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# Bacoside-A, an anti-amyloid natural substance, inhibits membrane disruption by the amyloidogenic determinant of prion protein through accelerating fibril formation

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ABSTRACT

Bacosides, class of compounds extracted from the *Bacopa monniera* plant, exhibit interesting therapeutic properties, particularly enhancing cognitive functions and putative anti-amyloid activity. We show that bacoside-A exerted significant effects upon fibrillation and membrane interactions of the amyloidogenic fragment of the prion protein [PrP(106–126)]. Specifically, when co-incubated with PrP(106–126), bacoside-A accelerated fibril formation in the presence of lipid bilayers and in parallel inhibited bilayer interactions of the peptide aggregates formed in solution. These interesting phenomena were studied by spectroscopic and microscopic techniques, which suggest that bacoside A-promoted fibrillation reduced the concentration of membrane-active prefibrillar species of the prion fragment. This study suggests that induction of fibril formation and corresponding inhibition of membrane interactions are likely the underlying factors for ameliorating amyloid protein toxicity by bacoside-A.



## 1. Introduction

Prion protein (PrP) is the pathogenic substance responsible for transmissible spongiform encephalopathies (TSE), fatal diseases characterized by loss of motor control, dementia, and paralysis [1]. The "prion hypothesis" proposes that native cellular protein PrP ($PrP^C$), a soluble protein rich in α-helical structures, transforms into the "scrapie form" ($PrP^{Sc}$), characterized by high β-sheet structures, consequently inducing further aggregation of the native protein [2]. According to this model, the fibrillar aggregates of PrP are responsible for disease initiation and progression [3]. The molecular factors associated with conversion of $PrP^C$ to $PrP^{SC}$ are still not fully resolved, however *cellular membranes* are believed to be intimately involved in the process [4,5]. Furthermore, membrane surfaces might constitute conduits for prion toxicity [6]. Previous studies reveal that membranes and membrane interactions intimately affect the misfolding pathways of amyloidogenic proteins' fibrillation [7–13]. In particular, lipid bilayers have been previously shown both to promote fibrillation of PrP fragments [14–16], as well as constitute a target for binding and interactions of prion aggregates, presumably oligomeric species [17–19].

While virtually all protein-misfolding diseases such as TSE are currently incurable, there is an intense search for therapeutic remedies. In particular, there have been significant efforts towards identification of *small molecules* that are capable of modulating protein aggregation and reduce amyloid protein toxicity [20–23]. *Bacoside A*, mixed saponins that are the active compounds of the medicinal plant *Bacopa monniera*, is used in traditional Indian medicine to treat various nervous disorders and contribute to memory enhancement [24,25]. Recent studies have suggested that bacoside-A might exhibit therapeutic effects against amyloid diseases, such as Alzheimer's disease [26,27].

Here, we investigate the interactions between bacoside-A and the 21-residue amyloidogenic determinant of the prion protein [termed PrP(106–126)] [14,16,17,28]. In particular, we assessed the consequence of bacoside-A/prion interactions upon ***membrane bilayers***. The experimental data reveal acceleration of PrP(106–126) fibril formation in the presence of lipid bilayers. Importantly, we found that he enhanced fibrillation of the peptide, induced by bacoside-A, went hand-in-hand with significantly reduced membrane interactions and bilayer disruption. This work demonstrates a direct relationship between externally-induced accelerated fibrillation and inhibition of membrane interactions, and points to possible use of bacoside-A and

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other aggregation modulators as potential therapeutic agents for TSE and amyloid diseases in general.

## 2. Experimental section

### 2.1. Materials

PrP(106–126) having the sequence KTNMKHMAGAAAAGAVVGGLG was purchased from Peptron (South Korea) in a lyophilized form at >95% purity (HPLC), bacoside A was purchased from Natural Remedies (Bangalore, India), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (DOPG), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DMPG), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (N-NBD-PE), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (N-Rh-PE) were purchased from Avanti Polar Lipids. Thioflavin T (ThT), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), sodium hydrosulfite, sodium phosphate monobasic and Dimethyl Sulfoxide (DMSO) were purchased from Sigma-Aldrich (Rehovot, Israel).

### 2.2. Sample preparation

PrP(106–126) was dissolved in HFIP at a concentration of 2.5 mM and stored at −20 °C until use to prevent aggregation. For each experiment, the solution was thawed, and the required amount was dried by evaporation for 6–7 h to remove the HFIP. The dried peptide sample was dissolved in 10 mM sodium phosphate, pH 7.4, at room temperature. Stock solutions of bacoside A prepared at 2 mM DMSO and diluted into the PrP(106–126) solutions at the required concentrations. All samples used in the experiments contained final DMSO concentration of 0.5% (v/v).

### 2.3. Thioflavin T (ThT) fluorescence assay

ThT fluorescence measurements were conducted at 25 °C using 96-well path cell culture plates on a Varioskan plate reader (Thermo, Finland). Measurements were made on samples containing 50 μM PrP(106–126) in the absence or presence of different concentrations of bacoside A and in the presence of lipid vesicles (final concentration 1 mM). A 192-μL aliquot of the aggregation reaction was mixed with 48 μL of 100 μM ThT in sodium phosphate, pH 7.4, in each well. The device was programmed to record fluorescence intensity every minute for 1 h. Excitation and emission wavelengths were 440 and 485 nm, respectively. The fluorescence curves were smoothed by using a five-point adjacent averaging.

### 2.4. Transmission electron microscopy (TEM).

Peptide aliquots (5 μL) from samples used in the ThT experiments (after 15 min incubation) were placed on 400-mesh copper grids covered with a carbon-stabilized Formvar film. Excess solutions were removed following 2 min of incubation, and the grids were negatively stained for 30 s with a 1% uranyl acetate solution. Samples were viewed in an FEI Tecnai 12 TWIN TEM operating at 120 kV.

### 2.5. Capillary assay

Samples containing 50 μM PrP(106–126) in the absence or presence of 100 μM bacoside A and in the presence of lipid vesicles (final concentration 1 mM) were prepared and immediately inserted into rectangular glass capillaries (CM Scientific, Silsden, UK), sealed and then monitored under light microscope. Images were recorded in time zero and after 30 min.

### 2.6. Förster resonance energy transfer (FRET)

Small unilamellar vesicles (SUVs, DMPC/DMPG at 1:1 molar ratio) were prepared by dissolving the lipid components in chloroform/ethanol and drying together under vacuum, followed by dissolution in sodium phosphate, pH 7.4, and sonication of the aqueous lipid mixture at room temperature for 10 min using a Sonics vibracell VCX130 ultrasonic cell disrupter. Prior to drying, the lipid vesicles were supplemented with (7-nitro-2-1,3-benzoxadiazol-4-yl)-phosphatydilethanolamine (N-NBD-PE) and *N*-Rhodamine-B-phosphatydilethanolamine (N-Rh-PE) at a 500:1:1 molar ratio, respectively. 50 μM PrP(106–126) in the absence or presence of bacoside-A at 1:2 PrP(106–126)/modulator concentration ratio, respectively, was added to the vesicles (final vesicle concentration 1 mM) at t = 0. Fluorescence emission spectra were acquired at different time points (λex = 469 nm) in the range of 490–650 nm using a Varioskan 96-well plate reader (Thermo, Finland).

To calculate the extent of FRET efficiency the following equation was used:

$$Efficiency = \frac{R_i - R_{100\%}}{R_0 - R_{100\%}} \times 100$$

in which R is a ratio of fluorescence emission of NBD-PE (531 nm)/Rhodamine B-PE (591 nm). Ri is the ratio in the peptide/vesicles mixtures, R100% was measured following the addition of 20% Triton X-100 to the vesicles, which causes complete dissolution of the vesicles, and $R_0$ corresponds to the ratio recorded for vesicles without any additives.

### 2.7. Giant unilamellar vesicles labeled with C-dot-DMPC

Amphiphilic carbon dots were prepared according to a published protocol. Briefly, the C-dot labeled lipids were prepared via a phosphorylation reaction between a chloride derivative of dimyristoylphosphatydilcholine (DMPC), a widely-studied membrane lipid [29] and C-dots that were synthesized through a hydrothermal method from 6-*O*-acylated fatty acid ester of D-glucose [30,31].

Giant unilamellar vesicles (GUVs) were prepared through the rapid evaporation method [32]. Briefly, GUVs comprising DOPC and DOPG (1:1 mole ratio) were prepared through dissolving the lipid constituents with 2 mg of C-dots-DMPC dissolved in 500 μL chloroform through vortexing and sonication. The mixture was then transferred to a 250-mL round-bottom flask and the aqueous phase (2.5 mL of 0.1 M sucrose, 0.1 mM KCl, 50 mM Tris solution, pH 7.4) was added carefully with a pipette and stirred gently for ~5 min. The organic solvent was removed in a rotary evaporator under reduced pressure (final pressure 20 mbar) at room temperature. After evaporation for 4–5 min an opalescent fluid was obtained with a volume of approximately 2.5 mL.

### 2.8. Confocal fluorescence microscopy

GUVs were imaged in the absence or presence of PrP(106–126), bacoside A, or their mixtures using a PerkinElmer UltraVIEW system equipped with an Axiovert-200 M (Zeiss, Germany) microscope and a Plan-Neofluar 63×/1.4 oil objective. The excitation wavelengths of 440 and 488 nm were generated by an Ar/Kr laser.

### 2.9. Fluorescence anisotropy

The fluorescence probe C-dot-DMPC was incorporated into the SUVs (DOPC/DOPG at 1:1 molar ratio) by adding the dye dissolved in chloroform (0.4 mg/mL) to the lipid and drying together under vacuum, followed by dissolution in sodium phosphate, pH 7.4, and sonication of the aqueous lipid mixture at room temperature for 10 min using a Sonics vibracell VCX130 ultrasonic cell disrupter. The fluorescence emission anisotropy of the C-dot-DMPC fluorescence was measured at