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Optimization of the SDS-PAGE gel slicing approach for identification of human liver microsomal proteins via MALDI-TOF mass spectrometry

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Abstract

Proteomics often exploits sequential cutting of the SDS-PAGE gel lane into slices with subsequent identification of proteins by peptide mass fingerprinting (PMF). In this paper, the influence of slice thickness on protein identification was investigated. Following the separation of the human liver microsomal fraction, the 37 to 75 kDa range of the gel lane was cut into 20 or 40 slices of 0.5 mm and 0.25 mm thickness, respectively. Identification of proteins after trypsinolysis was performed by MALDI-TOF mass spectrometry. A twofold reduction of slice thickness did not influence the number of peaks in PMF-spectra. It was established that at a tolerance of 0.15 Da the number of identified proteins in the 40 slices series exceeded by more than twofold the number of identifications in 20 slices series. It was shown that the decrease of slice thickness leads to a considerable enhancement of peptide peak's intensity and, also, to the changes of PMF-spectra which resulted gaining additional peptides for enrichment of sequence coverage.

Keywords: Human liver microsomes; SDS-PAGE; Peptide mass fingerprinting; Mass tolerance; Database search; Proteomic sample dilution; Slice series

Introduction

Although shotgun proteomics commonly employs the method based on multidimensional chromatographic separation of proteins (nLC-MS/MS), the SDS-PAGE has not as yet lost its popularity [1]. One important aspect of SDS-PAGE application is connected with its ability to analyse membrane proteins with enriched fractions – such as microsomes [2,3]; dorsal root ganglion plasma membrane [4]; enriched plasma membranes from embryos [5]; membrane proteins of yeast mitochondria [6] and human colon carcinoma [7]; and also, platelet from human blood [8].

In rather simple cases - e.g. with plasma membrane proteome of germinating barley embryos [5] or rat muscle [9] - after the separation procedure the stained bands were cut out and the identification of proteins occurring in these bands is performed. In complex mixtures the boundaries between bands are not distinct; in view of this, the slice-by-slice cutting is employed, i.e. the SDS-PAGE lane is cut into slices whereupon the protein content of each slice is analyzed by MS method [2,3,7]. Analysis of obtained slices by peptide mass fingerprinting (PMF) has shown that each slice often appears to be a mixture of 2 to 5 proteins [2,10]. Mixed spectra impeded identification, which necessitated the development of additional functions for the software; e.g. such an option as a signal-processing algorithm based on neural network improves the results of identification by Profound [10].

Ambiguity of results obtained by PMF compelled the researches to turn to LC-MS/MS for analyzing protein content in SDS-PAGE slices. Thus, Simpson et al. [7], while investigating membrane proteins of human colon carcinoma, cut the SDS-PAGE lane into 16 equal slices with the width ~3 mm each. Thiede et al. [11], in analyzing the proteins of Jurkat T-cells, cut sequentially the 150-mm gel lane into 100 slices, i.e. the width of each slice was about 1.5 mm. To identify the cytochrome P450 isoforms from rat [12] or human [13] liver microsomes, authors chose a definite interval of the gel lane (~0.8 cm) corresponding, in their case, to the appropriate molecular weight of cytochromes P450 (48 to 62 kDa) and cut this region into 5 or 6 slices, respectively. Thus, the width of each such slice varied between 1.3 - 1.6 mm.

Recently, we have demonstrated the advantage of cutting the gel into much thinner slices (about 0.2 mm thick) [14]. The mass spectrometric information from neighboring slices was found to contain different peptides belonging to one protein, which enabled to enhance the integral degree of sequence coverage by 12 ± 5% [2].

Although the sequential slicing of SDS-PAGE is used in many experiments, the choice of optimal slice width is yet an unsolved problem and requires additional systematic investigation. Using human liver microsomes (HLM) as an example, we have compared the results of PMF-based protein identifications that were obtained after cutting the fixed gel region into 40 and 20 slices and demonstrated that the lesser is the cutting step the greater number of proteins was identified. Besides, it was shown that the decrease of slice thickness leads to the 2 to 6-fold increase in the peptide peak's intensity in the mass spectrum.

Materials and Methods

Materials

Deionized water, HPLC grade acetonitrile, and ammonium bicarbonate were purchased from Acros Organics (USA); PMSF, 2.5-dihydroxybenzoic acid and EDTA were from Sigma-Aldrich

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(USA). Modified trypsin (part no. V511C) was obtained from Promega (Madison, WI, USA); trifluoroacetic acid and Coomassie Brilliant Blue G-250 were from Fluka (Germany). Other chemicals were purchased from RusChemBio (Russia).

Sample preparation and SDS-PAGE

Purified preparations of microsomal membranes were obtained as described previously [2]. In brief, a sample of human liver tissue (8-12 g) was obtained during surgical interventions conducted at the National Research Centre of Surgery as nontumorous tissue surrounding surgically removed liver metastases [14]. Informed consent was obtained from all patients. The protocol for this study was approved by the National Research Centre of Surgery, Russian Academy of Medical Sciences. The resulting pellet of microsomal membranes was suspended in 100 mM Na-phosphate buffer (pH 7.4) that contained 100 mM KCl, 1mM EDTA, 1mM dithiotreitol, and 20% glycerol (v/v) and used for SDS-PAGE separation. The protein concentration was determined by the Bradford assay using bovine serum albumin (BSA) as a standard [15]. Protein samples (110μg each) were mixed (ratio, 1:10) with a sample buffer (i.e. 0.06M Tris-HCl, pH 6.8, 100mM dithiothreitol, 2% β-mercaptoethanol, 2% SDS, 10% glycerol, 0.01% bromophenol blue), then boiled for 5 min in water bath at 95°C and loaded onto a 12% separation gel (size, 70 mm x 100 mm x 1 mm). Composition of the electrode buffer was 26mM Tris base, 195mM glycine, 3.5mM SDS in water. Electrophoresis was carried out at 150V until the bromophenol blue front reached the bottom of the gel. The proteins were stained with Coomassie Brilliant Blue G-250 as described previously [16]. Quantity One (version 4.6.1) was used to examine the relative quantity of the 37 to 75 kDa range (the quantity if this region was measured by its intensity and expressed as a percentage of the total intensity of the lane).

In-gel digestion

The procedure developed by Shevchenko et al. [17] was used for in-gel digestion. The gel was first pre-frozen, and then cut with a manual microtome into thin slices: for the chosen mass range (length, 10 mm) 20 or 40 slices were obtained. After that each slice was divided into three equal parts, and the middle piece of each slice (volume, 0.8 mm^3 or 0.4 mm^3) was sampled for in-gel trypsinolysis. For this purpose, the Coomassie-stained slices were destained with water and washed with a mixture that contained 50% acetonitrile in 100mM ammonium bicarbonate (v/v), pH 8.9, for 20 min at 50°C. They were then incubated for 20 min in 100% acetonitrile. After the acetonitrile had been removed and the gel pieces dried, 6.3 ± 2.0 μL of trypsin solution (25ng/μL modified trypsin in 50mM bicarbonate ammonium) were added and the mixture was incubated at 37°C overnight. Then 15μl of 0.7% trifluoroacetic acid were added to each gel piece and the samples were incubated for 2 h at room temperature. The extracted tryptic peptides were used for mass spectrometric analysis.

MALDI-TOF MS

Each mixture of proteolytic peptides (1μL) was spotted on a MALDI target (600/384 Anchor chip: Bruker Daltonics, Germany) in three replicates and dried in air. For ionization, a solution of 2.5-dihydrobenzoic acid (3mg/mL) in acetonitrile: 0.7% trifluoroacetic acid (1:1 v/v) was used. Mass spectra in the *m/z* range 600 to 4000 were manually acquired using FlexControl software (Bruker Daltonics) in the reflection/delayed extraction mode with an accelerating voltage of 25kV and a 135ns delay using Ultraflex II (Bruker Daltonics). All mass spectra represented signal averaging of 1000 laser shots from one location on sample spot. From each sample spot 4-6 mass spectra were acquired. Laser fluency was adjusted above the desorption threshold of the matrix to obtained the best resolution and the highest mass measurement accuracy. Signals with a S/N ratio > 6, a maximum of 100 peaks per spectrum, were considered to build peak lists using the SNAP algorithm (FlexAnalysis 2.0, Bruker Daltonics) and internally calibrated with trypsin autolysis products (*m/z* 842.5094 Da and 2211.1046 Da). Resulting peak lists were used to search against NCBI non-redundant human sequences database (NCBInr) (ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz), UniProtKB/Swiss-Prot (ftp://ftp.ebi.ac.uk/pub/databases/uniprot/current_release/knowledgebase/complete/, April 2010) or IPI_HUMAN (http://www.ebi.ac.uk/IPI/IPIhelp.html). Proteins were identified by their PMF using Mascot (Matrix Science, USA). During the database search, a maximum of one missed cleavage was allowed, considered trypsin cleavage; a mass tolerance of 0.15 Da was used, and variable modifications, such as methionine oxidation and cysteine modification with acrylamide, were taken into account.

Results and Discussion

The aim of the present paper was to investigate usefulness of SDS-PAGE thin slicing for enrichment of proteome coverage of the human liver microsomal fraction. Human liver microsomes (HLM) represent the most popular in vitro model for studying hydrophobic membrane proteins which display a large group of physiologically important receptors, transporters, and enzymes as well as key pharmacological targets. There are now multiple methods for analyzing of membrane proteomes. Fractionation of proteins by SDS-PAGE, followed by in-gel digestion of gel slices prior to MS of peptides has been widely used for the analysis of HLM proteome [13].

We have reported previously that combination of sequential thin slicing of SDS-PAGE gel lanes with consequent slice-by-slice mass-spectral protein identification can be used to differentiate between polymorphic variants of cytochromes P450 [2]. In the current report, the working hypothesis has been proposed that the decrease of gel slices' thickness enhances the number of identified membrane proteins under the same experimental conditions. To verify this hypothesis, the following experimental steps were performed. The proteins comprising human liver microsomal ghosts [14] were separated by SDS-PAGE. Equal amounts of protein (0.11mg) were deposited on two parallel gel lanes. After the separation, the 10-mm region of the gel lane, corresponding to molecular weights of 37 to 75 kDa (a prestained molecular mass marker was used to estimate the position of this region) was cut sequentially into slices: one lane was cut into 20 slices and another (parallel) lane, into 40 slices. Thus, the slices obtained differed in thickness by twofold, constituting 0.5 mm in case of 20 slices and 0.25 mm in case of 40 slices (Figure 1). Thus, slice no.1 in the 20-slice series approximately corresponds to slices nos. 1 and 2 in the 40-slice series. Accordingly, slice no. 2 (20-slice series) corresponds to slices nos. 3 and 4 (40-slice series) and so on. Each slice was divided into three fragments and the middle piece of each slice (piece sizes, 0.5 x 0.8 x 2 mm and 0.25 x 0.8 x 2 mm for 20- and 40-sliced gel lanes, respectively) was subjected to in-gel proteolysis by trypsin.

A series of pilot experiments was conducted with the aim to select the optimal amount of trypsin solution. It was established that the amount of required trypsin depends both on the amount of protein

loaded on the SDS-PAGE gel lane and on the volume of the gel slice taken for trypsinolysis. In our experimental conditions, the amount of trypsin solution varied within 6.3 ± 0.5μL. The average number of peptide peaks in the mass spectrum was calculated and found to be 96 ± 23 for 0.25-mm slices and 90 ± 26 for 0.5-mm slices. Difference in the peaks' number has no statistical significance, i.e. the decrease of the cutting step does not lead to the increase in the number of observed peptides; thus, irrespective of slice thickness, the susceptibility of proteins to hydrolysis was nearly the same.

Mass spectra for each slice were obtained in three replicates. From two to four MALDI-TOF mass spectra, taken from each of the three replicates (positions on the MALDI target) per one slice, the best one (i.e. the one with the greatest score for identified protein and the highest percentage of sequence coverage) was selected.

Protein identification was performed by PMF. Among several important parameters of PMF search engines (p*I*, taxonomy, cleavage enzyme, the number of peptides matched, the percentage of sequence coverage, etc.) peptide mass tolerance should receive a special attention [18-21]. For automated protein identification for a set of PMF-spectra the proprietary program PMFScan was developed to dispatch a batch of spectra with specific parameters to the local Mascot v.2.1 server. Using PMFScan, the tolerance between the theoretical and experimental peptide masses would screened over the range of values [20]. Figure 2 shows how the number of identified HLM proteins varied depending on the peptide mass tolerance in the range from 0.02 to 0.4 Da with a step of 0.01 Da. The asymmetric bell-shaped dependency with a widened maximum at 0.15 Da was observed for both 20 and 40 slices with two protein databases - UniProtKB/SwissProt and IPI_human. Upon cutting the gel lane into 20 slices, the number of identified proteins did not significantly differ – regardless of the database used. With 40 slices, the use of the IPI_human database yielded a 10% higher number of identifications than the use of UniProtKB/SwissProt. Such mass tolerance dependencies of database searching results are in qualitative agreement with the earlier reported data [18,21]. For example, Ossipova et al. [18] reported that human plasma protein identification results vary with tolerance value and achieved the maximum at 0.1 –0.15 Da.

Similar dependencies to those presented in Figure 2 were obtained when PMF was searched against NCBInr (data not shown). Overall,

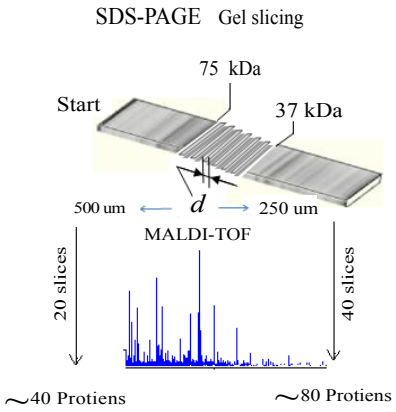

Figure 1: Schematic representation of the strategy for membrane protein analysis from human liver microsomes.

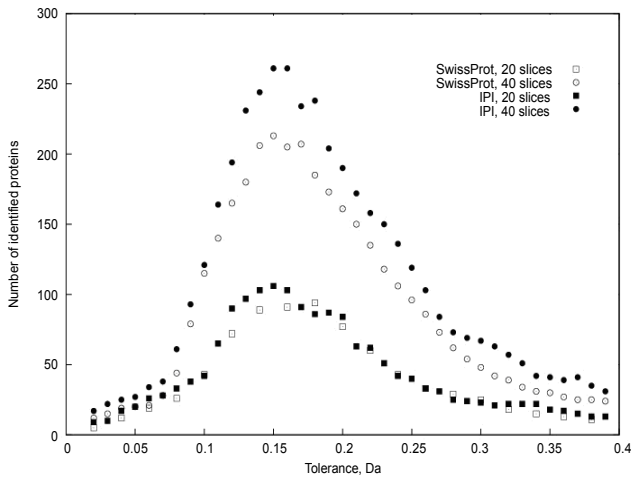

Figure 2: Number of identified proteins depending on the tolerance of PMF search. Mascot searches were performed against SwissProt and IPI databases.

Filled circles and squares indicate, respectively, the number of human liver microsomal proteins identified against IPI in 40 and 20 SDS-PAGE gel slices.

Open circles and squares denote the number of human liver microsomal proteins identified against SwissProt in 40 and 20 SDS-PAGE gel slices.

upon searching against NCBInr at the mass tolerance of 0.15 Da, 351 and 195 proteins were identified in 40 and 20 slices, respectively. Changing the value of mass tolerance from 0.15 Da led to reduction of the total number of identified proteins in both 40 and 20 slices. For example, at the mass tolerance 0.1 Da we could identify 241 proteins in 40 slices and only 119 proteins in 20 slices. It is to be noted that the total number of HLM proteins identified by use of the NCBInr database significantly surpasses the one obtained by UniProtKB/SwissProt and IPI_human. Such a difference is observed since there are over 10 mln entries in NCBInr whereas the other two databases, UniProtKB/SwissProt and IPI_human, contain a much lesser number of entries: 500 thousand and 250 thousand entries, respectively.

The majority of proteome studies performed via PMF do not involve detailed analysis of the number of identified proteins based on the parameters specified for the PMF search engine. For instance, tolerance values are taken according to the manufacturing characteristics assuming the quality of internal calibration for several spectra. These values are usually decelerated, which in turn may result in the rapid decrease in the number of identified proteins [18,20]. However, our results have shown that peptide mass tolerance values significantly influence the number of identified proteins and, hence, the tolerance value for the every protein search requires careful selection.

Further analysis of PMF search results for the UniProtKB/SwissProt database was used to refer, upon data interpretation, to the annotations of proteins presented therein; the mass tolerance value 0.15 Da was chosen for the detailed analysis of MS data on microsomal proteins in 20 and 40 slices of SDS-PAGE gel.

SDS-PAGE fractionation followed by in-gel tryptic digestion of 40 gel slices, derived from the gel region corresponding to cytochromes P450 (molecular weights about 50 - 60 kDa), with subsequent PMF searching against UniProtKB/Swiss-Prot database, enabled to identify (at a tolerance of 0.15 Da) 205 proteins – while in the 20-slice series from the same region only 91 proteins were identified. In both cases

№	Protein name	Swissprot accession number	MW, Da	% sequence coverage	
				20 slices	40 slices
1	Bile acyl-CoA synthetase	Q9Y2P5	75336	–	32±6
2	Liver carboxylesterase 1	P23141	62481	35±10	53±3
3	Chondroitin sulfate N-acetyl-galactosaminyl-transferase 1	Q8N6G5	62532	–	23
4	Dimethylaniline monooxy-genase [N-oxide-forming] 3	P31513	60033	–	34
5	Tyrosine-protein kinase Lyn	P07948	58537	23±2	33±3
6	Cytochrome P450 4A11	Q02928	59591	–	30±5
7	Cytochrome P450 4A22	Q5TCH4	59208	–	29±4
8	Cytochrome P450 1A2	P05177	58257	–	30±4
9	Cytochrome P450 3A4	P08684	57306	24±7	37±4
10	Cytochrome P450 2A13	Q16696	56665	33±4	39±3
11	Cytochrome P450 2E1	P05181	56812	–	39±2
12	Cytochrome P450 2A6	P11509	56509	49±3	48±4
13	Cytochrome P450 2B6	P20813	56242	–	25
14	Cytochrome P450 2C8	P10632	55825	40±1	–
15	Cytochrome P450 2C18	P33260	55711	34	35±4
16	Cytochrome P450 2F1	P24903	55466	–	34±2
17	Protein disulfide-isomerase	P07237	57116	58±4	44±4
18	Protein disulfide-isomerase A3	P30101	56747	40±6	47±4
19	Epoxide hydrolase 1	P07099	52915	41±1	56±10
20	Calreticulin	P27797	48112	37±2	61±2
21	Arylacetamide deacetylase	P22760	45719	–	35±4
22	Lactosylceramide 4-alpha-galactosyltransferase	Q9NPC4	40473	–	45
23	Heparan sulfate glucosamine 3-O-sulfotransferase 5	Q8IZT8	40383	–	35
24	Phosphatidylinositol N-acetylglu-cosaminyltransferase subunit A	P37287	54127	–	32
25	Phosphatidylinositol-5-phosphate 4-kinase type-2 beta	P78356	47348	–	26±4
26	Serine incorporator 1	Q9NRX5	50461	–	27±2
27	Ras-related protein Rab-21	Q9UL25	24332	41	43
28	78 kDa glucose-regulated protein	P11021	72288	43	–
29	Erlin-1	O75477	38926	–	42
30	Beta-1,3-galactosyl-O-glycosyl-glycoprotein beta-1,6-N-acetylglucosaminyltransferase	Q02742	49767	–	31
31	Keratin, type II cytoskeletal 1	P04264	66099	–	31
32	Synaptotagmin-like protein 3	Q4VX76	68560	37	28
33	Integrin beta-2	P05107	84726	20	–
34	Mitofusin-1	Q8IWA4	84047	–	25
35	ATP synthase subunit alpha, mitochondrial	P25705	59751	36±2	38
36	ATP synthase subunit beta, mitochondrial	P06576	56560	47±3	36±3
37	GRAM domain-containing protein 1C	Q8IYS0	76035	23	–
38	Transmembrane protein C3orf1	Q9NPL8	33317	19	–
39	Tyrosine-protein kinase STYK1	Q6J9G0	47516	27±3	–
40	FERM domain-containing protein 3	A2A2Y4	65023	27±2	–
41	Cytochrome b-c1 complex subunit 2, mitochondrial	P22695	48443	41	37
42	Long-chain fatty acid transport protein 4	P33121	77893	22	–
43	X-ray repair cross-complementing protein 5	P13010	82652	–	27
44	Amine oxidase [flavin-containing] A	P21397	59644	–	35±3
45	Amine oxidase [flavin-containing] B	P27338	58725	–	43±5
46	ADP-ribosil cyclase 1	P28907	34306	27	43

47	ADP/ATP translocase 4	Q9H0C2	34999	–	33
48	Trifunctional enzyme subunit beta, mitochondrial	P55084	51262	32	36±4
49	Electron transfer flavoprotein-ubiquinone oxidoreductase	Q16134	68452	–	24
50	Anthrax toxin receptor-like	A6NF34	62864	–	23
51	Inositol 1,4,5-triphosphate receptor-interacting protein-like 2	Q3MIP1	58409	–	18
52	LEM domain-containing protein 2	Q8NC56	56940	–	25±2
53	Creatine kinase U-type, mitoch.	P12532	47007	–	25
54	MOSC domain-containing protein 2, mitochondrial	Q969Z3	37999	–	36
55	RING finger and transmembrane domain-containing protein 1	Q5M7Z0	49677	–	20
56	Leucine-rich repeat transmembrane neuronal protein 3	Q86VH5	65854	–	18
57	Neuropilin and tolloid-like protein 2	Q8NC67	59354	–	36
58	MAGUK p55 subfamily member 6	Q9NZW5	61079	–	30±3
59	Transmembrane protease serine 11A	Q6ZMR5	47538	–	20
60	Probable palmitoyltransferase ZDHHC23	Q8IYP9	45953	–	15
61	Tyrosine-protein kinase ZAP-70	P43403	69827	–	34
62	Phosphate carrier protein, mitochondrial	Q00325	40069	–	34±4

Table 1: Summary table of human liver membrane proteins identified in the region 35-75 kDa on the SDS-PAGE gel lane.

more than 40% of identified proteins could be attributed to membrane proteins according to the UniProtKB/Swiss-Prot annotations (Table 1). Replicate PMF analyses of the gel slices showed that 68% membrane proteins from human liver microsomal ghosts occurred in each of the repeats. Table 1 presents a list of 62 identified membrane proteins with molecular weights in the 35 to 90 kDa range. Twenty-five proteins were identified in 20 SDS-PAGE gel slices, while in 40 SDS-PAGE gel slices 53 membrane proteins were identified; seventeen proteins were common to both slice series. Half of the proteins listed in Table 1 were located in the endoplasmic reticulum, a major constituent of microsomes; the rest were the proteins from mitochondria, as well as from the inner and outer cellular membranes but not from ribosome. The procedure for obtaining the human liver microsomal fraction employed in our study presupposed the presence in this fraction of all above-mentioned proteins. The data obtained provide evidence for the possible presence within the microsomal fraction of elsewhere-localized proteins. Sutton et al. [3] have shown that 82% of mouse liver microsomal proteins identified in the 42-72 kDa bands on SDS-PAGE gel lane were associated with endoplasmic reticulum, mitochondria, membranes and cytosol; while nuclear proteins were under-represented. The twofold increase in the number of slices (from 20 to 40) leads not only to the increase of the total number of identified proteins but also to the proportional (double) increase in the number of membrane proteins, which commonly reside in liver microsomes. As regards the core microsomal inhabitants, cytochromes P450 (CYPs), the experiment with 40 slices allowed us to identify 10 different CYP forms while with 20 slices only 5 CYPs were identified.

Twofold divergence in the total number of proteins identified in the 40 versus 20 gel slices occurred due to detection of additional proteotypic peptides in the thinner slice (Figure 3 and Figure 4). Figure 3 reports the mass spectra with nos. 18 (0.5-mm thickness) and 34 (0.25-mm thickness) obtained for arylacetamide deacetylase (AAAD), a specific protein from the microsomal fraction of liver cells that is involved in activation of arylamine substrates to ultimate carcinogens [22]. On cutting the gel into 20 slices, this protein was not identified by the search engine: overall, only nine matching *m/z* values were registered in slice 18 (Figure 3a) with low sequence coverage of 21% and Mascot score of 41, which was below significant threshold of 56 (Figure 4a). However, with cutting into 40 slices, the enzyme was identified in four slices (nos. 33-36). The PMF spectra of AAAD (Figure 3b) in slice no. 34 contained 13 masses and sequence coverage of 34% was obtained under these conditions (Mascot score: 84, Figure 4b). Among peaks in both spectra, eight masses were shared between slices of 0.25-mm and 0.5-mm thickness (Figure 3 and Figure 4c). The comparison between both spectra allowed us to identify five additional tryptic peptides with *m/z* 1069.494, 1143.546, 1236.638, 1911.977 and 2350.205 in slice no. 34 (0.25-mm thickness) as matching to peaks in slice no. 18 (0.5-mm thickness). Three peptides (1143.546, 1236.638 and 2350.205 Da, Figure 4d) were observed in more than 50% mass spectra in which the AAAD was identified, which allowed declaring these peptides as proteotypic [23].

Densitometry analysis of the protein region between 37 and 75 kDa demonstrated that this area contain 46.3 ± 3.2% of the total HLM protein loaded on the SDS-PAGE gel lane. As about 2 nmol of HLM protein were deposited on each parallel gel lanes, so one slice in 20-slices series contained in the region of 9.3 pmol of HLM protein, whereas one slice in 40-slices series contained approximately 4.6 pmol of HLM protein. After the in-gel trypsin digestion the concentration of HLM protein in the final solution volume was nearly equal in both slice series, and the concentration of matrix solution was 20 mg/mL in both cases. The final volume of mixture, consisting of proteolytic peptides and a matrix, spotted on each position on the MALDI target was 2.0μL. As a result a matrix/analyte molar ratio was nearly the same (around of 250:1) as in the case of 20-slice series and 40-slice series (with the assumption that the trypsin digestion was complete, all the peptides were extracted from the gel, and the concentration of the resultant peptide mixture was the same as the protein content per slice in both cases). Therefore our sample preparation conditions for both types of slices were close to optimal recommendations, according to which a final matrix : analyte molar ration must be at least 100:1 and a final volume of 0.5-2.0μL [24].

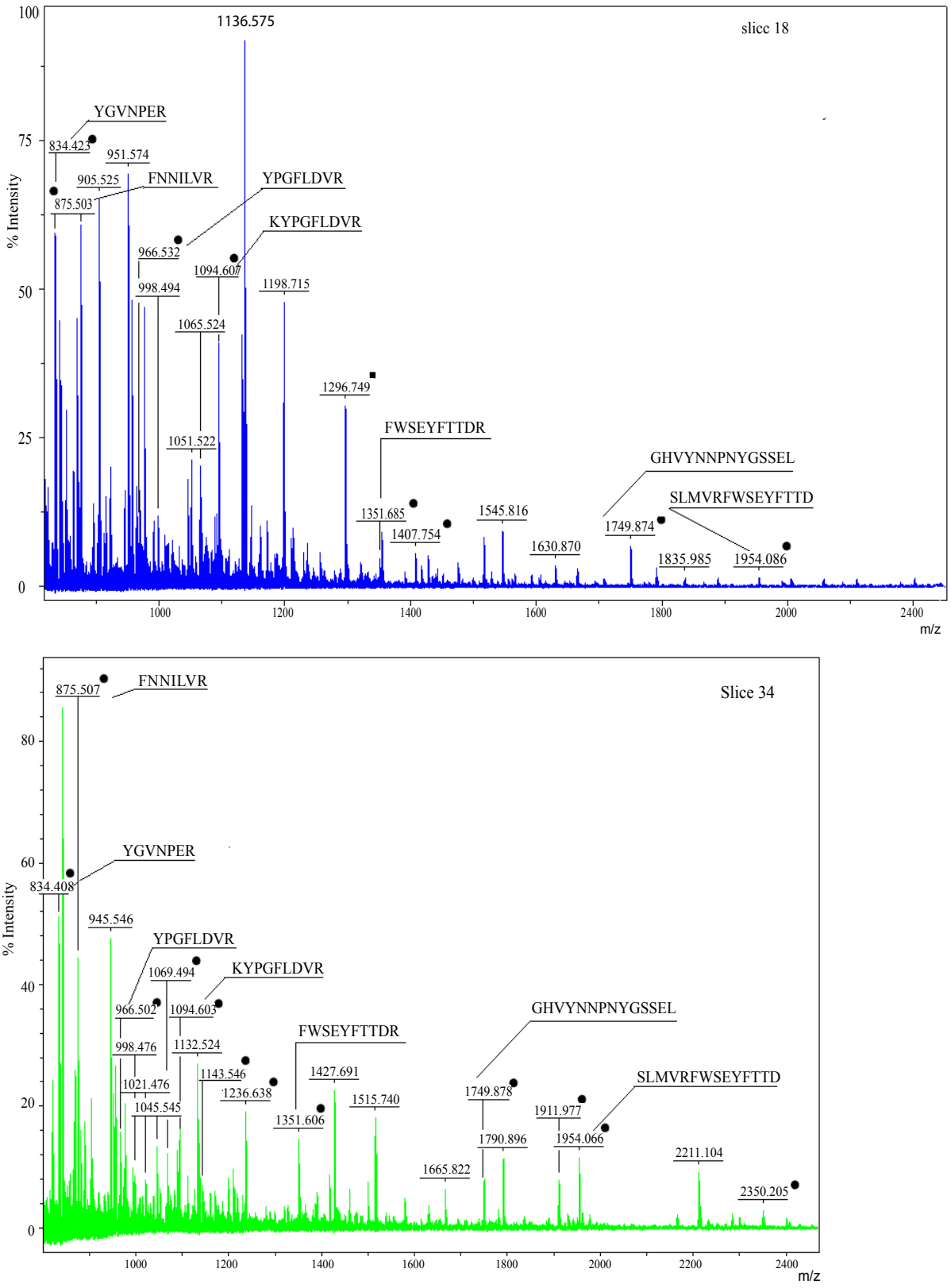

Figure 3: MALDI-TOF mass spectra of the tryptic digest of the SDS-PAGE gel slices 18 (0.5-mm thickness) and 34 (0.25-mm thickness). Labeled peaks (black circle (●) correspond to the matched peptides of arylacetamide deacetylase (AAAD, SWISS-PROT entry: P22760).

(a) AAAD in slice#18 (0.50mm) (Mascot Score 41)

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        10         20         30         40         50         60
MGRKSLYLLI VGILIAYYIY TPLPDNVEEP WRMMWINAHL KTIQNLATFV ELLGLHHFMD
        70         80         90        100        110        120
SFKVVGSFDE VPPTSDENVT VTETKFNNIL VRVYVPKRKS EALRRGLFYI HGGGWCVGSA
       130        140        150        160        170        180
ALSGYDLLSR WTADRLDAVV VSTNYRLAPK YHFPIQFEDV YNALRWFLRK KVLAKYGVNP
       190        200        210        220        230        240
ERIGISGDSA GGNLAAAVTQ QLLDDPDVKT KLKIQSLIYP ALQPLDVDLP SYQENSNFLF
       250        260        270        280        290        300
LSKSLMVRFW SEYFTTDRSL EKAMLSRQHV PVESSHIFKF VNWSSLLPER FILKGHVYNP
       301        320        330        340        350        360
NYGSSELAKK YPGFLDVRAA PLLADDNKLR GLPLTYVITC QYDLLRDDGL MYVTRLRNTG
       370        380        390
VQVTHNHVED GFHGAFSFLG LKISHRLINQ YIEWLKENL
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(b) AAAD in slice#34 (0.25mm) (Mascot Score84)

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        10         20         30         40         50         60
MGRKSLYLLI VGILIAYYIY TPLPDNVEEP WRMMWINAHL KTIQNLATFV ELLGLHHFMD
        70         80         90        100        110        120
SFKVVGSFDE VPPTSDENVT VTETKFNNIL VRVYVPKRKS EALRRGLFYI HGGGWCVGSA
       130        140        150        160        170        180
ALSGYDLLSR WTADRLDAVV VSTNYRLAPK YHFPIQFEDV YNALRWFLRK KVLAKYGVNP
       190        200        210        220        230        240
ERIGISGDSA GGNLAAAVTQ QLLDDPDVKT KLKIQSLIYP ALQPLDVDLP SYQENSNFLF
       250        260        270        280        290        300
LSKSLMVRFW SEYFTTDRSL EKAMLSRQHV PVESSHIFKF VNWSSLLPER FILKGHVYNP
       301        320        330        340        350        360
NYGSSELAKK YPGFLDVRAA PLLADDNKLR GLPLTYVITC QYDLLRDDGL MYVTRLRNTG
       370        380        390
VQVTHNHVED GFHGAFSFLG LKISHRLINQ YIEWLKENL
```

(c) Observed both in slice#18 (0.50mm) and slice#34 (0.25mm)

Start - End	Observed	Mr (expt)	Mr (calc)	Miss	Sequence
86 – 92	875.51	874.50	874.50	0	K.FNNILNR.V
176 – 182	834.44	833.43	833.40	0	K.YGVNPER.I
244 – 258	1954.05	1953.05	1952.91	1	K.SLMVRFWSEYFTTDR.S
249 – 258	1351.61	1350.60	1350.59	0	R.FWSEYFTTDR.S
294 – 309	1749.82	1748.81	1748.81	0	K.GHVYNNPNYGSSELAK.K
310 – 318	1094.59	1093.58	1093.59	1	K.KYPGFLDVR.A
311 – 318	966.51	965.50	965.50	0	K.YPGFLDVR.A

(c) Observed only in slice#34 (0.25mm)

Start - End	Observed	Mr (expt)	Mr (calc)	Miss	Sequence
33 – 41	1143.55	1142.54	1142.57	0	R.MMWINAHLK.T
42 – 63	2577.26	2576.25	2576.31	0	K.TIQNLATFVELLGLHHFMDSFK.V
64 – 85	2350.18	2349.17	2349.12	0	K.VVGSFDEVPPTSDENVTVTETK.F
136 – 146	1236.63	1235.62	1235.65	0	R.LDAVVVSTNYR.L

Figure 4: The sequence of arylacetamide deacetylase identified in the SDS-PAGE gel slices with nos. 18 (0.5-mm thickness, **a**) and 34 (0.25-mm thickness, **b**). Proteins score greater than 56 were significant. Amino acid residues definitively identified from analysis of fragment ions by MALDI-TOF PMF are in bold. Common peptides are shown in ©, and proteotypic peptides observed only in slice 34 are underlined in (**b**) and shown in (**d**) at the bottom.

In analyzing peptide mixtures derived from gel slices with different thicknesses, we, in fact, examined the effect of proteomic sample dilution (1:2) on hydrolysis peaks intensity upon identification of HLM membrane proteins. It was established that in thinner slices the intensity of theses peaks is 2 to 6 times higher (Figure 5). The intensities of 13 coincident hydrolysis peaks corresponding to the peptide fragments' masses of CYP2A6 (MW 56481) for 0.5-mm slice no. 15 and for 0.25-mm slice no. 30 are presented in Table 2. The intensity of peaks was enhanced; e.g. for the peak with *m/z* 2408 the accretion was about 600%. Only for two peaks (with *m/z* 1450 and 1474) the intensities from thinner slices were somewhat lower. Analogous results were obtained for liver carboxylesterase 1 and CYP 2C18 (data not shown). The hydrolysis peak's intensity for liver carboxylesterase 1 (MW 62481) in 0.25-mm slice no. 14 was 2 to 5.5 times higher than in 0.5-mm slice no. 7. For example, the accretion for the coincident peaks with *m/z* 1639 and *m/z* 949 was, respectively, 360% and 540%. Besides, in 0.25-mm slices the overall intensity of all peaks was, on average, 4.2 times higher than it was in 0.5-mm slices. Of note, the intensities of CYP 2C18 (MW 55711) peaks in one of 0.5-mm slices reached the maximum value of 3.7×10^5 while in 0.25-mm slice it was 1.5×10^6.

Regular search engines do not use peak intensities as parameter for protein identification [25] however, peak intensity is an inherent attribute of the experimental spectrum useful for protein identification [19]. Peak intensity is related to matrix : analyte ratio as well to the in-gel-concentration of the individual protein and to the complexity of the sample [26,27].

We assume that the influence of the sample complexity was the main reason for the increased MALDI peaks intensity for the thinner slices. For the soft ionization there exist selective effects that may lead to spectral suppression of distinct peptide ions. MALDI-TOF-MS reveals strong differences in signal intensity among peptide peaks [27], including the complete absence in spectra of the peaks for the relatively abundant peptide ions due to competitions for protons [28]. Due to cutting the protein bands on SDS-PAGE gel lane into the thinner gel slices leads to diminishing the complexity of the protein mixture in the piece, thus to declining the complexity of resultant peptide mixture from the thinner gel piece compared with the resultant peptide mixture from the thicker gel piece. Simplification of the peptide mixture created the preferential conditions for the matrix-assisted ionization, so we observed higher intensities of peaks and also the appearance of the proteotypic peaks, which trigger the identification of more proteins and enhance the sequence coverage.

Conclusions

Separation of liver microsomal membrane proteins can be achieved by SDS-PAGE, resulting in more than one protein per gel band. Several proteins are assumed to occur within each protein band, so the direct analysis of a peptide mixture resulting from proteolytic digestion often becomes a problem for peptide detection and protein identification. The simplified mixture can be obtained by cutting a protein band in to several slices. Our approach involved obtaining of the most possible number of thin slices in the selected region on the SDS-PAGE gel lane with subsequent MS identification of protein in each particular slice.

It was shown, that the number of identified proteins increased two-fold when the selected gel region was cut into the two times thinner slices. There were 53 membrane proteins positively identified when

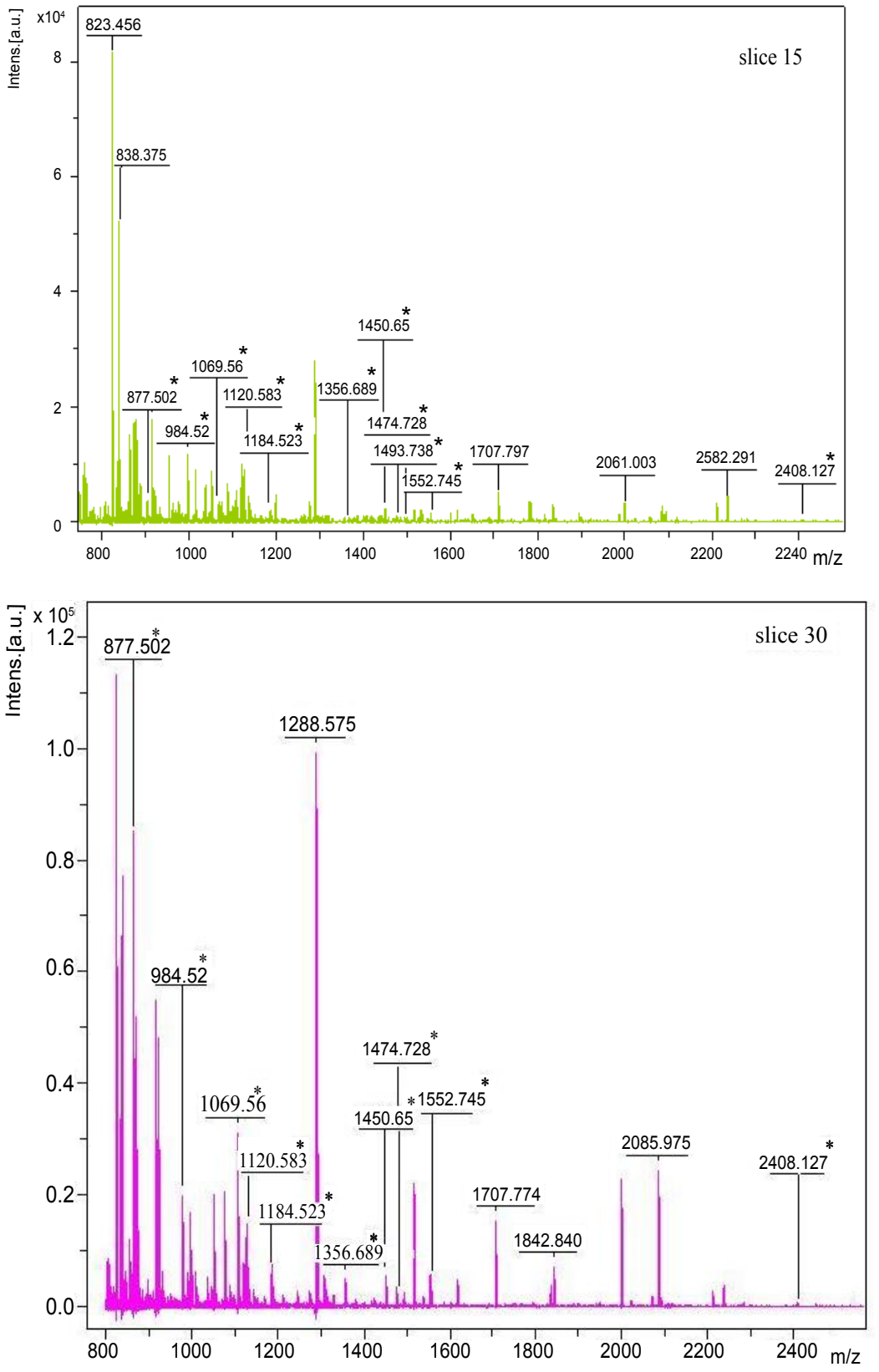

Figure 5: MALDI-TOF mass spectra of a tryptic mass fingerprint of SDS-PAGE gel slices 15 (0.5-mm thickness) and 30 (0.25-mm thickness), containing CYP2A6. Asterisk (*) indicates mass peaks corresponding to CYP2A6 tryptic peptides.

№	Mass values matched (m/z)	Intensity		
		1DE_20 slices (slice no. 15)	1DE_40 slices (slice no. 30)	Increase, %
1	877.502	4024.56	4937.833	123±31 (n=3)
2	984.52	1570.6	2753.69	175±65 (n=3)
3	1069.56	1416.17	2167.74	153
4	1120.583	4115.78	6495.025	157 ±34 (n=8)
5	1125.569	3135.9	13760.75	439±57 (n=8)
6	1128.514	3371.56	12690.56	376 ± 123 (n=8)
7	1184.523	1345.26	5684.024	422 ± 167 (n=8)
8	1356.689	1097.68	3929.68	358 ±118 (n=8)
9	1450.65	6719.11	5135.656	83±26 (n=8)
10	1474.728	3684.42	3041.747	82 ±15 (n=8)
11	1493.738	780.84	1717.41	220 ±55 (n=8)
12	1552.745	2813.98	4868.517	182 ±42 (n=8)
13	2408.127	177.95	1036.469	582 ±164 (n=8)

Table 2: Increase of intensity of *m/z* values matched to cytochrome P450 2A6 (SWISS-PROT entry: P11509).

1-cm long fragment of SDS-PAGE gel lane that was cut into 40 slices, comparing to 25 proteins identified in 20 slices, with 17 proteins overlapped. Analysis of three to six replicates of MALDI-TOF mass spectra revealed that although the total number of peaks was not changed, the mass fingerprints were different for thin and thick slices, the former demonstrating increased peak intensity. PMF spectra for thin slices were enriched by few interpretable peaks, picked up by the search engine for identifying, additional proteins and for increasing the sequence coverage. We conclude, that step of SDS-PAGE gel slicing sufficiently influences on the protein identification by peptide mass fingerprinting.

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