Inhibition of Amyloid-like Fibril Formation of Trypsin by Red Wines

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Abstract: The aim of the present study was to examine the potential role and applicability of dietary supplements in reducing the risk of development of amyloid diseases associated with the gastrointestinal tract, such as type II diabetes. Trypsin, a well-known serine protease was used as a model protein in our experiments. The effect of various red wines on the formation of amyloid-like fibrils of trypsin was studied *in vitro*, in aqueous ethanol, at pH 7.0. Turbidity measurements, aggregation kinetics experiments, Congo red binding assays and electronic circular dichroism spectroscopic measurements were used to follow the aggregation process in the presence or absence of various red wines. The results suggest that red wines effectively inhibit the formation of amyloid-like fibrils of trypsin and the inhibitory effect is dose-dependent. The extent of inhibition was found to be proportional to the total concentration of phenolic compounds.

Keywords: Amyloid-like fibrils; Electronic circular dichroism; Congo red; Natural polyphenols; Red wine; Trypsin

1. INTRODUCTION

Amyloid formation might be a general property of the polypeptide backbone as all proteins can form long-unbranched, β-sheet rich amyloid fibrils *in vitro* under appropriate conditions [1–2]. There are striking similarities in the aggregation behaviour of different peptides and proteins. Unfolded or partially unfolded proteins associate with each other to form small, soluble aggregates that undergo further assembly into protofibrils or protofilaments. These structures are commonly short, thin, sometimes curved, fibrillar species that are presumed to assemble into mature fibrils. Experiments *in vitro* indicate that the formation of such species is generally characterized by a lag phase, followed by a period of rapid growth. This phenomenon is associated increasingly with common and highly debilitating diseases. An increasing number of disorders, including Alzheimer's and Parkinson's diseases, spongiform encephalopathy and type II diabetes, are directly associated with the deposition of such aggregates in brain, heart and spleen tissue. Amyloid diseases predominantly involve the aggregation of specific proteins, such as the prion protein or the amyloid β-peptide, but fibrils can be formed by many other peptides and proteins [3]. Natural phenolic compounds, a long family of plant substances, are one of the most actively investigated categories of potential amyloid inhibitors [4–6]. More than 8,000 plant polyphenols are currently known and more than 4,000 flavonoids have been identified among them [7]. Red wine represents a rich source of polyphenols such as flavonoids (flavanol monomers, condensed tannins, flavonols, anthocyanins) and non-flavonoids (hydroxycinnamates, benzoic acids, hydrolyzable tannins, stilbenes (resveratrol)) [8]. Resveratrol, a polyphenolic red wine constituent, inhibits Aβ42 fibril formation and islet amyloid polypeptide aggregation in a dose-dependent manner [9-10]. The inhibitory effect of flavone derivatives is dependent on the number and position of hydroxyl groups around the flavone backbone [11]. The position of phenolic hydroxyl moieties on the aromatic rings is a major determinant potent anti-aggregation effect, while the number of hydroxyl groups is less important [12]. Natural phenolic and polyphenolic substances stabilize native states, or remodel and inactivate toxic amyloid oligomers [4]. Several observations suggest that polyphenols inhibit amyloid fibril formation through specific aromatic interactions with the amyloidogenic core [13]. Aromatic rings of polyphenolic compounds and the aromatic residues present in proteins may associate via π-π stacking interactions [14]. The hydrophobic and/or aromatic character of these compounds contributes significantly to the anti-amyloidogenic effect, whereas the antioxidative potency relates mostly to the destabilization of fibrils [15]. Studies have shown that the presence of vicinal dihydroxyphenyl moieties, irrespective of their position in the aromatic rings, play a key role in the inhibitory property of polyphenols [16–17]. Non-flavonoids showed higher anti-aggregation activity than flavonoids. Polyphenols containing a higher

number of aromatic rings, hydroxyl and keto groups and possess a high degree of planarity, have the largest inhibition activity [18]. A structure–function relationship study suggested that the presence of at least two phenolic rings with two to six atoms long linkers, along with a minimum number of three hydroxyl groups on the aromatic rings, are essential for efficient inhibition exerted by polyphenols [5]. The mechanism of inhibition of amyloid formation is not the same for all natural polyphenols. Some inhibit the formation of oligomers, but promote fibril formation, others inhibit the formation of fibrils, but not oligomers, and there are others, which inhibit both. Other polyphenols redirect amyloid fibrils from fibrillogenic forms to nonfibrillogenic oligomers [19]. Polyphenols induce conformational changes in the oligomer aggregate. These changes disrupt H-bonds and perturb the amyloid aggregate. Polyphenol molecules push the β-sheets apart, which leads to a loosely packed structure. H-bonding capacity of polyphenols is responsible for this behavior [20]. Flavonoids, phenols in particular, represent an important component of a normal human diet. Elevated intake of dietary polyphenols might be relevant to the prevention of amyloidosis. Nutraceutical strategies might become a way to reduce the risk of certain amyloid diseases [19, 21]. Many drugs used today are natural products or derivatives of natural products [22–23].

Earlier we have demonstrated that phenylmethylsulfonyl-trypsin (PMS-trypsin) forms amyloid-like fibrils in 60% (v/v) ethanol/10 mM phosphate buffer (PBS) at pH = 7.0 [24]. Here, we report that various red wines inhibit the formation of amyloid-like fibrils of PMS-trypsin dose-dependently.

2. MATERIALS AND METHODS

2.1. Materials

Bovine pancreas trypsin (EC 3.4.21.4), gallic acid and *N*-benzoyl-L-arginine ethyl ester (BAEE) were purchased from Sigma-Aldrich Company (Budapest, Hungary). Folin–Ciocalteu's phenol reagent was the product of Merck (Darmstadt, Germany). Red wines were purchased from commercially sources: La Bonita Cabernet Franc semi-sweet and Pincegyöngye Édes Vörös sweet red wines bottled by Helibor Ltd., H-6070 Izsák; Transdanubian Merlot sweet red wine, bottled by Szent Imre Wine-cellar Ltd., H-6223 Soltszentimre; La Fiesta Kékfrankos semi-sweet red wine, bottled by Grape-Vine Ltd., H-6120 Kiskunmajsa; Egri bikavér 2012 dry red wine, bottled by Ostoros-Novaj Bor Co., H-3327 Novaj; Portugieser 2014 dry red wine, bottled by Dolium Wine-cellar Ltd., H-7773 Villány.

2.2. General methods

Trypsin concentration was determined based on the UV absorbance of the constituent aromatic and cystine residues at 280 nm, using a calibration curve measured for trypsin in the 0-1 mg/ml concentration range. Spectroscopic samples have been corrected for respective solvent backgrounds in all experiments where applicable.

2.3. Assay of enzyme activity

Trypsin activity, with BAEE as a substrate, was determined using the method of Schwert and Takenaka [25]. The absorption increase at 253 nm was measured in a 3 ml reaction mixture, containing 46.7 mM Tris/HCl (pH 8.0) and 0.9 mM BAEE. The reaction was started by the addition of 20 µl enzyme sample (0.15 mg/ml).

2.4. Trypsin modification with phenylmethylsulfonyl fluoride (PMSF)

Chemical modification with PMSF inactivates trypsin irreversibly. Thus the autolysis of enzyme at pH = 7.0 does not affect the experiments. Solutions of 8.4 µl PMSF (100 mM) in 2-propanol and 20 µl trypsin (50 mg/ml) in 0.001 M HCl were added to 2 ml 0.05 M PBS (pH = 7.0) at 24 ºC while the solution was stirred. During the procedure the same amounts of reactants were added four times, repeatedly to the solution. The enzyme sample was incubated for 30 min. The reaction mixture was then filtered on a Sephadex G-25 column in 0.05 M PBS (pH = 7.0), to remove the unreacted inhibitor molecules. The degree of conversion was determined by enzymatic activity assays, of which results indicated that 95% of the trypsin had been modified.

2.5. Turbidity measurements

In order to estimate the amount of fibrillar material, turbidity of PMS-trypsin solutions was monitored by UV-vis spectrophotometry at 350 nm and 24 ºC, using a quartz cuvette of 10 mm path length. Turbidity experiments were performed in 0.13 mg/ml solutions of PMS-trypsin in 60% (v/v) ethanol/10 mM PBS (pH = 7.0) after 24 h incubation in the presence or absence of red wines at a 50-fold final dilution.

2.6. Determination of total phenolic content

The total phenolic content of red wine samples was determined utilizing the Folin-Ciocalteu reagent and the spectrophotometric method of Waterhouse [26]. For calibration, gallic acid solutions of different

concentrations (0–50 mg/l) were used. The total phenolic content was expressed as mg gallic acid equivalents (GAE) per l of red wine. The data presented are the average of three measurements.

2.7. Aggregation kinetics

Aggregation experiments were performed in 60% (v/v) ethanol/10 mM PBS (pH = 7.0) at 0.13 mg/ml PMS-trypsin concentration and various concentrations of red wine. The increase of UV absorption at 350 nm, indicative of the presence of large aggregated particles, was followed for 30 min at 24 °C and pH 7.0.

2.8. CR binding

Absorption spectra of Congo red (CR) were recorded in the range of 400–600 nm using a 10 mm path length quartz cuvette. Samples were prepared at a protein concentration of 0.285 mg/ml. 200 μl 1-day-aged PMS-trypsin samples in 60% (v/v) ethanol, in the absence or presence of Egri bikavér, were vortexed with 800 μl of a solution, which contained 4.9 μM CR, 5 mM PBS and 150 mM NaCl (pH 7.0) and incubated at room temperature for 15 min before the measurements. Difference spectra were obtained by subtracting the spectra of CR alone and PMS-trypsin alone from spectra of solutions containing both PMS-trypsin and CR.

2.9. ECD measurements

Electronic circular dichroism (ECD) measurements were carried out using a Jasco J-815 spectropolarimeter. Spectra were recorded in the range of 185–260 nm in a 1 mm path length cell at 24 °C. PMS-trypsin was dissolved in 60% (v/v) ethanol/10 mM PBS (pH 7.0) at a concentration of 0.15 mg/ml. Red wine was contained in the samples at a 50-fold dilution. Ellipticity was expressed in mdeg units.

3. RESULTS AND DISCUSSIONS

PMS-trypsin amyloid-like fibrils were prepared in aqueous ethanol according to our previous report [24]. To determine whether red wines affect fibril formation of PMS-trypsin, samples were incubated in aqueous ethanol at pH 7.0, in the presence and absence of different red wines. Aggregation of PMS-trypsin can be monitored by turbidity measurements. An increase in the UV absorbance at 350 nm indicates a greater degree of aggregation due to an increase in the scattering of light by aggregated particles [16]. In the absence of red wines, higher absorbance values were observed indicating higher degree of turbidity and that red wines have the ability to effectively inhibit PMS-trypsin fibril formation *in vitro* (Figure 1). These experiments revealed that the

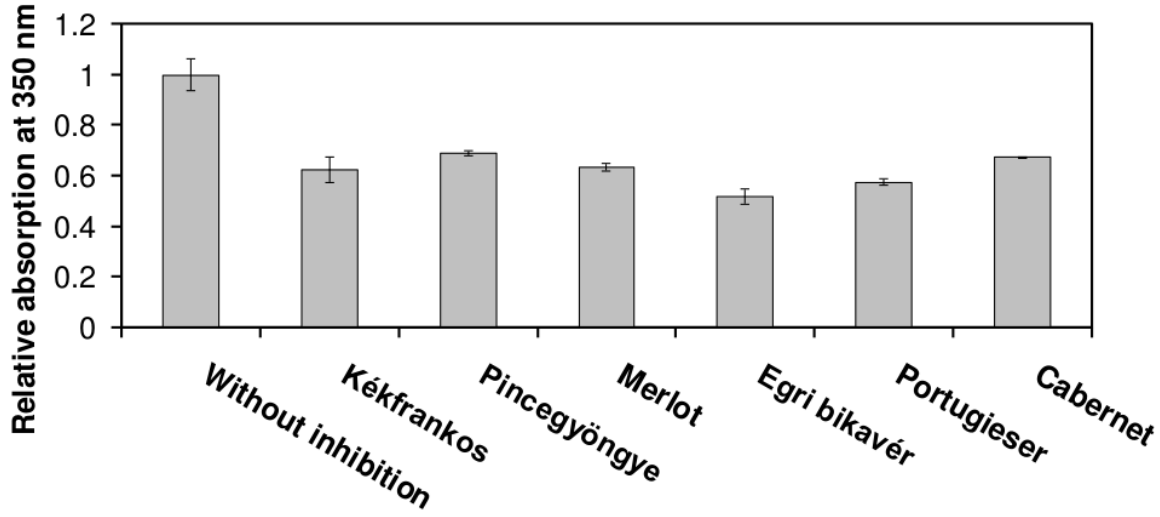

Figure 1. Turbidity changes of PMS-trypsin at 24 ºC in 60% (v/v) ethanol/10 mM PBS (pH = 7.0), monitored via the absorption at 350 nm after incubation for 24 h in the presence of various red wines at 50-fold dilution. The protein concentration was 0.13 mg/ml.

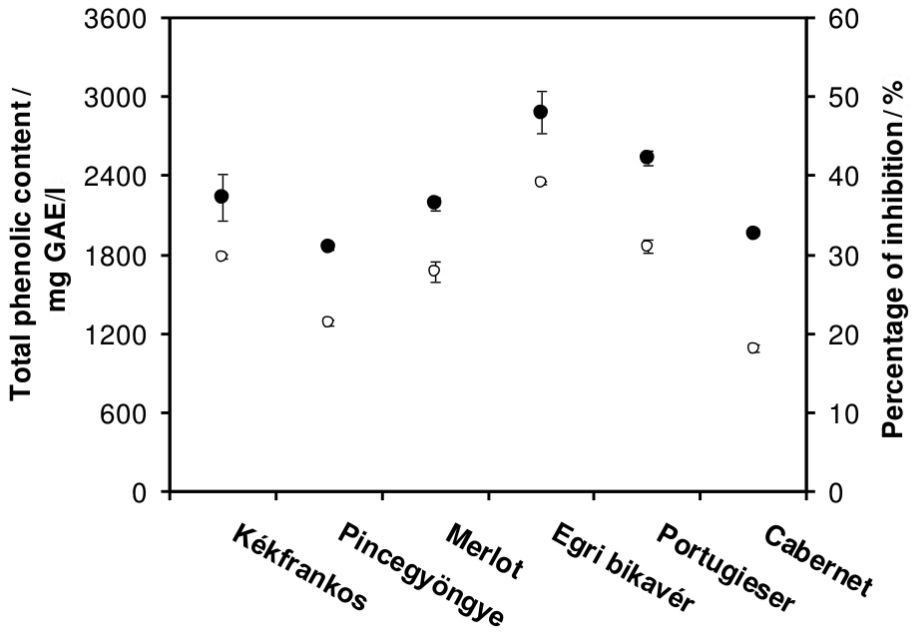

Figure 2. Inhibition of amyloid-like fibril formation as a function of total phenolic content. Percentage of inhibition in 60% (v/v) ethanol (●), total phenolic content (○). Red wines were diluted 50-fold in 60% (v/v) ethanol/10 mM PBS (pH = 7.0). The protein concentration was 0.13 mg/ml.

greatest effect was exerted by the red wine Egri bikavér in 60% (v/v) ethanol/10 mM PBS (pH = 7.0). The absorption at 350 nm decreased to 51.9% relative to the reference sample after incubation for 24 h in the presence of diluted Egri bikavér.

The total concentration of phenolic compounds of various red wines varied from 1,053 to 2,354 mg GAE/l (Figure 2). The degree of inhibition was found to be proportional to the total phenolic content. Figure 3 illustrates that red wine led to a dose-dependent decrease in the absorption.

CR is a planar, hydrophobic, diazo dye, which binds to the β-sheet of amyloid and amyloid-like fibrils selectively, therefore it is a commonly used agent to detect amyloid fibrils. Binding of CR to amyloid generally results in an increase and a characteristic red shift (from 490 nm to 540 nm) of light absorption [27–28]. A moderate red shift of the absorption maximum (from 490 nm to 510 nm) was observed in the visible spectra of CR in the presence of PMS-trypsin in 60% (v/v) ethanol/10 mM PBS (pH = 7.0) (Figure 4). Difference spectra indicate spectral changes of CR upon binding to PMS-trypsin amyloid fibrils in the absence or presence of 25 to 250-fold diluted red wine, Egri bikavér. This suggests that the aggregates have amyloid-like properties in 60% (v/v) ethanol/10 mM PBS (pH = 7.0) and the active components of Egri bikavér inhibit the formation of amyloid fibrils dose-dependently.

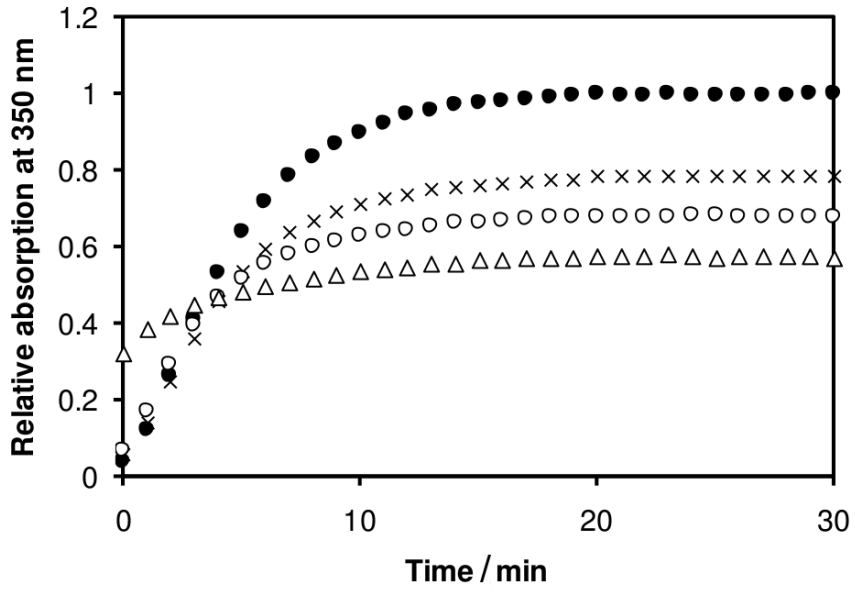

Figure 3. Kinetics of aggregation of PMS-trypsin without red wine (•) in 60% (v/v) ethanol/10 mM PBS (pH = 7.0), or in the presence of Merlot wine diluted 50- (Δ), 1000- (○), or 4000-fold (x), monitored via the time-dependent increase of UV absorption at 350 nm, at 0.13 mg/ml protein concentration.

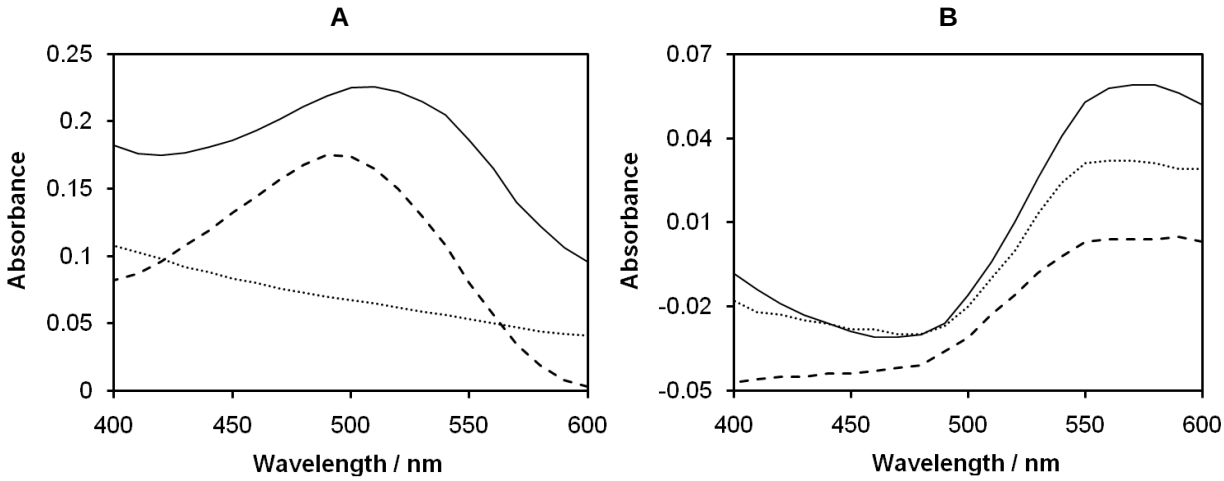

Figure 4. Visible absorption spectra of PMS-trypsin stained with CR in 60% (v/v) ethanol/10 mM PBS (pH = 7.0): PMS-trypsin + CR (**A**, solid line), CR alone (**A**, dashed line), PMS-trypsin alone (**A**, dotted line) and difference spectra in 60% (v/v) ethanol/10 mM PBS (pH = 7.0) without red wine (**B**, solid line) or in the presence of Egri bikavér wine in 25- (**B**, dashed line) and 250-fold (**B**, dotted line) final dilution. Spectra were recorded at 3.9 μM and 57 μg/ml dye and protein concentrations, respectively.

Changes in the secondary structure of PMS-trypsin were followed by ECD measurements. ECD spectra of PMS-trypsin in 60% (v/v) ethanol/10 mM PBS (pH = 7.0), measured in the presence or in the absence of 50-fold diluted Egri bikavér are shown in Figure 5. Measurements were carried out by placing the cuvette either in the middle of the sample compartment or next to the detector. For clear, homogenous samples the two measurements shall provide identical results. Remarkable change in the intensity and a blue shift of the 217 nm minimum was observed in the absence of red wine, upon moving the cuvette from the middle of the sample compartment towards the detector, indicating aggregated particles in the sample. Light dispersion of the sample was significantly decreased by the addition of 50-fold diluted Egri bikavér wine, suggesting that a lower amount of aggregates is present compared to the sample not containing the additive. It was shown previously, that high concentrations of ethanol promote structural transitions of PMS-trypsin, resulting in an increase of β-sheet structure of the protein [24]. Such structural features were not changed remarkably by the addition of diluted red wine. Conversely, ECD spectra recorded in different cuvette positions suggest, that addition of red wine do not arrest transition from native to β-sheet structure, but it may inhibit intermolecular associations and the formation of large aggregates.

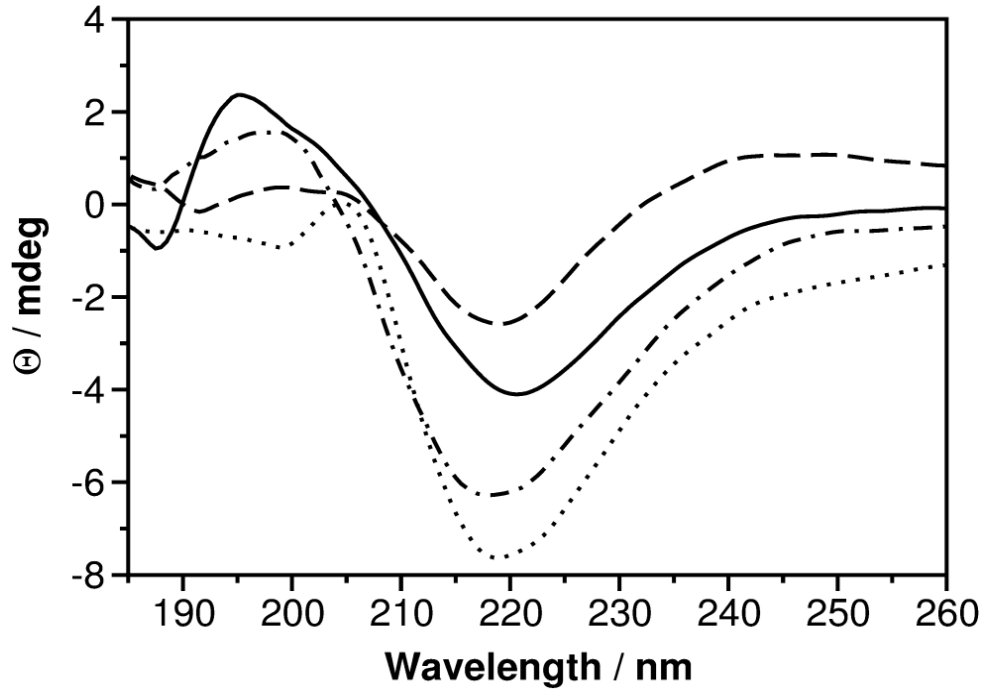

Figure 5. ECD spectra of PMS-trypsin in 60% (v/v) ethanol/10 mM PBS (pH = 7.0) in the absence (solid line, dashed line) and in the presence of 50-fold diluted Egri bikavér (dotted-dashed line, dotted line). The sample was set either in the middle of the sample compartment (solid line, dashed-dotted line), or next to the detector (dashed line, dotted line). PMS-trypsin concentration was 0.15 mg/ml.

CONCLUSIONS

Results of the present *in vitro* study demonstrated that red wines inhibit the aggregation of PMS-trypsin in 60% (v/v) ethanol/10 mM PBS (pH = 7.0) in a dose-dependent manner. The inhibitory effect was found to correlate with the amount of natural phenolic and polyphenolic substances contained in red wines. Although further studies are needed to verify direct applicability of red wine or its active components, our results suggest that moderate red wine consumption may constitute a part of a preventive dietary strategy against amyloidogenic diseases of the gastrointestinal tract. Among the examined red wines Egri bikavér demonstrated the strongest inhibitory effect on trypsin fibril formation.

LIST OF ABBREVIATIONS

BAEE, *N*-benzoyl-L-arginine ethyl ester; ECD, electronic circular dichroism; CR, Congo red; GAE, gallic acid equivalent; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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