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VARIETAL DIFFERENCES AND CHROMOSOME LOCATIONS OF MULTIPLE ISOFORMS OF STARCH BRANCHING ENZYME IN WHEAT ENDOSPERM

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Key Word Index—*Triticum aestivum* L.; Triticeae; wheat; starch branching enzyme; native-PAGE; enzyme activity staining; aneuploid.

Abstract—Starch branching enzyme isolated from wheat endosperm was fractionated into two distinct isoforms, BE I and BE II, using anion-exchange column chromatography on Q-Sepharose Fast Flow. Both BE I and BE II were separated precisely into several isoforms using native polyacrylamide electrophoresis coupled with enzyme activity staining, and varietal differences in the major BE I isoforms expressed in the endosperm 21 days after flowering were observed. Chromosome locations of genes controlling the expression of the three BE I isoforms of 'Chinese Spring' were determined using aneuploid analysis, which indicated that two isoforms, BE I-1 and BE I-2, are controlled by 1DL and the other isoform, BE I-4, is 7BL. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Starch branching enzyme (Q-enzyme, α-1,4-glucan: α-1,4-glucan 6-glucosyltransferase; EC 2.4.1.18), which catalyses the introduction of branches of α-1,6-glucosidic bonds into α-1,4 glucans, is involved in the synthesis of amylopectin. Starch branching enzyme is also reported to consist of two groups BE I and BE II in certain plants and organs, such as spinach leaf [1], maize endosperm [2–4], pea embryo [5] and rice endosperm [6]. The classification of these enzyme isoforms in all investigated species is essentially based on anion-exchange column chromatography elution patterns. The type of isoform variation influenced starch properties, particularly the molecular structure of amylopectin. The maize endosperm mutant of the *amylose extender*, for example, which lacks BE IIb, increased both amylose content and the average chain length of amylopectin [7–9]. Characteristic enzymatic differences between isoforms, such as substrate preference, were also found by *in vitro* amylopectin synthesis using purified isoforms. It has been proposed that BE I transfers preferentially the longer chains of α-1,4-glucan, rather than those of BE IIa and IIb [10, 11]. Each BE I and BE II in the rice endosperm may also be separated into several isoforms using native-PAGE followed by enzyme activity staining [6].

Little information is available, moreover, on wheat starch branching enzyme. In this paper, we report multiple isoforms of starch branching enzyme in wheat endosperm, particularly from the viewpoint of varietal differences and chromosome locations of genes controlling their expression.

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RESULTS AND DISCUSSION

Separation of multiple isoforms of starch branching enzyme by anion-exchange chromatography and native-PAGE

Two peaks denoting the activity of starch branching enzyme were eluted stepwise with NaCl at 50 and 300 mM using anion-exchange column chromatography on Q-sepharose Fast Flow (Fig. 1). The elution profile of starch branching enzyme, separated into two classes using anion-exchange chromatography, basically resembled those reported in other cereal endosperms such as maize [4,5] and rice [6]. The two classes of wheat endosperm starch branching enzyme, eluted at 50 and 300 mM NaCl, were designated BE I (fractions 27–34) and BE II (fractions 57–63), based on the classification in these reports.

The electrophoretic pattern of native-PAGE coupled with enzyme activity staining for samples of the crude extract of *Triticum aestivum* cv. 'Chinese Spring' and the deposited fractions of BE I and BE II are shown in Fig. 2. Both isoforms, BE I and BE II, were further separated using native-PAGE, and BE I

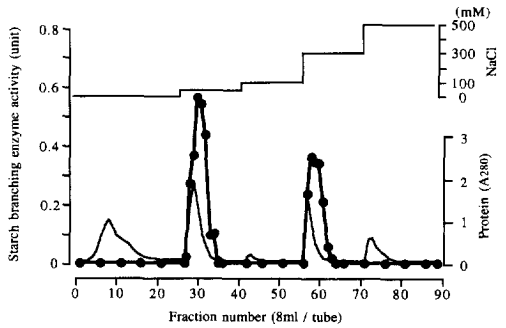

Fig. 1. Anion-exchange column chromatography of starch branching enzyme of wheat endosperm on Q-Sepharose FF. ●, Starch branching enzyme activity, BE I (fractions 27–34), BE II (fractions 57–63); ——, proteins (absorbance at 280 nm).

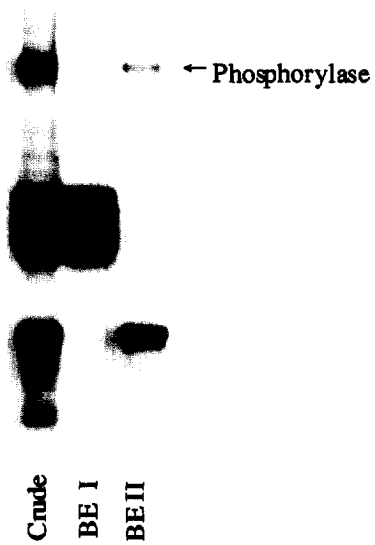

Fig. 2. Native-PAGE coupled with enzyme activity staining of isoforms of starch branching enzyme in fractions from Q-Sepharose column chromatography. Crude—crude enzyme extract of 'Chinese Spring'; BE I and BE II—Deposited fractions of BE I (fraction 27–34) and BE II (fractions 57–63) from Q-Sepharose column chromatography.

was found to consist of several isoforms having lower mobilities than BE II isoforms.

Enzyme activity gel staining detected the specific blue band of wheat endosperm phosphorylase, whose mobility is much lower than that of BE I. This detection was in addition to the purple to brown bands of starch branching enzyme. The wheat endosperm phosphorylase band was detected even in the absence of rabbit muscle phosphorylase *a* in the reaction solution (data not shown). The difference in band colour reflects the difference in the reaction mode of the two enzymes; the purple–brown bands stained with I_2/KI solution were based on branched α-glucan produced by starch branching enzyme, and the blue bands as linear α-1,4-glucan produced by phosphorylase.

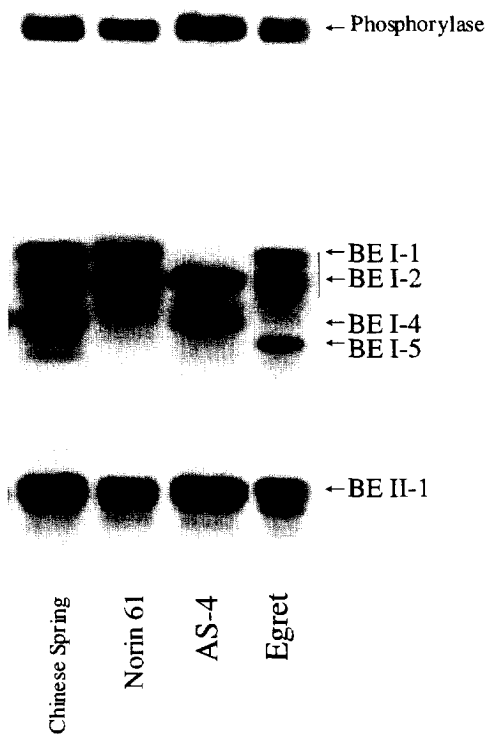

Fig. 3. Native-PAGE coupled with enzyme activity staining of starch branching enzyme of four cultivars having different BE I isoforms from wheat endosperm at 21 days after flowering.

Varietal differences in multiple isoforms of starch branching enzyme

Native-PAGE, followed by starch branching enzyme activity staining, revealed varietal differences in the major isoforms of starch branching enzyme present in endosperms at 21 days after flowering (DAF) (Fig. 3). All cultivars used in this experiment had major and minor isoforms in both BE I and BE II. Norin 61, for example, had BE I-1, I-2, and II-1 as major isoforms, and BE I-3, I-4, and I-5 as minor isoforms. The distinction of major and minor isoforms depended on band strength. Among the 10 cultivars used in the experiment, there were four types based on combinations of the major isoforms (Table 1). Varietal differences in the major isoforms were only found in BE I, but not in BE II. All cultivars used in

Table 1. Varietal differences of the major isoform compositions of starch branching enzyme in wheat endosperm

Major isoforms		Cultivars
BE I-1, I-2, I-4	BE II-1	Chinese Spring, Kanto 107, Norin 27, Igachikugo Oregon
BE I-1, I-2,	BE II-1	Norin 61, Norin 66, Saikai 168, Tozan 18
BE I-2, I-4	BE II-1	AS-4
BE I-1, I-2, I-5	BE II-1	Egret

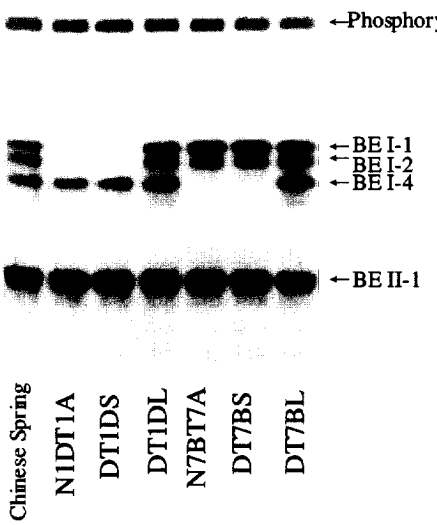

Fig. 4. Native-PAGE coupled with enzyme activity staining of starch branching enzyme of nullitetrasomics (NT) and ditelosomics (DT) of 'Chinese Spring'.

this experiment had BE II-1 as the major isoform and several minor BE II isoforms having higher mobilities than BE II-1.

In experiments on enzyme activity staining, some difficulties arose in obtaining reproducible zymograms. The synthesis of starch in the reaction medium but not in the gel, in particular, was often observed. These difficulties, were mostly overcome, however, by reducing the sample volume applied to the gel and by rinsing gel sufficiently before the enzyme reaction, as described in the Experimental. Such difficulties were thought to be caused by the leaching of starch branching enzyme from the gel into the reaction solution, in which the starch synthesis reaction should be advantageous compared with the inside of the gel in supplying both phosphorylase *a* as the co-working enzyme and glucose-1-phosphate as the substrate.

Chromosome locations of genes for isoforms of starch branching enzyme

At 21 DAF, the endosperm of the 'Chinese Spring' euploid had four major starch branching enzyme isoforms, designated BE I-1, BE I-2, BE I-4, and BE II-1 (Fig. 4). The analysis of nullitetrasomic and ditelosomic lines enabled us to determine the chromosome location of genes controlling the expression of all three BE I isoforms of 'Chinese Spring'. Genes for BE I-1 and BE I-2 were located on the long arm of 1D (Fig. 4), because deletion of the long arm of 1D chromosome eliminated BE I-1 and BE I-2. In the same way, BE I-4 was found to be controlled by the gene located on 7BL. On the other hand, no nullitetrasomics or ditelosomics lacked BE II-1.

Given the varietal differences and the independent chromosome locations of BE I isoforms, cross breeding should enable us to modify the major BE I isoform composition. The molecular structures of wheat amylopectin reportedly differ between cultivars [12]. Further studies on the relationship between BE I genotypes and their amylopectin structures would thus be important both for understanding the mechanism of amylopectin synthesis *in vivo* and for understanding the genetic modification of wheat starch properties.

EXPERIMENTAL

Plant materials. 10 common wheat (*Triticum aestivum* L.) cultivars, 35 'Chinese Spring' nullitetrasomics (NTs) [13] and 36 ditelosomics (DTs) [14, 15] were grown in a field of the National Agriculture Research Center, Tsukuba, Japan in 1994–1995. Developing seeds were harvested at 21 days after flowering (DAF) and stored at −20° until use in experiments, i.e. the separation of starch branching enzyme isoforms using anion-exchange column chromatography, the analysis of varietal differences in multiple isoforms, and the determination of chromosomes carrying the gene controlling the production of starch branching enzyme isoforms using native-PAGE coupled with enzyme activity staining.

Extraction of starch branching enzyme from wheat endosperm and anion-exchange column chromatography. Whole developing seeds (250 g) of 'Chinese Spring' were mashed to extract endosperms using a smooth roller mill with a roller interval set at 1 mm. The major portion of seed coats with embryos were then removed from endosperms by selection using specific gravity in ice-cold 50 and 80% satd $(NH_4)_2SO_4$ solns. Small pieces of the seed coats contaminated in the endosperm fraction were removed with tweezers.

The fractionated endosperm (110 g) was homogenized using a mortar homogenizer in 200 ml of 50 mM Tris–HCl buffer (pH 7.5) containing 5 mM EDTA. The homogenate was centrifuged at 5000 *g* for 20 min to obtain the supernatant, and the pellet was then resuspended and the above procedure repeated. The combined supernatant was brought to 70% satn with powdered $(NH_4)_2SO_4$ and allowed to stand overnight at 4°. The resulting ppt was collected at 10 000 *g* for 20 min, redissolved in 10 ml of the dissolution buffer (10 mM Tris–HCl (pH 7.5), 2 mM EDTA) and dialysed twice, each against 1 l of the same buffer for 12 hr. The dialysed extract was loaded onto a column (26 mm i.d. × 20 cm) of Q-Sepharose Fast Flow (Pharmacia) previously equilibrated with the dissolution buffer. The column was eluted stepwise at a flow rate of 1 ml min^{-1} with the dissolution buffer containing 0, 50, 100, 300 and 500 mM NaCl. Frs (8ml) were collected and measured for protein content (*A* at 280 nm) and starch branching enzyme activity using phosphorylase stimulation method [16]. One unit of enzyme activity was defined as an increase of one *A* unit at 540 nm per min. Fractions containing major portions of activity were combined and used for the native-PAGE experiment.

Native-PAGE and enzyme activity staining of starch

branching enzyme. To investigate varietal differences in multiple isoforms and the chromosome locations of isoform genes, the developing seeds of 10 cultivars and 'Chinese Spring' anueploids were used. Crude enzymes were extracted from 500 mg of the hand-prepared wheat endosperm. The endosperm was homogenized in 1 ml of extraction buffer (50 mM Tris–HCl (pH 6.8), 5 mM EDTA, 10% glycerol) and centrifuged at 10 000 *g* for 10 min to obtain the supernatant as the sample soln. Native-PAGE was conducted at 4° according to ref. [17]. Two to 20 μl of each sample soln was applied for each lane of the native polyacrylamide slab gel (separation gel:10%T, 2.66%C). The activity staining of starch branching enzyme was conducted using a modified method of ref. [6]. After electrophoresis, the gel was rinsed $\times 3$ (10 min each) in H_4O, then equilibrated $\times 3$ (10 min each) with 10 mM MES (pH 6.2), each for 10 min. In insufficient gel equilibration, the reaction buffer pH was shifted to alkaline. Activity band detection was scarcely possible if the reaction buffer pH at the end of incubation exceeded 7.4. After thorough equilibration, the gel was incubated in reaction buffer containing 50 mM MES (pH 6.2), 10 mM glucose-1-phosphate, 1 mM adenosine monophosphate, and 25 units of rabbit muscle phosphorylase *a* (Sigma), at 25° with rotary shaking at 33 rpm for 15–24 hr. After incubation, the starch bands formed in the gel were stained with 0.1% I_2/1% KI. To obtain clear zymograms, the reaction should be stopped 2 or 3 hr after the first appearance of insoluble solid starch in the reaction buffer. In such cases, some major bands were slightly visible as white starch bands even before gel staining.

REFERENCES

1. Hawker, J. S., Ozbun, J. L., Ozaki, H., Greenberg, E. and Preiss, J., *Archives of Biochemistry and Biophysics*, 1974, **160**, 530.
2. Boyer, C. D. and Preiss, J., *Biochemistry and Biophysics Research Communications*, 1978, **80**, 169.
3. Boyer, C. D. and Preiss, J., *Plant Physiology*, 1981, **67**, 1141.
4. Baba, T., Arai, Y., Ono, T., Munakata, A., Yamaguchi, H. and Itoh, T., *Carbohydrate Research*, 1982, **107**, 215.
5. Smith, A. M., *Planta*, 1988, **175**, 270.
6. Yamanouchi, H. and Nakamura, Y., *Plant Cell Physiology*, 1992, **33**, 985.
7. Boyer, C. D., Damewood, P. A. and Matter, G. L., *Starch/starke*, 1980, **32**, 217.
8. Ikawa, Y., Glover, D. V., Sugimoto, Y. and Fuwa, H., *Starch/starke*, 1981, **33**, 9.
9. Takeda, C., Takeda, Y. and Hizukuri, S., *Carbohydrate Research*, 1993, **246**, 273.
10. Takeda, Y., Guan, H. P. and Preiss, J., *Carbohydrate Research*, 1993, **240**, 253.
11. Guan, H. P. and Preiss, J., *Plant Physiology*, 1993, **102**, 1269.
12. Shibanuma, K., Takeda, T., Hizukuri, H. and Shibata, S., *Carbohydrate Polymers*, 1994, **25**, 111.
13. Sears, E. R., *Missouri Agricultural Experimental Station Research Bulletin*, 1954, **572**, 1.
14. Sears, E. R., *Chromosome Manipulations and Plant Genetics*, ed. R. Riley and K. R. Lewis. Oliver and Boyd, Edinburgh, 1966, p. 29.
15. Sears, E. R. and Sears, L. M. S., in *Proceedings of 5th International Wheat Genetics Symposium*. Indian Society of Genetics and Plant Breeding, Indian Agricultural Research Institute, New Delhi, 1978, p. 38.
16. Nakamura, Y., Yuki, K., Park, S. Y. and Ohya, T., *Plant Cell Physiology*, 1989, **30**, 833.
17. Davis, B. J., *Annals of the New York Academy of Science*, 1964, **121**, 404.