



Research paper

A sensitive sandwich ELISA to measure (1→3)-β-D-glucan levels in blood

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ARTICLE INFO

Article history:

Received 24 May 2010

Received in revised form 12 November 2010

Accepted 15 December 2010

Available online 22 December 2010

Keywords:

(1→3)-β-D-glucan

Horseshoe crab-clotting reaction

Sandwich ELISA

Recombinant protein

ABSTRACT

A highly sensitive (1→3)-β-D-glucan (β-glucan)-specific sandwich ELISA was developed using a fragment of recombinant horseshoe crab factor G protein. The factor G fragment, which was expressed in *Escherichia coli*, contains a QQWS motif, two β-glucan-binding domains, and an additional N-terminal cysteine residue. The sensitivity of our ELISA was comparable to a conventional (1→3)-β-D-glucan detection method using a horseshoe crab-clotting reaction such as an amebocyte lysate-based assay. In addition, the β-glucan levels measured by our sandwich ELISA in plasma samples showed a good correlation with those measured by the amebocyte lysate-based assay. In the case of our sandwich ELISA, it is not necessary to pre-inactivate interfering substances in plasma samples that is essential for the conventional amebocyte lysate-based assay. Moreover, the assay time of the ELISA method is much shorter than that of the amebocyte lysate-based assay. Because of these advantages, the ELISA system will be more suitable for high-throughput analysis in clinical laboratories using general clinical auto-analyzers.

β-glucan is a typical biomarker for fungal infections and the measurements of β-glucan levels by our ELISA could be useful for the diagnosis of fungal infections.

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1. Introduction

(1→3)-β-D-Glucan (β-glucan) concentration in peripheral blood is a diagnostic marker for a fungal infection (Obayashi et al., 1995). β-Glucan is a component of the cell wall of most pathogenic fungi, although there are some exceptions like *Zygomycetes* which do not have it (Obayashi et al., 2008). Detection or quantitative analysis of plasma β-glucan is generally carried out with assay systems based on the coagulation cascade reaction of the horseshoe crab amebocyte (Obayashi et al., 1995; Mori et al., 1997; Tamura et al., 1994; Moro et al., 2003; Ostrosky-Zeichner et al., 2005). The coagulation cascade consists of many protein factors. β-Glucan activates factor G, β-glucan sensitive serine protease zymogen, then activated factor G convert proclotting enzyme to clotting enzyme which convert coagulogen to coagulin, and this induces the gelation of the horseshoe crab blood (Muta et al., 1995). Conventional β-glucan analysis

systems utilize this cascade and use the semi-purified amebocyte extract, although the final detection method varies, i.e., turbidimetric method (Mori et al., 1997) and chromogenic method (Tamura et al., 1994; Moro et al., 2003).

Amebocyte lysate-based β-glucan detection systems have the sensitivity to detect the fungal infection in patients. However, these systems have some weak points due to the complexity of semi-purified amebocyte extract. For instance, human blood also has a similar clotting cascade consisting of similar serine proteases and protease inhibitors. These human factors interfere with the cascade reaction, and it is, therefore, necessary to pre-inactivate interfering substances in blood before running the assay. Moreover, since endotoxins also activate this cascade, it is necessary to silence the endotoxins in the sample to increase the specificity of the measurement.

To overcome these complications, we tried to construct a β-glucan assay system using purified proteins. There have been several reports on β-glucan ELISA systems using purified β-glucan-binding proteins such as mouse Dectin I (Brown and Gordon, 2001; Graham et al., 2006), anti-β-glucan antibody

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(Meikle et al., 1994; Sander et al., 2008), and *Tachypleus tridentatus* β -glucan-binding protein (T-GBP) which is different from factor G protein (Tamura et al., 1996; Tamura et al., 1997). The systems using anti- β -glucan antibody (Meikle et al., 1994; Sander et al., 2008) or mouse Dectin I (Brown and Gordon, 2001; Graham et al., 2006) could not detect even 100 pg/mL β -glucan. The detection limit of the assay using T-GBP (Tamura et al., 1996; Tamura et al., 1997) was also far from the cut-off value for diagnosis of fungal infection and five times higher than the required level. Consequently, the sensitivities of all these methods were inferior to that of the amebocyte lysate-based assay.

In this paper, we report the development of a highly sensitive and simple sandwich ELISA for β -glucan detection. Our ELISA system, using a recombinant factor G fragment, showed a sensitivity similar to that of the horseshoe crab amebocyte lysate-based systems and potential utility in the diagnosis of fungal infection.

2. Materials and methods

2.1. Materials

The recombinant mouse Dectin I was purchased from R&D Systems (USA), and anti- β -glucan monoclonal antibody (IgG, Kappa Light) was from Biosupplies (Australia). Lentinan (Ajinomoto, Japan), Shizophylan (Kaken Pharmaceutical, Japan), Curdlan (Wako, Japan), Barley β -glucan (Wako, Japan), Dextran (Wako, Japan), and mannan (Wako, Japan) were used for the sandwich ELISA. β -Glucan was dissolved in 0.1 M NaOH and diluted with β -glucan free water (Water for injection, Ohtsuka pharmaceutical, Japan).

2.2. Nucleotide and deduced amino acid sequence of *Limulus polyphemus* factor G-subunit α

Poly(A)+RNA was extracted from the amebocytes of horseshoe crabs (*Limulus polyphemus*). Full-length cDNA was obtained from amebocyte mRNA using rapid amplification of cDNA ends (RACE) PCR using the *Tachypleus tridentatus* sequence as a probe. cDNA of factor G-subunit α (*Limulus polyphemus*) was sequenced with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Japan). The primer was designed from the *Tachypleus tridentatus* factor G-subunit α sequence.

2.3. Expression of recombinant β -glucan-binding proteins

Poly(A)+RNA was extracted from Silkworms (*Bombyx mori*, Katakura, Japan), pyralidmoth (*Plodia interpunctella*, Sumika Technoservice, Japan) using mRNA purification kit (GE Healthcare, Germany). A cDNA library was constructed from the Poly(A)+RNA by reverse transcription and was used as a template for PCR. Regions of the β -glucan-binding domain of each protein are already reported, and we constructed β -glucan-binding protein fragments as follows: *Bombyx mori* (Bm β GBP); amino acid residues 1–479, deduced molecular weight 54 kDa (Ochiai and Ashida, 1988, 2000), *Plodia interpunctella* (Pi β GBP); amino acid residues 181–471, deduced molecular weight 33 kDa (Fabrick et al., 2003; Fabrick et al., 2004). cDNAs encoding the

designated regions were amplified by PCR. The designed primers were as follows:

5'-tacgaggcaccaccg-3' (Bm β GBP-F), 5'-gttaaagttttgcaata-3' (Bm β GBP-R),

5'-gaggtcaagtttcctgaag-3' (Pi β GBP-F), 5'-gtcagagtc-tatgcgtg-3' (Pi β GBP-R).

The PCR products were cloned into the pTrcHis2-TOPO expression vector and expressed in *Escherichia coli* (*E. coli*) strain DH5 α (Invitrogen, USA). The expression vector is designed to express target proteins with additional N-terminal three amino acid residues (Met-Ala-Leu) and C-terminal 29 amino acid tag residues (comprising the c-myc tag, the six-histidine tag, and linker regions). All β GBPs expressed here have these sequences. Bacteria were cultured in Luria-Bertani medium. The cultures were grown at 37 °C, and when OD_{600nm} of the cultures reached 0.8, protein synthesis was induced by the addition of isopropyl- β -D-thiogalactosylpyranoside to a final concentration of 1.0 mM. The cultures were incubated at 20 °C with vigorous shaking for 72 h, and bacterial cells were collected by centrifugation. To remove β -glucan of medium origin, cells were washed five times with 1/10 culture volume of 10 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl. Then, bacterial cells were suspended in 1/10 culture volume of 10 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 0.2% cationic detergent EX120 (Sanyo Chemical, Japan) and incubated at 20 °C for 30 min with gentle rocking. The supernatants were collected by centrifugation, and the β GBPs were purified according to the manufacturer's instructions using Ni-agarose (Wako, Japan). The Ni-agarose bound proteins were eluted with guanidine hydrochloride buffer, pH 7.5, and the buffer was exchanged to 20 mM Tris-HCl, pH 7.5 using Nanosep Omega (Pall, USA). β GBPs were labeled with peroxidase (POD) using Peroxidase Labeling kit-NH2 (DOJINDO Laboratories, Japan), according to the manufacturer's instructions. β GBP-PODs were purified by gel filtration on Wakosil 5Diol-200 (Wako, Japan). Labeling efficiency was approximately 15%.

2.4. Expression of recombinant factor G-subunit α and deletion constructs

Various fragmented molecules containing C-terminal region of *Limulus polyphemus* factor G-subunit α were designed and were expressed in *E. coli* as described in the earlier section. The isolated molecules were as follows: Fragment A (amino acid residues 252–668, deduced molecular weight 46 kDa), Fragment B (amino acid residues 406–668, deduced molecular weight 29 kDa), Fragment C (amino acid residues 543–668, deduced molecular weight 14 kDa) and Fragment D (amino acid residues 252–534, deduced molecular weight 31 kDa).

These constructs also had additional N-terminal three amino acids (Met-Ala-Leu) and C-terminal 29 amino acid residues comprising the c-myc tag, the six-histidine tag, and linker regions; both were derived from the expression vector. The N-terminally Cys added Fragment A (Cys/Fragment A) was also isolated which has Cys just after the vector derived N-terminal three amino acids. For the POD labeling of proteins, buffer was displaced with 20 mM Tris-HCl (pH 7.5), 1 mM (Tris

[2-carboxyethyl] phosphine HCl) using Nanosep Omega, and proteins were labeled with POD (Roche, Mannheim, Germany) by the maleimide method. POD-labeled fragment proteins were purified using Ni-agarose, and further purified on a DEAE-5PW column. Labeling efficiency was around 20% (Fragment A, B, C, D) and around 40% (Cys/Fragment A).

2.5. Protein analysis

Protein concentrations were determined by BCA™ (bicinchoninic acid) Protein Assay kit (ThermoFisher Scientific, USA) with bovine serum albumin as a standard. SDS-PAGE was performed in a 10–20% SuperSep™ (Wako, Japan) pre-cast gel under reducing conditions, and proteins were stained with Coomassie Brilliant Blue.

2.6. Determination of β -glucan concentration by ELISA system

We used lentinan as β -glucan standard. Microtiter ELISA plates (ThermoFisher Scientific, USA) were coated with 50 μ L of purified 0.1 μ M β -glucan-binding protein in 50 mM MOPS buffer, pH 7.0, and incubated for 1 h at 25 °C. β -Glucan samples (50 μ L) diluted in ELISA buffer (50 mM Tris–HCl, 150 mM NaCl, 50 mM MgCl₂, 0.5% HSA), were added, and the plates were incubated for 1 h at 25 °C. Plate wells were then treated with 0.1% HSA in 50 mM MOPS, pH 7.0, for 1 h at room temperature to block further protein adsorption. Unbound β -glucan was removed by washing with 0.05% Tween-20 in PBS (PBS-Tween) buffer, and then 50 μ L of POD conjugated recombinant protein (0.1 μ g/mL in ELISA buffer, pH 7.4, containing 1% skim milk) was added and incubated for 1 h at 25 °C. Unbound recombinant protein-POD was removed by washing three times with PBS-Tween, and 50 μ L of TMB peroxidase substrate (3,3', 5,5'-tetramethyl-benzidine, Wako, Japan) was added and incubated for 30 min at 37 °C. The reaction was stopped by 50 μ L of 1 M phosphoric acid, and analyzed on a Microplate Reader at 450 nm (Ultra evolution, TECAN, Switzerland).

To determine the lower detection limit, serial dilutions of standard were measured using five replicates, and the mean and the standard deviation (SD) of each dilution was calculated. The detection limit was defined as the lowest concentration at which the mean of the β -glucan sample minus 2SD did not overlap with the mean of a negative sample plus 2SD (Kagebayashi et al., 2009).

2.7. Measurement of plasma β -glucan

For the measurements of β -glucan level in human plasma, we used commercially available β -glucan-positive human plasma samples purchased from Discovery Life Sciences, Inc. The collection of human samples was declared to be carried out under the protocol approved by FDA. These plasma samples were stored at -20 °C. For the measurement of β -glucan content, samples were diluted 1/10 with ELISA buffer. The β -glucan level was also measured by β -glucan Test Wako, in combination with Toxinometer MT358 (Wako, Japan), which is based on the turbidimetric kinetic method. β -Glucan concentration in plasma was calculated by standard curve prepared with lentinan solution.

2.8. Preparation of culture supernatant

Culture supernatant of fungi and Gram-negative bacteria were used as crude β -glucan and crude endotoxin. *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Candida albicans* (Department of Biotechnology National Institute of Technology and Evaluation, Japan) were cultured in D-MEM medium, pH 6.0 (Wako, Japan) at 30 °C. *Pseudomonas aeruginosa* and *E. coli* (American Type Culture Collection, USA) were also cultured in D-MEM medium, pH 7.0, at 37 °C. To obtain β -glucan and endotoxin, mediums were collected at growing phase of the organism as reported previously (Miyazaki et al., 1995). After centrifugation, the supernatants were filtered through a 0.22 μ m sterilization filter.

3. Results

3.1. Screening of β -glucan-binding proteins

To construct a highly sensitive ELISA system for β -glucan detection, we screened several proteins which were reported to have β -glucan-binding activity. In this screening, we examined five β -glucan-binding proteins (β GBPs), i.e., Bm β GBP (beta-1,3-glucan recognition protein, 1–479 aa) from *Bombyx mori*, factor G-subunit α fragment (406–668 aa) from *Limulus polyphemus*, Pi β GBP (181–471 aa) from *Plodia interpunctella*, mouse Dectin I, and anti-glucan antibody. Three of them, Bm β GBP, factor G-subunit α fragment and Pi β GBP were recombinant proteins designed in our laboratory and all recombinant proteins contained β -glucan-binding domains.

We examined the potential of these five β GBPs for sandwich ELISAs, one for a capture protein on the microtiter well surface and one for a detection protein with a POD-label. All of the 25 combinations of these five β GBPs were assessed with absorbance value of POD activity and signal to noise (S/N) ratio (Fig. 1). A combination of factor G-subunit α fragment for both capture and detection was found to show a remarkably higher sensitivity than the other combinations with an absorbance of 412 (OD_{450nm} × 1000) that was 5.9 times higher than the next highest pair, and an S/N ratio 3.5 that was 2.5 times higher than the other pairs. For this reason, we selected the factor G-subunit α fragment for further development of sensitive assay system.

3.2. Selection of the most appropriate fragment of factor G-subunit α

We next investigated the variants of the factor G-subunit α from *Limulus polyphemus* to find more efficient fragments for the ELISA. We cloned the cDNA of *Limulus polyphemus* factor G-subunit α and determined the cDNA sequence and the amino acid sequence for the production of the recombinant factor G-subunit α and its variants. In Fig. 2, the amino acid sequences of factor G-subunit α of *Limulus polyphemus* and *Tachypleus tridentatus* are shown. The primary structure of *Limulus polyphemus* factor G-subunit α is a polypeptide of 668 amino acids (estimated molecular weight is 74.6 kDa) consisting of four domains, a glucanase like domain, a QQWS motif, glucan-binding domains 1 and 2 (Fig. 3A). It has 80.1% similarity in the total amino acids sequence and 82.5% similarity in the carboxyl-terminal glucan-binding domains

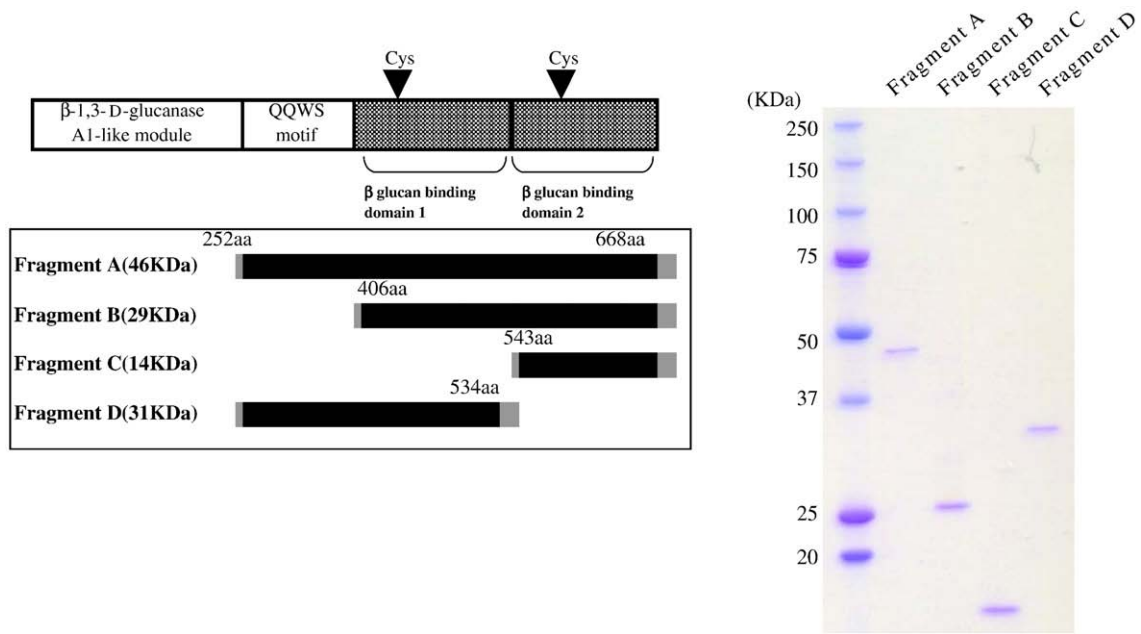


Fig. 3. Design of the truncated *Limulus polyphemus* factor G-subunit α molecules and its SDS-PAGE image. (A) Schematic representation of the factor G-subunit α and the truncated fragments. The positions of cysteine residue exist in each β -glucan-binding domain are indicated with (▼). The locations of the truncated fragments (Fragments A to D) in the factor G-subunit α are indicated by black bars. Shaded boxes indicate N and C-terminal additional sequences derived from the expression vector. (B) A SDS-PAGE image of the truncated fragments (Fragment A to D). Each protein (1 μ g) was applied to SDS-PAGE (10–20% gradient gel) and stained with Coomassie Brilliant Blue.

amino acid Tag sequence derived from the vector. A SDS-PAGE analysis of the purified fraction revealed that the all fragments consisted of a single major protein with an approximately expected size (Fig. 3B).

We examined the β -glucan-binding activity of the four fragments by a sandwich assay, and the results for all 16 combinations were displayed in Fig. 4. The most efficient combination was found to be a combination of Fragment A as both capture and detection molecule. Although the Fragment A (46 kDa) is the largest in four fragments and contains all of the

QQWS motif, β -glucan-binding domains of 1 and 2, the yield of purification from *E. coli* cell extract was the highest among the four fragments. Therefore, we chose this fragment for assembling our sandwich ELISA system as both immobilized capture molecule and POD-labeled detection molecule. The detection limit of the assembled sandwich ELISA against lentinan was about 1 ng/ml. This sensitivity was relatively high compared to the general ELISA assay but it was estimated to be more than 50 times less sensitive than that of conventional horseshoe crab amebocyte lysate-based assays.

		POD labeled Fragment							
		A		B		C		D	
		OD _{450 nm}	S/N	OD _{450 nm}	S/N	OD _{450 nm}	S/N	OD _{450 nm}	S/N
Plate coated Fragment	A	353	4.8	8	1.2	32	1.2	411	2
	B	270	3.1	3	1.1	132	1.5	258	3.2
	C	114	1.6	8	1.4	24	1.5	260	2.3
	D	55	1	84	1.6	51	1.1	340	1.9

Fig. 4. Screening of the most suitable factor G-subunit α fragment for the sandwich ELISA. The sensitivities of the ELISA constructed with the factor G-subunit α fragments (Fragments A to D) in all combinations were listed. The data were obtained using 1000 pg/mL lentinan as a model sample of β -glucan and the resultant absorbances at 450 nm (OD_{450nm} × 1000) and the signal to noise (S/N) ratios were indicated.

3.3. Development of a highly sensitive sandwich ELISA system

To optimize the ELISA assay system, we further investigated the property of “Fragment A.” At first, we examined the site of POD-conjugation in Fragment A. Since POD was expected to be introduced in either or both of two cysteine residues present in the β -glucan-binding domains of Fragment A (Fig. 3A), the introduced POD might cause the inhibition of β -glucan binding by structural hindrance. Therefore, we introduced an additional cysteine residue at the N-terminal of Fragment A to enhance the labeling efficiency and to reduce the possible binding inhibition. By introducing this modification, The POD-labeling efficiency of additional cysteine introduced Fragment A (Cys / Fragment A) was improved to twice of the original Fragment A. We extensively purified the POD-labeled Cys/Fragment A by Ni-NTA affinity chromatography and DEAE-5PW ion exchange chromatography so that neither free POD nor free Cys/Fragment A remain. We constructed a sandwich ELISA using the purified POD-labeled Cys/Fragment A as a detection molecule in combination with the parental Cys/Fragment A as a capture molecule.

The standard curve of the ELISA made with lentinan as standard β -glucan is shown in Fig. 5. As you can see in this concentration response curve, this ELISA system can measure wide range of β -glucan. The absorbance of 17 pg/mL lentinan minus $2 \times$ SD was still higher than that of negative control plus 2SD, and this concentration corresponds to the limit of detection for lentinan in this system. The detection limit of β -glucan Test Wako, a commercially available amoebocyte lysate-based assay was reported to be 17 pg/mL of lentinan. To our knowledge, our sandwich ELISA system reported here is the only β -glucan ELISA system with comparable sensitivity to the conventional horseshoe crab amoebocyte lysate-based assay.

3.4. Specificity of sandwich ELISA for β -glucan recognition

The assay systems described here have been evaluated using lentinan as a standard β -glucan. Lentinan is produced as

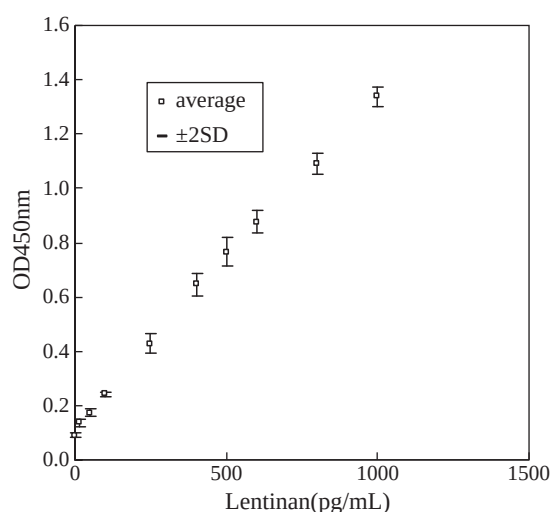


Fig. 5. Standard curve of the sandwich ELISA using Cys/Fragment A. Each spots were results from five measurements. The mean values (□) and the $2 \times$ standard deviations (2SD) (–) of each sample were indicated. The detection limit was determined to be the mean value of 5-replicates of zero standard plus 2SD value. The resultant detection limit of β -glucan level was determined to be 17 pg/mL.

pharmaceutical and is rather uniform in molecular size and structure. In contrast β -glucan contained in the blood sample of mycosis patient is derived from cell wall of pathogenic fungi and is a mixture of molecules with various molecular sizes and structures. Therefore, we investigated the reactivity of various β -glucans of different origin and structures. Hara et al. reported that representative fungi (*Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Candida albicans*) release β -glucan into culture medium during proliferation, and we used culture fluids of each fungi as crude β -glucan of pathogenic fungal origin. In addition, we also examined specificity of sandwich ELISA using purified glucans with different conformation.

As shown in Table 1, our sandwich ELISA was found to react specifically with glucans with (1 $\rightarrow$ 3)- β linkage (Lentinan, Schizophyllan, Barley β -glucan, Curdlan, crude β -glucan of *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Candida albicans*) and not to react with glucans without (1 $\rightarrow$ 3)- β linkage (Dextran, mannan) and crude endotoxin from *Pseudomonas aeruginosa* and *Escherichia coli*). This specificity was consistent with amoebocyte lysate-based assay.

3.5. Measurement of the native β -glucan in plasma samples

Next, we examined whether the sandwich ELISA can be adopted for the measurement of β -glucan in human plasma. The recoveries of β -glucan spiked into human plasma were examined to check the interfering effect of plasma component on the ELISA, and 72.8–93.9% of the input β -glucan was recovered by the assay (Table 2).

Conventional horseshoe crab amoebocyte lysate-based assays are widely used to measure blood β -glucan levels and they utilize the proteolytic enzyme cascade reaction similar to human blood coagulation cascade. Therefore sample pretreatment such as dilution-heating treatment or alkaline treatment is necessary to inactivate proteases and protease inhibitors in human blood. In contrast, since the sandwich ELISA assembled with a recombinant factor G fragment does not utilize proteolytic enzyme cascade reaction, sample pretreatment for inactivate proteases and protease inhibitor might be not necessary. The β -glucan level of human plasma samples was assayed by the sandwich ELISA

Table 1
Reactivity of the sandwich ELISA to various β -glucans and non- β -glucans.

Name	Glycosidic linkage	Absorbance (450 nm)*
Lentinan	β -(1 $\rightarrow$ 3),(1 $\rightarrow$ 6)	0.558 $\pm$ 0.017
Schizophyllan	β -(1 $\rightarrow$ 3),(1 $\rightarrow$ 6)	0.218 $\pm$ 0.014
Curdlan	β -(1 $\rightarrow$ 3)	0.412 $\pm$ 0.007
β -glucan (<i>Cryptococcus neoformans</i>)	β -(1 $\rightarrow$ 3),(1 $\rightarrow$ 6)	0.230 $\pm$ 0.022
β -glucan (<i>Aspergillus fumigatus</i>)	β -(1 $\rightarrow$ 3),(1 $\rightarrow$ 6)	0.323 $\pm$ 0.027
β -glucan (<i>Candida albicans</i>)	β -(1 $\rightarrow$ 3),(1 $\rightarrow$ 6)	0.407 $\pm$ 0.019
Dextran	α -(1 $\rightarrow$ 6)	0.075 $\pm$ 0.005
Mannan	α -(1 $\rightarrow$ 2),(1 $\rightarrow$ 3),(1 $\rightarrow$ 6)	0.066 $\pm$ 0.011
Endotoxin (<i>Pseudomonas aeruginosa</i>)	–	0.070 $\pm$ 0.003
Endotoxin (<i>Escherichia coli</i>)	–	0.089 $\pm$ 0.010

* Each value represents the means of replicated values $\pm$ SE and the experiment was independently repeated two times.

Table 2

Recovery test.

Added (pg/mL)	Measured (pg/mL)	Expected (pg/mL)	Recovery (%)
0	1.7		
50	40.1	51.7	77.6%
100	74.0	101.7	72.8%
200	148.1	201.7	73.4%
400	377.2	401.7	93.9%
1000	834.3	1001.7	83.3%

Low-level plasma was added with lentinan (50, 100, 200, 400, 1000 pg/mL).

with or without heat treatment at 70 °C for 10 min. In Table 3, the β -glucan levels obtained by the sandwich ELISA are shown. As expected, the pretreatment of samples did not influence the values of β -glucan; therefore, we can measure β -glucan in human plasma without sample pretreatment by the sandwich ELISA.

We also investigated the effect of heparin which is generally included in the plasma sample on the sandwich ELISA. Absorbance at 450 nm which represents the lentinan level in the ELISA was not changed significantly by the addition of heparin into the plasma sample proving not to interfere the sandwich ELISA. (Absorbance₄₅₀/heparin concentration (IU/mL): $0.477 \pm 0.031/0$ IU/mL, $0.416 \pm 0.018/3$ IU/mL, $0.499 \pm 0.062/6$ IU/mL, $0.497 \pm 0.034/12$ IU/mL). Since the results described above supported the potential use of our ELISA system for human plasma samples, we further compared the performance of our ELISA with that of the amebocyte lysate-based assay by doing a correlation study with β -glucan-positive human plasma samples. We compared β -glucan levels in various human plasma samples with the sandwich ELISA and β -glucan Test Wako, a conventional amebocyte lysate-based assay by turbidimetric kinetic method. As shown in Fig. 6, the measured β -glucan values showed a good correlation between these two methods.

These results strongly suggest the potential of our sandwich ELISA as an alternative β -glucan detection system for the clinical samples.

4. Discussion

In this article, we described the construction of a specific and sensitive sandwich ELISA for β -glucan. First, we screened proteins to find a β -glucan-binding molecule that is useful for the sandwich ELISA. We screened *Bombyx mori* β GBP, *Plodia interpunctella* β GBP, *Limulus polyphemus* factor G-subunit α , Dectin I, and anti- β -glucan antibody, and found that *Limulus*

Table 3

Effect of sample pretreatment.

	Absorbance (450 nm)*	
	Pretreatment(70 °C, 10 min)	Untreated
Plasma 1	0.079 ± 0.008	0.058 ± 0.004
Plasma 2	0.086 ± 0.004	0.079 ± 0.004
Plasma 3	0.624 ± 0.020	0.621 ± 0.025
Plasma 4	1.160 ± 0.099	1.160 ± 0.037
Plasma 5	1.040 ± 0.149	0.876 ± 0.034

*Each value represents the means of replicated values $\pm$ SE.

All plasma samples were diluted 10-fold before applying to the assay. The β -glucan levels of pretreated (70 °C for 10 min) samples were measured for the sandwich ELISA.

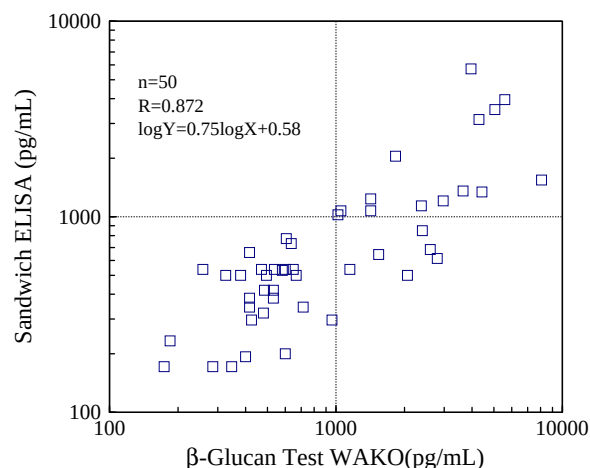


Fig. 6. Correlation of the detected levels of β -glucan in plasma samples between our sandwich ELISA and a conventional method, β -glucan Test WAKO. The β -glucan contents of a panel of 50 human plasma samples were measured by both the sandwich ELISA and the β -glucan Test WAKO, respectively. 1000 pg/mL lentinan corresponds to 35 pg/mL plasma β -glucan according to the instruction of β -glucan Test WAKO.

polyphemus factor G-subunit α , a component of the coagulation cascade of horseshoe crab amebocyte, has the highest binding activity. Then, we characterized the *Limulus polyphemus* factor G-subunit α by making deletion mutants and identified the region that is suitable for the ELISA assay. Within the deletion mutants, we found "Fragment A," a 46 kDa fragment with a QQWS region and two β -glucan-binding domains, is the best for detecting β -glucan. Molecules having more than two β -glucan-binding domains were supposed to be more potent in binding activity as reported previously (Markus et al., 1996). Therefore, we tried to make fragments with multiple binding domains but, despite the repeated trials, we could not obtain enough amounts of protein because of the extensive degradation of fragments. Finally, we introduced a cysteine residue in the N-terminal portion of the Fragment A (Cys/fragment A) to improve the POD-labeling efficiency and to reduce the possible β -glucan-binding inhibition by the coupled POD molecule. After optimizing the purification procedure of materials, we achieved a very low detection limit of 17 pg/mL β -glucan as lentinan in the sandwich ELISA using Cys/fragment A for capture and POD-labeled Cys/fragment A for detection. It has been thought that the high sensitivity of the horseshoe crab amebocyte lysate-based assay originates from the signal amplification by the multi-step cascade reaction. However, the detection limit achieved here is equal to that of the conventional horseshoe crab amebocyte lysate-based assay. Takaki et al. (2002) reported that a native factor G could bind to β -glucan with high affinity ($K_a = 1.5 \times 10^8 \text{ M}^{-1}$). These results suggest that the strong binding activity of factor G to β -glucan might be the key factor that is responsible for the high sensitivity of horseshoe crab amebocyte lysate assay and that high sensitivity can be achieved without the coagulation cascade reaction.

The sandwich ELISA has been evaluated with lentinan during development. However, the native β -glucan is known to be a mixture of diverse molecules with different molecular sizes and structures. Therefore, we tested our ELISA system against

several fungal β -glucans with divergent structures. As a result, we confirmed that our ELISA system could detect specifically the fungal β -glucan in the same way as the amebocyte lysate-based assay but without reacting with a negative control of endotoxin.

Furthermore, the procedure of the ELISA is simpler than the amebocyte lysate-based assay. Because the sandwich ELISA consists only two purified recombinant proteins and because the assay is accomplished without enzymatic cascade reactions, the pretreatment of the blood samples to inactivate the interfering factors is unnecessary. Indeed, there is no significant difference between β -glucan values measured with or without the pretreatment.

Finally, in order to evaluate our ELISA system, we compared the values of β -glucan in human plasma sample between the sandwich ELISA and β -glucan Test Wako, one of the commercially available amebocyte lysate-based assays. Overall, the values showed relatively good correlation ($r=0.87$), indicating the potential of our ELISA system as an alternative method for the β -glucan detection. To our knowledge, this is the only sandwich ELISA system for β -glucan having comparable sensitivity to amebocyte lysate-based assays. Although Tamura et al. (1997) reported a β -glucan ELISA system using T-GBP from *Tachypleus tridentatus*, sensitivity to pachyman was 100 pg/mL and was five times higher than the cut-off value for diagnosis of fungal infection (20 pg/mL for pachyman) (Obayashi et al., 2008). On the other hand, our sandwich ELISA achieved a very low detection limit of 17 pg/mL of lentinan, which is 1/18 of the cut-off value of β -glucan Test Wako (314 pg/mL in the case of lentinan). We believe the sensitivity of our system will be further enhanced by a highly purified binding protein or by further optimizing the blocking methods to reduce non-specific binding.

In addition to specificity and sensitivity, the simplicity and speed of the sandwich ELISA method make it potentially suitable for high-throughput diagnostic assays using an automated clinical analyzer, and this will increase the utility of this assay in the clinical laboratory.

Acknowledgements

We would like to acknowledge Dr. M. Warashina and Mr. T. Kitagawa for their helpful comments on earlier version of this manuscript.

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