Influence of the mannan binding module of β- mannanase *Cf*Man26A in the hydrolysis of mannan

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Bachelor Diploma Thesis (30hp), 2011

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Abstract

Hemicellulose is next to cellulose the most abundant polysaccharide on earth and as such an important renewable resource. Mannans are the major hemicellulose in softwodd and are found as storage polysaccharide in various plants. β-mannanases are the main mannan degrading enzymes. In this work the β-1,4-mannanase from the soil living bacteria *Cellulosomas fimi* was studied and special focus was put on the product profile.

Mannans are frequently used in the food-, paper- and textile industries and the field of possible applications is large. Deeper knowledge in the mannan degrading enzymes and the products formed from hydrolysis is required in order to enter this field and to optimize current utilization.

β-1,4-mannanase, CfMan26A, from *C. fimi* is an endo acting enzyme consisting of five domains. The first one is a glycosidehydrolase family 26 catalytic domain and the third is a family 23 carbohydrate binding domain (CBM) which binds mannans. Two variants of the enzyme were expressed, one fullength form comprising all five domains and one truncated form with only the first two domains, e.i. excluding the CBM. The hydrolysis products of mannohexaose and galactomannan (guar gum) from the two enzymes were studied with HPACD-PAD. The products from guar gum hydrolysis were also analyzed by the use of size exclusion chromatography. The results showed a striking difference in product profile for mannohexaose hydrolysis and a probable difference in the profile from guar gum hydrolysis, indicating that the CBM affected the product pattern.

The activity on galactomannan (locust bean gum and guar gum) was not significantly different between the two variants. But the results showed that both enzymes where restricted by the galactose side groups.

In addition the affinity of the fullength enzyme to soluble galactomannans was confirmed with affinity gel electrophoresis.

To conclude, this study showed that a CBM can affect the hydrolysis products of a β-1,4-mannanase without significantly affecting the rate of hydrolysis. This is of special importance when considering applications for the hydrolysis products. These findings can also be a further step in elucidating the action of the CBM.

AE	affinity electrophoresis
BSA	bovine serum albumin
CBM	carbohydrate binding module
CD	catalytic domain
*Cf*Man26A	endo-1,4-β mannanase from *Cellulosomas fimi*, fullength enzyme
*Cf*Man26-50K	truncated variant of endo-1,4-β mannanase from *Cellulosomas fimi* comprising the catalytic and Ig like domain
DNS	dinitrosalicylic acid
DP	degree of polymerization
FPLC	performance liquid chromatography
GG	guar gum
GH	glycoside hydrolase
HPAEC-PAD	high performance anion exchange chromatography with pulsed amperometric detection
HPLC	high performance liquid chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	luria broth
LBG	locust bean gum
M3	mannotriose
M6	mannohexaose
PMSF	phenylmethanesulfonyl fluoride
SEC	size exclusion chromatography
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Populärvetenskaplig sammanfattning

Växtcellväggar består av cellulosa, hemicellulosa och lignin. Hemicellulosa är, näst efter cellulosa, en av de vanligast förekommande polysackariderna på jorden. Med dagens krav på energi och resurser är uttnyttjandet av hemicellulosa av stort intresse. Till exempel blir hemicellulosa ofta en restprodukt vid upprening av cellulosa vilken hade varit önskvärd att utnyttja. För att kunna dra nytta av hemicellulosan krävs kunskap om de processer som bryter ner den och om de produkter som bildas. Nedbrytningen sker, som så mycket annat i naturen, med hjälp av enzym.

Hemicellulosa är en heteropolysackarid uppbyggd av olika sockerarter bland annat xylos, glucos, galactos och mannos. Sammansättningen varierar mellan växter och mellan växtdelar. Nedbrytningen är därför komplex och innefattar många olika enzym. En sorts enzym som bryter ner hemicellulosa är β-mannanase. De hydrolyserar bindingar på måfå mitt i mannanbaserade sockerkedjor. Många av β-mannanasen består av flera moduler varav den vanligaste icke-katalytiska är en som binder sockerkedjan, sk carbohydrate binding module, CBM. CBM ökar närheten mellan sockerkedjan och den katalytiska modulen och underlättar på så sätt nedbrytningen.

Tillämpningsområdet för β-mannanaser är stort. De används idag i stor utsträckning inom pappers- och livsmedelsindustrin och i mindre utsträckning inom textil- , olje/gas-, detergent- och läkemedelsindustrin.

I detta arbete studerades β-mannanaset, *Cf*Man26A, från den jordlevande bakterien *Cellulomonas fimi* med syftet att öka kunskapen om dess sätt att verka och dess produkter. *Cf*Man26A består av fem moduler varav en CBM som binder mannan baserade polysackarider. CBM har i vissa fall hos andra enzym visat sig påverka hastigheten av hydrolysen, men inte mycket är känt om hur de påverkar produktbilden. Fokus i arbetet lades på att se om CBM av *Cf*Man26A påverkar produktbilden. För att kunna studera detta uttrycktes två varianter av enzymet, en med CBM och en utan, i *E.coli.* En oligosackarid i form av mannohexaose samt en polysackarid i form av guar gum, hydrolyserades. De produkter som bildades vid olika långt gången hydrolys analyserades. Det visade sig att där var en skillnad i produktbilden av mannohexaose. Produktbilden från GG var mer komplex och behöver analyseras ytterligare för att kunna dra några slutsatser, men vid en första anblick såg det ut att vara en skillnad i produktbilden.

Resultatet visade att CBM inte bara kan påverka hastigheten av hydrolysen utan även vilka produkter som bildas. Detta är av särskilt intresse om man vill använda produkterna till något speciellt och kan öppna upp för nya tillämpningsområden för restprodukter från hemicellulosa nedbrytning. Noggrannare analys av produkterna skulle kunna ge en klarare bild av hur CBM verkar och hur enzymet fungerar i detalj.

1. Introduction

1.1 General introduction

Hemicellulose is next to cellulose the most abundant polysaccharide on earth and as such an important renewable resource. The increasing demand of energy, resources and sustainable processes of today gives an incitement for more extensive utilization of hemicellulose and the enzymes degrading it. Enzymes are nature's way of solving many complicated processes and are both specific and efficient. In addition they have the positive characteristic of being environmentally sustainable and are therefore of great interest to society. Enzymes degrading hemicellulose are produced foremost by prokaryotes but also by eukaryotes and archea[15, 36]. Much research is currently focusing on how hemicellulases can be used to increase the efficiency in cellulose refinery and to develop applications for the residues [8, 15]. One interesting field is the production of health promoting prebiotics from hemicellulose. Most research has been conducted on xylanases but some focus, especially in our group, has also been put on mannan degrading enzymes [23, 25]. Deeper knowledge in the action of and products produced by these enzymes can lead to better utilization of them for example in paper/pulp industry and food/feed industry where they already are frequently used [15] and for expansion into new fields of applications.

1.2.1 Hemicellulose

Wood and other kinds of biomass are made up of cellulose, hemicellulose and lignin. Hemicellulose is a heteropolysaccharide consisting of different sugar monomers; xylose, mannose, glucose, arabinose and galactose. The hemicellulose content and composition varies between different sources and also depends on cell type and age. The degree of polymerization is usually 100-200 [20].

Table 1. Hemicelluloses in hardwoods and softwoods. Data from Timell[29]

Hemicellulose	Amount (% of wood)
Hardwood	
O-acetyl-4-*O*-methylglucuronoxylan	10-35
Glucomannan	3-5
Softwood	
O-acetyl-galactoglucomannan	15-25
Arabnino-4-O-methylglucoronoxylan	10-15
Arabinogalactan*	10-20

* only in larchwood

The two largest groups of hemicellulose have a backbone of predominantly xylan or mannan linked with *O*-glycosidic linkages [23]. *O*-acetyl- galactoglucomannan is the major hemicellulose in softwood comprising up to 25% of the dry weight [16]. Mannan based hemicelluloses, mannan, galactomannan (GM) and galactoglucomannan, are also common as storage polysaccharides in seed, roots and bulbs [21].

1.2.2 Galactomannan

GM functions as a storage polysaccharide in seed endosperm in leguminous plants [10]. GM has a backbone of β- (1→4)- linked mannan chains with α- (1→6) linked galactose side groups (Figure 1). GMs are due to the galactose side groups water soluble. The degree of substitution determines the solubility and viscosity. Higher degree of substitution gives a less soluble and more viscous polysaccharide.

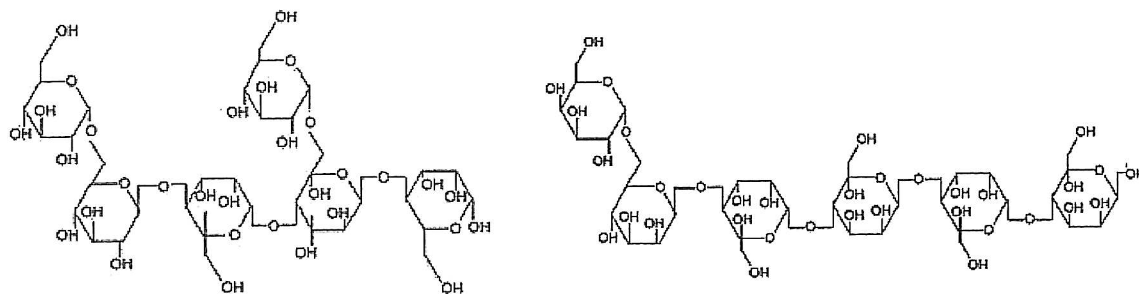

Figure 1. Schematic picture of galactomannan composed of β- (1→4)- linked mannan chains with α- (1→6) linked galactose side groups. Guar gum (left) has a mannosyl:galactosyl ratio of 2:1 and locust bean gum (right) has a ratio of 5:1 [24]. (Figure from http://sci-toys.com)

Two of the best characterized GM are locust bean gum (LBG) and guar gum, (GG). They have a mannosyl:galactosyl ratio of 5:1 and 2:1 and a degree of polymerization (DP) of 1500 and 900 respectively [24]. LBG and GG are due to their degree of substitution viscous and are therefore used as stabilizing agents in food and feed industry. They are also used in the pulp, textile and pharmaceutical industry [17]. GG is increasingly used as a health promoting additive in food as it decreases blood cholesterol and has positive effects on the colon microflora [32]. Manno-oligosaccharides have shown prebiotic effects but are not yet commercially exposed. Production of functional food from a cheap raw material such as GG or LBG would be very attractive.

1.3.1 Glycosidehydrolases and β- mannanases

Hemicellulose are complex polymers and full degradation requires the action of a number of different enzymes such as endo acting mannanases, exo acting glucosidases, mannosidases and galactosidases as well as estrases (Figure 2) [24].

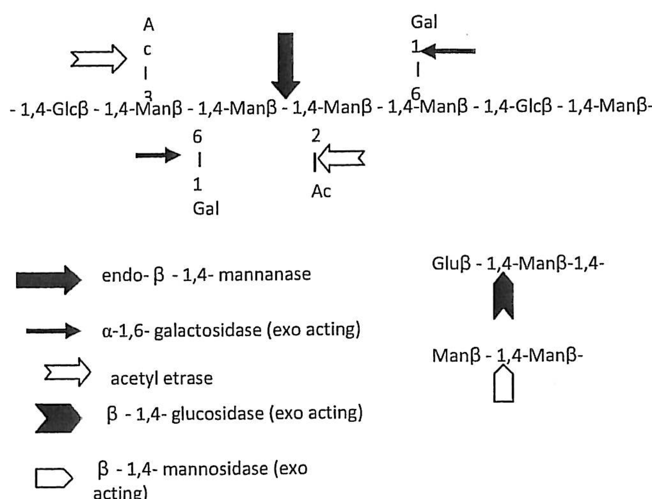

Figure 2. Schematic view of enzymes required for total degradation of *O*-acetyl- galactoglucomannan. (Figure after inspiration [11]).

All of these, except the estrases, are glycohydrolases (GH). A large number of GHs have been identified in all kinds of species. They are grouped into 122 families based on sequence similarity and further grouped into 14 clans based on 3D structures [http://www.cazy.org/Glycoside-Hydrolases.html] [10].

GHs catalyse the hydrolysis of glucosidic bonds by either an inverting mechanism, generating an inverted conformation of the anomer, or by a retaining mechanism generating a retained conformation of the same.

β- mannanases (mannan endo-1,4-β-mannosidase, EC 3.2.1.78.) [http://www.enzyme-database.org/query.php?ec=3.2.1.78] are enzymes that randomly hydrolyse the backbone of mannans, glucomannans and galactomannans. They belong to the largest clan, clan A, which all share the $(\beta/\alpha)_8$-TIMbarrel fold and are defined to GH family 5, 26, or 113. They function by a retaining mechanism and some, but not all, of the β- mannanases have been shown to have transglycosylating properties [15].

β- mannanases from eukaryotes and bacteria have been annotated to family 5 and 113 while, with the exception of a few fungal enzymes, only bacterial ones have been annotated to family 26 [12, http://www.cazy.org/Glycoside-Hydrolases.html.].

β- mannanases are mainly used for pulp bleaching in the paper industry and in the food industry to produce instant coffee and fruit juices. Other existing applications are found within the textile-, detergent- and gas industries. They are further used to increase the nutritional value in the food/feed industry [15]. During the last couple of years more focus has been put on the use of β- mannanases in production of biofuel, prebiotic oligosaccharides and novel materials [8, 32]. The list of possible areas of application of β- mannanases can be made long [31].

1.3.2 Carbohydrate binding modules

Many β- mannanases are modular enzymes. The most common non-catalytic domain are carbohydrate binding modules (CBM) which in many cases are connected to the catalytic domain (CD) with a proline rich linker sequence [7, 27, 35]. CBMs are carbohydrate binding regions found mostly in polysaccharide degrading enzymes. They were previously called cellulose binding domains (CBD) but after the discovery that some of them bound other polysaccharides apart from cellulose the name was changed to carbohydrate binding modules, CBM. CBMs are proposed to have three general roles in GHs; i) increase the enzyme concentration on the substrate, ii) target the enzyme to the proper substrate, iii) disrupt the polysaccharide structure [6]. Studies have also shown that some CBMs increase the thermo stability of the enzyme [26].

Increasing efficiency caused by CBMs is more pronounced for insoluble substrates compared to soluble ones but examples of both have been reported [4, 12, 26]

CBMs are, in analogy with GHs, grouped into families based on sequence similarity. Today 61 different families have been classified [http://www.cazy.org/Carbohydrate-Binding-Modules.html]. The CBMs are also grouped into types based on mode of binding. Type A bind insoluble and crystalline polysaccharides, Type B bind soluble polysaccharides and oligosaccharides down to DP 3, type C bind oligo and mono sugars. For all three types aromatic residues are essential for substrate binding and specificity by providing hydrophobic surfaces enabling stacking interactions with the hydrophobic face of the carbohydrate. For type B and C polar residues are also important and provide hydrogen bonding residues [6, 31].

Not very many structures of CBMs are available but structures of members of over half of the families have been solved and show that the most common fold is the β-sandwich fold [6].

As mentioned, CBMs have been identified in many polysaccharide degrading enzymes. It has also been shown possible to change specificity of the CD by adding or eliminating an adjacent CBM [4]. Unfortunately there is no obvious pattern in which CBM is attached to which CD [11].

CBMs are currently used as affinity tags for purification of other proteins. The field of possible applications is large especially within biotechnology [19, 34]. They are also promising candidates for new pharmaceuticals designed to modify cell-cell signalling which often is based on carbohydrate labelling of proteins [6].

1.4 β- mannanase CfMan26A from Cellulomonas fimi
Cellulomonas fimi is a soil living bacteria common in environments containing decaying plant material. *Cf*Man26A from *C. fimi* is an endo acting β- mannanase with a retaining mechanism and without transglycosylating property [14].

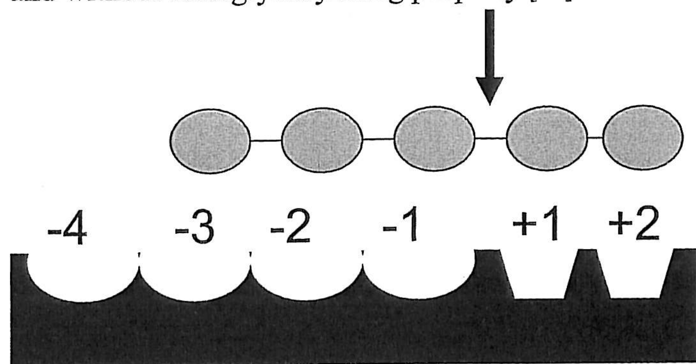

Figure 3. Schematic view of the active site of *Cf*Man26A. Six binding sites responsible for productive binding have been identified of which five are required for efficient hydrolysis [9]. Arrow indicates where cleavage occurs.

Like other endo acting enzymes the CD binds the substrate in a cleft. Six binding subsites have been identified, five of which are required for efficient hydrolysis [9] (Figure 3).

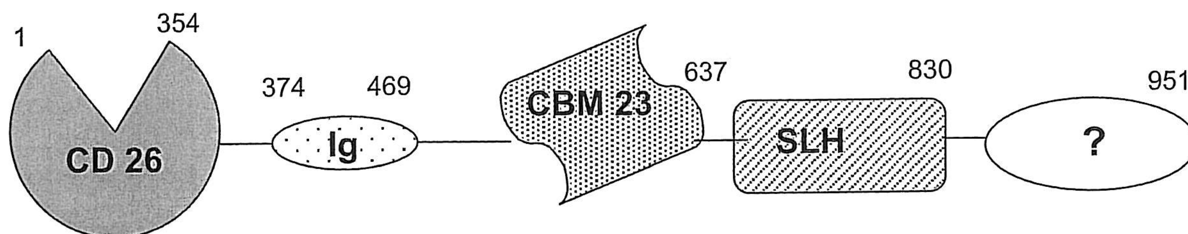

Figure 4. Schematic view of *Cf*Man26A with its five modules. Numbers indicate the positions of the amino acids in the sequence of the mature enzyme [14]. See text for further details.

*Cf*Man26A is comprised of five modules. A GH family 26 CD, an Ig like domain, a family 23 CBM a surface layer homology module (SLH) and a module of still unknown function (Figure 4). In contrast to many other modular polysaccharide degrading enzymes the domains of *Cf*Man26A are not separated by any obvious linker sequences [22].

Ig like domains are not very common in mannanases but have been reported from other GHs and is thought to stabilize the CD as the activity of CD is lost if the Ig-like domain is cleaved off [14]. The SLH is proposed to anchor the enzyme to the cell wall as this is a function of SLHs in other GHs [21].

The CBM of *Cf*Man26A belongs to family 23. This family contains mannan binding modules from bacteria and has only been found in β-mannanases. It binds soluble mannanas [22] and shares thereby properties with type B CBMs. *Cf*Man26A is so far the only known β-mannanase produced by *C. fimi* [23].

1.5 This work

It is known that CBMs can influence the hydrolysis of some substrates [12]. Most studies regarding this have been conducted on xylanases and not on mannanases. With the high interest in using enzymes in different industrial processes, and especially for the production of prebiotics, knowledge of enzyme products and actions is of value. Much research has been conducted on the truncated variant of *Cf*Man26A [10, 14] but far less on the full length variant. The aim of this project was to deepen the knowledge of *Cf*Man26A and the effect of the CBM by comparing the product profile from mannan hydrolysis by the full length variant (*Cf*Man26A) and the truncated variant (*Cf*Man26-50K). In order to do so, the full length and truncated form of the enzyme were expressed in *Escherichia coli*. Oligo substrates and the more complex GG were hydrolysed and the product profiles were compared by HPLC and size exclusion chromatography (SEC).

In addition the affinity of *Cf*Man26A to LBG and GG was studied by the use of affinity electrophoresis (AE).

2. Materials and methods

2.1 Protein expression and purification

Enzyme expression and purification was performed as described previously with minor changes [23]. The full length form of β-1,4- mannanase 26 from *Cellulomonas fimi* (*Cf*Man26A) and a truncated form consisting of the catalytic domain and the Ig like domain (*Cf*Man26-50K) were expressed as fusion proteins with a C-terminal His6-tag in *E. coli* BL21(DE3). The two strains hosted the plasmids pET27bMan26A or pET28bMan26CD, encoding the full length enzyme and the truncated form, comprising the CD and Ig-like domain, respectively. Prior to expression the pET28bMan26CD plasmid was isolated from *E. coli* XL-1 Blue and transformed into *E. coli* BL21 (DE3) by electroporation. Both plasmids were originally received from D. Stoll, University of British Columbia.

Overnight cultures of *E. coli* BL21 (DE3) were diluted 200 fold in LB media supplemented with kanamycin (50µg/ml) and grown at 37 °C, 200 rpm. Protein expression was induced by addition of IPTG to a final concentration of 0.4 mM when OD_{600} 1.3 was reached. Cultures were thereafter incubated at 15°C, 200 rpm another 24-28 h followed by harvesting by centrifugation (SA-11150/13350 rotor) at 4°C, 5000 rpm for 30 min. Pellets were frozen for storage before cell lysis and further purification. Thawed cell pellets were washed three times with QIAGEN NPI-5 buffer [5 mM imidazol, 300 mM NaCl, 50 mM sodium phosphate pH 7.9] and resuspended in the same supplemented with 0.1mM PMSF. Cells were ruptured three times using a French pressure cell. Debris and unbroken cells were removed by centrifugation 4 °C, 12000 rpm, 30 min (Beckman Ti-51-2 rotor) followed by filtration twice thru a 0.2 µm filter. Cleared lysate was run on SDS-PAGE and tested for enzyme activity (see below). Cleared lysate was loaded on a 1 ml Ni-NTA Superflow cartridge (QIAGEN) at a flow rate of 0.5 ml/min with UV-detection at 280 nm using a FPLC system (Pharmacia biotech). The column was washed with at least 10 column volumes of QIAGEN NPI-10 buffer [10 mM imidazol, 300 mM NaCl, 50 mM sodium phosphate pH 7.9] and eluted by a stepwise increase in percentage (20, 30, 50, 100%) of QIAGEN NPI-250 elution buffer [250 mM imidazol, 300 mM NaCl, 50 mM sodium phosphate pH 7.9] at a flow rate of 1 ml/min. 2.5 ml

fractions were collected and those containing protein were tested for purity on SDS-PAGE and activity using the DNS activity assay (see below). Fractions containing enzyme were pooled, concentrated and run on Ni-NTA column until sufficient purity was reached, where after the buffer was changed to 50mM citrate buffer, pH 6.0. Concentration of the samples and buffer change was conducted with the use of Amicon Ultra-15 spin columns or Amicon Stirred cell 8050, both with a 10 kDa cutoff.

2.2 Activity assay

β-mannanase activity was assayed according to the dinitrosalicylic acid- assay (DNS) described elsewhere [25]. In short enzyme was incubated with 0.5% locust bean gum, LBG, (Sigma G-0753) as substrate for 20 min at 37°C, the reaction was stopped by adding DNS and samples were boiled for 10 min. Liberated reducing sugars were thereafter detected by measuring the absorbance at 540 nm. The activity was calculated from a mannose (Megazyme) standard curve in DPX-Excell. Activity was assayed on uninduced cells, culture supernatant, lysed induced cells, flowthru and fractions collected after affinity chromatography.

2.3 Protein concentration

Protein concentration was determined with Pierce micro BCA kit according to manufactures' recommendations using the microplate procedure. Both samples and standards were run in triplicates. Absorbance at 562 nm was measured with the use of a Packard spectra count.

2.4 Protein purity

Filtered cell lysate, fractions after Ni-NTA column and pooled concentrated samples were loaded on 12% SDS-PAGE gels in Bio Rad systems with 0.75 mm spacers according to standard procedures [5] to control protein expression and protein purity. Samples were run at 200V for 50 min followed by staining with Coomassie blue over night for detection [18].

2.5 Kinetics and hydrolysis products of M6

Initial hydrolysis of mannohexaose (M6, Megazyme) was conducted as described previously [9] with 0.7-0.8 nM of *Cf*Man26A or 0.7nM *Cf*Man26-50K and varying concentrations of M6(0.5-4mM). Hydrolysis was performed at 37 °C and aliquots were withdrawn at 20, 40 and 60 min followed directly by boiling for 5 or 10 min. 2mM M6 was incubated separately for 24 and 96 h in order to study mid hydrolysis products. End products were obtained by incubating 2 mM M6 with 156 nM *Cf*Man26A or *Cf*Man26-50K. In addition hydrolysis of 4mM mannotriose (M3) was conducted in the same manner with 0.7 nM *Cf*Man26A or *Cf*Man26-50K and aliquots were withdrawn at 30, 60 and 90 min. A separate incubation with 2mM M3 for 24 hours was also conducted. Hydrolysis of 2mM M6 was repeated three times while only single hydrolysis were conducted for the other M6 concentrations. The products formed were analysed and quantified using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). A DIONEX CX 500 system (Dionex, Sunnyvale, CA) with a ED40 electrochemical detector and Carbo Pac PA-100 pre- and analytical columns was used. Elution was performed with 80 mM NaOH. Mixtures with different concentrations of manno-oligosaccharides M1-M5, 0.02-0.27 mM, (Megazyme) were run intermediate in order to produce standard curves (response versus concentration) in DPX-excell for quantification. Remaining amount of M6 was calculated by recalculating the M1-M5 formed. The specific activity was determined from the average value of three measurements at 2mM at a % M6 conversion of 4-7%. In order to determine kinetic parameters for M6 hydrolysis v0 versus [M6] was plotted in Kaleidagraph and Michaelis-Menten curves were fitted to the data

All hydrolysis experiments were conducted at 37 °C in 50mM sodium citrate buffer pH 6.0 with 0.1 mg/ml BSA (Sigma).

2.6 Activity on GG and LBG

The activity of both enzymes on LBG, or guar gum, GG (Sigma G-4129) was studied at 37°C using a DNS stopped assay method as described above. Aliquots were withdrawn at different time points 5-75 min followed by boiling 10 min. Specific activity was calculated from the values derived at 20 min.

2.7 Affinity gel electrophoresis

Binding characteristics of the two enzymes was studied by discontinuous affinity gel elctrophoresis with a Bio-Rad system with 0.75 mm spacers as described previously [23]. 1-9 µg protein was loaded in native loading buffer (2,0 g glycerol/l, 1g bromophenol blue/l, 125 mM Tris-HCl pH 8.8) on non-denaturing PAGE (7.5%) with a final concentration of 150 mM Tris-HCl pH 8.8. The separating gel was supplemented with LBG or GG to final concentrations of 0.01-0.2 w/v% of either LBG or GG. BSA and *Tr*Man5A were used as reference proteins. A native gel without polysaccharide was run simultaneously as reference. Electrophoresis was run at 150 V for 60-90 min at 4°C. Proteins were stained with coomassie blue over night for detection. The dissociation constants were determined by plotting $\frac{1}{(R-r)}$ versus $\frac{1}{c}$ and using equation 1. The intercept gives the negative reciprocal of the dissociation constant [21].

$$\frac{1}{(R-r)} = \frac{(1+K_d/c)}{(R_c-r)} \qquad \text{equation 1}$$

d = migration distance of protein (*Cf*Man26A)
D = migration distance of reference protein (BSA)
r = relative mobility (d/D) of *Cf*Man26A in the presence of mannan
R= relative mobility of *Cf*Man26A in the absence of mannan
R_c = relative mobility of protein- mannan complex in excess of mannan
K_d = dissociation constant of protein for affinity ligand.

2.8 End products from guar gum

End product hydrolysis of GG was conducted with 0.27% GG in 50 mM citiric acid buffer pH 6.0 with 0.1 mg/ml BSA and 20 nM enzyme. First a long term hydrolysis of GG was conducted in order to determine when the hydrolysis was complete. Samples were withdrawn at different time points and the liberated reducing ends were analysed by the use of DNS-assay described above. The hydrolysis was considered complete when the amount of reducing sugars of the last time points separated by 24 h did not differ more than 6 %.
The end products formed were analysed with HPAEC-PAD as described above and with size exclusion chromatography (SEC) using a FPLC-system (Pharmacia biotech) with refractory (RI) detector. In the SEC system two columns were connected in series, a Superdex 75 10/300 and a Superdex 200 10/300 (GE Healthcare). Dextran of Mw 1270, 5220, 11600, 23800 and 48600 were used for molecular mass determination. 500µl sample was loaded on the column at a flow rate of 0.5 ml/min using water as mobile phase.

3. Results

3.1 Protein expression and purification

Two variants of β-mannanase 26A from *Cellulomonas fimi* were successfully produced by heterologous expression in *Escherichia coli* BL21(DE3). One variant comprised the first 464 amino acids, corresponding to the CD and Ig-like domain, and the other comprised the entire enzyme of 951 amino acids, corresponding to five modules. Both variants were expressed as fusion proteins with a C-terminal 6His-tag in order to enable purification by affinity chromatography. The largest part of the enzyme activity was, as expected, found to be associated with the cell and not secreted into the media [23].

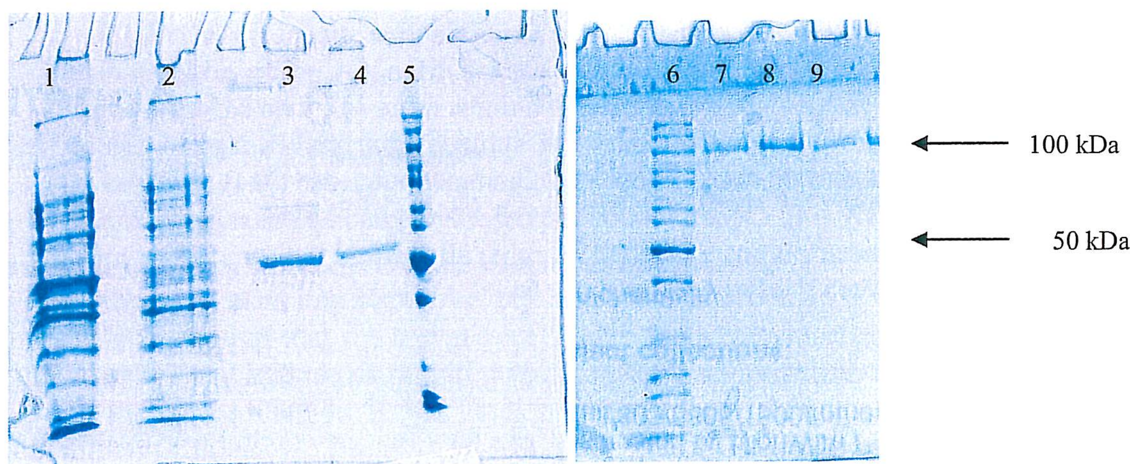

Figure 5 SDS-PAGE of sample from before and after purification with affinity chromatography of *Cf*Man26A and *Cf*26-50K. Numbered lanes 1) cell lysate from cells expressing *Cf*Man26-50K, 2) flowthru after Ni-NTA column, 3) *Cf*Man26-50K after Ni-NTA-column, 4) *Cf*Man26-50K after Ni-NTA column diluted x2, 5and 6) protein ladder fermentas prestained,7-9) *Cf*Man26A after Ni-NTA column in different dilutions.

The two enzymes were purified from cleared cell lysate on Ni- NTA column and eluted at approximately 80-100 mM imidazol. After purification two proteins appeared up as single bands on SDS-PAGE at approximately 50 and 100 kDa, (Figure 5).

3.2 Activity and kinetics

The specific activity for *Cf*Man26A and *Cf*Man 26-50K on LBG and GG was determined by a stopped DNS assay (Table 2). In this method the reducing sugars liberated by hydrolysis are detected as they react with DNS leading to an increase in absorbance at 540 nm proportionally to the concentration.

Table 2 k_{cat}/mol enzyme for both enzyme variants with LBG and GG 0.5 w/v% as substrate was determined with a stopped DNS assay[25].

	*Cf*Man26-50K	*Cf*Man26A
k_{cat}/mol LBG (s^{-1})	382 ± 65 (376*)	291± 26
k_{cat}/mol GG (s^{-1})	110 ±29 (84*)	191 ±86

*[11]

No significant difference in activity could be seen between the enzymes on the same substrate and both enzymes had lower activity on GG than on LBG.

The specific activities for *Cf*Man26-50K and *Cf*Man26A with M6 as substrate were calculated to 21.0 ± 4.2 and 29.3 ±5.3 s^{-1} respectively. K_m and k_{cat} for *Cf*Man26-50K was determined by non linear regression to 0.57 ± 0.19 mM and 28.0 s^{-1}. To be compared with 0.69±0.03 mM and 77 ±1 s^{-1} determined previously [14]. Attempts were made to fit Michaelis-Menten equations to kinetic data from hydrolysis by *Cf*Man26A as well but without success. If both the CBM and CD in *Cf*Man26A both bound M6 the reaction would no longer obey Michaelis-Menten kinetics which would explain the difficulties in fitting a Michaelis- Menten equation to the data.

3.3 M6 hydrolysis

In order to compare the action of hydrolysis between the two enzymes M6 was hydrolysed with similar enzyme load, this would give the same ratio of active site/substrate. A clear difference in profile of the initial products could be seen. *Cf*Man26A produced 1.5-6 times more M3 than M2 while *Cf*Man26-50K produced 2-6 times more M2 than M3 at 1.4- 5.1 % conversion. The product profile from *Cf*Man26-50K showed a fairly even distribution of products with a slight emphasis on mannobiose (M2) and mannotetrose (M4), this was in line with that reported previously [14]. *Cf*Man26A on the other hand had M3 as major product. Hydrolysis of different concentrations of M6 (0.5-4 mM) all showed the same difference in initial product profile between the two enzymes.

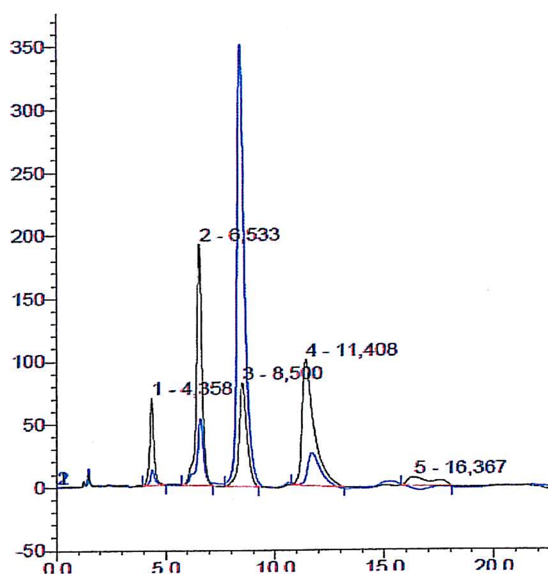

Figure 7. Early mid time products from hydrolysis of 2mM M6 incubated with 0.7 nM *Cf*Man26A (blue) or *Cf*Man26-50K (black) 24 hours corresponding to 31-37 % conversion. Chromatogram shows the analysis using HPAEC-PAD. Peaks 1-5 denote M1-M5. See text for further details.

This difference in product profile was maintained during the hydrolysis as could be seen when analysing the products from mid and end hydrolysis (Figure 7 and Table 4). The difference did however decrease as the hydrolysis prolonged and both enzymes had M1 and M2 as products after prolonged incubation with 156 nM enzyme (Figure 8).

Table 4 Product from late mid time hydrolysis corresponding to approximately 60 and 70% conversion of 2mM M6 by 0.7 nM *Cf*Man26-50K or *Cf*Man26A.

	*Cf*Man 26-50K 69.1 - 69.4 % conversion (N=3)	*Cf*Man 26A 57.2-63.6% conversion (N=3)
M1 (mM)	0.39 ± 0.09	0.6 ± 0.6
M2 (mM)	0.88 ± 0.03	0.4 ± 0.1
M3 (mM)	0.52 ± 0.02	1.3 ± 0.3
M4 (mM)	0.97 ± 0.03	0.4 ± 0.2
M5 (mM)	0.14 ± 0.02	0.1 ± 0.1

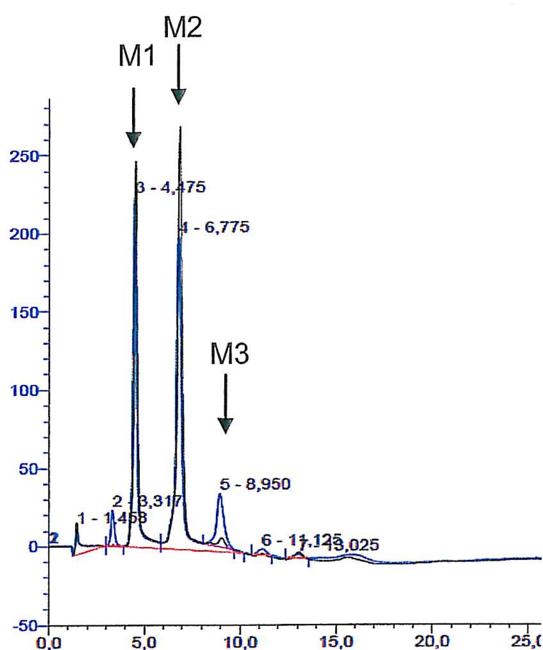

Figure 8. End products from hydrolysis of 2mM M6 incubated with 156 nM *Cf*Man26A (blue) or *Cf*Man26-50K (black) 96 hours. Chromatogram shows the analysis using HPAEC-PAD. Peaks 1-3 denote M1-M3. See text for further details.

Both enzymes showed low activity of M3 hydrolysis in accordance with previous studies [14]. It was not surprising that the end products were the same since both enzymes were able to hydrolyse M3 and hydrolysis would prolong until no more substrate could be hydrolysed or the enzyme turned inactive. It was likely to assume that *Cf*Man26-50K reached the end state earlier than *Cf*Man26A because as M4 was hydrolysed faster than M3 which were be proposed to be the major initial products of the two enzymes. The way this study was conducted did however not permit that to be settled. No significant difference in M3 hydrolysis could be detected (data not shown).

3.4 Polysaccharide hydrolysis

End products from hydrolysis of 0.3% GG with 20 nM of either enzyme, again giving the same ratio active site/substrate, were analysed with HPAEC-PAD to detect products with DP <7 (Figure 9) and with SEC to detect products with DP>1000.

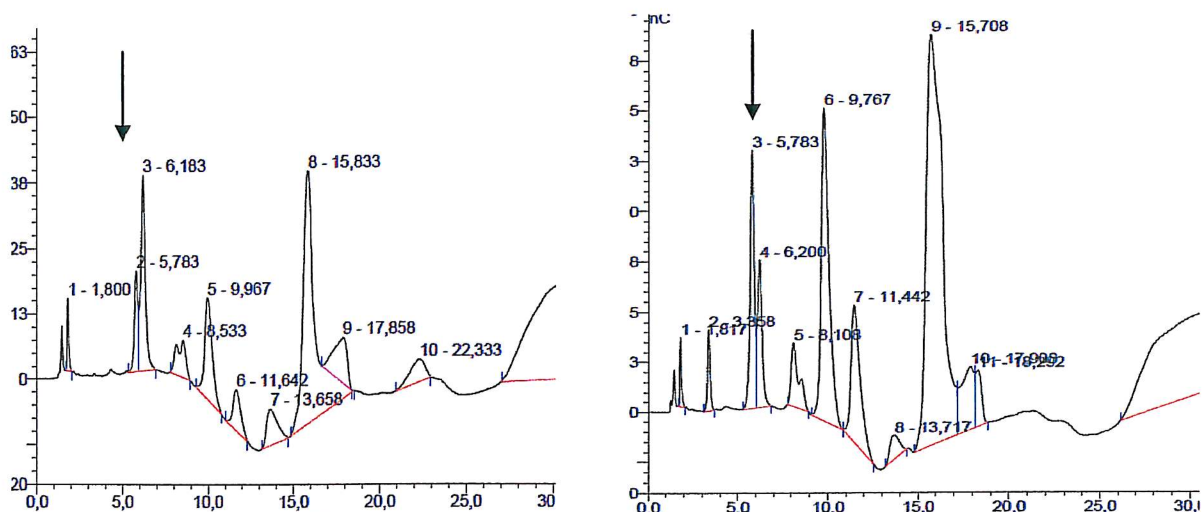

Figure 9. End products from 0.3% GG hydrolysed with 20 nM CfMan26-50K (left) and CfMan26A (right). Chromatogram shows the analysis using HPAEC-PAD. Small differences in products formed could be detected. Products indicated with arrow correspond to approximately DP 2.

Repeated analysis of the oligosaccharides produced indicated that there was a difference in product profile (Figure 9) but further analysis were required in order to draw any conclusions.

3.5 Affinity electrophoresis
AE is a method to measure the affinity of a protein to a substrate. If the substrate is incorporated in the gel a protein that binds to it will be hindered and migrate a shorter distance than if the substrate is not in the gel. The migration of a protein that does not bind to the substrate will not be affected by it in the gel. AE is a good method for detecting protein affinity to soluble polysaccharides [13, 28, 30]

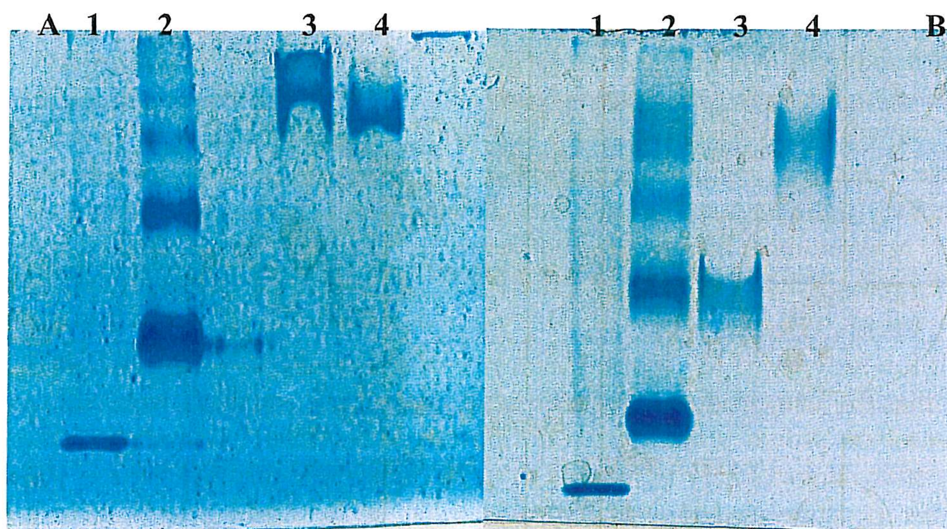

Figure 10. Affinity electrophoresis, 7.5% acrylmide, at 0.2% LBG (A) and at 0% as reference (B). Lane 1) *Cf*Man26-50K, 2) BSA, 3 *Cf*Man26A, 4) *Tr*Man5A. Gels were run at 150V, 70 min 4 °C.

Affinity of *Cf*Man26A to LBG has been studied by AE previously [2, 3] and that affinity was confirmed in this work (Figure 10). *Cf*Man26-50K lacking the CBM did not show any affinity for LBG. BSA and Man5A from *Tricoderma reesei*, both known to not bind to soluble mannans [12] where run as reference as well as a gel without LBG.

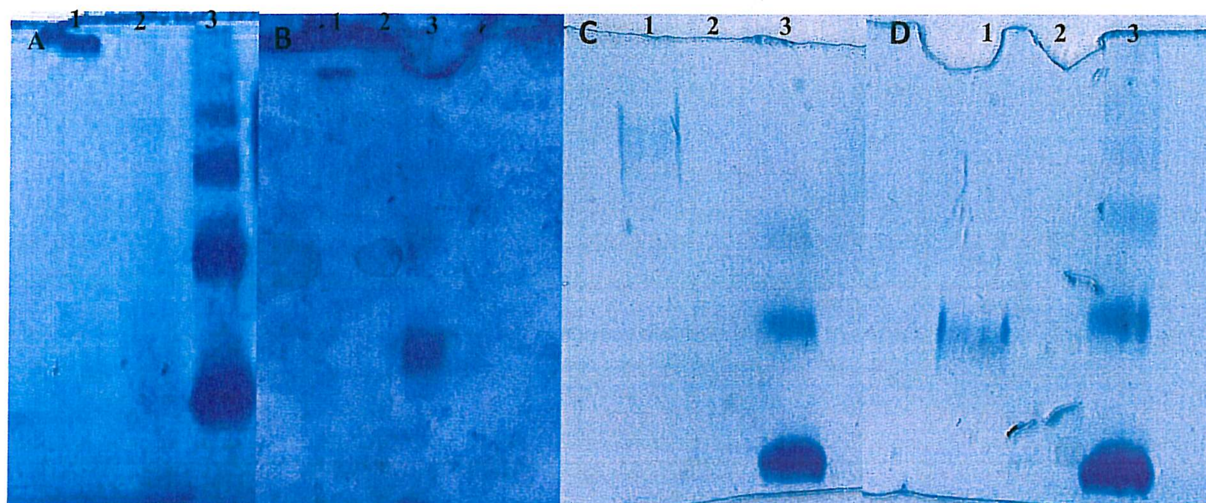

Figure 11. Affinity electrophoresis, 7.5% acrylamide, at 0.2% GG (A), 0.1% GG (B), 0.01% GG (C) and 0% GG as reference (D). Lane 1) *Cf*Man26A, 2) *Cf*Man26-50K, 3)BSA. BSA was used as reference protein. Gels were run at 150V, 70 min 4 °C. *Cf*Man26-50K ran out of the gel in almost every run.

Affinity *Cf*Man26A for GG has prior to this work been shown with other methods [3] and was here confirmed with AE. *Cf*Man26-50K lacking the CBM did not show any affinity in this case either (Figure 11). By the use of equation 1 (see experimental section) the dissociation constants for *Cf*Man26A for GG and LBG were calculated to be 6.0 * 10^{-3} and 6.5 * 10^{-3} % respectively to be compared with a K_d of 4.6 * 10^{-4} % previously determined for *Cf*Man26A to LBG [22].

4. Discussion

The initial product profile of *Cf*Man26-50K on M6 in this experiment as well as the end product profile for both enzymes was in line with that reported previously [14, 21]. M6 has been shown to bind at subsites -4 to +2 on the CD of *Cf*Man26-50K [9]. This binding gives M2 and M4 as major products. A change in binding to span subsites -3 to +3 would lead to more M3 being produced which was seen for *Cf*Man26A. If the CBM binding the substrate could cause such a shift this could be an explanation to the difference between the enzymes regarding initial and mid hydrolysis product profile presented in this work. This proposed binding did however not affect the activity of the enzymes on M6. It should be noted that M6 hydrolysis is not a simple one substrate → one product reaction but a process where the primary products are substrate in further hydrolysis reactions.

Due to problems with the HPAEC-PAD system the uncertainty in quantification was large which prohibited comparison of absolute concentration between runs but comparison of product ratios was feasible and indicated a clear difference in M2/M3 ratio between the enzymes throughout the hydrolysis.

Though the specific activity in this work was determined at 2mM, which is above K_m (0.69), the concentration of M6 may still be too low to make an accurate determination, therefore no conclusions could be draw based on the small difference seen in specific activity between the enzymes. The value of k_{cat} determined for *Cf*Man26-50K differed from that determined previously [14] which underlines the difficulty in determining kinetic parameters in this kind of hydrolysis and the uncertainty in the values for the specific activity, k_{cat} and K_m.

Comparison of the activity of *Cf*Man26A and *Cf*Man26-50K on LBG and GG showed that both enzymes were restricted by the galactose side groups as the activity for the more substituted GG was lower. This is a feature known for other GHs aswell [14]. From the results it was tempting to speculate that *Cf*Man26-50K was more active than *Cf*Man26A on LBG while the opposite would be the case for the more substituted GG. This could then be explained by the CBM increasing the proximity of substrate to active site as the CBM was not restricted by the side groups (see below). Due to the viscosity of LBG and GG the activity could not be determined with high precision and the differences were within experimental error and no conclusions could therefore be drawn. A more accurate determination of the specific activity would have to be conducted for this. It is unlikely that there is a difference as no difference has been detected in previous experiments with the two enzyme forms on 0.2w/v % LBG [21]

The results from AE confirmed previous results that the CBM binds soluble mannans [2, 22]. The K_d calculated in this work should only be considered as crude approximations as too few concentrations of polysaccharides were used. The K_d for LBG determined in this work was in order of magnitude larger than that determined previously implying that the affinity in this

work most likely was underestimated. The results indicated that the affinity for GG was greater than for LBG but as K_d for LBG varied from what had been reported before, the difference was small and few measurements were made no conclusions ought to be drawn regarding that. All gels run did however point in that direction. Despite this it was clear that the CBM was not restricted by the galactose side groups.

Differences in activity of β-mannanase with and without CBM have been studied before but not the product profile. This work points out that there can be a difference in product profile even when the activity is similar. This result is of special importance when seeking new applications for the rest products from hemicellulose degradation such as prebiotics. It would be of interest to compare the product profile of other substrates and do more exact analysis of the products. More exact determination of K_d to various polysaccharides could also be of value. The results give incitement to do similar experiments with other β-mannanases with and without CBM. Determining the affinity of CBM23 to M6 would be a natural next step in elucidating if binding of M6 to CBM that could be the cause of the noticed shift in product profile. Structure determination of *Cf*Man26A would also be of interest to shine light on if the CBM reason for the results presented in this work.

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