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Original Article

Investigation of the anti-amyloidogenic effect of quercetin and choline bitartrate

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ABSTRACT

Plants contain many anti-amyloidogenic molecules that can play a significant role in the prevention of currently incurable neurodegenerative diseases. Our experiments proved that quercetin and choline bitartrate are anti-amyloidogenic agents *in vitro*, as they effectively inhibit the formation of α-chymotrypsin amyloid fibrils at pH 7.0 in 55% ethanol. The effect of quercetin and choline bitartrate on protein aggregation and fibril formation was concentration-dependent based on turbidity measurements and Congo red binding assays.

KEYWORDS: choline bitartrate, Congo red, protein aggregation, quercetin, turbidity.

1. INTRODUCTION

As the average age increases, the frequencies of currently incurable protein conformational diseases, including neurodegenerative diseases, increase, and hence their prevention is extremely important [1-3]. Nowadays, there is great interest in compounds that can effectively inhibit protein misfolding [4, 5]. Currently, approximately 35 million people worldwide suffer from Alzheimer's disease [6]. Plant-based foods contain many natural molecules that may pay a significant role in the prevention of neurological disorders [7-9]. These molecules bind to soluble oligomers during aggregation, inhibiting further oligomerization and the formation of amyloid fibers [10]. Examples of such compounds are quercetin and choline.

Oxidative stress is common in many diseases [11]. Quercetin is an important natural polyphenol, a strong antioxidant and metal ion chelator, which is present in many foods and medicinal herbs. During the transformation of a protein into amyloid fibrils, the polyphenol can bind specifically to the amino acid residues tyrosine, phenylalanine and tryptophan. Quercetin inhibits insulin fibrillation and destabilizes mature insulin fibrils. The anti-amyloidogenic and fibril destabilizing effect of quercetin is due to hydrophobic interactions, aromatic stacking and hydrogen bonds [12]. Higher hydrophobicity facilitates aggregation [13]. Quercetin has been shown to alleviate behavioral and cognitive impairment in several neurodegenerative diseases. Quercetin improves learning and memory, reduces senile plaques and mitochondrial dysfunction [14]. Quercetin protects against aging. Because quercetin is a lipophilic molecule, it easily crosses the blood-brain barrier and exerts a neuroprotective effect [15]. Quercetin is an excellent candidate for the treatment of neurodegenerative diseases associated with protein misfolding and aggregation [16]. In the presence of phenolic hydroxyl groups, the electron density in the aromatic rings increases; therefore the binding of quercetin to the aromatic amino acids in the beta-sheet structure of the peptide may increase. The more hydroxyl groups in a molecule,

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the greater its anti-amyloidogenic activity [17]. Quercetin inhibits the formation of Aβ fibrils in a concentration-dependent manner and destabilizes already formed fibrils [18]. Quercetin inhibits the elongation of fibrils by not binding to Aβ monomers, but primarily to Aβ fibrils at the edge of growth [19]. Quercetin can bind to β-amyloid oligomers in the early stages of aggregation, resulting in the formation of modified oligomers. It inhibits the formation of β-sheet structures, potentially preventing the development of Alzheimer's disease [20]. Quercetin degrades prion fibrils and reduces fibril-induced cytotoxicity and oxidative stress [21]. According to thioflavin T binding, turbidimetric and transmission electron microscopy studies, quercetin can slow down amylin fibril formation [22]. It even promotes the transformation of already formed fibrils into monomers [23]. Quercetin prevents the fibrillation of human calcitonin, in which vicinal hydroxyl groups play an essential role. This structural motif may facilitate the formation of a covalent bond forming a Schiff base with human calcitonin, disrupting critical electrostatic and aromatic interactions in the process of amyloid formation [24]. Quercetin breaks superoxide dismutase 1 fibrils into shorter fragments, but does not depolymerize them completely into monomers. Quercetin prevents the linking of oligomers and non-native monomers formed during aggregation, thereby stopping the elongation of fibrils [25]. Quercetin effectively inhibits the formation of islet amyloid polypeptide amyloid fibrils [26].

Because choline is an essential nutrient, it must be obtained from the diet along with essential amino acids, fatty acids, vitamins, and minerals to maintain health. Choline is found in many foods, and hence a variety of diets can meet choline needs [27]. Foods known to be high in choline include eggs, beef, chicken, milk, fish and certain plant foods [28]. Choline has a neuroprotective effect and improves intellectual performance [29]. The severity of Alzheimer's disease-like symptoms in a mouse model of Alzheimer's disease can be significantly reduced by supplementing the maternal diet with choline during pregnancy and lactation [30]. The results showed that postnatal choline-containing compounds improve memory functions in memory-impaired populations [31]. Not only is this micronutrient critical for normal early brain growth and function, but it may have promising effects on brain function, delaying or mitigating cognitive decline with aging and in neurodegenerative diseases such as Alzheimer's disease [32]. Choline contains a hydroxyl-terminated alkyl chain, which increases its tendency to form H-bonds. The choline ion can not only bind to negatively charged residues, but can also come into contact with other amino acids of peptides and proteins. Examining the effect of choline on protein stability, it was established that the hydrophobic core of the unfolded proteins becomes accessible for interaction through the alkyl part of choline [33]. Choline O-sulfate is a very good inhibitor in preventing $A\beta_{16-22}$ amyloid aggregation [34]. Choline-O-sulfate suppresses the conformational change of human islet amyloid polypeptide from random coil to β-sheet structure, which results in inhibition of amyloid formation [35]. Choline bitartrate effectively inhibits the formation of trypsin amyloid fibrils in the presence of 60% ethanol at pH 7.0 [36].

2. MATERIALS AND METHODS

2.1. Materials

Lyophilized α-chymotrypsin (EC 3.4.21.1; type II from the bovine pancreas) and quercetin were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary). The choline bitartrate was the product of Vital-Trend Ltd. (Budapest, Hungary). All other reagents were of analytical quality.

2.2. *In vitro* α-chymotrypsin fibril formation

The α-chymotrypsin samples were incubated at a concentration of 0.15 mg/ml for 1 day at 24 °C in the presence of 55% ethanol/10 mM phosphate buffer at pH 7.0, in the presence or absence of different concentrations of quercetin or choline bitartrate.

2.3. Turbidity measurements

We can monitor the amount of protein aggregates by measuring turbidity [37]. Turbidity of α-chymotrypsin samples in the absence and presence of various concentrations of quercetin or choline bitartrate were monitored by measuring the absorption at 350 nm. A cuvette with a path length of 1 cm was used for the measurements.

Before the measurements samples were incubated at 24 °C for 24 h. During turbidity measurements, the protein concentration was 0.15 mg/ml in 55% ethanol/10 mM phosphate buffer at pH 7.0. The results of the turbidity measurement experiments were calculated from 3 parallel measurements. Blank corrections were made prior to all experiments.

2.4. Congo red binding assay

Congo red is an amyloid specific dye. The binding of Congo red to amyloid fibrils can be monitored by absorption spectroscopy. When the Congo red dye binds to the amyloid protein, the absorption intensity increases and the absorption maximum shifts to higher wavelengths [38, 39]. 200 µl of 1-day-aged samples containing 0.15 mg/ml α-chymotrypsin in 55% ethanol/10 mM phosphate buffer at pH 7.0 was mixed with 800 µl of Congo red (disodium-3,3'[[1,1-biphenyl]-4,4'-diylbis(azo)] bis(4-amino-naphthalin-1-sulfonate)) solution in 5 mM phosphate buffer/150 mM NaCl at pH 7.0. The absorption spectra of the samples were recorded between 400-600 nm in a 1 cm path length quartz cuvette after incubating them for 15 min at room temperature. Difference spectra were constructed by subtraction of spectrum of α-chymotrypsin alone and Congo red alone from the spectrum of α-chymotrypsin + Congo red. The maximum of the difference spectrum at 540 nm indicates the presence of amyloid fibrils.

2.5. Statistical analysis

The data obtained from turbidity measurements were represented as mean ± standard error (SEM) of the average of three independent measurements. Data were analyzed by one-way analysis of variance (ANOVA).

3. RESULTS AND DISCUSSION

The presence of certain polar organic solvents induces protein aggregation and the formation of amyloid fibrils [40, 41]. During the experiments, amyloid fibrillation of the well-known α-chymotrypsin model protein was performed *in vitro* by incubating the protein in 55% ethanol at room temperature for 24 hours at a pH of 7.0 before measuring the absorption of the samples [42]. The turbidity measurement is suitable for monitoring the anti-aggregation efficiency of an inhibitor [43, 44]. The sample without quercetin showed maximum absorption at 350 nm, while the presence of quercetin caused a significant decrease in the absorption value. According to turbidity measurments quercetin is very effective in inhibiting α-chymotrypsin aggregation *in vitro* (Figure 1). 0.2 mg/ml quercetin almost completely reduced the amount of aggregates compared to the sample without inhibition. Quercetin was effective even at a concentration of 0.025 mg/ml, as the amount of aggregates was reduced to 79% in its presence. The anti-aggregation efficiency of quercetin was proportional to its concentration present in the sample.

Turbidity values were measured both in the presence and absence of different concentrations of choline bitartrate. The measured turbidity values were inversely proportional to the concentration of choline bitartrate. Choline bitartrate showed aggregation-inhibiting activity *in vitro*. At a concentration of 4 mM choline bitartrate, the amount of aggregates decreased to 78% compared to the sample without the inhibitor (Figure 2).

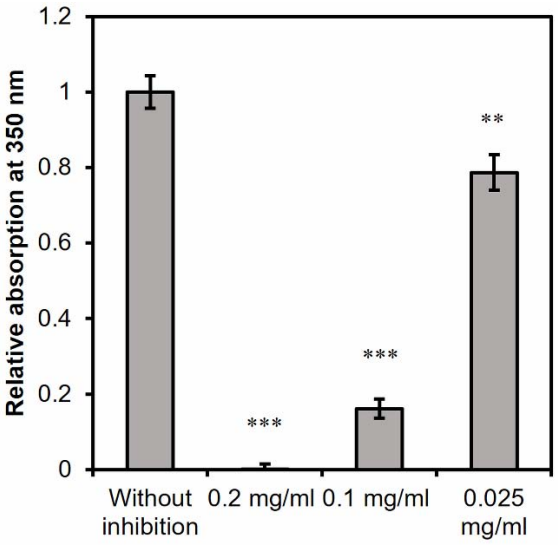

Figure 1. Turbidity measurements in the absence and presence of different concentrations of quercetin by recording the absorption after 24 h incubation at 350 nm in 55% ethanol at pH 7.0. α-chymotrypsin concentration: 0.15 mg/ml. Each bar represents the average of at least three independent measurements. All data are presented as mean ± standard error of the mean (SEM). Significance was defined as ***$P < 0.001$ and **$P < 0.01$.

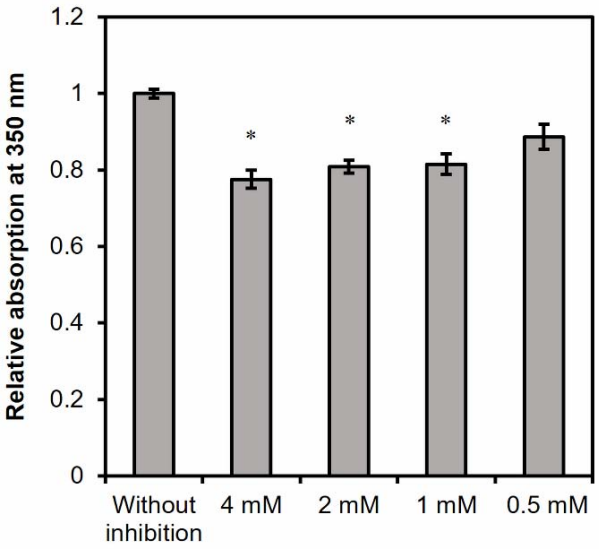

Figure 2. Turbidity measurements in the absence and presence of different concentrations of choline bitartrate by recording the absorption after 24 h incubation at 350 nm in 55% ethanol at pH 7.0. α-chymotrypsin concentration: 0.15 mg/ml. Each bar represents the average of at least three independent measurements. All data are presented as mean ± standard error of the mean (SEM). Significance was defined as $*P < 0.05$.

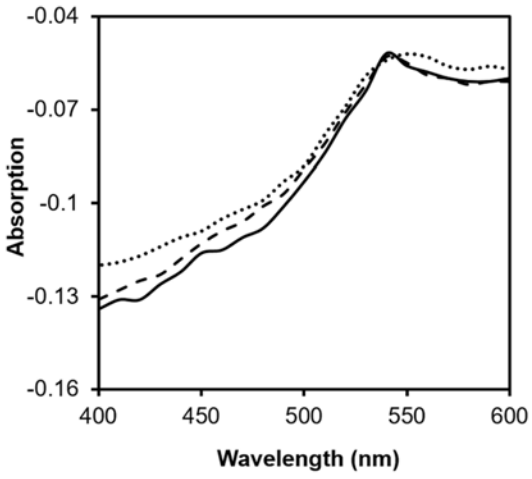

Figure 3. Congo red differential spectra in the absence (solid line) and presence of 0.5 mM (dashed line) and 4 mM (dotted line) choline bitartrate.

Congo red staining is a method used to identify amyloids [45]. When Congo red binds to the β-sheet of amyloid fibrils, light absorption increases and a characteristic red shift of the absorption maximum is observed [46]. Congo red binding experiments are suitable for showing whether an inhibitor inhibits amyloid fibril formation [47]. The presence of an effective inhibitor is accompanied by spectral changes; the slope of the difference spectrum decreases between 450 and 540 nm. In the presence of choline bitartrate, based on the shape change of the difference spectra, it can be established that the amount of fibrils decreased compared to the sample without the inhibitor. In the presence of 4 mM choline bitartrate, there are fewer fibrils in the sample than at a concentration of 0.5 mM, and thus the effect of choline bitartrate proved to be concentration dependent. (Figure 3). Our Congo red binding experiments confirmed that choline bitartrate is an anti-amyloidogenic compound.

4. CONCLUSION

The summary of our results is that quercetin and choline bitartrate effectively inhibit the aggregation of α-chymotrypsin and the formation of amyloid fibrils.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

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