

An Endo-Beta-1, 4-Glucan Hydrolase from Germinated Barley

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ABSTRACT

An endo-beta-1, 4-glucan hydrolase present in sodium chloride extracts of germinated barley was purified over 100-fold by dialysis, chromatography on DEAE-cellulose, redialysis, and rechromatography on DEAE-cellulose. Disc electrophoresis revealed one major and eight minor proteins. The activity of the preparation on carboxymethyl cellulose had the following characteristics: a pH optimum of 5.0, stimulation by sodium chloride with maximum stimulation at 0.08M, stable at 40°C., and rapidly inactivated at 60°C., inhibited by cellobiose, glucono-1,5-lactone, and N-bromosuccinimide, not inhibited by phenylmercuric nitrate and several other compounds. The preparation also hydrolyzed barley beta-D-glucan but showed no action on insoluble cellulose or laminarin.

Beta-1,4-glucan hydrolases that cause a rapid decrease in the viscosity but only a slow increase in reducing power of carboxymethyl cellulose (CMC) solutions are referred to as endo-beta-1,4-glucan hydrolases or as endo-carboxymethyl cellulases. Such enzymes have been observed in germinated barley (1,2). Several other beta-glucosidases have been found in germinated barley, such as endo-barley-beta-glucan hydrolases² (2,3), exo-barley-beta-glucan hydrolase² (4,5), laminarinase (6,7), cellobiase (5), and transglycosidase (8). The work on the beta-glucosidases of germinated barley has been well covered in reviews (9,10,11) and a book (12).

Endo-beta-1,4-glucan hydrolase (E-CMC) of germinated barley has been shown to be distinct from two endo-barley-beta-glucan hydrolases (EBG) of germinated barley on the basis of heat stability and column separations (1,2,13). In addition, the specificities of the two types of glucan hydrolases are different. EBG acts on barley beta-D-glucan but not on CMC nor laminarin while E-CMC acts on CMC and barley beta-D-glucan but not on laminarin. E-CMC is stable at 40°C. (2) and has a pH optimum of 5.0 as did a carboxyethyl cellulase reported by Sandegren and Enebo (14).

The purpose of this paper is to describe a method for purifying an endo-beta-1,4-glucan hydrolase from germinated barley and to describe some of its properties.

EXPERIMENTAL

Enzymes

Germinated Barley Enzymes. A 0.1M NaCl extract of germinated barley was prepared (crude extract) and dialyzed to prepare albumin according to the procedure of Luchsinger and Richards (13).

Cellulase

Technical grade cellulase (4,000 units per g., Nutritional Biochemicals Corp., Cleveland, Ohio) was dissolved in distilled water to yield 10 units per ml. It was found that one unit of this commercial cellulase was equivalent to 50 units of barley E-CMC activity.

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²The original authors referred to these enzymes as glucanases rather than glucan hydrolases.

Substrates

Crude barley beta-glucan (8.6 g.) (15) was added to 500 ml. of hot 0.09M NaCl and blended until dissolved. The pH was adjusted to 4.7.

Purified barley beta-glucan (1.2 g.) (16) was added to 100 ml. of hot 0.09M NaCl and blended until dissolved. The pH was adjusted to 4.7.

Carboxymethyl cellulose was a gift of the Hercules Powder Co., Wilmington, Delaware. When used as a substrate for E-CMC assays, 3.0 g. of CMC-7HP was dissolved by blending in 500 ml. of hot 0.09M NaCl. The pH was adjusted to 5.0. When used as a substrate for reducing power determinations, 1.9 g. of CMC-4MP was dissolved by blending in 100 ml. of hot 0.09M NaCl and the pH adjusted to 5.0.

Laminarinase activity was measured on a solution containing 0.714 g. of laminarin (Institute of Seaweed Research, Inveresk, Midlothian, Scotland) per 100 ml. of 0.077M NaCl, heated to 55°C. before use, and adjusted to pH 4.6.

The cellulose substrate employed was prepared from white paper blotters (Zenith blotters). Suspensions of the blotters were prepared by blending 5 g. of cellulose per 500 ml. of hot distilled water until a suitable suspension was obtained. Cellulase activity was measured by following the reducing power.

Ten grams of soluble starch was dissolved in 1 liter of 0.02M sodium acetate-0.06M NaCl, pH 4.8.

Protein Determination

Protein concentration was estimated from the 260 and 280 μ absorptions of the solutions with a formula suggested by Lowry and presented by Kalckar (17): using a 1 cm. light path, mg. protein per ml. = (1.45) (Abs. at 280) - (0.74) (Abs. at 260).

Enzyme Activity Determinations

Assay solutions consisted of 7 ml. of substrate, 2 ml. of 0.2M sodium acetate buffer pH 5.0, and 1 ml. of enzyme, and were incubated at 30°C. When studying the effects of salt or inhibitors on the reaction rates, 6 ml. of substrate, 2 ml. of buffer, 1 ml. of enzyme, and 1 ml. of the salt or inhibitor solution were used.

One unit of endo-activity is the change in the reciprocal specific viscosity of a 10-ml. reaction mixture per 30 min. measured in an Ostwald No. 200 viscosimeter. Reducing power was measured by a modified dinitrosalicylic acid method (18) and was expressed as milligrams of glucose per milliliter of enzyme solution. Laminarinase activity was expressed as the increase in the reducing power of a laminarin solution during 1 hr. of incubation with the enzyme. Reducing power on barley beta-glucan and on CMC-4MP (RP-BG and RP-CMC respectively) was expressed as the increase in reducing power for a 1-hr. incubation period.

Amylase activity was measured by following the increase in reducing power of a reaction mixture that consisted of 9 ml. of soluble-starch substrate and 1 ml. of the enzyme. Since this method will detect both alpha- and beta-amylase, beta-amylase was distinguished from alpha-amylase by hydrolyzing the starch for 3 days and separating the products by descending paper chromatography using Whatman No. 1 paper irrigated with n-butanol-pyridine-water (10:3:3) for 4 days. The appearance of a maltose spot but no glucose indicated that only beta-amylase was present. When both maltose and glucose appeared, it was concluded that alpha-amylase was present, although beta-amylase could also be present in this case.

Cellobiase activity was determined by the glucostat procedure described in a pamphlet by Worthington Biochemicals Corp., 1963, as modified by Luchsinger and Richards (19). Typically, cellobiase assay solutions contained 2.5 ml. of substrate (12 mg. per ml.), 1.0 ml. of 0.2M acetate buffer pH 4.7, and 1.0 ml. of enzyme in a total volume of 5.0 ml. One-milliliter aliquots were assayed for glucose at appropriate time intervals. Enzyme activity was expressed as milligrams of glucose produced in 60 min. per milliliter of enzyme.

Qualitative Determination of Products Formed during Hydrolysis of Substrates

Tests were carried out by incubating 1.0 ml. of enzyme with 1.0 ml. of substrate, 0.5 ml. of 0.2M acetate buffer pH 5.0, and 2 drops of toluene as a preservative. The mixtures were allowed to incubate for 3 days at 22° to 25°C. after which the reactions were stopped by heating for 15 min. in a boiling water bath. Any insoluble material was removed by centrifugation. Samples were desalted by electro dialysis and an amount containing 1.0 mg. of carbohydrate was spotted on Whatman No. 1 paper. Glucose, laminaribiose, cellobiose, gentiobiose, and maltose were used as guide sugars. The chromatograms were irrigated for 4 days in n-butanol-pyridine-water (10:3:3). The carbohydrates were located by spraying with a solution containing equal volumes of 2M AgNO₃ and concentrated ammonia and heating at 105°C. until the color developed.

Ion-Exchange Resins

P-Cellulose and DEAE-cellulose (Schleicher and Schuell Co.) were prepared as described by Peterson and Sober (20) except that the P-cellulose was washed with 0.1N HCl and 0.1N NaOH instead of 1N reagents, and the DEAE-cellulose was adjusted to approximately pH 12 with alkali, washed with distilled water until pH 8 was reached, and suspended in distilled water.

Electrophoresis

The disc electrophoretic apparatus used was patterned after Ornstein and Davis (Distillation Products Industry, Rochester, New York)³. The acrylic gel columns (65 mm. X 7 mm.) contained 7½% polyacrylamide gel (separating gel pH 8.9) and 4% polyacrylamide gel (spacer gel pH 6.7). Tris-glycine buffer (0.001M) pH 8.4 was used on the column which was subjected to a current of 5 mA. The tracking dye was 0.001% Bromophenol blue, and 0.7% Amidoschwartz was employed for staining the protein. This dye will detect as little as 4 to 10 γ of protein. Excess dye was removed by passing 8 mA and 7% acetic acid through the column for 2 hr. Samples ran from the cathode to the anode.

RESULTS

Preparation of E-CMC. Previous work indicated that ammonium sulfate fractionation of germinated barley extracts to concentrate the E-CMC activity in fractions precipitating at concentrations above 50% saturation (2) followed by chromatography of the fractions on P-cellulose (13) should result in highly purified E-CMC. However, separation of the ammonium sulfate fractions on P-cellulose was

³The disc electrophoresis apparatus was constructed by Vito Expisito and Valentine Ulrich of the Agronomy and Plant Genetics Department, West Virginia University.

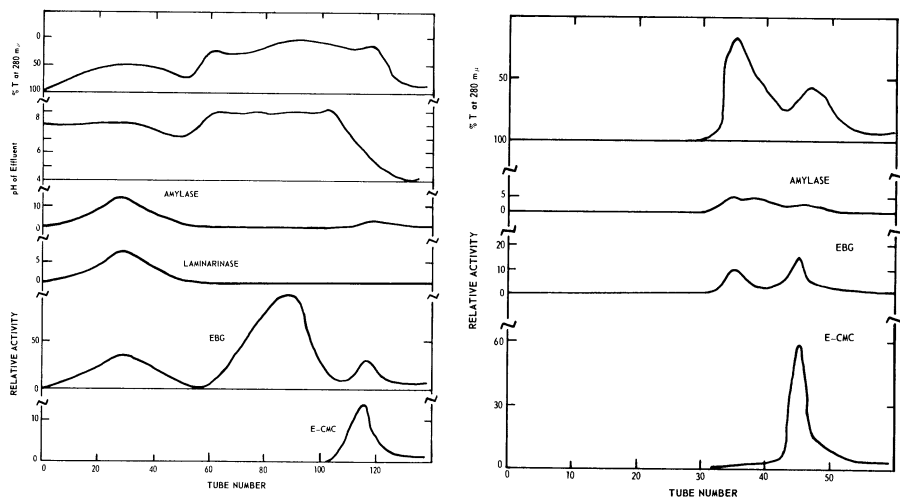


Fig. 1 (left). Chromatographic separation of albumin on DEAE-cellulose. Enzyme samples containing approximately 600 to 900 mg. of protein were applied on columns 2.0 cm. by 30 to 35 cm. Distilled water was used to elute the front (the first 280 $m\mu$ absorption peak). The first gradient consisted of 400 ml. of distilled water and 400 ml. of 0.1M NaCl and ran from tube 45 to 74. The second gradient consisted of 500 ml. of 0.06M NaCl and 500 ml. of 0.06M NaCl-0.1M sodium phosphate buffer pH 4.0 and ran from tube 74 to 130. A buffer of 0.25M sodium phosphate-0.06M NaCl pH 3.8 was used from tube 130 to the end of the run. Fifteen milliliters of effluent was collected in each tube.

Fig. 2 (right). Chromatographic separation of crude E-CMC on DEAE-cellulose. Samples containing approximately 100 to 125 mg. of protein were placed on columns of 1.2 cm. by 30 to 35 cm. The same gradients were employed as in Fig. 1, except that the first gradient was omitted. The second gradient was started at tube 15. Ten milliliters of effluent was collected in each tube.

not an effective way to produce E-CMC because the E-CMC was eluted over a wide range of tubes (21). An effective way to prepare E-CMC was to dialyze the ammonium sulfate fractions and then separate them on DEAE-cellulose (21).

A higher yield of enzyme was obtained by a different procedure which consisted of dialysis of crude extract, separation of the resulting albumin on DEAE-cellulose, redialysis and reseparation of the crude E-CMC on DEAE-cellulose. A typical separation of albumin on DEAE-cellulose is shown in Fig. 1. A column, 2 cm. i.d., was used. The albumin was mixed with DEAE-cellulose and added to a column already containing a DEAE-cellulose base of 7 cm. The total height of the DEAE-cellulose column was 35 cm. before elution of the enzymes. Enzyme activity measurements were made on every third or fourth tube. Tubes 110 to 120 were combined and labeled crude E-CMC.

The crude E-CMC was dialyzed against five 30-vol. aliquots of distilled water (approximately 8 hr. against each aliquot) and re-separated on DEAE-cellulose. A typical separation is shown in Fig. 2. A column, 1.2 cm. i.d., was used. The enzyme was mixed with DEAE-cellulose and placed on a 5-cm. DEAE-cellulose base to yield a total column of approximately 35 cm. before elution of the enzyme. The pH profile was similar to that in Fig. 1. Approximately 90% of the E-CMC activity was recovered from the column. Tubes 43 through 47 were assayed for E-CMC and EBG

TABLE I. PURIFICATION OF BETA-1,4-GLUCAN HYDROLASE ACTIVITY (E-CMC) FROM BARLEY MALT

Fraction	Beta-1,4-Glucan Hydrolase				Reducing Power Activity per ml. ^a Substrate	
	Units per ml.	mg. protein per ml.	Specific activity	Recovery, % of crude extract	Laminarin	Soluble starch
Crude extract	19.6	37.00	0.53	100	5.4	360.0
Albumin	11.2	5.30	2.10	75	4.3	33.3
Crude E-CMC	12.0	0.48	25.00	75	0.0	1.4
Reseparated E-CMC	8.7	0.13	67.00	68	0.0	1.0

^aReducing power activity on CMC, BG, and cellobiose was below the limit of the method during a 60-min. incubation; however, there was an increase in reducing power on all three of these substrates when longer incubation times were used.

activities. The ratio of E-CMC to EBG activity was approximately three in every tube showing the identical elution patterns for the two activities. Tubes 43 to 50 were combined, labeled reseparated E-CMC, and frozen. The enzyme was stable for at least three freezings and thawings and also for at least 4 days at 4°C. The specific activities of the E-CMC at various stages of the purification and the relative freedom of the preparation from laminarinase and amylase activities are shown in Table I.

Reseparated E-CMC was subjected to disc electrophoresis on polyacrylamide gel at a pH of 8.4. The proteins ran from the cathode to the anode. If 7 γ of protein was applied, one band was observed. If 40 γ of protein was applied and separated under the same conditions, one main band and eight minor bands were observed.

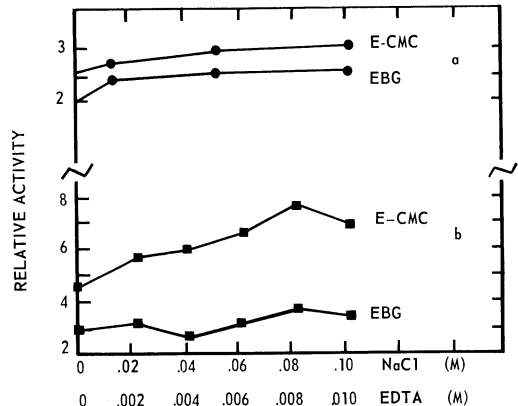
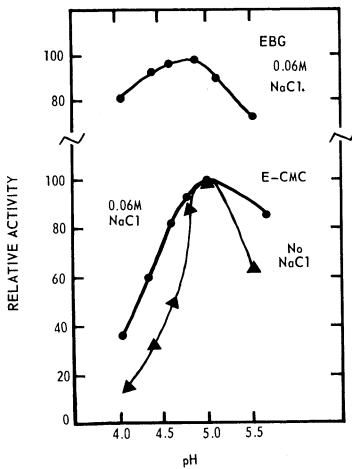


Fig. 3 (left). Effect of pH and NaCl on the E-CMC activities of the crude E-CMC. (●) With 0.06M NaCl. (▲) Without NaCl. The assay methods are described in the "Experimental" section.

Fig. 4 (right). Effect of NaCl and EDTA on the E-CMC and EBG activities. (a) Effect of EDTA on crude E-CMC. (b) Effect of NaCl on reseparated E-CMC. The assay methods are described in the "Experimental" section.

Effects of Various Agents and Conditions on E-CMC Activity

The effect of pH on the activity of crude E-CMC over a range of 4.0 to 5.5 is shown in Fig. 3. The effect of sodium chloride on the pH curve is also shown. When studying the effect of sodium chloride, enzyme samples were dialyzed five times vs. distilled water in the manner described in the experimental section, and the substrate was prepared without sodium chloride. The activities were measured in 0.04M sodium acetate buffer. The final pH of the reaction mixture was plotted.

The effects of sodium chloride on the E-CMC and EBG activities of resealed E-CMC, and the effects of EDTA (disodium ethylene diamine tetraacetate) on the E-CMC and EBG activities of the crude E-CMC are shown in Fig. 4. The effects of heating on the EBG and E-CMC activities of resealed E-CMC are shown in Fig. 5. The effects of inhibitors on the E-CMC and EBG activities of resealed E-CMC

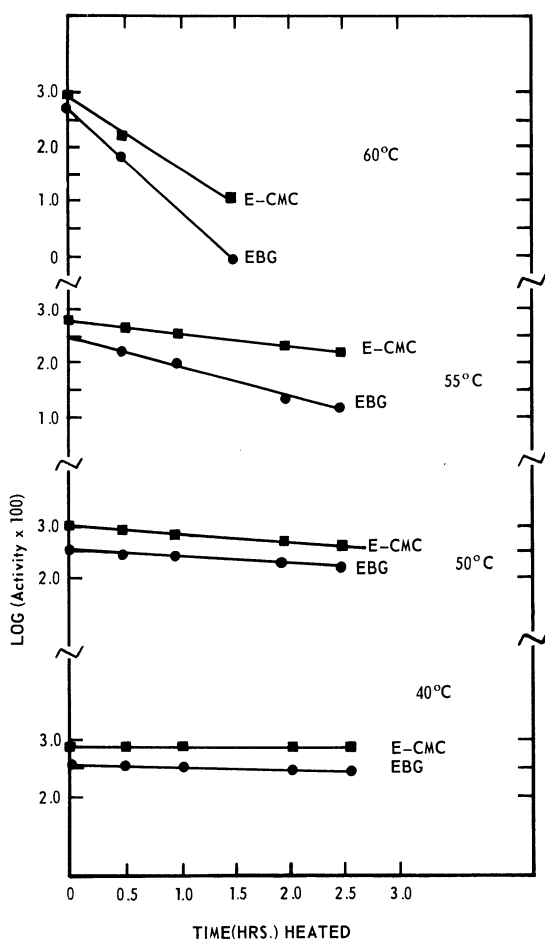


Fig. 5. Effect of temperature on the E-CMC and EBG activities of resealed E-CMC. The assay methods are described in the "Experimental" section.

TABLE II. EFFECTS OF INHIBITORS ON E-CMC AND EBG ACTIVITIES OF RESEPARATED E-CMC^a

Compound ^b	E-CMC	EBG
	% Inhibition	% Inhibition
Control	0	0
Cellobiose (1%)	65	22
Cellobiose (0.1%)	10	0
Glucono-1,5-lactone (10^{-1} M)	90	35
Glucono-1,5-lactone (10^{-2} M)	10	0
N-Bromosuccinimide (10^{-3} M)	95	100
N-Bromosuccinimide (10^{-4} M)	93	100

^aInhibitors were tested by incubating 6 ml. of substrate, 2 ml. of 0.2M acetate buffer of appropriate pH, 1 ml. of inhibitor of appropriate concentration, and 1 ml. of enzyme. The concentrations listed are the final concentrations in 10 ml. reaction mixture. There was no significant difference in the amount of inhibition that occurred when the effects of inhibitors were measured immediately or when the inhibitors were preincubated with the enzyme for a period of 60 min.

^bPhenyl mercuric nitrate (10^{-4} M and 10^{-5} M), p-chloromercuribenzoate (10^{-3} M), 8-hydroxyquinoline (10^{-3} M), sodium cyanide (10^{-3} M), cupferron (10^{-3} M), and $HgCl_2$ (10^{-3} M) and GSH (10^{-3} M) were also tested and produced essentially no inhibition or activation.

are shown in Table II. Table III shows the effects of varying the concentration of N-bromosuccinimide on the E-CMC and EBG activities of the preparation.

Action on Cellulose

The activities of resealed E-CMC, crude E-CMC, and a commercial cellulase were tested on insoluble cellulose (blotter cellulose). The action was followed by measuring the reducing power. No increase in reducing power was observed with crude E-CMC or resealed E-CMC after 3 days of incubation at 30°C. The commercial cellulase effected an increase in reducing power equivalent to 0.53 mg. of glucose in 3 days.

DISCUSSION

Ammonium sulfate fractionation resulted in a considerable purification of the E-CMC activity present in germinated barley extracts. About 30% of the initial E-CMC activity is concentrated in the 90% saturated ammonium sulfate fraction

TABLE III. THE EFFECT OF N-BROMOSUCCINIMIDE CONCENTRATION ON THE E-CMC AND EBG ACTIVITIES OF RESEPARATED E-CMC^a

N-Bromosuccinimide Concentration	% Inhibition	
	E-CMC	EBG
0	0	0
1×10^{-3} M	96	100
5×10^{-4} M	96	92
2.5×10^{-4} M	47	52
1.25×10^{-4} M	19	12
1×10^{-5} M	8	9
1×10^{-5} M	0	0

^aThe procedure described in Table II was used.

accompanied by small amounts of EBG and amylase activities (21). Attempts to refine the fractionation by removing precipitates at 70 and 80% saturated levels lowered the recovery of the E-CMC and did not significantly improve its purity with respect to EBG and amylase activities. Attempts to further purify the ammonium sulfate fractions by chromatography on P-cellulose ion exchange resin were unsuccessful because the E-CMC was eluted over a rather extensive area instead of in a well-defined peak. A further purification of E-CMC was effected by separation of the ammonium sulfate fractions on DEAE-cellulose columns; however, the yields were low (21).

Better yields were obtained by separating albumin on DEAE-cellulose. The major portion of the E-CMC was eluted from DEAE-cellulose at a pH of 6 and a NaCl concentration of 0.06M (Fig. 1) along with some EBG and amylase activities. Rechromatography of the crude E-CMC on DEAE-cellulose removed most of the amylase and part of the EBG. The distribution pattern of the EBG not separated by the second chromatographing was essentially identical to that of the E-CMC (Fig. 2). The over-all recovery of E-CMC activity beginning with the albumin fraction was 68%. Most of the loss occurred during the initial dialysis of the germinated barley extract (Table I). The specific activity of the E-CMC was increased over 100-fold during the fractionation.

Disc electrophoresis of the preparation showed the presence of one major component and eight minor components. Whitaker (22) suggested an explanation for the situations where highly purified cellulase systems were found to be heterogenous on electrophoresis. He suggested that sequential or conformational isomers may be present in systems where the components were found to be reactive toward one cellulose substrate. We encountered difficulties extracting and testing the individual components on carboxymethyl cellulose; thus, it is not known whether his interpretation applies in this case.

The enzyme properties of E-CMC and EBG activities were very similar. The optimum pH for E-CMC activity was near 5 (Fig. 3). The optimum for the associated EBG activity in the presence of 0.06M sodium chloride was 4.85 and the curve strongly resembled that of the E-CMC curve in the presence of sodium chloride (Fig. 3). E-CMC and EBG were both stable at 40°C. They were both slowly inactivated at 50°C., and at the same rate. At 55° and 60°C. EBG was inactivated at a slightly greater rate than E-CMC. Sodium chloride stimulated both activities, with E-CMC activity stimulated to a slightly greater degree.

N-bromosuccinimide inhibited both E-CMC and EBG activities to the same degree (Tables II and III). Cellobiose and glucono-1,5-lactone inhibited both activities but inhibited E-CMC to a greater extent than EBG. Thus the inhibition results appear contradictory, part of them suggests that the two activities are caused by a single enzyme and part of them suggests that the two activities are caused by separate enzymes. Cellobiose and glucono-1,5-lactone have been shown to be competitive inhibitors for beta-glucosidases (23,24). Whether or not these two inhibitors were acting competitively against the EBG and E-CMC activities could not be tested because of technical difficulties. The viscometric methods used to follow the two activities do not lend themselves to the calculation of kinetic constants. Reducing-power method results from which the constants could be calculated are not sensitive enough for the levels of activity present, or are not applicable because

the reagents form precipitates with the unchanged and partially degraded substrates.

If one assumes that the cellobiose and glucono-1,5-lactone were acting as competitive inhibitors of these two beta-glucosidase activities, the inhibitor results may in fact not be contradictory. Although both substrates have beta-1,4-glucosidic linkages, they have quite different over-all structures. CMC is cellulose solubilized by carboxymethylation and therefore has consecutive beta-1,4-glucosidic linkages with a certain number of the glucose moieties substituted with carboxymethyl groups. Barley beta-D-glucan contains both beta-1,3- and beta-1,4-glucosidic linkages with groups of two, three, and possibly four adjacent 1,4-linkages present. It follows that the substrate geometry presented to the enzyme would be expected to differ for the two substrates. Consequently, the differences in the degrees of inhibition of the two activities by cellobiose and glucono-1,5-lactone could simply reflect a difference in binding of the two substrates and does not in fact prove that two different enzymes or even two different active sites are required to exhibit the E-CMC and EBG activities. Further, since barley beta-glucan contains groups of two, three, and possibly four adjacent beta-1,4-glucosidic linkages, the barley beta-glucan could well be hydrolyzed by a random cleaving enzyme specific for the beta-1,4-glucosidic bonds. Thus the results of the inhibition experiments may be consistent with the concept that the action on the two substrates results from the same enzyme even though they do not prove the concept.

Results with other reagents suggest certain clues as to the nature of the enzyme itself. Metal chelators such as 8-hydroxyquinoline and EDTA did not inhibit the E-CMC or EBG activities of the purified preparation (Table II and Fig. 4). This suggests that the enzyme does not require a metal ion (5) for its activity. In addition, GSH (reduced) and p-chloromercuribenzoate did not significantly affect the activity of the enzyme (Table II), thereby suggesting that the enzyme does not require intact sulfhydryl groups for its activity. Although the enzyme can hydrolyze soluble substrates containing beta-1,4-glucosidic linkages, it apparently is not active on insoluble celluloses. It therefore resembles the "Cx" enzymes isolated from microorganisms (23,24).

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