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Original Article

Investigation of the anti-amyloidogenic effect of black chokeberry juice

Márta Kotormán* and Afrodité Szarvas

Department of Biochemistry and Molecular Biology, Faculty of Science and Informatics, University of Szeged, Középfasor 52, H-6726 Szeged, Hungary.

ABSTRACT

Many people's lives are affected by various protein misfolding diseases. Since these diseases are currently not curable, their prevention is very important. Fruit, including black chokeberries, contain effective fibril formation inhibitors and are recommended for consumption. We investigated the anti-amyloidogenic effect of black chokeberry juice using turbidity measurements, aggregation kinetics assay and Congo red binding experiments. We found, that black chokeberry juice was very effective in inhibiting α-chymotrypsin amyloid-like fibril formation in aqueous ethanol in a concentration dependent manner, since its total polyphenol content is much higher than that of most other fruit. Due to the intense blue color of the juice, it can be used as a natural food coloring, which has also a health-promoting property.

KEYWORDS: amyloid-like fibrils, *Aronia melanocarpa*, black chokeberry, Congo red, total polyphenolic content.

1. INTRODUCTION

The irreversible conversion of proteins to amyloid is of particular importance in many health disorders, such as Alzheimer's-, Huntington's-, Parkinson's-, Creutzfeldt-Jakob diseases, type 2 diabetes and amyotrophic lateral sclerosis. These diseases are increasingly common in our days, and today millions of people are affected worldwide [1, 2]. Cellular oxidative stress is common in diabetes mellitus and other disorders [3]. There is no cure now for neurodegenerative diseases [4, 5]; therefore it would be important to prevent them. Protein aggregation inhibitors can slow down or prevent the formation of amyloid fibrils. Among them there are many natural compounds [6, 7]. These may be potencial therapeutic agents for Parkinson's- [8] and Alzheimer's diseases [9]. Plants do not produce amyloid because they contain a number of aggregation inhibitors [10], which can stabilize proteins structurally [11]. This method of protein stabilization is much easier to accomplish than stabilization by chemical modification [12] or immobilization [13]. Proper nutrition is needed to optimize brain function and prevent cognitive decline [14]. Fruit contain several natural molecules that can help prevent these diseases [15, 16]. According to epidemiological information, there is a link between high fruit uptake and a low risk of various human diseases [17, 18]. The latest clinical research confirms that berries rich in phytochemicals prevent age-related neurodegenerative diseases [19]. Anthocyanin-rich fruit diets may also have beneficial effects in combating cognitive decline and neurodegeneration associated with ageing [20, 21].

Black chokeberry (*Aronia melanocarpa*) is rich in various bioactive compounds such as different polyphenols. These polyphenolic molecules are proanthocyanidins, anthocyanins (cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-xyloside, cyanidin-3-*O*-glucoside), phenolic acids (neochlorogenic and chlorogenic acids), flavonols

*Corresponding author
kotorman@expbio.bio.u-szeged.hu

and flavanols. The berries also contain vitamins (vitamins E and C), mineral elements (calcium, magnesium and potassium), carotenoids, organic acids, pectins and carbohydrates in smaller amounts. Consumption of chokeberry is nowadays growing because of its health-promoting properties; it has anti-inflammatory, gastroprotective, antidiabetic, cardioprotective and anticarcinogen effects [22-24]. With heavy metals causing oxidative stress, polyphenols in chokeberry can form complexes [25]. The juice of chokeberry has the highest antioxidant capacity, which was 4-10 times higher than that of other popular juices [26]. In addition to its many beneficial effects, no undesirable or toxic effects are known [27]. One of the strongest antioxidant in black chokeberry is quercetin [28], which is also an antiamyloidogenic agent [29-31], especially because it can get through the blood brain barrier [32]. Chlorogenic acid has been shown to be neuroprotective in both *in vitro* and *in vivo* models [33]. Epidemiological studies suggest that anthocyanins may be useful in the prevention of various neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases [34]. Anthocyanins extracted from *Aronia melanocarpa* protect SH-SY5Y cells against apoptosis induced by amyloid beta (1-42) [35]. Black chokeberry juice has a neuroprotective effect [36]. Results suggest that it can improve the learning and memory of young, healthy rats [37]. The protective effect of black chokeberry against the development of Alzheimer's disease has been shown [38, 39]. Much of the evidence focuses on polyphenols, but also on other components (e.g., carotenoids, terpenes, and fibers), which may play a role in their effect [40]. Due to its anthocyanides it can be used as a natural food coloring [23].

2. MATERIALS AND METHODS

2.1. Materials

The bovine pancreas α-chymotrypsin (EC 3.4.21.1, lyophilized, three times crystallized) was the product of Sigma-Aldrich Ltd. (Budapest, Hungary). Folin-Ciocalteu's phenol reagent was purchased from Merck Ltd. (Darmstadt, Germany). The fresh black chokeberry used was bought at the local market.

2.2. Making black chokeberry juice

The juice of ripe black chokeberry fruits was hand-pressed and centrifuged at 13000 rpm for 1 minute. Supernatants from the samples were stored at -20 ^{o}C until their use and diluted for further measurements, as necessary, with distilled water.

2.3. Preparation of α-chymotrypsin amyloid-like fibrils

The results suggest that different organic solvents can be used to produce different amounts or shapes of amyloid fiber [41]. The samples containing 0.15 mg/ml α-chymotrypsin and 55% ethanol/ 10 mM phosphate buffer at pH 7.0 were incubated for 1 day at 24 ^{o}C in the presence and absence of different concentration of black chokeberry juices.

2.4. Turbidity measurements

The absorption of the samples incubated for one day was measured at 350 nm using 1 cm pathlength quartz cuvette at 0.15 mg/ml protein concentration in the presence of 55 % ethanol/ 10 mM phosphate buffer at pH 7.0 in the presence and absence of different concentration of black chokeberry juices. Blank corrections were made for enzyme-free solutions in each sample.

2.5. Determination of total phenolic content

Determination of the total phenolic content of black chokeberry juice was made using Waterhouse method [42]. From the absorption of the samples incubated with Folin Ciocalteu reagent at 765 nm, all polyphenol contents were expressed in GAE mg/l using gallic acid calibration line.

2.6. Kinetics of aggregation

Kinetics measurements of aggregation were performed by reading the absorption at 350 nm per minute for 20 minutes. The protein concentration in the incubation mixtures was 0.15 mg/ml in 55% ethanol/10 mM phosphate buffer at pH 7.0.

2.7. Congo red (CR) binding assay

The binding of CR to amyloid fibrils causes an increase in the intensity of absorption and a characteristic redshift of the absorption maximum [43]. 800 μl of CR solution (in 5 mM phosphate buffer in the presence of 150 mM NaCl) was mixed with 200 μl of the incubated sample for one day. The spectra between 400 and 600 nm were recorded using a UV–visible spectrophotometer (Hitachi U 2000) after 15 minute incubation. The spectra of CR and α-chymotrypsin are subtracted

from the values obtained in the presence of CR and amyloid fibrils, resulting in a difference spectrum to monitor the efficacy of the inhibitor.

2.8. Statistical analysis

Independent turbidity measurements were repeated at least three times and were presented as mean ± standard error of the mean (SEM). Experimental data were analyzed by one-way ANOVA. Significance was defined as $P < 0.001$.

3. RESULTS AND DISCUSSION

In these experiments the well known serin protease, α-chymotrypsin was used, as model protein. The amyloid-like fibrils were made as we previously described, by incubating the enzyme in the presence of 55% ethanol for one day at pH 7.0 [44]. The effectiveness of an inhibitory agent can be monitored by turbidity measurement [45]. We found that the aggregation of α-chymotrypsin was effectively inhibited by chokeberry juice. Black chokeberry juice diluted 200 times reduced the aggregate content to 43.7% and 500 times to 71.3% relative to the sample without an inhibitory agent. The average of 3 replicates is shown in Figure 1. In other words, the effect of black chokeberry juice was proportional to its concentration.

For kinetic experiments 2000- and 1000-times dilutions of chokeberry juice were used, and measurements were also performed without an inhibitor. In the control sample, absorption first increased rapidly, then only moderately. At 1000-fold dilution the slope of the initial phase was significantly flattened, which proved to be more effective than the 2000-fold dilution. Kinetic measurements showed that chokeberry juice was so effective that it was effective even at 2000 times dilution (Figure 2), as its total polyphenol content is significantly higher than that of most fruits. Its total polyphenol content was measured. Three parallel measurements resulted in 9469.3 ± 306.9 GAE mg/l. The anti-amyloidogenic effect of black chokeberry juice is mainly due to its polyphenolic compounds. Earlier we had found that the anti-amyloidogenic activity of inhibitors was proportional with their total polyphenol content [46]. Natural polyphenols have been found to be promising inhibitors of α-synuclein fibril formation, which may be potential therapeutic agents for Parkinson's disease [8].

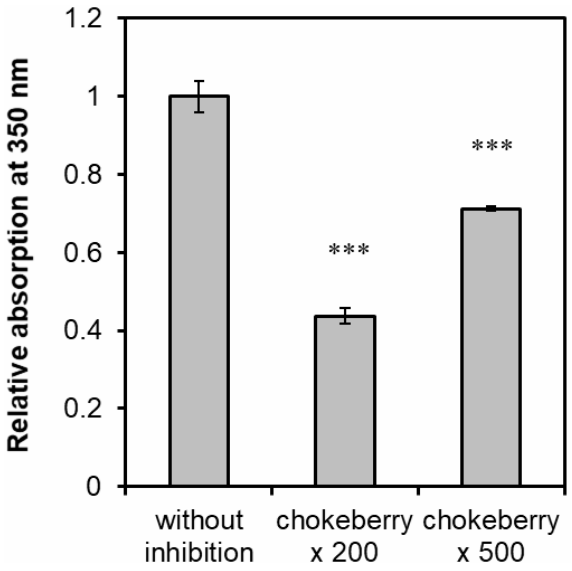

Figure 1. Turbidity measurements in the absence and presence of different concentration of the black chokeberry juice by recording the absorption after 24 h incubation at 350 nm at 0.15 mg/ml α-chymotrypsin concentration in 55% ethanol at pH 7.0. Each bar represents the average of at least three independent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

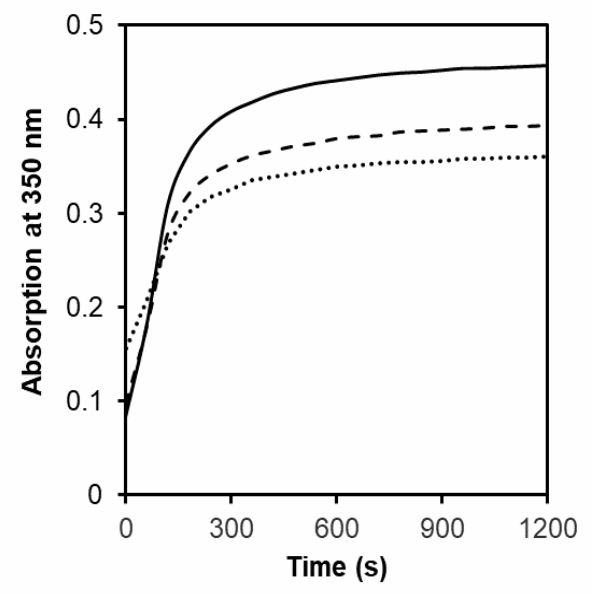

Figure 2. Kinetics of α-chymotrypsin aggregation in the absence (solid line) and presence of the black chokeberry juice diluted 1000 (dotted line) and 2000 times (dashed line) by recording the absorption for 20 minutes at 350 nm at 0.15 mg/ml protein concentration in 55% ethanol at pH 7.0.

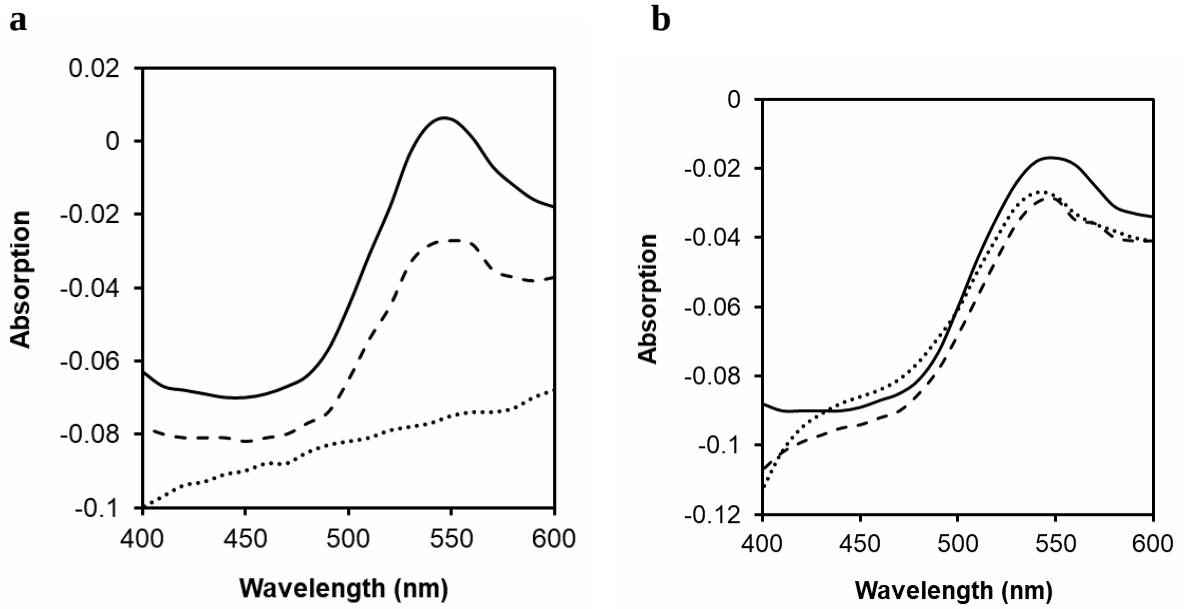

Figure 3. CR differential spectra of the samples in the absence (solid line) and presence of black chokeberry juice (**a**) diluted 500 (dashed line) and 200 times (dotted line), and chlorogenic acid (**b**) at 0.05 mg/ml (dashed line) and 0.1 mg/ml (dotted line).

CR dye can detect the presence of amyloid fibrils and monitor the effectiveness of an inhibitor [47, 48]. CR spectra were recorded in the presence and absence of black chokeberry juice (Figure 3a). In the control sample, a greater increase in absorption was observed in the presence of fibrils compared to the absorption in the presence of chokeberry juice. In the control sample, in the presence of fibrils, the absorption maximum shifted towards higher wavelengths, whereas in the presence of 500-fold diluted chokeberry juice, the amount of displacement decreased and in the presence of 200-fold dilution of inhibitor the absorption maximum was the same as that of the protein-free sample. The spectral changes are well reflected in the CR difference spectra. The difference spectrum has a peak at 550 nm without an inhibitor. The maximum value decreases in the presence of 500-fold dilution of chokeberry juice, indicating a decrease in amyloid fibrils. In the presence of 200-fold diluted chokeberry juice, the shape of the difference spectrum also changed, practically disappearing at the maximum at 550 nm. CR binding experiments have also shown that black chokeberry juice is an effective anti-amyloidogenic agent and its potency is proportional to its concentration.

We investigated the anti-amyloidogenic effects also of chlorogenic acid, which is present in black chokeberry using CR binding assay. According to the CR differential spectra we found that the chlorogenic acid is effective in inhibiting α-chymotrypsin amyloid-like fibril formation and its efficiency was concentration dependent (Figure 3b). It was found that chlorogenic acid suppressed the formation of human islet amyloid polypeptide oligomers [49].

Proper nutrition can contribute to the prevention of undesirable protein conformation disorders, in which the consumption of polyphenol-rich fruits, including black chokebrry, can play an important role. Thanks to the intense blue color of its juice, it can also be used as a natural food coloring.

CONCLUSION

In conclusion, black chokeberry juice is an efficient agent against α-chymotrypsin aggregation in aqueous ethanol at pH 7.0. Consumption of black chokeberry may help prevent amyloidogenic diseases.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

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