Article

N-terminal (1→3)-β-D-glucan recognition proteins from insects recognize the difference in ultra-structures of (1→3)-β-D-glucan

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Abstract: The recognition of (1→3)-β-D-glucans (BGs) by β-1,3-D-glucan recognition protein (BGRP) found in invertebrates plays a significant role in the activation of toll pathway and pro-phenol oxidase system in insect host defense against fungal invasion. To examine the structural diversity of BGs in BGRP interaction, the binding specificity of BGRPs cloned from four different insectswas characterized using ELISA. Recombinant BGRPs expressed as Fc-fusion proteins of human IgG1 bound to solid phase BGs. Because of the binding specificities, the BGRPs were categorized into two different ultrastructure- binding characters. The BGRPs from Silkworm and Indian meal moth bound to BGs containing triple-helical structure. Other BGRPs from red flour beetle and yellow mealworm beetle showed no binding to triple-helical BGs, but to alkaline-treated BGs, which have partially opened helical conformation. These evidences suggest that the innate immune system distinguishes different BG conformations and it is equipped for the diversity of BG structures.

Keywords: β-D-glucan; glucan binding protein; host defense; innate immunity

1. Introduction

Innate immune system is equipped in various organisms to recognize molecular patterns in pathogens [1]. To accomplish host defense mechanisms in invertebrates, it is important to discriminate the large number of potential pathogens from itself using a restricted number of germ-line encoded receptors and binding proteins. Insects possess unique pattern-recognition receptors, called peptidoglycan recognition proteins (PGRP) and BGRP against peptidoglycan (PG) and (1→3)-β-D-glucan (BG), respectively [2]. These recognition proteins initiate the activation of pro-phenoloxidase, which leads to melanin formation in addition to Toll and Imd pathways [3]. The interaction of BGRP with BG and PGRP with PG activates serine proteases cascade and subsequently alternate pro-phenoloxidase to phenoloxidase [4]. This reaction system can be applied in detecting BG and PG using body fluid obtained from Silkworm larvae [5]. However, it does not distinguish the content of BG and PG in a test sample, because the fluid contains both BGRP and PGRP [5].

The structural diversity of BGs has been reported in previous works. Water-soluble BGs generally possess (1→6)-β-D-glucopyranosyl branches with various frequencies and lengths on the (1→3)- β-D-glucan main chain. The ultrastructure of the branched (1→3)- β-D-glucans, such as sonifilan from *Schizophyllum commune* and laminarin from *Laminaria digitata*, is a triple helix [6, 7].

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The triple helical conformation can be transiently converted to single-strand random coiled form or helical form. For example, partially opened triple helix can be converted through serial treatment with alkaline-and-neutralization [8]. It is hypothesized that the innate immune system is able to recognize the structural diversity of BGs [9]. Therefore, in this study we have isolated four kinds of BGRPs from different insects and examined their binding specificity to different structures of BGs. As a result, we found that two classes of BGRPs from lepidopteran and coleopteran bound to the triple-helical conformation and the single-strand conformation of BGs, respectively. These evidences support that innate immune system in insect surveys different molecular patterns of polysaccharide with conformational alteration.

2. Results

2.1. *Evaluation of direct binding activity of BGRP-Fc proteins to solid phase BG by ELISA*

It has been well documented that SPG and laminarin form triple helical ultrastructure in physiological solution [6, 7]. The triple-helix conformation can be altered to partially opened helix conformation by treating with alkaline, and treating partially opened helix conformation with acid solution for renaturation after neutralization [10].

2.1.1. Binding of BGRPs to solid phase of SPG

To examine the binding ability of BGRP-Fc to structurally different β-glucans, Sonifilan (SPG), laminarin, and their alkaline-treated glucans, were tested by ELISA. *Bombyx mori*-derived BGRP (BmBGRP) and *Plodia interpunctera i.e.* Indian meal moth-derived BGRP (PiBGRP) showed significant binding to both SPG and alkaline-treated SPG (AT-SPG). (Figure 1 upper)

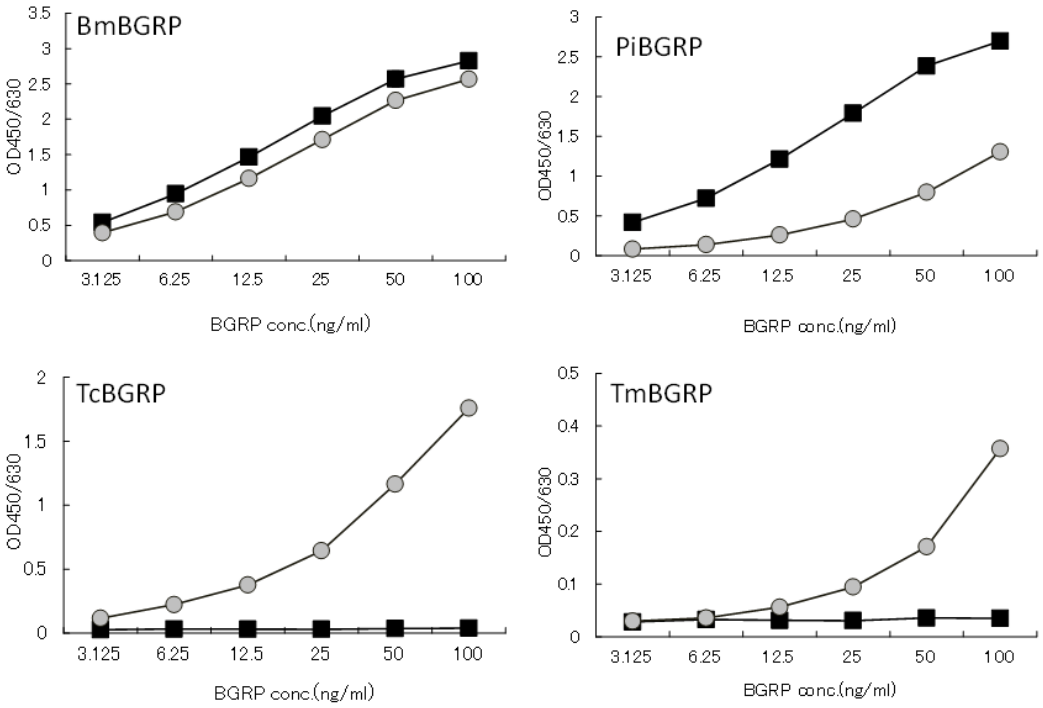

Figure 1. Binding activity of BGRP-Fc to solid-phase BGs. Binding of BGRP-Fc to SPG (black square) and AT-SPG (gray circle) were measured. SPG and AT-SPG were coated on ELISA plate at 1 μg/ml.

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There was no difference in the binding ability of BmBGRP to SPG and AT-SPG. However, PiBGRP showed higher binding to SPG than AT-SPG. In contrast, *Tribolium castaneum*–derived BGRP (TcBGRP) and *Tenebrio molita*–derived BGRP (TmBGRP) showed no binding to SPG, but bound to AT-SPG. (Figure 1 lower)

2.1.2. Binding of BGRPs to the solid phase of laminarin

To confirm that these specificities might have resulted from the conformational difference of BG, other BG, laminarin, which has lower MW and lower branching ratio for 1, 6-β-monoglucoside than SPG was applied to the binding assay. BmBGRP and PiBGRP bound well to undenatured laminarin. (Figure 2 upper) TcBGRP and TmBGRP showed no binding to laminarin, but significantly bound to AT-laminarin as well as AT-SPG. (Figure 2 lower)

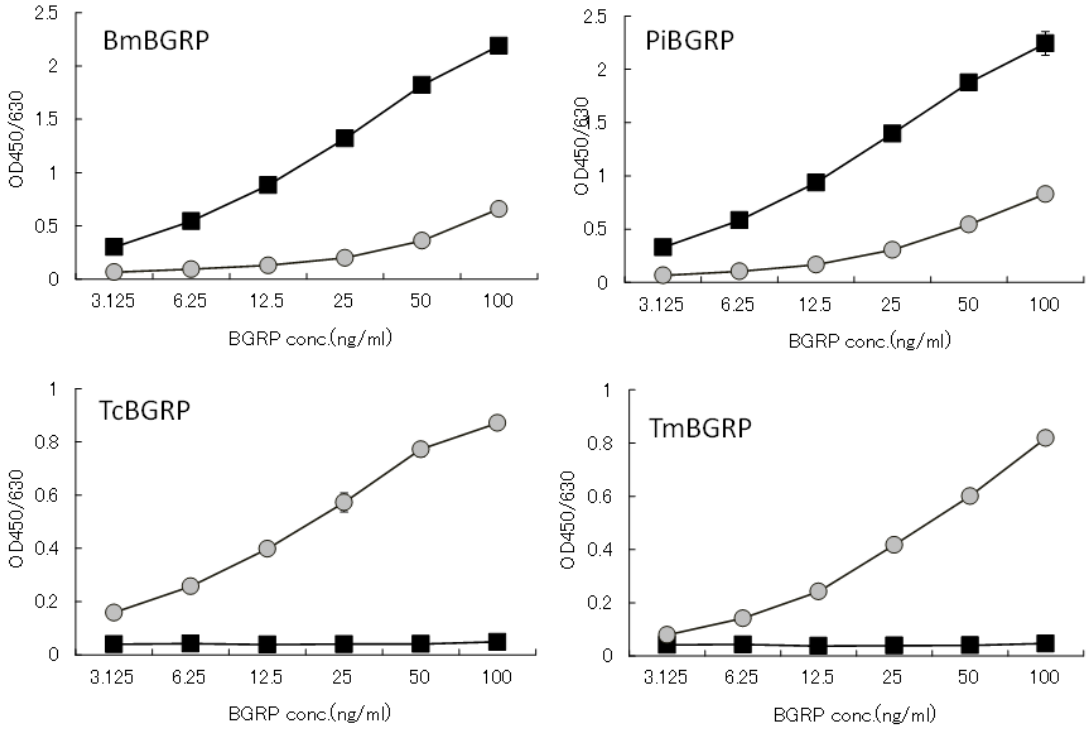

Figure 2. Binding activity of BGRP-Fc to solid-phase BGs. Binding of BGRP-Fc to laminarin (black square) and AT-laminarin (gray circle) measured by ELISA. Laminarin and AT-laminarin were coated on ELISA plate at 10 μg/ml.

2.2. Competitive effect of liquid phase BGs in ELISA

We have reexamined the binding specificity of these BGRPs to liquid phase BGs as a competitor against solid phase of BGs with different conformations.

2.2.1. Effect of liquid phase SPG and AT-SPG on BGRPs-binding to solid phase SPG and AT-SPG

First, the effect of liquid phase SPG and AT-SPG on BGRPs binding to SPG coated on ELISA plate was examined. Binding inhibition of BGRPs to SPG on plate was observed in Bm and Pi in the presence of untreated SPG in liquid phase. (Figure 3)

In competition with SPG, the binding of Bm and Pi to SPG was inhibited on ELISA plate. (Figure 3 upper) However, in the competition with AT-SPG, the binding of Tc and Tm to solid phase AT-SPG significantly reduced. (Figure 4 lower)

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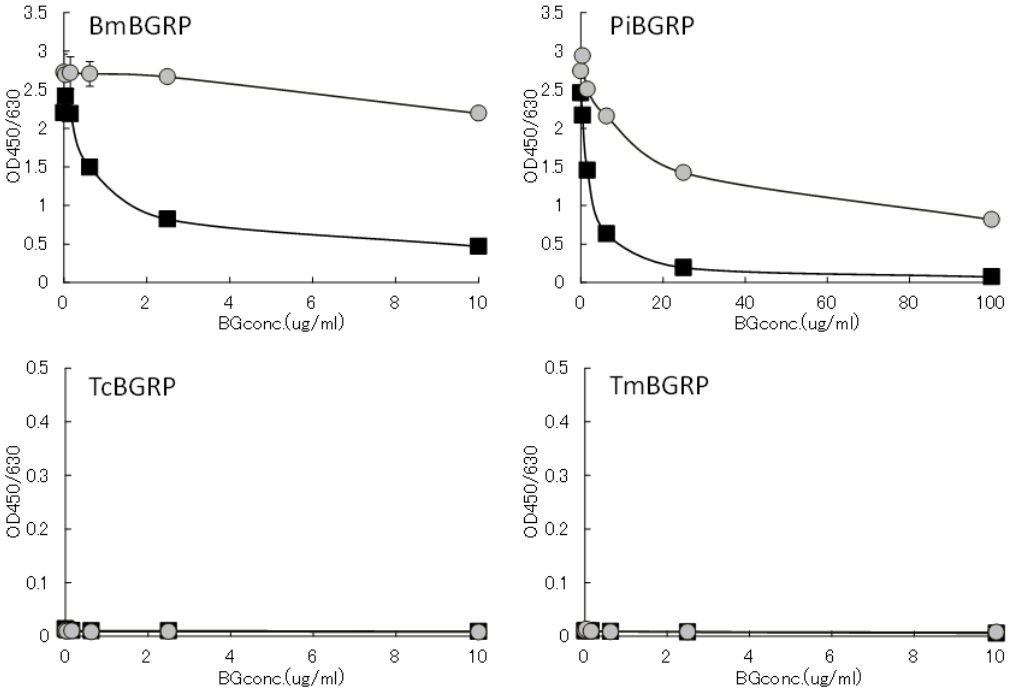

Figure 3. Binding activity of BGRP-Fc to SPG and AT-SPG in the liquid phase. Competition with liquid phase SPG (black square) and AT-SPG (gray circle) in the BGRP-Fc binding to SPG (10 µg/ml). TcBGRP-Fc and TmBGRP-Fc failed to bind solid phase SPG.

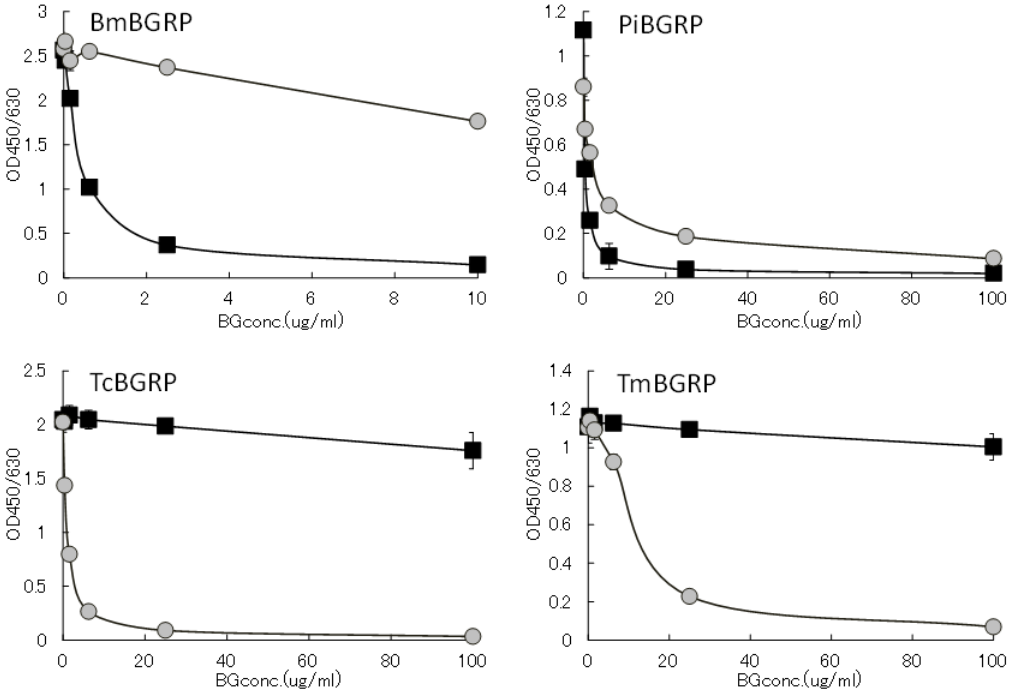

Figure 4. Binding activity of BGRP-Fc to SPG and AT-SPG in the liquid phase. Competition with liquid phase SPG (black square) and AT-SPG (gray circle) in the BGRP-Fc binding to AT-SPG (10 µg/ml).

2.2.2. Effect of liquid phase laminarin and AT-laminarin on BGRPs-binding to solid phase laminarin and AT-laminarin

Similar results were observed in the competition assay using laminarin and AT-laminarin. (Figure 5) Particularly, in case of Tc and Tm, binding inhibition with AT-laminarin in liquid phase was observed in BGRPs binding against AT-laminarin. (Figure 6)

The above results suggest that the binding of Bm and Pi tends to be high on triple helical BGs, although Tc and Tm have higher binding ability to AT-SPG and AT-laminarin, which forms partially opened helical conformation.

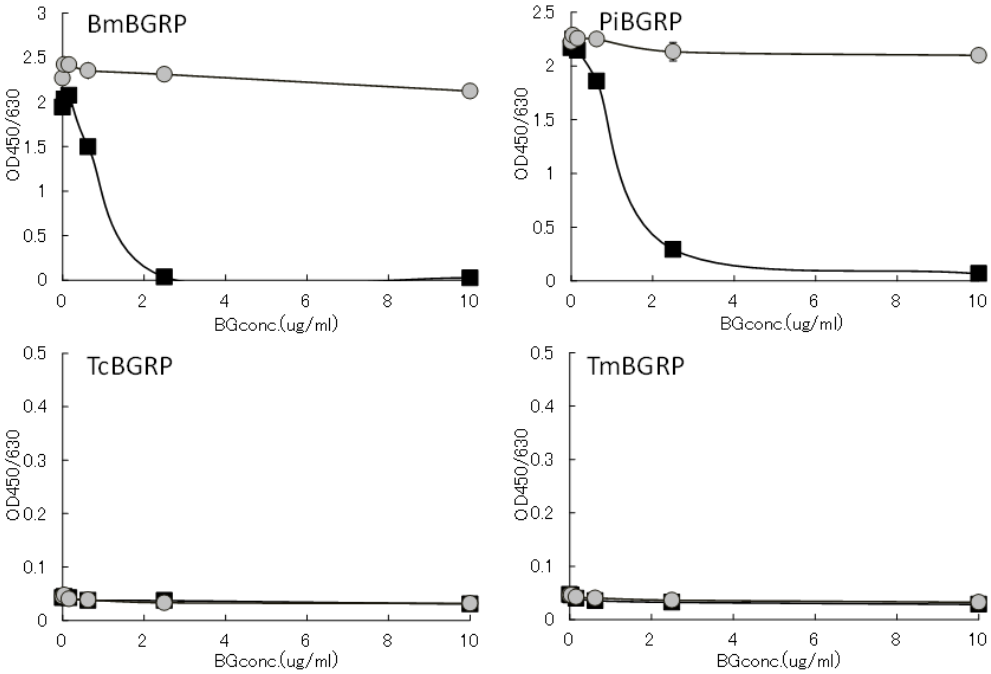

Figure 5. Binding activity of BGRP-Fc to laminarin and AT-laminarin in the liquid phase. Competition with liquid phase laminarin (black square) and AT-laminarin (gray circle) in the BGRP-Fc binding to solid phase laminarin (10 μg/ml). TcBGRP-Fc and TmBGRP-Fc failed to bind the solid phase laminarin (10 μg/ml).

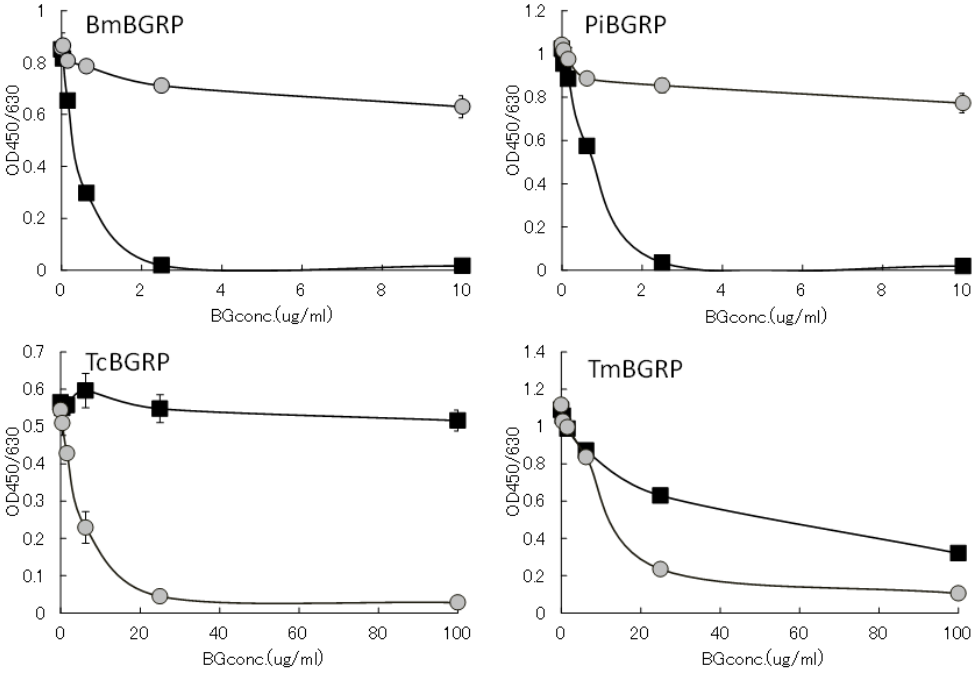

Figure 6. Binding activity of BGRP-Fc to laminarin and AT-Iaminarin in the liquid phase. Competition with liquid phase laminarin (black square) and AT-Iaminarin (gray circle) in the BGRP-Fc binding to solid phase AT-laminarin (10 μg/ml).

2.3. *Binding kinetics of BGRPs to SPG and AT-SPG*

The interaction of BmBGRP and TcBGRP with different conformers of SPG was determined and quantified, using biolayer interferometry (BLitz). A concentration-dependent increase in the binding of BmBGRP to SPG was observed. In contrast, the interaction of TcBGRP with SPG was quite lower than that of BmBGRP with SPG. However, the binding of TcBGRP was significantly higher than BmBGRP in the case of AT-SPG (Figure 7). The affinity (KD) of BmBGRP toward SPG and AT-SPG was calculated as 0.29 μM and 0.20 μM respectively. Rmax of BmBGRP to SPG and AT-SPG was 0.26 and 0.07, respectively (Table 1), suggesting that the binding site for BmBGRP on SPG was reduced by alkaline-treatment. However, the KD of TcBGRP toward SPG and AT-SPG was 1.77 and 0.71 μM respectively. This suggests that the affinity of Tc BGRP was improved by alkaline treatment of SPG. These results suggest that TcBGRP tends to interact with alkaline-denatured conformation of BG.

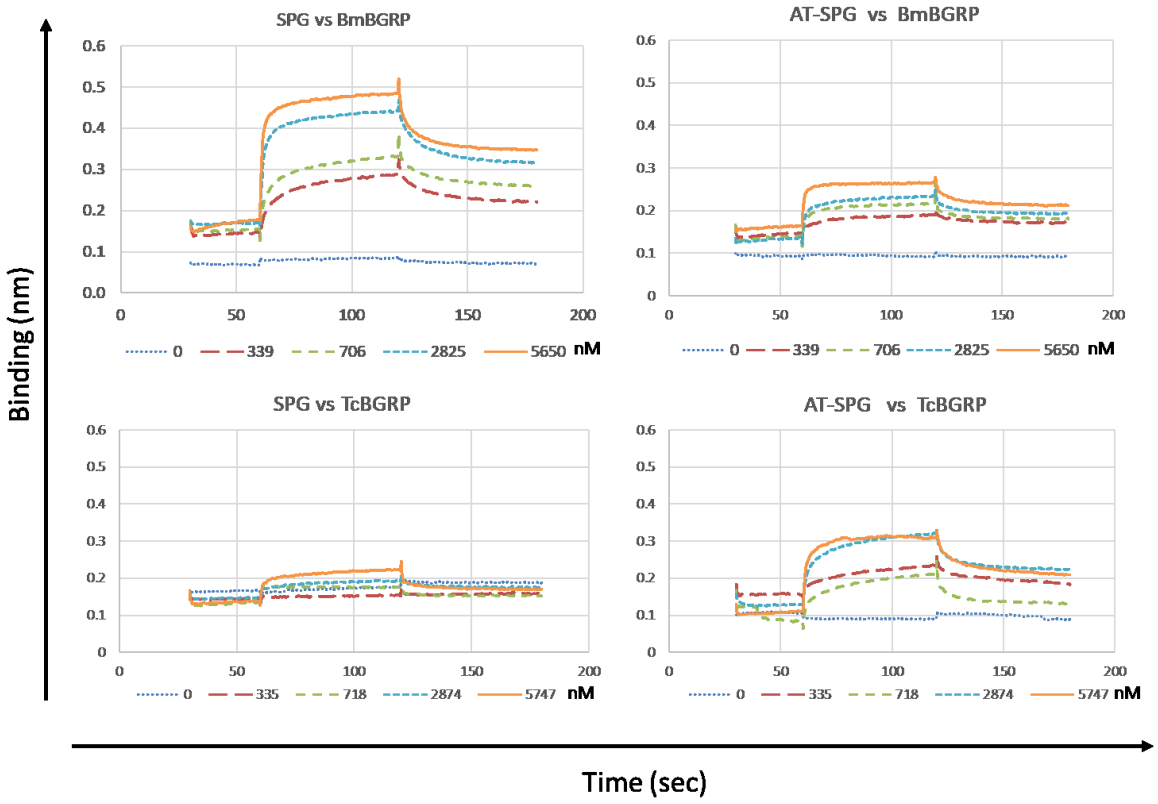

Figure 7. The binding affinity measurements of BmBGRP and TcBGRP to SPG or AT-SPG on BG-conjugated biosensors. KD, Ka, and Kd were measured and were calculated using the BLitz system. All experiments were performed in at least two independent occasions in duplicate readings. The X-axis and Y-axis depict the time in seconds and the binding in nm, respectively.

Table 1 Binding kinetics of BGRPs to SPG or AT-SPG

β-glucan	BGRP	Conc. (nM)	K_D (μM)	ka (1/Ms)	kd (1/s)	Rmax	R equilibrium
SPG	Bm	5650	0.29	2.09×10^{5}	6.16×10^{-2}	0.2626	0.2496
	Tc	5747	1.77	7.28×10^{4}	1.29×10^{-1}	0.09497	0.07264
AT-SPG	Bm	5650	0.20	3.40×10^{5}	6.79×10^{-2}	0.07618	0.07358
	Tc	5747	0.07	6.63×10^{4}	4.71×10^{-2}	0.1853	0.1649

The binding of BmBGRP and TcBGRP to sensor chip conjugated with SPG or AT-SPG was monitored using Bio-Layer Interferometry method. The binding of various concentrations of BGRPs from 339 nM to 5.7 μM, and dissociation of BGRP from SPG or AT-SPG was analyzed for 90 seconds in each period. The K_D was calculated by binding/dissociation kinetics at every concentration of BGRPs as shown in Figure 7.

3. Discussion

Bm and Pi showed consistently higher binding to native BGs, which have helix conformation. This binding specificity was confirmed by reverse experiment using BG competitors in liquid phase prior to interacting with solid-phase BGs on ELISA plate.

The binding ability of Bm and Pi to alkaline-treated BG was also observed either in solid phase or in liquid phase. Even the alkaline-treated BGs partially possess helical conformation in the solution [10]. Bm and Pi may bind to the scattered helical portion remaining in the AT-SPG and AT-laminarin. In contrast, Tm and Tc had no binding ability to the helical conformation. These results strongly suggest that Tm and Tc BGRP failed to recognize tightly spiraled glucosyl-linkage in the 1,3-β-D-glucan strands.

A report revealed that the ligand BG structure co-crystalized with PiBGRP was triple-helical in conformation [11]. This evidence is consistent with the present study that PiBGRP preferentially binds to triple helical conformation of BGs. Another report also supports the conformational dependency of BmBGRP, because silkworm (*Bombyx mori*) larvae fluid showed higher sensitivity to the triple helical BGs than the alkaline-treated BGs in melanin formation triggered by BG and BGRP interaction [12].

In case of TcBGRP and TmBGRP, these proteins bound to alkaline-treated BGs, but not to native BGs in both solution and solid-phase. It was reported that alkaline treatment would form partially opened helix conformation in BG [10, 13].

The conversion between helix and random coiled conformers can be mediated by different chemical or physical treatments [13]. Treatment of the helical SPG with NaOH has been used to prepare the disordered forms [13, 14]. Aketagawa et al. [15] suggested that treatment of SPG with NaOH alters the triple-helix to single chains [14]. This mechanism implies that immediately after treatment with NaOH, the molecular weight should be one-third of the untreated glucan. However, experimental evidence has shown that denatured SPG has the same molecular weight as untreated SPG [15]. An alternative explanation that is consistent with the experimental observations regarding molecular weight would be that NaOH treatment results in a partially disordered helix rather than completed strand separation.

It was reported that for glucans with different conformation but with the same degree of polymerization, the triple-helix is 100 to 1000 times less potent than the single-helix in the activation of limulus coagulation including factor G [15]. It was speculated that limulus amebocyte lysate (LAL) activity would be dependent on the degree of partial opening of the triple-helix after NaOH treatment. More stable conformers with different degrees of strands opened with aniline blue, and their relationship with LAL activation were analyzed [10]. These studies suggested that, for both low and high molecular weights of glucan, conformations with a higher degree of partial opening

(single helix structure) were more effective in activating the LAL assay. It was demonstrated that there might be a gradient of activity between conformers with a greater degree of opening and the triple-helix forms.

Although insects or invertebrates do not have acquired immunity against pathogens, they are able to recognize the molecules on the pathogenic microorganisms using pattern recognition receptor molecules [1]. LAL assay and silkworm larvae plasma (SLP) reagent set (Wako Pure Chemical Industries, Ltd.) used for the detecting (1→3)- β -D-glucan showed that BG-binding proteins and BGRP are among the well-known pattern recognition receptor molecules [5, 16]. Among the recognition receptor proteins, BGRPs are well-characterized molecules as their 3-D structures are observed clearly in X-ray crystallography and NMR [11, 17].

In the surface plasmon resonance (SPR) analysis, the NaOH-treated laminarin, which is a partially opened helical conformer, showed impaired affinity to β-GRP N-terminal protein from *Bombyx mori* [17], indicating that β-GRP N-terminal portion binds the triple-helical structure of β-1,3-glucan. They studied the time-dependent recovery of the binding affinity of NaOH-treated laminaran to β-GRP after neutralization, and confirmed that β-GRP preferably binds the refolded triple helical structure of laminarin [17].

In the present study, the binding specificity of Bm and Pi BGRPs was similar to the findings of a former study using β-GRP N-terminal protein from *Bombyx mori* [17]. However, a different specificity was observed by Tc and Tm in this investigation. The Tc and Tm BGRP showed less binding to the native form of laminarin and SPG, and preferential binding to NaOH-treated laminarin and SPG possessing partially opened helix conformation. In the BLitz analysis, the binding kinetics of BmBGRP and TcBGRP were examined by pre-loading SPG or AT-SPG to the biolayer interferometry sensor. The KD of BmBGRP to SPG and AT-SPG did not change, but Rmax of BmBGRP to SPG reduced from 0.26 to 0.07 in the interaction with AT-SPG (Table 1), suggesting that the binding site for BmBGRP on SPG reduced with alkaline-treatment. On the contrary, the KD of TcBGRP to SPG and AT-SPG was 1.77 and 0.71 μM, respectively, and Rmax of TcBGRP to SPG and AT-SPG increased from 0.09 to 0.18. These results suggest that NaOH treatment of triple helical BG resulted in conformational change, which is a good target for TcBGRP.

From the results of the amino acid sequence alignment of the four BGRPs tested, seven amino acid residues were obviously different and consisted of -DYFDGKNK- in TcBGRP and TmBGRP, while PiBGRP and BmBGRP had -IKDG- instead. It was reported that the amino acid residues of Y78, G83, G85, and R87 on PiBGRP interact with the glucose residues of triple helical (1→3)-β-D-glucan [11]. These amino acid residues reside around -IKDG- on Pi and Bm BGRPs, while Tc [18] and Tm [19] have -DYFDGKNK- (Figure 8). The binding specificities of Tc BGRP and Tm BGRP to the opened helical glucans might correlate with the stretched peptide portion sandwiched with Y78 and G85 of Pi BGRP corresponding to Y85 and G96 of Tc and Tm (Figure 8).

The preferential higher reactivity to NaOH-treated (1→3)-β-D-glucan is demonstrated in the other innate immune system in horseshoe crab [14]. The limulus amebocyte lysate (LAL) requires alkaline-treatment of the test samples before measuring water soluble (1→3)-β-D-glucans. LAL is less reactive to triple helical BG [20]. Therefore, it is likely that the innate immune system against (1→3)-β-D-glucan may have diverse recognition systems in response to different conformations of polysaccharides to accomplish host defense against various microorganisms.

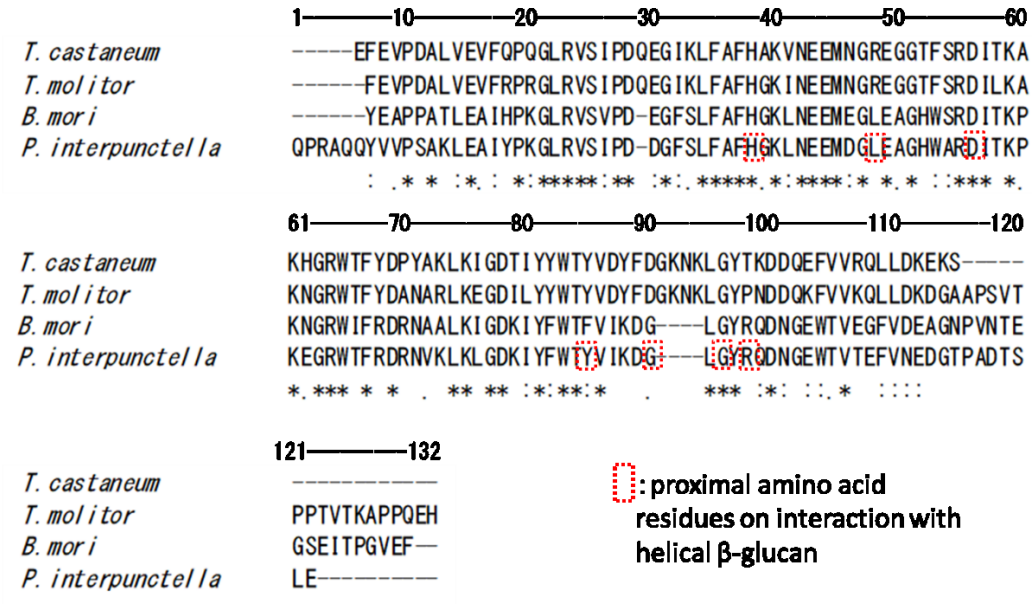

Figure 8. Sequence alignment of N-terminal domains of BGRP from *Plodia interpunctella (P. interpunctella)*, *Bombyx mori (B. mori)*, *Tenebrio molitor (T. molitor)*, and *Tribolium castaneum (T. castaneum)*

4. Materials and Methods

4.1. Insect larvae

Bombyx mori (Kinsho) and *Tenebrio molitor* were purchased from Kogensha (Nagano, Japan) and Asahi Pet (Yokohama, Japan), respectively. *Plodia interpunctella*, and *Tribolium castaneum* were kindly supplied by Dr. Akihiro Miyanoshita and Dr. Taro Imamura, National Food Research Institute (Tsukuba, Japan).

4.2. β-glucans

Sonifilan (Schizophyllan, SPG) and laminarin from *Laminaria digitata* were purchased from Kaken Pharmaceutical Co., Ltd and Sigma-Aldrich (St. Louis, MO). The laminarin solution was prepared by solubilizing in pyrogen–free distilled water at 10 mg/ml. For alkaline treatment, β-glucan neutral solutions, SPG, and laminarin, were mixed with equal volume of 1 M NaOH, followed by neutralization by diluting with 0.1 M Tris-HCl buffer (pH 8.0) to prepare 1 mg/ml of alkaline-treated β-glucans, referred as AT-SPG and AT-laminarin, respectively.

4.3. Preparation of BGRP-Fc molecules

We prepared the recombinant carbohydrate recognition domain of BGRP conjugated with human IgG Fc protein. The various BGRP cDNAs from *B. mori* [21], *T. molitor* [19], *P. interpunctella*[11], and *T. castaneum*[18] were amplified by PCR using KOD polymerase and specific oligonucleotide primers listed in Table 2. These cDNAs were inserted into pDisplay vector (Invitrogen), which was already ligated with human IgG1 Fc cDNA. The expression vectors were

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transduced into 293T cells using the Lipofectamine LTX (Invitrogen). BGRP-Fc proteins were isolated from a culture supernatant of 293T cells. The protein concentrations of the recombinant BGRP-Fc proteins were determined by sandwich ELISA using anti-human IgG-Fc (Jackson Laboratory), horseradish peroxidase (HRP)-conjugated anti-Hemagglutinin -tag monoclonal antibody (Santacruz) , and purified soluble dectin-1-Fc proteins possessing Hemagglutinin-tag as capture antibody, detection antibody, and standard protein, respectively.

Table 2 PCR primers for cDNA inserted to Fc-fusion expression vector plasmids

BGRP CRDs	Primers	Sequence
Bm	Forward	5′-CCAGATCTTACGAGGCACCACCGGCCAC-3′
	Reverse	5′-GCGGATCCGAATTCTACTCCTGGTGTTAT-3′
Pi	Forward	5′-GAGGATCCCAGCCGCGTGCGCAGCAGTAC-3′
	Reverse	5′-GACCTGCAGCCCTCGAGACTCGTGTCAGCCGG-3′
Tc	Forward	5′-GCGGATCCGAGTTTGAAGTTCCGGATGCT-3′
	Reverse	5′-GACCTCGAGCTAGACTTTTCTTTGTCTAGTAA-3′
Tm	Forward	5′-GCCAGATCTTTTGAGGTGCCAGATGCTTTG-3′
	Reverse	5′-GCCGGATCCGTGTTCTTGCGGTGGAGCCTT-3′

4.4. Preparation of BGRP-CRD

Hexahistidine-tagged carbohydrate recognition domain of BGRPs was prepared using cold shock-promoted protein expression by *E. coli* BL21. The PCR products of the N-terminal portion of BGRP were inserted into the multiple cloning site of pCold-I (TaKaRa Bio Inc., Japan). This vector is capable of expressing a target protein at low temperature (15 ℃) using a cold shock promoter (cspA). It was constructed by the insertion of BGRP cDNAs into the multiple cloning sites of a cold shock vector pCold-I (TaKaRa Bio Inc., Japan). The construct was composed of the $(His)_6$ - tag and BGRP. For expression of Bombyx mori BGRP, a DNA fragment encoding Tyr1-Phe119 was cloned into pCold-I vector. For *P. interpunctella*, Gln1-Glu117, *T. castaneum*, Glu1-Ser110, *T. molitor*, and Phe1-His126, each expression plasmid was transformed into the *E. coli* strain BL21(DE3). The transformed cells were grown in LB medium at 37 ℃ and induced with 0.1 mM isopropyl β-D-thiogalactoside (Wako) for 24 h at 15 ℃. The harvested cells were suspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl and then were sonicated. After centrifugation, the supernatants were collected and applied to a Co Sepharose column (Clontech) equilibrated with PBS (8 mM Na_2HPO_4, 1 mM KH_2PO_4, 137 mM NaCl and 3 mM KCl, pH 7.4). After washing the column with PBS, the proteins were eluted with PBS containing 500 mM of imidazole. *4.5. Binding assay of BGRP-Fc to β-glucans by ELISA*

SPG and AT-SPG, and laminarin and AT-laminarin were diluted with Tris-HCl buffered saline (pH 8.0) at 1 and 10 μg/ml respectively, then coated on 96 well ELISA plate (Nunc maxisorp plate, NUNC) by incubating overnight at 4 °C. The unbound excess β-glucans were washed off with PBS containing 0.05% of Tween 20 (PBST), and the plate was covered with PBS containing 0.5% of BSA (BPBS) for 2 h at room temperature (23℃±4℃). After blocking, various concentrations of BGRP-Fc proteins (0 to 100 ng/ml) were added to each well and incubated for 1 h at room

temperature. The bound BGRP-Fc proteins were detected with anti-human IgG Fc antibody conjugated with HRP and TMB substrate solution (Kirkegaard & Perry Laboratories, Inc.), and 1 M phosphoric acid. Absorbance at 450 nm after subtraction of OD630 was measured by microplate reader (Corona MTP-450, Tokyo, Japan).

4.6. Competitive ELISA using liquid phase of β-glucans

SPG and AT-SPG, and laminarin and AT-laminarin were diluted in Tris-HCl buffered saline (pH 8.0) at 10 μg/ml, then coated on 96 well ELISA plate (Nunc maxisorp plate, NUNC) by incubating overnight at 4 °C. The unbound excess β-glucans were washed off with PBST, and the plate was covered with PBS containing BPBS for 2 h at room temperature. In parallel, the β-glucans solution (0 to 100 μg/) in BPBS was mixed with each BGRP-Fc for 1 h at room temperature. The BGRP-Fc and β-glucan solution was added to the ELISA plate precoated with various β-glucans. The bound BGRP-Fcs to the solid phase β-glucans were probed with anti-human IgG Fc antibody conjugated with HRP. The enzyme activity was monitored by adding TMB substrate and 1 M of phosphoric acid. Absorbance at 450 nm after subtraction of OD630 was measured by micro plate reader (Corona MTP-450, Tokyo, Japan).

4.7. Binding affinity studies

Measurements of the association and dissociation rates of BGRP were carried out using direct binding of BGRPs to SPG-conjugated biosensors. The sensor chip was prepared by loading biotinylated SPG (Biotin-SPG) on the Streptavidin-biosensor (Fortebio, Cat no. 18-5095). AT-SPG biosensor was prepared by loading and neutralizing alkaline-treated SPG with 0.1 M of Tris-HCl buffer (pH 8.0) of biotin-SPG. All readings (KD, Ka, and Kd) were generated using Blitz system and binding graphs were re-plotted using Microsoft Excel 2010. KD was calculated automatically by the software where KD = Kd/Ka. Statistical error for Ka and Kd was calculated by the software based on the replicate experiments. The calculated KD readings did not show statistical error.

5. Conclusions

BGRPs from insects have at least two types of specificity for the different conformations of (1→3)-β-D-glucans. BGRPs from Lepidoptera, Bm, and Pi, tend to interact with the triple-helical conformation. On the contrary, BGRPs from Coleoptera, Tm, and Tc, preferentially bind to alkaline-denatured ultrastructure. These results suggest that innate immune system of insects distinguishes the ultrastructural changes of (1→3)-β-D-glucans.

Author Contributions: Y.A., K.I., D.Y., N.M., and N.O. designed the study. Y.A. and M.I. wrote the manuscript. M.I., T.K., and J.T., prepared the BGRP. Y.A. and M.I. checked the data and performed statistical analyses. Y.A. and M.I. contributed to data collection. All authors read and approved the final manuscript.

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