Alternative Assay Techniques for Protein Kinase A

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A protein kinase is an enzyme that aids in the transfer of a phosphate group from ATP to a protein substrate. Phosphorylating a protein can act as a switch in turning on or off an enzyme's activity. Protein kinase A (cAMP dependent protein kinase or PKA) has several functions in the cell including the regulation of glycogen, sugar, and lipid metabolism. Traditional PKA assays have involved the use of ^{32}P-ATP. These radioactive assays have safety, disposal, and cost issues associated with them. Finding a non radioactive technique, could potentially find wide application in the study of kinases. In this study ion chromatography (IC) will be used to directly measure the ADP produced in a PKA catalyzed reaction. The second substrate used here is a synthetically designed substrate called Kemptide. The ion chromatography system will separate the product of interest, ADP, from the reactant (ATP) based upon their net ionic change.

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INTRODUCTION

A protein kinase is an enzyme that aids in the transfer of a phosphate group from ATP to a protein substrate creating a final products of ADP and the phosphorylated protein. Within the protein substrate itself, specific amino acids are selectively phosphorylated, meaning a phosphate is added to a particular amino acid residue within the protein. A kinase will recognize an amino acid sequence and pick out a residue for phosphorylation. The most common amino acid for this covalent modification is serine, followed by threonine, and in some cases tyrosine. Phosphorylating a protein often acts as a switch in turning on or off an enzyme's activity. An enzyme is a protein which catalyzes a chemical reaction by lowering the activation energy needed for the reaction to occur. Without the aid of enzymes many reactions which take place in the body would be very lengthy, creating a highly unfavorable situation and making life impossible. Enzymes have thousands of functions in the human body, one of which is regulating the metabolic process. Protein kinase A (PKA or cAMP dependent protein kinase) has several functions in the cell including, being a key regulatory protein for the metabolism of glycogen, carbohydrates and lipids.

In the study of enzymes, it is necessary to develop an assay to measure an enzyme's activity. Traditional PKA assays have involved the use of ^{32}P-ATP. These radioactive assays have safety, disposal, and cost issues associated with them. Finding a non radioactive technique, could potentially find wide application in the study of kinases. In this study ion chromatography (IC) will be used to directly measure one of the products, ADP, produced in PKA catalyzed reactions. The other substrate or reactant used in this study is a designed synthetic substrate called Kemptide. Kemptide is a very small protein with the amino acid sequence Leu-Arg-Arg-Ala-Ser-Leu-Gly. It is the Serine residue which becomes phosphorylated. The loss of phosphate by the ATP produces ADP. The procedure used was modified from that described in Jiang et al.

cAMP is required for a PKA catalyzed reaction to occur. If the cAMP is absent PKA is an inactive tetramer consisting of two regulatory and two catalytic subunits, R_2C_2. cAMP binds to the regulatory subunit which causes dissociation between the regulatory and catalytic subunits. By binding to the regulatory subunit, cAMP allows PKA to be activated. For this reason the concentration of cAMP is very important to the

rate at which PKA will phosphorylate a protein substrate such as Kemptide.

In this reaction Mg^{2+} is also necessary for the assay to occur. It coordinates with the ATP and ADP molecules and therefore must be included in the assay mix. Many enzymes are metal activated and loosely bind metal ions from the solution. They help the catalytic process of enzymes in three different ways. These include binding to substrates to orient them properly, mediating oxidation-reduction reactions, and by shielding negative charges that might inhibit the reaction (Voet et al. 1999).

Buffer and EDTA (ethlyenediamine tetraacetic acid) are also two very important components of the assay mixture. The buffer used in this experiment was 20 mM potassium phosphate. A buffer resists change of pH in a solution. This is necessary because the pH (in this case pH 7) in an enzymatic experiment if very important. If there is a change in the pH the enzymes involved in the assay many not be as active or active at all. EDTA is used to coordinate or bind free diavalent metals.

The IC system will separate the product (ADP), from the substrate (ATP) based upon their net ionic change. A conductivity detector is used to detect the presence and concentration of any anions. The ADP and ATP carry several negative charges, so they will easily be separated by the ion exchange column, and recognized by the conductivity detector. A computer system then integrates the electrical signal, allowing a chromatogram to be produced. The chromatogram is then interpreted to find the net ADP produced. The concentration of ADP will be determined by comparing the chromatogram with a previously established ADP standard curve. This work will lead to the determination of the kinetic parameters, which describe PKA's enzymatic activity. Experiments such as this are important in understanding how kinases work. A better understanding of these enzymes can have an important impact on future studies in human physiology.

To better understand and study the enzyme PKA it is important to find the K_m and V_{max} in regards to the enzyme with Kemptide. Once those values are determined and compared to that of prior experiments performed with similar methods, it will verify that the study of enzymes by ion chromatography a reasonable alternative in some cases. K_m also known as the Michaelis constant is the point in a reaction where the reaction velocity is half of the maximum. K_m is of importance because an enzyme with a small K_m value will have maximum catalytic efficiency at low substrate concentrations (Voet et al. 1999). K_m also is a measure of the degree of affinity the enzyme has for a substrate. A small K_m indicates tight binding of the substrate. V_{max} is the maximal velocity of the reaction, or the point in which the enzyme is saturated. At this point the reaction can no longer gain in velocity no matter how great the substrate concentration.

In order to study the enzyme it is important to find the velocity in which the enzyme will phosphorylate a protein. This is done by measuring the amount of the product ADP, because the amount of ADP will be equivalent to the amount of Kemptide which was phosphorylated in the reaction. Researchers using other techniques have determined the K_m and $V_{o\,max}$ (initial velocity) to be 5 μM and 16 μmol min^{-1} mg^{-1} respectively (Kemp and Pearson 1991). This is the concentration targeted for Kemptide to be used in this study.

EXPERIMENTAL PROCEDURE

There were many steps that became important in developing an assay procedure, so that the enzyme kinetics could be determined through the use of ion chromatography. The following five steps were used to determine this information.

1. An assay was developed in order to study the activity of the enzyme, PKA.
2. A concentration standard curve for ATP and ADP was developed to determine the amount of ADP produced.
3. A technique was found to stop the assay at certain time intervals in order to find the initial velocity in which PKA phosphorylates a substrate.
4. Each separate time point was then analyzed by ion chromatography in order to determine the amount of ADP produced during the assay.
5. By adjusting with these techniques it is possible to find conditions which allow the study of enzyme kinetics of PKA through the use of ion chromatography.

Assay Procedure

The procedure used in this experiment was adapted from Jiang et al. The concentrations of reagents were taken from their procedure; however, most other parts of the procedure were adjusted to better fit the experiment at hand.

Materials

The assay mixture had a final volume of 500 μL. The following ingredients and their final

concentrations were used in the mixture: Kemptide at 3.75 mM, EDTA at 25 mM, $MgCl_2$ at 25 mM, ATP at 2.45 mM, cAMP at 10 uM, PKA 25 units, and Potassium Phosphate buffer.

Three methods were used to stop the assay at a certain time including: phosphoric acid at 150 mM, trichloroacetic Acid at 25%, and running the assay through the ion chromatograph at different time points without a stop solution.

The Kemptide, ATP, cAMP, and PKA were all made into aliquots at their respective concentrations and volumes. These solutions were frozen in order keep the solutions from breaking down. The EDTA, $MgCl_2$, potassium phosphate, phosphoric acid, and trichloroacetic acid solutions were all made prior to running the assay. One liter volume of potassium phosphate buffer at pH 7 was prepared first so that all solutions could be diluted with the buffer.

The first step in producing the assay was unthawing the aliquots. After each was unthawed each separate ingredient was added to the mixture with the PKA enzyme being the last reagent added. The length of time the assay ran depended on the type of experiment that was occurring at that time.

RESULTS

The first experiment run was to see if the there was detection of ADP and ATP by the ion chromatograph post assay. One solution was run with PKA and the other without, to see if there was conclusive evidence of a change in ADP concentration. Standard curves were then made for ADP and ATP by testing known concentrations in the ion chromatograph. The height in arbitrary units for each separate concentration is graphed versus time (see Figure 1). This will give the ADP concentration curve needed to compare the ADP in the assay to the previously established curve to determine the amount of ADP produced in the reaction.

The second experiment was used to determine when the enzyme PKA goes to completion. A time frame of 0, 7, 37, 67, and 97 minutes was used. No stop solution was used to end the reaction; instead each sample had the same starting point and was then placed in the automatic sampler. The sampler injects its solution at 7 minutes and then runs for another 30 minutes to separate the ions on the column. The amount of time it takes to separate the column depends on the method selected using the Dionex software system on the computer. The PKA catalyzed reaction goes to completion at approximately 7 minutes (see Figure 2).

A third experiment used the stop solution TCA to end the reaction. The time intervals for this experiment were 0, 10, 15, 20, 25, and 30 minutes. Each separate assay was stopped by adding 50 µL of TCA at each respective time. The solutions were then placed in the ion chromatograph for testing. No conclusive results occurred from this experiment.

The quality of the ATP was questioned. Fresh ATP was ordered and the following assay was performed. The fourth experiment ran with only one 500 uL assay. Out of this reaction mixture 100 uL aliquots were removed and transferred to a 100 uL TCA solution. Once the enzyme was placed in the mixture vial the assay could begin. After 30 seconds 100 uL of reaction mixture was added to the first vial of TCA. After one minute the second 100 uL of reaction mixture was added. This occurred at 1 minute intervals all the way to 4 minutes. It is thought that the TCA reacted with ADP breaking it down. For this reason no ADP was detected.

The fifth experiment used no stop solution, but instead each assay was produced as the previous one was running and then injected separately immediately following the end of the chromatography of the previous assay. For example, the first assay was put into the automatic sampler and 7 minutes later it was injected. 7 minutes before the end of the chromatograph the next assay was completed. It was then placed into the automatic sampler for injection. This assay ran for a total of 14 minutes. The next 21 followed by 28 minutes, and a blank which had no enzyme.

In an effort to determine the K_m and V_{max} of the Kemptide towards PKA an experiment was run in which phosphoric acid was used to stop the enzymatic reaction. The results are displayed in Figure 3. The amount of product (ADP peak height) is plotted against time. This gives a linear result indicating that steady state conditions exist. The slope of this line is the initial velocity for that particular Kemptide concentration.

Figure 4 shows a common chromatogram of the assay mixture. In this case the reaction was run to completion at 7 minutes. The important points on this chromatogram are peaks 6 and 7, which are ADP and ATP respectively. ATP is more negatively charged than ADP, for this reason ADP will be elueted from the column faster than ATP. The stronger affinity of ATP to the column makes it tougher to be removed so it is shown at a later time than ADP. One important thing to note here is the product ADP peak occurs due to the fact that the reactant ATP is losing a phosphate within the reaction. For this reason the ATP peak is smaller than it would be if no reaction had taken place.

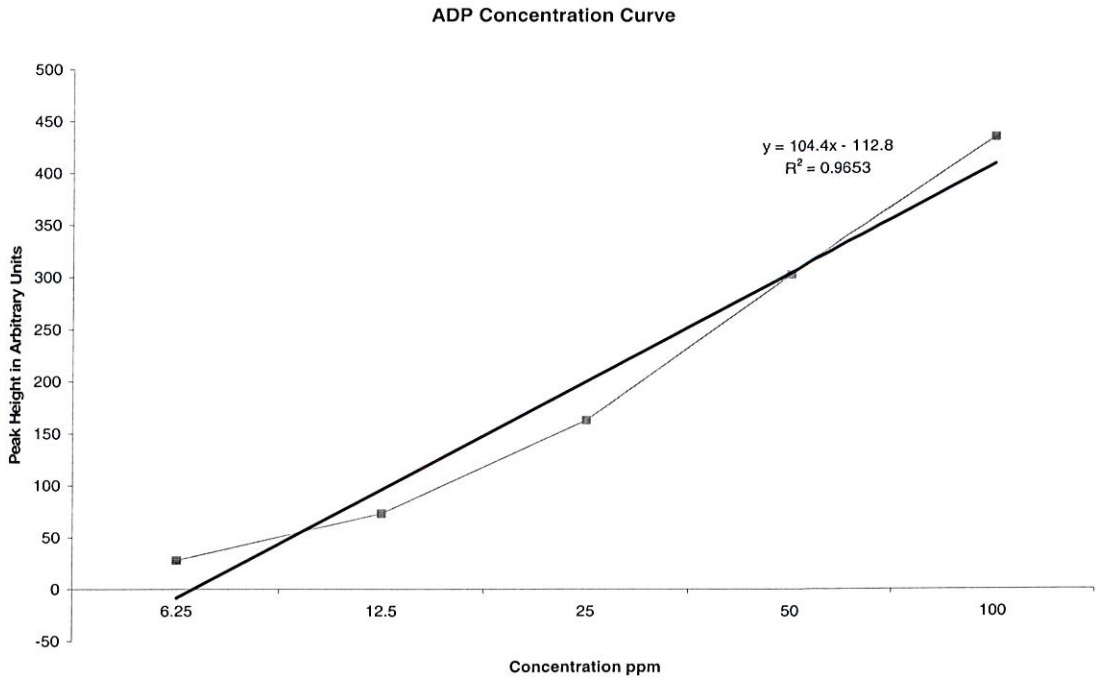

Figure 1. This graph shows the concentrations in parts per million (ppm) of ADP versus their chromatogram peak heights. This information serves as a standard curve, allowing the determination of how much ADP is produced in the PKA catalyzed reaction.

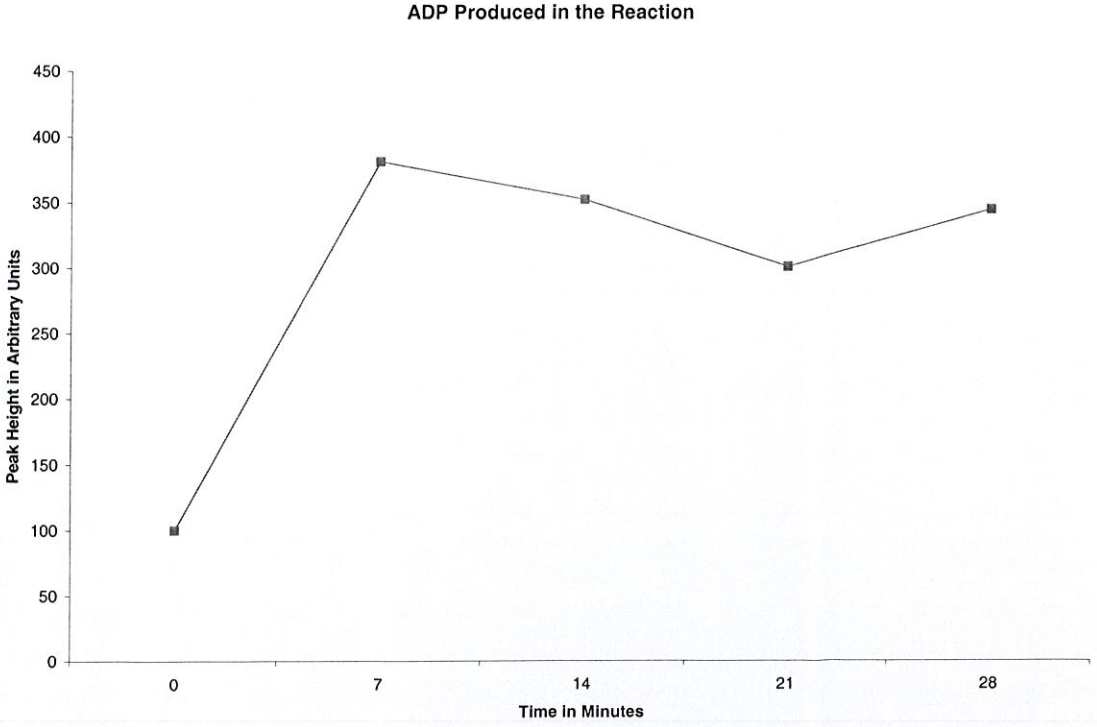

Figure 2. The amount of ADP produced over time. The time points are very large. After 7 minutes the production of ADP ceases and the concentration remains steady. This indicates that the PKA catalyzed reaction goes to completion in approximately 7 minutes. This graph shows the importance of studying PKA at a time of 7 minutes and before.

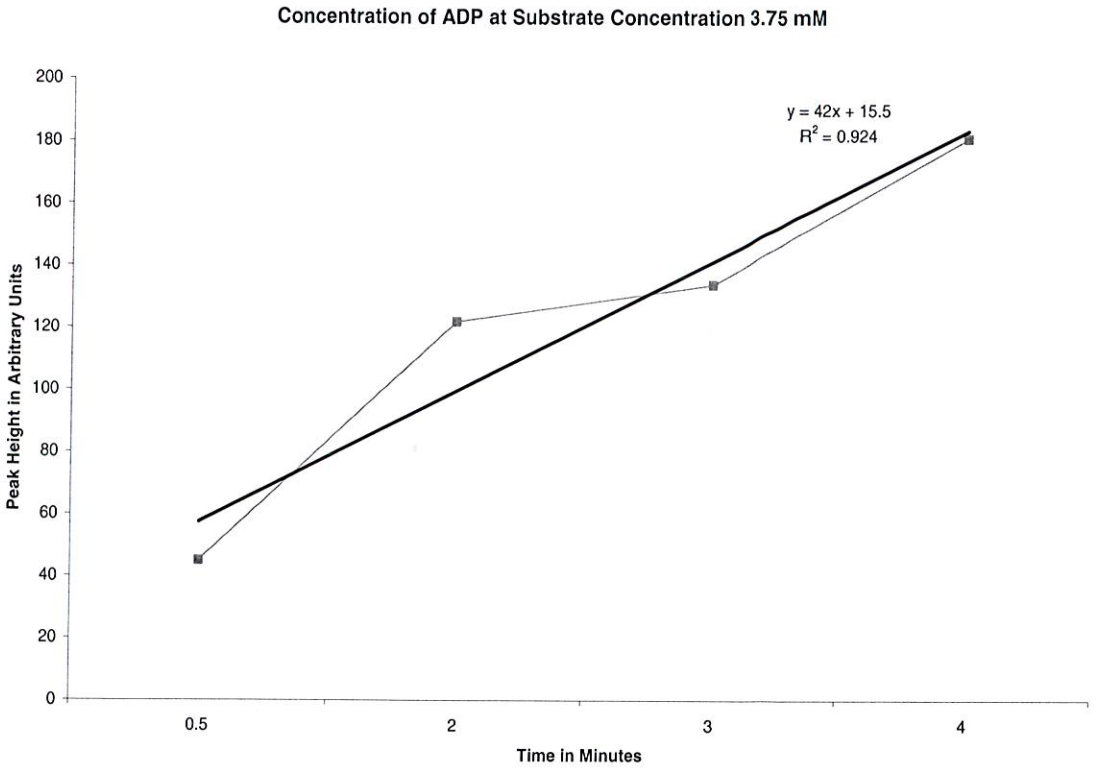

Figure 3. Measurement of enzyme activity with a Kemptide concentration of 3.75 mM. The data points were taken from time points ranging from 30 seconds to 4 minutes. Each time point was analyzed by ion chromatography to determine the amount of ADP produced.

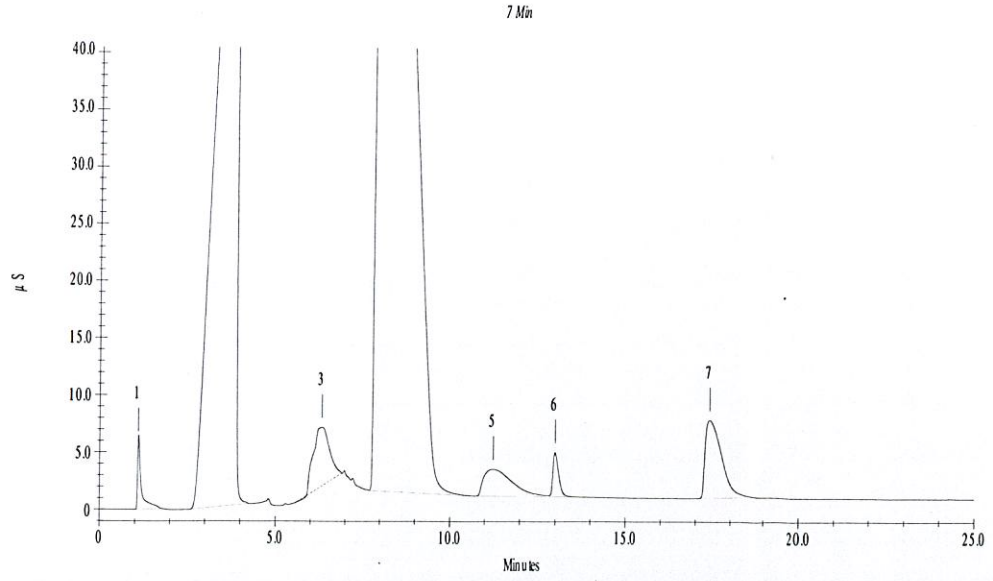

Figure 4. This figure shows a common chromatogram from the IC. Peaks numbered 6 and 7 are of ADP and ATP respectively. This chromatogram was conducted after completion of the assay at 7 minutes. Note the significant increase in ADP which would not be evident without the action of PKA.

DISCUSSION

This study shows that it is possible to measure the enzymatic activity of PKA using the synthetic substrate Kemptide. One of the first factors in recognizing these possibilities occurs with the results of the first experiment. ADP and ATP were both detectable by the system. A standard curve for ADP was made (see Figure 1). The r^2 is 0.97 which is very reasonable considering the conditions used. The assay originally had little or no ADP product when tested before the addition of the enzyme. When the PKA was added a significant increase in the product ADP, occurred. More evidence which shows the decline in the reactant ATP and increase in product ADP also is important in defining the capability of ion chromatography when studying enzymes.

Experimental results show that the enzymatic activity of PKA is completed in 7 minutes. This is an important point, because it allowed for a window in which to study the enzyme. Since the reaction was completed at 7 minutes it is important to study the enzyme between 0 and 7 minutes where the reaction rate is linear, so that the initial velocity can be determined.

The change in substrate concentration was also an important factor when studying PKA. With the addition of more substrate it was possible to see a larger portion of ADP being phosphorylated. This data helps formulate the K_m and V_{max} which are the two very important parameters used when studying and comparing the many different enzymes present in the living organisms. Research using other methods has found the K_m and V_{max} to be 5 μM and 16 μmol min^{-1} mg^{-1} respectively (Kemp, Pearson 1991). This was set as a target goal when considering the effectiveness of our technique. Finding the enzyme kinetics of PKA with the IC technique will require more experimentation with other Kemptide concentrations which are currently in progress.

Because of the rapid rate which the enzyme works, it was necessary to find a method to stop the reaction before it reached completion. For this reason, stop solutions are very important tools used when studying the enzyme. In the first experiments no stop solution was used. The reaction was ended when the assay mixture was injected on the IC column. This occurred 7 minutes after being placed in the automated sampler. This showed an obvious limitation when considering the fact that the reaction was at completion at about 7 minutes. This made stop solution a necessity. The first solution used was TCA. TCA will precipitate proteins, ending the reaction by precipitating the PKA. TCA didn't work as a stop solution for the IC because it apparently reacted with the ADP, causing the ADP to breakdown. No ADP was detected. The second stop solution used was phosphoric acid. Phosphoric acid works as a competitive inhibitor which ends the reaction. A competitive inhibitor competes with the enzyme by binding to the enzymes substrate binding site. In this case the phosphoric acid binds to the PKA not allowing the ATP to bind to the substrate. Phosphoric acid was found to be a good stop solution because not only did it end the reactions at the correct time, but it also did not interfere in the detection of ADP on the chromatograph.

Figure 4 is important because it shows a product, ADP. This product was not present before the enzyme was added to the assay. The ability to measure the concentration of product produced is the major goal of this study because with it brings the possibility to study the enzyme kinetics of PKA.

This study has shown some promising results in the study of PKA via the use of ion chromatography. It is possible to distinguish the amount of product produced in a PKA catalyzed reaction. This product ADP was easily distinguishable in the chromatograms. When using the synthetic substrate Kemptide, PKA will cause the reaction to go to completion within 7 minutes. This is of importance because it gives the relative time frame in which the assay must be studied. For this reason it is necessary to use a stop solution in order for the concentration of product to be determined at small time intervals. TCA was found ineffective for this study because it reacted with the ADP providing no results. Phosphoric acid however, was found to work well in stopping the reaction. The concentration of ADP could still be determined after adding this competitive inhibitor. The slope of Figure 3 provides the V_o. This is of great importance when developing the linear Lineweaver-Burk plot which will be used to find the K_m and V_{max} of the reactions. More research must be performed at different substrate concentrations in order for the Lineweaver-Burk plot to be complete. Once complete, these results can be compared with previous research to determine if ion chromatography is an effective technique in studying protein kinase A.

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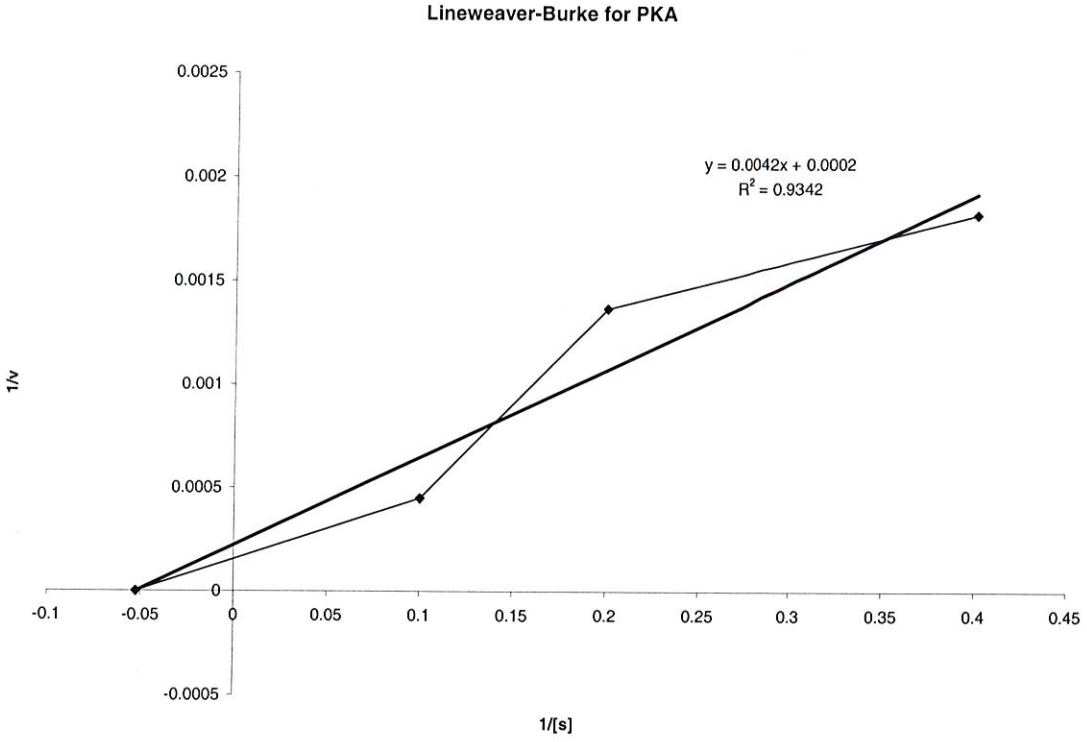
Results from Continued Research



Km = 19.0356 μM
Vmax = 450