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Grapefruit Seed Extract Inhibits the Formation of Amyloid-like Fibrils by Trypsin in Aqueous Ethanol

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Several natural compounds deriving from plants are known to be efficient anti-amyloid aggregation agents. In this study, anti-aggregation activity of grapefruit seed extract was investigated using trypsin as a model protein in aqueous ethanol at pH 7.0. Using turbidity measurement, Congo red (CR) binding assay, electronic circular dichroism (ECD) and transmission electron microscopy (TEM), we found that grapefruit seed extract has ability to inhibit trypsin amyloid-like fibril formation *in vitro,* and effectiveness increases with growing concentration of grapefruit seed extract. The total phenolic content of it was determined. The results showed that in addition to the polyphenolic compounds some other compounds are also responsible for the fibril formation inhibitory effect. We indicated it for the first time that limonin has anti-fibrillation effect.

Keywords: Amyloid-like fibrils, Congo red, Grapefruit seed extract, Inhibitory effect, Limonin, Polyphenols, Trypsin.

Proteins involved in conformational diseases contain great amounts of "intrinsic disorder" [1]. Growing interest and researches have been focused on finding potential fibrillation inhibitors as fibrillary protein aggregates are regarded to be closely associated with many diseases such as diabetes, Alzheimer's and Parkinson's disease [2,3]. Several natural compounds deriving from plants are known to be efficient anti-amyloid aggregation agents. There are many natural compounds that can bind to various amyloid species directly including oligomers and fibrils. These, in turn, can lead to conformational change in the β-sheet assembly to form nontoxic aggregates [4,5]. Grapefruit seed extracts are used in cosmetics, food supplements, and pesticides because they have antimicrobial properties [6]. It was demonstrated, that grapefruit seed extract reduces the ethanol- and stress-induced gastric damage [7]. There are large quantities of polyphenolic compounds in it [8,9]. Bioflavonoids with bioactive properties are present in grapefruit seed extract e.g., hesperitin and naringenin [10]. Grapefruit seeds contain 17-beta-D-glucopyranosides of limonin, nomilin, obacunone, deacetylnomilin, nomilinic acid and deacetylnomilinic acid. The concentration of nomilin glucoside is the highest among glucosides. The major neutral limonoid aglycones commonly found in citrus, namely limonin, nomilin, deacetylnomilin, obacunone and ichangin are also present in the seeds. These are highly oxygenated triterpenoids. Limonin is the predominant aglycone [11,12]. Nomilin and obacunone are furan rings containing limonoids with anticancer effect. They possess antiviral, antibacterial and antimalarial activities [13]. Naringenin can also be a useful chemopreventive agent against neurodegenerative diseases such as Alzheimer's disease [14]. The amount of aggregated Aβ is reduced by resveratrol [15]. Aromaticity plays an important role as it breaks the hydrophobic interaction between Aβ monomers. It is possible for aromatic compounds to interact with residues Phe19 and Phe20 of Aβ peptide *via* π-π stacking interactions [16]. Resveratrol effectively inhibits fibrillogenesis. It also destabilizes preformed fibrils of hen egg white lysozyme in a concentration-dependent manner [17,18]. The conversion of β-lactoglobulin from amyloid to amorphous aggregation is induced by resveratrol [19]. Flavanol and (−)-epicatechin are identified to be effective at reducing Aβ production [20]. The (−)-epicatechin interacts with *β*-lactoglobulin mainly *via* the residues, which are normally involved in *β*-sheet formation in the absence of (−)-epicatechin, thus (−)-epicatechin has the effect of both slowing down and reducing *β*-lactoglobulin fibril formation [21]. The aim of our study was to investigate the anti-fibrillation activity of grapefruit seed extract and that of limonin using phenylmethylsulfonyl trypsin (PMS-trypsin) as a model protein in 60% ethanol at pH 7.0. We also wanted to investigate whether there was some connection between the total phenolic content of the inhibitory agent and its anti-amyloidogenic efficacy.

In these experiments trypsin was used as a model protein modified with phenylmethylsulfonyl fluoride (PMSF). Amyloid-like fibrils were prepared using PMS-trypsin as previously reported [22]. The measurement of solution turbidity is a procedure for the detection of protein aggregates [23]. The inhibitory effect of grapefruit seed extract on PMS-trypsin aggregation was determined at different concentrations using turbidity measurements in 60% ethanol at pH 7.0. Grapefruit seed extract diluted 800 times reduced the absorption at 350 nm to 52.3% compared relative to the sample which contained no inhibitory agent after 24 h of incubation. At

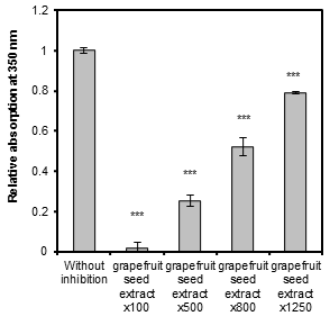

Figure 1: Turbidity at 350 nm of PMS-trypsin in 60% ethanol in the absence and presence of different concentrations of grapefruit seed extract. Protein concentration: 0.13 mg/mL. All data were presented as mean ± standard error of the mean (SEM) from three independent measurements. P values less than 0.001 are summarized with three asterisks.

100-fold dilution grapefruit seed extract almost eliminated the formation of aggregates. Anti-aggregation effect of the grapefruit seed extract was dose dependent (Figure 1).

The total phenolic content of the grapefruit seed extract was determined, and it was 495.5 mg GAE/l. Compared to other inhibitory agents the efficiency of inhibition of amyloid formation did not change proportionally with the total phenolic content in the presence of the grapefruit seed extract [24]. It is probable that polyphenolic compounds are not the only responsible factors for the fibril formation inhibitory effect, but also other compounds are involved. Grapefruit seed extract also contains limonoids [25], which have neuroprotective effects [26,27]. We determined the limonin concentration of the grapefruit seed extract, it was 16.8 ng/mL.

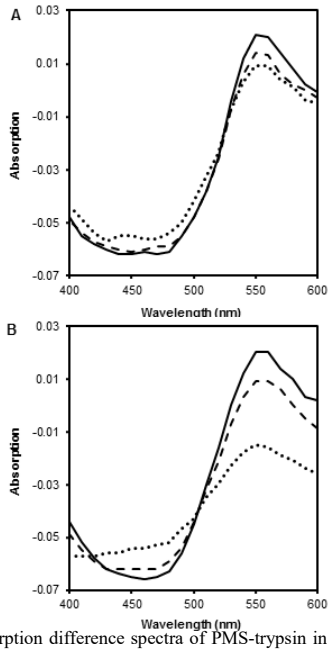

Figure 2: CR absorption difference spectra of PMS-trypsin in the absence (solid line) and presence of 34 nM (dashed line) and 340 nM limonin (dotted line) (A) and presence of grapefruit seed extract diluted 800 times (dashed line) and 100 times (dotted line) (B).

Congo red (CR) is an amyloid marker, which is generally used for the detection of amyloid fibrils [28]. The peak point of the difference spectrum for the Congo red binding assay at 541 nm is characteristic of amyloid fibrils [29]. To determine the presence of amyloid-like fibrils in our samples and the anti-amyloidogenic efficiency of limonin and grapefruit seed extract against PMS-trypsin amyloid-like fibrillation CR binding assay was used. As illustrated by Figure 2 the presence of amyloid-like fibrils was proved in the absence of any inhibitory agent, but limonin and grapefruit seed extract were able to exert inhibitory effects on fibrillation. The data suggest that limonin and grapefruit seed extract inhibit amyloid-like fibril formation of PMS-trypsin dose dependently. Our results indicated for the first time that limonin inhibits the fibril formation in a concentration dependent manner.

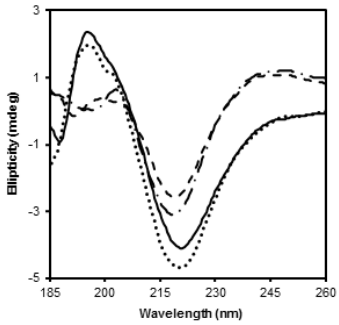

Figure 3: Far-UV CD spectra of PMS-trypsin in the absence (dashed line, solid line) and presence (dotted-dashed line, dotted line) of grapefruit seed extract diluted 500 times. The sample was set in the middle of the sample compartment (solid line, dotted line), or next to the detector (dashed line, dotted-dashed line). Protein concentration: 0.15 mg/mL.

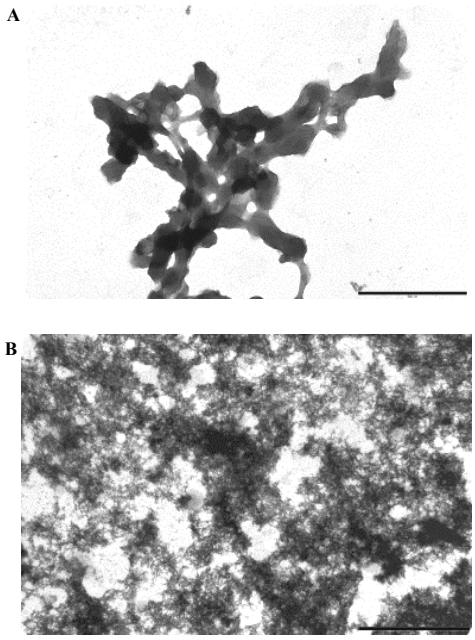

Figure 4: Transmission electron micrographs of PMS-trypsin samples in the absence (A) and presence (B) of grapefruit seed extract diluted 500 times in 60% ethanol at pH 7.0. Protein concentration: 0.13 mg/mL. The scale bar represents 500 nm.

We have shown successfully that the grapefruit seed extract inhibits the aggregation process of PMS-trypsin in 60% ethanol at pH 7.0. The inhibition of PMS-trypsin aggregation positively correlated with increased grapefruit seed extract concentration. Our results indicated for the first time that limonin effectively inhibited fibril formation.

Changes in the PMS-trypsin secondary structure can be revealed using far-UV ECD measurements in 60% ethanol in the absence or presence of grapefruit seed extract diluted 500 times (Figure 3). We made these experiments in two various sample positions: in the middle of the sample compartment, or next to the detector. For real

solutions, the two experiments must give the same results. In the presence of diluted grapefruit seed extract light dispersion decreased. This can be explained by the fact that the aggregation speed has decreased, or the size of the aggregates formed has decreased with grapefruit seed extract. ECD spectra recorded in various sample positions suggest that grapefruit seed extract is an effective anti-amyloidogenic agent.

Transmission electron microscopy was used to detect the morphological changes in the presence of grapefruit seed extract. TEM studies showed that there was no fibril formation in the presence of grapefruit seed extract diluted 500 times, indicating the inhibitory effect of grapefruit seed extract on the formation of amyloid-like fibrils by trypsin (Figure 4). The results showed that the grapefruit seed extract had significant inhibitory effect on amyloid-like fibril formation by trypsin.

Experimental

General experimental procedures: The absorbance of the samples was measured using a Cecil CE-5501 spectrophotometer. The CR absorption spectra (400–600 nm) were recorded with a UV–vis spectrophotometer (Hitachi U 2000). Electronic circular dichroism spectra were measured by 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 the iTEM software (Olympus).

Materials: Trypsin (EC 3.4.21.4; from bovine pancreas) and limonin (from citrus seeds) were purchased from Sigma-Aldrich Company (Budapest, Hungary). Grapefruit seed extract was distributed by Bioextra Zrt. 3000 Hatvan, Lőrinci út 20-22.

Turbidity measurements: Turbidity assay was performed as an indicator to suggest the formation of protein aggregates and fibrils [30,31]. The turbidities of PMS-trypsin samples incubated in 60% ethanol at pH 7.0 for 24 h at 24°C in the absence and presence of grapefruit seed extract at different concentrations were determined by monitoring the change in absorbance at 350 nm using a cuvette of 1 cm path length. The protein concentration was 0.13 mg/mL in these measurements. Respective blank corrections were done prior to all experiments. Significance was determined by a one-way analysis of variance (ANOVA).

Determination of total phenolic content: The total phenolic content of grapefruit seed extract was colorimetrically determined according to Waterhouse using Folin-Ciocalteu reagent [32]. The absorbances of the solutions were measured at 765 nm. The total phenolic contents were calculated from the calibration curve, and the results were expressed as gallic acid equivalent in milligrams per litre of samples (mg GAE/l).

Determination of limonin concentration using HPLC-MS: Liquid chromatographic separation was performed on a DionexUltimate 3000 UHPLC system equipped with a membrane degasser, a binary pump, a standard autosampler, a thermostated column compartment and a variable wavelength detector. The components of the sample were separated on a Gemini-NX C18 (3 µm, 150 x 2 mm) column (Phenomenex) equipped with a Gemini-NX C18 guard column (5 µm, 4 x 2 mm) thermostated at 25°C. Mobile phase A consisted of water containing 0.1% formic acid, while methanol containing 0.1 % formic acid served as mobile phase B. The gradient elution was performed as follows: 0 min, 20% B; 1 min, 20% B; 12 min, 95% B; 17 min, 95% B; 18 min, 20% B; 25.0 min, 20% B. The flow rate was set to 0.2 mL/min. The injection volume was 5 µl.

Mass analyses were performed on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer. Ionization of analytes was performed using heated electrospray interface (HESI) in negative electrospray ionization. The temperature of ion transfer capillary, spray voltage, sheath gas flow rate, auxiliary gas flow rate were set to 350°C, 3.5 kV, 35 and 10, respectively. Detection of limonin was achieved in PRM mode monitoring the characteristic fragment ion (m/z 469.2>249.1) of the deprotonated molecule. The acquired data were processed using Xcalibur™ version 2.2.1 and Trace Finder version 3.3 (Thermo Fisher Scientific).

Congo red binding assay: Congo red was dissolved in a 5 mM phosphate buffer (pH 7.0) containing 0.15 mM NaCl. The concentration of CR solution was determined using ε_M: 45,000 M^{-1} cm^{-1} at 498 nm [33]. 0.2 mL 1-day-old samples containing 60% ethanol were mixed with 0.8 mL CR solution before the experiments. Congo red and protein concentrations were 6 µM and 26 µg/mL, respectively. The absorption spectra of the samples were recorded in a 1 cm path length quartz cuvette after incubating them for 15 min at 24 °C. We calculated the difference spectra in the presence and absence of grapefruit seed extract or in the presence of limonin at different concentrations by subtracting the spectra of CR and PMS-trypsin from the spectra of samples containing both PMS-trypsin and CR.

Electronic circular dichroism: ECD spectra of PMS-trypsin in the presence or absence of 500-fold diluted grapefruit seed extract were measured at 0.15 mg/mL protein concentration in 60% ethanol at pH 7.0. The spectra were measured using a 0.01 cm quartz cell from 185 to 260 nm at 24°C. Spectra presented here are accumulations of 10 independent scans. The results of ECD measurements were plotted as ellipticity (millidegree) vs. wavelength (nm).

Transmission electron microscopy: Grapefruit seed extract was filtered through 0.02 mm Whatman inorganic membrane filter before use. 10 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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References

[1] Yiu CPB, Chen YW. (**2017**) From disorder to mis-order: Structural aspects of pathogenic oligomerization in conformational diseases. *Protein & Peptide Letters*, ***24***, 307–314.
[2] Khan E, Mishra SK, Kumar A. (**2017**) Emerging methods for structural analysis of protein aggregation. *Protein & Peptide Letters*, ***24***, 331–339.
[3] Nie RZ, Zhu W, Peng JM, Ge ZZ, Li CM. (**2016**) A-type dimeric epigallocatechin-3-gallate (EGCG) is a more potent inhibitor against the formation of insulin amyloid fibril than EGCG monomer. *Biochimie*, ***125***, 204–212.
[4] Porat Y, Abramowitz A, Gazit E. (**2006**) Inhibition of amyloid fibril formation by polyphenols: structural similarity and aromatic interactions as a common inhibition mechanism. *Chemical Biology & Drug Design*, ***67***, 27–37.

[5] Bu XL, Rao PPN, Wang YJ. (**2016**) Anti-amyloid aggregation activity of natural compounds: Implications for Alzheimer's drug discovery. *Molecular Neurobiology*, ***53***, 3565–3575.
[6] Ganzera M, Aberham A, Stuppner H. (**2006**) Development and validation of an HPLC/UV/MS method for simultaneous determination of 18 preservatives in grapefruit seed extract. *Journal of Agricultural and Food Chemistry*, ***54***, 3768–3772.
[7] Brzozowski T, Konturek PC, Drozdowicz D, Konturek SJ, Zayachivska O, Pajdo R, Kwiecien S, Pawlik WW, Hahn EG. (**2005**) Grapefruit-seed extract attenuates ethanol-and stress-induced gastric lesions via activation of prostaglandin, nitric oxide and sensory nerve pathways. *World Journal of Gastroenterology*, ***11***, 6450–6458**.**
[8] Xu WT, Qu W, Huang KL, Guo F, Yang JJ, Zhao H, Luo YB. (**2007**) Antibacterial effect of Grapefruit Seed Extract on food-borne pathogens and its application in the preservation of minimally processed vegetables. *Postharvest Biology and Technology*, ***45***, 126–133.
[9] Kim SY, Shin KS. (**2012**) Antimicrobial activity of trifoliate orange (*Poncirus trifoliate*) seed extracts on gram-negative food-borne pathogens. *Preventive Nutrition and Food Science*, ***17***, 228–233.
[10] Brorson O, Brorson SH. (**2007**) Grapefruit seed extract is a powerful *in vitro* agent against motile and cystic forms of *Borrelia burgdorferi sensu lato*. *Infection*, ***35***, 206–208.
[11] Ozaki Y, Fong CH, Herman Z, Maeda H, Miyake M, Ifuku Y, Hasegawa S. (**1991**) Limonoid glucosides in citrus seeds. *Agricultural and Biological Chemistry*, ***55***, 137–141.
[12] Gualdani R, Cavalluzzi MM, Lentini G, Habtemariam S. (**2016**) The chemistry and pharmacology of citrus limonoids. *Molecules*, ***21***, 1530.
[13] Fan Y, Chen Y, Chen J, Zhang F, Ondieki G, Li Y, He X. (**2017**) Inhibition of Cytochrome P450 by Nomilin and Obacunone and Potential Mechanism in Human Liver Microsomes. *Chinese Herbal Medicines*, ***9***, 295–298.
[14] Heo HJ, Kim DO, Shin SC, Kim MJ, Kim BG, Shin DH. (**2004**) Effect of antioxidant flavanone, naringenin, from *Citrus junos* on neuroprotection. *Journal of Agricultural and Food Chemistry*, ***52***, 1520–1525.
[15] Regitz C, Fitzenberger E, Mahn FL, Dussling LM, Wenzel U. (**2016**) Resveratrol reduces amyloid-beta ($A\beta_{1-42}$)-induced paralysis through targeting proteostasis in an Alzheimer model of *Caenorhabditis elegans*. *European Journal of Nutrition*, ***55***, 741–747.
[16] Singh SK, Srivastav S, Yadav AK, Srikrishna S, Perry G. (**2016**) Overview of Alzheimer's disease and some therapeutic approaches targeting Aβ by using several synthetic and herbal compounds. *Oxidative Medicine and Cellular Longevity*, *2016*, 7361613.
[17] Tu LH, Young LM, Wong AG, Ashcroft AE, Radford SE, Raleigh DP. (**2015**) Mutational analysis of the ability of resveratrol to inhibit amyloid formation by islet amyloid polypeptide: critical evaluation of the importance of aromatic-inhibitor and histidine-inhibitor interactions. *Biochemistry*, ***54***, 666–676.
[18] Shariatizi S, Meratan AA, Ghasemi A, Nemat-Gorgani M. (**2015**) Inhibition of amyloid fibrillation and cytotoxicity of lysozyme fibrillation products by polyphenols. *International Journal of Biological Macromolecules*, ***80***, 95–106.
[19] Ma B, Zhang F, Liu Y, Xie J, Wang X. (**2017**) Resveratrol induces the conversion from amyloid to amorphous aggregation of β-lactoglobulin. *Protein & Peptide Letters*, ***24***, 1113–1119.
[20] Cox CJ, Choudhry F, Peacey E, Perkinton MS, Richardson JC, Howlett DR, Lichtenthaler SF, Francis PT, Williams RJ. (**2015**) Dietary (-)-epicatechin as a potent inhibitor of βγ-secretase amyloid precursor protein processing. *Neurobiolology of Aging*, ***36***, 178–187.
[21] Carbonaro M, Di Venere A, Filabozzi A, Maselli P, Minicozzi V, Morante S, Nicolai E, Nucara A, Placidi E, Stellato F. (**2016**) Role of dietary antioxidant (-)-epicatechin in the development of β-lactoglobulin fibrils. *Biochimica et Biophysica Acta*, ***1864***, 766–772.
[22] Kotormán M, Simon LM, Borics A, Szabó MR, Szabó K, Szögi T, Fülöp L. (**2015**) Amyloid-like fibril formation by trypsin in aqueous ethanol. Inhibition of fibrillation by PEG. *Protein & Peptide Letters*, *22*, 1104–1110.
[23] Zhao R, So M, Maat H, Ray NJ, Arisaka F, Goto Y, Carver JA, Hall D. (**2016**) Measurement of amyloid formation by turbidity assay-seeing through the cloud. *Biophysical Reviews*, ***8***, 445–471.
[24] Kotormán M, Kasi PB, Halász L, Borics A. (**2017**) Inhibition of amyloid-like fibril formation of trypsin by red wines. *Protein & Peptide Letters*, ***24***, 466–470.
[25] Cvetnić Z, Vladimir-Knezević S. (**2004**) Antimicrobial activity of grapefruit seed and pulp ethanolic extract. *Acta Pharmaceutica*, ***54***, 243–250.
[26] Venkatesan R, Ji E, Kim SY. (**2015**) Phytochemicals that regulate neurodegenerative disease by targeting neurotrophins: a comprehensive review. *BioMed Research International*, *2015*, 814068.
[27] Yoon JS, Yang H, Kim SH, Sung SH, Kim YC. (**2010**) Limonoids from *Dictamnus dasycarpus* protect against glutamate-induced toxicity in primary cultured rat cortical cells. *Journal of Molecular Neuroscience*, ***42***, 9–16.
[28] Girych M, Gorbenko G, Maliyov I, Trusova V, Mizuguchi C, Saito H, Kinnunen P. (**2016**) Combined thioflavin T-Congo red fluorescence assay for amyloid fibril detection. *Methods and Applications in Fluorescence*, ***4***, 034010.
[29] Goda S, Takano K, Yamagata Y, Nagata R, Akutsu H, Maki S, Namba K, Yutani K. (**2000**) Amyloid protofilament formation of hen egg lysozyme in highly concentrated ethanol solution. *Protein Science*, ***9***, 369–375.
[30] Bhat SA, Bano B. (**2014**) Conformational behaviour and aggregation of chickpea cystatin in trifluoroethanol: Effects of epicatechin and tannic acid. *Archives of Biochemistry and Biophysics*, ***562***, 51–61.
[31] Fazili NA, Naeem A. (**2015**) Anti-fibrillation potency of caffeic acid against an antidepressant induced fibrillogenesis of human α-synuclein: Implications for Parkinson's disease. *Biochimie*, ***108***, 178–185.
[32] Waterhouse AL. (**2002**) Determination of Total Phenolics. In: *Current Protocols in Food Analytical Chemistry*; John Wiley and Sons, I1.1.1-I1.1.8.
[33] Chaturvedi SK, Alam P, Khan JM, Siddiqui MK, Kalaiarasan P, Subbarao N, Ahmad Z, Khan RH. (**2015**) Biophysical insight into the anti-amyloidogenic behavior of taurine. *International Journal of Biological Macromolecules*, ***80***, 375–384.