Noncovalent Interaction Between Amyloid-β-Peptide (1-40) and Oleuropein Studied by Electrospray Ionization Mass Spectrometry

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Beta amyloid peptide (Aβ) is the major proteinaceous component of senile plaques formed in Alzheimer's disease (AD) brain. The aggregation of Aβ is associated with neurodegeneration, loss of cognitive ability, and premature death. It has been suggested that oxidative stress and generation of free radical species have implications in the fibrillation of Aβ and its subsequent neurotoxicity. For this reason, it is proposed that antioxidants may offer a protective or therapeutic alternative against amyloidosis. This study is the first report of the formation of the noncovalent complex between Aβ or its oxidized form and the natural derived antioxidant oleuropein (OE) by electrospray ionization mass spectrometry (ESI MS). ESI MS allowed the real time monitoring of the complex formation between Aβ, OE, and variants thereof. Several experimental conditions, such as elevated orifice potential, low pH values, presence of organic modifier, and ligand concentration were examined, to assess the specificity and the stability of the formed noncovalent complexes. (J Am Soc Mass Spectrom 2006, 17, 568–575) © 2006 American Society for Mass Spectrometry

Alzheimer's disease (AD) is the most common form of senile dementia, which affects a great part of the world's population, with no cure yet known. Even though the cause of AD remains unknown, the formation of the amyloid β peptide containing plaques and the tau (τ)-protein containing neurofibrillary tangles are the main hypotheses that have prevailed [1]. Human AD neuritic plaques consist mainly of deposited β amyloid peptide (Aβ) [2], which derives from the proteolytic cleavage of the amyloid precursor protein (APP). Spontaneous conformational changes of Aβ lead to the formation of toxic protofibrillar intermediate species, rich in β-sheet, that convert to mature fibrils and eventually plaques [3]. The formation of the aforementioned fibrils is initiated by a multitude of risk factors, with oxidative stress and generation of free radicals being the main mechanisms [4]. Reactive oxygen species (ROS) can cause protein and lipid oxidation, which leads to loss of ion homeostasis and cell death, and are related to the aggregation and neurotoxicity of Aβ [5]. Even if ROS are a consequence rather than a cause of Aβ aggregation [6], the fact that oxidative stress can result in neurodegeneration and cell death [7] suggests that free radical scavengers [8] may be beneficial as a therapeutic and/or preventive approach against AD. In light of the suggested link between oxidative stress, inflammatory response [9] and AD, anti-inflammatory agents [10] have been employed as therapeutic approaches against AD, because Aβ has been shown to stimulate macrophages and microglia. Other therapeutic alternatives include inhibition of amyloid formation by controlling APP proteolytic cleavage [11, 12], control of Aβ degradation [13], immunization with Aβ [14, 15], and inhibition of Aβ aggregation or disaggregation of Aβ deposits. The last involves binding of small molecules [16, 17] to Aβ, which stabilize its structure, thus becoming potential inhibitors of amyloidosis. Approaches like this are being studied as therapeutic alternatives for disorders like AD [18], Creutzfeldt Jakob disease [19], and diabetes type II [20]. It is noteworthy that antioxidants, such as melatonin, have shown to form noncovalent complexes with Aβ [21] and exhibit anti-amyloidogenic properties.

Plants containing antioxidants and phytoestrogens, and more specifically substances with phenolic struc-

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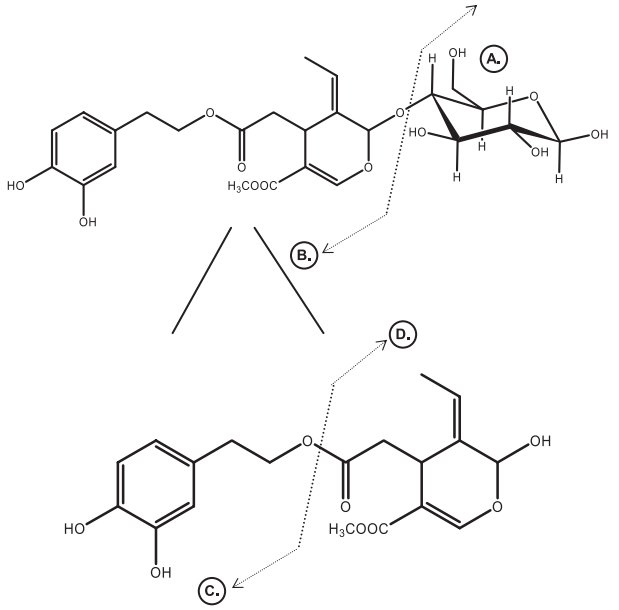

Figure 1. Molecular structure of oleuropein (OE). The molecule is a glucoside consisting of a glucose unit (**A**) and a moiety called oleuropein aglycon (**B**). The latter consists of two chemical subunits, the polyphenolic part hydroxytyrosol (**C**), and the lactone part elenolic acid (**D**).

tures, such as α-tocopherol (vitamin E) and 17β-estradiol, have been involved in the management of cognitive disorders such as AD [22]. In vitro [23] and epidemiological [24] studies pointed out the positive impact of natural extracted polyphenols on the incidence of age-related disorders, such as dementia. In this study, oleuropein (OE) (Figure 1), a bioactive polyphenol extracted from the fruits and leaves of *Olea Europea* L., was evaluated towards the decrease or even prevention of Aβ aggregation, which is inherent to AD. OE has been shown to possess a broad range of biochemical and beneficial pharmacological properties, such as antioxidant [25], anti-inflammatory [26], antiatherogenic [27], antibacterial [28], and anticancer [29]. The potential effect of OE on the brain function may be attributable, at least in part, to its significant cardioprotective effects. It has been reported that AD is analogous to atherosclerosis, because they both are age-dependent diseases in which abnormal accumulation of a normal metabolite (cholesterol and Aβ, respectively) precedes clinical symptoms and leads to disease [30, 31]. The link between heart disease, hypercholesterolemia, and AD [32] may involve similar mechanisms in the pathogenesis of these disorders. The circumstantial evidence that cholesterol-related interventions can alter Aβ deposition [33, 34], suggest that OE could be promising in the management of AD. Furthermore, the importance of inflammatory processes in the clinical manifestation of AD [9, 35], combined with the epidemiological evidence of a protective effect of anti-inflammatory agents [36] against AD, suggest that a polyphenolic natural extract, such as OE, could prove effective against age-dependent disorders such as AD. Furthermore, it has been reported that OE and its metabolites could be potent bioactive compounds against contemporary diseases, such as HIV [37], osteoporosis [38], and AD [39].

The aim of the present study was the establishment of the noncovalent interaction between Aβ (1-40) and OE by means of electrospray ionization (ESI) mass spectrometry. Mass spectrometry (MS) is a powerful tool for analyzing the primary structure of biomolecules and especially for monitoring their interactions [40]. In general, noncovalent interactions between proteins/peptides and ligands may be involved in triggering diseases. Thus, the elucidation of the structure and the formation mechanism of noncovalent complexes between proteins and ligands may lead to a better understanding of a disease process and the development of a therapeutic approach [41]. This is the first report of detecting noncovalent complexes between Aβ and naturally occurring compounds by ESI MS. The effect of OE concentration, solution pH and organic modifier content on the stability and specificity of the noncovalent interaction, as well as the possible effect of time on the conformation and aggregation of Aβ, in the presence or absence of OE, is also discussed.

Experimental

Sample Preparation

A 150 μL aliquot of a freshly prepared Aβ (1-40) solution 100 μM (M_r 4329.9; Bachem AG, Bubendorf, Switzerland) in deionized water was added into 150 μL of an equimolar OE solution, (M_r 540.5) in 1 mM ammonium acetate- (Merck, Darmstad, Germany) acetic acid 0.5%. OE was isolated from the leaves of *Olea europea* var. koroneiki, according to a previously described procedure [42] with greater than 99% purity, as it was assessed by NMR analysis. All solvents used were of analytical grade purity.

The prepared solutions were directly infused after 1 min vortexing, or analyzed after incubation at room temperature for 1 h, 3.5 h, 3 days, 5 days, 9 days, and 25 days. In another aliquot of the Aβ (1-40) solution an equal volume of the acetate buffer was added, whereas an equal volume of deionized water was added in an aliquot of the OE solution in acetate buffer and analyzed at each time interval. The stability of Aβ and OE under the experimental conditions was also checked. Post addition of OE in equimolar ratio to already aged Aβ solutions was also performed.

Mass Spectrometry

A Sciex API-III triple quadrupole instrument equipped with a standard atmospheric pressure ionization source (Sciex, Concord, ON, Canada) was used in the positive ion mode. Sample solution was directly infused at a flow rate of 5 μL/min using a Harvard syringe pump. A standard PPG solution was used for tuning and mass

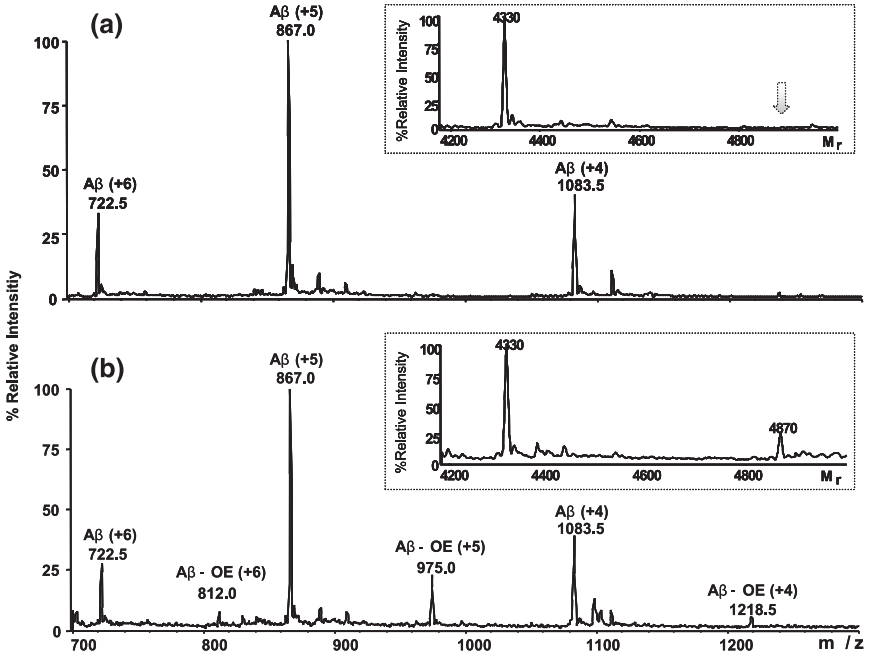

Figure 2. ESI mass spectra of (**a**) amyloid-β peptide (1-40) at a concentration level of 100 μM and (**b**) its mixture with oleuropein (1:1 molecular ratio, 100 μM), acquired by a triple quadrupole mass analyzer. The deconvoluted spectra of the Aβ peptide and the Aβ-OE noncovalent complex with measured M_r values 4330.0 and 4870.0, respectively, are presented in the respective insets.

calibration. The scanning mass range of the mass spectrometer was 300–1500 Da at a rate of 5000 Da/min at a step size of 0.5 Da. The acquired mass spectra were the average of 20–40 scans. Needle potential was set at 4000 V and the OR potential at 35 V. The curtain and nebulizer gas flow rates were 1.0 and 0.4–0.8 L/min, respectively.

Results and Discussion

ESI MS is a gentle ionization method, which allows the preservation of the fragile noncovalent interactions in the gas-phase provided careful optimization of the instrumental parameters. Therefore, ESI MS analysis represents an ideal approach to provide the M_r of the noncovalent complexes formed in solution, as well as the stoichiometry of the interacting species.

ESI-MS Analysis of Aβ and Aβ-OE

ESI mass spectrometric analysis of the Aβ (1-40) solution (Figure 2a) revealed a multiply charged ion envelope including signals at *m/z* 722.5 (+6), 867.0 (+5), 1083.5 (+4), and 1444.0 (+3). An average mass (M_r) of 4330.0 is derived after deconvolution of the raw ESI MS data (Figure 2a, inset), which correlates well with the theoretical value of 4329.9. ESI MS analysis of the Aβ solution containing OE generated a spectrum including the aforementioned Aβ envelope and a "bell-shaped" distribution of multiply charged ions at *m/z* 812.0, 975.0, and 1218.5 (Figure 2b), which corresponded to the +6, +5, and +4 charge states of the 1:1 Aβ:OE noncovalent complex, respectively. Deconvolution of the observed ion envelope gave rise to an M_r of 4870.0, which is in good agreement with the theoretical average mass of 4870.4 for the 1:1 Aβ:OE noncovalent complex (Figure 2b, inset). Thus, the electrospray ionization process and the experimental conditions maintained the existing interactions in solution, thereby showing that OE forms noncovalent complexes with Aβ with 1:1 stoichiometry.

Parameter Optimization for the Aβ-OE Noncovalent Complex Monitoring

The binding strength of the Aβ-OE noncovalent complex was tested under various critical conditions for its formation and maintenance. The stability of the complex, as well as nonspecific aggregation, can be assessed by calculating the ratio of the complex ion envelope intensity plots versus that of the peptide ion envelope in the spectrum (Figure 3). It is known that conformational changes of the peptide and denaturation of proteins occur in the presence of organic modifiers [43] or at low pH values. It is also known that nonspecific aggregation may occur at higher concentrations of peptide/ligand. Therefore, it was important to confirm that the Aβ-OE complex was the result of specific interactions. The stability and specificity of the Aβ-OE complex was assessed by varying the OR potential, the pH of the sample solution, the percentage of an organic modifier in the spraying solution [44] (Figure 3), and the OE concentration.

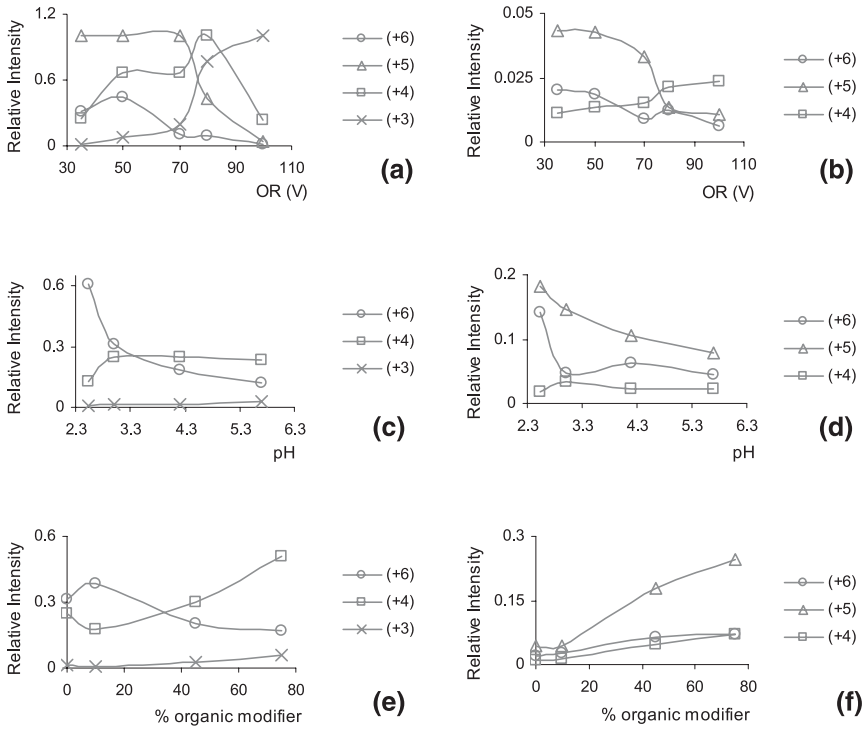

Figure 3. Relative signal intensity dissociation curves for Aβ (1-40) peptide (**a**, **c**, and **e**) and its complex with oleuropein (**b**, **d**, and **f**) in equimolar ratio, as a function of orifice potential (**a** and **b**), pH (**c** and **d**) and % of organic modifier in the sample (**e** and **f**). Each trend line corresponds to the different charge states of the Aβ or the Aβ-OE ion envelope. Relative intensity was calculated as the ratio of the signal intensity of each multiply charged ion versus the signal intensity of the most abundant peak in the spectrum, i.e., Aβ (+5) serving as internal standard. This peak shifted to lower charged states with increase in the OR potential.

Orifice Potential Effect on Aβ-OE Interaction

The collisional excitation effect of the ion-sampling interface may be incorporated to qualitatively study the binding strength of the peptide-ligand noncovalent complex. Elevating the OR potential, increases the internal energy of the noncovalent complex, which in turn affects its conformation and consequently can result in the dissociation of the complex. The energy required for breaking these complexes apart depends on the binding energy of the noncovalent interaction and may serve as an indication for the gas-phase complex stability and furthermore, as an indication for the solution phase noncovalent interactions.

The binding strength of the Aβ-OE noncovalent interaction was studied at OR potential settings ranging from 35 to 100 V (35, 50, 70, 80, and 100 V) (Figure 3a and b). In the ESI mass spectrum of the Aβ-OE solution, an increase in the OR potential shifted the *Aβ* peptide "bell-shaped" envelope to higher *m/z* values due to charge stripping. From 35 up to 70 V, the central charge state of the envelope was +5, while it shifted to +4 at 80 V and to +3 at 100 V (Figure 3a). In the ESI mass spectrum of Aβ-OE, an increase in the OR potential from 35 to 50 V resulted in a slight decrease of the signals corresponding to Aβ and Aβ-OE, whereas the Aβ-OE signals were still present at OR potential values >80 V. The multiply charged ion envelope of the Aβ-OE complex shifted to higher *m/z* values at 80 V and it was centered around the +4 charge state (Figures 3b and 4). At 100 V, the +4 charge state of the Aβ-OE complex could still be detected. The ratio of the [complexed:free] peptide slightly decreased with increasing OR potential values, i.e., from 0.23 (35 V) to 0.17 (80 V), thus indicating the strong stability of the complex (Figure 4).

Solution pH Effect on Aβ-OE Interaction

It is well established that the ESI process in the positive ion mode is more effective when using samples with low pH values because of a more efficient protonation and formation of multiply charged species. On the other hand, extreme basic or acidic pH values of a solution may denature a protein from its native conformation, which in turn may disrupt noncovalent interactions. Protein unfolding induced by low pH values can be revealed in mass spectra because of extensive protonation of the basic amino acids of a protein (e.g., Lys, Arg, His), which are easily accessed in the "open" conformation [44].

The stability of the Aβ-OE noncovalent complex was studied as a function of the sample solution pH (Figure 3c and d). The solvent pH was adjusted from 5.7 to 4.2,

3.0, and 2.5 by adding glacial acetic acid. Increase of the pH shifted the Aβ ion envelope to higher *m/z* values, since fewer protons are available for the protonation of the peptide (Figure 3c). On the other hand, lower pH values shifted the Aβ-OE ion envelope to lower *m/z* values (Figure 3d), due to a more extensive protonation. The ratio of the [complexed:free] peptide changed from 0.29 (pH 5.7) to 0.09 (pH 2.5) (Figure 5), which indicates substantial unfolding of the peptide and subsequent release of OE from its binding site at lower pH values.

Organic Solvent Percentage Effect on Aβ-OE Interaction

To efficiently generate gas-phase ions during the ESI process, an organic co-solvent is usually added to enhance evaporation of the charged droplets. The presence of an organic solvent, like methanol or acetonitrile, and the medium pH values needed for protonation enhance the ESI sensitivity and the ion signal stability. However, the addition of organic solvents may disrupt the tertiary structure of a protein or peptide and result in a more extended conformation, where a higher number of basic amino acids are available for protonation, as it was the case in low pH values. It should be mentioned that to maintain noncovalent interactions, the choice of solution conditions should be conserved as close to the protein's native-like solution environment as possible.

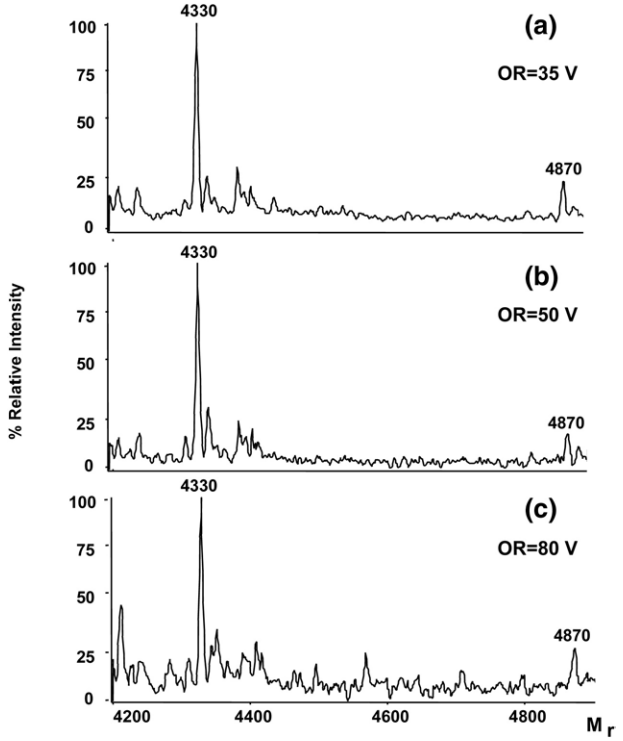

Figure 4. Deconvoluted ESI mass spectra of amyloid-β peptide (1-40) in a mixture with oleuropein in 1:1 molecular ratio (concentration level 100 μM) analyzed under different orifice potential values: (**a**) 35 V, (**b**) 50 V, and (**c**) 80 V. The slight decrease of the [complexed:free] peptide ratio (0.23, 0.19, and 0.17, respectively) with increasing orifice potential values indicates the high binding energy of the Aβ-OE noncovalent complex.

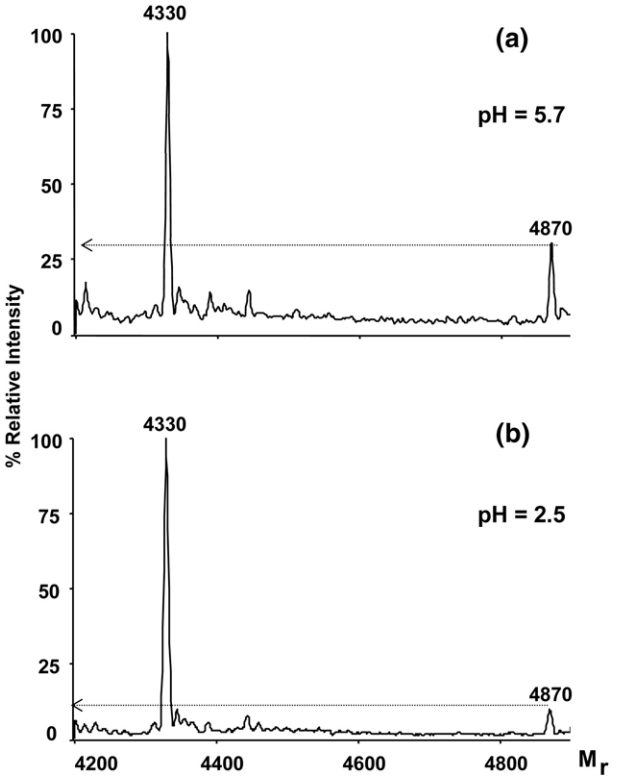

Figure 5. Deconvoluted electrospray ionization mass spectra of amyloid-β peptide (1-40) in a 1:1 mixture with oleuropein (concentration level 100 μM) at two different sample pH values: (**a**) 5.7 and (**b**) 2.5. The respective ratio of the [complexed:free] peptide changes from 0.29 to 0.09, due to substantial unfolding of the peptide and subsequent release of oleuropein from its binding site at lower pH values.

The effect of the organic solvent content on the preservation of the Aβ-OE noncovalent complex has been studied by preparing samples containing 0, 10, 45, and 75% MeOH (Figure 3e and f). The ratio of the [complexed:free] peptide changed from 0.25 (10% MeOH) to 0.40 (45% MeOH) (Figure 6). It was found that addition of 45% MeOH was optimum for observing higher abundance of the complex-derived signals, apparently due to better ESI ionization (Figures 3f and 6). Nevertheless, the Aβ-OE noncovalent complex is efficiently conserved even at extreme MeOH content values, i.e., 75%, even though the S/N of the ESI signals decreases and new peaks appear in the mass spectrum because of partial hydrolysis of the peptide (Figure 6c).

Solute Concentration Effect on Aβ-OE Interaction

Nonspecific complexes may be formed as artifacts of the ESI process. The prevalence of nonspecific aggregation in the gas-phase may be avoided by increasing the internal energy of the complex (e.g., by elevating the OR potential), or by reducing the solution concentration of the interacting species. The specificity of the Aβ-OE

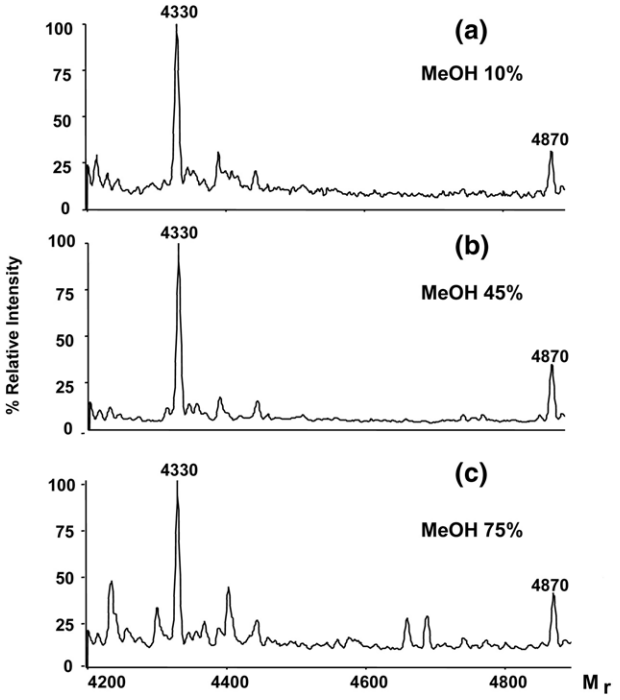

Figure 6. Deconvoluted electrospray ionization mass spectra of amyloid-β peptide (1-40) in a mixture with oleuropein (1:1 molecular ratio, concentration level 100 μM) in samples containing: (**a**) 10%, (**b**) 45%, and (**c**) 75% MeOH as organic co-solvent. The abundance of the Aβ-OE noncovalent complex decreases at high percentages of organic modifier as a result of peptide denaturation. However, the Aβ-OE complex detection, even when the MeOH content increases up to 75%, indicates the specificity and the stability of the formed noncovalent complex.

noncovalent interaction was evaluated at low concentration levels ranging from 5 to 100 μM (5, 10, 50, 100 μM). The Aβ:OE molecular ratio used was 1:1 at all concentration levels and consistent results were obtained, thus proving the specificity of the interaction. Molecular excess of 10:1 in OE:Aβ provided no difference in the stoichiometry of the complex, thus indicating a very specific interaction.

The presented data support that OE forms noncovalent complexes with Aβ (1-40). The stoichiometry of the complex (1:1) is not pH, organic solvent, or analyte concentration dependent. The optimum values of pH, OR potential, and % of organic modifier for the detection of the Aβ-OE noncovalent complex were found to be 4.2, 35 V, and 45%, respectively.

Time Effect on Aβ-OE Interaction

The spontaneous aggregational propensity of Aβ, as well as its complex formation ability with OE, was studied as a function of time. In this way, rapid equilibrium was studied, as well as possible structural modifications of the Aβ-OE complex and the effect of OE on Aβ aggregation.

The ESI mass spectra of the Aβ-OE samples included satellite signals at higher *m/z* values of the +4, +5, and +6 ion signals of the Aβ peptide, and were detected even when the analyzed samples had been incubated for some hours. These signals were consistent with the oxidation of Aβ at the methionine 35 (Met35) residue, and they became more pronounced with time, as shown in the deconvoluted spectrum of the 9- and 25-day old samples (Figure 7a and 7b, Table 1). Deconvolution of these ion signals gave rise to an M_r of 4346.0, which agrees well with the theoretical mass of 4345.9 for the Met35(O)-containing Aβ peptide ($Aβ_{ox}$). In addition, ESI signals corresponding to the noncovalent complex of OE with the oxidized form of Aβ ($Aβ_{ox}$) were also detected. The theoretical M_r of the 1:1 $Aβ_{ox}$-OE noncovalent complex (4886.4, Table 1) agrees well with the corresponding value derived from the deconvoluted spectrum of $Aβ_{ox}$-OE (Figure 7). The ion signals corresponding to the oxidized complex become more prominent with time, thus indicating an increase in the formation of the $Aβ_{ox}$ component. The ESI mass spectrum of the aged Aβ sample is significantly different than that of the Aβ:OE sample. In the mass spectrum of the 25 day-old Aβ sample the [Aβ:$Aβ_{ox}$] ratio is 2:1, while this ratio in the 25 day-old Aβ:OE sample is 1:1. This indicates that the presence of OE renders the Met35 residue more susceptible to oxidation in aged samples. This may be significant in terms of its possible role in attenuating the Aβ oligomerization, as it has been previously shown by Palmblad et al. [45]. The high proportion of Met sulfoxide present in the senile plaques [46] can be explained by the fact that the single Met35 residue of Aβ is critical to a free radical process of Aβ toxicity to neurons [6]. Met participates in unusual free radical reaction chemistry [47], and its oxidation leads to conformational switching [48] and adaptation of the β-sheet conformation, which is responsible for the Aβ toxicity [49]. However, $Aβ_{ox}$ was found to interact noncovalently with OE as well. Therefore, a detailed study of the topology of the binding is currently under way by mapping analysis of the detected Aβ-OE noncovalent complex.

It should be noted that no mass spectral signals attributed to oligomers, besides dimers, have been observed for samples incubated for 25 days. This is possibly due to Met35 oxidation, or occupation of a big surface of the peptide by OE, which blocks the oligomerization more efficiently. However, it has been reported that the aggregated forms of Aβ become weaker and ultimately absent in aged solutions as a result of a conformational shift from random coil to the more compact β-sheet conformation [50].

Post-Addition of OE in Aged Aβ Solution

In two aliquots of the 3-day old Aβ solution an equal volume of an equimolar OE solution and an equal volume of mobile phase was added, respectively, the latter being used as a control sample at this concentration level (0.05 mM). The samples were incubated for 2, 6, and 21 days at room temperature before analysis. In this way, conclusions could be derived regarding the inhibitory properties of OE on further fibrillation of Aβ,

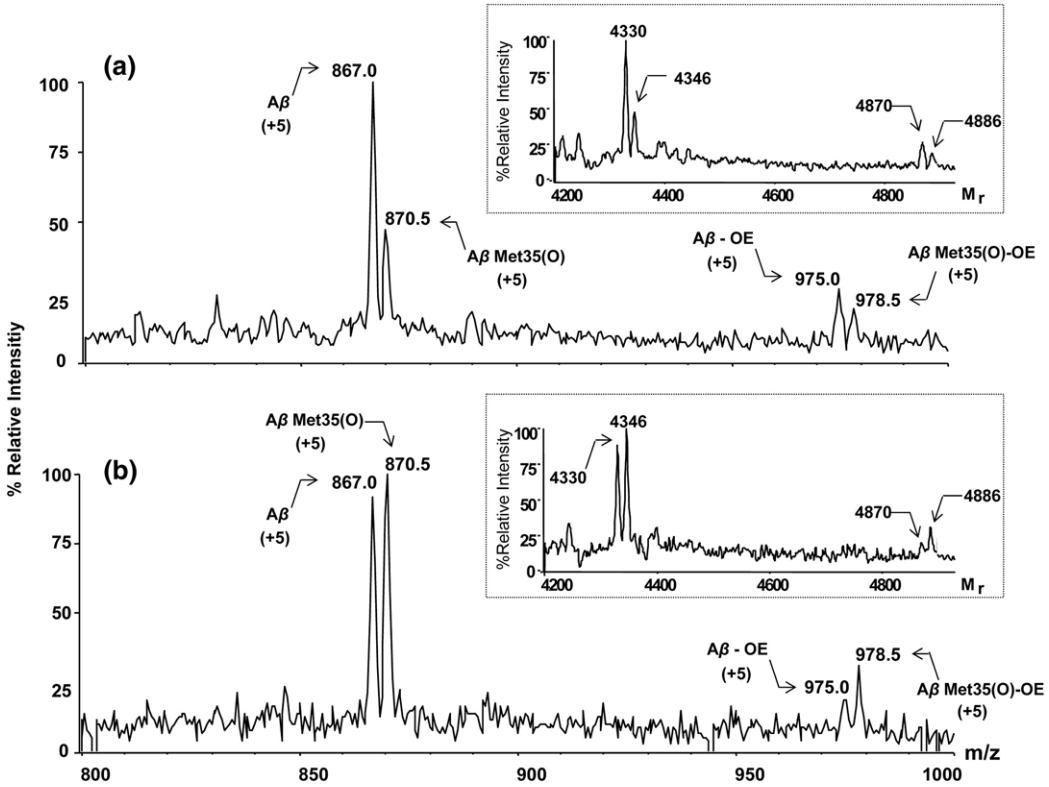

Figure 7. Electrospray ionization mass spectrum of amyloid-β peptide (1-40) in a mixture with oleuropein (1:1 molecular ratio, concentration level of 100 μM) acquired by a triple quadrupole mass analyzer after incubation at room temperature for (**a**) 9 days and (**b**) 25 days. The deconvoluted spectra in the insets depict the presence of Aβ (1-40) and its oxidized form Aβ_{ox}, with measured M_r values 4330.0 and 4346.0, respectively, as well as their respective noncovalent complexes with oleuropein, with measured M_r values 4870.0 and 4886.0, respectively.

its possible disaggregational properties, or its ability to form complexes not only with the monomers, but also with oligomers or higher order polymers. This could be attributed to different complex formation kinetics when the addition of the analyte is performed directly in freshly prepared Aβ solution, than when performed in Aβ solutions already containing some aggregates. Nevertheless, clear conclusions could not be drawn regarding the effect of OE on the aggregation of Aβ because no mass spectral signals attributed to oligomers, besides dimers, have been detected during the post-addition study.

Table 1. Theoretical and observed average mass values of the Aβ (1–40) peptide, its oxidized form Aβ (1–40) $_{ox}$ and their respective complexes with oleuropein

	Average mass (M_r)	
Species	Theoretical	Observed
Aβ (1–40)	4329.9	4330.0
Aβ (1–40)$_{ox}$	4345.9	4346.0
Aβ (1–40)-OE	4870.4	4870.0
Aβ (1–40)$_{ox}$-OE	4886.4	4886.0

Conclusions

This study demonstrates the utility of ESI MS in this area of neuroscience research. The successful detection of the noncovalent complex between Aβ and OE could be invaluable in a series of studies focused on screening the ability of several bioactive phytochemicals in terms of complexating Aβ and Aβ_{ox} and "locking" them in a non-toxic conformation, thus acting as potential anti-amyloidogenic agents. This may offer an ideal protective alternative against Aβ toxicity.

In conclusion, this ESI MS study shows that the stoichiometry of the noncovalent complex between Aβ (1-40) and OE is neither pH nor organic modifier sensitive. Even though the primary constituent of amyloid plaques is Aβ (1-42) peptide, Aβ (1-40) possesses a high degree of homology to Aβ (1-42) and is co-localized with it in the neuritic plaque . The Met35(O)-containing Aβ (1-40) variant (Aβ_{ox}) was also found to interact noncovalently with OE at 1:1 stoichiometry. The preservation of the Aβ-OE complex even at extreme conditions (i.e., high percentage of organic co-solvent or high OR values) is indicative of the high binding energy of the Aβ-OE interaction. The very high sensitivity of ESI MS-based methodologies enables the

recognition of possible biomarkers useful in the diagnosis of this disease and may shed some light into the mechanisms involved in AD pathology. Furthermore, the presented ESI MS methodology enabling the direct determination and real-time monitoring of all species in equilibrium could provide insights into structural analogs of OE with improved properties that may act as potential anti-amyloidogenic agents.

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References

1. Selkoe, D. J. Alzheimer's Disease: Genes, Proteins, and Therapy. *Physiol. Rev.* **2001,** *81*(2), 741–766.
2. Selkoe, D. J. Alzheimer's Disease: Genotypes, Phenotypes, and Treatments. *Science* **1997,** *275,* 630–631.
3. Koo, E. H.; Lansbury, P. T., Jr.; Kelly, J. W. Amyloid Diseases: Abnormal Protein Aggregation in Neurodegeneration. *Proc. Natl. Acad. Sci. U.S.A.* **1999,** *96,* 9989–9990.
4. Markesbery, W. R. Oxidative Stress Hypothesis in Alzheimer's Disease. *Free Radical Biol. Med.* **1997,** *23,* 134–147.
5. Glippingdale, A. B.; Wade, J. D.; Barrow, C. J. The Amyloid-β-Peptide and Its Role in Alzheimer's Disease. *J. Peptide Sci.* **2001,** *7,* 227–249.
6. Varadarajan, S.; Yatin, S.; Aksenova, M.; Butterfield, A. D. Alzheimer's Amyloid β-Peptide-Associated Free Radical Oxidative Stress and Neurotoxicity. *J. Struct. Biol.* **2000,** *130,* 184–208.
7. Behl, C. Alzheimer's Disease and Oxidative Stress: Implications for Novel Therapeutic Approaches. *Prog. Neurobiol.* **1999,** *57,* 301–323.
8. Grundman, M.; Grundman M.; Delaney, P. Antioxidant Strategies for Alzheimer's Disease. *Proc. Nutr. Soc.* **2002,** *61,* 191–202.
9. Markesbery, W. R.; Carney, J. M. Oxidative Alterations in Alzheimer's Disease. *Brain Pathol.* **1999,** *9,* 133–146.
10. McGeer, P. L.; Schulzer, M.; McGeer, E. G. Arthritis and Anti-Inflammatory Agents as Possible Protective Factors for Alzheimer's Disease: A Review of 17 Epidemiologic Studies. *Neurology* **1996,** *47,* 425–432.
11. Walter, J.; Haass, C. Secretases as Targets for β-Amyloid Lowering Drugs. *Drug Dev. Res.* **2002,** *56,* 201–210.
12. Walsh, D. M.; Klyubin, I.; Fadeeva, J. V.; Rowan, M. J.; Selkoe, D. J. Amyloid-β Oligomers: Their Production, Toxicity, and Therapeutic Inhibition. *Biochem. Soc. Trans.* **2002,** *30,* 552–557.
13. Chesneau, V.; Verkellis, K.; Rosner, M. R.; Selkoe, D. J. Purified Recombinant Insulin-Degrading Enzyme Degrades Amyloid β-Protein but Does Not Promote Its Oligomerization. *Biochem. J.* **2000,** *351,* 509–516.
14. Lahiri, D. K.; Farlow, M. R.; Greig, N. H.; Sambamurti, K. Current Drug Targets for Alzheimer's Disease Treatment. *Drug. Dev. Res.* **2002,** *56,* 267–281.
15. Selkoe, D. J. Deciphering the Genesis and Fate of Amyloid β-Protein Yields Novel Therapies for Alzheimer Disease. *J. Clin. Invest.* **2002,** *110,* 1375–1381.
16. Lansbury, P. T., Jr. Back to the Future: The "Old-Fashioned" Way to New Medications for Neurodegeneration. *Nat. Rev. Neurosci.* **2004,** *5,* S51–S57.
17. Lansbury, P. T., Jr. Following Nature's Anti-Amyloid Strategy. *Nat. Biotechnol.* **2001,** *19,* 112–113.
18. Rishton, G. M.; Retz, D. M.; Tempest, P. A.; Novotny, J.; Kahn, S.; Treanor, J. J. S.; Lile, J. D.; Citron, M. Fenchylamine Sulfonamide Inhibitors of Amyloid β Peptide Production by the γ-Secretase Proteolytic Pathway: Potential Small-Molecule Therapeutic Agents for the Treatment of Alzheimer's Disease. *J. Med. Chem.* **2000,** *43,* 2297–2299.
19. Kuner, P.; Bohrmann, B.; Tjernberg, L. O.; Näslund, J.; Huber, G.; Celenk, S.; Grüninger-Leitch, F.; Richards, J. G.; Jakob-Rötne, R.; Kemp, J. A.; Nordstedt, C. Controlling Polymerization of β-Amyloid and Prion-Derived Peptides with Synthetic Small Molecule Ligands. *J. Biol. Chem.* **2000,** *275,* 1673–1678.
20. Douglas, D. J.; Collings, B.A.; Numao, S.; Nesatyy, V. J. Detection of Noncovalent Complex Between α-Amylase and Its Microbial Inhibitor Tendamistat by Electrospray Ionization Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2001,** *15,* 89–96.
21. Bazoti, F. N.; Tsarbopoulos, A.; Markides, K. E.; Bergquist, J. Study of the Noncovalent Interaction Between Amyloid-β-Peptide and Melatonin Using Electrospray Ionization Mass Spectrometry. *J. Mass Spectrom.* **2005,** *40,* 182–192.
22. Howes, M. J. R.; Perry, N. S. L.; Houghton, P. Plants with Traditional Uses and Activities Relevant to the Management of Alzheimer's Disease and Other Cognitive Disorders. *J. Phytother. Res.* **2003,** *17,* 1–18.
23. Moosmann, B.; Behl, C. The Antioxidant Neuroprotective Effects of Estrogens and Phenolic Compounds are Independent from their Estrogenic Properties. *Proc. Natl. Acad. Sci. U.S.A.* **1999,** *96,* 8867–8872.
24. German, J. B.; Walzem, R. L. The Health Benefits of Wine. *Annu. Rev. Nutr.* **2000,** *20,* 561–593.
25. Visioli, F.; Poli, A.; Galli, C. Antioxidant and Other Biological Activities of Phenols from Olives and Olive Oil. *Med. Res. Rev.* **2002,** *22,* 65–75.
26. Visioli, F.; Bellosta, S.; Galli, C. Oleuropein, the Bitter Principle of Olives, Enhances Nitric Oxide Production by Mouse Macrophages. *Life. Sci.* **1998,** *62,* 541–546.
27. Carluccio, M. A.; Siculella, L.; Ancora, M. A.; Massaro, M.; Scoditti, E.; Storelli, C.; Visioli, F.; Distante, A.; De Caterina, R. Olive Oil and Red Wine Antioxidant Polyphenols Inhibit Endothelial Activation: Antiatherogenic Properties of Mediterranean Diet Phytochemicals. *Arterioscler. Thromb. Vasc. Biol.* **2003,** *23,* 622–629.
28. Fleming, H. P.; Walter, W. M., Jr.; Etchells, J. L. Antimicrobial Properties of Oleuropein and Products of Its Hydrolysis from Green Olives. *Appl. Microbiol.* **1973,** *26,* 777–782.
29. Owen, R. W.; Giacosa, A.; Hull, W. E.; Haubner, R.; Würtele, G.; Spiegelhalder, B.; Bartsch, H. Olive Oil Consumption and Health: The Possible Role of Antioxidants. *Lancet Oncol.* **2000,** *1,* 107–112.
30. Golde, T. E.; Eckman, C. B. Cholesterol Modulation as an Emerging Strategy for the Treatment of Alzheimer's Disease. *Drug Discov. Today* **2001,** *6,* 1049–1055.
31. Hofman, A.; Ott, A.; Breteler, M. M. B.; Bots, M. L.; Slooter, A. J.; van Harskamp, F.; van Duijn, C. N.; Van Broeckhoven, C.; Grobbee, D. E. Atherosclerosis, Apolipoprotein E, and Prevalence of Dementia and Alzheimer's Disease in the Rotterdam Study. *Lancet* **1997,** *349,* 151–154.
32. Refolo, L. M.; Malester, B.; LaFrancois, J.; Bryant-Thomas, T.; Wang, R.; Tint, G. S.; Sambamurti, K.; Duff, K. E.; Pappolla M. A. Hypercholesterolemia Accelerates the Alzheimer's Amyloid Pathology in a Transgenic Mouse Model. *Neurobiol. Dis.* **2000,** *7,* 321–331.
33. Refolo, L. M.; Pappolla, M. A.; LaFrancois, J.; Malester, B.; Schmidt, S. D.; Thomas-Bryant, T.; Tint, G. S.; Wang, R.; Mercken, M.; Petanceska, S. S.; Duff, K. E. A Cholesterol-Lowering Drug Reduces β-Amyloid Pathology in a Transgenic Mouse Model of Alzheimer's Disease. *Neurobiol. Dis.* **2001,** *8,* 890–899.
34. Jick, H.; Zornberg, G. L.; Jick, S. S.; Seshadri, S.; Drachman, D. A. Statins and the Risk of Dementia. *Lancet* **2000,** *356,* 1627–1631.
35. Heininger, K. A Unifying Hypothesis of Alzheimer's Disease. II. Pathophysiological Processes. *Hum. Psychopharmacol. Clin. Exp.* **1999,** *14,* 525–581.
36. Stewart, W. F.; Kawas, C.; Corrada, M.; Metter, E. J. Risk of Alzheimer's Disease and Duration of NSAID Use. *Neurology* **1997,** *48,* 626–632.
37. Lee-Huang, S.; Zhang, L.; Lin Huang, P.; Chang, Y. T.; Huang, P. L. Anti-HIV Activity of Olive Leaf Extract (OLE) and Modulation of Host Cell Gene Expression by HIV-1 Infection and OLE Treatment. *Biochem. Biophys. Res. Commun.* **2003,** *307,* 1029–1037.
38. Puel, C.; Quintin, A.; Agalias, A.; Mathey, J.; Obled, C.; Mazur, A.; Davicco, M. J.; Lebecque, P.; Skaltsounis, A. L.; Coxam, V. Olive Oil and Its Main Phenolic Micronutrient (Oleuropein) Prevent Inflammation-Induced Bone Loss in the Ovariectomized Rat. *Br. J. Nutr.* **2004,** *92,* 119–127.
39. Hashimoto, T.; Ibi, M.; Matsuno, K.; Nakashima, S.; Tanigawa, T.; Yoshikawa, T.; Yabe-Nishimura, C. An Endogenous Metabolite of Dopamine, 3,4-Dihydroxyphenylethanol, Acts as a Unique Cytoprotective Agent Against Oxidative Stress-Induced Injury. *Free Rad. Biol. Med.* **2004,** *36,* 555–564.
40. Loo, J. A. Electrospray Ionization Mass Spectrometry: A Technology for Studying Noncovalent Macromolecular Complexes. *Int. J. Mass Spectrom.* **2000,** *200,* 175–186.
41. Pramanik, B. N.; Ganguly, A. K.; Gross, M. L. Applied Electrospray Mass Spectrometry; Marcel Dekker: New York, 2002; p 361
42. Shasha, B.; Leibowityz, J. On the Oleuropein, the Bitter Principle of Olives. *J. Org. Chem.* **1961,** *26,* 1948–1954.
43. Shen, C. L.; Murphy, R. M. Solvent Effects on Self-Assembly of β-Amyloid Peptide. *Biophys. J.* **1995,** *69,* 640–651.
44. Ganguly, A. K.; Pramanik, B. N.; Huang, E.; Tsarbopoulos, A.; Girijavallabhan, V. M.; Liberles, S. Studies of the *Ras*-GDP and *Ras*-GTP Noncovalent Interactions. *Tetrahedron* **1993,** *49*(36), 7985–7996.
45. Palmblad, M.; Westlind-Danielsson, A.; Bergquist, J. Oxidation of Methionine 35 Attenuates Formation of Amyloid β-Peptide (1-40) oligomers. *J. Biol. Chem.* **2002,** *277,* 19506–19510.
46. Naslund, J.; Schierhorn, A.; Hellman, U.; Lanfelt, L.; Roses, A. D.; Tjernberg, L. O.; Siberring, J.; Gandy, S. E.; Winblad, B.; Greengard, P.; Nordstedt, C.; Terenius, L. Relative Abundance of Alzheimer's Aβ Amyloid Peptide Variants in Alzheimer Disease and Normal Aging. *Proc. Natl. Acad. Sci. U.S.A.* **1994,** *91,* 8378–8382.
47. Schoneich, C.; Zhao, F.; Madden, K. P.; Bobrowski, K. Side Chain Fragmentation of N-Terminal Threonine or Serine Residue Induced Through Intramolecular Proton Transfer to Hydroxy Sulfuranyl Radical Formed at Neighboring Methionine in Dipeptides. *J. Am. Chem. Soc.* **1994,** *116,* 4641–4652.
48. Dado, G. P.; Gellman, S. H. Redox Control of Secondary Structure in a Designed Peptide. *J. Am. Chem. Soc.* **1994,** *115,* 12609–12610.
49. Selkoe, D. J. Alzheimer's Disease: A Central Role for Amyloid. *J. Neuropathol. Exp. Neurol.* **1994,** *53,* 438–447.
50. Chen, X. G.; Brining, S. K.; Nguyen, V. Q.; Yergey, A. L. Simultaneous Assessment of Conformation and Aggregation of β-Amyloid Peptide Using Electrospray Ionization Mass Spectrometry. *FASEB J.* **1997,** *11,* 817–823.