JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Influence of Ethylenediaminetetraacetic Acid (EDTA) on the Structural Stability of Endoglucanase from *Aspergillus aculeatus*

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ABSTRACT: The effect of the chelating agent ethylenediaminetetraacetic acid (EDTA) on the structure and function of endoglucanase is studied. In the presence of 2 mM EDTA, endoglucanase showed an enhanced enzymatic activity of 1.5-fold compared to control. No further change in activity was observed with increase in the concentration of EDTA to 5 mM. The K_m values for control and in the presence of EDTA are 0.060 and 0.044%, respectively, and K_{cat} was 1.9 min^{-1} in the presence of EDTA. The kinetic parameters indicated a decrease in the K_m with an increase in the K_{cat}. Far-ultraviolet circular dichroism (far-UV-CD) results showed a 20% decrease in ellipticity values at 217 nm in the presence of EDTA compared to native enzyme. The apparent T_m shifted from a control value of 57 ± 1 to 76 ± 1 °C in the presence of EDTA (5 mM). The above results suggested that the enhanced activity in the presence of EDTA is due to an increase in the K_{cat} and flexible conformation of the enzyme. The stability of endoglucanase increased in the presence of EDTA.

KEYWORDS: endoglucanase, activity, EDTA, structure

INTRODUCTION

Cellulose is a linear polymer of glucosyl units connected by β-1,4 linkages. These linear chains vary in length and often consist of many thousands of units. Most of the organic material on earth is stored in the form of cellulose. Cellulose is readily degraded by a number of microbes producing many different cellulolytic enzymes. Due to the energy crisis, cellulose is an important alternative source of renewable energy.[1−3] Many bacteria and fungi produce cellulases to break down this complex substrate from its insoluble form to glucose. At present, commercial applications have been mainly limited to the detergent and textile industries. However, present environmental concerns have geared up enormous interest in the application of cellulases in biomass conversion.[4]

Aspergillus aculeatus is an important commercial source of inexpensive cellulase, not only in the food and textiles industries but also in pharmaceutical industries.[5] Endoglucanase is the main component for cellulose degradation from *A. aculeatus* and belongs to GH family 12. It is known to catalyze glycosidic bond cleavage with net retention of anomeric configuration.[6,7]

Ethylenediaminetetraacetic acid (EDTA) is a chelating agent used for many decades as a protease inhibitor and metal ion scavenger. It has been shown to be a noneffective molecule on cellulase activity. Studies with zinc binding proteins have shown that incubation of native cysteine/histidine-rich region 1 (C/H1) with EDTA produced changes in the circular dichroic spectra with a decrease in the α-helical content. The results were consistent with protein unfolding in the absence of Zn^{2+}, and addition of excess Zn^{2+} did not restore the native structure of C/H1, indicating that EDTA caused irreversible denaturation.[8] The present study describes the molecular characterization of endoglucanase in the presence of EDTA. This is investigated by activity measurements, fluorescence spectroscopy, circular dichroic, and thermal denaturation studies. These data would provide insight into protein−ligand interactions for better understanding of the structure−function relationship of the enzyme and proteins in general.

MATERIALS AND METHODS

Materials. Viscozyme, a commercial cellulase preparation of *A. aculeatus*, was obtained from Novozymes, Bangalore, India. Endoglucanase was purified as described earlier.[9] Carboxymethylcellulose (CMC, medium viscosity), EDTA, dithiothreitol (DTT), calcium chloride ($CaCl_2$), zinc chloride ($ZnCl_2$), copper chloride ($CuCl_2$), ferrous chloride ($FeCl_2$), sodium acetate, glucose, 1-anilino-8-naphthalenesulfonic acid (ANS), guanidine hydrochloride (GuHCl), and 2,4-dinitrosalicylic acid (DNS) were purchased from Sigma-Aldrich Co., St. Louis, MO. All of the chemicals used were of analytical grade.

Enzyme Assay. The endoglucanase activity was measured by incubating a solution of 0.1 mg/mL with 0.9 mL of 0.5% CMC (degree of substitution, 0.7% (w/v)) in 0.02 M sodium acetate buffer, pH 5.0, at 37 °C for 1 h. Reaction was stopped by the addition of 3 mL of DNS reagent. To determine the reducing sugar produced, the solution was boiled for 5 min in a boiling water bath and then cooled. Total volume was made up to 20 mL with distilled water and absorbance measured at 520 nm[10] using a Shimadzu UV1601 spectrophotometer (Shimadzu, Singapore). Glucose was used as a standard. One unit of enzyme activity corresponds to the release of 1 μmol equivalent of the reducing sugar (glucose) from the substrate per minute. To determine the effect of EDTA, endoglucanase solution was preincubated in the absence and presence of different concentrations of EDTA for 2 h, and then the reaction was started by the addition of 0.5% CMC solution. The enzyme activity in the absence of EDTA served as control, and the relative activity was calculated on the basis of its original activity.

Kinetic parameters were determined by incubating the enzyme in presence of EDTA (5 mM) in the assay mixture, when the enzyme

Received: October 6, 2010
Revised: June 9, 2011
Accepted: June 9, 2011
Published: June 09, 2011

activity was found to be maximum. The Michaelis−Menten constant K_m and K_{cat} of the enzyme were determined by employing the Lineweaver−Burk plot obtained by the initial velocity studies using CMC as the substrate (ranging from 0 to 1.5%) in sodium acetate buffer (eq 1). The K_m was calculated using the equation

$$1/V = 1/V_{max} + K_m/V_{max} \times 1/[S] \quad (1)$$

where V is the velocity, V_{max} is the maximum velocity, $[S]$ is the substrate concentration, and K_m is the Michaelis−Menten constant.

Effect of Chemical Reagents on Activity. Endoglucanase was incubated in the presence and absence of different concentrations of $CaCl_2$, $ZnCl_2$, DTT, $CuCl_2$, and $FeCl_2$ for 2 h at 37 °C, and the relative activities were determined using CMC as substrate in 0.02 M sodium acetate buffer, pH 5.0.

Fluorescence Studies. Fluorescence emission spectral measurements were done using a Shimadzu RF-5000 (Shimadzu Corp., Kyoto, Japan) automatic recording spectrofluorimeter at 25 °C and scanned. The temperature of the cell was maintained by circulating the water through the thermojacketed holder from a circulating water bath. The protein was excited at 280 nm and emission scanned from 300 to 400 nm.[11] The bandwidths for excitation and emission monochromators were fixed at 5 and 10 nm, respectively. The readings of fluorescence intensity were plotted versus EDTA concentrations.

ANS Binding Assay. The extent of exposure of hydrophobic surfaces in the enzyme was measured by its ability to bind the fluorescent dye ANS. A stock solution of ANS was prepared in methanol, and concentration was determined using an extinction coefficient of 5000 M^{-1} cm^{-1} at 350 nm. The protein solution was incubated with an excess (100-fold) of ANS for 30 min in the dark, and ANS fluorescence was measured. The protein concentration was 1 μM. It was excited at 380 nm, and emission spectra were scanned from 400 to 600 nm with slit widths of 5 and 10 nm for excitation and emission, respectively.

Thermal Transition Temperature. T_m was determined by using a Cary100 Bio UV−visible spectrophotometer (M/s Varian, Australia Pty Ltd.). Measurements were done at different temperatures ranging from 20 to 90 °C with 1 °C/min increment, and the spectra were recorded at 287 nm. A protein concentration of 2 μM was used for all of the experiments, and buffer with respective concentrations of EDTA was used in the reference cell. The apparent thermal transition temperature (apparent T_m) was calculated by monitoring the progress of denaturation followed by changes in the absorbance or van't Hoff plot.[12] The results are the average of three independent experiments.

Circular Dichroic (CD) Spectra. CD spectra were obtained by using a JASCO J-810 automatic recording spectropolarimeter (JASCO Corp., Tokyo, Japan) fitted with a 250 W xenon lamp. Samples were analyzed in 0.1 cm optical path length cells in the far-UV (below 250 nm) region. The secondary structure of endoglucanase was analyzed using the computer program of Yang et al.,[13] which calculates the structural component ratio of secondary structures for the protein by the least-squares method. The reported CD values were the average of at least three independent runs. Before the spectra were recorded, all samples were previously centrifuged and filtered through Millipore filters (0.45 μm pore diameter).

Statistical Analysis. Data analysis was carried out in triplicate, and each parameter indicated as the mean of three replicates. The corresponding standard deviations have been reported. The data were analyzed using an analysis of variance technique, and means were separated by using Duncan's new multiple-range test at the 5% probability level. The kinetic parameters were assessed for significant levels.[14]

RESULTS AND DISCUSSION

The effect of the reducing agent DTT, metal ions, and EDTA on the endoglucanase activity was investigated. The presence of DTT inhibited the endoglucanase activity (Figure 1). This result shows that disulfide linkages are involved in maintaining the active site conformation of the endoglucanase.[15] It was further observed that $CaCl_2$, $ZnCl_2$, $CuCl_2$, and $FeCl_2$ did not have any effect on the endoglucanase activity. The enzyme activity increased by 1.5-fold in the presence of 2−5 mM EDTA in 0.02 M sodium acetate buffer, pH 5.0, at 37 °C. An increase in enzyme activity was observed even after the removal of EDTA by dialysis against buffer overnight with three to four changes as compared to the control (absence of EDTA), thus showing that the presence of EDTA retains most of the activity and had a positive influence on enzyme activity. Different concentrations of metal ions (Ca^{2+}, Zn^{2+}, and Fe^{2+}) did not show any effect on the enzyme activity even after treatment of endoglucanase with EDTA, followed by dialysis for the complete removal of EDTA. The increase in activity due to EDTA remains as such and is not affected by metal ions. This confirmed that endoglucanase does not require metal ions for enhancing or inhibiting enzymatic activity.

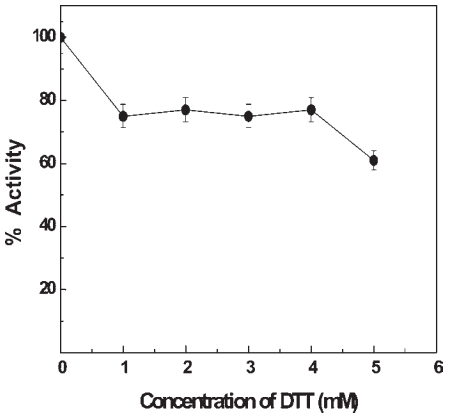

Figure 1. Effect of DTT on endoglucanase. The enzyme was preincubated with DTT and activity measured with 0.5% CMC in 20 mM sodium acetate buffer, pH 5.0, at 37 °C in the presence of different concentrations of DTT.

However, some authors have shown that Zn^{2+} and Cu^{2+} metallic ions had an inhibitory effect, which is a common feature of cellulases and xylanases[16] including plant cellulases.[17] Here the isolated endoglucanase from *A. aculeatus* did not show any effect on its activity in the presence of Zn^{2+} and Cu^{2+}, which shows that sulfhydryl groups are not involved in the activity or present at the active site. The enzyme lost some of its activity in the presence of DTT due to breakage of disulfide linkages, which are responsible for maintaining the active site or native conformation of the enzyme. Enzyme inhibition by metallic cations usually suggests the presence of at least one sulfhydryl group of cysteine in the active site, the oxidation of which by the cations destabilizes the conformational folding of the enzyme[18] or leads to the formation of disulfide bonds at an irregular position of the protein.[17] Fe^{2+} and Mn^{2+} had significant negative effects on endoglucanase II. Similar effects were also observed in endoglucanases purified from *Bacillus* strains.[19] Darlene et al.[20] have shown that endoglucanase from *Achlya ambisexualis* shows no inhibition by either EDTA or EGTA, indicating that no divalent cations are required. Aside from the thiol inhibitor Hg^{2+}, the only other ions tested that had significant effects on activity were Mn^{2+} and Ca^{2+}, both of which were mildly inhibitory. The patterns of ion inhibition and sensitivity to sulfhydryl-binding agents vary greatly among

Table 1. Kinetic Parameters of Endoglucanase in the Presence and Absence of EDTA[a]

sample	K_m (app) (%)	K_{cat} (min)	K_{cat}/K_m
control	0.060 a ± 0.004	1.33 a ± 0.2	22.16 a ± 1
EDTA	0.044 b ± 0.002	1.87 b ± 0.3	42.97 b ± 3.01
	±0.0018 (4DF)	±0.015 (4DF)	±1.29 (4DF)

[a] Activity measurements were done in 0.02 M sodium acetate buffer, pH 5.0, using CMC as substrate. Means in the same column followed by different letters differ significantly ($p < 0.05$), according to Duncan's new multiple-range test. DF, degrees of freedom.

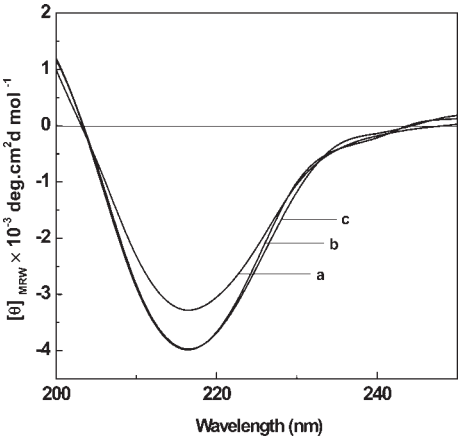

Figure 2. Far-UV-CD spectra of endoglucanase in the presence and absence of cofactors. The spectra were measured in different concentrations of EDTA after preincubation of the enzyme for 2 h: (a) control (in buffer only); (b) 2 mM; (c) 5 mM. The spectra were measured in 20 mM sodium acetate buffer, pH 5.0, at 25 °C. The concentration of enzyme was 0.24 mg/mL.

plant and microbial glucanases, and their significance, if any, is not understood. Thus, metal ion requirement or inhibition of cellulase activity is not universal and cannot be generalized.

The nature of activation of endoglucanase in the presence of EDTA was further characterized by steady state kinetics. Kinetic parameters of endoglucanase activity in the absence and presence of EDTA were determined using a Lineweaver–Burk plot (Table 1). The control (in the absence of EDTA) had an apparent K_m of 0.060%, and K_{cat} was 1.3 min^{-1}. In the presence of EDTA, K_m decreased to 0.044% compared to control and had a K_{cat} of 1.9 min^{-1}. The K_m and K_{cat} values in the presence and absence of EDTA differed significantly ($p < 0.05$), according to Duncan's new multiple-range test. The decrease in the K_m (high affinity for substrate) and increase in the K_{cat} (higher catalytic constant for conversion of substrate to product) suggests increased catalytic efficiency. The ratio of K_{cat}/K_m, a quantitative index of catalytic power, was 2-fold higher in the presence of EDTA. Popa et al.[21] in their work on viral neuraminidase have shown enhanced activity in the presence of EDTA, and the kinetic data obtained indicated modification of the enzyme such that the affinity for the substrate increased, which is evidenced by decreased K_m values.

The changes in the secondary structure of endoglucanase as a function of EDTA were followed by far-UV-CD measurements. The addition of EDTA causes significant changes in the CD spectra, suggesting structural changes of the enzyme due to

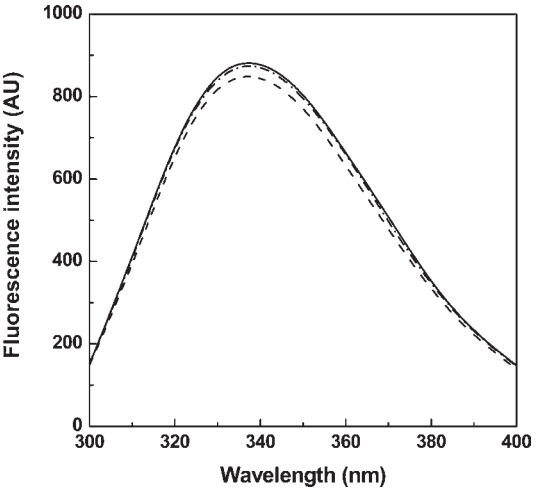

Figure 3. Effect of EDTA on fluorescence spectra of endoglucanase: (—) control; (— · —) 2 mM EDTA; (— —) 5 mM EDTA. The spectra were measured in 20 mM sodium acetate buffer, pH 5.0, at 25 °C by excitation at 280 nm.

EDTA. The far-UV-CD spectra of endoglucanase in the presence of different concentrations of EDTA are shown in Figure 2. There was a significant change in the intensity of the negative trough at 217 nm. The control had an ellipticity value of −3450 ± 30 deg·cm^2/dmol cm^2, which decreased to −4155 ± 50 deg·cm^2/dmol cm^2 in the presence of EDTA. There is a 20% increase in the ellipticity value at 217 nm in the presence of EDTA with a significant increase in the ordered structure, where the amount of β-sheet structure has increased. Enhanced endoglucanase activity can be correlated to the changes in the β-sheet structure. The far-UV-CD spectra of endoglucanase in the presence of zinc, copper, calcium, and iron did not show any change. Matt et al.[22] found that incubation of native C/H1 with EDTA produced CD spectra with significantly decreased α-helical component, consistent with protein unfolding in the absence of Zn^{2+}, which is typical of small zinc-binding motifs.[23] The addition of excess Zn^{2+} did not restore C/H1 to its native structure, suggesting that denaturation by EDTA was irreversible. No changes were observed in the relative fluorescence intensity (Figure 3) of the endoglucanase in the presence of EDTA. This indicates that there is no interaction between tryptophan and EDTA and also that there is no change in the tertiary structure of the endoglucanase. To date, very little is understood about the relationship between structure and function of the active site centers of the cellulolytic enzymes.

The presence of cofactors (zinc, calcium, copper, and iron) did not show any effect on the apparent thermal denaturation temperature of endoglucanase. Figure 4 shows typical thermal transition curves of endoglucanase in aqueous media containing EDTA. The apparent thermal transition temperature (T_m) increased from the control value of 57 °C to 66, 68, 70, and 76 °C in the presence of 1, 2, 3, and 5 mM EDTA, respectively. This shows that EDTA stabilizes the protein structure against the thermal denaturation. The exact mechanism for the increased stability is not clear and requires thorough investigation in this regard.

To further investigate the role of EDTA, we have monitored the exposure of hydrophobic surfaces in the presence of EDTA. The hydrophobic dye ANS reports hydrophobic surfaces on the

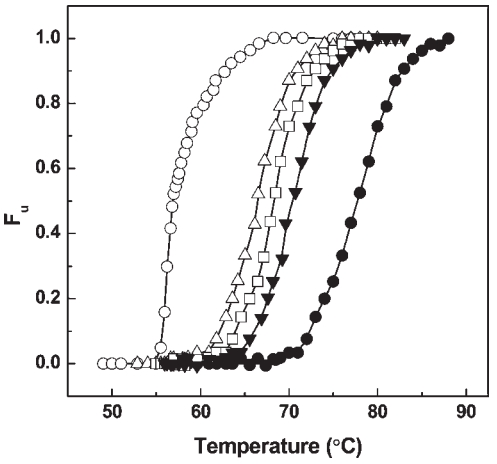

Figure 4. Apparent thermal transition temperature profile of endoglucanase in the presence of EDTA, in 20 mM sodium acetate buffer, pH 5.0, as a function of temperature in the range of 25−90 °C with a 1 °C/min increase in the temperature: (○) in the absence of EDTA (control); (△) 1 mM EDTA; (□) 2 mM; (▼) 3 mM; (●) 5 mM EDTA. The spectra were measured at 287 nm.

proteins.[24,25] Its fluorescence is sensitive to the polarity of its microenvironment, and upon binding to the apolar surfaces, depending on the extent of hydrophobicity of the surfaces, its emission intensity is increased. This property of the dye has been demonstrated to identify the intermediates on the unfolding pathways of proteins.[26] Figure 5A shows the fluorescence of ANS-bound endoglucanase in the presence and absence of EDTA. The fluorescence of ANS alone in buffer shows an emission maximum at 512 nm; ANS on binding to protein shows a shift in the emission maximum to 480 nm in the presence of EDTA with an increase in the fluorescence intensity. This shows that the enzyme exists in a flexible conformational state with exposed hydrophobic surfaces, which become more accessible to the hydrophobic dye ANS. The profile of emission intensity versus EDTA concentration is shown in Figure 5B. At 5 mM EDTA, there is maximum change taking place; EDTA concentration above 5 mM leads to quenching of the fluorescence intensity. Prolonged incubation of endoglucanase for >3 h with EDTA does not lead to any significant alteration in the profile. These results indicate that EDTA alters the structure of the endoglucanase such that its hydrophobic surfaces are exposed to a greater extent compared to the native enzyme. It could be that at higher concentrations of EDTA the hydrophobic residues that were exposed could be moving inside or away from the solvent, leading to decreased fluorescence intensity. Krishnan et al.[27] have shown using bovine carbonic anhydrase (BCA) that in the absence of EDTA, ANS reports a weak and broad transition at around 2 M GuHCl inducing unfolding of BCA. However, in the presence of 2 mM EDTA, the transition becomes sharp and stronger at 1.5 M GuHCl. These results suggests that the presence of EDTA does influence the unfolding behavior of BCA and leads to increased exposure of the hydrophobic surfaces of BCA at 1.5 M GuHCl. Similarly, we have previously shown with this enzyme that in the presence of denaturants the endoglucanase activity increased at lower concentrations and, further, that the activity gradually decreased as the concentration of denaturants was increased.[28]

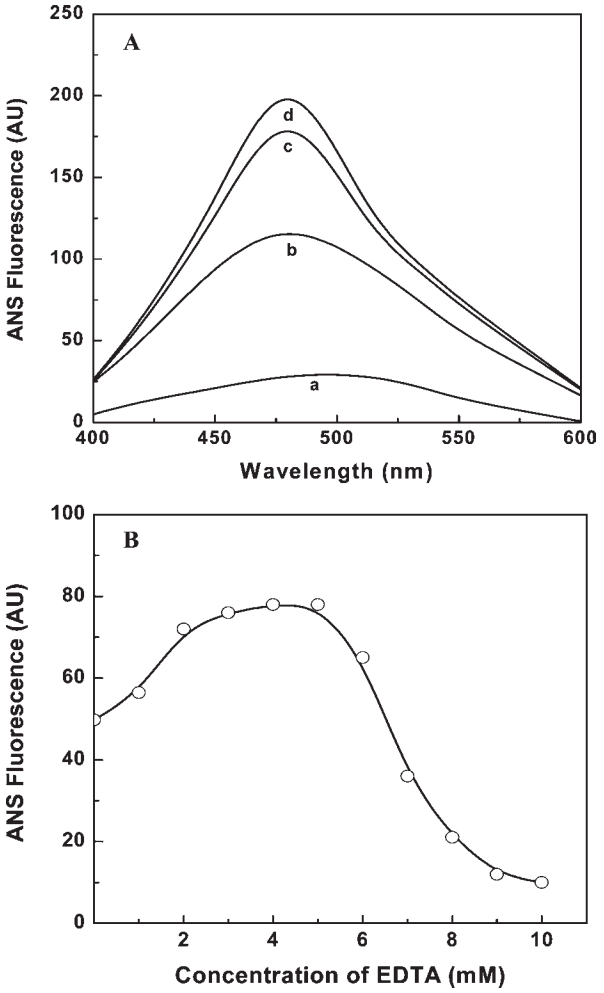

Figure 5. (A) Changes in the fluorescence emission spectra of ANS binding to endoglucanase as a function of wavelength: (a) buffer with ANS; (b) protein with ANS; (c) in the presence of 2 mM EDTA; (d) in the presence of 5 mM EDTA. The spectra were measured in 20 mM sodium acetate buffer, pH 5.0, at 25 °C in the wavelength range of 400−600 nm. The samples were incubated for 24 h at 25 °C. The spectra were measured in different concentrations of EDTA after preincubation of the enzyme for 2 h at 25 °C. (B) Changes in ANS fluorescence (480 nm) as a function of EDTA concentration.

The sharp transition in ANS binding (Figure 5A) has been shown to indicate the intermediate state.[26] This intermediate state is characterized by the presence of a substantial amount of secondary structure with no rigid tertiary structure of the molecule.[29−31] Near-UV-CD spectra are not shown as the enzyme does not show any significant tertiary structural patterns. An increase in apparent thermal transition temperature was observed in the thermal denaturation measurements. Folding and unfolding intermediates of the proteins, including intermediate state, expose hydrophobic surfaces and become more accessible to ANS binding. Here we show for the first time, in the case of endoglucanase, that the presence of EDTA can partially open the molecule to form an intermediate state, which can be implicated in protein substrate interactions. The opening of the molecule gives the enzyme a more favorable state for substrate interaction and may have a role in the activation and also stabilization of the molecule. Both are important properties for

an enzyme that finds tremendous applications in food-processing industries. Endoglucanase has the potential for commercialization to produce low-calorie food formulations and foods rich in soluble fibers.

These potential effects of EDTA on the structure and function of proteins should be taken into account in the interpretation of a number of other studies done in the presence of EDTA and should be considered in future studies.

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Funding Sources

G.S.N. is thankful to the Council for Scientific and Industrial Research, government of India, for providing financial support in the form of a research fellowship during the course of this work.

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; CMC, carboxymethylcellulose; UV-CD, ultraviolet circular dichroism; DTT, dithiothreitol; $CaCl_2$, calcium chloride; $ZnCl_2$, zinc chloride; NaCl, sodium chloride; $CuCl_2$, copper chloride; $FeCl_2$, ferrous chloride; DNS, 2,4-dinitrosalicylic acid; T_m, apparent transition temperature; ANS, 1-anilino-8-naphthalenesulfonic acid; GuHCl, guanidine hydrochloride; C/H1, cysteine/histidine-rich region 1.

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