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Eduscho Coffee Extract Effectively Inhibits the Formation of Amyloid-like Fibrils by Trypsin in Aqueous Ethanol

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In this work we used an *in vitro* trypsin aggregation model to show that certain commercial coffee extracts can inhibit protein aggregation. Aggregation experiments were performed using several spectroscopic methods and a dye binding assay, such as turbidity, Congo red (CR) and electronic circular dichroism (ECD), that was further supported by transmission electron microscopy (TEM). A correlation was found between the anti-aggregation properties and the total phenolic content of the coffee extracts. The results revealed that the greatest effect was exerted by the Eduscho coffee extract. It was found that the inhibitory effect of this extract was concentration dependent. Using size exclusion chromatography, we demonstrated that the inhibitory effect of the Eduscho coffee extract on the formation of amyloid-like fibrils was due to its capacity to stabilize the oligomeric form of the protein.

Keywords: Amyloid-like fibrils, Coffee, Congo red, Electronic circular dichroism, Inhibitory effect, Polyphenols, Trypsin.

Our knowledge of protein aggregation will be substantially expanded in the next few decades [1]. The attention of several researchers has been attracted to protein misfolding and its aberrant assembly into unbranched amyloid fibrils. It is typical for amyloids to have a characteristic cross β sheet structure. It has been reported that non-disease associated proteins may also form amyloid aggregates in humans under certain conditions, which suggests that polypeptides have an inherent ability to form amyloid fibrils, but their propensity to form pathological amyloid fibrils depends on their amino acid sequence [2, 3]. Amyloid fibrils are the main triggering cause of more than fifty serious human diseases [4]. The search for therapeutic agents that inhibit amyloid aggregation is a major challenge of our time [5]. Coffee is an antioxidant rich beverage, which is widely consumed worldwide [6]. There are nearly 1,000 phytochemicals in it [7]. Coffee intake has many beneficial health effects [8]. Much research, epidemiological studies, and meta-analyses concerning coffee consumption highlighted its inverse correlation with the occurrence of diabetes mellitus, various cancer types, and Parkinson's and Alzheimer's diseases. These health benefits are known to be due to coffee polyphenols [9]. The chemical structure of polyphenols includes two or more phenol rings with hydroxyl groups in *ortho* or *para* positions. Several natural polyphenols have been reported to possess potent inhibitory effects on amyloid fibril formation [10]. Coffee extracts have three main active components, i.e. caffeine, caffeic acid (CA), and chlorogenic acid (CGA). Earlier findings suggest that all components show different inhibitory effects on the formation of toxic human islet amyloid polypeptide (hIAPP) amyloids. CA shows the highest effect to delay the conformational transition of the hIAPP molecule with the most prolonged lag time, whereas caffeine represents the lowest potency [11]. It has been proved that lysozyme fibrillization is effectively inhibited by CA [12]. CA was also shown to inhibit $A\beta_{1-42}$ self-induced aggregation [13]. CA has the ability to pass through the blood-brain barrier and therefore, this compound has been suggested as an anti-amyloidogenic agent [14]. Caffeine can be found in many dietary sources including coffee (71–220 mg caffeine/150 mL), tea (32–42 mg/150 mL), cola (32–70 mg/330 mL), and cocoa (4 mg/150 mL). Caffeine was demonstrated to reduce levels of Aβ and Aβ-induced neurotoxicity both *in vitro* and *in vivo* [15] and, therefore, caffeine may prevent the development of Alzheimer's disease. Caffeine has the capacity to inhibit the formation of β-sheets by interacting with the peptide's aromatic side chains of the constituent amino acids. In addition, caffeine molecules form hydrogen bonds with peptide backbone thereby weakening interstrand hydrogen bonds of β-sheets [16]. The disruption of the blood-brain barrier happens at an early pathological stage in Alzheimer's disease. It may make it possible for Aβ to accumulate in the brain by allowing the transport of Aβ produced in the periphery. Caffeine administration protects the brain against Alzheimer's disease-associated blood-brain barrier disruption [17]. Trigonelline is also a coffee ingredient, and it is one of the most often consumed alkaloids. It was demonstrated by molecular modelling that trigonelline has high affinity to the A-β(1-42) peptide, and acts similarly to the anti-Alzheimer's disease drug candidate - cotinine [18]. The neuroprotective activities of CGA against Aβ-caused toxicity were also examined [19]. A recent epidemiological study suggested that higher caffeine consumption over decades decreases the risk of Alzheimer's disease [20].

The well-known serine protease trypsin is a globular protein, with mostly β-sheets and some α-helices in the secondary structure of the protein. Trypsin is composed of a double β-barrel. Phenylmethyl-sulfonyl-trypsin (PMS-trypsin) is catalytically inactive, so autolysis cannot alter the formation of amyloid-like fibrils. In our earlier paper we reported that the most fibrils of PMS-trypsin were formed in 60% ethanol at pH 7.0 [21]. Here, we report that Eduscho coffee extract inhibits the formation of amyloid-like fibrils of PMS-trypsin

dose-dependently. The PMS-trypsin amyloid aggregates were prepared according to our previous report by the incubation of the soluble protein in the presence of 60% ethanol at 24°C at pH 7.0 [21]. The turbidity measurements were performed to detect the effects of various coffee extracts on the aggregation of PMS-trypsin. These experiments revealed that the greatest effect was exerted by the Eduscho coffee extract. In the presence of Eduscho coffee extract diluted 250 times the absorption at 350 nm reduced to 18.1% after incubation for 24 h relative to the sample not containing coffee extract (Figure 1).

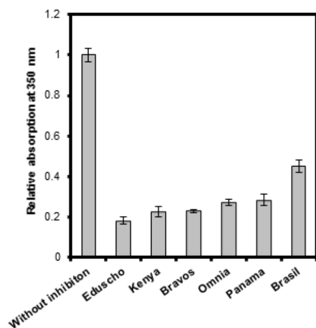

Figure 1: Turbidity measurement of samples by recording the absorption at 350 nm. PMS-trypsin (0.13 mg/mL) was incubated in 60% ethanol in either the absence or presence of different coffee extracts diluted 250 times at pH 7.0. Each bar represents the average of at least three independent measurements. All data were presented as mean ± standard error of the mean (SEM).

The total concentrations of phenolic compounds of different coffee extracts ranged from 4,942 to 7,702 mg GAE/L (Figure 2). The calculated Pearson's correlation coefficient values (r) fell in between 0.896 and 0.997. The degree of inhibition was found to change *pro rata* with the total concentrations of phenolic compounds.

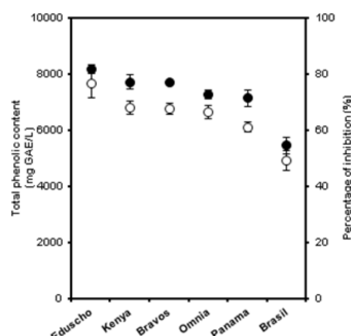

Figure 2: Change in the percentage inhibition in 60% ethanol (●) with the total phenolic content (o). Coffee extracts were diluted 250 times. Protein concentration was 0.13 mg/mL. All data were presented as mean ± standard error of the mean (SEM) from three independent measurements.

Congo red binding assay was used to determine if the aggregates formed in the PMS-trypsin sample were fibril-like. Congo red is a well-known amyloid-specific dye, which exhibits an increase of absorption with a characteristic red shift upon binding with the amyloids [22, 23]. The inhibitory effect of the Eduscho coffee on PMS-trypsin fibrillation was observed *via* the Congo red binding assay (Figure 3A). The Congo red absorption difference at 550 nm was found to decrease with incubation in the presence of the Eduscho coffee extract. The data suggest that the Eduscho coffee extract inhibited the aggregation of PMS-trypsin dose-dependently. Similar to our results, it had been found that coffee components had inhibited the formation of amyloid fibrils using human islet amyloid polypeptide [11]. To find out whether there is a competition between CR and polyphenols of the Eduscho coffee extract in the binding to the protein amyloid-like fibrils, we used the successive addition of CR and coffee extracts (Figure 3B). During the first experiment, CR was first added to the fibrils, followed by the coffee extract. In the second experiment the administration of these agents happened in reverse order. With this experiment we could show that there was no competition between the two agents to bind to the fibrils. So, the polyphenol compounds in the coffee extract do not bind to the amyloid-like protein fibrils or the polyphenols bind to a different site.

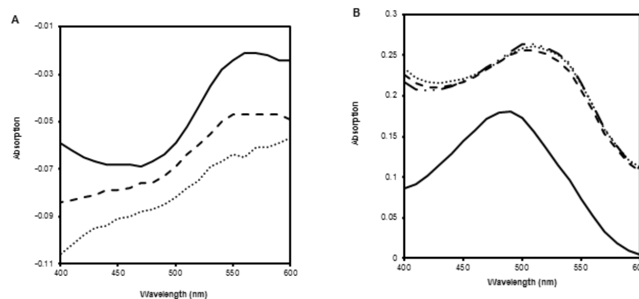

Figure 3: CR difference spectra in 60% ethanol of PMS-trypsin (A) without coffee extract (solid line) or in the presence of the Eduscho coffee extract diluted 250 times (dotted line) and diluted 500 times (dashed line). Absorption spectra by the successive addition of CR and the Eduscho coffee extract (B). CR alone (solid line), in the absence of the Eduscho coffee extract (dashed-dotted line), CR added first and then the Eduscho coffee extract to the protein (dotted line), and in the reverse order (dashed line).

ECD spectroscopy in the far UV wavelength range (185–260 nm) was used to detect the secondary structural changes in PMS-trypsin in 60% ethanol in the absence and presence of the Eduscho coffee extract. The sample was either set in the middle of the sample compartment, or at the right side, next to the detector (Figure 4). If the sample is a clear, real solution, containing no light-scattering aggregates, the two measurements should provide the same results. In contrast, a change in the intensity and blue shift was observed, when the sample was located next to the detector. This indicated the presence of large aggregates in the protein sample without the Eduscho coffee extract. Such a phenomenon was observed to a lesser extent when the Eduscho coffee extract was present in the samples. ECD spectra of PMS-trypsin were measured at lower concentrations of ethanol, in an attempt to follow the gradual conversion of the secondary structure preceding intermolecular association. Spectra were recorded in 10% ethanol at pH 7.0. In general, spectra measured at 10% ethanol concentration display minor α-helical contributions as opposed to those measured at 60% ethanol concentration, where spectral characteristics of the β-sheet structure are dominant [21]. In 10% ethanol/10 mM phosphate buffer the intensity of the shoulder appearing at 200 nm was found to increase to some extent over time, while the shoulder around 218 nm diminished (Figure 5A). Spectral changes were complete after 60 min incubation. This indicated slow helix to coil transition, which could be elucidated as a first structural transitional step of the aggregation process of trypsin. It was found that with increasing concentration of ethanol the helices of model 4-α-helix bundle protein unfolded completely [24]. Such structural changes were found to be slower and less pronounced in the presence of the Eduscho coffee extract (Figure 5B). From these findings we can conclude that the addition of the Eduscho coffee extract to the sample is effective in preventing PMS-trypsin from undergoing helix-to-coil transition.

To separate the particles of different size, gel filtration chromatography was used. The presence of the Eduscho coffee extract diluted 100 times increased the amount of the oligomers (Figure 6). With this experiment we demonstrated that the inhibitory effect of the Eduscho coffee extract on the formation of amyloid-like fibrils is due to its capacity to stabilize the oligomeric

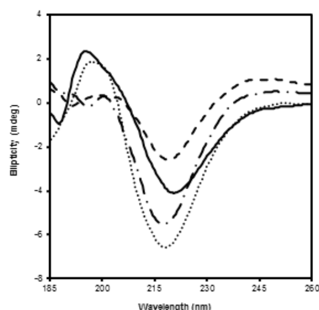

Figure 4: ECD spectra of PMS-trypsin in 60% ethanol at pH 7.0 without coffee extract (dashed line, solid line) and in the presence of the Eduscho coffee extract diluted 250 times (dotted-dashed line, dotted line). The sample was set in the middle of the sample holder (solid line, dotted line), or next to the detector (dashed line, dotted-dashed line). PMS-trypsin concentration was 0.15 mg/mL.

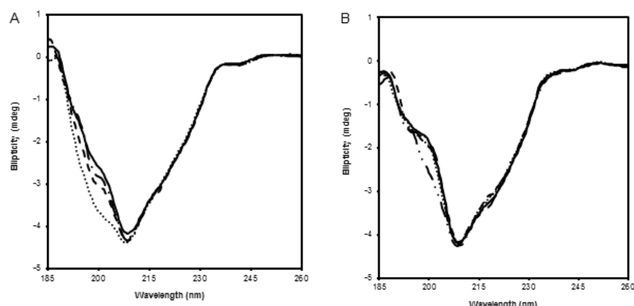

Figure 5: ECD spectra of PMS-trypsin in 10% ethanol at pH 7.0 without coffee extract (**A**) and in the presence of the Eduscho coffee extract diluted 250 times (**B**) at 0 min (solid line), 20 min (dotted-dashed line), 30 min (dashed line), 60 min (dotted line), and 120 min (double-dotted-dashed line). PMS-trypsin concentration was 0.15 mg/mL.

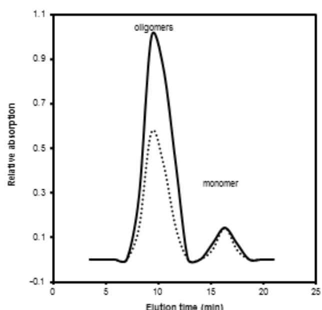

Figure 6: Size exclusion chromatography using Sephadex G-75 column in the presence of the Eduscho coffee extract diluted 100 times (solid line) and without it (dotted line).

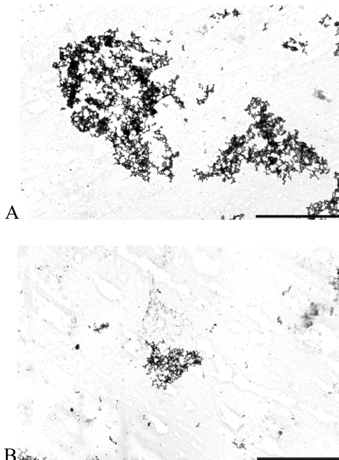

Figure 7: Transmission electron micrographs of PMS-trypsin in the absence (**A**) and the presence of the Eduscho coffee extract diluted 250 times (**B**). The scale bar indicates 10 μm. Enzyme concentration: 0.13 mg/mL.

form of the protein, because it contained polyphenols. Similarly to our results, phenyl ethyl alcohol had had an inhibitory effect on hen egg white lysozyme fibrillation by producing mainly oligomers [25]. The TEM image indicated an abundance of fibrils of PMS-trypsin after an incubation period of 24 h in 60% ethanol/10 mM phosphate buffer at pH 7.0. However, in the presence of the Eduscho extract, fibrillar structure was reduced significantly which, again, confirms the inhibitory property of the Eduscho coffee extract against PMS-trypsin fibrillation (Figure 7).

In view of all results it can be concluded that the Eduscho coffee extract is an efficient anti-amyloidogenic agent, as it arrests PMS-trypsin fibrillation in a concentration dependent manner.

Experimental

General experimental procedures: The absorbance of the samples was measured using a Cecil CE-5501 spectrophotometer. The absorption spectra were recorded with a UV–vis spectrophotometer (Hitachi U 2000). Electronic circular dichroism spectra were measured using a Jasco J-815 Circular Dichroism Spectrometer. Electronmicrographs were taken on a JEOL JEM-1011 transmission electron microscope (operating at 60 kV), using an Olympus Morada 11 megapixel camera and iTEM software (Olympus).

Materials: Bovine pancreatic trypsin (EC 3.4.21.4) was purchased from Sigma-Aldrich (Budapest, Hungary). Folin-Ciocalteu's phenol reagent was the product of Merck (Darmstadt, Germany). All the other reagents used were of analytical grade. The different ground, roasted coffees were obtained from commercial sources. Eduscho Wiener Extra was purchased from Tchibo Budapest Ltd., H-2040 Budaörs, Neumann János street 1, Douwe Egberts Omnia Classic from Douwe Egberts Hungary, 1132 Budapest, Váci road 22-24, Bravos Classic from Mocca Negra Co., 2000 Szentendre, Kalászi road 3, Brasil Santos, Panama arabica and Kenya Kiandi from Latin Negyed Ltd., Szeged, Tisza L. boulevard 36.

Preparation of the extracts: Three g of coffee was added to 22.5 mL hot distilled water and then boiled 3 times. After the samples cooled down to room temperature, they were centrifuged for 1 min at 13,000 rpm. The supernatant was used for measurements.

Turbidity measurements: Turbidity assay was performed to follow the formation of protein aggregates [14]. The measurements were performed in a quartz cuvette of 1 cm path length at 0.13 mg/mL PMS-trypsin concentration in 10 mM phosphate buffer at pH 7.0. The turbidity of the trypsin sample was determined by monitoring the change in absorption at 350 nm after incubation for 24 h in 60% ethanol. We calculated the percentage of inhibition from the turbidity measurements: the relative absorption for the given coffee extract was subtracted from 1 and then it was multiplied by 100.

Determination of total phenolic content: Analysis of the total phenolic content by means of the Folin Ciocalteu colorimetric method was performed according to the protocol of Waterhouse [26]. The reaction mixture was kept for 2 h at room temperature, and absorption was measured at 765 nm. We used different concentrations of gallic acid solutions (0–50 mg/L) for calibration. The total phenolic contents were expressed as mg gallic acid equivalents (GAE) per L of the coffee samples.

Congo red binding assay: CR was dissolved in a 5 mM phosphate buffer containing 150 mM NaCl (pH 7.0). For CR assays, 200 μL (0.13 mg/mL) aliquots of the 1-day-aged protein solution were withdrawn and mixed with 800 μL of a solution containing 6.3 μM CR. The absorption spectra were recorded in a 1 cm path length

cuvette after 15 min incubation. The CR difference spectra were obtained by subtracting CR and PMS-trypsin spectra from the CR + PMS-trypsin spectrum. By the successive addition of CR and the Eduscho coffee extract, 0.4 mg/mL PMS-trypsin concentration was used.

Electronic circular dichroism: ECD measurements were performed to explore the changes in the secondary structure of PMS-trypsin. Spectra were recorded in a 0.1 cm path length quartz cuvette at 0.15 mg/mL protein concentration. Corresponding solvent spectra, measured for mixtures containing all components but PMS-trypsin, were subtracted from the spectra of the protein. Spectra presented here are the accumulations of 10 independent scans. Ellipticity was expressed in mdeg units.

Size exclusion chromatography: The samples (0.13 mg/mL) were incubated for 1 day at 24°C, and then centrifuged for 1 min at 13,000 rpm. To calibrate the column lysozyme (14 kDa), trypsin (23.3 kDa) and bovine serum albumin (67 kDa) were used. The samples (0.5 mL) were filtered at 24°C in 25 mM phosphate buffer (pH=7.0) using a Sephadex G-75 column (10 x 300 mm^2). The absorption of the fractions was measured at 280 nm.

Transmission electron microscopy: The Eduscho coffee extract was filtered through a 0.02 mm Whatman inorganic membrane filter before use. Ten µL aliquots of the protein solutions were placed on carbon-coated 300-mesh nickel grids (Nisshin EM Co. Ltd. Tokyo) and stained with 2% (w/v) uranyl acetate.

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