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Araştırma Makalesi / Research Article

Effect of [H+] on the Activity of α-Amylase Isolated from *Bacillus subtilis*

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Abstract

Without enzyme pre-incubation at the appropriate enzyme concentration, at different pH's and at different substrate concentrations, separately for each and optimal pH values were determined after the enzyme was pre-incubated for 120 minutes with buffer at each pH. The optimal pH was found to be 7.12 in both conditions. To examine the effect of pH on enzyme stability, the enzyme was pre-incubated for 120 minutes at the previously determined optimal pH at a constant substrate concentration ([S] = 2.52 µg μL^{-1}). It was found that pH1 = pKa = 6.15 and pH2 = pKb = 8.68 and optimal pH = 7.415. KM values for enzyme (4.898; 4.7311; 5.004; 1.4615; 0.2483; 1.6666 µg μL^{-1}) by studying Michaelis-Menten kinetics at different pH's.and Vmax values (2.023; 2.431; 2.542; 0.952; 0.117; 0.438 µmol/min.) were found.

Keywords: Starch, enzyme, substrate, optimal pH, *Bacillus subtilis*

1. Introduction

Enzymes, which act as catalysts in biochemical reactions, are living components of biological systems. Enzymes, which have entered daily and economic life to be used for various purposes, have important metabolic functions in cells. Enzymes are high molecular weight protein molecules containing a non-protein prosthetic group. Many bacteria secrete extracellular enzymes instead of the nutrient media in order to benefit from the nutrients in the environment where they reproduce. One of the main functions of these enzymes, mostly hydrolases and proteases, is to provide usable nutrients to the microorganism. Extracellular proteolytic enzymes are widely used in the food industry and other biotechnological fields. (Taylor and Richardson, 1979; Boing, 1982; Kıran et al., 2006).

The term amylase was first used to describe enzymes that catalyze the hydrolysis of α-(1→4) glycosidic bonds in polysaccharides, such as starch or their degradation products. Especially, amylases produced by bacteria have a great commercial importance due to their heat resistance and are used in the starch industry. Amylases isolated from various organisms play an important role in the hydrolysis mechanism of starch, which is their substrate. amylases; They are hydrolytic enzymes of great importance that enable the breakdown of starch into products such as glucose, maltose, maltotriose, oligosaccharides and α-(1→6) glycosidic bonds. α-Amylases are extracellular enzymes that cleave α-(1→4) glycosidic bonds in straight amylose molecule and branched amylopectin molecule (Aunstrup, 1979; Kandra, 2003; Gubta et al., 2003; Vishnu et al., 2006; Taniguchi and Honda, 2009).

2. Materials and Methods

2.1 Chemicals and devices

α-amylase (E.C.3.2.1.1. Catalog No: 10070) from Fluka; citric acid, potassium dihydrogenphosphate, boric acid, diethylbarbutiric acid, sodium hydroxide, soluble starch, sodium potassium tartrate from Merck; 3,5-Dinitrosalicylic acid was obtained from Sigma. Deionized and bidistilled pure water was used in all experimental studies.

Spectrophotometer (U.V-160 Shimadzu U.V-Visible Recording Spectrophotometer), shaker (Memmert), Vortex (Fisons Whirli Mixer^{TM}), pH meter (Jenway 3040 Ion Analyzer) devices were used.

2.2 Preparation of 0.0286 m universal buffer

3.004 g of citric acid, 1949 g of potassium dihydrogenphosphate, 0.885 g of boric acid, 2.633 g of diethylbarbutiric acid were dissolved in approximately 200 mL of purified water. Finally, the volume of this solution was brought to 500 mL with distilled water. The pH of the buffers prepared according to Perrin and Dempsey was verified with a pH meter.

2.3 Preparation of bernfeld reagent

20 g of 3,5-Dinitrosalicylic acid are suspended in 400 mL of distilled water. 32 g 300 mL^{-1} sodium hydroxide solution is added drop by drop by mixing with a magnetic stirrer. A hot water bath is used if a clear solution is not obtained. 600 g of sodium potassium tartrate is added in small portions and the volume is made up to 2000 mL with distilled water.

2.4 Determination of appropriate enzyme concentration and experiment time

For this purpose, enzyme solutions at constant substrate concentrations, [E_1], [E_2], [E_3], [E_4], [E_5], The enzyme concentration and incubation period to be used in our study were determined depending on the results obtained from the activity determination by incubating at t_1, t_2, t_3, t_4, times.

2.5 Determination method of enzyme activity

Enzyme activity was determined by the Bernfeld method; For different pHs (5.05;

5.62; 6.15; 7.12; 8.02; 8.68; 9.29; 9.91) in 0.0286 M Universal buffer different concentrations (0.42; 0.83; 1.25; 1.67; 2.1; 2.52 and μg/μL) starch solutions were prepared. The enzyme solution (3.10^{-3} μg/μL) diluted in the same buffers as the starch solutions prepared from here was left to incubate at 30 °C for 15 minutes at a certain shaking speed (120 rpm). After this time, 500 μL 3,5-Dinitrosalicylic acid was added to stop the reaction and color it. The solution was incubated in a boiling water bath for 5 min. The reason for this is;This is because the reaction between 3,5-dinitrosalicylic acid and the reducing ends of the sugar units does not take place at low temperatures. Then, by diluting with 5 mL of distilled water, the absorbance was measured at 489 nm, where the maltose units absorb the maximum with 3,5-Dinitrosalicylic acid. The rate of formation was determined for each condition from the standard curve prepared using maltose.

2.6 Effect of pH on enzyme activity

Activity determination was made by using starch and enzyme solutions with fixed concentrations prepared at different pH. For this purpose, the activity was determined after the enzyme solution (3.10^{-3} μg/μL) in 0.0286 M Universal buffers was pre-incubated at 30 °C for 120 minutes.

2.7 Initial velocity as a function of $[E]_t$

Under ordinary in vitro experimental conditions, the enzyme is present in the reaction media in very small or catalytic amounts. It is usually found at a concentration of $[E]_t = 10^{-12}$-10^{-7} M, while $[S]_t = 10^{-6}$-10^{-2} M. Initial speed at any substrate concentration is given by the following equation (1):

$$V = \frac{V_{max}[S]}{K_M + [S]} = \frac{k_p[E]_T[S]}{K_M + [S]} = \frac{k_p}{\left[1 + \frac{K_M}{[S]}\right]}.[E]_T \quad (1)$$

Thus, the initial speed is directly proportional to $[E]_t$ at all substrate concentrations. The relationship between V and $[E]_t$ is linear when the correct initial velocities are measured, that is, the product formation rate should be constant throughout the entire time interval of the experiment. Since V varies with [*S*], the experiment time should be short enough to allow a small function of the substrate (about 5% or less) to be used.

2.8 Effect of pH on the rate of enzymatic reactions

The active center on the enzyme is generally composed of amino acid residues with ionic structure. This active center has a structure suitable for the binding of the substrate and the catalysis of its reaction. Changes in pH change the ionizability of these ionizable groups and thus the conformation of the active center. This also affects the rate of catalysis. Apart from that, the substrate molecule can contain ionizable groups and only one ionic form of the substrate can be bound to the enzyme and catalyzed. The severity of the changes in the ionization of functional groups in the catalytic mechanism may completely disrupt the reaction mechanism and cause irreversible inactivation. In this study, α-amylase, one of the extracellular enzymes, is widely used in the industry, especially in the detergent industry. The secretion of this enzyme by many Bacillus species, and therefore its easy availability, increases its industrial importance.

In this study, it is aimed to try to elucidate the reaction mechanism by detecting the effect of $[H^+]$ ions on the commercially available α-amylase enzyme,

which is purified from Bacillus subtilis, and accordingly, to intervene in industrial processes.

3. Results and Discussion

To determine the appropriate enzyme concentration, at pH 7, at a constant substrate concentration ([*S*] = 2.52 µg/µL) at different times (10; 15; 20 and 30 minutes) and different enzyme concentrations ($E_1 = 2.10^{-3}$; $E_2 = 2.4.10^{-3}$; $E_3 = 3.10^{-3}$; $E_4 = 4.10^{-3}$, $E_5 = 6.10^{-3}$ µg/µL) and the results obtained by the activity determination using soluble starch as substrate are given in Figure 1.

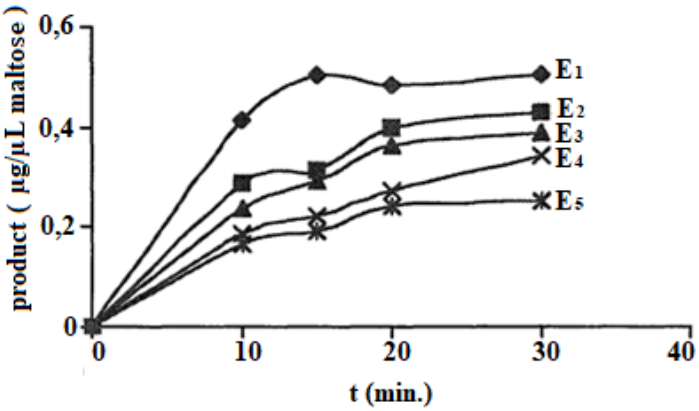

Figure 1. Determination of Appropriate Incubation Time at Different Enzyme Concentrations

At a constant enzyme concentration ([*E*] = 3.10^{-3} µg/µL) and a constant substrate concentration with a pH of 7.12 ([*S*] = 2.52 µg/µL), each enzyme studied was pre-incubated for 120 minutes and its activity was determined. Optimal pH value determined by this process is given in Figure 2 and K_M and V_{max} values are given in Table 1.

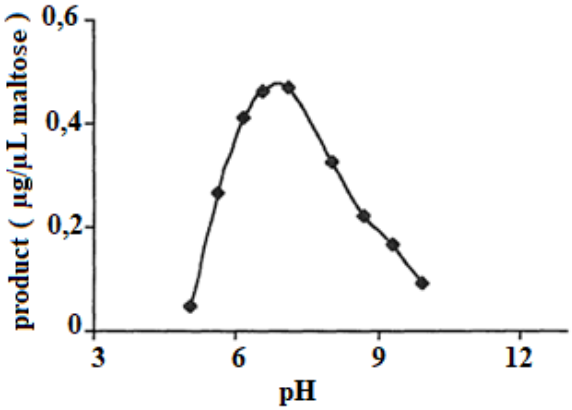

Figure 2. Activity values measured after two hours of pre-incubation at each pH studied at a certain substrate concentration (Optimal pH = 7.12)

The enzyme concentration was taken as 3.10^{-3} µg/µL.The enzyme was subjected to a 120-minute pre-incubation process with the buffer at the pH's to be worked.Then, after 15 minutes of incubation with the substrate at a concentration of 2.52 µg/µL,

the pH was determined to be 7.12 from the activity values measured. However, despite the same substance concentrations and reaction volume, the absorbance values measured under these conditions (for example, product 0.5 µg/µL at pH = 7.12), it was found to be approximately 4 times higher than the absorbance values measured in conditions without pre-incubation (product 0.13 µg/µL at pH = 7.12)(Figure 2). These results suggest that positive changes occur in the conformation of the enzyme during the pre-incubation, in the direction of increasing the activity. We believe that the high optimal pH for the commercially available and bacterial α-amylase we used is due to its non-pure crystalline structure. Although it is seen in Figure 2 that the optimal pH is 7.12, it does not give an idea why the reaction rate decreases in pH ranges above or below 7.12. This decrease in the graph may be the result of the formation of inappropriate ionic forms of the enzyme, substrate, and enzyme-substrate complex or inactivation of the enzyme. In other words, it may be the result of a combination of these effects.

Table 1 Obtained by Activity Determination at Different p**H**'s K_M , pK_M, V_{max} , log (V_{max}), log (V_{max} / K_M) values

	pH					
	5.05	**5.62**	**6.15**	**7.12**	**8.02**	**8.68**
K_M **(x10^{-2})**	4.898	4.7311	5.004	1.4612	0.2483	1.6666
pK_M	1.31	1.325	1.300	1.835	2.605	1.779
V_{Max}	2.023	2.431	2.542	0.952	0.117	0.438
Log(V_{max})	0.305	0.385	0.405	-0.021	-0.931	-0.358
Log(V_{max} / K_M)	1.616	1.711	1.706	1.814	1.674	1.419

One of the aims of our study is to determine the ionizing groups of the enzyme by using the kinetic parameters (K_M and V_{max}) and accordingly to make a claim about the reaction mechanism. However, since Michaelis-Menten curves are sigmoidal rather than hyperbolic, we could not determine the inhibition type from Lineweaver-Burk graphs.We determined the kinetic parameters of K_M and V_{max} for each pH with the Fig. P program running under Ms-DOS. Using these kinetic parameters, we drew the graphs suggested by Dixon and Webb with Excel 7.0 running under Microsoft Windows 95 (Figure 3-5).

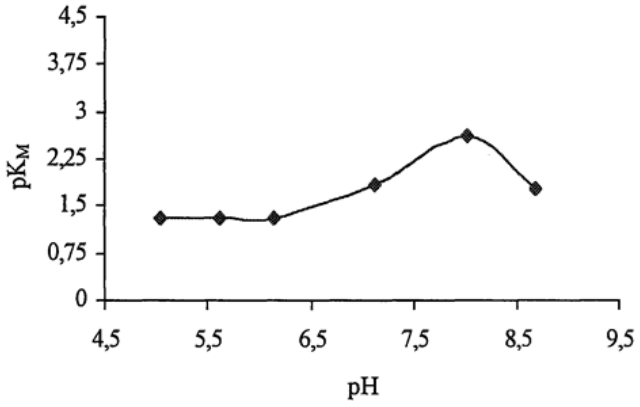

Figure 3. Computability of pK^E values of ionizable groups from pK_M - p**H** plot

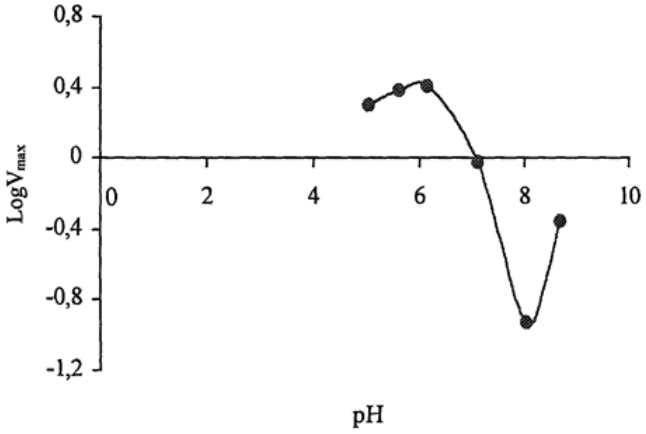

Figure 4. Computability of pK_A^{ES} and pK_B^{ES} values of ıonizable groups from the logV_{max} - p**H** graph

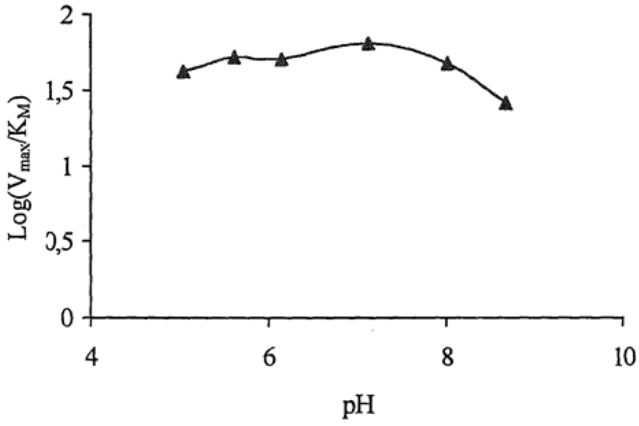

Figure 5. log (V_{max} / K_M) - computability of pK_A^E and pK_B^E values of ıonizable groups from the p**H** graph

Above pH = 8.68 or below pH = 6.15, decreased activity may be a result of irreversible inhibition of the enzyme. It can be said that the change occurring between pH = 7.12 and pH = 8.68 and pH = 7.12 and pH = 6.15 may be caused by inappropriate ionic forms of the enzyme or substrate. However, it can be said that the enzyme is stable between pH = 6.15 and pH = 8.68. The decrease in the enzyme activity at pHs below or above the optimal pH is due to the pH dependence of the K_M constant of the enzymatic reaction. These changes in pH affect the ionizability of the groups in the active centers of the enzymes. According to the results obtained, the enzyme should theoretically be inhibited reversibly at pHs below 6.15 and above 8.68. However, from the reaction kinetics examined, the catalytic activity of the studied enzyme did not change much in slightly acidic regions (pH = 5.05, 5.62, and 6.15), but at pH 8.02 and above, the catalytic efficiency of the enzyme decreased significantly,even the observation of inactivations can be considered as an indication of the presence of acidic amino acid residues in the active center of the enzyme.

The effect of the concentration of different hydrogen ions on the enzyme activity is similar to the effect of other activators or inhibitors, therefore the same theory and kinetic methods can be used to explain this effect. These investigations may yield useful results in elucidating the mechanism of the enzyme-catalyzed

reaction. However, a limited number of mechanisms are compatible with kinetic parameters. Because kinetic parameters are produced with simplified models. In other words, it is not possible to elucidate complex mechanisms by this method.

Dixon and Webb found that p*K* values of ionizing groups could be determined by plotting logarithms of kinetic constants (V_{max} and K_M) against pH. If this kinetic analysis is indexed by acidic region A and basic region B, for the free enzyme which contains basically only two ionizable groups for activity; pK_M = -log K_M or log (V_{max} / K_M), pK_A^E and pK_B^E values if plotted against pH, It is stated that if log V_{max} is plotted against pH, $pK_A^{ES}$and pK_B^{ES} values can be determined.

4. Conclusions

Although our results can be calculated as 6.15 for pK_A^E and 8.68 for pK_B^E when Log (V_{max} / K_M) is plotted against pH, they are not in good agreement with Tipton and Dixon's theoretical approaches. It has been determined that the derived theoretical relations are valid only for simplified systems, and that enzymes with oligomeric structure are not suitable models to confirm Tipton, Dixon and Webb theories with substrates in macromolecular structure.

Declaration of Author Contributions

The authors declare that they have contributed equally to the article. All authors declare that they have seen/read and approved the final version of the article ready for publication.

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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