH solution. A total of 9 unique hydrophobic peptides were identified in the FAPH solution as well as several large peptides containing missed cleavages that were not identified in 50% acetonitrile. Impressively, 49% sequence coverage was obtained by solubilization of the peptides in FAPH solution, and a total sequence coverage of 88% was obtained from peptides identified using both solutions from a single digest. One large hydrophobic peptide (7294 Da; GRAVY = 0.674) was not identified by either method. As discussed above, it may be the site of a post-translational modification since the FAPH solution was successful at solubilizing all other hydrophobic peptides. Alternatively, it is possible that the unidentified peptide from amino acids 206–271 was not efficiently digested because it is located in a very hydrophobic region of the protein that was not well solubilized in 10% acetonitrile or 60% methanol. Efficient digestion of proteins within the membrane has proved successful using these solvents, but additional methods or increased organic solvent may be needed for proteins that have been denatured and aggregated by TCA precipitation. VGLUT1 is significantly larger (65 kDa) and contains more transmembrane-spanning domains (10–12) than other membrane proteins that are digested in-solution with efficiency.

Conclusions

The success of any membrane protein purification protocol relies heavily upon the detergent used for extraction and purification, and a variety of detergents have been tested. SDS is a more economical detergent than most used for protein purification and allowed the efficient one-step purification of VGLUT1. Removal of the detergent after purification was simple and did not interfere with downstream analysis by mass spectrometry. The method presented here for extraction and purification of a membrane protein in 1% SDS, protein concentration, and detergent removal by TCA precipitation, followed by hydrophobic peptide solubilization in solutions containing 50% formic acid, can be applied to the analysis of any recombinant membrane protein by mass spectrometry. The method requires the use of trypsin digestion in an organic solvent. Since trypsin cleaves at only two amino acids and produces very few missed cleavages, mass spectrum analysis from trypsin digestion is significantly simplified compared to digestion by other enzymes such as pepsin and chymotrypsin and chemical methods like cyanogen bromide. This simplification is desirable when peptide analysis is combined with protein chemical modification and/or cross-linking methods, which required more detailed analysis of the mass spectra. The use of chemical modification and cross-linking combined with this method should be a useful for strategy for structural analysis of recombinant membrane proteins by mass spectrometry.

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Abbreviations

AMBIC	ammonium bicarbonate buffer
BSA	bovine serum albumin
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-propanesulfonate

CHCA	α -cyano-4-hydroxycinnamic acid
DDM	<i>n</i> -dodecyl- β -D-maltoside
FAPH buffer	50% formic acid, 25% acetonitrile, 15% isopropanol, 10% water
GRAVY	grand average hydropathy
IMP	integral membrane protein
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TCEP	tris-(2-carboxyethyl)phosphine
TFA	trifluoroacetic acid
VGLUT1	vesicular glutamate transporter 1

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MEFRQEEFRK LAGRALGRLH RLLEKRQEGA ETELELSADGR PVTTHTRDPP VVDCTCFGLP 60
  ---+-----+-----+-----+-----+-----+
RRYIIA[MSG] LGFCISFGIR CNLGVAIVSM VNNSTTHRGG HVVVQKAQFN WDPETVGLIH 120
+ -----+-----+-----+-----+-----+-----+
[SSFFWGYIVT] QIPGGFICQK FAANRVFGFA IVATSTLNML IPSAARVHYG CVIFVRILQG 180
-----+-----+-----+-----+-----+-----+
LVEGVITYPAC HGIWSKWAPP LERSRLATTA FCGSYAGAVV AMPLAGVLVQ YSGWSSVFYV 240
+++++++ ++++++ +
VGSFGIFWYL FWLLVSYESP ALHPSISEEE RKYIEDAIGE SAKLMNPVTK FNTPWRRFFT 300
+++++++ ++++++ ++++++ +
SMPVYAIIVA NFCRSWTFYL LLISQPAYFE EVFGFEISKV GLVSALPHLV MTIIVPIGQG 360
+++++++ +-----+-----+-----+-----+-----+
IADFLRSRHI MSTINVRKLM NCGGFGMEAT LLLVVGYSHS KGVAISFLVL AVGFSGFAIS 420
+++++++ ++ ++++++ --
GFNVNHLDIA PRYASILMGI SNGVGTLSGM VCPIIVGAMT KHKTREEWQY VFLIASLVHY 480
-----+-----+-----+-----+-----+ +-----+
SGVIFYGVFA SGEKQPWAEP EEMSEKCGF VGHDQLAGSD ESEMEDEVEP PGAPPAPPPS 540
-----+-----+-----+-----+-----+-----+
YGATHSTVQP PRPPPPVRDY AAASFLEQKL ISEEDLNSAV DHHHHHH 587
+++++++ ++++++ ++++++ ++++++ ++++++

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Figure 1.

Putative transmembrane topology of the recombinant VGLUT1 protein. Twelve transmembranous regions (67–91, 118–137, 146–165, 170–189, 210–229, 234–257, 299–323, 340–358, 379–398, 403–422, 435–458, 471–490) are shown as white letters in color backgrounds. +, regions identified in 50% acetonitrile; – regions identified in 50% formic acid; ± regions identified in both 50% acetonitrile and 50% formic acid (corresponding to Table 1). Approximately 87% (510/587) sequence coverage of the protein was obtained.

Figure 2.

Affinity purification of hexahistidine-myc-tagged VGLUT1. (A) Western blot probed with antimyc antibody to detect recombinant VGLUT1. Lane 1: 1:1000 dilution of column eluted fractions that contain VGLUT1. Lane 2: column flow-through showing no VGLUT1. Lane 3: crude yeast membranes precolumn contain VGLUT1. (B) Coomassie blue stained SDS-PAGE gel (20 μ L of the following samples). Lane 1: pooled VGLUT1-containing fractions eluted from the Ni^{2+} -chelating column. Lane 2: column flow-through. Lane 3: crude yeast membranes before column purification.

Figure 3.

Effect of residual SDS on peptide and protein analysis by MALDI-MS. Panels A and B: mass spectrum of intact BSA protein TCA precipitated from a solution containing 25 mM Tris buffer (A) or 1% SDS in Tris Buffer (B). Panels C and D: mass spectrum of peptides from a trypsin digest of BSA protein TCA precipitated from a solution containing 25 mM Tris buffer (C) or 1% SDS in Tris Buffer (D).

Figure 4.

MALDI mass spectrum of intact VGLUT1 protein. Affinity-purified VGLUT1 was TCA precipitated, resuspended in 50% acetonitrile and 0.1% TFA, and mixed 1:1 with sinapinic acid matrix. The spectrum results from accumulation of 500 laser shots in linear mode. Six individual spots were analyzed and averaged to determine the mass of the protein.

Figure 5. Mass spectrum of peptides from VGLUT1 digested with trypsin in 10% buffered acetonitrile. Peptides <2500 Da were identified in reflectron mode, and larger peptides were identified in linear mode. The m/z and assigned peptide sequence is labeled.

Figure 6. Mass spectrum of VGLUT1 peptides identified in 50% formic acid. The m/z and assigned peptide sequence in the protein is labeled. The spectrum depicts average masses acquired in reflectron mode but optimized for the acquisition of larger peptides.

Figure 7. GRAVY score correlation with peptides identified in acetonitrile and FAPH Buffer. Panel A: Mass spectrum of the large VGLUT1 peptides in 50% acetonitrile. Panel B: Mass spectrum of the large VGLUT1 peptides in FAPH buffer. The average m/z and GRAVY score of the identified peptides are shown. Mass spectra were acquired in reflectron mode optimized for larger peptides.

Table 1

^a

peptide position	m/z observed (Da)	GRAVY	50% acetonitrile	50% formic acid	missed cleavages	modification
002-018	2064.36 avg	-1.171		X	4	
005-009	708.33 mi	-2.440	X			
005-021	2038.37 avg	-1.094		X	4	
019-047	3315.72 avg	-0.983	X		3	
026-047	2425.62 avg	-1.127	X		1	
027-047	2268.12 mi	-0.967	X			
048-061	1632.75 mi	0.186	X			2 CAM
063-080	2019.48 avg	1.639		X		1 CAM
081-098	1972.97 mi	0.306	X			1 CAM
099-106	823.48 mi	0.150	X	X		
107-140	3915.47 avg	0.209		X		1 CAM
141-166	2740.24 avg	0.965		X	1	
146-166	2180.61 avg	1.271	X	X		
167-176	1249.65 mi	1.300	X			1 CAM
177-196	2229.61 avg	0.435	X	X		1 CAM
197-203	868.46 mi	-0.929	X	X		
206-271	7294.35 avg	0.674	N.D.			
273-283	1195.58 mi	-0.391	X			
284-290	802.45 mi	0.029	X			
291-296	820.41 mi	-1.400	X	X		
298-314	2037.45 avg	1.259	X	X		1 CAM
315-339	3016.47 avg	0.444		X		
340-366	2831.48 avg	1.396	X	X		
369-377	1058.54 mi	-0.311	X			
379-401	2485.94 avg	0.661		X		1 CAM
402-432	3190.72 avg	1.171		X		
433-461	2883.49 avg	1.169	X			
464-494	3568.07 avg	0.358		X	1	
466-494	3310.78 avg	0.562		X		

peptide position	m/z observed (Da)	GRAVY	50% acetonitrile	50% formic acid	missed cleavages	modification
495-507	1589.68 mi	-2.008	X			
508-558	5346.45 avg	-0.865	X			1 CAM
559-569	1242.60 mi	-0.409	X			
570-587	2126.97 mi	-1.122	X			

^ami = observed as a monoisotopic mass, avg = observed as an average mass, CAM = carbamidomethyl cysteine, N.D. = not detected.