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Catalytic and Thermodynamic Characterization of Endoglucanase (CMCase) from *Aspergillus oryzae* cmc-1

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Abstract Monomeric extracellular endoglucanase (25 kDa) of transgenic koji (*Aspergillus oryzae* cmc-1) produced under submerged growth condition (7.5 U mg^{-1} protein) was purified to homogeneity level by ammonium sulfate precipitation and various column chromatography on fast protein liquid chromatography system. Activation energy for carboxymethylcellulose (CMC) hydrolysis was 3.32 kJ mol^{-1} at optimum temperature (55 °C), and its temperature quotient (Q_{10}) was 1.0. The enzyme was stable over a pH range of 4.1–5.3 and gave maximum activity at pH 4.4. V_{max} for CMC hydrolysis was 854 U mg^{-1} protein and K_m was 20 mg CMC ml^{-1}. The turnover (k_{cat}) was 356 s^{-1}. The pK_{a1} and pK_{a2} of ionisable groups of active site controlling V_{max} were 3.9 and 6.25, respectively. Thermodynamic parameters for CMC hydrolysis were as follows: ΔH^*=0.59 kJ mol^{-1}, ΔG^*=64.57 kJ mol^{-1} and ΔS^*= −195.05 J mol^{-1} K^{-1}, respectively. Activation energy for irreversible inactivation '$E_{a(d)}$' of the endoglucanase was 378 kJ mol^{-1}, whereas enthalpy (ΔH^*), Gibbs free energy (ΔG^*) and entropy (ΔS^*) of activation at 44 °C were 375.36 kJ mol^{-1}, 111.36 kJ mol^{-1} and 833.06 J mol^{-1} K^{-1}, respectively.

Keywords CMCase · Activation energy · Enthalpy · Entropy · Gibbs free energy · Thermostability

Introduction

Cellulose is the most abundant and renewable source of energy on earth. The potential importance of cellulose hydrolysis in the context of conversion of plant biomass to fuels and chemicals is widely recognized. It is a linear condensation polymer consisting of D-anhydroglucopyranose joined together by β-1, 4-glycosidic bonds, while anhydrocellobiose

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is the repeating unit of cellulose, since adjacent anhydroglucose molecules are rotated at 180° with respect to their neighbors [1]. It can be converted into soluble sugars by either acid or enzymic hydrolysis; the latter is preferred due to the high yields of desired products with fewer by-products, and in commercial situations, it is considered to be economically favorable. Microorganisms capable of degrading crystalline cellulose extensively produce three main types of enzymes, namely, endoglucanase (1, 4-β-D-glucan glucanohydrolase; EC 3.2.1.4), exoglucanase (1, 4-β-D-glucan cellobiohydrolase; EC 3.2.1.91) and β-glucosidase (cellobiase or β-D-glucoside glucohydrolase; EC 3.2.1.21) [2–4]. Previous studies have demonstrated that endo- and exoglucanases act synergistically and promote the solubilisation of crystalline cellulose into soluble sugars [5], whilst β-glucosidase completes the hydrolysis by converting cellobiose and cello-oligosaccharides into glucose [6].

Cellulases have application in paper and pulp industry [7], 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, color care agents, stone washing [9], biopolishing, biofinishing, and smooth surfacing of cotton fabric [10, 11]. 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 [12–14].

The filamentous fungi of genus *Aspergillus* are important in food industry, medicine and agriculture, as they are qualified producers of useful enzymes. In fermentation food industries, such as sake, miso and soy sauce, *Aspergillus* species have been used from the earliest times [15–17]. Furthermore, *Aspergillus oryzae* and *Aspergillus niger* are the strains that are approved of Generally Regarded As Safe (GRAS) by FDA, with their long history of uses in production of fermented foods [18]. *A. oryzae* nia D300 [a nitrate reductase (EC 1.6.6.1) gene-deficient mutant derived from the wild-type strain RIB40] is also such a strain that is used for fermented foods, but this strain does not produce cellulases in a reasonable amount. Therefore, F1-carboxymethylcellulase (*cmc*1) gene of *Aspergillus aculeatus* was expressed in *A. oryzae* nia D300, which is the most abundant endoglucanase enzyme in the cellulase components of *A. aculeatus* [19]. F1-carboxymethylcellulase consists of a single polypeptide chain of 221 amino acid residues with a molecular mass of 24,002 Da [20]. The aim of current study was purification and characterisation of endoglucanase (CMCase) from the transgenic strain, i.e. *A. oryzae* cmc-1, with intention to evaluate the effect of expression cloning on kinetic and thermodynamic properties of expressed CMCase.

Materials and Methods

All the data values are mean±SE of three replicates. The ±S.E values are shown as error bars in the figures.

Fungal Strain, Enzyme Production, and Purification

Pure culture of *A. oryzae* cmc-1 was obtained from the Industrial Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan. A loop full of spores was transferred aseptically to 50 ml of fungal growth medium (FGM)

and was incubated at 30±1 °C on a rotary shaker at 120 rpm for 36 h. The cell suspension having 10^6–10^7 cells per milliliter was used as inoculum in the growth media for production of endoglucanase. The composition of FGM for inoculum preparation was: salt solution 50 ml/l, trace element solution 1 ml/l, ammonium tartrate 10 mM/l, glucose 20 g/l, pH 6.5, whereas the composition of salt solution was (% *w*/*v*): KCl, 2.6; $MgSO_4 \cdot 7H_2O$, 2.6; KH_2PO_4, 7.6, and the composition of trace element solution was (% *w*/*v*): $Mo_2O_{24} \cdot H_2O$, 0.11; H_3Bo_4, 1.11; $CoCl_2 \cdot H_2O$, 0.16; EDTA, 5; $FeSO_4 \cdot 7H_2O$, 0.5; $MnCl_2 \cdot 4H_2O$, 0.5; $ZnSO_4 \cdot 7H_2O$, 2.2.

After sterilization, the FGM containing wheat bran (2% *w*/*v*) instead of glucose was inoculated with 5% inoculum (*v*/*v*) and incubated at 30±1 °C for 96 h. The crude enzyme was extracted from growth media by filtration through Whatman filter paper. The extract was centrifuged at 10,000 rpm (15,300×*g*) for 15 min at 4 °C to remove the suspended particles. The enzyme in crude extract was purified to homogeneity by a combination of fractional precipitation, Hiload and Mono-Q anion exchange chromatography, hydrophobic interaction chromatography (HIC), and gel filtration chromatography on fast protein liquid chromatography (FPLC) unit as described earlier [21].

Endoglucanase (CMCase) Assay

Endoglucanase activity in 50 mM acetate buffer (pH 5.0) at 40 °C was determined using carboxymethylcellulose sodium salt (CMC) as a substrate [22, 23]. Appropriate amount of the enzyme was reacted with 1% (*w*/*v*) CMC solution in 50 mM Na acetate buffer (pH 5.0) at 40 °C for 30 min, and reducing sugars were estimated colorimetrically with 3,5-dinitrosalicylic acid reagent [24]. The reaction was quenched by placing the tubes in boiling water for 10 min and then immediately cooled on ice.

One unit is defined as "the enzyme amount that releases 1 μmol of glucose equivalent per minute from CMC under defined conditions of pH and temperature".

Protein Assay

Protein was estimated as described by Bradford [25] using bovine serum albumin as a standard.

Native and Subunit Molecular Mass Determination

Native molecular mass of the enzyme was determined on FPLC Gel filtration column as described previously [26]. Purity of the purified enzyme and its subunit molecular mass was determined by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [27]. Protein marker of Fermentas (# SM0661) with molecular mass ranging from 10 to 200 kDa was run as standard. The gel was stained with Coomassie brilliant blue R-250 for protein staining.

Characterization of Endoglucanase

Physiochemical, catalytic, and thermodynamic properties of endoglucanase from *A. oryzae* cmc-1 were determined as mentioned below:

Optimum Temperature, Activation Energy, and Temperature Quotient (Q_{10})

Optimum temperature and activation energy (E_a) of endoglucanase were determined by incubating appropriate amount of the enzyme with 1% CMC at various temperatures ranging

from 25–70 °C in 50 mM Na acetate buffer for 30 min at pH 5.0. E_a was calculated using Arrhenius plot as described earlier [28]. 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 using the equation as given by Dixon and Webb [29].

$$\begin{aligned} Q_{10} &= \text{antilog}_{\varepsilon}\left(E \times 10/RT^2\right) \\ E &= E_a = \text{Activation Energy} \end{aligned} \tag{1}$$

Optimum pH

The optimum pH was determined by measuring activity at 55 °C using various buffers: glutamic acid/HCl (pH 2–2.9), Na acetate/acetic acid (pH 3.2–5.3), MES/KOH (pH 5.6–6.5), MOPS/KOH (pH 6.8–7.4), HEPES/KOH (pH 7.7–8.3), Glycine/NaOH (pH 8.6–9.8), and CAPSO/NaOH (pH 10.1–11.0). The pK_{a1} and pK_{a2} of ionizable groups of essential active site residues involved in the catalysis were determined as described by Dixon and Webb [29].

Catalytic Constants for CMC Hydrolysis

The kinetic constants (V_{max}, K_m, k_{cat}, and k_{cat}/K_m) were determined by incubating fixed amount of endoglucanase with varied concentrations of CMC as a substrate ranging from 0.1 to 2.5% (*w*/*v*) at 55 °C, pH 4.4 as described previously [22].

Thermodynamics of CMC Hydrolysis

The thermodynamic parameters for substrate hydrolysis were calculated using the Eyring's absolute rate equation derived from the transition state theory [30].

$$k_{cat} = (k_b T/h) \times e^{\left(-\Delta H^*/RT\right)} \times e^{\left(\Delta S^*/R\right)} \tag{2}$$

where

k_b	Boltzmann's constant (R/N)=1.38×10^{-23} J K^{-1}
T	Absolute temperature (K)
h	Planck's constant=6.626×10^{-34} Js
N	Avogadro's number=6.02×10^{23} mol^{-1}
R	Gas constant=8.314 J K^{-1} mol^{-1}
ΔH^*	Enthalpy of activation
ΔS^*	Entropy of activation

$$\Delta H^* = E_a - RT \tag{3}$$

$$\Delta G^*(\text{free energy of activation}) = -RT\ln(k_{cat}h/k_b \times T) \tag{4}$$

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \tag{5}$$

The free energy of substrate binding and transition state formation was calculated using the following derivations:

$$\Delta G *_{E-S}\,(\text{free energy of substratrate binding}) = -RT1\text{n}K_a \tag{6}$$

where $K_a = 1/K_m$

$$\Delta G^*_{E-T}(\text{free energy for transition state formation}) = -RT1\text{n}(k_{cat}/K_m). \tag{7}$$

Thermodynamics of Enzyme Stability

Thermal inactivation of enzyme was determined by incubating the enzyme solution in 10 mM Tris–HCl buffer (pH 7) at various temperatures (44 °C, 47 °C, 50 °C, 53 °C, 56 °C) in the absence of substrate. Aliquots were withdrawn at different time intervals, cooled on ice for 30 min, and assayed for endoglucanase activity at 55 °C. The data were fitted to first-order plot, and inactivation rate constants (K_d) were determined as described previously [31, 32]. The energy of activation for irreversible thermal inactivation $E_{a(d)}$ was determined by Arrhenius plot.

Thermodynamics of irreversible inactivation of the endoglucanase was determined by rearranging the Eyring's absolute rate equation derived from the transition state theory [30].

$$K_d = (k_b T/h) \times e^{\left(-\Delta H^*/RT\right)} \times e^{\left(\Delta S^*/R\right)}. \tag{8}$$

ΔH^*, ΔG^*, and ΔS^* of irreversible inactivation were calculated by applying Eqs. 3, 4, and 5 with the modifications that in Eq. 3, $E_{a(d)}$ was used instead of E_a and in Eq. 4, K_d was used in place of k_{cat}.

Effect of Proteases and Urea

The effects of protease and urea on endoglucanase activity were determined by incubating the enzymes at 30 °C in 10 mM Tris/HCl buffer at pH 7 containing α-chymotrypsin (0.2 mg ml^{-1}) and urea (4 M) as described [33] in separate experiments. Different time course aliquots were withdrawn and immediately assayed for the enzyme activity. Half-life ($t_{1/2}$) and doubling time (t_d) were calculated using the relation $0.693/K_d$.

Results

The extracellular endoglucanase from *A. oryzae* cmc-1 grown on wheat bran (7.5 U mg^{-1} protein) under submerged growth conditions was purified to homogeneity level by ammonium sulfate precipitation and different column chromatography on FPLC system. The onset of precipitation occurred at 20%, while complete precipitation was observed at 40% saturation of ammonium sulfate at 0 °C. After ammonium sulfate precipitation, the dialyzed sample was subjected to Hiload anion exchange, HIC, Mono-Q anion exchange, and gel filtration chromatography on Pharmacia FPLC unit (Fig. 1a). The five-step purification protocol for endoglucanase (CMCase) resulted into 12.33-fold purification with recovery of 32.4% (Table 1). Specific activity of the purified enzyme was 92 U mg^{-1} protein. The enzyme was eluted at about 378 mM NaCl on Hiload anion exchange column, while on hydrophobic interaction column, it eluted at the end of ammonium sulfate gradient. On Mono-Q anion exchange column, the enzyme was eluted just at the onset of sodium chloride gradient.

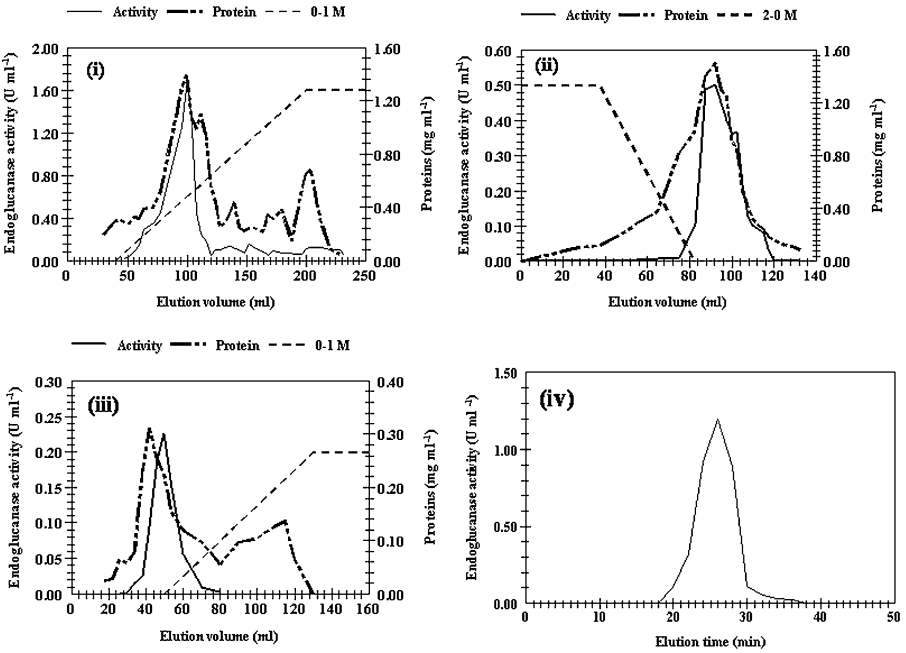


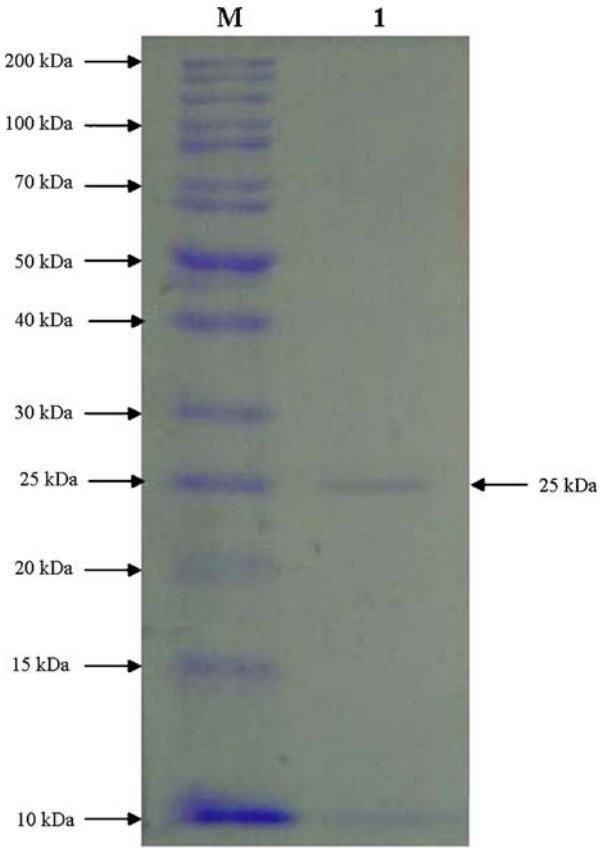
Fig. 1 **a** Fast protein liquid chromatography of endoglucanase: *i* Hiload anion exchange chromatography on Q-Sepharose column using 0–1 M NaCl gradient; *ii* Hydrophobic interaction chromatography on Phenyl Superose column using 2–0 M $(NH_4)_2$ SO_4 gradient; *iii* Mono-Q anion exchange chromatography using 0–1 M NaCl gradient; *iv* Gel filtration chromatography on Superose column. **b** 12.5% SDS-PAGE of purified endoglucanase of *A. oryzae* cmc-1. *Lane M* molecular markers, *Lane 1* purified endoglucanase

The native molecular mass of endoglucanase determined by gel filtration chromatography on Pharmacia FPLC unit and subunit molecular mass from SDS-PAGE was the same, i.e., 25 kDa, which provided an evidence for monomeric nature of endoglucanase (Fig. 1a,b).

Optimum temperature of the purified endoglucanase from *A. oryzae* cmc-1 for CMC hydrolysis was found to be 55 °C. The tri-phasic nature of Arrhenius plot explained that the enzyme exhibited two conformations up to optimum temperature, and beyond transition point at 55 °C, the enzyme activity was declined, indicating inactivation at higher temperatures (Fig. 2). The first transition state was at 40 °C where CMCase required very high activation energy (96.77 kJ mol^{-1}) to catalyze the reaction, while second conformation which occurred at optimum temperature (55 °C) was ideal and required very low activation energy, i.e. 3.32 kJ mol^{-1} for CMC hydrolysis. The temperature quotient (Q_{10}) for the enzyme was 1.0. The enzyme exhibited optimum activity in a pH range of 4.1–5.3, with the maximum activity at pH 4.4 (Fig. 3). Activity of the enzyme was inhibited rapidly below and above optimum pH range. The pK_{a1} and pK_{a2} values of acidic and basic limbs of the active site residues determined by Dixon plot were 3.9 and 6.25, respectively.

The K_m and V_{max} values determined through Lineweaver–Burk plot for the hydrolysis of CMC at 55 °C were 20 mg CMC ml^{-1} and 854 U mg^{-1} protein, respectively (Fig. 4). The k_{cat} was 356 s^{-1} and specificity constant (K_{cat}/K_m) was 17.8. The enthalpy (ΔH^*), Gibbs free energy (ΔG^*), and entropy (ΔS^*) of activation of CMC hydrolysis were found to be 0.59 kJ mol^{-1}, 64.57 kJ mol^{-1}, and −195.05 J mol^{-1} K^{-1}, respectively. The free energy for activation of substrate binding ($\Delta G^*_{E\text{-}S}$) and formation of activated (transition) complex ($\Delta G^*_{E\text{-}T}$) were 8.17 and −7.85 kJ mol^{-1}, respectively.

Fig. 1 (continued)



Thermostability represents the capability of an enzyme molecule to resist against thermal unfolding in the absence of substrate, while thermophilicity is the ability of an enzyme to work at elevated temperatures in the presence of substrate [34]. Monomeric endoglucanase was incubated at temperatures ranging from 44 °C to 56 °C and pseudo first-order plots were

Table 1 Purification of CMCases from *A. oryzae* cmc-1.

Treatment	Total units	Total protein (mg)	Specific activity (U mg^{-1})	Purification factor	Percent recovery
Crude	1,334	178	7.5	1.00	100
$(NH_4)_2$ SO_4 precipitation	1,001	75	13.35	1.78	75.03
	1,152[a]	72[a]	16[a]	2.13[a]	86.63[a]
FPLC Hiload® chromatography	671	21	31.95	4.26	50.30
	825[a]	20.5[a]	40.24[a]	5.36[a]	61.84[a]
Hydrophobic interaction chromatography	605	13	46.53	6.20	45.35
	665[a]	12[a]	55.42[a]	7.38[a]	49.85[a]
FPLC Mono-Q® chromatography	595	9	66.11	8.81	44.60
	610[a]	7[a]	87.14[a]	11.61[a]	45.72[a]
Gel filtration chromatography	432[a]	4.67[a]	92.50[a]	12.33[a]	32.38[a]

[a] Values after dialysis against distilled water

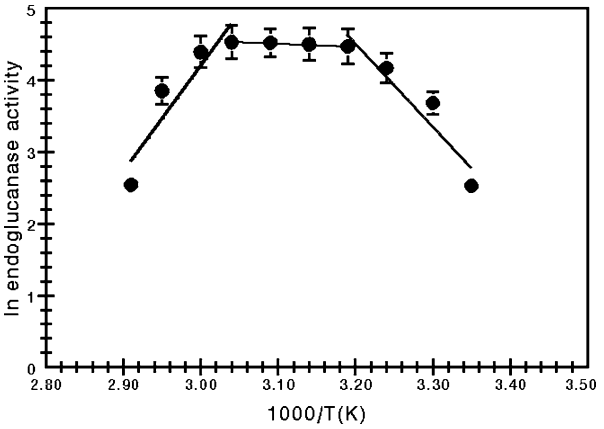


Fig. 2 Arrhenius plot for the effect of temperature on activity and determination of activation energy for CMcellulose hydrolysis by endoglucanase of *A. oryzae* cmc-1. Data presented are average values±SD of n=3 experiments

applied to determine the extent of thermal inactivation (Fig. 5). The enzyme was stable up to 47 °C and exhibited half-life of 138.6 min. The half-life ($t_{1/2}$) of an enzyme, at a given temperature, is the time it takes for the activity to reduce to half of its original/initial activity. At higher temperatures, half-life decreased sharply, i.e., 9.77 min at 53 °C and just 2.31 min at 56 °C (Table 2). Activation energy for irreversible inactivation '$E_{a(d)}$' of the endoglucanase was 378 kJ mol^{-1} (Fig. 6), and Gibbs free energy (ΔG^*) for activation of thermal unfolding of enzyme was 111.36 kJ mol^{-1}. With an increase in temperature, a decrease in free energy was observed. The enthalpy of activation of thermal unfolding (ΔH^*) of the enzyme at 44 °C was 375.36 kJ mol^{-1}. Its value remained almost the same up to 56 °C. The entropy of activation (ΔS^*) for unfolding of transition state of the endoglucanase was 833.06 J mol^{-1} K^{-1}, which was slightly increased at 56 °C (Table 2). At higher temperatures, decrease in ΔG^* made the enzyme thermally unstable.

The stability of endoglucanase against α-chymotrypsin and urea was also determined. The half-lives against these denaturants were 25.4 and 216 min, respectively, whereas the percent residual activities of the enzyme after 60 min treatment of α-chymotrypsin and urea at 30 °C were 75.5% and 17.3%, respectively (Fig. 7).

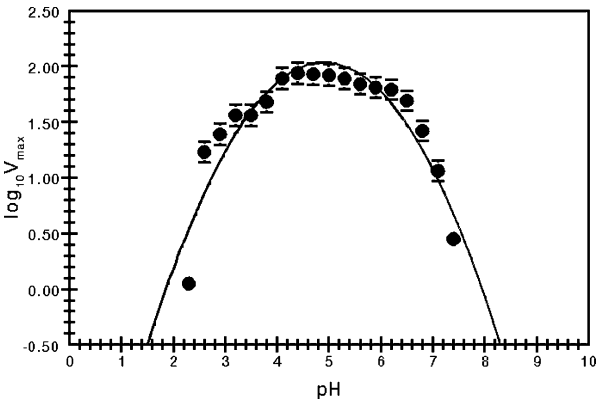


Fig. 3 Dixon plot for the effect of pH on activity and determination of pK_a of ionize able groups of active site residues of endoglucanase at 55 °C from *A. oryzae* cmc-1. Data presented are average values±SD of n=3 experiments

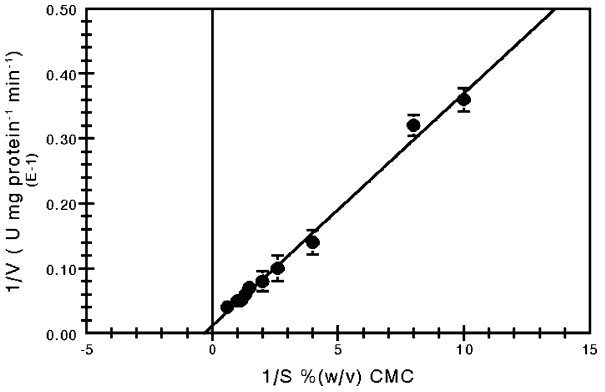


Fig. 4 Lineweaver–Burk plot for the determination of Michaelis kinetic constants (V_{max}, K_m) for CMcellulose hydrolysis at 55 °C, pH 4.4 by endoglucanase from *A. oryzae* cmc-1. Turnover (K_{cat})= $s^{-1}=V_{max}/[e]$, where [*e*] is the enzyme concentration=7.88 μg or 3.152×10^{-4} μ mol, K_m=mg CMC ml^{-1} and $K_{cat}/K_m=s^{-1}$ mg^{-1} CMC per milliliter. Data presented are average values±SD of n=3

Discussion

In one of our previous studies, we had expressed *cmc*1 gene of *A. aculeatus* in *A. oryzae* [19] with a hyper-expression system using an improved fungal promoter so that the transgenic *A. oryzae* (*A. oryzae* cmc-1) will be able to produce constitutively higher amounts of endoglucanase (CMCase) and the fungus itself can be used in fermented food industry and its biomass as single cell protein. For the production of CMCases, *A. oryzae* cmc-1 was grown on 2% wheat bran (cheap carbon source) in liquid phase conditions in which parameters can be closely monitored. After 96 h of incubation, 7.5 U mg^{-1} protein was obtained. The enzymes were then subjected to five-step purification protocol, i.e., ammonium sulfate precipitation, HiLoad anion exchange, hydrophobic interaction, Mono-Q anion exchange, and gel filtration chromatography on Pharmacia FPLC unit. The onset of CMCase precipitation occurred at 20% saturation of ammonium sulfate at 0 °C, while complete precipitation was observed at 40% saturation. The endoglucanase was highly hydrophobic in nature because on HIC column, the enzyme was eluted at the end of ammonium sulfate gradient, which indicated that the enzyme was strongly adsorbed. The purification protocol resulted into 12.33-fold purification with the recovery of 32.38%. The specific activity was increased to 95.2 U mg^{-1} protein (Table 1). Siddiqui et al. [21] have reported the precipitation of CMCases from *A. niger* between 45% and 65% ammonium sulfate saturation at 0 °C.

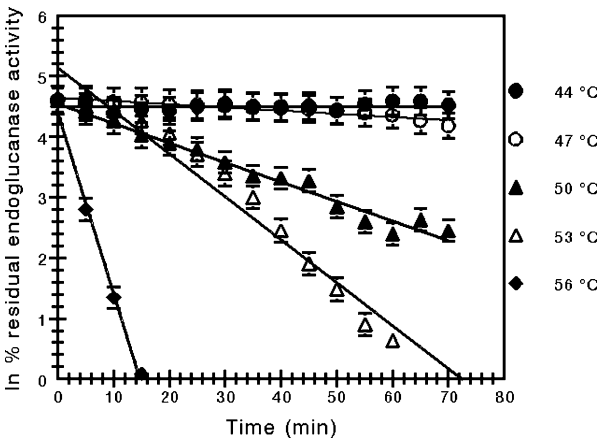


Fig. 5 Pseudo first-order plots for irreversible thermal denaturation of endoglucanase from *A. oryzae* cmc-1. The enzyme solution was incubated at various temperatures (44 °C to 56 °C) in 10 mM Tris–HCl buffer (pH 7). Data presented are average values±SD of *n*=3 experiments

Table 2 Kinetic and thermodynamic parameters for irreversible inactivation of endoglucanase from *A. oryzae* cmc-1.

Temp (°C)	Temp (K)	K_d (min^{-1})	$t_{1/2}$ (min^{-1})	ΔH^* (kJ mol^{-1})	ΔG^* (kJ mol^{-1})	ΔS^* (J mol^{-1} K^{-1})
44	317	0.0004	1732(t_d)	375.36	111.36	833.06
47	320	0.0050	138.6	375.34	104.54	846.25
50	323	0.0323	21.45	375.32	99.52	853.86
53	326	0.0709	9.77	375.29	97.42	852.36
56	329	0.3000	2.31	375.26	93.56	856.23

$E_{a(d)}$ was 378 kJ mol^{-1} (Fig. 6), K_d (first-order rate constant for inactivation) was taken from Fig. 5, $t_{1/2}$= half-life=0.693/K_d, all other parameters were calculated from Eqs. 3, 4, and 5 mentioned in "Materials and Methods".

CMCase from *Bacillus sphaericus* JS1 was purified 192-fold by $(NH_4)_2SO_4$ precipitation, ion exchange, and gel filtration chromatography, with an overall recovery of 23% [35]. The cellulase from bacterium strain AC1 was purified about 150-fold by ammonium sulfate fractionation, ion exchange, hydrophobic, and gel filtration chromatography, with a specific activity of 35 IU mg^{-1} [36]. The native and subunit molecular mass of *A. oryzae* cmc-1 endoglucanase was 25 kDa, showing that the enzyme is monomeric in nature (Fig. 1a,b). Carboxymethylcellulases are produced by a variety of microbes, and their molecular weights have been reported in the literature (Table 3) [37–48].

Temperature and pH optima of purified transgenic CMCase were 55 °C and 4.4, respectively. We have found that expression cloning resulted into an improvement of 5 °C in temperature optimum, as Swano et al. [20] had reported 50 °C as the optimum temperature of F1-CMCase from *A. aculeatus*, while pH optimum remained unchanged. The endoglucanases from different microbial origins have been reported to have temperature optima and pH optima in the range of 37–83 °C and 4.4–6.8, respectively (Table 3). It was postulated that two carboxyls are involved in the catalytic mechanism of most hydrolyses, one of which donates proton to the substrate while the other stabilizes it [33]. The comparison of pK_a values (pK_{a1}=3.9 and pK_{a2}=6.25) of the ionizable groups of amino acids in the present study revealed probable involvement of aspartate and glutamate (as proton donor) and histidine (as proton acceptor). Siddiqui et al. [21] have determined the active site residues for *A. niger* CMCases and found that both proton donating and receiving residues contain carboxyls as ionizable group with pK_a values of 3.5 and 5.5, respectively.

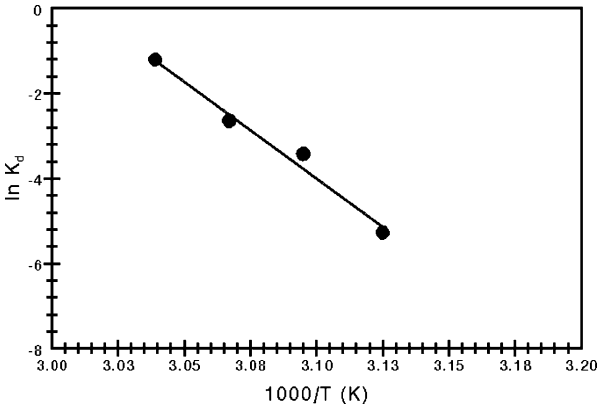


Fig. 6 Arrhenius plot to calculate activation energy '$E_a(_d)$' for irreversible thermal inactivation/denaturation of endoglucanase from *A. oryzae* cmc-1 using relationship ln K_d=−E_a/RT, where R (gas constant)=8.314 kJ mol^{-1}

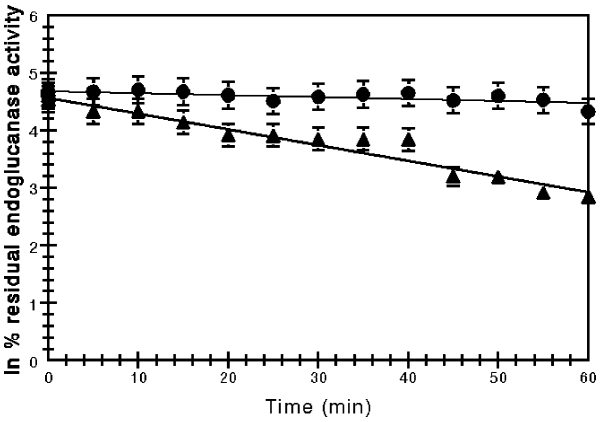


Fig. 7 Effect of α-chymotrypsin (0.2 mg ml^{-1}) and urea (4 M) on kinetic stability of endoglucanase from *A. oryzae* cmc-1 at 30 °C. α-chymotrypsin (*closed circle*) and urea (*closed triangle*). Data presented are average values±SD of $n=3$ experiments

The Arrhenius plot was tri-phasic, which explained that the CMCase has two conformations up to optimum temperature. The CMCase at first transition temperature (40 °C) required very high E_a (96.77 kJ mol^{-1}) for CMC hydrolysis as compared to the second transition state (55 °C), which was 3.32 kJ mol^{-1}. This explained that at higher temperature, i.e., 55 °C, the enzyme molecule was expanded, which resulted into an ideal conformation of the active site. The activation energies for CMCases from *A. niger* have also showed a tri-phasic trend where the values of E_a up to temperature optima were 53 and 18 kJ mol^{-1} [21]. CMCase from *Cellulomonas biazotea* have E_a 35 kJ mol^{-1} [47]. These results showed that the CMCase from *A. oryzae* cmc-1 requires very low energy (3.32 kJ mol^{-1}) to make activated enzyme substrate complex at optimum temperature. The lower value of E_a explains that the conformation of active site is favorable for ES* complex formation, hence requiring less energy. The temperature quotient for the enzyme was 1.0, which was close to the glucoamylase of *Humicola* sp. [26].

Table 3 Physiochemical properties of endoglucanase from various microbes.

Organism	Subunit molecular mass (kDa)	pH optima	Temperature optima (°C)	Reference
Aspergillus oryzae cmc-1	25	4.4	55	Current report
Acidothermus cellulolyticus	57.42, 74.58		81–83	[45]
Alternaria alternata	41		47	[46]
Aspergillus niger NIAB 280	36	4.4		[21]
Aspergillus niger	42, 45			[32]
Aspergillus niger & *Cellulomonas biazotea*	40, 23			[47]
Bacillus spp.	33	5.0	60	[37]
Chalara paradoxa CH 32	35		37	[48]
Clostridium thermocellum	83			[41]
Neurospora crassa	70			[38]
Ruminococcus flavefaciens	26, 25	6.8	55	[40]
Ruminococcus albus	43, 39	6.8	37	[42]
Streptomyces lividans	46	5.5	50	[43]
Thermomonospora curvata	100	6.0–6.5	70–73	[44]
Trichoderma viride	52, 42, 60, 38			[39]

The V_{max} and K_m values determined through Lineweaver–Burk plot for the hydrolysis of CMC at 55 °C were 854 U mg^{-1} protein and 20 mg CMC per milliliter, respectively (Fig. 4). The k_{cat} was 356 s^{-1} and specificity constant (K_{cat}/K_m) was 17.8. Theberge et al. [43] 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 per minute, 405.5 μmol glucose per hour, and 18 μmol glucose per minute per milligram 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 [44, 46, 49]; the endoglucanase from *Arachniotus citrinus* showed the V_{max} and K_m of 23 U ml^{-1} min^{-1} and 2.9% (*w*/*v*), respectively [22]. The comparison shows that the endoglucanase of *A. oryzae* cmc-1 hydrolysis the CMC more actively; however, it requires more substrate for saturation.

Thermodynamic parameters for CMC hydrolysis, i.e., the enthalpy of activation (ΔH^*), Gibbs free energy (ΔG^*), and entropy of activation (ΔS^*) were calculated as 0.59 kJ mol^{-1}, 64.57 kJ mol^{-1}, and −195.05 J mol^{-1} K^{-1}, respectively. The free energy for activation of substrate binding ($\Delta G^*_{E\text{-}S}$) and formation of activated complex ($\Delta G^*_{E\text{-}T}$) were 8.17 kJ mol^{-1} and −7.85 kJ mol^{-1}, respectively. Previously, we have reported 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}) [21]. The lower enthalpy and Gibbs free energy values of CMCase from *A. oryzae* cmc-1 as compared to CMCase of *A. niger* confirmed that the transgenic CMCase was highly active and efficient.

The ability to obtain stable enzymes is crucial for their application as biocatalysts. The development of methodologies that can increase enzyme stability is an important goal in enzyme technology. Catalytic protein molecules, like all other proteins, are only marginally stable due to the delicate balance of stabilizing and destabilizing interactions [50]. Detailed elucidation of the mechanisms responsible for stabilization and destabilization of enzymes especially at elevated temperatures is of special importance from both a scientific and a commercial point of view [51]. Interest in extremostable enzymes is mainly due to the fact that most industrial enzymes processes are carried out under abnormal physiological conditions, such as higher temperature, high pressures, extreme values of pH etc. Temperature is one of the most important environmental factors controlling the activities and evolution of organisms and is probably the best optimized physical variable in chemical reactions [52].

Thermal denaturation of enzymes is a two step process [32].

$$N \rightleftharpoons U* \rightarrow I$$

Where, 'N' is the native folded enzyme, 'U' is the unfolded inactive enzyme, which could be reversibly refolded on cooling and 'I' is the inactive enzyme formed after prolonged exposure to heat and therefore, cannot be recovered upon cooling [53]. The thermal denaturation of enzymes results in the breakage of non-covalent linkages including hydrophobic interactions with concomitant increase in the enthalpy (ΔH^*) of activation for denaturation. The opening up of the enzyme structure is accompanied by increase in the disorder or entropy (ΔS^*) of activation for denaturation [54].

The thermodynamic parameters of irreversible thermal inactivation of CMCase from *A. oryzae* cmc-1 at 44 °C, i.e., ΔH^*, ΔG^*, and ΔS^* were equal to 375.36 kJ mol^{-1}, 111.36 kJ mol^{-1}, and 833.06 J mol^{-1} K^{-1}, respectively (Table 2). Out of which ΔH^* and ΔG^* were decreased to 375.26 and 93.56 kJ mol^{-1}, respectively, at 56 °C, while ΔS^* was increased to 856.23 J mol^{-1} K^{-1}. The energy of activation $\{E_{a(d)}\}$ for irreversible thermal inactivation of CMCase from *A. oryzae* cmc-1 was 378 kJ mol^{-1} (calculated from Fig. 6). The pseudo first-order plots were linear. The enzyme showed activation on heating at 44 °C with doubling

time (t_d) of 1732 min. Swano et al. [20] has also reported the thermostability of F1-CMCase from *A. aculeatus* below 45 °C. The thermal denaturation at 45 °C of native endoglucanase from *A. citrinus* gave ΔS^* of −190 J mol^{-1} K^{-1}, ΔG^* of 86.2 kJ mol^{-1}, and ΔH^* of 25.3 kJ mol^{-1} [22]. The comparison indicates that the endoglucanase from *A. oryzae* cmc-1 showed more resistance to thermal denaturation as compared to *A. citrinus* endoglucanase. The extremely high ΔG^* of the *A. oryzae* cmc-1 enzyme (111.36 kJ mol^{-1}) as compared to that of *A. citrinus* explained that the thermostabilization was due to the higher free energy (functional energy), which enabled the enzyme to resist against unfolding of its transition state. Furthermore, higher ΔS^* value confirmed that the stabilization was not entropically driven and was due to higher free energy.

The stability of endoglucanase against α-chymotrypsin and urea after 60 min of treatment showed the half-lives of 25.4 and 216 min with the percent residual activities of 75.5% and 17.3%, respectively. The first-order plot for the stability of CMCases against chymotrypsin and urea was linear (Fig. 7). Siddiqui et al. [28] reported that 25% of endoglucanase activity of *A. niger* was lost after 32 h treatment of thermolysin. Urea is a strong denaturant. Unfolding of proteins by urea results into pushing of inner hydrophobic residues outward. The refolding of proteins depends upon the degree of unfolding. CMCases from *A. niger* and *C. biazotea* have been studied to check their stability against urea. The calculated half-lives were 125 and 89 min in 8 M urea at 37 °C and 40 °C, respectively [47].

Conclusions

We concluded that the endoglucanase produced by the transgenic *A. oryzae* cmc-1 was kinetically and thermodynamically very efficient and stable. In the light of our current and previous report [19] regarding the hyper and constitutive production of endoglucanase, we consider that this strain has high significance to be used in different food industrial processes.

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