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Research article

An extract of the marine alga *Alaria esculenta* modulates α-synuclein folding and amyloid formation

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HIGHLIGHTS

- *Alaria esculenta* extract and fractions alter α-synuclein thermal stability.
- *A. esculenta* fractions inhibit amyloid formation by α-synuclein.
- Results suggest interaction with unfolded α-synuclein.

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ABSTRACT

The conversion of α-synuclein from its natively unfolded and α-helical tetrameric forms to an amyloid conformation is central to the emergence of Parkinson's disease. Therefore, prevention of this conversion may offer an effective way of avoiding the onset of this disease or delaying its progress. At different concentrations, an aqueous extract from the edible winged kelp (*Alaria esculenta*), was shown to lower and to raise the melting point of α-synuclein. Size fractionation of the extract resulted in the separation of these distinct activities. The fraction below 5 kDa decreased the melting point of α-synuclein, whereas the fraction above 10 kDa raised the melting point. Both of these fractions were found to inhibit the formation of amyloid aggregates by α-synuclein, measured by thioflavin T dye-binding assays; this effect was further confirmed by transmission electron microscopy showing the inhibition of fibril formation. Circular dichroism analysis suggested that the incubation of α-synuclein under fibrillation conditions resulted in the loss of substantial native helical structure in the presence and absence of the fractions. It is therefore likely that the fractions inhibit fibrillation by interacting with the unfolded form of α-synuclein.

1. Introduction

Parkinson's disease (PD) is characterized by motor symptoms including balance impairment, rigidity, tremor and slowness, which in turn result from the lack of dopamine in the striatum. PD is the second most common neurodegenerative disorder [1] and, to date, there are no drugs that slow its course [2–4]. It is caused by the loss of dopaminergic neurons in the substantia nigra, a region of the brain involved with reward, addiction and movement. Death of these cells is associated with the presence of cytoplasmic inclusions called Lewy bodies and Lewy neurites.

The neuronal protein α-synuclein (αS) is the major component of Lewy inclusions and it was directly linked to PD when mutations in αS were shown to cause inherited forms of the disease [5]. Other diseases involving αS pathology include Lewy body dementia and multiple system atrophy [6]. The faulty folding (misfolding) of proteins has since emerged as an underlying cause of diverse neurodegenerative diseases [7]. In each of them, the proteins form amyloid, which is a tightly folded β-structure. It is only in this form that the proteins become toxic. Amyloids can range in size from

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oligomers to large aggregates. The amyloid aggregates of αS form the Lewy inclusions typical of PD [8]. The amyloid form of αS was found to have a direct role in the spread of Lewy pathology between neuronal cells [9] and between animals [10]. Self-templated propagation of the amyloid form of αS drives the spread of this protein conformation in a manner reminiscent of prions [11]. The neurotoxic effects of this aggregated αS include interference in vesicle docking on membranes [12], membrane permeabilization [13] and inflammation [14].

Several natural molecules, such as epigallocatechin 3-gallate (EGCG) and curcumin, have been found to alter protein folding and to reduce the accumulation of toxic amyloid forms of αS *in vitro* [15–17] and, as such, they offer promise for prevention of PD or delay of symptom onset. An ethanol/water extract from ginseng (*Panax ginseng*) reduced αS aggregates in a mouse model of PD and prevented locomotor deficits [18]. An Irish moss (*Chondrus crispus*) methanolic extract was shown to reduce αS fusion protein accumulation and motor symptoms in a transgenic *C. elegans* model [19], although amyloid formation and its modulation were not assessed.

Seaweed species can be cultivated along with salmon and invertebrates in integrated multi-trophic aquaculture (IMTA) systems [20]. This coordinated approach aims to enhance the environmental sustainability of aquaculture operations and it is therefore of interest to determine the nutritional and biological properties of these algal species. The prevention or mitigation of PD is an area of growing promise. Therefore, in the current study, extracts of the brown seaweed *A. esculenta* were evaluated for modulation of αS misfolding and amyloid formation.

2. Materials and methods

2.1. Algal samples, extracts and fractions

Samples were obtained from two species of macroalgae in the Bay of Fundy, New Brunswick, Canada, by members of the research team of Dr. Thierry Chopin (University of New Brunswick). The species sampled were *A. esculenta* and *Saccharina latissima* and each was obtained from Charlie Cove (IMTA) and Maces Bay, NB, Canada, on May 15, 2011. Samples were cleaned of dirt and other organisms, rinsed with distilled water, freeze-dried and then ground to a fine powder. Twenty grams of each seaweed powder was extracted with methanol/water (1:1) at 50 °C with stirring for 2 h at room temperature. These mixtures were then centrifuged to obtain clarified supernatants. The solvent was removed from the supernatants using rotary evaporation, then transferred to vials, freeze-dried and weighed. The salts in the extracts were then removed from 1 g of each extract by subjecting it to methanol trituration. Dried extracts were prepared to 50 mg/mL in 75% methanol/water and stored at −80 °C until analysis. The extracts contained a small amount of insoluble material. When aliquots were prepared for analysis, solutions were centrifuged briefly to remove that material and supernatants were transferred to new tubes. Following preliminary studies, extract E5 (*A. esculenta* from Charlie Cove, NB) was selected for further analysis.

2.2. Proteins and reagents

Recombinant human αS was purchased as a lyophilized powder from rPeptide (Bogart, GA, USA). A 2 mg/mL stock solution was prepared and stored in aliquots at −20 °C. Enzymes, buffer components and other reagents used were reagent or molecular biology grade. Water used was tissue-culture grade. Since αS contains no Trp residues, its concentration in solution was determined based upon an extinction coefficient at 275 nm of 5600 M^{-1} cm^{-1} [21].

2.3. Measurement of protein melting points (T_m)

Changes in the melting point of αS were measured by incubating it in 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0, with 1 mM SDS over a range of temperatures in the presence of SYPRO Orange (Sigma-Aldrich, St. Louis, MO, USA), which binds the exposed hydrophobic portions of the protein as it unfolds and undergoes a shift in fluorescence that can be quantified. Incubations were performed in white-walled microplates in a Roche (Laval, QC, Canada) LightCycler 480 real time PCR machine, which allowed fluorescence measurements over a series of temperatures. The excitation and emission wavelengths were 470 nm and 570 nm, respectively and the temperature range used was 25–85 °C. The ramp speed was rapid degrees for the preliminary algal extract screening, which was used to select extract E5 for the current project. The ramp speed was reduced to 1.26 ° per minute for the experiments reported here. This slow ramp speed was outside of the range that could be directly selected within the qPCR software; instead, it was achieved by setting the number of reads per degree sufficiently high to result in this reduced speed. The temperature of greatest rate of change of fluorescence was identified as the T_m of the protein. Changes in the melting point (thermal shifts) with respect to solvent-adjusted untreated αS were monitored in the presence of algal extracts, alongside positive controls containing the chemical chaperoning compound trimethylamine oxide (TMAO, 200 mM).

2.4. Acetone precipitation of the algal extract

Polypropylene microcentrifuge tubes were pre-treated to remove acetone-soluble impurities by soaking them twice for 24 h in glass-distilled acetone. For the precipitation, extract E5 was diluted to 2 mg/mL in water and 100 μL of the extract were added to 400 μL cold acetone in a treated tube, which was then vortexed and incubated for 1 h at −20 °C. After centrifugation for 10 min at 13,000 rpm, the supernatant was transferred to a second treated microcentrifuge tube and then dried under vacuum. Once dry, the material was dissolved in 100 μL of tissue culture water (Sigma-Aldrich, St. Louis, MO, USA). The pellet obtained from acetone precipitation was allowed to dry at room temperature for half an hour after removal of the supernatant and then dissolved in 100 μL of tissue culture water. Both resulting solutions were aliquoted and stored frozen at −20 °C until use.

2.5. Pepsin digestion of the algal extract

Immobilized pepsin beads (Thermo Scientific, Burlington, ON, Canada) were used according to the manufacturer's protocol. The beads were equilibrated in digestion buffer (20 mM sodium acetate, pH 4.5). An E5 extract sample was diluted to 1 mg/mL in tissue culture water and 100 μL were added to the pepsin beads and incubated at 37 °C for 4 h with shaking. The tube was then centrifuged for 5 min at 1000 rpm and the supernatant transferred to a new tube. To monitor the pepsin activity, an identical digestion was performed using bovine serum albumin, carbonic anhydrase II and the mixture of proteins present in the PageRuler™ Prestained Protein Ladder (Thermo Scientific, Burlington, ON, Canada). Starting material and digestion products were analyzed by SDS-PAGE to ascertain that the pepsin was active (results not shown).

2.6. Size fractionation of the algal extract

Fractions were prepared by ultrafiltration of the algal extract E5 at a concentration of 46 mg/mL in 75% methanol/water by making the volume up to 15 mL with the addition of 75% methanol/water and using Millipore Amicon ultrafiltration units

(Cedarlane, Burlington, ON, Canada) with 5 and 10 kDa mass cut-offs. The fraction retained on the 10 kDa cut-off filter was re-filtered on an identical filter in order to reduce possible carryover from the mixed portion between 5 and 10 kDa. The resulting fractions below 5 and above 10 kDa were designated fractions E5A and E5B, respectively.

2.7. Amyloid formation and sampling

Time course analysis of amyloid accumulation within samples of αS was performed using a single starting solution. Thus, for each replicate in a time course series, data were obtained by serially sampling a single solution. Tubes containing 100 mM MES, 1 mM SDS, and 1 mg/mL αS were prepared, containing 4 mg/mL of fraction E5A, E5 B or equivalent solvent (controls). Two 20 μL aliquots were removed from each incubation tube for time 0 and frozen. The tubes were then incubated at 37 °C, shaking at approximately 200 rpm, for specific time intervals, along with controls. Identical aliquots were removed from each tube over the course of the incubation for time series analyses.

2.8. Thioflavin T-binding assay

Thioflavin T (ThT) binding was carried out essentially as described previously [22], with minor changes. Ten μL from each aliquot of αS obtained over the course of the above incubation were added to the wells of a black, flat bottom 96-well plate (Costar) followed by 250 μL of the ThT dye solution. These, along with protein-free controls, were read on a SPECTRAmax® GEMINI XS dual-scanning microplate spectrofluorometer with excitation set to 450 nm and emission set to 482 nm.

2.9. Transmission electron microscopy

Transmission electron microscopy (TEM) was also undertaken on an aliquot of αS from the above incubation, essentially following the previously described procedure [22]. Drops (10 μL) from the samples were pipetted onto Parafilm. A carbon coated copper TEM grid (Canemco, Gore, QC, Canada) was placed on each drop for 5 min, removed and allowed to dry for 10 min, and then placed in 2% uranyl acetate, pH 4.0, for approximately 5 min under low light. The grids were then washed 4 times by floating on a series of drops of water for approximately 1 min each. Grids were then dried by touching them to the edge of a filter paper followed by air drying for approximately 10 min. Sample grids were then viewed by TEM (Hitachi, Mississauga, ON, Canada).

2.10. Circular dichroism analysis

Circular dichroism (CD) measurements were carried out using a Jasco J-810 spectropolarimeter (Easton, MD, USA). Aliquots of αS from the beginning and end of the above incubations were analyzed along with protein-free controls. The samples were placed in a 0.2 mm path-length quartz cuvette and three spectra were collected for each sample over the wavelength range of 190–260 nm and averaged. Since most secondary structure prediction methods based upon CD spectra require data for wavelengths below 200 nm, the data obtained in MES could not be used, as the buffer interferes with the spectra in the 190–200 nm region. Therefore, a method of analysis requiring the specific ellipticity value at 208 nm was used to predict the percentage of α-helix in the protein [23,24].

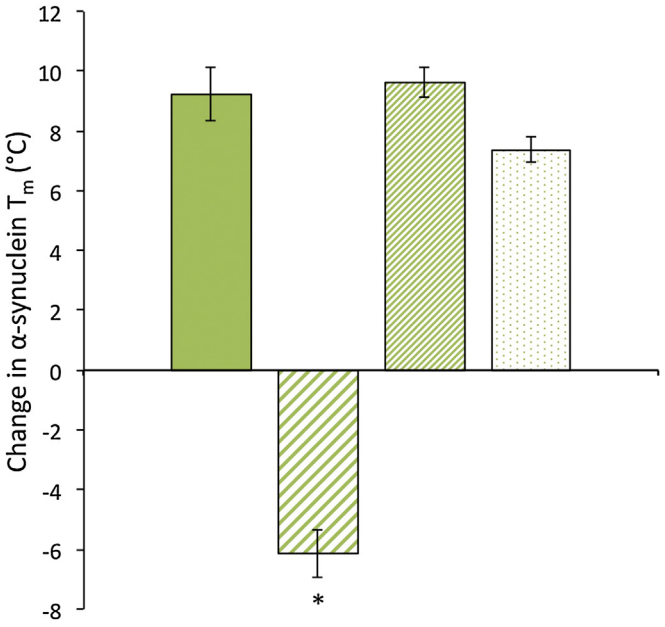

Fig. 1. Effect of chemical and enzymatic treatments on activity of *Alaria esculenta* extract E5 in modulation of αS melting temperature (T_m). Procedures were as indicated in the Materials and Methods. The change in T_m of αS was measured in the presence of untreated E5 (solid green), acetone-insoluble material from E5 (large hatched lines), acetone-soluble material from E5 (small hatched lines) and pepsin-digested E5 (stippled). Data shown are the changes in T_m of αS in the presence of E5 treated in various ways (means ± SEM, n = 3). Means that differ from the untreated extract (ANOVA with Dunnett's test, $p < 0.05$) are indicated by an asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.11. Statistical analysis

Data were processed using Excel (Microsoft, Mississauga, ON, Canada). Statistical analyses included T tests and one-way ANOVA with Dunnett's post test with $p < 0.05$ considered as significant and these analyses were conducted using Minitab statistical software (State College, PA, USA).

3. Results and discussion

3.1. *An* Alaria esculenta *extract and fractions shift the T_m of αS*

The thermal shift, which is a change in the T_m of a protein, was used to identify extracts and fractions thereof that modify the stability of αS. When a thermal shift assay is used to analyze a protein's T_m change, it is important to ascertain that exogenous protein is not present in the sample, as it may contribute to the observed shift or it can generate noise in the data. For this reason, extract E5 was treated in two ways to ensure that no protein was present. In one approach, the extract was treated with acetone, in which the vast majority of proteins are insoluble. In another, the extract was digested with pepsin. The pepsin activity was verified by digesting bovine serum albumin in parallel to the extract under identical conditions, showing complete disappearance of the intact protein (results not shown). The thermal shift of αS was evaluated in the presence of untreated algal extract E5 to determine thermal shift alongside acetone-soluble and precipitated extract components and pepsin-treated extract. Results showed a substantial increase in the T_m of αS in the presence of untreated E5, suggesting considerable stabilization of the αS protein (Fig. 1). The similar increases in the αS T_m in the presence of acetone-soluble and pepsin-treated E5 suggest that the stabilizing component is not a protein, as protein would be digested by the pepsin and

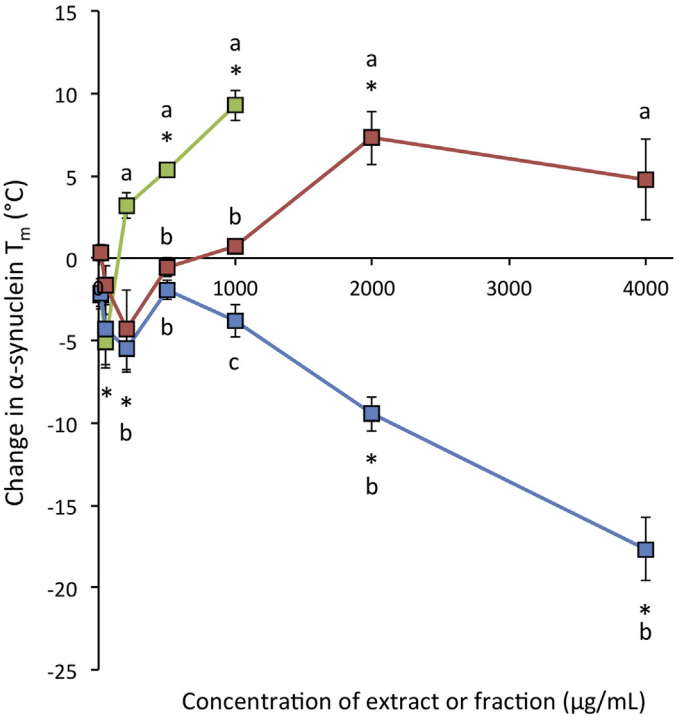

Fig. 2. Change in the melting temperature (T_m) of αS, as observed by SYPRO orange fluorescence as a function of the concentration of *Alaria esculenta* extract E5 and fractions E5A and E5B. Colours indicate E5 (green), E5A (blue) and E5B (red). Procedures were as indicated in the Materials and Methods. Data shown are means ± SEM, n = 3. Means different from the untreated protein (ANOVA with Dunnett's test, $p < 0.05$) are indicated by an asterisk; means that differ among extracts at a given treatment concentration are indicated by letters (T test, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

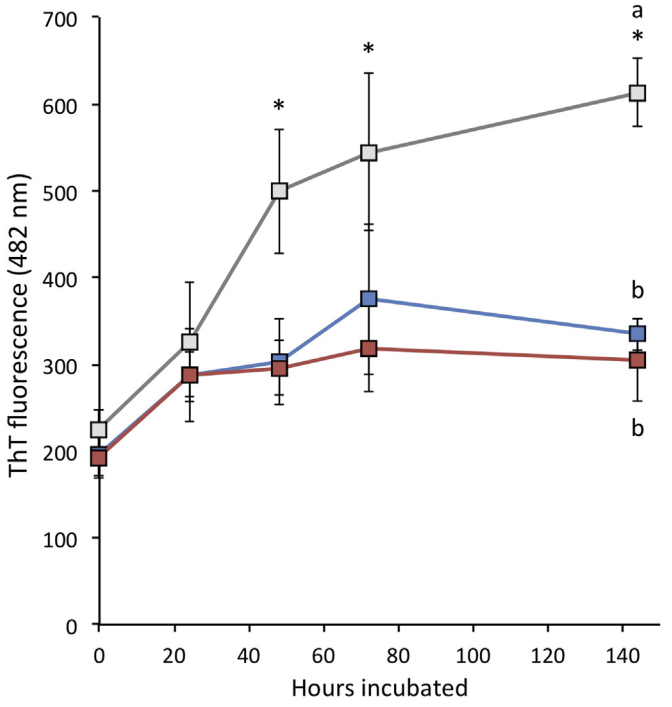

Fig. 3. Effect of *Alaria esculenta* extract fractions E5A and E5B on thioflavin T (ThT) fluorescence in solutions of αS. Procedures were as indicated in the Materials and Methods. Colours indicate αS controls with no algal material present (grey), with E5A (blue) and E5B (red). Means different from initial (pre-incubation) reading (ANOVA with Dunnett's test, $p < 0.05$) are indicated by an asterisk; means that differ among treatments at a specific incubation time are indicated by letters (T test, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the vast majority of proteins are insoluble in acetone. In contrast, the acetone-precipitated material appeared to destabilize the αS, as indicated by a negative shift in the T_m (Fig. 1). Thus, a minor component of the E5 extract, which is acetone-insoluble, has the opposite effect to the whole extract. It is unlikely that this component is another protein, as the presence of a second protein would be expected to increase the variation in the T_m shift and could possibly generate a second T_m peak, which was not observed in these studies (results not shown). However, the error for the acetone-insoluble E5 component-treated αS is consistent with those treated with whole extract and with other components.

The E5 extract and fractions thereof with molecular masses less than 5 (E5A) and greater than 10 kDa (E5B) were evaluated over a series of concentrations for stabilization of αS. At the lower concentrations tested, both extract E5 and fraction E5A brought about significant negative thermal shifts for αS (Fig. 2). The negative shift continued to increase in magnitude with increasing concentrations of E5A, whereas the shift became positive at higher concentrations of E5 and E5B (Fig. 2). These results suggest different concentration dependence of the destabilizing and stabilizing components of E5. Furthermore, the decreased T_m of αS in the presence of both the acetone-precipitated E5 material (Fig. 1) and E5A (Fig. 2) imply the presence of a destabilizing component with low molecular mass (<5 kDa) and low acetone solubility. Conversely, the increase in the T_m of αS when treated with the acetone-soluble portion of E5 or with E5B (Figs. 1 and 2, respectively) are consistent with the possibility of a stabilizing component with greater molecular mass (>10 kDa) and acetone solubility. These results suggest the presence of chemically distinct components within extract 5 (E5) that generate changes in αS stability. The size fractions are a promising first step toward the identification of the components with these contrasting effects.

Native αS can form α-helical tetramers or unfolded monomers [25]. The tetrameric form appears to resist amyloid fibril formation because it would most likely have to unfold before forming the predominantly β sheet structures in amyloid oligomers and fibrils. In agreement with this, an increase in the ratio of monomers to tetramers appears to favour pathogenesis [26]. Therefore an option for preventing Parkinson's disease development is to shift this equilibrium toward the stable folding of tetrameric α-helical αS. The divergent effects of the E5A andE5B extracts on αS thermal stability were intriguing in light of the fact that stable tetramers are suggested to preclude the amyloid transition. Nonetheless, the thermal shift only signifies a change in fold stability; less stably folded forms of a protein would have lower T_m values than those that are more stable. In this context, a positive thermal shift could accompany a transition toward a native helical tetramer or toward an amyloid conformation, either of which may have the greatest stability. A negative thermal shift would be consistent with some extent of unfolding or a more flexible structure. Since the protein was not incubated for amyloid formation prior to the thermal shift assay, it is reasonable to expect that the most stable folded structure adopted by the αS in the case of positive thermal shift was α-helix, but the implications of a negative thermal shift are less clear.

3.2. *Extract fractions from* Alaria esculenta *inhibit the formation of amyloid fibrils by αS*

The conversion of αS to its amyloid form appears to be the key process in the development of Parkinson's disease [1,10]; therefore, the effects of the extract fractions on amyloid formation were

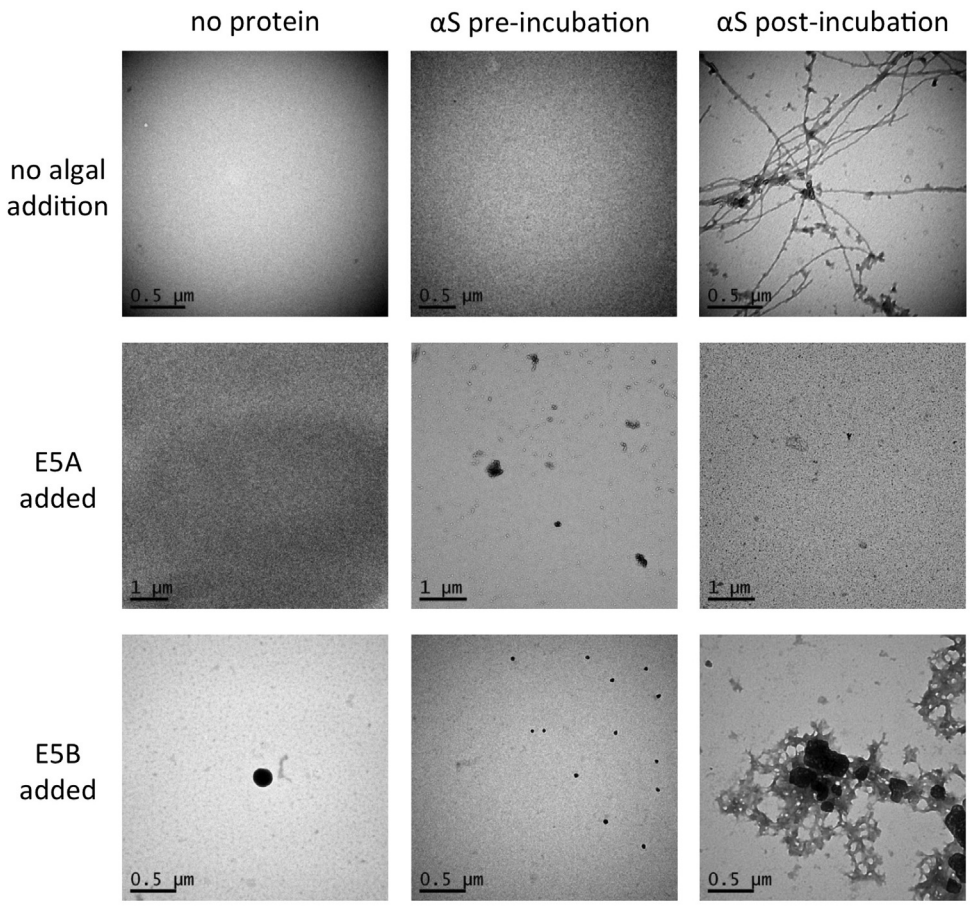

Fig. 4. TEM images of αS treated with *Alaria esculenta* extract fractions E5A and E5B. Procedures were as indicated in the Materials and Methods. Images obtained from the protein-free controls, and from the pre-incubation αS and post-incubation αS solutions are shown in consecutive columns. Consecutive rows are the algal-free controls, E5A-treated and E5B-treated samples. Scale bars are indicated on the images.

examined *in vitro*. The formation of amyloid fibrils by αS is carried out over several days and binding ThT dye is diagnostic of amyloid formation [27]. The incubation of αS at 37 °C with agitation resulted in a significant increase in ThT fluorescence after two days, indicative of amyloid fibril formation, whereas no significant increase in fluorescence occurred in the presence of E5A or E5B (Fig. 3). Although theses fractions had respectively decreased and increased the T_m of αS, they both appeared to inhibit the conversion to an amyloid form. TEM analysis of the products obtained after 6 days of incubation suggested divergence in the effects of these extracts. Although typical amyloid fibrils were visible in the untreated controls, there were no aggregates present in the presence of E5A and distinct aggregates devoid of fibrils were evident in the presence of E5B (Fig. 4). Both αS and aβ protein has been reported to form alternative non-amyloid aggregates in the presence of amyloid inhibitors [15,16,28,29]. Such a scenario may explain the different effects of the two extract fractions. E5A appears to inhibit aggregation completely, whereas E5B may be generating off-pathway non-amyloid aggregates.

3.3. *Secondary structure of αS in the presence of extract fractions from* Alaria esculenta

Under physiological conditions, αS exists both in an α-helical folded conformation and in an extended, natively unfolded structure [25,30], whereas amyloid fibrils are composed of β-sheet. Therefore, analysis of the secondary structure composition of αS offered insight into the folded structure of the protein under the conditions used in this study and in response to the algal fractions. The CD spectra of αS samples obtained prior to the incubation for fibril formation showed minima at 208 and 222 nm, consistent with the presence of substantial α-helix. The magnitudes of these minima were reduced in the presence of E5A and E5B, in comparison with those of control samples, suggesting that the extracts partially unfolded or otherwise destabilized the protein without introducing any change in overall structure (Fig. 5a). The spectra from samples obtained following the incubation had lower magnitudes and they revealed no minima consistent with predominant helix. These spectra each showed a single minimum between 217 and 221 nm, suggesting the possibility of increased β-sheet (Fig. 5b).

The MES buffer used in the fibril formation experiments was incompatible with data collection below 200 nm. The absence of those data precluded reliable deconvolution of the spectra to determine the proportions of secondary structures. Therefore, the proportion of α-helix was determined using the method of Greenfield and Fasman [23]. Prior to incubation, the helical contents of the αS in the presence of solvent alone, E5A and E5B were 32%, 30% and 27%, respectively. This shows that nearly a third of the protein in these samples was substantially helical. This further suggests that the structure that was observed (and melted) in the thermal shift assay was most likely native α-helix. Following incubation for fibril formation, αS showed helical contents of 22%, 20% and 19%, respectively. This reduction in αS α-helix content was observed in all samples after incubation, suggesting some extent of unfolding or β-sheet formation over the course of the incubation. Although the magnitude of the spectra differed among the untreated sample and those treated with E5A and E5B, they changed in the same manner with incubation. This suggests that the extract fractions

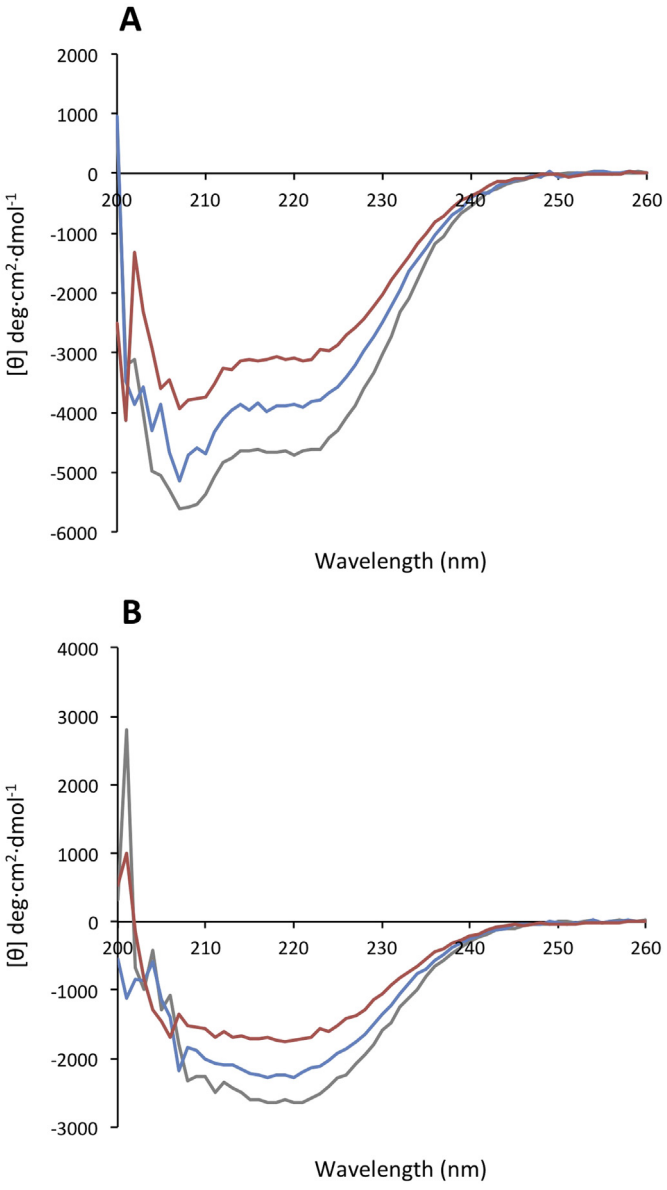

Fig. 5. Circular dichroism analysis of αS treated with *Alaria esculenta* extract fractions E5A and E5B. Procedures were as indicated in the Materials and Methods. Colours are as indicated in Fig. 3. Spectra were obtained for control αS solutions and those with E5A or E5B, both pre-incubation (panel A) and post-incubation (panel B).

reduce amyloid fibril formation by preventing aggregation of αS subsequent to the loss of substantial native helical structure, likely by interacting with the unfolded form.

4. Conclusions

The north Atlantic ocean is a largely unexplored resource in terms of organisms that have adapted to live in cold and variable environments. Yet, these are situations that are known to challenge protein folding. Therefore, they should be ideal for the discovery of molecules that prevent toxic protein misfolding. The findings here show that *A. esculenta* from the Bay of Fundy has molecules capable of altering αS protein folding and preventing its conversion to an amyloid conformation. This study also demonstrates a useful suite of assays allowing efficient evaluation of extracts from species in these environments that can be applied to any resource for protein folding studies.

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