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Published in:
Carbohydrate Polymers

DOI:
10.1016/j.carbpol.2021.117801

Carbohydrate Polymers 260 (2021) 117801

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Long chains and crystallinity govern the enzymatic degradability of gelatinized starches from conventional and new sources

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ARTICLE INFO

Keywords:
Starch
Enzymatic hydrolysis
Amylopectin
Extent of and length of side-chains
Structural features

ABSTRACT

Slowly digestible starches have received interest due to their lower increase of postprandial blood glucose and insulin levels and, hence, modification of starches towards slower digestibility has commercial interest. However, chemical characteristics driving enzymatic (digestive) degradation are not fully unraveled. The digestion properties of starches have been linked to their crystalline type, chain length distribution, amylose content or degree of branching, but content and length of relatively long side-chains in amylopectin has not been paid attention to. Therefore, this research focusses on the unique content and length of amylopectin side-chains from conventional and new starch sources (potato, corn, pea, and tulip) correlated to the enzymatic digestion. The rate of hydrolysis was found to be correlated with the crystalline type of starch, as previously suggested, however, the complete hydrolysis of all starches, independent of the crystalline type and source, was shown to be governed by the content of longer amylopectin chains.

1. Introduction

Starch is the main source of digestible carbohydrates in the human diet (Stephen et al., 2012) and can be found in most staple foods based on cereals, tubers, and legumes (Bajaj, Singh, Kaur, & Inouchi, 2018). This homopolysaccharide of α-D-glucosyl residues is composed of two main components: amylose and amylopectin. Amylose consists of linear chains of glucosyl units linked by α-(1→4)- bonds, while amylopectin is characterized by α-(1→6)-linked side chains connected to linear sections formed by α-(1→4) glycosidic linkages. Most starch granules consist of 70–80 % of amylopectin and 20–30 % amylose, but ranges from <2% amylose content in waxy starches to 65–80 % in high-amylose starches (Li, Gidley, & Dhital, 2019; Šárka & Dvořáček, 2017). The fine structure of amylopectin influences starch properties such as crystallinity, gelatinization, retrogradation, and pasting properties (Jane et al., 1999). Another important functionality of starch is its digestion behavior as consumption of starch with a high digestion rate leads to a rapid increase in the blood glucose level. A diet containing high amounts of rapidly digested starch causes fluctuations in the postprandial blood glucose, generating high stress to the glucose homeostasis regulatory system and has been associated with health complications (Marshall, 2006; Zhang, Sofyan, & Hamaker, 2008). Starch with lower digestion provides an extended glucose release with a low glycemic response and is thus desirable for commercial applications such as healthy ingredients in processed foods.

Therefore, decreasing the digestion rate of starches has been a growing interest in academic research (Ao et al., 2007; Lehmann & Robin, 2007; Zhang, Ao, & Hamaker, 2008). The susceptibility of starches to enzymatic hydrolysis has been linked to the starch structure (Kasprzak et al., 2012; Planchot, Colonna, Gallant, & Bouchet, 1995; Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005). Understanding the underlying factors governing the accessibility of starch to enzymatic hydrolysis, however, has proven to be a challenge. There have been a number of studies on starch digestion which typically described the digestion of variants of the same plant species (Syahariza, Sar, Hasjim, Tizzotti, & Gilbert, 2013; Zhang, Ao et al., 2008) or the effect of different pre-treatments, with e.g. heat (Shi, Gao, & Liu, 2018) or enzymes (Kasprzak et al., 2012; Li et al., 2014). However, comparison

Abbreviations: DP, Degree of polymerization; S_AF/LF ratio, ratio between short (DP 9-12) and long (DP 13-24) chains; F1-F4, fractions of enzymatic hydrolysis.

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https://doi.org/10.1016/j.carbpol.2021.117801
Received 22 June 2020; Received in revised form 5 February 2021; Accepted 6 February 2021
Available online 13 February 2021

of the digestion patterns of starches from a variety of different sources or existing fractions from the same source are sparse, especially regarding the overall accessibility of starch to enzymatic hydrolysis by comparing various starch structures is lacking. The main finding was a correlation between digestion and the crystalline type of the starches (Planchot et al., 1995; Srichuwong et al., 2005) which might be linked to the average chain length. In these digestive studies only 45 % (potato) to 70 % (rice) of the total starch structural features could be identified (Srichuwong et al., 2005) showcasing that a large fraction of starch wasn't digested and details on its structural features remain unclear. For modification purposes, such as decreasing the overall digestion rate, it is essential to understand the structural features of the entire material to allow future designed modifications. Hence, in this study we aimed to adapt the enzymatic hydrolysis time in order to identify and quantify amylopectin structures of various sources to their full extent.

A number of authors have attempted to depict the complex structure of starch, resulting in the proposal of different models for the amylopectin structure (Bertoft, 2017; Manners, 1989; Nikuni, 1978; Thompson, 2000). These models, however, generalized that all starches have similar amylopectin structures even though the starches from various botanical sources have shown distinct structural features (Bajaj et al., 2018; Jane et al., 1999; Srichuwong et al., 2005).

The hypothesis of this study was that not only amylopectin in general but specific structural features of amylopectin govern the rate and behavior of gelatinized starches during enzymatic hydrolysis. Moreover, understanding the structural features of entire starch material is needed for optimal modification. Therefore, the effect of various parameters on the different fractions of starch during enzymatic hydrolysis over time was studied by characterization of eight starches from various botanical sources (cereal, tuber, and legume) as well as several fractions from the same source. Analysis of their amylopectin fine structures and statistical analysis of the correlation between important structural parameters and the adapted enzymatic hydrolysis of entire starch structure was used to provide an adapted models of amylopectin structures of crystalline types of starches.

2. Material and methods

2.1. Material

Potato starch (food grade), waxy potato starch (Eliane C100), and corn starch were obtained from Avebe (Veendam, The Netherlands), waxy corn starch (Meritena 300) from Tereos Syral (Marckolsheim, France) and high amylose corn starch from Megazyme (Bray, Ireland). Pea and wrinkled pea seeds were purchased from a local supermarket and tulip bulbs from a local bulb farmer (Groningen, The Netherlands). α-amylase from porcine pancreas type IV-B (A3176-1MU, 9 U/mL) was obtained from Sigma Aldrich (St. Louis, Missouri, U.S.) and both amyloglucosidase from *Aspergillus niger* (E-AMGDF, 3260 U/mL) and isoamylase from *Pseudomonas* sp. (E-ISAMY, 200 U/mL) were purchased from Megazyme.

2.2. Starch extraction

The starch extraction was based on the method described by Shi et al. (2014). For wrinkled and pea starch, 10 g starch were steeped in 20 mL water for 24 h whereas for the starch extraction from tulip, the bulb was peeled and cut into small pieces. Then, the samples were ground with a blender (2 min) and filtered through a 112 μm sieve. The filtrate was washed with deionized water and the protein-rich layer was removed by four cycles of resuspension in 0.25 % NaOH (w/v) followed by centrifugation for 5 min at 150 × *g*. Subsequently, the starch was washed again with deionized water and freeze-dried.

2.3. Basic characterization of starch samples

Before use, all starches were freeze-dried overnight. All experiments were performed in duplicates.

The starch content was estimated by acid hydrolysis by first incubating starch in 12 M H_2SO_4 (16 mg/mL starch) for 1 h at 30 °C followed by a dilution to 1 M sulfuric acid and incubation for 3 h at 95 °C. The total starch content was determined using the D-Glucose assay kit (GOPOD Format, Megazyme).

The amylose content of the samples was measured with the amylose/amylopectin assay kit with high amylose corn starch as reference (both from Megazyme). Amylopectin was removed by Con A and the remaining starch was digested with α-amylase, analyzed with the GOPOD kit and compared with the total starch content.

The ash content was determined by igniting 1.2 mg starch in a muffle furnace overnight at 550 °C (Marshall, 2010). The weight of the crucible was recorded before and after the experiment and the ash content was calculated as follows.

Ash content (%) = Mass[ash]/(Mass[starch]*solids(%)/100)*100(1)

and for all samples amount of ash measured was about 1%.

For the iodine affinity, starch (25 mg/mL) was gelatinized in 90 % DMSO before being diluted 8x with sodium citrate buffer (50 mM, pH 6), mixed with iodine solution (final concentrations: 0.4 mg/mL starch, 0.13 % KI, 0.013 % I_2, 4.5 mM HCl) and measured with the spectrophotometer (450−750 nm, 2 nm steps, Molecular Devices, Model SpectraMax 384 Plus, Hampton, New Hampshire, U.S.).

The starch crystalline polymorphs were identified by X-ray diffraction (XRD) on a Bruker D8 ADVANCE diffractometer (Bruker Corporation; Billerica, Massachusetts, US). The angular range was 4−33° (2θ) with a step size of 0.02°(2θ) and acquisition time of 2 s per step. The background signal was subtracted from the spectra.

2.4. Degree of branching (reducing ends)

Starch (25 mg/mL in 50 mM sodium acetate buffer, pH 3.7) was gelatinized by incubation for 5 min at 100 °C followed by autoclaving for 15 min at 121 °C. The gelatinized starch was debranched in triplicates by incubating it with isoamylase (1 U/mg substrate) for 6 h at 40 °C. For the determination of the reducing ends before debranching, the starch samples were also incubated with heat inactivated isoamylase. Then, both sets of samples were measured using the pAHBAH assay by mixing 50 μl appropriately diluted sample with 200 μl pAHBAH solution (1/5 of 5% 4-hydroxybenzoic acid hydrazide in 0.5 M HCl and 4/5 of 0.5 M NaOH) and incubated for 30 min at 70 °C. The absorbance was measured with a spectrophotometer (SpectraMax Plus 384 Microplate Reader, Molecular Devices, Sunnyvale, U.S.) at 490 nm. Samples were measured in duplicates and D-glucose was used as a standard. The branching degree of the starches was calculated using the formula:

$$\text{Degree of branching (\%)} = (RE_{DBr} - RE_{Br})/ (TC - RE_{Br})*100 \quad (2)$$

RE_{DBr} = reducing ends concentration after debranching (mol)RE_{Br} = reducing ends concentration without debranching (mol)TC = total carbohydrate of starch after acid hydrolysis (mol)

2.5. Degree of branching (NMR)

Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy was used to determine the degree of branching. Sample preparation and NMR spectroscopy were conducted according to the methods established by Nilsson (Nilsson, Bergquist, Nilsson, & Gorton, 1996) with minor modifications. Samples were dissolved in deuterium oxide (D_2O, 99.9 atom % D, Sigma-Aldrich chemical) with constant stirring at 110 °C for 30 min, and then freeze-dried the samples. Two cycles of dissolution and freeze-drying were performed. A final concentration of 10 mg/mL samples in D_2O were used to run the spectroscopy. ^{1}H-NMR spectra were

recorded on a Varian 500 spectrometer (NMR center, University of Groningen) at a probe temperature of 80 °C by running 16 scans with a degree pulse angle of 90, a 25 s relaxation delay between scans was implemented. Spectra were analyzed in the software MestReNova 12.0.2 (Mestrelab research S.L.) and iNMR (http://www.inmr.net). The α-1,4 and α-1,6 signal were assigned at chemical shifts of 5.84 and 5.43 ppm. The percentage of α-1,6 glycosidic bonds was estimated from the ratio of the integrated peak areas corresponding to the α-1,4 and α-1,6 bonds types.

2.6. Starch chain length distribution

Starch (25 mg/mL in 20 mM sodium acetate buffer, pH 4.5) was gelatinized by first incubating the samples for 5 min at 100 °C followed by autoclaving for 15 min at 121 °C. The gelatinized starch samples were then debranched by incubating it with isoamylase (1 U/mg substrate) for 6 h at 40 °C before being diluted 10x and brought to a pH of ~8 with NaOH. The samples were analyzed with high performance anion exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD). The Dionex ICS-6000 HPAEC-PAD system (Thermo Fisher Scientific, Sunnyvale, CA, USA) was equipped with CarboPacTM PA-1 column (2 × 250 mm) and a CarboPacTM PA guard column (2 × 50 mm). The samples were eluted at a flow rate of 0.3 mL/min with an injection volume of 10 µl. Two mobile phases used were A (0.1 M NaOH) and B (1 M NaOAc in 0.1 M NaOH). The elution gradient profile was: 0−50 min, 5–40 % B; 50−65 min, 40–100 % B; 65−70 min, 100 % B. The system was re-equilibrated with 5% B in between runs. The data was collected using Chromeleon software, version 7.2.9. The response factor of highest available DP17 and DP18 (CarboExpert, Yuseong-gu, South Korea; Maltooctadecaose) was determined (025 mg/mL corresponding to the area of 47 nC*min) and was used to estimate higher DP material (DP > 27 area is estimated to amount to no higher than 0,5% of the total representation of chains in any of the analyzed starch samples).

2.7. In vitro enzymatic hydrolysis

Gelatinized starch (25 mg/mL in 50 mM sodium citrate buffer, pH 6 with 0.02 % sodium azide, 4 mM $CaCl_2$, 2.5 % DMSO) was digested with α-amylase (110 U/g substrate) and amyloglucosidase (130 U/g substrate) at 37 °C. Aliquots were taken at 20 min, 2 h, 3 h and 24 h. Reactions were stopped by incubation for 5 min at 95 °C. Time 0 min samples were obtained by incubating the samples with inactivated enzymes. The samples were diluted (50 x dilution for time 0 min and 100 × dilution for other samples) and analyzed by the GOPOD assay (Megazyme).

2.8. Statistical analysis

Data were presented as means of triplicates with standard deviations. The statistical analysis was performed using SPSS Statistics 25 (IBM Corp, USA). Statistically significant differences were determined by one-way analysis of variance (ANOVA) with post-hoc Tukey HSD (Honestly Significant Difference).

The correlation coefficients between the measured starch properties and digestibility were evaluated using Pearson correlation procedure. Correlations having $P \leq 0.05$ were accepted as statistically significant and $0.05 < P \leq 0.1$ as a tendency for significance. The degree of correlation was presented by linear correlation coefficient (r).

3. Results and discussion

3.1. Inherent starch properties

The aim of this study was to estimate the effect of important structural features of starches on their susceptibility to enzymatic digestion that are applicable to a large range of starch types. To ensure a wide validity, a collection of highly diverse starches had to be selected. A literature research (a summary is given in Table A1) provided an overview of the variety in amylopectin structures present in starches. Based on the gathered information, eight starches from different botanical sources were selected to represent a wide range in properties (crystallinity type, amylose content, and branching degree). All starch samples first underwent a basic characterization (Table 1) to verify that all detected differences were a result of their distinct structural features and not by e.g. variation in methods used. Further, the starches were gelatinized as the aim of this study was to correlate the functionality of starches with their molecular structure and not their granular morphology.

Generally, starches can be grouped according to the three types of polymorph observed in the XRD pattern of starch granules: A-, B- and C-type (Fig. A1), being closely packed polymorphs, more loosely packed and a mixture of both, respectively (Whistler, BeMiller, & Paschall, 1984). Normal and waxy corn starch were found to be of A-type starch whereas high amylose corn starch was classified as B-type starch (Table 1) which has been suggested to be related to the decreased crystallinity due to the lower amount of short chains present (Cheetham & Tao, 1998). The other studied starches from tuber (potato and tulip) and legume (green/wrinkled pea starch) were classified as B- and C-type starches, respectively, regardless of the amylose content which was in agreement with literature (Table A1). For tulip starch and wrinkled pea starch, the crystallinity could not be identified reliably due to high amounts of amorphous regions (Fig. A1) and was thus classified based on literature (Hizukuri, 1985; Ratnayake, Hoover, & Warkentin, 2002).

The estimated amylose contents were usually somewhat lower (≤10 %) than the reported values (Table A1) which could be due to different biological samples. However, they were still in good agreement with literature. Table 1 shows the large variation in amylose content, with about 20–30 % for native starches and ranging from about 2% (waxy potato starch) to 65 % (high amylose corn starch).

The degree of branching was determined with two separate methods, nuclear magnetic resonance (NMR) and via determining the reducing ends. Literature on the degree of branching was only available for waxy corn starch, potato starch and waxy potato starch, determined with NMR. These values, being 4.8 %, 3.0–3.4 % and 4.1 %, respectively (Nilsson et al., 1996) were in very good agreement with the here obtained data using NMR (Table 1, for Spectra see Fig. A3). In the second method, the degree of branching was estimated from the reducing ends detected in a sample before and after enzymatic debranching and has initially been developed for the determination of enzyme activity of e.g.

Table 1
General starch properties based on experimental values.

Crop group	Starch	Crystallinity type	Amylose w/w % dry matter	Branching % (reducing ends)	Branching % (NMR)
Cereal	corn starch	A	21.0 ± 1.1	4.7 ± 0.1	3.1 ± 0.5
	waxy corn starch	A	1.9 ± 0.4	6.0 ± 0.0	4.9 ± 0.2
	high amylose corn	B	64.6 ± 3.1	0.8 ± 0.0	too low
Tuber	tulip starch	B*	22.7 ± 1.3	3.2 ± 0.2	5.5 ± 0.1
	potato starch	B	19.5 ± 1.7	2.6 ± 0.1	2.8 ± 0.4
	waxy potato	B	1.9 ± 0.3	4.0 ± 0.0	3.8 ± 0.3
Legume	green pea starch	C	27.3 ± 1.5	3.0 ± 0.1	too low
	wrinkled pea starch	C*	63.6 ± 3.1	0.4 ± 0.0	too low

* indicates that the crystalline polymorph was determined based on literature (Hizukuri, 1985; Ratnayake et al., 2002).

branching enzymes (Utsumi et al., 2009). There were a number of differences between the values obtained from the two methods. While NMR is mostly vulnerable to low solubility of the sample, resulting in small signal-to-noise ratios and hence limited accuracy of peak integration, the reducing ends assay is influenced by the actual starch concentration and completeness of debranching as it is estimated from the number of reducing ends before and after debranching in relation to the total glucose content. It is therefore possible that corn starch and waxy corn starch were subject to partial hydrolysis during debranching, resulting in higher values for reducing ends than for NMR. Tulip starch, on the other hand, might not have been completely debranched and thus not all branches were detecting by the reducing ends determination. Due to the low solubility, no values could be detected using NMR for high amylose corn starch, green pea and wrinkled pea starch. Green pea did show a small peak (Fig. A3) but it was too small for determination. Generally, the degrees of branching derived from both methods show a negative correlation with the amylose content. Further, with exception of tulip starch, A-type starches had a higher degree of branching than B- and C-type starches with similar amylose contents.

3.2. Chain length distribution of amylopectin

The chain length distribution of the debranched starches was studied with high-performance anion-exchange chromatography (HPAEC) (Fig. 1). The detectable range of chain lengths was from DP 6 to about DP 40 (see Fig. A4 for raw data). Only minor peak areas of chains longer than DP 27 were detected (< 1 nC*min), indicating trace amounts of long chains in the debranched starches, and were thus excluded from further analysis. Estimation of the amounts of DP > 27 was made by using the response factor of the DP18 standard suggests that for these particular starch materials more than 95 % of the material is analyzed. For different starch sample, e.g. high amylopectin material composed of longer chains (such as obtained by the modification by branching enzymes) could in fact contain much more of the DP > 37 material. Within

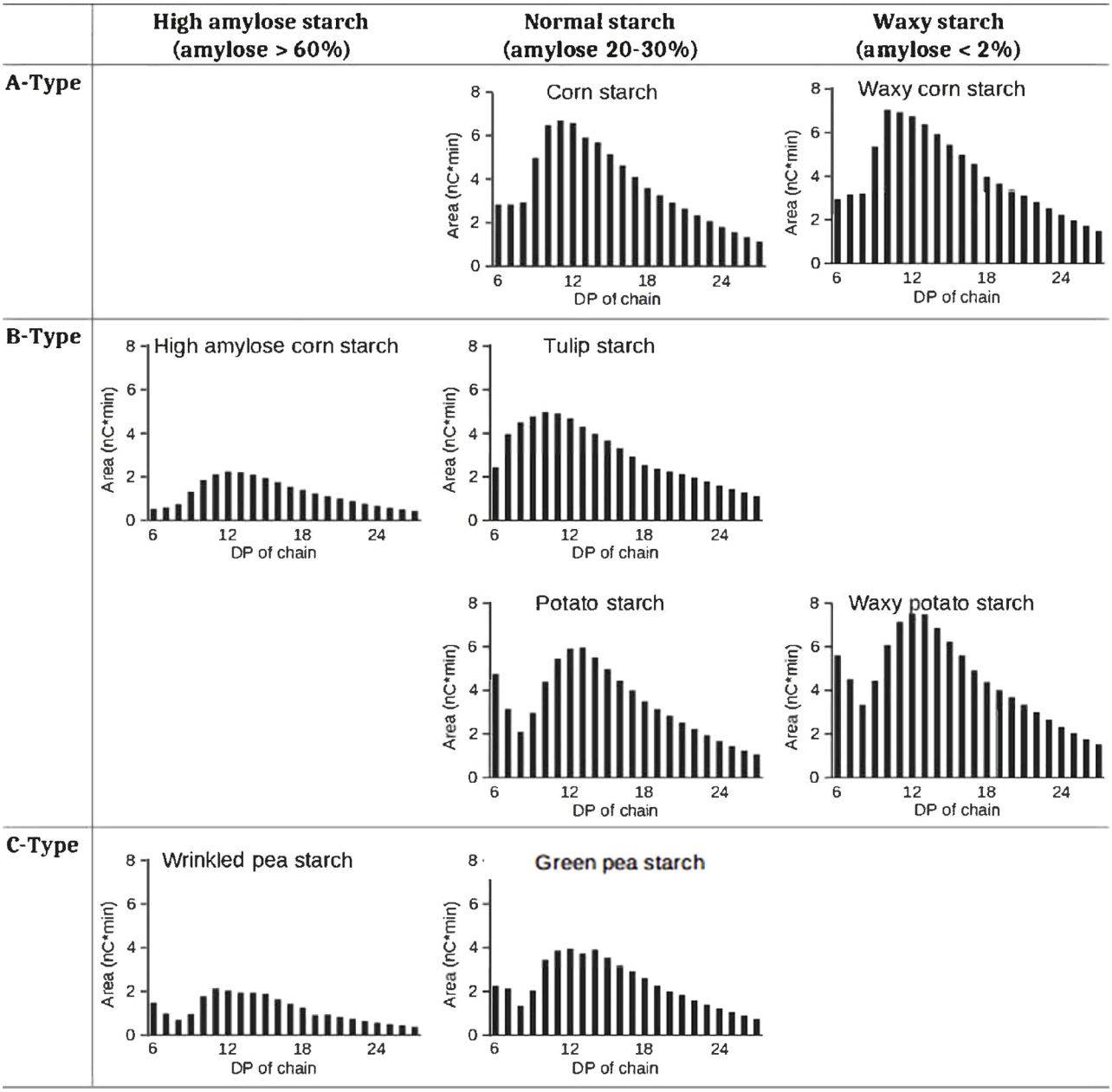

Fig. 1. Chain length distribution of amylopectin from various botanical sources categorized based on the crystalline polymorph types. The distribution was based on the total area of each chromatogram and respective relative area (%) of the signal of each detected peak.

the range, the studied starches exhibited different distribution profiles of the amylopectin chains. Overall, starches with a high amylose content (> 60 %) contained fewer detected chains which could be caused by the low solubility of amylose (Green, Blankenhorn, & Hart, 1975). Corn starch was the only starch showing some oligomers (DP 2-5). The very short chains (DP 6-8) are also known as the 'fingerprint' region as they are characteristic for starches of the same botanical source (Hanashiro, Abe, & Hizukuri, 1996; Koizumi, Fukuda, & Hizukuri, 1991) and thus showed similar patterns for the normal and waxy starches of e.g. corn and potato (Fig. 1). They were most prominent in (waxy) potato, followed by (waxy) corn, tulip and green pea starch. The corn starches showed similar populations in this range while both potato and pea starches exhibited local minima at DP 8, being characteristic for tuber starches (Srichuwong et al., 2005).

The short chain fraction (DP 9-12) was similarly distributed in (waxy) corn and tulip starch whereas the other starches showed an increase in population throughout this range. In all starches, the long chains fraction (DP 13-24) was described by a mostly linear decrease in amount of chains with small shoulders in waxy corn, waxy potato and tulip starches around DP 19. Reported chain length distribution profiles using HPAEC on the here presented starches were limited and are here reported for the first time for green pea starch, waxy potato starch and wrinkled pea starch, however, the profiles of corn and potato starch were in line with the literature (Hanashiro et al., 1996; Srichuwong et al., 2005).

The aforementioned classification into short external chains (SF, DP ≤ 12) and long substituted chains (LF, DP 13-24) was based on Zhang et al. (Zhang, Ao et al., 2008 but was extended by dividing the short fraction into very short (DP 6-8) and short (S_AF, DP 9-12) chains. This subdivision was conducted to exclude the 'fingerprint' region from the further analysis since it is specific for the botanical source (Silverio, Fredriksson, Andersson, Eliasson, & Åman, 2000; Srichuwong et al., 2005) but the target of this study was the identification of functional properties of the starch crystalline types. The ratio between short and long chains was then introduced as the S_AF/LF ratio and is shown in Table 2. A-type starches typically contained higher amounts of short chains (S_AF/LF 0.48−0.52, Table 2), followed by both C-type starches (0.40−0.43) and B-type starches (0.40−0.43) except for one outlier being tulip starch (0.53). The high ratio of tulip was supported by the high amorphous fraction observed during the crystallinity analysis (Fig. A1) as short chains are incapable of co-crystallization (Gidley & Bulpin, 1987). The general observed pattern was in line with the available literature where it has been described that A-type starches typically have chains that are on average slightly shorter than those of C-type starches and considerably shorter than those of B-type starches (Hanashiro et al., 1996; Hizukuri, 1985; Srichuwong et al., 2005).

Longer B chains (DP > 30) and the backbone chain (C-chain) the starches were further analyzed with iodine staining as they are very long for detection on HPAEC (Herrero-Martínez, Schoenmakers, & Kok, 2004). Iodine forms a colored complex with linear chains which gradually shifts from red to blue based on the length of the available linear chains (Bailey & Whelan, 1961; Tomasik & Schilling, 1998), providing insight into long internal chains. Generally, the obtained maximum absorbance value (λ_{max}) was positively correlated with the amylose content and negatively correlated with the degree of branching. Although the amylose content (23 %) and degree of branching (3.2 %) of tulip starch were similar to potato starch (20 %, 2.6 %, respectively), the S_AF/LF ratio was far higher (0.53 compared to 0.40), indicating a higher number of short chains. On the other hand, the λ_{max} of tulip starch (598 nm) was higher than of potato starch (582 nm), suggesting long internal chains and thus a unique structure of tulip starch of long internal chains with a high population of external short chains.

Table 2
Characteristics of amylopectin chain distribution and maximum absorption of starch-iodine complex.

Crystallinity type	Starch	S_AF/LF ratio[1,2]	λ_{max} (nm)
A	Corn starch	0.52 ± 0.01^{bc}	584 ± 3
A	Waxy corn starch	0.48 ± 0.00^{b}	527 ± 2
B	High amylose corn	0.42 ± 0.01^{a}	603 ± 9
B	Tulip starch	0.53 ± 0.01^{c}	598 ± 1
B	Potato starch	0.40 ± 0.00^{a}	582 ± 4
B	Waxy potato	0.43 ± 0.01^{a}	554 ± 7
C	Green pea starch	0.40 ± 0.01^{a}	607 ± 6
C	Wrinkled pea starch	0.43 ± 0.00^{a}	626 ± 14

[1] S_AF (short-A fraction) consists of DP 9–12 and LF (long fraction) contains chains of DP >13.
[2] Different lowercase letters indicate significant differences at P < 0.05.

3.3. Models of amylopectin structure

The analyzed properties were used to derive models for the amylopectin structures of corn, tulip, potato, and green pea starches which are presented in Fig. 2. The models were based on the widely accepted cluster model (Whistler et al., 1984) and included features such as the chain length and branch density. The amylopectin of corn starch had shorter chains and a high density of branches which have been previously found to be characteristic for the amylopectin structure in A-type starches (Kong, Corke, & Bertoft, 2009). The models for the B-type starches from potato and tulip showed long chains with a low branch density. The B-chains in tulip starch were estimated to be considerably longer than in potato starch based on the data obtained from HPAEC and iodine staining (Table 2). The amylopectin structure of green pea starch was modeled as an intermediate between corn and potato starch, due to its crystalline type and other properties typically being intermediates between A- and B-type starches. The amylopectin structure of green pea starch was more similar to corn starch. The chains were slightly longer than corn starch, but shorter than potato starch. The branching degree of green pea starch (2.4 %) was lower than corn starch (4%), hence the branches were slightly further apart from each other. Therefore, the amylopectin structure of green pea starch was an intermediate between corn and potato starch, with tendency for similarities with corn starch. It is a characteristic of type C starches to have an intermediate structure between the type A and B starches.

3.4. Digestion profile of gelatinized starches

In order to obtain accurate data for the starch characterization, such as the degree of branching and digestion pattern, the starch content of each sample was analyzed. As seen in Fig. 3, all samples consisted of >95 % starch, with exception of corn starch (88 %) and high amylose corn (85 %).

One of the most important functional properties of starches is their accessibility to digestive enzyme hydrolysis. Typically, the digestion behavior is studied *in vitro* using the Englyst method with α-amylase and amyloglucosidase (Englyst, Kingman, & Cummings, 1992). This method classifies the digestion fraction as Rapidly Digestible Starch (RDS, <20 min), Slowly Digestible Starch (SDS, 20−120 min) and Resistant Starch (RS, >120 min) (Englyst et al., 1992). In order to extend the digestion over 45–75 % (Srichuwong et al., 2005) achieved by the Englyst method and to provide information on the structures governing complete enzymatic two adjustments were made on the Englyst method. First, the time points (0 min, 20 min and 2 h) were extended by two additional time points (3 h and 24), resulting in four fractions (F1-F4). The second change was the decrease in enzyme concentration to decelerate the enzymatic hydrolysis and more accurately detect structural differences responsible for the rate of enzymatic hydrolysis. Therefore, the fractions shown here do not represent the respective levels of RDS and SDS. They do, however, allow comparison of the structural features of starches in response to enzymatic digestion until complete (>95 % starch) enzymatic hydrolysis highlighting the need for optimization of hydrolysis conditions for complete structural characterization of starches. Fig. 3

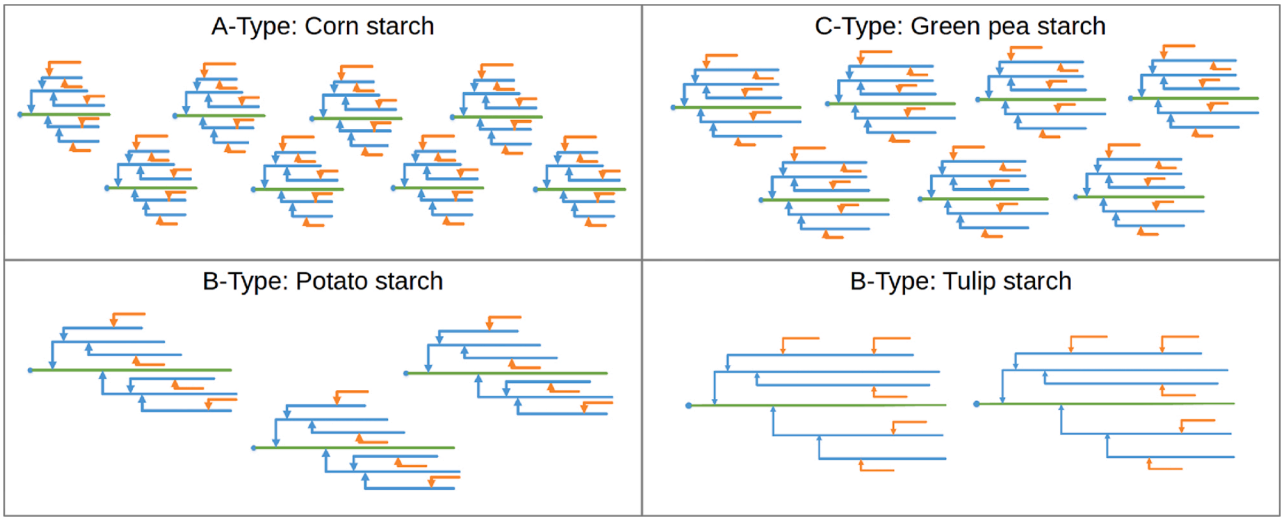

Fig. 2. Simplified models of amylopectin structure based on the results obtained on the starch characterization. Blue dots represent the reducing end of each amylopectin molecule, green lines indicate backbone chains carrying the reducing ends, blue lines show long, substituted branches (DP > 12), orange lines external branches chains (DP 6-12), and arrows branching points (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

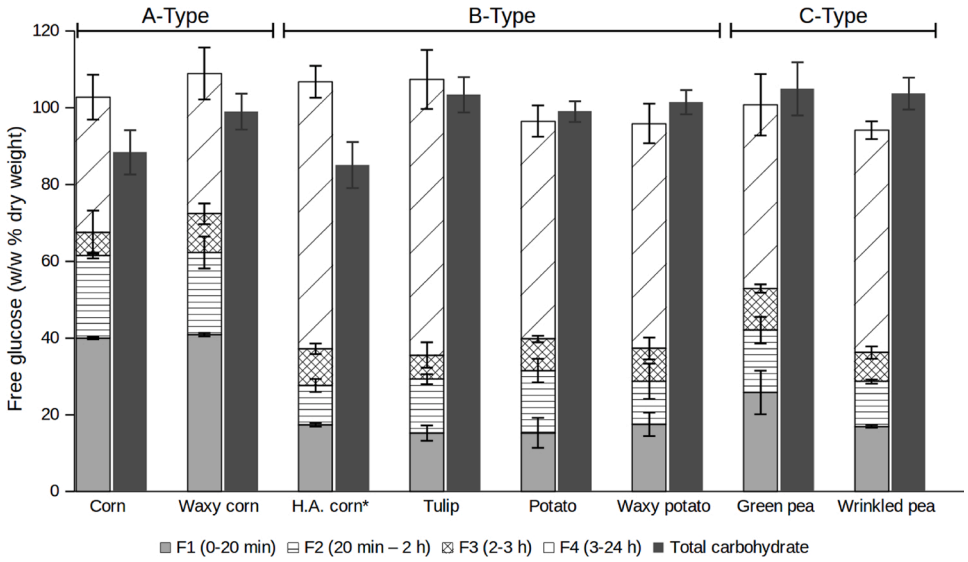

Fig. 3. The proportions of starch from various sources, classified into different hydrolysis fractions. The fraction are not equal to the Englyst method (Englyst et al., 1992) as the enzyme concentration is lower. * H.A. corn stands for high amylose corn starch.

shows that all of the starches were completely hydrolyzed after 24 h of *in vitro* digestion except for wrinkled pea starch (94 % hydrolysis) which could be due to minor retrogradation of long amylose chains during the enzymatic digestion of 24 h, hindering further hydrolysis of glycosidic bonds (Mir, Srikaeo, & García, 2013). The release of glucose during the digestion typically exhibited a sharp initial increase which decelerated as the reaction progressed (Fig. A2). Differences within the same crystallinity type were mainly found in the F3 and F4 fractions which can be attributed to the presence of long chains. Interestingly, starches of the same crystalline type showed similar digestion patterns, indicating the crystallinity to be more determining that the botanical origin or other parameters such as amylose content or the S_AF/LF ratio. This behavior was further supported by the observation that high amylose corn starch, classified as a B-type starch was digested similarly to the other B-type starches and not to the starches sharing its botanical origin (normal and waxy corn,) which were A-type starches (Fig. 3). Generally, A-type starches were digested at the fastest rate, followed by C- and B-type starches, respectively. The difference in rate was so pronounced that the enzymatic digestion of A-type starches led to a higher glucose release in 20 min (about 40 %) than the hydrolysis of B-type starches in 2 h (~30 %). Interestingly, hydrolysis of the long chains of starch (extended and similar resistant starch fraction) govern the complete hydrolysis of starch material and independently of the source and crystallinity type. This was shown to be particularly the case for the fraction beyond the 3 h hydrolysis point which appears to be hydrolyzed to a similar extent for all starch samples. Optimized enzymatic digestion achieving nearly complete hydrolysis of all starch samples hereby highlights the F4 fraction which could be of particular interest for modifications of aiming to obtain more slowly digestible starches.

The finding of the higher digestibility of A-type starches was in good agreement with literature (Planchot et al., 1995; Shi et al., 2018; Srichuwong et al., 2005) and has been speculated to be caused by the branch points in A-type starches being scattered over the crystalline and amorphous regions of the starch granule, leading to shorter internal

chains and branches and inferior crystalline structures which are more vulnerable to enzyme hydrolysis (Jane, Wong, & McPherson, 1997). Together with the here obtained data, the starch digestibility was likely related to the starch structure and more specifically to the properties of the crystallinity type rather than the botanical source of the starch.

Notably, some samples, especially from corn, were more susceptible to enzymatic digestion than to acid hydrolysis leading to a higher glucose recovery. This could be due to the fact that the starches were gelatinized for the enzyme treatment but not for the acid hydrolysis. Thus, it is possible that parts of the complex crystalline structure of the starch remained inaccessible for acid hydrolysis due granule size of the starch (Jane et al., 1997).

3.5. Structure-function relationship of gelatinized starches

The correlation between structural parameters of gelatinized starch and the digestion fractions were examined through statistical analysis based on Pearson correlation. Tulip starch was excluded from the statistical analysis as the derived data from iodine affinity indicated the presence of long chains which could not be detected using HPAEC, limiting the accuracy of its S_AF/LF ratio.

No significant correlation was found between digestibility and amylose content (Table 3). In other studies, amylose content was shown to influence digestibility, however, the starch samples were obtained from the same plant species (amaranth and corn) (Kong et al., 2015; Li et al., 2015; Syahariza et al., 2013).

Significant correlations were found between the degree of branching (reducing ends) and the digestibility with the fraction F1 and F2 showing positive correlations ($r = 0.750$, $P = 0.002$ and $r = 0.803$, $P = 0.001$, respectively) and F4 a negative correlation ($r=-0.749$, $P = 0.002$), indicating a faster digestion at a higher degree of branching. This observation was in contrast to the previous studies reporting a deceleration of digestion after introduction of new branches by enzymes (Kasprzak et al., 2012; Li et al., 2014). It is possible that a higher number of branches within the structure promotes the formation of the more open A-type and hence a higher susceptibility to enzymatic digestion. The structure of starches that have been subjected to enzymatic modification could differ substantially in position and density of branches. Therefore, it may be that enzyme treatment leading to a higher degree of branching could hamper the enzymatic digestion while starches with a high degree of branching have the opposite effect. Notably, the same trend was not observed for the degree of branching determined by NMR. A plausible explanation could be the lower sample size as NMR was only conducted in duplicates and three of the eight starches could not be determined. Additionally, the deviation between the replicates was higher for NMR than for the reducing ends assay. Thus, a more detailed study of the influence of the degree of branching determined by NMR on the digestibility of starches might find a correlation similar to the one observed here on the reducing ends assay. However, it is also possible, that the reducing ends assay gives a slightly distorted values, resulting in an overestimation of the correlation between branching and digestibility. Thus, further studies are required.

In addition to the correlation to the degree of branching (reducing ends), the fraction F1 exhibited a significant positive correlation with the S_AF/LF ratio (Table 3; $r = 0.763$, $P = 0.002$), indicating that a higher number of short (DP 9-12) than long (DP > 12) chains led to a faster digestion. This result was in good agreement with data obtained by Srichuwong et al. (2005) who reported the digestion rate to be positively correlated with the proportion of chains of DP 8-12 and negatively with the amount of chains of DP 16-26. A possible reason for the lower susceptibility of starches with longer amylopectin chains could be a decreased accessibility of the digestive enzymes to hydrolyze the glycosidic bonds. This is plausible as amylopectin chains interact with each other and with amylose through intramolecular and intermolecular hydrogen bonding as a result of e.g. retrogradation (Tako & Hizukuri, 2000).

Table 3
Pearson correlation coefficients for the structural parameters and digestion fractions of all studied starches except tulip starch.

	F1	F2	F3	F4
Amylose content	−0.396	−0.395	−0.013	0.482
Degree of branching (Reducing ends)	0.750*	0.803*	−0.140	−0.749*
Degree of branching (NMR)	0.460	0.178	0.228	−0.309
S_AF/LF ratio	0.763*	0.572	−0.509	−0.577

* Significant correlation ($P \leq 0.01$).

Since the significant correlations with the degree of branching (reducing ends) and the chain length were found regardless of the crystalline type, the branches themselves appeared to influence the digestibility. Comparing the obtained results with the models, it is apparent that a higher number of branches opens the structure and thus promotes enzymatic digestion. Even though the degree of branching determined by NMR did not show any significant correlation with the hydrolysis fractions, the trend is similar, supporting this finding. However, one should interpret this with caution due to method sensitivity.

4. Conclusions

Starches typically shared similarities in their source (cereal/tuber/legume), chain length distribution, and iodine affinity with a few exceptions such as corn amylose belonging to a different crystalline type than (waxy) corn. Structural characterization revealed that the A-type starch had shorter chains with high branch density, B-type starches had longer chains with less branch density, and the C-type starch was an intermediates of the two. Optimized enzymatic hydrolysis of all starch samples achieved >95 hydrolysis highlighting fractions resistant to standard digestion assays. The ratio between the short and long chains (S_AF/LF ratio) and the degree of branching analyzed by the reducing ends method showed significant correlation with the enzymatic hydrolysis. In contrast, the amylose content only showed a minor effect on the overall hydrolysis. Overall, the A-type starch, having a high proportion of short branches, were hydrolyzed faster, followed by C- and B-type starches. Fraction containing longer chains in all starches, irrespective to the source and crystalline type proved to be more resistant to enzymatic hydrolysis. Our research shows that modifications should be directed towards formation of long chains in amylopectin to result in a more slow digestibility.

CRediT authorship contribution statement

Aline L.O. Gaenssle: Formal analysis, Methodology, Visualization, Writing - original draft. **Caecilia A. Satyawan:** Investigation, Validation, Visualization. **Gang Xiang:** Investigation. **Marc J.E.C. van der Maarel:** Funding acquisition, Resources, Supervision. **Edita Jurak:** Conceptualization, Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

This research was supported by the Netherlands Organization for Scientific Research (NWO Groen program), The Netherlands, AVEBE, Veendam, The Netherlands. We further thank AVEBE for the financial support. We are grateful to Jacob Baas (University of Groningen) for use and training of the XRD instrument.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2021.117801.

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