

Partial Characterization of C_x Cellulase and Cellobiase from Ripening Tomato Fruits¹

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D. M. PHARR² AND D. B. DICKINSON

Department of Horticulture, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Cellulolytic enzymes were studied in extracts from the locular contents of ripening fruits of *Lycopersicon esculentum* var. KC-146. When acting on carboxymethyl cellulose, the enzyme preparations were capable of decreasing the viscosity of the reaction mixture and generating reducing groups, oligosaccharides, and glucose. Cellobiose celotriose, cellostreose, and cellopentose also served as substrates for glucose production.

These properties were due to the presence in the extracts of two cellulolytic enzymes— C_x cellulase (E.C. 3.2.1.4) and cellobiase—(E.C. 3.2.1.21). No evidence was found for the presence of exocellulase or C_1 , an enzyme which initiates enzymatic attack on insoluble cellulose.

Cellobiose was found to be weakly inhibitory to C_x cellulase. D-Glucono-1,5-lactone competitively inhibited cellobiase. The K_m for cellobiose was 0.34 mM, and the K_i for gluconolactone was 23 μ M.

Numerous physiological roles for cellulase in higher plants have been postulated. These include hormonally induced growth responses (6, 10), abscission of leaves (1, 2, 14, 19), pollen tube growth (16, 31), and softening of fruits as they ripen (7, 12, 33). However, little is known about the nature of cellulase from higher plants despite the probable physiological importance of cellulase in growth and development.

Microorganisms are known to produce at least four different types of enzymes which have distinctly different roles in the degradation of cellulose (15, 20). The least understood of these enzymes is the component which was termed " C_1 cellulase" by Reese and his associates (18). This enzyme was postulated to initiate enzymatic attack on insoluble cellulose. Conclusive evidence for the existence of C_1 cellulase as an entity distinct from other cellulolytic enzymes has been presented recently, and the mechanism of catalysis by this component is uncertain (15, 20, 32). In addition to C_1 , cellulolytic microorganisms produce hydrolytic enzymes termed C_x cellulase, exocellulase, and cellobiase. C_x cellulase is an endoenzyme (E.C. 3.2.1.4). This enzyme isolated from various sources is totally inactive or weakly active against insoluble cellulose but highly active

against soluble cellulose derivatives such as carboxymethyl cellulose. It catalyzes the hydrolysis of β -1,4-glucans of high molecular weight, yielding cellobiose and larger molecules as predominant products (3, 17, 31).

Glucose production by microbial cellulases is thought to be restricted primarily to two types of enzymes—cellobiases (E.C. 3.2.1.21) and exo- β -1,4-glucan hydrolases (20). Both enzymes have broad substrate specificities and substrates in common. However, they differ markedly in K_m for different substrates (20), mode of attack (15, 20), and susceptibility to inhibitors (28, 29). Thus, several means exist for distinguishing between the activities of exoglucanases and cellobiases in spite of the facts that their substrate specificities overlap and that the product of each reaction is glucose.

Most studies of cellulase in higher plants have attempted to correlate the activity of C_x cellulase with various physiological events. A few of these reports have provided information on the multiple enzymic nature of the cellulase complex of higher plants. Abeles (2) reported that extracts from plant abscission zones which exhibited C_x cellulase activity were not able to degrade cotton, thus emphasizing the lack of experimental evidence for the existence of C_1 cellulase in higher plants. Lewis and Varner (19) demonstrated the existence of two forms of C_x cellulase in abscission zones of aging bean explants. Extracts from pea epicotyls contain both C_x cellulase (6, 10) and cellobiase activity (5). Apparently the only suggestion of the existence of an exocellulase in higher plants was made without supporting data (13).

The present investigation was undertaken to provide a detailed characterization of cellulase from the locular tissues of ripening tomato fruits. In addition to studies of C_x , the possibility of the presence of other cellulolytic enzymes in tomato fruits was investigated.

MATERIALS AND METHODS

Fruit Source. Fruits of *Lycopersicon esculentum* var. KC-146 were used in all experiments. Plants were grown under both field and greenhouse conditions to furnish a supply of fruits during all seasons of the year. All fruits used in this study were about one-half ripe as estimated by external red coloration.

Carboxymethyl Cellulose. The substrate used for all C_x cellulase assays was the sodium salt of carboxymethyl cellulose. Technical literature described the polymer as having an average degree of polymerization of 500. No estimate of heterogeneity of molecular weight was available. The average degree of substitution was within the range 0.65 to 0.85. This figure, degree of substitution, refers to the average number of carboxymethyl groups per anhydroglucose residue. The maximal degree of substitution possible is 3.0 when all three of the

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² Present address: Department of Horticultural Science, North Carolina State University, Raleigh, N. C. 27607.

available hydroxyl groups of the anhydroglucose residues are substituted with carboxymethyl groups.

Other Materials. β -1,4-Glucosides were prepared by John Lola and were a gift from E. T. Reese of the U.S. Army Natick Laboratories, Natick, Mass. All other materials were available from commercial sources.

Enzyme Isolation. All enzyme isolations were made from the tissues within the fruit locules by a procedure similar to that suggested by Dickinson and McCollum (7). Locular tissue was cooled in a beaker placed in an ice bucket and then blended for 2 min in a Waring Blendor at high speed. The macerate was adjusted to pH 8.0 by slow addition of 1 N NaOH. After 30 to 45 min in an ice bucket, to allow adequate desorption of the enzyme, the macerate was centrifuged at 20,000g for 20 min. The supernatant fluid contained C_2 activity and was used directly for enzyme assays or was subjected to further treatment.

For cellobiase assays, proteins were precipitated from 100 ml of the pH 8.0 supernatant fluid by slow addition of 51.6 g of solid $(NH_4)_2SO_4$ with stirring. Precipitated material was collected by centrifugation at 20,000g at 4 C for 20 min. The pellets were combined and washed three times by suspension in 25 ml of cold 0.01 N Na-bicine buffer at pH 8.0 containing 0.516 g of $(NH_4)_2SO_4$ per ml in order to remove entrapped reducing substances. After each wash the proteins were collected as a tight pellet by 10 min of centrifugation at 20,000g at 4 C. The final washed pellet was resuspended in 25 ml of

0.01 N Na-bicine buffer at pH 8.0. These concentrated enzyme preparations were used without dilution for cellobiase assays.

In experiments in which the liberation of reducing groups was followed, dialysis of the enzyme preparations was necessary because $(NH_4)_2SO_4$ had an inhibitory effect on the reducing group method. Dialysis was carried out overnight against ice-cold 0.01 N Na-bicine at pH 8.0. Reducing groups were determined by the method of Nelson (26).

Preparation of Carboxymethyl Cellulose Solutions. CMC³ solutions were prepared by two different methods. In method A, 3 g of CMC were added to 250 ml of 0.1 N Na acetate buffer at pH 5.0 containing 1 μ g/ml of merthiolate as a preservative. Merthiolate is known not to inhibit tomato fruit cellulase (7, 33). The mixture was blended for 3 min in a Waring Blendor at high speed. Eight milliliters of this substrate were used in a total reaction volume of 10 ml. In method B 4 g of CMC were blended in 250 ml of the same buffer under identical conditions. Six milliliters of this substrate were used in a total reaction volume of 10 ml. Both substrates had about the same initial viscosity.

Viscosity Measurements. Viscosity was measured as efflux time from Ostwald viscometers (Sargent No. 300). Viscometers were submerged in a glass water bath held at 30 C and standardized by observing the efflux time of distilled water. These values were used for fluidity calculations. Viscometers were also standardized with viscosity standards supplied by Brookfield Engineering Laboratories, Inc., in order to convert efflux time directly into centipoise.

Glucose. Glucose was determined using Glucostat Special (Worthington Biochemical Corporation). This reagent was found to be contaminated with variable amounts of β -glucosidase, and procedures to be described elsewhere were used to overcome this problem (27).

Thin Layer Chromatography. Samples of reaction mixtures were chromatographed ascendingly in a solvent of formic acid-methyl ethyl ketone-tertiary butanol-water (15:30:40:30, v/v) on 5- \times 20-cm cellulose thin layer chromatograms. Sugar spots were visualized by spraying the chromatograms with Tollen's reagent—0.1 N $AgNO_3$ -5 N NH_4OH -2 N NaOH (1:1:2, v/v). Additional experimental details are described with individual experiments.

RESULTS AND INTERPRETATION

Assay Conditions for C_2 Cellulase. In Figure 1 data are shown from an experiment which tested proportionality between fluidity change of CMC reaction mixtures and concentration of crude pH 8.0 enzyme extract in the reaction mixtures. Fluidity of reaction mixtures was calculated according to a modification of the method of Levinson and Reese (18). The formula which was used to express fluidity is given below:

$$\text{Fluidity} = 1/\eta_{sp} = \frac{1}{t_{RM} - t_{H_2O}}$$

where $1/\eta_{sp}$ = the reciprocal of specific viscosity, t_{RM} = the efflux time of the reaction mixture from the viscometer, and t_{H_2O} = the efflux time of water. The readings taken at 6 min were subtracted from subsequent readings. Linear plots of increase in fluidity *versus* time were used to calculate rates of change in fluidity. These plots are shown in the lower graph (Fig. 1). A replot of fluidity change per min *versus* ml of enzyme in

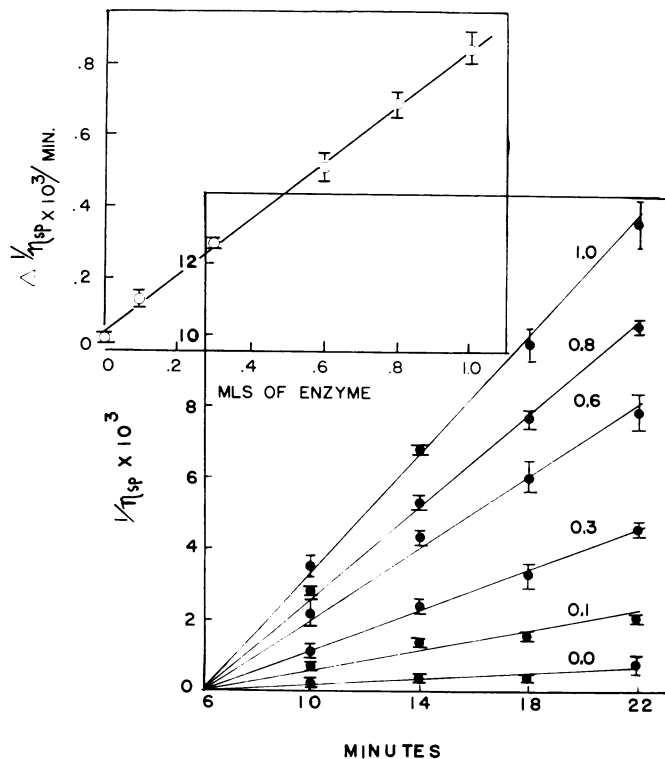


FIG. 1. Relationship between increase in fluidity of CMC reactions and enzyme concentration. The larger graph shows increase in fluidity as a function of time. Numbers above each line indicate the milliliters of enzyme in each reaction. The smaller graph shows the proportionality between rate of fluidity increase and milliliters of enzyme in the reaction. Vertical lines represent the range of duplicate assays. Eight ml of CMC, prepared by method A, were placed into viscometers. Final reaction volumes were 10 ml. Efflux times of the reaction mixtures were measured with a stopwatch 6 min after addition of enzyme and at subsequent 4-min intervals.

³ Abbreviation: CMC: carboxymethyl cellulose.

the reaction is shown in the upper graph in Figure 1. The rate of fluidity change was a good estimate of enzyme concentration. Proportionality was also obtained in numerous subsequent experiments in which 1 or 2 ml of enzyme were placed in the reaction mixture. The small increase in fluidity noted in the absence of enzyme in this experiment could not be consistently detected when boiled enzyme extract was added to reaction mixtures in subsequent experiments. Thus, no attempt was made to correct the data for any small increase in fluidity in the absence of enzyme.

Utilizing this assay system, it was established that the pH optimum was very near pH 5.0 in 0.1 N acetate buffer. All subsequent assays were conducted at pH 5.0.

Cellobiose Inhibition of C_x Cellulase. The effects of cellobiose and other sugars on C_x activity were studied using the viscometric assay. These data are shown in Figure 2. Cellobiose was the strongest inhibitor. Lactose and maltose were only weakly inhibitory. It is unlikely that this cellobiose inhibition is a nonspecific effect resulting from its high concentration. Glucose caused only slight inhibition, and its molar concentration was twice that of the disaccharides.

Cellobiose probably acted as an inhibitor of tomato C_x because of its structural similarity to CMC. Cellobiose has been shown to be a structural perturbant of fungal C_x cellulase (8), and it is known to be inhibitory to C_x enzymes from a number of organisms (24). Curiously, C_x cellulases from several fungi are strongly activated by cellobiose when acting on CMC as a substrate (24). Thus, inhibition of C_x enzymes by cellobiose is not a general phenomenon.

Partial Characterization of Reaction Products. It was desired to gain an insight into the type of reaction products formed as the tomato enzyme preparation attacked CMC. Reducing group production in the reaction mixtures was found to be at the lower limit of experimental detectability within the time course of the fluidity assays. Thus, reaction times were extended to 14 hr at 30 C in order to follow reducing group formation. During the incubation, viscosity of the reaction mixture was reduced from an initial value of 18 to 20 centipoise to a final value of 2 to 3 centipoise. An increase in reducing groups equivalent to 170 to 200 μg of glucose per ml of reaction mixture was detected. Reaction mixtures which contained enzyme inactivated by immersion in a boiling water bath for 10 min exhibited little decrease in viscosity—a decrease of about 0.9 centipoise in 14 hr. Reducing group production was not detectable in the inactive enzyme reaction mixture.

Ten-microliter aliquots taken from samples of 0 time, 8 hr, and 14 hr reactions were chromatographed and visualized under conditions described under "Materials and Methods." Authentic glucose and cellobiose were spotted as standards. A spot which cochromatographed with glucose was detected, but no other spots at or near the origin were seen which would suggest the presence of any oligosaccharides, including cellobiose. No spots were detected in the inactive enzyme control reaction. When CMC was spotted on the chromatograms and sprayed with Tollen's reagent, it did not give a clearly detectable reaction. Authentic cellobiose gave a much weaker reaction than glucose. It is possible that oligomers were present in the reaction mixture but were not detected owing to inadequate sensitivity.

In a single experiment it was noted that a concentrated enzyme preparation which was capable of degrading CMC did not release any soluble reducing compounds when incubated for 14 hr at 30 C with absorbent cotton or Whatman No. 1 filter paper in 0.1 N Na acetate buffer at pH 5.0. Thus, within

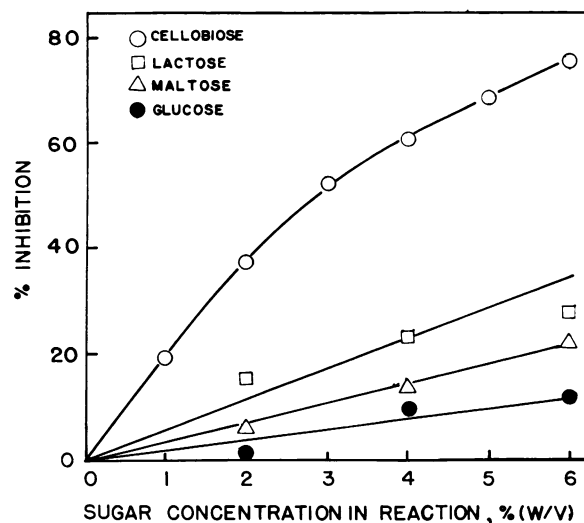


FIG. 2. Effect of sugars on C_x activity. Sugars were added to the reaction at the indicated concentrations prior to addition of enzyme. Substrate for these assays was CMC prepared by method B. The enzyme was prepared by (NH₄)₂SO₄ precipitation and concentration followed by extensive dialysis. Percentage inhibition of C_x activity (calculated from a control lacking inhibitor) was plotted as a function of sugar concentrations in the reaction. Initial C_x activity = 1.41 change in fluidity/min·ml of enzyme.

the limits of this test no activity was found against insoluble cellulose.

Glucose Production from Cellobiose. Preliminary experiments indicated that cellobiose could function as a substrate for the tomato enzyme concentrate (data not shown). These reactions were conducted by incubating the indicated amount of enzyme concentrate prepared as described in "Materials and Methods" in a 0.4-ml total reaction volume buffered at pH 5.0 with 0.1 N Na acetate containing 5 mM cellobiose. The reactions took place at 30 C for 50 min and were terminated by heating the covered tubes in a boiling water bath for 2 min. Precipitated proteins were removed by centrifugation, and Glucostat assays were conducted using 0.1 ml of the clear deproteinized supernatants. Boiled enzyme in appropriate amounts served as controls for the apparent initial glucose content of the reaction system. These values were subtracted from the values of assays containing active enzyme, and the difference in glucose level was attributed to hydrolysis of cellobiose by the tomato enzyme. It was noted that boiled tomato enzyme preparation in cellobiose substrate did not produce glucose under the assay conditions. Active enzyme in the absence of substrate did not increase in glucose content. Therefore, no further correction was necessary. Results of these assays indicated that glucose production was nearly proportional to enzyme concentration up to 0.1 ml of enzyme in the reaction. Additional experiments indicated that the rate of glucose production was linear with time up to about 60 min. Subsequently, assay tubes containing boiled enzyme and active enzyme were run in duplicate, and most assays were conducted using 0.1 ml of enzyme in the assay systems.

Differential Inactivation by Heat. The enzyme activities against CMC and cellobiose were differentially inactivated by heat (Fig. 3). Two conclusions may be drawn from these data. First, the C_x enzyme demonstrated greater heat stability than did the enzyme which produced glucose from cellobiose. Second, the viscosity-reducing enzyme behaved like C_x from other sources, having little, if any, capacity to hydrolyze cellobiose.

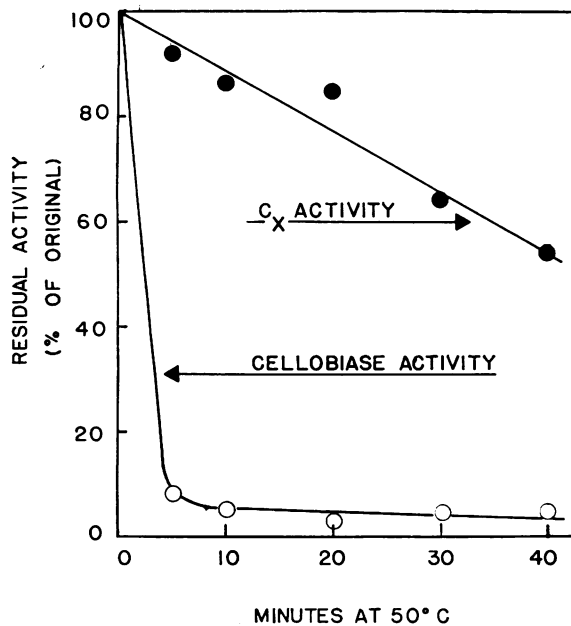


FIG. 3. Differential inactivation of C_x and cellobiase activity by heating at 50°C. A sample of tomato enzyme concentrate was subdivided into 2-ml aliquots in 13- × 100-mm test tubes. These tubes were placed in a water bath controlled at 50°C, removed at intervals up to 40 min, and cooled immediately in an ice water bath. Residual cellobiase and C_x cellulase activities were determined in each of the samples. The activities which remained were expressed as percentages of the activities in the nonheated control sample. Initial C_x activity = 4.0 change in fluidity/min·ml of enzyme. Initial cellobiase activity = 22 μ g of glucose formed/50 min·0.4 ml of reaction containing 0.2 ml of enzyme.

These results are in general agreement with other reports on heat inactivation which have been conducted with fungal enzymes at higher temperatures (20, 21).

Production of Glucose from Oligosaccharides. In order to establish the pattern of activity of the tomato enzyme preparation against β -1,4-glucosides, assays identical to those described for cellobiase were conducted except that substrates were different. The experiment was conducted three times with three different tomato enzyme concentrates (Fig. 4). The amount of glucose released decreased progressively as chain length of the substrate increased. The ratio of glucose produced from cellotetrose to glucose produced from cellobiose was 0.36 on the average. This ratio was reported to be near 0.2 for purified fungal cellobiase and 100 for purified fungal exoglucanase (28). Thus, the activity pattern for the tomato preparations suggested the presence of an enzyme similar in substrate specificity to fungal cellobiase.

Gluconolactone Inhibition and Some Kinetic Parameters. D-Glucono-1,5-lactone is a powerful competitive inhibitor of cellobiase but a very poor inhibitor of exoglucanases and endoglucanases (24, 28, 29). Preparatory to gluconolactone inhibition studies with the tomato enzyme, cellobiose substrate saturation experiments were conducted. From Woolf plots the K_m for cellobiose was estimated as 0.35 mM. This value is somewhat higher than the K_m (0.13 mM) reported for cellobiase from marine borer cecal tissue (22), but it is considerably lower than the K_m (5.4 mM) reported for a cellobiose hydrolase from human intestinal mucosa (25). The K_m of the tomato enzyme is also considerably lower than the K_m (2.2 mM) of *T. viride* exo- β -1,4-glucanase for cellobiose (20). A K_m of 19.0 mM was reported for endo- β -1,4-glucanase of *T. viride* acting on cellobiose as a substrate (20). While the sub-

strate range of enzymes within (E.C. 3.2.1.21) is very broad, only those enzymes which exhibit high activity on beta dimers of glucose are considered to be part of the cellulase complex (15). The tomato enzyme does exhibit reasonable apparent affinity for cellobiose.

The effects of a wide range of concentrations of D-glucono-1,5-lactone on tomato cellobiase activity were studied (data not shown). The study was conducted at a saturating level of cellobiose (5 mM). The inhibition curve was hyperbolic, and 50% inhibition of enzyme activity was obtained with a concentration of gluconolactone of less than 0.25 mM.

In order to determine the type of inhibition involved, substrate saturation experiments were conducted in the presence of increasing gluconolactone concentrations up to 0.2 mM (Fig. 5). The smaller graph shows saturation curves in the presence of different levels of inhibitor. Presence of the lactone did not change the hyperbolic shape of the substrate saturation curves. Inhibition of enzyme activity was higher at lower substrate concentrations. The larger graph of Figure 5 shows Woolf plots of the same data. The effect of inhibitor concentration on V_{max} and K_m was determined from these plots. These data are shown numerically in the upper right-hand corner of Figure 5. Increased inhibitor concentration resulted in a marked change in apparent K_m but little change in V_{max} . Considering the possibility for some experimental error, these data fit kinetic criteria for competitive inhibition (4).

In Figure 6, Dixon plots of these data are shown from which a graphic determination of K_i was derived. The value for K_i was 23 μ M. Thus, the apparent affinity of the tomato enzyme

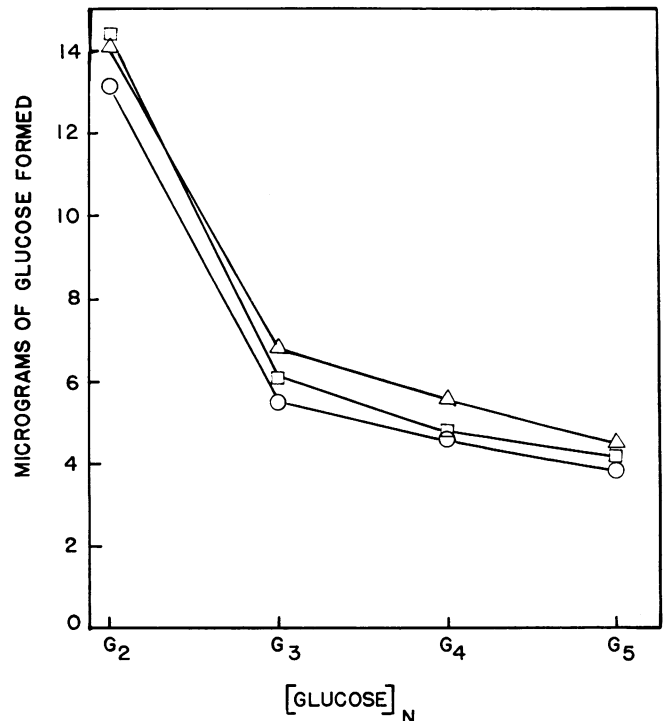


FIG. 4. Production of glucose from β -1,4-glucosides by tomato enzyme concentrates. □: Experiment 1; ○: experiment 2; △: experiment 3; G₂: cellobiose; G₃: cellotriose; G₄: cellotetrose; G₅: cellopento-
 se. Glucose production = μ g of glucose formed/50 min·0.4 ml reaction containing 0.1 ml of enzyme. Experiment 3 was conducted with 0.2 ml of enzyme, and glucose production data were divided by 2 in order to plot all data conveniently on the same scale. The initial concentrations of substrates in the 0.4-ml reaction mixtures were: cellobiose, 2 mg/ml; cellotriose, 3 mg/ml; cellotetrose, 4 mg/ml; and cellopento-

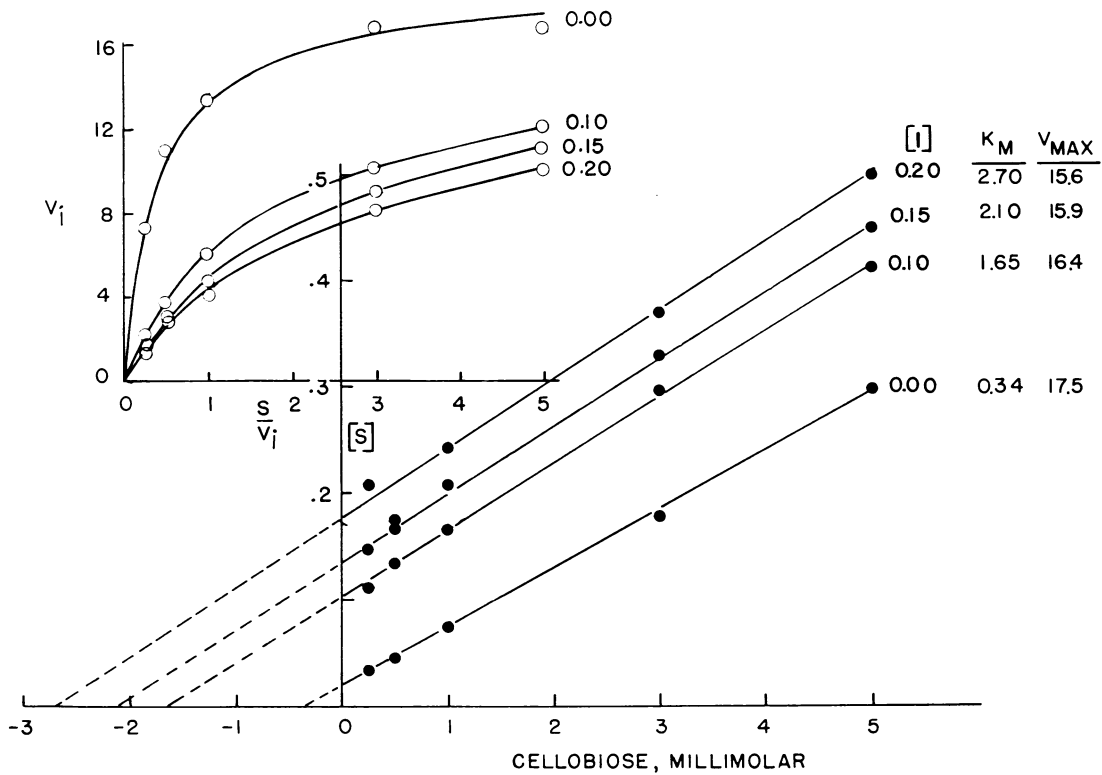


FIG. 5. Kinetic analysis of gluconolactone inhibition. Smaller graph shows substrate saturation curves in the presence of different mM concentrations of gluconolactone. Larger graph shows Woolf plots of the same data. V_i: initial velocity = micrograms of glucose formed/50 min · 0.4 ml reaction containing 0.1 ml of enzyme. See text for additional details.

for gluconolactone was on the order of 10 to 15 times as great as the apparent affinity for cellobiose.

Reduction of Glucose Production from CMC by Gluconolactone. Concentrations of gluconolactone as high as 20 mM had no effect on C_x activity when the enzyme was assayed viscometrically. However, as little as 3 mM gluconolactone eliminated glucose as a product in the CMC assay system. An experiment was conducted in which the effect of gluconolactone on viscosity loss, reducing group formation, and glucose production was followed simultaneously. CMC substrate was prepared by method B. Two reaction conditions were followed in Ostwald viscometers at 30 C. One reaction contained 3 mM gluconolactone, and the control reaction lacking inhibitor contained distilled water. Dialyzed enzyme concentrate was added to the substrate at a ratio of 1 ml to 4 ml. Larger reactions (30 ml) containing identical ratios of CMC, enzyme, and gluconolactone or distilled water were also incubated in the same water bath.

Data from this experiment are shown in Figures 7 and 8. From Figure 7 it may be seen that presence of the lactone did not affect the ability of the enzyme preparation to reduce the viscosity of the reaction mixture. However, gluconolactone markedly inhibited the formation of reducing groups.

Data in Figure 8 were obtained by assaying 0.1 ml of each of the time course samples for glucose content with Glucostat reagent. The difference in glucose produced within the reaction containing inhibitor and the reaction containing no inhibitor (about 110 μg by 14 hr) was adequate to account quantitatively for the difference in reducing group formation between the two reaction mixtures. It is reasonable to speculate from these data that C_x cellulase produced oligosaccharide products and that cellobiase degraded some of these products to glucose. The lactone would inhibit conversion of the oligomers to glucose, but it would not inhibit the activity of C_x. In a parallel ex-

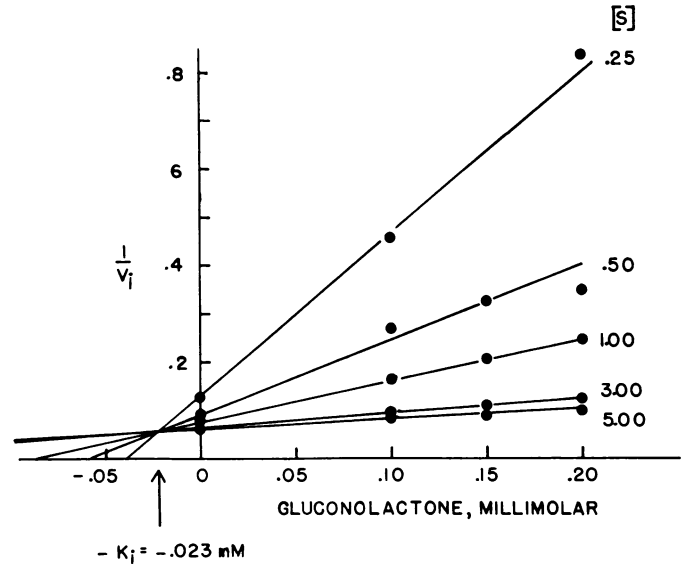


FIG. 6. Dixon plots of gluconolactone inhibition data for determination of K_i. Numbers to the right of each Dixon plot are mM cellobiose concentrations in the assays. See Figure 5 (smaller graph) for substrate saturation curves from which these data were derived. See text for additional details.

periment in which cellobiase was inactivated by heating the enzyme concentrate at 50 C for 10 min, rather than by inhibition with gluconolactone, similar results for viscosity loss, reducing group formation, and glucose production were obtained.

Finally, it may be seen from Figure 7 that reducing group formation exhibited a distinct rate change to a slower rate

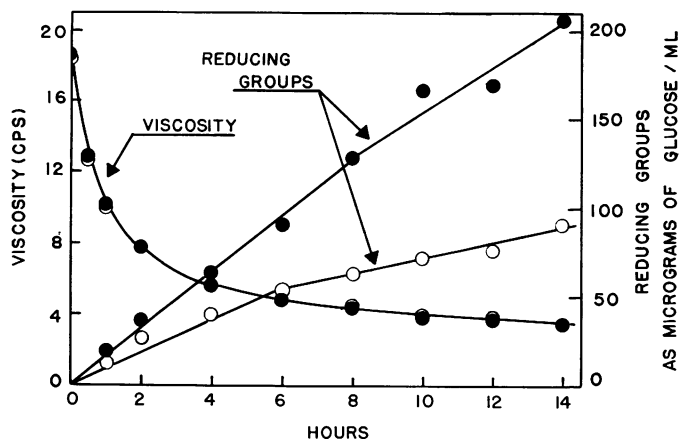


FIG. 7. Viscosity loss and reducing group formation from CMC reactions for reaction containing no gluconolactone and reaction containing 3 mM gluconolactone. Reducing group data were corrected for initial reducing group contents of reactions. ●: Control reaction; ○: reaction containing 3 mM gluconolactone. See text for experimental details.

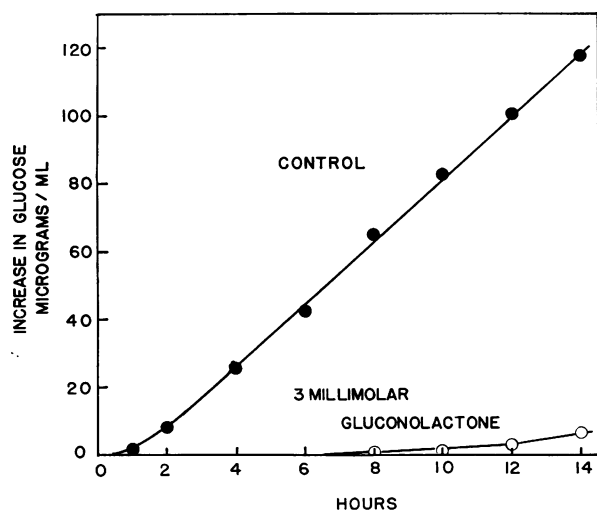


FIG. 8. Glucose production from CMC as measured by Glucostat reagent for reaction containing no gluconolactone and reaction containing 3 mM gluconolactone. See Figure 7 for viscosity loss and reducing group formation. Data were corrected for initial glucose content.

during the midcourse of the reaction. This rate change was typical of all long time course reactions. Such rate changes are characteristic of C_x cellulases acting on CMC (9). The more rapid initial rate is believed to be related to preferential hydrolysis of segments of the polymer chain which contain a series of adjacent unsubstituted glucosyl residues (9).

DISCUSSION

These experiments indicate that tissues within the locules of tomato fruits contain at least two cellulolytic enzymes (C_x cellulase and cellobiase), which are similar in general properties to cellulolytic enzymes of fungal origin (15). Their combined activity was adequate to explain the observed ability of the tomato fruit preparation to partially degrade CMC to glucose.

Within the limits of the present experimental approaches, no evidence was found for the existence of an exocellulase. The near absence of glucose as a reaction product from CMC

under conditions which inactivated or inhibited cellobiase activity suggested that the enzyme preparations did not contain an additional enzyme which could attack the polymer directly to produce glucose. The ability of the tomato enzymes to degrade β -1,4-linked oligosaccharides to glucose was correlated with the known substrate specificity of cellobiase rather than with that of exocellulase.

No enzyme activity was detected against insoluble forms of cellulose. This has been commonly observed for fungal enzyme preparations which contain C_x cellulase and cellobiase activity (15, 30) and is also true of other higher plant C_x cellulase preparations (2, 23).

Considerable evidence suggests that C_x cellulase plays important roles in higher plants, including the knowledge that *de novo* synthesis (6, 19) as well as secretion (1) of the enzyme is under hormonal control. It has been repeatedly suggested that this enzyme degrades higher plant cell walls. However, the potency of C_x cellulase in degrading insoluble cellulose is in question, and it remains to be demonstrated that the enzyme system described here is able to attack cell wall polymers directly. Since the present study clearly indicates the presence in higher plants of two enzymes of the cellulase complex, it will be of interest to learn whether senescence and hormonally induced changes involve changes in only one or both of the enzymes.

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