

INHIBITION OF CELLULASES¹

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Cellulolytic enzymes produced by microorganisms are involved in wood rotting, breakdown of cell walls, and maceration of plant tissues, and they may also play a role in invasion of plants by pathogens, intra- and inter-cellular penetration of host tissues (124, 132), and the development of wilts (9, 13, 38, 51, 53, 105). Cellulases also may aid in nourishment of pathogens, either directly by cellulose breakdown products or indirectly by release of nutrients from disrupted cells. Finally, cellulases may be essential to the maintenance of the pathogen during periods when it is living saprophytically.

Inhibition of cellulases may be a means of preventing pathogens from invading or spreading in host tissues, or of starving the pathogen when it is dependent on cellulose for food. Since, cellulose is not a food for higher plants, or nonruminant animals, a specific cellulase inhibitor could be a nontoxic weapon against phytopathogenic microorganisms. Furthermore, knowledge regarding cellulase inhibitors, particularly naturally occurring ones, may be useful in unravelling the complexities of natural host resistance to invaders.

In the past few years enzymic degradation of cellulose has been frequently reviewed (21, 35, 60, 81, 93, 100). The reaction between an enzyme and a complex polymeric substrate is not simple, nor is it as yet fully understood. The problem is even more complicated when the polymer is insoluble. The most difficult question involves the nature of the initial enzymic attack on crystalline portions of the cellulosic fiber.

As currently understood (32, 59, 62, 73, 99), the degradation of native cellulose involves two types of enzymes designated C_1 and C_x . C_1 acts on crystalline cellulose in such a way that subsequent action by C_x becomes possible. The nature of the action of C_1 is not yet understood. It seems to act by breaking or loosening the forces that hold the cellulose molecules together. This results in hydration of portions of the cellulose chain. Analogous mechanisms seem to be involved in wool degradation (65) and perhaps in lysis of some microbial walls. The exact relationship of C_1 to "enzyme A" that is responsible for tensile strength loss (106) and of "hydrocellulase" (32) that acts on suspensions of crystalline particles is not yet clear. C_x is a complex of enzymes hydrolyzing the β 1,4-glucosidic bonds in the cellulose molecule. While they are usually endoenzymes (random acting), they may occasionally be exoenzymes removing glucose (61, 133) or cellobiose (61, 117) succes-

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sively from the nonreducing end of the cellulose molecule in a manner analogous to that of glucamylase and β -amylase.

The C_x enzymes represent what is generally considered as the typical action of the enzymes that hydrolyze polysaccharides. In order to free the term "cellulase" for the combined action of C_1 and C_x , this group might preferably be called β 1,4-glucanases. These enzymes hydrolyze crystalline cellulose in the presence of C_1 . In the absence of C_1 they have the ability to degrade (a) cellulose derivatives (carboxymethyl cellulose), (b) celluloses which have been modified (swollen) by grinding, concentrated acid, or alkali, and (c) various glucans having mixed linkages including β 1,4-, such as lichenin and the β -glucans of oat and barley.

The cellulolytic microorganism does, of course, produce both C_1 and C_x as it grows on native cellulose. However, C_1 is not found in appreciable amounts in culture filtrates of most cellulolytic organisms, or in most commercial cellulase preparations. The best source of C_1 is *Trichoderma viride* (32, 45, 62). *T. viride* cellulase is easily produced (101) and has recently been made available by Japanese manufacturers (Meiji Seika Company, Kawasaki, or Kinki Yakult Manufacturing Company, Nishimiya) (120).

INDUCTION OF CELLULASE

One means of preventing cellulolytic action would be to repress the formation of cellulolytic enzymes. Cellulolytic fungi such as *T. viride* grow readily on a wide variety of carbon sources, but cellulase is produced only in the presence of cellulose or of cellulodextrins including cellobiose (4-0- β -D-glucosyl D-glucose). In a few exceptional cases lactose (4-0- β -galactosyl D-glucose) and sophorose (2-0- β -D-glucosyl D-glucose) are also inducers (68, 69, 71). Thus, cellulase is an adaptive enzyme. The substrate, cellulose, is insoluble, and the normal inducer appears to be cellobiose, the product of the enzyme action (69).

No specific repressor of cellulase formation has been found. Such a compound would be a most interesting tool for the plant pathologist. However, under conditions of rapid growth on sugars, including glucose and cellobiose, cellulase formation is strongly repressed, and even preformed enzyme may disappear from the medium (69, 118). For induction on cellobiose to occur, the sugar must be consumed slowly. In nature, this occurs when cellobiose is released from cellulose by enzyme action. Experimentally, cellobiose induction has been demonstrated when (a) cellobiose is slowly released from insoluble cellobiose octaacetate by action of an esterase, or (b) cellobiose consumption is slowed down by growing the fungus at suboptimal temperatures, with decreased aeration, with certain mineral deficiencies, or with an excess of cobalt in the medium (69).

It has been suggested that there are "low sugar" and "high sugar" diseases (26, 104). The presence of readily metabolizable substrates may well repress formation of enzymes active in pathogenesis.

CELLULOSE STRUCTURE AND ENZYME ACTION

The macromolecular, crystalline structure of cellulose greatly limits accessibility of the glucosidic bonds in cellulose to the hydrolytic C_x enzymes (62, 99, 125). This is the crux of the C_1 problem. The cellulose molecule (23, 49, 92, 103, 112) is a straight chain polymer of glucose units in the C_1 , chair, conformation, and having only $\beta 1,4$ linkages. The individual cellulose molecules differ in degree of polymerization, but may have an average of about 3000 glucose units. When very carefully isolated the chain may have 100,000 or more glucose units for a molecular length of 0.05 mm (112). Because the entire chain lies in a single plane, the cellulose molecules are strongly hydrogen bonded to each other resulting in a substance of extreme insolubility and low chemical reactivity. An individual cellulose molecule may pass through several regions of high crystallinity (micelles) as well as several amorphous regions where the chains are more loosely ordered. The crystalline micelle is about 600 Å long. This is equal to about 60 cellobiose units. The cellulose elementary fibril contains about 100 cellulose molecules for a cross sectional area of 3000 Å². A cellulose fiber contains about 7.5×10^6 elementary fibrils. The specific gravity (1.6) of the dry fiber indicates relatively little vacant space. Upon hydration the inter and intra fibrillar spaces increase to form a system of fluid filled cavities averaging 10 Å in width with some spaces up to 100 Å (112).

Only a few physical measurements have been made on the cellulase enzymes. The molecular weight may be as low as 10,000, but usually about 50 to 60,000 (24, 32, 86, 87, 127) and the dimensions on the order of 33 Å by 200 Å (127). A molecule of this size would have difficulty penetrating even the amorphous areas of cellulose and must be restricted largely to attack on exposed surfaces and loose chain ends. In crystalline areas, the hydrogen bonds as well as the glucosidic bonds must be broken before free sugar is released (109, 110).

Evidence that amorphous cellulose is more rapidly degraded than crystalline cellulose includes the increase in degree of crystallinity as enzyme action proceeds and the inverse correlation of degree of crystallinity with rate and extent of hydrolysis (99, 125). The accessibility of cellulose (and reactivity with enzymes) can be increased by swelling in acids or alkalis, by ball milling or other fine grinding, or by dissolving and reprecipitating (88, 125). Many workers have used water soluble cellulose derivatives as substrates. The introduction of substituent groups (methyl, carboxymethyl, hydroxyethyl, sulfate, etc.) at one or more of the free hydroxyls on carbons 2, 3, and 6 of the anhydroglucose units prevents chain aggregation, thus conferring water solubility on the cellulose and making the glucosidic linkages freely accessible to the enzyme.

Cellulase, however, hydrolyzes the linkages only between unsubstituted anhydroglucose units. When the number of substituents per anhydroglucose

unit exceeds one, the derivative becomes resistant to enzyme hydrolysis (94, 96). It appears probable that cellulase must bind to an unsubstituted cellobiose unit (85, 102) so that a uniform degree of substitution of 0.5 should give complete resistance. Nonuniform substitution accounts for the susceptibility of derivatives having a degree of substitution of around one. In practice a degree of substitution of 0.4 to 0.7 confers water solubility and high enzyme susceptibility. Methods of preparing and handling solutions of cellulose derivatives affect their susceptibility to enzyme action (72). Carboxymethyl cellulose (CMC) has been the most widely used soluble substrate for cellulose studies.

It has been noted that enzymic hydrolysis of insoluble cellulose does not follow first or zero order kinetics. The reaction rate decreases with the passage of time. This is true whether the reaction is followed by weight loss of residual substrate, by production of soluble products, or by decrease in turbidity of a cellulose suspension. It also is true whether the substrate is amorphous or crystalline. The effect is not due to product inhibition, as has been shown by action on a column where products were continually removed (62). This is considered an example of Schutz kinetics (27) which have been observed earlier for a number of enzymes acting on insoluble or colloidal substrates. The reasons for this type of kinetics are not well understood (27).

A substrate may be protected from an organism by inserting a barrier between them. This principle is utilized when wooden surfaces are painted, and when fabrics are coated with resins or other water proofing substances. For practical purposes there is a serious drawback. Once the barrier has been breached, the attack goes on unhindered.

A physical barrier is not always required to prevent the enzyme from coming into contact with its substrate. Cellulase action is also prevented when the enzyme is adsorbed on charcoal (95), clay (66), or cellulose (25, 32, 55, 62, 106).

Cellulose makes up 90 per cent of the cotton fiber, and about 36 to 54 per cent of most plant cell walls. Hemicelluloses may comprise 3 to 40 per cent, and pectic substances range from less than 1 per cent to 46 per cent of the wall. Lignin which is virtually absent in young parenchyma cells accounts for as much as 50 per cent of wood cell walls. Lignin, suberin, waxes, and cutin tend to encrust and water proof the cells, and lignin is deposited in the amorphous regions. Proteins, phenolics, and other substances may be present in trace amounts. The wall may thus be regarded as a complex interlocking system of high polymers. Cellulose, always present, and first to be deposited, provides form and support for this system and in turn is supported and protected by the other substances (23, 112).

The presence of other substances greatly affects the rate of enzyme attack on the cellulose wall. If the intermingled polymers are attacked by other enzymes, accessibility to the cellulose will be increased. Water proofing materials greatly limit access of hydrolytic enzymes to them, and many of the hydrophobic substances are resistant to microbial action.

When lignin is associated with the partially crystalline cellulose, the wood is extremely resistant to microbial attack (23, 34, 131). Fungi that can attack this complex are divided into "brown rotters" which are primarily cellulose consumers, and "white rotters" which consume both cellulose and lignin. In either case the residual lignin and cellulose show some degradation (22). The existence of a lignin cellulose bond has been proposed to account for the protective action of the lignin but the association is usually considered to be physical (23, 88). It is true that the resistance of cellulose to degradation increases with increasing lignin content, but if the effect were chemical, the addition of lignin to a cellulose culture would also inhibit cellulose breakdown, but it does not (34). Pew & Weyna (88), have shown that ball milling or fine grinding wood greatly increases its susceptibility to cellulolytic enzymes, presumably because of increased accessibility. However, ball-milled pure cellulose is also more susceptible to cellulase (99).

PHYSICAL FACTORS AFFECTING CELLULASE ACTION

Most of the work with cellulase has been done with crude enzyme preparations such as culture filtrates or cell homogenates. Perhaps this is the reason that cellulase has been found to be so tolerant of pH and temperature extremes.

Fungal cellulases, in general, are stable at 30° C from pH 3 to 8, active from 3.5 to 7, and usually show optimum activity at pH 4.0 to 5.5 in citrate, phosphate, or acetate buffers (72). The cellulase from *Curvularia lunata* shows a pH optimum of 4.6 to 5.0 (122), and the cellulases of *Irpex lacteus* and *T. viride* at pH 4.1 (46). Maximum activity of *T. viride* cellulase on food-stuffs was at pH 4.0 (120). The pH optimum for the C₁ of *T. viride* is at 4.0 when cotton is the substrate (73), and 4.8 when it acts on hydrocellulose (32). A cellulase from tomato fruit shows an optimum pH of 5.0 (25), and snail cellulase has a pH optimum of 5.6 (76). Bacterial cellulases show higher pH optima, often around 6.0 (43). Nematode cellulases have a broad pH optima from 5.5 to 8.0 (28).

Fungal cellulases are remarkably heat stable, at least in crude form, and must be heated to 100° C for 10 to 20 min for complete inactivation. The optimum temperature may be 70° C for brief assays, although 50° C is the usual temperature for reactions of 1 to 24 hr duration (72). It is possible to heat-inactivate cellobiase in an enzyme mixture without destroying all of the cellulase (63, 78, 79, 128). The cellulases of *I. lacteus* and *T. viride* retain 16 to 30 per cent of their original activity after 30 min at 99° C (46). A partially purified cellulase from *Poria vaillantii* lost 44 per cent of its activity in 10 min at 70° C (113). The optimum temperature for the C₁ of *T. viride* acting on cotton was 40° C (73), and 43° C when acting on hydrocellulose (32). Above 43° C hydrocellulase was rapidly inactivated. Fifty per cent of the C₁ activity was lost in 5 min at 60° C, and in 1 min at 70° C. C_x from the same preparation lost 50 per cent of its activity in 15 min at 60° C, but was quite stable at 50° C (62). The action of *Myrothecium* cellulase on in-

soluble cellulose at 50° C was only one half that of its action at 30° C (40). Myers & Northcote (76) found that the C_1 of purified snail cellulase was rapidly inactivated even at 30° C, and they used their enzyme at 25° C to digest cotton linters. C_x action for this preparation on cellofas B (CMC) was optimum at 37° C. The activity of aphid cellulase was sometimes doubled on heating to 50° C for 5 min, presumably due to destruction of a heat labile inhibitor (2). Crude cellulases from fungi can usually be precipitated by 66 per cent acetone or 80 per cent ethanol without loss of activity. They keep indefinitely at 5° C as solutions or as dry powders. The cellulase of rumen fluid is associated with the insoluble fraction (cells) and is highly unstable to attempts at purification or solubilization or to antiseptics. The C_1 component is particularly labile (40, 41).

The concentration of the reactants influences the rate of a reaction. For *T. viride* cellulase, the optimum concentration of CMC is 10 mg/ml (K_m 1.42 mg/ml) and the optimum concentration of cellulose sulfate is over 20 mg/ml (K_m 1.39 mg/ml) (72). *Aspergillus oryzae* cellulase has a K_m of 4.4 mM (56) acting on CMC and snail cellulase a K_m of 8.8 mM (76). The rate of cellulase action on insoluble celluloses is affected by adsorption of the enzyme on the substrate and by the amount of available surface. The amount of substrate hydrolyzed per unit of enzyme decreases rapidly as enzyme concentration is increased (44). This is another way of expressing the Schutz kinetics already discussed (62). Finally, the concentration of water may become limiting. Since cellulose is insoluble, the enzyme must diffuse to the cellulose molecule. Dry cellulosic materials are not degraded. Some fungi grow on cotton fiber at about 10 per cent moisture, well below the fiber saturation level (i.e., in the absence of any free water). Wood decay usually begins at, or just above the fiber saturation moisture content (24–32 per cent moisture) (23). For each gram of cellulose hydrolyzed, 0.11 grams of water are consumed but respiratory oxidation of the hydrolysis products produces 0.55 grams of water per gram of cellulose, so the process once begun is self sustaining as far as water is concerned (23).

Shaking has been reported to inactivate C_x , but not C_1 of *Myrothecium verrucaria* (42) and C_x of other fungi (5).

CHEMICAL INHIBITORS

The information on chemical inhibition of cellulases is scattered and tends to be confusing and contradictory. There are a number of reasons for this. Cellulases from plant, animal, and microbial sources have been used, frequently as crude preparations. Enzymes from different sources vary in inherent sensitivity and in the presence of complicating impurities. The various workers have used different substrates and assay conditions, and different methods of measuring cellulase activity. Frequently these involve large differences in the quantities of enzymes required. For example, a few random breaks in the chain will greatly reduce the viscosity of a solution of a cellulose derivative, but many glucosidic bonds must be broken before an

increase in reducing sugar can be detected. Relatively little hydrolysis occurs before reduction in optical density of a cellulose sol is detected, but a great deal before appreciable loss in weight of solid cellulose occurs. The older literature on chemical inhibition of cellulase has been covered in three reviews (35, 72, 81). This information is summarized below and recent data are reported in more detail.

The practical plant pathologist and the enzymologist both seek an inhibitor that is active at low concentration, specific for cellulase, unaffected by impurities, and indifferent to the source of enzyme or the method of measuring activity. They would be pleased if it were also stable and non-toxic. Such a compound has not yet been found.

Generally speaking, cellulases are inhibited by mercury, silver, copper, chromium, lead, and zinc salts at about 10^{-3} M (72). However, there are many exceptions. The above metals may be inactive or even stimulatory for some preparations. Merthiolate, widely used as a preservative for cellulase solutions, is not inhibitory at 0.01 per cent. Yet mercuric chloride is strongly inhibitory (113, 123). The inhibition by heavy metals is probably due to a nonspecific salt formation. The protection or stimulation of cellulase by sulfhydryl compounds such as cysteine or glutathione (32, 113, 123) or by proteins such as bovine plasma albumin, gelatin, and peptone (35, 72, 74, 126) may be due to reaction with toxic materials in the environment.

Cellulases are inhibited by large organic molecules such as acid or basic dyes, quaternary ammonium salts, or other detergents. The reaction involves ionic binding and is strongly affected by the pH of the reaction mixture (4, 84, 97). Acid dyes and anionic detergents inhibit at low pH; basic dyes and cationics become inhibitory at high pH's. Some cellulases such as that of *Pestalotiopsis westerdijkii* are particularly sensitive to these compounds over a wider pH range (97). Rather high levels (over 1 mg/ml) of this type compound are usually required for appreciable inhibition of cellulases. However, sodium keryl benzene sulfonate (Nacconal Z) is effective at 0.05 mg/ml against both C_1 and C_x of *T. viride* (73).

Cysteine, glutathione, cyanide, and sodium sulfide have occasionally been reported as cellulase inhibitors at from 10^{-2} to 10^{-3} M (35, 46, 67, 72). More frequently they are inactive or even stimulatory (see above). Since cellulases have been reported to lack sulfur containing amino acids (24, 97) the weak inhibitions reported for specific sulfhydryl inhibitors such as iodoacetate or *p*-chloromercuribenzoate must be due to reaction with other groups (72, 84).

Oxidizing agents can be strong inhibitors (97). Halogens and compounds that release active halogen such as hypochlorite, dichloramine B, N-chlorosuccinimide, N-bromosuccinimide, chloromelamine, and tetraglycine potassium periodide, may be active at 10^{-4} M. Organic compounds present in the reaction mixture exert a strong protective action. The instability of these compounds makes it unlikely that they will find practical use as enzyme inhibitors.

Phenol is not an inhibitor of cellulase, but a number of substituted phenols such as chlorophenols, saligenin, orthophenyl phenol and chlorophenyl phenols are moderately active against certain fungal cellulases (72). The cellulase of *Fomes marginatus* is strongly inhibited by a number of phenolic compounds including chlorophenols, tannin, pinosylvin monomethyl ether, pentachlorothiophenol, and 4,6-dinitro-o-cresol at 10^{-3} – 10^{-4} M (67).

A number of miscellaneous compounds have been tested against cellulases. Usually they are inactive. Of over 100 toxic fungicides, antibiotics, and proteolytic enzymes tested against several fungal cellulases, the only active compounds were saturated solutions of phenylmercuric acetate, octylgallate, 2,3-dichloro-1,4-naphthoquinone, and nitrosopyrazole. In addition ethylenebisdithiocarbamate at 10^{-4} M inhibited the cellulases of *T. viride* and *M. verrucaria*, but not that of *Pestalotiopsis westerdijkii*. We had thought that perhaps fungal attack might be prevented by specifically inactivating the extracellular enzymes. In fact, however, higher concentrations of chemical were required to inactivate the enzymes than to prevent growth of the fungus (97).

Saturated phenylmercuric nitrate inhibited the cellulase of *P. vailantii* (113). Phosphotungstic acid inhibited the cellulase of *I. lacteus* (46). The cellulase of *A. oryzae* was inhibited by dithionate, cupferron, *p*-benzoquinone, 8-hydroxyquinoline, β -indole acetic acid, quinine, adenine, caffeine, and betaine at high concentrations (10^{-1} – 10^{-3} M) (56). Diethylmalonate has been reported to inhibit cellulase, but not cellobiase of *C. lunata* and termite cellulase (3, 75). The cellulase of *Fomes marginatus* was inhibited by sodium arsenate, sodium arsenite, sodium azide, 8-hydroxyquinoline, and hydroxylamine at 10^{-3} – 10^{-4} M (67). Again the results of different workers do not always agree. For example, the cellulases of *T. viride*, *M. verrucaria*, and *P. westerdijkii* were not inhibited by 8-hydroxyquinoline or by other metal complexing agents such as versene (32, 72). Most authors agree that all of the above inhibitors are nonspecific protein reactants. There is no good evidence for participation of a metal or other cofactor in cellulase activity.

β -Glucosidases are competitively inhibited by glucose and by gluconolactone (19, 31, 80, 98). This inhibition is specific for enzymes attacking very short chains. Cellulases are not inhibited, except as the overall sugar production is decreased by failure of β -glucosidase to hydrolyze cellobiose and cellotriose to glucose (31, 98). A number of cellulases are competitively inhibited by methylcellulose (D.S. 1.2), and by the sugars lactose and cellobiose (72). The ratio of inhibitor to substrate by weight for effective inhibition is about 0.1 to 0.2 for methylcellulose and 2.0 for the sugars. Methylcellulose and cellobiose are also effective inhibitors of the C_1 of *T. viride* (32, 73).

NATURAL INHIBITORS

Compounds having the ability to inhibit enzymes occur in a wide variety of plants. Indeed they may be universal, varying only in amounts and degree

of inhibition. In 1917 a patent was granted for removal of inhibitors of bacterial amylases from grain by steeping in water (14). Since then water soluble amylase inhibitors have been reported from grain, potato, and banana, and inhibitors of proteinases from seeds of legumes (72). Natural inhibitors of pectic enzymes have been frequently reported from plant sources (15, 16, 72, 129, 132).

The naturally occurring enzyme inhibitors belong chiefly to the phenolics, tannins, or leucoanthocyanins. None of these has yet been crystallized or well characterized. They do not resemble the substrates of the enzymes inhibited; inhibition is not competitive and specificity is usually questionable. Less frequently the inhibitors are proteins. Some natural inhibitors of proteinases are proteins that combine stoichiometrically with the enzyme molecule and inhibit competitively.

A water-soluble cellulase inhibitor was extracted from leaves of the muscadine grape, *Vitis rotundifolia*. This extract also inhibited pectinase. The inhibitor was nondialyzable, heat-stable, unaffected by proteinases, and resistant to autoclaving at pH 5 or 9, but lost 50 per cent of its activity on drying. Marked variation in enzyme susceptibility was found, with 50 per cent inhibition of the cellulase of moldy cucumber flowers at 0.2 per cent inhibitor, while for 50 per cent inhibition of *A. niger* cellulase 4 per cent inhibitor was required (10-12, 29, 30). The inhibitor could be removed from leaf extracts by hide powder, caffeine, gelatin, or nicotine (90). It was isolated by precipitation with caffeine and extraction from the caffeine complex with chloroform without loss of activity. It was found to be a high molecular weight condensed tannin yielding a high percentage of phlobaphenes on acid treatment (91). An inhibitor of rumen cellulase with similar properties has been found in the leaves of sericea, *Lespedeza cuneata* (114). A water-soluble substance which inhibits the cellulase of the fungus, *Stachybotrys* and the termite, *Coptotermes*, has been reported from the wood of *Eucalyptus rostrata* (134).

About 500 plants have been screened for the presence of inhibitors of the cellulase of *T. viride* (70, 72) using methanol, water, and aqueous acetone extracts. The inhibitor content was measured in units, one inhibitor unit being the amount required to neutralize one cellulase unit (101). Seventeen per cent of the plants screened contained ten or more inhibitor units per gram dry weight of tissue extracted. Cellulase inhibitors were found in many plant families and in various parts of the plants including leaves, wood, flowers, fruits, and seeds. The most active sources are shown in Table I.

The bayberry inhibitor was soluble in water, methanol, aqueous alcohols, and aqueous acetone. It was nondialyzable, fairly heat and acid stable, but quickly inactivated at pH 8 and higher and was inactivated by gelatin or peptone. It appeared to be a polymeric leucoanthocyanin. Inhibitors from other plants had similar, but not identical properties. A polymeric leucoanthocyanin from perry pears (58) and a polymeric leucoanthocyanin from persimmon fruit showed similar properties. They strongly inhibited *T. viride* cellulase. The persimmon inhibitor inhibits the C_1 of *T. viride* (73). The

TABLE I
MOST ACTIVE SOURCES OF CELLULASE INHIBITORS

Species	Plant part	Inhibitor units per gram, dry weight
<i>Diospyros virginiana</i> , persimmon	Immature fruit	38,000
<i>Metrosideros polymorpha</i>	Wood	11,000
<i>Lespedeza cuneata</i> , sericea	Leaves	5,500
<i>Eucalyptus robusta</i>	Wood	4,000
<i>Eucalyptus rostrata</i>	Wood	3,000
<i>Musa paradisiaca</i> , Moko plantain	Immature fruit	2,000
<i>Vitis rotundifolia</i>	Leaves	1,700
<i>Myrica pennsylvanica</i> , bayberry	Leaves, fruit, twigs	1,700
<i>Vaccinium nitidum</i>	Leaves	1,200
<i>Psidium guajava</i> , guava	Leaves	1,200

inhibition of cellulase was noncompetitive and reversible by gelatin treatment even after 30 min (72).

Cellulases from different sources vary markedly in their resistance to these inhibitors. Cellulase preparations from *Streptomyces* sp, *M. verrucaria*, Basidiomycete QM 806, *Humicola fuscoatra*, *Penicillium helicum*, *Sporotrichum pruinosum*, and *Stachybotrys atra* were strongly inhibited by ten natural inhibitor extracts. Cellulase preparations from *Penicillium funiculosum* and *P. westerdijkii* showed an intermediate susceptibility. The cellulases of *Aspergillus luchuensis*, *Penicillium pusillum*, and some commercial cellulases were markedly resistant to the inhibitors. Other carbohydrases from these fungi behaved similarly, thus the β -1, 3-glucanases of *T. viride* and Basidiomycete QM 806 were strongly inhibited, the amylase and β -glucosidase of *Aspergillus luchuensis* were resistant.

The natural inhibitors have been partially purified by extraction in methanol, drying *in vacuo*, and extraction of inactive components with propanol, followed by dialysis. Such preparations are highly active, the purified inhibitor from bayberry has 54 μ /mg and the purified inhibitor from persimmon has 190 μ /mg. In contrast, natural tannins showed little or no activity; the best one, Quebracho oak tannin, has only 1 μ /mg. Tannic acid and simple phenols are inactive (72). We have tested astringent substances as inhibitors (unpublished results.) The fresh extract of polyphenol cells of unfermented cacao, rich in the dimer of leucocyanidin, was completely inactive even after attempts at polymerization. Likewise, extracts of Jarrah sapwood and heartwood, and *Rhizophora mucronata* catch were inactive.

We have also studied the occurrence of a cellulase inhibitor in persimmon (Unpublished results). Green fruits of astringent varieties (including the wild persimmon, *Diospyros virginiana*, a variety used for rootstocks, *D. sp*,

lotus type; and *D. kaki* varieties Yokung and K 1) have 200 or more inhibitor units per mg dry weight of fruit. As the fruit ripens, astringency decreases and inhibitor levels fall. Nonstringent varieties, (including *D. kaki* varieties Hyakume, Twentieth Century, and Jiro) have less than 20 inhibitor units per mg even when green. Leaves and twigs of persimmon also contain cellulase inhibitor, but at less than one inhibitor unit per mg, regardless of variety.

We have tested methanol, acetone, and aqueous extracts of 98 samples of woods of known resistance to termites, marine borers, and jungle decay (115) against *T. viride* cellulase. Forty-six per cent had less than 10 inhibitor units per gram dry weight, 28 per cent had 10 to 99 units per gram, 15 per cent had 100 to 199 units per gram, 11 per cent had 200 to 527 units per gram (unpublished results). There was no correlation evident between inhibitor content and resistance to decay, borers, or termites. These results are similar to those of our earlier studies with samples of decay resistant woods such as black locust and cypress that have no detectable inhibitor. Youatt (134) likewise found little inhibitor in the wood of *Eucalyptus regnans* and *E. roridum*, although both of these are very resistant to termites and decay.

Natural cellulase inhibitors have found a few practical uses. Grape leaves have long been added to dill pickle fermentations to prevent softening of the cucumbers. Hillis (48) reports the use of condensed tannin materials including Burma cutch from *Acacia catechu* heartwood, Malayan mangrove cutch from *Carapa obovata* and *Ceriops candolleana* barks, and Borneo mangrove cutch from *Ceriops candolleana*, *Rhizophora candelaria*, and *R. mucronata* barks throughout Indo-Pacific countries to preserve twine and fishing nets from decay. In Kyoto and in other places in Japan the juice of astringent varieties of persimmon is used to tan and preserve paper (111).

DISCUSSION

An adequate defense mechanism capable of dealing with foreign invaders is a requirement of the utmost importance for the survival of a species. Are natural inhibitors of cellulolytic enzymes such a defense mechanism? The idea is an old and appealing one. Cook, et al. (20) suggested in 1911 that polyphenol oxidases in green apples, green pears, and green walnut hulls form tannins from phenols in injured tissue, and that these tannins prevent entry of pathogenic organisms. Considerable work has been done on the possible role of substances toxic to microorganisms in disease resistance (64, 124) and durability of timbers (106). Recently, great interest has focussed on inactivation of pectic enzymes by phenolics as a major factor in disease resistance (16-18, 89, 129). General enzyme inhibition due to phenolics has been noted in cocoa (33) and tea (116) fermentations as well as in extraction of plant mitochondria (50). Cellulases may also be active in pathogenesis (1, 9, 13, 38, 39, 51-53, 105, 130, 132). The demonstration of cellulase in a host plant is difficult and the quantities reported by these investigators have not been impressive, but we are convinced that the cellulases do play a role in

invasion. Finally, cellulases are inhibited by phenolics (67, 72), and phenolics may increase in diseased tissue (121).

This picture is too simple. The amount of enzyme inhibitor extractable from a plant does not appear to be correlated with the resistance of that plant to insects and disease. Resistance must certainly be a summation of factors, one of which may be the presence of natural enzyme inhibitors.

The inhibitors we have found are not specific, but inhibit enzymes in general. Yet many cellulases display a remarkable resistance to inhibition (72). Since the inhibitors react also with other proteins and polysaccharides, the differences in resistance may be due largely to the presence of impurities. Because of their general reactivity, the inhibitors must be produced *in situ* and as needed, if they are to play a role in defense. Otherwise, they would be toxic to the host and inactivated before the pathogen appears.

The inhibitors appear to be polymerization products of phenols. The simple phenols are inactive (72, 89). Inhibitors of pectinase were produced from simple phenols by enzyme or autoxidation (89). In our studies we have failed to produce inhibitors of cellulase from simple phenols by this means (Unpublished results). Our active inhibitors were quickly inactivated by raising the pH to 8. This presumably caused oxidation and further polymerization to insoluble products. The chemical nature of the inhibitors remains elusive. They appear to be "condensed tannins" or polymerized leucoanthocyanins. The condensed tannins show three reactions with proteins: (a) the tanning reaction, (b) astringency, and (c) enzyme inhibition. We have studied natural and synthetic tanning agents and found no correlation of tanning ability and activity as enzyme inhibitors. We have done the same with astringent principles and found that while the same types of compounds are effective, monomers and dimers that are highly astringent do not inhibit enzymes. Somewhat larger molecules are required for enzyme inhibition than for astringency, although there is a range in which both properties are exhibited. As the molecules become very large they are neither astringent nor inhibitory to cellulase.

Work has been done on the nature of the monomer in persimmon leucoanthocyanin responsible for astringency (36, 54, 77) and the leucoanthocyanin of guava (107, 108), as well as the changes in molecular weight associated with ripening and loss of astringency (37, 57). Perhaps some of this work will cast light on the nature of the enzyme inhibitors.

Most of the work with enzyme inhibitors has dealt with the direct effect of extracted compounds. We may have been overlooking more important compounds which, although themselves inactive, yield active inhibitors as a result of hydrolysis or other changes brought about by the pathogenic organism.

The resistance of apple leaves to *Venturia inaequalis* (82, 83) is not correlated with content of phloridzin or of polyphenol oxidase, but resistant varieties have greater β -glucosidase activity. Fungus action triggers, in an unknown manner, the release of the β -glucosidase which hydrolyzes phlorid-

zin and releases phloretin. The phloretin is oxidized by phenol oxidases to form a transient inhibitor of spore germination. The inhibitor rather quickly becomes inactive on account of polymerization. No inhibitor is formed in the presence of gluconolactone which inhibits β -glucosidase, 4-chlororesorcinol which inhibits polyphenol oxidase, or sodium metabisulfite which prevents oxidation (82, 83). Similarly, resistance of pears to *Erwinia amylovora* is related not to arbutin levels, but to β -glucosidase content. The β -glucosidase hydrolyzes arbutin and releases toxic hydroquinone (47). Perhaps the clue to disease resistance lies in the unknown mechanism by which fungus invasion triggers hydrolysis of the glucoside, which in turn leads to formation of an inhibitor.

The presence of complex wall substances in bacteria may be a defense mechanism against the lytic enzymes of other organisms. Complex or unusual polymers may also occur in walls of higher plants (112) but they have not been reported to increase in disease conditions. However, resistance to polygalacturonase has been reported to increase in bean hypocotyls infected with *Rhizoctonia* with an increase in divalent cations, especially calcium. These cations are believed to form pectates which resist hydrolysis by the fungal enzymes (6-8). Formation of pectates in castor bean capsules has also been reported as a factor in resistance to *Botrytis* (119).

SUMMARY

Cellulases are remarkably stable to pH and temperature changes and to chemical inhibitors. They are competitively inhibited by cellobiose and methocel and inactivated by such protein reactants as halogens, heavy metals, and detergents. A type of polymeric leucoanthocyanin found in many plants, notably in the unripe fruit of persimmon, is a powerful, but nonspecific inhibitor of cellulase. The susceptibility of cellulases to chemical inhibitors increases with purification. The C_1 component, which allows cellulase to attack insoluble cellulose, is more labile than the hydrolytic C_x components. Formation of cellulolytic enzymes is repressed by growth on sugars.

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