FEBS Letters 494 (2001) 74–78 FEBS 24751

Trp122 and Trp134 on the surface of the catalytic domain are essential for crystalline chitin hydrolysis by *Bacillus circulans* chitinase A1

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Received 29 January 2001; revised 12 March 2001; accepted 13 March 2001

First published online 23 March 2001

Edited by Hans Eklund

Abstract From the 3D-structural analysis of the catalytic domain of chitinase A1, two exposed tryptophan residues (W122 and W134) are proposed to play an important role in guiding a chitin chain into the catalytic cleft during the crystalline chitin hydrolysis. Mutation of either W122 or W134 to alanine significantly reduced the hydrolyzing activity against highly crystalline β-chitin microfibrils. Double mutation almost completely abolished the hydrolyzing activity. On the other hand, the hydrolyzing activity against either soluble or amorphous substrate was not reduced. These mutations slightly impaired the binding activity of this enzyme. These results clearly demonstrated that the two exposed aromatic residues play a critical role in hydrolyzing the chitin chain in crystalline chitin.

Key words: Chitinase; Catalytic domain; Crystalline chitin; Aromatic amino acid residue; Site-directed mutagenesis

1. Introduction

Chitinases are classified into two different families, families 18 and 19, in the classification system of glycosylhydrolases, based on the amino acid sequence similarity of their catalytic domains [1]. The catalytic domains of family 18 chitinases have $(\beta/\alpha)_8$ barrel folds [2–6], whereas those of family 19 chitinases have high α-helical content and share structural similarity with chitosanase and lysozyme [7,8]. In addition to the difference in the 3D-structure, many differences have been found between the enzymatic features of the two families [9].

Chitinolytic bacteria generally produce multiple chitinases derived from different genes. Many chitinolytic bacteria produce only family 18 chitinases, while some other bacteria such as *Streptomyces* species also produce family 19 chitinases [10]. As we demonstrated previously, bacterial family 18 chitinases are further classified into three subfamilies, subfamilies A, B and C [11]. Chitinases in subfamily A have an insertion domain between the seventh and eighth β-strands of the $(\beta/\alpha)_8$ barrel basic structure, which is missing in chitinases of subfamilies B and C. *Bacillus circulans* WL-12 produces ChiA1, ChiC1 and ChiD1 as the initial products of the three chitinase genes, *chiA*, *chiB* and *chiC*, respectively [12]. ChiA1 and C1 have catalytic domains of subfamily A, and D1 has a catalytic domain of subfamily B [11]. Among the three chitinases, ChiA1 has the highest hydrolyzing activity against insoluble chitin. This chitinase contains the catalytic domain (CatD), two fibronectin type III-like domains (FnIIIDs) and the C-terminal chitin-binding domain (ChBD) [13]. The crystal structure of $CatD_{ChiA1}$ was determined recently at atomic resolution (1.13 Å) [4]. $CatD_{ChiA1}$ consists of an $(\beta/\alpha)_8$-TIM-barrel, and the two small insertion domains, β-domain 1 and β-domain 2, attached on top of the TIM-barrel provide a deep cleft for substrate binding. The catalytic site of ChiA1 is located almost at the bottom of the substrate-binding cleft. In addition to the crystal structure of CatD, solution structures of ChBD and the FnIIID were determined by NMR very recently ([14] and unpublished results). The ChBD makes a major contribution to the chitin-binding activity of ChiA1, and not only the ChBD but also the FnIIIDs are important for efficient hydrolysis of insoluble chitin [13].

In addition to the importance of domains in the C-terminal region, we found that the CatD itself has a special structure and mechanism essential for the degradation of crystalline chitin.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli JM109 was the host strain used throughout the construction of various recombinant plasmids. Recombinant plasmid pHT012 carries the entire *chiA* gene [15]. pCatD, which carries the truncated *chiA* gene encoding only CatD, was constructed by replacing the *Apa*I–*Hin*dIII DNA region in the pHT012 by the DNA fragment amplified by PCR using primers 5′-CGCATATGATAAATTTAAATAACACACTGC-3′ and 5′-AAGCTTACAGATCGGCCTTCAGTTT-3′. pHT002mut carrying an 826-bp *Apa*I–*Bam*H1 fragment from the *chiA* gene [15] was used as a template for site-directed mutagenesis by PCR.

2.2. Chemicals

Colloidal chitin and glycol chitin were prepared by the methods of Jeuniaux [16], and Yamada and Imoto [17], respectively. Chitin EX (powdered prawn shell chitin) used in the chitin affinity column and carboxymethyl chitin (CM chitin) were purchased from Funakoshi Chemical Co. (Tokyo). Highly crystalline β-chitin microfibrils from vestimentiferan, *Lamellibrachia satsuma*, were prepared as described previously [18]. Reduction of *N*-acetylglucosamine $(GlcNAc)_5$ was

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Abbreviations: GlcNAc, *N*-acetylglucosamine; CatD, catalytic domain; FnIIID, fibronectin type III-like domain; ChBD, chitin-binding domain

0014-5793/01/$20.00
PII: S0014-5793(01)02317-1

carried out as described [19]. $(GlcNAc)_5$ was obtained from Yaizu Suisan Chemical Co. Ltd. (Shizuoka, Japan).

2.3. Site-directed mutagenesis

Site-directed mutagenesis was carried out by using a QuikChange site-directed mutagenesis kit (Stratagene, CA). Primers used for the mutagenesis were 5′-CGGCGATCCAGCGATCGATACGGG-3′ and 5′-CCCGTATCGATCGCTGGATCGCCG-3′ for the W122A mutation, and 5′-CAGGGGATACGGCGGATCAGCCC-3′ and 5′-GGGCTGATCCGCCGTATCCCCTG-3′ for the W134A mutation. The mutant clones were selected after sequencing using an automated laser fluorescence DNA sequencer (Model 4000L; LI-COR).

2.4. Production and purification of ChiA1, $CatD_{ChiA1}$ and their mutants

Wild-type and mutant chitinases were produced in *E. coli* JM109 cells carrying pHT012, pCatD and their derivatives encoding various mutant chitinases. *E. coli* JM109 cells carrying a plasmid were grown in LB medium containing 100 μg/ml ampicillin for 20 to 23 h at 30°C. Then cells were collected by centrifugation, chitinases were extracted from the cells by a cold osmotic shock procedure [20], and collected by ammonium sulfate precipitation at 40% saturation for wild-type and mutant ChiA1, and at 60% saturation for wild-type and mutant $CatD_{ChiA1}$. Then, ChiA1 and its mutants were purified by chitin affinity column chromatography [21], and wild-type $CatD_{ChiA1}$ and its mutants were purified by high performance liquid chromatography (HPLC) with a Poros HS/M column (PerSeptive Biosystems, Framingham, MA, USA).

SDS–PAGE analysis of purified chitinases was conducted by the method of Laemmli [22].

2.5. Chitin-binding assay

Binding assay mixtures in 1-ml glass microtubes containing various concentrations of chitinase protein and 0.5 mg of β-chitin microfibrils in 0.5 ml of 20 mM sodium phosphate buffer (pH 6.0) were incubated on ice with occasional mixing. Each mixture was centrifuged at 4°C for 20 min at $10\,400 \times g$ to separate supernatant and β-chitin microfibrils with bound protein. The supernatant containing free protein was collected, and the protein concentration was determined. The

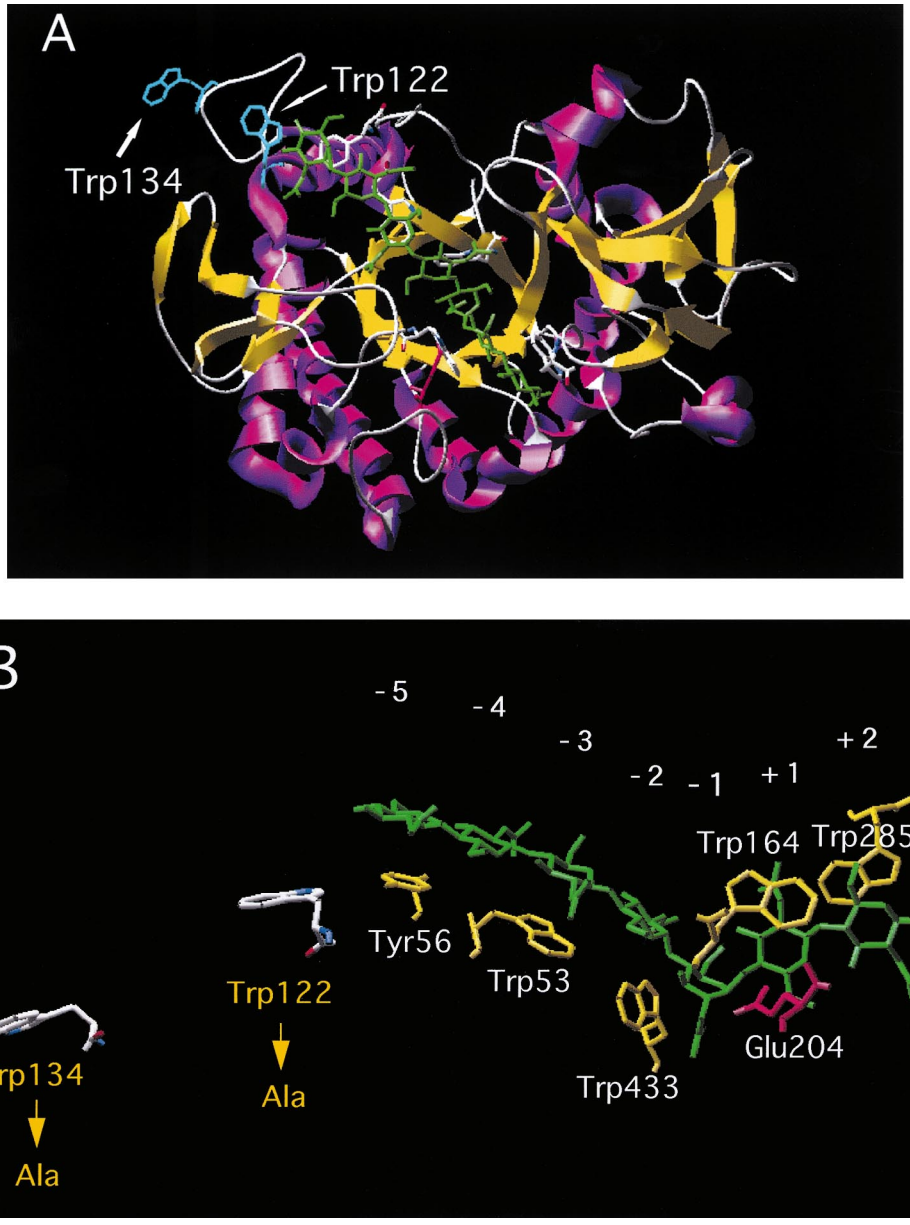

Fig. 1. Positions of W122 and W134. A: W122 and W134 are shown with a ribbon-drawing of the structure of inactivated $CatD_{ChiA1}$ complexed with $(GlcNAc)_7$. Green indicates bound $(GlcNAc)_7$. β-Strands are represented as yellow arrows and α-helices as purple spirals. B: W122 and W134 are shown with the bound $(GlcNAc)_7$ and aromatic residues in the substrate-binding cleft. Aromatic residues in the substrate-binding cleft are shown in yellow. Red indicates Q204, substituted for the catalytic residue E204.

amount of bound protein was calculated from the difference between the initial protein concentration and the free protein concentration after binding.

2.6. Enzyme and protein assay

Reducing sugar generated by the degradation of β-chitin microfibrils, reduced $(GlcNAc)_5$, glycol chitin, and CM chitin were measured by a modification of Schales' procedure using $(GlcNAc)_2$ as the standard [23]. Each assay mixture (total volume 350 μl) contained 150 μg (dry weight) of β-chitin microfibrils and enzyme in 0.1 M sodium phosphate buffer (pH 6.0).

The protein concentration was estimated either by measuring absorbance at UV 280 nm or by spectrofluorimetry at an excitation wavelength of 280 nm and an emission wavelength of 342 nm. The molar extinction coefficients (ε) used for the UV method were calculated from the amino acid compositions of wild-type and mutant chitinases [24].

3. Results and discussion

3.1. Positions of W134 and W122 on the surface of $CatD_{ChiA1}$

The crystal structure of inactivated $CatD_{ChiA1}$ complexed with $(GlcNAc)_7$ suggested that cleavage of the chitin chain occurs at the second linkage from the reducing end [4]. Seven subsites from −5 to +2 in the deep substrate-binding cleft were deduced from the structure. The oligomer chain bound to the cleft was bent and twisted at the third sugar ring (the position −1) and glutamine 204, substituted for the catalytic residue glutamic acid 204, was very closely located to the linkage between the second and third sugar rings from the reducing end. Four tryptophans, W285, 164, 433 and 53, in the substrate-binding cleft and one tyrosine, Y56, at the edge of the substrate-binding cleft were identified as the residues interacting with the GlcNAc units of the bound oligomer through stacking interactions.

In addition to the four tryptophans and one tyrosine in (or at the edge of) the substrate-binding cleft, two tryptophans, W122 and W134, were found on the surface of $CatD_{ChiA1}$. The two residues are aligned on the extension of the non-reducing end side of the bound $(GlcNAc)_7$, as shown in Fig. 1. The catalytic site of ChiA1 is located at the bottom of the deep substrate-binding cleft formed by the two insertion domains, β-domain 1 and β-domain 2 [4]. Therefore, to be hydrolyzed, a chitin chain must be introduced into the catalytic site, which is located inside of the protein. This is especially important for processive hydrolysis of crystalline chitin. Part of a chitin chain near its reducing end must be continuously peeled off from the crystalline chitin surface and introduced into the catalytic cleft to enable the continuous splitting off of chitobiose units from the reducing end of the chitin chain. W122 and W134 seem to be at the ideal positions for guiding and introducing a chitin chain into the substrate-binding cleft and, thus, to the catalytic site. Distances between W134 and W122 (approx. 15 Å) and between W122 and Y56 (approx. 10 Å) correspond to three GlcNAc units and two GlcNAc units of the chitin chain, respectively.

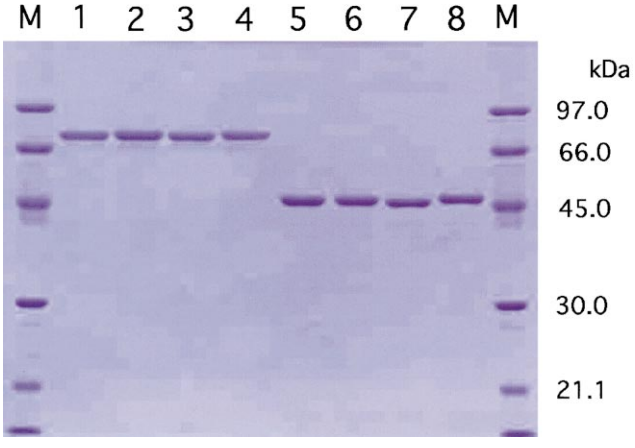

Fig. 2. SDS–PAGE analysis of the wild-type and mutant chitinases. Purified chitinases (5 μg each) were applied and protein bands were visualized by Coomassie brilliant blue R-250 staining. Lanes 1–4: ChiA1 and its mutants. Lanes 5–8: $CatD_{ChiA1}$ and its mutants. Lanes 1 and 5, wild-type; lanes 2 and 6, W122A; lanes 3 and 7, W134A; lanes 4 and 8, double mutants.

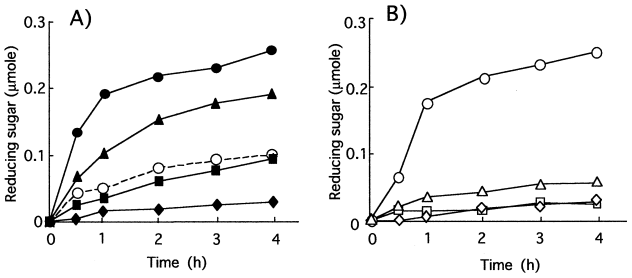

Fig. 3. Hydrolysis of β-chitin microfibrils. Reaction mixtures containing 150 μg (dry weight) of β-chitin microfibrils and either 51 pmol of ChiA1 (A) or 100 pmol of $CatD_{ChiA1}$ (B) were incubated at 37°C, and the amounts of released reducing sugar were measured. Closed symbols: ChiA1, open symbols: $CatD_{ChiA1}$. ●○, wild-type; ▲△, W134A; ■□, W122A, ◆◇, double mutant.

3.2. Effect of the mutations of W122 and W134 on the hydrolyzing activity

In order to examine the possible importance of W122 and W134 in the hydrolysis of crystalline chitin, the two aromatic residues were substituted by alanine and the effects of the mutations on the hydrolysis of highly crystalline β-chitin microfibrils were studied. It was shown that not only intact ChiA1 but also $CatD_{ChiA1}$ is able to hydrolyze the crystalline part of β-chitin microfibrils, although the efficiency was much lower than ChiA1 [18,25]. Sharp pointed tips formed at one end of microfibrils by the action of ChiA1 and $CatD_{ChiA1}$ are compatible with the hypothesis that the hydrolysis of β-chitin proceeds processively and unidirectionally [18,25].

Wild-type and mutant chitinases were produced in *E. coli* cells and purified either by chitin affinity column chromatography or by HPLC. As shown in Fig. 2, purified chitinases exhibited substantially single protein bands in SDS–PAGE analysis. Fig. 3A shows the time course of hydrolysis of β-chitin microfibrils by 51 pmol of chitinase A1 and its mutants. Either W122A or W134A mutation reduced the hydrolyzing activity significantly. The effect of mutation of W122, which is located closer than W134 to the substrate-binding cleft, was more severe than that of mutation of W134. Double mutation (W122A plus W134A) almost completely abolished the hydrolyzing activity. Surprisingly, the effect of double mutation was much more severe than that caused by the deletion of the ChBD and FnIIIDs. Hydrolysis of microfibrils was also examined with $CatD_{ChiA1}$ and its mutants as shown in Fig. 3B. Since the hydrolyzing activity against β-chitin microfibrils of $CatD_{ChiA1}$ was much lower than that of intact ChiA1, 100 pmol of each enzyme was used in this experiment. Interestingly, the mutations more severely affected $CatD_{ChiA1}$ than

Table 1
Specific hydrolyzing activities of wild-type and mutant chitinases against soluble substrates and an amorphous substrate

Enzymes	Specific hydrolyzing activity (units/nmol)[a]				
	Glycol chitin	CM chitin	4-MU-$(GlcNAc)_3$	Reduced $(GlcNAc)_5$	Colloidal chitin
ChiA1					
Wild-type	0.14 (100)	2.0 (100)	1.2 (100)	3.2 (100)	1.9 (100)
W122A	0.23 (164)	2.4 (120)	1.3 (108)	3.1 (97)	1.7 (89)
W134A	0.15 (107)	2.4 (120)	1.2 (100)	3.2 (100)	1.9 (100)
W122/134A	0.24 (171)	2.5 (125)	1.3 (108)	3.2 (100)	1.9 (100)
$CatD_{ChiA1}$					
Wild-type	0.13 (100)	2.5 (100)	1.3 (100)	3.9 (100)	1.3 (100)
W122A	0.22 (169)	2.7 (108)	1.4 (108)	3.8 (97)	1.4 (108)
W134A	0.16 (120)	2.8 (112)	1.4 (108)	3.9 (100)	1.5 (115)
W122/134A	0.22 (169)	2.7 (108)	1.4 (108)	3.9 (100)	1.3 (100)

[a]Relative specific activities (%) are shown in parentheses.

intact ChiA1. Even the W134A mutation, which reduced the hydrolyzing activity of ChiA1 less efficiently than the other mutations, reduced the activity of $CatD_{ChiA1}$ to approximately one-fifth of that of wild-type $CatD_{ChiA1}$. The effects of the two independent mutations of W122 and W134 were as severe as that of the double mutation. Therefore, it appeared that the presence of either the FnIIID or ChBD somehow partially compensated for the effect of W134A or W122A mutation.

The effects of mutations on the hydrolysis of soluble and amorphous substrates were also studied (Table 1). Neither the W122A nor the W134A mutation did significantly affect the hydrolyzing activity of the entire ChiA1 or $CatD_{ChiA1}$ against reduced $(GlcNAc)_5$. Interestingly, mutation of either of the two aromatic residues, especially W122, showed a tendency to increase the hydrolyzing activity against glycol chitin and CM chitin. This probably means that these aromatic residues might interfere with the entrance of these soluble and high molecular weight substrates to the substrate-binding cleft, but not with the entrance of the low molecular weight GlcNAc oligomer.

3.3. *Effect of the mutations on the binding activity to β-chitin microfibrils*

The C-terminal ChBD makes a major contribution to the chitin-binding activity of this enzyme, although a weak affinity of CatD for chitinous substrates was suggested [13,25]. However, it has not yet been clarified whether CatD is involved in the chitin-binding activity to any extent, especially when it is

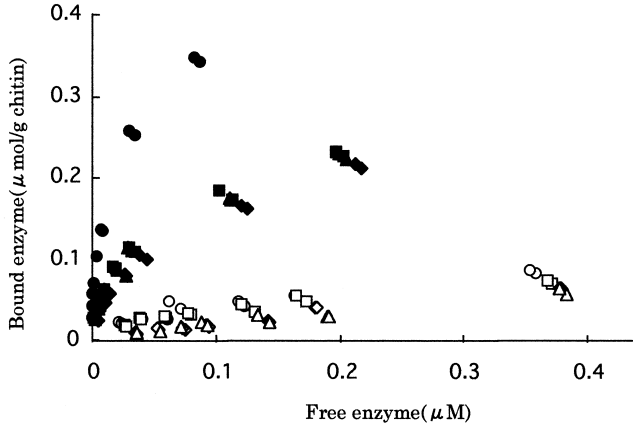

Fig. 4. The equilibrium isotherms of binding of wild-type and mutant chitinases to β-chitin microfibrils. Symbols are same as Fig. 3.

with ChBD as a part of the intact ChiA1 molecule. If W122 and W134 play a role in guiding and introducing chitin chain into the catalytic site, interaction of these residues with a single chitin chain would enhance the chitin-binding activity of the ChiA1 molecule.

To see whether the W122A and W134A mutations affect the binding of ChiA1, binding activities of wild-type and mutant ChiA1 to β-chitin microfibrils were compared. Fig. 4 shows binding isotherms of ChiA1, $CatD_{ChiA1}$, and their mutants. Binding activity of $CatD_{ChiA1}$ was much less significant than ChiA1 as expected from the previous results [13,25] and therefore effect of mutations on the binding activity of $CatD_{ChiA1}$ was not clearly detected. On the other hand, mutations of W122 and/or W134 significantly reduced the binding activity of ChiA1, although the binding activities of mutant ChiA1 proteins were still much higher than those of the truncated chitinase lacking ChBD and the FnIIIDs. These results clearly demonstrated that CatD itself is also involved in the binding of ChiA1 to crystalline chitin through the interaction between W122/W134 and substrate.

The results presented above clearly demonstrate that the two exposed aromatic residues are essential for the hydrolysis of crystalline chitin. Koivula et al. [26] reported that W272 of *Trichoderma reesei* cellobiohydrolase Cel6A is an essential determinant of crystalline cellulose hydrolysis activity. They described W272 as the first example of a site exclusively required for the degradation of crystalline cellulose. W272 is located close to the entrance of the enclosed catalytic tunnel. Therefore, the situation of W272 seems to be rather similar to that of Y56 in ChiA1, which is located at the edge of the entrance of the substrate-binding cleft. On the other hand, W122 and 134 of ChiA1 are located much farther from the catalytic groove. As far as we know, this is the first report demonstrating the critical role for crystalline substrate hydrolysis of the residues exposed on the surface of the CatD. The analogy with W272 of Cel6A suggests that Y56 of ChiA1 may be a residue which is also exclusively required for the degradation of crystalline chitin. To confirm this possibility, site-directed mutagenesis of Y56 together with all aromatic residues found in the substrate-binding cleft are now underway.

The 3D-structure of CatD of ChiA1 from *B. circulans* WL-12 is very similar to that of CatD of ChiA from *Serratia marcescens*. In the case of *Serratia* ChiA, the crystal structure of the entire molecule, consisting of two domains, CatD and the N-terminal domain (ChiN domain), was determined [3]. The ChiN domain is structurally similar to the FnIIIDs of

animal proteins, although no amino acid sequence similarity was found with the FnIIIDs of *B. circulans* ChiA1. Perrakis et al. found four conserved tryptophans in the ChiN domain which are located in ideal positions to facilitate interaction with an extended sugar chain modeled in the catalytic groove [27]. Therefore, it seems very interesting to examine whether the FnIIIDs of ChiA1 contain exposed tryptophans on its surface or not.

Acknowledgements: We are grateful to M. Shirakawa and T. Ikegami, Nara Institute of Science and Technology, for valuable discussions. This work was partly supported by a Grant-in-Aid for scientific research (12660070) from the Ministry of Education, Science and Culture of Japan.

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