Pak. j. life soc. sci. (2006), 4(1-2): 15-19

Pakistan Journal of Life and Social Sciences

Kinetic Study of Carboxymethyl Cellulase from *Trichoderma Harzianum*

Shabana Nawaz, M. Aslam Malana, Naheed Ikram, Samia Hafeez, M. Ishfaq Ghori and Amer Jamil[1].
Department of Chemistry, Bahauddin Zakariya University, Multan-Pakistan
[1]Department of Chemistry, University of Agriculture, Faisalabad-Pakistan

Abstract

Kinetic study of carboxymethylcellulase (CMCase) produced from *Trichoderma harzianum*, a filamentous fungus, was carried out in order to determine the K_M and Vmax of the enzyme. Energy of activation, enthalpy of activation and temperature coefficient Q_{10} were also calculated. The effect of different metal ions such as activation and inhibition, were studied. Kinetic investigations showed that K_M and Vmax of CMCase were 0.5mM and 1.25 µmol/ml/min, respectively. Energy of activation, Ea, for CMCase was observed to be 29.93 kJ $K^{-1}mol^{-1}$. Enthalpy of activation was found to be 2.48 kJ $K^{-1}mol^{-1}$, Temperature coefficient, Q_{10}, was 1.7 ºC. Ca^{2+} and Co^{2+} stimulated the enzyme activities while Fe^{2+}, Hg^{2+}, Cu^{2+}, Mg^{2+} inhibited the enzyme activities. Small value of K_M and substrate concentration showed a large affinity of the enzyme with substrate. Conclusively, the CMCase was found good catalytic agent to convert agricultural waste into glucose.

Introduction

The global interest in cellulose conversion to energy and chemicals is not surprising since cellulose is the most abundant carbohydrate produced in the biosphere (Suto and Tomita, 2001; Guedon *et al*., 2002). Cellulose can be converted to useful products by hydrolyzing with chemical, enzymatic or physical methods. It has been shown that the rate and extent of cellulose hydrolysis are influenced by the structural features of the cellulose substrate (Stone et al., 1969; Wood and Mc Crae, 1979; Fan *et al*., 1980). Cellulose in the form of cereal, grain residue, stalks, bagasse and saw dust is produced by photosynthesis. Such agricultural wastes are either thrown away or burnt which causes pollution in the environment. 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 materials into useful products must be found out.

Cellulose biodegradation is mediated by several enzymes which have been extensively studied because they are secreted in large quantities. The development of microbial strains, media composition and process control have contributed in the achievement of high level of extracellular accumulation of cellulases for subsequent application in industry (Gosh et al, 1984).

Cellulases are also used in the manufacturing of pharmaceuticals, beverages, paper and textiles. Bacterial and fungal cellulases now a days are used in animal feed industry, grain alcohol fermentation, brewing, malting and extraction of fruit and vegetable juices (Deshpande and Erickson, 1984; Kubicek *et al*., 1993)

There are three main types of enzymes that can degrade crystalline cellulose, viz, cellobiohydrolase (EC 3.2.1.91; 1,4-β-D glucan cellobiohydrolase) which releases cellobiose units from crystalline cellulose, endo 1,4-β glucan (EC 3.2.1.4; 1,4-β-D glucane glucanohydrolase) which degrades regions of amorphous cellulose, and β-glucosidase (EC 3.2.1.21; β-D-glucoside glucohydrase) or cellobiase which degrades short oligosaccharides such as cellobiose and cellotriose to glucose (Gilkes et al., 1984).

We isolated carboxymethyl cellulase (CMCase) from *Trichoderma harzianum* and subjected to kinetic studies. The enzyme showed good catalytic potential for conversion of agricultural wastes into soluble products.

Corresponding author: Amer Jamil
Department of Chemistry, University of Agriculture, Faisalabad-Pakistan
Email: amerjamil@yahoo.com

Materials and Methods

The enzyme, carboxymethyl cellulase was obtained from culture filtrate of *Trichoderma harzianum* (Ahmed et al., 2003).

CMCase assay

Carboxymethyl cellulase assay was performed according to Ahmed et al. (2003) by using carboxymethyl cellulose as substrate.

Kinetic Study

Optimum pH

Optimum pH for maximal activity of CMCase obtained from *Trichoderma harzianum* was determined by taking enzyme solution in buffer of different pH (2-9) as described by Roger *et al* (1985) and was assayed for the enzyme activity.

Optimum temperature

Optimum temperature for maximum activity of cellulase was determined as described by Sanyal *et al* (1988). Enzyme solution in buffer was assayed at different temperatures (20 °C to 40 °C with 10 °C intervals) for CMCase activity as described by Rajoka and Malik (1986).

Activation energy and enthalpy of activation (ΔH)

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

Ea = -Slope x R (Atkins, 1995)

Where R = Molar gas constant ($8.314 Jk^{-1}/mol$)

The enthalpy of activation ΔH, represents change in heat content required to form an activated enzyme substrate complex (Hunt *et al*., 1982). ΔH was calculated with the help of following equation: ΔH= 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 increase in temperature with the help of following equation.

$$\text{In Q10} = \frac{Ea}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)$$

Determination of V_{max} and K_M

CMCase was assayed with variable amounts of CMC as substrate. The data were plotted as Michaelis-Menton graph and Lineweaver-Burk plot. The values of Vmax and k_M were calculated from Lineweaver-Burk plot.

Effects of activators and inhibitors

Effect of activators/inhibitors on 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 $CaCl_2$, $CuCl_2$, $FeCl_3$, $HgCl_2$, $CoCl_2$. Three different concentrations of the activator / inhibitor viz., 0.5, 1.0, 1.5 m*M* were used to study their effect on the activity of CMCase with three different substrate amounts (0.5, 1.0, 1.5 mL) as given by Siddiqui *et al* (1997).

Results and Discussion

CMCase was used in the present study for the catalytic hydrolysis of carboxymethyl cellulose under different conditions of temperature, pH and substrate concentration.

Substrate effect

Different concentrations of substrate viz. 1 to 10 percent were used to assay carboxymethyl cellulase produced by *Trichoderma harzianum*. Maximum activity was found with 5 mM substrate concentration but after this activity became constant as shown in Fig. 1. By using the Lineweaver-Burk plot (1/V vs 1/[S]) (Fig. 2), the Vmax and K_M values obtained were 1.25 umol/ml/min and 0.5 mM respectively. Our results indicate small K_M value of CMCase which demonstrates high affinity of enzyme with the substrate (Palmer, 1991). Chahal et al. (1985) also observed this behavior of enzyme with different substrate concentrations. Our study is very much close to that of Ghori (2001) who observed the effect of substrate level on the cellulase activity of *Aspergillus niger* NRRL 567 with optimum substrate level at 4% CMC. Ortega (1981) observed maximum substrate level to be 6% with CMCase of *Aspergillus candidus*. The results presented by Duenas et al. (1995) also support our study. They used 5% substrate level with CMCase produced by mixed culture of *Trichoderma reesei* and *Aspergillus phoenicis*. Brimer et al. (1994) suggested that the difference in substrate levels used by different researchers for various cellulolytic microorganisms depend upon the difference in nature and composition of inducer substrate and optimum media in various studies.

Effect of pH

Buffers of varying pH viz 3 to 10 were used in order to check the effect of pH on CMCase from *Trichoderma harzianum*. Maximum activity was found at pH 6 (Fig. 3). Our results resemble with those of Puntambekar (1995) who obtained maximum CMCase activity from *Volvariella displosia* at pH 5.4. Whereas results of this study show contradiction with those of Ghori (2001) who showed maximum activity of CMCase at pH 3.5.

Effect of temperature

Maximum activity was observed at 30 ºC, then the activity started decreasing with increase in temperature (Fig. 4). The study showed thermophobic behavior of the enzyme. High temperature disturbed the configuration of enzyme and made it inactive towards substrate level. Our study is very much similar to that of Ghori (2001) and Vidya *et al* (1984), who reported the maximum activities of CMCase in their studies at 30 ºC.

Activation energy, enthalpy of activation (ΔH) and Q_{10}

Activation energy (Ea) for CMCase was 2.48 $kJK^{-1}mol^{-1}$ as calculated with help of Arrhenius plot (Fig. 5). At 30 ^oC CMCase had maximum catalysis for the conversion of CMC into glucose. After this temperature the enzyme started becoming denatured and showed less activity towards the conversion of substrate into product. Lower activation energy indicates a good relationship between enzyme and substrate. Enthalpy of activation was also calculated and found to be 2.48 $kJK^{-1}mol^{-1}$ for CMCase. This shows that kinetically, CMCase from *Trichoderma harzianum* was favorably good for the conversion of cellulose into glucose.

Increase in reaction rate for every 10 ^oC rise in temperature was also calculated for CMCase by employing the calculated activation energy. The Q_{10} value obtained for CMCase was 1.7. This value shows that there was, on average, 10 times increase in the reaction rate of the enzyme when temperature was increased from 20 ^oC to 30 ^oC. Lower Q_{10} values demonstrate high catalysis (Segal, 1975).

Effect of metal ions

The effect of metal ions viz Ca^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, Co^{2+}, on the activity of CMCase was studied on CMCase activity. The results are shown in Table 1. Ca^{+2} increased the enzyme activity up to 27% at 0.5 m*M* conc. and up to 93% at 1 m*M* concentration. However, the percent increase in activity was 53% at 1.5 m*M* conc. Sami et al. (1988), Ghori (2001) and Murtaza et al. (2003) reported that lower concentration of Ca^{+2} activated the CMCase while higher concentration had inhibitory effect on the enzyme activity. Addition of Co^{+2} also increased the enzyme activity. An increase of 73% was found at 0.5 m*M* Co^{+2} conc. A 60% increase in the activity was observed at 1.5 m*M*. Whereas, the activity was increased up to more than twice at 1.5 m*M* concentration of Co^{+2}. Sanyal et al (1988) and Murtaza et al (2003) also reported that Co^{+2} stimulated the CMCase activity. Cu^{+2} decreased the enzyme activity to 33, 38 and 46%, respectively at 0.5, 1.0 and 1.5 m*M* conc. Our results resemble to those of Akhtar and Akhtar (1995) who reported that Cu^{+2} worked as an inhibitor of CMCase. Decrease in enzyme activity was also observed with Fe^{+2}. A 9.19, 17.24 and 22.99 percent decrease in activity was found at 0.5, 1.0 and 1.5 m*M* Fe^{+2}. These results are very much in accordance with the work of Lin and Stutzenbergser (1995). Similarly, decreasing trend in the enzyme activity was observed at 0.5, 1.0 and 1.5 m*M* Hg^{+2} showing 29, 38 and 52% decrease respectively. Our results are similar to those of Romaniec et al. (1992) who reported that activity of CMCase was inhibited by Hg^{+2}.

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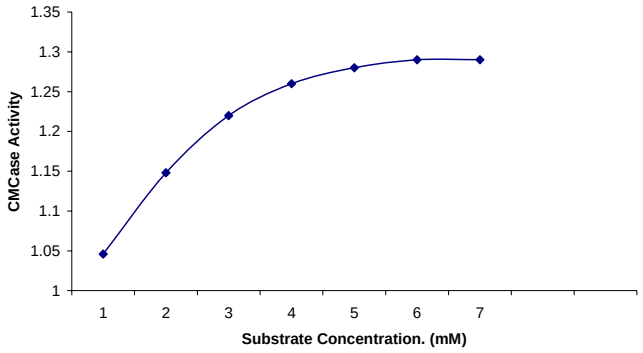

Figure 1. Michaelis-Menton graph showing effect of substrate concentration on CMCase activity

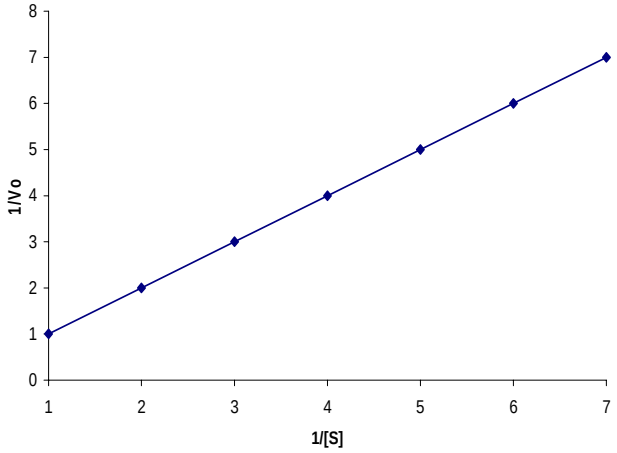

Figure 2. Lineweaver-Burk Plot for CMCase from *Trichoderma harzianum*

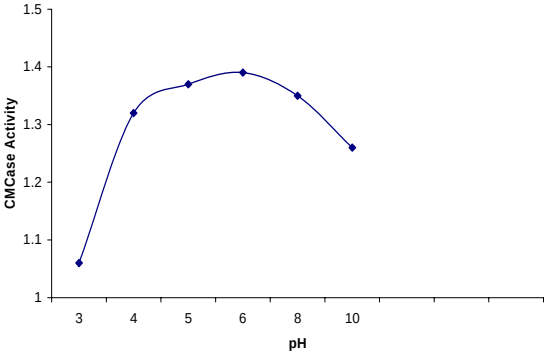

Figure 3. Effect of pH on CMCase activity from *Trichoderma harzianum*

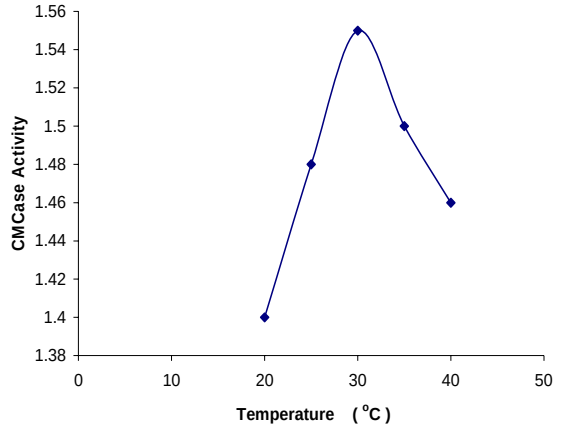

Figure 4. Effect of temperature on CMCase activity from *Trichoderma harzianum*

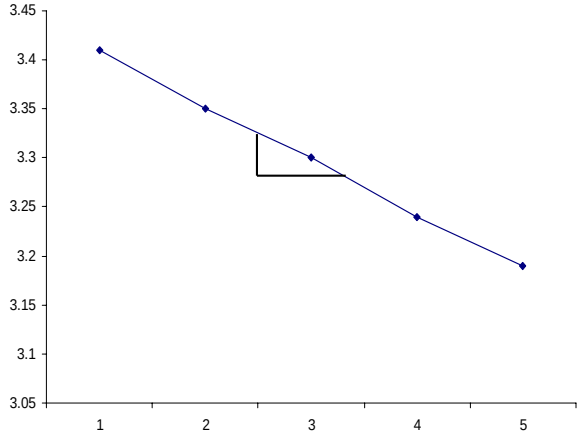

Figure 5. Arrhenius plot for activation energy of CMCase catalyzed reaction

Table 1. Effect of metal ions on CMCase activity from *Trichoderma harzianum*

Metal ion	Concentration	CMCase activity (IU/mL)
Ca^{2+}	0.0	1.0
	0.5	1.27
	1.0	1.93
	1.5	1.53
Co^{2+}	0.0	1.01
	0.5	1.73
	1.0	2.13
	1.5	1.6
Cu^{2+}	0.0	1.0
	0.5	0.67
	1.0	0.62
	1.5	0.54
Fe^{2+}	0.0	0.87
	0.5	0.79
	1.0	0.72
	1.5	0.67
Hg^{2+}	0.0	1.0
	0.5	0.71
	1.0	0.62
	1.5	0.48