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Abstract
Instrumentation was developed to monitor the volatile products of lipid peroxidation in near-realtime. This was accomplished using a miniature incubator with a cryofocusing inlet and gas chromatography with a time-of-flight mass spectrometer. The headspace above myoglobin-induced lipid peroxidation of arachadonic acid in phosphatidyl choline was sampled in order to quantitate hexanal, a known marker of lipid peroxidation. With an analysis time of less than four minutes, the rapid preconcentration of volatile products allowed for low detection limits in a short length of time. Reactions were sampled repetitively, allowing near-real-time monitoring of the reaction. When phospholipid samples contained no basal levels of hexanal, the production of hexanal was linear for approximately 40 minutes, and reached concentrations of approximately 1.5 ppm. For phospholipids samples which did contain basal levels of hexanal, the production was linear for approximately 25 minutes, and reached concentrations of around 5 ppm. The presence or absence of hexanal in the lipid sample prior to the addition of myoglobin was therefore essential in determining the linearity, initial rate, and extent of hexanal production during the reaction.

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The Measurement of Hexanal Production by Myoglobin-Induced Lipid Peroxidation Using Headspace Analysis and a Cryofocusing Inlet System with a Gas Chromatograph and Mass Spectrometer

By

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Abstract

Instrumentation was developed to monitor the volatile products of lipid peroxidation in near-real-time. This was accomplished using a miniature incubator with a cryofocusing inlet and gas chromatography with a time-of-flight mass spectrometer. The headspace above myoglobin-induced lipid peroxidation of arachadonic acid in phosphatidyl choline was sampled in order to quantitate hexanal, a known marker of lipid peroxidation. With an analysis time of less than four minutes, the rapid preconcentration of volatile products allowed for low detection limits in a short length of time. Reactions were sampled repetitively, allowing near-real-time monitoring of the reaction. When phospholipid samples contained no basal levels of hexanal, the production of hexanal was linear for approximately 40 minutes, and reached concentrations of approximately 1.5 ppm. For phospholipid samples which did contain basal levels of hexanal, the production was linear for approximately 25 minutes, and reached concentrations of around 5 ppm. The presence or absence of hexanal in the lipid sample prior to the addition of myoglobin was therefore essential in determining the linearity, initial rate, and extent of hexanal production during the reaction.

Introduction

Cell membranes are an integral part of any cell, as the regulation of import and export to the cell are the main responsibilities. The breakdown of this cell membrane, therefore, can be extremely hazardous to the cell. One portion of this cell membrane that can easily breakdown is the lipid bilayer, which can undergo oxidation. Such damage has been shown to interfere with signal transduction, maintenance of membrane potential, molecular recognition and transport, and cellular metabolism, each of which has the potential to initiate disease [1-5]. Specifically, lipid
peroxidation has been linked with a variety of diseases, such as diabetes [6], liver damage [7-9], heart disease [1, 10-15], cancer [16-20], and kidney failure [21-25]. The relationship between the peroxidation of the lipid bilayer and these diseases is poorly understood, as is the entire lipid peroxidation process. Therefore, in order to gain a full grasp of the connection between this breakdown and disease, it is first essential to more fully comprehend the complexities of lipid peroxidation.

There are a few methods by which lipid peroxidation is commonly studied. The most popular of these methods is the TBARS (thiobarbituric acid reactive substances) test. This is a spectrophotometric test which involves reacting thiobarbituric acid (TBA) with malondialdehyde, one of the major products of lipid peroxidation [26]. The colored product can then be analyzed spectrophotometrically to determine the concentration of malondialdehyde produced during the peroxidation reaction. However, malondialdehyde is not the only substance which can react with TBA. Moreover, the TBARS assay has been shown to overestimate malondialdehyde production from the lipid peroxidation reaction, as it is formed by the breakdown of larger molecules during the analytical procedure [27-29]. This lack of specificity and questionable reliability are definite disadvantages, yet its popularity remains strong due to the simplicity, sensitivity, and low detection limits of the experiment.

Other methods for the analysis of lipid peroxidation have also become more commonly used, such as methods involving derivatization of the peroxidation products, which can then be analyzed using chromatographic methods. These methods, like the TBARS test, provide sensitivity and low detection limits, but include the additional benefit of specificity [30-40].
Because many products are formed during the process of lipid peroxidation, chromatographic methods are especially useful because they allow many species to be examined simultaneously. This is in sharp contrast to the TBARS method, which allegedly only quantitates the malondialdehyde-TBA product. Unfortunately, these methods sacrifice simplicity, as the derivatizations can be difficult and time consuming. Both the TBARS test and derivatization procedures are destructive to the sample.

The determination of underivatized hexanal as a marker of lipid peroxidation via headspace gas chromatography has found wide application in the food industry [41-44]. Modifications of these analyses have been used to monitor lipid peroxidation in vitro and in vivo using static and dynamic headspace [45-47], and solid phase microextraction (SPME) [39]. These methods have a distinct advantage over other chromatographic procedures, as lengthy cleanup and derivatization steps are unnecessary, and have demonstrated the potential to elucidate kinetics, mechanisms, and products of lipid oxidation reactions. However, static and dynamic headspace methods do not, in general, yield the low limits of detection obtained through derivatization procedures, unless a preconcentration step is used. This limits the analyses to major products rather than a full profile. Sorbent trapping in the liquid or vapor phase yields lower limits of detection, though often at the expense of longer analysis times to allow for sample equilibration with the sorbent phase.

We have developed instrumentation that allows for the real-time measurement of the volatile products of lipid peroxidation using a miniature incubator in combination with a cryofocusing inlet system and gas chromatograph with a time-of-flight mass spectrometer (GC-TOFMS). Each
sample is contained in an individual enclosed chamber to which air/CO₂ can be provided for long-term viability of cell samples, or other gases if a modified atmosphere is desired. Headspace samples are drawn through a deactivated fused silica restrictor to a cryofocusing trap for preconcentration and injection into the GC-TOFMS. The preconcentration step is rapid; with a total analysis time of less than four minutes, multiple measurements can be made on a single sample as a reaction progresses. The preconcentration step also yields lower limits of detection than traditional static or dynamic headspace sampling. The entire system is under computer control and can be fully automated. Extensive studies have been conducted using this instrumentation with myoglobin-induced lipid peroxidation of phosphatidyl choline containing arachidonic acid as a model system.

Experimental

A miniature incubator, shown in Figure 1, was used to house the samples. The base (B) contained water which was kept at 37°C. Glass chambers (C), 6.5 cm in height, 2.4 cm in diameter, were held in fitted holes within the base, and were secured using O-rings (O). The samples were then placed inside the glass chamber, either directly, or within a small vial. The chamber was capped off using an aluminum insert (I) which was equipped with a self-sealing septum (S). The inserts were specially machined to provide an air-tight seal. Sampling could then be accomplished through the septum, using a needle placed around fused silica tubing.

The fused silica tubing lead to a cryofocusing apparatus, as shown in Figure 2. The sample (S) first ran through a 4-port valve (V1), which was computer controlled to either send sample into the system, or to connect it to vent. During sampling, the system was placed in “sampling”
position, shown in Figure 2 (a). Using a vacuum pump, sample was drawn through R1 and R3 onto the cryofocusing trap. The trap consisted of 15 cm of capillary dimension silicosteel, which was contained in an aluminum chamber and cooled to -90°C using cold nitrogen gas. Valve V2 was closed, so no sample flow occurred across R4. This allowed for a preconcentration of sample. Sample was drawn onto the trap in this manner for 25 seconds. Figure 2 (b) shows “flush” mode. This five-second period allowed for the tubing to be cleared of any residual sample, either by sweeping it onto the trap, or by sending it to vacuum. In this V1 is now connecting the system to room air, and the sample is connected to vent. A flow of gas now occurs across V2 in order to sweep sample through R3 and onto the trap. Because no gas flow occurred over R4 during sampling, no sample should be present, so any gas flow across it can go straight to vacuum. R1 is connected to room air, which is at a higher pressure than the chamber. Room air flows into the chamber until the pressure equilibrates. This sweeps residual sample in R1 back into the chamber, and prevents chamber-to-chamber cross-contamination. After a 30-second equilibration period, the system was placed into “analysis” mode, Figure 2 (c). The trap was “fired” using a 90V capacitative discharge. The valve connecting the system to vacuum, V3, is now closed, and the gas flow occurs towards the GCMS. The gas flow along R4 helps to force the sample from the trap toward the gas chromatograph where it can be separated and analyzed using the mass spectrometer.

2-arachidonyl-1-palmitoyl-sn-glycero-3-phosphocholine (GPC-AA) was obtained and stored at -80°C until used. Doubly-distilled, deionized water was used to dissolve the lipid to a concentration of 1 mg/mL. This sample was sonicated to disperse the lipid. 125-μL portions were divided and stored under nitrogen at -20°C until use. Horse heart myoglobin (Mb) was
stored at -20°C until use. It was dissolved in doubly-distilled deionized water to a concentration of 20 mg/mL. Pentanal and hexanal were stored at 0°C under nitrogen or argon.

In order to oxidize the phospholipid, appropriate Pipetmen were used to combine 25.0 μL of GPC-AA with 50 μL of 0.5 M sodium acetate buffer, pH 6.5, and 113 μL of distilled, deionized water. This solution was placed in a 4 mL vial, and vortexed. It was then placed in the closed incubation chamber to equilibrate at 37°C. After five minutes, the headspace was sampled, in order to determine basal levels of hexanal. Next, 62 μL of myoglobin were added to the vial, and it was again allowed to equilibrate. Beginning after five minutes, the headspace was sampled every five minutes. Sampling continued through 60 minutes, until hexanal concentrations became stable.

Hexanal produced in oxidation reactions was compared with standard samples which were run daily for calibration purposes. These standards were prepared by dissolving pure hexanal in 0.1 M sodium-acetate buffer, pH 6.5. A standard containing 100 ppm hexanal and 20 ppm pentanal was prepared and stored at -20°C. This solution was then diluted to a 10 ppm hexanal, 2 ppm pentanal solution, which could also be stored at -20°C. After the solution thawed and was vortexed, it was used to make a variety of standards daily, varying from 0.1 ppm to 5 ppm hexanal, which was approximately the range of concentrations that were found during lipid peroxidation reactions. A 250-μL portion of each standard was incubated for at least five minutes before sampling the headspace, in order to allow for equilibration.
To determine on-column hexanal concentrations, calibration data was compared with gas bag sampling. Neat hexanal was injected into a 100-L Tedlar bag using a Hamiltonian syringe. The bag was then filled with air. Aliquots of this standard were transferred to 1-L Tedlar bags with a gas tight syringe. From this, on-column hexanal concentrations could be determined.

**Results**

In general, we saw linear calibration data for the concentrations measured, 0.1 to 5 ppm (v/v in the liquid phase). Calibration sensitivity was $8 \times 10^5$ area units/ppm hexanal in the liquid phase. A compilation of three curves is shown in Figure 3. The data in this figure was collected on three different days over a two month period. During this time, the cryofocusing inlet was temporarily replaced by a liquid-sampling inlet for other experiments. Despite major instrumental changes, the repeatability and linearity of the data is quite strong. When regression lines were determined, correlation coefficients were generally observed to be around 0.98 to 0.99. Similarly, calibration curves were compiled for pentanal concentrations in the range of 0.02 to 5 ppm.

Oxidation reactions were measured using repetitive sampling, with 25 second sampling times. Figure 4 shows a representative chromatogram of the headspace of Mb-induced peroxidation of GPC-AA. The reaction was sampled sequentially every five minutes over the incubation period. Hexanal (H) and pentanal (P) have both been identified using mass spectral information, and are present in a 12:1 ratio. Figure 5 shows a graph of the concentration of hexanal production versus incubation time for many oxidation reactions. These experiments were conducted using a phospholipid sample which contained no basal levels of hexanal. The curves are linear through
approximately 40 minutes of incubation, and then level off. This was common in samples with no basal levels of hexanal. Each line represents an oxidation that was sampled repetitively for the duration of the experiments. The solid diamonds show samples that were allowed to equilibrate for a given length of time, and then were sampled only once. The fact that these points fall right in line with the repetitive sampling experiments shows that the reaction and equilibrium was left undisturbed during sampling. For samples with basal hexanal, such as Figure 6, the rate of production was much faster initially, and the curve became non-linear at around 25 minutes. The basal level of hexanal can be attributed to breakdown in storage. During this oxidative breakdown a number of large products are formed which are undetectable in the headspace. When the reaction begins, these products breakdown quickly to form hexanal, accounting for the rapid initial rate in these experiments. The final hexanal concentrations were much higher in samples such as this. Overall, final hexanal concentrations were approximately 1.5 ppm in samples with no basal levels of hexanal, and 5 ppm in samples with basal hexanal.

In order to confirm hexanal production through lipid peroxidation, Figure 7 was compiled. Figure 7A shows a typical run with myoglobin used as the oxidant. Figure 7B shows the reaction of GPC-AA with no myoglobin, being oxidized only by the air and light. Very little hexanal was formed during this reaction. Figure 7C shows hexanal production from phospholipid with myoglobin and butylated hydroxytoluene (BHT). BHT is a known oxidation inhibitor, and as shown in Figure 7, caused initial rates similar to that of the uninhibited reaction, but levels off after approximately 10 minutes.
Our procedure depends on the idea that a liquid-gas equilibrium is being established. To confirm that this equilibrium is not only established, but also left undisturbed during sampling, some experiments were conducted during which the sample was stirred continuously throughout the incubation period. This was accomplished using a miniature magnetic stirring bar in the reaction solution. While larger peak areas were observed, after calibration, hexanal concentration was found to be almost identical through both methods, as shown in Figure 8. This data was collected over four days, where the open squares represent stirred reactions, and the shaded diamonds represent unstirred reactions. It is clear that while there are slight differences in rates, they are no more dissimilar than the variation in all oxidations that we run, as can be seen when comparing Figure 8 with Figure 5. This is an important result, as our technology is currently being applied to cells, which cannot be stirred without causing damage.

For comparison purposes, neat hexanal was placed in gas-tight Tedlar bags, and they were sampled using the cryointegration system. From this, the on-column concentrations could be determined for oxidation reactions. Figure 9 shows the comparison between a 4 ppm hexanal standard (Figure 9a), and a $1 \times 10^{-13}$ g/mL gas bag sample (Figure 9b). The similarity in the peak area tells us that when we sample a standard which contains 4 ppm hexanal in the liquid phase, the concentration of the headspace is $1 \times 10^{-13}$ g/mL.

Figure 10 shows three chromatograms with different sampling times. Figure 10a was the typical 25 second sampling time. In Figure 10b, the sampling time was increased to 60 seconds, and in Figure 10c it was increased further to 120 seconds. This allowed for a higher pre-concentration of products in the trap. As the sampling time doubled, the peak area approximately doubled for
major products such as hexanal and pentanal. Minor products, which appeared in the noise during after short sampling times become apparent and in high enough concentrations to be quantitated. With further work, it should be straightforward to use the mass spectral information to determine the identities of these products, which may include species such as n-alkanals, alk-2-en-als, and 4-hydroxyalkenals. Even with the 120 second sampling time, the process is rapid, with the total analysis time under five minutes.

Conclusions

The miniature incubator with the cryofocusing inlet system and GC-TOFMS was an effective method of determining hexanal production in the myoglobin-induced peroxidation of arachidonic acid in phosphatidyl choline. The preconcentration step allows for low detection limits, while still providing specificity and rapid analysis. The nondestructive nature of the sampling gives this procedure a benefit over others. When phospholipid samples contained no basal levels of hexanal, the reaction was linear for approximately 40 minutes and reached concentrations of 1.5 ppm. In contrast, phospholipid samples which showed basal hexanal began with a faster initial rate, but became non-linear after only 25 minutes. The extent of oxidation was seen to be higher in these samples, with a final hexanal concentration of around 5 ppm. The applications of this procedure, including the oxidation of cells, are numerous, and the potential to quantitate and identify unknown minor products gives this technology the capability to be further developed in the future.

References


Figure 1: Miniature incubator with glass chambers (C) immersed in a circulating water bath (B) and secured with O-rings (O). Aluminum inserts (I) fit into the glass chambers and are equipped with self-sealing septa (S).
Figure 2: The cryofocusing apparatus showing sampling mode (a), flush mode (b), and analysis mode (c).
Figure 3: A compilation of three calibration curves collected over a two month span.
Figure 4: A typical chromatogram of a peroxidation reaction showing hexanal (H) and pentanal (P).
Figure 5: A compilation of oxidation reaction data from samples containing no basal levels of hexanal. The lines represent reactions which were sampled repetitively, while the solid diamonds show independent sampling.
Compilation of oxidation reactions in which the lipid sample contained basal levels of
Figure 7: A typical time course for hexanal production by Mb-induced lipid peroxidation of GPC-AA (A). Hexanal production in the absence of an oxidant (B). Hexanal production inhibited by the addition of the antioxidant BHT (C).
Figure 8: Time course data for unstirred reactions (shaded diamonds) and stirred reactions (open squares)
Figure 9: The comparison between hexanal peak areas for a 4ppm liquid standard (dashed line) and a $1 \times 10^{-13}$ g/mL gas phase sample (solid line).

Figure 10: Example chromatograms of peroxidation reactions when sample was drawn onto the trap for (a) 25 seconds, (b) 60 seconds, and (c) 120 seconds.