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Enzymatic Modification of Granular Potato Starch Using Isoamylase—Investigation of Morphological, Physicochemical, Molecular, and Techno-Functional Properties

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Granular potato starch is modified using the debranching enzyme isoamylase. The modification is performed in aqueous suspension (40% w/w) at 35 °C by grading the volume (100, 250, and 400 µL/50 g of starch, 200 U · mL^{-1}) of enzyme solution added. The starch products obtained are comprehensively investigated in terms of morphological (scanning electron microscopy), structural (X-ray diffraction), thermal (differential scanning calorimetry), techno-functional (solubility, hot paste viscosity, gel strength), and molecular properties (size exclusion chromatography-multi angle laser light scattering-differential refractive index detection). The granular integrity is basically preserved after modification and a molecular degradation predominantly of the amylopectin by debranching is proved. However, a slight reduction of the weight average molar mass of the amylose fraction is found too. In addition, the intended partial molecular degradation of the starch polysaccharides, the effect of the preparation procedure including washing with ethanol and grinding impacts several starch characteristics conspicuously.

1. Introduction

Potato starch (PS) is one of the most important starch types worldwide for food and non-food applications. The homoglucan consists of the two polysaccharide fractions, amylose (AM) and amylopectin (AP), at a ratio of about 25:75 (w/w).[1–3] AM is the largely linear polymer fraction consisting of α-D-1,4-glucosidic linked anhydroglucose units with a weight-average molar mass (M_w) between about 10^5 and 10^6 g · mol^{-1}.[4,5] In contrast, the AP fraction is characterized by the existence of additional branching points (α-D-1,4-glucosidic and α-D-1,6-glucosidic linkages, a highly branched molecule fraction). The M_w of the AP is up to about 10^8 g · mol^{-1}, whereas the average degree of polymerization (DP) of the AP branch chains fraction is about 20 to 35 with single chains types (B3 and B4) up to DP ≈ 100.[6] The AP fine structure causes the semi-crystalline nature consisting of alternating amorphous (branched regions of the AP) and crystalline (non-branched regions of the AP branch chains) lamellae and layers, respectively,[7,8] forming the granules. The AM is located in both amorphous and crystalline regions.[9]

Starch products are used in several food applications as, for example, a texturing or gelling agent.[10] However, the inherent properties of the native starch limit its industrial use for several reasons. To ensure the processability (technofunctional) on the one hand, and to achieve a distinct gelation of the starch as well as the formation of a mechanically stable gel structure (functional) on the other hand, a partial molecular degradation of the polymers is necessary, particularly of the AP-fraction. The most common modification approach with that objective is the acid hydrolysis of starch in its granular state (acid-thinning process, acid-thinned starch, thin-boiling starch). The semi-crystalline granular structure of the starch controls the accessibility for water and H_3O^+ ions, respectively, and hence basically determines the specificity of the molecular degradation. Moreover, the acid-induced cleavage take place on both α-D-1,4-glucosidic and α-D-1,6-glucosidic linkages. Since the acid penetrates into and acts randomly within the amorphous regions, the partial cleavage of the polymers occurs in both AP as well as AM.

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Altogether, the molecular degradation is not very specific in terms of the two different structure fractions. To form a gel, a transformation of the starch from the granular to the solution state is first required (preparation of a paste), which enables the subsequent partial retrogradation of the AM (formation of double helices, crystallization), thus promoting the associated development of a 3D AM network. The AP is embedded within the interspaces of the network,[11] which is the main reason for the necessity of the partial molecular size reduction. Based on that, a criterion of the (modified) starch for optimal gelation would be a reduced molecule dimension of the AP on the one hand, and an intact non-degraded AM fraction ensuring the comprehensive development of the network structure. These requirements are not fulfilled completely by acid-thinned starches, which are characterized by a compromise of favored degradation of the AP and acceptance of certain AM depolymerization.

A more specific alternative is the selective cleavage of the polymer branches (α-D-1,6-linkages) resulting in mostly partial AP degradation simultaneously without remarkable cleavage within the AM fraction. Besides pullulanase (PUL),[12] isoamylase (ISO; E.C.3.2.1.68, glycogen-6-glucanhydrolase) is an enzyme which acts exclusively on the α-1,6-glucosidic linkage of the starch polysaccharides.[13,14] ISO is obtained from different sources, for example, from *Pseudomonas* sp.,[15] *Pseudomonas amylodermosa*,[16] *Cytophaga* sp.,[17] *Flavobacterium* sp.,[18] or potato tubers,[14] which accounts for different conditions (pH, temperature etc.) for optimal action. A challenge and probably a disadvantage of the enzymatic modification in the granular state is the non-thermic termination, a requirement for the maintenance of the semi-crystalline structure.

The influence of ISO on selected starch properties was evaluated in previous studies. A decreasing AP portion with increasing enzyme concentration was reported on the one hand and simultaneously increasing contents of reducing sugars as well as AM on the other hand (cassava root meal starch; 0.01%, 0.02%, 0.03%, 0.04%, and 0.05% to 200 g of starch and 135 mL of water).[19] However, the effect of the selective partial degradation of starch polymers on the molecular composition seems to be more complex and requires a detailed examination and an appropriate analysis. The specific enzymatic molecular degradation of the AP by means of PUL impacts the techno-/functional properties of the converted sample. The specific hydrolysis improves properties like thin-boiling behavior and ability to form a gel or mechanical gel strength, respectively.[12] In addition, starch solubility can be enhanced owing to the enzymatic modification using PUL.[3]

Based on published results to date, a lack of scientific data regarding the correlation between the specific changes of the molecular composition (molecular level), physicochemical (pasting), flow (hot paste viscosity), and functional properties is evident. The present study aims for a selective partial modification of a PS by means of ISO according to a systematical experimental design and the subsequent comprehensive evaluation of different properties of the starch samples obtained. The study aims to identify a relationship between the modification process (i.e., concentration ISO) and resulting granule morphology (scanning electron microscopy, SEM) as well as swelling (differential scanning calorimetry, DSC), solution (rheological investigation), and restructuring properties (gel formation during cold storage). Moreover, the profound impact of the ethanol-treatment aiming to terminate the enzyme is discussed. An efficient alternative to conventional acid-thinned starches would have ecological and economic advantages due to the reduced demand for chemicals in the hydrolysis process, supposed reduced carbohydrate solubilization and reduced specific demand for the application (e.g., gelled sweets). However, a prerequisite for it is the inactivation of the enzyme without ethanol.

2. Experimental Section

2.1. Starch and Enzyme

A commercial native PS was used (Superior, batch number: L000292213, date of expiry: 12/04/2022, Emsland–Stärke GmbH, Emlichheim, Germany) as the basis for the modification. The dry matter (83.10% w/w), protein (≤0.1% w/w), fat (≤0.1% w/w), ash (≤0.5% w/w) and AM content (24.3% w/w) was reported elsewhere.[20] An enzyme solution (ISO) of high purity with a debranching activity of 200 U · mL^{-1} (cat. Number: E-ISAMY-200U, lot. number: 130101b, biological origin: Pseudomonas sp., EC 3.2.1.68, date of expiry: December 2020) was used for the modification (Megazyme Ltd., Bray, Co. Wicklow, Ireland). Deionized water was used for the experiments.

2.2. Enzymatic Modification Process

The enzymatic hydrolysis was performed according to Asiri et al.[18,12] and Li et al.[21] with modifications. A 40% w/w starch slurry (125 g in total) was prepared and heated to 35 °C under vigorous stirring (250 min^{-1}). The pH of the suspension was adjusted to 4.5 (1 M HCl solution) before adding the desired amount of ISO (100 µL, ISO-100; 250 µL, ISO-250; and 400 µL, ISO-400) to the starch suspension. The hydrolysis was stopped after 60 min by dewatering (first step; suction filtration, filter paper: 125 mm/100 unit ALBETLabScience, Hahnemuehle FineArt GmbH, Dassel, Germany) followed by suspending the granular starch immediately in ethanol (second step; 96% v/v, 160 mL) and vigorous stirring for 15 min at 35 °C. The ethanol was separated from the granular starch by suction filtration. Washing the starch in ethanol was performed in duplicate. A starch blank was prepared without the addition of enzyme (PS-B) and processed accordingly. The starch samples were stored at 20 ± 2 °C for about 24 h to remove the remaining ethanol and ground (pulverisette 14, FRITSCH GmbH, Idar–Oberstein, Germany). The starch samples were stored at 20 ± 2 °C in closed plastic containers.

2.3. SEM

The starch particle surface properties were investigated by means of SEM using a ZEISS Gemini SEM500 NanoVP microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a wolfram cathode. The samples were affixed with double-faced adhesive tape on the sample carrier and sputtered with gold (Au) with a layer thickness of about 3 nm (SCD 030, Balzers Union, Balzers, Liechtenstein). The micrographs were taken at a magnification of 1200.

2.4. X-Ray Scattering Experiments (X-Ray Diffraction)

The semi-crystalline structure of the starch samples was investigated on uniformly fine powders using X-ray diffraction (XRD). Random amounts of the powders were analyzed using a Bruker D2 PHASER diffractometer (Bruker Corporation, Billerica, MA, USA) from 3° to 80° 2θ using Cu-Kα radiation (30 kV/10 mA; step size 0.01° 2θ; time/step: 0.5 s).

2.5. Gelatinization Behavior (DSC)

The thermal gelatinization was examined using DSC (204 F1 Phoenix equipped with an intercooler, Netzsch, Selb, Germany) according to the description elsewhere.[18] Starch was weighted in an aluminum pan, suspended in water (about 5 mg starch and 20 µL water, ratio about 1:5) and the crucible hermetically sealed. The crucibles were stored at 20 ± 2 °C for 24 h before measurement. The scanning conditions included isothermal, heating, cooling segments. The cycle was as follows: the initial temperature was kept at 20 °C for 2 min, ramped to 95 °C at a heating rate of 10 K · min^{-1}, maintained at 95 °C for 2 min, and then cooled to 20 °C with a cooling rate of 40 K · min^{-1}. The obtained thermograms were evaluated (Netzsch Proteus thermal analysis-Version 7.0.1 software) in terms of gelatinization onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c), gelatinization range (ΔT_{gel}; $T_c - T_o$), and swelling enthalpy (ΔH_{gel}). The experiments were performed in repeated determination, and the arithmetic mean as well as the corresponding standard deviation were calculated.

2.6. Analysis using Size Exclusion Chromatography Techniques (Size Exclusion Chromatography)

2.6.1. Solution Preparation

Starch solutions were prepared by heating 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 shear-treatment (Ultra-Turrax T25, IKA-Werke GmbH & Co. KG, Staufen, Germany) at 24 000 min^{-1} for 2 min at about 80 ± 5 °C to ensure good solution state. The pastes were diluted 1:10 (v/v) in dimethyl sulfoxide (DMSO) and passed through 5 µm PTFE filters (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) before molecular analysis (size exclusion chromatography-multi angle laser light scattering-differential refractive index detection [SEC-MALS-DRI], starch total).

2.6.2. Enzymatic Debranching

Complete enzymatic debranching of the starch polysaccharides for analytical purpose was performed using PUL (biological origin: microorganism, 1350 NPUN · g^{-1}, 200 U · mL^{-1}; PromozymeD2, Novozymes A/S, Bagsvaerd, Denmark) as described elsewhere.[22]

The enzyme solution contained potassium sorbate and sodium benzoate as preservatives and sucrose as well as glucose as stabilizers. Before dilution of the aqueous solutions in DMSO, a volume of 187 µL enzyme formulation was added to an aliquot of 10 mL and the solution kept at 40 °C for 20 min under continuous stirring (400 min^{-1}). The hydrolysis was terminated by keeping the dispersion at 95 °C for 20 min. Before analysis (SEC-MALS-DRI and size exclusion chromatography-conventional calibration-differential refractive index detection [SEC-cal-DRI], AM, and AP branch chains), the solutions of the debranched samples underwent a stabilization and filtration process according to the previous description.

2.6.3. Chromatographic Systems

The separation and molecular characterization of the polydisperse solutions was carried out by means of SEC-MALS-DRI according to Ulbrich et al.[20] The separation was executed with an SEC-3010 module (WGE Dr. Bures GmbH & Co. KG, Dallgow–Doeberitz, 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 provide the concentration of the eluted solution and molar mass (MM) at each retention volume (M_i). The basis for the molecular characterization by means of SEC-MALS-DRI has been described elsewhere.[23,24]

The separation system was additionally calibrated (SEC-cal-DRI) using a set of 10 pullulan standards with a MM range between 342 and 805 000 g · mol^{-1} (PSS Polymer Standards Service GmbH, Mainz, Germany). 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 degree of polymerization (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.

2.6.4. Peak Separation Method

The SEC-chromatograms of the debranched starch samples were analyzed using peak separation and analysis software PeakFit Version 4.12 as described elsewhere.[25] Based on the fitted SEC chromatograms, single peaks representing the AM fraction, the AP branch chains fraction and the enzyme were calculated. The M_w of the AM fraction (M_w AM) was calculated by means of the correspondent separated chromatogram and the MM curve (fit) from the MALS-detector (SEC-MALS-DRI). The M_w of the

AP branch chain fraction (M_w AP branch chains) was determined based on the relevant chromatogram and the standard calibration curve (SEC-cal-DRI) according to the description elsewhere.[10,26]

2.7. Characterization of Concentrated Starch Systems

Concentrated starch pastes were prepared according to Asiri et al.[19] with modifications. Aqueous dispersions (7.5% w/w) were simultaneously heated and stirred at 250 · min^{-1} for 30 min using a water bath at 95 °C (1) or using an autoclave at 145 °C under stirring at 400 · min^{-1} for 30 min. After the thermal disintegration step, the pastes were cooled to 85 ± 5 °C and shear-treated at 24 000 · min^{-1} (Ultra-Turrax, T 25, IKA-Werke GmbH & Co. KG, Staufen, Germany) for 2 min. The freshly prepared solutions were immediately analyzed in terms of solubility and hot paste viscosity, and gels were casted.

2.7.1. Carbohydrate Solubility

The starch suspension (7.5% w/w) was diluted with water to 1% (w/w) and an aliquot of 30 g was centrifuged at 11 180 g for 15 min (Biofuge 28RS, Heraeus, Hanau, Germany). The mass of the supernatant was determined by weighing it and an aliquot of 0.5 mL was mixed with 4.5 mL DMSO (suspension-to-DMSO ratio of 1:10 v/v). The starch concentration was determined by means of SEC-MALS-DRI. The amount of starch dissolved (supernatant) was related to the total amount of starch within the 1% suspension multiplied by 100 and finally expressed as solubility [%].

2.7.2. Hot Paste Viscosity

The rheological measurements were carried out using a rotational rheometer (MCR 302, Anton Paar GmbH, Graz, Austria) with a cone-plate geometry (CP50-1/TG: 50 mm und 1°, True Gap). Based on the freshly prepared starch solutions, hot paste shear viscosity curves were determined in the range of shear rate 0.1–125 s^{-1} at 60 °C.

2.7.3. Gel Strength

Gels preparation and characterization were the same as those according to the description elsewhere with modifications.[27] After casting and storage for 24 h at 5.5 ± 1.5 °C, a fresh and planar surface was realized by cutting. The mechanical strength of the gel (diameter 30.0 mm, height 20.0 mm) 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 temperature of the starch gel at the beginning of the measurement was about 14 ± 1.5 °C. The peak force [N] of the first penetration was taken as gel strength. The gel preparation and hardness measurement were carried out in triple determination.

3. Results and Discussion

3.1. Supramolecular Structure

3.1.1. Structure of Starch Granules using SEM

The morphological properties of the starch granules were investigated by means of SEM (**Figure 1**). The surface of the granules of PS-N was found to be smooth and without visible defects, which is basically in line with the expectation.[10,18,28,29] However, the processing of the starch including heating and stirring the aqueous suspension, dewatering, and repeated washing in ethanol as well as drying and grinding finally changed the surface of single granules slightly (Figure 1, PS-B). In particular the washing in ethanol, which intends to deactivate the enzyme, and the effect on granular but also other starch properties, remained unappreciated to date.[21] Enzymatic modification visibly resulted in damage to the single granules (Figure 1, ISO-100,...,ISO-400). The comparison between PS-B and the ISO-samples clearly demonstrates the single impact of the debranching enzyme (Figure 1). In particular, some swelling and wrinkles on the surface are remarkable (Figure 1, ISO-100,...,ISO-400). Moreover, ISO-400 but also ISO-100 show some small cracks on the surface resulting from the treatment, particularly visible on large granules. Altogether, the special impact of the enzymatic treatment can be classified as minor. These findings are somewhat different to what was reported for various kinds of enzymes, modification conditions, and starch sources[30,31] with specific and remarkable damages. Granules of PS modified using α-amylase or pullulanase,[10,18] or barley starch modified using α-amylase and amyloglucosidase, respectively, were affected by the enzymatic treatment.[19,32,33] It was stated that the enzymatic modification causes primarily exo-corrosion initiated by the molecular degradation.

3.1.2. XRD

Figure 2 shows the XRD pattern of the starch samples. The diffraction pattern of the PS-N was identified to be of the B type, which is typical for a native regular PS,[34] having strong diffraction peaks at about 5.6° (1), 14.2–15.1° (2; double peak), 17.0° (3), 19.4° (4), 21.9° (5), and 23.8° (6),[35] as well as slight peaks at about 26.0° (7), 30.0–31.5° (8; double peak), and 34.1° (9).[36] The enzymatic modification (ISO-100,...,ISO-400) as well as the preparation steps including repeated washing with ethanol, drying, and grinding the starch granules (PS-B), did not change the crystalline type, since all diffraction patterns were found to be without a remarkable difference compared to the native sample (PS-N) with respect of position and relative area of the peak (Figure 2).

3.2. Thermal Properties (DSC)

The thermal gelatinization properties were determined using DSC technique, and the thermograms obtained were evaluated in terms of T_o, T_p, T_c, ΔT_{gel}, and ΔH_{gel} (**Figure 3**).

Regarding the characteristic temperatures, the differences between PS-N and PS-B were marginal. Obviously, processing the

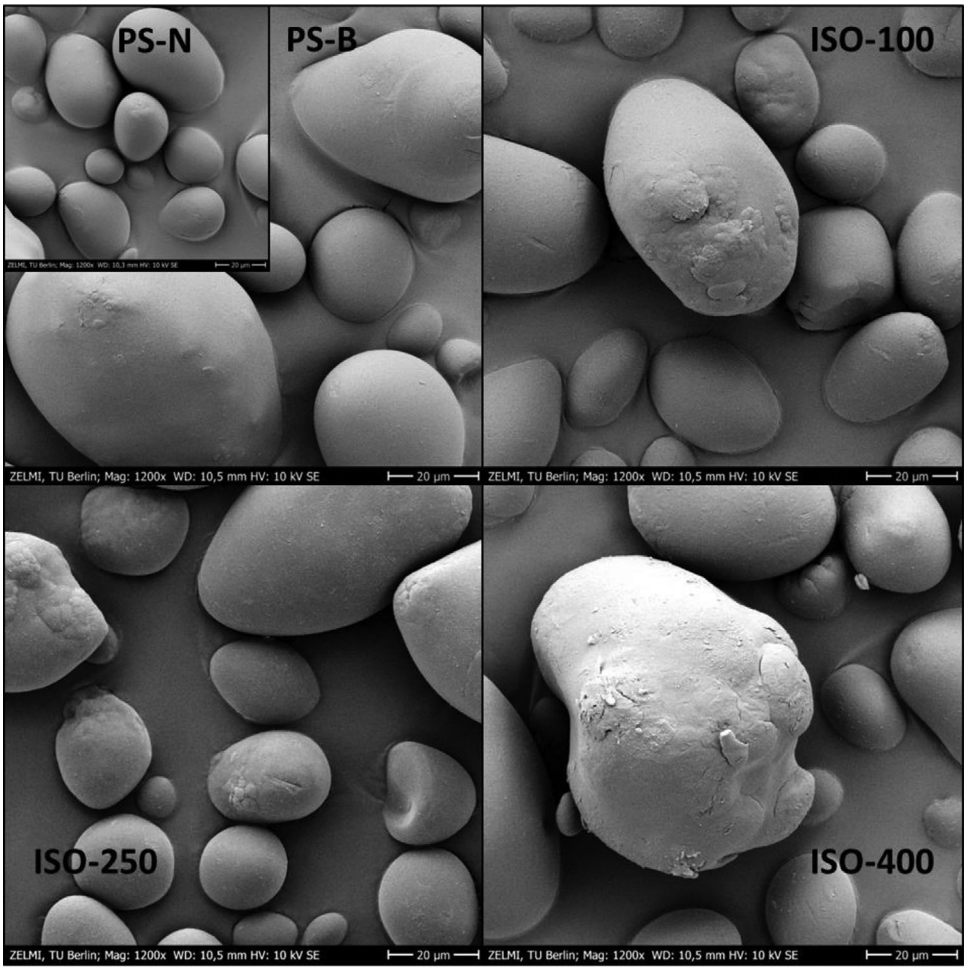

Figure 1. SEM micrographs of the starch samples (PS-N: native PS; PS-B: blank, processed without enzyme; ISO-100, ISO-250, and ISO-400: modified using 100, 250, and 400 µL ISO, respectively; magnification: 1200×; scale bar: 20 µm).

starch did not impact the semi-crystalline structure significantly (Figure 3). However, the partial enzymatic hydrolysis decreased the temperature range ΔT_{gel} significantly from about 16.5 K to 11 K, in particular due to reduction of T_c, whereupon an effect of the enzyme concentration could not found.

Compared to PS-N, the ΔH_{gel} decreased slightly but successively when processed, particularly increasing the enzyme concentration (Figure 3, PS-B,...,ISO-400), indicating certain changes of the structure due to specific cleavage within the amorphous areas of the semi-crystalline structure.

The reported findings accord well with that of previous studies using α-amylase or PUL, respectively, for modification. The level of treatment intensity impacted systematically in particular T_c and hence ΔT_{gel}, as well as ΔH_{gel}.[10,18] In contrast, Li et al.[27] reported increasing ΔT_{gel} owing to modification with PUL. A decrease in ΔH_{gel} can be attributed to the consecutive loss of the double-helical structures due to the partial chain cleavage and the overall modification procedure including probable carbohydrate loss during modification in slurry by leaching effects (removal of starch polymers associated with solubilization).[37,38]

3.3. Techno-Functional Properties

3.3.1. Solubility

Starch pastes with supposedly different solution states were prepared by varying the disintegration temperature (95 and 145 °C), and the solubility was determined based on centrifugation after dilution and analysis of the supernatant.

Both the disintegration method as well as the modification had significant impact on the solubility (**Figure 4**). A remarkable difference was found between PS-N and all processed starch samples (Figure 4, PS-B,...,ISO-400) for both disintegrated at 95 and 145 °C. When disintegrated at 95 °C the solubility of PS-N was found to be about 40% higher compared to the processed samples washed with ethanol. The repeated washing step of the granular starch at 35 °C with ethanol is probably the reason for the strongly limited solubility of PS-B and the hydrolyzed starch samples. Possibly, the granular integrity was at least partially preserved at the applied temperature and the granules were in a highly swollen state, despite thermal gelatinization accompanied by loss of the semi-crystalline structure. However,

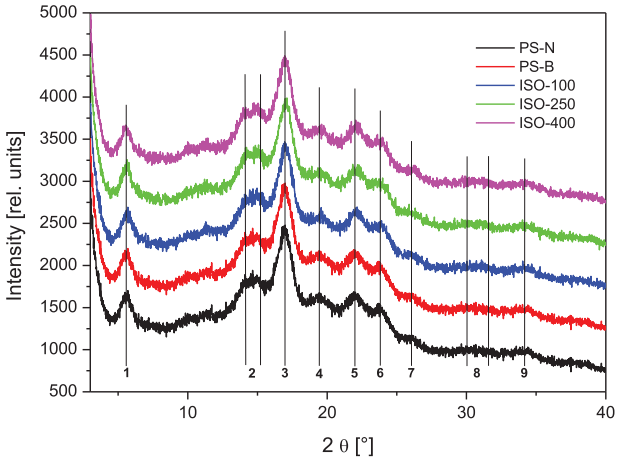

Figure 2. XRD patterns of the starch samples (PS-N: native PS; PS-B: blank, processed without enzyme; ISO-100, ISO-250, and ISO-400: modified using 100, 250, and 400 µL ISO).

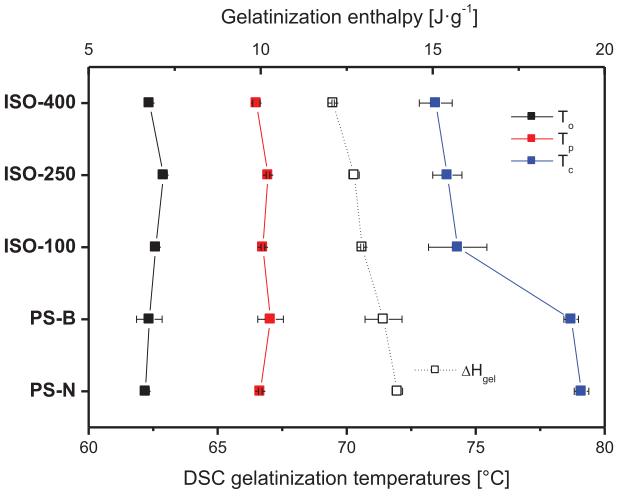

Figure 3. DSC gelatinization temperatures (T_o, T_p, T_c) and enthalpy (ΔH_{gel}) of the starch samples (PS-N: native PS; PS-B: blank, processed without enzyme; ISO-100, ISO-250 and ISO-400: modified using 100, 250, and 400 µL ISO, respectively).

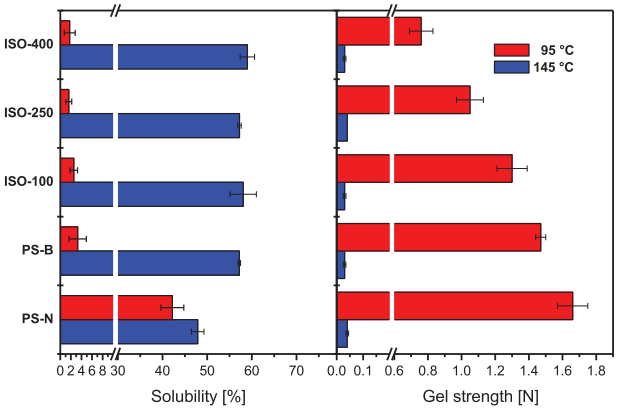

Figure 4. Solubility and gel strength of starch samples (PS-N: native PS; PS-B: blank, processed without enzyme; ISO-100, ISO-250, and ISO-400: modified using 100, 250, and 400 µL ISO, respectively) in dependence on the disintegration process (water bath: 95 °C, autoclave: 145 °C).

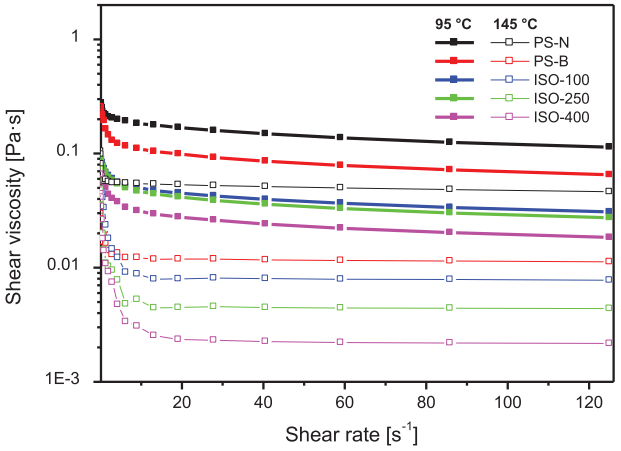

Figure 5. Hot paste viscosity of starch samples at 60 °C (PS-N: native PS; PS-B: blank, processed without enzyme; ISO-100, ISO-250, and ISO-400: modified using 100, 250, and 400 µL ISO, respectively) in dependence on the disintegration process (water bath: 95 °C, autoclave: 145 °C).

when disintegrated at 145 °C using the autoclaving process the solubility of the ethanol-processed samples (PS-B,...,ISO-400) was found to be about 55–60%, which is a significantly higher level compared to the PS-N (48%) on the one hand and to the disintegration at 95 °C on the other hand (between 1.5 and 3.5%, PS-B,...,ISO-400). Altogether it is assumed that the solubility of the starch samples is a function of both disintegration temperature and in particular repeated processing in ethanol at 35 °C during sample preparation (modification process). A remarkable impact of the partial enzymatic hydrolysis on the solubility or even the enzyme concentration applied is not evident from the data obtained (Figure 4). The findings are basically in line with previous investigations including enzymatic modification and subsequently stirring the granular starch in ethanol to terminate the reaction.[10] The very low carbohydrate solubility of the starch heated to 95 °C in a water bath cannot be attributed to the actual modification process but rather to washing with ethanol.

3.3.2. Hot Paste Viscosity

The viscosity curves of the starch samples are illustrated in **Figure 5** in dependence on the disintegration process and the temperature, respectively. Since the granular integrity is widely lost and the solution state is comparatively high when pasted at harsh conditions (145 °C), the viscosity of the hot dispersion is at a significantly lower level compared to the gentle pasting process (95 °C). The shear-rate dependence of the viscosity of the solutions prepared at 95 °C was basically distinct within the whole range (0.1–125 s^{-1}), which is typical. However, the improved solution state of the samples resulted in a loss of the shear-rate dependence at a shear rate higher than about 20 s^{-1}. This is consistent with previous findings in similar preparation and measurement conditions.[10] The differences between PS-N and PS-B, independent of the disintegration temperature, are attributed mainly to the impact of the ethanol washing step and associated influence on the solubility as discussed before (Section 3.3.1). However, the successive reduction of the viscosity level with increasing

Table 1. Molecular data for the starch, the AM-fraction, and the AP branch chains fraction.

	M_w starch total [10^6 g · mol^{-1}]	M_w AM [10^5 g · mol^{-1}]	M_w AP branch chains [10^3 g · mol^{-1}]	DP_w AP branch chains [/]
Method	SEC-MALS-DRI[a)]	SEC-MALS-DRI[b)]	SEC-cal-DRI[c)]	SEC-cal-DRI[c)]
PS-N	33.34 ± 0.07	7.70 ± 0.37	5.26 ± 0.66	32.28 ± 3.84
PS-B	14.10 ± 0.28	4.02 ± 0.13	4.71 ± 0.01	29.04 ± 0.04
ISO-100	10.51 ± 0.55	3.33 ± 0.13	4.29 ± 0.07	26.44 ± 0.04
ISO-250	7.97 ± 0.02	2.97 ± 0.18	4.42 ± 0.36	27.24 ± 2.22
ISO-400	7.02 ± 0.05	2.73 ± 0.08	4.15 ± 0.01	25.58 ± 0.04

[a)] Without debranching; [b)] after debranching; calculated from MALS-DRI signal (chromatogram and MM); [c)] after debranching and mathematical peak separation.

enzyme concentration (PS-B,...,ISO-400) is definitely ascribed to the systematically reduced polymer chain length, since the flow behavior of the hot solution is supposedly a function of the MM of the experimental condition applied (Section 3.4).

3.3.3. Gel Strength

The mechanical strength of the stored aqueous systems was determined and denoted as gel strength in general (Figure 4). The impact of the solution state, which was varied by the disintegration temperature and process, respectively, was significant in terms of the ability to form a gel structure. Paste preparation by means of pressure cooking, which resulted a good solution state, effectuated complete loss of capability to gel, independent of the starch sample investigated (PS-N, PS-B, or ISO-modified). This contradicts the experience from previous studies[39] on the one hand and surprisingly, because it is known that the molecular composition or the MM, respectively, controls the ability to gel as an inherent starch property[37,40] on the other hand. The mechanical strength of the gel prepared based on PS-N at 95 °C was about 1.6 N and met the expectation (Figure 4). However, the impacts by the processing (PS-B) first as well as the enzymatic modification (ISO-100,...,ISO-400) second, systematically reduced the gel strength to below 1 N. This was not expected, since the optimal M_w for partially molecular degraded PS (basis: regular PS, modification process: acid-thinning) in terms of best gel strength was found to be within the range 5–8 · 10^6 g · mol^{-1} in a previous study (paste preparation: 95 °C).[38] According to that, the samples ISO-250 and ISO-400 with a M_w of 8 · 10^6 and 7 · 10^6 g · mol^{-1}, respectively, should actually have similar properties regarding functionality. The specific effect, especially of the ethanol, is basically unknown. A detailed analysis of the latter in terms of inhibiting the gelation seems needful.

3.4. Molecular Characteristics

The molecular properties of the starch samples were determined based on SEC experiments. The preparation of molecularly dispersed solutions including pressure cooking, subsequent high-shear treatment of the dispersion and dilution in DMSO ensured good solution state without molecular degradation, and prevented microbial degradation and retrogradation. The SEC-recovery rate was between 80.0% and 82.4%. **Table 1** summarizes

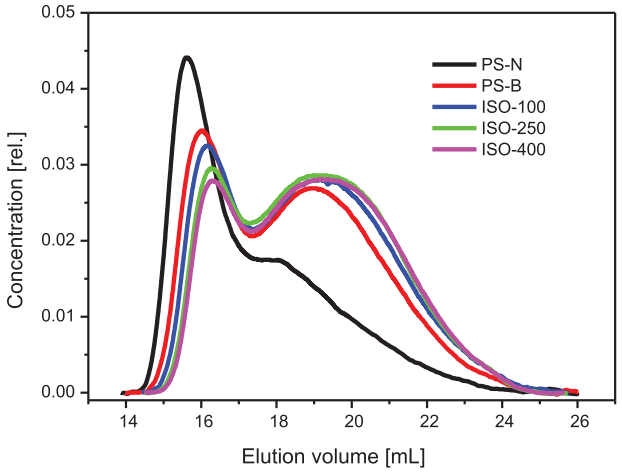

Figure 6. Chromatograms of the starch samples (starch total; PS-N: native PS; PS-B: blank, processed without enzyme; ISO-100, ISO-250, and ISO-400: modified using 100, 250, and 400 µL ISO).

the molecular data obtained for the starch, the AM-fraction as well as the AP branch chain fraction and the **Figures 6–8** the respective chromatograms. The difference between M_w starch total of PS-N and PS-B is remarkable and was actually not expected (Table 1). However, it underlines the importance of including so-called "blanks," which are actually not modified in the intended way (enzymatic hydrolysis) but completely processed in the same manner. Presumably, the comprehensive processing including exposure to ethanol, drying, and grinding in particular, governed a significant polymer chain cleavage. The chromatograms in Figures 7 and 8 clearly show the visible degradation of both AP and AM. Compared to PS-N, the chromatogram of PS-B is shifted to higher elution volume, indicating the cleavage of the AP with the formation of branched dextrins with reduced molecular size (Figure 6) and the simultaneously partial degradation of AM chains (Figure 7). Moreover, the slight but significant change of the chromatogram of the AP branch chains fraction (Figure 8) evidently shows that the degradation, induced by the processing (PS-B), is rather random within the amorphous regions around and directly on the branches as opposed to being specific and selective on the α-D-1,6-glucosidic linkages of the AP (Table 1, DP_w reduction from 32 to 29).

The molecular degradation of the starch induced by the ISO is also evident from the data in Table 1 and the corresponding

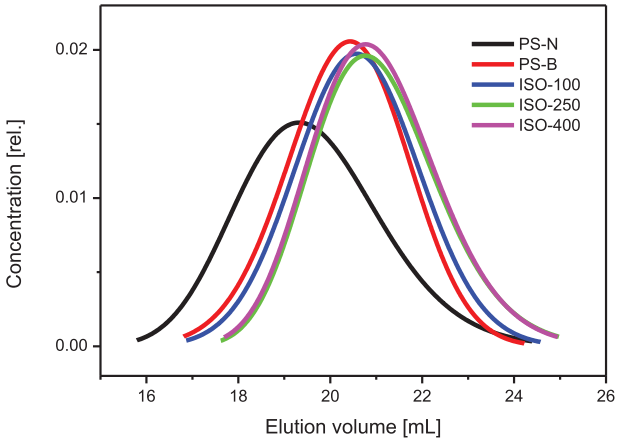

Figure 7. Chromatograms of the AM-fraction of the starch samples (PS-N: native PS; PS-B: blank, processed without enzyme; ISO-100, ISO-250, and ISO-400: modified using 100, 250, and 400 µL ISO).

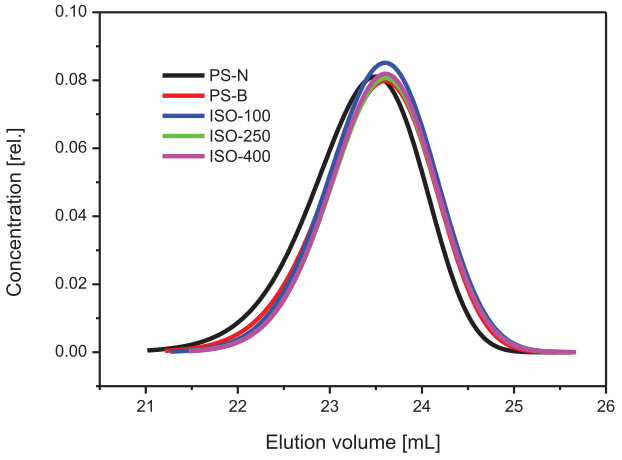

Figure 8. Chromatograms of the AP branch chain fraction of the starch samples (PS-N: native PS; PS-B: blank, processed without enzyme; ISO-100, ISO-250, and ISO-400: modified using 100, 250, and 400 µL ISO).

chromatograms (Figures 6 and 7). M_w of the starch total decreased systematically from about $14 \cdot 10^6$ to $7 \cdot 10^6$ g · mol^{-1} (reduction 50%) with increasing ISO concentration (PS-B,...,ISO-400). This is mainly attributed to the cleavage within the AP, since the M_w of the AM-fraction was not reduced to that extent (Table 1, M_w AM). However, a slight, successive ISO-induced cleavage within the AM-fraction with increasing ISO concentration can be deducted by the systematic shift of the chromatograms in Figure 7 (PS-B,...,ISO-400). This was not fundamentally expected since a high-purity debranching enzyme solution was used for the modification. Moreover, the gradation of DP_w of the AP branch chain fraction (Table 1) evidently demonstrates that the cleavage within the highly branched AP structure did not occur exclusively at α-D-1,6-glucosidic linkages but also nearby.

In summarizing the molecular investigation and concluding the information obtained, the specific molecular degradation by mainly debranching the AP using ISO seems possible. Considering this should include the fact that the effect of the processing followed by the actual ISO-induced modification can be generally impacted by the extent of the degradation controlled by the gradation of the enzyme concentration (PS-B,...,ISO), that is, the extent of the molecular degradation induced by the subsequent processing (washing with ethanol, drying, grinding) is probably controlled by the extent of the previous modification induced by the enzyme. In addition, marginal leaching effects could also play a part. An impact by the mechanical stress due to grinding is questionable but not completely impossible. However, this was not supported anyway by the SEM (Section 3.1.1) of the granular products.

4. Conclusions

The modification of granular PS using high-purity ISO aimed at a partial molecular degradation was successful in principle. The enzyme concentration can control the extent of the chain cleavage, which was evident from the molecular characteristics and the flow behavior of concentrated pastes. However, the non-thermal inactivation of the enzyme using ethanol as a lab scale preparative step is questionable, since some properties of the starch samples produced such as solubility and gelation behavior were conspicuous. In particular, the fact of the strongly reduced MM solely due to processing including stirring the aqueous suspension at 35 °C (1, annealing), repeated washing with concentrated ethanol (2), drying (3), and grinding to decollating the starch granules (4) and possible interactions of these methodical steps are not fully understood and need further investigation. Nevertheless, a partial degradation of the starch polymers with a high specificity is possible. Debranching enzymes like ISO and PUL could be an alternative to acid-involved processes since they act mostly selective within the AP.

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Conflict of Interest

The authors declare no conflict of interest.

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