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Gallic acid is the major component of grape seed extract that inhibits amyloid fibril formation

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ABSTRACT

Many protein misfolding diseases, for example, Alzheimer's, Parkinson's and Huntington's, are characterised by the accumulation of protein aggregates in an amyloid fibrillar form. Natural products which inhibit fibril formation are a promising avenue to explore as therapeutics for the treatment of these diseases. In this study we have shown, using in vitro thioflavin T assays and transmission electron microscopy, that grape seed extract inhibits fibril formation of kappa-casein (κ-CN), a milk protein which forms amyloid fibrils spontaneously under physiological conditions. Among the components of grape seed extract, gallic acid was the most active component at inhibiting κ-CN fibril formation, by stabilizing κ-CN to prevent its aggregation. Concomitantly, gallic acid significantly reduced the toxicity of κ-CN to pheochromocytoma12 cells. Furthermore, gallic acid effectively inhibited fibril formation by the amyloid-beta peptide, the putative causative agent in Alzheimer's disease. It is concluded that the gallate moiety has the fibril-inhibitory activity.

Amyloidosis, characterised by amyloid fibril accumulation, is associated with more than 20 diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD).[1–6] Amyloid fibrils are very stable, highly ordered aggregates predominantly composed of a cross β-sheet core structure.[7] In AD, the most prevalent age-related neurodegenerative disorder, two proteins aggregate to form amyloid fibrils, the amyloid-beta peptide (Aβ) (which forms plaques in extracellular space) and hyperphosphorylated tau protein (which polymerises to form intracellular neurofibrillary tangles).[2,8] Parametric imaging results have shown that plaques and tangles begin accumulating years before AD is clinically diagnosed.[9] An attractive approach to treatment of AD and other diseases associated with protein aggregation is to identify natural products that can prevent protein misfolding and the formation of amyloid fibrils and their β-sheet rich precursors.

Polyphenols are the most abundant form of antioxidants consumed in our diet. They are found in a vast array of foods, including dried legumes, chocolate, vegetables and cereals, but their richest sources are fruits and plant-derived drinks such as tea, juices, coffee and red wine.[10] Since the mid-1990s, research on the health benefits of polyphenols in disease prevention has dramatically increased. It is now well established that polyphenolic compounds can contribute to the prevention of chronic diseases such as cardiovascular diseases and cancers and also provide neuroprotective effects, potentially reducing the risk of developing age-related neurodegenerative diseases such as AD, PD, amyotrophic lateral sclerosis and polyglutamine diseases, for example, HD.[11–13]

Grape seed extract (GSE) is a powerful antioxidant product comprised of multiple polyphenolic compounds.[14] Recently, GSE was found to (i) inhibit amyloid fibril formation by Aβ both in vitro and in vivo, (ii) reduce Aβ levels in the mouse brain and also (iii) significantly improve the cognitive deterioration associated with AD.[15,16]

Our research group discovered that the reduced and carboxymethylated form of the milk protein, kappa-casein (RCM-κ-CN) forms amyloid fibrils routinely and reproducibly under physiological conditions.[17,18] It has been proposed that any protein, regardless of its primary structure, has the ability to form amyloid fibrils

Abbreviations: Aβ, amyloid-beta peptide; AD, Alzheimer's disease; DTT, 1,4-dithiothreitol; EGCG, epigallocatechin-3-gallate; GA, gallic acid; GSE, grape seed extract; HD, Huntington's disease; RCM-κ-CN, reduced and carboxymethylated kappa-casein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PC12, pheochromocytoma12; PD, Parkinson's disease; TEM, transmission electron microscopy; ThT, Thioflavin T.

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under appropriate conditions, and all amyloid fibrils possess a characteristic cross β-sheet secondary structure.[19,20] It seems logical that an effective amyloid fibril inhibitor should target the generic cross β-sheet secondary structure rather than the primary structure. Therefore, any inhibitors which can prevent RCM-κ-CN forming fibrils are likely to be also effective against disease-related, fibril-forming proteins.

The goals of the present study were (i) to investigate the anti-amyloid activity of commercially available GSE and its individual polyphenolic components (gallic acid, catechin, epicatechin and proanthocyanidins) in order to identify the active component in GSE capable of inhibiting the conversion of RCM-κ-CN and Aβ to their β-sheet rich structures in vitro and (ii) to determine whether this activity also invokes a change in the cellular cytotoxicity afforded by fibrillar RCM-κ-CN.[21]

Initially, the anti-amyloidogenic activity of commercial GSE was investigated using an in vitro thioflavin T (ThT) assay against RCM-κ-CN at physiological temperature and pH, that is, 37 °C, pH 7.4. ThT is a widely used dye that fluoresces markedly upon binding to β-sheet rich species such as amyloid fibrils. GSE was used in ThT assays after serial dilutions using reaction buffer (50 mM sodium phosphate buffer, pH 7.4), yielding a final dilution factor between 500 and 2000 times of the stock solution. As shown in Figure 1A, the ThT profile of RCM-κ-CN incubated in the absence of GSE increased steadily in fluorescence intensity after 1 h of incubation. When RCM-κ-CN was incubated in the presence of GSE (Fig. 1A), the change in ThT fluorescence with time was reduced in a concentration-dependent manner, that is, an increase in the relative GSE concentration resulted in a diminished change in ThT fluorescence intensity over the course of the assay.

To identify the active component(s) of GSE capable of preventing the conversion of RCM-κ-CN to amyloid fibrils, we incubated cyanidin rutinoside, catechin, epicatechin (individually and in combination with GA) and GA in the presence of RCM-κ-CN. These compounds are the major polyphenolics in GSE.[13] Again, in situ ThT assays were used to quantify the conversion of RCM-κ-CN to amyloid fibrils in the presence of these compounds. From these experiments, it was apparent that catechin and epicatchin did not exhibit amyloid fibril inhibition activity (see Supplementary data Fig. S1-2). However, two of the GSE polyphenols exhibited anti-amyloidogenic properties. At a 1:2 molar ratio of RCM-κ-CN to cyanidin rutinoside, amyloid fibril formation of RCM-κ-CN was inhibited by 54% (see Supplementary data Fig. S3). GA also prevented the conversion of RCM-κ-CN to amyloid fibrils in a concentration-dependent manner, but much more potently. At a 1:2 molar ratio, GA inhibited the amyloid fibril formation of RCM-κ-CN by 98% (Fig. 1B). At a 1:1 molar ratio, the inhibition percentage was 87%, while at 1.0:0.5 and 1.0:0.1 the inhibition percentage was reduced to 54% and 3%, respectively. Furthermore, the addition of GA to either catechin, epicatechin or cyanidin rutinoside did not alter the inherent ability of the compounds to prevent amyloid fibril formation by RCM-κ-CN (see Supplementary data Fig. S4) which indicates that those polyphenol compounds derived from GSE do not have synergistic effects against fibril formation.

TEM was used to provide qualitative information regarding the formation of RCM-κ-CN amyloid fibrils and to investigate whether a change in the morphology of the amyloid fibrils was induced by the presence of inhibitors (GSE or GA) during co-incubation. In the present study, in agreement with our previous results,[18] when RCM-κ-CN was incubated alone at 37 °C and pH 7.4, the mature amyloid fibrils were observed to be long, unbranched, rope-like

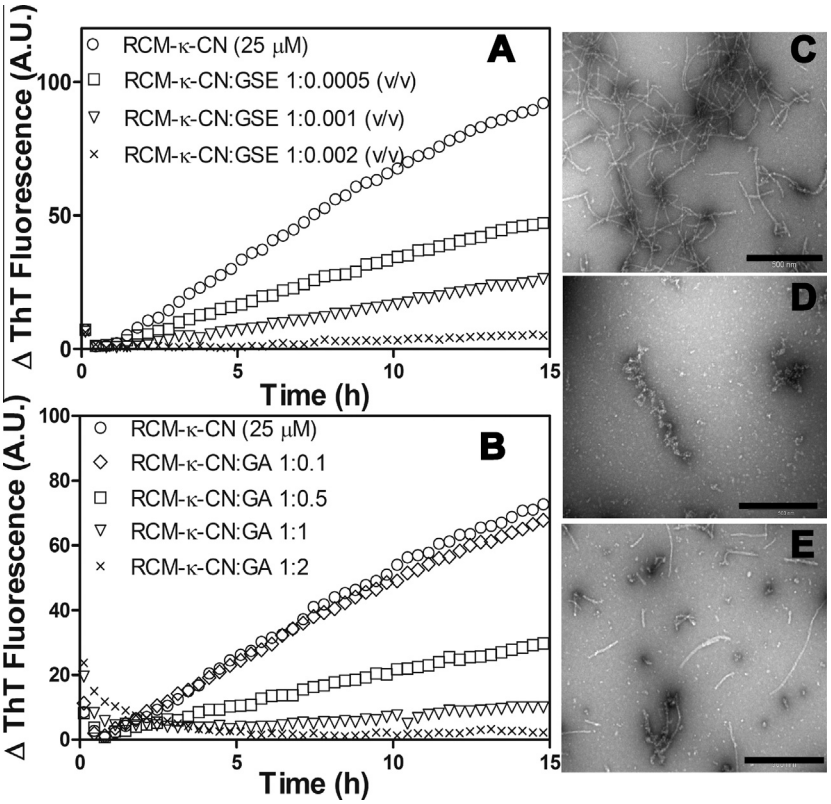

Figure 1. ThT fluorescence profiles of RCM-κ-CN (25 μM) incubated at 37 °C with increasing concentrations of (A) the GSE stock solution. Circle, square, triangle and cross represent RCM-κ-CN (25 μM) incubated without and with 2000×, 1000× and 500× diluted GSE solution, respectively and (B) GA monohydrate where circle, diamond and square, triangle, cross represent the indicated molar ratios of RCM-κ-CN:GA. TEM images of RCM-κ-CN after incubation in the (C) absence or (D) presence of GSE (0.0:0.002 (v/v)) or (E) GA (1:2 molar ratio) for 1000 min at 37 °C. In all cases the scale bar represents 500 nm.