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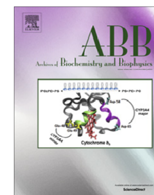

Conformational behaviour and aggregation of chickpea cystatin in trifluoroethanol: Effects of epicatechin and tannic acid

Sheraz Ahmad Bhat, Bilqees Bano *

Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh 202002, India

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Conformational alterations and aggregates of chickpea cystatin (CPC) were investigated upon sequential addition of trifluoroethanol (TFE) over a range of 0–70% v/v. CPC on 30% and 40% v/v TFE addition exhibited non-native β-sheet, altered intrinsic fluorescence, increased thioflavin T fluorescence, prominent red shifted shoulder peak in Congo red absorbance, and enhanced turbidity as well as Rayleigh scattering, suggesting the aggregate formation. TEM results confirmed the formation of fibrillar aggregates at 30% and 40% v/v TFE. On increasing concentration of TFE to 70% v/v, CPC showed retention of native-like secondary structure, increased intrinsic and ANS fluorescence. Thus our results show that favourable condition for fibrillation of CPC is in the range of 30–40% TFE. Moreover, anti-aggregational effects of polyphenols, epicatechin (EC) and tannic acid (TA) were analysed using ThT binding assay and other biophysical assays. EC and TA produced a concentration dependent decline in ThT fluorescence suggesting inhibition of the fibril formation. Furthermore, TA in comparison to EC, served as a more effective inhibitor against amyloid fibril formation of CPC. This work supports the universality of the amyloid-like aggregation not restricted to some special categories of protein and the fact that this aggregation can be prevented.

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Introduction

Protein aggregation is a common event during folding of many proteins and enzymes. It exists in competition with the normal folding pathway [1]. It is likely that in many cases when aggregation occurs from a solution of the native protein it is the partially unfolded intermediates in equilibrium with the native state that are the immediate precursors of the aggregates [2]. Aggregation is often irreversible, and aggregates commonly contain high levels of non-native, intermolecular β-sheet structures [3]. These protein aggregates lead to various neurodegenerative diseases and other disorders in humans such as Alzheimer's diseases, Parkinson's disease, type II diabetes mellitus and spongiform encephalopathies [4–6]. Identification of novel therapeutic ways to prevent or cure the diseases associated with protein aggregate deposition in tissues and resulting toxicity requires a rational understanding of the forces driving protein aggregation and transition of proteins into β-sheet rich aggregates [7].

Non-polar solvents like alcohols denature the tertiary and quaternary structures of several proteins while enhancing their helicity [8]. Trifluoroethanol (TFE),[1] the simplest alcohol with a CF3 group, and its derivatives are most commonly used as anaesthetics. It is toxic to blood, male reproductive system, brain, upper respiratory tract and eyes [9]. There are two main effects of TFE on peptides and proteins: first the stabilization of helical, β-turn and β-hairpin structures and second the disruption of the native tertiary structure of intact proteins leading to aggregates [10]. The strengthening of intermolecular H-bonds in proteins is large enough to account for the increase in aggregate propensities in the presence of TFE.

Cystatins or thiol proteinase inhibitors are a group of evolutionary related proteins collectively placed in the cystatin superfamily divided into three main groups the stefins, the cystatins and the kininogens depending on the presence of single or multiple 'cystatin domains', presence or absence of a signal sequence, carbohydrate content and sulfhydryl groups. Cystatins constitute a powerful regulatory system for cathepsins, besides this a plethora of biological activities are now ascribed to them [11]. Members of

* Corresponding author. Fax: +91 0571 2706002.
E-mail address: bilqeesbanos@gmail.com (B. Bano).

[1] *Abbreviations used:* ANS, 8-anilino-1-naphthalene-sulphonic acid; ATR-FTIR, attenuated total reflection Fourier transform infra-red spectroscopy; CPC, chickpea cystatin; CD, circular dichroism; CR, Congo red; EC, epicatechin; MG, molten globule; PFI, partially folded intermediate; SEM, scanning electron microscopy; ThT, thioflavin T; TFE, trifluoroethanol; TA, tannic acid; TEM, transmission electron microscopy.

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this family show predisposition for fibrillation and have recently been proven to be useful model system to study amyloidogenesis [12–14]. The aim this study was to investigate how a plant protein CPC (chick pea cystatin) isolated from chick pea behaves in the process of aggregation and fibrillation as only few reports are available on fibrillation of cystatins in general and no report is found on plant cystatins in particular. Furthermore, insights obtained only from some special cases often lead to misunderstanding or oversimplification.

There is a lack of potential anti-amyloidogenic agents till date. Polyphenols have been accredited with myriad of biological effects [15]. Their anti-fibrillation roles have emerged only lately [16]. An analysis of the effects of natural agents like epicatechin and tannic acid on fibrillation process can open new avenues for the treatment of protein misfolding diseases. Thus, investigation of the effects of these polyphenols epicatechin (EC) and tannic acid (TA) on CPC amyloid formation was also taken up.

In view of the above context, research to find out fibrous aggregation phenomena in a much wider range of protein species is of importance. This report intends to contribute to this point and to support a view that amyloid-like fibrous aggregation is a universal phenomenon not restricted to some special categories of protein [17,18], and that this aggregation can be prevented. This work thus adds to the spectrum of the proteins, other than those so far identified in specific diseases like neurodegenerative ones, undergoing amyloidogenesis under defined conditions *in vitro*. The study thus aims for a better understanding of how a protein (CPC) aggregates that will not only aid in deciphering the molecular mechanism of amyloid fibrillogenesis, but also shed light on a rational design of effective therapeutics for amyloidogenic diseases.

Materials and methods

Materials

Chick pea cystatin (CPC) was isolated as reported earlier [11]. Sodium phosphate monobasic and sodium phosphate dibasic were purchased from SRL (Mumbai, India). Trifluoroethanol and fluorescent dyes, viz., 8-anilino1-naphthalene sulphonic acid (ANS), Congo red (CR) as well as thioflavin-T (ThT) were obtained from Sigma (St. Louis, USA). Sodium phosphate mono and dibasic (pH 7) were used for buffer preparations.

Methods

Conformational study and amyloid fibril formation of the inhibitor

CPC in the concentration of 0.5 mg/ml in 50 mM sodium phosphate buffer (pH 7.4), sodium azide (0.02%) was incubated separately with varying concentrations of trifluoroethanol (0–70% v/v) for 4 h at 37 °C before any spectroscopic measurement was performed. All solutions were filtered through 0.22 μm syringe filters (sterilized, max. pressure 4.5 bars) under aseptic conditions before their use to remove traces of any aggregated material. All the measurements were carried out at room temperature. Three replicates for each set were analysed for the results.

Assay of antipapain activity

The anti-papain activity of native as well as treated CPC was measured according to the method of Kunitz [19]. The inhibitory activity of cystatin was assessed by its ability to inhibit Caseinolytic activity of papain. The activity was measured at the end of incubation period and the untreated CPC activity was taken as reference.

Intrinsic fluorescence measurements

The fluorescence spectra were recorded with a Shimadzu RF-5301 spectrofluorophotometer (Tokyo, Japan) in a quartz cell of path length 10 mm. The excitation wavelength was 280 nm and the emission was recorded in the range of 300–400 nm [20]. The final concentration of CPC was 20 μM.

Circular dichroism measurements

CD analysis was carried out with a JASCO J-815 spectropolarimeter calibrated with ammonium D-10-camphorsulfonate. Cells having path length 0.1 cm was used for scanning between 250 and 190 nm. Each spectrum was taken as the average of 4 scans for having a good signal to noise ratio. Concentration of CPC for the scans in far-UV region was 4 μM.

ANS (8-anilino 1-napthalene sulphonic acid) fluorescence measurements

ANS binding was measured by fluorescence emission spectra with excitation at 380 nm and emission was recorded from 400 to 600 nm. Typically, ANS concentration was 100 M excess of the protein concentration and protein concentration was in the vicinity of 4 μM [21].

Attenuated total reflection Fourier transformed infrared spectroscopy (ATR-FTIR)

FTIR spectra were recorded with Interspec 2020 FTIR spectrometer in deuterated water at room temperature. Sample aliquots were placed between CaF_2 windows separated by a 50 mm polyethylene terephthalate spacer. The sample compartment was thoroughly purged with dry nitrogen. Protein concentration was 6 μM. The scanning wave number was from 1500 to 1700 cm^{-1} [22].

Turbidimetric aggregation analysis

Turbidity assay was performed as an indicator to suggest the formation of protein aggregates [23]. Absorbance of control and the treated samples was carried out at 450 nm after the incubation period on a UV–vis spectrophotometer (UV–VIS 1700 Shimadzu, Japan). The concentration of the sample was 0.5 mg/ml.

Rayleigh scattering measurements

Rayleigh scattering measurement was performed on Shimadzu RF-5301 spectrofluorophotometer (Tokyo, Japan) in a quartz cell of path length 10 mm. The excitation wavelength was set at 350 nm and emission spectra were recorded from 300 to 400 nm. Fluorescence intensities at 350 nm were plotted. Both excitation and emission slits were 5 nm. The final concentration of CPC was 4.3 μM.

Thioflavin T fluorescence assay

Thioflavin T (ThT) fluorescence was measured to monitor aggregation of CPC. The following parameters were adjusted for monitoring ThT fluorescence assay during aggregation experiments: λ_{ex} = 440 nm, λ_{em} = 450–600 nm. The slit width was set at 5 nm both for excitation as well as emission while as the path length was 10 mm. Final concentration of protein in the sample was 4 μM while the concentration of ThT was 20 μM. Thioflavin T was prepared in 50 mM sodium phosphate buffer, pH 7 [24].

Congo red assay

The formation of aggregates was probed by measuring the increase and/or shift in absorbance of Congo red in the range between 400 and 700 nm. For this experiment, 50 μl (0.5 mg/ml, 20 μM) aliquots of the protein solutions were withdrawn and mixed with 250 μl of a solution containing 50 μM Congo red and the volume was made up to 0.5 ml with 50 mM sodium phosphate buffer, pH 7 [25] thus maintaining a ratio of 1:12.5 protein to CR. After 20–30 min of equilibration, absorbance was recorded [26].

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