

# Granule-Bound Starch Synthase I (GBSSI) in Quinoa (*Chenopodium quinoa* Willd.) and Its Relationship to Amylose Content

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## ABSTRACT

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The amylose concentration in starch from 16 quinoa (*Chenopodium quinoa* Willd.) genotypes grown under identical conditions was 4–20%. Based on the amylose content, a selection of six genotypes was made. Starch granule-bound proteins were extracted from six genotypes and analyzed using denaturing gel electrophoresis. Two major polypeptides with apparent molecular masses of 56 and 62 kDa were present in all genotypes. Both were identified as granule-bound starch synthase I

(GBSSI) using immunoblot analysis and internal peptide sequencing. The content of the two GBSSI isoforms in starch granules from the six genotypes, as determined by densitometry of the peptide bands, was positively correlated with the concentration of amylose in starch from mature seed. Starch synthase activity in developing seed was positively correlated to starch concentration in seed and amylose concentration in starch during seed development.

Quinoa (*Chenopodium quinoa* Willd.), a pseudocereal native to South America, was an important component in the diet of the Incan civilization (Galwey et al 1990). In the last decade, quinoa has attracted renewed interest because of its unique characteristics, mainly the high nutritional value of its protein (due to its high level of lysine) and its ability to grow under extreme conditions (Gonzalez et al 1989; Ruales and Nair 1992). However, research on genotype improvement, cultivation, and processing is necessary to further develop quinoa as a significant human food crop (Galwey et al 1990).

Starch granules consist of two glucose polymers: amylose, which is predominately linear, and amylopectin, a highly branched molecule with linear  $\alpha$ -(1,4)-linked glucan chains branched through  $\alpha$ -(1,6)-linkages. Most starches contain  $\approx$ 25% amylose and 75% amylopectin. The amylose-to-amylopectin ratio influences the physicochemical properties of starch and its end uses (Li et al 1994; Svegmarm et al 2002). Starches with extreme amylose or amylopectin concentrations exist in, or have been developed from, a number of species including rice, wheat, amaranth, corn, potato, and pea. Starch, the major component of quinoa, makes up  $\approx$ 55% of the seed and it is present in the form of small granules  $\approx$ 1.5  $\mu$ m in diameter (Chauhan et al 1992). No work has yet been published comparing the amylose concentrations in starches from different quinoa genotypes grown under identical conditions.

The final stages of starch synthesis take place in the amyloplast or chloroplast by the concerted action of ADP-glucose phosphorylase, starch-branching enzyme, starch synthase, and starch-debranching enzyme, all of which have specific roles in the formation of amylose and amylopectin (Smith 2001). From studies of mutants in which a low or undetectable amount of amylose is synthesized, like the *waxy*, *amf*, and *lam* mutations in cereals, potato, and pea (Nelson and Rhines 1962; Schwartz and Echt 1982; Hovenkamp-Hermelink et al 1987; Leij et al 1991; Denyer et al 1995; Nakamura et al 1998), it is known that one particular starch synthase, granule-bound starch synthase I (GBSSI), is responsible for the synthesis of amylose. The precise mechanism by which GBSSI synthesizes amylose remains unknown. Synthesis of amylose might proceed through elongation of amylopectin

chains followed by cleavage or by elongation of malto-oligosaccharides (Smith 2001).

The objectives of this research were to determine the amylose concentrations in starches from 16 quinoa genotypes and to investigate the relationship of amylose concentration to GBSSI concentration and starch synthase activity. Two isoforms of GBSSI are described in this report.

## MATERIALS AND METHODS

Quinoa seed was collected from 16 genotypes (Table I) grown in a greenhouse (supplemented light intensity to a minimum of 230  $\mu$ mol  $m^{-2} s^{-1}$ ; photoperiod 16 hr of light at  $20 \pm 1^\circ C$ ). Once plants reached physiological maturity in the greenhouse, the watering regime was reduced to approximate field conditions. Physiological maturity was defined as when the leaves senesced, seeds were well formed (plump), and the panicles were well formed.

To determine the starch synthase activity in developing seed, immature seed from three genotypes was harvested at four, six, and ten weeks after flowering. The plants were harvested without reduction in the watering regime. The panicles were directly frozen in liquid nitrogen after harvesting.

### Starch Isolation

Starch was isolated from mature seed as described by Zhao and Sharp (1996). Seed (500 mg) was soaked overnight in 40 mL of deionized water, drained, ground, and resuspended in 30 mL of water. The suspension was layered on 30 mL of cesium chloride (80% w/v) and centrifuged at  $6,000 \times g$  for 15 min. The pellet was resuspended and layered on cesium chloride and centrifuged as before. The pellet was washed twice with 9.5 mL of buffer (55 mM Tris-HCl, pH 6.8; 2.3% w/v SDS; 10% v/v glycerol; 5% v/v  $\beta$ -mercapto ethanol), three times with 20 mL of deionized water, and once with 20 mL of acetone. The pellet then was dried at room temperature under vacuum.

### Total Starch Determination

Total starch content was determined using the Megazyme (Bray, Ireland) total starch analysis procedure. Seed was milled in a coffee grinder and the milled seed powder (100 mg) was wetted with 0.2 mL of ethanol and then treated with thermostable  $\alpha$ -amylase and amyloglucosidase. The amount of glucose produced was measured and the starch content calculated as described by McCleary et al (1994). The analyses were done in triplicate.

### Amylose Determination

The amylose concentration in starch was determined by high-performance size-exclusion chromatography (HP-SEC) after debranching as described by Demeke et al (1999). A 3-mg starch

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sample was suspended in 3 mL of double-distilled water in a glass tube and incubated in a 130°C oven for 30 min. To debranch the starch, 1 mL of the starch solution, 55 µL of 1M sodium acetate (pH 4.0) and four units of isoamylase (200 units/2 mL of stock solution, Megazyme) were incubated at 40°C for 4 hr, after which the isoamylase was inactivated by boiling for 20 min. The sample was then freeze-dried. The debranched starch was dissolved in 200 µL of DMSO solution (99% w/v) and centrifuged at 15,000 × g for 10 min. The supernatant (40 µL) was analyzed by HP-SEC (Plgel 5µM MiniMix-C guard column [Polymer Laboratories Inc., Amherst, MA], Plgel Mini 4.6 mm, i.d., column, Waters model 600 controller [Waters Corporation, Milford, MA], Waters model 610 fluid unit, Waters model 717 plus auto sampler, Waters model 410 differential refractometer, with DMSO [99%] as the eluent at a flow rate of 0.2 mL/min). The analyses were done in triplicate.

### Isolation of Starch Granule-Bound Proteins

Starch granule-bound proteins (SGP) were isolated according to Demeke et al (1999) with slight modifications. Starch (4 mg) was dispersed in 600 µL of extraction buffer (62.5 mM Tris-HCl, pH 6.8; 2.3% w/v SDS; 5% v/v β-mercapto ethanol; 10% v/v glycerol; 0.0005% w/v bromophenol blue), boiled for 5 min, and cooled on ice. The suspension was centrifuged at 13,000 × g for 20 min at 4°C. The supernatant containing the SGP was decanted from the gelatinized starch pellet.

### Immunoblot Analysis

Starch granule-bound proteins were separated by SDS-PAGE (10% w/v polyacrylamide; 30:0.135 acrylamide and bisacrylamide) and visualized by silver staining (Bio-Rad Laboratories, Hercules, CA). The polypeptides were electrophoretically transferred at 4°C onto a PVDF membrane (Immobolin-P transfer membrane, Millipore, Billerica, MA) using transfer buffer (40 mM Tris-HCl, pH 7.4; 20 mM NaAc·3H<sub>2</sub>O; 2 mM EDTA; 20% v/v methanol; 0.05% w/v SDS) and the membrane was incubated for 2 hr in blocking buffer (5% w/v skim milk; 1× PBS; 0.1% v/v Tween) followed by incubation with primary antibodies (rabbit serum against starch synthase I (SSI) (Peng et al 2001) (1:8000 dilution), starch synthase II (SSII) (Gao and Chibbar 2000) (1:2000 dilution), starch branching enzyme I (SBEI) (Båga et al 2000) (1:5000 dilution), starch branching enzyme II (SBEII) (Nair et al 1997) (1:5000 dilution), and GBSSI (Matus-Cadiz 2000) (1:8000 dilution) from wheat. The excess of primary antibody was removed by four washes of 15 min each with blocking buffer. The washed membrane was incubated for 1 hr with secondary antibody (phosphatase-conjugated goat anti-rabbit serum, 1:5000 dilution) (Sigma-Aldrich, St. Louis, MO). The excess secondary antibody was removed by three 10-min washes with blocking buffer and three washes of 10 min each with Tris-sodium chloride buffer (50 mM Tris-HCl, pH

7.5; 150 mM NaCl). Immunoreactive polypeptides were detected as blue bands with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphatase (Stratagene, La Jolla, CA).

### Quantitative Determination of GBSSI

Starch granule-bound proteins, separated and visualized using immunoblot analysis as described above, were quantified with a Chemi-Doc imaging system using Quantity I software in duplicate (Bio-Rad Laboratories).

### Peptide Sequencing

Starch granule-bound proteins were extracted as described above from 10 mg of quinoa starch and resolved on a preparative SDS-PAGE gel (10% w/v polyacrylamide; 30:0.135 acrylamide and bisacrylamide). The migration of the two different GBSSI polypeptides was determined by Coomassie blue staining. The stained polypeptides were separated from the rest of the gel and subjected to internal peptide sequencing after trypsin digestion at the Genome BC Proteomics Centre, University of Victoria, Victoria, BC, Canada. The digests were compared with the NCBI database in a MS/MS ion search using carbamidomethyl as a fixed modification, oxidation as a variable modification, monoisotopic mass values, unrestricted protein mass, ±0.1 Da peptide as the mass tolerance, ±0.1 Da as the fragment mass tolerance, and 1 as the maximum number of mixed cleavages, as search parameters (Mascot, Matrix Science, v. 4.1).

### Starch Synthase Activity Using Incorporation of (U-<sup>14</sup>C)Glucose

Starch synthase (SS) activity was determined in quinoa according to Smith (1990) and Vos-Scheperkeuter et al (1986). The incubation conditions were optimized for substrate concentration, time, temperature, and pH to reach maximum starch synthase activity. As optimum conditions, an ADP-glucose concentration of 5.2 mM, an incubation time of 30 min, and a reaction temperature of 32°C at pH 7.5 were used. A positive and linear correlation was found between reaction time and (U-<sup>14</sup>C)glucose incorporated into the amylopectin. To avoid problems related to inactivation of enzymes during extraction of the starch, cell lysates from whole seeds in different stages of maturation were used instead of extracts from starch. The cell lysates were prepared by means similar to those described by Smith (1990) for the solubilization of SGP from starch. The assay mixture contained 20 µL of cell lysate (0.125 g/mL) in protein extraction buffer (100 mM Tris-acetate, pH 7.7; 100 mM N,N-bis-(2-hydroxyethyl)glycine; 0.5M Na-citrate, pH 7.5; 2.6 mM ADP(U-<sup>14</sup>C)glucose [Amersham, Little Chalfont, UK] at 2 GBq/mol; 1.5 mg of amylopectin [potato]; 100

TABLE I

Amylose Concentrations and Starch Contents of 16 Quinoa Genotypes

Genotype	Amylose (%)	Starch (% db)
Ames 21926	3.5 ± 1.0	52.4 ± 1.0
Baer	4.4 ± 0.5	56.7 ± 0.3
Appelawa	7.5 ± 0.3	53.5 ± 2.0
95Y	11.7 ± 1.5	54.2 ± 1.6
Ames 13746	11.9 ± 0.6	55.0 ± 2.3
Ames 13745	12.7 ± 0.2	53.2 ± 0.9
Tango	12.8 ± 0.3	50.0 ± 0.6
407 Dave	13.7 ± 0.3	57.3 ± 0.2
Diverse	14.4 ± 1.4	57.4 ± 3.3
Ames 13747	15.8 ± 0.5	53.6 ± 0.4
Ames 21935	17.1 ± 1.2	62.5 ± 1.2
NSL 92331	17.6 ± 0.4	53.0 ± 1.2
Ames 22154	17.8 ± 0.5	55.5 ± 0.7
NSL 106396	17.9 ± 0.6	49.4 ± 1.7
Ames 13732	18.2 ± 0.3	56.8 ± 0.9
Ames 22159	19.5 ± 0.2	48.3 ± 1.9

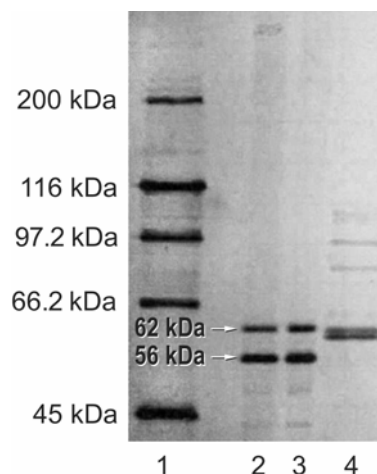


Fig. 1. SDS-PAGE gel of starch granule-bound proteins extracted from quinoa (Ames 22159) (lanes 2, 3) and wheat starch (lane 4).

mM KCl; 1 mM DTT; 1 mM EDTA; 5% v/v glycerol) and was incubated for 30 min at 32°C. The enzyme reaction was stopped by heating the assay mixture at 90°C for 2 min. The heat-inactivated reaction mixture (100 µL) was spotted on a membrane (943AH, 2.1 cm, Whatman, Brentford, UK) and dried. The unbound ADP-glucose was removed by washing the membranes four times for 30 min each time with 75% (v/v) methanol containing 1% (w/v) KCl. The washed membranes were dried and mixed with scintillation fluid in a scintillation vial. The radioactivity incorporated into the amylopectin was counted in a 1219 Rackbeta liquid scintillation counter (Perkin Elmer, Boston, MA). Enzyme activity was measured in triplicate and expressed as the amount of (<sup>14</sup>C)glucose incorporated into amylopectin in 30 min and expressed as counts per minute (cpm) per µL of cell lysate.

## RESULTS AND DISCUSSION

### Amylose and Starch Concentrations

All seed used in the analysis, with the exception of that used for the analysis of starch synthase activity, was visually inspected to ensure that only plump, well-filled seed was used. The amylose concentration in starch from 16 quinoa lines ranged from 3.5% (Ames 21926) to 19.5% (Ames 22159) (Table I). The amylose concentration in quinoa starch was reported previously to be 7–27% (Lorenz and Nyanzi 1989; Praznik et al 1999; Tang et al 2002). However, it was unclear from these earlier studies whether this variation was due to genotypic differences, the environment, the interaction of genotype and environment, or the accuracy of the amylose determination method used. The lines analyzed in this research were grown under identical conditions, and all amylose analyses were performed using the same methodology. Therefore, the variation in amylose reflects genotypic differences among

lines. Some lines contained waxy starch (Ames 21926 and Baer). However, no lines with high amylose starch were found (Table I). The starch concentration range in seed was 48.3–62.5%. The range of amylose contents in combination with the small granule diameter may make it possible to utilize quinoa starch in a wide range of food and nonfood products.

### GBSSI Identification

SDS-PAGE analysis of quinoa starch granule-bound proteins (SGP) revealed the presence of two predominant polypeptides with apparent molecular masses of 62 and 56 kDa, respectively (Fig. 1, lanes 2 and 3). In addition, a few smaller polypeptides were detected. The 62-kDa polypeptide comigrated with GBSSI polypeptides from wheat (Fig. 1, lane 4). Antibodies specific to wheat GBSSI were used in an immunoblot analysis and reacted with both the 62- and the 56-kDa polypeptides (Fig. 2). Antibodies against other wheat starch biosynthetic enzymes such as starch synthase I and starch-branching enzyme did not cross-react with the 62- and 56-kDa polypeptides from quinoa (results not

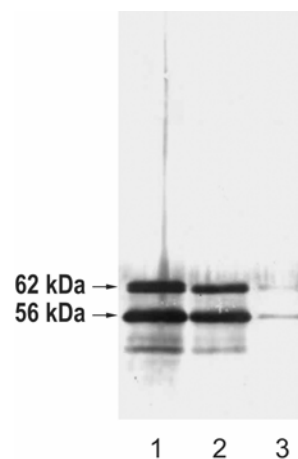


Fig. 2. Immunoblot detection using rabbit antibodies against wheat GBSSI of starch granule-bound proteins from quinoa. Lanes 1–3: Ames 22159 containing 20.5% amylose; 95Y containing 13.5% amylose; and Ames 21926 containing 3.5% amylose, respectively.

TABLE II  
Internal Peptide Sequences of GBSSI Proteins from Species  
Matching the Internal Peptide Sequences  
of 56- and 62-kDa Polypeptides from Quinoa

Polypeptide	Peptide Sequence	Matching Peptides	Score <sup>a</sup>
56 kDa	AGILESDR	GBSSI <i>Vauquelinia californica</i>	49
56 kDa	EALQAEVGLPIDR	GBSSI <i>Vauquelinia californica</i>	78
62 kDa	AGIIESDR	GBSSI <i>Perilla frutescens</i>	49
62 kDa	EALQAEVGLPVDR	GBSSI <i>Perilla frutescens</i>	72

<sup>a</sup> Score is  $-10 \times \log(P)$ , where  $P$  is the probability that the observed match is a random event. Individual ion scores  $>35$  indicate identity or extensive homology ( $P < 0.05$ ).

TABLE III  
Amylose Concentrations, Starch Concentrations,  
and Starch Synthase (SS) Activity of Three Quinoa Genotypes  
at Three Stages of Maturity (4, 6, and 10 weeks after flowering)

Genotype	Time After Flowering	Starch (% db)	Amylose (%)	SS Activity (cpm/µL) <sup>a</sup>
Ames 21926	4 weeks	7.3 ± 0.2	1.8 ± 0.2	0
Ames 21926	6 weeks	nd <sup>b</sup>	nd	nd
Ames 21926	10 weeks	52.4 ± 1.0	3.5 ± 0.4	46.8 ± 2.8
95Y	4 weeks	48.2 ± 1.8	6.5 ± 0.6	139.4 ± 19.5
95Y	6 weeks	52.7 ± 2.5	11.0 ± 0.7	234.5 ± 5.6
95Y	10 weeks	54.2 ± 1.3	13.5 ± 0.8	106.1 ± 3.7
Ames 22159	4 weeks	40.7 ± 1.5	15.8 ± 1.0	262.0 ± 4.7
Ames 22159	6 weeks	43.2 ± 1.9	20.5 ± 0.7	341.6 ± 7.2
Ames 22159	10 weeks	48.3 ± 1.9	20.7 ± 1.6	404.5 ± 16.6

<sup>a</sup> Amount of (<sup>14</sup>C)glucose incorporated into amylopectin in 30 min and expressed as counts per minute (cpm) per µL of cell lysate.

<sup>b</sup> Not determined due to shortage of material.

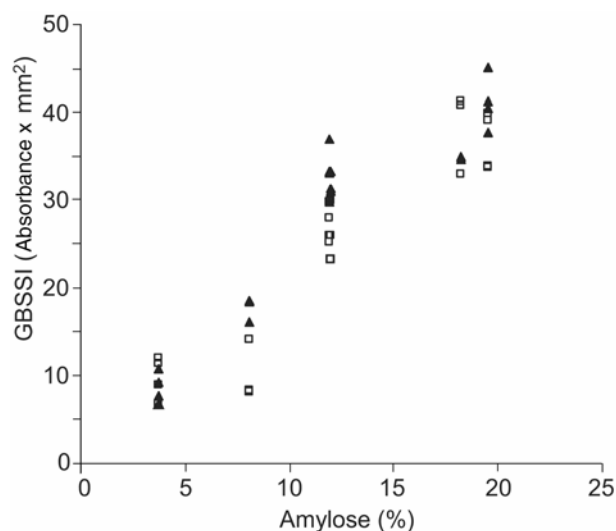


Fig. 3. Relationship between amylose concentration, as determined by HP-SEC, and GBSSI content, as separated by SDS-PAGE, visualized using immunoblot analysis and quantified with densitometry (quinoa genotypes Ames 21926, Appelawa, 95Y, Ames 13746, Ames 13732, Ames 22159). Amounts of the 56-kDa (▲) and 62-kDa (□) polypeptides are expressed in density units of the peptide bands on the immunoblot membrane.

shown). These antibodies did cross-react with larger (>70 kDa) polypeptides extracted from quinoa starch (results not shown).

Internal peptide sequence analysis of the 62- and 56-kDa polypeptides from quinoa starch revealed similar sequences for both polypeptides, and the sequences were similar to those of GBSSI from other species (Table II). These findings support the conclusion that the polypeptides were isoforms of GBSSI.

The presence of two forms of GBSSI differing in molecular mass is unusual but not unique to quinoa. Vos-Scheperkeuter et al (1986) detected multiple forms of GBSSI in both amaranth and maize starches. The major proteins that reacted with rabbit antibodies against the 60-kDa GBSSI from potato were a 65-kDa protein from amaranth and a 61-kDa protein from maize. In addition, one minor cross-reacting polypeptide was detected in each species; this suggested a minor isozyme of GBSSI. The two polypeptides might also be isozymes but we do not have further evidence to prove that this is the case. Another possible explanation is that the minor cross-reacting species in maize (65 kDa) was a precursor of GBSSI. A relationship between the two polypeptides is also indicated by the fact that both polypeptides are missing in *waxy* corn. It was suggested that the minor 61-kDa polypeptide in amaranth was an enzymatic digestion product of the major 65-kDa polypeptide (Vos-Scheperkeuter et al 1986). MacDonald and Preiss (1985) also reported the presence of a second isoform of GBSSI in extracts from normal maize starch.

### GBSSI Content and Activity in Quinoa and Its Relationship to Amylose Concentration

Immunoblot analysis, with anti-GBSSI antibodies, of the SGP from mature seed of three quinoa lines showed that the SGP fraction from quinoa starch with a relatively high amylose concentration (19.5%) had much larger and more distinct peptide bands than did the SGP fraction from quinoa starch of intermediate amylose content (11.7%) which, in turn, had larger and more distinct immunoreactive bands than did the SGP fraction from quinoa starch of low amylose content (3.5%) (Fig. 2). Densitometry analysis of the polypeptide bands confirmed that the intensities of the 62- and 56-kDa bands were proportional to the amylose contents of the starches from which they were extracted (Fig. 3). This positive correlation between amylose content and GBSSI is not surprising, considering that GBSSI is intimately involved in amylose synthesis (Nelson and Rhines 1962; Baldwin 2001).

The concentration of starch in developing quinoa seeds was low in the early stages of seed development but increased to near maximum levels after six weeks (Table III). The concentration of amylose in starch also increased during seed development (Table III), which has been observed in other species (Shannon and Garwood 1984). It appears that the synthesis of amylose is somewhat delayed compared with that of amylopectin. This is probably due to the timing of the biosynthesis of GBSSI, which appears later than some of the other starch synthases (Martin and Smith 1995).

Repeated attempts to determine specific GBSSI activity in vitro in SGP extracts from quinoa after prolonged periods of dialysis to renature the SGP proteins from starch were not successful (data not shown). Others have reported that GBSSI has low enzymatic activity in standard assays (Nelson et al 1978; MacDonald and Preiss 1985), so low that in some species it has been impossible to detect enzymatic activity in vitro (Smith 1990; Denyer et al 1995). Analysis of cell lysates by SDS-PAGE revealed that the two peptides identified as GBSSI were the major peptides present in the lysates. Therefore, a strong relationship would be expected between total starch synthase activity and GBSSI activity during seed development, hence total starch synthase activity rather than GBSSI activity was measured in cell lysates of developing quinoa seed.

The quinoa line with the lowest amylose concentration in mature seed (Ames 21926) exhibited the lowest starch synthase

activity during seed development, the medium amylose line (95Y) exhibited an intermediate activity, and the high amylose line (Ames 22159) exhibited the highest activity (Table III). These results indicate that the concentration of amylose in starch in the mature seed and starch synthase activity during seed development are related. In seed containing starch with a high amylose concentration (Ames 22159), an increase in starch synthase activity over the entire period of seed development was observed (Table III). The medium amylose line (95Y) exhibited maximum enzyme activity six weeks after flowering. A lack of seed of the low amylose line (Ames 21926) prevented any conclusions from being drawn regarding the time course of starch synthase activity during seed development.

Denyer et al (1997) found no correlation between the concentration of amylose in starch and the GBSSI concentration in different plant organs. Flipse et al (1996) found a nonlinear correlation between SGP activity and amylose concentration. At a certain level of SGP activity, a maximum amount of amylose was formed, but a further increase in SGP activity did not result in an increase in amylose concentration. Apart from GBSSI content and activity, other factors such as the presence of cofactors and the amylopectin matrix in which the GBSSI proteins are located might affect the amount of amylose synthesized (Koornhuyse et al 1996; Denyer et al 1997). The current study on quinoa did reveal a relationship between amylose content and both starch synthase activity and GBSSI concentration. However, other factors such as interactions between different biosynthetic enzymes, the physical characteristics of the enzymes (granule-bound or located in the soluble phase), and the availability of substrates and cofactors should be studied to further clarify the relationship between starch synthase activity, GBSSI concentration, and amylose content in quinoa starch.

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