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Note

Establishment of new method for analysis of starch contents and varietal differences in soybean seeds

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Aside from a report claiming that soybean seeds contain less than 1% starch, little is actually known about the genetic variation of the starch content in this important crop species. We used a starch-iodine test to identify varieties with high starch content in a soybean germplasm collection and found a total of 34 cultivars that showed a strongly positive reaction (dark color). For a more accurate quantitation of starch contents, we established a new method using a heat-resistant α -amylase and dinitrosalicylic acid (DNS) reagent. We compared the DNS method and the standard method using glucose-oxidase (GOD) with a known amount of starch as a standard. We showed that the DNS method generated results that were very highly correlated with the GOD method. In addition, we found the new method to be easier and faster to implement than the GOD method. Using the DNS method, we found several accessions in our soybean germplasm whose starch contents were 2–7%.

Key Words: starch content, soybean, iodine-starch, heat-resistant α -amylase, dinitrosalicylic acid.

Introduction

Starch is the most important carbohydrate in the human and animal diets and it also has numerous industrial applications. Rice, wheat, maize, potatoes and cassava are the major sources of food starch. Starch is widely used as a key material for processed foods such as bread, pancakes, cereals, noodles, pasta, porridge and tortilla. Increasing the starch content in food crops is one of the important targets for crop breeding.

Screening of germplasm, breeding and/or marker-assisted selection have been conducted to improve starch content in potato (Muth *et al.* 2008) and maize (Chander *et al.* 2008). In addition to conventional cross-breeding, starch content has been increased by over-expressing an important gene (identify the gene) involved in the accumulation of starch (Stark *et al.* 1992, Tobias *et al.* 1996, Geraldine *et al.* 2005).

Soybean (*Glycine max* (L.) Merr.) is widely used as raw material for animal feeds, dietary supplements, pharmaceuticals, food or industrial oils (www.soygrowers.com). Soybean seed mainly contains 43–48% of protein, 18–21% of lipid, and carbohydrate (Hildebrand and Hymowitz 1981, Poysa *et al.* 2002, Wilcox and Shibles 2001). The carbohydrate is mainly composed of sucrose (4.9–6.8%), raffinose (0.8–1.2%) and stachyose (3.5–4.3%). During seeds development, starch content reaches about 20% of seed weight

(Monma *et al.* 1991). Nevertheless, the starch contents in maturing seeds rapidly decreases to lower than 1% (Wilson *et al.* 1978). Monma *et al.* (1991) also reported that the decline in starch content in developing soybean seeds were due to higher levels of phosphorolysis. Consequently, soybean seeds have much lower starch content compared to the seeds of other legumes like mungbean, adzuki bean, and pea that contain 34% to 55% of starch (Wilson *et al.* 1978, Colonna *et al.* 1995). Soybean breeders have focused on improving the quality and quantity of proteins and lipids in soybean seeds. However, little is known about the genetic variation and mode of inheritance of starch content in this important crop species.

Increasing the starch content in soybean seeds is expected to have a large effect on the processing properties of soybeans. The soybeans having increased starch content may be suitable for processing nimame (boiled soybean) and miso (fermented soybean paste) especially because of their expected softness after boiling. They can also be used as edamame-like food because of their expected increased starch content at the late maturity stage. Thus, use of the high starch soybeans is expected to change the taste, flavor and processing property of soy food stuffs.

The determination of genetic variation in starch content in the soybean germplasm can be used for the development of new varieties with high starch content. The high-starch accessions may also be used to investigate and understand the mechanisms of starch synthesis and degradation during seed maturation.

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Starch content of crops or vegetables is usually evaluated using a color reaction of hydrolysate of starch with phenol-sulfuric acid or perchloric acid-anthrone (Michel *et al.* 1956, Clegg 1956). These reactions are conducted after the isolation of starch from plant materials and an enzymatic hydrolysis of starch. These processes are laborious and time consuming. An iodo-starch method is a rapid and simple procedure, but this is suitable for the qualitative analysis rather than quantitative analysis of starch. Starch content in soybean is usually evaluated using the glucose-oxidase (GOD) method (MacRae 1971, Monma *et al.* 1991). The starch in materials is converted to beta-D-glucose through multi-steps of denaturing in an autoclave and subsequent hydrolysis by an enzymatic reaction. The hydrogen peroxide form of beta-D-glucose becomes red by a reaction with phenol and 4-aminoantipyrine. The starch content is calculated by measuring the intensity of red. The GOD method provides an accurate starch content but this method requires a laborious and time consuming procedure as well as other methods involving by a color reaction.

Screening the 6,002 accessions of soybean germplasm for starch content required a simpler and easier procedure. We established a new method to assay the starch content in soybean seeds using a heat-resistant amylase and DNS reagent. Dinitrosalicylic acid (DNS) is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid. It is mainly used in the assay of alpha-amylase (Miller 1959). We used this method to identify several cultivars with high starch content.

Materials and Methods

Source of seed materials for screening

A total of 6,002 soybean [*Glycine max* (L.) Merr.] accessions were obtained from the gene bank of the National Institute of Agrobiological Sciences in Tsukuba, Japan and from Hokkaido University, the main branch of the National BioResource Project (*G. max*) Office, in Sapporo, Japan.

Rapid screening by iodine-starch reaction

We screened the 6,002 accessions in the soybean germplasm for high starch content in their seeds. At first, we used the rapid iodine-starch reaction to eliminate those with low starch contents. About 20 μ l of iodine solution (2% I_2 and 0.5% KI) was dropped on the exposed cotyledons of mature seeds. After 10-minutes incubation at room temperature, we classified the resultant color reaction in the cotyledons on a scale '1' to '4' based on the visible color intensity of reaction such as 'no reaction', 'light violet', 'violet', and 'dark violet' (Fig. 2A).

Developing a novel method for quantification of starch contents

We developed a more accurate method to quantitate the starch contents of the cultivars that scored 3 or 4 in the starch iodine test. We ground approximately 3 to 5 seeds with a

mortar and pestle transferred 50 mg of the soy-meal to a 2.0 ml tube containing 1.5 ml of 80% ethanol and sonicated the mixture with an ultra sonicator (USK-2, SND, Japan) at 38 KHz for 10 minutes to dissolve the soluble sugars. After centrifugation at 13,000 rpm for 10 minutes at 4°C, we removed the supernatant and air-dried the pellet for several minutes. We added 1.0 ml of water, 50 μ l of 2 M sodium acetate solution (pH 5.0) and 50 μ l of 10-fold heat-resistant amylase (Wako, Cat. No. 630-04401, Japan) and mixed them by a brief vortex and then incubated the mixture at 80°C for 30 minutes. After centrifuged at 13,000 rpm for 10 minutes at 4°C, we mixed 200 μ l of the supernatant with the same volume of dinitrosalicylic acid (DNS) reagent. After boiling the mixture for 5 minutes, we adjusted the final volume to 5 ml with water and measured the absorbance at 535 nm in a U-2800 spectrophotometer (HITACHI, Japan). We summarize the protocol in Fig. 1.

We used reducing sugars released from a known amount of starch as a standard for quantitation. We also examined the correlation between the absorbance data generated by the DNS and GOD methods with a known amount of starch (Wako, Cat. No. 439-90901, Japan).

Results and Discussion

In the initial screening, we analyzed the color intensity generated by the iodine-starch reaction in 6,002 cultivars of a soybean germplasm. Most of cultivars showed weak color reactions with a score of 1 (Fig. 2A and B). However, 34 cultivars generated strong color intensity ratings of 3 or 4 (Fig. 2). We quantitated the starch contents of these 34 cultivars with the DNS method using a heat-resistant α -amylase and dinitrosalicylic acid (DNS) reagent (Fig. 1). Before performing quantitative analysis, we compared GOD method with the new method from the point of view of work efficiency and the reliability.

Compared to the GOD method, the DNS method was

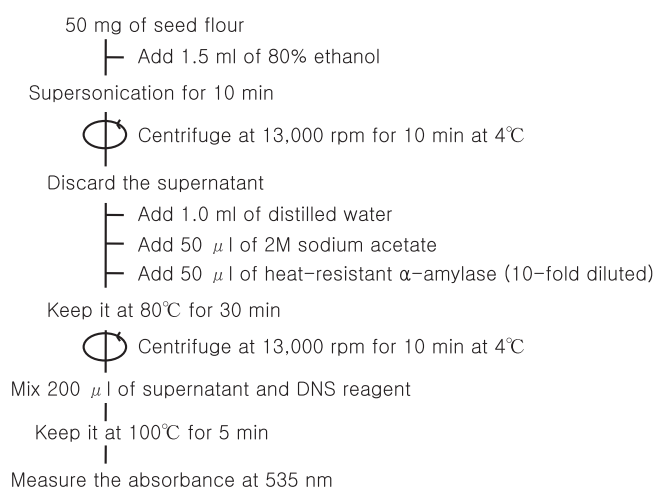


Fig. 1. Starch quantitation using the dinitrosalicylic acid (DNS) method.

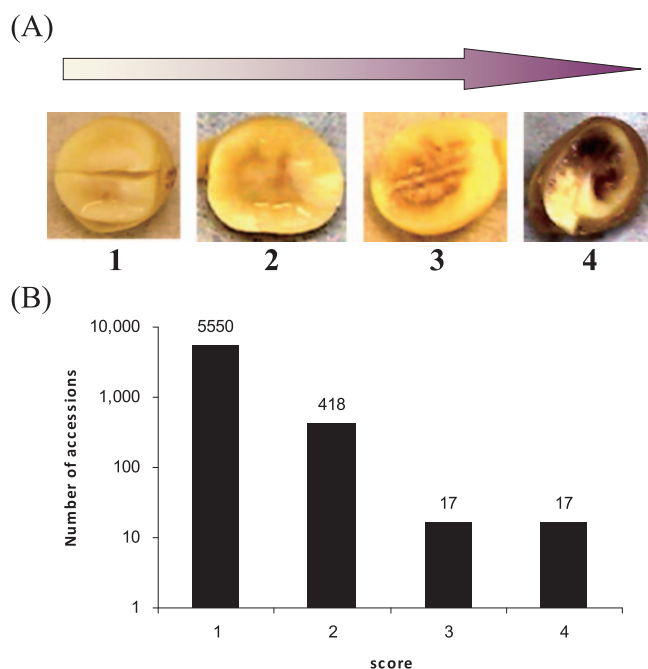


Fig. 2. Evaluation of starch content using the iodine-starch reaction. (A), Histochemical analysis in matured seeds. Reaction levels were evaluated by scoring '1' to '4' based on the color intensity. (B), Histograms of reaction levels in 6,002 soybean germplasm. At (B), the X-axis (score) corresponds to No. of (A) and the Y-axis (numbers of accessions) means total No. of each scoring at iodine-starch reaction.

labor saving and required less time. In the GOD methods (MacRae 1971, Monma *et al.* 1991), starch is heated in an autoclave to destroy its double helix structure for 20 minutes. The heat-denatured starch is subsequently hydrolyzed by gluco-amylase at 37°C for 1 hour. On the other hand, the use of a heat-resistant amylase allows these two processes to occur in one step in the DNS method. Therefore, the GOD method required 70 more minutes to process 24 samples while the DNS method required about 90 minutes to do it. The DNS method had a very high coefficient of determination (R^2) of 0.9973 to the GOD method (Fig. 3), indicating that the two methods have almost identical responses to varying starch levels. The absorbance values generated by the DNS method tended to be slightly higher than those produced with the GOD method (Fig. 3). This may be due to the different end products in each reaction and the wavelengths used to measure the absorbance of these end products. In the DNS method, glucose, maltose and dextrin are produced when starch is hydrolyzed by α -amylase. On the other hand, the GOD method produces only glucose after the gluco-amylase reacts with starch. End products of the DNS reaction were measured at 535 nm whereas the products of the GOD reaction were measured at 505 nm. The yellow-colored dinitrosalicylic acid changed to dark brown upon reaction with reducing sugars. Glucose-oxidase (GOD) turned pink when it reacted with glucose. The characteristics peculiar to each method account for their slightly different estimates of starch contents.

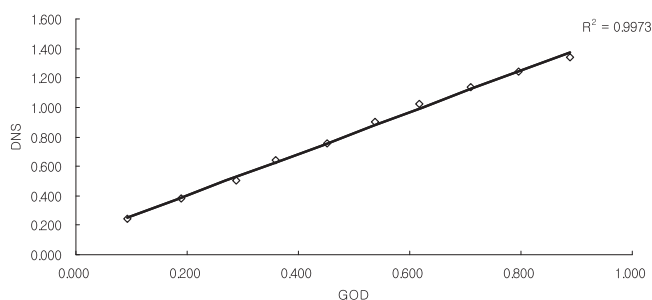


Fig. 3. Correlation between DNS and glucose-oxidase (GOD) methods upon reaction with a standard starch solution. Each value represents absorbance at 505 or 535 nm for the GOD and DNS methods, respectively. R^2 , the coefficient of determination, is the square the correlation coefficient and it is measure of the reliability of the correlation. The values were averaged from each of three replicates.

Table 1. DNS quantitation of starch content of germplasma with iodine-starch test scores 3 and 4 in Fig. 1. Ichihime was used as the control variety. The data represent means of three replicates $\pm$ SD

Cultivars (Conservation Lot No.)	Starch content (%) (DNS method)
Ichihime	0.76 $\pm$ 0.07
Aobata (32132)	5.26 $\pm$ 0.37
Aobatsu (31060)	6.59 $\pm$ 0.82
Aodaiz (30688)	6.00 $\pm$ 0.54
Kinkazan (31589)	4.41 $\pm$ 0.35
Shinanotaira (55117)	6.13 $\pm$ 0.76
Shiragiku (30769)	5.39 $\pm$ 0.41
Tanishidaizu (33279)	6.86 $\pm$ 0.36
Tanokurodaizu (31796)	4.06 $\pm$ 0.40
TO 1-0 (30633)	4.36 $\pm$ 0.49
Tokei 32 (111231)	5.04 $\pm$ 0.38
Tousan 86 (39566)	6.73 $\pm$ 0.87
Wasekurohikari (31705)	4.28 $\pm$ 0.29
Wasekuromame (31540)	5.53 $\pm$ 0.58
Wasemame (30464)	5.55 $\pm$ 0.17
Zairai 51-11 (32724)	5.91 $\pm$ 0.62

Consequently, these results demonstrated that DNS method developed in this study can be used to precisely and speedily quantitate starch contents. Theoretically, this method is useful to analyze the starch content of any crops. Using the DNS method, we found that the starch content in our soybean germplasm ranged from about 2 to 7%, and we identified the accessions containing much higher percentage of starch content (up to 7-fold) than the previously reported 0.19–0.91% (Wilson *et al.* 1978). Total 34 cultivars classified as '3' and '4' with iodine reaction showed about 2–7% of starch content. Especially the 15 cultivars that had more than 4% starch content are listed in Table 1. Their starch contents were much higher than that of standard cultivar.

By comparison, our control variety Ichihime was found to have only 0.76% of starch content (Table 1). In addition to Ichihime, we also found that the starch content of Fukuyutaka, Enrei and Williams 82 were 0.81%, 1.12% and 1.10%, respectively. Soybean cv. Ichihime has been used for

analysis of other seed component in our laboratory (Ujiie *et al.* 2005, Maria *et al.* 2007). Accordingly, we selected the Ichihime as a control. In this study, we successfully identified some cultivars with high starch contents that were 6–8 times than that of our control variety, Ichihime. However, further investigations in these cultivars are necessary to confirm the genetic stability for their high starch trait, because the starch content is often affected by environment factors such as temperature and humidity.

At present, we are developing the segregating progenies derived from several varieties with high starch contents. Further studies are under way to analyze the genetic control of starch accumulation in soybean seeds.

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