This is the final draft post-refereeing.

The publisher's version can be found at <http://dx.doi.org/10.1016/j.foodhyd.2016.06.026>

Please cite this article as: Dries, D.M., Gomand, S.V., Delcour, J.A. and Goderis, B. (2016). V-type crystal formation in starch by aqueous ethanol treatment: the effect of amylose degree of polymerization. Food Hydrocolloids 61, 649-661.

V-TYPE CRYSTAL FORMATION IN STARCH BY AQUEOUS ETHANOL TREATMENT: THE EFFECT OF AMYLOSE DEGREE OF POLYMERIZATION

D.M. Dries^{1,3,*}, S.V. Gomand^{1,3}, J.A. Delcour^{1,3} and B. Goderis^{2,3}

¹Laboratory of Food Chemistry and Biochemistry, KU Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium. E-mail: Sara_Gomand@hotmail.com; Jan.Delcour@biw.kuleuven.be

²Polymer Chemistry and Materials, KU Leuven, Celestijnenlaan 200F, B-3001 Leuven, Belgium. E-mail: Bart.Goderis@chem.kuleuven.be

³Leuven Food Science and Nutrition Research Centre (LForCe), KU Leuven, B-3001 Leuven, Belgium

*Corresponding author. Phone: (+32)-16-376776. Fax: (+32)-16-321997.

E-mail: Dorian.Dries@biw.kuleuven.be

KEYWORDS: starch, amylose, V-type crystallinity, size exclusion, X-ray diffraction, differential scanning calorimetry

CHEMICAL COMPOUNDS STUDIED IN THIS ARTICLE: amylopectin (PubChem CID: 439207); amylose (PubChem CID: 53477771); ethanol (PubChem CID: 702); maize starch (PubChem CID: 24836924); water (PubChem CID: 962).

ABBREVIATIONS: AP, amylopectin; AM, amylose; CHL, carbohydrate leaching; T_C , conclusion temperature of gelatinization; DSC, differential scanning calorimetry; 2θ , diffraction angle; dm, dry matter; ΔH , enthalpy of gelatinization; ETS_{x%}, starch treated with x% (v/v) ethanol; GCWSS, granular cold-water swelling starch; MW, molecular weight; DP_n , number average degree of polymerization; T_O , onset temperature of gelatinization; DP_{peak} , peak degree of polymerization; T_P , peak temperature of gelatinization; PI, polydispersity index; RI, refractive index; λ , wavelength; DP_w , weight average degree of polymerization; WAXD, wide angle X-ray diffraction

ABSTRACT

Maize, rice, cassava, potato and pea granular starches, representing the different starch polymorphs (A-, B- and C-types) and covering a broad range in amylose (AM) content and degree of polymerization (DP), were converted to V_H-type granular starch by aqueous ethanol treatment at 95 °C in a 68 to 48% ethanol (v/v) range. Microscopy, X-ray diffraction and calorimetric analyses showed that loss of native molecular order already occurred at the highest ethanol concentrations for starches containing the intrinsically less stable B-type crystals, whereas lower ethanol concentrations were necessary to induce native crystal melting in A-type starches. C-type starch, containing a mixture of A- and B-type crystals, exhibited features characteristic of both A- and B-type starches. No native crystals remained and granular products containing only V_H-type crystals were formed for all starches when using 48% (v/v) ethanol. However, the relative kinetics of V_H-type crystal formation depended on AM DP. For low DP AM starches (maize and rice), V_H-type crystal formation started during heating up to 95 °C once the native crystals were melting. The V_H-type crystallization went up to completion during the isothermal stay at 95 °C. For mid (pea) and high DP AM (potato and cassava) starches, V_H-type crystallization was initiated during holding at 95 °C and progressed further during subsequent cooling. The V_H-type crystallinity in the final product decreased with increasing DP of the AM fraction, likely as a result of progressively more disturbing entanglements. Surprisingly, there was no quantitative relation between V_H-type crystallinity and AM content.

1. INTRODUCTION

Starch serves as the principal carbohydrate reservoir in many higher plants. It is accumulated in a water insoluble granular form. From a molecular perspective, it consists primarily of two α -D-glucose polymers, amylose (AM) and amylopectin (AP) (Buléon, Colonna, Planchot, & Ball, 1998; Tester, Karkalas, & Qi, 2004). AM is essentially linear and has a molecular weight (MW) of 1×10^5 - 1×10^6 and a degree of polymerization (DP) on a number average basis (DP_n) ranging from 324 to 4,920 depending on the botanical species (Tester *et al.*, 2004). AP is highly branched and has a cluster-like organization (Peat, Whelan, & Thomas, 1956). It is much larger than AM and has a MW of 1×10^7 - 1×10^9 and DP_n varying between 9,600 and 15,900 depending on the botanical source (Tester *et al.*, 2004). The AM contents of normal (non-waxy) starches range from 14 to 29% (Waterschoot, Gomand, Fierens, & Delcour, 2015).

The outer chains of AP can form double helices which then can pack in regular arrays and create regions with crystalline register. The double helices are either organized in a monoclinic unit cell containing 4 water molecules or in a hexagonal unit cell containing a central channel with 36 water molecules which give rise to A- and B-type wide-angle X-ray diffraction (WAXD) patterns, respectively (Imberty, Chanzy, Pérez, Buleon, & Tran, 1988; Imberty & Pérez, 1988). The C-type WAXD pattern results from a superposition of the two aforementioned polymorphs. Cereal starches typically exhibit the A-type WAXD pattern, while most starches from roots and tubers display the B-type WAXD pattern (Zobel, 1988). An exception is cassava starch, for which literature data mention pure A- or C-type crystalline structures (Hoover, 2001). Legume starches have C-type crystallinity (Cairns, Bogracheva, Ring, Hadley & Morris, 1997).

In native starch granules, AM occurs mainly in an amorphous state intertwined with or located adjacent to AP (Jane, Xu, Radosavljevic, & Seib, 1992). However, potato starch AM is believed to take part in B-type crystal formation (Saibene & Seetharaman, 2010). An increasing AM

content has been associated with an overall decrease in degree of crystallinity in maize starch (Cheetham & Tao, 1998; Jenkins & Donald, 1995). In the presence of a suitable ligand, AM can adopt a single helical conformation, a structure generally known as V-type AM. When V-type AM helices stack parallel into crystals, they give rise to a V-type WAXD pattern (Zabar, Lesmes, Katz, Shimoni, & Bianco-Peled, 2009).

The use of starch as a functional viscosifying ingredient in food systems relies on its property to imbibe large amounts of water when heated above a characteristic temperature, *i.e.* the gelatinization temperature. At this temperature, the starch native crystals melt. Technologies have been developed to produce granular starches of enhanced cold-water swelling capacity, enabling viscosity build up at room temperature (Chen & Jane, 1994a, 1994b; Dries, Gomand, Goderis, & Delcour, 2014; Eastman, 1987; Eastman & Moore, 1984; Jane & Seib, 1991; Rajagopalan & Seib, 1992a, 1992b; Zhang, Dhital, Haque, & Gidley, 2012). Previous work (Dries *et al.*, 2014) reported on the structural and thermal transitions during the conversion of native maize starch into granular cold-water swelling starch (GCWSS) by aqueous ethanol treatment at elevated temperature. At balanced treatment temperatures and alcohol concentrations, granular starches with a V-type WAXD pattern are formed. Single helix formation is induced by the linear alcohol during starch plasticization, and the resultant product displays diffuse birefringence under polarized light. Crystals from V-type helices harvested after drying melt instantaneously when brought in contact with pure water, indicating that the helices are either empty (Rajagopalan *et al.*, 1992b) or that the ethanol remaining in the helix cavities is freely exchangeable with water (Brisson, Chanzy, & Winter, 1991). It was recently reported that the linear AM fraction is the primary responsible for V-type crystal formation and that the contribution of AP is negligible (Dries *et al.*, 2014).

GCWSS from different botanical origins exhibit specific granular morphologies and crystalline structures (Rajagopalan *et al.*, 1992b; Singh & Singh, 2003; Zhang *et al.*, 2012). This has been attributed to differences in molecular structure and supramolecular organization of native starch

(Zhang *et al.*, 2012). However, to the best of our knowledge, no research reports ever went beyond this qualitative statement. As AM DP strongly impacts on V-type crystal formation (Gelders, Duyck, Goesaert, & Delcour, 2005; Godet, Bizot, & Buleon, 1995; Le Bail, Bizot, Pontoire, & Buleon, 1995), it can reasonably be expected that it contributes to how a given starch responds to an aqueous ethanol treatment at elevated temperature.

In the present work, maize, rice, cassava, potato and pea starches were converted into V-type granular products by aqueous ethanol treatments at a predefined temperature (95 °C). This starch selection reflects the different polymorphs (A-, B- and C-types) known to starch and covers a broad range in AM content and DP. The impact of the latter on V-type crystal formation was studied. The influence of AM DP on V-type AM-lipid or AM-alcohol inclusion complex formation has been investigated earlier. Data were obtained for commercially available or extracted/synthesized AM samples with DPs ranging from 20 to 950 (Gelders, Vanderstukken, Goesaert, & Delcour, 2004; Godet *et al.*, 1995; Le Bail *et al.*, 1995). The present paper is believed to be the first to study V-type crystal formation with granular starches of varying AM contents and DPs and, in contrast to earlier work, also includes investigations on AM with a DP exceeding 950.

As V-type AM structures (V_H subtype) are obtained with linear alcohols (*e.g.* ethanol) and saturated fatty acids (Obiro, Ray, & Emmambux, 2012), the present work is also relevant in the framework of V-type AM-lipid inclusion complex formation.

2. EXPERIMENTAL SECTION

2.1 Materials

Maize, cassava and potato starches were from Cargill (Vilvoorde, Belgium) and rice and pea starches from Remy (Wijgmaal, Belgium) and Cosucra (Warcoing, Belgium), respectively. All reagents, solvents and chemicals were from Sigma-Aldrich (Bornem, Belgium) and of at least analytical grade. The ethanol used in this work is denaturated with up to 5% diethyl ether. The percentages of alcohol described further are those of commercial denaturated alcohol. Isoamylase (*Pseudomonas sp.*) was purchased from Sigma-Aldrich (catalog no I5284) and its units were as defined by the supplier.

2.2 Preparation of granular cold-water swelling starch

Native starches were gradually converted to GCWSS by aqueous ethanol treatment at 95 °C as described previously (Dries *et al.*, 2014). Samples [20.0 g dry matter (dm)] were dispersed in 180 g aqueous ethanol in pressure resistant Schott bottles with leak proof screw caps. The ethanol concentration was varied from 48 to 68% (v/v). The dispersions were heated for 30 min at 95 °C under continuous shaking. After cooling for 60 min at room temperature, they were Büchner filtered under vacuum. The filtrates were analyzed for soluble carbohydrates (*cf.* section 2.4). The starch pellets were washed with ethanol, air-dried and sieved as reported earlier (Dries *et al.*, 2014). Sample codes have the format ETS_{x%}, where ETS stands for ethanol treated starch and x% stands for the volume percentage of ethanol in the used aqueous ethanol. The subscript also indicates the botanical origin. Moisture contents of native starches and ETS were determined according to AACC approved method 44-15.02 (AACC, 1999).

2.3 Analysis of amylose contents

An AM content commercial assay kit (Megazyme, Wicklow, Ireland) based on Gibson and co-workers (1997) was used. In the method, starch is gelatinized by heating in 90% (v/v) dimethyl sulfoxide (DMSO) and subsequently defatted by adding 95% (v/v) ethanol to the gelatinized

dispersion. The defatted starch is separated from the supernatant by centrifugation (2,000 g, 5 min) and dispersed again in 90% (v/v) DMSO. AP is removed by precipitation with concanavalin A. The AM in the supernatant is hydrolyzed to glucose using a mixture of α -amylase and amyloglucosidase. The total starch in a separate aliquot before AP precipitation is similarly hydrolyzed to glucose. The proportion of AM (% dm) can be calculated as the ratio of glucose remaining in the supernatant to that of glucose derived from the hydrolysis of the total starch.

2.4 Carbohydrate leaching

The level of carbohydrate leaching (CHL) as a result of the aqueous ethanol treatments was determined in centrifuged (1,000 g, 10 min) filtrate (*cf.* section 2.2) by the method of Dubois *et al.* (1956) using glucose as standard and expressing the leached carbohydrate as starch ($0.9 \times$ glucose).

2.5 High performance size exclusion chromatography

2.5.1 Defatting, dispersing and debranching of starches

The DP of individual AM chains can be analyzed by prior enzymatic debranching (Wang, Hasjim, Wu, Henry, & Gilbert, 2014). Debranching allows separating the long AM chains from the short debranched AP chains. First, because maize and rice starches contain endogenous lipids, they were defatted prior to dispersion, based on the method of Umemoto *et al.* (1999), with slight modifications. The defatting procedure was also executed for the other starches to avoid discrepancies in final results due to differences in sample pretreatments. Native starch (10.0 mg) was accurately weighed in a test tube with screw cap, mixed with 6.0 ml ethanol and heated in a water bath at 100 °C for 10 min. Starch was separated by centrifugation (1,000 g, 10 min), the supernatant was discarded, the starch pellet washed with fresh ethanol and again separated by centrifugation. This defatting procedure was repeated two times. The obtained starches were enzymatically debranched based on the method of Umemoto *et al.* (1999) with

slight modifications. The defatted starch pellet was suspended in 5.0 ml deionized water and gelatinized by boiling for 60 min. To 1.0 ml of gelatinized starch suspension, 50 μ l 600 mM sodium acetate buffer (pH 4.0) was added and samples were placed in a water bath. When they reached a temperature of 40 °C, starches were hydrolyzed with isoamylase from *Pseudomonas* sp. [1250 U in 50 μ l 50 mM sodium acetate buffer (pH 4.0)] at 40 °C for 16 to 24 h. Next, the enzyme was inactivated by heating (100 °C, 10 min). Incubation with the same isoamylase was executed a second time at 40 °C for 16 to 24 h to ensure complete debranching. After enzyme inactivation (100 °C, 10 min), an aliquot (50 μ l) was transferred to an HPLC vial and immediately analyzed as described below.

2.5.2 Chromatographic separation and data analysis

High performance size exclusion chromatography (HPSEC) was used to estimate the (peak) DP value of the debranched AM fractions of the different starches with three columns in series (TSK gel G6000 PWXL, G4000 PWXL and G3000 PWXL) (Tosoh, Stuttgart, Germany). The system consisted of an online degasser (DGU-20A3), a column oven at 60 °C (CTO-20AC), a pump (LC-10AT) and an injector (SIL AC HT Autosampler), all from Shimadzu (Kyoto, Japan). Detection was with a differential refractive index (RI) detector (RID-10A, Shimadzu) at 40 °C. Elution was with 7.5 mM sodium acetate buffer (pH 4.0) at a flow rate of 0.5 ml/min. The system was calibrated with Shodex pullulan standards (Showa Denko, Tokyo, Japan). Their peak MWs and corresponding approximate DPs were 0.59×10^4 (DP 40), 1.18×10^4 (DP 70), 2.28×10^4 (DP 140), 4.73×10^4 (DP 290), 11.2×10^4 (DP 690), 21.2×10^4 (DP 1310), 40.4×10^4 (DP 2490), 78.8×10^4 (DP 4860) and 160×10^4 (DP 9880). A third order polynomial relation was fitted for the logarithm of the MWs of the standards and the elution volumes. The HPSEC-profile of debranched waxy maize starch allowed determining the elution volume from which onwards AP branches start to elute as being 27.0 ml. DP_{peak} is the DP corresponding to the highest intensity of the AM peak [$DP = (MW-18)/162$]. Finally, the polydispersity index

(PI) of the AM fraction of the starches is the ratio between the DP on a weight average basis (DP_w) and the DP_n (Gelders *et al.*, 2004; Wang *et al.*, 2014), with:

$$DP_w = \frac{\sum_{i=1}^{\infty} DP_i m_i}{\sum_{i=1}^{\infty} m_i}$$

$$DP_n = \frac{\sum_{i=1}^{\infty} DP_i (m_i/M_i)}{\sum_{i=1}^{\infty} (m_i/M_i)}$$

with m_i the mass concentration and M_i the MW of AM chains with a $DP = i$.

2.6 Light microscopy

A Nikon (Melville, NY, USA) Eclipse 80i epifluorescence microscope equipped with a charge-coupled device camera was used to study starch granule morphology, using both bright field and polarized light mode. Images were made for native starches and ETS suspended in ethanol and analyzed with NIS-Elements BR software (Nikon).

2.7 Wide angle X-ray diffraction

Wide angle X-ray diffraction (WAXD) sample preparation included overnight equilibration in a humidifier over saturated salt (NaCl) solution ($a_w = 0.75$) in (open) aluminum DSC pans (Perkin-Elmer, Waltham, MA, USA). Equilibrium moisture contents varied slightly amongst different botanical origins and were 16% for cassava and pea starches, 17% for rice starch and 19% for potato starch. DSC pans were sealed hermetically. WAXD patterns were collected using a XeuSS X-ray camera (Xenocs, Sassenage, France), comprising a GeniX 3D Molybdenum ultra-low divergence X-ray beam ($\lambda=0.71 \text{ \AA}$). The experimental setup and operating conditions were as described previously (Dries *et al.*, 2014). Silver behenate and polyethylene were used to calibrate diffraction angles. WAXD photographs were azimuthally averaged using ConeX software (Gommes & Goderis, 2010). Intensity patterns were corrected for empty holder scattering, taking into account the sample and holder transmission. WAXD patterns are represented here as a function of 2θ Cu K_α radiation ($\lambda = 1.54 \text{ \AA}$), with θ being half

the scattering angle, to allow comparison with literature data. An amorphous sample was prepared by gelatinizing native maize starch in excess water and freeze drying it. A scaling factor was applied to the amorphous pattern before subtraction from each starch scattering pattern. It therefore accounts for the amorphous contribution of X-ray scattering and is deduced for each sample separately (Cairns, Bogracheva, Ring, Hadley, & Morris, 1997). Then, the degree of crystallinity could be calculated as described earlier (Dries *et al.*, 2014):

$$\text{Degree of crystallinity (\%)} = \frac{Is - (Ia \times fs)}{Is} \times 100$$

with Is: integrated normalized intensity of the sample over the angular range 4.5 to 27 °2θ

Ia: integrated normalized intensity of an amorphous sample over the angular range 4.5 to 27 °2θ

fs: scaling factor corresponding to each particular sample

Furthermore, the proportions of A-, B- and V_H-type crystallinity were calculated by fitting a superposition of model patterns to the experimental data using a least-squares error minimization procedure as previously done for maize starch (Dries *et al.*, 2014). The scattering patterns of pure A- (waxy maize), pure B- (potato) and pure V_H-type starches (GCWSS) were used to model the scattering patterns of ETS. Modelling the scattering patterns of ETS_{potato} and ETS_{pea}, using the scattering pattern of native potato starch as B-type reference pattern led to unsatisfactory results because of B-type reflection broadening in the WAXD patterns of these samples. To overcome this problem, an additional ETS_{potato} was prepared as in section 2.2 but with a higher ethanol concentration [78% (v/v)]. This starch displayed a pure B-type diffraction pattern but also reflection broadening as in ETS_{potato} and ETS_{pea}. Therefore, its scattering pattern was suitable as B-type reference pattern in the model fitting.

2.8 Differential scanning calorimetry

2.8.1 Gelatinization in excess water

Differential scanning calorimetry (DSC) measurements were executed with a Q2000 DSC (TA Instruments, New Castle, DE, USA). Native starches and corresponding ETSs (2.50-4.00 mg) were accurately weighed in an aluminum pan (Perkin-Elmer, Waltham, MA, USA) and mixed with deionized water [1/3 (w/w) starch dm/water]. The pans were sealed hermetically and heated from 20 to 120 °C at 4 °C/min (together with an empty reference pan). The DSC system was calibrated with indium. DSC thermograms were analyzed for temperatures and enthalpies associated with starch gelatinization, using TA Universal Analysis software. The gelatinization onset (T_O), peak (T_P) and conclusion (T_C) temperatures, the gelatinization temperature range (ΔT) and gelatinization enthalpy (ΔH) were determined at least in triplicate.

2.8.2 Gelatinization, V_H -type crystal formation and melting in aqueous ethanol

Native starch (about 2.25 mg) was accurately weighed in a high pressure steel pan (Mettler-Toledo, Zaventem, Belgium), 48% (v/v) ethanol was added [1/9 (w/w) starch dm/solvent] and pans were hermetically sealed. In a first temperature protocol, samples were heated from 20 to 160 °C at 4 °C/min (first heating), cooled to 20 °C at the same rate, and reheated at the same rate to 160 °C (second heating). T_P and ΔH were determined during the first heating run. Peak temperatures and enthalpies related to the crystallization and melting of V_H -type crystals during the first heating run, the cooling segment and the second heating run are not tabulated and left to visual comparison because the complexity of these transitions (see result and discussion section) did not permit reliable integration. In a second temperature protocol, samples were heated from 20 to 95 °C at 4 °C/min (first heating), held at this temperature for 30 min (isothermal step), cooled to 20 °C at 4 °C/min, and reheated at the same rate to 160 °C (second heating). The relevance of the second temperature protocol will follow from the results and discussion section

(*cf.* section 3.5). Instrument calibration was as outlined above. Analyses were performed at least in duplicate.

2.9 Statistical analysis

Statistical analyses were performed with the software JMP Pro 11 (SAS Institute, Cary, NC, USA). It was verified whether mean values were significantly ($P < 0.05$) different using the one-way ANOVA procedure. Corresponding Tukey grouping coefficients are given.

3. RESULTS AND DISCUSSION

3.1 Amylose contents and degrees of polymerization

3.1.1 Amylose contents

Table 1 lists the AM contents of the different starches. The results are to a certain degree consistent with literature data: legume starches contain considerably higher levels of AM than (normal) starches from other botanical origins, while tuber starches contain the lowest (Wang *et al.*, 2014).

Table 1: Amylose (AM) contents of native starches, determined using the AM content Megazyme assay kit and the peak degrees of polymerization (DP_{peak}) and polydispersity indices (PI) of the AM fraction, determined using high performance size exclusion chromatography (HPSEC).

	Amylose content (% dm)	DP _{peak}	PI
Maize	23.7 (0.4)* ^a	1,710 (40) ^a	1.7 (0.0) ^a
Rice	18.1 (0.3) ^b	1,520 (70) ^a	1.7 (0.0) ^a
Cassava	17.3 (0.2) ^b	4,240 (180) ^b	2.4 (0.2) ^b
Potato	14.8 (0.3) ^c	3,520 (210) ^c	2.9 (0.0) ^c
Pea	26.8 (0.5) ^d	2,150 (60) ^d	2.1 (0.0) ^b

Results in the same column indicated with the same letter are not significantly different ($P < 0.05$).

*This value was previously published in Dries *et al.* (2014).

Pea starch had an AM content of 26.8% while Huang and co-workers (2007) noted an AM content of 31.2%. Rice starch contained 18.1% AM, which falls well within the range of AM contents for normal rice starches (10.6 to 28.1%) (Vandeputte, Vermeylen, Geeroms, & Delcour, 2003). Cassava starch had an AM level of 17.3% and thus close to the value (18.4%) reported by Gomand *et al.* (2010). The lowest AM content was found for potato starch (14.8%), which was lower than found by others (18-22%) (Gomand *et al.*, 2010; Waterschoot, Gomand, & Delcour, 2016). This could be due to co-precipitation of AM with AP, leading to an underestimation of the actual AM content (Vilaplana, Hasjim, & Gilbert, 2012). The AM content of the maize starch used in this work (23.7%) was reported earlier (Dries, Gomand, Goderis & Delcour, 2014).

3.1.2 Amylose degrees of polymerization

Figure 1 shows the elution of AM in the HPSEC profiles of debranched starches. Table 1 lists the corresponding DP_{peak} and PI values for the AM fraction. The present results are in line with literature data: AMs from cereal starches have a considerably lower DP than those from roots and tubers (Takeda, Hizukuri, Takeda, & Suzuki, 1987) whereas legume starches show intermediate values.

Figure 1: High performance size exclusion chromatography (HPSEC) profiles of debranched maize, rice, cassava, potato and pea starches. Results are shown as the refractive index (RI) detector signal (a.u.) as a function of the elution volume (ml). The degrees of polymerization (DPs) of the pullulan standards (■) are indicated.

Indeed, cassava and potato AM had the highest DP_{peak} values (4,240 and 3,520 respectively), followed by pea starch AM (2,150), and maize and rice starch AM (1,710 and 1,520, respectively). Starches containing AM with higher DP_{peak} also had the highest AM PI. Potato, cassava and pea AMs had PIs of 2.9, 2.4 and 2.1, respectively, while AMs from maize and rice had a PI of 1.7. Unfortunately, there is little published information on the DP of debranched AMs of various starch sources. Wang *et al.* (2014) recently reported that the AM DP_{peak} readings from SEC weight average chain length distributions of a series of debranched starches

decreases in the order potato (3,365), field pea (1,785), rice (1,740) and maize (1,550) AM. The present results agree quite well with these findings.

3.2 Amylose leaching and granular morphologies as a result of aqueous ethanol treatments

3.2.1 Amylose leaching

When the ethanol concentration equaled 68, 58 or 53% (v/v) during the production of ETS, no carbohydrate leaching was detected for maize, cassava, potato and rice starches. For pea starch, leached carbohydrate levels equaled 0.2, 0.2 and 0.3%, respectively. For the lowest ethanol concentration [48% (v/v)] employed during production of ETS, carbohydrate leaching was 0.1% for maize, cassava, potato and rice starches and 0.4% for pea starch. Thus, hardly any carbohydrate leached.

3.2.2. Granular morphologies

Figure 2 shows bright field and polarized optical microscopy images of native starches and ETS. A broad variation in size and shape of native starches of different botanical origins was noted (Figure 2, a1-d1): rice starch granules had polygonal shapes and ranged from 3 to 5 μm in size. The granules of cassava starch were spherical and approximately 20 μm in size. Pea starch had irregularly shaped granules with an average diameter of 35 μm . Potato starch showed oval granules with diameters up to 100 μm . The observed microscopic appearances are in agreement with literature (Eliasson, 2004; Ratnayake, Hoover, & Warkentin, 2002). Starch granules from all sources displayed birefringence and the typical Maltese cross pattern when viewed between crossed polarizers (Figure 2, A1-D1).

Figure 2: Bright field (lower case) and polarized light (uppercase) microscopy images as studied in ethanol of rice (A), cassava (B), potato (C) and pea (D) starches in their native form (1) and treated with 68% (2), 58% (3), 53% (4) and 48% (5) (v/v) ethanol at 95 °C. The scale bars (50 µm) in the top right corner hold for all images displayed in the same column (same botanical origin).

In general, treating these starches with aqueous ethanol of decreasing ethanol concentrations at 95 °C led to increasingly clear morphological changes, starting from the hilum, and a gradual disappearance of the Maltese cross pattern. However, the extent to which these changes occurred at each ethanol concentration varied with the botanical origin. After treatment with 68% (v/v) ethanol [Figure 2, a(A)2-d(D)2], rice and cassava starches still did not show any morphological changes and the intensity of birefringence and occurrence of the Maltese cross pattern remained unaffected. ETS_{potato68%} on the other hand showed some indentations starting from the hilum (Ratnayake *et al.*, 2002) and corresponding distortions in the center of the Maltese cross pattern. ETS_{pea68%} also showed a clear loss of birefringence in the center of the granules. When the ethanol concentration was decreased to 58% (v/v) [Figure 2, a(A)3-d(D)3], morphological deformations became also evident for rice and cassava starches and the intensity of their birefringence faded noticeably. At this point, some potato starch granules already had lost most of their native birefringence. ETS_{pea58%} still exhibited strong birefringence at the granule periphery. After treatment with 53% (v/v) ethanol [Figure 2, a(A)4-d(D)4], some rice starch granules still displayed the original Maltese cross pattern and appeared to be most resistant to the treatment. ETS_{cassava53%} no longer appeared birefringent under polarized light. ETS_{potato53%} still displayed birefringence at the edges of the granules although the intensity was weak. Remnants of the original Maltese cross pattern and strong birefringence were still observable in the outer parts of ETS_{pea53%} granules. Similar observations were made by Cai and Wei (2013) during *in situ* observation of the gelatinization process of pea starch in excess water and attributed to the presence of native B-type crystals in the center of the granule and more stable A-type crystals around the periphery (Bogacheva, Morris, Ring, & Hedley, 1998; Crochet, Beauxis-Lagrave, Noel, Parker, & Ring, 2005). Complete loss of both the native birefringence and the Maltese cross pattern became evident for all starches after treatment with 48% (v/v) ethanol at 95 °C [Figure 2, a(A)5-d(D)5]. Only for ETS_{rice48%}, a diffuse, homogeneous birefringence – similar to the microscopic appearance of ETS_{maize48%} reported

earlier (Dries *et al.*, 2014) – could be observed. As the intensity of birefringence depends on the degree of crystallinity [although the thickness of the granules and orientation of the crystallites play a role as well (French, 1984)], the stronger birefringence of ETS_{rice48%} must be due to a higher level of newly formed crystals. This was investigated by WAXD measurements (*cf.* section 3.3).

3.3 Crystalline structure as a result of aqueous ethanol treatments

Figure 3 shows the WAXD patterns of ETS and their native counterparts. Table 2 summarizes the corresponding levels of A-, B- and V_H-type crystallinity. It also includes earlier results for maize starch (Dries *et al.*, 2014). The latter are not discussed below. Native rice, potato and pea starches showed the A-, B- and C-type WAXD patterns characteristic for cereal, tuber and legume starches (Zobel, 1988) (Figure 3, A1, C1 and D1). The native cassava starch used in this work had a clear A-type crystallinity (Figure 3, B1) as reported earlier (Gomand *et al.*, 2010). Degrees of crystallinity were 35, 40, 38 and 25% for native rice, cassava, potato and pea starches respectively. Due to different starch moisture contents and alternative methods for subtracting amorphous background from the semi-crystalline WAXD pattern, starch crystallinity values reported in the literature vary rather widely. Zobel (1988) noted crystallinities of 38% for both rice and cassava starches. Huang *et al.* (2007) found yellow pea starch to have a crystallinity of 21%. The crystallinity of potato starch obtained here was considerably higher than the 24 and 28% readings reported by respectively Cooke and Gidley (1992) and Zobel (1988) but in good agreement with the value (43%) by Vermeulen *et al.* (2004). For rice starch, 2% of native crystals were of the V_H-type, due to the presence of AM-lipid inclusion complexes (Dries *et al.*, 2014; Morrison, Law, & Snape, 1993).

Figure 3: Wide-angle X-ray diffraction (WAXD) patterns of rice (A), cassava (B), potato (C) and pea (D) starches in their native form (1) and treated with 68% (2), 58% (3), 53% (4) and 48% (5) (v/v) ethanol at 95 °C. Experimentally obtained scattering patterns (open circles) are fitted with an amorphous (white), A-type (light grey), B-type (dark grey) and V-type (black) scattering pattern using a least-squared error fit model. The scattered intensities are given in arbitrary units (a.u.).

Table 2: Degrees of A-, B- and V_H-type crystallinity (%) for native and ethanol treated starch (ETS), determined using wide angle X-ray diffraction (WAXD). Different treatment concentrations of ethanol are indicated in the subscript. The degrees of V_H-type crystallinity for the granular cold-water swelling starches are indicated in bold.

Starch source	Crystal type	Native	ETS _{68%}	ETS _{58%}	ETS _{53%}	ETS _{48%}
Maize*	A	27	28	21	4	0
	V _H	2	2	12	17	18
Rice	A	33	34	27	16	0
	V _H	2	2	5	11	16
Cassava	A	40	35	20	0	0
	V _H	0	0	10	10	10
Potato	B	38	18	9	3	0
	V _H	0	0	0	7	10
Pea	A	13	14	11	4	0
	B	12	5	5	2	0
	V _H	0	0	3	6	13

*Data for maize starch were previously published in Dries *et al.* (2014).

For all starches, a gradual changeover from a native crystalline structure to a V_H-type crystalline structure was observed when gradually lowering the ethanol concentration from 68 to 48% (v/v). In line with the above microscopic observations, the crystallinities obtained at each ethanol concentration depended on the botanical origin. Treatment with 68% (v/v) ethanol (Figure 3, A2-D2), produced little of any changes to the A-type crystallinity of rice starch (34%) and decreased it from 40 to 35% for cassava starch. Under the same treatment conditions, potato and pea starches already showed a marked decrease in B-type crystallinity (from 38 to 18% and from 12 to 5%, respectively). The A-type crystallinity of pea starch remained practically unaffected (14%). Decreasing the ethanol concentration to 58% (v/v) (Figure 3, A3-D3) markedly reduced the A-type crystallinity in rice and cassava starches and was in line with the loss of birefringence at this point. B-type crystallinity of potato starch decreased further to 9% and remained constant for pea starch at 5%. At this point, V_H-type crystals were observed for all starches but potato starch. This may be because the AM in potato starch partially contributes to the B-type crystals (Saibene *et al.*, 2010). Hence, sufficient melting of B-type crystals is needed to liberate and induce mobility in the AM chains for V_H-type helix formation. When the ethanol concentration in the treatment was 53% (v/v) (Figure 3, A4-D4), 11% V_H-type crystals were

detected in rice starch but the A-type crystallinity was still 16%, explaining the persistent occurrence of the Maltese cross pattern (*cf.* section 3.2.2). At this point, ETS_{cassava53%} already showed a pure V_H-type (10%) WAXD pattern. In ETS_{potato53%}, only 3% native B-type crystals remained and 7% V_H-type crystals were detected. ETS_{pea53%} had approximately 4% A-type, 2% B-type and 6% V_H-type crystals. While at the lowest ethanol concentration [48% (v/v)] (Figure 3, A5-D5) pure V_H-type crystalline structures were detected for all starches, their degrees of crystallinity varied widely. The highest V_H-type crystallinity was found for ETS_{rice48%} (16%), which was close to that earlier reported for ETS_{maize48%} (18%) (Dries *et al.*, 2014). ETS_{potato48%} and ETS_{cassava48%} both had 10% V_H-type crystals. ETS_{pea48%} had 13% V_H-type crystals. Birefringence requires (nano) crystal orientation correlations over a long range, whereas crystallinity is picked up by WAXD irrespective of this long range order (Jenkins & Donald, 1997). The higher V_H-type crystallinity for ETS_{maize48%} (Dries *et al.*, 2014) and ETS_{rice48%} (Table 2) apparently induced some (moderate) crystal orientation correlation. Section 3.6 discusses the reason for the observed differences in V_H-type crystallinity.

3.4 Melting of residual native crystals

The above mentioned changes in crystalline structure and birefringence were also detectable by DSC. Figure 4 displays T_O, T_P, T_C and ΔH of native starches and ETS in excess water. Native A-type starches had lower ΔH (rice and cassava, 14.4 and 15.6 J/g, respectively) but higher T_P (76.2 and 69.3 °C, respectively) than B-type starch (potato, ΔH = 22.1 J/g and T_P = 61.7 °C) in line with literature data (Fredriksson, Silverio, Andersson, Eliasson, & Aman, 1998; Gomand *et al.*, 2010; Waterschoot *et al.*, 2015). The ΔH of pea starch was lower (13.2 J/g) than that of other starches and its T_P (68.3 °C) close to that of A-type starches as previously reported by Tananuwong and co-workers (2004). It should be noted that the magnitude of the gelatinization enthalpy (ΔH) (Figure 4) reflects but rarely corresponds quantitatively to the degree of native crystallinity as measured by WAXD (Table 2). Exothermic events may (partially) overlap with the gelatinization endotherm, thereby rendering the measured ΔH *smaller* than what can be

expected from the WAXD crystallinity. Relevant exothermic events include formation of amylose-lipid complexes (typically in cereal starches such as those of maize and rice) or of an AM network, as *e.g.* suggested by Putseys and co-workers (2011). The gelatinization endotherm may also be *larger* than expected from the WAXD crystallinity. Bogracheva *et al.* (2002) showed that in starch powders (moisture content: 11-15%) not all double helices – and this effect was more outspoken for B-type crystal containing starches – are in crystalline order. In other words, these disordered structures do not contribute to the intensity of the WAXD pattern and the resultant degree of crystallinity, although their dissociation and unwinding during a DSC run adds to the measured ΔH . Although WAXD measurements were performed after equilibrating starch powders in an closed container with high relative humidity, this could (partly) explain why potato starch has a considerably higher ΔH (22.1 J/g) than cassava starch (15.6 J/g), although the latter contained 2% more native crystals according to WAXD.

Treatment with 68% (v/v) ethanol at 95 °C affected neither the gelatinization characteristics of ETS_{rice68%}, nor its A-type crystallinity and granular morphology. For ETS_{cassava68%}, there was only a minor shift of T_P to higher temperatures and a small decrease in ΔH , both of which barely exceeded the error margins. T_P increased and ΔH decreased markedly for starches containing B-type crystals (*i.e.* potato and pea starches). Use of an ethanol concentration of 58% (v/v) during the treatment dramatically reduced ΔH for cassava and potato starches and only slightly for rice and pea starches, in line with the corresponding changes in birefringence and losses of native crystallinity. ETS_{cassava53%} no longer displayed a gelatinization endotherm, in agreement with its pure V_H -type crystalline structure. As mentioned before, V_H -type crystals melt upon first contact with pure water and as a result do not contribute to the DSC gelatinization enthalpy. Although ETS_{potato53%} still had 3% B-type crystals (Table 2), it no longer showed a gelatinization endotherm during heating in excess water. It is, however, reasonable to assume that in this particular case, based on the widening of the gelatinization endotherm for ETS_{potato68%} and ETS_{potato58%}, the signal could be vanishingly small and broad, and therefore not

detectable. At this point, rice and pea starches showed further decreases in ΔH and increases in T_p , in line with WAXD and microscopic observations. Finally, no gelatinization endotherm was detected for any of the starches after treatment with 48% (v/v) ethanol, in agreement with their pure V_H -type crystalline structures and loss of native birefringence.

Figure 4: Schematic overview of the gelatinization characteristics of native and ethanol treated starches (ETS), determined using differential scanning calorimetry (DSC). Starch botanical origin and different treatment concentrations of ethanol (v/v) are indicated in the subscript. Start and end points of the bars represent onset (TO) and conclusion (TC) temperatures of gelatinization. The vertical line inside the bar is the peak temperature of gelatinization (TP). Gelatinization enthalpies (J/g) are given with standard deviations between brackets.

The gradual loss of natural birefringence, melting of native crystals and decrease in post-treatment gelatinization enthalpy took place at higher ethanol concentrations for starches containing B-type crystals (potato and pea starches) than for those containing A-type crystals (maize, rice and cassava starches). This can be explained in terms of the molecular organization of the AP fraction. Whittam *et al.* (1990) demonstrated that A-type crystals are intrinsically more stable than B-type crystals [at water contents ranging from approximately 40 to 90% (w/w)]. This difference in stability seems to also hold in water:ethanol mixtures as, at a given treatment temperature, more water was required (lower ethanol concentrations) to ensure native A-type crystal melting than for B-type crystal melting.

3.5 Gelatinization, V_H-type crystal formation and melting in aqueous ethanol

Figure 5 shows DSC thermograms of native starches during the first heating (left panel), cooling (mid panel) and the second heating (right panel) in 48% (v/v) ethanol. Note that the temperature runs from high to low values in the mid panel. Black and grey lines represent the DSC curves during the first and second temperature protocols, respectively (*cf.* section 2.8.2). DSC profiles recorded during the first heating segment of protocol 1 showed a sharp endothermic transition at temperatures below 95 °C (*i.e.* the temperature of the aqueous ethanol treatment, indicated by the vertical dashed line in Figure 5) for all native starches, which was attributed to native crystal melting. These transitions are highlighted by the vertical full arrows in the left panel of Figure 5. Higher melting temperatures (T_p) were observed when starches were heated in 48% (v/v) ethanol (Table 3) than when heated in water (Figure 4). For maize, rice, cassava and pea starches, increases in T_p were accompanied by increases in ΔH (Table 3). Tan and coworkers (2004) made similar observations in their study of the gelatinization of maize starch in glycerol:water mixtures. They attributed the increase in ΔH to the decreased effectiveness of glycerol to gelatinize starch, which in turn was related to its lower hydrogen bonding capacity than that of water. A similar reasoning has been suggested to account for the gelatinization enthalpy increase seen for maize starch in aqueous ethanol (Dries *et al.*, 2014). The other

starches in the present work seem to follow this trend, except for potato starch: it showed a ΔH of 14.7 J/g in 48% (v/v) ethanol versus 22.1 J/g in pure water. Tan and coworkers (2004) also noted such decrease in ΔH for high AM maize starch in glycerol:water media. While no definitive explanation was given, it was suggested that differences in starch structures such as the occurrence of B-type rather than A-type crystals, might be at the origin. To our feeling, this allotropic difference might indeed be relevant when coupled to the exothermic mixing of water with ethanol or (ethanol-rich) binary ethanol:water systems (Peeters & Huyskens, 1993). Indeed, during (endothermic) melting of the native crystals, the water residing in the crystals mixes (exothermally) with the ethanolic medium. B-type crystals, as found in potato starch, contain considerably higher levels of water than A-type crystals (Imberty & Pérez, 1988). This may well lead to an increased exothermic heat of mixing and hence a lower overall endothermic ΔH at gelatinization. The difference in ΔH readings for pea starch in 48% (v/v) ethanol (Table 3) and pure water (Figure 4) barely exceeded the error margins, suggesting that the behavior of its A- and B-type crystals cancelled each other out.

Table 3: DSC based gelatinization peak temperatures (T_p) and associated melting enthalpies (ΔH) of native starches heated in 48% (v/v) ethanol and determined during the first heating run of the first thermal protocol. Results are shown with standard deviations between brackets.

Starch source	T_p (° C)	ΔH (J/g dm)
Maize	82.3 (0.8)	15.2 (0.4)
Rice	87.9 (0.3)	16.0 (0.2)
Cassava	83.7 (0.2)	18.4 (1.5)
Potato	76.1 (0.5)	14.7 (1.7)
Pea	85.1 (0.7)	14.6 (1.1)

For maize, rice and cassava starches, it seems that the endothermic gelatinization peak at the high temperature side precedes an exothermic signal (Figure 5, left panel, dashed arrows). It has been suggested that (exothermic) V_H -type crystal formation occurs in the same temperature interval as native crystal melting (Dries *et al.*, 2014; Jane, Craig, Seib, & Hosney, 1986). In

fact, although it is difficult to identify this signal, it can be argued that such exothermic event occurs (slightly) beyond gelatinization. In the present case, clear evidence for the formation of V_H -type crystals was found in their endothermic melting at higher temperatures during continued heating (Figure 5, left panel, tilted full arrows). For potato and pea starches (Figure 5, left panel, curves 4 and 5 respectively) on the other hand, neither V_H -type crystallization nor melting signals were detected. The V_H -type crystals of these starches (Figure 3) must thus either have been created during the holding phase at 95 °C or during the cooling segment of the aqueous ethanol treatment. Most likely, the share developed in the isothermal step is significant for maize, rice and certainly cassava starch, given that the exothermic signal prior to reaching 95 °C (indicated by the vertical dashed line in Figure 5) during the first heating run is rather small.

Figure 5: Differential scanning calorimetry (DSC) profiles (after a baseline slope correction) of maize (1), rice (2), cassava (3), potato (4) and pea (5) starches during heating (left panel), cooling (middle panel) and reheating (right panel) in 48% (v/v) ethanol. Black lines are the DSC thermograms recorded using the first temperature protocol (no isothermal step). Grey lines are the DSC thermograms recorded using the second temperature protocol (which includes an isothermal step – not shown). Relevant thermal transitions are indicated with arrows. All DSC traces are displayed to scale, implying that magnitudes of transitions can directly be compared.

The exothermic transition (highlighted by dashed arrows) in the DSC profiles recorded during cooling in the first thermal protocol (black lines in Figure 5, mid panel) indeed suggests that V_H crystals can be formed during cooling. This exothermic transition during cooling was observed for all starches when cooled from the fully non-crystalline state at 160 °C (first temperature protocol). However, its peak temperature depended on the starch botanical origin, and equaled 62.7, 57.4, 46.5, 44.9 and 59.6 °C for maize, rice, cassava, potato and pea starches, respectively. Section 3.6 rationalizes these differences in crystallization temperature. DSC thermograms recorded during cooling after holding the starches for 30 min at 95 °C (grey lines in Figure 5, mid panel) differed markedly from those recorded after continuous heating. Maize and rice starches no longer showed exothermic transitions indicating that V_H -type crystals fully developed during continuous heating to 95 °C and the subsequent isothermal step. For cassava, potato and pea starches, exothermic transitions during cooling in protocol 2 were still observable but decreased in size, shifted to higher temperatures and broadened considerably. To facilitate their identification, these peaks were shaded grey in Figure 5. The observations most likely can best be explained as resulting from slow and incomplete crystallization during the isothermal treatment at 95 °C and the further growth of V_H crystals during cooling.

The second heating runs in thermal protocol 1 (black lines in Figure 5, right panel) showed two endothermic peaks (labelled 1 and 2) with, at intermediate temperatures, an exothermic event (dashed arrow). The melting temperatures associated with endothermic peak 1 mirrored the crystallization temperatures recorded during cooling: when crystallization was observed at lower temperatures during cooling, the melting associated with peak 1 during reheating occurred at lower temperatures as well. It therefore seems that the stability (as reflected by the melting point) of V_H crystals formed at lower temperatures during cooling (*e.g.* potato and cassava) was lower than that of V_H crystals created at higher temperatures during cooling (*e.g.* maize, rice and pea). The melting points (peak 1) of these (rather unstable) crystals all were below those of the V_H crystals recorded during first heating, implying that the systems at that

stage were in a super-cooled state and that there was a driving force towards crystallization. Indeed, the exothermic signals right after peak 1 suggest recrystallization into more stable V_H crystals, which ultimately did melt in endothermic peak 2. This basic scenario seems to hold at least for maize, rice and pea starches, for which melting, recrystallization, and renewed melting events occurred in a rather narrow temperature interval. For potato and cassava starches, the first melting and recrystallization was at such low temperatures that the crystals formed at that point may again have been rather unstable and therefore melted and recrystallized a second time prior to ultimately melting at point 2. In fact, for these starches, a faint endothermic signal was spotted close to where melting peak 1 occurred for the other starches (not indicated in Figure 5). Melting point 2 (approximately 110-115 °C) was rather independent of the starch source. The melting temperature of V_H -type inclusion complexes is – at a constant starch to water ratio – predominantly related to the crystal size along the helical axis (Goderis, Putseys, Gommès, Bosmans, & Delcour, 2014). The present results therefore suggest that ethanol filled V_H -type crystals of different sizes parallel to the helical axis were created and that by melting and recrystallization during heating, a limiting (starch type independent) size was reached. Isothermal crystallization at 95 °C seems to have induced formation of such crystals since – at least for maize, rice and pea starches – no recrystallization was observed before melting for peak 2 (grey lines in Figure 5, right panel). No recrystallization was observed for potato and cassava starches either, although part of the crystals in protocol 2 was created during cooling at temperatures much lower than 95 °C. However, as these crystals were formed over a wide temperature range, a wide variety of stabilities may have been created, which melted and recrystallized at widely different temperatures such that reorganization transitions were obscured by overlaps. Irrespective of the initial crystal stability, crystals were formed during heating which ultimately melted at peak 2, much as in thermal protocol 1. It would be valuable to verify or deny the hypothesis of melting-recrystallization-remelting into progressively larger crystals by time resolved X-ray scattering experiments under DSC typical conditions.

3.6 Influence of the degree of polymerization of amylose on V-type crystal formation

Linear AM chains are at least in maize starch responsible for V_H-type crystal formation in GCWSS prepared by an aqueous ethanol treatment at elevated temperature (Dries *et al.*, 2014) and most likely also in the other cases presented here. Indeed, the obtained V_H-type crystallinities (Table 2) never exceeded the AM contents of the starches (Table 1). However, there was no quantitative relation between the levels of V_H-type crystallinity and the AM contents. Actually, while ETS_{rice48%} and ETS_{cassava48%} had similar AM contents, their V_H-type crystallinities were 16 and only 10%, respectively. While ETS_{pea48%}, had the highest AM content (26.7%), it did not have the highest V_H-type crystallinity (only 13%). However, there seems to be a relation between AM DP and the V_H-type crystallinity: the V_H-type crystallinity in the ETS (Table 2) decreased with increasing AM DP (Table 1). The higher the AM DP, the more strongly V_H-type crystal formation is hindered. This hypothesis finds support in the DSC experiments executed in 48% (v/v) ethanol (Figure 5).

The AM DP dependent ability to nucleate V_H crystals is well reflected in the DSC thermograms recorded during cooling from the fully molten state (black lines in Figure 5, mid panel). Starches containing high DP AM (cassava: DP_{peak} = 4,240; potato: DP_{peak} = 3,520) had significantly lower crystallization temperatures than those with low DP AM (maize: DP_{peak} = 1,710; rice: DP_{peak} = 1,520). Such MW-dependency is well-known in crystallization of synthetic polymers (Mathot & Pijpers, 1984) and is explained by the increased occurrence of entanglements for polymers of higher MW. In addition, these entanglements are part of the amorphous regions, which explains why starches with a higher AM DP have lower V_H-type crystallinity. The intermediate AM DP starch (pea: DP_{peak} = 2150) had a considerably higher crystallization point than that of the high DP AM starches (potato and cassava), though in terms of V_H-type crystallization kinetics during first heating in temperature protocol 1, its behavior was similar to that of the high DP AM starches. The higher AM content of pea starch (Table 1) most likely is at the base of this difference in crystallization temperature, since an increased

concentration of AM chains would favor crystallization and decrease the degree of supercooling necessary to induce crystal formation.

In spite of the fact that at 95 °C (*i.e.* the treatment temperature during the aqueous ethanol treatment) in 48% (v/v) ethanol all starches are above their native crystal melting point (T_P , Table 3) and below the (ultimate) V_H -type crystal melting point, only the low DP AM starches (maize and rice) crystallized up to completion during the isothermal step. At 95 °C, the supercooling with respect to the ultimate V_H crystal melting point (melting point 2 in Figure 5, right panel) is limited. According to classical nucleation theory, this implies that the driving force for crystal formation is small and that large nuclei need to be formed to outbalance the surface energy penalty associated with crystal formation. The formation of large nuclei is slow in particular for long, heavily entangled polymers. Therefore more mobile, short AM chains crystallize more readily than long AM chains.

When the time at high temperature is sufficient, such as 30 min at 95 °C, large nuclei can also be formed in starches with a high DP AM. Their number may be small and their growth limited since it equally requires chain conformational changes. For potato and to a lesser extent also for cassava and pea starches, 30 min appeared too short for completing the crystallization. Indeed, the crystallinity developed further during cooling (grey shaded exothermic areas in Figure 5, mid panel). At larger supercooling and thus lower temperatures, the driving force for crystallization increases. Smaller crystals can then be formed which require only shorter disentangled chain fragments. In other words, longer AM chains may crystallize into smaller V_H type crystals at lower temperatures, even if not fully disentangled. Since crystalline nuclei are present after 30 min at 95 °C, crystal growth (often thought to happen via a process of secondary nucleation) can proceed at higher temperatures during cooling compared to when cooled in absence of preformed primary nuclei, such as *e.g.* during cooling in thermal protocol 1.

CONCLUSIONS

At 95 °C, decreasing ethanol concentrations from 68 to 48% (v/v) induced a change from starch granules with a Maltese cross pattern to granules with diffuse or vanishing birefringence. These changes in microscopic appearance went along with a gradual decrease in the original crystallinity and an increase in V_H -type crystallinity. Starches with B-type crystallites underwent these structural changes already at higher ethanol concentrations than did A-type starches, due to the inherent lower thermal stability of B-type crystals in ethanol:water mixtures. Calorimetric measurements for starches in 48% (v/v) ethanol provided insights in the crystallization and melting kinetics of V_H -type AM crystals for starches of different botanical origin. For low DP AM starches, V_H -type structures were formed partially during heating once the native crystals had melted and went to completion during the isothermal incubation at 95 °C. For mid and high DP AM starches on the other hand, V_H -type crystal formation was triggered but incomplete during the isothermal incubation at 95 °C. The crystallization proceeded during cooling to room temperature. The lower degree of V_H -type crystallinity in GCWSS for starches with higher AM DP was suggested to result from an increased occurrence of entanglements. This work provides beyond-state-of-the-art information on the AM DP-dependent V-type crystal formation for granular starches with AM DPs exceeding 950. It is the first to report on the structural changes of starches of different botanical origins in response to aqueous ethanol treatment.

ACKNOWLEDGEMENTS

The Fonds Wetenschappelijk Onderzoek – Vlaanderen (FWO) is thanked for financial support. Luc Van Den Ende and Sarah Pycarelle are thanked for technical assistance. Special acknowledgements are offered to Andrés F. Doblado-Maldonado and Jung-Sun Hong for helpful discussions on the contents of this study. This study is part of the Methusalem programme ‘Food for the future’ (2007-2014). Jan A. Delcour is W.K. Kellogg Chair in Cereal Science and Nutrition at KU Leuven. Bart Goderis thanks the Hercules Foundation for supporting the purchase of equipment through project AKUL/09/0035 (molybdenum high-energy X-ray source for *in situ* diffraction studies of advanced materials and single crystals).

LITERATURE CITED

- AACC. (1999). Method 44-15.02, Moisture -- Air-Oven Methods. In *Approved methods of Analysis* (11th ed.). St. Paul, MN, USA: AACC International.
- Bogracheva, T. Y., Morris, V. J., Ring, S. G., & Hedley, C. L. (1998). The granular structure of C-type pea starch and its role in gelatinization. *Biopolymers*, 45(4), 323-332.
- Bogracheva, T. Y., Wang, Y. L., Wang, T. L., & Hedley, C. L. (2002). Structural studies of starches with different water contents. *Biopolymers*, 64(5), 268-281.
- Brisson, J., Chanzy, H., & Winter, W. T. (1991). The crystal and molecular structure of Vh amylose by electron diffraction analysis. *International Journal of Biological Macromolecules*, 13(1), 31-39.
- Buléon, A., Colonna, P., Planchot, V., & Ball, S. (1998). Starch granules: structure and biosynthesis. *International Journal of Biological Macromolecules*, 23(2), 85-112.
- Cai, C. H., & Wei, C. X. (2013). In situ observation of crystallinity disruption patterns during starch gelatinization. *Carbohydrate Polymers*, 92(1), 469-478.
- Cairns, P., Bogracheva, T. Y., Ring, S. G., Hadley, C. L., & Morris, V. J. (1997). Determination of the polymorphic composition of smooth pea starch. *Carbohydrate Polymers*, 32(3-4), 275-282.
- Cheetham, N. W. H., & Tao, L. P. (1998). Variation in crystalline type with amylose content in maize starch granules: an X-ray powder diffraction study. *Carbohydrate Polymers*, 36(4), 277-284.
- Chen, J., & Jane, J. (1994a). Preparation of granular cold-water soluble starches by alcoholic alkaline treatment. *Cereal Chemistry*, 71(6), 618-622.
- Chen, J., & Jane, J. (1994b). Properties of granular cold-water soluble starches prepared by alcoholic alkaline treatments. *Cereal Chemistry*, 71(6), 623-626.
- Cooke, D., & Gidley, M. J. (1992). Loss of crystalline and molecular order during starch gelatinization - origin of the enthalpic transition. *Carbohydrate Research*, 227, 103-112.
- Crochet, P., Beauxis-Lagrange, T., Noel, T. R., Parker, R., & Ring, S. G. (2005). Starch crystal solubility and starch granule gelatinisation. *Carbohydrate Research*, 340(1), 107-113.
- Dries, D. M., Gomand, S. V., Goderis, B., & Delcour, J. A. (2014). Structural and thermal transitions during the conversion from native to granular cold-water swelling maize starch. *Carbohydrate Polymers*, 114, 196-205.
- Eastman, J. E. (1987). Cold water swelling starch composition. U.S. patent 4,634,596.
- Eastman, J. E., & Moore, C. O. (1984). Cold-water-soluble granular starch for gelled food compositions. U.S. patent 4,465,702.

- Eliasson, A. (2004). *Starch in food. Structure, function and applications* (first ed.). Abington, Cambridge, England: Woodhead Publishing Limited.
- Fredriksson, H., Silverio, J., Andersson, R., Eliasson, A. C., & Aman, P. (1998). The influence of amylose and amylopectin characteristics on gelatinization and retrogradation properties of different starches. *Carbohydrate Polymers*, 35(3-4), 119-134.
- French, D. (1984). Organization of starch granules. In R. L. Whistler, J. N. BeMiller & E. F. Paschall (Eds.), *Starch: chemistry and technology* (second ed., pp. 183-247). New York, USA: Academic Press.
- Gelders, G. G., Duyck, J. P., Goesaert, H., & Delcour, J. A. (2005). Enzyme and acid resistance of amylose-lipid complexes differing in amylose chain length, lipid and complexation temperature. *Carbohydrate Polymers*, 60(3), 379-389.
- Gelders, G. G., Vanderstukken, T. C., Goesaert, H., & Delcour, J. A. (2004). Amylose-lipid complexation: a new fractionation method. *Carbohydrate Polymers*, 56(4), 447-458.
- Gibson, T. S., Solah, V. A., & McCleary, B. V. (1997). A procedure to measure amylose in cereal starches and flours with concanavalin A. *Journal of Cereal Science*, 25(2), 111-119.
- Goderis, B., Putseys, J. A., Gommès, C. J., Bosmans, G. M., & Delcour, J. A. (2014). The structure and thermal stability of amylose-lipid complexes: a case study on amylose-glycerol monostearate. *Crystal Growth & Design*, 14(7), 3221-3233.
- Godet, M. C., Bizot, H., & Buleon, A. (1995). Crystallization of amylose-fatty acid complexes prepared with different amylose chain lengths. *Carbohydrate Polymers*, 27(1), 47-52.
- Gomand, S. V., Lamberts, L., Derde, L. J., Goesaert, H., Vandeputte, G. E., Goderis, B., Visser, R. G. F., & Delcour, J. A. (2010). Structural properties and gelatinisation characteristics of potato and cassava starches and mutants thereof. *Food Hydrocolloids*, 24(4), 307-317.
- Gommès, C. J., & Goderis, B. (2010). CONEX, a program for angular calibration and averaging of two-dimensional powder scattering patterns. *Journal of Applied Crystallography*, 43, 352-355.
- Hoover, R. (2001). Composition, molecular structure, and physicochemical properties of tuber and root starches: a review. *Carbohydrate Polymers*, 45(3), 253-267.
- Huang, J. R., Schols, H. A., van Soest, J. J. G., Jin, Z. Y., Sulmann, E., & Voragen, A. G. J. (2007). Physicochemical properties and amylopectin chain profiles of cowpea, chickpea and yellow pea starches. *Food Chemistry*, 101(4), 1338-1345.
- Imberty, A., Chanzy, H., Pérez, S., Buleon, A., & Tran, V. (1988). The double-helical nature of the crystalline part of A-starch. *Journal of Molecular Biology*, 201(2), 365-378.
- Imberty, A., & Pérez, S. (1988). A revisit to the 3-dimensional structure of B-type starch. *Biopolymers*, 27(8), 1205-1221.

- Jane, J., Craig, S. A. S., Seib, P. A., & Hoseney, R. C. (1986). Characterization of granular cold water-soluble starch. *Starch-Starke*, 38(8), 258-263.
- Jane, J., & Seib, P. A. (1991). Preparation of granular cold water swelling soluble starches by alcoholic-alkali treatments. U.S. patent 5,057,157.
- Jane, J., Xu, A., Radosavljevic, M., & Seib, P. A. (1992). Location of amylose in normal starch granules. 1. Susceptibility of amylose and amylopectin to cross-linking reagents. *Cereal Chemistry*, 69(4), 405-409.
- Jenkins, P. J., & Donald, A. M. (1995). The influence of amylose on starch granule structure. *International Journal of Biological Macromolecules*, 17(6), 315-321.
- Jenkins, P. J., & Donald, A. M. (1997). Breakdown of crystal structure in potato starch during gelatinization. *Journal of Applied Polymer Science*, 66(2), 225-232.
- Le Bail, P., Bizot, H., Pontoire, B., & Buleon, A. (1995). Polymorphic transitions of amylose-ethanol crystalline complexes induced by moisture exchanges. *Starch-Starke*, 47(6), 229-232.
- Mathot, V. B. F., & Pijpers, M. F. J. (1984). Crystallization and melting behavior of polyethylene fractions obtained by various fractionation methods. *Polymer Bulletin*, 11(3), 297-304.
- Morrison, W. R., Law, R. V., & Snape, C. E. (1993). Evidence for inclusion complexes of lipids with V-Amylose in maize, rice and oat starches. *Journal of Cereal Science*, 18(2), 107-109.
- Obiro, W. C., Ray, S. S., & Emmambux, M. N. (2012). V-amylose structural characteristics, methods of preparation, significance, and potential applications. *Food Reviews International*, 28(4), 412-438.
- Peat, S., Whelan, W. J., & Thomas, G. J. (1956). The enzymic synthesis and degradation of starch. Part XXII. Evidence of multiple branching in waxy-maize starch. A correction. *Journal of the Chemical Society*, 3025-3030.
- Peeters, D., & Huyskens, P. (1993). Endothermicity or Exothermicity of Water-Alcohol Mixtures. *Journal of Molecular Structure*, 300, 539-550.
- Putseys, J. A., Gommers, C. J., Van Puyvelde, P., Delcour, J. A., & Goderis, B. (2011). In situ SAXS under shear unveils the gelation of aqueous starch suspensions and the impact of added amylose-lipid complexes. *Carbohydrate Polymers*, 84(3), 1141-1150.
- Rajagopalan, S., & Seib, P. A. (1992a). Granular cold-water-soluble starches prepared at atmospheric-pressure. *Journal of Cereal Science*, 16(1), 13-28.
- Rajagopalan, S., & Seib, P. A. (1992b). Properties of granular cold-water-soluble starches prepared at atmospheric-pressure. *Journal of Cereal Science*, 16(1), 29-40.
- Ratnayake, W. S., Hoover, R., & Warkentin, T. (2002). Pea starch: composition, structure and properties - a review. *Starch-Starke*, 54(6), 217-234.

- Saibene, D., & Seetharaman, K. (2010). Amylose involvement in the amylopectin clusters of potato starch granules. *Carbohydrate Polymers*, 82(2), 376-383.
- Singh, J., & Singh, N. (2003). Studies on the morphological and rheological properties of granular cold water soluble corn and potato starches. *Food Hydrocolloids*, 17(1), 63-72.
- Takeda, Y., Hizukuri, S., Takeda, C., & Suzuki, A. (1987). Structures of branched molecules of amyloses of various origins, and molar fractions of branched and unbranched molecules. *Carbohydrate Research*, 165(1), 139-145.
- Tan, I., Wee, C. C., Sopade, P. A., & Halley, P. J. (2004). Investigation of the starch gelatinisation phenomena in water-glycerol systems: application of modulated temperature differential scanning calorimetry. *Carbohydrate Polymers*, 58(2), 191-204.
- Tananuwong, K., & Reid, D. S. (2004). DSC and NMR relaxation studies of starch-water interactions during gelatinization. *Carbohydrate Polymers*, 58(3), 345-358.
- Tester, R. F., Karkalas, J., & Qi, X. (2004). Starch - composition, fine structure and architecture. *Journal of Cereal Science*, 39(2), 151-165.
- Umemoto, T., Nakamura, Y., Satoh, H., & Terashima, K. (1999). Differences in amylopectin structure between two rice varieties in relation to the effects of temperature during grain-filling. *Starch-Starke*, 51(2-3), 58-62.
- Vandeputte, G. E., Vermeylen, R., Geeroms, J., & Delcour, J. A. (2003). Rice starches. I. Structural aspects provide insight into crystallinity characteristics and gelatinisation behaviour of granular starch. *Journal of Cereal Science*, 38(1), 43-52.
- Vermeylen, R., Goderis, B., Reynaers, H., & Delcour, J. A. (2004). Amylopectin molecular structure reflected in macromolecular organization of granular starch. *Biomacromolecules*, 5(5), 1775-1786.
- Vilaplana, F., Hasjim, J., & Gilbert, R. G. (2012). Amylose content in starches: Toward optimal definition and validating experimental methods. *Carbohydrate Polymers*, 88(1), 103-111.
- Wang, K., Hasjim, J., Wu, A. C., Henry, R. J., & Gilbert, R. G. (2014). Variation in amylose fine structure of starches from different botanical sources. *Journal of Agricultural and Food Chemistry*, 62(19), 4443-4453.
- Waterschoot, J., Gomand, S. V., & Delcour, J. A. (2016). Impact of swelling power and granule size on pasting of blends of potato, waxy rice and maize starches. *Food Hydrocolloids*, 52, 69-77.
- Waterschoot, J., Gomand, S. V., Fierens, E., & Delcour, J. A. (2015). Production, structure, physicochemical and functional properties of maize, cassava, wheat, potato and rice starches. *Starch-Starke*, 67(1-2), 14-29.

- Whittam, M. A., Noel, T. R., & Ring, S. G. (1990). Melting behavior of A-type and B-type crystalline starch. *International Journal of Biological Macromolecules*, 12(6), 359-362.
- Zabar, S., Lesmes, U., Katz, I., Shimoni, E., & Bianco-Peled, H. (2009). Studying different dimensions of amylose-long chain fatty acid complexes: molecular, nano and micro level characteristics. *Food Hydrocolloids*, 23(7), 1918-1925.
- Zhang, B. S., Dhital, S., Haque, E., & Gidley, M. J. (2012). Preparation and characterization of gelatinized granular starches from aqueous ethanol treatments. *Carbohydrate Polymers*, 90(4), 1587-1594.
- Zobel, H. F. (1988). Molecules to granules - a comprehensive starch review. *Starch-Starke*, 40(2), 44-50.