Catalase Assay: Extracts and Inhibitors

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Experimental Significance

Part I: Extracts

- The first part of the experiment was established to measure the catalase activity of various tissues and compare them across the board to determine consistent trends between different organisms.

Part II: Inhibitor

- In the second part of the experiment, we measured the effectiveness of varying concentrations of Triton X-100 in inhibiting catalase activity

Catalase

Catalase is an enzyme found in nearly all living organisms; it is responsible for catalyzing the breakdown of hydrogen peroxide into water and oxygen.

H_20_2 itself is a harmful byproduct of many metabolic processes, which makes the role of catalase all the more important in functioning organisms.

Two stages:

1. $H_2O_2 + Fe(III)\text{-}E \rightarrow H_2O + O{=}Fe(IV)\text{-}E(.+)$
2. $H_2O_2 + O{=}Fe(IV)\text{-}E(.+) \rightarrow H_2O + Fe(III)\text{-}E + O_2$

History of Catalase

1811: Louis Jacques Thenard upon discovering the presence of hydrogen peroxide suggested its breakdown is caused by an unnamed substance.

1900: Oscar Loew finally coined the term “catalase” after discovering its presence in many plants and animals.

1937-1938: James Sumner and Alexander Dounce crystallized beef liver catalase and procured its molecular weight.

1981: The 3D structure of catalase was established.

Catalase Properties

Primary Structure:

Amino acid polypeptide chain, one heme group, one NADH.

Secondary Structure:

Coiling and folding of the polypeptide chain.

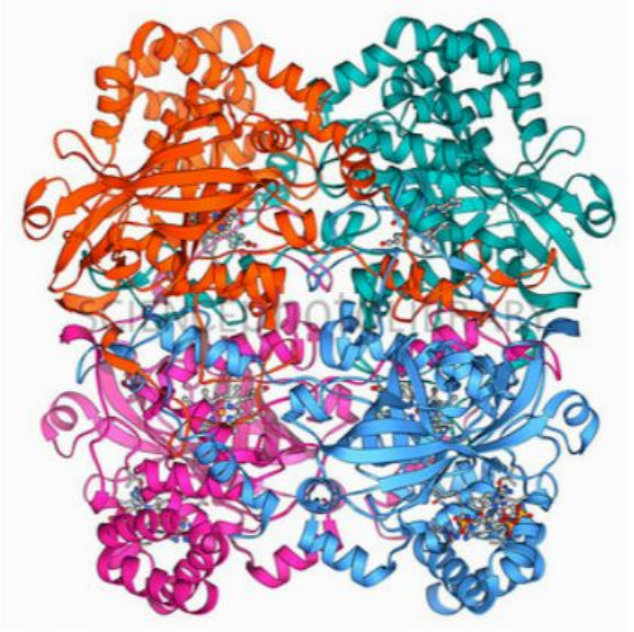
Alpha helix and beta pleated sheets (held together by H-bonds).

Tertiary Structure:

3-D structure of the polypeptide chain (catalase subunit).

Quaternary Structure:

Four subunits come together to form a functional catalase molecule.



Catalase Properties Continued

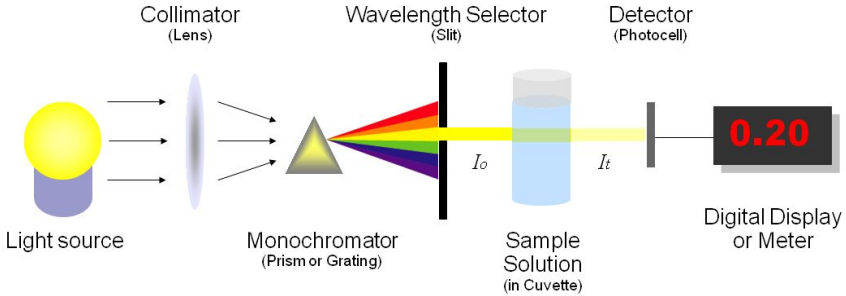
Catalase is a tetramer of four polypeptide chains composed of four heme groups which readily bind to hydrogen peroxide.

Each monomer of the catalase enzyme weighs about 57.5 kDA which means the entire molecule weighs close to 230 kDA.

In humans, the optimum condition for the catalase enzyme is at a pH of 7 and a temperature of 37 degrees Celsius. These properties vary among different organisms depending on their environments.

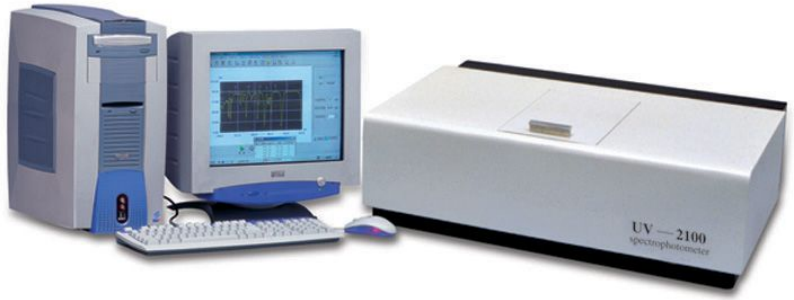
Spectrophotometer

- Lamp shines white light into a monochromator.
- Monochromator splits the light into colors.
- A specific wavelength of light is then shot at the sample and the detector behind the sample measures the transmittance (amount of light that passed through the sample) and the absorbance (amount of light the sample absorbs) of the sample at that wavelength.



Spec 20: (range generally 340 nm to 950 nm)

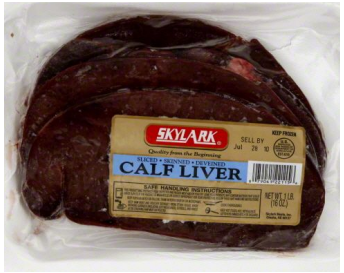
UV Vis: (range generally 200 nm to 700 nm)



Part I

Measuring Catalase Activity in Various Extracts

Extracts

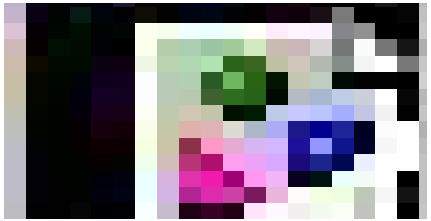


Calf Liver

Chicken Liver

Gala Apple

Clementine Orange



Lemon Leaf

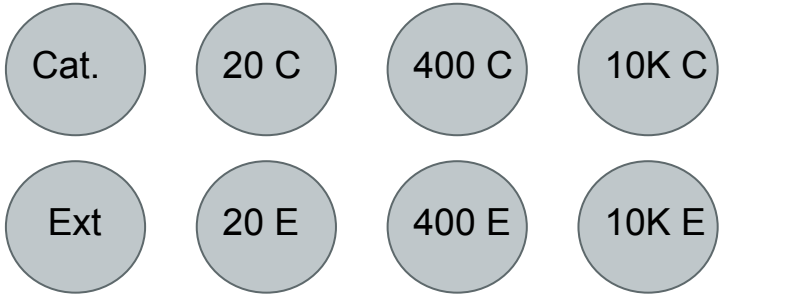
Assay Procedure (Day 1)

Extract Preparation:

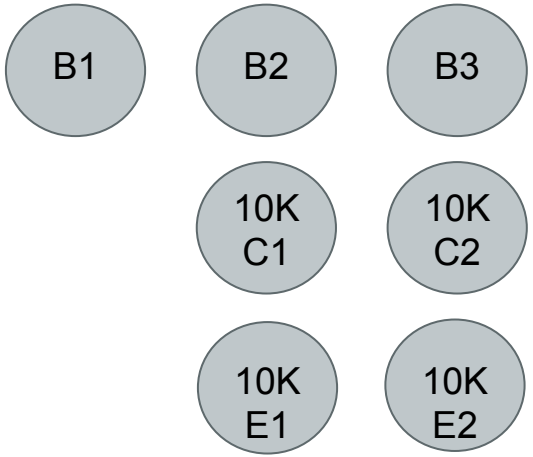
1. Mass out 1-2 grams of the sample.
2. Mash up the sample with a mortar and pestle as well as you can while adding 10 ml of PB.
3. Using the plastic pipettes, pipet 1.5 ml of the extract into 4 eppendorf tubes.
4. Put the 4 tubes into the centrifuge and spin them for 10 minutes at 14,000 RPM.
5. After the ten minutes elapse, pipet the supernatant out of the 4 eppendorf tubes into a clean glass test tube.
6. Obtain Bradford absorbance value.
7. Parafilm the glass tube and store it in the refrigerator for use on the next day.

Assay Procedure (Day 1) continued

1. Set up dilution tubes
   - Fill the 20 and 400 tubes with 380 λ of dH_2O each and the 10000 tubes with 480 λ of dH_2O each.



2. Set up 7 catalase reaction tubes.
   - B's should contain 225 λ of dH_2O each.
   - Rest should contain 219 λ of dH_2O each.

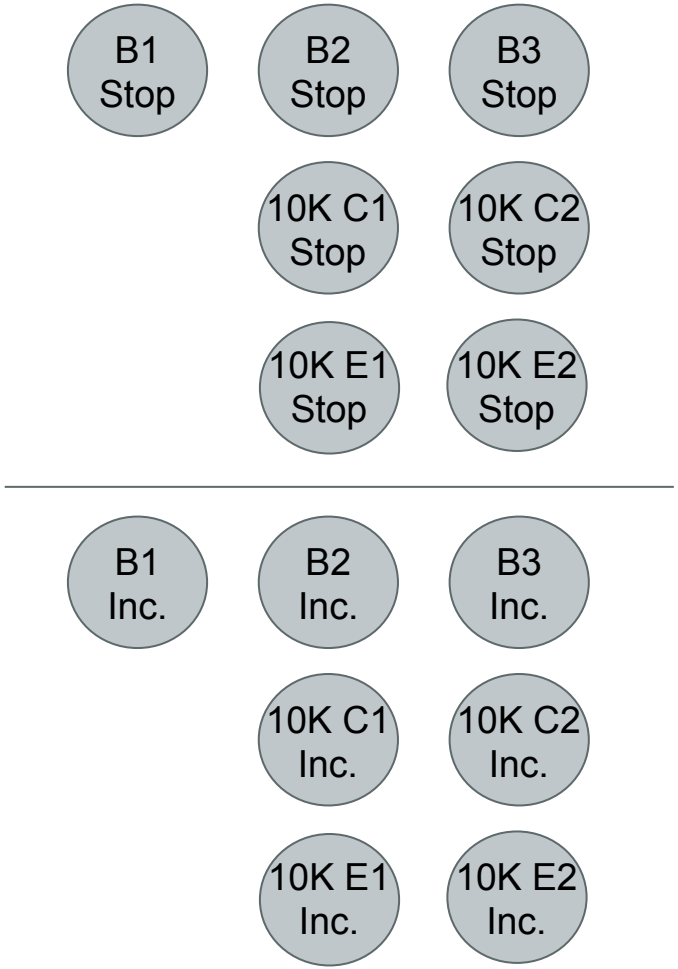


Assay Procedure (Day 1) continued

3. Set up 7 STOP eppendorf tubes
    - They should have 891 λ of dH_2O each and 9 λ of NaN_3 each.

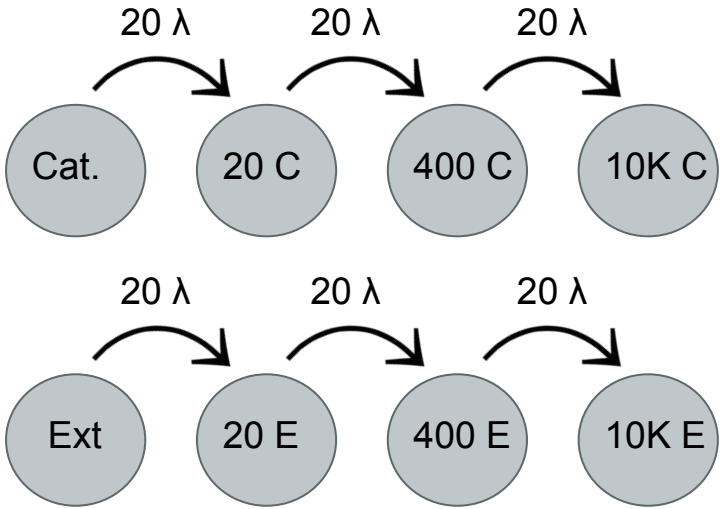
4. Set up 7 incubation tubes
    - Just label these tubes for now, they will be filled on the second day.

5. Fill one tube with 980 λ dH_2O and label it H_2O_2. Fill a second tube with 1000 λ of dH_2O and label it “balance”.

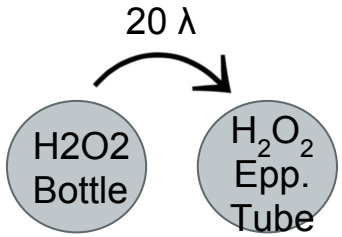


Assay Procedure (Day 2)

1. One group member should prepare the dilutions. Pipet 20 λ of catalase into the 20C dilution tube and 20 λ of the extract supernatant into the 20E dilution tube. Mix and bump. Then pipet 20 λ from those tubes to their respective 400 tubes. Mix and bump. Repeat with the 10000 tubes.

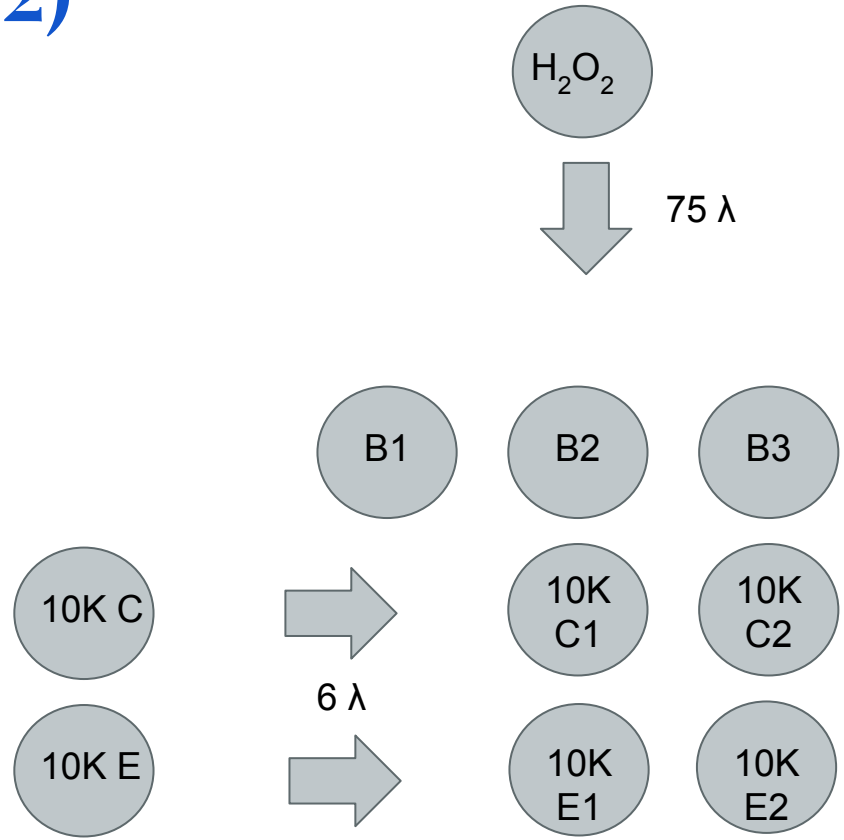


2. While dilutions are being made, have another group member pipet 20 λ of H_2O_2 into the H_2O_2 tube. Mix and bump the tube against the balance tube. Use the 1000 λ of dH_2O in the balance tube to blank the UV Vis three times using a glass cuvette, and measure the A_{240} of the H_2O_2 dilution.



Assay Procedure (Day 2) Continued

3. Transfer 75 λ of H_2O_2 from the H_2O_2 tube into each of the catalase reaction tubes. Transfer 6 λ of liquid from the 10000C tube into the 10KC catalase reaction tubes and do the same for the “E” tubes. Let the reaction tubes run for 4 minutes.



Assay Procedure (Day 2) Continued

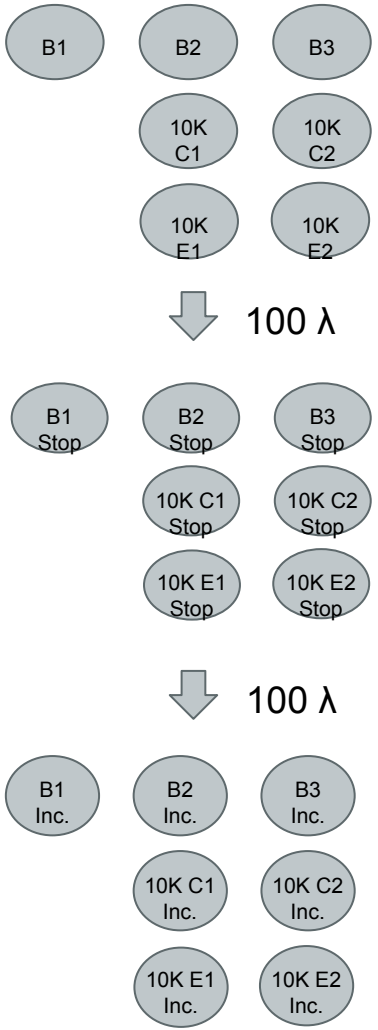
4. Transfer 100 λ from each of the reaction tubes into their respective STOP tubes.

5. Measure out roughly 10 ml of non-activated color reagent and pipet 10 λ of HRP into it. Mix and pipet 1 ml of the mixture into each of the incubation tubes.

6. Transfer 100 λ from each of the STOP tubes to their respective incubation tubes. Let the reaction run for 15 minutes.

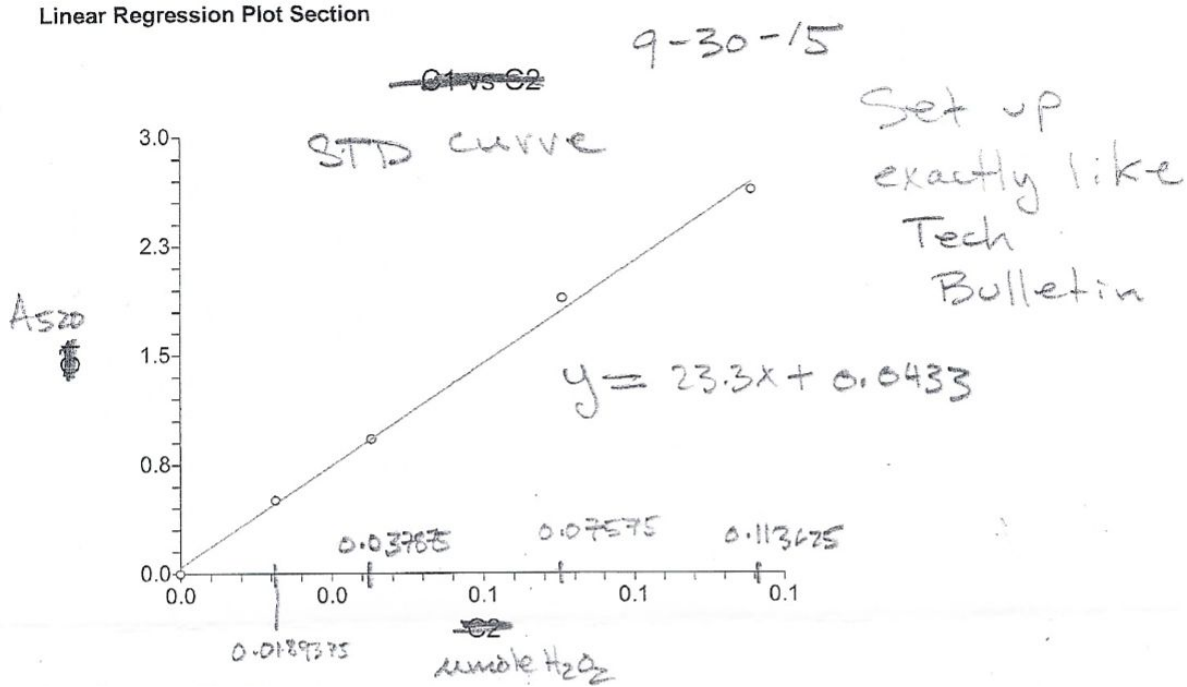
7. While the colorimetric reaction is running, transfer roughly 1 ml of solution from the incubation tubes into plastic cuvettes. Use unused color reagent as a blank.

8. Measure the A_{520} of each of the solutions on the UV Vis blanked against activated color reagent.



How to Find Activity

- Convert absorbances into micromoles of H_2O_2.
  - H_2O_2 Standard Curve



How to Find Activity Continued

- Perform these steps for both pure catalase and crude extract:
  - Find delta micromoles of H_2O_2 subtracting pure catalase or crude extract values from blank values.
  - Calculate activity using this equation:

$$\frac{(\Delta micromoles\ H2O2)(d1)(d2)}{(V)(T)} \quad \text{-->} \quad \frac{(\Delta micromoles\ H2O2)(10000)(100)}{(0.3)(4)}$$

= Activity (micromoles H_2O_2)/((ml)(min))

How to Find Specific Activity

- **Protein Quantitation (using Bradford)**
  - Using different concentrations of bovine serum albumin (BSA) in water and Bradford reagent
    - Dye creates a complex with the protein
    - Can measure absorbance at 596 nm.

  - High End: $y = 0.0076x + 0.5217$
  - Low End: $y = 1.9388x + 0.106$
- Use equations to determine catalase concentration.

How to Find Specific Activity Continued

- Activity divided by protein concentration in mg/ml
  - Pure Catalase: Given
  - Crude Extract: Bradford equations
    - Chicken Liver and Calf Liver
      - $y = 0.0076x + 0.5217$
    - Apple, Orange, and Lemon Leaf
      - $y = 1.9388x + 0.106$

Results

Extract	Average Volume (mL)	Total Protein (mg)	Average Activity (units)	Total Activity (units*mL)	Specific Activity (units/mg/ml)	Total Activity/ Total Protein (units*mL/mg)	Total Activity/ Wet Mass Tissue (units*mL/g)
Chicken Liver	5.5	3.889	6020.83	33114.57	8516.03	8514.93	27047.02
Calf Liver	5.5	3.184	1694.45	9319.48	2927.02	2926.97	7237.49
Apple	5.5	1.329	892.24	4907.32	3686.94	3692.49	3630.57
Orange	5.5	1.2	592.03	3256.17	2715.73	2713.48	2368.1
Lemon Leaf *	5.5	2.442	31.17	171.44	70.2	70.2	168.08

* Based on only one trial with a possibly unreliable protein concentration value.

Conclusions

- Total activity per gram of tissue:
  - Chicken Liver — Most metabolically active.
  - Calf Liver
  - Apple
  - Orange — Vitamin C - antioxidant
  - Lemon Leaf — Photosynthesis?
- Specific activity

Part II

Effects of Triton X-100 Inhibitor on Catalase

Purpose

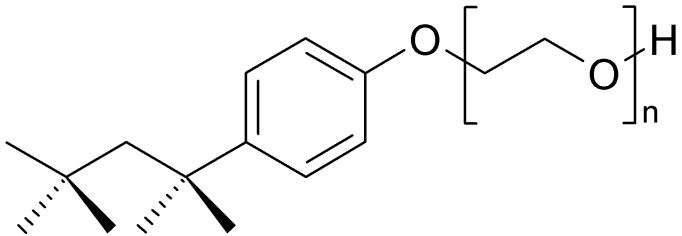
The purpose of part II of our catalase lab was to analyze the effect of the Triton X-100 inhibitor on the ability of catalase to break down H_2O_2.

History of Triton X-100

- Triton X-100 was originally a registered trademark of Rohm & Haas Co.
- It was then purchased by Union Carbide and then acquired by Dow Chemical Company

Triton X-100 Properties

- Noncompetitive inhibitor
- Mild detergent
- Because of the viscosity of Triton X, in order to prepare the stock solution, mass out a small amount of Triton X (0.028 g is how much we used), and fill up tube to 1 ml with dH_2O.
    - From this, we get the mass percent of the stock solution (2.8%), and we can use this to create working solutions of specific concentrations



Assay Procedure (Day 1)

- Set up tubes
- Pretty much the same as the Catalase assay, but with a few modifications:
  - No longer have dilution tubes for extract.
  - Replace extract experimental tubes with inhibitor experimental tubes.
  - Add specific concentrations of Triton X-100 to the different inhibitor reaction tubes.
    - 0.1%, 0.3%, 0.5%, 0.7%, 0.9%

Assay Procedure (Day 1) continued

- Calculate % mass:
    - (0.028/1) x 100% = 2.8%
  - Use this stock solution to make your inhibitor reaction tubes of specific % masses:
    - (x)(2.8) = (0.1)(300 λ)
      - x = 10.7 λ
- Adding Triton X-100 to inhibitor reaction tubes
  - Add the calculated amount of Triton X-100
  - Volume of Triton X-100 + Volume of dH_2O = 219 λ
    - Subtract volume of Triton X-100 from the original 219 λ dH_2O, and add the resulting volume of dH_2O to the reaction tube.

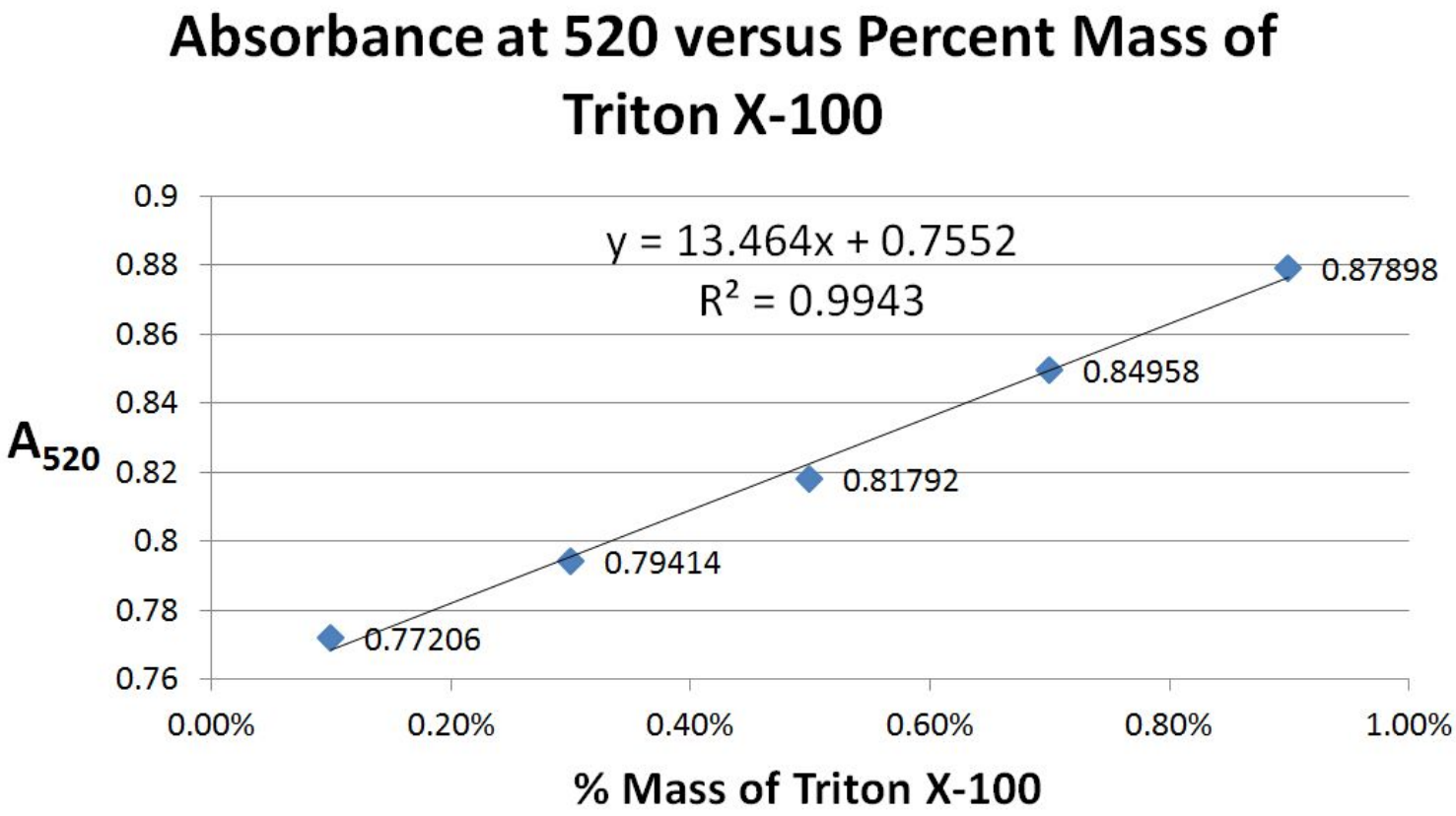
Assay Procedure (Day 2)

- Carry out the same procedure as the catalase assay with the few modifications indicated on the previous slides.
- We did two % masses for the first two trials and one % mass for the third trial.
- Extra measures taken to ensure relatively reliable results:
  - We conducted all the trials during the same week so that the concentration of catalase would remain constant.
  - The absorbances of H_2O_2 varied slightly for each of the three trials, but we tried to keep them as constant as possible and managed to keep the absorbances at around 0.66.

Results

Percent Mass of Triton X-100	Absorbance (520)
0.10%	0.77206
0.30%	0.79414
0.50%	0.81792
0.70%	0.84958
0.90%	0.87898

Results

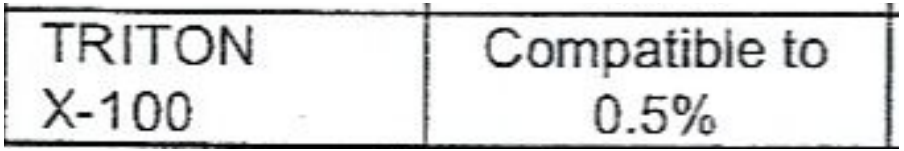

Absorbance at 520 versus Percent Mass of Triton X-100
y = 13.464x + 0.7552
R² = 0.9943
0.87898
0.84958
0.81792
0.79414
0.77206
A520
0.9
0.88
0.86
0.84
0.82
0.8
0.78
0.76
0.00%
0.20%
0.40%
0.60%
0.80%
1.00%
% Mass of Triton X-100

Conclusions

- Upward trend in absorbances
  - More H_2O_2 is present due to inhibition of catalase.
  - Triton X-100 does in fact inhibit catalase.
- Data does not completely match published results
  - Our Triton X-100 tubes' absorbances were not usually the same as our Blank tubes' absorbances.
  - Variation could be due to sources of error.

Effect of Substances on Colorimetric and UV assays

Substance	Colorimetric Assay	UV Assay	Expected Concentration in Undiluted Sample
Ascorbic acid	20% inhibition at 20 µM	Compatible up to 100 µM	29 µM in RBC; 77 µM in plasma
Albumin, bovine	Compatible at 50 mg/ml	Compatible at 1.5 mg/ml	35–50 mg/ml in blood
Sodium citrate	Compatible at 20 mM	Compatible at 5 mM	10–14 mM in blood
Tripotassium EDTA	Compatible at 4 mM	Compatible at 1 mM	3.4 mM in blood
Hemoglobin	Compatible at 0.8 mg/ml	Compatible at 1 mg/ml	120–180 mg/ml in blood
Heparin	Compatible at 14 units/ml	Compatible at 20 units/ml	1 unit/ml in blood
Glucose	Compatible to 5 mM	Compatible to 50 mM	17–44 mM in blood
TRITON X-100	Compatible to 0.5%	Compatible to 0.02%	Not normally present



Sources of Error

- Contamination
  - Scalpel
  - Tip of pipet
- Not reacting for exactly 4 minutes
- Low-end Bradford curve
- Time pressure
  - Forgetting to mix and bump
  - Forgetting to add H2O2
- Pipetting!

Acknowledgements

- We'd like to thank:
  - Dr. Pete for teaching us all the Chemistry we know, helping us plan out our procedures and make sense of our results, preparing the catalase and color reagent every day, making a very low-end Bradford curve for us to use, and providing some of his own lab equipment.
  - BASIS Chandler for providing us with facilities and equipment.
  - Ms. Terrell for assisting in the lab.

References

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