Plant Proanthocyanidins Bind to and Neutralize Bacterial Lipopolysaccharides

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Proanthocyanidins (PACs) are naturally occurring polymers derived from higher plants and they have recently been associated with several potential positive health benefits such as antibacterial, chemotherapeutic, and anti-atherosclerotic activities. Here we report on the binding of PACs from cranberries, tea, and grapes to lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria. LPS is the cause of several human illnesses, including sepsis and toxic shock syndrome. We show that in the case of cranberries, the majority of the LPS-binding activity is contained within a PAC fraction composed of polymers with an average degree of polymerization of 21. This PAC fraction modestly inhibits the binding of LPS to the surface of mammalian cells expressing the full complement of LPS receptors while it significantly abrogates the cellular internalization of LPS. Our results demonstrate PACs to be a new class of LPS-binding compound with potential utility in the removal of LPS from potable water sources and pharmaceutical preparations.

INTRODUCTION

During infection, bacteria cause damage to the human body in two ways: 1) they release toxins that harm and kill cells and 2) they perturb the immune system, leading to inflammation, which can be harmful in itself. In the case of Gram-negative bacteria (e.g., Escherichia coli), the primary trigger of the immune response is a molecule known as lipopolysaccharide (LPS), the major component of the bacterial outer cell membrane. LPS is the primary cause of sepsis, an inflammatory condition characterized by an overwhelming systemic response to bacterial infection. Sepsis has become the most common cause of death in intensive care units in the United States, causing 120,000 deaths annually and amassing $16.7 billion in associated healthcare costs. Commonly referred to as bacterial “endotoxin,” LPS is composed of three domains: 1) a bacterial membrane-proximal lipid A moiety and 2) a core oligosaccharide region which gives rise to 3) the O-antigen, a branched polysaccharide that extends from the core oligosaccharide. When released from bacteria, LPS circulates in the blood where it interacts with receptors (TLR4 and CD14) on the surface of our immune cells (dendritic cells and macrophages), eliciting the body’s immune response. While this LPS-induced immune response is necessary for the body to effectively combat bacterial infection, if it is left unchecked, the immune response can evolve into life-threatening sepsis.

To date, a range of LPS-binding compounds has been developed in an effort to prevent LPS-induced sepsis by inhibiting the interaction of LPS with its target receptors. These materials include peptides such as polymyxin B, hydrazones, and polyamines. Due to their toxicity and lack of specificity, however, these materials have demonstrated limited success in therapeutic settings; their use has largely been limited to in vitro applications where the removal of LPS from solutions is required. The need for the identification of novel LPS-binding compounds still remains.

This report describes our recent discovery that proanthocyanidins (PACs), naturally occurring polyphenolic compounds derived from higher plants, efficiently bind to and prevent the interaction of LPS with receptors on the surface of mammalian immune cells. This novel finding has identified PACs as a new class of LPS-binding compound with potential uses in applications requiring LPS purification and removal or the in vivo treatment of sepsis.

PROANTHOCYANIDINS: A NEW CLASS OF LPS-BINDING COMPOUND

Liquids containing PACs such as tea, red wine, and cranberry juice have recently been associated with several potential positive health benefits. Among the more notable of these benefits is the ability of cranberry juice to mitigate urinary tract infections by preventing the attachment of pathogenic bacteria to uroepithelial cells. This effect has been attributed to the ability of PACs to induce conformational changes in the cell surface proteins that bacteria use to attach themselves to epithelial cells.
PACs are composed chiefly of the monomeric flavan subunits (+)-catechin and (–)-epicatechin and their derivatives (Fig. 1(a)). The subunits are most commonly linked together via a single intermolecular bond as is the case in PACs from tea and grapes (known as B-type PACs). In some instances, the subunits are linked together by two intermolecular bonds (A-type PACs) as is observed in cranberries. Since the potential interactions of PACs with bacterial cell surface proteins did not seem adequate to explain all of the reported activities of cranberry juice, we hypothesized that PACs might interact with other molecules on the bacterial cell surface, particularly LPS.

In this study, we assessed the LPS-binding activity of PACs from cranberries, tea, and grapes. Figure 1(b) shows the relative abilities of PACs from these various sources to inhibit the binding of *E. coli* LPS to an immobilized receptor (polymyxin B) as a function of PAC concentration. Cranberry concentrate that had been enriched through dialysis to contain molecules greater than 6,000 molecular weight (MW) showed the lowest degree of LPS-binding with an inhibitory concentration (50%) (IC$_{50}$) of 10.5 µM. PACs from grapes and tea showed intermediate degrees of LPS-binding activity, with IC$_{50}$ values of 3.0 and 1.1 µM, respectively. However, PACs from fresh cranberries (enriched for PACs greater than 6,000 MW) showed the greatest ability to bind to LPS, with an IC$_{50}$ of 0.7 µM. A further comparison of the activities of cranberry PACs as a function of size showed that the polymers greater than 6,000 MW had the highest degree of LPS binding relative to smaller PACs (Fig. 1(c)).

An analysis of the degree of polymerization within each of the different sized “pools” of cranberry PACs verified enrichment for increased polymer length within each size fraction. The fraction with the greatest LPS-binding activity corresponded to polymers with an average degree of polymerization of 21 (Table 1). All subsequent experiments were performed using this fraction (referred to henceforth as cranberry PACs).

We also found that cranberry PACs bound not only to LPS from *E. coli* but also to a number of other

![Figure 1](image)

**FIGURE 1**
PACs interact with LPS to inhibit binding to a potent LPS-binding molecule, polymyxin B. (a) PACs are polymers commonly composed of (+)-catechin and (–)-epicatechin flavanoid subunits. (b) The LPS-binding activity of PACs from cranberry, tea, and grapes is concentration-dependent. Cranberry PACs greater than 6,000 MW show the greatest LPS-binding activity. (c) For cranberry PACs, the majority of the LPS-binding activity is contained within the fraction composed of polymers >6,000 MW (average degree of polymerization of 21).
Table 1 — Average Degree of Polymerization of Size-Fractionated PACs from Cranberry

<table>
<thead>
<tr>
<th>PAC Fraction</th>
<th>Average Degree of Polymerization</th>
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<tbody>
<tr>
<td>Non size-fractionated</td>
<td>12.6</td>
</tr>
<tr>
<td>&lt;2000</td>
<td>3.2</td>
</tr>
<tr>
<td>2000 – 3000</td>
<td>5.4</td>
</tr>
<tr>
<td>3000 – 6000</td>
<td>12.9</td>
</tr>
<tr>
<td>&gt;6000</td>
<td>21.4</td>
</tr>
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</table>

Gram-negative bacterial species (Table 2). Further, cranberry PACs bound very tightly to lipid A, the putative cell-binding domain on LPS, indicating that PACs predominantly bind to the lipid A region of the LPS molecule.

**PACS INHIBIT LPS INTERACTION WITH MAMMALIAN CELLS**

Based on cranberry PACs’ LPS-binding ability and its large degree of interaction with the cell-binding lipid A moiety, it followed that PACs could efficiently block the interaction of LPS with mammalian cells expressing cognate LPS receptors. Using a model system in which human epithelial cells were induced to express the LPS receptors CD14 and TLR4 (to emulate our native immune cells), we found that cranberry PACs caused a modest yet significant reduction (~15%) in the amount of LPS bound to these cell surface receptors (Fig. 2(a)). However, cranberry PACs substantially inhibited (~74% inhibition) the cells’ ability to internalize LPS (Fig. 2(b)). This result is consistent with the currently accepted model of LPS interaction with its cell surface receptors in which LPS binding to its receptors is required for internalization and the onset of the inflammatory response. This mechanism of action is depicted schematically in Fig. 3. Through this activity, PACs are able to effectively modulate the immune response and to keep the response in check.

**SUMMARY**

Our work has identified naturally occurring PACs as a new class of efficient LPS-binding compound. Given the limitations of some of the currently available LPS-binding substances, it is anticipated that PACs will find great utility in LPS removal and purification applications (Fig. 4). Important targets for use would include biological materials that cannot be heat- or UV-sterilized without degradation, including many pharmaceuticals and baby juices. PACs might also prove useful in therapeutic applications such as dialysis or the *in vivo* neutralization of LPS-induced inflammation.

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Table 2 — Binding of Cranberry LH20 PAC* to LPS and Lipid A

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Apparent IC₅₀ (µM)*b</th>
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<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td><em>Salmonella minn.</em></td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td><em>Escherichia coli</em> EH 100 (Ra mutant)</td>
<td>2.1 ± 0.7</td>
</tr>
<tr>
<td><em>Salmonella minn.</em> (Rc mutant)</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>3.4 ± 1.0</td>
</tr>
<tr>
<td>Lipid A*c</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
</tr>
</tbody>
</table>

*aCorresponds to PACs of greater than 6,000 molecular weight.
bApparent IC₅₀s are shown with their corresponding 90% confidence intervals. The IC₅₀ is the concentration at which the PAC blocked 50% of the binding of LPS to the immobilized LPS-binding molecule, polymyxin B.
cDiphosphoryl form of lipid A.
PACs slightly inhibit LPS binding to cell membranes and substantially inhibit cellular internalization of LPS. Cells expressing LPS receptors were incubated with LPS alone or with LPS plus cranberry PACs. LPS bound to the cell membrane (a) or internalized (b) was visualized using a fluorescein-conjugated antibody that binds to LPS (green). Cell nuclei are stained blue. (a) PACs slightly inhibit LPS binding to cells expressing LPS receptors on their surface. (b) PACs inhibit cell internalization of LPS. Symbols correspond to levels of significance relative to control (determined by Student’s t-test): [+] p < 0.1 and [¤] p < 0.001.

Mechanism of PACs’ inhibition of LPS interaction with immune cells. (a) In the absence of PACs, LPS binds to its receptor and is efficiently internalized, where it promotes the inflammatory response. (b) In the presence of PACs, binding of LPS to its receptor is partially blocked. In this instance, LPS internalization is greatly inhibited, thereby inhibiting the inflammatory response.
FIGURE 4
Use of immobilized PACs for LPS removal and purification. PACs immobilized to solid supports are added to an LPS-containing solution. After complex formation, the LPS-PAC complexes are sedimented via centrifugation, leaving the purified solution in the supernatant. The purified solution is removed leaving behind the separated LPS-PAC complexes. PACs’ LPS-binding activity can be used to purify LPS-contaminated solutions (e.g., potable water supplies, pharmaceutical preparations).
References

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JAMES B. DELEHANTY received his Ph.D. in cellular and molecular biology from Tulane University Health Sciences Center in New Orleans in 2001, where his work focused on elucidating the mechanisms of antibody recognition of metal-chelate complexes. Since joining NRL, his research interests have included the development of portable sensors for bio-threat agent detection, the investigation of novel high-throughput strategies for mammalian gene delivery, and the development of metal-chelate complexes as antimicrobial and antiviral agents. Currently his research is focused on expanding the biological sensing applications of semiconductor nanocrystals (quantum dots).

BRANDY J. JOHNSON received her Ph.D. in photonics from Oklahoma State University in 2004. Immediately following her graduate work, she joined the Center for Bio/Molecular Science and Engineering at NRL. Before coming to NRL, her work focused on protein- and porphyrin-based optical sensors. Her current work focuses on application of mesoporous materials to the pre-concentration, detection, and photocatalyzed destruction of chemical agents such as environmental contaminants and energetic agents. Other interests include studies on the interaction of naturally occurring plant metabolites and bacterial cells with a focus on the impact and potential application of those interactions. These studies include bacterial adhesion, biofilm formation, alteration of gene expression profiles, and neutralization of pathogens and toxins.
THOMAS E. HICKEY received his B.A. in microbiology and immunology from the University of California, Berkeley, in 1985, and his Ph.D. in immunology and biochemistry from the University of Iowa in 1991. He was commissioned a lieutenant in the U.S. Navy in 1991, and has served as a staff scientist at the Naval Medical Research Institute, Naval Medical Research Center, Naval Medical Research Detachment, Lima, Peru, and at the Uniformed Services University prior to coming to NRL. He is currently an assistant professor in the Preventive Medicine and Microbiology departments at the Uniformed Services University School of Medicine. His research has focused on the immunology of HIV, cholera, and Campylobacter pathogenesis in human systems. Current research efforts are devoted to the identification and evaluation of novel broad spectrum antiviral compounds.

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