J Ind Microbiol Biotechnol (2008) 35:515–524
DOI 10.1007/s10295-008-0310-4

ORIGINAL PAPER

Kinetics and thermodynamics of a novel endoglucanase (CMCase) from *Gymnoascella citrina* produced under solid-state condition

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Received: 14 September 2007 / Accepted: 11 December 2007 / Published online: 29 January 2008

Abstract *Gymnoascella citrina* produced two isoforms of endoglucanases (CMCase-I and -II) under solid-state condition. Purified CMCase-I was novel because it was apparently holoenzyme in nature. The enzyme was monomeric as its native and subunit mass were almost the same, i.e., 43 and 42 kDa, respectively. E_a for carboxymethylcellulose (CMC) hydrolysis was 36.2 kJ mol^{-1}. The enzyme was stable over a pH range of 3.5–6.5, while temperature optimum was 55 °C. V_{max}, K_m and k_{cat} for CMC hydrolysis were 39 U mg^{-1} protein, 6.25 mg CMC mL^{-1} and 27.5 s^{-1}, respectively. The pKa_1 and pKa_2 of ionizable groups of active site were 2.8 and 7.4, respectively. Thermodynamic parameters for CMC hydrolysis were as follows: $\Delta H^* = 33.5$ kJ mol^{-1}, $\Delta G^* = 70.42$ kJ mol^{-1} and $\Delta S^* = -114.37$ J mol^{-1} K^{-1}. The removal of metals resulted into complete loss of enzymatic activity and was completely recovered in the presence of 1 mM Mn^{2+}, whereas inhibition initiated at 5 mM. The other metals like Ca^{2+}, Zn^{2+} and K^{1+} showed no inhibition up to 7 mM, Co^{2+} completely inhibited the activity, while Mg^{2+} could not recover the initial activity up to 7 mM. So we are reporting for the first time, kinetics and thermodynamics of CMCase-I from *G. citrina*.

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Keywords Endoglucanase · Activation energy · Enthalpy · Entropy · Gibbs free energy

Introduction

Carboxymethylcellulases (β-1,4-D-glucan-4-glucanohydrolase, EC 3.2.1.4) are members of cellulase system, which is a consortium of enzymes mainly comprised of endoglucanases (EC 3.2.1.4), exoglucanases (EC. 3.2.1.91) and cellobiases (EC. 3.2.1.21). These enzymes act in synergy, though each has different profile [11, 45]. Cellulases have application in paper and pulp industry [17] as well as in alcohol and beverage industry [8]. Furthermore, cellulases have been widely used in detergents and in textile industry for desizing, stain removing, fabric softening, depilling, pilling prevention as anti-redepositors, colour care agents, stone washing, biopolishing, biofinishing and smooth surfacing of cotton fabric [15, 37]. Other uses of cellulases, which are of great ecological and commercial importance are: amelioration of municipal, forestry, agricultural and industrial wastes to control environmental pollution; biocomposting to produce natural organic fertilizers; production of food and feed supplements for cattle and poultry feed stocks; production of plant protoplast for genetic manipulation; preparations of pharmaceuticals; baking; malting and brewing; extraction of fruit juices and processing of vegetables; botanical extraction for maximum oil yield; processing of starch and fermentating tea and coffee [6, 11, 29].

Although there are many reports on isolation and characterization of cellulases but on an average less than 1% of the potential microbes have been identified [13, 29]. So the need to isolate and identify organisms, which are either hyper-producers and/or sufficiently robust to withstand

conditions of the intended application and/or are producers of novel enzymes is highly significant. In terms of enzyme novelty from an applications perspective, interest is focused on not only finding enzymes, which could break down lignocellulose much more rapidly but also enzymes, which could withstand pH, temperature and inhibitory agents more resiliently depending on the intended application [16].

Enzymes may require metal ions for their maximal catalytic activity and the enzymes requiring cofactors in the form of tightly bound metals for their activity are termed as holoenzymes. Therefore, chelation of metals results into complete loss of their activity, however, addition of metals reactivate them. The ions most commonly found in metalloenzymes are the transition metals such as iron, zinc, copper, manganese and cobalt, etc. [43]. Metal ions may be essential for the enzyme-catalyzed reactions to proceed at a measurable rate (essential activators). Alternatively, activators may act to promote a reaction, which is capable of proceeding at an appreciable rate in its absence (non-essential activators). The tightness of binding depends on conditions of pH and temperature used, and in addition activator may combine with other components of the system [7].

Gymnoascella citrina (synonym *Arachniotus citrinus*) is a filamentous mesophilic ascomycetous fungus [1] isolated from sheep dung and dog dung in Pakistan. It is a novel strain as there are few reports on enzymes from fungi belonging to the genus *Gymnoascella*. Previously, we successfully immobilized the glucoamylase and endoglucanase of *G. citrina* using gel entrapment technique [28, 35]. Solid state fermentation is gaining great interest from researchers as it offers several economical and practical advantages, e.g., higher product concentration, improved product recovery, very simple cultivation equipment, reduced waste water output, lower capital investment and plant operation costs [24, 36, 37]. Hence, we are reporting for the first time purification, kinetic and thermodynamic characterization of a novel apparently metal requiring CMCase (endoglucanase) produced under solid-state growth condition by *G. citrina*.

Materials and methods

All chemicals used were of analytical grade and mostly purchased from Sigma Chemical Company, Missouri, USA.

Microbial strain

Gymnoascella citrina was obtained from Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan. The culture was maintained on Malt extract peptone agar slants, which were prepared according to the method 90 of DSM-catalogue of strains [9].

Inoculum preparation

Vogel's medium (100 mL) was added in 500 mL conical flask having about 20 chromic acid washed marble gravels to break the fungal mycelia and autoclaved for 20 min at 121 °C (1.1 kg cm^{-2}). Glucose stock (50% w/v) was autoclaved at 121 °C (1.1 kg cm^{-2}) for 5 min and was aseptically transferred to each flask to get a final concentration of 2% (v/v). Platinum wire loop full of spores of *G. citrina* was transferred aseptically to each flask and the flasks were kept on orbital shaker at 110 rpm at 30 °C for 1 day [31].

Alkali treatment of corn cobs

Corn cobs obtained from CPC Rafhan, Faisalabad, Pakistan, were grinded to 40 mesh size and soaked for 24 h in aqueous solution of NaOH (2% w/v) at a ratio of 1:5 (w/v) and filtered through muslin cloth. The treated corn cobs were washed thoroughly with tap water and rinsed with distilled water till neutrality (pH: 7) and dried in oven at 70 °C for 48 h.

Production of CMCases

Gymnoascella citrina was grown under solid-state growth conditions as reported [26]. Briefly 50 conical flasks of 500 mL capacity, containing 30 g of wheat bran (40 mesh size) and alkali treated corn cob at a ratio 1:1 (w/w) were used for the production of CMCases. The substrates were soaked with 60 mL of distilled water and the flasks were plugged with cotton, covered with aluminum foil and autoclaved at 121 °C (1.1 kg cm^{-2}) for 20 min. Afterwards, 10 mL of inoculum was sprinkled aseptically on the surface of carbon source and incubated at 30 °C. Thick fungal mat covered whole carbon source on fourth day.

Isolation of crude enzyme

The crude CMCases were harvested after 1 month by adding 30 mL of distilled H_2O and shaken vigorously on orbital shaker at 100 rpm for 1 h. The crude enzyme was filtered through muslin cloth, centrifuged at 15,600×*g* at 4 °C for 30 min and further centrifuged at 39,200×*g* at 4 °C for 10 min to increase the clarity and then

concentrated by freeze drying. The concentrate was dialyzed using 15 kDa cut-off cellulosic dialysis tubing. Total proteins and CMCase activity was determined before and after dialysis.

Protein estimation

Total proteins were estimated by Bradford assay [2] and bovine serum albumin (BSA) was used as standard.

CMCase assay

Appropriate amount of CMCase (100 µl) was incubated for 15 min at 40 °C in the presence of 1 mL Carboxymethyl cellulose sodium salt solution (CMC) [1.5% CMC (w/v) in 50 mM sodium acetate buffer: pH 5]. The reducing sugars were determined by adding 3 mL of DNS reagent and the reaction mixture was boiled in a water bath for 10 min, cooled on ice and absorbance was measured at 550 nm. Glucose (50–400 µg) was used to prepare standard curve [41].

CMCase units were calculated as follows:

$$\text{U ml}^{-1}\text{min}^{-1} = \frac{\Delta A_{550} \text{ of sample} \times \text{Glucose standard factor } (500) \times \text{Total vol. of reaction mixture } (2.1\text{ ml})}{\text{Enzyme vol } (0.1\text{ ml}) \times \text{Incubation time } (15\text{ min}) \times \text{Reaction mix vol for color development } (2.1\text{ ml})}$$

Glass cuvettes with different volume capacity and brands showed variations in results. Therefore, we recommend that whole study may be carried out with the cuvette of same specification.

One unit of CMCase activity was defined as "µmol of glucose equivalent liberated min^{-1} under defined conditions".

Purification of CMCase

The harvested crude enzyme was subjected to five-step purification procedure comprising of fractional precipitation by ammonium sulphate, Hiload anion exchange, Hydrophobic interaction, Mono-Q anion-exchange and Gel filtration chromatography on fast protein liquid chromatography (FPLC) system [26, 30, 41].

Ammonium sulfate precipitation

Solid ammonium sulfate was added to 1 mL of crude, dialyzed, concentrated CMCase in eppendorf tubes to get 10–90% saturation at 0 °C and vertimixed. The tubes were left at 4 °C for about 5 h and centrifuged at 12,000 rpm for 15 min. The supernatant was assayed for residual endoglucanase (CMCase) activity. After optimization, the crude enzyme concentrate was placed on ice and solid ammonium sulfate was dissolved bit by bit to attain initially 35% saturation at 0 °C and left overnight at 4 °C. Later on, it was centrifuged at 18,000 rpm (39,200×g) for 30 min at 4 °C and the pellet was discarded, and more solid ammonium sulfate was added in supernatant to attain 65% saturation at 0 °C. Again kept for a night at 4 °C, centrifuged as mentioned before and supernatant was discarded. The pellet was dissolved in water and dialyzed extensively against distilled water for 24 h, with four changes of equal intervals. Total proteins and CMCase activity was determined before and after dialysis. The volume of enzyme solution was reduced by freeze drying [26].

Hiload anion-exchange chromatography

After ammonium sulfate precipitation the CMCases were loaded on FPLC Hiload-Q Sepharose column, using superloop of 50 mL at a rate of 2 mL min^{-1}. The linear gradient of NaCl (0–1 M) in 20 mM Tris/HCl pH 7.5 was used as elution buffer. The fractions (4 mL fraction^{-1}) containing endoglucanase activity were pooled and dialyzed against distilled water. The pools were assayed for enzyme activity and total proteins.

Hydrophobic-interaction chromatography (HIC)

Active fractions of CMCase-I from Hiload column were mixed with ammonium sulfate to get final concentration of 2 M and then filled in superloop by peristaltic pump and loaded on Phenyl Superose column at a rate of 0.5 mL min^{-1}. The elution was carried out with a linear gradient of ammonium sulfate (2–0 M) in 50 mM phosphate buffer pH 7. Active fractions (2.5 mL fraction^{-1}) were pooled, dialyzed and assayed for enzyme activity and total proteins.

Mono-Q anion-exchange chromatography

The dialyzed, purified CMCase from HIC was loaded on Mono-Q column at a flow rate of 1 mL min^{-1} and a linear gradient of NaCl (0–1 M) in 20 mM Tris/HCl pH 7.5 was used as elution buffer. Active fractions (2 mL fraction^{-1})

were pooled, dialyzed and assayed for total enzyme activity and proteins.

Gel filtration chromatography

The purified CMCase-I after Mono-Q was loaded on Superose column to get purification to homogeneity level and to determine the native molecular weight. The sample (200 µl run^{-1}) was loaded using "loop TMS program" of FPLC and 100 mM Tris/HCl, pH 7 having 0.15 M NaCl was used as elution buffer at a flow rate of 0.5 mL min^{-1}. A measure of 1 mL size fractions were collected.

Native molecular mass

The purified CMCase and different marker proteins were applied separately on FPLC gel filtration chromatography and native molecular mass was determined as described [36].

Sub-unit molecular mass

Sub-unit molecular mass of CMCase was determined by using sodium dodecyl-sulphate polyacrylamide gel electrophoresis (10% SDS-PAGE) [20], which was performed using BRL apparatus and gels were stained by Coomassie blue R-250. Standard curve, which was drawn between R_f values of standard protein bands versus their log molecular weight was used to determine the exact molecular mass.

Temperature and pH optimum

CMCase-I was assayed at different temperatures ranging from 10 to 80 °C and energy of activation (E_a) was determined by applying Arrhenius plot. The effect of temperature on the rate of reaction was expressed in terms of temperature quotient (Q_{10}), which is the factor by which the rate increases due to a raise in the temperature by 10 °C. Q_{10} was calculated by rearranging the equation given by Dixon and Webb [7].

$$Q_{10} = \text{antilog}_{\varepsilon}\,(E \times 10/RT^2) \quad (1)$$

where $E = E_a$ = activation energy.

Activity of the enzyme was also determined at 40 °C against different pH, ranging from 2 to 9.6 and Dixon plot was applied to determine the pKa of ionizable groups of active site residues [41].

Effect of metals

The holoenzyme nature of CMCases of *G. citrina* was determined by chelating the bound metals. Apo-CMCase was made by dialyzing the enzyme against 5 mM EDTA dissolved in 50 mM "MOPS"/KOH pH 7.0 for 20 h with three changes. The EDTA was removed by dialyzing apoenzyme intensively against 30 L of distilled deionised water in 24 h (four changes) [43]. Activity of apo-CMCase was determined in the presence of different metals: Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} and K^{1+}.

Effect of substrate

Michaelis–Menten kinetic constants (V_{max}, K_m) were determined by assaying the enzyme at different CMC concentrations ranging from 0.5 to 2.5% (w/v) and Lineweaver–Burk plot was applied [41]. Similarly, effect of 1.5 mM Mn^{2+} on kinetic constants for CMC hydrolysis by CMCase-I was also determined.

Thermodynamics of CMC hydrolysis

The thermodynamic parameters (ΔG^*, ΔH^* and ΔS^*) for CMC hydrolysis were calculated using the Michaelis constants determined under effect of substrate by rearranging the Eyring's absolute rate equation derived from the transition state theory [41].

$$K_{cat} = (K_b T/h)\, e^{(-\Delta H^* P/RT)}\, e^{(\Delta S^*/R)} \quad (2)$$

where

h Planck's constant = 6.63×10^{-34} J s
K_b Boltzman's constant (R/N) = 1.38×10^{-23} J K^{-1}
R gas constant = 8.314 J K^{-1} mol^{-1}
N Avogadro's No = 6.02×10^{23}
T absolute temperature.

$$E_a = -\text{Slope} \times R \quad (3)$$

$$\Delta H^* = E_a - RT \quad (4)$$

where ΔH^* is the enthalpy of activation of CMC hydrolysis

$$\Delta G^* = -RT \ln(K_{cat} h/K_b T) \quad (5)$$

where ΔG^* is the free energy of activation of CMC hydrolysis.

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \quad (6)$$

where ΔS^* is the entropy of activation of CMC hydrolysis.

$$\Delta G^{*}_{E-S} = -RT \ln K_a \text{(free energy of CMC binding)} \quad (7)$$

where $K_a = 1/K_m$

$$\Delta G^{*}_{E-T} = -RT \ln K_{cat}/K_m \text{ (free energy of transition state formation)} \quad (8)$$

Results and discussion

Solid-state fermentation offers several economical and practical advantages such as: higher product concentration, improved product recovery, etc. Therefore, *G. citrina*, a mesophillic fungus was grown under solid-state growth conditions. The production of CMCases after 30 days at 30 °C was 4 units mg^{-1} protein.

Purification of CMCases

Crude enzyme was purified to homogeneity level after subjecting to ammonium sulfate precipitation, Hiload anion exchange, hydrophobic interaction, Mono-Q anion exchange and gel filtration chromatography on Pharmacia FPLC unit. The five-step purification procedure resulted into 27.3-fold purification and final recovery of CMCase-I was 25.5% (Table 1). The purity of CMCase-I was apparently to homogeneity level, which was confirmed on 10% SDS-PAGE. Three step purification procedure for CMCase of *Cellulomonas biazotea* and four step procedure for that of *A. niger* resulted into an increase in specific activity of 9- and 12-fold, respectively [40].

Ammonium sulfate precipitation

The onset of CMCases precipitation occurred at 35% saturation of ammonium sulfate at 0 °C, while complete precipitation was at 65% saturation. The cellulases were 1.73-fold purified after precipitation. The CMCases from *A. niger* precipitated between 45 and 65% ammonium sulfate saturation at 0 °C [41].

Hiload anion exchange chromatography

Partially purified CMCases, after ammonium sulfate precipitation were further purified on FPLC Hiload anion exchange chromatography and two isoforms (CMCase-I and -II) were recovered. Majority of CMCases belonged to CMCase-I, which eluted just at the onset of sodium chloride gradient, while CMCase-II present in minute quantity eluted at about 500 mM NaCl. The elution pattern indicated that CMCase-I was slightly acidic in nature (Fig. 1). Endoglucanases from *A. niger* elute at 550 mM NaCl on Hiload column [41]. The production of CMCase-II was very low with respect to total CMCases, so CMCase-I was further purified for characterization.

Hydrophobic interaction chromatography

Partially purified CMCase-I from Hiload column was further purified by applying on phenyl superose column and was eluted at 286 mM ammonium sulphate (Fig. 2). The elution of CMCase-I from HIC column showed that it was tightly adsorbed on the column and hence had highly hydrophobic surface. On the other hand, the CMCases from *A. niger* elute at about 95 mM $(NH_4)_2SO_4$ on HIC [41]. Ammonium sulfate inhibited CMCase activity, which was recovered by dialysis against distilled water.

Mono-Q anion exchange chromatography

Purified CMCase-I from HIC was further purified on FPLC Mono-Q anion exchange column and was recovered before the start of NaCl gradient (Fig. 3). The *A. niger*

Table 1 Purification of CMCases from *Gymnoascella citrina*

Treatment	Total units	Total protein (mg)	Specific activity (U mg^{-1})	Purification factor	% Recovery
Crude	3,418	810.0	4.2	1.0	100
$(NH_4)_2$ SO_4 precipitation	2,941	403.0	7.3	1.7	86
FPLC Hiload® chromatography	1,343	43.4	30.9	7.3	39
Hydrophobic interaction chromatography	1,211	22.8	53.2	12.6	35
FPLC Mono-Q® chromatography	1,071	14.2	75.4	17.9	31
Gel filtration chromatography	874	7.6	115	27.3	25.5

Where, all quoted values were taken after dialysis against distilled water

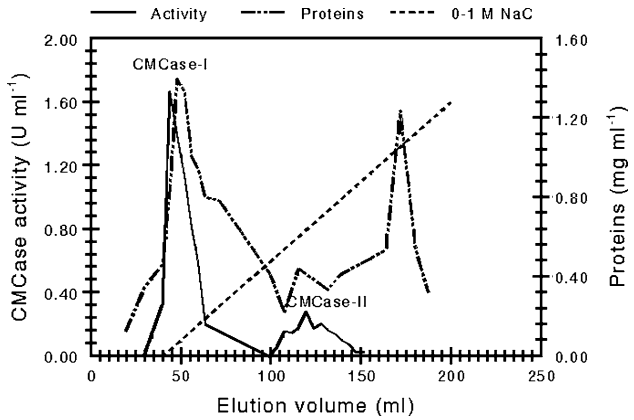

Fig. 1 FPLC Hiload anion exchange chromatography of CMCases on Q-Sepharose column using 0–1 M NaCl gradient for elution

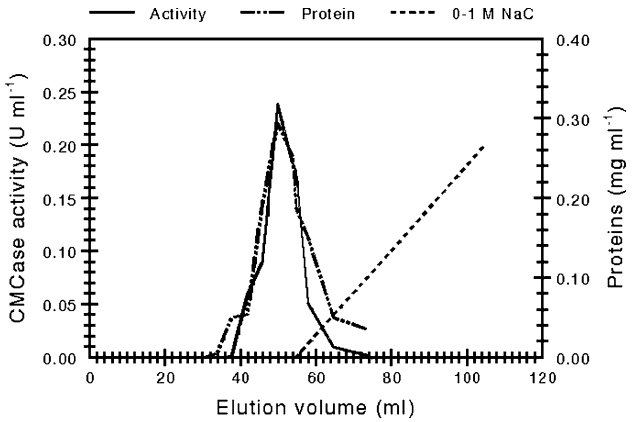

Fig. 3 FPLC Mono-Q anion exchange chromatography of CMCase-I using 0–1 M NaCl gradient

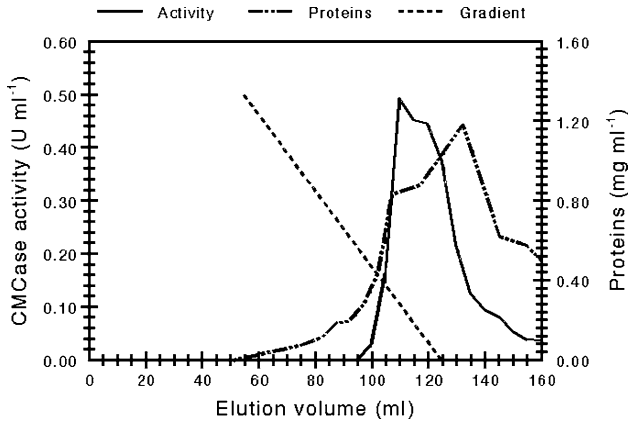

Fig. 2 FPLC Hydrophobic interaction chromatography of CMCase-I on FPLC Phenyl Superose column using 2–0 M $(NH_4)_2$ SO_4 gradient

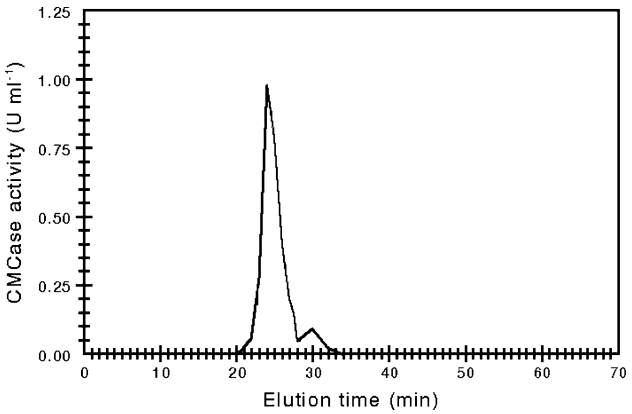

Fig. 4 FPLC Gel filtration chromatography of CMCase-I on Superose column

endoglucanases were eluted at about 640 mM NaCl on Mono-Q column [41].

Gel filtration chromatography

The purified CMCase-I was finally applied on gel filtration column (Fig. 4) and purity was at homogeneity level on SDS-PAGE (Fig. 5). The CMCase-I was about 27-fold purified (Table 1).

Molecular mass

The CMCase-I was monomeric in nature because its native (43 kDa) and sub-unit (42 kDa) molecular masses were almost the same. Molecular weights of CMCases produced by variety of microbes have been reported, e.g. native and SDS-PAGE molecular weight of *Bacillus* sp. was 33 kDa [38], *Neurospora crassa* have CMCases with 70 kDa molecular mass [46] and the endoglucanases obtained from *Trichoderma viride* had multiple forms with molecular weights 38, 42, 52 and 60 kDa [18].

Effect of temperature

Temperature optimum of CMCase-I was 55 °C. Arrhenius plot for energy of activation (E_a) showed a biphasic trend (Fig. 6) and ES-complex formation at optimum temperature (55 °C) required E_a of 36.2 kJ mol^{-1}. The activation energies for CMCases from *A. niger* showed a triphasic trend and E_a up to temperature optima were 53 and 18 kJ mol^{-1} [41] while CMCase from *C. biazotea* have E_a 35 kJ mol^{-1} [40]. It was found that removal of non-covalently bound polysaccharides from CMCases of *A. niger* have changed the activation energy profile and E_a of polysaccharides free and complexed were 17 and 55, and 19 and 21 kJ mol^{-1}, respectively [39]. Endoglucanase isolated from recombinant strain of *E. coli* has 38.9 kJ mol^{-1} E_a between temperatures 23 and 53 °C [23]. We have also calculated the temperature quotient of the enzyme, which was 1.01.

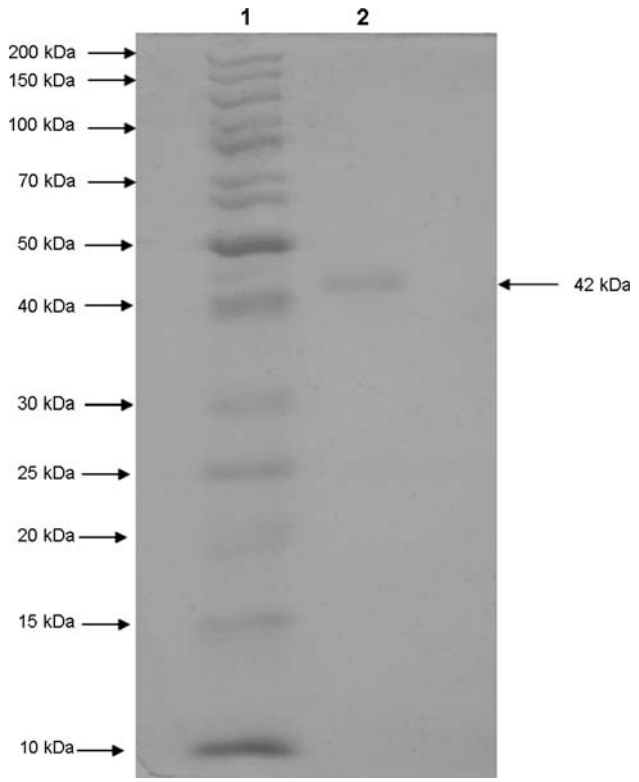

Fig. 5 10% SDS-PAGE of CMCase-I from *Gymnoascella citrina*: Fermentas protein marker #SM0661 10–200 kDa (*lane-1*) and CMCase-I (*lane-2*)

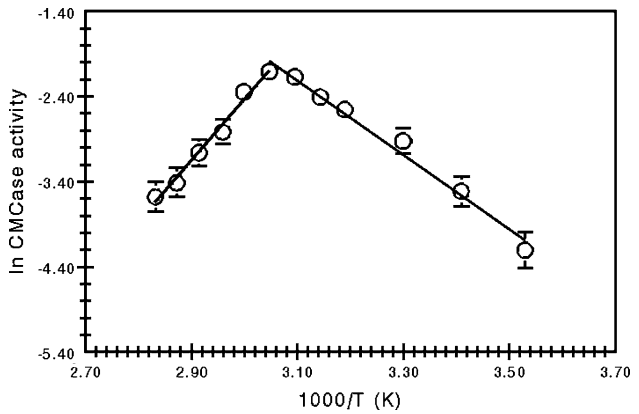

Fig. 6 Arrhenius plot for effect of temperature on activity and determination of activation energy for CMcellulose hydrolysis by CMCase-I of *Gymnoascella citrina.* Data presented are average values ± SD of $n = 3$ experiments

Effect of pH

CMCase-I had pH optima in the range of 3.5–6.5 and Dixon plot was applied to determine p*K*a of ionizable groups of the active site residues. It was found that CMCase-I involved two polar ionizable residues in catalysis (Fig. 7). The pKa_1 for proton donating ionizable group was 2.8, which showed that carboxyl groups may be acting as proton donor

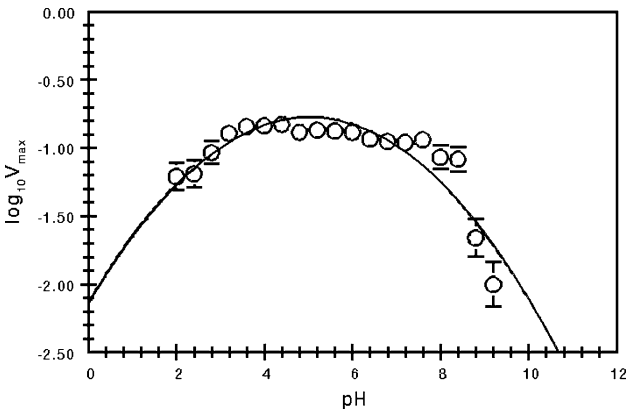

Fig. 7 Dixon plot for the effect of pH on activity and determination of p*K*a of ionize able groups of active site residues of CMCase-I from *Gymnoascella citrina*. Data presented are average values ± SD of $n = 3$ experiments

whereas, pKa_2 for proton receiving residue was 7.4, which on comparison with p*K*a values for amino acids present in proteins indicated that proton receiving group may be imidazole [14]. Hakamada et al. [12] deduced active site residues of a thermostable alkaline endoglucanases from an alkaliphilic *Bacillus* sp. by site directed mutagenesis and declared that histidine, glutamic acid, arginine and tyrosine are playing important role in catalysis. The active site residues for *A. niger* CMCases have been determined and found that both proton donating and receiving residues contain carboxyls as ionizable group with p*K*a values of 3.5 and 5.5, respectively [41]. Endoglucanases from *Schizophyllum commune* involve the amino acid residues with p*K*a values of 3.7 and 6.1 for CMC catalysis [29].

Effect of metals

The removal of metals from CMCase-I resulted into almost complete loss of enzymatic activity and apoenzyme of CMCase-I displayed only about 3.4% residual activity. The activity of apo-CMCase-I was completely recovered in the presence of 1 mM Mn^{2+}. We consider that the apo-CMCase-I was not absolutely free from all metals due to which it gave minute activity. Due to limited lab facilities we could not evaluate the quality of apoenzyme. Therefore, based on % residual activity, we believe that CMCase-I was apparently metallo in nature. The enzymes requiring cofactors in the form of tightly bound metals for their activity are termed as holoenzymes and chelating metals results into complete loss of their activity, however, addition of metals reactivate them. Hence, holoenzymes contain firmly bound metal ions at their active sites. Therefore, we conclude that CMCase-I is apparently holoenzyme and this property made it novel in nature.

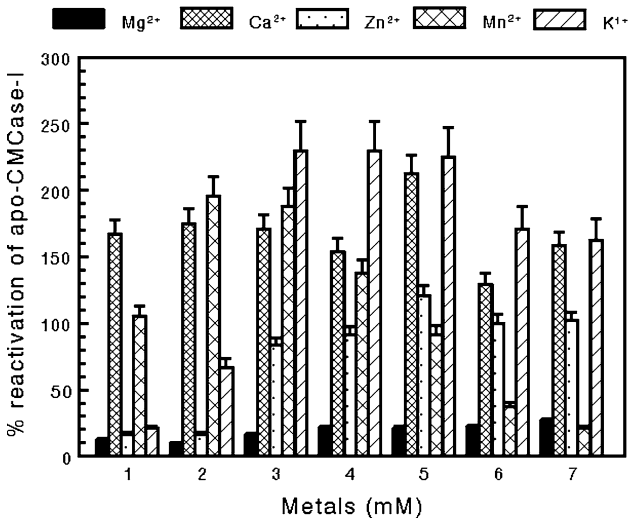

Fig. 8 Effect of metals on the reactivation of apo-CMCase-I from *Gymnoascella citrina*. *Error bars* represent the standard deviation

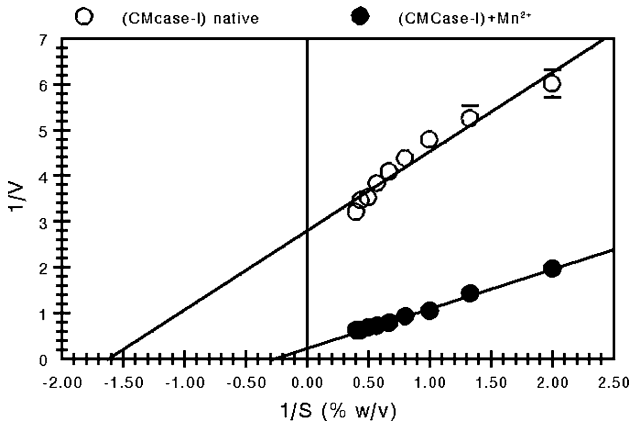

Fig. 9 Lineweaver–Burk plot for the determination of Michaelis kinetic constants (V_{max}, K_m) for CMcellulose hydrolysis at 50 °C, pH 5 by CMCase-I of *Gymnoascella citrina*. Turn over (K_{cat}) = s^{-1} = $V_{max}/[e]$, where [*e*] is the enzyme concentration = 2.143×10^{-4} µmol, K_m = mg CMC mL^{-1} and K_{cat}/K_m = s^{-1} mg^{-1}CMC mL^{-1}. Data presented are average values ± SD of $n = 3$ experiments

The effect of different metal ions like Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, $Co^{2+}$and K^{1+} on the reactivation of apo-CMCase-I was also determined. No inhibition up to 7 mM of Ca^{2+}, Zn^{2+} and K^{1+} was observed while Mn^{2+} showed onset of inhibition at 5 mM. On the other hand Mg^{2+} could not recover the initial activity up to 7 mM while Co^{2+} completely inhibited the activity (Fig. 8). Endoglucanases from *Cellulomonas uda* activated by 1 mM Mn^{2+} [37], while, that of *C. biazotea* and *A. niger* showed activation at 1.5 mM Mn^{2+} [40]. Furthermore, 1.25 mM Hg^{2+} completely inhibited and Pb^{2+} partially inhibited the endoglucanases of *Arthrobotrys oligospora* [19]. Endoglucanase from *Chalara paradoxa* were inhibited by Hg^{2+}, Ag^{1+} while, partial inhibited by 10 mM Zn^{2+}, Fe^{2+}, Mg^{2+} but stimulated by Mn^{2+} [22].

Effect of substrate

CMCase-I was assayed in the presence of different CMC concentrations at 50 °C, pH 5.0. The V_{max} was 39 U min^{-1} mg^{-1} protein and K_{cat} was 27.5 catalytic events s^{-1}, while K_m was 6.25 mg CMC mL^{-1}. The Specificity constant (V_{max}/K_m) was 6.24, while the enzyme concentration used was 2.143×10^{-4} µmol or 9 µg (Fig. 9).

The native CMCase-I was twelve fold activated by 1.5 mM Mn^{2+}. Kinetic rate constants in the presence of Mn^{2+} were: V_{max} = 454 U min^{-1} mg^{-1} protein, K_{cat} = 318 s^{-1}, K_m = 40 mg CMC mL^{-1} and V_{max}/K_m = 11.35. The Michaelis constant (K_m) value in the presence of Mn^{2+} become very high which confirmed that the affinity of CMC in the presense of Mn^{2+} was seriously affected and the enzyme required high amount of CMC to saturate it. The K_{cat} values confirmed that the Mn^{2+} binding has activated the conversion of transition complex into products.

Theberge et al. [42] found that endoglucanase from *Streptomyces lividans* IAF 74 have V_{max} of 24.9 U mg^{-1} and K_m of 4.2 mg mL^{-1}. CMCases of *Thermomonospora curcata*, *T. reesei* and *Alternaria alternata* hydrolyze CMC substrate with V_{max} of 833 µmol glucose min^{-1}, 405.5 µmol glucose h^{-1} and 18 µmol glucose min^{-1} mg^{-1} protein, respectively whereas, their K_m for CMC were 7.33 mg mL^{-1}, 1.32% (w/v) and 0.43 mg mL^{-1}, respectively [10, 21, 45]. The endoglucanase from *E. coli* harboring the genes of endoglucanase of *Fibrobacter succinogenes* S85 displayed V_{max} of 152 IU mg^{-1} and K_m of 0.49% w/v [4]. CMCases isolated by Wittmann et al. [44], Rashid and Siddiqui [32], Siddiqui et al. [41] and Lucas et al. [22] from *S. lividans* IAF9, *A. niger* and *C. paradoxa* had V_{max} of 110 IU mg^{-1}, K_{cat} of 1,000 min^{-1} and V_{max} of 1.1 µmol min^{-1}, respectively whereas, their K_m was 1.3, 70 and 8.3 mg mL^{-1}, respectively.

Thermodynamics of CMC hydrolysis

Thermodynamic parameters for CMC hydrolysis by CMCase-I of *G. citrina* were determined as described [41]. The enthalpy (ΔH^*) of activation of CMC hydrolysis was 33.5 kJ mol^{-1}. Gibbs free energy of substrate binding ($\Delta G^*_{E\text{-}S}$) was 4.92 kJ mol^{-1}, while change in free energy ($\Delta G^*_{E\text{-}T}$) for the formation of activated complex (ES*) was −3.98 kJ mol^{-1}. The conversion of transition state to products exhibited ΔG^* of 70.42 kJ mol^{-1} and the entropy of activation of CMC hydrolysis (ΔS^*) was −114.37 J mol^{-1} K^{-1}. Previously, we report about thermodynamics

of CMcellulose hydrolysis by native CMCase of *A. niger*, which hydrolyzed CMC with the following thermodynamic parameters: ΔG^* (69 kJ mol^{-1}), $\Delta G^*_{E\text{-}T}$ (−13 kJ mol^{-1}), $\Delta G^*_{E\text{-}S}$ (5.1 kJ mol^{-1}), ΔH^* (50 kJ mol^{-1}) and ΔS^* (−61 J mol^{-1} K^{-1}) [41]. The lower enthalpy value of CMCase from *G. citrina* as compared to CMCase of *A. niger* showed that the formation of transition state or activated complex between enzyme-substrate was very efficient.

Conclusion

In the light of our results, we concluded that endoglucanase of *G. citrina* was apparently metallo in nature. The capability of CMCase-I to resist against high concentration of metals (7 mM) signifies their importance for possible applications in industries like textile, detergents, and paper and pulp, etc.

Acknowledgments The project was funded by Pakistan Atomic Energy Commission (PAEC), Islamabad. Technical assistance of G. A. Waseer is appreciated. We are thankful to Dr. M. Sajjad Mirza (Plant Micro-biotechnlogy Division, NIBGE) for proof reading of the manuscript. Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan, is highly acknowledged for providing the culture of *Gymnoascella citrina*.

References

1. Bisby FA, Ruggiero MA, Wilson KL, Cachuela-Palacio M, Kimani SW, Roskov YR, Soulier-Perkins A, Hertum JV eds (2005). Species 2000 & ITIS Catalogue of Life: 2005 Annual Checklist, UK
2. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
3. Busto MD, Ortega N, Perez-Mateos M (1996) Kinetics and stability of cellulases of *T. reesei*. Biosource Technol 57:187–192
4. Cho KK, Kim SC, Woo JH, Bok JD, Choi YJ (2000) Cloning and expression of endoglucanase gene from *Fibrobacter succinogenes* in *Escherichia Coli*. Enzyme Microb Technol 27:475–481
5. Clark AJ, Yaguchi M (1985) The role of Carboxyl groups in the function of endo-ß-1,4-glucanase from *Schizophyllum commune*. Eur J Biochem 49:233–238
6. Coughlan MP, Wood TM, Montenecourt BS, Mendels M (1985) Cellulase: production, properties and applications. Biochem Soc Trans 13:405–416
7. Dixon M, Webb EC (1979) Enzyme kinetics and enzyme inhibition and activation. In: Enzymes, vol 3. Academic Press, New York, pp 47–467
8. Doran JB, Aldrich HC, Ingram LO (1994) Saccharification and fermentation of sugarcane bagasse by *Klebsiela*. Biotechnol Bioeng 44:240–247
9. DSM-Deushe Sammlung Von Mikroorganism und Zullkurturen GmbH (1989) (German collection of microorganisms and cell cultures.) Catalogue of strains, 4th edn. Braunschweig, Federal Republic of Germany, p 286
10. Eshel D, Ben AR, Dinoor A, Prusky D (2000) Endoglucanase of *Alternaria alternata*. Phytology 90:1256–1262
11. Godfrey T, West S (1996) Industrial enzymology. Macmillan, New York
12. Hakamada Y, Hatada Y, Koike K, Yoshimatsu T, Kawai S, Kobayashi T, Ito S (2000) Deduced amino acid sequence and possible catalytic residues of a thermostable, alkaline cellulase from an alkaliphilic *Bacillus* strain. Biosci Biotech Biochem 64:2281–2289
13. Handelsman J, Rondon MR, Brady SF, Clardy J, Goodman RM (1998) Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chem Biol 5:245–249
14. Horton HR, Moran LA, Och RS, Rawn JD, Scrimgeour KG (1996) Principles of biochemistry. Simmon and Schuster/A Viacom Company, NJ, pp 54–66
15. Hoshino E, Ito S (1997) Application of alkaline cellulases. In: Enzyme in detergency. Marcel Dekker, New York, pp 149–175
16. Howard RL, Abotsi E, Jansen van Rensburg EL, Howard S (2003) Lignocellulose biotechnology: issues of bioconversion and enzyme production. Afr J Biotechnol 2:602–619
17. Kibblewhite RP, Wong KKY (1999) Modificaiton of a commercial radiata pine kraft pulp using carbohydrate degrading enzyme. Appita J 52:300–304
18. Kim DW, Jeong YK, Jang YH, Lee JK (1994) Purification and characterization of endoglucanase and exoglucanase from *T. viride*. J Ferment Bioeng 7:363–369
19. Kumble KD, Trivedi PN, Jaffar MB (1990) Purification and characterisation of two endoglucanases from *Arthrobotrys oligospora*. Indian J Biochem Biophys 27:146–150
20. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227:680–685
21. Lin SB, Stutzenberger FJ (1995) Purification and characterization of the major β–1,4-endoglucoanase from *Thermomonospora curcata*. J Appl Bacteriol 79:447–453
22. Lucas R, Robles A, Garcia MT, Alvarez De Cienfuegos G, Galvez A (2001) Production, purification and properties of an endoglucanase produced by the hyphomycete *Chalara* (Syn. *Thielaviopsis*) *paradoxa* CH32. J Agric Food Chem 49:79–85
23. Melnik MS, Gerner ML, Rabinovich ML (1999) A low molecular weight endoglucanase from *Clostridium thermocellum* similar to endoglucanase-C. The specificity effects on synthetic substrates and amino acide composition. Appl Biochem Microbiol 35:548–555
24. Muniswaran PKA, Charyulu NCLS (1994) Solid substrate fermentation of coconut coir pith for cellulase production. Enzyme Microb Technol 27:33–36
25. Nakamura K, Kitamura K (1983) Purification and some properties of cellulase active on crystalline cellulose from *Cellulomonas uda*. J Ferment Technol 61:379–382
26. Niaz M, Ghafoor MY, Jabbar A, Rasul E, Wahid A, Ahmed R, Rashid MH (2004) Isolation and purification of glucoamylases from *Gymnoascella citrina* under solid phase growth conditions. Int J Biol Biotechnol 1:15–23
27. Pace NR (1997) A molecular view of microbial diversity and the biosphere. Science 276:734–740
28. Perveen R, Rashid MH, Saleem M, Khalid AM, Rajoka MI (2006) Kinetic and thermodynamic properties of an immobilized glucoamylase from a mesophilic fungus, *Arachniotus citrinus*. Protein Peptide Lett 13(7):665–671
29. Petre M, Zarnea G, Adrian P, Gheorghiu E (1999) Biodegradation and bioconversion of cellulose waste using bacterial and fungal cells immobilized in radiopolymerized hydrogels. Resour Conserv Recycl 27:309–332
30. Rashid MH, Siddiqui KS (1997) Purification and characterization of β-glucosidase from *Aspergillus niger* NIAB 280. Folia Microbiol 42:544–550

31. Rashid MH, Rajoka MI, Siddiqui KS, Shakoori AR (1997) Effect of different substrates on the simultaneous production of β-glucosidase and polysaccharides by *Aspergillus niger* NIAB-280. Pak J Zool 29:175–179
32. Rashid MH, Siddiqui KS (1998) Carboxyl group modification: high temperature activation of charge neutralized and charge reversed β-glucosidase from *Aspergillus niger*. Biotechnol Appl Biochem 27:231–237
33. Rashid MH, Shakoori AR, Rajoka MI, Siddiqui KS (1998) Monitoring of carboxyl group modification of β-glucosidase from *Aspergillus niger* NIAB-280 by native enzyme mobility shift assay (NEMSA). Pak J Zool 30:99–104
34. Rehm HJ, Reed G (1999) Enzymes for industrial application. In: Biotechnology. Wiley-VCH, Germany, pp 191–215
35. Saleem M, Rashid MH, Jabbar A, Perveen R, Khalid AM, Rajoka MI (2005) Kinetic and thermodynamic properties of an immobilized endoglucanase from *Arachniotus citrinus*. Process Biochem 40(2):849–855
36. Shankaranand VS, Ramesh MV, Lonsane BK (1992) Idiosyncrasies of solid state fermentation system in the biosynthesis of metabolites by some bacterial and fungal cultures. Process Biochem 27:33–36
37. Shankaranand VS, Lonsane B (1994) Ability of *Aspergilus niger* to tolerate metal ions and minerals in sold state fermentation system for the production of citric acid. Process Biochem 29:29–39
38. Sharma P, Gupta JK, Vadehra DV, Dube DK (1990) Purification and properties of endoglucanase from *Bacillus* isolates. Enzyme Microb Technol 12:132–137
39. Siddiqui KS, Azhar JJ, Rashid MH, Rajoka MI (1996) Activity and thermostability of carboxymethylcellulase from *Aspergillus niger* is strongly influenced by non-covalently attached polysaccharides. World J Microbiol Biotechnol 12:213–216
40. Siddiqui KS, Azhar MJ, Rashid MH, Ghuri TM, Rajoka MI (1997) Purification and effect of Mn^{2+} on the activity of carboxymethylcellulases from *A. niger* and *C. biazotea*. Folia Microbiol 42:303–311
41. Siddiqui KS, Najums Saqib AA, Rashid MH, Rajoka MI (2000) Carboxyl group modification significantly altered the kinetic properties of purified Carboxymethylcellulase from *Aspergillus niger*. Enzyme Microb Technol 27:467–474
42. Theberge M, Lacaze P, Shareck F, Morosoli R, Kluepfel D (1992) Purification and characterization of an endoglucanase from *Streptomyces lividans* 66 and DNA sequence of the gene. Appl Environ Microbiol 58:815–820
43. Wagner FW (1988) Preparation of metal-free enzyme. In: Methods in enzymology, part-A. Academic Press, New York, pp 158
44. Wittmann S, Shareck F, Kluepfel D, Morosoli R (1994) Purificaiton and characterization of Celb endoglucanase from *Streptomyces lividans*-66 and DNA sequence of the encoding gene. Appl Environ Microbiol 60:1701–1703
45. Wood TM (1985) Properties of cellulolytic enzyme systems. Biochem Soc Trans 13:407–410
46. Yazdi MT, Radiford A, Keen JN, Woodward JR (1990) Purification of cellulytic enzymes. Enzyme Microb Technol 12:120–123