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Crowded milieu tuning the stability and activity of stem bromelain

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ABSTRACT

Proteins *in vivo* are under an extremely crowded environment because of the presence of bulky and large biological macromolecules (known as crowders). These crowders affect the proper functioning and structure of proteins in a cell. During *in vitro* studies, we often ignore the effect of macromolecular crowding on protein stability. However, if a large concentration of crowder is used to examine protein stability, its effects on the functioning of protein inside the cell in a confined environment, as stated, can be understood. Keeping this in context, we investigated the effects of macromolecular crowding on stem bromelain (BM) with the help of different crowding agents of varying molecular weights such as dextran (40 kDa and 6 kDa) and ficoll (70 kDa). Activity and stability of BM was examined using UV-vis, fluorescence and circular dichroism (CD) spectroscopy. Furthermore, docking methods are used to complement the crowding effects on the stability of BM. We found that stability and activity of BM are dependent on the surrounding crowder molecules. Thermal flouresence results showed that, thermal stability of BM decreses with incresing concentration of crowder except dextran40. It was observed that the decrese in stability and activity can be related to the presence of soft interactions between crowder and BM. Thus,crowding does not always stabilize the native structure, instead, it depends on degree of disorder of protein structure and on two competing effects: the excluded volume, which favors compact states, and soft interactions, which favor extended conformers.

1. Introduction

The stability of protein inside a cell is governed by various factors such as cell environment, membrane composition, temperature, pH, chemical modifications, and binding of other molecules [1]. The major stress through which the protein undergoes within the cellular environment is that it has to suffer extremely crowded environment due to the presence of naturally occurring high molecular weight molecules like carbohydrates etc. In studies involving biological molecules, it is necessary to add crowding agents along with controlling pH and ionic strength to mimic physiologically relevant conditions [1].

Macromolecular crowding is steadily gaining importance as it describes the fact that total concentration of macromolecules inside the cell is so high that a significant proportion of the space is physically unavailable to other molecules [2]. Recent studies emphasize on the contribution of soft interactions in addition to hardcore repulsion. The interplay of both soft interactions and hardcore repulsion determine the excluded volume effect. Despite their non-specific and weak nature, the large number of soft interactions present under crowded conditions can sometimes overcome the stabilizing excluded volume effect [3]. The term soft interaction encompasses hydrogen bonding as well as hydrophobic and electrostatic interactions. Some of the electrostatic destabilizing interactions are recognized as pH dependent phenomenon [4].

Model macromolecular crowding agents such as polyethylene glycol (PEG), dextran and ficoll polymers, are generally used to mimic cell environment *in vitro* [2]. Using protein as crowders instead of synthetic polymers will give us more physiologically relevant information. However, observing a test protein under crowded conditions where its concentration is very less compared to the total protein concentration is extremely difficult [4]. Therefore, for the present work we have focussed on synthetic crowders only.

Macromolecular crowding is known to affect a multitude of processes in the biological systems. Studies have revealed that high concentration of crowders significantly modulates the catalytic efficiency of biologically important enzymes, DNA ligase [5], enterobactin-specific isochorismate synthase [6] and Ras (member of the class of small GTPases) [7] etc. Additionally, the presence of high concentration of crowding agents is also found to increase the thermal stability of proteins such as cytochrome c [8], apoflavodoxin [9] and creatine kinase [10] etc. At moderately acidic pH (pH 4), ficoll70 had a stabilizing influence on RNase A and α-lactalbumin [11].

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Stem bromelain (BM), a cysteine proteinase is used in the present work to explore the crowding effects on its structural stability and activity. BM contains 212 amino acid residues with molecular mass 23.8 kDa containing three disulfides and a single free cysteine (Cys) residue and is extensively used in chemical and pharmaceutical industry [12]. It reversibly inhibits platelet aggregation, angina pectoris, bronchitis, sinusitis, thrombophlebitis, and pyelonephritis, and enhances absorption of drugs, particularly antibiotics. It is also used in meat processing tenderization and as a dietary supplement [13–16]. It belongs to the α + β class of protein and its hydrodynamic radius is reported to be ~2.3 nm at 1 mg/mL concentration [13]. The increase in radius with increasing BM concentration is related to increase in the polydispersity value under denaturing conditions [16]. BM has six phenylalanine (Phe), five tryptophan (Trp) and fourteen tyrosine (Tyr) aromatic residues [11]. It also contains single oligosaccharide chain, but is not the part of the enzyme's active site [17].

The available literature identifies BM as a kinetically stable biomolecule based on its properties like β-sheet rich structure, resistance to proteolysis and sodium dodecyl sulfate (SDS) binding and high energy of activation against denaturation [14–16]. It was also discovered that cross-linked BM leads to formation of soluble aggregates which displays better storage with high thermal stability [18]. Preference selection of BM in the present study, for *in vitro* stability studies in crowded environment, is because of its stability and a wide effective range of activity in both acidic and alkaline conditions that allow it to remain active in a variety of biological environments.

In the past, our research group has studied the effect of osmolytes on the stability and activity of BM which was found to be largely stabilizing and in some cases even counteracting the deleterious effect of denaturants on BM structure [19–22]. Most of the *in vitro* protein studies are carried out in a buffer under low protein concentration (1 mg/mL). The concentration of biological macromolecules inside cells is estimated to be in the range of 80–400 g/L (5%–40% of volume occupancy) and creates a crowded medium, with considerably restricted amounts of free water [2]. Hence, despite being a widely studied enzyme, impact of macromolecular crowding on BM is still unexplored and devoid of any significant literature. Understanding the role of macromolecular crowding is not only relevant for protein stability *in vivo* but also contributes in assessing their role as drugs for various types of diseases. Therefore, studying the effect of model crowding agents, such as dextran (40 kDa and 6 kDa) and ficoll70, on the structural stability and activity of BM is extremely important from a clinical perspective.

Herein, macromolecular crowding effects on BM in the presence of Ficoll70 (f70), dextran40 (d40) and dextran6 (d6) under different concentration (10–300 g/L) is explored. Synthetic crowding agents used in the current study are polymers with carbohydrate moieties as repeating units, which are readily soluble in water. UV–vis spectroscopy, steady-state and thermal fluorescence, circular dichroism spectroscopy (CD) and enzyme assay are employed to ascertain the stability and activity of BM in a crowded environment. Furthermore, molecular interaction of crowders with BM is also described using the MolDock method.

2. Materials and methods

2.1. Materials

Stem bromelain (BM) from Ananas comosus (E.C. 3.4.22.32), dextran 40 (d40), dextran 6 (d6) and ficoll70 (f70), were purchased from Sigma-Aldrich, U.S.A. Highly pure anhydrous sodium phosphate monobasic, sodium phosphate dibasic dehydrate, casein (Hammarsten), trichloroacetic acid (TCA), sodium acetate, and acetic acid were purchased from Sisco Research Lab (SRL), India. All chemicals were of high purity and of analytical grade.

2.2. Sample preparations

10 mM sodium phosphate buffer (pH 7) prepared by using distilled deionized water with a resistivity of 18.3 Ω cm, was used for the preparation of all samples. The concentration of protein was fixed to be 0.5 mg/mL, for all experimental studies. Throughout the procedure, all samples were incubated for 1 h at 25 °C prior to experimental studies. Crowding agents d6, f70 and d40 were dissolved in phosphate buffer to get desired concentration (10–300 g/L).

2.3. Absorption measurements of BM in presence of crowders

Absorption spectra for BM in the absence and presence of d40, d6 and f70 at different concentrations were recorded on Shimadzu UV- 1800 (Japan) spectrophotometer with the highest resolution (1 nm) using matched 1 cm path length quartz cuvette at 25 °C. Protein concentration was determined using extinction coefficient $\varepsilon^{1\%} = 20.1$ (at 280 nm) [14]. Respective blank measurements were taken for every sample and subtraction results were analyzed.

2.4. Steady state fluorescence measurements of BM in presence of crowders

Steady-state fluorescence spectroscopy was performed at 25 °C using Cary Eclipse spectrofluorometer (Varian optical spectroscopy instruments, Mulgrave, Victoria, Australia) equipped with an intense xenon flash lamp as the light source equipped with a Peltier type temperature controller with a precision of ±0.05 °C, using the excitation wavelength at 295 nm with a slit width of excitation and emission at 5 nm and 5 nm, respectively. All emission spectra were recorded for all samples of BM in the presence of d40, d6 and f70 at different concentrations. The excitation wavelength of 295 nm was used in order to prevent Tyr residues from transferring the radiation energy to the Trp residues. Respective blank in each case was subtracted from the sample and then analyzed.

Quenching of emission of BM at 348 nm was examined using following Stern–Volmer (S–V) equations-

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \quad (1)$$

$$\frac{F_0}{F} = 1 + K_{sv}[Q]\, e^{-V[Q]} \quad (2)$$

where K_{sv} is the quenching constant and F_0, F is the fluorescence intensities in the absence and presence of crowders, respectively. [Q] is the crowder (i.e quencher) taken as g/L. Linear S–V plots (F_0/F vs [Q]) were modeled according to Eq. (1), while plots showing upward curvature (positive deviation) were analyzed on the basis of sphere-of action model given as Eq. (2), where V being the volume of the sphere surrounding the fluorophore wherein for quenching to occur no diffusion needs to take place [23].

To evaluate K_{sv} for different crowders, Eq. (2) can be modified to the following equation [24–26]:

$$\frac{1 - \frac{F}{F_0}}{[Q]} = K_{sv}\left(\frac{F}{F_0}\right) + \frac{1 - e^{-V[Q]}}{[Q]} \quad (3)$$

The modified S–V plots $(1\text{-}F/F_0)/[Q]$ against F/F_0 for steady-state fluorescence were plotted and found to be linear. The S–V quenching constant (K_{sv}) was obtained using least square fit method by the slopes.