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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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19 ABSTRACT

20 High-amylose potato starches were produced through genetic modification resulting in changed

21 granule morphology and composition with higher amylose content and increased chain-length of

22 amylopectin. The increased amylose content and structural changes in amylopectin gave

23 improved film forming behavior and better barrier and tensile properties in starch films. The

24 molecular structure in these starches was related to film-forming properties. Solution-cast films

25 of high-amylose starch revealed a homogeneous structure with increasing surface roughness at

26 higher amylose content, possibly due to amylose aggregation. Films exhibited significant higher

27 stress and strain at break compared to films of wild-type starch, which could be attributable to

28 the longer chains of amylopectin being involved in the interconnected network and more

29 interaction between chains as shown using transmission electron microscopy. The oxygen

30 permeability of high-amylose starch films was significantly decreased compared to wild-type

31 starch. The nature of the modified starches makes them an interesting candidate for replacement

32 of non-renewable oxygen and grease barrier polymers used today.

33 KEYWORDS

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 &

59 MacMasters, 1955). Extensive amylose breeding programs, such as that by National Starch

60 (Forsyth, IL., USA) have resulted in commercial crops with up to 90% amylose (Sidebottom,

61 Kirkland, Strongitharm & Jeffcoat, 1998). However, in many plants, for example potato, no

62 natural mutants have been described and genetic modification has been used to introduce the

63 high-amylose trait. Starches from potato have a distinctive quality, with a high amount of

64 phosphate and low content of lipids, which is beneficial for many applications where good

65 clarity, neutral taste or high swelling is needed. They are therefore of considerable industrial

66 interest and potato is an important target crop for modified starch qualities (Zeeman, Kossmann

67 & Smith, 2010). In potato breeding studies, intense focus has been placed on modifying the

68 expression of enzymes involved in the synthesis of amylose and amylopectin to change the

69 starch structure. Schwall et al. (2000) down-regulated two starch branching enzymes, *SBE A* and

70 *SBE B*, resulting in high-amylose producing potatoes (Schwall et al., 2000). These starches

71 exhibited up to 56% amylose, a low amount of high molecular weight (M_W) amylopectin and an

72 increased number of long chains in starch which may represent an amylose population with long-

73 branches which is normally minor in starch.. Hofvander and coworkers (2004) also produced

74 several high-amylose starches from four potato cultivars with amylose contents ranging between

75 40 and 86%, the starch with the highest amylose content also having a low degree of branching

76 (0.31%) compared with wild-type potato (about 2.3%) (Hofvander, Andersson, Larsson &

77 Larsson, 2004). In earlier studies on high-amylose maize starches, amylose fractions appeared to

78 be unaffected in structure in all aspects except the average M_W (Banks, Greenwood & Muir,

79 1974). However, the amylopectin fraction of these starches differed in structure, showing greater

80 average chain length, higher beta-limit values and higher retrogradation tendency in concentrated

81 solution (Banks, Greenwood & Muir, 1974).

82   It has been shown that commercially available amylose in a mixture with amylopectin exhibits

83   different physicochemical properties from wild-type starches with the same

84   amylose/ amylopectin ratio. It has also been reported that purified amylose added to starch had a

85   higher crystallinity than predicted from wild-type starch probably due to lower M_W (Rindlav-

86   Westling, Stading & Gatenholm, 2001). In addition, it is known that M_W influences the

87   mechanical properties (Lourdin, Valle & Colonna, 1995; Wolff, Davis, Cluskey, Gundrum &

88   Rist, 1951). However, not only the effect of high amylose content or high M_W but also structural

89   differences in amylose and amylopectin molecules might give different network microstructure

90   and influence mechanical and barrier properties.

91   In this study, starches with different amounts of amylose from three genetically modified potato

92   lines were characterized in order to understand the interrelation between molecular structure and

93   physical properties and predict the properties of these starches in materials such as films and

94   coatings. All three high-amylose starches were used to prepare solution-cast films to examine

95   their film-forming ability and their physical properties, i.e. barrier and tensile properties.

96   Different microscopy techniques were used to study the microstructure of the starches and films.

97   Properties of films were correlated to their microstructure and possible applications are

98   discussed.

99

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

123 *2.2.1 Light microscopy for characterization of starch granule shape and size*

124 Size and shape of starch granules were determined using a light microscope (Nikon Eclipse Ni-U

125 microscope, Tokyo, Japan) and images were captured with a Nikon DS-Fi2-U3 Camera (Nikon

126 Corporation, Japan). Polarized light was used to show crystallinity and iodine staining to detect

127 amylose using 100 mg starch dispersed in 2 mL water and 50µL Lugol`s solution (1g KI + 0.1g

128 I_2 in 50mL water).

129

130 *2.2.2 Starch content determination with thermostable α-amylase*

131 The purity of the starch was determined enzymatically as described elsewhere (Åman,

132 Westerlund & Theander, 1994). In brief, starch was treated with thermostable α-amylase (EC

133 3.2.1.1; 3000 U/mL; Megazyme, Wicklow, Ireland) in a boiling water bath for 30 min.

134 Afterwards an amyloglucosidase (EC 3.2.1.3, 260 U/mL, Megazyme, Wicklow, Ireland)

135 solution was added and the sample allowed to stand overnight in a 60 °C water bath. At the next

136 day, GOPOD reagent (Glucose oxidase plus peroxidase and 4-aminoantipyrine, Megazyme,

137 Wicklow, Ireland) was added and absorbance measured at 510 nm. Starch content was calculated

138 in terms of glucose concentration using a two-point calibration with a D-glucose standard (ready

139 to use solution from Megazyme). All chemicals used were of analytical grade.

140

141 *2.2.3 Amylose content in modified and wild-type starches*

142 The amount of amylose was measured colorimetrically using the iodine complex formation

143 method described elsewhere (Chrastil, 1987). Calibration of amylose content was performed on

144 different mixtures of potato amylose standard (type III, Sigma Chemical CO., MO, USA) and

145   granular potato amylopectin starch (Lyckeby Starch AB, Kristianstad, Sweden) with

146   concentrations of amylose from 10-70% (R^2=0.972).

147

148   *2.2.4 Separation of amylose and amylopectin*

149   To study the fine structure of high-amylose starches, amylose and amylopectin were isolated

150   according to Klucinec and Thompson (1998) with minor modifications (Klucinec & Thompson,

151   1998). About 2 g starch were dissolved in 56 mL 90% DMSO at 90 °C under nitrogen stream. A

152   mixture of n-butanol:isoamyl alcohol:water (372 mL; 23.5:23.5:325; v:v:v) was added drop-wise

153   at 80 °C under nitrogen stream (ca 1.5 h) and the sample slowly cooled to room temperature. The

154   solution was centrifuged at 13000 g at 4 °C for 2 h. The supernatant with amylopectin was

155   decanted and the solvent evaporated. The amylopectin and amylose fractions were dissolved

156   separately in hot water (about 30 mL) and 3 volumes of methanol were added. The solution was

157   allowed to rest for 1 h at room temperature. Methanol was then decanted after centrifugation

158   (6600 g, 4 °C and 2 h) and the remaining pellet once more dissolved in hot water (30 mL) and

159   treated likewise with ethanol (3 volumes). The amylose and amylopectin were freeze-dried and

160   checked for purity using gel permeation chromatography (GPC; Sepharose CL-6B) after

161   debranching as described below.

162

163   *2.2.5 Molecular size distribution and amylose content using CL-6B columns after debranching*

164   Size distribution of whole potato starch was measured using GPC on a Sepharose CL-6B column

165   (90 cm x 1.0 cm) after debranching with isoamylase (glycogen 6-glucanohydrolase; EC 3.2.1.68;

166   from *Pseudomonas* sp.; specific activity 280 U/mg) and pullulanase (amylopectin 6-

167   glucanohydrolase; EC 3.2.1.41; from Klebsiella planticola; specific activity 42 U/mg) as

168   described elsewhere (Bertoft & Spoof, 1989). Fractions of 0.5 mL were collected and analyzed

169   with phenol-H_2SO_4 reagent (DuBois, Gilles, Hamilton, Rebers & Smith, 1956). Granular potato

170   amylopectin starch (Lyckeby Starch AB, Kristianstad, Sweden) was used to indicate the elution

171   volume where chains from amylopectin elute from the column (85 mL) and was used as limit to

172   calculate amylose content (relative area under the curve eluting before 85 mL). Purity and size

173   distribution of separated starch components, i.e. amylose and amylopectin, were determined

174   accordingly.

175

176   *2.2.6 Chain length distribution using high-performance anion-exchange chromatography*

177   *(HPAEC)*

178   Chain length distribution of amylopectin was determined using a Dionex HPAEC system

179   (Dionex, Sunnyvale, CA, USA) with a CarboPac PA-100 column (4 × 250 mm) coupled with a

180   pulsed amperometric detector. A gradient of NaOAc in 0.15 M NaOH was used to elute starch

181   chains, as described in detail elsewhere (Koch, Andersson & Åman, 1998).

182

183   *2.2.7 Pasting properties using rapid visco analysis (RVA)*

184   Starch pasting properties were analyzed using RVA (Newport Scientific, Warriewood, NSW,

185   Australia). The standard method 1 according to the manufacturer was used, where 25.0 mL water

186   was added into an aluminum canister and 2.0 g starch (dry weight) was added. The paddle

187   rotation was kept constant at 160 rpm throughout the analysis. The suspension was equilibrated

188   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

189   50 °C at a rate of 11.8 K/ min.

190

191   *2.2.8 Solution-casting of starch films*

192   Aqueous starch solutions (180 mg dry weight/ 6 mL water) were heated to 140 °C in a closed

193   tube and held for 45 min under permanent stirring in a Pierce Reacti-Therm heating/ stirring

194   module. The solutions were allowed to cool to around 70 °C and 3.5 mL of the solution were

195   transferred to a Petri dish (5.5 cm diameter). Bigger Petri dishes (8.5 cm diameter and 8.4 mL

196   solution) were used to produce films for barrier properties and tensile testing. The solvent was

197   evaporated at 23 °C overnight and allowed to dry for 48 h.

198

199   *2.2.9 Microstructural study of solution-cast films*

200   Scanning electron microscopy (SEM) was carried out using a Philips XL 30 ESEM operated at

201   10 kV, to observe the surface of the starch films. Samples were previously gold-coated using an

202   Emitech K550X sputter device (Quorum Technologies Ltd, Ashford, Kent, UK).

203   For TEM, fragments of approximately 2 mm^2 were cut out of the films and embedded in TAAB

204   812 resin (epoxy) without prior fixation and dehydration. Thin sections (about 75 nm) were cut

205   using a diamond knife on a Leica EM UC6 ultramicrotome and collected on gold grids. Sections

206   were stained according to the periodic acid-thiosemicarbazide-silver proteinate method described

207   elsewhere (Thiéry, 1967) prior to examination with a Philips CM/12 transmission electron

208   microscope using an accelerating voltage of 80 kV.

209

210   *2.2.10 Material properties of solution-cast films*

211   Oxygen transmission rate (OTR) was measured in a Mocon Ox-Tran 2/21 (SH) in duplicate

212   according to standard method ASTM F1927 – 07 and oxygen permeability (OP) was calculated

213   based on film thickness (measured at six random places within the film). Prior to OTR

214   measurements, samples were conditioned at 23 °C and 50% RH. Tensile testing of starch films

215   was performed according to ASTM D882-02. Before punching out dumbbell shaped specimens,

216   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.
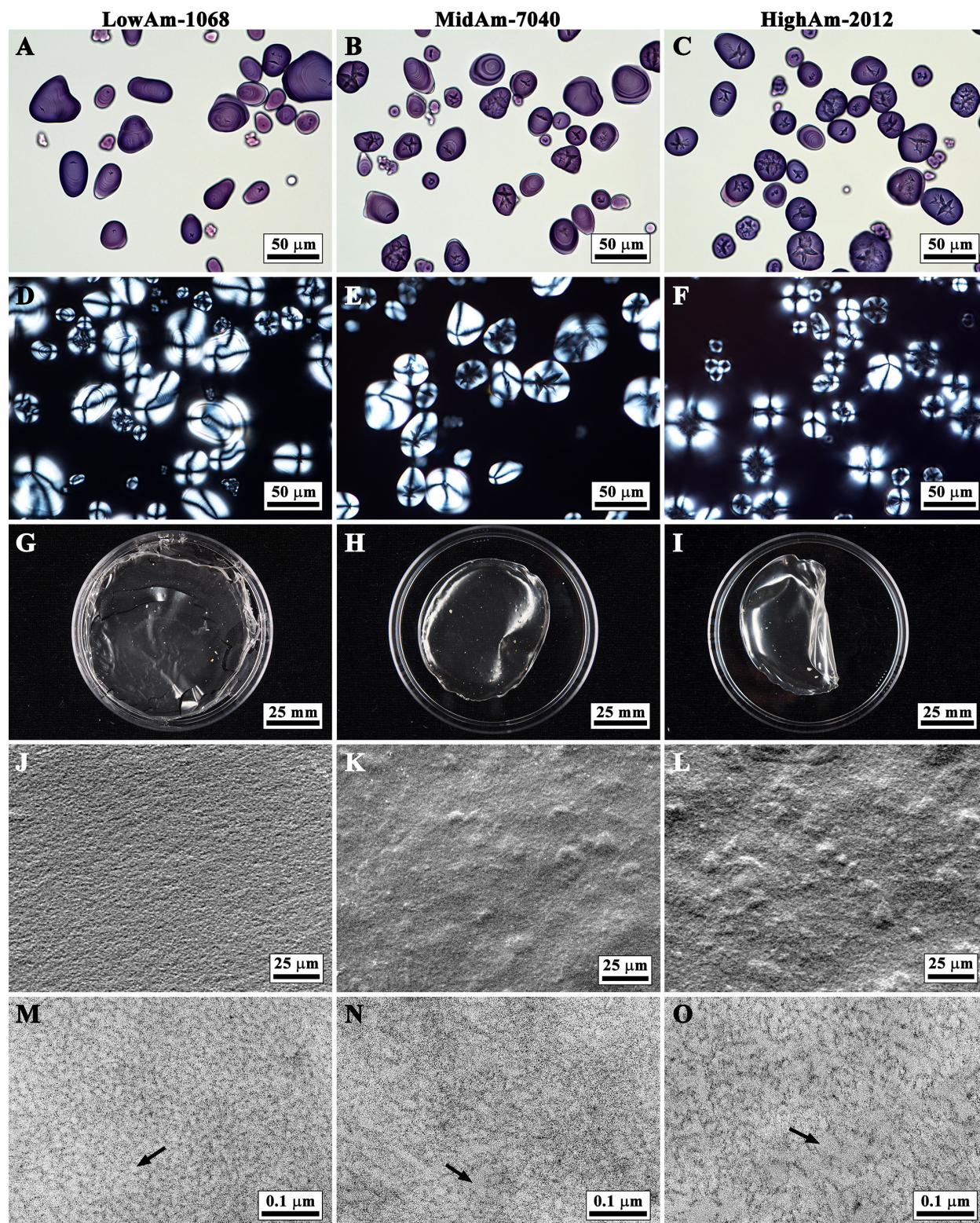
3.1 Light microscopy for morphology and birefringence of starch granules

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.

240 There was no loss of birefringence, indicating no major changes in the order and alignment of

241 starch molecules within the granule. This is in contrast to previous studies producing high-

242 amylose potato starches by inhibition of starch branching enzymes where starch granules of

243 high-amylose lines displayed less birefringence (Schwall et al., 2000).

244 Besides the irregular shape, high-amylose lines exhibited granules with overlapping of two or

245 more crosses. A theory has been proposed that the altered shape of the granules is a result of

246 failed amyloplast separation and thus the division of the amyloplast is retarded during

247 development because of the fusion of two granules by amylose interaction, forming helices

248 (Jiang, Horner, Pepper, Blanco, Campbell & Jane, 2010). Hence, rod and triangular shaped

249 granules are prevalent in potato starches with higher amylose content.

250

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

254   TEM images of microstructural network (M to O, arrows indicate pores) from high-amylose

255   lines LowAm-1068, MidAm-7040 and HighAm-2012.

256

257   **3.2 Amylose content by colorimetry and gel permeation chromatography**

258   Amylose content was determined based on iodine complex formation. The amylose content was

259   about 30% for the wild-type starches (Table 1), which was slightly higher than expected for

260   common potato starches using iodine (Morrison & Laignelet, 1983). This could be due to an

261   overestimation of amylose when using an extracted amylose standard for calibration which

262   probably has a lower chain length than in native starches. As expected, the three high-amylose

263   lines exhibited elevated amylose content, from 45-89%. The basis for colorimetric amylose

264   determination is the property of amylose to form a colorful complex with iodine depending on

265   the chain length of the polymer. No differentiation can be made on interference from color

266   formation by long-chain amylopectin molecules with iodine to a complex, and hence the

267   technique can give an overestimation of the amylose content (Morrison & Laignelet, 1983;

268   Vilaplana, Hasjim & Gilbert, 2012).

269

270   **Table 1.** Tuber yield, dry matter of fresh weight, starch purity and amylose content of wild-type

271   starches (Kuras, Verba, Dinamo) and their high-amylose lines (LowAm-1068, MidAm-7040,

272   HighAm-2012) determined colorimetrically using iodine binding and by gel permeation

273   chromatography (GPC) after debranching.

Sample	Tuber yield	Dry matter of fresh weight	Starch purity	Amylose content [%]	
	[%][a]	[%]	[%][b]	Colorimetric[c]	GPC[d]

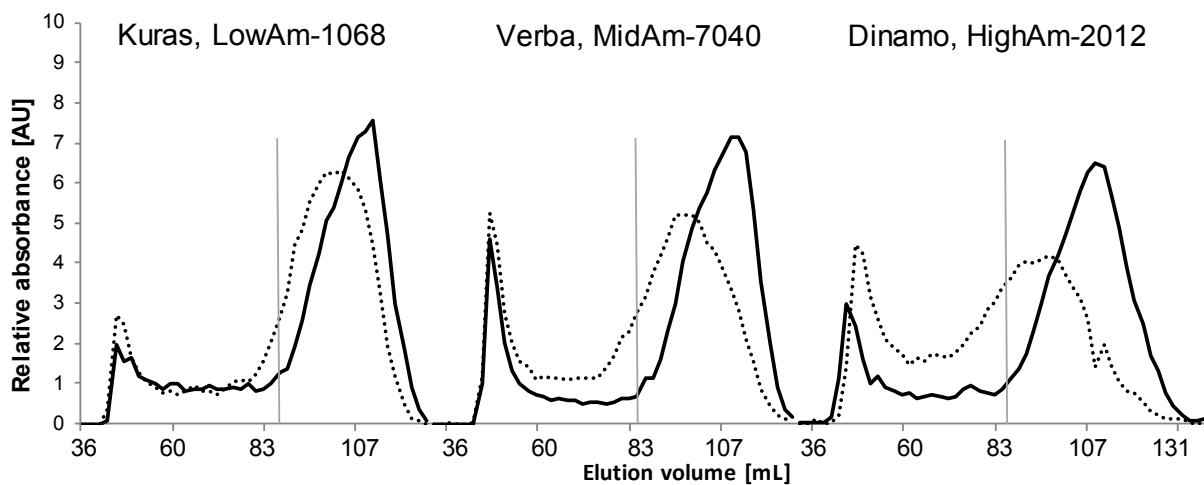|					
Kuras	100	31	86.3 ± 1.35	30 ± 0.3	22 ± 4.0
LowAm-1068	169	25	81.5 ± 0.88	45 ± 0.9	26 ± 0.8
Verba	100	31	87.9 ± 1.59	31 ± 1.9	23 ± 1.2
MidAm-7040	154	29	77.7 ± 2.23	70 ± 2.4	39 ± 0.8
Dinamo	100	31	85.3 ± 1.26	30 ± 0.8	23 ± 2.0
HighAm-2012	219	18	75.6 ± 2.76	89 ± 1.2	49 ± 5.8

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

277 Molar mass distribution of intact starches was carried out on Sepharose CL-2B columns

278 (supplementary material) and showed a typical pattern of potato starch with two peaks

279 corresponding to amylopectin with a typical wavelength at maximum absorbance of 560 nm and

280 an amylose peak with a typical wavelength at maximum absorbance of about 640 nm.

281 Separation using GPC of debranched molecules can give a better estimate of amylose content. A

282 granular potato amylopectin starch standard was used to indicate the elution volume where the

283 first chains of debranched amylopectin elute from the column (at elution volume 85 mL;

284 Figure 2). In order to compare the starches, the amylose content was determined as the relative

285 area under the curve from 40 to 85 mL. The corresponding amylose content of the three wild-

286 type starches was about 23%, while their high-amylose lines LowAm-1068, MidAm-7040 and

287 HighAm-2012 contained about 26%, 39% and 49% of amylose, respectively. Not only was the

288 amount of amylose (first peak in chromatogram; Figure 2) increased, but the composition of

289 chain lengths in amylose and amylopectin changed, resulting in a shift in the chromatogram

290 (Figure 2, dotted lines). The elution profile of the debranched starches showed that with

291 increasing amylose content, the separation between the two components was less obvious. This

292   indicates that amylopectin molecules of the high-amylose line starches exhibited a higher amount

293   of long chains and/or that amylose molecules were slightly branched. As discussed below, it was

294   shown that amylopectin molecules had longer chains and that amylose molecules were not

295   changed. This is in agreement with previous studies on genetically modified high-amylose potato

296   starches, reporting an increase in average chain length in amylopectin (Blennow, Hansen,

297   Schulz, Jørgensen, Donald & Sanderson, 2003; Hofvander, Andersson, Larsson & Larsson,

298   2004; Schwall et al., 2000).

299

300

301

302   **Figure 2**. Gel permeation chromatograms on Sepharose CL-6B of wild-type potato starches

303   Kuras, Verba, Dinamo (solid line) and their high-amylose lines LowAm-1068, MidAm-7040,

304   HighAm-2012 (dotted line) after debranching with isoamylase/ pullulanase. Gray vertical lines

305   represent division of amylose and amylopectin for amylose content calculation, based on a

306   standard of granular amylopectin potato starch.

307   Recently, there have been many efforts to define amylose content and validation of methods to

308   determine amylose content (Vilaplana, Hasjim & Gilbert, 2012; Vilaplana, Meng, Hasjim &

309 Gilbert, 2014). However, with high-amylose starches with altered structure, the definition

310 becomes more complicated and it is well known that assays based on iodine binding can

311 overestimate amylose content (Cheetham & Tao, 1997; Hofvander, Andersson, Larsson &

312 Larsson, 2004; Schwall et al., 2000; Shi, Capitani, Trzasko & Jeffcoat, 1998; Vilaplana, Hasjim

313 & Gilbert, 2012). In a recent study by Vilaplana et al. (2014), two-dimensional distributions

314 based on molecular size and branch chain length revealed the complex topologies of high-

315 amylose maize starch showing the presence of distinct intermediate compounds with the

316 molecular size of amylose, but branching structure similar to that of amylopectin, albeit having

317 longer chain length (Vilaplana, Meng, Hasjim & Gilbert, 2014).

318

319 **3.3 Separation of amylose and amylopectin using n-butanol-amylose complex formation**

320 To study the changed structure of the high-amylose starches in more detail, amylopectin and

321 amylose were isolated through chemical fractionation using n-butanol. The purity of each

322 fraction was determined using GPC of debranched samples. All isolated amylopectin fractions of

323 wild-type starches showed ≤ 3.5% amylose and were hence considered pure (Table 2 and Figure

324 3). The amylopectin isolated from the high-amylose lines exhibited up to 18% amylose

325 (calculated at the same separation volume for division of the two components at 85 mL shown in

326 Figure 2).

327 **Table 2.** Yield and purity of amylose (AM) and amylopectin (AP) after separation with n-

328 ButOH/ iso-amylalcohol (1st separat.) and second separation of the amylose fraction (AM

329 separat.)

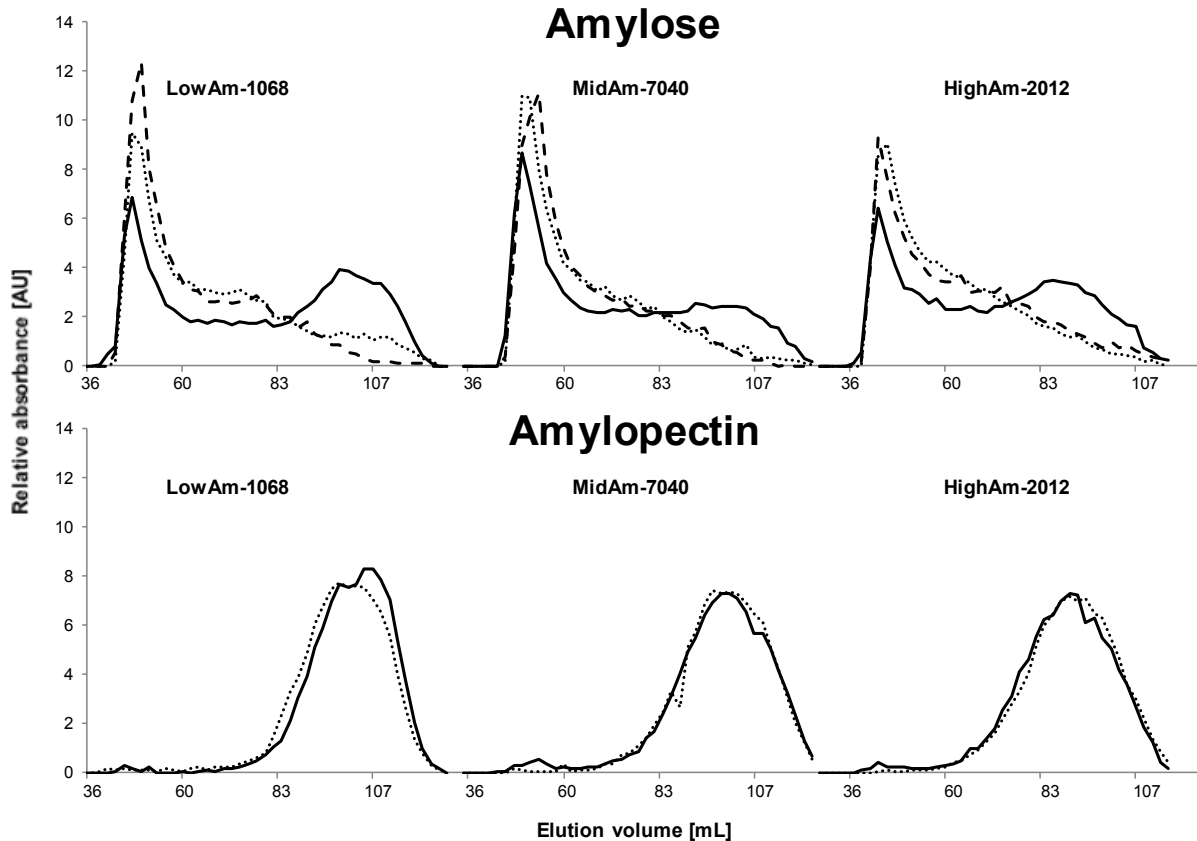| Sample	Yield after ButOH[a]	Purity[b]	
		1st separat.	AM separat.

	1st separat.	AM separat.	AP	AM	AP	AM
Kuras	91.9	54.1	97.9	62.9	93.3	91.4
LowAm-1068	87.5	31.3	95.6	52.2	93.7	78.2
Verba	96.3	62.4	96.5	65.3	97.5	92.8
MidAm-7040	97.5	55.0	88.2	66.7	89.3	86.5
Dinamo	79.5	51.3	98.7	62.7	96.2	75.3
HighAm-2012	102.0	26.7	81.6	59.1	85.0	84.7

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

333 However, the chromatograms showed that the amylopectin structure was changed compared to

334 the wild-type starches, displaying an increased amount of longer chains rather than an impurity

335 from amylose. Hence, the division between amylopectin and amylose was adjusted to be at

336 elution volume 65 mL resulting in $\geq 95\%$ purity of isolated amylopectin fractions (data not

337 shown). Isolated amylose fractions were less pure and contained a considerable amount of

338 amylopectin (Figure 3, solid line). Hence, those isolated amylose fractions were precipitated with

339 n-butanol once more. The second isolate of amylose (Figure 3, dotted line) showed no

340 amylopectin peak in the elution profile, but rather a long tailing amylose peak. Amylopectin

341 isolated from the amylose fraction showed the same elution profile as amylopectin isolated from

342 whole starch (Figure 3, dotted line) and hence was considered to reflect remnants from the

343 separation. In order to distinguish whether only linear amylose, low-branched amylose or low-

344 M_W amylopectin with long-chains was present in the amylose fractions, the samples were run

345 without debranching using GPC (Figure 3, dashed line). A change in the chromatogram would

346 have indicated branching. However, it was found that all isolated amylose fractions consisted of

347 linear chains. Similar results have been reported for high-amylose maize starches, i.e. low-M_W

348 fractions were not branched, as debranching with isoamylase did not show any differences in the

19

349   gel permeation chromatogram (Shi, Capitani, Trzasko & Jeffcoat, 1998). A small difference was

350   seen for the amylose fraction of LowAm-1068 (Figure 3, dashed line), where a minor peak

351   probably indicated some impurity from amylopectin molecules.

352

353

354   **Figure 3.** Gel permeation chromatogram on Sepharose CL-6B after debranching of high-

355   amylose potato lines LowAm-1068, MidAm-7040 and HighAm-2012: isolated amylose and

356   amylopectin fractions (solid line = first separation, dotted line = second separation, dashed line =

357   second separation without debranching).

358   The elution profiles of the isolated compounds demonstrated that there was little difference in the

359   amylose fractions of the high-amylose lines, as reported elsewhere (Banks, Greenwood & Muir,

360   1974). However, the amylopectin fraction showed a profile shift to longer chains. Hence, further
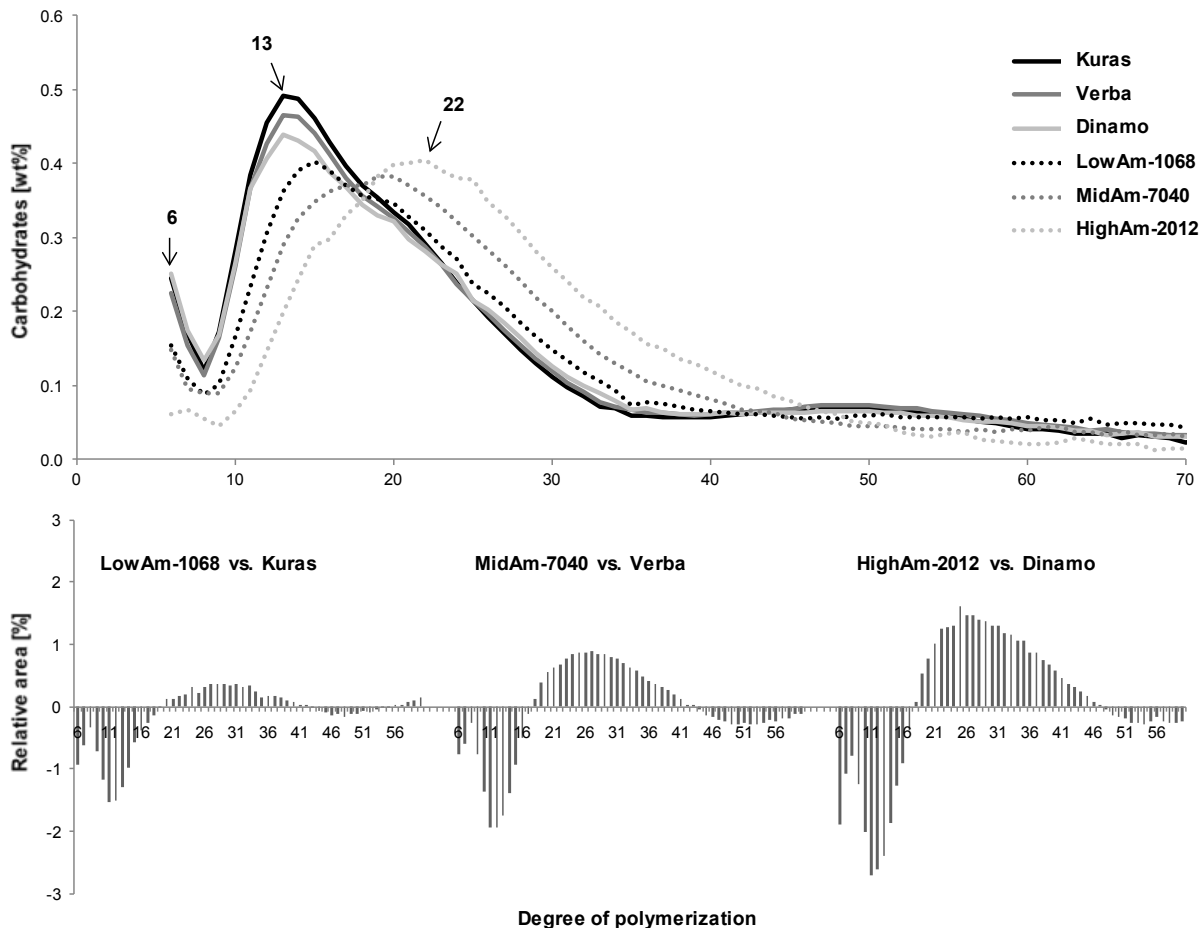
361 investigations on the chain length distribution were carried out and are discussed in more detail

362 below.

363

364 **3.4 Chain length distribution of amylopectin**

365 Since the distribution and size of linear amylose molecules was considered to be similar in all

366 three starches, no further investigations were carried out on these samples. Instead, amylopectin

367 was investigated in more detail. Chain length distribution of isolated amylopectin was

368 determined using HPAEC-PAD and the unit chain profiles were plotted (Figure 4).

369

370

371 **Figure 4.** Chain length distribution after debranching of amylopectin of wild-type starches

372 Kuras, Verba, Dinamo (solid line) and their high-amylose lines LowAm-1068, MidAm-7040,

373   HighAm-2012 (dotted line) using HPAEC-PAD and difference between the relative areas in the

374   chain length distribution of high-amylose line minus wild-type starch (lower part).

375   The amylopectin average chain length of all wild-type starches was about 35, with a peak at

376   degree of polymerization (DP) 13 (Figure 4). Similar profiles of regular potato amylopectin have

377   been reported by others (Jane et al., 1999; Koch, Andersson & Åman, 1998). In contrast, molar

378   proportions of chains with DP 18-37 increased in all high-amylose lines. The peak DP peak

379   shifted to 22 for HighAm-2012. The changes in the individual unit chains between the

380   amylopectin of wild-type starch and their corresponding high-amylose lines are visualized in the

381   lower part of Figure 4. In the high-amylose lines, chains with DP 6-8 were particularly

382   decreased. These chains have been shown to possess typical profiles depending on the crop and

383   are probably outermost chains in the amylopectin (Bertoft, 2004). The proportion of short chains

384   (DP 6-18) in amylopectin of HighAm-2012 was decreased by 50%, that of MidAm-7040 by at

385   least 32% and that of LowAm-1068 by about 13% (Figure 4). More thorough investigations of

386   the fine structure of amylopectin, i.e. β-limit and φ,β-limit dextrin, would give a better

387   understanding of which chain categories, outer and inner chains, are involved in the molecular
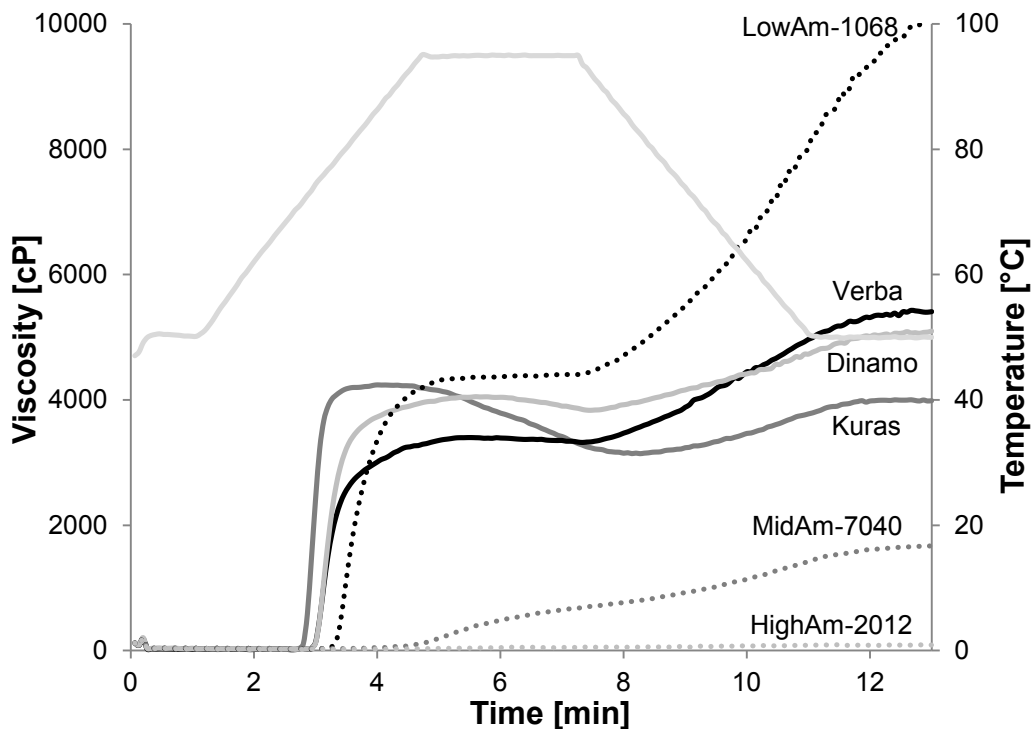
388   network formation (Bertoft, 2007).

389

390   **3.5 Pasting properties of starches**

391   Starch pasting properties provide information on the ability to form a paste after heating and

392   cooling. Native potato starch does not solubilize at temperatures lower than 50 °C, but when

393   heated in water starch granules start absorbing water and swell. Wild-type potato starches started

394   to swell at around 72-75 °C, seen as an increase in viscosity (Figure 5). As the temperature

395   increases, starch granules rupture and more soluble amylose can leak out resulting in a small

396 decrease of the viscosity (breakdown). During the holding time, the material is subjected to high

397 temperature and mechanical shear which further disrupted the granules. The holding strength is

398 the ability of a material to withstand shear stress and heat, which was high in all wild-type potato

399 starches. During the cooling phase, re-association of molecules, especially amylose, occurred in

400 all wild-type starches resulting in the formation of a gel structure increasing viscosity (setback).

401 The low setback region indicated a low rate of starch retrogradation. Even though there were

402 differences in pasting behaviour between wild-type starches, our main focus was laid on the

403 high-amylose starches. As seen from the RVA amylographs in Figure 4, there were large

404 differences between the three high-amylose starches which showed a completely different profile

405 compared to their wild-type counterparts.

406

407

23

408 **Figure 5.** Pasting profiles of wild-type starches Kuras, Verba, Dinamo (solid line) and their

409 high-amylose lines LowAm-1068, MidAm-7040, HighAm-2012 (dotted line) measured using

410 RVA. (Upper light grey line indicates temperature profile)

411 The high-amylose starch LowAm-1068 had a higher pasting temperature (79 °C) and final

412 viscosity and a substantially higher setback compared to wild-type starches. The higher pasting

413 temperature indicates a higher swelling capacity of granules. During the holding time, there was

414 only a small breakdown detectable in LowAm-1068 which indicates that the material could

415 withstand heat and shear forces and that not all granules were disrupted. Light microscopy

416 images were taken after the RVA run (data not shown) and revealed a mixture of swollen

417 granules and fragments of swollen granules within a continuous network. During cooling,

418 viscosity increased with a final viscosity that was twice as high compared with wild-type

419 starches, probably due to the slightly increased amylose content in LowAm-1068. Amylose re-

420 associates during cooling until a gel structure is formed, which increases viscosity.

421 The two starches with highest amylose content showed very low or no granule swelling (Light

422 microscopy images, data not shown) and viscosity (Figure 5) under the conditions used. It is

423 primarily the amylopectin that is responsible for granule swelling, while the high amylose

424 content alters the pasting properties and restricts swelling (Tester & Morrison, 1990). The

425 inhibited granule swelling of starches with >55% amylose (colorimetric determination with

426 iodine) has been reported previously (Karlsson, Leeman, Björck & Eliasson, 2007; Schwall et

427 al., 2000).

428

429 **3.6 Microstructural characterization of solution-cast films**

430   As starches from high-amylose lines needed higher gelatinization temperatures, the starch water

431   slurry was heated to 140 °C during vigorously stirring and then cast into Petri dishes. From the

432   images of solution-cast films (Figure 1 G-I), it can be seen that film-formation was different for

433   each high-amylose line. MidAm-7040 and HighAm-2012 gave cohesive but curled-up films;

434   LowAm-1068 gave partly fragmented films. All three solution-cast films were homogeneous and

435   no granule-shaped remnants or phase separation was seen (Light microscopy images, data not

436   shown). The difference in film-formation of the three starches of the high-amylose lines could be

437   due to the difference in amylose and amylopectin ratio as well as the difference in amylopectin

438   structure (described below). Amylose is thought to be the better film-former resulting in cohesive

439   films, whereas the higher amount of amylopectin in LowAm-1068 could result in the

440   fragmentation of the film. It has been shown that high temperature and longer heating are

441   necessary to completely disrupt starch granules of high-amylose starches (Bengtsson, Koch &

442   Gatenholm, 2003; Koch, Gillgren, Stading & Andersson, 2010). On the other hand, harsh

443   conditions could degrade amylose and amylopectin affecting the film cohesiveness negatively

444   (Koch, Gillgren, Stading & Andersson, 2010). However, we did not expect a severe degradation

445   of starch during heating to 140 °C as in the study of Koch et al. (2010) 150 °C were needed to

446   significantly degrade starch. There is little known about time and temperature conditions to

447   produce homogenous solution cast films (Koch, Gillgren, Stading & Andersson, 2010) and the

448   effect on molecular structure, microstructure and material properties. Hence, further

449   investigations are required. A general problem is that most studies are not explicit on the

450   conditions to solubilize starch to produce solution cast films; i.e. in this study it took 45 min to

451   heat up the sample tube to the holding temperature of 140 °C.

452

453 SEM images have shown a more uneven surface texture for films of starches with higher

454 amylose content (Figure 1 J-L). Similar findings have previously been attributed to remnants of

455 granules after gelatinization and/or fast aggregation of amylose during the cooling process,

456 resulting in a rougher surface (Bengtsson, Koch & Gatenholm, 2003; Koch, Gillgren, Stading &

457 Andersson, 2010). However, in our study, light microscopy images (iodine staining and

458 differential interference contrast) did not reveal any granule-shaped remnants.

459 The microstructure of solution-cast films was studied using TEM. TEM micrographs (Figure 1

460 M-O) showed mainly darkly stained starch molecules and brighter pore areas (indicated by

461 arrows). Since individual amylopectin molecules are different from amylose molecules in size

462 and structure and vary between the high-amylose lines, the TEM-images were expected to show

463 morphological differences. At first sight, all films looked similar showing a rather homogenous

464 and dense structure. However, there were distinct differences between films regarding pore size

465 and shape. TEM micrographs of LowAm-1068 films (Figure 1 M) revealed small pores

466 (indicated by arrows) of about 10 nm (through a few manual measurements) which were mainly

467 round-shaped and representing an amylopectin-like branched network structure (Leloup,

468 Colonna, Ring, Roberts & Wells, 1992). TEM micrographs of MidAm-7040 films showed a

469 more heterogeneous pore distribution with less regular shaped pores up to 30-40 nm in size.

470 HighAm-2012 films exhibited a more open but also fractal-structure with pore sizes up to 60 nm

471 which could be argued to be more amylose-like and revealing some rod-like strands. TEM

472 micrographs of the films LowAm-1068 and MidAm-7040 did not evidently reveal the dense

473 amylose-like structure as reported previously for pure amylose gels and films (Leloup, Colonna,

474 Ring, Roberts & Wells, 1992; Richardson, Kidman, Langton & Hermansson, 2004; Rindlav-

475 Westling, Stading, Hermansson & Gatenholm, 1998). The morphology of the HighAm-2012

476   film, however, showed some indication of a more open structure with stiff rod-like strands and

477   was more alike pure amylose gels and films. As discussed above, the genetic modification of the

478   potato resulted in a change in amylopectin structure besides an increase in amylose content

479   (Figure 2). We therefore suggest that not only amylose but also long chains of amylopectin or

480   intermediate components can contribute to the change in microstructure, e.g. physical

481   entanglements between longer branches and amylose chains, observed using TEM. However,

482   further investigations are needed to better understand the created starch network structure seen in

483   TEM images.

484

485   **3.7 Barrier properties and tensile strength of solution-cast films**

486   Oxygen permeability (OP) was studied in solution-cast films of all three high-amylose lines and

487   compared with the wild-type Dinamo (Table 3). It was found that all three high-amylose lines

488   exhibited lower OP ($P<0.01$) than native potato starch films. In addition, OP was much lower

489   than in materials made from most synthetic polymers. In a previous study on amylose films,

490   better barrier properties were attributed to higher crystallinity compared with amylopectin films

491   (Rindlav-Westling, Stading, Hermansson & Gatenholm, 1998). This would decrease the

492   solubility of oxygen, resulting in a lower oxygen transmission rate.

493   Starch films of the high-amylose lines exhibited higher stress at break compared with the wild-

494   type starch film Dinamo (Table 3; $P<0.05$). The stiffness and higher strength of these films

495   compared with wild-type starch could be attributable to the longer chains in amylopectin being

496   involved in the interconnected network and increasing the interaction between chains.

497   Furthermore, strain at break increased in all high-amylose lines (Table 3; $P<0.05$). It has been

498   shown previously for starch films of different amylose and amylopectin mixtures that higher

499   amylose content increases elongation (Lourdin, Valle & Colonna, 1995). As compared to

500   commonly used oxygen barriers, such as EVOH (poly ethylene-co-vinylalcohol), the stress at

501   break is considerable higher but the strain at break is lower when measured at the same

502   conditions (23 °C and 50% RH). The high standard deviations are due to the strong polar bonds

503   and a high surface energy. This facilitates fracture propagation from, e.g. a small flaw in the

504   surface, as easily introduced when cutting the samples for the tensile test. The standard deviation

505   decreases when testing hundreds of samples, but the interesting effect of such materials is that

506   they may facilitate easy-to-open packages. However, the films or coatings in a real package

507   would be supported by a water barrier and sealant layer of, e.g. renewable polyethylene that also

508   would give the main contribution to the mechanical properties of the package.

509

510   **Table 3.** Oxygen permeability (OP), stress at break and strain at break of parental starch Dinamo

511   and high-amylose lines, high-amylose maize starch, low-density polyethylene (LDPE) and poly

512   ethylene-co-vinylalcohol (EVOH)

Sample	OP[a]	Stress at break[b]	Strain at break[b]
	[cc mm/ m^2 24h atm]	[MPa]	[%]
Dinamo	0.170 ± 0.01	34.1 ± 9.5	1.5 ± 0.3
LowAm-1068	0.089 ± 0.04	42.2 ± 3.7	2.8 ± 0.4
MidAm-7040	0.100 ± 0.03	45.0 ± 12.0	2.3 ± 0.8
HighAm-2012	0.085 ± 0.03	46.0 ± 12.4	3.4 ± 2.2
High-amylose maize starch[c]		40	1.9
LDPE[d]	1900	7-16	100-800
EVOH[e]	0.01-12	20-210	20-330

28

513   [a] mean ± standard error (n=2), [b] mean ± standard deviation (n=6), moisture content was between
514   10.2 to 10.4 % in the starch films, [c] from Koch, Gillgren, Stading and Andersson (2010), [d] from
515   Doak (1986), [e] from Lange and Wyser (2003)

516

517   **4. Conclusions**

518   Targeted gene suppression of *SBE1* and *SBE2* through RNA interference in three different potato

519   cultivars resulted in high-amylose starches, which were characterized in detail and used to relate

520   film performance to molecular structure. These high-amylose lines revealed starches with

521   changed granular and molecular structures, pasting properties and film performance. The

522   amylose content was increased to 45, 70 and 89% using iodine binding-based measurements.

523   However, GPC revealed a more reliable amylose content of 26, 39 and 49% as the chain length

524   of amylopectin was increased in addition to amylose content affecting the measurement when

525   using the colorimetric method. The genetic modification produced starches with increasing

526   amount of irregular shaped granules yielding basically no pasting at 95 °C. At high temperature,

527   140 °C, all three starches were gelatinized. Highest amylose content and amylopectin with the

528   longest chains resulted in cohesive films with a rough surface and improved physical properties.

529   The improved oxygen barrier provided by the starches from high-amylose potato and their

530   superior mechanical properties in terms of stronger films and increased strain at break indicate

531   that they have the potential for interesting commercial applications such as in films or coatings.

532   They are thought to have a particularly interesting future as barrier coatings, as the presently

533   used industrial facilities (e.g. blade and curtain coaters) possibly could be used when applying

534   them on boards or polymer films**.** However, they may have the same shortcomings as poor water

535   barriers and must, just like the currently used oxygen barrier polymers, be encapsulated between

536   two hydrophobic layers, that could for example be renewable polyethylene and tie layers.

537

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