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POLYPHEMIN: A TEICHOIC ACID-BINDING LECTIN FROM THE HORSESHOE CRAB, LIMULUS POLYPHEMUS

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SUMMARY: A Staphylococcus aureus-agglutinating lectin, capable of binding to N-acetyl-D-glucosamine, was isolated from the serum of Limulus polyphemus. The monosaccharide alone was incapable of inhibiting bacterial agglutination by this lectin. Quantitative precipitation studies with purified cell wall-derived teichoic acids, either devoid of or containing N-acetyl-D-glucosamine, confirmed the carbohydrate-binding specificity of the lectin and suggested that secondary, non-specific interactions contribute to binding biomolecules containing this sugar. The agglutination pattern with various S. aureus strains having N-acetyl-D-glucosamine-associated teichoic acid, teichoic acid without this sugar, and no teichoic acid indicated that this cell wall component is not the sole binding site for the lectin on intact S. aureus cells. Affinity gel chromatography, using N-acetyl-D-glucosamine-associated teichoic acid as the specific absorbent, has been used to isolate this lectin from Limulus serum.

A Staphylococcus aureus-binding agglutinin from Limulus polyphemus hemolymph has been previously described [1]. When recovered by affinity chromatography with commercially prepared agarose (a linear polysaccharide of alternating D-galactose and 3,6-anhydro-L-galactose units), the agglutinin appeared to be distinct from the N-acetylneuraminic acid-binding lectin, limulin, and to be specific for galactose or galactose-associated molecules. This agglutinin may bind more strongly to other carbohydrate forms, however, based on its weak affinity for agarose and the complexity of carbohydrate constituents present on bacterial cell surfaces. Teichoic acid is a significant cell surface component of S. aureus; previous studies have shown that this organism is agglutinated by whole Limulus serum [2]. This report describes the teichoic acid-binding capacity of an agglutinin from Limulus serum, and a specific affinity absorbent containing teichoic acid used to isolate this protein.

Abbreviations. GlcNAc - N-acetyl-D-glucosamine; HEPES - N-2-hydroxy-ethyl-piperazine-N'-2-ethane-sulfonic acid

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MATERIALS AND METHODS

Preparation of teichoic acid. S. aureus strains H, 52A2, and 52A5 were obtained from Rivka Bracha, Weizmann Institute of Science, Rehovot, Israel. Cell walls from S. aureus strains H and 52A2 were prepared by mechanically disrupting lyophilized bacteria with glass beads [3]: 250 ml of cold distilled water, 15 g of bacteria (dry weight), and 250 g of glass beads (d = 0.15 mm) were mixed and agitated with a Sorvall Omni-Mixer for 45 min at 4°C. Cell walls were harvested from the fluid phase by centrifugation at 25,000 xg for 30 min and the collected material washed 6X with 5 vol of 0.1 M phosphate buffer, pH 7.0, and 6X with cold distilled water. The recovered cell walls were then lyophilized. Teichoic acid was extracted from S aureus H and 52A2 cell walls using trichloroacetic acid [4]. The precipitates from the first and second extractions were recovered separately. Teichoic acid from the second extraction step was used for all analyses in this study.

Chemical analysis of teichoic acid. The teichoic acids (4 mg each) from S. aureus H and 52A2 were each hydrolyzed with 2 N HCl (2 ml) at 100°C for 4 h in sealed tubes. The hydrolysates were dried by evaporation at 40°C and reconstituted in 50 µl of 10% isopropanol for analysis by paper chromatography. Descending chromatography was carried out on Whatman 3MM paper using n-butyl alcohol:pyridine:water (6:4:3) and n-butyl alcohol:acetic acid:water (67:23:10) as solvent systems [5]. For detection of nitrogenous sugars and amino acids, 15 µl of glucosamine, galactosamine, alanine, glycine, glutamic acid, lysine, and aspartic acid, each at a concentration of 5 mg/ml, and 15 µl of the hydrolysate were spotted 1.5 cm apart on the chromatography paper. After development, the papers were dried at room temperature, sprayed with ninhydrin, and heated at 60°C for 10 min. Teichoic acid hydrolysates and glucosamine, ribitol, 1,4-anhydro-ribitol, and glycerol control samples, each at a concentration of 1.0 mg/ml, were spotted on a second paper as above for detection of polyols and reducing sugars after development using alcoholic silver nitrate [6].

Buffers. Many systems in this study utilized a buffer containing 0.01 M $CaCl_2$, 0.14 M NaCl, and 0.01 M HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethane-sulfonic acid), pH 7.4 at 4°C, hereafter referred to as HEPES buffer. HEPES-citrate buffer contained 0.01 M HEPES and 0.02 M Na citrate, pH 7.4 at 4°C [1].

Preparation of the teichoic acid-Sepharose 6B affinity absorbent. N-acetyl-D-glucosamine(GlcNAc)-associated teichoic acid from S. aureus H was coupled to epoxy-activated Sepharose 6B (Pharmacia Fine Chemicals) by the method of Vretblad [7]. The gel was swollen and washed with distilled water on a glass filter for 1 h. To 6 ml of the wet gel in 0.1 N NaOH was added a 6-ml solution of teichoic acid (500 mg) in 0.1 N NaOH, and the mixture was incubated at 45°C in a shaking waterbath for 15 h. After incubation, the gel was washed with 0.1 N NaOH, incubated in 1.0 M ethanolamine for 4 h at room temperature to block excess oxirane groups [8], and washed consecutively with water, 0.1 M borate buffer (pH 8.0 and containing 0.5 M NaCl), and 0.1 M acetate buffer (pH 4.0 and containing 0.5 M NaCl). The gel was then equilibrated with HEPES buffer prior to use.

Isolation of teichoic acid-binding lectin. Limulus hemolymph was incubated with the affinity gel for 12 h. Unbound material was removed by washing the gel extensively with HEPES buffer. Lectin was subsequently eluted from the affinity gel with HEPES-citrate buffer at a flow rate of 8 ml/h and collected in 3 ml fractions. All fractions containing protein were consolidated, dialyzed with HEPES buffer, and concentrated to 1.0 ml by ultrafiltration. Protein concentration was measured by the method of Bradford [9], and the biologic activity was assessed by bacterial agglutination.

Bacterial agglutination and inhibition of agglutination. Stock suspensions of S. aureus H, 52A2, and 52A5 were heat-killed at 60°C for 2 h and the cells

were washed 3X with saline. Working dilutions of these test bacteria were made in HEPES buffer containing 0.01% safranin O, and these were standardized by absorbance at 550 μm. All bacterial agglutination studies were performed in microtitration assays as previously described [10]. For the sugar inhibition studies, 25 μl of a predetermined sugar concentration in HEPES buffer (adjusted to pH 7.4 with 0.1 N NaOH in the case of acidic sugars) were placed in the experimental wells containing the serially diluted lectin and allowed to incubate 30 min at room temperature prior to the addition of 25 μl of the S. aureus test cells. All titers were recorded following a 12-h incubation period at 4°C.

Quantitative precipitation assays. Precipitation tests were performed by a modified method of Goldstein et al. [11]. Designated amounts of teichoic acid from S. aureus H and 52A2 were dissolved in HEPES buffer and 200 μl from each dilution were dispensed into separate 1.5-ml polypropylene tubes. An equal volume of lectin at 5 mg/ml was added to each tube to make a final volume of 400 μl. The tubes were covered, mixed, and incubated at room temperature for 30 min, then at 4°C for 4 days. Precipitated protein was collected by centrifugation at 8000 xg for 5 min and washed 2X in HEPES buffer. The precipitates were digested in 200 μl of 0.1 N HCl and assayed for protein.

RESULTS

None of the teichoic acid preparations used in this study had detectable nucleic acid, as determined spectrophotometrically, or protein. Chemical analyses of the teichoic acids from S. aureus H and 52A2 were consistent with published descriptions [5].

Limulus lectin, recovered by affinity chromatography with immobilized teichoic acid, was eluted as a single peak by HEPES-citrate. As determined by agglutination of S. aureus H cells, the purified lectin, termed polyphemin, had a specific activity twelve times that seen in whole hemolymph on a per-milligram-protein basis (Table 1).

Results of agglutination assays with S. aureus H, 52A2, and 52A5 revealed similar agglutination patterns for whole hemolymph and polyphemin (Table 2).

TABLE 1. Isolation of Polyphemin from Limulus Hemolymph

Source of agglutinin	Volume (ml)	Protein (mg/ml)	Titer	Specific activity*	Total activity*	Purification
Whole hemolymph	10	95	32	0.33	320	1
Purified lectin	0.8	4	16	4	12.8	12

* Specific activity = $\frac{\text{titer}}{\text{mg protein/ml}}$

** Total activity = titer · volume

TABLE 2. Bacterial Agglutination by Limulus Serum and Components

Source of agglutinin	Strains of Staphylococcus aureus		
	H	52A2	52A5
Whole serum	5.3 ± 0.41*	2.3 ± 0.49	3.3 ± 0.49
Serum adsorbed with			
S. aureus H	-0-	0.4 ± 0.77	0.2 ± 0.63
Purified polyphemin	4.8 ± 0.69	1.1 ± 0.69	2.4 ± 0.62

* Expressed as Log_2 values ± 1 standard error

Both agglutination samples reacted best with the S. aureus H strain, which contains GlcNAc-associated teichoic acid and only weakly with the strain devoid of this sugar on its teichoic acid (52A2). The strain lacking this cell wall polymer altogether (52A5) was agglutinated to a somewhat greater degree than S. aureus 52A2. As expected, hemolymph depleted of this lectin by adsorption exhibited little or no agglutinating activity for these staphylococci.

In an effort to define the specificity of polyphemin, we individually tested ten different monosaccharides (glucose, galactose, mannose, the amino-sugar counterparts of each, the N-acetylaminosugar counterparts of each, and N-acetylneuraminic acid) for their ability to inhibit S. aureus H agglutination. None of these sugars at concentrations as high as 100 mM effected a visible decrease in the agglutination activity of this lectin. In the quantitative precipitations assays, however, good precipitation was evident using GlcNAc-associated teichoic acid compared to the teichoic acid without this sugar, which at comparable amounts did not precipitate the lectin (Fig. 1). No precipitated protein was detected in control samples containing the lectin and HEPES buffer alone.

DISCUSSION

Having well characterized cell-wall teichoic acids, S. aureus strains H and 52A2 were useful aids in characterizing the agglutinating activity of Limulus polyphemus serum against certain Gram-positive organisms. Teichoic acid from S. aureus H cell walls consists of a ribitol-phosphate polymer with D-alanine

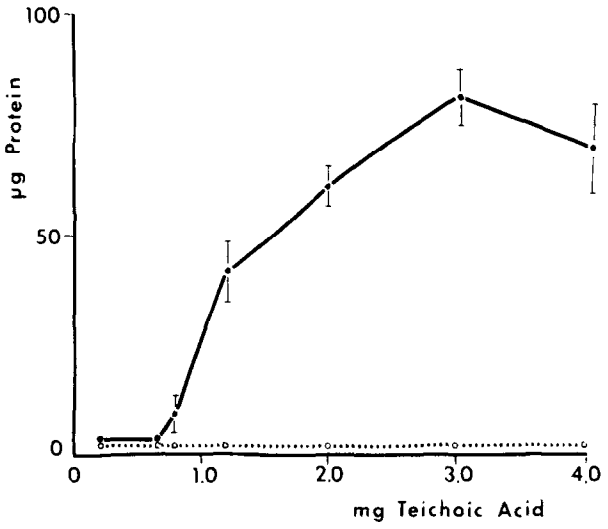

FIGURE 1. Quantitative precipitation of S. aureus H teichoic acid (●—●) and S. aureus 52A2 teichoic acid (o---o) by the lectin polyphemin.

esterified at C-2 or C-3 of about half the ribitol residues, and GlcNAc in β linkage with C-4 of the ribitol. The cell-wall teichoic acid of the mutant strain 52A2 differs in that it contains no GlcNAc [5] (Fig. 2). A significant difference observed in the reactivity of Limulus serum with these two teichoic acids in bacterial agglutination assays (Table II) suggested an agglutinin reacting with GlcNAc; thus, a method for recovering the lectin by affinity chromatography was developed using GlcNAc-associated teichoic acid as the affinity ligand.

Successful generation of an affinity absorbent requires alkali (0.1 N NaOH) for immobilization onto epoxy-activated Sepharose 6B; although the D-alanyl

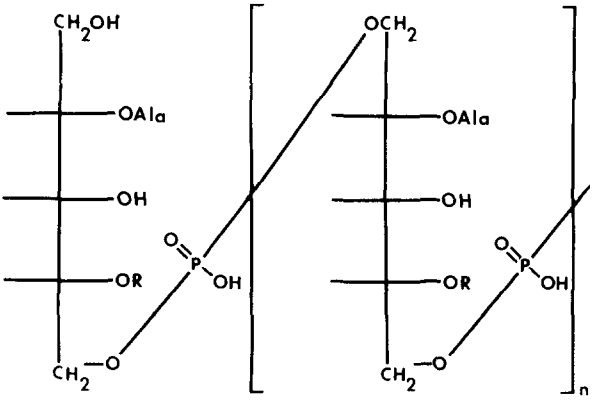

FIGURE 2. Teichoic acid from S. aureus H (R = N-acetyl-D-glucosamine) and S. aureus 52A2 (R = H)

residues are lost at this pH, the glycosidic linkage between GlcNAc and ribitol is unaffected. Moreover, concentrations of NaOH up to 0.5 N have been used for extraction of teichoic acids from certain Staphylococcus, Bacillus, and Lactobacillus species [4]. Those teichoic acids containing GlcNAc-1-phosphate linkages as an integral part of the polymer chain have a characteristically high lability to acids and alkali and are consequently not suitable for such treatments [12].

Affinity chromatography with immobilized teichoic acid achieved a twelvefold purification of the S. aureus-binding agglutinin, polyphemin, from Limulus serum (Table I). Calcium ions were required for restoration of the biological activity of this lectin following elution with citrate. Quantitative precipitation assays (Fig. 1) led to two assumptions on the carbohydrate-binding specificity of this lectin. First, polyphemin appears to bind specifically to GlcNAc residues, as precipitation of the lectin occurred with GlcNAc-associated teichoic acid, but not with teichoic acid devoid of this sugar. Second, free monosaccharides including GlcNAc failed to inhibit agglutination of S. aureus H cells by polyphemin suggesting that secondary, non-specific interactions may contribute to binding of the lectins to this sugar.

Interestingly, polyphemin also agglutinated bacterial cells lacking teichoic acid altogether (S. aureus 52A5; Table II). This is likely due to the presence of GlcNAc residues in the underlying peptidoglycan. Thus, bacterial cells agglutinated by polyphemin have accessible GlcNAc residues on the teichoic acid or in the peptidoglycan. S. aureus 52A2 cells, on the other hand, were not appreciably agglutinated probably due to the GlcNAc-deficient teichoic acid masking the peptidoglycan layer. Similar interactions with bacterial cells and cell-wall polymers have been reported for wheat germ agglutinin [13].

The S. aureus-binding agglutinin described by Gilbride and Pistole was shown to contain a significant amount of copper, indicating the metalloprotein may be at least partially composed of hemocyanin [14]. Preliminary evidence (E.R. Brandin, unpublished data) suggests that polyphemin is indeed associated with the copper-containing hemocyanin proteins of Limulus. Further investiga-

tion is needed, however, to better characterize the nature and significance of this association.

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