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Original Article

The anti-amyloidogenic effect of hydroxypropyl-β-cyclodextrin and trans-ferulic acid

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ABSTRACT

Protein conformational diseases make the lives of millions miserable worldwide. Therefore, it is very important to search for molecules that inhibit protein misfolding. In our experiments, we investigated the anti-amyloidogenic effect of hydroxypropyl-β-cyclodextrin and trans-ferulic acid using α-chymotrypsin as a model protein at neutral pH in the presence of 55% ethanol. Both inhibitors proved to be effective in inhibiting aggregation and their effect was exerted in a concentration-dependent manner. Trans-ferulic acid has proven to be a very powerful anti-aggregation agent, as it almost completely eliminated the presence of aggregates in the sample at a concentration of 10 mM.

KEYWORDS: α-chymotrypsin, hydroxypropyl-β-cyclodextrin, protein aggregation, trans-ferulic acid, turbidity.

1. INTRODUCTION

The number of people suffering from diseases caused by misfolding of proteins is now estimated at more than 500 million [1]. Therefore, it is very important to search for molecules that inhibit protein misfolding [2, 3], since these diseases cannot be cured [4]. Natural molecules can be new drugs [5-7]. β-cyclodextrins are cyclic oligosaccharides built from 7 D-glucose units. β-cyclodextrins bind to the hydrophobic part of the protein surface, increasing its stability, thus inhibiting misfolding and aggregation. Higher hydrophobicity facilitates the aggregation. β-cyclodextrin inhibited amyloid formation by inhibiting the misfolding of the monomeric form of transthyretin [8, 9]. β-cyclodextrin inhibits alcohol-induced trypsin aggregation. β-cyclodextrin interacts with the native protein, and its inhibitory effect is concentration-dependent. The cavity of β-cyclodextrin can enclose certain amino acid side chains of the target protein through hydrophobic interaction and prevent the subsequent transformation into β-sheets, providing conformational stability to the protein. However, the exact molecular mechanism of the interaction between cyclodextrins and proteins remains unexplored [10]. The combined effect of naturally occurring polyphenols with β-cyclodextrin on the aggregation of α-synuclein was investigated. Polyphenol-β-cyclodextrin combinations not only inhibited protein aggregation, but were also effective in breaking down already formed fibrils [11]. 2-Hydroxypropyl-β-cyclodextrins are produced by condensation of β-cyclodextrin and propylene oxide. The β-cyclodextrin molecule has 21 hydroxyl groups, all of which can condense with propylene oxide. 2-Hydroxy-β-cyclodextrins are actually mixtures of variously substituted derivatives. The number of substituted hydroxyl groups is determined by the conditions of the synthesis [12]. 2-Hydroxypropyl-β-cyclodextrin, administered as eye drops for three months, reduced the amount of Aβ in the retina of aged

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mice by 65% [13]. The internally disordered protein AIMP1/p43 was more stable in complexes with hydroxypropyl-β-cyclodextrin than the free protein [14]. Hydroxypropyl-β-cyclodextrin is nontoxic to cells and greatly inhibits Aβ fibrillation and reduces Aβ-induced toxicity in a concentration-dependent manner. At a 1:2 molar ratio of protein:cyclodextrin, the amount of fibrils was reduced by 60% [15].

Since oxidative stress plays a role in the pathogenesis of many diseases [16, 17], the use of antioxidants may be important for prevention. Ferulic acid is an antioxidant found in plant cell walls that has anti-inflammatory and free radical scavenging properties. Ferulic acid proved to be effective in inhibiting Aβ aggregation [18]. Ferulic acid can also destabilize preformed Aβ fibrils [19]. The dissociation of the peptides from the fibrils probably takes place along the axis of the fibrils by breaking the hydrogen bonds of the backbone and the Asp-Lys salt bridges [20]. At the beginning of aggregation, ferulic acid interacts with Aβ40, resulting in the formation of non-fibrillar amorphous aggregates, which have a β-structured conformation, but tend to turn into monomers [21]. Ferulic acid influences Aβ peptide folding by increasing helical and decreasing non-helical propensities [22]. Experimental results support that ferulic acid can be useful in the prevention and treatment of Alzheimer disease in certain doses [23]. A 10-month administration of ferulic acid *via* food has been shown to reduce Aβ deposits in Alzheimer's disease-transgenic mice [24]. Ferulic acid has been found to be a promising drug candidate as it ameliorates cognitive impairment and ameliorates neuropathological features in the brains of animal models of Alzheimer's disease [25]. Ferulic acid has been shown to be a strong anti-aggregation agent in the case of glucagon [26]. Ferulic acid inhibits collagen fibrillation. The anti-fibrillation effect of this natural phenolic compound is due to the aromatic interactions between ferulic acid and collagen [27]. Polyphenols can bind to proteins mainly by forming hydrogen bonds and hydrophobic interactions [28, 29]. By protecting the native structure of insulin, ferulic acid inhibits the formation of amyloid fibrils [30]. Stabilizing the native structure of proteins with natural polyphenols is much easier [31] than with chemical modification [32] or immobilization [33]. The anti-amyloidogenic property of a phenolic compound is mostly determined by the position of the hydroxyl groups on the aromatic ring, not their number [34]. Ferulic acid may be a potential therapeutic agent in both the food and pharmaceutical industries [35].

2. MATERIALS AND METHODS

2.1. Materials

Lyophilized, three times crystallized α-chymotrypsin from the bovine pancreas (EC 3.4.21.1; type II), hydroxypropyl-β-cyclodextrin and trans-ferulic acid were obtained from Sigma-Aldrich Ltd. (Budapest, Hungary). All other chemicals used were of analytical grade.

2.2. Aggregation of α-chymotrypsin

The presence of polar organic solvents promotes the formation of amyloid fibrils [36]. The α-chymotrypsin was incubated in 55% ethanol/ 10 mM potassium phosphate buffer (pH 7.0) at a concentration of 0.15 mg/ml for one day in the absence and presence of different concentrations of hydroxypropyl-β-cyclodextrin or trans-ferulic acid. The samples were incubated at room temperature.

2.3. Turbidity measurements

Amyloid formation can be measured by the turbidity assay, an inexpensive method based on the intrinsic light scattering properties of aggregates [37]. Turbidity increases with time during protein aggregation [38]. Aggregation of α-chymotrypsin was determined by monitoring the absorbance at 350 nm wavelength using a 1 cm long quartz cuvette, as previously reported [39]. The samples were incubated in the presence of 55% ethanol at pH 7.0 at a protein concentration of 0.15 mg/ml for one day before the measurements. For each sample, blank corrections were made for protein-free solutions. All tests were performed in triplicate.

2.4. Congo red binding assay

The amyloid-specific Congo red dye is suitable for testing the effectiveness of an agent that inhibits fibril formation [39]. 0.2 ml of the protein

sample incubated for one day was added to 0.8 ml of Congo red solution, which also contained 0.15 mM NaCl in 5 mM phosphate buffer. The spectra of the obtained samples were taken after 15 minutes of incubation in the interval between 400 and 600 nm. The difference spectrum was calculated by subtracting the spectra obtained for the protein-only and Congo red-only samples from the spectrum of the sample containing both Congo red and protein. If the difference spectrum has a maximum at 540 nm, this indicates the presence of amyloid fibrils.

2.5. Statistical analysis

Data obtained from three independent turbidity measurements were expressed as the mean ± standard error (SEM). For comparison among groups, one-way analysis of variance (ANOVA) was used. Significance was defined as ***P < 0.001, **P < 0.01 and *P < 0.05.

3. RESULTS AND DISCUSSION

We used the model protein α-chymotrypsin for our experiments. When α-chymotrypsin is incubated in the presence of 55% ethanol for up to one day at pH 7.0 under *in vitro* condition, amyloid fibrils are formed [40]. Turbidity measurement is a suitable method for monitoring the anti-aggregation efficiency of an inhibitor [41, 42]. The decrease in the turbidity value experienced in the presence of the inhibitor indicates the effectiveness of the inhibitor. Hydroxypropyl-β-cyclodextrin inhibition of α-chymotrypsin aggregation was monitored by turbidity measurements at 350 nm. The aggregation-inhibiting effect of hydroxypropyl-β-cyclodextrin proved to be concentration-dependent (Figure 1). At a concentration of 6.5 mM, it reduced the amount of aggregates to 81.8% compared to the sample without inhibitor. Hydroxypropyl-β-cyclodextrin also inhibited Aβ fibrillation in a concentration-dependent manner [15].

Trans-ferulic acid proved to be a more effective inhibitor of α-chymotrypsin aggregation than hydroxypropyl-β-cyclodextrin. Figure 2 shows the change in turbidity during the formation of α-chymotrypsin aggregates in the presence and absence of trans-ferulic acid at different concentrations (10, 5, 2.5, 1.25 mM). The presence

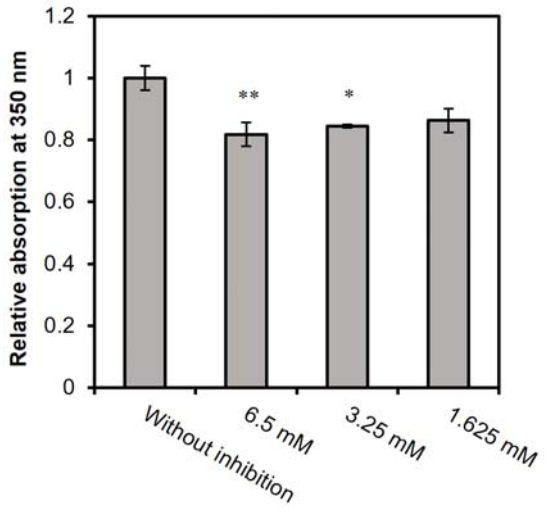

Figure 1. Turbidity measurements in the absence and presence of different concentrations of the hydroxypropyl-β-cyclodextrin by recording the absorption after 24 h incubation at 350 nm in 55% ethanol at pH 7.0. α-chymotrypsin concentration: 0.15 mg/ml. Each bar represents the average of at least three independent measurements. All data are presented as mean ± standard error of the mean (SEM). Significance was defined as **P < 0.01 and *P < 0.05.

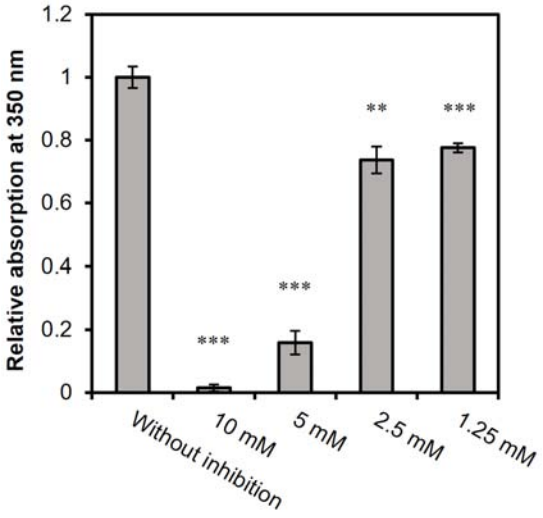

Figure 2. Turbidity measurements in the absence and presence of different concentration of the trans-ferulic acid by recording the absorption after 24 h incubation at 350 nm in 55% ethanol at pH 7.0. α-chymotrypsin concentration: 0.15 mg/ml. Each bar represents the average of at least three independent measurements. All data are presented as mean ± standard error of the mean (SEM). Significance was defined as ***P < 0.001 and **P < 0.01.

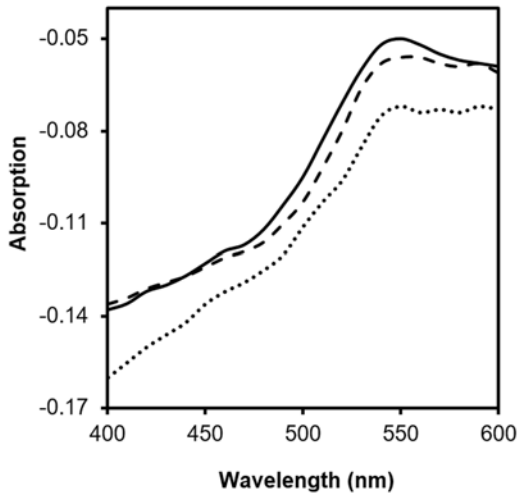

Figure 3. Congo red differential spectra in the absence (solid line) and presence of 2.5 mM (dashed line) and 3.1 mM (dotted line) trans-ferulic acid.

of 10 mM trans-ferulic acid reduced the amount of protein aggregates to 1.5%, while 2.5 mM to 73.7%, that is, the more concentrated trans-ferulic acid was more effective. Ferulic acid was also effective in inhibiting the formation of insulin amyloid fibrils [30].

In the presence of amyloid fibrils, characteristic changes can be observed in the Congo red binding absorption spectra, a red shift of the absorption maximum and an increase in the peak intensity [43]. When examining Congo red binding, the spectra were recorded between 400 and 600 nm in the presence and absence of different concentrations of trans-ferulic acid. Congo red difference spectra well reflect the concentration-dependent anti-amyloidogenic effect of trans-ferulic acid (Figure 3). The decrease of the maximum measured at 540 nm in the difference spectrum indicates a decrease in the amount of amyloid fibrils in the presence of an inhibitor.

According to our experimental results, trans-ferulic acid is promising as a potential inhibitor of amyloid aggregation.

CONCLUSION

By measuring turbidity and Congo red binding, we found that trans-ferulic acid very effectively inhibits α-chymotrypsin aggregation *in vitro*, making it a promising anti-amyloidogenic agent.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

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