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Research Article

Kinetics of the Enzymatic Hydrolysis of Sweet Cassava Starch, Bitter Cassava, and *Gadung* (*Dioscorea hispida Dennst*) Flours at Low Temperature

Hargono Hargono , Bakti Jos, Andri Cahyo Kumoro*

Department of Chemical Engineering, Faculty of Engineering, Diponegoro University
Jl. Prof. Soedarto, SH, Kampus Undip Tembalang, Semarang 50275, Indonesia

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Abstract

Starch is a potential substrate for this purpose, but the extra cost is needed to hydrolyze it into reducing sugar. As an alternative to the expensive and energy demanding conventional hydrolysis process, the low-temperature hydrolysis is being studied. Granular starch hydrolyzing enzyme (GSHE) was used in the process to degrade starch into reducing sugar at 30 °C and pH 4. The substrates included sweet cassava starch, bitter cassava, and *gadung* (*Dioscorea hispida Dennst*) flours. Starch concentrations studied were in the range 50-400 g/L, respectively, while the concentration of enzyme was maintained at 1.5 % (w/w). The optimum productivity of reducing sugar (Q_{rs}) of sweet cassava starch, bitter cassava, and *gadung* flours were 4.11, 3.10, and 0.52 (g/L.h), respectively. The Michaelis-Menten constants (K_m) for these three substrates were determined as 139.84 g/L, 141.43 g/L, and 140.92 g/L for sweet cassava starch, bitter cassava, and *gadung* flours, respectively. Increasing of cyanide concentration during hydrolysis process of sweet cassava starch, bitter cassava, and *gadung* flours decreased $V_{max,}$ significantly. Based on Lineweaver-Burk plot for sweet cassava starch, bitter cassava, and *gadung* flours (50-400 g/L) with the cyanide concentration of 42, 168, and 176 mg/kg, can be classified as noncompetitive inhibition with K_I value of 0.0317.

Keywords: GSHE; Low temperature hydrolysis; Inhibition kinetics; Reducing sugar

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1. Introduction

Cassava is a starchy root crop that is grown almost entirely in the low land tropics. Cassava roots are an excellent source of carbohydrates. The roots are very starchy, and the young leaves are a good source of protein. As a defense mechanism against attack by a predator, cassava produces two cyanogenic glucosides, linamarin, and a small of lotaustralin [1]. Cassava cultivars can be classified into high cyanide (bitter) and low cyanide (sweet) [2]. Lambri *et al*. [3] reported that the total cyanide content of cassava root for sweet white and bitter white was 374 and 442 mg/kg (d.w), respectively. Djazuli and Bradbury [4] reported of 14 samples of cyanogens content of cassava starch in Indonesia, obtained by the acid hydrolysis method

* Corresponding Author.
E-mail: andrewkomoro@che.undip.ac.id (Kumoro, A.C.)
Telp.: +6224-7460058; Fax.: +6224-76480675

[5]. The maximum value of cyanogens content in cassava starch and cassava flours were 12 ppm and 149 ppm. Toxicity of cyanide in the tubers depending on the form of granules. The degree of toxicity of cyanogens in starch < chip < flour. The World Health Organization (WHO) has set the safe level of cyanogens in cassava flour at 10 ppm [5].

Bitter Yam (*Dioscoreae hispida* Dennst) is commonly found in secondary forest, grows under shaded areas or near sreams, and locally known as *gadung* [6]. However, some studies have also pointed out that this yam tuber also contains some toxic compound, which can seriously impact the health of the people who consume the tuber. Among the yam species, *Dioscoreae hispida* was considered as one of the most underutilized species because of the presence of poisonous alkaloids known as dioscorin and cyanogens as anti-nutrient [7].

Recently, inhibitory kinetics of the granular starch hydrolyzing enzyme (GSHE) by inhibitor have not fully studied. The effect of cyanide on GSHE activity has been studied. Adam [8] reported that cyanide, probably in the undissociated acid form, it was shown that cyanide is a weak competitive inhibitor of green bean lipoxygenase. Parashar and his coworker [9] reported that the toxicity of cyanide is hitherto attributed to bind to the heme proteins active site and thereby inhibit their activity. The reason for this probably lies in the presence of components in various preparations which react with cyanide and reduce its effectiveness as GSHE inhibitor.

The objective of this study is to investigate the effect of cyanide in the sweet cassava starch, bitter cassava, and *gadung* flours on the activity of GSHE. Competitive, noncompetitive, and uncompetitive inhibition mathematical models were applied to quantify the inhibitory effect of this cyanogenic compound.

2. Materials and Methods

2.1. Materials

2.1.1. Cassava and *gadung* tubers

Ten months old of bitter cassava was called Pandemir (*Manihot glaziovii*) and the sweet cassava (*Manihot esculenta*) tubers were obtained from Wonogiri district in Indonesia, while *gadung* tuber with 9 months old was obtained in Godean district in Indonesia.

2.1.2. Sweet cassava starch extraction

The sweet cassava tuber was selected, washed, peeled, and grated to finer particles. The starch was extracted from the grated pulp by sieving while the fiber was retained. The fiber retained was repeatedly washed for at least four times with distilled water on the cloth screen. The fiber, still contains some unrefined starch, was pressed by hand and dried in the sun. The powdery starch was then stored in an air tight container to prevent contamination and moisture.

2.1.3. Bitter cassava and gadung flours extraction

The tuber of bitter cassava and *gadung* were washed to remove dirt, peeled, sliced, air-dried, milled, and sieved to obtain 100 mesh size raw flour. Analysis of physicochemical properties of the sweet cassava starch, bitter cassava, and *gadung* flours are presented in Table 1.

2.1.4. Reagents

Cyanide (as Potassium cyanide) was the product by MERCK, potassium sodium tartrate tetrahydrate and 3,5-dinitrosalicylic acid (Sigma-Aldrich), NaOH (98 %, Merck), Na_2SO_3 (98.5 %, Merck), H_2SO_4 (98.5 %, Merck), sodium acetate buffer (Sigma-Aldrich), glucose (99.5 %, Sigma-Aldrich).

2.1.5. Enzyme

The enzymes were a granular starch hydrolyzing enzymes, Stargen™ 002, which is a mixture of α-amylase and glucoamylase produced by Genencor (Palo Alto, USA). The activity and optimal pH range declared by the producer are 570 GAU g/L and 4.0-4.5, respectively. A Glucoamylase Unit (GAU) is the amount of enzyme that releases 1 g of reducing sugar calculated as glucose per hour from the soluble

Table 1. Properties of sweet cassava starch, bitter cassava, and *gadung* flours

Parameters	native cassava starch (control)	sweet cassava starch	bitter cassava flour	*gadung* flour
Moisture content (%), w/w	10.22	10.25	10.80	12.2
Starch (%), w/w	85.84	83.12	82.70	26.38
Total cyanide, mg/kg	0	42	168	176

starch substrate under the conditions of the assay [10].

2.2. Reversible inhibition

One common type of reversible inhibition is called competitive [11]. Three modes of reversible enzyme inhibition can be distinguished by their effects on different inhibitor. Double-reciprocal plot data collected in the presence of different concentration of an inhibitor reveal the value of K_I, the dissociation constant of the inhibitor from the enzyme. The inhibition effect of K_I on the reaction kinetics is reflected on the normal K_m and V_{max} observed in Lineweaver-Burk plots [12]. The rate equations of three simple types of inhibition [13] are:

Competitive inhibition:

$$\upsilon_0 = \frac{v_{\max}[S]}{K_m\left[1+\frac{I}{K_I}\right]+[S]} \quad (1)$$

Noncompetitive inhibition:

$$\upsilon_0 = \frac{v_{\max}[S]}{(K_s+S)[1+K_I]} \quad (2)$$

Uncompetitive inhibition:

$$\upsilon_0 = \frac{v_{\max}[S]}{K_m+[S]\left[1+\frac{1}{K_I}\right]} \quad (3)$$

and in the double-reciprocal equation becomes:

Competitive inhibition:

$$\frac{1}{\upsilon_0} = \frac{K_m}{v_{\max}}\left\{1+\left[\frac{1}{K_I}\right]\left[\frac{1}{S}\right]\right\}+\frac{1}{v_{\max}} \quad (4)$$

Noncompetitive inhibition:

$$\frac{1}{\upsilon_0} = \frac{K_m}{v_{\max}}\left[\frac{1}{S}\right]+\frac{1}{v_{\max}}\left(1+\frac{1}{K_I}\right) \quad (5)$$

Uncompetitive inhibition:

$$\frac{1}{\upsilon_0} = \frac{K_m}{v_{\max}}\left\{1+\left[\frac{1}{K_I}\right]\left[\frac{1}{S}\right]\right\}+\frac{1}{v_{\max}}\left\{1\left[\frac{1}{K_I}\right]\right\} \quad (6)$$

where υ_0 is initial velocity (g/L.h), V_{max} is maximum reaction velocity (g/L.h), [S] is substrate concentration remaining at time t (g/L), K_m is Michaelis-Menten constants, K_I is inhibitor constant from the enzyme, and [I] is inhibitor concentration (mg/L).

The plot of the [$1/\upsilon_0$] versus 1/[S] at different cyanide in tubers was constructed to confirm the reversibility of cyanide mediated inhibition, the straight line all passed through the origin, and the slope was decreased with increasing concentration of inhibitor [11].

2.3. Determination of inhibitory type

The double-reciprocal plot offers and easy way of determining whether an enzyme inhibitor is competitive, noncompetitive or uncompetitive. Figure 1 show profile of the Lineweaver-Burk plot for determination of three common types of reversible kinetic constants [11,13].

2.4. Enzymatic hydrolysis

The sweet cassava starch, biter cassava and the *gadung* flours slurries of 50, 100, 150, 200, 250, 300, 350, and 400 g/L concentration were used in this research. The slurry was adjusted to pH 4 (in 50 mM sodium acetate buffer), and Stargen™ 002 (1.5 % v/w) was added, mixed and incubated at a temperature of 80 °C for 15 min at 100 rpm. The slurry was out cooled to

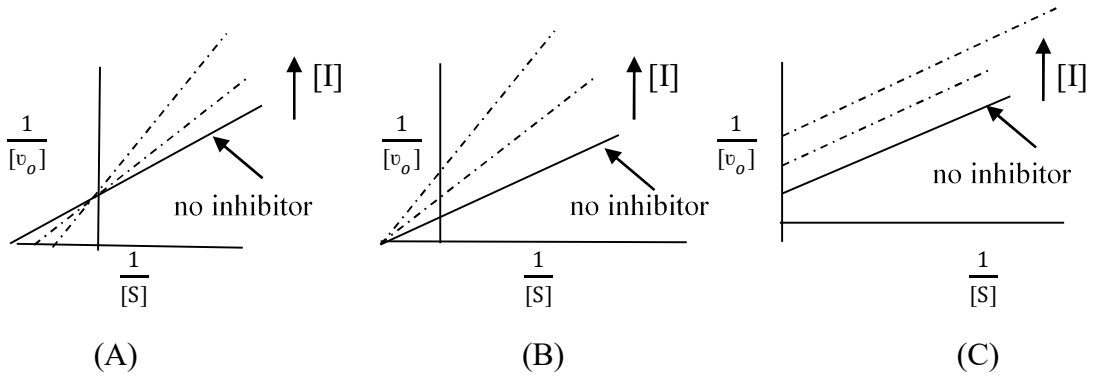

Figure 1. Lineweaver-Burk plot for (A) competitive inhibition, (B) noncompetitive inhibition, and (C) uncompetitive inhibition

room temperature (30±1 °C) and incubation was continued for 24 h. The samples were periodically withdrawn from the flask at 6 h interval and substantially subjected to reducing sugar. Before the samples were analyzed by a spectrophotometer, they were centrifuged (100 Hz, 4 °C, and 10 min) to obtain the filtrate.

2.5. Analytical methods

The starch content was determined by AQAC method [14]. The water content in cassava starch was determined by standard drying method in an oven at 105 °C to constant mass [15]. The total cyanide analysis by the acid hydrolysis method [5]. During the cassava starch hydrolysis, the content of reducing sugar was measured using dinitro salicylic acid method [16]. The reagent consisting of an aqueous solution of 1 % 3,5-dinitrosalicylic acid, 0.05 % Na sulfite, 20 % Na-K tartrate, and 1 % NaOH solution was added in the ratio 3:1 to the samples in glass tubes, shaken in incubated in a boiling water bath for 8 min. The reacted samples were cooled in an ice water bath for 5 min, prior to measuring absorbance at 540 nm by using a UV/visible spectrophotometer (UV-160A, SHIMADZU, Kyoto, Japan). Glucose (0 to 60 g/L) was used as a standard, therefore, reducing sugar concentrations was reported as g/L.

2.6. Productivity of reducing sugar (Q_{rs})

The productivity of reducing sugar (Q_{rs}) in the first hour of hydrolysis, which is calculated by equation (7):

$$Q_{rs}(t) = \frac{C_{rs,t} - C_{rs,t=0}}{t} \quad (7)$$

where Q_{rs} is the productivity of reducing sugar (g/L.h), $C_{rs,t}$ is the mass concentration of remaining reducing sugar (g/L) when productivity is calculated, $C_{rs,t=0}$ is the mass concentration of reducing sugar before the enzyme are added to the medium, and t (h) is the time for which for productivity is calculated, being $t = 0$ defined as the moment when the enzyme blend are added to the medium [17].

2.7. Determination of kinetic parameters

The amount of reducing sugar at different substrate concentration [S] is plotted as a function of time. The initial velocity of sweet cassava starch, a bitter cassava and *gadung* flours at a concentration of 50-400 g/L is determined from the slope of curve the relationship between reducing sugar versus time at beginning of a reaction [11]. The characteristic constant V_{max} and K_m can be determined experimentally by incubating the enzyme with different concentrations substrate. The results can be plotted as a graph of initial of velocity, υ_0 against initial concentration of substrate [S_o]. Based on equation (4-6) when we plot of $1/\upsilon_0$ versus $1/[S_0]$ a straight line is obtained Y intercept (for determining V_{max}) and X intercept (for determining K_m) as described in Figure 1 [11,13].

2.8. Determination of the inhibitor constants for a noncompetitive inhibitor

The value of dissociation constant (K_I) can be determined as the intercept of the slope by plotted as a function of inhibitor concentration [I]. For this plot, the x-intercept is equal to $-K_I$ [18].

3. Results and Discussion

3.1. Determination of reducing sugars by GSHE from sweet cassava starch, bitter cassava, and *gadung* flours hydrolysis and determination of productivity of reducing sugar

The reducing sugars obtained from sweet cassava starch, bitter cassava flour, and *gadung* flour by GSHE with concentration of 1.5 % (w/w), and starch concentration of 200 g/L at 30 °C and pH 4 during hydrolyzing time of 0 to 24 h are given in Figure 2. At time of 0

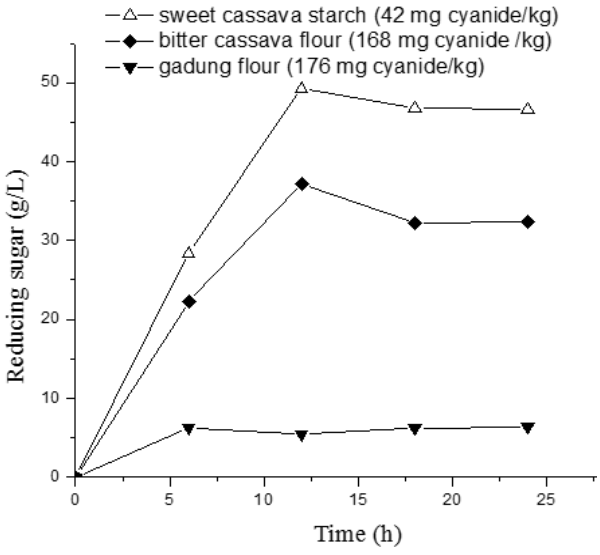

Figure 2. Hydrolysis profile of sweet cassava starch, bitter cassava, and *gadung* flours at substrate concentration of 200 g/L, enzyme concentration of 1.5 % (w/w), pH 4, and temperature of 30 °C

to 24 h, product of the reducing sugar of sweet starch was higher than the bitter and *gadung* flours. At hydrolyzing time of 12 h, a maximum reducing sugar (sweet cassava starch of 40.98, bitter cassava flour of 37.21, and *gadung* flour of 5.36 g/L), respectively, is appeared. Furthermore after 12 hours, the hydrolysis of both types of cassava decelerated, and then followed by constant rate at 18 to 24 h. Based on eq. (7), the optimum productivity of reducing sugar (Q_{rs}) of sweet cassava starch, bitter cassava, and *gadung* flours were 4.11, 3.10, and 0.52 (g/L.h), respectively. The starch content in *gadung* tuber is much lower than sweet cassava and bitter cassava tubers, so the cyanide content in the *gadung* tuber is much higher than sweet cassava and bitter cassava tubers, as a consequence the cyanide may inhibit the enzyme leading to decrease of enzyme activity. Shanavas *et al.* [19] reported the reducing sugar formed from cassava starch by varying level of Stargen, at pH 4.5 and temperature of 30 °C. It was found that the reducing sugar of 98.3 g/L could be hydrolyzed by Stargen level 100 mg on 10 % (w/v) starch. Also reported by Yussof *et al.* [20], that the hydrolysis time of 8 to 24 h hydrolysis native tapioca starch increases reducing sugar, as indicated by increasing dextrose equivalent (DE) 18 to 35.7 %.

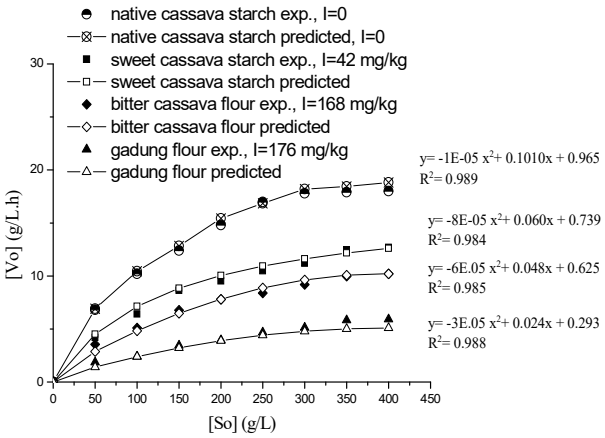

Figure 3. Effect of initial sweet cassava starch, bitter cassava and *gadung* flours at concentration (the range 50-400 g/L) on initial velocity of enzyme (pH 4, 30°C)

3.2. Effect of sweet cassava starch, bitter cassava, and *gadung* flours on initial activity of enzyme

Effect of initial sweet cassava starch, bitter cassava, and *gadung* flours at concentration of 50-400 g/L on initial activity of enzyme (pH 4, 30 °C) was investigated. As shown in Figure 3, the relationship of initial activity of enzyme to substrate concentration frequently assumes the form of saturation kinetics. It was observed that the initial activity of enzyme increased with increasing initial sweet cassava starch, bitter cassava and *gadung* flours concentration up to 400 g/L. The initial activity of the enzymes increased from 4.64 to 12.66 g/L.h (sweet cassava starch), 3.56 to 10.20 g/L.h (bitter cassava flour), and 1.99 to 5.75 g/L.h (*gadung* flour). The straight lines all passed then origin, and the slope was decreased with increasing concentration of cyanide in tubers. These result suggested that the inhibition of cyanide on GSHE activity was reversible. Adam [8] were also reported that the cyanide, probably in the undissociated, is shown to be a weak competitive inhibitor with inhibitor constant $K_I = 1.54\times10^{-3}$ M KCN.

3.3. Determination of kinetics parameters and types of reversible inhibitions

The Michaelis-Menten equation can be linearized in double-reciprocal form [21]. A plot of $1/\upsilon_0$ versus $1/[S_0]$ yields a linear line with a slope of K_m/V_{max} and y-axis intercept $1/V_{max}$. As shown in Table 2, the maximum rate (V_{max}) and saturation constant (K_m) of sweet cassava starch, bitter cassava and *gadung* flours were obtained. The effect of cyanide on the activity of GSHE was investigated by the equations (4-6) that were derived for competitive, non-competitive and uncompetitive inhibition. The computer program Excel 7.0 was used to solve the equations (4-6) by minimizing the mean square of residuals. The inhibition constants for the sweet cassava starch, bitter cassava

Table 2. Comparison of the maximum velocity and the saturation constans obtained in the sweet cassava starch, bitter cassava and *gadung* flours at pH 4 and temperature of 30°C

Substrates	K_m/V_{max}, (h)	V_{max} (g/L.h)	K_m (g/L)	Percentage of error (%)
Sweet cassava starch	8.25	16.95	139.84	2.85
Bitter cassava flour	10.61	13.33	141.43	2.84
Gadung flour	18.89	7.46	140.92	4.05

and *gadung* flours of cyanide content 42, 168, and 176 mg/kg from experimental for the activity of GSHE data were calculated. The non-competitive inhibition model described the experimental activity of GSHE rate better than competitive and uncompetitive inhibition models. A plot of $1/\upsilon_0$ versus $1/[S_0]$ gives a family straight line with a positive intercept at the y-axis, which indicates that the cyanide is a noncompetitive inhibitor of the enzyme (Figure 4).

The above results suggest that the inhibitor only binds to free enzyme rather than enzyme-substrate complex, the relative percentage error between the experimental and predicted activity of GSHE rate using non-competitive inhibition model for sweet cassava starch was obtained to be 2.85 %, while for bitter cassava and *gadung* flours can be shown in Table 2. To obtained the value of K_I can be determined as the intercept in Figure 5. The K_I value was 0.0317.

4. Conclusions

The results clearly showed that the cyanide gives an inhibitory effect on the enzymatic hydrolysis of sweet cassava starch, bitter cassava and *gadung* flours using GSHE at pH 4 and temperature of 30 °C. Increasing of cyanide concentration during hydrolysis process of sweet cassava starch, bitter cassava, and *gadung* flours decreased V_{max} significantly. The Michaelis-Menten constants (K_m) for these three substrates were determined as 139.84, 141.43, and 140.97 g/L for sweet cassava starch, bitter cassava, and *gadung* flours, while the value of K_m/V_{max} was calculated as 8.25, 10.61, and 18.89 h, respectively. Based on the Lineweaver-Burk plot, the sweet cassava starch, the bitter cassava, and the *gadung* flours (50-400 g/L) with cyanide concentration of 42, 168, and 176 mg/kg, respectively can be classified as a noncompetitive inhibition, with K_I value of 0.0317.

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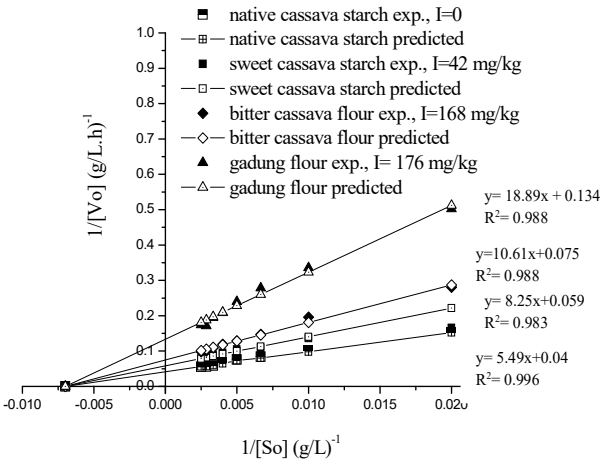

Figure 4. Lineweaver-Burk plots for sweet cassava starch, bitter cassava and *gadung* flours at enzyme concentration of 1.5% (w/w), pH 4, and temperature of 30 °C

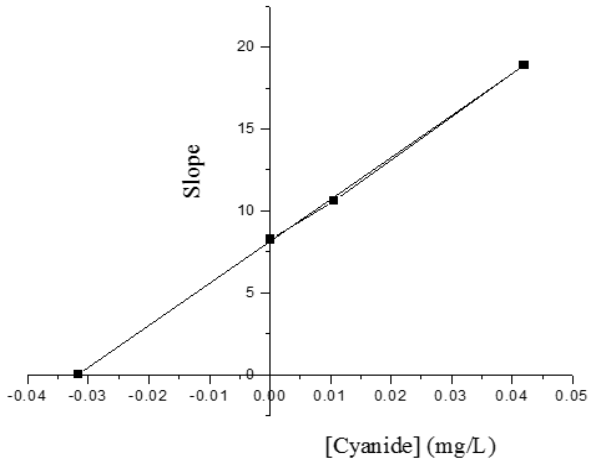

Figure 5. Determination of the inhibitor constants (K_I) for a noncompetitive inhibitor and the value of K_I is determined from the x intercept of the line

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