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Proanthocyanidin-based Endotoxin Removal

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14. ABSTRACT The application of proanthocyanidins (PACs) to the capture and removal of bacterial lipopolysaccharide (LPS) from solution has been demonstrated. PACs from whole cranberries, grape juice, black tea, and cranberry juice were purified and immobilized onto thiol-activated Sepharose beads. This material was used in pull-down type assays for the capture of LPS. The binding of LPS by PACs has been shown to compete with that of polymyxin B, which is known to bind the lipid A component of LPS. The removal of LPS from solution using a column of a PAC-modified resin is also demonstrated over a range of pH values and ionic strengths. The performance of the resin in protein solutions as compared to that of polymyxin B is evaluated. PACs were also immobilized onto Luminex beads and applied to the capture of <i>E. coli</i> and <i>B. globigii</i> .					
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PROANTHOCYANIDIN-BASED ENDOTOXIN REMOVAL

INTRODUCTION

Bacterial endotoxin, or lipopolysaccharide (LPS), is the major component of the outer membrane of Gram-negative bacteria. LPS is shed by bacterial cells during their life cycle and released in large amounts during degradation of the outer membrane following cell death. Commonly, clinical treatment of bacterial infections involves the use of compounds that disrupt bacterial membranes, resulting in the release of LPS. Contamination by LPS also poses a risk in a number of therapeutic settings: plasmid DNA preparations used for gene therapy, therapeutic recombinant protein preparations, and implanted medical devices all face the need for removal of LPS contamination. Current FDA recommendations indicate the introduction of no more than 5.0 Endotoxin Units (EU; 0.5 ng LPS) per kilogram of body weight [1].

The need for cost-effective, safe methods of LPS neutralization and removal has resulted in work directed at the synthesis or purification of a variety of compounds with LPS-binding activities [2–11]. Polymyxin B (PMB) is the most commonly employed agent for removal of LPS contamination. PMB is obtained from *Bacillus polymyxa* culture filtrates [12] and has been applied to the removal of LPS in a variety of formats [13–15]. Several LPS-binding peptides such as cecropins, sapecin, and a lactoferrin-based peptide (LF11) have been identified [2–4]. LPS-binding and lipid A-binding drugs and their structural analogs have been described for LPS removal. Other small molecules such as spermine-sulfonamide analogs, acyl-polyamines, and bis-guanyldrazones have been shown to possess potent LPS-binding characteristics, as have biosurfactants such as surfactin C [5–7]. These various compounds present a range of limitations. Antimicrobial peptides tend to be somewhat unstable, though there are several ongoing efforts attempting to address this issue. Biosurfactants are typically purified from bacterial cultures, as is PMB. The purification of biosurfactants requires multiple steps, including adsorption or thin-layer chromatography, which present limitations on the scalability of production. Other materials present complications related to such factors as toxicity, specificity, and a decreased efficacy upon immobilization.

Proanthocyanidins (PACs) are abundant, naturally occurring plant secondary metabolites. They are polymers composed of monomeric flavanoid subunits such as catechin and epicatechin (Fig. 1). The basic monomer structures consist of a phenyl ring bound to a bicyclic benzopyran structure. This structure is modified by several hydroxyl groups often in the 3, 5, and 7 positions of the bicyclic ring as well as at the 3' and 4' positions on the phenyl ring. Polymers of 2 to 50 subunits are possible and may contain varying ratios of different subunits. Polymers joined by a single bond between the 4 and 8 or 4 and 6 carbons are classified as B-type. A-type PACs contain an additional inter-subunit ether linkage between oxygen-7 and carbon-2. Typical plant sources of PACs include fruits, leaves, seeds, roots, and bark [16].

A wide range of health benefits have been ascribed to PACs including antiviral [17–19] and antibacterial activities [20–23] and inhibition of microbial adhesion [24–33]. The interaction of PACs from cranberry and black tea with lipopolysaccharide from several bacterial species has been described [34]. That report indicated that PACs specifically bound and neutralized the endocytosis of LPS by blocking its interaction with cognate LPS receptors. Inhibition of this interaction effectively prevented

LPS-induced activation of the transcription factor, NF- κ B, and suggested that PACs may be useful as a component of in vivo treatments for Gram-negative bacterial infections. The work presented in this report demonstrates that immobilized PACs from cranberry, black tea, and grape juice can be used for the removal of LPS from solution. We further show that the binding affinities of the immobilized PACs for LPS are comparable to those of immobilized polymyxin B [34]. We further demonstrate the removal of LPS using a column of a PAC-modified resin over a range of pH values and ionic strengths.

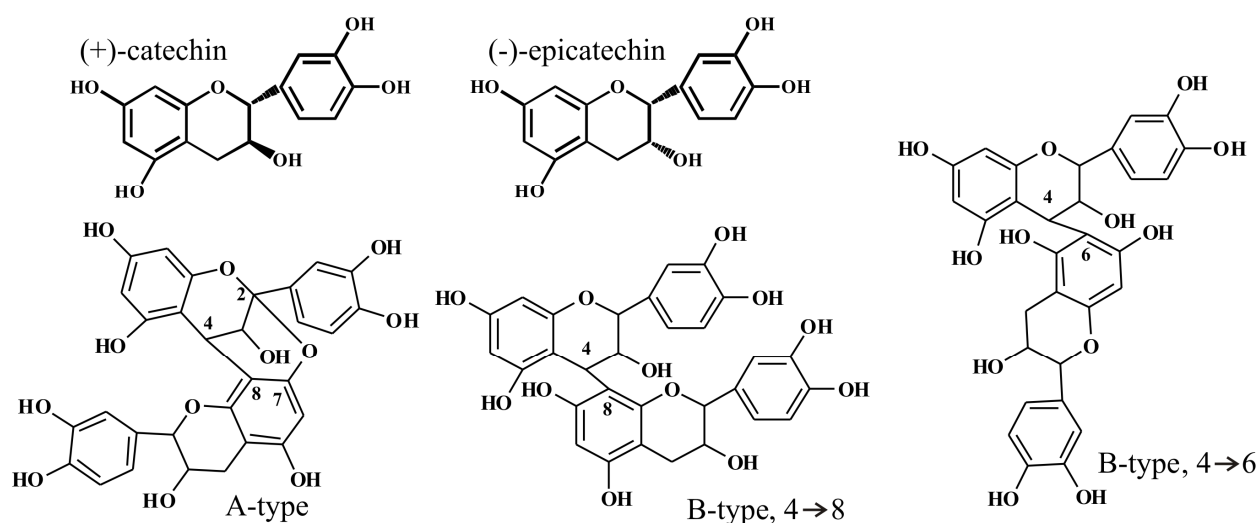


Fig. 1 — Structures of the monomeric flavonoid subunits catechin and epicatechin and the common PAC linkages.

APPROACH

Proanthocyanidins were obtained by purification from Mountain Sun pure unsweetened cranberry juice (Celestial Group, Inc., Melville, NY), Welch's 100% red grape juice, Lipton black tea, whole cranberries, chocolate, coffee, and green tea. Purification of proanthocyanidins through hydrophobic adsorption chromatography has been described previously [35–37]. Whole juice (cranberry or grape) was reduced by rotary evaporation (60 °C) to a minimum volume. Rotary evaporation (40 °C) was used to remove all acetone, and extracts were resolubilized in 75% ethanol to twice the original volume. Cranberries and chocolate were masticated using a Waring blender (100 g) with 75% ethanol in water (60 mL). Vacuum filtration was used to separate the solids from the resulting liquid. Solids were resuspended in 70% acetone (300 mL), sonicated for 30 min, and filtered [38]. Resuspension, sonication, and filtration of insoluble material were repeated three times and all liquid was combined. The solution was reduced by rotary evaporation to remove all acetone and resolubilized in 75% ethanol to twice the original volume. Tea was extracted using sonication of one family-sized tea bag in 70% acetone (60 mL). The resulting solution was reduced by rotary evaporation (40 °C) and resolubilized in 75% ethanol (120 mL).

Extract solutions in 75% ethanol were applied to a Sephadex LH20 column in batches equal to the bed volume. Elution with ethanol equivalent to five times the bed volume was used to remove small phenolics and other material. PACs were then eluted in three bed volumes of 70% acetone and reduced by rotary evaporation (40 °C) to a minimum volume followed by drying to powder under a nitrogen stream. Thiolytic and HPLC analysis [39–42] of purified materials indicated the absence of low molecular weight species following LH20 purification. PACs used in these experiments were considered to be devoid of

sugars, acids, and low molecular weight contaminants. PACs recovered from cranberry juice were subsequently dialyzed against water containing 25% ethanol using 6,000 molecular weight cut-off (MWCO) tubing (Spectra/Por MWCO 6-8,000) to obtain a material enriched for high molecular weight polymers (HMW-PAC). This separation by dialysis is not expected to provide fully isolated components but rather to enrich for the indicated fraction. Following purification, the average degree of polymerization for each material was determined by combining the modified vanillin and acid butanol assays [37,43]. Results of the acid butanol assay are dependent upon the reactivity of the interflavanoid bonds. As purified standards are not available, the method provides only an estimate of the degree of polymerization of the materials. The method is, however, valid for determination of variations in fractions from a given species as in the case of the dialyzed material. The average degrees of polymerization (dp) for PACs were determined to be: cranberries (dp = 12.6), cranberry juice (dp = 8.9), grape juice (dp = 7.2), tea (dp = 4.1), and HMW-PAC (dp = 21.7). Radial diffusion assays were used to determine PAC concentrations (in tannic acid equivalents; TAE) for each material [37,44]. The following PAC concentrations were obtained for 1 mg/mL samples of each type: cranberry (63.4 μ M), grape juice (29.9 μ M), cranberry juice (39.0 μ M), tea (49.8 μ M), and HMW-PAC (38.3 μ M).

Purified PACs were immobilized onto activated thiol-Sepharose® 4B (Sigma-Aldrich, St. Louis, MO) via an N-[*p*-Maleimidophenyl]isocyanate crosslinker (PMPI; Pierce Biotechnology, Rockford, IL) as previously described [34]. Sepharose was swelled in deionized water and washed with water followed by ethanol. The PMPI crosslinker was incubated with the rinsed Sepharose material in ethanol for 1 hour using a 10-fold molar excess of PMPI over thiol groups. The modified Sepharose was then incubated overnight with PAC in 50% ethanol at a concentration 10-fold higher than the concentration of PMPI used. The PAC resin was then rinsed and resuspended in 0.02% sodium azide in water. Materials were stored in the dark at 4 °C until use. PAC concentrations for bead sets were determined by Prussian blue (PB) assay [45]. Tannic acid equivalents were assigned based on PB assay results for the beads as compared to those of the same soluble PAC material. The tannic acid equivalents obtained for the bead stocks were 642 nmol PAC per gram beads (P/g) for cranberry, 616 nmol P/g for tea, 560 nmol P/g for grape juice, 496 nmol P/g for cranberry juice, and 560 nmol P/g for the HMW-PAC from cranberry.

Fluorescence-based pull-down assays for lipopolysaccharide were conducted in 50 mM Tris (pH 8.0) using either Sepharose-immobilized PACs or agarose-immobilized PMB (Sigma-Aldrich, St. Louis, MO) as the LPS-capture reagent. Immobilized capture molecules and LPS were incubated at room temperature for 1 hour with constant agitation followed by washing and transfer to a 96-well plate. LPS and capture molecule concentrations are given in the text and figure captions. The fluorescence of the bound fluorescein isothiocyanate-labeled LPS (FITC-LPS; from *E. coli* 055:B5; Sigma-Aldrich) was measured using a Tecan Safire monochromometer-based micro plate reader at 495 nm excitation and 520 nm emission (5 nm bandwidths). Lipid A from *E. coli* and LPS variants from *Salmonella minnesota* (wild-type and Rc mutant), *E. coli* (Ra and Rc mutants), and *Shigella flexneri* were obtained from Sigma-Aldrich.

All column format experiments were conducted under gravity flow using a 0.2 mL bed volume. Materials were packed in disposable polypropylene columns 0.8 cm in diameter by 4 cm in length (Bio-Rad Laboratories, Hercules, CA). LPS binding was determined based on the fluorescence of fluorescein isothiocyanate-labeled LPS (FITC-LPS; from *E. coli* 055:B5; Sigma-Aldrich) as measured using a Tecan Safire monochromometer-based micro plate reader at 495 nm excitation and 520 nm emission (5 nm bandwidths). Protein concentrations were determined based on absorbance measurements collected using an Agilent 8453 UV/vis spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA). Agarose-immobilized PMB (Sigma-Aldrich) was used for comparison to the PAC resin. Myoglobin, bovine serum albumin, and cytochrome *c* were used to assess nonspecific adsorption by the PAC resin and were obtained from Sigma-Aldrich.

Escherichia coli ATCC® 35218 and *Bacillus subtilis* (formerly *Bacillus globigii*) ATCC® 49760 were obtained from and propagated as directed by American Type Culture Collection (Manassas, VA). Rabbit polyclonal antibody to *E. coli* was obtained from Abcam Inc. (Cambridge, MA). Rabbit and goat antibodies to *Bacillus globigii* were gifts from Naval Medical Research Center (NMRC; Bethesda, MD). The tracer antibodies were fluorescently labeled with Cy3 (Amersham Biosciences, Buckinghamshire, UK) as directed except that 3 mg of protein were labeled with the amount of the dye intended for 1 mg. Fluorescently labeled protein was separated from excess dye by gel chromatography using a Bio-Gel P-10 (Medium) column (BioRad, Hercules, CA). Luminex beads sets (developmental 181, 198, 121, 154, 196, 117, 158, 119) were obtained from Luminex Corp. (Austin, TX). For immobilization of the PACs onto Luminex beads, 100 μ L of the bead set (1.25×10^7 beads/mL) was rinsed with 400 μ L sodium phosphate buffer (100 mM) at pH 6.5 followed by rinsing with 400 μ L 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (100 mM) at pH 4.2. The beads were then suspended in 385 mL of the MES buffer with 250 μ g (3-[(2-aminoethyl)dithio]propionic acid hydrochloride) (AEDP) and 500 μ g 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC). Following incubation for 2 h with sonication, the beads were triple rinsed with 400 μ L borate buffer (100 mM) at pH 8.5 and incubated in the borate buffer with 700 μ g tris(2-carboxyethyl)phosphine hydrochloride (TCEP) with sonication (10 min). The triple rinse with borate buffer was repeated followed by triple rinse with ethanol. The beads were then resuspended in ethanol with 500 μ g *N*-(*p*-maleimidophenyl)isocyanate (PMPI) and incubated 1 h with sonication. Two 400 mL ethanol rinses were followed by resuspension in 400 μ L 50% ethanol to which 2.5 mg of PAC in 400 μ L 50% ethanol was added. This solution was incubated for 1 h with sonication and removed to 4 °C overnight. The samples were triple rinsed with 400 μ L sodium phosphate buffer (100 mM, pH 7) and stored in that solution at 4 °C (final volume 1 mL; approx. 1.25×10^6 beads/mL). Each type of PAC was immobilized onto a different bead set. An additional set was functionalized with catechin using the same protocol.

RESULTS AND DISCUSSION

The ability of PACs from multiple plant sources to inhibit the interaction of LPS with its cognate receptors on mammalian cells has been demonstrated. This activity was attributed to the ability of PACs to efficiently bind to LPS in solution [34]. This study sought to assess the ability of immobilized-PACs to serve as effective LPS-capture reagents for the removal of LPS from solution [35]. PAC beads for use in pull-down assays were generated by immobilizing PACs from cranberry, cranberry juice, tea, and grape juice onto thiol-activated Sepharose beads. An additional bead set was generated by immobilizing HMW-PAC from cranberry onto Sepharose beads. Assays conducted using these beads demonstrated that all five bead sets could be used to bind FITC-labeled LPS from solution (50 mM Tris, pH 8.0; Fig. 2). Beads functionalized with catechin monomer displayed minimal non-specific binding of FITC-LPS to catechin, the Sepharose, or the crosslinker (Fig. 2). Concentration dependence curves for the capture of LPS using beads with immobilized PACs from tea and cranberries demonstrated that these PAC species were nearly identical in their LPS-capture efficiency (Fig. 3). Capture of FITC-LPS by the immobilized PACs was dose-dependent, however, it did not conform to a standard binding isotherm. Though relatively low concentrations of free PAC were not found to quench the fluorescence of FITC-LPS, higher PAC concentrations did result in fluorescence quenching. Immobilized PACs represent a locally high concentration and, therefore, likely cause quenching of the FITC-LPS fluorescence. This may explain the trends shown in Fig. 3 where the increase in fluorescence intensity with increasing target concentration cannot be described by a standard model.

As demonstrated in Fig. 4, the binding of LPS to the PAC-functionalized Sepharose beads was not related to non-specific interactions. A concentration-dependent decrease in the amount of LPS captured by immobilized PACs was observed as increasing amounts of soluble PACs were added. In these assays, the soluble competitive inhibitor and the immobilized capture reagent were paired (e.g., soluble PACs from tea were used to inhibit LPS binding to PAC-tea beads). Immobilized capture molecule

concentration in the two assay types was 5.5 μM for PAC-tea beads and 6.0 μM for PAC-cranberry beads. Inhibition of 50% of LPS binding (IC_{50}) to the PAC-tea beads was achieved at 6.5 μM soluble tea PAC while 50% inhibition of LPS binding to PAC-cranberry beads occurred at 9.8 μM soluble cranberry PAC.

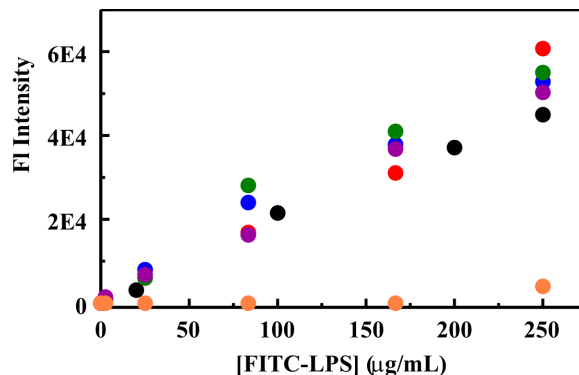


Fig. 2 — The FITC-LPS binding activity of the PAC bead sets. Results for Sepharose beads functionalized with PACs from grape juice (blue), cranberry juice (purple), tea (red), cranberries (black), and the high molecular weight component of PACs from cranberries (HMW-PAC; green). Capture molecule concentrations are between 5.5 and 6.0 μM for all bead sets. Also shown is the binding of FITC-LPS by catechin-functionalized Sepharose beads (orange).

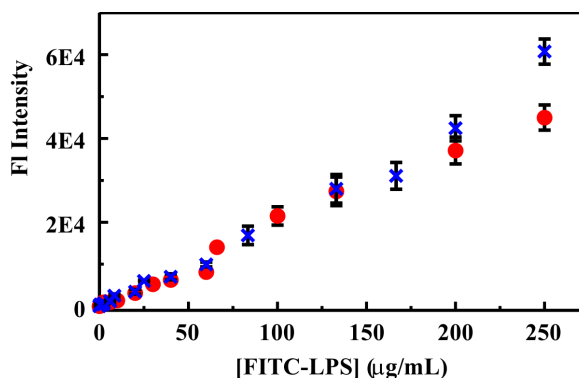


Fig. 3 — Proanthocyanidin capture of FITC-LPS. Sepharose-immobilized PACs from cranberry (red) and tea (blue) bind LPS in solution, resulting in increased fluorescence intensity in the pull-down assay. Capture molecule concentration is 5.5 μM for PAC from tea and 6.0 μM for PAC from cranberry.

The discrepancies between the capture molecule concentrations and the free PAC concentrations at which 50% inhibition occurs may be the result of actual or observed differences. First, the quenching of FITC-LPS fluorescence by PACs as described above may be a contributing factor. Second, capture molecule concentrations on the beads have been estimated using the PB assay. The estimate was made by comparing the results of the assay from the PACs immobilized onto Sepharose beads to the results obtained using free PACs from the same source. This comparison assumes that all components of the

immobilized polyphenolics are accessible to the reagents in a manner similar to those in free in solution. The analysis also assumes that all degrees of polymerization were immobilized equally so that the degree of polymerization achieved on the Sepharose beads was similar to that observed in solution. The potential for self-quenching of FITC-LPS fluorescence at high concentrations was considered and eliminated as a contributing factor due to the absence of this effect on PMB-beads.

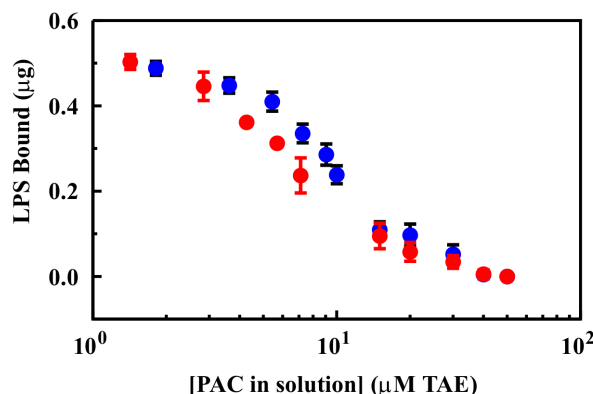


Fig. 4 — Competitive binding assays. The presence of PAC in solution inhibits the binding of LPS to PAC immobilized on Sepharose beads. FITC-LPS concentration was 100 µg/mL (~100 nM) and capture molecule concentration was 5.5 µM for PACs from tea (red) and 6.0 µM for PACs from cranberries (blue).

Polymyxin B is a cationic peptide that is known to interact specifically with the lipid A portion of LPS through electrostatic interactions between the peptide and phosphate groups present on the lipid A moiety [46]. Lipid A is responsible for the high toxicity of LPS and it is highly conserved across a wide array of bacterial species. Previously, we used a pull-down assay based on agarose-immobilized PMB to demonstrate that PACs from tea, grape juice, cranberry, and cranberry juice inhibited the binding of LPS by polymyxin B with IC_{50} values of approximately 1 to 3 µM [34]. Though the inhibition of LPS binding to PMB indicated the interaction of PACs with LPS, it did not guarantee the interaction of PACs with the lipid A moiety. To further investigate this potential interaction, the binding of other LPS variants by the PAC functionalized beads was assessed. Figure 5 demonstrates the inhibition of FITC-LPS binding by LPS mutants from *E. coli*. These LPS mutants possess shorter polysaccharide chains than the wild type LPS from *E. coli*. LPS variants with shortened polysaccharide chains are referred to as Ra through Re. The Re mutant has the smallest polysaccharide region consisting of only lipid A and 3-deoxy-D-mannooctulosonic acid. The Ra mutant has the longest polysaccharide chain of these variants; the length of the Rc polysaccharide region falls between that of Re and Ra. Here, the concentrations of FITC-LPS and capture molecule were held constant while the concentration of the LPS variant was altered. The IC_{50} obtained for FITC-LPS binding was consistent with the two targets having similar binding affinities. These results further implicated the lipid A region of LPS in the interaction with the PACs. LPS variants from other species provided similar results (Fig. 6).

Similarly, lipid A was used in the competitive assay as described in Fig. 5. Here, the PAC- and PMB-functionalized beads were equilibrated with varying concentrations of lipid A prior to incubation with FITC-LPS (final FITC-LPS concentration 3 µg/mL (~3 nM)). The presence of lipid A inhibited FITC-LPS binding by immobilized PACs with IC_{50} values of 100 nM and 500 nM for PACs from tea and cranberry, respectively (Fig. 7). The addition of lipid A concentrations as high as 100 µM, however,

failed to result in inhibition of greater than 80% FITC-LPS binding. It is unclear whether this is a result of the unavailability of lipid A, which may form micelles or precipitate at the higher concentrations, or the fact that binding interactions between LPS and the immobilized PACs are not limited to the lipid A portion of LPS. The binding of FITC-LPS by polymyxin B was also inhibited at a lower than expected level following equilibration of PMB beads with lipid A.

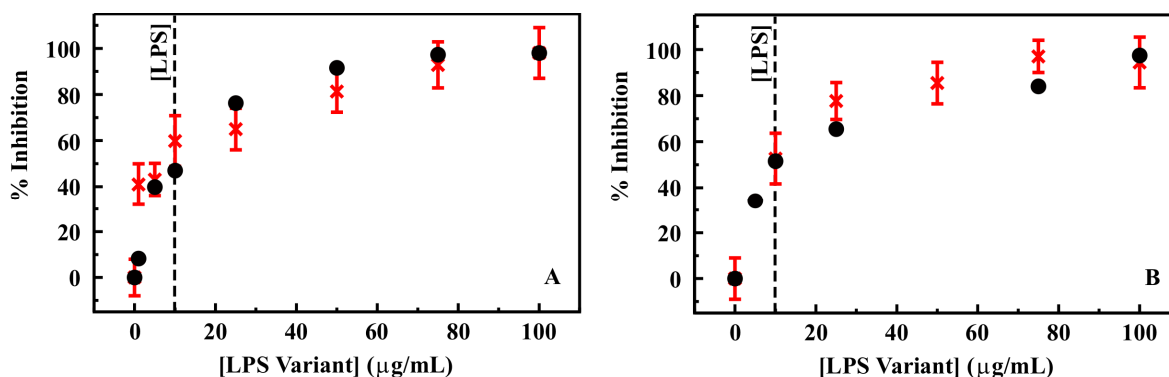


Fig. 5 — Competition by LPS variants. Addition of varying Ra (black) and Rc (red) mutants of LPS from *E. coli* to samples containing 10 $\mu\text{g/mL}$ FITC-LPS resulted in a reduction of FITC-LPS binding to the PAC beads. The concentrations at which 50% inhibition of FITC-LPS binding occurred indicated similar binding affinities of the immobilized PACs for the three LPS variants. Capture molecule concentrations were 6.0 μM for PACs from cranberries (panel A) and 5.5 μM for PACs from tea (panel B). The dashed line indicates the FITC-LPS concentration used.

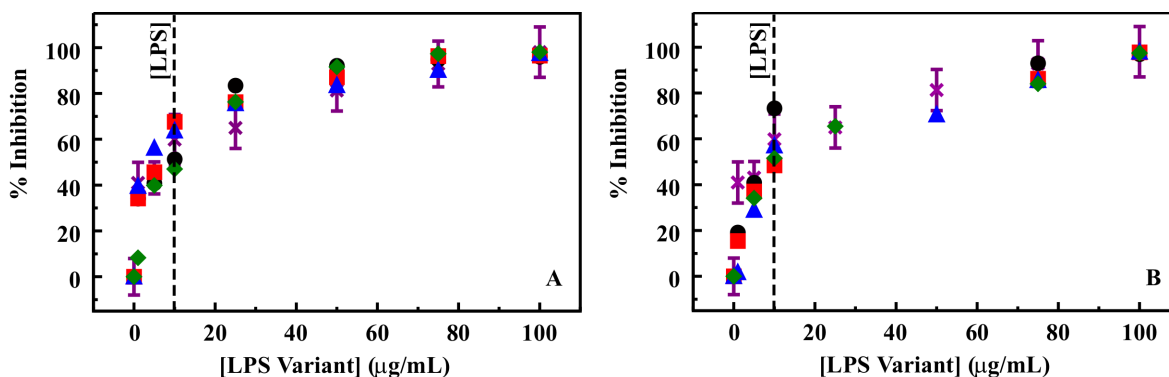


Fig. 6 — Other LPS variants. Shown here are the results of a competitive assay in which the concentrations of FITC-LPS (from *E. coli* O55:B5; 10 $\mu\text{g/mL}$) and capture molecule (6.0 μM PAC-cranberry in Panel A and 5.5 μM PAC-tea in Panel B) are held constant while the concentration of an LPS variant (no fluorescent label) is altered: *S. minnesota*, wild type (red) and Rc mutant (black); *E. coli*, Ra (green) and Rc (purple) mutants; *S. flexneri*, wild type (blue). The dashed line indicates the FITC-LPS concentration used.

In order to evaluate the utility of Sepharose-immobilized PACs relative to the materials currently employed for LPS capture, a side-by-side comparison was made to commercially available agarose-immobilized PMB. Figure 8 presents the amount of LPS captured as a function of the concentration of capture molecule for PMB and PACs from tea and cranberry. Quenching of the FITC-LPS fluorescence

intensity upon interaction with the immobilized PACs presented a difficulty in direct comparison of the capture materials. This issue was addressed by measuring the fluorescence intensity of the FITC-LPS remaining in solution after incubation with the capture material. The amount of captured LPS was determined by comparing the fluorescence remaining after incubation with capture material to an identical sample that contained no capture molecule. Both types of PAC beads performed similarly to the PMB beads. This result was expected based on the inhibition of polymyxin B binding of LPS by the proanthocyanidins observed in the previous study [34].

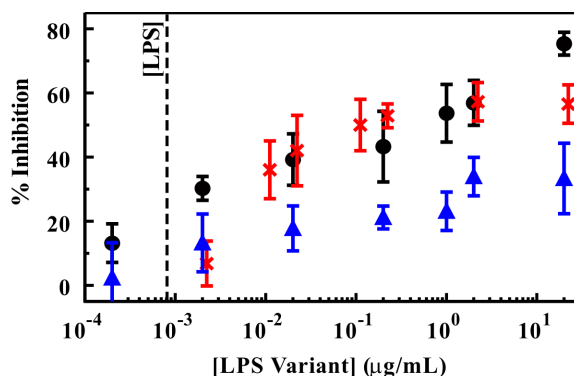


Fig. 7 — Competition by lipid A. Equilibration of beads with lipid A inhibits the binding of FITC-LPS to immobilized PACs or polymyxin B (PMB). FITC-LPS concentration was 3 $\mu\text{g/mL}$ (~ 3 nM) and capture molecule concentration was 5.5 μM for PACs from tea (red); 6.0 μM for PACs from cranberries (black); and 9 μM for PMB (blue). The dashed line indicates the FITC-LPS concentration used.

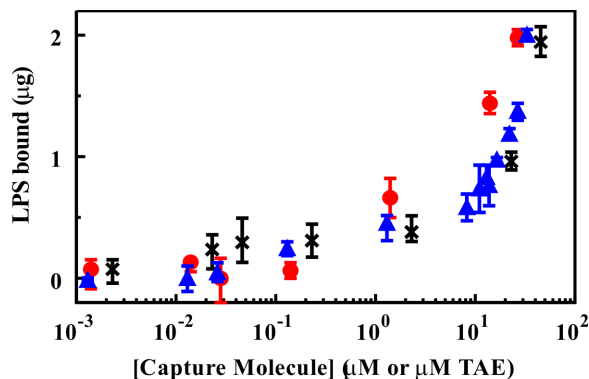


Fig. 8 — Comparison of proanthocyanidins to polymyxin B. Immobilized PMB (black); PAC from tea (red); and PAC from cranberry (blue) show similar binding affinities for FITC-LPS when compared in side-by-side assays. FITC-LPS concentration was 100 $\mu\text{g/mL}$ (~ 100 nM).

Column formats provide the potential for continuous or large batch separation of desirable and undesirable components. With this type of application in mind, LPS breakthrough curves were generated for columns of the PAC resin generated using PACs from cranberry and polymyxin B agarose. Figure 9

shows application to the columns of 1 mL followed by repeated 0.5 mL applications. A stock 100 $\mu\text{g/mL}$ FITC-LPS solution in 50 mM pH 8.0 sodium phosphate buffer (NaPi) was used (ionic strength 0.15). The PAC resin adsorbed 44% of the LPS from the initial 1 mL addition while PMB adsorbed 24%. PMB adsorbed none of the LPS from subsequent additions demonstrating a capacity of 120 μg LPS per mL of the material. The PAC resin continued to adsorb part of the LPS from each subsequent addition through 9 mL for a capacity of 385 μg LPS per mL of the material.

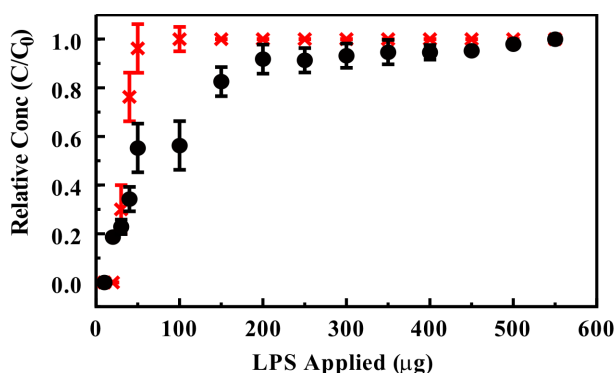


Fig. 9 — Breakthrough curves. Shown here are breakthrough curves for the two LPS removal resins being compared: PAC-Sepharose (black) and PMB-agarose (red). C/C_0 indicates the ratio of the final LPS concentration of the solution as compared to the concentration of the starting solution.

The adsorption of proteins by the columns was evaluated using myoglobin (myo), bovine serum albumin (BSA), and cytochrome *c* (cyt *c*) to provide an assessment of the potential for separation of targets using PAC functionalized resin. The three proteins were selected based on the differences in their pI values. BSA has a slightly acidic pI of 4.9; myo has a neutral pI of 6.8; and cyt *c* has a basic pI of 10.6. Proteins were applied as 1 mL samples of 500 $\mu\text{g/mL}$ solutions in 50 mM pH 8.0 NaPi (ionic strength 0.15). Figure 10 shows the recovery of proteins from each of the column materials in the effluent. The data indicate a slight increase in protein adsorption by the PAC resin for cyt *c* as compared to BSA and myo. The material retained 20% of the BSA, 20% of the myo, and 28% of the cyt *c*. The PMB resin showed decreasing protein adsorption with increasing pI, retaining 54% of the BSA, 20% of the myo, and 6% of the cyt *c*. Also shown in Fig. 10 is the myoglobin elution profile for each of the columns. The elution profile from the PAC resin was found to be slightly narrower than that of the PMB resin. This indicates the potential for reduced dilution of the protein during removal of LPS.

The absorbance of LPS and proteins by the resins was evaluated at varying pH and ionic strength (Fig. 11). Aliquots (0.5 mL) of each sample were applied to the two resins, and the retention of LPS and myoglobin was measured as described for the experiments above. Samples contained 100 $\mu\text{g/mL}$ LPS and 500 $\mu\text{g/mL}$ myoglobin. For the varied pH experiments, the ionic strength was held constant at 0.15 through addition of sodium chloride. The pH was varied from 4.8 to 9.0. A decrease in LPS retention was observed for the PAC resin at pH 9. The PAC resin was also found to retain more myoglobin at lower pH values. The PMB resin retained only 5% of myoglobin and 4% of LPS at pH 4.8. Above pH 5.8, the retention of myoglobin was consistent for all pH values. The retention of LPS, however, was found to be optimal at pH 7.0 and to fall off as the pH was increased or decreased from that value. For experiments with varied ionic strength, the pH was held constant at 8.0 while ionic strength was altered through addition of sodium chloride to obtain a range from 0.15 to 0.85. The retention of LPS by the PAC and

PMB resins was found to decrease slightly as the ionic strength of the buffer was increased. Retention of myoglobin also decreased slightly.

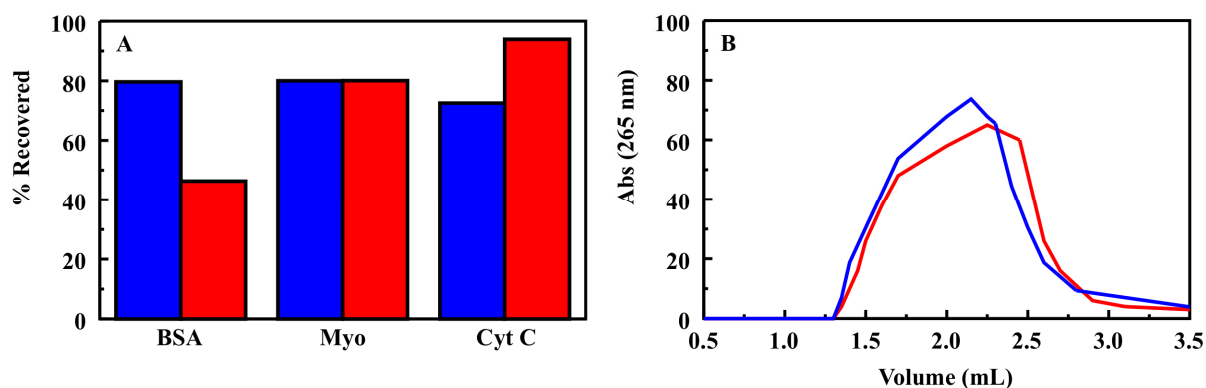


Fig. 10 — Protein retention. Panel A: Shown here is the recovery of protein in the first milliliter eluted from the two columns: PAC-Sepharose (blue) and PMB-agarose (red). Data are presented as percent recovered of the total applied. Panel B: Shown here are the myoglobin elution profiles for each of the column materials.

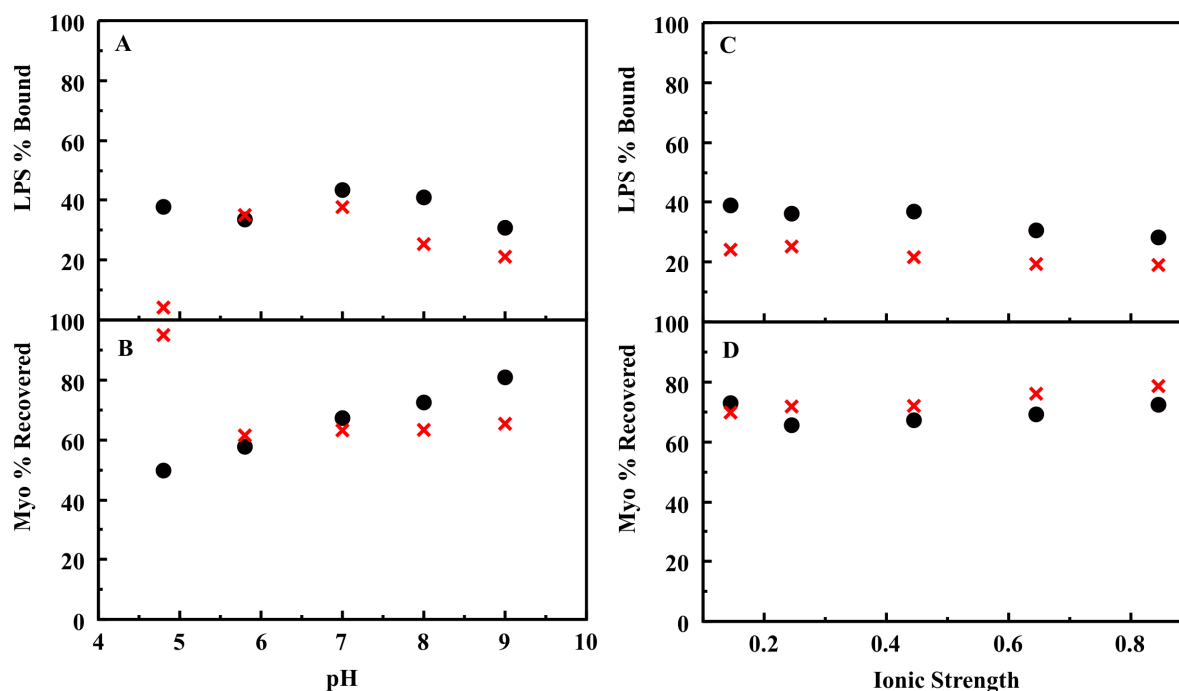


Fig. 11 — Varying buffer composition. Shown here is the impact on retention of LPS and myoglobin of varying buffer pH or ionic strength; PAC-Sepharose (black) and PMB-agarose (red). Panel A: The percent LPS bound from samples of varying pH containing myoglobin. Panel B: The percent myoglobin recovered from samples of varying pH containing LPS. Panel C: The percent LPS bound from samples of varying ionic strength containing myoglobin. Panel D: The percent myoglobin recovered for samples of varying ionic strength containing LPS.

PMB-agarose can be regenerated through rinsing with a 1% deoxycholate solution. In order to determine the potential for regeneration of the PAC resin, a column was repeatedly exposed to a 0.5 mL sample containing 100 $\mu\text{g/mL}$ LPS in 50 mM pH 8.0 NaPi (ionic strength 0.15). The column was then rinsed with 3 mL 1% deoxycholate followed by 5 mL of water. The retention of LPS over nine cycles is shown in Fig. 12. Retention is reduced only slightly over repeated application and regeneration using this method. This reduced retention is similar to that observed with PMB-agarose over repeated regeneration cycles.

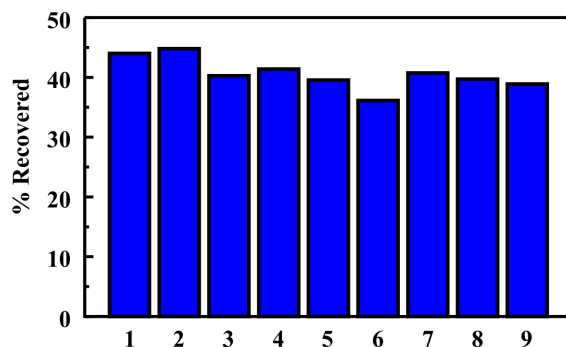


Fig. 12 — Regeneration. Shown here is the impact on LPS retention of repeated regeneration of the PAC resin using a 1% deoxycholate solution.

The Luminex system offers an alternative for array based sensing [47–49]. Based on the performance of the PAC functionalized beads in capture and removal of LPS, it was of interest to determine the potential for use of these compounds in the capture, detection, or removal of bacterial cells. In each Luminex assay, the total volume was 108 μL including 8 μL of the as prepared bead solution (approx. 1000 beads). Cells were taken from fresh overnight cultures. PAC functionalized Luminex beads were incubated with cells for 1 h with agitation followed by centrifuging to spin cells and beads down into a minimal volume. The sample was then resuspended and tracer was added, 10 μg for *E. coli* and 5 μg for *B. globigii*. In the case of *E. coli*, this was the cy3-labeled rabbit antibody. For *B. globigii*, the tracer consisted of a 50/50 mixture of cy3-labeled goat and rabbit antibodies. Figure 13 presents the results of assays conducted at varied concentrations of the bacterial cells. Beads functionalized using PACs from black tea showed the greatest *E. coli* capture. PACs from chocolate also performed significantly better than the other materials. PACs from cranberry and HMW-PAC showed similar performance, significantly less than either chocolate or black tea. PACs from chocolate and black tea and the HMW-PAC also showed the greatest capture of *B. globigii*. Counts for these assays are lower due to the use of less tracer; however, capture of this Gram-positive bacterium is also much less efficient than capture of *E. coli*, a Gram-negative bacterium. This difference is expected based on the proposed interaction of PACs with the LPS component of the cell membrane [34]. Overall, the PACs provided poor capture of both bacterial species when compared with antibodies or even antimicrobial peptides with detection limits for *E. coli* in the range of 5×10^6 cfu/mL.

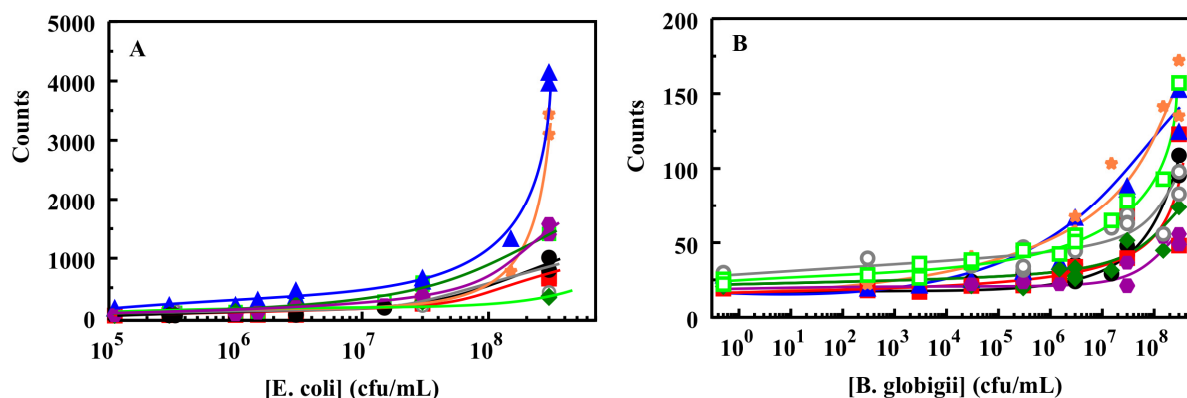


Fig. 13 — Luminex assay. Shown here are the results for assays conducted using the PAC functionalized Luminex beads for capture of *E. coli* (panel A) and *B. globigii* (panel B): green tea (black), catechin (red), black tea (blue), grape juice (green), cranberry (purple), chocolate (orange), coffee (gray), and HMW-PAC (chartreuse). Data presented are from three separate assays.

CONCLUSIONS

Lipopolysaccharide, the major component of the outer membrane of Gram-negative bacteria, is the primary cause of sepsis, an inflammatory syndrome involving an overwhelming systemic response to bacterial infection. Because potential bacterial contamination poses great risk in a number of therapeutic settings (e.g., plasmid DNA preparations used for gene therapy, therapeutic recombinant protein preparations, implanted medical devices), robust yet facile ways to remove LPS contamination are needed. Currently, polymyxin B is the most commonly employed agent for removal of LPS contamination. PMB was identified from *Bacillus polymyxa* culture filtrates [12] and is currently obtained from those cultures. It has been used in bead-based applications as well as in column formats. PMB has also been employed as an extracorporeal column for the treatment of sepsis [50]. PMB nonapeptide was investigated as an alternative but, while it was found to be less toxic, it was also found to be much less potent than the parent molecule as an anti-endotoxin [51].

In this report, we have described new materials that can serve as alternatives to PMB for the removal of LPS from solution. Sepharose-immobilized proanthocyanidins bound LPS similarly to immobilized polymyxin B. PAC binding of LPS via the lipid A component was implicated, though other mechanisms have not been completely eliminated. When applied as column resins, the PAC-resin shows a higher capacity for LPS than the standard PMB resin used for comparison. Protein retention by the PAC-resin was demonstrated to be low and showed less dependence on protein pI than that observed for the PMB-resin. While binding of LPS and retention of proteins was only slightly impacted by changes in ionic strength, the PAC-resin was found to retain less protein at higher pH values. Regeneration of the material was demonstrated over several cycles with little impact on the performance of the resin.

PACs are widely available from a range of plant sources, are purified with relative ease, and can be covalently attached to solid supports. As such, they represent a readily available, low-cost, alternative LPS-binding material. Purification of these compounds is relatively simple involving hydrophobic adsorption chromatography. For the work described here, acetone, ethanol, and water were employed as solvents during purification. Due to the simplicity of the purification protocol, it is likely that the acetone could be replaced with a solvent of lower environmental impact. These features combine to make PACs an attractive alternative to the traditionally employed peptides. In addition, many studies have reported the low toxicity of PACs [52–55]. There is a large amount of ongoing research into the potential health benefits and applications of these materials. While the work presented here demonstrates the potential

utility of PACs in the removal or concentration of bacterial LPS, the absence of bactericidal activity described for PACs from some sources [32] also provides a potential extension of the application to concentration, separation, or removal of bacteria from solution while maintaining viability.

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