Journal of Cereal Science 56 (2012) 464–469

Contents lists available at SciVerse ScienceDirect

Journal of Cereal Science

journal homepage: www.elsevier.com/locate/jcs

Starch isolation method impacts soft wheat (*Triticum aestivum* L. cv. Claire) starch puroindoline and lipid levels as well as its functional properties

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A R T I C L E I N F O

Article history:
Received 4 January 2012
Received in revised form
31 May 2012
Accepted 4 June 2012

Keywords:
Wheat hardness
Puroindolines
Swelling behavior
Gelatinization behavior

A B S T R A C T

Wheat (*Triticum aestivum* L.) kernel hardness is a major quality characteristic, which has been ascribed to the presence of puroindolines a and b. These proteins occur in higher levels at the surface of water-washed starch granules from soft wheat cultivars than at that of starch from hard wheat cultivars. In the present study, prime starch was isolated from flour from soft wheat (cultivar Claire) using a dough ball or batter based separation method. Starch isolated with the dough ball method contained lower levels of puroindolines, as well as of other starch granule associated proteins and lipids than that isolated with the batter method. Similar patterns of puroindoline and lipid levels after starch isolation can presumably be related to (polar) lipid binding by puroindolines. Both isolated starch fractions showed comparable differential scanning calorimetry thermograms, whereas higher levels of starch surface associated components restricted starch swelling. Necessary controls demonstrated that the observed differences did not arise from artefacts associated with hydration, fractionation or freeze-drying in the experimental protocols. Apparently, proteins and lipids at the starch granule surface impact water absorption and, as such, starch swelling, but they do not affect starch granule internal phenomena such as melting of the crystalline amylopectin chains.

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1. Introduction

Wheat (*Triticum aestivum* L.) kernel endosperm hardness is a key quality trait affecting its milling behavior, the properties of the resulting flour and the quality of products made thereof. What determines wheat hardness is still not fully clear, but a soft texture has been attributed to the presence of the basic and cysteine rich proteins puroindoline a (PIN-a) and b (PIN-b). These proteins are gene products of the wild-type alleles *Pina-D1a* and *Pinb-D1a*, respectively, which are located on the *Hardness* (*Ha*) locus on the short arm of chromosome 5D (Morris, 2002). The absence of and/or a point mutation in one or both genes have been linked to hard endosperm texture. Durum wheat (*Triticum turgidum* L. ssp. *durum*), the hardest of all wheat species, has no puroindolines (PINs) (Bhave and Morris, 2008). PINs are believed to play a role in wheat hardness through stabilization of the amyloplast membrane during kernel ripening and desiccation (Feiz et al., 2009). PIN-a and PIN-b have a unique tryptophan-rich domain, which is thought to be responsible for their lipid binding capacity. As such, they can become inserted into membranes such as that of the amyloplast (Douliez et al., 2000; Kooijman et al., 1997).

PINs are the major components of friabilin (Morris et al., 1994), which occurs in higher levels at the surface of water-washed starch granules of soft wheat cultivars than at that of hard wheat cultivars (Greenwell and Schofield, 1986). This can probably be explained by different lipid binding properties of the mutant PIN-b proteins in hard wheat cultivars (Clifton et al., 2007). However, Jolly et al. (1993) showed that only a fraction of the PINs in whole wheat is recovered at the surface of water-washed starch granules and that PIN levels in whole meal of soft and hard wheat cultivars are similar. This points to a difference in the level of association of PINs with the starch granule surface in hard and soft wheat cultivars and/or to a difference in levels of PINs at the granule surface of water-washed starch as a result of the applied starch isolation

Abbreviations: C^*, close packing concentration; cv., cultivar; dm, dry matter; DSC, differential scanning calorimetry; ΔH, enthalpy; MM, molecular mass; PAGE, polyacrylamide gel electrophoresis; PBS-T, phosphate buffered saline containing Tween 20; PIN-a, puroindoline a; PIN-b, puroindoline b; PINs, puroindolines; RVA, Rapid Visco Analyser; SDS, sodium dodecyl sulfate; SP, swelling power; %SS, percentage solubilized starch; Tris, tris-(hydroxymethyl)-aminomethane; T_o, onset temperature; T_p, peak temperature; T_c, conclusion temperature; T_c–T_o, temperature range.

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http://dx.doi.org/10.1016/j.jcs.2012.06.003

method. The latter is explored here by studying the impact of starch isolation methods on the PIN levels at the starch granule surface. In addition, we further examine whether the used starch isolation methods affect the swelling and pasting properties as well as the gelatinization behavior of the resultant starches. We here report on the outcome of our work.

2. Materials and methods

2.1. Materials

Soft wheat [cultivar (cv.) Claire, containing both wild-type PINs] was from Limagrain (Rilland, The Netherlands). Wheat grains were conditioned to 16.0% moisture and subsequently milled with a Bühler MLU-202 laboratory mill (Bühler AG, Uzwil, Switzerland) with the milling flow scheme depicted in Delcour et al. (1989). Total flour yield was 64.3% and consisted of the first two break and reduction fractions. The last break and reduction fractions were not used. Molecular mass (MM) markers for regular SDS-PAGE (LMW-SDS Marker Kit) and for immunoblotting (SeeBlue Plus2 Pre-stained Standard) were from GE Healthcare (Uppsala, Sweden) and Invitrogen (Meerbeke, Belgium), respectively. Durotest-P antibodies were from R-Biopharm AG (Darmstadt, Germany). Casein from bovine milk (technical grade; catalog No. C7078) and albumin from human serum (catalog No. A1653) were from Sigma–Aldrich (Bornem, Belgium). All other chemicals, solvents and reagents were from Sigma–Aldrich and of analytical grade, unless specified otherwise.

2.2. Experimental

2.2.1. Moisture content

Kernel and flour moisture contents were determined according to AACC Approved Method 44-19.01 (AACC, 1999) with slight modifications. Kernels and flour were dried at 130 °C for 16 and 2 h, respectively.

2.2.2. Starch isolation

Prime starch was isolated from wheat flour cv. Claire using either a dough ball (Finnie et al., 2010; Wolf, 1964) or a batter method (Finnie et al., 2009; Knight and Olson, 1984). In the dough ball method, 96.6 g flour (11.0% moisture level) was mixed with 52.0 mL deionized water in a pin-mixer (National Manufacturing, Lincoln, NE, USA) to form optimal dough, as estimated by mixography (National Manufacturing) [Approved Method 54-40.02 (AACC, 1999)]. Starch was washed from the dough with deionized water until the washing water was clear (*ca.* 500 mL). In the batter method, 33.7 g flour (11.0% moisture) was gently suspended in 140 mL deionized water to form a thin batter. After a 10 min rest, the batter was centrifuged (100 g; 5.0 min) to obtain gluten protein micro agglomerates (Van Der Borght et al., 2005). The starch slurries from both methods were purified in a similar way, *i.e.* sieved (38 μm sieve; Retsch, Haan, Germany), centrifuged (3570 g; 10 min) and the obtained supernatants were discarded. The starch pellets were washed twice with deionized water (*ca.* 400 mL) and the supernatants were discarded each time. Starch tailings in the upper layer of the pellet were carefully removed with a spatula. The obtained prime starch fractions (*i.e.* the lower layers of the pellets) were freeze-dried before further analysis. To evaluate the combined impact of hydration, fractionation method and subsequent freeze-drying on starch functional properties, dough and batter were prepared from Claire flour as described above and immediately freeze-dried, *i.e.* without further fractionation. The latter was done to ensure that the interpretation of our results would not be confounded by artefacts arising from the isolation methodology.

2.2.3. Physico-chemical characterization of flour and starch fractions

Protein level of Claire flour was determined using the Dumas combustion method, an adaptation of the AOAC Official Method (AOAC, 1995) to an automated Dumas protein analysis system (EAS Vario Max CN, Elt, Gouda, The Netherlands) with 5.7 as nitrogen to protein conversion factor. To determine protein level of the isolated starches, starch granule associated proteins were extracted based on Jolly et al. (1993). Thus, starch was extracted with 1.0% (w/v) sodium dodecyl sulfate (SDS) [50 mg starch in 250 μL 1.0% (w/v) SDS; 60 min] at room temperature. Following centrifugation (10900 g; 3.0 min) and proper dilution, protein levels in the supernatants were determined according to Lowry et al. (1951) and expressed using a human serum albumin standard curve. Starch content was calculated as 0.9 × the glucose level estimated by gas chromatography following acid hydrolysis and conversion to alditol peracetates (Courtin et al., 1999). Ash levels were measured according to Approved Method 08-01.01 (AACC, 1999) and damaged starch levels according to Approved Method 76-31.01 (AACC, 1999) using the “Starch damage assay procedure” of Megazyme (Bray, Ireland). Particle size distribution was analyzed with laser light diffractometry (dry analysis) with a Mastersize S device (Goffin/Meyvis, Hoeilaart, Belgium) according to Pareyt et al. (2008). Protein, ash and damaged starch levels were determined at least in triplicate, particle size distribution in duplicate.

2.2.4. Electrophoretic separation and immunoblotting

Starch granule surface associated proteins were extracted with 1.0% (w/v) SDS at room temperature as outlined above. Following centrifugation (10900 g; 3.0 min), four volumes of supernatant were diluted with one volume of sample buffer [12.5 mM tris-(hydroxymethyl)-aminomethane (Tris); 4.0% (w/v) SDS; 30% (v/v) glycerol; 0.004% (w/v) bromophenol blue; adjusted to pH 6.8 with 1.0 M HCl]. After boiling (5.0 min) and centrifugation (10900 g; 3.0 min), the proteins in the supernatant were separated by SDS polyacrylamide gel electrophoresis (PAGE; 20% polyacrylamide) under non-reducing conditions with a PhastSystem (GE Healthcare), according to the GE Healthcare separation technique file 110. Gels were stained with sensitive silver-staining as described in the GE Healthcare development technique file 210.

PINs were quantified in triplicate by immunoblotting using a mini-PROTEAN 3 system (Bio-Rad Laboratories, Hercules, CA, USA). Gels (140 × 160 × 1.5 mm) consisted of a stacking gel (4.0% polyacrylamide, according to the manufacturer's instructions) on top of a separating gel (17.0% polyacrylamide). Sample preparation was in triplicate and similar to that for the electrophoretic separation described above, except that the supernatant was diluted 40 times in 1.0% (w/v) SDS. After boiling and centrifugation, a sample aliquot (35.0 μL) was loaded onto the gel. Electrophoretic separation was carried out at 160 V for 90 min. After equilibration in semi-dry blot buffer [48 mM Tris; 39 mM glycine; 20% (v/v) methanol; pH 9.2] for 30 min, the separated proteins were electroblotted (15 V; 35 min) onto an activated Protran™ nitrocellulose membrane (0.45 μm pore size; Schleicher and Schuell, Dassel, Germany). Free binding sites were blocked by overnight incubation in a 1.0% (w/v) casein solution in phosphate buffered saline (1.8 mM KH_2PO_4, 10.0 mM Na_2HPO_4, 2.7 mM KCl, 137.0 mM NaCl, pH 7.4) containing 0.01% (v/v) Tween 20 (PBS-T). After three washing steps in PBS-T (10 min each), the blot membranes were incubated for 60 min with primary Durotest-P antibodies (dilution 1:15000 in PBS-T). They were washed again in PBS-T (3 × 10 min) before incubation (60 min) with secondary goat anti-mouse antibodies conjugated with horseradish peroxidase (dilution 1:6250 in PBS-T). After three washing steps in PBS-T (10 min each), the blot membranes were incubated with 3,3′,5,5′-tetramethylbenzidine solution as substrate

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