Variation of cassava genotypes based on physicochemical properties of starches and resistant starch content

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Abstract. Cassava has a large variation in their qualitative trait, such as variation in resistant starch content and physicochemical properties. Characteristics of cassava, such as physicochemical properties, are very important for its application in food industries and will affect the quality of the end products. The properties of starch could indicate the appropriateness of starch for various uses. Our paper aims to study the variation of the resistant starch content of several cassava starch and its physicochemical properties. Tapioca starch produced from LIPI cassava collection were used as material. The variation of resistant starch content was determined by using Megazyme kit protocol. Variation of physicochemical properties is determined based on the value of resistance to freeze thawing, swelling power and starch solubility, and paste clarity. Variation of cassava genotypes was determined by using SSR markers. The result showed that Manggu has the highest percentage of resistant starch (7.32%). The percentage of starch resistance to freeze thawing varies from 0.153 to 1.173. Swelling power of different starch samples was varies from 8.59 to 11,605 g/g, and the solubility of starch have a slight ranges from 0.293 to 0.437 g/g at 90°C. The value of paste clarity varies from 0.135-0.543. Dendogram generated based on UPGMA cluster analysis showed that all the cassava genotypes were distributed in two main clusters.

1. Introduction

Cultivated cassava (Manihot esculenta Crantz) is one of the highly heterozygote species that belong to the genus of Manihot. As one of the most important staple food in the world, cassava is cultivated mainly for its starchy roots in South America, Africa, and Asia. In industry, cassava tuber is processed into food products or some intermediate products, such as tapioca, cassava flour and modified cassava flour (mocaf). While in some other industries, starch is processed again into some end products, such as sweeteners, ethanol, glue and organic acids. Starch application influences the texture of various foods. It is generally used in industry as a thickener, colloidal agent, stabilizer, gelling agents, bulking agents, water retention agent and adhesive.

Cassava have a large variation of the qualitative traits, such as variation in physicochemical properties and starch characteristics [1], variation of macronutrient content of fat and crude protein and variation in micronutrient of carotene, Fe, Na, K, Cu, and Zn, and vitamins [2,3,4], disease resistance, roots quality and storage root bulking [2,5], variation in morphological, agronomic [6,7] and molecular characteristics [8], variation in dry matter, amylose, starch and resistant starch content [9]. And current market trends demand functional food with health benefit such as resistant starch, a part of starch that undigested by amylase enzyme in the small intestine and passes to the colon to be
fermented by microorganisms [10]. This kind of starch have a similar function with dietary fibers and show similar health benefits such as probiotic [11].

There are some important properties of starch affecting its application in food processing or the quality of the end products, such as solubility, swelling power, water and oil binding capacity, syneresis, rheological behaviour of pastes and gels, granule size, paste clarity, starch gelatinization and retrogradation [12,13,14]. Water binding capacity, swelling power, and solubility of starch influence the starch viscosity [14]. Starch granule characteristics will affect the hydration rate and the swelling power during the processing of starch into food products [14,15]; and paste clarity improves the visual appearance of food product [14,16]. Therefore, information about physicochemical properties will give a big advantage for food product development in industries.

The objective of our study are obtaining more conclusive data regarding to variation of starch and resistant starch content, and also physicochemical properties of cassava (such as resistance to freeze thawing, swelling power and solubility, paste clarity) from our cassava collection at Research Centre for Biotechnology, Indonesia Institute of Sciences (LIPI), together with the information about DNA profiling of cassava genotypes based on single sequence repeat (SSR) marker.

2. Materials And Methods

2.1. Material
Several cassava starches were used to evaluate the resistant starch content, and physicochemical properties. Their leaves were also used for DNA isolation. As control, we used commercial starches of Tapioca Rose Brand. All starches were produced from nine month old cassava genotypes grown in Research Center for Biotechnology, Indonesia Institute of Sciences (LIPI), Cibinong, Bogor, West Java, Indonesia.

2.2. Methods

2.2.1. Starch extraction
Cassava starch was extracted from each cassava genotype by mixing gently one kg of grated cassava flesh from each cassava genotypes with 3 liters of water (1:3), and these process is repeated for five times. The starch is then filtered and precipitated for 6 hour. The wet starch obtained is dried under the sunshine to a maximum humidity of 14%.

2.2.2. Resistant starch content of several different genotypes of cassava
Resistant starch content was determined as described in the protocol of Megazyme kit. About 100±5 mg of the sample was weighed into a 15 mL centrifuge tube and 4 mL of 1,0 M sodium maleate buffer (pH 6,0) containing pancreatic α-amylase (10 mg/ml) and amylglucosidase (3 U/ml) was added. The tube was covered tightly with bootle cap, mixed and placed horizontally in a shaking oven (200 rps). The solution was incubated at 37°C with continuous shaking for 16 hrs. To the solution was added 4 mL of 99% ethanol to precipitate the starch and mixed vigorously on a vortex mixer. It was centrifuged at 30000 rpm for 10 mins. The supernatant was decanted and the residue rinsed twice with 8 mL 50% ethanol, followed by centrifugation at 3,000 rpm for 10 mins. The residue was re-suspended with 2 mL of 2 M potassium hydroxide in an ice bath with stirring for 20 mins and 8 mL of 1,2 M sodium acetate buffer (pH 3,8) was added with 0,1 mL of amylglucosidase (3300 U/ml). The sample was mixed and incubated at 50°C with continuous shaking for 30 mins. The sample was then diluted with water and centrifuged at 3,000 rpm for 10 mins. The glucose was quantified with glucose oxidase/peroxidase reagent (GOPOD), which gave a measure of the RS content of the sample [17,18].

2.2.3. Variation of cassava based on SSR marker
DNA was isolated from cassava leaves. PCR reactions were carried out in 20-µl volumes containing 50–100 ng cassava genomic DNA, 0.2 µM of each forward and reverse primers, 1xPCR buffer, 1.5 mM MgCl2, 200 mM of each dNTP and about 1 U Taq DNA polymerase. The temperature cycling profile was: an initial denaturation step for 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C or 45°C for 2 min and primer extension at 72°C for 2 min; a final extension cycle of 5 min at 72°C was added. Between 10 µl of the PCR reaction was electrophoresed on 12% polyacrylamide gels for 3 h at 60 W, and DNA was visualized ethidium bromide [8]. Four SSR primer was used for SSR analysis, such as SSRY 100, SSRY 103, SSRY 51, SSRY 181.

2.2.4. Resistance to freeze thawing
Resistance of starch to freeze thawing was measured based on Demiate and Kotovicz. Ten percent of starch solutions were cooked in a water bath at 100 °C for 10 minutes until the starch gelatinized. The pastes were stored in freezer with -18 °C for 90 hours, and then continued by thawed in oven at 45˚C for 3 hours. The pastes were centrifuged at 4000 rpm for 15 minutes [19]. Resistance to freeze thawing was determined based on percentage of the weight of water liberated from the initial paste mass.

2.2.5. Paste Clarity
Paste clarity was measured according to Demiate and Kotovicz (0. One percent of starch/flour solutions were heated to boiling in a water bath at 100 °C for 30 minutes to complete gelatinization. The transmittance of the paste was measured using a spectrophotometer at the wavelength of 650 nm [19]. The results are averages of three repetitions.

2.2.6. Swelling power and solubility
The starch suspensions were heated on centrifuge tubes for 30 minutes under agitation. After this period, the tubes were centrifuged at 2,000 g for 10 minutes in order to separate the swollen granules from the soluble starch fraction. The soluble supernatant fraction was collected and dried on oven for quantification. The gel pellet was weighed and the swelling power expressed as times of weight increment in relation to the amount of starch in the initial dispersion. The solubility was calculated as percentage (w/w) of the initial amount of starch in the dispersion. Two repetitions were made for these analyses [19].

2.2.7. Statistical analysis
Three replications were used to obtain average values and standard deviations for all tests. All results were analyzed with SAS version 9.2 statistical software (SAS Institute Inc., Cary, NC). ANOVA with Duncan’s Multiple Range test was used to evaluate the differences in resistant starch content and starch properties. Probability (P) ≤ 0.05 indicates significance. Clustering analysis was conducted by using UPGMA analysis.

3. Results and Discussion

3.1. Starch Content
The results of cassava starch analysis showed that each cassava has a different starch content. From the twelve cassava starches analyzed, the Gajah genotype had the highest starch content (28%), while the lowest were Gebang and Darul Hidayah. Gajah is local cassava genotype that have high starch and yield, while Adira 1 and Adira 4 are national varieties that have been released by the Ministry of Agriculture because of their high yield and starch. So if converted to the needs of the starch industry, then Adira 1, Adira 4 and Gajah can be used as a good source for starch industry [20,21,22]. While Iding, although the starch is high, the yield is lower, making it less economical to be used as a source of starch. Some cassava genotypes from other collection have a higher starch content comparing to our cassava genotypes, such as some cassava genotypes from China have a starch content up to 31.9%, and in Tanzania, some genotypes are up to 34.3%.
Figure 1. Percentage of starch content from several cassava genotypes

3.2. Resistant starch content of several cassava starches

Table 1 shows the resistant starch content of several cassava genotypes. All of our cassava genotypes are naturally have resistant starch content. The data obtained from the statistical analysis showed a significant differences in resistant starch content at P<0.05 among the different genotypes that were tested. The level of resistant starch in the cassava starch ranged from 0.05 to 7.68%. Manggu genotype showed the highest resistant starch content, while the lowest was Gebang genotype (Table 1).

| Genotypes         | % Resistant starch content |
|-------------------|---------------------------|
| Adira 1           | 5.78±0.30^a               |
| Adira 4           | 1.66±0.05^b               |
| Apuy              | 4.01±0.49^d               |
| Gajah             | 2.80±0.34^bc              |
| Gebang            | 0.05±0.03^a               |
| Iding             | 2.66±0.08^bc              |
| Kristal Merah     | 6.57±0.03^ef              |
| Manggu            | 7.68±0.87^f               |
| Mentega 2         | 5.83±0.03^g               |
| Menti             | 4.59±0.22^d               |
| Ubi Kuning        | 6.86±0.73^ef              |

Notes: Mean value with different letters denote significant differences, while the same superscript are not significantly different (Duncan, α = 0.05).

Cassava is naturally containt resistant starch that varied up to 7% [23]. We need around 6 mg of resistant starch for daily intake [11]. Kristal Merah, Manggu and Ubi Kuning are cassava genotypes that can be used to meet daily needs of resistant starch. However, the amount of resistant starch can be improved to get more benefit by improving the amount of resistant starch type 3. RS type 3 or commonly known as retrograded gelatinized starch [24], can be produced through modification by physic with thermal process or heat moisture treatment [25,26,27]. Starch is composed by linear amorphous molecule of amylose and branched semi crystalline structures of amylopectin. Each cassava genotype have a different composition and structure of both amylose and amylopectin, and it
cause the difference in its properties and functions [28,29]. Amylose molecules have a great tendency to form double helices, particularly near refrigeration temperatures (4–5°C) and with adequate moisture content. Retrograded amylose has high gelatinization temperatures, up to 170°C, and cannot be dissociated by cooking. The gelatinization temperature of retrograded amylose, however, decreases with shortening of the amylose chain length. After starchy foods are stored, particularly in a refrigerator, amylose molecules and long branch chains of amyllopectin form double helices and lose their water-binding capacity. The double helices of starch molecules do not fit into the enzymatic binding site of amylase, thus they cannot be hydrolyzed by this enzyme.

3.3. Variation of Cassava genotypes based on SSR marker

A total of 12 cassava genotypes were used in the clustering analysis of cassava genetic variations based on five SSR primers. Dendogram analysis results showed that cassava genotypes were distributed in two main clusters, namely clusters 1A and 1B at genetic distance 25. In cluster 1A there was only the Kristal Merah genotype, and the rest were in the IB cluster. The IB cluster is divided into three sub-clusters, namely IIA, IIB and IIC clusters at a genetic distance of about 23. In Subcluster IIA there are genotypes of Menti, Manggu, Mentega 2, Gebang, Iding and Gajah. While in sub-cluster IIB there was only the Adira 4 genotype, and in the IIC sub-cluster there were Ubi Kuning, Adira 1 and Apuy. The results of cluster analysis show that there is no clear grouping between cassava genotypes that have higher starch or resistant starch content. And we found that there is none specific marker that have a correlation to the have higher starch or resistant starch content.

![Figure 2. Dendogram of cassava based on UPGMA analysis](image)

3.4. Resistance to freeze-thawing

Table 2 shows the resistance to freeze thawing of several cassava genotypes. The data obtained from the statistical analysis showed a significant differences in resistance to freeze thawing at p<0.05 among the different genotypes that were tested. The value of freeze thawing resistance were varied from 0.033-1.207. Resistance level of starch and flour to freeze thawing could be differ into three group: resistance group (Gajah, Gebang, Manggu, Mentega 2), moderate resistance group (Adira 4, Apuy, Iding, Kristal Merah, Menti, Ubi Kuning, and tapioca of Rose Brand)), and susceptible group
(Adira 1). Resistance group were suitable to use for frozen food, while the susceptible one were not recommended.

Resistance to freeze thawing is one of the important properties of starches. Susceptibility to freeze thawing is indicated by water liberation from pastes after cooling for certain time. During storage of starch paste or gel in low temperature, amylose will precipitates as well as amylopectin. Acceleration of retrogradation will increased by repeated cycles of freezing and thawing of starch gel. Small granules are less susceptible to this process than large granules [30].

| Samples          | % of resistance to freeze and thawing |
|------------------|---------------------------------------|
| Adira 1          | 1,173±0,33 b                           |
| Adira 4          | 0,527±0,09 ab                          |
| Apuy             | 0,297±0,17 ab                          |
| Gajah            | 0,153±0,07 a                           |
| Gebang           | 0,157±0,05 a                           |
| Iding            | 0,480±0,11 ab                          |
| Kristal Merah    | 0,863±0,30 ab                          |
| Manggu           | 0,160±0,05 a                           |
| Mentega 2        | 0,213±0,15 a                           |
| Menti            | 0,327±0,16 ab                          |
| Ubi Kuning       | 0,397±0,10 ab                          |
| Tapioka Rose Brand | 0,317±0,17 ab                       |

Notes: Mean value with different letters denote significant differences, while the same superscript are not significantly different (Duncan, α = 0,05).

3.5. Swelling Power and Solubility of Starches

Table 3 shows the swelling power and solubility of several cassava genotypes. The data obtained from the statistical analysis showed unsignificant differences in swelling power and solubility at p<0,05 among the different genotypes that were tested. The swelling power and solubility of starch depend on the capacity of the starch molecule to hold water through hydrogen bonding after gelatinization. Both swelling power and solubility describes the interaction between water molecules and glucan chain. At the low temperature during the gelatinization, only a small amount of starch granules were swelled carbohydrate solubilized, but at higher temperatures (>70°C), a rapid increase in swelling power will observed, and at 90°C, more the starch granule were swelled and large amount of carbohydrate leaks from the granular structure.

When starch paste is heated, the crystalline structure of starch will relaxed, the groups of amylose and amylopectin associate with water molecules through hydrogen bondings and it causes an increase in the swelling power and in the solubility of the granules. By increasing the temperature, the starch granules will disrupted and amylose will released from the amylopectin.

There were differences in swelling power among the cassava genotypes, attributed to disparities in bonding forces within the starch granule. Swelling power of cassava starch varied from 8,295 to 13,0575 g/g as shown in Table 3. The swelling power and solubility provide evidence of the magnitude of interaction between starch chains within the amorphous and crystalline domains. The swelling behaviour of starch depends mainly on the amylose content, structure of amylose and amylopectin, and presence of non-carbohydrate substances, especially in the presence of lipids acting as inhibitor of swelling. Solubility of starch is an indicator of the degree of starch granules dispersion after cooking. Solubility increased with increase in temperature. The solubility could imply to the amount of amylose leaching out from starch granule when swelling, therefore the higher the solubility the higher will be the amylose leaching. Difference in solubility could also be attributed to different chain length distribution in the starch [31].
Starches have been classified as high swelling, moderate swelling, restricted swelling, or highly restricted swelling. High-swelling starches have swelling power of approximately 30 or higher at 95 °C. Their granules swell enormously and the internal bonds become fragile toward shear when the starch is cooked in water, Restricted-swelling starches have swelling power in the range of 16 to 20 at 95 °C. The cross-linkages in their granules reduce swelling and stabilize them against shearing during cooking in water. Therefore, the resulting swelling power indicated that the starch extracts obtained were highly restricted type [32].

Table 3. Swelling power and solubility of cassava starch

| Sample of starch       | Swelling Power | Solubility  |
|------------------------|----------------|-------------|
| Adira 1                | 8.590±0.064 a  | 0.361±0.053 a |
| Adira 4                | 10.183±0.279 a | 0.311±0.048 a |
| Apuy                   | 11.605±0.384 a | 0.350±0.047 a |
| Gajah                  | 9.826±0.153 a  | 0.364±0.047 a |
| Gebang                 | 11.893±0.499 a | 0.368±0.054 a |
| Iding                  | 10.420±0.288 a | 0.437±0.057 a |
| KM Cimanggu            | 10.342±0.240 a | 0.315±0.050 a |
| Manggu                 | 11.454±0.348 a | 0.388±0.052 a |
| Mentega 2              | 10.182±0.089 a | 0.301±0.047 a |
| Menti                  | 9.018±0.037 a  | 0.362±0.069 a |
| Ubi Kuning             | 10.447±0.328 a | 0.349±0.049 a |
| Tapioka Rose Brand     | 9.905±0.214 a  | 0.293±0.048 a |

Notes: Mean value with different superscript letters denote significant differences, while the same superscript are not significantly different (Duncan, α = 0.05).

3.6. Paste Clarity

Table 4 shows the starch paste clarity of several cassava genotypes. The data obtained from the statistical analysis showed a significant differences in paste clarity of starch at p<0.05 among the different genotypes that were tested. The paste clarity were varied from 0.135 to 0.543. The most transparent starch is obtained from the Carvita 25, while the most opaque one is from the control of tapioca Rose Brand. More soluble the starch, more transparent the paste. Light absorbance indicates the clarity of a starch paste. Transparent paste is preferred to be used to thicken food products such as fruit pies, while opaque paste is preferred to applied in salad dressing.

Table 4. Paste clarity of cassava starch

| Starch samples   | Paste clarity |
|------------------|---------------|
| Adira 1          | 0.462±0.002 f |
| Adira 4          | 0.282±0.004 c |
| Apuy             | 0.383±0.001 d |
| Gajah            | 0.431±0.017 d |
| Gebang           | 0.369±0.002 e |
| Iding            | 0.441±0.001 ef|
| Kristal Merah    | 0.226±0.020 a |
| Manggu           | 0.252±0.012 b |
| Mentega 2        | 0.362±0.002 c |
| Menti            | 0.245±0.001 ab|
| Ubi Kuning       | 0.245±0.001 ab|
| Tapioka Rosebrand| 0.543±0.002 g |
Mean value with different superscript letter denote significant differences, while the same superscript are not significantly different (Duncan, \( \alpha = 0.05 \)).

4. Conclusion
According to our result, we make a conclusion that cassava have a great variety in their starch and resistant starch content and its physicochemical properties. Manggu is one of the cassava genotypes with the highest content of resistant starch (7.32%). The percentage of starch resistance to freeze thawing varies from 0.153 to 1.173, with the highest resistance to freeze thawing are Gajah, Gebang, Manggu and Mentega 2. Swelling power of different starch samples was varies from 8.59 to 11.605 g/g, and the solubility of starch have a slight ranges from 0.293 to 0.437 g/g at 90°C. Among all genotypes, there are none difference on solubility and swelling power between the genotypes. The value of paste clarity varies from 0.135-0.543, and the clearest paste is belong to Kristal Merah. Dendogram generated based on UPGMA cluster analysis showed that all the cassava genotypes were distributed in two main clusters, and three subclusters.

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