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Identification of Granule Bound Starch Synthase (GBSS) Isoforms in Wheat

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ABSTRACT

Granule bound starch synthase (GBSS), also known as the "waxy protein", is responsible for the synthesis of amylose in the amyloplasts of cereal crops. In hexaploid wheat (*Triticum aestivum* L.), GBSS is involved in amylose synthesis and rolls as an important factor to determine flour quality and end-use quality in food products. Genes on three *Wx* loci have been found to encode GBSS in common wheats. We developed techniques for the purification and separation of GBSS in wheat. Three major GBSS isoforms, which were encoded by the genes on three loci, *Wx-A1*, *Wx-B1*, and *Wx-D1* migrating differently by one dimensional SDS-polyacrylamide gel electrophoresis (1D SDS-PAGE), were identified. GBSS from 66 Korean hard and soft winter wheats were purified and determined for their *Wx* loci and four of them were identified possessing a null allele either at the *Wx-A1* and *Wx-B1* loci. With help of identification of three GBSS isoforms using 1D SDS-PAGE system, we are able to identify and monitor *Wx* gene expressions in breeding materials for developing waxy or partial waxy wheats without experiencing consecutive selecting generations.

Key Words : granule bound starch synthase (GBSS), *Wx*, wheat (*Triticum aestivum* L.), one dimensional SDS-PAGE (1D SDS-PAGE).

Starch is one of the major storage components in the plant and is used in food or non-food industries. Granule bound starch synthase (GBSS) is responsible for the synthesis of amylose in the amyloplasts of plant storage organs (Shannon & Garwood, 1984; Tsai, 1974). A role for GBSS in amylose synthesis has been described for some plant species, such as barley (Rohde et. al., 1988), rice (Sano et. al., 1986), and maize (MacDonald & Preiss, 1985). Waxy potatoes and rice have been developed through inhibition of GBSS expression by antisense RNA (Kuipers et al., 1994).

In hexaploid wheat (*Triticum aestivum* L.), three loci *Wx-A1*, *Wx-B1* and *Wx-D1*, located on the chromosome arms 7AS, 4AL and 7DS, respectively, encode GBSS (Yamamori et. al., 1994). A spontaneous waxy wheat could occur only through simultaneous recessive mutations at all three loci. To date, no spontaneously occurring waxy wheat has been isolated. However, waxy wheats (all three null) have been produced via hybridizations of lines carrying null (non-functional) alleles at the individual *Wx* loci (Nakamura et al., 1995).

Wheat lines carrying one or two null alleles were designated "partial waxy"; the starch of such lines often possessed reduced-amylose, when compared to wild type (possesses 3 functional *Wx* alleles) wheats (Yamamori et al., 1994). Null alleles at *Wx-A1* and *Wx-B1* were found to be fairly common among wheats of Japanese and Australian origin (personal comm. with Dr. R.A. Graybosch).

Both amylose-free and reduced-amylose wheat starch would have potential commercial utilities. Waxy maize starch has found numerous applications in both the food and non-food industries (Alexander, 1992; Kirby, 1992). Waxy maize starch forms the basis of a number of fat replacement products (Alexander, 1992) and is used in the paper industry (Kirby, 1992). Food products from starches with higher amylopectin contents retrograde at a slower rate in baked products (Biliaderis, 1992). The availability of waxy or reduced-amylose wheat starch can allow the development of food products especially noodle which is one of the major manufactured food products processed from soft wheat in Korea, Japan, and China.

Proteins isolated from the starch granules have been classified into two types: one is embedded within the starch granule and the other is distributed on the surface of the starch granule during maturation or preparation of the starch granule (Schofield & Greenwell, 1988). The predominant GBSS from the starch granule has a molecular weight of 59~60 kDas. The absence of this protein content from the starch granule results in the waxy or amylose-free phenotype in maize (Shure et al., 1983). Difficulties in dissolving the granule have limited the study of GBSS that are not on the surface of starch granules. However, the extraction of this protein from normal and waxy maize by mechanical disruption of the granule and α-amylase treatment reveals that the 60 kDa protein is a granule bound starch synthase (MacDonald & Preiss, 1985). The observation that waxy mutants from barley, maize, rice, and potato lack GBSS activity demonstrates that this enzyme activity is responsible for the synthesis of the amylose fraction of starch (Sivak et al., 1993). Nakamura et. al. (1993a) developed a two dimensional electrophoretic separation system that allowed separation and identification of three subunit groups of

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polypeptides encoded by the genes located on three different *Wx* loci, and surveyed wheat collections and identified wheat lines revealing null alleles. Use of two dimensional electrophoresis is more complicated and difficult to score, and requires more time to run than a 1D electrophoresis system.

The objectives of this research is to develop wheat GBSS extraction methods and one dimensional SDS-PAGE system for identifying and investigating genetic differences in the major proteins in starch granule and employ this method to evaluate Korean wheat cultivars and experimental lines.

MATERIALS AND METHODS

Plant materials

Seed stocks of the 66 Korean wheat cultivars and experimental lines used in the investigation of GBSS isoform variation were kindly provided by National Crop Experiment Station, RDA, Suwon, Korea. Plants were grown in the field during 1995~1996 and grains were stored at 4℃. The wheat entries were given in Table 1. Three check lines, 'BaiHuo' (null *Wx-D1*), 'Kanto 107' (null *Wx-A1* and *Wx-B1*), and 'Norin 67' (null *Wx-A1* and *Wx-B1*), which were originally provided by China and Japan were also provided by National Crop Experiment Station, RDA, Suwon, Korea. Four waxy breeding lines, waxy 1, waxy 2, waxy 3, and waxy 4 were used as GBSS references. Two U.S.A. check cultivars, 'Ike' (null *Wx-A1* and *Wx-D1*) and 'TAM 200' (null *Wx-D1*), were provided by Dr. R.A. Graybosch (USDA/ARS, Univ. of Nebraska, U.S.A). Genotypic information of *Wx* loci for check lines are also shown in Table 1.

Starch granule preparation

Starch granules were prepared by 1 ml extraction buffer (0.055 M Tris-HCl, pH 6.8, 2.6% SDS, 10% glycerol, 2% β-mercaptoethanol) from 25 mg flour samples. Extraction was done by shaking for 30 minutes followed by centrifugation at 14,000×g for 30 seconds. After decanting supernatant, another extraction was done by adding 1 ml extraction buffer and vortexing. After second extraction, wash was done by adding 1 ml water and vortexing the sample. After centrifugation at 14,000×g for 2 minutes, repeated pellet washes were done with 2 changes of acetone. After final wash and centrifugation, the pellet was air dried.

GBSS sample preparation

In order to purify GBSS, 10 mg of purified starch prepared by the starch granule preparation method was transfered to a new 1.5 ml micro-tube. Extraction was done by boiling tube containing 100 μl lane buffer (2% SDS, 10% glycerol, 0.06 M Tris-HCl pH 8.8, 0.002 M ethylenediaminetetraacetic acid) and 10 μl dithiotheitol (from stock solution of 60 mg/ml) until sample was gelatinized. After boiling, additional 200 μl 4-vinylpyridine solution (2.2 μl 4-vp / 1 ml lane buffer) was applied. After vortexing, the sample was placed on dry bath set at 55℃ for 30 min. After additional 10 μl DTT solution was added, sample was vortexed and reheated at 55℃ for 15 minutes. A small amount of bromophenol blue solution was added to the sample as a tracking dye.

One dimensional SDS-PAGE analysis

The GBSS was analyzed by 17% resolve gel. Tris-HCl (0.375 M) was used to adjust pH of resolve gel at pH 8.4. Preparation of acrylamide stock solution of 30% acrylamide/1.0% bis-acrylamide for stax solution followed the methods as described by Graybosch & Morris (1990). Samples ran at 20 watts for 5 hours. After GBSS migration in the electrofield, the gel was fixed and stained as described by Graybosch & Morris (1990) with some modification. Briefly a gel was transferred to fixing solution (50% ETOH, 10% HOAC) and was shaken overnight at room temperature. After fixing, the gel was transferred to a second solution (10% ETOH, 5% HOAC) and shaken for 30 minutes. After decanting the solution, the gel was washed by adding double distilled water and shaking for 20 minutes. After washing, the gel was transferred to glutaraldehyde solution (10% glutaraldehyde) and shaken for 30 minutes. After repeating decanting and adding water 2 more times, dithioerythritol solution (0.23 mM) was added and the gel was shaken for 30 minutes. The gel was transferred to silvernitrate solution (0.13 mM) and shaken for 30 minutes. After shaking, the gel was transferred to the developer solution (0.037% formaldehyde, 0.4 mM Na_2CO_3). When the GBSS subunit fractions stained optimally, the gel was transferred to a stop solution (1% HOAC).

RESULTS

The list of Korean wheat varieties and experimental lines and the results of one dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE) analysis of granule bound starch synthase (GBSS) extracted from wheat flour is shown in Table 1. Allelic designations and types were based on the work of Yamamori et al. (1994): Wild-type or functional alleles, e. g. those producing gene products, are designated "a" and null alleles are designated "b". Most Korean wheat cultivars exhibited all three functional *Wx* alleles. Lines with null alleles were very rare in Korean wheats tested.

The results of presence or absence of the wheat waxy proteins from 66 Korean wheat lines showed somewhat different patterns than that found in Japanese and Australian cultivars in which the abundancy of either single or both null at the *Wx-A1* and *Wx-B1* alleles (Miura & Tanii, 1994) were found. Among 66 wheat lines, 'Suwon 252' was the only line that did not produce

Table 1. Identification of three GBSS isoforms in Korean wheat cultivars and experimental lines using one dimensional SDS polyacrylamide gel electrophoresis. Hexaploid wheats used as check materials were evaluated for their alleles of *Wx-A1*, *Wx-B1* and *Wx-D1* proteins.

Ⅰ. Korean wheat cultivars and experimental lines	Genotypes
Urimil, Tapdongmil, Gobunmil, Geumgangmil, Eunpamil, Geurumil, Chokwang, Namhaemil, Cheonggemil, Olmil, Alchanmil, Olgeurumil, Jaeraesomaec, Yukseong 3, Shinkwang, Naemil, Yungkwang, Kyungkwang, Jaeraesomaec, Jaeraejong I, Somaecjaerae, Jaeraeneulmil, Jaeraemil, Chungnamjaerae, Tongmil, Jaeraejong, Jaeraejong 2, Suwon 85, Suwon 86, Suwon 185, Suwon 205, Suwon 207, Suwon 209, Suwon 210, Suwon 211, Suwon 213, Suwon 218, Suwon 225, Suwon 229, Suwon 230, Suwon 234, Suwon 236, Suwon 239, Suwon 241, Suwon 243, Suwon 244, Suwon 245, Suwon 246, Suwon 249, Suwon 258, Suwon 259, Suwon 260, Suwon 261, Suwon 263, Suwon 264, Suwon 265, Suwon 266, Suwon 268, Suwon 269, Suwon 270, Suwon 271, Suwon 272	*Wx-A1a*† *Wx-B1a* *Wx-D1a*
Dahongmil, Changkwang 1, Jinpoong	*Wx-A1a* *Wx-B1b* *Wx-D1a*
Suwon 252	*Wx-A1b* *Wx-B1a* *Wx-D1a*
Ⅱ. Check varieties and breeding lines	**Genotypes**
Bai Huo, TAM 200	*Wx-A1a Wx-B1a Wx-D1b*
Kanto 107, Norin 67	*Wx-A1b Wx-B1b Wx-D1a*
Waxy 1, Waxy 2, Waxy 3, Waxy 4	*Wx-A1b Wx-B1b Wx-D1b*
Ike	*Wx-A1b Wx-B1a Wx-D1b*

† "a" is designed for wild type or functional *Wx* allele and "b" is designed for mutant or null allele.

Wx-A1 protein in silver-stained gels, showing that it had a null allele (*Wx-A1*b) for the *Wx-A1* protein.

Since Suwon 252 is a soft winter wheat (grad. #5 by Single Kernel Characterization System 4100, Perten, Sweden), hybridization between Suwon 252 and lines carrying two null waxy (*Wx-B1*, *Wx-D1*) will be more advantages to produce waxy or partial waxy wheats for noodle applications. Three varieties, 'Dahongmil', 'Changkwang 1' and 'Jinpoong' showed that they did not possess functional genes for the *Wx-B1* locus. Limited genetic variations for the *Wx* loci in Korean wheat might indicate that there might be other factors which should be considered prior to selecting wheats with low amylose contents in their breeding programs.

Fig. 1 shows 1D SDS-PAGE patterns of granule bound starch synthase extracted from flours of Korean wheat lines. All Korean wheat cultivars except Dahongmil at lane 5 exhibited all three GBSS isoforms. Dahongmil showed absence of *Wx-B1* protein. In addition to the protocols described in materials and methods, gel concentration, boiling temperature, gel running conditions and developing time are all critical factors for getting clear GBSS isoform fractionation. Since each waxy proteins bound to starch granules were not extracted and separatedly easily, the gelatinization of the granule was thought to be required in order to disrupt these polypeptide-polysaccharide interactions. With all factors considered, the presence of each *Wx-A1*, *Wx-B1* and *Wx-D1* protein could be detected by using 1D SDS-PAGE without the additional efforts of running 2D PAGE.

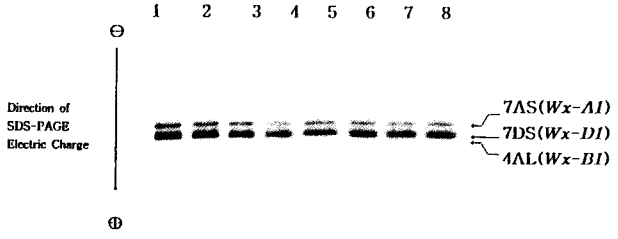

Fig. 1. One dimensional SDS-PAGE patterns of GBSS isolated from flours of Korean wheats. Genotypes of three GBSS isoforms encoded by genes on *Wx-A1*, *Wx-B1*, and *Wx-D1* loci are indicated. Lane 1 ; Geurumil, lane 2 ; Chokwang, lane 3 ; Namhaemil, lane 4 ; Cheonggemil, lane 5 ; Dahongmil(null *Wx-B1*), lane 6 ; Olmil, lane 7 ; Alchanmil, lane 8 ; Olgeurumil.

Although unclear separation of *Wx-D1* and *Wx-B1*

proteins which are encoded by the genes on chromosome 7D and 4A, respectively, occurred in the case of few lines, because of overlapping and sequence homology between these two subunits, we are still able to distinguish both products by 1D SDS-PAGE system.

All three *Wx* proteins encoded by the genes located on *Wx-A1*, *Wx-B1* and *Wx-D1* loci were detected and the relative amount of waxy protein levels (as measured by intensity of band) were various. From the wheat lines with all three GBSS fractions, *Wx-A1* protein usually stained less than other two bands. Each subunit group which had similar migration pattern as they were nearly identical size showed almost same thickness and intensity throughout the materials tested.

Genotypic variations of GBSS isoforms extracted from starch granule of check varieties or breeding lines are depicted in Fig. 2. The GBSS isoforms were detected as the *Wx-A1* protein, *Wx-B1* protein and *Wx-D1* protein and the 60 kDa ranges by the 1D SDS-PAGE system. Chinese cultivar BaiHuo which was known to possess null *Wx-D1* allele also showed deficiency of functional *Wx-D1* allele. Kanto 107 which was soft red spring wheat with the lowest amylose content among accessions of Japanese wheats and was widely used in Japanese wheat breeding programs for high quality noodle (Miura & Tanii, 1994) showed null *Wx-A1* and *Wx-B1* alleles. Our result of Kanto 107 agreed with previously report (Nakamura et al., 1993b) that it was deficient for the *Wx-A1* and *Wx-B1* alleles and only the *Wx-D1* gene was functional to produce *Wx-D1* protein. Norin 67 exhibited a particular banding pattern, being characterized by a lack of the *Wx-A1* and *Wx-B1* proteins. Four waxy lines, waxy 1, 2, 3, and 4 showed that they were lacked all three *Wx* proteins. We conducted SDS-PAGE analysis of glutenin and 70% ethanol-soluble proteins (gliadin) for these waxy lines and found identical protein subunit patterns among them.

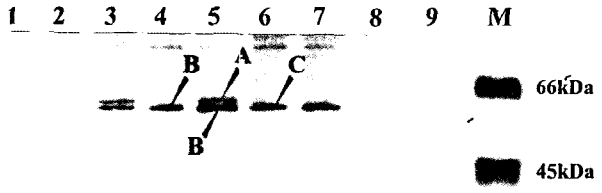

Fig. 2. Genotypic variations of three GBSS isoforms isolated from flour from check varieties and breeding lines. lane 1 ; waxy 1, lane 2 ; waxy 3, lane 3 ; TAM200, lane 4 ; Ike, lane 5 ; BaiHuo, lane 6 ; Kanto 107, lane 7 ; Norin 67, lane 8 ; waxy 4, lane 9 ; waxy 2, M ; molecular size marker. Characters with arrow indicate each GBSS isoform encoded by *Wx* gene : A; *Wx-A1*, B; *Wx-B1*, C; *Wx-D1*.

Nakamura et al. (1993a) used the two-dimensional polyacrylamide gel electrophoresis to identify the waxy proteins as three subunit groups because mobility of *Wx-B1* and *Wx-D1* proteins are overlapping. Use of 2D electrophoresis is tedious and ambiguous to score the result and time-consuming. This experiment clearly demonstrated that, 1D SDS-PAGE method with *Wx* isoforms overcome those problems associated with 2D electrophoresis.

Although a limited number of Korean cultivars was assessed in this study, it should be emphasized that introduction of waxy mutant genes into Korean wheat genetic background to develop wheats for high quality noodle and other high quality food products. The abundancy of null *Wx-B1* alleles in Australian and Japanese cultivars for noodle making that was ascribed by the fact that the complete lack of the *Wx-B1* gene (Miura & Tanii, 1994) should be noted in this regard.

In lines with one or two functional allele, the amount of GBSS produced per unit starch may be reduced. Thus the absence of multiple loci encoding GBSS in wheat allows the production of both amylose-free and reduced-amylose starch, through manipulation of the number of active alleles. Hybridization of lines carring the three null alleles has resulted in the production of amylose- free common and durum wheats. Therefore, this 1D SDS-PAGE analysis will be very useful to screen for least one null waxy gene in their breeding materials and will assist developing both reduced-amylose and amylose-free wheats by identifying plants with either normal or null *Wx* gene(s) from the early breeding stages. The clear banding patterns shown from the Fig. 1 and 2 indicated the easy detection of waxy protein information in an early generation when cross hybridization were made. Since only few seeds can be used in this technique, this method provides the potential for selecting partial waxy or waxy genotypes from large populations.

DISCUSSIONS

The primary aim of this research was to develop simple methods that can be applied to identify three GBSS isoforms which were encoded by the genes located on the *Wx-A1*, *Wx-B1*, and *Wx-D1* loci. The results showed that the three GBSS isoforms were clearly detected by 1D SDS-PAGE system with silver staining. Biochemical analysis of the granule bound starch synthase showed that GBSS from 7AS presented relatively lower than those from 7DS and 4AL. The GBSS isoforms were found to be rarely varied among Korean wheat lines tested.

Advantages of using this method are high repeatability of the result and reduced running costs and time that would be required for conducting consecutive 2D PAGE analysis. Nakamura et al. (1993a, b) used two-dimensional electrophoresis to detect three *Wx* loci according to their isoelectric points and molecular weights. However, we are able to identify three distinct GBSS isoforms of hexaploid wheat using one dimensional SDS-PAGE sys-

tem. Use of 1D electrophoresis is more simple and easy for scoring results and requires less time to run than 2D electrophoresis.

Wheat starch which is composed of amylose and amylopectin is one of the major factors affecting the quality of end-use food products, especially noodles in Asia. The relative amounts of amylose and amylopectin deposited in the wheat endosperm are key factors that influence noodle quality (Oda et al., 1980). It has been known that low amylose cultivars provide better quality in noodle making (Miura & Tanii, 1994). Therefore, selection for partial waxy or waxy wheat would be of benefit as a breeding strategy. Non-waxy and waxy phenotypes are distinguishable by staining of starch in the kernels with iodine, as non-waxy starch stains dark blue while waxy starch stains reddish brown. Waxy and non-waxy pollen grains stained differently in iodine solution, the former ones being reddish brown whereas the latter ones stained blue. These iodine staining technique may be used advantageously to distinguish waxy and non-waxy plant types. However, this is only useful to identify between waxy or non-waxy type. Therefore, using 1D SDS-PAGE system, we are able to provide an easy method to identify partial waxy mutant which could be used as parentages for producing waxy mutant wheat lines. Since no naturally existing waxy wheat has been found yet, genetic manipulation through recombination and cross hybridization with consecutive backcrosses after intermate between Korean wheat lines and partial waxy wheats may be required to obtain waxy or partial waxy wheats. Mutagenic treatments may not be suitable for producing waxy mutant wheat because of the low frequency of mutants in hexaploid wheats. Although transgenic plants with free amylose production is a potential way to obtain waxy wheat, hybridization between preexisted wheats with null *Wx* gene in their counter part could be the simplest way. To achieve this goal, it is necessary to establish a more discriminating method to identify expression of GBSS encoded by the genes on three *Wx* loci. These genes are located on the homoeologous chromosomal parts 7AS, 7DS, and 4AL in which part of segment are translocated from 7BS (Liu et al., 1992). Therefore, use of such simple 1D SDS-PAGE system is fit to it's purpose.

Comparison of the deduced amino acid sequences showed that there was a large degree of similarity between the mature peptides of the GBSS proteins, even more than 83% identity of proteins shared in cereal proteins (Ainsworth et al., 1993). Therefore, the simplified 1D SDS-PAGE might be applied in different cereals for identifying and quantifying waxy proteins.

In this study, although a limited number of Korean cultivars were assessed, it should be emphasized that introduction of waxy mutant genes into Korean wheat genetic background to develop wheats for high quality noodle and other proper end-use qualities can be accommodated through the use of *Wx* isoform electrophoresis. The effect of GBSS from three *Wx* loci on changing amylose content may different based on their genetic backgrounds and the contribution of each null *Wx* allele on reducing amylose content has not been elucidated yet. In order to develop wheats with diverse starch composition, we also need to survey wheats from foreign countries and introduce different types of null *Wx* genes into Korean wheat resources.

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