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Short Communication

DIRECT IDENTIFICATION OF CYTOCHROME P450 ISOZYMES BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME OF FLIGHT-BASED PROTEOMIC APPROACH

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ABSTRACT:

The main targets of our investigation were cytochrome P450 isozymes (P450), the key enzymes of the hepatic drug-metabolizing system. Current research approaches to the identification of individual P450 forms include specific P450 inhibitors or substrates, antibody-based identification, and mRNA-based expression profiling. All of these approaches suffer from one common disadvantage—they all are indirect methods. On the other hand, current developments in mass spectrometry provide a direct and reliable approach to protein identification with sensitivity in the femtomole or low picomole range. In this study we have used high-accuracy, matrix-assisted laser desorption/ionization time of flight (MALDI TOF)-based peptide mapping to perform direct identification of distinct P450 isozymes in various rat and rabbit liver microsomes. For the first time, the P450 isozyme composition of clofibrate-induced rat and phenobarbital-induced rabbit liver microsomes was determined by peptide mass fingerprinting (PMF). Application of MALDI TOF-based PMF allows differential identification of such highly homologous P450s as CYP2B1 and CYP2B2. We have found that CYP2A10 previously reported only in rabbit olfactory and respiratory nasal mucosa is present in phenobarbital (PB)-induced rabbit liver microsomes. Two other rabbit P450s, earlier identified only by screening a cDNA library, were found to be present in PB-induced rabbit liver microsomes. In summary, direct identification of P450s by proteomic technique offers advantages over other methods with regard to identification of distinct P450 isozymes and should become a standard approach for characterizing microsomes.

Cytochrome P450 isozymes (P450) are the key enzymes of the hepatic drug-metabolizing system. Eukaryotic P450s are membrane proteins that are expressed in varying amounts, and many forms differ very little in their amino acid sequence and catalytic properties. Currently the number of sequenced and named distinct P450s exceeds 1925 (dnelson.utmem.edu/CytochromeP450.html). Since individual P450 isozymes exhibit a broad, often overlapping substrate specificity, knowledge of the P450 composition in a particular type of microsomes is critical in predicting drug/substrate interactions and formation of reactive intermediates. Current research approaches to the identification of individual P450 forms include: specific P450 inhibitors or substrates (Halpert et al., 1994; Kobayashi et al., 2002), antibody-based identification (Shou et al., 2000), and mRNA-based analysis (Chow et al., 1999). However, only a limited number of almost 2000 P450s is characterized in terms of substrate specificity and has available antibodies, not to mention the fact that, to the best of our knowledge, there is no known substrate or inhibitor that is absolutely specific for only one P450 isozyme. As for antibody-based techniques, the main problem is a very high degree of sequence homology characteristic for P450 isozymes. For example, neither polyclonal nor monoclonal antibodies can distinguish CYP2B1 and CYP2B2 (Wilson et al., 1987). On the whole, the use of these techniques is restricted to laboratories having large libraries of P450 antibodies, specific substrates, and/or inhibitors. Finally, the application of a quantitative analysis of mRNA for the assessment of P450 isozyme expression is questionable, too. A comparative analysis of the correlation between mRNA levels and protein expression demonstrated that for a majority of liver proteins there is no statistically significant correlation between protein and mRNA expression (Anderson and Seilhamer, 1997; Chen et al., 2002).

On the other hand, tryptic peptide mass fingerprinting (PMF) in conjunction with MALDI TOF mass spectrometry has become the main analytical tool in protein identification due to its ability to analyze picomole quantities of gel- or HPLC-separated proteins in short time (Kuster and Mann, 1998). Particularly important in case of P450s identification is the fact that mass determination of several peptides from the same protein results in verification of a significant part of the protein sequence. Even more attractive is that this technique allows identification of several proteins in the same sample (Jensen et al., 1997). Therefore, proteomic approach could be applied for the purposes of direct identification screening of P450 isozyme composition. In addition to identification, proteolytic PMF provides structural information and can be used in search and characterization of unknown P450s.

In this study we have used MALDI TOF-based peptide mass fingerprinting to perform a detailed direct identification of distinct P450 isozymes in various rat and rabbit liver microsomes and have shown that identification of P450s by proteomic technique offers advantages over other methods with regard to identification of distinct P450 isozymes and should become a standard approach for characterizing microsomes.

[1] Abbreviations used are: P450, cytochrome P450; PMF, peptide mass fingerprinting; PB, phenobarbital; CF, clofibrate; amu, atomic mass units; HA, hydroxyapatite; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight; 1DE, one-dimensional electrophoresis; 2DE, two-dimensional electrophoresis; Kpi, potassium phosphate.

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TABLE 1

Comparison of in-gel and in-solution tryptic digests of purified CYP2B1

MH^+ (Calculated)	Start	End	Peptide Sequence	In-Solution Digest	In-Gel Digest	
					Purified CYP2B1	Microsomal CYP2B1
2350.492	1	21	MEPTILLLLALLVGFLLLLVR	–	–	–
2284.293	28	48	GNFPPGPRPLPLLGNLLQLDR	+	+	+
1235.649	49	59	GGLLNSFMQLR	+	+	+
2676.345	62	85	YGDVFTVHLGPRPVVMLCGTDTIK	+	–	–
1378.660	86	98	EALVGQAEDFSGR	+	+	+
1187.704	99	109	GTIAVIEPIFK	+	+	+
1254.612	110	120	EYGVIFANGER	+	+	+
825.429	127	133	FSLATMR	+	+	+
1617.779	146	158	IQEEAQCLVEELR	+	+	+
816.353	188	193	FDYTDR	+	+	–
1360.665	188	197	FDYTDRQFLR	+	+	+
953.546	198	204	LLELFYR	+	+	–
2405.199	205	225	TFSLLSSFSSQVFEFFSGFLK	–	–	–
847.422	226	232	YFPGAHR	+	+	+
1783.959	237	251	NLQEILDYIGHIVEK	+	+	+
927.490	254	262	ATLDPSAPR	+	+	+
1155.605	263	271	DFIDTYLLR	+	+	+
984.559	309	316	YGFLLMLK	+	–	–
842.439	317	323	YPHVAEK	+	+	+
1153.597	327	336	EIDQVIGSHR	+	+	+
829.442	337	343	LPTLDDR	+	+	–
1572.784	346	358	MPYTDAVIHEIQR	+	+	+
1336.738	359	370	FSDLVPIGVPHR	+	+	+
1201.556	423	433	SEAFMPFSTGK	+	+	–
989.509	435	443	ICLGEGIAR	+	+	+
801.436	467	473	DIDLTPK	+	+	–
1453.715	480	491	IPPTYQICFSAR	+	+	+

Materials and Methods

Preparation of Microsomes. Hepatic microsomes from adult male Sprague-Dawley rats and male New Zealand rabbits (2–2.5-kg body weight) were prepared by differential centrifugation as previously described (Alterman et al., 1993), pyrophosphate-washed, suspended in 100 mM KPi buffer, pH 7.4, containing 20% glycerol, aliquoted, and stored at −70°C prior to use. Phenobarbital (PB) and clofibrate (CF) induction were performed as described elsewhere (Alterman et al., 1995). Livers were excised from animals sacrificed by exposure to carbon dioxide.

Enzyme Preparations. Electrophoretically homogeneous cytochrome P450 2B1 was isolated as described elsewhere (Alterman et al., 1995).

SDS-Polyacrylamide Gel Electrophoresis. Electrophoresis was performed as described previously (Galeva and Alterman, 2002).

Enzymatic Digestion. In-solution digestion was performed according to (Stone and Williams, 1996) and electrophoretically separated protein bands were excised by hand and in-gel digested as previously described (Rosenfeld et al., 1992). The resulting peptide mixtures were desalted using ZipTips C_{18} and eluted onto the sample plate with the matrix solution (10 mg/ml of α-cyano-carboxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid).

MALDI-TOF Mass Spectrometry and Protein Identification. Mass spectra were recorded on a Voyager-DE STR (Applied Biosystems, Foster City, CA). The instrument was operated in positive reflector mode at following parameters: accelerating voltage 20,000 V, grid voltage 75%, mirror voltage ratio 1.12, guide wire 0.002%, extraction delay time 180 ns. Acquisition mass range was 700 to 3000 Da, and a resolution of 7,000 to 10,000 was achieved within that mass range. Internal mass calibration was performed using trypsin autolysis peaks (MH^+ 842.5021 and 2211.0968). The peptide mass fingerprinting data were analyzed by ProFound (The Rockefeller University edition, http://129.85.19.192/profound_bin/WebProFound.exe) and MS-Fit (The University of California, http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). The SWISS-PROT database (Swiss Institute of Bioinformatics, http://us.expasy.org/) was used to identify the proteins. The parameters used in the search were as follows: peptide mass tolerance 30 ppm, 1 missed cleavage and carboxymethylated cysteine.

Results

Peptide Mass Mapping of Purified CYP2B1. Differential identification by PMF of such highly homologous proteins as cytochrome(s) P450 is greatly dependent on achieving maximal sequence coverage. To this end we have compared peptide patterns of in-gel and in-solution proteolytic digests using CYP2B1 as a template (Table 1). No significant differences in sequence coverage were found. From a total of 27 theoretical proteolytic peptides in the mass range 700 to 2500 amu, 23 peptides, representing approximately 50% of the sequence, were seen in reflector mode (Fig. 1). Addition of linear mode MALDI TOF analysis brings sequence coverage to 77% (data not shown), but mass accuracy significantly drops. For this reason we did not take into account data acquired in a linear mode. Accordingly, in further work we used both in-gel and in-solution digests.

Peptide Mass Mapping of Rat Liver Microsomes. Liver microsomes from untreated and CF-induced rats were separated on 12% SDS-polyacrylamide gel electrophoresis. Eight individual protein bands as well as apparently unstained gel areas covering the mol. wt. range from approximately 45 to 60 kDa were excised and subjected to tryptic peptide mass fingerprinting. Table 2 lists proteins identified in different rat liver microsomes.

A number of P450s was reported to be present in untreated microsomes. Waxman et al. (1985) using Western blot identified the presence of CYP2C6, CYP2C11, CYP3A2, CYP2A1, CYP1A2, and CYP2B in untreated rat liver microsomes. Later, Schulz-Utermoehl et al. (1999) reported Western blot identification of CYP2D1, CYP2D2, CYP2D4, and CYP2D5 in untreated hepatic microsomes. We identified the presence of four P450 isozymes in the following untreated microsomes: CYP2C11, CYP2D2, CYP2D5, and CYP2A1 (Table 2). Interestingly, the most abundant isozymes in untreated microsomes according to Waxman et al. (1985) are CYP2C6 and CYP2C11 whereas the amount of CYP2A1 is about three times less. In our study, using PMF we identified the presence of CYP2C11 and CYP2A1 and haven't seen any traces of CYP2C6. That fact might suggest that the inconsistencies in P450 isozyme composition might be due to differential expression of P450s based on some environmental or physiological conditions.

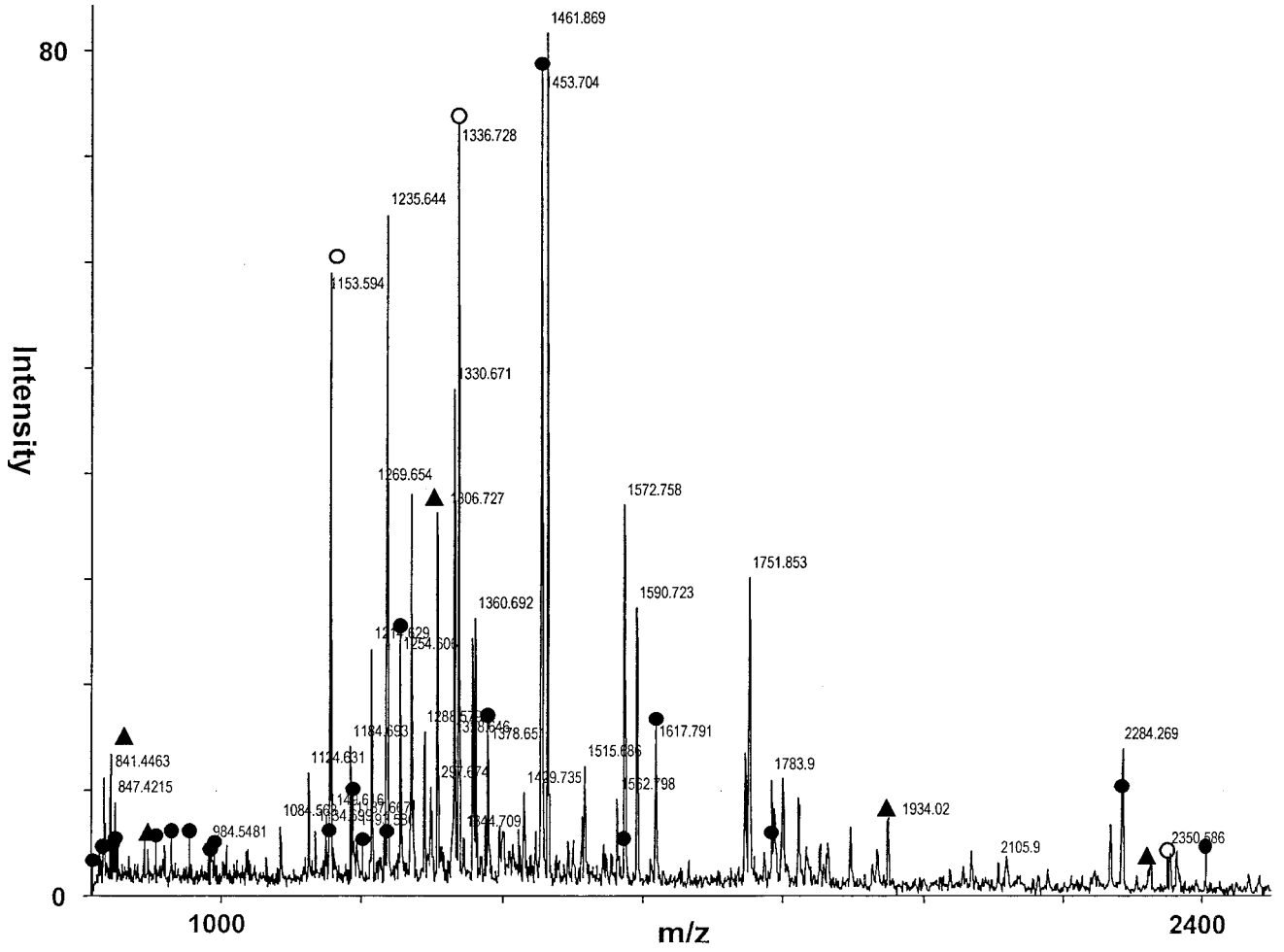

FIG. 1. *Peptide mass fingerprint of 1DE band containing CYP2B1/2B2.*

Filled circles denote mass peaks corresponding to common CYP2B1/2B2 tryptic peptides; open circles denote CYP2B1-specific tryptic peptides and triangles CYP2B2-specific tryptic peptides.

TABLE 2

Proteins identified by peptide mass fingerprinting in rat liver microsomes

Protein	SWISS-PROT Accession Number	pI	Molecular Weight	Untreated Microsomes[a]		CF-Induced Microsomes[a]	
				Number of Matched Peptides	Sequence Coverage	Number of Matched Peptides	Sequence Coverage
			kDa		%		%
Liver carboxylesterase 10	P16303	6.3	62.14	8	22	9	26
Protein disulfide isomerase A3, ERP60	P11598	5.9	56.62	16	36	14	27
Sulfite oxidase	Q07116	5.8	54.35	8	20		
Protein disulfide isomerase, PDI	P04785	4.8	56.95	10	24		
UDP-glucuronosyltransferase B12	P36511	7.8	61.04			14	29
Calreticulin	P18418	4.3	47.98	8	27		
ATP synthase alpha chain, mitochondrial	P15999	9.2	58.83	16	36		
Cytochrome P450 4A3	P20817	9.4	58.23			16	41
Cytochrome P450 2D5	P12939	6.3	57.08	9	22	6	16
Cytochrome P450 2D2	P10634	7.0	56.68	13	27	12	26
Cytochrome P450 4A1	P08516	9.4	58.20			15	33
Cytochrome P450 2C11	P08683	7.8	57.18	9	23	8	20
Cytochrome P450 2B1	P00176	7.0	55.93			9	19
Cytochrome P450 2B2	P04167	6.8	55.93			10	23
ATP synthase beta chain, mitochondrial	P10719	5.2	56.35	12	36		
Cytochrome P450 2A1	P11711	9.2	56.01	8	19	9	21
UDP-glucuronosyltransferase 2B3	P08542	8.8	60.51			8	18
L-Gulonolactone oxidase	P10867	6.8	50.48	9	26	6	15
Epoxide hydrolase	P07687	8.6	52.58	20	31	15	35

[a] Peptide mass fingerprinting data obtained from gel pieces excised from 1DE gel area covering the apparent molecular weight range from 45 to 66 kDa. The proteins are arranged according to their position on the gel.

TABLE 3

Proteins identified by peptide mass fingerprinting in PB-induced rabbit liver microsomes.

Protein	SWISS-PROT Accession Number	pI	Molecular Weight	PB-Induced Microsomes, 1DE Separation		PB-Induced Microsomes, LC-Separation	
				Number of Matched Peptides	Sequence Coverage	Number of Matched Peptides	Sequence Coverage
			kDa		%		%
UDP-Glucuronosyltransferase B13	P36512	8.8	60.53	9	18	8	18
Cytochrome P450 1A1	P05176	9.7	58.32	8	16	10	23
Cytochrome P450 1A2	P05176	8.8	58.26	7	15	11	25
Cytochrome P450 2A10	Q05555	9.3	57.17	10	20	12	23
Cytochrome P450 2B4*	P00178	9.3	55.73	14	32	12	33
Cytochrome P450 2C1	P00180	9.0	54.59			11	21
Cytochrome P450 2C14	P17666	9.0	55.70			11	19
Cytochrome P450 4B1	P15128	9.0	58.60	8	15	10	21
Epoxide hydrolase	P04068	7.3	52.49	22	46		

*Traces of CYP2B5 (only one of seven isozyme-specific tryptic peptides was found, at the same time five out of six isozyme-specific tryptic peptides for CYP2B4 were found)

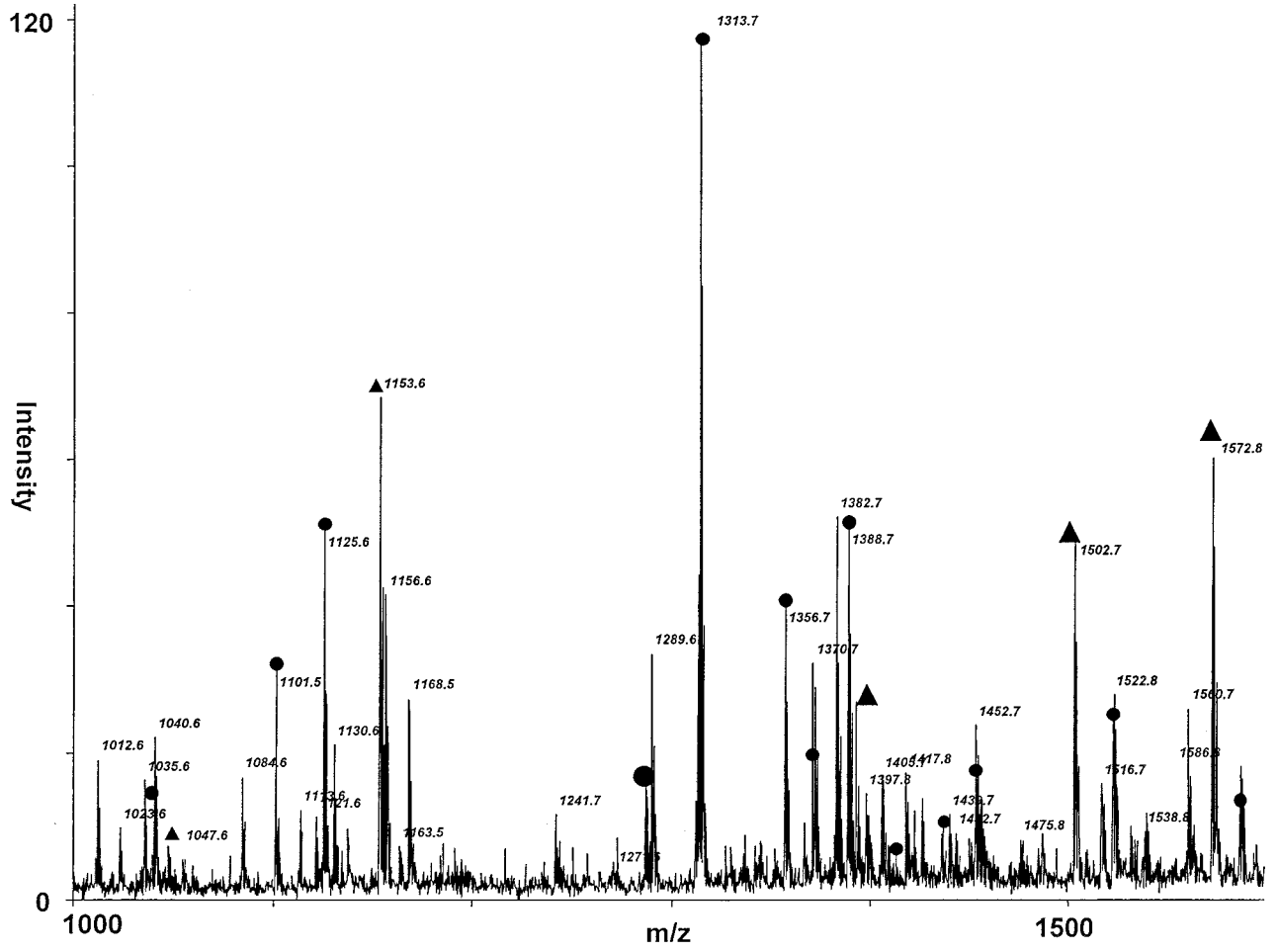

FIG. 2. *Expanded view (1000 to 1600) of the peptide mass fingerprint of 300 mM phosphate buffer fraction eluted from HA-Ultragel column.*

Black dots denote mass peaks corresponding to CYP2A10 tryptic peptides and triangles denote mass peaks corresponding to CYP2B4.

Peptide mass fingerprint of CF-microsomes revealed, as expected, the presence of CYP4A1 and CYP4A3. In addition, six more P450 isozymes were positively identified: CYP2A1, CYP2B1, CYP2B2, CYP2C11, CYP2D2, and CYP2D5 (Table 2). Application of PMF allows differential identification of such highly homologous P450s as CYP2B1 and CYP2B2 (homology >97%) differing in only 14 amino acids out of 491. Their theoretical tryptic digests differ in 7 peptides, and 4 of these 7 peptides fall within our working range 700 to 2500 amu. Mass peaks corresponding to isozyme-specific tryptic peptides characteristic for CYP2B1 and CYP2B2, respectively, were found in our experimental digests (Fig. 1).

Peptide Mass Mapping of Rabbit Liver Microsomes. The results of peptide mass fingerprinting of PB-induced rabbit liver microsomes obtained by application of 1DE separation are shown in Table 3. Five P450 isozymes were identified by in-gel digest of the following five excised gel pieces: CYP1A1, CYP1A2, CYP2B4, CYP4B1, and CYP2A10. Presence of CYP2A10 in PB-induced rabbit liver microsomes was unexpected, since previously this form was reported only

in rabbit olfactory and respiratory nasal mucosa (Peng et al., 1993). This finding is an excellent example of the advantage of PMF application for the analysis of P450 composition.

To enhance the detection of minor P450 isozymes, we performed a chromatographic separation of P450s from PB-induced rabbit microsomes. The microsomes were solubilized by Na cholate, and the solubilizate was loaded on the DEAE-Sepharose column. All uncoupled material was applied to the hydroxyapatite (HA) column that was then washed successively with increasing concentrations of potassium phosphate buffer. P450 isozymes identified in fractions eluted from hydroxyapatite are listed in Table 3, and an expanded peptide mass fingerprint view of one of the fractions is presented on Fig. 2. Two additional rabbit P450 isozymes were identified in HA-fractions, CYP2C1 and CYP2C14. Both isozymes are presumed to be PB-inducible and were previously identified only by screening a cDNA library (Leighton et al., 1984). The material bound to the DEAE-column was eluted by addition of 200 mM NaCl to the phosphate buffer and contained two P450 isozymes, CYP4B1 (LM5) and CYP1A1.

Surprising was the absence of CYP2B5 in rabbit PB-microsomes. Two rabbit PB-inducible P450 isozymes, CYP2B4 and CYP2B5, are highly homologous to rat PB-inducible CYP2B1 and 2B2. Like CYP2B1 in rats, CYP2B4 is the major PB-induced form of cytochrome P450 in rabbits and has a closely related homologous form CYP2B5 differing in only 11 amino acids. However, unlike rat PB-microsomes, in which both CYP2B1 and CYP2B2 were induced by PB and identified in microsomes (Galeva and Alterman, 2002), PMF of rabbit PB-microsomes reveals the presence of only CYP2B4 but not CYP2B5. These data could possibly point to some differences in the mechanism of phenobarbital induction between rats and rabbits.

In conclusion, this article describes a detailed proteomic analysis of the P450 isozymes composition in various rat and rabbit liver microsomes. The data obtained clearly show that this approach has a great potential for the direct identification of differentially expressed cytochrome(s) P450 and should become a standard technique for characterizing microsomes.

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