

The difference in affinity between two fungal cellulose-binding domains is dominated by a single amino acid substitution

Markus Linder^{a,b,*}, Gunnar Lindeberg^c, Tapani Reinikainen^a, Tuula T. Teeri^a, Göran Pettersson^b

^aVTT Biotechnology and Food Research, Box 1500, FIN-02044 VTT, Espoo, Finland

^bDepartment of Biochemistry, University of Uppsala, Box 576, S-751 23 Uppsala, Sweden

^cDepartment of Medical and Physiological Chemistry, University of Uppsala, Box 575, S-751 23 Uppsala, Sweden

Received 17 July 1995; revised version received 13 August 1995

Abstract Cellulose-binding domains (CBDs) form distinct functional units of most cellulolytic enzymes. We have compared the cellulose-binding affinities of the CBDs of cellobiohydrolase I (CBHI) and endoglucanase I (EGI) from the fungus *Trichoderma reesei*. The CBD of EGI had significantly higher affinity than that of CBHI. Four variants of the CBHI CBD were made in order to identify the residues responsible for the increased affinity in EGI. Most of the difference could be ascribed to a replacement of a tyrosine by a tryptophan on the flat cellulose-binding face.

Key words: Cellulose-binding domain; Synthetic peptide; Protein–carbohydrate interaction; Cellulase; *Trichoderma reesei*

1. Introduction

Most cellulolytic enzymes have a domain organization, consisting of a catalytic domain and a cellulose-binding domain (CBD). The CBDs of fungal enzymes are very similar in their amino acid sequence and size [1]. These small domains are responsible for most of the enzyme's affinity for cellulose [2–5]. The cellulose binding mediated by the CBD is essential for the enzymatic activity on crystalline cellulose, but has no effect on the hydrolysis of soluble substrates. The most thoroughly studied fungal CBD is that of cellobiohydrolase I (CBHI) from the filamentous fungus *Trichoderma reesei*. This is the major cellulolytic enzyme of the fungus, and it is capable of efficient hydrolysis of crystalline cellulose. The three-dimensional structure of the CBHI CBD has been solved [6], permitting detailed structure–function studies [3,4,7]. So far three tyrosines, one glutamine and one asparagine have been proposed to participate in the binding. Together they form a distinct flat binding face of the folded peptide. The presence of tyrosyl or other aromatic residues in the binding face is typical for carbohydrate–protein interactions [8].

In the hydrolysis of cellulose CBHI acts synergistically with a homologous endoglucanase, EGI. Despite the close similarity between their CBDs, some results suggest that the EGI CBD has a higher affinity towards cellulose [9]. Here we have carried out a detailed comparison of the two CBDs, and by means of specific amino acid substitutions investigated the molecular basis of the difference between the CBDs.

*Corresponding author. Fax: (358) (0) 455-2103.

Abbreviations: CBD, cellulose-binding domain; CBHI, cellobiohydrolase I; EGI, endoglucanase I.

2. Materials and methods

2.1. Peptides

Peptides were synthesized using Fmoc chemistry and purified as described in [10]. The purified peptides were characterized by time-of-flight plasma desorption mass-spectroscopy and amino acid analysis.

2.2. Adsorption of peptides to cellulose

Lyophilized peptide was dissolved in 50 mM sodium acetate buffer, pH 5.0, containing 50 mM NaCl. The peptide concentration was determined by amino acid analysis and UV absorbance at 280 nm. Dilutions of the peptide were made with the same buffer. A suspension (2 mg/ml) of tunicate cellulose (kindly provided by Daicel Co., Japan) in Milli-Q water and an equal volume of peptide in was mixed for 20–25 h at 4°C with a magnetic stirrer. The suspension was then centrifuged (4000 rpm, 10 min) and the concentration of peptide in the supernatant was determined by analytical reversed phase high performance liquid chromatography (HPLC). A water/acetonitrile gradient with 0.1% trifluoroacetic acid was used for elution.

3. Results and discussion

The binding isotherms for the wild-type CBHI and EGI CBD peptides clearly show that the affinity of the EGI CBD is markedly higher than that of the CBHI CBD (Fig. 1). As seen in Fig. 2 the amino acid sequences of the two CBDs are very similar, only 9 out of the 36 amino acids are different. The amino acid differences are indicated on the structure of the CBHI CBD, revealing their occurrence in three or four clusters (Fig. 3). Only one of the substitutions, tyrosine-5 of CBHI to a tryptophan in EGI, clearly changes a part of the binding face (Fig. 3). The changes P30(D), L28(S), V27(Y) occur near the tip of the wedge-shaped CBD. In the structure of CBHI CBD, the side chain of L28 points towards the flat face, and that of V27 towards the rough face and P30 forms the tip. The cluster with changes T17(K), V18(T) and A20(T) is found on the rough face, with the side chain of A20 in CBHI pointing away from it. Apparently, the cysteine in the EGI CBD at position 16 forms a disulfide bridge with another cysteine near the N-terminus. As the sequences of the two CBDs are so similar, their three-dimensional structures are presumably also conserved [11]. Assuming that the positions of the residues in EGI CBD are the same as those in the CBHI CBD, we designed a set of four variants of the CBHI CBD. The hybrids constructed were T17K:V18T:A20T, P30D, V27Y:L28S:P30D and Y5W. Their binding to crystalline cellulose was determined in order to identify residues responsible for the increased affinity of the EGI CBD. It should be noted that the CBDs used here were made synthetically, and that we have demonstrated earlier, in the case of CBHI that there is no difference in binding between the natural and synthetic CBDs [12].

The effects of the amino acid substitutions are shown in the

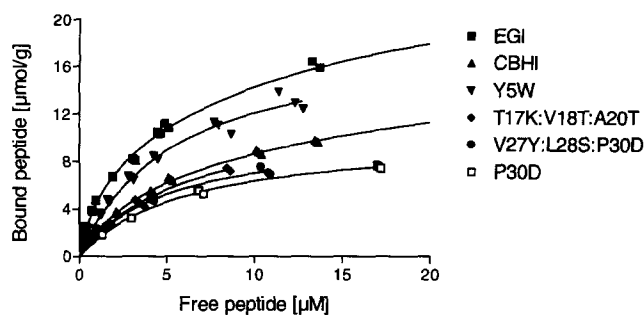


Fig. 4. Low concentration region of the adsorption isotherms of the variant and wild-type CBDs.

and S28 together compensate part of the decrease observed when P30 was changed alone.

Fig. 1 shows that a saturating level of CBD on cellulose was not quite achieved. However, both wild-type isotherms seem to converge to some value in excess of 25 $\mu\text{mol/g}$ cellulose. Using the approximate dimensions of $3 \times 2 \text{ nm}$ for the CBHI CBD [6] and assuming a monolayer, a minimum covered area of 90 m^2/g cellulose can be calculated. Accessible crystalline surface areas are not easily measured for cellulose, but some calculations can be made on the basis of the microfibril dimensions obtained from electron micrographs. The diameter of tunicin microfibrils has been estimated at 10 nm [18] and the density of I_β cellulose has been calculated to be 1.6 g/cm^3 [19]. From these values a theoretical maximum crystalline surface area of 250 m^2/g can be calculated. These calculations thus indicate that somewhat less than half of the surface would be covered by CBDs, provided that the binding itself does not change the surface. It should be noted that the surface of crystalline cellulose is equally divided between two distinctly different types, potentially providing two types of binding sites [20].

The results are in good agreement and support earlier conclusions that the flat face binds to cellulose and that the rough face is not involved in binding. It is noteworthy that the CBHI enzyme could, by a single amino acid substitution acquire a higher affinity towards cellulose. If a higher affinity offered CBHI a selective evolutionary advantage, i.e. better productivity, such a mutation could easily have occurred during evolution. Since this has not happened it is tempting to assume that the affinity of CBHI is balanced for optimum performance of the enzyme. Indeed, our preliminary data obtained with a hybrid CBHI with the EGI CBD instead of its own, revealed improved binding but no changes in the enzymatic activity of

CBHI [9]. On the other hand, mutations decreasing the affinity of the CBHI CBD do decrease its catalytic activity on crystalline cellulose [3,4]. Therefore, it is possible that beyond a certain, relatively high affinity of the CBD slight increases will no longer improve the enzymatic activity on crystalline cellulose.

References

- [1] Gilkes, N., Henrissat, B., Kilburn, G., Miller, R. and Warren, R.A.J. (1991) *Microbiol. Rev.* 55, 303–315.
- [2] van Tilbeurgh, H., Tomme, P., Clayssens, M., Bhikhabhai, R. and Pettersson, G. (1986) *FEBS Lett.* 204, 223–227.
- [3] Reinikainen, T., Ruohonen, L., Nevanen, T., Laaksonen, L., Kraulis, P., Jones, T.A., Knowles, J. and Teeri, T. (1992) *Proteins* 14, 475–482.
- [4] Reinikainen, T., Teleman, O. and Teeri, T. (1995) *Proteins* (in press).
- [5] Ståhlberg, J., Johansson, G. and Pettersson, G. (1991) *Bio/Technology* 9, 286–290.
- [6] Kraulis, P., Clore, G., Nilges, M., Jones, T.A., Pettersson, G., Knowles, J. and Gronenborn, A. (1989) *Biochemistry* 28, 7241–7257.
- [7] Linder, M., Mattinen, M.-L., Kontteli, M., Lindeberg, G., Ståhlberg, J., Drakenberg, T., Reinikainen, T., Pettersson, G. and Annala, A. (1995) *Prot. Sci.* 4, 1056–1064.
- [8] Vyas, N. (1991) *Curr. Opin. Struct. Biol.* 1, 732–740.
- [9] Srisodsuk, M. (1994) Thesis, Helsinki University, VTT Publications 88, Espoo, Finland, pp. 64–69.
- [10] Lindeberg, G., Bennich, H. and Engström, Å. (1991) *Int. J. Peptide Protein Res.* 38, 253–259.
- [11] Hoffrén, A.M., Teeri, T. and Teleman, O. (1995) *Protein Eng.* (in press).
- [12] Johansson, G., Ståhlberg, J., Lindeberg, G., Engström, Å. and Pettersson, G. (1989) *FEBS Lett.* 243, 389–393.
- [13] Klyosov, A.A., Mitkevich, O.V. and Sinitsyn, A.P. (1986) *Biochemistry* 25, 450–452.
- [14] Quijcho, F.A. (1993) *Biochem. Soc. Trans.* 21, 442–448.
- [15] Engle, A., Purdie, N. and Hyatt, J. (1994) *Carbohydr. Res.* 265, 181–195.
- [16] Radzica, A. and Wolfenden, R. (1988) *Biochemistry* 27, 1664–1670.
- [17] Hunter, C. and Sanders, J. (1990) *J. Am. Chem. Soc.* 112, 5525–5534.
- [18] Belton, P.S., Tanner, S.F., Cartier, N. and Chanzy, H. (1989) *Macromolecules* 22, 1615–1617.
- [19] Heiner, A.P., Sugiyama, Y. and Teleman, O. (1995) *Carbohydr. Res.* (in press).
- [20] Henrissat, B., Vigny, B., Buleon, A. and Perez (1988) *FEBS Lett.* 231, 177–182.
- [21] Shoemaker, S., Schweickart, V., Ladner, M., Gelfand, D., Kwok, S., Myambo, K. and Innis, M. (1983) *Bio/Technology* 1, 691–696.
- [22] Penttilä, M., Letovaara, P., Nevalainen, H., Bhikhabhai, R. and Knowles, J. (1986) *Gene* 45, 253–263.
- [23] Bernstein, F.C., Koetzle, T.F., Williams, G.J.B., Meyer, E.F., Brice, M.D., Rodgers, J.R., Kennard, O., Shimamouchi, T. and Tasumi, M. (1977) *J. Mol. Biol.* 112, 535–542.