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Pretreatment of chemically-synthesized $\text{A}$$\beta_{42}$ affects its biological activity in yeast

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Pretreatment of chemically-synthesized Aβ42 affects its biological activity in yeast

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Keywords: Alzheimer’s disease, Candida glabrata, peptide conformation, quiescent cells, Saccharomyces cerevisiae, synthetic Aβ42

The tendency of amyloid β (Aβ42) peptide to misfold and aggregate into insoluble amyloid fibrils in Alzheimer’s disease (AD) has been well documented. Accumulation of Aβ42 fibrils has been correlated with abnormal apoptosis and unscheduled cell division which can also trigger the death of neuronal cells, while oligomers can also exhibit similar activities. While investigations using chemically-synthesized Aβ42 peptide have become common practice, there appear to be differences in outcomes from different preparations. In order to resolve this inconsistency, we report 2 separate methods of preparing chemically-synthesized Aβ42 and we examined their effects in yeast. Hexafluoroisopropanol pretreatment caused toxicity while, ammonium hydroxide treated Aβ42 induced cell proliferation in both C. glabrata and S. cerevisiae. The hexafluoroisopropanol prepared Aβ42 had greater tendency to form amyloid on yeast cells as determined by thioflavin T staining followed by flow cytometry and microscopy. Both quiescent and non-quiescent cells were analyzed by these methods of peptide preparation. Non-quiescent cells were susceptible to the toxicity of Aβ42 compared with quiescent cells (p < 0.005). These data explain the discrepancy in the previous publications about the effects of chemically-synthesized Aβ42 on yeast cells. The effect of Aβ42 on yeast cells was independent of the size of the peptide aggregates. However, the Aβ42 pretreatment determined whether the molecular conformation of peptide resulted in proliferation or toxicity in yeast based assays.

Introduction

Soluble oligomers of amyloid β (Aβ42) peptide have been found to be highly neurotoxic and important in the etiology of AD in comparison to fibril forms.1 However, it is still unknown which form of the aggregates are more dangerous; oligomers and protofibrils or fibrils and filaments.2-4 Despite these uncertainties aggregation studies in search for chemo-preventatives that block Aβ42 oligomer and fibril formation using synthetic forms of Aβ42 have become common practice.5-8

The preparation of Aβ42 peptide can affect its amyloidogenicity6 yet pretreatments are required in order to produce monomers.9 Lioudyno et al.10 reported that pretreatment with 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) enhanced the amyloidogenicity of the Aβ42 peptide as well as its interaction with biological membranes.

Complex mechanisms contribute to development of AD. Syndromic loss due to plaque formation11 and neuronal death due to oligomeric forms of Aβ42 are all hallmarks of the disease.12 It has been reported that different conformations of Aβ42 induce neurotoxicity by distinct mechanisms in human cortical neurons.13,14 The longer variants have higher propensity to aggregation than the shorter form.13 The mechanism of Aβ42 toxicity is still unknown, although it is suggested that amyloidogenic protein aggregates on neuronal cells cause permeabilisation of lipid membranes,15-17 and increase oxidative stress,18,19 mitochondrial dysfunction,20-22 and endoplasmic reticulum stress23,24 which ultimately leads to apoptosis.

Yeast exhibit some responses to Aβ42 peptide that indicate they are good models for studying the effects of on neuronal cells.25 However, some limitations are expected since yeast lack receptors that have been proposed for Aβ42 binding. Putative receptors include the insulin receptor,26 the alpha7 nicotinic receptor,27 the metabotrophic glutamate receptor,28 and cellular prion protein.29 Chacińska and colleagues30 showed an induction of proliferation in the presence of synthetic Aβ42 in exponentially growing Saccharomyces cerevisiae, while Bharadwaj et al.31 showed toxicity of synthetic Aβ42 with a different preparation method on Candida glabrata cells. In this study we report on the preparation of Aβ42 peptide with 2 different methods and treatments of identical yeast cell cultures. The two pretreatments resulted in totally opposite outcomes.

Results

Pretreatment determines the activity of Aβ42 peptide

HFIP pretreatment of Aβ42 was previously shown to cause toxicity to C. glabrata cells.31 This study confirms significant toxicity of HFIP pretreated Aβ42 to C. glabrata (Fig. 1A) and to

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In order to examine the presence of amyloidogenic proteins within 24 h in the presence of amyloid peptide whereas younger Stationary phase fractions of \( S.\text{cerevisiae} \) of incubation. Conversely, NH4OH pretreatment resulted in a propensity ThT stained proteins on their surface (\( m \)-fluorescence. on the surface of yeast cells ThT treated cells were visualised for propensity Fig. 2 cells were affected by the toxicity after 48 h of incubation (see Fig. 2A) more than doubled in number (\( P < 0.001 \)), and \( S.\text{cerevisiae} \) (Fig. 1B) numbers significantly increased over the time (\( P < 0.005 \)).

Susceptibility of quiescent and non-quiescent cells to toxic \( \text{A} \beta_{42} \)
Stationary phase fractions of \( C.\text{glabrata} \) were tested for their susceptibility to 2 \( \mu \text{M} \) \( \text{A} \beta_{42} \) generated by HFIP pretreatment method. Results indicated that quiescent daughter cells (Fig. 2A) are more resistant to the toxic effect of \( \text{A} \beta_{42} \) than older non-quiescent mother cells (Fig. 2B). The older cells lost their viability within 24 h in the presence of amyloid peptide whereas younger cells were affected by the toxicity after 48 h of incubation (see Fig. 2).

Amyloid staining on the yeast cell surface and aggregation propensity
In order to examine the presence of amyloidogenic proteins on the surface of yeast cells ThT treated cells were visualised for fluorescence. \( S.\text{cerevisiae} \) exhibited presence of a high level of ThT stained proteins on their surface (Fig. 3). Yeast treated with HFIP prepared \( \text{A} \beta_{42} \) on the other hand have more aggregation propensity, causing the cells to adhere and attach to each other (see Figs. 3G, I). Cell aggregation appears absent in those cells treated with NH4OH prepared \( \text{A} \beta_{42} \) (Figs. 3D, F). This result indicates that peptide produced by each method of preparation interacts differently with the yeast cell walls.

Flow cytometry for analysis of fluorescence intensity associated with peptide
The fluorescence intensity associated with ThT level on the surface of the yeast cells treated with HFIP- and NH4OH-pretreated amyloid peptide was measured by flow cytometry. Data showed an increase in the fluorescence intensity (FI) in the presence of HFIP pretreated peptide (Fig. 4C) compared with the NH4OH method (Fig. 4D), and untreated controls (Fig. 4A): fluorescence intensity was measured to be 8.4%, 5.4% and 3.7% respectively (see Figs. 4C, D). It seems the peptide conformation generated from the HFIP method has a greater propensity to induce aggregation and may cause amyloid deposition on the surface of yeast cells.

TEM analysis of peptide conformers generated by pretreatment
The \( \text{A} \beta_{42} \) prepared for viability counts was analyzed by TEM. The NH4OH method resulted in more uniform aggregate free peptide solution (Fig. 5A) compared with HFIP (Fig. 5C). The latter showed the presence of protofibrils structures at 0 h. After 24 h incubation at 37°C short fibrils were observed in NH4OH prepared peptide (Fig. 5B) while, the HFIP method (Fig. 5C) resulted in long and twisted fibrils (Fig. 5E).

Effect of fibrils on the yeast cells
The toxicity of fibrils, formed after prolonged incubation, was examined. Fibrils generated from HFIP and NH4OH pretreatment were both toxic to yeast cells (Fig. 6). However, HFIP prepared \( \text{A} \beta_{42} \) fibrils exhibited greater toxicity compared to those fibrils prepared with NH4OH.

Discussion
The current study indicates that different preparations of \( \text{A} \beta_{42} \) peptide can lead to different biological activity toward yeast. The recently introduced NH4OH pretreatment of synthetic \( \text{A} \beta_{42} \) reportedly results in formation of higher levels of monomeric peptide\(^{32}\) and has not previously been tested on yeast. Soluble

![Figure 1](image1.png)

**Figure 1.** Effect of HFIP and NH4OH pretreated synthetic \( \text{A} \beta_{42} \) on the yeast cells. 2 \( \mu \text{M} \) peptide was added to exponential phase \( C.\text{glabrata} \) (A) and \( S.\text{cerevisiae} \) (B) cells, suspended in water. Samples were incubated at 30°C for 48 h. Viability was determined as percentage of untreated cells (control). (*\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.005 \), and ****\( P < 0.001 \)). Data are shown as Mean ± SEM.

![Figure 2](image2.png)

**Figure 2.** Effect of \( \text{A} \beta_{42} \) on the quiescent and non-quiescent fractions of \( C.\text{glabrata} \). The quiescent daughter cells (A) were more resistant to \( \text{A} \beta_{42} \) prepared with HFIP method than non-quiescent mother cells (B) in the first 24 h of incubation. After 48 h of incubation both cell fractions were susceptible to the toxicity effect of \( \text{A} \beta_{42} \) (***\( P < 0.005 \)).
monomers of the Aβ42 prepared by NH4OH caused cell proliferation and an increase in the number of colonies in both S. cerevisiae and C. glabrata, regardless of the type of solvent (H2O or dilute NaOH). With prolonged incubation more fibrils were formed and these were toxic to S. cerevisiae cells.

In contrast, the Aβ42 peptide prepared by the HFIP method contained high levels of aggregates and caused cell death within the first 24 h as well as after this time. Older cells were shown to be more susceptible to the effects of Aβ42. The Aβ42 peptide conformation appears to be the major determinant of its effect on yeast cells. Previous studies showed that Aβ42 peptide treatment, there was some ThT staining which may be attributed to proteins such as Flo1p, Als1p, Als5p, Muc1p and Bgl2p. While there is clear evidence for peptides from Flo1p, Als1p, Als5p, and Muc1p to be amyloid forming, the evidence for these proteins forming amyloid on the yeast surface is currently incomplete and limited to the birefringence and ThT staining that they cause. While it is tempting to speculate that Aβ42 may be forming amyloid on the yeast cell surface, proof of amyloid formation requires analysis of the resistance to an ionic detergent. This evidence is still lacking for other yeast cell surface proteins.

HFIP-treated Aβ42 resulted in peptide with long strand fibril morphology compared with the NH4OH method. It is known that HFIP pretreatment method does not efficiently generate aggregate free solutions. This HFIP pretreatment results in peptide that has a high propensity to adhere to the yeast cell wall. In this form the peptide in any size, monomeric, oligomeric and even fibrils are toxic to yeast cells. On the other hand, Aβ42 that is freshly pretreated with NH4OH caused cell proliferation due to

![Figure 3. Staining of amyloid proteins present on S. cerevisiae cell walls by thioflavin T. First row (A–C) shows untreated S. cerevisiae cells in presence of ThT in comparison with cells treated with 5 μM NH4OH (D–F) and HFIP (G–I) prepared Aβ42 for 24 h in H2O. ThT staining comparison of untreated (control), NH4OH and HFIP pretreated cells are shown in panels A, D and G respectively. The HFIP pretreatment of Aβ42 results in higher fluorescence intensity and adhesion of peptide to the S. cerevisiae yeast cell wall protein. Scale bar = 50 μm.](image-url)

The attachment of the Aβ42 peptide to the yeast cell surface has been demonstrated by Bharadwaj (2011) who demonstrated direct binding of fluorescein-labeled Aβ42 to the cell surface by fluorescence microscopy. This finding was further confirmed by electron microscopy and western blotting of sub-cellular fractions. In this study we showed that the Aβ42 peptide caused increased ThT staining of the yeast cell surface. Without Aβ42 peptide treatment, there was some ThT staining which may be attributed to proteins such as Flo1p, Als1p, Als5p, Muc1p and Bgl2p. While there is clear evidence for peptides from Flo1p, Als1p, Als5p, and Muc1p to be amyloid forming, the evidence for these proteins forming amyloid on the yeast surface is currently incomplete and limited to the birefringence and ThT staining that they cause. While it is tempting to speculate that Aβ42 may be forming amyloid on the yeast cell surface, proof of amyloid formation requires analysis of the resistance to an ionic detergent. This evidence is still lacking for other yeast cell surface proteins.

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to monomeric and oligomeric forms while prolonged incubation led to a fibrillar conformation which was toxic to S. cerevisiae cells.

The data presented here show that different methods for the preparation of Aβ42 can lead to different biological activities in in vitro and in yeast biological assays. This work should serve as a caution in working with and interpreting the outcomes of biological assays.

Materials and Methods

Yeast strains and growth conditions

C. glabrata (ATCC90030) and S. cerevisiae BY4743 (a/α his3Δ/bis3Δ leu2Δ/leu2Δ +/lys2Δ met15Δ/+ ura3Δ/ura3Δ) were utilised for experiments and were grown in YEPD (1% yeast extract, 2% peptone, and 2% D-glucose) with aeration at 30°C. For fractionation of quiescent and non-quiescent cells, growth in the YEPD media continued for 7 d with shaking (200 rpm). Cells were subsequently pelleted and washed for further analysis. For exponential phase of growth, yeast cells were grown overnight and were harvested by centrifugation the next day and resuspended in 20 ml fresh media. Then one ml was incubated into 80 ml of fresh media and incubated at 30°C with shaking (200 rpm) for another 2-3 h to reach an OD600 of 205 1.0.

Fractionation of stationary phase C. glabrata and S. cerevisiae

Percoll density gradients (GE Healthcare, United Kingdom) were prepared according to the manufacturer’s preformed gradient protocol and method described by Allen et al.40 with some modification. Briefly, Percoll was diluted 9:1 (v/v) with 1.5 M NaCl. To form a gradient 10 ml of the Percoll solution was added to 15 ml Corex tubes and centrifuged for 15 min at 20°C in a Beckman Coulter centrifuge (SW41Ti rotor). Stationary phase yeast cells which were grown for 7 d at 30°C with shaking were then pelleted and resuspended in 1 ml Tris buffer (50 mM, pH 7.5). Around 2 ml of cell suspension was overlaid onto preformed gradient, and centrifuged at 400 g for 60 min in a Beckman Coulter centrifuge. Fractions of quiescent (lower fraction) and non-quiescent cells were collected and washed twice in 40 ml Tris buffer to remove any Percoll residue, pelleted and then resuspended in ddH2O.

Preparation of Aβ42 peptide

Synthetic human Aβ42 peptide was purchased from Keck laboratories (Yale University, New Haven, CT). The peptide was synthesized and purified using tBOC chemistry with DCC and HOBT coupling reagents in the form of lyophilized powder. All solvents used for the preparation of Aβ42 solutions were filtered and centrifuged to minimise the presence of debris that could induce aggregation of the peptide.

Preparation of Aβ42 using NH4OH

Peptide solutions were prepared according to the protocol of Ryan et al.32 Briefly, 20 mg peptide was dissolved in 40 ml of 10% NH4OH to final concentration of 0.5 mg/ml solution (w/v). The solution was then left at room temperature for 10 min and then sonicated for 5 min. For yeast treatments, the peptide was prepared immediately prior to use. Each 0.5 mg aliquot of Aβ pellet was dissolved in 60 µl of ddH2O and then vortexed to promote the dissolving of the peptide. The solution was then sonicated for 5 min and centrifuged at 14,000 g for 5 min to...
pellet any undissolved peptide. The supernatant was carefully recovered for use in subsequent assays. Peptide concentration was determined from the \( A_{214\text{nm}} \) measurement of the solution in a quartz cuvette (BioMate™ Spec, Thermo Scientific, UK).

**Preparation of A\( \beta_{42} \) using HFIP**

A\( \beta_{42} \) was dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) according to the method described by Crescenzi and colleagues.\(^{39}\) Briefly the peptide film was dissolved in HFIP to a concentration of 1 mg/ml, sonicated for 5 min on ice and dried overnight to form a clear film. The film was subsequently dissolved in 60 \( \mu \)l of ddH\( _2\)O, sonicated for 5 min and centrifuged to remove insoluble aggregates. Peptide concentrations were determined as described above.

**Effect of prolonged incubation of A\( \beta_{42} \) on the survival of yeast**

The A\( \beta_{42} \) samples from each pretreatment method were incubated at 35\( ^\circ \)C for 6 d. Exponentially growing \( S.\ ceriseiae \) were washed and suspended in H\( _2\)O and then were treated with 5 \( \mu \)M of HFIP and NH\( _4\)OH pretreated peptide and incubated overnight. Treatment and viability were determined as follows.

Exponentially growing yeast cells were washed twice and resuspended in ddH\( _2\)O. After estimation of cell numbers by a haemocytometer, cells were transferred into 96-well culture plates at a concentration of \( 10^3 \) cells/ml, treated with 2-5 \( \mu \)M of A\( \beta_{42} \) peptide in a final volume of 200 \( \mu \)l per microtiter well and incubated overnight at 30\( ^\circ \)C with shaking. The next day 100 \( \mu \)l aliquots of cell suspension were plated onto YEPD plates and incubated for 2 days at 30\( ^\circ \)C. Colonies were counted for each treatment and the viability was estimated as percentage of untreated cells. All treatments were performed in triplicate.

**Flow cytometry analyses after Thioflavin T staining of treated cells**

Exponentially growing \( S.\ ceriseiae \) were washed and incubated in H\( _2\)O in the presence and absence of pretreated HFIP and NH\( _4\)OH peptide. After 16 h of incubation, Thioflavin T (ThT) was added to each sample to a final concentration of 20 \( \mu \)M and incubated for 10 min.\(^{40}\) Around 10,000 cells per sample were analyzed by flow cytometry (FACS, Canto II-BD™) using a pacific blue filter with 351 nm excitation and collecting fluorescent emission at 450/65. Data were recorded and saved as FCS3 files. All data were analyzed by WEASEL (WEHI, Melbourne, Australia) software. ThT fluorescence intensity of multiple experimental samples was calculated as percentage of unstained yeast cells.

**Confocal microscopy after Thioflavin T staining of treated cells**

Confocal fluorescence and differential interference contrast (DIC) microscopy was performed using a Nikon® Eclipse Ti...
Transmission electron microscopy

Freshly prepared aliquots of Aβ42 peptide with both pretreatment methods were used for imaging the starting peptide mixture. The peptide samples were then incubated overnight at 35°C for analysis of peptide conformation after 24 h. Samples from 0 and 24 h were gently agitated before transferring a 4 μL aliquot onto the grids. After 30 sec adsorption time, the excess sample was drawn off using Whatman® 541 filter paper. The grids were stained subsequently with 2% w/v potassium phosphotungstate (pH 7.2) for 10 sec. The grids were then air dried before examination. The samples were examined using a Tecnai™ Microscope (FEI, Eindhoven, The Netherlands) at an operating voltage of 120 kV. Images were recorded using a Megaview III CCD camera and AnalySIS camera control software (Olympus Australia).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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