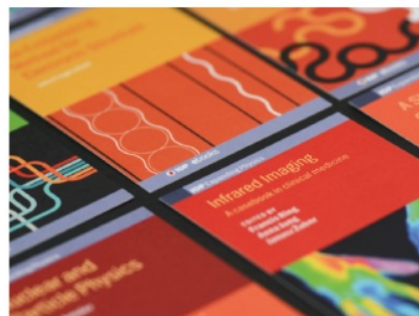# Effect of glutaraldehyde addition on the stability of the α-amylase from Bacillus subtilis ITBCCB148

By Y. Witazora

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Effect of glutaraldehyde addition on the stability of the α-amylase from *Bacillus subtilis* ITBCCB148

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Abstract. α-Amylase is widely used in industry because of its ability to hydrolyze starch to glucose. Limited enzyme activity in extreme pH and temperature makes it necessary to increase enzyme stability. The purpose of this study was to improve the stability of the α-amylase from the bacteria *Bacillus subtilis* ITBCCB148 by chemical modification using glutaraldehyde (GA). The results showed that modified enzymes using glutaraldehyde 0.01; 0.03 and 0.05% have an optimum pH of 5.5; optimum temperature of 55°C; K_M of 4.74; 5.03 and 3.87 mg/mL substrate; the V_{max} of 285.71; 270.27 and 212.77 μmol mL^{-1} min^{-1}; k_i of 0.0183; 0.0111 and 0.0160 min^{-1}; half-life ($t_{1/2}$) 37.87; 62.43 and 43.31 minutes; ΔG_i 102.656; 104.047 and 103.038 kJ mol^{-1}, respectively. Chemical modification of the α-amylase from *B. subtilis* ITBCCB148 using glutaraldehyde can increase thermal stability by 1.6-2.7 times which can be seen from a decrease in the value of k_i, an increase in half-life and ΔG_i.

Keywords: α-amylase, *B. subtilis* ITBCCB148, glutaraldehyde, enzyme stability

1. Introduction

The α-amylase (EC 3.2.1.1) is able to break down starch by hydrolyzing the 1,4-glycosidic polysaccharide bond resulting in short chain dextrin. This enzyme can be obtained from plants, animals and microorganisms [1]. The isolation of amylase from microbia is preferred because it is easy to grow and its growth is faster, relatively lower production costs, production conditions do not depend on seasonal and time changes and are easy to optimize and modify. *B. subtilis* has been known as a producer of thermostable α-amylase needed in industrial processes [2].

The selective and specific nature of enzymes in hydrolyzing starch is able to reduce the formation of unwanted products, the gaining of higher glucose through a easier process, without the use of harmful solvents so that it is more environmentally friendly [3]. Specificity, thermostability and enzyme pH response are very important factors for industrial use [4]. In general, enzymes are not stable under conditions of high temperature and extreme pH. According to Daba *et al*.[5] enzymes can be stabilized through several ways such as chemical modification, immobilization, directed mutagenesis and solvent engineering. Mozhaev *et al*. suggested the use of chemical modification to increase the stability of enzyme [6].

Some advantages of chemical modification compared to immobilization are in the interaction of enzymes with the substrate. Chemical modification does not use an insoluble matrix such as immobilization so that the interaction of enzymes with the substrate is not disturbed and the decrease in enzyme activity can be suppressed. In addition, in the immobilized process, the mechanism of action of enzymes used in the clinical field during interactions with receptors or other components of cellular membranes, is likely to change due to the presence of a supporting matrix [7].

Chemical modification with bifunctional reagents can increase the stability of enzymes through inter- and intramolecular cross-bonds producing in a stable tertiary structure. The most popular bifunctional reagents used are dimethyladipimidate and glutaraldehyde because both of these compounds are readily available and dissolve in aqueous solvents. Modifications with dimethyladidimate have been reported by Kazan *et al*.[8] against the penicillin G-acylase enzyme produced 9 times increase in thermal stability compared to the native enzyme. Whereas Yandri *et al*.[9] made modifications using dimethyladipimidate against α-amylase to produce enzymes with thermal stability of 1.5-3.5 times higher than the native enzymes. Daba *et al*.[5] reported that modification with glutaraldehyde on the β-amylase was able to modify the specific residues of the enzyme and provide better stability compared to the addition of trinitrobenzenesulfonic acid (TNBS) and methylpolyethyleneglycol (mPEG). The β-amylase modified with GA was able to maintain the remaining 50% activity at 50°C while the unmodified enzyme had lost the remaining 50% activity at 50°C.

In this work, α-amylase was used from local bacteria isolate ITBCCB148. Modification with glyoxylic acid even though the optimum pH and temperature did not change, but the stability of the modified enzyme was better at higher temperatures of 65 and 70°C [10] and modification with cytraconic anhydride resulted in better thermal stability at 70 and 80°C [11].

Based on the results of research by Daba *et al*. which showed the potential of glutaraldehyde in increasing enzyme stability and has never been applied to α-amylase from ITBCCB148, it is necessary to do a research on chemical modification on α-amylase isolated from local bacteria isolate *B. subtilis* ITBCCB148 through cross-linking with glutaraldehyde (GA). This is expected to increase the stability of the native α-amylase.

2. Experimental
2.1 Materials
The α-amylase enzyme for the modification procces was the purified enzyme from the crude extract of the bacterial isolate *B. subtilis* ITBCCB148 obtained from the Laboratory of Microbiology and Fermentation Technology ITB. The chemicals used are distilled water, NaH_2PO_4, Na_2HPO_4, I_2, KI, HCl 1N, dinitrosalicylic acid (DNS), NaOH, Na-K tartrat, fenol, Na_2CO_3, $CuSO_4 \cdot 5H_2O$, *folin ciocelteau* reagent, *Bovine Serum Albumin* (BSA), glutaraldehyde solution (50%) from SIGMA and glucose.

The equipment used in this study were glassware, analytical balance Sartorius-Germany, micropipette Eppendorf, pH meter Fisher-Canada; spectrophotometer Shimadzu UV-Vis and magnetic stirrer NUOVA II-USA.

2.2 Modification of the native α-amylase
Modification of the native enzyme using glutaraldehyde [5, 12]. A total of 10 mL of enzyme in 0.01 M phosphate buffer pH 8 were added with glutaraldehyde in phosphate buffer pH 8 with various concentrations of 0.01; 0.03 and 0.05% produced a modified mixture of enzyme-glutaraldehyde 0.01% (GA 0.01%), enzyme-glutaraldehyde 0.03% (GA 0.03%) and enzyme-glutaraldehyde 0.05% (GA 0.05%). The mixture was stirred with a magnetic stirer for 1 hour at 4°C.

2.3. Characterization of the native and modified α-amylase
Characterization of the native and modified α-amylase was carried out by determination several variables such as optimum pH, optimum temperature, enzyme kinetics data (K_M and V_{max}), thermal stability test, determination of half-life ($t_{1/2}$), constant inactivation rate (k_i) and energy changes due to denaturation (ΔG_i).

Determination of the optimum pH of the native and modified enzymes was carried out by varying the pH of the substrate in 0.1 M phosphate buffer, namely 4.5; 5.0; 5.5; 6.0; 6.5; 7.0; 7.5 and 8.0 for 30 min. The temperature was kept constant at 60°C. The enzyme activity was measured by the Mandels method [13] and the remaining activity was determined by comparing the activity of each pH variation with the highest activity produced.

Determination of the optimum temperature of the native and modified enzymes was carried out by varying the incubation temperature, namely 50; 55; 60; 65; 75 and 80°C at their optimum pH. The enzyme activity was measured by the Mandels method [13] and the remaining activity was determined by comparing the activity of each pH variation with the highest activity produced.

Determination of enzyme kinetics data (K_M and V_{max}) was carried out by varying the substrate concentration (starch solution), namely 0.1; 0.2; 04; 0.6; 0.8; 1% at 60°C for 30 minutes. The data on the relationship between the enzyme reaction rate and the substrate concentration were plotted into the Lineweaver-Burk curve.

Determination of the thermal stability of the native and modified enzymes was carried out by measuring the enzyme activity every 10 minutes intervals during 80 minutes of heating at the optimum temperature and pH. The residual activity was measured by comparing the enzyme activity at 10 minutes intervals with the initial activity of the enzyme (without heating). The initial activity of the enzyme is given a value of 100% [14].

Determination of half-life ($t_{1/2}$), inactivation rate constant (k_i), and energy changes due to denaturation (ΔG_i): changes in the value of k_i (inactivation rate constant) of the native amylase and after modification were carried out using the first order inactivation kinetics equation [8] as in Equation 1:

$$\ln (E_i/E_0) = -k_i\, t \qquad (1)$$

The energy changes due to denaturation (ΔG_i) of the native α-amylase and after modification, it was carried out using the Equation 2 [8]:

$$\Delta G_i = -\, RT \ln (k_i\, h/k_B\, T) \qquad (2)$$

Where:
R = gas constant (8.315 J. K^{-1} mol^{-1})
T = absolute temperature (K)
ki = thermal inactivation rate constant
h = Planck's constant (6.63 x 10^{-34} J.s)
kB = Boltzmann's constant (1.381 x 10^{-23} $J.K^{-1}$)

3. Results and Discussion

3.1 Determination of the optimum pH of the native and modified enzymes

Figure 1 shows that the native and modified enzymes have the same optimum pH, namely 5.5. This shows that the addition of additives does not change the optimum pH of the enzyme. At pH 6.5 - 8.0 the modified enzyme with glutaraldehyde 0.01% has better stability than the native enzyme. This shows that the enzyme with modification (GA 0.01%) is able to work over a wider pH range, especially at neutral to slightly alkaline pH. The enzyme with the addition of 0.01% glutaraldehyde had the highest residual activity of 46% compared to the native enzyme which was only 31%.

The existance of an optimum pH indicates that the protein charge plays an important role in the intermolecular cross-links necessary for the formation of an insoluble protein layer. The reaction of GA with various proteins varies depending on pH. The optimum pH for GA insolubilization varies from protein to protein. Tong *et. al.* [15] reported crosslinking of GA with BSA at a pH of 4.7 (nearly the same as the isoelectric point of BSA) resulting in a BSA microcapsule with pH-controlled permeability. Permeability transition at pH 10 is associated with swelling of the capsule as a result of charge repulsion. The charge on the protein can regulate the maximum cross-linking when the repulsion charge is minimal. The ability of an enzyme to maintain its activity at a pH far beyond its optimum pH range is affected by electrostatic equilibrium and hydrogen bonding which causes protein molecules to be opened [8].

The stability of the modified enzyme over a wide pH range indicates that cross-linking with glutaraldehyde produces the right form of ionization in the amino groups on the enzyme surface so that electrostatic equilibrium and protein bonds can be maintained to produce a stable structure.
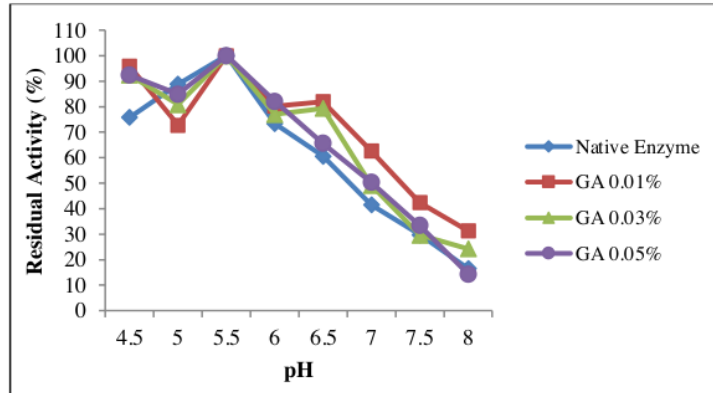
Figure 1. The optimum pH of the native and modified α-amylase

3.2 Determination of the optimum temperature of the native and modified enzymes
Figure 2 shows the optimum temperature of the native and modified enzymes that do not experience a shift, which is st 55ºC. However, the stability of the enzyme modified with glutaraldehyde 0.01; 0.03 and 0.05% were much higher than the native enzyme.
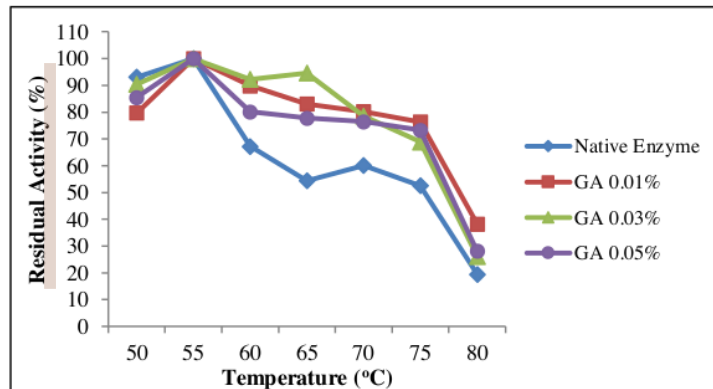
Figure 2. The optimum temperature of the native and modified α-amylase

The modified enzyme with GA 0.01 and 0.05% showed more stable results in the temperature range 55-75ºC, while the modified enzyme with GA 0.03% showed the best stability at 55-70ºC. Although there was still a decrease in activity at 80ºC, the modified enzyme with glutaraldehyde 0.01; 0.03 and 0.05% still had higher residual activity, namely 38, 26, and 28%, respectively; while the native enzyme had residual activity of only 19%. This shows that the modification with glutaraldehyde produces an enzyme that is more stable over a wide temperature range than the native enzyme. According to Singh [16], the cross-linking of enzyme molecules with glutaraldehyde produces a stiffer structure so that it is resistant to denaturation.

Effect of modification on thermal stability

Based on the graph in Figure 3, it is known that the residual activity (%) of the enzyme modified with glutaraldehyde (0.01; 0.03 and 0.05%) after 80 minutes of incubation was 24, 41 and 28%, respectively.
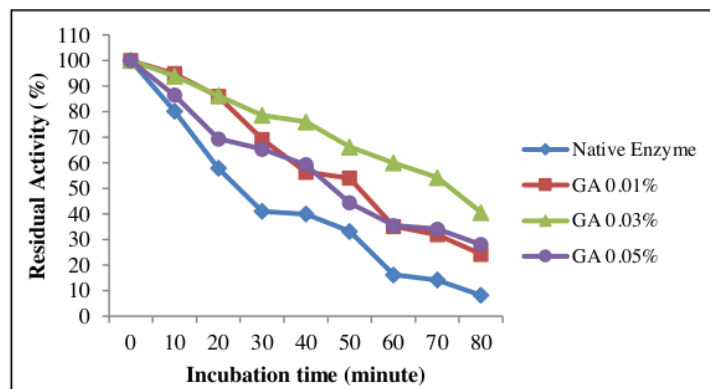

Figure 3. Thermal stability of the native and modified α-amylase

This value is higher than the remaining activity of the native enzyme by only 8%. This shows that the cross-linking of glutaraldehyde with the lysine residue of the enzyme molecule is able to protect the active center of the enzyme from the influence of extreme temperatures so that the modified enzyme is more stable than the native enzyme. Daba *et al*.[5] reported that crosslinking of β-amylase with glutaraldehyde had a residual activity of 33% after 30 minutes of incubation.

Torchilin *et al*.[17] stated that the thermal stability of an enzyme can be increased through cross-linking because the presence of intra- and intermolecular cross-links results in stiffer enzyme molecules so that they are more resistant to changes in conformation. The covalent bonds formed during crosslinking are stable even in the presence of substrates and solutions of high ionic strength. The two aldehyde functional groups that are owned can bind the amine group (- NH_2) on the lysine residue of the amylase on the surface, so that it can reduce the contact of the hydrophilic lysine residue with the solvent, thus the hydrophobic group between the non-polar amino acid residues in the enzyme gets stronger and forms a structure that is rigid. However, the addition of glutaraldehyde also reduces enzyme activity. This result is similar to that reported by Daba *et al*. [5] that binding with glutaraldehyde increases the stability of the β-amylase enzyme but decreases the enzyme catalytic activity.

The modified enzyme was also able to maintain its activity during the inactivation process against time so that there was no drastic decrease compared to the native enzyme. The modified enzyme with glutaraldehyde 0.01; 0.03 and 0.05% produced enzymes that were more stable than the native enzyme. The best enzyme stability is shown by a green graph, which is the result of modification with 0.03% glutaraldehyde. The addition of glutaraldehyde concentration to 0.05% did not increase the stability of the enzyme but decreased it.

According to Chui and Wan [18], enzyme activity is inversely proportional to the concentration of glutaraldehyde. This is because the cross-linking that occurs extensively in the enzyme molecule can cause a distortion of the enzyme structure (for example in the conformation of the active side of the enzyme). By this distortion, the ability to access and accommodate the substrate can be reduced and this affects the biological activity of the enzyme. Based on the graph in Figure 3, modification with 0.01% glutaraldehyde results in a lower stability effect. At low glutaraldehyde concentrations, the glutaraldehyde molecule cannot provide sufficient aldehyde groups to cross-link with the amine groups on the enzyme surface.

At higher glutaraldehyde concentrations, the cross-linking is sufficient to form a tight enzyme structure by releasing water molecules to form an insoluble enzyme. At low concentrations of enzymes and glutaraldehyde tend to cause intramolecular cross-linking by increasing the likelihood that the glutaraldehyde functional group will react with the same enzyme molecule. Therefore, choosing the right enzyme and substrate concentration needs to be considered. In the results of this study, the appropriate addition of glutaraldehyde to produce higher enzyme stability is at the glutaraldehyde concentration of 0.03%.

3.4 Effect of modification on enzyme kinetics data (K_M and V_{max})
Various substrate concentrations were used to determine their interactions with the enzim, namely 0.1; 0.2; 0.4; 0.6; 0.8 and 1%. The relationship of substrate concentration ([S]) to enzim activity (V) can be seen in Lineweaver-Burk in Figure 4. The K_M and V_{max} values of the native and modified enzymes can be seen in Table 1.
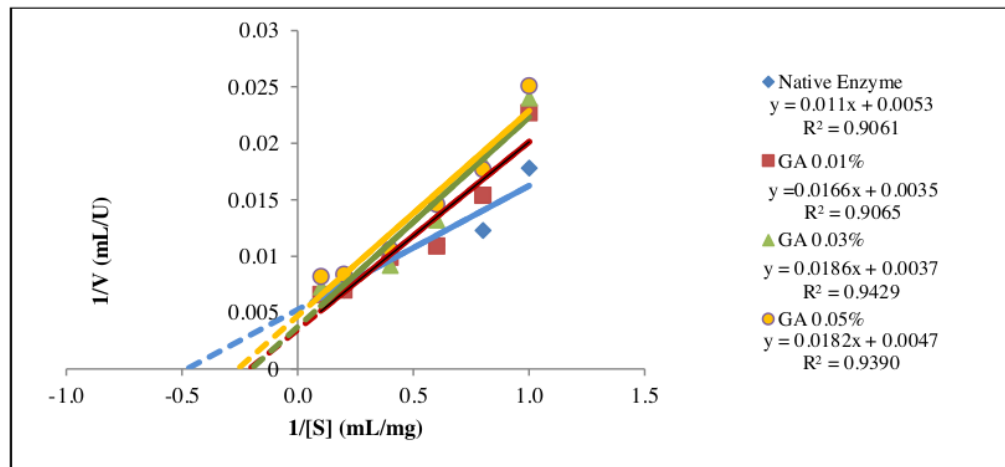

Figure 4. Kinetics data of the native and modified enzyme with glutaraldehyde in various concentration of 0.01; 0.03 and 0.05%.

Table 1. Values of K_M and V_{max} of the native and modified enzymes with glutaraldehyde in various concentration 0.01; 0.03 and 0.05%.

Enzim	K_M (mg mL^{-1} substrate)	V_{max} (μmol mL^{-1} min^{-1})
Native	2.08	188.68
Modified with		
GA 0.01%	4.74	285.71
GA 0.03%	5.03	270.27
GA 0.05%	3.87	212.77

Table 1 shows an increase in the K_M value of the modified enzyme. The increase in the K_M value of the modified enzyme showed enzyme affinity to the substrate is reduced. This occurs due to changes in the enzyme structure, especially in the active central site of the enzyme. According to Regan *et al*. [19], the kinetics of enzymes can change due to the role of individual enzyme molecules which can be

modified via different amino groups and leads to the ability to be exposed to different catalytic centers. According to Selvarajan $et\ al.$[20], the K_M value that increases after β-galactosidase is cross-linked with glutaraldehyde due to limited diffusion to substrates with high molecular weight. In addition, the increase in K_M can also occur due to changes in the microenvironment of enzyme molecules which depend on the enzyme tertiary structure. Mignaeault $et\ al.$[21] reported an increase in K_M after trypsin was crosslinked with glutaraldehyde. The crosslinking procedure results in more limited enzymes.

The V_{max} values of all the modified enzymes have increased compared to the native enzyme, which means that there is a conformational change on the active site of the enzyme due to cross-linking which causes the ability of enzyme-substrate complex formation to change. Selvarajan $et\ al.$[20] reported an increase in K_M and V_{max} after β-galactosidase-ZnO was cross-linked with glutaraldehyde, which showed the enzyme was able to catalyze faster and increase the hydrolysis ability of lactose.

3.5 Constants of thermal inactivation rate (k_i), half-life ($t_{1/2}$) and energy changes due to denaturation (ΔG_i) of the native and modified enzymes

The constant values of thermal inactivation rate (k_i), half-life ($t_{1/2}$) and energy changes due to denaturation (ΔG_i) of the native and modified enzymes are shown in Table 2.

Table 2. The values of k_i, $t_{1/2}$, and ΔG_i of native and modified α-amylase

Enzyme	k_i (min^{-1})	$t_{1/2}$ (min)	ΔG_i (kJ mol^{-1})
Native	0.0298	23.26	101.402
Modified with			
GA 0.01%	0.0183	37.87	102.656
GA 0.03%	0.0111	62.43	104.047
GA 0.05%	0.0160	43.31	103.038

The data in Table 2 above shows that the k_i values of the modified enzyme are lower than the k_i value of the native enzyme. The same result was also reported by Daba [5] after chemical modification of β-amylase using glutaraldehyde. According to Daba [5], the decrease in inactivation rate was due to the decrease in activation energy. The kinetic stability depends on the energy barrier to unfolding (partial unfolding in a transitional form is not irreversible). The activation energy between the folding and transition forms will decrease as the value of k_i decreases and enzyme stabilization occurs entropically. The lower k_i value indicates that the modified enzyme is less flexible in water because the cross-linking with glutaraldehyde causes the enzyme structure to be stiffer and its stability increases. The lowest k_i value is produced in the modification of the enzyme with 0.03% glutaraldehyde, indicating that the stability of the enzyme the best in these conditions.

The value of ΔG_i indicates the amount of energy required to denaturate an enzyme or the energy required to convert 1 mol L^{-1} of the substrate into 1 mol L^{-1} of the product. Increasing the value of ΔG_i in the modified enzyme indicates an increase in enzyme stability, this is due to the enzyme structure becoming increasingly rigid and inflexible. With its stiffer nature, the enzyme structure becomes stronger so that it takes a lot of energy to denature the enzyme.

The half-life ($t_{1/2}$) of the modified enzyme was 2.68 times that of the native enzyme. The half-life of modified enzymes (0.01; 0.03 and 0.05%) increased from 23.26 minutes to 37.87; 62.43; and 43.31 minutes, respectively (Table 2). The highest half-life obtained from modification with 0.03% glutaraldehyde, an increase of 2.68 times compared to the native enzyme. The half-life is the time when the enzyme activity reaches 50% of its original activity. The longer the half-life of the enzyme, the better the stability of the enzyme [20].

4. Conclusions

Based on the discussion above, the chemical modification using glutaraldehyde for α-amylase can increase the stability of the enzyme to determine the optimum pH, optimum temperature, increase the value of K_M and V_{max}, decrease the value (k_i), increase the half-life $(t_{1/2})$ and ΔG_i. This shows that the modified enzyme is more stable than the native enzyme.

5. Acknowledgments

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