Optimization of Exonuclease Digestion for Isolation of DNA Adducts

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Abstract:

With many researchers concentrating on a cure for cancer, there are some focusing on detecting genetic predispositions early enough for preventative measures. The Environmental Cancer Research Program (ERCP) is one such place. Specific research is being done on DNA adducts, however the bigger bulkier adducts are having low yields. This research focused on optimizing the conditions of the enzyme used to detach the DNA adducts from DNA. The data showed that extended incubation of the enzyme at 45°C does not diminish the enzymatic rate, and increasing the concentration of Mg$^{2+}$ in the buffer results in a lower enzymatic rate after extended incubation at 55°C.
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Abstract

With many researchers concentrating on a cure for cancer, there are some focusing on detecting genetic predispositions early enough for preventative measures. The Environmental Cancer Research Program (ERCP) is one such place. Specific research is being done on DNA adducts, however the bigger bulkier adducts are having low yields. This research focused on optimizing the conditions of the enzyme used to detach the DNA adducts from DNA. The data showed that that extended incubation of the enzyme at 45°C does not diminish the enzymatic rate, and increasing the concentration of Mg\(^{2+}\) in the buffer results in a lower enzymatic rate after extended incubation at 55°C.

DNA Adducts

What if a simple blood test could tell you if you were at risk for developing cancer? This is a feasible concept within the near future. DNA adducts are chemicals that attach to DNA and cause genetic damage. When they attach to DNA, some cause mistakes during replication, which could result in cancer. Assessment of the presence and concentration of DNA adducts has been used as biomarkers to determine exposure to chemical carcinogens (1, 2). Over a decade ago, the methodologies that could measure DNA adducts in blood and tissue were developed. The clinical applications and methodologies are still being perfected at such places as the Environmental Cancer Research Program (ECRP) of Northeastern University.

At present the process used in this lab to extract DNA adducts consists of five steps: obtaining DNA from a biological sample, hydrolyzing DNA to mononucleotides by nuclease P1 and snake venom phosphodiesterase (SVPDE), tagging the phosphate of
the adduct with benzoyl-histamine, conducting chromatography and mass spectrometry. The process has been refined, but with large adducts such as benzo[a]pyrene, hydrolysis to mononucleotides is less than ideal (only 10-20% yields). This research set out to optimize the enzymatic activity of phosphodiesterase I from *Crotalus adamanteus* venom (SVPDE). Primary research and literature reviews were used to find the ideal conditions for the reaction. The conditions focused upon were thermo stability of the enzyme over time and magnesium concentration in the buffer.

**Properties of Phosphodiesterase I from Crotalus adamanteus venom**

- Enzyme Commission #: 3.1.4.1
- Also known as calcium- and calmodulin-dependent phosphodiesterase
- Hydrolyzes 5’mononucleotides from 3’hydroxy-terminated ribo- and deoxyribo-oligonucleotides (The turnover is dependent upon the size of the DNA adduct and experimental conditions)
- MW 115,000 (3)
- Glycoprotein which binds concanavalin A
- Optimal pH for enzymatic activity: 9.8-10.4 (3)
- Inhibitors:
  - Reducing agents- glutathione, cysteine, ascorbic acid(4)
  - Complete inhibitors- 5 mM EDTA (5)
  - Partial inhibitors- ATP, ADP, AMP (5)
- Activators: Absolute requirement for Mg$^{2+}$ (3)
- Specificity: Nonspecific with respect to base or sugar of nucleotides. Nucleosides in the syn conformation are not recognized. (5)
- Reaction: ADP-ribosylated proteins are cleaved at the pyrophosphate linkages to generate a phosphoribosyl-AMP. (3)

**Assay and Reaction**

To assess the enzymatic activity under each of the various conditions, the rate of the reaction as SVPDE hydrolyzes p-nitrophenyl thymidine-5’-phosphate is measured as the solution progresses from nearly colorless to yellow, the yellow compound being nitrophenol. ADP-ribosylated proteins are cleaved at the pyrophosphate linkages to
generate a phosphoribosyl-AMP. Figure 1 illustrates the reaction. The enzymatic rate is determined by the change in absorbance at 400 nm over time. The methodology follows that set forth by Worthington labs (5). According to Worthington labs, one unit of SVPDE hydrolyzes one micromole of p-nitrophenyl thymidine-5’-phosphate per minute at pH 8.9 and 25°C, at the conditions set forth in their manual. The SVPDE was prepared in a stock of 0.1 unit/μl and the substrate was prepared at a concentration of 5 mM. It was stored in small aliquots of 30 μl at -80°C, as to prevent any repetitive freeze-thaw complications. The stock buffer was prepared as 0.11 M TrisHCl (pH 8.9) with 0.11 M NaCl and 15 mM MgCl₂. For each trial 90 μl buffer, 10 μl of substrate and 5 μl of enzyme were used.

Each trial was performed at room temperature (25°C) and each solution was allowed to reach temperature equilibrium before experimentation.

<table>
<thead>
<tr>
<th>Table A: Assay Timing (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:00 Clean cuvette with methanol</td>
</tr>
<tr>
<td>0:15 Clean cuvette with distilled water</td>
</tr>
<tr>
<td>0:30 Wipe dry with kimwipes</td>
</tr>
<tr>
<td>0:45 Blank spectrometer with air</td>
</tr>
<tr>
<td>0:50 Place cuvette in spectrometer</td>
</tr>
<tr>
<td>1:00 Add buffer and enzyme solution- allow to thermally stabilize</td>
</tr>
<tr>
<td>1:55 Add substrate and mix with pipette</td>
</tr>
<tr>
<td>2:00 Begin readings at 400 nm</td>
</tr>
</tbody>
</table>
illustrates the timing of the assay. Such precision was taken for cleaning cuvettes to eliminate false data from impurities on or within the cuvette that could mix with experimental solutions. For each experiment, readings were taken every fifteen seconds for five minutes.

**Testing the Thermo stability of SVPDE**

Previous research has tested the effect of various temperatures on enzymatic rate. Phillips found that linearity of rate of was best at 25°C, but that hydrolyzing was highest at 45°C (3). In essence, the rate remained constant for longer at lower temperatures than high, but the actual yield of mononucleotides hydrolyzed was higher at 45°C. Since the highest output is the focus of this research, the stability of the enzyme over time in a 45°C water bath was tested.

**Methods**

Assays were performed and compared of enzyme that had been incubated for four and twenty-four hours. A standard was performed sans incubation to establish a base rate before incubation for comparison. After incubation, the solutions were allowed to equilibrate to the room temperature of 25°C. The rates of each incubation were established using the standard assay with readings every fifteen seconds for five minutes. For each incubation trial 5 µl of enzyme and 90 µl of buffer or distilled water were combined in a closed cuvette covered with Parafilm. The alternation between buffer and distilled water was meant to test the stability of the buffer in the hot water baths. Buffer was added for the assays to ensure inclusion of all necessary reaction components. All
were incubated in a 45°C water bath, floating at the top of the bath. Two trials were performed of each of the following in one series: 4 hours in buffer, 4 hours in distilled water, 24 hours in buffer, and 24 hours in distilled water. On a separate day, two additional trials were performed of the 24-hour buffer condition.

**Results and Discussion**

The raw data was converted using the first reading as a zero point for each trial and recalculating each point thereafter. For raw data points that exceeded 3.0 AU on the spectrometer, plotting ceased as reliability decreases after that point and spectrometer is unable to take consist readings above 3.0 AU. The rates for each trial were calculated using the equation from Worthington labs (5). This equation is as follows:

\[
\text{Rate (units/mg/minute)} = \frac{\Delta A_{400/min}}{16 \times (\text{mg}_{\text{enzyme}}/\text{ml}_{\text{solution}})}
\]

*16 is the extinction coefficient for phosphodiesterase I as determined by Worthington labs (5)

Figure 2 shows each of the trials that were incubated in buffer, their relative spectrometer readings being plotted over time. The best-fit line is included with each extrapolated through the zero. All the best-fit lines were polynomial; however, the rate for each was calculated from the initial linear readings.
Figure 2: Relative absorbencies over time for buffer condition at 45 °C

*The black and red points are of the 24 hour trials, and the orange points are from the 4-hour trials. The black points were done on one day, and the red points were the trials run on a separate day. The letters denote different trials within the conditions. The trial (4 hr B) is believed to be an outlier. At the conclusion of the assay, the color was concentrated in the top of the cuvette, possibly due to insufficient mixing of substrate into buffer and enzyme.

Statistically an additional trial of four-hour incubation may be warranted, however it is a comparison point. Because the twenty-four hour trials show repeatability with the trials performed on the later date, additional four-hour incubation was not found to be necessary. The trials done on a separate day are believed to have higher consistency because of improved lab techniques.

In the following figure, the rates are shown, calculated using the equation previously listed. The averages for the twenty-four hour trials were calculated and the error bars are the average’s standard deviations. The actual rates for the twenty-four incubation trials A, B, C, and D are as follows: 0.633, 0.277, 0.229 and 0.221
units/mg/minute. Trial B of the four-hour incubation was not included in this chart, because it was previously deemed an outlier.

Figure 3: Reaction rates of Buffer incubated trials

The conclusions made from this experiment depend upon the assumptions made of the data. Assuming that trial 4-hour B is an outlier due to insufficient mixing, and trial 24-hour A is an outlier because its vast difference from the norm of Trials B, C, and D, then it can be concluded that there is no significant change in rate between four and twenty-four hours incubation at 45°C. However, if Trial A is not thought to be an outlier, then it could be concluded the incubation at 45°C increases the enzymatic rate with prolonged incubation. Both conclusions are to the benefit of ERCP. Prolonged incubation does not decrease the enzymatic activity of phosphodiesterase I, whether it remains the same or increases would need to be concluded from further research.
**Increased Thermo stability at 55°C with an increased [Mg$^{2+}$]**

Previous research has shown that not only is Mg$^{2+}$ a necessary cofactor, but it increases enzyme stability in purification procedures. It was first reported that ultrafiltration of SVPDE in collodium bags with no Mg$^{2+}$ yielded inactive protein. Phillips later rebutted that in the presence of 10 mM Mg$^{2+}$, activity is retained (6, 3). Phillips also found that increasing the [Mg$^{2+}$] above the recommended 15 mM does not increase the enzymatic activity (3). The metal binding site on SVPDE is believed to be the location of stabilization, and possibly responsible for activation of hydroxide which mediates catalysis. Using this knowledge, the experiment tested whether increasing the [Mg$^{2+}$] would result in a higher rate of enzymatic activity after four hours incubation at 55°C. This temperature was chosen, because above 45°C the enzyme begins to denature until 75°C where the enzyme is completely denatured. To ensure the results were not solely that of the change from ideal temperature, trials were performed at 45°C.

**Methods**

An enzyme sample was incubated at 55°C for four hours, after which time an assay was performed, taking readings every 15 seconds for five minutes. In all samples, there was 90 µl buffer and 5 µl SVPDE. As in the previous experiment, the sample was allowed to equilibrate to room temperature before the assay was performed. In the control’s buffer the [Mg$^{2+}$] was 15 mM, and in the experimental condition the [Mg$^{2+}$] in the buffer was increased to 35 mM. The same volumes and assay procedure were used from the previous experiment. The procedure previously described was used for the trials performed at 45°C. The only alteration was the dilution of the solution with 100 µl of
distilled water. This was done because previous attempts with the procedure yielded absorbencies above 3.0 AU after less than fifteen seconds. Because no conclusions could be made from this data, the dilution was performed. This was not necessary for the trials done at 55°C, the raw data was usable without dilution.

Results and Discussion

The same analysis was used for the raw data of this experiment as the last. Figure 4 shows the relative absorbencies of the trials over time for the incubations at 55°C.

The best-fit lines for each of the trials were polynomial, but the rates were again calculated from the initial linear data using the equation previously given. The rate of the control was 0.391 units/mg/minute. For experimental trials 1 and 2, the rates were
calculated to be 0.197 and 0.181 units/mg/minute respectively. This data shows that with 35 mM [Mg$^{2+}$] after fours of incubation at 55°C, there is a decrease in enzymatic activity.

Figure 5 shows the results from the trials run at 45°C. Although the data cannot be compared directly, since the trials performed at 45°C were diluted, the conclusions from each condition can be compared. For 45°C, increasing the [Mg$^{2+}$], results in an increase of enzymatic rate. This is opposite of the findings from the previous condition.

![Figure 5: Relative absorbencies over time of 55°C incubation](image)

The hypothesis that increasing the [Mg$^{2+}$] in the buffer at 55°C would result in an increase in enzymatic rate is not supported by these results. However, at 45°C the enzymatic rate appears to be higher with a [Mg$^{2+}$] of 35 mM, but additional trials would be necessary to support this finding.
Stability of SVPDE over time in storage (-80°C)

Although a specific experiment was not planned for this comparison, the use of two different batches of enzyme yielded comparable data. Worthington labs recommends storage of the enzyme at -80°C, however they do not state a shelf life of the enzyme under this condition. There is no mention of a change in enzymatic activity after prolonged storage or repeated freeze and thaw. Enzyme from both batches was used under the same conditions, yielding vastly different data. Two controls were initially used for [Mg$^{2+}$] experiment. Both trials had 90 µl buffer (15mM [Mg$^{2+}$]) and 5 µl SVPDE incubated for four-hours in a Parafilm sealed cuvette at 55°C. The old enzyme had been in storage for over 6 months, while the new enzyme had been in storage for two weeks. Figure 6 shows the relative data, analyzed using the same procedure as all previous experiments.

Figure 6: Comparison of enzymatic activity after various storage length
The rate in units/mg/minute for the old enzyme was determined to be 0.043 and that of the new to be 2.72. That is over sixty-three times greater in rate. Therefore, the conclusion could be made that extended storage at -80°C decreases the enzymatic rate. However, although this comparison shows a difference in enzymatic rate, the cause cannot be explicitly attributed to length of storage at -80°C; other factors such as different batches may be responsible for the difference.

**Conclusion**

For the research being performed in the ECRP labs involving bulky DNA adducts, the conditions found during this research concluded that there is no decrease in enzymatic activity over time with incubation at 45°C. In addition, the possible conclusion that 35 mM [Mg$^{2+}$] increases the enzymatic rate at 45°C could be tested to see if there is an increase in yield of mononucleotides hydrolyzed. Additional research would be necessary to test whether these improved conditions increase the yield of bulky DNA adducts. Other research that may build upon these experiments is whether enzyme aggregation plays a role in the low standard rate of the thermo stability experiment. Aggregates may have time to settle during incubation that were unable for the standard. Finally, the reason the two experiments could not be compared were due to the findings of the difference in rates between the two batches. Whether the difference is due to batch characteristics, lengthened storage or other factors would need to be determined in further research.
References