ELSEVIER

Carbohydrate Research 295 (1996) 103–116

CARBOHYDRATE RESEARCH

Purification and characterization of a (1 → 3)-β-D-glucan-binding protein from horseshoe crab (*Tachypleus tridentatus*) amoebocytes

Hiroshi Tamura [a,b], Shigenori Tanaka [b], Toshio Oda [b], Yayoi Uemura [b], Jun Aketagawa [b], Yohichi Hashimoto [a,*]

[a] *Department of Biochemistry, Faculty of Science, Saitama University, 225 Shimo-okubo, Urawa 338, Japan*
[b] *Tokyo Research Institute, Seikagaku Corporation, Tateno 3-1253 Higashiyamato-shi, Tokyo 207, Japan*

Received 2 April 1996; accepted in revised form 29 August 1996

Abstract

A novel (1 → 3)-β-D-glucan-binding protein (T-GBP) has been purified from the amoebocyte lysate of the Japanese horseshoe crab, *Tachypleus tridentatus*. It is a basic protein (pI 9.2) which appears to be a homotetramer composed of subunits with an apparent mol wt of 168 000 and with an amino-terminal sequence (20 residues) KSGFILTAPKSLTLGRNNRL. T-GBP exerted an inhibitory effect on the (1 → 3)-β-D-glucan-initiated coagulation cascade reconstituted with purified preparations of factor G and the proclotting enzyme from the lysate. The binding of (1 → 3)-β-D-glucans to T-GBP was evaluated by measuring the residual amidolytic activity of the clotting enzyme, the product of the coagulation cascade, using Boc-Leu-Gly-Arg-4-nitroanilide as the chromogenic substrate. The binding specificity of a wide range of (1 → 3)-β-D-glucans and other polysaccharides towards T-GBP was expressed by the relative inhibition (%) of the activation of factor G, the first protease zymogen in the pathway, which is activated by binding to (1 → 3)-β-D-glucans. T-GBP was found to have a high affinity for linear (1 → 3)-β-D-glucans, e.g. pachyman, curdlan, and paramylon. It was able to bind to (1 → 3)-β-D-glucans with side-chain branches and mixed linkage such as schizophyllan, lentinan, laminarins, yeast β-D-glucan, and (1 → 3),(1 → 4)-β-D-glucans such as lichenin and barley β-D-glucan. Binding of pachyman to T-GBP was demonstrated by an enzyme-linked immunosorbent assay using a specific antibody (rabbit IgG) raised against T-GBP. © 1996 Elsevier Science Ltd.

Keywords: (1 → 3)-β-D-glucan; Horseshoe crab; Amoebocyte; (1 → 3)-β-D-glucan-binding protein

* Corresponding author. Tel.: +81-(0)48-858-3399; Fax.: +81-(0)48-858-3698.

PII S0008-6215(96)00214-5

1. Introduction

In 1968, Levin and Bang [1] reported that trace amounts of Gram-negative bacterial endotoxins were able to coagulate the amoebocyte lysates from the American horseshoe crab, *Limulus polyphemus*. Later, Iwanaga et al. [2], using an amoebocyte lysate of the Japanese horseshoe crab, *Tachypleus tridentatus*, established the biochemical mechanism of the coagulation process. The endotoxin-initiated coagulation pathway is believed to play a crucial role as a defense system against Gram-negative bacteria in these crustaceans.

More recently, Morita et al. [3] described an alternative coagulation pathway in *T. tridentatus* amoebocyte lysate, initiated by (1 → 3)-β-D-glucans, major cell wall constituents of many fungi, and operating as a hemostatic and defensive system against infection by fungi. They isolated and characterized the responsible protease zymogen, designated factor G from the lysate, which undergoes the β-D-glucan-dependent activation leading to the production of the clotting enzyme, which is a component of the endotoxin-initiated pathway [4]. This finding led us to develop a highly sensitive chromogenic assay for (1 → 3)-β-D-glucan using the crustacean protease cascade reconstituted with purified preparations of factor G and the proclotting enzyme (ProCE) [5].

In this paper, we report the purification and characterization of a novel (1 → 3)-β-D-glucan-binding protein (T-GBP), which was found during fractionation of *T. tridentatus* amoebocyte lysate. This high molecular-weight protein is a potent inhibitor for factor G activation by (1 → 3)-β-D-glucans. Several pieces of evidence are presented to demonstrate specific binding of (1 → 3)-β-D-glucans to T-GBP.

2. Experimental

Materials.—To remove trace amounts of (1 → 3)-β-D-glucans that would convert factor G to its fully active form, aluminum-capped test tubes and pipettes were heated at 260 °C for 2 h in an oven, and heat-stable reagents were autoclaved at 121 °C for 20–120 min. Endotoxin- and (1 → 3)-β-D-glucan-free distilled water was used for all experiments. Possible contaminations with endotoxin and (1 → 3)-β-D-glucan were checked using Endospecy, an endotoxin-specific chromogenic limulus reagent (Seikagaku, Tokyo, Japan) and Gluspecy, a (1 → 3)-β-D-glucan-specific chromogenic limulus reagent (Seikagaku). An endotoxin- and (1 → 3)-β-D-glucan-free reagent was prepared by charcoal treatment; activated charcoal (acid washed with phosphoric and sulfuric acids, Sigma, St. Louis, MO, USA) was added to the reagent solution in a ratio of 1 g/50 mL, and mixed. The mixture was then filtered through a Millex-GS sterilizing filter (0.22 μm; Millipore, Bedford, MA, USA).

Pachyman, a (1 → 3)-β-D-glucan, was purified from powder of sclerotic *Poria cocos* by the method of Saitô et al. [6] and dissolved in 0.1 M NaOH. The solution was further diluted with 0.01 M NaOH before being used as the standard activator of factor G. Other linear (1 → 3)-β-D-glucans used were curdlan from *Alcaligenes faecalis* var. *myxogenes* (Wako Pure Chemical Industries, Osaka, Japan), and paramylon from *Euglena gracilis*, a gift of Dr. B.A. Stone, Melbourne University, Australia. Other β-D-glucans tested

were *Laminaria digitata* laminarin, a (1 → 3),(1 → 6)-β-D-glucan, *Cetraria islandica* lichenin and barley β-D-glucan, (1 → 3),(1 → 4)-β-D-glucans (Sigma), *Eisenia arborea* laminarin, a (1 → 3),(1 → 6)-β-D-glucan (Nakarai Tesque, Kyoto, Japan), schizophyllan, a (1 → 3),(1 → 6)-β-D-glucan (Kaken, Tokyo), and lentinan, a (1 → 3),(1 → 6)-β-D-glucan (Ajinomoto, Tokyo). Water-insoluble β-D-glucans were dissolved in 0.1 M or 0.3 M NaOH, then neutralized with HCl. Partially carboxymethylated (CM-) curdlan (degree of substitution, 0.63) was prepared by the method of Clarke and Stone [7]. Lipopolysaccharides (LPSs) of *Escherichia coli* 0111:B4 and *Salmonella minnesota* R595 (Re), and Lipid A from *S. minnesota* R595 (Re) were obtained from List Biological Laboratories (Campbell, CA, USA). Linear (1 → 3)-β-D-glucans and branched and mixed linkage (1 → 3)-β-D-glucans containing different types of linkage were used to investigate the binding specificity of (1 → 3)-β-D-glucans based on the ability of T-GBP to inhibit the β-D-glucan-dependent factor G activation.

Other polysaccharides used were a carboxymethyl cellulose, a (1 → 4)-β-D-glucan (Nakarai Tesque). A *Leuconostoc* sp. dextran, a (1 → 6)-α-D-glucan (Seikagaku), *Pullularia pullulans* pullulan, a (1 → 4),(1 → 6)-α-D-glucan (Hayashibara Biochemical, Okayama, Japan), and yeast α-D-mannan, a mixed linkage α-D-mannan (Sigma). Gyrophoran, a (1 → 6)-β-D-glucan, was prepared from the frond powder of the lichen, *Gyrophora esculenta* [8]. A (1 → 3)-β-D-xylan was prepared from *Caulerpa brachypus* according to Iriki et al. [9].

Preparation of coagulation factors.—*T. tridentatus* amoebocyte lysate was prepared by the method of Nakamura et al. [10]. Coagulation factors B, C, G, and ProCE were prepared from the lysate by fractionation on a dextran sulfate-Sepharose CL-6B column [11] by elution with a stepwise increase of NaCl concentration, and identified by measuring the amidolytic activity of the clotting enzyme as described previously [12–14]. T-GBPs in the fractions were detected by inhibition of factor G activation as described below. Fractions found to contain factor G were pooled as the stock solution at −40 °C.

Preparation of (1 → 3)-β-D-glucan-conjugated bovine serum albumin (BSA).—(1 → 3)-β-D-Glucan (pachyman)-conjugated BSA was prepared by the method of Tabata et al. [15]. Pachyman (7 mg) was coupled with BSA (40 mg, Miles, Kankakee, IL, USA) in the presence of CNBr (47 mg) in alkaline condition (50 mL), and after blocking CNBr with 0.2 mM glycine (80 mL), the resulting pachyman-conjugated BSA was purified by gel permeation chromatography (GPC) on Sephacryl S-200 HR (Pharmacia Biotech, Sweden).

Assay for activation of factor G.—(1 → 3)-β-D-Glucan-dependent activation of factor G was assayed by measuring the amidolytic activity of clotting enzyme produced in the reconstituted (1 → 3)-β-D-glucan-initiated cascade, to which purified pachyman (2.5 pg) was added as the standard factor G activator. The reaction mixture (total volume, 200 μL) contained Tris–HCl buffer, pH 8.0 (20 μmol), MgSO4 (10 μmol), *t*-butoxycarbonyl (Boc)-Leu-Gly-Arg-4-nitroanilide (Boc-LGR-4NA, Peptide Institute, Osaka, Japan, 0.1 μmol), factor G (40 μL, $A_{280} = 0.9$), ProCE (20 μL, $A_{280} = 2.5$), and pachyman (2.5 pg) at 37 °C for 20 min. The amount of 4-nitroaniline released was measured at 545 nm after diazo-coupling [16].

Assay for inhibitory ability of T-GBP.—Addition of T-GBP to the reconstituted (1 → 3)-β-D-glucan-initiated cascade was expected to cause a measurable decrease in the formation of activated factor G by competitive binding of the activator, e.g. pachyman to T-GBP. Hence, to assay the inhibitory ability of T-GBP, the sample to be tested was added to the reaction mixture (total volume, 200 μL) of the same composition as above, and the amount of 4-nitroaniline released was determined after 20-min incubation at 37 °C. The relative inhibition (%) was calculated by dividing the amidolytic activities obtained with reaction mixtures containing varying amounts of T-GBP by that of the control, to which water was added instead of the sample. One unit of inhibitory ability was defined as the amount of T-GBP that is able to reduce the activation of factor G by 50% under the standard assay condition.

A fixed amount of T-GBP (50 μL, 34 units/mL) was mixed with varying amounts (2.5–2500 pg) of pachyman, and after 10-min incubation at 37 °C, the residual inhibitory ability was measured and expressed as the relative inhibition (%).

Assay for binding ability of various (1 → 3)-β-D-glucans to T-GBP.—A sample (50 μL, 10^{-11}–10^{-6} g/mL) of a (1 → 3)-β-D-glucan was mixed with T-GBP (50 μL, 1.6 units), and incubated at 37 °C for 10 min. The mixture was added to the solution (100 μL) of the same composition as that used for the assay of the inhibitory ability except for pachyman which was excluded. After 20 min-incubation at 37 °C, the amount of 4-nitroaniline released was measured as described above.

Effects of polysaccharides on the inhibitory ability of T-GBP.—A sample (25 μL, 10^{-11}–10^{-6} g/mL) of a polysaccharide to be examined was mixed with T-GBP (50 μL, 1.6 units), and incubated at 37 °C for 10 min. To the mixture, pachyman (25 μL, 100 pg/mL) was added, mixed, and incubated at 37 °C for 10 min. The procedures thereafter were the same as those used for (1 → 3)-β-D-glucan.

Estimation of apparent molecular weight (mol wt) of T-GBP.—The apparent mol wt of T-GBP was estimated by gel permeation chromatography using a Sephacryl S-300 HR (Pharmacia Biotech) column (1.4 × 95 cm) pre-equilibrated with 50 mM Tris–HCl buffer, pH 8.0, containing 500 mM NaCl and 4 mM $CaCl_2$. The column was calibrated using a high-mol-wt-gel filtration kit (Pharmacia Biotech) consisting of aldolase (mol wt, 158 000), catalase (mol wt, 232 000), ferritin (mol wt, 440 000), and thyroglobulin (mol wt, 669 000) as the marker proteins. The sample was eluted with the buffer. The V_0 and V_t values were measured using blue dextran 2000 and NaCl. A linear relation was obtained between the K_{av} values and the logarithmic mol wt's of the marker proteins.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of T-GBP.—Purified T-GBP was concentrated to 2 mg/mL and desalted by centrifugation using an Ultrafree C3LCC (mol-wt-cut, < 5000; Millipore, Bedford, MA, USA) at 4500 g for 60 min. A 30-μL portion of the sample was made up to 100 μL with 125 mM Tris–HCl buffer, pH 8.0, containing 4% (w/v) SDS, 14% (v/v) glycerol, and water or 0.01% (w/v) 2-mercaptoethanol. T-GBP (12 μg) in the buffer (20 μL) was heated at 100 °C for 5 min and separated by SDS-PAGE [17] at a constant current of 3 mA for 1 h. Protein bands in the gel were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 and destained with 10% (v/v) MeOH–7% (v/v) CH_3COOH. The positions of T-GBP bands were compared in parallel gels under reducing and non-reducing conditions.

Isoelectric point (pI).—The pI for T-GBP was determined using isoelectric focusing polyacrylamide electrophoresis (IEF-PAGE) [18] using a Phast Gel IEF with a Phast System (Pharmacia Biotech). A pI-marker protein kit (Pharmacia Biotech) was used as the standard. Protein bands in a gel were visualized as above.

The amino-terminal sequence.—The amino-terminal sequence of purified T-GBP (7.5 μg) was determined by the method of Matsudaira [19] using a Shimadzu PPSQ-10 gas-phase amino acid sequencer.

Preparation of antisera.—Samples (500 μL each) containing T-GBP (400 μg/mL) were emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into the backs of two rabbits. Four injections were made every other week and the samples (100 μg/mL each) of T-GBP were intravenously injected as a booster. The rabbits were bled one week after the final injection. The titers of the antisera were examined by Ouchterlony-immunodiffusion technique. Double diffusion was conducted in a 1% (w/v) agarose (immunodiffusion grade, ICN Biomedicals, Costa Mesa, CA, USA) layer containing sodium barbiturate buffer, pH 8.6 ($\mu = 0.05$) and 0.1% (w/v) NaN_3 on a glass slide. Aliquots (10 μL) of antisera were placed in a centre well surrounded by regularly spaced wells containing the antigen (2.5 μg in water, 20 μL). Diffusion was allowed to occur in a moist atmosphere for 12–24 h. Preimmune sera were collected before the immunization. Anti-T-GBP antibody (IgG) was purified from the antiserum (titer = 1:64) by the method of Ricardo et al. [20]. Immunoblotting was conducted to confirm the specificity according to Towbin et al. [21]. A single spot was detected exactly at the position of T-GBP on SDS-PAGE by staining with horseradish peroxidase-conjugated goat anti-rabbit IgG.

Inhibition of inhibitory ability of T-GBP by the antibody.—Varying concentrations (0.1–1000 μg/mL) of purified anti-T-GBP IgG were added to the inhibition assay reaction mixture, and the residual amidolytic activity was measured as described in the Experimental section. Relative inhibition (%) was calculated using the control with added non-specific rabbit IgG.

Enzyme-linked immunosorbent assay (ELISA) using pachyman-conjugated BSA was performed to demonstrate specific binding of (1 → 3)-β-D-glucan to T-GBP [15]. Aliquots (100 μL) of the (1 → 3)-β-D-glucan–BSA conjugate (1.5 μg) were placed in wells of microtiter plates [endotoxin- and (1 → 3)-β-D-glucan-free 96-well microplate, Toxipetplate 96F, Seikagaku] and were allowed to settle at 4 °C for 12 h. Following aspiration of the supernatant, the wells were washed three times with phosphate buffered saline (PBS) (200 μL; 0.01 M phosphate buffer, pH 7.4, containing 0.13 M NaCl), and 5% (w/v) BSA in PBS was added to wells and incubated at 37 °C for 2 h to block non-specific antibody-binding sites. The blocking solution was removed by aspiration and the wells were washed three times with PBS containing 0.1% (w/v) Tween-20 (PBST). Aliquots (100 μL) of T-GBP (1 μg/mL) were added to the wells and incubated at 37 °C for 2 h. After aspiration of T-GBP solution, the wells were washed three times with PBST. The immobilized antigen in the wells was then incubated with the solution (100 μL) of 1:5000, 1:10000, and 1:50000 diluted anti-T-GBP IgG with PBS at 37 °C for 2 h. After washing the wells successively with PBST and PBS, a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Rockland, PA, USA) diluted 1:1000 with PBS (50 μL/well) was added and incubated at 37 °C for 2 h. After

washing the wells, a substrate solution (100 μL) containing 0.1% (w/v) tetramethylbenzidine and 0.006% (v/v) H_2O_2 in 50 mM acetate buffer, pH 5.0, was added to each well and left standing at room temperature for 10 min. The reaction was stopped by addition of 2 M H_2SO_4 (50 μL), and the absorbance at 450 nm was read using a microplate reader (Wellreader SK 601, Seikagaku). The values obtained with preimmune IgG were taken as the controls.

Determination of protein.—Protein was measured by the method of Lowry et al. [22] using BSA as the standard. Proteins in chromatography eluates were monitored at 280 nm.

Purification.—All procedures were carried out at 4 °C or below. Prior to chromatography, columns were washed thoroughly until the inhibitory ability of the effluents became negative. The amoebocyte lysate (900 mL; protein content, 4502 mg) was fractionated on a dextran sulfate-Sepharose CL-6B column (5 × 23 cm) pre-equilibrated with 20 mM Tris–HCl buffer, pH 8.0. The T-GBP was eluted from the column with 0.45 M NaCl in the buffer. The column was further eluted with the buffer containing 0.2 M NaCl [5]. Fractions (2.5 mL) were collected at a flow rate of 120 mL/h and monitored for activation of factors G, B, and C, inhibition by T-GBP of factor G activation, and protein content. The pooled fraction (510 mL; protein content, 60.8 mg) containing T-GBP was freeze-dried, dissolved in distilled water (25 mL), and applied to a Sephacryl S-300 HR column (2.2 × 95 cm) pre-equilibrated with 50 mM Tris–HCl buffer, pH 8.0, containing 0.5 M NaCl and 4 mM $CaCl_2$, which was also used as the eluent. Fractions (2.5 mL) were collected at a flow rate of 18 mL/h and monitored for inhibitory ability and protein content. The pooled fractions (47.5 mL) were freeze-dried, dissolved in water (8 mL), and rechromatographed on a Sephacryl S-300 HR column (1.4 × 95 cm) under the same elution conditions as above. Fractions (1 mL) were collected at a flow rate of 4.5 mL/h, and the pooled fraction (15 mL) of T-GBP was stored at −80 °C.

3. Results and discussion

Inhibition of activation of factor G by T-GBP.—Experiments, summarized in Table 1, were conducted to provide unequivocal evidence for the role of T-GBP in the inhibition of factor G activation. There was no appreciable inhibitory effect when factor G was fully activated by the standard (1 → 3)-β-D-glucan, pachyman, prior to the addition of T-GBP (expt 1). In contrast, as seen from expt 3, no measurable amidolytic activity could be detected when T-GBP was preincubated with pachyman, because there is apparently a complete suppression of the activation of factor G. When T-GBP and factor G were incubated simultaneously with pachyman (expts 2 and 4), there was a partial inhibition, indicating that a fraction of factor G was not bound to T-GBP. Thus the binding of (1 → 3)-β-D-glucan to T-GBP causes an inhibitory effect on the activation of factor G, resulting in a significant decrease in amidolytic activity of clotting enzyme due to the lowered activation of the ProCE [2] (Fig. 1). No appreciable inhibition of factor G activation could be found in the plasma fraction of *T. tridentatus* hemolymph (data not shown).

Table 1
Procedures demonstrating the inhibition of factor G activation by competitive binding of (1 → 3)-β-D-glucan to T-GBP

Expt	Procedure [a]	Inhibition (%)
1	FG + BG + ProCE (37 °C, 20 min) → + T-GBP + S (37 °C, 3 min)	0
2	FG + T-GBP (37 °C, 10 min) → + BG + ProCE + S (37 °C, 20 min)	37.5
3	BG + T-GBP (37 °C, 10 min) → + FG + ProCE + S (37 °C, 20 min)	100
4	FG + BG + T-GBP + ProCE + S (37 °C, 20 min)	33.4

[a] Abbreviations: FG, factor G; BG, pachyman, a linear (1 → 3)-β-D-glucan; ProCE, proclotting enzyme; S, substrate, Boc-LGR-4NA; T-GBP, the pooled fraction containing T-GBP (0.45 M NaCl fractions from dextran sulfate-Sepharose CL-6B). Amidolytic activity in the final reaction was assayed as described in the Experimental section. After incubation at 37 °C for the indicated time, the amount of 4-nitroaniline released was measured at 545 nm. Relative inhibition (%) of factor G activation in each experiment was calculated using the amidolytic activity obtained without T-GBP as the control.

The inhibitory ability of T-GBP increased proportionally depending on the amounts of T-GBP from 10 to 80 μg/mL, and the inhibition curve decreased proportionally with increasing concentrations (2–20 ng/mL) of the standard (1 → 3)-β-D-glucan, pachyman (data not shown). T-GBP was shown to be totally inactive on the LPS (endotoxin)-initiated coagulation pathway when the amidolytic activities were measured using Endospecy, to which T-GBP (160 units) and an *E. coli* 0111:B4 LPS or a *S. minnesota*

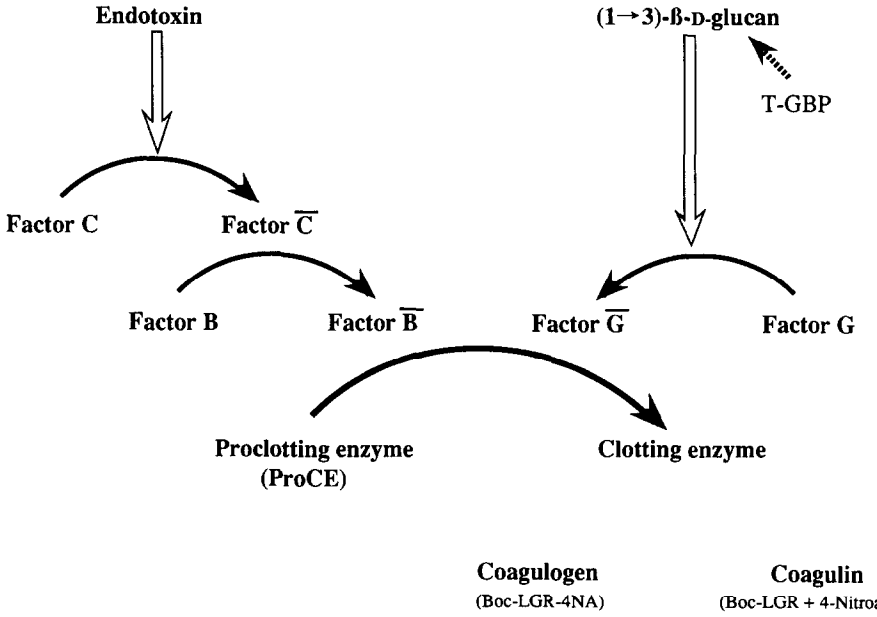

Fig. 1. Endotoxin- and (1 → 3)-β-D-glucan-dependent coagulation cascades in *T. tridentatus* amoebocyte lysate. Factors C̄, B̄, and Ḡ indicate their corresponding activated forms of factors C, B, and G, respectively. The amidolytic activity of the clotting enzyme was measured using Boc-LGR-4NA as the substrate.

R595(Re) LPS or its lipid A as the activator were added. The absence of inhibitory effect on the pathway indicates no binding affinity of T-GBP to either endotoxins or factors C and B and their activated forms.

Purification of T-GBP.—The elution profile of *T. tridentatus* amoebocyte lysate proteins from a dextran sulfate-Sepharose CL-6B with stepwise increase in NaCl concentration in 20 mM Tris–HCl buffer, pH 8.0, is shown in Fig. 2A. There is a clear separation of three peaks, I (ProCE), II (factor G and coagulogen), and III (factor B, C, and T-GBP). Gel permeation chromatography of peak III on Sephacryl S-300 HR separated T-GBP completely from factors B and C in the pooled fraction (Fig. 2B). Rechromatography on Sephacryl S-300 HR eluted T-GBP as a single peak in which those fractions showing inhibitory ability and absorption at 280 nm were condensed. The final purification factor for T-GBP was 730 with a yield of 56.7% (Table 2).

Properties.—Purified T-GBP eluted as a single peak on Sephacryl S-300 HR, which coincided with that of protein mol wt 580 000. It migrated as a single band on SDS-PAGE, is apparently homogeneous (Fig. 3A), and is a basic protein with an approximate pI of 9.2 (Fig. 3B). Dissociation in reducing conditions suggests that the native protein is most likely a tetramer in which the subunits (mol wt, 168 000) are linked through disulfide bonds. The discrepancy between the T-GBP mol wt obtained from sephacryl chromatography and that calculated from the sum of the subunit mol wt may be due to a weak affinity of the native T-GBP to the gel matrix causing its retarded migration. The pI values and apparent mol wts of (1 → 3)-β-D-glucan-binding proteins (GBP) from other arthropods have been reported as follows: a single polypeptide chain M_r 38 kDa on SDS-PAGE from great wax moth (*Galleria mellonella* L.) haemocytes [23]; a monomeric protein M_r of 62 kDa and a pI of 4.3 from silkworm haemolymph [24]; a glycoprotein M_r 91 000 on SDS-PAGE under non-reducing conditions from a cockroach (*Blaberus craniifer*) haemocyte, shown to be a heterodimer consisting of two subunits M_r 63 000 and 52 000, respectively, on SDS-PAGE under reducing conditions [25]; a monomeric protein with a pI of 5.0 and M_r 100 kDa from plasma of a crayfish (*Pacifastacus leniusculus*) [26]. Compared with these GBPs, it is evident that T-GBP isolated from *T. tridentatus* amoebocyte lysate is unique in its high-mol-wt-oligomeric form as well as in its basic nature. The amino-terminal sequence (20 residues) of T-GBP monomer was KSGFILTAPKSLTLGRNNRL.

Binding specificity.—Table 3 shows the comparison of various (1 → 3)-β-D-glucans in their binding abilities towards T-GBP, expressed as their relative inhibition (%). It is

Fig. 2. Elution profiles of T-GBP. (A) Elution of T-GBP from a dextran sulfate-Sepharose CL-6B column by stepwise increase in NaCl concentration. Each coagulation factor was estimated by measuring the amidolytic activity of the clotting enzyme induced in the presence of LPS or (1 → 3)-β-D-glucan. Factors G, B, and C were eluted with the buffer; 0.2 M and 0.45 M NaCl in the buffer as indicated by arrows, ProCE (●), factors B and C (△), factor G (○). Absorbance at 405 nm due to 4-nitroaniline released from Boc-LGR-4NA was measured directly and corrected for the control. The amidolytic activity of the clotting enzyme in the coagulation cascades involving activated factors G, B, and C was expressed as the specific activity ($\Delta A405$ nm/min · mL) [5]. Coagulogen (▲) was detected by the turbidity due to trypsin-induced clot formation [5]. The inhibitory activity (■) of T-GBP was expressed as the relative inhibition (%) of factor G activation, as described in the Experimental section. (B) Separation of T-GBP and factors B and C by GPC on Sephacryl S-300 HR.

apparent that T-GBP is able to inhibit almost completely factor G activation induced by pachyman, curdlan, and paramylon, indicating a high binding specificity of T-GBP for these high-mol-wt linear-(1 → 3)-β-D-glucans. Interestingly, it was found that *L. digitata* and *E. arborea* laminarins, low-mol-wt (1 → 3),(1 → 6)-β-D-glucans, exhibited comparable binding affinities to T-GBP to those of linear (1 → 3)-β-D-glucans. How-

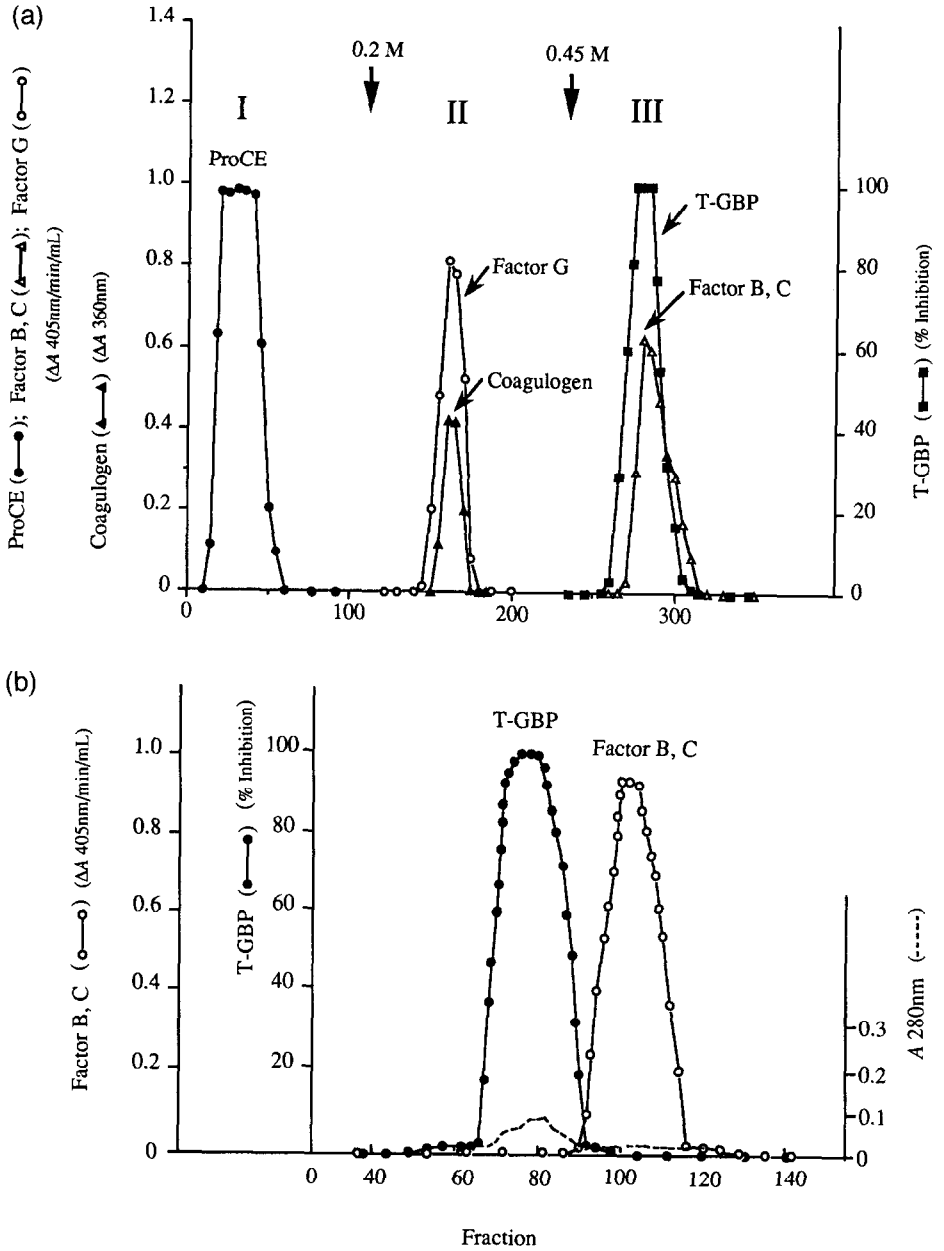

Table 2
Purification of T-GBP from *T. tridentatus* amoebocyte lysate

Step	Total protein (mg)	Total activity (units) [a]	Specific activity (U/mg) [a]	Yield (%)	Purification (fold)
Extract	4502.0	1050	0.2	100	1
Dextran sulfate-Sepharose CL-6B	60.8	2130 [b]	35.0	202.9	150
Sephacryl S-300 HR (1st)	11.9	631	53.0	60.1	228
Sephacryl S-300 HR(2nd)	3.5	595	170.0	56.7	730

[a] Activity: the ability of T-GBP to inhibit the activation of factor G using pachyman as the activator. One unit of the inhibitory ability of T-GBP was defined as the amount of T-GBP required to reduce the activity of factor G to 50% under the standard assay conditions.
[b] The increased activity is probably due to the removal of the contaminating factor G that competes with T-GBP for pachyman added to the reaction mixture as the activator.

ever, T-GBP is less effective in inhibiting the activation of factor G by schizophyllan and lentinan, which bear one and two (1 → 6)-β-D-glucopyranosyl residues at about every third and fifth residue along the main chain and take the form of a triple helix in aqueous solution [27,28]. Similar degrees of the inhibitory ability were obtained with these branched (1 → 3)-β-D-glucans in aqueous and neutralized solutions, where they tend to adopt a single helical conformation when they were dissolved in 0.3 M NaOH

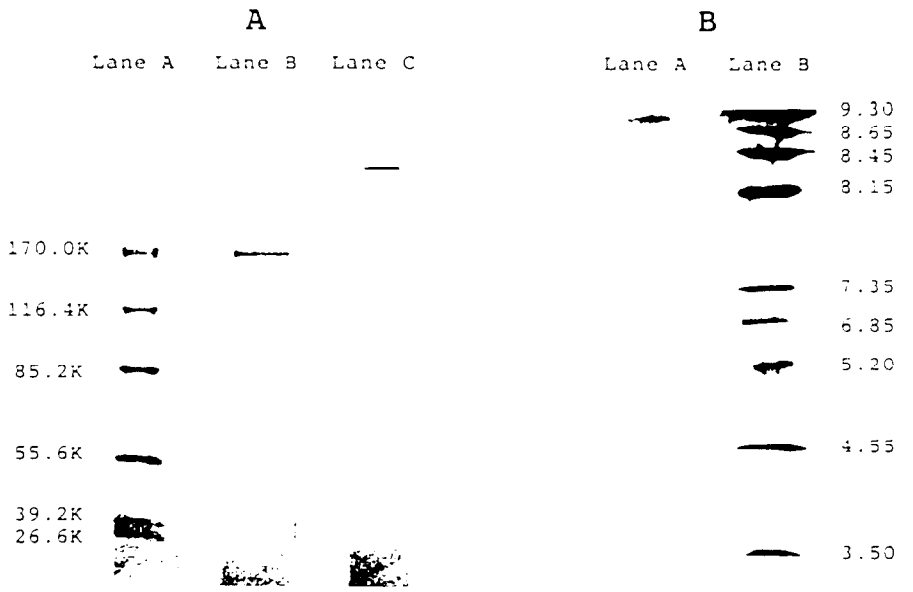

Fig. 3. SDS-PAGE and electrofocusing of T-GBP in gel. (A) SDS-PAGE of T-GBP. Lane A, mol-wt marker proteins; lane B, T-GBP (12 μg) in the presence of 2-mercaptoethanol; lane C, T-GBP (12 μg) under non-reducing conditions. (B) Determination of isoelectric point (pI). Lane A, T-GBP (10 μg); lane B, pI marker proteins.

Table 3
Binding abilities of various (1 → 3)-β-D-glucans to T-GBP

β-D-Glucan	Type of linkage	Mol wt (M_n) [a]	Concentration [b] (g/mL)	Inhibition [c] (%)
Pachyman	(1 → 3)-β-D	80000	5×10^{-11} (0.1 M NaOH)	99.0
Curdlan	(1 → 3)-β-D	> 136000	5×10^{-11} (0.1 M NaOH)	98.5
CM-curdlan	(1 → 3)-β-D	> 95000	5×10^{-11} (H_2O)	99.2
Paramylon	(1 → 3)-β-D	> 118000	2.5×10^{-11} (0.3 M NaOH)	99.2
Laminarins				
from *Laminaria digitata*	(1 → 3),(1 → 6)-β-D	5850	1×10^{-7} (H_2O)	95.8
from *Eisenia arborea*	(1 → 3),(1 → 6)-β-D	16800	1×10^{-7} (H_2O)	95.2
Schizophyllan	(1 → 3),(1 → 6)-β-D	76800	1×10^{-9} (0.3 M NaOH)	63.6
	(1 → 3),(1 → 6)-β-D		2×10^{-6} (H_2O)	60.6
Lentinan	(1 → 3),(1 → 6)-β-D	94700	2×10^{-10} (0.3 M NaOH)	59.4
	(1 → 3),(1 → 6)-β-D		2×10^{-8} (H_2O)	57.5
Lichenin				
from *Cetraria islandica*	(1 → 3),(1 → 4)-β-D	22000	7.5×10^{-7} (H_2O)	40.7
Barley β-D-glucan	(1 → 3),(1 → 4)-β-D	> 23100	1.25×10^{-7} (H_2O)	36.2

[a] Number-average mol wt (Mn) was determined as described previously [16].
[b] Water-insoluble β-D-glucans were dissolved in 0.1 M or 0.3 M NaOH and neutralized with equivalent concentrations of HCl.
[c] The binding ability of (1 → 3)-β-D-glucan to T-GBP was assayed as described in the Experimental section and expressed as the relative inhibition (%) of each sample using the amidolytic activity obtained without T-GBP as the control.

and neutralized [29]. Thus, it can be postulated that T-GBP is able to bind to the branched (1 → 3)-β-D-glucans regardless of their helical conformation, contrasting with a conformational requirement of factor G whose activation can be triggered efficiently with forms of the single helical conformation in preference to those of a triple helix [30]. These findings imply that T-GBP is a carbohydrate-binding protein capable of binding specifically to consecutive (1 → 3)-β-D-glucopyranosyl residues, and that its binding ability is not influenced noticeably by conformational change in the structure of the β-D-glucans. (1 → 3)-β-D-Oligosaccharides were not included in the inhibition tests because they bind tightly to factor G, resulting in a complete blocking of factor G activation by (1 → 3)-β-D-glucans of longer chain lengths [31]. The inhibitory ability of T-GBP was much lower when the (1 → 3),(1 → 4)-β-D-glucans, *C. islandica* lichenin and barley β-D-glucan, were tested as activators of factor G, owing to their structural feature being devoid of consecutive (1 → 3)-β-D-glucopyranosyl residue [32]. It is of interest that the barley β-D-glucan also be recognized by the human monocyte receptor [33]. These results suggest that the binding of the branched and mixed linkage β-D-glucans to T-GBP may be essentially related to the length of a main (1 → 3)-β-D-glucopyranosyl linkage, since the values of the relative inhibition (%) in the binding of (1 → 3)-β-D-glucans to T-GBP resemble closely those of the activators of factor G, established by extensive survey of linear (1 → 3)-β-D-glucans with different chain lengths as well as of those with branched and mixed linkage structures [16].

Only a very weak blocking (%) of inhibitory ability of T-GBP was obtained with the following polysaccharides at concentrations (g/mL) comparable to (1 → 3)-β-D-glucans,

Table 4
Enzyme-linked immunosorbent assay for binding of pachyman to T-GBP [a]

Antibody	Dilution of antibody	Absorbance at 450 nm (Δ450)
Anti-T-GBP IgG	1:5000	2.86 ± 0.05
	1:10000	2.63 ± 0.05
	1:50000	0.75 ± 0.05
Preimmune IgG (control)	1:5000	0.11 ± 0.01
	1:10000	0.07 ± 0.01
	1:50000	0.02 ± 0.00

[a] Enzyme-linked immunosorbent assay was performed as described in the Experimental section, and absorbance at 450 nm due to bound horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was recorded, and the binding of pachyman to T-GBP was expressed as the absorbance (Δ450) corrected for that obtained with IgG from rabbit preimmune serum as the control.

as shown in parentheses: CM-cellulose (1.5%, 1×10^{-7}, in water), gyrophoran (1.1%, 2×10^{-11}, in 0.1 M NaOH), dextran (1.0%, 1×10^{-7}, in water), pullulan (1.0%, 1×10^{-7}, in water), yeast α-D-mannan (1.2%, 1×10^{-8}, in 0.3 M NaOH), and *C. brachypus* (1 → 3)-β-D-xylan (1.1%, 1×10^{-7}, in 0.3 M NaOH). Obviously, none of these polysaccharides is able to fulfil the structural requirements needed to compete with (1 → 3)-β-D-glucans for the binding site(s) of T-GBP.

Proof of binding of (1 → 3)-β-D-glucan to T-GBP.—Immunological evidence is presented to verify the binding of a (1 → 3)-β-D-glucan (pachyman) to T-GBP by ELISA using a specific rabbit antibody (IgG) raised against T-GBP. The amounts of T-GBP bound to pachyman-conjugated BSA fixed on wells were determined by measuring the activity of horseradish peroxidase-conjugated goat anti-rabbit IgG at 450 nm. The absorbance at 450 nm (Δ450) due to the bound horseradish peroxidase was summarized in Table 4. The large differences between the values obtained with added T-GBP and those of the controls indicate tight binding of T-GBP to (1 → 3)-β-D-glucan. Furthermore, full activation of factor G was found when the amidolytic activity was measured with a reaction mixture to which rabbit anti-T-GBP IgG (100 μg/mL) was added under the standard assay conditions for the inhibitory ability; the preimmune IgG was entirely inactive. The inhibitory ability of T-GBP decreased proportionally with increasing concentration (1–10 μg/mL) of IgG added, and the minimal amount of the antibody required for 50% inhibition was determined to be 7.0 μg/mL.

General remarks.—GBPs purified from the plasma fractions of silkworm and crayfish haemolymphs by precipitation with (1 → 3)-β-D-glucan have been implicated as components of the (1 → 3)-β-D-glucan-dependent protease cascade which leads to the activation of prophenol oxidase in haemocyte lysates or in GBP-free plasma fractions [24,26].

Recently, Katsumi et al. [34], working with a plasma fraction of haemolymph of silkworm, reported the characterization of a 39 kDa zymogen of a serine protease, which was converted to an active form (38 kDa) by limited proteolysis triggered by zymosan, a yeast cell wall preparation containing (1 → 3)-β-D-glucan. The binding specificity, the mechanism of activation, and the physiological role of the protease precursor remain unclear. Immunostaining and immunoelectron microscopy methods revealed that a

38-kDa GBP from haemocyte lysate of the great wax moth was localized on the cell surface of plasmatocytes, which play macrophage-like roles in recognizing and phagocytizing foreign particles [23]. T-GBP is the first GBP that has been characterized with respect to binding specificity towards linear as well as branched (1 → 3)-β-D-glucans and (1 → 3),(1 → 4)-β-D-glucans (Table 3). From the data, it may be speculated that T-GBP plays not only an opsonic function in self-defense against invading fungi but also a regulatory role in the (1 → 3)-β-D-glucan-initiated coagulation pathway of amoebocytes. Studies on the subcellular location of T-GBP in amoebocytes are needed to elucidate its physiological function in relation to other factors of the horseshoe crab [35].

References

[1] J. Levin and F.B. Bang, *Thromb. Diath. Haemorr.*, 19 (1968) 186–197.
[2] S. Iwanaga, T. Morita, T. Nakamura, and J. Aketagawa, *J. Protein Chem.*, 5 (1986), 255–268.
[3] T. Morita, S. Tanaka, T. Nakamura, and S. Iwanaga, *FEBS Lett.*, 129 (1981), 318–321.
[4] S. Tanaka, T. Nakamura, T. Morita, and S. Iwanaga, *Biochem. Biophys. Res. Commun.*, 105 (1982), 717–723.
[5] T. Obayashi, H. Tamura, S. Tanaka, M. Ohki, S. Takahashi, M. Arai, M. Masuda, and T. Kawai, *Clin. Chim. Acta*, 149 (1985) 55–65.
[6] H. Saitô, A. Misaki, and T. Harada, *Agr. Biol. Chem.*, 32 (1968) 1261–1269.
[7] A.E. Clarke and B.A. Stone, *Phytochemistry*, 1 (1962), 175–188.
[8] Y. Shibata and T. Fukimbara, *J. Ferment. Technol.*, 50 (1971) 388–396.
[9] Y. Iriki, T. Suzuki, K. Nishizawa, and T. Niwa, *Nature*, 187 (1960) 82–83.
[10] S. Nakamura, S. Iwanaga, T. Harada, and M. Niwa, *J. Biochem.*, 80 (1976), 1011–1021.
[11] L.-O. Anderson, H. Borg, and M. Miller-Anderson, *Thromb. Res.*, 7 (1975), 451–459.
[12] T. Nakamura, T. Horiuchi, T. Morita, and S. Iwanaga, *J. Biochem.*, 99 (1986) 847–857.
[13] T. Nakamura, T. Morita, and S. Iwanaga, *Eur. J. Biochem.*, 154 (1986) 511–521.
[14] T. Nakamura, T. Morita, and S. Iwanaga, *J. Biochem.*, 97 (1985) 1561–1574.
[15] K. Tabata, W. Itoh, A. Hirata, I. Sugawara, and S. Mori, *Agric. Biol. Chem.*, 54 (1990) 1953–1959.
[16] S. Tanaka, J. Aketagwa, S. Takahashi, Y. Shibata, Y. Tsumuraya, and Y. Hashimoto, *Carbohydr. Res.*, 218 (1991) 167–174.
[17] U.K. Laemmli, *Nature*, 227 (1970) 680–685.
[18] R.C. Allen, C.A. Saravis, and H.R. Maurer (Eds.), *Gel Electrophoresis and Isoelectric Focusing of Proteins*, Walter de Gruyter, Berlin and New York, 1984, pp 236–240.
[19] P. Matsudaira, *J. Biol. Chem.*, 262 (1987) 10035–10038.
[20] M.J. Ricardo, R.L. Trouy, and D.T. Grimm, *J. Immunol.*, 127 (1981) 946–951.
[21] H. Towin, T. Staehelin, and J. Gordon, *Proc. Natl. Acad. Sci.*, USA, 76 (1979) 4350–4354.
[22] O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J Biol. Chem.*, 193 (1951) 265–275.
[23] V. Matha, L. Grubhoffer, F. Weyda, and L. Hermanová, *Cytobios*, 64 (1990) 35–42.
[24] M. Ochiai and M. Ashida, *J. Biol. Chem.*, 263 (1988) 12056–12062.
[25] K. Söderhäll, W. Rögener, I. Söderhäll, R.P. Newton, and N.A. Ratcliffe, *Insect Biochem.* 18 (1988) 323–330.
[26] B. Duvic and K. Söderhäll, *J. Biol. Chem.*, 265 (1990) 9327–9332.
[27] T. Norisuye, T. Yanaki, and H. Fujita, *J. Polym. Sci., Polym. Phys. Ed.*, 18 (1980) 547–558.
[28] H. Saitô, Y. Yoshioka, M. Yokoi, and J. Yamada, *Biopolymers*, 29 (1990) 1689–1698.
[29] H. Saitô, Y. Yoshioka, N. Uehara, J. Aketagawa, S. Tanaka, and Y. Shibata, *Carbohydr. Res.*, 217 (1991) 181–190.
[30] J. Aketagwa, S. Tanaka, H. Tamura, Y. Shibata, and H. Saitô, *J. Biochem.*, 113 (1993) 683–686.

[31] S. Tanaka, J. Aketagawa, S. Takahashi, Y. Shibata, Y. Tsumuraya, and Y. Hashimoto, *Carbohydr. Res.*, 244 (1993) 115–127.
[32] J.R. Woodward, G.B. Fincher, and B.A. Stone, *Carbohydr. Polymers*, 3 (1983) 207–225.
[33] M.J. Janusz, K.F. Austen, and J.K. Czop, *J. Immunol.*, 3 (1989) 959–965.
[34] Y. Katsumi, H. Kihara, M. Ochiai, and M. Ashida, *Eur. J. Biochem.*, 228 (1995) 870–877.
[35] T. Shigenaga, Y. Takaenoki, S. Kawasaki, N. Seki, T. Muta, Y. Toh, A. Ito, and S. Iwanaga, *J. Biochem.*, 114 (1993) 307–316.