Original Article

Investigating the inhibitory effects of Seidlitzia rosmarinus extract on the amyloid fibril formation of κ-casein in the presence of dextran

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Abstract

Background: Formation of amyloid fibrils has been associated with different protein aggregation diseases. Many studies indicate that many proteins can be converted in vitro into amyloid structures. Isolated κ-casein (κ-CN) spontaneously forms amyloid fibrils under physiological conditions, so it is a convenient model for researching generic aspect of fibril formation. The aim of this study was to test in vitro the ability of S. rosmarinus extract to inhibit the formation of amyloid fibrils in κ-CN. **Materials and Methods:** In this study the effect of aqueous extract of S. rosmarinus on the amyloid formation of κ-CN in the presence and absence of crowding agent, dextran, have been examined using Thioflavin T binding (ThT) assay, fluorescence spectroscopy, and circular dichroism (CD) spectroscopy. **Results:** ThT binding assay showed that dextran increased the rate of amyloid fibril formation and S. rosmarinus extract retarded the amyloid fibril formation in κ-CN. In the presence of dextran however, the effect of S. rosmarinus extract on the amyloid formation of κ-CN was less than in its absence. Fluorescence spectroscopy results also demonstrated that dextran led to unfolding and increased the exposure hydrophobic area in κ-CN. S. rosmarinus extract efficiency decreased the exposure of hydrophobic regions in κ-CN, whereas in the presence of dextran this effect of extract was reduced. CD spectroscopy results exhibited that incubation of κ-CN with S. rosmarinus extract prevented a structural transition to a β-sheet. CD spectroscopy results also indicated that by adding dextran to reduced κ-CN β-sheet structures observed, which indicates structural change. S. rosmarinus extract however, prevented transition to β-sheet structural. **Conclusion:** In conclusion our finding suggests that S. rosmarinus extract prevents amyloid fibril formation in κ-CN, although this effect decreased in the presence of dextran.

Keywords: *S. rosmarinus, κ-casein, Aggregation, amyloid.*

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Introduction

The failure of proteins to fold appropriately, or to remain correctly folded, is associated with a large number of cellular malfunctions that give rise to many disease (1). The aggregates usually consist of fibers containing misfolded protein with a β-sheet conformation, termed amyloid (2). There are various causes of protein misfolding that can lead to amyloid formation. Depending on stress conditions such as temperature, pH, the presence of reducing agents,

disordered (amorphous) or ordered (amyloid fibril) aggregation will occur (3). Amyloid fibril formation is often associated with a wide range of diseases. However, amyloid fibril formation is not confined to disease-related proteins. Studies have shown that non-disease related proteins can also assemble into amyloid fibrils (4).

Seidlitzia rosmarinus is a perennial woody plant grown mostly along the banks of salt marshes and in soils with high saline water tables. This plant being a halophyte and is very well adapted to grow in dry and salt affected desert soils (5). It plays an important role in both soil preservation and maintenance. The leaves, stems and seeds harvested in fall are used as fodder for livestock. The dried leaves powder is used as detergent for washing cloths and dishes. It has also many industrial applications such as dyeing, making soaps, pottery and ceramics among others (6). s.rosmarinus extracts contain many bioactive components including phenolic mono-terpenes (α-pinene, camphene, limonene), diterpenes (carnosic acid, carnosol), flavones (genkwanin, isoscutellarein 7-O-glucoside), and caffeolyl derivatives (rosmarinic acid). The highest accumulation of these groups of compounds occurs in leaves and it is related to young stages of plant development. S.rosmarinus extracts in both aqueous and lipid medium have been shown to possess antioxidant activity, which is due to the presence of a catechol group in the aromatic ring of the phenolic terpenes, and cathecols conjugated with a carboxylic acid group in rosmarinic acid (7).

Caseins, milk proteins, are composed of αS1, αS2, β, and κ-casein, and have nutritional function for the neonate by providing a rich source of protein and calcium (8).

The κ-casein is a specific milk phosphoglycoprotein which present only one phosphoserine residue per molecule and contain 10% of bovine milk (9). The monomeric form of κ-casein has two cysteine residues (cys11-cys11 and cys88-cys88,) which are joined together by an interchain disulfide bond. These cysteines are also capable of forming polymers by participating in interchain disulfide linkages (10). Thus κ-casein in aqueous solution forms large spherical polymers with an average molecular mass of 1.18 MDa ranging from monomers to decamers. The relatively unstructured nature of the monomeric form of κ-casein may be the reason for inherent propensity to form amyloids (11). It has been previously shown that the both reduced form and native (nonreduced) κ-casein form amyloid fibrils under condition of physiological pH and temperature (8, 12, 13).

The chaperon ability of S. rosmarinus extract in preventing protein aggregation has been investigated (14) .To mimic the chaperon activity of S. rosmarinus extract in vivo, this study explored the effect of S. rosmarinus extract on amyloid fibril formation of κ-casein in the presence of the crowding agent, dextran. This aim has been done by ThT binding assay. fluorescence spectroscopy. In addition, the structural change in κ-casein were examined by CD spectroscopy.

Methods

Chemical and proteins. κ-casein (19 kDa), dextran (68 kDa), 1,4-dithiothreitol (DTT), NaN3, and 1-anilino-8-naphthalene sulfonic acid (ANS), thioflavin T (ThT), Na2HPO4 and dextran were obtained from Sigma-Aldrich (St. Louis, USA).

Plant materials. S. rosmarinus was collected from Zabol, located in Sistan and Baluchestan province of Iran in Nov 2011. The collected plants were dried in the shade and aerial parts were separated.

Preparation of the aqueous extract. Fifteen grams of plant were ground into powder and extracted with 150 mL distilled water by magnetic stirring (4,000 rpm) for 24 h at room temperature. The extracts were filtered through filter paper (Whatman no.42). After filtration, the mixture was centrifuged at 12,000 g for 20 min to remove the debris. The solvent evaporated and the residues were freeze-dried. The extracts were sealed in glass bottles and stored at +4 ˚C until use.

ThT binding assay. Amyloid fibril formation of κ-casein (2.5 mg/mL) in the absence and presence of crowding agent, dextran and S. rosmarinus extract (in a 1:1 weight ratio) was investigated by incubation of κ-casein in 50 mM Na2HPO4, 20 mM DTT, pH 7.4 at 37 ˚ C in an incubator (A-Q Germany). Samples were shaken at 210 rpm for acceleration of fibril formation. Fibrillation of κ-casein under different

conditions was monitored using a Cary Eclipse spectro-fluorimeter (Varian, USA). Aliquots of 10µL were sampled and amyloid fibril formation was detected by the increase in ThT fluorescence (0.4 µM in 50 mM Na2HPO4, 0.05% (w/v) NaN3, pH 7.4). The excitation and emission wavelengths were set to 378 and 479 nm, with a 2.5 and 5 nm slit width, respectively.

Intrinsic Fluorescence Spectroscopy. The Intrinsic fluorescence intensity was monitored on samples containing native and reduced κ-casein, in the presence and absence of dextran and S. rosmarinus extract to investigate the effect of S. rosmarinus extract on the environment of the tryptophan residues of κ-casein. Samples containing κ-casein (2.5 mg/mL), 10% w/v dextran, and S. rosmarinus extract (in a 1:1 weight ratio) with 20 Mm DTT were incubated in a 50 Mm sodium phosphate buffer (pH 7.4), 0.05% (w/v) NaN3 for 3h. Fluorescence spectra were obtained on a Cary Eclipse fluorescence spectrofluorimeter (Varian USA). Tryptophan residues were excited at 295 nm using a 2.5 nm slit width, and emission spectra were recorded from 300-400 nm with a 5 nm slit width. The spectrofluorometer was set to 700 V with a scan speed of 240 nm.min-1.

ANS Binding Assay. An ANS binding assay was used to assess changes in hydrophobicity as a result of interaction between κ-casein and S. rosmarinus extract in the absence and presence of the crowding agent, dextran. Samples containing κ-casein (2.5 mg/mL), S. rosmarinus extract (in a 1:1 weight ratio), 10% w/v dextran with 20 mM DTT, were incubated in a 50 mM sodium phosphate buffer, 0.05% (w/v) NaN3 and pH 7.4. The ANS fluorescence was monitored on a spectrofluorimeter. spectrofluorometer The excitation and emission wavelengths were set to 400-600 nm, with 2.5 and 5nm slit widths, respectively. The fluorescence emission intensity was measured in a 10 nm path length quartz cuvette in 1mL samples titrated with 3 µL aliquots of a 10 mM ANS stock solution in a 50 mM phosphate buffer, pH 7.4 and 0.05% (w/v) NaN3, with 1 min of stirring after each addition. Titration was continued until the fluorescence intensity reached a plateau.

Circular Dichroism Spectroscopy (CD). In order to examine the changes in the secondary structure of κ-casein in the absence and presence of 10% dextran and S. rosmarinus extract, Far -UV CD experiment was carried out with 0.2 mg/ml κ-casein in 10 mM phosphate buffer, pH 7.0, at 37 ˚ C. the measurements were taken in a 1 cm path length cuvette using a Varian Aviv spectropolarimeter.

Results

ThT Binding. Studies κ-casein contains extensive intermolecular disulfide bridging in its native state and is therefore potentially susceptible to destabilization of its structure by reducing agents (15). In this study, fibril formation in reduced κ-casein in the absence and presence of 10% dextran and S. rosmarinus extract were measured.

Figure 1 shows that an increased in fluorescence intensity is observed in κ-casein sample after addition of DTT and reduction its disulfide bonds which enhanced in the presence of dextran. The first order rate constant also proves this i.e. in the absence of dextran the rate constant of κ-casein was (2.7±0.0001) ×10-2min-1 while in the presence of dextran it increased to (4.9±0.005) ×10-2min-1.

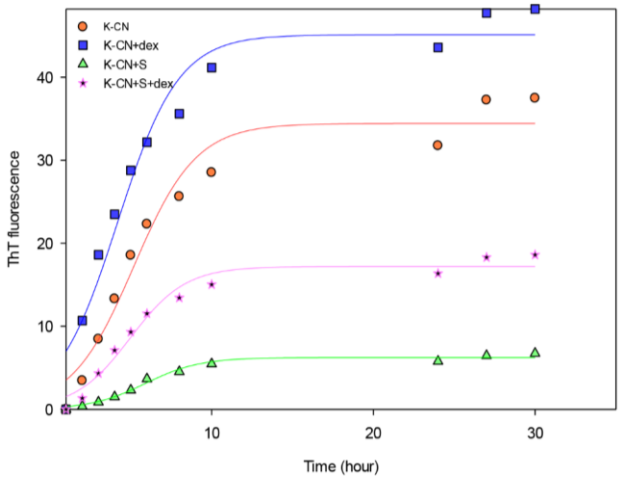

Figure 1. Amyloid formation of reduced κ-casein (2.5 mg/ml) in the absence and presence of 10% v/w dextran and S. rosmarinus extract (1:1 w: w ratio). Protein was in 50 mM sodium phosphate buffer, 0.05%(w/v) NaN3, pH 7.4, and the incubation temperature was 37 °C. The experiment was done twice and fitted using sigmaplot software.

Aggregation of reduced κ-casein was decreased by the addition of S. rosmarinus extract. In the presence of dextran, however, S. rosmarinus extract was less capable to prevent amyloid formation of κ-casein

compared with in the presence of dextran. This is also obvious from the rate constant in the way that in the absence of dextran the rate was (2.2±0.016) ×10-2 min-1 while in the presence of dextran it increased to (3.4±0.02) ×10-2 min-1 (Table1).

Table1. Summary of rate constants for κ-casein in thioflavinT binding assays. The rate constants were calculated by fitting exponential function to thiolavin T binding data using sigmaplot software.

Sample components	Rate constant × 10^{-2} (min^{-1})
κ-casein	2.7 ± 0.0001
κ-casein + 10% (w/v) dextran	4.9 ± 0.005
κ-casein + *S. rosmarinus* extract	2.2 ± 0.016
κ-casein +10% (w/v) dextran + *S. rosmarinus* extract	3.4 ± 0.002

Fluorescence Spectroscopy Studies. The intrinsic fluorescence of κ-casein resulting from its single tryptophan residue at position 97, which can provide information about changes in protein structure (16). Figure 2 shows a comparison of maximum fluorescence intensity of κ-casein in the absence and presence of S. rosmarinus extract and/or dextran (10%w/v). The results showed that adding dextran to κ-casein increased its fluorescence intensity to about 30%, which indicates that protein has unfolded. In the presence of S. rosmarinus extract, the maximum fluorescence in κ-casein decreased about 75.23%. This suggests that S. rosmarinus extract prevent conformational changes in the environment of Trp97 which led to decrease the access of tryptophan residue to solution. In the presence of dextran, however S. rosmarinus extract decreased the fluorescence intensity in κ-casein by 55.04%, which reflects that dextran extenuated the extract effects.

From statistical point of view, the t value calculated using SPSS software between columns related to different (w:w) ratio of κ-casein, S. rosmarinus extract are less than the T critical value, indicates the difference is significant with >95% confidence

ANS binding assay. In this study binding of the hydrophobic dye, ANS, to κ-casein in the absence

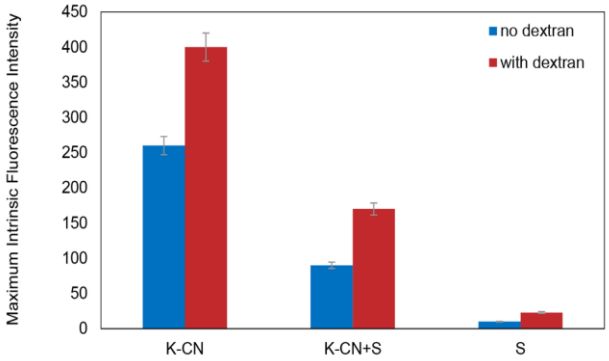

Figure 2. The maximum intrinsic fluorescence of κ-casein (2.5 mg/ml), S. rosmarinus extract (1:1 w: w ratio) and 10% w/v dextran. The experiments were incubated in a 50 mM phosphate buffer, 0.05% NaN3 and pH 7.4 at 37 °C in 3 h. The error bars are absolute values of maximum calculated errors. Data are shown as mean±SD.

and presence of S. rosmarinus extract and 10% w/v dextran were examined in order to investigate the changes in the exposed hydrophobic aria of κ-casein (Fig 3). Reduced κ-casein alone showed high ANS binding. The level of ANS fluorescence intensity in the presence of 10% w/v dextran increased about 25% while in the presence of S. rosmarinus extract the fluorescence intensity decreased by 70%. This reduction in fluorescence intensity indicates the effect of extract in prevention of structural change in κ-casein and thus expose the hydrophobic area of the protein.

Samples containing κ-casein and S. rosmarinus extract show higher fluorescence in the presence of dextran compare to its absence, which demonstrate that dextran effects on conformation of κ-casein and decreasing the chaperone ability of S. rosmarinus extract.

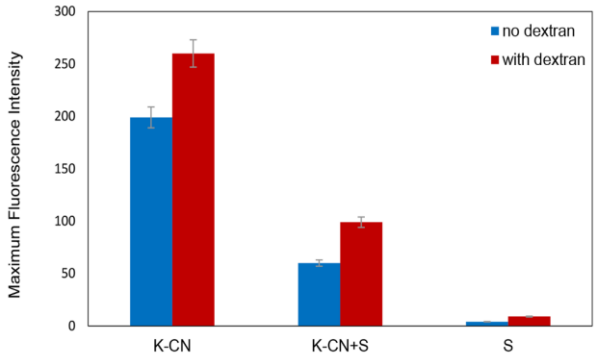

Figure 3. The average maximum fluorescence intensity for binding of ANS to κ-casein (2.5 mg/ml) in the absence and presence of S. rosmarinus extract (1:1 w: w ratio) and dextran (10% w/v). All experiments were conducted in 50 mM phosphate buffer, 0.05% NaN3, 20 mM DTT and pH 7.4 at 37 °C. Data are shown as mean±SD.

Circular dichroism (CD) spectroscopy. To examine the effect of S. rosmarinus extract on the secondary structure of DTT induced κ-casein, far-UV CD spectrum was performed. Far-UV CD measurement of native κ-casein showed spectra with negative ellipticity in 208 and 224 nm which is characteristic of the α-helix structure (Fig. 4) (17). By adding DTT, the spectrum shows a decrease in α-helix related negative ellipticity and appearance of a negative ellipticity at the range of 218 nm, which is characteristic of β-sheet structures. Adding dextran to reduced κ-casein also showed negative ellipticity in the 218 nm region which reflects the presence of β-sheet structure (Fig. 4).

In the presence of S. rosmarinus extract an increase in the size of negative CD signal over the range of 208nm and 222nm observed. Fig 4 also shows that in the sample containing κ-casein and S. rosmarinus extract after adding dextran, an increase of negative CD signal was observed which indicates decreasing in stability of the helical region in the protein κ-casein.

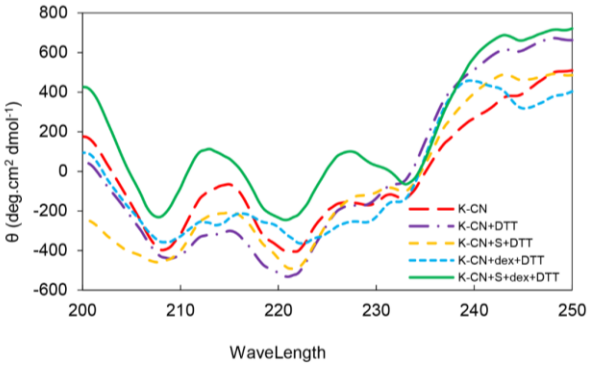

Figure 4. Far-UV spectra of the native state, the reduced state and in the presence of S. rosmarinus extract (w: w ratio) and 10% v/w dextran. Protein concentration was 0.2 mg/ml in 10 mM phosphate buffer, pH 7.0, and 37° C in a J.180 spectropolarimeter with a 1cm path length cell.

Discussion

It has previously shown that S. rosmarinus extract has chaperone property against aggregation of ovotransferrin, insulin, and α-lactalbumin (14). Considering that intracellular environment contains high concentration of macromolecules which create a crowded environment to investigate protein aggregation in in vivo condition, the intracellular environment can be simulated using macromolecular crowding agent, such as dextran (18).

In this study, the protective effect of S. rosmarinus aqueous extract against aggregation and amyloid formation of κ-casein in the presence of dextran as a macromolecular crowding agent, was investigated.

ThT binding results in our study revealed that reduced κ-casein at natural pH forms amyloid fibrils as shown in previous studies (19, 20). Dextran increased the fluorescence of ThT which indicates an increase in the formation of amyloid fibrils in the κ-casein. This is in agreement with the results of previous studies that have shown the effects of this crowding agent on the formation of amyloid in various proteins (18). This effect may be due to the following reasons: a) dextran increases the rate of amyloid fibril formation of κ-casein b) decxtran reduces the stability of κ-casein and thus facilitates the formation of amyloid in this protein.

ThT binding results indicated that S. rosmarinus extract decreased the rate and the extent of amyloid fibril formation. This could be a result of interaction between the phenolic compound in the S. rosmarinus and aromatic residues in the amyloeidogenic sequence of κ-casein (21). However, the effect of S. rosmarinus on reducing amyloid fibrils decreased in the presence of dextran. Therefore, fluorescence spectroscopy was done.

The results of intrinsic fluorescence assay showed that upon the addition of dextran, the intensity of intrinsic fluorescence increased. This effect is probably due to the increased polarity of the environment around reduced tryptophan following a structural change in κ-casein by dextran(22) . In the presence of S. rosmarinus extract, the intrinsic intensity of κ-casein was reduced which can be due to the binding of the compounds in the extract to the unfolded regions of reduced κ-casein, and lead them to refolding.

In the presence of dextran, S. rosmarinus extract, was not able to prevent unfolding as well as it was in the absence of dextran.

ANS binding assay results also showed that presence of dextran led to the exposure of hydrophobic regions in κ-casein. This indicates the effect of dextran to facilitate the loss of stability of this protein. S.

rosmarinus extract reduced hydrophobic exposure of κ-casein probably due to the interaction between S. rosmarinus extract and κ-casein although its effect decreased in the presence of dextran.

Consistent with the intrinsic and ANS fluorescence results, the results from far-UV spectrum of κ-casein also indicated that the presence of dextran increased the content of β-sheet structure in the protein, indicating an increase in aggregation of protein, while the S. rosmarinus extract preserved the native structure of κ-casein, by protecting the α-helix structures and reducing the β-sheet structure contain. The suggested mechanism could be the non-covalent interaction of OH groups in aromatic ring of polyphenol component in S. rosmarinus extract with β-sheet structures (21). In the presence of dextran, the S. rosmarinus extract was less effective. The possible cause is that dextran increased the rate of amyloid fibril formation in κ-casein thus the S. rosmarinus extract was more efficient in preventing the amyloid fibril formation of κ-casein in the absence of dextran than in its presence.

In summary, the results of this study indicate that, dextran decreases the stability and facilitates the formation of amyloid in κ-casein, which affects the structure of protein and increases the rate of amyloid formation. S. rosmarinus extract however, has a protective effect against the aggregation and formation of amyloid in κ-casein. These results also showed that in the presence of dextran and simulating intracellular condition, S. rosmarinus extract continues to play its protective role, although this crowding agent somewhat reduces the effect of S. rosmarinus extract. Therefore, this study demonstrated the potential protective effect of S. rosmarinus extract in preventing the amyloid formation of κ-casein and in future this effect can be studied on various proteins, including proteins involved in amyloidogenic diseases.

Conflicts of Interest

The authors have no conflict of interest to declare.

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