Volume 231, number 1, 177-182 FEB 05691 April 1988

Possible adsorption sites of cellulases on crystalline cellulose

Bernard Henrissat*, Béatrice Vigny, Alain Buleon and Serge Perez

Laboratoire de Biochimie des Protéines and Laboratoire de Physicochimie des Macromolécules, Institut National de la Recherche Agronomique, rue de la Géraudière, F-44072 Nantes, France

Received 7 January 1988

The possible adsorption sites of cellulases on crystalline cellulose were investigated by molecular graphic representation of a crystal of cellulose and estimation of the accessibility of the various glycosidic bonds to enzymatic attack. The results show that only certain glycosidic bonds of certain surface cellulose chains are susceptible to enzymatic hydrolysis. These preferential sites correlate well with previous electron microscopy observations of the adsorption sites of 1,4-β-D-glucan cellobiohydrolase I (CBHI) from *Trichoderma reesei* on *Valonia* cellulose.

Cellulase; Cellulose crystal; Adsorption site; Molecular graphics

1. INTRODUCTION

The enzymatic degradation of crystalline cellulose involves the action of a cellulase complex composed of two major components: 1,4-β-D-glucan glucanohydrolase (endoglucanase, EG, EC 3.2.1.4) and 1,4-β-D-glucan cellobiohydrolase (cellobiohydrolase, CBH, EC 3.2.1.91). These enzymes show multiple-type synergy when acting on cellulose [1]. The individual cellulase components differ not only in their mechanism of action and substrate specificity, but also in their adsorption characteristics on cellulose [2]. For instance, the filamentous fungus *Trichoderma reesei* produces one cellobiohydrolase, namely CBHI, which binds strongly to highly crystalline celluloses such as Avicel [3], cotton fibers [4] or *Valonia* cellulose microcrystals [5,6]. The reasons for the unusual and almost irreversible adsorption of CBHI at the surface of cellulose are not understood yet and they are poorly documented although they are most likely related to the enzyme mode of action. The exact mechanism by which CBHI breaks down crystalline cellulose is still speculative, but it is reasonable to assume that the process involves the specific adsorption of the enzyme on the substrate followed by directional hydrolysis along a crystallographic direction [6-8]. Chanzy et al. [6] have shown by electron microscopy that CBHI adsorbs specifically on certain faces/edges of cellulose microcrystals from *Valonia*. The present work shows that molecular graphics helps in finding possible reasons for such a 'crystalline specificity' by examination of the exposed/buried glycosidic bonds of a cellulose crystal.

Correspondence address: B. Henrissat, Laboratoire de Biochimie des Protéines, Institut National de la Recherche Agronomique, rue de la Géraudière, F-44072 Nantes, France

2. METHODS

2.1. *Construction of the microfibril model*

Valonia cellulose is one of the most perfect native cellulose materials [9] with each microfibril being a single crystal [10,11] with a parallel chain-packing [7,12,13]. A model microfibril containing 16 chains (A 'real' microfibril of *Valonia* cellulose has a $\sim 20 \times 20$ nm square cross-section [11] and contains 1200-1400 chains [15]. For convenience, our model was built with only 16 chains, but this does not interfere with the evaluation of the intrinsic accessibility of the surface chains.) of 6 anhydroglucose residues was generated from the coordinates of the two-chains unit cell proposed for native cellulose [12] with $a = 0.818$ nm, $b = 0.784$ nm, c (fiber axis) $= 1.038$ nm and gamma (monoclinic angle) $= 97.04°$. The hydroxylic hydrogens were omitted since their location is not known. Two morphologies could be constructed for the microfibril cross-section (see fig.1). However, electron microscopy observations of

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

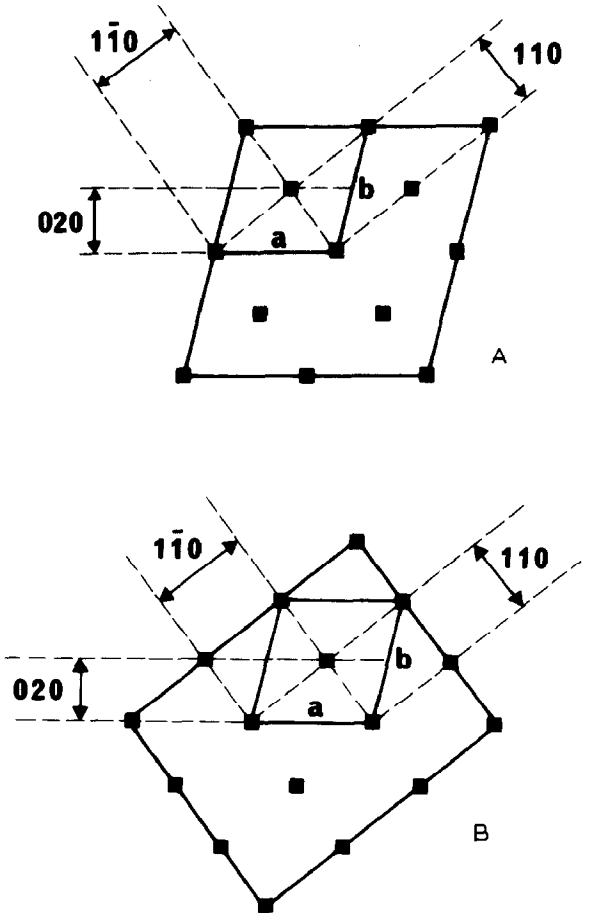

Fig.1. Schematic representation of the *a,b* projection of the two possible models for a microcrystal of cellulose. The main lattice spacings are indicated with their Miller indices.

cross-sections in bright field contrast [11], micro-diffraction [14] as well as lattice imaging [15], have proven that the morphology of *Valonia* microfibrils corresponds to the model in fig.1B and we have therefore constructed our microfibril following that model.

2.2. *Lattice energy calculations*

The energy of interaction between a given cellobiose repeating unit and the neighboring molecules was computed as described [16] starting from the known atomic coordinates. The interaction energy between two molecules was considered to be the sum over all pairwise atom-atom interactions. Such interaction was calculated according to 6-12 potential functions where short-range repulsive and attractive interactions were taken into account. In performing the interchain energy calculations, we have used a cutoff distance such that $d_{ij} \leq 1.5\ R_{ij}$, where R_{ij} is the appropriate van der Waals radii sum [17]. The number of interatomic contacts satisfying this particular condition was also computed and referred to as the number of 'short contacts' (table 1): this indicates the complementarity of the shapes of the interacting units.

2.3. *Computer graphics tools*

A modified version of the FRODO crystallographic program [18] was used on the laboratory PRIME 750 super-minicomputer connected to an interactive color display (Evans and Sutherland PS 350). Accessibility of the various atoms was estimated by generating their van der Waals surfaces.

3. RESULTS AND DISCUSSION

Table 1 shows that, as far as packing energy is concerned (H-bonds and van der Waals contacts), the cellulose chains located at the obtuse corners of the microfibril appear to be much less stabilized than the other surface chains. Since enzymatic hydrolysis occurs at the glycosidic oxygens, it is reasonable to assume that the more exposed the glycosidic oxygens, the more likely they will undergo hydrolysis. The accessibility of the glycosidic bonds of the chains was therefore evaluated by molecular graphics modelling. The various surface chains of the microfibril featuring the van der Waals radii of the constitutive atoms around the glycosidic bonds are displayed in fig.2. Examination of these views reveals that the surface chains can be classified into 3 major groups on the basis of availability of the glycosidic linkages. The most exposed glycosidic bonds belong to the two edge chains located at the obtuse angles of the microfibril. The surface chains of the (110) face of the microfibril come next in accessibility of their glycosidic bonds whereas the chains of the (1 – 1 0) face, with the exception of the corner chains, show very little (if any) exposure of their glycosidic

Table 1

Packing features of the various cellobiose units at the surface of a crystal of cellulose

	Inter-molecular H-bonds	van der Waals contacts	Packing energy (kcal)
Cellobiose unit on a 110 face	1	408	−18.8
Cellobiose unit on a 1 – 1 0 face	1	491	−19.5
Cellobiose unit on an obtuse corner	0	275	−8.8
Cellobiose unit on a sharp corner	1	312	−14.7

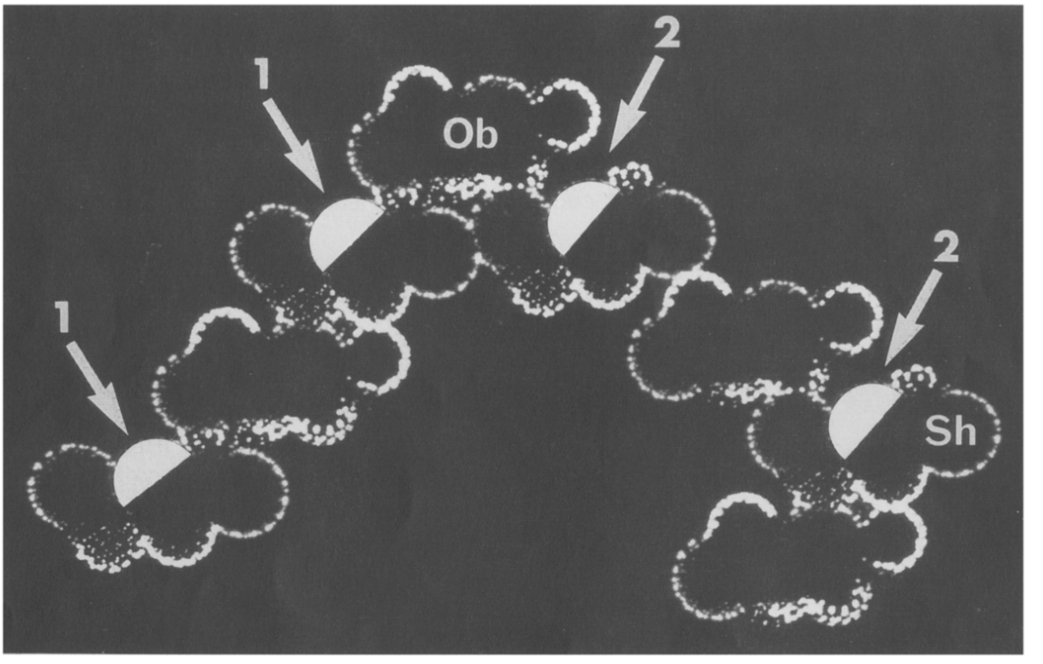

Fig.2. Molecular graphic representation of a cross-section of the model microfibril of cellulose. The van der Waals surfaces are represented by dots. The glycoside oxygens of interest are filled in white. Ob and Sh denote, respectively, the obtuse and sharp corners of the microfibril. Arrows 1 point to the glycosidic oxygens of the (1 1 0) face whereas arrows 2 point to those of the (1 − 1 0) face).

bonds. The two edge chains at the sharp angle of the microfibril have an accessibility similar to those of the (1–10) face.

It is quite remarkable that this classification of the exposed/buried glycosidic oxygens of the surface chains of the microfibril correlates precisely with electron microscopy observations which have shown that CBHI from *Trichoderma reesei* adsorbs specifically at the corners and/or the (1–10) faces of *Valonia* cellulose microcrystals [6]. It is also interesting to note that Takai et al. [19] have observed a selective decrease of the (1–10) diffraction line of bacterial cellulose after cellobiohydrolase action.

Examination of the glycosidic oxygens of the surface chains also shows that, along a given chain, the exposed glycosidic oxygens alternate with buried ones at every anhydroglucose unit. This is the case even for the most accessible chains at the obtuse corners of the microfibril (fig.3) where one of every two glycosidic oxygens is well exposed whereas the other is completely buried. Enzymatic hydrolysis of a glycosidic bond with a lysozyme-type mechanism proceeds through protonation of the glycosidic oxygen by an acidic amino acid residue [20,21] and stabilization of the resulting carbonium ion by another amino acid residue. It is therefore very unlikely that hydrolysis of the buried glycosidic bonds can occur. In these conditions, hydrolysis of two successive exposed glycosidic bonds should release a cellobiose unit and this may explain why cellobiose is the major product released by cellulase digestion of crystalline cellulose [22,23].

Another consequence of the inaccessibility of one of every two glycosidic bonds to enzymatic attack concerns the tentative mechanism proposed [24,25] for explaining the co-operativity between two cellobiohydrolases. A consequence of such a mechanism would be the enzymatic attack of a buried glycosidic oxygen by one of the two cellobiohydrolases; this would be possible if the two enzymes have different molecular mechanisms or if the enzymes are able to twist the cellulose chains to expose the buried glycosidic oxygen.

'Classical' interpretations of enzyme catalysis

involve flexibility of the substrate which could adopt, in the active center of the enzyme, a conformation similar to that of the transition state. However, in the case of a crystalline fibrous polymer such as cellulose where the extended chains are held together by a tight network of hydrogen bonds, such a flexibility is highly restricted and therefore the enzyme must compensate somehow the conformational limitations of the substrate. The adsorption of cellulase onto cellulose surface is necessarily the first stage of the process. Several authors have reported that the adsorption of CBHI is almost irreversible [2–5,26,27] but, to the best of our knowledge, there has been no conclusive study

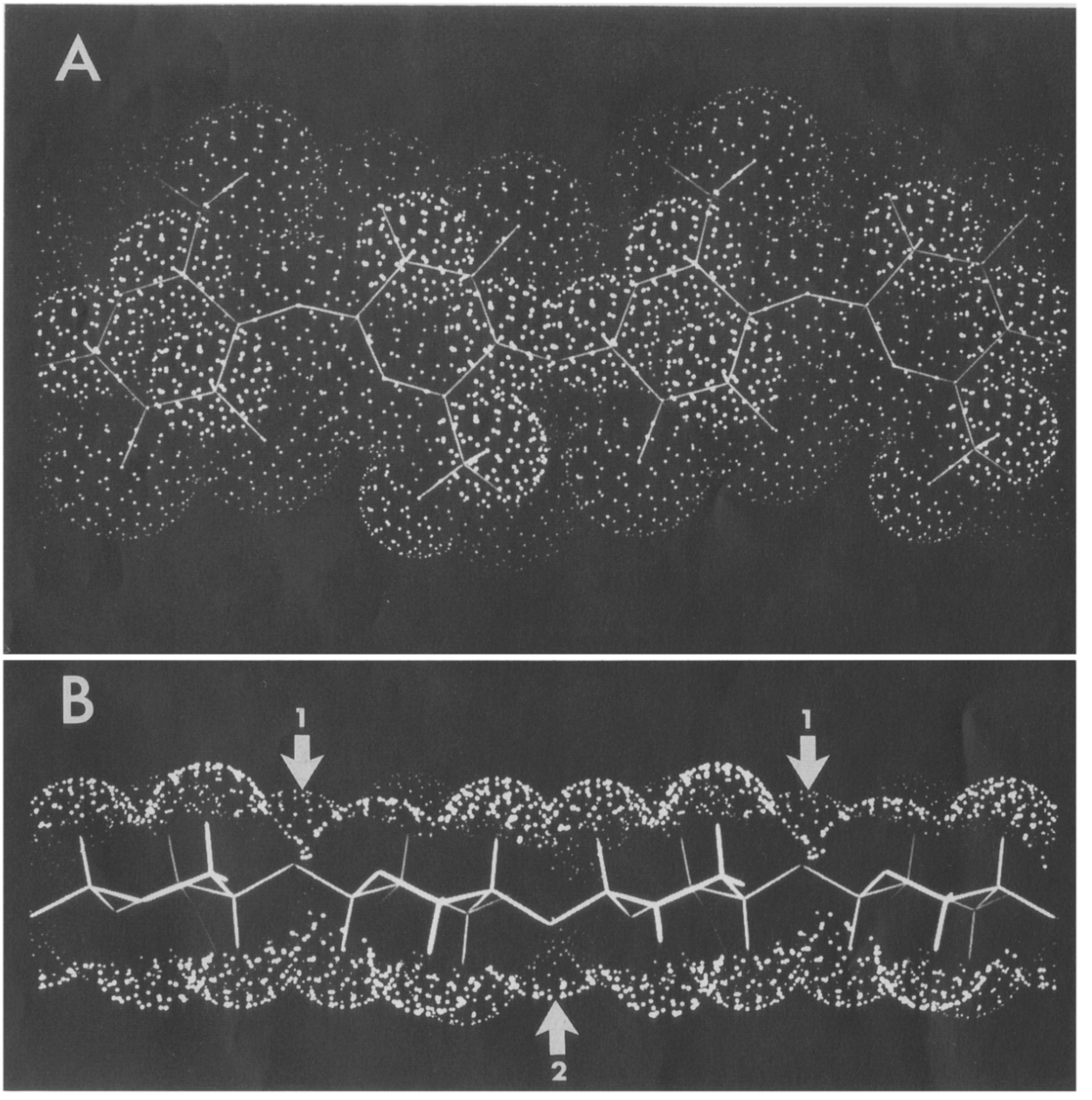

Fig.3. Molecular graphic representation of the cellulose chain located at the obtuse corner of the microfibril. (A) View from the top; (B) view from the side. Arrows 1 and 2 show respectively exposed and buried glycosidic oxygens.

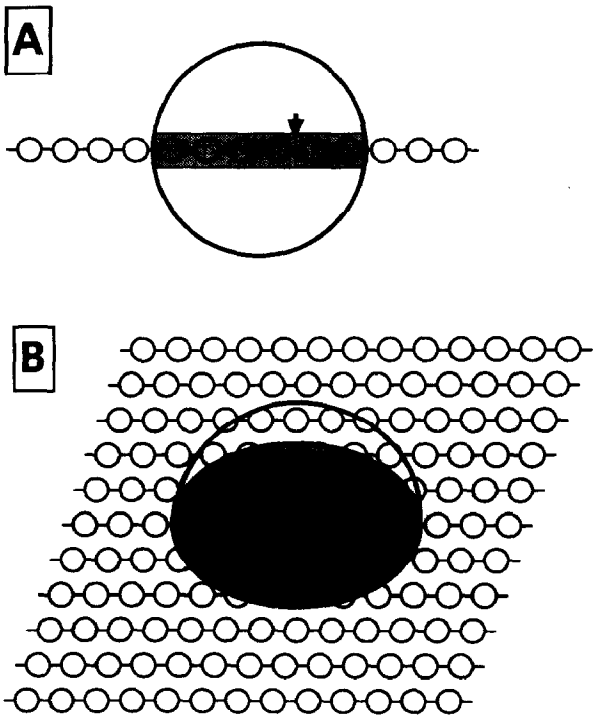

Fig.4. Schematic representation of the interaction of an enzyme with (A) a single chain substrate and (B) a multichain substrate such as cellulose.

of the molecular aspects of such a behavior. It has been proposed that the carboxy-terminus of CBHI [28] and the amino-terminus of CBHII [29] may represent the cellulose-binding domain of these enzymes. Such a domain should most likely exhibit a high stereochemical complementarity with the substrate. A number of enzymes degrading soluble polysaccharides have been found to have an active center composed of several subsites, each of which interacting with one of the building units of the substrate. This binding of the protein to a relatively large portion of a linear polysaccharide (typically 4–7 monosaccharide units) involves numerous individually weak interactions (van der Waals, H-bonds) which sum into a resulting overall binding energy (see fig.4A). In the case of the crystal of cellulose, composed of tightly packed chains, the enzyme is not facing a single unidimensional chain but rather several chains, i.e. a surface. In such a case, the number of weak interactions involved in the enzyme-substrate complex is greatly increased since the enzyme interacts most likely with several adjacent chains and the resulting binding energy would be higher than that resulting from a single chain interaction (see fig.4B). This could explain the unusual tight binding of CBHI onto cellulose which is perhaps necessary to compensate the low flexibility of the substrate.

Acknowledgements: The authors wish to thank Drs Buisson and Duée (CEN Grenoble) for implementing FRODO on the PRIME computer. Anne Imberty (CERMAV-CNRS Grenoble) kindly performed the lattice energy calculations.

REFERENCES

[1] Henrissat, B., Driguez, H., Viet, C. and Schülein, M. (1985) Bio/Technology 3, 722–726.
[2] Rabinovitch, M.L., Nguen Van Viet and Klesov, A.A. (1982) Biokhimiya 47, 465–477.
[3] Reese, E.T. (1982) Proc. Biochem. 17, 2–6.
[4] Beltrame, P.L., Carniti, P., Focher, B., Marzetti, A. and Cattaneo, M. (1982) J. Appl. Polym. Sci. 25, 3493–3502.
[5] Chanzy, H., Henrissat, B., Vuong, R. and Schülein, M. (1983) FEBS Lett. 153, 113–118.
[6] Chanzy, H., Henrissat, B. and Vuong, R. (1984) FEBS Lett. 172, 193–197.
[7] Chanzy, H. and Henrissat, B. (1985) FEBS Lett. 184, 285–288.
[8] Henrissat, B. and Chanzy, H. (1986) in: Cellulose: Structure, Modification and Hydrolysis (Young, R.A. and Rowell, R.M. eds) pp. 337–347, Wiley, New York.
[9] Kulshreshtha, A.K. and Dweltz, N.E. (1973) J. Polym. Sci., Polym. Phys. Ed. 11, 487–497.
[10] Bourret, A., Chanzy, H. and Lazaro, R. (1972) Biopolymers 11, 893–898.
[11] Revol, J.F. (1982) Carbohydr. Polym. 2, 123–134.
[12] Gardner, K.H. and Blackwell, J. (1984) Biopolymers 13, 1975–2001.
[13] Sarko, A. and Muggli, R. (1974) Macromolecules 7, 486–494.
[14] Revol, J.F. and Goring, D.A.I. (1983) Polymer 24, 1547–1550.
[15] Sugiyama, J., Harada, H., Fujiyoshi, Y. and Uyeda, N. (1985) Planta 166, 161–168.
[16] Mackie, W., Sheldrick, B., Akrigg, D. and Pérez, S. (1986) Int. J. Biol. Macromol. 8, 43–51.
[17] Scaringe, R.P. and Pérez, S. (1987) J. Phys. Chem. 91, 2394–2403.
[18] Jones, T.A. (1978) J. Appl. Cryst. 11, 268–272.
[19] Takai, M., Hayashi, J., Nisizawa, K. and Kanda, T. (1983) J. Appl. Polym. Sci., Appl. Polym. Symp. 37, 345–361.
[20] Capon, B. (1969) Chem. Rev. 69, 407–498.
[21] Paice, M.G. and Jurasek, L. (1979) Adv. Chem. Ser. 181, 361–374.
[22] Wood, T.M. and McCrae, S.I. (1972) Biochem. J. 128, 1183–1192.
[23] Fägerstam, L.G. and Pettersson, L.G. (1980) FEBS Lett. 119, 97–100.

[24] Wood, T.M. (1985) Biochem. Soc. Trans., 407–410.
[25] Wood, T.M. and McCrae, S.I. (1986) Biochem. J. 234, 93–99.
[26] Castanon, M. and Wilke, C.R. (1980) Biotechnol. Bioeng. 22, 1037–1053.
[27] Ryu, D.D.Y. and Mandels, M. (1984) Biotechnol. Bioeng. 26, 488–496.
[28] Van Tilbeurgh, H., Tomme, P., Claeyssens, M. Bhikhabhai, R. and Pettersson, G. (1986) FEBS Lett. 204, 223–227.
[29] Chen, C.M., Gritzali, M. and Stafford, D.W. (1987) Bio/Technology 5, 274–278.