Free Radical Biology & Medicine 51 (2011) 1–4

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Methods in Free Radical Biology and Medicine

A method for detection of 4-hydroxy-2-nonenal adducts in proteins

Chika Wakita [a], Kazuya Honda [a], Takahiro Shibata [a], Mitsugu Akagawa [b], Koji Uchida [a,*]

[a] Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464–8601, Japan
[b] Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai 599–8531, Japan

ARTICLE INFO

Article history:
Received 3 January 2011
Revised 27 January 2011
Accepted 14 February 2011
Available online 30 March 2011

Keywords:
4-Hydroxy-2-nonenal
Pyridylamination
Reductive amination
Free radicals

ABSTRACT

We developed a procedure to measure 4-hydroxy-2-nonenal (HNE)–amino acid adducts using the fluorescent probe 2-aminopyridine (2-AP). The method is based on the fact that HNE forms Michael addition-type amino acid adducts possessing an aldehyde functionality, which upon reaction with 2-AP in the presence of $NaBH_3CN$ can be converted to their pyridylaminated derivatives. The HNE–amino acid adducts, namely Michael addition-type HNE–cysteine, HNE–histidine, and HNE–lysine adducts, after pyridylamination were resistant to conventional acid-hydrolysis conditions for protein (6 N HCl/110 °C/24 h) and could be detected by HPLC with a fluorescence detector. The reductive amination-based fluorescent labeling of HNE adducts is a simple and accurate technique that may be widely used to reveal increased levels of covalently modified proteins with HNE and its related aldehydes during aging and disease.

Several lines of evidence indicate that oxidative modification of proteins and the subsequent accumulation of the modified proteins have been found in cells during aging, under oxidative stress, and in various pathological states including premature diseases, muscular dystrophy, rheumatoid arthritis, and atherosclerosis [1,2]. The important agents that give rise to the modification of a protein may be represented by reactive aldehydic intermediates, such as ketoaldehydes, 2-alkenals, and 4-hydroxy-2-alkenals [3]. These reactive aldehydes are considered important mediators of cell damage because of their ability to covalently modify biomolecules, which can disrupt important cellular functions and can cause mutations [4,5]. Furthermore, the adduction of aldehydes to apolipoprotein B in low-density lipoproteins (LDLs)[1] has been strongly implicated in the mechanism by which LDL is converted to an atherogenic form that is taken up by macrophages, leading to the formation of foam cells [6,7]. 4-Hydroxy-2-nonenal (HNE), among the reactive aldehydes, is a major product of lipid peroxidation and is believed to be largely responsible for the cytopathological effects observed during oxidative stress [4,5]. HNE exerts these effects because of its facile reactivity with biological materials, including proteins [4,5]. The protein-bound HNE has been shown to serve as one of the most useful biomarkers for the occurrence of oxidative stress/lipid peroxidation [5]. Upon reaction with protein, HNE specifically reacts with nucleophilic amino acids, such as cysteine, histidine, and lysine, to form their Michael addition adducts possessing a carbonyl functionality.

Among a wide variety of protein modifications, introduction of carbonyl groups into amino acid residues is a hallmark of oxidative damage to proteins [8,9]. Carbonyl groups are introduced into proteins by a variety of modification pathways in vivo and in vitro, particularly metal-catalyzed oxidation of specific amino acid residues [10–12] and also adduction of lipid peroxidation-derived aldehydes [5,13–16] or carbohydrates (i.e., the Maillard reaction or glycation) [17]. Carbonyl derivatives can be measured by convenient methods using 2,4-dinitrophenylhydrazine, which reacts with carbonyl groups to generate dinitrophenylhydrazones with characteristic absorbance maxima at 360–390 nm [18]. Using these methods, it has been confirmed that carbonyl derivatives accumulate on tissue proteins during aging and disease development such as Alzheimer disease [19,20], rheumatoid arthritis [21], amyotrophic lateral sclerosis [22], diabetes mellitus [23], and Parkinson disease [20]. Nevertheless, the methods are unfortunately limited to measuring total carbonyl derivatives formed by various nonspecific pathways, and information on chemical structures and formation mechanisms is barely provided. Therefore, more specific methods for the determination of carbonyl derivatives are required to understand the chemical nature, oxidation pathway, and distribution level in vivo.

Principles

To analyze HNE–amino acid adducts in proteins, we adapted a reductive amination-based pyridylamination using 2-aminopyridine (2-AP) and sodium cyanoborohydride ($NaBH_3CN$; Fig. 1). This method was originally developed for detection of reducing sugars [24]. Our strategy for the fluorescent labeling of protein-bound HNE is illustrated in Fig. 2. The method is based on the fact that HNE forms Michael addition adducts possessing an aldehyde functionality, which

Abbreviations: 2-AP, 2-aminopyridine; BSA, bovine serum albumin; HNE, 4-hydroxy-2-nonenal; LDL, low-density lipoprotein.

* Corresponding author. Fax: +81 52 789 5741.
E-mail address: uchidak@agr.nagoya-u.ac.jp (K. Uchida).

0891-5849/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2011.02.037

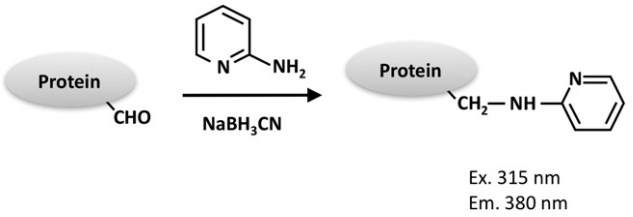

Fig. 1. Pyridylamination of protein-bound aldehydes.

upon reaction with 2-AP in the presence of $NaBH_3CN$ can be converted to the pyridylaminated derivatives. They are supposed to be very stable because of their resonance, which lowers the potential energy of the substance and thus makes it more stable than any of the contributing structures. Thus, it was anticipated that the HNE–amino acid adducts after pyridylamination must be resistant against acid-hydrolysis conditions (6 N HCl/110 °C/24 h).

Materials

Sodium cyanoborohydride (Cat. No. 15,615-9), N^{α}-acetylcysteine (Cat. No. 7250), and N^{α}-acetyllysine (Cat. No. A2010) were obtained from Sigma–Aldrich (St. Louis, MO, USA). N^{α}-acetylhistidine (Cat. No. 100092) was obtained from MP Biomedicals. Sequence grade modified trypsin (Cat. No. V5111) was obtained from Promega (Madison, WI, USA). 2-Aminopyridine (Cat. No. 011–14181), bovine serum albumin (BSA; Cat. No. 017–15141), dithiothreitol (Cat. No. 045–08974), and iodoacetoamide (Cat. No. 093–02152) were obtained from Wako Pure Chemical Industries (Osaka, Japan). The stock solutions of HNE were prepared by the acid treatment (1 mM HCl) of 4-hydroxy-2-nonenal dimethylacetal, which was synthesized according to the procedure of De Montarby et al. [25].

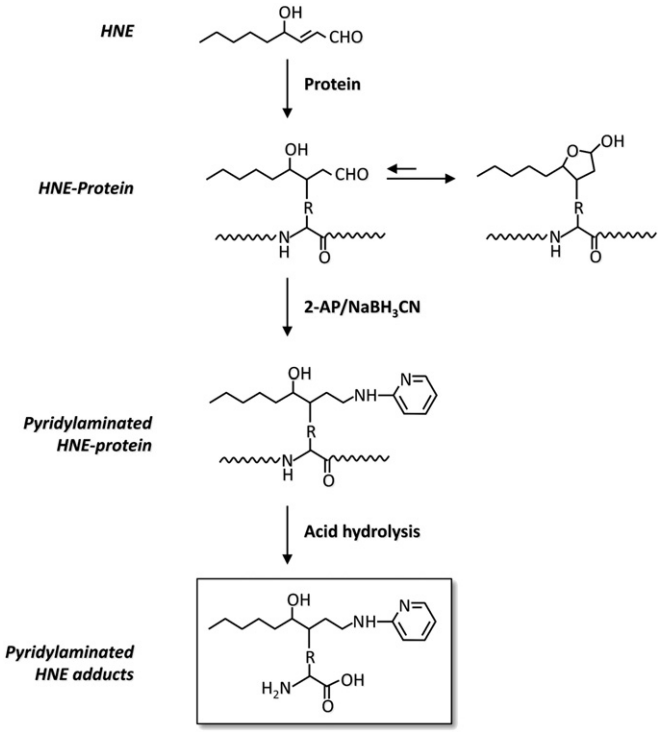

Fig. 2. Strategy for the fluorescent labeling of HNE–amino acid adducts in proteins.

Instrumentation

The quantitative analyses were performed using a Jasco HPLC system (Jasco, Tokyo, Japan) that consisted of a PU2080 pump and an FP-1520S fluorescence detector.

Protocol

Preparation of authentic HNE adducts

The reaction mixture (10 ml) containing 20 mM N^{α}-acetyl derivatives of cysteine, histidine, or lysine was incubated with 20 mM HNE in 50 mM sodium phosphate buffer (pH 7.2). After incubation for 24 h at 37 °C, the Michael addition-type HNE adducts were purified by reversed-phase HPLC on a Develosil ODS-HG-5 column (4.6 × 250 mm i.d.; Nomura Chemicals, Aichi, Japan): HNE–N^{α}-acetylhistidine and HNE–N^{α}-acetylcysteine were eluted with 25% acetonitrile in 0.1% trifluoroacetic acid, and HNE–N^{α}-acetyllysine was eluted with 20% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 2.5 ml/min. The elution profiles were monitored by absorbance at 220 nm. The products eluted between the starting materials (N^{α}-acetyl amino acids and HNE) are considered to be the HNE adducts, which can be entirely collected and used as the authentic HNE Michael adducts.

Note. Because of the introduction of new chiral centers at C-2, C-4, and C-5, the Michael addition-type HNE adducts composed of at least eight configurational isomers may give multiple peaks on the HPLC analysis [26,27]. The HNE–lysine Michael adduct is predominantly produced in the presence of a large excess of lysine over HNE. However, the non-Michael adducts, such as the pyrrole adduct [28], Schiff base Michael adduct 1:2 cross-links [29], and 3-hydroxy-3-imino-1,2-dihydropyrrole derivative [30,31], could also be generated. Therefore, before the isolation by HPLC, we recommend the use of LC–MS to identify the Michael adduct.

Pyridylamination of authentic HNE adducts

The authentic HNE adducts (1 mM) were evaporated to dryness in vacuo and treated with 5 μl of 450 mM 2-AP dissolved in 0.42 N HCl and 2 μl of 315 mM $NaBH_3CN$, and the mixture was allowed to react at 37 °C for 24 h in the dark. The pyridylaminated HNE–*N*-acetylcysteine adducts were hydrolyzed in vacuo with 6 N HCl for 24 h at 110 °C. The hydrolysate was evaporated to dryness at room temperature in vacuo followed by reconstitution in 200 μl of distilled water. The hydrolysates were then analyzed by reverse-phase HPLC.

Pyridylamination of HNE adducts in protein samples

The protein samples were dialyzed at 4 °C using a microdialysis cassette (e.g., Slide-A-Lyzer; Pierce, Rockford, IL, USA) or an ultrafiltration device (e.g., Microcon YM; Millipore, Bedford, MA, USA). The dialysis was continued with at least three changes in phosphate-buffered saline containing 1 mM diethylenetriaminepentaacetic acid. After the protein concentration was measured using a commercial kit, 180 μl of the protein solution was transferred to a vial. Ten microliters of 6.8 M 2-AP dissolved in 6 N HCl and 10 μl of 315 mM $NaBH_3CN$ were added to the protein sample and the mixture was allowed to react for 24 h at 37 °C with shaking in the dark. After the reaction, the protein was precipitated by the addition of 200 μl of cold 20% (wt/vol) trichloroacetic acid (TCA). After standing for 30 min in an ice bath, the mixture was centrifuged at 17,000 *g* for 15 min at 4 °C, and the pellet of the precipitated protein was separated. The pellet was washed twice with 0.5 ml of cold 20% (wt/vol) TCA and 0.5 ml of cold acetone and then centrifuged as before. After the pellet was allowed to air dry, the resulting protein was hydrolyzed for 24 h at 110 °C with 1.5 ml of 6 M HCl. The hydrolysate was evaporated to dryness at room temperature in

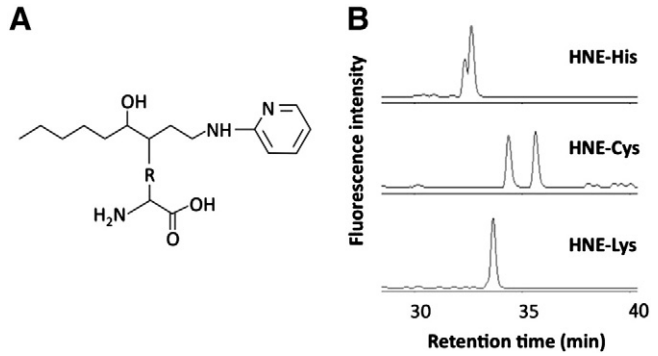

Fig. 3. HPLC analysis of the pyridylaminated HNE adducts. (A) The structure of pyridylaminated HNE adducts. R, cysteine, histidine, or lysine residue. (B) Detection of the pyridylaminated HNE adducts. The authentic HNE adducts of *N*-acetylhistidine, *N*-acetylcysteine, and *N*-acetyllysine were pyridylaminated with 2-AP/$NaBH_3CN$ followed by the acid hydrolysis. The hydrolysates were then analyzed by reverse-phase HPLC.

vacuo followed by reconstitution in 200 μl of distilled water containing 0.1% TFA. The hydrolysates were then analyzed by reverse-phase HPLC.

HPLC analysis of pyridylaminated HNE adducts

The hydrolysates of the pyridylaminated samples were analyzed by reverse-phase HPLC on a Sunniest C18 column (4.6×250 mm i.d.; ChromaNik, Japan). The samples were eluted with a gradient of water containing 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing 0.1% trifluoroacetic acid (solvent B) (0–40 min at 95–60% A, 40–45 min at 60–0% A), at a flow rate of 0.8 ml/min. The elution profiles were monitored by absorbance at 230 nm and by fluorescence intensity (ex. 315 nm, em. 380 nm). The pyridylaminated HNE adducts were identified by comparing the elution times to those of the authentic samples. The next sample was injected when the baseline was restabilized.

Calculations and expected results

Various methods for determination of lipid peroxidation-derived products have been developed. A colorimetric assay using 2-thiobarbituric acid for detection of malondialdehyde is probably the easiest way of evaluating lipid peroxidation. A highly sensitive and selective liquid chromatography assay has also been recently utilized to accurately estimate the biological levels of malondialdehyde in UV-treated human plasma [32]. An alternative and probably more popular approach for the detection of lipid peroxidation products in biological samples is the use of antibodies. Antibodies are usually raised by immunizing animals with carrier proteins, such as keyhole limpet hemocyanin, that had been treated with lipid peroxidation products and target specific antigenic structures (lipid peroxidation-specific epitopes) generated on the amino acid side chains of proteins. Taking advantage of the fact that the lipid peroxidation-specific epitopes are excellent immunogens that are capable of stimulating an adaptive immune response, a number of monoclonal antibodies against these epitopes have been developed. Because excellent antibodies directed against the HNE–histidine epitopes are commercially available, Western blot, dot blot, immunocytochemical, and ELISA techniques are accessible.

On the other hand, because of the lack of specific and reliable methods for the determination of HNE adducts, very few studies have so far quantitatively demonstrated their formation in proteins. To quantitatively analyze HNE adducts generated in proteins, we adapted a reductive amination-based approach. The treatment of the authentic HNE adducts with 2-AP/$NaBH_3CN$ followed by acid hydrolysis gave the fluorescent products corresponding to their pyridylaminated derivatives (Fig. 3). Under the HPLC conditions used, three of the pyridylaminated HNE adducts, namely HNE–cysteine, HNE–histidine, and HNE–lysine, were separately detected. Multiplicity of the pyridylaminated HNE–cysteine and HNE–histidine adducts is due to the presence of their closely related forms (configurational isomers). Stoichiometry between the concentrations of HNE–N^{α}-acetylhistidine adducts used for the derivatization and the increase in peak area of the products showed a linear correlation (data not shown). Using this pyridylamination method, we have previously detected the HNE–cysteine adducts in sulfhydryl proteins, such as thioredoxin, exposed to HNE [28]. To further evaluate the formation of the HNE amino acid adducts, six proteins, including BSA, enolase, lysozyme, GAPDH, angiotensin, and lactoglobulin, were incubated with 1 mM HNE for 24 h at 37 °C and the HNE adducts were analyzed by the pyridylamination method. Of interest, in all the proteins tested, the HNE–histidine adducts were detected as the major products (Fig. 4A). The yield of the HNE–histidine adducts in the HNE-modified BSA excelled all the others (Fig. 4B). The concentration of the HNE–histidine adducts reached about eight molecules per protein (BSA) molecule, which accounted for about 50% of the histidine residues that had disappeared. The reactivity of BSA toward HNE, generating the highest yield of HNE–histidine adducts, may be due to its binding property with an extraordinarily diverse range of hydrophobic compounds. Moreover, when we analyzed the HNE adducts in Cu^{2+}-oxidized LDL, the HNE–histidine adduct was also detected as the major product (Fig. 5), whereas other HNE-derived Michael adducts, including HNE–lysine and HNE–cysteine adducts, and 2-alkenal-derived Michael adducts were barely detected in the oxidized LDL. The reductive amination method

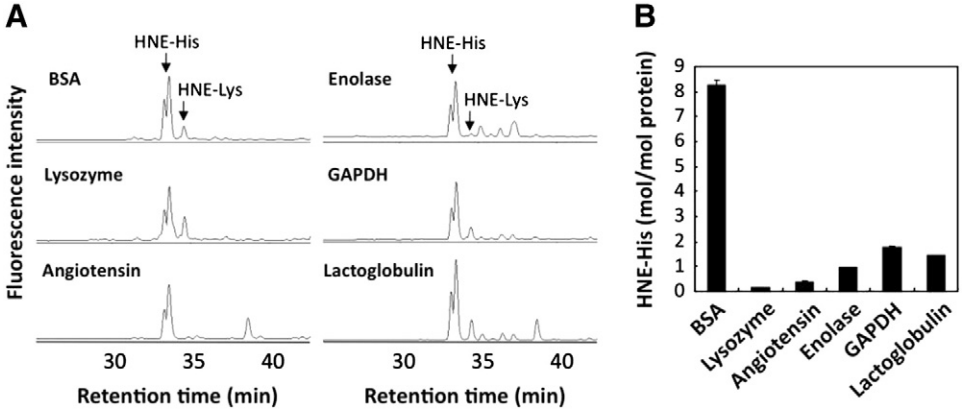

Fig. 4. Detection of HNE adducts in proteins. The proteins were incubated with 1 mM HNE for 24 h at 37 °C and the HNE adducts were analyzed by the pyridylamination method. (A) HPLC analysis of HNE adducts in proteins treated with HNE. (B) Quantification of HNE–histidine adducts in proteins treated with HNE.

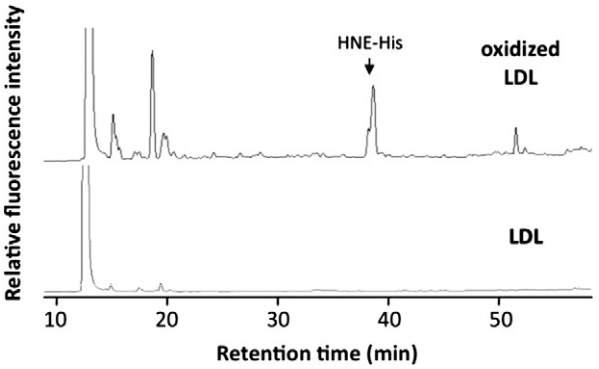

Fig. 5. Detection of HNE–histidine adducts in oxidized LDL. LDLs incubated with or without $CuSO_4$ (5 uM) for 24 h at 37 °C were analyzed by the pyridylamination method.

could also be utilized for detection of HNE adducts in biological samples, for which combination with liquid chromatography–mass spectrometry may be essential. On the other hand, the reductive amination-based fluorescence labeling method can be used for gel-based analysis of protein carbonyls. Our preliminary experiments have shown that the reductive amination of HNE-modified proteins with an appropriate fluorescent probe resulted in the appearance of labeled protein bands in SDS–PAGE (C. Wakita and K. Uchida, unpublished data). Thus, this reductive amination-based method may form the basis for the detection and identification of target proteins possessing an aldehydic functionality by two-dimensional gel electrophoresis-based proteomics.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas (Research in a Proposed Research Area), Japan (K.U.).

References

[1] Stadtman, E. R.; Levine, R. L. Protein oxidation. *Ann. N.Y. Acad. Sci.* **899:**191–208; 2000.
[2] Shacter, E. Quantification and significance of protein oxidation in biological samples. *Drug Metab. Rev.* **32:**307–326; 2000.
[3] Uchida, K. Role of reactive aldehyde in cardiovascular diseases. *Free Radic. Biol. Med.* **28:**1685–1696; 2000.
[4] Esterbauer, H.; Schaur, R. J.; Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. Med.* **11:** 81–128; 1991.
[5] Uchida, K. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Prog. Lipid Res.* **42:**318–343; 2003.
[6] Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Low-density lipoprotein and atherogenesis. *N. Engl. J. Med.* **320:**915–924; 1989.
[7] Steinberg, D. Role of oxidized LDL and antioxidants in atherosclerosis. *Adv. Exp. Med. Biol.* **369:**39–48; 1995.
[8] Dalle-Donne, I.; Rossi, R.; Giustarini, D.; Milzani, A.; Colombo, R. Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta* **329:**23–38; 2003.
[9] Nyström, T. Role of oxidative carbonylation in protein quality control and senescence. *EMBO J.* **24:**1311–1317; 2005.
[10] Levine, R. L. Carbonyl modified proteins in cellular regulation, aging and disease. *Free Radic. Biol. Med.* **32:**790–796; 2002.
[11] Stadtman, E. R. Protein oxidation and aging. *Science* **257:**1220–1224; 1992.
[12] Stadtman, E. R. Protein oxidation in aging and age-related diseases. *Ann. N.Y. Acad. Sci.* **928:**22–38; 2001.
[13] Uchida, K.; Szweda, L. I.; Chae, H. Z.; Stadtman, E. R. Immunochemical detection of 4-hydroxy-2-nonenal modified proteins in oxidized hepatocytes. *Proc. Natl. Acad. Sci. U. S. A.* **90:**8742–8746; 1993.
[14] Furuhata, A.; Nakamura, M.; Osawa, T.; Uchida, K. Thiolation of protein-bound carcinogenic aldehyde: an electrophilic acrolein–lysine adduct that covalently binds to thiols. *J. Biol. Chem.* **277:**27919–27926; 2002.
[15] Ichihashi, K.; Osawa, T.; Toyokuni, S.; Uchida, K. Endogenous formation of protein adducts with carcinogenic aldehydes: implication for oxidative stress. *J. Biol. Chem.* **276:**23903–23913; 2001.
[16] Yamada, S.; Kumazawa, S.; Ishii, T.; Nakayama, T.; Itakura, K.; Shibata, N.; Kobayashi, M.; Sakai, K.; Osawa, T.; Uchida, K. Immunochemical detection of a lipofuscin-like fluorophore derived from malondialdehyde and lysine. *J. Lipid Res.* **42:**1187–1196; 2001.
[17] Liggins, J.; Furth, A. J. Role of protein-bound carbonyl groups in the formation of advanced glycation endproducts. *Biochim. Biophys. Acta* **1361:**123–130; 1997.
[18] Levine, R. L.; Williams, J. A.; Stadtman, E. R.; Shacter, E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* **233:**346–357; 1994.
[19] Choi, J.; Malakowsky, C. A.; Talent, J. M.; Conrad, C. C.; Gracy, R. W. Identification of oxidized plasma proteins in Alzheimer's disease. *Biochem. Biophys. Res. Commun.* **293:**1566–1570; 2002.
[20] Choi, J.; Rees, H. D.; Weintraub, S. T.; Levey, A. I.; Chin, L. S.; Li, L. Oxidative modifications and aggregation of Cu, Zn-superoxide dismutase associated with Alzheimer and Parkinson diseases. *J. Biol. Chem.* **280:**11648–11655; 2005.
[21] Mantle, D.; Falkous, G.; Walker, D. Quantification of protease activities in synovial fluid from rheumatoid and osteoarthritis cases: comparison with antioxidant and free radical damage markers. *Clin. Chim. Acta* **284:**45–58; 1999.
[22] Bowling, A. C.; Schulz, J. B.; Brown Jr., R. H.; Beal, M. F. Superoxide dismutase activity, oxidative damage, and mitochondrial energy metabolism in familial and sporadic amyotrophic lateral sclerosis. *J. Neurochem.* **61:**2322–2325; 1993.
[23] Grattagliano, I.; Vendemiale, G.; Boscia, F.; Micelli-Ferrari, T.; Cardia, L.; Altomare, E. Oxidative retinal products and ocular damages in diabetic patients. *Free Radic. Biol. Med.* **25:**369–372; 1998.
[24] Hase, S.; Ikenaka, T.; Matsushima, Y. Structure analyses of oligosaccharides by tagging of the reducing end sugars with a fluorescent compound. *Biochem. Biophys. Res. Commun.* **85:**257–263; 1978.
[25] De Montarby, L.; Mosset, P.; Gree, R. Sorbic acid iron tricarbonyl complex as resolving agent: chiral syntheses of 4-hydroxy nonenal and cariolic acid. *Tetrahedron Lett.* **29:**3895; 1988.
[26] Hashimoto, M.; Shibata, T.; Wasada, H.; Toyokuni, S.; Uchida, K. Structural basis of protein-bound endogenous aldehydes—chemical and immunochemical characterizations of configurational isomers of a 4-hydroxy-2-nonenal-histidine adduct. *J. Biol. Chem.* **278:**5044–5051; 2003.
[27] Wakita, C.; Maeshima, T.; Yamazaki, A.; Shibata, T.; Ito, S.; Akagawa, M.; Ojika, M.; Yodoi, J.; Uchida, K. Stereochemical configuration of 4-hydroxy-2-nonenal-cysteine adducts and their stereoselective formation in a redox-regulated protein. *J. Biol. Chem.* **284:**28810–28822; 2009.
[28] Sayre, L. M.; Arora, P. K.; Iyer, R. S.; Salomon, R. G. Pyrrole formation from 4-hydroxynonenal and primary amines. *Chem. Res. Toxicol.* **6:**19–22; 1993.
[29] Nadkarni, D. V.; Sayre, L. M. Structural definition of early lysine and histidine adduction chemistry of 4-hydroxynonenal. *Chem. Res. Toxicol.* **8:**284–291; 1995.
[30] Itakura, K.; Osawa, T.; Uchida, K. Structure of a fluorescent compound formed from 4-hydroxy-2-nonenal and Na-hippuryllysine: a model for fluorophores derived from protein modifications by lipid peroxidation. *J. Org. Chem.* **63:** 185–187; 1998.
[31] Xu, G.; Sayre, L. M. Structural characterization of a 4-hydroxy-2-alkenal-derived fluorophore that contributes to lipoperoxidation-dependent protein cross-linking in aging and degenerative disease. *Chem. Res. Toxicol.* **11:**247–251; 1998.
[32] Breusing, N.; Grune, T.; Andrisic, L.; Atalay, M.; Bartosz, G.; Biasi, F.; Borovic, S.; Bravo, L.; Casals, I.; Casillas, R.; Dinischiotu, A.; Drzewinska, J.; Faber, H.; Fauzi, N. M.; Gajewska, A.; Gambini, J.; Gradinaru, D.; Kokkola, T.; Lojek, A.; Luczaj, W.; Margina, D.; Mascia, C.; Mateos, R.; Meinitzer, A.; Mitjavila, M. T.; Mrakovcic, L.; Munteanu, M. C.; Podborska, M.; Poli, G.; Sicinska, P.; Skrzydlewska, E.; Vina, J.; Wiswedel, I.; Zarkovic, N.; Zelzer, S.; Spickett, C. M. An inter-laboratory validation of methods of lipid peroxidation measurement in UVA-treated human plasma samples. *Free Radic. Res.* **44:**1203–1215; 2010.