Stability of Antibacterial Agent Hypoiodous Acid Against Time and Temperature of Storage

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Abstract. Hypoiodous acid (HIO) could be obtained by reacting two substrates: hydrogen peroxide (H$_2$O$_2$) and potassium iodide (KI), by the presence of peroxidase enzyme. This product has been well developed as antimicrobial compound and could be utilized in food products, however this product has not receive much attention in the durability. Therefore, this research was conducted to determine the final product reaction of HIO stability against time and temperature of storage. The product of HIO was composed using reaction mixture containing H$_2$O$_2$ and KI at 2 and 20 mM and the final product of HIO was stored for 60 minutes in temperature of 25–40°C. The pH and the remaining of H$_2$O$_2$ substrate was observed. The measurement of remaining H$_2$O$_2$ was done using 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as substrate. As results, gradual decrease in pH value was found in the beginning of 10 minutes of storage and during this 10 minutes, remarkable decrease was found after 6 minutes of reaction process. The elevation in the temperature of storage provided remarkable effect to the pH value and remaining concentration of substrates changes. Thus, this study concluded that HIO formation reaction is obstructed by high temperature.

1. Introduction

It has been well documented that antimicrobial agent attracted to researcher that was addressed to the application in industries, health, and food sector. One example of a potential antimicrobial agent for food products is hypoiodous acid (HIO) that could be obtained from a chemical reaction which is catalyzed by the presence of peroxidase using hydrogen peroxide and potassium iodide. This enzyme could be obtained from natural plant sources. Many types of plants have been known contain a lot of peroxidase in their roots [1]. Some of them are Moringa oleifera, Nicotiana tabacum, Raphanus sativus, Solanum ningrum, Brassica oleracea var. Botrytis and var. Maraton [2]. Meanwhile in animal sources, peroxidase is found in milk in the form of lactoperoxidase [3].

Peroxidase has a role as a catalyst in HIO formation from a reaction mixture of hydrogen peroxide and potassium iodide. Naturally, HIO is produced in our body because of the enzymatic reaction which is involving myeloperoxidase in the form of saliva [4]. HIO as an antibacterial agent may inhibit various...
cell functions through reaction with sulfhydryl group of pathogenic microbes [5]. Stability of HIO itself can be seen through its constituent compounds approach including peroxidase enzyme as the catalyst. In general, peroxidase activity can be identified with spectrophotometer UV-Vis with a wavelength of 412 nm using 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as substrate [6], because ABTS will be oxidized along with H₂O₂ and form metastable cation radical compounds [7].

Therefore, this study was conducted to explore the reaction of final product and the stability of HIO against time and temperature of storage which was measured by observing the changes in pH in the reaction solution, the H₂O₂ residue with ABTS using spectrophotometer. Thus, HIO can be determined the optimal use against time of reaction in food products as antibacterial agent.

2. Materials and Methods

2.1 Chemicals and enzyme
H₂O₂ at the concentration of 20 and 2 mM, KI at concentration of 20 and 2 mM, 2,2'-azino-bis [3-ethylbenzothiazoline-6-sulphonic acid] (ABTS) were obtained from Roche (Germany). Daikon radish as the source of peroxidase enzyme was obtained freshly from a modern market in Tembalang, Semarang, Indonesia. Aquadest and phosphate buffer were obtained from UPT Integrated Laboratory, Diponegoro University, Semarang, Indonesia.

2.2 Preparation of HIO
Measuring the residue of H₂O₂ was conducted using a reaction mixture containing 225 μl of 20 mM H₂O₂, 225 μl of 20 mM KI, and 50 μl of peroxidase enzyme. This reaction mixture was reacted gently then measured using ABTS as substrate. HIO solution for pH analysis was conducted using a reaction mixture of 40 ml solution that was consisted of 18 ml of 20 mM H₂O₂, 18 ml of 20 mM KI, and 4 ml of peroxidase enzyme. Solution then measured using pH meter. The mixture was determined as HIO solution and was measured every minute for 10 minutes, then the next 30 and 60 minutes. This method was adopted and modified from previous study [8]. HIO solution was stored in four different temperatures, i.e. 25, 30, 35 and 40°C. For storage temperature of 30-40°C, HIO solution was stored in a water bath. Same method was also applied to HIO 2 mM which was made of 2 mM H₂O₂ mixed with 2 mM KI and peroxidase enzyme.

2.3 Preparation of peroxidase
Preparation of peroxidase enzyme from Daikon radish was derived from previous study [9] to obtain crude extract of the enzyme. Daikon radish was washed and cut into small pieces then weighed and blended with phosphate buffer (0.01 M, pH 7) in ratio of 1:4. Blended radish was filtered with filter cloth and centrifuged in 1700 rpm for 10 minutes. Supernatant and sediment were separated with filter cloth. Supernatant could be used as peroxidase enzyme.

2.4 Changes in pH of HIO observation method
A 40 ml of HIO solution was measured by pH meter every minute for first 10 minutes. Changes in pH of HIO solution was also measured after 30 and 60 minutes of storage in temperature of 25, 30, 35 and 40°C.

2.5 Residue H₂O₂ with ABTS method
A 450 μl of HIO solution was added to a reaction mixture containing 450 μl of 0.27 mM ABTS and 100 μl of Daikon radish peroxidase. ABTS as substrate in the measurement of hydrogen peroxide residue during reaction process using spectrophotometer at wavelength of 412 nm. This method was adopted from previous study [3].
2.6. Data analysis
Data was obtained from 2 replications and calculated using Microsoft Excel 2010. This study used a quantitative descriptive analysis.

3. Results and Discussion

3.1 Changes in pH of HIO
The changes in pH should be occurred since the reaction between KI and H₂O₂ generating HIO compounds. When H₂O₂ was reacted to KI, the HIO and KOH would be produced. This reaction is shown as follow:

\[ \text{I}^- + \text{H}_2\text{O}_2 \rightarrow \text{HIO} + \text{OH}^- \] [10]

The changes pH value with different temperature might be occurred by the presence of the enzyme in which the acceleration of the HIO formation depended on the optimum temperature of the enzyme activity. The high temperature could inhibit the action of the enzyme and decreased the acceleration of HIO formation. Decreasing HIO formation might affect the change in pH value. The changes of pH at different concentrations of HIO at the first ten minutes of reaction using various incubation temperatures can be seen in the Fig 1. and Fig. 2.

![Figure 1. Changes in pH in 20 mM HIO reaction solution at during 10 minutes](image1)

![Figure 2. Changes in pH in 2 mM HIO reaction solution during 10 minutes](image2)
The changes of pH level for 10, 30, and 60 minutes of reaction time can be seen in Fig. 3 and Fig. 4. The various concentration might be affected to the enzyme activity and the amount of reaction product. Based on this study, pH value at a range from 6.185 to 7.1 was able to be determined at 20 mM reaction solution of HIO while a range from 6.795 to 7.04 was detected at the reaction solution of HIO at 2 mM. According to previous study, HIO was one of reactive iodine species which has near neutral pH value [11].

Figure 3. Changes in pH of 20 mM HIO Solution at 60 Minutes

Figure 4. Changes in pH of 2 mM HIO solution at 60 minutes

3.2. Residue of H$_2$O$_2$
Residue of H$_2$O$_2$ was measured using absorbance value of HIO reaction solution with wavelength of 412 nm and using ABTS as substrate. This method has been used widely to determine the formation HIO by the reduction of H$_2$O$_2$ and the residue of H$_2$O$_2$ during reaction time in various storage temperature as shown at Fig. 5 to Fig 8. The addition of ABTS was used to produce green color caused by the reaction between ABTS and H$_2$O$_2$ [12]. Green color that was displayed on reaction mixture showed the presence of hydrogen peroxide residues in HIO solution.
Based on Fig. 5, it can be seen that the absorbance value of HIO 20 mM in temperature of 40°C was the lowest among other temperatures. There was also similarity in trend between HIO 20 mM in 30 and 40°C where both didn’t show any decrease. This is allegedly caused by the peroxidase enzyme activity which were affected by temperature. This is in accordance with other researcher study[13] which stated that peroxidase enzyme derived from vegetable sources (radish, turnip, pepper and cabbage) has maximum temperature to maintain its activity at 30°C. Therefore, peroxidase enzyme stored in temperature of 40°C had lower activity than that of 30°C. This is shown in Fig. 5 and Fig. 6 that the absorbance value didn’t have any decrease in both concentrations of HIO solution. It is also demonstrated that HIO solution was not stable in high temperature. Fig. 7 and Fig. 8 showed the absorbance value of different concentrations of HIO solution in 60 minutes of storage.

**Figure 5.** Residue H₂O₂ in 20 mM HIO Solution at 10 Minutes

**Figure 6.** Residue H₂O₂ in 2 mM HIO solution at 10 minutes
Absorbance value of H₂O₂ residue most likely to be fluctuating in the first 10 minutes of storage and more stable in the next 60 minutes. It was also shown that HIO solution of 20 mM was likely formed in the fifth minute in temperature of 25 and 35°C. Meanwhile, HIO solution with concentration 2 mM was likely formed in the sixth minutes. The sudden increase of absorbance value in several incubation time had not yet explained. There are several factors that affect the formation reaction of HIO. According to previous study, redox reaction might be occurred only if the enzyme had superior reduction capability than the substrate [4]. Besides that, anion size, anion access, and anion binding ability might also affected redox reactions [14].

Figure 7. Residue H₂O₂ in 2 mM HIO solution at 60 minutes

Figure 8. Residue H₂O₂ in 2 mM HIO solution at 60 minutes
4. Conclusion
Gradual decrease in pH value was found in the beginning of 10 minutes reaction and the decrease in remaining substrates concentration was also found but in the beginning of 6 minutes reaction. The elevation in the temperature of storage effected to the pH changes and remaining concentration of substrates changes. Thus, this study concluded that HOI formation reaction was obstructed by high temperature.

5. References

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