Role of caffeine and alcohol in the formation of glucose from starch-α-Amylase reaction: Application of a two-step model

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ABSTRACT

α-Amylase is an enzyme present in our saliva and pancreatic secretion and helps in the digestion of starch by breaking it down into sugar (glucose) molecules. The sugar molecules then go into our blood stream and provide energy. Very recently it was shown by us that caffeine inactivates the enzyme, and the amount of inactivation is increased as the caffeine concentration is increased before it levels off. The inactivation of the enzyme results in a decreased amount of sugar formation and may benefit those who are prediabetic or diabetic and want to control blood sugar level. Interestingly, alcohol (ethanol) works just in the reverse manner and resists the thermal inactivation of the enzyme, and hence, provides stability resulting in an increased amount of sugar formation. A two-step inactivation mechanism developed by us has been applied to explain these results.

Keywords: Starch-amylase reaction, effect of caffeine, effect of ethanol, glucose formation, amylase inactivation.

1. INTRODUCTION

α-Amylase is a metallo-enzyme that contains at least 1 mol of calcium ion[1-4] per mole of protein. The strength of the binding of calcium ions to the protein varies according to the source of the enzyme, and for all α-amylases the presence of calcium ions increases the stability of the enzyme toward denaturation by heat and other chemicals.[2] This enzyme can be obtained from bacterial (bacillus), fungal or various animal sources. Irrespective of the source, the enzyme exhibits very similar[5-7] properties. These include rapid fragmentation of starch molecules to reducing sugars, and the inactivation reaction of the enzyme initiated by dissociation of Ca^{2+} ions followed by denaturation by heat.[8,9] Temperature, pH, and various chemical substances are already known to influence the α-amylase activity and its inactivation rates in aqueous solutions [ref. 8-10, and references therein]. Although the effects of various chemical substances on the enzyme have been studied extensively, much fewer studies were carried out involving a food additive or a natural component of food like caffeine. Very recently we reported our thorough studies on the influence of caffeine on α-amylase activity.[10] The results were quite interesting and may have health benefits. We plan to discuss these results in sections 3 and 4.

Although innumerable studies have been carried out in aqueous solutions, there are situations in which we require enzyme to function in organic/non-aqueous media. Much of the current focus in enzyme technology involves enhancement of enzyme activity and stability under non-conventional conditions of organic solvents.[11-29] The non-aqueous medium has the advantage of having an increased solubility of nonpolar substrates favoring synthesis over hydrolysis. Besides, non-aqueous medium is likely to suppress water dependent side reactions, and thus, can promote high selectivity, high specificity and catalytic rates under milder experimental conditions. Exploiting of such advantages is often limited by the loss of activity of enzymes in these systems. For instance, α-chymotrypsin is less active in most organic solvents[14-16] and has activities 104–105 times lower in anhydrous octane than in water. While there are enzymes like α-chymotrypsin that show loss of activity in nonaqueous solvents, there are also other enzymes that show stabilization.[14-16] Our study on α-amylase in alcohol-water mixture shows that as the alcohol concentration is increased in water-alcohol mixed solutions, the enzyme inactivation is suppressed, and hence, a more active enzyme is obtained. This finding is in contrast with what has been observed for α-chymotrypsin.

The present article discusses our results on caffeine-amylase[10] and alcohol-amylase[11] studies and provides explanations of the results by applying the inactivation theory that we put forward earlier.

2. Theory of Inactivation of α-Amylase

Over 20 years ago[8,9] we reported a two-step inactivation model[8] for α-amylase. The first reversible stage (eq 1) involves a forward reaction that forms an inactive apoenzyme, E^{2-}, from the active enzyme, CaE, with a rate constant of k_1 and a reverse reactivation reaction involving the combination of E^{2-} with calcium ions with a rate constant of k_{-1}. The second irreversible stage (eq 2) of reaction forms a denatured form of the enzyme, EI^{2-}, from E^{2-} with a rate constant of k_2. Even though both EI^{2-} and E^{2-} are the inactive forms of the enzyme, the reversibly inactivated form, E^{2-}, can be quickly transformed into the active form, CaE, by adding calcium ions. On the other hand, the EI^{2-} is the denatured form of the enzyme that

cannot be reactivated by adding calcium ions.

$$CaE \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Ca^{2+} + E^{2-} \quad \text{..... (1)}$$

$$E^{2-} \xrightarrow{k_2} EI^{2-} \quad \text{..... (2)}$$

Based on these inactivation steps, the following equations can be derived:

$$XD = 1 - e^{-k_3 t} \quad \text{...(3)}$$

$$\text{Where, } k_3 = 1 - \frac{k_1 k_2}{k_{-1}[Ca^{2+}] + k_2} \quad \text{..... (4)}$$

The expression XD (eqn 3) gives the fraction of enzyme inactivated at a time, t, after the inactivation process begins, and k_3, given by equation 4, is a function of calcium ion concentration, and the rate constants, k_1, k_{-1}, and k_2 and hence, a function of temperature. From the expression 3 one can readily obtain the percentage of the active enzyme as follows:

$$\text{Percent Active Enzyme} = (1\text{-}XD)\ 100 = 100\ e^{-k_3 t} \quad \text{... (5)}$$

A detailed derivation of the fraction of enzyme inactivated (XD) has been presented in reference 8.

3. Determining the enzyme activity and inactivation values

Since both the caffeine-amylase and alcohol-amylase studies involve the measurements of activity and inactivation values, the next three sections describe how these measurements were made.

3.1. Activity Measurements:

Activity was measured at 25 ^{o}C by allowing the enzyme to react with an amylose solution (0.186 g/L) for 2 min before stopping the reaction with a cold iodine solution, which also converts unhydrolyzed amylose into amylose – iodine (AI) or starch – iodine blue complex (absorbance peak at 615 nm). The addition of iodine not only provides information about the amount of unhydrolyzed amylose, it also stops the enzymatic hydrolysis reaction.[8,9] The resulting solution was then placed in a thermostated bath for 10 min to reach 25 ^{o}C before measuring its absorbance at 615 nm. A more active enzyme leaves behind a

smaller amount of unhydrolyzed amylose, and hence, gives a smaller absorbance value at 615 nm. On the other hand, an inactivated enzyme will have a larger amount of unhydrolyzed amylose and will give a larger absorbance value at 615 nm.

3.2. Inactivation Experiments with alcohol

The enzyme solution with ethanol was prepared by adding 6.85 mL of concentrated protein (1.60 μM), 1 mL of pH 4.0 buffer, absolute ethanol, and water for a total volume of 100 mL in a volumetric flask. The temperature was maintained at 30 ^{o}C for 60 minutes (in circulating water bath) during each thermal inactivation experiment involving alcohol. At specific time intervals an aliquot of the protein solution (30 ^{o}C) was withdrawn and quickly cooled by adding cold water (20 ^{o}C), and then, the temperature was raised to 25 ^{o}C before determining its activity by adding the amylose solution. A blank test was done by leaving out enzyme from the solution, and its absorbance reading was taken (at 615 nm and 25 ^{o}C) after adding cold iodine, followed by warming to 25 ^{o}C, as was discussed under the previous section. The blank value is proportional to the initial amylose concentration (before the enzymatic hydrolysis) and was used in the calculations of protein activity values. In these studies, the pH of 4.0 was selected as the starch – iodine method (applied for activity measurements) shows high sensitivity at this pH and allows the measurement of enzyme activity over a wider range of inactivation time. Even though the percent amylose hydrolyzed values are lower at pH 4.0 as compared to pH 7.0, the values of the fraction of enzyme inactivated (XD) at two different pH values are quite comparable.[8]

Before the inactivation of the enzyme at 30 ^{o}C, an activity measurement was made at 25 ^{o}C soon after its preparation to ensure that the amount of enzyme inactivated was negligibly small. This also provides an activity value for a fully active enzyme at 25 ^{o}C.

3.3 Inactivation Experiments with caffeine

The concentrations of caffeine in the α-amylase solutions range from 0.00% - 0.30% with an increment of 0.05%. The enzyme solutions were prepared by adding 6.85 mL of concentrated protein (1.60 μM), 1 mL of pH 4.0 buffer, desired amount of caffeine, and water for a total volume of 100 mL in a volumetric flask and was kept at 25 ^{o}C. For each caffeine concentration, an aliquot of the protein solution was withdrawn before determining its activity by adding the amylose solution. A blank test was done by leaving out enzyme from the solution, and its absorbance reading was taken (at 615 nm and 25 ^{o}C) after adding cold iodine, followed by warming to 25 ^{o}C. As in activity measurements, these studies also maintained a pH of 4.0. In addition to having high sensitivity of the method at this pH, this study took advantage of determining the effect of caffeine in an acidic solution of pH 4.0. In the future, one can extend these studies to a higher pH (7.4, blood pH) so that the effect of caffeine is known as the pH changes from acidic to neutral or slightly basic.

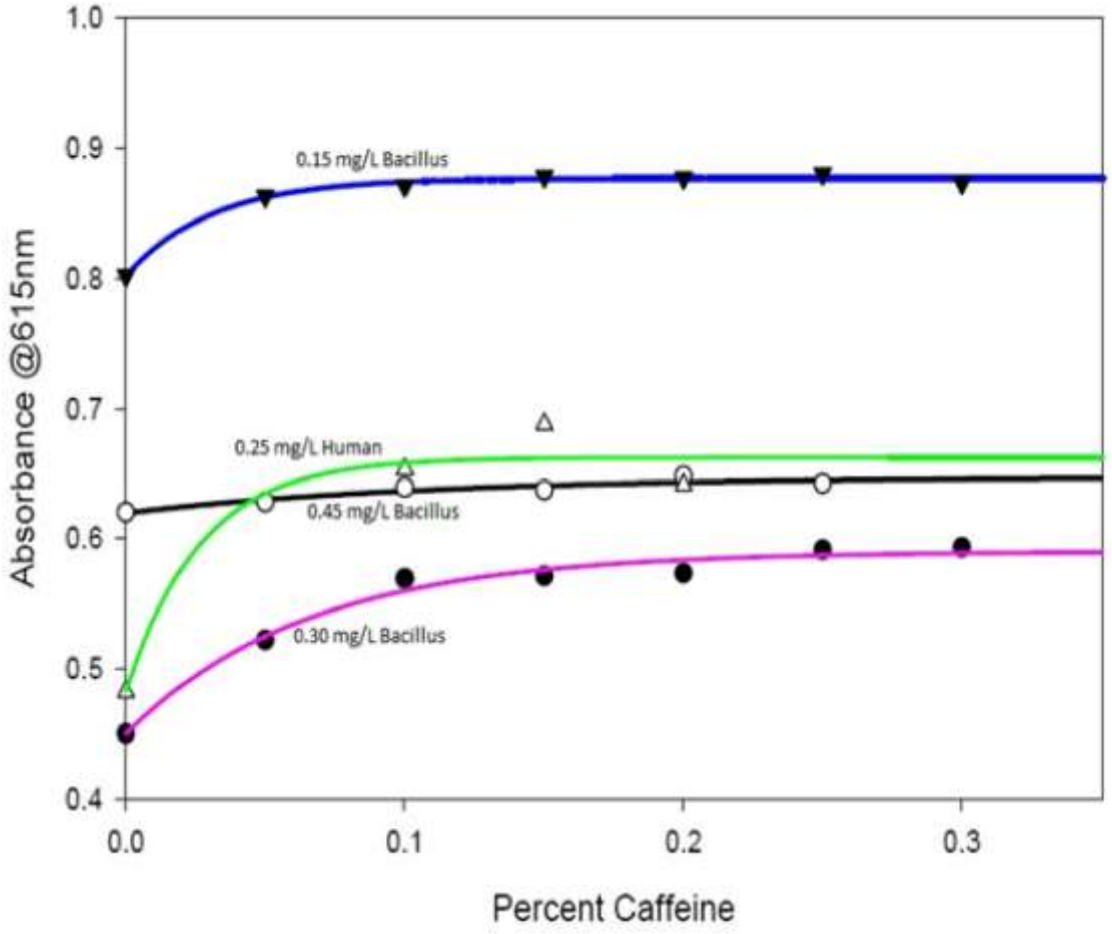

Figure 1: Percent caffeine against Absorbance values at 615 nm are plotted for different enzyme concentrations.

4. Results of Caffeine-Amylase interaction

Figure 1 represents caffeine concentration vs. absorbance values at 615 nm. The four curves represent four different enzyme concentrations ranging from 0.15 to 0.45 mg/L. Each curve shows an increasing trend in the absorbance value and suggests a gradual inactivation of the enzyme with an increased caffeine concentration that leaves behind a larger quantity of unhydrolyzed amylose, and hence, a larger absorbance value in iodine solution. The absorbance values, presented in Figure 1, are converted to percent active enzyme by applying eqns. 6 and 7. In eqn 6, A_0 is the absorbance value in the absence of enzyme (blank), A is the absorbance value in the presence of the enzyme with varied amount of caffeine (0-0.3% caffeine), and AH represents the percent amylose hydrolyzed in 2 minutes of reaction time applied for activity measurements.

$$\mathbf{AH} = \left(\frac{A_\circ - A}{A_\circ}\right) 100 \qquad \ldots. (6)$$

$$\textbf{Percent active} = \left(\frac{AH_c}{AH_\circ}\right) 100 = (1 - XD)\ 100 \quad \ldots. (7)$$

In eqn 7, the AH_0 represents the percent of amylose hydrolyzed by the fully active enzyme (no caffeine being added), and AH_c represents the amount of amylose hydrolyzed due to an inactivated enzyme as a result of the addition of caffeine. This is how Figure 2 was obtained from the absorbance values of Figure 1 and represents percent active enzyme for different caffeine concentrations. The second curve from the bottom represents the results from human enzyme, and the other three are those from the bacterial sources. In each case the results show the same trend; as the percent caffeine is increased in the enzyme solution, the percent of active enzyme drops. Also, the effect is most pronounced at lower enzyme concentrations than at higher concentrations. The bottom curve (blue) represents results from the weakest enzyme concentration with a sharp drop in the activity value, whereas the upper most curve is due to the most concentrated enzyme solution with much less pronounced effect of caffeine concentration. For the bottom curve, the largest drop in the value takes place from 0.0% to 0.1% of caffeine, and then, shows almost no change in the value at higher concentrations of caffeine.

Based on the two-step inactivation model (eqns 1 & 2) one can provide a possible explanation for the inactivation of the enzyme by caffeine. There is a possibility that a caffeine molecule binds with the apo-enzyme (**E^{2-}**), and thus, promotes the metal dissociation from the active enzyme (eqn 1), and hence, causes inactivation. As caffeine concentration is increased, at some point the caffeine molecules saturate the enzyme binding sites, causing the maximum inactivation effect, and beyond that point, no significant caffeine effect can be noticed. This can be noticed for the bottom curve of Figure 2 (0.15 mg/L of bacterial enzyme) that levels off at around 0.1% caffeine, whereas the third curve from the bottom with more concentrated enzyme (0.30 mg/L of bacterial enzyme) levels off at around 0.2% of caffeine.

5. Results of Alcohol-Amylase interaction

Figure 3 represents inactivation time vs. absorbance (at 615 nm) plots for different percentages of ethanol in ethanol-water solution of α-amylase at 30^{o}C. The six curves represent six different ethanol concentrations ranging from 1% to 50% (top to bottom curve). Each curve shows an increasing trend in the absorbance values and suggests a gradual inactivation of the enzyme with time that leaves behind a larger quantity of unhydrolyzed amylose, and hence, a larger absorbance value in iodine solution. The absorbance values of Figure 3 are converted to percent amylose hydrolyzed (AH) by applying eqns. 6 followed by the calculation of percent active enzyme by eqn 7. In eqn 6, A_0 is the absorbance value in the absence of enzyme (blank) and A is the absorbance value in the presence of the enzyme that has been inactivated at 30^{o}C for a time period (t) ranging from 0 to 60 minutes.

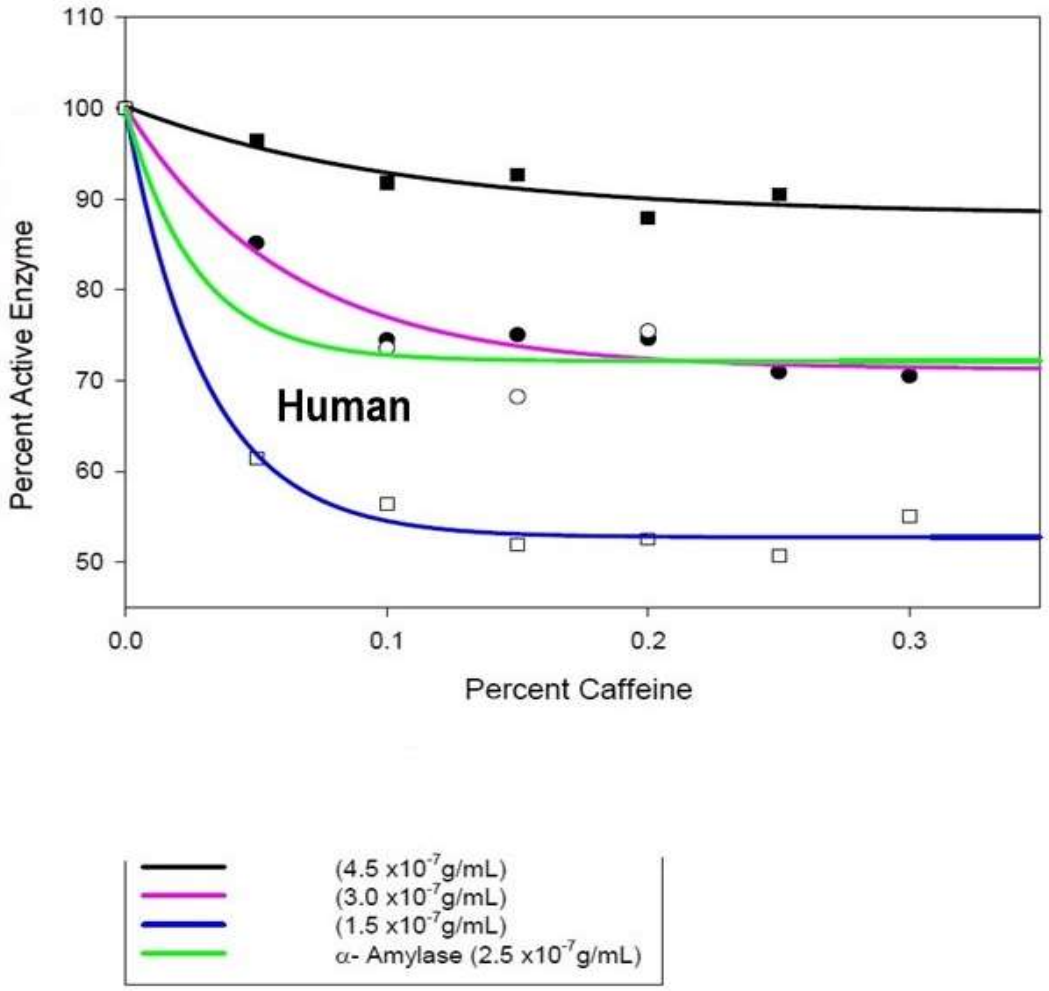

Figure 2: Percent caffeine against Percent active enzyme values are plotted for both human (second green curve from the bottom) as well as bacterial enzyme with protein concentrations ranging from 0.15 to 0.45 mg/L. The caffeine effect is the largest for the most dilute enzyme solution, and the smallest for the most concentrated enzyme solution.

$$\mathbf{AH = \left(\frac{A_\circ - A}{A_\circ}\right) 100} \qquad \mathbf{\ldots. (6)}$$

$$\mathbf{Percent\ active = \left(\frac{AH_t}{AH_\circ}\right) 100 = (1 - XD)\ 100} \qquad \mathbf{\ldots. (7)}$$

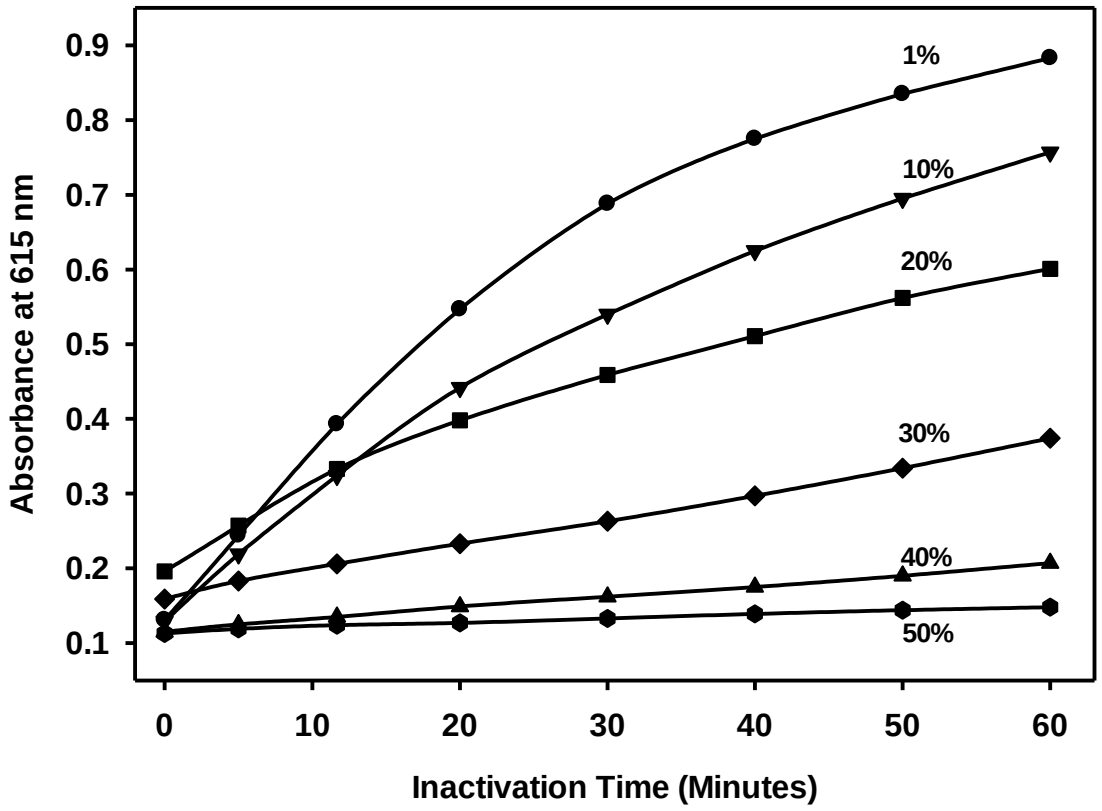

Figure 3: Increase in absorbance values at 615 nm is shown for different percentages of ethanol in ethanol-water solutions. Larger absorbance values mean a larger amount of unhydrolyzed amylose and hence, a greater inactivation of the enzyme. The symbols represent experimental data points and solid lines are cubic fit primarily to guide eyes to the trend of the data.

The percent of amylose hydrolyzed, AH, is proportional to the concentration of the active enzyme in the solution.[8,9] In eqn 7, AH_0 represents the amount of amylose hydrolyzed due to a fully active enzyme (t = 0 min) and AH_t represents the amount hydrolyzed after the enzyme was inactivated for t minutes. This is how Figure 4 was obtained from the absorbance values of Figure 3 and represents percent active enzyme at different inactivation time. In Figures 3 and 4 various symbols are used to represent results from different alcohol-water solutions. For example, the filled circles represent the solution with 1% alcohol (bottom curve in Fig 4) and filled hexagons for 50% alcohol (upper most curve in Fig 4) solutions, etc. As the inactivation reaction continues, the percent enzyme remaining active is sharply decreased within an hour of inactivation time when alcohol concentration is low (like 1% or 10%), and almost no such decrease in activity is noticed for a high alcohol concentration (like 50%) in the solution. In other words, at 50% alcohol concentration, the enzyme inactivation is very minimum (around 5%) while at 1% alcohol concentration the inactivation is by around 90% in 1 hour (Fig 4) suggesting a systematic stabilization of

the enzyme with the increased proportion of alcohol. The solid curves in Figure 4 are obtained by fitting eqn 5 of the inactivation model. The excellent fit suggests the validity of the two-step inactivation model that incorporates irreversible thermal inactivation[8] with time (eqn 3). This inactivation cannot be due to an aggregation of the enzyme molecules as there was no precipitate formation or unusual absorbance (or activity) change from that observed for thermal inactivation reactions in the absence of nonaqueous solvents.[8,9] The k_3 values, obtained from the theoretical fit decrease from 4.54×10^{-2} to 8.43×10^{-4} (s^{-1}) when the alcohol concentration is increased from 1% to 50%. A substantial drop (by 54 times) in the k_3 value is noticed when alcohol concentration is increased from 1% to 50%. A larger k_3 value means a faster inactivation reaction and a smaller value means a slower reaction or a greater stabilization. Because of a very low k_3 value, the enzyme shows almost no inactivation in 1 hr of inactivation time for 50% alcohol. On the basis of inactivation reactions 1 and 2, one can reach the same conclusion when the nonaqueous solvent concentration is increased in the aqueous-nonaqueous mixed solution. Since the products of reaction 1 are ionic, a nonaqueous solvent, because of its low polarity will suppress the ionization reaction reducing the amount of the apo-enzyme (E^{2-}) formation, and hence, suppress the subsequent reaction leading to irreversible inactivation reaction 2. Thus, a larger proportion of ethanol is expected to show a larger extent of stabilization. These results are quite different from those of other enzymes like α-chymotrypsin, which show an increasing instability with an increased proportion of nonaqueous solvent in the solution [ref 12,13 and references therein]. The inactivation of these enzymes follows a mechanism quite different from that of α-amylase. Although there is no mathematical model presented for these inactivation processes, some authors [ref 13, 15, references therein] suggest that the inactivation of these enzymes is due to unfolding and aggregation of protein molecules promoted by nonaqueous solvents.

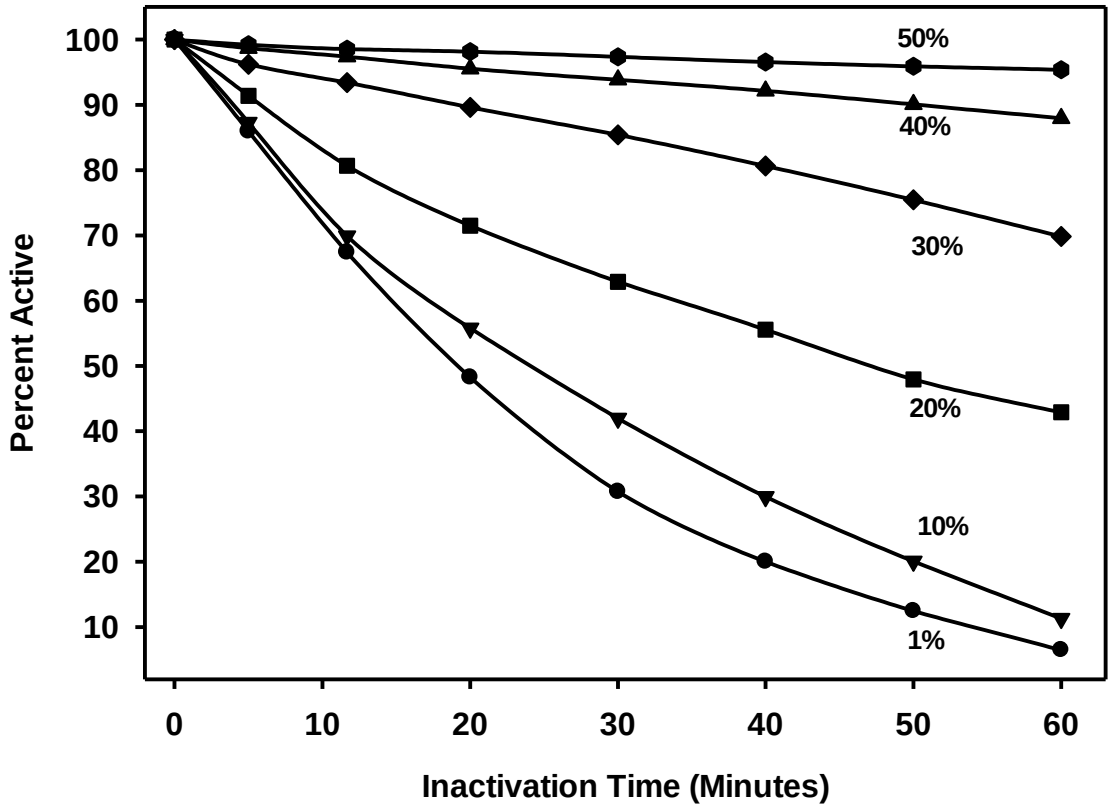

Figure 4: This figure was obtained by converting the absorbance values of Figure 3 to percent active enzyme by using equations 6 and 7. The enzyme solution with 1% ethanol shows the maximum decrease in activity in 60 minutes while the 50% alcohol shows almost no decrease in activity within that time at 30°C. The symbols represent the experimental data points and the solid lines represent theoretical fits to eqn 3. This is how k_3 values (s^{-1}) were obtained.

6. Preparation of Solutions

Iodine solution: The cold iodine solution (around 2^{o}C) of 126.9 mg/L was made from a solution containing the same volume each of 0.05 M iodine solution and 5% potassium iodide solution (Fisher Scientific).

Amylose solution: The amylose solution (~ 1.50 g/L) was made by first dissolving potato amylose (Sigma Chemical) in 2 M NaOH solution followed by the addition of an equal volume of 2 M HCl and then heating to a clear solution before adjusting its pH by adding acetic acid-acetate buffer solution. Dilute solutions of 0.186 g/L were prepared during experiments.

Caffeine solution: The caffeine solutions with desired concentrations were made by dissolving solid in water.

Alcohol solution: These solutions were made by adding desired amounts of absolute alcohol to aqueous solutions of α-amylase.

7. Concluding comments

Caffeine inactivates α-amylases from various sources. As the caffeine concentration is increased, the amount of inactivation is also increased, which finally levels off. As the enzyme is inactivated, the hydrolysis of starch liberates a smaller amount of sugar. This finding may indicate a positive health benefit of caffeine. About the effect of alcohol, this enzyme exhibits a stabilization effect when the proportion of alcohol is increased.

It should be noted that the experiments described in this paper were done in the chemistry lab setting without the involvement of any human subject. Besides, the temperatures maintained in the caffeine and alcohol inactivation experiments were 25 and 30 ^{o}C respectively, lower than the human body temperature of 37 ^{o}C. Since the inactivation model, applied here, is valid at both lower and higher temperatures,[8,9] one can expect that the caffeine-enzyme and alcohol-enzyme inactivation behavior observed at a lower temperature may also be observed at a higher temperature (the body temperature). Future research involving human subjects is needed to confirm the findings on caffeine-amylase and alcohol-amylase interactions.

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