Folia Microbiol. 42 (4), 303–311 (1997)

Purification and the Effect of Manganese Ions on the Activity of Carboxymethylcellulases from *Aspergillus niger* and *Cellulomonas biazotea*

K.S. SIDDIQUI*, M.J. AZHAR, M.H. RAŚHID, T.M. GHURI and M.I. RAJOKA

National Institute for Biotechnology and Genetic Engineering (NIBGE), P.O. Box 577, Faisalabad, Pakistan

Received October 15, 1996

ABSTRACT. Carboxymethylcellulases (CMCases) from *Aspergillus niger* and *Cellulomonas biazotea* were purified by a combination of ammonium sulfate precipitation, anion-exchange and gel-filtration chromatography with a 12- and 9-fold increase in the purification factor. The native and subunit molar mass of CMCase from *A. niger* were 40 and 25–57 kDa, respectively, while those from *C. biazotea* were 23 and 20–30 kDa, respectively. Low concentrations of Mn^{2+} activated the enzymes from both organisms (mixed activation) with apparent activation constants of 0.80 and 0.45 mmol/L of CMCases from *A. niger* and *C. biazotea*, respectively, while at higher CMC concentrations Mn^{2+} inhibited the enzymes (mixed and partial uncompetitive inhibition). The reason for this complex behavior is that more than one Mn^{2+} bind to the same enzyme form with the apparent average inhibition constants of 2.7 and 1.3 mmol/L for CMCases from *A. niger* and *C. biazotea*, respectively.

Carboxymethylcellulase (endo-β-1,4-glucanase, EC 3.2.1.4) is one of the component enzymes of the cellulase system which hydrolyzes the 1,4-β-D-glycosidic bonds within the cellulose molecules. Carboxymethylcellulases (CMCases) have been purified and characterized from a variety of fungi and bacteria, such as *Aspergillus niger* (IFO31125) (Akiba *et al.* 1995), *A. japonicus* (Kundu *et al.* 1988), *Cellulomonas flavigena* (Sami and Akhtar 1993), *Thermomonospora fusca* (Calza *et al.* 1985), *C. uda* (Nakamura and Kitamura 1983) and *Bacillus circulans* (Kim 1995). No work has yet been reported on the enzymology of CMCases from *C. biazotea*. In this paper we report on the purification and comparative effects of Mn^{2+} on the activity of CMCases from a fungus, *Aspergillus niger* NIAB280, and a bacterium, *C. biazotea* NIAB442. This is the first report of detailed kinetic analysis of the effect of Mn^{2+} on the activity of CMCases with a view to understanding the structure-function relationship of these enzymes.

MATERIALS AND METHODS

Organisms. Aspergillus niger strain NIAB280 and *Cellulomonas biazotea* strain NIAB442 were maintained using standard procedures.

CMCase production. C. biazotea was allowed to grow in optimized liquid salt medium containing Sigmacell 100 as a carbon source in shake flask cultures at 30 °C (Rajoka and Malik 1986). After 3 d of fermentation, the matter was removed by centrifugation at 3800 ***g*** for 5 min. The culture (4 L working volume) was then centrifuged at 25 900 ***g*** for 30 min at 4 °C and the cell pellet was discarded. The supernatant containing extracellular CMCase was dialyzed in Amicon hollow fiber cartridge (cut-off size 10–15 kDa) against 4 volumes of distilled water.

A. niger was grown in shake flasks at 30 °C for 15 d (Gokhale *et al.* 1988) using a mixture of (2 %, *W/V*) Sigmacell 100 and wheat bran as substrates in a 1:1 (*W/W*) ratio. At the end of the fermentation, the insoluble material was separated by centrifugation at 15 300 ***g*** for 1 h, the supernatant containing CMCase was dialyzed to remove low-molar-mass compounds and concentrated using an Amicon hollow fiber cartridge.

CMCase assay. All CMCase assays were performed in 50 mmol/L MOPS buffer (pH 7) at 40 °C for 30 min using 1 % (*W/V*) carboxymethylcellulose, sodium salt (CMC) as substrate, and an appropriate amount of the enzyme (Wood and Bhat 1988). The pH of the assay mixture changed after the addition of CMC, and it was readjusted to pH 7. After 30 min the reaction was stopped by adding 3 mL of 3,5-dinitrosalicylic acid (DNS) reagent, the mixture was boiled for 15 min, cooled in ice and absorbance at 550 nm was determined (Ng and Zeikus 1988). One unit of CMCase activity is defined as

*Corresponding author.

μmol glucose equivalents liberated per min. Unless stated otherwise all CMCase assays in this study were performed as described above.

Protein estimation. Total protein was estimated using bovine serum albumin as standard (Bradford 1976).

CMCase purification

Removal of polysaccharides. Noncovalently bound polysaccharides were removed from the enzymes of *A. niger* including CMCase by subjecting the crude extract to compartmental electrophoresis (Siddiqui *et al.* 1994). After compartmental electrophoresis, the sample from the anodic compartment containing polysaccharide-free CMCase was again dialyzed. The complete removal of polysaccharides from CMCase was checked as described earlier (Siddiqui *et al.* 1994).

Ammonium sulfate precipitation. In the case of *A. niger* solid ammonium sulfate was added to 735 mL of total concentrate to give a final $(NH_4)_2SO_4$ concentration of 30 % (*W/V*) and the solution was left overnight at 4 °C. After centrifugation at 15300 ***g*** for 15 min, the pellet of precipitated proteins was discarded while the supernatant was treated with 20 % (*W/V*) more solid $(NH_4)_2SO_4$ to give a final concentration of 50 %. The solution was kept overnight at 4 °C and once again centrifuged. This time the supernatant was discarded while the pellet containing CMCase was dialyzed to remove salts and freeze-dried.

In the case of *C. biazotea* solid ammonium sulfate was added to 500 mL of total concentrate to give a final concentration of 10 % and the solution was left overnight at 4 °C. After centrifugation at 15300 ***g*** for 15 min, the pellet of precipitated proteins containing CMCase was redissolved in water, dialyzed against water to remove salts and freeze-dried.

Fast protein liquid (FPLC) anion-exchange chromatography of crude extract (after ammonium sulfate precipitation) was performed as described by Rossomando (1990). The pooled fractions were dialyzed to remove salts.

FPLC gel filtration chromatography. The protein extract from each organism after FPLC ion-exchange chromatography was subjected to FPLC gel filtration chromatography (Stellwagen 1990) with the dual purpose of determining the purity and native molar mass of CMCases from both organisms. Different molar mass markers (carbonate dehydratase 29 kDa, chicken egg albumin 45 kDa, bovine serum albumin 66 kDa, alkaline phosphatase 100 kDa and alcohol dehydrogenase 150 kDa) were used for calibration.

Sodium dodecylsulfate (SDS) denaturing-renaturing polyacrylamide gel electrophoresis (SDS–DR–PAGE). Subunit molar mass of CMCases from both organisms was determined by subjecting *A. niger* and *C. biazotea* nonpurified crude extracts to SDS–PAGE (See and Jackowski 1989) using Hoeffer Mighty Small apparatus with the modification that CMCases were incubated in sample buffer containing 1 % (*W/V*) SDS at 50 °C for 60 min in the presence of 2-mercaptoethanol before loading on the gel. After electrophoresis, the gel was incubated for 45 min in 50 mmol/L Tris-HCl (pH 7) buffer containing 20 % 2-propanol for the removal of SDS. This step was repeated. 2-Propanol was then removed by incubating the gel in 50 mmol/L Tris-HCl (pH 7) buffer for 90 min. The gel was then stained for CMCase activity (Coughlan 1988). The part of the gel containing different molar mass markers was stained, after removing SDS, with Coomassie-R250 stain (Merril 1990).

Effect of Mn^{2+}. The CMCases from both organisms were dialyzed against the assay buffer containing 10 mmol/L EDTA to remove metal ions. The enzymes were then dialyzed against the assay buffer to completely remove EDTA. The type of CMCase activation/inhibition by Mn^{2+} was determined by varying CMC (%, *W/V*) concentration at different fixed Mn^{2+} concentrations (Dixon and Webb 1979). The data were plotted according to Lineweaver and Burk.

The type as well as the inhibition constants of Mn^{2+} for CMCases were determined by varying Mn^{2+} concentration at different fixed CMC concentrations (Dixon and Webb 1979). The data were plotted according to Dixon and Webb (1979). The apparent activation constants of Mn^{2+} for CMCases were determined by measuring the enzyme activity at different Mn^{2+} concentration and plotting the data after Rangarajan and Hartely (1992) and Siddiqui *et al.* (1993). It was observed that Mn^{2+} gave a light color with the DNS reagent which also absorbed at 550 nm; therefore, for each concentration of Mn^{2+} there was a corresponding reagent blank containing the same amount of Mn^{2+}.

RESULTS AND DISCUSSION

CMCase purification. The three-step purification procedure for the *C. biazotea* CMCase and four-step procedure for the *A. niger* CMCase (an additional step of compartmental electrophoresis for the removal of extracellular polysaccharides) resulted in an increase in specific activity of 9- and 12-fold, respectively (Table I). The CMCases from *Sclerotium rolfsii* (Lindner 1988), *C. uda* (Nakamura and Kitamura 1983), *C. flavigena* (Sami and Akhtar 1993) and *A. japonicus* (Kundu *et al.* 1988) were purified by four-step procedures with only 0.4-, 2.4-, 6-, and 9-fold increases in specific activity. CMCase from *C. biazotea* was sparingly soluble in buffer as it was salted out by only 10 % ammonium sulfate as compared with CMCase from *A. niger* (Fig. 1). CMCases from *A. niger* and *C. biazotea* (Fig. 2) eluted at 0.3 and 0.6 mmol/L NaCl, respectively, from Mono-Q anion-exchange column, thus indicating that the former enzyme is less negatively charged. It is reported that microbial CMCases tend to aggregate and even bind with gel matrices (Calza *et al.* 1985; Sami *et al.* 1988).

Table I. Purification of CMCases from *A. niger* and *C. biazotea*

Procedure	Volume mL	Concentration U/mL	Total units	Recovery %	Protein mg/mL	Specific activity, U/mg	Total protein
Crude (centrifuged)							
A. niger	3600	0.81	2911	100	0.38	2.15	1357
C. biazotea	2900	0.90	610	100	0.18	5.00	522
Concentrated and dialyzed							
A. niger	620	3.91	2421	83	1.77	2.21	1095
C. biazotea	500	1.00	516	84	0.46	2.20	230
Compartmental electrophoresis							
A. niger	735	2.13	1568	54	0.46	4.63	338
C. biazotea	—	—	—	—	—	—	—
$(NH_4)_2SO_4$ precipitation							
A. niger	72	5.93	427	15	0.64	9.26	46.1
C. biazotea	6	18	109	18	2.95	6.17	17.7
Ion-exchange chromatography							
A niger	9	5.67	51	1.7	0.32	17.7	2.89
C. biazotea	9	4.33	39	6.4	0.25	17.3	2.25
Gel filtration chromatography							
A. niger	2	5.68	11	0.4	0.22	26.3	0.43
C. biazotea	2	4.55	9	1.5	0.17	26.8	0.34

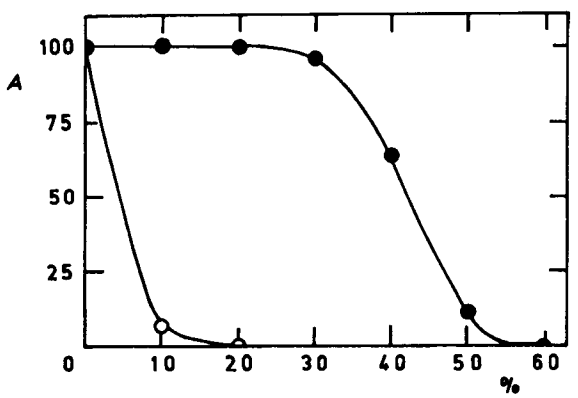


Fig. 1. The effect of varying ammonium sulfate concentration (%, *W/V*) on the precipitation of CMCases from *A. niger* (*closed circles*) and *C. biazotea* (*open circles*); A — CMCase activity, %.

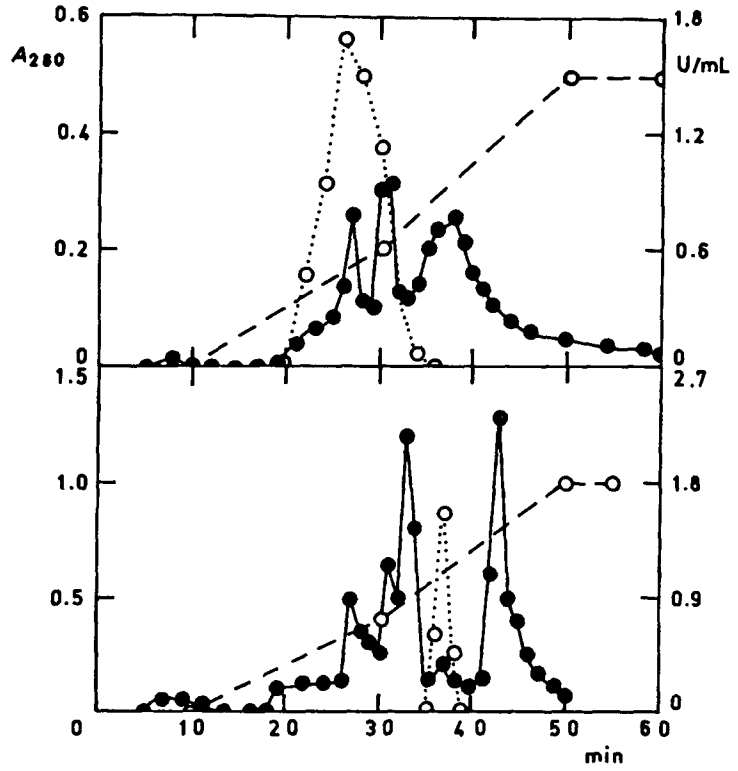


Fig. 2. FPLC anion-exchange chromatography of extracellular extract from *A. niger* (*top*) and *C. biazotea* (*bottom*) on a Mono-Q column at a flow rate of 1 mL/min. Buffer A: 25 mmol/L Tris-HCl (pH 7.5) + 1 mol/L NaCl; one mL fractions were collected. Fractions number 24 to 30 for *A. niger* and 36 to 38 for *C. biazotea* were pooled. *Solid line* — total proteins (A_{280}), *dotted line* — CMCase activity (U/mL).

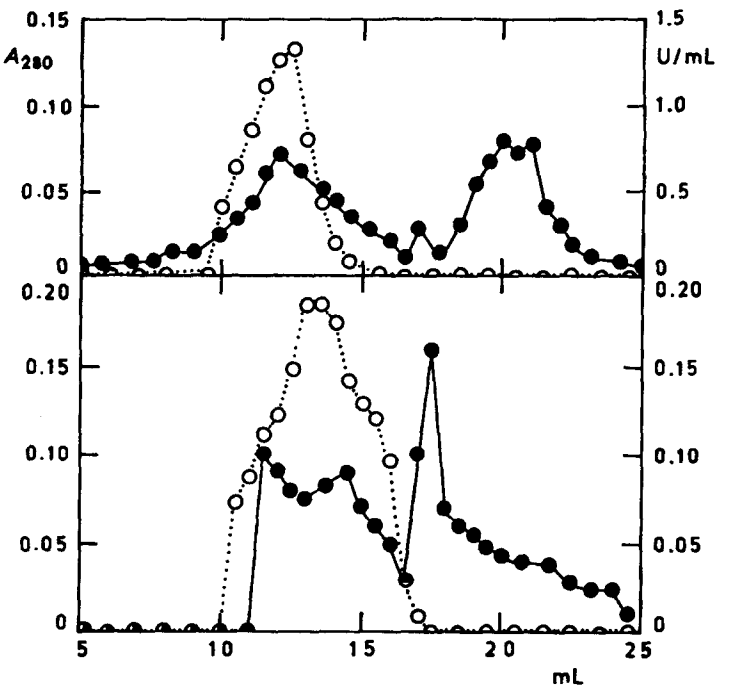


Fig. 3. FPLC gel filtration chromatography of extracellular extract from *A. niger* (*top*) and *C. biazotea* (*bottom*) on a Superose column in 50 mmol/L Tris-HCl (pH 7) at a flow rate of 0.5 mL/min. The distribution coefficient (KD) = $(V_e - V_o)/(V_i - V_o)$ where V_e are the retention volumes (mL) of CMCase from *A. niger* (12.5 mL) and *C. biazotea* (13 mL), V_o is the retention volume of blue dextran (7.9 mL), and V_i is the retention volume of tyrosine (21.2 mL). *Solid line* — total proteins (A_{280}), *dotted line* — CMCase activity (U/mL).

Native and subunit molar mass. The native molar mass of CMCases from the two organisms was determined from the gel filtration chromatogram (Fig. 3) and was found to be 23 and 40 kDa for *C. biazotea* and *A. niger*, respectively. The results regarding the native molar mass of CMCases, especially from *C. biazotea*, should be interpreted with caution due to possible interaction between gel matrix and CMCase.

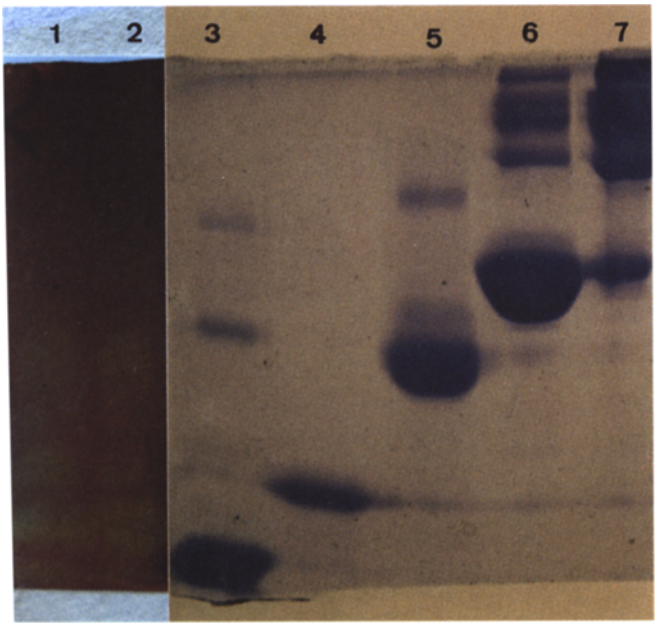


Fig. 4. SDS-denaturing-renaturing 7.5 % PAGE of extracellular protein extract from *A. niger* stained for CMCase activity for the determination of subunit molar mass. Lane 1 and 2 — CMCases (from *top* to *bottom*: 57, 42, 30 and 25 kDa). From lane 3 to 7 — molar mass markers stained by Coomassie Brilliant Blue. Lane 3 — trypsinogen (24 kDa), lane 4 — chymotrypsinogen (28), lane 5 — chicken egg albumin (45), lane 6 — monomer of bovine serum albumin (66), lane 7 — dimer of bovine serum albumin (132).

The denaturing-renaturing PAGE for the determination of subunit molar mass gave four bands ranging from 25 to 57 kDa for CMCases from *A. niger* (Fig. 4), whereas *C. biazotea* yielded two bands of 20 and 30 kDa (*result not shown*). These multiple bands could be the result of proteolytic cleavage (Sami *et al.* 1988) as 0–9 mmol/L TUG–PAGE of *A. niger* crude extract gave only two bands (*our unpublished results*). The CMCases from both organisms seem to be monomeric proteins. The molar mass of CMCases is 20.4 kDa for *C. flavigena* (Sami and Akhtar 1993), 75–82 kDa for *Bacillus circulans* (Kim 1995), 41 and 48 kDa for *Clostridium cellulolyticum* (Fierobe *et al.* 1993), 40 kDa for *A. niger* (Akiba *et al.* 1995), 45 kDa for *Sclerotium rolfsii* (Lindner 1988), and 57 kDa for *A. japonicus* (Kundu *et al.* 1988). All the above enzymes were also monomeric.

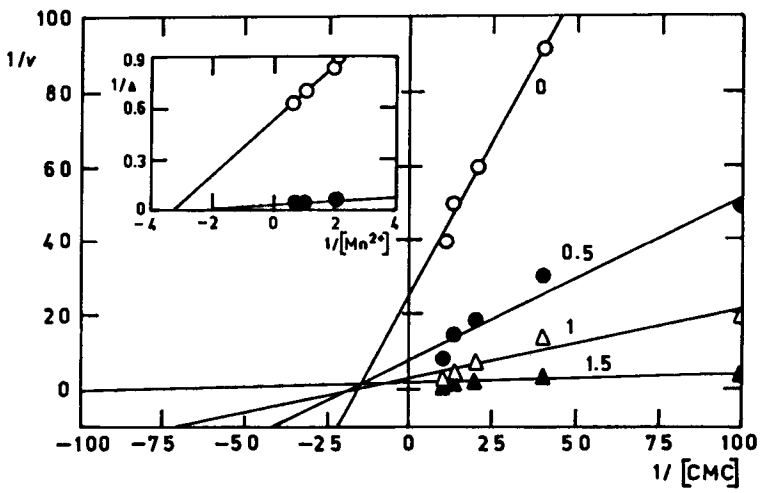


Fig. 5. Lineweaver–Burk plot of *A. niger* CMCase activation by Mn^{2+} (concentration, mmol/L, *numbers at lines*) showing mixed type of activation. Velocity *v* (U/mL) and [CMC] — % (*W/V*). *Inset graph*: secondary plots of reciprocal values of the change in slope (*open symbols*) and *y*-axis intercept (*closed symbols*) against $1/[Mn^{2+}]$ (both expressed as $1/\Delta$ where Δ is defined as slope or intercept in the absence of Mn^{2+} minus that in its presence). Both plots are linear, implying partial activation.

Effect of Mn^{2+}. Many workers have studied the effect of metal ions on the activity of CMCases from different organisms. These workers tested the enzyme activity at a single metal ion concentration. Some metal ions activated while others inhibited the CMCase activity. Nickel (Ni^{2+}) and cobalt (Co^{2+}) activated CMCase from *Cellulomonas* sp. (Sexana *et al.* 1992) while they had no effect on the enzyme from *A. niger* IFO31125 (Akiba *et al.* 1995). Similarly, 1 mmol/L Mn^{2+} activated the enzyme from *C. uda* (Nakamura and Kitamura 1983) but inhibited CMCase from *A. japonicus* (Kundu *et al.* 1988). We studied the effect of varying Mn^{2+} and CMC-Na concentration on the activity of CMCases from both organisms in order to determine the type, as well as activation/inhibition constants. The CMCases were activated at lower and inhibited at higher Mn^{2+} concentrations.

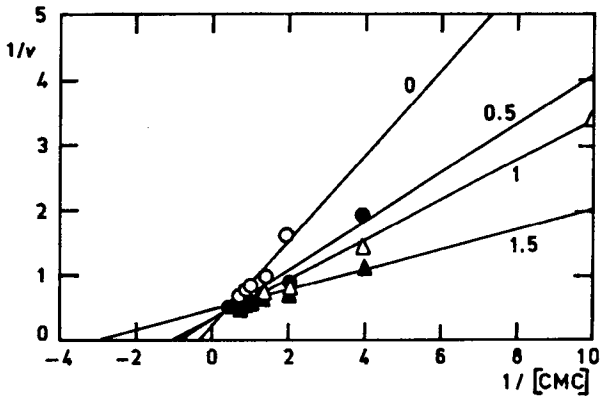


Fig. 6. Lineweaver–Burk plot of *A. niger* CMCase inhibition by (Mn^{2+}) at high CMC concentrations, showing partial uncompetitive type of inhibition with *v* given in U/mL) and [CMC] in %; *numbers at lines* — Mn^{2+} concentration, mmol/L.

CMCase from *A. niger* was activated up to 1.5 mmol/L Mn^{2+} (partial mixed type) at low CMC concentrations (Fig. 5) but, at the same Mn^{2+} concentration and higher CMC concentrations the enzyme activity was inhibited (partial uncompetitive type; Fig. 6). This shows that at high CMC concentration Mn^{2+} cannot combine with free enzyme but only with the enzyme-substrate complex (Dixon and Webb 1979). This seems reasonable as Mn^{2+} could act as a bridge between carboxyl groups of the substrate and enzyme. This line of reasoning is also strengthened because it is reported that lower concentrations of Ca^{2+} activated while higher concentrations inhibited CMCase activity from *Cellulomonas flavigena* and had no effect on the avicelase activity (Sami *et al.* 1988). Moreover, the thermostability of CMCases from both organisms was not affected in the presence of 0.5 mmol/L Mn^{2+} (*our unpublished results*), also indicating that Mn^{2+} only bind to the active-site pocket in the presence of CMC.

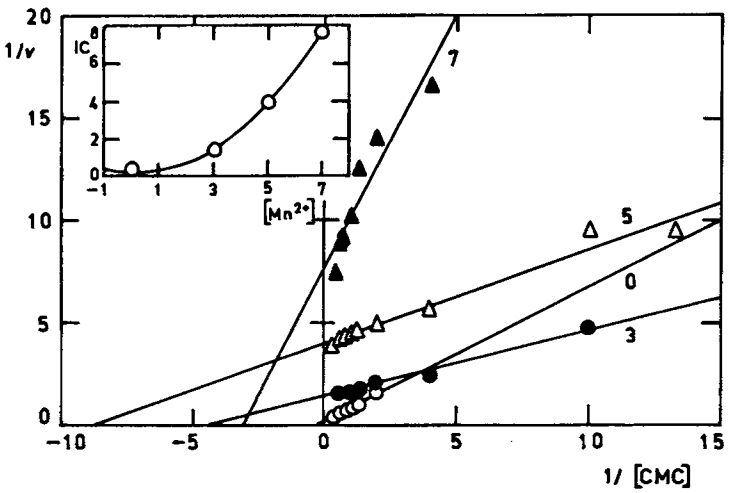


Fig. 7. Lineweaver–Burk plot of *A. niger* CMCase inhibition by high concentrations of Mn^{2+} (mmol/L, *numbers at lines*), at high CMC concentration showing a mixture of partial uncompetitive and mixed type of inhibition where *v* (U/mL) and [CMC], %. *Inset*: secondary plot of intercept on 1/*v* axis (IC) *vs.* [Mn^{2+}] gives a parabola showing that more than one Mn^{2+} binds to the same form of the enzyme.

At both high Mn^{2+} (3–7 mmol/L) and CMC concentrations the inhibition became complex and showed a mixture of partial uncompetitive and mixed types (Fig. 7). The reason is apparent if we look at the secondary graph (*inset graph*, Fig. 7), which shows a parabola, thus indicating that more than one Mn^{2+} atoms bind to the same enzyme form (Dixon and Webb 1979). A Dixon plot of *A. niger* CMCase indicated a mixed type of inhibition with an apparent average inhibition constant of 2.7 mmol/L for Mn^{2+} (Fig. 8). The apparent average activation constant of *A. niger* CMCase for Mn^{2+} is 0.8 mmol/L (Fig. 9).

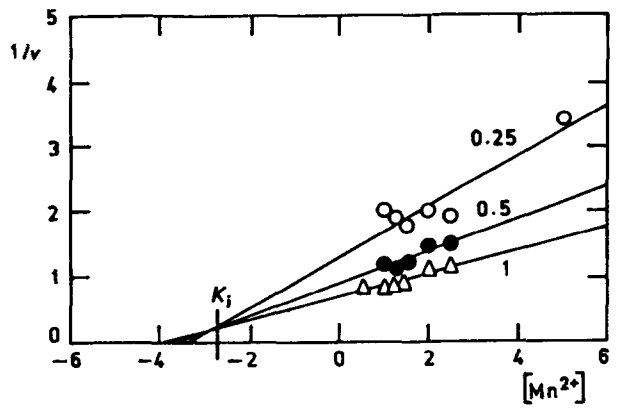


Fig. 8. Dixon plot of *A. niger* CMCase (*%, numbers at lines*) inhibition by Mn^{2+} showing a mixed type of inhibition. The intersection point above [Mn^{2+}] axis corresponds to the inhibition constant (K_i).

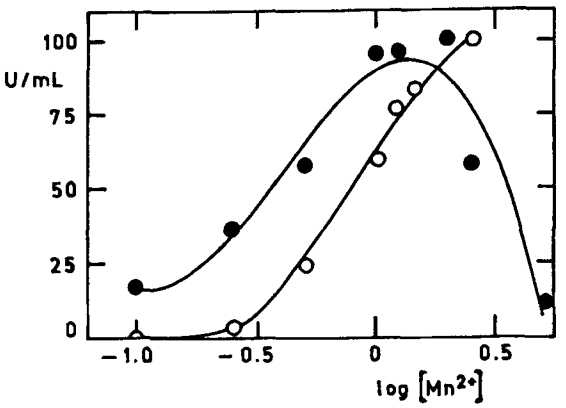


Fig. 9. Apparent activation constants of CMCases from *A. niger* (*open circles*) and *C. biazotea* (*closed circles*). The activation constant corresponds to that Mn^{2+} concentration which gives a 50 % increase in CMCase activity (U/mL, % of activity) as compared with the enzyme without any Mn^{2+}.

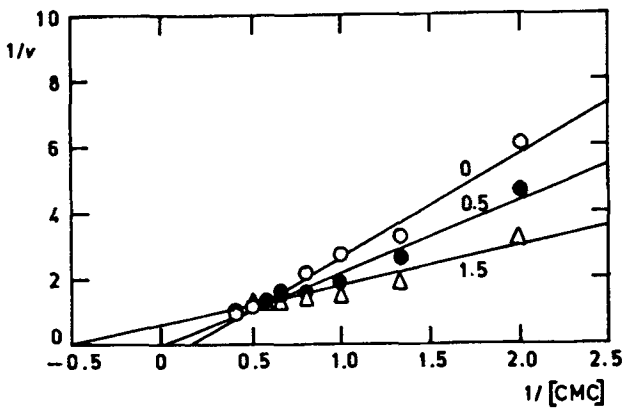


Fig. 10. Lineweaver–Burk plot of *C. biazotea* CMCase activation and inhibition by low concentrations of Mn^{2+} (mmol/L, *numbers at lines*) at high [CMC], showing partial uncompetitive type of activation/inhibition where *v* (U/mL) and [CMC], %.

The CMCase from *C. biazotea* was activated at 0.5 mmol/L Mn^{2+} and inhibited at 1.5 mmol/L (partial uncompetitive type; Fig. 10). At higher Mn^{2+} concentrations (3 and 5 mmol/L) the inhibition was of partial mixed type with $K_i > K_i'$ because the lines intersect below the 1/[CMC] axis (Dixon and Webb 1979) (Fig. 11 and Scheme I). This also shows that Mn^{2+} has a greater affinity for the enzyme-substrate complex (ES) than the free enzyme. The secondary graph (*inset graph*, Fig. 11) is again parabolic, implying that more than one Mn^{2+} binds to the same enzyme form.

A Dixon plot of the *C. biazotea* CMCase indicated an uncompetitive type of inhibition with an apparent average inhibition constant (K_i) of 1.3 mmol/L (Fig. 12). The apparent average activation constant of *C. biazotea* CMCase for Mn^{2+} was 0.45 mmol/L (Fig. 9).

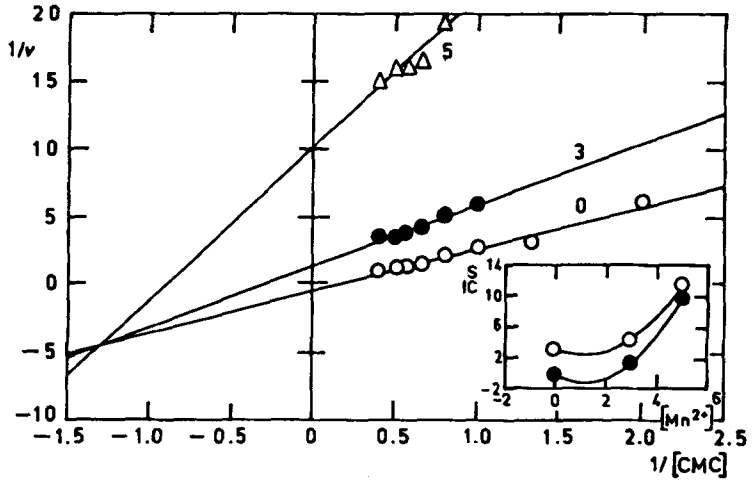
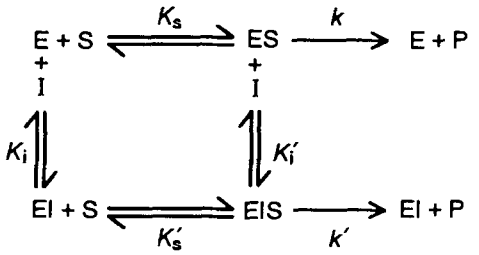


Fig. 11. Lineweaver–Burk plot of *C. biazotea* CMCase inhibition by high concentrations of $[Mn^{2+}]$ (mmol/L, *numbers at lines*) at high [CMC], showing partial mixed type of inhibition where v (U/mL) and [CMC], %. *Inset:* secondary plots of intercept on $1/v$ axis (IC, *closed circles*) and slopes (S, *open circles*) *vs.* $[Mn^{2+}]$ gives a parabola showing that more than one Mn^{2+} binds to the same form of the enzyme.



Scheme I. E = CMCase; S = CMC; I (inhibitor = Mn^{2+}); P = product; k and k' are rate constants and K_i and K_i' are inhibition constants; in this case of partial mixed inhibition $K_i > K_i'$ and $k' < k$.

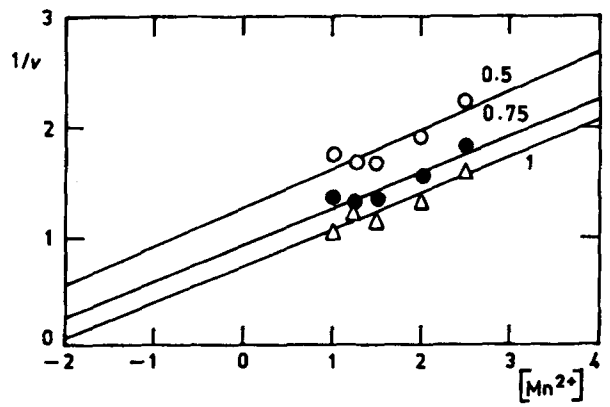


Fig. 12. Dixon plot of *C. biazotea* CMCase (*numbers at lines* — CMCase concentration, %) inhibition by Mn^{2+} (mmol/L) showing uncompetitive inhibition. The slope $= 1/V \cdot K_i = 0.34$.

The K_m of CMCase from *A. niger* at low substrate concentrations was 0.07 % (Fig. 5). The abnormally high K_m (5 mmol/L) of CMCase from *C. biazotea* (Fig. 10 and 11) could be explained on the basis of inhibition by phenolics secreted in the medium by the organism as reported by Bae *et al.* (1993) for CMCase from *Fibrobacter succinogenes* S85. Lupo and Stutzenberger (1988) have also reported abnormally high K_m values, ranging from 1.3 to 8.3 % for CMCases from *Thermomonospora fusca*.

It could be concluded that Mn^{2+} first binds at a site away from the active site of CMCases from both organisms, thereby changing the conformation of the enzyme and hence activating the

enzyme. Furthermore, binding of at least one Mn^{2+} at the active site requires that CMC is already bound, the $E-Mn^{2+}-CMC$ complex then producing inhibition by blocking active-site carboxyls.

The work is a part of the MSc project of Mr. Javed Azhar and was financed by PAEC. We wish to acknowledge Director *NIBGE*, Dr. K.A. Malik, for providing research facilities. We are thankful to Mr. Tanvir Ahmad for his help in computer graphics. The technical assistance of Mr. G.A. Wasser is appreciated.

REFERENCES

AKIBA S., KIMURA Y., YAMAMOTO K., KUMAGAI H.: Purification and characterization of a protease-resistant cellulase from *Aspergillus niger*. *J.Ferment.Bioeng.* **79**, 125–130 (1995).

BAE H.D., MCALLISTER T.A., YANKE J., CHENG K.J., MUIR A.D.: Effects of condensed tannins on endoglucanase activity and filter paper digestion by *Fibrobacter succinogenes* S85. *Appl.Environ.Microbiol.* **59**, 2132–2138 (1993).

BRADFORD M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal.Biochem.* **72**, 248–254 (1976).

CALZA R.E., IRWIN D.C., WILSON D.B.: Purification and characterization of two β-1,4-endoglucanases from *Thermomonospora fusca*. *Biochemistry* 24, 7797–7804 (1985).

COUGHLAN M.P.: Staining techniques for the detection of the individual components of cellulolytic enzyme system. *Methods Enzymol.* **160**, 135–144 (1988).

DIXON M., WEBB E.C.: Enzyme inhibition and activation, pp. 332–467 in *Enzymes*. Academic Press, New York 1979.

FIEROBE H.P., BAGNARATARDIF C., GAUDIN C., GUERLESQUIN F., SAUVE P., BELAICH A., BELAICH J.P.: Purification and characterization of endoglucanase-C from *Clostridium cellulolyticum* — catalytic comparison with endoglucanase-A. *Eur.J. Biochem.* **217**, 557–565 (1993).

GOKHALE D.V., PUNTAMBEKAR U.S., DEOBAGKAR D.N., PEBERDY J.F.: Production of cellulolytic enzyme by mutants of *Aspergillus niger* NCIM1207. *Enzyme Microb.Technol.* **10**, 442–445 (1988).

KIM C.: Characterization and substrate specificity of an endo-β-1,4-D-glucanase 1 (avicelase 1) from an extracellular multienzyme complex of *Bacillus circulans*. *Appl.Environ.Microbiol.* **61**, 959–965 (1995).

KUNDU R.K., DUBE S., DUBE D.K.: Extracellular cellulolytic enzyme system of *Aspergillus japonicus*. 3. Isolation, purification and characterization of multiple forms of endoglucanase. *Enzyme Microb.Technol.* **10**, 100–109 (1988).

LINDNER W.A.: Carboxymethyl cellulase from *Sclerotium rolfsii*. *Methods Enzymol.* **160**, 376–382 (1988).

LUPO D., STUTZENBERGER F.: Changes in endoglucanase patterns during growth of *Thermomonospora curvata* on cellulose. *Appl.Environ.Microbiol.* **54**, 588–589 (1988).

MERRIL C.R.: Gel staining techniques. *Methods Enzymol.* **182**, 477–488 (1990).

NAKAMURA K., KITAMURA K.: Purification and some properties of a cellulase active on crystalline cellulose from *Cellulomonas uda*. *J.Ferment.Technol.* **61**, 379–382 (1983).

NG T.K., ZEIKUS J.G.: Endoglucanase from *Clostridium thermocellum*. *Methods Enzymol.* **160**, 351–355 (1988).

RAJOKA M.I., MALIK K.A.: Comparison of different strains of *Cellulomonas* for production of cellulolytic and xylanolytic enzymes from biomass produced on saline lands. *Biotechnol.Lett.* **8**, 753–756 (1986).

RANGARAJAN M., HARTLEY B.S.: Mechanism of D-fructose isomerization by *Arthrobacter* D-xylose isomerase. *Biochem.J.* **283**, 223–233 (1992).

ROSSOMANDO E.F.: Ion-exchange chromatography. *Methods Enzymol.* **182**, 309–317 (1990).

SAMI A.J., AKHTAR M.W.: Purification and characterization of two low-molecular weight endoglucanases of *Cellulomonas flavigena*. *Enzyme Microb.Technol.* **15**, 586–592 (1993).

SAMI A.J., AKHTAR M.W., MALIK N.N., NAZ B.A.: Production of free and substrate-bound cellulases of *Cellulomonas flavigena*. *Enzyme Microb.Technol.* **10**, 626–631 (1988).

SEE Y.P., JACKOWSKI G.: Estimating molecular weights of polypeptides by SDS-gel electrophoresis, pp. 1–21 in *Protein Structure: A Practical Approach* (T.E. Creighton, Ed.). IRL Press, Oxford (UK) 1989.

SEXANA S., BAHADUR J., VERMA A.: Effect of cobalt and nickle on growth and carboxymethylcellulase activity of *Cellulomonas* spp. *Biometals* 5, 209–212 (1992).

SIDDIQUI K.S., LOVINY-ANDERTON T., RANGARAJAN M., HARTELY B.S.: *Arthrobacter* D-xylose isomerase: chemical modification of carboxyl groups and protein engineering of pH optimum. *Biochem.J.* **296**, 685–691 (1993).

SIDDIQUI K.S., RASHID H., SHEMSI A.M., RAJOKA M.I.: A simple and nondestructive method for the separation of polysaccharides from β-glucosidase produced extracellularly by *Aspergillus niger*. *Enzyme Microb.Technol.* **16**, 912–917 (1994).

STELLWAGEN E.: Gel filtration. *Methods Enzymol.* **182**, 317–328 (1990).

WOOD T.M., BHAT K.M.: Methods for measuring cellulase activities. *Methods Enzymol.* **160**, 87–112 (1988).