Inhibitor Effect (Lipid and Protein) in Starch Hydrolysis to Produce Glucose by using Amyloglucosidase

Munira¹,²,³, Padil²,³, Sarto³, M Hidayat³

¹Chemical Engineering Department, Universitas Muslim Indonesia, Jl Urip Sumoharjo KM 5, Makassar, Indonesia 90231
²Chemical Engineering Department, Universitas Riau Kampus Bina Widya, HR. Subrantas Km.12,5, Simpang Panam, Pekanbaru, Indonesia
³Department of Chemical Engineering, Universitas Gadjah Mada, Grafika 2 Street, Yogyakarta, Indonesia
Email : mhidayat@ugm.ac.id

Abstract. Microalgae contains approximately 24 % of starch which is potential for bioethanol feedstock by enzymatic hydrolysis. The problem is that starch in microalgae is accumulated with protein and lipid, while the interaction of starch-protein and starch-lipid might inhibit enzymatic hydrolysis of starch. Native starch, isolate protein and palmitic acid are used to investigate the interaction effects of starch-protein and starch-lipid in microalgae. This study aimed to investigate effects of protein and lipid concentration (1:0, 1:0.05, 1:0.1, 1:0.25, 1:0.5, 1:1) (w/w) added into native starch hydrolysis by amyloglucosidase enzyme were incubated at 45°C for 3 hours. The product of hydrolyzed was determined as glucose with spectrophotometer UV-Vis at 540 nm. The result shows that the increasing of protein and lipid concentration added into starch granule will significantly (P<0.05) decrease concentration of glucose product. The kinetic parameters are evaluated by Michaelis-Menten models for uncompetitive inhibition.

Keywords : Amyl glucosidase, Inhibitor Effect, Glucose

1. Introduction

Starch is one of the macromolecules that consisting of two types of polysaccharides is amylose and amylpectin. They consist of glucose monomers which formed by alpha 1,4-glycosidic bonding and alpha 1,6-glycosidic bonding of glucose. Starch, the main composition is produced by plants to stock excessive nutrition as a photosynthesis product for a long time, can be used as the bioethanol feedstock. Microalgae is one of the plants that contain numerous starches. In microalgae, starch is found in the cell with the variety of content that is depending on the species of microalgae, such as microalgae Tetraselmis chuii contains 19.62 % starch or approximately 24% of its total carbohydrate.

Generally, the producing of bioethanol from microalgae is processed by two steps. The first is converted starch to glucose then produced bioethanol from glucose. Enzymatic hydrolysis is one of the methods to produce glucose from starch that due to its numerous advantages of the step, operating at low temperature and high selectivity but consuming long time. Amyloglucosidase enzyme from Aspergillus niger can be used to hydrolysis of starch by breaking alpha 1,4-glycosidic bonding and alpha 1,6-glycosidic bonding then glucose was produced. In microalgae, starch is found in the cell which is accumulated with protein, lipid, and others. The lipid content in dry biomass microalgae approximately 50 % [5]. One of the microalgae species, Tetraselmis chuii, contained lipid which was 10.7% (weight of dry biomass) which its free fatty acids content was 5.4%, as well as 50 % of its free fatty acids, was saturated fatty acid with the content of most fatty acid was palmitic acid. Furthermore, protein contained in microalgae approximately 6-70 % (weight of dry biomass). This protein consists of amino acids that have a high nutritional quality. According to a well-balanced protein that recommended by WHO/FAO with comparing the amino acid profile of various microalgae with some food items showed the amino acid profile of microalgae has the nutritional quality that is comparable to egg and soy protein.
Lipid and protein, accumulated with starch, might inhibit enzymatic hydrolysis of starch. Glucose yield was significantly decreasing by adding the fatty acid such as lauric acid, meristic acid, palmitic acid, oleic acid acted as inhibitors in starch hydrolysis by \textit{alpha-amylose} and amylase enzymes. The interaction of starch-lipids formed an inclusion complex, called amylase-lipid complex (ALC), can affect the characteristics of starch. As well as the presence of protein in starch was found in starch granule surface that can affect of texture, mechanical properties and digestion of starch by enzyme [14], owing to the interaction of starch-protein of hydrogen bonds might be formed between anionic groups of starch with cationic groups of protein that forming a protein matrix around the starch granules which can block access of enzyme to substrate. In this study, native starch, palmitic acid, and isolated soy protein are used as an approach to investigated of starch-lipid interaction.

The experiment aimed to investigate limit of palmitic acid and protein concentration added into native starch hydrolysis by amylase enzymes which decreases of glucose yield and to study the inhibit mechanism as well as kinetic parameters.

2. Method

2.1. Materials

Native Corn Starch and Palmitic Acid were obtained from Sigma Aldrich, Amyloglucosidase enzyme from \textit{Aspergillus niger} (E.C 3.2.1.3) with enzyme activity 150,000 U/mg was purchased from Suntaq Enzyme, isolate soy protein, n-hexane, CH$_3$COOH, NaCH$_3$COOH and Nelson Reagen were obtained from local suppliers.

2.2. Preparation

2.2.1. Preparation of Starch-Lipid. Starch-lipid mixture used in preparing to blend [16]: Starch was added into the palmitic acid solution which is dissolved in hexane. The component was stirred at 100 rpm at room temperature for overnight. Hexane in mixture component was removed by added N$_2$.

2.2.2. Preparation of Starch-Protein. Amount of starch and protein with a specific ratio was mixed in dry sample container then homogenized [13].

2.3. Hydrolysing Starch-lipid and Starch-protein

Different concentration of lipid and protein to starch were applied in this experiment. The initial ratio of lipid and protein to native starch were varied from 1:0, 1:0.05, 1:0.1, 1:0.25, 1:0.5 and 1:1. The ratio of enzyme to substrate ([E]/[S]) is constant ([E]/[S] = 0.1). Hydrolysis process was incubated at pH 4.5, 45$^\circ$ C for 180 minutes. Samples were collected after every 30 minutes interval and immediately immersed in water bath at 90 $^\circ$C for 10 minutes and then cooled to -30 $^\circ$C for 1 hour in order to stop the enzyme activity. The product of hydrolysis the at 4000 rpm for 15 minutes a supernatant contains glucose which was analyzed using Nelson-Simongy method.

3. Results

When native starch as a control sample (without adding other materials, 0%) was hydrolyzed with amylase enzymes for 3 hours at pH 4.5 and temperature 45$^\circ$C, 47% substrate was converted to glucose. By adding of inhibiting materials, palmitic acid as lipid and isolated soy protein as protein, in the different ratio to starch (1:0.05; 1:0.1; 1:0.25; 1:0.5; 1:1) show decreasing of glucose production by increasing of inhibiting concentration (Figure.1).
Figure 1. Glucose concentration as a hydrolysis of starch production by amyloglucosidase enzyme at 45 °C, pH 4.5. (a) Starch-Lipid (palmitic acid) component, (b) Starch-Protein (Isolate soy protein) component. *1:0.0 sample is a native starch 100 % (without lipid or protein)

Indeed, both palmitic acid and protein might be decreased of the glucose production dramatically. According of t-test analysis with confident index 95% (P<0.05) which compare of glucose production by control sample and starch-inhibit materials sample in various of ratio shows glucose concentration significantly (P<0.05) decrease in ratio of native starch-palmitic acid 1:0.5 (50% w/w palmitic acid) and native starch-soy protein 1:1 (100% w/w isolate soy protein).

The substrate conversion by adding 50% (w/w) of palmitic acid can decrease ~30% from control sample result, instead of the presence of protein on native starch hydrolysis decrease ~20% of substrate conversion (Figure.2). To study about characteristic of inhibitors was determined as uncompetitive inhibitors for all adding materials (lipid and protein). Then, the kinetic parameters were estimated with Equation (1) as Michaelis-Menten models for uncompetitive inhibition. This model was also used in another experiment for hydrolysis by alpha-amylases in different process conditions and different additives.

\[
\frac{dP}{dt} = \frac{V_{\text{max}}(S)}{K_m + \left(1 + \frac{(f)}{K_I}(S)\right)}
\]

Figure 2. Compared of starch hydrolysis of the control sample, starch-lipid (1:0.5) and starch-protein (1:1) in each time. Significantly differences (P<0.05)
The Equation (1) is used to simulate the experimental data with MATLAB program (Figure 3). The kinetic constant for all additive in starch hydrolysis by amyloglucosidase shows the inhibition degree $\alpha = ([I]/K_I)$ increase with increasing inhibitors concentration (Table 1).

![Graph](https://via.placeholder.com/150)

**Figure 3.** Starch (S) and glucose (P) concentration experiment data vs starch,glucose concentration simulation by Michaelis Menten uncompetitive model profile at processing time. a) Starch:Lipid (1:0.5), b) Starch:Protein (1:1)

### 4. Discussion

The result shows palmitic acid as a lipid and soy protein as protein can inhibit a hydrolysis process. Inhibit mechanism formed because there is an interaction of starch with palmitic acid as lipid and between starch and protein. As we know that the amylose chain in starch shaped twists with helix configuration composed of six anhydroglucose units in each partition of the hydroxyl group of the glucosyl residues located on the outside of the helix and hydrophobic groups located on the inside along the tube amylose [18], [15] and the presence of hydrophobic palmitic acid will be trapped in hydrophobic areas of amylose helix, hydrophobic groups in the amylose formed because the circle

| Table 1. Estimated kinetic parameters from simulation for glucose concentration |
|-------------------------------------|
| Substrate | $V_{max}$ (g/L/min) | $K_M$ (g/L) | $K_I$ (g/L) | $\alpha = ([I]/K_I)$ |
| Control (100% Starch) | 2.9358 | 466.6401 | - | - |
| Starch:Protein | | | | |
| 1:0.05 | 1.5878 | 449.7586 | 37.1707 | 0.0137 |
| 1:0.1 | 1.5748 | 450.2702 | 37.2149 | 0.0271 |
| 1:0.25 | 1.5598 | 450.3054 | 37.2384 | 0.0672 |
| 1:0.5 | 1.4837 | 450.3794 | 37.3187 | 0.1342 |
| 1:1 | 1.3979 | 450.2627 | 37.4490 | 0.2673 |
| Average | 1.5208 | 450.2007 | 37.2783 | 0.1019 |
| Starch:Lipid | | | | |
| 1:0.05 | 1.4042 | 416.5459 | 10.0184 | 0.0055 |
| 1:0.1 | 1.4928 | 407.2019 | 10.1693 | 0.0988 |
| 1:0.25 | 1.3769 | 432.8306 | 10.1023 | 0.2524 |
| 1:0.5 | 1.2386 | 457.8503 | 10.3739 | 0.5306 |
| 1:1 | 1.2693 | 440.2651 | 10.4438 | 0.9572 |
| Average | 1.3724 | 430.9406 | 10.2215 | 0.3689 |
The data has confirmed that increasing of lipid concentration added on starch hydrolysis, decreasing of glucose production were significant in ratio 1:0.5 or adding 50% lipid (w/w). On other hand, the effect of lipid adding significantly in 10 % of lipid added on a cooking process of starch [9].

By Heating pretreatment will expand starch granules and separates amylose from starch structure. This process will accelerate amylose-lipid interaction. But in order to minimize energy consumption, the heating treatment is eliminated from this study so the yield of glucose is relatively low.

Hydrolysis process is incubated at pH 4.5, this condition denatured protein, which is a tersier bond of protein denatured became a cation of amino acid which makes interaction with glucosyl bond of starch. The bonding is a hydrogen bond around starch granule which can block enzyme access.

The data shows that the significant effect of glucose production in adding 100 % of protein (ratio starch-protein 1:1). Protein has not significantly effective for inhibiting starch to glucose in lower concentration.

Kinetic parameters from simulation experiment data with Michaelis-Menten uncompetitive model has order in magnitude with hydrolysis of rice starch by adding glucose, maltose and glycerol [17]. The simulation shows the inhibitor degree of lipid are greater than protein. it concluded that lipid is the most effective inhibitor

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