Substrate-binding domains of glycanases from *Streptomyces lividans*: characterization of a new family of xylan-binding domains

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The substrate-binding domains of six glycanases from *Streptomyces lividans* were investigated to determine their specificity towards cellulose and xylan. Based upon amino acid sequence similarities, four of the six domains could be assigned to existing cellulose-binding domain families. However, the binding domains

of xylanase A and arabinofuranosidase B could not be classified in any of the known families and should therefore be classified as members of a new family. Evidence is also presented that this new family is one of true xylan-binding domains.

INTRODUCTION

The complete degradation of polymeric substrates such as xylan and cellulose requires the action of several glycanases and, in some cases, of debranching enzymes. Such enzymes are produced by many lignocellulolytic microorganisms, both prokaryotic and eukaryotic. Nine enzymes involved in the degradation of cellulose and hemicellulose by *Streptomyces lividans* have been cloned and characterized. These are xylanases A, B, C (XlnA: #322292, XlnB: #322293, and XlnC: #541495) [1–3], cellulases A and B (CelA: #121809, CelB: #439694) [4,5], arabinofuranosidases A and B (AbfA: #1703047, AbfB: #1850908) [6,7], mannanase A (ManA: #421656) [8] and acetylxylan esterase A (AxeA: #2055282) [9]. [The accession numbers given are from the National Centre for Biotechnology Information (NCBI) data-bank.] Six of these share a modular organization, consisting of a catalytic domain linked to a substrate-binding domain, found in many glycanases. Based on amino acid sequence analyses, these substrate-binding domains were often identified as cellulose-binding domains (CBDs), whether or not they are associated with an endoglucanase. Recently, it was found that some xylan-degrading enzymes, such as xylanases and acetylxylan esterases, have binding domains that are specific for xylan but not for cellulose [9,10] and are therefore true xylan-binding domains (XBDs). To date, over 120 domains have been identified by amino acid sequence similarities and classified into ten different families of CBDs [11].

The general function of these substrate-binding domains is to allow proper alignment of the soluble enzyme with the insoluble polysaccharide, thus increasing enzyme concentration at the point of enzymic attack. Nevertheless, they are not essential for hydrolysis of the polymeric substrate, and some of the polysaccharide substrate-binding domains, identified by sequence similarities, are even associated with enzymes that are not glycanases. So far, their role has not been clearly defined [11].

In this paper we investigate the specificity of the different substrate-binding domains found in cellulases and hemicellulases from *S. lividans* and propose a new family to be added to the existing classification of Tomme et al. [11].

MATERIALS AND METHODS

Insoluble substrates

Insoluble oat spelt xylan was prepared by suspending 35 g of oat spelt xylan (Sigma) in 350 ml of distilled water which was boiled for 1 h and centrifuged for 30 min at 9000 *g* at 4 °C. The sediment was dried with acetone and kept at room temperature. Cellulose Avicel® PH-105 (FMC Corporation) was extensively washed with distilled water and dried with acetone.

Enzyme purification

The *S. lividans* enzymes CelB, AxeA and AbfB were produced and purified as previously described [2,5,7,9]. XlnA₁ [12], its truncated form XlnA₂ [13] and XlnB [2] were produced as described in the respective references, and purified according to the method of Roberge et al. [14]. The XBD from XlnA₁ of *S. lividans* was purified by solubilizing the ammonium sulphate precipitate from a culture filtrate in 20 mM Tris/HCl buffer, pH 8.5, and extensively dialysing at 4 °C in the same buffer, with a SpectraPor (Spectrum) dialysis membrane having a molecular-mass cut-off of 6–8 kDa. The dialysed fraction was filtered through a 0.45 µm membrane and loaded on an AP2 DEAE 15 HR column (20 × 100 mm; Waters). The fractions containing the 17 kDa fragment were pooled, extensively dialysed against Milli-Q water and lyophilized. The powder was then solubilized in 100 mM sodium phosphate buffer, pH 7.0, at a concentration of 16 mg/ml and filtered through a 0.45 µm membrane. Final purification was obtained by separation on a Superdex HR75 beaded column (3 × 60 cm; Pharmacia) with 100 mM sodium phosphate, pH 6.0, as the eluent.

Substrate-binding assays

Substrate binding was determined by suspending 10 mg of the insoluble polysaccharide in 500 µl of 25 mM sodium phosphate buffer, pH 7.0, containing 50 mM NaCl and 5 IU of enzyme. The mixture was kept under constant agitation in an ice-bath for periods of up to 15 min. At intervals, an Eppendorf tube containing the reaction mixture was withdrawn and centrifuged

Abbreviations used: AbfB, α-L-arabinofuranosidase; AxeA, acetylxylan esterase; CBD, cellulose-binding domain; CelB, cellulase B; DTT, dithiothreitol; PAHBAH, *p*-hydroxybenzoic acid hydrazide; XBD, xylan-binding domain; XlnA₁, XlnB₁, xylanase A₁, B₁ with their binding domains; XlnA₂, XlnB₂, catalytic domain of xylanase A₁, B₁.

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at maximum speed (16000 g) for 2 min. The supernatants were kept frozen at -20°C until determination of the residual enzyme activity.

In order to exclude non-specific binding of proteins, the substrates were pre-treated in the same manner with 10 mg of a non-hydrolytic protein having a pI similar to that of the glycanase being investigated. After treatment, the substrates were washed three times with sodium phosphate buffer containing NaCl (pH 7) and immediately used for the binding assay. The following proteins were used: trypsinogen, pI 9.3, for XlnB₁, B₂ and AxeA; glucose oxidase, pI 4.2, for CelB; human haemoglobin, pI 7.1, for AbfB; and β -lactoglobulin, pI 5.2, for XlnA₁, A₂. All these proteins were purchased from Sigma.

Enzyme assays

Enzyme activities were determined by the *p*-hydroxybenzoic acid hydrazide method (PAHBAH, ICN Biochemicals) [15], using the following substrates (all from Sigma) at a concentration of 1% (w/v): birchwood xylan for XlnA₁, A₂ and XlnB₁, B₂; carboxymethylcellulose (low viscosity) for CelB; and oat spelt xylan for AbfB. Fully acetylated birchwood xylan, prepared according to the method of Johnson et al. [16], was used for AxeA assays. The reaction mixtures, composed of 900 μl of the appropriate substrate in buffer solution with 100 μl of supernatant, were incubated for 10 min at 60°C in the cases of XlnA₁, A₂ and XlnB₁, B₂. For CelB and Abf, incubation was at 55°C . The reaction was stopped by adding 100 μl of the reaction mixture to 300 μl of a 0.25% (w/v) PAHBAH solution in 0.5 M NaOH and by heating at 95°C for 5 min. After cooling in an ice-bath, the released reducing sugars were determined by spectrophotometry at 450 nm in an ELISA plate reader. In the case of AxeA, activity was determined as previously described [9], except that the released acetic acid was measured at 210 nm with a UV detector. Enzyme activities are expressed in international units (IU), where one unit corresponds to the amount of enzyme that releases 1 μmol of product per min.

Free thiol determination

Free thiol determination for the *S. lividans* XBD was performed with Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] (Sigma) [17] using 3.5 nmol of protein in 100 mM phosphate buffer, pH 8.0, in the absence and presence of 6 M guanidine hydrochloride. Thiol concentration was calculated from the molar absorbance of the 2-nitro-5-thiobenzoic acid anion at 412 nm with a Carry 118 spectrophotometer. ϵ_{412} values of

1.415×10^4 and $1.37 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ were used for XBD in phosphate buffer and in phosphate buffer containing 6 M guanidine hydrochloride respectively [18].

Protein analysis

Protein purity was assessed by SDS/PAGE [19] and Western blot analysis with antibodies raised against the different enzymes [20], and the protein content of the enzyme preparations was determined according to the method of Lowry et al. [21] with BSA fraction V (Bio-Rad) as standard. Analytical SDS/PAGE of XBDs was performed with a 16.5% T/3% C Tris/tricine system [22], where *T* represents the total concentration of monomer and *C* is the percentage of total monomer *T* which is due to the cross-linking agent. XBD samples (4 μg) were reduced with 50 mM dithiothreitol (DTT) in 400 mM Tris/HCl buffer, pH 8.0, at 40°C for 60 min. For alkylated samples, 25 mM iodoacetamide was added and the reaction tubes were protected from light.

RESULTS AND DISCUSSION

Sequence similarity

According to the recent classification of CBDs by Tomme et al. [11], the amino acid sequences of the substrate-binding domains found in the glycanases of *S. lividans* allow classification into two groups. The binding domains of the two cellulases, CelA and CelB, are typical for binding to cellulose and belong to subfamily IIa of the CBDs. Those of AxeA and XlnB₁ can be classified into subfamily IIb, whereas the binding domains of XlnA₁ and AbfB cannot be attributed to any of the existing families. Analysis of their primary binding-domain sequences by the BLAST algorithm [23] showed a high degree of similarity to several proteins listed in the GenBank Database. Figure 1 shows an alignment of the proteins with a high percentage of similarity. Other putative binding domains were found for Xln II, a xylanase belonging to the family 10 glycanases produced by an *Actinomyces* sp. (GenBank accession number U08894), and for two β -1,3-glucanases, one from *Arthrobacter* (GlcI) (GenBank accession number D23668) and the other from *Oerskovia xanthineolytica* (GluI) [24], as well as for a protease from *Rarobacter faecitabidus* [25]. The consensus deduced from the alignment in Figure 1 is clearly different from all those reported by Tomme et al. [11]. This finding justifies the introduction of a novel family of glycan-binding domains. With the exception of AbfB, the proteins in this new family have their binding domains

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1  XlnA      nggdssseppadgggqikgvgsgrCldVpdastsDgtqlQlwdChsgtnQqwaatdagelrvyGdKCLDaaG
2  AbfB      .....agsnrCldVlgsqDgallQlydCwggtnQqwtstdtgrltvyGdKCLDvpg
3  XlnII     .gtsepppasdagtikgvgsgrCldVpnastsDgvqlQlwdChggtnQqwtysdsqelrvyGnKCLDaaG
4  GlcI/GluI qpptpgglpagtggalrigstlCldVpwadptDtnqvQlatCsgnaaQqwtgrtgdgtvralG.KCLDvar
5  RPI       wklsltvtstdvttsyvqgyqnnCldVpnsdftDgkqlQvwnCngtnaQkvsfhpdgltlrinG.KCLDdarw
6  Consensus -----C-DV-----D---Q---C-----Q-----G-KCLD---

7  XlnA      ..TsnGskVqiysCwGgdnQkWrlnsdgsvv.gvqsglCldDavngt.anGtliQLYtCsngsnQrWtrt
8  AbfB      haTapGtrVqiwsCsGgrnQqWrnsdgtvv.gvesglCldEaagagt.pnGtavQLWtCnggggQkWtgl
9  XlnII     ..TgnGtkVqi
10 GlcI/GluI sgTadGtaVwiytCnGtgaQkWtydsatkalrnpqsgkCldDaqggaplrdGqkvQLWtCnqteaQrWtl.
11 RPI       awThnGteVqlmnCnGhiaQkFtlngagdlv.nvhankCvDvkdwgg..qGgklQLWecsgganQkWwrr
12 Consensus --T--G--V---C-G---Q-W-----C-D-----G---QLW-C---Q-W---
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Figure 1 Amino acid alignment of substrate-binding domains of *S. lividans* XlnA and AbfB and putative binding domains from a xylanase of *Actinomyces* sp. XlnII, β -1,3-glucanases of *Arthrobacter* sp. (GlcI) and *Oerskovia xanthineolytica* (GluI) and from a protease of *Rarobacter faecitabidus* (Rpi)

Figure 2 Digitalized image of SDS/PAGE of purified *S. lividans* XBD

Lane 1, molecular-mass standards; lane 2, XBD reduced with DTT; lane 3, XBD non-reduced; lane 4, XBD reduced with DTT and alkylated with iodoacetamide; lane 5, molecular-mass standards.

located at the C-terminus of the molecule. The presence of six cysteine residues in all of them suggests an important structural feature. This arrangement has also been found in some, but not all, CBD families. In order to verify whether these cysteines form disulphide bridges, the binding domain of XlnA₁ was purified from a culture filtrate of *S. lividans* IAF18 and the presence of free thiol groups was investigated with Ellman's reagent under native and denaturing conditions. In both cases, no free thiol groups could be detected, indicating that all six cysteine residues are involved in disulphide bridges (results not shown). Furthermore, SDS/PAGE analysis of the reduced and non-reduced XBDs showed different electrophoretic mobilities, supporting the conclusion that these residues are involved in disulphide bridges (Figure 2).

As the binding domains found in both XlnA₁ and AbfB of *S. lividans* were different from those of known CBD families, we postulated the existence of a specific XBD. To verify our hypothesis, binding studies were carried out on Avicel and on insoluble oat spelt xylan with all the cellulases and hemicellulases of *S. lividans*. So far, studies on the binding of cellulases and

xylanases reported in the literature have only been done with substrates such as Avicel or bacterial microcrystalline cellulose.

Binding assay on insoluble substrates

A comparison of the substrate-binding capabilities of both XlnA₁ and AbfB (Figures 3A and 3B) with those of XlnB₁ and AxeA (Figures 4A and 4B) clearly showed distinct absorption patterns, thus confirming their classification into different families of glycan-binding domains. This is not surprising, since the amino acid sequences of the binding domains are almost identical in each of these pairs of enzymes. Figures 3(A) and 3(B) show the results obtained with XlnA₁ and AbfB, where binding to insoluble xylan was almost immediate (within less than 1 min of incubation) and remained unaffected for more than 15 min. Moreover, neither of these enzymes adsorbed to Avicel pre-treated with non-hydrolysing protein, whereas xylan, prepared in the same manner, fully retained the enzymes. This behaviour clearly demonstrates the specific binding of XlnA₁ and AbfB to their natural substrate. Further evidence of the importance of the XBD in the enzyme's interaction with its substrate was shown by binding assays carried out with XlnA₂, the truncated form containing only the catalytic domain. While this protein is adsorbed to a significantly lesser degree by the untreated xylan, the association is reduced by pre-treatment with a non-specific protein, such as β -lactoglobulin, and competition with twice the amount of β -lactoglobulin still further decreases the substrate association of XlnA₂ (results not shown).

To our knowledge, the only other reported specific XBD is that of XylD, a xylanase produced by *Cellulomonas fimi* which also contains a CBD [10]. The XBD of this enzyme shows significant similarity to the binding domains of XlnB and AxeA of *S. lividans* and has been classified into subfamily IIB of CBDs [11]. Therefore, similar substrate-binding studies were carried out with AxeA and XlnB₁ and the results are shown in Figure 4. The substrate-binding domains of these enzymes showed a clear preference for insoluble xylan. They rapidly attached to this substrate and remained so during the entire course of the experiment. When mixed with Avicel, about 50–60 % of AxeA was bound within 10 min, while XlnB₁ adsorbed immediately. However, upon pre-treatment of the cellulose with trypsinogen, an unspecific and non-hydrolytic protein, both hemicellulases failed to attach, in a significant manner, to the substrate. This

Figure 3 Relative activities of (A) AbfB, (B) XlnA₁ and (C) XlnA₂ associated with insoluble substrate after incubation at 4 °C

Enzymes were incubated in the presence of: ●, no substrate; ■, insoluble xylan; ◆, insoluble xylan saturated with non-hydrolytic protein as described in the Materials and methods section; ▲, Avicel; ▼, Avicel saturated with non-hydrolytic protein as described in the Materials and methods section.

Figure 4 Relative activities of (A) AxeA, (B) XlnB₁ and (C) XlnB₂ associated with insoluble substrate after incubation at 4 °C

Enzymes were incubated in the presence of : ●, no substrate; ■, insoluble xylan; ◆, insoluble xylan saturated with non-hydrolytic protein as described in the Materials and methods section; ▲, Avicel; ▼, Avicel saturated with non-hydrolytic protein as described in Materials and methods section.

Figure 5 Relative activity of CelB associated with insoluble substrate after incubation at 4 °C

Enzyme incubated in the presence of : ●, no substrate; ■, insoluble xylan; ◆, insoluble xylan saturated with non-hydrolytic protein as described in the Materials and methods section; ▲, Avicel; ▼, Avicel saturated with non-hydrolytic protein as described in the Materials and methods section.

shows that neither AxeA nor XlnB₁ possess a significant affinity for cellulose. The same pre-treatment of insoluble xylan did not affect the binding of either XlnB₁ or AxeA to this substrate. This is proof that attachment to substrates is due to specific interactions inherent in the binding domains of the enzymes. We previously reported that the catalytic domain of AxeA alone, without its substrate-binding domain, does not associate with either xylan or cellulose [9]. The same result was obtained with the XlnB₂ catalytic domain (Figure 4C) and similar results have been reported for two other xylanases harbouring subfamily IIb CBDs. Thus, XylD of *C. fimi* does not attach to either cellulose or xylan without its two binding domains [10], whereas the N-terminal fragment of XynD from *Thermomonospora fusca*

shows little affinity for xylan [26]. In the latter case, the low affinity is likely to be due to non-specific adsorption to the substrate. A similar result was obtained with AxeA, which has no catalytic activity towards either xylan or cellulose (Figure 4A). Therefore, substrate-binding by XlnB₁ and AxeA of *S. lividans* is due to specific CBDs, without affinity for cellulose. This is also the case for XylD from *C. fimi* [10] and it is in contrast with the other two xylanases containing domains that are classified in subfamily IIb, which are reported to attach to cellulose [25]. However, since the substrate had not been pre-treated in either case, it is possible that binding to cellulose was the result of non-specific interactions. Should this be the case, family II would have to be re-defined as a family of CBDs and of XBDs. Another example of this distinction can be deduced from the binding studies carried out with CelB of *S. lividans*, a representative of CBD subfamily IIa. Results clearly show that CelB does not bind to insoluble xylan, but attaches almost instantly to both the saturated and the unsaturated Avicel (Figure 5). This is behaviour consistent with that of a true, specific CBD and is characteristic for subfamily IIa CBDs [11,27].

In this study we have shown that only the catalytic domains of the hemicellulases produced by *S. lividans* do not bind specifically to their substrate (Figures 3 and 4). Therefore, the observed difference in specificity between subfamilies IIa and IIb arises from differences in the substrate-binding domains themselves. We suggest that enzymes belonging to subfamily IIa are true CBDs and those in subfamily IIb are true XBDs, and not dual CBD/XBDs. The differences in primary structures between subfamily IIa and IIb are subtle. The main differences reside in two small deletions of nine and six amino acids at the C-terminal of the binding domains for subfamily IIb [11]. In the light of the results presented here, these two small stretches of amino acids appear to be responsible for the different substrate specificity of the two subfamilies, but this will have to be verified experimentally in future studies.

In conclusion, the evidence presented here justifies the addition of a new family, family XI, of substrate-binding domains to the existing classification of Tomme et al. [11], and shows that the members of this new family are specific XBDs. Furthermore, we demonstrated that subfamily IIb, in its present form, is composed of binding domains with distinct specificity towards xylan.

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