Effect of Cocoyam Modified Starch (Xanthosoma sagittifolium), Beetroot Juice, Cocoyam Modified Starch Adsorbing Beetroot on Plasma Selenium and Glutathione Peroxidase of Pre-Diabetic Rat

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Abstract. This research was aimed to identify effect of cocoyam (Xanthosoma sagittifolium) and beetroot on plasma Glutathione Peroxidase (GPx) and selenium of pre-diabetic rats. The pre-diabetic rats were fed with cocoyam modified starch, beetroot juice and cocoyam modified starch adsorbing beetroot for 3 weeks. The plasma selenium and GPx were analyzed. The blood were withdrawn once in a week during 3 weeks. Selenium levels in pre-diabetic rats consumed each of feeding treatment were not significantly different as compared to control feeding (AIN). Plasma GPx activity in pre-diabetic rats consumed cocoyam modified starch and cocoyam modified starch adsorbing beetroot tended to have higher values than control in every week. Plasma GPx activity of pre-diabetic rats consumed beetroot juice tended to have higher values than control at the first and the second week.

1. Introduction

Type 2 Diabetes Mellitus (T2DM) is one of metabolic syndromes which the body cannot control blood sugar caused by inadequate or non-functioning insulin hormone secretion in the pancreas, resulted in hyperglycemia. In general, T2DM patients experience complications due to oxidative stress continually [1]. Oxidative stress is a condition in the body which the amount of free radicals exceed the amount of available primary antioxidants such as glutathione peroxidase, super oxidase dismutase, and catalase. In hyperglycemia condition, free radicals are produced in large quantities through various mechanisms such as glucose and GAPDH autooxidation, polyol pathway, hexosamine pathway, formation of AGEs (Advanced Glycation End-products), and PKC activity [2].

T2DM patients have decreased one of the primary antioxidants namely glutathione peroxidase (GPx) along with its enzyme cofactor, selenium. GPx is a group of selenoprotein enzyme and its structure
contains selenium [3]. Diabetic patients have lower GPx activity compared to healthy people [4]. In addition, GPx activity in the liver, kidneys, and pancreas of diabetic rats is lower than in healthy rats [5]. In condition of excessive free radicals, the consumption of selenium by the GPx enzyme increases, which caused selenium levels to decrease [6]. GPx activities depend on selenium, so when selenium levels decrease, GPx activity also decreases. Consequently, additional intake is needed to increase the activity of GPx and selenium in order to reduce the risk of diabetes complications due to oxidative stress.

Beetroot has antibacterial and antioxidant bioactivities due to high phytonutrients content such as flavonoids, betalain, and phenolic acids [7]. Previous studies have shown that beetroot can increase levels of GPx antioxidant activity in \textit{in vivo} experiments [8-10]. In addition, \textit{in vitro} experiment showed that beetroot has a high antioxidant activity [7, 9, 11]. Therefore, beetroot is potential as a functional food with antioxidative property to suppress free radicals in T2DM individuals.

Cocoyam (\textit{Xanthosoma sagittifolium}) tubers have high anti-hyperglycemia and antioxidant abilities [12-14]. However, there is still no research on the effect of cocoyam bioactive carbohydrate, especially the role of modified starch as antioxidants \textit{in vivo} such as GPx and its co-factor, selenium. Referring to various things that have been stated before, cocoyam and beetroot have antioxidant and anti-hyperglycemia potential to reduce oxidative stress that occurs in T2DM people. Therefore, it is necessary to study the effect of beetroot, modified cocoyam starch, and its combination against one of the oxidative stress biomarkers in the body, namely GPx activity and its cofactor, selenium.

2. Methodology
2.1 Materials
Beetrots were purchased from All Fresh in Alam Sutera, South Tangerang, Indonesia. Cocoyam starch samples were obtained from Banguntapal, Bantul, Yogyakarta. Several chemicals were obtained from many sources: Streptozotocin (Sigma, St Louis, USA), Xylazine 2% (Interchemie LA Waalre, Netherlands), Ketamine 10% (Kepro, ZA Deventer, Netherlands), and Nicotinamide (Sigma, St Louis, USA). HCl, HNO₃, HClO₄, KH₂PO₄, K₂HPO₄, NaN₃, GSH, NADPH, GR were obtained from Waco (Tokyo, Japan). EDTA.2Na, EDTA, 2-3, diaminonaphthalene (DAN), and cyclohexane were obtained from Dojindo (Tokyo, Japan). t-ButhylOOH and sodium selenite (Sigma, St. Louis, MO, USA). Trace element serum L-2 (Seronorm, Billingstad, Norway) was used.

2.2 Animal Experiment
The animal experiment protocol was approved by the University of Indonesia Faculty of Medicine Ethics Committee (Protocol Number: 18-09-1045). The animals used were 6-week-old male Sprague Dawley (SD) rats purchased from the National Agency of Drug and Food Control (BPOM), Central Jakarta. SD rats were treated in individually closed system cages at a temperature 24 °C and RH 70% and dark and light cycle of 12 hours, respectively. SD rats were given drinking water ad libitum access. The animal experiment consists of acclimatization, induction, feed adaptation, treatment, and termination phase. The acclimatization period was conducted for two weeks to reach ± 200 grams of bodyweight by commercial feeding obtained from local market in Central Jakarta.

In the induction stage, nicotinamide (NC) with a dose of 120 mg/kg was dissolved in 0.9% NaCl solution, then injected into SD rats, followed by streptozotocin (STZ) with a dose of 60 mg/kg dissolved into buffer solution (pH 4), then injected to SD rats. After three days commercial feeding. SD rats were examined for fasting blood glucose with glucometer (Easy Touch, GCU). The rats with fasting blood glucose in the range of 100-125 mg/dl, considered as pre-diabetes [15]. If there are SD rats have not reached the pre-diabetic condition, they were re-induced by STZ and NC with a dose of 40 mg/kg and 120 mg/kg, respectively. The maximum re-induction is once, otherwise the rats was excluded.
The pre-diabetic rats were divided into four groups of treatment (n = 3), namely control, beetroot juice, modified cocoyam starch, and modified cocoyam starch adsorbing beetroot. Beetroot juice was given to pre-diabetic rats by gavage feeding at 3 ml per day and was given control feeding. At the first 3 days, pre-diabetic rats were in a phase of feeding adaptation period with the following feeding ratios: first day 75% and 25%; second day 50% and 50%; third day 25% and 75% of commercial and treatment feeding, respectively. On the fourth day, they were given 100% feeding treatment for each group for 3 weeks and plasma bloods were collected as the baseline.

Feed consumption of pre-diabetic rats were observed every day. The bodyweight of pre-diabetic rats were measured twice a week. Fasting blood glucose and non-fasting blood glucose were measured with glucometer (EasyTouch, GCU) once a week. Pre-diabetic rats were first anesthetized with ketamine 10% and xylazine 2%. Then the bloods were collected retro-orbitally once a week by using hematocrit and the bloods were stored in EDTA tubes, centrifuged at a rotational speed of 3500 rpm for 10 minutes to obtain the plasma and then transferred to a new tube and stored at -80 °C. At the end of animal experiment, pre-diabetic rats were terminated by cardiac puncture. Pre-diabetic rats were anesthetized with ketamine 10% and xylazine 2%. Then, the syringe needle was inserted through the chest cavity until it reaches the heart, blood was collected by cardiac puncture.

2.3 Glutathione Peroxidase Activity
Glutathione peroxidase activity analysis use Paglia method [16]. Blood plasma samples were dissolved into phosphate buffer (pH 7.4) in a ratio of 1: 9. The sample solution (25 μl), blank, and reference were dissolved in 200 μl of the reaction mixture solution consisting of 75 μl phosphate buffer, 25 μl 10 mM NaN3, 25 μl 10 mM EDTA.2Na, 25 μl 10 mM GSH, 25 μl 10 U / ml GR, and 25 μl 3 mM NADPH into microplate wells. The microplate was incubated in spectramax plus 384 at 37 °C for 10 minutes. After incubation, 30 μl 6 mM t-BuOOH solution was added to each microplate wells. Then NADPH oxidation was measured with absorbance at 340 nm every 12 seconds for 10 minutes at 37 °C using spectramax plus 384 plate reader (Molecular Device, USA). GPx activity was calculated based on changes in absorbance of NADPH and expressed by changes in NADPH concentration per minute (ΔmM NADPH per minute) [16].

2.4 Selenium Content
Selenium content was assessed by modified Watkinson method [16]. Amount of 50 μl blood plasma samples were added into 2 ml of mixed acid solution (HNO3 and HClO4 at a ratio of 2:1) by heating at 50 °C for 1 hour, at 100 °C for 1 hour, at 140 °C for 2 hours, at 170 °C for 3.5 hours, and at 190 °C for 0.5 hour by using programmed heating blocks. After the samples were cooled, they were reheated at 150 °C for 10 minutes with 0.5 ml of 12 N HCl solution added. After 10 minutes, the sample was left at room temperature for cooling.

About 1 ml of EDTA 0.1 M was added into the sample solutions. 0.1 ml of 1% thymol blue was added to the sample solutions until it turns red or purple. 25% NH4OH was added into the sample solutions until it turns yellow or blue. 2 N HCl was added into the sample solutions until turns pink or red. 1 ml of 0.1 N HCl and 0.1% and were added to the sample solutions. Sample solutions were heated with a water bath shaker at 50 °C for 10 minutes. After 10 minutes, the sample was cooled down. Then, 2 ml of cyclohexane Luminazoel was added to the sample solution and were shaken with a shake shaker (Direct Mix TS-50) for 2 minutes. After 2 minutes, the samples solutions were centrifuged for 5 minutes at 2500 rpm. The sample solutions were measured the fluorescence intensity with fluorescence spectrophotometer F-2700 (Hitachi, Japan) with exitation 378 nm and 525 nm emission for selenium content analysis. The accuracy of selenium analysis was verified by using reference material serum L-2 (seronorm trace element serum L-2, Sero, Bilingstad, Norway).
2.5 Statistical Analysis
Statistical analysis was conducted by independent t-test to analyze the significance between the control group and each treatment group on GPx activity and selenium content in pre-diabetic rat plasma bloods. Pairwise t-test analysis was performed to analyze the significance of an increase or decrease between week 0 on each week on body weight, percent consumptions, and fasting blood glucose and non-fasting blood glucose in pre-diabetic rats. One-way ANOVA analysis was conducted on selenium levels in rat feeds. All statistical analysis tests were performed with IBM SPSS statistics version 23.

3. Results and Discussion
3.1 Bodyweight of rats
Overall, the bodyweight of pre-diabetic rats (Table 1) was increased every week for three weeks. Pre-diabetic rat bodyweight was gained significantly in the control feeding group, modified cocoyam starch, and beetroot juice at the second and third week as compared baseline (p <0.05), means that the pre-diabetic rats were grown normally during the animal experiment. In addition, it can be concluded that the feeding gave positive effect on the body weight of pre-diabetic rats, especially in control, modified cocoyam starch, and beetroot juice group. Moreover, the modified cocoyam starch adsorbing beetroot have stable bodyweight than other groups as shown by not significant bodyweight gain for three weeks.

| Samples | Week 0     | Week 1     | Week 2     | Week 3     |
|---------|------------|------------|------------|------------|
| Control | 265.75±23.76 | 287.38±26.61* | 314.75±20.95* | 327.38±17.46* |
| CMS     | 264.00±23.06 | 284.00±28.24  | 322.17±30.81* | 337.50±31.00* |
| CMSB    | 269.67±31.07 | 279.80±12.91  | 316.67±10.76  | 327.33±13.95  |
| JB      | 278.67±31.89 | 303.5±28.16*  | 312.67±38.09* | 326.67±21.78* |

Data are means ± SD, n =3. Statistical analyses were performed by the t-test pairwise. *p< 0.05. Data are significantly different from the week-0. CMS = modified cocoyam starch; CMSB = Modified cocoyam starch adsorbing beetroot; JB = beetroot juice.

3.2 Feeding Consumption
Feeding consumption of each treatment (Table 2) is calculated from the difference between the weight of the feed given and the feed remained. Inversely from the body weight of pre-diabetic rats, the feed consumptions of pre-diabetic rats in each group decreased weekly with 20% decreased as compared to baseline. The consumption of modified cocoyam starch adsorbing beetroot and control group at week three was significantly less than at baseline (p <0.05). However, the pre-diabetic rats in each group were in normal conditions and did not experience bodyweight loss during the entire animal experiment.

| Feeding Treatment | Week 0     | Week 1     | Week 2     | Week 3     |
|------------------|------------|------------|------------|------------|
| Control          | 73.78±13.33 | 70.74±11.81 | 55.67±8.85* | 49.64±13.79* |
| CMS              | 71.09±16.71 | 65.11±8.1   | 55.01±7.14  | 55.43±8.76  |
| CMSB             | 76.88±21.55 | 63.14±13.52 | 55.61±9.61  | 51.2±11.5*  |
| JB               | 72.83±23.98 | 64.44±14.04 | 51.83±11.31 | 42.92±16.1 |

Data are means ± SD, n =3. Statistical analyses were performed by the t-test pairwise. *p< 0.05 means data are significantly different as compared to baseline.
3.3 Fasting Blood Glucose
Figure 1 shows fasting blood glucose in each group for three weeks. The range of fasting blood glucose for pre-diabetic is 100-125 mg/dl [15]. Modified cocoyam starch and the control group tended to have stable fasting blood glucose in every week. In beetroot group, fasting blood glucose tended to decrease until the second week (127 mg/dl into 102 mg/dl), but in the third week fasting blood glucose tended to increase (102 mg/dl to 118 mg/dl). The fasting blood glucose of pre-diabetic rats which fed with modified cocoyam starch adsorbing beetroot tended to decrease in every week. There was no significant difference between every week of observation as compared to baseline for all groups.

3.4 Non-Fasting Blood Glucose
Figure 2 shows the non-fasting blood glucose of all groups. The pre-diabetic rats have non-fasting blood glucose in a range of 140-199 mg/dl [15]. In control and beetroot juice groups, non-fasting blood glucose decreased at first week (150 mg/dl to 122 mg/dl and 133 mg/dl), respectively, but at the second week to the third week tended to be stable. Whereas non-fasting blood glucose in the modified cocoyam starch and modified cocoyam adsorbing beetroot tended to be unstable, at the first week was decreased, but at the third week was increased. There was no significant difference at every week of observation as compared to baseline of each group.
3. Plasma Selenium of Rats

Figure 3 shows the selenium content in all groups after three weeks administration of feeding treatment and showed relatively stable plasma selenium in every week. No significant difference of plasma selenium between each treatment group compared with the control group in every week of observation.

3.6 Glutathione Peroxidase Activity

The GPx activity of treatment and control group are shown in Figure 4. GPx activity of rats fed with modified starch of cocoyam tended to be higher than control for three weeks. Therefore, modified starch of cocoyam may have the potential to help in reducing free radicals in the Pre-diabetic rats. There is no reference on high antioxidative ability of modified cocoyam starch to be compared with this study. However, some studies revealed that cocoyam flour has a high antioxidant ability. According to Nishanthini and Mohan [12], the cocoyam flour extracted with methanol has a higher antioxidative ability as compared to vitamin C, due to bioactive compounds such as flavanoids and phenolics as antioxidant [12]. Hence, modified starch of cocoyam may contain antioxidant compounds such as phenolic and flavonoid, which can suppress free radicals in pre-diabetic rats.
Figure 4. GPx activities of treatment and control group

GPx activity of rats fed with beetroot juice tended to be higher than in control group at first and second week. Rats exposed to gamma-irradiation, the GPx activity value dropped significantly [10]. However, after being given betalaine from beetroot orally for 30 days at a dose of 5 mg/kg, 20 mg/kg, and 80 mg/kg, GPx activity in pancreatic organs had a higher value than control group. The higher the dose, the higher the value of GPx activity in the pancreas.

At third week, beetroot juice group, the GPx activity tended to be the same value as compared control group. This might be due to the degradation of antioxidant components in beetroot juice during storage. The beetroot juice stored at 4 °C for 2, 4, 8, 16, and 32 days showed a significant decrease in the content of phenolic compounds [17]. Moreover, antioxidant analysis by DPPH method showed that antioxidant activity of beetroot juice decreased by 31% at 4 °C after 48 hours and stored up to 32 days, the value of antioxidant activity was reduced by 62%. The decrease in phenolic content and antioxidant activity is triggered by the oxidation of phenolic compound by enzymatic browning such as phenylalanine ammonia-lyase (PAL), and polyphenol oxidase (PPO) [18]. In this study beetroot juice was stored in a refrigerator at ± 4 °C. At third week beetroot juice has been stored for more than 20 days so that there was a possibility of a decrease in the content of antioxidant components and antioxidant activity. Therefore, at the third week beetroot juice did not show significant effect on pre-diabetic rats due to prolonged storage. Standard deviations of the GPx activity are relatively high, as a consequence of high deviation, the GPx activity after 3 weeks administration tended to increase. GPx activity of modified cocoyam starch adsorbing beetroot tended to be higher than the control group for 3 weeks. Starch has ability to adsorb phytonutrient bioactive compounds such as polyphenols, and interacts with polyphenol by forming non-covalent bonds which include hydrogen bonds, hydrophobic interactions, electrostatic, and ion interactions [19]. Phytonutrients adsorbed on starch may influence the digestibility of starch and may also influence non-fasting blood glucose [19-21]. Moreover, starch had ability to act as encapsulant of bioactive compounds to protect and stabilize the bioactive compound [22-24].

Conclusion

Pre-diabetic rats administrated with modified cocoyam starch, and modified cocoyam starch adsorbing beetroot tended to have higher GPx activity value as compared to control feeding after three weeks administration. Pre-diabetic rats administrated with beetroot juice tended to have higher GPx activity than
control feeding after one and two weeks supplementation. For further recommendation, prolong storage more than two weeks of beetroot juice needs to be stored in frozen form to preserve bioactive compounds in beetroot juice.

Acknowledgments
The authors would like to thank INSINAS RISTEK DIKTI, contract no: 030A/VR.RTT/IV/2019, and Public Health department Gunma University, Japan, for grants in aid, and the laboratory facilities provided. The authors also would like to thank Ms Yoshizawa Chiho for technical assistance during Se and GPs assessments.

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The 3rd International Conference on Eco Engineering Development
IOP Conf. Series: Earth and Environmental Science 426 (2020) 012184
doi:10.1088/1755-1315/426/1/012184

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