Pak. j. life soc. sci. (2003), 1(2): 87-91

Pakistan Journal of Life and Social Sciences

Kinetic Study of Carboxymethylcellulase from *Aspergillus niger*

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Abstract

Kinetics of carboxymethylcellulase (CMCase) isolated from Aspergillus niger was studied. The enzyme showed maximum activity at pH 5.0 and temperature 30oC. Low value of Michaelis constant (Km 0.0133) obtained from Line weaver-Burk plot is indicative of high affinity of the enzyme for the substrate. Vmax of the enzyme was found to be 0.427. Energy of activation was calculated from Arrhenius plot and found to be 15kJK^{-1}mol^{-1} that shows good relationship between enzyme and the substrate. Enthalpy for the hydrolysis of cellulose by the enzyme at optimum temperature was 12.5kJK^{-1}mol^{-1}. Low value of Q10 (1.18) shows high catalytic activity of the enzyme. Effect of metal ions on the enzyme activity was also studied. Mn^{2+} and Ca^{2+} enhanced the activity whereas Cu^{2+} and Hg^{2+} inhibited the enzyme activity.

Key words: CMCase, Assay, *Aspergillus niger*

Introduction

Cellulose biodegradation refers to the breakdown of cellulose into its component glucose units through the action of enzymes. This process has attracted scientific attention because of the complexity of enzymes involved and the environmental as well as economic significance of the process. Cellulose, the major structural polysaccharide of plants, is a hydrophilic linear glucose polymer with the anhydroglucose units bonded by β-1,4 glucocidic linkage. The depolymerization of cellulose into monomeric sugars either by strong acids or by commercial enzymes remains a major economic dilemma, which has stimulated investigations into the structure and function of microbial cellulases and development of improved strains with increased cellulase production.

Enzymatic conversion of cellulose to metabolizable sugars is an essential step if further conversion to useful products is required such as ethanol production (Rajoka *et al.,* 1992), which is valuable in many respects. It is used as solvent in industries (Moo Young, 1985), in the preparation of medicines, resins, perfumes etc.(Routh *et al.,*). Apart from production of ethanol, cellulases perform various other functions in industry e.g., starch production, waste treatment, baking, production of plant protoplasts for genetic manipulation, wine production, preparation of pharmaceuticals etc. (Esterbauer *et al.,* 1991).

Conversion of agricultural residues to useful products is also an attractive option as a remedy for air pollution, energy production and other environmental concerns (Doran *et al.,* 1994). Furthermore fast growing population of the world is becoming a permanent threat to the natural resources (Yaqub 1990,1996). Under such conditions we have to look for alternative strategies to meet our future energy demands. Therefore, efficient methods of recycling of waste material into useful products must be found out.

Cellulose biodegradation is mediated by several enzyme systems. The more studied are the extra cellular cellulose systems in fungi that have three components: Endoglucanases (EC 3.2.1.4), cellobiohydrolases (3.2.1.9.1) and β-glucosidases (EC 3.2.1.2.1) (Coughlan and Ljungdahl, 1988).

Endoglucanases, also referred to as CM-cellulases, attack on amorphous cellulose by random cleavage of β-glucosidic linkages. CMCase (endoglucanase) converts the polymeric form of cellulose into oligosaccharide form, then cellobiohydrolase (Exoglucanse) separates cellubiose by acting on non-reducing end. Finally, β glucosidase changes cellobiose into glucose units.

Characterization and kinetic studies of enzymes are important for their utilization in different processes. Various kinetic parameters were determined for carboxy methyl cellulose from *Aspergillus niger* and discussed in this paper.

Materials and Methods

CMCase Assay

CMCase isolated from *Aspergilluus niger* was investigated to study the different kinetic parameters. Carboxy methyl cellulose was used as substrate that is hydrolyzed by CMCases to produce free carboxy methyl glucose units. The free carboxy methyl glucose form a colored complex, which may be detected spectrophotometrically at 550 nm (Gadgil *et al.,* 1995). Appropriately diluted enzyme solution (1 ml) was incubated for 30 minutes with 1 ml of 1.0 percent CMC and 1 ml of acetate buffer (pH 5) at 30°C.The reaction was terminated by adding 3 ml of DNS reagent and

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mixture was boiled for 10 minutes and cooled in ice. After cooling the absorbance was noted at 550 nm. Enzyme activity was determined by using standard factor obtained from glucose standard curve. (Fig.1).

Optimum pH and Temperature

Optimum pH for maximal activity of CMCase obtained from *Aspergillus niger* was determined by taking enzyme solution in buffer of different pH (2-9) and was assayed with 1 %CMC solution. Optimum temperature for maximal activity of CMCase was determined as described by Sanyal *et al.,* (1988). Enzyme solution in buffer was assayed at different temperatures (10°C to 70°C) with 10°C intervals for CMCase activity as described by Rojaka and Malik (1986).

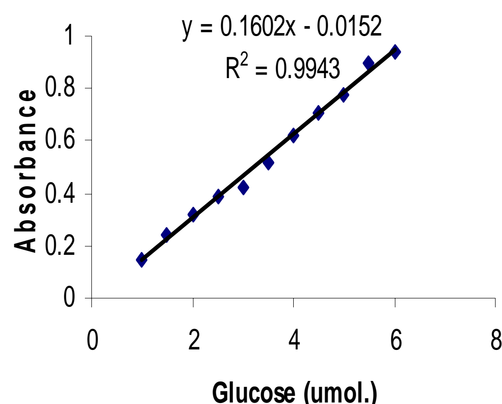

Fig. 1: Standard curve for glucose used for the determination of CMCase activity

Activation Energy and Enthalpy of Activation (ΔH)

Activation energy of CMCase was determined by using the data for optimum temperature in the following equation:

Ea = Slope * R (Atkins, 1995)

Where R= Molar gas constant (I8.314 JK^{-1} mol^{-1})

The enthalpy of activation ΔH, represents the change in heat content required to form an activated enzyme substrate complex (Hunt *et al.,* 1982).

ΔH was calculated by equation:

$$\Delta H = Ea - RT$$

Increase in Reaction Rate per 10°C Rise in Temperature (Q_{10})

The value of activation energy was also used to calculate the increase in reaction rate for every 10°C rise in temperature with the help of equation described by Rajoka and Malik (1986):

1n Q10 = Ea/R(1/T2-1/T1)

Effect of Enzyme Concentration

Effect of different enzyme concentrations was determined by adding (10,20,30,40,50,60,70,80,90 and 100µL) of enzyme solution to 1ml CMC solution , and 1mL buffer of pH 5.0. These solutions were incubated for 30 min. at 30°C. To these solutions 3mL DNS reagent was added, heated in boiling water bath for 10 min, cooled in ice-cold water and absorbance was noted at 550 nm.

Effect of Substrate

The effect of substrate on CMCase was studied to calculate the values of Vmax and Km.

Determination of Vmax and Km

CMCase was assayed in acetate buffer (pH 5.5) with variable amounts of 1% CMC as substrate. The values of Vmax and Km were calculated from the intercept and the slope respectively from the plot of 1/V vs 1/ [S] (Line weaver –Burk plot).

Effect of Activators and Inhibitors

Effect of activators/inhibitors on the CMCase was studied as described by Ghosh *et al.,* (1991). The enzyme activities were determined after adding activators and inhibitors (Rajoka and Malik, 1986). Various activators or inhibitors used were $MnCl_2$, $CaCl_2$, $CuSO_4$ and $HgCl_2$. Three different concentrations of the activators / inhibitors viz., 0,0.5,1.0,1.5 mM were used to study their effect on the activity of CMCase with three different substrate concentrations (0.5, 1.0, 1.5 ml) as given by Siddiqui *et al.,* (1997)

Results and Discussion

Optimum pH and Temperature

The optimum pH of CMCase was determined in buffers of pH range 2-9. The optimum pH of CMCase found was 5 from Fig 2. This value is in good agreement as given in literature. The experiments at varying temperatures viz 10,20,30,40,50,60 and 70°C were performed to find out the optimum temperature for CMCase activity. It was observed that optimum temperature for the enzyme was 30°C (Fig 3). At 20°C activity of the enzyme was lower; it was increased gradually per 10°C rise in temperature. At 30°C enzyme showed maximum activity, which was further decreased at 60°C and 70°C. Our results are in close agreement with Rajoka and Malik (1984) who reported temperature optima of different enzymes from *C. biozota* mainly between 30-50°C. The enzyme retained 100% original activity up to 50°C. All enzymes showed a tendency to decrease activity above 50°C and to a great extent at 60°C. Esen (1992) further stated that β-glucosidase activity decreased above 55°C.

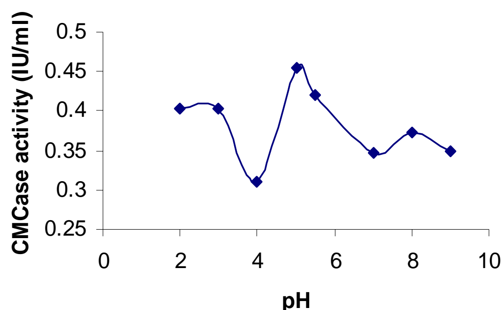

Fig. 2: Effect of pH on CMCase activity

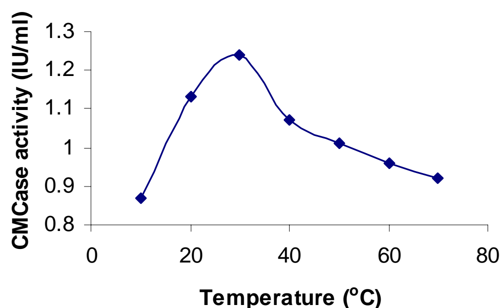

Fig. 3: Effect of Temperature on CMCase activity

Energy of Activation and Enthalpy of Activation (ΔH)

Energy of activation (Ea) for CMCase was 15KJ $K^{-1}mol^{-1}$ as calculated with the help of Arrhensius plot (Aktin, 1995). The slope is shown in Fig 4. It was observed that at 30°C CMCase had maximum catalysis in the conversion of CMC into glucose. After this temperature the enzyme starts becoming denatured and show less activity towards the conversion of substrate into product. This small amount of activation energy indicates a good relationship between enzymes and substrates. Enthalpy of activation (ΔH) was also calculated and found to be (12.25 KJ K^{-1}/mol^{-1}) for CMCase. It was interpreted that kinetically CMCase from *Aspergillus niger* was favorably good for the conversion of cellulose into glucose.

Increase in Reaction Rates Per 10°C (Q_{10}) Rise in Temperature

Increase in reaction rate for every 10°C rise in temperature was calculated for CMCase with the help of activation energy. The Q_{10} value obtained for CMCase was 1.18. This value shows that there was on average, 1.18 times increase in the reaction rate of this enzyme when the temperature was increased from 20°C to 30°C. Lower Q_{10} values demonstrated high catalysis, as a distinctive feature of enzyme catalysis is that the Q_{10} of a catalyzed reaction is lower as compared to the same reaction uncatalyzed (Segel, 1975).

Effect of Enzyme Concentration

Using increasing amounts of enzyme (10µl-100µl), the CMCase activity was determined at pH 5.0 and temperature 30°C. Up to 90µl, activity was enhanced but after this it became almost constant (Fig 5). This showed that all the available substrate had been converted into product at 100µl.

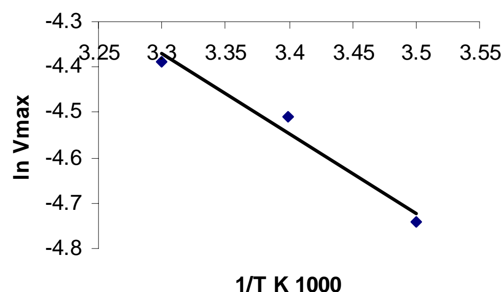

Fig. 4: Arrhenius plot for activation energy of CMCase catalyzed reaction.

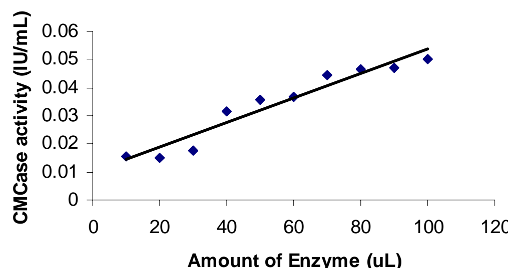

Fig. 5: Effect of enzyme concentration on CMCase activity

Effect of Substrate Concentration

The dependence of the reaction rate on the concentration of carboxy methyl cellulose (0.5 and to 0.05 ml) was calculated using the Michaelis-Menton equation. The reaction followed first order kinetics up to 0.45 ml substrate but after this, the activity became constant. By using Line weaver-Burk plot (1/V vs 1/ [S]) (Fig 6), the Vmax and Km values obtained were 0.427 µmol/ml/min and 0.0133 respectively. Our results indicate small Km values of CMCase. This demonstrate high affinity of the enzyme with the respective substrate (Ghori and Malana, 2001; Palmer, 1991).

Effect of Metal Ions

The effect of metal ions viz. Mn^{2+}, Ca^{2+}, Cu^{2+} and Hg^{2+} on the activities of CMCase was studied at pH 5.0 and temperature 30°C. The results are summarized in Table 1.

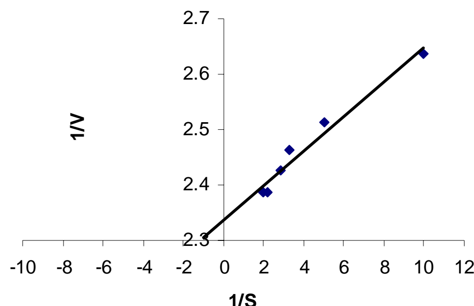

Fig. 6: Line-weaver Burk Plot for CMCase from *Aspergillus niger*

Experiments having varying concentration of metal ions viz 0.5,1.0, 1.5mM were performed to determine their effect as activator or inhibitor on the enzyme. The substrate levels for enzyme in all treatments were also varied from 0.5 to 1.5 mL of 1% substrate solution.

Effect of Mn^{2+}

CMCase was activated by 1.0 mM Mn^{2+} concentration and its high level inhibited the CMCase activity. Our results correlate with Nakeamura and Kitamura (1983) who reported that 1 mmol/L Mn^{2+} activated the cellulase from *C. nuda*. Dixon and Webb (1979) observed that at higher CMCase concentration Mn^{2+} could not combine with the free enzyme and the type of inhibition was complex. Mn^{2+} acted as a bridge between carboxyl group of substrate and enzyme3. Present result are also supported by Siddiqui *et al.,* (1997) and Ghori and Malana, (2001). However, these results differ from that of Kundun *et al.,* (1998) who reported that CMCase produced from *A.Japanicus* was inhibited by Mn^{2+}.

Effect of Ca^{2+}

Our study has indicated that Ca^{2+} acted as an activator metal ion and it increased the activity up to 0.5mM concentration. By using concentration of 1.0 mM it also caused an increase in the activity but showing no remarkable difference. These findings are favored by the work of Sami *et al.,* (1988) who reported that lower concentration of Ca^{2+} had activated the CMCase while higher concentrations had an inhibitory effect. These results are also supported by Ghori and Malana, (2001) and Sharma *et al.,* (1991). They produced CMCase from *Aspergillus* sp. and reported that activity was totally inhibited by Hg^{2+} partially inhibited by Ca^{2+} and Co^{2+}.

Effect of Cu^{2+}

The present study has proved that Cu^{2+} acts as an inhibitor increase of CMCase. Increased concentration of Cu^{2+} has inhibited the enzyme activity. Our results are comparable to that of Akthar and Akthar (1995) who reported that CMCase activity produced from *Bacillus* was increased by Mg^{2+}, Zn^{2+}, Ca^{2+}, while Hg^{2+} and Cu^{2+} decreased the activity

Effect of Hg^{2+}

The results are in good agreement with Akthar and Akthar (1995), Ghori and Malana (2001) and Romanice *et al.,* (1992) who reported that activity of CMCase was inhibited by Hg^{2+}. Sharma *et al.,* (1990) and Akhtar and Akhtar (1995) reported that CMCase was in activated by Hg^{2+}.

Table 1: Effect of varying concentrations of Mn^{2+}, Ca^{2+}, Cu^{2+} and Hg^{2+} on CMCase activity with varying substrate levels

S. No.	Substrate (mL)	Metal Ion Conc. (mM)	Mn^{2+} Activity (U/mL)	Ca^{2+} Activity (U/mL)	Cu^{2+} Activity (U/mL)	Hg^{2+} Activity (U/mL)
1	A (0.5 mL)		0.67	0.995	0.60	0.58
2	B (1.0 mL)	0.0	0.71	1.15	0.61	0.59
3	C (1.5 mL)		0.78	1.25	0.62	0.61
4	A (0.5 mL)		0.70	1.02	0.574	0.57
5	B (1.0 mL)	0.5	0.788	1.18	0.571	0.58
6	C (1.5 mL)		0.79	1.30	0.58	0.579
7	A (0.5 mL)		0.77	1.03	0.53	0.55
8	B (1.0 mL)	1.0	1.01	1.19	0.55	0.579
9	C (1.5 mL)		0.92	1.22	0.57	0.579
10	A (0.5 mL)		0.97	1.01	0.55	0.52
11	B (1.0 mL)	1.5	0.94	1.16	0.547	0.55
12	C (1.5 mL)		0.92	1.20	0.56	0.56

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