JOURNAL OF
EMERGING INVESTIGATORS

Article

How ethanol concentration affects catalase catalysis of hydrogen peroxide

Chen-Chih Liu[1], Michael Edgar[1]
[1]Milton Academy, Milton, Massachussetts

SUMMARY

Catalase is a critical enzyme in the human body because it is capable of converting potentially dangerous hydrogen peroxide into water and oxygen. We sought to determine whether ethanol affects its activity as alcohol consumption has been often linked to hepatitis occuring in the liver, where catalase level is especially high, and ethanol is known to be capable of denaturing proteins. We tested different concentrations of ethanol and found that higher concentrations reduced the activity of catalase. We infer that this is due to ethanol's ability to replace intramolecular hydrogen bonds within catalase with intermolecular hydrogen bonds between its hydroxyl group and catalase. We also conjecture that catalase catalyzes ethanol oxidation, which consumes hydrogen peroxide and thus decreases the amount of remaining hydrogen peroxide that undergoes conversion by catalase. This work has important implications on the negative effects of ethanol on metabolism, in which catalase plays an important role, and protein function more broadly.

INTRODUCTION

Catalase is an enzyme that catalyzes the reaction and conversion of hydrogen peroxide into water and oxygen, as in the reaction $2H_2O_2 \rightarrow 2H_2O + O_2$ (1). When it is active, catalase has a certain 3D structure that contains a channel through which hydrogen peroxide can diffuse, and this channel contains an iron heme group (1). In the first step of the reaction, hydrogen peroxide oxidizes the heme group from Fe(III) to Fe(IV) (1). At the second step, the second hydrogen peroxide molecule reduces the enzyme back to Fe(III), releasing water and oxygen (2). Catalase is a critical antioxidant enzyme because excessive oxygen can become converted into hydrogen peroxide, which can evolve into hydroxyl radicals that can mutate DNA and cause disease (3). By converting hydrogen peroxide to water and oxygen, catalase mitigates these dangerous effects.

Alcohol consumption, which is often linked to diseases such as hepatitis, leads to alcohol-induced oxidative stress (4). In the body, metabolism of ethanol results in an increased level of nicotinamide adenine dinucleotide (NADH), an enzyme essential to carrying electrons during metabolism, that increases respiration rate and the intake of oxygen (4). The antioxidant catalase then steps in to combat the effect of excessive oxygen. Due to its structure containing a hydroxyl group with the chemical formula -OH (oxygen and hydrogen), ethanol can denature a protein such as catalase by forming intermolecular hydrogen bonds, or the bonding between H and electronegative atoms such as O or nitrogen (N), with the protein that replace the original intramolecular hydrogen bonds within the protein (5).

This study aimed to further investigate the relationship between ethanol and catalase by studying the effect of ethanol concentration on catalase activity. An *ex vivo* study done by Konkuk University showed that an increase in the ethanol consumption of rats decreases catalase activity in the liver, demonstrating the negative effect of alcohol on the antioxidant system (4). We sought to confirm this relationship by conducting an *in vitro* experiment adopting the disc flotation method as described by Choinski and Patterson (6). To measure catalase activity, we recorded the time it took for a catalase-soaked disc to float inside hydrogen peroxide and thus indirectly measured the amount of oxygen produced by the reaction of catalase with hydrogen peroxide (6). Based on previous studies as well as the general principle that ethanol at high concentrations denatures proteins, we predicted that high concentrations of ethanol would reduce catalase activity (7).

RESULTS

To determine the effect of ethanol concentration on catalase activity, we submerged paper discs soaked with potato catalase and varying ethanol concentrations of 0%, 3.96%, 7.92%, 11.88%, and 15.83% into 10 mL of hydrogen peroxide (**Figure 1**). Catalase activity was measured as the time elapsed before each paper disc rose to the surface of the hydrogen peroxide solution, as the conversion of hydrogen peroxide to oxygen by catalase allowed the paper disc to float.

To test the validity of our experimental setup, we generated a standard curve, measuring time-to-rise using increasing concentrations of potato catalase. A linear model of the standard curve exhibited a high R^2 value of 0.96 and illustrated as predicted that, as the catalase concentration increased by 1%, the time it took for the disc to rise decreased by 1.16 seconds (**Figure 2**). A proportional relationship between catalase concentration to time-to-rise demonstrated that time-to-rise could be inferred as a valid representation of catalase activity.

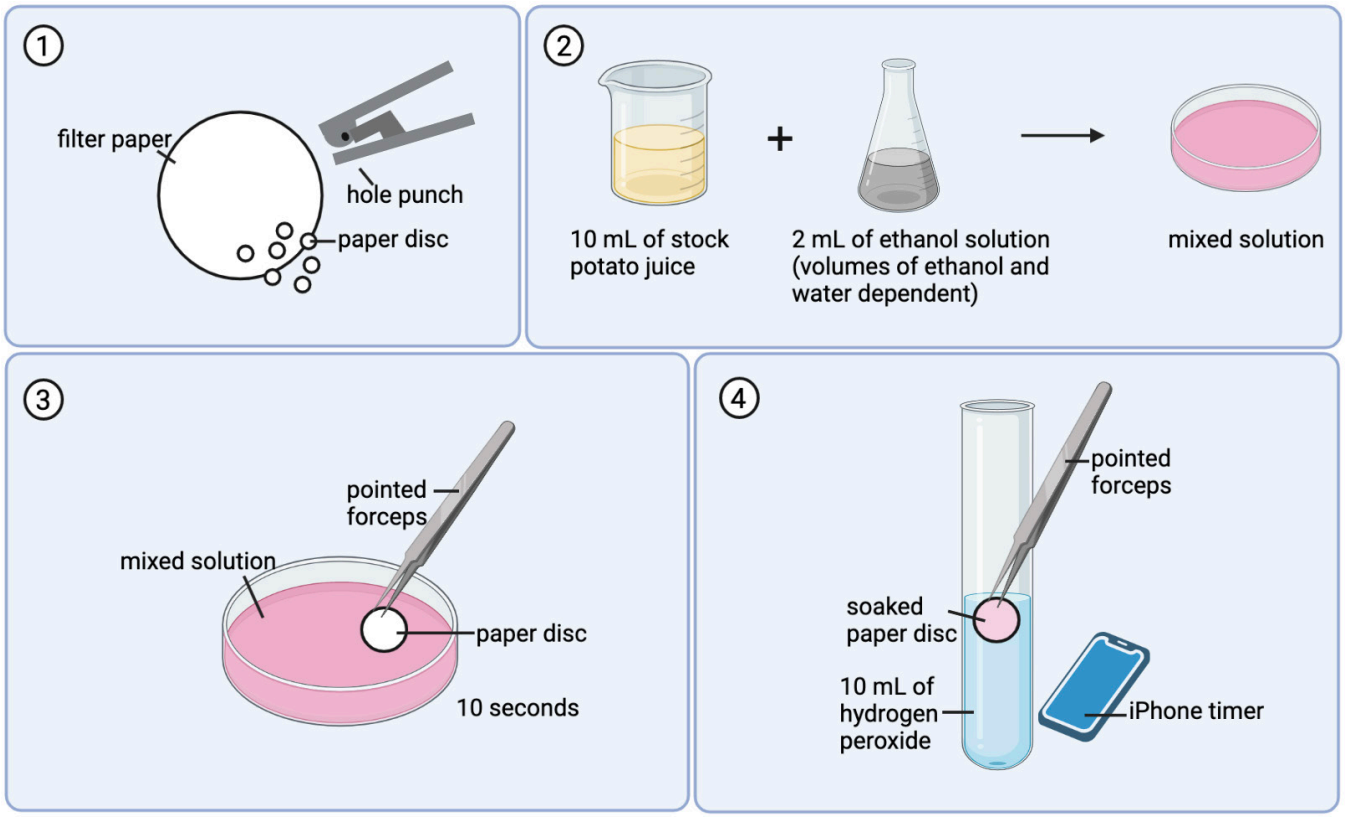

Figure 1. Overview of the experimental setup. Step **1)** Paper discs were made. Step **2)** Testing solution was made from potato juice and ethanol solution of varying concentrations (0%, 3.96%, 7.92%, 11.88%, 15.83%). Step **3)** Paper disc was soaked in the testing solution. Step **4)** The stopwatch was started as soon as the disc was dropped inside hydrogen peroxide then stopped when the disc rose back up to hit the surface. Created with BioRender.com.

As controls, discs soaked completely with water or ethanol, and no catalase, did not float when left observed for over five minutes. Discs in the negative control group, which were soaked in potato catalase and no (0%) ethanol, rose to the surface in 13 seconds. Every treatment group that tested discs soaked with ethanol exhibited a time-to-rise greater than 13 seconds (**Figure 3**), demonstrating that the presence of ethanol increased time-to-rise (0% ethanol = 13 seconds, 3.96% = 17 seconds, 7.92% = 18 seconds, 11.88% = 22 seconds, 15.83% = 27 seconds). We measured a rate of 0.839 seconds per every 1% increment of ethanol concentration, exhibiting a high R^2 value of 0.95 and indicating that a linear model closely fit our data (**Figure 3**). Increasing percent ethanol concentration linearly increased the time it took for a filter paper disc to rise to the surface of hydrogen peroxide. A one-way analysis of variance (ANOVA) performed on our dataset demonstrated significant variation among our tested conditions ($p < 0.01$). A post hoc Tukey's Test revealed that, except for the combination of 3.96% and 7.92%, mean time-to-rise significantly increased ($p < 0.0001$) for all concentrations (T-test: 0%+3.96% ($p = 0.0002$), T-test: 3.96%+11.88% ($p = 0.0001$), T-test: 11.88%+15.83% ($p = 4.7\times10^{-5}$)).

After comparing the 5 trials obtained from discs soaked in stock potato juice made from bottled and tap water, the differences were statistically insignificant, indicating that the type of water used was not a significant variable.

DISCUSSION

Our results showed that an increase in ethanol concentration significantly increased the time it took for a paper disc to float. The standard curve that we generated confirmed the validity of the disc flotation method by showing that higher catalase activity led to lower oxygen production and extended the time it took for the disc to rise. In addition, the inability of water-soaked or ethanol-soaked discs (without potato catalase) to float indicated that catalase was required for this buoyant effect. We therefore conclude that our results provide *in vitro* support for the study from Konkuk University (4).

Ethanol is a common agent of protein denaturation. In a protein's secondary structure, hydrogen bonding forms the peptide bond between the amino group and the carboxyl group on each side of an amino acid (8). In the tertiary structure, hydrogen bonding occurs between polar side chains, leading to specific folding that dictates the protein's shape (8). With a hydroxyl group, ethanol forms hydrogen bonds between its hydrogen atom H, which has a slightly positive charge, and an atom with a slightly negative charge from the protein (5).

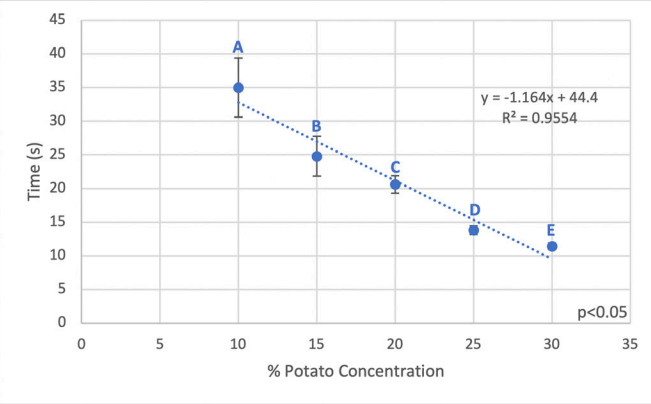

Figure 2. Effect of percent potato concentration on the time it took for a paper disc to rise to the surface of hydrogen peroxide. Potato concentration of 30% was made by blending 60 grams of potato with 200 mL of store-bought bottled water, and concentrations of 10%, 15%, and 20% were diluted from 25% stock made from blending 50 grams of potato with 200 mL of water. The total time-to-rise was measured after soaking each disc and then submerging it in hydrogen peroxide. The mean of 5 replicates is plotted for each of the conditions. The error bars indicate standard deviation. The dotted line indicates the linear trendline. Points with different letters are significantly different, determined by *p*-values less than 0.05 from post hoc Tukey's Test. Overall *p* denoted was calculated using ANOVA.

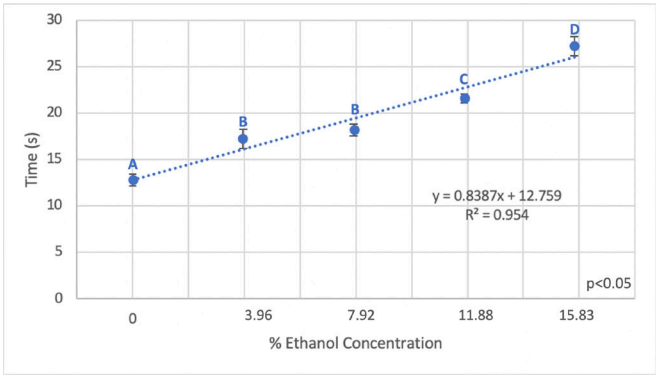

Figure 3. Effect of percent ethanol concentration on the time it took for a paper disc to rise to the surface of hydrogen peroxide. Ethanol concentrations of 0%, 3.96%, 7.92%, 11.88%, and 15.83% were tested by mixing 2 mL of differing ethanol solutions with 10 mL of potato juice. The total time-to-rise was measured after soaking each disc and then submerging it in hydrogen peroxide. The mean of 5 replicates is plotted for each of the conditions. The error bars indicate standard deviation. The dotted line indicates the linear trendline. Points with different letters are significantly different, determined by *p*-values less than 0.05 from post hoc Tukey's Test. Overall *p* denoted was calculated using ANOVA.

By replacing the intramolecular hydrogen bonds originally in the protein, these bonds denature the protein, or break the protein's structure, affecting its function. With respect to catalase, ethanol may result in the disappearance of the hydrogen peroxide channel or the iron heme group active site and therefore disable the ability of catalase to react with hydrogen peroxide. A higher ethanol concentration increases such denaturation, reducing catalase activity overall. An alternative interpretation could be the fact that hydrogen peroxide, as found in a study from the University of Pennsylvania, was consumed in the ethanol-oxidation reaction by catalase (9). Researchers found that catalase is an important enzyme in catalyzing ethanol oxidation and that 45% of hydrogen peroxide was utilized by the reaction (9). With a higher concentration of ethanol solution, more hydrogen peroxide is diverted to partake in ethanol oxidation, and thus less hydrogen peroxide is available for its conversion to water and oxygen. The decrease in oxygen therefore leads to an increase in the time for the disc to rise. It is possible that at a lower concentration such as the 3.96% or 7.92% groups, we did not witness a significant difference because the effect of ethanol was not large enough to either denature catalase or catalyze ethanol oxidation to result in a detectable difference.

The design of the experiment included a few sources of uncertainty. However, none of them prevented us from making a scientific conclusion. As potato juice was made by blending potatoes, it was possible that potato enzymes other than catalase, such as other types of peroxidases, may have contributed to the experimental results (10). Further testing could be made more conclusive by isolating catalase from potatoes. Another source of uncertainty arose in the exposure of potato juice to air. As the final testing was conducted in the span of around two hours using the same stock, reaction with oxygen in the air might have changed the composition of the potato juice in later trials with higher concentrations. To avoid this, a new potato solution could be made for every trial, however this could introduce another source of error with potential differences in catalase concentration using different potatoes. The dependent variable should then be changed to the percentage difference as compared to each control.

Regarding the broader implications of this study, our study explains the harms of alcohol use in impairing catalase's detoxifying role in metabolism. Our experiment suggests that ethanol decreases catalase activity and thus impedes the conversion of potentially dangerous hydrogen peroxide into water and oxygen. It may be interesting to explore potential treatments that could, for example, promote the activity of catalase in the setting of an alcohol overdose. We could also examine higher concentrations of ethanol to investigate if the linear relationship between catalase activity and ethanol concentration is preserved. We could test even higher ethanol concentrations, although the concentration of potato juice would need to be altered for the disc to successfully float (our preliminary testing demonstrated that higher ethanol concentrations combined with the present potato juice concentration prevented floating). Determining the maximum concentration of ethanol in which the enzyme can still function could also provide chemical insight. Overall, the results of this study show that an increase in ethanol concentration reduces the activity of catalase and the conversion of hydrogen peroxide to water and oxygen.

METHODS AND MATERIALS

Paper discs

Paper discs with diameters of 6.00 mm and thickness of 102 medium were collected by hole-punching the Lab Nerd filter paper (**Figure 1**).

Preparing control groups

Control groups, which helped establish that soaked discs without potato catalase did not float, consisted of Kirkland Signature Purified Drinking Water and Taiwan Sugar Corporation's 95% ethanol, respectively.

Disc flotation method

A solution was poured into a petri dish, then a paper disc was dipped inside for 10 seconds, using pointed forceps. This interval was controlled to ensure that every disc absorbed the same amount of solution and that an increased catalase activity, for instance, was not a result of increased absorption time. The disc was taken out of the solution, then the stopwatch was started as soon as the disc was dropped into 10 mL of hydrogen peroxide inside a test tube. This volume was controlled so that each disc would have the same amount of substrate (hydrogen peroxide) to react with and that the distance a disc would have to travel remained constant. The timing ended once the disc floated up and hit the surface (**Figure 1**).

Generating a standard curve

Stock potato juice with a concentration of 25% was made by blending 50 grams of peeled potato with 200 mL of tap water. Potato juice with 20%, 15%, and 10% concentrations were made by diluting 12 mL of the stock with 3 mL, 8 mL, and 18 mL of store-bought bottled water, respectively. Potato juice with a 30% concentration was made by blending 60 grams of peeled potato with 200 mL of store-bought bottled water. Concentrations were determined to ensure that time-to-rise was measurable. The disc flotation method was repeated 5 times for each of these 5 concentrations, changing test tubes in between each trial.

Preparing ethanol solutions

A control group 0% ethanol solution consisted of 2 mL of tap water and guaranteed that the experiment measured catalase activity specifically resulting from differing ethanol concentrations. A 23.75% ethanol solution was made by mixing 0.5 mL of 95% ethanol with 1.5 mL of water inside a syringe. The 47.5% ethanol solution was made by mixing 1 mL of ethanol with 1 mL of water. The 71.25% ethanol solution was made by mixing 1.5 mL of ethanol with 0.5 mL of water. The 95% ethanol solution was made with 2 mL of ethanol. Two mL was determined as the maximum amount of ethanol as higher volumes prevented discs from successfully reaching the surface.

Testing final solutions

Final solutions were made by mixing 10 mL of stock potato juice with ethanol solutions of varying concentrations - 0%, 23.75%, 47.5%, 71.25%, 95% - inside a conical tube, resulting in final ethanol concentrations of 0%, 3.96%, 7.92%, 11.88%, and 15.83%. The disc flotation method was repeated 5 times for each of these 5 concentrations, changing test tubes in between each trial.

Statistical testing

One-way ANOVA using Stat Plus was performed on the standard curve by selecting the entire dataset with 5 trials for each of the 5 potato concentrations - 10%, 15%, 20%, 25%, 30% (11). The ANOVA was performed and followed by a post hoc Tukey's Test using Stat Plus on the experimental dataset with 5 trials for each of the 5 ethanol concentrations - 0%, 3.96%, 7.92%, 11.88%, and 15.83% (11). The overall trend was considered significant when the p-value of ANOVA was less than 0.05, and treatment groups were considered significantly different from each other when the comparison's adjusted *p*-value from the post hoc test was less than 0.05. Microsoft Excel was employed to produce best-fit linear trends (12). ANOVA was also performed by selecting 5 trials of stock potato juice (0% ethanol) respectively made from tap and bottled water.

Received: July 2, 2021
Accepted: October 7, 2021
Published: November 15, 2021

REFERENCES

1. "How does catalase break down hydrogen peroxide?" *UCSB ScienceLine*, 26 Sept. 2002, scienceline.ucsb.edu/getkey.php?key=166.
2. Karakus, Yonca Yuzugullu. "Typical Catalases: Function and Structure." *IntechOpen*, 6 Feb. 2020, www.intechopen.com/books/glutathione-system-and-oxidative-stress-in-health-and-disease/typical-catalases-function-and-structure.
3. Goodsell, David. "Catalase," *PDB-101*, September 2004, pdb101.rcsb.org/motm/57.
4. Pyun, Chang-Won, *et al*. "Effect of Chronic Alcohol Consumption on Phosphatidylcholine Hydroperoxide Content of Rat Liver and Brain." *Tropical Journal of Pharmaceutical Research*, vol. 14, no. 7, July 2015, pp. 1225-30, http://dx.doi.org/10.4314/tjpr.v14i7.15.
5. Ophardt, Charles E. "Denaturation of Proteins." *Virtual Chembook*, Elmhurst College, 2003, chemistry.elmhurst.edu/vchembook/568denaturation.html.
6. Choinski, J. S., Jr., and J. W. Patterson. "A simple and inexpensive method for studying the kinetics of the enzyme catalase." *Journal of Biological Education*, vol. 27, no. 1, 1993, pp. 7-9, https://doi.org/10.1080/00219266.1993.9655294.
7. "Chemical Disinfectants." *Center for Disease Control and*

Prevention, 18 Sept. 2016, www.cdc.gov/infectioncontrol/guidelines/disinfection/disinfection-methods/chemical.html.
8. "Protein Structure." *Scitable by Nature Education*, Nature, 2014, www.nature.com/scitable/topicpage/protein-structure-14122136/.
9. Oshino, Nozomu, *et al*. "The Characteristics of the 'Peroxidatic' Reaction of Catalase in Ethanol Oxidation." *Biochem J.*, vol. 131, no. 3, Mar. 1973, pp. 555-63, https://doi:10.1042/bj1310555.
10. Whitson, Mary Kathryn. "Enzymes." Northern Kentucky University, www.nku.edu/~whitsonma/Bio150LSite/Lab%2011%20Enzymes/Bio150LEnzymes.html.
11. "StatPlus:mac." AnalystSoft, www.analystsoft.com/en/products/statplusmac/.
12. "Microsoft Excel." Microsoft, www.microsoft.com/en-us/microsoft-365/excel.