Yüksek sekans içerikli fare beyin zarından VDAK'ın (voltaja dayalı anyon kanalları) kütle spektroskopi haritalandırılması

Natalia AKENTIEVA[1]

Öz

Voltaj bağımlı anyon kanalları (VDAK) spesifik olmayan difüzyon gözenekleri veya mitokondriyal membran aracılı substratların taşınması için özel sistemler olarak hizmet veren entegre membran proteinleridir. VDAK'ın fonksiyonel rolü birçok çalışmada araştırılmış ve VDAK'ın farklı işlevleri gösterilmiştir. VDAK'ın post-translasyon modifikasyonları regülasyonu için önemlidir. VDAK'ın post-translasyon modifikasyonları regülasyonu için önemlidir. Araştırmamızın amacı, VDAK'ların post-translasyonel ve diğer zar proteinlerinin modifikasyonları ve primer yapının karakterizasyonu için yaklaşım geliştirmektir. Fare beyin zarları diferansiyel santrifüjleme ile fare beyninden izole edildi. Fare beyin zarlarından gelen mitokondriyal izoform VDAK'ın primer yapısı tripsin ile proteolitik yıkımdan sonra peptit karışımlarının LTQ-FTMS haritalama ve SDS-PAGE 'nin kombinasyonu ile neredeyse tamamı (%95, 283 amino asitin 258'i) tanımlanmıştır. VDAK'ın bulunan her bir peptid dizisi doğru kütle, izotopik dağılım ve MS / MS tandem analizine göre analiz edilmiş ve doğrulanmıştır. VDAK'ın peptitlerinin post-translasyon modifikasyonları gösterilmiştir. VDAK'ın yüksek dizi içeriği 11 transmembran alanı dahil olmak üzere elde edilmiştir. 30-34 kDa'da diğer bazı proteinler için geniş çaplı sekans içeriği de tespit edilmiştir. Sırasıyla; östrojen reseptorü baskılayıcısı aktivitesinin içeriğinin %76'sı, malat dehidrogenazın %55'i, sintaksin 1A ve sintaksin 1B2'nin %60 ve %65'i belirlenmiştir. Bu sonuçlar, kütle spektrometrik haritalamanın ana yapı membran proteinlerinin karakterizasyonu ve bunların post-translasyonel modifikasyonlarının tanımlanması için güvenilir ve duyarlı bir yaklaşım olduğunu göstermiştir.

Anahtar Kelimeler: VDAK, ana yapı, sekans içeriği

Yayın Bilgisi

Gönderi Tarihi:10.09.2018

Kabul Tarihi:02.10.2018

Online Yayın Tarihi:31.12.2018

DOI: 10.26453/otjhs.458683

Sorumlu Yazar

Natalia AKENTIEVA

Mass-spectrometric mapping of VDAC (voltage dependent anion channel) from mouse brain membranes with high sequence coverage

Natalia AKENTIEVA[1]

Abstract

Voltage dependent anion channels (VDAC) are integral membrane proteins serving as nonspecific diffusion pores or as specific systems for the transport of substrates through mitochondrial membranes. The functional role of VDAC has been investigated in many studies, and different functions of VDAC have been shown. Posttranslational modifications of VDAC are significant for its regulation. The aim of our research was to develop approach for characterization of primary structure and posttranslational modifications of VDACs and other membrane proteins. Mouse brain membranes were isolated from mouse brains by differential centrifugation. Primary structure of mitochondrial isoform VDAC1 from mouse brain membranes has been identified almost completely (95%, 258 of 283 amino acids) by combination of SDS-PAGE and LTQ-FTMS mapping of peptide mixtures after proteolytic degradation with trypsin. Sequence of each found peptide of VDAC has been analysed and confirmed according to accurate mass, isotopic distribution and MS/MS tandem analysis. Posttranslational modifications of VDAC's peptides have been shown. High sequence coverage of VDAC has been obtained, including 11 transmembranes domains. Extensive sequence coverage has been also detected for some other proteins at 30-34 kDa. A repressor of estrogen receptor activity has been identified with 76% coverage, malate dehydrogenase with 55 % sequence coverage, syntaxin 1A and syntaxin 1B2 have been sequenced with 60% and 65% coverage, respectively. These results demonstrated that mass spectrometric mapping is reliable and sensitive approach for characterization of primary structure membrane proteins and identification of their posttranslational

Article Info

Received:10.09.2018

Accepted:02.10.2018

Online Published:31.12.2018

DOI: 10.26453/otjhs.458683

Corresponding Author

Natalia AKENTIEVA

modifications.

Keywords: VDAC, primary structure, sequence coverage

[1] Institute of Problems of Chemical Physics Russian Academy of Sciences, 142432, Moscow region, Chernogolovka, prospect Akademika Semenova d.1.

INTRODUCTION

VDAC (voltage dependent anion channel) is involved in the choice the cells make to survive or die, which is particularly relevant to cancer cells. For these reasons, VDAC has become a potential therapeutic target to fight cancer but also other diseases, in which mitochondrial metabolism is modified. Mitochondrial VDACs (or so called "mitochondrial porins") are a small, 30-34 kDa proteins, originally discovered in the outer membrane of mitochondria, where they constitute the major pore-forming proteins.[1, 2] The role of VDACs is very different and significant for cells. For instance, recent studies strongly suggest, that VDAC is involved in release of apoptogenic factors, such as cytochrome c.[3-6] Moreover, VDAC acts as a platform for many proteins and in so doing supports glycolysis and prevents apoptosis by interacting with hexokinase, or members of the Bcl-2 family, respectively. Thus, the mitochondrial expressed VDAC presents itself as a core component that can be targeted in tumors, which has the potential to debilitate the aberrant metabolic fluxes of tumors and initiate apoptotic signaling cascades.[7] Due to its large pore, VDAC is presumed to play important role as a controlled passage for adenine nucleotides and other metabolites to and from mitochondria.[8, 9]

Mitochondrial VDACs have no primary sequence homology, or very minor, with bacterial channel porins. A large number of bacterial porins have been isolated and purified from bacterial outer membrane of *Rhodobactercapsulatus*, and the proteins functionally characterized in reconstitution experiments.[10] It has been demonstrated, most porins are trimeric and the structure of porin consists of a barrel formed by 16 tilted anti parallel beta-sheets.[11] The porin from *R. capsulatus* has been characterized by MALDI-

MS determination of peptides, while the total sequence coverage of porin *in-situ* gel digestion has been identified by 56%. The large protein fragment has not been characterized because it poorly eluted from the gel matrix.[12]

In contrast to bacterial porins, which primary and third dimensional structure has been well identified, the mammalian mitochondrial VDACs are not so well characterized. For example, the partial protein sequence for two fragments of the 36 kDa polypeptide has been detected by isolation of cDNA clones from a rat hippocampal library.[13] Five human cDNA clones have been identified to date showing 75-94% homology, however, only two mammalian proteins have been isolated and identified.[14,15] The mammalian VDAC is also a β barrel, but it is monomer. The crystal structure of mitochondrial VDAC is not available and, therefore, new studies about structure of VDAC are so important.

It is possible, that the activity of VDAC can be regulated by posttranslational modifications. Moreover, different VDAC isoforms may have various posttranslational modifications, which can effect on their specialized functions. Hence, the identification of posttranslational modifications of VDAC is very important to understand the molecular mechanism of its regulation and role in cell function. Therefore, it would be important to develop approach for rapid and total characterization of primary structure and posttranslational modifications of VDACs from cell's lysates.

Many different methods of mass-spec analysis exist, but the FTICR is the most effective approach to measure exact masses of peptides and mapping the sequence protein.[16]Mass spectrometric peptide mapping, particularly, by rapid-acquisition LTQ-FTMS, has recently been shown to be an efficient tool for characterization the primary structure of proteins. In combination with *in-situ* proteolytic digestion of proteins separated by one/two SDS-PAGE, mass spectrometric mapping permits identification of proteins from complex mixtures such as cell's lysates. LTQ-FTMS allows to perform accurate mass measurements (2-5 ppm) with greater sensitivity, accuracy and speed.[17]

Traditionally, proteomics analyses of complex protein samples involve the resolution of proteins using two-dimensional gel electrophoresis followed by the identification of resolved proteins by mass spectrometry. The limitations of this approach for membrane proteins are well documented. The major obstacle remains solubility for two reasons: first, many hydrophobic proteins are not solubilized in the non-detergent isoelectric focusing sample buffer; and second, solubilized proteins are prone to precipitation at their isoelectric point.

Therefore, in the present study we used the one-dimensional SDS-PAGE and coupled it with mass spectrometry for protein identification. We have studied the membrane protein–VDAC from MBM lysate, its primary structure by peptide mapping upon digestion in-gel matrix.

We have applied electrospray ionisation-MS/LTQ-FTMS to identify total sequence coverage of mitochondrial VDAC from the MBM. LTQ-FTMS analysis allowed the complete characterization the primary structure of this membrane protein. This result confirms that LTQ-FTMS can be used to identify the primary structure of membrane proteins in crude cell's lysates.

MATERIAL AND METHODS

Material

Reagents were of analytical grade and were used without further purification. Male mouse brains [VDAC$^{(+/+)}$ (*Mus.musculus,* strain CD1)] have been used for membrane preparation. Mouse brains were purchased from "BrainBits" (USA) and stored until use at −20 °C.

Reagents for SDS-PAGE and Western blot were purchased from "Amersham Biosciences" (USA) and "Calbiochem" (USA). Sypro Ruby staining kit was from "Invitrogen" (USA). The anti-Porin 31HL (Ab-1) mouse mAb (anti-VDAC1 isoform) monoclonal antibody were purchased from "Calbiochem" (USA). Goat anti-mouse IgG –HRP antibody were from "Santa Cruz Biotechnology" (USA).

Membrane Preparation

The membrane preparation for mouse brain regions has been followed.[18] Frozen mouse brain regions were homogenized in 0.32 M

sucrose and centrifuged for 10 min at 1000 *g* at 0-4 °C. The supernatant was centrifuged again at 100.000 *g* for 45 min. This pellet was disrupted osmotically in the distilled water, then washed twice with wash buffer (20 mM K_2HPO_4/KH_2PO_4 buffer with 50 mM KC1, pH 7.5), and stored in frozen state. For lysate preparation, mouse brain membranes were thawed, pellet and washed twice with wash buffer. The membranes were recovered by centrifugation at 100.000 *g* for 20 min, washed once again with wash buffer and re-suspended in lysis buffer (50 mM Tris-HCl buffer with 150 mM NaC1, pH 7.5, 1mM EDTA, 1% Triton X-100) to a final protein concentration of 1 mg/ml, incubated for 60min at 4 ^{0}C. The suspension was then centrifuged at 16.000 *g*, and the supernatant (lysate) loaded on 10% one-dimensional SDS-PAGE.

Electrophoresis

Polyacrylamide gel electrophoresis was performed using 10% polyacrylamide SDS-PAGE, under reducing conditions.[19]

After electrophoresis, the gel was stained or used for Western blot. Proteins from the SDS-PAGE gel were transferred onto Tropifluor™ polyvinylidenedifluoride membrane. Then the membrane was blocked with 5% dried milk for 1h, washed three times with Tris-buffered saline (0.05%)-Tween 20 and incubated overnight with anti-Porin 31HL (Ab-1) mouse mAb (anti-VDAC-1 isoform) monoclonal antibody (1:500).The membrane was then incubated for 1 h with goat anti-mouse IgG-HRP antibody (1:10000). Immuno-reactive bands were visualized using the ECL-plus Western blotting detection system.

Digestion in SDS-PAGE matrix (in-situ gel digestion)

Proteolytic digestion in the SDS gel matrix was carried out according to the procedure.[20] Staining and distaining of the gel were kept to a minimum, because both steps lead to partial fixing of the protein to the gel. The protein bands were excised from the gel by robotic instruments and washed for 1h in 40% (v/v) aqueous acetonitrile in order to remove staining dye, gel buffers and SDS. The distained gel plugs were dried at room temperature in a vacuum centrifuge and swollen in 100 µl 50

mM NH_4HCO_3, containing 12.5 ng / µl modified trypsin in an ice bath. After the gel plug had reached its original size, the supernatant was removed, and 100 µl 50 mM NH_4HCO_3 was added. Proteolytic digestion was carried out for 24h at 37 ^{0}C under gentle shaking. Peptides were extracted with a 1:1 mixture of acetonitrile and 50 mM NH_4HCO_3 for 24 h and lyophilized to dryness. The lyophilized extracts were dissolved in UHQ water, and the peptides were precipitated by the addition of 10% TCA. After 30 min at 0 ^{0}C the samples were centrifuged for 10 min at 15.000 *g*. After the pellet was washed with cold acetone, it was dried and stored at –20 ^{0}C.

Molecular mass determinations by Electrospray Ionization Mass Spectrometry (ESI-MS)

ESI-MS was used to identify the sequence coverage of VDAC. Following enzymatic degradation with trypsin, peptides were dissolved in 0.12% (vol / vol) TFA/water and analysed by LTQ-FTMS (Thermo Fisher, San Jose, CA). The mass spectrometer was set up in nanospray configuration with a Pico Viewnanospray source (New Objective, Woburn, MA) and coupled to a 1D plus nano-LC system (Eksigent, Livermore, CA) running at a flow rate of 200 nl/min. Sample injection was performed with an autosampler (Endurance, Spark, Plainsboro, NJ). The column was a C-18 PicoFrit (75m × 10cm) (New Objective). The mobile phases were HPLC grade water (Fisher Scientific, Pittsburgh, PA), containing 1% formic acid (Sigma-Aldrich, St. Louis, MO) (Solvent A) and acetonitrile were (Honeywell, Burdick & Jackson, Muskegon, MI) containing 1% formic acid (Solvent B). The sample (5 L) was loaded at 600 nl/min at 5% B for 10 min. The flow was then decreased to 200 nl/min with isocratic elution for 5 min followed by linear increase in Solvent B (2%/min) for 45 min. The LTQ-FTICR (7T) mass spectrometer was operated in the data dependent mode. The survey scans (*m/*z = 450-1500) were acquired using the FTICR-MS with resolution of ~100.000 at *m/z* = 421.75 after ion accumulation in the trap to a value of ~1.000.000. The MS/MS isolation width was 2.5 Da, and the normalized collision energy was 35%.

Electrospray ionization was accomplished with a spray voltage of 2.8-3.1kV without sheath gas. The ion transfer tube was 70V and the capillary temperature was 250 ^{0}C.

Data Analysis

Theoretical lists of the masses for singly, doubly and triply unmodified tryptic peptides for VDAC1 (gi code: 56207177) was prepared by *in silico* digestion using Protein Prospector.[21] Missed trypsin cleavages (0, 1, 2, 3, and 4) and the following variable modifications were considered: acetylation (N-term), carbamidomethylation of cysteine (C), oxidation of methionine (M), Pyro-glu (N-term Q) and phosphorylation of serine, threonine, and tyrosine residues.

The MS data were collected in the profile mode as were the MS/MS spectra. The "raw" files were processed using LCQ-DTA software (Thermoelectron), and the resulting text files were exported to MASCOT 1.9.05 software (Matrix Science, Oxford, U.K.). The LCQ-DTA settings were as follows: grouping tolerance, 0.0001 Da; "intermediate scans", 1; and "minimum number of scans per group", 1. The tandem MS data were searched using the following settings and databases (considering 0, 1, 2, 3 or 4 missed trypsin cleavages): (1) enzyme, trypsin , MS tolerance 3 ppm, MS/MS tolerance 0.8Da, NCBI nonredundant database (July 26, 2005), and carbamidomethylation of cysteines and oxidation on methionines as "variable modifications"; (2) no enzyme, MS tolerance 3ppm, MS/MS tolerance 0.8 Da, VDAC1 (gi code: 56207177), and carbamidomethylation of cysteines, oxidation on methionines and phosphorylation of all serine, threonine, and tyrosine residues as variable modifications.

RESULTS AND DISCUSSION

The protein profiles of mouse brain membranes are shown in Fig.1A.

The presence of protein band, which cross-reacted with monoclonal antibodies, directed against VDAC1 in these membranes, as detected by SDS-PAGE and Western blot analysis, is shown in Fig.1C. Only one protein band at 30-34 kDa was found to cross-react with

the VDAC1-monoclonal antibody. Localization of this protein band is correlated with molecular mass of VDAC. Nevertheless, it is possible, that the available monoclonal VDAC antibodies to the N-terminus of human VDAC1 are not be highly specific. Therefore, multiple proteins of around 30-34 kDa could cross react with VDAC antibody and were visible by Western blot. As example, it has been reported recently to label proteins in the plasma membrane despite the absence of VDAC there.[22-25]

In our case, the procedure of preparation MBM is supposed the content of enriched fraction of mitochondrial membranes. Therefore, immunoreactive band at 30-34 kDa is belong to the mitochondrial VDAC1. In addition to, the mass spec analysis of the digests of detected band definitely identified the amino acid sequence of mammalian VDAC1.

The stained band corresponding to that detected by Western blotting has been selected and spots have been excised by robotic instrument (Fig.1B), digested with trypsin and examined by LTQ-FTMS analysis. At first, we investigated the utility of finding MS/MS spectra of VDAC peptides in this large data set using the accurate mass measurements. We determined the overlap (within 3ppm) of m/z values (+1, +2 and +3 charge states) for tryptic peptides (0.1-4 missed cleavages and variable modifications for carbamidomethylation of cysteines, oxidation of methionines and phosphorylation of all serine, threonine, and tyrosine residues for $VDAC^{(+/+)}$ from MBM.

The MS/MS spectrum of sufficient quality signal (m/z value) has been interpreted and found to be attributable to the peptides of VDAC1. The predicted M.w. of VDAC1 should be 30756 D, according to MS-Digest Search Results (http://prospector.ucsf.edu). We detected the experimental M.w. of VDAC1, it was 30754 D. This M.w. has been found by MASCOT Search Results Program and perfectly correlated with predicted molecular mass.

Then we analysed all possible theoretical m/z values of the singly, doubly, triply charged species from peptides. We analysed the FTMS spectra according to the accurate peptide masses of VDAC1 peptides in either +1, +2 or +3

charge states. Every FTMS spectrum, containing signals corresponding to these accurate m/z values, was then inspected to confirm the theoretical charge state and isotopic distribution. The observed peptides have shown in Table 1, they have obtained from MS/MS spectra and deduced the peptide sequence of VDAC1.

In addition, Table 1 compares the theoretical and observed (experimental) masses of the VDAC1 peptides produced after analysis with nano-FTMS. The theoretical masses of the VDAC1 peptides have been found, using Protein Prospector Program (http://prospector.ucsf.edu). Observed masses of the VDAC1 peptides were detected as singly, doubly and triply charged ions, using MASCOT search program.

All VDAC1 peptides have been found as singly, doubly and triply charged peptides and tandem MS/MS spectra have been observed for each peptide. Fig. 2A shows the total spectrum of single liquid chromatography spectrum (time detection from 0-45 min).

Eluted peptides were analysed by MASCOT Search Program. For example, Fig. 2B shows the MS/MS spectrum of the peptide eluting at 20.05 min. The theoretical mass of this peptide (947.5202), which is correspond to singly charged state with the sequence $_{275}$LGLGEFQA$_{280}$. We calculated delta, the difference between theoretical, predicted mass and observed, experimental mass (947.5202-946.5223=0.0021). A delta value (-0.0021/947.5202) divided on theoretical mass, it gave value of tolerance. The tolerance for peptide $_{275}$LGLGEFQA$_{280}$ was–2.2 ppm, which is less than 5 ppm. All peptides with tolerance less than 5 ppm have been chosen as real peptides, corresponding to theoretical, predicted sequences. The theoretical mass of this peptide corresponded to singly charged peptides with the sequence $_{275}$LGLGEFQA$_{280.}$ To confirm the sequence of peptide, we compared the experimental MS/MS spectrum with theoretical pattern fragmentation, found from Prospector Program. The MS/MS spectrum of this peptide showed almost the complete suite of y- and b-

ions, which confirmed the theoretical sequence of peptide (Fig. 3).

The complete set of b-ions and y-ions for interpretation the sequence $_{275}$LGLGEFQA$_{280}$ is shown in Fig. 4. Some of other ions, such as a, b-NH_3, b-H_2O and y-NH_3 ions were also observed.

We were not detected b_1- and y_1-, y_2- ions, because of low mass values. Sequence of each found peptide has been analysed and confirmed according to accurate mass, isotopic distribution and MS/MS tandem analysis.

As a matter of fact, we identified two peptides, modified by phosphorylation. The first peptide $_{94}$GLKLTFDSSFSPNTGKKNAKIK$_{115}$ (M.m=2781.1508) is belong to transmembrane domain and contains 5 PO_4 groups. The second peptide is $_{111}$NAKIKTGYKREHINLGCDVDFDIAGPSIR$_{139}$ (M.m.=3310.6405). It contains 29 amino acids, which belong to soluble loop and transmembrane part. This peptide has one phosphorylated residue.

It has been shown that PKA can phosphorylate VDAC.[26,27] Moreover, *in vitro* studies demonstrated that PKA can directly bind and phosphorylate VDAC1 and modulate its function. In addition to, the selective tyrosine phosphorylation of VDAC1 and VDAC2 isoforms have been shown following hypoxic stimulus.[28,29] However, the number of added phosphates per VDAC molecule has not been detected. In the present study, we identified phosphorylated peptides and found, at least, 6 phosphorylation sites on VDAC1, which is consistent with previous studies showing that PKA can phosphorylate this protein. We can suggest, that phosphorylation of VDAC1 can modulate of VDAC function and its interactions with other membrane proteins.

Our data showed, that the complete structural characterization and high identification (95%, 258 of 283 amino acids) sequence coverage of VDAC1 was obtained by LTQ-FTMS mapping of peptide mixtures after proteolytic degradation with trypsin (Fig. 5).

It has been shown earlier, that VDACs are very abundant proteins. They have been isolated and purified from *Torpedo* electric fish, wheat plant, from mitochondrial membranes *Arabidopsis*,

from *Saccharomyces cerevisiae* and mammalian cells, including human B lymphocytes and bovine skeletal muscle, human heart mitochondria, mouse liver mitochondria.[30-36] However, the sequence coverage of many VDAC proteins has been found by the isolation of cDNA clones.[15-17]

Moreover, the sequence coverage of mitochondrial VDAC proteins was not very high in mass spectrometric studies. For example, the rat liver mitochondrial outer membranes have been purified and loaded on SDS-PAGE. VDAC bands have been electroeluted from gel, reduced/alkylated, trypsin digested and analyzed by MALDI-TOF MS.[18,36] Byusing peptide mass fingerprinting, sequence coverage of VDAC1 was identified only of 49%. Analysis the tryptic digest by ESI-MS/MS showed the 85% sequence coverage VDAC1 protein.[36] In addition, just combination of MALDI-TOF analysis with ESI-MS/MS experiments of the tryptic and proteinase K digestions have increased the total coverage of VDAC to 99%.[36]

Mass spectrometry methods including electrospray ionization and matrix-assisted laser desorption/ionization (MALDI-TOF) have been used broadly for protein sequencing over the past ten years. The four primary advantages of mass spectrometric sequencing include: the high sensitivity, the rapid speed of the analysis, the large amount of information generated in each experiment and the ability to characterize posttranslational modifications. The developments of accurate mass-based proteomics and gas fragmentation methods have provided huge specificity for database-assisted protein identification and characterization of posttranslational modifications of proteins.[37-39]

In or study, we identified 95% (258 of 283 amino acids) of total sequence coverage the primary structure of VDAC1, using just one approach-LTQ-FTMS analysis. In addition to, we identified some other proteins in this molecular weight range (30-34 kDa excised band) with high sequence coverage. For example, repressor of estrogen receptor activity (M.w: 33276 D) was identified with 76% coverage (227 of 299 amino acids sequenced).

Syntaxin 1A (M.w: 33034 D) and syntaxin 1B2 (M.w: 33224 D) have been sequenced with 60% and 65% coverage, respectively. For malate dehydrogenase (EC.1.1.1.37, M.w: 35588 D) 185 of 338 amino acids were detected with 55% total coverage. Our results show, that LTQ-FTMS is very sensitive, accurate and powerful tool to characterize primary structure of membrane proteins. It allows obtain high sequence coverage of membrane proteins from crude cell's lysates, without additional purification steps.

It is known already, that VDACs of *Neurosporacrassa* are formed from a single layer of protein, consisting of one alpha-helix and 12 beta strands, curved into a cylinder that forms the pore.[40] The structure of five bacterial porins has been determined by X-ray crystallography: *Rodobactercapsulatus*, *Escherichia coli* OmpF and PhoE, *Rhodopseudomonasblastica*, and maltoporin from *E.coli*.[41-44]

The secondary structure of typical VDACs is a transmembrane β-barrel. It has been detected in eukaryotic VDACs based on computer programs.[45,46] VDAC folding pattern has been generated and supported by analysis of regular alternation of polar and non-polar residues of proposed beta strands.[47] The topological model of VDAC presented in this work is the 12β-strand/one α-helix model designed for yeast.[48] We used this model to show the sequence coverage of transmembrane domains of VDAC1 from MBM. The transmembrane 12 beta strands produce hydrophobic peptides during the proteolytic digestion. These hydrophobic peptides sticky, they are binding at the active site in the polyacrylamide. It is not easy extract them from gel and identify by mass spec analysis. However, in our case the LTQ-FTMS analysis allowed to identify 11 transmembrane strands (Fig. 6), just one strand $_{140}$ALVGYEGWLAGYQMNFETSKSR$_{160}$ has not been detected.

We determined 137 of 147 amino acids, which belong to the transmembrane strands. This result indicates, that LTQ-FTMS analysis is a very accurate and effective approach to identify the transmembrane hydrophobic domains.

CONCLUSIONS

In summary, we make conclusion, that the combination of SDS-PAGE and mass-spectrometric mapping of the specific gel bands should be developed as a powerful tool for the identification primary structure of membrane proteins. The great advantage of mass spec peptide mapping is ability to identify sequence coverage of interest protein in protein mixture. This approach is also useful in detection of posttranslational modifications membrane proteins and receptors. Furthermore, the mass spec mapping of peptides combined with photolabeling approach will allow study the structure of ligand-protein sites.

The work was supported by the FASE (State Registration Number No. 01201361874).

REFERENCES

1. Colombini M. A candidate for the permeability pathway of the outer mitochondrial membrane. Nature. 1979;279(5714):643-645.PMID:450112.
2. De Pinto V, Messina A, Accardi R, et al.New functions of an old protein: the eukaryotic porin or voltage dependent anion selective channel (VDAC). Ital. J. Biochem. 2003;52(1):17-24. PMID: 12833633.
3. Tsujimoto Y, Shimizu S. Biochimie. The voltage-dependent anion channel: an essential player in apoptosis. 2002;84(2-3):187-193. PMID: 12022949.
4. Crompton M, Barksby E, Johnson N, et al. Mitochondrial intermembrane junctional complexes and their involvement in cell death. Biochimie. 2002;84(2-3):143-152. PMID: 12022945.
5. Crompton M. The mitochondrial permeability transition pore and its role in cell death. Biochem J. 1999;341(Pt2):233-249. PMID: 10393078.
6. Lawen A, Ly JD, Lane DJ, et al. Voltage-dependent anion-selective channel 1 (VDAC1)-a mitochondrial protein, rediscovered as a novel enzyme in the plasma membrane. Internat. J. Biochemistry and Cell Biology. 2005;37(2):277-282. DOI: 10.1016/j.biocel.2004.05.013.
7. Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature. 1999;399(6735):483–487. DOI: 10.1038/20959.
8. Rostovtseva T, Colombini M. VDAC channels mediate and gate the flow of ATP: implications for the regulation of mitochondrial function. Biophys. J.

1997;72(5):1954-1962. DOI: 10.1016/S0006-3495(97)78841-6.

9. Hodge T, Colombini M. Regulation of metabolite flux through voltage gating of VDAC channels. J. Membr. Biol. 1997;157(3):271-279. PMID: 9178614.
10. Schiltz E, Kreusch A, Nestel U. et. al. Primary structure of porin from Rhodobacter capsulatus. Eur. J. Biochem. 1991;199(3),587-594. PMID: 1651239.
11. Przybylski M, Glocker MO, Nestel U, et al. X-ray crystallographic and mass spectrometric structure determination and functional characterization of succinylated porin from Rhodobacter capsulatus: implications for ion selectivity and single-channel conductance. Protein Sci. 1996;5(8):1477-1489. DOI: 10.1002/pro.5560050804.
12. Buhler S, Michels J, Wendt S, et al. Mass spectrometric mapping of ion channel proteins (porins) and identification of their supramolecular membrane assembly. Proteins. 1998; Suppl.2:63-73. PMID: 9849911.
13. Bureau MH, Khrestchatisky M, Heeren MA, et al. Isolation and cloning of a voltage-dependent anion channel-like Mr 36.000 polypeptide from mammalian brain. J. Biol. Chem. 1992;267(12):8679-8684. PMID: 1373732.
14. Blachly-Dyson E, Baldini A, Litt M, et al. Human genes encoding the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane: mapping and identification of two new isoforms. Genomics.1994; 20 (1): 62-67. DOI: 10.1006/geno.1994.1127.
15. Ha H, Hjajek P, Bedwell DM, et al. A mitochondrial porincDNA predicts the existence of multiple human porins. J. Biol.Chem.1993; 268 (16):12143-12149. PMID: 7685033.
16. Zhang H, Li XJ, Martin DB, et al. Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. Natl. Biotechnol. 2003;21(6):660-666. DOI: 10.1038/nbt827.
17. Chalmers MJ, Hakansson K, Johnson R, et al. Protein kinase A phosphorylation characterized by tandem Fourier transform ion cyclotron resonance mass spectrometry. Proteomics. 2004;4(4):970-981. DOI: 10.1002/pmic.200300650.
18. Sapp DW, Witte U, Turner DM, et al. Regional variation in steroid anesthetic modulation of [35S] TBPS binding to gamma-aminobutyricacid A receptors in rat brain. J.Pharmacol. Exp.Ther. 1992;262(2):801-808. PMID:1323664.
19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4.Nature. 1970;227(5259):680-685. PMID: 5432063.

20. Shevchenko A, Jensen ON, Podtelejnikov AV, et al. A strategy for identifying gel-separated proteins in sequence databases by MS alone. Proc. Natl. Acad. Sci. 1996;93(25):14440-14445. PMID: 8962070.
21. Clauser KR, Baker P, Burlingame AL.Role of accurate mass measurement (+/- 10 ppm) in protein identification strategies employing MS or MS/MS and database searching. Anal. Chem. 1999;71(14):2871-2882. PMID: 10424174.
22. Dermietzel R, Hwang TK, Buettner R, et al. Cloning and in situ localization of a brain-derived porin that constitutes a large-conductance anion channel in astrocytic plasma membranes. Proc. Natl. Acad. Sci. U S A. 1994;91(2):499-503. PMID: 7507248
23. Sabirov RZ, Sheiko T, Liu H, et al. Genetic demonstration that the plasma membrane maxianion channel and voltage-dependent anion channels are unrelated proteins. J. Biol. Chem. 2006;281(4):1897-904. DOI: 10.1074/jbc.M509482200.
24. Yu WH, Forte M. Is there VDAC in cell compartments other than the mitochondria? J. Bioenerg. Biomembr. 1996;28(2):93-100. PMID: 9132422.
25. Rostovtseva TK, Tan W, Colombini M. On the role of VDAC in apoptosis: fact and fiction. J. Bioenerg Biomembr. 2005;37(3):129-142. DOI: 10.1007/s10863-005-6566-8.
26. Bera AK, Ghosh S. Dual mode of gating of voltage-dependent anion channel as revealed by phosphorylation. J. Struct. Biol. 2001;135(1):67-72. DOI: 10.1006/jsbi.2001.4399.
27. Bera AK, Ghosh S, Das S. Mitochondrial VDAC can be phosphorylated by cyclic AMP-dependent protein kinase. Biochem. Biophys. Res. Commun. 1995;209(1):213-217. DOI: 10.1006/bbrc.1995.1491.
28. Baines ChP, Song ChXu, ZhengYuT, et al. Protein kinase C interacts with and inhibits the permeability transition pore in cardiac mitochondria. Circ. Res. 2003;92(8):873-880. DOI: 10.1161/01.RES.0000069215.36389.8D.
29. Liberatori S, Canas B, TaniCh, et al. Proteomic approach to the identification of voltage-dependent anion channel isoforms in guinea pig brain synaptosomes. Proteomics. 2004;4(5):1335-1340. DOI: 10.1002/pmic.200300734.
30. Shafir I, Feng W, Shoshan_Barmataz VV. Voltage-dependent anion channel proteins in synaptosomes of the Torpedo electric organ: immunolocalization, purification, and characterization. J. Bioenergetics and Bomembranes. 1998; 30 (5): 499-510. PMID: 9932652.
31. Blumental A, Kahn K, Beja O, et al. Purification and characterization of the voltage-dependent anion-selective channel protein from wheat mitochondrial

membranes. Plant Physiol. 1993;101(2):579-587. PMID: 12231713.

32. Brugiere S, Kowalski S, Ferro M, et al. The hydrophobic proteome of mitochondrial membranes from Arabidopsis cell suspensions. Phytochemistry. 2004;65(12):1693-1707. DOI: 10.1016/j.phytochem.2004.03.028.
33. Prinz T, Muller J, Kuhn K, et al.Characterization of low abundant membrane proteins using the protein sequence tag technology. J. Proteome Res. 2004;3(5):1073-1081. DOI: 10.1021/pr049925u.
34. Florke H, Thinnes FP, Winkelbach H, et al. Channel active mammalian porin, purified from crude membrane fractions of human B lymphocytes and bovine skeletal muscle, reversibly binds adenosine triphosphate (ATP). Biol. Chem. Hoppe Seyler. 1994;375(8):513-520. PMID: 7529026.
35. Taylor S, Warnock D, Glenn G, et al. An alternative strategy to determine the mitochondrial proteome using sucrose gradient fractionation and 1D PAGE on highly purified human heart mitochondria. J. Proteome Res. 2002;1(5):451-458. PMID: 12645917.
36. Da Cruz S, Xenarios I, Langbridge J. et al. Proteomic analysis of the mouse liver mitochondrial inner membrane. J. Biol. Chem. 2003;278 (42):41566-41571. DOI: 10.1074/jbc.M304940200.
37. Bogdanov B, Smith RD. Proteomics by FTICR mass spectrometry: top down and bottom up. Mass Spectrom. Rev. 2005;24(2):168-200. DOI: 10.1002/mas.20015.
38. Aebersold R, Mann, M. Mass spectrometry-based proteomics. Nature. 2003;422(1):198-207. DOI: 10.1021/bc034150l
39. Distler A, Kerner J, Peterman S, et al. A targeted proteomic approach for the analysis of rat liver mitochondrial outer membrane proteins with extensive sequence coverage. Analytical Biochemistry. 2006;356(1):18-29. DOI: 10.1016/j.ab.2006.03.053.
40. Song J, Midson C, Blachy-Dyson E, et al. The sensor regions of VDAC are translocated from within the membrane to the surface during the gating processes. Biophys. J. 1998;74 (6):2926-2944. DOI: 10.1016/S0006-3495(98)78000-2.
41. Weiss M, Kreusch A, Schiltz E, et al. The structure of porin from Rhodobacter capsulatus at 1.8 A resolution. FEBS Lett. 1991;280(2):379-382. PMID: 1707373.
42. Cowan S, Schirmer T, Rummel G, et al. Crystal structures explain functional properties of two E. coliporins. Nature. 1992;358(6389):727-733. DOI: 10.1038/358727a0.
43. Kreusch A, Neubuser A, Schiltz E, et al. Structure of the membrane channel porin from Rhodopseudomonasblastica at 2.0 A

resolution. Protein Sci. 1994; 3 (1):58-63. DOI: 10.1002/pro.5560030108.

44. Schirmer T, Keller T, Wang Y, et al. Structural basis for sugar translocation through maltoporin channels at 3.1 A resolution. Science. 1995;267(5197):512-512. PMID: 7824948.
45. Forte M, Guy HR, Mannella CA. Molecular genetics of the VDAC ion channel: structural model and sequence analysis. J. Bioenerg. Biomembr. 1987;19(4):341-350. PMID: 2442148.
46. Rauch G, Moran O. On the structure of mitochondrial porins and its homologies with bacterial porins. Biochem. Biophys. Res. Commun. 1996;200(2):908-915. DOI: 10.1006/bbrc.1994.1536.
47. Blachly-Dyson E, Peng SZ, Colombini M, et al. Selectivity changes in site-directed mutants of the VDAC ion channel: structural implications. Science. 1990;247(4947):1233-1236. PMID: 1690454.
48. Song J, Midson C, Blachy-Dyson E, et al. The sensor regions of VDAC are translocated from within the membrane to the surface during the gating processes. Biophys. J.1998;74(6):2926-2944. DOI: 10.1016/S0006-3495(98)78000-2.

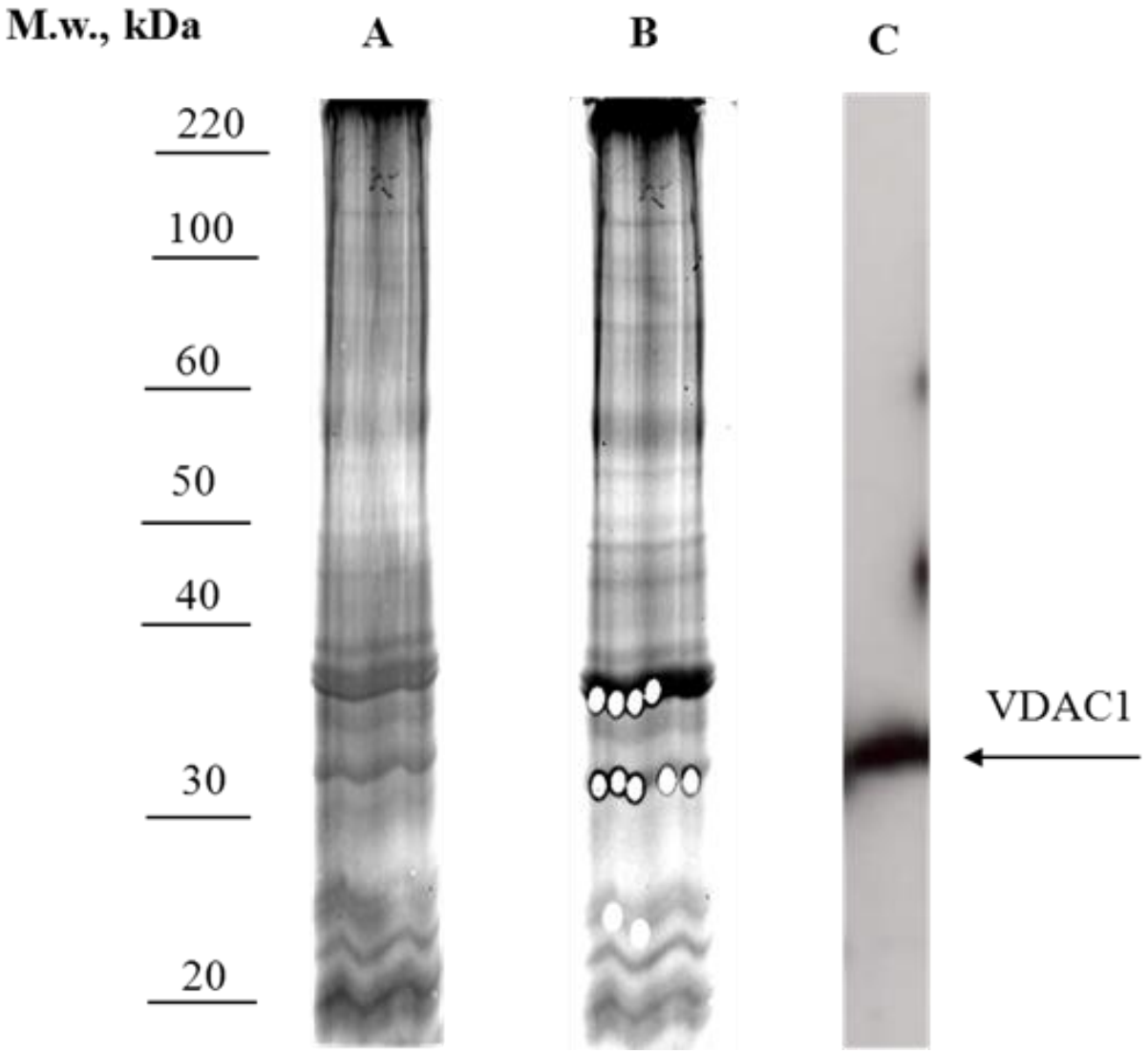

Figure 1. SDS-PAGE electrophoresis gel the membrane lysate (mouse brain), submitted for mass-spec analysis. (A) Sypro Ruby stained gel, (B) Cut gel by robotic instruments, (C) Cross-reactivity of proteins band with the anti-VDAC1 antibody. Marker proteins (left) are indicated, with their molecular weights in kDa. Arrow points the localization of VDAC1.

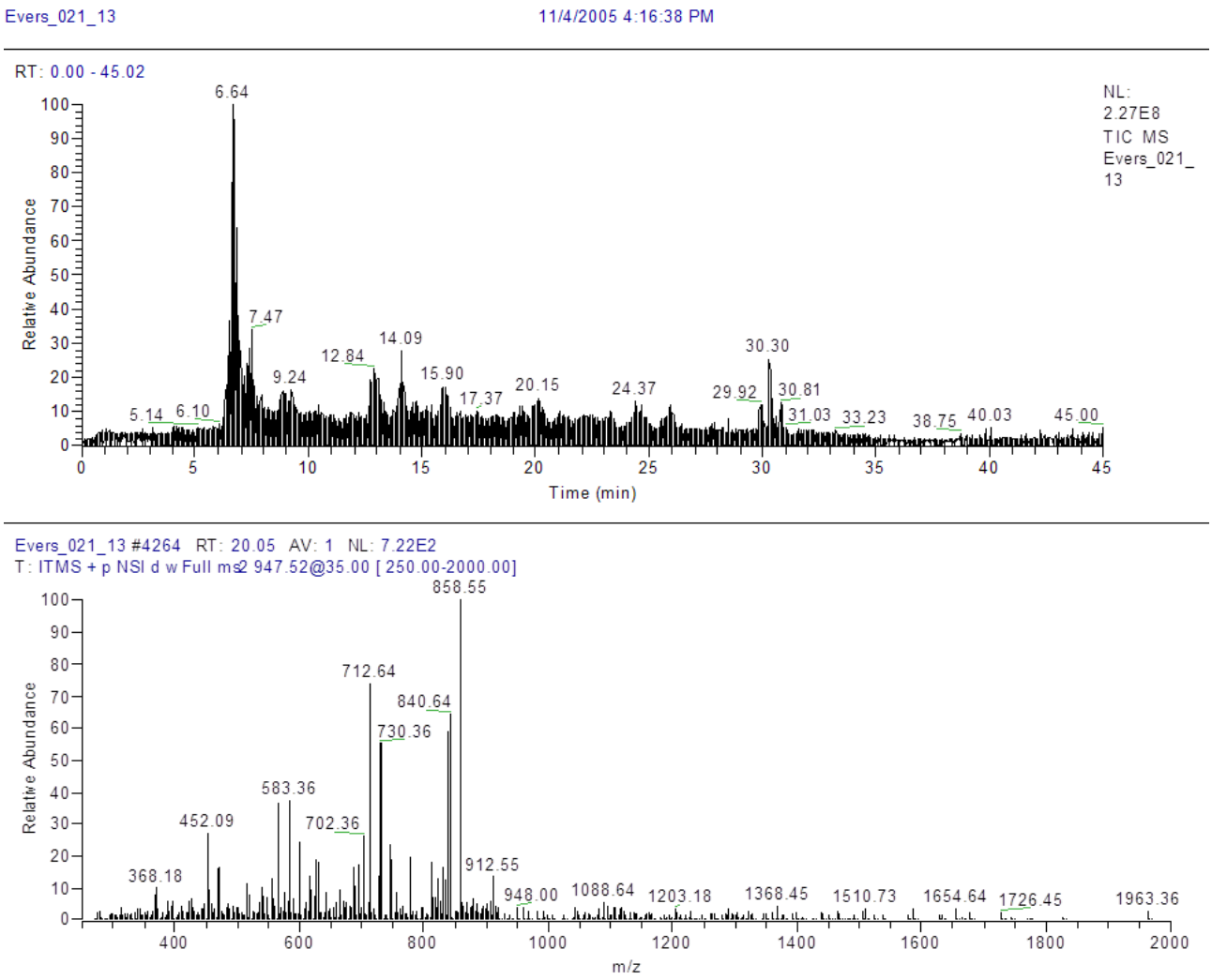

Fig.2. LTQ-FTMS analysis of tryptic digestion of VDAC1 mouse brain. Panel A, total profile of liquid chromatography of tryptic-cleaved peptides of VDAC1. Panel B, total profile of MS/MS fragmentation spectrum of the peptide eluting at 20.05 min.

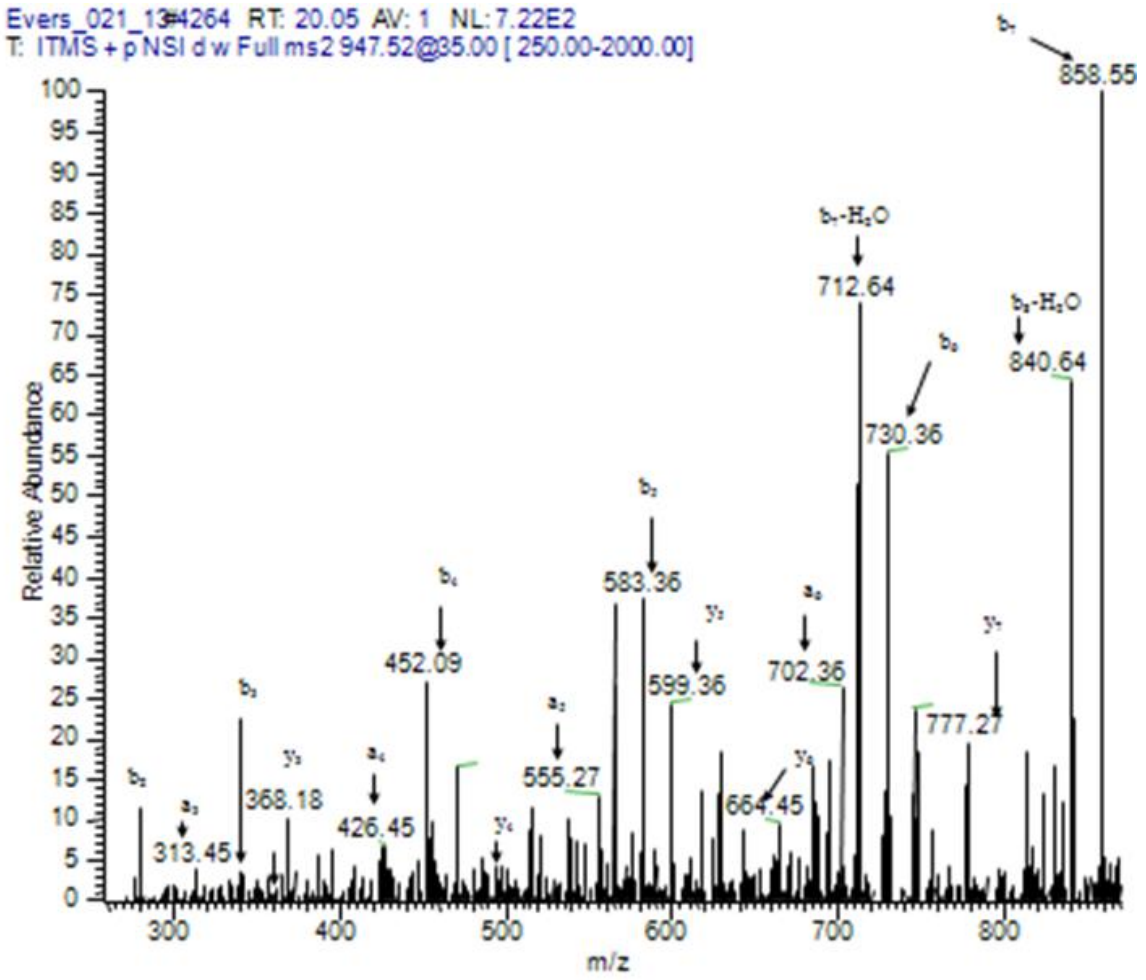

Fig.3. Selected region of MS/MS fragmentation spectrum of the [M+1H]+=947.5223 with tr =20.05 min from 275 LGLGEFQA280 acquired by LTQ-FTMS as described in the Experimental Section.

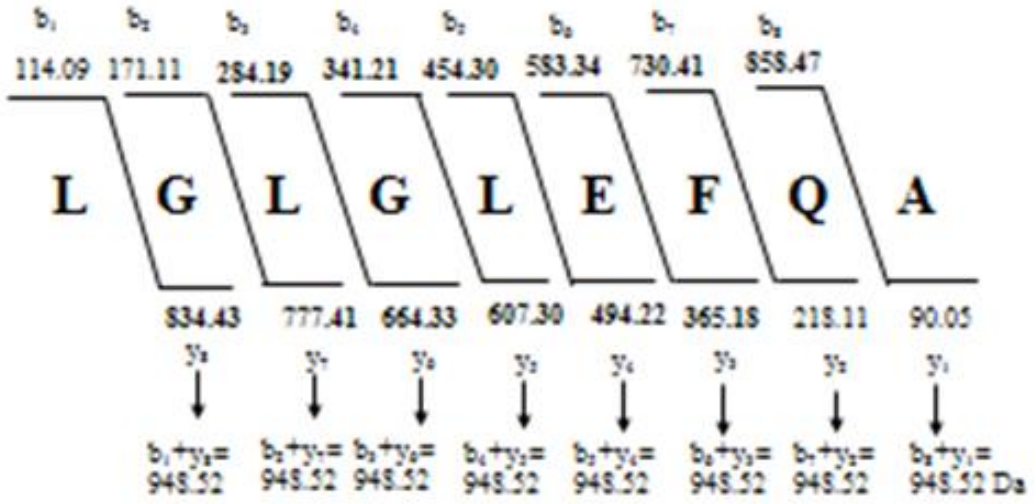

Fig.4. A summary of the interpretation of the product ion spectrum shown in Fig. 3. Specifically, the calculated m/z of the b-ions are shown above the amino acid sequence and the calculated m/z of the y-ions are shown below the amino acid sequence. All values refer to the monoisotopic m/z of the product ion in its singly protonated form. The calculated m/z are shown to two decimal place. The calculated b-and y-ions series of predicted sequence are shown in bold.

1	10	20	30	40	50	60
MAVPPTYADL	GKSARDVFTK	GYGFGLIKLD	LKTKSENGLE	FTSSGSANTE	TTKVNGSLET	
70	80	90	100	110	120	
KYRWTEYGLT	FTEKWNTDNT	LGTEITVEDQ	LARGLKLTFD	SSFSPNTGKK	NAKIKTGYKR	
130	140	150	160	170	180	
EHINLGCDVD	FDIAGPSIR**G**	**ALVLGYEGWL**	**AGYQMNFETS**	KSRVTQSNFA	VGYKTDEFQL	
190	200	210	220	230	240	
HTNVNDGTEF	GGSIYQKVNK	KLETAVNLAW	TAGNSNTRFG	IAAKYQVDPD	ACFSAKVNNS	
250	260	270	280	283		
SLIGLGYTQT	LKPGIKLTLS	ALLDGKNVNA	GGHKLGLGLE	FQA		

Fig.5. Sequence coverage VDAC1 from mouse brain deduced from LTQ-FTMS data. The sequence for VDAC1 identified from the 34kDa band is shown. The sequence of the protein is shown with those residues not covered by LTQ-FTMS highlighted in bold. The total coverage of VDAC1 was 95%.

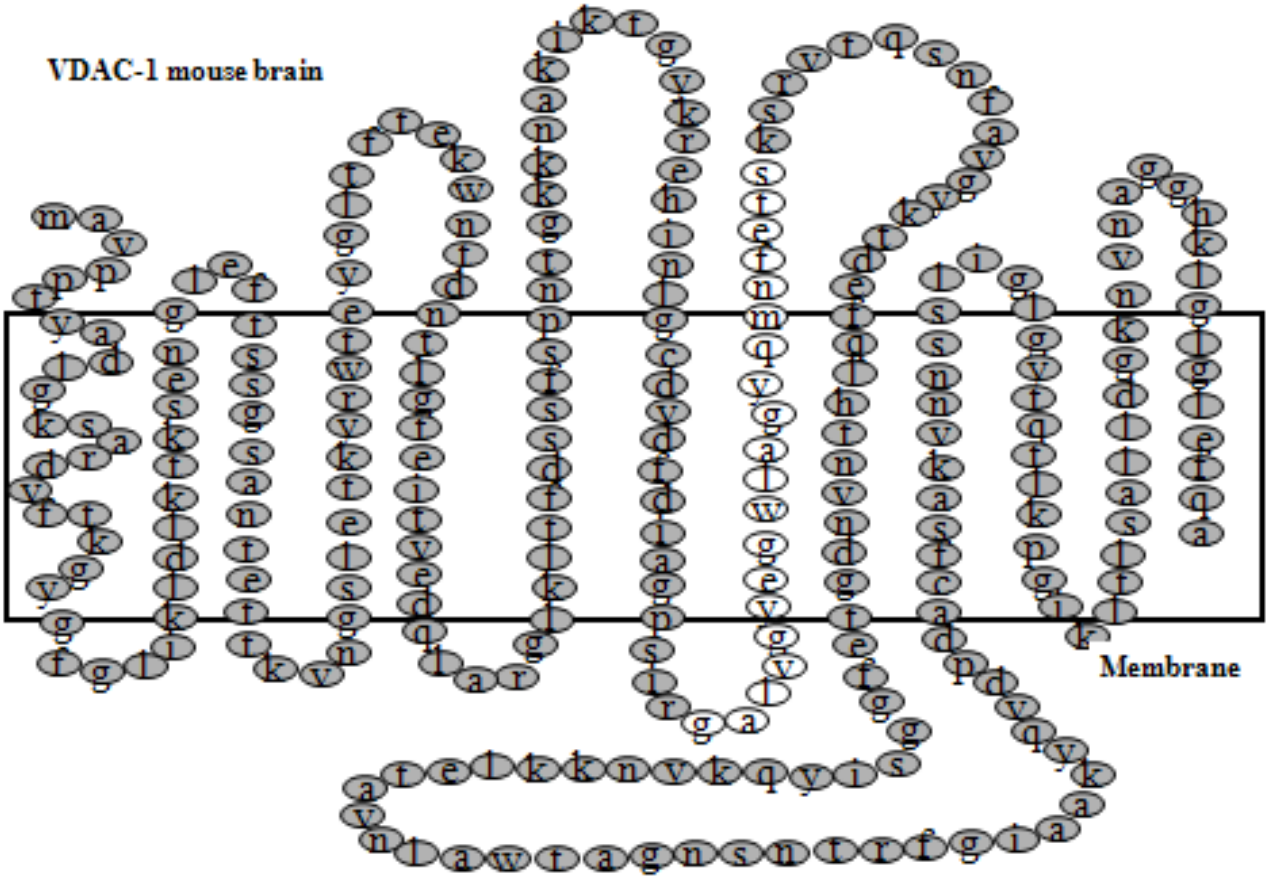

Figure 6.Coverage map of VDAC1 isoform. The topological model of eukaryotic VDACs designed for yeast [40]. Identified amino acids (gray circles), non-identified (white circles). The lines show the boundary of the hydrophobic part of the membrane.

Table.1. Peptides identified in VDAC1 tryptic digestions of VDAC1 using LTQ-FTMS. All sequences were confirmed from manual interpretation of the MS/MS spectra. Calculated masses were determined from the observed m/z values of singly, doubly, triply charged species.

Sequence	start-end	missed cleavages	theoretical mass	calculated mass
MAVPPTYADLGKSAR	1--15	1	1624,8382	1624,8383
AVPPTYADLGK	2--12	0	1172,6054	1172,6077
DVFTKGYGFLIKLDLK	16-32	2	1913,2282	1913,2261
GYGFGLIK	21-28	0	853,6854	853,6897
GYGFGLIKLDLK	21-32	1	1323,1122	13223,1137
TKSENGLEFTSSGSANTETTK	33-53	1	2187,109	2187,1093
SENGLEFTSSGSANTETTK	35-53	0	1959,6714	1959,6705
VNGSLETK	54-61	0	846,4454	846,4447
SENGLEFTSSGSANTETTKVNGSLETK	35-61	1	2788,0882	2788,0845
TKSENGLEFTSSGSANTETTKVNGSLETK	33-61	2	3016,8682	3016,8672
YRWTEYGLTFTEK	62-74	1	1692,3982	1692,4047
WTEYGLTFTEK	64-74	0	1373,4406	1373,4403
WNTDNTLGTEITVEDQLAR	75-93	0	2175,9772	2175,9744
GLKLTFDSSFSPNTGK	94-109	1	1698,7509	1698,7524
GLKLTFDSSFSPNTGRKNAKIKTGYK	94-119	5	2858,4982	2858,4949
LTFDSSFSPNTGK	97-109	0	1399,6654	1399,6619
LTFDSSFSPNTGKK	97-110	1	1527,6802	1527,6769
KNAKIKTGYKREHINLGCDVDFDIAGPSIR	110-139	4	3406,0582	3405,0585
NAKIKTGYKREHINLGCDVDFDIAGPSIR	111-139	4	3317,7985	3317,7901
REHINLGCDMDFDIAGPSIR	120-139	0	2225,2286	2225,2251
EHINLGCDMDFDIAGPSIR	121-139	0	2118,0654	2118,0651
SRVTQSNFAVGYK	160-172	1	1456,5982	1456,5947
VTQSNFAVGYK	162-172	0	1212,9265	1212,9238
SNFAVGYK	165-172	0	912,7254	912,7253
TDEFQLHTNVNDGTEFGGSIYQK	173-195	0	2599,5309	2599,5326
KLETAVNLAWTAGNSNTR	199-216	0	1945,9282	1945,9217
LETAVNLAWTAGNSNTR	200-216	0	1817,3654	1817,3667
FGIAAKYQVDPDACFSAK	217-234	1	2028,0082	2028,0063
VNNSSLIGLGYTQTLKPGIK	235-254	1	2102,2056	2102,2035
LTLSALLDGK	255-264	0	1029,2254	1029,2271
LTLSALLDGKNYNAGGHK	255-271	1	1806,8482	1806,8451
NVNAGGHKLGLEFQA	265-280	1	1724,1382	1724,1305
LGLGLEFQA	274-280	0	946,5202	946,5223