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NMR analysis of substrate binding to a two-domain chitinase: Comparison between soluble and insoluble chitins

Tomoya Takashima, Takayuki Ohnuma, Tamo Fukamizo*

Department of Advanced Bioscience, Kindai University, 3327-204 Nakamachi, Nara 631-8505, Japan

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ABSTRACT

CJP-4 is a two-domain chitinase from Japanese cedar (*Cryptomeria japonica*) pollen, consisting of an N-terminal CBM18 domain and a GH19 catalytic domain. The substrate binding to an inactive mutant protein of full-length CJP-4, in which the catalytic acid Glu108 was mutated to glutamine, CJP-4(E108Q), was analyzed by NMR spectroscopy. Based on the chemical shift perturbations of ^{1}H-^{15}N HSQC signals of Gly26 (CBM18 domain) and Trp185 (GH19 domain), the association constants for individual domains of CJP-4(E108Q) toward soluble chitin hexamer $(GlcNAc)_6$ were determined to be 2300 and 3500 M^{-1}, respectively. Isothermal titration calorimetry provided a similar association constant for $(GlcNAc)_6$ (1980 M^{-1}) with the one-site binding model. One $(GlcNAc)_6$ molecule appeared to bind to a single binding site of CJP-4(E108Q), spanning from CBM18 to GH19 domains. When chitin nanofibers, insoluble chitinase substrate, were added to the CJP-4(E108Q) solution, strong line-broadening was observed for the majority of the backbone resonances in CBM18 domain but not in GH19 domain, indicating a binding preference of CBM18 domain to the insoluble chitin. We here demonstrated importance of CBM18 domain in insoluble chitin recognition based on the NMR binding data obtained for full-length CJP-4. Chitin nanofibers were found to be useful for spectroscopic observation of insoluble chitin binding to proteins.

1. Introduction

Plant chitinases are defensive enzymes that hydrolyze β-1,4-glycosidic linkages of chitinous components localized to the cell wall of fungal pathogens [1,2]. The enzymes are classified into either GH18 or GH19 family based on their amino acid sequences (http://www.cazy.org/Glycoside-Hydrolases.html). Plant GH19 chitinases are further subdivided into at least three classes, namely class I, class II, and class IV, according to domain organization and loop deletions [3]. Class I chitinases consist of two domains, an N-terminal hevein domain belonging to the CBM18 family and a GH19 catalytic domain, while class II chitinases only have a catalytic domain homologous to those of class I enzymes. Class IV chitinases are also two-domain enzymes composed of a CBM18 hevein domain and a GH19 catalytic domain, but are smaller due to the deletions of three internal loops (Loop II, Loop IV, and Loop V) and a C-terminal loop. The substrate-binding cleft of the GH19 catalytic domain has been intensively studied and fully characterized by X-ray crystallography and NMR spectroscopy [4–9]. The chitin recognition mechanism of the CBM18 hevein domain has been similarly elucidated by NMR spectroscopy [10–13]. However, experimental data on substrate recognition by full-length proteins of multi-domain chitinases have been very limited. Since the linker regions between the domains are often labile, expression, purification, and crystallization of full-length proteins with a multi-domain structure are challenging. Thus, most studies on the multi-domain chitinases were conducted using recombinant proteins of individual domains separately produced [14–16].

Japanese cedar (*Cryptomeria japonica*) pollen is a major source of air-borne allergens in Japan that cause pollinosis. An important

Abbreviations: CBM18, carbohydrate-binding module family 18; CJP-4, a class IV chitinase from Japanese cedar pollen; CJP-4-Cat, a GH19 catalytic domain of CJP-4; CJP-4(E108Q) and CJP-4(E108Q)-Cat, inactive mutants derived from CJP-4 and CJP-4-Cat, respectively, in which Glu108 is specifically mutated to glutamine; GH19, glycoside hydrolase family 19; GlcNAc, *N*-acetylglucosamine; $(GlcNAc)_n$, β-1,4-linked oligosaccharide of GlcNAc with polymerization degree of n; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherent spectroscopy; ITC, isothermal titration calorimetry.

* Corresponding author. Biochemistry-Electrochemistry Research Unit, School of Chemistry, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand.

E-mail address: tamo0111fuka@gmail.com (T. Fukamizo).

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allergen in the cedar pollen is CJP-4 [17], a class IV chitinase that is composed of 247 amino acids (molecular mass, 25.7 kDa). As characterized for the other class IV chitinases, CJP-4 consists of CBM18 hevein and GH19 catalytic domains, connected by a serine/threonine–rich linker region, as shown in Fig. 1. This figure also shows a significant sequence similarity with other class IV chitinases identified to date and two conserved loop structures [boxed regions; Loop I (58–68) and Loop III (121–134)] as a molecular signature for class IV enzymes. Recently, we have successfully conducted backbone resonance assignments of an inactive mutant of CJP-4, CJP-4(E108Q), using three-dimensional NMR techniques [18]. This two-domain chitinase is now ready for analyzing the interaction with the substrate by NMR spectroscopy.

On the other hand, most studies on the chitinase-substrate interaction were conducted using pseudo-substrates, soluble chitin oligosaccharides [19], which enable more quantitative analysis as compared with the natural substrate, insoluble chitin. However, it is uncertain if these experimental data obtained from the oligosaccharide substrates indeed reflect the chitinase-substrate interaction in nature. Ifuku first reported the production and characterization of chitin nanofibers [20], which represent higher dispersibility in aqueous medium, without losing its crystallinity. Chitin nanofibers were already used as the substrate for chitinase, and the enzymatic reactions were successfully monitored by turbidimetry using this insoluble polymer [21]. This material may also be useful for analyzing chitinase interaction with insoluble substrate in aqueous solution.

To gain insights into the molecular basis of interaction of multi-domain chitinase with its substrate, the present study employed nuclear magnetic resonance (NMR) spectroscopy and isothermal titration calorimetry (ITC) using an inactive mutant of two-domain chitinase CJP-4(E108Q) and its CBM18-truncated protein, CJP-4(E108Q)-Cat. Chitin hexamer $(GlcNAc)_6$ and chitin nanofibers were used as soluble and insoluble substrates, respectively.

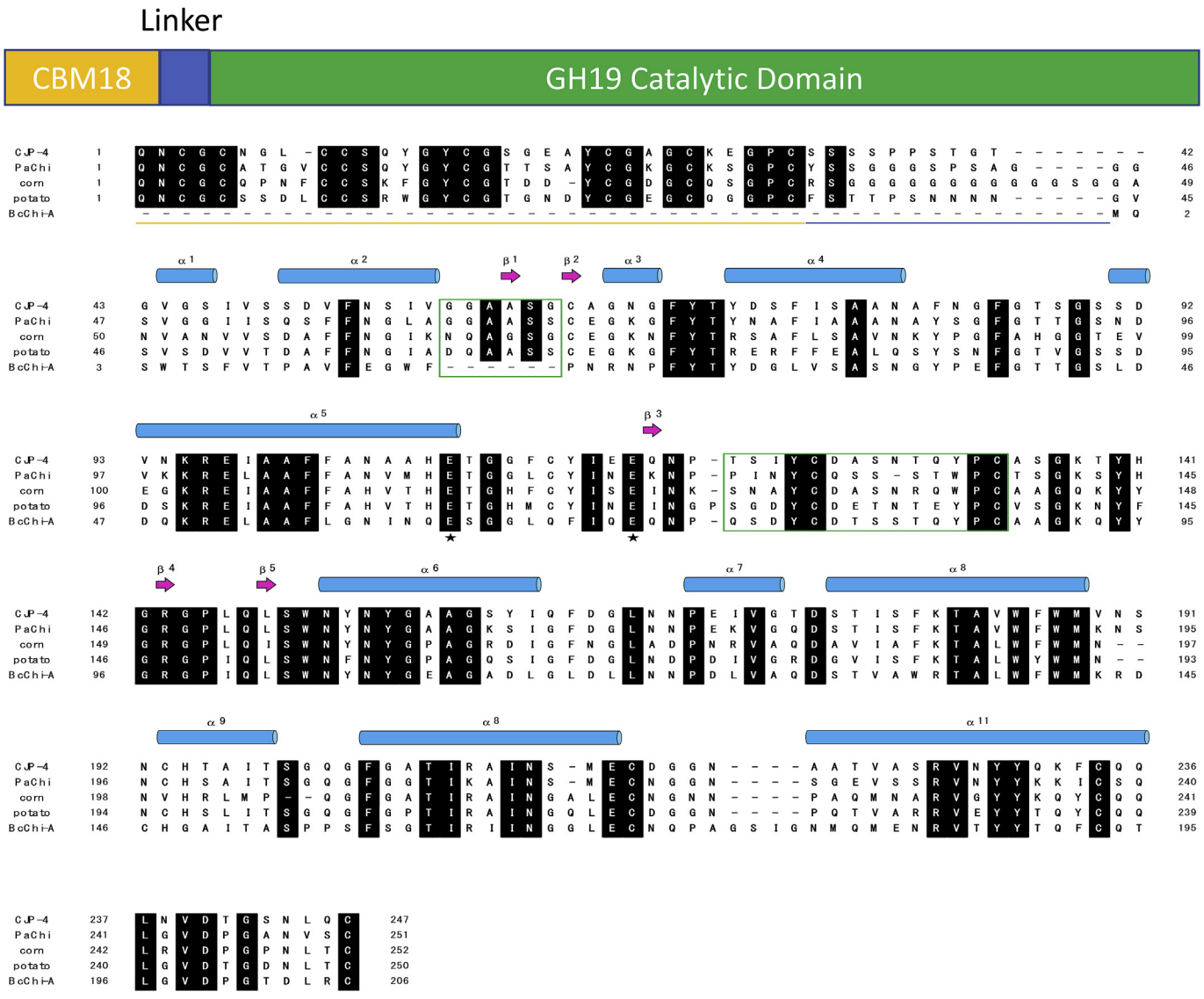

Fig. 1. Amino acid sequence alignment of plant class IV chitinases. PaChi, a class IV chitinase from *Picea abies* [31]; corn, a class IV chitinase from *Zea mays* [32]; potato, a class IV chitinase from *Solanum tuberosum* (DBLINK, BioProject: PRJNA225997; DBSOURCE REFSEQ: accession XP_006359038.1); and BcChi-A, a class II chitinase from *Bryum coronatum* [33]. Amino acids completely conserved are highlighted by white characters with black backgrounds. Sequences for the N-terminal CBM18 domain and linker regions are underlined in yellow and blue, respectively. Secondary structures estimated from backbone chemical shifts (Takashima et al., 2017 [18]) are shown above the sequences, and labeled with α1-α11, for α-helices, and β1-β5 for β-strands. Asterisks indicate the putative catalytic residues. The boxed regions of 58–68 and 121–134 represents loop structures, Loop I and Loop III, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)