米粒の生長並びに米デンプン合成に関する研究(2)

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Development of Endosperm and Synthesis of Starch in Rice Grain

II. Synthesis of starch*

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Abstract : The brown rice grains (*Japonica* c.v. Koshihikari) fed with $^{14}CO_2$ in different ripening stages were separated into the outer layer and inner portion by the wet-milling method, and starch granules were prepared. The specific radioactivity was higher in the inner portion starch when $^{14}CO_2$ was fed in the early ripening stage, but in the outer layer starch in the late ripening stage. Specific radioactivity of amylose and amylopectin was similar in the inner portion starch when $^{14}CO_2$ was fed in the early ripening stage. As grains ripened, however, the specific radioactivity of amylose became higher than that of amylopectin. No difference was found in the specific radioactivity between the inner chains and exterior chains of starch molecules. Most of the ^{14}C fed in the early ripening stage existed in the portion resistant to glucoamylase and hydrochloric acid, and that in the late ripening stage was found in the digested fraction.

Key Words : Amylose, Amylopection, Ripening, Rice grain, Specific radioactivity, Starch granules, Wet-milling method.

米粒の生長並びに米デンプン合成に関する研究　第2報　デンプンの合成：何　光存・木暮　秩・鈴木　裕（香川大学農学部）

要　旨：米デンプンの性質は米粒の部位によって違うことが知られている．^{14}C を取り込ませた米粒の外部と内部からデンプンを分離し，それらのデンプン粒の構造及び分子成分の形成と米粒登熟との関係を検討した．前報で得られた ^{14}C 標識した玄米を湿式搗精法で外部と内部画分に分け，ドデシルベンゼンスルホン酸ナトリウムで洗浄によりデンプンを精製した．デンプンの比放射活性は全粒としては出穂後18日目に最も高かった．これを外部と内部で比べると，登熟前期（11日まで）には内部の方が高いが，18日目以降では外部の方が高くなった．ゲルろ過により分離したアミロースとアミロペクチンの比放射活性は登熟前期には大差がないが，後期にはアミロースの方が高かった．β-アミラーゼで分解の結果，放射活性はデンプン分子内に均一に分布していることが分かった．生デンプン粒のグルコアミラーゼで分解しにくい部分は登熟前期に，分解しやすい部分は後期に比放射活性が高かった．また塩酸分解した部分と残った部分も比放射活性が異っていた．

キーワード：アミロース，アミロペクチン，湿式搗精法，デンプン粒，登熟，比放射活性，米粒．

In the previous paper, we reported that the ^{14}C-labeled assimilates distributed throughout the whole rice endosperm when $^{14}CO_2$ was fed about the 5th day after heading (DAH), then accumulation occurred early in the inner portion and later in the peripheral layer. Based on the percentage of ^{14}C distributed among various components and the radioactivity peak period, we proposed that starch synthesis was the critical process during the active development of endosperm, and synthesis was more active in the middle ripening period[7].

There are many uncertain points about starch synthesis such as the relationship between amylose and amylopectin and their disposition onto starch granule. Radioisotope is a useful method for the study of starch synthesis, and has been applied by many researchers[12,13,15]. Starches from different portions of rice grains differed in physicochemical properties presumably due to physiological gradient[9]. In this paper, we discuss the synthesis of starch in different portions of rice grain and the relation between starch composition and rice grain ripening.

Materials and Methods

1. *Rice grains*

The brown rice grains (*Japonica*. c.v. Koshihikari) administrated with $^{14}CO_2$ during the ripening period were obtained as in the previous experiment[7].

2. *Separation of the outer layer of rice grains*

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and preparation of starch granules

Separation of the outer layer was performed by the wet milling method that had been described[8]. In the present experiment, 25 g brown rice were soaked in water, rather than in 60% ethanol, at 10°C for 12 h because there was no crack on the surface of the grain. The soaked rice grains were milled in a small scale wet milling equipment with a 300 ml beaker and an emery disk of diameter 7 cm. Preliminary experiment had demonstrated that the milling result was as desirable as that of larger scale sample wet-milled in ethanol. To get 25% of outer layer and 75% inner portion, it took about one hour. The outer layer and inner portion were separated by sieve then homogenized by Waring blender. Starch granules from two parts were purified by washing with 1.2% sodium dodecylbenzenesulfonate.

3. *Fractionation of amylose and amylopectin*

Amylose and amylopetin were fractionated by gel filtration. Thirty mg rice starch were suspended in 4.5 ml water and gelatinized in a boiling water bath for 3 min. After the solution was cooled, 0.5 ml of 5N NaOH was added and left at 5°C overnight. About 15 mg gelatinized starch was applied on Sepharose CL-2B column (2.6×50 cm) and eluted with 0.05N NaOH containing 0.02% NaN_3 at 10 ml per h. Each 4 ml fraction was collected. After being neutralized by 1N acetic acid, 0.5 ml was taken for carbohydrate determination, 0.5 ml for radioactivity, and the remaining for absorption peak maximum (λmax) of glucosan-iodine complex.

4. *Analytical methods*

The carbohydrate content was determined by phenol-sulfuric acid method[4]. Radioactivity was measured by a liquid scintillation counter as described in the previous report[7]. Specific radioactivity was expressed as dpm per mg. For determining the properties of eluted glucosan. 0.2 ml of I_2-KI solution was added into the fractions. After standing in room temperature for 20 min, the testing mixture was scanned from 500 nm to 650 nm on the Hitachi 150-20 spectrophotometer.

5. *β-amylolysis*

Thirty mg gelatinized starch were hydrolyzed by 50 units of β-amlase (Sigma, Type 1-B) in 10 ml of 0.02M acetate buffer (pH 4.8) for 12 h. Reaction was stopped by putting in a boiling water bath, and the β-amylolysis limit dextrin was precipitated by adding 3 volume ethanol. The dextrin was washed with ethanol once, then dissolved in 1N NaOH. The supernatant was recovered as the maltose fraction. Both fractions were measured for specific radioactivity.

6. *Digestion of starch granules by glucoamylase*

Thirty mg starch granules were incubated with 3 units of glucoamylase (Seikagaku Kogyou, from *Rhizupos niveus*) in 0.02M acetate buffer (pH 4.8) at 40°C for 4 h (3 h for outer layer starch). After centrifugation at 3, 000 rpm for 15 min, the superanatant was used for measuring specific radioactivity in the digested fraction. The remained granule residue was washed with water then gelatinized, and specific radioactivity was measured.

7. *Digestion of starch granules by HCl*

Thirty mg starch granules were incubated in 5 ml 2.2N HCl at 35°C for 4 days. After contrifugation, both the degraded and remain-

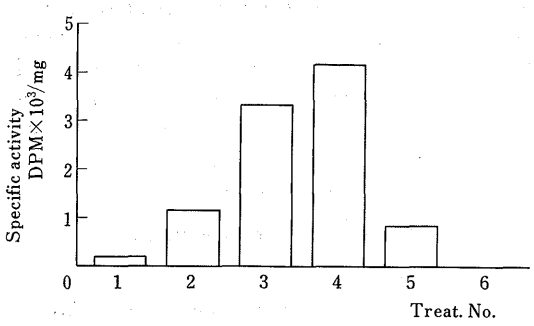

Fig. 1. Specific radioactivity of starches from ^{14}C-fed rice grains. For Treat. No., See the Table 1 in the previous report[7].

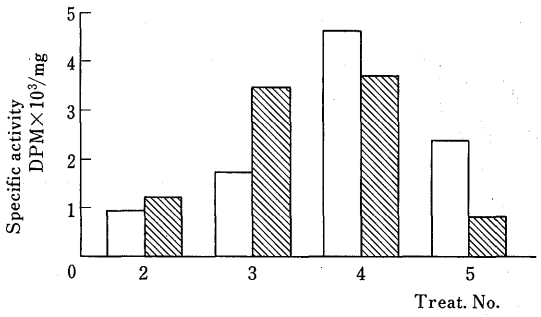

Fig. 2. Specific radioactivity of starches from the outer layer (□) and inner portion (▨) of the ^{14}C-fed rice grains. For Treat. No., see legend to Fig.1.

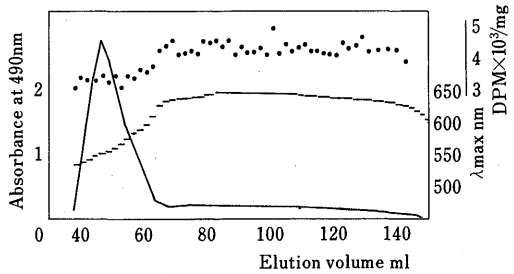

Fig. 3. Elution profile of the inner portion starch from grains of Treat. No.4 on Sepharose CL-2B column. ——— absorbance at 490 nm for estimating carbohydrates content, ---------- absorption λmax of glucosan-iodine complex, ····· specific radioactivity.

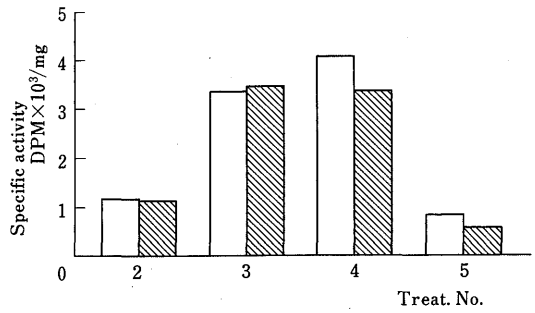

Fig. 4. Specific radioactivity of amylose (□) and amylopetin (▨) of starch from the inner portion of ^{14}C-labeled rice grains. For Treat. No., see legend to Fig.1.

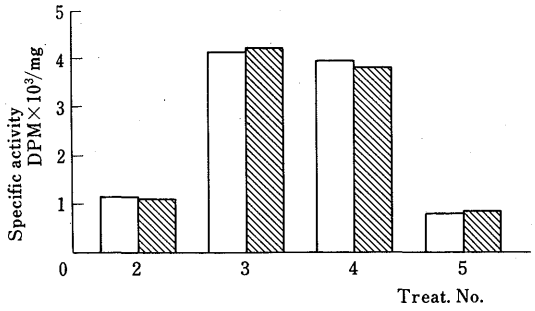

Fig. 5. Specific radioactivity of maltose fraction released by β-amylolysis (□) and β-amlase limit dextrin (▨), the starches used were from the inner portion of ^{14}C-labeled rice grains. For Treat. No., see legend to Fig.1.

ing fractions were recovered and specific radioactivity was determined.

8. *Scanning electronic microscope (SEM) observation of starch granules*

The starch granules digested by glucoamylase and HCl were freeze-dried and dusted onto the double sided cellotape adhering to the surface of SEM Stub. All specimens were coated with platinum and observed on Hitachi S-800 SEM at 8 kV accelerating voltage.

Results

1. *Starch of whole grains*

Result shown in Fig. 1 indicated that the specific radioactivity of starch from whole rice grains changed with the ripening stages when $^{14}CO_2$ was fed. The specific radioactivity of starch obtained from the mature grains was 200 dpm/mg when $^{14}CO_2$ was fed on the 5th day before heading (DBH). It rose after heading and reached 4200 dpm/mg in grains fed with $^{14}CO_2$ on the 18th day after heading (DAH). The specific radioactivity dropped in the late ripening stage and was not detected in starch granules of grains fed on 32 DAH.

2. *Starches from the outer layer and inner portion of rice grains*

Specific radioactivity of the outer layer and inner portion starches from brown rice grains fed with $^{14}CO_2$ on 5, 11, 18 and 25 DAH are shown in Fig.2. The activity was higher in the inner portion starch than that in the outer layer starch when $^{14}CO_2$ was fed in the early ripening stage (5 and 11 DAH). However, the activity in the outer layer starch became higher after 18 DAH. It is obvious that the starch in the inner portion was synthesized fast in the early ripening stage and that in outer layer fast in the late ripening stage.

3. *Components of starch*

Gel filtration is an effective method for fractionation of starch components[2]. In this experiment, amylopectin was eluted out near the void volume and amylose was eluted out later by Sepharose CL-2B gel filtration, as detected by λmax of starch-iodine complex (Fig.3). Specific activity of amylopectin fraction was about 3.1-3.5 dpm/μg and above 4 dpm/μg for amylose fraction, though the deviation was large because starch was diluted. The inner portion starches fed with $^{14}CO_2$ on 5, 11, 18 and 25 DAH were subjected to gel filtration, and the fractions from 30-62 ml

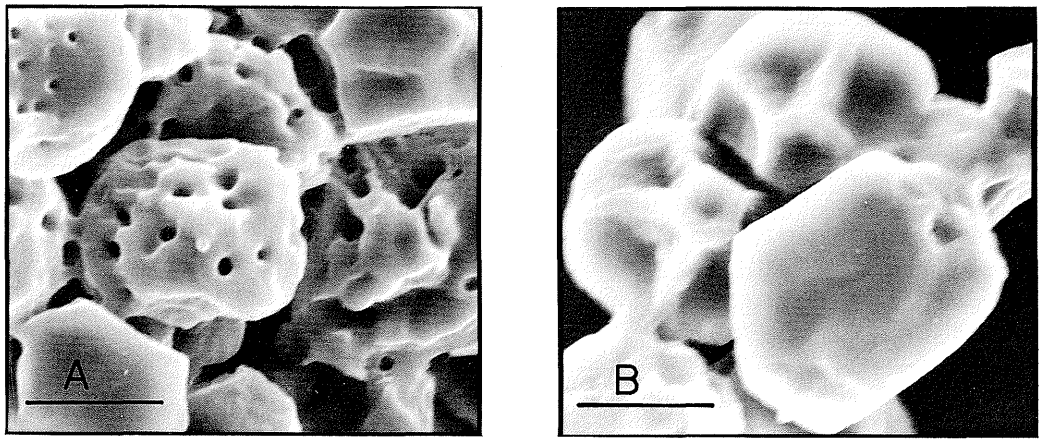

Fig. 6. Starch granules of rice, A : digested by glucoamylase and B : degraded with hydrochloric acid (bar=3μm).

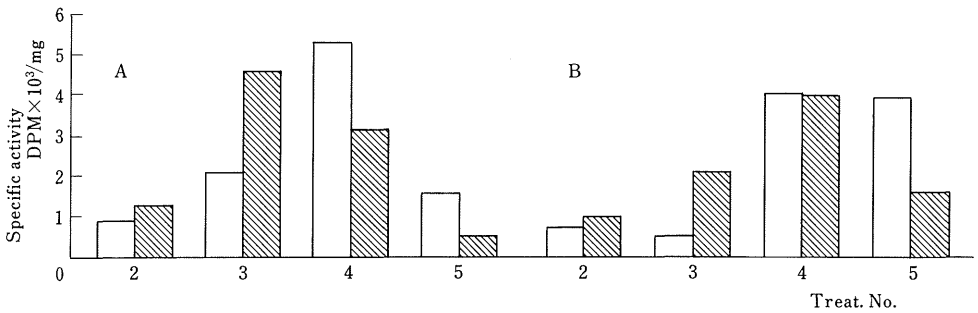

Fig. 7. Specific radioactivity of the digested fration (□) and remained residue (▨) of starch granules digested by glucoamylase. A : inner portion starch, B : outer layer starch. For Treat. No., see legend to Fig.1.

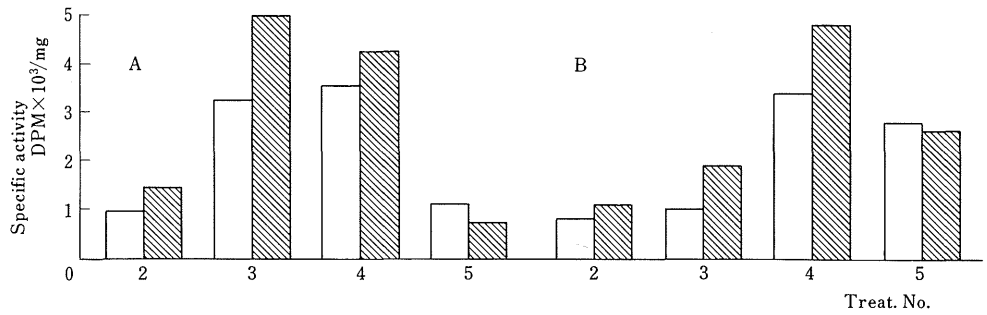

Fig. 8. Specific radioactivity of the degraded fraction (□) and the remained residue (▨) of starch granules treated with HCl. A : inner portion starch, B : outer layer starch. For Treat. No., see legend to Fig.1.

with λmax below 580 nm was pooled as amylopectin and 66-140 ml with λmax above 620 nm were pooled as amylose. The specific radioactivity of both fractions are shown in Fig. 4. In the early ripening stage, the activities of both fractions were essentially similar though that of amylopectin appeared a little higher by feeding on 11 DAH. However, specific radioactivity in amylose fraction turned to be higher than that in amylopectin after feeding on 18 DAH, suggesting that amylose accumulation was accelerated in the inner portion starch in the late ripening stage.

β-amylase cut the α-1,4-glucoside bond

from the nonreducing end and stopped near the α-1,6 branch, so ^{14}C contained in the outer chain and inner chain of starch molecule can be separated after β-amylase hydrolysis. The specific radioactivity of the released maltose fraction and the limit dextrin fraction was not significantly different among the inner portion starches of the four tested samples, suggesting that ^{14}C scattered throughout the starch molecules and not affected by the stages of $^{14}CO_2$ feeding (Fig.5).

4. *Starches digested by glucoamylase*

When raw rice starch granules were subjected to 3-4 h digestion by glucoamylase, about 27% was eroded. Scanning electron microscope revealed that the many medium size holes were made as well as surface erosion observed on the glucoamylase-digested granules (Fig.6-A). The specific radioactivity of the digested and the remained fractions changed with the times of $^{14}CO_2$ feeding (Fig.7). For the inner portion starches, the radioactivity was relatively high in the remained fraction when $^{14}CO_2$ was fed on 5 and 11 DAH, and in the digested fraction when $^{14}CO_2$ was fed on 18 and 25 DAH. The outer layer starches also had a relatively high specific radioactivity in the remained fraction when $^{14}CO_2$ was fed on 5 and 11 DAH, but activity in both fractions was similar by feeding on 18 DAH and turned to be higher in the digested fraction by feeding on 25 DAH feeding. The result indicated that the remained fraction was synthesized earlier than the digested fraction and there seemed to be a turning stage for synthesizing the two fractions which was earlier in the inner portion starch than the outer layer starch.

5. *Lintnerized starch*

About 35% weight was lost from the starch granules in 4 days lintnerization. Scanning electron microscope observation of the lintnerized starch granules showed that little erosion occurred on the surface, though some granules looked like empty bags with concave facets, suggesting corrosion occurred interior in granules (Fig.6-B). Both the inner portion and outer layer starches showed higher specific radioactivity in the residue portion for grains fed with $^{14}CO_2$ from 5 to 18 DAH, and higher in the degraded fraction for grains fed with $^{14}CO_2$ on 25 DAH (Fig.8). The earlier synthesized portion of granule seemed to be more resistant to HCl erosion.

Discussion

Since starch occupies nearly 70% weight of rice grains, the increase in weight of rice grains during the ripening is mainly due to starch accumulation. Starch content in rice grains increased until 20 days after flowering and the size of granule increased most rapidly during the period of 10-18 DAH[3,11]. The amount of ^{14}C-labeled assimilates incorporated into starch for whole rice grain was high during 11-18 DAH, suggesting starch accumulation was fast in this period. When the grains were separated into outer layer and inner portion, the amounts of ^{14}C in starches prepared from the two portions were not the same. In the eary ripening period, the ^{14}C-labeled assimilates were mainly transported into the inner portion for starch synthesis. The place of starch synthesis was moving to the peripheral layer of rice grain as the ripening proceeded. The specific radioactivity of outer layer starch, however, was much higher than that in the inner portion starch when $^{14}CO_2$ was fed on 25 DAH. The starches from the outer layer and inner portion differed in physicochemical properties, presumably due to the physiological gradient in rice grains[9], which was confirmed by the results of this study. Properties of rice starch were affected most by environment in the early ripening stage[1]. The variation might reflect mainly the characteristics of the inner portion starch, which corresponds to most part of the total starch and is synthesized in the early ripening stage. The outer layer starch may be susceptible to the environmental condition in period different from the inner portion starch. This subject will be discussed in our next report.

Starch is composed of two types of D-glucopyranose polysaccharides, the linear or sparsely branched amylose and the highly branched amylopectin. Amylose content determines physical properties of starch and eating quality of rice. How amylose and amylopectin be synthesized and make up the granule structure is not yet clearly known. A popular hypothesis is that phosphorylases and starch synthases catalyzed the addition of glucose to primer to form linear molecule, amylose; Q-enzyme and other alike enzymes cleave a fragment from the linear polymer and transfer it to C-6 position to produce amylopectin[14].

Therefore, amylose is thought to be an intermediate for amylopectin. In wheat, some evidences have been obtained that amylopectin was formed from amylose by using the ^{14}C tracer technique[12,15]. However, results in maize are contrary to above conclusion, in that the specific radioactivity of amylose and amylopectin increased at the same rate during 1-6 h after plant was exposed to $^{14}CO_2$[13]. Data in Fig.4 suggested that amylopectin perhaps came from amylose for the inner portion starch similarly to the situation in wheat. In the early ripening stage, a portion of amylose formed is soon transformed into amylopectin in a fixed rate, and the specific radioactivity is similar in both components. After certain time, the transformation activity declined and more amylose was stocked so that specific radioactivity in amylose was higher than that in amylopectin. The consequence is that amylose content in rice starch increased with ripening period[3]. The radioactivity scattered throughout the entire molecule of amylose and amylopectin in maize[13]. In this experiment, specific radioactivity was found similar in the inner and exterior chains in rice starch despite of $^{14}CO_2$ feeding in different stages.

Starch granules have complex structure and are composed of amorphous and crystal portions and show layer structure by enzyme digestion and microscopic observation. Rice starch has compound granules, they showed various erosion types when digested by amylase[5]. Many holes and surface erosion were observed on the glucoamylase-treated granules in the present experiment. This may reflect characteristics of the glucoamylase used[10]. Since ^{14}C radioactivity fed in the early stage related mainly to the fraction not digested by glucoamylase and that in the late stage was present in the degraded fraction, it can be considered that the early-formed starches are resistant, and the late-formed starches are susceptible to the glucoamylase. This conclusion is also consistent with our result that glucoamylase digested starches from the outer layer faster than those from the inner portion in grains of four varieties[10].

Rice starch granules showed an alternate erosion type when degraded by HCl. Little surface erosion was observed suggesting internal corrosion. The crystallinity was enhanced after potato starch granules was lintnerized, suggesting HCl selectively degraded the amorphous portion of the starch granules[6]. Data in Fig.8 indicate that the portion resistant to HCl degradation was formed earlier than the susceptible portion. There are two possible explanations for such differences in resistance to glucoamylase and acid. One is that amyloplast of different physiological age not only synthesizes different ratio of amylose and amylopectin but also forms different granule structure. Another possibility is that the starch granules formed still take some structural changes during the ripening period. Elucidation of this problem will help to understand starch formation.

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* In Japanese with English summary.

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