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# Isolated fungal cellulase terminal domains and a synthetic minimum analogue bind to cellulose

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The cellulose-binding properties of the highly conserved terminal region which is common to several fungal cellulases were studied. Domains were prepared by proteolytic cleavage of *Trichoderma reesei* CBH1 and the corresponding enzyme from *Sporotrichum pulverulentum*, and a peptide corresponding to residues 462–497 (the C-terminal part) of *Trichoderma* CBH1 was synthesized. The three peptides showed similar binding behavior, whereas reduced and *S*-carboxymethylated *T. reesei* fragment was inactive. This region thus appears to serve as an independent functional domain in which the C-terminal part is responsible for the binding, which in turn requires an intact three-dimensional structure.

Cellulase; Binding domain; Synthetic domain; Cellulose binding; Fluorescence; (*Trichoderma reesei, Sporotrichum pulverulentum*)

## 1. INTRODUCTION

Studies on four different cellulases from the fungus *Trichoderma reesei* [1–6] have revealed a common structural organization (fig.1), consisting of a central catalytically active 'core', a highly conserved terminal region, 'A', stabilized by internal disulfide bridges, and an interconnecting, highly glycosylated region, 'B', rich in serine, threonine and proline. Two of the proteins have their A-region at the N-terminus, whereas two have it at the C-terminal end. The absolute polarity of the A-region sequence is always the same. Physicochemical studies on enzymes with and without a B-A-region [7,8] show that it constitutes a protruding part of the intact enzyme and can be regarded as a separate domain. The role of the whole B-A-region is to enhance the activity towards the solid substrate [9–11]. The importance of this structural organization is demonstrated by the fact that it also occurs in two cellulases from the unrelated bacterium *Cellulomonas fimi* [12]. Data obtained on glycoamylases [13] suggest that this organization may be general for enzymes that degrade insoluble polysaccharides.

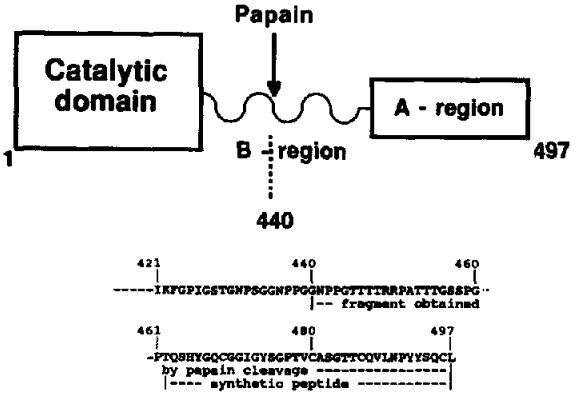


Fig.1. Structural organization of Tr CBH-1.

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*Abbreviations:* Tr, *Trichoderma reesei*; Sp, *Sporotrichum pulverulentum*; Boc, *t*-butyloxycarbonyl; Bzl, benzyl; Br-Z, 2-bromobenzyloxycarbonyl; Me-Bzl, 4-methylbenzyl; Dnp, 2,4-dinitrophenyl; DMF, dimethyl formamide; DCM, dichloromethane; TFA, trifluoroacetic acid

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Here, we present data on the functional (cellulose-binding) properties of (i) the B-A-region from the main cellulase CBH1 from *T. reesei*, (ii) the B-A-region from the corresponding and strongly homologous [14,15] enzyme from *Sporotrichum pulverulentum* and (iii) a synthetic 'minimum' A-region of Tr CBH1.

## 2. MATERIALS AND METHODS

Cellulose powder (Avicel PH-101, Fluka) was washed with water to decrease background fluorescence. Carboxypeptidases A, Y and papain were from Sigma. Protected amino acids were from Peninsula except Boc-His(Dnp) which was a Bachem product. TFA, DCM and DMF were from Rathburn. Other chemicals for peptide synthesis, including the starting resin, were from Applied Biosystems. Chelating Sepharose® was a gift from Pharmacia. Other chemicals were of analytical grade. Fluorescence spectra were recorded with an Aminco SPF-500 spectrophotofluorimeter.

Amino acid compositions were determined using an LKB Alpha Plus analyzer after hydrolysis for 24 h at 110°C in 6 M HCl containing 2 mg/ml phenol.

Automated Edman degradation [16] was carried out in an Applied Biosystems 470A gas-liquid phase sequencer equipped with an Applied Biosystems 120A PTH analyzer.

C-terminal analysis was performed on 0.1 $\mu$M reduced and carboxymethylated [17] protein in 0.1 M pyridine acetate buffer, pH 5.6, containing 1% SDS at 22°C using both carboxypeptidases A and Y (2 nM each). Released amino acids were analysed on a Biotronik LC5001 amino acid analyzer.

Total sugar content was estimated using the anthrone/sulfuric acid method with mannose as standard [18].

Plasma desorption mass spectrometry was carried out in a Bioion 20 instrument (Bioion Nordic, Uppssala) [19].

### 2.1. *Preparation of natural peptides*

The B-A-region of Tr CBH 1 and that of Sp Exo were prepared according to [11] and purified by gel filtration on Biogel P-150, respectively.

### 2.2. *Peptide synthesis*

A peptide corresponding to region 462–497 of Tr CBH 1 was assembled by the solid-phase procedure [20] using an Applied Biosystems 430 A synthesizer. The amount of the starting Boc-Leu-$OCH_2$-PAM-resin (0.77 mmol/g) was 0.5 mmol. $\alpha$-Boc protected amino acids were coupled as symmetrical anhydrides in 2-fold molar excess, except for Asn and Gln, which were used as 1-hydroxybenzotriazole esters (4 equiv.). Double couplings followed by capping with acetic anhydride were used throughout the synthesis. Side-chain-protecting groups were: Bzl for Ser and Thr, Br-Z for Tyr, Me-Bzl for Cys and Dnp for His. A part of the peptide resin (0.5 g) was treated overnight with 0.5 M thiophenol/DMF in order to remove most of the Dnp groups and, after washing with DMF and DCM, with 50% TFA/DCM for 30 min to deprotect the $\alpha$-amino group. The peptide was liberated from the resin by reaction with HF (10 ml) in the presence of *p*-cresol (0.5 ml), *p*-thiocresol (0.5 ml) and dimethyl sulfide (0.2 ml) for 1 h at 0°C. The resin obtained after removal of HF in vacuo was washed with EtOAc, $CHCl_3$ and ether containing 0.5% $\beta$-mercaptoethanol. The peptide was then extracted into 1% $\beta$-mercaptoethanol/TFA (3 × 3 ml), and the resulting solution was concentrated in a stream of dry nitrogen to a volume of about 2 ml. Addition of dry ether precipitated the product which was collected by centrifugation, washed with ether and dried in vacuo. A part of the crude product (10 mg) was dissolved in 1.2 ml of 0.5 M Tris-HCl, pH 8.0, containing 6 M guanidine-HCl, and nitrogen was bubbled through the solution for 5 min. $\beta$-Mercaptoethanol (0.2 ml) was added, and the solution was kept for 24 h at 37°C to accomplish reduction and removal of remaining Dnp groups, if present. The reduced material was desalted on Sephadex G-25 (PD-10 column) using 0.1 M acetic acid as

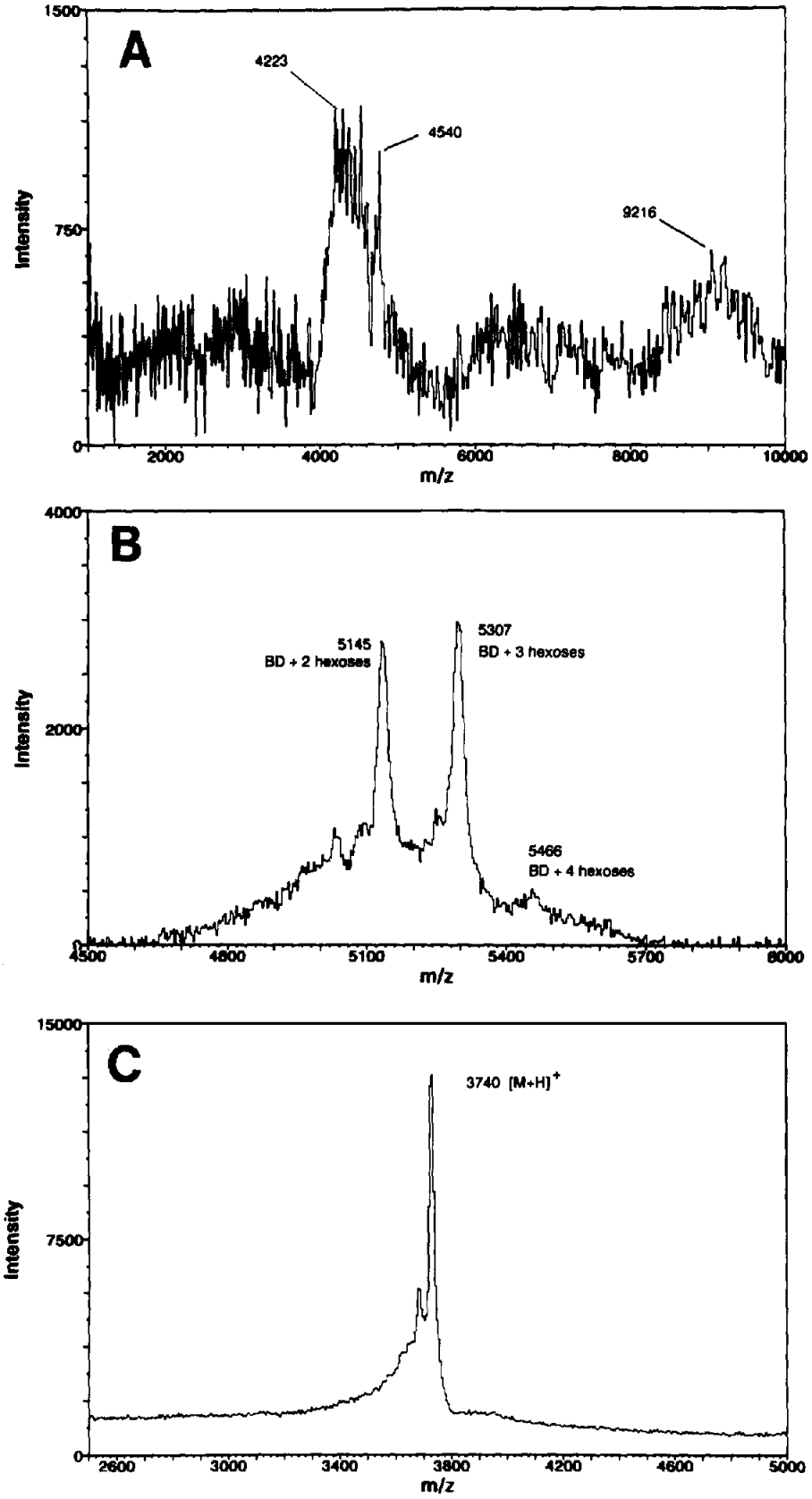
Table 1

Amino acid composition of the isolated B-A peptides from *Trichoderma* CBH 1 and *Sporotrichum* EXO and the compositions of the corresponding regions based on the known sequences [1,2,15]

| Amino acid | *Trichoderma* (440–497) | | *Sporotrichum* (471–516) | |
|---|---|---|---|---|
| | Found[a] | Expected | Found[b] | Expected |
| Cys | –[d] | 4 | 3.5 | 4 |
| Asx | 2.1 | 2 | 1.1 | 1 |
| Thr | 10.5 | 11 | 6.7 | 8 |
| Ser | 6.1 | 6 | 5.1 | 4 |
| Glx | 4.1 | 4 | 3.2 | 4 |
| Pro | 7.2 | 7 | 8.2 | 6 |
| Gly | 9.8 | 10 | 6.1 | 6 |
| Ala | 2.1 | 2 | 1.1 | 1 |
| Met | 0 | 0 | 0 | 0 |
| Val | 1.9 | 2 | 2.0 | 3 |
| Ile | 0.9 | 1 | 1.0 | 1 |
| Leu | 2.0 | 2 | 1.1 | 1 |
| Tyr | 3.8 | 4 | 4.7 | 5 |
| Phe | 0 | 0 | 0 | 0 |
| His | 1.0 | 1 | 1.0 | 1 |
| Lys | 0 | 0 | 0 | 0 |
| Arg | 2.3 | 2 | 0 | 0 |
| Trp | 0[c] | 0 | 1.2[c] | 1 |
| Total | 54.0 | 58 | 46.0 | 46 |

[a] Normalized to 54 residues
[b] Normalized to 46 residues
[c] Tryptophan estimated spectrophotometrically
[d] Not determined

Fig.2. $^{252}$Cf-PDMS spectra of the studied peptides. (A) B-A domain from Tr CBH-1. (B) B-A domain from Sp 'Exo'. (C) Synthetic peptide corresponding to Tr CBH-1 462–497.


A
4223
4540
9216
Intensity
1500
750
0
2000
4000
6000
8000
10000
m/z
B
5145
BD + 2 hexoses
5307
BD + 3 hexoses
5466
BD + 4 hexoses
Intensity
4000
2000
4500
4800
5100
5400
5700
6000
m/z
C
3740 [M+H]+
Intensity
15000
7500
0
2600
3000
3400
3800
4200
4600
5000
m/z

eluent. The peptide-containing fractions (3 ml) were added to a solution consisting of 40 mg reduced and 10 mg oxidized glutathione in 50 ml of 0.2 M Tris-acetate, pH 8.2. Oxidation of the peptide was allowed to proceed overnight at room temperature [21]. The peptide was recovered by adsorption onto a small column of BondElut $C_{18}$ (200 mg) which was further washed with 0.1% TFA and $H_2O$. The product was eluted with 3 × 0.5 ml of a 1:1 mixture of acetonitrile and 0.1 M sodium phosphate, pH 7.0, then applied onto a column (1 × 2 cm) of chelating Superose® ($Cu^{2+}$ form) [22]. Elution was performed with 20–100 mM sodium phosphate containing 0.1 M NaCl using a pH gradient (pH 7.0–3.8). The fractions corresponding to the main peak were further purified by RP-HPLC. Mass analysis of the purified material showed the expected molecular mass (3741 + 3 Da) (fig.2C).

### 2.3. *Binding to cellulose*

Peptide solutions (5 μM) were incubated in 2 ml sodium acetate, pH 5, for 2 h at 40°C with various concentrations of cellulose powder under continuous agitation. The cellulose powder was pelleted by centrifugation and the free peptide concentration was determined by fluorescence measurement.

Fluorescence emission spectra were recorded for pure peptides and for incubation supernatants using 239 nm as excitation wavelength for the Tr peptide and its synthetic counterpart and 280 nm for the Sp peptide. The peptide concentration in the supernatants was calculated by comparing the emission intensity after background corrections to that of cellulose-free 5 μM solutions of each peptide.

## 3. RESULTS

### 3.1. *Characterization*

Amino acid compositions of the isolated B-A peptides from Tr CBH1 and Sp EXO are presented in table 1 together with compositions corresponding to the known sequences of Tr CBH1 [1,2] EXO [14]. Edman degradation of the Tr peptide gave the result Gly-Asn-Pro-Pro-Gly-X-X-X...., which correlates well with the sequence Gly - Asn - Pro - Pro - Gly - Thr(CHO) - Thr(CHO) - Thr(CHO) found in the region 440– of the CBH1 (glycosylated Ser and Thr usually give no signal in Edman degradation). The B-A peptide thus corresponds to residues 440–497 of CBH-1 with a net formula mass of 5800 Da. A carbohydrate content of 30–40% [10] increases the total molecular mass to around 9000 Da. Mass analysis (fig.2A) shows a small peak at about 9000 Da/e and a larger (double ionization) peak at about 4500 Da/e. The peaks are broad, probably due to heterogeneous glycosylation. Carboxypeptidase digestion of the Sp B-A fragment indicated a sequence corresponding to the C-terminal end of the protein [14]. A combination of amino acid analysis, carbohydrate and mass analysis (fig.2B) revealed that the fragment corresponds to the last 46 (471–516) residues of the protein. The amino acid composition found (table 1) is in good agreement with this localization of the B-A peptide.

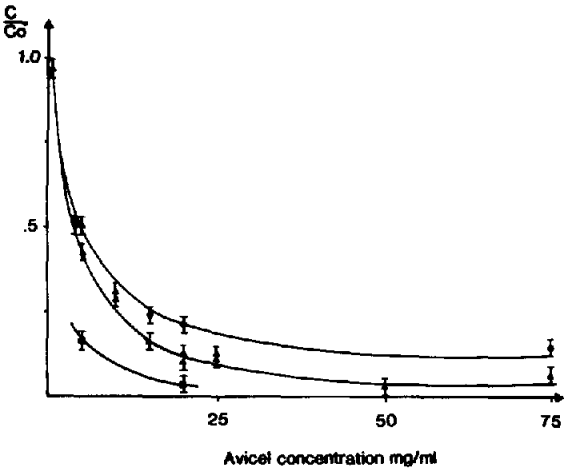



Fig.3. Adsorption of Tr B-A domain (▲—▲), Sp B-A domain (■—■) and synthetic Tr A domain (●—●) as a function of the cellulose powder concentration.

### 3.2. *Binding measurements*

In fig.3 the Avicel-binding properties of the Tr B-A domain, synthetic 'Tr A' and Sp B-A domain are compared. The patterns for the synthetic peptide and proteolytic Tr fragment are virtually identical, with the exception of a somewhat higher concentration of the synthetic peptide at high Avicel concentration, probably due to a low content (<5%) of inactive components. The Sp fragment has a similar behavior, although with somewhat stronger binding. Reduction and *S*-carboxymethylation of the Tr B-A region eliminate the binding ability (not shown).

## 4. DISCUSSION

Tr B-A peptide, Sp B-A and synthetic Tr A all bind to Avicel, whereas reduced and carboxymethylated Tr B-A peptide has lost this ability. The A-region peptide thus appears to be responsible for the binding, which also requires the native, disulfide-bonded conformation. This rules out the previously discussed possibility that the carbohydrate moiety might be important for the

binding. It is also evident that the correct disulfides were spontaneously formed in the synthesis procedure, which in turn demonstrates that the B-A-region has the properties of a functional domain, wherein the A part directly interacts with the cellulose and the B-region is a flexible arm protected from proteolytic attack by glycosylation and/or a high proline content.

The role of an extra binding region, flexibly connected to the catalytic enzyme 'core' is not clear, but comparison between *T. reesei* EIII with and without the A-B-region shows that the difference in catalytic efficiency toward Avicel parallels the difference in binding to the substrate [9]. A similar enhancement of the binding efficiency involving only the catalytic site would have a negative effect on the turnover number of the hydrolysis. It is also possible that the binding of the A-domain effects some physical disruption of the regular cellulose structure, thereby facilitating the hydrolytic process.

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