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Extraction of ADP-Heptose and Kdo_2-Lipid A from *E. coli* Deficient in the Heptosyltransferase I Gene

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Featured Application: This paper highlights details for the improved extraction of ADP-Heptose and Kdo_2-Lipid A, plus the subsequent deacylation to yield O-deacylated Kdo_2-Lipid A.

Abstract: The enzymes involved in lipopolysaccharide (LPS) biosynthesis, including Heptosyltransferase I (HepI), are critical for maintaining the integrity of the bacterial cell wall, and therefore these LPS biosynthetic enzymes are validated targets for drug discovery to treat Gram-negative bacterial infections. Enzymes involved in the biosynthesis of lipopolysaccharides (LPSs) utilize substrates that are synthetically complex, with numerous stereocenters and site-specific glycosylation patterns. Due to the relatively complex substrate structures, characterization of these enzymes has necessitated strategies to generate bacterial cells with gene disruptions to enable the extraction of these substrates from large scale bacterial growths. Like many LPS biosynthetic enzymes, Heptosyltransferase I binds two substrates: the sugar acceptor substrate, Kdo_2-Lipid A, and the sugar donor substrate, ADP-L-*glycero*-D-*manno*-heptose (ADPH). HepI characterization experiments require copious amounts of Kdo_2-Lipid A and ADPH, and unsuccessful extractions of these two substrates can lead to serious delays in collection of data. While there are papers and theses with protocols for extraction of these substrates, they are often missing small details essential to the success of the extraction. Herein detailed protocols are given for extraction of ADPH and Kdo_2-Lipid A (KLA) from *E. coli*, which have had proven success in the Taylor lab. Key steps in the extraction of ADPH are clearing the extract through ultracentrifugation and keeping all water that touches anything in the extraction, including filters, at a pH of 8.0. Key steps in the extraction of KLA are properly lysing the dried down cells before starting the extraction, maximizing yield by allowing precipitate to form overnight, appropriately washing the pellet with phenol and dissolving the KLA in 1% TEA using visual cues, rather than a specific volume. These protocols led to increased yield and a higher success rate of extractions thereby enabling the characterization of HepI.

Keywords: Heptosyltransferase I; lipopolysaccharide biosynthesis; ADP-Heptose; Kdo_2-Lipid A

Citation: Milicaj, J.; Castro, C.D.; Jaunbocus, N.; Taylor, E.A. Extraction of ADP-Heptose and Kdo_2-Lipid A from *E. coli* Deficient in the Heptosyltransferase I Gene. *Appl. Sci.* **2021**, *11*, 8314. https://doi.org/10.3390/app11188314

Academic Editor: Gohar Khachatryan

Received: 20 August 2021
Accepted: 3 September 2021
Published: 8 September 2021

1. Introduction

Lipopolysaccharide (LPS) makes up approximately 30% of the outer membrane (Figure 1) of Gram-negative bacteria and is a multipurpose cellular component necessary for cellular motility, surface adhesion, and intermolecular interactions in a bacterial biofilm. LPS is also known for causing an immunological response in vivo, and is therefore, commonly referred to as an endotoxin [1–3]. LPS can also be utilized by bacteria to prevent the cell permeation of hydrophobic molecules, thereby serving as a bacterial antibiotic resistance mechanism [4,5].

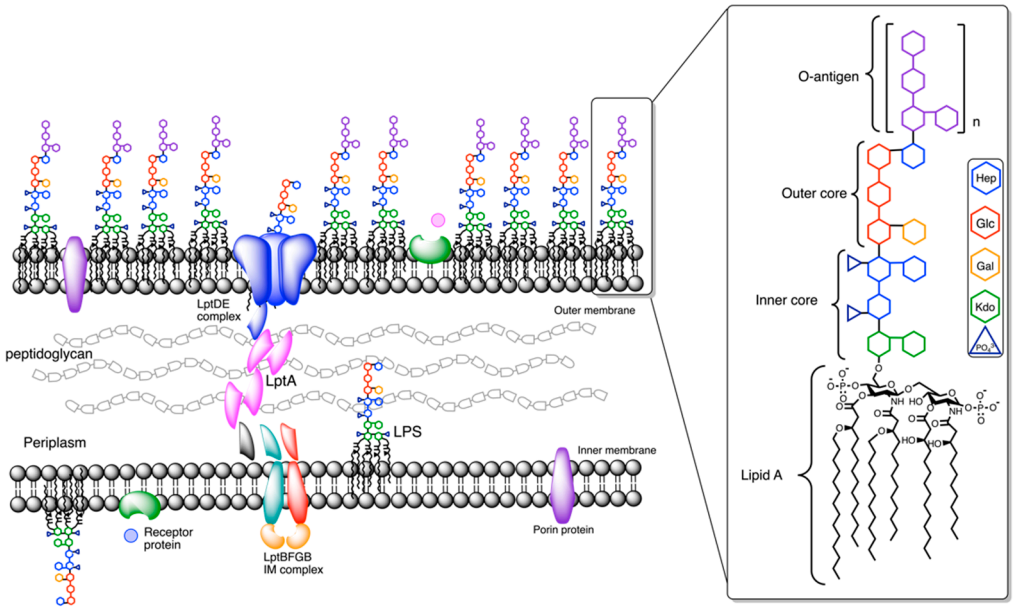


Figure 1. Bacterial lipid bilayer with LPS transfer protein complex and the full LPS structure (inset).

Furthermore, xenobiotics including Polymyxin B and metal ions, have been shown to trigger alterations of LPS structure to further fortify the outer membrane [6,7]. Additionally, previous studies have shown that removing genes involved in the inner core oligosaccharide biosynthesis, including Heptosyltransferase I (HepI), results in increased bacterial antibiotic susceptibility and an expression of a deep-rough LPS morphology [8], which makes HepI a target of interest for Gram-negative antibacterial therapeutic design. As shown in Figure 2, HepI transfers a heptose moiety from ADP-L-*glycero*-D-*manno*-heptose (ADPH) to Kdo_2-Lipid A (KLA) in a nucleophilic substitution reaction. Kinetic characterization of the HepI reaction, which is enabled by detection of the ADP produced during the reaction via a UV-Vis coupled spectrophotometric assay with putative inhibitors, as well as biophysical characterization of HepI including circular dichroism spectroscopy, small-angle x-ray scattering, intrinsic tryptophan fluorescence, and isothermal titration calorimetry all require significant quantities of purified ADPH and KLA [9–12].

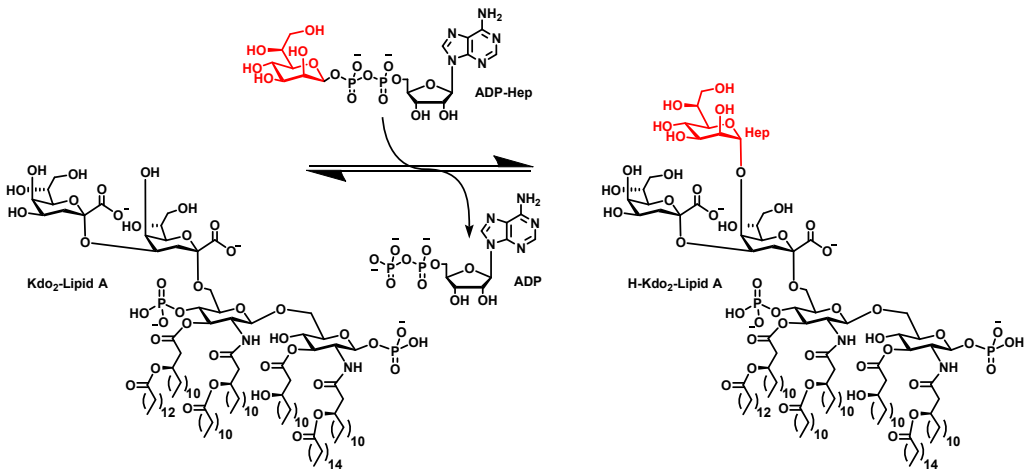


Figure 2. Reaction of HepI with ADPH and KLA showing the transfer of the heptose moiety (red).

In addition to their described use with HepI, ADPH and KLA have a variety of other applications in biological and biochemical investigations. ADPH and KLA have both been shown to stimulate an immune response in vivo [13,14]. A 2018 study done by Pfannkuch et al. showed that organic extracts from *H. pylori* were abundant in ADPH,

which was determined to be a pathogen-associated molecular pattern (PAMP) [15]. PAMPs are molecules found in a variety of infectious species that are detected by host cells and trigger immune pathways and inflammatory mediators. ADPH is also a substrate for HepII–IV [2,16] making it a vital compound for multiple steps of LPS biosynthesis. Previous work by Durka et al. demonstrated the synthesis of a variety of ADPH analogs in an attempt to determine potential inhibitors for Heptosyltransferases [17]. The first liganded crystal structure of HepI was published using a fluorine functionalized form of ADPH as it stabilized the compound for crystallization [18]. Dr. Kosma at the Institute of Organic Chemistry has published a library of work [15,19,20] pertaining to the synthesis of ADPH analogs and is interested in the recognition of heptoses by surfactant proteins. KLA has also a wide variety of applications that are on the frontier of therapeutic design models. A review by Wang et al. describes the application of KLA in drug development and its role as an endotoxin [21]. Raetz et al. explains how KLA exhibits equivalent immune stimulating effects to a fully formed LPS structure which is known to stimulate an aggressive immune response [22]. KLA is also a substrate for another enzyme known to modify KLA structures, *S. typhimurium* Lipid A deacylase LpxR [23].

There are a notable variety of uses for ADPH and KLA, and therefore the need for proper protocol to successfully extract pure compound as an extremely cost effective and reliable option is critical to helping continue the projects described above. Here we have described, in excessive detail, the steps required to obtain these substrates as well as visual and analytical data for comparison. Provided as well are troubleshooting techniques to prevent the loss of product, to assist in increasing efficiency and yield of these processes.

2. Materials and Methods

2.1. General Materials and Equipment

E. coli WBB06 cells (an *E. coli* strain deficient in HepI and HepII) were obtained from Brabetz et al. [24]. Amicon Ultra-15 (30 kDa, 10 kDa and 3 kDa Molecular Weight Cut-Off (MWCO)) centrifugal concentrators, tryptone and yeast extract were obtained from ThermoFisher Scientific (Pittsburg, PA, USA). Sodium chloride, calcium chloride, sodium hydroxide, tetracycline (Tet), 200 proof Ethanol, 98% phenol, diethyl ether, acetone (HPLC grade), triethylamine, acetonitrile, petroleum ether and chloroform were acquired from Sigma (St. Louis, MO, USA). All UV-Vis measurements were taken using Cary 100 Bio UV-Vis from Agilent (Santa Clara, CA, USA). All ESI-MS spectra were collected using a Thermo Scientific (Waltham, MA, USA) ESI spectrometer. Lyophilizations were performed using a VirTis Benchtop 4 k Series Freeze Dryer.

2.2. ADPH Isolation and Purification

ADPH isolation has been previously reported [25,26], and optimizations of that protocol are described below. Luria-Bertani (LB) media was prepared freshly or within 24 h of use; 2 L portions were made in 4 L flasks. Overnight cultures of *E. coli* WBB06 (HepI and HepII knockout *E. coli* strain) were started by inoculating 10 mL of LB-Tet with WBB06 cells from a glycerol stock stored at −80 °C. A minimum of 8 L of LB-Tet media supplemented with 5 mM $CaCl_2$ [27] was inoculated with the overnight culture (1 mL of overnight growth per 2 L of media) and allowed to grow at 37 °C to an OD_{600} of 1 (approximately 10–12 h), then centrifuged for 10 min at 5000 rpm to pellet the cells. The cell pellets were scraped into a 250 mL beaker to which a stir bar and 80 mL of 50% ethanol (10 mL of 50% ethanol per 1 L of growth) were added and allowed to stir on ice for 2 h. A total of 50% ethanol was prepared using 200 proof ethanol and pH 8 water in an effort to prevent breakdown of sugar nucleotides at pH's lower than 7. Cellular debris was removed by centrifugation in 30 mL Nalgene tubes at 6000 rpm for 20 min at 4 °C. The supernatant was decanted into 150–200 mL Erlenmeyer flask with a ground joint neck for Schlenk-line vacuum attachment. The ethanol was evaporated from the supernatant by stirring under vacuum on ice and monitoring changes in volume; it should reduce to about half its original volume in approximately 1 h. The extract was further clarified through ultracentrifugation at 40,000 rpm for

1 h at 4 °C. The clarified extract was filtered successively using Amicon Ultra-15 30 kDa and 3 kDa centrifugal filters to remove proteins and other large nucleotide species. An additional centrifugal filter at 10 kDa MWCO can be used in between the 30 kDa and the 3 kDa to reduce filtration time. The pH of the flow through was analyzed to ensure it remained at pH 8 for the duration of the extraction process, if not, adjustments should be made at this point. pH adjustments can be made simply by using 0.2 M HCl or 0.2 M NaOH (1 M solutions should not be used to prevent overshooting the solution pH which could be detrimental to the small molecule integrity). The extract was purified by placing it over a 64 mL DEAE column using a triethylamine bicarbonate (pH 8) gradient from 1–500 mM using pH 8 ultrapure water. The purification method was established on an Akta Purifier 10 with a flow rate of 3 mL/min and a pressure setting at 3 MPa (according to manufacturer's recommendations). Fractionation was on a peak dependent setting where peaks were seen from UV traces at 254 nm. Further, 10–15 mL fractions were collected in labelled 15 mL falcon tubes and approximately 500 µL of each fraction were flash frozen and lyophilized. All fractions were stored at −80 °C until characterization was complete to prevent breakdown. ESI-mass spectrometry was used to determine fractions that contained pure ADPH by observation of a peak at (m/z^{-1} = 619) in a 50:50 acetonitrile:water solution (Figure S1). Fractions confirmed to contain ADPH were lyophilized, combined into a smaller volume of pH 8 water and re-lyophilized to remove traces of triethylamine. Presence of ADPH can also be determined with a variety of other characterization techniques (discussed below).

2.3. Kdo_2-Lipid A Isolation

Kdo_2-Lipid A isolation has been previously reported [28–30], and optimizations of that protocol are described below. Kdo_2-Lipid A was extracted from a minimum of 8 L of frozen or fresh WBB06 cells grown as described for ADPH extraction (above). The cell pellet was resuspended in 80 mL of water in a 250 mL beaker and the mixture was divided into 30 mL Kimble glass tubes (10 mL per tube) and centrifuged for 10 min at 5000 rpm. The supernatant was discarded, and cells were washed with the following solvents, twice each: ethanol (160 mL total), then acetone (160 mL total), then diethyl ether (160 mL total). After the addition of each solvent, the cell pellets were completely resuspended, then centrifuged to pellet the cells (10 min, 5000 rpm), and finally the supernatant was discarded before the next wash. Each wash step involved the addition of ~20 mL of solvent, then manual agitation of the cell pellet and pulverizing of large pieces using a metal spatula, capping the glass tube with a septum and vortexing the sample aggressively for at least 2 min. This process is critical to ensuring an even resuspension of the cells in each wash step, since the more clumped the cell pellet remains in between steps the more contaminated the Kdo_2-Lipid A will be upon extraction. After the final diethyl ether wash, cells were then left to dry in the hood at room temperature at least 1 h (up to overnight) in a large weigh boat. The dried cells were weighed, pulverized into a fine powder, and distributed evenly among a set of clean 30 mL glass tubes at ~1 g per tube. Pulverizing the cells is a necessary step and can be done with a variety of implements including a mortar and pestle, manual glass homogenizer, the bottom of a beaker or a spatula, all which have been successful in this step. To each tube, 20 mL of a 2:5:8 80% phenol:chloroform:petroleum ether solution was added (this solution can be prepared using liquid phenol that has been diluted or solid phenol that is melted into a liquid phase and diluted with the correct quantities of hot deionized water; the remaining solvents are added after the phenol has been diluted and returned to room temperature; the solution will not be homogeneous and will require a vigorous shaking before use, this is normal and does not affect the extraction process). The tube containing the dried cells and phenol:chloroform:petroleum ether suspension was capped with a septa and vortexed for 3–5 min, left on a nutator for 10 min, then centrifuged at 5000 rpm for 10 min. The KLA containing supernatant was gravity filtered using fluted standard grade filter paper and the phenol:chloroform:petroleum ether extraction was repeated. The diethyl ether and chloroform were removed *in vacuo* at room temperature

from the supernatant which reduces the volume by approximately half. A mixture of 75 mL acetone, 15 mL diethyl ether and ~5–10 drops of deionized water was added to the solution and left to sit for 45 min-overnight to precipitate the Kdo_2-Lipid A. The solution was centrifuged at 10,000 rpm for 25 min in clean 30 mL glass tubes, and the supernatant was decanted off, gently. The lipid pellets were washed a minimum of 4 times with ~2–3 mL 80% phenol and diethyl ether, each separately; pellets were dislodged from the side of the tube with a spatula and swirled vigorously in each wash step. The lipid suspensions were centrifuged in between washes and the supernatant was removed and discarded prior to the next wash. The pellets will dramatically diminish over the course of the washes and become more colorless. The pellets may remain in the glass tubes for ~1 h to continue drying under the hood. The dried pellets were then dissolved in 1% triethylamine aqueous solution and flash frozen for lyophilization (yield ~100–150 mg). The amount of 1% TEA is not specified, as the yield will vary per extraction, thus there is not a single volume that will work irrespective of the yield; however, it is recommended to start with 30 mL and to increase volume as needed until all the solid is fully dissolved. Dissolving the solid into solution may take some time and therefore can be assisted by continual agitation on a nutator or orbital shaker prior to flash freezing.

2.4. Kdo_2-Lipid A Deacylation

O-deacylation of Kdo_2-Lipid A as shown in Figure 3 has previously been reported [25], and optimizations of that protocol are described below. To a double necked 100 mL round-bottom flask, 50 mg of solid Kdo_2-Lipid A was added followed by a stir bar. The reaction flask was placed in a water bath at 37 °C with a water-jacketed condenser, where in the spare neck is sealed with a septum, and 5 mL of hydrazine (carefully extracted from bottle under nitrogen) was added via syringe. Per 10 mg of Kdo_2-Lipid A, 1 mL of hydrazine is required. The reaction was refluxed for 1 h with vigorous stirring, with the reaction being stopped by placing the flask on ice for 10 min. The lipid was precipitated by slowly adding 50 mL of very cold acetone (kept in the −80 °C freezer for at least a half hour) using roughly 10 mL of acetone per 1 mL of hydrazine. O-deacylated Kdo_2-Lipid A (ODLA) was then centrifuged for 30 min at 11,000 rpm. The pellet was washed three times with cold acetone and three times with diethyl ether; solution was centrifuged at 11,000 rpm for 10 min in between washes and the supernatant was carefully decanted. After allowing the diethyl ether that remained after decanting to evaporate for ~10 min with the tube on its side under the fume hood, the pellets were dissolved in deionized water, pooled together, flash frozen and lyophilized. Deacylated product was confirmed by ESI-mass spectroscopy in 50:50 acetonitrile:water by observation of the half mass (m/z^{-2} = 695) (Figure S2).

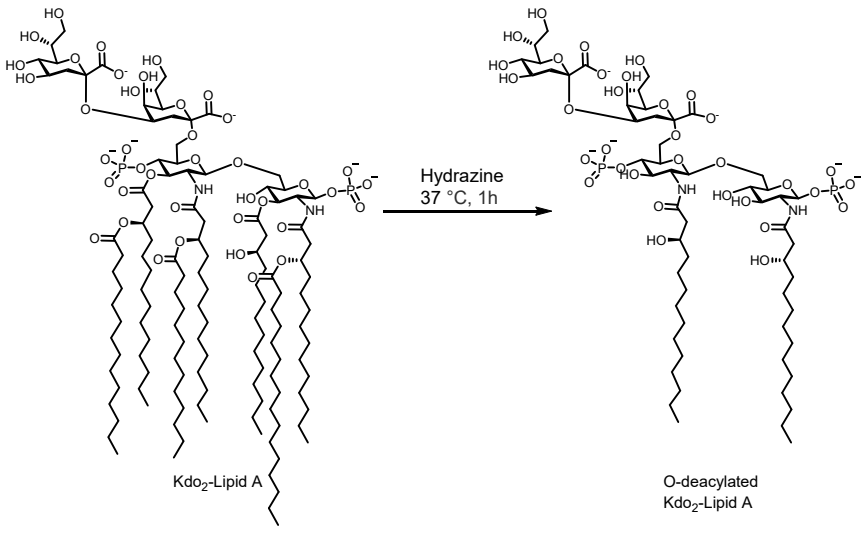


Figure 3. Reaction of Kdo_2-Lipid A with hydrazine to selectively remove O-attached acyl chains.

3. Results

3.1. ADPH Extraction and Purification

The extraction of ADPH from the cell pellet using 50% ethanol directly yields a yellow solution with a suspension of cell debris that may appear "clumpy", resulting from their lysis in ethanol. Once the cellular debris has been pelleted the supernatant will contain an amalgamation of nucleotides, sugars, small soluble peptides, nucleic acids, etc., the majority of which are removed in the filtration steps. It is common in the ultracentrifugation step to produce a second pellet because of the particulate matter and larger proteins that may sediment at that speed; this step is critical to making the subsequent filtration steps efficient, otherwise the extended time spent on those filtrations will increase the likelihood for product breakdown. The crude ADPH found in the flowthrough may become slightly acidic, which can enhance the undesired hydrolysis of ADPH, therefore, even a small change in pH should be adjusted back to eight. The purified fractions off the DEAE column that appear most yellow in color are likely to be the ones containing ADPH. The elution profile off the DEAE column (detecting at UV_{280} and UV_{254}) will vary with each extraction, however, typically four to five distinct species will elute in the 95-fraction run (Figure 4).

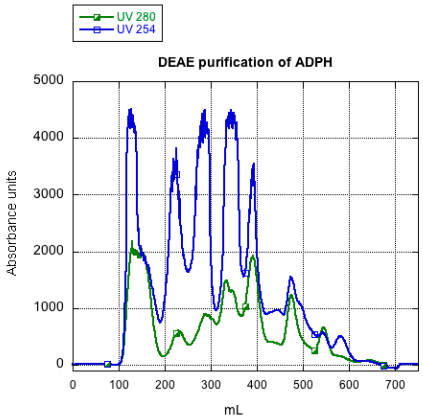


Figure 4. Trace from the AKTA purifier of DEAE purification of crude ADPH.

Oftentimes, these species include AMP, ADP, ATP, and ADPH, which should each appear as distinct peaks. After lyophilization, the final solid stored at −80 °C will appear flaky opaque and light yellow in color (Figure S3); because of the ionic nature of ADPH, when dried it can also become a high static solid and therefore weighing out the compound onto aluminum foil or a metal vessel can aid in making an accurate measurement. ESI-MS can be difficult to run with these samples since they are sugars and can caramelize in the high temperature capillary. Tips for how to handle this are described in the discussion below.

3.2. Kdo_2-Lipid A Extraction and Deacylation

Drying down the cells yields an off-white to beige colored powdery solid that is quite dry (Figure 5). Once pulverized, the sample did not appear significantly different, however, the particles were noticeably finer and more powdery. As mentioned earlier, the extraction solution will appear inhomogenous however an aggressive shake or vortexing before proceeding will ensure success; remember that shaking solvents will generate pressure which will need to be vented immediately after shaking. The extraction solution will also appear yellow in color but mostly contains membrane bound proteins, lipids, polysaccharides, etc., which can be discarded. When rotovapping, it is possible to rotovap the chloroform and petroleum ether quickly and inevitably begin to rotovap some phenol and it is critical that this does not happen since the lipid is soluble in the phenol solution. Keeping a close eye will ensure minimal loss of product. Once KLA is precipitated, the lipid in solution will appear like dust floating in solvent, it is difficult to notice its formation at times, but the longer it sits the more will compound will precipitate out of the solution. The use of ice

did not assist in the process and inevitably made it more difficult to get the lipid out of the solution. The steps for washing will dramatically reduce the overall yield while removing unnecessary lipidic components.

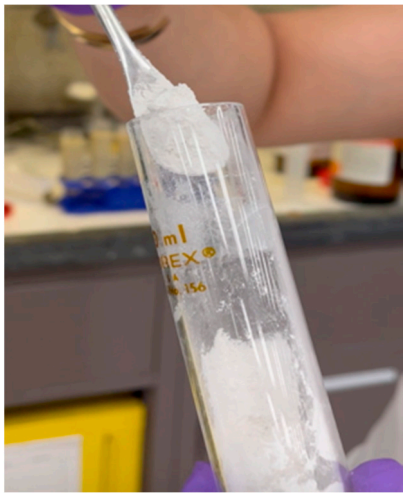
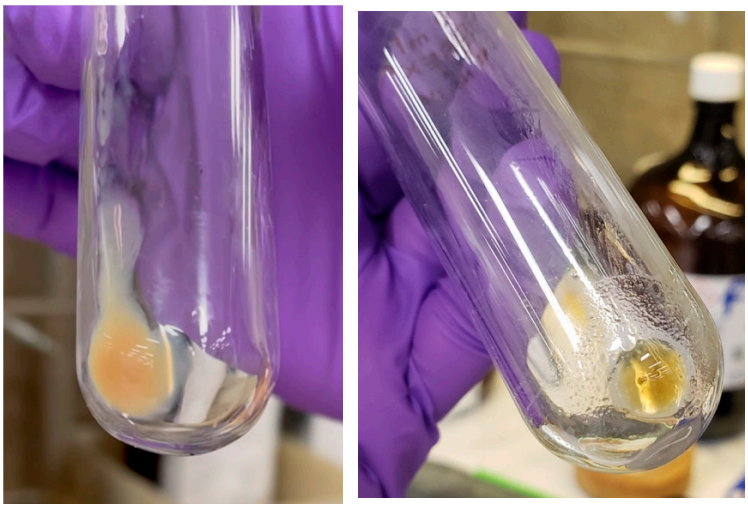


Figure 5. WBB06 cells dried down after drying under the hood.

The pellet starts off yellow and opaque (Figure 6A) and after washes with phenol, the pellet loses its opacity but remains quite yellow (Figure 6B). Washes with ether will make the pellet less colored and change its overall texture from a gummy oil like substance to a flaky solid. The final product after lyophilization is a white fluffy crystal with a cotton-like texture (Figure 7A).



(**A**) (**B**)

Figure 6. (**A**) Kdo_2-Lipid A pelleted after precipitation; (**B**) Kdo_2-Lipid A after washes with phenol.

Before precipitation of the O-deacylated Kdo_2-Lipid A, the protocol states to put the round bottom flask on ice. This is crucial as the addition of cold acetone will make the reaction mixture, smoke and sputter which can be dangerous. The solid being washed has very light, flaky, and white to off-white color and can easily be lost in the decanting process, so gently pouring off the wash solution will prevent product loss. Dissolving of the ODLA will take more water than it may seem; be sure to be generous with the volume of water, within reason, as it will take far longer to lyophilize a larger volume. If successfully made, ODLA will dissolve in water with little required agitation. The final product should be a

white flaky solid and is also quite static when trying to weigh some for a substrate sample (Figure 7B).

(**A**) (**B**)

Figure 7. (**A**) Kdo_2-Lipid A after lyophilization; (**B**) O-deacylated Kdo_2-Lipid A after lyophilization.

4. Discussion

4.1. ADPH Extraction and Purification

All the steps in the ADPH extraction and purification have been designed largely to prevent the breakdown of the compound before it can be isolated and carefully stored. The entire process needs to be done all in one day to ensure a minimal amount of hydrolysis occurs before the purification is complete. ADPH in any solution phase is not very stable and will break down readily which is why as many steps as possible are done on ice. All the centrifuge tubes, or samples of ADPH that are being processed must be kept on ice through the duration of the extraction and purification steps. It is stored as a solid at −80 °C and is made into 1 mL stock solutions directly before use. When broken down, the once yellow solution will become very obviously pink. To prepare the purification elution buffer, triethylamine bicarbonate, a 1 L bottle of 750 mL H_2O and 125 mL TEA is stirred with constant bubbling of CO_2 (g) until the solutions combine thoroughly which typically takes about an hour. Unless there are extreme variations in the growing process, the extraction remains relatively consistent with little variability found in the purification. DEAE resin used must be cleaned and equilibrated between runs to maintain consistency which can be done according to the manufacturer's specifications. Oftentimes, purifications set on FPLC can be done without supervision however, it is crucial that once the fractions have been collected that they are immediately stored at −80 °C until ready for lyophilization. When determining which fractions have ADPH by using mass spectrometry, there may be some complications with the instrument due to the sugars caramelizing in the electrospray chamber. To avoid this, run as few samples as possible; this is done by picking one or two fractions that represent the peak maxima for each species that elutes. In addition, it will help to flush the injection port with a 50:50 water:methanol mixture between samples to minimize clogs in the capillary. Thorough cleanings of the mass spectrometer sample chamber after using to assay for ADPH content should be done as well to avoid contamination of other samples. Successive lyophilization with pH 8 water has shown to decrease triethylammonium bicarbonate traces on the solid that can alter certain experimentation if introduced. It is also possible to determine the presence of ADPH in the samples using ^{31}P NMR or ^{1}H NMR [31–33]. The high throughput screening methods of ESI-MS make it far more suitable for ADPH extraction, however, without access to a mass spectrometer an NMR will be more than sufficient. ADPH can be stored as a solid at −80 °C for upwards of a year with semi-regular checks to ensure small-molecule integrity has been maintained. It will last about three months as an aqueous stock at −80 °C however, enduring freeze-thaw cycles will decrease its shelf life considerably with about 5–8 freeze-thaws before total product breakdown occurs.

4.2. Kdo_2-Lipid A Extraction and Deacylation

As discussed in the protocol the cell dry-down and lipid extraction can be done from fresh or frozen cells. Cell pellets can be frozen at −80 °C for up to one month prior to use due to the stability of the Kdo_2-Lipid A. It is emphasized in the protocol to fully resuspend the cells when drying them down in each of the solvents before centrifugation. This is intended to make sure that all the cells are exposed to the solvent on their cell surface, since the drier the cells, the more successful the extraction will be. If care is not taken during the dry-down step, it will yield a clumpy beige/grey cell solid that is difficult to work with. Lipid extracted from these cells is gummy and requires a lot more wash steps than usual which inevitably reduces overall yield. Once successfully dried, the cells can be stored in a Falcon tube or capped vial at 4 °C for about a month before the extraction. The storage environment must be a dry because exposure of the dried pellet to moisture will cause molding of the cells. Pulverizing the dried down cells is a necessary step prior to the lipid extraction, however, pulverizing the cells will also generate more surface area to ensure efficient resuspension in the extraction solution. Cell resuspension is done initially by spatula, then by aggressive vortex, and finally by circulation on a nutator. The extracted solution should appear clear and yellow with no floating particulate matter, and if necessary, a second filtration step can be performed to remove any visible solids as they will also negatively impact this process. Removal of petroleum ether and chloroform by way of rotary evaporator is a critical step that if overdone will breakdown the lipid in solution before it can be successfully precipitated. To avoid this, high vacuum conditions, with rapid rotation in a room temperature water-bath for ~10–15 min will remove the more volatile solvents leaving a yellow crude extract that is deeper in color, but not dark. Be sure to give the solution sufficient time to allow full precipitation of the lipid from solution, a minimum 45 min is recommended but to obtain a much higher yield the mixture should stand overnight at room temperature before centrifugation. As mentioned previously, a large volume of the pellet will dissipate as the washes continue; this is to be expected as the extracted pellet contains components of the lipid bilayer as well as other membrane bound lipids which are undesirable in the final product. If there is any undissolved lipid in the 1% TEA, it will not lyophilize properly and therefore will not deacylate to completion, which is why it is important to add a little solution at a time until the lipid dissolves fully. The KLA solid will be a fluffy white solid that appear cotton-like in texture; these characteristics are a good visual cue of purity as well as characterization via mass spec. Due to the highly charged nature of the compound, it will often be detected as the doubly or triply charged species, and have a correspondingly smaller observed m/z. The compound can also be characterized via NMR if it is to remain in it is fully acylated state. Kdo_2-Lipid A is not fully water soluble and will require the use of detergent for experimental purposes. Chaps and Triton X-100 are two commercially available detergents that have been shown to work well with HepI and similar proteins that will solubilize the Kdo_2-Lipid A [34]. Experimentally, it has been shown that selective deacylation generates a hydrophilic lipid A moiety that does not differ greatly from the fully acylated version in it is binding and kinetic interactions with the protein [25].

The deacylation reaction is straightforward and dependable, as hydrazine is a potent reactant if stored properly and extracted from the reagent bottle under nitrogen while not introducing any water contamination. The acetone washes after deacylation are helpful for removing trace quantities of hydrazine and the diethyl ether washes will help to dissolve acyl chain contaminants from the product. If the lipid does not dissolve in water readily, add more water dropwise and vortex to help with dissolving. Similar to the Kdo_2-Lipid A lyophilization, dissolving ODLA fully is critical to the outcome of the crystal after freeze drying. ODLA and Kdo_2-Lipid A can be stored as a solid, sealed, at 4 °C indefinitely. As aqueous solutions, they will only last about two weeks prior to significant signs of breakdown.

All three solids (ADPH, KLA and ODLA) are charged and thus experience a static cling when weighing them out for a stock solution and the powdery sample will fly off the

spatula faster than it can be remotely retrieved. To avoid the frustration of losing product due to its intrinsic properties, a safe introduction of water works well to cut the electrostatic tension by way of a kimwipe soaked in water in the corner of a weighing chamber on an analytical balance.

5. Conclusions

Overall, through a process of careful optimization of the protocols for extraction of ADPH and KLA, increases in the yield of these processes can be significantly enhanced. Overall, since both ADPH and KLA are useful for both HepI reactions, as well as other immunological assays, the need for optimized methods will likely only increase in years to come.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11188314/s1, Figure S1: ESI-MS spectrum fraction from DEAE purification containing ADPH (m/z^{-1}: 619), Figure S2: ESI-MS spectrum O-deacylated Kdo_2-Lipid A (m/z^{-1}: 1391, m/z^{-2}: 695), Figure S3: ADPH after lyophilization.

Author Contributions: Conceptualization, J.M., C.D.C. and E.A.T.; methodology, J.M. and C.D.C.; validation, J.M., C.D.C. and N.J.; writing—original draft preparation, J.M. and C.D.C.; writing—review and editing, J.M., C.D.C., N.J. and E.A.T.; visualization, N.J.; supervision, project administration, funding acquisition, E.A.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Institute of Health, Grant Number 1R15AI119907-01.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We would like to thank Cassie Lui, Frank Tucci, Cody Hecht, Mackenzie Schlosser and Maya Marshall for working through these protocols to evaluate their steps in more detail. We would also like to thank Joy M. Cote and Daniel Czyzyk for establishing these protocols in the Taylor lab for use in HepI experiments.

Conflicts of Interest: The authors declare no conflict of interest.

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