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Dimerisation of *N*-acetyl-L-tyrosine ethyl ester and Aβ peptides via formation of dityrosine

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Abstract
Alzheimer's disease (AD) is characterised by the formation of amyloid deposits composed primarily of the amyloid β-peptide (Aβ). This peptide has been shown to bind redox active metals ions such as copper and iron, leading to the production of reactive oxygen species (ROS) and formation of hydrogen peroxide (H_2O_2). The generation of H_2O_2 has been linked with Aβ neurotoxicity and neurodegeneration in AD. Because of the relative stability of a tyrosyl radical, the tyrosine residue (Tyr-10) is believed to be critical to the neurotoxicity of Aβ. This residue has also been shown to be important to Aβ aggregation and amyloid formation. It is possible that the formation of an Aβ tyrosyl radical leads to increased aggregation via the formation of dityrosine as an early aggregation step, which is supported by the identification of dityrosine in amyloid plaque. The role of dityrosine formation in Aβ aggregation and neurotoxicity is as yet undetermined, partly because there are no facile methods for the synthesis of Aβ dimers containing dityrosine. Here we report the use of horseradish peroxidase and H_2O_2 to dimerise *N*-acetyl-L-tyrosine ethyl ester and apply the optimised conditions for dityrosine formation to fully unprotected Aβ peptides. We also report a simple fluorescent plate reader method for monitoring Aβ dimerisation via dityrosine formation.

Keywords: *Alzheimer's disease, amyloid β peptide, horseradish peroxidase, metal catalyzed oxidation, oxidative stress, dityrosine*

Abbreviations: *ATEE, N-acetyl tyrosyl ethyl ester; AD, Alzheimer's disease; Aβ, Amyloid β peptide; APP, β amyloid precursor protein; Boc, t-Butyloxycarbonyl; DOPA, 3, 4-dihydroxyphenylalanine; ECL, enhanced chemiluminescence; ESI-MS, Electrospray-ionization mass spectrometry; HFIP, hexafluoroisopropanol; MCA, multichannel analysis mode; MCO, Metal catalysed oxidation; NFT, intracellular neurofibrilar tangles; OH, hydroxyl radical; PBS, Phosphate buffered saline; ROS, Reactive oxygen species; RP-HPLC, Reversed phase-high performance liquid chromatography; SDS, sodium dodecyl sulphate; TR, Tyrosyl radical*

Introduction

There is growing support for the involvement of free radicals in Aβ associated neurotoxicity in Alzheimer's disease (AD) [1]. Recent work has identified specific amino acids in the Aβ sequence that are involved in free radical generation, either via an ability to bind metals such as Cu^{2+}, or through their ability to stabilise or transfer an electron [2]. For example, the (His)XXX(Tyr)XX(His) motif in the Aβ peptide sequence (Figure 1) appears to be crucial for oxidative damage to the β amyloid precursor protein (APP) in the initial stages of free-radical-based pathogenesis in AD[3]. The sole tyrosine residue in the Aβ sequence is within this motif (Tyr 10) and is in close proximity to the Cu^{2+} binding site containing the histidines residues [3,4]. Tyr 10 has been identified as an

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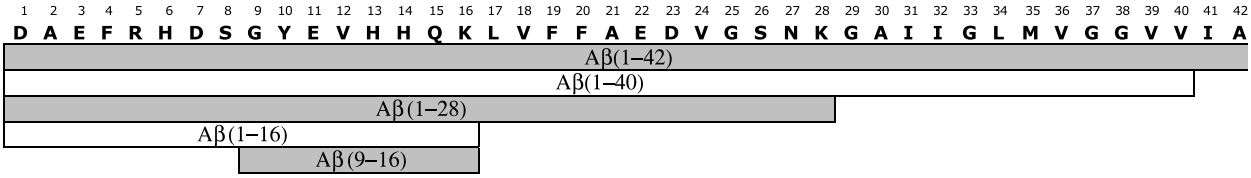


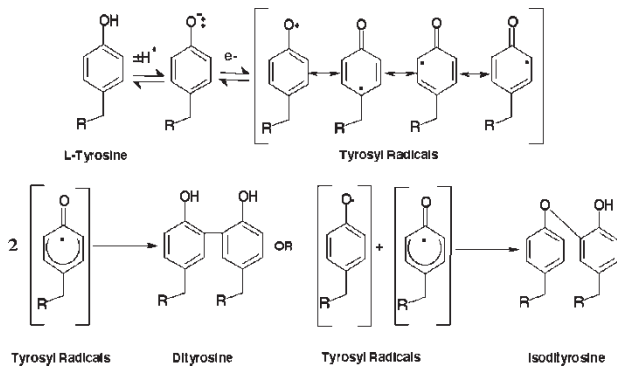
Figure 1. Amino acid sequence of Aβ peptides. Both tyrosine-10 and methionine-35 play a role in ROS formation and histidines 6, 13 and 14 are the metal binding sites.

important amino acid in the aggregation and amyloidogenicity of Aβ [5,6].

The importance of Tyr 10 in Aβ amyloid formation and associated neurotoxicity may be partly due to the ability of Tyr 10 to form a stable radical during metal catalysed oxidation (MCO), since the proximity of Tyr 10 to the neighbouring histidines residues allows a redox active metal ion such as Cu^{2+} to extract one electron to form a tyrosine radical [7–9]. The most stable structures resulting from tyrosine radical coupling include formation of protein-bound dityro sine (C_{ortho}–C_{ortho} coupling) and isodityrosine (C_{ortho}–O coupling) [10,11]. The principles of this dimerisation are shown in Scheme 1.

Dityrosine bridges in peptides and proteins are formed by many ubiquitous processes in biological systems leading to protein degradation and cellular damage [12], including ultraviolet irradiation of calmodulin [13–15], peroxidation of membrane lipids [16], the inflammatory response by the action of some peroxidase enzyme systems like myeloperoxidase (MPO) and Hydrogen Peroxide (H_2O_2) [17–25] or by polymorphonuclear leukocytes [26], and by the action of an enzymatic alternative to peroxidase inside the mitochondrion [27,28]. Dityrosine has also been chemically identified in tissues associated with some diseases including AD [29,30]. There are few studies that attempt to relate the oxidation of tyrosine to senile plaque formation in AD [9,31–32], although there is some data indicating that Aβ dityrosine formation occurs during amyloid formation and may be important in Aβ associated neurotoxicity and associated neurodegeneration [29,33]. The formation of Aβ dimers through the phenolic coupling of tyrosine could be an early event critical to fibril formation and Aβ deposits in AD [31].

There have been limited studies of dityrosine, in particular, dityrosine-containing dimeric peptides, partly because of the difficulty in synthesising these systems. Synthesis and study of dityrosine-containing Aβ dimeric peptides could lead to a better understanding of the importance of Aβ oxidative modification and dimerisation in amyloid formation and AD associated neurodegeneration. In the current study, we have modified a method for dimerising peptide-bound tyrosine residues in reasonable yield using chemically unprotected peptides [6]. The method uses horseradish peroxidase in the presence of H_2O_2 and is derived from a method applied to forming dityrosine in unprotected cyclic tetrapeptides [34]. We initially applied this method to the dimerisation of the model compound *N*-acetyl-L-tyrosine ethyl ester (ATEE) and subsequently to Aβ(9–16), an Aβ model peptide containing Tyr-10 plus two of the oxidatively sensitive histidine residues. After obtaining high yields of dimeric peptides for Aβ(9–16) we applied the same conditions to the dimerisation of Aβ(1–28) and Aβ(1–40) and used fluorescent and antibody detection methods to monitor the formation of dimers and higher oligomers.



Scheme 1.

Experimental procedures

Chemicals and materials

BDH Chemicals (Poole, England) was the source of H_2O_2 (30%), sodium phosphate, ethyl acetate, ammonium carbonate and other buffers and salts (Analar grade). BDH (Poole, England) and Merck (Darmstadt, Germany) supplied the organic solvents (HiPerSolv for HPLC). ATEE, 2-mercaptoethanol, Chelex 100 and DEAES Sephadex were purchased from Sigma Chemical Co. (St Louis, USA). All other reagents were of the highest grade. HRP was compared to commercially available HRP type VI-A (purchased from Sigma Chemical Co.) and resulted in higher dityrosine formation.

Electrophoretic molecular weight markers and reagents for enhanced chemiluminescence (ECL) were purchased from Amersham Pharmacia Biotech (Sydney, Australia). Electrophoretic reagents and

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Trans-Blot nitrocellullose membrane were obtained from BioRad Laboratories (Sydney, Australia).

Aβ(9–16), Aβ(1–28) and Aβ(1–40) were synthesized utilizing manual solid-phase chemistry as described by He and Barrow [35] using *N-tert*-butyloxycarbonyl (Boc) amino acid chemistry and PAM resin. After hydrogen fluoride cleavage of the peptides from the resin the crude peptide was purified by HPLC. Peptide purity and identity were confirmed by HPLC, ESI-MS and amino acid analysis.

Instrumentation

RP-HPLC was performed using Beckman System Gold model 126-pump and Beckman System Gold Diode array detector Module 168 (Fullerton, USA) and recorded using Nouveau Gold software package. UV–Vis and fluorescence spectra were acquired in a 10 or 4 mm quartz cuvette using a Shimadzu UV-2401PC UV–Vis recording spectrophotometer (Kyoto, Japan) and a Varian Cary 50 Bio UV–Vis spectrophotometer (Melbourne, Australia), and a Varian Cary Eclipse fluorescence spectrophotometer (Melbourne, Australia). Some samples were analysed using fMax Fluorescence Microplate Reader with incubator and SOFT maxPRO software (Sunnyvale, USA). Fluorescence plate reader measurements were carried at 25°C using filters of (λ_{ex}355 nm/λ_{em}460 nm, λ_{ex}485 nm/λ_{em}538 nm, λ_{ex}544 nm/λ_{em}590 nm and λ_{ex}584 nm/λ_{em}612 nm). ESI-MS were acquired using a Micromass Quattro II triple quadrupole instrument (Manchester, UK) in Positive Ion Mode, connected to a Hewlett Packard (HP1100) LC system (Palo Alto, USA). Samples were dissolved in 1% formic acid and buffer solutions of either 0.1% formic acid or ammonium formate in MilliQ water: acetonitrile (50:50), were fed at 40 μl/min to the MS probe. ^{1}H and ^{13}C NMR spectra were recorded using a Varian (Palo Alto, USA) Unity plus spectrometer operating at 400 MHz for protons. Infrared spectra were acquired on a Bio-Rad FTS-165 Fourier-transform infrared spectrophotometer (Hercules, USA).

Dimerisation of *N*-acetyl-L-tyrosine ethyl ester (ATEE)

ATEE (0.2045 g, 0.8 mmole) was dissolved in borate buffer (100 ml, 0.1 M, pH 9.1). The sealed reaction vessel was immersed in a 37°C water bath under an argon or nitrogen atmosphere. Then 250 μl of freshly prepared HRP solution (4 mg/1 ml) was added while stirring. Three aliquots of 100 μl of the solution were transferred immediately with a syringe to a 1 ml vial and 2 μl of 2-mercaptoethanol was added to stop the reaction and the solution freeze–dried. These aliquots were used as the zero time reaction controls. To the remaining reaction mixture was added 85 μl of 30% of H_2O_2 solution (0.8 mmole). After 2 min, the reaction was quenched by addition of 2-mercaptoethanol (25 μl). Another three aliquots of 100 μl were transferred and tested as end reaction samples. All samples were frozen in liquid nitrogen and freeze dried. The product was desalted using flash C18 chromatography, eluting with methanol and water (1:1) and the reaction mixture analysed by RP-HPLC using a Zorbax 300SB column. Peaks were observed corresponding to ATEE, diATEE, isodiATEE and triATEE, eluting in that order (Figure 2). The major product, diATEE, was purified by semi-preparative RP-HPLC. A yield of 45% diATEE was obtained, based on weight of desalted product. Analytical RP-HPLC on the purified diATEE indicated a purity of greater than 99%.

The overall yield of diATEE from tyrosine was 29 (±3)%. ESI-MS *m/z* 501 $[M+H]$. ^{1}H NMR (400 MHz, CD_3OD): 1.08 (6H, t), 1.81 (6H, s), 2.81 (2H, dd, J = 8 Hz), 2.95 (2H, dd, J = 6 Hz), 4.01 (4H, q), 4.49 (2H, q), 6.73 (2H, d, J = 8 Hz), 6.92 (1H, d, J = 2 Hz), 6.94 (3H, m) (Figure 4(b)) ^{13}C NMR (125 MHz, CD_3OD): 15.7, 23.5, 50.2, 56.8, 63.3, 118.5, 128.5, 130.7, 132.3, 135.8, 155.4, 174.4. IR ν_{max} 3434, 1736, 1643, 1564 cm^{-1}.

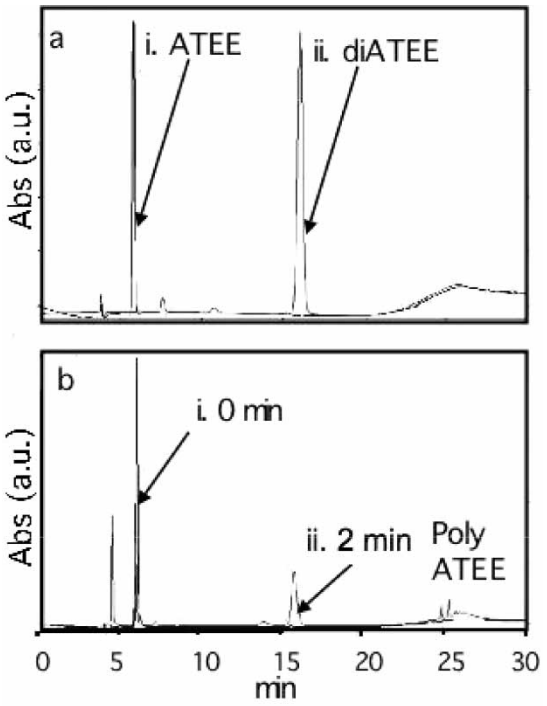

Figure 2. Chromatogram of: (a) ATEE and diATEE subjected to HPLC analysis on a RP C_{18} column (Zorbax 300SB, 250 × 4.6 mm, 5 m) using 1 ml/min flow rate, 45% methanol and 0.1% of trifluoroacetic acid in water for 15 min, followed by 5 min linear gradient to 80% methanol fixed for 5 min and then returned back to initial conditions within 5 min using linear gradients (where (i) and (ii) are 1.6 mM ATEE and 0.8 mM diATEE standards, respectively) and (b) the crude reaction mixture of ATEE at (i) the start or 0 min, and (ii) the end after 2 min of reaction.

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Time course monitoring of ATEE dimerisation

A closed vial containing 7.5 mg of ATEE (30 μmole) dissolved in 4 ml of either phosphate buffer pH 7.4 or borate buffer pH 9.1 (10 mM) was placed in a water bath at 37°C. While stirring, the vial was purged with nitrogen for 10 min. Then 10 μl of freshly prepared HRP solution in MilliQ water (0.3 mg/10 μl) was added. A measured quantity of 100 μl of the solution was removed from the closed vial using a syringe and transferred to a 1 ml Eppendorf vial. The reaction was stopped by the addition of 1 μl of 2-mercaptoethanol, the sample freeze dried and used as the zero time reaction. To the remaining reaction mixture was added 34 μl of 3% of H_2O_2 (30 μmole). Hundred microlitres of the reaction mixture was removed at a specific time intervals (2, 4, 6, 10, 60 min and 24 h). After freeze-drying, the samples were analysed by HPLC to monitor the formation of the dimer.

Dimerisation of Aβ(9–16)

Aβ(9–16) (10 mg, 10 μmole) was dissolved in borate buffer (40 ml, 0.1 M, pH 9.1). The sealed reaction vessel with a magnetic stirrer was immersed in a 37°C water bath and purged with nitrogen gas for 10 min. Two hundred microlitres of freshl prepared HRP solution (0.2 mg/200 μl) was added while stirring. Hundred microlitre of the solution were transferred immediately to use as the zero time reaction, 2 μl of 2-mercaptoethanol added to stop the reaction and then freeze-dried. To the remaining reaction mixture was added 0.15% w/v H_2O_2 solution freshly prepared from a 30% H_2O_2 stock solution (114 μl, 5 μmole). After 15 min, the reaction was stopped by addition of 2-mercaptoethanol (30 μl). The reaction product was analysed by RP-HPLC with diAβ(9–16) being the major product. Aβ(9–16) was purified using semi-preparative RP-HPLC and recovered with an overall yield from starting peptide of 22% yield. ESI-MS confirmed the presence of a dimer and amino acid analysis was consistent with the expected product. ESI-MS *m/z* 1992.8 ([$M + H$] expected 1993.2); Amino acid analysis: Gln/Glu 4.8 (4), Gly 1.87 (2), His 3.7 (4), Lys 2.0 (2), Tyr 0.0 (0), Val 2.0 (2).

Time course monitoring of Aβ(9–16) peptide dimerisation

The same procedure as for the Aβ(9–16) dimerisation reaction was followed on a 1 mg scale. Two hundred and fifty microlitres of aliquots of the reaction solution were taken at times 0, 1, 2, 3, 4, 5, 10, 15, 20, 30, 40, 60 and 80 min and each quenched with 1 μl of 2-mercaptoethanol. The remaining solution was left for a further 80 min and quenched with 5 μl of 2-mercaptoethanol. All samples were frozen in liquid nitrogen and freeze dried. The samples were analysed for yield of dimer by RP-HPLC.

UV–visible and fluorescence monitoring of Aβ(9–16) reaction

Samples were dissolved in MilliQ water and 5 μl aliquots were injected into 245 μl of 0.5 mM borate buffer in a 4 mm quartz cell, and analysed at room temperature using Cary Eclipse UV–Vis and fluorescence spectrophotometers. The absorbance between 180 and 800 nm were measured for UV–Vis spectra. The fluorescence spectra were scanned at different excitation values: λ_{ex}274 nm/λ_{em} scanned from 280–550 nm and λ_{ex}325 nm/λ_{em} scanned from 330–640 nm using 5 nm slit width.

Dimerisation of Aβ(1–40) and Aβ(1–28)

Solutions of Aβ(1–28) and Aβ(1–40) were dimerised as described in Table I. ATEE was used as a control to monitor the formation of dimers. The reaction was started by the addition of 100 μl of (30 μmole) H_2O_2, and all vials were incubated at 37°C for 6 h. The samples were then frozen under liquid nitrogen, freeze dried and analysed using ESI-MS, fluorescence plate reader and Western blot gel (WO2).

Gel electrophoresis monitoring of Aβ(1–28) and Aβ(1–40) dimerisation reactions

To monitor the dimerisation of Aβ(1–28) and Aβ(1–40), gel electrophoresis-polyacrylamide gel electrophoresis was performed in the presence of SDS (SDS-PAGE). The dried samples were diluted × 10 (2 μl in 20 μl) using sample buffer (8 M urea, 8%

Table I. Composition of reaction solutions for dimerisation of Aβ(1–40) and Aβ(1–28).

	Volume of solutions (μl)				
Reaction Solution	Acetate buffer⋆	HRP†	Aβ(1–28)‡	Aβ(1–40)¶	ATEE§
Aβ(1–28)	750	100	50	–	–
Aβ(1–40)	765	100	–	35	–
ATEE	700	100	–	–	100

⋆0.1 M acetate buffer pH 7.4. †0.4 mg/ml of HRP in buffer. ‡0.2 mg/ml of Aβ(1–28)in ?hexafluoroisopropanol (HFIP). ¶0.4 mg/ml of Aβ(1–40)? in HFIP. §0.07 mg/ml of ATEE in buffer.

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SDS, 30% glycerol, 100 mM tricine, 0.01% phenol red and 10% mercaptoethanol), then gel electrophoresis was run according to the procedure of Cherny et al. [36]. Using the primary monoclonal mouse antibody, WO2 in conjunction with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako, Denmark), and visualized using chemiluminescence (ECL, Amersham Pharmacia Biotech) [36]. The epitope for WO2 is residues 5–8 of Aβ [37].

Results and discussion

Dimerisation of N-*acetyl-*L*-tyrosine ethyl ester*

ATEE was used as a model for the dimerisation of peptide tyrosine residues to enable easier analysis of reaction products while developing reaction conditions for peptide dimerisation. Chemical reaction conditions were tested initially, including MCO systems such as Cu^{2+} plus H_2O_2 with and without methionine [38]. However, these chemical conditions gave very low reaction yields of dimers and most resulted mainly in recovery of unreacted starting material. The use of the enzyme horseradish peroxidase in the presence of H_2O_2 under appropriate conditions did lead to the formation of ATEE dimers in relatively good yield. The method was developed based on a method previously employed to produce dimeric cyclic peptides [39]. The reactivity of HRP was previously shown to be optimum at alkaline pH. Alkaline pH has been shown to decrease the aggregation of Aβ peptides and increase their solubility. However, too alkaline, a pH can result in destruction or racemisation of sensitive residues in some peptides. Therefore, we compared the dimerisation of ATEE at neutral pH and slightly basic pH to determine if pH significantly impacted the products formed in addition to the reaction rate. The results of a time course study of ATEE dimerisation at pH 7.4 and pH 9.1 are shown in Figure 3.

The reaction at pH 7.4 did not result in a significant slowing of reaction rate relative to the reaction at pH 9.1, as determined by RP-HPLC monitoring of starting material peak height. In both cases, most of the starting reagent had reacted in the first few minutes. However, the reaction at pH 7.4 never provided a high yield of dityrosine-containing dimer (diATEE) as this dimer appeared to rapidly polymerise further before a significant yield could build-up. The reaction at pH 9.1, however, resulted in a rapid build-up of diATEE in the first two minutes, after which this product further reacted forming larger polymers (Figure 3). This time-course study clearly shows that diATEE can be formed in good yield if the reaction is not left too long. The optimum ratio of ATEE to HRP was found to be about 1:220.

To purify diATEE from the reaction mixture, the sample was desalted using flash C18 chromatography

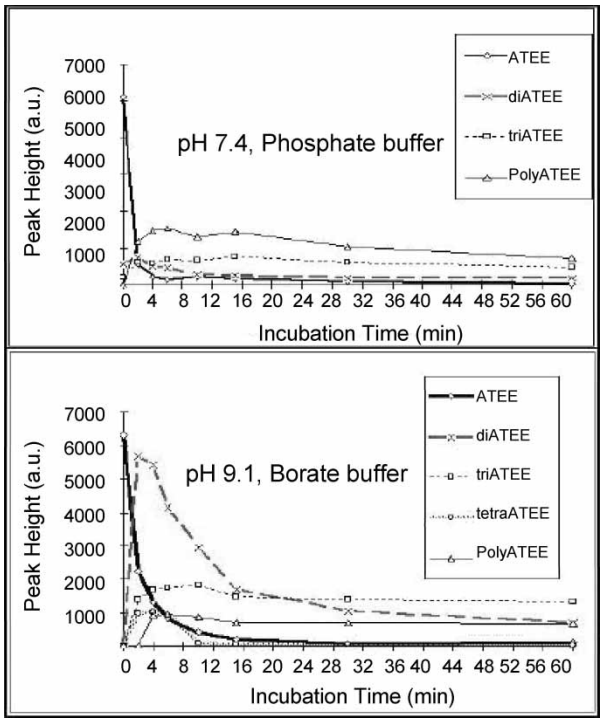

Figure 3. Time course study of dimerisation of ATEE using HRP at pH 7.4 and 9.1. Data are from the peak height at λ280 nm from HPLC.

and then applied to RP-HPLC. The aqueous RP-HPLC phase required a lower level of TFA (0.01%) than that normally used for peptides, to avoid hydrolysis of the C-terminal ester bond. ^{1}H NMR resonances from the aromatic region (6.8–7.4 ppm) of the purified diATEE product are consistent with formation of a new carbon–carbon bond at the ortho positions of both tyrosine residues. Assignments are shown in Figure 4. The ^{1}H NMR values are in agreement with those previously reported for dityro sine [21] and theoretical values calculated using the ACD-HNMR (Toronto, Canada) integrated software package with a confidence of 95%.

Dimerisation of Aβ(9–16)

Dimerisation of Aβ(9–16) was achieved under similar conditions to those used for ATEE. The reaction was monitored using a combination of RP-HPLC and ESI-MS. The reaction appeared to occur slower than for ATEE. Monitoring by RP-HPLC indicated that dimer formation reached a maximum at approximately 15 min, after which other polymeric reaction products slowly appear and the dimer yield decreases. It is not clear why the peptide reaction is significantly slower than that of ATEE under the same conditions, but it may be related to increased steric hindrance in the peptide due to the presence of other bulky amino acids.

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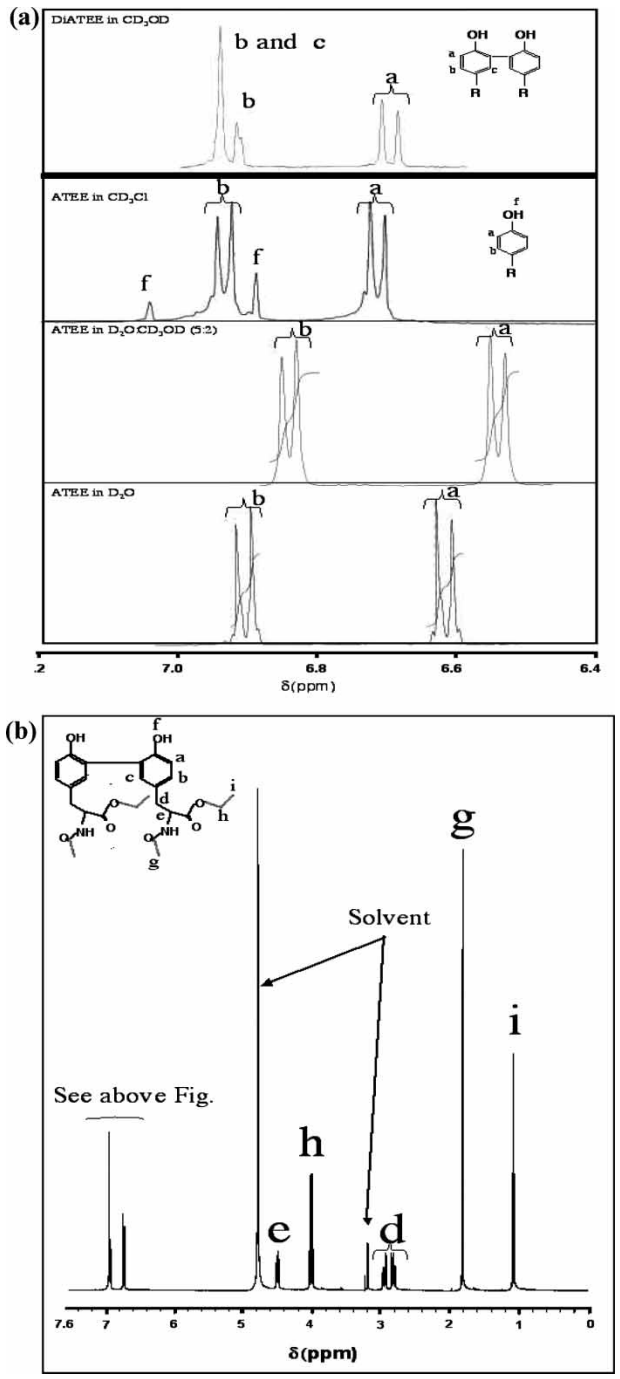

Figure 4. (a) Aromatic region of 400 MHz ^{1}H NMR spectra of diATEE and ATEE in different solvents and (b) 400 MHz ^{1}H NMR spectrum of diATEE in CD_3OD.

The slower reaction time of Aβ(9–16) relative to ATEE makes the peptide reaction more controllable and leads to an overall higher yield. Although, the yield after RP–HPLC purification was 22% by weight from tyrosine starting material, this yield is low due to losses during chromatography. The actual yield before purification was estimated by HPLC to be 61 (±6)%. This yield was obtained by creating standard concentration vs peak area curves at 220 nm for both Aβ(9–16) and dimeric Aβ(9–16), using purified samples of both. Since the HPLC peaks were narrow, similar results were obtained either by peak height or area. The standard curves were then applied to quantitate both Aβ(9–16) and dimeric Aβ(9–16) in optimised reaction mixtures, to enable accurate quantitation of both compounds by RP-HPLC.

ESI–MS of the purified dimeric Aβ(9–16) product confirmed the presence of a dimer. A parent ion was observed at 1993.2 Da, consistent with the expected molecular weight of 1992.8 Da for $[M+H]^+$. Multiple charged species including 2^+(997.1 Da), 3^+(665.1 Da), 4^+(499.1 Da) and 5^+(399.4 Da) were also observed in the ESI–MS, confirming that the dimer observed was not one that was formed in the mass spectrometer. The UV and fluorescence spectrum of the product was also consistent with that observed for the dityrosine species observed for ATEE, supporting the formation of an ortho-ortho linkage between the two peptide tyrosines. For example, for both diATEE and dimeric Aβ(9–16), the new UV maxima appeared at 251 and 294 nm. Observed λ_{em}, max at 274 and 325 nm were similar for both diATEE and dimeric Aβ(9–16) and consistent with that observed by Heinecke et al. for dityrosine [20]. Amino acid analysis of the purified dimer confirmed that all amino acids other than tyrosine were intact. No tyrosine was detected, also supporting that this residue had dimerised.

Dimerisation of Aβ(1–28) and Aβ(1–40)

Reactions of Aβ(1–40) and Aβ(1–28) were carried out as above using the HRP/H_2O_2 system. A fluorescence plate reader with two specific filters for tyrosine oxidation (λ_{ex}355 nm/λ_{em}460 nm, and λ_{ex} 485 nm/λ_{em}538 nm [40]) was used to monitor the formation of dityrosine oligomers. These filters were used to monitor dityrosine formation as other oligomeric products have higher λ_{ex} and λ_{em}, and using λ_{ex}325/λ_{em}407 nm, normally used for dityrosine, is not recommended due to interference from His and Phe oxidation products [40]. The plate reader results showed formation of dityrosine-like products (Figure 5), as indicated from the elevation in fluorescence with both filters. Results were similar to those obtained for the ATEE control. The formation of Aβ(1–40) dimers has also been confirmed by Yoburn et al. [5,6] using similar reaction methods but much higher concentrations of reactants.

The HRP/H_2O_2 reaction with Aβ(1–28) and Aβ(1–40) was also monitored using SDS-PAGE and antibody detection (WO2). The Gel/WO2 results (Figure 6) showed the formation of higher oligomer and indicate that the dimerisation promotes further aggregation into larger oligomers. Several attempts were made to ensure that the gel was not over-loaded but the peptides appeared to grossly oligomerize resulting in an almost continuous band and, in fact,

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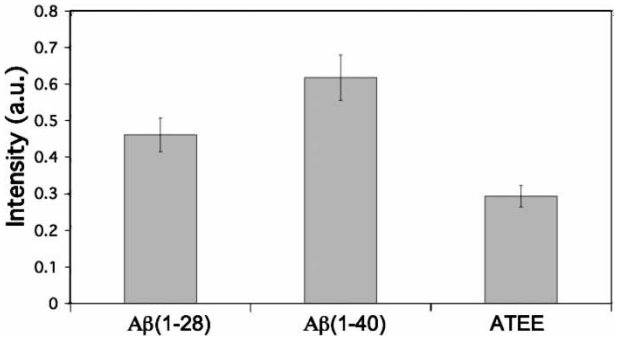

Figure 5. Results of HRP reaction with Aβ(1–28), Aβ(1–40) and ATEE monitored using a fluorescence plate reader (λ_{ex}485 nm/λ_{em}538 nm). The results from three replicates are shown with error bars. (a.u. is arbitrary units).

the WO2 antibody appeared to be more sensitive to dityrosine formation in Aβ(1–40) than the other Aβ peptides. It is not known whether these larger oligomers are the result of covalent polymerisation or non-covalently linked peptide aggregates where aggregation is promoted by the initial formation of a covalent dityrosine dimer. Further work is needed to determine the biological significance of this increased aggregation. The gel results show that Aβ modified by formation of dityrosine still binds WO2 antibodies. This demonstrates that dityrosine formation between Aβ molecules has no effect on the detection of Aβ by WO2 (epitope of WO2 is Aβ(5–8)).

Our results show that ATEE can be used as a model for dityrosine formation in peptides. NMR analysis of the reaction product from ATEE indicates that the major dimer results from a new carbon–carbon bond linking the ortho positions of two tyrosine residues. This dityrosine formation is not reversible and results in the formation of a new amino acid (dityrosine) that is fluorescent. We also have shown that Aβ fragment dimers can be synthesised in relatively high yield using a procedure similar to Yoburn et al. [5,6], who recently published a method to dimerise Aβ(8–14) and Aβ(1–40). In addition, we demonstrate that Aβ peptides, including Aβ(1–40), can be dimerised and the time course of the reaction monitored by fluorescence spectroscopy and with a fluorescent plate reader. Gel electrophoresis indicated that formation of Aβ(1–28) and Aβ(1–40) dimers resulted in increased aggregation for these peptides. These dimeric Aβ peptides may be useful for studying both the importance of early dimerisation in amyloid formation and the role of dityrosine formation in Aβ neurotoxicity.

The neurotoxicity of these dimeric peptides remains to be determined. Tyr-10 is known to be important in amyloid formation and neurotoxicity, and a tyrosine to phenylalanine substitution is one of the three residue differences between the human and mouse Aβ sequence. Non-transgenic mice do not form amyloid plaque and this is believed to be due to differences in their Aβ sequence relative to humans, including the tyrosine to phenylalanine substitution. The formation of dityrosine-linkages is known to occur in a number of peptides and proteins [38,41], and is associated with aging [42–44]. Dityrosine-linkage may play a role in forming higher oligomers of Aβ and may be important in the pathogenesis associated with AD. MPO has been reported to be present in human AD plaques [45] and further study is necessary to determine if MPO could induce dityrosine formation under more physiological conditions. The results presented here provide methods for the synthesis and monitoring of dityrosine-containing peptides and, in particular, Aβ peptides. These methods may be useful

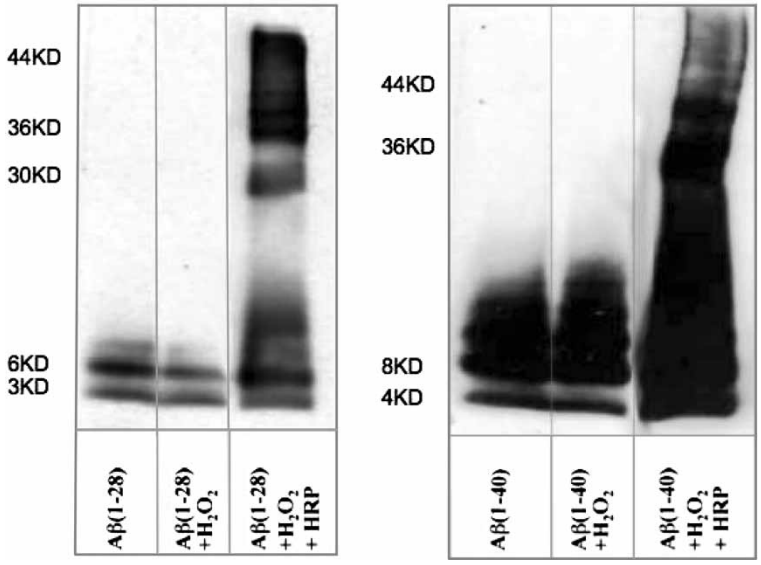

Figure 6. Gel/WO2 electrophoresis results of Aβ HRP/H_2O_2 reaction, showing the formation of multiple oligomers of Aβ.

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for investigating the role of Aβ metal binding, oxidative damage and dityrosine formation in amyloid formation and neurodegeneration in AD.

Conclusions

In this study, we report chromatographic and spectroscopic techniques for the preparation, isolation and analysis of dityrosine as either a protected amino acid or as part of a peptide. Optimum conditions for dimerisation of ATEE and Aβ(9–16) peptide dimers using HRP are described. The method does not require protection and deprotection of functional groups and demonstrates the utility of peroxidases for formation of o,o′-dityrosine. Dimeric Aβ(9–16) was obtained in reasonable yield and purity, without modification of other residues, notably histidine and phenylalanine (Figure 7).

Our results also demonstrate that dityrosine-linkage can lead to oligomerisation of Aβ peptides. Dityrosine modification could be an early pathological event in Aβ deposition in AD. A recent study has shown that the substitution of tyrosine with alanine in the Aβ sequence inhibits the ability of this peptide to catalyse the production of H_2O_2 and to cross-link, as well as inhibiting Aβ neurotoxicity [8]. The role of dityrosine-linkages in the aggregation and neurotoxicity of Aβ needs further investigation both *in vitro* and *in vivo*. The application of the methods described here for the formation and monitoring of Aβ dityrosine formation should be useful for exploring the role of Aβ dimerisation and dityrosine formation in amyloid formation and neurodegeneration in AD.

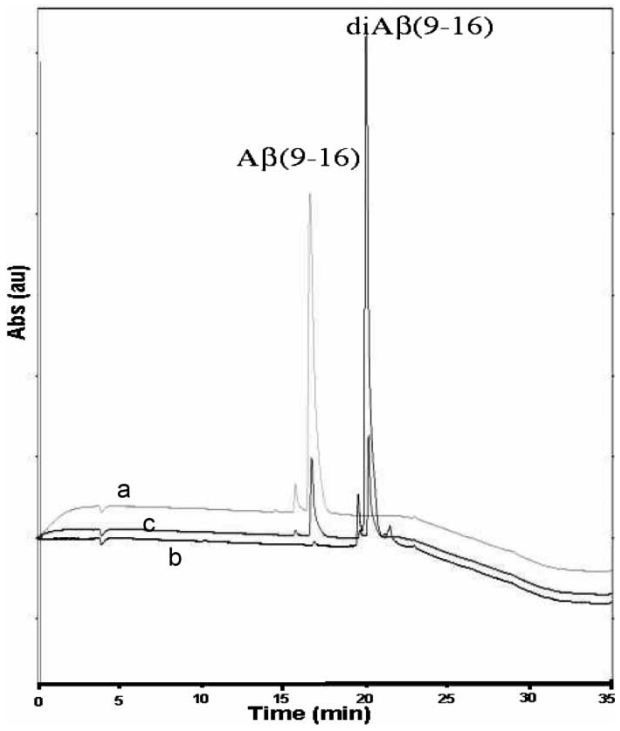

Figure 7. The chromatograms of Aβ(9–16) and Aβ(9–16) dimer (diAβ(9–16)) standards; (a) 1 mg/ml of Aβ(9–16), (b) diAβ(9–16) and (c) mixture of 0.2 mg/ml of both Aβ(9–16) and diAβ(99–16).

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