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High Amylose Corn Starch Gels – A Molecular Investigation of the Network Constituting Polymers

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The relationship between microstructural features and mechanical strength of aqueous starch gels is investigated. The gels are prepared by systematic variation of the factors starch type (high amylose [AM] corn starches [HACS] having about 50 and 70% w/w AM; HACS-50/-70), starch concentration (6 and 9% w/w) and storage time (1 and 14 days). The gel matrices are separated by means of a centrifugation method, and two phases are obtained account for the dissolved starch (liquid phase) and the network constituting starch (swollen phase). Subsequently, the latter starch fraction is partially digested using two amylases (AMY) and in combination with pullulanase (PUL) [α-AMY, α-AMY-PUL, β-AMY, β-AMY-PUL], too. The starch samples (dissolved and network constituting) and the degradation products (polysaccharides after partial enzymatic hydrolysis) are characterized molecularly by means of size exclusion chromatography (SEC)-techniques, and amounts and molecular properties of specific fractions and molecule segments, respectively, are determined. A clear correlation between the specific involvement and contribution of the starch polymers (e.g., absolute amount, state, function) in the gel network and the mechanical gel strength is found. Particularly, the starch's AM content and the polymer concentration of the gel are evident as controlling factors in developing the gel's firmness.

1. Introduction

Starch is a versatile polysaccharide with many food and non-food applications. It normally consists of two different structure fractions, AM and amylopectin (AP). The polymers are based on α-D-glycosidic linked anhydroglucose units (AGU), at which AM is the largely non-branched (α-1,4-glycosidic linked AGU; M_w about 10^5–10^6 g•mol^{-1}) and AP the highly branched fraction (α-1,4-glycosidic linked AGU building the linear molecule sections, and additional α-1,6-glycosidic linkages forming the molecule branches; M_w about 10^6–10^9 g•mol^{-1}). The existence of an intermediate structure fraction (IM), which cannot be clearly assigned to both AM and AP, is commonly accepted for different starches (normal CS,[1] HACS,[2,3] different CS[4,5] pea.[6,7] The AM/AP ratio of common starches is about 20/80 to 30/70,[8,9] and pea starches have AM contents higher than 30% w/w.[10] However, so-called starch genotypes exhibit AM contents, which are highly different compared to normal starches (e.g., waxy or high AM varieties[11,12]). This inherent special feature, in particular in terms of the molecular composition of the starch, remarkably impacts the specificity of different important techno-/functional properties like hot paste viscosity and achievable mechanical gel strength.

The starch polymers are synthetized in the form of semicrystalline granules with alternating amorphous and crystalline structures. The fact of the supramolecular structure (granular state) accounts for the requirement of a disintegration process (e.g., pressure cooking) to convert the polymers in the presence of water to an aqueous starch paste, which is a prerequisite for most applications. Particularly, the utilization as a gelling agent requires a very high degree of disintegration of the starch polymers (first step), i.e., the complete loss of the starch's granular integrity, since the gelation is based mostly on retrogradation of dissolved starch polysaccharides, a partial rearrangement of polymer chain sections (preferentially of AM) upon cooling (second step).[13] The micro-/macro structures and associated gel characteristics (e.g., achievable mechanical strength) depend on several and partly interacting factors like starch source,[14,15] AM/AP-ratio[16,17] and molecular composition as well as polymer concentration,[14,18] solution state,[15] or the presence of lipids.[19] Moreover, with retrogradation, the mechanical properties are essentially controlled by the storage conditions.[16] The development of the gel matrix, including rigidity, molecular re-order, and increasing crystallinity is necessarily ascribed to AM (irreversible, short-term changes) and also the relative amount of AM available,[20] respectively, and long-term changes of the gel strength (enhancement) are attributed to reordering and ongoing crystallization processes mainly within the AP fraction (reversible).[21,22]

Altogether, crystallization of the AM fraction plays the major role in the sol-to-gel transition and formation of a mechanically stable gel structure,[21] which makes particularly high AM

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genotypes interesting for research involving the study of detailed gel buildup. AM gel structures show a dynamic nature (non-equilibrium), i.e., the diffusion-controlled aggregation processes govern the mechanical properties of the hydrated network.[21] Besides the formation of an elastic gel-network, the gelation is characterized by a development of opacity generally attributed to further chain aggregation, resulting in crystalline areas detectable by X-ray diffraction (XRD) experiments (slow process). The network formation of AM can be regarded as initiated by a phase-separation (demixing, fast process) in the homogenous sol, which yields to polymer-rich regions interspersed with polymer-deficient regions. The enhancement of local AM concentration in the polymer-rich regions enables interchain-associations; the partial crystallization within the polymer-rich regions occurs at a much slower rate than the demixing.[21] However, nucleation and limited growth of rod-shaped microcrystals occurring promptly during network formation were assumed elsewhere.[23] The specific cross-linking involves the cooperative molecular interaction of many residues from each participating AM chain and the formation of double-helices and interhelix interactions, respectively. These conformationally ordered segments show a B-type crystalline structure (XRD, immobile "solid-like" structures). They are interconnected by possibly more mobile amorphous single-chain segments with a conformation similar to those present in solution and "elastically active."[24] In general, growth and coarsening of AM gels may depend on molecular size of involved polymers, concentration of the system, and the overall gelation conditions.[25,26] The macromolecular organization within the 3-D microstructure of AM gels was described by Leloup et al.[27] (continuous model), including interconnecting network strands, with the crystalline portion (crystallites) embedded in an amorphous matrix. The molecule chain segments constituting the crystallites are disposed oblique to the microfiber axis building the infinite 3-D network. The network strands, which probably consist of continuous associated blocks and involved double helices, are linked to others by loops of amorphous amylose segments extent into the pores.[27]

The gel strength is an important application characteristic of starch when used as a gelation agent. The achieved mechanical gel firmness is generally adjusted by many factors such as starch type and the different specific molecular properties (e.g., AM content), the starch concentration in the aqueous system and the storage conditions. However, strictly speaking, the gel strength is supposed to be essentially dependent directly on the macromolecular organization, i.e., possibly the portion of crystalline structures. The absolute amounts (portion) and molecular properties (chain length, molecule branches) as well as the state (crystalline, amorphous) of molecule segments constituting the polymer-rich region of the gel matrix seem to govern the firmness in a complex way. Aiming to specify the (detailed) macromolecular contribution to the gel microstructure and a correlation to the mechanical strength, an elaborate method was used, including i.a. gel preparation (disintegration by pressure cooking; varied parameters starch [HACS-50/-70], starch concentration [6 and 9% w/w] and storage time [1 and 14 d]), separation of the gel matrix (polymer-deficient and polymer-rich phase, centrifugation), specific enzymatic digestion of the network constituting phase (α-AMY, α-AMY-PUL, β-AMY, β-AMY-PUL), and molecular characterization of the products (SEC-techniques). This is the second of a series of three publications.

2. Experimental Section

2.1. Starch Genotypes

Commercial native HACS genotypes (HYLON V [HACS-50] and HYLON VII [HACS-70], Ingredion Germany GmbH, Hamburg, Germany) were used for the gel preparation. 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 were 88.46% w/w (HACS-50) and 88.36% w/w (HACS-70).[28] Deionized water was used for all experiments.

2.2. Enzymes

Different enzymes were used for the examination, an α-AMY (powder, Optizym A 16 126 [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), and a PUL (solution, PromozymeD2 [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. Gel Preparation

Dispersions of the starches (HACS-50/-70) with different concentrations (6 and 9% w/w) were prepared according to Ulbrich and Flöter[29] based on pressure cooking and subsequent high-shear treatment. Gels were casted (containers: 30.0 mm diameter, 20.0 mm height) and stored at 5.5±1.5°C for two different durations (1 and 14 days).[30]

2.4. Gel Characterization

The methodical approach for the gel characterization including gel preparation, investigation of the mechanical strength, separation of the gel matrix phases, and molecular characterization of the gel network constituting starch before and after specific enzymatic digestion is represented schematically in **Figure 1**.

2.4.1. Mechanical Gel Strength

The examination was made according to the description elsewhere[30] with modifications. After storage, a fresh and planar surface was realized by cutting the gels. The mechanical strength of the gel matrix was determined by compression using a texture analyzer (Test Control II, Z1.0, 1kN, Zwick/Roell, Ulm, Germany) equipped with a cylindrical penetration probe (diameter 25.4 mm). The peak force [N] of the first penetration was taken as the gel strength. The experiments were carried out in triple determination; and the arithmetic average and the corresponding standard deviation were calculated.

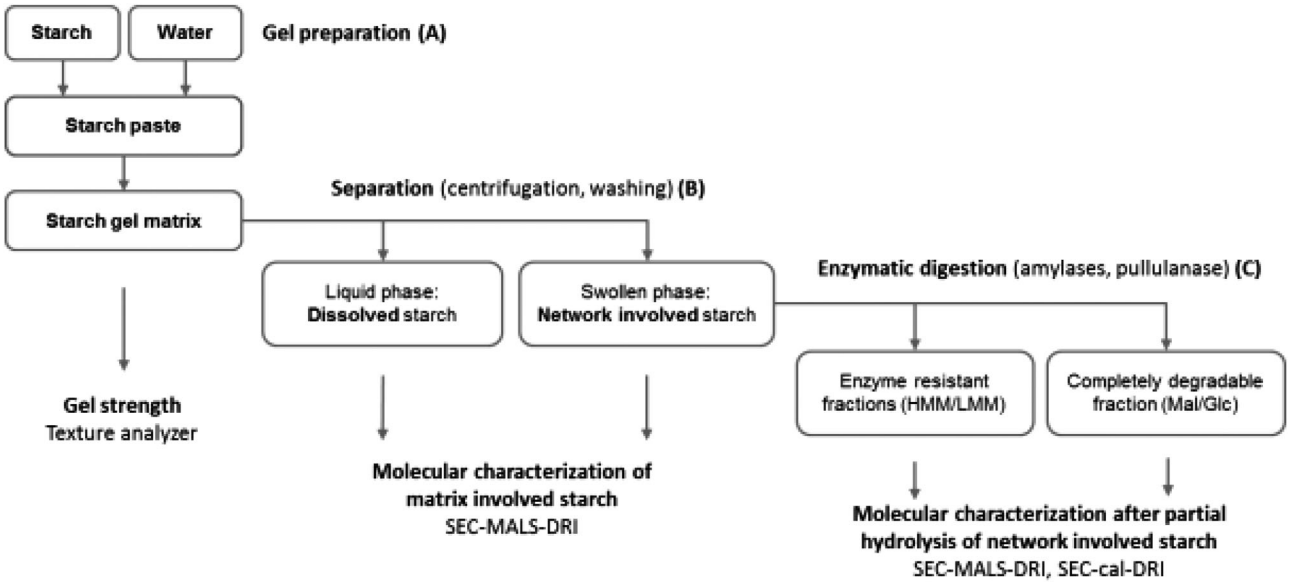

Figure 1. Schematic representation of the methodical approach of the starch gel preparation (A), the separation of the starch gel matrix into different constituent phases (B; networked polymers and dissolved polymers), and the isolation of enzyme resistant polymer residues from the gel network involved starch fraction (C).

2.4.2. Separation of the Gel Matrix and Molecular Characterization of the Phases

Preparation of Phases: In order to analyze the starch gels molecularly by means of SEC-techniques (SEC-MALS-DRI, SEC-cal-DRI), the samples were processed according to Ulbrich et al.[31] The gel matrix was diluted 1:1 (w/w) with water and subjected to a high-shear treatment (11 000 min^{-1}, 30 s) with an Ultra-Turrax T25 (IKA-Werke GmbH & Co. KG, Staufen, Germany). Afterwards, an aliquot of the sample was centrifuged (10 000 min^{-1}, 15 min; Biofuge 28RS, Heraeus, Hanau, Germany) for separation. The supernatant (SUP) was decanted (liquid phase; dissolved starch), and an aliquot stabilized by dilution 1:10 (w/w) in preheated DMSO. The sediment (SED) was blended with twice the amount of water (2500 min^{-1}, 30 s; mini shaker/vortex mixer, MS2, IKA-Werke GmbH & Co. KG, Staufen, Germany), and centrifuged again (10 000 min^{-1}, 15 min). The washed SED was obtained (swollen phase; network involved starch), and the SUP was discarded. Afterwards, 40 mL water was added to the SED and premixed (mini shaker/vortex mixer, 5 s) in order to completely obtain the SED out of the centrifuge cup. The sample (swollen phase) was subjected to a high-shear treatment (8000 min^{-1}, 30 s), and an aliquot of the homogenized sample was stabilized by dilution 1:10 (w/w) in preheated DMSO, and heated at 90°C for 48 h under continuous stirring for complete dissolving. The stabilized solutions (liquid phase, swollen phase) were passed through 5 µm PTFE filters (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) before analysis (SEC-MALS-DRI; 2.4.2.2). All calculations of the relative and absolute amounts were based on known concentrations of the gels, known dilution steps and amounts of phases obtained (e.g., after centrifugation, weighing) as well as determination of the concentration by means of SEC-techniques (detection of carbohydrate concentration).

Separation Technique: The molecular characterization of the 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 λ = 635 nm and equipped with seven detectors at angles ranging from 35° to 145°. The DRI was a SEC-3010 RI detector operating at λ = 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.[32,33]

The separation system was additionally calibrated (SEC-cal-DRI) using a set of 10 pullulan standards as well as glucose with a MM range between 180 and 805 000 g•mol^{-1} (PSS Polymer Standards Service GmbH, Mainz, Germany). 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.

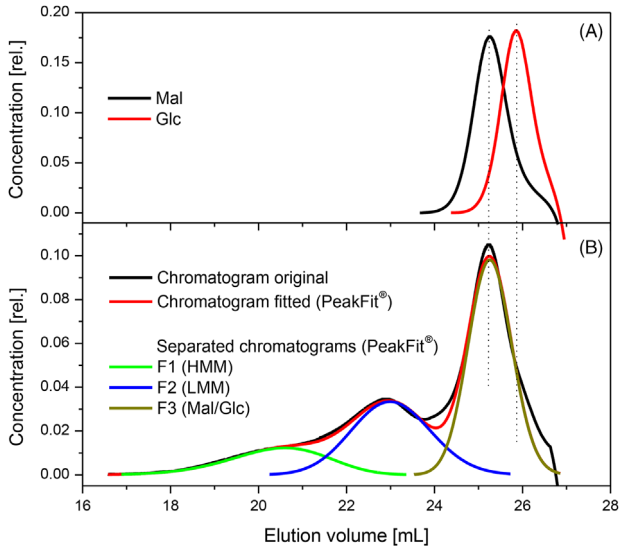

Figure 2. SEC-chromatograms of Mal and Glc (A), and the SEC-chromatogram of the gel structure (SED) of HACS-50 (9% starch concentration, 14 days storage time) after enzymatic digestion with α-AMY (original), the corresponding chromatogram fit, and the chromatograms obtained by means of mathematical peak separation (PeakFit) corresponding to different molecule fractions (F1-F3; B).

2.4.3. Partial Enzymatic Digestion of the Network-Involved Starch and Molecular Characterization

A mass of 10 g of the homogenized sample (swollen phase; 2.4.2.1) was taken, and the respective volume of the specific enzyme solution added (α-AMY: 500 µL α-AMY solution, α-AMY-PUL: 500 µL α-AMY solution and 200 µL PUL solution, β-AMY: 160 µL β-AMY solution, β-AMY-PUL: 160 µL β-AMY solution and 200 µL PUL solution). Hydrolysis was performed at 40°C for 45 min while continuously stirring, and the dispersion subsequently heated at 95°C for 20 min for enzyme deactivation. The solution was stabilized by dilution 1:10 (w/w) in preheated DMSO, and heated at 90°C for 48 h under continuous stirring for complete dissolving. The stabilized solutions were passed through a 5 µm PTFE filter before analysis (SEC-MALS-DRI and SEC-cal-DRI; 2.4.2.2).

The SEC-chromatograms of the enzymatically digested swollen phase (α-AMY/-PUL, β-AMY/-PUL) were advanced analyzed using peak separation and analysis software PeakFit Version 4.12 as described elsewhere.[34] According to **Figure 2** (exemplarily shown for HACS-50/9% w/w starch concentration/14 days storage time/digestion with α-AMY), single peaks (chromatograms) representing different fractions were identified. The SEC-chromatogram originating from the respective enzyme solution/formulation/preparation was subtracted,[28] and the relative chromatogram area of each separated fraction was taken for the calculation of the relative amount/portion. 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; F1 [HMM fraction]) or the standard calibration curve (SEC-cal-DRI; F2 [LMM fraction]), respectively, according to the description elsewhere.[28] The portion of F3, which corresponds to mostly Mal/Glc,[28] was taken as the degradable fraction.

2.5. Statistical Analysis

The impact of the different parameters (starch type [HACS-50/-70], starch concentration [6 and 9% w/w], storage time [1 and 14 days]) on different properties was investigated statistically using Statgraphics Plus 5.0 software (first experimental design: gel strength and properties of gel network involved starch [absolute amount and M_w], second experimental design: absolute amounts of resistant [F1 and F2] and degradable fractions [F3] after specific enzymatic digestion [α-AMY, α-AMY-PUL, β-AMY, and β-AMY-PUL]). The values for the probability of error (p-value) were listed in the ANOVA tables (analysis of variance; Tables 2 and 3). With a p-value less than 0.05, the factor investigated had a statistically significant effect with a 95.0% level of significance (indicated by boldface type in the ANOVA tables).

3. Results and Discussion

3.1. Gel Network Constituting Starch

The starch gels were separated by centrifugation aiming to obtain a gel network constituting starch fraction (swollen phase, superlattice) and the starch fraction dissolved in the liquid phase (enclosed, not molecularly bound, or entangled). **Figure 3** shows the weighted SEC-chromatograms (chromatogram area corresponds to the quantitative contribution within the respective gel phase) of the isolated starch fractions. Most starch polymers were found to be involved in the gel network (gel structure, SED; ≥ 95% w/w), and just a marginal portion was dissolved in the liquid phase (soluble polymers, SUP; ≤ 5% w/w). Since the predominant part of the starch used was network involved, the molecular composition of this fraction was very similar compared to the starting material. The M_w of HACS-50 and HACS-70 is about $7.54 \cdot 10^6$ and $3.63 \cdot 10^6$ g•mol^{-1}, respectively.[28] In the case of HACS-50, particularly the starch concentration of the gel impacted the M_w of the network involved starch systematically, which was lower at 6% w/w (about $6.7 \cdot 10^6$ g•mol^{-1}) and marginally higher at 9% w/w (about $7.65 \cdot 10^6$ g•mol^{-1}). For HACS-70, M_w of the network involved starch isolated was slightly lower ($2.8...3.3 \cdot 10^6$ g•mol^{-1}) compared to the initial starch (starting material used). Since the carbohydrate concentration was very low, the M_w of the respective dissolved starch fraction could not be calculated via the SEC-techniques. However, both phases when weighted should – theoretically – yield the same total molecular composition as the initial starch, which is effectively the case estimated from the measurements. Within the experimental design (starch varieties, concentration and storage time), the gel network constituting starch broadly equates the initial starch both quantitatively and qualitatively. This fact is, however, absolutely independent of the nature of the detailed involvement on a molecular and supramolecular level, namely, e.g., crystalline or amorphous state, which is assumed to impact the functional properties directly.

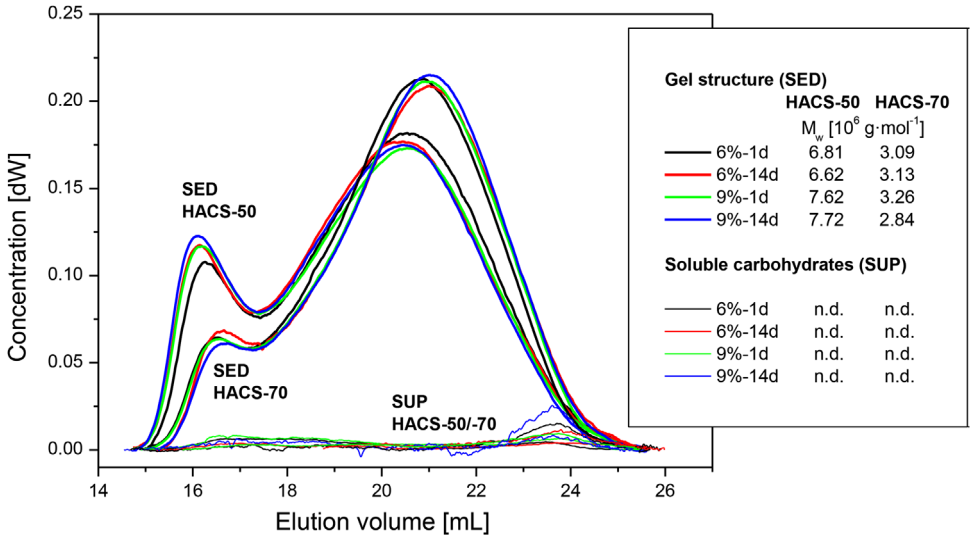

Figure 3. Weighted SEC-chromatograms of the starch involved in the gel network (SED) and starch polymers dissolved (SUP). The chromatogram areas correspond to the quantitative contribution within the respective gel phase. Gels were prepared by variation of the starch type (HACS-50/-70), the concentration (6 and 9% w/w) and the storage time (1 and 14 days).

Table 1. Enzymatic treatment (α-/β-AMY/-PUL), molecular fraction obtained (F1-F3, M_w range), and SEC-technique applied for detailed molecular characterization (MALS detection, conventional calibration).

Fractions obtained		F1 HMM[a)] [g•mol^{-1}]	F2 LMM[b)] [g•mol^{-1}]	F3 Mal/Glc[c)] [g•mol^{-1}]
Enzymes				
	α-AMY	130.000...432.000	8.780...16.900	●[d)]
	α-AMY-PUL	–	8730...11.800	●
	β-AMY	429.000...1.35•10^6	16.500...27.700	●
	β-AMY-PUL	–	15.600...21.700	●
enzyme combinations		SEC-MALS-DRI	SEC-cal-DRI	SEC-cal-DRI

[a)] High molar mass fraction; [b)] Low molar mass fraction; [c)] Fraction largely assigned to maltose and glucose, containing other structures is possible; [d)] Peak position determined accords to maltose.

3.2. Detailed Involvement of Molecule Fraction/Fragments within the Gel Network Fine Structure

As shown and discussed in Section 3.1 (see also Figure 3), most of the starch was involved in the gel network (≥ 95% w/w), and just a slight portion was dissolved in the liquid phase (≤ 5% w/w). For analytical purposes, the network constituting starch fraction was digested enzymatically using different enzymes (α-AMY, α-AMY-PUL, β-AMY, and β-AMY-PUL) and characterized chromatographically aiming for information in particular about the starch's state. Different molecular fractions (F1, F2, and F3) were identified and classified according to the MM level (**Table 1**), where F1 (HMM fraction) and F2 (LMM fraction) are molecule remnants not completely degraded to Mal/Glc, and F3 the product from (nearly) complete hydrolysis to Mal/Glc. In particular, the release of isomaltose is also possible for the digestion with α-AMY. The respective relative portions of the different degradation products are summarized in **Figure 4**.

The partial resistance to complete molecular cleavage (F1 and F2) is mainly attributed to the (inherent) molecular structure (1; linear [α-1,4-glycosidic linked AGU] or branched [α-1,4-glycosidic linked AGU and α-1,6-glycosidic linkages]), the specificity of the applied enzyme/enzyme combination (2; endo-/exo-/debranching enzyme), the participation or existence of molecule segments in highly ordered structures (3; double helical structures and crystallization [retrogradation], AM-lipid-complexes [resistant to amylolytic enzyme hydrolysis][35]) protecting the respective molecule sections from/to enzymatic cleavage of the glycosidic linkages,[36,37] and the interrelation of 1–3. In contrast, F3 is released from molecule segments, which are accessible and not resistant to the respective enzymatic attack (not protected by both molecule fine structure and supramolecular structure).

The HMM fraction F1 was one of the products owing to digestion of the gels with α- and β-AMY, respectively (Table 1). The origin is most likely the IM/AP fraction exclusively, and the relative portions ranged about 10–15% (α-AMY) and 5–20% (β-AMY), respectively. The M_w range of F1 (α-AMY) of about 130.000...430.000 g•mol^{-1} was significantly higher compared to a fraction ascribed to α-LDs in a previous study with 90.000 g•mol-1 (HACS-50) and 70.000 g•mol^{-1} (HACS-70).[28] This indicates

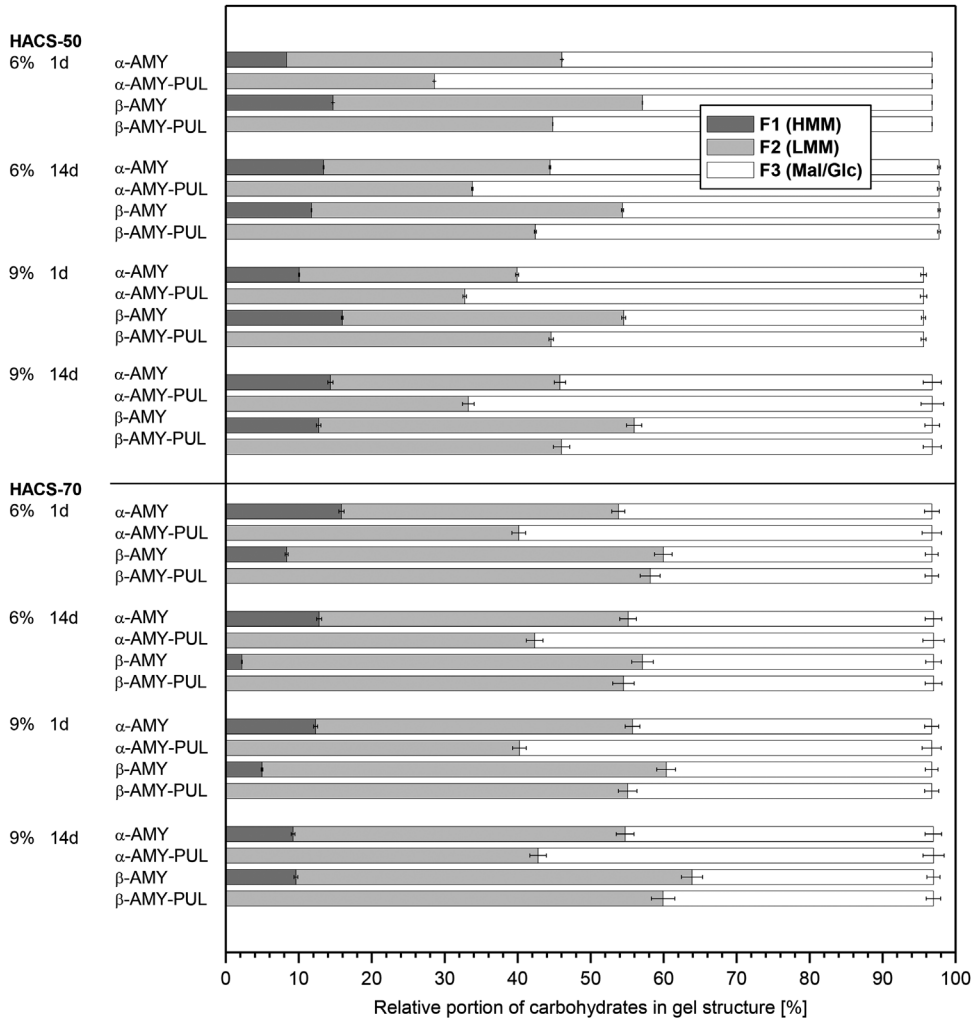

Figure 4. Relative portion of different molecule fractions (F1: HMM fraction, F2: LMM fraction, and F3: Mal/Glc resulting from complete hydrolysis) derived after specific enzymatic digestion (α-AMY, α-AMY-PUL, β-AMY, and β-AMY-PUL) of the starch gel structure.

a specific and enhanced protection of the respective polymers to enzymatic hydrolysis due to the involvement in the gel matrix. A different relationship was found for the HMM fraction F1 after digestion with β-AMY. Compared to the respective β-LDs (HACS-50: 800.000 g•mol^{-1}, HACS-70: 900.000 g•mol^{-1}),[28] the M_w of F1 was basically in the same range or slightly lower (430.000...1.35•10^6 g•mol^{-1}, one exception). Besides F1, the degradation of the gel network with α-/β-AMY resulted in a LMM fraction F2 with a M_w range of 8.780...16.900 g•mol^{-1} (α-AMY) and 16.500...27.700 g•mol^{-1} (β-AMY), representing molecule sections which are supposed to be largely constituent of crystalline regions within the gel network. The origin is presumably mostly the AM fraction, however, contribution of the branched molecule fractions isn't impossible and even likely, respectively.

The LMM fraction F2 after digestion with the enzyme combinations (α-AMY-PUL and β-AMY-PUL) represents the only enzyme resistant polymer fraction of the gel network involved starch. The M_w range is 8.730...11.800 g•mol^{-1} (α-AMY-PUL) and 15.600...21.700 g•mol^{-1} (β-AMY-PUL), respectively, and in particular the lower limits accord well with that of the F2 after α-/β-AMY treatment discussed before. F2, owing to digestion with α-AMY-PUL, ultimately represents the fraction of the polymer sections existent in the crystalline form or completely embedded in a crystalline block and hence are protected. The DP_w was calculated to be between 54 and 73, which accords basically well with literature data,[38] and the relative portion was about 29...34% (HACS-50) and 40...43% (HACS-70) of the total starch (Figure 4), which is remarkable. In contrast, F2 released after β-AMY-PUL digestion consists of longer chains on average (DP_w 96...134). Presumably, each chain is preserved by both the partial involvement in crystalline sections by itself (protected by supramolecular structure) and the limitation due to the fact that β-AMY is an exo-enzyme cleaving exclusively α-1,4-glycosidic linkages (every second) from the non-reducing end of the chain (protected by the enzyme's specificity). The relative portion of F2 (β-AMY-PUL) was about 43...46% (HACS-50) and 55...60% (HACS-70) of the starch (Figure 4), which is in average about 13% (HACS-50) and 16% (HACS-70) higher when compared to degradation with α-AMY-PUL. The differences regarding the relative portions (F2 of α-AMY-PUL as well as β-AMY-PUL)

Table 2. ANOVA of the impacts starch type (HACS-50/-70), starch concentration (6 and 9% w/w), and storage time (1 and 14 days) have on gel strength and starch in the gel network (boldface type: factor investigated had a statistically significant effect with a 95.0% level of significance).

Impact		Starch in gel network	
	Gel strength	Amount	M_w
Starch type	**0.0089**	0.4788	**0.0001**
Starch concentration	**0.0003**	**0.0000**	0.1775
Storage time	0.0647	0.1748	0.6899

between HACS-50 and HACS-70 are deeply rooted in the varying AM/AP-ratio. The higher AM content of the starch increases the relative portion of polymers or rather polymer segments, which are involved in crystalline structures.

F3 quantitatively represents the gel network involved starch and starch polymer chain sections, respectively, which were unrestrictedly accessible for the enzymes (*α*-AMY, *α*-AMY-PUL, *β*-AMY, and *β*-AMY-PUL) to complete hydrolysis to Mal/Glc. This fraction was calculated to be about 40–68% (HACS-50) and 33–57% (HACS-70), respectively, and represents the minimum of the starch within the gel network existing in the amorphous state.

3.3. Absolute Portions/Amounts of Different Molecule Fractions and Correlation to Gel Strength

The relative composition of the gel network involved starch and the state assigned to it were discussed in the above Section 3.2. However, the absolute content of the dissimilar existing starch (state) is more important, since a direct correlation to the resulting mechanical gel strength was assumed.

Tables 2 and **3** summarize the data from the statistical analysis (ANOVA), and **Figure 5** shows the gel strength and the absolute portions/contents of the enzyme resistant (F1 and F2) as well as the corresponding degradable starch (F3). Starch type and starch concentration are statistically significant impacts on the gel strength (ANOVA, Table 2 [gel strength], Figure 5A). Increasing AM content of the starch and increasing starch concentration in the gel enhance the mechanical strength remarkably. Higher storage time also increased gel firmness, but the impact was not found to be statistically significant at a 95% confidence level. Since nearly the total amount of the starch used for the gel was bound to the network, independent of the starch concentration adjusted in the system (6 or 9% w/w), the impact was highly significant (ANOVA, Table 2 [amount]). However, starch type (which includes the different AM contents of the genotypes) and storage time did not impact the amount of starch bound to the gel network. The fact that almost all starch was bound to the network explains that the molecular composition of the gel network bound starch was exclusively controlled by the starch type (HACS-50/-70), which is obvious from the ANOVA (Table 2 [M_w]) and Figure 3 (Section 3.1).

The impact of the gel's starch concentration on the mechanical strength is explicit (Figure 5A); the gel strength increases remarkably up to about 40 (HACS-50) and 50 N (HACS-70), respectively, due to enhancement from 6 to 9% w/w. However, the simultaneously strong influence of the starch type (varying AM content) indicates the different effect of both non-branched (AM) and branched (IM and AP fraction) on contributing to the development of a supramolecular structure and corresponding firmness. Moreover, a respective impact of the IM fraction,[28] which exists most likely in both starch varieties, seems self-evident. Digestion of the gel matrix with *α*-AMY caused about 1.0 % w/w (absolute) of the HMM fraction F1 (Figure 5B), independently on the parameters varied (ANOVA, Table 3). In contrast, the amount of the resistant HMM fraction F1 after degradation with *β*-AMY was impacted with statistical significance by the starch type (ANOVA, Table 3). The lower AM content of the HACS-50 resulted in a higher portion of resistant branched structures (Figure 5B). Since F1, which is allocated to structures similar to *α*-/*β*-LDs, does not exist after digestion in combination with PUL (*α*-AMY-PUL, *β*-AMY-PUL), the fraction is supposed to be present in the amorphous state, acting probably as a filler retaining water.

The portion of the LMM fraction F2 was found between about 2.0 and 4.0% w/w (absolute, digestion with *α*-AMY/-PUL) and between about 2.5 and 5.0% w/w (absolute, digestion with *β*-AMY/-PUL, Figure 5C), respectively. Both starch type and starch concentration were statistically significant factors in the respective resistant portions (Table 3). Increasing AM content of the starch as well as increasing starch concentration of the gel enhanced the portions. F2 isolated after digestion with *α*-/*β*-AMY-PUL, but particularly after *α*-AMY-PUL treatment, accounts for the molecule fraction largely present in the double helical and the crystalline state. A direct correlation between the absolute amount of the LMM fraction F2 and the gel strength is obvious from Figure 5A and C. The origin of the resistant fraction F2 is assumed to be largely the AM, since the significant involvement of (unbranched) chain segments with DP_w higher than 50 (Table 1) based on AP would be impossible. The participation of the IM fraction[4] is questionable because of the branched nature. The amounts of F2 (Figure 5C) are in all cases not higher than the (theoretical) accounted for AM in the respective gel systems, which are also indicated in Figure 5C.

The amount of the degradable fraction F3, which was determined to about 2.5-5.5% w/w (*α*-AMY/-PUL) and about 2.0-5.0% w/w (*β*-AMY/-PUL), respectively, is dependent on both the starch type and the starch concentration of the gel (Figure 5D). The factors are statistically significant (Table 3, exception: starch type/*β*-AMY). On the one hand, fraction F3 decreases with an increasing amount of AM in the system, since the unbranched AM is more protected from the enzymatic degradation due to large scale involvement in the double helical and crystalline areas as discussed in the previous section. Simultaneously, the content of branched polymers (IM, AP), which are more exposed to complete degradation to Mal/Glc due to the amorphous state and associated accessibility, is lower. On the other hand, the increased amount of starch in the gel system (enhancement of the starch concentration) increases the amount of degradable material (Figure 5D). Compared to the treatment with *α*-/*β*-AMY, the amount of F3 increased generally when digested using the enzyme combination (*α*-/*β*-AMY-PUL). That increase of F3 (*α*-/*β*-AMY-PUL) basically accounts for the complete disappearance of F1, the digestion of the branched fraction associated with *α*-/*β*-LDs as discussed before. Moreover, the relationship of F2 and F3 is very interesting when comparing the degradation based on *α*-/*β*-AMY. The higher

Table 3. ANOVA of the impacts starch type (HACS-50/-70), starch concentration (6 and 9% w/w), and storage time (1 and 14 days) on absolute amounts of resistant (F1 and F2) and degradable fractions (F3) after specific enzymatic digestion (α-AMY, α-AMY-PUL, β-AMY, and β-AMY-PUL) of the starch gel structure (boldface type: factor investigated had a statistically significant effect with a 95.0% level of significance).

Enzyme	α-AMY			β-AMY		
Fraction	resistant		degraded	resistant		degraded
	F1 (HMM[a])	F2 (LMM[b])	F3 (Mal/Glc[c])	F1 (HMM)	F2 (LMM)	F3 (Mal/Glc)
Impact						
Starch type	0.8237	**0.0380**	**0.0079**	**0.0253**	**0.0064**	0.0520
Starch concentration	0.1743	**0.0117**	**0.0010**	0.0583	**0.0014**	**0.0018**
Storage time	0.7496	0.8626	0.7561	0.5499	0.5081	0.8782

Enzyme	α-AMY-PUL		β-AMY-PUL		
Fraction	resistant	Degraded	resistant		degraded
	F2 (LMM)	F3 (Mal/Glc)		F2 (LMM)	F3 (Mal/Glc)
Impact					
Starch type	**0.0005**	**0.0004**		**0.0037**	**0.0032**
Starch concentration	**0.0001**	**0.0000**		**0.0004**	**0.0010**
Storage time	0.0527	0.1114		0.7605	0.9870

[a] High molar mass fraction; [b] Low molar mass fraction; [c] Fraction largely assigned to maltose and glucose, containing other structures is possible.

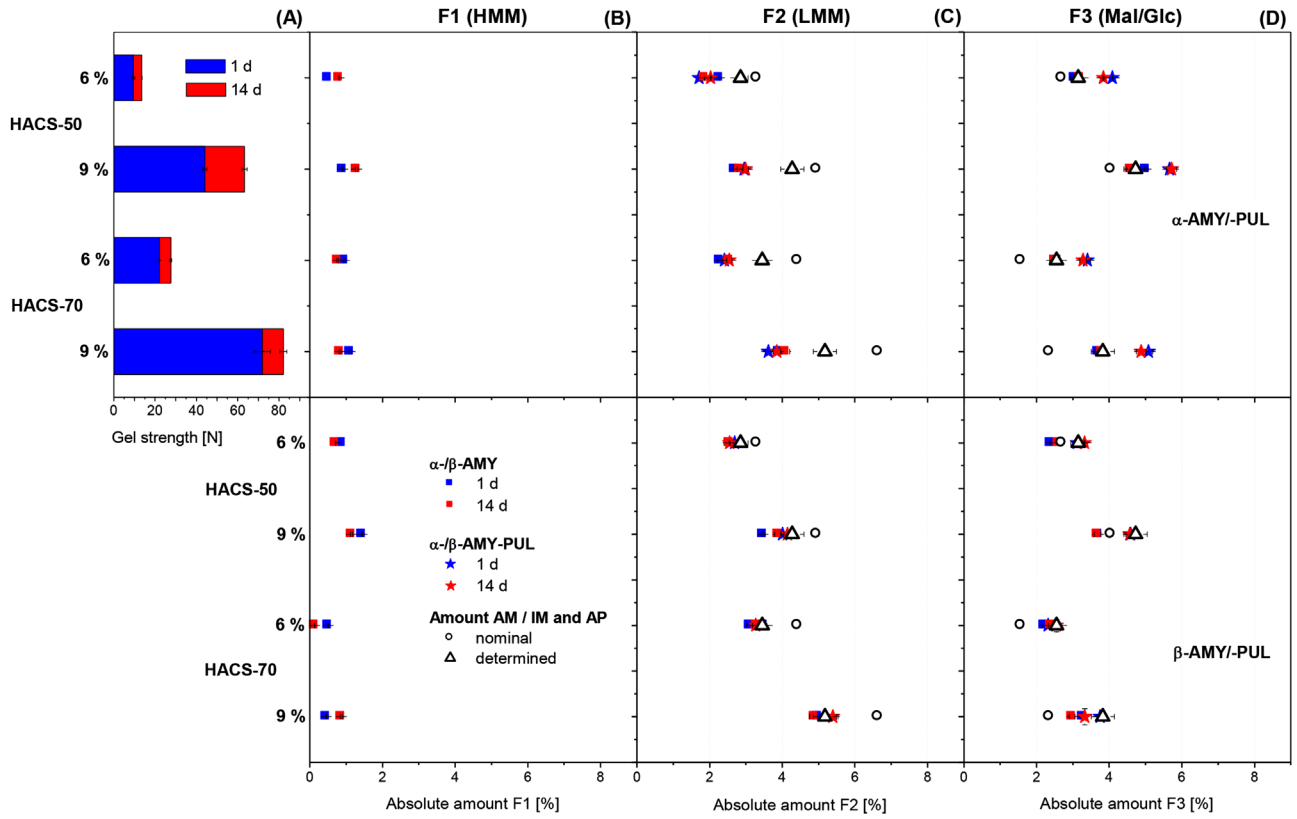

Figure 5. Gel strength (A) dependent on starch type (HACS-50/-70), starch concentration (6 and 9% w/w), and storage time (1 and 14 days), and absolute contents of different resistant molecule fractions (B and C: HMM F1 and LMM F2) as well as the degraded fraction (D: F3, completely digested to Mal/Glc) derived after specific enzymatic digestion of the gel matrix (α-AMY, α-AMY-PUL, β-AMY, and β-AMY-PUL). The calculative amounts of AM (indicated in C) and AP/IM (indicated in D) in the gel are included (determination in Ulbrich et al., 2021).

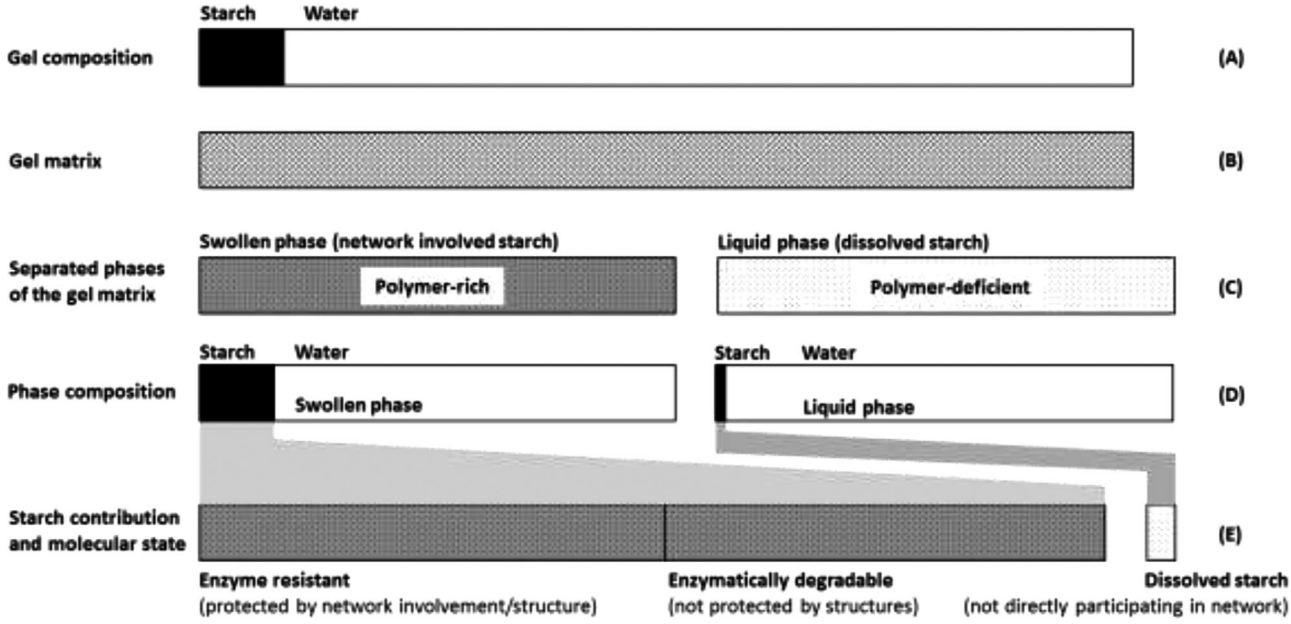

Figure 6. Starch gel composition (schematic).

amount of enzyme resistant fraction F2 results in a lower amount of F3, and vice versa.

3.4. Gel Microstructure – Contribution of the Starch Polysaccharides within the Gel Matrix

Aqueous dispersions of the starch genotypes (HACS-50/-70; detailed molecular investigation in a previous study[28]) with different concentrations (6 and 9% w/w) were prepared by pressure cooking and subsequent high-shear treatment of the paste (**Figure 6**A). Any granular remnants within the aqueous system were largely precluded by the procedure. The gels were casted and stored (1 and 14 days). For analytical purposes, the gel matrix (Figure 6B) was separated by centrifugation into a liquid and a swollen phase (Figure 6C), where about 5% of the starch were proved to be dissolved in the liquid phase (SUP; low polysaccharide concentration, polymer-deficient), and about 95 % were found to exist as a highly hydrated starch based matrix (SED; high polysaccharide concentration, polymer-rich) (Figure 6C and D). Since the starch used, independent of the varied parameters, was almost completely bound to the matrix (or entangled; existent in the swollen phase), the molecular composition of the latter accorded well with that of the initial starch, i.e., both branched (AP/IM fraction) as well as largely non-branched (AM) polymer fractions are effectively matrix-constituting.

Within the gel matrix (network), the branched polymer fraction (AP/IM) appears to exist entirely in the amorphous state (exposed to enzymatic digestion), most likely connected via intermolecular associations to the microscopic network somehow or other or embedded within. In contrast, the largely non-branched structure fraction (AM) most probably constitutes the network strands (filament, microfiber), which are of semicrystalline character containing crystalline blocks (preserved from enzymatic digestion) (Figure 6E). These crystalline sections are estimated to be about 20–26 nm in length,[27] since the molecule segments inside the crystallites possess a DP_w between 54 and 73 (double helices or B-type crystalline, 6 AGU per helical turn [left-handed, 6-fold helices], pitch per helical turn about 2.10 nm[39]). A direct relationship between the absolute portion of the enzyme resistant carbohydrate fraction (particularly when digested with α-AMY-PUL) and the gel strength is evident. In contrast, the dissolved starch within the liquid phase, which is not associated with the network, is most likely irrelevant concerning gel firmness. A foam-like structure of the gel is conceivable. In addition to a wide variety of reasons, the comparatively high degree of retrogradation caused the high turbidity of the gel (light transmittance 0.0%, results not amplified).

4. Conclusions

A very complex method for the microstructural examination of starch gels was applied and advanced, and the methodical approach was found to be suitable regarding the study's specific issue. The starch polymer fraction included within the swollen gel phase – constituting the actual matrix network – was analyzed in detail, particularly with respect to the state of different existing segments (enzymatically degradable/resistant; amorphous/crystalline) and the molecular properties. An adaptive classification was conducted, and by means of calculation of weighted amounts, both the relative involvement of the starch polysaccharides within the organization levels of the starch's network and the absolute amounts of distinct fractions within the aqueous starch gel were considered. The correlation of the apparent gel strength to quantities of specific network-constituting starch polysaccharide structures is impressive. The increase of the gel firmness from 10 (HACS-50, 6% w/w, 1 d) to about 80 N (HACS-70, 9% w/w, 14 d) is accompanied by an enhancement of the AP/IM fraction (branched, amorphous) from about 3 to 4% w/w and concurrently, of the double helical/crystalline

portion from about 2 to 4% w/w in the gel microstructure, which illustrates the dependence of functional properties on molecular parameters and the superior structure (supramolecular) of the network. The latter are governed by the inherent molecular composition of the starch, the concentration as well as the storage conditions. The results of the study provide a new insight in the starch gels microstructure on a molecular and supramolecular level, and can support the specific industrial application of starch products as a gelling agent.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M.U. contributed to Conceptualization, Methodology, Software, Validation, Writing - Original Draft, Writing - Review - Editing, Visualization, Supervision, Project administration. F.S. contributed to Data curation, Validation, Formal analysis. E.F. contributed to Head of department, Resources.

Data Availability Statement

Research data are not shared.

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