ORIGINAL RESEARCH

A proteomic approach to determination of the significance of protein oxidation in the ageing of mouse hippocampus

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Abstract: The increase in oxidised forms of protein including carbonyls, methionine sulphoxide and cross-links has been reported by many groups in addition to the accumulation of heat-labile and less-active enzymes in aged animal tissues. Since oxidised forms of proteins are thermodynamically unstable, the partially unfolded proteins are assumed to be more susceptible to irregular proteolysis in aged animal tissues. Here, we report a decrease in the relative abundance of calmodulin, UCH-L1 and nm23-M1 in aged mouse hippocampus detected by two-dimensional gel electrophoresis and quantitative image analysis. To investigate the involvement of oxidation in the down-regulation of these proteins, the level of methionine sulphoxide was analysed by high-throughput MALDI-TOF mass spectrometry with post-source decay fragmentation. As a result of proteomic analysis, increased methionine oxidation was detected in those proteins in aged mouse hippocampus. Our observation suggests that the oxidation of calmodulin, UCH-L1 and nm23-M1 might be involved in the down-regulation of these proteins in aged mouse hippocampus.

Keywords: proteome, protein oxidation, ageing, mouse hippocampus, calmodulin

Introduction

Alzheimer's disease (AD) is characterised by neurofibrillary tangles, plaques of amyloid-beta peptide and neuronal cell loss (Henderson and Finch 1989). The onset of dementia is usually found to occur around 40–60 years for familial AD (FAD), and over 60 years for sporadic AD (SAD) (Harman 2002). The different categories of AD may be due to varying genetic backgrounds augmenting the rate of neuronal cell ageing, which may be closely related to superoxide radical formation in mitochondria (Forman and Kennedy 1975) and accumulation of oxidative cell damage.

Washburn first suggested the role of free radical reactions in the molecular pathology of bio-degenerative processes (Washburn 1973). Later, in 1981, Harman proposed the free radical theory of ageing (Harman 1981). Reactive oxygen species (ROS) produced in mitochondria may generate 'altered' proteins, for example, protein carbonyls (Nakamura and Goto 1996), disulphide bridges (Barron et al 1988), nitrotyrosine (Good et al 1996) and methionine sulphoxide (Garner and Spector 1980). Carbonylation of lysine, arginine and histidine residues may cause a drift in isoelectric point and the loss of cleavable sites for trypsin-like proteinases. However, the isoelectric point is not affected by oxidation at the sites of cysteine, tyrosine and methionine residues. In any case, the expression level of oxidation-sensitive proteins that are preferentially damaged with ROS is assumed to decrease in aged animal tissues, since oxidised proteins are conformationally unstable and more susceptible to proteolysis.

In this field of research, we first searched target proteins for which the relative abundance decreased with ageing of the mouse hippocampus. The level of oxidised forms of these proteins was estimated semi-quantitatively by using a coupled method of peptide mass fingerprinting (PMF) (Ji et al 1994) combined with MALDI-TOF/MS (Pappin 1997) and neutral loss (NL) analysis by MALDI-PSD-TOF/MS/MS (Hoffmann et al 2001).

Materials and methods

Apparatus and chemicals

The CoolPhoreStar system for two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was purchased from Anatech (Tokyo, Japan). The Molecular

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Imager FX, ProteomeWorks Spot Cutter and PDQuest software for 2-D gel image analysis were supplied by Bio-Rad (Richmond, California, USA). An AXIMA-CFR MALDI-TOF mass spectrometer (Shimadzu Biotech, Kyoto, Japan) was utilised for PMF and NL analysis. Immobiline DryStrip and Pharmalyte 3–10 were obtained from Amersham Bioscience (Piscataway, New Jersey, USA). Urea, thiourea, acrylamide, N,N-methylenebisacrylamide, TEMED, SDS, Triton X-100 and DTT were products of Sigma (St Louis, Missouri, USA). Silicon oil KF-96L-5CS was supplied by Shin-Etsu Chemicals (Tokyo, Japan). SYPRO Ruby Protein Gel Stain was purchased from Genomic Solutions (Ann Arbor, Michigan, USA).

Sample preparation

C57BL/6 male mice at various ages were supplied by our laboratory animal facility. The whole brain tissues were quickly removed from 4 heads per age group of C57BL/6 mice, after dipping the whole body into liquid nitrogen. Regions of hippocampus were removed from the frozen tissue slices that were kept below –20 °C during the whole process of dissection. The tissue samples were separately homogenised using a protein extraction reagent containing 8.5 M urea, 0.2% (w/v) SDS, 2% (v/v) Triton X-100, 3% (v/v) 2-ME and 2% (v/v) Pharmalyte, pH 3–10. The supernatant was removed by centrifugation at 100 000 × *g* for 10 min at 4 °C.

2-D polyacrylamide gel electrophoresis

High-resolution 2-D PAGE performed in combination with isoelectric focusing (IEF) on an immobilised pH gradient (IPG) (Westermeier et al 1983) and SDS polyacrylamide gel electrophoresis (SDS-PAGE) on a vertical gel was slightly modified for our differential protein analysis. The first-dimensional IEF was carried out on the Immobiline DryStrip (18 cm long, pH 4–7) in the CoolPhoreStar horizontal electrophoresis chamber. The strip was rehydrated in a swelling buffer containing 6 M urea, 2 M thiourea, 13 mM DTT, 1% (v/v) Pharmalyte, 2.5 mM acetic acid and 2% (v/v) Triton X-100 at 20 °C overnight before use. A 20-µl aliquot of the brain tissue extract was applied to the gel strip using a small piece of filter paper. Electrofocusing was started at 500 V for 2 h, and followed by 700 V for 1 h, 1000 V for 1 h, 1500 V for 1 h, 2000 V for 1 h, 2500 V for 1 h, 3000 V for 1 h and subsequently at 3500 V for 10 h using a PowerPhoreStar Pro3800.

Following the electrofocusing, the strip was equilibrated with 10 ml of SDS-treatment solution containing 6 M urea, 33 mM DTT, 25 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 25% (v/v) glycerol and 0.005% (w/v) bromophenol blue (BPB) for 45 min. The treatment with iodoacetamide for alkylation of free thiol groups was skipped to avoid artificial protein oxidation during incubation.

The second-dimensional SDS-PAGE was carried out under a constant current (20 mA per gel) until the dye front of BPB reached almost the end of the gel slab in a vertical electrophoresis chamber (CoolPhoreStar SDS-PAGE Tetra-200) using a 7.5% T, 3% C polyacrylamide gel slab. Tris-Tricine buffer (0.1 M Tris, 0.1 M Tricine) and Tris-HCl buffer (0.2 M Tris-HCl, pH 8.8) were used for the cathodic and anodic electrolyte solutions, respectively. The 2-D PAGE mapping of mouse hippocampus protein was repeated 3 times for every tissue sample to assure reproducibility.

Staining of the protein spots and quantitative image analysis

After the second-dimensional gel electrophoresis, the gel slab was immersed in a fixing reagent (40% methanol, 7% acetic acid 1% 2-ME) for 30 min, and incubated in a SYPRO Ruby staining solution supplemented with 1% 2ME for 90 min. Excess dye was removed by rinsing in 10% methanol, 7% acetic acid for 30 min. 2-D gel images of protein spots were acquired using the Molecular Imager FX laser scanning fluorometer. Spot detection, matching and quantification were carried out using the PDQuest software version 7.01. The statistical analysis of protein variations in relative abundance (ppm in whole protein spots) was carried out using 12 frames of 2-D gel images per age group (triplicate 2-DE for every tissue sample obtained from 4 heads of mice per age group). The *P* values of the non-paired *t* test were calculated to evaluate the significance of variations.

Spot cutting and in-gel digestion

Protein spots were punched out from the gel slab using an automated spot excising system (Bio-Rad Spot Cutter) equipped with a 1.5-mm diameter needle. The excess dye of SYPRO Ruby stain was removed by rinsing twice in 60% methanol, 50 mM ammonium bicarbonate, 5 mM DTT for 15 min, and twice in 50% acetonitrile, 50 mM ammonium bicarbonate, 5 mM DTT for 7 min. The gel piece was dehydrated in absolute acetonitrile, and reswollen with an in-gel digestion reagent containing

30% acetonitrile, 50 mM ammonium bicarbonate, 5 mM DTT and 10 μg/ml sequencing grade trypsin (Promega V5111). Digestion was carried out overnight at 30 °C.

PMF for identification of protein and determination of methionine sulphoxide-containing peptides

Following the tryptic digestion, a 1 μl aliquot of the reaction mixture was removed and mixed with 1 μl of matrix solution (10 mg/ml alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 40% methanol, 0.1% TFA) on a stainless steel MALDI target plate. The MALDI-TOF/MS analysis was performed using the Shimadzu Biotech AXIMA-CFR mass spectrometer operated in reflectron positive ion mode. Database searches were carried out using the MS-Fit database search engine in ProteinProspector (http://prospector.ucsf.edu/) in which methionine oxidation was considered as a possible modification for the search query.

NL analysis by MALDI-PSD-TOF/MS/MS

In the parameter setting of the Shimadzu Biotech LaunchPad software version 2.2.1 for the MALDI-TOF/MS seamless PSD analysis on AXIMA-CFR, the mass range for the ion gating was set to be 14-Da wide, positioning the mass of a selected parent ion at the centre. The laser power for NL analysis was adjusted to be 20%–50% higher than the optimal level for a regular MALDI-TOF/MS.

Results

Age-related protein variations in mouse hippocampus

The proteins extracted from hippocampus of C57BL/6 male mice at 6, 12 and 24 months after birth were separated

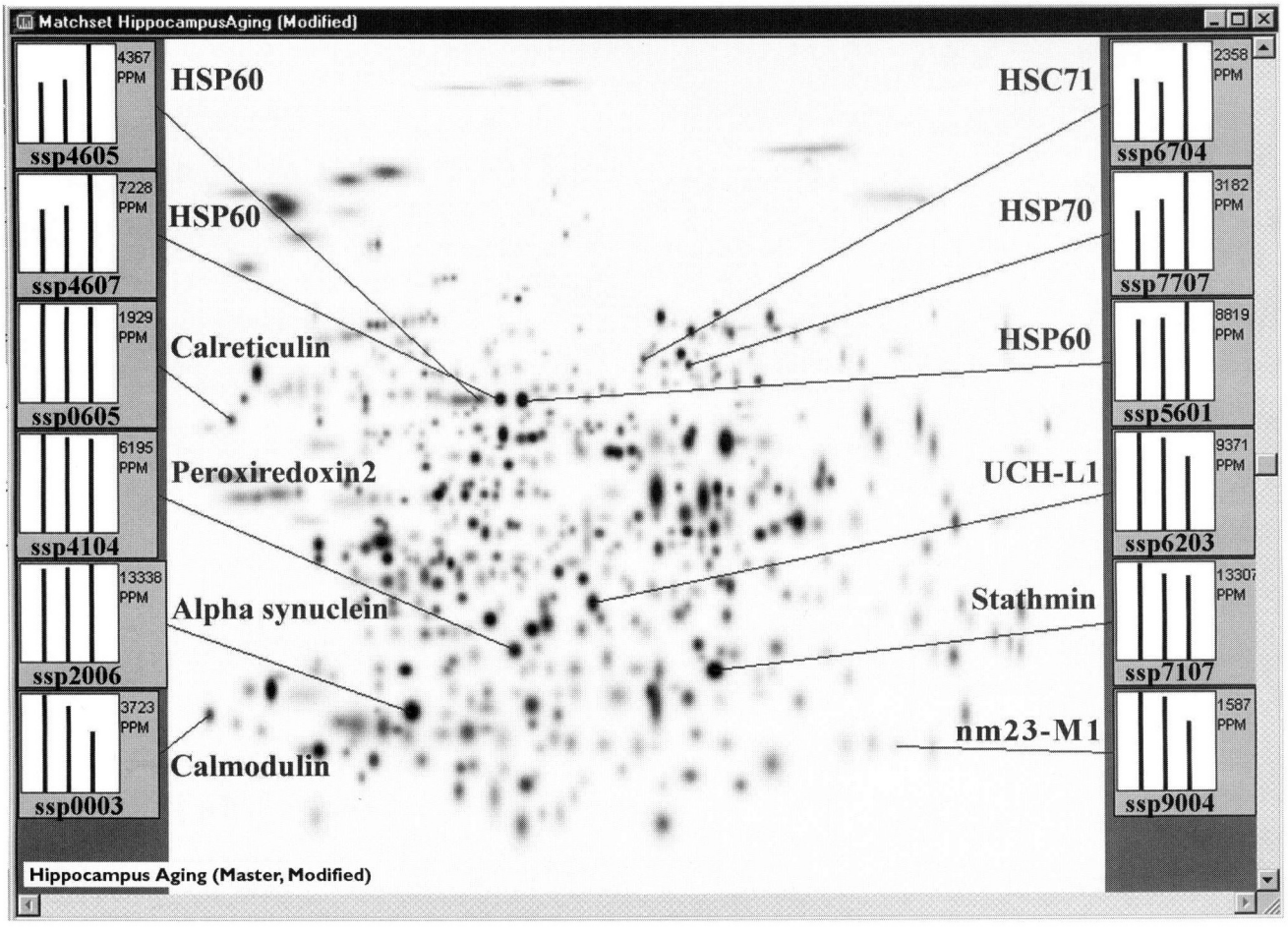
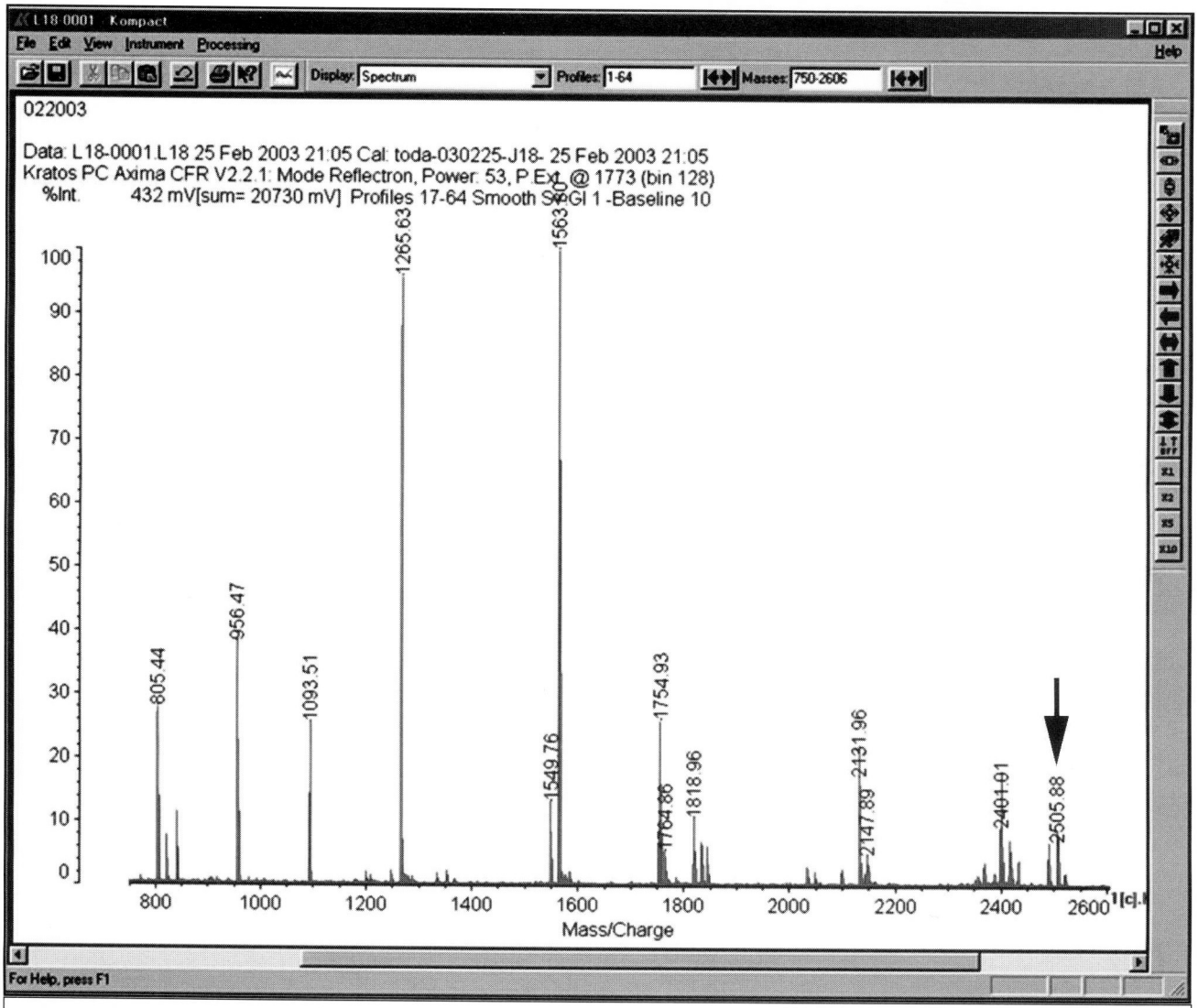


Figure 1 Analysis of the age-related protein variations in the mouse hippocampus by 2-D gel electrophoresis. More than 550 spots were detected on the 2-D gels by SYPRO Ruby staining and completely matched among gels for comparative image analysis. Increase in the relative abundance of ssp4605, ssp4607, ssp6704 and ssp7707, and decrease in that of ssp0003, ssp6203 and ssp9004 were detected by the quantitative 2-D gel image analysis. The margin graphs indicate the average levels of the relative abundance of those spot proteins in hippocampus of four heads of mice per age group (6, 12 and 24 months after birth from the left to the right in the bar graph).



1. 9/13 matches (69%).
Acc #: P02593 **Species:** HUMAN **Name:** Calmodulin
Index: 112263 **MW:** 16838 Da **pI:** 4.1

m/z Submitted	MH^+ Matched	Delta Da	Modifications	Start	End	Missed Cleavages	Database Sequence
805.4400	805.4242	0.016		32	38	0	(K)ELGTVMR (S)
956.4700	956.4729	–0.0029		15	22	0	(K)EAFSLFDK (D)
1093.5100	1093.4649	0.045		79	87	0	(K)DTDSEEEIR (E)
1265.6600	1265.6126	0.047		96	107	0	(K)DGNGYISAAELR (H)
1563.8000	1563.7542	0046	AcetN	1	14	0	(–)ADQLTEEQIAEFK(E)
1754.8800	1754.8713	0.0087		92	107	1	(R)VFDKDGNGYISAAELR (H)
1844.8600	1844.8918	–0.032		15	31	1	(K)EAFSLFDKDGDGTITTK (E)
2489.9300	2490.0805	–0.15		128	149	0	(R)EADIDGDGQVNYEEFVQMMTAK (–)
2505.8800	2506.0754	–0.20	1 Met–ox ←	128	149	0	(R)EADIDGDGQVNYEEFVQMMTAK(–)

Figure 2 Primary screening of methionine sulphoxide-containing peptides by peptide mass fingerprinting. The tryptic peptides of calmodulin prepared from mouse hippocampus at 24 months after birth were subjected to peptide mass fingerprinting. As the result of a conventional MALDI-TOF/MS, the mass spectrum shown in this figure was obtained. The methionine oxidation in the peptide 128–149 was suggested in the result of the ProteinProspector MS-Fit database search as shown below the mass spectrum.

by 2-D gel electrophoresis as described earlier. Protein spots visualised by staining with SYPRO Ruby on the gel slabs were analysed using the PDQuest software. The quantitative image analysis indicated that the expression levels of most proteins in hippocampus were well-regulated to be within 30% of variation throughout all age groups of the mice. On the 2-D gel protein maps prepared from four heads of mice per age group, significant decrease was detected in proteins of ssp0003, ssp6203 and ssp9004 in aged mouse hippocampus (Figure 1). In contrast to the down-regulation, increase was detected in proteins of ssp4605, ssp4607, ssp5601, ssp6704 and ssp7707 in hippocampuses of aged mice at 24 months after birth.

To confirm the significance of the down-regulation, statistical analysis was performed among the data obtained from the four heads of mice per age group. The *P* values of the non-paired *t* test were summarised in the Table 1. The results suggest that the down-regulation of ssp0003, ssp6203 and ssp9004 in hippocampus of mouse at 24 months may be significant.

Identification of protein and detection of oxidised methionine by MALDI-TOF mass spectrometry

Protein spots including ssp0003, ssp4505, ssp4607, ssp5601, ssp6704, ssp7107, ssp7707 and ssp9004 were excised from the SYPRO Ruby-stained 2-D gel, and subjected to identification by peptide mass fingerprinting as described in Materials and methods. From the results of the MS-Fit database search, ssp4505, ssp4607 and ssp5601 were all assigned to heat-shock protein 60 (HSP60). Proteins of ssp6704, ssp7707, sp0003, ssp6203 and ssp9004 were identified as heat-shock cognate 71 (HSC71), HSP70, calmodulin, ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) and nm23-M1, respectively. The results suggest that the relative abundances of calmodulin, UCH-L1 and nm23-M1 are down-regulated, whereas the stress-inducible proteins, such as HSP60, HSP70 and HSC71 are up-regulated in the aged mice hippocampus, in the same area of aged mouse brain (Figure 1).

Detection and semi-quantitative determination of methionine sulphoxide in the down-regulated proteins in aged mouse hippocampus

Methionine sulphoxide in tryptic peptides was primarily detected by PMF and then confirmed by NL analysis. Figure 2 shows a typical result of the PMF of ssp003 calmodulin. The mass spectrum of PMF suggested that the methionine residue in the peptide at m/z = 2505.88 might be oxidised as indicated with arrows. To confirm the existence of methionine sulphoxide in the peptide, NL analysis was performed. Figure 3 shows the results of NL analysis of the candidate peptide, detected at m/z = 2505.88 and its unmodified precursor form, detected at m/z = 2489.88 as a control peptide. A mass signal 64 Da lower than the parent peptide ion produced by *cis*-elimination of methionine sulphoxide was detected as the neutral loss of the candidate peptide ion gated at m/z = 2505 (Figure 3, B). Conversely, the MALDI- TOF/MS/PSD spectrum of the control peptide ion gated at m/z = 2489.88 showed no significant neutral loss (Figure 3, C). A similar neutral loss was detected in another calmodulin peptide gated at m/z = 2521.88, peptide of calmodulin, UCH-L1 peptide

Table 1 Statistical analysis of age-related variation of proteins in mouse hippocampus

Relative abundance of calmodulin (mean +/– SEM [pmm])	*P values of unpaired t test* 6 months	*12 months*	*24 months*
6 months (3723 +/– 101.5 [ppm])		$P = 0.0209$	$P = 0.0003$
12 months (3310 +/– 85.77 [ppm])	$P = 0.0209$		$P = 0.0016$
24 months (2487 +/– 125.2 [ppm])	$P = 0.0003$	$P = 0.0016$	
Relative abundance of UCH-L1 (mean +/– SEM [pmm])			
6 months (9371 +/– 140.3 [ppm])		$P = 0.2766$	$P = 0.0007$
12 months (9098 +/– 179.8 [ppm])	$P = 0.2766$		$P = 0.0027$
24 months (7686 +/– 226.1 [ppm])	$P = 0.0007$	$P = 0.0027$	
Relative abundance of nm23-M1 (mean +/– SEM [pmm])			
6 months (1587 +/– 33.20 [ppm])		$P = 0.4880$	$P = 0.0002$
12 months (1545 +/– 46.71 [ppm])	$P = 0.4880$		$P = 0.0007$
24 months (1134 +/– 44.88 [ppm])	$P = 0.0002$	$P = 0.0007$	

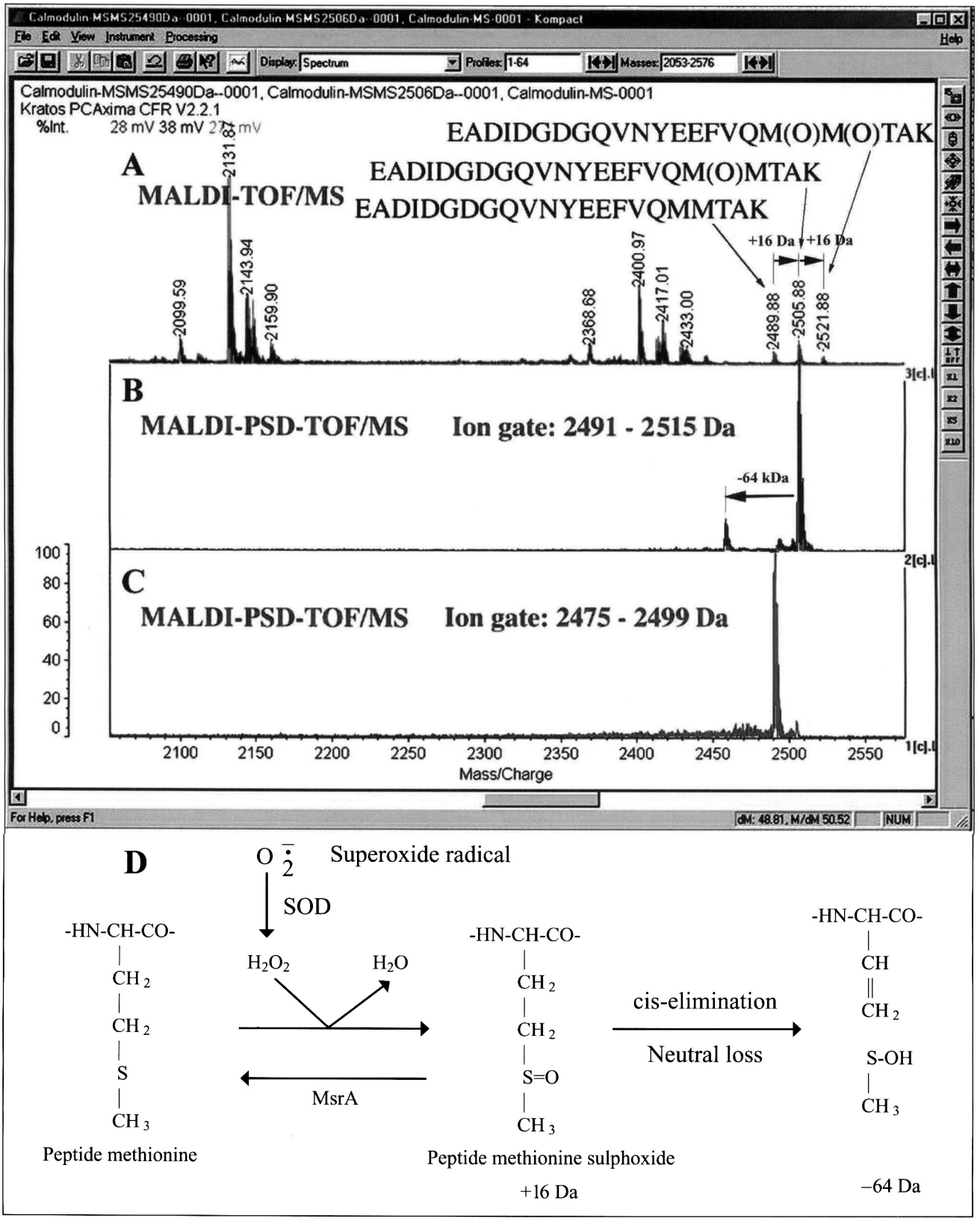


Figure 3 Detection and confirmation of methionine sulphoxide in aged mouse calmodulin by mass spectrometry. Methionine sulphoxide-containing peptides were simply detected in signals of 16 Da-higher masses than their un-oxidised form by the primary MALDI-TOF/MS (A). Methionine oxidation in the 2505.88-Da peptide was confirmed by detecting 64-Da 'neutral loss' in the secondary MALDI-PSD-TOF/MS (B). No neutral loss was observed in MALDI-PSD-TOF/MS of the 2489.88-Da peptide which was an un-oxidised form of the 2505.88-Da peptide (C). The chemical fomulae represent the structural alterations in oxidation and the neutral loss of methionine residues (D).

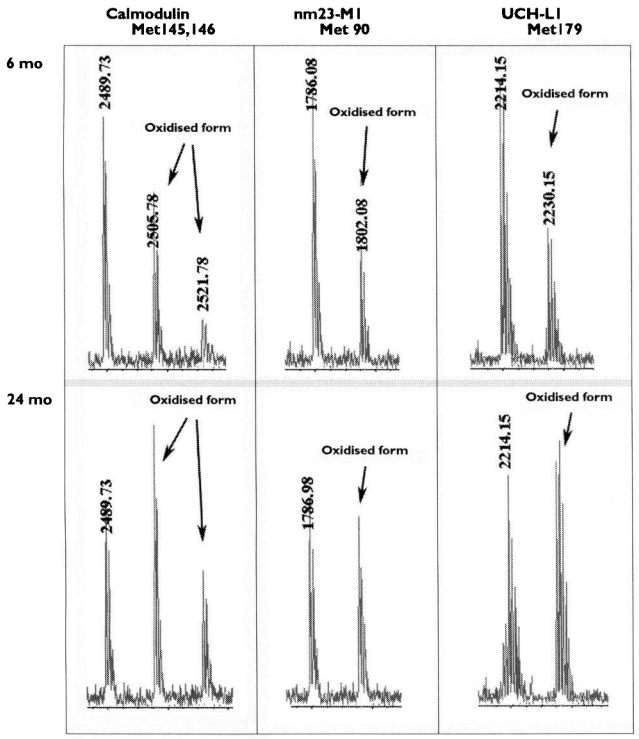


Figure 4 MALDI-TOF/MS analysis of the level of methionine sulphoxide in calmodulin. Calmodulin was isolated from hippocampus of young adult mice aged (6 months) and (24 months) by 2-D gel electrophoresis. The tryptic peptides obtained by in-gel digestion were subjected to the MALDI-TOF/MS. The step of alkylation was skipped to avoid artificial oxidation of methionine in the procedure. All of the reagents used in the gel treatment were supplemented with DTT or 2-ME to keep the protein in a reductive condition. It has been confirmed that those reducing reagents do not affect the level of methionine sulphoxide in the protein in our preliminary experiment. The MS signals indicate that the level of methionine sulphoxide in calmodulin is elevated in the mouse hippocampus at the late stage of ageing.

gated at m/z = 2230.15, peptide of UCH-L1 and nm23-M1 peptide gated at m/z = 1802.08 (data not shown).

Subsequently, the MS signals of corresponding protein spots on 2-D gels prepared from mouse hippocampus at 6 and 24 months after birth were compared to analyse the increase in the level of methionine sulphoxide in those down-regulated proteins. The results of the MS analysis indicated that the level of methionine oxidation in calmodulin, nm23-M1 and UCH-L1 increased in aged mouse hippocampus (Figure 4). Such increase in methionine oxidation was not detected in other protein spots on the 2-D gels examined.

Discussion

Decline in memory function and behavioural dysfunction accompanies normal ageing in mammals. Accumulation of oxidative damages during the natural process of brain ageing has been suggested to be a major cause of age-related onset of neurodegenerative disorders by many groups (Smith et al 1991; Berlett and Stadtman 1997; Ramassamy et al 1999; Villeponteau et al 2000; Giasson et al 2002). However, the physiological significance of protein oxidation in normal brain ageing still remains unclear. In this paper, we intended to take a new approach to determine the significance of protein oxidation in ageing of mouse hippocampus by using the methods of proteomics. Initially, we looked for the target proteins demonstrating age-related variations in relative abundance on 2-D gel protein maps of mouse hippocampus. The result of the comparative 2-D gel image analysis indicated a decrease in the abundance of calmodulin, UCH-L1 and nm23-M1 in the mouse hippocampus, as well as an increase in HSP60, HSP70 and HSC71 at the late stage of ageing. Subsequently, we found the increased methionine sulphoxide in calmodulin, UCH-L1 and nm23-M1. As the loss of conformational stability in oxidised calmodulin has been already reported (Gao et al 1998), the increase of methionine oxidation in calmodulin might be a cause of down-regulation of the protein in aged mouse hippocampus. Although the methionine oxidation is reversible since methionine sulphoxide reductase (MsrA) can remove the oxidised form of proteins (Sun et al 1999), we speculate that the activity of MsrA is not sufficient to repair all of the oxidised methionine in aged mouse hippocampus. In fact, Gabbita et al reported a decrease in the activity of MsrA in Alzheimer's disease brain (Gabbita et al 1999).

Castegna et al have reported UCH-L1 as a target of protein oxidation in human AD brain (Castegna et al 2002). The correlation of protein oxidation and age-dependent alteration in calcium homeostasis has already been discussed (Squier and Bigelow 2000). Recently, the oxidation of methionine residues at 144 and 145 in calmodulin was reported as a regulatory factor of calmodulin dependent activation of plasma membrane Ca-ATPase (Bartlett et al 2003). The results of our proteomic analysis suggest that the methionine oxidation in calmodulin, UCH-L1 and nm23-M1 might be involved in the age-related deterioration of hippocampus function through abnormal calcium signalling, impaired recycling of ubiquitin and decline in GTP-related signal transduction in aged mouse hippocampus.

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