Conversion of Plant Cell Walls to Sugars by *Clostridium cellulovorans* Cellulosomes

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Some approaches to improve conversion of biomass to biofuel

1. **Engineer plants to produce more starch, oils or wax.**

2. **Breed plants with reduced lignin content and modified wood composition to allow milder pretreatment.**

3. **Insert lignocellulolytic genes into plants to cause self disassembly.**

4 **Isolate microorganisms with more active lignocellulolytic enzymes.**

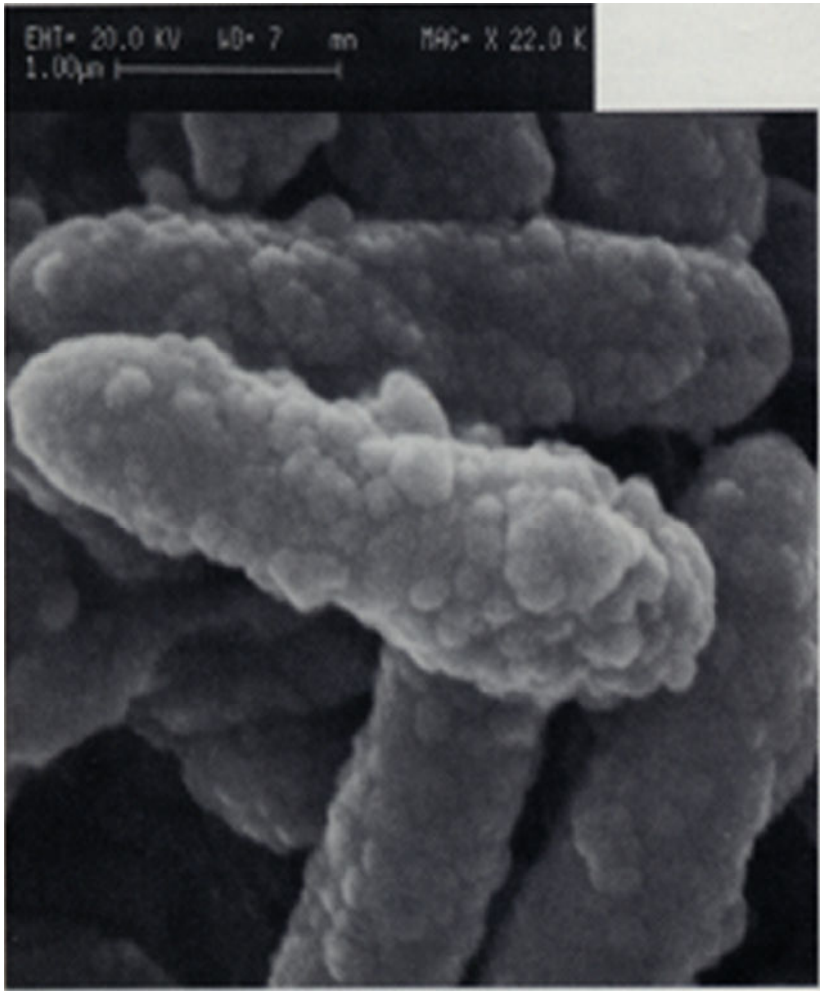
5. **Determine the biochemical properties of lignocellulolytic enzymes and their synergistic relationships.**

6. **Improve the activity of lignocellulolytic enzymes.**

Characteristics of *Clostridium cellulovorans*

Growth	obligate anaerobe
Optimum growth temperature	37 C
Optimum pH for growth	7.0
Fermentation	cellulose, xylan, pectin cellobiose, glucose, fructose, galactose, mannose, sucrose, lactose, mannose
Fermentation products	H_2, CO_2, acetate, butyrate, formate, lactate, ethanol
Mol % G+C	26 to 27

Sleat et al., 1984



***C. cellulovorans* grown on cellulose**

QuickTime?and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

***C. cellulovorans* grown on cellobiose**

From Blair and Anderson

What makes *Clostridium cellulovorans* such an efficient plant cell wall degrader? They have cellulosomes.

What is a cellulosome?

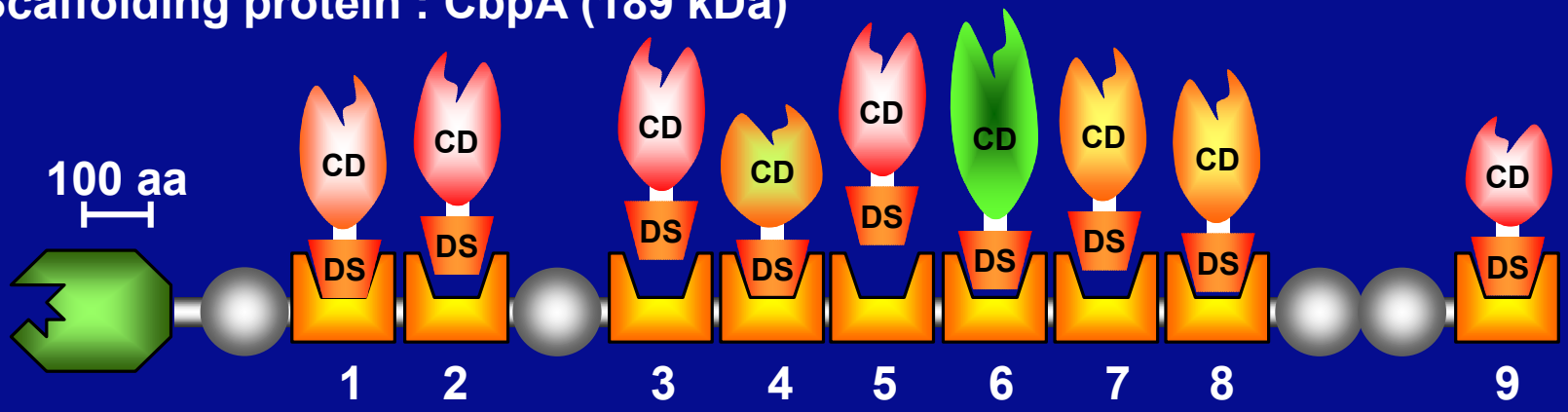
It is a large extracellular enzyme complex that is capable of degrading plant cell walls. It consists of two major components:

1) A large non-enzymatic scaffolding protein.

2) A large number of cellulases and hemicellulases

Structure of *C. cellulovorans* Cellulosome Scaffolding Protein CbpA

Scaffolding protein : CbpA (189 kDa)



Cellulose Binding Domain (CBD) : It has the role of binding CbpA to cellulose.

Surface Layer Homology Domain (SLH): It has the dual role of anchoring CbpA to the *C. cellulovorans* cell surface and of binding the cellulosome to the substrate.

Cohesin Domain (100 amino acids): Cohesin domains are highly conserved, very hydrophobic. The CbpA interacts with the cellulosomal enzyme subunits through its cohesin domains.

Cellulosomal enzyme subunit with dockerin domain (DS)

Important features of the cellulosome

Two major components

1. The scaffolding protein (CbpA)

a. Cellulose binding domain (CBD or CBM)

b. Cohesins or enzyme binding sites

2. Many cellulases and hemicellulases with dockerins.

Cohesin-dockerin interactions lead to the assembly of the cellulosome.

Three reasons why *C. cellulovorans* is a good plant cell wall degrader:

1. **CBD of the cellulosome brings many enzymes to the point of attack on the complex substrate.**
2. **Multiple cohesins and large number of cellulosomal cellulases and hemicellulases result in a large heterogeneous population of cellulosomes with a multiplicity of degradative functions.**
3. **Synergy of cellulosomal enzymes exists and interaction of the cellulosome with non-cellulosomal cellulases and hemicellulases occurs.**

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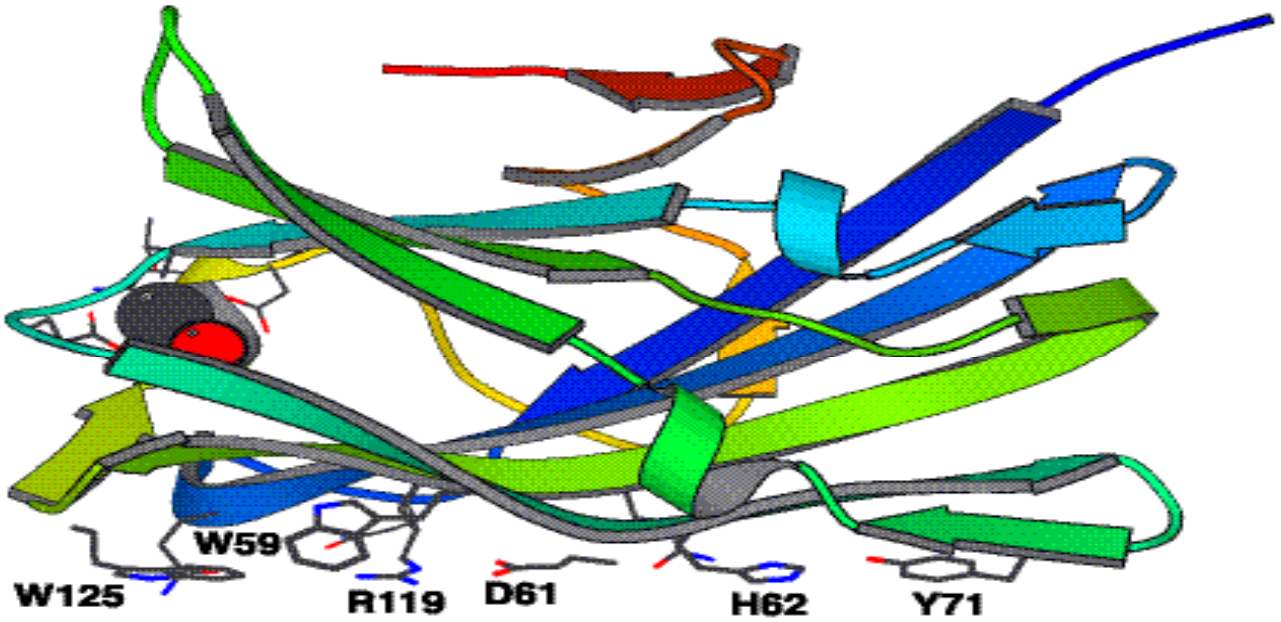
How does the CBD of CbpA bind to the substrate?

CBD was subcloned and expressed in large quantities in

***E. coli*, purified, crystallized and analyzed for its structure.**

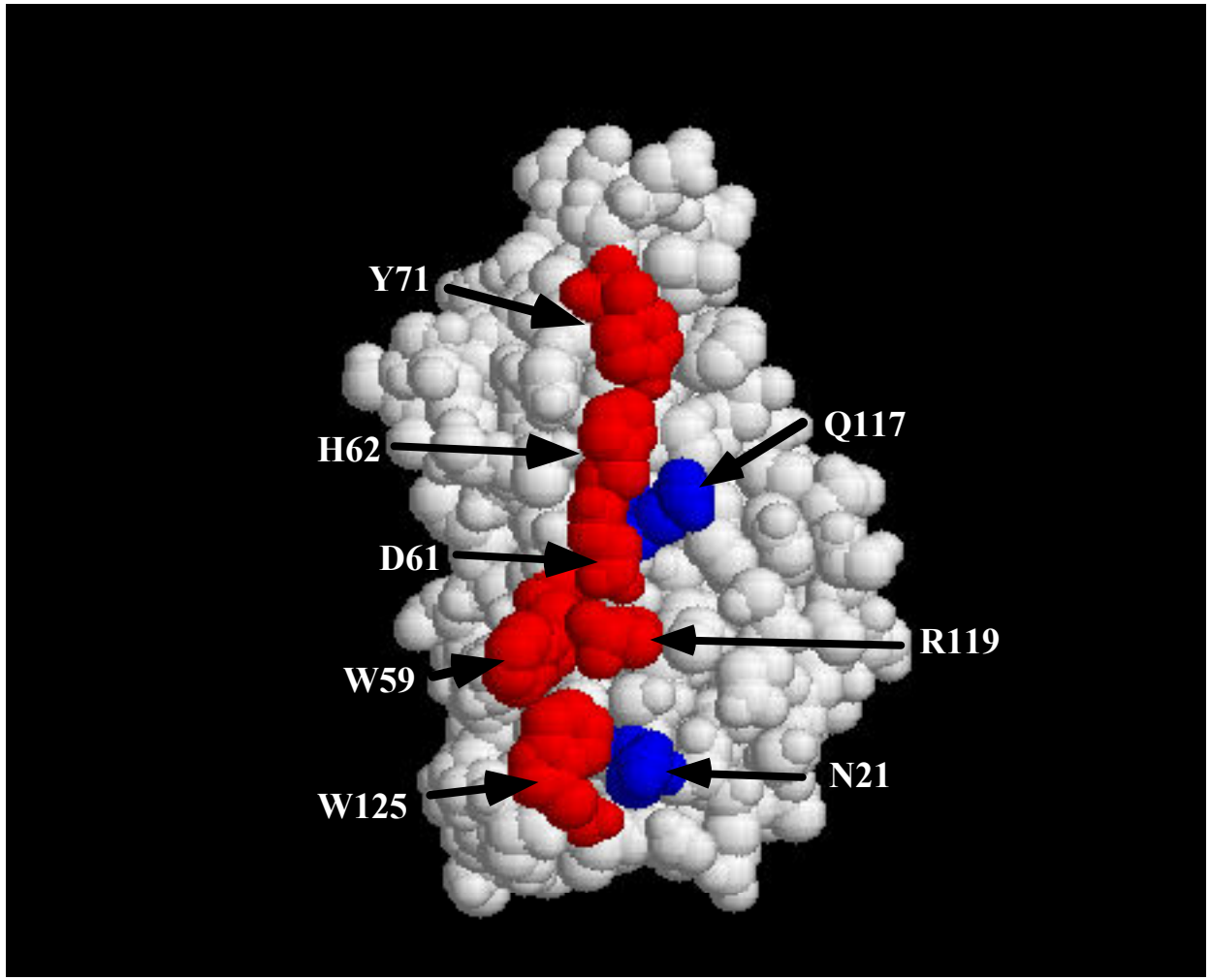
This was done in collaboration with Andy Fisher.

Ribbon structure of CBD from CbpA



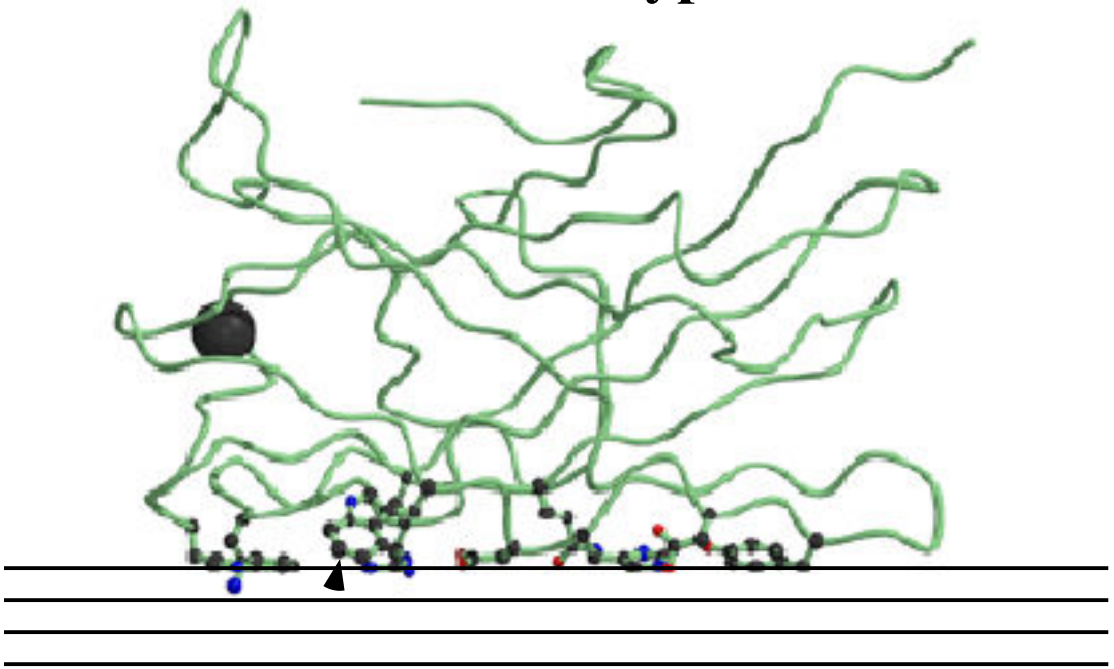
Dissociation Constants of Mutated MBP-CBDs

Protein	Dissociation Constants Kd (mM)	Position of Mutated Residue
MBP-CBD	0.4	Wild Type
MBP-CBD N21A	1.5	Anchor strip
MBP-CBD W59A	10.6	Planar strip
MBP-CBD D61A	16.2	Planar strip
MBP-CBD H62A	14.4	Planar strip
MBP-CBD Y71A	19.5	Planar strip
MBP-CBD Q117A	0.9	Anchor strip
MBP-CBD R119A	10.7	Planar strip
MBP;CBD W125A	12.8	Planar strip
MBP	107.9	
BSA	128.7	
CBD	0.6	



Positions of mutated amino acid residues on the space filling model of CBD

CBD wild type



Murashima et al. J. Bacteriol. 187:7146 (2005).

CBD binds the cellulosome to the substrate and concentrates the cellulosomal enzymes at a target site on substrate.

Although cellulosomal enzymes can be dissociated from the cellulosome, these “free” cellulosomal enzymes are incapable of attacking crystalline forms of cellulose.

On the other hand cellulosomes are capable of degrading crystalline cellulose.

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2. **Multiple cohesins and large number of cellulosomal cellulases and hemicellulases result in a large heterogeneous population of cellulosomes.**
3. **Synergy of cellulosomal enzymes and interaction of the cellulosome with non-cellulosomal cellulases and hemicellulases.**

With 9 cohesin sites on the CbpA and perhaps 50 different cellulosomal enzymes, there is the possibility of 50^9 (2×10^{15}) different types of cellulosome. This is based on the assumption that all 50 types of the enzymes are present at equal amounts and there was random and equal binding of the enzymes to the 9 cohesin sites on the CbpA.

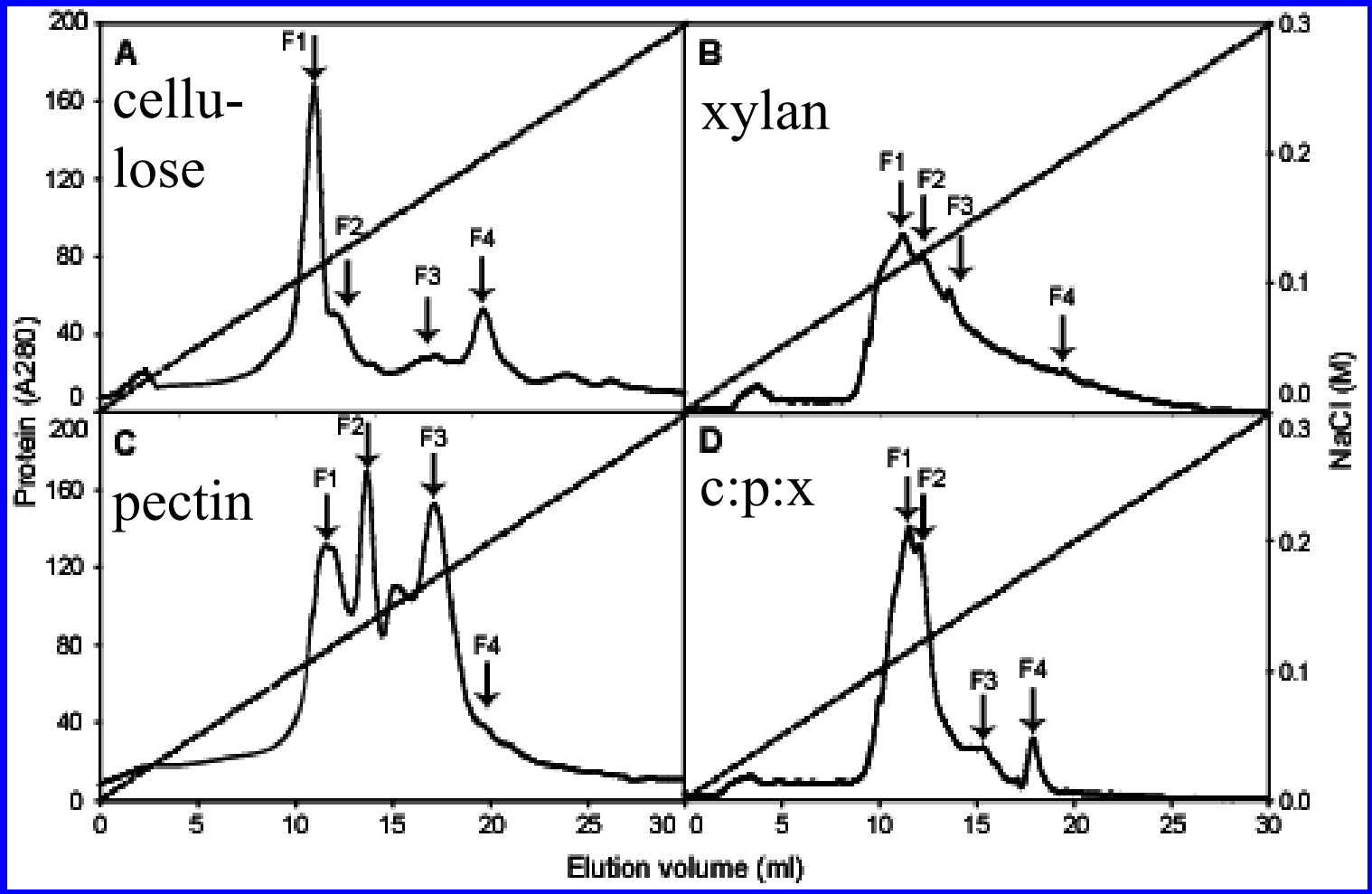
It is unlikely that all 50 enzymes are present at the same time in equal amounts and that they bind with equal efficiency to CbpA.

In fact, the examination of mRNA made during growth on different carbon substrates indicated that not all cellulase genes were expressed equally nor were all genes expressed at the same time (Han et al. J. Bacteriol. 185: 6067, 2003).

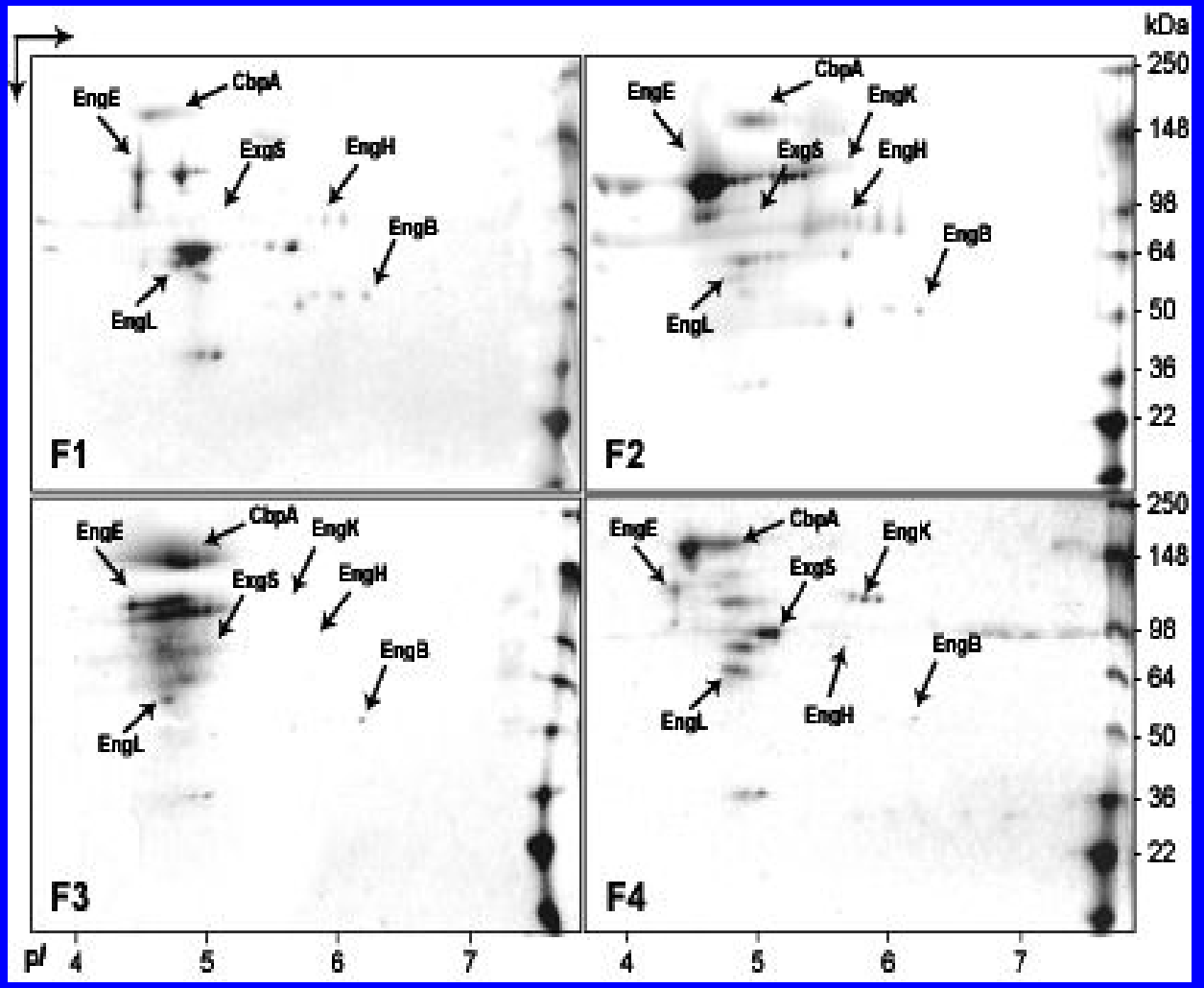
Moreover, an examination of the cellulosomal proteins from cells grown on different carbon sources showed that different proteins were expressed and that there was heterogeneity within the cellulosome population (Han et al. Microbiology 151:1491, 2005).

Pattern of cellulosomes from cells grown with different carbon sources: cellulose, xylan, and pectin

Anion exchange chromatography of purified cellulosomes



2-D Subunit Patterns of Cellulosomes from Cells Grown on Cellulose-Pectin-Xylan



The heterogeneous enzymatic composition of cellulosomes allows the bacterium to degrade plant cell walls with different structure and composition and under varying environmental conditions.

The analysis of cellulosomal enzymes that are produced with a specific natural substrate may provide a good strategy for making designer cellulosomes that can attack specific natural substrates.

For instance, an analysis of the enzymes found in cellulosomes from cells grown on corn stalk, may provide the optimum cocktail of enzymes to degrade corn stalks to sugars.

This strategy can be applied to other specific substrates such as rice straw, switch grass, sugar cane bagasse, etc. to develop designer cellulosomes.

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3. **Synergy of cellulosomal enzymes and interaction of the cellulosome with non-cellulosomal cellulases and hemicellulases.**

Cellulosomal subunits of *Clostridium cellulovorans*

Gene products	Modular structure	GH families	Number of residues	Molecular weight
CbpA	CBD - SLH1- Coh (1- 2) - SLH2 - Coh (3- 8) - SLH 3-4 - Coh9	-	1,848	189,149
HbpA	SLH-Coh	-	240	24,930
EngE	(SLH)3-GH-X-DS	GH 5	1,030	111,796
EngK	CBD IV-Ig-GH-DS	GH 9	892	97,024
EngM	CBD IV-Ig-GH-DS	GH 9	876	96,373
ExgS	GH-DS	GH 48	727	80,485
EngH	GH-CBD III-DS	GH 9	715	79,321
EngL	GH-DS	GH 9	522	57,629
EngB	GH-DS	GH 5	441	48,823
EngY	GH-DS	GH 9	738	80,241
ManA	GH-DS	GH 5	425	47,156
PelA	PL1-X-PL-DS	PL 4	914	94,458
XynA	GH-DS-NodB	GH 11	520	57,023
XynB	CBD IV-GH-DS	GH 10	607	65,976

Why is there an apparent redundancy of cellulolytic enzymes in cellulosomes?

There are many family 5 and family 9 endoglucanases in cellulosomes. Why is it necessary to have so many endoglucanases?

C. cellulovorans family 9 enzymes

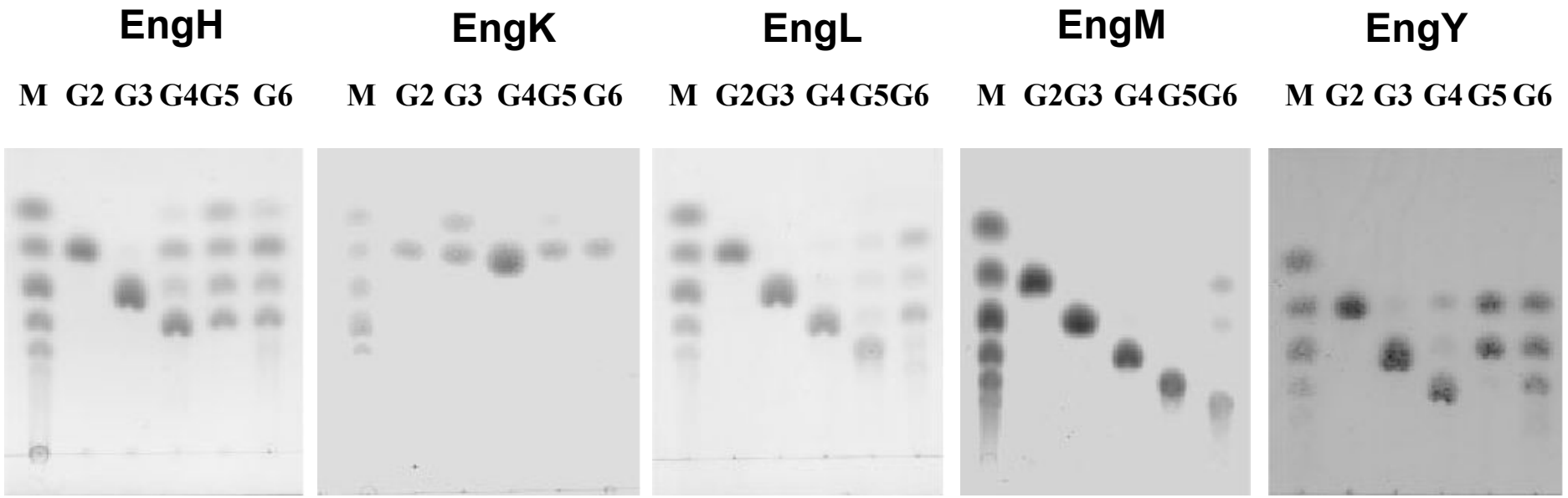
Enzyme	Structure	Identity of catalytic domains
EngK		100%
EngM		61%
EngY		29%
EngH		22%
EngL		20%

Catalytic module family 9
Ig-like module
CBM family 3
CBM family 4
CBM family 30
Dockerin module
Signal peptide

Activity of family 9 cellulosomal enzymes

Substrate	rEngH	rEngK	rEngL	rEngM	rEngY
CMC	5.875	0.021	0.632	0.096	5.423
Avicel	0.014	0.018	0.002	0.006	0.003
ASC	0.065	0.057	0.009	0.001	0.016
β-glucan	1.018	0.038	ND	NT	0.197
Lichenan	0.025	ND	ND	0.104	0.031
Laminarin	ND	ND	ND	ND	ND
Oat spelts xylan	ND	ND	ND	ND	ND
Birchwood xylan	ND	ND	ND	ND	ND

Products of family 9 enzymes of *Clostridium cellulovorans*



The substrate specificity and the products produced are different for each of the family 9 enzymes.

Arai et al., Appl. Microbiol. Biotechnol. 71:654-660 (2006).

The presence of multiple cellulases and hemicellulases with different specificities for substrates makes the cellulosome population more efficient and versatile in degrading various plant cell walls under various environmental situations.

Three reasons why *C. cellulovorans* is a good plant cell wall degrader:

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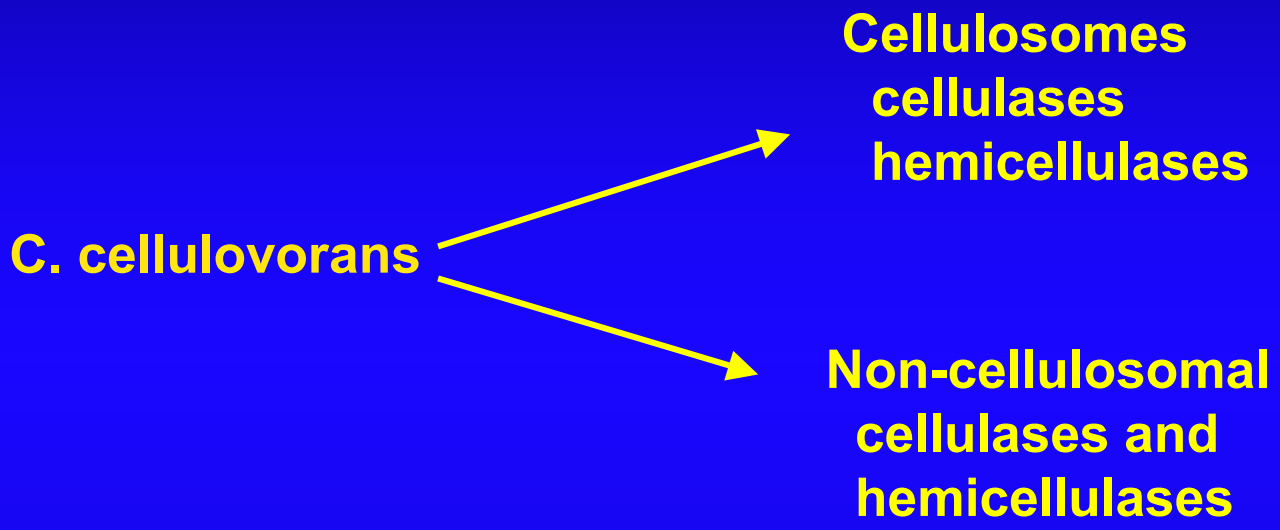
3. Synergy of cellulosomal enzymes and interaction of the cellulosome with non-cellulosomal cellulases and hemicellulases.

Synergy: When two or more enzymes are used together,

the activity is greater than the sum of their individual activities.


C. cellulovorans
Cellulosomes
cellulases
hemicellulases
Non-cellulosomal
cellulases and
hemicellulases

C. cellulovorans **produces both cellulosomes and non-cellulosomal enzymes. The non-cellulosomal fraction also contains cellulases and hemicellulases that are independent enzymes.**

Cellulosomes are efficient enzyme complexes because of synergy between the various enzymes present in the complex and also because the cellulosomes cooperate synergistically with the enzymes in the non-cellulosomal fraction.

Non-cellulosomal enzymes subunits of *Clostridium cellulovorans*

Gene products	Modular structure	Function	GH family	Number of residues	Molecular weight
EngD	GH-CBD II	Endo 1,4 β-glucanase	GH 5	515	55,976
EngF	GH-CBD XVII	Endo 1,4 β-glucanase	GH 5	557	60,130
EngO	CBD4-9-Ig-GH	Endo 1,4 β-glucanase	GH 9	724	79,474
XylA	GH-RICIN	β-Xylosidase	GH 39	549	59,958
ArfA	GH	α-Arabinofuranosidase	GH 51	492	55,731
BgaA	GH	β-Galactosidase	GH 42	659	76,464
BglA	GH	β-glucan glucohydrolase	GH 1	445	51,565

Synergy between cellulosomal cellulases

Enzymes	Activity (U/μmol)	Synergy
ExgS	0.089	--
EngE	0.054	-
EngH	0.359	-
ExgS + EngE	0.245	1.71
ExgS + EngH	0.995	2.22
EngE + EngH	0.648	1.57
ExgS + EngE + EngH	1.260	2.81

Substrate = Avicel. Enzymes are associated with mini-CbpA to form minicellulosomes. The optimum ratios of enzymes were used.

Synergy between cellulosomal cellulases and cellulosomal XynA

Enzyme	μmol/ml of Sugar Liberated	Degree of Synergy
No enzyme	0.007 ± 0.003	-
Cellulases	0.300 ± 0.003	-
XynA	0.087 ± 0.004	-
Cellulase + XynA	0.622 ± 0.014	1.61

Cellulases = 1 nmol each of EngE, EngH, & ExgS; 0.5 nmol of XynA;

Substrate = corn cell wall; Incubation at 37°C for 15 h.

Synergy of cellulosomal XynA and non-cellulosomal enzymes

	Synergy Coefficients		
Substrate	X + A	X + B	X + A + B
Arabinoxylan	1.8	0.8	1.2
Arabinogalactan	0.9	1.6	1.2
Corn Fiber Gum	2.0	1.2	2.3
Corn Stem Powder	2.7	1.2	2.9

X = cellulosomal xylanase A (XynA)

A = α-L-arabinofuranosidase

B = β-galactosidase

Synergistic effects between cellulosome and non-cellulosomal fraction during degradation of acid swollen cellulose

Enzyme fraction	Amount of released sugar(mg/ml)
No enzyme	0.0
Cellulosome fraction (CS)	506.9 ± 16.6
Non-cellulosomal fraction (nCS)	209.5 ± 17.7
CS and nCS	1.555.3 ± 15.9 (2.2) ←
CS and BglA	695.4 ± 22.5 (1.3) ←
nCS and BglA	238.5 ± 10.8
rBglA	24.1 ± 5.9

Incubation for 16 h at 50°C.

Three reasons why *C. cellulovorans* is a good plant cell wall degrader:

1. CBD binds the cellulosome to the substrate and concentrates the enzymes to a fixed point.

2. There is a heterogeneous population of cellulosomes with a multiplicity of degradative functions.

3. There is synergy of the cellulosomal and non-cellulosomal enzymes.

Current thoughts:

Non-cellulosomal lignocellulolytic enzymes initially attack plant cell walls and prepare them for subsequent degradation by cellulosomes.

Current activities:

Improving cellulase activity by DNA shuffling techniques.

Converting *Bacillus subtilis* into a cellulosome producer with *C. cellulovorans* genes.

Current Postdocs & Lab Members

Hyunju Cha
Chung-Yi Wang
Helen Chan

Former Postdocs & Visiting Scientists

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Tetsuo Hamamoto
Masahiro Takagi
Seiichi Hashida
Akihiko Ichiishi
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