Purification of a Peptidoglycan Recognition Protein from Hemolymph of the Silkworm, Bombyx mori

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A method was developed for obtaining a homogeneous silkworm hemolymph protein (peptidoglycan recognition protein, PGRP) which has affinity for peptidoglycan and the ability to trigger the prophenoloxidase cascade upon its binding to peptidoglycan. The purified PGRP had a molecular mass of about 19 kDa and is composed of a single polypeptide with an isoelectric point of 6.5. It bound to peptidoglycan in the absence of divalent cation, whereas its binding to β1,3-glucan and chitin was not detected. N-Acetyl-D-glucosaminyl-(β1–4)-N-acetyl muramyl-L-alanyl-D-isoglutamine did not inhibit purified PGRP to bind insoluble peptidoglycan, but fragmented soluble peptidoglycan did. PGRP seemed to require peptidoglycan as a possible ligand to keep its glycan portion consisting of at least two or more of the repeating unit. PGRP did not have any detectable lysozyme activity, and its amino acid composition and amino-terminal sequence of 20 amino acid residues were shown to be different from those of silkworm lysozyme. PGRP seems to be a hitherto unknown protein. In the absence of PGRP, the prophenoloxidase cascade in the plasma fraction of hemolymph could not be triggered by peptidoglycan, indicating that some type of activity, capable of activating the cascade, is generated upon their binding. However, the exact nature of this activity is not yet known. The purified PGRP bound to peptidoglycan did not hydrolyze significantly any of the 26 commercially available peptidyl-7-amino-4-methylcoumarins, substrates for various proteases.

Insects have effective defense mechanisms against microorganisms such as bacteria and fungi. The major defense mechanisms in the hemocoel are either cellular (phagocytosis, hemocyte aggregation (nodule formation) and formation of multicellular hemocyte capsules (encapsulation)) (1) or humoral effects by immune proteins, lectins, and the prophenoloxidase cascade (2–6). Recognition of microorganisms as non-self is apparently involved when the insect defense mechanisms are set in motion. As stated by Janeway (7), clonally non-self is apparently involved when the insect defense mechanism is activated upon their binding. Thus, anumber of molecules with affinity for particular structures of bacterial or fungal cell walls have been reported from insect hemolymph and they are thought to be potential recognition molecules for foreignness. It is reasonable that recognition molecules for foreignness have to be expressed constitutively and to be present in the plasma or cell surface before invasion by foreign objects. Hemolin (8, 9), lectins with various ligand specificities (4, 10–13) and proteins with affinity to β1,3-glucan (14–16), lipo polysaccharide (17), and peptidoglycan (18) have been suggested as recognition molecules. However, the biological activities of these proteins are far from being fully understood. Proteins with affinity to β1,3-glucan and peptidoglycan were originally discovered in the course of studies on prophenoloxidase cascade.

The prophenoloxidase cascade is present in insect and crustacean hemolymph (5, 6). Very recently the chitinous cuticle of the insect exoskeleton was also found to contain a prophenoloxidase cascade (19). The hemolymph prophenoloxidase cascade consists of prophenoloxidase, serine proteasezymogens, and proteins with specific affinity to bacterial or fungal cell wall components. It is activated by various non-self materials naturally or artificially introduced into the hemocoel. Under the conditions where the cascade is triggered, increased phagocytosis and hemocyte movement have been observed (20, 21). After activation, phenol oxidase catalyzes melanin synthesis from phenolic substances. Melanization is thought to facilitate sequestration of pathogens in nodules or multicellular capsules (22), and more recently it has been speculated that intermediate compounds (quinones) in melanin synthesis from mono- or diphenols are highly toxic to living cells including infectious microorganisms (23). Thus, the prophenoloxidase cascade is considered an integral part of insect defense mechanisms.

Previously we predicted the presence of two kinds of hemolymph molecules which have specific affinity for peptidoglycan (a bacterial cell wall component) and β1,3-glucan (a fungal cell wall component), respectively (18). Both molecules have been supposed to have ability to trigger prophenoloxidase cascade upon binding to their respective ligands. We proposed to name them β1,3-glucan recognition protein (βGRP) (14) and peptidoglycan recognition protein (PGRP) (14). The postulated βGRP has been isolated from silkworm, cockroach, and crayfish (14–16). cDNA of crayfish β1,3-glucan binding protein (which has been

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1 The abbreviations used are: βGRP, β1,3-glucan recognition protein; PGRP, peptidoglycan recognition protein; GMDP, N-acetyl-D-glucosaminyl-(β1–4)-N-acetyl muramyl-L-alanyl-D-isoglutamine; MDP, N-acetyl muramyl-L-alanyl-D-isoglutamine; peptidyl-NH-Mec, peptidyl-NH-Mec, peptidyl-7-amino-4-methylcoumarin; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; HPLC, high performance liquid chromatography; MES, 2-(N-morpholinio)ethanesulfonic acid; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid).
shown to have similar function to silkworm βGRP was cloned and the deduced amino acid sequence of the protein was reported (24).

Peptidoglycan has been shown to have various biological functions both in mammals (25) and insects (26). Specific binding proteins or receptors have been reported from mammals (27–29), but such molecules have not been isolated from invertebrates. Previously, a plasma fraction of silkworm hemolymph, which had been passed through Sepharose 4B coupled to peptidoglycan in the absence of divalent cation, was shown to have all phenoloxidase cascade components except for the putative PGRP. This plasma fraction (referred to as plasma-PG) was proposed to be used for assaying PGRP in the course of its purification (18).

We now describe a method for assaying PGRP by using plasma-PG and a procedure for obtaining homogeneous PGRP, as well as preliminary characterization of the molecule.

MATERIALS AND METHODS

Animals—Silkworm (Bombyx mori) larvae were reared on an artificial diet as described (30). Purification of Peptidoglycan and Soluble Fragmented Peptidoglycan—Peptidoglycan was prepared from Micrococcus luteus cell walls according to the method of Araki et al. (31).

For the preparation of soluble fragmented peptidoglycan, peptidoglycan was digested with egg white lysozyme (chicken) and fractionated by chromatography on a Sephadex G-50 SF column as described (18). Fractions (numbers 57 to 81) obtained in the chromatography were pooled and lyophilized. The lyophilized powder was dissolved in a small volume of 0.2M potassium phosphate buffer, pH 6.5, containing 1 mM EDTA, 1 mM 1,10-phenanthroline, 1 mM phenylmethanesulfonyl fluoride, 5 mM phenylthiourea, and 1% ethanol. The suspension was stirred for 2 h, then followed by centrifugation at 5,800 g for 20 min. Ammonium sulfate was added to the supernatant (69 g/500 ml of the supernatant) and the mixture was stirred for 2 h. Precipitated material was then collected by centrifugation and dissolved in 100 ml of 0.1 M potassium phosphate buffer, pH 6.5, containing additives as above. The solution was dialyzed for 30 h against the same buffer (1.9 liter) followed by dialysis against two changes of 1 M potassium phosphate buffer, pH 6.5.

The dialyzed solution was applied at a flow rate of 20 ml/h to a peptidoglycan-Sepharose 4B column (5 × 2.5-cm inner diameter) according to the method of Yoshida et al. (18) except that lysozyme-digested peptidoglycan was without fractionation by column chromatography on Sephadex G-50. The column was then sequentially eluted at 20 ml/h with the following eluants: 50 ml of 0.1 M potassium phosphate buffer, pH 6.5; a linear gradient of KCl from 0 to 2 M in a total volume of 120 ml of 0.1 M potassium phosphate buffer, pH 6.5; 60 ml of 5 M MES, pH 5.5, containing 2 M KCl. The final elution was carried out with 10 ml of 5 M acetic acid buffer, pH 4.5, containing 0.5 M sodium acetate at a flow rate 220 ml/h. Thirty-mi fractions were collected in containers containing 1.2 ml of 0.5 M Pipes, pH 7.0. All of the fractions were dialyzed separately against 3 liters of 10 mM potassium phosphate buffer, pH 6.5, for 18 h with a change of buffer.

The following column chromatography was performed at room temperature on a fast protein liquid chromatography system (FPLC; Pharmacia LKB Biotechnology Inc.). The active fractions (numbers 8–12 in Table I) obtained in the previous step were applied at a flow rate of 1 ml/min to a hydroxyapatite column (100 × 7.8-mm inner diameter; Koken Ltd., Tokyo) for high pressure liquid chromatography, previously equilibrated with 10 mM potassium phosphate buffer, pH 6.5, followed by washing the column with 10 ml of the same buffer. The adsorbed proteins were eluted at a flow rate of 1.0 ml/min with the following linear gradients of potassium phosphate buffer, pH 6.5, from 10 to 144 mM and from 144 to 1 M with concentration incremental rates of 2.48 and 93 mM/min, respectively. Fraction volume was 1.5 ml. Fractions eluted between 190 and 198 min from the beginning of the application of phosphate gradient were pooled (Fig. 1) and dialyzed overnight against 2 liters of 10 mM triethanolamine-HCl buffer, pH 7.5.

The dialyzed fractions were applied to a Mono Q column (HR 5/5) (Pharmacia LKB Biotechnology Inc.), equilibrated with the same buffer as that used for dialysis. Adsorbed proteins were eluted with a linear salt gradient in the same buffer (Fig. 2). The flow rate was maintained at 1 ml/min and 1.5-ml fractions were collected. The fraction with the highest PGRP activity (Fig. 2) was used as the PGRP preparation for study.

For the amino acid sequence analyses, the purified PGRP was passed through a reversed phase cyano-propyl-derived silica high performance liquid chromatography column (4.6 mm inner diameter × 250 mm, pore size = 300 Å) as follows: 0.5 ml of PGRP solution (about 40 μg of protein/ml of 10 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl) was applied to the column which was then eluted with a gradient of CH3CN from 30 to 70% in 0.1% CF3COOH/H2O at a flow rate of 0.4 ml/min. It took 50 min to finish the gradient. The only protein peak appeared at 29.69 min from the beginning of the gradient elution. Protein contained in the peak was pooled and lyophilized.

Purification of Silkworm Lysosome Lysozyme activity was assayed by measuring turbidity of Micrococcus lysodeikticus (Seikagaku Kogyo Ltd., Tokyo) suspension according to the manufacturer’s instruction manual.

The supernatant at 65% saturation of the ammonium sulfate fractionation of silkworm hemolymph during the first step of PGRP purification was dialyzed against 2.5 liters of 10 mM phosphate buffer, pH 6.5, for 40 h with three changes of the buffer. The dialyzed solution was applied to a CM-cellulose column (180 × 21-mm inner diameter) equilibrated with the same buffer at a flow rate of 100 ml/h. Adsorbed proteins were eluted at a flow rate 10 ml/h with a linear gradient from 0 to 1.0 M KCl in 200 ml of 10 mM potassium phosphate buffer, pH 6.5. Active fractions eluted at about 0.5 M KCl were pooled and dialyzed against 3 liters of 3 mM potassium phosphate buffer, pH 6.5, for 24 h with a change of the buffer, followed by chromatography on hydroxyapatite column (100 × 7.8-mm inner diameter; Koken Ltd., Tokyo) in the fast protein liquid chromatography. A gradient with an incremental rate of phosphate buffer, pH 6.5, concentration, 1 mM/min was applied at a flow rate of 3 ml/min to the column. A major peak eluted at 0.28 M KCl was used as purified silkworm lysozyme.

Determination of the Molecular Weight of Native PGRP—The molecular weight of native PGRP was estimated with two methods. The PGRP (A280 nm = 0.45) in 40 mM potassium phosphate buffer, pH 6.5, containing 0.2 mM NaCl was subjected to sedimentation equilibrium ultracentrifugation which was conducted by the method of Yphantis (33) using aHitachi analytical ultracentrifuge (Model 202 equipped with a Hitachi ultracentrifuge processor (Model-7)).

The native PGRP (15 μg) was chromatographed at a flow rate of 0.5 ml/min on Superose 12 column equilibrated with 10 mM Tris-HCl.
buffer, pH 7.5, containing 0.15 M NaCl. Egg white ovalbumin (chicken, 
45 kDa), myoglobin (horse skeletal muscle, 17 kDa), and cytochrome c 
(horse heart, 12.4 kDa) were used as proteins for molecular mass 
standard. Two-hundred μl of each standard protein solution containing 
200 μg of protein was subjected to chromatography on the Superose 12 
column under the same conditions as PGRP. A plot of their retention 
times against the elution volumes of their molecular masses gave a straight line. The molecular mass corresponding to the retention time of PGRP was 
read from the line.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Isoelectric 
Focusing-Polyacrylamide Gel Electrophoresis (IEF-PAGE)—SDS-
PAGE was carried out in a 1-mm thick slab gel according to Laemmli 
(34). IEF-PAGE was performed according to the method of Wrigley (35). Gel 
containing 5% acrylamide, 0.25% bis-acrylamide, and 2% Ampho-
line pH 3.5–10, was prepared in a column (110 × 2.5 mm inner diam-
eter). After 4 μg of PGRP had been electrophoresed for 3 h at 200 V, the 
gel was treated with 12% trichloroacetic acid and then stained for protein 
with Coomassie Brilliant Blue R-250. The gel was calibrated 
with the following isoelectric point markers: amyloglucosidase (pI 3.50), 
glucose oxidase (pI 4.15), soybean trypsin inhibitor (pI 4.55), β-lacto-
globulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human 
carbonic anhydrase B (pI 6.55), horse myoglobin (pI 6.85 and pI 7.35), 
lentil lectin (pI 8.15, pI 8.45, and pI 8.65), trypsinogen (pI 9.30).

Tests for Ability of Purified PGRP to Bind Peptidoglycan, Chitin, and 
β1,3-Glucan and the Effect of Some Constituents of Peptidoglycan and 
Chitin on the Ability—One mg of peptidoglycan was dispersed in 1 ml of T-M buffer and washed with 1 ml of this buffer by four cycles of sedimentation and suspension by centrifuga-
tion at 12,600 × g for 5 min. The sedimented peptidoglycan was 
suspended in 0.5 ml of T-M buffer. Forty-
l of the mixture of PGRP and peptidoglycan suspension and extract 
was then centrifuged at 12,600 × g for 5 min. The sedimented peptidoglycan was washed with 300 
ml of T-M buffer as above except that all 

The reactivity was 

The reactivity was
Peptidoglycan Recognition Protein from Insect Hemolymph

TABLE I

| Fraction number | Volume (ml) | Activity (units/ml) | A_{280} |
|-----------------|-------------|---------------------|---------|
| 1               | 16          | 0                   | 0.073   |
| 2               | 16          | 0                   | 0.106   |
| 3               | 16          | 0                   | 0.222   |
| 4               | 16          | 0                   | 0.120   |
| 5               | 16          | 1                   | 0.330   |
| 6               | 16          | 1                   | 0.150   |
| 7               | 24          | 1                   | 0.166   |
| 8               | 30          | 1                   | 0.047   |
| 9               | 30          | 2                   | 0.028   |
| 10              | 30          | 3                   | 0.031   |
| 11              | 30          | 4                   | 0.040   |
| 12              | 30          | 1                   | 0.020   |
| 13              | 30          | 0                   | 0.012   |
| 14              | 30          | 0                   | 0.011   |

* Fraction numbers 1-7 were obtained by salt gradient elution, fraction numbers 8 and 9 by elution with pH 5.5 buffer, and fraction numbers 10-14 by elution with pH 4.5 buffer.

In IEF-PAGE, the PGRP preparation gave a single band, the position of which corresponded to about pl 6.5 (data not shown). The amino acid composition of PGRP is presented in Table I together with that of silkworm lysozyme for comparison. No amino sugar was detected in the amino acid analysis of PGRP. Peroxidase-conjugated lectins (concanavalin A, Lantil seed agglutinin A, D. biflorus agglutinin, P. vulgaris agglutinin E4, A. hypogaea agglutinin, U. europaea agglutinin 1, and wheat germ agglutinin) were not reactive to PGRP under the experimental conditions as described under “Materials and Methods.” The absence of amino sugar and the non-reactivity of the lectins corroborate the possibility that PGRP is not a glycoprotein, although more thorough studies are necessary to demonstrate unambiguously the absence of sugar moieties in PGRP.

Native PGRP was sedimented to equilibrium at 23,000 rpm. A plot of ln(A_{280}) versus (radius)^2 gave a straight line. If the partial specific volume was assumed to be 0.75 ml/g, the slope of the line corresponded to that of protein with a molecular mass of 14 kDa. Native PGRP eluted as a symmetrical peak from the Superose 12 column with a retention time corresponding to that of a protein of 17 kDa when the column was calibrated with ovalbumin (chicken), myoglobin (horse skeletal muscle), and cytochrome c (horse heart) for molecular mass determination (data not shown). These values are smaller than the molecular mass of PGRP observed in SDS-PAGE. The reason for the inconsistency was unclear, but we concluded that native PGRP is likely to exist as monomer.

The restoration of reactivity of the prophenoloxidase cascade to peptidoglycan in plasma-PG supplemented with varied amounts of purified PGRP is shown in Fig. 4. A decreasing lag period was observed as the concentration of PGRP increased. Once activation of prophenoloxidase is initiated, however, the rate of conversion of prophenoloxidase to phenol oxidase seems to be independent of the amount of PGRP added. The kinetics for the activation of prophenoloxidase must reflect the underlying molecular mechanism for triggering prophenoloxidase cascade by peptidoglycan.

Purified PGRP was shown to bind to peptidoglycan, but not to chitin or β1,3-glucan (Fig. 5A). GMDP, MDP, N-acetylglucosamine, and N-acetylgalactosamine, all of which are the constituents of peptidoglycan, did not inhibit appreciably the binding of PGRP to peptidoglycan. However, soluble fragmented peptidoglycan inhibited appreciably for PGRP to bind to insoluble peptidoglycan (Fig. 5B). As our preliminary experiments indicated that the glycan portion of peptidoglycan has the ability to trigger prophenoloxidase cascade, it is probable that PGRP binds to the glycan portion of peptidoglycan.

A fraction (0–50 fraction), which has been shown to have all the components of prophenoloxidase cascade except prophenoloxidase, was prepared to examine whether proteins other than PGRP with affinity to peptidoglycan are present in silkworm hemolymph. A few polypeptides in the fraction were detected to bind to peptidoglycan (Fig. 6). It remains, however, to be investigated whether each of them can be adsorbed directly onto peptidoglycan by itself.

The sequence of 20 amino acid residues from the NH₂ terminus of PGRP was determined as: H-Asp-X-Asp-Val-ValSer-Lys-Lys-Gln-Trp-Asp-Gly-Leu-Ile-Pro-Val-His-Val-Ser-Tyr-. The

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2 K. Kinoshita and M. Ashida, unpublished observation.
second residue could not be identified.

Amidase activity of PGRP bound to peptidoglycan was examined using 26 commercially available peptidyl-NH-Mecs. None of the substrates were hydrolyzed significantly (data not shown), suggesting that PGRP is not an inactive protease which becomes active after binding to peptidoglycan.

**DISCUSSION**

We have previously proposed that the insect hemolymph prophenoloxidase cascade includes a βGRP and a PGRP, which have specific affinity to β1,3-glucan and peptidoglycan, respectively (18). These molecules were proposed to trigger the cascade upon binding to their respective ligands. Ochiai and Ashida (14) reported a method to obtain a homogeneous preparation of the putative βGRP. The postulated PGRP, capable of binding to peptidoglycan has been demonstrated, purified, and characterized in the present study.

The purified PGRP preparation was shown to be homogeneous by SDS-PAGE, IEF-PAGE, reversed-phase HPLC on cyanopropyl-derived silica column, and the determination of a 20-amino acid NH2-terminal sequence. The molecules are capable of restoring the reactivity of the prophenoloxidase cascade to peptidoglycan in plasma-PG which is assumed to contain all components of prophenoloxidase cascade except for PGRP (Fig. 4). These results indicate that the purified protein is PGRP. The molecular weight, isoelectric point, and amino acid composition of PGRP are different from those of βGRP (14). So, it is now proved unambiguously that the prophenoloxidase cascade in insect hemolymph has two points of initiation where PGRP and βGRP interact with their respective ligands.

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**TABLE II**

| Summary of purification of PGRP | Volume | Protein concentration | Total protein | Activity | Total activity | Yield | Specific activity |
|-------------------------------|--------|-----------------------|---------------|----------|----------------|-------|------------------|
| Hemolymph                     | 250    | 78                    | 19,500        | _b       | _b             |       |                  |
| Ammonium sulfate              | _c     | _c                    | _c            | _b       | _b             |       |                  |
| Peptidoglycan-Sepharose 4B    | 120    | 0.0075                | 0.900         | 2.5      | 300            | 100   | 0.333            |
| Hydroxyapatite                | 6.5    | 0.0573                | 0.372         | 21.5     | 140            | 47    | 0.376            |
| Mono Q                        | 1.5    | 0.189                 | 0.283         | 80       | 120            | 40    | 0.424            |

a Yield was calculated based on the total activity of peptidoglycan-Sepharose 4B fraction.
b Because hemolymph and ammonium sulfate precipitate contained unidentified factor(s) which causes activation of prophenoloxidase in plasma-PG in the absence of peptidoglycan, PGRP activity in these fractions could not be quantified.
c Not determined.

**TABLE III**

| Comparison of amino acid compositions of PGRP and silkworm lysozyme |
|--------------------------|-----------------|-----------------|
| Amino acid | Recovered amino acid | |
|      | PGRP | Lysozyme |   |
| Asx  | 100 | 142 |   |
| Thr  | 33  | 54  |   |
| Ser  | 71  | 78  |   |
| Gix  | 111 | 70  |   |
| Gly  | 115 | 78  |   |
| Ala  | 69  | 45  |   |
| Cys/2| 21  | 58  |   |
| Val  | 90  | 35  |   |
| Met  | 10  | 8   |   |
| Ile  | 48  | 32  |   |
| Leu  | 82  | 66  |   |
| Tyr  | 37  | 31  |   |
| Phe  | 23  | 35  |   |
| Lys  | 28  | 107 |   |
| His  | 39  | 41  |   |
| Trp  | 16  | 28  |   |
| Arg  | 63  | 65  |   |
| Pro  | 43  | 25  |   |

PGRP and lysozyme were analyzed for amino acid composition as described under “Materials and Methods.”

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**FIG. 3.** SDS-PAGE of purified PGRP, silkworm lysozyme, and egg white lysozyme (chicken). About 3.5 μg of protein was applied to each lane. Other experimental details are described under “Materials and Methods.” a, peptidoglycan recognition protein; b, silkworm (B. mori) lysozyme; c, egg white lysozyme; d, marker proteins lined from top to bottom in a following order (numbers in parentheses show polypeptide molecular weights): phosphorylase a (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), lysozyme (14, 400).

**FIG. 4.** Effect of the supplemented PGRP on activation of the prophenoloxidase cascade by peptidoglycan in plasma-PG. Each reaction mixture consisted of 10 μl of serially diluted PGRP solution, 110 μl of plasma-PG containing 7.27 mM CaCl2 and 10 μl of peptidoglycan solution (1.0 mg/ml). Reaction mixtures were incubated at 25°C, and at intervals an aliquot was assayed for phenol oxidase activity to monitor the activation of prophenoloxidase cascade. Concentrations of PGRP in reaction mixtures (μg of protein/ml of reaction mixture): ●, 0.018 μg; □, 0.090 μg; △, 0.180 μg; ○, 0.900 μg; ◊, 10.0 μg.
The ammonium sulfate fraction. Such nonspecific activation of prophenoloxidase cascade by peptidoglycan was prepared from silkworm plasma (36). Proteins adsorbed onto insoluble peptidoglycan in the fraction were investigated as described under “Materials and Methods.” The adsorbed proteins were subjected to SDS-PAGE together with 0–50 fraction. a, adsorbed proteins from 260 μg of protein of 0–50 fraction; b, 104 μg of 0–50 fraction; m, marker proteins (see Fig. 3).

and one may expect that they share some common properties. However, molecular weight, amino acid composition, isoelectric point, and NH₂-terminal sequence of PGRP were all different from those of insect lysozyme (43). The purified PGRP preparation did not show any appreciable lysozyme activity. We are currently undertaking by molecular cloning the entire primary structure of PGRP, to determine whether PGRP and lysozyme are related.

When the prophenoloxidase cascade in plasma-PG supplemented with PGRP was triggered with peptidoglycan, prophenoloxidase was activated in such a way that higher concentrations of the recognition protein reduced the lag time. However, the maximum velocity of prophenoloxidase activation seemed not to depend on the PGRP concentration (Fig. 4). A similar relationship between concentration of βPGRP and activation of prophenoloxidase in plasma-PG (plasma devoid of βPGRP) was observed (14). Furthermore, as was observed for βPGRP, PGRP bound to peptidoglycan did not show any significant activity to hydrolyze 26 commercially available substrates for proteases. These observations suggest that the basic mechanisms for the activation of prophenoloxidase cascade by peptidoglycan and βGLuc are similar. It would be necessary to isolate the components and to reconstruct the prophenoloxidase cascade in vitro to analyze the mechanism by which it is triggered by peptidoglycan or βGLuc.

Proteins with ability to bind to peptidoglycan were looked for by using a fraction (0–50 fraction) prepared from silkworm hemolymph plasma. The 0–50 fraction had advantages over non-fractionated plasma in such an investigation. 1) It did not have lysozyme activity which was likely to interfere with the present method for examining the binding of protein to insoluble peptidoglycan. 2) The fraction has been shown to contain all the components of prophenoloxidase cascade except prophenoloxidase (36) of which activation in plasma is known to cause the formation of aggregates of proteins (44). As is seen in Fig. 6, some polypeptides including the one with mobility corresponding to that of PGRP seemed to bind to peptidoglycan. This result, however, does not necessarily mean that each of them independently has ability to bind to peptidoglycan by
itself. It should be noted that we have observed that a purified polypeptide from silkworm hemolymph did not bind to peptidoglycan, but did it in the presence of PGRP.3 Thus, there remains a possibility that the polypeptides other than PGRP shown to have apparent affinity to peptidoglycan in Fig. 6 did not bind directly to peptidoglycan.

The inability of PGRP to bind to β1,3-glucan and chitin and the fact that the PGRP which had been incubated with soluble fragmented peptidoglycan barely bound to insoluble peptidoglycan corroborate our contention that the binding of PGRP to peptidoglycan is specific. Furthermore, the present observation is in accordance with our previous survey on the substances with elicitor activity for triggering prophenoloxidase cascade in silkworm plasma (45). No constituents of peptidoglycan (GMDP, MDP, N-acetylgalactosamine, and N-acetylmuramic acid), however, inhibited appreciably PGRP to bind to insoluble peptidoglycan. This result seems to indicate that structure with two or more repeating units of the glycan portion of peptidoglycan is recognized by PGRP. It is desirable to study further the structural requirements for PGRP to bind to peptidoglycan for our understanding on the mechanism of activation of prophenoloxidase cascade by peptidoglycan.

Peptidoglycan is known to have various biological activities, such as potentiation of the immune system, production of fever, promotion of slow wave sleep (25), and macrophage activation in mammals (27) and induction of synthesis of bactericidal substance in insect (46–48). Undoubtedly peptidoglycan interacts with specific ligands or receptors when it elicits such activities in living organisms. Isolation of peptidoglycan receptor on cell surface or peptidoglycan binding protein (or receptor) from insect hemolymph and possess affinities for microbial cell wall components.

We extend sincere thanks to Dr. M. Ochiai at the Institute of Low Temperature Sciences, Hokkaido University, for help on the analyses of the NH₂-terminal amino acid sequence of PGRP and to Y. Kozumi for skilful technical assistance.

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