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The Cellulosome of *Clostridium thermocellum*

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I. Introduction

The accelerated interest in microbial cellulases stems from their potential industrial application. This area is an excellent example in which basic and applied science are closely interlinked: the better understood the mechanism of cellulase action, the better will be the applicative value as a biotechnological process. In spite of significant advances in the area, we are still far away from both the scientific and practical goals.

Although current concepts of cellulase action explain to a reasonable extent the phenomenon of cellulose degradation, new hypotheses have recently been introduced. These have been postulated due to the necessity to account for various physical features of cellulolytic organisms that cannot be explained by the nature of the purified extracellular enzymes. These include the observed adherence of various microbes and their cellulases to cellulose, and the extremely high affinity of the cell-associated system versus the cell-free system. In particular, the rate of cellulose catabolism by intact cells is in most systems significantly higher than that of the cell-free cellulase system. These observations have stimulated the formulation of new ideas on the mechanism of cellulose degradation.

In this review, we present a new concept concerning the mode of cellulose degradation. According to our findings (using the anaerobic thermophilic bacterium *Clostridium thermocellum* as a model organism), many of the required enzymes for efficient degradation of crystalline cellulose form a defined, multicomponent, high molecular weight complex which we have termed the *cellulosome*. In the model organism, the cellulosome is anchored to the cell surface in polycellulosomal centers, which also mediate the adherence of the bacterium to the insoluble substrate.

Biomass in the form of cellulose is the major constituent of plant matter, thereby comprising the most abundant organic resource in the world. As such it provides a very appealing renewable raw material for the production of food and energy. However, before using this resource, it is necessary to convert it into a more usable form such as gaseous (e.g., methane) or liquid (e.g., ethanol) feedstock by means of an economically feasible technological process.

One of the potential industrial approaches to biomass utilization is the enzyme-catalyzed hydrolysis of cellulose to soluble sugars that can in turn serve as substrates for fermentation to fuels and chemicals. This particular approach has been the subject of intensive research over the past several decades, and although some of the biochemistry concerning the enzymatic hydrolysis of cellulose has been clarified, the development of a viable biotechnological process has thus far been stymied. In order

to better understand the considerations involved in the design of such a process, it is instructive to review briefly some of the major chemical and structural features of cellulose as well as some of the important characteristics and historical findings concerning its degradation by cellulase enzymes.

A. Cellulose and Cellulases

Cellulose is composed of repeating units of cellobiose, 4-O-(β-D-glucopyranosyl)-D-glucopyranose, a simple disaccharide (Fig. 1). Despite its relatively simple primary structure, the tertiary (or quaternary) structure of cellulose is extremely complicated (Cowling and Kirk, 1976). The basic molecular structure is a linear polymer consisting of up to 10^4 D-glucose moieties which are arranged in fibrils. The fibrils consist of several parallel cellulose molecules stabilized by hydrogen bonds. Although individually the hydrogen bonds are relatively weak, collectively they become a strong associative force as the degree of polymerization increases. The fibrils are organized into a "paracrystalline" state, thus adding to the structural rigidity of cellulose. Over three-quarters of the cellulose structure is considered to exist in these enzymatically resistant, crystalline regions and the remainder comprises the relatively easily hydrolyzable "amorphous" areas.

The utilization of cellulose as a substrate for bioconversion processes is determined by its susceptibility to cellulase enzymes, and the availability of high activity enzymes is the basis for the design of a successful process for the enzymatic conversion of cellulose. However, the action of cellulases on cellulose is not a straightforward phenomenon, and the quality of high activity with reference to cellulase must be further discussed.

The enzymatic hydrolysis of cellulose is a heterogeneous reaction system which involves either water-soluble, particulate, or cell-bound enzymes attacking a water-insoluble substrate. Due to the complicated

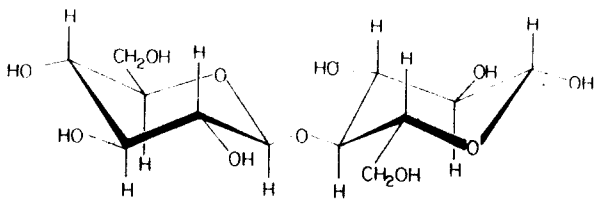

FIG. 1. The structure of cellobiose, the repeating unit of the cellulose polymer.

substrate structure, enzymatic hydrolysis of cellulose to simple sugars is equally complicated. A single enzyme cannot accomplish the task of extensive cellulose degradation, and multiple enzyme systems are required. Consequently, microorganisms that successfully grow on cellulose as a substrate are capable of doing so by producing a collection of different cellulases [1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4] including endoglucanases and exoglucanases [exo-cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91) and β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21)] which act cooperatively to hydrolyze the intricate structure of the cellulose matrix.

The overall facility with which a particular enzyme system acts on cellulose may depend upon many factors, including the following:

1. The recognition and binding of the soluble, particulate, or cell-bound enzyme onto cellulose.
2. The diffusion into or motility of the cellulases on the solid cellulosic matrix.
3. The hydrolytic reactions necessary to form soluble sugars.
4. The relative efficiency of hydrolysis of crystalline regions or the capacity to converty crystalline regions to amorphous regions.
5. Product inhibition characteristics of the various cellulase enzymes.

The final industrial design may take into account other factors, which are not directly related to cellulose hydrolysis, such as the following:

1. Subsequent diffusion of the soluble degradation products from the cellulose matrix.
2. Catabolic conversion of soluble sugars to other desirable fermentation products, e.g., ethanol, acetone, acetic acid, butanol.
3. Tolerance of these enzymes and/or of the cells to high levels of these products.

Thus, in considering an appropriate enzyme system for potential industrial saccharification of cellulose in the classical sense, a stable cell-free enzyme preparation with adequate levels of all essential components of the enzyme is required.

B. Mechanism of Cellulase Action

A mechanism for the conversion of native cellulose to soluble sugars was first suggested by Reese and colleagues (1950). Their model was based on a two-step sequential process which was characterized by a C_1 component and a group of components collectively referred to as C_x.

The C_1 component was considered to consist of a "defibrillating factor" which severed the hydrogen bonds necessary to form the crystalline

structure of cellulose. Initially, the hydrolysis of covalent bonds was not attributed to the C_1 component. Although evidence has been accumulated for the existence of this component type in the multimembered cellulase enzyme system of "true" cellulolytic microorganisms, the specificity of the C_1 component(s) is still in controversy. Various laboratories (Halliwell and Griffin, 1973; Wood and McCrae, 1979) have isolated C_1 activity which was identified as a cellobiohydrolase. On the basis of these findings, Reese's original position concerning the C_1 was modified, and in later publications the C_1 component was also considered to have the capacity to degrade portions of cellulose (Reese, 1976). This particular controversy has not been settled and may eventually prove superfluous.

In any event, in the original hypothesis, the C_1 component was envisaged to cause first the alteration or "activation" of the cellulose structure, which then enabled further hydrolysis by the C_x enzyme(s). The latter were considered to comprise cellulolytic enzymes (both endo- and exoglucanases) in the conventional sense where covalent bonds are broken.

The original hypothesis was important for many reasons. First, it served as a working model which stimulated a wide variety of subsequent studies. It also served to describe on the molecular level many of the observed phenomena associated with cellulase hydrolysis of cellulose. For example, the interplay between the C_1 and the C_x components offered a reasonable explanation for the synergy among cellulase components. Also, according to the hypothesis, microorganisms that grow on soluble cellulose derivatives would synthesize only C_x components, whereas microorganisms that grow on highly ordered forms of cellulose would produce both C_1 and C_x.

More recent evidence strongly supports the mechanism of enzymatic degradation first suggested by Eriksson (1969). According to this mechanism, amorphous regions in the cellulose fiber are first attacked by endoglucanases, thereby exposing free chain ends. Exoglucanases then come into action by hydrolytically removing cellobiose from the chain ends. The free cellobiose is hydrolyzed further to glucose through the action of β-glucosidase(s).

The reigning concept now states that endoglucanases act randomly over the cellulose chain and that exoglucanases act on exposed chain ends, the endo- and exoglucanases having a strong synergistic action. Though synergism between exo- and endoglucanases has clearly been established, extensive hydrolysis of more crystalline forms of cellulose has been shown to be accomplished only by certain pairs of hydrolytic enzymes (Wood and McCrae, 1979). These pairs of endo- and exoglucanases have been suggested to form loose complexes on the cellulose surface. Such a phenomenon might be necessary to allow for rapid sequential action of

these enzymes, although simultaneous action of exo- and endoglucanases may also occur. Complex formation and consequent degradation within crystalline regions would prevent potential reformation of the glycosidic bond after single-point cellulase-induced cleavage. Despite attempts to demonstrate directly by electron micoscopy the presence of such complexes attached to cellulose fibers (White and Brown, 1981), definitive proof awaits further experimentation.

The phenomenon of product inhibition is noteworthy to mention in this context. Cellobiose has been implicated as a potent inhibitor of many cellulolytic systems. The operational activity of these systems can be increased by increasing the cellobiase (β-glucosidase) activity which converts cellobiose to the less inhibitory glucose.

C. Microbial Degradation of Cellulose

The mechanism described above was derived mainly from studies on aerobic fungi such as *Trichoderma* and *Sporotrichum* species. Relatively little has been done with other cellulolytic systems, notably those of anaerobic bacteria, although it has been assumed (perhaps without sufficient basis) that the same principles hold.

Indeed, many fungi and bacteria are known that can degrade cellulose (Table I). However, in many cases, the products of growth are microbial cells and various metabolic products which at present are not industrially applicable.

Simple growth of a successful cellulolytic organism on cellulose will produce a variety of products, some of which are potentially useful industrially and others are not. For example, among the preferred bacterial products are soluble sugars, ethanol, acetone, butanol, butanediol, and the like. Among the undesirable products are acetic acid, lactic acid, and other organic acids which are difficult to separate and process further. In addition, only a few fungal and bacterial species have been reported that produce high activity cellulases capable of extensively degrading insoluble cellulose to soluble sugars *in vitro*. In this context, many organisms are known to degrade only soluble forms of cellulose such as carboxymethylcellulose. Moreover, many organisms do not produce large amounts of cellulases in the soluble form despite the fact that they grow effectively on insoluble cellulose, the degradation of which must take place extracellularly.

One microorganism which has been considered seriously for potential industrial application is *Clostridium thermocellum*, an anaerobic thermophilic cellulolytic bacterium, which we have used as a model organism in our investigations. In the remaining sections of the review, we trace the historical and contemporary studies that have led to the

TABLE I

SOME REPRESENTATIVE CELLULOLYTIC MICROORGANISMS

Microorganism	Reference
BACTERIA	
Anaerobic strains	
Gram positive	
Ruminococcus albus	Sijpesteijn (1949); Hungate (1947)
Ruminococcus flavefaciens	Sijpesteijn (1951)
Eubacterium cellulosolvens	Holdeman and Moore (1972)
Clostridium cellulovorans	Sleat *et al.* (1984)
Clostridium stercorarium	Madden (1983)
Clostridium thermocellum	Viljoen *et al.* (1926)
Gram negative	
Bacteroides cellulosolvens	Guiliano and Khan (1984); Murray *et al.* (1984)
Bacteroides succinogenes	Hungate (1950)
Acetivibrio cellulolyticus	Patel *et al.* (1980); Saddler and Khan (1980)
Aerobic strains	
Gram positive	
Bacillus sp.	Robson and Chambliss (1984)
Streptomyces flavogriseus	Ishaque and Kluepfel (1980)
Cellulomonas fimi	Whittle *et al.* (1982)
Thermomonospora sp.	Henssen (1957)
Gram negative	
Cellvibrio gilvus	Cole and King (1964); Carpenter and Barnett (1967); Breuil and Kushner (1976)
Cellvibrio fulvus	Mullings and Parish (1984); Berg *et al.* (1972a,b)
Pseudomonas fluorescens var. *cellulosa*	Yamane *et al.* (1965)
FUNGI	
Anaerobic strain	
Neocallimastix frontalis (Phycomycetes)	Orpin (1975); Bauchop (1979); Mountfort and Asher (1985)
Aerobic strains	
Trichoderma reesei	Montenecourt (1983)
Sporotrichum pulverulentum	Eriksson (1979)
Myrothecium verrucaria	Halliwell (1961)
Penicillium iriense	Boretti *et al.* (1973)
Phanerochaete chrysosporium	Eriksson (1981)

formulation of the cellulosome concept. The characterization of the cellulosome in *C. thermocellum* is treated in detail, and recent advances using other cellulolytic systems are discussed within the framework of this model.

II. *Clostridium thermocellum*

A. Isolation of the Organism and Catabolic Steps

A species of *C. thermocellum* capable of fermenting cellulose to ethanol was first described by Viljoen *et al.* (1926). However, stable pure cultures of anaerobic thermophilic cellulolytic bacteria were initially isolated by McBee (1948, 1950) and later by several other investigators. Although the cellulolytic properties of several of the latter strains remain poorly defined, many are similar to the *C. thermocellum* strains described by McBee (1950). Certain physiological features of *C. thermocellum* have been sources for conflicting reports. For example, McBee (1948, 1950) was unable to grow *C. thermocellum* on glucose while Patni and Alexander (1971) obtained good growth of the same organism on either glucose or xylose. These authors also demonstrated the presence of an inducible hexokinase along with some other glycolytic enzymes. Later, Lee and Blackburn (1975) described the cellulolytic properties of an isolate similar to *C. thermocellulaseum* (this strain is currently considered a derivative of *C. thermocellum*) that proliferated on cellulose, glucose, and numerous other mono- and disaccharides.

Ng *et al.* (1977) described several isolates which grew on cellulose and its degradation products, but not on glucose. Lamed and Zeikus extensively studied the catabolic pathways of *C. thermocellum* and identified some unusual enzymes including a regulatory alcohol dehydrogenase (Lamed and Zeikus, 1980) and an ammonium-activated malic dehydrogenase (Lamed and Zeikus, 1981).

One of the distinctive features of *C. thermocellum* is its production of very high levels of an extracellular cellulase system. The extracellular endoglucanase activity was found to be constitutive, namely, independent of whether the cells were grown on cellulose, cellobiose, or glucose (Garcia-Martinez *et al.*, 1980; Lamed and Zeikus, 1980). Growth of *C. thermocellum* on a soluble substrate such as cellobiose was more rapid than growth on the insoluble substrate (cellulose), indicating that the solubilization of cellulose is a rate-limiting step for growth. Of note is a report (Ng *et al.*, 1977) that the specific activity of both endoglucanase and exoglucanase remains constant throughout the course of fermentation, suggesting the growth-linked production of extracellular cellulase.

Transport systems for both cellobiose and glucose are present in *C. thermocellum*. Glucose is apparently transported in this organism by an ATP-dependent permease only in glucose-adapted cells (Hernandez, 1982), whereas cellobiose is incorporated by both glucose- and cellobiose-grown cells (Ng and Zeikus, 1982). One plausible explanation for the observed phosphorylation of the residual glucose moiety is that cellobiose transport may be linked to phosphorylation by membrane-bound enzymes (both cellobiose phosphorylase and a hypothetical hexokinase, for example) which together funnel the phosphorylation reaction to completion.

The enzymatic pathways in *C. thermocellum* responsible for the catabolism of cellulose degradation products have not been entirely elucidated. The lack of clarity, especially regarding the initial steps of transport and activation of soluble sugars, may be due to the fact that, among the published works, a variety of different strains were used. In some cases, the cultures may not have been entirely pure.

In most cellulolytic microorganisms, cellobiose is cleaved by β-glucosidases to glucose. In some cellulolytic bacteria, however (e.g., *Cellvibrio gilvus* and in particular *C. thermocellum*), cellobiose is converted into glucose 1-phosphate and glucose by the enzyme cellobiose phosphorylase (Alexander, 1968, 1972; Swisher *et al.*, 1964). The latter mechanism causes the bacterium to exhibit preferential growth on cellobiose (versus glucose) as an energy source, perhaps due to conservation of the energy contained in the glycosidic linkage of cellobiose. Another possible explanation for the preference for cellobiose is the low level (Hernandez, 1982) or lack (Lamed and Zeikus, 1980) of soluble glucokinase in this organism. If hexokinase is indeed absent, it remains unclear how one of the glucose units in cellobiose becomes phosphorylated (Ng and Zeikus, 1982), since cellobiose phosphorylase activity which has been demonstrated in *C. thermocellum* gives glucose 1-phosphate and glucose as products (Alexander, 1968). An isomerase converts glucose 1-phosphate to glucose 6-phosphate. Both glucose units, however, were shown to be metabolized via glucose 6-phosphate, in spite of the absence of demonstrable levels of hexokinase (Ng and Zeikus, 1982) and phosphoenolpyruvate phosphotransferase system (Hernandez, 1982; Lamed and Zeikus, 1980).

B. Industrial Potential

The advantages and disadvantages of anaerobic thermophilic fermentation, with particular relevance to ethanol production, have been reviewed recently (Sonnleitner and Fiechter, 1983; Esser and Karsch, 1984; Slapack *et al.*, 1986). On the positive side, the application of a thermophilic organism would reduce energy costs required for refrigeration of fermentors during culture of mesophilic organisms; circulating tap

water is sufficient for temperature maintenance in thermophilic cultures. In addition, fermentation under anaerobic conditions would obviate problematic and costly aeration procedures. Moreover, thermophiles are capable of fermenting a broad range of substrates, product distillation costs are less expensive, the danger of contamination (particularly by pathogens) is low, and the thermophilic enzymes produced are unusually stable to a variety of harsh conditions.

These advantages are offset by a spectrum of limiting factors which have curbed the industrial application of anaerobic thermophilic strains. These organisms usually exhibit relatively low productivity and low tolerance to high concentrations of desired products (such as ethanol). Fermentation at high temperatures is deleterious to biotechnological equipment, causing extensive deterioration of biosensors and the leaching of inhibitory trace metals from fermentation vessels. Consequently, there are serious problems in scale-up procedures and in the industrial applicability of these relatively uncharacterized strains.

Despite these limitations, various potential strategies have been considered in order to obtain desired products from cellulose. One approach would be to apply either a crude or purified cellulase preparation to obtain soluble sugars which can, in turn, serve as substrates for further fermentations. A recently described variation of this approach was the use of a resting cell suspension capable of cellulose degradation (Giuliano and Khan, 1985). An alternative approach would be the direct fermentation of cellulose to preferred products. This approach would theoretically use a cellulolytic organism that effectively produces high levels of a desired product under optimal conditions. Such a strain has yet to be described, and for this reason the application of cocultures has been considered.

Coculture fermentation involves the growth of two or more compatible and complementary organisms, one capable of effective cellulolysis and the second (or additional strains) capable of competing successfully for the resulting sugars, thereby producing a preferred product(s) which predominates (Zeikus *et al.*, 1983). A combination of the two above-described approaches has recently been described and has been called simultaneous saccharification fermentation (Ooshima *et al.*, 1985). Using this procedure, cellulose is hydrolyzed enzymatically via a given cellulase system, and the resultant sugars are then fermented by a suitable microorganism (i.e., bacteria or yeast). A related, but potentially more elegant, approach would be to genetically incorporate the essential cellulase enzymes into the sugar-fermenting bacterium or yeast.

C. Strategies Involving *C. thermocellum*

The concept of direct cellulose fermentation to ethanol has been studied actively by several research groups. As mentioned above, in this approach soluble sugars produced by extracellular cellulolysis would be

assimilated secondarily by a high ethanol-yielding organism which is grown either in coculture or in succession with the cellulolytic species (the latter having an unfavorable product pattern). The idea behind this approach is based on the successful modification of the product pattern of *C. thermocellum* from mixed acid–ethanol production to almost strictly ethanolic fermentation when another clostridial species with high ethanol yield is cocultured.

In this context, *C. thermocellum* has been studied specifically together in culture with either *Clostridium thermohydrosulfuricum* (Ng *et al.*, 1981) or *Clostridium thermosaccharolyticum* (Avgerinos and Wang, 1980; Wang *et al.*, 1983; Saddler and Chan, 1984). Despite the fact that theoretical ethanol yields were attained in these systems, the approach was hampered by the problem of adapting *C. thermocellum* to high ethanol concentrations (4–5%). In addition, difficulties were encountered in fermenting cellulose levels greater than a few percent of the insoluble substrate. This was even more striking when natural substrates (corn stover, wood shavings, etc.) were fermented. Furthermore, toxic substances were produced which adversely affected growth and product pattern in pretreated corn stover. The latter was studied as a substrate in the "MIT process" with genetically improved *C. thermocellum* and *C. thermosaccharolyticum* (Leuschner *et al.*, 1983). Attempts to recycle cellulase also failed to improve the process significantly, perhaps due to the "large molecular nature" of the cellulase of this organism (see following section).

There have also been attempts to isolate wild-type or mutant strains of *C. thermocellum* exhibiting improved ethanol production or improved adaptability to high concentrations of ethanol (Herrero and Gomez, 1980). Unfortunately, several of these strains (which were selected during growth on pure cellulose and which produced lower levels of undesirable organic acids in small-scale experiments) behaved differently upon fermentation in larger vessels or upon growth on "natural" substrates. For example lignin, which is not degradable under anaerobic conditions, severely interferes with cellulose degradation. In addition, the property of resistance to ethanol concentrations greater than 2% is difficult to acquire and the trait is usually genetically unstable.

D. "True" Cellulase in *C. thermocellum*

The ability of *C. thermocellum* to grow efficiently on α-crystalline cellulose has long been recognized (McBee, 1948). "True" cellulase activity, the ability to dissolve crystalline cellulose completely (Johnson *et al.*, 1982b), has also been demonstrated in cell-free preparations, and this feature is probably the major reason why *C. thermocellum* remains such an attractive organism. Such activity has been shown to depend