ISSN 2303-4521
Periodicals of Engineering and Natural Sciences *Original Research*
Vol. 10, No. 2, February 2022, pp.18-29

Testing temperature and pH stability of the catalase enzyme in the presence of inhibitors

Amna Kumašin[1], Lejla Mahmutović[2], Altijana Hromić-Jahjefendić[3]
[1] Genetics and Bioengineering Department, Faculty of Engineering and Natural Sciences, International University of Sarajevo, Bosnia and Herzegovina

ABSTRACT

The catalase enzyme is analyzed under different conditions in order to get a better understanding of its function, purpose and benefit to organisms. This enzyme resides in all living organisms that have exposure to oxygen. It uses hydrogen peroxide (H_2O_2) as its substrate, and is responsible for breaking down H_2O_2 into oxygen and water, therefore neutralizing oxidative stress in the cells. Maintaining the levels of oxidative stress is highly important because of the impact that the reactive oxidative species (ROS) have on the cells. ROS damage cells by targeting DNA and proteins leading to various complications and illnesses such as cancer, diabetes, neurodegenerative diseases and they even have an impact on the process of aging. To determine the activity of the catalase enzyme and test its stability, different temperatures and pH were employed, along with examining the catalase behavior under the presence of ascorbic acid as an inhibitor. Three samples were used for this experiment: animal sample, plant sample and microorganisms. The indicator of the reaction which aided in the process of determining whether catalase is performing its function was the formation of gas bubbles in the test tubes, and the quantity of that indicator assisted in drawing conclusions about the enzyme activity. This study revealed that the optimum conditions for catalase enzyme activity tend to be 37 °C at a pH of 7, especially present in liver and yeast samples. Ascorbic acid has proven to be a valuable inhibitor of the catalase enzyme. Extremely high or low temperature, along with highly acidic or basic environments tend to alter the enzyme activity disallowing it to perform its key role.

Keywords: Catalase enzyme, H_2O_2, Oxidative stress, Temperature, pH, Inhibitor, Ascorbic acid (Vitamin C).

Corresponding Author:

Dr. Altijana Hromić-Jahjefendić
Genetics and Bioengineering Department
International University of Sarajevo
Hrasnička cesta 15
E-mail: ahromic@ius.edu.ba

1. Introduction

1.1. Catalase (H_2O_2 oxidoreductase, EC 1.11.1.6; CAT)

The catalase enzyme has a variety of applications ranging from food industry to medical chemistry. Catalase is an enzyme characterized as a ubiquitous heme protein localized in the peroxisomal matrix, due to it being mostly present in the organelles known as peroxisomes, especially when considering mammalian cells. The molecule itself is a tetramer, containing four identical tetrahedral subunits. These subunits consist out of specific groups such as prosthetic group and protoheme [1]. Aside from heme, the core of the active site includes a tyrosine ligand placed on the proximal side of the molecule, along with a conserved histidine and aspartate on the distal side. The structure of the mammalian catalase incorporates a beta-barrel, enclosing four distinct domains. These domains include: alpha-helical domain, an anti-parallel eight – stranded beta-barrel domain, an amino – terminal arm and a wrapping domain [2]. The molecular mass of the enzyme is estimated to be approximately 220-270 kDa. Depending on whether the organism is prokaryotic or eukaryotic, catalase can be

© The Author 2022. This work is licensed under a Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/) that allows others to share and adapt the material for any purpose (even commercially), in any medium with an acknowledgement of the work's authorship and initial publication in this journal.

classified into three groups when examining its structure: monofunctional heme-containing catalases, heme-containing catalases- peroxidases, and manganese – containing catalases [3]. The main function of the enzyme is the catalytic role, meaning that the heme will be oxidized in the presence of hydrogen peroxide, which will yield the formation of an oxyferryl species. Another segment of catalase functionality is displayed by its peroxidative reactions, resulting in the removal of hydrogen peroxide, along with the particular substrate being oxidized in the process. The main reaction involves breaking down of hydrogen peroxide into oxygen and water [2, 3].

$$2\ H2O2 = O2 + 2\ H2O \qquad (1)$$

1.2. Oxidative stress

Oxidation is a normal process that happens to all living cells in nature. The reaction of oxidation is a result of oxygen – producing a change in the cells by exposing them to free radicals [4]. The ongoing effect of free radical exposure leads to the formation of oxidative stress. When molecules become damaged, meaning their structural form and metabolic functions are no longer in their healthy state, this usually implies a deficiency of an electron, or perhaps having a spare electron, and the ultimate result is that molecules become unstable [5]. This unstable form of a molecule is called a free radical. Free radicals are highly reactive chemicals that have a purpose to harm cells by targeting the DNA, leading up to the formation of mutations [6]. These radicals are constantly being created in the body through breading, exercising, performing food digestion, etc. The mechanism behind the functioning of free radicals is explained as a form of a chain reaction. The overall mechanism revolves around the fact that free radicals come into contact with neighboring molecules causing them to become free radicals themselves, due to the spare electron donation [4, 6].

1.2.1. Reactive oxygen species

The categorization of free radicals places them in the group of ROS (Reactive Oxygen Species). These are chemically reactive molecules containing oxygen. The most widely known examples of ROS would include: superoxide, hydrogen peroxide and hydroxyl radical [7]. When examining the biochemical pathway behind ROS molecules, it is evident that it begins with molecular oxygen which is being reduced, meaning it gains a spare electron, and as a result superoxide radical is formed. The superoxide radical can be converted to hydrogen peroxide, and the conversion of hydrogen peroxide is followed by the production of either a water molecule, or in other cases the formation of a hydroxyl radical [8]. The ROS molecules can be generated through two distinct pathways. The endogenous pathway includes scenarios that may happen inside of an organism, ranging from oxidative phosphorylation, respiratory bursts by the cells of the immune system, and various inflammation processes. On the other hand, the exogenous pathway is more concerned with the external environment which could pose threat to an organism. These examples include external toxins, air pollution, UV rays and radiation, stress, alcohol, cigarette smoke, and unhealthy diet [9]. The effects of ROS molecules are numerous. In lipid peroxidation, occurring in the plasma membrane of the cell, the hydroxyl radical may donate the extra electron causing the lipid bilayer to experience disbalance. The osmolarity disbalance will impact the cell, potentially inducing cell lysis. Reactive oxidative molecules can damage proteins and enzymes by reducing or oxidizing specific groups in the protein structure, leading to an impairment in the protein function [10]. The hydroxyl radical can cause various mutations of the DNA molecule. If this mutation were to be found in the germ cells, and is not repaired by a certain DNA repair mechanism, it could be passed on to future generations. The myelin sheath of the neurons when impacted by the ROS molecules results in a reduced action potential transmission which thereby decreases neuronal functionality [11]. When the reactive oxidative species attack mitochondria, they mark the start of the caspase cascade and the leaking of cytochrome C, which ultimately leads to the process of cell death known as apoptosis [8, 10, 11].

1.2.2. Hydrogen peroxide

Hydrogen peroxide is a chemical compound with the formula H_2O_2. It is a nonpolar, chiral molecule defined as an inorganic peroxide, containing two hydroxyl groups that are covalently bonded by oxygen-oxygen bonding. Hydrogen peroxide can be broken down to water and oxygen [12]. It is a colorless solution. Under the influence of light, it tends to decompose, so it is preferable to store it in dark containers. H_2O_2 occurs naturally in the air, however only in small amounts. The Agency of Toxic Substances and Diseases has reported that hydrogen peroxide is widely used in households at low concentration for various medical applications ranging from its use as an antiseptic to alternative medicine, and as clothes and hair bleach. In industries it is used as a component of rocket fuel, as bleach for textiles and paper and for producing foam rubber and organic chemicals [13]. Hydrogen peroxide can be very toxic if ingested, inhaled or if it comes in contact with the skin or eyes. Some of the effects of hydrogen peroxide exposure would include mild ocular irritation along with respiratory irritation [14]. The inhalation of vapors from concentrated solutions may result in severe pulmonary irritation. Ingestion of dilute solution of hydrogen peroxide can result in mild gastrointestinal irritation, gastric distension, and on rare occasions gastrointestinal erosions and embolism [15]. Skin contact with H_2O_2 causes irritation and temporary bleaching of the skin, however more concentrated solutions may cause severe skin burns with blisters [14, 15].

1.2.3. Antioxidants

Antioxidants are substances that are crucial in fighting free radicals and oxidative stress, since they have the ability to inhibit oxidation [16]. These molecules can donate an electron to a free radical, therefore neutralizing the free radical and terminating the chain reaction between the molecules. Antioxidants are present in fruits, vegetables, pulses, seeds, and some supplements [17]. For the proper functioning of the human organism, antioxidants are highly required. Essential antioxidants are the ones that need to be sourced from food, because the body does not make it on its own. Examples of essential antioxidants would include: vitamin E, vitamin C, beta-carotene and selenium [17, 18]. The variety of antioxidants allows for the reduction of free radicals in different tissues, since each of them functions in different cell types. Antioxidants are a valuable weapon for fighting reactive oxidative species, especially in cases where the body's natural defensive system, which are enzymes, cannot perform their key role [17, 18].

1.3. Factors influencing enzyme activity - Enzyme inhibition by Ascorbic acid / Vitamin C

Vitamin C is a water-soluble vitamin that is normally occurring in a variety of food, mostly comprising fruit and vegetables. Structurally, vitamin C is an acid, often referred to as ascorbic acid, due to one of the hydrogen atoms being loosely bound [19]. Vitamin C has two main functions. The primary function of vitamin C is to act as an antioxidant. As an antioxidant, vitamin C donates an electron and neutralizes free radicals, therefore helping the cells with inflammation processes and oxidative stress. The second function of vitamin C is to serve as a cofactor for enzymes such as collagen, carnitine, and neurotransmitters by aiding in their synthesis [20]. The biochemistry behind the structure and mechanism of functioning of vitamin C allows for the potential inhibiting nature of this molecule. Vitamin C breaks down and, therefore, generates hydrogen peroxide which is a reactive oxidative species that harms cells via accumulation, furthermore, forming oxidative stress which ultimately results in cancer. Cancer studies suggest that by taking vitamin C intravenously, instead of orally, the concentration of vitamin C will raise in the blood cells [21, 22]. This high concentration is the fundamental aspect in the fight against cancer cells, due to their poor defensive mechanism against ROS. Such experiments are crucial for the improvement of chemotherapy. When assessing the activity of vitamin C, it is evident that it has the ability to target cancer cells alone, leaving normal cells to continue with their functioning [22]. The catalase enzyme has a key role to fight off oxidative stress in living organisms, leading to the conclusion that cells with low catalase levels are more prone to cell death when exposed to high levels of vitamin C. The

combined knowledge of catalase levels in tumors and vitamin C levels in the body can generate future advanced models for cancer treatment therapy [23]. In reference to the catalase enzyme, vitamin C serves as a reversible inhibitor, meaning it does not permanently affect the enzyme. There are no alternations made to the shape of the enzyme, nor its active site. Once vitamin C leaves the catalase, the enzyme may continue with its primary function [24].

2. Material and Methods

Chemicals used for the experiment would include: 6M HCl (100mL stock solution), 2M NaCl (200 mL stock solution), 1M NaOH (100 mL stock solution), dH_2O (3L), 30 % H_2O_2 and ascorbic acid /vitamin C (50 mg powder form);

2.1. Preparation of sample

Three distinct sources of catalase were utilized for the experiment. The animal sample included chicken liver (300g). For the plant sample, potatoes were peeled and used. Microorganisms were represented in the form of beaker yeast (8g). Each of the samples was prepared by primarily cutting it into smaller pieces, so that they may fit into the test tubes.

2.2. Preparation of experiment – theoretical background

Preparation of experiment: The first part of the experiment was testing catalase enzyme activity at 37 °C. Primarily, each sample needed to be cut into smaller, but equal pieces. Three beaker flasks (1000 mL each), were used and filled up with dH_2O. The water bath was then set at 37 °C for 15 minutes in order for it to warm up, before proceeding with the experiment [25]. Nine clean test tubes, each labeled with its sample name and pH value, were filled with different solutions, in order to test the stability of the catalase when exposed to different pH (neutral, acidic and basic solutions were applied). NaCl was set at pH 7 (neutral), HCl at the pH of 2 (acidic), and NaOH at the pH of 12 (basic). 800 µL of each solution was placed into the test tubes. The tubes filled with the distinct solutions were placed into the water bath which has reached 37 °C and left to warm up for 10 minutes, before introducing the samples [25]. The samples were left for approximately 20 minutes in the water bath. The final step of this part of the experiment involved adding 530 µL of H_2O_2 into each tube, followed by an observation of the overall reaction rate and catalase activity expression indicated by the formation of bubbles [25]. The second part of the experiment included testing the catalase activity at the temperature of 4 °C. The sample preparation and the chemicals, along with their concentrations that were applied remained the same as in the first part of the experiment. Instead of using the water bath, in this part of the experiment, the refrigerator was used in order to obtain the temperature of 4 °C. The test tubes, each labeled with the name of the sample and distinct pH carrying only the solution was placed into the refrigerator for 10 minutes, before the pieces of samples were introduced into the test tubes [25]. After 10 minutes, the sample pieces were placed into the tubes and left for cooling for approximately 20 minutes, followed by their removal from the fridge and the observing of the catalase activity. The third phase of the experiment was examining catalase activity by applying high temperatures. The activity was observed at the temperature of 50 °C. The procedure follows the steps of the first part of the experiment (at 37 °C), only instead of the body temperature, the temperature of 50 °C was applied. The indicator of the reaction in each of the three phases came at the end of the experiment and it involved the creation of gas bubbles [25].

Effect of temperature: The activity of the enzyme catalase was examined at three distinct temperatures, presented in the range from 4-37-50 °C, with the goal to assess the potential enzyme active site alternations, and determine the optimum temperature conditions for its functioning [25].

Effect of pH: The activity of the enzyme catalase was observed over the pH range 2.00-7.00-12.00. The activity was assessed by the preparation and testing of different solutions, providing neutral (control), acidic and basic environments inside of test tubes filled with three different sample types, including animal, plant and

microorganisms. The solutions used were NaCl at the pH of 7 (neutral pH – control), HCl at the pH of 2 (acidic conditions), and NaOH set at the pH of 12 (alkaline conditions). The experiment was further carried out by the addition of 30% hydrogen peroxide, at three distinct temperatures (4-37-50 °C) [25].

Inhibition studies: Ascorbic acid (vitamin C) was tested as a potential inhibitor of the catalase enzyme. After determining the overall functioning of the enzyme at different temperatures and pH, the experiments were repeated with the addition of an extra step in the procedure, which involved placing vitamin C in powder form inside of the test tubes, and observing the reaction rate [25].

3. Results

The results were conducted by observing the gas bubble formation inside of the test tubes as a reaction indicator. It is noted that the higher the content of the gas bubbles, the greater the activity of the catalase enzyme.

3.1. Effects of temperature and pH on the animal catalase activity

Table 1. Effects of temperature and pH on the animal catalase activity

pH	4°C	37°C	50°C
2	No enzyme activity detected	No enzyme activity detected	No enzyme activity detected
7	High content of gas bubbles, low activation energy, fast reaction rate	Reaction rate plateau reached	High content of gas bubbles, low activation energy, fast reaction rate
12	Low content of gas bubbles, high activation energy, slow reaction rate	Low content of gas bubbles, high activation energy, slow reaction rate	Low content of gas bubbles, high activation energy, slow reaction rate

By observing Table 1. it can be concluded that pH values had a bigger influence on the enzyme activity than the temperatures. The animal catalase sample reached the reaction plateau at the temperature of 37°C and the pH value of 7. An alkaline environment (pH 12) has also proven to be quite favorable for the enzyme function, however a slow reaction rate with low gas bubble content was demonstrated. On the other hand, acidic conditions were detrimental for the catalase performance.

3.2. Effect of temperature and pH on plant catalase activity

Table 2. Effect of temperature and pH on plant catalase activity

pH	4°C	37°C	50°C
2	No enzyme activity detected	No enzyme activity detected	No enzyme activity detected
7	Low content of gas bubbles, high activation energy, slow reaction rate	Low content of gas bubbles, high activation energy, slow reaction rate	Low content of gas bubbles, high activation energy, slow reaction rate
12	No enzyme activity detected	High content of gas bubbles, low activation energy, fast reaction rate	Low content of gas bubbles, high activation energy, slow reaction rate

In Table 2. it is noted that an overall plateau of the reaction has not been achieved. Compared to the animal catalase sample, the plant catalase demonstrated higher sensitivity when exposed to changes in temperature. A temperature around 37 °C can be taken as a favorable temperature for the functioning of this enzyme, however only if combined with a proper pH value. The preferable pH value would range between 7 and 12, taking into

account that an alkaline environment has proven to be more significant regarding the efficiency of the plant catalase activity.

3.3. Effect of temperature and pH on the yeast catalase activity

Table 3. Effect of temperature and pH on the yeast catalase activity

pH	4°C	37°C	50°C
2	No enzyme activity detected	No enzyme activity detected	Low content of gas bubbles, high activation energy, slow reaction rate
7	High content of gas bubbles, low activation energy, fast reaction rate	Reaction rate plateau reached	High content of gas bubbles, low activation energy, fast reaction rate
12	Low content of gas bubbles, high activation energy, slow reaction rate	High content of gas bubbles, low activation energy, fast reaction rate	High content of gas bubbles, low activation energy, fast reaction rate

In Table 3. the yeast catalase activity was tested when exposed to different temperatures and pH values. The results indicate that the overall functioning of the yeast catalase is more efficient at a higher temperature (50 °C), however the influence of pH is crucial when evaluating the catalase activity. Low temperature (4 °C) has proven to cause an impaired enzyme function, and if combined with acidic conditions it will lead to a complete loss of yeast catalase function. On the other hand, an alkaline environment (pH 12) and a neutral environment (pH 7) are the optimum conditions for the functioning of the yeast catalase enzyme.

3.4. Effect of Inhibitor on Catalase Activity

Ascorbic acid / vitamin C was tested as a reversible, competitive inhibitor of the catalase enzyme. Since the results of inhibition are consistent for all samples (animal, plant and microorganisms) under all temperatures (4 °C, 37 °C, and 50 °C), and all pH values (2, 7 and 12), it is noted that the catalase enzyme activity will be inhibited when exposed to ascorbic acid. Tables 1, 2 and 3 list certain cases where the reaction did not occur, meaning that regardless of the substrate or inhibitor concentration, the catalase enzyme activity was not detected. In Figure 1., due to the consistency of the behavior of the catalase enzyme, the three samples are marked as the red line, and the ascorbic acid is marked with a green line depicting the effect of reversible inhibition. In order for ascorbic acid / vitamin C to act as a reversible inhibitor, the substrate concentration would need to be increased so that the inhibitor cannot attach to the active site of the enzyme, and therefore, catalase can proceed with its function of converting hydrogen peroxide into oxygen and water. The effects of inhibition are highly dependent on substrate concentration and enzyme concentration.

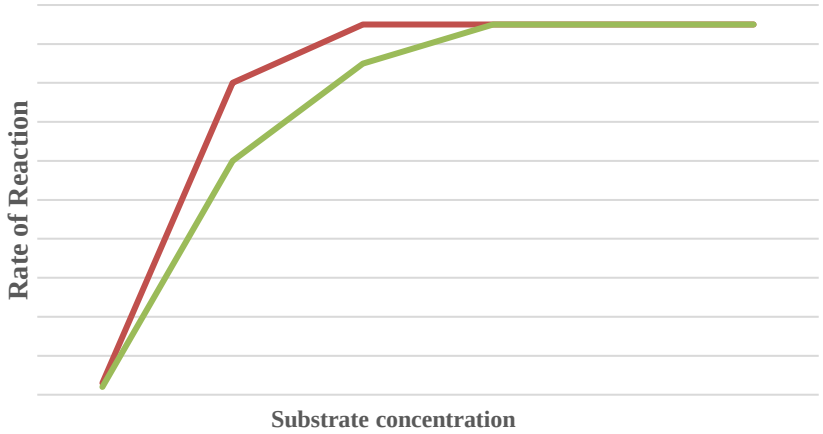

Figure 1. Effect of inhibitor on catalase activity

4. Discussion

An enzyme's structure is highly significant in terms of the function that it will perform. Animal, plant and yeast catalase differ slightly in the structure of the catalase molecule, which greatly affects the activity of that particular enzyme [26]. It is noted that animal cells produce more catalase enzyme compared to plant cells, hence the increased reaction rate that has been proven experimentally. Animal catalase resembles human catalase, therefore, making it clear as to why its optimum functioning is set at 37 ℃ and the neutral pH of 7 [27]. Plant catalase needs to be examined by taking into consideration the organism itself, meaning that plants are placed in the soil, and that location directs further functioning of enzymes [28]. Optimal conditions such as temperature and pH of the soil oscillate, compared to the laboratory environment where the experiment was conducted, furthermore, yielding results which included either a slow reaction rate, or no display of the reaction at all. Plant catalase tends to function at its optimum when exposed to a temperature of 20 ℃ to a maximum of 30 ℃, and at a pH of 7, taking into account that the plant catalase prefers an alkaline environment for its functioning as well (pH 12) [29]. On the other hand, yeast as organisms have a specific way of surviving and functioning. Yeast tend to divide when exposed to high temperatures, while when exposed to low temperatures they do not die, in fact they remain dormant [30]. These characteristics are a valuable insight into the metabolic functioning of the organism, making a direct correlation to the yeast enzyme kinetics. The yeast catalase has proven to be very flexible in terms of dealing and recovering from harsh conditions to which it was exposed, experimentally. The favorable conditions for the yeast catalase would include a pH ranging from 7-12, and the temperature of approximately 40 ℃. Extremely low temperatures tend to be detrimental for the yeast catalase performance [31].

Enzyme kinetics was used for a more complete comprehension of the inhibition properties of vitamin C on the catalase enzyme activity. The most important aspect of the enzyme kinetics includes the functioning of the region known as the active site. When the substrate binds to the active site an enzyme-substrate complex is formed [32]. When the complex reacts, some of the chemical bonds along with the substrate begin to weaken, causing the enzyme and substrate to merge. Eventually, the chemical reaction at the active site leads to the creation of a different molecule. This is referred to as a product. Once the reaction has been completed, the product is released from the active site and the enzyme returns to its original state and is free to react again with another potential set of substrates [33].

In the case of catalase enzyme, the substrate is hydrogen peroxide, and the products include oxygen and water. One of the most crucial concepts when discussing the ability of enzymes to speed up a certain chemical reaction is the activation energy. Basically, these biological catalysts physically bring the reactants together, increasing the efficiency of the reaction. When there is less energy required, meaning the activation energy is lowered, the reaction will occur at a faster rate [34]. Primarily, enzymes use binding energy. This means that free energy is released by the formation of a large number of weak interactions between a complementary enzyme and its substrate. Furthermore, free energy that is released by binding is therefore, called binding energy [35]. Ultimately, this means that the maximum binding energy is released when substrate is converted into its transition state, so the energy released by the interactions between the enzyme and the substrate is highly required for the lowering of the activation energy. The region of the enzymes known as the active site is the key property for the functioning of these molecules. If it were to be occupied by another molecule, the action of that enzyme would be restricted or inhibited. In competitive inhibition the velocity (v_{max}) is going to remain exactly the same, however the affinity value will experience changes [24].

The affinity (km value) of a substrate to the enzyme is lower, due to the fact that usually the enzyme is going to be with the inhibitor rather than the substrate [36]. Basically, the velocity will stay the same as long as there is enough substrate that can overpower the inhibitor for the active site of an enzyme. It is approximated that if in

an experiment, 50% of an inhibitor is added along with adding 50% of a substrate, the inhibitor will take over the active site of an enzyme. In order to overcome competitive inhibition, the concentration of a substrate needs to be increased [35, 37]. If there is enough substrate to outweigh the inhibitor, concentration-wise, the active site of an enzyme will then be occupied by the substrate. This is the case with ascorbic acid, serving as a competitive, reversible inhibitor of the catalase enzyme. The effect of vitamin C inhibition decreases over time, and the functioning of the catalase active site can be restored. This effect can be further highlighted if an increased substrate concentration is applied to the sample [37].

The substrate concentration is depicted by using the Michaelis-Menten curve that was plotted as the concentration of substrate or product vs time, respectfully [38]. As the substrate is consumed, the concentration of the substrate decreases over time, however, the product concentration will increase over time. By taking the slope of the graph, it is possible to obtain the rate of the reaction. The rate of the reaction is the change in concentration of substrate or product per unit time. As substrate concentration decreases, a negative sign should be placed when performing the calculations, while in the case of products a positive sign is used. The unit of the rate of a reaction will be concentration per unit time. This is known as the velocity of a reaction as the concentration is changing with respect to time [39].

$$V = - d(S)/dt = d(P)/dt. \quad (2)$$

Velocity of the reaction changes when the substrate concentration changes. An increase in the substrate concentration will lead to the velocity of the reaction being increased, until it reaches a plateau, which is called the maximum velocity (v_{max}) for a particular enzyme [40]. When the maximum velocity is divided by two (½ v_{max}), this will allow for the determining of the substrate concentration at which the process is occurring. Another value should be properly estimated when aiming to comprehend the catalytic efficiency of a certain enzyme. The Km value is defined as the substrate concentration at which ½ v_{max} is achieved. Therefore, the ratio of *kcat/Km* represents the efficiency of an enzyme [41]. More precisely, this ratio is called the specificity constant. This yields a conclusion stating that the higher the ratio is achieved, the more efficient a certain enzyme will be [33, 37, 41].

Many studies have been conducted on the topic of the antioxidant capacity of ascorbic acid / vitamin C and the catalase enzyme activity. One of those studies was focused on the antioxidant activity of vitamin C when consumed in different quantities [42]. For approximately one month, guinea pigs were feed with vitamin C in two concentrations. The low concentration was set at 33 mg of vitamin C/kg of diet, and the other one was a high concentration set at 13, 200 mg of vitamin C/kg of diet [42]. Animals in both groups showed a 50% inhibition rate of the enzyme activity by vitamin C. The results further suggested that there has been a decrease in the MDA (malondialdehyde), which serves as a marker for oxidative stress proving that vitamin C possesses an increased antioxidant characteristic, therefore, regulating oxidative damage in the cells [42]. Another study that was performed on this topic was tightly bound to the investigation of toxic effects of chemicals on tissues. More precisely, bromobenzene, which is a highly toxic substance that is known to cause liver dysfunction known as induced hepatocellular injury [43]. Since hepatocytes have various roles in the body, some of them being detoxification and protein synthesis, the catalase enzyme along with ascorbic acid and vitamin E were tested to measure their antioxidant ability against the toxin. On a molecular scale it was noted that bromobenzene - induced hepatocellular injury causes single – stranded breaks in the DNA when there is a build – up of ROS (Reactive Oxygen Species) in the cells, and this imbalance could be regulated by exposure to vitamin C, E and the catalase enzyme activity [43]. Just as in this study which focuses on animal catalase, the effect of ascorbic acid was put to test when the liver sample was exposed to hydrogen peroxide, creating results which would suggest that vitamin C does possess antioxidant characteristics, however the other two studies have taken a comprehensive approach and highlighted the impact of the combined effect of the catalase enzyme and ascorbic

acid (case-dependent), which would result in an impactful antioxidant capacity in regards to regulating oxidative damage in the cells. It has also been suggested that among all of these antioxidants, vitamin E has shown the best results due to it having the strongest inhibition of the oxidative damage [43].

The plant catalase activity has also been investigated in a variety of plants to ensure proper germination and plant growth. One study suggested that the germination of oil seeds can be more efficient if treated by ascorbic acid, pyridoxine, hydrogen peroxide and the catalase enzyme [44]. The production, proper germination and storage of oil seeds is of great importance to living beings due to their high fatty acid and energy content, along with being rich in vitamins D and E. The germination of oil seeds is quite a demanding process, because gas and water exchange inside of the seeds can be easily impaired by many mechanisms, especially the so called hydrophobic – film formation [44]. In this study the dual nature of ascorbic acid / vitamin C has been investigated. The oil seeds that were used for the experiment included sunflower, rape seed, and safflower. In the case of sunflower and rape seed, ascorbic acid seemed to have promoted protein preservation and lipid peroxidation, therefore, stimulating growth [44]. On the other hand, ascorbic acid had no effect on seed germination in safflower. The effect of ascorbic acid on various plant types has not yet been examined to full length, however the catalase activity will be inhibited by vitamin C in a great number of plant samples, as it is suggested in this study where ascorbic acid successfully inhibited the catalase enzyme in the potato sample, and in the case of safflower, due to a more extensive study approach, it was established that hydrogen peroxide increased the growth and aided the germination process by inhibitor oxidation [44].

A study concerning Saccharomyces cerevisiae, a form of brewer's yeast gave a beneficial insight once more on the antioxidant capacity of ascorbic acid and the overall catalase activity. Yeast often serve as a model for in vivo studies and are highly rich in vitamin B and proteins [45]. Brewer's yeast is known to promote glucose tolerance, therefore, assisting in the prevention of diabetes type 2, and boosts both the nervous and immune system [45]. For this particular study, paraquat was used to test the antioxidant ability of ascorbic acid. Paraquat is a toxic substance known as a plant killer chemical [45]. Different types of yeast were involved in the experiment, such as: wild-type strain EG103 (SOD) Saccharomyces cerevisiae, isogenic mutant strains deficient in cytosolic superoxide dismutase (sod1Δ), and mitochondrial superoxide dismutase (sod2Δ) [45]. Results of the Saccharomyces cerevisiae study suggested that samples that have been treated with ascorbic acid prior to paraquat exposure have displayed a higher survival rate compared to the ones that have not been treated with ascorbic acid and were only dependent on the catalase activity [45]. Utilizing various yeast forms, including baker's yeast that was used in this study have demonstrated a rapid reaction rate of the catalase activity when exposed to oxidative stress, creating more space for the investigation of the antioxidant capacity of ascorbic acid when the organism is attacked by various sources of toxic substances. An overall conclusion that can be drawn from the brewer's yeast study is that ascorbic acid / vitamin C tends to intervene in regulating the levels of the catalase enzyme inside of the cells, and their combined effect has a beneficial effect on the yeast samples survival rate [45].

5. Conclusion

The main role of the catalase enzyme is to regulate oxidative stress in the cells, however due to structural differences of the enzyme, the functioning of the active site may vary depending on the conditions and environment to which it is exposed. The enzyme activity was compared by using different samples (animal, plant and yeast), along with applying different temperatures and exposing it to distinct pH values to examine enzyme stability. Depending on the organism, the enzyme structure varies, therefore, the function of the catalase enzyme displayed different results regarding its activity. The optimum conditions for the functioning of the animal catalase have been estimated to be at 37 °C and the pH of 7. On the other hand, the plant catalase showed a much bigger sensitivity when it comes to pH, yielding a conclusion that the favorable conditions for the plant catalase activity would include an alkaline environment combined with the temperature between 20-37 °C.

Finally, the yeast catalase has proven to function at its best when exposed to either neutral or alkaline conditions combined with a temperature range between 37-50 °C. Inhibitor of the catalase were also put to verification to test the behavior of the enzyme. Ascorbic acid testing was conducted with the purpose to give an insight on reversible inhibition. Vitamin C proved its valuable role as inhibitor of catalase in regards to all organisms that were tested, therefore, creating space for future work to be done on the investigation of vitamin C inhibition as a novel medical therapy. The assessment of all of these parameters gave a more complete picture on the detailed functioning of the catalase enzyme, which is particularly important when looking upon the variety of roles that this enzyme undertakes, ranging from pasteurization of food products, immunomodulatory potential, oxidative stress regulation, tumor growth inhibition, enzymatic polymerization, biocorrosion prevention, etc.

References

[1] H. Kirkman and G. Gaetani, "Catalase: a tetrameric enzyme with four tightly bound molecules of NADPH," vol. 50, no. 8, pp. 114-120, 2021.

[2] M. M. Goyal and A. Basak, "Human catalase: looking for complete identity," *Protein and cell,* vol. 1, no. 10, pp. 888-897, 2010.

[3] M. Zamocky and F. Koller, "Understanding the structure and function of catalase: clues from molecular evolution and in vitro mutagenesis," *Progress in Biohysics and Molecular Biology,* vol. 72, no. 1, pp. 19-66, 1999.

[4] L. Michaelis and M. P. Schubert, "The theory of reversible two-step oxidation involving free radicals," *Chemical Reviews,* vol. 22, no. 3, pp. 437-470, 1938.

[5] E. T. Denisov and I. Khudyakov, "Mechanisms of action and reactivites of the free radicals of inhibition," *Chemical Reviews,* vol. 87, no. 6, pp. 1313-1357, 1987.

[6] V. Lobo, A. Patil, and N. Chandra, "Free radicals, antioxidants and functional foods: Impact on human health.," *Pharmacognosy Reviews* , vol. 4, no. (8), p. 118, 2010.

[7] Y. Li and M. Trush, "Defining ROS in Biology and Medicine. Reactive Oxygen," vol. 1, no. (1), 2016.

[8] D. J. Betteridge, "What is oxidative stress?," *Metabolism,* vol. 49, no. 2, pp. 3-8, 2000.

[9] J. M. Dos Santos, S. Tewari, and R. H. Mendes, "The role of oxidative stress in the development of diabetes mellitus and its complications," 2019.

[10] J. Goff, "Mineral absorption mechanisms, mineral interactions that affect acid-base and antioxidant status and diet considerations to improve mineral status," *Journal of Dairy Science* , vol. 101, no. 4, pp. 2763-2813, 2018.

[11] B. P. Tseng, T. Suarez, K. Tran, and C. L. Limoli, "Functional consequences of radiation-induced oxidative stress in cultured neural stem cells and the brain exposed to charged particle irradiation.," *Antioxidants & redox signaling,* vol. 20, no. 9, pp. 1410-1422, 2014.

[12] A. Reed, "A Statistical Manual for Chemists, 2nd ed.," *Clinical Chemistry,* vol. 18, no. 5, pp. 494-494, 1972.

[13] J. M. Campos-Martin, G. Blanco, and J. L. Fierro, "Hydrogen peroxide synthesis: an outlook beyond the anthraquinone process.," *Angewandte Chemie International Edition,* vol. 45, no. 42, pp. 6962-6984, 2006.

[14] B. E. Watt, A. T. Proudfoot, and J. A. Vale, "Hydrogen peroxide poisoning," *Toxicological reviews,* vol. 23, no. 1, pp. 51-57, 2004.

[15] S. J. Cina, J. C. Downs, and S. E. Conradi, "Hydrogen peroxide: a source of lethal oxygen embolism. Case report and review of the literature.," *The American journal of forensic medicine and pathology, ,* vol. 15, no. 1, pp. 44-50, 1994.

[16] I. S. Young and J. V. Woodside, "Antioxidants in health and disease.," *Journal of clinical pathology,* vol. 54, no. 3, pp. 176-186, 2001.

[17] C. Rice-Evans and N. J. Miller, "Antioxidants–the case for fruit and vegetables in the diet," *British food journal,* 1995.

[18] B. Ames, M. Shigenaga, and T. Hagen, "Oxidants, antioxidants, and the degenerative diseases of aging," *Proceedings Of The National Academy Of Sciences,* vol. 90, no. 17, pp. 7915-7922, 1993.

[19] M. B. Davies, J. Austin, and D. A. Patridge, Vitamin C: its chemistry and biochemistry. royal society of chemistry., London: The Royal Society of Chemistry, 1991.

[20] M. Traber and J. Stevens, "Vitamins C and E: Beneficial effects from a mechanistic perspective. Free Radical Biology And Medicine," *Free Radical Biology And Medicine,* vol. 51, no. 5, pp. 50-62, 2011.

[21] A. R. Ness, D. Chee, and P. Elliott, "Vitamin C and blood pressure–an overview," *Journal of human hypertension,* vol. 11, no. 6, pp. 343-350, 1997.

[22] E. Cameron and L. C. Pauling, Cancer and vitamin C: a discussion of the nature, causes, prevention, and treatment of cancer with special reference to the value of vitamin C, Corvallis, Oregon: Linus Pauling Institute of Science and Medicine, 1979.

[23] E. Pawlowska, J. Szczepanska, and J. Blasiak, "Pro- and Antioxidant Effects of Vitamin C in Cancer in correspondence to Its Dietary and Pharmacological Concentrations," *Oxidative Medicine And Cellular Longevity,* vol. 1, no. 1, pp. 1-18, 2019.

[24] A. G. Marangoni, Enzyme kinetics: a modern approach, John Wiley & Sons., 2003.

[25] E. Blinderman, "Catalase - Biology LibreTexts," Mercer County Community College, 1 August 2021. [Online]. Available: https://bio.libretexts.org/@go/page/24158.

[26] A. M. Vetrano, D. E. Heck, T. M. Mariano, V. Mishin, D. L. Laskin and J. D. Laskin, "Characterization of the oxidase activity in mammalian catalase.," *Journal of Biological Chemistry,* vol. 280, no. 42, pp. 35372-35381, 2005.

[27] H. Aebi, " Catalase. In Methods of enzymatic analysis," *Academic press,* vol. III, no. 12, pp. 673-684, 1974.

[28] H. Mitsuda and K. Yasumatsu, "Studies on plant catalase.," *Journal of the Agricultural Chemical Society of Japan,* vol. 19, no. 3, pp. 208-213, 1955.

[29] H. Mitsuda and A. Nakazawa, "Comparative Researches in the Stabilities of Plant Catalase and Animal Catalase," *Bulletin of the Institute for Chemical Research, Kyoto University,* vol. 32, no. 1, pp. 19-24, 1954.

[30] T. C. M. Seah and J. G. Kaplan, "Purification and properties of the catalase of bakers' yeast," *Journal of Biological Chemistry*, vol. 248, no. 8, pp. 2889-2893, 1973.

[31] C. L. Brown, "A Study of Yeast Catalase.," *ACTA CHEMICA SCANDINAVICA*, vol. 7, no. 2, pp. 435-440., 1953.

[32] A. Cornish-Bowden, Fundamentals of enzyme kinetics., John Wiley & Sons, 2013.

[33] G. WILKINSON, " Statistical estimations in enzyme kinetics," *Biochemical Journal*, vol. 80, no. 2, pp. 324-332., 1961.

[34] A. Cornish-Bowden, Principles of enzyme kinetics, Elsevier., 2014.

[35] P. F. Cook and W. W. Cleland, Enzyme kinetics and mechanism, Garland Science., 2007.

[36] P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, " Selective enzyme purification by affinity chromatography," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 61, no. 2, p. 636, 1968.

[37] E. ES, Enzyme kinetics., 1975.

[38] S. Lopez, J. France, W. J. J. Gerrtis, S. Dhanoa, and J. Dijkstra, " A generalized Michaelis-Menten equation for the analysis of growth," *Journal of animal science*, vol. 78, no. 7, pp. 1816-1828, 2000.

[39] A. Cornish-Bowden, Principles of enzyme kinetics, Elsevier, 2014.

[40] D. Vasic-Racki, U. Kragl, and A. Liese, "Benefits of enzyme kinetics modelling," *Chemical and biochemical engineering quarterly,*, vol. 17, no. 1, pp. 7-18, 2003.

[41] S. Schnell and P. K. Maini, " A century of enzyme kinetics: reliability of the KM and vmax estimates," *Comments on theoretical biology,*, vol. 8, no. 2-3, pp. 169-187, 2003.

[42] S. Cadenas, C. Rojas, R. Pérez-Campo, M. López-Torres, and G. Barja, " Effect of dietary vitamin C and catalase inhibition of antioxidants and molecular markers of oxidative damage in guinea pigs," *Free Radic Res*, vol. 21, no. 2, pp. 109-118, 1994.

[43] W.U. Jian, K. Karlsson, and A. Danielsson, "Effects of vitamins E, C and catalase on bromobenzene- and hydrogen peroxide-induced intracellular oxidation and DNA single-strand breakage in Hep G2 cells," *Journal of Hepatology*, vol. 26, no. 3, pp. 669-677, 1997.

[44] A. DOLATABADIAN, M. Sanavi, S.A.M, "Effect of the Ascorbic Acid, Pyridoxine and Hydrogen Peroxide Treatments on Germination, Catalase Activity, Protein and Malondialdehyde Content of Three Oil Seeds," *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, vol. 36, no. 2, pp. 61-66.

[45] J. Saffi, L. Sonego, Q. D. Varela, and M. Salvador, " Antioxidant activity of L-ascorbic acid in wild-type and superoxide dismutase deficient strains of Saccharomycescerevisiae," *Redox Repor*, vol. 11, no. 4, pp. 179-184, 2006.