Isolation and Characterization of Fatty Acid Content of Shrimp Shell Chitin

Biomarkers are biologically produced organic compounds that can be used to identify sources of organic matter in geologic materials (soils, sediments, rocks). This information allows scientists to track important environmental and ecological changes over time and space. Some of the most commonly used biomarkers are a class of lipids called fatty acids. These compounds resist degradation and are diverse in structure, making them valuable indicators of carbon sources. Most fatty acids can be extracted from sediments using organic solvents. However, a significant fraction cannot be extracted in this way and must first be liberated by hydrolysis of ester or amide bonds. The understanding of the sources of these “bound lipids” is limited, but multiple studies have speculated about a relationship between fatty acids and chitin, a biopolymer found in arthropods and fungi. Analyses of chitin from several sources (Red crab shells, commercial chitin standard) have indicated that there are fatty acids associated with the material. However, it is not known how this association occurs, or whether it is natural or an artifact of the chitin preparation process. As a member of Neal Blair’s organic geochemistry lab, this summer I am interested in investigating this property of chitin and gaining valuable research experience. Therefore, the purpose of this study is to attempt a new method for the isolation of chitin in order to determine if fatty acids are naturally associated with it.

Chitin is the second most abundant biopolymer on earth. It is a β-1,4 linked polymer of N-acetylglucosamine, and is found mainly in arthropod exoskeletons and fungal cell walls (Fig. 1). Chitin is an insoluble, high molecular weight polymer with an extensive hydrogen bonding network, and it is naturally strengthened and functionalized by associated minerals and proteins. This makes it a challenge to purify without compromising the polymer structure, and it is thus difficult to analyze natural fatty acid content.

Preliminary analyses in our lab indicated that a chitin standard from Sigma Aldrich chemical company contained bound fatty acids. The use of the methylating reagent tetramethyl ammonium hydroxide (TMAH) with Gas Chromatography/Mass Spectroscopy (GCMS) identified the presence of C16 and C18 fatty acids in the chitin (Fig. 2). Even though this chitin was intended for laboratory use, the bound fatty acid content was most likely not considered in purity analyses. Due to this, it is uncertain as to whether the fatty acids are naturally bound to the chitin structure, or accidentally added by the purification process. Accordingly, I would like to investigate the natural presence of fatty acids with an alternative purification technique.

Thus far, no one has attempted to study the lipid content of chitin purified with ionic liquids. Ionic liquids are organic salts with relatively low melting points (<100°C), and some have been found to disrupt the hydrogen bonds in chitin and allow it to dissolve. This releases proteins and minerals contained in the chitin, which can be re-precipitated and separated. Since ionic liquids have a strong ionic character and a low melting point, dissolution can be achieved in pH neutral solutions at low temperatures. These conditions are gentler and yield chitin with a higher purity than traditional isolation methods, and thus should preserve the polymer structure and any covalently-bound fatty acid associations.

In this project, I want to analyze the difference in lipid content between chitin prepared by the Sigma method (using methanesulfonic acid) and chitin isolated with an ionic liquid. My objective is to compare the different extraction methods to see if I get the same lipid results, thus providing additional evidence that fatty acids might be a natural part of the chitin structure.
I have developed a method for the extraction of free lipids from shrimp shell and Sigma chitin using a microwave extraction step. This ensures that the following purification will not inadvertently attach free lipids to the polymer, and that fatty acids identified in TMAH analysis will not be free lipids. It also allows for comparison between the original Sigma standard and the extracted sample, so I can identify the fatty acids in the original standard as free or bound.

Once the free lipids have been extracted with organic solvent (Dichloromethane and methanol), I will purify chitin using the Sigma technique of methanesulfonic acid as detailed by Hirano.\textsuperscript{10} This is an effort to re-create Sigma chitin with minimal fatty acid contamination. A parallel test will be performed using ionic liquids. Shrimp shells will be dissolved in 1-ethyl-3-methyl-imidazolium acetate ionic liquid ([C2mim]OAc, Fig. 3), and re-precipitated according to the procedure by Ying Qin, et al.\textsuperscript{8} I hypothesize that fatty acids in the purified chitins will be similar to each other, suggesting that their presence is not an artifact of purification and that they may be covalently bound to the polymer. If the fatty acid contents of the two chitins are dramatically different, it is likely that the fatty acids are in fact purification artifacts. The next step in the project will depend on these preliminary results.

It is a challenge to separate chitin from associated proteins and minerals, and several techniques are needed to characterize the effectiveness of purification. Elemental Analysis (EA) will provide elemental compositions and will be used to constrain the purity of the chitin. In pure chitin monomers, the percent by mass of carbon, nitrogen, and hydrogen are 47.3\%, 6.9\%, and 6.4\%, respectively. Additional identification will be provided by $^1$H NMR. In the spectrum of pure chitin, a peak at 2.3 ppm represents acetamide protons and a peak at 4.5 ppm corresponds to the proton on C1 (Fig. 1).\textsuperscript{11} However, there will also be multiple peaks between 3.5 and 4.0 ppm from the hydroxyl hydrogens. These could be similar to peaks resulting from protein residues, so EA data will be correlated with the spectrum to identify this potential contamination.

 Furthermore, the identity of the chitin will be verified with FTIR, which will show key chitin functional groups such as an amide I band around 1660 cm\textsuperscript{-1}, an amide II band at 1560 cm\textsuperscript{-1}, and broad O-H and N-H stretches in the 3400 cm\textsuperscript{-1} region.

TMAH analysis will determine the presence of bound lipids in the chitin. A sample of chitin will be heated to 300\° C in the presence of the TMAH reagent, which will methylate functional groups containing oxygen. This generates methyl esters of fatty acids that were originally bound as esters or amides. It is expected that there will be a significant presence of fatty acids C16 (Palmitate) and C18 (Stearate), which were two of the most prominent peaks in the gas chromatograph of the Sigma chitin standard. Glucosamine should also be identified, which will verify the chitin structure (Fig. 2).

If fatty acids are found to survive isolation, a future study might involve an enzymatic degradation of chitin followed by liquid chromatography to screen for sugar-fatty acid monomers. If there are fatty acids naturally bound to chitin as amides, our understanding of the role of chitin in lipid sequestration in sediments will be fundamentally impacted.

Using an Academic Year grant, this winter I developed a microwave extraction method and began the first round of free-lipid extractions. Next quarter I will proceed with TMAH analyses and further free lipid extractions. The two chitin isolation procedures and characterization of the products will be performed this summer.

I view this immediate project as a first step to my long term goal of characterizing the role of chitin in the carbon cycle, and I plan to continue working in the Blair lab during subsequent years at Northwestern. Ultimately, I will pursue a PhD and research career in environmental biochemistry.
Figure 2: Gas chromatogram of Sigma chitin standard TMAH derivatives. Peaks are identified by mass spectrometry and a NIST library. Notable peaks include the methyl esters of 1) the straight chained saturated C16 fatty acid, 2) an unsaturated C18 fatty acid, and 3) the straight chained saturated C18 fatty acid, as well as the methylated derivative of glucosamine (4).

Figure 3: Chemical structure of 1-ethyl-3-methylimidazolium acetate ([C2mim]OAc) ionic liquid
References


8. Ying Qin, Xingmei Lu, Ning Sun and Robin D. Rogers, Dissolution or extraction of crustacean shells using ionic liquids to obtain high molecular weight purified chitin and direct production of chitin films and fibers. Royal Society of Chemistry: Green Chemistry 2010, (12), 968-971.

