A protein which bound to $^{125}$I-labeled peptidoglycan (PGN) was isolated from hemolymph of silkworm larvae. The N-terminal amino acid sequence and the molecular weight of the protein were in accord with those described for Promoting Protein (PP) from the silkworm. The binding of the protein to $^{125}$I]PGN was competitively inhibited by various β-glucans. The binding kinetics of PGN and chitin to the protein were analyzed in a biosensor.

Key words: silkworm; promoting protein; β-glucan binding protein; peptidoglycan

Insects respond to a microbial infection by the production of various humoral immune proteins, including a battery of antibacterial proteins. Most of these immune genes are normally silent and are induced upon bacterial infection. Bacterial cell wall components such as PGN and LPS are effective elicitors for the induction of the genes. We have found that PGN fragments of some definite structure are recognized as a signal molecule and elicit the synthesis of antibacterial proteins. Since these findings suggest the presence of a PGN recognition system in the silkworm, Bombyx mori. Since these findings suggest the presence of a PGN recognition system in the silkworm, we have tried to isolate PGN binding proteins from the hemolymph. One of the proteins isolated was identified as promoting protein, PP. PP is a basic protein with 136 amino acid residues recently isolated from the silkworm hemolymph as a factor that promotes the replication of BmNPV in silkworm cells cultured in vitro. Although the biological function of PP in the silkworm larvae is totally obscure, it has been suggested that the protein facilitates virus entry into the cells by reacting with the cell surface.

Silkworm strain C108 was reared aseptically on an artificial diet (Nihon Nosan Kogyo) at 28℃, as described previously. Hemolymph was collected from the fifth instar larvae 24 h after injection of the larvae with UV-killed Bacillus licheniformis (1 x 10$^7$ cells). Uncross-linked linear PGN was isolated from Micrococcus luteus culture medium incubated with penicillin, as described previously. The linear PGN was reacted with ASD (sulfosuccinimidyl 2-p-azide-salicylamido-1,3'-dithiopropionate; Pierce) to form ASD–PGN, then iodinated with carrier-free Na$^{125}$I (Dupont) with Iodo-Gen Iodination Reagent system (Pierce). PGN-binding activity was detected by photoaffinity crosslinking of $^{125}$I]labeled-PGN to proteins as described, and the labeled protein was separated by SDS–PAGE. Gels were exposed to an Imaging Plate (Fuji Film) and the image was visualized with a fluoro-image analyzer (Fuji Film FLA-2000). The binding kinetics of PGN and oligo chitin to PP was analyzed in a biosensor (IAsys Plus, Affinity Sensors). Cell wall PGNs were isolated and solubilized as described previously. LPS and laminarin were purchased from Sigma Chemicals. Oligo chitin (hexa-N-acetyl-chitohexaose) was purchased from Seikagaku Kogyo.

For isolation of PGN-binding protein, ammonium sulfate was added to the larval hemolymph to 60% saturation, and the precipitate was collected and dialyzed against 0.05 M phosphate buffer, pH 6.5. The dialyze was incubated with UV-killed B. licheniformis cells for 20 min at 4℃. After washed the bacterial cells with 20 mM phosphate buffer, pH 6.5, containing 0.1 M NaCl, the proteins bound to the bacterial cells were eluted with the same buffer containing 1 M NaCl. The eluate was dialyzed against 20 mM Tris–HCl, pH 7.5, and loaded onto a DEAE-Toyopearl 650S column (16 x 85 mm). The unadsorbed fraction was applied to a TSKgel CM-SPW column (7.5 x 75 mm) and eluted with a linear gradient formed with 0 and 1.0 M NaCl in 20 mM Tris–HCl, pH 7.5. A peak fraction with PGN-binding activity was collected.

The purified protein showed single bands on both native and SDS–PAGE, and a single peak by gel filtration chromatography on a Superdex 75HR column (10 x 300 mm). The sequence of N-terminal 20 amino acids was EFNVVTRTLCREVDASACTV, which matched perfectly with that of PP. The molecular mass of the isolated protein estimated by the Superdex...
column was 13 kDa, and that by SDS–PAGE was 17 kDa. These values and the characteristics of the protein giving a lower value with gel filtration are also shared with PP. These results indicated the PGN-binding protein was PP.

Binding of \[^{125}\text{I}]\text{ASD-PGN to PP was competitively inhibited by unlabeled PGNs, LPS, oligo chitin and laminarin (Fig. 1). Binding was not inhibited by GlcNAc, MurNAc (Fig. 1) or soluble starch (data not shown). Both linear PGN and oligo chitin bound to PP immobilized on a IAsys Amino cuvette in a dose-dependent manner (Fig. 2). The }K_D\text{ values for PGN and oligo chitin were calculated to be 1.55 and 0.87 mg/ml, respectively.}

The results suggest that PP binds oligo- or poly-\(\beta\)-glucans including PGN of various structures, LPS, chitin (\(\beta\)-1,4-glucans) and laminarin (\(\beta\)-1,3-glucan), but not the monomers or \(\alpha\)-glucans. PP has been isolated as a protein factor that stimulates BmNPV replication in vitro. The protein was constitutively synthesized in the larval fat body, and the concentration in the hemolymph was maintained at relatively high levels (10 mg/ml or higher) throughout the fifth instar stage. Since it is difficult to assume, however, that the silkworm prepares for the entry of a virus to stimulate its growth, PP might have some other original role(s) in the silkworm. Involvement of PP in chitin metabolism during cuticle degradation is another possibility.

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References


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