Nonradiolabeling Assay for WaaP, an Essential Sugar Kinase Involved in Biosynthesis of Core Lipopolysaccharide of *Pseudomonas aeruginosa*

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**waaP** is present in the lipopolysaccharide (LPS) core gene clusters of a wide range of gram-negative bacteria, and is an essential gene in *Pseudomonas aeruginosa*. The WaaP protein is a sugar kinase that adds phosphate to heptose I in the core oligosaccharide. This study describes the standardization and utility of a chemiluminescence-based enzyme-linked immunosorbent assay for the detection of WaaP kinase activity. Important features of the assay include high sensitivity, the preparation of dephosphorylated LPS as a substrate, and the use of monoclonal antibody 7-4 that specifically recognizes phosphate substituents in the LPS core.

**waaP** is a conserved gene that encodes a sugar kinase involved in the phosphorylation of heptose I of the inner core of lipopolysaccharide (LPS) in *Pseudomonas aeruginosa* (12), *Escherichia coli*, and *Salmonella enterica* serovar Typhimurium (15). Our laboratory has further shown that **waaP** of *P. aeruginosa* is an essential gene, since a mutation in this gene is lethal to the bacterium (12). Intriguingly, WaaP of *P. aeruginosa* also possesses eukaryote-like protein tyrosine kinase activity (16). Therefore, this protein offers an attractive target for the development of novel drugs to control *P. aeruginosa* infections.

In general, the measurement of kinase activities utilizes radiolabeled [32P]ATP or [33P]ATP (1, 11). Yethon and Whitfield developed a radiolabeling kinase assay to determine the activity of WaaP from *E. coli* (14). However, automation of a radiolabeling assay for the purpose of high-throughput screening of novel antimicrobial compounds would be difficult and labor-intensive (4). Thus, the aim of this study was to develop a sensitive, chemiluminescence-based, enzyme-linked immunosorbent assay (ELISA) for measuring the sugar kinase activity of *P. aeruginosa* WaaP.

Since a mutation in **waaP** is lethal in *P. aeruginosa*, no **waaP** mutant was available for preparing phosphate-deficient LPS. To prepare dephosphorylated LPS as the substrate for the WaaP assay, LPS extracted from *P. aeruginosa* PAO1 cultures using the standard hot-phenol method described by Westphal and Jann (13) (PAO1-LPS) was treated with 48% hydrofluoric acid (HF) (Fisher Scientific, Nepean, Ontario, Canada) at 4°C for 48 h (9). Following HF treatment, the samples were diluted 1:1 in H2O, dialyzed extensively against 50 mM Tris-HCl (pH 8.0) and then against H2O, and lyophilized to recover the dephosphorylated LPS, which was designated HF-LPS. To determine the effectiveness of HF treatment, HF-LPS, PAO1-LPS, and WaaP-reconstituted HF-LPS were assayed for inorganic phosphate according to the method described by Ames and Dubin (2), using a standard curve of 0 to 40 nmol of K2HPO4. No phosphate could be detected from 250 μg of HF-LPS, whereas 2.18 μg of phosphate (∼0.87%) could be detected from 250 μg of PAO1-LPS.

The phosphate assay was also performed on HF-LPS following reconstitution with purified WaaP. The procedures for the overexpression and purification of WaaP with a C-terminal six-His tag (WaaPHisC) and the methods for analyzing the proteins, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting, were as described by Zhao and Lam (16). The 50-μl reconstitution reaction mixture contained 100 ng of HF-LPS, 20 mM MgCl2, 5 mM dithiothreitol, 250 μM ATP, and 20 mM Tris-HCl buffer, pH 7.8, and the reaction was started by the addition of 5 μg of enzyme (purified WaaPHisC in 20 mM Tris-HCl, pH 7.5). The mixtures used for phosphate assay or processed for ELISA were placed in 96-well plates incubated at 37°C for 30 min and quenched by the addition of 60 μl of chloroform/ethanol (1:10) solution. The samples were centrifuged at 12,000 × g at 4°C for 10 min, and 100-μl aliquots of supernatant were transferred to 96-well plates, left at room temperature in the fume hood overnight, and subjected to ELISA the next morning. Approximately 20 nmol of Pi/ng of LPS was detected in the reconstituted P-HF-LPS compared to 190 nmol of K2HPO4. Functional and capable of restoring approximately 10% of the phosphate content in HF-LPS.

SDS-PAGE and Western immunoblotting analyses were used to characterize HF-LPS and to determine if monoclonal antibody (MAb) 7-4 (6) recognizes the phosphate substituents in the LPS core. The core oligosaccharide bands of both HF-LPS and PAO1-LPS migrated similarly (Fig. 1A, arrow), except that the lowest-molecular-weight core oligosaccharide band of HF-LPS migrated slightly faster than that of the control (Fig. 1A, lane 2). This suggests that no sugar residue was cleaved off from the core region of PAO1-LPS after HF treatment and that the slightly faster migration of the core LPS band in HF-LPS corresponds to the cleavage of phosphates by the HF treatment. No reaction was detected in Western im—

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munoblots between MAb 7-4 and the HF-LPS (Fig. 1B, lane 2). In contrast, MAb 7-4 reacted strongly with the core LPS of untreated PAO1-LPS (Fig. 1B, lane 1). Since HF treatment is known to remove phosphates from LPS (8, 10), the loss of the recognition of HF-LPS by MAb 7-4 provided the evidence that the phosphate substituents in the LPS core are part of the epitope for MAb 7-4. Dephosphorylation of PAO1-LPS by HF treatment had no effect on the antigenicity of B-band O-antigen polymers (Fig. 1C, lane 2), but a slight degradation of high-molecular-weight B-band LPS resulted in the appearance of a slightly higher proportion of semirough LPS (Fig. 1A, lane 2, arrow). HF treatment also abrogated the reactivity of A-band-specific MAb N1F10 (Fig. 1D, lane 2). Nonetheless, a strong reactivity of the low-molecular-weight LPS bands with MAb 5c101 (outer-core specific) implied that the core oligosaccharide was intact (Fig. 1E, lane 2). These antibodies have been described previously (3, 5, 6).

Once we established that MAb 7-4 specifically recognizes the phosphate substituent in the LPS core, it was used as the primary antibody in the ELISA for the detection of WaaP kinase activity. ELISA procedures based on what was described by Bantroch et al. (3) were used with minor modifications. The antigens added to the 96-well plates included PAO1-LPS, HF-LPS, and reconstituted P-HF-LPS. Transparent Nunc-Immuno F16 Maxisorp 96-well plates were used for the colorimetric reading (VWR, Mississauga, Canada) and opaque plates were used for the chemiluminescence reading (Costar; Corning, Puslinch, Ontario, Canada). The chloroform-ethanol evaporation procedure (3) was used for antigen coating. For the standard colorimetric method, p-nitrophenyl phosphate (Sigma Chemicals, St. Louis, Mo.) was used as the substrate, which was diluted in 1 M diethanolamine buffer (pH 9.8) containing 0.5 mM MgCl2, and the ELISA was developed at 37°C for 2 h and read at 405 nm using a microplate reader (Flow Laboratories, Mississauga, Ontario, Canada). For chemiluminescence development, 100 lL of a 1:5 (vol/vol) dilution of the chemiluminescence substrate CDP-Star Ready-to-Use with Emerald-II (CDP*) (contains 0.25 mM CDP* diluted in 1 M diethanolamine buffer) (Applied Biosystems, Bedford, Mass.) was added to each well, incubation was at 20°C for 20 min, and chemiluminescence response was measured on a 1420-VICTOR2 Multilabel Counter (Wallac, Montreal, Quebec, Canada).

The conventional ELISA allowed the detection of 50 ng of PAO1-LPS with an absorbance of 0.3 at 405 nm (Fig. 2A). The sensitivity of the assay improved significantly when the chemiluminescence substrate CDP* was used and as little as 5 ng of LPS could be detected, producing a signal of 2000 chemiluminescence units above the control blank (Fig. 2B). Thus, the chemiluminescence-based assay is at least 1,000 times more sensitive than the conventional ELISA. The latter assay was further optimized by simultaneously adding primary and secondary antibodies to obtain a linear response for detecting 0 to 80 ng of LPS, compared to the loss of linearity at 50 ng of LPS.
when the primary and secondary antibody were added consecutively (data not shown). Using the chemiluminescence ELISA to compare the reactivity of MAb 7-4 to HF-LPS and PAO1-LPS, respectively, a dose-dependent linear curve was obtained for 80 to 200 ng of PAO1-LPS while no reactivity could be detected with 1 to 200 ng of HF-LPS (Fig. 2C).

In a separate study, the chemiluminescence ELISA of WaaP was shown to be remarkably versatile. The assay was used in determining the kinetic parameters on the phosphorylation of HF-LPS by the purified WaaP (16), and these parameters can be summarized as the following: $K_m$ of WaaP was 0.22 mM for ATP and 14.4 μM for HF-LPS and $k_{cat}$ was 27.23 min$^{-1}$.

In conclusion, a sensitive and nonradio-labeling ELISA for WaaP was developed that was suitable for determining kinase activity of this protein (16) and is appropriate for high-throughput screens for antimicrobial compounds that are inhibitory to the activity of WaaP.

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REFERENCES


