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Identification and characterisation of soluble epoxide hydrolase in mouse brain by a robust protein biochemical method

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Summary. The central nervous system is an important potential target for certain environmental prototoxins, but relatively little is known regarding brain-specific expression of biotransformation enzyme systems. On the other hand, developments in the field of molecular biology and advances in high-throughput screening methods continue to increase the number and amounts of available proteins. We used thus a robust and reliable technique, two-dimensional gel electrophoresis coupled to matrix assisted laser desorption/ionisation mass spectroscopy followed by tandem mass spectrometry and identified for the first time soluble epoxide hydrolase and added other biotransformation enzymes in the hippocampal region of mouse brain. Soluble epoxide hydrolase has an Mr of 61.5 kDa, pI of 5.9, twenty-six matching peptides and sequence coverage of 56% and was unambiguously identified by MS/MS. Since localised biotransformation events in regions of the central nervous system may account for pathologies and/or toxicities initiated by exposure to certain endogenous and/or environmental chemicals, identification of these enzymes would present an opportunity for developing novel therapeutic targets or would have critical toxicologic significance.

Keywords: Soluble epoxide hydrolase – Mouse brain – Two-dimensional electrophoresis – Mass spectrometry – Biotransformation enzymes

Introduction

Brain protection against chemicals is mainly provided by specific properties of cerebral microvessels forming the blood-brain barrier. In addition, several xenobiotic metabolizing enzymes (XMEs) have been evidenced both in brain tissue and in capillaries that provide a metabolic barrier to this organ against penetrating lipophilic substances (Minn et al., 1991; Ghersi-Egea et al., 1992). XMEs are involved in the biotransformation of hydrophobic compounds to more water-soluble and readily excretable products through functionalisation and conjugation reactions, and result in detoxification. However, xenobiotic metabolism can also possibly lead to formation of pharmacologically active or toxic products.

Epoxides are organic three-membered oxygen compounds that arise from oxidative metabolism of endogenous as well as xenobiotic compounds via chemical and enzymatic oxidation processes, including the cytochrome P450 monooxygenase system. The resultant epoxides are typically unstable in aqueous environments and chemically reactive (Fretland and Omiecinski, 2000). Biocatalytic epoxide conversion can proceed by hydrolysis, conjugation to thiol-cofactors and through nucleophilic ring opening using a variety of enzymes, including epoxide hydrolase, haloalcohol dehalogenase, glutathione-S-transferase (GST) and CoM-transferase (de Vries and Janssen, 2003). Epoxide hydrolases (EC 3.3.2.3) belong to the α/β-hydrolase fold family of enzymes and catalyze the hydration of chemically reactive epoxides and arene oxides to their corresponding diols. There are at least five epoxide hydrolases known in mammals; cholesterol epoxide hydrolase, hepoxillin A3 hydrolase, leukotriene A4 hydrolase, soluble or cytosolic epoxide hydrolase (SEH) and microsomal epoxide hydrolase (MEH) (Fretland and Omiecinski, 2000; Wixtrom and Hammock, 1988). Whilst the first three hydrate cholesterol 5, 6 α &$/\beta$ oxides, hydroxy-epoxide derivatives of arachidonic acid and leukotriene A4, respectively, the latter two utilize a broad spectrum of substrates and at times display complimentary substrate selectivity. MEH appears to be mainly involved in trans-addition of water to a broad range of xenobiotic epoxides, such as naphthalene epoxide and

polycyclic aromatic hydrocarbon class of chemical carcinogens (Phillips and Grober, 1994; van Bladeren et al., 1985). SEH, on the other hand, not only participates in metabolism of xenobiotics but also in endogenously derived fatty acid epoxides (Du Teaux et al., 2004).

Since the documentation of the existence of SEH as a different enzyme from the MEH (Ota and Hammock, 1980), its expression profile, physiological substrates and roles have been the focus of considerable research. SEH has been described at mRNA (Johansson et al., 1995), activity (Schladt et al., 1986) and protein expression (Oesch et al., 1986) level in different mammalian tissues, including the brain. Although immunochemical methods can give an idea on the presence and abundance of a particular protein, the methods by no means unequivocally identify a given protein in a particular tissue. Non-specific bindings may arise that make interpretation difficult at times and there are also a whole range of artifacts, endogenous pigments and enzymes that activate the detection system giving rise to false positive and false negative results. Here, we report for the first time identification and analysis of SEH and add other related detoxification enzymes in mouse brain using the high throughput screening technique, two-dimensional gel electrophoresis (2-DE) coupled to matrix assisted laser desorption/ionisation (MALDI) time-of-flight (TOF) mass spectrometry (MS) analysis followed by tandem mass spectrometry (MS/MS). This method allows unambiguous identification of SEH independent of antibody availability and specificity.

Materials and methods

Animals

Nine male FVB/N mice, about three-months-old, obtained from the Department of Biochemistry, Université 7, Paris, France were kept in well-controlled (humidity, temperature and light/dark cycle) environment with free access to food and water *ad libitum*, were used for the experiments. Animals were sacrificed by decapitation and the hippocampi were dissected at -20°C and kept at -80°C until time of analysis. The freezing chain was never interrupted until use.

Sample preparation

Hippocampus tissues were suspended in 1 ml of sample buffer consisting of 40 mM Tris, 5 M urea (Merck, Darmstadt, Germany), 2 M thiourea (Sigma, St. Louis, MO, USA), 4% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate) (Sigma), 10 mM 1,4-dithioerythritol (Merck), and 1 mM EDTA, protease inhibitors cocktail (Roche), 1 mM phenylmethylsulfonyl chloride, and 1 μg of each pepstatin A, chymostatin, leupeptin, and antipain. The suspension was sonicated for approximately 30 sec and centrifuged at 150,000 g for 60 min. The protein content in the supernatant was determined by the Coomassie blue method (Bradford, 1976).

Two-dimensional electrophoresis (2-DE)

2-DE was performed in triplicate as reported elsewhere (Weitzdoerfer et al., 2002). 750 μg of protein was applied on immobilised pH 3–10 non-linear gradient strips in sample cups at their basic and acidic ends. Focusing was started at 200 V and the voltage was gradually increased to 5000 V at a rate of 3 V/min and then kept constant for a further 24 h (approximately 180.000 Vh totally). After the first dimension, strips (18 cm) were equilibrated for 15 min in the equilibration buffer containing 6 M urea, 20% glycerol, 2% SDS, 2% 1,4-dithioerythritol and then for 15 min in the same equilibration buffer containing 2.5% iodoacetamide instead of 1,4-dithioerythritol. After equilibration, strips were loaded on 9–16% gradient SDS gels for second-dimensional separation. The gels ($180 \times 200 \times 1.5$ mm) were run at 40 mA per gel. Immediately after the second dimension run, gels were fixed for 18 h in 50% methanol, containing 10% acetic acid, the gels were then stained with Colloidal Coomassie Blue (Novex, San Diego, CA) for 12 h on a rocking shaker. Molecular masses were determined by running standard protein markers (Biorad Laboratories, Hercules, CA) covering the range 10–250 kDa. pI values were used as given by the supplier of the immobilised pH gradient strips (Amersham Bioscience, Uppsala, Sweden). Excess of dye was washed out from the gels with distilled water and the gels were scanned with ImageScanner (Amersham Bioscience). Electronic images of the gels were recorded using Adobe Photoshop and Microsoft Power Point Softwares.

MALDI-MS(/MS)

Spots were excised with a spot picker (PROTEINEER sp^{TM}, Bruker Daltonics, Germany) and placed into a 384-well microtiter plate. In-gel digestion and sample preparation for MALDI analysis were performed by an automated procedure (PROTEINEER dp^{TM}, Bruker Daltonics) (Yang et al., 2004). Briefly, spots were excised and washed with 10 mM ammonium bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate. After washing, gel plugs were shrunk by addition of acetonitrile and dried by blowing out the liquid through the pierced well bottom. The dried gel pieces were re-swollen with 40 ng/μl trypsin (Promega, Madison, USA) in enzyme buffer (consisting of 5 mM Octyl β-D-glucopyranoside, and 10 mM ammonium bicarbonate) and incubated for 4 hrs at 30°C. Peptide extraction was performed with 10 μl of 1% trifluoroacetic acid in 5 mM Octyl β-D-glucopyranoside. Extracted peptides were directly applied onto a target (AnchorChip^{TM}, Bruker Daltonics) that was loaded with α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics) matrix thinlayer. The mass spectrometer used in this work was an Ultraflex^{TM} TOF/TOF (Bruker Daltonics) operated in the reflector mode for MALDI-TOF peptide mass fingerprint (PMF) or LIFT (a pecial technique introduced to increase the efficiency of UltraFlex-TOF–TOF instruments) mode for MALDI-TOF/TOF with a fully automated mode using the FlexControl^{TM} software. An accelerating voltage of 25 kV was used for PMF. Calibration of the instrument was performed externally with $[M+H]^+$ ions of angiotensin I, angiotensin II, substance P, bombesin, and ACTH (clip 1–17 and clip 18–39). Each spectrum was produced by accumulating data from 200 consecutive laser shots. Those samples which were analysed by PMF from MALDI-TOF were additionally analysed using LIFT-TOF/TOF MS/MS from the same target. A maximum of three precursor ions per sample were chosen for MS/MS analysis. In the TOF1 stage, all ions were accelerated to 8 kV under conditions promoting metastable fragmentation. After selection of jointly migrating parent and fragment ions in a timed ion gate, ions were lifted by 19 kV to high potential energy in the LIFT cell. After further acceleration of the fragment ions in the second ion source, their masses could be simultaneously analysed in the reflector with high sensitivity. PMF and LIFT spectra were interpreted with the Mascot software (Matrix Science Ltd, London, UK). Database searches, through Mascot, using combined PMF and MS/MS datasets were performed via BioTools 2.2 software (Bruker). A mass tolerance of 100 ppm and 1 missing cleavage sites for PMF and MS/MS tolerance of 0.5 Da and 1 missing

cleavage sites for MS/MS search were allowed and oxidation of methionine residues was considered. The probability score calculated by the software was used as criterion for correct identification. The algorithm used for determining the probability of a false positive match with a given mass spectrum is described elsewhere (Berndt et al., 1999).

Results

Among the spots analysed from a region of 2-DE covering a molecular size range of 10–250 kDa and a pI of 3–10, eight spots were identified as proteins involved in the detoxication pathway of the mouse brain by PMF and MS/MS (Fig. 1 and Table 1). The proteins include SEH, Glutathione-S-transferases (GST, Mu1, Mu5 & P2) and peroxiredoxins (II, III & VI). MS/MS data are presented only for SEH, since this is the protein of major interest for the present work. PMF analysis revealed sequence coverage of 56% (Fig. 2) and a Mascot score of 214. Following identification by PMF, the workflow control software automatically selected two peaks (m/z 1333.71 and m/z 2002.09) from the MS spectrum (Fig. 3) to generate an MS/MS spectrum, as MS/MS analysis of one or two available peptides can provide unambiguous identification of a protein in question. Indeed, the generated LIFT-TOF/TOF spectrum of m/z 1333.71 (Fig. 4a) and m/z 2003.10 (Fig. 4b) was nicely matched to peptide 273–284 (YQIPALAQAGFR) and peptide 5–24 (VAAFDLDGVLALPSIAGAFR), respectively, confirming unambiguous assignment of the spot to SEH. The goal of a high-throughput proteomics approach is to determine the identity of proteins present in the sample. However, because MS/MS spectra are produced from

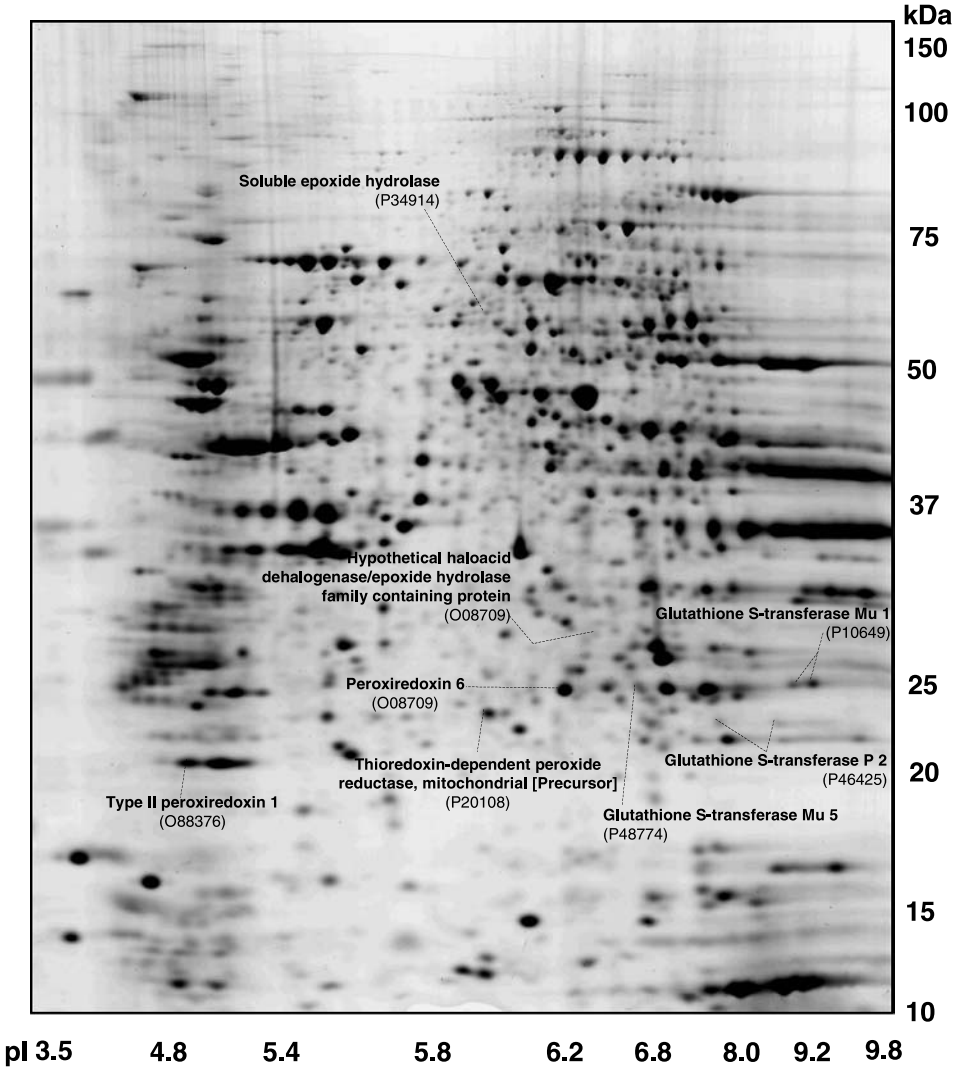

Fig. 1. 2-DE gel image of biotransformation enzymes, including SEH: Proteins were extracted and separated on an immobilised pH 3–10 non-linear gradient strip followed by separation on a 9–16% gradient polyacrylamide gel. Gels were stained with Coomassie blue and spots were analysed by MALDI-MS and MS/MS

Table 1. List of identified biotransformation enzymes in mouse brain

Accession number	Entry name	Protein name	Peptide matches	Score[a]	Theoretical/Observed	
					p*I*	M_r (Da)
P10649	GTM1_MOUSE	Glutathione S-transferase Mu 1	27	241	8.14/9.00 ~ 9.20	25.84/25.10
P48774	GTM5_MOUSE	Glutathione S-transferase Mu 5	17	139	6.82/6.65	26.63/25.00
P46425	GTP2_MOUSE	Glutathione S-transferase P 2	13	318	8.13/7.80 ~ 8.20	23.41/23.20
O08709	PDX6_MOUSE	Peroxiredoxin 6	17	278	5.72/6.20	24.74/24.95
P20108	PDX3_MOUSE	Thioredoxin-dependent peroxide reductase, mitochondrial [Precursor]	14	141	7.15/5.95	28.13/24.00
O88376	O88376	Type II peroxiredoxin 1	16	396	5.20/4.95	21.79/20.80
P34914	HYES_MOUSE	Soluble epoxide hydrolase	26	214	5.85/5.90	62.52/61.50
Q9CYW4	Q9CYW4	Hypothetical haloacid dehalogenase/epoxide hydrolase family containing protein (35% identity to Q6LBD3, hydrolase of HAD-superfamily)	12	71	6.31/6.30	28.03/28.00

Brain proteins were separated by 2-DE and identified by MALDI-TOF/TOF, following in-gel digestion with trypsin. [a] Score is $-10 * \text{Log}(P)$, where P is the probability that the observed match is a random event (MASCOT, http://www.matrixscience.com)

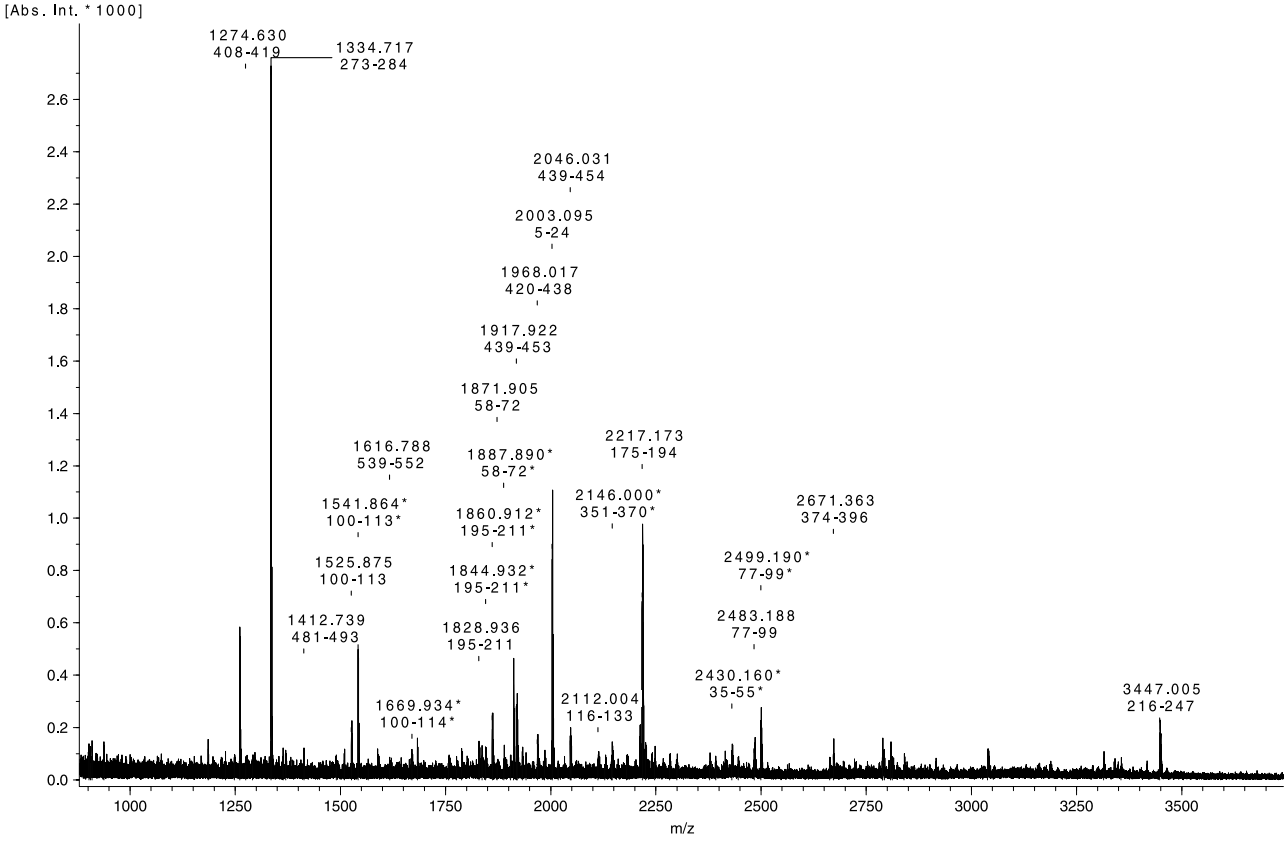

Fig. 2. Peptide mass fingerprint (PMF) of SEH. Matched peptides were shown and annotated. Peaks with * letter contained methionine oxidation in their peptides

peptides and not proteins, all conclusions drawn about the protein content of the original sample are based upon the identification of peptides. The connectivity between peptides and proteins is usually quite straightforward when analysis is based on digestion of proteins, leading to unambiguous identification (Fountoulakis, 2001).

The database search approach only enables identification of those peptides that are present in the searched

1 MALRVAAFDL DGVLALPSIA GAFRRSEEAL ALPRDFLLGA YQTEFPEGPT
51 EQLMKGKITF SQWVPLMDES YRKSSKACGA NLPENFSISQ IFSQAMAARS
101 INRPMLQAAI ALKKKGFTTC IVTNNWLDDG DKRDSLAQMM CELSQHFDFL
151 IESCQVGMIK PEPQIYNFLL DTLKAKPNEV VFLDDFGSNL KPARDMGMVT
201 ILVHNTASAL RELEKVTGTQ FPEAPLPVPC NPNDVSHGYV TVKPGIRLHF
251 VEMLCPALCL CHGFPESWFS WRYQIPALAQ AGFRVLAIDM KGYGDSSSPP
301 EIEEYAMELL CKEMVTFLDK LGIPQAVFIG HDWAGVMVWN MALFYPERVR
351 AVASLNTPFM PPDPDVSPMK VIRSIPVFNY QLYFQEPGVA EAELEKNMSR
401 TFKSFFRASD ETGFIAVHKA TEIGGILVNT PEDPNLSKIT TEEEIEFYIQ
451 QFKKTGFRGP LNWYRNTERN WKWSCKGLGR KILVPALMVT AEKDIVLRPE
501 MSKNMEKWIP FLKRGHIEDC GHWTQIEKPT EVNQILIKWL QTEVQNPSVT
551 SKI

Fig. 3. Protein sequence of SEH with peptide matched (bold letter) demonstrating sequence coverage of 56%. Boxed peptides were analysed by MS/MS

sequence database. It cannot therefore identify peptides derived from post-translationally modified proteins. However, prediction of O-& N-glycosylation as well as phosphorylation by submission of identified peptide sequences to the database revealed one O-glycosylation (NetOGlyc 3-1 Server), three N-glycosylation (NetNGlyc 1.0 Server) and twenty-three potential phosphorylation (NetPhspho 2.0 Server) sites.

Discussion

During the course of mammalian evolution, a myriad of catabolic pathways have evolved to defend against harmful environmental chemicals and their metabolites. Aromatic hydrocarbons such as styrene, stilbene or benzo[a]pyrene can be oxidised *in vivo* to form mutagenic epoxides that readily alkylate nucleic acids (Argiriadi et al., 1999). The first line of chemical defense against xenobiotic-derived epoxides is the enzyme epoxide hydrolase, which exists as SEH and MEH. To the best of our knowledge, this is the first report to describe protein expression of SEH together with seven other detoxifying enzymes in the mouse brain using MS/MS. Failure to detect MEH could probably be attributed to its microsomal prevalence, a notion supported by absence of any of the isoforms of cytochrome P (CYP) 450 enzymes, which are representative of microsomal proteins.

The mouse SEH is encoded on chromosome 14, which is homologous to human chromosomes 8, 13 and 14 (Grant et al., 1994). The precise biological function of SEH is still largely unknown. Besides degradation of potential toxic epoxides, an accumulating body of evidence indicates that SEH may also play a role in determining steady-state levels of physiological mediators (Grant et al., 1993; Arand et al., 2003). CYP450 enzymes metabolize arachidonic acid to biologically active eicosanoids, such as hydroxyeicosatetraenoic acids and epoxyeicosatrienoic acids (EETs). The two eicosanoids have opposing action within the vasculature and imbalance in their levels is associated with certain pathological conditions, including hypertension and vascular inflammation (Kroetz and Zeldin, 2002; Sarkis et al., 2004). Whilst hydroxyeicosatetraenoic acids are potent vasoconstrictors, EETs have potent vasodilatory properties. EETs are thought to modulate ion transport and gene expression, probably through activation of Gα or Src signal transduction pathways, thereby producing vasorelaxation, anti-inflammatory and pro-fibrinolytic effects (Spector et al., 2004). The formation of these eicosanoids is tightly controlled not only by the expression and activity of CYPs but also by SEH, which catalyzes the hydrolysis of EETs to dihydroxyeicosatrienoic acids. Thus, identification of SEH by this robust and reliable technique will allow many pharmacological as well as toxicological studies to be performed on the protein. More importantly, as bioactive eicosanoids are detected in the brain (Yue et al., 2004) and the role of EETs in cerebral angiogenesis (Zhang and Harder, 2002) and regulation of body temperature (Nakashima et al., 2001) is beginning to be elucidated, such identification would provide a tool for manipulation of SEH for better understanding of the cellular role of the biologically active metabolites in the brain.

Haloacid dehalogenases belong to the haloacid dehalogenase super family, to which magnesium-dependent phosphatases and P-type ATPases also belong (de Jong and Dijkstra, 2003). They catalyze the hydrolytic dehalogenation of small haloalkanoic acids to yield the corresponding hydroxyalkanoic acids. Since the beginning of the last century, halogenated hydrocarbons have been extensively applied in industry and agriculture and most of them are highly toxic. Although these enzymes elaborated mainly by bacteria, detection of a hypothetical protein similar to haloacid dehalogenases indicates that there are proteins with potential ability to dehalogenate toxic haloacids in mouse brain. Glutathione plays a primary role in protecting cells from oxidative stress and in detoxifying foreign compounds. Many xenobiotics, including the neurotoxic solvent styrene have shown to cause glutathione depletion in the brain (Trenga et al., 1991), thereby disrupting the expression and activity of glutathione conjugating enzymes. GSTs are phase II enzymes that catalyze nucleophilic attack of glutathione on electrophilic

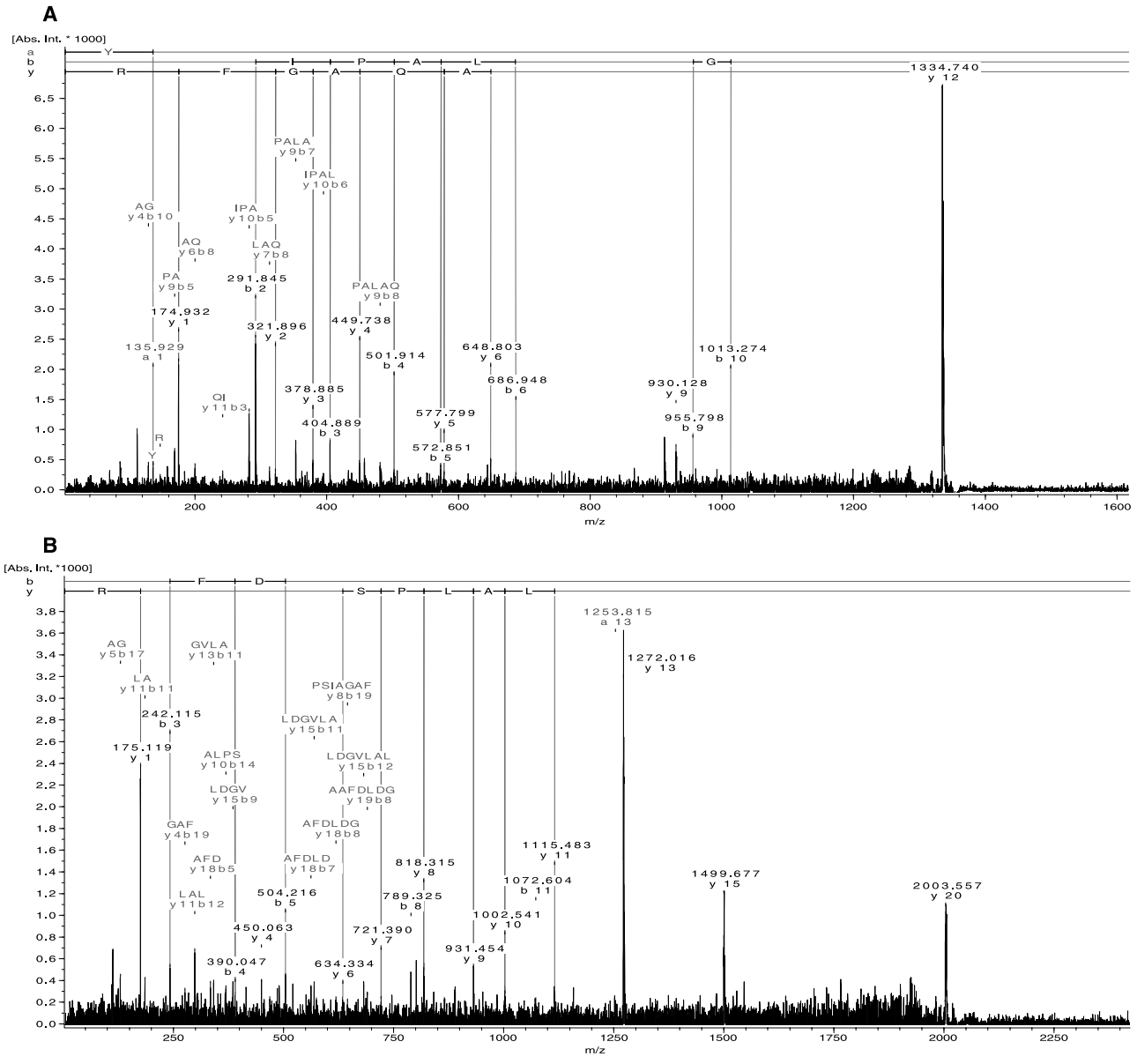

Fig. 4. LIFT-TOF/TOF (MS/MS) spectrum of SEH: the parent ions (m/z 1333.71 and m/z 2003.10) were selected for further analysis by MS/MS and the amino acid sequences YQIPALAQAGFR (**A**) and VAAFDLDGVLALPSIAGAFR (**B**) were unambiguously assigned to SEH

centers in a wide variety of organic molecules, including epoxides, organic hydroperoxides and activated alkenals, resulting from oxidative metabolism (Mannervik et al., 1985). Although glutathione transferases exist both as soluble and membrane-bound enzymes, the detected isoforms Mu1, Mu5, and P2 are cytosolic enzymes, lending further support for the view that membrane-bound proteins are less likely to be detected. The peroxiredoxins define an emerging family of peroxidases that are able to protect biomolecules by reducing hydrogen peroxide and alkyl hydroperoxides with the use of reducing equivalents derived from thiol-containing donor molecules, such as thioredoxin, glutathione, trypanothione and AhpF. To date, six distinct groups of mammalian peroxiredoxins (I–VI) have been identified, out of which three of them (II, III & VI) were detected in the present work, and are implicated in a host of cellular processes (Krapfenbauer et al., 2003).

To sum up, the MALDI LIFT-TOF/TOF mass spectrometer enables PMFs and MS/MS spectra to be acquired from the same sample with high throughput and sensitivity. Detection of SEH in hippocampus of mouse brain by such method supports the different suggested biological roles in this tissue. Regional variation in expression of

detoxifying enzymes appears to underlie susceptibility to xenobiotic-mediated insults. Identification of different types and/or isoforms of the enzyme using this technique thus also enable one to determine regional differences in sensitivity to these insults. Furthermore, the results establish that the brain actively expresses a number of different biotransformation enzymes and suggest that for brain, which exhibits low regeneration capacity, such expressions would be of critical toxicological importance.

We here show unambiguous protein chemical characterisation and identification of SEH, independent of antibody availability and specificity and propose an analytical tool.

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