A multifunctional ELISA to measure oxidised proteins: oxPin1 in Alzheimer's brain as an example

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A B S T R A C T

Background: Oxidative stress occurs in many neurodegenerative diseases including Alzheimer’s disease (AD) and evidence suggests that specific proteins are oxidised in individual diseases. Thus measures of oxidised proteins such as in human biological samples could represent potential disease-specific biomarkers. Protein carbonylation is considered to be an important marker of oxidative stress. In AD in particular, the peptidyl prolyl isomerase, Pin1, has been shown to be sensitive to metal-catalysed oxidation with the addition of carbonyl side-chains.

Methods: Based on this protein modification we developed a novel, enzyme-linked sandwich immunoassay for the quantification of oxidised Pin1 (oxPin1) in human brain tissue samples.

Results: We successfully developed an ELISA for the measurement of oxidised Pin1 in biological samples and measured oxPin1 in hippocampal tissue extracts from controls and AD, which showed an increased ratio of oxPin1 to total Pin1 in patients with early AD pathology compared with controls.

Conclusions: We show that oxidised proteins, in this case oxPin1, can be measured using the developed ELISA. In addition, our results support the presence of increased oxidative stress in the early stages of AD pathology and show that the oxPin1/Pin1 ratio could indicate early stage pathology. This warrants further investigation in other biological fluids.

General significance: Importantly, further development and adaption of the assay design will enable multi-functional use for the quantification of oxidised proteins in tissues and biological fluids that may be used in investigating the role of oxidised proteins in a range of neurodegenerative diseases, particularly in which disease-specific protein oxidation has been implicated.

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1. Introduction

The brain uses more oxygen than any other organ of the body, making it prone to damage by reactive oxygen species (ROS) produced by its aerobic activity. ROS are produced continuously and play an important role in biological processes such as signal transduction, immune responses and ageing [1]. An imbalance between endogenous pro-oxidants and antioxidant defences leads to unregulated production of ROS and subsequent oxidative stress [2] and is implicated in the progression of neurodegenerative diseases including Alzheimer’s disease (AD) [3].

One of the most important measures currently used to measure oxidative stress is the quantification of the oxidative state of glutathione (GSH), the most prevalent antioxidant in the brain. In particular, the ratio between GSH and oxidised glutathione (GSH/GSSG) has been shown to be decreased in affected regions of AD brain compared with controls [4]. However, the use of GSH as a measure of oxidative stress has recently been questioned [5]. Furthermore, GSH/GSSG is a broad measure of oxidative stress, which is a feature of a wide range of diseases, and is not a marker for a specific disease.

Several alternative methods of measuring specific types of oxidative stress such as metal-catalysed oxidation, nitration, and methionine oxidation are also available but, again, these are generally not specific for an individual disease [6]. Since there is now increasing evidence that specific proteins are oxidised in specific neurodegenerative diseases [7–9], the measurement of specifically oxidised proteins may represent an alternative with promise for the development of disease-specific (oxidised protein) biomarkers. The role of oxidative stress in AD has been rather extensively studied and several proteins that appear to be specifically oxidised have been identified [7,8]. However, the measurements currently used, such as 2D gel analysis, chromatography...
and mass spectrometry, are often specialised and time-consuming. Therefore, a simple effective measure of individually oxidised proteins would be of significant value. Therefore, we aimed to develop a simple effective method for measuring oxidised proteins and investigated its use in the detection of specifically oxidised proteins in AD as an example.

The oxidation of many proteins results in the addition of carbonyl groups (aldehydes and ketones) to protein side chains (predominantly Pro, Arg, Lys, and Thr) [10,11]. In AD, the proteins that appear most vulnerable to oxidative stress include the peptide-prolyl isomerase, Pin1 and glutamine synthetase (GS) which, when oxidised contain carbonyl side chains that can be measured [11,12]. Pin1 is of particular importance to AD, as it binds to phosphorylated tau (p-tau), amyloid precursor protein (APP) and glycogen synthase kinase-3β (GSK3β), all of which play a significant role in the pathogenesis of AD. Pin1 binds specifically to proteins with phosphorylated serine (Ser) or threonine (Thr) residues preceding a proline residue. Upon binding, Pin1 catalyses the isomerisation of the protein at the proline residue, twisting it from cis to trans conformation [13]. Upon Pin1 binding at Thr231, tau is isomerised to trans conformation, facilitating its dephosphorylation by the trans-specific protein phosphatase 2A (PP2A) [14]. Similarly, processing of APP via the non-amyloidogenic pathway is favoured when APP is in trans-conformation and binding of Pin1 to APP at Thr668 reduces production of Aβ42 [15]. Pin1 is also responsible for inhibition of glycogen-synthase kinase 3β (GSK3β), which is involved in the phosphorylation of both APP and tau [16,17]. By binding to GSK-3β, Pin1 reduces phosphorylation of both APP and tau and thus further reducing amyloidogenic processing of APP and hyperphosphorylation of tau. When Pin1 is oxidised, its activity is restrained and can no longer bind to its substrates. The absence of Pin1 may thus correlate with enhanced amyloidogenic APP processing and increased Aβ42 production together with hyper-phosphorylation of tau and subsequent formation of neurofibrillary tangles (NFTs) [7,12].

In this study, we aimed to develop simple, effective means for measuring levels of specifically oxidised proteins in human biological material. As an example, we developed an ELISA for the measurement of oxidised Pin1 (oxPin1) and used it to measure levels of oxPin1 in brain tissue at different stages of Alzheimer’s disease pathology compared with cognitively normal controls.

2. Materials and methods

2.1. Tissue samples

Frozen human hippocampal tissues from control and AD patients were obtained from the Department of Pathology (Radboud UMC) and were collected using standard protocols. Left over material from autopsies was used according to institutional guidelines which were in line with the Dutch national law. Additional hippocampal samples were obtained from The Netherlands Brain Bank (NNB, Netherlands Institute for Neuroscience Amsterdam), collected from donors for or from whom a written informed consent for a brain autopsy and the use of the material and clinical information for research purposes had been obtained by the NBB. Tissue samples were taken as quickly as possible after autopsy and rapidly frozen in liquid nitrogen. Post-mortem diagnosis and grading were performed and judged by a neuropathologist, according to the criteria established by Braak & Braak (all samples) and CERAD (for Radboud UMC patients only) [18–20]. Clinical diagnosis of AD was based on the clinical criteria of probable AD [21,22]. Patient characteristics for all patients are described in Table 1.

Protein extraction from brain tissue was performed by suspending serial cryosections of hippocampus or cortex (10 μm thickness) in cold 5 M guanidine hydrochloride (GuHCl)/50 mM Tris–HCL, pH 8.0 containing a protease inhibitor cocktail (Complete Mini, EDTA free, Merck Millipore, Darmstadt, Germany) and vortexing until the tissue was fully lysed. Samples were centrifuged for 20 min (16,000 × g at 4 °C) and the supernatant was stored at −80 °C in clean polypropylene tubes. Total protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

2.2. Production and purification of recombinant Pin1 protein

Recombinant Pin1 protein was produced using a construct of Pin1 cDNA cloned in the pET-46 EK expression vector (Novagen, Madison, WI) and OneShot® BL21 (DE3) Star™ cells (Life Technologies, Carlsbad, CA, USA) by isopropyl-β-D-thiogalactoside (ITPG) induction. His-tagged recombinant protein was purified under native conditions using a Nickel Nitrotriacetic acid (Ni-NTA) chromatography column (Qiagen, Hilden, Germany) and elution with imidazole. The protein was cleared of imidazole using dialysis with phosphate buffered saline and subsequently concentrated using a 9 kDa cut-off protein concentrator according to the manufacturer’s instructions (Thermo Fisher Scientific, Rockford, IL, USA). The final protein concentration was determined using the BCA protein assay kit.

2.3. Oxidation of recombinant Pin1 protein

Purified Pin1 protein (80.8 μl; 0.5 μg/mL; estimated purity ~95% based on SDS-PAGE gel analysis) was incubated with an oxidation solution containing 0.2 mM Fe(II)SO₄, 10 mM H₂O₂ and 0.3 mM EDTA in PBS (pH 7.4) in a final volume of 100 μl for 3 h [12]. One microliter of 40 μM butylated hydroxytoluene in methanol was added to stop the reaction giving a final (theoretical) protein concentration of 0.4 μg/mL. The mixture was dialysed against PBS (pH 7.4) at 4 °C using a mini slide-a-lyser cassette (2 kDa cut-off; Thermo Scientific, Rockford, IL, USA) for 2 h and then overnight in fresh, cold PBS. Following dialysis, the sample was divided into aliquots and stored at −80 °C.

2.4. SDS-PAGE analysis of Pin1

Recombinant Pin1 protein (1 μg) and an equal volume of oxidised recombinant Pin1 protein were mixed with 4× reducing sample buffer (25% (w/v) glycerol, 2% (w/v) SDS, 62.5 mM Tris–HCl, pH 6.8, 32 mM DTT and 0.005% (w/v) bromophenol blue) and PBS to a final volume

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<th>Table 1: Patient demographics and protein levels.</th>
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<td>Post-mortem delay (h)</td>
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<td>Total protein (μg/mL)</td>
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Values are presented as mean (standard deviation) except where otherwise indicated; ns = non-significant.

a Additional clinical information is provided in Supplementary Table S1. Advanced AD = Braak stage 5–6; early AD = Braak stage 3–4 and controls = Braak stage 0–2.

b p-Values obtained using ANOVA.
of 16 μL. Samples were then heated to 95 °C for 5 min, loaded on to SDS-PAGE gels with 4% acrylamide stacking gel and 12% acrylamide running gel, and electrophoresed at 200 V. Gels were stained with Coomassie Brilliant Blue (R250, Serva) stain for 45 min. The Precision Plus Protein™ Standard (All Blue) molecular weight marker (10–250 kDa; BioRad Laboratories, Inc., California, USA) was used to approximate protein size. Protein bands were scanned using a BioRad Gel Doc 2000 apparatus and density measurements were used to calculate oxPin1 concentration in comparison to non-oxidised protein (Quantity One programme — BioRad v.4.2.1).

2.5. Direct ELISA for carbonylated proteins

A direct ELISA for the measurement of carbonyl content in the protein samples with minor modifications from the original protocol [23] was used to confirm protein carbonylation as a measure of protein oxidation. Protein samples derivatised with 2,4-dinitrophenyl hydrazine (5 μM DNPH; 45 min) in 6 M GuHCl, pH 2.4 (1:3 ratio) were absorbed onto an ELISA plate. The hydrazone adducts, 2,4-dinitrophenyl (DNP), were detected using a biotinylated antibody directed against DNP (Anti-Dinitrophenol-KLH, Rabbit IgG Fraction, Biotin-XX Conjugate; Molecular Probes®, Oregon, USA), followed by 20 min incubation with 100 μL of streptavidin-HRP complex (1:200 R&D DuoSet kit). The colour reaction was developed using TMB substrate (30 min at RT), stopped with 0.5 M H2SO4 (50 μL) and read at 450 nm (Tecan Sunrise ELISA plate reader and Magellan data analysis software). The extent of protein oxidation was assessed by comparison with an oxidised BSA standard. Oxidised BSA and reduced BSA were prepared as previously described [15].

2.6. OxPin1 sandwich ELISA

OxPin1 levels in brain tissue were quantified using a novel homemade sandwich ELISA with oxidised recombinant Pin1 as standard. ELISA plates (F96 cert. MaxiSorp™ Immuno-plate: Nunc, Roskilde, Denmark) were incubated overnight at 4 °C with polyclonal rabbit anti-DNP (2 μg/mL in 1 M NaHCO3, pH 9.6; Life Technologies) and washed (5× 300 μL, 0.05% Tween-20/phosphate buffered saline (PBST)) between all incubation steps. Plates were blocked with 0.1% BSA/PBS, pH 7.4 (250 μL). Samples, standards (oxPin1, stock = 0.2 μg/μL) and blank (PBS) were derivatised with 5 μM DNPH in 6 M GuHCl, pH 2.4 (1:3 ratio), vortexed, incubated for 45 min in the dark, and subsequently diluted in sample diluent (1 mM EDTA, 0.5% Triton X-100 in PBS). The highest standard (50 ng/mL) was serially diluted in sample diluent to obtain a 6-point standard curve. Standards, blank (PBS) and tissue samples (100 μL/well) were incubated for 2 h at room temperature (RT) with agitation (650 rpm). An additional wash step (1:1 ethanol:PBS) was used to ensure the removal of unbound DNPH that could react with the detection antibody. Detection antibody (250 ng/mL biotinylated polyclonal goat anti-Pin1; R&D Pin1 DuoSet, #DYC2294) was applied for 1 h followed by 20 min incubation with 100 μL of streptavidin-HRP complex (1:200 R&D DuoSet kit). The colour reaction was developed using TMB substrate (30 min at RT), stopped with 0.5 M H2SO4 (50 μL) and read at 450 nm (Tecan Sunrise ELISA plate reader and Magellan data analysis software).

2.7. Total Pin1 sandwich ELISA

Pin1 levels in brain tissue (diluted 1:25) were measured using a commercially available ELISA kit (R&D DuoSet® IC for total Pin1; Abingdon, UK) according to the manufacturer’s instructions.

2.8. Statistical analysis

Between groups comparison was performed using Kruskal–Wallis tests for non-Gaussian distributed data (oxPin1) and one-way analysis of variance (ANOVA) or Student’s t-test for Gaussian-distributed data.

Analysis of covariance (ANCOVA) was performed to control for covariant influences such as age, gender and post-mortem delay.

3. Results

3.1. Confirmation of Pin1 oxidation

Several oxidation conditions were tested and we found that oxidation using 0.2 mM Fe(II)SO4, 10 mM H2O2 and 0.3 mM EDTA gave the optimal results with respect to oxidation level versus protein degradation. Still, SDS-PAGE gel analysis (Fig. 1A) suggested that Pin1 was partially degraded after oxidation. We noted a more slowly migrating band for oxised Pin1 than non-oxidised Pin1 (Fig. 1A) as has been observed previously for other oxidised proteins [24–26] and we were able to confirm the band as Pin1 in western blot analyses using both polyclonal and monoclonal anti-Pin1 antibodies (Fig. 1B). Using band density measurements we calculated the concentration of full-length oxidised Pin1 to be 0.2 μg/μL. We derivatised proteins (either from recombinant source or from brain extracts) with DNPH and immunoprecipitated these using anti-Pin1-coated protease G beads. The derivatised proteins were detected with the anti-DNP antibody and in this way we observed oxPin1 in the form of a detectable band at the correct molecular weight expected for Pin1. However, the results for the brain extract proteins gave inconsistent results when repeated (data not shown). Oxidation of Pin1 was confirmed using a direct ELISA for detection of carbonyl side chains. Compared with untreated Pin1, reduced BSA, and PBS treated with DNPH, high OD450 values in the direct ELISA obtained for oxPin1 and oxidised BSA confirmed their oxidation status (Fig. 1C). Furthermore, the OD450 value for reduced Pin1 did not differ significantly from untreated Pin1 suggesting that untreated Pin1 was not oxidised.

3.2. Validation of the sandwich ELISA for oxPin1

A typical standard curve for oxPin1 is shown in Fig. 2. Linearity was observed up to 50 ng/mL. The lower limit of detection was 2.7 ng/mL. Consistent with the results of direct ELISAs, PBS and non-oxidised recombinant Pin1 (up to 50 ng/mL) treated in the derivatisation reaction were detected at only very low levels (OD450 value consistently less than 0.2; data not shown). Omission of capture antibody, detection antibody or antigen gave absorbance values resembling the blank confirming the absence of cross-reaction of either capture or detection antibodies with other proteins or each other. It also indicated that neither the biotinylated Pin1 antibody nor brain tissue samples bind non-specifically to the plate.

3.3. Levels of total protein, Pin1 and oxPin1 in brain extracts

Grouped patient demographics and protein levels are reported in Table 1. Patients were divided into groups and analysed according to Braak stage pathology as follows: Controls (Braak 0–2), early AD pathology (Braak 3–4) and advanced AD pathology (Braak 5–6) [27]. Levels of Pin1 and oxPin1 reported in the main text are reported per mg of total protein. The clinical characteristics of individual patients are provided in Supplementary Table S1.

Analysis of hippocampal tissue according to the degree of pathology showed that Pin1 tended to be correlated with post-mortem delay in controls (r = 0.872, p = 0.054) and was correlated with age in advanced AD (r = 0.789, p = 0.012). No other correlations between protein measurement and age, gender or post-mortem delay were observed. When using ANCOVA to control for both age and post-mortem delay, Pin1 levels, but not oxPin1 levels, were significantly higher in advanced AD pathology compared with both early pathology (p < 0.01) and controls (p < 0.05; Fig. 3A and B). The oxPin1:Pin1 ratio was significantly higher in early pathology than controls (p < 0.05) and advanced AD pathology (p = 0.01; Fig. 3C).
4. Discussion

We designed a novel sandwich ELISA for the purpose of measuring specifically oxidised proteins using measures of oxPin1 in biological samples as an example. We demonstrate the successful application of this novel sandwich ELISA to assess the levels of oxPin1 in human hippocampal samples from AD patients and healthy controls. In addition, we show evidence of an increased ratio of oxPin1 to Pin1 in early AD pathology which substantiates the need for further investigation of the role of Pin1 and oxPin1 in the pathophysiology of AD. The design of this ELISA has potential applications for the measurement of other proteins that are specifically oxidised to produce carbonyl side chains in AD and a wide range of neurodegenerative diseases that represent potential biomarkers of disease.

In the current study, the ratio of oxPin1:Pin1 seemed more informative than individual measurement of Pin1 or oxPin1 alone. When we analysed the ratio of oxPin1:Pin1 in human hippocampal samples, we observed an increased ratio in the hippocampi of patients with early AD pathology (Braak stage 3–4) compared with controls (Braak stage 0–2; p = 0.032). This is consistent with the notion that Pin1 is oxidised in AD and that this occurs early in the disease process particularly since we did not observe an increase in the ratio of oxPin1:Pin1 in the patients with advanced AD pathology. This contradicts previous reports [7,12], but we classified our samples according to Braak pathology to investigate the relationship between levels of oxPin1 and the pathological features of AD whereas the earlier studies used clinical diagnosis of MCI and AD established by neurological and psychological assessment. This may provide some explanation for differences between the studies.

We also noted that post-mortem delay tended to be correlated with levels of Pin1 in the control samples. This could also potentially explain differences between studies and should be further explored in future studies.

The finding of increased oxPin1:Pin1 in the hippocampus of early AD pathology is relevant in consideration of other research showing the involvement of Pin1 in both the direct regulation of tau phosphorylation and the indirect regulation via GSK3β and PP2A. Together, the observation that Pin1 is oxidised early in AD, and likely leads to its dysfunction, helps to confirm that oxidation of Pin1 may lead to enhanced formation of NFTs as it can no longer bind to and dephosphorylate the tau protein.

Interestingly, we observed an ~40% increase in Pin1 levels in the hippocampus of patients with advanced AD pathology compared with controls and an ~19% decrease in early stage AD pathology compared with controls. The increase in Pin1 levels in the advanced pathology samples was surprising as we expected from previous research that Pin1 levels would be generally decreased in AD [12,28]. However, a
In the current study, the use of a GuHCl protocol to extract proteins from the brain tissue should have ensured the total extraction of Pin1 from all cell fractions including any that may have been accumulated abnormally in granules but potentially could have affected Pin1 stability. We do not believe that the latter was the case as repeated measures of oxPin1 following freeze-thawing were consistent. In earlier experiments, we also measured levels of oxPin1 in brain tissue extracted using other extraction buffers, including RIPA carbonate, and AC/ BT (1.5 M aminocaproic acid, 75 mM Bis-Tris) buffers. However, levels of oxPin1 in these samples were much lower than those measured in the GuHCl extract and were often at, or below, the limit of detection.

We acknowledge that relatively small numbers of hippocampal tissues limit our ability to generalise the findings to other AD patients. In this study oxPin1 was only measured in brain tissue and showed only moderate differences between the groups thus limiting its current potential for use as a clinical biomarker. The main purpose of demonstrating the use of our developed ELISA for the measurement of oxPin1 in human hippocampal samples has been, however, adequately achieved. The utility of the ELISA will need to be further examined in studies including measures of Pin1 and oxPin1 not only in larger numbers of patients but should also compare hippocampal levels with levels in another brain area in which no, or only minimal, changes might be expected (e.g. cerebellum). We were able to show that specifically oxidised proteins can be measured in biological material. The design of our ELISA using an anti-DNP antibody to capture all carboxylated proteins and specifically detect a target protein using a protein-specific detection antibody enables its adaptation for measurement of other potential disease-specific oxidised proteins such as alpha-synuclein in Parkinson’s disease [30–32], and glutamine synthetase in AD [7]. In addition, the use of antibodies to specific proteins reduces the likelihood of measuring background interference from other carboxylated molecules (e.g. nucleic acids and lipids), which can otherwise give interference [6].

Other adaptations using antibodies to measure alternative forms of oxidative modification (e.g. cysteine or methionine oxidation) might be of high relevance for the measurement of some proteins. For example, the oxidation of DJ-1 at its cysteine (Cys) residues has been implicated in the pathogenesis of Parkinson’s disease and might be of interest for future studies. We have preliminary evidence that assays adapted for the measurement of Cys-oxidised DJ-1 shows promise. For this assay we used purified recombinant Fab antibody fragments specifically directed against Cys-oxidised DJ-1 as the capture antibody and a biotinylated goat anti-human DJ-1 antibody for detection and could use this assay to measure Cys-oxidised DJ-1 in brain tissue extracts and some CSF samples but not in serum. However, these ELISAs will require further optimisation to improve inter-assy reproducibility and consistency in the background signal. The development of purified antibodies directed against a specific form of oxidised protein would be of benefit in developing optimal ELISAs in future studies. Such studies may reveal if protein oxidation is a global process in early stages of neurodegenerative pathology.

5. Conclusions

We have developed a novel sandwich ELISA for the measurement of oxPin1 in human brain tissue and shown that the ratio of oxPin1:Pin1 is increased in the hippocampus of early stage AD pathology compared with both controls and advanced AD pathology. Further development and modification of the assay design will enable multi-functional use for the quantification of oxidised proteins in tissues and biological fluids that may be used in investigating the role of oxidised proteins in a range of neurodegenerative diseases, particularly in which disease-specific protein oxidation has been implicated.

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