The effect of *Curcuma longa* extract on the rate of aggregation and concentration of proteins in albumen.

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Technical Note

ABSTRACT

Protein aggregation may cause the loss of drug potency, alter pharmacokinetic profiles or induce unwanted immunogenicity. Albumen contains a variety of proteins and exhibits non-reversible aggregation in the presence of denaturing stimuli. The effectiveness of *Curcuma longa* extract, containing the principal curcuminoid curcumin, in preventing or decreasing the rate of aggregation and retaining functional proteins in albumen was tested using a laser and light sensor. The presence of *C. longa* extract in the albumen decreased the rate at which the solution became opaque when denatured through the application of heat. The data suggests that *C. longa* extract decreases the rate of aggregation of the proteins present in albumen. To evaluate if the proteins remaining in the albumen solution after heating were functional, a Bio-Rad protein assay was performed. The results suggest that in the presence of *C. longa* extract more functional proteins remain in solution.

KEY WORDS: Curcumin, protein aggregation, neurodegenerative diseases, Alzheimer's disease

INTRODUCTION

Amyloidosis is the general term for conditions that stem from the accumulation of amyloids or insoluble fibrous protein aggregates. These conditions exist in Type II Diabetes, Alzheimer's and Parkinson's diseases. These diseases are a result of either unwanted or unnatural protein aggregation or as a result of mis-folded proteins (1). Protein aggregation may also occur during drug manufacture or storage. In general, aggregated proteins provoke stronger immune responses than soluble proteins reducing the efficacy of the drug. Immunogenicity to protein drug aggregation is particularly dangerous since it may cause the body to become unresponsive to the administered protein drug and change vital functions (2). Thus aggregation is detrimental to clinical outcomes and may cost pharmaceutical companies time and money.

Protein aggregation is a complex process that occurs in several ways. Most of the mechanisms of aggregation are caused by mis-folding and subsequent non-covalent or covalent attractive

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forces or bonds that prevent the formation of the native folded structure. Mis-folding creates unnatural structural motifs including oligomers, protofibrils, fibrils and finally, amyloids. The hydrophobic core of a protein may be exposed to other hydrophobic patches which can cause aggregation. Immature aggregates are the most toxic as the hydrophobic patches can come into contact with, and damage certain cell components (3).

The literature is replete with studies that have attempted to address protein aggregation. Using a protein from the nematode *Caenorhabditis elegans,* Thioflavin T was shown to extend the lifespan by enhancing protein homeostasis by binding to amyloid plaques (4). Curcumin has similar protein binding properties to Thioflavin T (5) having similar effects on *C. elegans*, but curcumin is less potent (6). However, Thioflavin T is expensive and has adverse side effects if used in higher concentrations. If turmeric (*C. longa*) extract, which contains high percentages of curcumin, is able to reproduce the aggregation preventive results of Thioflavin T, it could be a cheaper, safer alternative (7).

A study on the effect of curcumin on the α-synuclein protein (previously identified as a cause of aggregation in Parkinson's) showed that curcumin was able to decrease the rate of aggregation and clumping and increase the rate of reconfiguration of the backbone in these proteins (8). Thus, more folded, functional proteins from a solution of unfolded proteins were observed in the presence of curcumin.

Albumen contains a variety of proteins and exhibits non-reversible aggregation in the presence of denaturing stimuli. Thus, albumen can be used as a model protein to study the effect of substances from which drugs can be derived for various diseases where protein aggregation is a cause. In this study, the effect of *C. longa* extract on the rate of aggregation of albumen and the concentration of functional proteins was investigated. The rate of aggregation measures the rate at which the proteins become structurally dissimilar while the concentration measures the remaining functional proteins after heat exposure. Heat-denatured proteins clump together, while functional proteins remain in the solution. A measure of the concentration of functional proteins in a solution containing curcumin *versus* one without, is indicative of the benefits of curcumin in preventing aggregation. The greater the concentration of functional proteins that remain after heat exposure, the more instrumental the addition of curcumin is in preventing clumping (9). The objective of the study was to evaluate curcumin's effectiveness in reducing the rate of aggregation while retaining the protein structure and function after denaturation.

MATERIALS AND METHODS

Materials

The albumen was extracted from eggs obtained from Pete and Gerry's® Organic Eggs (Monroe, NH) and used without further modification. An alcoholic extract of turmeric whole rhizome was obtained from Herb Pharma (Williams, OH) and used as is (turmeric to solvent ratio was 1:4, 63-73% organic grain alcohol) (10).

Methods

Preparation of the albumen samples for both experiments

Undiluted samples of albumen were prepared by extracting the albumen from 14 eggs and whisked 15 times. The solution was poured into two bowls. Each bowl contained 120 ml of the albumen preparation. Then 12 drops of *C. longa* extract were added to one bowl. The solutions in both bowls were whisked 7 times and then divided into 10 ml samples. For each experiment 24 samples were prepared, 12 with albumen alone and 12 with albumen plus *C. longa* extract.

Rate of Aggregation

A sample was placed on a hot plate stirrer (Corning PC 320, Marshall Scientific, Brentwood, NH) at 0 RPM at 80°C. A laser beam (λ=650 nm) emanating from a red laser pointer (5mW, GNWE, AAA Accessories from Amazon) was passed through the sample to a

Figure 1 A sample of albumen during heating (Note: the light blocking assembly has been removed to show the details of the equipment set up)

light sensor/detector (Vernier Thermometer Probe, Light Sensor, and Logger Lite Software, Vernier Software and Technology, Beaverton, OR). Outside light was prevented by covering the experimental set up with a PVC light blocking assembly. The experimental setup without the light blocking assembly is shown in Figure 1.

The positions of the sensor and laser were adjusted so that the maximum light was captured by the detector as recorded by the Vernier Logger Lite software. Data was recorded for the 10 ml samples of albumen in a 25 ml beaker placed between the laser and sensor in a fixed position on the hotplate. Slight adjustments were made to maximize light capture.

The data collection parameters in the Logger Lite Software were set to 60 readings per minute for 8 minutes. When the opacity leveled off (no significant changes for ≥5 seconds) the beaker was removed and the recording stopped.

The lux data from the Logger Lite software was analyzed using Microsoft Excel. The range of data, from the highest recorded lux value immediately after the beaker was placed on the hotplate until the lowest value (immediately before the beaker was removed) (±0.1 lux) per sample was fitted to an exponential equation. Although, in most cases, the aggregation followed bi-exponential kinetics, the data was modeled using a single exponential since this adequately reflected relative rates of aggregation. Since opacity was used as a measure of aggregation, the coefficient of x in the regressed exponential equation was used as a measure of the slope and of the rate of aggregation.

Concentration

The above prepared samples containing only albumen, and those containing albumen with *C. longa* extract were used. All the samples were heated for 5 minutes at 80°C with no stirring. The contents of the beakers were poured into separate centrifuge tubes and centrifuged at 500 g for 15 minutes (Ultra 8V Centrifuge, Irvine Scientific, Lawrenceville, GA).

The Quick Start™ Bradford protein assay (Bio-Rad, Hercules, CA) was prepared using the manufacturer's recommended procedure (9).

12 samples of the 100 µl mixtures from the center of each centrifuge tube were prepared (a total of 24 samples, 12 with albumen alone and 12 with albumen and the *C. longa* extract). 6 ml Bio-Rad protein assay was added to each cuvette. After 5 minutes, each cuvette was covered with Parafilm and manually agitated. A Spectronic 20 D+ Spectrophotometer (ThermoFisher, Madison, WI) was adjusted to 595 nm and the absorbance values were recorded for each sample. Finally a sample of *C. longa* extract was tested using the same procedure. The absorption was plotted along the Bovine Serum Albumin curve (standard graph comparing absorption and concentration using equation 0.8506(x) + 0.1102) and the concentrations were recorded. The Bovine Serum Albumin was manufactured by the Bio-Rad Laboratories (9). The molecular weight of the Bovine Serum Albumin is ~66 kDa. (11).

The results were analyzed using a one-tailed *t*-test. Differences were considered significant at $p<0.05$.

RESULTS

Aggregation

As previously discussed, the values for the slope of aggregation were determined by fitting the data to exponential functions (see Figure 2) using Microsoft Excel, and taking the slope as the x coefficient from the regressed equation in the form $y = a*e^{mx}$.

Figure 2 shows how each rate of aggregation was determined. It shows the first experiment using the albumen solution. The same process was used to obtain values for all the other experiments.

Concentration

The equation used to relate the recorded spectrophotometric values to concentrations was 0.8506(x) + 0.1102 where x is the absorbency.

The absorbency of the albumen solution containing the *C. longa* extract was adjusted for the absorbency of the *C. longa* extract. As shown in Table 1, the concentration of proteins in the albumen solution containing the *C. longa* extract greater than for albumen alone.

The results for both series of experiments are summarized in Table 1. The results are expressed as the mean ± standard deviation.

Table 1 Rate of Aggregation (lux/minute) and Concentration of Proteins (µg/ml) in Albumen (n=12), $p<0.05$

AVERAGE RATE OF AGGREGATION DURING HEAT DENATURATION (lux/min)		AVERAGE CONCENTRATION OF PROTEINS REMAINING AFTER HEAT DENATURATION (µglml)	
Without extract	**With extract**	**Without extract**	**With extract**
-3.953±2.458	-1.646±1.024	0.937±0.182	1.253±0.212

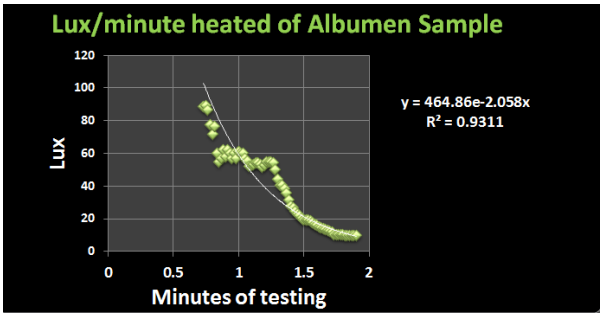

Figure 2 Lux *versus* Time graph for Albumen Solution Displaying exponential Regression Line and Equation. The measure of the slope in Experiment Number 1 was -2.058 lux/min.

DISCUSSION

Consistent with previous literature, specifically the paper reporting the effect of curcumin on α-synuclein (8), curcumin in this study was able to reduce the aggregation of proteins in albumen (p=0.005).

It should be noted that the data for the concentration of functional proteins in albumen with *C. longa* extract may be underestimated. (note that concentration is measured based on dye penetration, but by the time the dye is added, the curcumin has bound to the ovalbumin and the dye may be unable to bind to its maximum extent with the ovalbumin). This may have resulted in a lesser concentration of functional proteins (than actual one) in the albumen containing the *C. longa* extract solution. However, even with this underestimation, the concentration of the functional proteins in the albumen containing the *C. longa* extract was significantly greater (p=0.005) than their concentration in the solution without the extract.

Further experiments are needed to confirm these results. To investigate the hypothesis that the backbone reconfiguration of proteins is faster in the presence of *C. longa* extract, the diffusion coefficient of ovalbumin with, and without, *C. longa* extract present should further be investigated.

CONCLUSIONS

The rate of aggregation of egg-white proteins in the presence of *Curcuma longa* extract was significantly less (p=0.005) than without *C. longa*. The concentration of functional proteins in egg-white that remained in the presence of *C. longa* extract was significantly greater when the albumen was subjected to heat denaturation (p=0.005). Curcumin, in its solubilized state, may be effective in decreasing protein aggregation.

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