Year 3 Final Report for Grant No. DE-FG02-10ER16076
"Dissecting the functional significance of non-catalytic carbohydrate binding modules in the deconstruction of plant cell walls"

Michael G. Hahn
University of Georgia
Complex Carbohydrate Research Center

Overview of the research programme:
The project seeks to investigate the mechanism by which CBMs potentiate the activity of glycoside hydrolases against complete plant cell walls. The project is based on the hypothesis that the wide range of CBMs present in bacterial enzymes maximize the potential target substrates by directing the cognate enzymes not only to different regions of a specific plant cell wall, but also increases the range of plant cell walls that can be degraded. In addition to maximizing substrate access, it was also proposed that CBMs can target specific subsets of hydrolases with complementary activities to the same region of the plant cell wall, thereby maximizing the synergistic interactions between these enzymes. This synergy is based on the premise that the hydrolysis of a specific polysaccharide will increase the access of closely associated polymers to enzyme attack. In addition, it is unclear whether the catalytic module and appended CBM of modular enzymes have evolved unique complementary activities.

Objectives
The key objectives of this proposal were to test the following hypotheses:
1. The biological rationale for the diversity of bacterial CBMs found in nature is to maximize the range of plant cell walls that can be degraded by the cognate enzymes.
2. The essential synergy between glycoside hydrolases that display complementary activities is promoted by CBMs through the targeting of these biocatalysts to the same region of the plant cell wall.
3. Specific CBMs, which target the ends of polysaccharide chains, direct glycoside hydrolases to regions of cell walls that are particularly susceptible to enzyme attack.
4. The catalytic module linked to a specific CBM has evolved biochemical properties that are complementary to the targeting function of the appended CBM.

I. Project outcomes:

Construction of protein hybrids: To facilitate the objectives outlined above appropriate hybrid enzymes comprising appropriate CBMs and catalytic modules needed to be generated. In preliminary data, the majority of the constructs had been made prior to the start of the project. However, the remaining hybrid proteins were synthesized during the course of the program. It should be emphasized that we reduced our focus on cellulose degradation and thus this line of investigation was limited to fusing the crystalline cellulose binding CBM (CBM3a) to a novel endoglucanase Cel124A. Instead, we focused more extensively on xylan and mannan degradation. Thus, we also fused CBM2b-1-2 and CBM15 to Xyn10D, CBMX14 (now referred to as CBM60) to four xylanases, CBM35-Xyl to *Cj*CBM62A, CBM35-Pel to *Cj*Xyn10B for studies of xylan degradation. In addition, we fused

CBM3a, CBM27 and CBM35 to *Cj*Man5a and *Cj*Man26a for studies of mannan degradation and esterases. The activities of esterases were studies by fusing CBM2b-1-2 and CBM27 to *Cj*CE2B, and CBM3a and CBM27 *Cj*CE2C. Finally, we fused CBM9 and CBM27 to GFP for use as cell wall probes.

CBMs and cellulose degradation: As stated above, our initial experiments indicated that a range of cellulases had little effect on the cell wall, suggesting that this component of the project would be difficult. Thus, when the project was funded, at a reduced cost, we were invited to redefine our objectives. To this end, we reduced our work on cellulases. We did, however, pursue the activity of a novel cellulase against the plant cell, and its capacity to act in synergy with Cel48S, the major cellobiohydrolase expressed by *Clostridium thermocellum*. To explore the capacity of *Ct*Cel124 to disrupt the plant cell wall, the catalytic module was incubated with sections of Arabidopsis stem, which were subsequently stained by Calcofluor White that binds predominantly to cellulose, and CBM9 fused to green fluorescent protein. CBM9 binds to the reducing end of cellulose and xylan chains and thus provides a direct read out of cellulose hydrolysis. The Calcofluor White staining data show that the primary cell walls were considerably thinner and significantly disrupted after cellulase treatment. Although CBM9 did not bind to untreated cell walls, after incubation with *Ct*Cel124$_{CD}$, the protein stained the secondary and primary cell walls of both plant species. These data indicate that *Ct*Cel124 is able to attack cellulose embedded in cell walls. These promising data suggest that combining *Ct*Cel124 with other *exo*- and other *endo*-acting cellulases may have a significant effect on cellulose degradation within intact cell walls.

It is well established that *exo*- and *endo*-acting cellulases act in synergy to hydrolyze cellulose. In addition, these enzymes normally contain cellulose-specific CBMs that potentiate catalysis by recruiting the cellulases to the surface of the insoluble substrate. In the *C. thermocellum* cellulosome, the most abundant *exo*-acting cellulase is Cel48S, while the crystalline cellulose-specific CBM (CBM3a) is supplied by the non-catalytic scaffoldin CipA. To explore the possible synergy between *Ct*Cel124 and Cel48S, the capacity of the enzymes, individually and in combination, to release reducing sugar from Avicel was assessed. The data, showed that 1.3-fold more reducing sugar was released when the two enzymes were used in combination, compared to the additive value when the two enzymes were used in isolation. These data indicate that *Ct*Cel124 and Cel48S exhibit a degree of synergy when acting on highly crystalline cellulose. When both enzymes were appended to CBM3a, which binds to crystalline cellulose, more extensive synergy (1.9-fold) was observed between the two cellulases. Thus, it is possible that the CBM may target Cel48S and *Ct*Cel124to similar regions of Avicel and, by so doing, potentiate the synergy between the two enzymes.

A striking feature of the substrate binding cleft of *Ct*Cel124 is the different topologies displayed by its positive and negative sub-sites, respectively. Sub-sites -4 to -1 form a deep narrow cleft in which the bound trisaccharide is significantly twisted. In contrast, sub-sites +1 to +3 display a more open topology, and the conformation of the bound trisaccharide adopts an approximate two-fold screw axis. The twisted structure of cellotriose is adopted by cellooligosaccharides in solution, while the linear conformation adopted by cellotriose bound to the distal positive sub-sites is

similar to the structure of the glucan chains in crystalline cellulose. Thus, it is likely that the substrate binding cleft of *Ct*Cel124 is tailored to recognize specific sub-structures of cellulose, which are at the interface between crystalline and paracrystalline (or amorphous) regions of cellulose. Many cellulose-degrading bacteria express a large number of *endo*-β-1,4-glucanases, exemplified by *C. thermocellum*, that has the potential to synthesize ~30 endoglucanases. The biological rationale for the expansion in this enzyme activity in *C. thermocellum* and other organisms is currently unclear. Cellulose, although chemically invariant, displays very different topologies ranging from highly crystalline structures to isolated highly twisted glucan chains (amorphous cellulose). It is possible that at least some of the endoglucanases expressed by a single organism are tailored to recognize specific cellulose substructures found in nature. *Ct*Cel124, by targeting the boundary between crystalline and amorphous regions of cellulose, may generate reaction products that comprise substrates for *exo*-acting cellulases that act on the non-reducing end of crystalline cellulose and the reducing end of isolated cellulose chains, respectively.

CBMs active on xylans and pectins: This project was ongoing when the original grant was submitted. The initial paper derived from this work, does not include the post-docs employed on the grant, but did involve intellectual input from the original PI, H.J. Gilbert. A brief summary of the data are as follows: Pectic HG is abundant in the cell walls of parenchymal tissues of tobacco stems, as evidenced by JIM5 antibody binding. HG degradation could be quantified by determining the depletion of JIM5 immunofluorescence. After a 1-h treatment with 10 nM *Cellvibrio japonicus* pectate lyase Pel10A, 50 ± 3% of JIM5 binding was lost compared with the control, in which no enzyme was added. When crystalline cellulose-directed CBMs from families 3a and 2a were appended to the lyase, there was an increase in the depletion of JIM5 fluorescence; the levels of JIM5 fluorescence were reduced to <25% and <15% of control levels, respectively. In these experiments, the remaining JIM5 fluorescence was restricted to the corners of intercellular space at the junctions of adhered and unadhered cell walls, examples of which are shown for CBM2a·-Pel10A. In contrast, there was no increase in activity when xylan-binding CBMs from family 15 or 2b were appended to the pectate lyase. This is consistent with the observation that no xylan has been detected in tobacco stem pith parenchymal cell walls, and these CBMs bind strongly to the secondary cell walls of the vascular tissues in other regions of the stem. These studies indicate that there are distinct regions of primary cell walls that contain different amounts of HG and/or the tissue location of the pectic polysaccharide influences its susceptibility to degradation by pectate lyase. The pectic HG in adhered cell walls is most readily lost, followed by cell wall regions lining intercellular space and, finally, the corners of intercellular space at the junctions between adhered and unadhered regions of adjacent cells. The appending of a crystalline cellulose-directed CBM to the pectate lyase can promote the degradation of pectic HG in all these regions of primary cell walls.

Wheat endosperm cell walls are typified by a high level of arabinoxylan and a low level of cellulose. The capacity of xylan and cellulose-directed molecular probes to bind to cell walls of wheat grain was assessed by immunohistochemistry. Thus, the monoclonal antibody LM10 (raised against unsubstituted xylan) has been demonstrated previously to bind only to no- or low substituted xylans and showed no recognition of the cell walls in the wheat grain endosperm. The GH51

arabinofuranosidase, Abf51A, from *C. japonicus* releases O2 and O3 linked arabinofuranose side chains from monosubstituted backbone residues in xylan and arabinan. To study the impact of appended CBMs on arabinofuranosidase action, hybrid enzymes were generated by fusing the cellulose binding CBM2a or the xylan-binding CBM2b-1-2 to the catalytic module of Abf51A. Although some LM10 binding to wheat grain central endosperm cell walls was observed after treatment with 100 nM Abf51A, the binding was sparse. The Abf51A derivatives containing CBM2a and CBM2b-1-2 were more active in generating the LM10 epitope than the catalytic module alone when used at equimolar concentrations. Appending the cellulose (CBM2a)- and xylan (CBM2b-1-2)-binding modules to Abf51A resulted in 4- and 20-fold increases in LM10 epitope detection, respectively. These data indicate that LM10 is an effective probe for arabinofuranosidase action on endosperm cell wall arabinoxylan. The substantial potentiation of arabinofuranosidase activity by the xylan-binding module and the lesser impact of the cellulose-binding module on side chain removal reflect the relative abundance of these polysaccharide ligands in the endosperm

A previous study has shown that the enzymatic treatment of tobacco stem sections with xylanases Xyl10B or Xyl11A reduced but did not completely abolish xylan epitopes in secondary cell walls, indicative of the recalcitrant nature of these composite structures. Variants of Xyl10B and Xyl11A containing the xylan-binding modules CBM15 and CBM2b-1-2 and the crystalline cellulose binding modules CBM2a and CBM3a were generated. Equimolar amounts of enzyme were incubated with a series of tobacco stem sections, and the removal of xylan was determined using CBM2b-1-2 appended to GFP. The catalytic modules of Xyl10B and Xyl11A, as discrete entities, resulted in 37 ± 5% and 36 ± 5% reductions in CBM2b1-2–mediated fluorescence, respectively. Appending the CBMs to the two xylanases generally resulted in an increase in the capacity of the enzymes to remove xylan from secondary cell walls; only Xyl10B linked to CBM15 did not display an increase in activity compared with Xyl10B. CBM2b-1-2, which binds to a broad range of xylans, potentiated the activities of both xylanases by decreasing the fluorescence intensities by $<$2-fold compared with the respective catalytic modules alone. That CBM15 only enhanced Xyl11A activity may reflect the specificity of CBM15 for highly exposed xylans, which are readily removed by the Xyl10B catalytic module on its own (discussed further below). Both cellulose-directed CBM2a and CBM3a increased the catalytic activities of the two enzymes to a similar extent as CBM2b-1-2 ($<$2- to 4-fold decrease in fluorescence compared with the catalytic modules alone). There was a general trend that appending CBMs had an increased impact on Xyl11A activity relative to Xyl10B. It is possible that the xylan-binding CBM2b-1-2 and the two cellulose-binding modules target their attached catalytic modules to distinct xylan substructures or contexts in the cell walls. If this hypothesis is correct, one might expect enhanced xylan degradation when the CBM3a- and CBM2b-1-2–linked xylanases are coincubated with cell walls. To address this question, tobacco stem sections were treated with equal quantities of CBM3a- and CBM2b-1-2–containing enzymes, either individually or in combination, while keeping the total concentration of the catalytic module constant. No significant additional xylan degradation by the CBM enzyme mixtures was observed compared with incubations containing CBM3a·Xyl10B or CBM2b-1-2·Xyl10B. These data suggest that the different CBMs are not directing the enzyme to xylans that are in differing cell wall contexts.

The role of CBMs in mannan degradation: Although the project was mainly centered on the role of CBMs in xylan degradation, results obtained during the course of the grant caused us to examine mannan degradation as well. We focused our research on two mannanases, *Cj*Man5A (GH5) and *Cj*Man26A (GH26), which differ in their enzymatic activities. The GH26 mannanase targets mannan primarily, while the GH5 enzyme displays similar activity against galactomannan and glucomannan. The objective was to use highly characterized enzymes and CBMs to create artificial enzymes whose activities could be tested on different intact cell walls to gain insight into whether the specificity of the CBMs or the topology of the catalytic modules are the primary drivers for the action of these enzymes against cell walls. The results of these studies (published in a paper in the Journal of Biological Chemistry (Zhang et al., 2014)) strongly suggest that context of the targeted polysaccharide within the intact wall influences the function of the CBMs. Thus, in tobacco cell walls, where cellulose is the major polysaccharide, a CBM targeted to cellulose enhanced mannan degradation by a mannanase, which also implies that cellulose and mannan are in very close proximity to each other in the tobacco cell wall. In contrast, in the walls of the moss, *Physcomitrella patens*, mannans are the predominant polysaccharides, and thus a mannan-directed CBM was more effective in potentiating mannanase activity in these walls. These results suggest that the reason for the combinatorial multiplicity of hydrolases and their appended CBMs in microbes is to deal with the large diversity of cell wall complexity encountered in the biosphere. A further conclusion from these studies is that the activity of hydrolases observed in vitro against isolated polysaccharides is probably a poor predictor of their activity against targets within the structurally complex cell wall.

Ability of CBMs to modulate polysaccharide esterase activity: Since mannans, like xylans, are frequently acetylated in plant cell walls, we also examined esterases that can release acetate from native mannans and xylans, *Cj*CE2B and *Cj*CE2C, both from CE2. These enzymes were fused to CBMs targeting mannan (CBM27 and CBM35), crystalline cellulose (CBM3a), and xylan (CBM2b-1-2). Fusing CBM27 to the esterases markedly enhanced their ability to release acetate from mannan-rich *Physcomitrella* (moss) walls, while fusion of CBM3a had no impact on the esterase activity against these walls. CBM3a-esterase fusions also had no enhanced activity against xylan-rich walls of tobacco or *Miscanthus* stems, suggesting that acetylated xylans in tobacco walls are not in close proximity to crystalline cellulose domains. In contrast, fusion of CBM2b-1-2 to the esterases abolished the esterase activity against tobacco stem cell walls and markedly reduced the esterase activity against *Miscanthus* walls. The most plausible reason for this outcome is that CBM2b-1-2 is selective for deacetylated domains on xylans, and hence would target the esterase enzyme to xylan regions where the target substrate is absent.

Characterization of CBM60: In addition to evaluating role of CBMs in enzyme action against complete cell walls, we have also explored the biochemical properties and crystal structure of a novel family of CBMs (CBM60) that are located in xylanases. Uniquely, the proteins display broad ligand specificity, targeting xylans, galactans, and cellulose. Some of the CBM60s display enhanced affinity for their ligands through avidity effects mediated by protein dimerization. The crystal structure of *v*CBM60 reveals that ligand recognition is conferred primarily by polar interactions between a protein-bound calcium ion and the O2 and O3 of a single sugar. The

observation that ligand is exclusively a β-linked sugar that contains equatorial hydroxyls at C2 and C3, explains the broad ligand specificity displayed by *v*CBM60. The capacity of the tandem *v*CBM60-*v*CBM60 protein to bind to ligands in intact cell walls of tobacco stem sections was assessed using immunohistochemistry. This analysis indicated that the protein bound extensively to secondary cell walls of the xylem elements and phloem fibers. Although only weak recognition of the primary cell walls was observed in cortical parenchyma, pith parenchyma and epidermal tissues, this likely reflects the thinness of the walls. The thickened vascular secondary walls contain a large amount of xylan, as well as cellulose. In primary cell walls the cellulose microfibrils are embedded in a matrix consisting mainly of pectins that include galactans, albeit at low concentrations. The binding profile of *v*CBM60-*v*CBM60 to tobacco stems is broadly similar to other xylan-specific CBMs (such as CBM2b-1–2), although CBM2b-1–2 did not display any binding to primary cell walls. The lack of cellulose recognition (CBMs that bind crystalline cellulose bind extensively to both secondary and primary cell walls likely reflects the extensive inter and intra-chain hydrogen bonds between the hydroxyl groups within the microfibrils, which are therefore unable to make polar contacts with *v*CBM60. The weak binding to primary cells also indicates that either galactan is present at low concentrations in these walls or is not accessible to *v*CBM60. Thus, although *v*CBM60 appears to display broad ligand specificity *in vitro*, in an *ex vivo* setting this module targets mainly cell walls rich in xylan, consistent with the location of family 60 CBMs predominantly in xylanases.

Overall summary

To summarize, in four structurally distinct cell walls, the appending of CBMs to enzymes can promote the activity of the respective catalytic modules in some cases, while having no or even negative impacts in other instances. To be effective, the CBM may target the substrate hydrolyzed by the catalytic module or non-substrate polysaccharides in close proximity to target substrates in the same cell wall. Thus, the effectiveness of CBM to potentiate the activity of hydrolase catalytic domains is strongly dependent on both the composition and overall structure of the plant cell walls being attacked. In those cases where CBMs have a positive effect, it is apparent that appended CBMs can provide an advantage to enzyme action when cell walls present tightly packed structures with restricted access to the polysaccharides. Even the degradation of HG, generally viewed as the most accessible polymer in primary cell walls, can be potentiated by CBMs, consistent with the occurrence of these modules in a small subsection of pectate lyases. However, a component of the pectic polymers found in intractable cell wall regions, where they are potentially attached to cellulose and/or hemicelluloses polymers, is likely to be difficult to degrade. The experiments presented here are consistent with the view that CBMs, which are targeted to nonpectic polysaccharides in these regions, can lead to enhanced degradation of these recalcitrant populations of HG polysaccharides. The use of cellulose-poor wheat endosperm cell walls as the target for arabinofuranosidase action on arabinoxylan and mannan-rich *Physcomitrella* walls as the target for mannanase action emphasize the relevance of the appropriate CBMs for the cell wall context. It therefore seems likely that in the context of intact cellulose-rich plant cell walls, the possession of a cellulose-directed CBM would confer a selective advantage for many polysaccharide-degrading enzymes, while in

cell walls enriched in other polysaccharides, other CBMs would offer greater advantage. In both cases, the advantage would occur by allowing the enzyme to remain in intimate contact with cell wall materials. In this proposed mechanism, the enzyme is bound to the cell wall through its specific CBM, whereas the catalytic module is able to access its target substrate, which must be in close association with the polysaccharide target of the CBM. Thus, the CBM greatly increases the concentration of the enzyme in the vicinity of the substrate, leading to the observed increase in polysaccharide hydrolysis.

II. Estimate of unexpended funds:

There were no unexpended funds at the end of the funding period.

III. Publications supported by DOE

Zhang X, Rogowski A, Zhao L, Hahn MG, Avci U, Knox JP, Gilbert HJ (2014) Understanding how the complex molecular architecture of mannan-degrading hydrolases contributes to plant cell wall degradation. *J.Biol.Chem.* **289:** 2002-2012.

McKee LS, Peña MJ, Rogowski A, Jackson A, Lewis RJ, York WS, Krogh KBRM, Viksø-Nielsen A, Skjøt M, Gilbert HJ, Marles-Wright J (2012) Introducing endo-xylanase activity into an exo-acting arabinofuranosidase that targets side chains. *Proc.Natl.Acad.Sci.USA* **109: 6537-6542.

Urbanowicz BR, Peña MJ, Ratnaparkhe S, Avci U, Backe J, Steet HF, Foston M, Li H, O'Neill MA, Ragauskas AJ, Darvill AG, Wyman C, Gilbert HJ, York WS (2012) 4-*O*-methylation of glucuronic acid in *Arabidopsis* glucuronoxylan is catalyzed by a domain of unknown function family 579 protein. *Proc.Natl.Acad.Sci.USA* **109:14253-14258.

Correia MAS, Mazumder K, Brás JLA, Firbank SJ, Zhu YP, Lewis RJ, York WS, Fontes CMGA, Gilbert HJ (2011) Structure and function of an arabinoxylan-specific xylanase. *J.Biol.Chem.* **286:** 22510-22520.

Bras JL, Cartmell A, Carvalho AL, Verze G, Bayer EA, Vazana Y, Correia MA, Prates JA, Ratnaparkhe S, Boraston AB, Romao MJ, Fontes CM, Gilbert HJ (2011) Structural insights into a unique cellulase fold and mechanism of cellulose hydrolysis. *Proc Natl Acad Sci USA* **108:** 5237-5242.

*Herve C, Rogowski A, Blake AW, Marcus SE, Gilbert HJ, Knox JP (2010) Carbohydrate-binding modules promote the enzymatic deconstruction of intact plant cell walls by targeting and proximity effects. *Proc Natl Acad Sci USA* **107:** 15293-15298.

Montanier C, Flint JE, Bolam DN, Xie H, Liu Z, Rogowski A, Weiner DP, Ratnaparkhe S, Nurizzo D, Roberts SM, Turkenburg JP, Davies GJ, Gilbert HJ (2010) Circular permutation provides an evolutionary link between two families of calcium-dependent carbohydrate binding modules. *J Biol Chem* **285:** 31742-31754.

*Note that no postdoctoral researcher employed on the DOE grant is an author of the Herve et al. paper. However, the PI on the DOE grant at that time provided intellectual input and assisted in writing the paper. Thus, as his time is costed on the grant, it is reasonable to claim it as an output from the DOE project.

**Several authors on these papers were funded off of this grant, so it is reasonable to claim them as outputs from this DOE project.