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Chromatographic Study of High Amylose Corn Starch Genotypes – Investigation of Molecular Properties after Specific Enzymatic Digestion

Marco Ulbrich, Fanni Scholz, and Eckhard Flöter*

Two high amylose corn starches (HACS; HYLON V and HYLON VII) are dissolved completely and subjected to specific enzymatic degradation by means of different amylases (AMY). The starches are digested using α-, β-, and γ-AMY (single) as well as in combination with the debranching enzyme pullulanase (PUL; α-AMY-PUL and β-AMY-PUL), and the products are characterized by means of size exclusion chromatography (SEC)-techniques including multi angle laser light scattering-differential refractive index detection (SEC-MALS-DRI) and conventional calibration-differential refractive index detection (SEC-cal-DRI). Enzymolysis is resulted in largely maltose (Mal; α- and β-AMY and respective combinations with PUL) or glucose (Glc; γ-AMY; almost complete digestion) as the major fraction, but also other residual fractions of higher molar mass (MM), i.a. α- and β-limit dextrins (α-/β-LDs). The quantity (relative portion) and quality (specific molecular properties) of the reaction products are found to be strongly dependent on both the enzymatic treatment by itself (kind of enzyme/-combination and associated specificity of action) and the molecular composition of the initial starch (portion and specific molecular properties of the amylose [AM], intermediate [IM], and amylopectin [AP] fraction), which is further investigated in detail.

1. Introduction

The polysaccharide starch is a mixture of different structure fractions. It consists of the mostly linear amylose (AM) with anhydroglucose units (AGU) linked via α-1,4-glucosidic bonds (weight average MM [M_w] between 10^5 and 10^6 g mol^{-1}), and the amylopectin (AP), which is highly branched (α-1,4- and α-1,6-glucosidic linkages, M_w up to several 10^7 g mol^{-1}). Normal starches (isolated from, e.g., potato, wheat, or corn) feature an AM content of about 20–30% w/w.[1] However, e.g. most pea starches possess a higher AM content (e.g., 33% w/w [smooth pea], 71% w/w [wrinkled-pea]).[2,3] In contrast to normal or regular starches, respectively, starch varieties vary strongly with respect to the AM/AP-ratio. On the one hand, waxy varieties consist of AP (marginal AM content),[4,5] on the other hand, the so-called high AM starches have AM contents higher than, e.g., 50% w/w,[6,7] in either case significantly higher compared to the regular ones. The existence of an intermediate (IM) fraction is commonly accepted).[8–10] The starch type (source, variety) defines not only the AM/AP-ratio, but rather the molecular features of the polymer fractions specified by parameters like MM and the correspondent MM distribution (MMD), degree of branching, AP branch chain (BC) length, complexing with iodine and supramolecular properties like type (crystal pattern) and degree of crystallinity (granule characteristics) (Cheetham and Tao[11]). Moreover, minor components like fat/lipids, protein, and water vary strongly related to their relative portion depending on the type of starch. For example, the fat/lipids content of corn starches is positively correlated with the AM content,[12–14] and potato AP is characterized by a significant portion of phosphate ester groups bound covalently.[15–19] The determination of the AM content and impacts of the different methods (iodine complexing/colorimetric, concanavalin A precipitation, 1D size exclusion chromatography (SEC) fully branched, 1D SEC debranched, and 2D structural distributions from multidimensional SEC × SEC) on the values obtained were comprehensively discussed by Vilaplana et al.[20]

These molecular starch properties determine inevitably the behavior of a solution, physicochemical, and techno-/functional properties like hot paste viscosity or the ability to form firm gels. Since the AM fraction is mainly responsible for the development of a 3-D gel network, high AM starches are basically suitable for this purpose.[21,22] An appropriate solution state of such starches is an essential requirement (Vesterinen et al.[23]). The inherent starch properties, in particular on the molecular level, govern the enzymatic digestion, which is of great importance for both starch analytical problems and industrial processing. The characteristics of molecules and molecule fractions (specific amount, molecular properties), respectively, originated from the specific

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enzymatic digestion of a dissolved starch, are strongly dependent on the composition of the initial starch material and, of course, the specificity of the respective amylolytic enzyme.

Four groups of starch converting enzymes are differentiated, the endo-AMY (1), the exo-AMY (2), debranching enzymes (3), and transferases (4) (van der Maarel et al.[24]). The α-AMY (EC 3.2.1.1) is an endo-AMY, which is able to cleave α-1,4-glycosidic linkages present in the inner part (endo-) of the molecule chain producing oligosaccharides with varying chain length and α-limit dextrins (α-LDs), which constitute branched oligosaccharides. Hydrolysis of granular starches (HACS, waxy CS [WxCS], normal CS, wheat starch, wrinkled pea starch) using α-AMY (*Aspergillus fumigatus*) results in minor amounts of maltose (Mal) and maltotriose, and the major product is glucose (Glc) (Planchot et al.[25]). The release of Mal, maltotriose, and a resistant amylodextrin (porcine-pancreatic α-AMY, human-salivary α-AMY) and maltotriose, maltopentaose, maltohexaose, and a resistant amylodextrin (Bacillus subtilis α-AMY) was reported by Jane and Robyt.[26] The formation of α-LDs by hydrolysis of AP was reported elsewhere.[27,28] The β-AMY (EC 3.2.1.2) is an exo-AMY and cleaves exclusively α-1,4-glycosidic linkages (every 2nd) from the non-reducing end of the chain. Since the digestion is terminated on the polymer branches (α-1,6-linkages in AP, IM fraction, and AM), Mal as well as β-LDs are released,[29] which should have a degree of polymerization (DP) of approximately 50% compared to the AP fraction.[30] Landerito and Wang[31] reported contents of β-LDs between about 39% and 57% after hydrolysis for different CS (WxCS, common CS, HYLON V, and HYLON VII). The fine structure of β-LDs is based on chain length fractions of average DP 50, 25, 15, 10, 7, 3, and 2 (WxCS) (Derde et al.[32]). The γ-AMY (glucoamylase, amyloglucosidase; EC 3.2.1.3) catalyzes the hydrolysis of α-1,4- and α-1,6-glucosidic linkages from the non-reducing end of the polymer chain (exo-AMY) to release predominantly β-D-Glc and related poly- and oligosaccharides.[33] The debranching enzyme PUL hydrolyzes starch by cleavage of the α-1,6-linkages (PUL type I; release of AP BC and basically BC from other branched polymer fractions [IM and AM]; Li et al.,[34] Hii et al.[35]). In particular, the combined application of PUL (debranching) and, e.g., α-AMY and β-AMY, respectively, enables the degradation of the starch polymer structure for the most part with the release of predominantly mono- and/or disaccharides.

A prerequisite for the (theoretically) complete enzymatic digestion according to the particular specificity of the enzyme is the accessibility to the respective sites, which is ensured by an optimal solution state. Retrograded regions, but also chemical modification, can decrease the extent of the total hydrolysis or impede the hydrolysis at least partially. This resistance of starch polymer areas to molecular degradation is based on double helical structures and associated formation of crystalline regions (retrogradation), which would be expected in particular for the AM fraction but also for smaller unbranched residues originating, e.g., from longer AP BC (e.g., after starch is debranched). The expected partial resistance of starch polymers to enzymatic digestion due to being protected by the higher organization level (retrogradation), which is evidentially the case in starch gels, can be potentially utilized for analytical purposes. Specific enzymatic degradation of starch polysaccharides in a gel, which are not protected by the structure (double helical, crystalline) and accessible to enzymatic cleavage, respectively, and subsequent special characterization of the resistant residues by means of SEC-techniques (size exclusion chromatography-multi angle laser light scattering-differential refractive index detection, SEC-MALS-DRI, size exclusion chromatography-conventional calibration-differential refractive index detection, SEC-cal-DRI) could contribute to better understanding of the gel structure. Hence, comprehensive knowledge regarding the molecular properties of two HACS (HYLON V and HYLON VII), and their specific enzymatic degradation (single: α-AMY, β-AMY, γ-AMY, combination: α-AMY-PUL and β-AMY-PUL) in the solution state (complete dissolved) was derived by SEC-techniques in the present study, which is a necessary requirement for the successful and reliable analysis of the gel fine structure in a second step. This is the first of a series of publications.

2. Experimental Section

2.1. Starch Genotypes

Two commercial native HACS genotypes (HYLON V [HACS-50] and HYLON VII [HACS-70], Ingredion Germany GmbH, Hamburg, Germany) were provided for the examination. The specifications indicated are supplier information (HACS-50: 55% w/w AM [nominal], 1.0% w/w total fat, 0.5% w/w protein, ≤0.1% w/w ash; HACS-70: 73.8% w/w AM [colorimetric method], 0.58% w/w protein). The dry matter contents (DM; HACS-50: 88.46% w/w, HACS-70: 88.36% w/w) were determined using a moisture analyzer (MA 30, Sartorius, Göttingen, Germany).

2.2. Starch Degrading Enzymes

Different starch degrading enzymes were used, an α-AMY (powder, Optizym A 16126 [21 000 U g^{-1}], SternEnzym GmbH & Co. KG, Ahrensburg, Germany), a β-AMY (solution, Secura [5000 BAMU g^{-1}], Novozymes A/S, Bagsvaerd, Denmark), a γ-AMY (solution, AMIGASE MEGA L [≥36,000 AGI g^{-1}], DSM Food Specialties B.V., Delft, Netherlands) and a PUL (solution, Promozyme D2 [200 U mL^{-1}], Novozymes A/S, Bagsvaerd, Denmark). A solution of the α-AMY was prepared by dispersion of the powder in water (addition of 9 g water to 1 g enzyme; freshly prepared before each experiment).

2.3. Molecular Characterization Using SEC-Techniques

Methodical steps of the comprehensive molecular characterization of the starch genotypes as well as the products after enzymatic digestion are summarized schematically in **Figure 1**.

2.3.1. Preparing Starch Solutions

Starch solutions were prepared by heating aqueous dispersions of 2.5% w/w in an autoclave (Model I, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to 145 °C under continuous stirring (300 min^{-1}) for 30 min and subsequent high-shear-treatment using an Ultra-Turrax T25 (IKA-Werke GmbH & Co. KG, Staufen, Germany) at 24,000 min^{-1} for 2 min at about 80 ± 5 °C. An

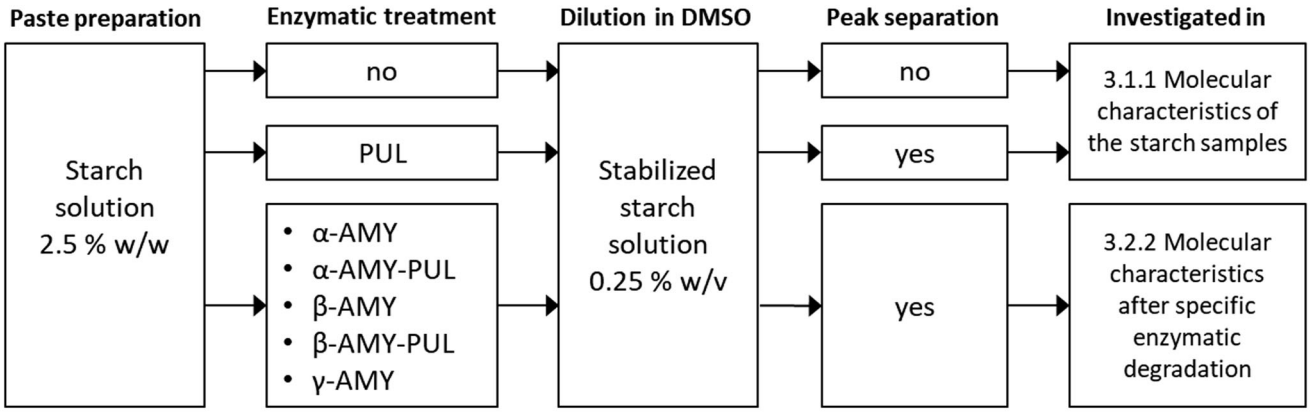

Figure 1. Schematic representation of the different preparation steps for the SEC based molecular characterization.

aliquot of the dispersion was diluted 1:10 (v/v) in preheated DMSO (2.5 mg mL^{-1}), and the stabilized solution was passed through a 5 µm PTFE filter (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) before analysis (SEC-MALS-DRI, no enzymatic treatment).

2.3.2. Specific Enzymatic Degradation

A volume of 10 mL of the freshly prepared starch solution (2.5% w/w) was tempered to 40 °C and the respective volume of the enzyme solution was added (α-AMY: 150 µL, β-AMY: 100 µL, γ-AMY: 150 µL, PUL: 187 µL). For the degradation using enzyme combinations (α-AMY-PUL and β-AMY-PUL) the respective volumes of both enzymes were added. The dispersion was gently stirred at 40 °C for 20 min. After enzymatic digestion, the solution was heated to 95 °C and tempered for 20 min for termination of the enzyme. The solution was diluted 1:10 (w/v) in preheated DMSO and passed through a 5 µm PTFE syringe filter (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) before analysis.

2.3.3. Separation Technique

The molecular characterization of the polydisperse solutions was carried out by means of SEC-MALS-DRI. The separation was executed with an SEC-3010 module (WGE Dr. Bures GmbH & Co. KG, Dallgow-Döberitz, Germany) including degasser, pump, and auto sampler connected to a MALS detector and a differential refractive index detector (DRI). The MALS detector was a Bi-MwA (Brookhaven Instruments Corporation, Holtsville, NY, USA) fitted with a diode laser operating at $\lambda = 635$ nm and equipped with seven detectors at angles ranging from 35° to 145°. The DRI was a SEC-3010 RI detector operating at $\lambda = 620$ nm. Three columns in a row were used: AppliChrom ABOA DMSO-Phil-P-100 (100–2500 Da), P-350 (5–1500 kDa), and P-600 (20 to >20 000 kDa) (Applichrom, Oranienburg, Germany). The samples were eluted with degassed DMSO (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) containing 0.1 M $NaNO_3$ at a flow rate of 0.5 mL min^{-1} and a temperature of 70 °C. During the sample run on the SEC-MALS-DRI system (single determination), the data from the MALS and DRI detectors were collected and processed using ParSEC Enhanced V5.61 chromatography software to give the concentration of the eluted solution and MM at each retention volume (M_i). The basis for the molecular characterization by means of SEC-MALS-DRI has been described elsewhere.[36,37]

The separation system was additionally calibrated (SEC-cal-DRI) using a set of 10 pullulan standards (800k, 400k, 200k, 110k, 50k, 22k, 10k, 6k, 1.3k, and 342) as well as Glc with a MM range between 180 and 805 000 g mol^{-1} (PSS Polymer Standards Service GmbH, Mainz, Germany). The calibration limit (800k) is indicated in **Figure 2**A. The standards were dissolved in DMSO (2.5 mg mL^{-1} w/v) and gently stirred 24 h at 80 °C. The standard solutions were measured and the elution volume at the position of the peak maximum was used as the reference for the particular M_i and the calculation of the calibration curve. The calibration related to the DP was calculated from the M_i divided by 162. The weight average DP (DP_w) was calculated from the M_w divided by 162.

2.3.4. Mathematical Peak Separation

The SEC-chromatograms (starch without enzymatic treatment [Section 3.1.1], enzymatically debranched by means of PUL [Section 3.1.1], digested with α-/β-/γ-AMY and α-/β-AMY-PUL [Section 3.2.2]) were advanced analyzed using peak separation and analysis software PeakFit Version 4.12 as described elsewhere.[38] Based on the fitted SEC-chromatograms, single peaks (chromatograms) representing different fractions were identified and calculated. The chromatograms originating from the enzyme solution (Section 3.2.1) were subtracted, and the relative chromatogram area of each separated fraction was taken as the relative amount. The values of M_w and DP_w were calculated by means of the correspondent separated chromatogram and the MM curve (fit) from the MALS-detector (SEC-MALS-DRI) or the standard calibration curve (SEC-cal-DRI), respectively, according to the description elsewhere,[39,40] Specific band broadening, induced by the injected volume or the injected polymer concentration, respectively, was excluded based on preliminary experiments. SEC-cal-DRI was used in Section 3.1.1 (characterization of the AP BC fraction [**Figure 3**]) and 3.2.2 (characterization of the obtained fractions [α-AMY: F2 and F4,

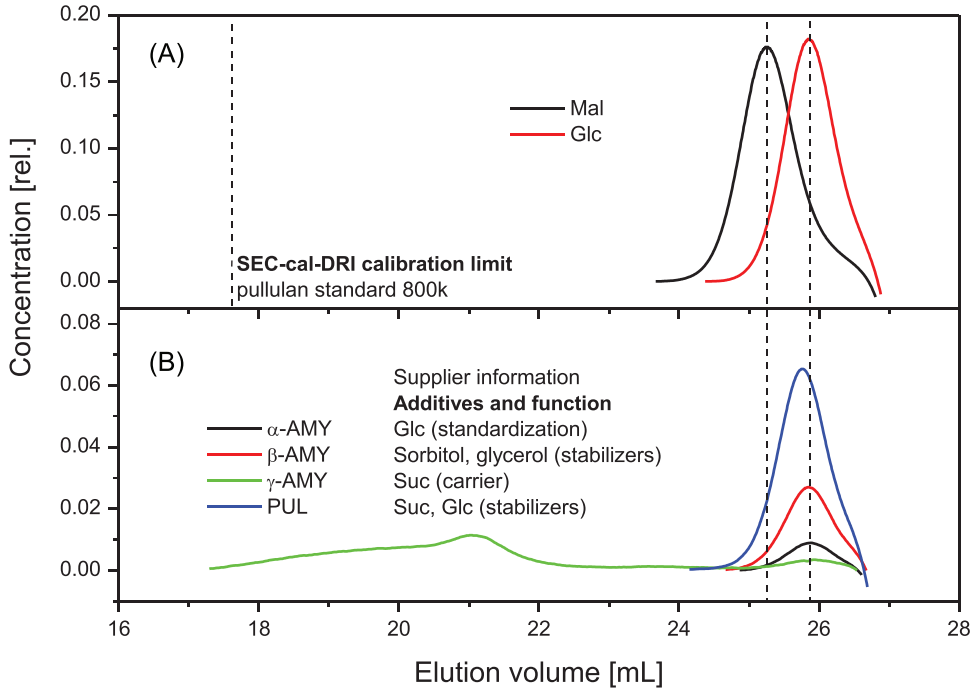

Figure 2. SEC-chromatograms of (A) Mal and Glc and (B) of the enzyme formulations (α-, β-, and γ-AMY as well as PUL) taking as reference or blank, respectively. The calibration limit (SEC-cal-DRI) is indicated.

α-AMY-PUL: F3, β-AMY: F3, β-AMY-PUL: F3; **Figures 4** and **5**]). The applied method was indicated properly.

2.4. Determination of the AM Content

The AM content of the starch genotypes was determined by means of different methods, which are summarized in **Table 1**.

2.4.1. BV Method and Calibration Curve

Methods 1–4: The blue value (BV) was determined according to the description elsewhere,[1,41] at 20±2 °C after defatting with EtOH (except method 4). The spectrophotometer used was a Jenway 6505 UV/visible (absorbance at $\lambda = 635$ nm). The calibration curve for the determination of the AM content of the starch samples was prepared over a range of 2–64% w/w AM using mixtures of a commercial WxCS (estimated AM content: 2% w/w, DM: 88.40% w/w; C*Gel 04201, Cargill B.V., Sas van Gent, Netherlands) and a HACS (nominal AM content: 64% w/w, DM: 88.40% w/w; Megazyme International Ireland, Wicklow, Ireland). Mixtures were 100:0 (2% w/w AM), 80:20, 60:40, 40:60, 20:80, and 0:100 (64 % w/w AM). There was a linear correlation (R^2 = 0.9953, n = 18) between the BV_{20} and the AM content [% w/w] (BV_{20} = 0.0399•AM [%] + 0.1763).

2.4.2. Defatting Using Organic Solvents

Methods 2–4: Partially defatted samples were prepared by treatment using two different solvent systems (TCM-MeOH [mixing ration 1:2 v/v] and BuOH). An aqueous starch suspension (50% w/w, 100 g) was prepared and a volume of 400 mL of the respective organic solvent system added. The system was stirred at 300 min^{-1} for 24 h at 50 °C. After an initial centrifugation step (10 min, 3000 min^{-1}), the obtained sediment fraction was again suspended in 200 mL of the solvent system and subsequently centrifuged (10 min, 3000 min^{-1}). After suspending the starch again in 200 mL of the solvent system, the starch was achieved by suction filtration (filter paper: DP 1574 125, ALBETLabScience, Hahnemuehle FineArt GmbH, Dassel, Germany). The samples were dried in a climate cabinet at 40 °C and about 35% RH for 24 h, and subsequently ground (pulverisette 14, Fritsch, Idar-Oberstein, Germany; 200 µm mesh), bottled in closed containers and stored at 20 ± 2 °C.

Method 4: The starch samples partially defatted using TCM-MeOH were disintegrated (Section 2.3.1). After high-shear-treatment (Ultra-Turrax), an aliquot of the starch dispersion was diluted appropriately and the BV determined (Section 2.4.1).

2.4.3. Peak Areas of SEC-Chromatograms

Methods 5 and 6: The peak areas of the separated chromatograms (Sections 2.3.4 and 3.1.1) were evaluated. On the basis of the non-debranched starch, three peaks were separated (chromatogram 1: AP fraction [F1], chromatogram 2: IM fraction [F2], and chromatogram 3: AM fraction [F3]), and F3/(F1+F2) × 100% w/w was taken as the AM content (method 5, **Figure 6**C,D). On the basis of the debranched starch, two peaks were separated and investigated (chromatogram 1: AM fraction [F1] and chromatogram 2:

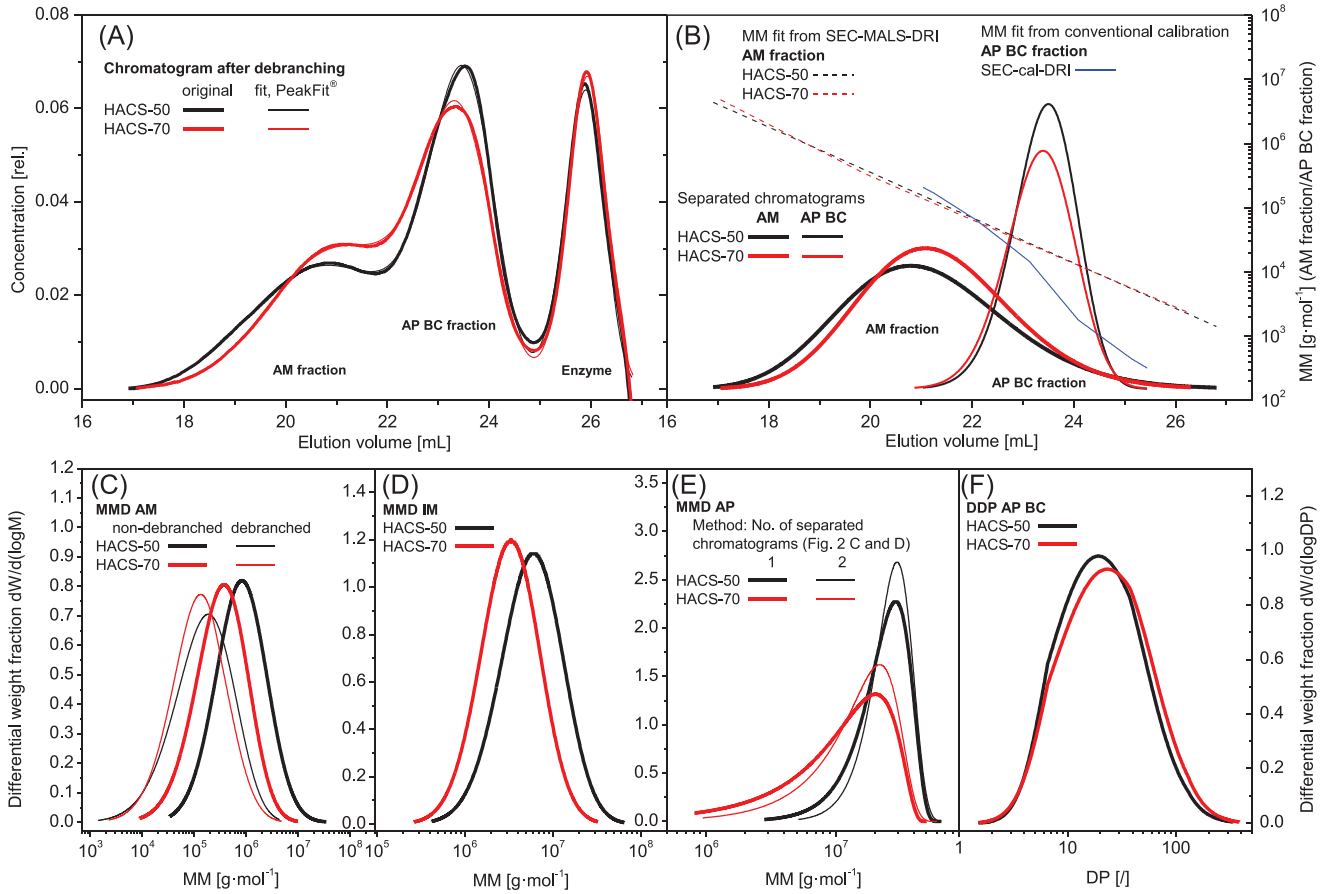

Figure 3. Molecular characterization of HACS-50 and HACS-70 after complete debranching. A) SEC-chromatograms after complete enzymatic debranching and respective fits (PeakFit), B) separated chromatograms for the AM fraction and the AP BC fraction based on A (PeakFit), C) calculated MMD curves of the AM fraction (derived from the Figure 6C,D without debranching and Figure 3B), D) calculated MMD of the IM fraction (without debranching, derived from the Figure 6C,D), E) MMD curves of the AP fraction (without debranching, derived from the Figure 6C,D), and F) DDP curve of the AP BC fraction (derived from the Figure 3B).

AP BC fraction [F2]), and F1/(F1+F2) × 100% w/w was taken as the AM content (method 6, Figure 3B).

3. Results and Discussion

3.1. Molecular Characteristics of the Starch Genotypes

3.1.1. Chromatographic Investigation

Molecularly dispersed solutions of both HACS-50 and HACS-70 were investigated by means of SEC-MALS-DRI. Figure 6A shows the chromatograms and the related MM fits of the genotypes. The first chromatogram peak between about 15 and 17.5 mL corresponds presumably for the most part to the AP fraction, and the different specific chromatogram areas and height, respectively, are ascribed to the different AP contents of the corn starches. However, the elution of AP of lower MM is strongly assumed to be concomitant with the AM of higher MM. The slightly different positions of the MM fits indicate differences regarding molecule structure as well as solution state, i.e., the possible existence of small particles. The MM fit of the HACS-70 is lower, most probably due to the significantly higher AM content of the sample eluting concomitantly with the highly branched polymer molecules. The high degree of branching and the compact structure, respectively, result in a higher MM of the polymers eluting at a specific elution volume. The huge differences between the corn starch varieties are clearly obvious in the calculated MMD curves in Figure 6B. The MMD of HACS-70 is broader and shifted to lower MM (PDI: 9.27 HACS-50 and 12.60 HACS-70), indicating lower MM in particular of the AM fraction. Since the AM content of the HACS-70 (colorimetric method, supplier: 73.8% w/w) is supposed to be significantly higher compared to the HACS-50 (nominal, supplier: 55% w/w), the corresponding M_w of the samples were strongly different (HACS-50: 7.54×10^6 g mol^{-1}, HACS-70: 3.63×10^6 g mol^{-1}; **Figure 7**).

Based on the SEC-chromatograms (Figure 6A), separated peaks were identified by means of deconvolution (Figure 6C: HACS-50, and D: HACS-70). Two different approaches resulted in two and three chromatograms, respectively, where F1 represents the AP fraction, and F2 an IM fraction (branched). Fraction F3 represents an AM containing polymer fraction (in the case of two separated chromatograms) and the AM fraction (in the case of three separated chromatograms), respectively. The chromatograms calculated for F1 using the different methods

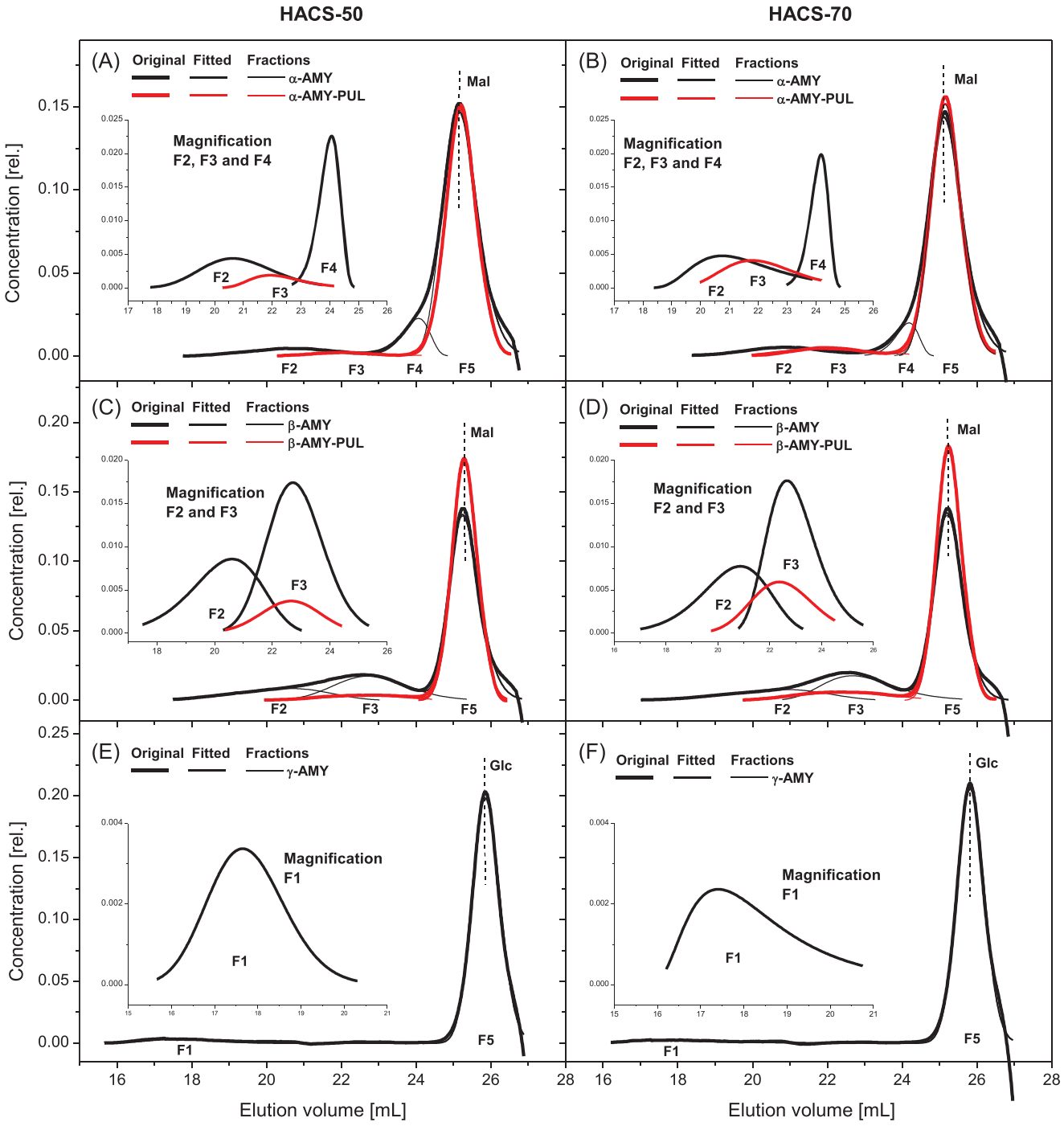

Figure 4. Molecular characterization of HACS-50 (A, C, and E) and HACS-70 (B, D, and F) after degradation using different enzymes/combinations (A and B: α-AMY and α-AMY-PUL, C and D: β-AMY and β-AMY-PUL, E and F: γ-AMY). SEC-chromatograms after treatment of the starch dispersion with enzyme/combination and subtraction of the respective blank (original), the chromatogram fits (PeakFit), and the separated chromatograms of the fractions F1–F5 calculated by means of mathematical deconvolution. Fractions F1–F5 correspond to elution volume at peak maxima of the chromatograms of about 17–18 mL (F1), 20–21 mL (F2), 22–23 mL (F3), 24 mL (F4), and 25–26 mL (F5; Mal/Glc).

were similar, and used for further investigation. Moreover, the existence of an IM branched fraction (F2) was obvious for both starches, and likewise, the remarkable AM fraction (F3). The AM contents determined using different methods is presented in Section 3.1.2.

For the purpose of an advanced and comprehensive molecular examination, the completely enzymatically debranched starch samples were investigated, too. Figure 3A shows the SEC-chromatograms of debranched HACS-50/-70 (original) and the respective chromatogram fits (PeakFit). Based on the fits,

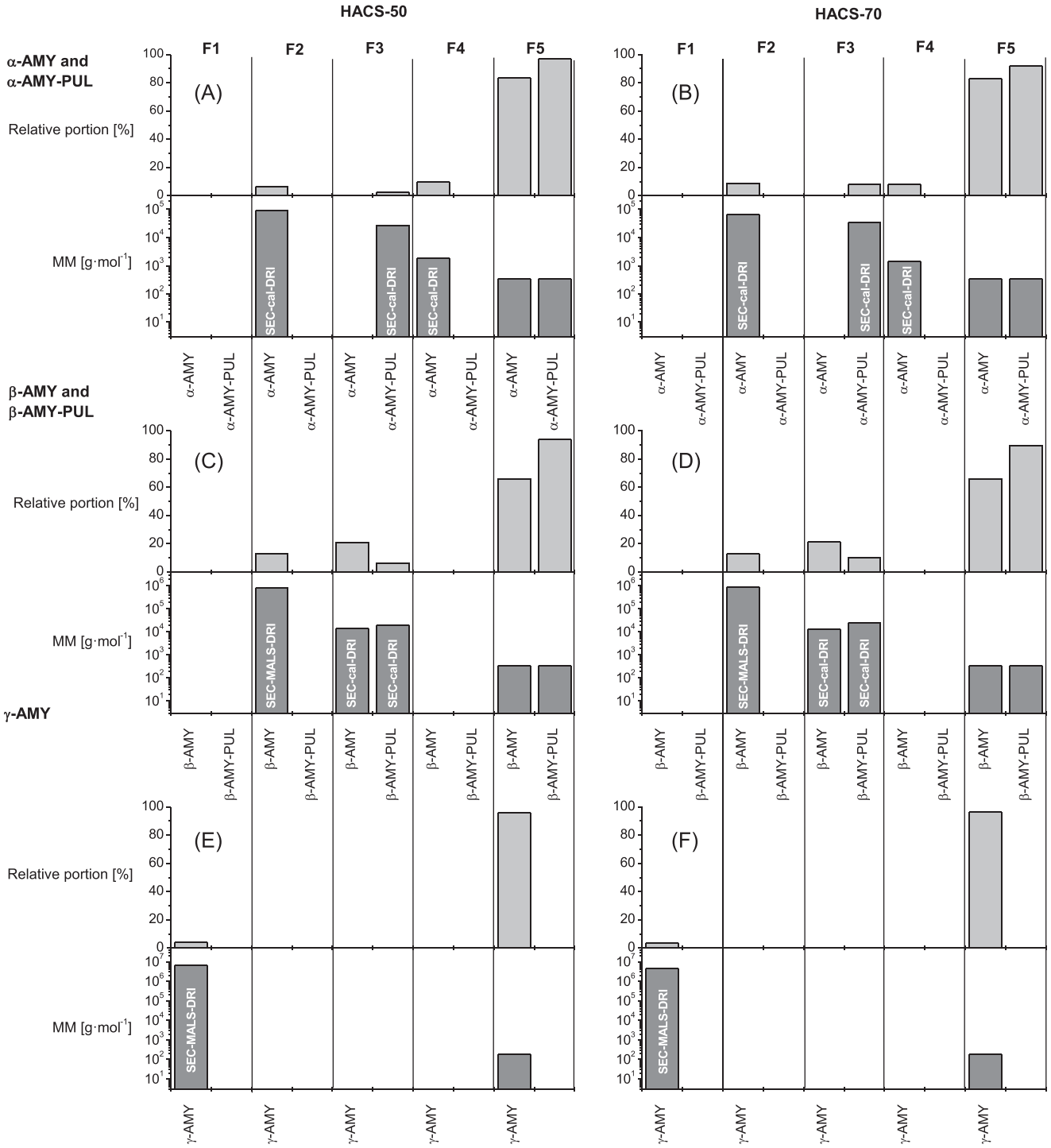

Figure 5. Relative portion and corresponding M_w of the different fractions generated by degradation of HACS-50 and HACS-70 using different enzymes/combinations (A and B: α-AMY and α-AMY-PUL, C and D: β-AMY and β-AMY-PUL, E and F: γ-AMY). SEC-technique (SEC-MALS-DRI, SEC-cal-DRI) used for calculation of M_w was indicated (F1–F4), and attribution based on the respective peak maximum elution volume of Mal and Glc, respectively (F5).

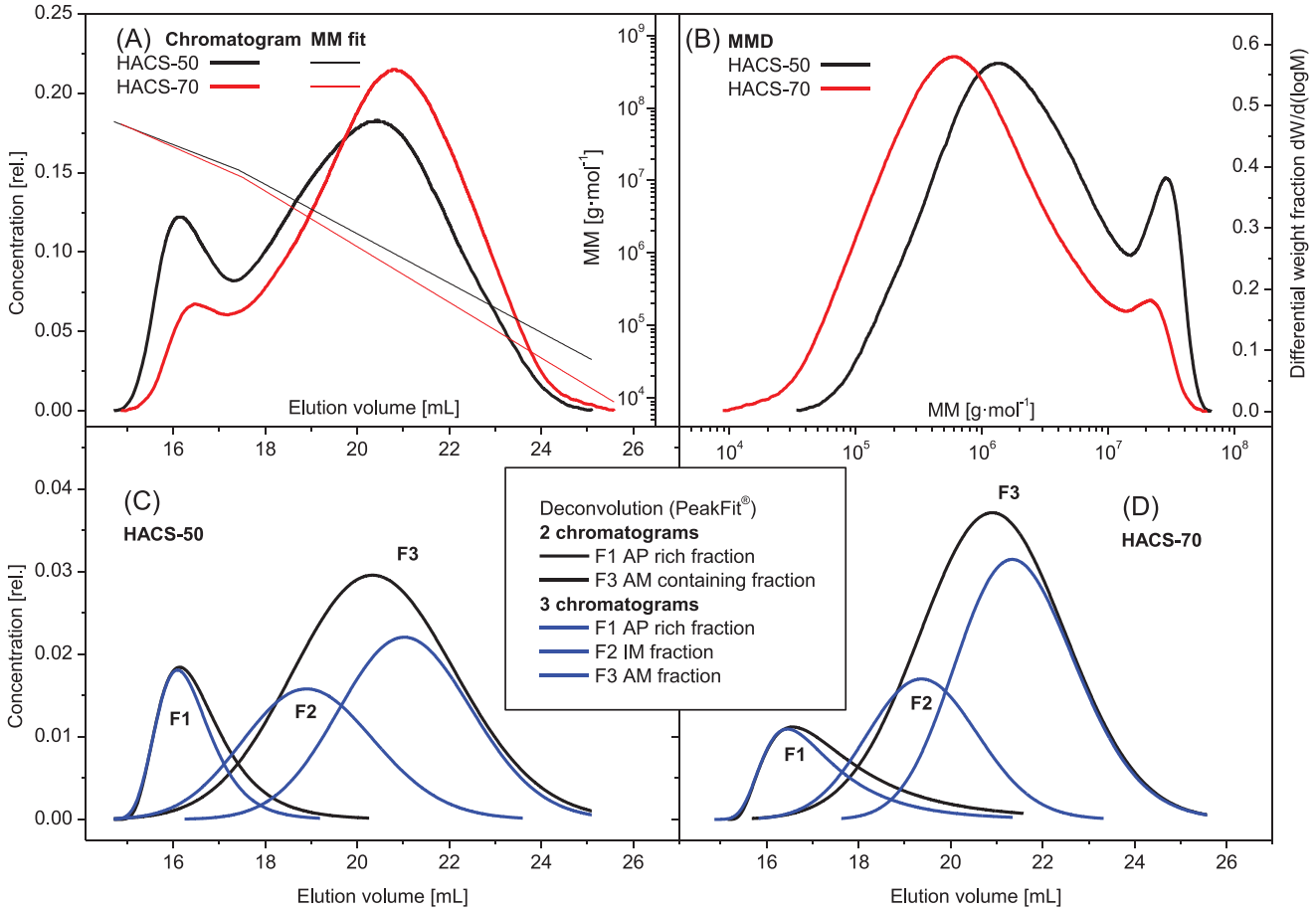

Figure 6. Molecular characterization of HACS-50 and HACS-70. (A) SEC-chromatograms of the starch samples and corresponding MM fits, (B) MMD curves of the starch samples, (C) separated SEC-chromatograms of HACS-50 (PeakFit), (D) separated SEC-chromatograms of HACS-70 (PeakFit).

Table 1. Methods (1–6) for the determination of the AM content with variation of the basic principle (colorimetric and SEC-technique), the defatting with EtOH, the starch basis (native and partially defatted), and starch state (granular and dispersed).

	Method	1	2	3	4	5	6
BV		•	•	•	•		
Including defatting/EtOH		•	•	•			
Native starch		•				•	•
Partially defatted starch	TCM-MeOH		•		•		
	BuOH			•			
Granular		•	•	•			
Dispersion/Autoclave					•	•	•
SEC technique (deconvolution)	Non-debranched					•	
	Debranched						•

chromatograms of the fractions ascribed to the AM, the AP BC as well as the enzyme solution (sucrose [Suc], Glc) were obtained by deconvolution (Figure 3B; separated chromatogram arose from enzyme solution is not shown).

By means of the MM fits (Figures 6A and 3B), specific molecular data like MMD curves (AM fraction in Figure 3C [SEC-MALS-DRI], IM fraction in Figure 3D [SEC-MALS-DRI], AP fraction in Figure 3E [SEC-MALS-DRI]) and differential DP (DDP) curves (AP BC fraction in Figure 3F [SEC-cal-DRI]), and corresponding M_w and DP_w (Figure 7, molecular properties), respectively, were calculated.

AM: The molecular properties of the AM fraction strongly depended on both method used and starch genotype in itself (Figure 7). The M_w AM of HACS-70 was found to be significantly lower (non-debranched: 5.84×10^5 g mol^{-1}, after debranching: 2.21×10^5 g mol^{-1}) compared to HACS-50 (non-debranched: 1.44×10^6 g mol^{-1}, after debranching: 3.05×10^5 g mol^{-1}), which is supported by the respective shifts of the MMD curves (Figure 3C). The existence of a very small amount of comparatively high MM polymers up to 4×10^6 g mol^{-1} within the AM fraction even after debranching is remarkable (Figure 3C). Since there is a significant difference between the M_w AM derived from the non-debranched and the debranched starch sample, the data suggest a (slightly) branched structure of the AM fraction. However, the respective portions calculated on the basis of the chromatogram areas were basically comparable (HACS-50: about 48% w/w, HACS-70: about 57 % w/w; Figure 7, relative portion).

IM: An IM structure fraction was identified as a single peak by means of deconvolution of the chromatogram of the non-debranched starch resulting in three fractions (F1–F3), and it is

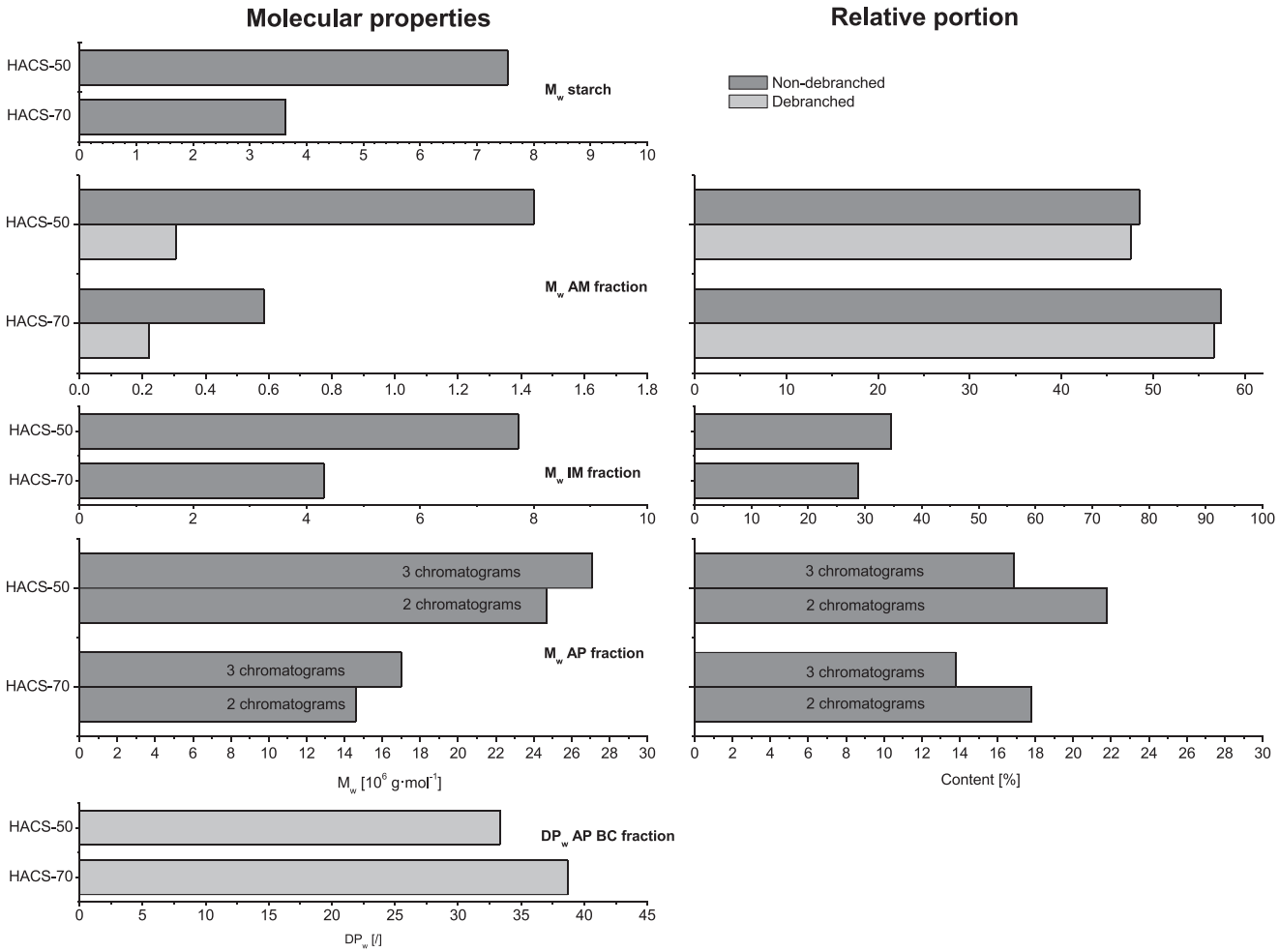

Figure 7. Molecular data of the HACS-50/70 (M_w starch [Figure 6A; SEC-MALS-DRI], M_w AM fraction: based on the respective chromatogram calculated by mathematical peak separation of the chromatogram derived from the non-debranched [Figure 6C,D; F3 with deconvolution to three chromatograms]/debranched starch [Figure 3B; SEC-MALS-DRI], M_w IM fraction, and M_w AP fraction: based on the respective chromatogram calculated by mathematical peak separation of the chromatogram derived from the non-debranched starch [Figure 6C,D; SEC-MALS-DRI], DP_w AP BC: based on the respective chromatogram calculated by mathematical peak separation of the chromatogram derived from the debranched starch [Figure 3B; SEC-cal-DRI]) from SEC experiments.

consequently a constituent of F3 (besides AM) resulting in two fractions using this method (Figure 6C,D). The IM fraction is of branched nature, and it differs in terms of molecular properties and relative amount dependent on the genotype (Figure 7). HACS-50, which has a lower AM content compared to HACS-70, has a slightly higher portion of the IM polymer fraction (about 35% w/w) compared to HACS-70 (30% w/w) exhibiting concurrently a remarkably higher MM (HACS-50: about 7.5×10^6 g mol^{-1}, HACS-70: about 4.3×10^6 g mol^{-1}). A differentiation between AP and the IM fraction is permissible owing to significantly different values of M_w. However, after complete debranching, the separated BC fraction was assigned to AP (Figure 3F).

AP: Since the AM content of the HACS-70 was higher compared to HACS-50 (nominal and determined), a respective lower AP content was expected, regardless of the existence of the IM fraction. Depending on the method used (Figure 6C,D), the AP content was estimated to be about 18 or 14% w/w (HACS-70) and 22 or 17% w/w (HACS-50) (Figure 7, relative portion). M_w AP of HACS-50 was found to be significantly higher ($25...27 \times 10^6$ g mol^{-1}) compared to HACS-70 ($15...17 \times 10^6$ g mol^{-1}). Moreover, differences between both genotypes were also found with respect to the AP fine structure. The DP_w of the AP BC fraction (Figure 7) of HACS-70 was higher (38.7) compared to HACS-50 (33.3) and the DDP curves differed (Figure 3F). Surprisingly, a small amount of BC was calculated to have a DP of up to 200–300 (Figure 3F), which is likely accountable to very long B- and C-chains. Since this is not confirmed by data published elsewhere,[42–44] the contribution of small molecules from the AM fraction is also conceivable.

3.1.2. AM Content Determined via Different Methods

Differences with respect to molecular properties of the genotypes (HACS-50/-70) are not only limited simply to the AM/AP

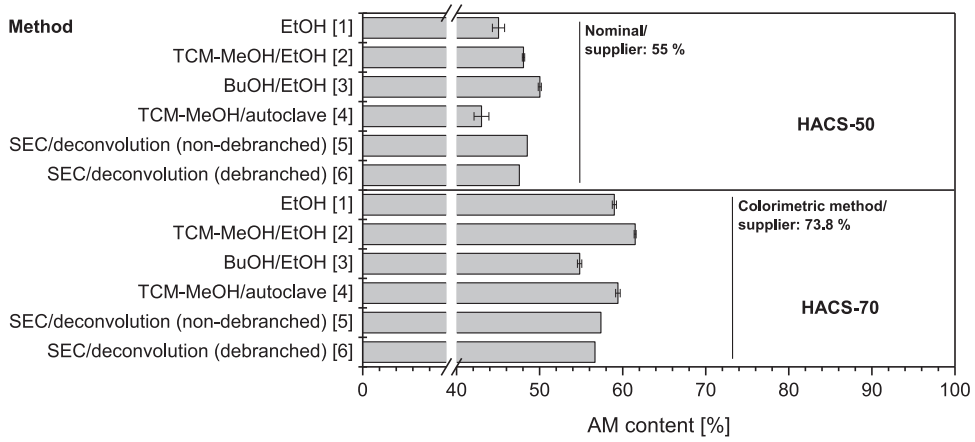

Figure 8. AM contents of HACS-50/-70 determined using different approaches: BV method including a defatting step using EtOH based on native/non defatted starches[1] and partially defatted starches[2,3], BV method based on partially defatted starches after disintegration by means of pressure cooking[4] without additional defatting using EtOH, and determination based on examining (including deconvolution) SEC-chromatograms of the native/non defatted starches non-debranched[5] and completely debranched[6].

ratio, but rather to all molecular parameters analyzed (Section 3.1.1). However, the AM content of the starch varieties was hereinafter comprehensively examined, since it is the most important attribute. The AM contents were determined using the BV method,[1,41] including a defatting step by means of EtOH (method 1–3, Table 1). Because such genotypes normally contain comparatively high amounts of lipids (Chen and Jane[12]), two differently defatted samples (TCM-MeOH/EtOH and BuOH/EtOH) of each genotype were included in addition to the respective native samples (method 2–3, Table 1). In addition, the determination was performed via BV after disintegration (method 4, Table 1) as well as based on examination of the SEC-chromatograms before (method 5, Table 1 [non-debranched]; Figure 6C,D) and after enzymatic degradation (method 6, Table 1 [debranched], Figure 3B).

The values calculated are summarized in **Figure 8**. All AM contents determined were lower compared to the expectation based on the supplier information (HACS-50: 55% w/w, HACS-70: 73.8 % w/w; nominal values) with a maximum of about 50% w/w for the HACS-50 and about 62% w/w for the HACS-70, respectively. Reasons are versatile and could be i.a. an incomplete dispersion of the starch polysaccharides by processing according to the method applied (method 1–3), despite the dissolving step using DMSO. In an aqueous system (HYLON VII, 1% w/w, heating from 50 to 95 °C, heating rate of 5 K min^{-1}), the maximum values for solubility as well as degree of gelatinization were found to be about 15 g g^{-1} and about 35%, respectively,[45] which is comparatively low. In particular, for HYLON VII (aqueous dispersion) it was suggested, that even temperatures of 120 as well as 140 °C are not high enough to gelatinize and disrupt the starch granules completely, even with continuous mechanical stirring (Wu et al.[46]). The high AM content and accordingly low AP content are surely responsible for the restricted swelling and prevention of complete disintegration owing to the preparation steps (Table 1, methods 1–3). Moreover, existing lipid remnants, despite the different defatting procedures applied, probably impact the formation of the AM-iodine complexes reducing the BV. In addition, the tendency of high AM starches to comparatively fast and comprehensive retrogradation could also influence the analysis of the AM content.[47,48] Consequently, the calculated AM contents would be quasi underestimated. Shi et al.[6] determined AM contents of 56.8±0.4 (HYLON V) and 71.0±1.6% w/w (HYLON VII) by means of potentiometric iodine titration method. They concluded possibly i) overestimation in the presence of branched molecules with long side chains which bind iodine, or ii) underestimation in the presence of linear low MM molecules which bind less iodine than normal AM. The AM contents reported by Duan et al.[49] for the same starches were slightly lower (HYLON V: 55.1 ± 0.6% w/w [potentiometric titration], 41.3 ± 5.6% w/w [colorimetric method]; HYLON VII: 66.5 ± 0.6% w/w [potentiometric titration], 54.3 ± 0.6% w/w [colorimetric method]), but accord reasonably with the values of the present study. Wu et al.[46] reported an AM content of 51.8% w/w for HYLON V and 66.2% w/w for HYLON VII (based on Con A). The impact of the analysis method on the calculated AM content is huge. Schwall et al.[7] investigated several high AM potato starches very systematically and found strongly different AM contents, which were up to 19% w/w higher with the potentiometric compared to the colorimetric method. The disintegration step using pressure cooking did not result in higher calculated AM contents (method 4, Figure 8), which supports the assumption of a significant impact of structural features. Shi et al.[6] proved a remarkably higher portion of branched structures (high MM and IM MM fraction) and corresponding lower portion of linear polymers in both starch varieties than the expected AP content would suggest. The portions of the linear fraction (HYLON V: 41.8%, HYLON VII: 54.4%) were in the same magnitude as the AM contents calculated in the present study as well as when reported by Duan et al.[49] based on the colorimetric method. Despite the fact of certain deviation dependent on the method used for examination of the AM contents (methods 1–4, Figure 8), the estimation of the AM content based on the analysis of SEC-chromatograms of the non-debranched (method 5, Figure 8) and the completely debranched starch (method 6, Figure 8) seems to provide reasonably reliable information. The branched nature of the AM fraction of the investigated corn starch varieties was evidenced.

Based on the results of the molecular characterization (3.1.1), both genotypes were evaluated as very different starches, which don't differ simply with regard to the AM/AP-ratio (Jane et al.[50]). In contrast, remarkable differences of the respective structure fractions were pointed out such as AM, which was found to be branched to a certain extent having a higher MM and degree of branching with lower content in the starch (Cheetham and Tao[51]). The existence of a branched IM fraction (Shi et al.[6]), which is not clearly associated with both either AM or AP, could be assumed. Moreover, the AP structure differed in terms of MM and fine structure (Jane et al.[50]). Higher AM content of the starch was found to be associated with lower M_w of the AP fraction and with presumably lower degree of branching. It is hypothesized, that the highly different starch polysaccharide compositions and structures have enormous impact on the enzymatic degradation and resulting specific products, respectively, as well as functional properties.

3.2. Enzymatic Degradation of the Dissolved Starch Samples

3.2.1. Blank Enzymes

Aiming for the detailed analysis of the degradation products after treating the starch genotypes (molecularly dispersed solution) with different types of commercial enzymes (*α*-, *β*-, *γ*-AMY, PUL), the specific contribution of each enzyme formulation to the SEC-chromatogram was investigated, since the solutions contain different low MM substances (e.g., Glc) for stabilizing effects. The specific contribution (chromatogram area) due to the additives of the enzyme solutions was considered as blank (*α*-, *β*-, and *γ*-AMY) in the following experiments, which was deducted directly from the chromatogram of the respective enzyme treated sample or separated by means of mathematical deconvolution after complete debranching (PUL; Section 3.1.1, Figure 3A,B). The latter is an appropriate methodical approach when debranching (Ulbrich et al.[52]), since the cleavage of the *α*-1,6-glycosidic bonds in the starch (basis, e.g., native starch, not depolymerized by modification) results (polymer) molecules (AM fraction, AP BC fraction [DP_w about 35]) significantly differ from mono- and disaccharides.

Figure 2 shows the SEC-chromatograms of the disaccharide Mal and the monosaccharide Glc A) as well as the enzyme formulations B). The chromatograms in Figure 2B having a peak maximum between about 25.8 and 26.0 mL elution volume are very probably assigned to Glc (*α*-AMY), sorbitol/glycerin (*β*-AMY), Suc (*γ*-AMY) as well as Suc/Glc (PUL). However, the broad and distinctive chromatogram region between 17.5 and 22.5 mL elution volume in the case of the *γ*-AMY is presumably related to a high MM polymer fraction ($M_w > 10^6$ g mol^{-1}) also contained in the solution (enzyme formulation).

3.2.2. Molecular Characterization after Specific Enzymatic Degradation

The starch genotypes were digested by means of the different enzymes (*α*-, *β*-, and *γ*-AMY) and enzyme combinations (*α*-AMY-PUL and *β*-AMY-PUL), and the resulting solutions were characterized using SEC. The respective blanks were subtracted from the chromatograms (Figure 4, SEC-chromatograms original) and based on the chromatogram fits (Figure 4, PeakFit), different molecule fractions generated due to specific enzymatic cleavage of the glyosidic bonds were obtained by deconvolution (Figure 4, fractions). Amount and molecular composition of the fractions generated were strongly dependent on both the starch genotype (HACS-50 and -70) and particularly the type of enzyme (*α*-AMY, *α*-AMY-PUL, *β*-AMY, *β*-AMY-PUL, and *γ*-AMY) digested with it. The classification of the fractions is presented in **Table 2**.

Table 2. Residual polymer/molecule fractions (F1–F5) owing to digestion using different enzymes/combinations (*α*-AMY, *α*-AMY-PUL, *β*-AMY, *β*-AMY-PUL, and *γ*-AMY) assigned to distinctive elution volume at peak maximum.

Range of elution volume at $peak_{max}$ [mL]		17–18	20–21	22–23	24	25–26
Enzyme/combination	Fraction	F1	F2	F3	F4	F5
α-AMY			•		•	•
α-AMY-PUL				•		•
β-AMY			•	•		•
β-AMY-PUL				•		•
γ-AMY		•				•

Degradation by means of *α*-AMY resulted at least three fractions (Figure 4A,B), at which F5 is attributed predominantly to generated Mal (relative portion about 83%, Figure 5A,B). Dependent on the genotype, slight differences were obvious for F2 and F4 (chromatograms Figure 4A,B), which is supported by the data with respect to relative portion and M_w (Figure 5A,B). In particular M_w or DP_w, respectively, of both fractions was slightly lower with lower AP content of the initial starch sample (F2: DP_w 555–405, F4: DP_w 11.4–8.6). Reasons for this are assumed to be the remarkably different molecular properties of both varieties. Particularly the AP and the IM fraction (branched structures) can cause small branched residuals (*α*-LDs) after degradation using *α*-AMY. Since both the M_w AP and M_w IM of HACS-50 were significantly higher compared to HACS-70, the MM of the respective *α*-LDs was accordingly. Fraction F4 could probably also be released from (slightly) branched structures, possibly from the AM. However, the existence of a high MM fraction (F3) after digestion using the enzyme combination (*α*-AMY-PUL) was not actually expected (Figures 4A,B and Figure 5A,B). Characterizing *α*-LDs derived from a WxCS, Lee and Hamaker[53] identified three sub-fractions (I–III) of branched *α*-LDs besides the linear fraction consisting of maltooligosaccharides.

Degradation using *β*-AMY resulted in at least three fractions (Figure 4C,D), similar to the treatment with *α*-AMY. Fraction F5 corresponds to the Mal, which is typically released due to specific cleavage of every 2nd *α*-1,4-glycosidic linkage starting on the non-reducing end of the polymer molecules and ending near the *α*-1,6-glycosidic linkages, the branches within the molecule structure (relative portion of Mal of about 66% for both HACS-50 and -70, Figure 5C,D). Fraction F2, having a relative portion of about 13% for both HACS-50 and -70 (Figure 5C,D), corresponds most likely to the *β*-LDs, the "backbone" of the AP. This is confirmed

by the high M_w calculated to be about $8–9 \times 10^5$ g mol^{-1}, although the level is low compared to literature data (Tester and Qi[30]). An accordingly lower portion of the β-LDs fraction was initially expected for HACS-70, since the AP content and the overall amount of branched molecules (AP and IM fraction) of HACS-70 is lower compared to HACS-50 (Figure 7). In particular, the ratio (contents) of β-amylolyse limits (F2 and F3, Figures 4C,D and 5C,D) and initial branched fractions (F1 and F2, Figure 6C,D) was in accordance with data published elsewhere.[54,55] Fraction F3 could originate from the IM fraction (branched), which means that it is another β-LDs fraction. A higher relative portion of F3 (about 21%, Figure 5) compared to F2 supports this assumption, since the ratio of IM and AP fraction is similar. Particularly F3 can also contain residues of partly retrograded AM, residues of small AM chains as well as residues of comparatively small branched dextrins (Zhao et al.,[56] and Manelius et al.[57]). Moreover, the contribution of AM chains degraded up to the first branch point considered from the non-reducing end is also possible or even likely in F3. The digestion using the enzyme combination (β-AMY-PUL) resulted in about 6% (HACS-50) and 10% (HACS-70), respectively, of a low MM fraction (F3), and Mal (F5; 94% and 90%), which is obvious from Figure 5C,D. Analogous to α-AMY-PUL, the existence of such fragments and also residues having α-1,6-glycosidic linkages are supposable.

Since γ-AMY, acting as an endo-enzyme (non-reducing end), hydrolyses both α-1,4-glycosidic as well as α-1,6-glycosidic linkages of each AGU, the most important product on a quantity basis was expected to be Glc, which is represented by F5 in Figure 4E,F with a percentage of about 96% for both starches (Figure 5E,F). However, a very small fraction (F1, relative portion <4%, Figures 4E,F and 5E,F) having a comparatively high MM was identified, too. The origin of F1 from the starch seems absolutely implausible, since residues after digestion having such high MM contradicts completely the mechanism and the specificity of the enzymatic hydrolysis. It is rather supposed, that the chromatogram area is caused by the enzyme formulation itself (indication in Figure 2B), since marginal variation in terms of the enzyme concentration or volume added, respectively, for both digestion experiment and preparation of the corresponding blank could not excluded.

The treatment with the specific enzymes resulted in an extensive molecular degradation of the starch polymers. Compared to the digestion with pure α-/β-AMY, which results in appreciable portions of α-/β-LD in addition to the release of Mal, the combined degradation with a specific debranching step included (α-/β-AMY-PUL) minimized residual polymers in the solutions investigated. Comparing the "pattern" after enzymatic digestion (resistant fractions), e.g., in terms of portion and molecular properties, can give an indication for the molecular structure of the degraded polymers on the one hand, and deliver possibly valuable information on the residual molecules protected from degradation by its structural features on the other hand.

4. Conclusions

A specific method including pressure cooking and subsequent high shear treatment was appropriate for preparing molecularly disperse solutions of the HACS, which is an essential prerequisite for the analysis via SEC. The comprehensive molecular investigation of the genotypes in the first part of the study, mainly by means of SEC-techniques and special processing of the chromatograms, provided new insights regarding the molecular composition. The differences are not limited simply to the AM/AP-ratio of the genotypes, but refer to basically all quantitatively (relative amount of different structure fractions) and qualitatively (e.g., MM, branched/not branched) analyzed molecular characteristics. The molecular properties of the initial starch seriously define the enzymatic digestibility of the starch polymers using different specific starch degrading enzymes and the properties of the resulting reaction products, respectively. This is important and should be considered, particularly when using the enzymatic digestion as part of a special and complex analysis method based on SEC experiments. The findings of the present study can conceivably support the investigation of microstructural features of aqueous starch gels, particularly the challenging interpretation of SEC-chromatograms of enzymatically degraded gel matrices, which is presented in a separate study.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Keywords

amylose content, enzymatic degradation, high amylose corn starch, molecular properties, size exclusion chromatography

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