Role of the Interdomain Linker Peptide of *Trichoderma reesei* Cellobiohydrolase I in Its Interaction with Crystalline Cellulose*

(Received for publication, March 8, 1993, and in revised form, June 17, 1993)

Malee Srisodsuk, Tapani Reinikainen, Merja Penttilä, and Tuula T. Teeri

From the VTT, Biotechnical Laboratory, SF-02151 Espoo, Finland

Cellobiohydrolase I (CBH I), the major component of *Trichoderma reesei* cellulolytic system, is comprised of a catalytic core domain joined to a cellulose binding-domain (CBD) by an extended O-glycosylated interdomain linker peptide. Two internal deletions were introduced to the linker in order to investigate its function particularly in the hydrolysis of crystalline cellulose. Deletion of the first one-third of the linker, including a putative hinge region, reduces the binding capacity of CBH I in high enzyme coverage but does not affect its enzymatic activity on crystalline cellulose. The longer deletion removing practically all of the linker dramatically reduces the rate of crystalline cellulose degradation even though the enzyme still binds to the substrate. We conclude that sufficient spatial separation of the two domains is required for efficient function of CBH I. It is evident that the presence of a functional CBD is increasingly important for CBH I toward higher enzyme to cellulose ratios. Our data suggest that the putative hinge removed by the first deletion facilitates CBD-driven binding and dense packing of the wild type enzyme on the cellulose surface.

Comparison of known β-1,4-glycanases shows that most have a multidomain structure (1–5). Cellobiolytic and many xylanolytic enzymes characteristically consist of a relatively large catalytic domain with a defined specificity toward small soluble carbohydrates, and a significantly smaller substrate binding linker peptide. relatively long, glycosylated linker peptides of 6–59 amino acids. The linker sequences from different enzymes rarely share any apparent sequence homology but their amino acid composition is typically rich in proline and hydroxyamino acids. The linker sequences from different enzymes rarely share any apparent sequence homology but their amino acid composition is typically rich in proline and hydroxyamino acids (5, 7). The proline and hydroxyamino acid content is different among the linkers.

The widespread occurrence of these linker sequences in carbohydrate-degrading enzymes emphasizes their importance on the enzyme function. Based on their similarity to the proline-rich linkers from other proteins, such as immunoglobulin A1 and ribosomal protein L12, it has been suggested that cellulase linkers represent extended, flexible hinges between the two domains facilitating their independent function (9, 10). It has also been observed that some cellulase linkers are susceptible to proteolytic attack in certain culture conditions (11, 12).

The two domain structure of four different *Trichoderma reesei* cellobiohydrolases has been clearly demonstrated (2, 6). Detailed crystal structures of the cellulolytic core domains of the two cellobiohydrolases (CBH I and CBH II) (13) and an NMR structure of the cellulose-binding domain of CBH I (14) have already been solved. However, so far it has not been possible to obtain structures of intact cellobiohydrolases and therefore only approximate structural information is available on the linker peptides. The linkers identified in *T. reesei* cellobiohydrolases are approximately 30–44 amino acids in length, rich in prolines, glycines, serines, and threonines, and highly O-glycosylated (6, 15, 16). Small angle x-ray studies of the two cellobiohydrolases suggest an extended structure with possible flexibility within the linker peptide (17–20).

The purpose of the present study was to investigate the importance of the length of the interdomain linker peptide of *T. reesei* cellobiohydrolase I for its binding and activity on crystalline cellulose. Two internal deletions were introduced to the linker, one removing a putative flexible hinge region but preserving most of the O-glycosylation sites and the other removing practically all of the linker peptide binding the two domains very closely to each other. Implications of these deletions on the interactions of CBH I with crystalline cellulose will be discussed.

**EXPERIMENTAL PROCEDURES**

Strains and Vectors—*Escherichia coli* strain DH5α (F−, endA1, hsdR17(RK−,MK+), supE44,thi-1, h-), recA1, gyrA96, relA1, Δ(argF lacZ)M15) was used as the bacterial cloning host. The *T. reesei* strain VTT-D-93201, used as the expression host, is a derivative of the Zkansformants-1transformation vector pEM-F5, a derivative of pUC18 containing cbh1 cDNA linked to the cbh1 promoter and terminator sequences. A phleomycin selection plasmid pAN8-1 was used in selection of the *Trichoderma* transformants.

Preparation of Bacterial Microcrystalline Cellulose—Acetobacter xylinum was grown on peptone/yeast extract/glucose medium (23), and the cellulose was extracted basically as described elsewhere (8).

Construction of the Deletion Mutants—CBH I linker region was deleted using a polymerase chain reaction as described by Ho et al. (24, 25). A BseIDII/SmaI restriction fragment in pEM-F5 was exchanged with the polymerase chain reaction fragment containing the mutated region, and the resulting plasmid was transformed to *E. coli* DH5α. The nucleotide sequence of the whole fragment subjected to polymerase chain reaction was confirmed. Standard methods were used in the DNA manipulations (26).

Transformation and Screening of the Transformants—Transformation was carried out as described by Penttilä et al. (27) using co-transformation with the selection plasmid pAN8-1. The transformants were screened twice by streaking on selection plates containing

1 The abbreviations used are: CBH I, cellobiohydrolase I; CBH II, cellobiohydrolase II; amdS, *Aspergillus nidulans* gene encoding aceticamidase; PAGE, polyacrylamide gel electrophoresis; CNPL, 2′-chloro-4′-nitrophenyl-β-D-galactoside; CBD, cellulose-binding domain.
2 A. Jones, personal communication.
3 E. Margolles and M. Penttilä, manuscript in preparation.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of a Finnish International Development Agency (FINNIDA) scholarship.
‡ To whom correspondence should be addressed: VTT, Biotechnical Laboratory, P.O. Box 202, SF-02151, Espoo, Finland. Tel.: 358-0-4565136; Fax: 358-0-4552028.
0.25 mg/ml phleomycin. Spores of phleomycin resistant colonies were picked on microtiter plate wells containing 200 μl of medium A (1.5% distillers' spent grain, 1.5% KH₂PO₄, 0.5% (NH₄)₂SO₄, 5 mg/liter FeSO₄·7H₂O, 1.6 mg/liter MnSO₄·H₂O, 1.4 mg/liter ZnSO₄·7H₂O, and 3.7 mg/liter CoCl₂·6H₂O). 1 mm sporophore was added after 1 day of incubation to induce cellulase production. After incubation for 7 days at 28 °C in humidified bath, the plate was centrifuged (4000 rpm, 10 min), and the supernatants were analyzed on SDS-PAGE (28) and Western blot.

Protein Purification—One slant of a selected positive single spore culture was incubated in 50 ml of medium containing 2% Solka Floc and 1% distillers' spent grain. After 1 day incubation in a shake flask at 28 °C, the culture was added to 500 ml of the same medium, and the cultivation was continued for 7 days whereafter the protein was purified as described previously (29). CBH I catalytic core domain was produced by papain digestion (6).

Western Blotting—The proteins were transferred electrophoretically onto nitrocellulose membrane (30). Cellulases were detected with monoclonal antibodies raised against CBH I (CI-89, C271 (31), and H-4 (32)), and thereafter recognized by commercial alkaline phosphatase-labeled goat anti-mouse proteins (Promega).

Protein Concentration Determination—CBH I concentration was calculated from UV absorbance at 280 nm using a molar extinction coefficient, ε = 73,000 scm⁻¹ cm⁻¹ (1).

Enzyme Activity Measurement—2.5 mM 2-chloro,4'-nitrophenyl β-lactoside (CNPL) (33) was used to determine the enzyme activity on soluble substrates. The end product, chloronitrophenol, was measured at 405 nm and quantitated from a chloronitrophenol standard curve. A. xylinum microcrystalline cellulose was used to determine the enzyme activity on crystalline cellulose. Cellulose suspension was shaken with the enzyme solution for 3 h at 50 °C. The activity was determined from the liberated reducing sugar as measured by dinitrosalisylic acid method (34) using glucose as a standard.

Adsorption Studies—0.2-10 μ of enzyme solution was mixed with the cellulose and incubated at 4 °C in end-over-end mixer (Clinicon, AG 20) for 90 min. The bound enzyme was separated by filtration through 0.45-μm Durapore membrane (Millipore). The free enzyme was quantitated by spectrophotometry at 280 nm or by spectrophotometry (Shimadzu, Japan) at emission wavelength 350 nm/ band width 20 nm and excitation wavelength 280 nm/ band width 3 nm using standard curve prepared from the same enzyme. The bound enzyme concentration was calculated from the difference between initial enzyme and free enzyme concentration.

RESULTS

Characteristics of the Deletion Mutants—The length of the CBH I linker region up to the papain cleavage site shown in Fig. 1 is 31 amino acids. Based on the CBH I core structure recently solved by Alwyn Jones and co-workers there is no apparent linker structure present on the core side of the papain cleavage site. Although the amino acid composition of the linker sequences of Trichoderma cellulases is very similar, no obvious sequence homology can be detected. The N terminus of the linker peptide which links to the core domain contains a glycine- and proline-rich, three times repeated sequence GNPFP/SQG. The rest of the linker shows an abundance of serine and threonine residues providing 10 putative sites for O-glycosylation. The C-terminal end of the linker contains two glycines and two prolines (Fig. 1). Two deletions were introduced to the linker peptide, the code of each mutant refers to the position of the last residue still included in the deletion. Deletion of the amino acids 434–444 (ΔG-444) removes approximately one-third of the linker including the repeated sequence but leaving all of the putative O-glycosylation sites intact. Deletion of the residues 434–460 (ΔG-460) removes practically all of the linker (Fig. 1). CBH I catalytic domain (core) produced by papain digestion was used in comparative activity and binding experiments.

Production and Purification of the Enzymes—The mutant proteins were expressed in a T. reesei strain lacking the original cbh1 gene. 500 mg of mutant enzymes were secreted into a liter of culture medium under the conditions used for cultivation.

The proteins were purified to homogeneity by DEAE ion-exchange chromatography followed by affinity chromatography as described previously (29). Both of the mutant enzymes produced separately into two partially overlapping peaks in the DEAE ion-exchange chromatography (data not shown). The two forms (denoted A and B in Fig. 2 and Table I) were separately pooled, purified to homogeneity, and shown to differ slightly in their electrophoretic mobility in SDS-PAGE (Fig. 2). The observed difference in mobility corresponded to approximately a 2-kDa difference in molecular mass. The origin of the two isoenzymes is not clear but could be caused by differential glycosylation sometimes observed in Trichoderma cellulase cul-

![Fig. 1. Schematic representation of the domain structure of CBH I. The amino acids contained in the linker region are shown in boldface letters and the papain cleavage site between the core and the linker of CBH I (6) is indicated by an arrow. ΔG-444 = deletion of residues 434–444, ΔG-460 = deletion of residues 434–460, all of the indicated residues included. The zigzag lines represent putative flexible hinge regions and the thick bar with Y represents the O-glycosylated sticklike structure.](image-url)
Enzyme activity of different forms of mutant enzymes against soluble substrate (CNPL) and crystalline cellulose substrate

For bacterial cellulose, the enzyme to substrate ratio was 1.7 μmole/g. Enzyme activities were determined as described under "Experimental Procedures." The values shown are the mean ± S.E. of three different incubations (CNPL) and two independent duplicate determinations (bacterial cellulose).

| Enzyme  | Substrate         | Bacterial cellulose |  
|---------|-------------------|---------------------|
| ΔG-444-A | CNPL              | 54 ± 1              |
| ΔG-444-B | CNPL              | 55 ± 1              |
| ΔG-460-A | CNPL              | 52 ± 1              |
| ΔG-460-B | CNPL              | 53 ± 2              |
|          | Bacterial cellulose | 20 ± 1              |

a katal/mol = moles of product/mole enzyme.

b A and B refer to the two partially overlapping peaks from DEAE ion-exchange chromatography (see text).

tivations. However, the activities of both forms of the two deletions on soluble substrate (CNPL) and crystalline cellulose were found to be identical (Table I), and further characterization was carried out with only one form (A) of each mutant (Fig. 2, lanes 1-7). Deletion of the residues 434-444 and 434-460 results in 1.6- and 3.6-kDa reduction in molecular mass, respectively. This corresponds well to molecular masses estimated from SDS-PAGE (Fig. 2). Western blotting with two monoclonal antibodies specific for the CBD (31, 32) confirmed the presence of an intact CBD in both mutants (data not shown).

Adsorption Properties of the Deletion Mutants—The adsorption studies were performed by varying the degree of cellulose saturation from 5-9% to 90-100% by incubating bacterial cellulose suspensions at 1 mg/ml with 0.2-10 μm enzyme solutions. No reducing sugar formation was detected under conditions used in the adsorption experiments (80 min at 4 °C).

The equilibrium adsorption isotherms for the CBH I wild type, core, and the deletion mutants are shown in Fig. 3A. All of the Scatchard plots (Fig. 3B) derived from the corresponding adsorption isotherms were concave, indicating complex interaction. The affinities of the enzymes to cellulose were estimated from the initial slopes of the adsorption isotherms (Fig. 4). The resulting “distribution coefficients” are summarized in Table I. Both of the deletions introduced to the CBH I linker peptide reduce only slightly the enzyme affinity on crystalline cellulose. The shape of the binding isotherm of the core protein was different from the others and the affinity could not be accurately estimated.

The amount of enzyme bound on crystalline cellulose depends on the initial enzyme concentration used as shown in Fig. 5A. At an initial enzyme concentration of 0.2 μm (5-9% of saturation) practically no difference is observed between the different forms of CBH I. At an initial enzyme concentration greater than 1 μm (20-40% of saturation), a decrease in the binding capacity of the core domain relative to the other three forms becomes apparent. Greater binding of the wild-type CBH I over the deletion mutants become more pronounced as the initial enzyme concentration approaches 10 μm. The deletion mutants bind better than the core domain at enzyme concentrations of 1 μm and beyond. At very high protein concentration the two deletion mutants and the core domain of CBH I found less available binding sites on cellulose than the wild-type CBH I (Table II).

Catalytic Activity of the Wild-type and Modified CBH I—All four enzymes had equal activities on the small soluble oligosaccharide, CNPL (Table III). Their activities on crystalline cellulose depend on the concentration ratio between the enzyme and the substrate. The specific activities calculated per total amount of protein added to the reaction mixture are increased at higher enzyme concentrations (Fig. 5B). In all concentrations studied the core domain has 40% and the ΔG-460 has approximately 50% of the native CBH I activity. The ΔG-444 is as active as or slightly more active than the wild-type.
TABLE II
Relative distribution coefficients and binding capacities for binding to bacterial cellulose

Distribution coefficients (relative affinities) were estimated from the initial slope of the adsorption isotherms. Binding capacities were calculated from the adsorption isotherms by extrapolating the amount of bound protein to infinity. The values shown are the mean ± S.E. of duplicate determinations. Several additional independent experiments gave similar results.

| Enzyme  | Distribution coefficient | Binding capacities |
|---------|--------------------------|---------------------|
|         | liters/g                 | µmol/g              |
| CBH I   | 30                       | 4.2 ± 0.1           |
| ΔG-444  | 20                       | 2.0 ± 0.1           |
| ΔG-460  | 20                       | 2.6 ± 0.2           |
| Core    | ND                       | 1.7 ± 0.4           |

* ND, not determined.

CBH I, also in longer incubations of up to 72 h (data not shown).
The productivity of the enzymes was evaluated by calculating specific activities of the enzymes actually bound on cellulose (Fig. 5C). While some differences in binding in the lower (4 °C) and higher (50 °C) temperatures used for binding and catalysis (respectively) cannot be ruled out, it is likely that the relative order of the enzymes in terms of binding capacities is not changed. Somewhat surprisingly the ΔG-444 exhibits higher productivity than the wild-type CBH I while the ΔG-460, lacking the linker peptide, has the lowest productivity. Also the core domain has higher productivity than the wild-type CBH I under conditions used. In all cases the productivity is decreased at higher enzyme concentrations.

DISCUSSION

The interaction of cellulases with crystalline cellulose is a dynamic process which involves adsorption, desorption, and rearrangements of the two domains on the cellulose surface. The breakdown process of the highly ordered crystalline substrate apparently requires coordinated action of both domains of the enzymes by a mechanism which is still poorly understood. In addition to the catalytic events also the binding of the enzymes onto the heterogeneous surface of native cellulose must be understood. A number of studies have addressed this problem using mixtures of cellulases or purified enzymes (6, 8, 29, 35–40).

The adsorption process has turned out to be a very heterogeneous phenomenon, partly due to the properties of the substrate. Even a perfect cellulose crystal has at least two non-equivalent crystal faces and corners possibly with different affinities to the enzyme (41). Native cellulose also contains imperfect crystals, chain ends, and nicks generating more amorphous regions on the surface. As recently pointed out by Gilkes et al. (8), the concept of overlapping potential binding sites almost certainly applies also to the crystalline surface of cellulose. Therefore simple models of binding assuming a Langmuir type of adsorption or two binding sites (37) may not adequately represent the complexity of the binding events. We approach the problem assuming that the cellulose surface contains a number of potential binding sites which may overlap and exhibit different affinities to the enzyme.

Earlier the role of the interdomain linker peptide has also been studied experimentally on Pseudomonas fluorescens subspecies cellulosa xylanase A (42) and Cellulomonas fimi endoglucanase A (43). Deletion of the entire linker peptide between the xylanolytic core domain and a cellulose binding domain had no effect on the xylanolytic activity of xylanase A (42). In the case of endoglucanase A, deletion of the linker peptide did not change its binding to cellulose but was shown to alter its elution properties and the relative orientations of the two domains. Direct comparison of endoglucanase A and CBH I is,

FIG. 5. Adsorption (A), total enzymatic activity (B), and productivity (C) of different forms of CBH I at various initial enzyme concentrations of 0.2–10 µM. All adsorption experiments were performed at 4 °C, 90 min, and the enzymatic activities were measured at 50 °C for 3 h. The productivity of each form of CBH I was calculated per mol of enzyme bound on cellulose.
TABLE III  
Enzyme activity against soluble model substrate, CNPL

| Enzyme | CNPL (katal/mol<sup>*</sup>) |
|--------|-------------------------------|
| CBH I  | 275 ± 20                      |
| AG-444 | 269 ± 20                      |
| AG-400 | 271 ± 25                      |
| Core   | 280 ± 10                      |

<sup>*</sup> katal/mol = moles of product/mole enzyme.

however, difficult due to the differences in the structure and function of bacterial and fungal cellulases. First, unlike fungal cellobiohydrolases, the bacterial enzymes are relatively inefficient in solubilizing highly crystalline cellulose. Second, while fungal catalytic domains bind to and degrade crystalline cellulose, known bacterial core domains do not (8, 43). Finally, the size of the bacterial CBD is approximately three times that of the fungal CBD, and it is doubtful whether they function identically.

Here we have investigated the binding and activity of T. reesei CBH I and its truncated forms on bacterial crystalline cellulose. At very low enzyme concentration the amount of native CBH I bound was linearly dependent on the free protein concentration (Fig. 4) as found earlier by many authors (35, 36). Therefore, the initial slopes of the binding isothersms were used to estimate relative affinities or “distribution coefficients” of the native and deleted forms of CBH I. The binding capacity reflecting the number of all available binding sites for CBH I on the cellulose surface was calculated from the adsorption isothersms by extrapolating the amount of bound protein to infinity. Furthermore, the productivity of the enzymes was estimated by calculating the activity per enzyme actually bound on the substrate in given enzyme concentrations assuming that the putative temperature dependence of binding does not significantly change the relative order of the productivities obtained.

CBH I core domain is capable of binding to crystalline cellulose (6, 29, 37) (Figs. 3 and 5). Although the affinity could not be calculated accurately due to the sensitivity of measurement, at very low enzyme concentration (< 0.1 μM), it is the same order of magnitude as that of the other forms of CBH I studied here (Fig. 4). In very low initial enzyme concentration there is little difference in the binding capacities of the different forms of CBH I. In agreement with the results of Stålbärg et al. (37), the need of a CBD becomes apparent toward higher enzyme concentrations where the number of binding sites available for the core is less than a third of that for native CBH I (Fig. 5A). Somewhat surprisingly, the core domain exhibits significantly higher productivity than native CBH I in lower enzyme concentrations. This suggests that, at least in the beginning of the degradation, the sites available for binding the core domain are readily accessible for hydrolysis.

The deletion mutant AG-460 finds less binding sites on the cellulose surface than wild-type CBH I but many more than the core. Its specific activity is, however, only slightly above that of the core domain. In addition, the productivity of AG-460 is lowest of all forms of CBH I studied here indicating that a certain distance between the two domains is required for optimal activity. In conditions of high initial enzyme concentration the mutant AG-444 binds to only about half the binding sites occupied by wild-type CBH I. In spite of this, toward crystalline cellulose, AG-444 is as good an enzyme as the wild-type CBH I. It is possible that the reduced binding capacity of AG-444 is compensated by better turnover leading to improved productivity (see Fig. 5C). Alternatively, and perhaps more likely, the amount of enzyme bound productively is approximately the same for the two forms of CBH I but, unlike AG-444, wild-type CBH I also binds to catalytically inaccessible regions on the cellulose surface. The clearly decreasing productivity of wild-type CBH I but not of AG-444 toward higher enzyme coverage would indeed seem to support the latter hypothesis.

The deletion AG-444 removes practically all of the triple repeat, GNPNPS/G in the CBH I linker. Proline has less conformational freedom than any other amino acid while glycine can adopt many different conformations. It is therefore possible that the repeated sequence provides CBH I with a long flexible hinge with glycines responsible for the flexibility and prolines for the necessary length. The sequence immediately following this repeat contains 10 threonine or serine residues approximately 7 of which are likely to be O-glycosylated (44). This locally abundant glycosylation may provide the linker with rigidity needed to maintain sufficient distance between the two functional domains (45).

Based on our results we propose that in low enzyme concentration all forms of CBH I bind to easily accessible sites on the cellulose surface and there is no competition for the binding sites between the enzyme molecules. In high concentration, however, the number of binding sites becomes limiting and the molecules with a functional CBD find more binding sites than the core, presumably because of their ability to interact with highly crystalline regions. Native CBH I with a functional hinge may be able to bind predominantly via its CBD permitting close packing of the enzyme molecules on the cellulose surface. Binding of the core domain takes place as soon as new binding sites become available by the action of other, productively bound molecules of CBH I, other cellulases or perhaps the CBD of CBH I itself. While no data has yet been obtained on the putative crystal-breaking activity of the CBH I CBD, it is interesting that non-catalytic, defibrillating activity of the Cen A CBD has been demonstrated (46).

In the case of the two CBH I deletion mutants removal of the putative hinge region leads to limited flexibility of the molecule forcing the enzyme to bind simultaneously with both of its domains. In spite of this, the overall performance of AG-444 is similar to that of native CBH I.

In conclusion, the present study suggests that the interdomain linker peptide of CBH I has a dual role in providing the necessary distance between the two functional domains and facilitating the dynamic adsorption process led by the CBD. The presence of an extended, partially flexible linker allows the CBD to adsorb to crystalline regions of cellulose and diffuse laterally along the surface leading the core domain to new, enzymatically accessible sites.

Acknowledgments—We thank Emilio Margolles for the expression vector pEM-F5, Riitta Nurmi for construction of T. reesei cbh I negative strain, and Lauri Ruohonen for help with the fungal transformation technique. We are grateful to Dr. Siruuka Keränen for many fruitful discussions and advice and to Drs. Jonathan Knowles and R. A. J. Warren for helpful comments concerning the manuscript.

REFERENCES
1. Van Tilbeurgh, H., Tomme, P., Claysensena, M., Bhukhambhai, R., and Petersen, G. (1986) FEBS Lett. 204, 225–230
2. Teeri, T. T., Lehtovaara, P., Kauppinen, S., Salevoori, I., and Knowles, J. (1987) Gene 51, 43–52
3. Gilkes, N. R., Henriassa, B., Warren, R. A. J., Miller, R. C., Jr., and Kilburn, D. G. (1988) J. Biol. Chem. 263, 10401–10407
4. Beguin, P. (1990) Annu. Rev. Microbiol. 44, 219–248
5. Gilkes, N. R., Henriassa, B., Kilburn, D. G., Miller, R. C., Jr., and Warren, R. A. J. (1991) Microbiol. Rev. 55, 303–315
6. Tomme, P., Van Tilbeurgh, H., Petersen, G., Van Damme, J., Vandenbroecke, J., Knowles, J., Teeri, T., and Claysenena, M. (1988) Eur. J. Biochem. 170, 575–581
7. Claysenena, M., and Tomme, P. (1989) in Trichoderma reesei Cellulases: Biochemistry, Genetics, Physiology and Application (Kubicek, C. P., Eweleigh,
Linker Peptide Deletion of Fungal Cellulobiohydrolase I

20761

D. E. Esteban, R. W. Kubec, Prazn, E. M., ed) pp. 1–11, Proceedings, Tricell (1989) Royal Society of Chemistry
8. Gils, N. R., Jervis, E., Hennessy, R., Takan, B., Miller, R. C., Jr. Warren, R. A. J., and Kilburn, D. G. (1990) J. Biol. Chem. 265, 6743–6749
9. Burton, J., Wood, S. G., Peckrzok, A., and Siemiez, I. Z. (1989) Biophys. Chem. 33, 19–45
10. Bushnov, V. N., Gudkov, A. T., Lifjas, A., and Sepsev, N. P. (1989) J. Biol. Chem. 264, 4498–4505
11. Knowles, J., Tere, T. T., Lehtovaara, P., Penttila, H., and Saloheimo, M. (1988) in Biochemistry and Genetics of Cellulose Degradation (Aubert, J. P., Beguin, P., and Milet, J., eds) pp. 183–169, Academic Press, London
12. Miller, R. C., Gils, N. R., Jeeberg, B., Kilburn, D. G., Langsford, M. T., and Warren, R. A. J. (1988) in Biochemistry and Genetics of Cellulose Degradation (Aubert, J. P., Beguin, P., and Milet, J., eds) pp. 235–248, Academic Press, London
13. Roos, J., Bengt, T., Tere, T., Knowles, J. K. C., and Jones, T. A. (1990) Science 249, 380–386
14. Kraulis, P. J., Clare, G. M., Nilges, M., Jones, T. A., Pettersson, G., Knowles, J., and Gremien, A. M. (1989) Biochimica Biophysica Acta 884, 7241–7257
15. Fagerstrom, L. G., Pettersson, L. G., and Engelsman, J. A. (1984) FEBS Lett. 167, 309–315
16. Gun, E. K., and Brown, R. D., Jr. (1976) Biochem. Biophys. Acta 446, 371–386
17. Schmuck, M., Pilz, I., Hayn, M., and Esteban, H. (1988) Biotechnol. Lett. 10, 397–402
18. Abuja, P. M., Pilz, I., Clausens, M., and Tomme, P. (1988) Biochem. Biophys. Res. Commun. 166, 189–185
19. Abuja, P. M., Schmuck, M., Pilz, I., Tomme, P., Clausens, M., and Esteban, H. (1988) Eur. Biophys. J. 16, 339–342
20. Abuja, P. M., Pilz, I., Clausens, M., and Esteban, H. (1989) Biochem. Biophys. Res. Commun. 166, 615–623
21. Tere, T. T., Kumair, T., Lehtovaara, P., and Knowles, J. K. C. (1987) Anal. Biochem. 162, 504–507
22. Mattern, I. E., Punt, P. T., Unklaas, S., Powell, P. H., and van der Hofden, C. A. M. J. (1987) Abstracts of the 19th Lauter Lectures on Molecular Biology, p. 14, University of the Netherlands
23. Henniss, S. (1963) in Methods in Carbohydrate Chemistry (Whistler, R. L., ed) Vol. 3, pp. 4–9, Academic Press, New York
24. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene 84, 245–246
25. Sambrook, J., Fristch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Penttila, M., Nevalainen, H., Ratto, M., Salminen, E., and Knowles, J. (1987) Gene 61, 155–164
27. Lasnilla, U. K. (1970) Nature 227, 689–685
28. Reinikainen, T., Russe, L., Nevanen, T., Laaksonen, L., Kraulis, P., Jones, T. A., Knowles, J. K. C., and Tere, T. T. (1992) Proteins 14, 475–482
29. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
30. Aho, S., Oikkonen, V., Jalkla, T., Palobrini, M., Buhler, R., Niki-Paavola, M. L., Bamford, D. H., and Korkoh, M. (1991) Eur. J. Biochem. 200, 643–649
31. Mischa, H., Huf, F., Messerer, R., Wiesinger, E., Hayn, M., Tomme, P., Esteban, H., Küchler, E., Claeyssens, M., and Kubick, C. P. (1989) Biochim. Biophys. Acta 1005, 1–7
32. Claeyssens, M. (1988) in Biochemistry and Genetics of Cellulose Degradation (Aubert, J. P., Beguin, P., and Milet, J., eds) pp. 393–397, Academic Press, London
33. Miller, G. L. (1989) Anal. Chem. 61, 425–428
34. Oshino, J., Sakata, M., and Harnos, Y. (1983) Biotechnol. Bioeng. 25, 3103–3114
35. Kyriacou, A., and Neufeld, R. J. (1988) Enzyme Microb. Technol. 10, 675–681
36. Stühlinger, J., Johansson, G., and Pettersson, G. (1991) Bio/Technology 9, 286–290
37. Hoshino, K., Kanda, T., Sasaki, Y., and Nisizawa, K. (1992) J. Biochem. 111, 600–605
38. Kim, D. W., Kim, T. S., Jeong, Y. K., and Lee, J. K. (1992) J. Ferment. Bioeng. 78, 461–466
39. Tere, T. T., Reinikainen, T., Russeven, L., Jones, T. A., and Knowles, J. K. C. (1993) J. Biotechnol. 24, 169–176
40. Henniss, B., Vigny, B., Buléon, A., and Perez, S. (1988) FEBS Lett. 231, 177–182
41. Ferrari, I. M. A., Durrant, A. J., Hall, J., Hazelwood, G. P., and Gilbert, H. J. (1990) Biochem. J. 268, 261–264
42. Shen, H., Schmuck, M., Pilz, I., Gils, N. R., Kilburn, D. G., Miller, R. C., Jr., and Warren, R. A. J. (1991) J. Biol. Chem. 266, 11334–11340
43. Saloheimo, M. (1987) Biosynthesis of an indigestible glycosylated secretory enzyme (CBH1) of Trichoderma reesei, Doctoral thesis, Technical Research Centre of Finland Publication 34, Espoo
44. Jenas, F. (1990) Trends Biotechnol. Sci. 15, 291–294
45. Din, N., Gils, N. R., Takan, B., Miller, R. C., Jr., Warren, R. A. J., and Kilburn, D. G. (1991) Bio/Technology 9, 1096–1099