INDIGO-BINDING DOMAINS IN CELLULASE MOLECULES

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Study of cellulase adsorption on indigo particles and insoluble cellulose, as well as experiments on indigo interaction with immobilized amino acids together with theoretical analysis of three-dimensional structures of enzyme molecules, provided an evidence that certain cellulases, which have hydrophobic domains (clusters of closely located aromatic and non-polar residues) on their surface, may bind significant amounts of indigo and thus act as emulsifiers helping the dye to float out of cellulose fibers to the bulk solution in the process of enzymatic denim treatment. Only those cellulases, which had such indigo-binding domains, could efficiently remove indigo from the denim fabric providing high abrasive effects on the surface of the material.

Cellulases found applications as components of detergents, animal feed additives and as biocatalysts used for textile treatment [1]. In cotton textiles, the major cellulase applications are biopolishing the fabrics and denim garment treatment carried out to achieve the stonewashed look of the material [1, 2].

We have reported that the denim-washing performance of cellulases (both crude preparations and purified enzymes) differs dramatically [3, 4]. We have not observed any correlation between the washing performance of enzymes and their hydrolytic activity measured by traditional methods based on the analysis of reducing sugars. Even in those cases when different enzyme samples were equalized by cellulase activity, the abrasive effects induced on the surface of fabric by the enzyme action varied by several times. However, the reasons for some cellulases to demonstrate much higher abrasive activity remained unclear.

One of the problems arising in the process of enzymatic denim treatment may be the redeposition of indigo dye on the white yarn of the material (so called backstaining). Our previous studies indicated that the high adsorption ability of cellulases on cellulose is the major factor causing high indigo backstaining [5]. In this case, the basic mechanism of indigo redeposition should involve binding the dye to the enzyme molecules adsorbed on the surface of cellulose fibers. Such kind of mechanism implies that the enzyme molecules must have surface sites capable to bind indigo.

The aim of the present work was to study the interaction of indigo with several purified cellulases in order to find out if the higher ability to bind indigo may be responsible for higher denim-washing performance of certain cellulases.

Experimental

Enzymes. Endoglucanases (EG I and EG II) and cellobiohydrolases (CBH I and CBH II) were purified from *Trichoderma reesei* culture ultrafiltrate using chromatofocusing on a Mono P HR 5/20 column (Pharmacia, Sweden) as described previously [5]. Core domain (CD) of CBH I was obtained by limited proteolysis with papain [6]. Endoglucanases Eg3 and Eg5 were isolated from culture filtrates of *Penicillium verruculosum* and *Chrysosporium lucknowense*, respectively, using purification schemes described elsewhere [7, 8]. All enzymes were homogeneous according to the data of isoelectrofocusing and electrophoresis in polyacrylamide gels.

Abrasive activity (denim-washing performance) of purified cellulases was estimated using the model microassay [9]. The activity was defined as a difference in color intensity of denim swatches treated in the absence (buffer) and in the presence of enzyme, and it was expressed in arbitrary units per mg of protein. Protein was analyzed by the Lowry method [10] using bovine serum albumin as a standard.

Study of protein adsorption. 0.2 ml of indigo (Sojuzchimexport, Russia) or Avicel cellulose (Serva, Germany) suspension in distilled water (5 mg/ml) were added to 0.8 ml of enzyme solution (0.25 mg/ml) in 0.1 M acetate buffer, pH 5.0, and the mixture was agitated on a magnetic stirrer at room temperature for 30 min. Then, the suspension was centrifuged at 15 rpm for 4 min, and residual protein in supernatant was analyzed by the Lowry method [10]. Control experiments, where 0.8 ml of the acetate buffer without protein were mixed with 0.2 ml of the suspension, were also carried out since the indigo suspension gave some background absorbance at 750 nm, when analyzed with reagents used in the Lowry assay.

Study of indigo interaction with immobilized amino acids. Amino acids immobilized on cross-linked 4% beaded agarose from Sigma (USA) were used. CNBr-activated cross-linked agarose and cross-linked carboxymethyldextran (CM-Sephadex C-50) from Pharmacia (Sweden) were used as controls. Experiments on indigo incorporation in polysaccharide gels with immobilized amino acids were carried out as follows. Gels (0.2 ml) were placed into 2-ml test tubes and washed 3 times with 1.5 ml of water. Then, 1 ml of indigo colloidal suspension (0.5 mg/ml) in 0.01 M acetate buffer, pH 5.0, was added, and the mixture was agitated for 30 min on a magnetic stirrer. After

sedimentation of gel particles, liquid above the gel was removed, gel was washed two times with 1.5 ml of water for 5 min on a magnetic stirrer, and then indigo incorporated into the gel was extracted with DMSO. Three extractions with 1 ml of DMSO for 10 min on a magnetic stirrer were carried out. The absorbance of combined fraction of extracts (A_{620}) was measured, and then the amount of indigo incorporated into a gel was calculated from the extinction coefficient of indigo. For each amino acid, the experiment was carried out in duplicate.

Enzyme structure modeling. Three-dimensional structures of Eg3 and Eg5 were calculated using SWISS-MODEL protein modeling program available from the Swiss Institute of Bioinformatics via the Internet (http://www.expasy.ch) [11–13]. Five templates with 32–40% sequence identity were used for modeling the structure of Eg3. *Humicola insolens* EG V core domain (64% identity to Eg5) was used as a template for modeling the structure of Eg5. Analysis of cellulase surface residues was carried out using Swiss-PdbViewer (http://www.expasy.ch/spdbv/).

Results and Discussion

The adsorption of six purified cellulases on indigo and Avicel cellulose was studied (Fig. 1). In the case of CBH I from *T. reesei*, the adsorption of both the whole enzyme and its catalytic core domain (CD) without cellulose-binding domain (CBD) was measured. Albumin was used as a control in the adsorption study. As expected, only those cellulases, which had CBD, were adsorbed on cellulose to a notable extent (CBH I, CBH II, EG I, EG II). However, all proteins including albumin were capable to bind to indigo. Much higher amount of protein was bound to indigo particles than to cellulose in all cases. The quantity of bound protein varied from practically zero to 62 μg/mg carrier in the case of adsorption on cellulose, and from 57 to 111 μg/mg in the case of adsorption on indigo particles. These data indicate that, although protein adsorption on indigo is much less specific than that on cellulose, different proteins have different affinity to the dye.

It has been reported that aromatic amino acid residues (Tyr, Trp, and Phe) in cellulase CBDs play a pivotal role
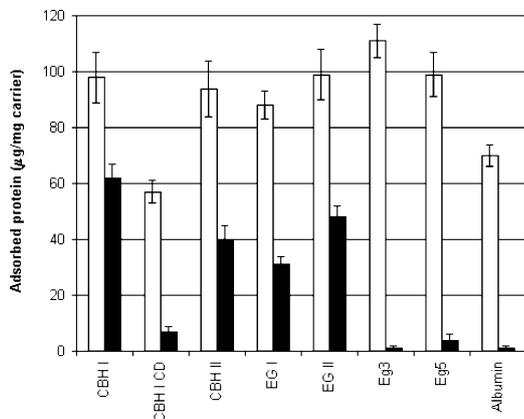

Fig. 1. Adsorption of purified cellulases on indigo (left bars) and Avicel cellulose (right bars).

in protein-cellulose interactions [14, 15]. For example, three Tyr residues in the CBD of CBH I form a planar strip which stack on glucose rings in a cellulose chain [14]. Since after removing the CBD CBH I not only almost completely lost its ability to bind to cellulose but also the ability to bind to indigo to a significant degree (Fig. 1), the same aromatic residues seem to play a major role in binding indigo. Indigo is a hydrophobic compound (insoluble in water), and its aromatic rings may interact with the aromatic rings of Tyr, Trp and Phe, as well as with side chains of other non-polar amino acids, via the hydrophobic interactions. Another mechanism of indigo binding may involve forming the hydrogen bonds between protein amino acid residues and NH and =O groups of the dye molecule.

In order to find out if non-polar side chains of amino acids may bind indigo, interaction of indigo with several amino acids immobilized on cross-linked agarose was studied (Table 1). CNBr-activated 4% beaded agarose, treated with monoethanolamine to remove active groups of the matrix, was used as a control, since most of the amino acids were immobilized on CNBr-activated carrier. Since all the amino acids were immobilized via the α-amino group and they contained free carboxyl group, in order to find out how carboxyl groups affect the penetration of indigo molecules into polysaccharide-based gels, cross-linked carboxymethyldextran was used as a second control.

Table 1

Interaction of indigo with amino acids immobilized on cross-linked 4% beaded agarose

Amino acid / carrier	Indigo bound (μg)	Hydrophobicity index ΔG-Miller (kcal/mol)*
CNBr-activated agarose**	137 ± 2	
Carboxymethyldextran	35 ± 1	
Gly/CNBr-activated agarose	77 ± 3	-0.06
Trp/CNBr-activated agarose	84 ± 1	-0.45
Leu/CNBr-activated agarose	94 ± 7	-0.65
Phe/CNBr-activated agarose	120 ± 8	-0.67
Tyr/Epoxy-activated agarose	154 ± 16	0.22

* An empirical measure of hydrophobicity of side chains introduced by Miller *et al.* [16]. More hydrophobic side chain gives more negative value.
** Gel was treated with 1 M monoethanolamine at pH 9 for 2 h to block active groups and then washed 4 times with water.

Data presented in Table 1 indicate that cross-linked agarose itself (control 1) accumulated indigo. The polysaccharide matrix, less polar than water and rich in OH groups capable to form hydrogen bonds, seems to be more advantageous environment for the dye than the water phase outside of gel particles. However, when carboxyl groups were introduced into the polysaccharide matrix (control 2), the lowest amount of indigo was incorporated in gel indicating that charged groups negatively affect the indigo binding. So, when amino acids, covalently immobilized in polysaccharide gels, were used, two opposite tendencies took place. On the one hand, free carboxyl groups of immobilized amino acids affected negatively the accumulation of indigo in gels. On the other hand, side chains of the amino acids could bind indigo positively affecting the dye incorpo-

ration. As expected, the amount of indigo incorporated in a gel increased with the increase in the hydrophobicity of side chains in the following order: Gly < Trp < Leu < Phe. Rather unexpectedly, the highest level of bound indigo provided Tyr-agarose gel. Although Tyr contains the aromatic ring in a side chain, its hydrophobicity is lower than that of other amino acids tested. Perhaps, the hydrogen bond formation between the hydroxyl group of Tyr and =O group of indigo, combined with the possibility of hydrophobic interactions between the aromatic structures of the molecules, provided the highest indigo binding effect.

Then, using Swiss-PdbViewer program, we carried out visual analysis of surface hydrophobic amino acid residues (exposed to solvent) in cellulase molecules. Three-dimensional structures of CBH I, CBH II and EG I core catalytic domains from *T. reesei* were taken from Brookhaven Protein Data Bank (http://www.pdb.bnl.gov). The structures of Eg3 and Eg5 were calculated using programs for protein modeling available via the Internet. Since it is problematic whether residues inside the enzyme active site cleft (tunnel) may be accessible to indigo molecules, only surface residues exposed to solvent out of the active site were considered. The results of the analysis are summarized in Table 2. Specific abrasive activities (washing performance) of the enzymes are also given in the table.

Table 2

Abrasive activities and aromatic (Tyr + Phe + Trp) or overall non-polar (Tyr + Phe + Trp + Val + Leu + Ile + Pro + Met) amino acid residues on the surface of cellulase molecules

Enzyme	Abrasive activity (units/mg)	Q-ty of aromatic residues	% of surface residues from the total	
			aromatic	non-polar
CBH I	6	5	1.2	5.8
CBH II	16	6	1.7	11.8
EG I	21	6	1.6	9.7
Eg3	107	11	5.0	13.1
Eg5	70	7	3.4	13.2

Analyzing data presented in Table 2, one may conclude that a certain relationship exists between the washing performance of enzymes and (i) quantity (percentage) of aromatic amino acid side chains exposed to solvent on the surface of protein globules or (ii) overall percentage of the surface non-polar residues. Two enzymes (Eg3 and Eg5) had higher content of both aromatic (Tyr+Phe+Trp) and overall non-polar (Tyr+Phe+Trp+Val+Leu+Ile+Pro+Met) residues, and only these enzymes could remove indigo from the denim fabric efficiently. The abrasive activity of Eg3 and Eg5 was 107 and 70 units/mg, respectively, whereas the activity of other enzymes was in the range of 6–21 units/mg. Aromatic side chains seem to be more important for indigo binding since the difference in (Tyr + Phe + Trp) content between the first (CBH I, CBH II, EG I) and second (Eg3 and Eg5) group of enzymes was more distinct than the difference in (Tyr + Phe + Trp + Val + Leu + Ile + Pro + Met) content.

Together with the general high content of non-polar amino acid residues exposed to solvent, a very important factor may be the position of these residues on the surface of

enzyme globule. An example of cellulase three-dimensional structure (Eg3 model) is shown in Fig. 2. Some of the essential aromatic residues are marked. One may discriminate at least two domains (clusters of closely located aromatic residues) on the surface of enzyme, where indigo molecules and aggregates could be bound. One domain, formed by Tyr-9, Trp-24, Tyr-114, and Tyr-118, is located at the bottom of enzyme molecule. Another domain, formed by Tyr-151, Phe-188, together with several other non-polar residues (which are not marked), can be seen on the right side of the enzyme. Since four aromatic residues are located close to the enzyme active site cleft (two residues on each side of the cleft), they may play an important role also in primary binding of the enzyme to cellulose. Eg3 has no CBD, so the same aromatic residues (Tyr and Trp), which are important for the adsorption of CBDs on cellulose [14, 15], may be responsible for the initial (weak) protein-cellulose interaction and enzyme orientation on cellulose fibers.
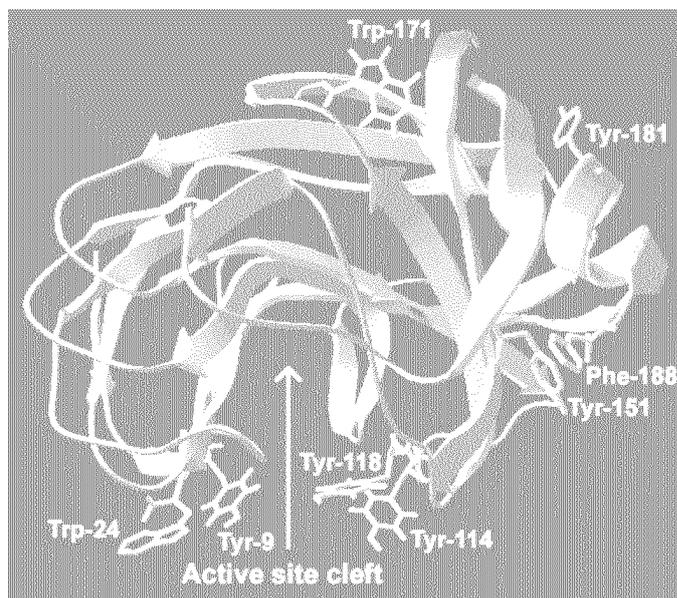

Fig. 2. Ribbon model structure of Eg3 from *P. verruculosum*.

Data obtained provide an evidence that the molecules of certain cellulases, which have hydrophobic domains (clusters of closely located aromatic and non-polar residues) on their surface, may bind significant amounts of indigo and thus act as emulsifiers helping the dye to float out of cellulose fibers to the bulk solution in the process of denim treatment. Only those cellulases, which had such indigo-binding domains on the surface of protein globule, could efficiently remove the dye from the denim fabric.

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