

A Comparative study on the chaperone-like activity of camel and bovine β -caseins

Mehran Miroliaei^{1,*}, Mozhgan Shirazi², Reza Yousefi³

¹ Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, Iran

² Department of Biology, Research and Science Branch, Islamic Azad University, Tehran, Iran

³ Department of Biology, Faculty of Sciences, University of Shiraz, Shiraz, Iran

* Corresponding Author: email address: mmiroliaei@yahoo.com (M. Miroliaei)

ABSTRACT

Molecular chaperones are characterized by a general behavior, arresting the exposed hydrophobic surfaces of denaturing substrate proteins. In the present study, the capacity of β -caseins (β -CN) from camel and bovine milk in suppression of thermal aggregation process of apo-yeast alcohol dehydrogenase (YADH) was assessed. Apo-I enzyme was prepared by removal of the structural zinc; while apo-II-protein was obtained by depleting conformational and catalytic zinc atoms. Fluorescence spectroscopy using ANS probe revealed greater hydrophobic surface in apo-II ADH. Considerable decrease in aggregation of the heat treated protein molecules was observed upon exposing to β -CNs (camel, bovine). Bovine β -CN afforded more adverse effects on thermal aggregation. A direct correlation between casein's chaperone activity and structural stability of the substrate proteins was displayed. Moreover, an association between casein source and chaperone-like activity is suggested.

Keywords: β -casein; Aggregation; Yeast Alcohol Dehydrogenase; Apo-Enzyme; Aggregation; 8 Anilino-1-Naphthalenesulfonic Acid (ANS).

INTRODUCTION

What makes protein aggregation among the issues of great impact is the importance in hampering the production, storage and use of many proteins and peptides in a wide range of biotechnological and pharmaceutical applications. Protein aggregates have certain features indicating a common underlying mechanism of toxicity [1]. Of the employed strategies by nature in prevention of protein aggregation, molecular chaperones are of considerable interest. It is believed that protein aggregation originates from the intermolecular hydrophobic interactions. In this respect, molecular chaperones retard aggregation through a mechanism by which the exposed hydrophobic regions of denaturing protein are sequestered. Environmental temperature is among the most common factors leading to protein aggregation. Anti-aggregation ability of chaperones is vital for living organisms since protein aggregation causes many so called conformational diseases such as Alzheimer's, Parkinson's, and Huntington

diseases and type II diabetes [2-4]. Therefore, chaperoning machinery is necessary to rescue living cells from toxicity of unfolded proteins and their harmfulness aggregates. Recently, a novel function for caseins has been proposed as "molecular chaperones" protecting many proteins including whey proteins against heat, chemical and UV light-induced aggregation [5].

Bovine β -casein (β -CN) is a highly amphiphilic protein containing two distinct segments in its primary structure with a hydrophilic (ψ) N-terminal part and a hydrophobic (ϕ) C-terminal part, along with a cluster of five phosphoryl residues in the N-terminal domain [6-8]. As a member of intrinsically unstructured protein (IUP) family, β -casein (β -CN) possesses relatively high amount of prolyl residues, and adopts non-compact and flexible structure. Meanwhile, as a rheomorphic (from the Greek rheos - stream and morph - form) protein, β -CN is a dynamic protein with flexible conformation [9].

In the present study, chaperone-like properties of bovine and camel β -CN were compared in a heat-induced aggregation system including native yeast alcohol dehydrogenase (YADH, EC.1.1.1.1) and its apo-forms (I & II) as the substrate proteins. YADH is a tetrameric metalloprotein giving a molecular mass of 150 kDa with two zinc atoms per subunit (catalytic and structural) [10]. The enzyme acquires extremely sensitive and delicate entity toward heat stress [11]. Apo-I YADH prepared by removal of the structural zinc retaining enzyme catalytic activity, whereas apo-II YADH obtained via depleting both catalytic and structural zinc from the enzyme leading to whole loss of functionality. Diverse forms of YADH (native, apo-I and apo-II) are differed in surface hydrophobicity; thereby, exhibiting different interactions with a chaperone molecule [12]. Since exposed hydrophobic sites of unfolded proteins are recognized for preferential interaction with molecular chaperones, these model proteins are believed to be good candidates for qualification of chaperone properties of β -casein. Such studies advance our understanding of the mechanism underlying the chaperone activity of milk β -caseins.

MATERIALS AND METHODS

Yeast Alcohol Dehydrogenase, NAD^+ , dithiothreitol (DTT), and ANS (1-anilinonaphthalene-8-sulfonate) were purchased from Sigma (St. Louis, MO, U.S.A.). Ethanol (96% purity), EDTA (Ethylene Diamine Tetraacetic Acid) were obtained from Merck (Dormstadt, Germany). Tri-sodium citrate dehydrate was purchased from Scharlau Chemie (Spain). Bovine and camel β -caseins (97% purity) received from Institute of Biochemistry and Biophysics (IBB) of Tehran University (Iran) as a generous gift.

Preparation of apo-enzymes:

Apo-YADH was prepared as described by Yang et al. [13]. In brief, the native enzyme (2–3 mg/ml) was dissolved in 0.1M Tris-HCl buffer, pH 6.5 containing 0.1 M NaCl and 100 mM DTT, for 1–2 h at 4°C. DTT-Zn and excess DTT were removed by gel filtration on Sephadex G-25 (fine) column (19 cm \times 1.5 cm). Apo-II YADH was prepared in the same condition except 4

hours incubation at 25 °C with 20 mM sodium citrate buffer, pH 7.0 containing 250 mM EDTA.

Zinc content determination

Determination of zinc content of native and apo-YADH were performed by atomic absorption spectroscopy on a Chem Tech Analytical CTA-2000 ($\lambda = 214 \text{ nm}$). Samples were aspirated directly into an air-acetylene flame, and determinations were made on each sample by standard curve [14].

Enzyme activity studies

Enzyme activity measurements were performed in the presence of 200 mM ethanol (as a substrate) and 1.65 M NAD^+ (as a coenzyme) in a UV-Vis spectrophotometer (Shimadzu 160 model) at 25 °C. The production of NADH was monitored by the increase of absorbance at 340 nm [15].

Determining the chaperone-like activity

Chaperone-like activity of β -caseins was measured by the capacity of preventing heat induced aggregation of targeted proteins. Aggregation at 50°C was monitored as turbidity at 360nm, taking the maximal turbidity as 100% aggregation. The final concentration of YADH in 50 mM potassium phosphate buffer pH 7.8 was 25 $\mu\text{g/ml}$. The light absorbance of each sample was recorded automatically every 1 min.

Fluorescence Studies

Fluorescence emission spectra in the absence and presence of β -caseins were recorded on a Cary-Eclipse spectrofluorimeter. 460 μl of enzyme solution (1 mg/ml) was added to 100 μl of the β -casein solution and incubated for 1 hour at 50 °C. ANS solutions (10 mM) were used as a common extrinsic fluorescence probe. The excitation wavelength was 390 nm and all the spectra were recorded in the range of 400–600 nm.

RESULTS

Figure 1 shows the time evolution of the heat aggregation for YADH (native, apo-forms) and in presence of β -caseins (camel, bovine). Obviously, in the holo-YADH sample, aggregation process is initiated gradually after 200 seconds of exposure to 50 °C (Figure 1A, a), while for the other two

samples, apo-I (Figure 1B) and apo-II (Figure 1C), it follows a hyperbolic kinetic pattern with no significant lag period and reaches sharply a maximum, indicating an aggregation process undergoing quickly in the absence of β -casein. Such a trend was efficiently suppressed by the presence of β -caseins (Figure 1 B & C [a-c]). Although camel β -CN is less effective than the bovine casein, both suppressed the whole aggregation of holo-enzyme (Figure 1 A, e) at 1:1 (w/w) ratio of ADH/ β -casein. This phenomenon is more evident for other two

apo forms. However, 1:1 (w/w) ratio of enzyme: β -casein display more capacity to delay heat aggregation of ADH than the 3:1 (w/w) ratio of ADH to β -casein for all the samples. As indicated, the extent of aggregation obeys a similar trend in both apo-forms being more pronounced for apoII-ADH after 20 min (Figure 1C). It is noteworthy that the anti-aggregation activity of used caseins follows a concentration dependent manner.

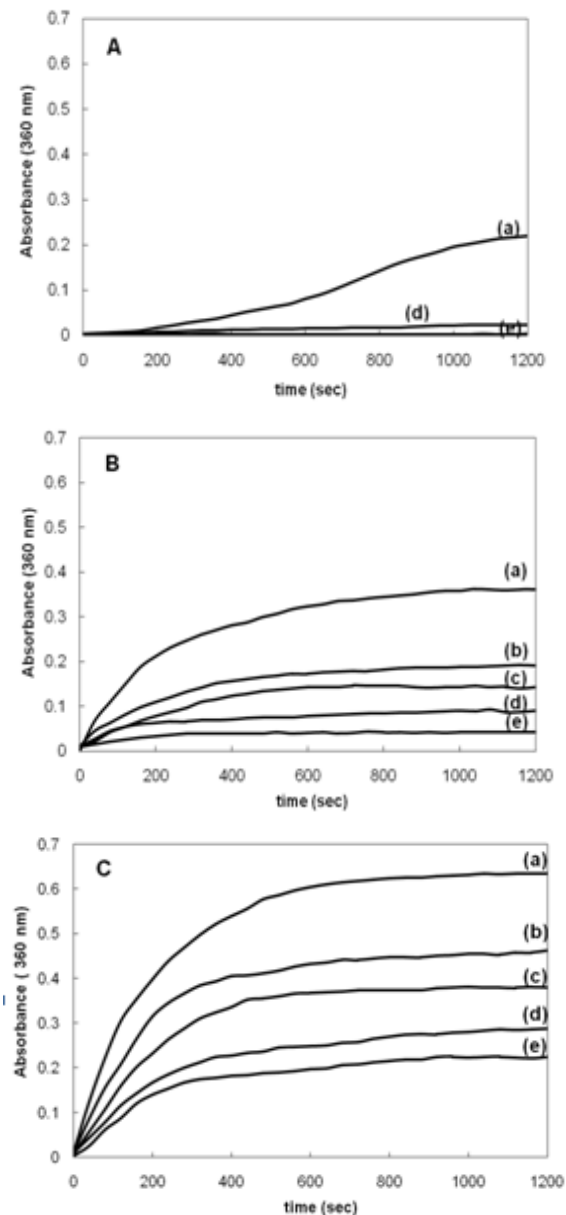


Figure 1. Thermal aggregation of native (A), apo-I (B) and apo-II (C) YADH in the presence of bovine and camel β -CNs at 50 °C. (a) control sample, (b) 3:1 ratio of enzyme/camel β -CN, (c) 3:1 ratio of enzyme/bovine β -CN, (d) 1:1 ratio of enzyme/camel β -CN, (e) 1:1 ratio of enzyme/bovine β -CN

It is believed that only aggregation-prone conformers of reactant (proteins/enzymes) molecules are susceptible to interact with molecular chaperones, and that chaperones do not show any affinity toward native or previously aggregated proteins [19]. An experiment was performed to determine whether this assumption is through for β -CN when encountered with some proteins of different hydrophobicity. To this end, β -CNs were added to the reaction mixtures after 300 seconds of initial thermal aggregation process, then the extent of aggregation was measured for the next 1500 seconds in the

same experimental conditions. Figure 2 (A & B) implies a similar progress of apo-I and apo-II in the heat induced aggregation process, with respect to the point that apo-II is more sensitive than apo-I. In particular, Figure. 2A shows the time evolution of aggregation for apo-I which turned downward after adding β -CNs (bovine, camel), being less prominent for apo-II (Figure 2B). Bovine β -casein afforded more suppression than camel β -casein. To ensure the validity of this assumption, the following experiment was performed.

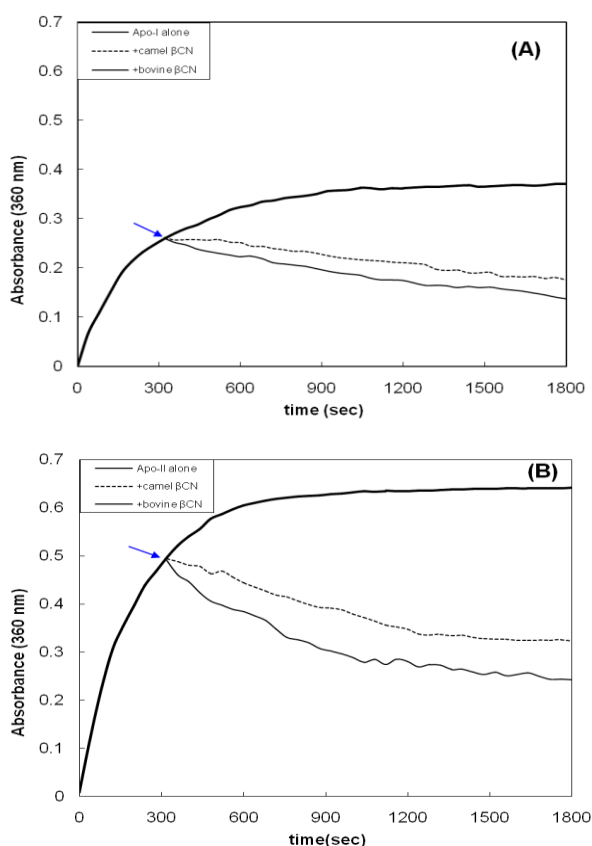


Figure 2. Influence of bovine and camel β -CNs on partially aggregated apo-I (A) and apo-II (B) ADH at 50 °C.

The fluorescence emission of ANS is known to increase when the dye binds to the hydrophobic regions of a protein [21, 22]. The probe was used as an extrinsic indicator to verify the exposure of hydrophobic clusters on protein surface of apo- and holo-YADH. As reported in Figure 3, apo-II YADH binds ANS more efficiently than apo-I and the native enzyme. The fluorescence spectra indicate different changes in tertiary

structure, an indication of increased hydrophobic regions in zinc depleted apostates than the native molecules. However, enhancement of observed fluorescence was diminished by the presence of β -CNs; again bovine β -CN was more prominent (Figure 4A and B). The effectiveness of β -CNs in fluorescence signals is in accordance with the ability to prevent aggregation of native YADH, reported earlier [20].

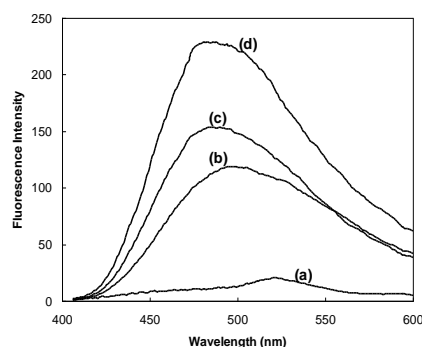


Figure 3. Fluorescence spectra of YADH in aqueous buffer (sodium citrate 20 mM, pH 7.0) (a), native state (b), apo-I (c) and apo-II (d) after treatment at 50°C. Protein concentration was 0.1 mg/ml in the solution containing 40 μ M ANS. Excitation wavelength: 390 nm

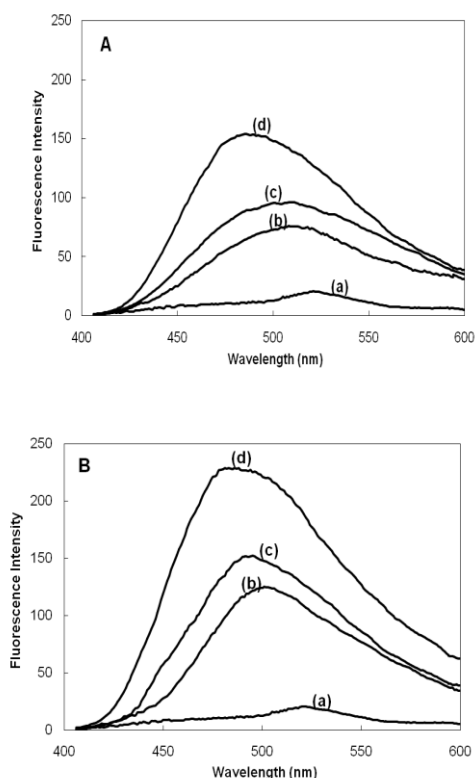


Figure 4. Extrinsic fluorescence of apo-YADH I (A) and apo-II (B). ANS in aqueous sodium citrate buffer (20 mM pH 7.0) (a) contains enzyme with bovine β -CN (b), with camel β -CN (c), and without caseins (d). All experiments were performed in solutions containing 40 μ M ANS, and 0.1 mg/ml enzyme. Excitation wavelength was 390 nm

DISCUSSION

Disulfide bond formation has been implicated to play a major role in assisting thermal aggregation of YADH [16]. The enzyme has been shown to contain nine cysteine residues per subunit [17]. Of these, two residues are linked to the catalytic zinc, four to the structural zinc, two in a disulfide bridge formation and one free [17]. Our results (Figure. 1 B & C) clearly showed that the apo-states (control) are much more

sensitive toward aggregation than the holo-enzyme, and that β -casein could efficiently suppress this event. The inhibitory effects of β -CNs may result from the ability to suppress conformational changes which results from newly exposed cysteine residues that previously linked to zinc atoms in native enzyme, and are susceptible to induced disulfide bonds exchange in apo-YADHs. This is in accordance with the previous findings in preventive effects of sugars on

thermal aggregation of YADH, which were suggested to possess chaperone-like activities [16]. Whole molecule of native YADH has been shown to possess an intra-molecular chaperone-like activity, which evolved by the presence of a particular region, YSGVCHTDLHAWHGDWPLPVK [40–60]-sequence of the original structure [18]. Accordingly, the entire suppression of aggregation induced by caseins may be partly assisted by this intra-molecular property of the native enzyme. This is true for the apo-forms of alcohol dehydrogenase, to a lesser extent.

In order to monitor the extent of aggregation for all the samples, turbidity measurements were performed as a function of time. The obtained results (Figure 2) indicated that both used β -caseins could solubilize the already-aggregated ADH molecules to a small degree. This could be ascribed to the higher affinity of bovine β -CN in interacting with the aggregation-prone molecular conformers. A similar effect has been observed for the native enzyme when treated with bovine β -casein [20]. The following explanation may ensure the validity of this assumption. Changes in the fluorescence spectra in the course of heating at 50 °C indicate that the tertiary structure of apo forms is mainly dependent on temperature (Figure 3). This trend was evidenced by changes in the fluorescence spectra which demonstrated that different ADH-forms exist in different states of distinct stability (Figure 4). They differ in surface hydrophobicity, to be considered as a key determinant for rescuing chaperoning action. Greater hydrophobicity of bovine β -CN has been proven to bestow its stronger interactions with the aggregation-prone molecular species such as present substrates [23]. Surface hydrophobicity affects intermolecular interactions, such as binding of small ligands or associations with other macromolecules, including protein–protein or protein–lipid interactions.

The results presented in this communication indicate bovine β -casein is more efficient than camel β -casein in improving the structural stability of apo-YADH by protecting the exchange of disulfide bonds liable for the protein thermal

aggregation. Furthermore, these findings furnished additional mechanistic reasons in relation to prevention of irreversible aggregation of native YADH provided by β -casein. Taken together, bovine β -CN displayed greater protection than camel β -CN. A direct correlation between casein's chaperone activity and the inherent substrate stability is also suggestive.

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