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STARCH BIOSYNTHESIS OF AMYLOMAIZE DURING ENDOSPERM DEVELOPMENT

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Key Word Index—*Zea mays*; Gramineae; maize; starch biosynthesis; *amylose-extender*; amylose; amylopectin.

Abstract—In an attempt to investigate starch biosynthesis of amylomaize, the endosperm starches from four genotypes with different doses of *amylose-extender* (*ae*) between 0 and 3 were isolated at 14, 18, 22, 28, and 36 days after pollination, and their properties were examined. Gene effects of *ae* on the endosperm starches were found in the apparent amylose content from measurements of iodine binding capacity and in the elution pattern from fractionation of starches debranched by isoamylase on Sephadex G-75. With increasing doses of *ae*, the lengths of two side-chain fractions of amylopectin in the original starch granule and the carbohydrate content of shorter side-chain fraction both increased. However, the length of each side chain fraction varied little between 14 and 36 days in all genotypes. In addition, the amylose content in *ae* homozygous at the 14-day stage was *ca* 24% lower than the value of amylose and intermediate fractions in the original starch granule.

INTRODUCTION

Among well-known endosperm marker genes of maize (*Zea mays*), *amylose-extender* (*ae*) is characterized by an increased amylose component in the starch synthesized [1]. Recently, the *ae* starch has been investigated by many workers [2–10]. Wolff *et al.* [2] reported that *ae* amylopectin, which was non-complexing with BuOH, had longer inner and outer branches than those of normal amylopectin. They proposed that the *ae* amylopectin was intermediate in structure between normal amylose and amylopectin. Greenwood *et al.* [3] reported that *ae* starch contained amylose of a lower degree of polymerization than normal amylose and an unusually high proportion of intermediate material fitting neither the definition of amylose nor amylopectin. Although they isolated normal amylopectin from the intermediate material, they were unable to separate the linear material from branched component of the intermediate material. While Boyer *et al.* [4] found either that amylopectin of *ae wx* (*amylose-extender waxy*) was a loosely branched amylopectin with an inner chain length of 52 glucose units, or that its outer chain was longer in length and fewer in number per mg of starch than that of *wx*. They proposed that the *ae wx* starch was similar to the anomalous amylopectin of *ae* starch. Although there are many reports on the *ae* starch, enzymatic or physiological reports on the *ae* starch biosynthesis have been few [11–15]. Therefore, it has not been well explained how the gene effects of *ae* relate to the mechanism of the *ae* starch biosynthesis enzymatically or physiologically.

Manners and Rowe [16] reported that extracts of sweet corn contained a mixture of debranching enzymes; R-enzyme (EC 3.2.1.41) and isoamylase (EC 3.2.1.68). On the basis of their report, Ayers and Creech [17] suggested that *ae* might function by allowing increased activity of a debranching enzyme, and short-chain amylose might be accumulated as the result. They further suggested [18] either that the dominant *Ae* codes for the production of a suppressor, or that *ae* codes for an activator of a debranching enzyme. However, no direct evidence for the debranching theory for *ae* gene has been reported yet.

In this paper, in order to elucidate effects of *ae* in starch biosynthesis, we have used four endosperm starches with different doses of *ae* between 0 and 3 as experimental material, and have examined some properties of the whole starches and the debranched starches at various stages of endosperm development. The purpose of the present investigation was to clarify the mechanism of the *ae*-specific starch biosynthesis during endosperm development.

RESULTS

Endosperm starches with different doses of *ae* between 0 and 3 were isolated at various stages of endosperm development, and values of iodine binding, amylose content, and λ_{max} of the starches is shown in Table 1. As a general tendency in all four genotypes, the iodine binding value was low at the 14-day stage, and increased rapidly between 14 and 18 days, and then the rate of increase slowed down up to the 36-day stage. Likewise, the amylose content and λ_{max} showed the same tendency. The amylose content at a certain stage increased with increasing doses of *ae*, and the maximum values obtained at 36-day stage with increasing doses of *ae* from 0 to 3 were 25.4, 29.8, 37.9, and 65.9%, respectively.

Fig. 1 illustrates elution patterns of endosperm starches with different doses of *ae* at the 14-day stage after debranching. With increasing doses of *ae*, the elution patterns of Frs. II and III became broader, and the peaks of the fractions shifted to the higher MW side. The same tendency of the elution pattern was also observed at other stages of endosperm development. Results of fractionations of debranched starches between 14 and 36 days after pollination are shown in Table 2. Percentages of Fr. I in 0, 1, and 2 doses of *ae* at the 14-day stage were lower than those at other stages, and increased rapidly between

Table 1. Iodine binding, amylose content, and λ_{max} of endosperm starches with different doses of *ae*

DAP* (days)	Dosage of *ae*	Iodine binding (mg iodine/100 mg sample)	Amylose content (%)	λ_{max} (nm)
	0	2.1	12.7	565
14	1	2.4	14.1	570
	2	2.8	16.7	575
	3	5.9	35.0	580
	0	3.8	22.5	568
18	1	4.4	26.1	575
	2	5.2	30.8	578
	3	10.0	59.6	582
	0	4.0	23.5	570
22	1	4.6	27.3	578
	2	6.0	35.8	580
	3	10.7	63.3	585
	0	4.2	24.9	570
28	1	4.6	27.6	578
	2	6.1	36.3	583
	3	10.8	63.9	585
	0	4.3	25.4	570
36	1	5.0	29.8	580
	2	6.4	37.9	588
	3	11.1	65.9	592

*Days after pollination.

14 and 18 days, and then the increase slowed down up to 36-day stage. In case of 3 doses of *ae*, the percentage remained almost constant throughout endosperm development. The percentage of Fr. I increased with increasing doses of *ae* at any stages, and these values agreed with those of the amylose contents except for 14-day stage (Tables 1 and 2). Percentages of Fr. II in all genotypes were similar throughout endosperm development, although average chain length of the peak became longer with increasing doses of *ae*. As to Fr. III, the percentage showed quite the reverse tendency against that of Fr. I by increase of *ae* and endosperm development. Average chain length of the peak of Fr. III became somewhat longer with increasing doses of *ae*.

The percentage value of each fraction was converted to mg starch per endosperm, and the result is shown in Fig. 2. The content of each fraction was low at the 14-day stage in all genotypes, and increased up to the 36-day stage. Dosage of *ae* showed significant effects on endosperm starches of Frs. I and III during endosperm development, while there was no noticeable effect on the starch of Fr. II by dosage of *ae*.

DISCUSSION

Mechanism of starch biosynthesis in *ae* endosperm became somewhat distinct by using endosperm starches with different doses of *ae* and different ages of development. Recently, Ikawa *et al.* [5] reported on fractionations on Sephadex G-75 of debranched starches from various maize mutants including amylomaize. They separated the elution pattern to three fractions, and showed the percentage content of each fraction. As pointed out by them [5, 6], Fr. I in our examination corresponded to the amylose and *ae*-specific intermediate fractions, and Frs. II and III corresponded to side-chain fractions of amylopectin in the original starch granule. Therefore, there were three remarkable effects on the endosperm starch by the *ae* allele during endosperm development; amylose content, lengths of two side-chain fractions, and content of shorter side-chain fraction.

Ikawa *et al.* [5] reported that in normal and *wx* maize starch, the content of Fr. I agreed with the amylose percentage measured by potentiometric iodine titration. However, in the case of *ae* starch, the value of Fr. I was 10–15% lower than the amylose percentage. They suggested that this reduction was due to the fact that amylomaize starch contained an intermediate fraction different from typical amylose and amylopectin. In our results, the value of Fr. I agreed with the amylose content measured by amperometric iodine titration except for 1, 2, and 3 doses of *ae* at the 14-day stage (Table 1 and 2). This may be due to the fact that the range of Fr. I was $\lambda_{max} > 600$ nm, and the Fr. I contained *ae*-specific intermediate fraction. The values of Fr. I for 1, 2, and 3 doses of *ae* at the 14-day stage were *ca* 6, 8, and 24% higher than the amylose contents, respectively. In addition, values of β-amylolysis limits of whole endosperm starches in normal and *ae* homozygous were 58.4 and 74.7% at the 14-day stage, respectively (unpublished data). The value of *ae* was *ca* 7% higher than other stages, while in normal 5% lower. These results suggest that biosynthesis of *ae*-specific intermediate fraction which is composed of an anomalous amylopectin and/or an amylose of a lower degree of polymerization, as pointed out by Greenwood *et al.* [3], is very vigorous at early stage of endosperm development.

The length of longer side chain in *ae* amylopectin was longer (about 13 glucose units) than that in normal, and

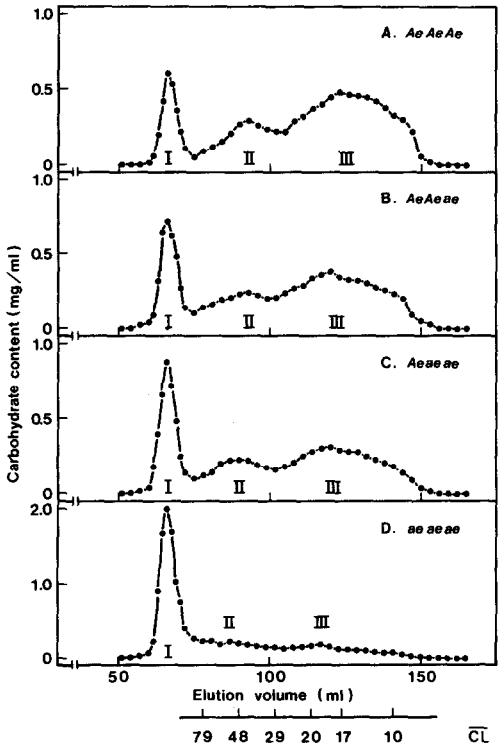

Fig. 1. Elution patterns on a column of Sephadex G-75 of four endosperm starches debranched by isoamylase. Endosperm starches with different doses of *ae* allele between 0 and 3 at 14-day stage were debranched, applied on a column of Sephadex G-75 (1.6 × 75 cm), and eluted with 0.02 M NaOH containing 0.02% NaN_3. Amounts of carbohydrate and reducing end group in each tube were measured by the phenol–sulfuric acid method and the Nelson's reducing sugar method, respectively. Average chain length ($\overline{CL}$) was calculated from the ratio of the carbohydrate and the reducing ends. Endosperm genotypes examined were *Ae Ae Ae* (A), *Ae Ae ae* (B), *Ae ae ae* (C), and *ae ae ae* (D) in the triploid endosperm.

did not change during endosperm development (Table 2). However, the content of the side-chain fraction was not influenced by the *ae* allele, which was different from the result reported by Ikawa *et al.* [5]. As to the shorter side-chain fraction, the content was decreased by *ae* during endosperm development, although the chain length in *ae* homozygous was somewhat longer (about 2 glucose units). These results indicated that the structure of amylopectin either in normal, or in *ae* did not change throughout its biosynthesis. In addition, Ayers and Creech [17, 18] suggested that the high amylose accumulation in amylomaize might be due to debranching of the original starch. If their suggestion is correct, it would be expected that the side chain of amylopectin would be very long at a period when the amylose content increases rapidly. However, although the amylose content in *ae* homozygous increased rapidly between 14 and 18 days after pollination, the lengths of two side chains of amylopectin fraction changed very little at the period of endosperm development. Therefore, the results of the present experiments do not support the suggestion of a debranching theory.

It is important to find starch synthetase (EC 2.4.1.1) and branching enzyme (EC 2.4.1.18) which characteristically participated in amylomaize starch biosynthesis. The findings should be necessary in the final understanding of the role of the recessive *ae* allele in starch biosynthesis.

EXPERIMENTAL

Materials. The inbred M-14 homozygous seeds for either *ae* or its nonmutant were obtained from The Maize Genetic Cooperation and Dr. R. W. Briggs in Brookhaven Laboratory NY, respectively, and they have been maintained in the National Institute of Genetics (Misima, Japan). They were grown in 1979 at The Tsukuba Agricultural Technical Center. 1 and 2 doses of *ae* were obtained by crossing in either direction between the two homozygous lines, and 0 and 3 doses of *ae* by self-pollination in each of the homozygous lines. Developing kernels of each genotype were harvested at 14, 18, 22, 28, and 36 days after pollination. The ears were immediately placed on dry ice in the field and stored at −20° until used. The endosperm genotypes for 0, 1, 2, and 3 doses of *ae* were *Ae Ae Ae*, *Ae Ae ae*, *Ae ae ae*, and *ae ae ae*, respectively. Crystalline *Pseudomonas* isoamylase (EC 3.2.1.68) was purchased from Hayashibara Biochemical Laboratory (Okayama, Japan), and all other reagents were of highest purity.

Isolation of endosperm starches. Endosperm tissues were prepared by removing embryo and pericarp from kernels at 4°, and their starches were isolated and purified according to the method of Boyer *et al.* [4, 11]. For each genotype and for each harvest date, at least starches from 4 ears were examined for each analysis.

Measurement of amperometric iodine binding and amylose content of starches. Amperometric iodine binding of starches was measured according to the method of ref. [19]. Defatted starches (20–60 mg wet wt basis) were dissolved in 2 ml of 5 M KOH, and then filled up to 20 ml with H_2O. An aliquot (10 ml) was transferred to a 200 ml of titration vessel fitting with a vigorous stirrer, platinum electrode, and a circulation of H_2O kept at 10°. Further, 75 ml of H_2O, 10 ml of 1 M HCl, and 5 ml of 0.4 M KI, chilled to 10°, were added to the vessel. After allowing to stand for 5 min, the soln was titrated with 1.57 mM KIO_3 at the rate of 533 μl per min. Amperometric variation was recorded, and the titration value was determined from a calibration curve without starch. The amount of starch in the soln titrated was measured by the PhOH–H_2SO_4 method [20], and the I_2 binding value of the starch was calculated. Amylose content of the starch was calculated from the I_2 binding capacity of the starch and corresponding amylose [19].

Measurement of iodine absorption spectra. The absorption spectra of the I_2 starch complex were measured by the procedure of Krisman [21]. Starch soln, which had been diluted to obtain a final conc. of 2–5 mg per 100 ml, was added to the I_2 reagent soln. said with $CaCl_2$. The spectra of the soln were recorded by Hitachi model 100–50 spectrophotometer over the range of 400–700 nm.

Fractionation of starches debranched by isoamylase on Sephadex G-75. Starches were debranched by the method of Mercier and Kainuma [22] with some modifications. Starch granules (100 mg wet basis) were suspended in 4.4 ml of H_2O, and the starch suspension was incubated for 15 min at 100°, and then autoclaved for 1 hr at 125°. To the defatted starch soln, 5000 U of crystalline isoamylase and 0.5 ml of 0.2 M NaOAc buffer (pH 3.5) were added. The mixture was incubated for 24 hr at 40°. After the incubation, 10-fold vols of EtOH were added and allowed to stand 15 hr at room temp. Starches ppts were collected by centrifugation at 10000 *g* for 10 min, and suspended in 1 ml of H_2O, and then dissolved with 0.5 ml of 1 M NaOH. The soln was made up to a final vol. of 10 ml with H_2O. Four ml of the debranched starch soln were applied on a column of Sephadex G-75 (1.6 × 75 cm) previously equilibrated with 0.02 M NaOH containing 0.02% of NaN_3 to prevent bacterial growth, and

Table 2. Percentages of Fr. I, II, and III, and average chain length of each peak of Fr. II and III

DAP* (days)	Dosage of *ae*	Fr. I (%)	Fr. II (%)	Fr. III (%)	Fr. II + III (%)	Peak of Fr. II (CL)†	Peak of Fr. III (CL)†
	0	15.5	22.6	61.9	84.5	41	17
14	1	20.5	20.7	57.2	77.9	42	17
	2	25.3	23.1	51.6	74.7	45	18
	3	59.3	19.6	21.1	40.7	51	19
	0	21.3	22.7	56.0	78.7	41	17
18	1	26.8	22.9	50.3	73.2	43	17
	2	30.1	22.0	47.9	69.9	45	18
	3	61.6	21.2	17.2	38.4	53	19
	0	21.8	23.9	54.3	78.2	41	17
22	1	27.2	23.3	49.5	72.8	45	18
	2	32.3	23.1	44.6	67.7	47	18
	3	60.6	21.9	17.5	39.4	54	19
	0	23.3	23.5	53.2	76.7	42	17
28	1	28.0	22.9	49.1	72.0	44	17
	2	34.3	20.9	44.8	65.7	47	18
	3	61.1	20.9	18.0	38.9	54	19
	0	24.9	22.9	52.2	75.1	41	17
36	1	28.8	21.6	49.6	71.2	44	18
	2	34.3	21.8	43.9	65.7	47	18
	3	61.0	20.7	18.3	39.0	54	19

*Days after pollination.
†Average chain length.

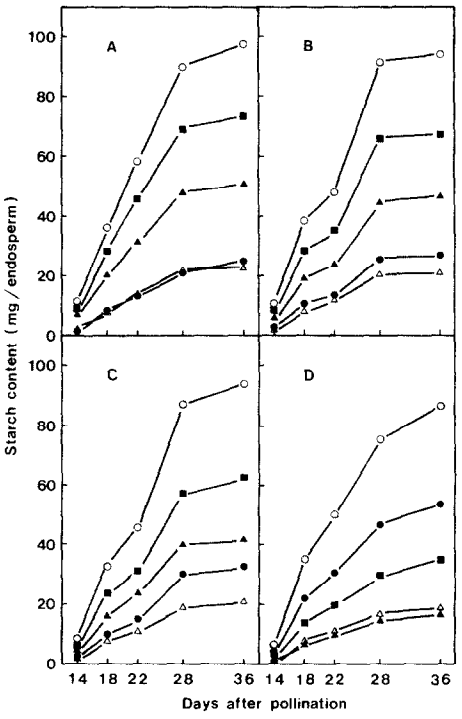

Fig. 2. Dosage effects of *ae* allele on each fraction of endosperm starch during endosperm development. The value of each starch fraction in four endosperm genotypes, *Ae Ae Ae* (A), *Ae Ae ae* (B), *Ae ae ae* (C), and *ae ae ae* (D), was calculated from the percentage obtained in Table 2. (○——○) total endosperm starch; (●——●) Fr.I; (△——△) Fr.II; (▲——▲) Fr.III; (■——■) Fr.II + III.

eluted with the same soln. Each fraction (1.5 ml) was collected at the rate of 20 ml per hr at room temp, and neutralized with 1 M HCl. Under these conditions, *waxy* starch was debranched completely, and the reovery of carbohydrates charged on a column was more than 90%.

The amounts of carbohydrates and reducing end-groups in each fraction were measured by the PhOH–H_2SO_4 method [20] and the Nelson's reducing sugar method [23], respectively. The average chain length ($\overline{CL}$) was calculated from the ratio of the carbohydrate and reducing ends. Each fraction eluted was further divided by λ_{max} of absorption spectra of I_2–starch complex according to the method of Ikawa *et al.* [6]. The ranges of Fr. I, II, and III were $\lambda_{max} \geq 600$ nm, 600 nm $> \lambda_{max} \geq 540$ nm, and 540 nm $> \lambda_{max}$, respectively.

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