Novel sugar-binding specificity of the type XIII xylan-binding domain of a family F/10 xylanase from *Streptomyces olivaceoviridis* E-86

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Received 7 August 2000; revised 11 September 2000; accepted 12 September 2000

Edited by Takashi Gojobori

Abstract The type XIII xylan-binding domain (XBD) of a family F/10 xylanase (FXYN) from *Streptomyces olivaceoviridis* E-86 was found to be structurally similar to the ricin B chain which recognizes the non-reducing end of galactose and specifically binds to galactose containing sugars. The crystal structure of XBD [Fujimoto, Z. et al. (2000) J. Mol. Biol. 300, 575–585] indicated that the whole structure of XBD is very similar to the ricin B chain and the amino acids which form the galactose-binding sites are highly conserved between the XBD and the ricin B chain. However, our investigation of the binding abilities of wt FXYN and its truncated mutants towards xylan demonstrated that the XBD bound xylose-based polysaccharides. Moreover, it was found that the sugar-binding unit of the XBD was a trimer, which was demonstrated in a releasing assay using sugar ranging in size from xylose to xyloheptaose. These results indicated that the binding specificity of the XBD was different from those of the same family lectins such as the ricin B chain. Somewhat surprisingly, it was found that lactose could release the XBD from insoluble xylan to a level half of that observed for xylobiose, indicating that the XBD also possessed the same galactose recognition site as the ricin B chain. It appears that the sugar-binding pocket of the XBD has evolved from the ancient ricin super family lectins to bind additional sugar targets, resulting in the differences observed in the sugar-binding specificities between the lectin group (containing the ricin B chain) and the enzyme group.

Key words: Type XIII xylan-binding domain; Carbohydrate-binding motif; Ricin super family; Xylanase; *Streptomyces olivaceoviridis*

1. Introduction

β-Xylanase (EC 3.2.1.8) hydrolyzes β-1,4-glycosidic linkages within the xylan backbone to yield short-chain xylo-oligosaccharides of varying length. Interest in the application of xylanases in the pulp and paper industry has increased in recent years. Xylanases are used in the pulp-prebleaching process to remove hemicelluloses, which bind to the pulp. The hydrolysis of pulp-bound hemicellulose releases the lignin in the pulp, reducing the amount of chlorine required by conventional chemical bleaching, thus minimizing the output of toxic, chloro-organic waste. In general, xylanases are composed of a catalytic domain, and are divided into two families (F/10 and G/11) based on amino acid sequence similarities [1], and they also have several functional domains, most of which are carbohydrate-binding domains [2]. These carbohydrate-binding domains bind to the polysaccharides in plant cell walls and are important for the efficient hydrolysis of cellulosic substances.

A family F/10 xylanase (FXYN; 45 kDa) was previously purified from *Streptomyces olivaceoviridis* E-86 and the substrate specificity of the enzyme was well characterized [3,4]. We have recently succeeded in crystallizing the intact FXYN and have isolated the xylanase gene, which consists of a family F/10 catalytic domain and a type XIII xylan-binding domain (XBD) [5,6]. Most recently, the crystal structure of FXYN including XBD was identified at 1.9 Å resolution [7]. According to an analysis of amino acid similarities and structural data, it was suggested that the XBD folds in the same manner as the ricin toxin B chain (RTB), a member of the 'ricin super family' lectins which binds specifically to galactose [8]. The 'ricin super family' lectins are classified into two groups; the lectin group (containing RTB) and the enzyme group, as described by Hirabayashi et al. [8]. Closer examination of the RTB structure revealed that most of the members of the lectin group consist of two domains and each domain is divided into three homologous subdomains; α, β and γ, with ~40 amino acid residues comprising each core peptide [8–11]. Notably, all members of the lectin group are known to bind specifically to galactose/*N*-acetylgalactosamine except for the *Streptomyces sieboldina* agglutinin, and both of domains possess carbohydrate-binding activity which serves to increase the binding specificity [8]. In comparison, members of the enzyme group of proteins such as the type XIII XBD possess only one domain, which consists of the same three subdomains (α, β and γ) as the lectin group proteins, but their carbohydrate-binding properties have not been reported in the literature to date. Therefore, determinations of the structure–function relationships of the enzyme group proteins are of considerable interest.

Recently, we reported on the mechanistic aspects of the catalytic domain of the FXYN [12–14], but to date there have been no reports on the XBD of FXYN. In this paper,

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PII: S0014-5793(00)02067-6

we focus on the characterization of the type XIII XBD that is located in the C-terminus of the catalytic domain of the FXYN from *S. olivaceoviridis*. To our knowledge, this is the first report that deals with the detailed biochemical characterization of the type XIII XBD. We demonstrate that the sugar-binding unit of the XBD is a trimer, a novel finding for a member of the lectin family.

2. Materials and methods

2.1. Production and purification of truncated FXYN derivatives

To express FXYN and its truncated derivatives (Cat, ΔXBD1 and ΔXBD2), the appropriate DNA sequences were amplified by the polymerase chain reaction (PCR) (see Fig. 1) and the resulting fragments were cloned into the pQE60 vector (Qiagen GmbH, Hilden, Germany) as described previously [6]. Each DNA fragment was amplified using a set of primers which included either the *Nco*I or the *Bam*HI restriction sites, enabling the PCR products to be cloned into *Nco*I/*Bam*HI-restricted pQE60. Full-length and truncated derivatives of FXYN with fused 6×His tags at the C-terminus were expressed and purified from cell-free extracts using a HisTrap chelating column (Pharmacia, Uppsala, Sweden) as described previously [6]. Elution of the enzyme was monitored by SDS-PAGE [15]. A single enzyme peak was detected and corresponding fractions were pooled and concentrated using a Centriprep (Amicon, Inc., Beverly, MA, USA) to give a final volume of 1.0 ml. The concentrated solution was loaded on a HiTrap desalting column (Pharmacia) to remove imidazole.

2.2. Enzyme assay

All enzyme activities were measured at 45°C in 80 mM sodium phosphate buffer, pH 5.7 with 1 mM *p*-nitrophenyl-β-D-xylobioside (pNP-X_2) as the substrate. pNP-X_2 was synthesized from xylobiose, which was purified from a xylobiose mixture (Suntory Ltd., Osaka, Japan), as described by Takeo et al. [16].

2.3. Hydrolysis of insoluble oat spelt xylan

Insoluble xylan was prepared from oat spelt xylan (Fluka) as described previously [6]. 50 g of oat spelt xylan were suspended in 500 ml of distilled water, microwaved for 5 min, and centrifuged for 30 min at 9000 rpm at 4°C. After centrifugation, the supernatant was lyophilized and kept at room temperature (soluble xylan). The precipitate after centrifugation was washed three times with distilled water and lyophilized (insoluble xylan). 500 μl of a reaction mixture, consisting of insoluble oat spelt xylan (2 or 10 mg/ml) in McIlvaine buffer (pH 7.0) containing 0.1% BSA and enzyme (0.01 mg/ml), was incubated at 30°C for 1, 2, 3, 6, 10, or 24 h. The concentration of reducing power generated from the presence of xylan was determined as described by Somogyi-Nelson [17].

2.4. Binding assay of FXYN and its truncated derivatives

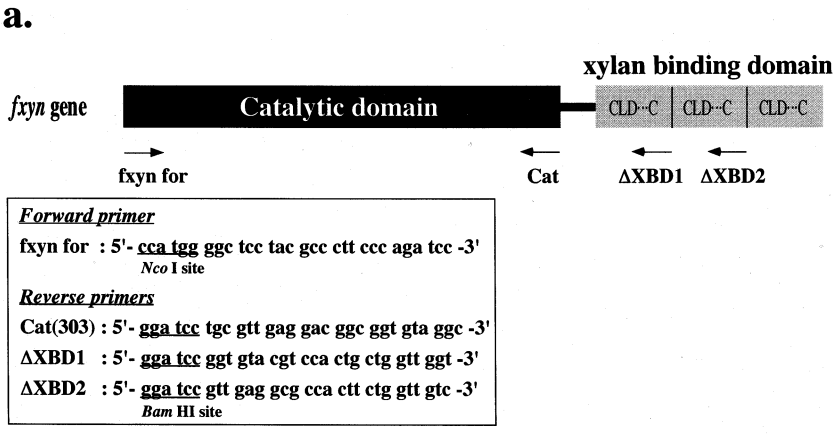
The binding abilities of the purified FXYN, and its truncated derivatives toward insoluble xylan were measured as follows: 5 μl of purified enzyme (1.0 mg/ml or 0.1 mg/ml) was mixed with 45 μl of polysaccharide solution, containing insoluble xylan at various concentrations, in 80 mM sodium phosphate buffer (pH 5.7) and 0.1% BSA, at 4°C for 10 min and then centrifuged to remove the insoluble xylan–xylanase complex. Unbound enzyme was quantified by measuring residual activity in the supernatant. The binding ability of an enzyme was defined as follows:

$$[ES]/[E]_0 = -B_{1/2}/(B_{1/2} + S) + 1$$

$B_{1/2}$: the amount of insoluble xylan added to bind half of the enzyme.

2.5. FXYN releasing assay

To determine the amount of FXYN released from insoluble xylan by the addition of several different mono-, di- and oligosaccharides: xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4) (Megazyme Ltd), xylose (X_1), glucose, galactose, maltose, sucrose (Wako Pure Chemical Industries, Osaka, Japan), lactose, cellobiose (Sigma Chemical Co., USA), xylopentaose (X_5), xylohexaose (X_6), and xyloheptaose (X_7), releasing assays were performed as follows. 5 μl of purified



b.

XBD	α	317	GV-GSGRCLDVPNASTTDGTQVQLYDCH--SATNQQWTYTDA	355
	β	360	-V-YGDKCLDAA--GTGNGTKVQIYSCW--GGDNQKWRLNSD	395
	γ	400	GV-QSGLCLDAVGGGTANGTLIQLYSCS--NGSNQRWTRT	436
Ricin B1α☆		15	G--RNGLCVDVRDGRFHNGNAIQLWPCKSNTDANQLWTLKRD	54
	1β☆	59	S--NG-KCLTTY--GYSPGVYVMIYDCNTAATDATRWQIWDN	95
	1γ	100	NP-RSSLVLAAT--SGNSGTTLTVQTNI--YAVSQGWLPTNN	136
Ricin B2α		146	G--LYGLCLQAN--S----GQVWIEDCSS-EKAEQQWALYAD	178
	2β	183	PQQNRDNCLTS-DSN-IRETVVKILSCGPA-SSGQRWMFKND	221
	2γ☆	226	NL-YSGLVLDVRA-SDPSLKQIILYPLH--GDPNQIWLPLF	136

Fig. 1. a: Structure of wt FXYN and its truncated derivatives are shown as follows: black, catalytic domain; bold line, linker region; gray, XBD. The primers used for amplification of DNA fragments containing FXYN truncated derivatives are shown as arrows. b: Topological sequence alignment of the XBD with the ricin B-chain. The amino acid residues corresponding to the sugar-binding site are framed by a shaded box (■). The subdomains identified as the sugar-binding pockets are depicted as stars (☆).

enzyme (1.0 mg/ml) was mixed with 45 μl of a polysaccharide solution containing 0.5 mg/ml of insoluble oat spelt xylan and 0.1% BSA in 80 mM sodium phosphate buffer (pH 5.7), for 20 min at 4°C and the mixture was centrifuged at 15 000 rpm for 5 min. After removal of the supernatant, 50 μl of an oligosaccharide solution (in 80 mM phosphate buffer (pH 5.7) containing 0.1% BSA) was added and then mixed for 10 min. Enzyme released into the supernatant, after removal of the insoluble compounds by centrifugation, was quantified by determining the residual enzyme activity in the supernatant.

2.6. Model building of the XBD subdomain 1α-sugar complexes

A manual model building of the XBD (accession number 1xyf) was conducted with the program QUANTA as described by Fujimoto et al. [7]. The model of the XBD subdomain 1α-lactose complex was build by the superposition of the XBD and ricin–lactose complex. The structure of xylotriose in the binding pocket of the XBD subdomain α was built based on the X-ray structure of β-1,4-xylan hydrate and fitted into the binding site.

3. Results

3.1. Binding abilities of wt FXYN toward insoluble xylan

The hydrolysis rate of insoluble xylan by the truncated mutant Cat was only one half of that of the FXYN, even though the environment of the catalytic center of the enzyme was maintained after removal of the XBD, as described previously [6]. Therefore, the binding ability of FXYN and Cat toward insoluble xylan was investigated to assess whether the XBD was able to bind directly to insoluble xylan to promote its overall hydrolysis. As shown in Fig. 2a, the binding ability ($B_{1/2}$) of the Cat was 3 mg/ml and 12 mg/ml at enzyme concentrations of 0.01 mg/ml and 0.1 mg/ml, respectively. In contrast, the $B_{1/2}$ of the FXYN was 0.05 mg/ml and 0.5 mg/ml at enzyme concentrations of 0.01 mg/ml and 0.1 mg/ml, respectively. The relative binding abilities of FXYN and Cat for insoluble xylan under the condition of our previously described analysis (where the enzyme and substrate concentrations were 0.0 and 5 mg/ml, respectively), showed that the FXYN could bind to insoluble xylan at twice the level of the Cat mutant (>90 vs. $<50\%$ respectively) (see Fig. 2a and [6]). These data indicate that the XBD in FXYN binds directly to insoluble xylan to promote the overall hydrolysis of the insoluble substrate. Indeed, the hydrolysis rate of insoluble xylan by the Cat mutant was similar to that of the FXYN at the higher substrate concentration however, at the lower concentration it was markedly different (see Fig. 2b,c).

3.2. Effect of a single subdomain on the hydrolysis rate of FXYN

The XBD consists of three repeat subdomains (α, β and γ). The subdomains are homologous to each other and appear to

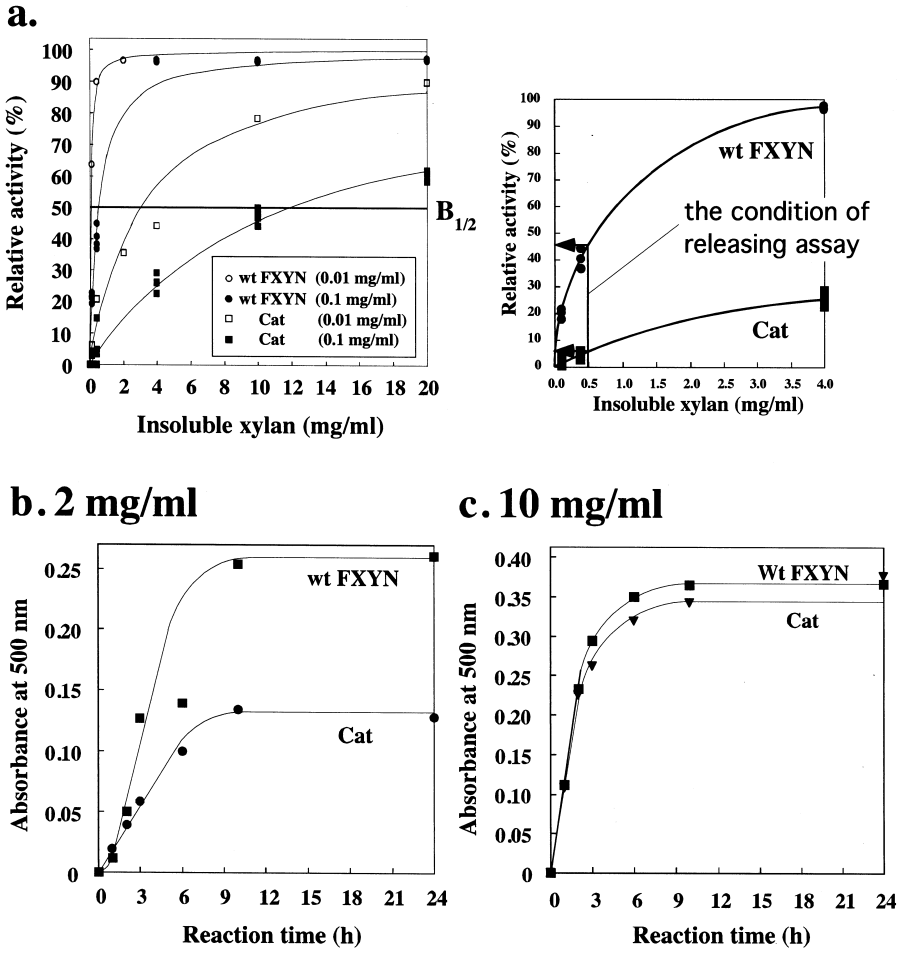


Fig. 2. a: Binding isotherm for FXYN and its truncated derivative, Cat, on insoluble oat spelt xylan. Identical amounts of FXYN and Cat (0.1 mg/ml or 0.01 mg/ml) were incubated with various concentrations of insoluble xylan. Unbound enzyme was quantified by measuring residual activity in the supernatant. Time course of hydrolysis of insoluble oat spelt xylan by FXYN and Cat using 2 mg/ml (b) or 10 mg/ml (c) of insoluble xylan. Reducing sugars were determined by the method of Somogyi-Nelson.

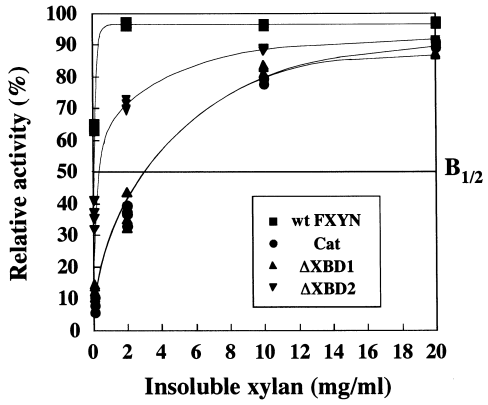


Fig. 3. Binding isotherm for FXYN and its truncated derivatives, ΔXBD1 and ΔXBD2, on insoluble oat spelt xylan.

contain possible sugar-binding sites, which could be predicted from the amino acid sequence of the RTB (Fig. 1b), suggesting that all three subdomains in the XBD have sugar-binding potential. Therefore, we constructed two truncated derivatives of FXYN that included only one or two subdomains designated as ΔXBD1 or ΔXBD2 respectively (Fig. 1a). The binding abilities of ΔXBD1 and ΔXBD2 toward insoluble xylan were investigated to confirm that a single subdomain could still retain the ability to bind the insoluble xylan. The binding ability ($B_{1/2}$) of ΔXBD1 was the same as that of Cat; in contrast, ΔXBD2 did bind slightly to the insoluble xylan (Fig. 3). The hydrolysis rates of xylan by ΔXBD1 and ΔXBD2 were the same as that of the Cat (data not shown). These findings indicate that all three the subdomain (α, β and γ) in the XBD are necessary to bind the linear sugar backbone of sugars such as β-1,4-xylan to enhance the hydrolysis rate of the FXYN. Essentially, this means that the whole structure of the XBD constituting three subdomains is important in the recognition of polysaccharides.

3.3. Minimum sugar unit in the sugar-binding pocket of the XBD

The XBD selectively binds to insoluble xylan as described above but the minimum sugar unit in the sugar-binding pocket has not yet been determined. Therefore, a releasing assay of FXYN was performed using xylo-oligosaccharides of varying lengths (X_1–X_7). Under the substrate concentration (0.5 mg/ml) for the releasing assay, the amounts of Cat and FXYN

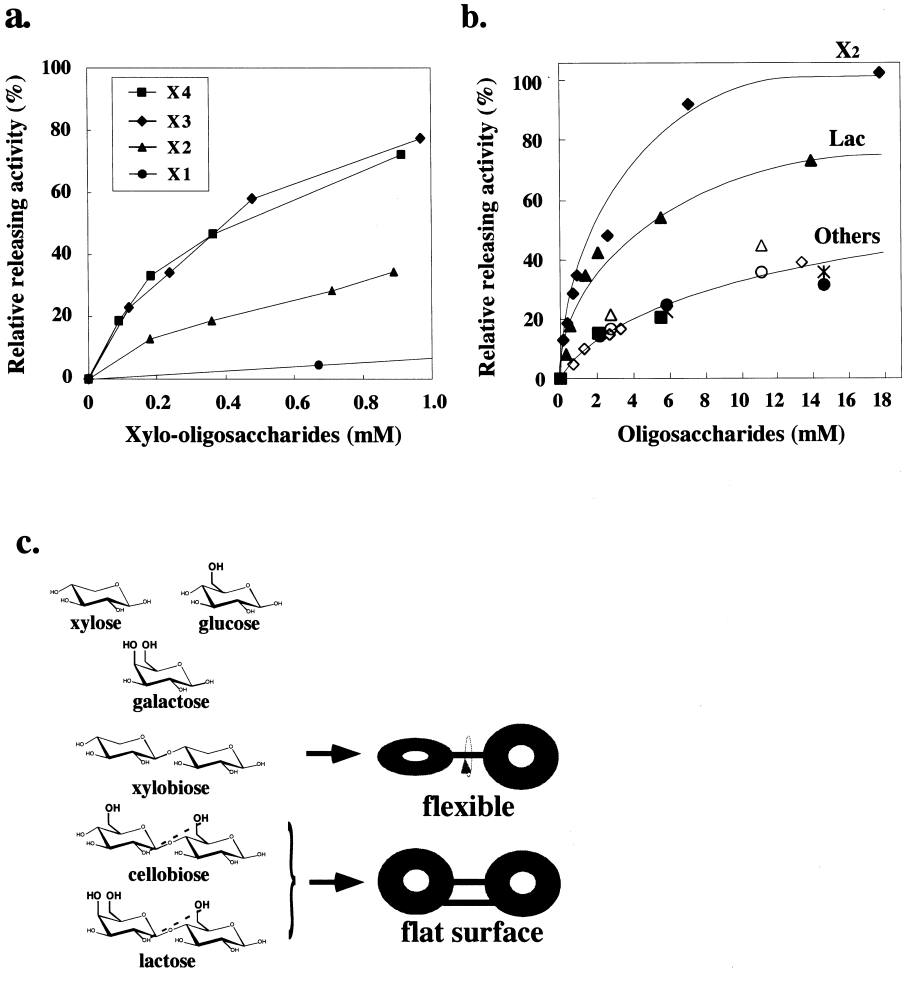


Fig. 4. a: Releasing assay of FXYN from insoluble oat spelt xylan by xylo-oligosaccharides of varying lengths (X_1–X_4). X_1–X_4 in this figure indicates xylo-mono-, di-, tri-, and tetrasaccharides, respectively. b: Releasing assay of FXYN from insoluble oat spelt xylan by selected mono- and disaccharides as follows: (◇): xylose, (○): glucose, (△): galactose, (◆): xylobiose, (●): cellobiose, (▲): lactose, (■): maltose, (*): sucrose. c: The structures of some of the mono- and disaccharides.

bound to insoluble xylan were 5 and 45%, respectively (Fig. 2a). Furthermore, almost the all of Cat bound to the insoluble xylan was released even when the binding complex was washed by buffer before adding the oligosaccharide solution (data not shown). Thus, the data were reflected in the binding properties of the XBD because the binding affinity of the Cat for insoluble xylan was negligible under the experimental conditions used. In fact, these binding properties are different from the size of the subsite in the catalytic domain of the family FXYNs, which is longer than a penta-oligosaccharide [18–20]. As shown in Fig. 4a, the amounts of FXYN released from insoluble xylan increased in relation to the increasing length of the oligosaccharide, up to a degree of polymerization (DP) of three sugar units, whereby the releasing ability of the tetramer was no different to that of the trimer. The releasing abilities of the pentamer to heptamer were almost same as tetramer (data not shown). To our knowledge, this finding is the first observation which demonstrates that the minimum binding sugar unit in the sugar-binding pocket of the XBD is a trimer linked by β-1,4 glycosidic bonds.

3.4. Binding specificity of the XBD

A releasing assay of FXYN from insoluble xylan by a range of mono- and disaccharides was performed to determine the sugar-binding specificities of the XBD in FXYN. In this assay, carbohydrates were selected which were considered to be able to release insoluble xylan. The differences between the xylo-oligosaccharides and the selected carbohydrates can be summarized as follows; (i) the existence of a C6 hydroxyl group in the sugar ring; (ii) the configuration of the C4 hydroxyl group at the non-reducing end sugar; or (iii) the sugar linkage between the disaccharides.

As shown in Fig. 4b, the releasing abilities of all of the monosaccharides (xylose, glucose and galactose) were quite similar. In the case of the following disaccharides: cellobiose, maltose and sucrose, FXYN was released from the insoluble xylan to almost the same degree as the monomeric xylose (Fig. 4b) whereas xylobiose released a much higher level of FXYN than any of the other disaccharides. Based on these results, the substrate recognition of the XBD can be summarized as follows: (i) an additional C6 atom relative to xylose causes steric hindrance; (ii) the glycosidic bond must be a β-1,4 linkage; (iii) the configurations of both the C2 and C3 hydroxyl groups must be equatorial to allow entry to the binding pocket. Contrary to expectation, in addition to xylobiose, lactose was also found to be a good releasing compound. The releasing ability of lactose was intermediate between xylobiose and the other disaccharides. The differences in structure between xylobiose and lactose are the configuration of the C4 hydroxyl group and the presence or absence of the C6 atom. Given that such marked structural differences exist between lactose and xylobiose, it appears extremely unlikely for lactose to be able to bind to the XBD. However, the fact that lactose released the FXYN enzyme almost as well as xylobiose strongly suggests that the binding of lactose with the XBD is occurring in a different manner to that of xylobiose.

4. Discussion

Carbohydrate-recognition proteins belonging to the 'ricin super family' having the Gly-X-X-X-Gln-X-Trp motif have been classified into two groups, the lectin group (containing RTB) and the enzyme group, as described by Hirabayashi et al. [8]. A family FXYNs from *S. olivaceoviridis* E-86 is known to possess a XBD belonging to the 'ricin super family' at its C-terminus. According to similarities in the amino acid sequences between the XBD and the RTB, all of the essential amino acid residues of the RTB required for sugar-binding were well conserved in the XBD (Fig. 1b) [21–23]. Moreover, the whole structure of the XBD was similar to that of the RTB as described by Fujimoto et al. [7]. In the structure of the RTB subdomain 1α-lactose complex (see Fig. 5a and [20]), Asp-22 of the RTB hydrogen-bonds to the C3 and C4 hydroxyl groups of galactose and this interaction is stabilized by a hydrogen bond from Nε2 of the conserved amide Gln-47. The Asn-46 of the RTB is also a key residue, hydrogen bonding at the C3 hydroxyl group of the galactose. The aromatic ring of residue Trp-37 of the RTB forms affinity stacking with the sugar ring by van der Waals forces. As the binding pocket in the XBD of FXYN is indeed similar to that of the RTB, the XBD should be able to recognize the C3 and C4 hydroxyl

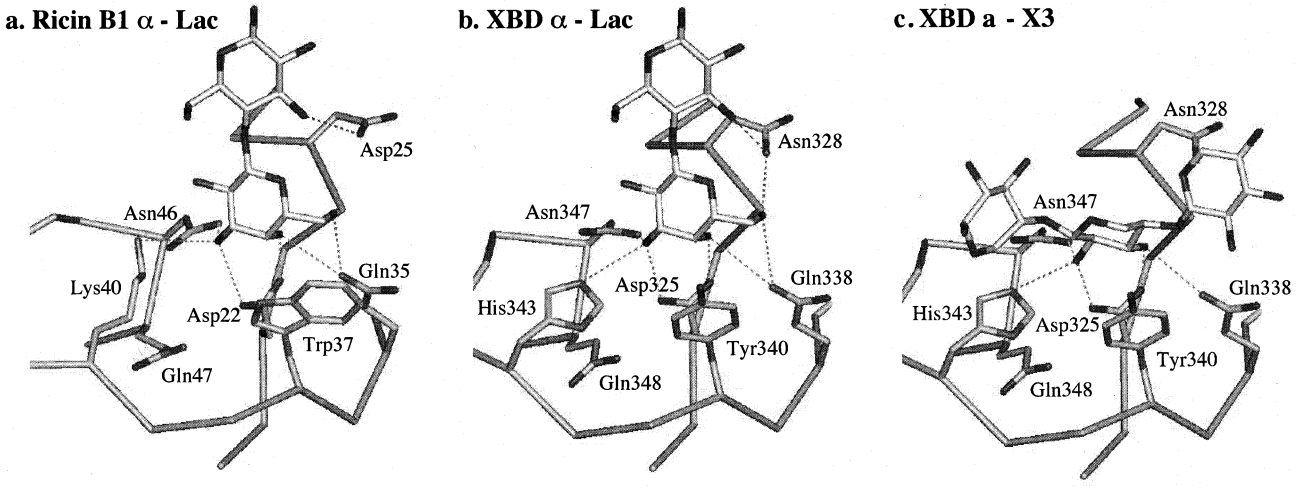


Fig. 5. Models of the ricin B chain subdomain 1α-lactose (1aai; [21]) (a), the XBD subdomain α-lactose (b) and the XBD subdomain α-xylotriose (X_3) (c) complexes. Key residues in each subdomain for binding to lactose are indicated. Lactose or xylotriose are shown as white sugar rings.

groups of xylose located at the non-reducing end of xylan (Fig. 5a,b) [11,21]. However, according to the model of the RTB–lactose or the XBD–lactose complexes (Fig. 5a,b), the C4 hydroxyl group of xylose at the non-reducing end of xylobiose is in an equatorial configuration, in contrast to that of galactose at the non-reducing end of lactose, resulting in the loss of a hydrogen bond to both the Asp-325 and Gln-338 of the XBD. Moreover, the C4 hydroxyl group of xylose at the non-reducing end of xylobiose is so close to the aromatic ring of residue Tyr-340 of the XBD that it is not possible for it to form affinity stacking with the sugar ring. These structural data indicate that xylobiose cannot bind to the binding pocket of XBD in the same manner as lactose. Therefore, we speculated that the sugar-binding ability of the XBD allowing it to specifically bind to xylo-oligosaccharides (see Fig. 4a) must be different from that of the RTB. The crystal structure of XBD indicates that the XBD is structurally able to bind to xylo-oligosaccharides at the different manner from lactose, if the location between the C3 and C4 hydroxyl groups of lactose is same as that of the C2 and C3 hydroxyl groups of xylose, there is no steric hindrance between xylo-oligosaccharides and the binding pocket of the XBD (see Fig. 5b,c and [7]).

Biochemical analyses of the XBD supported the structural studies described above. The XBD specifically bound to insoluble xylan and was subsequently released from it by adding various xylo-oligosaccharides (see Fig. 4a). The XBD was more able to recognize the oligomeric carbohydrates such as xylotriose rather than monosaccharides, which are the minimum recognition units of the RTB. If the binding pocket of the XBD was similar to that of the RTB, the XBD would not be able to recognize oligosaccharides because we could not find any amino acid residues in the binding pocket of the RTB capable of binding to the trisaccharide (Fig. 5a). These results indicate that the way in which xylobiose binds with the XBD is different from that of lactose binding with the RTB. Interestingly, cellobiose was not good as a releasing compound of the FXYN enzyme from insoluble xylan. These observations can be explained by the structural differences existing between xylobiose and cellobiose. The glycosyl bonds in cellobiose are anchored by a hydrogen bond between the C2 hydroxyl group of the non-reducing sugar and the C6 hydroxyl group of the reducing sugar, allowing the two sugar rings in cellobiose to form a flat surface (Fig. 4b,c). In contrast, the glycosyl bond in xylobiose is flexible owing to the absence of the C6 hydroxyl group. It was therefore considered that this flexibility in the glycosidic bond was an important factor in determining the insertion of the substrate into the binding pocket of the XBD (Fig. 5c). From this point of view, it was expected that lactose would not compete with insoluble xylan for the binding of the FXYN since lactose forms the same flat ring surface as cellobiose. Contrary to this expectation, lactose was in fact a good releasing compound, inhibiting the binding of insoluble xylan with the FXYN. These findings indicate that not only does the XBD have the binding pocket allowing it to bind to xylo-oligosaccharides but it also has the same binding pocket as the RTB to allow it to bind to lactose (Fig. 5b,c). In the case of the enzyme group lectins (such as the XBD), the sugar-binding pocket of ancient galactose-binding ricin super family lectins appears to have acquired an ability to bind and hydrolyze a linear sugar backbone, resulting in the differences observed in the sugar-binding specificities between the lectin group (containing RTB) and the enzyme group.

In conclusion, a type XIII XBD was characterized, indicating that the sugar-binding unit of the XBD was a trimer, which was in complete contrast to other lectins from the same family, such as the RTB. All of the subdomains (α, β and γ) in the XBD were found to be necessary to bind the linear sugar backbone of polysaccharides such as xylan and to enhance the hydrolysis rate of the FXYN enzyme. We first show that the XBD also binds to lactose at the same manner as that of RTB. These results were clearly consistent with our prediction from the structural analysis of the XBD.

Acknowledgements: The authors thank SUNTORY Ltd. for the supply of 'xylobiose mixture' and Dr. Yasuo Gama, NIMC, AIST, for his help in the synthesis of pNP-X_2. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences and by a Grant of Rice Genome Project PR-2209, MAFF, Japan.

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