

Inhibitory Effects of Cyanide on the Activity of Granular Starch Hydrolyzing Enzyme (GSHE) during Hydrolysis of Cassava (*Manihot Esculenta Crantz*) Starch

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Abstract

The kinetics and inhibitory effects of cyanide on the granular starch hydrolyzing enzyme (GSHE) activity during hydrolysis of cassava (*Manihot esculenta* Crantz) starch at low temperature were studied. The substrates included native cassava starch at various concentrations (100-400 g/L) and native cassava starches with added cyanide at various concentrations (50-150 mg/kg), while the concentration of enzyme was 1.5% (w/w). A decrease in reducing sugar concentration during hydrolysis of cassava starch indicated that the cyanide reduced the enzyme activity. Lineweaver-Burk plot of Michaelis-Menten equation was used to study the inhibition kinetics. The maximum velocity (V_{max}) value was higher for native cassava starch than that of native cassava starch with added cyanides. The presence of cyanide was found to reduce the V_{max} values. No significant different of the saturation constant (K_m) value between native cassava starch and native cassava starch with added cyanides was observed. Based on the inhibition type analysis, the effect of cyanide in the cassava starch can be classified as a noncompetitive inhibition, with the K_i value of 0.33 mg/L.

Keywords

cyanides, inhibitory, enzyme activity, kinetics, hydrolysis

1 Introduction

Cassava is a high-yielding crop that can grow well in the tropical and subtropical regions, such as Africa, South America, India and Southeast Asia. Indonesia is one of the cassava leading producers in the world. Cassava has many superior growing characteristics over the other crops that include high drought and flood-tolerance and ability to grow on light sandy soils and medium texture soils [1]. Another advantage of cassava is related to its higher starch content than many other stem tuber plants. The conversion of cassava starch into reducing sugar is expected to increase its economic potentials, especially applied to bitter cassava. Cassava starch can be converted into glucose syrup (for food applications) and to ethanol (for fuel application) through hydrolysis and fermentation processes. Based on its starch content, it is estimated that 7.2 tons of fresh cassava can produce 1.9570 ton of glucose or 1 ton of ethanol [2].

Cassava plant produces two cyanogenic glucosides (CG), namely linamarin and a small portion of lotaustralin [3]. Linamarin produces the toxic hydrogen cyanide

compound, which can be hazardous to consumers [4]. Based on the CG content, cassava can be classified into high cyanide (bitter) and low cyanide (sweet) cassavas [5]. Lambri et al. [6] reported that the total cyanide content of sweet white and bitter white cassava roots were 374 and 442 mg/kg (d.w.), respectively. Earlier, Djazuli and Bradbury [7] found the cyanogen content of cassava starch in Indonesia to be around 12 mg/kg (d.w.). Recently, Hargono et al. [8] investigated the effect of cyanide on hydrolysis of sweet cassava and bitter cassava starches using granular starch hydrolyzing enzyme (GSHE). It was observed that cyanide was an inhibitor for the enzyme. Tung et al. [9] studied the effect of the presence of 20 mg/L cyanide on the fermentation of cassava starch using *Aspergillus oryzae* IFO 30113 strains for 2-12 days. The cyanide was found to cause a decrease of both biomass and glucose concentration. Cyanide also decreased the catalase (CAT) enzyme activity (5.27% and 6.61%) in the brain tissues of the fish exposed to 0.5 mg/L concentration of cyanide for 24 and 72 h, respectively [10]. Several authors had investigated

the enzymatic hydrolysis of numerous tuber starches. These studies included the hydrolysis profiles of native and mildly heat-treated tapioca and sweet potato starches at sub gelatinization temperature [11], direct conversion of cassava and sweet potato roots slurry into glucose and high fructose syrups [12], and the production of glucose and high fructose syrup from various tuber starches [13]. The hydrolyzed starches were chemically analyzed for their degree of hydrolysis, which was also further physically proven by the presence of distinct pores penetrating deep into the starch granules [14]. However, the effect of cyanide that probably contained in the starch on the enzyme activity was not studied. The Michaelis-Menten equation provides an easy way of determining whether an enzyme inhibitor is either competitive, noncompetitive or uncompetitive [15].

The objectives of this study are to investigate the inhibition mechanism of GSHE activity by cyanide during hydrolysis of cassava (*Manihot esculenta* Crantz) starch and to obtain the kinetics parameters. The competitive, noncompetitive and uncompetitive inhibition kinetics models were used to quantify the inhibitory effect of this cyanogenic compound. The model parameters (K_m and V_{max}) obtained from this study is expected to be used for the scale up of the hydrolysis process into pilot plant or even the commercial scale.

2 Materials and Methods

2.1 Materials

Ten months age of cassava (*Manihot esculenta* Crantz) tubers were obtained from commercial farm located in Wonogiri district in Indonesia. The extraction procedure, cyanide content and physicochemical properties of the cassava used in this study were the same as those used in the previous research conducted by Hargono [8]. Cyanide (as Potassium cyanide) was the product of MERCK, potassium sodium tartrate tetrahydrate and 3,5-Dinitrosalicylic acid, sodium hydroxide (98%, Merck), sodium sulfite (98.5%, Merck), sulfuric acid (98.5%, Merck), sodium acetate buffer, glucose (99.5%), were purchased from their authorized distributor. Granular starch hydrolyzing enzyme as Stargen™ 002, contains *Aspergillus kawachi* alpha-amylase expressed in *Trichoderma reesei* and a glucoamylase from *Trichoderma reesei* that work synergistically to hydrolyze granular starch substrate to glucose was produced by Genencor (Palo Alto, USA). The activity and optimal pH range declared by the producer are 570 GAU/g and 4.0-4.5, respectively [16].

2.2 Enzymatic Hydrolysis

Cassava starch slurry of 100, 150, 200, 250, 300, 350 and 400 g/L concentration were used in this research. The slurry was adjusted to pH 4 by addition of 50 mM sodium acetate buffer. Then, the Stargen™ 002 enzyme was gently added to the slurry at 1.5% w/w concentration. The mixture was incubated at $80 \pm 1^\circ\text{C}$ in a thermostatic water bath heater with continuous stirring of 100 rpm for 15 min. The slurry was out cooled to room temperature ($30 \pm 1^\circ\text{C}$) and incubation was continued for 36 h. Sample were collected at 6 h interval and were subjected to reducing sugar and initial velocity determination. To study the inhibitory effect of cyanide on enzyme activity, cyanide with various concentration 50, 100 and 150 mg cyanide/kg of starch was added to the starch slurry. Samples were periodically withdrawn from the flask at 6 h interval and were substantially subjected to reducing sugar and kinetic parameters determinations. Prior to reducing sugar determination through Spectrophotometry, the samples were centrifuged (100rpm, 4°C and 10 min) to obtain the clear filtrates.

2.3 Analytical Methods for Determination of Reducing Sugar

The reducing sugar was measured using dinitrosalicylic acid (DNS) method described by Miller [17]. Reagent consisting of aqueous solution of 1% DNS, 0.05% sodium sulfite, 20% potassium sodium tartrate tetrahydrate and 1% sodium hydroxide solution was added in the ratio 3:1 to the samples in the glass tubes, and followed by shaken and incubated them in a boiling water bath for 8 min. Prior to measuring the absorbance at 540 nm by using a UV/visible spectrophotometer (UV-160A, SHIMADZU, Kyoto, Japan), the reacted samples were cooled in an ice water bath for 5 min. Glucose solutions (0 to 10 g/L) were used as standard. Therefore, the reducing sugar concentrations obtained were reported as g/L.

2.4 Determination of Kinetic Parameters

In general, the Michaelis and Menten [15] equation has proven to be the simplest yet a powerful approach to describe the kinetics of most enzymatic reaction, as shown below:

$$v_o = \frac{V_{max} [S]}{K_m + [S]} \quad (1)$$

where V_o is the initial reaction velocity, $[S]$ is the initial substrate concentration, V_{max} is the maximum rate of reaction at infinite substrate concentration, and K_m is the Michaelis-Menten constant of the substrate.

Cyanogenic inhibition can be characterized by end product inhibition and the enzyme kinetics using a set of linear plots, often of Lineweaver-Burk type [18]. This simplified approach in the kinetics experiments is to measure the initial reaction velocity, designated V_o . The amount of the product formed at different substrate concentration $[S_o]$ is plotted as a function of time. The initial reaction velocity $[V_o]$ for each substrate concentration is determined from the slope of the curve at beginning of the reaction [19]. The characteristic constants, namely V_{max} , K_m and K_i can be determined experimentally by incubating the enzyme with different substrate concentrations. The results can be plotted as a graph of the initial reaction velocity, V_o against the initial substrate concentration, $[S_o]$. When we plot of $1/V_o$ versus $1/[S_o]$, a straight line should be obtained with the y intercept (for determining V_{max}) and the x intercept (for determining K_m) [20-22]. The value of dissociation constant (K_i) can be determined using the Dixon plot, where the slopes of the Lineweaver-Burk plots are plotted as function of inhibitor concentrations $[I]$. For this plot, the x intercept represents the value of $-K_i$ [23].

2.5 Inhibitory Kinetic Analysis

The noncompetitive inhibition mechanism can be described by the Lineweaver-Burk equation in double-reciprocal form and can be written as follow:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \left[1 + \frac{[I]}{K_i} \right] \frac{1}{[S]} + \frac{1}{V_{max}} \left[1 + \frac{[I]}{\alpha K_i} \right] \quad (2)$$

and the secondary plot can be constructed as:

$$\text{slope} = \frac{K_m}{V_{max}} + \frac{K_m(I)}{V_{max}K_i} \quad (3)$$

$$\text{Y-intercept} = \frac{1}{V_{max}} + \frac{1}{\alpha K_i V_{max}} \quad (4)$$

where V is the enzyme reaction rate in the absence and presence of cyanide. K_i and K_m are the inhibition constant and Michaelis-Menten constant respectively. α is the ratio of the uncompetitive inhibition constant to competitive inhibition constant, and the value of α is 1 as noncompetitive inhibition. $[I]$ and $[S]$ are the concentration of inhibitor and substrate, respectively. The secondary plot of slope versus $[I]$ are linearly fitted, assuming a single inhibition site [23].

3 Result and Discussion

3.1 Effect of Cyanide on Reducing sugar

The presence of cyanide at 50, 100 and 150 mg/kg, during enzymatic hydrolysis of starch at concentrations (100-400 g/L) for 36 h, with concentration of enzyme 1.5% (w/w), pH 4 and 30°C resulted in a significant decrease of reducing sugar concentration as shown in Table 1. It was observed that the reducing sugar concentration increased with increasing initial concentration native cassava starch. However, in addition of cyanide (50-150 mg cyanide/kg) into the slurry of various starch concentrations (100-400g/L) decreased the reducing sugar concentrations. This condition indicates that cyanide being possibly an enzyme inhibitor; therefore it needs to be proven further. Masiero et al. [24] studied the hydrolysis of fresh potato (200 g/L) with Stargen™ 002 (45 GAUg⁻¹) for 62 h. After the first 20 h, the glucose concentration liberated was 30 g/L. Shanavas et al. [25] investigated a hydrolysis of cassava starch (200 g/L), by Stargen™ 002 with concentration 0.4% (w/w) for 24 h at 30°C. The reducing sugar concentration obtained was 185 g/L. Although, the same substrate concentration of 200 g/L and same enzyme were used in this research and other research groups, different glucose and reducing sugar concentrations were obtained during hydrolysis of starch. This phenomenon can be attributed to the difference in enzyme activity, enzyme concentration, reaction time and type of starch.

3.2 Effect of initial native cassava starch and cyanide concentrations on initial reaction velocity

Effect of the addition of cyanide at concentration (50-150 mg/kg) on the initial reaction velocity of enzyme was investigated. As shown in Fig. 1, the initial reaction velocity increases with the increase of substrate concentration to some extent. However, further increase in substrate concentration may cause saturation kinetics. Therefore, addition more substrate under this condition would not change the initial reaction velocity. Hence the slope of the plot of initial reaction velocity, V_o versus $[S_o]$ would approach zero.

A simple visualization of the enzyme-catalyzed reaction scheme is given in Fig. 2 [26]. At concentration of cassava starch $[S_o]$ up to 100 g/L the concentration of ES would be directly proportional to $[S_o]$. Hence, the velocity would depend on $[S_o]$. At very high concentration of S , practically all of the enzyme would be present in the form of the ES complex.

Table 1 The reducing sugar concentrations obtained from enzymatic hydrolysis of cassava starch with and without addition of cyanide

Cassava starch	Reducing sugar, g/L	Reduction of reducing sugar, %
NCS 100 g/L	51.74	-
NCS 100 g/L + 50 mg cyanide/kg	49.06	5.18
NCS 100 g/L + 100 mg cyanide/kg	46.21	10.69
NCS 100 g/L + 150 mg cyanide/kg	42.01	18.82
NCS 200 g/L	59.4	-
NCS 200 g/L + 50 mg cyanide/kg	55.8	6.06
NCS 200 g/L + 100 mg cyanide/kg	52.6	11.45
NCS 200 g/L + 150 mg cyanide/kg	50.1	15.66
NCS 300 g/L	69.11	-
NCS 300 g/L + 50 mg cyanide/kg	64.12	7.22
NCS 300 g/L + 100 mg cyanide/kg	61.35	11.22
NCS 300 g/L + 150 mg cyanide/kg	58.43	15.45
NCS 400 g/L	69.56	-
NCS 400 g/L + 50 mg cyanide/kg	65.98	5.15
NCS 400 g/L + 100 mg cyanide/kg	61.29	11.89
NCS 400 g/L + 150 mg cyanide/kg	56.22	19.18

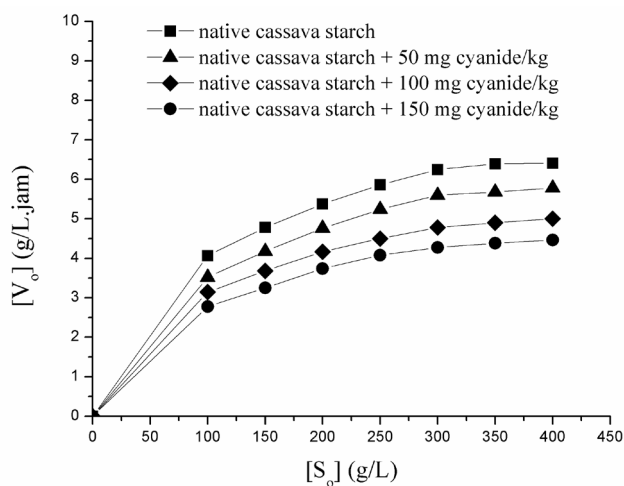


Fig. 1 Effect of native cassava starch with and without the presence of cyanide at various concentrations (50-150 mg/kg) at pH 4 and 30°C.

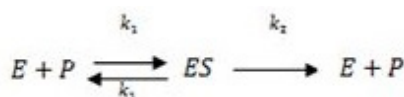


Fig. 2 Enzyme-catalyzed reaction

Dhital et al. [24] studied the inhibition of α -amylase activity by cellulose. They hydrolyzed maize starch at concentrations of 5 to 40 mg/mL using α -amylase with the presence of 0 to 30 mg/mL of cellulose. The V_{max} values in the absence of cellulose were 0.125 mM/min. On the other hand, the values of V_{max} with the presence of

5 to 30 mg/mL of cellulose were ranged from 0.009 to 0.036 mM/min. This finding clearly indicates that cellulose does indeed inhibit α -amylase activity over the range of concentration studied. This finding clearly indicates that cellulose does indeed inhibit α -amylase activity over the range of concentration studied.

At a given reaction time, the concentrations of reducing sugar produced from hydrolysis of native cassava starch without the presence of cyanide were higher than that of with cyanide. This observation indicates higher hydrolysis rate of native cassava starch without the presence of cyanide as compared to that with cyanide. Therefore, we recommend to prefer the use of free cyanide cassava starch in enzymatic hydrolysis instead of those with considerable cyanide content, so that the concentration of reducing sugar obtained can be higher.

3.3 Determination of Kinetic Parameters

The mode of kinetic inhibition of the cyanide was determined by Lineweaver-Burk plot. The plot of $1/V_0$ versus $1/[S_0]$ yields a linear line with a slope of K_m/V_{max} and intercept of $1/V_{max}$ as shown in Fig. 3.

The results show that double reciprocal plots straight lines were intercepted at single point in the second quadrant. This plot clearly indicated the pattern of the noncompetitive inhibition. Table 2 summarizes the comparison of the maximum reaction velocity (V_{max}) and the saturation

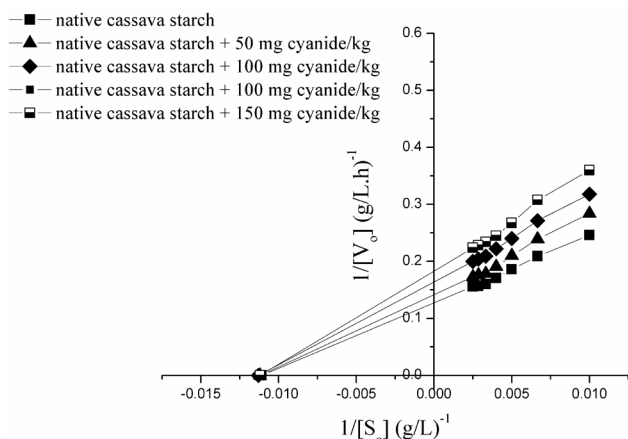


Fig. 3 Lineweaver-Burk plots for native cassava starch with and without presence of cyanide at various concentrations (50-150 mg/kg) at pH 4 and 30°C.

constants (K_m) obtained in hydrolysis of native cassava starch and native cassava starch added with cyanide at various concentrations (50-150 mg/kg) at pH 4 and 30°C. Subsequently, the inhibitor constant, K_i , was found to be 0.33 mg/L, as shown in Fig 4. This result is well suited to the noncompetitive inhibition model shown in Fig. 5. The S and I are not mutually exclusive, but EIS is catalytically inactive. When S binds, the enzyme undergoes a conformational change with aligns the catalytic centre, with the susceptible bond of S; I interferes with the conformational change, but no effect on S binding. Adam [27] also reported cyanide, probably in the undissociated form to be competitive inhibitor for green bean lipoxygenase with inhibitor constant $K_i = 1.54 \times 10^{-3} \text{M KCN}$.

Zołnierowicz et al. [28] studied the effect of KCN of mitochondrial malic enzyme from human placenta. The study showed that KCN is an uncompetitive inhibitor of this enzyme. In contradiction to the mitochondrial enzyme, the cytosolic malic enzymes from other sources were only slightly affected by KCN and under the same conditions the effect of azide was negligible. Dhital et al. [29] investigated the α -amylase hydrolysis of maize starch in the presence of cellulose. They found that the inhibition is a mixed mode inhibition or non-competitive inhibition. The dissociation constant for EIS complex obtained in their study was 6 mg/mL.

Recently, Hargono et al. [8] investigated the kinetics of the enzymatic hydrolysis of sweet cassava starch and bitter cassava and gadung (*Dioscorea hispida* Dennst) flours using GSHE at low temperature. The Michaelis-Menten constant (K_m) for these three substrates were 137.74, 141.64 and 140.84 g/L, respectively. Based on the inhibition type

Table 2 Comparison of the maximum velocity (V_{max}) and the saturation constants (K_m)

Substrate	V_{max} (g/L.h)	K_m (g/L)
NCS	7.87	91.13
NCS + 50 mg cyanide/kg	6.90	91.42
NCS+100 mg cyanide/kg	6.02	90.54
NCS+150 mg cyanide/kg	5.44	91.68

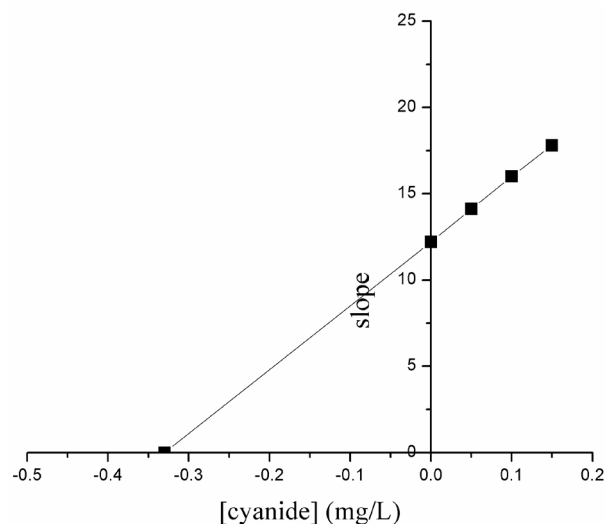


Fig. 4 The plot for the determination of the inhibitor constant for noncompetitive inhibition

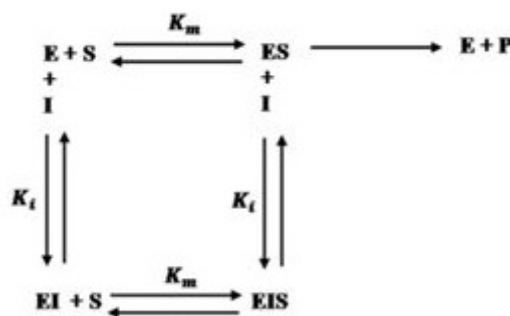


Fig. 5 Type of noncompetitive inhibition

analysis, the effect of cyanide in the sweet cassava starch, bitter cassava and gadung flours enzymatic hydrolysis can be classified as a noncompetitive inhibition.

4 Conclusion

Based on the experimental and modeling results, it is clearly shown that cyanide gives an inhibitory effect on the enzymatic hydrolysis of cassava starch using GSHE. Addition of cyanide (50-150 mg cyanide/kg) during hydrolysis of native cassava starch decreased the V_{max} values significantly. However, addition of cyanide did not significantly change the Michaelis-Menten constants (K_m) of

the system. Based on the Lineweaver-Burk plot for native cassava starch at various concentrations (100–400 g/L) and the effect of cyanide concentrations (50–150 mg/kg),

the cyanide inhibits the cassava starch hydrolysis according to a non-competitive inhibition, with the K_i value was 0.33 mg/L.

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