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Trifluoroethanol and acetonitrile induced formation of the molten globule states and aggregates of cellulase

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ABSTRACT

A systematic investigation on the effects of trifluoroethanol and acetonitrile at various concentrations on cellulase (EC 3.2.1.4) was studied by enzyme assay, intrinsic fluorescence, ANS binding, circular dichroism and ATR-Fourier transform infra red spectroscopy. The results show the presence of molten globule states at 3% (v/v) TFE and 80% (v/v) ACN. Cellulase aggregates at 25% (v/v) TFE and 90% (v/v) ACN, as detected by decrease in intrinsic and ANS fluorescence, loss in tertiary structure and enzyme activity, increase non-native β-sheet structure as evident from far-UV CD and FTIR spectra, increase in Thioflavin T fluorescence and shift in Congo red assay.

1. Introduction

Protein aggregation is one of the most exciting new frontiers in protein chemistry as well as in molecular medicine. In the past decades, attention was being focused on the study of self-assembling peptides and their relevance in biology, protein aggregation, and their application in biotechnology [1,2]. There are many α-helix proteins that can form non-pathogenic β-sheet rich protein aggregates under carefully designed in vitro conditions irrespective of their native structures, has led to the idea that formation of aggregates is a basic asset of the polypeptides [3,4]. The transition of α-helix to β-sheet turns the normal protein to amyloidogenic resulting in various protein conformational diseases, such as prion and Alzheimer's [5–7]. The hydrophobic microenvironment is generally a key factor in these helix/sheet transitions.

Destabilization by addition of organic solvents is one of the most common strategies employed in proteins for their alteration in aggregation. Non-polar solvents like alcohols denature the tertiary and quaternary structures of several proteins while enhancing their helicity [8], while acetonitrile induces β-sheets in some proteins [9]. Trifluoroethanol (TFE), 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 [10]. 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 [11]. The strengthening of intermolecular H-bonds in proteins is large enough to account for the increase in aggregate propensities in the presence of TFE. ACN, also known as cyanomethane, is an organic solvent that is toxic in nature. Exposure usually occurs in the industries where this solvent is produced or handled. It is also exposed to general population by breathing, by taking contaminated water or food, by skin or eye contact with vapours. Its common uses are: assessing effectiveness and impurity levels of drugs in pharmaceutical industries, extracting fatty acids from animal and vegetable oils [9].

Microbial cellulase enzymes have attracted considerable research and commercial interest as they are very important tools of biotechnological industries. They are also used in the textile, laundry, pulp and paper industry [12]. *Aspergillus niger* account for approximately 20% of total cellulase production. The tendency of an enzyme to self-organise impulsively during their native state is a foremost problem for biotechnologists as no evidences regarding structural properties of aggregates of cellulase peptides has been reported [13]. Various studies such as viscosity measurements have revealed that cellulase follows the complex aggregation behaviour involving the presence of complex structures due to association of peptides of this enzyme with altered physiological conditions [14]. The well known fact, that the specific catalytic activity of enzymes in organic solvents is generally lower than in aqueous solutions; is the main attraction to biotechnologists. Cellulase is an exocellular protein, strongly suggests that in vivo the unfolded nascent polypeptide chains are transiently in contact with amphipathic compounds such as membrane phospholipids. The presences of

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amphipathic compounds facilitate the exposure of hydrophobic surfaces that increase the yield and the rate of aggregation process. Here in our study TFE and ACN were used to mimic the local cellular environment of cellulase resulting in the formation of molten globule state and aggregates. Thus, this paper reports the in vitro characterization of the cellulase under conditions mimicking the in vivo environment.

2. Experimental

2.1. Materials

Cellulase from *Aspergillus niger*, acetonitrile (ACN), sodium phosphate monobasic, sodium phosphate dibasic were purchased from SRL, Mumbai, India. Trifluoroethanol (TFE) and fluorescent probes, viz., 8-anilinonaphthalene, 1-sulfonic acid ammonium salt (ANS), Congo Red (CR) as well as Thioflavin T (ThT), were obtained from Sigma (St. Louis). Sodium phosphate mono and dibasic (pH 7) was used for buffer preparations.

2.2. Determination of protein concentration

Concentration of protein was determined by the method of Lowry et al. [15].

2.3. Preparation of protein samples for biophysical studies

Cellulase was dissolved in 20 mM phosphate buffer of pH 7 to give a stock solution of 5 mg/ml. In all the experiments, the final protein concentration was 0.1 mg/ml. The protein samples were incubated separately with varying concentrations of trifluoroethanol and acetonitrile for 4 h at 37 °C.

2.4. Cellulase activity assay

Cellulase activity was assayed by using 200 mg cellulose powder as a substrate in 500 ml of protein solutions prepared in 20 mM sodium phosphate buffer, pH 7. The amount of reducing sugar released was determined by phenol/H_2SO_4 method of Dubois et al. [16].

2.5. Intrinsic fluorescence measurements

The fluorescence spectra were recorded on a Shimadzu RF-5301 spectrofluorophotometer (Tokyo, Japan) in a 10 mm path length quartz cell. The excitation wavelength was 280 nm and the emission was recorded in the range of 300–400 nm [17]. The final concentration of cellulase was 2.22 μM.

2.6. ANS (8-anilino 1-napthalene sulphonate) 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 molar excess of the protein concentration and protein concentration was in the vicinity of 2.22 μM [18].

2.7. Circular dichroism spectroscopy

CD spectra of protein samples were recorded on a J-810 Jasco CD spectropolarimeter calibrated with ammonium D-10-camphorsulfonate. Cells of path lengths 0.1 and 1 cm were used for scanning between 250–200 nm and 300–250 nm. Each spectrum was the average of 4 scans [19]. Base lining and analysis were done using Jasco J-720 software. Protein concentration for the scan was 0.5 mg/ml for far-UV and 1 mg/ml for near-UV CD. The results were expressed as the mean residue ellipticity (MRE in degree cm^2 $dmol^{-1}$), which was defined as:

$$\text{MRE} = \frac{\theta_{\text{obs}}}{10 \times n \times \text{Cp} \times l}$$

where θ_{obs} was the observed ellipticity in degrees (°), Cp is the molar fraction, and l is the length of light path in cm.

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

FTIR spectra were recorded with Interspec 2020 FTIR spectrometer in deuterated water. Protein concentration was 2.22 μM. The scanning wave number was from 1000 to 4000 cm^{-1} [20].

2.9. Thioflavin T fluorescence assay

Thioflavin T (ThT) fluorescence was measured to monitor aggregation of cellulase in a 1 cm path length quartz cell. The following parameters were adjusted for monitoring ThT fluorescence intensity during aggregation experiments: λ_{ex} = 440 nm, λ_{em} = 450–600 nm. Final concentration of protein in the sample was 2.22 μM while the concentration of ThT was 15 μM. Thioflavin T was prepared in 20 mM sodium phosphate buffer, pH 7 [21].

2.10. Congo red assay

The formation of aggregates was probed by measuring the shift in absorbance of Congo red at 400–700 nm. For this experiment, 240 μl (0.2 mg/ml) aliquots of the protein solutions were withdrawn and mixed with 260 μl of a solution containing 20 μM Congo red and 20 mM sodium phosphate buffer, pH 7 [22].

3. Results

3.1. Cellulase activity assay

Before evaluating the biophysical properties of cellulase on addition of organic solvents, its ability to hydrolyze cellulose was monitored. Fig. 1a and b shows the effect of TFE and ACN on cellulase activity respectively. The native enzyme (in the absence of co solvents) had the maximum activity. On increasing concentration of TFE, the activity of cellulase did not change significantly up to 5% (v/v) TFE whereas on further increasing the concentrations, enzyme activity decreases gradually and reaches minimum at 25% (v/v) TFE (Fig. 1a). In the presence of ACN there is gradual decrease in activity up to 80% (v/v) and reaches minima at 90% (v/v) (Fig. 1b). In presence of organic solvent at higher concentration, the enzyme activity becomes lower because of the diminished surface area of protein. Cellulase in 3% (v/v) TFE retains ~90% of the activity, while at 80% (v/v) ACN, it retains up to ~42% of the activity. On the basis of enzyme assay, it can be concluded that cellulase retain its activity at low solvent concentration.

3.2. Intrinsic fluorescence

Tryptophan fluorescence is a sensitive probe in detecting of the polarity of the microenvironment [17]. There are six tryptophan residues present in *A. niger* cellulase [23]. Changes in fluorescence intensity of cellulase on varying concentration of TFE and ACN are shown respectively in Fig. 2a and b. From 0 to 3% (v/v) TFE and 0 to 80% (v/v) ACN, the fluorescence intensity increases gradually, indicating the structural alterations in cellulase. On further increasing the concentration of these respective solvents, fluorescence intensities were dropped down radically up to 25% (v/v) in TFE and 90%