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Improved material properties of solution cast starch films: effect of varying amylopectin structure and amylose content of starch from genetically modified potatoes

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ABSTRACT

High-amylose potato starches were produced through genetic modification resulting in changed granule morphology and composition with higher amylose content and increased chain-length of amylopectin. The increased amylose content and structural changes in amylopectin gave improved film forming behavior and better barrier and tensile properties in starch films. The molecular structure in these starches was related to film-forming properties. Solution-cast films of high-amylose starch revealed a homogeneous structure with increasing surface roughness at higher amylose content, possibly due to amylose aggregation. Films exhibited significant higher stress and strain at break compared to films of wild-type starch, which could be attributable to the longer chains of amylopectin being involved in the interconnected network and more interaction between chains as shown using transmission electron microscopy. The oxygen permeability of high-amylose starch films was significantly decreased compared to wild-type starch. The nature of the modified starches makes them an interesting candidate for replacement of non-renewable oxygen and grease barrier polymers used today.

KEYWORDS

high-amylose potato starch, film forming, molecular structure, chain-length, amylopectin, microscopy
1. Introduction

Starch is well known for its excellent film forming and oxygen barriers capabilities and thus a potential replacement of synthetic polymers in food packaging. Starch consists of two major components, mainly linear amylose and branched amylopectin, both of which consist of glucose residues but differ in physicochemical properties. Already in the early 1950s much attention was given to amylose starches because of their good film-forming properties and their manifold of applications (Wolff, Davis, Cluskey, Gundrum & Rist, 1951). Amylose is used in coatings of food products, sweets or fibre-fortified foods, and as gelling agent because of its ability to create high-strength gels, e.g. to enhance the structure of food products such as tomato paste (Richardson, Jeffcoat & Shi, 2000; Van Patten & Freck, 1973). There have been many attempts to use high-amylose starches in, e.g. composites together with waxes (Muscat, Adhikari, McKnight, Guo & Adhikari, 2013) or chitosan (Feng, Hu & Qiu, 2013) or were extruded (Li et al., 2011) and plasticized together with glycerol (Muscat, Adhikari, Adhikari & Chaudhary, 2012) to produce packaging material from natural resources. Since amylose consists mainly of linear chains, a tight and stable network with strong molecular orientation can be created in films (Rindlav-Westling, Stading, Hermansson & Gatenholm, 1998). Amylose films are capable of being greaseproof (Banks, Greenwood & Muir, 1974) and have been shown to perform better in terms of mechanical and oxygen barrier properties than amylopectin films (Liu, 2005; Lourdin, Valle & Colonna, 1995; Myllarinen, Partanen, Seppala & Forssell, 2002; Rankin, Wolff, Davis & Rist, 1958; Rindlav-Westling, Stading, Hermansson & Gatenholm, 1998). Hence, there have been several efforts to find natural mutants or to develop crops with high-amylose starch in tubers and grains which would be beneficial from a techno-functional point of view (Banks, Greenwood & Muir, 1974; Walker & Merritt, 1969; Wolff, Hofreiter, Watson, Deatherage &
MacMasters, 1955). Extensive amylose breeding programs, such as that by National Starch (Forsyth, IL., USA) have resulted in commercial crops with up to 90% amylose (Sidebottom, Kirkland, Strongitharm & Jeffcoat, 1998). However, in many plants, for example potato, no natural mutants have been described and genetic modification has been used to introduce the high-amylose trait. Starches from potato have a distinctive quality, with a high amount of phosphate and low content of lipids, which is beneficial for many applications where good clarity, neutral taste or high swelling is needed. They are therefore of considerable industrial interest and potato is an important target crop for modified starch qualities (Zeeman, Kosssmann & Smith, 2010). In potato breeding studies, intense focus has been placed on modifying the expression of enzymes involved in the synthesis of amylose and amylopectin to change the starch structure. Schwall et al. (2000) down-regulated two starch branching enzymes, SBE A and SBE B, resulting in high-amylose producing potatoes (Schwall et al., 2000). These starches exhibited up to 56% amylose, a low amount of high molecular weight (M_W) amylopectin and an increased number of long chains in starch which may represent an amylose population with long-branches which is normally minor in starch. Hofvander and coworkers (2004) also produced several high-amylose starches from four potato cultivars with amylose contents ranging between 40 and 86%, the starch with the highest amylose content also having a low degree of branching (0.31%) compared with wild-type potato (about 2.3%) (Hofvander, Andersson, Larsson & Larsson, 2004). In earlier studies on high-amylose maize starches, amylose fractions appeared to be unaffected in structure in all aspects except the average M_W (Banks, Greenwood & Muir, 1974). However, the amylopectin fraction of these starches differed in structure, showing greater average chain length, higher beta-limit values and higher retrogradation tendency in concentrated solution (Banks, Greenwood & Muir, 1974).
It has been shown that commercially available amylose in a mixture with amylopectin exhibits different physicochemical properties from wild-type starches with the same amylose/amylopectin ratio. It has also been reported that purified amylose added to starch had a higher crystallinity than predicted from wild-type starch probably due to lower $M_W$ (Rindlav-Westling, Stading & Gatenholm, 2001). In addition, it is known that $M_W$ influences the mechanical properties (Lourdin, Valle & Colonna, 1995; Wolff, Davis, Cluskey, Gundrum & Rist, 1951). However, not only the effect of high amylose content or high $M_W$ but also structural differences in amylose and amylopectin molecules might give different network microstructure and influence mechanical and barrier properties.

In this study, starches with different amounts of amylose from three genetically modified potato lines were characterized in order to understand the interrelation between molecular structure and physical properties and predict the properties of these starches in materials such as films and coatings. All three high-amylose starches were used to prepare solution-cast films to examine their film-forming ability and their physical properties, i.e. barrier and tensile properties. Different microscopy techniques were used to study the microstructure of the starches and films. Properties of films were correlated to their microstructure and possible applications are discussed.
2. Materials and methods

2.1 Materials

Development and growth of high-amylose potato lines

High amylose potato lines were generated essentially as described previously (Andersson, Melander, Pojmark, Larsson, Bulow & Hofvander, 2006). In brief, 200 bp of two starch branching enzymes, SBE1 and SBE2, were synthetically produced in tandem (Eurofins Genomics, Ebersberg, Germany), flanked by attB sites, and cloned into the binary vector pGWIWgbss via pDonor221 using the Gateway® system (Invitrogen, Carlsbad, CA, USA). pGWIWgbss is a modified version of the pK7GWIWG2(II) (Karimi, Inze & Depicker, 2002) vector, in which the 35S promoter has been replaced by a granular bound starch synthase (GBSS) promoter of Solanum tuberosum origin. Three potato cultivars, Kuras, Verba and Dinamo, were transformed using Agrobacterium tumefaciens strain AGLO harboring the RNAi construct. Transformation, regeneration and selection of lines were performed as described by Andersson et al. (2006) with the modification that 50 mg/L kanamycin was used as the selection agent (Andersson, Trifonova, Andersson, Johansson, Bulow & Hofvander, 2003). High-amylose lines were grown in the greenhouse with 16 hours day length, 18/15 °C day/night temperature and 75% relative humidity for 5 months. Starch was extracted from mature tubers using a protocol described elsewhere (Larsson, Hofvander, Khoshnoodi, Ek, Rask & Larsson, 1996). In brief, tubers were peeled and homogenized at 4 °C with a fruit juicer. Tris-HCl (pH 7.5, 50 mM), Na-dithionite (30 mM) and EDTA (10 mM) was added and starch granules were allowed to sediment and washed four times with buffer and three times with acetone before drying overnight at room temperature.

2.2 Methods
2.2.1 Light microscopy for characterization of starch granule shape and size

Size and shape of starch granules were determined using a light microscope (Nikon Eclipse Ni-U microscope, Tokyo, Japan) and images were captured with a Nikon DS-Fi2-U3 Camera (Nikon Corporation, Japan). Polarized light was used to show crystallinity and iodine staining to detect amylose using 100 mg starch dispersed in 2 mL water and 50µL Lugol’s solution (1g KI + 0.1g I₂ in 50mL water).

2.2.2 Starch content determination with thermostable α-amylase

The purity of the starch was determined enzymatically as described elsewhere (Åman, Westerlund & Theander, 1994). In brief, starch was treated with thermostable α-amylase (EC 3.2.1.1; 3000 U/mL; Megazyme, Wicklow, Ireland) in a boiling water bath for 30 min. Afterwards an amylglucosidase (EC 3.2.1.3, 260 U/mL, Megazyme, Wicklow, Ireland) solution was added and the sample allowed to stand overnight in a 60 °C water bath. At the next day, GOPOD reagent (Glucose oxidase plus peroxidase and 4-aminoantipyrine, Megazyme, Wicklow, Ireland) was added and absorbance measured at 510 nm. Starch content was calculated in terms of glucose concentration using a two-point calibration with a D-glucose standard (ready to use solution from Megazyme). All chemicals used were of analytical grade.

2.2.3 Amylose content in modified and wild-type starches

The amount of amylose was measured colorimetrically using the iodine complex formation method described elsewhere (Chrastil, 1987). Calibration of amylose content was performed on different mixtures of potato amylose standard (type III, Sigma Chemical CO., MO, USA) and
granular potato amylopectin starch (Lyckeby Starch AB, Kristianstad, Sweden) with concentrations of amylose from 10-70% \((R^2=0.972)\).

2.2.4 Separation of amylose and amylopectin

To study the fine structure of high-amylose starches, amylose and amylopectin were isolated according to Klucinec and Thompson (1998) with minor modifications (Klucinec & Thompson, 1998). About 2 g starch were dissolved in 56 mL 90% DMSO at 90 °C under nitrogen stream. A mixture of n-butanol:isoamyl alcohol:water \((372 \text{ mL}; 23.5:23.5:325; \text{v:v:v})\) was added drop-wise at 80 °C under nitrogen stream (ca 1.5 h) and the sample slowly cooled to room temperature. The solution was centrifuged at 13000 g at 4 °C for 2 h. The supernatant with amylopectin was decanted and the solvent evaporated. The amylopectin and amylose fractions were dissolved separately in hot water (about 30 mL) and 3 volumes of methanol were added. The solution was allowed to rest for 1 h at room temperature. Methanol was then decanted after centrifugation \((6600 \text{ g}, 4 °C \text{ and } 2 \text{ h})\) and the remaining pellet once more dissolved in hot water \((30 \text{ mL})\) and treated likewise with ethanol \((3 \text{ volumes})\). The amylose and amylopectin were freeze-dried and checked for purity using gel permeation chromatography \((\text{GPC; Sepharose CL-6B})\) after debranching as described below.

2.2.5 Molecular size distribution and amylose content using CL-6B columns after debranching

Size distribution of whole potato starch was measured using GPC on a Sepharose CL-6B column \((90 \text{ cm x 1.0 cm})\) after debranching with isoamylase \((\text{glycogen 6-glucanohydrolase; EC 3.2.1.68; from Pseudomonas sp.}; \text{specific activity } 280 \text{ U/mg})\) and pullulanase \((\text{amylopectin 6-glucanohydrolase; EC 3.2.1.41; from Klebsiella planticola}; \text{specific activity } 42 \text{ U/mg})\) as
described elsewhere (Bertoft & Spoof, 1989). Fractions of 0.5 mL were collected and analyzed with phenol-H$_2$SO$_4$ reagent (DuBois, Gilles, Hamilton, Rebers & Smith, 1956). Granular potato amylopectin starch (Lyckeby Starch AB, Kristianstad, Sweden) was used to indicate the elution volume where chains from amylopectin elute from the column (85 mL) and was used as limit to calculate amylose content (relative area under the curve eluting before 85 mL). Purity and size distribution of separated starch components, i.e. amylose and amylopectin, were determined accordingly.

2.2.6 Chain length distribution using high-performance anion-exchange chromatography (HPAEC)

Chain length distribution of amylopectin was determined using a Dionex HPAEC system (Dionex, Sunnyvale, CA, USA) with a CarboPac PA-100 column (4 × 250 mm) coupled with a pulsed amperometric detector. A gradient of NaOAc in 0.15 M NaOH was used to elute starch chains, as described in detail elsewhere (Koch, Andersson & Åman, 1998).

2.2.7 Pasting properties using rapid visco analysis (RVA)

Starch pasting properties were analyzed using RVA (Newport Scientific, Warriewood, NSW, Australia). The standard method 1 according to the manufacturer was used, where 25.0 mL water was added into an aluminum canister and 2.0 g starch (dry weight) was added. The paddle rotation was kept constant at 160 rpm throughout the analysis. The suspension was equilibrated at 50 °C for 1 min, heated at a rate of 12.2 K/ min to 95 °C and kept for 3.5 min before cooling to 50 °C at a rate of 11.8 K/ min.
2.2.8 Solution-casting of starch films

Aqueous starch solutions (180 mg dry weight/ 6 mL water) were heated to 140 °C in a closed tube and held for 45 min under permanent stirring in a Pierce Reacti-Therm heating/stirring module. The solutions were allowed to cool to around 70 °C and 3.5 mL of the solution were transferred to a Petri dish (5.5 cm diameter). Bigger Petri dishes (8.5 cm diameter and 8.4 mL solution) were used to produce films for barrier properties and tensile testing. The solvent was evaporated at 23 °C overnight and allowed to dry for 48 h.

2.2.9 Microstructural study of solution-cast films

Scanning electron microscopy (SEM) was carried out using a Philips XL 30 ESEM operated at 10 kV, to observe the surface of the starch films. Samples were previously gold-coated using an Emitech K550X sputter device (Quorum Technologies Ltd, Ashford, Kent, UK).

For TEM, fragments of approximately 2 mm² were cut out of the films and embedded in TAAB 812 resin (epoxy) without prior fixation and dehydration. Thin sections (about 75 nm) were cut using a diamond knife on a Leica EM UC6 ultramicrotome and collected on gold grids. Sections were stained according to the periodic acid-thiosemicarbazide-silver proteinate method described elsewhere (Thiéry, 1967) prior to examination with a Philips CM/12 transmission electron microscope using an accelerating voltage of 80 kV.

2.2.10 Material properties of solution-cast films

Oxygen transmission rate (OTR) was measured in a Mocon Ox-Tran 2/21 (SH) in duplicate according to standard method ASTM F1927 – 07 and oxygen permeability (OP) was calculated based on film thickness (measured at six random places within the film). Prior to OTR
measurements, samples were conditioned at 23 °C and 50% RH. Tensile testing of starch films was performed according to ASTM D882-02. Before punching out dumbbell shaped specimens, samples were kept in an air-conditioned climate chamber at 23 °C and 50% RH.
3. Results and discussion

Three high-amylose potatoes from three cultivars Kuras, Verba and Dinamo were produced by suppression of the starch branching enzymes $SBE1$ and $SBE2$ and are denoted LowAm-1068, MidAm-7040 and HighAm-2012, respectively. All high-amylose lines resulted in higher tuber yield but lower dry matter which is attributed to lower starch content (Table 1), a feature reported previously for high-amylose potato lines (Hofvander, Andersson, Larsson & Larsson, 2004). The extracted starch from each potato wild-type cultivar and their high-amylose lines were characterized in detail and their film-forming ability studied in terms of microstructure and physical properties.

3.1 Light microscopy for morphology and birefringence of starch granules

Starch granules from high-amylose lines exhibited different shapes and sizes compared to wild-type potato (Figure 1 A-C). Those in wild-type potatoes were typically oval, round or even irregular shaped (data not shown), whereas with increasing amount of amylose, the starches had an increasing amount of very small, rod-shaped granules which did not exist in wild-type starches. Also, starch granules of high-amylose lines showed fissures in the middle of the granules. This has been reported before by others (Schwall et al., 2000), but seems to appear only when starch granules are in contact with water as for preparation for light microscopy imaging.

Using polarised light, starch granules display birefringence, shown as a Maltese cross (Figure 1 D-F). This property indicates the radial order centred at the hilum, the growth centre of the granule, and is correlated with the orderly alignment of the starch molecules. There were some unevenly shaped granules in all three high-amylose starches displaying one or more overlapping crosses, but this was most obvious for HighAm-2012 containing the highest amount of amylose.
There was no loss of birefringence, indicating no major changes in the order and alignment of starch molecules within the granule. This is in contrast to previous studies producing high-amylose potato starches by inhibition of starch branching enzymes where starch granules of high-amylose lines displayed less birefringence (Schwall et al., 2000).

Besides the irregular shape, high-amylose lines exhibited granules with overlapping of two or more crosses. A theory has been proposed that the altered shape of the granules is a result of failed amyloplast separation and thus the division of the amyloplast is retarded during development because of the fusion of two granules by amylose interaction, forming helices (Jiang, Horner, Pepper, Blanco, Campbell & Jane, 2010). Hence, rod and triangular shaped granules are prevalent in potato starches with higher amylose content.
Figure 1. Light microscopy images of starch granules (A to C: stained with iodine, D to F: under polarised light), photos of solution-cast films (G to I), SEM images of film surface (J to L) and
TEM images of microstructural network (M to O, arrows indicate pores) from high-amylose lines LowAm-1068, MidAm-7040 and HighAm-2012.

3.2 Amylose content by colorimetry and gel permeation chromatography

Amylose content was determined based on iodine complex formation. The amylose content was about 30% for the wild-type starches (Table 1), which was slightly higher than expected for common potato starches using iodine (Morrison & Laignelet, 1983). This could be due to an overestimation of amylose when using an extracted amylose standard for calibration which probably has a lower chain length than in native starches. As expected, the three high-amylose lines exhibited elevated amylose content, from 45-89%. The basis for colorimetric amylose determination is the property of amylose to form a colorful complex with iodine depending on the chain length of the polymer. No differentiation can be made on interference from color formation by long-chain amylopectin molecules with iodine to a complex, and hence the technique can give an overestimation of the amylose content (Morrison & Laignelet, 1983; Vilaplana, Hasjim & Gilbert, 2012).

Table 1. Tuber yield, dry matter of fresh weight, starch purity and amylose content of wild-type starches (Kuras, Verba, Dinamo) and their high-amylose lines (LowAm-1068, MidAm-7040, HighAm-2012) determined colorimetrically using iodine binding and by gel permeation chromatography (GPC) after debranching.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tuber yield [%]a</th>
<th>Dry matter of fresh weight [%]</th>
<th>Starch purity [%]b</th>
<th>Amylose content [%]</th>
<th>Colorimetricc</th>
<th>GPCd</th>
</tr>
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<td></td>
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<tr>
<td>Variety</td>
<td>Amylase</td>
<td>Starch</td>
<td>Molar Mass Distribution</td>
<td>Amylose Content</td>
<td></td>
<td></td>
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<td>--------</td>
<td>-------------------------</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Kuras</td>
<td>100</td>
<td>31</td>
<td>86.3 ± 1.35</td>
<td>22 ± 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LowAm-1068</td>
<td>169</td>
<td>25</td>
<td>81.5 ± 0.88</td>
<td>26 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verba</td>
<td>100</td>
<td>31</td>
<td>87.9 ± 1.59</td>
<td>23 ± 1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MidAm-7040</td>
<td>154</td>
<td>29</td>
<td>77.7 ± 2.23</td>
<td>39 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dinamo</td>
<td>100</td>
<td>31</td>
<td>85.3 ± 1.26</td>
<td>23 ± 2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HighAm-2012</td>
<td>219</td>
<td>18</td>
<td>75.6 ± 2.76</td>
<td>49 ± 5.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) relative to wild-type potato cultivar, \(^b\) Starch content based on dry matter (mean ± stdev., n=3) determined enzymatically according to Åman, Westerlund and Theander (1994), \(^c\) based on starch content (mean ± stdev., n=4), \(^d\) mean and standard error

Molar mass distribution of intact starches was carried out on Sepharose CL-2B columns (supplementary material) and showed a typical pattern of potato starch with two peaks corresponding to amylopectin with a typical wavelength at maximum absorbance of 560 nm and an amylose peak with a typical wavelength at maximum absorbance of about 640 nm.

Separation using GPC of debranched molecules can give a better estimate of amylose content. A granular potato amylopectin starch standard was used to indicate the elution volume where the first chains of debranched amylopectin elute from the column (at elution volume 85 mL; Figure 2). In order to compare the starches, the amylose content was determined as the relative area under the curve from 40 to 85 mL. The corresponding amylose content of the three wild-type starches was about 23%, while their high-amylose lines LowAm-1068, MidAm-7040 and HighAm-2012 contained about 26%, 39% and 49% of amylose, respectively. Not only was the amount of amylose (first peak in chromatogram; Figure 2) increased, but the composition of chain lengths in amylose and amylopectin changed, resulting in a shift in the chromatogram (Figure 2, dotted lines). The elution profile of the debranched starches showed that with increasing amylose content, the separation between the two components was less obvious. This
indicates that amylopectin molecules of the high-amylose line starches exhibited a higher amount of long chains and/or that amylose molecules were slightly branched. As discussed below, it was shown that amylopectin molecules had longer chains and that amylose molecules were not changed. This is in agreement with previous studies on genetically modified high-amylose potato starches, reporting an increase in average chain length in amylopectin (Blennow, Hansen, Schulz, Jørgensen, Donald & Sanderson, 2003; Hofvander, Andersson, Larsson & Larsson, 2004; Schwall et al., 2000).

Figure 2. Gel permeation chromatograms on Sepharose CL-6B of wild-type potato starches Kuras, Verba, Dinamo (solid line) and their high-amylose lines LowAm-1068, MidAm-7040, HighAm-2012 (dotted line) after debranching with isoamylase/pullulanase. Gray vertical lines represent division of amylose and amylopectin for amylose content calculation, based on a standard of granular amylopectin potato starch.

Recently, there have been many efforts to define amylose content and validation of methods to determine amylose content (Vilaplana, Hasjim & Gilbert, 2012; Vilaplana, Meng, Hasjim &
Gilbert, 2014). However, with high-amylose starches with altered structure, the definition becomes more complicated and it is well known that assays based on iodine binding can overestimate amylose content (Cheetham & Tao, 1997; Hofvander, Andersson, Larsson & Larsson, 2004; Schwall et al., 2000; Shi, Capitani, Trzasko & Jeffcoat, 1998; Vilaplana, Hasjim & Gilbert, 2012). In a recent study by Vilaplana et al. (2014), two-dimensional distributions based on molecular size and branch chain length revealed the complex topologies of high-amylose maize starch showing the presence of distinct intermediate compounds with the molecular size of amylose, but branching structure similar to that of amyllopectin, albeit having longer chain length (Vilaplana, Meng, Hasjim & Gilbert, 2014).

3.3 Separation of amylose and amyllopectin using n-butanol-amylose complex formation

To study the changed structure of the high-amylose starches in more detail, amyllopectin and amylose were isolated through chemical fractionation using n-butanol. The purity of each fraction was determined using GPC of debranched samples. All isolated amyllopectin fractions of wild-type starches showed ≤ 3.5% amylose and were hence considered pure (Table 2 and Figure 3). The amyllopectin isolated from the high-amylose lines exhibited up to 18% amylose (calculated at the same separation volume for division of the two components at 85 mL shown in Figure 2).

Table 2. Yield and purity of amylose (AM) and amyllopectin (AP) after separation with n-ButOH/ iso-amylalcohol (1st separat.) and second separation of the amylose fraction (AM separat.)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Yield after ButOH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Purity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt; separat.</td>
</tr>
</tbody>
</table>

---

<sup>a</sup> Yield after n-ButOH/ iso-amylalcohol.

<sup>b</sup> Purity determined by GPC of debranched samples.
<table>
<thead>
<tr>
<th></th>
<th>1st separat.</th>
<th>AM separat.</th>
<th>AP</th>
<th>AM</th>
<th>AP</th>
<th>AM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kuras</td>
<td>91.9</td>
<td>54.1</td>
<td>97.9</td>
<td>62.9</td>
<td>93.3</td>
<td>91.4</td>
</tr>
<tr>
<td>LowAm-1068</td>
<td>87.5</td>
<td>31.3</td>
<td>95.6</td>
<td>52.2</td>
<td>93.7</td>
<td>78.2</td>
</tr>
<tr>
<td>Verba</td>
<td>96.3</td>
<td>62.4</td>
<td>96.5</td>
<td>65.3</td>
<td>97.5</td>
<td>92.8</td>
</tr>
<tr>
<td>MidAm-7040</td>
<td>97.5</td>
<td>55.0</td>
<td>88.2</td>
<td>66.7</td>
<td>89.3</td>
<td>86.5</td>
</tr>
<tr>
<td>Dinamo</td>
<td>79.5</td>
<td>51.3</td>
<td>98.7</td>
<td>62.7</td>
<td>96.2</td>
<td>75.3</td>
</tr>
<tr>
<td>HighAm-2012</td>
<td>102.0</td>
<td>26.7</td>
<td>81.6</td>
<td>59.1</td>
<td>85.0</td>
<td>84.7</td>
</tr>
</tbody>
</table>

a Yield in % calculated gravimetrically based on starch content (sum of amylose and amylopectin after n-butanol fractionation), b Determined in % using GPC on Sepharose CL-6B after debranching, point of division at 85 mL elution volume.

However, the chromatograms showed that the amylopectin structure was changed compared to the wild-type starches, displaying an increased amount of longer chains rather than an impurity from amylose. Hence, the division between amylopectin and amylose was adjusted to be at elution volume 65 mL resulting in ≥95% purity of isolated amylopectin fractions (data not shown). Isolated amylose fractions were less pure and contained a considerable amount of amylopectin (Figure 3, solid line). Hence, those isolated amylose fractions were precipitated with n-butanol once more. The second isolate of amylose (Figure 3, dotted line) showed no amylopectin peak in the elution profile, but rather a long tailing amylose peak. Amylopectin isolated from the amylose fraction showed the same elution profile as amylopectin isolated from whole starch (Figure 3, dotted line) and hence was considered to reflect remnants from the separation. In order to distinguish whether only linear amylose, low-branched amylose or low-MW amylopectin with long-chains was present in the amylose fractions, the samples were run without debranching using GPC (Figure 3, dashed line). A change in the chromatogram would have indicated branching. However, it was found that all isolated amylose fractions consisted of linear chains. Similar results have been reported for high-amylose maize starches, i.e. low-MW fractions were not branched, as debranching with isoamylase did not show any differences in the
gel permeation chromatogram (Shi, Capitani, Trzasko & Jeffcoat, 1998). A small difference was seen for the amylose fraction of LowAm-1068 (Figure 3, dashed line), where a minor peak probably indicated some impurity from amylopectin molecules.

Figure 3. Gel permeation chromatogram on Sepharose CL-6B after debranching of high-amylose potato lines LowAm-1068, MidAm-7040 and HighAm-2012: isolated amylose and amylopectin fractions (solid line = first separation, dotted line = second separation, dashed line = second separation without debranching).

The elution profiles of the isolated compounds demonstrated that there was little difference in the amylose fractions of the high-amylose lines, as reported elsewhere (Banks, Greenwood & Muir, 1974). However, the amylopectin fraction showed a profile shift to longer chains. Hence, further
investigations on the chain length distribution were carried out and are discussed in more detail below.

3.4 Chain length distribution of amylopectin

Since the distribution and size of linear amylase molecules was considered to be similar in all three starches, no further investigations were carried out on these samples. Instead, amylopectin was investigated in more detail. Chain length distribution of isolated amylopectin was determined using HPAEC-PAD and the unit chain profiles were plotted (Figure 4).

Figure 4. Chain length distribution after debranching of amylopectin of wild-type starches Kuras, Verba, Dinamo (solid line) and their high-amylase lines LowAm-1068, MidAm-7040, HighAm-2012.
HighAm-2012 (dotted line) using HPAEC-PAD and difference between the relative areas in the chain length distribution of high-amylose line minus wild-type starch (lower part).

The amylopectin average chain length of all wild-type starches was about 35, with a peak at degree of polymerization (DP) 13 (Figure 4). Similar profiles of regular potato amylopectin have been reported by others (Jane et al., 1999; Koch, Andersson & Áman, 1998). In contrast, molar proportions of chains with DP 18-37 increased in all high-amylose lines. The peak DP peak shifted to 22 for HighAm-2012. The changes in the individual unit chains between the amylopectin of wild-type starch and their corresponding high-amylose lines are visualized in the lower part of Figure 4. In the high-amylose lines, chains with DP 6-8 were particularly decreased. These chains have been shown to possess typical profiles depending on the crop and are probably outermost chains in the amylopectin (Bertoft, 2004). The proportion of short chains (DP 6-18) in amylopectin of HighAm-2012 was decreased by 50%, that of MidAm-7040 by at least 32% and that of LowAm-1068 by about 13% (Figure 4). More thorough investigations of the fine structure of amylopectin, i.e. β-limit and φ,β-limit dextrin, would give a better understanding of which chain categories, outer and inner chains, are involved in the molecular network formation (Bertoft, 2007).

3.5 Pasting properties of starches

Starch pasting properties provide information on the ability to form a paste after heating and cooling. Native potato starch does not solubilize at temperatures lower than 50 °C, but when heated in water starch granules start absorbing water and swell. Wild-type potato starches started to swell at around 72-75 °C, seen as an increase in viscosity (Figure 5). As the temperature increases, starch granules rupture and more soluble amylose can leak out resulting in a small
decrease of the viscosity (breakdown). During the holding time, the material is subjected to high temperature and mechanical shear which further disrupted the granules. The holding strength is the ability of a material to withstand shear stress and heat, which was high in all wild-type potato starches. During the cooling phase, re-association of molecules, especially amylose, occurred in all wild-type starches resulting in the formation of a gel structure increasing viscosity (setback). The low setback region indicated a low rate of starch retrogradation. Even though there were differences in pasting behaviour between wild-type starches, our main focus was laid on the high-amylose starches. As seen from the RVA amylographs in Figure 4, there were large differences between the three high-amylose starches which showed a completely different profile compared to their wild-type counterparts.
**Figure 5.** Pasting profiles of wild-type starches Kuras, Verba, Dinamo (solid line) and their high-amylose lines LowAm-1068, MidAm-7040, HighAm-2012 (dotted line) measured using RVA. (Upper light grey line indicates temperature profile)

The high-amylose starch LowAm-1068 had a higher pasting temperature (79 °C) and final viscosity and a substantially higher setback compared to wild-type starches. The higher pasting temperature indicates a higher swelling capacity of granules. During the holding time, there was only a small breakdown detectable in LowAm-1068 which indicates that the material could withstand heat and shear forces and that not all granules were disrupted. Light microscopy images were taken after the RVA run (data not shown) and revealed a mixture of swollen granules and fragments of swollen granules within a continuous network. During cooling, viscosity increased with a final viscosity that was twice as high compared with wild-type starches, probably due to the slightly increased amylose content in LowAm-1068. Amylose re-associates during cooling until a gel structure is formed, which increases viscosity.

The two starches with highest amylose content showed very low or no granule swelling (Light microscopy images, data not shown) and viscosity (Figure 5) under the conditions used. It is primarily the amylopectin that is responsible for granule swelling, while the high amylose content alters the pasting properties and restricts swelling (Tester & Morrison, 1990). The inhibited granule swelling of starches with >55% amylose (colorimetric determination with iodine) has been reported previously (Karlsson, Leeman, Björck & Eliasson, 2007; Schwall et al., 2000).

### 3.6 Microstructural characterization of solution-cast films

...
As starches from high-amylose lines needed higher gelatinization temperatures, the starch water slurry was heated to 140 °C during vigorously stirring and then cast into Petri dishes. From the images of solution-cast films (Figure 1 G-I), it can be seen that film-formation was different for each high-amylose line. MidAm-7040 and HighAm-2012 gave cohesive but curled-up films; LowAm-1068 gave partly fragmented films. All three solution-cast films were homogeneous and no granule-shaped remnants or phase separation was seen (Light microscopy images, data not shown). The difference in film-formation of the three starches of the high-amylose lines could be due to the difference in amylose and amylopectin ratio as well as the difference in amylopectin structure (described below). Amylose is thought to be the better film-former resulting in cohesive films, whereas the higher amount of amylopectin in LowAm-1068 could result in the fragmentation of the film. It has been shown that high temperature and longer heating are necessary to completely disrupt starch granules of high-amylose starches (Bengtsson, Koch & Gatenholm, 2003; Koch, Gillgren, Stading & Andersson, 2010). On the other hand, harsh conditions could degrade amylose and amylopectin affecting the film cohesiveness negatively (Koch, Gillgren, Stading & Andersson, 2010). However, we did not expect a severe degradation of starch during heating to 140 °C as in the study of Koch et al. (2010) 150 °C were needed to significantly degrade starch. There is little known about time and temperature conditions to produce homogenous solution cast films (Koch, Gillgren, Stading & Andersson, 2010) and the effect on molecular structure, microstructure and material properties. Hence, further investigations are required. A general problem is that most studies are not explicit on the conditions to solubilize starch to produce solution cast films; i.e. in this study it took 45 min to heat up the sample tube to the holding temperature of 140 °C.
SEM images have shown a more uneven surface texture for films of starchy grains with higher amylose content (Figure 1 J-L). Similar findings have previously been attributed to remnants of granules after gelatinization and/or fast aggregation of amylose during the cooling process, resulting in a rougher surface (Bengtsson, Koch & Gatenholm, 2003; Koch, Gillgren, Stading & Andersson, 2010). However, in our study, light microscopy images (iodine staining and differential interference contrast) did not reveal any granule-shaped remnants.

The microstructure of solution-cast films was studied using TEM. TEM micrographs (Figure 1 M-O) showed mainly darkly stained starch molecules and brighter pore areas (indicated by arrows). Since individual amylpectin molecules are different from amyllose molecules in size and structure and vary between the high-amylose lines, the TEM-images were expected to show morphological differences. At first sight, all films looked similar showing a rather homogenous and dense structure. However, there were distinct differences between films regarding pore size and shape. TEM micrographs of LowAm-1068 films (Figure 1 M) revealed small pores (indicated by arrows) of about 10 nm (through a few manual measurements) which were mainly round-shaped and representing an amylpectin-like branched network structure (Leloup, Colonna, Ring, Roberts & Wells, 1992). TEM micrographs of MidAm-7040 films showed a more heterogeneous pore distribution with less regular shaped pores up to 30-40 nm in size. HighAm-2012 films exhibited a more open but also fractal-structure with pore sizes up to 60 nm which could be argued to be more amylose-like and revealing some rod-like strands. TEM micrographs of the films LowAm-1068 and MidAm-7040 did not evidently reveal the dense amylose-like structure as reported previously for pure amylose gels and films (Leloup, Colonna, Ring, Roberts & Wells, 1992; Richardson, Kidman, Langton & Hermansson, 2004; Rindlav-Westling, Stading, Hermansson & Gatenholm, 1998). The morphology of the HighAm-2012
film, however, showed some indication of a more open structure with stiff rod-like strands and was more alike pure amylose gels and films. As discussed above, the genetic modification of the potato resulted in a change in amylopectin structure besides an increase in amylose content (Figure 2). We therefore suggest that not only amylose but also long chains of amylopectin or intermediate components can contribute to the change in microstructure, e.g. physical entanglements between longer branches and amylose chains, observed using TEM. However, further investigations are needed to better understand the created starch network structure seen in TEM images.

3.7 Barrier properties and tensile strength of solution-cast films

Oxygen permeability (OP) was studied in solution-cast films of all three high-amylose lines and compared with the wild-type Dinamo (Table 3). It was found that all three high-amylose lines exhibited lower OP (P<0.01) than native potato starch films. In addition, OP was much lower than in materials made from most synthetic polymers. In a previous study on amylose films, better barrier properties were attributed to higher crystallinity compared with amylopectin films (Rindlav-Westling, Stading, Hermansson & Gatenholm, 1998). This would decrease the solubility of oxygen, resulting in a lower oxygen transmission rate.

Starch films of the high-amylose lines exhibited higher stress at break compared with the wild-type starch film Dinamo (Table 3; P<0.05). The stiffness and higher strength of these films compared with wild-type starch could be attributable to the longer chains in amylopectin being involved in the interconnected network and increasing the interaction between chains. Furthermore, strain at break increased in all high-amylose lines (Table 3; P<0.05). It has been shown previously for starch films of different amylose and amylopectin mixtures that higher amylose content increases elongation (Lourdin, Valle & Colonna, 1995). As compared to
commonly used oxygen barriers, such as EVOH (poly ethylene-co-vinylalcohol), the stress at break is considerable higher but the strain at break is lower when measured at the same conditions (23 °C and 50% RH). The high standard deviations are due to the strong polar bonds and a high surface energy. This facilitates fracture propagation from, e.g. a small flaw in the surface, as easily introduced when cutting the samples for the tensile test. The standard deviation decreases when testing hundreds of samples, but the interesting effect of such materials is that they may facilitate easy-to-open packages. However, the films or coatings in a real package would be supported by a water barrier and sealant layer of, e.g. renewable polyethylene that also would give the main contribution to the mechanical properties of the package.

Table 3. Oxygen permeability (OP), stress at break and strain at break of parental starch Dinamo and high-amylose lines, high-amylose maize starch, low-density polyethylene (LDPE) and polyethylene-co-vinylalcohol (EVOH)

<table>
<thead>
<tr>
<th>Sample</th>
<th>OP(^a) [cc mm/ m(^2) 24h atm]</th>
<th>Stress at break(^b) [MPa]</th>
<th>Strain at break(^b) [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dinamo</td>
<td>0.170 ± 0.01</td>
<td>34.1 ± 9.5</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>LowAm-1068</td>
<td>0.089 ± 0.04</td>
<td>42.2 ± 3.7</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>MidAm-7040</td>
<td>0.100 ± 0.03</td>
<td>45.0 ± 12.0</td>
<td>2.3 ± 0.8</td>
</tr>
<tr>
<td>HighAm-2012</td>
<td>0.085 ± 0.03</td>
<td>46.0 ± 12.4</td>
<td>3.4 ± 2.2</td>
</tr>
<tr>
<td>High-amylose maize starch(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDPE(^d)</td>
<td>1900</td>
<td>7-16</td>
<td>100-800</td>
</tr>
<tr>
<td>EVOH(^e)</td>
<td>0.01-12</td>
<td>20-210</td>
<td>20-330</td>
</tr>
</tbody>
</table>
mean ± standard error (n=2), \(^b\) mean ± standard deviation (n=6), moisture content was between 10.2 to 10.4% in the starch films, \(^c\) from Koch, Gillgren, Stading and Andersson (2010), \(^d\) from Doak (1986), \(^e\) from Lange and Wyser (2003)

4. Conclusions

Targeted gene suppression of SBE1 and SBE2 through RNA interference in three different potato cultivars resulted in high-amylose starches, which were characterized in detail and used to relate film performance to molecular structure. These high-amylose lines revealed starches with changed granular and molecular structures, pasting properties and film performance. The amylose content was increased to 45, 70 and 89% using iodine binding-based measurements. However, GPC revealed a more reliable amylose content of 26, 39 and 49% as the chain length of amylopectin was increased in addition to amylose content affecting the measurement when using the colorimetric method. The genetic modification produced starches with increasing amount of irregular shaped granules yielding basically no pasting at 95°C. At high temperature, 140°C, all three starches were gelatinized. Highest amylose content and amylopectin with the longest chains resulted in cohesive films with a rough surface and improved physical properties. The improved oxygen barrier provided by the starches from high-amylose potato and their superior mechanical properties in terms of stronger films and increased strain at break indicate that they have the potential for interesting commercial applications such as in films or coatings. They are thought to have a particularly interesting future as barrier coatings, as the presently used industrial facilities (e.g. blade and curtain coaters) possibly could be used when applying them on boards or polymer films. However, they may have the same shortcomings as poor water barriers and must, just like the currently used oxygen barrier polymers, be encapsulated between two hydrophobic layers, that could for example be renewable polyethylene and tie layers.
Acknowledgement

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References


