Evaluating the Reversibility of Thermal Reconformation of Amylase

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Abstract

Thermal denaturing of the enzyme Amylase and the reversibility of this denaturing was evaluated by submerging samples of the enzyme into heated baths. These samples were then combined with a starch solution to evaluate the sample's ability to facilitate the decomposition of starch into simple sugars. Iodine was then used as an indicator of this decomposition. It was found that at temperatures of 90 degrees Celsius, Amylase undergoes reversible denaturing, but complete denaturing was undetectable by our admittedly crude experimental apparatus.

1   Introduction

Enzymatic activity is known to be highly dependent on temperature but inherits an added complexity from its ability to undergo a conformational change in two different forms: complete thermal denaturing and reversible thermal denaturing. In both cases, the binding site of the protein is sufficiently altered such that the enzyme can no longer facilitate a chemical reaction. The key difference comes in the reversibility of the change; reversible thermal denaturing forms an equilibrium state with the enzyme's proper conformation, whereas complete thermal denaturing renders the enzyme too damaged to convert back to the native state [1].

The ability for a protein to revert to its proper conformation is clearly invaluable in any biological context, but it is of particular worth in the human digestive system, where the ability to digest cooked, and therefore hot, foods is a hallmark of the species. Amylase is an enzyme found in human saliva that aids in the decomposition of starches in simpler sugars. The location of amylase lends it to most commonly be exposed to temperatures outside of the typical human body temperature range.

This investigation, therefore, attempts to quantify the ability for human amylase to resist complete denaturing in favor of reversible denaturing as a function of temperature.

2   Background

2.1   Amylase

The α-amylase (AAMY) is an enzyme present in the tissues of animals, plants, and some microorganisms that catalyzes the chemical break down of polysaccharides, such as starch and glycogen, into glucose monomers that can be absorbed by the body. Ptyalin is an isoform of amylase found in human saliva, and it functions best at a temperature of $37°$ C, which is conveniently body temperature, and within a pH range of 5.6-6.9 [3]. The amylase enzyme in saliva softens food while in the mouth and continues to digest starch into glucose until the food reaches the stomach, where the pH level is too low for it to continue to be active.

2.2   Temperature Dependence

Activity rates of salivary amylase are heavily affected by changes in temperature. A rise in temperature generally denotes increased activity, but only up until the optimum temperature of $37°$ C. Once the temperature is raised past that, the effectiveness of the enzyme begins to decrease [4]. When the enzyme temperature is raised past the optimum, it has surpassed its equilibrium point and will go into a reversible state of inactivity. The inactive form of the enzyme no longer serves its catalytic purpose, but it is not yet denatured. Evidence shows that the shift between active and inactive states is caused by localized conformational changes in the enzyme rather than permanent changes in enzyme structure [1]. If the temperature is lowered back down to optimum, the enzyme should be able to return to its active state. Once the temperature is raised too far above optimum, that is when the denaturation reaction begins to occur. Denaturation permanently alters the chemical bonds within the enzyme structure, and all catalytic activity is lost [4].

3 Setup

3.1 Experimental Solutions

3.1.1 Lugol's Iodine Solution

The form of iodine used is known as Lugol's solution and is a solution of 10g potassium iodide, 5g iodine crystals, and 100ml distilled water [2]. This solution was then diluted in 40 ml of water to be .03 times as concentrated. The iodine is used in this investigation as an indicator for starch. Iodine turns black in the presence of starch, but the amylase would theoretically have decomposed the starch by the time the iodine was added, and hence stay yellow instead.

3.1.2 Starch

Food grade corn starch was used in this experiment. It is advertised as "100 pure", which is debatable, but we have no reason to believe the corn starch contains anything other than corn starch. The solutions used for these experiments was 200mg corn starch per 100ml of water.

3.1.3 Spit

The researchers provided the spit for these experiments. It was not purified in any way, and presumably contains a host of contaminants, including but not limited to other enzymes, food particles, and bacteria.

3.1.4 Resultant Solutions

In each experimental trial, 10 ml of spit was combined with 10ml of starch solution. In the control groups, 20 ml of starch solution was used on its own. These solutions were subsequently mixed with the Lugol's iodine solution 1 ml at a time after they had been submerged in their respective water baths.

3.2 Temperature Control

The experimental setup consists of a test tube within an insulated bath, with a heating element at the top of the bath. The position of the heating element produced a temperature gradient on the order of tens of kelvins on the length scale of the height of the test tube. An aquarium bubbler was used to homogenize the temperature throughout the system to great success. Temperature changes were mirrored at the top and bottom of the bath

with deviations of less than a tenth of a degree, and delays on the order of seconds at most, as shown in Figure 1.

A proportional–integral–derivative (PID) configuration was used for the temperature feedback control. Such a configuration corrects for changes in temperature by considering three factors: the difference between the current temperature and the target temperature, the rate of change of the temperature, and the heat saturation of the heating element.[1] The algorithm weighted these in the following fashion:

- The proportional term, expressed as $C_1(T_{curr} - T_{target})$ where T_{curr} is the current temperature, T_{target} is the target temperature, and C_1 is a proportionality constant that is a function of the thermal susceptibility of the system and the power output of the heating element.

- The integral term that accounts for the heat stored in the heating element. This term was expressed as $C_2(1 - e^{-\tau t})(T_o - T_{curr})$, where τ is a time constant representing the rate at which the heating element fully heats up, C_2 is a proportionality constant representing the heat storage capacity of the heating element, and T_o is the fully heated temperature of the heating element.

- The derivative term is, in theory, meant to buffer the system by changing the state of the heating elements slightly before the system passes the target temperature. However, the heating element is so intense and as a result is switched on for such a small amount of time that this term is negligible.

These culminate into the following equation:

$$0 > C_1(T_{curr} - T_{target}) - C_2(1 - e^{-\tau t})(T_o - T_{curr}) \quad (1)$$

When the equation is true, the heating element in turned on.

This algorithm results in a reduction in the standard deviation in temperature by 67% at 80 degrees Celsius, from
1.21 degrees to .40 degrees Kelvin.

3.3 Procedure

Two baths were prepared for each trial; a bath at the experimental temperature, (90° C), and a bath at body

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[1] The code was inspired by the heater code provided in module 3, and even used the same OneWire package, but the majority of the code is my own.

temperature (37° C). Each trial consisted of 3 test tubes: a tube to remain in the experimental bath, a tube to remain in the body temperature bath, and a tube to be switched between the two. Each tube was left in a bath for a total of 20 minutes. The switched tube spent 10 minutes in the experimental bath, followed by 10 minutes in the body temperature bath. During this phase, the tubes only contain spit.

After the heating, the spit was mixed with the starch solution. The tubes were then returned to their respective baths; the switched tube was placed in the body temperature bath. They were left in this state for 10 minutes.
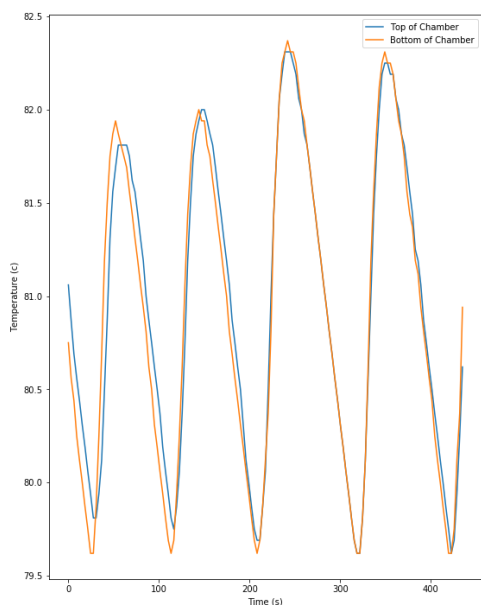

Figure 1: Temperature vs. Time of a system with a targeted temperature of 95 degrees. Note that this data was taken using the Module 3 temperature control code, not the configuration used in the experiment themselves.

Before introducing any iodine into the solutions, the buffer limit of the spit was evaluated explicitly for the current batch of spit. The evaluation was done by introducing iodine into the spit, one ml at a time, and attempting to observe any change in the coloring over a 5-minute time scale. Once this limit was found, this amount of iodine was immediately added to each mixture. Iodine was then introduced to each mixture 1 ml at a time, with photo documentation after each addition.

This procedure was conducted at experimental temperatures of 90, 70, and 55 degrees Celsius. There were intentions to conduct further trials at finer temperature

differences where deemed fruitful, but these trials did not come to fruition, as will be explained in the Data section.

4   Hypothesis

At high temperatures, it is expected that the majority of the enzyme will denature permanently. As a result, the immersion in the body temperature bath following immersion in the high temperature bath would yield results similar to those found without subsequent immersion in the body temperature bath. That is, lowering the temperature of the solution after exposing it to high temperatures will not result in substantial recovery of enzyme activity.

Figure 2: All trials and controls after introducing 7ml of iodine. From the left: switched trial, 90 degrees, 37 degrees, only water and iodine, only starch and iodine, only spit and iodine.
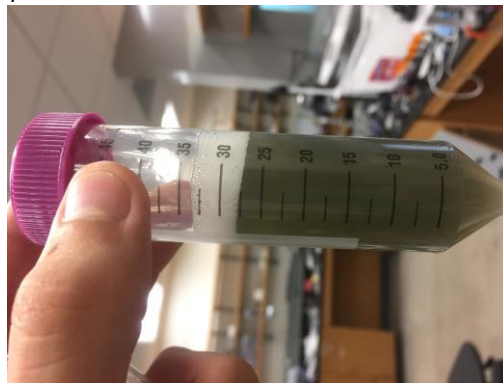

Figure 3: The trial that was immersed in the 90 degree bath, and subsequently immersed in the 37 degree bath. 7ml of iodine was introduced into the solution.

5 Complications

5.1 Iodine Bleaching

There appears to be a depletable bleaching mechanism in human spit. When mixed with the spit, the iodine gradually undergoes a process that renders the iodine colorless. Fortunately, it was discovered through testing that there is a limit to this bleaching, and a unit of spit can only render a certain amount of iodine colorless. Although the absolute limit of this bleaching is unclear, the solution of iodine and spit was observed to maintain its color on a time scale of tens of minutes after introducing a ratio of 1 part diluted iodine solution to 3 parts spit. The nature of this process is unclear, and may warrant future study, but could not be well explained in the course of our research.
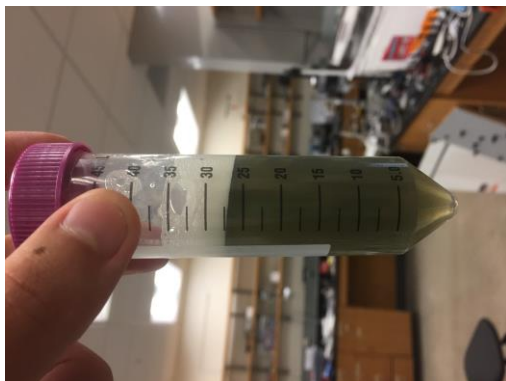

Figure 4: The trial that was immersed in the 37 degree bath. 7ml of iodine was introduced into the solution.

Figure 5: The trial that was immersed in the 90 degree bath. 7ml of iodine was introduced into the solution.

6 Data

Figure 2 shows all controls and trials. From the left, the trials are ordered as follows:

- Switched from 90 degree bath to 37 degree bath. Figure 4.

- 90 degree bath, full duration. Figure 5.

- 37 degree bath, full duration. Figure 3.

- Control; only water and iodine

- Control; starch, water, and iodine

- Control; spit and iodine

The trial that was removed from the 90 degree bath and subsequently moved to the 37 degree bath is indistinguishable from the trial that remained in the 37 degree bath after introducing 7 ml of iodine, 4 ml more than the buffer. The trial immersed in the 90 degree bath is clearly much darker, but it has also adopted a blue hue, contrary to the control tube with only starch and iodine, which is a jet black.

all other trials were effectively identical to this trial. The 37 degree trial and the switched trials were indistinguishable from one another, with the 90 degree trial exhibiting differing degrees of blueness and similarity to the other two tubes.

Subsequent addition of iodine only added a yellow hue to the solutions.

7 Analysis

The similarities between the switched trial and the body temperature trials indicate that a minimal amount of

irreversible denaturing occurred. The majority of the enzyme was able to return to its proper conformation, thereby decomposing the starch into sugar in a manner and efficiency very similar to that of the body temperature trial.

The clear and observable differences between the body temperature trial and the high-temperature trial supports the notion that the high temperatures had an effect on the amylase. The darker tone indicates there is a higher presence of starch in the solution, presumably because the amylase has denatured, either reversibly or entirely. However, the high-temperature trials have a blue/purplish hue to it that is not present in the trials containing only starch and iodine. The hue is likely due to a contaminant in the spit, or perhaps the starch itself was changed in the process of heating.

Following the realization that little complete denaturing was occurring at the highest temperature reasonably attainable, it was deemed unfruitful to continue the investigation with a more refined temperature scale.

8   Conclusions

This investigation was incapable of detecting any level of complete denaturing of the enzyme amylase at the temperature of 90 degrees Celsius. The results support the notion that the enzyme undergoes reversible denaturing that is quickly counteracted by reverting the temperature of its environment to body temperature, 37 degrees Celsius.

It should be noted that this investigation simply could not detect any denaturing, not that it did not occur at all. The methods described here are quite crude. It is entirely possible and plausible that the coloration of the sample is not responsive enough to changes in the concentration of starch.

The darker coloration of the sample kept at 90 degrees Celsius could indicate that more starch is present in the solution, but the blue hue of the sample is worrisome. The absence of this coloration in the sample only containing starch and iodine suggests that a fundamental change has occurred in either the spit or the starch. If the amylase was simply inactivated with no other effects, the sample would appear to be identical to the sample containing only starch. It dawns upon the researcher now that he should have simply heated the bath of starch to attempt to observe the blue coloration without the spit present. This researcher is a fool.

In any interpretation, these results do not support the initial hypothesis that the high temperatures would completely denature the Amylase. The sample that was switched from 90 degrees to 37 degrees certainly showed some enzymatic activity, and by our metrics, this activity was indistinguishable from the sample kept at 37 degrees. Should our metrics, namely these metrics being the coloration of the sample after the introduction of iodine, be correct, it can be said that the amylase undergoes very little complete denaturing in favor of reversible denaturing.

This conclusion can be supported anecdotally in real life, as the mouth is often exposed to high temperature foods, but this line of thought is hardly scientific.

While this investigation was somewhat poorly conducted, it introduces some interesting questions that are of value to study. The rate at which proteins denature completely is ill understood, and moreover the biological contexts which separate those who are hardy versus those who are sensitive to changes in temperature deserves closer inspection. Moreover, some interesting chemical reactions were observed that this investigation was incapable of explaining: the blue hue of the 90 degree sample, and the bleaching of iodine caused by the spit are two such examples. Although the use of spit itself was convoluting for the purposes of this investigation, a better analysis of how spit may affect denaturing and enzymatic activity is a fascinating topic that was completely glossed over by this investigation.

This investigation ultimately, but crudely, supports the notion that amylase is resistant to complete thermal denaturing, but admits its methods may simply not have been sensitive enough to observe the effects of the denaturing.

References

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