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ISOLATION OF CELLULASE AND HEMICELLULASE ENCODING GENES FROM *THERMOBIFIDA FUSCA*

Abstract of the thesis

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Introduction

The complexity of cell wall-degrading enzyme systems is a consequence of the complex nature of plant cell wall. Cellulose as the major component of the lignocellulose is composed of a homogeneous backbone of β-1,4-linked glucose residues. The hydrolysis of cellulose requires the activity of three types of enzymes: endoglucanases (EC 3.2.1.4) catalyze the random hydrolysis of the β-1,4-glucosidic backbone of the main cellulose chain, exoglucanases (also known as cellobiohydrolases) (EC 3.2.1.91) hydrolyse β-1,4-linked cellobiose residues from the reducing and nonreducing ends. Finally, cellobiases (also known as β-glucosidases) (EC 3.2.1.21) hydrolyse cellobiose into glucose. Hemicelluloses act as linkers between lignin and cellulose. The high percentage of hemicellulose fraction in the cell wall of higher plants makes this material the second most abundant biopolymer in nature. Besides xylan, mannan is the other major hemicellulose constituent. Galactomannan is composed of a homogeneous backbone of β-1,4-linked mannose residues. The complete conversion of galactomannan into galactose and mannose requires the activity of three types of enzymes, namely endomannanases (EC 3.2.1.78), α-galactosidases (EC 3.2.1.22), and β-mannosidases (EC 3.2.1.25). Endomannanases catalyze the random hydrolysis of the β-1,4-mannosidic backbone of the main mannan chain, α-galactosidases cleave the terminal α-1,6-linked D-galactosyl residues, and β-mannosidases hydrolyze β-1,4-linked mannose residues from the nonreducing ends of various oligosaccharides.

Thermobifida spp. are gram-positive, compost- and soil-inhabiting bacteria with broad degradative activity on plant cell wall constituents. *Thermobifida fusca*, the most extensively studied species of this genus is the model organism of thermophilic, aerobic cellulolytic bacteria. This actinomycete utilizes various plant cell wall polymers, including cellulose as the major carbon source and secretes several different cellulases: three endoglucanases (Cel9B, Cel6A, Cel5A, formerly named as E1, E2, and E5), two exoglucanases (Cel6B and Cel48A, formerly E3 and E6), an endo/exoglucanase (Cel9A, formerly E4), and an intracellular β-glucosidase, named BglC of this organism have till now been characterized in detail. While there are ample data on the cellulolytic system of *T. fusca*, the hemicellulolytic enzyme system of this species is still poorly characterized. Only two endoxylanase- and an endomannanase-encoding gene have been cloned to date, although the

hemicellulolytic enzyme system of *T. fusca* may be as complex as the well characterized cellulase system of this organism.

During a previous survey we isolated a number of thermophilic actinomycetes from the hot core of manure compost and determined their endoglucanase, cellobiohydrolase, endoxylanase, β-mannosidase, protease, amylase, as well as lignin solubilization activities. One of the strains of *T. fusca*, designated TM51 was found to show an outstanding cellulolytic-hemicellulolytic activity. Preliminary zymogram analysis of the cellulolytic enzyme system of this strain revealed a more complex endoglucanase pattern than that, described previously in *T. fusca*.

In order to find whether these additional proteins are encoded by unknown cellulase genes, or their appearance is a consequence of post-translational modifications, more detailed studies were initiated. The aim of this work was to obtain a better insight into the hemicellulose degrading enzyme system of *T. fusca*, the model organism of thermophilic, aerobic cellulose-degrading bacteria, as only two endoxylanase–and a single endomannanse–encoding gene of this system has been cloned from this species to date.

Methods

Strains and plasmids

Identification of *Thermobifida fusca* TM51, based on standard procedures (Kukolya et al., 1997) was confirmed by partial 16S rDNA sequencing and DNA-DNA hybridization; *T. fusca* ATCC 27730 served as reference strain. *Streptomyces lividans* TK24 was used to prepare an expression library from *T. fusca* TM51. DNA fragments from the promising cellulase-positive clones were subcloned in *Escherichia coli* DH5α. Plasmids pIJ699 and pBluescript KS+ (Stratagene) were used for constructing the expression library and subcloning DNA fragments in *E. coli*, respectively.

Construction of a genomic library of *T. fusca* TM51

Total DNA isolated from *T. fusca* TM51 was partially digested with *Sau*3AI. The excised ~15-kb fragments were ligated into plasmid pIJ699 according to the method of Blanco et al., (1997). *Streptomyces lividans* TK24 protoplasts were transformed with the vector, and transformants were selected on R2-agar plates by the method of Hunter (1985), using thiostrepton (100 μg ml^{-1}) as a positive selection marker. The plates were incubated for 3 days at 30°C, and transformants were

screened for β-mannosidase (on metilumbelliferil-β-mannopiranoside, MUβM) and endoglucanase activities (on agar plates containing 0.5% (w/v) CM-cellulose; endoglucanase activities were visualized with Congo red staining).

PCR identification of *T. fusca* endoglucanases in *S. lividans* transformants

Oligonucleotide primers based on cellulase sequences known from *T. fusca* were designed and used for PCR-identification of the cloned sequences

Sequencing of the new endoglucanase and β-mannosidase genes

DNA sequencing was performed by Gene Analyzer (Applied Biosystems 3100). DNA sequence analysis, comparisons and G+C content determination of the cellulases of *T. fusca* were carried out by using the GCG software. The Pfam site (http://www.sanger.ac.uk/Software/Pfam/) was used to determine the domain structure and composition of enzymes. The CAZY internet server (http://afmb.cnrs-mrs.fr/~pedro/CAZY) was used to assign the new enzymes into the appropriate glycosyl hydrolyse family (Henrissat and Bairoch, 1993).

Production and purification of recombinant enzymes

The β-mannosidase and endoglucanase positive *Streptomyces lividans* clones were grown on sucrose containing basal medium supplemented with 5 μg of thiostrepton ml^{-1} for 72 h. The β-mannosidase enzyme (ManB) was purified with fast-performance liquid chromatography (FPLC, Pharmacia) by anion-exchange chromatography on a Mono-Q column (buffer A: 50 mM Tris-HCl, pH 7.4; buffer B: buffer A plus 1 M NaCl) and by gel chromatography on an FPLC Superose-12 column (50 mM Tris-HCl, pH 7.4).

The endoglucanase enzyme (Cel5B) was purified with FPLC (Pharmacia) by cellulose affinity chromatography on microcrystalline cellulose column. Linear gradient elution was performed using NaCl (1 M NaCl, 50 mM Tris-HCl, pH 7.4) and SDS (0.1%, w/v, 50 mM Tris-HCl, pH 7.4) solutions. The activity and purity of the enzymes were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) zymography and silver staining.

Characterization of the enzymes

Substrate-specificity of the endoglucanase was assayed on low viscosity CM-cellulose, MN300 cellulose, Avicel, oat-spelt xylan, birch wood xylan, LBG-mannan, lichenin, pustulan and laminarin as polysaccharide substrates, and para-nitrofenil(*p*NP-)-cellobioside, -glucoside, -xyloside, and -mannoside as oligosaccharide substrates at 70^{o}C for 20 min. The reaction mixture contained 0.25 μg purified Cel5B enzyme and 10 mg of each polysaccharides or 20 mM of each *p*NP-glucosides, respectively in 1 ml phosphate buffer (0.1 M, pH 7.0). When polysaccharide substrates were used, enzyme activities were determined by measuring the liberated reducing sugars by the dinitrosalicylic acid assay (Miller, 1959). In activity tests on *p*NP-substrates, the reaction was terminated by adding equal volume of borate buffer (0.2 M, pH 10.0) to the reaction mixture, the liberated *p*-nitrophenol was measured at 400 nm.

The endoglucanase mode of action of Cel5B was demonstrated by viscosimetry according to Irwin et al. (1993). Endoglucanase activity of the recombinant enzyme was determined by using Remazol brilliant blue-dyed cellulose (Löewe Biochemica) substrate, according to the manufacturer's recommendations.

Substrate specificity of ManB was determined by using different *p*NP-glycosides: *p*NP-β-D-mannopyranoside (*p*NPβM), *p*NP-α-D-mannopyranoside (*p*NPαM), *p*NP-β-D-glucopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-β-D-xylopyranoside and *p*NP-α-L-arabinopyranoside according to the method described below.

β-D-mannosidase activity was assayed by using 100 μl of 0.005 M *p*NPβM in 0.1 M sodium phosphate buffer (pH 7.0) and an appropriate dilution of the enzyme solution in a 1-ml final volume of 0.1 M sodium phosphate buffer (pH 7.0) at 50°C for 10 min. The reaction was stopped by the addition of 2 ml of 0.2 M sodium borate buffer (pH 10.0), and the release of *p*NP was monitored at 400 nm. Blank solutions always contained the same components except for the enzyme.

Transferase activity was examined under different reaction conditions in a 0.5-ml final reaction volume. The enzyme was incubated in 0.1 M sodium phosphate buffer (pH 7.0) with *p*NPβM at a concentration of 2.5 mM, at room temperature in the presence of methyl-, ethyl-, propyl-, and butyl-alcohol added at 10 and 20% (vol/vol) concentrations. β-mannosidase was also incubated using 25 mM *p*NPαM as acceptor and 25 mM *p*NPβM as donor compounds in 0.1 M sodium phosphate buffer (pH 7.0), containing 0, 10, and 20% (vol/vol) dimethyl sulfoxide at room temperature. Samples taken after

1, 12, 24, and 48 h were analyzed by high-performance liquid chromatography (HPLC). The separation was performed on a Hypersil APS (aminopropylsilica) column (4.6 mm by 100 mm).
Temperature and pH optima of enzymes were determined according to the method of Kurakake and Komaki (2001).

Nucleotide sequence accession number

The GenBank accession numbers for the nucleotide sequences of *manB* and *cel5B* are AF489440 and AY298814, respectively.

Results and discussion

Altogether 2000 thiostrepton resistant *Streptomyces lividans* transformants were tested for endoglucanase and β-mannosidase activities. Screening of transformants resulted in the identification of one β-mannosidase positive (J9) and 16 endoglucanase positive clones. Among the endoglucanase positive transformants, one colony, named E61 produced a ~67 kDa endoglucanase positive band as determined in zymogram analysis. This clone yielded no PCR product with any of the *Thermobifida* endoglucanase specific primers, indicating that this transformant contained a new endoglucanase encoding gene, not known from *T. fusca* thus far.

manB, a new β-mannosidase encoding gene from *Thermobifida fusca*

The ORF of *manB* is composed of 2523 nucleotides and codes for a protein of 840 amino acids (aa), with a calculated molecular mass of 94.194 kDa and a predicted pI of 4.87. A potential ribosome-binding site, AAGAG, was located 63 bp upstream from the start codon, GTG. An AACCGGTT motif was found at position -115; the same motif was present in two copies at the promoter region of the endomannanase-encoding gene of *T. fusca* (Hilge et al., 1998).

The N-terminal part of the putative β-mannosidase protein (ManB) contained no signal peptide sequence characteristic of extracellular enzymes. Computer-assisted homology analyses using the National Library of Medicine Retrieval System (http: //www.ncbi.nlm.nih.gov) and the BLAST algorithm to scan GenBank and other databases indicated that ManB is a modular protein. It contains an N-terminal sugar binding domain (aa 79 to 157) with a jelly roll fold, an immunoglobulin-like β-

sandwich domain (aa 208 to 312), and a TIM barrel domain (aa 329 to 483). At the C terminus of the protein there is a domain of unknown function (aa 671 to 816).

ManB was found to show high similarities to β-mannosidases grouped into family 2 of the GHs (Coutinho and Henrissat, 1999). Two glutamate residues have been identified in the catalytic site of family 2 GHs; the identity of Glu 519 as a nucleophile partner was confirmed experimentally in the Man2A protein (Stoll et al., 2000). In ManB, the predicted nucleophile is Glu 530, whereas the acid base catalyst is Glu 443.

ManB can be classified as a typical soluble, cytoplasmic enzyme. The intracellular location of ManB is explained by its role in mannan decomposition, i.e. this protein acts as a terminal component of the mannan-degrading enzyme system. The β-glucosidase enzyme of *T. fusca* is also intracellularly localized, as demonstrated by Spiridonov and Wilson (2001). *Thermobifida*s and other compost-inhabiting actinomycetes produce extracellularly large variety of cellulases, xylanases, and mannanases. On the other hand, their cellobiases, β-xylosidases, and β-mannosidases, which catalyze the final steps of polysaccharide decomposition, are often intracellularly located (Stoll et al., 1999). This strategy helps to prevent the consumption of the released sugars by other compost-inhabiting microorganisms that are unable to utilize cellobiose, xylobiose, or mannobiose as they lack the appropriate enzyme and transport systems. *T. fusca* seems to have the appropriate transport system, as the *bglABC* operon, which has recently been described for this organism (Spiridonov and Wilson, 2001), encodes two types of sugar permeases, sharing substantial homology with CebG, a cellobiose-cellotriose transport protein known from *Streptomyces reticuli* (Schlosser and Schrempf, 1996). Xylobiose and mannobiose permeases are known from *Aureobasidium pullulans* (Lubomir and Peter, 1998) and a similar mechanism may support the mannobiose uptake in *Thermobifida*s as well.

The pH and temperature optima of the enzyme produced in *S. lividans* were 7.17 and 53°C, respectively. The thermal stability of ManB was relatively low, as 50% of the enzyme activity was destroyed after 10 min of incubation at 60°C and the half-life of its activity was 30 h at 40°C. The relative heat sensitivity of ManB, produced by an otherwise typical thermophilic organism can be explained by the intracellular location of the enzyme. These data on pH and temperature optima are almost identical to those of BglC, a β-glucosidase enzyme (7.0 and 50°C), another intracellular member of the cellulolytic enzyme system of *T. fusca* (Spiridonov and Wilson, 2001).

The kinetic parameters determined by using *p*NPβM as substrate were as follows: K_m=0.18 mM and V_{max}=5.96 μmol min^{-1} mg^{-1}. The inhibition constant for mannose is K_i=5.5 mM. Glucono-lacton had no effect on the enzyme activity.

Due to its theoretical and practical importance we investigated the transglycosylase activity of ManB. Transglycosylase activity was observed, when *p*NPβM and *p*NPαM were incubated with ManB, resulting in transfer of the mannosyl group from *p*NPβM to *p*NPαM. The characteristic transglycosylation activity of ManB indicates that the enzyme cleaves the β-glycoside bond via retention, a mechanism constituting the prerequisite of the transglycosylation activity. This transglycosylation activity seems to be a common feature among β-mannosidases of microbial origin belonging to family 2 of GHs. Creating β-mannosidic bonds is still one of the most difficult issues in oligosaccharide chemistry. However, recent investigations on chemoenzymatic syntheses promise an efficient approach for solving this problem. The narrow substrate specificity and the extremely low K_i value of ManB make this protein an excellent candidate for chemoenzymatic utilization. Due to these outstanding parameters, ManB could be a promising subject of site directed mutagenesis aiming at modifying its catalytic nucleophile to enhance the transmannosidase activity. According to recent reports the glycosynthase product yields have successfully been improved by this type of mutagenesis (Moracci et al., 2001). Withers (2001) produced a mutant *Agrobacterium* β-glucosidase (Abg) which converted 90% of the monosaccharides into oligosaccharides, demonstrating the efficiency of enzyme engineering in this field of research.

Tf cel5B*, a new endoglucanase encoding gene from *Thermobifida fusca

Sequence analysis of *Tf cel5B* revealed an ORF of 1851 nucleotides, encoding a putative protein of 616 aa, with a calculated molecular mass of 67.665 kDa and an estimated pI of 4.22. The ORF had a G+C content of 65.47%, not significantly different from the overall 67% G+C content determined for the whole genome of *T. fusca* (Kukolya et al., 2002). A potential ribosome binding site (AGGA) was identified 22 nucleotides upstream of the translation initiation codon. All cellulase genes from *T. fusca* contain a 14-bp inverted repeat sequence, TGGGAGCGCTCCCA in their 5' regulatory regions, which serves as the binding site for CelR, a transcriptional regulator protein (Spiridonov and Wilson, 1998) providing the coordinated regulation of these enzymes. In this new, putative endoglucanase encoding gene an imperfect copy of the CelR binding sequence, CGGGAGCGCACCCT was identified 67 nucleotides before the translational start codon.

The enzyme (Cel5B) has a signal peptide of 42 aa preceeding the catalytic domain, which is similar to other actinomycete signal sequences both in size and aa composition. The signal peptide cleavage site was predicted between aa 30 and 31 by the SignalP program (Nielsen et al., 1997). Further computer searches for protein homologies confirmed that Cel5B contains a family 5 catalytic domain located between aa 43 and 385 and a 78 aa long CBD III type cellulose binding domain, starting from aa 476. A putative linker region, separating the catalytic and the cellulose binding domains was identified between aa 385 and 476. The sequence of this linker is quite different from that of the linkers found in the other glucanases and contains multiple Pro, Ser and Thr residues, as well as an unique triplicate motif of PPTEPTE. The length of this region is 91 aa, exceeding significantly the length of the linker sequences of other cellulases known from *T. fusca*.

The GH5 family enzymes are known to exert cellulose 1,4-β-cellobiosidase, β-mannosidase, glucan 1,3-β-glucosidase, licheninase, glucan endo-1,6-β-glucosidase, mannan endo-1,4-β-mannosidase, and endo-1,4-β-xylanase activities; therefore we tested a number of different enzymes to determine the range of activity of Cel5B. This newly described enzyme was found to hydrolyze only cellulose and showed no activity on xylan, mannan or other hemicellulosic substrates.

Measuring viscosity reduction of CM-cellulose-containing solutions is a widely used method to distinguish endo- and exo-acting cellulolytic activity. By using this assay, Irwin et al. (1993) demonstrated that Cel6B, an exoglucanase of *T. fusca* caused no significant reduction of viscosity, Cel9A, the processive endoglucanase of this organism caused only slight reduction, whereas adding small amounts of Cel5A and Cel6A, the two true previously described endoglucanase enzymes of *T. fusca* to a CM-cellulose-containing solution resulted in a rapid and strong decrease of viscosity. In the present experiments, Cel5B was found to decrease viscosity as efficiently as Cel5A and Cel6A, indicating that this protein is also a typical endo-acting glucanase enzyme, the fourth one known to be produced by this thermophilic actinomycete.

The thermostable, alkalitolerant nature of this extracellular enzyme could be explained by the natural habitat of its producer organism. The composted horse manure had a pH around 8.0 and temperature of 75^{o}C, when sampled for isolating *T. fusca* TM51. The enzyme was stable up to 66^{o}C then, at higher temperatures its stability rapidly decreased. Irrespective of this relative instability, the temperature optimum of Cel5B was ~77^{o}C owing to the stabilizing effect of the CM-cellulose substrate. Similar substrate mediated stabilization events have previously been observed by Mawazda et al. (2000) in studies on endoglucanases from *Bacillus* sp. strains CH43 and HR68. This 77^{o}C

temperature optimum of Cel5B falls in the temperature optimum range of 70 – 80°C of the other endoglucanase, endoxylanase and endomannanase enzymes of *T. fusca* (Irwin et al., 1994, Hilge et al., 1998) suggesting that this newly described endoglucanase has similar perspectives of industrial utilization as the other thermostable hydrolyses of this thermophilic microorganism.

Cellulases of *T. fusca* described thus far belong to four different GH families, including GH5, GH6, GH9 and GH48. GH5, GH6 and GH9 families seem to be represented by two cellulase enzymes in this organism. Theoretically, this cellulase system, composed of pairs of enzymes that belong to the same GH family may be evolved in *T. fusca* as results of repeated gene duplications. Some previous studies provide supports to such assumptions. For example, the XYLA enzyme of *Neocallimastix patriciarum* contains two homologous, duplicated catalytic domains (Gilbert et al., 1992), the two GH2 family endoglucanases from *Bacillus* sp. strain N4 share 77% sequence identity (Fukumori et al., 1986). Furthermore, CelK and CbhA, two cellobiohydrolases from *Clostridium thermocellum* also show more than 80% homology (Kataeva et al., 1999; Zverlov et al., 1999) indicating that these pairs of sequences originate from gene duplication events. In other cases, however horizontal gene transfers were postulated to contribute to the complexity of the cellulase systems. Based on the sequence similarities found between Cel9B of *T. fusca* and CbhA of *C. thermocellum,* Zverlov et al. (1998) have already suggested that such an event might happened between these two organisms. In the present study, where the catalytic domain sequences of the seven known cellulase enzymes of *T. fusca* were compared with the same sequences of 35 cellulases of families GH5, GH6, GH9 and GH48 from 18 organisms, we clearly demonstrated, that meanwhile the enzyme pairs of the same GH family in *T. fusca* are not closely related to each other, they show in many cases significant similarities to various cellulase enzymes of taxonomically distinct organisms. The close similarity, observed between several enzymes of *T. fusca* and *C. fimi* is especially worthy of mentioning.

Sources and chronologies of horizontal gene transfers can be estimated by comparing the G+C content and the codon usage patterns of the whole genome and that of the suspectedly acquired genes (Garcia-Vallvé et al., 1999). Such comparisons have recently been used to demonstrate the horizontal transfer of an endoglucanase gene from the rumen bacterium, *Fibrobacter succinogenes* to *Orpinomyces joyonii*, a rumen inhabiting fungus by Garcia-Vallvé et al. (2000). In *T. fusca*, no significant differences were observed in the G+C contents of the seven cellulase genes and that of the whole genom, indicating that all these cellulases were acquired by this thermophilic actinomycete in the ancient times or they arrived from closely related organisms. By comparing the G+C contents at the

third codon position only *cel9A* was found to differ significantly, indicating that this component of the cellulase system of *T. fusca* arrived most lately, but still in early times. Potential sources of horizontal gene transfer are other compost inhabiting, lignocellulose degrading actinomycetes, like *Cellulomonas* or *Streptomyces* spp. which have an average G+C content of 70%, slightly higher than that of the genome of *T. fusca*. These organisms, while sharing the same ecosystem have to compete for lignocellulose, a most hardly digestible source of carbon and energies. Successful competition could only be achieved by building up highly efficient lignocellulose degrading enzyme systems. Plant debris, composed mainly by lignocellulose creates a virtually closed microcosm, which could exclusively be colonized by species, capable of utilizing this hard food. Such closed system provides, at the same time an ideal room for the transfer of genetic material among those organisms that could gain a footing there.

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