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Equilibrium studies of cellulase aggregates in presence of ascorbic and boric acid

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ABSTRACT

The aggregate formation of cellulase was detected at 300 and 10 mM ascorbic and boric acid respectively. These aggregates showed reduced enzyme activity, loss in near-UV signal, decrease tryptophan and ANS fluorescence. They possess increase in non-native β-sheet structure as evident from far-UV CD and FTIR spectra, large hydrodynamic radii, increase thioflavin T fluorescence and shift in Congo red. Cellulase at 90 mM ascorbic acid exists as molten globule with retention of secondary structure, altered tryptophan environment, high ANS binding and loss in tertiary structure. Ascorbic acid acts as an antioxidant up to 90 mM and beyond this as a pro-oxidant.

1. 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 folded 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]. The aggregation of insoluble polypeptide chains has seriously confined the scale of protein marketed by the biotechnology industry. These protein aggregates lead to various neurodegenerative diseases in humans such as Alzheimer's diseases, Parkinson's disease, type II diabetes mellitus and spongiform encephalopathies has been linked to protein aggregates [4–6]. Identification of novel therapeutic ways to prevent or cure the diseases associated with protein aggregates 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].

Ascorbic acid or vitamin C ($C_6H_8O_6$) is distributed widely in both the plant and animal kingdoms. Vitamin C is also present in many other biological systems and multivitamin preparations, which are commonly used to supplement inadequate dietary intake. It functions in collagen synthesis by changing the charge of iron so that it can be absorbed. It serves many functions ranging from antiseptic as well as antibacterial applications to insecticides. It is widely used as an antioxidant. The antioxidant property of ascorbic acid is often considered responsible for its protective effects against cardiovascular disease and certain types of cancers. Similar to these, ascorbic acid has also been shown to exhibit pro-oxidant properties that can alter protein functions. Relatively high concentrations of ascorbic acid are able to induce apoptosis in various tumour cell lines. Consequently, it has been shown to induce cell death, nuclear fragmentation and internucleosomal DNA cleavage in human myelogenous leukaemia cell lines. Boric acid is mildly toxic to humans, with a median lethal dose at 2660 mg/kg of body mass through internal ingestion or inhalation. While this median lethal dose in humans is just barely more toxic than table salt for one-time exposure it does not rule out long term toxicological effects. Boric acid, applied foliarly on field-grown nodulated soybeans, caused up to a 10-fold increase in allantoate concentration in treated leaf tissue.

Cellulase efficiently and economically achieves the conversion of cellulose to β D-glucose. Glucose produced from cellulose substrate can be directly used in animal and human foods. These glucose containing products can be further used as substrate for subsequent fermentation or other processes which can yield valuable end products such as methanol, ethanol, amino acids, organic acids, single cell protein and other bioreactors. Cellulase can also be used to increase digestibility of foods enriched in high fibre content and to enhance food flavour, texture and quantity. *Aspergillus niger* has three classes of enzymes endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) [8]. Endoglucanase is the most important constituent produced by *Aspergillus* for cellulose hydrolysis. The purpose of this study is to

Abbreviations: ANS, 8-anilino-1-naphthalene-sulphonic acid; ATR-FTIR, attenuated total reflection Fourier transform infrared spectroscopy; CD, circular dichroism; MRE, mean residual ellipticity; ThT, thioflavin T.

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investigate the effect of organic acid (e.g. ascorbic acid) and inorganic acid (e.g. boric acid) on cellulase used as a model enzyme in vitro.

2. Materials and methods

2.1. Materials

Cellulase from *Aspergillus niger*, ascorbic acid, boric acid, sodium phosphate monobasic, sodium phosphate dibasic were purchased from SRL (Mumbai, India). Cellulose 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.

Cellulase was dissolved in 20 mM phosphate buffer of pH 7 to give a stock solution of 5 mg/ml and then dialyzed in the same buffer. Concentration of protein was determined by the method of Lowry [9].

2.2. Effect of ascorbic and boric acid on cellulase

Samples of cellulase in ascorbic and boric acid were prepared separately with varying concentration, i.e. 0–500 mM in phosphate buffer, pH 7 and then these samples were incubated for 4 h before performing spectroscopic measurements. All the measurements were carried out at room temperature. Three replicates for each set were analyzed for the results.

2.3. Assay of cellulase activity

Cellulase activity was assayed by using 200 mg cellulose powder as a substrate in 500 μl of enzyme solution prepared in 20 mM sodium phosphate buffer, pH 7 as native and in the presence of ascorbic and boric acid. The amount of reducing sugar released was determined by phenol/H_2SO_4 method of Dubois et al. [10].

2.4. Comet assay

Isolated lymphocytes were exposed to cellulase with 300 mM ascorbic acid in a total reaction volume of 1.0 ml. Incubation was performed at 37 °C for 1 h. After incubation, the reaction mixture was centrifuged at 4000 rpm, the supernatant was discarded and pelleted lymphocytes were resuspended in 100 μl of sodium phosphate buffer and processed further for Comet assay. Alkaline conditions are necessary to perform this assay [11].

2.5. Intrinsic fluorescence measurements

The fluorescence spectra were recorded on a Shimadzu RF-5301 spectrofluorophotometer (Tokyo, Japan) in a 1 cm path length quartz cell. The excitation wavelength was 280 nm and the emission was recorded in the range of 300–400 nm [12]. The final concentration of cellulase was 4.5 μM.

2.6. Acrylamide quenching

In the acrylamide-quenching experiments, aliquots of 5 M acrylamide stock solution were added to a protein stock solution (15 μM) to achieve the desired acrylamide concentration. Excitation was set at 295 nm in order to excite tryptophan fluorescence only, and the emission spectrum was recorded in the range 300–400 nm. The slit width was set at 10 nm for both excitation and emission. The decrease in fluorescence intensity at λ_{max} was analyzed according to the Stern–Volmer equation [13]:

$$\frac{F_0}{F} = 1 + K_{SV}[Q]$$

where F_0 and F are the fluorescence intensities at an appropriate wavelength in the absence and presence of a quencher (acrylamide), respectively, K_{SV} is the Stern–Volmer constant for the collisional quenching process, and [Q] is the concentration of the quencher.

2.7. 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 molar excess of the protein concentration and protein concentration was in the vicinity of 4.5 μM [14].

2.8. 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, respectively. Each spectrum was the average of 4 scans [15]. Protein concentration for the scan was 11 μM for far-UV and 18 μM for near-UV CD. The results were expressed as the mean residue ellipticity (MRE in $cm^2\ dmol^{-1}$), which was defined as:

$$\text{MRE} = \frac{\theta_{obs}(m^\circ)}{10 \times n \times \text{Cp} \times l}$$

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

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

FTIR spectra were recorded with Interspec 2020 FTIR spectrometer in deuterated water. For amide I spectroscopy, protein samples are often dissolved in D_2O and gently heated for a few hours in order to partially denature the protein and exchange the labile protons with deuterons. The sample is then lyophilized and redissolved in pure D_2O. Solutions are placed between two CaF_2 windows with a spacer. Since the D_2O bend vibration absorbs strongly below 1500 cm^{-1} the path length must be kept to a minimum, therefore sample concentrations must be relatively high. Protein concentration was 117 μM. The scanning wave number was from 1000 to 4000 cm^{-1} [16].

3. Size exclusion chromatography

SEC experiments were carried out on a Sephadex G 200 (76 9 1.15) cm column. The column was pre-equilibrated with 20 mM phosphate buffer pH 7 and in the presence of ascorbic and boric acid. Two millilitres of 5 mg/ml native and cellulase in the presence of ascorbic and boric acid were applied to the column and eluted at 20 ml/h. The eluted fractions were read at 280 nm. The molecular weight marker used were glucose oxidase [GOD] (160,000), Concanavalin A [con A] (104,000), bovine serum albumin [BSA] (66,700), α1 antitrypsin (52,000), cellulase (46,000), lactoglobulin (33,000), chymotrypsinogen [Chy] (25,500), soyabean trypsin inhibitor [SBI] (20,000) and cytochrome c [cyt c] (12,500).